



Original Research Article

Effects of electrolyte balance on intestinal barrier, amino acid metabolism, and mTORC1 signaling pathway in piglets fed low-protein diets

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ABSTRACT

A proper dietary electrolyte balance (dEB) is essential to ensure optimal growth performance of piglets. In the low-protein diet, this balance may be affected by the reduction of soybean meal and the inclusion of high levels of synthetic amino acids. The objective of this experiment was to evaluate the optimal dEB of low-protein diets and its impact on the growth performance of piglets. A total of 108 piglets (initial age of 35 d) were randomly divided into 3 groups with 6 replicates of 6 pigs each as follows: low electrolyte diet (LE group; dEB = 150 milliequivalents [mEq]/kg); medium electrolyte diet (ME group; dEB = 250 mEq/kg); high electrolyte diet (HE group; dEB = 350 mEq/kg). Results indicated that the LE and HE diet significantly decreased the average daily gain, average daily feed intake, and crude protein digestibility ($P < 0.05$) in piglets. Meanwhile, LE diets disrupted the structural integrity of the piglets' intestines and decreased jejunal tight junction protein (occludin and claudin-1) expression ($P < 0.05$). Additionally, the pH and HCO_3^- in the arterial blood of piglets in the LE group were lower than those in the ME and HE groups ($P < 0.05$). Interestingly, the LE diet significantly increased lysine content in piglet serum ($P < 0.05$), decreased the levels of arginine, leucine, glutamic acid, and alanine ($P < 0.05$), and inhibited the mammalian target of rapamycin complex 1 (mTORC1) pathway by decreasing the phosphorylation abundance of key proteins. In summary, the dietary electrolyte imbalance could inhibit the activation of the mTORC1 signaling pathway, which might be a key factor in the influence of the dEB on piglet growth performance and intestinal health. Moreover, second-order polynomial (quadratic) regression analysis showed that the optimal dEB of piglets in the low-protein diet was 250 to 265 mEq/kg.

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1. Introduction

Since the shortage of protein resources has always been one of the important factors restricting the development of swine husbandry (Cao and Li, 2013; Ouyang et al., 2013), low-protein dietary strategies with amino acid balance in pig production have been widely accepted. Previous researchers have reported that supplementing with crystalline amino acids instead of soybean meal can improve nitrogen utilization, and reduce feed costs and nitrogen excretion without compromising pig growth performance (Wang et al., 2018). Meanwhile, it has also been discovered that low-protein diets decrease the growth performance of piglets (Gómez et al., 2002; Zhang et al., 2013). Interestingly, our previous

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research found that low-protein diets could lead to dietary electrolyte imbalance (not yet published). Thus, it is speculated that the electrolyte imbalance in the diet was one of the main reasons for the impact of low-protein diets on piglet growth performance, while little data were reported about the interaction between low-protein diets and electrolyte balance.

Dietary electrolyte balance (dEB) is a parameter that is important for pig growth but is not commonly used in feed formulation for pig nutrition (Lei et al., 2017). The dEB generally refers to the content of monovalent mineral cations and anions that are present in the diet and is defined as milliequivalents (mEq) of $\text{Na} + \text{K} - \text{Cl}$ ions (Chrystal et al., 2020). Meanwhile, the dEB has been reported to affect the acid–base balance, growth performance, and nutrient digestibility of pigs (Dersjant-Li et al., 2001; Guzmán-Pino et al., 2015). Therefore, maintaining a proper dEB is essential for optimal pig growth performance. There have been many studies on the optimal dEB to improve the growth performance of piglets (Guzmán-Pino et al., 2015; Lei et al., 2017). Nevertheless, the optimal dEB for pigs in the literature is inconsistent. Lei et al. (2017) concluded that weaned pigs achieved the optimal growth rate when fed diets containing 166 to 250 mEq/kg dEB. Jones et al. (2019) observed that in stages 1 and 2, the optimal dEB for weaned piglets was 243 and 199 mEq/kg, respectively. At present, data about the optimal dEB for low-protein diets in piglets have not been found until now.

Research has found that there may be adverse effects on the digestibility of some amino acids from the reduction in the dEB (Chrystal et al., 2020; Ravindran et al., 2008). Amino acids are one of the important regulators of the mammalian target of rapamycin complex 1 (mTORC1) signaling pathway in cells by nutrients (Bar-Peled and Sabatini, 2014). This signaling pathway in mammalian cells regulates various aspects of cell growth, proliferation, autophagy, and protein synthesis (Jewell et al., 2015). Cells employ this pathway to sense changes in energy, hormones, and nutrients from the internal and external sources. Through two downstream molecules, ribosomal protein S6 kinase 1 (S6K1) and eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1), which participate in the protein translation process when mTORC1 is phosphorylated by amino acid activation, it promotes protein synthesis (Huang and Fingar, 2014; Peterson et al., 2010). It is hypothesized that the dEB could regulate the mTORC1 signaling pathway by affecting amino acid metabolism.

Therefore, in this experiment, the optimal dEB under low-protein diet conditions was investigated to improve growth performance, nutrient digestibility, and intestinal health in piglets. In order to better apply the low-protein diet to swine husbandry and provide a reference for further research, the optimal electrolyte levels of piglets on the low-protein diet and its regulatory molecular mechanisms were investigated by setting three electrolyte levels in this experiment.

2. Materials and methods

2.1. Animal ethics statement

The experiment was approved by the Zhejiang University Animal Care and Use Committee (No. ZJU20230265).

2.2. Animals and experimental design

One hundred and eight piglets (Duroc × Landrace × Yorkshire; initial age of 35 d; initial BW of 9.80 ± 0.23 kg) were randomly divided into 3 dietary treatments with 6 replicates of 6 pigs each replicate according to initial BW, gender, and litter. Dietary treatments included the following: (1) low electrolyte diet (LE; CP = 16.5%,

dEB = 150 mEq/kg), (2) medium electrolyte diet (ME; CP = 16.5%, dEB = 250 mEq/kg), (3) high electrolyte diet (HE; CP = 16.5%, dEB = 350 mEq/kg). The dEB was calculated using the following formula: $\text{dEB (mEq/kg)} = (\text{Na} \times 434.98) + (\text{K} \times 255.74) - (\text{Cl} \times 282.06)$ (Chrystal et al., 2020). The calculated and detected values of the composition and nutrient levels of the experimental diet are shown in Table 1. The net energy value of the diet was calculated based on the data provided in the China Feed Composition and Nutritional Value Table (Tables of Feed Composition and Nutritive Values in China in Chinese, 2020). The dry matter (DM) was obtained by oven-drying at 105 °C. The measurement methods of CP (GB/T 6432-2018), crude fat (EE; GB/T 6433-2006), crude fiber (CF; GB/T 6434-2022), ash (GB/T 23742-2009), amino acids (GB/T 18246-2019), Ca (GB/T 6436-2018), P (GB/T 6437-2018), Na (GB/T 13885-2017), K (GB/T 13885-2017), and Cl (GB/T 6439-2007) in diet are all based on China National Standards, respectively. According to the recommendation of NRC (2012), excepting the CP level of the low-protein diet, all other nutritional requirements of piglets meet the requirements of 11 to 25 kg pigs.

2.3. Growth performance and diarrhea incidence

Each piglet was weighed on d 1 and 30 of the experiment. Feed intake for each pen pig was recorded daily. The values of average daily feed intake (ADFI), average daily gain (ADG), and the ratio of weight gain to feed intake (G:F) were calculated. The formula for calculating the diarrhea incidence was as follows: $\text{diarrhea incidence (\%)} = \frac{\sum (\text{the number of pigs with diarrhea per pen} \times \text{days of diarrhea})}{(\text{total number of pigs} \times \text{number of experimental days})} \times 100$ (Tang et al., 2022).

2.4. Samples collection and treatment

On the 30th day of the experiment, 6 pigs (1 pig per pen) were selected from each treatment group. Blood samples were collected from the vena cava of the piglets with a centrifuge tube without anticoagulant and was left to stand at room temperature for 30 min. Serum was collected after centrifuging at $1500 \times g$ for 10 min and was stored at -80 °C until analysis. Small intestine samples were collected after the piglets were sacrificed. After washing with phosphate-buffered saline (PBS; pH = 7.2 to 7.4), intestinal tissues from the duodenum, jejunum, and ileum (approximately 20 cm per tissue) were collected. One segment (approximately 3 cm per tissue) was fixed in 4% paraformaldehyde (BL539A, Biosharp, Guangzhou, China) for paraffin embedding, three segments (approximately 1 cm per tissue) to 2.5% glutaraldehyde (BL910A, Biosharp, Guangzhou, China) for electron microscopy detection, and the remaining intestinal segment for mucosal collection. Mucosal tissue samples were immediately frozen in liquid nitrogen and stored at -80 °C until analysis.

2.5. Apparent total tract nutrient digestibility analysis

The digestibility of DM, CP, EE, CF, ash, Ca, and P was determined by the Cr_2O_3 indicator method. At the beginning of the experiment, feed samples were collected and stored at -20 °C for nutritional analysis. After the feeding experiment, 6 pigs (1 pig per pen) were selected from each group and used Cr_2O_3 (analytical reagent, 10006918, Macklin, Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) as an exogenous indicator for the digestion test, and 0.5% Cr_2O_3 was mixed in the feed. The pre-trial period was 4 d and the trial period was 3 d. The feces collection time was 14:00 to 15:00 every day, and samples from each piglet were collected and stored separately using a rectal feces collection method for three consecutive days. After the fecal sample was collected, it was immediately mixed with 10% HCl in

Table 1
Ingredient and chemical composition of the basal diet (air-dry basis, %).

Ingredients	Treatment			Nutrient levels ¹	Treatment		
	LE	ME	HE		LE	ME	HE
Soybean meal	15.00	15.00	15.00	NE, kcal/kg	2455.13	2434.92	2413.64
Whey powder	3.00	3.00	3.00	DM	89.68	89.56	90.23
Rapeseed meal	3.00	3.00	3.00	CP	16.81	16.74	16.67
Fermented yellow wine lees	3.00	3.00	3.00	EE	3.20	3.21	3.20
Wheat bran	3.00	3.00	3.00	CF	2.71	2.71	2.70
Fish meal	2.00	2.00	2.00	Ash	4.36	4.34	4.31
Soybean oil	2.00	2.00	2.00	Lys	1.21	1.20	1.19
Sodium chloride	0.30	0.30	0.30	Met + Cys	0.71	0.69	0.66
Choline chloride (98%)	0.10	0.10	0.10	Thr	0.67	0.67	0.66
L-Lysine HCl	0.70	0.70	0.70	Try	0.23	0.22	0.20
DL-Met	0.20	0.20	0.20	Val	0.74	0.72	0.73
L-Thr	0.20	0.20	0.20	Leu	1.20	1.21	1.19
L-Try	0.05	0.05	0.05	Ile	0.64	0.61	0.60
L-Val	0.19	0.19	0.19	Ca	0.69	0.70	0.70
Ile	0.10	0.10	0.10	P	0.59	0.57	0.56
Limestone	0.60	0.60	0.60	Na	0.21	0.30	0.39
Calcium phosphate dibasic	0.70	0.70	0.70	K	0.72	0.96	1.18
Sodium bicarbonate	0.00	0.38	0.75	Cl	0.44	0.44	0.44
Potassium sulfate	0.00	0.45	0.90	dEB, mEq/kg	150.01	250.74	350.85
Premix ²	0.50	0.50	0.50				

DM = dry matter; EE = crude fat; CF = crude fiber; LE = low electrolyte diet (150 milliequivalents [mEq]/kg dietary electrolyte balance [dEB]); ME = medium electrolyte diet (250 mEq/kg dEB); HE = high electrolyte diet (350 mEq/kg dEB).

¹ Except for NE, which is a calculated value, all other nutritional levels are measured values.

² The premix provides following per kilogram of diet: vitamin A, 1750 IU; vitamin D₃, 200 IU; vitamin E, 11 IU; vitamin K₃, 0.5 mg; biotin, 50 µg; choline, 400 mg; folacin, 0.3 mg; niacin, 30 mg; pantothenic acid, 9 mg; riboflavin, 3 mg; thiamin 1 mg; vitamin B₆, 3 mg; vitamin B₁₂, 15 µg; Fe, 100 mg; Zn, 80 mg; Cu, 5 mg; I, 0.14 mg; Se, 0.25 mg; Mn, 3 mg.

a volume ratio of 5:1, sealed at -4°C , dried at $70 \pm 1^{\circ}\text{C}$, and crushed for testing. Finally, the fecal samples were dried at 65 to 70°C in an oven in the laboratory and stored at -20°C for chemical analysis. According to Chinese standards, Cr₂O₃ (GB/T 13088-2006) and nutritional components in the samples were analyzed. All contents were calculated by the following formula: Digestibility (%) = $100 - [(feed\ Cr_2O_3\ content/fecal\ Cr_2O_3\ content) \times (fecal\ nutrient\ content/feed\ nutrient\ content) \times 100]$.

2.6. Blood gas parameters and serum analysis

On the 30th day of the experiment, arterial blood was analyzed for pH, pCO₂, pO₂, bicarbonate (HCO₃⁻), and base excess (BE) by a blood gas analyzer (248 pH/Blood Gas Analyzer, Ciba Corning Diagnostics Ltd., Halstead, Essex, U.K.). The levels of Na⁺, K⁺, and Cl⁻ in serum were determined with commercial kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China). All assays were performed according to the manufacturer's instructions.

2.7. Detection of amino acids in serum

Amino acids analysis was performed using high-performance liquid chromatography (Hitachi L-8800 Amino Acid Analyzer, Tokyo, Japan), using pre-column derivatization with o-phthalaldehyde (CE12045, ChemeGen, Shanghai, China) (Dai et al., 2014). All chromatographic procedures were performed at room temperature.

2.8. Intestinal morphology

The duodenum, jejunum, and ileum were fixed and immersed in 4% paraformaldehyde, followed by dehydration and paraffin embedding of the fixed sample. Samples were cut into 5-µm sections and then stained with hematoxylin and eosin. All specimens were examined under a light microscope (Nikon Eclipse E-400 equipped with a digital camera head [DS-5M] and camera control

unit [DS-L1], Nikon, Tokyo, Japan). Villus height, villus width, and crypt depth were measured using an image analysis system.

2.9. Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) experiment

The TEM experiment was carried out as previously described (Shuting et al., 2019). The specimens of jejunum were first fixed for 1 to 2 h using 2.5% glutaraldehyde and 1% osmium tetroxide (OsO₄). Following dehydration, infiltration, embedding, ultrathin sectioning, and staining, images were captured in the TEM (Model H-7650, Hitachi, Japan). The sample undergoes the same processing steps as those for the SEM, starting with fixation and ending with dehydration. After dehydration and coating, the sample was examined using SEM (Model SU-8010, Hitachi, Japan).

2.10. Immunofluorescence staining

After deparaffinizing, the jejunal tissue segment was washed for 5 min with distilled water. Ethylenediaminetetraacetic acid (EDTA; E8008, Sigma–Aldrich, Shanghai, China) was then used to extract antigens from tissue sections. Sections were blocked with 3% bovine serum albumin before being incubated overnight with rabbit anti-zonula occludens-1 (ZO-1; 21773-1-AP, Proteintech, Wuhan, China), rabbit anti-occludin (27260-1-AP, Proteintech, Wuhan, China), and rabbit anti-claudin-1 (28674-1-AP, Proteintech, Wuhan, China) polyclonal antibodies at 4°C , respectively. The sections were rinsed 3 times for 5 min each with PBS (pH 7.4) before being treated with goat anti-rabbit IgG-FITC secondary antibody (FITC-30000, Proteintech, Wuhan, China) and were then left to incubate at room temperature for 50 min in the dark. The 4',6-diamidino-2-phenylindole (DAPI; C1006, Beyotime Biotechnology, Shanghai, China) stain was added and was allowed to incubate for 10 min at room temperature after tissue sections were washed with PBS (pH = 7.4). Finally, a confocal scanning microscope (NIKON ECLIPSE TI-SR, Nikon, Japan) was used to detect the

fluorescence of the sections, and NIKON DS-U3 software was used to capture the images.

2.11. Western blotting analysis

The relative protein levels of mTOR, regulatory associated protein of mTOR (Raptor), S6K1, Sestrin-1, and 4EBP1 in the duodenum and jejunum, and ZO-1, occludin, claudin-1 in the jejunum, were determined using the protein blotting technique described earlier (Yu et al., 2019). The primary antibodies used in the present study were as follows: anti-p-mTOR (#5536) and anti-mTOR (#2983) were purchased from Cell Signaling Technology (Danvers, USA); anti-p-S6K1 (ET1608-53), anti-S6K1 (ER3125), anti-Raptor (ER1802-57), anti-Sestrin-1 (ER191466), anti-p-4EBP1 (RT1004), anti-4EBP1 (ER1706-64) and anti- β -actin (M1210-2) were purchased from HUABIO (Hangzhou, China), while anti-ZO-1, anti-occludin and anti-claudin-1 were purchased from Proteintech (Wuhan, China). SDS-PAGE was used to separate proteins. Afterward, the proteins were blotted onto the PVDF membrane (IPVH00010, Merck Millipore Ltd., Tullagreen, Ireland). The membrane was sealed in milk, incubated with blotted, and developed using enhanced chemiluminescence (ECL) kit (P00185) purchased from Beyotime Biotechnology (Shanghai, China).

2.12. Data analysis

Comparisons among groups were analyzed by one-way analysis of variance (ANOVA) followed by Student's *t*-test or Duncan's multiple range tests (SPSS 26.0, SPSS Inc., Chicago, IL, USA). Second-order polynomial (quadratic) regression analysis was performed using SPSS to obtain the curve regression equation. Data were expressed as mean \pm SD. These values were considered highly significant when $P < 0.01$, significant when $P < 0.05$, and trending when $0.05 \leq P < 0.10$.

3. Results

3.1. Growth performance

Growth performance data of piglets fed different dEB diets are summarized in Table 2. Compared to piglets fed a 250 mEq/kg dEB diet, piglets fed 150 and 350 mEq/kg dEB diets had lower ADG and ADFI ($P < 0.05$). Meanwhile, the G:F of piglets in the ME group was significantly higher than that in the LE group ($P < 0.05$). As shown in Fig. 1, the ADG, G:F, and ADFI exhibited a quadratic variation with dEB, and the results showed that the dEB corresponding to the best ADG, G:F, and ADFI were 255.24, 257.14 and 249.29 mEq/kg, respectively. In addition, the diarrhea rate of piglets in the LE group

was significantly higher than that of the other two groups ($P < 0.05$).

3.2. Nutrient digestibility

The effects of dEB levels on nutrient digestibility are presented in Table 3. The digestibility of CP for piglets fed a diet with a dEB of 250 mEq/kg was greater than for pigs fed diets with dEB of 150 and 350 mEq/kg ($P < 0.05$). While the digestibility of DM, EE, CF, ash, Ca, and P was not affected by changing dEB throughout the experiment ($P > 0.05$). The results from quadratic regression curve analysis of CP digestibility vs. dEB showed that the optimal CP digestibility occurred at 250.68 mEq/kg dEB (Fig. 1).

3.3. Intestinal morphology and tightly junction protein distribution and abundance

Histopathological analysis showed that the dEB affected the morphology of small intestinal, and 150 mEq/kg dEB caused villi shortening, necrosis, and intestinal epithelial cell loss (Fig. 2A). Upon quantitative analysis, the results found that the LE diet significantly decreased villus height and the ratio of villus height to crypt depth (V:C) of the duodenum and jejunum ($P < 0.05$) (Table 4). Moreover, the SEM (Fig. 2B) and the TEM (Fig. 2C) of the jejunum showed that 150 mEq/kg dEB led to a decrease in the number of intestinal epithelial microvilli, an irregular alignment, and a deepening of the intestinal surface grooves. The results of the quadratic curve regression analysis are shown in Fig. 3. When the villus height and V:C of duodenum and jejunum were optimal, the dEB was 243.06, 255.95, 255.82, and 264.2 mEq/kg, respectively. Additionally, the distribution and abundance of tightly junction proteins (ZO-1, occludin, and claudin-1) located in the intestinal epithelium were measured by immunofluorescence and Western blotting analysis. The results found that occludin and claudin-1 staining in the jejunum of piglets in the LE group were diffuse, and expression decreased in the intercellular tight junction region (Fig. 4A). Western blotting detection showed that the expression of occludin and claudin-1 proteins in piglets in LE group was significantly decreased ($P < 0.05$, Fig. 4B).

3.4. Acid–base balance and serum electrolyte concentration

The results of the pH, pCO₂, pO₂, HCO₃⁻ and BE in the arterial blood of piglets, as well as the concentrations of electrolytes in the serum are shown in Table 5. Compared to the LE group, the arterial blood pH and HCO₃⁻ of piglets in the ME and HE groups were significantly decreased ($P < 0.05$). Meanwhile, there were no significant differences in pCO₂, pO₂ and BE in the arterial blood of piglets among groups ($P > 0.05$). Moreover, the content of K⁺ in the

Table 2
Effect of dietary electrolyte balance on the growth performance of piglets.

Item	Dietary treatment			P-value
	LE	ME	HE	
Initial BW, kg	9.80 \pm 0.21	9.91 \pm 0.07	9.90 \pm 0.13	0.327
Final BW, kg	26.01 \pm 0.62 ^b	27.46 \pm 0.55 ^a	26.36 \pm 0.56 ^b	0.002
ADG, g	540.19 \pm 21.63 ^b	585.05 \pm 18.76 ^a	548.72 \pm 16.12 ^b	0.002
ADFI, g	696.21 \pm 14.33 ^b	717.04 \pm 11.31 ^a	695.67 \pm 11.47 ^b	0.014
G:F	0.78 \pm 0.03 ^b	0.82 \pm 0.02 ^a	0.79 \pm 0.02 ^{ab}	0.035
Diarrhea rate, %	14.54 \pm 5.14 ^a	10.74 \pm 5.45 ^b	10.19 \pm 5.23 ^b	0.003

ADG = average daily gain; ADFI = average daily feed intake; G:F = the ratio of weight gain to feed intake.

LE = low electrolyte diet (150 milliequivalents [mEq]/kg dietary electrolyte balance [dEB]); ME = medium electrolyte diet (250 mEq/kg dEB); HE = high electrolyte diet (350 mEq/kg dEB).

The data were expressed as mean \pm SD ($n = 6$).

^{a,b}Data with different superscript letters in a row indicated that the differences between different treatment groups were statistically significant ($P < 0.05$).

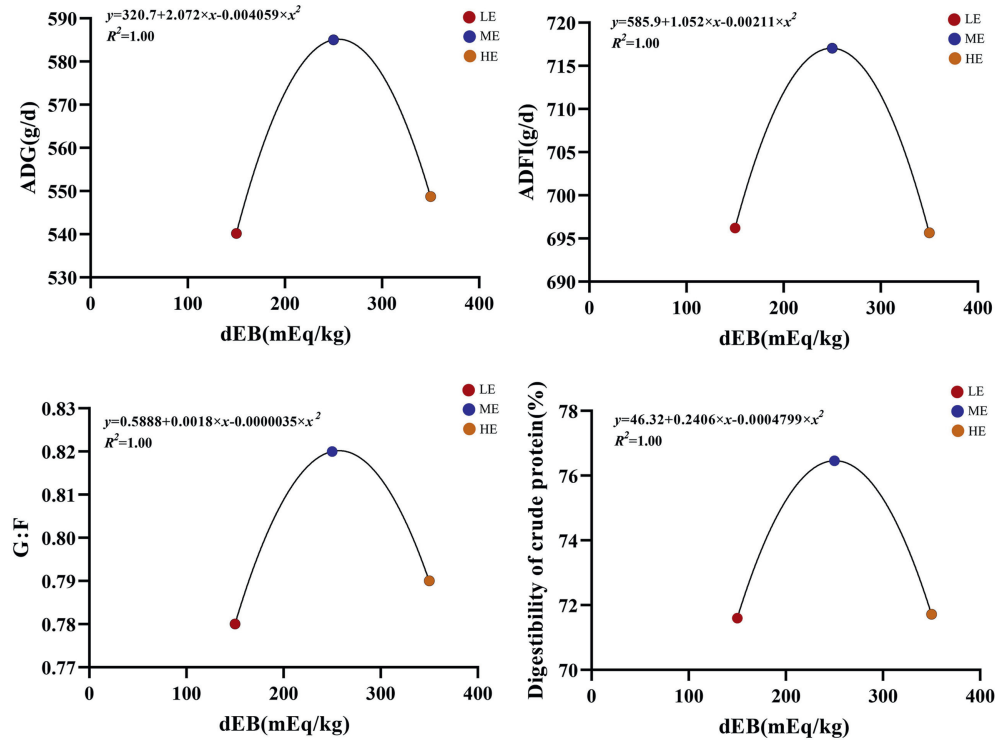


Fig. 1. Quadratic curve regression analysis of dietary electrolyte balance and piglet growth performance. ADG = average daily gain; ADFI = average daily feed intake; G:F = the ratio of weight gain to feed intake; dEB = dietary electrolyte balance; mEq = milliequivalents. LE = low electrolyte diet (150 mEq/kg dEB); ME = medium electrolyte diet (250 mEq/kg dEB); HE = high electrolyte diet (350 mEq/kg dEB).

Table 3
Effect of dietary electrolyte balance on nutritional digestibility of piglets, %.

Item	Treatments			P-value
	LE	ME	HE	
DM	87.54 ± 5.51	87.84 ± 5.65	87.52 ± 5.33	0.994
CP	71.60 ± 2.92 ^b	76.46 ± 3.02 ^a	71.72 ± 3.13 ^b	0.021
EE	65.32 ± 2.36	65.82 ± 3.16	65.04 ± 3.73	0.909
CF	39.93 ± 4.31	40.57 ± 4.70	39.23 ± 5.91	0.900
Ash	86.91 ± 5.40	87.27 ± 6.04	86.51 ± 6.08	0.975
Ca	41.35 ± 3.07	42.79 ± 3.55	41.42 ± 3.21	0.699
P	46.76 ± 3.64	47.79 ± 4.79	46.10 ± 5.09	0.813

DM = dry matter; EE = crude fat; CF = crude fiber. LE = low electrolyte diet (150 milliequivalents [mEq]/kg dietary electrolyte balance [dEB]); ME = medium electrolyte diet (250 mEq/kg dEB); HE = high electrolyte diet (350 mEq/kg dEB).

The data were expressed as mean ± SD (n = 6).

^{a,b}Data with different superscript letters in a row indicated that the differences between different treatment groups were statistically significant (P < 0.05).

serum of piglets fed a diet in the HE group was significantly higher than that in the LE group, while Cl⁻ was significantly lower than that in the LE group (P < 0.05).

3.5. Serum free amino acids concentration

Serum free amino acids concentrations are shown in Table 6. Serum arginine, leucine, glutamic acid, and alanine concentrations were decreased in piglets fed the LE diet compared with those fed the ME diet (P < 0.05). Interestingly, the content of lysine in the serum of piglets in the LE group was significantly higher than that in the ME and HE groups (P < 0.05). The remaining 12 free amino acids were not significantly affected by dietary electrolyte levels (P > 0.05).

3.6. Abundance of mTORC1 pathway proteins

As presented in Fig. 5, when compared with the ME group, the relative protein abundance of p-mTOR, p-S6K1, and p-4EBP1 in the duodenal and jejunal mucosa of piglets in the LE and HE groups decreased (P < 0.05), with no significant difference between the dietary LE and HE groups when compared with one another (P > 0.05). Meanwhile, the protein abundance of Sestrin-1 in the duodenal and jejunal mucosa of piglets in the ME group was significantly decreased (P < 0.05). Additionally, the relative expression of Raptor protein in the ME group was significantly higher than that in the LE group in the jejunal mucosa (P < 0.05).

4. Discussion

It is advantageous to reduce the crude protein level in the piglet diet to save protein resources, such as soybean meal. Recent research indicated that even with essential amino acid (EAA) supplementation, piglet growth performance may be negatively impacted by a significant reduction in the amount of soybean meal in piglet diets (Batson et al., 2021; Che et al., 2017; Soto et al., 2019). Soybean meal is characterized by a high K⁺ content, and a decrease in its use leads to a significant reduction in the dEB in the diet (Royall et al., 2022). It is hypothesized that this might be one of the reasons that the low-protein diet affects piglet growth performance. It is well documented that the dEB may affect the growth performance and nutrient digestibility of weaned and growing pigs (Guzmán-Pino et al., 2015; Lei et al., 2017). In this study, the results showed that the dEB could affect piglets ADG, ADFI, and G:F and the ME group had the best performance, in line with earlier findings. The experiment obtained a quadratic regression equation through quadratic curve regression analysis of the growth performances and dEBs. It was found that in order to obtain the optimal ADG,

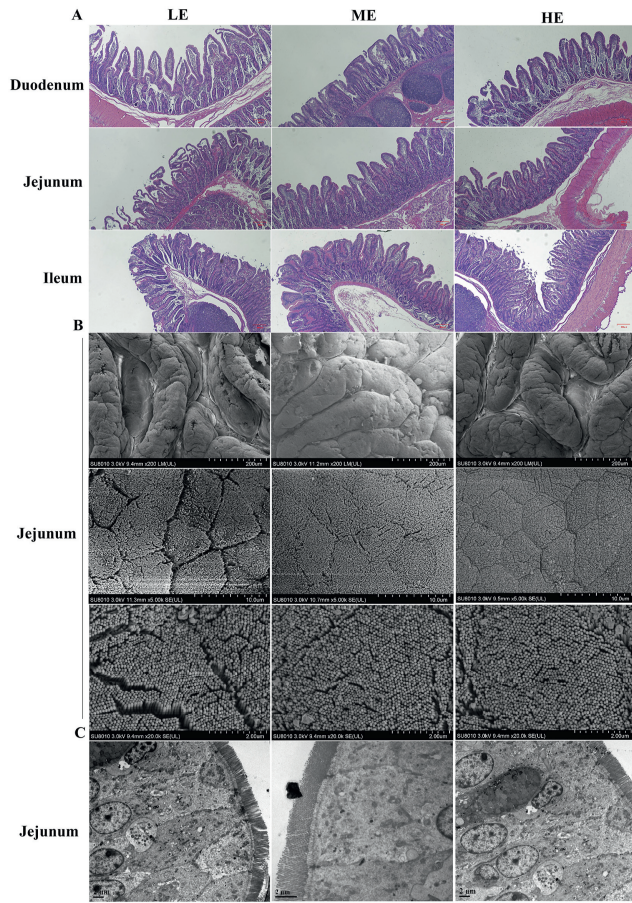


Fig. 2. Effect of dietary electrolyte balance on the morphology of the intestine. (A) Duodenum, jejunum and ileum were stained with H&E, and microscopically examined. (B) Representative scanning electron microscopy (SEM) of the jejunum in the LE, ME and HE groups. The scale bars are 200 μm (magnification, 200 \times), 10 μm (magnification, 5000 \times), and 2 μm (magnification, 20,000 \times), respectively. (C) Representative transmission electron microscopy (TEM) of the jejunum of the LE, ME and HE groups (scale bar, 2 μm ; magnification, 10,000 \times). LE = low electrolyte diet (150 milliequivalents [mEq]/kg dietary electrolyte balance [dEB]); ME = medium electrolyte diet (250 mEq/kg dEB); HE = high electrolyte diet (350 mEq/kg dEB).

ADFI, and G:F, the electrolyte values were 255.24, 249.29, and 257.14 mEq/kg, respectively. Lei et al. (2017) suggested that

weanling piglets achieved optimal growth when fed diets with a dEB in the range of 166 to 250 mEq/kg. NRC (2012) reported that the optimal dEB for pigs is approximately 250 mEq/kg. Furthermore, it has been discovered that dietary electrolytes affect changes in animal feed intake (Helm et al., 2021), which may be the reason that the dEB affects the ADG and G:F of piglets. Meanwhile, the digestibility of DM, CP, EE, CF, ash, Ca, and P was assayed and found that piglets in the 250 mEq/kg dEB group had higher CP digestibility compared to piglets in the 150 mEq/kg and 350 mEq/kg dEB groups, which was similar to previous findings (Jones et al., 2019; Lei et al., 2017). The results of the quadratic regression equation showed that when the electrolyte was 250.68 mEq/kg, the CP digestibility of piglets was the highest, which was consistent with the growth performance of piglets.

In order to further investigate the effect mechanism of dEB on the piglet growth performance, three treatments consisting of LE, ME, and HE treatments were selected to determine the intestinal health of piglets. The structure and function of the intestine are reflected through the morphology of the intestine (Moeser et al., 2007; Stokes, 2017). In the current experiment, it was found that the ME diet improved the morphological and structural integrity of the duodenum and jejunum in piglets compared to the LE diet and the HE diet, which was consistent with previous studies (Deng et al., 2021). The regression results showed that the dEB corresponding to the highest villous height and V:C of duodenum and jejunum were 243.06, 255.95, 255.82 and 264.2 mEq/kg, respectively. Shortening of villi and deepening of crypts may reduce the surface area for intestinal absorption of nutrients, thereby decreasing nutrient utilization, which is consistent with the results for growth performance. Additionally, it was found that 150 mEq/kg dEB diet can cause diarrhea in piglets. As is well known, various intestinal pathogens can induce permeability defects in the intestinal epithelium by altering the distribution of tight junction proteins (Tong et al., 2016). Tight junctions, consisting of cytoplasmic scaffold folding proteins such as ZO-1, occludin, claudins, and adhesion molecules, play a key role in maintaining intestinal permeability (Dokladny et al., 2016; Wang et al., 2016). In the study, occludin and claudin-1 staining were diffuse in the jejunum of LE group piglets, with reduced staining in the intercellular tight junction area and significantly decreased protein expression. This indicates that a 150 mEq/kg dEB can disrupt the jejunal tight junction in piglets, which may be the cause of piglet diarrhea. Thus far, no data are available on the effect of dEB on intestinal tight junction in piglets. The above results of this study

Table 4
Effect of dietary electrolyte balance on intestinal morphology of piglets.

Organs	Dietary treatment			P-value
	LE	ME	HE	
Duodenum				
Villus height, μm	193.55 \pm 31.04 ^b	277.65 \pm 19.89 ^a	214.11 \pm 19.00 ^b	<0.001
Crypt depth, μm	55.37 \pm 8.86	51.45 \pm 10.33	47.19 \pm 9.51	0.360
V:C	3.97 \pm 0.81 ^b	5.56 \pm 1.08 ^a	4.30 \pm 1.26 ^{ab}	0.049
Jejunum				
Villus height, μm	187.41 \pm 14.79 ^b	209.07 \pm 5.57 ^a	191.94 \pm 5.94 ^b	0.004
Crypt depth, μm	58.52 \pm 18.73	45.42 \pm 6.25	48.52 \pm 3.62	0.158
V:C	3.42 \pm 0.84 ^b	4.68 \pm 0.64 ^a	3.98 \pm 0.36 ^{ab}	0.014
Ileum				
Villus height, μm	193.26 \pm 18.31	208.27 \pm 7.54	193.06 \pm 16.57	0.414
Crypt depth, μm	57.67 \pm 16.41	56.05 \pm 7.65	51.39 \pm 12.14	0.675
V:C	3.58 \pm 1.01	3.79 \pm 0.84	3.93 \pm 0.91	0.801

V:C = the ratio of villus height to crypt depth.

LE = low electrolyte diet (150 milliequivalents [mEq]/kg dietary electrolyte balance [dEB]); ME = medium electrolyte diet (250 mEq/kg dEB); HE = high electrolyte diet (350 mEq/kg dEB).

The data were expressed as mean \pm SD (n = 6).

^{a,b}Data with different superscript letters in a row indicated that the differences between different treatment groups were statistically significant (P < 0.05).

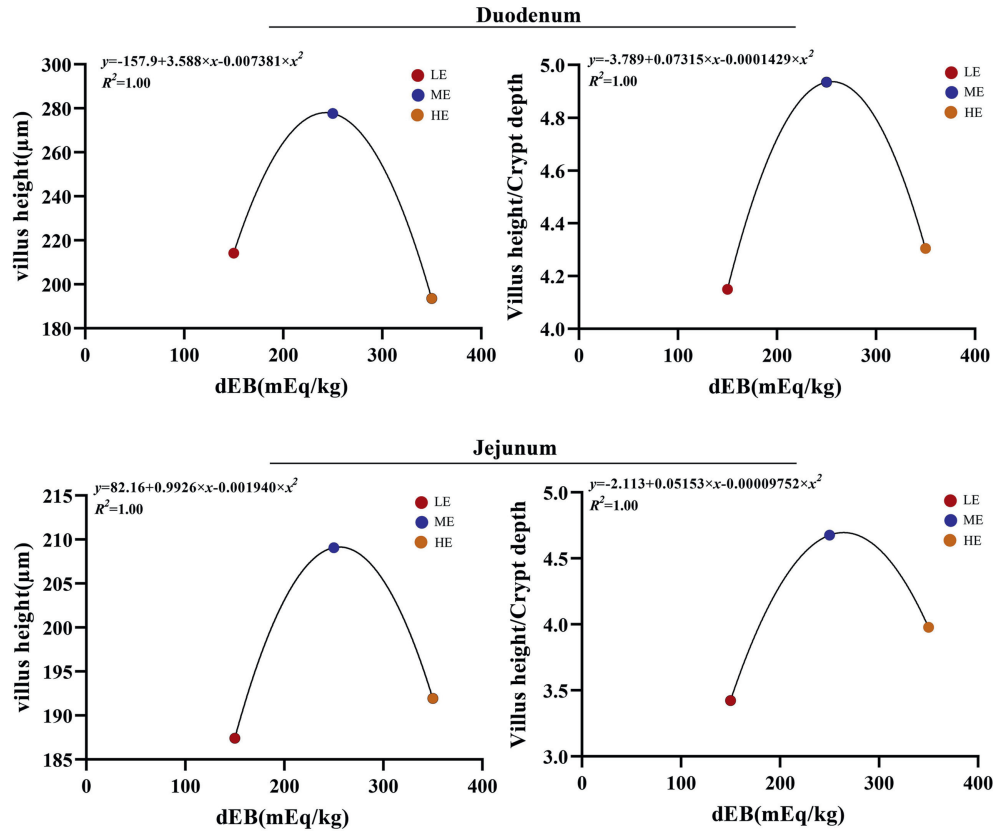


Fig. 3. Quadratic curve regression analysis of dietary electrolyte balance and intestinal morphology structure in piglets. dEB = dietary electrolyte balance; mEq = milliequivalents. LE = low electrolyte diet (150 mEq/kg dEB); ME = medium electrolyte diet (250 mEq/kg dEB); HE = high electrolyte diet (350 mEq/kg dEB).

suggest that the dEB can affect the integrity of intestinal structure in piglets by altering their intestinal tight junction structure, thereby regulating the digestion and absorption of nutrients.

Almost all physiological mechanisms in the body rely on maintaining an appropriate acid–base balance to function normally (Quade et al., 2021). It is widely believed that the dEB might affect acid–base status and mineral metabolism, thereby inhibiting food intake and nutrient metabolism (Deng et al., 2020). This study found that pH and HCO_3^- in arterial blood were decreased in the LE group compared to the ME and HE groups, corresponding to observations in the literature (Bournazel et al., 2020; Quade et al., 2021). To maintain the extracellular fluid electrically neutral, an equivalent amount of Cl^- must be expelled for every increase in HCO_3^- in the plasma, and vice versa (Quade et al., 2021). This was consistent with the results of this experiment, which showed that the serum Cl^- level was significantly higher and the K^+ level was significantly lower in the LE group compared to the HE groups. However, the serum Na^+ concentration did not change over the three treatments, which was consistent with Guzmán-Pino et al. (2015). They also found that Na^+ concentrations in pig serum were not affected by dEB. Altogether, the above results showed that the dEB affected the acid–base balance in piglets.

The metabolism of amino acids is closely related to the acid–base balance. Research has found that the acid–base balance impacts the growth performance of livestock as well as the metabolic fate of certain nutrients, such as amino acids (Ibrahim et al., 2023). Amino acid metabolism in the intestine usually varies with the dEB, and amino acids are essential for intestinal development and function (Adedokun et al., 2017; Chrystal et al., 2020). In this study, compared with the ME group, piglets fed the LE diet had

significantly lower serum arginine, leucine, glutamic acid and alanine levels. This is similar to previous findings, where Chrystal et al. (2020) found that a decrease in dietary electrolyte levels from 230 to 120 mEq/kg led to decreased amino acid digestibility. Interestingly, it was found the highest lysine levels in the serum of piglets in the LE group. The increase in serum lysine levels in piglets may be due to the low-protein diet causing a high Cl^- and low K^+ environment in piglets, which was conducive to lysine absorption (Mushtaq and Pasha, 2013). Therefore, considering the potential effect on amino acid digestibility, the dEB should be considered in low-protein diet feeding studies and adjusted to obtain optimal growth performance.

The mTORC1 regulates multiple anabolic and catabolic processes, including protein synthesis and autophagy, in response to various environmental inputs, such as amino acid concentrations (Bar-Peled et al., 2013). Raptor and mTOR are members of the mTORC1. Among these, the mTOR (a serine-threonine-specific kinase) is a cellular energy sensor that integrates growth factors and nutrient signaling (Chen et al., 2022). Raptor, as a pivotal element of mTORC1, is a phosphorylation recruitment substrate that is essential for all of mTORC1's functions (Duan et al., 2016). When mTORC1 is activated, phosphorylation of its downstream effector S6K and 4E-BP promotes synthetic metabolic processes such as protein, lipid, and nucleotide synthesis, which induces cell growth and proliferation (Rabanal-Ruiz et al., 2017). The research results found the LE diet and HE diet not only significantly inhibited mTOR, S6K1, and 4EBP1 phosphorylation in the duodenum and jejunum, but also decreased the expression of Raptor protein in the jejunum. Moreover, Sestrin 1 was found to participate in mTOR inhibition (Xue et al., 2017). In this study, a significant increase in the protein

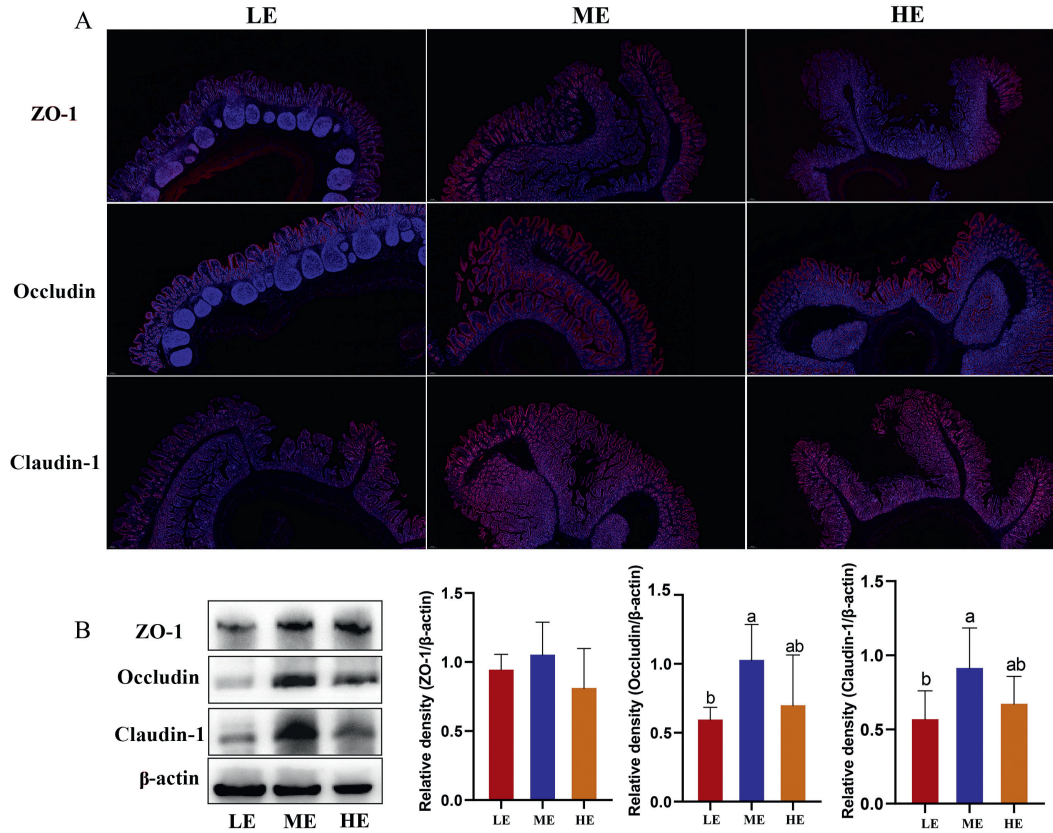


Fig. 4. Effect of dietary electrolyte balance on tight junction protein distribution and abundance. (A) Representative immunofluorescent images for detection of ZO-1 (red), occludin (red), claudin-1 (red), and DAPI (blue). (magnification, 200×). (B) Western blotting analysis of protein expression of ZO-1, occludin and claudin-1. Lanes 1, 2, and 3 represent the treatment of the LE, ME, and HE diet, respectively. ZO-1 = zonula occludens-1 LE = low electrolyte diet (150 milliequivalents [mEq]/kg dietary electrolyte balance [dEB]); ME = medium electrolyte diet (250 mEq/kg dEB); HE = high electrolyte diet (350 mEq/kg dEB). Data are expressed as means ± SD (n = 6). ^{a,b}Bars with different letters are significantly different among dietary protein treatments (P < 0.05).

Table 5
Arterial blood gases and serum electrolyte contents in samples obtained during the collection period.

Item	Treatments			P-value
	LE	ME	HE	
pH	7.02 ± 0.22 ^b	7.34 ± 0.19 ^a	7.36 ± 0.29 ^a	0.039
pCO ₂ , mmHg	39.55 ± 3.31	43.65 ± 4.23	39.92 ± 3.01	0.120
pO ₂ , mmHg	85.35 ± 4.31	82.77 ± 5.85	86.99 ± 4.21	0.341
HCO ₃ ⁻ , mmol/L	29.84 ± 2.66 ^b	33.79 ± 2.61 ^a	34.14 ± 2.60 ^a	0.022
BE, mmol/L	8.06 ± 0.44	8.33 ± 0.64	8.15 ± 0.64	0.637
Na ⁺ , mmol/L	135.47 ± 17.35	137.34 ± 16.46	142.86 ± 19.28	0.759
K ⁺ , mmol/L	4.04 ± 0.66 ^b	4.52 ± 0.65 ^{ab}	5.23 ± 0.66 ^a	0.021
Cl ⁻ , mmol/L	121.09 ± 10.09 ^a	114.34 ± 9.33 ^{ab}	105.08 ± 10.42 ^b	0.048

BE = base excess.
LE = low electrolyte diet (150 milliequivalents [mEq]/kg dietary electrolyte balance [dEB]); ME = medium electrolyte diet (250 mEq/kg dEB); HE = high electrolyte diet (350 mEq/kg dEB).
The data were expressed as mean ± SD (n = 6).
^{a,b}Data with different superscript letters in a row indicated that the differences between different treatment groups were statistically significant (P < 0.05).

expression of Sestrin-1 in the small intestine of piglets was discovered for the LE and HE groups. In this regard, it has been found that low amino acid availability inhibits mTORC1 activity for nutrient recovery. For example, mTORC1 can sense leucine by leucine-tRNA synthase and glutamine levels (Kar et al., 2017). The above results have shown that dietary electrolyte imbalance activates the expression of Sestrin-1 protein, reducing the binding of mTOR to Raptor, inhibiting the enrichment of mTOR in lysosomes,

Table 6
Effect of dietary electrolyte balance on serum amino acid concentration in piglets (mg/L).

Item	Dietary treatment			P-value
	LE	ME	HE	
Lysine	42.85 ± 3.87 ^a	36.80 ± 3.67 ^b	36.96 ± 3.97 ^b	0.024
Methionine	16.09 ± 0.97	15.83 ± 0.80	15.95 ± 1.02	0.893
Threonine	78.61 ± 3.96	77.22 ± 5.02	79.35 ± 4.89	0.728
Leucine	29.59 ± 3.09 ^b	33.84 ± 2.15 ^a	31.59 ± 2.61 ^{ab}	0.044
Isoleucine	18.79 ± 1.02	18.45 ± 0.69	18.80 ± 1.06	0.771
Phenylalanine	30.99 ± 2.90	30.88 ± 2.83	32.16 ± 1.63	0.629
Valine	34.03 ± 1.61	34.26 ± 1.89	35.62 ± 1.22	0.211
Glycine	101.68 ± 5.05	110.25 ± 6.35	106.93 ± 5.58	0.058
Cysteine	8.48 ± 0.41	8.32 ± 0.69	8.50 ± 0.33	0.795
Aspartic acid	24.42 ± 1.92	24.56 ± 1.55	23.80 ± 1.27	0.688
Arginine	37.97 ± 2.90 ^b	44.12 ± 3.41 ^a	40.39 ± 2.91 ^{ab}	0.012
Serine	24.64 ± 1.33	25.38 ± 2.05	24.82 ± 1.64	0.737
Glutamic acid	94.35 ± 3.20 ^b	101.18 ± 5.95 ^a	96.35 ± 3.36 ^{ab}	0.043
Alanine	90.04 ± 6.60 ^b	101.52 ± 6.52 ^a	95.25 ± 5.72 ^{ab}	0.022
Tyrosine	27.91 ± 2.24	29.74 ± 1.31	28.30 ± 1.96	0.241
Histidine	18.66 ± 1.65	20.31 ± 2.03	18.74 ± 1.59	0.224
Proline	46.33 ± 2.48	46.73 ± 2.36	46.13 ± 2.87	0.919

LE = low electrolyte diet (150 milliequivalents [mEq]/kg dietary electrolyte balance [dEB]); ME = medium electrolyte diet (250 mEq/kg dEB); HE = high electrolyte diet (350 mEq/kg dEB).
The data were expressed as mean ± SD (n = 6).
^{a,b}Data with different superscript letters in a row indicated that the differences between different treatment groups were statistically significant (P < 0.05).

and inhibiting eukaryotic translation and ribosome synthesis. This may be related to the fact that the LE diet significantly decreases the content of amino acids such as arginine and leucine in piglet serum,

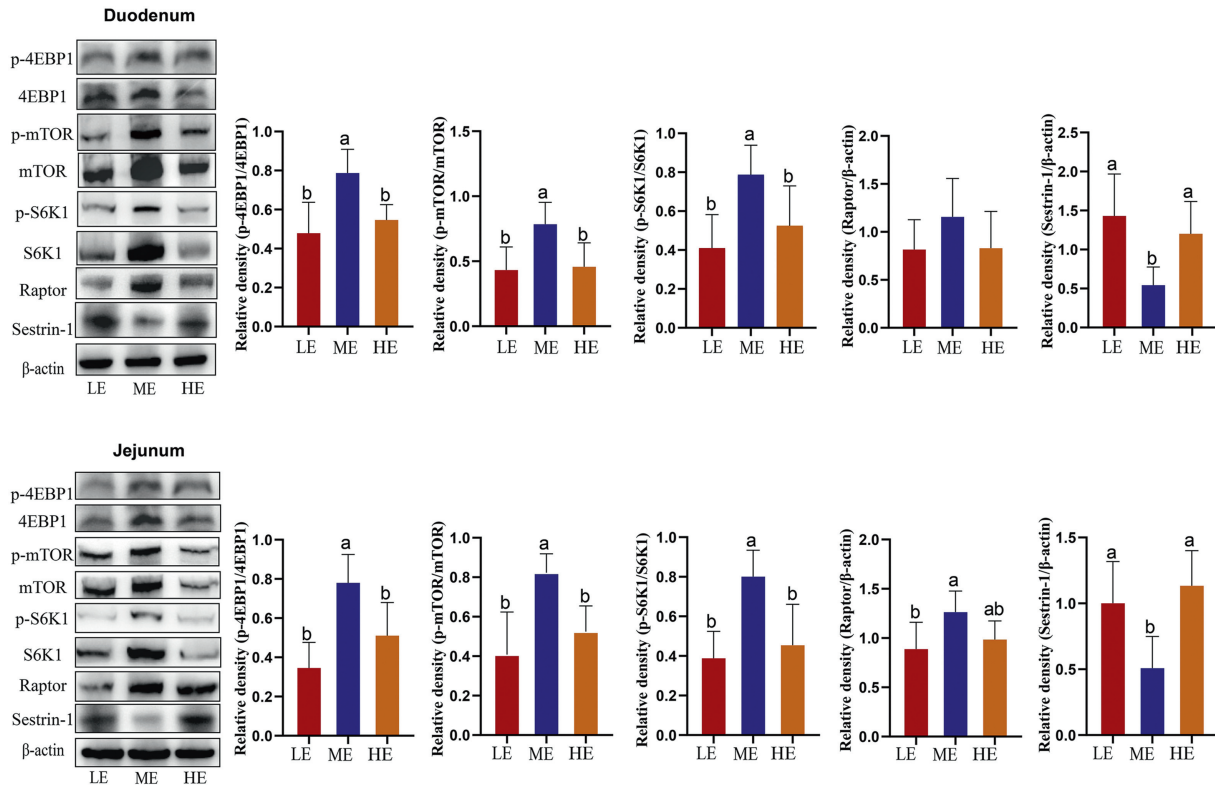


Fig. 5. Effect of dietary electrolyte balance on protein abundance of key molecules of the duodenal and jejunal mTORC1 signaling pathway in piglets. Western blotting analysis of protein expression of p-4EBP1, 4EBP1, p-mTOR, mTOR, p-S6K1, S6K1, Raptor, and Sestrin-1. Lanes 1, 2, and 3 represent the treatment of the LE, ME, and HE diet, respectively. S6K1 = ribosomal protein S6 kinase 1; 4EBP1 = eukaryotic translation initiation factor 4E-binding protein 1; mTORC1 = mammalian target of rapamycin complex 1. LE = low electrolyte diet (150 milliequivalents [mEq]/kg dietary electrolyte balance [dEB]); ME = medium electrolyte diet (250 mEq/kg dEB); HE = high electrolyte diet (350 mEq/kg dEB). Data are expressed as means \pm SD ($n = 6$). ^{a,b}Bars with different letters are significantly different among dietary protein treatments ($P < 0.05$).

resulting in amino acid starvation (Ögmundsdóttir et al., 2012; Tsun et al., 2013). It's worth noting that further research is required to understand how it is regulated.

In summary, our research has shown that the dEB significantly affects the intestinal health and growth performance of piglets, as well as the metabolism of nutrients such as amino acids. Therefore, when widely using low-protein diets to improve the growth performance of piglets, the dEB needs to be considered. This study found that the optimal electrolyte level of piglets under low-protein diets was 250 to 265 mEq/kg. In addition, it was found that dietary electrolyte imbalance can affect amino acid metabolism, reduce amino acid levels in piglet serum, and inhibit the mTORC1 signaling pathway, which may be one of the key mechanisms affecting growth performance.

Author contributions

Qian Lin: Conceptualization, Investigation, Methodology, Formal analysis, Writing original draft, Writing-review & editing. **Xiaodian Tu, Xin Li and Feiyang Gou:** Investigation, Methodology, Formal analysis, Data curation. **Lin Ding, Zeqing Lu and Jie Feng:** Conceptualization, Writing-review & editing. **Yongfei Ying and Caihong Hu:** Conceptualization, Writing-review & editing, Supervision, Project administration, Funding acquisition. All authors read and approved the final manuscript.

Data availability statement

The authors confirm that the data supporting the findings of this article are available.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can 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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