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ORIGINAL ARTICLE

The effect of aspartate supplementation on the microbial composition and innate immunity on mice

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Abstract The study was conducted to investigate the changes of intestinal microbiota composition and innate immunity with different dietary dosages of aspartate (Asp) supplementation. Thirty-six female ICR mice were divided randomly to four groups and thereafter fed the basal diets (controls) or those supplemented with additional 0.5, 1.0 and 2.0% aspartate. After 2 week feeding, microbial composition in ileum and feces, gene expression of pro-inflammatory cytokine, and innate immune factors in ileum were determined. The ratio of *Firmicutes*: *Bacteroidetes* in ileum and feces decreased in 0.5 and 1.0% Asp-supplemented groups, whereas this ratio increased in feces in 2.0% Asp-supplemented group. Meanwhile, the gene expression of IL-17 and IFN-γ in ileum decreased in 1.0% Asp-supplemented group; the gene expression in ileum of Muc2 decreased in 0.5 and 1.0% Asp-supplemented groups. Dietary supplementation with 2.0% Asp enhanced the expression of pIgR and Crp1 as compared to the other three groups. The results indicated that dietary 1.0% Asp supplementation lowers the ratio of *Firmicutes*:*Bacteroidetes*, which affects the innate immunity by decreasing the gene expression of IL-17, IFN-γ, and Muc2 in ileum.

Keywords Aspartate · Intestinal microbiota · Innate immunity

Abbreviations

iNOS	Inducible nitric oxide synthase
TLR	Toll-like receptor
Asp	Aspartate
IL-17	Interleukin-17
IFN-γ	Interferon-gamma
pIgR	Polymeric immunoglobulin receptor
Muc2	Mucin-2
Muc4	Mucin-4
Muc6	Mucin-6
Crp1	Cryptdins-1
Crp4	Cryptdins-4
Crp5	Cryptdins-5
Lzy2	Lysozyme 2
SCFA	Short-chain fatty acid
NOD	Nucleotide-binding oligomerization domain protein

Introduction

Aspartate is a non-essential amino acid in the mammals. As a member of arginine family, aspartate can be transformed into other arginine family of amino acids including Arginine, Glutamine, and Glutamate to exert its beneficial function (Wu et al. 2007). It is also crucial for the proliferation of lymphocytes by serving as a substrate for the synthesis

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of purine and pyrimidine nucleotides (Newsholme and Calder 1997). In addition, aspartate is conductive to regulating the immune function through the synthesis of arginine and asparagine (Li et al. 2007). In the synthesis of arginine, aspartate helps to maintain an adequate intracellular concentration of arginine in response to immunological challenges. As the direct product of aspartate (Wu 2009), asparagine increased the expression and activity of ornithine decarboxylase for polyamine synthesis in thymocytes and inducible nitric oxide synthase (iNOS) in activated macrophages (Li et al. 2007). Accumulating evidence indicates that aspartate plays a crucial role in nutrition (Wu 2010, 2013), metabolism (Barrio et al. 1982; Blachier et al. 2013), immunity (Li et al. 2007), and oxidative stress (Duan et al. 2016).

Intestinal microbiota and innate immunity is important in the maintenance of mucosal homeostasis (Lee and Hase 2014; Ivanov and Dan 2011; Hodin et al. 2012). Our previous studies demonstrated that dietary supplementation with arginine and glutamine altered intestinal microbiota and modulated innate immunity (Ren et al. 2014a, b). The interaction of intestinal microbiota and immune system keeps intestinal homeostasis, when the intestinal dysbiosis occurs, the abnormal changes affect multiple signaling pathways and thus result in the secretion of pro-inflammatory cytokines (Ren et al. 2014b). Intestinal pro-inflammatory cytokines (e.g. IL-17, IFN-γ), which regulated by intestinal microbiota and toll-like receptor (TLR) signaling pathway, are closely related to the host defense (Jin et al. 2012; Cao et al. 2014).

Thus, we hypothesized different levels of aspartate supplementation may change the composition of microbiota in the ileum and feces and modulate the expression of pro-inflammatory cytokines and innate immune factors in ileum.

Materials and methods

Experiment design

Thirty-six female ICR (Institute for Cancer Research) mice (6 weeks of age) were obtained from SLAC Laboratory Animal Central (Changsha, China). The mice were housed in pathogen-free colonies (temperature, 20–30 °C, relative humidity, 50–60 °C, 12 h dark/12 h light) and had free choice of standard rodent feed and drinking water. After 3 days of acclimation, mice were divided into four groups (the control group, 0.5% Asp-supplemented group, 1.0% Asp-supplemented group, and 2.0% Asp-supplemented group, $n = 9$ per group) according to a completely randomized design. Mice in the control group were fed with a basal diet; mice of 0.5% Asp-supplemented group were fed with a basal diet supplemented with 0.5% aspartate (0.5% Asp + basal diet); mice of 1.0% Asp-supplemented group were fed with a basal diet supplemented with 1.0% aspartate (1.0% Asp + basal diet); mice of 2.0% Asp-supplemented group were fed with a basal diet supplemented with 2.0% aspartate (2.0% Asp + basal diet). All mice were killed after 2-week aspartate supplementation, and the feces and the content of ileum were collected at necropsy; the samples were stored at −80 °C for analysis. The ileum tissues were frozen in liquid nitrogen and stored at −80 °C for further analysis.

16 s DNA sequencing with illumina MiSeq sequencing

DNA was extracted from the luminal content of ileum and feces using the Qiagen QIAamp DNA stool Mini Kit according to the protocol for isolating DNA. Equal amounts of DNA from six different mice were pooled to generate one common sample for each type of sample. Illumina MiSeq sequencing and general data analysis were performed by a commercial company (Biotree, Shanghai, China).

Rt-PCR

Total RNA of ileum was isolated from liquid nitrogen-frozen ileum using TRIZOL (Invitrogen, USA) and then treated with DNase I (Invitrogen, USA) according to the manufacturer's instruction. The concentration of total RNA in each sample was determined spectrophotometrically at 260 nm. Primer sequences used in this study were according to previous reported (Ren et al. 2014c, d) (Table 1). For controlling to normalize target gene transcript levels, β-actin was used as an internal control. The amplification reactions were carried out in an ABI Prism 7900 HT sequence detection system (Applied Biosystems, Foster, CA, USA). The relative levels of genes were expressed as a ratio of the target gene to the control gene using the formula $2^{-(\Delta\Delta Ct)}$, where $Ct = \left(Ct_{target} - Ct_{\beta-actin}\right)_{treatment} - \left(Ct_{target} - Ct_{\beta-actin}\right)_{control}$

Statistical analysis

Data are presented as means ± SEMs, were analyzed statistically using IBM Statistics SPSS 22.0 software. Differences among treatment means were determined using the Duncan and LSD multiple comparison test. A P value less than 0.05 were taken to indicate statistical significance.

Table 1 Primer pairs used in the RT-PCR

Gene	ID	Nucleotide sequence of primers (5′–3′)	Product length
β-Actin	NM_007393.3	F: GTCCACCTTCCAGCAGATGT R: GAAAGGGTGTAAAACGCAGC	117
IL-17	NM_010552.3	F: TACCTCAACCGTTCCACGTC R: TTTCCCTCCGCATTGACAC	119
Ifn-γ	NM_008337.4	F: ATGAACGCTACACACTGCATCTTGGCTT R: CCTCAAACTTGGCAATACTCATGAATGC	361
pIgR	NM_011082.3	F: AGTAACCGAGGCCTGTCCTT R: GTCACTCGGCAACTCAGGA	66
J-chain	NM_152839.3	F: GAACTTTGTATACCATTTGTCAGACG R: CTGGGTGGCAGTAACAACCT	88
Muc2	NM_023566.3	F: CCCAGAAGGGACTGTGTATG R: TTGTGTTCGCTCTTGGTCAG	276
Muc4	NM_080457.3	F: GTCTCCCATCACGGTTCAGT R: TGTCATTCCACACTCCCAGA	281
Crp1	NM_010031.2	F: CTAGTCCTACTCTTTGCCCT R: TTGCAGCCTCTTGATCTACA	206
Crp4	NM_010039.1	F: GTCCAGGCTGATCCTATCCA R: GGGGCAGCAGTACAAAAATC	222
Crp5	NM_007851.2	F: GTCCAGGCTGATCCTATCCA R: GATTTCTGCAGGTCCCAAAA	202
Lyz2	NM_013590.4	F: GAATGGAATGGCTGGCTACT R: CGTGCTGAGCTAAACACACC	62

Results

Asp influenced intestinal microbiota

The taxonomy of the intestinal microbiota was assessed by the taxon-dependent analysis. In the four groups, six phyla were detected in the ileum contents and nine phyla were detected in feces. *Firmicutes* dominated the ileum microbiota with a percentage of 99.32% in the control group, 99.17% in 0.5% Asp-supplemented group, 99.29% in 1.0% Asp-supplemented group, and 99.35% in 2.0% Asp-supplemented group (Fig. 1a). For the fecal microbiota, the three most abundant phyla were *Bacteroidetes*, *Firmicutes,* and *Proteobacteria*. In the control group, the abundances of *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* accounted for 62.5, 31.93, and 2.00%, respectively. In the 0.5% Asp-supplemented group, the abundances of *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* accounted for 76.18, 21.17, and 2.04%, respectively. In the 1.0% Asp-supplemented group, the abundances of *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* accounted for 77.74, 19.40, and 1.54%, respectively. In the 2.0% Asp-supplemented group, the abundances of *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* accounted for 52.77, 39.35, and 4.28%, respectively (Fig. 1b). In feces, the ratio of *Firmicutes: Bacteroidetes* was lower in the 0.5% Asp-supplemented and 1.0% Asp-supplemented groups than in the control group, with a percentage of 23.24 and 26.07%, respectively. Interestingly, the ratio of *Firmicutes* to *Bacteroidetes* was increased in the 2.0% Asp-supplemented group than in the control group, with a percentage of 83.03% (Fig. 1c).

Ten orders were detected in the ileum contents in all the four groups, *Lactobacillales* dominated the ileum microbiota with a percentage of 96.42% in the control group, 98.04% in 0.5% Asp-supplemented group, 98.33 in 1.0% Asp-supplemented group, and 98.18 in 2.0% Asp-supplemented group (Fig. 1d). Eighteen orders were detected in the fecal microbiota, and the three most abundant orders were *Bacteroidales*, *Lactobacillales*, and *Clostridiales*. In the control group, *Bacteroidales*, *Lactobacillales,* and *Clostridiales* were 62.55, 26.47, and 4.60%, respectively. In the 0.5% Asp-supplement group, *Bacteroidales*, *Lactobacillales*, and *Clostridiales* were 76.14, 13.29, and 7.24%, respectively. In the 1.0% Asp-supplement group, the percentages were *Bacteroidales* (77.73%), *Lactobacillales* (10.42%) and *Clostridiales* (8.43%). In the 2.0% Asp-supplement group, the percentages were *Bacteroidales* (39.34%), *Lactobacillales* (27.45%), and *Clostridiales* (22.33%) (Fig. 1e).

Collectively, aspartate supplementation affects the intestinal microbiota in mice, especially affecting the ratio of *Firmicutes*: *Bacteroidetes* in feces. Dietary 0.5% Asp and 1.0% Asp supplementation lowers the ratio of *Firmicutes*:*Bacteroidetes*, whereas the dietary 2.0% Asp increased the ratio of *Firmicutes*: *Bacteroidetes*.

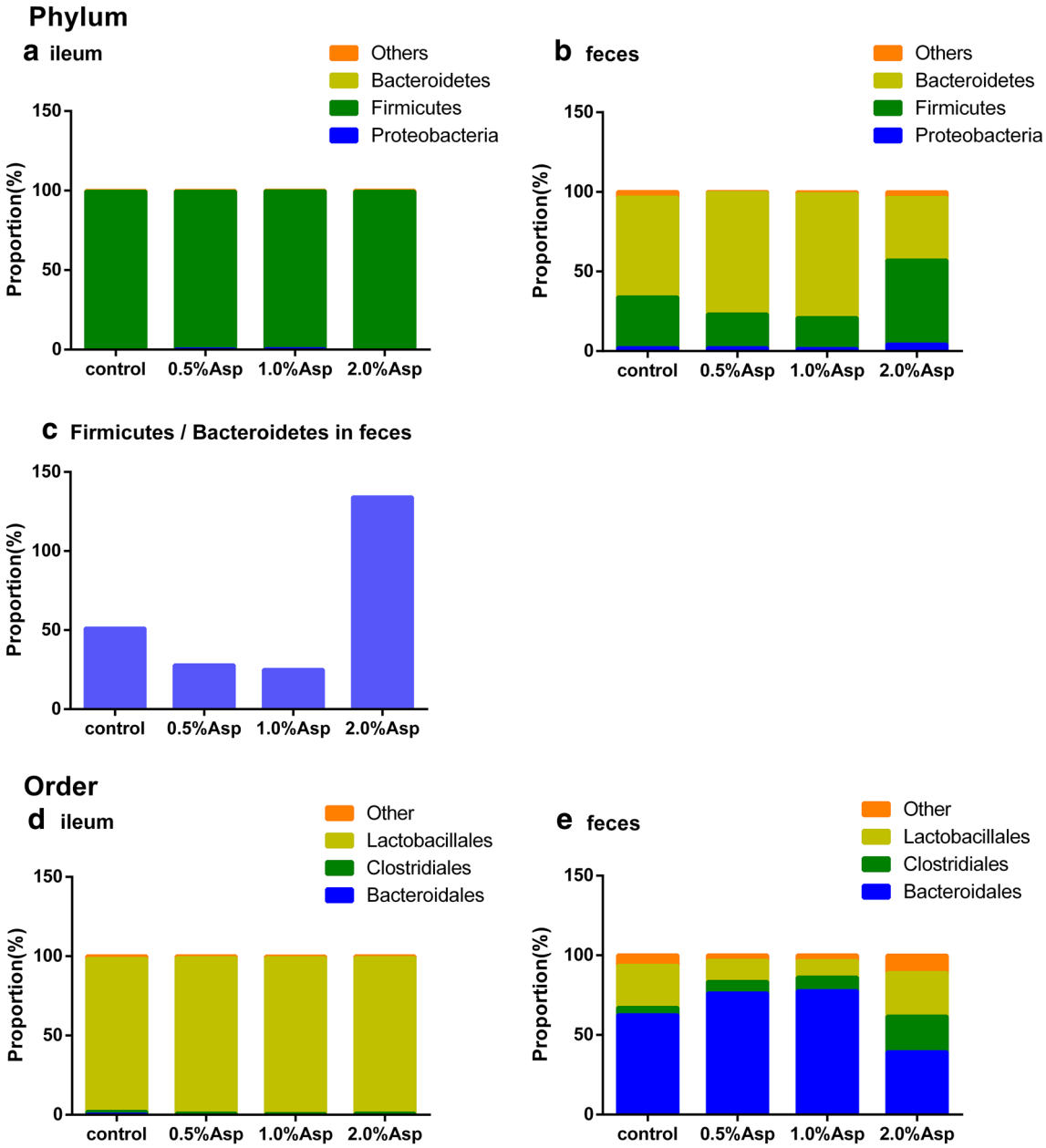

Fig. 1 Aspartate supplementation influences intestinal microbiota. **a–b** Microbial composition in the ileum and feces in the control group, 0.5% Asp group, 1.0% Asp group and 2.0% Asp group in the phylum. **c** Ratio of *Firmicutes:Bacteroidetes* in the feces of the control group, 0.5% Asp group, 1.0% Asp group, and 2.0% Asp group. **d–e** Microbial composition in the ileum and feces in the control group, 0.5% Asp group, 1.0% Asp group, and 2.0% Asp group in the order. Mice in control group received basal diet, while mice in 0.5% Asp group received basal diet + 0.5% Asp; mice in 1.0% Asp group received basal diet + 1.0% Asp; mice in 2.0% Asp group received basal diet + 2.0% Asp

Asp affected intestinal innate immunity

We analyzed the gene expression of intestinal pro-inflammatory cytokines [i.e., interleukin-17 (IL-17) and interferon–gamma (IFN-γ)], and innate immune factors, such as J-chain of secretory IgA (J-chain), polymeric immunoglobulin receptor (pIgR), mucin-2 (Muc2), mucin-4L(Muc4), cryptdins-1 (Crp1), cryptdins-4 (Crp4), cryptdins-5 (Crp5), and lysozyme 2 (Lzy2) in the ileum in this study. The results showed that dietary supplementation with 1.0% Asp decreased the expression of IL-17 and IFN-γ as compared to the control group and 2.0% Asp-supplemented group ($P < 0.05$) (Fig. 2a). In addition, dietary supplementation with 1.0% Asp and 2.0% Asp lowered the mRNA level of

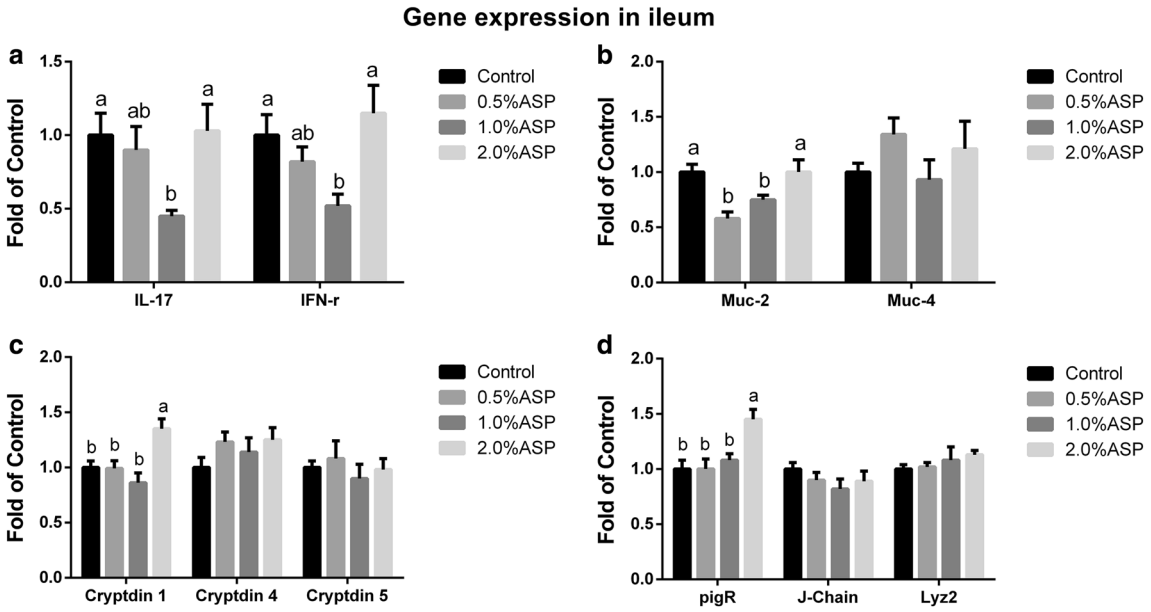

Fig. 2 Asp supplementation affects intestinal innate immunity may through intestinal microbiota. **a** Expression of pro-inflammatory cytokines (Il-17, IFN-γ). **b** Expression of mucin (Muc2, Muc4). **c** Expression of α-defensins (Crp1, Crp4, and Crp5). **d** Expression of other innate immune factors (pIgR, J-chain, Lzy2). Mice in control group received basal diet, while mice in 0.5% Asp group received basal diet + 0.5% Asp; mice in 1.0% Asp group received basal diet + 1.0% Asp; mice in 2.0% Asp group received basal diet + 2.0% Asp. The values having different superscript letters are different ($P < 0.05$)

Muc2 compared with the control group ($P < 0.05$) and 2.0% Asp-supplemented group ($P < 0.05$) (Fig. 2b). Interestingly, dietary supplementation with 2.0% Asp enhanced the expression of pIgR and Crp1 as compared to the other three groups (Fig. 2c, d). Nevertheless, there were no statistical differences in the expression of Muc4, Crp4, Crp5, Lzy2, and J-chain among the four groups (Fig. 2b–d).

Discussion

Intestinal microbiota and innate immunity are important factors in the maintenance of mucosal homeostasis. Researches enunciated that aspartate enhances intestinal integrity and contributes to the modulation of immune responses (Li et al. 2007; Pi et al. 2014). Our previous studies have demonstrated that dietary arginine or glutamine supplementation in mice altered the intestinal microbiota, contributing to the activation of intestinal innate immunity (Ren et al. 2014a, b). In this study, we explored the effects of different levels of Asp supplementation on the innate immune responses and ileum and fecal microbiota.

In general, the *Firmicutes*-to-*Bacteroidetes* ratio is an important indicator in gut microbiota composition (Mariat et al. 2009). As *Firmicutes* can provide additional energy for host by fermenting polysaccharide to short-chain fatty acid (SCFA) (Ley et al. 2006), the lower ratio of *Firmicutes* to *Bacteroidetes* may be linked to lose weight (Xiao et al. 2016). In addition, enhanced population of *Bacteroidetes* promotes an intimate interaction between the microbiota and host enterocytes by consuming glycans released from the mucus (Sonnenburg et al. 2005). As shown in our results, 0.5 and 1.0% supplementation of Asp decreased the ratio of *Firmicutes* to *Bacteroidetes* in feces. In contrast, 2.0% Asp supplementation increased the ratio of *Firmicutes* to *Bacteroidetes* in feces. This result indicates that dietary supplementation with a low dose of aspartate increases the abundance of *Bacteroidetes* or decreases the abundance of *Firmicutes* in the intestinal. However, dietary supplementation with 2.0% Asp plays an opposite role. As diet is an external factor that directly affects the composition of microbiota (Hildebrandt et al. 2009), and aspartate supplementation regulated the metabolism and utilization of amino acids by bacteria in small intestinal (e.g., *Clostridium perfringens, Bacteroides fragilis,* and *Acidaminococcus fermentans*) (Dai et al. 2011), thus alters the composition and activity of some microorganism. Furthermore, this change may have some connection with the dose of aspartate supplementation. This study provides an insight that the dietary with suitable does of aspartate may change the results of the microbiota-associated diseases (e.g., obesity).

Emerging evidence showed that aspartate played a crucial role on the intestine physiological functions

and innate immune response (Pi et al. 2014). Activation of TLR4 and nucleotide-binding oligomerization domain protein (NOD) signaling pathway are related to the defensive responses of the host via the production of pro-inflammatory cytokines and inflammatory response. However, the collateral host-tissue injury could also be caused by the abnormal activation of TLR4 and NOD signaling (Akira 2012; Coll and O'Neill 2010). Therefore, negative regulator of the TLR4 and NOD signaling is also necessary for the immunologic balance. It is reported that Asp down-regulated the mRNA expression of TLR and NOD signaling-related genes (Wang et al. 2016) and thus decreased the expression of the pro-inflammatory cytokines, and this regulation may be related to the dose of aspartate supplementation. In this study, 1.0% supplementation of aspartate inhibited the expression of IL-17 and IFN-γ compared with the other three groups. IL-17 is a kind of pro-inflammatory cytokine which produced by mostly produced by Th-17 cells, followed by gd T cells, NKT cells, and NK cells, it can stimulate the epithelial cells, endothelial cells, and fibroblasts to release multiple cytokines that lead to inflammation. IFN-γ is produced by Th-1 cells, NKT cells, NK cells, and some cytotoxic CD8 T cells, and it plays a key role on immune regulation approach stimulating the macrophage producing pro-inflammatory cytokines and activating natural killer cell. Our results indicate that the suitable dose of aspartate in dietary has potential to treat the intestinal chronic inflammatory diseases which involved the gene expression of IL-17 and IFN-γ.

Mucins play a role in the protection of digestive tract against bacterial attachment and penetration (Kim et al. 2008), whereas the excessive secretion of mucin destroys the mucus barriers. It is reported that the absence of the intestinal mucus layer activates the mucosal immune system (Hartmann et al. 2015), and Muc2 as the major component of the intestinal mucus layer indicated that the decreased gene expression of Muc2 activated the mucosal immune system. In this study, 0.5% Asp-supplemented and 1.0% Asp-supplemented groups decrease the expression of Muc2 significantly compared with the control and 2.0% Asp-supplemented group, but there is no statistical difference in the gene expression of Muc4 and Muc6 between the four groups. One possible explanation for this observation is that the reduction of IFN-γ decreased the expression of Muc2, as IFN-γ upregulate the expression of Muc2 (Lee et al. 1937). Although there is no significant difference between control group and 0.5% Asp-supplemented group, a tendency shows that 0.5% aspartate supplementation decreases the gene expression IFN-γ. Our result suggests that dietary with 0.5 and 1.0% supplementation of aspartate inhibits the expression of Muc2 and thus keeps the balance of mucus barriers.

IgA is the predominant immunoglobulin class in mucosal secretions, and pIgR plays crucial role in the transportation of IgA (Peters et al. 2003). How aspartate effects the expression of pIgR in the ileum is unknown. However, the previous studies suggested that the cytokines produced by epithelial cell (e.g., IFN-γ and TNF-α) up-regulated the expression of pIgR (Reséndizalbor et al. 2010). α-defensins are broad-spectrum antimicrobial peptides produced by the Paneth cells, and they are regarded as an important component of innate immunity in the small intestine (Ouellette and Bevins 2001). It is reported that Crp 1 induce TLR4-independent IL-6 production and increase the intestinal inflammatory response (Vandenbroucke et al. 2014). Our results indicate that 2.0% Asp supplementation increases the expression of Crp1. One possible reason is that 2.0% aspartate supplementation alters the intestinal microbiota composition, thus increases the expression of Crp1, and promotes the release of antimicrobial peptides (Nakamura et al. 2015). As 0.5 and 1.0% aspartate supplementation also altered the intestinal microbiota, whereas it is no change in Crp1 gene expression, it may be related to the changed microbial species (e.g., *Firmicutes* and *Bacteroidetes*). As 0.5 and 1.0% Asp-supplemented group decreased the *Firmicutes* and increased *Bacteroidetes*, whereas 2.0% Asp-supplemented group is opposite.

In summary, dietary with 0.5 or 1.0% aspartate supplementation lowered the ratio of *Firmicutes*:*Bacteroidetes* in feces, and decreased the expression of pro-inflammatory cytokines (IL-17, IFN-γ) and Muc2. In addition, dietary with 2.0% aspartate supplementation increases the expression of pIgR and Crp 1. This study is the first to investigate the effect of different doses of supplemental aspartate to the intestinal microbiota and intestinal immunity. This research will help guiding the use of aspartate supplementation.

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Author contributions G.L., R.H., and Y.Y. conceived the experiment(s), P.B. and S.L. conducted the experiments, P.B., S.L., and G.L. analyzed the results. P.B., G.L., and C.A.H. prepared the manuscript. All authors reviewed the manuscript.

Compliance with ethical standards

Ethical approval The protocol for this study was approved by the Committee on the Ethics of Animal Experiments of Institute of Subtropical Agriculture, Chinese Academy of Sciences, and it was conducted out in accordance with the recommendations in the Guide for

the Care and Use of Laboratory Animals of Institute of Subtropical Agriculture, Chinese Academy of Sciences.

Conflict of interest The authors declare that there is no conflict of interest regarding the publication of this article.

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