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Original Research Article

Serum biochemical parameters and amino acids metabolism are altered in piglets by early-weaning and proline and putrescine supplementations

Jing Wang [a, b, c], Yuxin Xiao [b, c], Jianjun Li [c], Ming Qi [c, d], Bie Tan [b, c, *]

[a] Hunan International Joint Laboratory of Animal Intestinal Ecology and Health, Laboratory of Animal Nutrition and Human Health, College of Life Sciences, Hunan Normal University, Changsha 410081, China
[b] College of Animal Science and Technology, Hunan Agricultural University, Changsha 410128, Hunan, China
[c] Laboratory of Animal Nutritional Physiology and Metabolic Process, Key Laboratory of Agroecological Processes in Subtropical Region, National Engineering Laboratory for Pollution Control and Waste Utilization in Livestock and Poultry Production, Institute of Subtropical Agriculture, Chinese Academy of Sciences, Changsha 410125, Hunan, China
[d] University of Chinese Academy of Sciences, Beijing 10008, China

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ABSTRACT

The study was to investigate the effect of early-weaning stress and proline (Pro) and putrescine (Put) supplementations on serum biochemical parameters and amino acids (AA) metabolism in suckling and post-weaning pigs. Blood and small intestinal mucosa were harvested from suckling piglets at 1, 7, 14, and 21 d of age and piglets on d 1, 3, 5, and 7 after weaning at 14 d of age, as well as from piglets received oral administration of Pro and Put from 1 to 14 d old. In suckling piglets, the serum glucose, albumin and total cholesterol levels were increased ($P < 0.05$) with increasing age, whereas the serum globulin, urea nitrogen (BUN), alkaline phosphatase (ALP) and aspartate aminotransferase (AST) levels were lowered ($P < 0.05$). The concentrations of most serum AA and the AA transporters related gene expressions were highest in 7-d-old piglets ($P < 0.05$), whereas the phosphorylation status of the mammalian target of the rapamycin (mTOR) signaling pathway in the small intestine increased in piglets from 1 to 21 d old ($P < 0.05$). Weaning at 14 d old increased ($P < 0.05$) the BUN and triglycerides levels in serum, as well as jejunal solute carrier family 7 member 6 (*SLC7A6*), ileal *SLC36A1* and *SLC1A1* mRNA abundances at d 1 or 3 post-weaning. Weaning also inhibited ($P < 0.05$) the phosphorylation levels of mTOR and its downstream ribosomal protein S6 kinase 1 (S6K1) and 4E-binding protein-1 (4EBP1) in the small intestine of weanling pigs. Oral administration of Put and Pro decreased ($P < 0.05$) serum ALP levels and increased ($P < 0.05$) intestinal *SLC36A1* and *SLC1A1* mRNA abundances and mTOR pathway phosphorylation levels in post-weaning pigs. Pro but not Put treatment enhanced ($P < 0.05$) serum Pro, arginine (Arg) and glutamine (Gln) concentrations of weaning-pigs. These findings indicated that early-weaning dramatically altered the biochemical blood metabolites, AA profile and intestinal mTOR pathway activity, and Pro and Put supplementations improved the AA metabolism and transportation as well as activated the intestinal mTOR pathway in weanling-pigs. Our study has an important implication for the broad application of Pro and Put in the weaning transition of piglets.

1. Introduction

A pig is born with an immature digestive system that must develop rapidly to adapt the switch from processing dilute amniotic fluid to digesting milk during the first 2 d after birth (Buddington, 1994). However, the intestinal digestive capacities are never in great excess and cannot meet the growing needs for additional

* Corresponding author.
E-mail address: bietan@isa.ac.cn (B. Tan).
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nutrients and energy of piglets (Buddington, 1994). When piglets are weaned at a very early stage (14 d old), their gastrointestinal tract is too immature to cope with the physiological and psychological stresses induced by changes in their feed composition and feeding conditions, which consequently results in intestinal disorder and adversely affects feeding efficiency and disease resistance of post-weaning piglets (Burrin and Stoll, 2003a; Tang et al., 2005). Variations of feed compositions and additives supplementations have been tested to be feasible options for facilitating advanced intestinal maturity during the suckling period and optimizing the weaning transition, such as functional amino acids (AA) and their metabolites (Yang et al., 2013; Fang et al., 2016; Lv et al., 2018; Hu et al., 2019).

Amino acids, which are major nutrients for pigs, are not only absorbed and used by the host to synthesize protein and other important substances, but also perform additional functions in growth, health and disease (Liu et al., 2017; Yang and Liao, 2019). For example, proline (Pro), one of the most abundant AA in sow's colostrum and milk, plays an important role in cellular energy sensing, cell differentiation and could serve to reduce the redox status of the cell (Brunton et al., 2012; Kang et al., 2014). It is also important to note that Pro is the major precursor of polyamines in the small intestine of neonatal piglets, and the Pro requirements substantially increase during young pig development (Wu et al., 2011). Further, polyamines, including putrescine (Put), spermidine and spermine, also play essential roles in intestinal maturation and remodeling (Wu et al., 2000). Supplemental crystalline free AA are absorbed more rapidly and completely than protein-bound AA in pigs (Yang and Liao, 2019), and the core process for absorption of dietary AA is mainly mediated by the specific transporters (Broer, 2008). Among the AA transporters, the L-type AA transporter 1/2 (solute carrier family 7 member 7 [SLC7A7]), SLC7A6, proton-assisted AA transporter 1 (SLC36A1) and excitatory AA transporter 3 (SLC1A1) have been identified as the major intestinal transporters for neutral, basic and acidic AA (Broer, 2008). The mammalian target of rapamycin (mTOR) is known as a master regulator of protein synthesis in cells (Liao et al., 2008). The mTOR signaling pathway is responsive to stimulation by growth factors and certain nutrients, including AA, phosphorylates eIF4E-binding protein-1 (4EBP1), and ribosomal protein S6 kinase-1 (S6K1), thereby promoting the initiation of polypeptide formation (Liao et al., 2008; Sancak et al., 2008). In our previous study, we demonstrated the age-related developmental changes of intestinal morphology and polyamines profile during the suckling and weanling periods (Wang et al., 2016a, 2016b), as well as the beneficial effect of Pro and Put on intestinal morphology, epithelial restitution and barrier function of piglets after weaning-stress injury (Wang et al., 2015a).

The current study mainly aimed to investigate the developmental changes in AA profile, intestinal AA transporters and the mTOR signaling pathway phosphorylation of piglets during suckling and post-weaning periods. Based on ours and others' findings, we hypothesized that oral administration with Pro and Put during the suckling period would improve the AA metabolism by regulating the mTOR signaling pathway in the intestine of post-weanling-piglets. Research in this field may provide potentially valuable information to fully understand the AA metabolism in suckling and weanling piglets, as well as the roles of Pro and Put in improving the adaptation to weaning in piglets.

2. Materials and methods

All animals used in this study were managed according to the Chinese Guidelines for Animal Welfare. The experimental protocol was approved by the Animal Care and Use Committee of the Chinese Academy of Sciences (Beijing, China).

2.1. Animals and experimental design

Sixty-four neonatal piglets (Duroc × Landrace × Large Yorkshire) from 8 litters (8 piglets per litter) were assigned to 8 groups (8 piglets per group) on the basis of different litter origins and similar body weights. Thirty-two piglets were nursed by sows until they were 21 d old (suckling piglets), while the other 32 piglets were weaned at 14 d of age (weanling piglets) and then housed in the same farrowing cage without a sow and fed creep feed (Artificial milk 101, Anyou Feed, China). Eight piglets from each litter were slaughtered on d 1, 7, 14, and 21 of age during the suckling period and on d 1, 3, 5, and 7 post-weaning (Fig. 1, Trial 1).

Another 18 neonatal piglets (Duroc × Landrace × Large Yorkshire) from 3 litters (6 piglets per litter) were assigned to 3 groups, which received oral administration of equal volumes of saline (control), Put (5 mg/kg body weight) or Pro (25 mg/kg body weight) twice daily from day 1 to 14. The Put dosage was based on the results reported by Grant et al. (1990), and the dosage of Pro was chosen to double the dosage obtained with sow's milk (Wu and Knabe, 1994). Piglets were weaned at 14 d of age and fed creep feed. At day 3 post-weaning, the most severe stage of intestinal impairment of weanling pigs, all piglets were slaughtered (Fig. 1, Trial 2).

Five milliliters of blood were collected aseptically in aseptic capped tubes from a jugular vein 2 h after feeding, centrifuged at 3,000 × *g* for 10 min at 4 °C to obtain serum samples then stored at −80 °C for AA and biochemistry parameters analysis. After electrical stunning, piglets were killed, and all the urine was collected aseptically in aseptic capped tubes from bladders and stored at −80 °C for further analysis. The small intestines were rinsed thoroughly with ice-cold physiological saline. Samples of jejunal and ileal mucosa were scraped and immediately snap-frozen in liquid nitrogen then stored at −80 °C for RNA and protein extraction.

2.2. Analysis of serum biochemical parameters

Serum biochemical parameters, including blood glucose (GLUC), total protein (TP), triglycerides (TG), total cholesterol (TC), high density lipoprotein (HDL), low density lipoprotein (LDL), very low density lipoprotein (VLDL), albumin (ALB), alkaline phosphatase (ALP), aspartate aminotransferase (AST), blood urea nitrogen (BUN), and creatinine (CRE), were measured using spectrophotometric kits following the manufacturer's instructions (TBA-120FR, Toshiba Medical Systems Corporation, Japan) and determined using an Automatic Biochemistry Radiometer (Au640, Olympus).

2.3. Analysis of serum AA profile

Seventeen AA in serum were determined by LC–MS/MS (HPLC Ultimate 3000 and 3200 QTRAP LC–MS/MS) as described previously (Ruan et al., 2013). Amino acid contents were determined from the serum of suckling and weanling piglets. Briefly, 1 mL of the serum sample and 2.5 mL of 7.5% trichloroacetic acid solution were mixed thoroughly and centrifuged at 12,000×*g* at 4 °C for 15 min. The supernatant was analyzed for the AA content by an ion-exchange AA analyzer (Hitachi, Japan) (Wang et al., 2008).

2.4. Real-time quantitative reverse transcriptase PCR

The expressions of *SLC7A6*, *SLC7A7*, *SLC36A1* and *SLC1A1* mRNA in the jejunal and ileal mucosa of piglets were determined by real-time RT-qPCR according to the procedure described by Wang et al. (2015a). Primers are shown in Table 1, and β-actin was used as a housekeeping gene to normalize target gene transcript levels. The comparative Ct value method was used to quantify expression levels of target genes relative to those of β-actin.

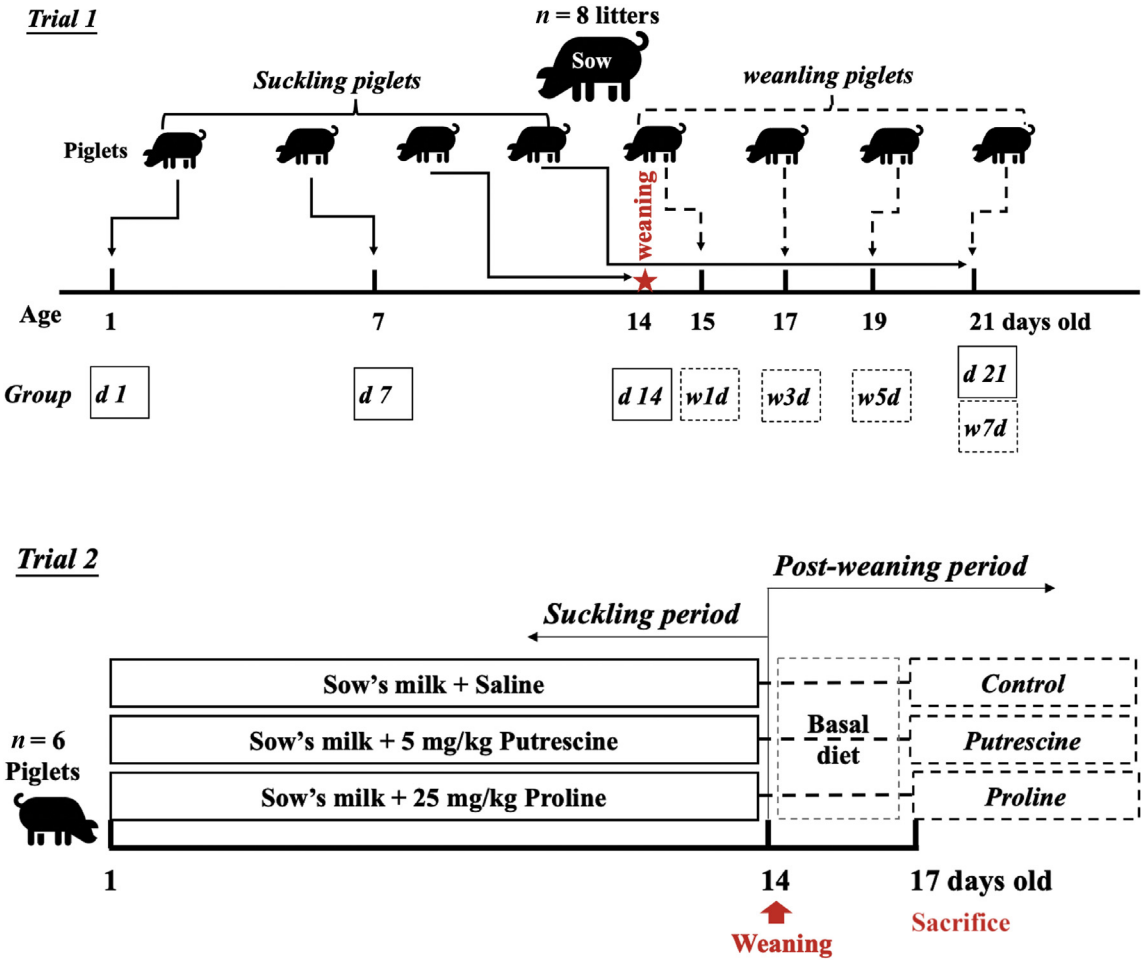

Fig. 1. The study design of trail 1 and trial 2. w = post-weanling.

2.5. Western blot analysis

Jejunal mucosa samples were homogenized, and protein concentrations were measured using the bicinchoninic acid assay method with bovine serum albumin (BSA) as standard (Beyotime Institute of Biotechnology, Shanghai, China). All samples were adjusted to an equal concentration (70 μg protein). The supernatant fluid (containing tissue proteins) was then diluted with 5 × sodium dodecyl sulfate (SDS) sample buffer and heated in boiling water for 5 min (Xiao et al., 2013). After the solution was cooled on ice, it was used for Western blot analysis (Wang et al., 2016a). The following first antibodies were used for protein quantification: mTOR (1:1,000; Cell Signaling Technology, USA), phosphorylated mTOR (p-mTOR (Ser2448); 1:1,000; Cell Signaling Technology, USA), phosphoprotein 70 S6K (p70S6K; 1:400; Abcam, UK), phosphorylated p70S6K (p-p70S6K (Thr389); 1:1,000; Cell Signaling Technology, USA), 4EBP1 (1:1,000; Cell Signaling Technology, USA), phosphorylated 4EBP1 (p-4EBP1 (Ser65); 1:1,000; Cell Signaling Technology, USA) and β-actin (1:1,000; Cell Signaling Technology, USA) as well as secondary antibodies (horseradish peroxidase-conjugated goat anti-rabbit IgG; 1:5,000; Boster Biological Technology, Wuhan, China). The images were detected by chemiluminescence (Millipore, Billerica, MA). Each Western blot was subjected to multiple exposures to ensure that the chemiluminescence signals were linear. Western blots were quantified by measuring the intensity of correctly sized bands using software (Alpha Imager 2200 Software; Alpha Innotech Corporation, San Leandro, CA, USA) and protein measurement was normalized to β-actin.

Table 1
Primers used for quantitative reverse transcription-PCR[1].

Gene	Accession no.	Primers
β-Actin	XM_003124280.3	F: 5′-GGATGCAGAAGGAGATCACG-3′ R: 5′-ATCTGCTGGAAGGTGGACAG-3′
SLC7A6	XM_021094156.1	F: 5′- TCTGTTGTGGGTGCCCTTTG-3′ R: 5′- GACGGCTGGATGATGTAGTTGG-3′
SLC7A7	NM_001110421.1	F: 5′- TTTGTTATGCGGAACTGG-3′ R: 5′- AAAGGTGATGGCAATGAC-3′
SLC36A1	XM_003134140.5	F: 5′- TGTGGACTTCTTCCTGATTGTC-3′ R: 5′- CATTGTTGTGGCAGTTATTGGT-3′
SLC1A1	NM_001164649.1	F: 5′- GGCACCGCACTCTACGAAGCA-3′ R: 5′- GCCCACGGCACTTAGCACGA-3′

[1] *SLC7A6* and *SLC7A7* = solute carrier family 7 member 6 and member 7; *SLC36A1* = proton-assisted AA transporter 1; *SLC1A1* = excitatory AA transporter 3.

2.6. Statistical analysis

All statistical analyses were conducted by one-way ANOVA using IBM SPSS Statistics version 23. Tukey–Kramer multiple comparison procedure was used for Post-hoc comparisons. The Kruskal–Wallis test was used when data were not normally distributed. The correlation between serum AA and mRNA levels of AA transporters was assessed with Pearson's correlation. Differences were declared as statistically significant at $P < 0.05$. Data were expressed as mean ± SEM.

3. Results

3.1. Serum biochemical parameters of suckling and weanling piglets

The serum metabolites levels and hepatic enzyme were analyzed as shown in Figs. 2 and 3, in order to monitor the general health status of piglets during suckling and weanling, as well as treatment with Put and Pro. The blood glucose concentration of 1-d-old suckling piglets was significantly lower ($P = 0.004$) than those in piglets at 7, 14, and 21 d old. Albumin level at the end of the suckling period (d 21) was higher than that of the initial ones (d 1), whereas the serum globulin was highest at birth and then decreased from 1 to 21 d old ($P < 0.001$). Weaning at 14-d-old did not affect the blood glucose, albumin and globulin levels in piglets. No significant differences were observed in total serum protein (data not shown) and creatinine levels among suckling and weanling piglets. Significantly higher serum AST, ALP activities were found at d 1 and 7, along with higher BUN level found at d 1 of

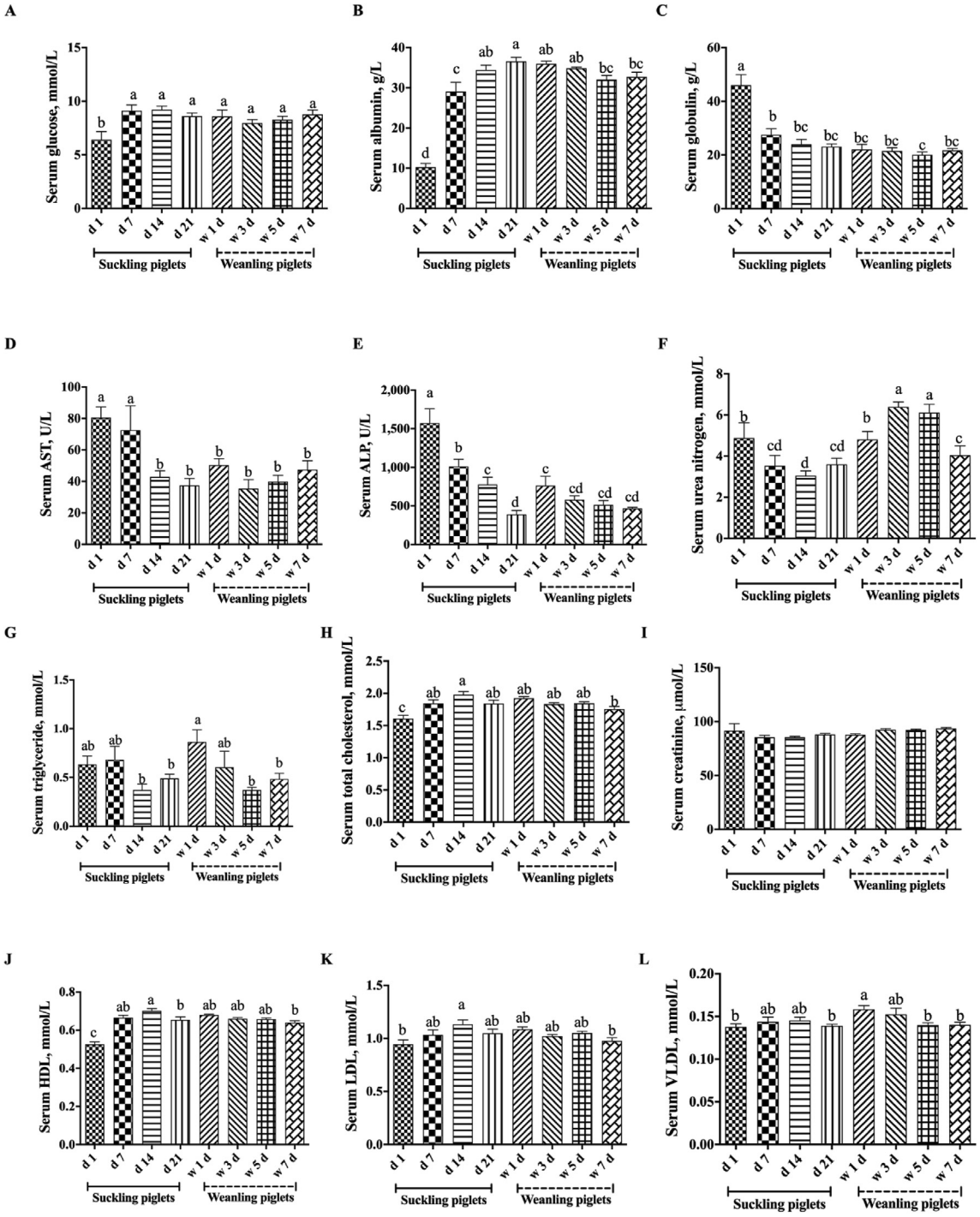

Fig. 2. Concentrations of serum biochemical metabolites and enzymes in suckling and weanling piglets. (A) glucose, (B) albumin, (C) globulin, (D) aspartate aminotransferase (AST), (E) alkaline phosphatase (ALP), (F) urea nitrogen, (G) triglycerides, (H) total cholesterol, (I) creatinine, (J) high density lipoprotein (HDL), (K) low-density lipoprotein (LDL), (L) very low-density lipoprotein (VLDL). Data are expressed as means ± SEM, $n = 8$. $^{a-d}$ Bars with different letters are significantly different ($P < 0.05$).

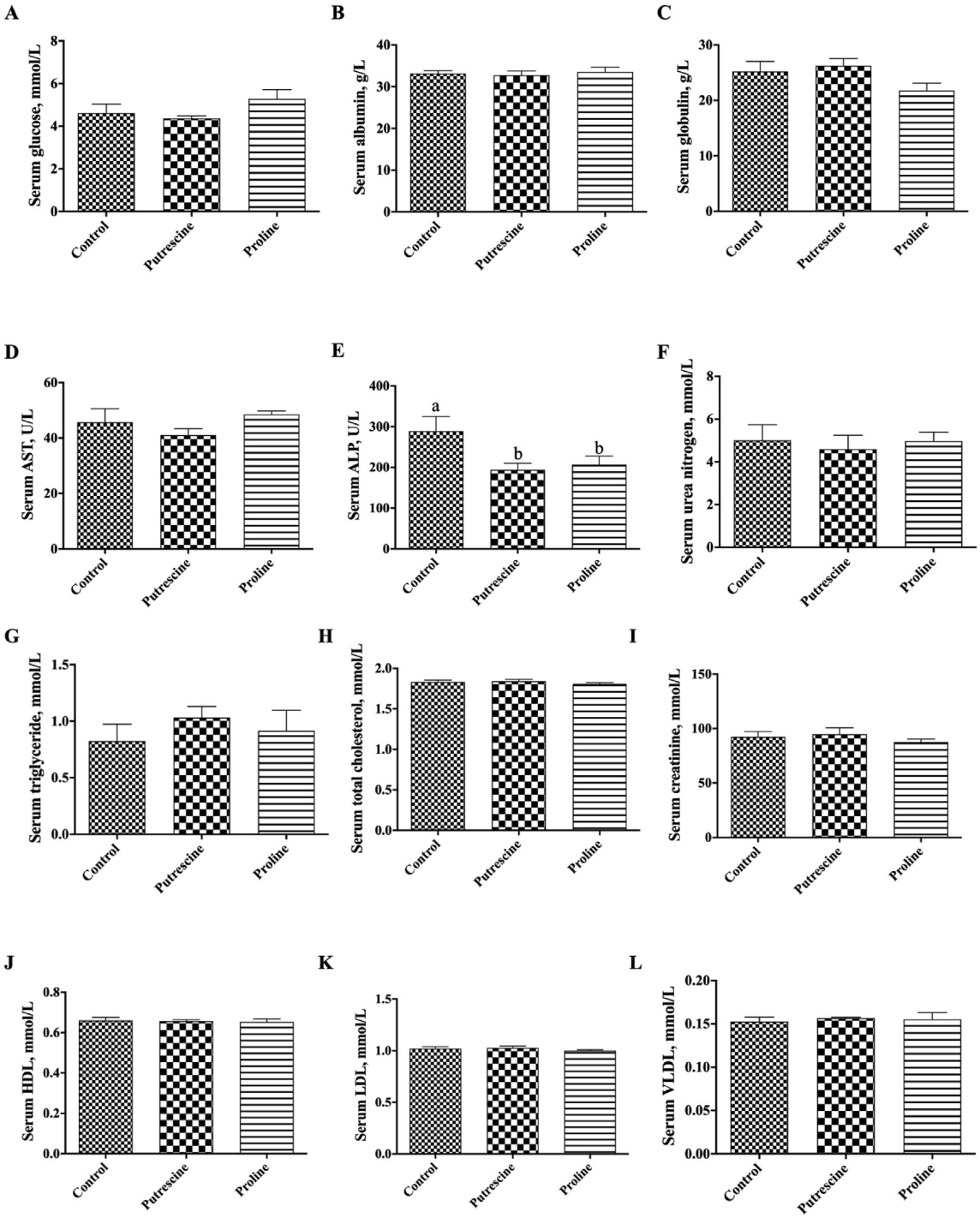

Fig. 3. Effects of putrescine and proline supplementation on serum biochemical metabolites and enzyme levels in piglets. (A) glucose, (B) albumin, (C) globulin, (D) aspartate aminotransferase (AST), (E) alkaline phosphatase (ALP), (F) urea nitrogen, (G) triglycerides, (H) total cholesterol, (I) creatinine, (J) high density lipoprotein (HDL), (K) low density lipoprotein (LDL), (L) very low-density lipoprotein (VLDL). Data are expressed as means ± SEM, $n = 6$. [a, b] Bars with different letters are significantly different ($P < 0.05$).

age compared to piglets at d 14 and 21 of age ($P = 0.006$). Weaning at 14 d old did not affect serum AST and ALP activities. However, BUN at d 1 post-weaning was increased compared to that in 14-d-old suckling piglets, and it reached the highest values at d 3 and 5 post-weaning then decreased at d 7 post-weaning ($P = 0.006$). Differences regarding the triglycerides and VLDL values in the serum of suckling piglets were statistically insignificant, whereas weaning induced an increase in triglycerides at d 1 post-weaning and then it decreased with age ($P = 0.034$). The levels of total cholesterol ($P = 0.020$), HDL ($P = 0.017$), and LDL ($P = 0.036$) in serum at d 14 postnatal were higher than these of piglets at birth. Early weaning had no effect on serum total cholesterol, HDL, and LDL levels of piglets after weaning.

At d 3 post-weaning, there were no significant differences between control, Put and Pro groups in respect to the majority of the mentioned serum metabolites and hepatic enzymes, apart from the ALP activities that were significantly decreased ($P = 0.044$) in Put and Pro groups compared to those in the control group (Fig. 3E).

Table 2
Serum amino acids concentrations in suckling and weanling piglets[1].

Item	Suckling				Post-weaning				Pooled-SEM	*P*-value
	d 1	d 7	d 14	d 21	w 1 d	w 3 d	w 5 d	w 7 d		
Essential amino acids, μg/mL										
Threonine	7.83	10.45	5.47	7.11	7.21	5.34	8.98	4.82	0.559	0.142
Valine	9.29	11.46	6.33	9.58	9.86	7.33	7.65	5.41	0.654	0.308
Methionine	2.20	4.94	2.58	3.71	3.66	2.23	2.40	2.09	0.267	0.051
Isoleucine	3.80	6.75	4.38	6.34	6.55	5.56	6.55	5.02	0.412	0.494
Leucine	11.30	20.07	13.03	18.86	19.47	16.54	19.47	14.93	1.224	0.494
Tryptophan	1.51	4.06	3.22	3.28	3.34	2.56	2.67	2.50	0.206	0.083
Phenylalanine	3.02	5.36	3.48	5.03	5.20	4.42	5.20	3.99	0.327	0.494
Lysine	12.30	13.88	6.90	13.16	10.95	4.53	6.58	8.92	0.923	0.089
Histidine	3.55	6.22	2.75	4.57	3.27	3.54	5.18	3.76	0.314	0.092
Conditionally essential amino acids, μg/mL										
Arginine	4.25^{b}	12.80^{a}	6.19 ^{ab}	7.46 ^{ab}	9.03 ^{ab}	5.06^{b}	7.59 ^{ab}	5.17^{b}	0.655	0.014
Glutamine	10.55	18.74	10.65	17.62	18.19	15.45	18.19	13.95	1.154	0.370
Glycine	13.02	33.47	15.60	19.23	19.13	14.96	22.91	22.15	1.719	0.066
Cysteine	2.05 ^{ab}	2.49^{a}	1.37 ^{bcd}	1.07 ^{cd}	1.84 ^{abc}	1.01^{d}	0.72^{d}	0.94^{d}	0.118	<0.01
Proline	1.60	2.84	1.84	2.67	2.76	2.34	2.97	2.11	0.174	0.403
Non-essential amino acids, μg/mL										
Aspartic acid	0.99^{b}	3.12^{a}	1.54^{b}	1.83 ^{ab}	1.36^{b}	1.38^{b}	1.52^{b}	1.22^{b}	0.180	0.022
Serine	6.52 ^{ab}	8.92^{a}	3.91^{b}	5.63 ^{ab}	4.11^{b}	3.20^{b}	5.05^{b}	4.02^{b}	0.452	0.022
Alanine	14.10^{b}	28.12^{a}	13.69^{b}	20.98 ^{ab}	12.53^{b}	10.92^{b}	17.56 ^{ab}	15.89^{b}	1.401	0.035

w = post-weanling.
$^{a-d}$ Values with different letters within the same row are significantly different ($P < 0.05$).
[1] $n = 8$.

3.2. Serum AA profiles of suckling and weanling piglets

Nutritionally, AA are classified as essential or nonessential for animals based on their traditional role in protein synthesis. Table 2 shows the AA profiles in serum of suckling and weanling piglets. There were no significant differences in essential AA concentrations of serum among suckling and weanling piglets at a different age. The concentrations of aspartic acid (Asp) ($P = 0.022$), serine (Ser) ($P = 0.022$), alanine (Ala) ($P = 0.035$), cysteine (Cys) ($P < 0.001$), and arginine (Arg) ($P = 0.014$) in serum increased at d 7 postnatal and then decreased with age in suckling piglets, and weaning at 14 d old did not affect these AA concentrations in serum with the exception of Cys. Within the first week after weaning, serum Cys concentration at d 1 post-weaning was higher than those at d 3, 5, and 7 post-weaning ($P < 0.05$).

Table 3
The effects of oral administration with putrescine and proline on serum amino acids concentrations in piglets[1].

Item	Control	Putrescine	Proline	Pooled-SEM	*P*-value
Essential amino acids, μg/mL					
Threonine	31.05	32.68	32.16	1.292	0.884
Valine	27.99	28.71	28.10	1.214	0.970
Methionine	6.46	6.67	6.74	0.330	0.945
Isoleucine	15.11	16.31	15.96	0.680	0.781
Leucine	20.92	19.57	21.59	0.933	0.693
Tryptophan	8.33	7.28	8.33	0.423	0.528
Phenylalanine	14.15	15.48	14.20	0.756	0.742
Lysine	30.85	29.52	31.82	0.923	0.622
Histidine	10.85	10.81	10.11	0.452	0.774
Conditionally essential amino acids, μg/mL					
Arginine	19.63^{b}	23.30 ^{ab}	27.22^{a}	1.122	0.012
Glutamine	59.06^{b}	55.12^{b}	69.42^{a}	2.343	0.025
Glycine	46.34	49.88	48.24	1.973	0.785
Cysteine	13.53	15.05	12.63	0.831	0.513
Proline	10.41^{b}	10.67^{b}	17.82^{a}	0.961	<0.01
Non-essential amino acids, μg/mL					
Aspartic acid	4.26	4.59	4.42	0.265	0.890
Serine	11.75	11.17	11.93	0.575	0.867
Alanine	44.45	42.54	44.32	2.097	0.926

$^{a, b}$ Values with different letters within the same row are significantly different ($P < 0.05$).
[1] $n = 6$.

Table 3 shows the effect of oral administration with Put and Pro on the serum AA profile in weanling piglets. Administration with Pro enhanced the glutamine (Gln) ($P = 0.025$) and Pro ($P < 0.001$) levels in serum as compared to these in control and Put groups. Proline treatment also showed higher ($P = 0.012$) Arg concentration in serum than that in the control group. However, Put administration did not affect the AA profile in serum.

3.3. Amino acid transporters gene expressions in the small intestine of suckling and weanling piglets

The gene expression levels of AA transporters in the small intestine are presented in Figs. 4 and 5. The *SLC7A6* mRNA levels of jejunal ($P < 0.001$) and ileal ($P < 0.001$) mucosa, as well as the ileal mucosa *SLC7A7* mRNA levels ($P < 0.001$) at d 1 and 7 postnatal were significantly higher and then decreased with age. Suckling piglets at birth (d 1) had lower mRNA levels of *SLC7A7* ($P < 0.001$) in jejunal mucosa compared with those on d 7. Within the first week after weaning, the mRNA expression level of *SLC7A6* in jejunal mucosa significantly decreased ($P < 0.001$) from d 1 to 5 post-weaning. The mRNA level of *SLC7A7* in jejunal mucosa was lowest at d 3 post-weaning, but recovered to pre-weaning level at d 7 after weaning ($P < 0.001$). Further, the *SCL7A6* mRNA abundance in jejunal mucosa at d 7 of weanling piglets was higher than ($P < 0.001$) that in 21-d-old suckling piglets. Considerable increases ($P = 0.005$) of *SLC36A1* mRNA levels in ileal mucosa were observed at d 1 and 7, and then these were reduced with age. Changes of *SLC36A1* mRNA abundances in jejunal mucosa remained insignificant during the suckling period. In the first 7 d after weaning, the relative mRNA abundances of *SLC36A1* in ileal mucosa were significantly increased ($P = 0.005$) at d 3, then were followed by a decrease at d 7 post-weaning. There was a lower ($P = 0.005$) mRNA level of *SCL36A1* in jejunal mucosa observed in weanling piglets at d 7 post-weaning in comparison with that in 21-d-old suckling piglets. The jejunal mucosal *SLC1A1* mRNA abundance at d 7 postnatal and the ileal mucosal *SLC1A1* mRNA abundance at d 1 were markedly higher ($P = 0.0016$) and then declined until d-21 in suckling piglets. Weaning at 14 d of age increased ($P = 0.0016$) the mRNA abundances of *SCL1A1* in jejunal and ileal mucosa at d 1 post-weaning when compared to these in

14-d-old suckling piglets. Subsequently, the *SCL1A1* mRNA levels were reduced with age.

As shown in Fig. 5, Put and Pro treatments did not affect *SCL7A6* and *SLC7A7* mRNA levels in jejunal and ileal mucosa. Compared to the control group, Put administration increased the jejunal ($P < 0.001$) and ileal ($P < 0.001$) mucosal *SCL36A1* mRNA abundances, and Pro administration increased the ileal mucosal *SCL36A1* mRNA abundance ($P < 0.001$). Administration of Put and Pro increased the *SLC1A1* mRNA abundances of jejunal ($P = 0.009$) and ileal ($P < 0.001$) mucosa of piglets as compared to these in the control group.

Additionally, Appendix Tables 1 and 2 show the correlation between serum AA concentrations and their transporters in the small intestine. A significant positive correlation was found between *SLC7A6* mRNA levels in ileal mucosa and serum Cys ($r = 0.58$) concentration in suckling and weanling pigs. Jejunal mucosal *SLC7A7* mRNA abundances positively correlated with methionine (Met), tryptophan (Trp), lysine (Lys), Arg, glycine (Gly), Cys, Asp and Ala levels in the serum of suckling and weanling pigs ($r = 0.33$, 0.39, 0.37, 0.33, 0.33, 0.43, 0.33, 0.32, respectively). Ileal mucosal *SLC36A1* mRNA levels positively correlated with serum concentrations of threonine (Thr), Cys, Asp and Ser ($r = 0.41$, 0.41, 0.45, 0.36, respectively). In jejunal mucosa, a significant positive correlation was found between *SLC1A1* and serum levels of Thr, Met, Lys, histidine (His), Arg, Gly, Cys, Asp, Ser, and Ala ($r = 0.34$, 0.38, 0.32, 0.31, 0.40, 0.40, 0.49, 0.44, 0.42, 0.34, respectively). In weanling pigs, following Put and Pro supplementation, jejunal or ileal mucosa *SLC7A6* mRNA abundances negatively correlated with serum levels of valine (Val) and Met ($r = -0.48$, -0.67, -0.65, respectively). A negative correlation was observed between ileal mucosal *SLC7A7* mRNA abundances and Thr and Gly concentrations in serum ($r = -0.51$, -0.50). A positive correlation was found in jejunal mucosal *SLC36A1* mRNA levels and serum concentration of Arg ($r = 0.47$). Ileal mucosal *SLC1A1* mRNA abundances positively correlated with Arg, Gln and Pro concentrations in serum ($r = 0.50$, 0.51, 0.72, respectively).

3.4. mTOR signaling pathway in jejunal mucosa of suckling and weanling piglets

In order to study the developmental changes in intestinal protein synthesis of suckling and weanling piglets, and to test whether oral administration of Put and Pro in the suckling period affects the protein synthesis in the gut of weanling-pigs, the mTOR, p70S6K and 4EBP1 protein abundances were determined and these are shown in Fig. 6. The ratio of p-mTOR to mTOR ($P < 0.001$) and the ratio of p-p70S6K to p70S6K ($P < 0.001$) in jejunal mucosa significantly increased from d 1 to 21, whereas the ratio of p-4EBP1 to 4EBP1 of jejunal mucosa at d 14 was lower than ($P < 0.001$) that in d 1, 7 and 21 of suckling piglets. Compared with the values in 14-d-old suckling piglets, the mTOR and 4EBP1 phosphorylation statuses were remarkably reduced at d 3 and d 5 post-weaning, respectively, and then phosphorylated mTOR and 4EBP1 gradually increased until d 7 post-weaning but were still lower than these in 21-d-old suckling piglets. Although weaning at 14 d did not decrease the p70S6K phosphorylation at d 1 and 3 post-weaning, the p70S6K phosphorylation significantly increased ($P < 0.001$) at d 5 and 7 post-weaning and was found to be higher than that in 21-d-old suckling piglets.

Oral administration with Pro increased the phosphorylated levels of mTOR ($P < 0.001$), p70S6K ($P = 0.002$) and 4EBP1 ($P < 0.001$) in jejunal mucosa as compared to control and Put-treated groups. Compared to the control group, Put treatment enhanced the phosphorylated levels of mTOR ($P < 0.001$) and 4EBP1 ($P < 0.001$), whereas it did not affect the ratio of p-p70S6K to p70S6K in jejunal mucosa of weaning piglets.

4. Discussion

In a commercial setting, suckling piglets are weaned at 15 to 28 d of age to optimize whole herd production (McGlone and Pond, 2003). Early-weaning could decrease the intestinal digestive and absorptive capacities, which induces malabsorption of nutrients and

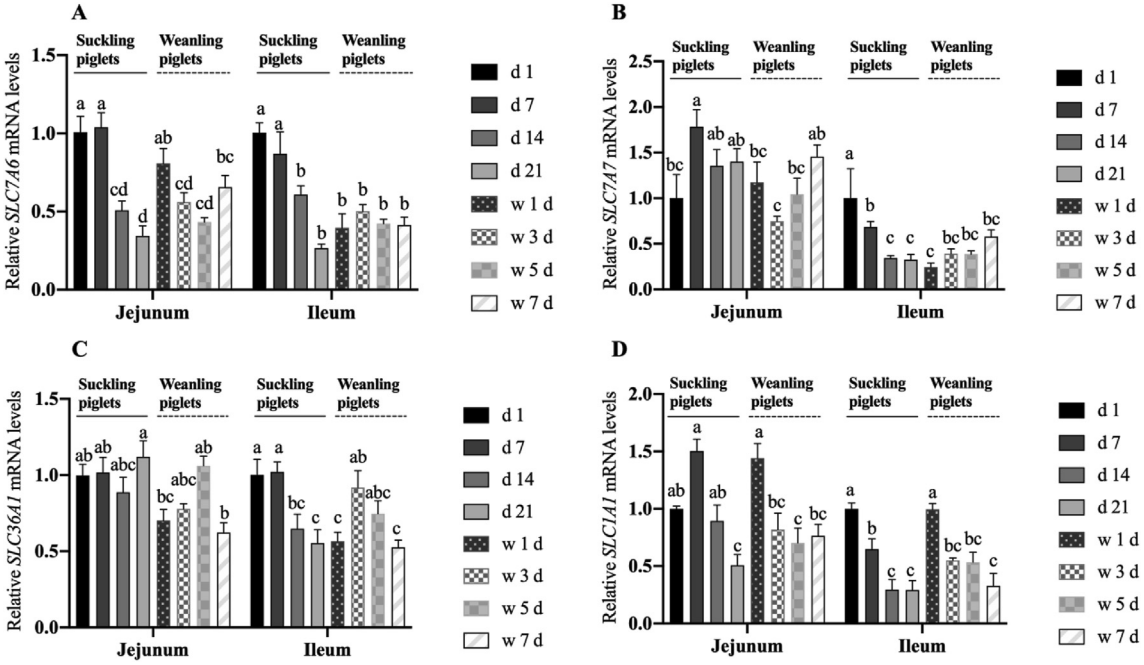

Fig. 4. Relative mRNA levels of amino acids transporters in the jejunal and ileal mucosa of suckling and weanling pigs. (A) solute carrier family 7 member 6 (*SLC7A6*), (B) *SLC7A7*, (C) proton-assisted AA transporter 1 (*SLC36A1*), (D) excitatory AA transporter 3 (*SLC1A1*). Data are expressed as means ± SEM, $n = 8$. w = post-weaning. $^{a-d}$ Bars with different letters are significantly different ($P < 0.05$).

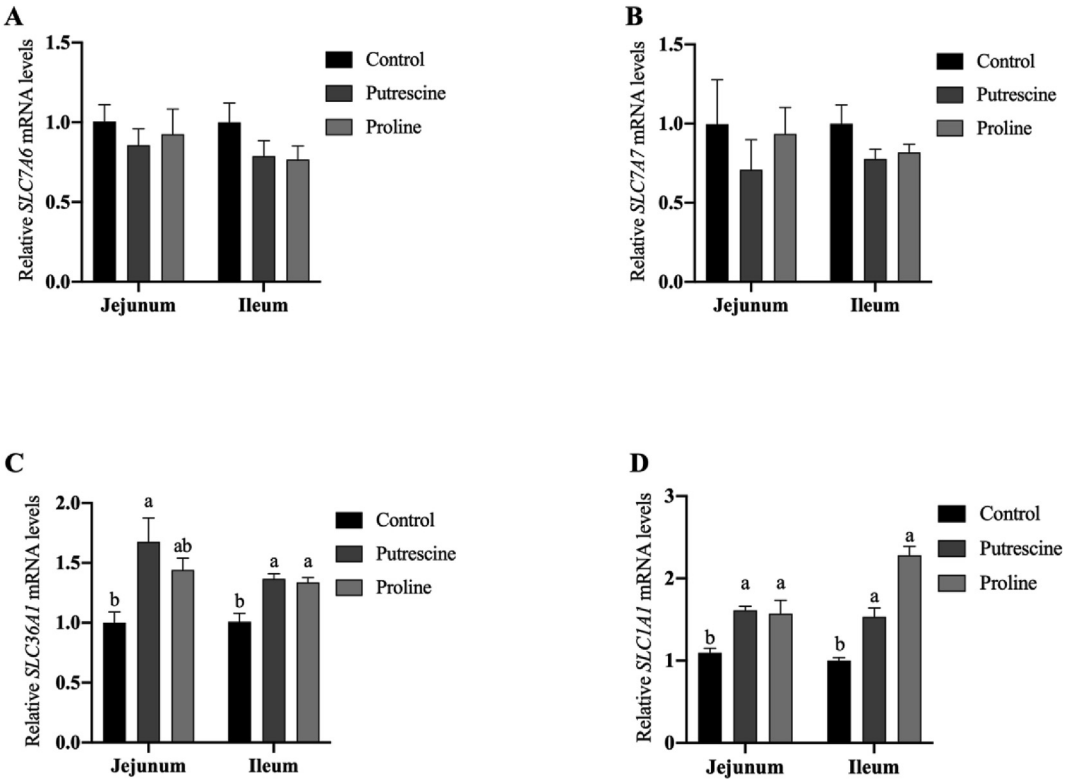

Fig. 5. Effects of putrescine and proline supplementation on AA transporter mRNA levels of jejunal and ileal mucosa in piglets. (A) solute carrier family 7 member 6 (*SLC7A6*), (B) *SLC7A7*, (C) proton-assisted AA transporter 1 (*SLC36A1*), (D) excitatory AA transporter 3 (*SLC1A1*). Data are expressed as means ± SEM, $n = 8$. [a, b] Bars with different letters are significantly different ($P < 0.05$).

growth retardation (Yang et al., 2013). Optimal nutritional strategies after weaning are necessary to guarantee intestinal development and growth performance (Mou et al., 2019). Some functional AA and their metabolites are key regulators of cell signaling and gene expression (Wu, 2009; Nesterov et al., 2020). A thorough understanding of the process of AA metabolism in suckling and weanling period is therefore very important will help optimize the weaning transition of piglets. The results of the present study indicated that the serum AA profile, intestinal AA transporters and mTOR signaling pathway activity in piglets were altered by the early-weaning stress. The most severe impairment in the intestinal barrier was observed from d 3 to 5 post-weaning (Wang et al., 2016b). In the current study, Pro and Put administration during the suckling period improved the AA metabolism by activating the mTOR pathway of the gut in piglets 3 d post-weaning.

The neonatal piglets gradually establish their metabolic mechanism and undergo intensive growth of bone, muscle, fat, and gut (Kabalin et al., 2017). The values of serum biochemical parameters of suckling piglets change continuously until weaning, whereas weaning leads to alternations in many nutritional metabolism processes, such as protein synthesis (Lallès et al., 2004), AA metabolism (Petersen, 1994) and lipid metabolism of the visceral organ (Gu and Li, 2003). In this study, the serum glucose and albumin levels were lower at birth and then gradually increased until the end of the suckling period, whereas the highest value of immunoglobulin was found in newborn piglets and was followed by a significant decrease until 21 d old. Systemic glucose can be oxidized to provide energy and is involved in the synthesis of fatty acids (Uyeda and Repa, 2006). Serum albumin and immunoglobulin might reflect hepatic protein metabolic status and immune status of young piglets (Wang et al., 2011). Our results were consistent with the Kabalin et al. (2017) study, which indicated that the increase of glucose and albumin levels were due to the established gluconeogenesis pathway after colostrum intake, as well as the most intensive liver protein synthesis and faster muscle growth during the first two weeks after birth. Neonatal piglets received immunoglobulin from maternal colostrum to ensure the passive systemic immunity, whereas the absorption of immunoglobulin through the intestine only occurs up to 18 to 36 h after birth. After that, no more immunoglobulin or other macro-molecules can be absorbed (de Passillé et al., 1988). The significant decrease of immunoglobulin in our study may have occurred because of the "gut-closure" of newborn pigs and the degradation of maternal immunoglobulin. Consistent with this result, Szymeczko et al. (2008) reported that total globulin concentration in serum of 24-h-old piglets amounted to 53.39 ± 1.01 g/L, and significantly decreased on 7th day to 33.69 ± 0.78 g/L. The BUN levels in the blood, the main dead-end product of protein metabolism, is an indicator of AA utilization efficiency (Wang et al., 2011; Lv et al., 2018). Increased BUN concentration is related to decreased protein synthesis and increased protein catabolism. The high levels of BUN at birth pointed to faster catabolism of proteins to cover energy needs and sustain growth (Kabalin et al., 2017), and then BUN levels reduced with increasing volume sow's milk intake, liver protein synthesis and blood glucose concentrations. Weaning induced the elevated BUN level from d 1 to 5 post-weaning, which might be explained by the decreased feed intake during the first 48 h after weaning (Brooks et al., 2001) and the impaired protein turnover in various tissues during the most severe stage of weaning stress (Deng et al., 2009; Suryawan and Davis, 2014; Yang et al., 2016b). The activities of AST and ALP in serum have been considered as good markers of soft tissue damage, including alternations

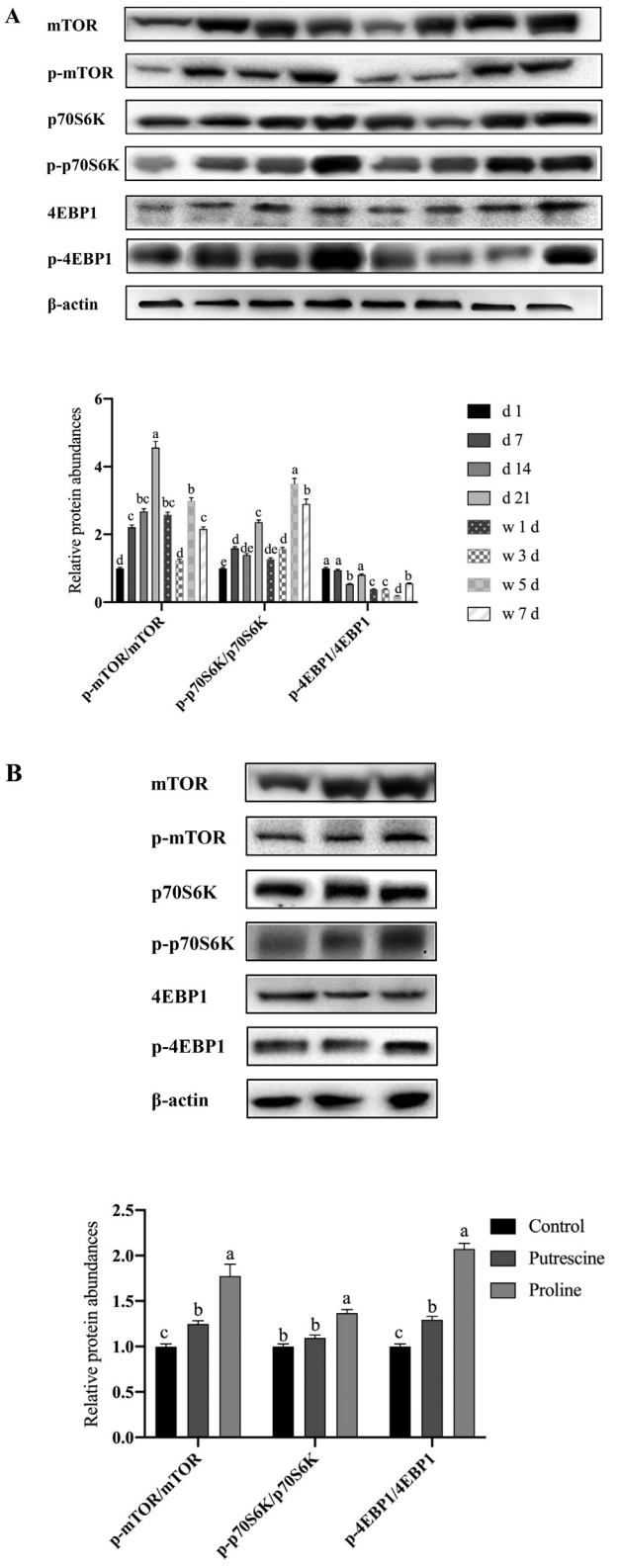

Fig. 6. Protein abundances of mTOR signaling pathway in jejunal mucosa of piglets. (A) Representative western blots and relative protein abundances of mTOR, p-mTOR, p70S6K, p-p70S6K, 4EBP1, p-4EBP1, and β-Actin in jejunal mucosa of suckling and weanling piglets. (B) Representative western blots and relative protein abundances of mTOR, p-mTOR, p70S6K, p-p70S6K, 4EBP1, p-4EBP1, and β-Actin in jejunal mucosa of piglets treated with putrescine and proline; $n = 6$. mTOR = mammalian target of rapamycin; p-mTOR = phosphorylation mTOR; p70S6K = phosphoprotein 70 ribosomal protein S6 kinase-1; 4EBP1 = eIF4E-binding protein-1; p-4EBP1 = phosphorylation 4EBP1; w = post-weanling; Data are expressed as means ± SEM, $n = 8$. $^{a-e}$ Bars with different letters are significantly different ($P < 0.05$).

of membrane permeability and consequent release of enzymes into the extracellular fluid (Nyblom et al., 2004; Obaleye et al., 2007). The neonatal pig intestine undergoes rapid growth and reorganization, which would induce dramatic changes in intestinal structure and barrier function (Buddington, 1994; Zhang et al., 1997). The high concentrations of AST and ALP in serum during the first week after birth indicated the neonatal pig liver and gut were not fully formed and mature, exhibited by higher membrane permeability (Ramsay et al., 2018), whereas the levels of AST and ALP in serum were reduced with the development of piglets and their organs. Liver and adipose tissue are the main organs for serum triglycerides synthesis and storage (Wang et al., 2011). Serum total cholesterol, including free cholesterol and cholesterol ester, is mainly synthesized and stored in the liver (Wang et al., 2011). Similar to changes of serum triglycerides levels observed in our study, Kabalin et al. (2017) also found that the level of serum triglycerides was 2.57 mmol/L in 1-d-old piglets and then reduced to 1.34 mmol/L in 21-d-old piglets. They suggested that the decreased blood triglycerides concentration might have resulted from the establishing of synthesis and storing fat which is very intensive during the first week of suckling, and may also be explained by the triglycerides levels in sow's milk decreasing with lactation (Kabalin et al., 2017). Weaning at 14 d old enhanced the concentration of serum triglycerides at first-day post-weaning, indicating that the efficiency of lipids utilization was negatively affected by weaning stress (Liu et al., 2018). Additionally, serum total cholesterol, HDL, LDL, and VLDL gradually increased during the suckling period, which also reflected that the lipometabolic status of neonatal piglets was altered with increasing age. Notably, oral administration of Put and Pro in this study did not affect the values of most of the blood biochemical parameters but decreased the serum ALP level of weaning pigs. The increased concentrations of ALP in blood exhibited an increased intestinal permeability (Bilski et al., 2017; Celi et al., 2019). Results in the current study were comparable with our previous study that Put and Pro could maintain intestinal integrity and decrease gut permeability (Wang et al., 2015a).

Observation of the dynamic profile of blood AA is important to study the changes in AA metabolism of piglets during suckling and post-weaning. In the present study, the concentrations of most AA (Asp, Ser, Ala, Cys, and Arg were significantly different) in serum were highest at 7-d-old of suckling piglets, which was supported by previous researches reported by Yin et al. (2011) and Thongsong et al. (2019). Although Asp, Ser, Ala, Cys and Arg are nutritionally non-essential AA, these AA do have a significant effect on systemic homeostasis and metabolism of young pigs (Rezaei et al., 2013). For example, Arg is currently considered nutritionally essential for neonatal piglets, and could stimulate the secretion of insulin and growth hormone, as well as nitric oxide and polyamines, thereby playing an important role in regulating the protein accretion, white fat accretion and cell proliferation (Flynn et al., 2000; Rezaei et al., 2013; Liu et al., 2017). Cys is extensively utilized by the gut and is involved in the bio-synthesis of the mucosal epithelial proteins, including glutathione and mucin (Bauchart-Thevret et al., 2011). Asp and Ala are abundant in sow's milk (Rezaei et al., 2016), and Asp is one of the major energy sources for mammalian enterocytes (Wu, 2013). Several studies have demonstrated that Asp contributes to improving the intestinal mucosal energy status, enhancing the barrier morphology in piglets (Wang et al., 2015b; Chen et al., 2016). Serine has been suggested as playing key roles in protein synthesis, one-carbon metabolism, and cell signal transduction in pigs (Rezaei et al., 2013; Zhou et al., 2018). The change of serum AA pattern in piglets may suggest the extensive requirement of certain AA and their metabolites and utilization (Bengtsson, 1971; Mou et al., 2019) in the early stage after birth. The absorption of AA mainly depends on their respective transporter systems on the membrane of the

enterocyte (Yin et al., 2014). *SLC7A6* and *SLC7A7* are the Na^+-independent transporters for both cationic AA and neutral AA, such as Arg, Gln, and Lys (Yang, 2011; Thongsong et al., 2019). *SLC36A1* is classically described as the system IMINO mediated Na^+-dependent transporter that can transport imino AA such as Pro (Yang, 2011). SLC1A1 is a primary Na^+-dependent Glu transporter expressed in small intestine epithelial cells and is also capable of Asp and Cys transport (Wu et al., 2015; Wang et al., 2017). In the suckling period, the mRNA abundances of *SLC7A6*, *SLC7A7*, *SLC36A1* and *SLC1A1* in jejunal or ileal mucosa increased in 1- or 7-d-old piglets, which positively correlated with serum Asp, Arg, Cys, Ser and Ala concentrations. This is consistent with the observations from previous studies that the rate of absorption of AA was increased from d 1 to 7 after birth and then gradually declined or remained unchanged (Xiao, 2005; Fu et al., 2013). The changes in mRNA abundances of *SLC7A7* and *SLC1A1* post-weaning were comparable with the Xiao (2005) study that AA transporters mRNA expression levels increased during the first day after weaning and then decreased to the value at weaning by d 21. These findings were not in line with the results of Wang et al. (2017) who suggested weaning decreased the SLC1A1 abundance in the small intestine.

Another interesting finding is that Pro supplementation increased serum Gln, Arg and Pro concentrations, as well as the mRNA levels of *SCL36A1* and *SLC1A1* in the small intestine of weaning piglets. Our results confirmed that supplementing Pro for suckling pigs could increase blood Pro level in weanling-pigs (Wu et al., 2010; Brunton et al., 2012) and Pro plays an important role in the synthesis of Arg and Gln in enterocytes of the small intestine (Wilkinson et al., 2004; Wu et al., 2011; Liu et al., 2017). Although Put changed the *SCL36A1* and *SLC1A1* gene, it did not affect the AA levels in the serum of weanling pigs. As Fang et al. (2019) described, supplementation of spermine, which could be converted from Put, enhanced the AA transporters mRNA abundances in the ileum of piglets during d 3 to 9 after weaning at 12 d old. However, the increased serum AA levels in the spermine treatment group were also found in the Fang et al. study, which is not consistent with our result, possibly because their piglets were fed with liquid formula milk after weaning rather than a weaning diet. Ewtushik et al. (2000) fed weanling piglets with a solid diet supplemented with polyamines and found that polyamines treatment did not affect the AA concentrations in the blood of weanling piglets.

In addition to the AA profile in blood and their transporter gene expressions in the small intestine, the mTOR signaling pathway, a major regulator of protein synthesis, was investigated in the current study. The weight and total protein of small intestine in neonatal pigs markedly increase 6- or 3-fold between birth and 3 weeks of age, respectively (Aumaitre and Corring, 1978; Burrin and Stoll, 2003b). We failed to detect the protein synthesis rate in the small intestine, but in our study the activation of mTOR and its downstream p70S6K and 4EBP1 in the small intestine dramatically increased with the increasing age of suckling pigs. mTOR is a well-known mediator of cell growth and proliferation, and its activity is regulated by a variety of factors such as nutrients, growth factor, and cellular stress (Kim et al., 2013; Huang and Fingar, 2014). The present study also identified the protein expression of mTOR pathway in the small intestine was inhibited by weaning (Yang et al., 2016a). The dynamic changes of mTOR signaling pathway with age coincided with the changes in cell differentiation and proliferation of small intestinal mucosa in our previous study (Wang et al., 2016b). Both Pro and Put play essential roles in promoting cell proliferation and protein activity (van Meijl et al., 2010; Kong et al., 2014) as well as being involved in repairing intestinal damage caused by weaning (Wang et al., 2015a), which is corroborated by up-regulated mTOR pathway phosphorylation levels of the small intestine in Pro and Put-treated weanling pigs in the current study.

5. Conclusion

Our results revealed that blood biochemical parameters, AA profiles, AA transporters gene expressions and mTOR pathway protein abundances were age-dependent changes in suckling piglets between birth to 21 d old. Weaning at 14 d old altered the protein metabolism, and blood triglyceride concentration, as well as the phosphorylation of intestinal mTOR pathway in piglets. Oral administration of Put and Pro during the suckling period may improve the membrane permeability of the intestine, and Pro is critical for the regulation of AA metabolism and transportation in weanling piglets. The beneficial effects of Pro and Put were partially mediated by activation of mTOR pathway in the gastrointestinal tract. Therefore, the results of this work may provide helpful insights into the developmental changes of AA metabolism and its related mTOR signaling pathway in suckling and post-weanling pigs, as well as the role of Pro and polyamines in improving weaning adaption and transition.

Author contributions

Jing Wang: investigation, visualization, writing - original draft; **Jianjun Li**: data curation; **Ming Qi**: investigation; **Yuxin Xiao**: investigation; **Bie Tan**: supervision, review & editing.

Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that might inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2020.11.007.

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