# Effects of supplemental β-carotene on growth performance, immune function and intestinal mucin in weaned piglets

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# Abstract

Background Weaning stress causes intestinal immune system disorders. β-carotene displays anti-inflammatory,which can prevent the development of inflammatory diseases. The aim of this study was to determine the effect of supplemental β-carotene on growth performance, immune function and intestinal mucin in weaned piglets.

Results A total of 45 piglets with the average body weight of 4.62 ± 0.06 kg (14 d of age) were randomly assigned to 3 treatments with 5 replicate pens per treatment and 3 pigs per pen and weaned at 21 d. The control group (CG) fed the basal diets from 14 d to 24 d of age. The low-dose group (LG) and the high-dose group (HG) fed the basal diets supplemented with 40 or 80 mg/kg β-carotene from 14 d to 24 d of age, respectively. Compared with the CG, t he average daily gain (ADG) and average daily feed intake (ADFI) of piglets in the HG were increased ($p<0.05$). And the IgA and IL-10 levels in serum in the HG were also higher than those in the CG ($p<0.05$). The IgM, TNF-α and IL-6 levels in serum in the β-carotene groups were lower than those in the CG ($p<0.05$). Periodic Acid-Schiff stain results showed that the content of mucopolysaccharide and the number of goblet cells in the intestine in the β-carotene groups were more than those in the CG ($p<0.05$). The results of immunofluorescence showed that the expression of MUC1 in the intestine in the β-carotene groups was lower than that in the CG ($p<0.05$). And the expression of mucin1 mRNA in the intestine in the β-carotene groups was also lower than that in the CG ($p<0.05$). However, compared with the CG, the expression of mucin2 mRNA in the intestine in the HG was increased ($p<0.05$).

Conclusion Supplemental β-carotene could improve growth performance, immune function and intestinal mucin in weaned piglets. It is suspected that our pre-protection of β-carotene in weaned piglets may help weaned piglets safely through the weaning period.


# Background

Weaning causes stress syndrome in weaned piglets, resulting in digestive dysfunction, growth retardation, diarrhea, enteritis and even death [1]. Hur and Lee [2] pointed out that weaning reduced feed intake, feed conversion rate and slower growth of piglets. Additionally, after weaning, the bacteria, LPS or toxins in the intestinal tract can disrupt immune system disorder and cause inflammation. Kelly et al [3] showed that weaning stress reduced antibody levels and inhibits cellular immunity. Pie et al [4] found that weaning increased gene expression of IL-6, TNF-α and IL-1β in jejunum of piglets. Hu et al [5] also reported that the expression of proinflammatory cytokines IL-6, TNF-α and IFN-γ mRNA in piglets was increased by weaning, but the expression of TGF-β and IL-10 mRNA was not significant. And other studies had reported that mucin1 (MUC1) and MUC2 were associated with the development of colitis [6–8]. It proves that intestinal mucin is closely related to intestinal inflammation.

β-carotene is a precursor of vitamin A (VA) activity. In previous research, β-carotene is shown to prevent the development of inflammatory diseases in the rats, calves and poultry. Chew and Jean Soon [9] found

that β-carotene stimulated the proliferation of lymphocytes in animals and enhanced cell-mediated humoral immune responses, which has a positive impact on immune response. Blum [10] reported that supplementation with VA and β-carotene enhanced the immune system in neonatal calves. Cucco et al [11] found that supplementation with β-carotene to poultry promoted growth and improved immunity. Another study showed that β-carotene enhanced the induction of mucosal IgA in weaned mice [12]. Additionally, Hwa and Shiau [13] pointed out that rats fed diets containing agar with 0 and 0.3 mg/l00 g diet β-carotene had higher mucinase activity than those fed diets witbout agar in fecal. To date, the effect of β-carotene on weaned piglets has not been reported. In our experiment, we aimed to investigate the effects of β-carotene on growth performance, immune function and intestinal mucin in weaned piglets.

# Results

### Effects of β-carotene on growth performance of weaned piglets

From 21 d to 24 d of age, the ADG and ADFI of weaned piglets in the HG were higher than those in the CG ($p$<0.05). But, the FCR of weaned piglets in the HG was lower than that in the CG ($p$<0.05). There was no significant difference in the ADG, ADFI and FCR between the LG and the CG ($p$>0.05) (Table 3).

### Effects of β-carotene on immunoglobulin and immune factors in serum of weaned piglets

At 24 d of age, the IgA and IL-10 levsls in serum in the HG were significantly higher than those in the CG ($p$<0.05) (Figure 1 A and G). However, compared with the CG, the supplementation of β-carotene significantly reduced the IgM and TNF-α levels in serum ($p$<0.05) (Figure 1 C and D). And the IL-6 level in serum in the HG was lower than that in the CG ($p$<0.05) (Figure 1 E). Supplementation with β-carotene had no significant effect on IgG and IL-8 ($p$>0.05) (Figure 1 B and F).

### Effects of β-carotene on the number of goblet cells and mucopolysaccharide secretion in the small intestine of weaned piglets

To verify the effect of β-carotene on MUC2, we investigated the effects of β-carotene on PAS staining of goblet cells and mucopolysaccharide. The results showed that the secretion of mucopolysaccharide in the small intestine in the β-carotene groups was significantly higher than that in the CG at 24 d of age (Figure 2 A). And the number of goblet cells in the duodenum, jejunum and ileum in the β-carotene groups was more than that in the CG ($p$<0.05). And the number of goblet cells in the ileum in the HG was significantly more than that in the CG ($p$<0.01) (Figure 2 B).

### Effects of β-carotene on the expression of MUC1 protein in the small intestine of weaned piglets

Using the immunofluorescence method confirmed the effect of β-carotene on the MUC1 expression in the small intestine. The results showed that supplementation of β-carotene significantly reduced the expression of MUC1 in the duodenum, jejunum and ileum compared with the CG (Figure 3).

### Effects of β-carotene on the expression of MUC1 and MUC2 mRNA in the small intestine of weaned piglets

The real-time fluorescence quantitative results showed that the expression of MUC1 mRNA in the duodenum, jejunum and ileum in the β-carotene groups was lower than that in the CG ($p$<0.05) (Figure 4 A). However, the expression of MUC2 mRNA in the duodenum, jejunum and ileum in the HG was higher than that in the CG, and the expression of MUC2 mRNA in the duodenum of the weaned piglets in the LG was higher than that in the CG ($p$<0.05) (Figure 4 B).

## Discussion

Weaning stress causes diarrhea, decreased growth performance, and even death in piglets, which brings huge economic losses to the pig breeding industry. Our results showed that the ADG and ADFI of weaned piglets in the HG were significantly higher than those in the CG. But, the FCR of weaned piglets in the HG was lower than that in the CG. The effect of β-carotene on animal growth performance is mainly concentrated on poultry and mice, and everyone has different opinions. Cucco et al [11] had confirmed that supplementation with β-carotene to poultry promoted growth. However, Nishiyama, Y et al [15] showed that feeding 50 mg/kg β-carotene to weaned mice had no effect on daily weight gain in mice. We suspect that the results may be different due to the different animal species studied, the experimental period and the dose of supplementation.

In addition, weaning stress causes intestinal immune system disorders. Pié et al [4] reported that weaning stress up-regulated expression of proinflammatory cytokines (TNF-γ, IL-6 and IL-1β) in the intestine of 28-d-old weaned piglets. And Hu et al [5] showed that weaning stress increased expression of TNF-α and IL-6 mRNA on 3 d and 7 d of post-weaning. By detecting the immunoglobulins and inflammatory factors in serum of weaned piglets, we found that compared with the CG, the IgA and IL-10 levels in serum in the HG were increased after weaning, but the levels of IgM, TNF-α and IL-6 in serum of weaned piglets were reduced at 24 d, and the IgM level in serum in the LG was reduced. Nishiyama, Y et al [16] found that newborn mice fed with 50 mg/kg β-carotene significantly increased the IgA level in serum of 14 d and 28 d mice. Keita et al [12] had showed that supplemental β-carotene in weaning mice was effective to enhance mucosal IgA induction in the jejunum or ileum. In previous study, Boon [17] also showed that β-carotene promoted lymphocyte proliferation and stimulated the production of various cytokines, thereby enhancing the body's immune response. The results suggested that β-carotene enhanced immune function in weaned piglets.

Other study had pointed out that mucins play an important role in the activation of immune cells [18]. Our results showed that the supplementation with β-carotene significantly increased the secretion of mucopolysaccharides and the number of goblet cells in the duodenum, jejunum and ileum, but supplementation with β-carotene significantly reduced the expression of MUC1 protein in the duodenum, jejunum and ileum compared with the CG. The results of the expression of MUC1 protein was similar with expression of MUC1 mRNA. But the expression of MUC2 mRNA in the duodenum, jejunum and ileum in

the HG were higher than that in the CG. In the early study, it was also found that mucin secretion and expression were associated with the development of inflammatory bowel disease [19]. Piel et al [20] found that feeding carboxymethyl cellulose to post-weaning piglets increased ileal mucin secretion and goblet cell number, thereby alleviating intestinal function damage caused by weaning. In addition, studies had shown that when the intestine was infected with pathogens, it caused up-regulation of MUC1 protein levels, thereby inhibiting the inflammatory response caused by pathogens [21, 22]. But Liu et al [23] showed that the supplementation with high-dose zinc oxide (2425 mg/kg zinc) to the diet enhanced the intestinal mucosal function of weaned piglets by up-regulating the expression of MUC1, MUC2 mRNA in the colon 7 days after weaning. The result about MUC1 was contrary to our results, maybe the tissue and date which we selected were different. Otherwise, Kosuke et al [24] also pointed out that during the early stage of inflammation, MUC1 expression is relatively low, and TNF-α produced during the ongoing inflammatory response up-regulates MUC1 expression. This result was consistent with our study. Therefore, we hypothesized that β-carotene might alleviate immune dysfunction caused by weaning stress by regulating the expression of mucin.

# Conclusion

Some studies had shown that β-carotene had immunoregulatory functionsin the rats, calves and poultry [9,11], but whether β-carotene could alleviate immune dysfunction caused by weaning in piglets has not been reported. Our experiment demonstrated that β-carotene increased the levels of IgA and IL-10 in serum , reduced the levels of IgM, TNF-α and IL-6 in serum, up-regulated the expression of MUC2 and down-regulated the expression of MUC1 in the small intestine of weaned piglets. Based on these findings, we propose that supplementation of β-carotene might relieve immune function disorders in piglets caused by weaning.

# Methods

### Animals and experimental design

A total of 45 Junmu No.1 White piglets ( Sanjiang white Pig × Seghers hybrid ) with the average body weight of 4.62 ± 0.06 kg (14 d of age) were randomly assigned to 3 treatments with 3 pigs per pen and 5 replicate pens per treatment and weaned at 21 d. Piglets were purchased from the original breeding farm of Jilin University, Changchun City, Jilin Province. The control group (**CG**) fed the basal diets from 14 d to 24 d of age. The low-dose group (**LG**) and the high-dose group (**HG**) fed the basal diets supplemented with 40 or 80 mg/kg β-carotene from 14 d to 24 d of age, respectively. The basal diet was prepared according to the NRC (2012) nutritional requirements of piglets. The ingredients of the basal diets are shown in Table 1. All piglets were housed in a temperature-controlled nursery (32-34°C) and were offered *ad libitum* access to water and creep feed.

### Sample collection

At 21 d and 24 d of age, the body weights of the piglets were recorded. At 24 d of age, all piglets were euthanatized by intravenously injection of pentobarbital sodium (50 mg/kg body weight) and bled via jugular venipuncture. The piglets abdominal walls were opened using a scalpel and the complete intestine was removed to observe the intestinal mucin. Approximately a 2 cm of at the midpoint of the duodenum, jejunum and ileum was collected, fixed in 4% paraformaldehyde and kept at 4 °C for Periodic Acid-Schiff (**PAS**) stain immunofluorescence. Section of the midpoint of the duodenum, jejunum and ileum were collected, rinsed with normal saline, blotted dry with filter paper, frozen in liquid nitrogen, and stored at -80 ℃ for real-time PCR.

### Growth performance

The body weight (**BW**) of piglets and amount of the feed were recorded at 21 d and 24 d of age. The average daily feed intake (**ADFI**), average daily gain (**ADG**) and d feed conversion rate (**FCR**) were calculated during the experiment period.

ADFI [g/days] = Total consumption [g]/(Test days [day] × Total number of piglets).

ADG [g/day] = (Final BW [g] − Initial BW [g])/(Test days [day] × Total number of piglets)

FCR [%] = ADFI/ADG

### Detection of immunoglobulins and inflammatory factors in serum

The blood samples were placed into RNAase free tubes and then left to stand at the room temperature for 30 minutes. The blood was centrifuged at 3000 rpm for 15 minutes and then the serum was placed in a 1.5 ml centrifuge tube for the test.

Immunoglobulins (IgA、IgG and IgM) and inflammatory factors (TNF-α, IL-6, IL-8 and IL-10) were measured using a pig ELISA quantification kit (Bethyl Laboratories, Montgomery, TX, USA) and ELISA starter accessory package (Bethyl Laboratories) according to the manufacturer's instructions. The plates were read at 450 nm with a micro plate reader (Multiskan FC; Thermo Fisher Scientific, Waltham, MA, USA).

### Intestinal tissue PAS stain

The 4% paraformaldehyde-fixed and paraffin-embedded sections of the duodenum, jejunum and ileum were used for PAS stain. Firstly, sections of 5-mm thickness were deparaffinized and rehydrated then treated with 0.5% periodate solution for 10 minutes. Secondly, the sections were rinsed with running water for 5 min. Thirdly, the sections were then incubated in Schiff reagent for 20 min in the dark then washed with sulfite rinse for 2min and rinsed with running water for 10 min. Finally, The sections were hematoxylin counterstaining, dehydration, transparent and neutral gum seal. Images of immunofluorescent sections were captured using an Eclipse Ti-SR microscope with a DS-U3 Image-Pro system (Nikon).

### Immunofluorescence

The 4% paraformaldehyde-fixed and paraffin-embedded sections of the duodenum, jejunum and ileum were used for immunofluorescence. Firstly, sections of 5-mm thickness were deparaffinized and rehydrated then processed for antigen retrieval. Secondly, the sections were incubated in hydrogen dioxide for 10 min in the dark. Then, the sections were incubated with primary antibody MUC1 (1:200 dilution). Cy3-conjugated Affinipure Goat Anti-Rabbit IgG(H+L) antibody was used as secondary antibody (1:50 dilution). Nuclei were stained with Hochest (Solarbio, Beijing). Images of immunofluorescent sections were captured using an Eclipse Ti-SR microscope with a DS-U3 Image-Pro system (Nikon). MUC1 antibody purchased from Biorbyt. Secondary antibody purchased from Proteintech.

### Analysis of mRNA abundance by real-time PCR

Total RNA was extracted from jejunum tissue using RNAiso Plus (TaKaRa Code:9109). The yield and purity of the RNA were evaluated using a NanoDrop 2000 (Thermo Scientific). RNA (1 μg) was used to generate cDNA (PrimeScript$^{TM}$ RT reagent kit with gDNA Eraser, TaKaRa Cat# RR047A) in a volume of 20 μL. Real-time polymerase chain reaction was performed in a total volume of 20 μL using SYBR® Premix Ex Tap$^{TM}$ II (TaKaRa Cat# RR820A). The primer sets used were designed with software ( Sangon Biotech Co., Ltd, Shanghai) and the sequences are listed in Table 2. Glyceraldehyde-3-phosphate dehydrogenase (**GAPDH**) and β-actin were used as housekeeping genes in this study. The relative mRNA expression of the target genes was determined using the $2^{-\Delta\Delta CT}$ method [14].

### Statistical analysis

SPSS (SPSS GmbH, Munich, Germany) version 17.0 was used for data analysis. The statistical differences between groups were analyzed via a one-way ANOVA followed by a post-hoc least significant difference test. The results are given as the means ± SEM. Statements of statistical significance were based on $P \leq 0.05$.

## Abbreviations

ADFI: Average daily feed intake; ADG: Average daily gain; BW: Body weight; CG: control group; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; HG: high-dose group; LG: low-dose group; MUC1: Mucin1; MUC2: Mucin2; PAS: Periodic Acid-Schiff; SEM: standard error of the mean; VA: vitamin A.

## Declarations

### Ethics approval and consent to participate

The protocols for this study concerning animal use were reviewed and approved by the Animal Ethical Committee of Jilin University (No. 201705001).

### Consent for publication

Not applicable.

### Availability of data and material

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

### Competing Interest

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.


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### Authors' contributions

XZ, WL, RL and MW designed the experiment and overall coordinated the project; HG, QC and TW helped in statistical analysis; WL have drafted the work. All authors read and approved the final manuscript.


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# Tables

**Table 1**. Ingredient composition and nutrient levels of the basic diets (air-dry basis,%)

| Item | amount |
|---|---|
| Ingredients | air-dry basis [%] |
| Corn | 57.0 |
| Soybean meal | 24.5 |
| Whey powder | 6.0 |
| Soybean oil | 2.2 |
| Puffed soybeans | 4.0 |
| Fish meal | 3.0 |
| Calcium hydrogen phosphate | 1.3 |
| Stone powder | 0.7 |
| Salt | 0.3 |
| Composite premix[1] | 1.0 |
| Total | 100 |
| Nutrient content: | |
| Digestive energy (MJ·kg-1) | 13.6 |
| Crude protein | 19.7 |
| Lysine | 1.2 |
| Calcium | 0.85 |
| Phosphorus | 0.64 |

[1]The compound premix provides per kilogram of feed: Vitamin A 12000 IU, Vitamin D 31700 IU, Vitamin E 45 IU, Vitamin K 4.5 mg, Vitamin B1 4 mg, Vitamin B2 12 mg, Vitamin B6 7.5 mg, Vitamin B12 35 Mg, Biotin 150 μg, Pantothenic Acid 30 mg, Folic Acid 2.5 mg, Niacinamide 50 mg, Choline

chloride 560 mg, Iron 220 mg, Zinc 210 mg, Copper 180 mg, Manganese 55 mg, Selenium 0.3 mg, Iodine 0.3 mg , Co 0.3 mg.

**Table 2.** Sequences of primers used for real-time PCR

| Gene | Accession number | Sequences (5'→3') | Size(bp) |
|---|---|---|---|
| MUC1 | XM_021089730 | F:5’CCTGCTGGTGCTGGTCTGTATTC<br>R:5’GTGGCTGCCAGGTTCGAGTAAG | 68 |
| MUC2 | XM_021082584 | F:5’CTGCTCCGGGTCCTGTGGGA<br>R:5’CCCGCTGGCTGGTGCGATAC | 100 |
| GAPDH | NM_008084.3 | F: GAGAAACCTGCCAAGTATGATGAC<br>R: TAGCCGTATTCATTGTCATACCAG | 212 |
| β-actin | NM_007393.5 | F: GTGACGTTGACATCCGTAAAGA<br>R: GCCGGACTCATCGTACTCC | 245 |

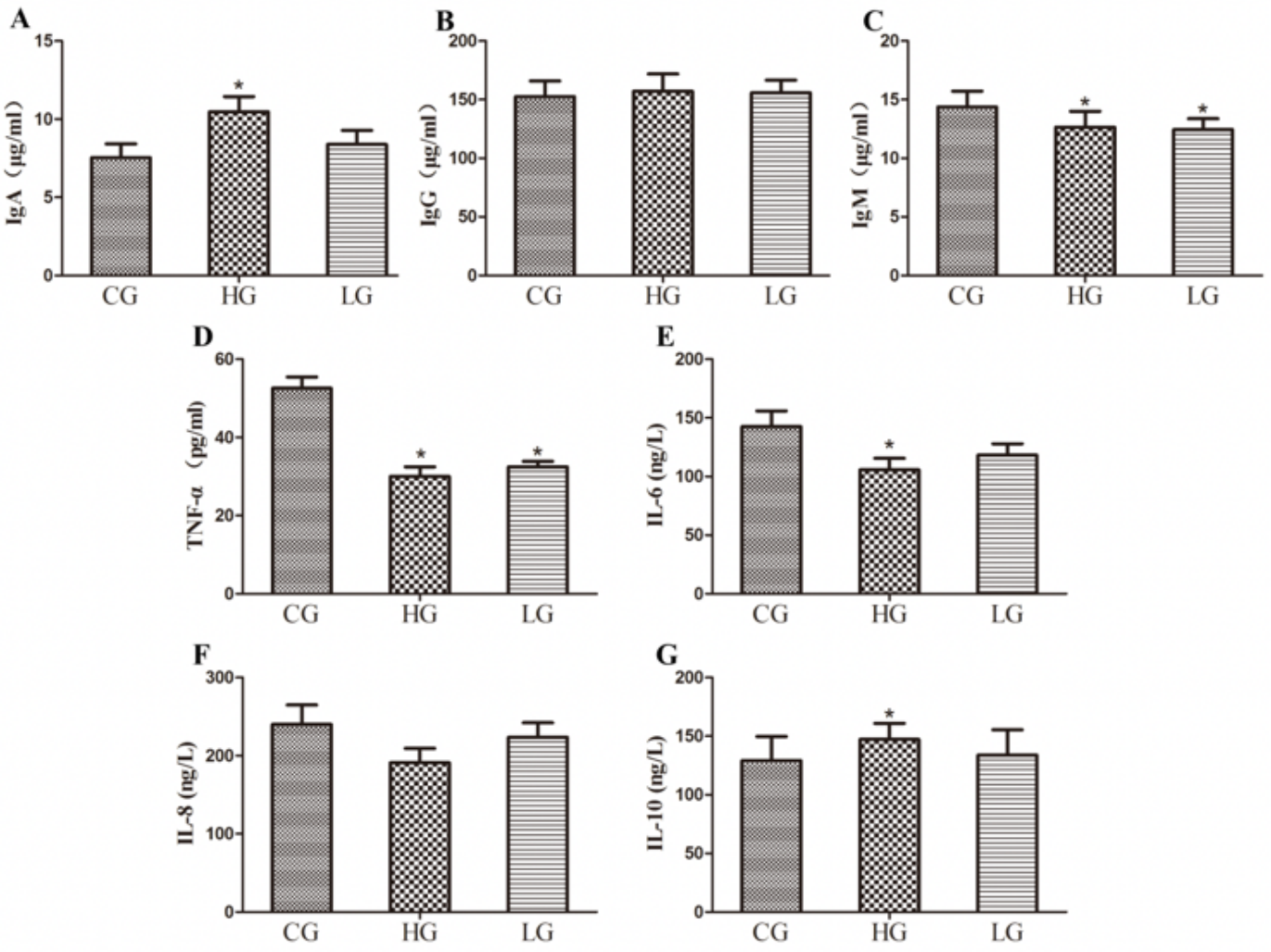
Note: MUC1: mucin 1; MUC2: mucin 2; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

**Table3.** Effect of β-carotene on growth performance in weaned piglets

| Variables | CG | HG | LG |
|---|---|---|---|
| ADG (g/d) | 61.67±3.92 | 108.3±12.14* | 80.01±7.28 |
| ADFI (g/d) | 181.25±15.34 | 243.47±18.23* | 217.82±16.06 |
| FCR (ADFI/ADG) | 2.94±0.18 | 2.25±0.15* | 2.72±0.16 |

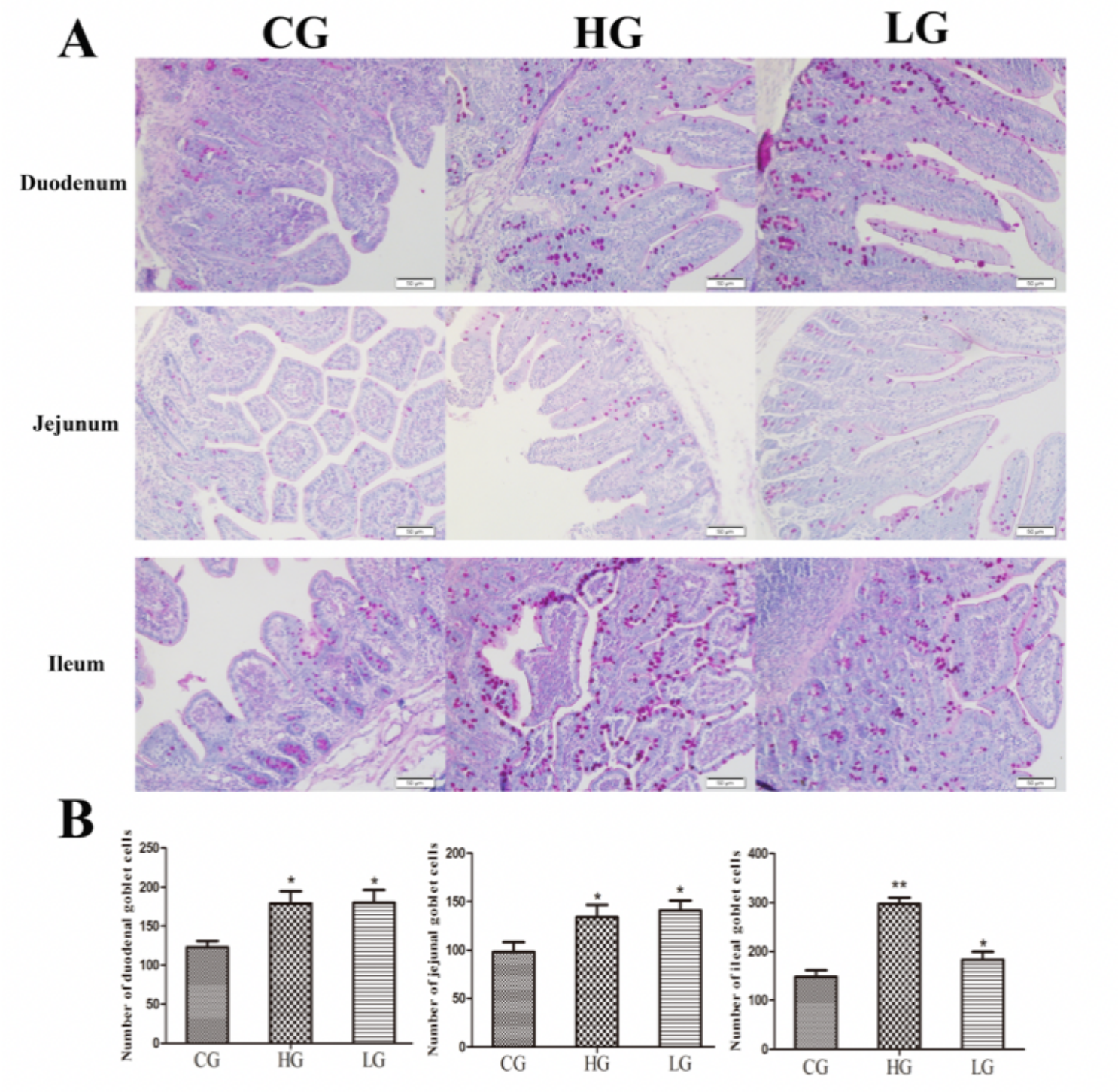
Note: *p<0.05, compared with the CG. Each value represents the mean ± SEM (n=5). SEM: standard error of the mean; CG: control group; HG: high-dose group; LG: low-dose group; ADFI: average daily feed intake; ADG: average daily gain; BW: body weight; FCR: feed conversion rate; CG: control group; HG: high-dose group; LG: low-dose group.

# Figures



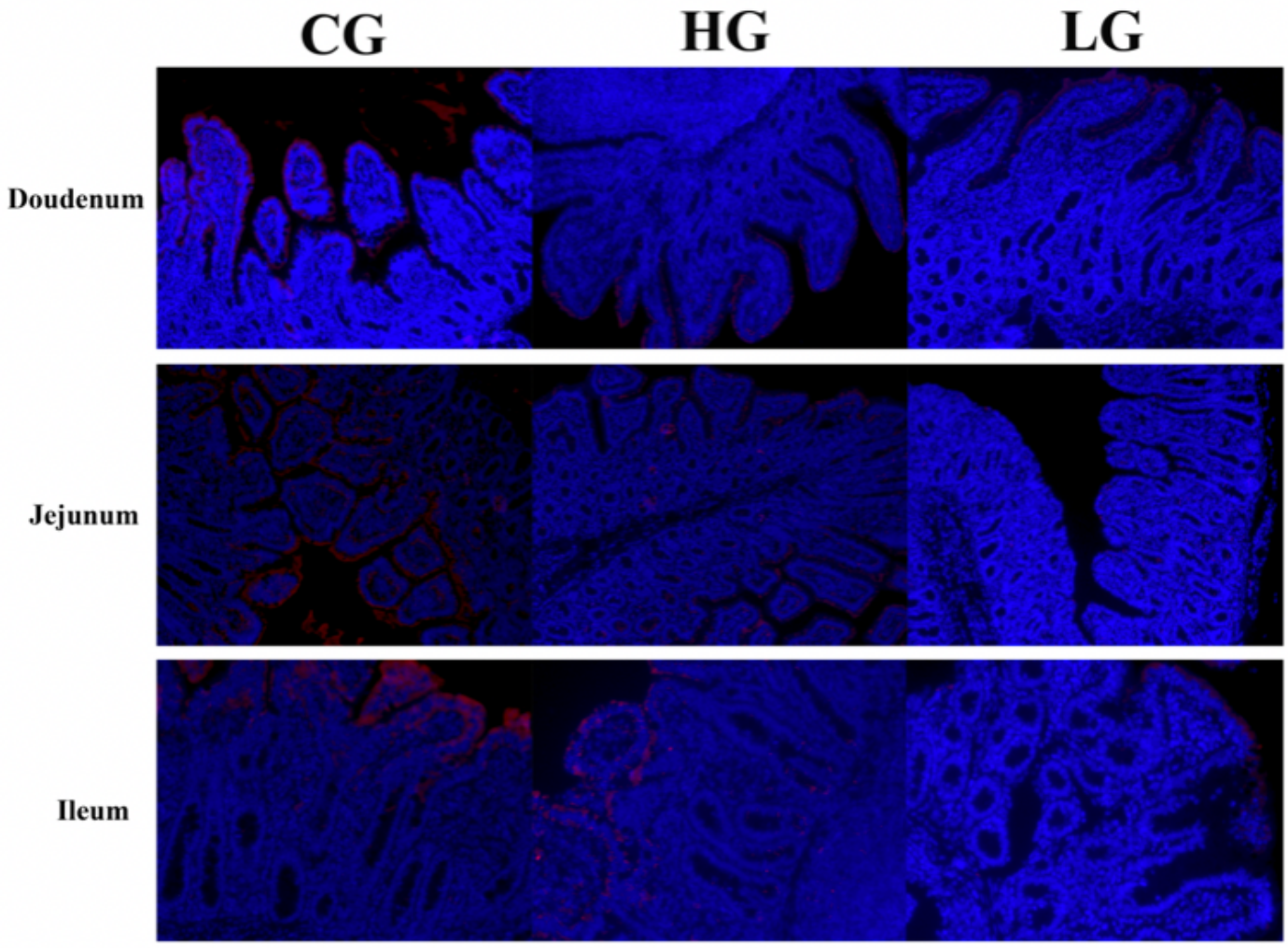

## Figure 1

Effect of β-carotene on immunoglobulin and immune factors in serum of weaned piglets. (A-C) Serum immunoglobulin (IgA, IgG and IgM) levels of weaned piglets. (D-G) Serum immune factors (TNF-α, IL-6, IL-8 and IL-10) levels of weaned piglets. The data are represented as the mean ± SEM (n = 5); *p<0.05 compared with the CG. CG: control group; HG: high-dose group; LG: low-dose group



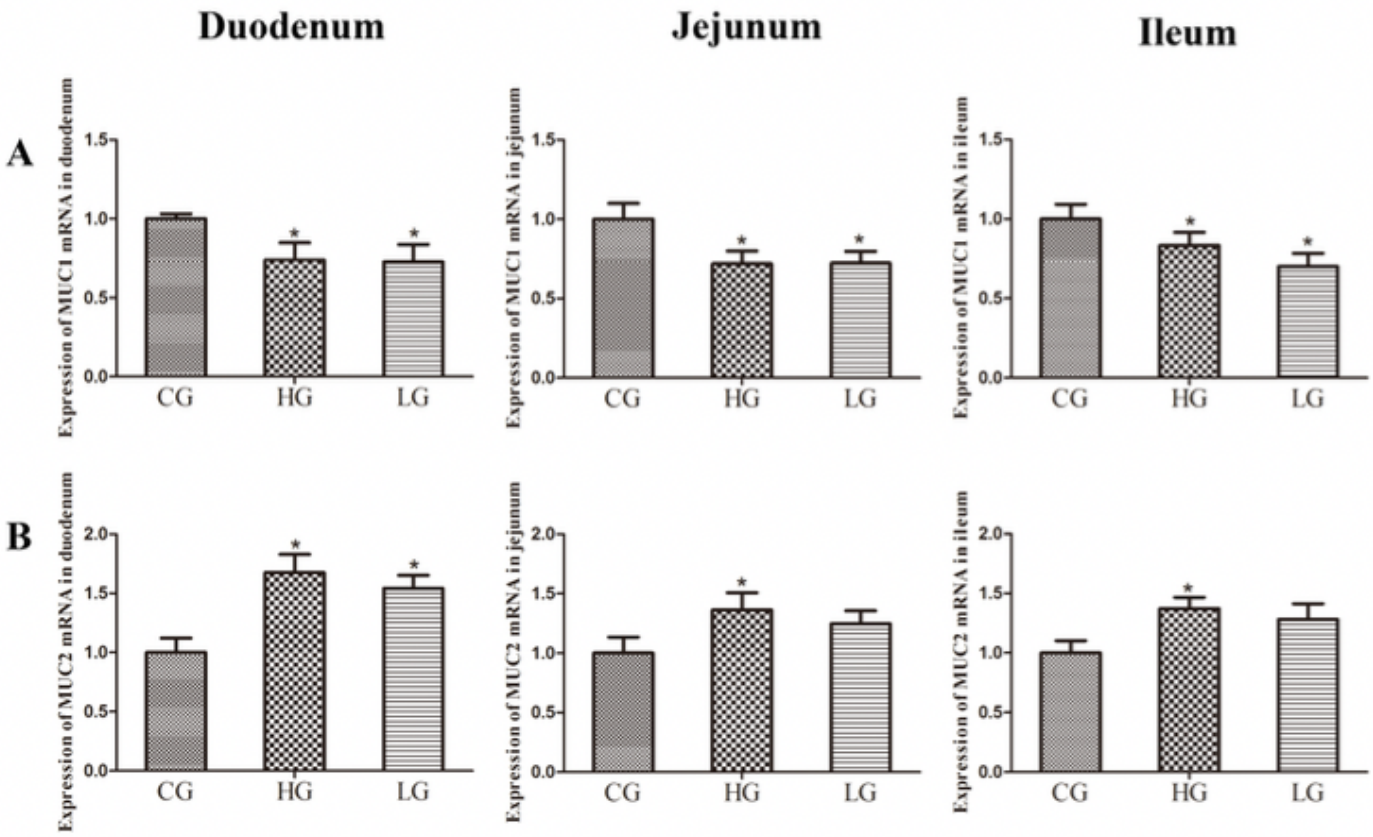

## Figure 2

The mucopolysaccharide secretion and the number of goblet cells in the small intestine. (A) Detection of mucopolysaccharide secretion by PAS method (original magnifification 200x; scale bar, 50 μm). (B) The number of goblet cells in the small intestine of weaned piglets. The data are represented as the mean ± SEM (n = 5); *p<0.05, **p<0.01 compared with the CG. CG: control group; HG: high-dose group; LG: low-dose group




## Figure 3

Immunofluorescence detection of MUC1 protein expression in small intestine of weaned piglets (original magnifification 200x). CG: control group; HG: high-dose group; LG: low-dose group




## Figure 4

Effect of β-carotene on the expression of MUC1 and MUC2 mRNA in the small intestine. (A) The expression of MUC1 in the small intestine of weaned piglets. (B) The expression of MUC2 in the small intestine of weaned piglets. The data are represented as the mean ± SEM (n = 5); *p<0.05 compared with the CG. CG: control group; HG: high-dose group; LG: low-dose group