

# Supplementation with branched-chain amino acids to a low-protein diet regulates intestinal expression of amino acid and peptide transporters in weanling pigs

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**Abstract** This study determined the effects of dietary branched-chain amino acids (AA) (BCAA) on growth performance, expression of jejunal AA and peptide transporters, and the colonic microflora of weanling piglets fed a low-protein (LP) diet. One hundred and eight Large White × Landrace × Duroc piglets (weaned at 28 days of age) were fed a normal protein diet (NP, 20.9 % crude protein), an LP diet (LP, 17.1 % crude protein), or an LP diet supplemented with BCAA (LP + BCAA, 17.9 % crude protein) for 14 days. Dietary protein restriction reduced piglet growth performance and small-intestinal villous height, which were restored by BCAA supplementation to the LP diet to values for the NP diet. Serum concentrations of BCAA were reduced in piglets fed the LP diet while those in piglets fed the LP + BCAA diet were similar to values for the NP group. mRNA levels for Na<sup>+</sup>-neutral AA exchanger-2, cationic AA transporter-1, b<sup>0,+</sup> AA transporter, and 4F2 heavy chain were more abundant in piglets fed the LP + BCAA diet than the LP diet. However, mRNA and protein levels for peptide

transporter-1 were lower in piglets fed the LP + BCAA diet as compared to the LP diet. The colonic microflora did not differ among the three groups of pigs. In conclusion, growth performance, intestinal development, and intestinal expression of AA transporters in weanling piglets are enhanced by BCAA supplementation to LP diets. Our findings provide a new molecular basis for further understanding of BCAA as functional AA in animal nutrition.

**Keywords** Intestinal morphology · Functional amino acids · Gut microflora · Swine

## Abbreviations

ADFI	Average daily feed intake
ADG	Average daily gain of body weight
ASCT2	Na <sup>+</sup> -neutral AA exchanger 2
BCAA	Branched-chain amino acids
B <sup>0</sup> AT1	System B <sup>0</sup> neutral AA transporter
CAT1	Cationic amino acid transporter 1
CP	Crude protein
DGGE	Denaturing gradient gel electrophoresis
4F2hc	4F2 heavy chain
LP	Low protein
mTOR	Mammalian target of rapamycin
NP	Normal protein
PepT-1	Peptide transporter 1
rBAT	Related to b <sup>0,+</sup> amino acid transporter
RT-PCR	Real-time polymerase chain reaction
VFA	Volatile fatty acids

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## Introduction

Diarrhea in neonatal and early weaned piglets caused high mortality and morbidity and impaired the growth

performance and growth potential of survived piglets, which hampered swine industry and resulted in huge economic losses in swine production (Armstrong and Cline 1976; Rezaei et al. 2013a, b). Reducing dietary protein level is a useful strategy to control the frequency and severity of diarrhea in weanling piglets (Opapeju et al. 2009; Wu et al. 2010). However, it may lead to an unbalanced supply of amino acids, causing the impairment of pig performance (Wu 2010a, b).

Dietary supplementation with certain amino acids has been reported to increase the efficiency of utilization of dietary protein (Wang et al. 2009; Wu et al. 2011a, b), regulate tissue protein synthesis through the mTOR signaling pathway (Escobar et al. 2006; Yin et al. 2010), enhance the synthesis and secretion of hormones in metabolism, participate in oxidative stress (Li et al. 2011a), modulate the immunity system, and decrease the susceptibility of animals to disease (Li et al. 2007). Dietary amino acids also play critical roles in maintaining gut health and preventing gut diseases through serving as the primary fuels for intestinal mucosa and as substrates for synthesis of intestinal protein and other biological products such as nitric oxide, polyamines and glutathione (Bergen and Wu 2009; Wang et al. 2009). For instance, glutamine could stimulate cell proliferation, inhibit cell apoptosis and is necessary for the integrity of tight junction in enterocytes (Rhoads and Wu 2009; Wu et al. 2011a). Arginine could regulate intestinal cell migration and antioxidative responses through activating the mTOR signaling pathway as well as synthesis of nitric oxide and polyamines (Tan et al. 2010; Wang et al. 2012b; Wu et al. 2013a). Threonine is of great importance for the maintenance of the intestinal mucosal barrier functions through modulating intestinal protein synthesis as well as production and secretion of mucins (Bertrand et al. 2013; Wang et al. 2010). However, the function of the branched-chain amino acids (BCAA) in the intestine is largely unknown.

Dietary amino acids will exert their effects on extra-intestinal tissues after being absorbed by the small intestine (Baker 2009; Rezaei et al. 2013a). Therefore, increasing attention has been paid to effects of different diets on intestinal metabolism and function (Dai et al. 2010; Gilbert et al. 2008). Amino acids are transported into the cell in the free form by specific transporters (Palacín and Kanai 2004) or in a dipeptide or tripeptide form by peptide transporters (Leibach and Ganapathy 1996). Different amino acid transporters have unique substrate specificities (Wu 2013a). For example, transporters have been characterized that are specific for basic amino acids (CAT1,  $\gamma^+$ LAT/4F2hc), neutral amino acids (B<sup>0</sup>AT1, ASCT2 and rBAT/b<sup>0,+</sup>AT) and acidic amino acids (EAAT1, EAAT2) (Kanai and Hediger 2003). Recent work has shown that changes in nutrient content can affect the levels of amino acid

transporters in the small intestine, skeletal muscle, and mammary tissue (Gilbert et al. 2008; Laspiur et al. 2009). However, at present little is known about effects of BCAA on the absorption of amino acids in the small intestine of weaning piglets. We hypothesized that dietary supplementation with BCAA to a low-protein diet can regulate intestinal expression of amino acid and peptide transporters in animals.

Dietary composition is one of the major factors that can influence the microbial population in the gastrointestinal tract (Blachier et al. 2010; Yin et al. 2009). It seems likely that reducing dietary protein levels and supplementing amino acids to low-protein diets may affect available amounts of substrates for bacterial proliferation in the gastrointestinal tract (Dai et al. 2011). However, whether BCAA can modulate the microbial population is still unknown. Identifying amino acids that can modulate nutrient transporters and the intestinal microflora in weaning piglets fed an LP diet is of enormous nutritional importance. The primary objective of the current study was to determine the effects of feeding an LP diet supplemented with BCAA on piglet growth performance, expression of amino acid and peptide transporters in the small intestine, and changes in intestinal microflora populations and metabolism in the large intestine of weaning piglets.

## Materials and methods

### Animals, housing, and experimental design

This study was approved by the Animal Care and Use Committee of China Agricultural University (Beijing, China). A total of 108 piglets (Large White  $\times$  Landrace  $\times$  Duroc) weaned at 28 days of age, with an average initial body weight of  $7.97 \pm 0.11$  kg, were assigned randomly to one of 18 pens on the basis of sex and initial body weight. Each treatment had 6 pens ( $1.8 \times 2.1$  m<sup>2</sup>), with three for castrated males and three for females. Pigs were fed three times a day (8:00 a.m., 2:00 p.m. and 6:00 p.m.) using a single-sided 3-hole feeder. Pigs had free access to water via a nipple drinker.

Three diets based on maize, soybean meal and whey powder were prepared for this study (Table 1). The diets comprised a normal protein (NP) diet (containing 20.9 % crude protein) supplemented with lysine, methionine, threonine and tryptophan. A low-protein (LP) diet (17.1 % crude protein) was formulated by decreasing the soybean-meal content, increasing the maize content, and by supplementing the four limiting amino acids mentioned above. In addition, an LP + BCAA (17.9 % crude protein) diet was formulated by supplementing leucine, isoleucine and valine to the LP diet to meet the recommendations of the

**Table 1** The composition and analyzed nutrient content of the experimental diets

	Diets		
	Normal protein	Low protein	Low protein + branched chain AA
Ingredients (%)			
Maize	54.41	65.01	63.95
Dehulled soybean meal	20.20	8.40	8.40
Fish meal	4.00	4.00	4.00
Whey powder	10.00	10.00	10.00
Concentrated soybean protein	5.00	5.00	5.00
Soybean oil	2.10	2.40	2.80
Salt	0.34	0.35	0.35
Limestone	0.83	0.65	0.65
Dicalcium phosphate	1.10	1.40	1.40
Zinc oxide	0.30	0.30	0.30
Vitamin–mineral premix <sup>a</sup>	1.00	1.00	1.00
L-Lysine HCl	0.42	0.81	0.82
DL-Methionine	0.07	0.20	0.21
L-Threonine	0.17	0.35	0.36
L-Tryptophan	0.06	0.13	0.13
L-Isoleucine	0.00	0.00	0.19
L-Leucine	0.00	0.00	0.10
L-Valine	0.00	0.00	0.34
Nutrient composition (% analyzed) <sup>b</sup>			
Crude protein	20.9	17.1	17.9
Arginine	1.24	0.83	0.87
Histidine	0.59	0.45	0.45
Isoleucine	0.84	0.60	0.80
Leucine	1.61	1.26	1.38
Lysine	1.45	1.40	1.38
Methionine + cystine	1.09	0.89	0.91
Phenylalanine	0.95	0.69	0.72
Threonine	0.94	0.90	0.89
Tryptophan	0.27	0.28	0.29
Valine	0.97	0.74	1.01
Total calcium	0.88	0.85	0.85
Total phosphorus	0.65	0.61	0.60
Calculated nutritional value			
ME, MJ/kg	13.46	13.46	13.46

<sup>a</sup> Provided per kilogram of complete diet: vitamin A, 9,000 IU; vitamin D3, 3,000 IU; vitamin E, 64 IU; vitamin K3, 3 mg; vitamin B12, 12 µg; riboflavin, 5.5 mg; pantothenic acid, 15 mg; niacin, 40 mg; choline chloride, 551 mg; folacin, 0.8 mg; vitamin B1 1.5 mg; vitamin B6 3 mg; biotin, 100 µg; Mn, 40 mg; Fe, 100 mg; Zn, 100 mg; Cu, 150 mg; I, 0.3 mg; Se, 0.3 mg

<sup>b</sup> Values are the means of a chemical analysis conducted in duplicate

National Swine Nutritional Guide for BCAA requirements (NSNG 2010). The three diets were formulated to meet or exceed National Research Council (NRC 1998) recommendations for all other nutrients and were supplied in the mash form. Pigs were weighed at the beginning and end of the experiment. The amount of feed in the feeder was monitored and an attempt was made to ensure that feed was always available in the feeder. Feed was weighed before being added to the feeder. Any wasted feed or feed refusals were also weighed and this amount was subtracted from the amount of feed added to the feeder to determine feed

disappearance. These values were used to calculate average daily gain of body weight (ADG), average daily feed intake (ADFI), and feed:gain ratio. The nursery-room temperature was initially set at 30 °C and was progressively decreased by 1 °C each week.

#### Slaughter, tissue and digesta sample collection

Blood samples were collected randomly from six pigs per treatment at the end of the experiment. Pigs were bled via puncture of the jugular vein before the morning feeding.

Blood samples were collected into heparin-free vacutainer tubes (Becton–Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA). After blood collection, all samples were transferred to centrifuge tubes and centrifuged for 15 min at  $8,000\times g$  at 25 °C. The serum was carefully removed and subdivided into two plastic vials, then stored at  $-80$  °C.

At the end of the 14-day experiment, the 18 pigs used for blood sampling were killed and samples were collected to evaluate intestinal morphology, bacteria in digesta and expression of the intestinal nutrient transporters. The small intestine was dissected from the mesentery and immediately placed on ice. The duodenum was considered as the part of the intestine from the pylorus to the ligament of Treitz, a segment 10 cm proximal to the ileocecal junction was considered as the ileum, and the remainder of the small intestine was considered the jejunum (Wang et al. 2008). Segments (3 cm in length) of mid-duodenum, mid-jejunum, and mid-ileum (the entire alimentary canal without digesta) were fixed in 10 % neutral buffered formalin for subsequent histological measurement. In addition, 3-cm mid-jejunum segments (the entire alimentary canal without digesta) were also immediately flushed with PBS and the digesta collected into a sterile Eppendorf tubes for further RT-PCR and Western blot analysis. Each tube was immediately frozen in liquid nitrogen and stored in a freezer at  $-80$  °C. The proximal colon contents were gently squeezed into the sterile Eppendorf tubes and snap-

frozen in liquid nitrogen, then stored at  $-80$  °C for both genomic DNA isolation and analysis of volatile fatty acids.

### Intestinal morphology

Samples from each intestinal segment were embedded in paraffin and cut into 5- $\mu$ m serial sections, and five non-successive sections from each tissue sample were selected and stained with hematoxylin–eosin for identification (Wang et al. 2008). Five well-oriented villi (determined as the distance between the crypt openings and the end of the villi) and their associated crypt (measured from the crypt-villous junction to the base of the crypt) per section were selected and measured under a light microscope (CK-40, Olympus, Tokyo, Japan) at  $40\times$  magnification and analyzed with an Image Analyzer (Lucia Software, Lucia, Za Drahov, Czechoslovakia). The means of these measurements were calculated to yield a single value per piglet. These procedures were processed by an observer unaware of the dietary treatments.

### RNA isolation and RT-PCR analysis

Total RNA was isolated from a 300 mg of jejunum segment (the entire alimentary canal without digesta) using a RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNA (1  $\mu$ g) was reverse-

**Table 2** Primers used for real-time PCR

Genes	Primers	Sequences (5'–3')	Size (bp)	T <sub>m</sub> (°C)	Accession no.
ASCT2	Forward	GCCAGCAAGATTGTGGAGAT	206	60	DQ231578
	Reverse	GAGCTGGATGAGGTTCCAAA			
B <sup>0</sup> AT1	Forward	CACAACAACCTGCGAGAAGGA	155	60	DQ231579
	Reverse	CCGTTGATAAGCGTCAGGAT			
CAT-1	Forward	TGCCCATACTTCCCGTCC	192	59	NM_001012613
	Reverse	GGTCCAGGTTACCGTCAG			
b <sup>0,+</sup> AT	Forward	ATCGGTCTGGCGTTTTAT	144	59	NM_001110171
	Reverse	GGATGTAGCACCCCTGTCA			
y <sup>+</sup> LAT1	Forward	GCCCATTGTCACCATCATC	216	59	NM_001110421
	Reverse	GAGCCCACAAAGAAAAGC			
4F2hc	Forward	CTCGAACCACCAAGGAC	174	59	XM_003361818
	Reverse	GAGGTGAGACGGCACAGAG			
Pept-1	Forward	CCCAGGCTTGCTACCCAC	144	60	NM_214347
	Reverse	ACCCGATGCACTTGACGA			
rBAT	Forward	TTCCGCAATCCTGATGTTC	146	59	NM_001123042
	Reverse	GGGTCTTATCACTTGGGTC			
$\beta$ -Actin	Forward	TGCGGGACATCAAGGAGAAG	216	60	XM_003357928
	Reverse	AGTTGAAGGTGGTCTCGTGG			

ASCT2 Na<sup>+</sup>-neutral AA exchanger, B<sup>0</sup>AT1 system B<sup>0</sup> neutral AA transporter, CAT-1 cationic amino acid transporter 1, b<sup>0,+</sup>AT related to b<sup>0,+</sup> amino acid transporter, y<sup>+</sup>LAT1 y<sup>+</sup>L amino acid transporter-1, 4F2hc 4F2 heavy chain, Pept-1 intestinal peptide transporter, rBAT basic amino acid transporter, T<sub>m</sub> melting temperature

transcribed to complementary DNA (cDNA) using a PrimeScript 1st Strand cDNA Synthesis Kit (Takara, Otsu, Japan) according to the manufacturer's protocol. Primers for the selected genes (Table 2) were designed using Oligo 7.0 Software. Real-time PCR was performed using an Applied Biosystems 7500 Real-Time PCR System (Foster City, CA, USA) with SYBR Green PCR Master Mix (Takara, Otsu, Japan) containing MgCl<sub>2</sub>, dNTP, and Hotstar Taq polymerase. Quantification of target mRNA was conducted using a relative standard curve generated by a serial dilution (1:10<sup>7</sup> to 1:1) of amplification products. The PCR system consisted of 5.0 µL of SYBR Green qPCR mix, 1.0 µL of cDNA, 3.6 µL of double distilled water, and 0.4 µL of primer pairs (25 µM forward and 25 µM reverse) in a total volume of 10 µL. The protocols for all genes included a denaturation program (1 min at 95 °C), amplification and quantification program repeated for 35 cycles (5 s at 95 °C, 30 s at 58 °C), followed by the melting curve program at 60–95 °C with a heating rate of 0.1 °C per second and continuous fluorescence measurement. Six samples were used for each treatment and each sample was measured in triplicate.

#### Detection of volatile fatty acids in colonic digesta

The concentrations of short chain fatty acids were determined by a gas chromatographic method following the procedures of Franklin et al. (2002) with modifications. Approximately, 1.5 g of thawed colonic digesta was suspended in 1.5 mL of distilled water in a screw-capped tube. The entire sample was centrifuged at 15,000×*g* for 10 min at 4 °C. After that, 1 ml of supernatant was transferred into an ampoule and mixed with 200 µL of metaphosphoric acid (HPO<sub>3</sub>). The ampoules were placed in an ice bath for 30 min then centrifuged again for 10 min. The sample was injected into a HP 6890 Series Gas Chromatograph (Hewlett Packard, PA, California) equipped with a HP 19091N-213 column with 30.0 m × 0.32 mm i.d. (Agilent, PA, California). The injector and detector temperatures were 185 and 210 °C, respectively.

#### Western blot analysis

Relative protein levels for CAT-1, rBAT, 4F2hc and PepT-1 obtained from a whole homogenized section of a jejunum segment were determined by the western blotting technique. The frozen jejunum samples were powdered under liquid nitrogen, and lysed in RIPA buffer (150 mM NaCl, 1 % Triton X-100, 0.5 % sodium deoxycholate, 0.1 % SDS, 50 mM Tris-HCl at pH 7.4, plus a protease inhibitor cocktail purchased from Apply Gene (Beijing, China). Protein concentrations were determined using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Equal amounts of proteins (30 mg) were electrophoresed on SDS polyacrylamide gels. Prestained protein markers (Fermentas, Waltham, MA, USA) were analyzed in each gel. Proteins were electrotransferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA) and blocked with 5 % nonfat dry milk overnight at 4 °C. The transfer efficiency was assessed by gel staining with Coomassie Blue. Samples were incubated with corresponding primary antibodies (1:500 dilution for 2 h at 25 °C or overnight at 4 °C) against CAT-1, rBAT, 4F2hc and PepT-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The primary antibodies have been validated for use in swine by the manufacturer. After being washed with Tris-Tween-20 buffer (pH 7.4), membranes were incubated with a secondary antibody (rBAT: horseradish peroxidase-conjugated goat anti-rabbit IgG; CAT-1, PepT-1 and 4F2hc: horseradish peroxidase-conjugated rabbit anti-goat IgG) (Zhongshan Gold Bridge, Beijing, China) at a 1:7,000 dilution for 1 h at room temperature. The membrane was exposed to X-ray film for 1 min. Band densities were detected with the western blot luminance reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and quantified using AlphaImager 2200 (Alpha Innotech, San Leandro, CA, USA).

#### DNA isolation and PCR-DGGE analysis

DNA extraction of the colonic digesta for DGGE analysis was performed according to the instructions of a DNA

**Table 3** Growth performance of weanling pigs fed a normal protein diet, a low-protein diet, or a low-protein diet supplemented with BCAA for 14 days

	Diets			SEM	<i>P</i> value
	Normal protein	Low protein	Low protein + BCAA		
ADG (g/day)	303 <sup>a</sup>	174 <sup>b</sup>	286 <sup>a</sup>	16.48	<0.01
ADFI (g/day)	366 <sup>a</sup>	300 <sup>b</sup>	383 <sup>a</sup>	14.55	<0.01
Feed:gain ratio	1.21 <sup>a</sup>	1.73 <sup>b</sup>	1.34 <sup>a</sup>	0.07	<0.01

Values are means of six pens of six pigs per diet

Means within a row with different superscripts are significantly different (*P* < 0.05)

SEM standard error of the mean

Stool Mini Kit (Qiagen, Hilden, Germany). The bacterial universal V3 region of the 16S RNA gene was amplified according to primers published by Muyzer et al. (1993). In brief, the PCR mixture contained 125 ng of genomic DNA, 25 pM of each primer and 25 mL SYBR Green PCR Master Mix 2× (Promega, Madison, WI, USA). The final volume was adjusted to 50 µL with sterile deionized water. The PCR analysis was conducted in a 50 µL volume as described in the routine PCR procedure. The procedure included 5 min at 95 °C; 29 cycles of 30 s at 95 °C, 30 s at 58 °C, and 30 s at 72 °C. The PCR products were analyzed by electrophoresis on 1.0 % (w/v) agarose gel in SYBR green I (Sigma Chemicals, St. Louis, MO, USA) to verify a single product of the expected size. Nucleic acid concentrations were measured using a Gel Doc™ XR<sup>+</sup> camera with Image Lab™ Software (Bio-Rad, Hercules, CA, USA).

DGGE was performed for separation of PCR amplicons on 8 % polyacrylamide gels with a vertical gradient of denaturants of 45–60 %. Electrophoresis began with pre-running for 5 min at 200 V and then for 16 h at 85 V in 0.5 × Tris–acetate–EDTA buffer at a constant temperature of 60 °C using a DCode™ Universal Mutation Detection System (Bio-Rad). DGGE gels were stained by SYBR Green I (Sigma Chemicals) for 10 min at 25 °C, and photographed with a Gel Doc™ XR<sup>+</sup> with Image Lab™ Software (Bio-Rad) and analyzed by Quantity One Basic

4.6.6 Software for Windows (Bio-Rad). Interesting bands in the denaturing gradient gel electrophoresis profiles were excised with a sterile needle, re-amplified and purified with a Takara Mini Best DNA Fragment Purification Kit (Takara, Dalian, China) according to the manufacturer's protocol.

The PCR products were cloned into the vector pMD-19T according to the manufacturer's instructions (Takara) and transformed into *Escherichia coli* DH5α cells (Tiangen, Beijing, China) via chemical transformation. The transformants were grown on Luria–Bertani agar in the presence of 100 µg/mL ampicillin. The positive recombinants were screened and identified by PCR and sent for sequencing. Homology searches were performed using the BLAST program in the NCBI database (<http://blast.ncbi.nlm.nih.gov>).

#### Chemical analyses

The experimental diets were analyzed for crude protein, calcium, and phosphorus according to AOAC (2003) procedures. Dietary amino acids except methionine, cystine, and tryptophan were determined by ion-exchange chromatography using a Hitachi L-8800 AA Analyzer (Tokyo, Japan) after acid hydrolysis with 6 N HCl (reflux for 24 h at 110 °C). Methionine and cystine were determined after oxidation with performic acid and subsequent hydrolysis

**Table 4** Serum amino acid concentrations in weanling pigs fed a normal protein diet, a low-protein diet, or a low-protein diet supplemented with BCAA for 14 days

	Diets			SEM	P value
	Normal protein	Low protein	Low protein + BCAA		
Essential amino acids					
Arginine	304 <sup>a</sup>	143 <sup>b</sup>	139 <sup>b</sup>	28	<0.01
Histidine	89 <sup>a</sup>	34 <sup>b</sup>	24 <sup>b</sup>	8.7	<0.01
Isoleucine	174 <sup>a</sup>	40 <sup>b</sup>	182 <sup>a</sup>	20	<0.01
Leucine	254 <sup>a</sup>	153 <sup>b</sup>	242 <sup>a</sup>	16	<0.01
Lysine	578	588	574	48	0.98
Methionine	121	123	131	6.4	0.58
Phenylalanine	115 <sup>a</sup>	67 <sup>b</sup>	69 <sup>b</sup>	7.7	<0.01
Threonine	529	517	534	35	0.98
Tryptophan	81	80	85	4.1	0.84
Valine	253 <sup>a</sup>	101 <sup>b</sup>	267 <sup>a</sup>	31	<0.01
Nonessential amino acids					
Alanine	769	785	824	40	0.83
Asparagine	229	185	176	16	0.30
Cysteine <sup>A</sup>	9.2	6.4	6.7	0.64	0.12
Glutamate	362	350	368	34	0.97
Glutamine	369 <sup>a</sup>	217 <sup>b</sup>	229 <sup>b</sup>	25	<0.01
Glycine	1,228	1,155	1,256	95	0.89
Proline	304	259	277	17	0.57
Serine	263	222	217	12	0.16

Values (µmol/L) are means for six pigs per diet with one pig chosen from each pen ( $n = 6$ )

Means within a row with different superscripts are significantly different ( $P < 0.05$ )

SEM standard error of the mean

<sup>A</sup> Free cysteine; not including cystine

with 6 M HCl, and the tryptophan content was determined after alkaline hydrolysis at 120 °C for 16 h (AOAC 2003) and separated by reversed-phase HPLC (Agilent 1200, Santa Clara, CA, USA).

Serum AA concentrations were determined by ion-exchange chromatography with physiological fluid analysis conditions (S-433D AA Analyzer, Sykam, Germany). Frozen serum samples were first thawed at 4 °C and then deproteinized with 120 mg of salicylic acid/mL of serum. After samples were placed in an ice bath for 20 min, the reaction system was adjusted for pH by adding lithium hydroxide solution (2 mol/L) and then centrifuged at 12,000g (L-80 XP, Beckman, USA) for 30 min. The supernatant fluid was collected and then passed through a filter (0.1 µm) before use for amino acid analysis.

### Statistical analysis

Statistical analyses were performed using the statistical software SAS Version 9.2. Data were analyzed by ANOVA according to the GLM procedure of SAS. Log transformation of variables was performed when variance of data was not homogenous among treatment groups, as assessed using the Levene's test (Wei et al. 2012). Differences among treatment means were determined using Student–Newman–Keuls multiple range test (Fu et al. 2010). Differences at  $P < 0.05$  were considered significant.

## Results

### Growth performance of weanling piglets

The growth performance of weanling pigs fed the different diets is presented in Table 3. ADG and ADFI of pigs fed the NP treatment were higher than those fed the LP treatment ( $P < 0.05$ ). The ADG and ADFI of pigs in the LP + BCAA group were similar to those in the NP group ( $P > 0.05$ ). The feed:gain ratio was greater for pigs fed the LP diet, as compared with pigs in the NP and LP + BCAA groups ( $P < 0.05$ ).

### Serum-free AA concentrations

Serum-free AA concentrations are shown in Table 4. There were significant decreases in the serum concentrations of arginine, histidine, isoleucine, leucine, phenylalanine and valine in pigs fed the LP diet, as compared with those fed the NP diet ( $P < 0.05$ ). Serum concentrations of arginine, histidine and phenylalanine were also lower in pigs fed the LP + BCAA diet, compared with the NP diet ( $P < 0.05$ ), while serum concentrations of isoleucine, leucine and valine were higher in pigs fed the LP + BCAA diet,

compared with the LP diet ( $P < 0.05$ ), and did not differ from those in the NP group ( $P > 0.05$ ).

### Intestinal morphology

Pigs fed the LP diet had significantly ( $P < 0.05$ ) lower villus height in the duodenum, jejunum and ileum than pigs fed the NP diet (Table 5). Supplementation with BCAA to the LP diet restored villous height to that for the NP diet in the jejunum and ileum and partially restored villous height in the duodenum ( $P < 0.05$ ). Crypt depth and villous height to crypt depth ratio in the jejunum and ileum were not affected by reduction of dietary protein ( $P > 0.05$ ). However, in the duodenum, the crypt depth of pigs fed the LP diet was lower than for pigs fed the NP diet ( $P < 0.05$ ). The crypt depth for pigs fed the LP + BCAA was intermediate to that for the other two treatments.

### mRNA levels for amino acid and peptide transporters in the jejunum

The abundance of ASCT2, B<sup>0</sup>AT1, CAT-1, rBAT, 4F2hc, b<sup>0,+</sup>AT, y<sup>+</sup>LAT and PepT-1 mRNAs in the small intestine of piglets is shown in Fig. 1. There was a significant ( $P < 0.05$ ) decrease in intestinal ASCT2 mRNA levels in pigs fed the LP diet, compared with the NP diet; however, no difference was detected between the LP and the LP + BCAA groups. The intestinal CAT-1, rBAT and 4F2hc mRNA levels in pigs fed the LP diet were lower compared with the LP + BCAA group ( $P < 0.05$ ). The abundance of these mRNAs in the NP group was intermediate to the other two treatments. In contrast, intestinal PepT-1 mRNA level in the LP group was higher than that in the LP + BCAA group ( $P < 0.05$ ), while PepT-1 mRNA level in the NP group was again intermediate to the other two treatments. Intestinal mRNA levels for B<sup>0</sup>AT1, b<sup>0,+</sup>AT and y<sup>+</sup>LAT mRNA did not differ among the three treatment groups ( $P > 0.05$ ).

### Protein expression of amino acid and peptide transporters in the small intestine

Results of the western blot analysis indicated the presence of CAT-1, rBAT, 4F2hc and PepT1 in the small intestine (Fig. 2). These four transporters were chosen for western blot analysis because their mRNA levels were significantly affected by dietary CP content and BCAA supplementation. Compared to the NP group, the protein abundance of intestinal PepT-1 was increased in pigs fed the LP diet ( $P < 0.05$ ). No difference in the PepT-1 protein was detected between the NP and the LP + BCAA groups ( $P > 0.05$ ). The protein abundance of rBAT-1 was decreased in pigs fed the LP diet ( $P < 0.05$ ) while there

was no difference between the NP and the LP + BCAA groups. The protein abundance of the CAT-1 and the 4F2hc did not differ among pigs fed the different diets ( $P > 0.05$ ).

#### Volatile fatty acid concentrations in the colon

Concentrations of volatile fatty acid were all significantly influenced by dietary protein levels (Table 6). Specifically, amounts of acetic acid, propionic acid, butyric acid, pentanoic acid, isobutyric acid and isovaleric acid were decreased in pigs fed the LP diet, compared with the NP group ( $P < 0.05$ ). However, there was no difference between the LP and the LP + BCAA groups ( $P > 0.05$ ).

#### DGGE fingerprints

Representative results of the DGGE fingerprints are shown in Fig. 3. Most bands among the three groups of pigs were similar. There was no difference in the numbers of bands, Simpson's Index of Diversity and Dice's Coefficient of Similarity ( $P > 0.05$ ) (Table 7). The bands which differed between the NP and the LP groups were excised from the gels and their species identified by sequence analysis. Compared to the LP group, an increase in the *Clostridium*-like phenotype was observed in the NP group (Bands 1 and 4, Fig. 3).

## Discussion

Low-protein diets are often fed to weanling piglets, which have a compromised ability to digest solid diets (largely of

plant origin), to alleviate adverse effects on the intestine (Wang et al. 2009). Specifically, by reducing dietary protein levels, amounts of amino acids available for bacterial proliferation in the gastrointestinal tract are reduced, thereby decreasing the incidence of diarrhea (Rezaei et al. 2013b). In the present study, making the LP and the NP diets isonitrogenous would negate the very reasons for conducting the experiment. The LP and the LP + BCAA diets were essentially isonitrogenous as only small amounts of BCAA were added to the LP diet and the protein content of the two diets was similar (Table 1). Therefore, we believe that any differences observed were due to the presence of BCAA and not due to differences in the dietary protein levels between the LP and LP + BCAA groups.

Both reduced feed intake and metabolic disturbances in piglets fed an LP diet may result from an imbalance in amino acid composition (Wu 2009). While the content of BCAA is relatively high in swine feedstuffs (Hou et al. 2012; Li et al. 2011b; Tan et al. 2012), dietary requirements of these amino acids for protein synthesis in young pigs are also high (Wu et al. 2013b). In mammals, BCAA are degraded via interorgan cooperation, with skeletal muscle as the major site for their transamination and with the liver as the primary organ for the oxidation of their carbon skeletons (Le Plenier et al. 2012; Lei et al. 2012a, b, 2013; Murakami et al. 2012). In the piglet small intestine, BCAA are also degraded by both enterocytes (Chen et al. 2007, 2009) and luminal microorganisms (Dai et al. 2012a, b, 2013). In our study, both the LP (17.1 % crude protein) and the LP + BCAA diets were deficient in protein (NRC 1998). In the LP diet, only lysine, methionine, threonine

**Table 5** Intestinal morphology in weanling piglets fed a normal protein diet, a low-protein diet, or a low-protein diet supplemented with BCAA for 14 days

	Diets			SEM	P value
	Normal protein	Low protein	Low protein + BCAA		
<b>Duodenum</b>					
Villous height ( $\mu\text{m}$ )	413 <sup>a</sup>	326 <sup>c</sup>	368 <sup>b</sup>	12	<0.01
Crypt depth ( $\mu\text{m}$ )	194 <sup>a</sup>	156 <sup>b</sup>	178 <sup>ab</sup>	6.1	0.02
Villous height:crypt depth	2.15	2.10	2.09	0.06	0.90
<b>Jejunum</b>					
Villous height ( $\mu\text{m}$ )	362 <sup>a</sup>	264 <sup>b</sup>	333 <sup>a</sup>	16	<0.01
Crypt depth ( $\mu\text{m}$ )	167	136	153	7.8	0.26
Villous height:crypt depth	2.21	1.91	2.21	0.08	0.19
<b>Ileum</b>					
Villous height ( $\mu\text{m}$ )	323 <sup>a</sup>	243 <sup>b</sup>	313 <sup>a</sup>	13	<0.01
Crypt depth ( $\mu\text{m}$ )	148	130	136	6.0	0.47
Villous height:crypt depth	2.19	1.90	2.35	0.09	0.10

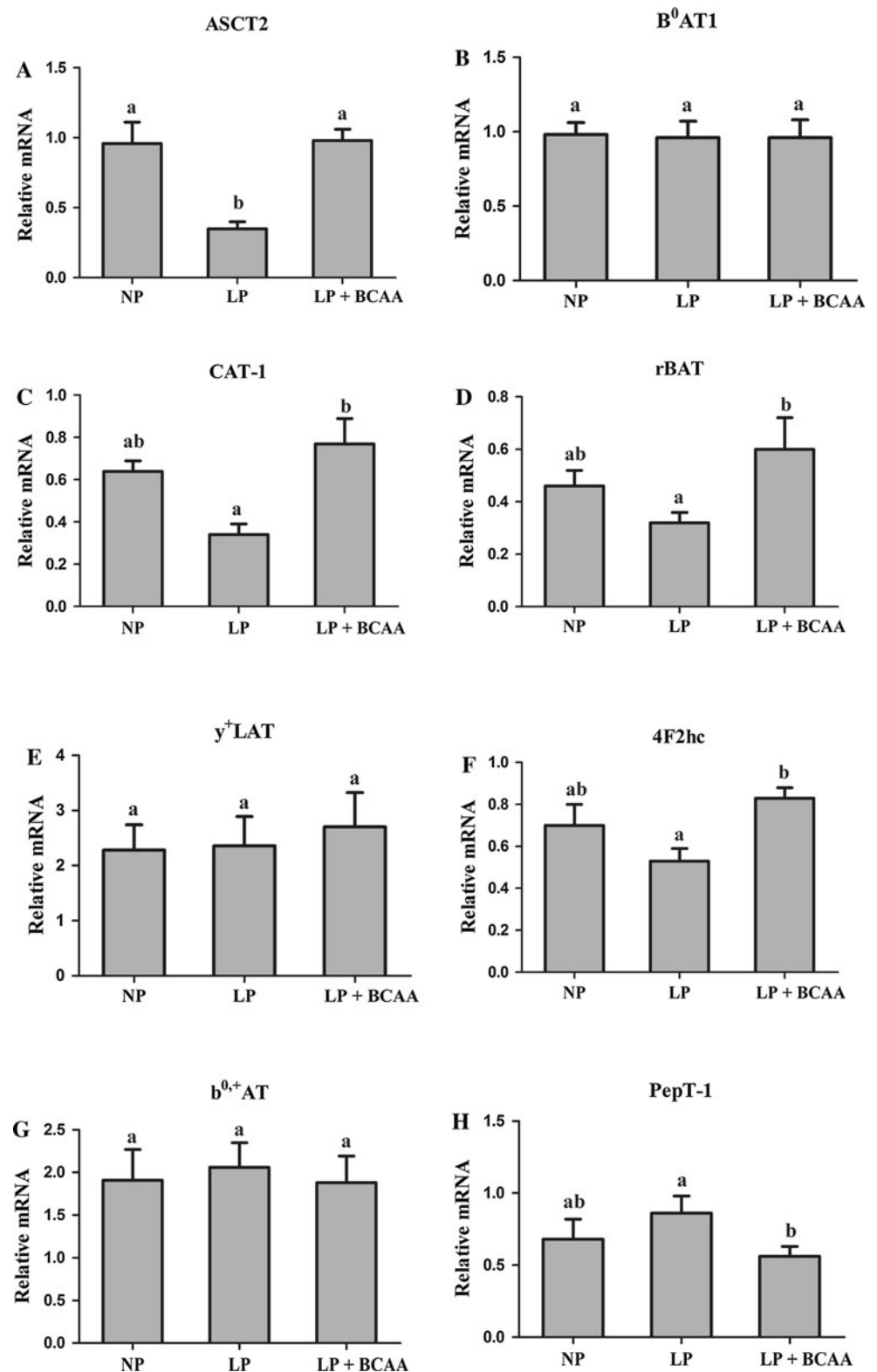
Values are means of six pigs per diet with one pig chosen from each pen ( $n = 6$ )

Means within a row with different superscripts are significantly different ( $P < 0.05$ )

SEM standard error of the mean



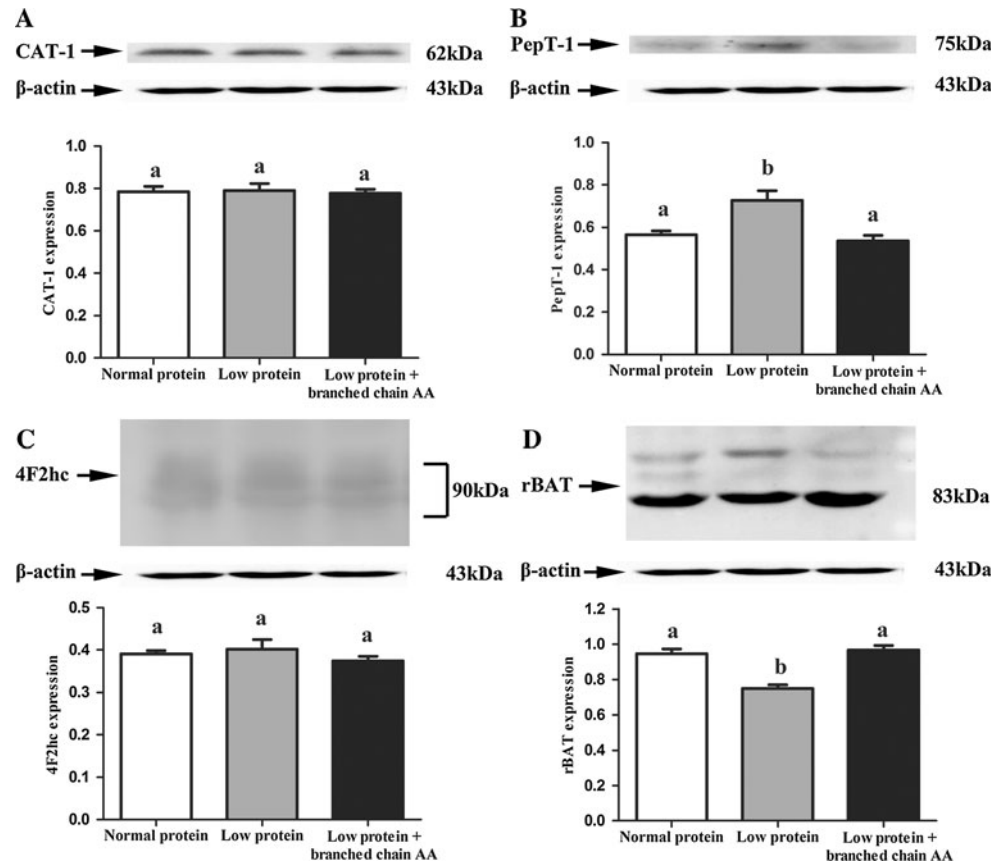
**Fig. 1** Effect of normal protein (20.9 % crude protein, *NP*), low protein (17.1 % crude protein, *LP*) or low protein + BCAA (17.9 % crude protein, *LP + BCAA*) diets on **a** ASCT2, **b** B<sup>0</sup>AT, **c** CAT-1, **d** rBAT, **e** y<sup>+</sup>LAT, **f** 4F2hc, **g** b<sup>0,+</sup>AT and **h** PepT-1 mRNA expression in a whole homogenized jejunum segment obtained from piglets 14 days after weaning ( $n = 6$ ).  $\beta$ -Actin was used as an internal standard to normalize the signal. Means without a common letter differ significantly ( $P < 0.05$ )



and tryptophan were supplemented, while the LP + BCAA diet provided a better balance of amino acids by simultaneously supplementing leucine, valine and isoleucine (Dillon 2013). Therefore, these two diets represent a

reasonable model for evaluating the effects of BCAA on nutrient transporters and intestinal microbiota in the gut. Among the three BCAA, only leucine is known to activate the mTOR signaling pathway and stimulate tissue protein

**Fig. 2** Western blot analysis of the proteins CAT-1 (a), PepT-1 (b), 4F2hc (c), and rBAT (d) obtained from a whole homogenized section of a jejunum segment obtained from piglets fed a normal protein (20.9 % crude protein), low protein (17.1 % crude protein) or low protein + BCAA (17.9 % crude protein) diet for 14 days after weaning ( $n = 6$ ).  $\beta$ -Actin was used as an internal standard to normalize the signal. Means without a common letter differ significantly ( $P < 0.05$ )



**Table 6** Concentrations of VFA in the colon of weaning pigs fed a normal protein diet, a low-protein diet, or a low-protein diet supplemented with BCAA for 14 days

Volatile fatty acid	Treatments			SEM	<i>P</i> value
	Normal protein	Low protein	Low protein + BCAA		
Acetic acid	25.38 <sup>a</sup>	15.55 <sup>b</sup>	14.67 <sup>b</sup>	1.42	<0.01
Propionic acid	9.27 <sup>a</sup>	5.24 <sup>b</sup>	5.24 <sup>b</sup>	0.57	<0.01
Butyric acid	1.06 <sup>a</sup>	0.41 <sup>b</sup>	0.37 <sup>b</sup>	0.14	0.04
Pentanoic acid	6.93 <sup>a</sup>	3.56 <sup>b</sup>	3.25 <sup>b</sup>	0.50	<0.01
Iso-butyric acid	1.13 <sup>a</sup>	0.50 <sup>b</sup>	0.46 <sup>b</sup>	0.12	0.02
Isovaleric acid	1.54 <sup>a</sup>	0.47 <sup>b</sup>	0.41 <sup>b</sup>	0.21	0.02

Values (mmol/kg colonic content) are means of six pigs per diet with one pig chosen from each pen ( $n = 6$ )

Means within a row with different superscripts are significantly different ( $P < 0.05$ )

SEM standard error of the mean

synthesis (Li et al. 2011a; Suryawan and Davis 2011), isoleucine and valine were added to the LP diet along with leucine to maintain an adequate balance among these amino acids (Suryawan et al. 2013).

Results of the present study showed that reducing the dietary protein level impaired growth performance of weaning pigs, as previously reported by Deng et al. (2009). The lower feed intake observed in piglets that consumed the LP diet may be related to a deficiency in some essential amino acids, whose metabolites regulate the

production of neurotransmitters and thus food consumption (Wu 2013b). The ADG of piglets was also decreased likely due to the reduced feed intake. Supplementation with BCAA to the LP diet improved the growth performance of piglets to a level similar to that for the NP diet which is consistent with previous studies showing that BCAA improved the growth performance and feed efficiency in pigs fed an LP diet (Brudevold and Southern 1994; Figueroa et al. 2003; Lordelo et al. 2008; Yin et al. 2010). These improvements likely result from an increase in tissue

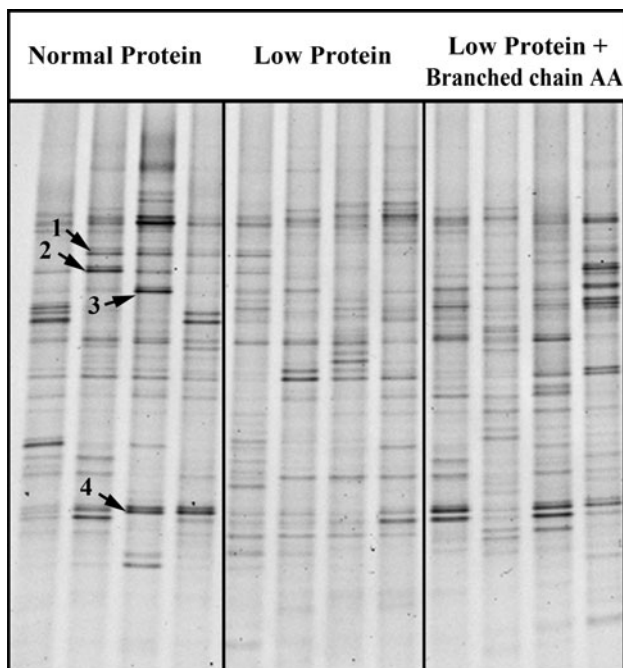
protein synthesis (Yin et al. 2010). Note that deposition of 1 g protein in the body is associated with the retention of 3 g water (Wu 2013a). Thus, protein growth in skeletal muscle is a major determinant of feed efficiency in young animals.

In the current study, diets with a reduced protein level but supplemented with BCAA cannot sustain adequate concentrations of nutritionally essential amino acids, such as arginine, histidine and phenylalanine in the serum. In young pigs, serum concentrations of some amino acid levels may reflect their dietary intake, but this is not true for certain amino acids due to the complex compartmentalization of

their metabolism in vivo (Wu 2013a). BCAA are not synthesized by animal cells and rates of BCAA catabolism are not augmented by an LP diet (Rezaei et al. 2013a). Therefore, serum concentrations of leucine, isoleucine and valine were lower in the LP diet than those in the NP diet. Supplementing BCAA to the LP diet increased the concentrations of leucine, isoleucine and valine in the serum of weanling piglets.

A deficiency of BCAA in diets impairs intestinal protein synthesis in young pigs (Yin et al. 2010). Thus, the LP diet resulted in reduced intestinal mucosal mass in weanling piglets. The folds in the intestine called villi are an important exchange area for digestion and absorption (Pluske et al. 1996). Villous height and crypt depth are critical factors affecting the piglet's ability to absorb nutrients (Kelly et al. 1991). In the current study, reducing the dietary protein level resulted in a significant reduction of villous height in the duodenum, jejunum and ileum as well as a decline of crypt depth in the duodenum in the LP group. Similar findings were reported for weanling pigs fed a lysine-deficient diet (He et al. 2013). The reduction in villus height would reduce the surface area available for absorption of nutrients and this may partially explain the reduced growth performance and reduced feed efficiency in pigs fed the LP diet. In contrast, the villus height was similar for pigs fed the NP diet and the LP + BCAA diet, indicating an increased surface area for nutrient absorption enhanced growth and the efficiency of nutrient utilization in pigs fed a BCAA-supplemented LP diet.

To provide a molecular mechanism for BCAA to improve intestinal function, additional measurements were made to evaluate the abundance of transporters of peptides and free amino acids in response to dietary amino acid availability. It is well known that small peptides or amino acids are mainly absorbed in the small intestine (Bröer 2008). Intestinal absorption of amino acids and small peptides is mediated, respectively, by amino acid and peptide transporters, which vary in type and specificity (Closs et al. 2006; Prasad et al. 1999). Based on the notion that different nutrient levels in the diet can regulate nutrient transporters in the small intestine (Gilbert et al. 2008;



**Fig. 3** Example of DGGE fingerprints of PCR products of V3 regions of 16S rDNA from colonic digesta samples taken from piglets 14 days after weaning. Piglets received normal protein (20.9 % crude protein), low protein (17.1 % crude protein) or low protein + BCAA (17.9 % crude protein) diets. Four representative samples from each group are shown. Arrows (1–4) indicate excised bands that were re-amplified (see Table 8)

**Table 7** The microbial communities in the colon of weanling piglets fed a normal protein diet, a low-protein diet, or a low-protein diet supplemented with BCAA for 14 days

Items	Diets			SEM	P value
	Normal protein	Low protein	Low protein + BCAA		
Number of bands	27.8	27.8	28.2	0.81	0.96
Simpson's index of diversity	3.2	3.3	3.3	0.03	0.37
Dice's coefficient of similarity	57.5	60.0	60.2	1.48	0.61

Values are means of four pigs per diet

SEM standard error of the mean

Suryawan and Davis 2011), we hypothesized that the small intestine expresses different levels of amino acid and peptide transporters in response to different intakes of dietary protein and amino acids.

Available evidence shows that, in the small intestine, rates of small-peptide transport by PepT1 are higher than rates of free amino acid transport by amino acid transporters (Daniel 2004). Among the gene encoding for peptide transporters evaluated in this study, PepT1 was the only gene that exhibited a greater expression level in the small intestine of pigs fed the LP diet than piglets which consumed the NP or the LP + BCAA diet. In the piglet small intestine, PepT1 mainly transports dipeptides and tripeptides from the digestion of dietary proteins (Daniel 2004). The lower feed intake influenced by the LP diet may explain the change in intestinal PepT-1 abundance. Ihara et al. (2000) reported that when rats were starved for 4 days, semi-starved (50 % amount of control) for 10 days, or given total parenteral nutrition for 10 days, PepT1 mRNA levels were increased by 179, 161 and 164 % in the starved, parenterally fed, and semi-starved rats, respectively. Furthermore, Chen et al. (2005) observed that PepT-1 mRNA abundance was higher in feed-restricted chickens than ad libitum fed chickens. Therefore, compared with the NP diet, the reduction in feed intake caused by the LP diet may have led to the increase in PepT-1 expression, so as to efficiently transport small peptides from the lumen of the small intestine into enterocytes. This may aid in explaining why bioavailability of dietary amino acids is higher in animals fed an LP diet than an NP diet (Rezaei et al. 2013a). Key regulators of intestinal PepT-1 expression may be BCAA. In support of this view, the abundance of the intestinal PepT-1 protein in pigs fed the LP + BCAA diet returned to the level for the NP group. Therefore, the increased expression of PepT1 in the LP diet may provide a mechanism for conserving dietary protein and compensating for decreased feed intake.

Amino acid transporters that are related to the absorption of BCAA by the small intestine were measured in this study. It would be ideal to analyze the expression of all genes responsible for intestinal amino acid transport in pigs using the currently available technology. However, the

DNA sequences of many porcine amino acid transporters are still unknown and were, therefore, unavailable for use in the present experiment. B<sup>0</sup>AT1, belonging to the B<sup>0</sup> system, is the major apical neutral amino acid transporter in the kidney and the small intestine (Bröer 2008). ASCT2 mediates high-affinity transport of neutral amino acids except for aromatic AA. In this research, some amino acid transporters related to the transportation of cationic amino acids (lysine, arginine and histidine) were also quantified at both mRNA and protein levels. For example, rBAT/b<sup>0,+</sup>AT is a transporter for cationic amino acids and cystine, which is coupled to the efflux of neutral amino acids. In addition, CAT-1 transports cationic amino acids in the kidney and the small intestine, whereas y<sup>+</sup>LAT1/4F2hc transporter is a mediator of cationic amino acid efflux from epithelial cells (Bröer 2008). All of these amino acid transporters play a vital role in intestinal absorption of amino acids. Our results indicate that dietary intake of protein or BCAA regulates intestinal expression of amino acid transporters to efficiently absorb amino acids from the lumen of the small intestine into enterocytes.

Some researchers have demonstrated the special function of specific amino acid levels in modulating the expression of the amino acid transporters. For example, Torras-Llort et al. (1998) reported that b<sup>0,+</sup>-like and y<sup>+</sup>-like transport systems were upregulated in the chicken intestine by increasing dietary lysine levels. Similarly, Wang et al. (2012a) found that different lysine levels could regulate CAT-1 expression in the pig small intestine. The function of the BCAA in the regulation of the amino acid transporters has also been reported in recent years (Laspiur et al. 2009). In particular, Drummond et al. (2010) demonstrated that an increase in dietary leucine intake upregulated the expression of amino acid transporters in human skeletal muscle by stimulating the mTOR pathway. Thus, like other functional amino acids, such as glycine (Wang et al. 2013), glutamate (Brosnan and Brosnan 2013; San Gabriel and Uneyama 2013), glutamine (Ren et al. 2013), tryptophan (Fernstrom 2013), cysteine (Hou et al. 2013), and arginine (Satterfield et al. 2013), BCAA have both nutritional and regulatory roles in animal metabolism. We surmise that supplementing BCAA to the LP diet may increase the expression of intestinal amino acid transporters in weanling piglets through activating the mTOR signaling pathway. Further studies are required to test this hypothesis.

Short chain fatty acids are the major end products from the fermentation processes of gut microflora in the large intestine (Bergen and Wu 2009), and are significantly affected by the type of diet and microbial composition in the gut (van Beers-Schreurs et al. 1998). In our study, we observed that the VFA levels in the colon of pigs fed the NP diet were much higher compared with pigs fed the LP

**Table 8** Clone relatives to sequences retrieved from the DGGE bands from the colon digesta of weanling piglets

Band No.	Closest relative in GenBank	GenBank accession no.	% identity
1	<i>Clostridium</i> sp.	JX575996.1	100
2	Uncultured bacterium clone	GU241441.1	100
3	Uncultured bacterium clone	EU774357.1	99
4	<i>Clostridium</i> sp.	HF559183.1	100

and the LP + BCAA diets, and a relative increase in numbers of the *Clostridium*-like phenotype was also observed in the NP group. Galfi and Bokori (1990) obtained similar results in which the concentrations of VFA and *Clostridium* populations were increased when high-protein diets were fed. Also, Zentek et al. (2004) reported that increasing dietary protein levels can increase *Clostridium* content in the gut. *Clostridium* and other bacteria are also known to produce VFA (Dai et al. 2011). We speculate that undigested protein in the NP diet that reached the lower intestine of pigs may increase the numbers of *Clostridium*, leading to increased concentrations of VFA in the colonic digesta.

In conclusion, our data demonstrate that BCAA may be necessary to maintain normal intestinal development and physiological absorption of amino acids via regulating expression of intestinal amino acid and peptide transporters (especially rBAT and PepT-1) in the small intestine. As a functional amino acid, leucine may play an important role in modulating intestinal expression of genes responsible for efficient utilization of dietary protein and maintaining whole-body nitrogen homeostasis. Further studies are needed to reveal the influence of dietary LP or BCAA levels on the microflora in the colon.

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**Conflict of interest** The authors declare no conflicts of interest.

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