NON RUMINANT NUTRITION

Effects of vitamin B6 on the growth performance, intestinal morphology, and gene expression in weaned piglets that are fed a low-protein diet¹

Lanmei Yin,^{*,†,2} Jun Li,^{*,†,2} Huiru Wang,^{*,†} Zhenfeng Yi,^{*,†} Lei Wang,^{*,†} Shuo Zhang,[‡] Xiaozhen Li,[‡] Qiye Wang,^{*,†} Jianzhong Li,^{*,†} Huansheng Yang,^{*,†,3} and Yulong Yin^{*,†,‡,||}

^{*}Hunan International Joint Laboratory of Animal Intestinal Ecology and Health, Laboratory of Animal Nutrition and Human Health, College of Life Sciences, Hunan Normal University, Changsha 410081, China, [†]Hunan Provincial Key Laboratory of Animal Nutritional Physiology and Metabolic Process, Scientific Observing and Experimental Station of Animal Nutrition and Feed Science in South-Central, Ministry of Agriculture, Hunan Provincial Engineering Research Center for Healthy Livestock and Poultry Production, Key Laboratory of Agro-ecological Processes in Subtropical Region, Institute of Subtropical Agriculture, Chinese Academy of Sciences, Changsha 410125, China, [‡]Yunnan Yin Yulong Academician Workstation, Yunnan Xinan Tianyou Animal Husbandry Technology Co., Ltd, Kunming 650032, China, ^{||}Academics Working Station at The First Affiliated Hospital of Changsha Medical University, Changsha 410219, China

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²These authors contributed equally to this work.

³Corresponding author: yhs@hunnu.edu.cn

ORCID number: 0000-0003-1164-5771 (H. Yang).

Abstract

Vitamin B6 (VB6), which is an essential functional substance for biosome, plays an irreplaceable role in animal health. However, there are few studies that focus on the correlation between VB6 and intestinal health in weaned piglets. This study was conducted to investigate the effects of VB6 on the growth performance, intestinal morphology, and inflammatory cytokines and amino acid (AA) transporters mRNA expression in weaned piglets that are fed a low crude-protein (CP, 18%) diet. Eighteen crossbred piglets with initial body weights of 7.03 ± 0.17 kg (means $\pm$ SEM), weaned at 21-d age, were randomly assigned three diets with 0, 4, and 7 mg/kg VB6 supplementation, respectively. The experimental period lasted 14 days. Our results showed that there were no significant differences in growth performance, diarrhea rate, and biochemical parameters among the three treatments. In the jejunum, dietary VB6 supplementation did not affect the morphology and positive Ki67 counts. Dietary supplementation with 4 mg/kg VB6 decreased the mRNA expression of COX-2, IL-10, and TGF- β ($P < 0.05$). Dietary supplementation with 7 mg/kg VB6 increased the mRNA expression of SLC7A1, SLC7A6, SLC16A14, and SLC38A5 ($P < 0.05$) and 4 or 7 mg/kg VB6 decreased SLC36A1 mRNA expression ($P < 0.05$). In the ileum, VB6 supplementation did not affect positive Ki67 counts but significantly decreased villus area ($P < 0.05$) and tended to decrease villus height ($P = 0.093$). Dietary supplementation with 4 mg/kg VB6 had significantly increased the mRNA expression of IL-1 β , TNF- α , COX-2, IL-10, and TGF- β ($P < 0.05$). Dietary supplementation with 4 or 7 mg/kg VB6 had significantly decreased SLC6A20, SLC7A1, SLC7A6, SLC16A14, and SLC38A5 mRNA expression ($P < 0.05$). These findings suggest that dietary supplementation of VB6 mainly down-regulated inflammatory cytokines and up-regulated AA transporters mRNA

expression in jejunum, while up-regulated (4 mg/kg) inflammatory cytokines and down-regulated AA transporters mRNA expression in ileum, which may provide a reference for the intestinal development of weaned piglets that are fed a low-CP diet.

Key words: amino acid transporters, inflammation cytokines, intestine, low crude protein, vitamin B6, weaned piglets

Introduction

Weaned piglets are extremely sensitive to dietary crude protein (CP) levels and the CP level in the diet of weaned pigs is closely associated with their growth performance, feed efficiency, and gastrointestinal health (Acciaioli et al., 2011; Gallo et al., 2014). Indigested high-level dietary CP in the intestine could contribute to the production of pathogenic bacteria and increase the risk of digestive disorders (Ball et al., 1987). Many studies have shown that feeding weaned piglets a lower-CP diet could reduce feed costs, decrease plasma urea nitrogen, ammonia nitrogen, affect intestinal morphology, and intestinal amino acid (AA) transporters expression, cause lower ammonia concentrations in the small intestine, and volatile fatty acids in the ileal digesta (Bikker et al., 2006; Nyachoti et al., 2006; Zhang et al., 2013; Liu et al., 2014; Recktenwald et al., 2014; Ren et al., 2014). The small intestine is a major site for extensive absorption and metabolism of AAs in humans and animals (Stoll et al., 1998; Wu, 1998). Amino acids play an important role in intestinal development and function, and AAs metabolism in intestine is often changed along with the change of dietary CP levels and AAs supplement (Rhoads et al., 1997; Van Goudoever et al., 2000; Yue and Qiao, 2008). Low-CP diet may affect intestinal morphology and function by regulating the AAs metabolism.

Vitamin B6 (VB6) has long been recognized as a cofactor for many enzymes in various metabolic reactions, most of which are involved in the metabolism of AAs and protein, including nucleotide and protein synthesis (Axelrod and Trakatellis, 1964). VB6 is also an essential factor in the growth and differentiation of various immune-response cells through regulating AAs metabolism and then alter immune response (Chandra and Newberne, 1977). Low-CP diet with VB6 supplementation may affect intestine health by regulating the AAs metabolism. Moreover, VB6 could reduce the proteolytic maturation of IL-1 β by inhibiting NLRP3 activation (Zhang et al., 2016). Inflammatory bowel disease is often accompanied by a low levels of plasma VB6. VB6 supplementation could reduce the index of cell proliferation in mice colon while have no effects on colonic epithelium apoptosis (Komatsu et al., 2001). Even though VB6 is an important coenzyme in intestinal AA metabolism, its effect on intestinal health and function is not clear in weaned piglets. Therefore, we hypothesized that dietary supplementation with VB6 would improve intestinal health in weaned piglets that are fed a low-protein diet. The objective of the current study was to determine how VB6-supplement in a low-protein diet fed to weaned piglets affect growth, intestinal morphology, inflammatory factors expression, and AA transporters expression.

Materials and Methods

The experimental design and procedures that were used in this study were reviewed and approved (approval number 2016-093) by the Animal Care and Use Committee of Hunan Normal University, Changsha City, Hunan, China (Yan et al., 2018).

Animals and Experimental Treatments

Eighteen crossbred piglets (Duroc $\times$ Landrace $\times$ Large White), weaned at 21 d of age, were grouped by initial body weight of 7.03 ± 0.17 (means $\pm$ SEM) kg and randomly assigned to three treatment groups fed a low-CP diet with 0, 4, and 7 mg/kg VB6 supplementation, respectively. Each group had six replicates. Corn-soybean meal basal diet contained similar nutrient levels but differed in the VB6 concentrations that were provided to piglets. The background content of VB6 in basal diet (Table 1) was 7.58 mg/kg which slightly exceeded NRC (2012) nutrient requirement (7 mg/kg) for 5- to 11-kg BW piglets. The VB6 product that was used in our study was obtained from Royal DSM Vitamins Co., Ltd (Shanghai, China) and it was 99% pure. With zeolite powder as the carrier, VB6 was added to VB6-free

Table 1. Basal diet composition and nutritional component

Component	Content, %
Corn	42.85
Extruded corn	20.00
Soybean meal	10.00
Soy protein concentrate powder	2.88
Whey powder	10.00
Fish meal	3.00
Spray-dried plasma protein	5.00
Lys	0.55
Met	0.12
Thr	0.13
Trp	0.04
Soybean oil	2.55
Limestone	1.08
Dicalcium phosphate	0.70
Choline chloride	0.10
Antioxidant	0.05
Citric acid	0.30
Vitamin and mineral premix*	0.65
Total	100
Calculated composition	
CP, %	18.00
ME, MJ/kg	14.23
Ca, %	0.80
AP, %	0.36
Lys, %	1.35
Met, %	0.39
Met + Cys, %	0.74
Thr, %	0.79
Trp, %	0.22
VB6, mg/kg	7.58

*Vitamin-mineral premix supplied per kilogram of feed: 2,200 IU of vitamin A, 220 IU of vitamin D3, 16 IU of vitamin E, 0.5 mg of vitamin K3, 0.0175 mg of vitamin B12, 3.5 mg of riboflavin, 30 mg of niacin, 10 mg of D-pantothenic acid, 0.05 mg of biotin, 0.3 mg of folic acid, 1.0 mg of thiamine, 150 mg of Fe (FeSO₄), 100 mg of Zn (ZnSO₄), 30 mg of Mn (MnSO₄), 25 mg of Cu (CuSO₄), 0.5 mg of I (KIO₃), 0.3 mg of Co (CoSO₄), 0.3 mg of Se (Na₂SeO₃), and 4.0 mg of ethoxyquin.

vitamin premix using step-by-step dilution. Then, the vitamin premixes with different VB6 contents were used for producing a compound feed. Piglets were housed individually caged and the ambient temperature was maintained at 29 ± 1 °C. The experimental diets were offered on an ad libitum basis for 14 d, and water was available throughout the experimental period. Any sign of sickness or abnormal behavior were noted during the experimental period. At the end of study, individual piglets were weighed. Average daily gain (ADG), average daily feed intake (ADFI), feed conversion rate (FCR), and diarrhea rate (DR) were calculated according to Yang et al. (2013). The diarrhea rate of piglets was recorded daily and calculated as follows: Diarrhea rate (%) = the number of pigs suffering from diarrhea $\times$ diarrhea days / (the total number of pigs $\times$ experiment days) $\times$ 100%, where the “total number of pigs with diarrhea” was defined as the number of pigs with diarrhea observed on each day (Liu et al., 2008).

Sampling

Before slaughter, the piglets were fasted overnight and blood samples were collected by vein puncture from each piglet on the 14th day of the experiment, then centrifuged at $3,000 \times g$ and 4 °C for 10 min to recover serum. Serum samples were stored at -80 °C until for biochemistry analysis. Piglets were then held under general anesthesia with an intravenous injection of 4% sodium pentobarbital solution (40 mg/kg BW), followed by exsanguination (Kong et al., 2007). Intestinal tissues from middle sections of the duodenum, jejunum, and ileum were isolated using sterilized instruments, flushed with ice-cold PBS. Approximately 2 cm long (duodenum, jejunum, and ileum) was fixed in 10% neutral formalin and kept at 4 °C for morphometric analysis. The remaining of jejunum and ileum (approximately 20 cm long) was used for the collection of mucosa on a glass slide. Those mucosa samples were immediately frozen in liquid nitrogen and stored at -80 °C until it was required for further analysis of mRNA expression.

Analysis of Serum Biochemical Parameters

Serum concentrations of total protein, urea nitrogen, ammonia, total cholesterol, high-density lipoprotein, low-density lipoprotein, immunoglobulin G, immunoglobulin M, and glucose were determined using spectrophotometric kits in accordance with the manufacturer's instructions (Nanjing Jiangcheng Biotechnology Institute, Jiangsu Province, China) by an Automatic Biochemistry Radiometer (Hitachi Co., Tokyo, Japan).

Intestinal Histomorphology

The fixed intestinal tissues were prepared according to standard paraffin-embedding techniques. Three cross sections of 4- μ m thickness were cut from the paraffin blocks, dried at 37 °C, and stained with hematoxylin and eosin. The villus height (VH), villus width (VW), and crypt depth (CD) of each intestinal segment were determined under a microscope using a 100 $\times$ -combined magnification along with an image processing and analysis system (Version 1, Leica Imaging Systems Ltd., Cambridge, UK). At least 20 well-oriented intact villi and their associated crypts were examined in each intestinal section of each piglet. The villus height to crypt depth ratio (VH/CD) and villus area (VA) were calculated (De Vos et al., 2014; Zong et al., 2018).

Immunohistochemistry

The 4- μ m thickness sections were cut from the paraffin-embedded samples and processed according to standard

immunohistochemistry protocols with minor modifications as follows: 1) Deparaffinized in xylene and rehydrated in a descending ethanol series. 2) Microwave-heated twice in an aqueous sodium citrate buffer to retrieve antigen epitopes. 3) Blocked endogenous peroxidase in 3% hydrogen peroxidase for 10 min in the dark. 4) Incubated in diluted bovine serum albumin in a moist chamber, at 37 °C for 30 min. 5) Incubated in a moist chamber with the following primary antibody: anti-Ki-67 (Abcam, Cambridge, UK, dilution 1:800; incubation 90 min, 37°C). 6) Treated with a secondary antibody (zsbio, Beijing, China) at 37 °C to bind the primary antibody for 50 min (zsbio). 7) Visualized with diaminobenzidine (DAB) (ZSGB-BIO, Beijing, China) for positive cells. 8) Counterstained with hematoxylin, then dehydrated, and mounted. Except for blocking, each step was followed by four 3-min PBS washes. Lastly, Ki67 scores (the number of positive cells per field under 400 $\times$ magnification) were evaluated using Image-Pro Plus 6.0 software (Media Cybernetics; San Diego, CA).

RNA Extraction, cDNA Synthesis, and Quantitative Real-Time PCR

Total RNA was extracted from the frozen jejunal and ileal mucosa using Trizol reagent (Invitrogen), according to the manufacturer's instructions. The quality and yield of extracted RNA were determined by a NanoDrop ND2000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE) with A260/280 of 1.8 to 2.0. The integrity of the total RNA was further checked on 1% agarose gel electrophoresis with the visualization of complete 28S and 18S bands. The complementary DNA (cDNA) was then synthesized from 1 μ g total RNA using a PrimeScript RT reagent kit with gDNA eraser (Takara Biotechnology Co., China) for the removal of genomic DNA contaminations. Quantitative real-time PCR (qRT-PCR) for gene expression was performed in duplicate using a SYBR Premix Ex TaqTMIIqPCR kit (TaKaRa Biotechnology Co.) on the QuantStudio 5 Real-Time PCR System analyzer (Applied Biosystems). The specificity of the amplification was confirmed by the melting curve. The primers were designed using Primer Premier 5.0 software (Applied Biosystems) and were synthesized by Tsingke Biological Technology (Changsha, China). The primer sequences and product lengths of target genes are listed in Table 2. The fold change of the target genes was normalized to the housekeeping gene (β -actin) and calculated using the $2^{-\Delta\Delta C_t}$ method.

Statistical Analysis

The experimental data were examined by one-way ANOVA using the GLM procedure of SAS (Version 9.3; SAS Institute, Inc., Cary, NC). Values were represented as mean values. Differences were considered significant when $P < 0.05$ and tendencies when $0.05 \leq P < 0.10$.

Results

Growth Performance, Diarrhea Rate, and Serum Biochemical Parameters

Table 3 shows that no significant differences in growth performance and DR were observed among experimental treatments ($P > 0.05$). Table 4, which contains the biochemical parameters, shows that no differences existed in the concentration of total protein, urea nitrogen, ammonia, total cholesterol, high-density lipoprotein, low-density lipoprotein,

Table 2. Primer pairs used in the study

Gene*	Sequence, 5'–3'	Product length, bp
β -actin	F: AGTTGAAGGTGGTCTCGTGG R: TCGGGGACATCAAGGAGAAG	216
<i>IL-1β</i>	F: CTGGACCTTGGTTCTCT R: GGATTCCTCATCGGCTTCT	123
<i>TNF-α</i>	F: ACAGGCCAGCTCCCTCTTAT R: CCTCGCCCTCCTGAATAAAT	102
<i>COX-2</i>	F: CCAGGTTTAAGATCTGATGTGGGA R: TGCCCTTCCATCATTACGAATCCTT	165
<i>IL-10</i>	F: GGCTATTTGTCTGACTGC R: GGGCTCCCTAGTTTCTCTTCC	105
<i>TGF-β</i>	F: CGAGCCCTGGATACCAACTA R: AGGCTCCAGATGTAGGGACA	164
<i>SLC6A20</i>	F: CCAATGGCCTGGTGATCATG R: CGACACTGGCAAAGATGGAG	216
<i>SLC7A1</i>	F: GACCGGACCAAAACACCAATAAT R: GAGCGTGCCAATGGACATGA	114
<i>SLC7A6</i>	F: CAGGACGCCTTTGAGGGTTC R: TTCTGGGTTTTTGATTCTTCTGTT	126
<i>SLC16A14</i>	F: CCAGGATTGGTACTCGGGCT R: TGGGAGGTGAATGAAAGGGA	124
<i>SLC36A1</i>	F: CAACTGCCACAACAACGAGA R: AGATCATGACCAGGCTGACC	173
<i>SLC38A5</i>	F: GTATGCTGTTTTCTCATTTCCG R: CTTGAGGGGCCCTTACTCTCC	100

**IL-1 β* : interleukin-1 β , *TNF- α* : tumor necrosis factor- α , *COX-2*: cyclo-oxygenase-2, *IL-10*: interleukin-10, *TGF- β* : transforming growth factor- β , *SLC6A20*: solute carrier family 6 member 20, *SLC7A1*: solute carrier family 7 member 1, *SLC7A6*: solute carrier family 7 member 6, *SLC16A14*: solute carrier family 16 member14, *SLC36A1*: solute carrier family 36 member 1, *SLC38A5*: solute carrier family 38 member5.

Table 3. Effects of VB6 on growth performance and diarrhea rate*

Items ¹	VB6, mg/kg			Pooled SEM	P-value
	0	4	7		
IBW, kg	7.03	7.03	7.02	0.17	0.999
FBW, kg	9.37	9.24	9.27	0.21	0.971
ADG, g	166.67	145.71	160.71	6.69	0.469
ADFI, g	299.90	292.46	288.92	8.03	0.860
FCR, g/g	0.56	0.49	0.56	0.02	0.219
DR, [†] %	4.76	2.38	3.57	1.76	0.873

¹IBW, initial body weight; FBW, final body weight; ADG, average daily gain; ADFI, average daily feed intake; FCR, feed conversion rate.

*Data are analyzed by one-way ANOVA using the GLM procedure of SAS and presented as mean values. The pooled SEM represent pooled standard error of the mean ($n = 6$).

[†]Diarrhea rate (%) = total number of pigs with diarrhea/(total number of pigs $\times$ experimental days) $\times$ 100%.

immunoglobulin G, immunoglobulin M, and glucose among all the experimental treatments ($P > 0.05$).

Intestinal Morphology and Immunohistochemistry

Table 5 on histological evaluation showed that no effects on VH, VW, CD, VA, and VH/CD were observed in the duodenum and jejunum among the experimental treatments ($P > 0.05$). In the ileum, VB6 supplementation significantly decreased VA ($P = 0.027$) and tended to decrease VH ($P = 0.093$). Intestinal cell proliferation was evaluated using immunohistochemistry

Table 4. Effects of VB6 on serum biochemical parameters*

Item ¹	VB6, mg/kg			Pooled SEM	P-value
	0	4	7		
TP, g/L	45.35	41.68	45.70	1.43	0.471
BUN, mmol/L	1.57	1.87	1.91	0.15	0.629
NH ₃ L, μ mol/L	362.62	326.25	320.07	13.03	0.377
CHOL, mmol/L	1.85	1.82	1.85	0.07	0.984
HDL, mmol/L	0.69	0.74	0.79	0.04	0.593
LDL, mmol/L	0.97	1.04	1.04	0.04	0.786
IGG, g/L	1.22	1.02	1.25	0.05	0.141
IGM, g/L	0.43	0.37	0.33	0.02	0.126
GLU, mmol/L	4.62	4.01	4.85	0.19	0.215

¹TP, total protein; BUN, blood urea nitrogen; NH₃L, ammonia; CHOL, cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; IGG, immunoglobulin G; IGM, immunoglobulin M; GLU, glucose.

*Data are analyzed by one-way ANOVA using the GLM procedure of SAS and presented as mean values. The pooled SEM represent pooled standard error of the mean ($n = 6$).

Table 5. Effects of VB6 on small intestine morphological indicators*

Items	VB6, mg/kg			Pooled SEM	P-value
	0	4	7		
Duodenum					
VH, μm	289.41	280.96	292.93	8.75	0.889
VW, μm	143.12	149.06	142.02	4.09	0.403
VA, mm	0.132	0.133	0.131	0.01	0.994
CD, μm	363.61	432.53	402.38	13.33	0.288
VH/CD, $\mu\text{m}/\mu\text{m}$	0.82	0.65	0.75	0.03	0.325
Jejunum					
VH, μm	356.48	321.23	356.53	14.91	0.567
VW, μm	129.72	120.28	116.37	3.07	0.194
VA, mm	0.146	0.122	0.132	0.01	0.495
CD, μm	236.36	242.46	235.33	7.19	0.918
VH/CD, $\mu\text{m}/\mu\text{m}$	1.51	1.35	1.53	0.08	0.613
Ileum					
VH, μm	304.24a	266.81b	259.98b	9.06	0.093
VW, μm	131.68	123.46	119.39	2.58	0.140
VA, mm	0.126a	0.104b	0.097b	0.01	0.027
CD, μm	266.52	256.70	237.13	7.61	0.292
VH/CD, $\mu\text{m}/\mu\text{m}$	1.15	1.04	1.12	0.04	0.576

*Data are analyzed by one-way ANOVA using the GLM procedure of SAS and presented as mean values. The pooled SEM represent pooled standard error of the mean ($n = 6$). ^{a,b}Values within a row with different superscripts differ significantly at $P < 0.05$ or show a tendency toward differing at $0.05 \leq P < 0.10$.

for Ki67. The Ki67-positive cells per crypt in the jejunum and ileum were counted. VB6 did not affect Ki67-positive cells in the jejunum and ileum ($P > 0.05$) (Figure 1).

The mRNA Expression of Inflammatory Cytokines in Intestinal Mucosa

The mRNA expression of inflammatory cytokines in intestinal mucosa was measured. In the jejunum (Figure 2A), the 4 mg/kg VB6 supplementation notably reduced the relative mRNA expression of COX-2, IL-10, and TGF- β ($P < 0.05$) and the 7 mg/kg VB6 supplementation only notably reduced the relative mRNA expression of IL-10, as compared with the 0 mg/kg group ($P < 0.05$). No difference was observed for

Figure 1. Post-B6 supplementation weaned piglet intestinal cell proliferation. (A, B) The representative images of immunohistochemistry (IHC) staining with Ki67 antibody in weaned piglet jejunum and ileum shown (400×; $n = 6$). (C, D) The statistical analysis of Ki67-positive cells in each crypt from images shown on the A and B. Column data are analyzed by one-way ANOVA using the GLM procedure of SAS and presented as mean values. The bars represent SEM (SEM, standard error of the mean; $n = 6$). There are significant differences among treatment groups for bars not labeled with the same letter ($P < 0.05$).

the relative mRNA expression of IL-1 β . In the ileum (Figure 2B), the 4 mg/kg VB6 supplementation notably increased the relative mRNA expression of IL-1 β , TNF- α , COX-2, IL-10, and TGF- β ($P < 0.05$) and the 7 mg/kg VB6 supplementation only increased the relative mRNA expression of IL-1 β ($P < 0.05$), as compared with the 0 mg/kg group.

The mRNA Expression of AA Transporter in Intestinal Mucosa

The mRNA expression of AA transporters was detected in the intestinal mucosa. In the jejunum (Figure 3A), the 7 mg/kg VB6 supplementation significantly increased SLC7A1, SLC7A6, SLC16A14, and SLC38A5 ($P < 0.05$) mRNA expression and decreased SLC36A1 ($P < 0.05$) mRNA expression, as compared with the 0 mg/kg group. No difference was observed for the relative mRNA expression of SLC6A20 ($P > 0.05$). In the ileum (Figure 3B), the 4 mg/kg VB6 supplementation notably decreased the relative mRNA expression of SLC6A20, SLC7A1, SLC7A6, SLC16A14, SLC36A1, and SLC38A5 ($P < 0.05$) and the 7 mg/kg VB6 supplementation notably decreased the relative mRNA expression of SLC6A20, SLC7A1, SLC7A6, SLC16A14, and SLC38A5 ($P < 0.05$), as compared with the 0 mg/kg group.

Discussion

Many studies have shown that the accurate VB6 content in a diet for maximizing growth performance was recommended

as 2 to 5 mg/kg of mix feed for weaned piglets (Kosters and Kirchgessner, 1976). Growth and metabolic criteria suggested optimal responses at dietary and parenteral daily levels as 2 and 10 times higher, respectively, than those that are currently recommended (NRC 1998; Woodworth et al., 2000; Matte et al., 2001). The dietary VB6 requirement for piglets is 7 mg/kg in NRC (2012). Matte et al. (2001) reported that the basal level of dietary VB6 (7.7 mg/kg), although sufficient to prevent deficiency symptoms of VB6, is insufficient to cover the metabolic needs during the 2 wk following the weaning of piglets. Further studies are necessary to determine the precise optimal dietary level of VB6. According to our knowledge, no reports about the influence of VB6 on the incidence of diarrhea in weaned piglets have been published. In the present study, no significant differences were observed in ADG, ADFI, FCR, and DR among piglets at 2-wk post-weaning that were fed a low-CP diet with VB6 supplementation. This result is in line with Matte et al. (2001) and the effect of relevant metabolic processes require further research. Previous studies have shown that the content of ovalbumin-dependent antibody IgG and IgE were suppressed by a VB6-excess diet (Inubushi et al., 2000). The serum antibody (IgG, IgM) production in VB6-deficient mice decreased and could be recovered to normal level after a short-term supplementation of VB6 (Kumar and Axelrod, 1968). Our study results showed that VB6 supplementation had no significant impact on total protein, urea nitrogen, ammonia, total cholesterol, high-density lipoprotein, low-density lipoprotein, immunoglobulin G, immunoglobulin M,

Figure 2. Effects of dietary supplementation of VB6 on mRNA expression of jejunal (A) and ileal (B) inflammatory cytokines in weaned piglets. The mRNA expression abundances of IL-1 β , TNF- α , COX-2, IL-10, and TGF- β are normalized using β -actin as an internal control. Column data are analyzed by one-way ANOVA using the GLM procedure of SAS and presented as mean values. The bars represent SEM (SEM, standard error of the mean; $n = 6$). There are significant differences among treatment groups for bars not labeled with the same letter ($P < 0.05$).

and glucose among the three treatments. This is not consistent with previous studies on rats. This discrepancy might have been caused by the differences in species or the dose of VB6 supplementation.

The absorption of intestinal nutrients, which contains AAs, fatty acids, and glucose, is closely linked to intestinal histomorphology reflected by VH, VW, VA, CD, and the ratio of VH/CD (Hedemann et al., 2003). Higher VH has been generally believed to be a result of an increased surface area that is capable of greater absorption of the available nutrients. Deeper CD indicates a rapid cell turnover in the villus renewal. In the present study, we observed a significant decrease in VA and a tendentious decrease in VH in the ileum when our piglets were fed with dietary VB6 for 14 d post-weaning, thereby demonstrating that VB6 may cause a negative impact on the ileal morphology

of weaning piglets. Intestinal epithelial cell proliferation has recently been showed to have a positive connection with VH and CD in weaning piglets (Wang et al., 2018). Epithelial cells that are positive for Ki67 were mostly present in crypts. Cell proliferation of the gastrointestinal tract epithelium was evaluated by counting Ki67-positive cells (De Lange et al., 2010). No researches that we are aware of illustrating a relationship between VB6 and intestinal epithelial cell production. Our study results showed that VB6 supplementation had no significant impact on the Ki67-positive cells, neither in the jejunum nor in the ileum.

Over the last decade, it has become rather apparent that a robust relationship exists between VB6 and inflammation (Friso et al., 2001; Martha Savaria et al., 2010). A moderation of VB6 might mediate immune signal transduction or regulate

Figure 3. Effects of dietary supplementation with VB6 on mRNA expression of jejunal (A) and ileal (B) AA transporters in weaned piglets. The mRNA expression abundances of SLC6A20, SLC7A1, SLC7A6, SLC16A14, SLC36A1, and SLC38A5 are normalized using β -actin as an internal control. Column data are analyzed by one-way ANOVA using the GLM procedure of SAS and presented as mean values. The bars represent SEM (SEM, standard error of the mean; $n = 6$). There are significant differences among treatment groups for bars not labeled with the same letter ($P < 0.05$).

immune cell differentiation and cytokine production, together with other signal molecules, to reach an immune homeostasis. Lower plasma VB6 is associated with increased levels of pro-inflammatory cytokine TNF- α (Talwar et al., 2003). In patients with rheumatoid arthritis, high dose of VB6 supplementation (100 mg/d) suppresses TNF- α level (Huang et al., 2010). Both low and high plasma VB6 were associated with a significant suppression in TNF- α , IL-6, and COX-2 mRNA expression in the colon (Selhub et al., 2013). In our study, the 4 mg/kg VB6 supplementation notably reduced the jejunal mRNA expression of COX-2, IL-10, and TGF- β , while it increased the ileal mRNA expression of IL-1 β , TNF- α , COX-2, IL-10, and TGF- β , as compared

with the 0 mg/kg group. These results indicated that VB6 played an important role in intestinal inflammation response and there existed different mechanisms, which were necessary for their distinctive functions and their various microbial environments, for the jejunum and ileum. However, the molecular mechanisms were not yet fully understood and needed further research.

Amino acids not only played an important role in protein synthesis and turnover in mammalian intestine but also in cellular growth, proliferation, and inflammatory response (Naomoto et al., 2005; He et al., 2018). The low-CP diet could provide sufficient amounts of AAs to maintain basal rates of protein synthesis in the gut. Additionally, protein synthesis in

the small intestine did not decrease with the reduced protein intake for a short period of time (Deng et al., 2009). Amino acids in the enteron are mainly transported into the enterocyte in free form by specific AA transporters (Palacin and Kanai, 2004). Many reports suggested that VB6 participated in many phases of protein and AA metabolism and played a role in the active process of cellular uptake of AAs (Christensen et al., 1954). In the present study, the 7 mg/kg VB6 supplementation increased mRNA expression of SLC7A1, SLC7A6, SLC16A14, and SLC38A5 and decreased mRNA expression of SLC36A1 in the jejunal mucous. The 4 mg/kg VB6 supplementation decreased mRNA expression of SLC6A20, SLC7A1, SLC7A6, SLC16A14, SLC36A1, and SLC38A5 in the ileal mucous. It is suggested that VB6 may mediate the metabolism of protein metabolism and absorption of AAs by altering the transportation of AAs in weaned piglets. Combined with the mRNA expression of inflammatory factors in the jejunum and ileum, we found an interesting phenomenon: the mRNA expression of inflammatory factors was down-regulated in the jejunum, while it was up-regulated in the ileum and the mRNA expression of AA transporters were up-regulated in the jejunum, while they were down-regulated in the ileum after VB6 supplementation. Meanwhile, the mRNA expression of inflammatory factors was down-regulated, while the expression of AA transporters was up-regulated in the jejunum and the mRNA expression of inflammatory factors were up-regulated, while the expression of AA transporters was down-regulated in the ileum. We speculate that there exists an interaction between AA transportation and inflammatory response with dietary VB6 supplementation in the jejunum and ileum, but further study is necessary to determine the specific correlation.

In conclusion, dietary supplementation with VB6 mainly down-regulated inflammatory cytokines and up-regulated AA transporters mRNA expression in jejunum, while mainly up-regulated (4 mg/kg) inflammatory cytokines and down-regulated AA transporters mRNA expression in ileum, which may provide a reference for intestinal development of weaned piglets that are fed a low-CP diet. Further studies are required to test the mechanism that is involved in gene regulation and to determine whether other genes that are related to nutrient absorption are also regulated by VB6 supplementation.

Conflict of interest statement

The authors declare no real or perceived conflicts of interest.

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