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ORIGINAL ARTICLE

Dietary L-leucine supplementation enhances intestinal development in suckling piglets

Yuli Sun[1] · Zhenlong Wu[1] · Wei Li[1] · Chen Zhang[1] · Kaiji Sun[1] · Yun Ji[1] · Bin Wang[1] · Ning Jiao[1] · Beibei He[1] · Weiwei Wang[1] · Zhaolai Dai[1] · Guoyao Wu[1,2]

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Abstract L-Leucine is a signaling amino acid in animal metabolism. It is unknown whether supplementing L-leucine to breast-fed neonates may enhance their small-intestinal development. This hypothesis was tested with a piglet model. Seven-day-old sow-reared pigs with an average birth weight of 1.45 kg were assigned randomly to the control or leucine group ($n = 30$/group). Piglets in the leucine group were orally administrated with 1.4 g L-leucine/kg body weight, whereas piglets in the control group received isonitrogenous L-alanine, twice daily for 14 days. The supplemental L-leucine amounted to 200 % of L-leucine intake from sow's milk by 7-day-old pigs. At the end of the 2-week experiment, tissue samples were collected for determining intestinal morphology, expression of genes for intestinal leucine transporters (real-time RT-PCR and western blot analysis), and plasma metabolites and hormones. L-leucine administration increased ($P < 0.05$) villus height in the duodenum, an elevated ratio of villus height to crypt depth in the duodenum and ileum, plasma concentrations of leucine, glutamine and asparagine, as well as body-weight gains. mRNA levels for L-leucine transporters (*SLC6A14, SLC6A19* and *SLC7A9)* and the abundance of the $ATB^{0,+}$ protein were increased ($P < 0.05$) but those for *SLC7A7* mRNA and the LAT2 protein were decreased ($P < 0.05$) in the jejunum of leucine-supplemented piglets, compared with the control. Plasma concentrations of ammonia, urea, triglycerides, and growth-related hormones did not differ between the control and leucine groups. Collectively, these results indicate that L-leucine supplementation improves intestinal development and whole-body growth in suckling piglets with a normal birth weight.

Keywords Swine · Gene expression · Milk · Nutrition · Small intestine

Abbreviations

AA	Amino acids
BCAA	Branched-chain amino acids
BW	Body weight
IGF-1	Insulin-like growth factor 1
mTOR	Mammalian target of rapamycin
RT-PCR	Reverse-transcription polymerase chain reaction

Introduction

Branched-chain amino acids (BCAA; leucine, isoleucine and valine) are nutritionally essential for both humans and animals (Li et al. 2011a; Wu et al. 2014). Emerging evidence shows that L-leucine has a signaling role in the small intestine to activate the mammalian target of rapamycin (mTOR), thereby stimulating protein synthesis and inhibiting proteolysis (Rhoads and Wu 2009). Similar functions of L-leucine have also been reported for other tissues, including skeletal muscle (Columbus et al. 2015; Davis et al. 2010; Escobar et al. 2006) and mammary gland tissue (Lei et al. 2011, 2013). Thus, either dietary supplementation with L-leucine or provision of leucine-rich meal leads to increased protein accretion in tissues (Li et al. 2011a; Yin et al. 2010). Similarly, L-leucine supplementation enhanced intestinal development and whole-body growth in weaned piglets

✉ Zhenlong Wu
bio2046@hotmail.com

[1] State Key Laboratory of Animal Nutrition, Department of Animal Nutrition and Feed Science, China Agricultural University, Beijing 100193, China

[2] Department of Animal Science, Texas A&M University, College Station, TX 77843, USA

fed a low-protein diet (Zhang et al. 2013). Milk contains a high content of L-leucine (Davis et al. 1994; Wu and Knabe 1994). Effects of supplementing L-leucine to milk on breast-fed neonates are unknown (Rezaei et al. 2013; Wu 2014).

The intestinal development of neonates depends on the supplies of nutrients, growth factors, and hormones (Lei et al. 2013; Wu et al. 2013; Zabielski et al. 2008). Glutamate, glutamine and aspartate are major metabolic fuels for the small intestine to maintain its digestive function and to protect the integrity of the intestinal mucosa in piglets (Wu 2009). BCAA donate the amino group for the synthesis of glutamate and aspartate in the small intestine and other tissues, and glutamate is utilized to form glutamine in extra-intestinal tissues (Wu 2013a). Results of recent studies show several new features of BCAA metabolism and nutrition. First, 35–40 % of BCAA in the sow's milk are catabolized by the small-intestinal epithelial cells and bacteria in pigs (Chen et al. 2009; Dai et al. 2010), which might limit neonatal intestinal development. Second, the rate of muscle protein synthesis declines with the increased age, and adequate provision of L-leucine at an earlier stage may reverse this trend (Davis et al. 2010). Third, as noted previously, L-leucine can regulate expression of genes and signaling pathways to affect absorption and metabolism of dietary nutrients in animals (Yin et al. 2010; Zhang et al. 2013).

Based on the foregoing, we hypothesized that L-leucine supplementation to breast-fed neonates may promote their intestinal development. This hypothesis was tested in the present study with a piglet model. Because previous studies on neonatal piglets focused mostly on short-term parenteral nutrition through the use of intravenous infusion technique (Escobar et al. 2006; Suryawan et al. 2008), it is important to determine whether enteral supplementation with L-leucine may enhance intestinal development and whole-body growth in the suckling neonates.

Materials and methods

Animals and diets

Thirty crossbred (Large White × Landrace × Duroc) piglets with the birth weight (BW) of 1.45 ± 0.02 kg were selected and randomly allotted to either alanine (the isonitrogenous control) or L-leucine-supplemented group. These piglets had normal intrauterine growth before birth. During the entire experimental period, lactating sows had free access to a corn- and soybean meal-based diet (Table 1). Piglets were orally administrated with L-leucine (1.4 g/kg BW) or L-alanine (0.95 g/kg BW) in 10 mL saline twice daily at approximately 0800 and 1600 h, immediately after suckling. The total supplemental doses of L-leucine and L-alanine were 2.8 and 1.9 g/kg BW per day, respectively. The amount of leucine supplemented to piglets was calculated based on: (1) their milk intake (Kim and Wu 2004; Wu et al. 2000, 2004) to provide 200 % of L-leucine intake from sow's milk by 7-day-old piglets; and (2) results of our pilot study. Namely, in our preliminary study, we found that oral administration of 0.35 and 2.1 g L-leucine/kg BW to 7- to 21-day-old sow-reared piglets twice daily (0.70 and 4.2 g L-leucine/kg BW per day) had no effect ($P > 0.05$) on their daily BW gains (239 ± 6.3 and 238 ± 6.2 g/day, respectively; mean ± SEM, $n = 20$), compared with the daily BW gain (236 ± 6.5 g/day; mean ± SEM, $n = 20$) of piglets receiving an isonitrogenous amount of L-alanine (the control group), while oral administration of 0.7 and 1.4 g L-leucine/kg BW to 7- to 21-day-old sow-reared piglets twice daily (1.4 and 2.8 g L-leucine/kg BW per day) increased ($P < 0.05$) their daily BW gains by 10.6 and 11.9 % (261 ± 6.8 and 264 ± 7.0 g/day, respectively; mean ± SEM, $n = 20$), in comparison with the control group. L-Alanine was used as the isonitrogenous control, because it is not toxic and can be extensively catabolized by pigs (Kim and Wu 2004). All piglets had free access to sow's teats and drinking water throughout the experiment. They suckle approximately every 1.5 h. At 7, 14 and 21 days of age, milk intakes of piglets were measured using the weigh–suckle–weigh technique, as described by Wu et al. (2000). Body weights of piglets were recorded on the same days.

Table 1 Composition of the diet for lactating sows

Ingredient	Percentage (%; as-fed basis)
Corn	65.00
Soybean meal	26.00
Wheat bran	2.00
Soybean oil	2.00
Lysine–HCl (98.8 %)	1.00
Dicalcium phosphate	1.86
Limestone	0.94
NaCl	0.60
Vitamin–mineral premix[a]	0.60

Containing 90.0 % dry matter, 17.0 % crude protein, 1.64 % L-leucine, 0.76 % isoleucine, 0.84 % valine, and 3147 kcal metabolizable energy/kg diet

[a] Providing the following per kilogram of complete diet: 46.7 mg of Mn as manganous oxide; 75 mg of Fe as iron sulfate; 103.8 mg of Zn as zinc oxide; 9.5 mg of Cu as copper sulfate; 0.72 mg of I as ethylenediamine dihydroiodide; 0.23 mg of Se as sodium selenite; 7556 IU of vitamin A as vitamin A acetate; 825 IU of vitamin D3; 61.9 IU of vitamin E; 4.4 IU of vitamin K as menadione sodium bisulfate; 54.9 μg of vitamin B12; 13.7 mg of riboflavin; 43.9 mg of D-pantothenic acid as calcium pantothenate; 54.9 mg of niacin; and 1650 mg of choline as choline chloride

Tissue collection and processing

On day 14 of the trials, one hour after the last administration of L-leucine or L-alanine, blood samples were collected from

the anterior vena cava of piglets into vacuum tubes containing ethylenediaminetetraacetic acid (EDTA). Plasma was obtained by centrifugation at 3000×*g* for 5 min at 4 °C and frozen at −80 °C until analysis. Immediately after blood sampling, piglets were euthanized to obtain and weight tissues, as described by Wang et al. (2014a). The intestinal segments of the distal duodenum, mid-jejunum, and mid-ileum were flushed with ice-cold phosphate-buffered saline (PBS) and fixed in 10 % neutral buffered formalin for histological analysis (Wang et al. 2014a). A piece of the duodenum, jejunum, ileum and visceral organs (heart, liver, spleen, lung, kidneys) were weighed and snap frozen in liquid nitrogen and stored at −80 °C until analysis.

Biochemical analysis

Plasma concentrations of urea, glucose, triglycerides, total cholesterol, and ammonia were measured using colorimetric methods of commercial kits. Plasma insulin, insulin-like growth factor 1 (IGF-1), leptin and growth hormone were analyzed using commercial ELISA kits. All the assay kits were purchased from Jian Cheng Biotech (China) and used according to the instructions provided by the manufacturer.

Analysis of plasma amino acids by HPLC

Concentrations of amino acids (AA) in plasma were determined by reversed-phase HPLC after derivatization with *o*-phthaldialdehyde, as described previously described (Wu and Meininger 2008; Dai et al. 2014). Briefly, frozen samples (0.1 ml) were thawed and were acidified with 0.1 ml of 1.5 M $HClO_4$, followed by neutralization with 0.05 ml of 2 M K_2CO_3. The extract was used directly for AA analysis by HPLC.

Intestinal morphology examination

Morphological analysis was performed on formalin-fixed intestinal samples that were embedded in paraffin, sectioned, and stained with hematoxylin and eosin, as previously described (Wu et al. 1996). The sections were visualized using a light microscope (CK-40, Olympus, Tokyo, Japan) at 40× magnification. Villus height and crypts depth were measured and analyzed using an Image Analyzer (Lucia Software, Za Drahou, Czechoslovakia). Ten well-oriented villi and crypts were used for measuring villus height and crypts depth (Wang et al. 2014a). The villus height to crypt depth ratio was calculated.

Real-time RT-PCR analysis of mRNA expression levels for AA transporters

The general procedures for real-time RT-PCR analysis were performed as we described (Wang et al. 2015). Total RNA was extracted from jejunal tissue samples using Trizol reagent (CWBIO, Beijing, China) and reverse transcribed into cDNA in a volume of 10 μL using a high-capacity cDNA archive kit (TaKaRa, Japan) according to the manufacturer's protocol. The primer sequences used in this study are shown in Table 6. β-Actin was used as the internal control, which was not affected by L-leucine supplementation. Quantitative PCR was performed with SYBR Green (TaKaRa, Japan) using the ABI 7500 Real-time PCR system according to instructions of the manufacturer.

Western blot analysis

The general procedures for western blot analysis were performed as described by Wang et al. (2014b). Briefly, a fraction of the frozen jejunum (40 mg) was weighed and homogenized in liquid nitrogen. The homogenate was treated with RIPA buffer [50 mmol/L Tris–HCl (pH 7.4), 150 mmol/L NaCl, 1 % NP-40, 0.1 % SDS, 1.0 mmol/L PMSF, 1.0 mmol/L Na_3VO_4, 1.0 mmol/L NaF], which contained PMSF and a mixture of protease inhibitors (Roche Applied Science). The supernatant fluid was collected after centrifugation at 12,000×*g* for 15 min at 4 °C. Protein concentration was determined by the BCA protein Assay kit. Equal amounts of proteins (30 μg) were electrophoresed on 10 % SDS polyacrylamide gels and then were electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). The PVDF membranes were blotted with 5 % non-fat milk at 25 °C for 1 h and then were incubated with a primary antibody for 2 h at 25 °C or overnight at 4 °C. After wash, the membranes were incubated with the horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at 25 °C. The protein bands were developed by an enhanced chemiluminescence kit (Applygen Technologies Inc., Beijing, China) using the ImageQuant LAS 4000 mini system (GE Healthcare). Quantification of band density was determined using the Quantity One software (Bio-Rad Laboratories).

Statistical analysis

Data were analyzed using the independent-samples *T* test procedure of the SPSS statistical software. Values are expressed as mean ± SEM. *P* values < 0.05 were considered statistically significant (Assaad et al. 2014).

Results

Milk intake, body weights and intestinal morphology of piglets

Milk intakes of piglets at 7 days of age were 310 ± 22 and 306 ± 24 ml/kg BW/day in the control and leucine groups,

Table 2 Body weights of sow-reared piglets receiving oral administration of L-leucine or L-alanine between days 7 and 21 of age

Variable	Treatment		*P* value
	Alanine	Leucine	
BW on day 7, kg	2.88 ± 0.03	2.87 ± 0.04	0.842
d 14	4.60 ± 0.09	4.78 ± 0.06	0.101
d 21	6.21 ± 0.11	6.58 ± 0.08	0.012
Average daily weight gain (g/d)			
d 7-14	245 ± 9.0	271 ± 8.0	0.038
d 14-21	230 ± 9.3	257 ± 8.1	0.044
d 7-21	238 ± 6.2	265 ± 5.8	0.004

Values are mean ± SEM, $n = 30$ piglets/group

BW body weight

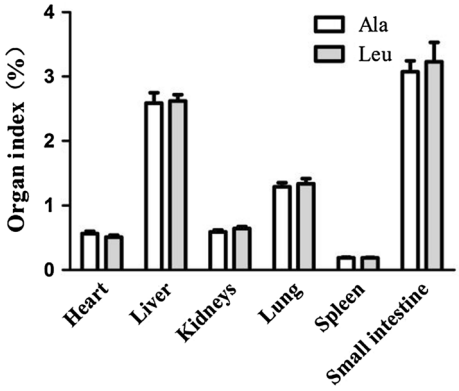

Fig. 1 Organ indexes for the heart, liver, kidneys, lung, spleen and small intestine (i.e., tissue weight/body weight) in 21-day-old suckling piglets that were administrated orally with 1.4 g/kg BW L-leucine or 0.95 g/kg BW L-alanine twice daily between 7 and 21 days of age. Values are mean ± SEM, $n = 6$ piglets/group

respectively ($P > 0.05$; mean ± SEM, $n = 10$). Milk intakes of piglets at 14 days of age were 251 ± 17 and 249 ± 18 ml/kg BW/day in the control and leucine groups, respectively ($P > 0.05$; mean ± SEM, $n = 10$). Milk intakes of piglets at 21 days of age were 180 ± 10 and 178 ± 12 ml/kg BW/day in the control and leucine groups, respectively ($P > 0.05$; mean ± SEM, $n = 10$). Based on our data that the sow's milk on days 7–21 of lactation contained 4.5 g L-leucine/L whole milk, as analyzed by high-performance liquid chromatography (Dai et al. 2014), leucine intake from the milk was 1.40 and 1.38 g/kg BW/day in the control and leucine groups, respectively, at 7 days of age; 1.13 and 1.12 g/kg BW/day in the control and leucine groups, respectively, at 14 days of age; and 0.81 and 0.80 g/kg BW/day in the control and leucine groups, respectively, at 21 days of age;. Thus, supplemental L-leucine amounted to 200, 250, and 350 % of L-leucine intake from the sow's milk by 7-, 14-, and 21-day-old piglets, respectively.

Table 3 Intestinal morphology in 21-day-old sow-reared piglets receiving oral administration of L-leucine or L-alanine between 7 and 21 days of age

Variable	Treatment		*P* value
	Alanine	Leucine	
Duodenum			
Villus height (μm)	423 ± 24	514 ± 20	<0.01
Crypt depth (μm)	195 ± 8.1	182 ± 8.4	0.269
Villus height: crypt depth	2.16 ± 0.06	2.86 ± 0.05	<0.01
Jejunum			
Villus height (μm)	651 ± 23	691 ± 16	0.165
Crypt depth (μm)	211 ± 5.9	212 ± 5.7	0.850
Villus height: crypt depth	3.11 ± 0.09	3.28 ± 0.08	0.165
Ileum			
Villus height (μm)	309 ± 5.6	319 ± 9.3	0.391
Crypt depth (μm)	134 ± 1.1	120 ± 0.85	<0.01
Villus height: crypt depth	2.31 ± 0.04	2.66 ± 0.08	<0.01

Values are mean ± SEM, $n = 6$ piglets/group

All piglets showed no signs of illness or abnormal behavior. The effects of leucine administration on BW gain between 7 and 21 days of age were determined. As shown in Table 2, the BW of piglets administrated with leucine was 6 % heavier ($P < 0.05$) than that in the control group at 21 days of age. In comparison with the age-matched piglets in the control group, daily weight gain between days 7 and 21 of age was 11.3 % higher ($P < 0.05$) in the leucine-supplemented piglets. The organ indexes (organ weight/BW × 100 %) for the heart, liver, kidneys, lung, spleen and small intestine did not differ between the two groups (Fig. 1). L-Leucine administration increased ($P < 0.01$) villus height and the ratio of villus height to crypt in the duodenum compared with controls (Table 3). The villus height and the crypt depth in jejunum were not affected by leucine, whereas the ratio of villus height to crypt depth was increased ($P < 0.01$) by L-leucine in the ileum (Table 3).

Concentrations of AA, other metabolites, and hormones in the plasma

Leucine supplementation increased plasma concentrations of leucine ($P < 0.01$) and asparagine ($P < 0.05$) by 62 and 24 %, respectively, but had no effect on those of other AA (including isoleucine, valine, glutamine, glycine, and lysine), as compared with the control group (Table 4). As expected, the plasma concentration of alanine was greater ($P < 0.01$) in piglets receiving oral administration of

Table 4 Plasma concentrations of amino acids in 21-day-old sow-reared piglets receiving oral administration of L-leucine or L-alanine between 7 and 21 days of age

Amino acids	Treatment		*P* value
	Alanine	Leucine	
Arg	142 ± 35	132 ± 29	0.577
Gly	973 ± 91	989 ± 118	0.910
His	61.9 ± 6.3	69.1 ± 7.5	0.103
Ile	71.5 ± 7.8	60.4 ± 7.0	0.208
Leu	192 ± 14	311 ± 29	<0.01
Lys	179 ± 24	163 ± 19	0.612
Met	36.3 ± 4.5	44.1 ± 3.0	0.532
Phe	57.0 ± 4.0	63.2 ± 6.7	0.440
Thr	114 ± 10	108 ± 19	0.896
Trp	45.8 ± 4.8	51.9 ± 3.5	0.352
Tyr	109 ± 8.7	118 ± 6.7	0.138
Val	153 ± 11	134 ± 11	0.325
Ala	985 ± 95	652 ± 42	<0.01
Asn	56.8 ± 3.7	70.2 ± 4.3	<0.05
Asp	38.8 ± 5.7	35.8 ± 3.4	0.114
Cit	97.9 ± 8.3	90.3 ± 7.0	0.831
Gln	471 ± 41	521 ± 55	0.361
Glu	123 ± 13	112 ± 11	0.532
Orn	104 ± 3.3	103 ± 2.3	0.802
Ser	197 ± 16	233 ± 31	0.286
Taurine	169 ± 44	163 ± 32	0.714

Values, expressed as μM, are mean ± SEM, $n = 6$ piglets/group

Table 5 Plasma concentrations of metabolites and hormones in 21-day-old sow-reared piglets receiving oral administration of L-leucine or L-alanine between 7 and 21 days of age

Variable	Treatment		*P* value
	Alanine	Leucine	
Urea (mmol/L)	1.68 ± 0.14	1.72 ± 0.12	0.791
Glucose (mmol/L)	6.15 ± 0.24	6.50 ± 0.19	0.276
Ammonia (μmol/L)	84.2 ± 4.8	75.8 ± 5.1	0.251
Triglycerides (mmol/L)	0.89 ± 0.07	0.94 ± 0.09	0.623
Total cholesterol (mmol/L)	3.27 ± 0.07	3.27 ± 0.07	0.941
Growth hormone (μg/L)	16.3 ± 0.21	16.5 ± 0.48	0.727
IGF-1 (μg/L)	12.6 ± 0.17	12.3 ± 0.15	0.32
Insulin (mIU/L)	55.9 ± 0.75	55.9 ± 0.51	0.955
Leptin (ng/L)	1.40 ± 0.04	1.40 ± 0.02	0.920

Values are mean ± SEM, $n = 6$ piglets/group

alanine as the isonitrogenous control, in comparison with the leucine group. Plasma concentrations of urea, glucose, ammonia, triglycerides, total cholesterol, growth hormone, insulin, IGF-1, or leptin did not differ ($P > 0.05$) between the control and leucine groups (Table 5).

mRNA levels and protein abundances of AA and peptide transporters in the jejunum

The mRNA levels for leucine transporters in the jejunum of piglets are shown in Fig. 2. Intestinal mRNA levels for *SLC6A14, SLC6A19*, and *SLC7A9* were increased ($P < 0.05$), whereas those for *SLC7A7* were lowered ($P < 0.05$) by oral administration of L-leucine to sow-reared piglets. L-leucine had no effect on the mRNA levels for *SLC1A5, SLC7A1, SLC15A1*, or *ATP1A1* in the jejunum, as compared with the control group. To determine whether the transcriptional alterations affected protein abundance for intestinal AA transporters, Western blot analysis was performed using specific antibodies. The results indicated that the protein abundance of $ATB^{0,+}$ (encoded by *SLC6A14*) was increased ($P < 0.05$), whereas that for LAT2 was lowered ($P < 0.05$) by leucine (Fig. 3). L-leucine supplementation did not affect the protein abundance of ASCT2 (encoded by *SLC1A5*), rBAT (encoded by *SLC3A1*), CAT1 (encoded by *SLC7A1*), or Na^+–K^+–ATPase (encoded by *ATP1A1*) in the jejunum of leucine-supplemented piglets (Fig. 3).

Discussion

L-Leucine is not synthesized by animal cells and, therefore, must be provided in diets for nonruminant mammals, birds, fish, and other animal species (Wu et al. 2014). As a functional AA (Wu 2013a, b), L-leucine activates the MTOR signaling pathway to regulate the synthesis and catabolism of proteins, including enzymes involved in biochemical reactions (Davis et al. 2010; Escobar et al. 2006). Although leucine is usually abundant in plant or animal proteins (Li et al. 2011b; Wu 2014), an increase in its exogenous provision beyond that from enteral foods may enhance lean-tissue growth in young animals (Wu et al. 2014). To our knowledge, little research has been done to determine effects of dietary supplementation with L-leucine to dam-reared neonates with either a normal- or a low-birth weight.

The postnatal development of the gastrointestinal system, which is dependent upon the actions of various bioactive substances (e.g., hormones, growth factors, and peptides in milk or food), is critical for intestinal and whole-body health in humans and animals (Wang et al. 2010; Zabielski et al. 2008). A positive association between a high content of L-leucine in the sow's milk (Wu and Knabe 1994) and the rapid intestinal development in neonatal pigs (Wu et al. 2000) suggests that L-leucine may be a biologically active nutrient that can regulate the development and maturation of the small intestine. Recent studies show that epithelial absorptive cells (Chen et al. 2009) and the intestinal bacteria (Dai et al. 2010) of the small intestine

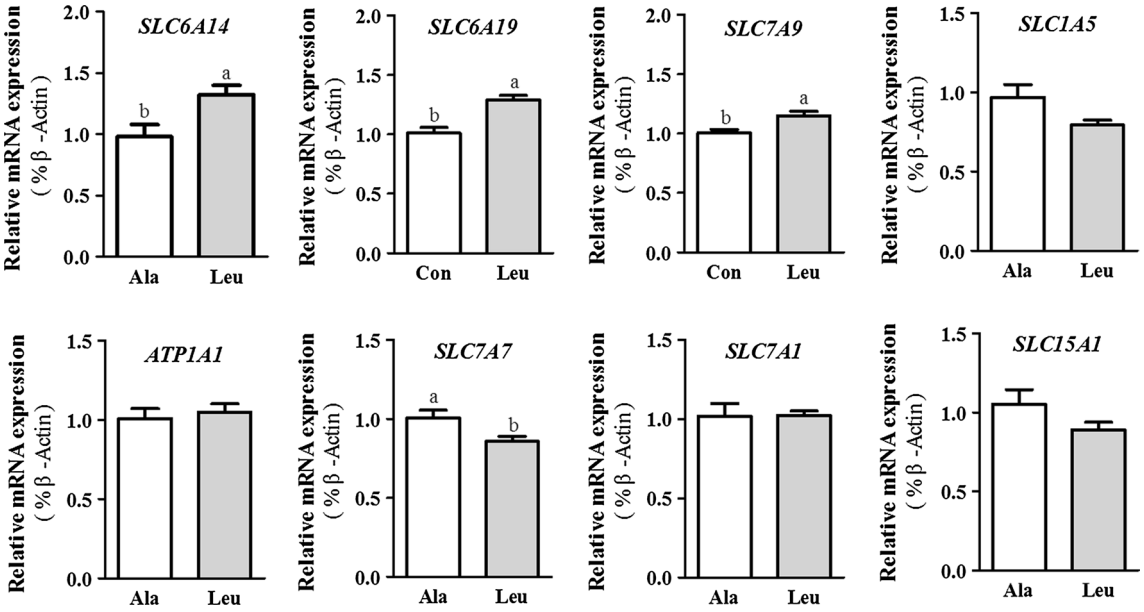

Fig. 2 Effects of L-leucine supplementation on mRNA levels of AA transporters (SLC6A19, SLC6A14, SLC1A5, SLC7A1, SLC7A7, and SLC7A9), ATP1A1, and PepT1 in the jejunum of 21-day-old suckling piglets that were supplemented with 1.4 g/kg BW L-leucine or 0.95 g/kg BW L-alanine twice daily between 7 and 21 days of age. Values are mean ± SEM, $n = 6$. Means without a common letter differ, $P < 0.05$

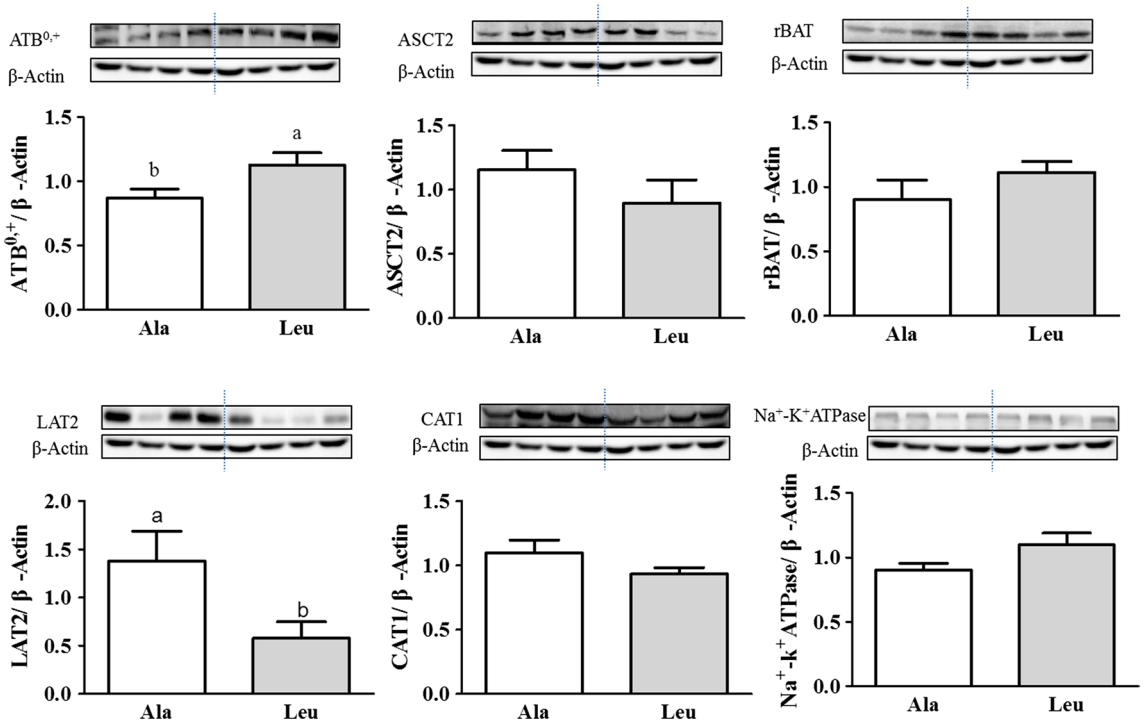

Fig. 3 Effects of L-leucine supplementation on the abundances of $ATB^{0,+}$, ASCT2, rBAT, LAT2, CAT1, and Na^+-K^+ ATPase proteins in the jejunum of 21-day-old suckling piglets that were supplemented with 1.4 g/kg BW L-leucine or 0.95 g/kg BW L-alanine twice daily between 7 and 21 days of age. Values are mean ± SEM, $n = 6$. Means without a common letter differ, $P < 0.05$

extensively degrade approximately 40 % of dietary L-leucine, thereby reducing the entry of dietary leucine into the portal circulation (Wu et al. 2014). Although growing evidence shows a beneficial effect of leucine in stimulating muscle protein biosynthesis in rats, pigs and humans (Suryawan et al. 2008, 2012), it is unknown whether

Table 6 Sequences of primers used for quantitative real-time PCR

Gene	Protein		Primer sequences (5′–3′)
SLC6A19	B^0AT1	F	CACAACAACTGCGAGAAGGA
		R	CCGTTGATAAGCGTCAGGAT
SLC6A14	$ATB^{0,+}$	F	CCGTGGTAACTGGTCCAAAAA
		R	CCAATCCCACTGCATATCCAA
SLC1A5	ASCT2	F	GCCAGCAAGATTGTGGAGAT
		R	GAGCTGGATGAGGTTCCAAA
SLC7A1	CAT1	F	TGCCCATACTTCCCGTCC
		R	GGTCCAGGTTACCGTCAG
SLC7A7	y^+LAT1	F	GCCCATTGTCACCATCATC
		R	GAGCCCACAAAGAAAAGC
SLC3A1	rBAT	F	TTTCCGCAATCCTGATGTTC
		R	GGGTCTTATTCACTTGGGTC
SLC7A9	$b^{0,+}AT$	F	ATCGGTCTGGCGTTTTAT
		R	GGATGTAGCACCCTGTCA
SLC15A1	PepT1	F	CCCAGGCTTGCTACCCAC
		R	ACCCGATGCACTTGACGA
ATP1A1	Na^+/K^+ ATPase	F	ATCGCAAATACGGAACGGACT
		R	GCCGACAGAACTTGACCCAT
β-actin		F	TGCGGGACATCAAGGAGAAG
		R	AGTTGAAGGTGGTCTCGTGG

B^0AT1 system B^0 neutral AA transporter, *$ATB^{0,+}$* system $B^{0,+}$ neutral AA transporter, *ASCT2* Na^+-neutral AA exchanger, *CAT1* cationic amino acid transporter 1, *y^+LAT1* y^+ L amino acid transporter-1, *rBAT* basic amino acid transporter, *$b^{0,+}AT$* related to $b^{0,+}$ amino acid transporter, *PepT1* intestinal peptide transporter

L-leucine supplementation may up-regulate expression of genes for AA transport in the small intestine of milk-fed neonates.

In the present study, 7- to 21-day-old suckling piglets were orally administrated with 1.4 g L-leucine/kg BW twice daily for 14 days. The amount of leucine was based on its provision from the sow's milk (Kim and Wu 2004; Wu et al. 2000). We found that leucine supplementation increased villus height in the duodenum, but not in the jejunum or ileum (Table 3). It is unknown whether this effect of leucine may result from stimulation of pancreatic secretions into the duodenum. Nonetheless, leucine appears to have a positive effect on the proliferation of enterocytes in the duodenum of piglets. In addition, the crypt depth of the ileum was lower in leucine-supplemented piglets, as compared with the control group (Table 3), because of enhanced migration of epithelial cells in the crypt via activating the mTOR signaling (Rhoads et al. 2008). It is unlikely that leucine supplementation acts by affecting plasma concentrations of growth hormone, IGF-1, insulin, and leptin in piglets, because these variables did not differ between the control and leucine-supplemented groups (Table 5).

Amino acids released from the hydrolysis of dietary proteins and peptides in the lumen of the small intestine are transported across cell membranes by a complex system of multiple amino acid transporters (Poncet and Taylor 2013; Taylor 2014). A number of L-leucine transporters have been identified on the apical surface of the mammalian small intestine that is responsible for the intestinal absorption of L-leucine. Among the transporters examined in our present study, the jejunal mRNA levels for SLC6A14, SLC6A19, and SLC7A9 were increased, and those for SLC7A7 were decreased by L-leucine supplementation. In contrast, the protein abundance of $ATB^{0,+}$ (encoded by SLC6A14) were enhanced, while that for LAT2 (encoded by SLC7A8) was decreased, by L-leucine supplementation. The inconsistency between the mRNA and protein levels for a transporter may be explained by different mechanisms responsible for the regulation of gene transcription and mRNA translation in the small intestine. Additional studies are required to test this hypothesis.

Beginning at 7 days of age, production of milk by sows becomes a limiting factor for maximal growth of piglets (Rezaei et al. 2013). It is noteworthy that L-leucine supplementation increased the growth of sow-reared piglets between 7 and 21 days of age (Table 2). Thus, although milk is rich in leucine (Wu and Knabe 1994), its provision is insufficient for realizing the genetic potential of piglets for maximum protein deposition. Similar results have been reported for glutamine supplementation to sow-reared piglets (Haynes et al. 2009). Of note, oral administration of L-leucine to breast-fed piglets even at 2.8 g/kg BW per day between 7 and 21 days of age enhanced their daily BW gain only by 11.3 % (Table 2). It is possible that the low content of L-arginine and glycine in sow's milk (Wu and Knabe 1994) limits maximal responses of suckling piglets to L-leucine supplementation. L-Leucine supplementation did not affect the plasma concentrations of isoleucine, valine, ammonia, urea, glucose, triglycerides, and total cholesterol during the entire experimental period (Tables 4, 5, 6). These results were not consistent with those reported for weaned piglets (Yin et al. 2010; Zhang et al. 2013), adult mice (Newgard 2012), and rats (Newsholme et al. 2014). This discrepancy might be due to the differences in experimental protocols, such as dietary intakes of AA (including leucine), supplemental doses of leucine, species, and ages of the animals. We suggest that dietary supplementation of L-leucine that amounted to 200–350 % of L-leucine intake from the sow's milk did not result in antagonism among the three BCAA in young pigs.

In conclusion, L-leucine supplementation in suckling piglet improves intestinal development as indicated by increased villus height in the duodenum, an elevated ratio of villus height to crypt depth in the duodenum and ileum, and enhanced expression of leucine transporters

in the jejunum. These effects of L-leucine supplementation resulted in improved absorption of dietary nutrients and growth performance of the young animals. L-Leucine is a functional AA to augment protein deposition in sow-reared piglets.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethics statement The use of animals for this research was approved by the Institutional Animal Care and Use Committee of China Agricultural University.

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