Dietary Supplementation of β-Carotene on Growth Performance, Jejunal Permeability and Tight Junction Proteins in Weaned piglets

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Research

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Abstract

Background: Weaning causes stress syndrome in the newborns, while it can lead to many intestinal diseases. The aim of this study was to determine the dietary supplementation of β-carotene on growth performance, jejunal permeability and tight junction proteins in weaned piglets, and explore the underlying mechanisms by which β-carotene regulates tight junction proteins. The positive control group was fed sow’s milk throughout the trial. The negative control group was weaned at d 21 without any supplementation. Two experimental groups of piglets were weaned at d 21 and fed 40 mg/kg or 80 mg/kg β-carotene-supplemented diets from d 14 to d 24, respectively. Blood and the jejunum were collected at d 24.

Results: The results showed that β-carotene at 80 mg/kg increased initial and final body weight and average daily feed intake, improved the villus height at d 24 ($p<0.05$) but reduced crypt depth in weaned piglets at d 24 ($p<0.05$). Additionally, β-carotene protected intestinal morphology. Supplemental 80 mg/kg β-carotene reduced the concentrations of D-lactic acid and diamine oxidase in serum ($p<0.05$) but enhanced the mRNA expression of claudin-3, occludin ($p<0.05$), and zonula occludens protein-1 ($p>0.05$) and the protein levels of claudin-3 ($p<0.01$), occludin ($p<0.05$), and zonula occludens protein-1 ($p>0.05$) in the jejunum at d 24. Using intestinal epithelial cells and a Rac1 inhibitor in vitro, we demonstrated that the β-carotene-induced higher tight junction proteins might depend on the Rac1 pathway.

Conclusions: β-carotene improved growth performance and attenuated jejunal permeability and tight junction proteins in weaned piglets, suggesting that β-carotene at 80 mg/kg may be an effective way for keeping healthier intestine of weaning piglets due to weaning.

Background

Weaning causes stress syndrome in newborns, resulting in a poor appetite, digestive dysfunction, growth retardation, diarrhoea and even death [1]. Additionally, studies have pointed out that weaning stress impairs intestinal barrier function [2–5]. The intestinal epithelial barrier is mainly composed of intestinal epithelial cells and tight junctions (TJs) [6]. Intestinal epithelial cells form physical barriers to separate substances that regulate body homeostasis from the intestinal lumen and are not permeable to most hydrophilic substances [7]. If the intestinal epithelial cells are damaged, the intestinal permeability changes, and thus the pathogens and inflammatory mediators of the intestinal lumen penetrate into the tissue through the intercellular space, impairing intestinal function [8]. TJs are located at the top of the intercellular structure and play a critical role by regulating the paracellular permeability and cell polarity of the intestinal epithelium [9]. The integrity of intestinal morphological structures and TJs are the structural basis of the intestinal epithelial barrier [10]. The intestinal barrier function and active absorption rate decrease when pigs are weaned at 3 weeks of age or earlier. However, when weaned at 4 weeks of age or later, the barrier function is less affected, and active absorption is not affected or is increased [5]. Therefore, we decided to wean the piglets at d 21 of age and collected samples when the piglets were at d 24 of age.
Tight junctions are linked to actin cytoskeleton via intracellular adaptor proteins such as ZO-1. Additionally, some studies have pointed out that the activation or inactivation of small GTPases of the Rho (ras homology) family whose best characterized members are RhoA, Rac1, and Cdc42 play a key role in the regulation actin dynamics and paracellular permeability [11–13]. Schlegel et al [14] have reported that the inactivation of RhoA and Rac1 caused occludin and claudin-1to be largely absent from Caco2 epithelial monolayers borders. Therefore, we hypothesized that Rac1 may be a key factor in the regulation of tight junction proteins.

β-carotene (BC) is a precursor of vitamin A (VA). Additionally, cell model testing has demonstrated that β-carotene can enhance the ability of the gap junctional intercellular communication (GJIC) [15]. Supplementation with VA and β-carotene enhanced the immunity in neonatal calves [16]. Moreover, supplementation with VA decreased the incidence of diarrhoea and mortality in malnourished children [17]. Therefore, increasing the effects of VA in protecting and proliferating enterocyte membrane cells may be critical at weaning stage. The aim of this study was to investigate the effects of β-carotene on intestinal permeability, intestinal morphological development, TJ protein, and gene expression in weaned piglets, and to determine the underlying mechanism by which β-carotene regulates tight junction proteins.

Materials And Methods

The methods were carried out in accordance with the relevant guidelines and regulations of the Animal Ethics Committee of Jilin Agricultural University. All experiments were performed in accordance with relevant guidelines and regulations of the Animal Ethics Committee of Jilin Agricultural University and all experimental protocols were approved by the Animal Ethics Committee of Jilin Agricultural University.

Animals and experimental design

A total of 24 male Junmu No.1 White piglets (Sanjiang white Pig × Seghers hybrid) with the similar body weight were randomly assigned to 4 treatments with 6 replicated piglets per treatment. The piglets were purchased from the original breeding farm of Jilin University, Changchun city, Jilin Province. The positive control (MILK) group was fed sow’s milk throughout the trial process. The negative control (0BC) group was weaned at d 21 without any supplementation. Another two groups were weaned at d 21 and administered oral 40 mg/kg (40BC) or 80 mg/kg β-carotene (80BC) from d 14 to d 24 of age, respectively. The duration of the experiment was 10 days. β-carotene powder was dissolved in 5 ml of corn oil (obtained from Sigma-Aldrich) for utilization in this experiment. β-carotene was fed once a day and stored at 4 °C. All piglets were offered ad libitum access to water and housed in a temperature-controlled room (32–34 °C). During the trial, all piglets were fed a basal diet. 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.
| Item                        | Amount   |
|-----------------------------|----------|
| Ingredients                 | air-dry basis [%] |
| Corn                        | 57.0     |
| Soybean meal                | 24.5     |
| Whey powder                 | 6.0      |
| Soybean oil                 | 2.2      |
| Extruded soybeans           | 4.0      |
| Fish meal                   | 3.0      |
| Dicalcium phosphate         | 1.3      |
| Limestone                   | 0.7      |
| Salt                        | 0.3      |
| Vitamin and mineral premix¹ | 1.0      |
| Total                       | 100      |

Nutrient levels:

| Item                        | Amount   |
|-----------------------------|----------|
| Digestive energy (MJ·kg⁻¹)  | 13.6     |
| Crude protein               | 19.7     |
| Lysine                      | 1.2      |
| Calcium                     | 0.85     |
| Phosphorus                  | 0.64     |

¹The 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.

Sample collection

At d 24, all piglets were anaesthetized by an intramuscular injection of 4% sodium pentobarbital solution (40 mg/kg body weight). After collecting the blood from the anterior vena cava, all piglets were then euthanized by exsanguination. The abdominal wall opened by using a scalpel, and we used anatomical features to identify the jejunum: the final meeting point of the duodenum and the pancreas was considered as the boundary between the duodenum and the jejunum, and the yolk stalk served as the boundary between the jejunum and the ileum. Then, the central ~ 2 cm of the jejunum was collected, fixed
in 4\% paraformaldehyde or 4\% glutaraldehyde, and kept at 4 ℃ for histology and scanning electron microscopy (SEM) analyses. Sections of the jejunum were collected, rinsed with normal saline, blotted dry with filter paper, frozen in liquid nitrogen, and stored at -80 ℃ for Western blot analysis and real-time PCR.

**Growth performance**

At d 21 and d 24, the initial body weight (IBW) and final body weight (FBW) of the piglets were recorded, respectively. And total consumption of the piglets were also recorded from d 21 to d 24. The average daily gain (ADG), average daily feed intake (ADFI) and feed conversion rate (FCR) were calculated.

\[
\text{ADG} \, [\text{g/day}] = \frac{\text{Final BW} \, [\text{g}] - \text{Initial BW} \, [\text{g}]}{\text{Test days} \, [\text{day}] \times \text{Total number of piglets}}.
\]

\[
\text{ADFI} \, [\text{g/days}] = \frac{\text{Total consumption} \, [\text{g}]}{\text{Test days} \, [\text{day}] \times \text{Total number of piglets}}.
\]

\[
\text{FCR} = \frac{\text{ADFI}}{\text{ADG}}.
\]

**Detection of D-lactic acid and diamine oxidase in serum**

Blood was obtained from the anterior vena cava. The serum was centrifuged at 3000 rpm for 15 minutes and placed in a 1.5 ml centrifuge tube. D-lactic acid and diamine oxidase (DAO) concentrations were measured using a pig D-lactic acid and DAO ELISA quantification kit (Bethyl Laboratories, Montgomery, TX, USA) and an ELISA starter accessory package (Bethyl Laboratories) according to the manufacturer’s instructions. The plates obtained from the procedures were measured at 450 nm with a micro plate reader (Multiskan FC; Thermo Fisher Scientific, Waltham, MA, USA).

**Histology**

The jejunum, which was fixed in 4\% paraformaldehyde was trimmed, dehydrated and then embedded in paraffin. Afterwards, the microtome was cut into approximately 5-μm-thick slices, which were placed on glass slides and stained with haematoxylin and eosin to observe intestinal histomorphology. Villus height was measured from the crypt mouth to the villus tip, and the associated depth of the crypts was measured from the base to the crypt mouth by a computer-aided light microscope (Nikon, Tokyo, Japan). A total of 100 villi measurements per piglet were calculated and used in the statistical analysis.

**SEM analysis of the jejunal morphology**

For SEM, the test procedure is as described previously [18]. All villi were observed with an SEM (PW-100-011, The LASER Company, Ireland). Using SEM, we observed whether the intestinal epithelium at the top of the villus was damaged. Five images from each piglet were observed.

**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, USA). RNA (1 μg) was used to generate cDNA (PrimeScript™ 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 Taq™ II (TaKaRa Cat# RR820A) following the manufacturer’s instructions. RNA integrity was detected by agarose gel electrophoresis. The RT-PCR programme was as follows: 30 s at 95 °C, 40 cycles of 5 s at 95 °C and 30 s at 60 °C, 5 s at 65 °C and 5 s at 95 °C. A melting curve was used to systematically analyse all samples. The primers were designed using software (Sangon Biotech Co., Ltd, Shanghai), and the sequences are listed in Table 2. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The relative mRNA expression levels of the target genes were determined using the $2^{-\Delta\Delta CT}$ method [19].

| Gene         | Accession number | Sequence (5'→3')                              | Size (bp) |
|--------------|------------------|-----------------------------------------------|-----------|
| GAPDH        | NM_001206359     | F:5'CTCAACCGGGAAGCTCACCTGGR:5'TGATCTCATCATACTTGGCATGTT | 100       |
| Claudin−3    | NM_001160075     | F:5'TGTGGATGAACTCGTGGTGGTGCGTGTCR:5'ATCTTGCTTTGGCGTGTC | 143       |
| Occludin     | NM_001163647.2   | F:5'CGTGGGCTTGGTGCTATG TGCTATG R:5'AGAATCCCTTTGC TGCTATG | 101       |
| ZO−1         | XM__003353439.2  | F:5'AAGCCCTAAGTTC AATCACAATCT R:5'ATCAAACTCAGGAGGC | 133       |

**Table 2**
Sequences of primers used for RT-qPCR amplification

**Western blot analysis**

First, the jejunal tissue was removed from the − 80 °C freezer and then thawed on ice. Soon afterwards, the samples were mechanically homogenized with RIPA lysate, deacetylase inhibitor mixture, protease phosphatase inhibitor mixture and PMSF and then centrifuged at 10,000 rpm for 15 minutes at 4 °C to collect the supernate. The total protein concentrations were determined by BCA kits.

Equal amounts of protein (25 µg) were added to SDS-PAGE gels, with electrophoresis initially at 80 V for 30 min and then switched to 120 V until the end. Then proteins were then transferred to polyvinylidene fluoride (PVDF) membranes for 140 min at 220 mA. The membranes were blocked with 5% nonfat milk for 1 h at room temperature. Then the membranes were washed in TBST buffer three times and subsequently incubated overnight at 4 °C with the primary antibodies for β-actin, occludin, claudin-3 and
ZO-1 (1:5000, 1:1000, 1:500, 1:500, diluted with TBST). The next day, the membranes were washed in TBST buffer and then incubated with HRP-conjugated secondary antibody (1:3000, diluted with TBST) at room temperature for 1 h. The membranes were treated with an enhanced chemiluminescent HRP substrate (Millipore, USA) and detected by a Gene Genome bioimaging system. The protein bands were quantified by Quantity One® software (Quantity One 4.61, Bio-Rad, Hercules, CA, USA). The primary antibodies for β-actin and the secondary antibody (HRP-conjugated goat anti-rabbit and anti-mouse) were purchased from Sigma (USA). The primary antibodies for occludin were purchased from Bioss. Claudin-3 was purchased from Abcam, and ZO-1 was purchased from Novus Biologicals. The RIPA kit and Difco non-fat milk were obtained from Pierce (USA). The PVDF membranes were purchased from Millipore (Bedford, MA, USA). We used 3 biological replicates, each representing 3 technical replicates.

**Intestinal epithelial cell culture**

The intestinal porcine epithelial cell line J2 from the jejunum (IPEC-J2) was obtained from ATCC and the cells were cultured in DMEM supplemented with 10% FBS (Life Technologies), 100 IU/mL penicillin and 100 µg/mL streptomycin. The cells were incubated at 37 °C in a humidified 5% CO₂ incubator. Monolayers were grown, and the cells were passaged using trypsin. Then, 13 × 10⁷ cells were cultured in each well of six-well plates. After incubation for 24 h, the IPEC-J2 cells were divided into four groups. Group I: control group. Group Ⅱ: β-carotene group. Group Ⅲ: NSC 23766 (specific Rac1 inhibitor) group. Group IV: β-carotene + NSC 23766 group. The cells in groups Ⅰ and IV were treated with 150 µg L⁻¹ β-carotene for 3 h, and the cells in groups Ⅱ and IV were treated with DMEM for 3 h. Our laboratory has previously conducted tests on β-carotene concentration and time using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide and thiazolyl blue tetrazolium bromide (MTT). After 3 h, phosphate buffered saline (PBS) was used to wash the cells. Then, groups Ⅱ and IV were treated with 50 mM NSC 23766 (specific Rac1 inhibitor) for 12 h, and the other two groups were treated with DMEM. The cells were then collected and treated with lysis buffer to extract the total protein for Western blotting. The remainder of the cells were used to extract total RNA for real-time PCR using RNAiso Plus (TaKaRa Code: 9109).

**Statistical analysis**

SPSS 21.0 (IBM Corporation, Armonk, NY, USA) was used for data analysis. The significant differences were analysed via one-way ANOVA followed by a post hoc least-significant difference (LSD) test. Statements of statistical significance were based on P ≤ 0.05.

**Results**

**The growth performance of piglets from d 21 to d 24**

At d 21, compared with the MILK group, the IBW was no significant difference in the 0BC group (p > 0.05). However, the IBW in the 80BC group was higher than that in the 0BC (p < 0.05). At d 24, the FBW in the 0BC group was lower than that in the MILK group (p < 0.01) Supplementation with 80 mg/kg β-carotene
increased FBW, ADG and ADFI of the weaned piglets compared with the 0BC group ($p < 0.05$; Table 3). There was no significant difference in the FCR between the four groups ($p > 0.05$; Table 3).

**Table 3**

| Variables | MILK | 0BC  | 40BC | 80BC |
|-----------|------|------|------|------|
| IBW (kg)  | 6.36 | 6.08 | 6.35 | 6.53* |
| FBW (kg)  | 7.12 | 5.74## | 6.71* | 7.09** |
| ADG (g)   | 253  | -113 | 120  | 187*  |
| ADFI (g)  | 493  | 183# | 204  | 293*  |
| FCR       | 1.94 | 1.62 | 1.70 | 1.57  |

Note: # $p < 0.05$, ## $p < 0.05$ compared with the MILK group; * $p < 0.05$, ** $p < 0.01$ compared with the 0BC group, $n = 6$. MILK: positive control group; 0BC: negative control group; 40BC: 40 mg/kg β-carotene group; 80BC: 80 mg/kg β-carotene group; IBW: initial body weight; FBW: final body weight; ADG: average daily gain; ADFI: average daily feed intake; FCR: feed conversion rate.

**Detection of D-lactic acid and DAO in serum**

At d 24, the D-lactic acid and DAO concentrations in serum of the 0BC group were significantly higher than those in the MILK group ($p < 0.05$; Fig. 1A and B). However, supplemental 80 mg/kg β-carotene significantly reduced the concentrations of D-lactic acid and DAO in serum compared with the 0BC group ($p < 0.05$), but there were no significant differences between the 0BC and 40BC groups ($p > 0.05$; Fig. 1A and B).

**Jejunal histomorphology**

At d 24, in the MILK group, the villus morphology was intact. In the 0BC group, the villi were slightly broken. In the 40BC and 80BC group, the damaged villi were repaired to different degrees (Fig. 2). The villus height and the ratio of villus height/crypt depth (V/C) in the MILK group were higher than those in the 0BC group. However, the crypt depth in the MILK group was lower than that in the 0BC group ($p < 0.05$; Table 4). Supplemental 80 mg/kg β-carotene increased villus height ($p < 0.05$) and improved V/C (Table 4).
Table 4  
Effects of β-carotene on jejunal morphology in weaned piglets

| Item                | MILK   | 0BC    | 40BC   | 80BC   |
|---------------------|--------|--------|--------|--------|
| Villus height (µm)  | 447 ± 21.4 | 319 ± 18.7# | 336 ± 20.2 | 380 ± 19.1* |
| Crypt depth (µm)    | 293 ± 18.3 | 312 ± 19.3# | 308 ± 18.9 | 297 ± 22.4 |
| villus/crypt (V/C)  | 1.52 ± 0.123 | 1.02 ± 0.214# | 1.09 ± 0.271 | 1.28 ± 0.0912* |

Note: #p < 0.05 compared with the MILK group; *p < 0.05 compared with the 0BC group. Each value represents the mean ± SEM of 6 piglets. MILK: positive control group; 0BC: negative control group; 40BC: 40 mg/kg β-carotene group; 80BC: 80 mg/kg β-carotene group.

At d 24, using SEM to observe the surface of the villi, the results showed that the villi in the jejunum in the 0BC group had denuded tips and that their lamina propria were exposed to the lumen; however, the villi in the MILK, 80BC and 40BC groups were intact (Fig. 3).

**β-carotene modulated the expressions levels of TJ genes and TJ proteins in weaned piglets**

At d 24, the relative mRNA expression levels of TJ genes in the 0BC group were significantly lower than those in the MILK group (p < 0.05; Fig. 4I). However, supplemental 80 mg/kg β-carotene significantly increased the mRNA expression levels of claudin-3 and occludin compared with the 0BC group (p < 0.05; Fig. 4I. A and B). Supplemental β-carotene had no significant effect on the mRNA expression of ZO-1 (p > 0.05; Fig. 4I. C).

At d 24, the expression levels of TJ proteins in the 0BC group were lower than in the MILK group (Fig. 4II. A). Supplemental 80 mg/kg β-carotene enhanced the abundance of claudin-3 (p < 0.01; Fig. 4II. B) and occludin (p < 0.05; Fig. 4II. C) compared with the 0BC group. However, supplemental β-carotene had no significant effect on the ZO-1 protein expression (p > 0.05; Fig. 4II. D).

**β-carotene regulated the expression of TJ genes and TJ proteins in intestinal epithelial cells in vitro**

We investigated the effects of β-carotene on the relative expression of TJ mRNA and proteins in intestinal epithelial cells in vitro and the underlying mechanism (Fig. 5). The results showed that in the presence of a selective Rac1 inhibitor (NSC 23766), the increase in occludin and ZO-1 relative mRNA expression mediated by β-carotene were suppressed (Fig. 5I. B and C). The relative mRNA expression of claudin-3 was not influenced by the Rac1 inhibitor (Fig. 5I. A). Furthermore, the expression of tight junction proteins is shown in Fig. 5II A. The results showed that Rac1 expression was suppressed (Fig. 5II. B). The TJ protein expression results were consistent with the relative mRNA expression results (Fig. 5II. C-E).

**Discussion**
Weaning stress causes poor appetite, digestive dysfunction, growth retardation, diarrhoea and even death [1]. The intestine is a crucial organ for the digestion and absorption of nutrients in piglets [20]. Our findings showed that in the OBC group, weaned piglets had a lower the FBW than the IBW. Supplemental 80 mg/kg β-carotene significantly increased the initial and final body weight of weaned piglets compared with 0BC group, and the ADG and ADFI of the piglets in the 80BC group were higher than that of 0BC group. Cucco et al [21] had reported that supplementation with β-carotene in poultry promotes growth. This result was consistent with our experimental results. Compared with the MILK group, the villi became shorter and the crypts were deeper in the 0BC group. The surface of the villi was damaged in the 0BC group. Supplemental 80 mg/kg β-carotene increased the height of the villi, villus/crypt and reduced the crypt depth. The effects of β-carotene on intestinal morphology are rarely reported. As we all konw, β-carotene (BC) is a precursor of vitamin A. Rojas-García and Rønnestad [22] found that supplementation with VA could increase intestinal villus height and decrease intestinal crypt depth in mouse pups. Additionally, previous studies demonstrated that insufficient supplementation with VA could cause impaired intestinal development in young mice and decreased AKP activity [23] and that VA deficiency caused intestinal villus growth disorders [24]. Studies also reported that insufficient VA would impede intestinal villus growth and, subsequently that supplementation with VA would promote the recovery of intestinal villi in weaned rats [25]. So, we hypothesized that β-carotene might have the same function as VA.

Intestinal permeability is an important factor reflecting the function of the intestinal mucosal barrier, and alteration of the expression of TJ proteins could cause intestinal diseases and increase intestinal permeability [26]. Plasma D-lactic acid levels can reflect the degree of intestinal mucosal injury and changes in permeability over time [27]. DAO is a highly active intracellular enzyme in the villi of mammalian intestinal mucosa. Increased plasma DAO could also indicate intestinal barrier disruption [28]. In addition, D-lactic acid and DAO concentrations in the plasma have been used as indicators of the extent of intestinal barrier injury [29, 30]. Our results showed that the D-lactic acid and DAO concentrations in the serum of the 0BC group were significantly higher than those of the MILK group. However, supplemental β-carotene reduced the concentrations of D-lactic acid and DAO in serum. The previous study had reported that the intestinal barrier was damaged, mucosal permeability began to increase, and a large amount of D-lactic acid and DAO entered the peripheral circulation [29, 30]. Our current study indicated that β-carotene could maintain the intestinal permeability of piglets by reducing the concentrations of D-lactic acid and DAO in serum. Additionally, Rodriguez et al showed that weaning stress led to increased intestinal permeability, which damaged intestinal barrier function [31]. Therefore, we thought that β-carotene might repaired weaning-induced intestinal permeability.

TJ proteins are located at the top of the intercellular structure and play critical roles in regulating epithelial barrier function and paracellular permeability [32]. Epithelial cells close the intercellular space through occludin and bind to ZO-1 to form a tight junction [33]. The disruption of TJ proteins is the initial event that is associated with the pathogenesis of many gastrointestinal diseases. Previous studies have shown that weaned piglets likely reduce the expression of TJ proteins [34]. Our results showed that weaning leads to mucosal desquamation through the SEM. We also found that after weaning TJ protein
expression in the 0BC group was significantly lower than that in the MILK group. Wheeler et al [35] found that under stress conditions, the relative expression levels of ZO-1 and occludin mRNA decreased, findings that were basically consistent with the results of our study. However, the expression levels of occludin and claudin-3 proteins increased in the 80BC group compared with the 0BC group. A previous study showed that β-carotene can promote gap junctional intercellular communication [15]. Another study also demonstrated that Vitamin A recovered LPS-induced inflammation-mediated intestinal barrier dysfunction and re-boosted LPS-induced decreases of ZO-1, Occludin and Claudin-1 at the tight junctions in IPEC-J2 cells [36]. Therefore, these data suggest that β-carotene increases TJ protein expression and improves epithelial barrier function in the intestine.

The underlying mechanisms of TJ protein regulation are undefined and implicate several signalling pathways including Rho, MAPK and PI3K/Akt et al. Many studies have shown that Rac1 activation enhances epithelial barrier function [14, 37]. Studies have also shown that Rac1 is necessary to maintain cell adhesion and tight junctions [38, 39]. For instance, human β-defensin-3 promotes Rac1 activation and increases TJ protein expression [40]. Moreover, CWA increased TJ protein expression and enhanced intestinal epithelial barrier function via a Rac1-dependent pathway [41]. Our results showed that when Rac1 inhibitors were added to intestinal epithelial cells, β-carotene was inhibited in enhancing occludin and ZO-1 proteins. These results suggest that β-carotene could increase TJ protein expression via the Rac1 signalling pathway, and by enhancing epithelial barrier function in the intestine.

**Conclusions**

Our study demonstrated that β-carotene reduced serum D-lactic acid and DAO levels, improved intestinal morphological development and increased the expression of TJ proteins in weaned piglets. Furthermore, it was verified that β-carotene increased TJ protein expression via the Rac1 signalling pathway. Based on these findings, we propose that supplementation with β-carotene at 80 mg/kg diets can be an effective nutritional strategy to improve growth performance, jejunal permeability and tight junction proteins of piglets.

**Abbreviations**

ADFI: average daily total feed intake; ADG: average daily gain; DAO: diamine oxidase; FBW: final body weight; FCR: feed conversion rate; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; IBW: initial body weight; MILK: positive control; PBS: phosphate buffered saline; PVDF: polyvinylidene fluoride; SEM: scanning electron microscopy; TJs: tight junctions; VA: vitamin A; V/C: villus height/crypt depth; ZO-1: zonula occludens protein-1; 0BC: negative control; 40BC: 40 mg/kg β-carotene; 80BC: 80 mg/kg β-carotene.

**Declarations**

Ethics approval and consent to participate
All experiments were performed in accordance with relevant guidelines and regulations of the Animal Ethics Committee of Jilin Agricultural University and all experimental protocols were approved by the Animal Ethics Committee of Jilin Agricultural University.

**Consent for publication**

Not applicable

**Availability of data and material**

All data generated or analyzed during this study are included in this published article.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors’ contribution**

XZ obtained financial support and oversaw this study; WL, RL, MW and XZ designed research; WL, RL, HG, QC and TW conducted research; WL, HG, QC and TW analysed data; WL wrote the paper; XZ holds primary responsibility for the final content. All authors read and approved the final manuscript.

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**Figures**

![Figure 1](image-url)

**Figure 1**

Effects of β-carotene on the concentrations of D-lactic acid and DAO in serum of weaned piglets. (A) Serum D-lactic acid levels of weaned piglets. (B) Serum DAO levels of weaned piglets. The data are represented as the mean ± SEM (n = 6; #p< 0.05 compared with the MILK; *p< 0.05 compared with the 0BC). MILK: positive control group; 0BC: negative control group; 40BC: 40 mg/kg β-carotene group; 80BC: 80 mg/kg β-carotene group.
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Figure 2

Jejunum of weaned piglets stained by H&E. Intestinal morphology shown by H&E staining the jejunum tissues of weaned piglets. Images of intestinal morphology at 100× magnification are shown. MILK: positive control group; 0BC: negative control group; 40BC: 40 mg/kg β-carotene group; 80BC: 80 mg/kg β-carotene group.
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Figure 3

Effect of β-carotene on the jejunal villi surface of weaned piglets by SEM. Intestinal morphology of the jejunum tissues of weaned piglets by SEM. Images of intestinal morphology at 250× magnification are shown. MILK: positive control group; 0BC: negative control group; 40BC: 40 mg/kg β-carotene group; 80BC: 80 mg/kg β-carotene group.
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Effect of β-carotene on the jejunal villi surface of weaned piglets by SEM. Intestinal morphology of the jejunum tissues of weaned piglets by SEM. Images of intestinal morphology at 250× magnification are shown. MILK: positive control group; 0BC: negative control group; 40BC: 40 mg/kg β-carotene group; 80BC: 80 mg/kg β-carotene group.
Figure 4

Effects of β-carotene on the relative expression levels of TJ mRNA and proteins in weaned piglets. (I) The relative mRNA expression levels of TJ genes in weaned piglets. (II) The TJ protein expression levels in weaned piglets. The data are represented as the mean ± SEM (n = 6; #p < 0.05 compared with the MILK group; *p < 0.05 compared with the 0BC group). MILK: positive control group; 0BC: negative control group; 40BC: 40 mg/kg β-carotene group; 80BC: 80 mg/kg β-carotene group.
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Figure 5

Effects of β-carotene on the relative expression of TJ mRNA and proteins in IPEC-J2. (I) The relative mRNA expression levels of TJ genes in IPEC-J2. (II) The TJ protein expression levels in IPEC-J2. The data are represented as the mean ± SEM (n = 6; #p < 0.05 compared with Group Ⅰ) *p < 0.05 compared with Group Ⅲ).
Figure 5

Effects of β-carotene on the relative expression of TJ mRNA and proteins in IPEC-J2. (I) The relative mRNA expression levels of TJ genes in IPEC-J2. (II) The TJ protein expression levels in IPEC-J2. The data are represented as the mean ± SEM (n = 6; #p < 0.05 compared with Group ü) *p < 0.05 compared with Group ï).