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ORIGINAL ARTICLE

# Dietary L-glutamine supplementation modulates microbial community and activates innate immunity in the mouse intestine

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**Abstract** This study was conducted to determine effects of dietary supplementation with 1 % L-glutamine for 14 days on the abundance of intestinal bacteria and the activation of intestinal innate immunity in mice. The measured variables included (1) the abundance of Bacteroidetes, Firmicutes, *Lactobacillus*, *Streptococcus* and *Bifidobacterium* in the lumen of the small intestine; (2) the expression of toll-like receptors (TLRs), pro-inflammatory cytokines, and antibacterial substances secreted by Paneth cells and goblet cells in the jejunum, ileum and colon; and (3) the activation of TLR4-nuclear factor kappa B (NF-κB), mitogen-activated protein kinases (MAPK), and phosphoinositide-3-kinases (PI3K)/PI3K-protein kinase B (Akt) signaling pathways in the jejunum and ileum. In the jejunum, glutamine supplementation decreased the abundance of Firmicutes, while increased mRNA levels for antibacterial substances in association with the activation of NF-κB and PI3K-Akt pathways. In the ileum, glutamine supplementation induced a shift in the Firmicutes:Bacteroidetes ratio in favor of Bacteroidetes, and enhanced mRNA levels for *Tlr4*, pro-inflammatory cytokines, and antibacterial substances participating in NF-κB and JNK signaling pathways. These results indicate that the effects of glutamine on the intestine vary with its segments and compartments. Collectively, dietary glutamine supplementation of mice beneficially alters intestinal bacterial community and activates the innate immunity in the small intestine through NF-κB, MAPK and PI3K-Akt signaling pathways.

**Keywords** Glutamine · Paneth cell · Firmicutes · NF-κB · Innate immunity

**Abbreviations**

| | |
|---|---|
| AKT | Protein kinase B |
| *Ang4* | RNase angiogenin 4 |
| *Crs* | Cryptdin-related sequence |
| *Ifn* | Interferon |
| *Il* | Interleukin |
| *Lyz2* | Lysozyme 2 |
| MAPK | Mitogen-activated protein kinases |
| *Muc* | Mucin |
| *Myd88* | Myeloid differentiation factor-88 |
| NF-κB | Nuclear factor kappa B |
| PI3K | Phosphoinositide-3-kinases |
| *Reg3γ* | Regenerating islet-derived 3 gamma |
| *Spla2* | Secretory group II A phospholipase A2 |

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*Tnf* Tumor necrosis factor
*Tlrs* Toll-like receptors

## Introduction

L-Glutamine is the most abundant free amino acid in the plasma of humans and many other mammals (including mice) (Curthoys and Watford 1995), and a conditionally essential amino acid under certain stress conditions (Mondello et al. 2010; Rezaei et al. 2013a; Wu et al. 2011). Besides its multiple nutritional functions (Wu 2013a, b), glutamine is also an important regulator for immune responses (Li et al. 2007). Recent studies have demonstrated that glutamine supplementation promotes the immune response in virus- or bacteria-infected mice (Ren et al. 2012b, 2013c, d). As the small intestine of most mammals (e.g., mice, pigs, and humans) is a major site for the utilization of glutamine (Dai et al. 2010, 2012a, b; Wu 2009), there is increasing interest in finding the effects of glutamine on intestinal cell-signaling (Rhoads and Wu 2009), health (Chen et al. 2014; Ren et al. 2014a) and immunity (Ruth and Field 2013). Of note, Dai et al. (2013) have reported that glutamine modulates the metabolism of other amino acids in jejunal and ileal bacteria. Previous studies have also suggested that glutamine regulates intestinal inflammation via various signaling pathways, such as nuclear factor kappa B (NF-κB), mitogen-activated protein kinases (MAPK), and phosphoinositide-3 kinases (PI3K)-protein kinase B (Akt) (Ren et al. 2013c), as reported for arginine (Ren et al. 2014b). Based on these findings, we hypothesized that glutamine could modulate the intestinal microbiota and intestinal innate immune system. However, direct evidence for supporting this hypothesis is missing.

Intestinal innate immunity, which tolerates commensal bacteria and prevents pathogens from invading into the body, acts as the first line of defense (Battersby and Gibbons 2013). Under normal conditions, intestine homeostasis is sustained by the harmonious interaction between the intestinal microbiota and immune system (King and Dekaney 2013; Clevers 2012; Forman et al. 2012). When intestinal dysbiosis occurs (e.g., a change in the ratio of Firmicutes to Bacteroidetes), pattern recognition receptors (PRRs) [e.g., toll-like reporters (TLRs)] sense these abnormal changes (Rosenstiel 2013) to trigger various signaling pathways [e.g., NF-κB and MAPK (Ben et al. 2012; Schirbel et al. 2013)], leading to the secretion of pro-inflammatory cytokines from cells of the innate immune system (Zanello et al. 2011) and the secretion of bioactive substances (e.g., defensins and mucins) from Paneth cells and goblet cells (Clevers 2012; Chu et al. 2012; Garcia-Miguel et al. 2013). These secreted molecules have strong local antimicrobial activity, while enhancing the intestinal integrity and barrier function to repair the intestinal dysbiosis (Jager et al. 2013). The dysfunction of intestinal innate immunity has been widely associated with various intestinal diseases, such as enteric infection (Charania et al. 2013) and inflammatory bowel diseases (Geremia et al. 2014). Therefore, it is of importance to determine the effects of glutamine supplementation on the intestinal microbiota and innate immunity.

Based on the foregoing, the major objective of this study is to investigate the abundance of intestinal bacteria, the activation of innate immunity, including the expressions of TLRs, pro-inflammatory cytokines, and antimicrobials, in the jejunum, ileum or colon of mice fed a standard rodent diet supplemented with or without 1 % glutamine for 14 days.

## Materials and methods

### Antibodies

Antibodies against p85 (Sc-1637), p-Akt (Sc-7985-R), JNK (Sc-571), p-JNK (Sc-12882) and TLR4 (Sc-10741) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA). Antibodies against ERK1/2 (CST 4695), p-ERK1/2 (CST 4370), p-38 (CST 8690), p–p-38 (CST 4511) and p65 (CST 6956) were procured from Cell Signaling Technology (Danvers, MA, USA). The antibody against lysozyme (3349-1) was obtained from Epitomics (Burlingame, CA, USA).

### Experimental design

One hundred female ICR (Institute for Cancer Research) mice (6-week old) were purchased from the SLAC Laboratory Animal Center (Hunan, China). The mice were housed in a pathogen-free animal colony (temperature, 25 °C; relative humidity, 53 %; 12-h dark/12-h light) and had free access to a standard rodent diet (Ren et al. 2014b) and drinking water. Animals were randomly divided into one of two groups ($n = 50$ per group): (1) mice were fed the basal diet (Ren et al. 2014b); (2) mice were fed the basal diet supplemented with 1.0 % L-glutamine (Ajinomoto Inc., Tokyo, Japan). The basal diet was analyzed for total amino acids as described previously (Dai et al. 2014; Rezaei et al. 2013b) and was found to contain 1.93 % glutamine and 1.81 % glutamate. The content of other amino acids in the basal diet was reported previously (Ren et al. 2012a, 2014b). Dietary supplementation with 1.0 % L-glutamine was based on our published studies indicating a beneficial effect on enhancing immunity in virus-infected mice (Ren et al. 2013a, b, c, d). Like previous studies (Ren et al. 2014b), we did not use alanine as an isonitrogenous control because alanine might affect the mouse immune system in vivo. The amount of supplemental glutamine nitrogen represented only 5.0 % of total nitrogen content in the diet. After 2 weeks of glutamine supplementation, mice were killed to collect the jejunum, ileum and colon for biochemical analysis. We determined the changes of intestinal innate immunity after 2 weeks of glutamine supplementation because the biological impacts of glutamine supplementation in vivo have been observed at this time point (Ren et al. 2013a, b, c). The luminal contents in the jejunum and ileum were collected for bacterial analysis. This study was approved by the Animal Welfare Committee of the Institute of Subtropical Agriculture, Chinese Academy of Sciences.

### Immunoblotting analysis

Western blot analysis was conducted according to a previous study (Ren et al. 2014b). Briefly, the equal amounts of proteins obtained from cytoplasmic or nuclear fractions were separated by a reducing SDS-PAGE electrophoresis (Hou et al. 2012, 2013). The proteins were transferred onto PVDF membranes (Millipore, MA, USA) and blocked with 5 % non-fat milk in Tris-Tween buffered saline buffer (20 mM Tris, pH 7.5, 150 mM NaCl, and 0.1 % Tween-20) for 3 h. The primary antibodies were incubated overnight at 4 °C; the HRP-conjugated secondary antibodies were subsequently incubated for 1 h at room temperature before developing the blots using Alpha Imager 2200 software (Alpha Innotech Corporation, CA, USA). We digitally quantified the resultant signals and normalized the data to the proliferating cell nuclear antigen (PCNA) or actin abundance. PCNA or actin was used as an internal loading control for the nuclear or cytoplasmic protein fraction, respectively.

### Luminal content collection and DNA extraction

Luminal content from the jejunum and ileum was separately collected for analysis of bacteria and DNA extraction, as we described (Ren et al. 2014b). DNA was extracted using the Tiangen stool mini kit (TianGen, Beijing, China) according to the manufacturer's instructions, and DNA concentration was determined using spectrophotometry (Nanodrop).

### Gene expression and bacterial abundance analysis

The small intestine was obtained after its lumen was cleaned with saline (Zhang et al. 2013b). Total RNA was isolated from the liquid nitrogen-frozen jejunum, ileum and colon using TRIZOL regent (Invitrogen, USA) (Wang et al. 2008) and then treated with DNase I (Invitrogen, USA) according to the manufacturer's instructions. Primers used in this study were presented in the previous study (Ren et al. 2014b). Real-time PCR was performed as previously described (Ren et al. 2013e). Briefly, 1-μl cDNA or DNA template was added to a total volume of 10 μl containing 5 μl SYBR Green mix, 0.2 μl Rox, 3 μl dd-H2O, and 0.4 μl each of forward and reverse primers. We used the following protocol: (1) pre-denaturation program (10 s at 95 °C); (2) amplification and quantification program, repeated 40 cycles (5 s at 95 °C, 20 s at 60 °C); (3) melting curve program (60–99 °C with a heating rate of 0.1 °C/s and fluorescence measurement). Relative expression was normalized to the value for the house-keeping gene (Zhang et al. 2013a, b).

### Statistical analysis

Values, expressed as mean ± SEM, were analyzed statistically using the SPSS 16.0 software (Chicago, IL, USA). Comparison of measured variables between control and glutamine-supplemented mice was made by the unpaired *t* test. Within-group comparison was made by the paired *t* test. Log transformation of variables was performed when variance of data was not homogenous among treatment groups, as assessed by the Levene's test (Wei et al. 2012). Probability values <0.05 were taken to indicate statistical significance.

## Results

### Changes in bacterial composition in the lumen of the small intestine

First, we investigated the impacts of glutamine supplementation on the prevalence of the Bacteroidetes and Firmicutes, which are the most abundant phyla of commensal bacteria in the small intestine (Eckburg et al. 2005). The content of Firmicutes in the jejunum (Fig. 1a) and ileum (Fig. 1b) of glutamine-supplemented mice was lower ($P < 0.05$) than that in the control group, whereas no difference in the abundance of Bacteroidetes in the jejunum (Fig. 1a) or ileum (Fig. 1b) between the two groups of animals ($P > 0.05$) was detected. The proportional representation of these phyla was markedly shifted ($P < 0.05$) in the ileum after glutamine supplementation, in favor of Bacteroidetes (Fig. 1b), while no change occurred in the jejunum (Fig. 1a).

Next, we assessed the population of *Lactobacillus* and *Streptococcus* (belonging to Firmicutes), as well as *Bifidobacterium* in the small intestine after glutamine supplementation. In the jejunum, glutamine supplementation increased ($P < 0.05$) the abundance of *Streptococcus* and *Bifidobacterium*, but did not affect the abundance of

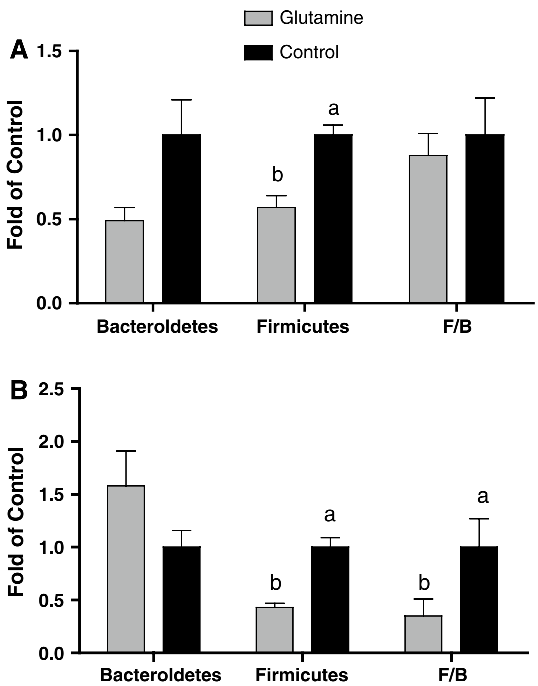


**Fig. 1** Composition of the microbiota in the lumen of the mouse small intestine. Mice were fed a standard rodent diet supplemented with or without 1.0 % L-glutamine for 2 weeks. **a** Relative abundance of Firmicutes and Bacteroidetes, and the ratio of Firmicutes:Bacteroidetes in the jejunum. **b** Relative abundance of Firmicutes and Bacteroidetes, and the ratio of Firmicutes:Bacteroidetes in the ileum. Values are mean ± SEM, $n = 6$, with *a–b* used to indicate a statistically significant difference ($P < 0.05$)

*Lactobacillus* (Fig. 2a). In the ileum, glutamine supplementation decreased ($P < 0.05$) the abundance of *Streptococcus* and *Lactobacillus*, as compared to the controls (Fig. 2b).

## Activation of TLRs in the ileum

Based on our observation that glutamine supplementation affected the small-intestinal bacterial abundance, we determined mRNA levels for *Tlrs* in the small intestine because TLRs are key components of innate immunity to recognize microbes in the intestine, resulting in the activation of immune response (Marques and Boneca 2011). In the jejunum, glutamine supplementation had little effect on mRNA levels for *Tlrs*, such as *Tlr2* and *Tlr 4* (Table 1). Similarly, glutamine supplementation did not affect the protein abundance of TLR4 in the jejunum (Fig. 3a, b). In the ileum, glutamine supplementation increased ($P < 0.05$) the mRNA level for *Tlr4*, decreased ($P < 0.05$) *Tlr5* expression, and had little effect on mRNA levels for other *Tlrs* (Table 2). The *Myd88* mRNA was detected because the MyD88-dependent pathway is distinct in the gut, which is used by all TLRs but TLR3 (Wu et al. 2013a). Glutamine

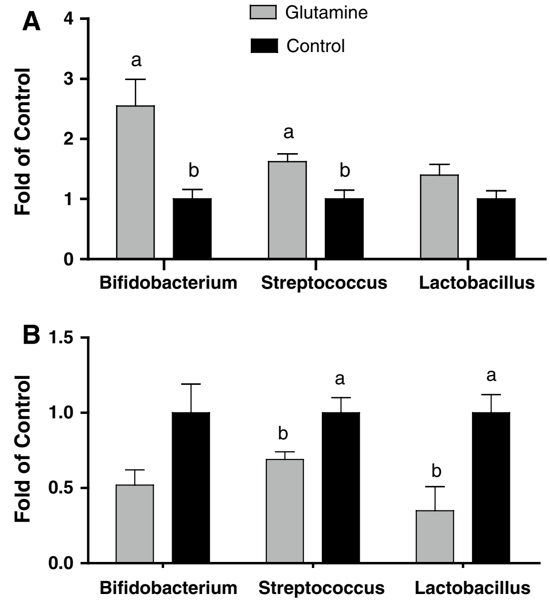


**Fig. 2** Relative abundance of *Lactobacillus*, *Streptococcus*, and *Bifidobacterium* in the intestine. Mice were fed a standard rodent diet supplemented with or without 1.0 % L-glutamine for 2 weeks. **a** Relative abundance of *Lactobacillus*, *Streptococcus* and *Bifidobacterium* in the jejunum. **b** Relative abundance of *Lactobacillus*, *Streptococcus* and *Bifidobacterium* in the ileum. Values are mean ± SEM, $n = 6$, with *a–b* used to indicate a statistically significant difference ($P < 0.05$)

**Table 1** mRNA levels for toll-like receptors (*Tlrs*) in the jejunum of mice supplemented with or without glutamine

| Gene | 1 % Glutamine | Control |
|---|---|---|
| *Tlr1* | 0.83 ± 0.10 | 1.00 ± 0.09 |
| *Tlr2* | 1.07 ± 0.16 | 1.00 ± 0.15 |
| *Tlr4* | 1.00 ± 0.21 | 1.00 ± 0.10 |
| *Tlr5* | 0.99 ± 0.11 | 1.00 ± 0.16 |
| *Tlr6* | 0.82 ± 0.12 | 1.00 ± 0.11 |
| *Tlr7* | 1.09 ± 0.22 | 1.00 ± 0.15 |
| *Tlr8* | 1.03 ± 0.12 | 1.00 ± 0.15 |
| *Tlr9* | 0.72 ± 0.16 | 1.00 ± 0.15 |
| *Myd88* | 1.15 ± 0.12 | 1.00 ± 0.12 |

Mice were fed a standard rodent diet supplemented with or without 1.0 % L-glutamine for 2 weeks. Values are mean ± SEM, $n = 6$

*Myd88* myeloid differentiation factor-88, *Tlrs* toll-like receptors

supplementation had little effect on the mRNA level of *Myd88* in the jejunum (Table 1) or ileum (Table 2).

## Inhibition of the NF-κB pathway in the small intestine

The NF-κB pathway plays a major role in activating host pro-inflammatory responses after the activation of TLRs by intestinal microbes (Karin and Lin 2002; Senftleben and

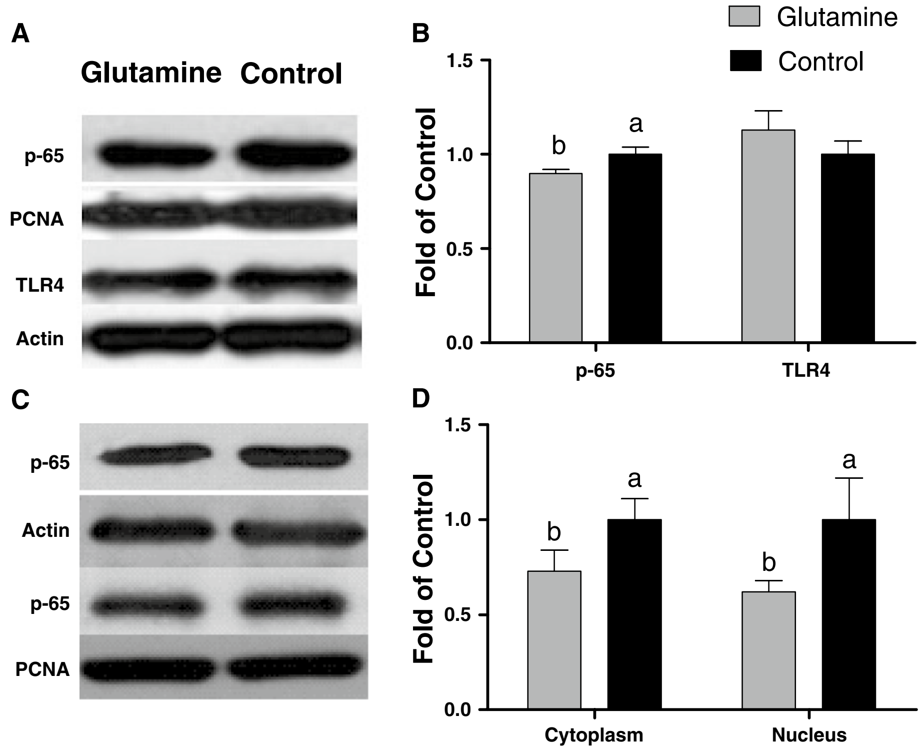


**Fig. 3** Activation of the TLR4-NF-κB p65 pathway in the mouse small intestine. Mice were fed a standard rodent diet supplemented with or without 1.0 % L-glutamine for 2 weeks. **a** Immunoblotting of nuclear p65 and of TLR4 in the jejunum after glutamine supplementation for 14days. **b** Quantification of relative p65 and TLR 4 abundance from data shown in (**a**). **c** Immunoblotting of cytoplasmic and nuclear p65 in the ileum after glutamine supplementation for 14days. **d** Quantification of relative cytoplasmic and nuclear p65 abundance from data shown in (**c**). Data are presented as mean ± SEM, $n = 6$, with *a–b* used to indicate a statistically significant difference ($P < 0.05$). NF-κB, nuclear factor kappa B; TLR, toll-like receptor

**Table 2** mRNA levels for toll-like receptors (*Tlrs*) in the ileum of mice supplemented with or without glutamine

| Gene | 1 % Glutamine | Control |
|---|---|---|
| *Tlr1* | 1.25 ± 0.12 | 1.00 ± 0.11 |
| *Tlr2* | 1.10 ± 0.19 | 1.00 ± 0.17 |
| *Tlr4* | 1.37 ± 0.08* | 1.00 ± 0.13 |
| *Tlr5* | 0.61 ± 0.09* | 1.00 ± 0.10 |
| *Tlr6* | 1.21 ± 0.17 | 1.00 ± 0.14 |
| *Tlr7* | 1.21 ± 0.09 | 1.00 ± 0.08 |
| *Tlr8* | 1.16 ± 0.07 | 1.00 ± 0.08 |
| *Tlr9* | 0.96 ± 0.17 | 1.00 ± 0.21 |
| *Myd88* | 0.93 ± 0.10 | 1.00 ± 0.13 |

Mice were fed a standard rodent diet supplemented with or without 1.0 % L-glutamine for 2 weeks. Values are mean ± SEM, $n = 6$

*Myd88* myeloid differentiation factor-88, *Tlrs* toll-like receptors

* Different from the control, $P < 0.05$

Karin 2002). In general, NF-κB p65 and p50 proteins are sequestered in the cytosol by inhibitory kappa B protein (IκB). After activation and the degradation of IκB proteins, p65 and p50 dimers translocate to the nucleus and bind to the cognate NF-κB sequences of target genes to produce pro-inflammatory cytokines (Ren et al. 2012b, 2013c, d). To determine the effects of glutamine supplementation on this pathway, we measured NF-κB p65 protein levels in the nucleus of epithelial cells in the jejunum. Glutamine supplementation decreased ($P < 0.05$) the abundance of p65 protein in the nucleus in the jejunum (Fig. 3a, b). We also determined the abundance of p65 in the cytoplasm and nucleus of epithelial cells in the ileum. The abundance of p65 protein in the cytoplasm and nucleus in the ileum was decreased ($P < 0.05$) by glutamine supplementation (Fig. 3c, d).

### Activation of the MAPK pathway in the ileum

Besides NF-κB, mitogen-activated protein kinases (MAPKs; e.g., p38 MAPK, ERK1/2, and JNK) also regulate intestinal innate immunity. To determine the effects of glutamine supplementation on p38 MAPK, ERK1/2 and JNK activation, we determined MAPK activation by quantifying the activation-associated phosphorylation of p38 MAPK, ERK1/2 and JNK using phospho-specific antibodies. In the jejunum, glutamine supplementation had little effect on p38 MAPK, ERK1/2 or JNK activation based on the ratios of phosphorylated p38 to total p38, phosphorylated ERK1/2 to total ERK1/2, and phosphorylated JNK to total JNK (Fig. 4a–d). In the ileum, glutamine supplementation did not affect p38 MAPK or ERK1/2 (Fig. 5a, b, d), but promoted ($P < 0.05$) JNK activation by increasing the ratio of phosphorylated JNK to total JNK (Fig. 5a, c).

### Inhibition of PI3K-Akt pathway in the jejunum

The PI3K-Akt pathway regulates intestinal innate immunity by phosphorylating IKKs to activate the NF-κB pathway, by inhibiting Raf1 (v-Raf1 murine leukemia viral oncogene homolog-1) to activate the ERK pathway, and by

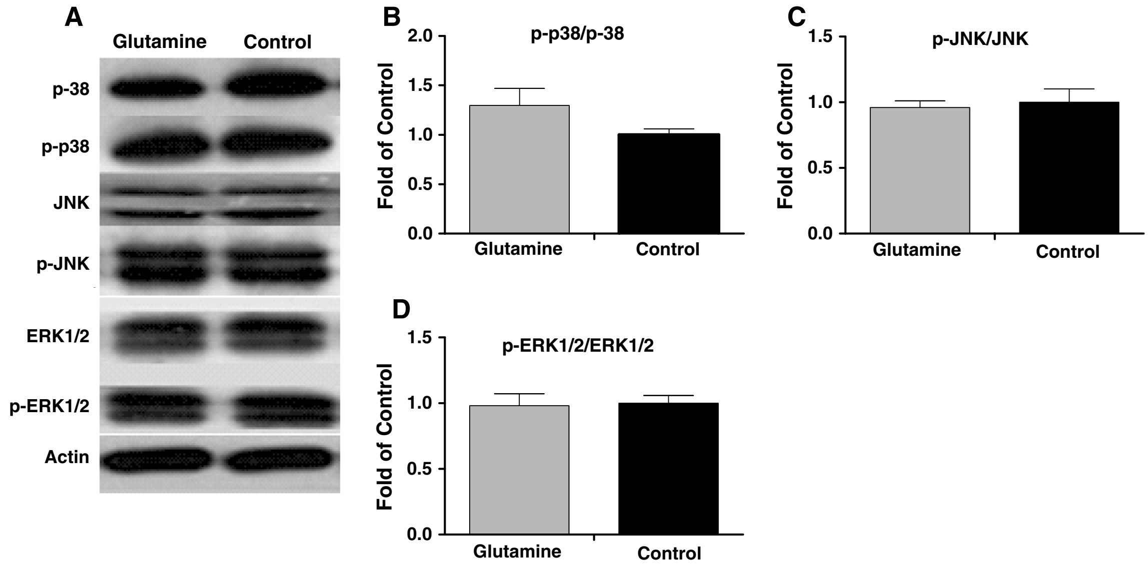


**Fig. 4** Activation of the MAPK pathway in the mouse jejunum. Mice were fed a standard rodent diet supplemented with or without 1.0 % L-glutamine for 2 weeks. **a** Immunoblotting of total and phosphorylated p38, JNK, and ERK1/2 in the jejunum after glutamine supplementation for 14days. **b** Quantification of ratio of relative phosphorylated to total p38 MAPK abundance from data shown in (**a**). **c** Quantification of ratio of relative phosphorylated to total JNK MAPK abundance from data shown in (**a**). **d** Quantification of ratio of relative phosphorylated to total ERK1/2 MAPK abundance from data shown in (**a**). Data are presented as mean ± SEM, $n = 6$

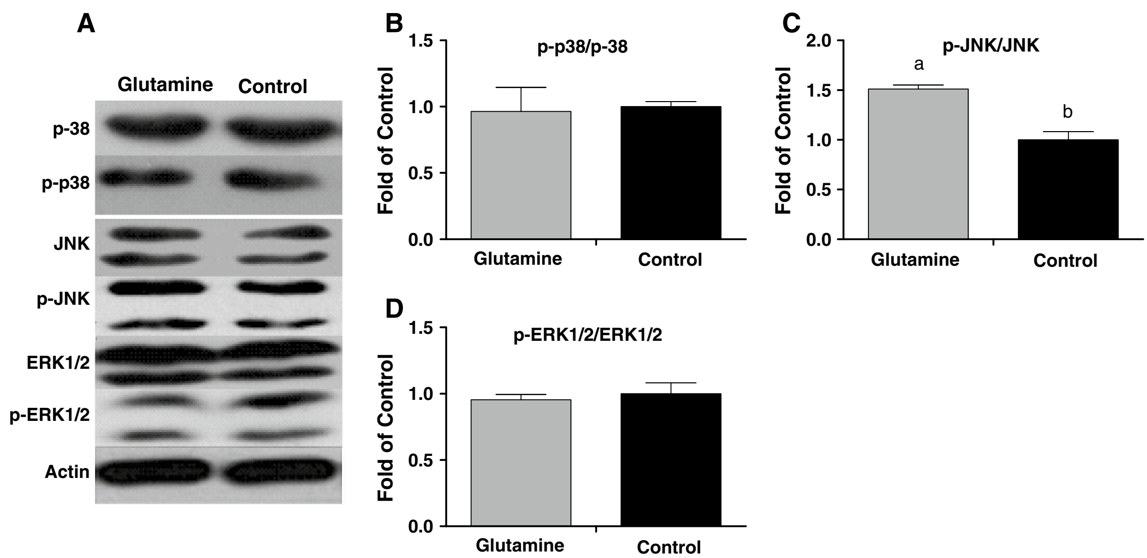


**Fig. 5** Activation of the MAPK pathway in the mouse ileum. Mice were fed a standard rodent diet supplemented with or without 1.0 % L-glutamine for 2 weeks. **a** Immunoblotting of total and phosphorylated p38, JNK, and ERK1/2 in the ileum after glutamine supplementation for 14days. **b** Quantification of ratio of relative phosphorylated to total p38 MAPK abundance from data shown in (**a**). **c** Quantification of ratio of relative phosphorylated to total JNK MAPK abundance from data shown in (**a**). **d** Quantification of ratio of relative phosphorylated to total ERK1/2 MAPK abundance from data shown in (**a**). Data are presented as mean ± SEM, $n = 6$, with *a–b* used to indicate a statistically significant difference ($P < 0.05$)

phosphorylating ASK1 (Apoptosis signal-regulating kinase 1) to inhibit the JNK pathway (Chen et al. 2003). Glutamine supplementation did not affect the abundance of PI3K or p-Akt in the ileum (Fig. 6a, b), but reduced ($P < 0.05$) the abundance of PI3K and p-Akt in the jejunum (Fig. 6c, d).

## Expression of pro-inflammatory cytokines in the ileum

Intestinal pro-inflammatory cytokines, whose production is affected by the intestinal microbiota and TLRs signaling, play a central role in intestinal inflammatory disease, i.e.,

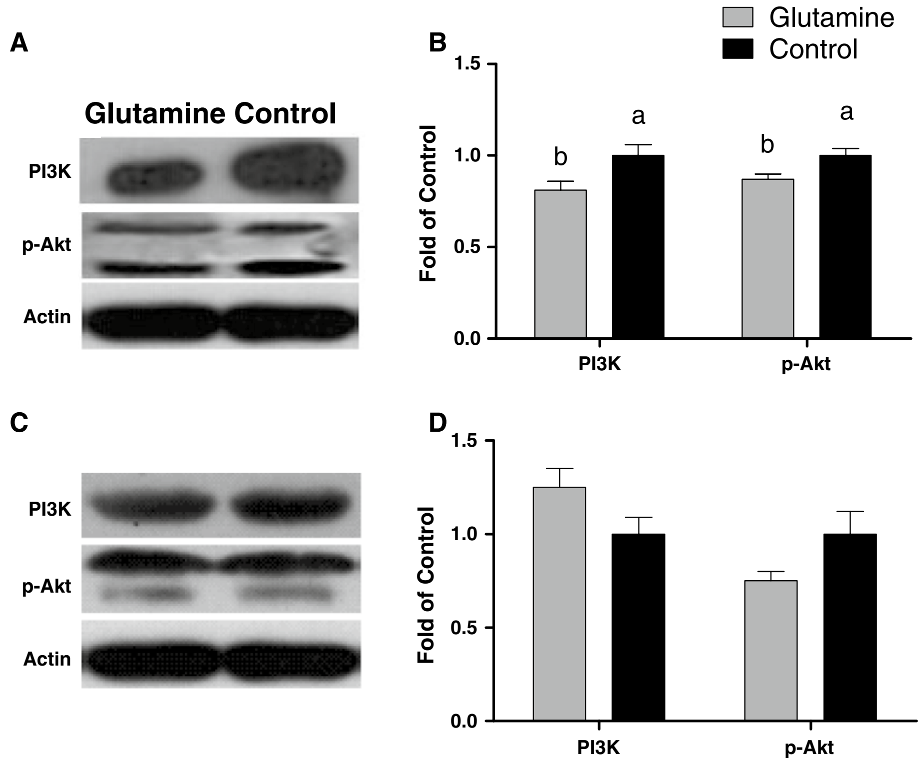


**Fig. 6** Abundance of PI3K and p-Ak proteins in the intestine. Mice were fed a standard rodent diet supplemented with or without 1.0 % L-glutamine for 2 weeks. **a** Immunoblotting of PI3K and p-Ak in the jejunum after glutamine supplementation for 14days. **b** Quantification of relative PI3K and p-Ak abundance from data shown in (**a**). **c** Immunoblotting of PI3K and p-Ak in the ileum after glutamine supplementation for 14days. **d** Quantification of relative PI3K and p-Ak abundance from data shown in (**c**). Data are presented as mean ± SEM, $n = 6$, with *a–b* used to indicate a statistically significant difference ($P < 0.05$). Akt, protein kinase B; PI3K, phosphoinositide-3-kinases

inflammatory bowel disease (Gyires et al. 2013). Thus, we investigated the effects of glutamine supplementation on mRNA levels for pro-inflammatory cytokines, including *Il-1β*, *Il-17*, *Ifn-γ* and *Tnf-α*. In the jejunum, glutamine supplementation did not affect mRNA levels for *Il-1β*, *Il-17*, *Ifn-γ* or *Tnf-α* (Fig. 7a). In contrast, glutamine supplementation markedly enhanced ($P < 0.05$) mRNA levels for *Il-1β*, *Il-17* and *Tnf-α*, and had no effect on the *Ifn-γ* mRNA level in the ileum (Fig. 7b).

Goblet cells and Paneth cells

To identify alterations in the intestinal innate immunity associated with the observed dysbiosis, we determined the expression of antimicrobial substances produced by Paneth cells or goblet cells, which play important roles in the maintenance of intestinal homeostasis (Bevins and Salzman 2011; Bergstrom et al. 2010). These substances include mucin 2 and 4, α-defensins, cryptdin-related sequence (CRS) peptides, lysozyme C, secretory group IIA phospholipase A2 (sPLA2), C-type lectins (REG3γ) and RNase angiogenin 4 (ANG4). In the jejunum, glutamine supplementation increased ($P < 0.05$) the mRNA levels for *Mucin 4*, *Cryptdin-1*, *4* and *5* and *Reg3γ*, compared to the controls (Table 3). In contrast, glutamine supplementation decreased ($P < 0.05$) the *Crs-4C* mRNA level and did not affect mRNA levels for *Mucin* 2, *Ang 4*, *Pla* 2, and *Lyz2* (Table 3). Likewise, glutamine supplementation had little effect on the abundance of the lysozyme protein in the jejunum (data not shown). Similar results were obtained for the ileum (Table 4). Finally, we found that glutamine supplementation did not affect mRNA levels for most antimicrobial substances in the colon, except *Reg3γ* and *Ang4* (Table 5).

## Discussion

Intestinal innate immunity serves as the first line of defense in animals in that it tolerates commensal bacteria and acts against the invasion of pathogens (Garcia-Miguel et al. 2013). Because of its key roles in maintaining intestinal homeostasis, identifying new means to enhance intestinal innate immunity has become a focus of active research on nutritional immunology (Ruth and Field 2013). As a functional amino acid (Wu 2013b), glutamine has regulatory roles in the metabolism and immune responses in mammals, such as mice, pigs and humans (Mondello et al. 2010; Ren et al. 2013a; Ruth and Field 2013; Zhong et al. 2012). Many ongoing investigations have shown that dietary glutamine supplementation enhances intestinal immunity (Ewaschuk et al. 2011) and influences immune development in newly weaned piglets (Johnson et al. 2006). However, less is known about the functions of dietary glutamine supplementation in intestinal innate immunity. In current study, dietary glutamine supplementation alters the intestinal bacterial community, enhances intestinal innate

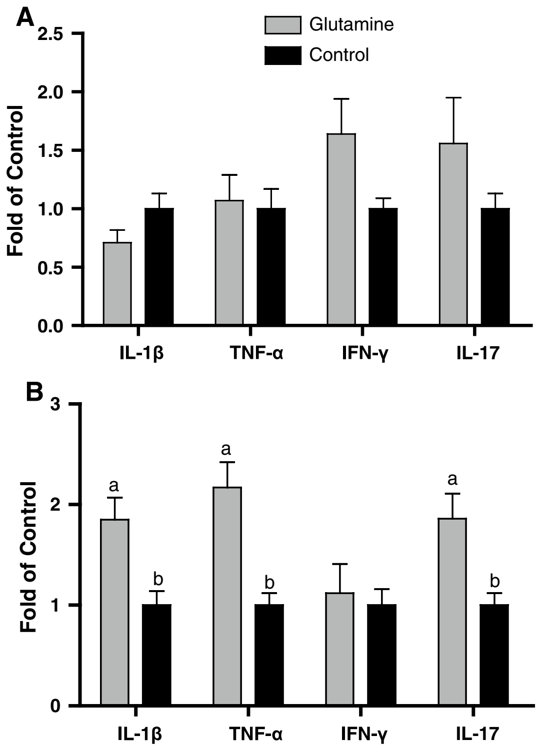


**Fig. 7** mRNA levels for pro-inflammatory cytokines in the mouse small intestine. Mice were fed a standard rodent diet supplemented with or without 1.0 % L-glutamine for 2 weeks. **a** The mRNA expression of pro-inflammatory cytokines in the jejunum. **b** The mRNA expression of pro-inflammatory cytokines in the ileum. Values are mean ± SEM, $n = 6$, with *a–b* used to indicate a statistically significant difference ($P < 0.05$). *Ifn* interferon, *Il* interleukin, *Tnf* tumor necrosis factor

**Table 3** mRNA levels for factors involved in innate immunity in the jejunum of mice supplemented with or without glutamine

| Gene | 1 % Glutamine | Control |
|---|---|---|
| *Mucin2* | 1.17 ± 0.36 | 1.00 ± 0.11 |
| *Mucin 4* | 1.76 ± 0.29* | 1.00 ± 0.09 |
| *Cryptdin-1* | 7.25 ± 1.29* | 1.00 ± 0.19 |
| *Cryptdin-4* | 4.76 ± 0.81* | 1.00 ± 0.13 |
| *Cryptdin-5* | 4.75 ± 0.84* | 1.00 ± 0.10 |
| *Crs-1C* | 1.55 ± 0.13 | 1.00 ± 0.12 |
| *Crs-4C* | 0.54 ± 0.10* | 1.00 ± 0.15 |
| *Spla2* | 0.62 ± 0.42 | 1.00 ± 0.33 |
| *Ang4* | 1.32 ± 0.27 | 1.00 ± 0.16 |
| *Reg3γ* | 2.32 ± 0.50* | 1.00 ± 0.13 |
| *Lyz2* | 1.14 ± 0.17 | 1.00 ± 0.52 |

Mice were fed a standard rodent diet supplemented with or without 1.0 % L-glutamine for 2 weeks. Values are mean ± SEM, $n = 6$

*Ang4* RNase angiogenin 4, *Crs* cryptdin-related sequence, *Lyz2* lysozyme 2, *Muc* mucin, *Reg3γ* regenerating islet-derived 3 gamma, *Spla2* secretory group IIA phospholipase A2

* Different from the control, $P < 0.05$

**Table 4** mRNA levels for factors involved in innate immunity in the ileum of mice supplemented with or without glutamine

| Gene | 1 % Glutamine | Control |
|---|---|---|
| *Mucin2* | 1.23 ± 0.18 | 1.00 ± 0.23 |
| *Mucin 4* | 1.13 ± 0.11 | 1.00 ± 0.19 |
| *Cryptdin-1* | 1.87 ± 0.38 | 1.00 ± 0.34 |
| *Cryptdin-4* | 2.61 ± 0.80* | 1.00 ± 0.34 |
| *Cryptdin-5* | 1.78 ± 0.58 | 1.00 ± 0.13 |
| *Crs-1C* | 1.30 ± 0.28 | 1.00 ± 0.24 |
| *Crs-4C* | 1.38 ± 0.27 | 1.00 ± 0.19 |
| *Spla2* | 3.21 ± 0.38 | 1.00 ± 0.16 |
| *Ang4* | 3.89 ± 0.63 | 1.00 ± 0.13 |
| *Reg3γ* | 3.65 ± 0.27* | 1.00 ± 0.10 |
| *Lyz2* | 1.03 ± 0.18 | 1.00 ± 0.18 |

Mice were fed a standard rodent diet supplemented with or without 1.0 % L-glutamine for 2 weeks. Values are mean ± SEM, $n = 6$

*Ang4* RNase angiogenin 4, *Crs* cryptdin-related sequence, *Lyz2* lysozyme 2, *Muc* mucin, *Reg3γ* regenerating islet-derived 3 gamma, *Spla2* secretory group IIA phospholipase A2

* Different from the control, $P < 0.05$

**Table 5** mRNA levels for factors involved in innate immunity in the colon of mice supplemented with or without glutamine

| Gene | 1 % Glutamine | Control |
|---|---|---|
| *Mucin2* | 1.25 ± 0.10 | 1.00 ± 0.18 |
| *Mucin4* | 1.26 ± 0.15 | 1.00 ± 0.16 |
| *Cryptdin-1* | 3.30 ± 1.62 | 1.00 ± 0.22 |
| *Cryptdin-4* | 0.66 ± 0.14 | 1.00 ± 0.22 |
| *Cryptdin-5* | 2.35 ± 0.91 | 1.00 ± 0.36 |
| *Crs-1C* | 0.63 ± 0.11 | 1.00 ± 0.11 |
| *Crs-4C* | 1.07 ± 0.28 | 1.00 ± 0.16 |
| *Spla2* | 0.81 ± 0.14 | 1.00 ± 0.16 |
| *Ang4* | 0.30 ± 0.12* | 1.00 ± 0.09 |
| *Reg3γ* | 2.78 ± 0.81* | 1.00 ± 0.23 |
| *Lyz2* | 1.17 ± 0.19 | 1.00 ± 0.22 |

Mice were fed a standard rodent diet supplemented with or without 1.0 % L-glutamine for 2 weeks. Values are mean ± SEM, $n = 6$

*Ang4* RNase angiogenin 4, *Crs* cryptdin-related sequence, *Lyz2* lysozyme 2, *Muc* mucin, *Reg3γ* regenerating islet-derived 3 gamma, *Spla2* secretory group IIA phospholipase A2

* Different from the control, $P < 0.05$

immunity, and affects the NF-κB, MAPK and PI3K-Akt signaling pathways.

The intestinal microbiota has critical roles in host health and is also a target of dietary nutrients (Hodin et al. 2012). The abundance and composition of intestinal bacteria can be easily affected by various dietary factors, such as food intake (Rist et al. 2012) and route of nutrient supply (enteral versus total parental nutrition) (Hodin et al. 2012). In this study, we found that glutamine supplementation affects the intestinal microbiota in the ileum and has little effect on the intestinal microbiota in the jejunum, as assessed from the ratio of Firmicutes to Bacteroidetes. They are the most abundant phyla of commensal bacteria in the intestine (Eckburg et al. 2005). The exact mechanism responsible for the decrease in intestinal Firmicutes after glutamine supplementation is unknown. One possible reason is that glutamine supplementation changes the intestinal microenvironment, thereby altering the composition of the intestinal microbiota (Dai et al. 2011). For example, glutamine supplementation regulates amino acid utilization and metabolism in small-intestinal bacteria (Dai et al. 2013), which may in turn affect the activity and number of certain microorganisms (Dai et al. 2012a, b). The discovery of the current study provides new insight into a role for the utilization of glutamine as an adjuvant therapy for various intestinal microbiota-associated diseases (e.g., obesity), which has high levels of intestinal Firmicutes (Ley et al. 2005).

Intestinal microbiota profoundly shapes intestinal innate immunity (Hooper et al. 2012). Based on our finding that glutamine supplementation does not affect the ratio of Firmicutes to Bacteroidetes in the jejunum, it is not surprising that no change in jejunal expression of the genes for TLRs is detected in mice receiving glutamine supplementation. In contrast, glutamine supplementation alters the ratio of Firmicutes to Bacteroidetes in the ileum and increases the ileal expression of *Tlr4*, which recognizes lipopolysaccharide in Gram-negative bacteria. However, it is unknown why glutamine supplementation decreases the expression of *Tlr5*, which detects flagellin from motile bacteria. Likewise, we have reported that glutamine supplementation decreases the mRNA level of *Tlr5* in the lung of *Pasteurella multocida*-infected mice (Ren et al. 2013d). In accordance with the increased expression of *Tlr4*, the expression of other intestinal innate factors also increases in the ileum, including pro-inflammatory cytokines, and antimicrobial substances produced by goblet cells and Paneth cells. This observation is similar to the previous report that arginine supplementation changes the intestinal microbiota, contributing to the activation of TLRs signaling, the enhanced expression of pro-inflammatory cytokines, and the activation of intestinal goblet cells and Paneth cells (Ren et al. 2014b). Similarly, Hodin et al. (2012) demonstrated that a shift in the Firmicutes to Bacteroidetes ratio in the intestine is associated with the activation of Paneth cells. In addition to inducing the changes in the abundance of Firmicutes, *Streptococcus* and *Bifidobacterium* in the jejunum, glutamine supplementation enhances the jejunal expression of the genes for antimicrobial substances produced by goblet cells and Paneth cells.

In the intestine, the activated pattern recognition receptors, like TLRs, trigger intestinal immune responses through various downstream signal transductions, such as NF-κB, MAPK and PI3K-pAkt (Ren et al. 2014a, b). In the present work, we found that glutamine supplementation has no effect on the expression of TLRs in the jejunum, enhances the expression of *Tlr4* in the ileum, and decreases the protein abundance of nuclear p65 in the jejunum and ileum. Thus, it is worthy to investigate the impacts of glutamine supplementation on the activation of other pattern recognition receptors, like NOD-like receptors (NRLs), which are pivotal cytoplasmic receptors for the maintenance of microbial communities in the gut (Rosenstiel 2013). Indeed, previous investigations have demonstrated that glutamine inactivates the NF-κB pathway at cellular and molecular levels (Haynes et al. 2009; Ren et al. 2013c, d). In the jejunum, glutamine supplementation inhibits the activation of PI3K-Akt, but has little effect on the MAPK. In contrast, glutamine supplementation activates the JNK in the ileum, which could, at least, partially explain the enhanced expression of pro-inflammatory cytokines in the ileum. The discrepancy in the effects of glutamine supplementation on MAPK and PI3K-Akt pathways between this and previous studies (Ren et al. 2013d) could result, in part, from the different animal models. Although glutamine supplementation exerts an inhibition on NF-κB and PI3K-Akt activation, there is no change in the expression of intestinal pro-inflammatory cytokines in the jejunum. Thus, it is interesting to determine the effects of glutamine supplementation on other intestinal inflammatory signaling pathways, such as signal transducer and activator of transcription (STAT), peroxisome proliferator-activated receptor-γ (PPARγ), and activating protein-1 (AP-1). Collectively, the finding that glutamine supplementation inhibits the NF-κB pathway has important implications for explaining beneficial roles for glutamine in intestinal inflammatory diseases. This supports the notion that animals have requirements for dietary glutamine to sustain optimal immune responses and health (Wu et al. 2013b; Wu 2014).

Intriguingly, the effects of glutamine supplementation on the intestinal innate immune response differ among the jejunum, ileum and colon. Similar results have also been reported in previous studies involving amino acid metabolism in intestinal bacteria (Dai et al. 2010, 2012a, b), as well as dietary supplementation with arginine and proline (Ren et al. 2013e, 2014a). Such differential responses may be related to different microenvironments among the various segments of the gut at molecular and cellular levels (Dai et al. 2011; Hou et al. 2014). Glutamine is mainly absorbed in the proximal region of the small intestine, resulting in little entry, if have, into the colon (Wu 2009; Wu et al. 2014). Another possible reason is that the intestinal microbiota in the large intestine is more complex than those in the small intestine, resulting in differences in concentrations of microbial metabolites (e.g., short-chain fatty acids and ammonia) (Bergen and Wu 2009). In support of this view, results of previous studies indicate that the responses of animals to dietary supplementation differ among different tissues (Ren et al. 2013e, d; Wu 2009).

In conclusion, glutamine supplementation induces the alteration of the intestinal microbiota and the activation of the TLRs signaling pathway. Consequently, these changes increase the expression of pro-inflammatory cytokines and the activity of goblet cells and Paneth cells through NF-κB, MAPK, and PI3K-pAkt signaling pathways. The effects of glutamine supplementation on the intestinal microbiota and innate immunity are dependent on the segment of the intestine with different populations of bacteria. Our results aid in enhancing our understanding of basic knowledge about the nutrition, physiology and immunology of glutamine as a functional amino acid in animals.

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**Conflict of interest** The authors declare that they have no conflict of interests.

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