Asparagine attenuates intestinal injury, improves energy status and inhibits AMP-activated protein kinase signalling pathways in weaned piglets challenged with *Escherichia coli* lipopolysaccharide

Xiuying Wang, Yulan Liu*, Shuang Li, Dingan Pi, Huiling Zhu, Yongqing Hou, Haifeng Shi and Weibo Leng

*Hubei Key Laboratory of Animal Nutrition and Feed Science, Hubei Collaborative Innovation Center for Animal Nutrition and Feed Safety, Wuhan Polytechnic University, Wuhan 430023, People’s Republic of China*

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**Abstract**

The intestine requires a high amount of energy to maintain its health and function; thus, energy deficits in intestinal mucosa may lead to intestinal damage. Asparagine (Asn) is a precursor for many other amino acids such as aspartate, glutamine and glutamate, which can be used to supply energy to enterocytes. In the present study, we hypothesised that dietary supplementation of Asn could alleviate bacterial lipopolysaccharide (LPS)-induced intestinal injury via improvement of intestinal energy status. A total of twenty-four weaned piglets were assigned to one of four treatments: (1) non-challenged control; (2) LPS + 0% Asn; (3) LPS + 0.5% Asn; (4) LPS + 1.0% Asn. On day 19, piglets were injected with LPS or saline. At 24 h post-injection, piglets were slaughtered and intestinal samples were collected. Asn supplementation improved intestinal morphology, indicated by higher villus height and villus height:crypt depth ratio, and lower crypt depth. Asn supplementation also increased the ratios of RNA:DNA and protein:DNA as well as disaccharidase activities in intestinal mucosa. In addition, Asn supplementation attenuated bacterial LPS-induced intestinal energy deficits, indicated by increased ATP and adenylate energy charge levels, and decreased AMP:ATP ratio. Moreover, Asn administration increased the activities of key enzymes involved in the tricarboxylic acid cycle, including citrate synthase, isocitrate dehydrogenase and α-ketoglutarate dehydrogenase complex. Finally, Asn administration decreased the mRNA abundance of intestinal AMP-activated protein kinase-α1 (AMPKα1), AMPKα2, silent information regulator 1 (SIRT1) and PPARγ coactivator-1α (PGC1α), and reduced intestinal AMPKα phosphorylation. Collectively, these results indicate that Asn supplementation alleviates bacterial LPS-induced intestinal injury by modulating the AMPK signalling pathway and improving energy status.

**Key words:** Asparagine; Intestine; Energy status; Weaned piglets; Lipopolysaccharide

The intestine is not only important for digestion and absorption of dietary nutrients, but also plays a key role in defence against harmful bacteria-derived endogenous and exogenous agents. Many factors such as infection and inflammation can cause intestinal damage and dysfunction. Recently, research has shown that the intestine requires a high amount of energy to maintain its health and function; thus, energy deficits in intestinal mucosa are closely related to various degrees of injury in the intestine. Amino acids can serve as a central fuel source for intestinal mucosa. Accumulating evidence suggests that amino acids play a critical role in intestinal integrity, function and health in animals and humans.

Thus, nutritional regulation (especially dietary addition of amino acids) targeting intestinal energy metabolism may hold great promise for intestinal disease prevention and improvement of animal and human health.

Asparagine (Asn) is a non-essential amino acid. As has been stated in a recent review, Asn, aspartate, glutamine, glutamate, arginine, citrulline, ornithine and proline are interconvertible via complex interorgan metabolism in most mammals. Asn can be converted to aspartate via deamination, and glutamate can subsequently be generated from α-ketoglutarate and aspartate by a transamination reaction. Emerging evidence has demonstrated that glutamine, aspartate and glutamate...
are the major sources of ATP in mammalian enterocytes\(^{(5)}\). In addition, Asn contributes to mounting an effective immune response in normal subjects, but can also contribute to abnormal lymphoblastic growth in leukaemia patients\(^{(9)}\). Newburg \textit{et al.}\(^{(10)}\) reported that deletion of Asn from diet formulations led to significant growth deficits in weanling rats. Moreover, Asn stimulates ornithine decarboxylase and cell proliferation in all kinds of cells, including intestinal cells\(^{(11,12)}\). However, to our knowledge, only a few studies have focused on the protective effect of Asn in the intestine.

Recent studies have shown that AMP-activated protein kinase (AMPK), silent information regulator 1 (SIRT1) and PPAR\(\gamma\) coactivator-1\(\alpha\) (PGC1\(\alpha\)) play key roles in the regulation of cellular energy metabolism\(^{(12,13)}\). AMPK is a heterotrimeric serine/threonine kinase. AMPK activation can restore energy status in human cells with mitochondrial dysfunction\(^{(14,15)}\). Several lines of evidence indicate that AMPK can increase SIRT1 activity by increasing cellular NAD\(^+\) levels, culminating in the modulation of downstream target activity\(^{(16)}\). In addition, AMPK and SIRT1 have been found to have a direct impact on the activity of PGC-1\(\alpha\) via phosphorylation and deacetylation, respectively\(^{(12)}\). These processes can initiate catabolic pathways including fatty acid oxidation and glycolysis to produce ATP, while synchronously inhibiting anabolic processes including fatty acid synthesis and gluconeogenesis to utilise energy\(^{(17)}\).

Accordingly, we hypothesise that Asn could improve intestinal integrity by regulating energy status through the modulation of the AMPK signalling pathway. In the present study, we established an acute model of intestinal injury by injecting \textit{Escherichia coli} lipopolysaccharide (LPS)\(^{(3,18)}\). Furthermore, we used a piglet model, which is an excellent animal model for studying the potential nutritional role of Asn in humans\(^{(19,20)}\). The aim of the present study was to investigate whether Asn could attenuate negative changes caused by LPS challenge in the intestine, and to elaborate its molecular mechanisms.

Materials and methods

\textit{Animal care and experimental design}

All the experimental procedures were approved by the Animal Care and Use Committee of Hubei Province, China. A total of twenty-four weaned, castrated barrows (Duroc \times Large White \times Landrace, 35 (\textit{SEM} 1) d old, 8.9 (\textit{SEM} 0.1) kg initial body weight (BW)) were randomly divided into four treatment groups (six replicate pens per treatment). Piglets were individually caged in a 1.80 $\times$ 1.10 m pen equipped with a feeder and a nipple drinker to allow \textit{ad libitum} access to feed and water. All piglets were housed in an environmentally controlled room. The basal diet (Table 1) was formulated to meet NRC\(^{(21)}\) requirements for all nutrients.

The four treatment groups were as follows: (1) non-challenged control (CONTR) group (piglets fed a control diet and injected with 0.9\% NaCl solution); (2) LPS + 0\% Asn treatment group (piglets fed the same control diet and injected with \textit{E. coli} LPS (\textit{Escherichia coli} serotype 055: B5); Sigma Chemical, Inc.); (3) LPS + 0.5\% Asn treatment group (piglets fed a 0.5\% Asn diet and injected with LPS); (4) LPS + 1\% Asn treatment group (piglets fed a 1.0\% Asn diet and injected with LPS). The Asn doses (purity >99\%; Amino Acid Biochemical Company Limited) were selected on the basis of our previous studies\(^{(22)}\). Our previous investigations showed that before the administration of LPS challenge, dietary supplementation of 0.5 and 1.0\% Asn did not affect growth performance, total and differential leucocyte counts, and serum biochemical parameters of weanling pigs (X Wang, Y Liu, S Li, D Pi, H Zhu, Y Hou, H Shi and W Leng, unpublished results; see online Supplementary Tables S1–S3), indicating that the Asn level of the basal diet was adequate to maintain growth performance and physiological function in weanling pigs under normal physiological conditions. However, our previous studies also showed that after the administration of LPS challenge, dietary supplementation of 0.5\% Asn attenuated weight loss, and both 0.5 and 1.0\% Asn supplementation

\begin{table}[h]
\centering
\small
\begin{tabular}{l|c|c}
\hline
\textbf{Ingredients} & \textbf{Content (g/kg)} \\
\hline
Maize & 570.5 \\
Soyabea meal (44\% CP) & 224 \\
Wheat middling & 73 \\
Fishmeal & 36 \\
Soya protein concentrate & 14 \\
Fat powder* & 20 \\
Milk replacer powder & 30 \\
Limestone & 9.4 \\
Dicalcium phosphate & 12.2 \\
Salt & 3.4 \\
Ala† & 13.5 \\
Acidifier‡ & 2.0 \\
\textit{L}-Lys HCl (78.8 \% Lys) & 2.7 \\
L-Met (99 \% Met) & 1.0 \\
L-Thr (98 \% Thr) & 0.8 \\
Butylated hydroquinone & 0.5 \\
Vitamin and mineral premix§ & 10 \\
\hline
\textbf{Nutrient composition} & \\
\hline
Digestible energy|♀| (MJ/kg) & 13.6 \\
Crude protein** & 203 \\
Crude fat** & 44 \\
Ca** & 8.0 \\
Total P|♀| & 7.0 \\
Total Lys|♀| & 11.1 \\
Total Met+L-Cys|♀| & 6.5 \\
\hline
\end{tabular}
\caption{Ingredients and composition of the experimental diets (as-fed basis)}
\end{table}

\begin{flushleft}
\textit{*} Rumen-stable fat powder, purchased from Berg + Schmidt. \\
\textit{†} In the 0.5\% asparagine diet, 1.35\% alanine was replaced by 0.5\% asparagine, 0.68\% alanine and 0.17\% maize starch. In the 1.0\% asparagine diet, 1.35\% alanine was replaced by 1.0\% asparagine and 0.35\% maize starch. All diets were isonitrogenous. \\
\textit{‡} A compound acidifier including lactic acid and phosphoric acid, provided by Wuhan Fanhua Biotechnology Company. \\
\textit{|♀|} Vitamin and mineral premix (defatted rice bran as the carrier) provided the following amounts per kg of complete diet: retinol acetate, 2700 $\mu$g; cholecalciferol, 62.5 $\mu$g; DL-\textit{w}-tocopheryl acetate, 20 mg; menadione, 3 mg; vitamin B\textsubscript{2}, 18 mg; riboflavin, 4 mg; niacin, 40 mg; pantothenic acid, 15 mg; choline chloride, 400 mg; folic acid, 700 $\mu$g; thiamin, 1.5 mg; pyridoxine, 3 mg; biotin, 100 $\mu$g; Zn, 80 mg (ZnSO\textsubscript{4} $\cdot$ 7H\textsubscript{2}O); Mn, 20 mg (MnSO\textsubscript{4} $\cdot$ 5H\textsubscript{2}O); Fe, 83 mg (FeSO\textsubscript{4} $\cdot$ H\textsubscript{2}O); Cu, 25 mg (CuSO\textsubscript{4} $\cdot$ 5H\textsubscript{2}O), I 0.048 mg (KI); Se, 0.36 mg (Na\textsubscript{2}SeO\textsubscript{3} $\cdot$ 5H\textsubscript{2}O). \\
\textit{|♀|} Based on diets containing maize starch. \\
\textit{‡} Calculated. \\
\textit{§} Analysed.
\end{flushleft}
attenuated the changes in total and differential leucocyte counts and serum biochemical parameters induced by LPS challenge in weanling pigs\(^{(22)}\), indicating the importance of exogenous Asn supply under pathological conditions. Thus, in the present experiment, we focused our investigation upon the effect of 0·5 and 1·0 % dietary Asn supplementation on intestinal variables in LPS-challenged piglets, but did not investigate the effect of Asn in non-LPS-challenged piglets. To obtain isonitrogenous diets, we added 1·35, 0·68 and 0 % alanine (purity > 99 %; Amino Acid Bio-Chemical Company Limited) to the control, 0·5 % Asn and 1·0 % Asn diets, respectively. Feed consumption and BW were recorded on day 1 and day 19 before the administration of saline or LPS injection. After 19 d of feeding the control, 0·5 % Asn and 1·0 % Asn diets, the challenged groups were treated with the same volume of 0·9 % NaCl solution. The LPS dose was chosen in accordance with our previous experiments\(^{(3,18)}\), in which this dose caused no significant difference in feed intake (266, 258, 270 g/kg BW, and the non-challenged group was treated with the same volume of 0·9 % NaCl solution. The LPS dose was chosen in accordance with our previous experiments\(^{(3,18)}\), in which this dose caused acute intestinal injury in weaned pigs. To avoid the potential effects of LPS-induced feed intake reduction on intestinal variables, all piglets were fed the same amount of feed per kg BW at 24 h following the administration of saline or LPS injection, causing no significant difference in feed intake (266, 258, 270, and 276 g, respectively) among the four treatment groups. According to the feed intake of LPS-challenged piglets, the amount of feed per kg BW was determined at 24 h after LPS challenge in our preliminary study. Piglets were supplied water ad libitum.

### Table 2. Specific primer sequences used for real-time PCR

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward (5′–3′)</th>
<th>Reverse (5′–3′)</th>
<th>Efficiency (%)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMPKα1</td>
<td>AAATCGGCGCCACTACATCCTG</td>
<td>GGATGCCCTGAAAAAGTTTAGAG</td>
<td>97</td>
<td>Oliver &amp; Miles(^{(30)})</td>
</tr>
<tr>
<td>AMPKα2</td>
<td>AACATTGCAAGGGTTGAAAG</td>
<td>CGCAGAAACTCACCCTCTGGA</td>
<td>99</td>
<td>Oliver &amp; Miles(^{(30)})</td>
</tr>
<tr>
<td>SIRT1</td>
<td>CTGGAACAGTGGTAGGAGGAG</td>
<td>CCTAGGACATCGAGGAACCA</td>
<td>101</td>
<td>Weber et al(^{(31)})</td>
</tr>
<tr>
<td>PGC1α</td>
<td>GATGTGTCGCTCCTGTCTGTTC</td>
<td>CATCCTTGGGCTGTTTAGAG</td>
<td>99</td>
<td>Weber et al(^{(31)})</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CGTGCCTGAGACCCAGATGGAG</td>
<td>GCTTGGACTGGCCTGGGAAGAT</td>
<td>100</td>
<td>Liu et al(^{(10)})</td>
</tr>
</tbody>
</table>

To obtain isonitrogenous diets, we added 1·35, 0·68 and 0 % alanine (purity > 99 %; Amino Acid Bio-Chemical Company Limited) to the control, 0·5 % Asn and 1·0 % Asn diets, respectively. Feed consumption and BW were recorded on day 1 and day 19 before the administration of saline or LPS injection. After 19 d of feeding the control, 0·5 % Asn and 1·0 % Asn diets, the challenged groups were treated with the same volume of 0·9 % NaCl solution. The LPS dose was chosen in accordance with our previous experiments\(^{(3,18)}\), in which this dose caused acute intestinal injury in weaned pigs. To avoid the potential effects of LPS-induced feed intake reduction on intestinal variables, all piglets were fed the same amount of feed per kg BW at 24 h following the administration of saline or LPS injection, causing no significant difference in feed intake (266, 258, 270, and 276 g, respectively) among the four treatment groups. According to the feed intake of LPS-challenged piglets, the amount of feed per kg BW was determined at 24 h after LPS challenge in our preliminary study. Piglets were supplied water ad libitum.

### Table 3. Effects of asparagine (Asn) supplementation on serum amino acid concentrations in weaned piglets at 24 h after the administration of *Escherichia coli* lipopolysaccharide (LPS) challenge

<table>
<thead>
<tr>
<th>Serum Amino Acid Concentrations</th>
<th>Mean Values with their Pooled Standard Errors, n=6 (One Piglet per Pen)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Treatment</td>
</tr>
<tr>
<td></td>
<td>Test</td>
</tr>
<tr>
<td></td>
<td>P</td>
</tr>
<tr>
<td>Asp (μmol/l)</td>
<td>64</td>
</tr>
<tr>
<td>Asn (μmol/l)</td>
<td>82</td>
</tr>
<tr>
<td>Glu (μmol/l)</td>
<td>249</td>
</tr>
<tr>
<td>Gln (μmol/l)</td>
<td>608</td>
</tr>
<tr>
<td>Arg (μmol/l)</td>
<td>189</td>
</tr>
<tr>
<td>Cit (μmol/l)</td>
<td>84</td>
</tr>
<tr>
<td>Orn (μmol/l)</td>
<td>97</td>
</tr>
</tbody>
</table>

\(^1\)CONTR, non-challenged control group (piglets fed a control diet and injected with 0·9 % NaCl solution); Cit, citrulline; Orn, ornithine.

\(^2\)LPS-challenged (0 % Asn) piglets were compared with CONTR piglets to determine the effects of LPS challenge. Linear and quadratic polynomial contrasts were used to determine the response to Asn supplementation among the LPS-challenged piglets.

**Blood and intestinal sample collection**

At 24 h post-injection, blood samples were collected into uncoated vacuum tubes (Becton Dickinson Vacutainer System) and centrifuged (3500 g, 10 min, 4°C) to obtain serum samples. Serum was stored at −80 °C until analysis. After the collection of blood samples, piglets were humanely euthanised with pentobarbital, and sections were cut at the mid-jejunum (3 cm and 10 cm) and mid-ileum (3 cm and 10 cm), respectively\(^{(3)}\). The 3 cm sections were flushed, and then placed in 10 % neutral buffered formalin for the analysis of intestinal morphology\(^{(3)}\). The 10 cm sections were opened and the contents were flushed\(^{(3)}\). Then, mucosal samples were collected with a sterile glass slide, and immediately frozen in liquid N\(_2\) and stored at −80 °C for further analysis\(^{(3)}\). Previous experiments have shown that at 24 h post-injection, LPS induced changes in intestinal energy metabolism and intestinal damage\(^{(24)}\). Thus, the time point of 24 h after the administration of LPS or 0·9 % NaCl solution was selected for the experimental measurements.

**Serum amino acid concentrations**

A volume of 50 μl serum was deproteinised in 50 μl of 1·5 M-perchloric acid. After 2 min, the samples were neutralised with 25 μl of 2 M-potassium carbonate and 1·125 ml double-distilled water. Then, the samples were centrifuged at 10 000 g for 1 min, and the supernatant was used for amino acid analysis. Serum concentrations of Asn and associated amino acids were measured by HPLC methods involving pre-column derivatisation with o-phthalaldehyde, as described previously\(^{(24)}\).

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**Table 2. Specific primer sequences used for real-time PCR**

- **AMPKα1**: AAATCGGCGCCACTACATCCTG
- **AMPKα2**: AACATTGCAAGGGTTGAAAG
- **SIRT1**: CTGGAACAGTGGTAGGAG
- **PGC1α**: GATGTGTCGCTCCTGTCTGTTC
- **GAPDH**: CGTGCCTGAGACCCAGATGGAG

**References**

- Oliver & Miles\(^{(30)}\)
- Weber et al\(^{(31)}\)
- Liu et al\(^{(10)}\)

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<tbody>
<tr>
<td></td>
<td>Treatment</td>
</tr>
<tr>
<td></td>
<td>Test</td>
</tr>
<tr>
<td></td>
<td>P</td>
</tr>
</tbody>
</table>

\(^1\)CONTR, non-challenged control group (piglets fed a control diet and injected with 0·9 % NaCl solution); Cit, citrulline; Orn, ornithine.

\(^2\)LPS-challenged (0 % Asn) piglets were compared with CONTR piglets to determine the effects of LPS challenge. Linear and quadratic polynomial contrasts were used to determine the response to Asn supplementation among the LPS-challenged piglets.
Intestinal morphology

After fixation for 24 h, intestinal samples were dehydrated, embedded in paraffin, sectioned, and stained with haematoxylin and eosin (H&E). Villus height and crypt depth were measured according to the methods described in our previous study.

Intestinal mucosal protein, DNA and RNA contents

Frozen mucosal samples were homogenised in ice-cold NaCl solution at a 1:10 (w/v) ratio, followed by centrifugation at 2500 rpm for 10 min at 4°C to collect the supernatant. The supernatant was used for the measurement of protein, RNA and DNA contents. Intestinal mucosal protein content was measured according to the method of Lowry et al. DNA content was measured by a fluorometric assay. RNA content was measured by spectrophotometry with a modified Schmidt–Tannhauser method.

Intestinal mucosal disaccharidase activities

Disaccharidase activities in the supernatant of intestinal mucosa were determined according to the methods described by Liu et al. using glucose kits (A082-1 for lactase, A082-2 for sucrase and A082-3 for maltase; Nanjing fiancheng Bioengineering Institute). In brief, 10 μl double-distilled water, glucose standard solution (5.55 mmol/l) or test samples were added to a test-tube and incubated with 20 μl of respective substrate for 20 min at 37°C. Then, 10 μl of terminating agent and 1000 μl of chromogenic agent were added and incubated at 37°C for 15 min. Double-distilled water was used to set zero at 505 nm, followed by the reading of the optical density value of each tube. One unit (U) of enzyme activity was defined as 1 nmol substrate hydrolysed/min under assay conditions (37°C, pH 6.0).

Intestinal mucosal ATP, ADP and AMP concentrations

Frozen intestinal samples (0.10–0.20 g) were homogenised in 2 ml of pre-cooled 1.5 M-perchloric acid. The homogenates were centrifuged at 3000 g for 10 min at 4°C, and the supernatants were collected. A volume of 1 ml supernatant was neutralised with 0.4 ml of 2 M-potassium carbonate, followed by centrifugation at 3000 g for 5 min at 4°C. The supernatant was stored at −80°C until analysis. ATP, ADP and AMP concentrations were measured using HPLC, according to the method proposed by Hou et al. Total adenine nucleotide and adenylate energy charge (AEC) levels were calculated by the following equations:

\[
\text{TAN} = \text{ATP} + \text{ADP} + \text{AMP},
\]

\[
\text{AEC} = (\text{ATP} + 0.5 \text{ADP}) / (\text{ATP} + \text{ADP} + \text{AMP}).
\]

Key enzyme activities of the tricarboxylic acid cycle in intestinal mucosa

The activities of key enzymes including citrate synthase (CS), isocitrate dehydrogenase (ICD) and α-ketoglutarate...
Table 4. Effects of asparagine (Asn) supplementation on intestinal mucosal protein, DNA and RNA contents in weaned piglets at 24 h after the administration of *Escherichia coli* lipopolysaccharide (LPS) challenge

(Mean values with their pooled standard errors, n 6 (one piglet per pen))

<table>
<thead>
<tr>
<th>Items</th>
<th>S</th>
<th>CONTR</th>
<th>LPS+0 % Asn</th>
<th>LPS + 0.5 % Asn</th>
<th>LPS + 1.0 % Asn</th>
<th>SEM</th>
<th>T</th>
<th>S</th>
<th>T x S</th>
<th>CONTR v. LPS + 0 % Asn</th>
<th>P†</th>
<th>Linear</th>
<th>Quadratic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (mg/g tissue)</td>
<td>Jejunum</td>
<td>64.6</td>
<td>48.7</td>
<td>55.4</td>
<td>59.8</td>
<td>4.3</td>
<td>0.040</td>
<td>0.635</td>
<td>0.069</td>
<td>0.001</td>
<td>0.001</td>
<td>0.109</td>
<td>0.278</td>
</tr>
<tr>
<td></td>
<td>Ileum</td>
<td>64.8</td>
<td>59.0</td>
<td>54.5</td>
<td>54.1</td>
<td>2.1</td>
<td></td>
<td></td>
<td></td>
<td>0.006</td>
<td>0.001</td>
<td>0.102</td>
<td>0.264</td>
</tr>
<tr>
<td>RNA:DNA</td>
<td>Jejunum</td>
<td>7.34</td>
<td>4.60</td>
<td>6.81</td>
<td>6.39</td>
<td>0.66</td>
<td>&lt;0.001</td>
<td>0.003</td>
<td>0.001</td>
<td>0.010</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Ileum</td>
<td>329</td>
<td>222</td>
<td>333</td>
<td>322</td>
<td>21</td>
<td></td>
<td></td>
<td></td>
<td>0.005</td>
<td>0.001</td>
<td>0.002</td>
<td></td>
</tr>
</tbody>
</table>

T, treatment; S, segment; CONTR, non-challenged control group (piglets fed a control diet and injected with 0.9 % NaCl solution).

* P values were obtained using treatment as the main effect and by analysing the data from the jejunum and ileum as repeated measures.

† LPS-challenged (0 % Asn) piglets were compared with CONTR piglets to determine the effects of LPS challenge. Linear and quadratic polynomial contrasts were used to determine the response to Asn supplementation among the LPS-challenged piglets.
Samples (0.15–0.20 g) were homogenised in 1 ml of lysis buffer containing protease inhibitors, and centrifuged at 12 000 g for 15 min at 4°C to collect supernatants for Western blot and protein assay. The protein contents of the supernatants were determined using the bicinchoninic acid reagent\(^\text{18,55}\). Equal amounts of intestinal mucosal proteins were loaded onto 10% polyacrylamide gels, separated through SDS-PAGE, and then transferred to blotting membranes. Immunoblots were blocked with 3% bovine serum albumin in TBS (Tris-HCl-buffered saline, including Tris-HCl, NaCl and KCl)/Tween-20 buffer for 60 min at room temperature. Then, the membranes were incubated overnight at 4°C with primary antibodies, followed by incubation with a secondary antibody for 120 min at room temperature. Specific primary antibodies included rabbit anti-phosphorylated AMPK\(\alpha\) (pAMPK\(\alpha\), Thr172, 1:1000, #2535; Cell Signaling Technology, Inc.), rabbit anti-total AMPK\(\alpha\) (tAMPK\(\alpha\), 1:1000, #2532; Cell Signaling Technology, Inc.) and mouse anti-\(\beta\)-actin (1:10 000, #A2228; Sigma-Aldrich, Inc.). Secondary antibodies included goat anti-rabbit IgG HRP (1:5000, #ANT019; Antgene Biotech) and goat anti-mouse IgG HRP (1:5000, #ANT019; Antgene Biotech). In our previous study, these antibodies were validated in weanling pigs\(^\text{33}\). Blots were developed using an enhanced chemiluminescence Western blotting kit (Amersham Biosciences), and visualised using a Gene Genome Bioimaging System (Alpha Innotech). Bands were analysed by densitometry using GeneTools software (Syngene). pAMPK\(\alpha\) was normalised to the total protein content of AMPK\(\alpha\).

### Statistical analysis

Experimental data were analysed by variance specific for repeated measures using the mixed procedure of SAS (SAS Institute, Inc.), with treatments as the between-animal effect and gut segment (jejunum and ileum) as the within-animal effect according to the following model:

\[
y_{ijk} = \mu + \alpha_i + \omega_j + (\alpha\omega)_{ij} + \mu_k + e_{ijk},
\]

where \(\alpha_i\) is the effect of the treatment (\(i = \text{CONTR, LPS} + 0\%\) Asn, LPS + 0.5% Asn and LPS + 1.0% Asn); \(\omega_j\) is the segment (jejunum and ileum); \(\alpha\omega\) is the interaction between treatment and segment; \(\mu_k \sim N(0, \tau^2)\) accounts for repeated measures made on the same individual, thereby rendering these observations correlated. The error term \(e_{ijk} \sim N(0, \sigma^2)\) represents unexplained variation. The variance interaction between segment and diet was described as random by using

\[
\alpha\omega \sim N(0, \sigma^2) (type = \text{arh}(1)).
\]

When a significant interaction between treatment and segment occurred, comparisons were made among the treatments in each segment (jejunum or ileum). LPS-challenged piglets (0% Asn) were compared with CONTR piglets to determine the effect of LPS challenge. Linear and quadratic polynomial contrasts were used to determine the response to dietary Asn supplementation among the LPS-challenged piglets. Results are expressed as means with their pooled standard errors. \(P \leq 0.05\) was considered as statistically significant, and \(0.05 < P \leq 0.10\) indicated a trend.
Table 6. Effects of asparagine (Asn) supplementation on intestinal adenylate purines in weaned piglets at 24 h after the administration of Escherichia coli lipopolysaccharide (LPS) challenge
(Mean values with their pooled standard errors, n 6 (one piglet per pen))

<table>
<thead>
<tr>
<th>Items</th>
<th>S</th>
<th>CONTR</th>
<th>LPS + 0 % Asn</th>
<th>LPS + 0.5 % Asn</th>
<th>LPS + 1.0 % Asn</th>
<th>SEM</th>
<th>T</th>
<th>S</th>
<th>T × S</th>
<th>CONTR v. LPS + 0 % Asn</th>
<th>Linear</th>
<th>Quadratic</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP (µg/g wet weight)</td>
<td>Jejunum</td>
<td>119</td>
<td>134</td>
<td>203</td>
<td>161</td>
<td>18</td>
<td>0.055</td>
<td>0.694</td>
<td>0.002</td>
<td>0.373</td>
<td>0.162</td>
<td>0.072</td>
</tr>
<tr>
<td></td>
<td>Ileum</td>
<td>195</td>
<td>108</td>
<td>168</td>
<td>161</td>
<td>18</td>
<td>0.055</td>
<td>0.022</td>
<td>0.005</td>
<td>0.398</td>
<td>0.854</td>
<td>0.948</td>
</tr>
<tr>
<td>ADP (µg/g wet weight)</td>
<td>Jejunum</td>
<td>124</td>
<td>115</td>
<td>127</td>
<td>94</td>
<td>17</td>
<td>0.541</td>
<td>0.653</td>
<td>0.367</td>
<td>0.001</td>
<td>0.005</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Ileum</td>
<td>150</td>
<td>110</td>
<td>101</td>
<td>122</td>
<td>22</td>
<td>0.005</td>
<td>0.022</td>
<td>0.005</td>
<td>0.398</td>
<td>0.854</td>
<td>0.948</td>
</tr>
<tr>
<td>AMP (µg/g wet weight)</td>
<td>Jejunum</td>
<td>223</td>
<td>271</td>
<td>209</td>
<td>236</td>
<td>19</td>
<td>0.415</td>
<td>0.001</td>
<td>0.559</td>
<td>0.041</td>
<td>0.110</td>
<td>0.291</td>
</tr>
<tr>
<td></td>
<td>Ileum</td>
<td>342</td>
<td>350</td>
<td>348</td>
<td>297</td>
<td>35</td>
<td>0.005</td>
<td>0.001</td>
<td>0.004</td>
<td>0.010</td>
<td>0.002</td>
<td>0.012</td>
</tr>
<tr>
<td>AMP:ATP</td>
<td>Jejunum</td>
<td>2.01</td>
<td>2.08</td>
<td>1.14</td>
<td>1.53</td>
<td>0.23</td>
<td>0.008</td>
<td>0.001</td>
<td>0.025</td>
<td>0.084</td>
<td>0.038</td>
<td>0.024</td>
</tr>
<tr>
<td></td>
<td>Ileum</td>
<td>1.87</td>
<td>3.35</td>
<td>2.28</td>
<td>1.89</td>
<td>0.30</td>
<td>0.001</td>
<td>0.001</td>
<td>0.025</td>
<td>0.010</td>
<td>0.002</td>
<td>0.012</td>
</tr>
<tr>
<td>TAN† (µg/g wet weight)</td>
<td>Jejunum</td>
<td>466</td>
<td>520</td>
<td>539</td>
<td>491</td>
<td>35</td>
<td>0.829</td>
<td>0.001</td>
<td>0.731</td>
<td>0.891</td>
<td>0.713</td>
<td>0.694</td>
</tr>
<tr>
<td></td>
<td>Ileum</td>
<td>647</td>
<td>608</td>
<td>617</td>
<td>580</td>
<td>60</td>
<td>0.062</td>
<td>0.001</td>
<td>0.026</td>
<td>0.667</td>
<td>0.074</td>
<td>0.225</td>
</tr>
<tr>
<td>AEC§</td>
<td>Jejunum</td>
<td>0.38</td>
<td>0.37</td>
<td>0.49</td>
<td>0.43</td>
<td>0.03</td>
<td>0.001</td>
<td>0.001</td>
<td>0.025</td>
<td>0.025</td>
<td>0.036</td>
<td>0.118</td>
</tr>
</tbody>
</table>

T, treatment; S, segment; CONTR, non-challenged control group (piglets fed a control diet and injected with 0.9 % NaCl solution); TAN, total adenine nucleotide; AEC, adenylate energy charge.

* P values were obtained using treatment as the main effect and by analysing the data from the jejunum and ileum as repeated measures.

† LPS-challenged (0 % Asn) piglets were compared with CONTR piglets to determine the effects of LPS challenge. Linear and quadratic polynomial contrasts were used to determine the response to Asn supplementation among the LPS-challenged piglets.

‡ AEC = (ATP + 0.5ADP)/(ATP + ADP + AMP).

Results

During the entire 19 d feeding trial (pre-challenge), there were no differences in initial BW (99, 99, 99 and 100 kg, respectively); average daily feed intake (733, 741, 703 and 724 g, respectively); average daily gain (477, 459, 492 and 524 g, respectively) and feed:gain ratio (1.54, 1.64, 1.43 and 1.34, respectively). During the LPS challenge, compared with CONTR piglets, LPS-challenged (0 % Asn) piglets had decreased glutamine, glutamate, ileal and jejunal RNA:DNA and protein:DNA ratios (linear, £0.001; quadratic, £0.05). Compared with CONTR piglets, LPS-challenged (0 % Asn) piglets tended to have decreased villus height and villus height:crypt depth ratio (VCR) in the jejunum and ileum (linear, £0.05; quadratic, £0.05). Among the LPS-challenged piglets, Asn supplementation increased villus height and crypt depth in the jejunum (linear, £0.001; quadratic, £0.05). Significant treatment × segment interactions were observed for villus height and VCR in the ileum (linear, £0.05). Among the LPS-challenged piglets, Asn (linear, £0.001; quadratic, £0.05) and ornithine (linear, £0.05; quadratic, £0.05) supplementation increased the concentrations of aspartate and ornithine (linear, £0.05; quadratic, £0.05).
Effects of asparagine (Asn) supplementation on intestinal activities of key enzymes in the tricarboxylic acid cycle in weaned piglets at 24 h after the administration of *Escherichia coli* lipopolysaccharide (LPS) challenge* (Mean values with their pooled standard errors, n = 6 (one piglet per pen))

<table>
<thead>
<tr>
<th>Items</th>
<th>S CONTR</th>
<th>LPS + 0 % Asn</th>
<th>LPS + 0.5 % Asn</th>
<th>LPS + 1.0 % Asn</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jejunum CS (μU/mg protein)</td>
<td>215</td>
<td>159</td>
<td>240</td>
<td>165</td>
<td>17</td>
</tr>
<tr>
<td>Ileum CS (μU/mg protein)</td>
<td>144</td>
<td>102</td>
<td>171</td>
<td>151</td>
<td>15</td>
</tr>
<tr>
<td>Jejunum ICD (μU/mg protein)</td>
<td>25-2</td>
<td>19-6</td>
<td>30-9</td>
<td>20-0</td>
<td>1-7</td>
</tr>
<tr>
<td>Ileum ICD (μU/mg protein)</td>
<td>16-8</td>
<td>11-3</td>
<td>17-7</td>
<td>16-6</td>
<td>1-1</td>
</tr>
<tr>
<td>Jejunum α-KGDHC (ng/mg protein)</td>
<td>389</td>
<td>275</td>
<td>459</td>
<td>282</td>
<td>26</td>
</tr>
<tr>
<td>Ileum α-KGDHC (ng/mg protein)</td>
<td>409</td>
<td>304</td>
<td>473</td>
<td>374</td>
<td>22</td>
</tr>
</tbody>
</table>

T, treatment; S, segment; CONTR, non-challenged control group (piglets fed a control diet and injected with 0.9 % NaCl solution); CS, citrate synthase; ICD, isocitrate dehydrogenase; α-KGDHC, α-ketoglutarate dehydrogenase complex.

* International unit (IU) was defined as 1 μmol substrate hydrolysed/min under specified assay conditions.

† P values were obtained using treatment as the main effect and by analysing the data from the jejunum and ileum as repeated measures.

‡ LPS-challenged (0 % Asn) piglets were compared with CONTR piglets to determine the effects of LPS challenge. Linear and quadratic polynomial contrasts were used to determine the response to Asn supplementation among the LPS-challenged piglets.
Increased mRNA abundance of ileal AMPKa1 and AMPKa2 (P<0.05). Among the LPS-challenged piglets, Asn supplementation decreased the mRNA abundance of jejunal and ileal AMPKa1, and ileal AMPKa2 (linear, P<0.05; quadratic, P<0.05).

No significant treatment × segment interaction was observed for the mRNA abundance of SIRT1 and PGC1α. Compared with CONTR piglets, LPS-challenged (0 % Asn) piglets had increased mRNA abundance of PGC1α (P<0.05). Among the LPS-challenged piglets, Asn supplementation decreased the mRNA abundance of SIRT1 (linear, P<0.05) and PGC1α (linear, P<0.05; quadratic, P<0.05).

Protein phosphorylation and abundance of AMP-activated protein kinase α

The pAMPKα:tAMPKα ratio in the jejunum were lower than that in the ileum (P<0.05), and the protein abundance of tAMPKα in the jejunum was higher than that in the ileum (P<0.001) (Fig. 2). A trend for the treatment × segment interaction was observed for the pAMPKα:tAMPKα ratio (P=0.073). Compared with CONTR piglets, LPS-challenged (0 % Asn) piglets had increased ileal pAMPKα:tAMPKα ratio (P<0.05). Among the LPS-challenged piglets, Asn supplementation decreased the ileal pAMPKα:tAMPKα ratio (linear, P<0.05; quadratic, P<0.05).

Discussion

LPS is the main constituent of the outer membrane of Gram-negative bacteria (3). Increasing evidence indicates that LPS is a cofactor in intestinal injury (3,18). Intestinal alterations including morphological injury, increased mucosal permeability and bacterial translocation have often been reported after intraperitoneal injection of LPS (3). The pathogenesis of LPS-induced intestinal injury is viewed as a complex event, and it has been correlated with increased inflammation caused by LPS challenge that leads to the expression of pro-inflammatory cytokines (34,35). In addition, LPS is known to cause significant damage to gastrointestinal oxygen metabolism and mitochondria dysfunction, leading to decreased ATP concentrations and eventually intestinal damage (36). Our previous studies have suggested that dietary supplementation with 0.5 % Asn alleviated growth suppression, and both 0.5 and 1.0 % dietary Asn supplementation attenuated the changes in total and differential leucocyte counts and serum biochemical parameters in weaned piglets after the administration of LPS challenge (22). Therefore, we extended the finding to the intestine to explore the effect of Asn supplementation on intestinal injury. To our knowledge, this is the first study to evaluate whether dietary Asn supplementation could attenuate intestinal injury in weaning piglets challenged with LPS.

Villus height, crypt depth and VCR were used to measure intestinal morphology (36). Mucosal protein contents, RNA:DNA and protein:DNA ratios are important biochemical indices for intestinal development (3). Mucosal disaccharidases, namely lactase, maltase and sucrase, are directly involved in the energy supply of organism, and can mirror intestinal
digestive function\(^{(37)}\). In the present study, villus height and disaccharidase activities in the jejunum were higher than those in the ileum, which is similar to the report of Rubio et al.\(^{(38)}\). LPS challenge decreased villus height, VCR, mucosal protein content, RNA:DNA and protein:DNA ratios, and increased disaccharidase activities linearly and quadratically. These data indicate that Asn protected the intestine from damage. Until now, little is known about the nutritional significance of Asn in the intestine. Previous studies have found that Asn stimulates enterocyte proliferation in the small intestine of pigs\(^{(8,11)}\). However, the protective mechanism of Asn remains unknown. Rhoads et al.\(^{(9)}\) reported that Asn can be converted to aspartate, and glutamate can subsequently be generated from α-ketoglutarate and aspartate. In the present study, Asn supplementation to the LPS-challenged pigs increased serum concentrations of aspartate, Asn, glutamine and ornithine linearly and quadratically. In this way, it is possible that Asn may be a precursor for many other amino acids to be produced on demand to meet the requirements of enterocytes.

The intestine takes up a high amount of energy to sustain its integrity, function and health\(^{(39,40)}\), and energy deficits in intestinal mucosa may relate to intestinal injury\(^{(23)}\). Most cellular processes need energy and are driven directly or indirectly by hydrolysing ATP to ADP and phosphate, or less frequently by pyrophosphate. The AMP:ATP ratio is a sensitive indicator of cellular energy state\(^{(41)}\). In comparison with the level of a single nucleotide, the energy charge of the adenyl pool is a better way to measure the energy status of a tissue\(^{(25)}\). In the present study, LPS challenge decreased ATP concentrations and AEC levels, and increased AMP:ATP ratios. Similarly, Hou et al.\(^{(25)}\) reported that LPS challenge altered the cellular energy status in intestinal mucosa. Asn supplementation to the LPS-challenged pigs increased ATP concentrations linearly and AEC levels linearly and quadratically, and decreased AMP:ATP ratios linearly and quadratically. These data support the notion that Asn supplementation attenuated LPS-induced intestinal damage possibly via modulating the adenine nucleotide pool.

The tricarboxylic acid cycle is a central route for energy production in the intestine\(^{(24)}\). Key enzymes involved in the tricarboxylic acid cycle include CS, ICD and α-KGDHC. The enzyme CS catalyses the first step of the tricarboxylic acid cycle by taking molecules of acetate and attaching them to oxaloacetate\(^{(43)}\). ICD is responsible for catalysing the oxidative decarboxylation of isocitrate into α-ketoglutarate and CO\(_2\)\(^{(44)}\). α-KGDHC is a multi-enzymatic complex that converts α-ketoglutarate to succinyl-CoA\(^{(45)}\). In the present study, Asn supplementation to the LPS-challenged pigs attenuated the decrease in the activities of the key enzymes in the tricarboxylic acid cycle linearly and quadratically. This may due to the conversion of Asn to aspartate\(^{(46)}\), which can be converted to tricarboxylic acid cycle intermediates (such as oxaloacetate)\(^{(46)}\). In the present study, it is possible that dietary supplementation with Asn improved intestinal energy status by enhancing the key enzyme activities of the tricarboxylic acid cycle.

AMPK is an energy regulator whose primary role involves maintaining the intracellular energy balance in eukaryotic evolution\(^{(12)}\). It can be activated by mechanisms including phosphorylation upon allosteric activation by increasing the AMP:ATP ratio\(^{(47)}\). To restore the cellular energy status, the activation of AMPK can switch on ATP-producing processes.
Asparagine improves intestinal energy status

while synchronously switching off ATP-consuming processes\(^{(47)}\). In addition, AMPK could chronically promote cellular ability to produce ATP and diminish potentially adverse cellular events\(^{(48)}\). Several reports have revealed that AMPK can enhance the activity of SIRT1 by increasing cellular NAD\(^+\) level\(^{(36)}\). SIRT1 was found to be a major regulator of muscle adaptation to nutrient availability\(^{(49-50)}\). When activated, SIRT1 also enhances mitochondrial oxidative function and leads to selective nutrient utilisation to regulate energy balance\(^{(53)}\). Furthermore, SIRT1 deacetylation has been proposed to be a potential activator for the transcriptional activity of PGC1\(\alpha\)\(^{(51)}\). D’Errico et al.\(^{(52)}\) reported that PGC1\(\alpha\) modulates mitochondrial biogenesis and function. In the present study, LPS challenge increased the mRNA abundance of AMPKa1, AMPKa2 and PGC1\(\alpha\), and the phosphorylation of AMPKa. In agreement with the results of the present study, Hou et al.\(^{(53)}\) reported that LPS challenge increased intestinal AMPKa phosphorylation. Asn supplementation to the LPS-challenged pigs decreased intestinal AMPKa1, AMPKa2, SIRT1 and PGC1\(\alpha\) mRNA abundance, and decreased ileal AMPKa phosphorylation linearly and quadratically. In the present study, consistent with reduced intestinal AMP:ATP ratios and increased ATP concentrations, dietary Asn supplementation inhibited intestinal AMPK signalling pathway in response to LPS treatment. It is possible that the reduced intestinal AMP:ATP ratios and the increased ATP concentrations in enterocytes might be enough to inhibit the AMPK signalling pathway in LPS-challenged piglets fed the Asn diet. Future research is needed to elucidate the mechanisms for the inhibitory effect of Asn on the AMPK signalling pathway.

In the present study, only two doses of Asn (0·5 and 1·0 % Asn) were used. The higher concentration of Asn (1·0 %) had a worse effect on some parameters characteristic for energy status compared with the lower concentration (0·5 %). We speculate that the different effects of the two doses of Asn might be due to the following mechanisms. First, Asn can be converted to glutamine via complex interorgan metabolism\(^{(31)}\). In the present study, Asn supplementation to the LPS-challenged pigs increased serum glutamine concentrations. Holecek\(^{(55)}\) reported that enhanced glutamine intake competed with intestinal absorption of a number of amino acids, which might affect amino acid and protein metabolism in the gut, liver and whole body. Second, as with all other nutrients, it is possible that excessive amount of Asn in diets can cause amino acid imbalances and toxicity. In addition, we used an acute model of LPS challenge, which is different from a more chronic LPS challenge situation (e.g. during 8–15 d post-weaning). Thus, future studies including more Asn doses are needed to better understand the effects of Asn supplementation in an acute or chronic LPS challenge situation. Moreover, the effect of dietary Asn supplementation on some variables differed between the jejunum and the ileum. This might be related to different microenvironments among various segments of the gut at molecular and cellular levels\(^{(30)}\). In summary, dietary supplementation of Asn mitigates intestinal injury and improves intestinal energy status of weaned piglets challenged by LPS. In addition, Asp supplementation modulates intestinal AMPK signalling pathway. These novel findings not only contribute to the understanding of the mode of action of Asn in the intestine of pigs, but also hold great significance for improving infant nutrition.

Supplementary material

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S0007114515001877

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The authors declare that they have no conflict of interest.

References

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