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Original Research Article

Low-protein diet supplemented with 1% L-glutamine improves growth performance, serum biochemistry, redox status, plasma amino acids, and alters fecal microbiota in weaned piglets

Jun Li, Jun Bai, Ying Yang, Zhenlong Wu*

State Key Laboratory of Animal Nutrition and Feeding, Department of Companion Animal Science, China Agricultural University, Beijing 100193, China

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ABSTRACT

Glutamine, one of the most abundant amino acids in the body, has been shown to exert various beneficial effects in pigs. However, knowledge regarding the role of dietary glutamine in low-protein diet-fed piglets remains scarce. The present study aimed to investigate the effects of different levels of L-glutamine on growth performance, serum biochemistry parameters, redox status, amino acids, and fecal microbiota in low-protein diet-fed piglets. A total of 128 healthy crossbred piglets (Landrace × Yorkshire) were randomly allocated into 4 groups of 4 replicate pens, with 8 piglets per pen. Piglets in the 4 groups were fed with corn and soybean meal-based low-protein diets (crude protein level, 17%) that contained 0%, 1%, 2%, and 3% L-glutamine, respectively, for 28 d. Pigs administered 1% L-glutamine had greater body weight on d 28 and average daily gain (ADG, $P < 0.01$), whereas a lower feed to gain ratio (F:G) from d 1 to 28 ($P < 0.01$), compared to the other three groups. Besides, lower body weight on d 14 and 28, ADG, average daily feed intake, and higher F:G from d 15 to 28 and d 1 to 28 were observed in response to 2% and 3% L-glutamine treatments than 0% and 1% L-glutamine treatments ($P < 0.01$). Moreover, 1% L-glutamine reduced serum glucose, malondialdehyde, hydrogen peroxide concentrations and inhibited aspartate aminotransferase, alanine aminotransferase, myeloperoxidase activities in low-protein diet-fed piglets on d 14, with concomitantly upregulated catalase, total superoxide dismutase activities and glutathione level ($P < 0.05$). However, dietary 3% L-glutamine enhanced blood urea nitrogen content in pigs on d 14 ($P < 0.05$). Further investigation revealed that 1% L-glutamine upregulated the serum glutamine, lysine, methionine, tyrosine, and reduced plasma valine content ($P < 0.05$). Additionally, 1% L-glutamine upregulated the abundance of *p_75_a5*, *Clostridium*, *Lactobacillus*, *Prevotellaceae_Prevotella*, and *Gemmiger* in the stool of piglets on d 14, with the *Streptococcus* level being concomitantly reduced ($P < 0.05$). Collectively, dietary 1% L-glutamine enhances the growth performance and improves serum physiochemical parameters and antioxidative capacity in low-protein diet-fed piglets at an early age, which are associated with an increased synthesis of glutathione by modulating amino acid levels, and the optimization of gut microbiota.

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1. Introduction

Low-protein diets, namely having a reduction in the crude protein level than the recommended level (NRC, 2012) followed by meeting the amino acid (AA) requirements for pigs, have gradually been applied in swine production due to multiple beneficial effects like reducing feed costs and nitrogen excretion. Previous studies have demonstrated that reducing crude protein levels by 2% to 4%, followed by the appropriate addition of essential AA, is an effective strategy to reduce nitrogen emissions, the risk of gut disorders, and

* Corresponding author.
E-mail addresses: bio2046@hotmail.com, wuzhenlong@cau.edu.cn (Z. Wu).
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feed costs, without compromising animal performance (Gloaguen et al., 2014; Jiaqi et al., 2020; Wu et al., 2015). It has been generally recognized that non-essential amino acids (NEAA) also play a vital role in achieving maximum growth and production performance, despite more emphasis on essential AA in the past (Wu et al., 2013). Some NEAA have higher utilization rates than synthesis rates in some circumstances and therefore are categorized as conditionally essential AA (Wu, 2009). The conditionally essential AA for weaned piglets are glutamic acid (Glu), glutamine (Gln), arginine, proline, glycine (Gly), and taurine (Upadhaya et al., 2022). With the increased availability of crystalline AA, formulating low-protein diets with a balanced AA content has become possible. However, knowledge regarding the effects of dietary supplementation of conditionally essential AA on growth performance in low-protein diet-fed piglets is limited.

Gln, one of the conditionally essential AA for pigs (Wang et al., 2015a), is the most abundant AA in sows' milk during a certain stage of lactation (22 to 29 d) and is also rich in plasma, body fluid, and skeletal muscle (Wu and Knabe, 1994; Wu et al., 2013). Exhaustion of Gln would give rise to considerable detrimental effects like villus atrophy, downregulation of the abundance of tight junction proteins, and increased paracellular permeability, and this phenomenon could be alleviated by Gln supplementation (Kim and Kim, 2017). Moreover, inadequate intake of Gln is closely related to the breakdown of the intestinal barrier and the development of diseases, which could be improved by Gln supplementation (Bertrand et al., 2016; Chaudhry et al., 2016; Zhou et al., 2010). Of note, Gln has been recommended as a feed supplement to reduce weaning stress in piglets (Ji et al., 2019), and the use of Gln in animals is safe and efficacious according to the European Food Safety Authority (Bampidis et al., 2020). Our previous results demonstrated that dietary 1% L-Gln increased the body weight and improved intestinal histomorphology and barrier function in weaned piglets (Wang et al., 2015b). We also found that L-Gln could prevent high-fat diet-induced metabolic disorders in rats (He et al., 2022), enhance tight junction integrity via Ca^{2+}/calmodulin-dependent protein kinase (CaMK)-adenosine monophosphate-activated protein kinase (AMPK) signaling (Wang et al., 2016), and ameliorate 4-hydroxy-2-nonenal-induced apoptosis by modulating glutathione (GSH)-related redox homeostasis (Liu et al., 2018). However, information regarding the effects of dietary Gln in piglets under low-protein diets remains limited.

In recent years, the gut microbiota has attracted the attention of many scientists as it exerts diverse beneficial effects on hosts like the fermentation of carbohydrates, maintenance of intestinal normal functions, protection from pathogenic bacteria, and modulation of immunity (Buffie and Pamer, 2013; Kamada et al., 2013). The swine gut microbiota is a complicated ecosystem exhibiting dynamic composition and diversity that shifts with time and along the entire gastrointestinal tract (Isaacson and Kim, 2012), and is implicated with the physiological status and developmental stage, nutritional composition, pathogen infection, and so on (Ji et al., 2017). Gut microbiota disruption is generally supposed to be one of the key factors resulting in postweaning diarrhea during the weaning transition, and the rehabilitation or reconstruction of the gut microbiota at an early age is critical to the host's health (Gresse et al., 2017). Importantly, recent data have revealed that the AA metabolism can alter the gut microbiota (Heianza et al., 2019). To the best of our knowledge, however, the studies regarding the effect of dietary Gln on gut microbiota in low-protein diet-fed piglets are limited. Given the multiple beneficial effects of Gln, we explored the regulatory effects of different levels of dietary L-Gln on growth performance, serum profile, redox status, and gut microbiota in low-protein diet-fed piglets.

2. Materials and methods

2.1. Animal ethics statement

All experiments were approved by the Animal Care and Use Committee of China Agricultural University (AW91012202-1-1).

2.2. Animal experimental design and sample collection

A total of 128 healthy crossbred piglets (Landrace × Yorkshire) were weaned at 24 d of age. After 7 d of adaption, piglets were randomly allocated into 4 groups of 4 replicate pens, with 8 piglets per pen. Piglets in the 4 groups were fed with corn and soybean meal-based low-protein diets (crude protein, 17%) that contained 0%, 1%, 2%, and 3% L-Gln for 28 d, respectively (Table 1). The diets were formulated according to the National Research Council (2012) to meet nutrient requirements for the pigs, and the crude protein, Ca, and phosphorus in feed were also assayed according to the national standard of China GB/T 6432-2018, GB/T 6436-2018, and GB/T 6437-2018, respectively. The net energy of L-Gln was estimated to be 3500 kcal/kg by averaging those for lysine, methionine, and threonine, and the net energy of other ingredients was set according to the nutrient requirements of swine (GB/T 39235-2020). Piglets were raised in cement-floored pens (1.5 m × 2.0 m) in a temperature-controlled room and had free access to the diets and water. On d 14 and 28, body weight was documented after 12 h-starvation and the feed intake was calculated via the offered and residual feed, on a cage basis. The blood samples were collected from the anterior vena cava, centrifuged at 3500 × *g* for 15 min, and kept at −80 °C for further study. L-Gln was purchased from Meihua Biotechnology Group Co., Ltd. (Zhengzhou, China).

2.3. Evaluation of serum physiochemical parameters

Serum glucose, creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and albumin were assayed by commercial kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) according to the manufacturer's instructions. The total protein was determined by bicinchoninic acid methods.

2.4. Determination of serum lipid metabolites, ammonia and urea nitrogen

The serum total cholesterol, triglyceride, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), ammonia, and urea nitrogen were measured using a colorimetric assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.5. Evaluation of serum oxidative stress

The serum oxidative stress-related parameters including hydrogen peroxide (H_2O_2), malondialdehyde (MDA), myeloperoxidase (MPO), total antioxidant capacity (T-AOC), total superoxide dismutase (T-SOD), and catalase (CAT) were determined by using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the protocol from the manufacturer.

2.6. Measurement of AA in feed and serum

Both alkaline hydrolysis and acidic hydrolysis of proteins were conducted to assay tryptophan and other AA in feed, respectively, according to the method of Dai et al. (2014). For serum AA,

Table 1
The composition and nutrient levels of the low-protein diet (as-fed basis, %).

Item	Diets			
	0 Gln	1 Gln	2 Gln	3 Gln
Ingredients				
Corn	54.77	56.65	58.68	60.70
Puffed corn	20.00	20.00	20.00	20.00
Soybean meal	11.75	9.09	6.41	3.72
Fish meal	4.00	4.00	4.00	4.00
Extruded soybean	3.20	3.20	3.20	3.20
L-Glutamine	0.00	1.00	2.00	3.00
Soybean oil	2.00	1.59	1.07	0.54
L-Lysine hydrochloride	0.77	0.86	0.94	1.03
Limestone	1.00	1.00	1.00	1.00
Premix[1]	1.00	1.00	1.00	1.00
Dicalcium phosphate	0.59	0.62	0.65	0.69
L-Threonine	0.30	0.34	0.38	0.42
Salt	0.40	0.40	0.40	0.40
DL-Methionine	0.12	0.13	0.15	0.16
L-Tryptophan	0.10	0.11	0.13	0.14
Total	100.00	100.00	100.00	100.00
Calculated nutrient levels				
Net energy, Mcal/kg	2.74	2.74	2.74	2.74
Total phosphorus	0.47	0.46	0.45	0.45
Available phosphorus	0.30	0.30	0.30	0.30
Crude protein	17.00	17.00	17.00	17.00
SID lysine	1.30	1.30	1.30	1.30
SID methionine	0.39	0.39	0.39	0.39
SID methionine + cysteine	0.67	0.65	0.64	0.62
SID threonine	0.76	0.76	0.76	0.76
SID tryptophan	0.23	0.23	0.23	0.23
Analyzed nutrient levels				
Crude protein	17.04	17.54	17.99	18.06
Ca	0.73	0.73	0.67	0.68
Phosphorus	0.44	0.44	0.42	0.41
Alanine, g/kg	8.93	8.54	8.59	8.06
Arginine, g/kg	8.01	7.60	7.64	6.33
Aspartate + aspartate, g/kg	14.07	13.63	13.39	11.31
Glutamate + glutamine, g/kg	28.59	38.64	48.15	59.26
Glycine, g/kg	5.88	5.71	5.55	4.94
Histidine, g/kg	4.45	4.16	4.45	3.76
Isoleucine, g/kg	6.85	6.37	6.48	5.71
Leucine, g/kg	14.96	13.96	14.30	13.33
Lysine, g/kg	12.31	13.05	15.13	14.24
Methionine, g/kg	5.11	5.18	5.30	5.13
Phenylalanine, g/kg	7.49	7.00	7.21	6.45
Serine, g/kg	7.01	6.48	6.59	5.60
Threonine, g/kg	9.05	9.53	9.41	9.75
Tryptophan, g/kg	2.26	2.43	2.40	2.37
Tyrosine, g/kg	3.17	3.04	3.04	2.79
Valine, g/kg	7.96	7.51	7.50	6.78

SID = standardized ileal digestibility.
0 Gln = low-protein diet; 1 Gln = low-protein diet supplemented with 1% L-glutamine; 2 Gln = low-protein diet supplemented with 2% L-glutamine; 3 Gln = low-protein diet supplemented with 3% L-glutamine.
[1] Premix provide the following per kilogram of diets: vitamin A, 2200 IU; vitamin D, 220 IU; vitamin E, 16 IU; vitamin K, 0.5 mg; biotin, 0.08 mg; choline, 600 mg; folacin, 0.3 mg; niacin, 30 mg; pantothenic acid, 12 mg; riboflavin, 4 mg; thiamin, 1.5 mg; vitamin B_6, 7 mg; Cu (from $CuSO_4$), 6 mg; I (from KI), 0.14 mg; Fe (from $FeSO_4$), 100 mg; Mn (from $MnSO_4$), 4 mg; Se (from Na_2SeO_3), 0.3 mg; Zn (from $ZnSO_4$), 100 mg.

approximately 0.05 mL plasma was mixed with 0.2 mL of 1.5 M $HClO_4$, followed by preservation at 4 °C for 30 min. After centrifugation (21,000 × *g*, 4 °C for 15 min), the supernatant was transferred into a new Eppendorf tube, and 0.1 mL of 2 M K_2CO_3 was added. The mixture was centrifuged at 21,000 × *g*, 4 °C for another 15 min to gain the final supernatant, which was subjected to high-performance liquid chromatography (HPLC) for analysis.

2.7. Analysis of 16S rDNA sequencing

Fecal bacterial DNA was extracted by using a FastDNA SPIN kit (Qiagen, Hilden, Germany) with the manufacturer's instructions. The quality of DNA was subsequently validated by agarose gel electrophoresis. The V3 to V4 region of the 16S ribosomal RNA gene in each sample was amplified, and the amplicons were sequenced by using an Illumina MiSeq platform (Illumina, San Diego, USA). Sequences with ≥97% similarity were filtered and classified into the same operational taxonomic units (OTU).

2.8. Statistical analysis

Data were analyzed using one-way ANOVA followed by Duncan's multiple comparison method in SPSS 20.0 software. Polynomial contrasts were conducted to analyze the linear and quadratic effects of dietary L-Gln levels. The significantly changed microbiota in feces was analyzed by the Kruskal–Wallis H test. All data were shown as mean ± SEM, and $P < 0.05$ was deemed a statistically significant difference.

3. Results

3.1. Effects of L-Gln supplementation on growth performance in low-protein diet-fed piglets

As shown in Table 2, supplementing L-Gln compromised the growth performance of piglets in a linear and quadratic fashion. Specifically, dietary L-Gln supplementation linearly and quadratically reduced body weight on both d 14 and 28 ($P < 0.01$), and average daily gain (ADG) across the experiment ($P < 0.01$), as well as average daily feed intake (ADFI) in piglets from d 1 to 14 ($P < 0.05$), while linearly and quadratically increasing feed to gain ratio (F:G) from d 1 to 28 ($P < 0.01$). Moreover, supplementing L-Gln linearly reduced ADFI from d 15 to 28 and d 1 to 28 ($P < 0.01$), whereas enhanced F:G ratio linearly from d 1 to 14 and d 15 to 28 ($P < 0.01$). Notably, piglets fed a low-protein diet containing 1% L-Gln had greater body weight and ADG on d 28, whereas a lower F:G ratio from d 1 to 28 when compared with pigs in the control group ($P < 0.01$). These results indicate that dietary 1% L-Gln exerts beneficial effects on low-protein diet-fed piglets and high levels of L-Gln may be detrimental for piglets.

3.2. Effects of L-Gln supplementation on serum physiochemical parameters in low-protein diet-fed piglets

The effects of dietary L-Gln inclusion on serum physiochemical parameters in low-protein diet-fed piglets are present in Table 3. L-Gln administration upregulated serum total protein level in a linear manner on d 14 ($P < 0.01$) and linearly caused a decrease in serum glucose ($P < 0.01$) and AST activity ($P = 0.024$) on d 28. Moreover, piglets fed with a low-protein diet containing 1% L-Gln had lower concentrations of serum glucose, and activities of serum AST and ALT on d 14 ($P < 0.05$), when compared with the control group. However, the serum creatinine and albumin levels didn't differ among the treatments ($P > 0.05$).

3.3. Effects of L-Gln supplementation on serum lipid metabolites in low-protein diet-fed piglets

Compared with the control group (Table 4), piglets fed L-Gln had lower concentrations of triglyceride (linear, $P < 0.05$), HDL-C (linear and quadratic, $P < 0.05$), and LDL-C (quadratic, $P < 0.05$) on d 28. However, the contents of serum total cholesterol, triglyceride, HDL-C, and LDL-C were observed to be similar in pigs fed low-protein diets with or without L-Gln on d 14 ($P > 0.05$), indicating that low-protein diet inclusion with L-Gln brings negligible beneficial effects to piglets at an early age.

Table 2
Effects of low-protein diet supplemented with different levels of L-glutamine on growth performance of piglets.

Item	Diets				SEM	*P*-value	Polynomial contrast	
	0 Gln	1 Gln	2 Gln	3 Gln			Linear	Quadratic
Initial body weight, kg	7.79	7.73	7.70	7.73	0.030	0.777	0.482	0.467
Day 14 body weight, kg	10.6^{a}	11.0^{a}	10.2^{b}	9.73^{c}	0.131	<0.001	<0.001	0.005
Day 28 body weight, kg	16.9^{b}	17.8^{a}	14.5^{c}	13.4^{d}	0.47	<0.001	<0.001	0.002
Day 1 to 14								
ADG, g	208^{ab}	230^{a}	177^{b}	143^{c}	10.1	0.001	<0.001	0.021
ADFI, g	329^{a}	349^{a}	294^{b}	275^{b}	8.8	<0.001	<0.001	0.039
F:G	1.58^{b}	1.52^{b}	1.68^{b}	1.94^{a}	0.055	0.011	0.005	0.057
Day 15 to 28								
ADG, g	445^{a}	488^{a}	308^{b}	261^{c}	25.1	<0.001	<0.001	0.012
ADFI, g	685^{a}	658^{a}	537^{b}	464^{b}	27.2	0.001	<0.001	0.481
F:G	1.54^{b}	1.35^{b}	1.74^{a}	1.78^{a}	0.053	0.002	0.002	0.103
Day 1 to 28								
ADG, g	324^{b}	359^{a}	243^{c}	202^{d}	16.8	<0.001	<0.001	0.002
ADFI, g	506^{a}	507^{a}	416^{b}	369^{b}	17.3	<0.001	<0.001	0.211
F:G	1.56^{b}	1.41^{c}	1.71^{a}	1.83^{a}	0.045	<0.001	<0.001	0.007

SEM = standard error of the mean; ADG = average daily weight gain; ADFI = average daily feed intake; F:G = feed to gain ratio.
0 Gln = low-protein diet; 1 Gln = low-protein diet supplemented with 1% L-glutamine; 2 Gln = low-protein diet supplemented with 2% L-glutamine; 3 Gln = low-protein diet supplemented with 3% L-glutamine.
[a−d]Values with different superscripts within a row means statistically significant ($P < 0.05$).

Table 3
Effects of low-protein diet supplemented with different levels of L-glutamine on physiochemical parameters of piglets.

Item	Diets				SEM	*P*-value	Polynomial contrast	
	0 Gln	1 Gln	2 Gln	3 Gln			Linear	Quadratic
Day 14								
Glucose, mmol/L	6.56^{a}	5.36^{b}	6.48^{a}	6.15^{a}	0.133	0.001	0.891	0.034
Creatinine, μmol/L	103.1	92.9	100.1	105.8	4.57	0.797	0.724	0.415
AST, U/L	58.8^{a}	18.5^{b}	34.1^{ab}	38.0^{ab}	4.85	0.022	0.226	0.015
ALT, U/L	99.9^{a}	66.5^{b}	83.1^{ab}	92.8^{a}	4.11	0.014	0.874	0.005
Albumin, g/L	23.7	22.5	23.2	23.9	0.33	0.460	0.721	0.157
Total protein, g/L	50.9^{b}	52.3^{b}	54.5^{ab}	58.0^{a}	0.80	0.004	<0.001	0.397
Day 28								
Glucose, mmol/L	9.15^{a}	7.30^{b}	6.63^{b}	6.78^{b}	0.313	0.007	0.002	0.060
Creatinine, μmol/L	80.0	82.9	71.9	77.3	3.44	0.735	0.554	0.867
AST, U/L	42.9	46.2	25.2	26.3	3.60	0.064	0.024	0.864
ALT, U/L	117.5	89.1	100.8	134.7	6.90	0.092	0.276	0.023
Albumin, g/L	21.5	24.3	23.8	26.1	1.00	0.479	0.163	0.906
Total protein, g/L	56.6	54.3	57.8	60.8	1.20	0.285	0.139	0.264

SEM = standard error of the mean; AST = aspartate aminotransferase; ALT = alanine aminotransferase.
0 Gln = low-protein diet; 1 Gln = low-protein diet supplemented with 1% L-glutamine; 2 Gln = low-protein diet supplemented with 2% L-glutamine; 3 Gln = low-protein diet supplemented with 3% L-glutamine.
[a,b]Values with different superscripts within a row means statistically significant ($P < 0.05$).

Table 4
Effects of low-protein diet supplemented with different levels of L-glutamine on serum lipid metabolism of piglets.

Item	Diets				SEM	*P*-value	Polynomial contrast	
	0 Gln	1 Gln	2 Gln	3 Gln			Linear	Quadratic
Day 14								
Total cholesterol, mmol/L	2.42	2.08	2.24	2.22	0.051	0.122	0.318	0.112
Triglyceride, mmol/L	0.438	0.391	0.441	0.423	0.0152	0.665	0.961	0.642
HDL-C, mmol/L	2.24	2.11	2.20	2.27	0.058	0.817	0.738	0.436
LDL-C, mmol/L	1.21	1.17	1.24	0.95	0.065	0.406	0.232	0.367
Day 28								
Total cholesterol, mmol/L	2.75	2.57	2.39	2.58	0.070	0.385	0.293	0.205
Triglyceride, mmol/L	0.527^{a}	0.447^{ab}	0.311^{b}	0.420^{ab}	0.0273	0.026	0.044	0.058
HDL-C, mmol/L	2.86^{a}	1.89^{b}	1.92^{b}	2.28^{b}	0.110	0.001	0.027	<0.001
LDL-C, mmol/L	1.53	1.22	1.01	1.47	0.082	0.087	0.573	0.019

SEM = standard error of the mean; HDL-C = high-density lipoprotein cholesterol; LDL-C = low-density lipoprotein cholesterol.
0 Gln = low-protein diet; 1 Gln = low-protein diet supplemented with 1% L-glutamine; 2 Gln = low-protein diet supplemented with 2% L-glutamine; 3 Gln = low-protein diet supplemented with 3% L-glutamine.
[a,b]Values with different superscripts within a row means statistically significant ($P < 0.05$).

3.4. L-Gln improved serum antioxidative capacity in low-protein diet-fed piglets

As presented in Table 5, piglets fed low-protein diets containing L-Gln had lower serum H_2O_2 (linear and quadratic, $P < 0.05$) and MDA (linear, $P < 0.05$) levels on d 14. Moreover, L-Gln treatments exhibited a quadratic increase in serum MPO and T-SOD activities ($P < 0.05$). Specifically, 1% L-Gln administration significantly reduced serum MPO activity, whereas enhanced serum T-SOD activity on d 14 ($P < 0.05$), with the T-SOD activity also being increased in response to 2% L-Gln treatment as compared to the control ($P < 0.01$). We also investigated the serum GSH level since L-Gln can transfer into Glu, the precursor material for synthesizing GSH. As expected, a low-protein diet supplemented with 1% L-Gln significantly enhanced the serum GSH concentration on d 14 ($P = 0.032$), exhibiting a robust antioxidative capacity by dietary inclusion of 1% of L-Gln. However, L-Gln treatments displayed minimal positive effects on antioxidative parameters in piglets on d 28, indicating L-Gln exerted its strongest antioxidative capacity in piglets at an early age.

3.5. Effects of L-Gln supplementation on serum AA in low-protein diet-fed piglets

To further investigate the role between improved antioxidative capacity and AA, we assayed AA contents by HPLC in the serum of piglets on d 14 (Table 6). As expected, low-protein diets supplemented with L-Gln linearly enhanced serum L-Gln contents ($P < 0.05$). Of note, compared with the control, 1%, 2% and 3% L-Gln administration reduced serum Glu by 19.0%, 16.1%, and 28.0% (linear, $P < 0.05$), respectively. Similarly, the Gly concentrations were reduced linearly by 14.6%, 20.1%, and 26.3% in response to 1%, 2%, and 3% L-Gln treatment ($P < 0.05$), respectively. Moreover, piglets fed the low-protein diet containing L-Gln had higher levels of serum histidine, lysine, and threonine (linear, $P < 0.05$), and linearly caused a decrease in β-alanine, isoleucine, serine, and valine ($P < 0.05$). In addition, 1% L-Gln enhanced serum tyrosine concentration as compared to the control ($P = 0.050$).

3.6. Effects of L-Gln supplementation on serum ammonia and urea nitrogen in low-protein diet-fed piglets

We next detected the serum ammonia and urea nitrogen (Fig. 1) since they are related to the metabolism of AA. L-Gln treatments linearly reduced serum ammonia level ($P < 0.05$), and increased blood urea nitrogen linearly in piglets on d 14 ($P < 0.05$), with these parameters being statistically significant in 3% L-Gln treatment ($P < 0.05$) when compared with the control group. However, the serum ammonia and urea nitrogen didn't differ among the treatments in pigs on d 28 ($P > 0.05$), indicating that piglets may not metabolize the AA in time in the diet supplemented with 3% L-Gln at an early age.

3.7. Effects of L-Gln supplementation on fecal microbiota composition in low-protein diet-fed piglets

As present in Fig. 2A and B, the Chao1 index, Shannon index, and Simpson index didn't differ among the treatments ($P > 0.05$), and the principal coordinate analysis (PCoA) of d 14 demonstrated that the treatments were not distinct from each other. Moreover, the PCoA analysis (Fig. 2C and D) showed that the diversity of fecal microbiota in the 3% L-Gln treatment was distinct from the control and the 1% L-Gln treatment on d 28, which may be related to the reduced feed intake induced by L-Gln, despite the alpha diversity on d 28 remaining similar ($P > 0.05$). We further revealed the significantly changed microflora at the genus level in the stool of piglets (Fig. 3A to D). On d 14, *p_75_a5*, *SMB53*, *Clostridium*, and *Lactobacillus* were the predominant bacteria at the genus level, and dietary L-Gln increased the *p_75_a5*, *Gemmiger*, and *Lactobacillus* level, whereas reduced the abundance of *SMB53*, and *Blautia* in the stool of piglets. Moreover, piglets fed diets supplemented with 1% L-Gln had a higher abundance of *Clostridium*, and *Prevotellaceae_Prevotella*, with the abundance of *Streptococcus* being concomitantly downregulated. On d 28, the predominant bacteria at the genus level were still *Lactobacillus*, *Clostridium*, *SMB53*, and *p_75_a5*, and dietary supplementation of 1% L-Gln upregulated the genus abundance of *p_75_a5* and *Gemmiger*, while 2% L-Gln

Table 5
Effects of low-protein diet supplemented with different levels of L-glutamine on serum redox status of piglets.

Item	Diets				SEM	*P*-value	Polynomial contrast	
	0 Gln	1 Gln	2 Gln	3 Gln			Linear	Quadratic
Day 14								
GSH, mg/L	7.61^{b}	9.12^{a}	8.33^{ab}	7.30^{b}	0.250	0.032	0.407	0.008
H_2O_2, mmol/L	44.3^{a}	24.9^{b}	24.8^{b}	23.3^{b}	2.56	0.003	0.002	0.034
MDA, nmol/mL	3.68^{a}	2.13^{b}	2.19^{b}	2.24^{b}	0.230	0.035	0.028	0.058
MPO, U/L	163^{a}	129^{b}	157^{a}	170^{a}	5.1	0.015	0.209	0.013
T-AOC, U/L	1.05	1.31	1.03	1.04	0.066	0.401	0.591	0.367
T-SOD, U/mL	37.9^{c}	43.6^{ab}	47.3^{a}	41.3^{bc}	1.10	0.013	0.103	0.004
CAT, U/mL	59.1^{b}	93.5^{a}	46.4^{b}	52.7^{b}	5.62	0.006	0.111	0.129
Day 28								
GSH, mg/L	6.54^{ab}	7.80^{a}	5.84^{b}	5.35^{b}	0.300	0.019	0.022	0.098
H_2O_2, mmol/L	12.0	15.0	10.5	9.3	0.94	0.170	0.134	0.252
MDA, nmol/mL	2.50	3.18	2.10	2.36	0.096	0.482	0.562	0.155
MPO, U/L	170	181	170	165	6.8	0.904	0.706	0.591
T-AOC, U/L	1.24	1.14	1.17	1.23	0.077	0.967	0.987	0.628
T-SOD, U/mL	53.0	52.7	51.0	53.1	1.32	0.946	0.906	0.679
CAT, U/mL	32.3	49.6	50.3	50.2	3.63	0.220	0.098	0.229

SEM = standard error of the mean; GSH = glutathione; H_2O_2 = hydrogen peroxide; MDA = malondialdehyde; MPO = myeloperoxidase; T-AOC = total antioxidant capacity; T-SOD = total superoxide dismutase; CAT = catalase.
0 Gln = low-protein diet; 1 Gln = low-protein diet supplemented with 1% L-glutamine; 2 Gln = low-protein diet supplemented with 2% L-glutamine; 3 Gln = low-protein diet supplemented with 3% L-glutamine.
[a–c]Values with different superscripts within a row means statistically significant ($P < 0.05$).

Table 6
Effects of low-protein diet supplemented with different levels of L-glutamine on serum amino acids in piglets on d 14 (μmol/L).

Item	Diets				SEM	*P*-value	Polynomial contrast	
	0 Gln	1 Gln	2 Gln	3 Gln			Linear	Quadratic
Alanine	602	609	580	623	23.6	0.938	0.877	0.715
Arginine	147	162	137	130	5.0	0.125	0.088	0.255
Asparagine	66.7	77.9	78.3	71.3	2.60	0.343	0.543	0.090
Aspartate	52.0	42.1	45.5	40.1	2.05	0.182	0.080	0.577
β-Alanine	43.6^{a}	35.0^{ab}	32.3^{b}	29.1^{b}	1.82	0.028	0.004	0.413
Citrulline	124^{ab}	137^{a}	105^{b}	123^{ab}	4.2	0.040	0.340	0.721
Glutamate	422^{a}	342^{ab}	354^{ab}	304^{b}	15.3	0.044	0.011	0.596
Glutamine	467^{b}	617^{a}	615^{a}	637^{a}	23.8	0.046	0.016	0.148
Glycine	1561^{a}	1333^{ab}	1248^{b}	1150^{b}	52.6	0.036	0.005	0.501
Histidine	38.9^{b}	39.4^{b}	42.4^{b}	57.8^{a}	2.13	0.001	0.001	0.038
Isoleucine	73.0^{a}	70.1^{ab}	54.7^{bc}	39.9^{c}	3.54	0.001	<0.001	0.280
Leucine	135	139	138	118	4.1	0.215	0.140	0.139
Lysine	231^{c}	444^{a}	294^{bc}	392^{ab}	23.5	0.001	0.042	0.109
Methionine	72.2^{b}	111.8^{a}	87.3^{ab}	75.4^{b}	5.31	0.027	0.729	0.012
Ornithine	96	103	85	128	6.3	0.097	0.157	0.139
Phenylalanine	84.5	85.6	81.5	78.7	1.85	0.561	0.207	0.606
Serine	247	243	211	200	8.2	0.092	0.016	0.828
Taurine	275	235	212	242	9.1	0.115	0.133	0.055
Threonine	365	551	545	630	43.9	0.170	0.047	0.550
Tryptophan	36.5	62.6	50.7	53.8	3.74	0.086	0.204	0.107
Tyrosine	55.5^{b}	72.7^{a}	64.9^{ab}	53.9^{b}	2.81	0.050	0.601	0.011
Valine	110.5^{a}	86.3^{b}	83.7^{b}	77.9^{b}	4.00	0.013	0.003	0.195

SEM = standard error of the mean.
0 Gln = low-protein diet; 1 Gln = low-protein diet supplemented with 1% L-glutamine; 2 Gln = low-protein diet supplemented with 2% L-glutamine; 3 Gln = low-protein diet supplemented with 3% L-glutamine.
$^{a-c}$Values with different superscripts within a row means statistically significant ($P < 0.05$).

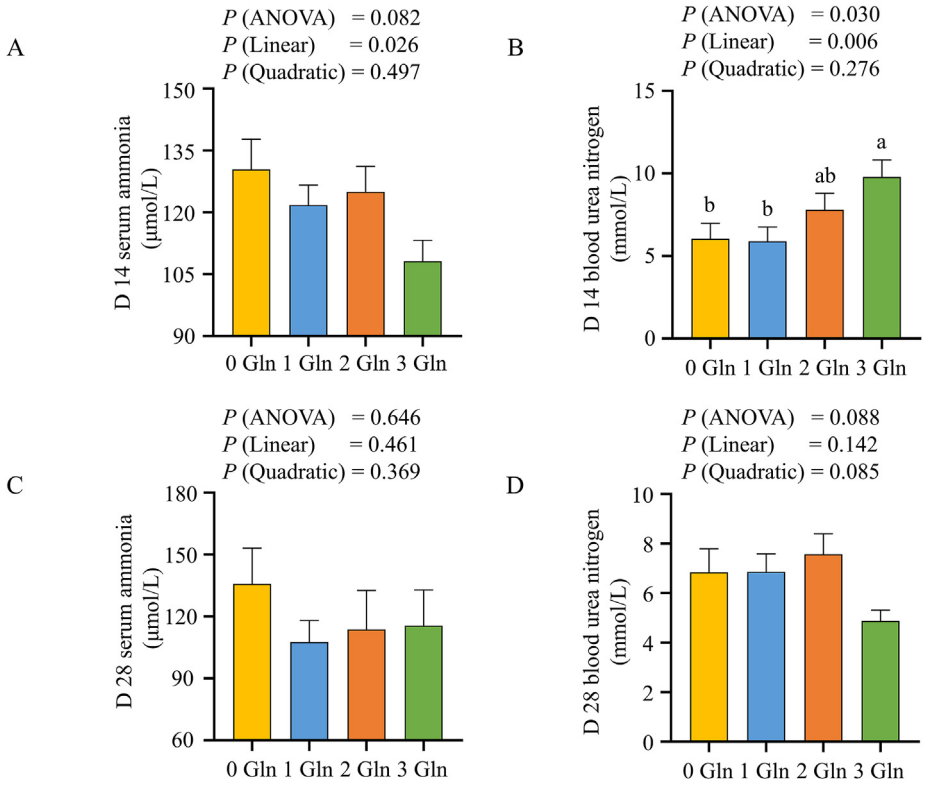

Fig. 1. Effects of low-protein diet supplemented with different levels of L-glutamine on serum ammonia and urea nitrogen in piglets. (A) Serum ammonia on d 14. (B) Blood urea nitrogen on d 14. (C) Serum ammonia on d 28. (D) Blood urea nitrogen on d 28. 0 Gln = low-protein diet; 1 Gln = low-protein diet supplemented with 1% L-glutamine; 2 Gln = low-protein diet supplemented with 2% L-glutamine; 3 Gln = low-protein diet supplemented with 3% L-glutamine. ^{a,b}Bars with different superscripts mean statistically significant ($P < 0.05$).

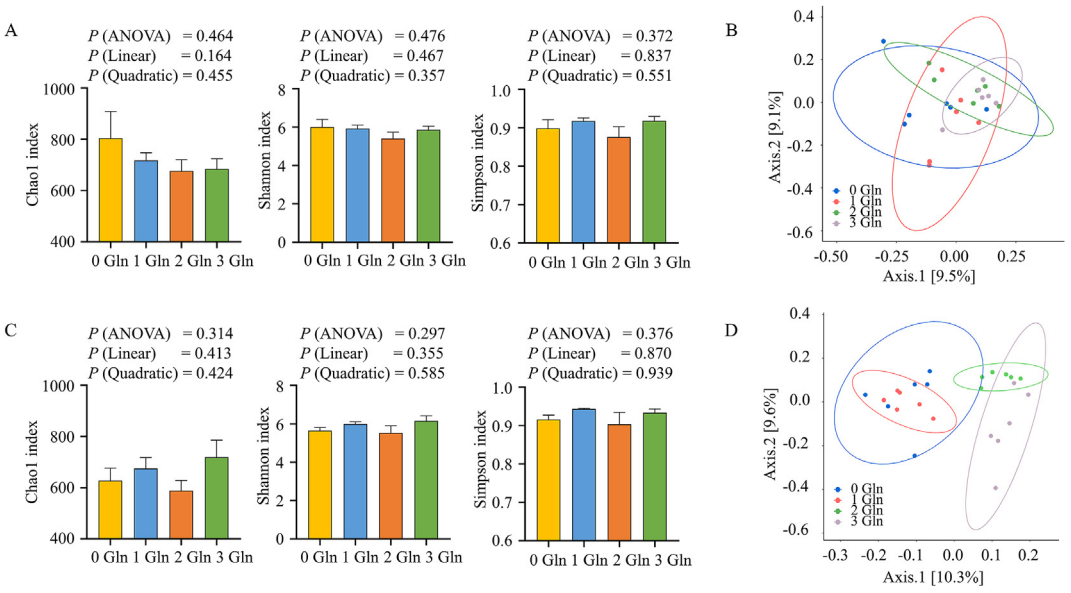

Fig. 2. Effects of low-protein diet supplemented with different levels of L-glutamine on fecal microbial diversity in piglets. (A) Alpha diversity on d 14 was estimated by using Chao1, Shannon, and Simpson indices. (B) PCoA of variation based on unweighted UniFrac distances on d 14. (C) Alpha diversity on d 28 was estimated by using Chao1, Shannon, and Simpson indices. (D) PCoA of variation based on unweighted UniFrac distances on d 28. PCoA = principal coordinate analysis. 0 Gln = low-protein diet; 1 Gln = low-protein diet supplemented with 1% L-glutamine; 2 Gln = low-protein diet supplemented with 2% L-glutamine; 3 Gln = low-protein diet supplemented with 3% L-glutamine.

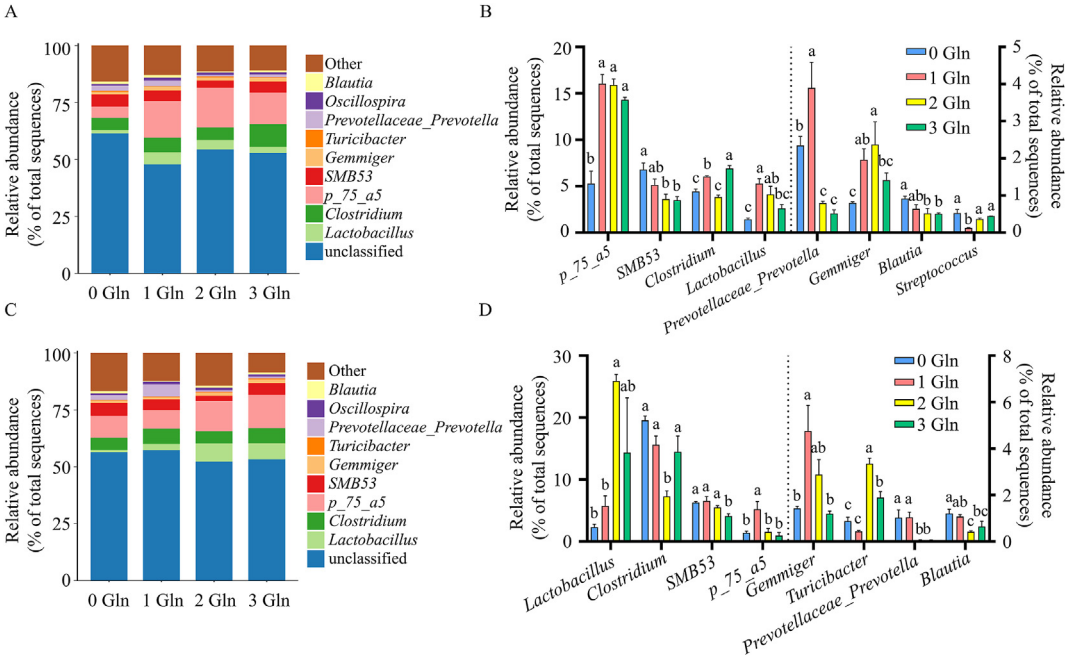

Fig. 3. Effects of low-protein diet supplemented with different levels of L-glutamine on fecal microbiota at the genus level. (A and B) The difference in fecal microflora on d 14 at the genus level in each group. (C and D) The difference in fecal microflora on d 28 at the genus level in each group. 0 Gln = low-protein diet; 1 Gln = low-protein diet supplemented with 1% L-glutamine; 2 Gln = low-protein diet supplemented with 2% L-glutamine; 3 Gln = low-protein diet supplemented with 3% L-glutamine. $^{a-c}$Bars with different superscripts mean statistically significant ($P < 0.05$).

induced incremental increases in *Lactobacillus*, *Turicibacter*, and a reduction of *Clostridium*, *Prevotellaceae_Prevotella*, and *Blautia*.

3.8. Effects of L-Gln supplementation on metabolism pathways of gut microbial community at KEGG level 3

On d 14 (Fig. 4), the functions of DNA replication, RNA degradation, D-Gln, D-Glu, and pyrimidine metabolism were enhanced by L-Gln administration, while functions of apoptosis, biofilm formation, p53 signaling pathway, arginine, proline, and butanoate metabolism, and diverse disease occurrence were dampened by L-Gln when compared with the control. On d 28 (Fig. 5), compared with the control group, 2% and 3% L-Gln inhibited the functions of arginine, proline, cysteine, and methionine metabolism, whereas they promoted the functions of lipopolysaccharide biosynthesis, metabolism of xenobiotics by cytochrome P450, methane

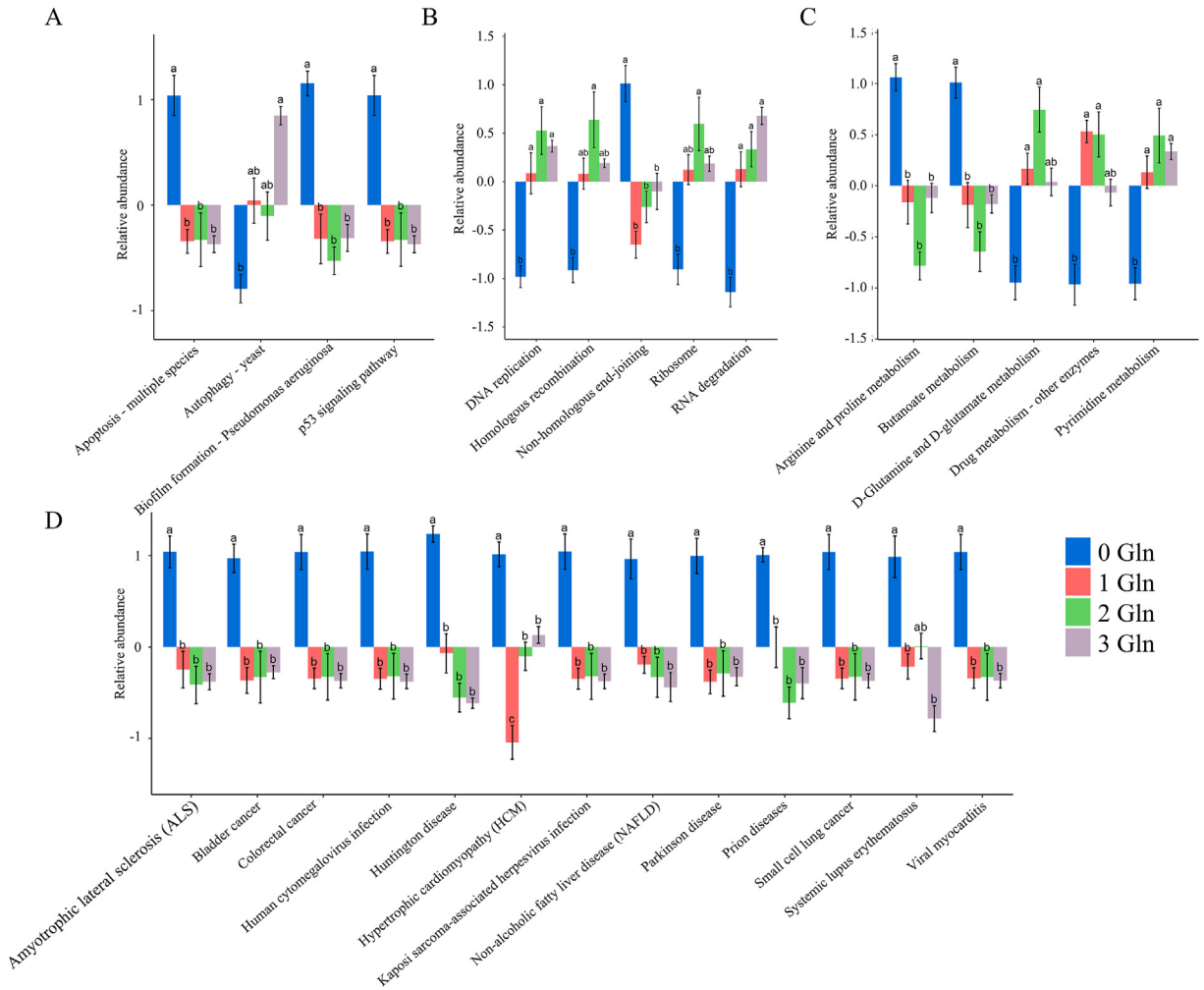

Fig. 4. The significantly altered pathways of different microbiota abundance after L-glutamine administration in low-protein diet-fed weaned piglets on d 14 at KEGG level 3. (A) Cellular processes. (B) Genetic information processing. (C) Metabolism. (D) Human diseases. 0 Gln = low-protein diet; 1 Gln = low-protein diet supplemented with 1% L-glutamine; 2 Gln = low-protein diet supplemented with 2% L-glutamine; 3 Gln = low-protein diet supplemented with 3% L-glutamine. $^{a-c}$Bars with different superscripts means statistically significant ($P < 0.05$).

metabolism, other glycan degradation, retinol metabolism, sphingolipid metabolism, steroid and steroid hormone biosynthesis. Of note, 2% and 3% L-Gln treatments enhanced the functions of human diseases including hypertrophic cardiomyopathy, pathogenic *Escherichia coli* infection, platinum drug resistance, and *Shigellosis*. These data suggest that a low-protein diet supplemented with 2% or 3% L-Gln is detrimental and not suitable for piglets.

4. Discussion

Gln is a conditionally essential AA for pigs and has been recommended as a feed supplement to reduce weaning stress in piglets due to its multiple beneficial effects (Ji et al., 2019). Substantial studies have investigated the effects of dietary Gln on growth in pigs. Yi et al. (2005) found that 2% (as-fed basis) Gln improved ADG and gain to feed ratio in weaned pigs at 48 h after *E. coli* K88 infection. Moreover, Kitt et al. (2002) found that Gln improved feed efficiency from d 14 to 21 in piglets during the early post-weaning period. Likewise, Wu et al. (1996) reported that 1% of Gln supplementation not only enhanced the gain to feed ratio by 25% but also prevented jejunal atrophy. Weanling pigs fed the 1% L-Gln diet had higher ADG and gain to feed ratio than those fed the control diet during d 0 to 14 and d 1 to 28 (He et al., 2016). In the present study, we advanced the knowledge that a low-protein diet supplemented with 1% L-Gln enhanced growth performance, while 2% and 3% L-Gln dramatically inhibited growth performance in low-protein diet-fed piglets, suggesting that the concentration of L-Gln should be less than 2% in low-protein diet-fed piglets. Our further investigation demonstrated that piglets fed diets containing 2% or 3% of L-Gln couldn't metabolize the extra L-Gln as evidenced by the increased blood urea nitrogen. Besides, supplementation of 2% or 3% L-Gln may also induce the antagonism of other AA like arginine and proline, which, in turn, inhibits growth performance (Holecek, 2013). Of note, a recent study demonstrated that pigs fed diets (normal protein level) with Gln inclusion improved growth performance after weaning when compared with the control, with 0.4% Gln being the most effective level among the treatments (con, 0.2%, 0.4%, 0.6%, 0.8% and 1% of L-Gln) (Duttlinger et al., 2020), indicating the optimum concentration of L-Gln in low-protein diet may be similar. More studies regarding the effects of dietary L-Gln on growth performance in low-protein diet-fed piglets need to be conducted.

Serum physiochemical parameters like AST, ALT, and glucose can reflect the physiological status of the organism to some degree. AST plays a vital role in modulating cell proliferation by participating in AA metabolism, especially Gln metabolism (Song et al., 2022).

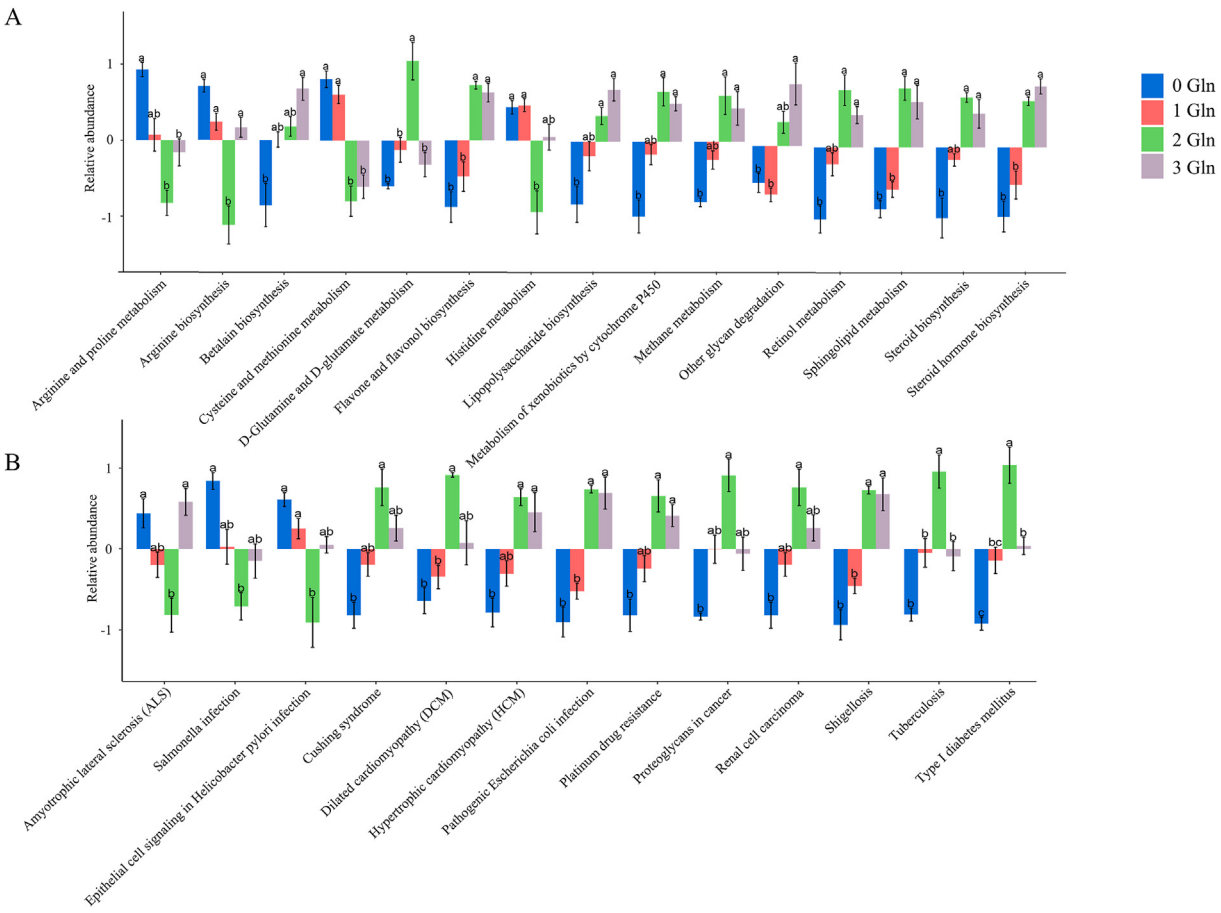

Fig. 5. The significantly altered pathways of different microbiota abundance after L-glutamine administration in low-protein diet-fed weaned piglets on d 28 at KEGG level 3. (A) Metabolism. (B) Human diseases. 0 Gln = low-protein diet; 1 Gln = low-protein diet supplemented with 1% L-glutamine; 2 Gln = low-protein diet supplemented with 2% L-glutamine; 3 Gln = low-protein diet supplemented with 3% L-glutamine. $^{a-c}$Bars with different superscripts means statistically significant ($P < 0.05$).

Briefly, when Gln enters the cytoplasm, it is subsequently hydrolyzed into Glu and ammonia (Wise et al., 2008). The Glu converts into α-ketoglutaric acid and is further catabolized in the tricarboxylic acid (TCA) cycle. On the other hand, Glu can also formulate aspartate by AST 2, then the aspartate from the mitochondrial matrix undergoes AST 1-catalyzed reaction in the cytoplasmic matrix to generate oxaloacetate, and finally converts into pyruvate, during which nicotinamide adenine dinucleotide phosphate (NADPH) is also produced (Li and Le, 2018). Moreover, ALT, also known as glutamic pyruvic transaminase (GPT), is a gluconeogenesis enzyme that catalyzes conversions between alanine and pyruvic acid. ALT, along with AST is often used as a blood biomarker of hepatic damage. In the current study, piglets on d 14 had lower activities of serum AST and ALT in response to 1% L-Gln administration, and our further study found that a low-protein diet supplemented with L-Gln enhanced serum Gln content, whilst reducing serum aspartate and β-alanine levels linearly, indicating that the cellular energy pathway regarding Gln converting into Glu is inactive. The elevation of serum Gln levels may be attributed to an augmented uptake of Gln by the small intestinal epithelium, which subsequently acts as a compensatory mechanism to offset Gln catabolism. Consistently, the serum glucose was observed to be lower in response to 1% L-Gln; these results together suggested that 1% Gln exerted other functional roles beyond supplying energy in low-protein diet-fed piglets at an early age. Intriguingly, previous studies demonstrated that a normal protein diet supplemented with 1% Gln increased ALT activity, with no significant effect on AST (Xiao et al., 2012), which may be related to the protein level in the diet, the age, and the physiological state of piglets.

The antioxidative function is one of the multiple beneficial effects of Gln since Gln can convert into Glu, which is the precursor substance for the synthesis of GSH. GSH is an important regulator of the cellular redox state protecting cells from damage caused by lipid peroxides, reactive oxygen and nitrogen species, and xenobiotics (Kennedy et al., 2020). H_2O_2 is an endogenous reactive oxygen species (ROS) that leads to oxidative stress directly as a molecular oxidant and indirectly through free radical generation (Murphy and Friedman, 2019), which can be hydrolyzed water and oxygen by CAT, and SOD is another important antioxidant enzyme in scavenging ROS. Moreover, the MDA level is the main product of lipid peroxidation by ROS and is therefore deemed a lipid peroxidation biomarker (Placer et al., 1966). He et al. (2016) demonstrated that feeding a 1% L-Gln diet (protein level 20.4%) increased SOD activity while decreasing the serum MDA content on d 28 in weanling pigs. Likewise, higher activities of jejunal mucosa glutathione peroxidase (GSH-Px) and T-SOD were observed in 1% Gln-fed piglets, with the MDA concentration being simultaneously reduced (Zhang et al., 2017a). Consistently, in the present study, a low-protein diet supplemented with 1% L-Gln reduced serum MDA and H_2O_2 levels, and MPO activity, whilst enhancing activities of CAT and T-SOD, and GSH content in pigs on d 14. Of note, there is a negative correlation between serum Gln contents and mucosal barrier function (Wang et al., 2008), suggesting there may be similar relationships between serum Gln or other AA like Gly. Our studies further revealed

that the L-Gln administration reduced serum Glu and Gly levels in piglets, which may account for the increase in GSH since both Glu and Gly are precursors of GSH. The inconsistency of antioxidative status in piglets among L-Gln treatments may be related to the differences like feed intake, blood urea nitrogen and other factors that ultimately result from Gln treatments since the organism is complicated. Together, L-Gln improved the antioxidative capacity in piglets by an increase in the synthesis of GSH.

Gut microflora play a critical role in regulating host health, and the disruption of gastrointestinal microbial structure may result in a series of metabolic disorders or even diseases (Thaiss et al., 2016). Intestinal microbiota can be affected by multiple factors like the use of antibiotics, diseases, weaning and dietary changes, during which the structure of the gut microbiota may alter, making pigs vulnerable and predisposed to infections (Summers et al., 2019). In the present study, weaned piglets fed low-protein diets supplemented with L-Gln, a conditionally essential AA, had similar results regarding alpha diversity among the treatments, indicating that L-Gln administration exerted little effect on microbial composition diversity. We also found that piglets fed a diet with 1% L-Gln had higher abundance of *p_75_a5*, *Gemmiger*, *Lactobacillus*, *Clostridium*, and *Prevotellaceae_Prevotella*, with the abundance of *Streptococcus* being concomitantly downregulated on d 14. Likewise, dietary 1% L-Gln upregulated the genus abundance of *p_75_a5* and *Gemmiger* in the stool of piglets on d 28, indicating 1% L-Gln administration optimized the microbial structure as *Gemmiger* and *Lactobacillus* have been widely considered probiotics (Zhou et al., 2023), and Prevotellaceae has been shown to ameliorate PD-1/PD-L1 inhibitor-related cardiotoxicity in colonic macrophages by producing butyrate (Chen et al., 2022). The inconsistencies in microbe alterations at genus level among the L-Gln treatments may be related to feed intake, both directly and indirectly. In agreement with our study, Xu et al. (2021) demonstrated that Gln ameliorated DSS-induced reduction of *Lactobacillus* in the colonic content of mice. Consistently, pregnant sows fed a diet supplemented with 1.0% Gln had higher abundance of Bacteroidales, and *Prevotella* (Zhang et al., 2017b), suggesting that Gln has a regulatory effect on microbial metabolism in the gastrointestinal tract. KEGG pathways further revealed that L-Gln administration could decrease cell apoptosis, promote DNA replication, enhance the metabolism of Gln, Glu, and pyrimidine, and prevent various human diseases like cancer, cardiovascular disease, endocrine and metabolic disease, infectious disease, and neurodegenerative disease on d 14. Consistently, Gln could promote DNA synthesis of intestinal epithelial cells by activating the ornithine decarboxylase (Wu, 1998). However, the functions of hypertrophic cardiomyopathy, platinum drug resistance, pathogenic *E. coli* and *Shigellosis* infection were promoted in response to 2% and 3% L-Gln administration on d 28. Among them, *E. coli* and *Shigellosis* infection are critical factors in intestinal disease etiology, indicating a high level of L-Gln could bring detrimental effects to piglets. Still, the role of gut microflora mediated by L-Gln is lacking in the context of low-protein diets. Therefore, the regulatory mechanism of L-Gln on gut microbiota in piglets fed with low-protein diets needs to be investigated in the future.

5. Conclusion

In summary, we found that supplementing 1% L-Gln improved the growth performance, serum physiochemical parameters, and antioxidative capacity in the low-protein diet-fed piglet at an early age. These beneficial effects of L-Gln were associated with an increased synthesis of GSH by modulating AA levels, and the optimization of intestinal microbial structure (like upregulating the abundance of *Gemmiger* and *Lactobacillus* and reducing the relative level of *Streptococcus*) instead of microbial composition diversity. Moreover, 1% L-Gln prefers to exert its functional effects on antioxidation and modulation of intestinal microbiota rather than be a single fuel in low-protein diet-fed pigs at an early age. Of note, a low-protein diet supplemented with 2% or 3% L-Gln compromised the growth performance in weaned piglets, which is related to the accumulation of blood urea nitrogen, inhibitory metabolism of arginine and proline, and increased susceptibility to *E. coli* and *Shigellosis* infection.

Author contributions

Jun Li: Conceptualization, Investigation, Writing – original draft, Writing – review & editing. **Jun Bai:** Investigation, Software. **Ying Yang:** Methodology, Data curation. **Zhenlong Wu:** Conceptualization, Supervision, Funding acquisition, Writing – review & editing. All authors read and approved the final manuscript.

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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