



## Original Research Article

# Glutamine in suppression of lipopolysaccharide-induced piglet intestinal inflammation: The crosstalk between AMPK activation and mitochondrial function



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## ABSTRACT

The study was conducted to investigate the regulatory mechanism of glutamine (Gln) on intestinal inflammation in an *Escherichia coli* lipopolysaccharide (*E. coli* LPS)-induced in vivo and in vitro models. Piglets ( $n = 8$ ) weaned at 21 d of age were fed a basal diet (control and LPS groups) or 1% Gln diet (Gln + LPS group) ad libitum for 4 weeks. On d 22, 24, 26 and 28, piglets in the LPS and Gln + LPS groups were intraperitoneally injected with *E. coli* LPS. Intestinal porcine epithelial cells (IPEC-J2) ( $n = 6$ ) induced by LPS were used to assess related mechanisms and compound C was used to inhibit adenosine 5'-monophosphate-activated protein kinase (AMPK) activity. Our current results showed that compared with the LPS treatment, the Gln + LPS treatment had better growth performance and greater villus height ( $P < 0.05$ ), and the Gln + LPS treatment reduced the rate of diarrhea by 6.4% ( $P < 0.05$ ); the Gln + LPS treatment decreased serum tumor necrosis factor (TNF- $\alpha$ ), interleukin-6 (IL-6),  $K^+$ , cortisol and insulin levels, whereas increased ( $P < 0.05$ ) serum immunoglobulin M and epidermal growth factor levels; the Gln + LPS treatment increased ( $P < 0.05$ ) the expression of aquaporins and AMPK pathway-associated targets in the jejunum and ileum of piglets, whereas decreased the expression of ion transporters ( $P < 0.05$ ). The in vitro results showed that 4 mmol/L Gln administration could inhibit ( $P < 0.05$ ) cell apoptosis and interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-6 and TNF- $\alpha$  secretion in LPS-induced IPEC-J2 cells, promote ( $P < 0.05$ ) mitochondrial respiratory metabolism and increase ( $P < 0.05$ ) the number of mitochondria and mitochondrial membrane potential. The activity of AMPK was elevated by 70% to 300% in Gln-treated IPEC-J2 cells under LPS challenge or normal conditions. Our results indicate that pre-administration of Gln to piglets suppresses intestinal inflammation by modulating the crosstalk between AMPK activation and mitochondrial function.

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## 1. Introduction

Glutamine (Gln), as an important energy substrate for enterocytes (He et al., 2016; Ren et al., 2019), has beneficial effects on maintaining the barrier structure and function of the intestine, promoting the growth, repair and integrity of mucosa, improving the immune function of intestine and maintaining homeostasis of microecology (Beutheu et al., 2014; Ren et al., 2019). In various stress conditions, Gln is influential in protein synthesis, energy

supply, and immunity in the intestine and regulates the pathogenesis of various intestinal diseases, including ulcerative colitis, diarrhea, and necrotizing enterocolitis (Kim and Kim 2017; Son et al., 2018; Ren et al., 2013). Examples of the protective effects of Gln administration include the utilization of cell energy (Kim and Kim 2017), maintenance of tight junctions in intestinal epithelium (Ewaschuk et al., 2011), and alleviation of intestinal damage (Ren et al., 2016).

Previous studies have reported that the process of maintaining the intestinal barrier is energy-consuming, and the impairment of the function of mitochondria as the main energy generator in enterocyte, is associated with gut inflammation (Ho et al., 2018). Mediators of mitochondrial signaling include molecules ATP and reactive oxygen species and associated with pathways such as adenosine 5'-monophosphate-activated protein kinase (AMPK), peroxisome proliferator-activated receptor gamma coactivator 1- $\alpha$  (PGC-1 $\alpha$ ) (Rath et al., 2018), can contribute to regulating mechanisms of the intestine by helping to survey the luminal environment of the host (Klos and Dabrowski 2021). Thus, it is crucial to maintain mitochondrial function through the control of intestinal barrier, including osmotic pressure, tight junctions, and signal molecules. It is clear that intestinal inflammation could cause the change of water and ion flow and result in osmotic pressure imbalance (Guttman et al., 2007). Many studies have reported that transmembrane aquaporins (AQP) and ion channels function in the transport and absorption of water, ions, and some macromolecules, and maintain the homeostasis of intestinal osmotic pressure to alleviate various stresses, especially in response to cell migration (Ribatti et al., 2014), inflammation (Peplowski et al., 2017), and oxidative stresses (Piskounova et al., 2015). These findings are strong indications of a link between water and ion channels and intestinal health. However, the exact involvement of intestinal AQP and ion transporters in the regulatory functions of Gln in piglets under immune stress conditions remains unknown, and the potential mechanisms involved in the AMPK pathway and mitochondrial function are also poorly understood. Here we examined the effects of Gln supplementation on the distribution and functions of intestinal AQP and ion transporters, as well as mitochondrial function in piglets injected intraperitoneally with *Escherichia coli* lipopolysaccharide (*E. coli* LPS). Additionally, the related mechanism was also explored in LPS-induced intestinal porcine epithelial cells (IPEC-J2).

## 2. Materials and methods

### 2.1. Animals and experimental design

All animals used in the study were approved by the Animal Welfare Committee of the Institute of Subtropical Agriculture of the Chinese Academy of Sciences (2013020, Changsha, China).

A total of 24 healthy piglets (Duroc  $\times$  Landrace  $\times$  Large Yorkshire) with a similar body weight (BW) of  $6.04 \pm 0.15$  kg were weaned at 21 d of age, and were assigned to 3 groups ( $n = 8$  per group). Two groups (control and LPS) were fed a corn and soybean meal-based diet, and the third group (Gln + LPS) was fed the basal diet supplemented with 1% Gln (Wuhan Yuancheng Gongchuang Technology Co., Ltd., Wuhan, China; purity  $\geq 99.2\%$ ). The ingredient and nutrient levels of the different diets without antibiotics met the nutrient specifications for pigs with BW of 5–10 kg are presented in Supplementary Table. The formal experiment began after 3 d adaptation, and at 08:00 on d 22, 24, 26, and 28 of the feeding periods, weaned piglets in the LPS and Gln + LPS groups were intraperitoneally injected with *E. coli* LPS (*E. coli* serotype O55: B5; L2880, Sigma Aldrich, Inc., St Louis, USA, 100  $\mu$ g/kg BW), whereas pigs in the control group were injected intraperitoneally with the same volume

of sterile saline. The administration dosages of LPS and Gln were adopted according to the previous experiments (He et al. 2016, 2017b). On d 28, serum samples were collected from the jugular vein of all piglets after 4 h of LPS or saline challenge and then were anesthetized as previously described (He et al., 2020b). Samples of jejunum and ileum were collected and immediately snap-frozen in liquid nitrogen and then stored at  $-80^\circ\text{C}$  for RNA and protein extraction. One intestinal segment was fixed in 10% neutral buffered formalin for examination of intestinal morphology. We monitored and recorded the BW of piglets and feed intake throughout the experiment period, and then calculated average daily gain (ADG), average daily feed intake (ADFI) and gain-to-feed (G:F) ratio.

### 2.2. Cell culture

IPEC-J2 cells were used in subsequent experiments to assess the impact of Gln in AMPK activation and mitochondrial function. Cells were seeded ( $5 \times 10^6$  cells/flask) and grown in uncoated plastic culture flasks (25  $\text{cm}^2$ ) in glucose Dulbecco's modified Eagle's medium (DMEM-H, Gibco, USA) containing 10% fetal bovine serum (FBS, Gibco, USA), 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin (basal medium). When the cells grow to about 80%, the culture mediums were replaced with basal media (without FBS) supplemented with different dosages of Gln (0, 2, 4, and 8 mmol/L) for 24 h. Gln with 4 mmol/L was selected as the optimal treatment concentration and 24 h as the optimal time through cellular respiratory metabolism analysis (He et al., 2018).

### 2.3. Cell treatment

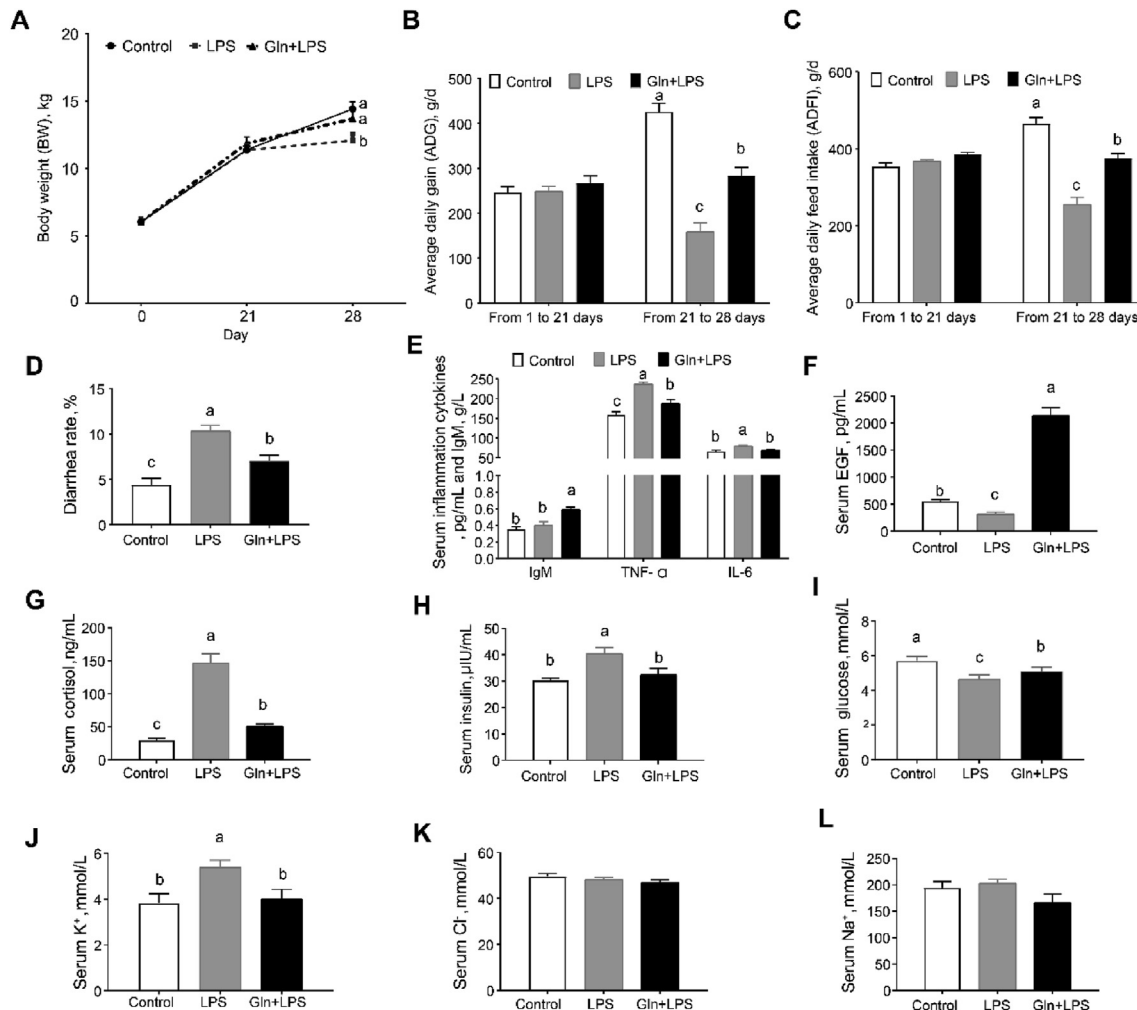
Cells were plated at a density of  $5 \times 10^5$  cells/well in a 6-well plate with 2 mL DMEM-H basal medium and were grown overnight. At approximately 70% to 80% confluence, Gln (0 or 4 mmol/L) was cultured in fresh basal medium without FBS, then cells were incubated for 18 h. To build the stress injury model, 20  $\mu$ g/mL LPS was added to the culture medium (containing 0 and 4 mmol/L Gln) for an exposure of 6 h. This dosage of LPS (20  $\mu$ g/mL) was found to achieve the greatest inhibitory effect on stress injury. Thereafter, the conditioned medium and cells were harvested for subsequent analysis. To inhibit AMPK activity, 15  $\mu$ mol/L of the AMPK inhibitor compound C (CAS 866405-64-3, Sigma-Aldrich, Shanghai, China) was added to the medium for 15 min, after which the cells were harvested.

### 2.4. Diarrhea index and intestinal morphology

After the LPS challenge, defecation events (e.g. stool color and shape) of piglets were observed and recorded. According to our observations and the criteria for the diagnosis of diarrhea, pig fecal samples were scored as previously described (He et al., 2017b), and then diarrhea index was calculated. Intestinal morphology was defined as previously described (He et al., 2017b). The values of villus height (VH), crypt depth (CD), mucosal thickness (MT), and villus height to crypt depth ratio (V:C) were derived and calculated using Image J software version 1.51.

### 2.5. Physiological and biochemical parameters and inflammatory cytokines

Serum glucose, chloride ( $\text{Cl}^-$ ), immunoglobulin M (IgM), potassium ( $\text{K}^+$ ) and sodium ( $\text{Na}^+$ ) were determined using spectrophotometric kits (Nanjing Jiangcheng Biotechnology Institute, Jiangsu, China) in accordance with the manufacturer's instructions (He et al., 2017b). The concentrations of epidermal growth factor (EGF), cortisol, insulin, interleukin-1 $\beta$  (IL-1 $\beta$ ), interleukin-6 (IL-6), and tumor necrosis factor (TNF- $\alpha$ ) were detected using ELISA kits



**Fig. 1.** The growth performance, diarrhea rate, and serum physiological and biochemical parameters in piglets. (A) Body weight, (B) average daily gain, (C) average daily feed intake, (D) diarrhea rate, (E) immunoglobulin M, interleukin-6 and tumor necrosis factor, (F) epidermal growth factor, (G) cortisol, (H) insulin, (I) glucose, (J) potassium, (K) chlorine, and (L) sodium. Values are presented as mean  $\pm$  SEM,  $n = 8$ . <sup>a, b, c</sup> means with different lowercase superscripts mean significant difference ( $P < 0.05$ ).

(Jiangsu Meimian industrial Co., Ltd., Jiangsu, China) according to the manufacturer's instructions.

## 2.6. Cell apoptosis

Cell apoptosis of IPEC-J2 cells was determined by the Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) flow cytometry kit. Briefly, the harvested cells were treated with apoptosis detection reagents (BD556547, USA) according to the manufacturer's instructions. The ratio of early to late apoptosis was quantified as a percentage of cells by flow cytometry (Beckman Coulter, USA).

## 2.7. Mitochondrial membrane potential and mitochondria number assay

Mitochondrial membrane potential was detected by double fluorescence staining with JC-1 (green fluorescent J-monomers or red fluorescent J-aggregates). Briefly, cells were seeded into confocal dishes and treated with different medium, and then JC-1 (10  $\mu$ g/mL, Invitrogen) was added into the medium for 30 min in the dark or MitoTracker probes (200 nmol/L, Invitrogen) was added into the medium for 30 min. Finally, cells were washed twice with

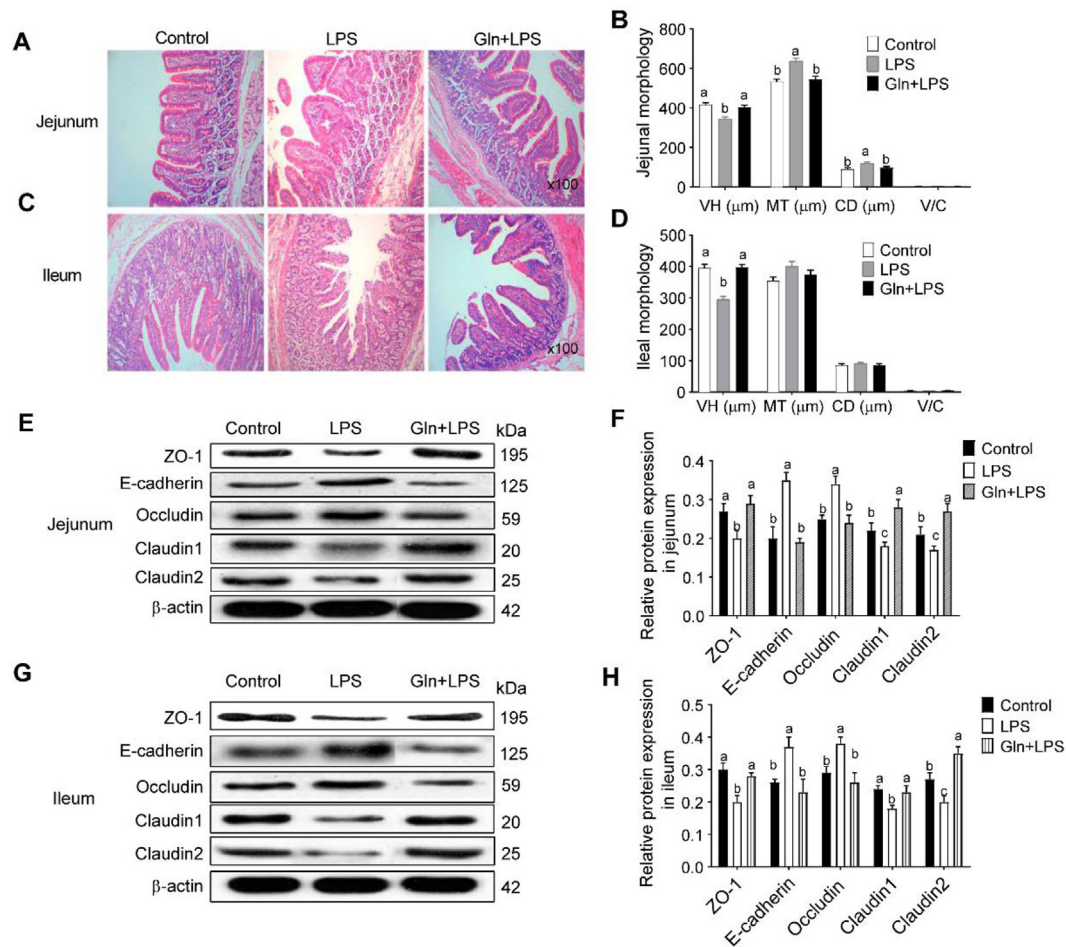
PBS and treated with an anti-fluorescence quenching agent and images were obtained with a Zeiss LSM880 confocal microscope.

## 2.8. Cell respiration metabolism

Cell mitochondrial respiration was detected using a Cell Mito Stress Test Kits (101706-100, Agilent Technologies, Inc., California, USA) in accordance with the manufacturer's instructions. IPEC-J2 cells were seeded at a density of  $3 \times 10^5$  cells/well in 24-well culture plates, incubated and treated with different treatments for 24 h. Then the cells were washed twice and culture media was used with an XF Assay medium containing 1.0 mmol/L sodium pyruvate, 4.5 g/L glucose, 4 mmol/L Gln (pH adjusting to 7.4 with 1 mol/L NaOH). Oxygen consumption rates (OCR) were performed using the Seahorse Bioscience XF-24 Extracellular Flux Analyzer (Agilent Technologies, Inc. USA) (He et al., 2020a). The maximum respiratory capacity (Max), spare respiratory capacity (SRC), non-mitochondrial respiratory capacity (Non-mito), ATP production (ATP), and mitochondrial proton leakage (PL) were calculated.

## 2.9. Quantitative real-time PCR

Total RNA was extracted from jejunum, ileum, and IPEC-J2 cells and was reverse-transcribed into cDNA using reverse transcriptase



**Fig. 2.** The effects of glutamine supplementation on intestinal morphology and tight junctions in the small intestine of weaned piglets. (A–B) Jejunal morphology, (C–D) ileal morphology, (E–F) protein expression level of jejunal tight junctions, and (G–H) protein expression level of ileal tight junctions. Values are presented as mean  $\pm$  SEM,  $n = 8$ . <sup>a, b, c</sup> Means with different lowercase superscripts mean significant difference ( $P < 0.05$ ).

and Prime Script RT reagent Kit (Takara, Tokyo, Japan) as previously described (He et al., 2018). Quantitative real-time PCR analysis was performed using the SYBR green method and the LightCycler 480II real-time PCR system. Primer sequences used in this study are showed in the previous reports (He et al., 2017b). The  $\beta$ -actin gene was used as the internal control.

## 2.10. Western blot analysis

Cytomembrane protein and total protein were extracted from jejunum, ileum, and IPEC-J2 cells, using a membrane and cytosol protein extraction kit (Beyotime) and RIPA Lysis Buffer (Beyotime) in accordance with the manufacturer's instructions. Cytomembrane protein was used to determine the relative protein levels of AQP3 (sc-20811, Santa Cruz, TX, USA), AQP4 (ab46182, Abcam, LON, UK), AQP8 (sc-14984, Santa Cruz), epithelial sodium channel alpha subunit ( $\alpha$ -ENaC, ab65710, Abcam), sodium-hydrogen exchanger 3 (NHE3, sc-28757, Santa Cruz),  $\text{Cl}^-/\text{HCO}_3^-$  exchanger (DRA/PAT1, sc-161150, Santa Cruz),  $\beta$ -ENaC (14134-1-AP, Proteintech, CA, USA), and  $\beta$ -ATPase  $\text{Na}^+/\text{K}^+$  antibody (ab254025, Abcam). Total protein was used to measure the relative protein levels of zonula occludens-1 (ZO-1, 21773-1-AP, Proteintech), occludin (ab31721, Abcam), E-cadherin (Proteintech), claudin-1 (#13995, Cell Signaling Technology, Boston, USA), claudin-2 (ab53032, Abcam),

AMPK $\alpha$ 1 (10929-2-AP, Proteintech), AMPK $\alpha$ 2 (18167-1-AP, Proteintech), PGC-1 $\alpha$  (ab106814, Abcam), target of rapamycin complex 2 (TORC2, 12497-1-AP, Proteintech), silent information regulator T1 (SIRT1, 13161-1-AP, Proteintech), and  $\beta$ -actin (60008-1-Ig, Proteintech). These proteins were detected by the Western blotting technique as previously reported (Tan et al., 2010).

## 2.11. Statistical analysis

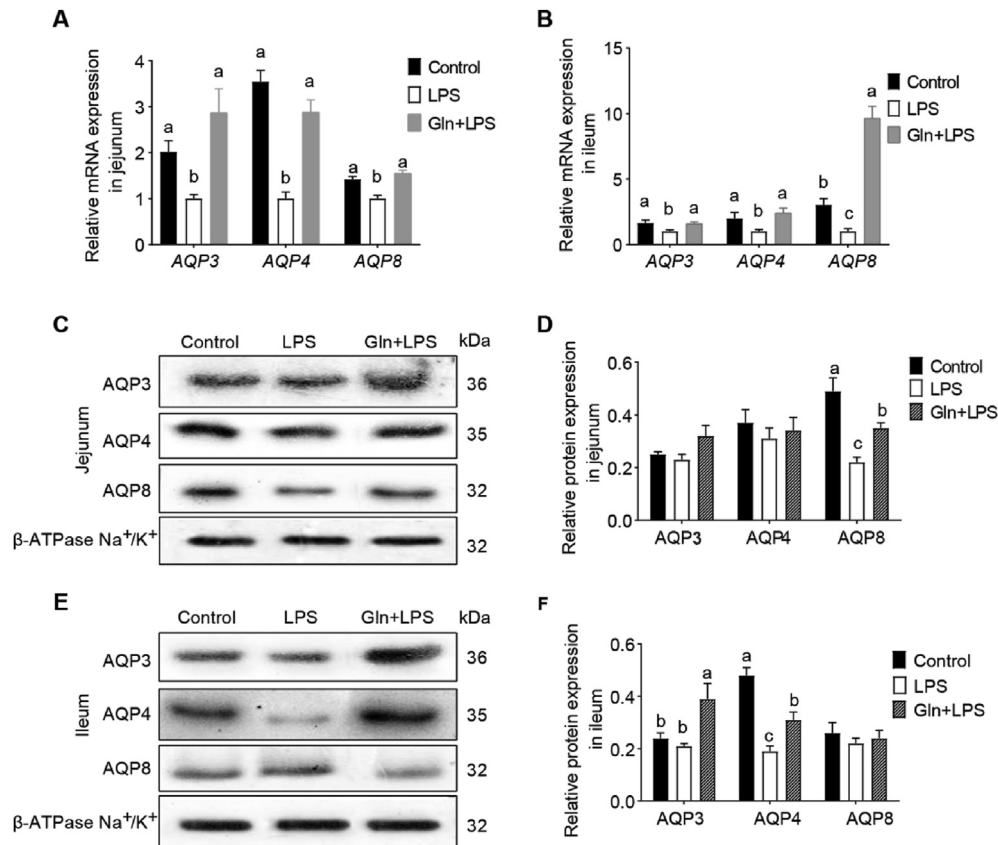
Statistical analysis was performed by one-way ANOVA using the SAS software version 9.2 package (SAS Institute Inc., Cary, NC, USA). All the in vivo and in vitro data are presented as means  $\pm$  standard error (SEM).  $P \leq 0.05$  indicated statistical significance.

## 3. Results

### 3.1. Growth performance, diarrhea index and serum parameters in piglets

Before LPS challenge, there were no significant differences in 21 d final BW, ADG and ADFI between piglets fed the Gln diet or basal diet (Fig. 1A–C). After LPS challenge, the final BW, ADG and ADFI at 28 d in the LPS group were decreased and were different from other groups. A remarkable increase in diarrhea index was





**Fig. 3.** The effects of glutamine administration on the gene and protein expression of aquaporins in the small intestine of piglets. (A) The mRNA expression of aquaporins in jejunum, (B) mRNA expression of aquaporins in ileum, (C–D) protein expression of aquaporins in jejunal epithelial membrane, and (E–F) protein expression of aquaporins in ileal epithelial membrane. Values are presented as mean  $\pm$  SEM,  $n = 8$ . <sup>a, b, c</sup> Means with different lowercase superscripts mean significant difference ( $P < 0.05$ ).

observed by LPS challenge. Compared with the LPS treatment, Gln + LPS treatment decreased ( $P = 0.002$ ) the diarrhea ratio, but the diarrhea rate was still greater than in the control ( $P = 0.047$ ) (Fig. 1D). After the LPS challenge, the serum IgM concentration was higher in the Gln + LPS group than in other groups ( $P = 0.021$ ) (Fig. 1E). Gln treatment decreased the contents of serum IL-6 ( $P = 0.035$ ) and TNF- $\alpha$  ( $P = 0.024$ ) in the LPS-induced piglets. Except for serum  $\text{Cl}^-$  and  $\text{Na}^+$ , the concentrations of serum cortisol, insulin and  $\text{K}^+$  were greater in the LPS group than in other treatments ( $P < 0.05$ ), but the concentration of serum EGF and glucose in the Gln + LPS group increased ( $P < 0.05$ ) compared to the LPS treatment (Fig. 1F–L).

### 3.2. Intestinal morphology and tight junction protein expression in weaned piglets

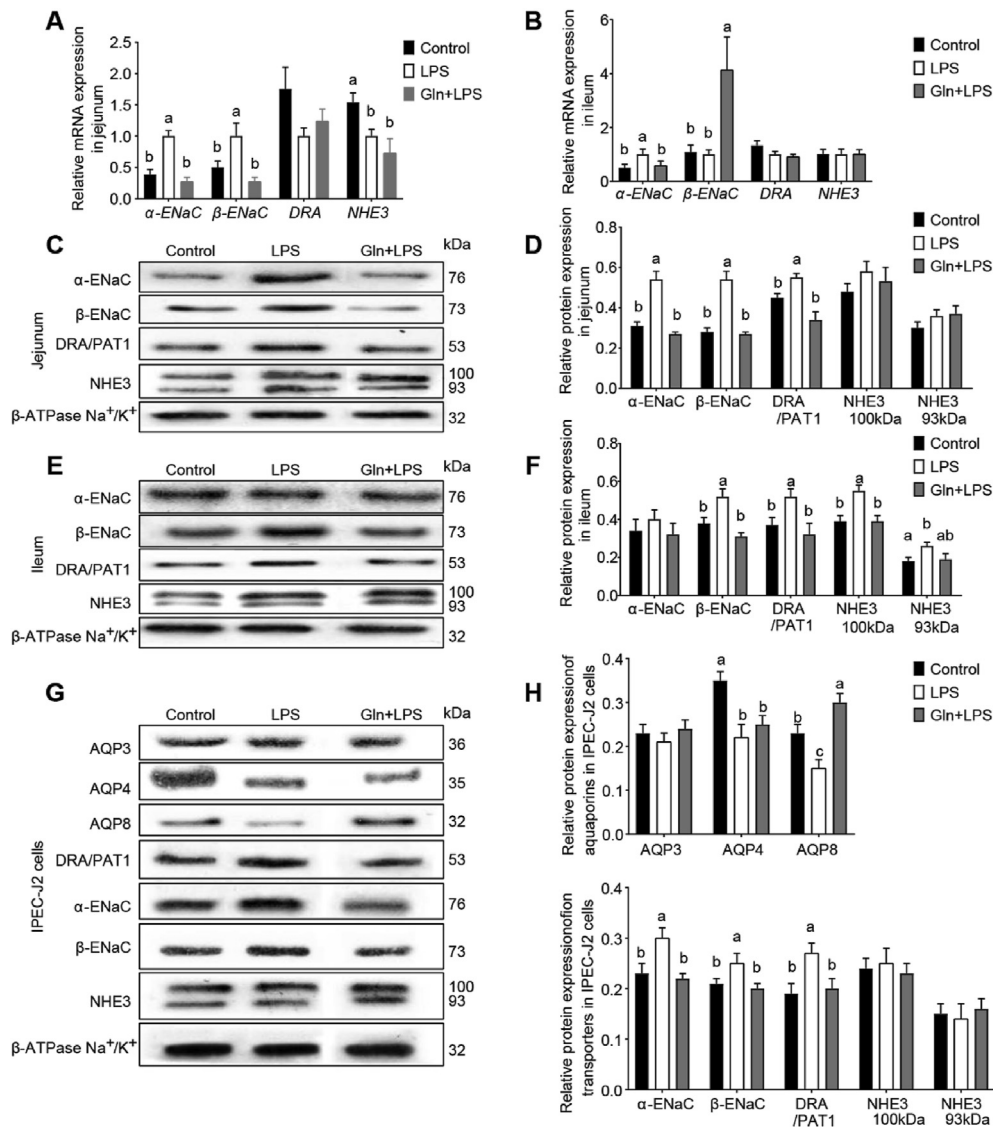
Compared with the LPS treatment, the VH in the Gln + LPS group was remarkably enhanced both in the jejunum and ileum (Fig. 2A–D) and the MT and CD values were decreased ( $P = 0.047$ ) in the jejunum. LPS treatment decreased expression levels of ZO-1 in jejunum and ileum ( $P = 0.035$ ), whereas Gln + LPS treatment was the same as the control treatment (Fig. 2E–H). In contrast, Gln + LPS treatment significantly increased the protein relative expression of claudin 1 and claudin 2 in the jejunum relative to the control ( $P < 0.05$ ), as well as claudin 2 expression in the ileum. For E-cadherin and Occludin, LPS treatment increased ( $P = 0.028$ ) their protein expression relative to the control and Gln + LPS treatments.

### 3.3. Gene and protein expression of aquaporins and ion transporters in piglets and IPEC-J2 cells

Compared to the control group, the mRNA abundance of AQP3, 4, and 8 in the jejunum and ileum in the LPS group were markedly decreased, whereas no differences in the mRNA abundance AQP3, 4, and 8 of small intestine (except for AQP8 in the ileum) were observed between the control and Gln + LPS treatments (Fig. 3A–B). In the jejunum, the protein expression of AQP3 and AQP4 were not altered by these treatments, whereas the AQP8 expression level was decreased ( $P = 0.016$ ) by both the Gln + LPS and LPS treatments (Fig. 3C–D). In the ileum, the Gln + LPS treatment did increase ( $P = 0.029$ ) the relative expression of AQP3, whereas LPS treatment did not affect the AQP3 expression level relative to the control. As for AQP4 expression in the ileum, Gln + LPS treatment was greater than LPS treatment ( $P = 0.029$ ) (Fig. 3E–F).

In the jejunum, the mRNA abundance of  $\alpha$ - and  $\beta$ -ENaC in the LPS treatment was greater ( $P < 0.05$ ) than the other groups, but NHE3 mRNA abundance in control group was increased ( $P < 0.05$ ) (Fig. 4A). In the ileum, the  $\alpha$ - and  $\beta$ -ENaC mRNA abundance in the LPS treatment was markedly different from the Gln + LPS treatment (Fig. 4B). Moreover, the LPS treatment enhanced ( $P < 0.05$ ) the protein expression of jejunal  $\alpha$ - and  $\beta$ -ENaC, DRA/PAT1, and ileal  $\beta$ -ENaC, DRA/PAT1, and NHE3 (100 kDa) relative to the control and Gln + LPS treatments (Fig. 4C–F).

In the LPS-induced IPEC-J2 cells stress injury model, the Gln + LPS treatment had no effect on the protein expression of



**Fig. 4.** The effects of glutamine administration on the gene and protein expression of ion transporters in the small intestine of piglets, as well as on the protein expression of aquaporins and ion transporters in IPEC-J2 cell membrane. (A) The mRNA expression of ion transporters in jejunum, (B) mRNA expression of ion transporters in ileum, (C–D) protein expression of ion transporters in jejunal epithelial membrane, (E–F) protein expression of ion transporters in ileal epithelial membrane, and (G–H) protein expression of aquaporins and ion transporters in IPEC-J2 cell membrane. Values are presented as mean  $\pm$  SEM,  $n = 8$  (piglets) or 6 (IPEC-J2). <sup>a, b, c</sup> Means with different lowercase superscripts mean significant difference ( $P < 0.05$ ).

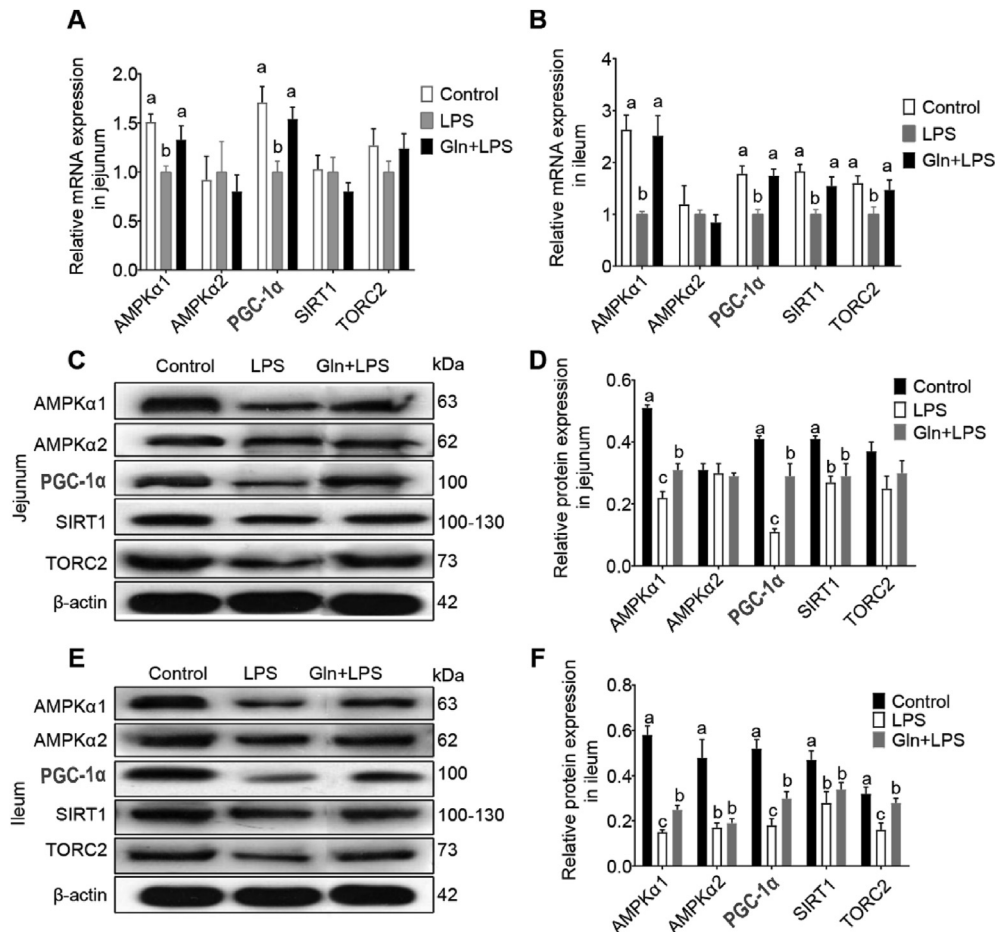
AQP3 and AQP4 comparing with LPS treatment ( $P > 0.05$ ), whereas increasing the AQP8 expression ( $P = 0.028$ ) (Fig. 4G–H). Except for NHE3, the protein expression of  $\alpha$ - and  $\beta$ -ENaC and DRA/PAT1 in LPS treatment was greater ( $P < 0.05$ ) than that in control and Gln + LPS treatments (Fig. 4G–H).

### 3.4. Gene and protein expression of AMPK pathway related key targets in piglets and IPEC-J2 cells

Compared with the control and Gln + LPS treatments, LPS treatment reduced ( $P < 0.05$ ) the mRNA abundance of *AMPK $\alpha$ 1* and *PGC-1 $\alpha$*  in the jejunum of piglets, as well as reduced ( $P < 0.05$ ) the mRNA abundance of ileal *AMPK $\alpha$ 1*, *PGC-1 $\alpha$* , *SIRT1*, and *TORC2* (Fig. 5A–B). Compared with the LPS treatment, the protein expression of jejunal *AMPK $\alpha$ 1*, *PGC-1 $\alpha$* , and ileal *AMPK $\alpha$ 1*, *PGC-1 $\alpha$* , and *TORC2* in the Gln + LPS treatment was increased ( $P < 0.05$ ) in piglets (Fig. 5C–F).

The IPEC-J2 cell experiments showed that the LPS treatment decreased the mRNA abundance of *AMPK $\alpha$ 1*, *PGC-1 $\alpha$* , *SIRT1*, and *TORC2* relative to the Gln + LPS treatments (Fig. 6A). The mRNA abundance of *SIRT1*, *PGC-1 $\alpha$* , and *TORC2* in the Gln + LPS treatment was lower than that in the control, whereas *AMPK $\alpha$ 1* mRNA abundance was highest ( $P = 0.007$ ) in the Gln + LPS treatment.

To further testify whether the activity of AMPK was activated by Gln, AMPK inhibitor compound C was used, the results showed that the activity of AMPK was decreased ( $P = 0.014$ ) by compound C under LPS challenge or normal conditions. Compared with the Gln treatment, LPS treatment decreased ( $P < 0.05$ ) the *AMPK $\alpha$ 1* and  $\alpha 2$  activity in IPEC-J2 cells, but the activity of *AMPK $\alpha$ 1* and  $\alpha 2$  in the Gln + LPS treatment was increased ( $P < 0.05$ ) compared to the LPS treatment (Fig. 6B–C). The mRNA expressions of *AMPK $\alpha$ 1*, *AMPK $\alpha$ 2*, and *PGC-1 $\alpha$*  in the Gln + LPS and Gln + LPS + compound C treatments were decreased ( $P < 0.05$ ) relative to the Gln treatment, whereas there was no discrepancy in the mRNA expression of



**Fig. 5.** Effects of glutamine administration on the expression of AMPK pathway related key targets in the small intestine of piglets. (A) The mRNA expression of AMPK pathway related key targets in jejunum, (B) mRNA expression of AMPK pathway related key targets in ileum, (C–D) protein expression of AMPK pathway related key targets in jejunum, and (E–F) protein expression of AMPK pathway related key targets in ileum. Values are presented as mean  $\pm$  SEM,  $n = 8$ . <sup>a, b, c</sup> Means with different lowercase superscripts mean significant difference ( $P < 0.05$ ).

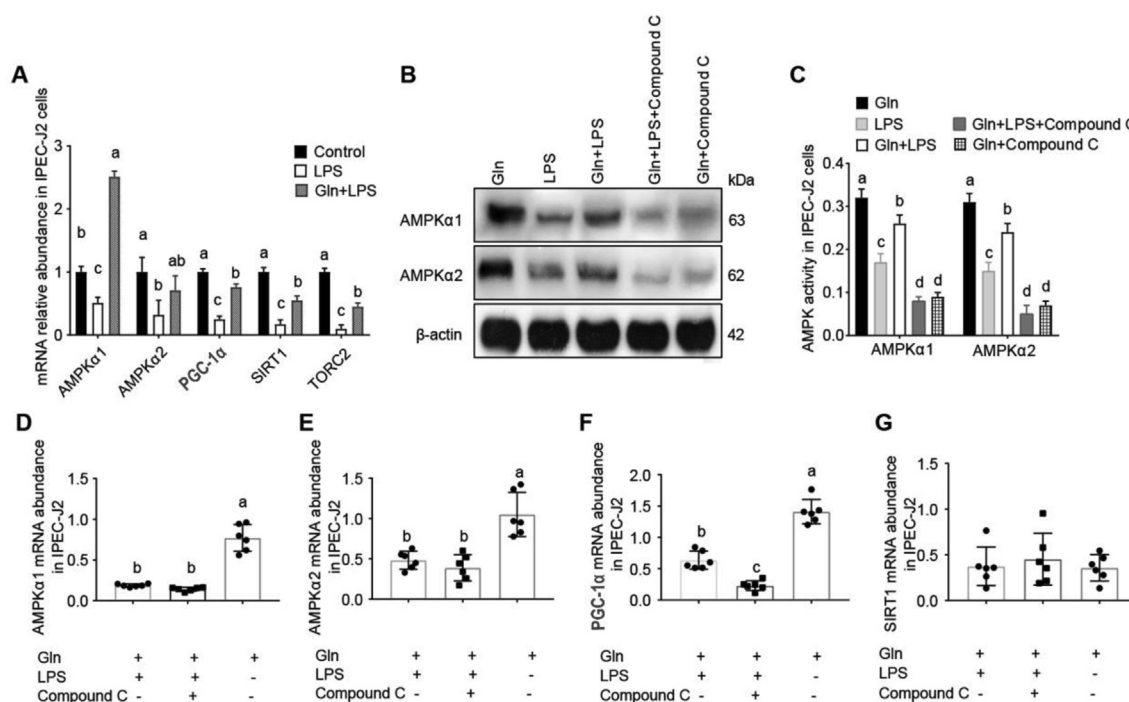
AMPKα1, AMPKα2, and SIRT1 between Gln + LPS and Gln + LPS + compound C treatments (Fig. 6D–G).

### 3.5. Cellular mitochondrial function, cytokines, and cell apoptosis in IPEC-J2 cells

Different dosages (0, 2, 4, and 8 mmol/L) of Gln had different effects on cellular mitochondrial respiration in IPEC-J2 cells (Fig. 7A). Notably, exposure to 4 mmol/L Gln markedly increased the mitochondria basal OCR, Max, and ATP comparing with other treatments (Fig. 7B). In the LPS-induced stress injury model, the Gln + LPS treatment increased ( $P < 0.05$ ) the basal OCR, Max, SRC, Non-mito, and ATP relative to the LPS treatment, whereas decreasing ( $P = 0.038$ ) PL (Fig. 7C–D). Moreover, the Gln + LPS treatment reduced ( $P < 0.05$ ) the concentrations of IL-1β, IL-6 and TNF-α in the culture medium relative to the LPS treatment (Fig. 7E). The LPS treatment increased ( $P < 0.05$ ) the ratio of both early and late apoptotic cells, as well as death cells relative to the Control, while the Gln + LPS treatment decreased ( $P < 0.05$ ) the ratio of apoptotic and death cells relative to the LPS treatment (Fig. 7F–G). Additionally, the LPS treatment decreased ( $P = 0.009$ ) the mitochondrial membrane potential (Fig. 7H–I) and the number of mitochondria relative to the Gln + LPS treatment (Fig. 7J–K), Gln administration decreased mitochondrial fragmentation.

## 4. Discussion

Recent reports have highlighted the role of increasing dietary Gln to replace antibiotics in the growth development and health of piglets (Duttlinger et al., 2020). However, there are no data concerning the regulatory mechanism of Gln on the transport and absorption of water and ions across the intestinal mucosa in piglets under stress injury conditions. Weaned piglets are vulnerable to stress damages because of an immature immune system, which can lead to diarrhea and growth retardation (Tang et al., 2020). Our results showed that Gln supplementation did not affect the growth performance of weaned piglets under normal conditions. After the LPS challenge, the values of BW, ADG and ADFI in piglets were markedly increased by Gln administration, indicating that Gln could alleviate LPS-induced stress injury in weaned piglets while it had no growth-improving impacts under normal conditions. Our results also showed that Gln administration decreased the ratio of diarrhea and increased the jejunal and ileal villus height in piglets. Our results are consistent with other reports (Duttlinger et al., 2020; McConn et al., 2020), confirming that Gln can maintain intestinal integrity against *E. coli* LPS infection, and that there may be a possible link between Gln and pig intestinal health (Chen et al., 2014; Dai et al., 2013). Furthermore, we found that the addition of Gln increased the concentrations of serum EGF and glucose, but



**Fig. 6.** Effects of glutamine on the gene and protein expression of AMPK pathway related key targets in IPEC-J2 cells. (A) The mRNA expression of AMPK pathway related key targets in LPS-induced IPEC-J2 cells, (B–C) protein expression of AMPKα1 and AMPKα2 under Gln or compound C treatment, (D) mRNA expression of AMPKα1, (E) mRNA expression of AMPKα2, (F) mRNA expression of peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC-1α), and (G) mRNA expression of silent information regulator T1 (SIRT1). Values are presented as mean ± SEM,  $n = 6$ . <sup>a, b, c, d</sup> Means with different lowercase superscripts mean significant difference ( $P < 0.05$ ).

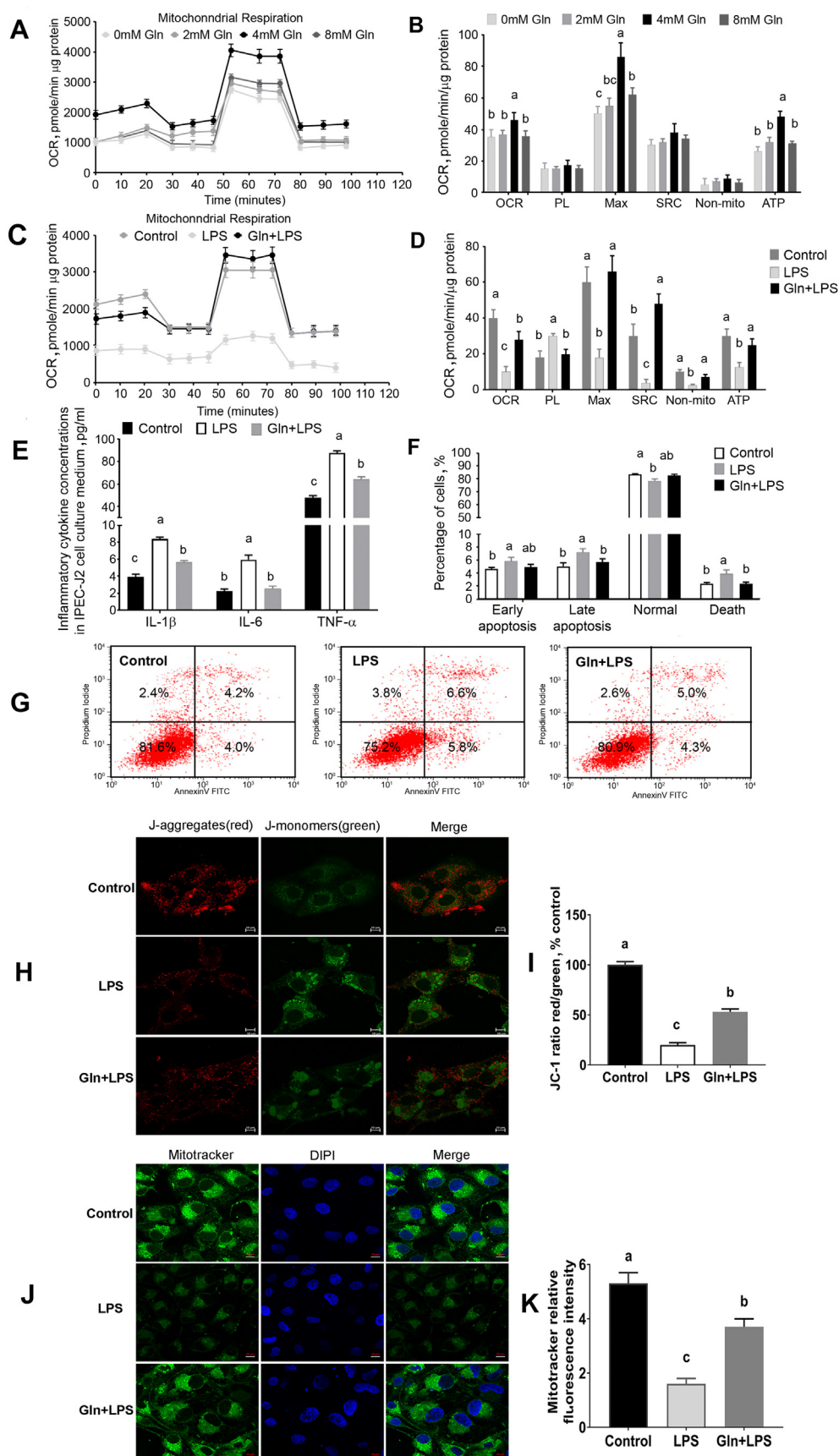
reduced cortisol and insulin levels under LPS challenge. These results suggest that LPS challenge caused metabolic disorders in piglets while Gln supplementation can regulate the secretion of hormones and ion balance in response to intestinal inflammation. This is consistent with previous studies that reported the activity of key stress hormones in piglets to change appreciably when intestinal absorption is impaired (Wang et al., 2019; Merlot et al., 2019). Gln administration made no difference in the levels of serum  $\text{Na}^+$  and  $\text{Cl}^-$  in the LPS-induced piglets, but decreased serum  $\text{K}^+$  level. It is evident that the high dosage of the  $\text{K}^+$  would lead to dysfunction of the digestive system, abdominal pain, nausea, and vomiting in piglets (He et al., 2017b; Sikder et al., 2018). The correlation between serum ion concentrations and mucosal barrier function implicates Gln as a potential protective nutrient protect the homeostasis of osmotic pressure against inflammation.

It is known that ions, water, and some macromolecules traverse tight junctions through either of the 2 major pathways (non-restrictive and restrictive pathways) (De Santis et al., 2015). The present results show that Gln administration enhanced the relative protein expression of jejunal and ileal ZO-1, claudin 1, and claudin 2 in the LPS-induced piglets whereas decreasing the expression of jejunal and ileal E-cadherin and Occludin. This may be associated with the redistribution of transport channel proteins regulated by Gln. It has been reported that Gln is not only a source of energy to maintain intestinal epithelial monolayer integrity, but also modulate the structure of the intestinal tract under the continuous interaction of the intestine with *E. coli* LPS (Robinson et al., 2015). Our results are consistent with recent studies demonstrating that Gln improves the regulation of nutritional transporter activity, tight junction permeability, and immune functions (De Santis et al., 2015). This is further confirmed by our results reported that the addition of Gln could increase the content of serum IgM and inhibited the secretion of cytokines (TNF-α, IL-6, IL-1β) in piglets

and IPEC-J2 cells. In addition, a significant reduction in the ratio of cell apoptosis and death was observed by Gln treatment.

Our results also demonstrated that Gln administration enhanced the mRNA abundance of jejunal and ileal AQP3, AQP4, and AQP8 in the LPS-induced weaned piglets, and altered the protein expression of jejunal AQP8 and ileal AQP3 and AQP4. One reason may be that Gln regulates enterocyte proliferation and affects tight junctions to preserve paracellular permeability, thereby upregulating the expression of AQP in the intestine. Some studies have reported that the expression of AQP (e.g. AQP3, AQP4, and AQP8) could be suppressed by acute injuries or chronic disease (Zou et al., 2019). These findings support our results that LPS treatment reduced the expression of AQP3, AQP4 and AQP8 in piglets. However, Gln administration decreased the protein expression of jejunal α/β ENaC, DAR/PAT1, and ileal β-ENaC, DAR/PAT1, and NHE3 in the LPS-challenged piglets, indicating the increased flow of water and ions both into and out of enterocytes under Gln treatment. It has been reported that α subunit of ENaC with the β subunit forms channels with distinct single channel kinetics and ion selectivity (Zhong et al., 2014). These findings are consistent with our in vitro experiments and suggest that exogenous Gln can help rebuild the structure of AQP and ion transporters and transport  $\text{Na}^+$  and  $\text{K}^+$  or stabilize the subunit in the cell membrane against stress injury. Further, the variation in the expression of the ion transporters (particularly DAR/PAT1 and NHE3) in the jejunum and ileum of piglets most likely represents an adaption to the dietary availability of Gln. Under LPS challenge conditions, these ion transporters may mediate Gln uptake across the brush-border membrane with the involvement of amino acid transporters or some AQP (He et al., 2017b). The presence of Gln could thus be of physiological relevance, as Gln may be a catalytic substrate that accelerates the rate of both forward and backward conversion of water and ions across cell membranes (Kim and Kim





**Fig. 7.** Mitochondrial respiration metabolism, cytokines, apoptosis, mitochondrial membrane potential and mitochondrial number in IPEC-J2 cells. (A) Oxygen consumption rates (OCR) in IPEC-J2 cells treated with different levels of glutamine; (B) the values of mitochondrial respiration parameters in IPEC-J2 cells treated with different levels of glutamine; (C)

OCR in IPEC-J2 cells treated with either 0 or 4 mmol/L glutamine and either 0 or 20 µg/mL LPS; (D) the values of mitochondrial respiration parameters in IPEC-J2 cells treated either 0 or 4 mmol/L glutamine and either 0 or 20 µg/mL LPS; (E) interleukin-1β, interleukin-6 and tumor necrosis factor in IPEC-J2 cell medium culture, (F–G) cell apoptosis; (H–I) mitochondrial membrane potential determined by JC dye staining (red, aggregate; green, monomer); and (J–K) mitochondrial number and fragmentation determined by MitoTracker probes. Values are presented as mean ± SEM, *n* = 6. <sup>a, b, c</sup> Means with different lowercase superscripts mean significant difference (*P* < 0.05).

2017). This could lead to locally enhanced expression of AQP but reduced expression of ion transporters in enterocytes.

Gln metabolism can speed up the production of ATP to support the transport of water and ions (Teuwen et al., 2019). This study showed that Gln administration markedly regulated the abundance of AMPKα1, peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC-1α), SIRT1, and TORC2 in the LPS-induced piglets and IPEC-J2 cells. A possible explanation is that Gln is similarly to α-ketoglutarate and serves as a major metabolic fuel to maintain enterocyte metabolism or activate the AMPK pathway to maintain intestinal osmotic homeostasis (He et al., 2017b). It is evident that the activation of AMPK plays a vital role in modulating glucose, mitochondrial biogenesis, inflammation, and apoptosis (He et al., 2017a). PGC-1α and SIRT1 are a central regulator of mitochondrial biogenesis, which promote the transcription and replication of the mitochondrial genome to maintain intestinal homeostasis (Rath et al., 2018). Confirmation was further provided by the in vitro observation that the addition of different levels of Gln markedly improved mitochondrial respiration metabolism. Exposure to 4 mmol/L Gln increased the basal OCR and ATP production in LPS-induced IPEC-J2 cells, suggesting that Gln could promote cellular mitochondrial function to alleviate inflammation. Further, Gln administration increased the mitochondrial membrane potential and the number of mitochondria, indicating that Gln could maintain the balance of mitochondrial metabolism and reduce mitochondrial fragmentation. It is evident that decreased mitochondrial membrane potential promotes mitochondrial membrane permeability transition pore opening and activates caspase-mediated apoptosis signaling pathway. This was consistent with our findings as demonstrated that LPS challenge could significantly increase cell apoptosis. Mechanically, most (90%) of the supplemented Gln can be metabolized in the intestine, with only a small amount entering the mitochondria. None other than this small part can play an important role in promoting the activation of AMPK in enterocyte (Ren et al. 2016, 2019). Interestingly, our in vivo results were consistent with our in vitro findings, showing that the Gln administration could activate AMPK activity under LPS challenge or normal conditions. It is conceivable that LPS challenge disrupts the original structure and function of intestinal AQP and ion transporters, with the result that the activation of AMPK by Gln is promptly mobilized for cellular metabolism including mitochondrial respiratory metabolism, movement of water and ions, and hormone secretion, to maintain the distribution of AQP and ion transporters and then alleviate intestinal inflammation. Our cellular mitochondrial respiration results also confirmed that with the supply of Gln, the mitochondria have sufficient energy reserve to handle acute stress injury, thereby maintaining osmotic pressure homeostasis and structural functions of water and ion channels. Thus, the AMPK pathway-mediated regulation of water and ion channels may be a promising focal point to deduce the mechanisms through which Gln improves intestinal mitochondrial function in inflammatory conditions.

## 5. Conclusion

While our present study focuses on the role of Gln in piglet health, it does raise the question of whether Gln is an intermediary link between the involvement of intestinal mitochondrial function and regulatory pathway. Our results demonstrate a possible mechanism by which Gln could alleviate intestinal inflammation,

potentially via the involvement of intestinal water and ion channels and the activation of the AMPK pathway. Gln supplementation promoted mitochondrial function and cell proliferation and inhibited the secretion of inflammatory cytokines to improve intestinal health. While future work is required to determine the exact role of Gln in mitochondrial metabolism and specific water and ion channels, these studies provide an interesting direction for future work exploring the relationship between water and ion homeostasis, intestinal stress injury, and their associated pathologies.

## Author contributions

**Liuqin He:** Investigation, Visualization, Writing – original draft. **Xihong Zhou:** Data curation, Review & editing. **Ziping Wu:** Investigation. **Yanzhong Feng:** Investigation. **Di Liu:** Investigation. **Tiejun Li:** Investigation, Review & editing. **Yulong Yin:** Supervision, 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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## Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.aninu.2022.03.001>.

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