Check for updates

Potential nutritional healthy-aging strategy: enhanced protein metabolism by balancing branched-chain amino acids in a finishing pig model

Lingyu Zhang, [ID] [a,b,c] Qiuping Guo,[a] Yehui Duan,[a] Wenlong Wang,[a] Yuhuan Yang,[d] Yunju Yin,[d] Saiming Gong,[d] Mengmeng Han,[a,b] Fengna Li [ID] *[a,b] and Yulong Yin[a]

Branched-chain amino acids (BCAAs) have key physiological roles in the regulation of protein synthesis, metabolism, food intake and aging. This study aimed to investigate the protective effect of balanced BCAAs on healthy aging by increasing skeletal muscle mass and muscle fiber composition in a finishing pig model. A balanced BCAA ratio (Leu : Ile : Val = 2 : 2 : 1) significantly activated the mTOR pathway and upregulated the expression of amino acid transporters, such as ASCT2, SNAT2, LAT1, PAT1, and SLC38A9, simultaneously modulating mitochondrial function and muscle fiber composition, thereby inhibiting inflammatory cytokines, such as IL-6 and TNF-α, regulating amino acid metabolism, and ultimately increasing skeletal muscle mass. Overall, our results suggest that a BCAA ratio around 2 : 2 : 1 may be a promising candidate for healthy aging in humans and animals.

1. Introduction

Aging is an inevitable, universal, multifactorial and complex process, which is defined as complicated by the occurrence of various diseases that modify body functions and tissue structures, associated with vulnerable injuries and cardiovascular, neural, immune and hormonal diseases.[1] It is presumed that the global population aged 60 or over is projected to grow by 16% and more than 21% in 2030 and 2050, respectively.[2] Despite the growing elderly population, healthy aging is key to longevity and avoiding aging-related diseases.[3]

Of interest, sarcopenia of aging is noted in humans, which is a condition characterized by age-related and gradual loss of skeletal muscle mass.[4,5] Meanwhile, an age-related decline in the synthesis rate of skeletal muscle myosin heavy chain (MyHC) has been reported in humans, leading to a decline in locomotor function in elderly life, as MyHC is a key contractile

protein.[6] It is reported that fiber type distribution changed towards a decrease in the percentage of MyHC II fibers in the elderly, where the MyHC II fiber ratio was inversely related to MyHC I fibers.[4,7] Both the declined total number of muscle fibers and the specifically atrophic MyHC II fibers contribute to the loss of skeletal muscle mass, resulting in a reduced proportion of MyHC II muscle fibers in the skeletal muscle tissue in the elderly.[8] Therefore, healthy-aging strategies would focus on how to reverse the lower muscle mass, as well as to promote the composition of MyHC II in skeletal muscle.

The maintenance of skeletal muscle mass is a dynamic and complex equilibrium between muscle protein synthesis and deposition. The influx of amino acids (AAs) and the resulting increasing protein synthesis associated with the intake of dietary proteins or AAs counteracts the efflux of AAs from muscle protein breakdown that occurs during daily and exercise consumption.[9] AAs not only constitute protein, but they are also important nutrients as signaling molecules, which are able to trigger anabolic pathways and affect protein synthesis and deposition in skeletal muscle.[10] It is also reported that, except for strengthening exercise, increasing the dietary protein intake, especially the intake of essential AAs (EAAs), is very important for maintaining the muscle mass of the elderly.[11] Of all the EAAs, branched-chain amino acids (BCAAs), leucine (Leu), isoleucine (Ile), and valine (Val), accounting for almost 50% of muscle protein EAAs, are emphasized as critical regulators of metabolic health,

[a]Key Laboratory of Agro-Ecological Processes in Subtropical Region, Institute of Subtropical Agriculture, Chinese Academy of Sciences, Hunan Provincial Engineering Research Center for Healthy Livestock and Poultry Production, Changsha 410125, China. E-mail: lifengna@isa.ac.cn; Fax: +86-731-8461-2685; Tel: +86-731-8461-9767
[b]College of Advanced Agricultural Sciences, University of Chinese Academy of Sciences, Beijing 100049, China
[c]National Engineering Laboratory for Rice and By-Product Deep Processing, Central South University of Forestry and Technology, Changsha 410004, China
[d]College of Animal Science and Technology, Hunan Agricultural University, Changsha 410128, China

Paper

especially for skeletal muscle mass. It has been widely accepted that BCAAs, in particular Leu,[12] act as activators of protein synthesis *via* the mammalian target of rapamycin (mTOR) signaling, increasing skeletal muscle mass in turn.[13,14] Besides, the transport of BCAAs requires the aid of AA transporters such as sodium-dependent neutral AA transporter 2 (SNAT2), L-type AA transporter 1 (LAT1), and proton-assisted amino acid transporter 1 (PAT1).[15,16] These transporters exhibit a dual transporter and receptor function, sensing BCAA availability, relaying nutrient signals to the cell interior, regulating the uptake and efflux of BCAAs, and regulating the dynamic balance of protein synthesis and deposition in mammals, subsequently affecting the growth of muscle fibers, increasing skeletal muscle mass, and ultimately playing the role of anti-aging agents in skeletal muscles.

In addition to aiming at increasing skeletal muscle mass, changing the muscle fiber composition may be another target of a healthy-aging nutritional strategy. In fact, oxidative fibers (MyHC I) have slower contraction rates, while glycolytic fibers (MyHC II) have fast contraction speeds and high strength. Generally, muscles with higher proportions of glycolytic fibers generally have greater amounts of glycogen, as well as higher glycolytic enzyme activity, such as that of hexokinase (HK) and lactate dehydrogenase (LDH). As aging is a process that reduces the proportion of MyHC II muscle fibers in the skeletal muscle tissue, healthy-aging may also be related to the increase in the glycolytic fibers with higher abundance of glycolytic enzymes.[17] Dietary BCAAs, especially Leu, have been reported to promote mitochondrial function through mTOR signaling,[18,19] which may alter muscle fiber composition, increase bone metabolism and contractile function, and ultimately achieving the goal of our nutritional strategy: healthy aging in elderly.[20]

There have been increasing applications of BCAAs as a potential source of functional food ingredients for health promotion in recent years,[21–23] as BCAAs are particularly important for protein metabolism in muscle tissues, and are essential substrates and important regulators for protein synthesis.[24,25] Many studies report the relationships between dietary manipulation of BCAAs with age-related changes in body composition, sarcopenia, obesity, insulin and glucose metabolism, and aging biology itself.[3,26,27] However, there are few studies of the relationship between the ratios of balanced intake of BCAAs and the healthy-aging process. It is worth noting that the balance of BCAAs plays a pivotal role in skeletal muscle health and maintains the normal physiological functions of mammals, as antagonism between BCAAs might happen if one of the BCAAs is excessive. Indeed, three BCAAs share the same two key enzymes in the first step of BCAA catabolism, namely BCAA aminotransferase (BCAT) and branched chain α-keto acid dehydrogenase (BCKD);[26] thus the three BCAAs may compete for the two enzymes or the same AA transporter, which further verified the fundamentality of controlling the balance of BCAAs. Although BCAAs are powerful nutritional factors for increasing protein metabolism and muscle growth,[28] it remains unclear as to how to balance BCAAs to avoid the subsequent antagonism caused by the imbalance of BCAAs and to affect protein metabolism efficiently.

In addition, a number of studies have shown that pigs and humans have many similarities in physiological structure and function, including eating mode, metabolic mode, kidney structure and function, pulmonary vascular structure, aging trends, respiratory rate and behavior.[29,30] Secondly, pigs have great similarities with humans in nutrition and physiological metabolism,[31] and the genome of pigs is highly consistent with humans.[32] Therefore, finishing pigs are the closest model animals to humans, and are used to research healthy ageing strategies in this study.

In order to dig deeper into the above issues, this study focuses on balanced BCAAs and protein metabolism, and is part of a series of experiments to investigate the optimal ratio of BCAAs in a finishing pig model (60–100 kg body weight). We previously reported that protein-restricted diets supplemented with a balanced BCAA ratio (Leu : Ile : Val = 2 : 1 : 1–2 : 1 : 2) contribute to reduce lipid deposition while improving growth performance.[26] We thus hypothesized that balanced BCAAs could simultaneously modulate protein metabolism in skeletal muscles, which could be a potential nutritional strategy to resist aging.

2. Materials and methods

2.1. Animals, experimental design and sample collection

Complied with the Chinese guidelines on experimental protocols and animal welfare, a total of 160 Duroc × Landrace × Large White castrated male pigs were fed a corn and soybean meal-basal diet (Table 1), which met the NRC nutritional requirements (2012). Before the beginning of the experiment, all pigs were fed the same diet for 1 week, and then the pigs with an average initial body weight of 59.11 ± 0.46 kg (mean ± standard error of mean, SEM) were randomly assigned to one of five treatments (8 pens per treatment, 4 pigs per pen): a basal 16% crude protein (CP) diet (positive control), a restricted 12% CP diet (negative control) and three experimental groups were fed a restricted 12% CP diet supplemented with BCAAs to the ratio (Leu : Ile : Val) 2 : 1 : 1, 2 : 2 : 1, and 2 : 1 : 2. The total amount of BCAAs in the three experimental groups was equal. The pigs were housed in pens (3.5 × 2.8 m) equipped with feed intake recording equipment, thus pigs had *ad libitum* access to the feed and water. The pigs were labeled with individual electronic ear markers. The experiment lasted for 43 days until the average final body weight of pigs was 95 kg, and was approved by the Animal Protection Committee of the Institute of Subtropical Agriculture, Chinese Academy of Sciences.

At the end of the feeding trial, the diets were removed 12 h before slaughter. Blood samples were obtained from the jugular vein using 10 mL centrifuge tubes containing sodium heparin, centrifuged at 3000*g* and 4 °C for 15 min to recover plasma, and then stored at −20 °C. After collection of blood samples, pigs were slaughtered under commercial conditions using electrical stunning (120 V, 200 Hz) and exsanguination.

Table 1 Composition of the experimental diets (as-fed basis)

Item[a]	16% CP	12% CP	12% CP BCAA2 : 1 : 1	12% CP BCAA2 : 2 : 1	12% CP BCAA2 : 1 : 2
Ingredient (%)					
Corn	66.88	80.84	81.18	81.18	81.18
Soybean meal	23.90	11.75	9.80	9.80	9.80
Wheat bran	6.00	3.00	3.00	3.00	3.00
Soybean oil	0.88	1.44	2.20	2.20	2.20
Lysine	—	0.37	0.42	0.42	0.42
Methionine	—	0.03	0.05	0.05	0.05
Threonine	—	0.11	0.13	0.13	0.13
Tryptophan	—	0.03	0.03	0.03	0.03
Leucine	—	—	0.29	0.02	0.02
Isoleucine	—	—	0.26	0.65	0.14
Valine	—	—	0.20	0.08	0.59
$CaHPO_4$	0.50	0.60	0.60	0.60	0.60
Limestone	0.54	0.53	0.54	0.53	0.54
Salt	0.30	0.30	0.30	0.30	0.30
Premix[b]	1.00	1.00	1.00	1.00	1.00
Total	100.00	100.00	100.00	100.00	100.00
Nutrient content (%)					
Digestible energy (MJ kg^{-1})	14.21	14.23	14.22	14.22	14.22
Crude protein	16.04	12.03	12.07	12.07	12.07
SID Lys	0.73	0.73	0.73	0.73	0.73
SID Met + Cys	0.51	0.42	0.42	0.42	0.42
SID Thr	0.52	0.47	0.46	0.46	0.46
SID Trp	0.17	0.13	0.13	0.13	0.13
SID Leu	1.30	1.04	1.27	1.00	1.00
SID Ile	0.59	0.40	0.62	1.00	0.50
SID Val	0.63	0.46	0.62	0.51	1.00
Total Ca[c]	0.51	0.50	0.50	0.50	0.50
Total P[c]	0.45	0.40	0.39	0.39	0.39
Available P	0.20	0.20	0.19	0.19	0.19

[a] Diet treatment: 16% CP, 16% crude protein level diet (NRC, 2012); 12% CP, 12% crude protein level diet; BCAA2 : 1 : 1, 12% CP diet supplemented with BCAAs to the ratio (Leu : Ile : Val) 2 : 1 : 1; BCAA2 : 2 : 1, 12% CP diet supplemented with BCAAs to the ratio 2 : 2 : 1; BCAA2 : 1 : 2, 12% CP diet supplemented with BCAAs to the ratio 2 : 1 : 2. [b] Supplied per kg of diet: vitamin A, 10 800 IU; vitamin D3, 4000 IU; vitamin E, 40 IU; vitamin K3, 4 mg; vitamin B1, 6 mg; vitamin B2, 12 mg; vitamin B6, 6 mg; vitamin B12, 0.05 mg; biotin, 0.2 mg; folic acid, 2 mg; niacin, 50 mg; D-calcium pantothenate, 25 mg; Cu (as copper sulfate), 150 mg; Fe (as ferrous sulfate), 100 mg; Mn (as manganese oxide), 40 mg; Zn (as zinc oxide), 100 mg; I (as potassium iodide), 0.5 mg; and Se (as sodium selenite), 0.3 mg. [c] Measured values.

Approximately 10 g of longissimus dorsi muscle (LDM) between the 6–7th ribs on the right side of each carcass was snap-frozen in liquid nitrogen and stored at −80 °C.

2.2. Carcass characteristics

After slaughter, carcass weight was analyzed using slaughter segmentation and recorded immediately. The loin eye area was measured via a transverse cut at the sixth and seventh rib regions of the loin from the right side of each carcass by tracing the area and subsequently measuring using a vernier scale. Thus, it was estimated according to the equation: loin eye height and width (loin eye area (cm^2) = loin eye height (cm) × width (cm) × 0.7). Fat-free lean weight was estimated according to the equation: fat-free lean weight = 0.95 × [7.231 + (carcass weight, pound) × 0.437] + (loin eye area, square inch) × 3 (NRC, 1998). The lean mass index is obtained by dividing the lean weight by the body weight.

2.3. Muscle histology

Samples of the LDM were embedded in paraffin and cut into 5 μm serial sections after being cut into 3.0 × 0.5 × 0.5 cm^3 cubes, and tissue samples were stained with hematoxylin–eosin (H&E) for identification. The muscle fiber numbers and area of the LDM were measured using a light microscope (CK-40; Olympus, Tokyo, Japan) at ×200 magnification and analyzed using an Olympus BX43 microscope with an Olympus SC30 color camera attachment and Olympus cellSens software. Approximately 50 fibers were evaluated per sample. The means of these measurements were calculated to yield a single value per animal.

2.4. Blood biochemical assessments

Blood samples were harvested from the anterior vena cava and sera were separated after centrifugation at 300 rpm for 10 min at 4 °C. The concentrations of serum creatinine (CREA), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and glucose (GLU) were analyzed using commercial kits (Leadman Biotech Limited, Beijing, China) and biochemical analytical instruments (Beckman CX4; Beckman Coulter, Germany). The concentrations of serum interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) were measured using commercial porcine ELISA kits (Cusabio Life Science, Inc., Wuhan, China).

2.5. Muscle biochemical assessments

The concentrations of muscle glycogen (MG), hexokinase (HK), lactate dehydrogenase (LDH) and citrate synthase (CS) were analyzed using commercial kits (Jiancheng, Nanjing, China). Total protein concentrations were determined using the BCA assay (Jiancheng, Nanjing, China). All experiments were performed according to the manufacturer's instructions. The values obtained were normalized to the total cellular protein content and were expressed as micromoles per gram of protein.

2.6. Muscle crude protein content analysis

Chemical analysis of the crude protein content was measured to detect the physicochemical properties of the LDM, according to the methods of the Association of Official Analytical Chemists (AOAC, 2007). Experiments were performed for all the LDM samples in triplicate to reduce the experimental and manual errors.

2.7. Serum and muscle free amino acid profile

Blood samples were harvested from the anterior vena cava and sera were separated after centrifugation at 300 rpm for 10 min at 4 °C. 0.5 g of muscle samples was homogenized with 5 ml of 0.01 M hydrochloric acid and centrifuged at 5000 rpm for 5 min, and then 0.5 ml of supernatants was mixed with 8% salicylsulfonic acid for one night at 4 °C. The mixtures were further centrifuged at 12 000 rpm for 10 min twice. The final supernatants were used for amino acid analysis using a High-speed Amino Acid Analyzer L-8900 (Japan).

2.8. RNA extraction and quantitative real-time PCR

Total RNA was extracted from the LDM (approximately 50 mg of each) using TRIzol™ reagent (Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. An ND-1000 NanoDrop® (Thermo Fisher Scientific, Wilmington, DE, USA) was used to measure the 260/280 nm ratios of the samples, which were between 1.8 and 2.0, and the RNA integrity was determined by 1% agarose gel electrophoresis. All the samples used in this study were complete RNA samples, as the agarose gels clearly showed the 5S, 18S, and 28S rRNA bands. A 20 μL reaction volume containing 1 μg of RNA was used for cDNA synthesis, using the PrimeScript® first-strand cDNA synthesis kit (Takara, Osaka, Japan) according to the manufacturer's instructions. The cDNA was then stored at −80 °C until further analysis.

Gene-specific primers used for PCR analyses are listed in Table 2. A regular PCR was performed to test primer specificity and verify the amplified products for the selected genes. Total reaction volumes (10 μL) contained 2 μL cDNA template, 5 μL SYBR® Green PCR master mix (Thermo Fisher Scientific, Inc., Waltham, MA, USA), 2.2 μL ddH$_2$O, and 0.4 μL of each primer. The relative expression of each target gene was determined by real-time (RT)-PCR using an ABI 7900HT system (Applied Biosystems, Carlsbad, CA, USA) with three replicates per biological sample. The RT-PCR conditions were: 10 min incubation at 95 °C, followed by 40 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 20 s. A melting curve program (60–99 °C with a heating rate of 0.1 °C s^{-1}) and fluorescence measurements were performed to generate melting curves for each sample, and to verify primer specificity and ensure PCR product purity. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize the mRNA levels of the selected genes. The relative mRNA expression levels were calculated according to the following formula: $R = 2^{-\Delta\Delta Ct}$, as previously described.[26]

2.9. Immunoblotting analysis

Total protein extracted from the LDM was used to measure the relative protein levels of system ASC amino acid transporter 2 (ASCT2) (5345S, Cell Signaling Technology, USA), MYHC1 (67299-1-Ig, Proteintech, USA), MYH4 (20140-1-AP, Proteintech, USA), MYH2 (SC-71632, Santa Cruz, USA), MYH7 (SC-53089, Santa Cruz, USA), P-4EBP1 (9451S, Cell Signaling Technology, USA), 4EBP1 (9452S, Cell Signaling Technology, USA), P-P70S6K1 (9208S, Cell Signaling Technology, USA), P70S6K1 (14485-1-AP, Proteintech, USA), P-mTOR (5536S, Cell Signaling Technology, USA), and mTOR (2983S, Cell Signaling Technology, USA) using the western blotting technique as previously described.[26] Briefly, about 50 μg of protein of each

Table 2 Real-time PCR primer sequences

Genes	Primer sequences (5′–3′)	Size (bp)
ASCT	F: GATTGTGGAGATGGAGGATGTGG R: TGCGAGTGAAGAGGAAGTAGATGA	128
CAT1	F: TGCCCATACTTCCCGTCC R: GGTCCAGGTTACCGTCAG	192
PAT1	F: TGTGGACTTCTTCCTGATTGTC R: CGTTGTTGTGGCAGTTGTTGGT	125
SNAT2	F: TGAAGAAGACCGAAATGGGA R: TGGTGGGGTATGAGTAGTTG	96
SLC38A9	F: CTTTGGGCAGTGGTCGAGTCTTC R: CTTTGGGCAGTGGTCGAGTCTTC	190
TAT1	F: GCCCATTGCCTTCGAGTTAG R: AGCGAGGTAGAATGCCACAT	154
LAT1	F: TTTGTTATGCGGAACTGG R: AAAGGTGATGGCAATGAC	155
MSTN	F: GTCCCGTGGATCTGAATG R: TTCCGTCGTAGCGTGATA	293
MuRF1	F: AGCACGAAGACGAGAAAATC R: TGCGGTTACTCAGCTCAGTC	150
MEF2a	F: TGAATACCCAGAGGATAAGCAGTT R: TAATCGGTGTTGTAGGCGG	133
MyoG	F: AGGCTACGAGCGGACTGA R: GCAGGGTGCTCCTCTTCA	230
IL-6	F: TGCAATGAGAAAGGAGATGTGTG R: CCCAGATTGGAAGCATCCGT	121
TNF-α	F: AACCTCAGATAAGCCCGTCG R: ACCACCAGCTGGTTGTCTTT	129
OPA1	F: GATGGTGCTTGTTGACTTAC R: TTGCTGAATCCTGCTTGG	274
MFN2	F: CATCGCCATATAGAGGAAGG R: CACAGTTGAGGTCGTAGC	193
PCG-1α	F: GCCCAGTCTGCGGCTATTT R: GTTCAGCTCGGCTCGGATTT	265
GAPDH	F: CAAAGTGGACATTGTCGCCATCA R: AGCTTCCCATTCTCAGCCTTGACT	123

samples, with a pre-stained protein ladder (Thermo Fisher Scientific, Rockford, IL, USA), was used for sodium dodecyl sulfate polyacrylamide gel electrophoresis. Subsequently, the protein was electrotransferred to a polyvinylidene fluoride membrane and blocked in 5% (w/v) bovine serum albumin at room temperature for 1 hour and the membranes were incubated with the corresponding primary antibodies at 4 °C overnight. The primary antibodies were diluted to 1 : 1000. After three washes, the membranes were incubated with DyLight 800-labeled secondary antibodies (HRP goat anti-mouse IgG/ HRP goat anti-rabbit IgG, SA00001-1/SA00001-2, Proteintech, USA) and diluted to 1 : 5000, respectively. And tubulin (2146S, Cell Signaling Technology, USA) diluted at 1 : 1000 was used as an internal control, to explore the alteration of protein metabolism.

2.10. Statistical analysis

Data were analyzed by one-way ANOVA using SAS 9.2 software (SAS Institute, Inc., Cary, NC, USA). Duncan's multiple-range test for homogeneity of variance was used to determine the effects of the treatments. GraphPad Prism 8.0 (San Diego, CA, USA) was used to plot the images. The results were expressed as means and differences between means were considered statistically significant at $P < 0.05$.

3.   Results

3.1.   Stimulative effect of BCAAs on skeletal muscle carcass characteristics, fiber size and crude protein content

Our previous studies have shown that low protein caused a decrease in protein deposition.[28] To assess the role of BCAAs in protein deposition, we set up a negative control group (12% CP) to verify whether BCAAs mitigate the low protein-induced effects. At the same time, on the basis of low protein (12% CP), we added BCAAs to reach three different ratios (Leu : Ile : Val = 2 : 1 : 1/2 : 2 : 1/2 : 1 : 2), and explored which balanced BCAA ratio achieved the best performance, even exceeding the positive control group (16% CP).

As shown in Fig. 1, loin eye area, lean weight, and lean mass ratio in the 12% CP group were lower than those in the 16% CP group, while the three BCAA groups showed varying degrees of improvement. Compared to the 12% CP group, loin eye area, lean weight, and lean mass ratio were simultaneously significantly decreased ($P < 0.05$) in the BCAA supplemented group, indicating that in terms of carcass traits, a balanced BCAA ratio enhanced skeletal muscle mass.

In addition, the slices of skeletal muscle stained with H&E could verify whether the morphology of muscle fibers is consistent with the results of carcass traits. For the growth of muscle fibers, the cross-sectional area and total number of skeletal muscle fibers are of great significance. As shown in Fig. 1D, compared to the 12% CP group, muscle fiber area of the BCAA groups was increased, while their fiber number was decreased, which is consistent with the results of carcass

traits. In particular, muscle fiber area increased in all three BCAA groups compared to the 12% CP group.

The amount of muscle tissue is consistent with the crude protein content present in the skeletal muscle. As shown in Fig. 1F, muscle crude protein content in the three BCAA ratios were completely higher than those in the 12% CP group, especially the ratio of 2 : 2 : 1 and 2 : 1 : 2 ($P < 0.05$). Overall, together with the results of carcass traits and H&E slices, it can be seen that BCAAs do increase muscle deposition, thus we wanted to explore which ratio is more appropriate and investigate its mechanism further.

3.2.   BCAAs enhanced serum biochemical enzyme activity and inhibited low protein-induced inflammatory effects on cytokines

Changes in serum biochemical indicators can reflect the impact of dietary nutrition on energy metabolism. As shown in Fig. 2, compared to the 16% CP group, 12% CP group reduced the levels of serum creatinine (CREA), aspartate transaminase (ALT), and alkaline phosphatase (ALP) to varying degrees. Surprisingly, all three BCAA groups ameliorated this downward trend and enhanced the activities of these enzymes, reflecting that BCAAs could improve amino acid metabolism, thus enhancing the synthesis and decomposition of proteins.

In addition, we tested two serum cytokines associated with inflammation, interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α). As shown in Fig. 2F and G, compared to the 16% CP group, IL-6 and TNF-α were significantly higher in the 12% CP group, indicating that a low protein diet may enhance inflammation. In the current study, compared to the 12% CP group, all three BCAA groups decreased the abundance of IL-6 and TNF-α. Then we further detected *TNF-α* expression in the LDM; the results also showed that compared to the 12% CP group, all three BCAA groups significantly reduced ($P < 0.05$) the expression level of *TNF-α* (Fig. 2H), which is consistent with the above-mentioned ELISA results.

3.3.   Effects of BCAAs on serum and muscle amino acid composition

Based on the results of serum biochemical enzymes, we suspected that BCAAs increased muscle deposition by affecting AA metabolism. Thus, we further detected the composition of serum and muscle free AAs. The results regarding the serum free AA profile of the LDM are revealed in Table 3. The concentrations of most serum AAs were strongly influenced by the dietary BCAAs. Specifically, relative to the negative control (12% CP), BCAA groups increased the concentrations of lysine (Lys), alanine (Ala), arginine (Arg), and proline (Pro) ($P < 0.05$). Similarly, compared to the 12% CP group, the concentrations of leucine (Leu) and threonine (Thr) ($P = 0.05$) tended to change in the BCAA groups. Diet treatments had no effect on other AAs. Meanwhile, the concentrations of valine (Val), total EAAs, and total AAs in the BCAA 2 : 1 : 2 group were significantly higher than those of the control ($P < 0.01$).

The concentration of free AAs in skeletal muscle can directly reflect the physiological condition and the nutritional
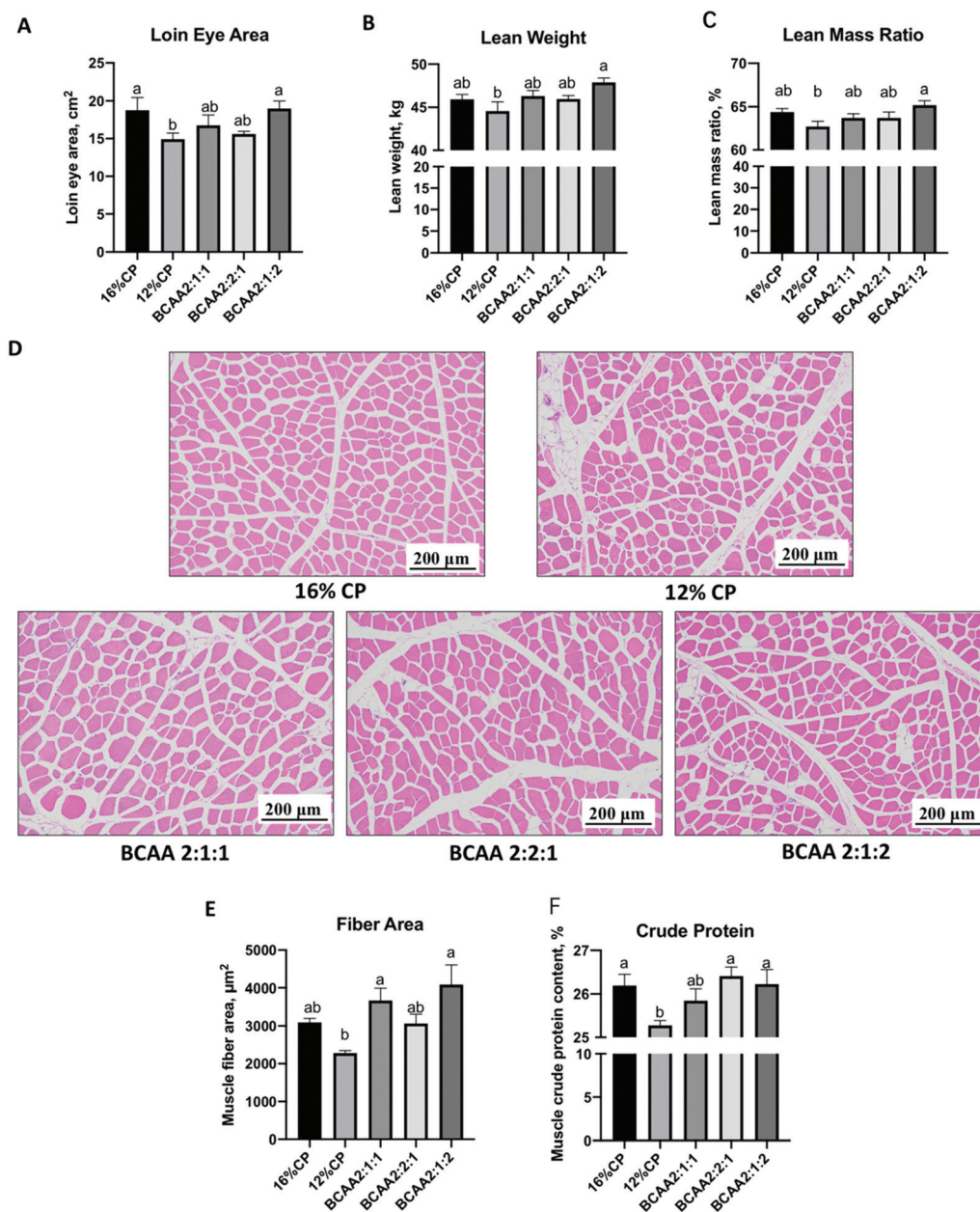
Fig. 1 Stimulative role of BCAAs on skeletal muscle carcass characteristics, fiber size and crude protein content. Carcass traits for loin eye area (A), lean weight (B), and the lean mass ratio (C) in finishing pigs offered five diets; analysis of hematoxylin and eosin slices of longissimus dorsi muscle in finishing pigs offered five diets (D), and the area (E) of fibers in the muscle slices of finishing pigs; and muscle crude protein content of longissimus dorsi muscle in finishing pigs offered five diets (F). 16% CP, 16% crude protein level diet (NRC, 2012); 12% CP, 12% crude protein level diet; BCAA2 : 1 : 1, 12% CP diet supplemented with branched-chain amino acids to the ratio (Leu/Ile/Val) 2 : 1 : 1; BCAA2 : 2 : 1, 12% CP diet supplemented with branched-chain amino acids to the ratio 2 : 2 : 1; BCAA2 : 1 : 2, 12% CP diet supplemented with branched-chain amino acids to the ratio 2 : 1 : 2. Values with different letters (a and b) indicate a significant difference among different treatments ($P < 0.05$). Values are the mean $\pm$ SEM ($n = 8$).

level. As shown in Table 4, in the LDM, compared with the 12% CP group, most AA levels (especially for histidine, Ile, Lys, tryptophan, Val, Arg, glutamic acid, glycine, and Pro) increased ($P < 0.05$). Similarly, compared to the 12% CP group, the concentrations of Leu ($P = 0.10$), Thr ($P = 0.19$), aspartic acid (Asp) ($P = 0.17$), and methionine (Met) ($P = 0.06$) tended to change in the BCAA groups. However, no significant change in response to diet treatments was detected in other AAs.

3.4. BCAAs enhanced the expression of skeletal muscle amino acid transporters, growth factors, and the mTOR pathway to promote protein deposition

Our results showed that BCAAs significantly affected the composition of AAs in serum and muscle, thus we suspected that the direct cause may be changes in the capacity of muscle cells for sensing and the intake of AAs. Moreover, the intake of AAs
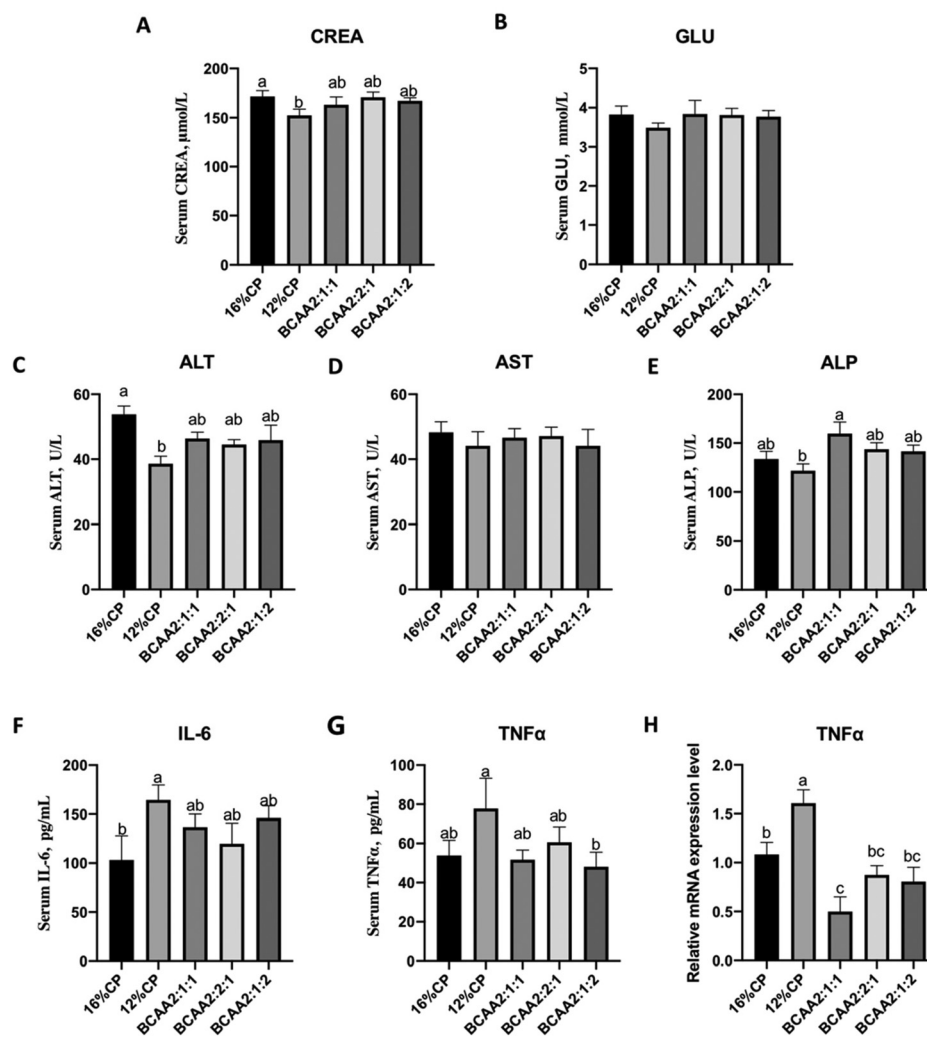
Fig. 2 BCAAs enhanced serum biochemical enzyme activity and inhibited low protein-induced inflammatory effects on cytokines. Activities of serum biochemical indicators for creatinine (A), glucose (B), alanine transaminase (C), aspartate transaminase (D), and alkaline phosphatase (E), and serum cytokines for interleukin 6 (F) and tumor necrosis factor alpha (G) in finishing pigs offered five diets, and the relative mRNA levels of tumor necrosis factor alpha in the longissimus dorsi muscle of finishing pigs offered five diets (H). 16% CP, 16% crude protein level diet (NRC, 2012); 12% CP, 12% crude protein level diet; BCAA2 : 1 : 1, 12% CP diet supplemented with branched-chain amino acids to the ratio (Leu/Ile/Val) 2 : 1 : 1; BCAA2 : 2 : 1, 12% CP diet supplemented with branched-chain amino acids to the ratio 2 : 2 : 1; BCAA2 : 1 : 2, 12% CP diet supplemented with branched-chain amino acids to the ratio 2 : 1 : 2. Values with different letters (a–c) indicate a significant difference among different treatments ($P < 0.05$). Values are the mean $\pm$ SEM ($n = 8$).

is closely related to the physiological function of the corresponding transporters. In fact, AA transporters not only have the transport function, but also serve as receptors to send the signal to downstream molecules, especially the mTOR pathway. Therefore, we measured the expression of AA transporters and the mTOR pathway in the LDM. As shown in Fig. 3, compared to the 12% CP group, BCAAs significantly improved the expression of *ASCT*, *CAT1*, *PAT1*, *SNAT2*, *LAT1*, and *SLC38A9* ($P < 0.5$). Among the three BCAA groups, BCAA 2 : 2 : 1 group performed the best, and the protein expression result of ASCT is consistent with it. To expand the understanding of low-protein diets with BCAA supplementation on muscle mass, we measured the mRNA expression levels of the muscle growth-related genes (*MyoG*, *MEF2a*, and *MSTN*) and

muscle protein degradation-related gene (*MuRF1*). Compared to the 12% CP group, the BCAA2 : 2 : 1 group significantly improved the expression of *MyoG* and *MEF2a* ($P < 0.5$), while it significantly decreased the expression of *MSTN* and *MuRF1* ($P < 0.5$). In terms of the mTOR pathway, we surprisingly found that, compared to the negative control (12% CP group), both BCAA2 : 2 : 1 and BCAA2 : 1 : 2 groups significantly increased the expression of P-4EBP1/4EBP1, P-P70S6K1/P70S6K1, and P-mTOR/mTOR ($P < 0.5$), even exceeding the positive control (16% CP group).

In the aging process, the aerobic ability of skeletal muscle is weakened, which is mainly manifested by a decrease in mitochondrial function in skeletal muscle.[33] Meanwhile, it is reported that mTOR is a central sensor for the regulation of

Table 3 Serum amino acid content of finishing pigs fed 12% CP diets supplemented with branched-chain amino acids[a,b]

Amino acids (AA) (%)	16% CP	12% CP	12% CP			SEM	P
			BCAA2 : 1 : 1	BCAA2 : 2 : 1	BCAA2 : 1 : 2		
Essential AAs							
Histidine	11.23[ab]	9.79[bc]	9.56[c]	11.50[a]	9.31[c]	0.28	0.02
Isoleucine	16.53	15.69	15.99	18.84	18.13	0.50	0.20
Leucine	27.49[ab]	25.33[b]	28.44[ab]	27.70[ab]	31.31[a]	0.67	0.05
Lysine	37.65[ab]	32.49[b]	38.27[ab]	33.36[b]	43.24[a]	1.01	<0.01
Methionine	5.06	4.91	5.33	5.65	5.38	0.15	0.56
Phenylalanine	15.30	15.52	14.69	16.63	15.45	0.33	0.49
Threonine	19.69[ab]	18.79[b]	18.04[b]	22.77[a]	19.67[ab]	0.53	0.05
Tryptophan	10.87	10.13	8.86	10.52	10.32	0.36	0.47
Valine	34.21[b]	34.03[b]	34.45[b]	35.89[b]	53.58[a]	1.54	<0.001
Total EAAs	175.69[b]	170.50[b]	172.02[b]	181.53[b]	204.62[a]	3.59	<0.01
Nonessential AAs							
Alanine	44.84[a]	31.97[b]	39.39[a]	41.92[a]	38.98[a]	1.12	<0.01
Arginine	35.52[bc]	33.30[c]	34.90[bc]	37.73[ab]	41.23[a]	0.74	<0.01
Aspartic acid	2.61	3.03	2.64	3.01	2.12	0.15	0.32
Cysteine	5.91	4.80	5.92	5.55	6.15	0.24	0.43
Glutamic acid	39.46	37.18	39.88	42.07	36.37	1.26	0.65
Glycine	61.03	62.06	67.36	73.57	72.59	2.77	0.50
Proline	25.37[ab]	23.14[b]	25.05[ab]	26.60[a]	26.28[a]	0.38	0.03
Serine	12.29	12.10	12.79	13.22	13.15	0.23	0.45
Tyrosine	16.24	15.99	15.54	15.99	16.29	0.31	0.95
Total NEAAs	243.26[ab]	218.12[b]	249.45[a]	264.24[a]	255.74[a]	4.97	0.05
Total AAs	418.95[c]	394.48[bc]	415.48[bc]	440.76[ab]	457.18[a]	5.67	<0.01

[a] Different letters (a , b and c) within a row indicate the significant difference among different treatments ($P < 0.05$), $n = 8$. [b] Diet treatment: 16% CP, 16% crude protein level diet in 2012 NRC; 12% CP, 12% crude protein level diet; BCAA2 : 1 : 1, 12% CP diet supplemented with branched-chain amino acids to the ratio (Leu : Ile : Val) 2 : 1 : 1; BCAA2 : 2 : 1, 12% CP diet supplemented with branched-chain amino acids to the ratio 2 : 2 : 1; BCAA2 : 1 : 2, 12% CP diet supplemented with branched-chain amino acids to the ratio 2 : 1 : 2.

Table 4 Muscle amino acid content of finishing pigs fed 12% CP diets supplemented with branched-chain amino acids[ab]

Amino acids (AA) (%)	16% CP	12% CP	12% CP			SEM	P
			BCAA2 : 1 : 1	BCAA2 : 2 : 1	BCAA2 : 1 : 2		
Essential AAs							
Histidine	15.00[a]	10.39[b]	14.75[a]	13.56[ab]	12.60[ab]	0.54	0.03
Isoleucine	17.30[a]	12.07[b]	16.10[ab]	17.62[a]	17.93[a]	0.73	0.04
Leucine	38.93[ab]	32.24[b]	42.64[ab]	46.91[a]	46.06[a]	1.99	0.10
Lysine	34.78[a]	21.62[b]	33.09[ab]	37.63[a]	38.70[a]	2.08	0.04
Methionine	18.25[abc]	14.85[c]	15.97[bc]	20.79[ab]	21.78[a]	0.92	0.06
Phenylalanine	29.13	23.53	31.47	33.08	34.20	1.57	0.18
Threonine	46.12[ab]	40.58[b]	49.67[a]	46.40[ab]	48.71[ab]	1.20	0.19
Tryptophan	4.84[ab]	3.44[b]	4.74[ab]	5.33[a]	5.97[a]	0.26	0.04
Valine	32.85[b]	25.59[c]	33.41[b]	30.66[b]	38.46[a]	1.03	<0.01
Nonessential AAs							
Alanine	146.79	138.50	156.81	148.78	144.95	3.44	0.59
Arginine	54.45[bc]	47.99[c]	59.27[ab]	55.47[bc]	66.15[a]	1.71	0.01
Aspartic acid	3.53[ab]	1.38[b]	4.64[ab]	4.94[ab]	5.79[a]	0.55	0.17
Cysteine	5.25	4.95	6.95	5.05	4.98	0.53	0.71
Glutamic acid	51.98[ab]	40.53[b]	51.56[ab]	58.32[a]	60.58[a]	2.28	0.04
Glycine	67.96[ab]	55.81[b]	71.33[ab]	70.41[ab]	85.86[a]	3.25	0.02
Proline	35.62[ab]	31.95[b]	38.52[a]	35.52[ab]	40.53[a]	0.93	0.03
Serine	35.03	31.70	33.31	32.91	31.96	1.37	0.95
Tyrosine	29.22	29.90	27.61	30.74	30.20	0.88	0.85

[a] Different letters (a, b and c) within a row indicate the significant difference among different treatments ($P < 0.05$), $n = 8$. [b] Diet treatment: 16% CP, 16% crude protein level diet in 2012 NRC; 12% CP, 12% crude protein level diet; BCAA2 : 1 : 1, 12% CP diet supplemented with branched-chain amino acids to the ratio (Leu : Ile : Val) 2 : 1 : 1; BCAA2 : 2 : 1, 12% CP diet supplemented with branched-chain amino acids to the ratio 2 : 2 : 1; BCAA2 : 1 : 2, 12% CP diet supplemented with branched-chain amino acids to the ratio 2 : 1 : 2.
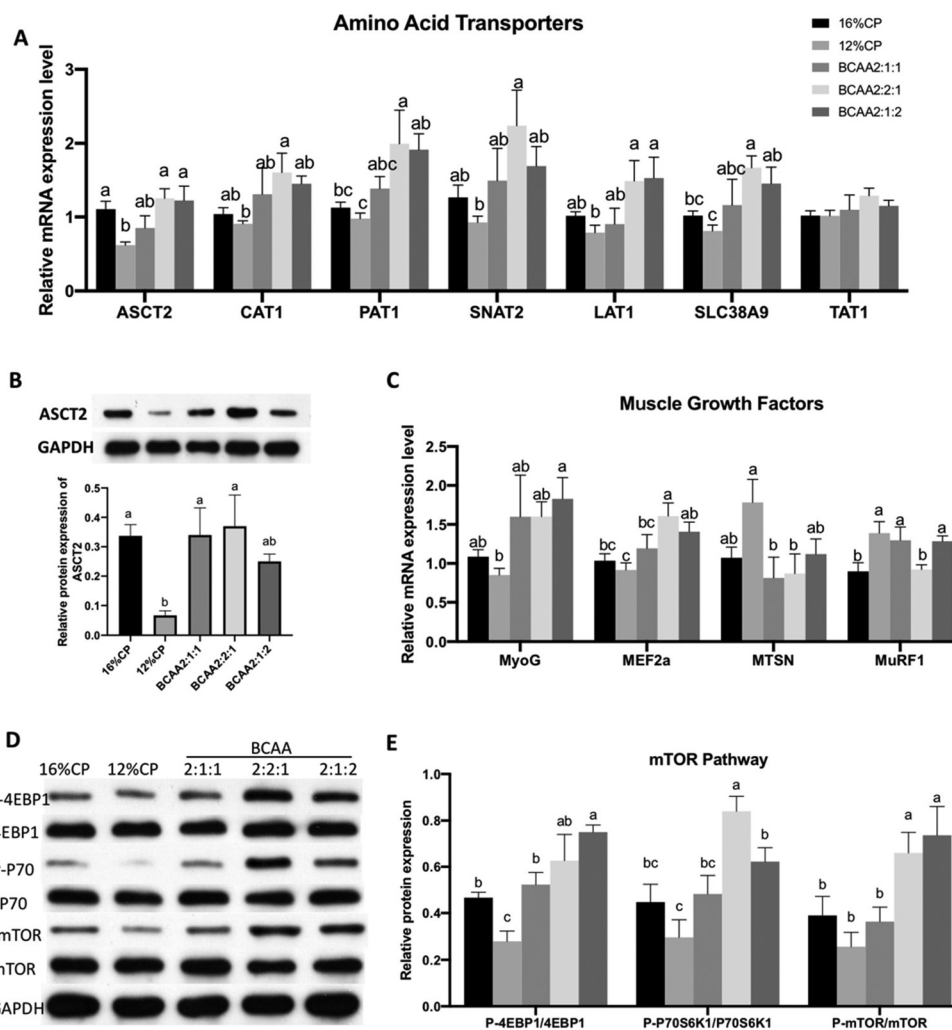
Fig. 3 BCAAs enhanced the expression of skeletal muscle amino acid transporters, growth factors, and the mTOR pathway to promote protein deposition. Relative mRNA levels of amino acid transporters (A) and muscle growth factors (C), and the relative protein levels of ASCT2 (B) and the mTOR pathway (D and E) in the longissimus dorsi muscle of finishing pigs offered five diets. 16% CP, 16% crude protein level diet (NRC, 2012); 12% CP, 12% crude protein level diet; BCAA2:1:1, 12% CP diet supplemented with branched-chain amino acids to the ratio (Leu/Ile/Val) 2:1:1; BCAA2:2:1, 12% CP diet supplemented with branched-chain amino acids to the ratio 2:2:1; BCAA2:1:2, 12% CP diet supplemented with branched-chain amino acids to the ratio 2:1:2. Values with different letters (a–c) indicate a significant difference among different treatments ($P < 0.05$). Values are the mean $\pm$ SEM ($n = 8$).

energy metabolism,[34] and multiple signaling pathways associated with it are involved in regulating mitochondrial function.[35] Therefore, the mitochondrial function-related genes (*OPA1*, *MFN2*, and *PGC-1α*) were analysed. As shown in Fig. 4G, compared to the negative control, the BCAA 2:2:1 group significantly improved the expression of *OPA1*, *MFN2*, and *PGC-1α* ($P < 0.5$), even more than the 16% CP group.

3.5. Protective effects of BCAAs on the composition of fiber types in skeletal muscle

In the skeletal muscle slices stained with H&E, we found that different BCAA ratios improved muscle fiber cross-sectional area to varying degrees. As the cross-sectional area of muscle fiber is associated with the muscle fiber types, therefore, we

further explored the expression of muscle fiber type in the LDM.

Specifically, the cross-sectional area of glycolytic muscle fibers is larger than that of oxidized muscle fibers. Since glycolytic fibers contain a larger number of glycogen (MG) and glycolytic enzymes, such as lactate dehydrogenase (LDH) and hexokinase (HK), and rely more on glycolysis to generate ATP than do oxidative fibers, which prefer oxidative phosphorylation, we detected the content of glycogen and activity of biochemical enzymes in the LDM. As shown in Fig. 4, compared to the 12% CP group, the BCAA 2:2:1 group significantly increased the abundance of MG, HK, and LDH ($P < 0.5$), even exceeding the 16% CP group. On the other hand, we detected citrate synthase (CS) in the LDM, which is abundant in oxidative fibers. The results showed that compared to the positive control, the
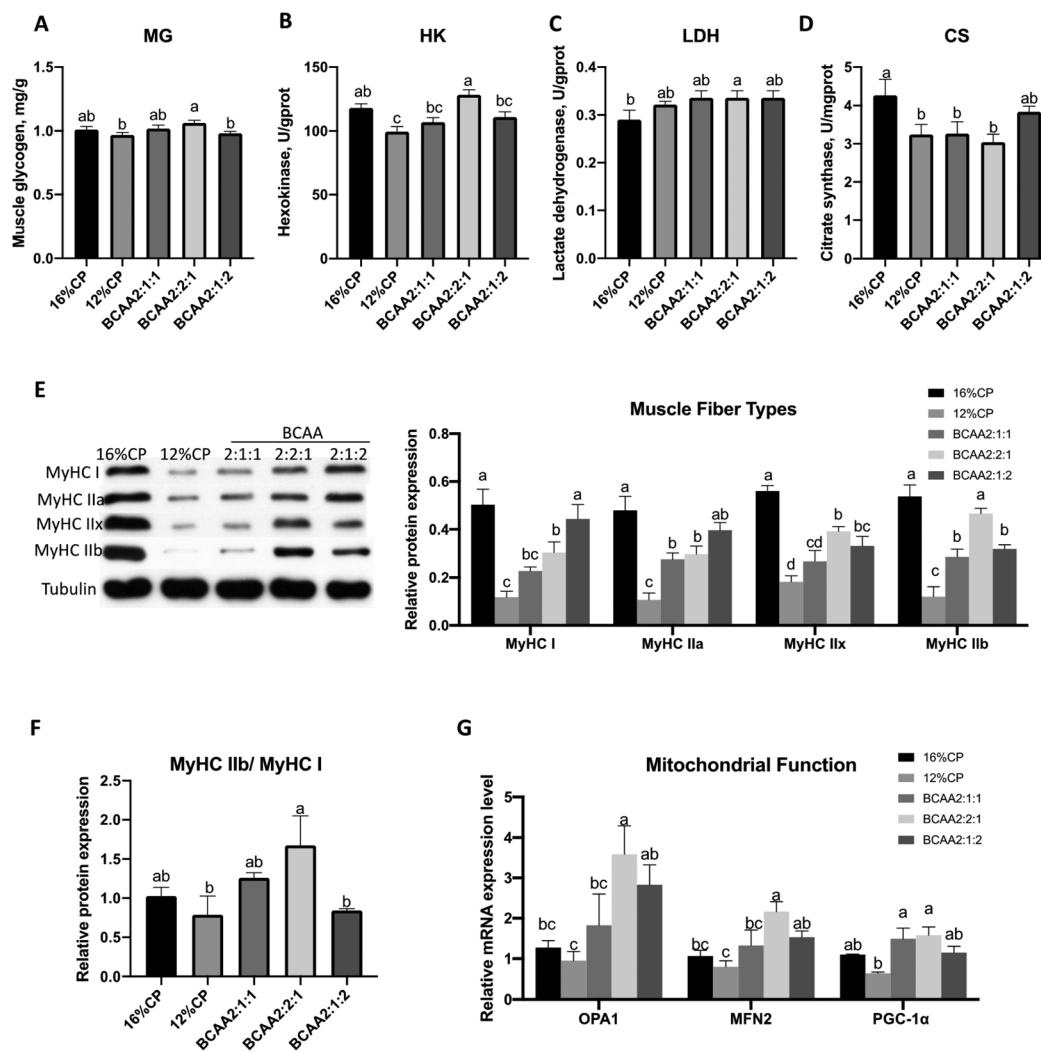
Fig. 4 Protective effects of BCAAs on the composition of fiber types and mitochondrial function in skeletal muscle. Activities of muscle biochemical indicators for muscle glycogen (A), hexokinase (B), lactate dehydrogenase (C), and citrate synthase (D), and the relative protein levels of muscle fiber types (E), the ratio of the relative protein level of MyHC IIb to the relative protein level of MyHC I (F), and the relative mRNA levels of mitochondrial function-related genes (G) in the longissimus dorsi muscle of finishing pigs offered five diets. 16% CP, 16% crude protein level diet (NRC, 2012); 12% CP, 12% crude protein level diet; BCAA2 : 1 : 1, 12% CP diet supplemented with branched-chain amino acids to the ratio (Leu/Ile/Val) 2 : 1 : 1; BCAA2 : 2 : 1, 12% CP diet supplemented with branched-chain amino acids to the ratio 2 : 2 : 1; BCAA2 : 1 : 2, 12% CP diet supplemented with branched-chain amino acids to the ratio 2 : 1 : 2. Values with different letters (a–d) indicate a significant difference among different treatments ($P <$ 0.05). Values are the mean $\pm$ SEM ($n = 8$).

BCAA 2 : 2 : 1 group also decreased the abundance of CS significantly ($P < 0.5$).

Then we further detected the protein expression of MyHC I, MyHC IIa, MyHC IIx, and MyHC IIb by western blotting; the results showed that compared to the 12% CP group, the three BCAA groups significantly increased the expression of four muscle fiber types ($P < 0.5$). Among them, compared to the 12% CP group, BCAA 2 : 1 : 2 significantly increased the expression of MyHC I the most, while BCAA 2 : 2 : 1 significantly increased the expression of MyHC IIb the most, even similar to the 16% CP group (Fig. 4E). Moreover, we calculated the ratio of expression of MyHC IIb to MyHC I. Among the five groups, the ratio of BCAA 2 : 2 : 1 was the highest (Fig. 4F), wherein the ratio varied the most for the composition of muscle fibers.

4. Discussion

In the present study, we found that, through activating the mTOR pathway, balanced BCAAs, especially the ratio of 2 : 2 : 1, upregulate the expression of AA transporters and muscle growth factors, simultaneously modulating mitochondrial function and muscle fiber type composition, thereby inhibiting inflammation, regulating amino acid metabolism, increasing skeletal muscle deposition, and ultimately combating aging effectively.

BCAA supplementation significantly reversed limited loin eye areas, lean weight, and the lean mass ratio caused by low protein diet, indicating that BCAAs benefit a higher skeletal muscle mass. Not only the carcass traits, skeletal muscle his-

tology and crude protein content showed the same trend. In terms of muscle histology, the determination of the number of muscle fibers mainly occurs during the pregnancy of the mother, thus the total number of muscle fibers changes slightly after birth. Correspondingly, the cross-sectional area of muscle fibers is basically unchanged during pregnancy, but it will increase rapidly after birth, regulated by the nutrition. Therefore, for the growth of muscle fibers, the regulation of the cross-sectional area of skeletal muscle fibers is of great significance. The increase in the cross-sectional area of muscle fibers is directly related to the increase in protein deposition, both of which can be regulated by the BCAA intake.

The impact of dietary nutrition on energy metabolism is directly reflected in the changes of serum biochemical indicators. Serum CREA is a breakdown product of creatine phosphate derived from skeletal muscle, which is usually produced at a relatively constant rate by the body depending on the absolute amount of muscle mass,[36] thus it can ubiquitously serve as a well-established biomarker of skeletal muscle mass in human subjects.[37] Our results showed that dietary BCAAs enhanced the activity of CREA, indicating a higher muscle mass, which is consistent with the result of the carcass traits. ALP is an important indicator of calcium and phosphorus metabolism in osteoblasts, whose activity can reflect the active state of osteoblasts. Our study found that the serum ALP activity of BCAAs was higher than that in the control group, suggesting that dietary BCAAs promoted the activity of osteoblasts, strengthened the bone formation process, and resisted the symptoms of osteoporosis during the aging process. Another two important serum metabolites, AST and ALT, play important roles in transamination, reflecting the synthesis and decomposition of proteins. Our results showed that dietary BCAAs enhanced the activity of ALT, thereby improving amino acid metabolism.

The decline in the immune system is one of the signs of aging, and maintaining optimal immune activation is the best way to delay the aging process. During the aging process, there will be a chronic inflammatory state, that is, inflamm-aging.[38] It is usually accompanied by an abnormal increase in the systemic specific inflammatory response and a decrease in anti-inflammatory response ability, and is closely associated with the occurrence and development of many age-related diseases, such as Alzheimer's disease, atherosclerosis, Parkinson's disease, and osteoporosis. It is found that pro-inflammatory cytokines play a central role in the inflammatory senescence induced by chronic and imbalanced inflammation.[39] IL-6 is considered to be the inflammatory response factor most strongly associated with senile diseases and physical disability, and its serum level can be used as a predictor of inflamma-ging.[40] TNF-α is also an important pro-inflammatory factor that can promote local and systemic inflammatory responses.[41] The elevated serum levels of IL-6 and TNF-α in the elderly are related to disease, disability and mortality. Surprisingly, though low-protein diets increased the serum IL-6 and TNF-α, BCAA supplementation reversed it. Besides, TNF-α also has an important effect on muscle protein syn-

thesis, which may impair muscle protein synthesis by inactivating protein phosphorylation in the intracellular signal transduction pathway of mTOR. Thus, low abundance of TNF-α in BCAA supplementation may contribute to protein deposition.

Protein metabolism is regulated by the concentration of muscle free AAs, which can reflect the nutritional status, and is closely related to the biological function of tissues. On the other hand, the distribution of free AAs in serum reflects the total metabolic flow of nutrients and their metabolites from all tissues and organs.[42] The composition of serum AAs is affected by a variety of factors, including the synthesis and degradation of protein, as well as the transport and metabolism of amino acids. Our results showed that serum free AA levels of BCAAs supplemented to a low protein diet (12% CP) were similar to those of the positive control (16% CP), which is consistent with the result of the carcass traits and muscle histology. That is to say, dietary BCAAs regulated protein metabolism in the muscle tissue. The possible mechanism is based on a change in the composition of AAs available in the serum, which acts as a signal to initiate the protein synthesis process in muscles. Among the three BCAAs, leucine is the main nitrogen source for glutamine synthesis in muscle tissues. It is reported that a low protein diet may lead to a deficiency of BCAAs, especially Ile and Val.[28] In this experiment, the content of most AAs in the muscles under a low-protein diet was detected to have decreased, while balanced BCAA supplementation, especially the ratio of $2:2:1$, and $2:1:2$, reversed most of the reduction trend, which suggested that an appropriate BCAA ratio compensated for the deficiency of Ile or Val, thus promoting the synthesis of proteins and affecting the growth and development of muscle deposition.

The concentration of free AAs in skeletal muscles is associated with the level of the AA transporters. Before playing a protein regulatory role, BCAAs must first enter the cell under the activity of the coordinated AA transporters. At the same time, as 'transceptors', they sense the availability of AAs, transmit nutritional signals, and control the absorption and outflow of AAs, thus triggering a series of cascading responses that display dual transmitter and receptor functions in protein metabolism.[28] The expression of AA transporters in skeletal muscle is related to the interaction between cell growth regulation and AA availability. Indeed, ASCT2 (also known as SLC1A5), CAT1 (also known as SLC7A1), SNAT2 (also known as SLC38A2), LAT1 (also known as SLC7A5), PAT1 (also known as SLC36A1), and SLC38A9 have been reported to exhibit the characteristic of transceptors and participate in the activation of mTOR complex 1 (mTORC1).[43–45] In the current study, BCAAs are absorbed by AA transporters, thus the expression levels of AA transporters are increased apparently by a dietary balanced BCAA ratio of $2:2:1$. This indicated that an appropriate ratio of dietary BCAAs may increase its bioavailability by increasing the activity of those amino acid transporters. In addition, the altered trend of AA transporter abundances was almost similar to that of free AAs in skeletal muscles, which

reaffirms that an appropriate BCAA ratio can compensate for the deficiency of Ile or Val. Therefore, a BCAA ratio of 2 : 2 : 1 in low-protein diets appear to enhance the activity of AA transporters, thereby promoting protein metabolism and skeletal muscle mass.

High abundances of AA transporters are involved with the overactivation of the nutrient sensing signaling pathway, mTOR.[46] Indeed, BCAAs are not only the substrate of protein synthesis, but also participate as a signaling molecule in the regulation of mTOR, which can be activated by certain AAs to stimulate protein synthesis. In addition, mTOR activation also inhibits proteolysis and autophagy, preventing the breakdown of muscle protein that is being synthesized. In general, the effects of dietary BCAAs on protein synthesis were attributed to the direct regulation of the AA dependent mTOR signaling pathway. The mTOR signaling pathway can sense changes in upstream amino acids, growth factors, energy and environmental pressure, activating the downstream effectors P70S6K1 and 4EBP1, thereby regulating protein metabolism and muscle growth. In the present study, the expressions of mTOR and its two key downstream effectors (P70S6K1 and 4EBP1) in muscle were affected by dietary protein levels and the supplementation of a balanced BCAA ratio, where the ratios of 2 : 2 : 1 and 2 : 1 : 2 performed the best. Collectively, supplementation of balanced BCAAs substantially activated the mTOR pathway, thus increasing muscle deposition and resisting the aging process, which seemed to be linked with the higher abundances of AAs and AA transporters in skeletal muscle.

Meanwhile, the expression of myogenic regulatory factors, including MyoG, MEF2a, MSTN, and MuRF1, indicate the growth and development of muscle fibers. The synergies between MyoG and MEF2a exert a major role in processing myogenesis, whose expression levels have been viewed as an indicator of muscle development. In contrast, MSTN is a negative mediator of skeletal muscle mass. Recent high-profile studies have reported MSTN regulated muscle regeneration effects and may play a regulatory role in aging.[47] Besides, MuRF1 play a crucial role in muscle protein degradation, which may be a key factor in the negative modulation of muscle mass. In the present study, the altered trend in muscle growth factor expression was similar to the change in the abundances of free AA compositions, AA transporters, and mTOR signal activation. Therefore, we surmise that low-protein diets supplemented with balanced BCAAs may increase muscle fiber growth and decrease muscle protein degradation, which was in accordance with the activation of the mTOR pathway, even exerting an anti-aging role.

Not only increasing skeletal muscle mass, our healthy-aging strategies also considered the composition of muscle fiber types in skeletal muscles. The structural changes in the aging process not only include a reduction in muscle mass and muscle fibers, but also a shift of muscle fibers toward MyHC I fibers.[6] Therefore, the nutritional strategy of MyHC plasticity also exerts a key role in the healthy-aging of skeletal muscle. Different skeletal muscle fiber types preferentially rely on different metabolic pathways to meet their energy requirements, thus showing signs of oxidative or glycolytic metabolism, the two main energy metabolism pathways in skeletal muscle to obtain ATP.[48] Indeed, skeletal muscle comprises a spectrum of fast-twitch glycolytic fibers (MyHC IIb), which use glycogen as the main source of energy for anaerobic metabolism to fuel short and intense activity, and slow-twitch oxidative fibers (MyHC I), which are used for prolonged low-intensity activity driven primarily by fuels such as blood glucose and fatty acids. Glycolytic fibers contain a larger number of glycogen (MG) and glycolytic enzymes, and rely more on glycolysis to generate ATP than do oxidative fibers, which prefer oxidative phosphorylation. In mammals, MG is utilized locally to help fuel muscular activity, while over-accumulation of skeletal muscle glycogen affects oxidative metabolism, leading to functional impairment and metabolic rearrangement.[49] In addition, the remodeling of diverse muscle fiber types is essential for muscular energy metabolism and activities of glycolytic enzymes, such as LDH and HK, which can indirectly alter the muscle fiber type composition.[50] Besides, it is reported that slow-twitch oxidative muscle fibers have higher oxidative enzyme activity, such as CS, the first enzyme in the tricarboxylic acid cycle, and plays a key role in energy metabolism. In the current study, we found that though low-protein diets decreased the activity of glycolytic enzymes, BCAA supplementation reversed it, especially the ratio of 2 : 2 : 1, which seemed like to be a balanced ratio. In addition, our western blotting results showed a similar trend, where the dietary BCAA ratio of 2 : 2 : 1 showed a high expression of glycolytic fibers, and seemed to help with healthy-aging.

The mitochondrion also plays a crucial and proactive role in healthy-aging.[33] To further understand the molecular basis of how healthy-aging effects induced by BCAAs were activated, the mitochondrial function-related genes (*OPA1*, *MFN2*, and *PGC-1α*) were analyzed. It was reported that the expression of OPA1 and MFN2 in skeletal muscle decreased in elderly skeletal muscle,[51,52] and can be rescued by increasing mitochondrial mass through overexpression of the mitochondrial biogenesis activator PGC-1α.[20] Combined with a previous study, we suggest that BCAA supplementation increased the expression of mitochondrial function-related genes, especially the ratio of 2 : 2 : 1, consequently exerting an anti-aging role. Since the trend in mitochondrial expression is consistent with that of muscle fiber composition, we speculated that a balanced BCAA ratio modulated the composition of muscle fiber through the regulation of mitochondrial function, which may be regulated by the mTOR signaling pathway[53] and required further study. Overall, although both BCAA 2 : 2 : 1 and 2 : 1 : 2 ratios showed the advantage of increasing skeletal muscle mass, the ratio of 2 : 2 : 1 significantly promoted the expression of glycolytic fibers, which may be more beneficial for anti-aging. Therefore, it is recommended to balance BCAA at 2 : 2 : 1 as a potential nutritional strategy for healthy aging.
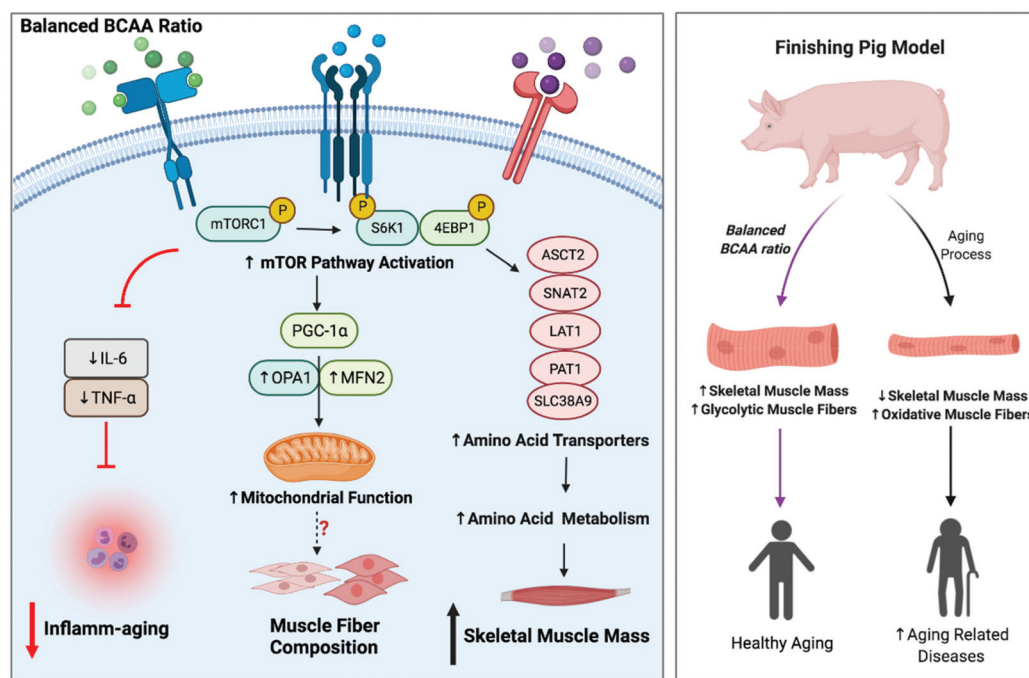
Fig. 5 Graphical representation of the anticipated mechanism of the effects of a balanced BCAA ratio on the healthy aging process of skeletal muscle in a finishing pig model. A balanced BCAA ratio (Leu : Ile : Val = 2 : 2 : 1) significantly activated the mTOR pathway and upregulated the expression of amino acid transporters, such as ASCT2, SNAT2, LAT1, PAT1, and SLC38A9, simultaneously modulating mitochondrial function and the muscle fiber type composition, thereby inhibiting inflammatory cytokines, such as IL-6 and TNF-α, regulating amino acid metabolism, and ultimately increasing skeletal muscle mass.

5. Conclusion

In conclusion, a balanced BCAA ratio (Leu : Ile : Val = 2 : 2 : 1) activates the mTOR pathway to upregulate the expression of AA transporters, resulting in increasing skeletal muscle mass, as well as contributing to the composition of glycolytic muscle fibers, exerting healthy-aging roles in skeletal muscles, which may be linked with higher mitochondrial function (Fig. 5). Therefore, our results suggest that a BCAA ratio around 2 : 2 : 1 could potentially be used for healthy aging.

Conflicts of interest

The authors have no conflicts of interest to declare.

Acknowledgements

References

1 V. Fanjul, I. Jorge, E. Camafeita, A. Macias, C. Gonzalez-Gomez, A. Barettino, B. Dorado, M. J. Andres-Manzano, J. Rivera-Torres, J. Vazquez, C. Lopez-Otin and V. Andres, Identification of common cardiometabolic alterations and deregulated pathways in mouse and pig models of aging, *Aging Cell*, 2020, **19**, e13203.

2 G. Das, S. Paramithiotis, B. Sundaram Sivamaruthi, C. H. Wijaya, S. Suharta, N. Sanlier, H. S. Shin and J. K. Patra, Traditional fermented foods with anti-aging effect: A concentric review, *Food Res. Int.*, 2020, **134**, 109269.

3 D. G. Le Couteur, S. M. Solon-Biet, V. C. Cogger, R. Ribeiro, R. de Cabo, D. Raubenheimer, G. J. Cooney and S. J. Simpson, Branched chain amino acids, aging and age-related health, *Ageing Res. Rev.*, 2020, **64**, 101198.

4 L. B. Verdijk, R. Koopman, G. Schaart, K. Meijer, H. H. C. M. Savelberg and L. J. C. van Loon, Satellite cell content is specifically reduced in type II skeletal muscle fibers in the elderly, *Am. J. Physiol.: Endocrinol. Metab.*, 2007, **292**, E151–E157.

5   A. J. Cruz-Jentoft and A. A. Sayer, Sarcopenia, *Lancet*, 2019, **393**, 2590.

6   K. S. Nair, Aging muscle, *Am. J. Clin. Nutr.*, 2005, **81**, 953–963.

7   L. Larsson, B. Sjodin and J. Karlsson, Histochemical and Biochemical Changes in Human Skeletal-Muscle with Age in Sedentary Males, Age 22-65 Years, *Acta Physiol. Scand.*, 1978, **103**, 31–39.

8   X. Chen, Y. Guo, G. Jia, H. Zhao, G. Liu and Z. Huang, Ferulic acid regulates muscle fiber type formation through the Sirt1/AMPK signaling pathway, *Food Funct.*, 2019, **10**, 259–265.

9   K. Dideriksen, S. Reitelseder and L. Holm, Influence of Amino Acids, Dietary Protein, and Physical Activity on Muscle Mass Development in Humans, *Nutrients*, 2013, **5**, 852–876.

10   W. K. Mitchell, D. J. Wilkinson, B. E. Phillips, J. N. Lund, K. Smith and P. J. Atherton, Human Skeletal Muscle Protein Metabolism Responses to Amino Acid Nutrition, *Adv. Nutr.*, 2016, **7**, 828S–838S.

11   E. Volpi, H. Kobayashi, M. Sheffield-Moore, B. Mittendorfer and R. R. Wolfe, Essential amino acids are primarily responsible for the amino acid stimulation of muscle protein anabolism in healthy elderly adults, *Am. J. Clin. Nutr.*, 2003, **78**, 250–258.

12   Y. T. Shih, T. N. Huang, H. T. Hu, T. L. Yen and Y. P. Hsueh, Vcp Overexpression and Leucine Supplementation Increase Protein Synthesis and Improve Fear Memory and Social Interaction of Nf1 Mutant Mice, *Cell Rep.*, 2020, **31**, 107835.

13   C. J. Fuchs, W. J. H. Hermans, A. M. Holwerda, J. S. J. Smeets, J. M. Senden, J. van Kranenburg, A. P. Gijsen, W. K. H. W. Wodzig, H. Schierbeek, L. B. Verdijk and L. J. C. van Loon, Branched-chain amino acid and branched-chain ketoacid ingestion increases muscle protein synthesis rates in vivo in older adults: a double-blind, randomized trial, *Am. J. Clin. Nutr.*, 2019, **110**, 862–872.

14   J. Chen, Y. Ou, R. Luo, J. Wang, D. Wang, J. Guan, Y. Li, P. Xia, P. R. Chen and Y. Liu, SAR1B senses leucine levels to regulate mTORC1 signalling, *Nature*, 2021, **596**, 281–284.

15   T. Pearson, O. Wendowski and P. P. Powell, Enhanced small neutral but not branched chain amino acid transport after epigenetic sodium coupled neutral amino acid transporter-2 (SNAT2) cDNA expression in myoblasts, *J. Cachexia Sarcopenia Muscle*, 2021, **12**, 811–822.

16   Y. Yamamoto, R. Sawa, I. Wake, A. Morimoto and Y. Okimura, Glucose-mediated inactivation of AMP-activated protein kinase reduces the levels of L-type amino acid transporter 1 mRNA in C2C12 cells, *Nutr. Res.*, 2017, **47**, 13–20.

17   A. R. Konopka, B. Jemiolo, S. W. Trappe and M. P. Harber, Myosin Heavy Chain Plasticity in Aging Skeletal Muscle with Aerobic Exercise Training, *J. Gen. Intern. Med.*, 2012, **27**, 291–291.

18   X. Sun and M. B. Zemel, Leucine modulation of mitochondrial mass and oxygen consumption in skeletal muscle cells and adipocytes, *Nutr. Metab.*, 2009, **6**, 26.

19   A. Morio, R. Tsutsumi, T. Kondo, H. Miyoshi, T. Kato, S. Narasaki, S. Satomi, E. Nakaya, M. Kuroda, H. Sakaue, T. Kitamura and Y. M. Tsutsumi, Leucine induces cardio-protection in vitro by promoting mitochondrial function via mTOR and Opa-1 signaling, *Nutr., Metab. Cardiovasc. Dis.*, 2021, **31**, 2979–2986.

20   C. E. Fealy, L. Grevendonk, J. Hoeks and M. K. C. Hesselink, Skeletal muscle mitochondrial network dynamics in metabolic disorders and aging, *Trends Mol. Med.*, 2021, **27**, 1033–1044.

21   L. Genton, M. Pruijm, D. Teta, I. Bassi, P. D. Cani, N. Gaia, F. R. Herrmann, N. Marangon, J. Mareschal, G. G. Muccioli, C. Stoermann, F. Suriano, A. Wurzner-Ghajarzadeh, V. Lazarevic and J. Schrenzel, Gut barrier and microbiota changes with glycine and branched-chain amino acid supplementation in chronic haemodialysis patients, *J. Cachexia Sarcopenia Muscle*, 2021, **12**, 1527–1539.

22   I. Buondonno, F. Sassi, G. Carignano, F. Dutto, C. Ferreri, F. G. Pili, M. Massaia, E. Nisoli, C. Ruocco, P. Porrino, C. Ravetta, C. Riganti, G. C. Isaia and P. D'Amelio, From mitochondria to healthy aging: The role of branched-chain amino acids treatment: MATeR a randomized study, *Clin. Nutr.*, 2020, **39**, 2080–2091.

23   G. D'Antona, M. Ragni, A. Cardile, L. Tedesco, M. Dossena, F. Bruttini, F. Caliaro, G. Corsetti, R. Bottinelli, M. O. Carruba, A. Valerio and E. Nisoli, Branched-chain amino acid supplementation promotes survival and supports cardiac and skeletal muscle mitochondrial biogenesis in middle-aged mice, *Cell Metab.*, 2010, **12**, 362–372.

24   H. Wei, Y. L. Shi, Y. Cheng, Y. F. Dong, R. Miao, W. Cao and J. Q. Qiu, Bcaa Alleviates Rats Skeletal Muscle Protein Degradation After Eccentric Exercise By Reducing KLF15 Expression, *Med. Sci. Sports Exercise*, 2021, **53**, 270–270.

25   K. Matsumoto, M. Mizuno, T. Mizuno, B. Dilling-Hansen, A. Lahoz, V. Bertelsen, H. Munster, H. Jordening, K. Hamada and T. Doi, Effect of BCAA intake during exercise on skeletal muscle protein metabolism in young individuals, *Med. Sci. Sports Exercise*, 2004, **36**, S194–S194.

26   L. Zhang, F. Li, Q. Guo, Y. Duan, W. Wang, Y. Yang, Y. Yin, S. Gong, M. Han and Y. Yin, Different Proportions of Branched-Chain Amino Acids Modulate Lipid Metabolism in a Finishing Pig Model, *J. Agric. Food Chem.*, 2021, **69**, 7037–7048.

27   A. Hiraoka, M. Hirooka, M. Abe, T. Ninomiya, Y. Hiasa and K. Michitaka, Efficacy of Branched-Chain Amino Acid Supplementation and Walking Exercise for Preventing Sarcopenia in Patients with Liver Cirrhosis, *Hepatology*, 2018, **68**, 1162a.

28   Y. Duan, Q. Guo, C. Wen, W. Wang, Y. Li, B. Tan, F. Li and Y. Yin, Free Amino Acid Profile and Expression of Genes Implicated in Protein Metabolism in Skeletal Muscle of Growing Pigs Fed Low-Protein Diets Supplemented with

Branched-Chain Amino Acids, *J. Agric. Food Chem.*, 2016, **64**, 9390–9400.

29 C. Lanzas, P. Ayscue, R. Ivanek and Y. T. Grohn, Model or meal? Farm animal populations as models for infectious diseases of humans, *Nat. Rev. Microbiol.*, 2010, **8**, 139–148.

30 M. M. Swindle, A. Makin, A. J. Herron, F. J. Clubb and K. S. Frazier, Swine as Models in Biomedical Research and Toxicology Testing, *Vet. Pathol.*, 2012, **49**, 344–356.

31 E. R. Miller, The Pig As A Model For Human Nutrition, *Annu. Rev. Nutr.*, 1987, **7**, 361–382.

32 H. Ellegren, B. P. Chowdhary, M. Johansson, L. Marklund and L. Andersson, A primary linkage map of the porcine genome reveals a low rate of genetic recombination, *Genetics*, 1994, **137**, 1089–1100.

33 C. E. Fealy, L. Grevendonk, J. Hoeks and M. K. C. Hesselink, Skeletal muscle mitochondrial network dynamics in metabolic disorders and aging, *Trends Mol. Med.*, 2021, **27**, 1033–1044.

34 N. Shimizu, N. Yoshikawa, N. Ito, T. Maruyama, Y. Suzuki, S. Takeda, J. Nakae, Y. Tagata, S. Nishitani, K. Takehana, M. Sano, K. Fukuda, M. Suematsu, C. Morimoto and H. Tanaka, Crosstalk between Glucocorticoid Receptor and Nutritional Sensor mTOR in Skeletal Muscle, *Cell Metab.*, 2011, **13**, 170–182.

35 A. Ramanathan and S. L. Schreiber, Direct control of mitochondrial function by mTOR, *Proc. Natl. Acad. Sci. U. S. A.*, 2009, **106**, 22229–22232.

36 S. B. Heymsfield, C. Arteaga, C. Mcmanus, J. Smith and S. Moffitt, Measurement of Muscle Mass in Humans – Validity of the 24-Hour Urinary Creatinine Method, *Am. J. Clin. Nutr.*, 1983, **37**, 478–494.

37 S. S. Patel, M. Z. Molnar, J. A. Tayek, J. H. Ix, N. Noori, D. Benner, S. Heymsfield, J. D. Kopple, C. P. Kovesdy and K. Kalantar-Zadeh, Serum creatinine as a marker of muscle mass in chronic kidney disease: results of a cross-sectional study and review of literature, *J. Cachexia Sarcopenia Muscle*, 2013, **4**, 19–29.

38 C. Franceschi, M. Bonafe, S. Valensin, F. Olivieri, M. De Luca, E. Ottaviani and G. De Benedictis, Inflamm-aging. An evolutionary perspective on immunosenescence, *Ann. N. Y. Acad. Sci.*, 2000, **908**, 244–254.

39 F. Olivieri, M. Bonafe, S. Giovagnetti, R. Stecconi, M. Cardelli, L. Cavallone, L. Spazzafumo, F. Marchegiani, G. Carrieri, E. Mugianesi, C. Giampieri, M. Centurelli, R. Moresi, S. Tesei, R. Lisa, C. Viticchi, L. Falsetti, S. Salvioli and C. Franceschi, In vitro IL-6 production by EBV-immortalized B lymphocytes from young and elderly people genotyped for -174 C/G polymorphism in IL-6 gene: a model to study the genetic basis of inflamm-aging, *Mech. Ageing Dev.*, 2003, **124**, 549–553.

40 D. Di Bona, S. Vasto, C. Capurso, L. Christiansen, L. Deiana, C. Franceschi, M. Hurme, E. Mocchegiani, M. Rea, D. Lio, G. Candore and C. Caruso, Effect of interleukin-6 polymorphisms on human longevity: A systematic review and meta-analysis, *Ageing Res. Rev.*, 2009, **8**, 36–42.

41 M. De Martinis, C. Franceschi, D. Monti and L. Ginaldi, Inflamm-ageing and lifelong antigenic load as major determinants of ageing rate and longevity, *FEBS Lett.*, 2005, **579**, 2035–2039.

42 H. Garcia-Villalobos, A. Morales-Trejo, B. A. Araiza-Pina, J. K. Htoo and M. Cervantes-Ramirez, Effects of dietary protein and amino acid levels on the expression of selected cationic amino acid transporters and serum amino acid concentration in growing pigs, *Arch. Anim. Nutr.*, 2012, **66**, 257–270.

43 S. Wang, Z. Y. Tsun, R. L. Wolfson, K. Shen, G. A. Wyant, M. E. Plovanich, E. D. Yuan, T. D. Jones, L. Chantranupong, W. Comb, T. Wang, L. Bar-Peled, R. Zoncu, C. Straub, C. Kim, J. Park, B. L. Sabatini and D. M. Sabatini, Metabolism. Lysosomal amino acid transporter SLC38A9 signals arginine sufficiency to mTORC1, *Science*, 2015, **347**, 188–194.

44 R. Zoncu, L. Bar-Peled, A. Efeyan, S. Y. Wang, Y. Sancak and D. M. Sabatini, mTORC1 Senses Lysosomal Amino Acids Through an Inside-Out Mechanism That Requires the Vacuolar H$^+$-ATPase, *Science*, 2011, **334**, 678–683.

45 J. W. Jung, S. J. Y. Macalino, M. Cui, J. E. Kim, H. J. Kim, D. G. Song, S. H. Nam, S. Kim, S. Choi and J. W. Lee, Transmembrane 4 L Six Family Member 5 Senses Arginine for mTORC1 Signaling, *Cell Metab.*, 2019, **29**, 1306–1319.

46 L. Y. Zhang, Y. H. Duan, Q. P. Guo, W. L. Wang and F. N. Li, A selectively suppressing amino acid transporter: Sodium-coupled neutral amino acid transporter 2 inhibits cell growth and mammalian target of rapamycin complex 1 pathway in skeletal muscle cells, *Anim. Nutr.*, 2020, **6**, 513–520.

47 X. L. Fan, U. Gaur, L. Sun, D. Y. Yang and M. Y. Yang, The Growth Differentiation Factor 11 (GDF11) and Myostatin (MSTN) in tissue specific aging, *Mech. Ageing Dev.*, 2017, **164**, 108–112.

48 P. A. Dutchak, S. J. Estill-Terpack, A. A. Plec, X. Zhao, C. Yang, J. Chen, B. Ko, R. J. Deberardinis, Y. Yu and B. P. Tu, Loss of a Negative Regulator of mTORC1 Induces Aerobic Glycolysis and Altered Fiber Composition in Skeletal Muscle, *Cell Rep.*, 2018, **23**, 1907–1914.

49 G. Testoni, J. Duran, M. Garcia-Rocha, F. Vilaplana, A. L. Serrano, D. Sebastian, I. Lopez-Soldado, M. A. Sullivan, F. Slebe, M. Vilaseca, P. Munoz-Canoves and J. J. Guinovart, Lack of Glycogenin Causes Glycogen Accumulation and Muscle Function Impairment, *Cell Metab.*, 2017, **26**, 256–266.e254.

50 M. Xu, X. Chen, Z. Huang, D. Chen, M. Li, J. He, H. Chen, P. Zheng, J. Yu, Y. Luo and B. Yu, Effects of dietary grape seed proanthocyanidin extract supplementation on meat quality, muscle fiber characteristics and antioxidant capacity of finishing pigs, *Food Chem.*, 2021, **367**, 130781.

51 C. Tezze, V. Romanello, M. A. Desbats, G. P. Fadini, M. Albiero, G. Favaro, S. Ciciliot, M. E. Soriano, V. Morbidoni, C. Cerqua, S. Loefler, H. Kern, C. Franceschi, S. Salvioli, M. Conte, B. Blaauw, S. Zampieri, L. Salviati, L. Scorrano and M. Sandri, Age-Associated Loss of OPA1 in Muscle Impacts Muscle Mass, Metabolic Homeostasis,

Systemic Inflammation, and Epithelial Senescence, *Cell Metab.*, 2017, **25**, 1374–1389.

52 E. Marzetti, R. Calvani, M. Lorenzi, F. Tanganelli, A. Picca, M. Bossola, A. Menghi, R. Bernabei and F. Landi, Association between myocyte quality control signaling and sarcopenia in old hip-fractured patients: Results from the Sarcopenia in HIp FracTure (SHIFT) exploratory study, *Exp. Gerontol.*, 2016, **80**, 1–5.

53 J. S. You, K. Kim, N. D. Steinert, J. Chen and T. A. Hornberger, mTORC1 mediates fiber type-specific regulation of protein synthesis and muscle size during denervation, *Cell Death Discovery*, 2021, **7**, 74.