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Original Research Article

High-level L-Gln compromises intestinal amino acid utilization efficiency and inhibits protein synthesis by GCN2/eIF2α/ATF4 signaling pathway in piglets fed low-crude protein diets

Jun Li [a], Yinfeng Chen [a], Yang Yang [a], Ying Yang [a], Zhenlong Wu [a, b, *]

[a] State Key Laboratory of Animal Nutrition and Feeding, Department of Companion Animal Science, China Agricultural University, Beijing 100193, China
[b] Beijing Advanced Innovation Center for Food Nutrition and Human Health, China Agricultural University, Beijing 100193, China

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ABSTRACT

Gln, one of the most abundant amino acids (AA) in the body, performs a diverse range of fundamental physiological functions. However, information about the role of dietary Gln on AA levels, transporters, protein synthesis, and underlying mechanisms in vivo is scarce. The present study aimed to explore the effects of low-crude protein diet inclusion with differential doses of L-Gln on intestinal AA levels, transporters, protein synthesis, and potential mechanisms in weaned piglets. A total of 128 healthy weaned piglets (Landrace × Yorkshire) were randomly allocated into four treatments with four replicates. Pigs in the four groups were fed a low-crude protein diet containing 0%, 1%, 2%, or 3% L-Gln for 28 d. L-Gln administration markedly (linear, $P < 0.05$) increased Ala, Arg, Asn, Asp, Glu, Gln, His, Ile, Lys, Met, Orn, Phe, Ser, Thr, Tyr, and Val levels and promoted trypsin activity in the jejunal content of piglets. Moreover, L-Gln treatment significantly enhanced concentrations of colonic Gln and Trp, and serum Thr (linear, $P < 0.01$), and quadratically increased serum Lys and Phe levels ($P < 0.05$), and decreased plasma Glu, Ile, and Leu levels (linear, $P < 0.05$). Further investigation revealed that L-Gln administration significantly upregulated *Atp1a1*, *Slc1a5*, *Slc3a2*, *Slc6a14*, *Slc7a5*, *Slc7a7*, and *Slc38a1* relative expressions in the jejunum (linear, $P < 0.05$). Additionally, dietary supplementation with L-Gln enhanced protein abundance of general control nonderepressible 2 (GCN2, $P = 0.010$), phosphorylated eukaryotic initiation factor 2 subunit alpha (eIF2α, $P < 0.001$), and activating transcription factor 4 (ATF4) in the jejunum of piglets ($P = 0.008$). These results demonstrated for the first time that a low crude protein diet with high-level L-Gln inclusion exhibited side effects on piglets. Specifically, 2% and 3% L-Gln administration exceeded the intestinal utilization capacity and compromised the jejunal AA utilization efficiency, which is independent of digestive enzyme activities. A high level of L-Gln supplementation would inhibit protein synthesis by GCN2/eIF2α/ATF4 signaling in piglets fed low-protein diets, which, in turn, upregulates certain AA transporters to maintain AA homeostasis.

1. Introduction

Reducing the crude protein levels in pig diets is a recognized and efficient approach for mitigating feed costs and nitrogen excretion. Decreasing pig diet crude protein levels by no more than 4% whilst concomitantly supplementing appropriate proportions of essential amino acids (AA) to satisfy the AA gap will not only maintain the growth performance, but also exert diverse beneficial effects including reducing risk of gut disorders, feed costs, and nitrogen emissions (Gloaguen et al., 2014; Wu et al., 2015). In recent years, non-essential AA have been recognized for their importance in animal production (Chen et al., 2021; He et al., 2019; Yang et al.,

* Corresponding author.
E-mail address: wuzhenlong@cau.edu.cn (Z. Wu).
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2022), some of which have even been classified into conditionally essential amino acids (CEAA). For piglets, Glu, Gln, Arg, Pro, Gly, and Tau belong to the CEAA (Wu, 2009). Our previous study indicated that CEAA (e.g., Glu and Gln) are indispensable for weaning piglets to activate the translation initiation factors and thus maintain optimal protein synthesis, particularly during the weaning transition period (Wu et al., 2014). In low-crude protein diets, CEAA levels are also lower leading to a greater need for nitrogen due to endogenous protein synthesis (Gloaguen et al., 2014). Similarly, CEAA are required for maintaining the activation of translation initiation factors and protein synthesis in piglets fed an extremely low-protein level diet (12.7% crude protein) (Deng et al., 2009). Recent advances in pig nutrition research has shown that dietary Glu could ameliorate the negative impacts on performance of weaned piglets fed low-protein diets (Upadhaya et al., 2022). Of note, Gln can be converted into Glu and ultimately participate in the citric acid cycle and function as a substrate for protein synthesis and energy (Berres et al., 2010), indicating Gln may also have beneficial effects on piglets fed low-protein diets. However, little is known regarding the effects of a low crude protein diet with L-Gln inclusion on piglets.

Gln, one of the most abundant and versatile AA in plasma, milk and tissue, is critical to intermediate metabolism, pH homeostasis, and nitrogen exchange among tissues (Cruzat et al., 2018). Gln can be utilized as a substrate for the synthesis of nicotinamide adenine dinucleotide phosphate (NADPH) and nucleotides, which are involved in antioxidative function and cellular integrity function in almost every cell (Cruzat et al., 2014; Curi et al., 2016). The Gln level in serum from weaned piglets varies from 400 to 800 μmol/L, with the level ranging from 2300 to 5000 μmol/kg in liver and muscle tissues (Roth, 2008). Gln deficiency has been linked to various detrimental effects and intestinal diseases, including intestinal villus atrophy, dysfunction of tight junctions, and upregulation of paracellular permeability (Bertrand et al., 2016; Chaudhry et al., 2016; Kim and Kim, 2017). Notably, Gln has been proposed as a potential feed supplement for ameliorating weaning stress in piglets (Ji et al., 2019), and our previous study demonstrated that 1% L-Gln administration improved the jejunal tight junction in piglets (Wang et al., 2015). It is unknown whether a higher level of L-Gln in low-protein diets exerts stronger effects on biological function, thus warranting further study.

Protein synthesis is of paramount importance to sustaining physiological processes concerned with AA concentration, sensing and transportation, translation, and so on (Merrick and Pavitt, 2018). Both in vivo and in vitro studies have demonstrated the ability of AA to stimulate protein synthesis (Escobar et al., 2005; O'Connor et al., 2003). A sufficient supply of AA is a prerequisite for protein synthesis since AA are the fundamental components of proteins and function as substrates in the process of protein synthesis (Kimball, 2002). Mechanisms of regulating protein synthesis are typically associated with the general control nonderepressible 2 (GCN2) pathways. GCN2, an AA sensor responsible for regulating biosynthesis based on nutrient availability, is also the eukaryotic initiation factor 2 subunit alpha (eIF2α) kinase and can repress translation initiation by phosphorylating the translation initiation factor eIF2α if AA are inadequate (Hinnebusch, 2005; Nofal et al., 2022). The phosphorylated eIF2α is able to further activate downstream genes, such as activating transcription factor 4 (ATF4), ultimately reducing global protein synthesis (Ma et al., 2017). Notably, inhibiting Gln degradation has been shown to facilitate protein synthesis in intestinal porcine epithelial cells, indicating Gln's possible crucial role in the process of protein synthesis. The uptake and transportation of Gln, followed by subsequent glutaminolysis are critical for the GCN2 nutrient-sensing pathway (van Geldermalsen et al., 2016). However, knowledge about the effects of dietary L-Gln on AA transporters, protein synthesis, and underlying mechanisms in the intestine of piglets fed low-crude protein diets is unavailable. Therefore, the present study aims to investigate the effects of low-crude protein diet supplementation with 0%, 1%, 2%, and 3% L-Gln on AA levels, AA transporters, protein synthesis, and underlying mechanisms in piglets. The findings of this study may improve understanding of the role of L-Gln in protein synthesis and its effects in piglets fed low-crude protein diets.

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. Animals and experimental design

One hundred and twenty-eight healthy male weaned piglets (Landrace × Yorkshire) aged 31 d and with an initial weight of 7.74 ± 0.12 kg were randomly assigned to four groups, with each group consisting of four pens and eight piglets in each pen. The piglets in the control group were fed a low-crude protein diet (17% crude protein), while the three treatments were fed low-crude protein diets supplemented with 1%, 2%, and 3% L-Gln (Meihua Biotechnology Group Co., Ltd., Zhengzhou, China) for 4 weeks. The diets (Table 1) were formulated in line with the nutritional requirements for pigs outlined by the National Research Council (2012), and the composition of the diets was in accordance with our previous study (Li et al., 2024). The curde protein, calcium (Ca), and phosphorus (P) in feed were measured according to China National Satnadard (Cude protein: GB/T 6432-2018, Ca: GB/T 6436-2018, and P: GB/T 6437-2018).

2.3. Sample collection

On the morning of d 28, six piglets from each treatment were sacrificed, and one piglet closest to the average body weight of piglets in each pen was chosen. The 5th and 6th closest piglets to the average body weight of the pen were randomly selected from the first two pens and the latter two pens, respectively. Blood samples were collected from the anterior vena cava after a feed withdrawal period of 12 h. The serum was obtained after centrifuging at 3500 × *g* for 15 min. Subsequently, piglets were sacrificed and dissected. The mid-jejunum and mid-colon tissues were excised, and the jejunal and colonic content collected. After that, the mid-jejunal tissue was flushed with ice-cold sterile phosphate-buffered saline and opened longitudinally. The chyme on the surface layer of jejunal mucosa was removed by sterile glass microscope slide, after which the jejunal mucosa sample was scratched using a sterile glass microscope slide and immediately frozen in liquid nitrogen. All samples were stored at −80 °C for further analysis.

2.4. Measurement of AA in feed

The levels of Trp and other AA in the feed were assayed using alkaline hydrolysis and acidic hydrolysis of the feed, respectively, in accordance with the methods described by Dai et al. (2014). For Trp measurement, about 50 mg feed was weighed and placed into a 2-mL screw-cap tube. Next, approximately 40 of mg hydrolyzed potato starch was added, followed by pipetting 1 mL 4.2 mol/L NaOH and 0.02 mL 1-octanol (Sigma–Aldrich, USA). The mixture was subjected to ultrasound for 10 min, and then nitrogen was injected into the tube and placed at 105 °C for 20 h. After cooling, the

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

Item	Diets[1]			
	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[2]	1.00	1.00	1.00	1.00
Dicalcium phosphate	0.59	0.62	0.65	0.69
L-Thr	0.30	0.34	0.38	0.42
Salt	0.40	0.40	0.40	0.40
DL-Met	0.12	0.13	0.15	0.16
L-Trp	0.10	0.11	0.13	0.14
Total	100.00	100.00	100.00	100.00
Calculated nutrient levels[3], %				
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 Lys	1.30	1.30	1.30	1.30
SID Met	0.39	0.39	0.39	0.39
SID Met + Cys	0.67	0.65	0.64	0.62
SID Thr	0.76	0.76	0.76	0.76
SID Trp	0.23	0.23	0.23	0.23
Nutrient levels[4]				
Crude protein, %	17.04	17.54	17.99	18.06
Ca, %	0.73	0.73	0.67	0.68
P, %	0.44	0.44	0.42	0.41
Ala, g/kg	8.63	8.24	8.53	8.09
Arg, g/kg	7.79	7.08	7.32	6.50
Asp + Asn, g/kg	13.65	12.51	12.79	11.41
Glu + Gln, g/kg	29.60	37.11	47.53	58.14
Gly, g/kg	5.62	5.34	5.40	4.78
His, g/kg	4.35	4.03	4.05	3.82
Ile, g/kg	6.50	6.03	6.32	5.67
Leu, g/kg	14.36	13.60	14.17	13.34
Lys, g/kg	12.25	13.03	15.64	14.51
Met, g/kg	5.12	4.61	5.06	5.37
Phe, g/kg	7.22	6.70	7.03	6.52
Ser, g/kg	6.90	6.27	6.34	5.81
Thr, g/kg	9.00	9.24	9.36	9.67
Trp, g/kg	2.24	2.43	2.45	2.51
Tyr, g/kg	3.120	2.92	2.98	2.89
Val, g/kg	7.62	7.12	7.28	6.77

SID = standardized ileal digestibility.
[1] 0 Gln, a low-protein diet without L-Gln; 1 Gln, 2 Gln, or 3 Gln, the low-protein diet with 1% L-Gln, 2% L-Gln, or 3% L-Gln, respectively.
[2] Premix provided 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.
[3] The calculation of these indexes were in accordance with NRC (2012).
[4] The nutrient levels of crude protein, Ca, phosphorus, and amino acids were actually measured.

mixture was vortexed and centrifuged at 12,000 × *g* for 1 min. Subsequently, 600 μL of the supernatant was pipetted and filtered through a 0.22 μm needle filter and transferred into a new 1.5 mL Eppendorf tube. The solution was diluted 50 times with water and subjected to high-performance liquid chromatography (HPLC) for further analysis.

For the measurement of other AA, approximately 0.5 g of feed was weighed and put into a 15 mL glass tube. Then 10 mL of 6 mol/L HCl was added to the tube, followed by ultrasonic treatment for 10 min. Nitrogen was injected into the glass tube for about 30 s and the cap was instantly screwed on. The tube was then heated at 110 °C for 24 h. After heating, the mixture was vortexed and transferred into a 50-mL tube. The 15-mL glass tube was washed with water twice, and the washings were also transferred into the same 50-mL tube. Next, 600 μL of the supernatant was filtered through a 0.22 μm needle filter and transferred into a new 1.5 mL Eppendorf tube. The solution was diluted a further 20 times with water and analyzed by HPLC.

2.5. AA levels in jejunal, and colonic contents, and serum of piglets

About 0.05 mL of plasma/jejunal content or 50 mg of colonic contents were mixed with 0.2 mL 1.5 mol/L $HClO_4$ and incubated at 4 °C for 30 min. The supernatant was harvested by centrifuging at 21,000 × *g*, 4 °C for 15 min, and transferred into a new Eppendorf tube, followed by adding 0.1 mL of 2 mol/L K_2CO_3. The final supernatant was obtained by centrifugation at 21,000 × *g*, 4 °C for another 15 min, which was then analyzed by HPLC to determine the AA concentrations.

2.6. Quantitative real-time polymerase chain reaction (qPCR)

To extract total RNA from the mucosa of the jejunum, a commercial TRIzol reagent (Aidlab Biotechnology, Beijing, China) was employed. After verification by 1% agarose gel electrophoresis, the RNA concentrations were assayed by a Nanophotometer P-330 (IMPLEN, German). The RNA was subsequently transferred into cDNA by reversing transcription with a commercial kit (Aidlab Biotechnology, Beijing, China). qPCR was performed using SYBR Premix (Aidlab Biotechnology, Beijing, China) on a qPCR machine (ABI-Prism 7500). The primer sequences of the target genes used in the current study are presented in Table S1. *GAPDH* was used as an internal control, and the results of the qPCR were calculated using the $2^{-\Delta\Delta Ct}$ method.

2.7. Western blot analysis

The protein of jejunal mucosa was extracted using RIPA buffer, and the protein solution was obtained by centrifuging at 12,000 × *g* for 15 min. The protein solution was then denatured at 99 °C for 6 min and subsequently subjected to an SDS-PAGE gel to separate the target protein bands. The separated bands were then transferred onto a polyvinylidene fluoride membrane (Millipore, Billerica, USA) and incubated with the respective antibodies. The protein bands were visualized using an Image Quant LAS 4000 system (GE Healthcare Bio-sciences AB, Inc., Sweden) after combination with a chemiluminescence kit (Amersham Biosciences). Anti-GCN2 (3302S) was purchased from Cell Signaling Technology, and anti-mTOR (#66888), anti-p-eIF2α (#28740), anti-eIF2α (#11170), and anti-ATF4 (#10835) were gained from Proteintech Group.

2.8. Jejunal digestive enzymes

The jejunal activities of amylase, trypsin, and lipase were determined with commercial kits according to the methods provided by the manufacturer (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.9. Statistical analysis

The data, exhibited as mean and SEM, were analyzed by using SPSS 20.0 software using the model:

$$Y_{ijkl} = \mu + T_i + P_j + C_k + S_l + e_{ijkl}$$

where Y_{ijkl} represents the observation of the dependent variable; μ, the mean value of population; T_i, the fixed effect of treatment i; P_j, the fixed effect of period j; C_k, the random effect of the piglets k; S_l, the effect of square l; e_{ijkl}, the error associated with the observation. Polynomial contrasts were carried out to analyze the linear and quadratic effects of dietary L-Gln levels. $P < 0.05$ is considered statistically significant.

3. Results

3.1. AA concentrations in feed

We firstly analyzed the AA content of the diets (Table 1). The sum of Glu and Gln was given since both cannot be discriminated after acid hydrolysis. As expected, the levels of Glu and Gln in feeds with 0%, 1%, 2%, and 3% L-Gln were found to be 28.59, 38.64, 48.15, and 59.26 g/kg, respectively. The differences in Glu and Gln levels between adjacent groups (0% vs. 1% L-Gln, 1% vs. 2% L-Gln, 2% vs. 3% L-Gln) were 10.05, 9.51, and 11.11 g/kg, respectively, which equated to 1.01%, 0.95%, and 1.11%. Additionally, the concentrations of other AA including Ala, Arg, Asp, and Asn, Gly, His, Ile, Leu, Lys, Met, Phe, Ser, Thr, Trp, Tyr, and Val were found to be similar among the treatments, indicating that the feeds were well-suited for the following animal feeding experiments.

3.2. Jejunal AA concentrations

As presented in Table 2, low-crude protein diet supplementation with L-Gln linearly increased Ala, Arg, Asn, Asp, Glu, Gln, His, Ile, Leu, Lys, Met, Orn, Phe, Ser, Thr, Tyr, and Val levels in jejunal contents of piglets ($P < 0.05$). However, the β-Ala, Cit, Gly, Tau, and Trp levels did not differ among the treatments ($P > 0.05$).

Table 2
Effects of L-Gln on the concentrations of amino acids in the jejunum content of piglets fed low-crude protein diets (μmol/L).

Item	Gln levels[1]				SEM	*P*-value	
	0 Gln	1 Gln	2 Gln	3 Gln		Linear	Quadratic
Ala	4122	4807	6303	8985	634.7	0.004	0.356
Arg	2462	2646	3527	5840	489.7	0.009	0.218
Asn	1083	1361	1886	3193	261.7	0.002	0.225
Asp	1825	1911	3255	5001	425.0	0.002	0.227
β-Ala	27.8	34.9	28.1	32.7	3.34	0.805	0.868
Cit	137	120	197	234	26.2	0.132	0.616
Glu	4627	4694	7397	10,549	764.1	0.001	0.192
Gln	1723	1781	2540	4750	382.1	0.001	0.073
Gly	9229	11,030	8889	11,754	1097.3	0.602	0.819
His	873	812	1123	2219	181.6	0.004	0.037
Ile	1667	2062	2768	4605	402.3	0.005	0.294
Leu	3364	3640	5182	7331	605.3	0.014	0.396
Lys	3213	3594	4896	7329	595.5	0.009	0.328
Met	1588	1943	2505	4223	368.9	0.006	0.287
Orn	281	320	505	638	62.3	0.026	0.692
Phe	1398	1789	2087	3527	302.3	0.009	0.325
Ser	2380	2643	3873	7104	591.7	0.001	0.107
Tau	1292	1449	1592	1504	136.6	0.565	0.678
Thr	1615	2356	3046	4874	398.0	0.002	0.396
Trp	478	463	563	868	74.3	0.056	0.273
Tyr	1255	1556	1878	2702	217.6	0.017	0.512
Val	1909	2593	3373	5141	427.0	0.004	0.455

[1] 0 Gln, a low-protein diet without L-Gln; 1 Gln, 2 Gln, or 3 Gln, the low-protein diet with 1% L-Gln, 2% L-Gln, or 3% L-Gln, respectively.

3.3. Digestive enzymes of jejunal content

The increased AA levels may be related to intestinal digestive dysfunction. We next assayed the activities of amylase, trypsin, and lipase in the jejunal contents (Table 3). Notably, L-Gln treatment linearly increased jejunal trypsin activities ($P = 0.006$). However, the activities of amylase and lipase did not differ in jejunal contents ($P > 0.05$), suggesting dietary supplementation with a high level of L-Gln had no detrimental effects on digestive enzymes in piglets.

3.4. Colonic AA concentrations

To further investigate whether the intestinal AA was effectively utilized, we next determined the AA levels in colonic content (Table 4). As expected, diets supplemented with L-Gln linearly increased colonic Gln and Trp levels ($P < 0.01$), suggesting that supplementing with 2% or 3% L-Gln may exceed the utilization capacity of intestinal Gln in weaned piglets.

3.5. Plasma AA concentrations

Plasma AA are absorbed from the intestinal content and are important building blocks for protein synthesis in the body. Apart from utilization by intestinal epithelial cells, AA within cells are released into the bloodstream via active AA transporters and utilized by other tissues. Hence, we investigated the concentrations of AA in the serum (Table 5). L-Gln treatment linearly increased plasma Thr content ($P = 0.003$), whereas it lowered blood Glu, Ile, and Leu (Linear, $P < 0.05$). Moreover, L-Gln administration quadratically increased serum Lys and Phe concentration. These data suggest that a high level of L-Gln administration is likely to compromise the utilization of essential AA, such as Ile and Leu, in piglets fed low-protein diets.

3.6. Jejunal AA transporters

We next analyzed the gene expressions of jejunal AA transporters (Table 6). Consistent with our previous results, L-Gln administration linearly upregulated jejunal *Atp1a1*, solute carrier family 1a5 (*Slc1a5*), *Slc3a2*, *Slc6a14*, *Slc7a5*, *Slc7a7*, and *Slc38a1* relative abundance ($P < 0.05$). However, the Gln importer, *Slc38a2*, did not differ among the treatments ($P = 0.941$).

3.7. L-Gln activated GCN2/eIF2α/ATF4 signaling pathway

The nutrient sensor mechanistic target of rapamycin (mTOR) and GCN2 are closely involved in sensing AA and regulating protein synthesis. As shown in Fig. 1, L-Gln administration significantly increased the protein abundance of mTOR (linear and quadratic, $P = 0.014$). Moreover, dietary L-Gln linearly increased the protein GCN2, p-eIF2α/eIF2α, and ATF4 relative abundance ($P \leq 0.01$). These results indicated that 2% and 3% L-Gln were likely to compromise

Table 3
Effects of L-Gln on digestive enzymes of jejunal content in piglets fed low-crude protein diets (U/mg prot).

Item	Gln levels[1]				SEM	Polynomial contrast	
	0 Gln	1 Gln	2 Gln	3 Gln		Linear	Quadratic
Amylase	0.91	1.06	1.72	1.99	0.303	0.171	0.921
Trypsin	246	310	644	953	99.2	0.006	0.471
Lipase	2.90	3.04	4.45	5.30	0.603	0.118	0.776

[1] 0 Gln, a low-protein diet without L-Gln; 1 Gln, 2 Gln, or 3 Gln, the low-protein diet with 1% L-Gln, 2% L-Gln, or 3% L-Gln, respectively.

Table 4
Effects of L-Gln on the concentrations of amino acids in the colonic content of piglets fed low-crude protein diets (μmol/kg).

Item	Gln levels[1]				SEM	Polynomial contrast	
	0 Gln	1 Gln	2 Gln	3 Gln		Linear	Quadratic
Ala	725	986	918	863	52.6	0.465	0.145
Arg	96.7	70.8	88.9	74.3	7.42	0.471	0.717
Asn	6.31	9.35	11.7	11.3	1.23	0.133	0.484
Asp	536	455	468	464	32.7	0.517	0.589
β-Ala	48.5	39.2	50.0	38.3	3.74	0.569	0.878
Cit	138	240	180	206	14.3	0.236	0.157
Glu	1075	1359	1183	1132	76.0	0.995	0.302
Gln	18.1	21.5	39.4	37.8	3.02	0.002	0.621
Gly	178	210	236	231	11.8	0.088	0.453
His	70.3	70.4	71.1	82.4	7.46	0.606	0.724
Ile	115	153	141	115	11.7	0.928	0.198
Leu	183	241	235	200	18.3	0.788	0.233
Lys	581	814	800	717	47.7	0.350	0.108
Met	145	203	211	178	12.6	0.322	0.078
Orn	360	453	623	379	52.9	0.626	0.124
Phe	347	178	522	109	64.0	0.491	0.309
Ser	160	229	231	202	15.0	0.344	0.117
Tau	39.1	14.1	151.1	24.0	21.07	0.596	0.196
Thr	150	187	187	181	9.6	0.298	0.289
Trp	4.93	2.30	9.83	9.54	0.946	0.003	0.403
Tyr	76.2	105.6	90.8	86.0	6.65	0.810	0.219
Val	161	215	198	189	14.8	0.633	0.321

[1] 0 Gln, a low-protein diet without L-Gln; 1 Gln, 2 Gln, or 3 Gln, the low-protein diet with 1% L-Gln, 2% L-Gln, or 3% L-Gln, respectively.

Table 5
Effects of L-Gln on plasma amino acids in piglets fed low-crude protein diets (μmol/L).

Item	Gln levels[1]				SEM	Polynomial contrast	
	0 Gln	1 Gln	2 Gln	3 Gln		Linear	Quadratic
Ala	611	613	551	532	21.5	0.138	0.805
Arg	130	126	115	142	6.3	0.689	0.252
Asn	67.2	63.1	69.0	52.6	3.57	0.248	0.402
Asp	43.8	41.6	34.1	32.2	2.80	0.106	0.980
β-Ala	33.8	36.4	34.8	30.5	1.29	0.338	0.201
Cit	92.2	104	103	129	7.4	0.107	0.641
Glu	381	344	275	260	17.9	0.006	0.727
Gln	487	416	531	400	19.0	0.329	0.379
Gly	1232	1147	1166	1026	57.7	0.263	0.824
His	30.8	32.9	30.5	35.8	1.83	0.459	0.694
Ile	59.5	58.2	38.7	41.8	3.09	0.005	0.671
Leu	130	119	108	98	5.7	0.045	0.997
Lys	179	251	220	192	12.4	0.952	0.046
Met	53.3	50.3	61.9	53.1	2.37	0.610	0.559
Orn	76.6	109	98.2	78.0	7.73	0.920	0.102
Phe	60.5	76.0	72.9	64.9	2.52	0.628	0.019
Ser	202	206	246	210	9.7	0.459	0.313
Tau	186	182	201	226	11.1	0.183	0.533
Thr	295	289	516	512	36.7	0.003	0.986
Trp	33.2	40.2	40.3	40.3	2.60	0.382	0.531
Tyr	68.2	61.2	57.2	61.7	3.48	0.479	0.434
Val	92.0	98.6	64.8	79.8	5.19	0.113	0.662

[1] 0 Gln, a low-protein diet without L-Gln; 1 Gln, 2 Gln, or 3 Gln, the low-protein diet with 1% L-Gln, 2% L-Gln, or 3% L-Gln, respectively.

protein synthesis by the GCN2/eIF2α/ATF4 signaling pathway in the jejunum of piglets fed low-protein diets.

4. Discussion

AA hold fundamental significance in cellular biology, serving as pivotal constituents in the synthesis of novel proteins and precursors for metabolic pathways. During digestion, the gut

Table 6
Effects of low-protein diet supplemented with different levels of L-Gln on amino acid transporters in the jejunal mucosa of piglets.

Item	Gln levels[1]				SEM	*P*-value	
	0 Gln	1 Gln	2 Gln	3 Gln		Linear	Quadratic
Atp1a1	1.00	1.92	3.31	5.91	0.601	0.002	0.400
Slc1a5	1.00	1.41	2.07	2.01	0.206	0.046	0.556
Slc3a2	1.00	1.15	1.07	2.56	0.153	<0.001	<0.001
Slc6a14	1.00	1.13	8.54	5.77	0.929	0.003	0.287
Slc7a5	1.00	1.45	3.13	2.38	0.229	0.001	0.063
Slc7a7	1.00	1.02	0.93	1.75	0.125	0.049	0.091
Slc38a1	1.00	1.48	12.32	5.23	1.402	0.032	0.119
Slc38a2	1.00	0.94	0.85	1.01	0.068	0.941	0.459

Atp1a1 = ATPase, Na^+/K^+ transporting subunit alpha 1; *Slc1a5* = solute carrier family 1 member 5; *Slc3a2* = solute carrier family 3 member 2; *Slc6a14* = solute carrier family 6 member 14; *Slc7a5* = solute carrier family 7 member 5; *Slc7a7* = solute carrier family 7 member 7; *Slc38a1* = solute carrier family 38 member 1; *Slc38a2* = solute carrier family 38 member 2.
[1] 0 Gln, a low-protein diet without L-Gln; 1 Gln, 2 Gln, or 3 Gln, the low-protein diet with 1% L-Gln, 2% L-Gln, or 3% L-Gln, respectively.

enzymatically degrades the proteins present in ingested food into AA, and these AA are absorbed by enterocytes. AA within the cells are released into the bloodstream, where they are efficiently funneled to various tissues and organs stationed throughout the body. In the present study, we formulated low-crude protein diets with graded doses of L-Gln. We conducted AA analysis of the feeds and observed an increase in Glu and Gln concentrations with increasing doses of L-Gln, while other AA were similar among the groups. The Ala, Arg, Asn, Asp, Glu, Gln, His, Ile, Leu, Lys, Met, Orn, Phe, Ser, Thr, Tyr, and Val concentrations were increased with concomitant increases in L-Gln dosage in the jejunal content of piglets fed low-crude protein diets containing L-Gln. Interestingly, we also found that L-Gln administration linearly promoted trypsin activity without compromising amylase and lipase activities. Notably, high-level L-Gln administration reduced the average daily feed intake in our previous study (Li et al., 2024). Therefore, these increased AA were attributed to the lowered utilization efficiency caused by L-Gln since the AA levels in feed among the groups were similar and the digestive enzyme activities were not inhibited, while the average daily intake was even reduced in response to 3% L-Gln treatment (Li et al., 2024). Consistently, Holecek (2013) demonstrated that long-term Gln administration may impair AA absorption in the gut and kidneys and even AA distribution in tissue. Additionally, the Gln and Trp concentrations in the colonic contents were higher in piglets fed low-protein diets containing 2% and 3% L-Gln, suggesting that more than 2% L-Gln could not be effectively utilized.

Importantly, the AA concentrations in the gut play an important role in the corresponding levels in the bloodstream. Normally, when the gut boasts an ample supply of AA, this is reflected in the bloodstream with a corresponding increase in plasma AA levels. In contrast, in cases of insufficient AA supply in the gut, the bloodstream exhibits a reduction in AA levels, reflecting the limited supply. In the current study, we found that low-crude protein diets supplemented with L-Gln linearly reduced plasma Glu, Ile, and Leu in weaned piglets, while linearly enhancing Thr level. Intriguingly, a higher plasma Gln content was only found in piglets fed diets with 2% L-Gln instead of 3% L-Gln, which may be related to the reduction in feed intake in the 3% L-Gln group (Li et al., 2024). The plasma AA results indicated that high levels of L-Gln (2% or 3% L-Gln) may exert a synergistic effect on the utilization of Thr while showing antagonism towards Ile and Leu since these AA cannot be synthesized in vivo. Consistently, a previous study demonstrated that long-term Gln supplementation reduced Val, Leu, Ile, Gly, Ser, and

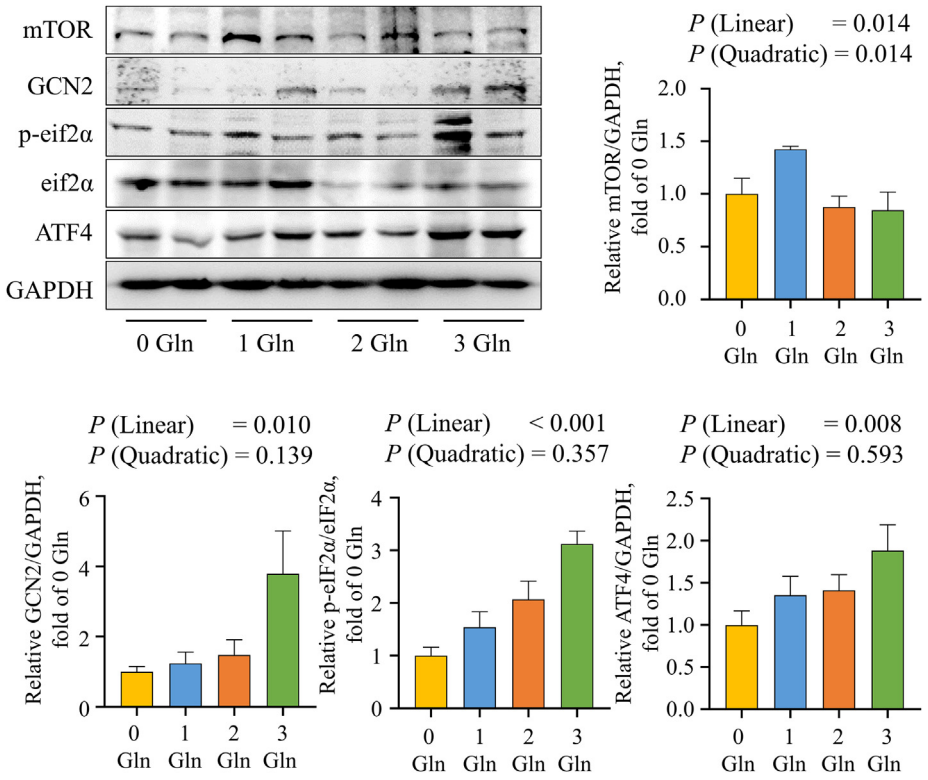

Fig. 1. L-Gln compromised protein synthesis by activating the GCN2/eIF2α/ATF4 signaling pathway in the jejunum of pigletes fed low crude protein diet. The protein abundances of GCN2, p-eIF2α/eIF2α, and ATF4 in the jejunal mucosa. 0 Gln, a low-protein diet without L-Gln; 1 Gln, 2 Gln, or 3 Gln, the low-protein diet with 1% L-Gln, 2% L-Gln, or 3% L-Gln, respectively. GCN2 = general control nonderepressible 2; p-eIF2α = phosphor eukaryotic initiation factor 2 subunit alpha; ATF4 = activating transcription factor 4; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; mTOR = mechanistic target of rapamycin kinase.

Pro in the blood (Holecek, 2013). Elevated concentrations of various Gln metabolites, notably Glu, Orn, Cit, Arg, Asp, Ser, and Ala, have been frequently observed in blood plasma following acute administration of Gln (Dechelotte et al., 1991; Melis et al., 2005). Nonetheless, this augmentation appears transitory, as with prolonged administration of Gln the levels of these AA appear to remain stable or even decrease (Holecek, 2011; Kalhan et al., 2005; Ockenga et al., 2005; Valencia et al., 2002).

The transportation of AA in the lumen of the small intestine involves a complex series of processes facilitated by specialized transporters that meet the diverse physiological demands of the body. Normally, the levels of intracellular AA are higher or at least equal to those in extracellular fluid since active transport is employed to concentrate numerous AA within the cell (Hyde et al., 2003). In mammals, the uptake of AA into the cell may concern many AA transporters, such as Gln transporters *Slc1a5*, *Slc3a2*, *Slc6a14*, *Slc7a5*, *Slc7a7*, *Slc38a1* and *Slc38a2* (Broer, 2002; Freidman et al., 2022). *Slc38a1* and *Slc38a2* import AA with thermodynamic Na^+ influx to catalyze AA transportation (Broer, 2002). *Atp1a1* plays an important role in maintaining the transmembrane gradient for Na^+ away from equilibrium, and is therefore critical for cellular functions like transporting AA (Henriksen et al., 2013). *Slc3a2*, *Slc7a5*, and *Slc7a7* are AA exchangers, which could couple the uptake of required AA with the efflux of cytoplasmic AA (Meier et al., 2002). In addition, *Slc6a14* transports its AA substrates in a single direction, which leads to the net influx of AA (Sniegowski et al., 2021). These AA transporters are moderated by the GCN2 regulator, responsible for Gln uptake and metabolism to Glu (Luo et al., 2018; Shen et al., 2021). In the present study, L-Gln administration linearly upregulated the relative expressions of *Atp1a1*, *Slc1a5*, *Slc3a2*, *Slc6a14*, *Slc7a5*, and *Slc7a7* in the jejunum of piglets fed low-protein diets. Specifically, piglets fed a low-crude protein diet with 2% L-Gln upregulated jejunal *Slc6a14*, *Slc7a5*, and *Slc38a1* relative expression, and 3% L-Gln promoted relative abundance of *Atp1a1*, *Slc3a2*, *Slc6a14*, *Slc7a5*, and *Slc7a7*. The upregulated AA carriers in the jejunum may be related to less available AA within the cells, as evidenced by elevated extracellular AA levels in jejunal contents, which may further activate nutrient sensors and potential signaling pathways. Consistently, dietary inclusion with an AA blend (Glu:Gln:Gly:Arg:N-acetylcysteine = 5:2:2:1:0.5) upregulated the relative gene expression of *Slc7a9*, which transports Arg, Lys, Orn, and cystine in the jejunum of piglets, with the carrier *Slc1a5* being not statistically significant (Yi et al., 2018). Likewise, our previous study demonstrated that dietary supplementation with 1.4 g/kg body weight L-Leu upregulated the *Slc6a19*, *Slc6a14*, and *Slc7a9*, with the Leu carrier *Slc7a7* also being reduced in the jejunum of 21-day-old piglets (Sun et al., 2015). Notably, 3% L-Gln treatment did not upregulate *Slc1a5* and *Slc38a2* relative expressions; both were demonstrated to be Gln importers (Willems et al., 2013; Zhang et al., 2023). These findings suggest that a high level of dietary L-Gln may compromise the utilization efficiency of many AA, leading to the upregulation of AA transporters to import higher levels of other AA instead of Gln into cells, while appropriate (1%) L-Gln administration did not affect the Gln carrier. Still, further studies are needed to advance the knowledge regarding how these transporters function with enhanced L-Gln in piglets or other animal models.

Cells and organisms need to integrate information from their surroundings to ensure that they grow under optimal conditions. The precise control of cell growth in response to nutrient availability is orchestrated by sensors such as GCN2. GCN2, an eIF2 kinase, is capable of sensing AA limitation by binding uncharged

tRNAs due to its domain homologous to histidyl-tRNA synthetases (Sonenberg and Hinnebusch, 2009). Specifically, GCN2 senses AA deficiency and phosphorylates eIF2α at its Ser 51 residue (Hu and Guo, 2021; Murguia and Serrano, 2012), resulting in the induction of its downstream genes, including ATF4, ultimately reducing global protein synthesis (Li et al., 2023). In the current study, we found that L-Gln supplementation linearly upregulated protein GCN2, p-eIF2α/eIF2α, and ATF4 relative abundance. Consistently, mice had lower serum and hepatic Leu levels within the first 6 h of feeding a diet deficient in Leu, corresponding with GCN2-dependent increases in *Atf4* mRNA translation and the induction of *Slc7a11* and *Slc7a5*, which transport large neutral AA like Leu (Jonsson et al., 2022). These results suggested that dietary supplementation with a higher level (2% or more) L-Gln compromised AA utilization efficiency and inhibited protein synthesis by the GCN2/eIF2α/ATF4 signaling pathway, which, in turn, upregulated certain AA transporters to maintain AA homeostasis by enhancing the intracellular AA (Cordova et al., 2022; Kilberg et al., 2009; Menchini and Chaudhry, 2019; Pakos-Zebrucka et al., 2016).

5. Conclusion

In conclusion, our findings demonstrated for the first time that dietary 2% and 3% L-Gln compromises jejunal AA utilization in piglets fed low-crude protein diets, which is independent of digestive enzymes activities, despite most studies demonstrating the beneficial effects of L-Gln administration. Moreover, administration of 2% or 3% L-Gln exceeded the intestinal utilization capacity. Additionally, high level L-Gln supplementation inhibits protein synthesis by GCN2/eIF2α/ATF4 signaling in piglets fed low-protein diets, which, in turn, upregulates certain AA transporters to maintain AA homeostasis. Our study not only advances the knowledge regarding the application of Gln in low protein diets but also provides guidance for practical pig production.

Credit author statement

Jun Li: Conceptualization, Investigation, Writing - original draft, Writing - review & editing; **Yinfeng Chen** and **Yang Yang**: Investigation, Software; **Ying Yang**: Methodology, Data curation; **Zhenlong Wu**: Conceptualization, Supervision, Funding acquisition, Writing - review & editing.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2024.06.008.

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