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L-Leucine supplementation reduces growth performance accompanied by changed profiles of plasma amino acids and expression of jejunal amino acid transporters in breast-fed intra-uterine growth-retarded piglets

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Abstract

Previously, we provided an evidence that L-Leucine supplementation facilitates growth performance in suckling piglets with normal birth weight. However, it remains hitherto obscure weather breast-fed piglets displaying intra-uterine growth restriction (IUGR) show a similar effect in response to L-Leucine provision. In this study, 7-d-old sow-reared IUGR piglets were orally administrated with L-Leucine (0, 0·7, 1·4 or 2·1 g/kg BW) twice daily for 2 weeks. Increasing leucine levels hampered the growth performance of suckling IUGR piglets. The average daily gain of IUGR piglets was significantly reduced in 1·4 g/kg BW and 2·1 g/kg BW L-Leucine supplementation groups ($P < 0·05$). Except for ornithine and glutamine, the plasma concentrations of other amino acids were abated as L-Leucine levels increased ($P < 0·05$). Leucine supplementation led to reduction in the levels of urea, blood ammonia, blood glucose, TAG and total cholesterol, as well as an elevation in the level of LDL-cholesterol in suckling IUGR piglets ($P < 0·05$). In addition, 1·4 g/kg BW of L-Leucine enhanced the mRNA expression of $ATB^{0,+}$, whereas decreased the mRNA abundances of *CAT1*, $y + LAT1$, *ASCT2* and $b^{0,+}$AT in the jejunum ($P < 0·05$). Concomitantly, the jejunum of IUGR piglets in L-Leucine group contains more $ATB^{0,+}$ and less SNAT2 protein than in the control ($P < 0·05$). Collectively, L-Leucine supplementation impairs growth performance in breast-fed IUGR piglets, which may be associated with depressed nutritional conditions and alterations in the uptake of amino acids and the expression of amino acid transporters in the small intestine.

Keywords: **Leucine: Intra-uterine growth restriction: Amino acids: Piglets: Transporter**

Intra-uterine growth restriction (IUGR) is a major determinant of infant mortality and morbidity in human obstetrics and domestic animal production[1]. In addition to showing a high rates of perinatal mortality, surviving neonates with IUGR display retarded effects on postnatal development, feed utilisation efficiency, and intestinal development and maturity[2]. Moreover, emerging evidence suggests that IUGR contributes to a broad range of metabolic disorders and chronic diseases in adults, including cardiovascular disorder, hypertension, obesity, insulin resistance and diabetes type II[3,4]. It is generally believed that maternal, placental or fetal origin risk factors adversely affect fetal growth and contribute to the development of IUGR[5]. However, nutritional strategies to improve the growth of IUGR piglets are not available due to the lack of knowledge about the mechanisms of IUGR in humans and animals.

Aside from serving as the substrate for protein synthesis, L-Leucine, a nutritionally essential amino acid for both humans and animals[6,7], has a signalling role to activate the mechanistic target of rapamycin, thereby stimulating protein synthesis and inhibiting proteolysis in the small intestine[8], skeletal muscle[9–12] and mammary gland tissue[13,14]. Dietary supplementation with L-Leucine or a provision of leucine-rich meal leads to enhanced intestinal development and increased protein accretion in piglets[7,15,16]. Despite the fact that sow's milk contains a high proportion of L-Leucine[17,18], our previous study showed that L-Leucine supplementation improved intestinal development and whole-body growth in normal suckling piglets[19]. This result indicates that the extra provision of L-Leucine is required for maximal growth in piglets due to the catabolism of L-Leucine by small intestinal epithelial cells and bacteria in pigs[20,21].

IUGR piglets are characterised by continuous impairment of intestinal, liver and muscle development[22–24]. Using temporal proteomic approaches, Wang *et al.* demonstrated that alterations in amino acid metabolism might be one of the underlying mechanisms responsible for the impaired fetal development in IUGR fetuses[22]. Further studies show that the concentration of L-Leucine in the umbilical vein[25] was lowered in IUGR piglets compared with that of control. The plasma branched-chain

Abbreviation: IUGR, intra-uterine growth restriction.

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amino acid concentrations of umbilical artery and vein were lower in growth-restricted neonates than in normal body weight controls[26–30]. Consistently, branched-chain amino acid-supplemented diet partially improved fetal growth restriction caused by maternal undernutrition in mice[31]. Supplementing weaned IUGR piglets with L-leucine improved muscle protein synthesis and aided in weight gain, as shown in a study by Xu *et al.*[32]. These findings imply that L-leucine might be a nutritionally vital factor in the growth and development of IUGR neonates. However, it remains unknown whether supplementation of L-leucine improves the growth of IUGR piglets during the suckling period. Neonatal piglets serve as an excellent model for addressing the scientific problem related to newborn human beings and provide evidence on the regulation role of L-leucine on early growth and metabolism of suckling piglets. Thus, we investigated the impact of L-leucine supplementation on IUGR suckling piglets. Our data indicate that L-leucine administration presents an adverse effect in breast-fed IUGR piglets, with a significant reduction in average daily gain and plasma free amino acid levels. Additionally, we profiled the blood biochemistry and the expression of amino acid transporters in the small intestine.

Materials and methods

Animals and experimental design

All the animal treatment procedures were approved by the Institutional Animal Care and Use Committee of China Agricultural University and conducted strictly following the Guide for the Care and Use of Laboratory Animals of the Chinese Association for Laboratory Animal Science and Use. A total of sixty crossbred (Large White × Landrace × Duroc) female neonatal IUGR piglets (it has been pointed out that female newborns are more prone to develop IUGR[33]) weighing 0·85 (SEM 0·02) kg were chosen and randomly divided into five groups assigned to five lactating multiparous sows in parity 3. Twelve IUGR pigs fed by each sow were randomly allocated to four treatment groups (0, 0·7 1·4, 2·1 g/kg BW of L-leucine supplementation) with three piglets per group. Thus, fifteen piglets in total were included in each group. All the piglets and lactating sows were maintained in a farrowing pen with temperature at 19–21°C. The obstetric tables for lactating sows were equipped with steel piglet isolation guardrail and floating guardrails. The isolation guardrails assisted the piglets to more effectively contact the sow's teats. The floating guardrails function to prevent piglets from being crushed by sows. People who drenched the piglets were not blind to the treatment. To achieve an isonitrogenous feeding, 1·43, 0·95, 0·48 and 0 g/kg BW of L-alanine were mixed into the 10 ml of L-leucine-containing solutions. All the breast-fed IUGR piglets were given orally twice per day at 0800 and 1600 hours with the prepared saline solution containing L-leucine and/or L-alanine for 14 consecutive days (from 7 to 21 d of age) (Fig. 1). To ensure that the isonitrogenous L-leucine/L-alanine supplements were added in equal volume, plastic medical graduated injector syringes were used to quantify the volume of solution. These liquid preparations were injected into the throat of the piglets. The feed formula for lactating sows was designed following the nutrition requirements provided by National Research Council, which was the same as our previous report[19]. Based on intakes of milk and body weight, the amount of leucine supplemented to the piglets was 100 %, 200 % or 300 % of the leucine consumed by 7-d-old piglets. All piglets were afforded free access to teats of sow (the frequency was approximately every 1·5 h) and drinking water, and milk intake was recorded on 14 and 21 d of age using the weigh–suckle–weigh technique[34]. Milk intake did not differ among the four groups of piglets. Body weight was recorded at 0700 hours on 7, 14 and 21 d of age.

At the end of the test period (day 21 of age), piglets undergoing the last L-leucine and/or L-alanine supplementation were subjected to blood collection 1 h later. Blood samples obtained from the anterior vena cava of piglets were collected into EDTA anticoagulant tubes. Plasma separation was implemented by centrifugation at 3000 ***g*** for 5 min at 4°C and frozen at −80°C before analysis. For sampling, six piglets were randomly selected from the control and the 1·4 g/kg BW L-leucine treatment group. We chose piglets from 1·4 g/kg BW L-leucine owing to this dose of L-leucine showed an obvious effect on growth performance of IUGR piglets. In addition, we have previously reported that 1·4 g/kg BW L-leucine improved intestinal development and growth in normal suckling piglets[35], so as to make a contrast with the suckling IUGR piglets. The piglets were anaesthetised with Zoletil (10 mg/kg BW) and then euthanised via exsanguination in a slaughterhouse. Thereafter, the heart, liver, spleen, lung and kidney tissues were removed from the body and weighed. Each segment (duodenum, mid-jejunum and mid-ileum) of small intestine was flushed using ice-cold PBS (0·137 M NaCl, 2·7 mM KCl, 0·01 M Na_2PO_4, 1·8 mM KH_2PO_4, pH 7·4), followed by drain off excess fluid and weight record. Approximately 1 cm of segments from distal duodenum, mid-jejunum and mid-ileum samples, respectively, was snap frozen in N_2 and stored at −80°C for the subsequent assays.

Blood biochemical analysis

Plasma was separated by centrifugation at 3000 ***g*** for 5 min at 4°C and stored at −80°C until analysis. Plasma contents of urea, glucose, ammonia, TAG, total cholesterol, HDL-cholesterol and LDL-cholesterol were analysed by colorimetric methods using commercial kits purchased from Nanjing Jiancheng Bioengineering Inc. All the assay methods were carried out in accordance with the protocols provided by the manufacturers. The biochemical reaction principles for urea determination are as follows: Urea is hydrolysed by urease, resulting in the production of ammonia and carbon dioxide. In an alkaline medium, ammonia and phenol chromogenic reagents produce a blue substance. The amount of this substance is proportional to the urea content and can be evaluated colorimetrically at 640 nm. Quantification of blood glucose was performed using a glucose oxidase method. Glucose oxidase catalyses the formation of gluconic acid and hydrogen peroxide (H_2O_2) from glucose. H_2O_2 reacts with peroxidase to yield o-tolidine, which leads to the generation of a blue substance. The absorbance of this coloured substance was measured at 630 nm. Determination of blood ammonia was based on the following principle: The rate of

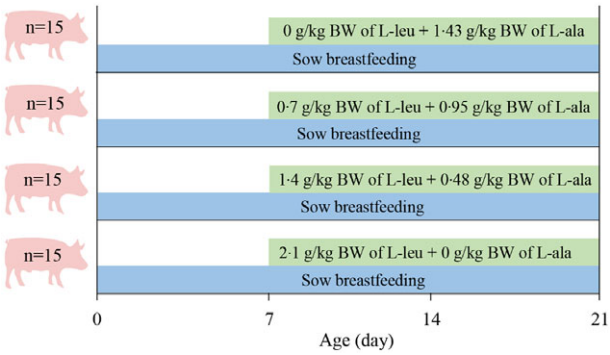

Fig. 1. Experimental design showing intra-uterine growth restriction (IUGR) piglets supplemented with L-leucine or L-alanine from 14 to 21 d of age. Sixty female IUGR piglets weighing 0·85 (SEM 0·02) kg were randomly assigned to five lactating sows in parity 3. Twelve IUGR pigs fed by each sow were randomly allocated to one of four treatment groups, as indicated. Each group contained a total of fifteen piglets. To achieve isonitrogenous feeding, L-alanine was added to 10 ml of L-leucine-containing solutions. All of the breast-fed IUGR piglets were given orally twice a day for 14 consecutive days (from 7 to 21 d of age). L-ala, L-alanine; L-leu, L-leucine.

enzymatic reaction, that is, the conversion of NAD(P)H to $NAD(P)^+$, was proportional to the concentration of ammonia in the reaction system in the presence of excess α-ketoglutarate, NAD(P)H and sufficient glutamate dehydrogenase. TAG levels were quantified by GPO-PAP method. TAG were hydrolysed into glycerol and NEFA by lipoprotein lipase. Glycerol is converted to 3-phosphoglycerol by ATP and glycerol kinase, which is then oxidised by phosphoglycerol oxidase to produce phosphoric acid. Subsequently, phosphoric acid is converted into dihydroxyacetone and H_2O_2 by glycerophosphate oxidase. H_2O_2 together with 4-aminoantipyrine and 4-chlorophenol is catalysed by peroxidase to form red quinone compounds, the absorbance of which at 510 nm is proportional to the concentration of TAG. The content of total cholesterol was determined by COD-PAP method. Cholesteryl ester is hydrolysed to free cholesterol by cholesterol ester hydrolase, which is oxidised to cholestenone by cholesterol oxidase to generate H_2O_2, followed by a catalysation process by peroxidase in the presence of 4-aminoantipyrine and phenol to generate a red quinoneimine pigment. HDL-cholesterol and LDL-cholesterol were assessed by surfactants treatment methods. HDL-cholesterol forms soluble complexes in the presence of surfactants, allowing HDL-cholesterol to react directly with enzyme reagents to produce H_2O_2. The red quinone compounds of 4-(p-benzoquinone-monoimino)phenazone are synthesised by oxidase in the presence of H_2O_2, 4-amino-antipyrazoline and phenol. The absorbance of 4-(p-benzoquinone-monoimino)phenazone at 546 nm was proportional to the content of HDL-cholesterol. Amphoteric surfactants selectively protect LDL-cholesterol, while non-LDL-cholesterol are eliminated by cholesterol enzymatic reagents. Cholesterol is released from LDL-cholesterol and participates in the Trinder reaction, which produces a coloured substance measured at 546 nm.

Analysis of free amino acids in plasma and small intestine

A known value of 100 μl of plasma samples was acidified with 100 μl of 1·5 M $HClO_4$, followed by neutralisation with 50 μl of 2 M K_2CO_3. The extract was obtained by centrifugation (21 000 ***g***, 10 min) and subjected to derivatisation with o-phthaldialdehyde and analysis of amino acids. For small intestine tissue, ~40 mg powdered sample was weighed and acidified with 200 μl of 1·5 M $HClO_4$ and then was mixed with 100 μl of 2 M K_2CO_3. After a derivatisation with o-phthaldialdehyde, free amino acid levels in plasma and small intestinal tissues were detected by reversed-phase HPLC using a HPLC apparatus (Waters Inc.) equipped with an analytical column (C18; 4·6 mm × 15 cm, 3 μm) protected by a guard column (C18; 4·6 mm × 5 cm, 20–40 μm) and a Model 2475 Multi λ fluorescence detector as previously described[36].

Detection of mRNA expression of transporters for amino acids and peptides

Jejunal tissues (~40 mg per sample) were pulverised in N_2, and total RNA extraction from the tissues was performed by using a commercial Trizol reagent (CWBIO). After a quality evaluation, the total RNA was subjected to reverse transcription by a PrimeScript RT Master Mix kit (TaKaRa) following the instructions provided by manufacturer. Reverse transcription solution system was prepared in a 20 μl final volume containing 2·0 μl of 5 × PrimeScript RT Master Mix, 2·0 μl of RNA (25 ng/μl) and RNA enzyme-free water. The reaction condition was 37°C 15 min, 85°C 5 s, followed by cooling at 4°C. The cDNA products were preserved at −20°C. Real-time quantitative PCR was conducted by using SYBR Green reagent (TaKaRa) executing the procedure as follows: (1) 95°C, 30 s; (2) 95°C, 5 s; 60°C, 34 s; 40 cycles. The reaction system was composed of template cDNA, forward primer (200 nM), reverse primer (200 nM), SYBR Mix, ROX and PCR grade water. The primer sequences used for real-time PCR are listed in Table 1. *β-actin*, which was stably expressed in the jejunum of piglets exposed to the present treatment condition, was selected as internal reference. Gene expression relative to *β-actin* was normalised by comparative Ct ($2^{-\Delta\Delta Ct}$) method[37].

Determination of protein abundance of amino acid transporters

Approximately 40 mg of frozen jejunum samples was pulverised in N_2 and subjected to protein extraction using RIPA buffer (50 mM Tris-HCl (pH 7·4), 150 mM NaCl, 1 % NP-40, 0·1 % SDS, 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF) added with a protease inhibitors cocktail (Roche Applied Science). Bicinchoninic acid assay was carried out in order to quantify the protein concentration for each samples. Thirty microgram of proteins was electrophoretic separated by 10 % SDS polyacrylamide gels, followed by transfer to a polyvinylidene difluoride membrane (Millipore). After blocking with 5 % non-fat milk at room temperature for 1 h, the membranes were incubated with a primary antibody overnight at 4°C and then were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The following primary and secondary antibodies were used: anti-*β*-actin (1:2000, Santa Cruz Biotechnology, sc-47778), anti-$ATB^{0,+}$ (1:500, Santa Cruz Biotechnology, sc-169348), anti-CAT1 (1:500, Santa Cruz Biotechnology, sc-33087), anti-PAT1 (1:500, Santa Cruz Biotechnology,

Table 1. Primers sequences used for quantitative real-time PCR

Gene	Primer sequences(5' to 3')
B^0AT1	F: CACAACAACTGCGAGAAGGA R: CCGTTGATAAGCGTCAGGAT
ATB^{0+}	F: CCGTGGTAACTGGTCCAAAAA R: CCAATCCCACTGCATATCCAA
ASCT2	F: GCCAGCAAGATTGTGGAGAT R: GAGCTGGATGAGGTTCCAAA
CAT1	F: TGCCCATACTTCCCGTCC R: GGTCCAGGTTACCGTCAG
y^+LAT1	F: GCCCATTGTCACCATCATC R: GAGCCCACAAAGAAAAGC
rBAT	F: TTTCCGCAATCCTGATGTTC R: GGGTCTTATTCACTTGGGTC
$b^{0+}AT$	F: ATCGGTCTGGCGTTTTAT R: GGATGTAGCACCCTGTCA
PepT1	F: CCCAGGCTTGCTACCCAC R: ACCCGATGCACTTGACGA
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; $b^{0,+}AT$, $b^{0,+}$ amino acid transporter; PepT1, intestinal peptide transporter.

sc-161150), anti-rBAT (1:500, Santa Cruz Biotechnology, sc-32930), anti-SNAT2 (1:500, Santa Cruz Biotechnology, sc-166366), anti-xCT (1:500, Santa Cruz Biotechnology, sc-79359), horseradish peroxidase-AffiniPure goat anti-rabbit IgG (1:5000, Huaxingbio Co., Ltd., HX2031), horseradish peroxidase-AffiniPure goat anti-mouse IgG (1:5000, Huaxingbio Co., Ltd., HX20321) and horseradish peroxidase-AffiniPure rabbit anti-goat IgG (1:5000, Huaxingbio Co., Ltd., HX2030). The protein bands were visualised by using the ImageQuant LAS 4000 mini system (GE Healthcare) and quantitative analysed by Quantity One software (Bio-Rad Laboratories).

Statistical analysis

The statistical unit was the pig because all of the pigs were reared in the same testing room. The sow was not included in the statistical model. Data were analysed by one-way ANOVA followed by Student–Newman–Keuls multiple comparison or the independent-samples *t*-test procedure within the SPSS statistical software. Values are expressed as mean values with their standard error of the mean. $P < 0{\cdot}05$ indicates statistically significant.

Results

Effects of L-leucine on growth performance and organ weight of suckling intra-uterine growth restriction piglets

We observed no obvious stress in all the piglets respond to drenching act. Importantly, a recent publication demonstrates that the act of drenching does not impose additional stress to low birth weight piglets[38], providing a support on the handling method used in our study. The effects of L-leucine on the growth performance of IUGR newborn piglets are shown in Table 2. At the end of the trial, the body weight of piglets receiving 0·7, 1·4 and 2·1 g/kg BW of L-leucine supplementation was decreased by 5·0, 11·4 and 5·8 %, respectively, compared with the control group ($P < 0{\cdot}05$). The average daily gain of piglets was lower by 19·3, 24·2 and 19·8 %, respectively, with the increased level of L-leucine from day 7 to 21 of age. An increasing dose of L-leucine significantly reduced the average daily gain of IUGR suckling piglets for the entire trial period by 8·6, 18·9 and 11·7 %, respectively ($P < 0{\cdot}01$). Although a reduction in growth performance was observed, the organ index (organ weight/body weight) of the heart, liver, kidney, lung, spleen and small intestine of IUGR newborn piglets was not affected by L-leucine supplementation (Fig. 2).

Effects of L-leucine levels on blood biochemistry in newborn piglets with intra-uterine growth restriction

As shown in Table 3, L-leucine supplementation significantly reduced the contents of urea, blood ammonia, glucose, TAG and total cholesterol in plasma ($P < 0{\cdot}01$), with a linear relationship with the increase of L-leucine levels. Concretely, dietary leucine levels reduced the plasma urea in IUGR suckling piglets significantly by 17·6, 24·7 and 26·1 % ($P < 0{\cdot}05$), respectively. Ammonia content in plasma was decreased by 5·9, 33·1 and 31·7 % with the increase of L-leucine supplement ($P < 0{\cdot}05$). Plasma glucose concentration significantly decreased by 9·3, 11·5 and 8·5 % in leucine-treated group compared with the control ($P < 0{\cdot}05$). Piglets subjected to increased L-leucine supplementation displayed declining plasma TAG by 15·8, 25·8 and

Table 2. The growth performance of breast-fed intra-uterine growth restriction piglets supplemented with varying levels of L-leucine (Means values with their pooled standard errors of the mean, *n* 15)*

	L-leucine supplementation (g/kg BW)					
Items	0	0·7	1·4	2·1	SEM	*P*
Body weight (kg)						
Day 7	1·88	1·89	1·90	1·90	0·02	0·996
Day 14	3·33	3·06	3·00	3·06	0·05	0·157
Day 21	4·99[a]	4·74[a,b]	4·42[b]	4·65[a,b]	0·07	0·026
Average daily weight gain (g/d)						
Day 7–14	207[a]	167[b]	157[b]	166[b]	6·37	0·022
Day 14–21	236	240	203	227	5·04	0·233
Day 0–21	222[a]	203[a,b]	180[b]	196[b]	4·97	0·003

* Piglets were supplemented with L-leucine twice daily.
[a,b,c,d]Mean values within a column sharing different superscript letters differ ($P < 0{\cdot}05$).
[d]0 represents 7 d of age.

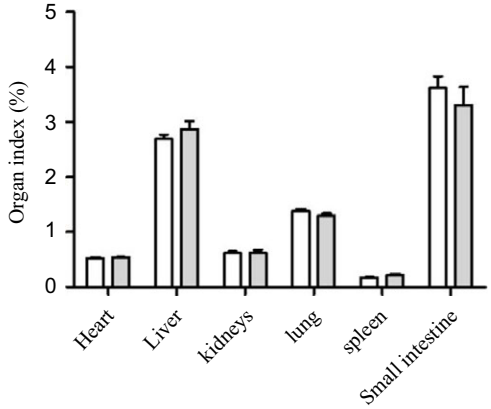

Fig. 2. Effect of 1·4 g/kg BW L-leucine on organ index (tissue weight/ body weight) of breast-fed intra-uterine growth restriction piglets. Values are means with their standard error of the mean, *n* 6. Piglets were supplemented with 1·4 g/kg BW L-leucine or 0·95 g/kg BW L-alanine (the isonitrogenous control) twice daily for 2 weeks. □, Ctl; ▭, Leu.

30 %, respectively. No differences were observed in regard to plasma HDL for IUGR piglets supplemented with different levels of L-leucine ($P > 0·05$). The plasma LDL content was increased by 13·6, 18·1 and 22·0 % with the increase of L-leucine supplementation ($P < 0·05$).

Amino acid profiles in plasma and intestinal tissues in suckling piglets with intra-uterine growth restriction receiving L-leucine

The data on plasma free amino acid concentrations of breast-fed IUGR newborn piglets receiving different levels of L-leucine are shown in Table 4. L-leucine supplementation significantly increased the blood leucine content of 21-d-old newborn piglets compared with that of the control group ($P < 0·01$). With increasing L-leucine supplementation, the plasma levels of the essential amino acids, including histidine, isoleucine, methionine, phenylalanine, threonine and valine, were decreased significantly ($P < 0·01$). Similarly, the contents of non-essential amino acids including alanine, arginine, aspartic acid, asparagine, citrulline, glutamine, glutamic acid, glycine, serine, taurine and tyrosine also showed a marked reduction, as the level of leucine supplement increased ($P < 0·01$). The amino acid profiles in the duodenum, jejunum and ileum tissues of IUGR suckling piglets orally administered with L-leucine are listed in Table 5. L-leucine treatment (1·4 g/kg BW) significantly increased the leucine content in all segments of small intestine including duodenum, jejunum and ileum of IUGR suckling piglets ($P < 0·05$). The contents of aspartate, glutamic acid, glutamine and glycine in duodenum of IUGR suckling piglets were reduced in response to supplementation with L-leucine ($P < 0·05$). In the jejunum, glycine and taurine in L-leucine-treated group decreased significantly ($P < 0·01$) by 20·2 and 23·3 %, respectively. The levels of glycine, citrulline and taurine in ileum were significantly decreased ($P < 0·05$), whereas the contents of glutamate were significantly increased in piglets supplemented with 1·4 g/kg BW leucine compared with those given 0 g/kg BW leucine ($P < 0·05$).

Regulation of the expression of transporters for amino acids and peptides by L-leucine in the jejunum of suckling piglets with intra-uterine growth restriction

The effects of 1·4 g/kg BW of L-leucine on the mRNA levels of genes encoding amino acid transporters in the jejunum of IUGR suckling piglets are shown in Fig. 3. In comparison with the control group, L-leucine markedly increased the mRNA level of $ATB^{0,+}$, whereas decreased the mRNA expression of *ASCT2*, y^+*LAT1*, $B^{0,+}$*AT* and *CAT1* ($P < 0·05$). Consistently, the mRNA level of oligopeptides transporter *PepT1* in L-leucine treatment group was also lower than that of the control ($P < 0·05$). However, no significant difference in the expression of B^0*AT1* and *rBAT* genes was observed between the two groups ($P > 0·05$). The abundances of amino acid transporters at protein level in the jejunum of IUGR suckling piglets were detected by Western blot. As shown in Fig. 4, L-leucine supplementation significantly increased the level of $ATB^{0,+}$ protein in the jejunum ($P < 0·05$), whereas decreased the abundance of SNAT2 ($P < 0·01$), compared with the control. However, L-leucine treatment showed no significant impact on the levels of CAT1, PAT1, rBAT and xCT ($P > 0·05$).

Discussion

It has been well established that IUGR fetuses present an elevation in the risk of fetal death or perinatal mortality and morbidity compared with normal fetuses[39,40]. Of note, piglets exposed to weaning are highly predisposed to pathogenic infection and disease[41,42], particularly metabolically abnormal IUGR piglets. Thus, nutritional reinforcement prior to weaning may contribute

Table 3. The biochemical parameters of plasma from breast-fed intra-uterine growth restriction piglets at 21 d of age*
(Means values with their pooled standard errors of the mean, *n* 15)

	L-leucine supplementation (g/kg BW)					
Items	0	0·7	1·4	2·1	SEM	*P*
Urea, mM	2·98[a]	2·45[a,b]	2·24[b]	2·20[b]	0·22	0·032
Blood ammonia, μM	67·9[a]	63·9[a]	45·4[b]	46·4[b]	2·82	0·001
Glucose, mM	8·86[a]	8·04[b]	7·84[b]	8·11[b]	0·13	0·006
TAG, mM	1·20[a]	1·01[a,b]	0·89[b]	0·84[b]	0·04	0·004
Total cholesterol, mM	3·79[a]	3·29[b]	3·13[b]	3·14[b]	0·07	<0·001
HDL-cholesterol, mM	1·26	1·29	1·18	1·28	0·02	0·362
LDL-cholesterol, mM	1·77[b]	2·01[a,b]	2·09[a]	2·16[a]	0·05	0·054

* Piglets were supplemented with L-leucine twice daily.
[a,b,c]Mean values within a row sharing different superscript letters differ ($P < 0·05$).

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Table 4. Effect of L-leucine supplementation on concentrations of amino acids in the plasma of breast-fed intra-uterine growth restriction piglets at 21 d-old (μM)*
(Means values with their pooled standard errors of the mean, *n* 15)

	L-leucine supplementation (g/kg BW)					
Amino acids	0	0·7	1·4	2·1	SEM	*P*
Histidine	122[a]	108[a,b]	94·9[b]	74·8[c]	4·67	0·001
Isoleucine	108[a]	75·4[b]	63·1[b,c]	50·6[c]	4·62	<0·001
Leucine	228[c]	362[b]	444[a]	466[a]	20·5	<0·001
Lysine	222[a]	216[a]	201[a]	172[b]	6·42	0·026
Methionine	29·8[a]	26·9[a,b]	23·1[b]	21·8[b]	1·01	<0·001
Phenylalanine	72·1[a]	54·9[b]	50·5[b]	40·9[c]	2·41	<0·001
Threonine	167[a]	161[a]	106[b]	80·4[c]	8·29	<0·001
Tryptophan	46·4[a]	43·9[a]	31·1[b]	28·2[b]	1·82	<0·001
Valine	210[a]	147[b]	122[c]	113[c]	8·18	<0·001
Alanine	1246[a]	1054[b]	507[c]	476[c]	75·8	<0·001
Arginine	201[a]	148[b]	147[b]	115[c]	6·56	<0·001
Asparagine	70·3[a]	65·0[a,b]	58·8[b,c]	50·7[c]	1·91	<0·001
Aspartate	19·6[a]	13·8[b]	14·3[b]	10·6[c]	0·73	<0·001
Citrulline	90·8[a]	86·1[a]	81·1[ab]	71·0[b]	2·31	0·012
Glutamine	457[b]	495[a]	427[b]	437[b]	7·98	0·009
Glutamate	167[a]	128[b]	121[b]	103[c]	5·74	<0·001
Glycine	714[a]	701[a]	630[b]	553[c]	15·7	<0·001
Ornithine	143	149	148	142	2·67	0·771
Serine	291[a]	258[a]	215[b]	189[b]	10·4	<0·001
Taurine	142[a]	154[b]	152[a]	96·5[b]	5·44	<0·001
Tyrosine	130[a]	131[a]	102[b]	108[b]	3·73	0·002

* Piglets were supplemented with L-leucine twice daily.
[a,b,c]Mean values within a row sharing different superscript letters differ ($P < 0·05$).

Table 5. Effects of L-leucine supplementation on amino acid concentrations of small intestine in intra-uterine growth restriction breast-fed piglets (μmol/g tissue)
(Means values with their pooled standard errors of the mean, *n* 6)*

	Duodenum				Jejunum				Ileum			
Amino Acid	Ctl	Leu	SEM	*P*	Ctl	Leu	SEM	*P*	Ctl	Leu	SEM	*P*
Aspartate	829	748	20·1	0·03	812	857	19	0·149	1448	1547	53·9	0·271
Glutamate	4104	3678	59·3	0·002	4329	4176	153	0·506	6854	7431	153	0·039
Asparagine	157	185	12·1	0·165	322	360	13·9	0·12	241	207	10·7	0·106
Serine	504	491	13·4	0·564	877	981	85·8	0·451	679	561	32·4	0·125
Glutamine	416	369	11·9	0·045	606	700	60·3	0·343	579	462	36·4	0·069
Histidine	52·3	59·5	5·19	0·396	66·1	70·3	3·14	0·394	78·2	78·9	3·98	0·918
Glycine	2033	1779	59·4	0·028	4056	3237	123	0·003	3612	2662	228	0·035
Threonine	170	211	11·7	0·052	338	410	23·1	0·06	199	189	8·43	0·507
Citrulline	237	208	6·58	0·07	268	309	36·7	0·469	111	75	4·73	0·002
Arginine	533	547	12·5	0·445	710	652	50·9	0·464	579	477	32·5	0·078
Taurine	2355	2322	96·8	0·859	3498	2683	58	<0·001	2133	1662	91·8	0·013
Alanine	2337	1801	115	0·034	2829	2573	81·4	0·073	1787	1525	66·3	0·04
Tyrosine	228	224	8·95	0·78	343	308	20·1	0·263	196	174	16·6	0·411
Tryptophan	34·2	33·3	1·45	0·67	56·3	50·9	3·62	0·424	33·2	26·5	2·13	0·096
Methionine	89	101	8·62	0·376	152	152	6·75	0·996	88·4	76·3	4·5	0·112
Valine	346	275	19·6	0·134	485	473	17·2	0·65	339	257	14·8	0·008
Phenylalanine	170	176	5·56	0·422	260	248	11·2	0·452	148	131	10	0·335
Isoleucine	201	180	8·82	0·148	311	328	20·2	0·632	210	166	12·5	0·055
Leucine	597	895	34·4	0·001	727	835	20·7	0·01	496	489	26·4	0·85
Ornithine	56·6	63·6	2·97	0·165	78·2	69·6	3·36	0·165	77·3	64·1	5·01	0·111
Lysine	384	387	15·7	0·905	594·3	544·4	41·9	0·437	397	390	13·6	0·769

* Piglets were supplemented with 1·4 g/kg BW L-leucine twice daily.

to the growth and health of pigs. Interestingly, a comparatively low level of leucine in the umbilical vein of IUGR during late gestation has been noted[25]. This prompted us to propose a hypothesis that L-leucine supplementation during the suckling period may improve the growth performance of IUGR piglets. Unexpectedly, contrary to the growth promotion effect of leucine on normal suckling piglets, administration of leucine led to decreased production performance in IUGR piglets during suckling period, accompanied by lower plasma amino acid levels, changes in plasma biochemical parameters and the expression of amino acid transporters in the jejunum. Previous studies on weaned IUGR piglets (14–35 d of age) fed a basal diet

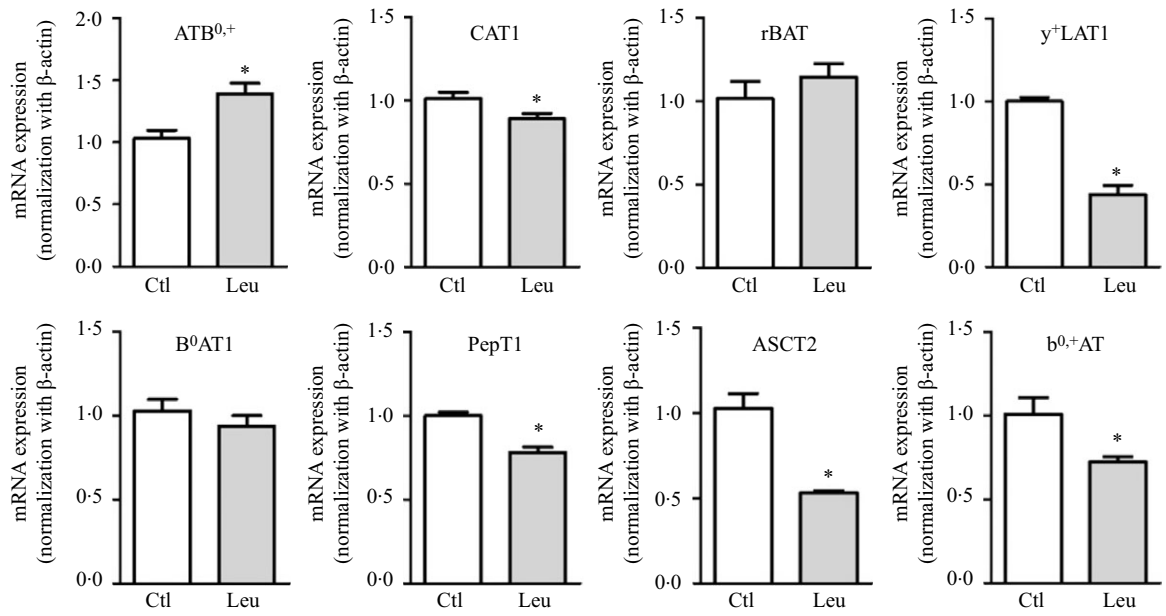

Fig. 3. The mRNA levels of amino acid transporters (*ATB$^{0,+}$*, *CAT1*, *rBAT*, *y^{+}LAT1*, *B^{0}AT1*, *ASCT2* and *b$^{0,+}$AT*) related to leucine uptake, and small peptide transporter *PepT1* in the jejunum of 21-d-old suckling intra-uterine growth restriction (IUGR) piglets. The IUGR piglets were provided with 1·4 g/kg BW L-leucine or 0·95 g/kg BW L-alanine (the isonitrogenous control) for 2 weeks between 7 and 21 d of age. Values are means with their standard error of the mean, *n* 6. *$P < 0·05$.

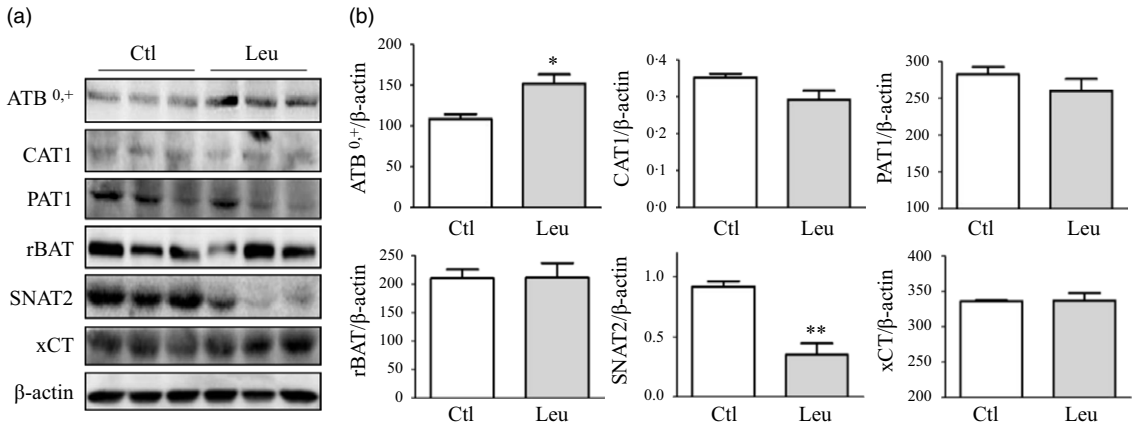

Fig. 4. The abundances of ATB$^{0,+}$, CAT1, PAT1, rBAT, SNAT2 and xCT proteins in the jejunum of 21-d-old suckling piglets born with intra-uterine growth restriction (IUGR). (a) Representative immunoblotting bands. (b) The statistical analysis of protein abundance evaluated by gray value using Image J (NIH). The two groups as shown indicate IUGR piglets orally supplemented with 0·95 g/kg BW L-alanine (the isonitrogenous control) or 1·4 g/kg BW L-leucine, respectively, between 7 and 21 d of age. Values are means with their standard error of the mean, *n* 6. *$P < 0·05$.

supplemented with 0·3–0·35 % L-leucine have shown that L-leucine improves growth performance and glycolipid metabolism[32,43]. These evidence suggests that L-leucine is beneficial to IUGR piglets in response to weaning stress. Here, the present study on breast-fed IUGR piglets prior to weaning (14–21 d of age) demonstrated that L-leucine (0·7–2·1 g/kg BW) exhibited a negative effect on whole-body growth. IUGR piglets may be difficult to adapt to the extra addition of L-leucine during a normal feeding period of breast milk. Hence, although L-leucine is in favour of supporting the growth of normal suckling piglets and IUGR piglets post-weaning, it is not recommended to be supplied to IUGR piglets during a pre-weaning stage following the doses provided in this study. However, as L-leucine at lower dose exerted a supportive effect on the growth of weaned IUGR piglets and high L-leucine supplementation has been shown to facilitate the catabolism of valine and isoleucine[32,44,45], whether leucine at a lower dose (< 0·7 g/kg BW) or in combination with valine and isoleucine is beneficial to the growth of breast-fed IUGR requires additional study in the future.

Urea is a predominant form of elimination for amino groups deriving from amino acids[46]. A low urea level has been implicated in amino acid malnutrition[47]. In this experiment, the plasma urea concentration of IUGR piglets decreased with the increasing L-leucine, which evinces an aggravating amino acid malnutrition. Indeed, L-leucine supplementation reduced amino acid transport in the jejunum of IUGR pigs, resulting in a fall in plasma levels of a variety of amino acids and a corresponding decrease in urea. In parallel, a reduction in plasma glucogenic

amino acids, which can be converted to glucose, may engender a decrease in blood glucose levels of breast-fed IUGR piglets provided with L-leucine supplementation. Blood ammonia is a product originating from amino acid deamination[48]. Thereby, the decline in plasma amino acids in response to the increased delivery of L-leucine occurs accompanied by a reduction in ammonia. Additionally, it has been confirmed that IUGR piglets exhibit impaired intestinal morphology and nutrient absorption capacity, as well as decreased intestinal tissue protein abundance and aminopeptidase activity in comparison with normal piglets[23,49]. The addition of L-leucine in this experiment may exacerbate the intestinal burden of suckling IUGR piglets.

Amino acids serve not only as the basic unit of protein synthesis but also act as precursors for various bioactive molecules and metabolic energy substrates[50]. It has been noted in suckling piglets with normal weight that a 2-week L-leucine supplementation increased the levels of lysine and methionine in plasma[35]. In contrast, this study revealed that L-leucine treatment significantly suppressed the plasma contents of lysine and methionine in IUGR suckling piglets. Lysine, as the first limiting amino acid of pigs fed with typical grain-based diet, is essential for the synthesis of muscle proteins, hormones, Ig and Hb as well as the regulation of nutrients metabolism and gene expression[51]. A deficiency of dietary lysine has been observed to induce growth retardation, alteration in amino acids metabolism and impairment of immune function[52,53]. In general, methionine is regarded as the second or third limiting amino acid in typical diets of pig production. Beyond protein synthesis, methionine has been functioned in providing methyl groups and the synthesis of multiple bioactive molecules[54]. Given that the two limiting amino acids are indispensable and crucial for the growing development and physiological function of piglets, a reduction in lysine and methionine induced by leucine supplementation undoubtedly results in slow growth in IUGR suckling piglets.

Compared with normal piglets, piglets with IUGR display an abnormal concentration of plasma amino acids[55], which may induce a distinct metabolic response to leucine supplementation. Our previous study with normal newborn piglets indicated that leucine supplementation significantly increased the glycine levels in plasma and ileum[35]. Conversely, administration of leucine, in the present study, reduced the contents of glycine and serine (biosynthetically linked with glycine) in the plasma from suckling IUGR piglets. Glycine is abundant in the plasma of suckling piglets and serves to facilitate glutathione biosynthesis, protein synthesis and maximal growth of piglets[56–58]. Additionally, glycine and serine appear as the donors of one carbon unit and participate in the biosynthesis of purine, pyrimidine and s-adenosylmethionine[59,60]. Given the particular importance of glycine as abovementioned, a reduction in glycine levels in plasma and small intestine, as observed in this study, may be part of the reasons for the decreased growth performance of IUGR suckling piglets fed with L-leucine. Furthermore, as shown by our previous study, L-leucine increased the intestinal mucosa levels of glutamine and glutamate which provides energy source and thus promotes the growth of normal suckling piglets[35]. Nevertheless, L-leucine treatment significantly reduced the contents of glutamine and glutamate in intestinal tissues from IUGR suckling piglets. These amino acids are known as the crucial energy substrate for intestinal mucosa metabolism[61], which may be the underlying reason in part for the slow growth of suckling IUGR piglets in response to the additional L-leucine. It is worth noting that supplementing with L-leucine significantly decreased plasma levels of valine, isoleucine and tryptophan. Evidence has suggested that L-leucine provision dose-dependently accelerates the catabolism of valine and isoleucine and decreases available tryptophan[44,45], which may be an additional essential factor for the depressed performance of breast-fed IUGR piglets in this trial and conduces to clarifying the reason for the reduction of valine levels in the ileum in response to L-leucine supplementation.

The protein and polypeptide are hydrolysed into amino acids in the cavity of small intestine, after which the amino acids are transferred through a series of transporters. Following the observations from IUGR in primate model and humans, a decreased activity of key amino acid transporters was identified in placenta[62,63]. It has been recognised that maternal protein malnutrition leads to down-regulation of nutrient transporters and contributes to the development of IUGR[64]. Changes in nutrient absorption capacity of IUGR piglets after birth suggest a potential dependence on the expression of transporters for nutrients, particularly for amino acids[65,66]. Thus, we determined the impact of leucine on the expression of amino acid transporters in intestinal tissues of suckling IUGR piglets. Individual amino acid can be transported by multiple amino acid transporters. In mammals, L-leucine transporters are mainly located at the apical membrane of small intestinal epithelium[67]. Na^+-dependent neutral amino acid transporter $ATB^{0,+}$ appears as one of the key transporters responsible for L-leucine absorption in the jejunum[68], and up-regulation of its expression promotes the uptake of L-leucine. In agreement with our results from suckling piglets with normal weight[35], L-leucine supplementation significantly increased the expression of $ATB^{0,+}$ in the jejunum of suckling IUGR piglets. ASCT2, y^+LAT1, $B^{0,+}$AT and CAT1 belong to the ASC, y^+L, $b^{0,+}$ and y^+ system, respectively, and their expression in the jejunum of IUGR suckling piglets was suppressed in response to L-leucine administration. These transporters are known to be involved in the transport of the following amino acids: ASCT2 (glutamine, serine, cysteine, alanine, threonine and valine)[69], y^+LAT1 (arginine, lysine, histidine, glutamine, leucine, alanine, cysteine, methionine)[70], $B^{0,+}$AT (neutral amino acids)[71] and CAT1 (arginine, histidine, lysine, ornithine)[72]. By comparison with our previous study[35], despite the fact that down-regulation of y^+LAT1 was observed in both IUGR and normal suckling piglets following L-leucine supplementation, the expression of B^0AT1 and $b^{0,+}$AT was dramatically enhanced in normal breast-fed piglets while showing no effect and being significantly lowered in IUGR suckling piglets, respectively. These findings partially shed light on the discrepancy between the responses of normal and IUGR suckling piglets to L-leucine supplementation at the same dose. Na^+-coupled neutral amino acid transporters 2 (SNAT2) belongs to system A transporters that extensively distributed in most tissues including small intestine[73]. The expression of this transporter is modulated by multiple factors such as nutritional status (amino acids level) and stressors[74]. The present experiment indicated that SNAT2 level was reduced in the jejunum of IUGR suckling piglets supplemented with 1·4 g/kg BW

L-leucine, implying SNAT2 may respond to the low plasma amino acid levels induced by leucine. In addition, L-leucine inhibited the expression of PepT1, which is responsible for the transport of amino acids in the form of small peptides[75], in the jejunum of IUGR piglets during suckling period. Together, the reduction in plasma amino acid concentrations in IUGR suckling piglets supplemented with 1·4 g/kg BW L-leucine is possibly linked to the regulation of intestinal transport function, which is mediated by a diverse array of transporters for amino acids and peptides.

In conclusion, a provision of L-leucine (0·7–2·1 g/kg BW) impairs the growth performance in IUGR suckling piglets along with depressed nutritional status and plasma amino acids levels, as well as changed expression of amino acid transporters in the jejunum. These findings hint that supplementation of leucine presents an adverse impact on breast-fed piglets with IUGR in the conditions described in this study. Since neonatal piglets well mimic human infant features, a range of doses of L-leucine supplementation in low-birth weight infants at suckling period may also present an adverse effect on growth.

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The authors declare no conflict of interests.

References

1. Wu G, Bazer FW, Wallace JM, *et al.* (2006) Board-invited review: intrauterine growth retardation: implications for the animal sciences. *J Anim Sci* **84**, 2316–2337.
2. Wu G, Bazer FW, Cudd TA, *et al.* (2004) Maternal nutrition and fetal development. *J Nutr* **134**, 2169–2172.
3. Barker DJ, Eriksson JG, Forsen T, *et al.* (2002) Fetal origins of adult disease: strength of effects and biological basis. *Int J Epidemiol* **31**, 1235–1239.
4. Park JH, Stoffers DA, Nicholls RD, *et al.* (2008) Development of type 2 diabetes following intrauterine growth retardation in rats is associated with progressive epigenetic silencing of Pdx1. *J Clin Invest* **118**, 2316–2324.
5. Faraci M, Renda E, Monte S, *et al.* (2011) Fetal growth restriction: current perspectives. *J Prenat Med* **5**, 31–33.
6. Wu G, Bazer FW, Dai Z, *et al.* (2014) Amino acid nutrition in animals: protein synthesis and beyond. *Annu Rev Anim Biosci* **2**, 387–417.
7. Li F, Yin Y, Tan B, *et al.* (2011) Leucine nutrition in animals and humans: mTOR signaling and beyond. *Amino Acids* **41**, 1185–1193.
8. Rhoads J & Wu G (2009) Glutamine, arginine, and leucine signaling in the intestine. *Amino Acids* **37**, 111–122.
9. Columbus DA, Fiorotto ML & Davis TA (2015) Leucine is a major regulator of muscle protein synthesis in neonates. *Amino Acids* **47**, 259–270.
10. Escobar J, Frank JW, Suryawan A, *et al.* (2005) Physiological rise in plasma leucine stimulates muscle protein synthesis in neonatal pigs by enhancing translation initiation factor activation. *Am J Physiol Endocrinol Metab* **288**, E914–E921.
11. Wilson FA, Suryawan A, Gazzaneo MC, *et al.* (2010) Stimulation of muscle protein synthesis by prolonged parenteral infusion of leucine is dependent on amino acid availability in neonatal pigs. *J Nutr* **140**, 264–270.
12. Escobar J, Frank JW, Suryawan A, *et al.* (2006) Regulation of cardiac and skeletal muscle protein synthesis by individual branched-chain amino acids in neonatal pigs. *Am J Physiol Endocrinol Metab* **290**, E612–E621.
13. Appuhamy JA, Knoebel NA, Nayananjalie WA, *et al.* (2012) Isoleucine and leucine independently regulate mTOR signaling and protein synthesis in MAC-T cells and bovine mammary tissue slices. *J Nutr* **142**, 484–491.
14. Lei J, Feng D, Zhang Y, *et al.* (2013) Hormonal regulation of leucine catabolism in mammary epithelial cells. *Amino Acids* **45**, 531–541.
15. Yin Y, Yao K, Liu Z, *et al.* (2010) Supplementing L-leucine to a low-protein diet increases tissue protein synthesis in weanling pigs. *Amino Acids* **39**, 1477–1486.
16. Zhang S, Qiao S, Ren M, *et al.* (2013) Supplementation with branched-chain amino acids to a low-protein diet regulates intestinal expression of amino acid and peptide transporters in weanling pigs. *Amino Acids* **45**, 1191–1205.
17. Wu G & Knabe DA (1994) Free and protein-bound amino acids in sow's colostrum and milk. *J Nutr* **124**, 415–424.
18. Davis TA, Nguyen HV, Garcia-Bravo R, *et al.* (1994) Amino acid composition of human milk is not unique. *J Nutr* **124**, 1126–1132.
19. Sun Y, Wu Z, Li W, *et al.* (2015) Dietary L-leucine supplementation enhances intestinal development in suckling piglets. *Amino Acids* **47**, 1517–1525.
20. Dai ZL, Zhang J, Wu G, *et al.* (2010) Utilization of amino acids by bacteria from the pig small intestine. *Amino Acids* **39**, 1201–1215.
21. Chen L, Li P, Wang J, *et al.* (2009) Catabolism of nutritionally essential amino acids in developing porcine enterocytes. *Amino Acids* **37**, 143–152.
22. Wang J, Chen L, Li D, *et al.* (2008) Intrauterine growth restriction affects the proteomes of the small intestine, liver, and skeletal muscle in newborn pigs. *J Nutr* **138**, 60–66.
23. Wang X, Lin G, Liu C, *et al.* (2014) Temporal proteomic analysis reveals defects in small-intestinal development of porcine fetuses with intrauterine growth restriction. *J Nutr Biochem* **25**, 785–795.
24. Liu C, Lin G, Wang X, *et al.* (2013) Intrauterine growth restriction alters the hepatic proteome in fetal pigs. *J Nutr Biochem* **24**, 954–959.
25. Lin G, Liu C, Feng C, *et al.* (2012) Metabolomic analysis reveals differences in umbilical vein plasma metabolites between normal and growth-restricted fetal pigs during late gestation. *J Nutr* **142**, 990–998.
26. Cetin I, Marconi AM, Bozzetti P, *et al.* (1988) Umbilical amino acid concentrations in appropriate and small for gestational age infants: a biochemical difference present in utero. *Am J Obstet Gynecol* **158**, 120–126.

27. Cetin I, Marconi AM, Corbetta C, *et al.* (1992) Fetal amino acids in normal pregnancies and in pregnancies complicated by intrauterine growth retardation. *Early Hum Dev* **29**, 183–186.
28. Ogata ES, Bussey ME & Finley S (1986) Altered gas exchange, limited glucose and branched chain amino acids, and hypoinsulinism retard fetal growth in the rat. *Metab Clin Exp* **35**, 970–977.
29. Karsdorp VH, van Vugt JM, Jakobs C, *et al.* (1994) Amino acids, glucose and lactate concentrations in umbilical cord blood in relation to umbilical artery flow patterns. *Eur J Obstet Gynecol Reprod Biol* **57**, 117–122.
30. Wu G, Pond WG, Ott T, *et al.* (1998) Maternal dietary protein deficiency decreases amino acid concentrations in fetal plasma and allantoic fluid of pigs. *J Nutr* **128**, 894–902.
31. Mogami H, Yura S, Itoh H, *et al.* (2009) Isocaloric high-protein diet as well as branched-chain amino acids supplemented diet partially alleviates adverse consequences of maternal undernutrition on fetal growth. *Growth Horm IGF Res* **19**, 478–485.
32. Xu W, Bai K, He J, *et al.* (2016) Leucine improves growth performance of intrauterine growth retardation piglets by modifying gene and protein expression related to protein synthesis. *Nutrition* **32**, 114–121.
33. Radulescu L, Ferechide D & Popa F (2013) The importance of fetal gender in intrauterine growth restriction. *J Med Life* **6**, 38–39.
34. Wu G, Flynn NE & Knabe DA (2000) Enhanced intestinal synthesis of polyamines from proline in cortisol-treated piglets. *Am J Physiol Endocrinol Metab* **279**, E395–E402.
35. Sun Y, Wu Z, Li W, *et al.* (2015) Dietary L-leucine supplementation enhances intestinal development in suckling piglets. *Amino Acids* **47**, 1517–1525.
36. Wu G & Meininger CJ (2008) Analysis of citrulline, arginine, and methylarginines using high-performance liquid chromatography. *Methods Enzymol* **440**, 177–189.
37. Livak KJ & Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* **25**, 402–408.
38. Van Tichelen K, Prims S, Ayuso M, *et al.* (2021) Handling associated with drenching does not impact survival and general health of low birth weight piglets. *Animals* **11**, 404.
39. Sharma D, Shastri S & Sharma P (2016) Intrauterine Growth Restriction: antenatal and Postnatal Aspects. *Clin Med Insights Pediatr* **10**, 67–83.
40. Malhotra A, Allison BJ, Castillo-Melendez M, *et al.* (2019) Neonatal Morbidities of Fetal Growth Restriction: pathophysiology and Impact. *Front Endocrinol* **10**, 55.
41. Dou S, Gadonna-Widehem P, Rome V, *et al.* (2017) Characterisation of early-life fecal microbiota in susceptible and healthy pigs to post-weaning diarrhoea. *PLOS ONE* **12**, e0169851.
42. McLamb BL, Gibson AJ, Overman EL, *et al.* (2013) Early weaning stress in pigs impairs innate mucosal immune responses to enterotoxigenic E. coli challenge and exacerbates intestinal injury and clinical disease. *PLOS ONE* **8**, e59838.
43. Zhang J, Xu W, Han H, *et al.* (2019) Dietary leucine supplementation restores serum glucose levels, and modifying hepatic gene expression related to the insulin signal pathway in IUGR piglets. *Animals* **9**, 1138.
44. Wessels AG, Kluge H, Hirche F, *et al.* (2016) High leucine diets stimulate cerebral branched-chain amino acid degradation and modify serotonin and ketone body concentrations in a pig model. *PLOS ONE* **11**, e0150376.
45. Bertocchi M, Bosi P, Luise D, *et al.* (2019) Dose-response of different dietary leucine levels on growth performance and amino acid metabolism in piglets differing for aminoadipate-semialdehyde synthase genotypes. *Sci Rep* **9**, 18496.
46. Weiner ID, Mitch WE & Sands JM (2015) Urea and ammonia metabolism and the control of renal nitrogen excretion. *Clin J Am Soc Nephrol* **10**, 1444–1458.
47. Benabe JE & Martinez-Maldonado M (1998) The impact of malnutrition on kidney function. *Miner Electrolyte Metab* **24**, 20–26.
48. Krebs HA (1935) Metabolism of amino-acids: deamination of amino-acids. *Biochem J* **29**, 1620–1644.
49. Wang T, Huo YJ, Shi F, *et al.* (2005) Effects of intrauterine growth retardation on development of the gastrointestinal tract in neonatal pigs. *Biol Neonate* **88**, 66–72.
50. Wu G (2013) Functional amino acids in nutrition and health. *Amino Acids* **45**, 407–411.
51. Liao SF, Wang T & Regmi N (2015) Lysine nutrition in swine and the related monogastric animals: muscle protein biosynthesis and beyond. *Springerplus* **4**, 147.
52. Han H, Yin J, Wang B, *et al.* (2018) Effects of dietary lysine restriction on inflammatory responses in piglets. *Sci Rep* **8**, 2451.
53. Yin J, Han H, Li Y, *et al.* (2017) Lysine restriction affects feed intake and amino acid metabolism via gut microbiome in piglets. *Cell Physiol Biochem* **44**, 1749–1761.
54. Yang ZY, Htoo JK & Liao SF (2020) Methionine nutrition in swine and related monogastric animals: beyond protein biosynthesis. *Anim Feed Sci Tech* **268**, 114608.
55. MacKay DS, Brophy JD, McBreairty LE, *et al.* (2012) Intrauterine growth restriction leads to changes in sulfur amino acid metabolism, but not global DNA methylation, in Yucatan miniature piglets. *J Nutr Biochem* **23**, 1121–1127.
56. Flynn NE, Knabe DA, Mallick BK, *et al.* (2000) Postnatal changes of plasma amino acids in suckling pigs. *J Anim Sci* **78**, 2369–2375.
57. Wang W, Wu Z, Lin G, *et al.* (2014) Glycine stimulates protein synthesis and inhibits oxidative stress in pig small intestinal epithelial cells. *J Nutr* **144**, 1540–1548.
58. Wang W, Dai Z, Wu Z, *et al.* (2014) Glycine is a nutritionally essential amino acid for maximal growth of milk-fed young pigs. *Amino Acids* **46**, 2037–2045.
59. Reina-Campos M, Diaz-Meco MT & Moscat J (2020) The complexity of the serine glycine one-carbon pathway in cancer. *J Cell Biol* **219**, e201907022.
60. Wang W, Wu Z, Dai Z, *et al.* (2013) Glycine metabolism in animals and humans: implications for nutrition and health. *Amino Acids* **45**, 463–477.
61. Blachier F, Boutry C, Bos C, *et al.* (2009) Metabolism and functions of L-glutamate in the epithelial cells of the small and large intestines. *Am J Clin Nutr* **90**, 814S–821S.
62. Pantham P, Rosario FJ, Weintraub ST, *et al.* (2016) Down-regulation of placental transport of amino acids precedes the development of intrauterine growth restriction in maternal nutrient restricted baboons. *Biol Reprod* **95**, 98.
63. Roos S, Jansson N, Palmberg I, *et al.* (2007) Mammalian target of rapamycin in the human placenta regulates leucine transport and is down-regulated in restricted fetal growth. *J Physiol* **582**, 449–459.
64. Jansson N, Pettersson J, Haafiz A, *et al.* (2006) Down-regulation of placental transport of amino acids precedes the development of intrauterine growth restriction in rats fed a low protein diet. *J Physiol* **576**, 935–946.

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65. Lin G, Wang X, Wu G, *et al.* (2014) Improving amino acid nutrition to prevent intrauterine growth restriction in mammals. *Amino Acids* **46**, 1605–1623.
66. Ji Y, Wu Z, Dai Z, *et al.* (2017) Fetal and neonatal programming of postnatal growth and feed efficiency in swine. *J Anim Sci Biotechnol* **8**, 42.
67. Buddington RK, Elnif J, Puchal-Gardiner AA, *et al.* (2001) Intestinal apical amino acid absorption during development of the pig. *Am J Physiol Regul Integr Comp Physiol* **280**, R241–R247.
68. Samluk L, Czeredys M, Skowronek K, *et al.* (2012) Protein kinase C regulates amino acid transporter ATB(0,+). *Biochem Biophys Res Commun* **422**, 64–69.
69. Ni F, Yu WM, Li Z, *et al.* (2019) Critical role of ASCT2-mediated amino acid metabolism in promoting leukaemia development and progression. *Nat Metab* **1**, 390–403.
70. Cleal JK, Brownbill P, Godfrey KM, *et al.* (2007) Modification of fetal plasma amino acid composition by placental amino acid exchangers *in vitro*. *J Physiol* **582**, 871–882.
71. Jando J, Camargo SMR, Herzog B, *et al.* (2017) Expression and regulation of the neutral amino acid transporter B0AT1 in rat small intestine. *PLOS ONE* **12**, E0184845.
72. Chen C, Yin Y, Tu Q, *et al.* (2018) Glucose and amino acid in enterocyte: absorption, metabolism and maturation. *Front Biosci* **23**, 1721–1739.
73. Broer S (2008) Amino acid transport across mammalian intestinal and renal epithelia. *Physiol Rev* **88**, 249–286.
74. Mando C, Tabano S, Pileri P, *et al.* (2013) SNAT2 expression and regulation in human growth-restricted placentas. *Pediatr Res* **74**, 104–110.
75. Spanier B (2014) Transcriptional and functional regulation of the intestinal peptide transporter PEPT1. *J Physiol* **592**, 871–879.

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