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Non Ruminant Nutrition

NON RUMINANT NUTRITION

Dietary vitamin A affects growth performance, intestinal development, and functions in weaned piglets by affecting intestinal stem cells

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ABSTRACT:

Vitamin A (**VA**) is an important nutrient for weaning piglets. It plays a significant role in the normal formation, development, and maintenance of epithelial cells. Previous studies have shown that VA supplements could improve the host's intestinal barrier function. Therefore, we hypothesized that VA supplements can affect intestinal function in weaned piglets by regulating intestinal stem cells. Thirty-two 21-d-old weaned [(Yorkshire × Landrace) × Duroc] piglets with an average weight of 8.34 ± 0.13 kg were randomly divided into 4 treatment groups, with 1) 2 mg/kg (control), 2) 4 mg/kg, 3) 8 mg/kg, and 4) 16 mg/kg doses of VA, respectively. The experiment lasted for 14 d. Weaned piglets were given ad libitum access to food and water during the test. The ADG (linear, $P = 0.020$) and G:F (linear, $P = 0.005$) of the piglets were found to increase significantly from days 8 to 14. The *Lgr5⁺* gene expression ($P = 0.012$) in the jejunum mucosa of the 16 mg/kg VA group was increased. The jejunum villus height ($P = 0.027$) and villi surface area ($P = 0.035$) were significantly increased in the 4 mg/kg VA treatment group. The crypt depth increased significantly in the 4 and 8 mg/kg VA treatment groups (quadratic, $P = 0.043$), and the ratios of villus height to crypt depth significantly increased in the 16 mg/kg VA group (quadratic, $P = 0.015$). The maltase ($P = 0.032$), sucrose ($P = 0.041$), and alkaline phosphatase activity (linear, $P = 0.024$) were significantly increased when further supplemented with 4 mg/kg VA. *Slc2a2* mRNA abundance was significantly increased in the 2 mg/kg VA group (linear, $P = 0.024$). Moreover, the budding rates, buddings number per organoid, and *Chromogranin A* and *Muc2* expression of piglet intestinal organoids were significantly reduced ($P < 0.05$) by VA and its metabolites (retinoic acid). Compared with the control group, the expression of *Spp1* and *Trop2* increased. These results indicated that VA may increase the stemness of intestinal stem cell in vitro. This study suggested that VA could affect growth performance and intestinal function by regulating intestinal stem cells in the jejunum of weaned piglets.

Key words: enzyme activity, growth performance, intestinal development, intestinal stem cells, vitamin A, weaning piglets

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Abbreviations

CD	crypt depth
RE	relative expression
TSAP	tissue-specific phosphatase
VA	vitamin A
VH	villus height
VSA	villus surface area
VW	villus width

Introduction

The weaning transition is a critical period for piglet growth. The piglets' growth and development after weaning is very sensitive to a variety of stressors, which has an adverse effect on intestinal health and leads to huge economic losses in swine husbandry (van Beers-Schreurs and Bruininx, 2002). Weaning stress usually affects the intestinal stem cells in weaned piglets, resulting in negative effects such as diarrhea (Yang et al., 2016a). At the same time, this phase is accompanied by significant changes in gastrointestinal physiology, microbiology, and immunology (Hampson, 1986; Pluske et al., 1997). Hence, it is necessary for piglets to supplement some essential nutrients (such as vitamin A [**VA**]) to relieve weaning stress.

Vitamin A is an important nutrient for piglets. It plays a key role in vision, fetal development, reproduction, and epithelial cells (Blomhoff, 1994; Balmer and Blomhoff, 2002; Blomhoff and Blomhoff, 2006). There is evidence that VA can be involved in the synthesis of glycoproteins (Wolf et al., 1979). This is important for the normal formation, development, and maintenance of epithelial cells. VA deficiency leads to reduced synthesis of serum glycoprotein in rats (Wolf et al., 1979). β-Carotene, an antioxidant, is a form of VA that plays an important role in preventing lipid peroxidation and tumor formation and delaying aging (Sarkar et al., 1995; Bhatia, 1998). Previous studies have shown that VA supplements could improve the host's intestinal barrier function (Lima et al., 2010). Weaning stress in piglets causes severe intestinal damage in the first 2 wk after weaning (Montagne et al., 2007). We hypothesized that dietary VA supplements can improve intestinal development, morphology, and mucosal enzyme activity in weaning piglets. This study focused on the effects of VA on growth performance, organ index, intestinal morphological structure, intestinal digestive enzyme activity, blood biochemical parameters, and intestinal stem cells in weaned piglets.

Materials and methods

The experimental design and procedures in this study were approved by the Animal Care and Use Committee of the Hunan Normal University, Changsha City, Hunan, China (Chen et al., 2019).

Animals and experimental treatments

This animal experiment was carried out in the animal house of the Institute of Subtropical Agroecology, Chinese Academy of Sciences. Thirty-two piglets [(Yorkshire × Landrace) × Duroc] at 8.34 ± 0.13 kg average BW were weaned at the age of 3 wk and randomly assigned to 1 of the 4 basic diets containing 2 (control group), 4, 8, or 16 mg/kg VA (Royal DSM NV, Shanghai, China). The VA in the diet was added in the form of retinol acetate. The basic diet (Table 1) met the nutritional requirements of the National Research Council (NRC, 2012) for piglets weighing 7 to 11 kg, and it contained 0.20 mg/kg VA. The piglets were fed for 14 d—8 replicate pens per treatment group and 1 piglet in each pen. The piglets were free to feed and water throughout the experimental period. They were regularly observed for illness, diarrhea, or other abnormal behavior. Following previously described methods (Yin et al., 2001), the piglets' growth performance was determined by calculating the ADG, ADFI, and the ratio of ADG to ADFI (G:F) throughout the trial period.

Table 1. Ingredient and chemical composition of weaning piglet diets, as-fed basis

Items	Dietary vitamin A, mg/kg			
	2	4	8	16
Ingredient, %				
Corn	40.14	40.14	40.14	40.14
Extruded corn[1]	20	20	20	20
Soybean meal	9	9	9	9
Fish meal	7	7	7	7
Plasma protein powder	5	5	5	5
Whey powder	9	9	9	9
Glucose[2]	3	3	3	3
Soybean oil	3.8	3.8	3.8	3.8
Stone powder	1.05	1.05	1.05	1.05
Choline chloride	0.1	0.1	0.1	0.1
Antioxidants	0.05	0.05	0.05	0.05
Citric acid	0.5	0.5	0.5	0.5
Salt	0.1	0.1	0.1	0.1
L-Lys HCl	0.45	0.45	0.45	0.45
DL-Met	0.2	0.2	0.2	0.2
L-Thr	0.14	0.14	0.14	0.14
L-Trp	0.02	0.02	0.02	0.02
Mineral premix[3]	0.15	0.15	0.15	0.15
Vitamin premix[4]	0.3	0.3	0.3	0.3
Total	100	100	100	100
Calculated composition				
CP, %	18.65	18.65	18.65	18.65
ME, kcal/kg	3,407	3,407	3,407	3,407
VA, mg/kg	2.20	4.20	8.20	16.20
Ca, %	0.80	0.80	0.80	0.80
Total P, %	0.56	0.56	0.56	0.56
Available P, %	0.38	0.38	0.38	0.38
L-Lys[5], %	1.37	1.37	1.37	1.37
Thr[5], %	0.81	0.81	0.81	0.81
Trp[5], %	0.22	0.22	0.22	0.22
SAA[5,6], %	0.75	0.75	0.75	0.75
DCAD[7], mEq/kg DM	2.32	2.32	2.32	2.32

[1]Bulk density is 0.3 to 0.5 kg/L, puffing temperature is 100 to 150 °C. The moisture content of the finished product is 8% to 10%, and the degree of gelatinization is above 90%.
[2]Dextrose monohydrate.
[3]Mineral premix per kilogram of feed: 150 mg Fe ($FeSO_4$), 100 mg Zn ($ZnSO_4$), 30 mg Mn ($MnSO_4$), 25 mg Cu ($CuSO_4$), 0.5 mg I (KIO_3), 0.3 mg Co ($CoSO_4$), and 0.3 mg Se (Na_2SeO_3).
[4]Vitamin premix supplied per kilogram of feed: 220 IU vitamin D_3; 16 IU vitamin E; 0.5 mg vitamin K_3; 17.5 μg vitamin B_{12}; 3.5 mg riboflavin; 30 mg niacin; 10 mg D-pantothenic acid; 0.05 mg biotin; 0.3 mg folic acid; 1.0 mg thiamine; 7 mg pyridoxine; and, 4.0 mg ethoxyquin.
[5]Standardized ileal digestible.
[6]SAA = Met + Cys.
[7]DCAD = dietary cation–anion difference, which was calculated according to the formula: [(%Na/0.023 + %K/0.039)] − [(%Cl/0.0355 + %S/0.016)].

Sample collection and measurements

At 14 d, after the piglets had been made to fast overnight, approximately 10 mL of blood samples was collected from them

Table 2. Primers used for real-time PCR analysis

Genes	Primers	Primers sequences, 5′ to 3′	Size, bp	NCBI accession number
Lgr5⁺	Forward	GCCTTTGTAGGCAACCCTTC	121	NM_001315762.1
	Reverse	AGGCACCATTCAAAGTCAGTG		
Slc15a1/PepT1	Forward	CATCGCCATACCCTTCTG	144	NM_214347.1
	Reverse	TTCCCATCCATCGTGACATT		
Slc7a1/CAT1	Forward	CAACGACCGGACCAAAACAC	193	NM_001012613.1
	Reverse	CTGGTACACCATGTTCGGCT		
Slc6a19/B⁰AT1	Forward	CCTGACGCTTATCAACGGGT	137	XM_003359855.4
	Reverse	AGTTCATGTCGCAGGTCTGG		
Slc1a1/EAAT3	Forward	GGCACCGCACTCTACGAAGCA	177	NM_001164649.1
	Reverse	GCCCACGGCACTTAGCACGA		
Slc2a2/GLUT2	Forward	GACACGTTTTGGGTGTTCCG	149	NM_001097417.1
	Reverse	GAGGCTAGCAGATGCCGTAG		
Slc27a4/FATP4	Forward	AGACACACGTTGGACCTTCC	188	XM_021069609.1
	Reverse	GCAGGTTGGTGTTGATGAGC		
Chromogranin A	Forward	ACTCCGAGGAGATGAACGGA	205	NM_001164005.2
	Reverse	CTTGGAGGACGCCTCTTCTG		
Muc2	Forward	GCTCCAGAGAGAAGGCAGAACC	171	XM_021082584.1
	Reverse	CTCAGGTGCACAGCGAACTC		
Spp1	Forward	GCCTCTGCCCTTCCAGTTAAA	210	NM_214023.1
	Reverse	CTCAGGGCTTTCGTTGGACT		
Trop2	Forward	CATTACGAGCACCCCACCAT	239	XM_003127967.4
	Reverse	GTGAGGCGCTTCATGGAGAA		
β-Actin	Forward	AGTTGAAGGTGGTCTCGTGG	215	XM_003124280.5
	Reverse	TGCGGGACATCAAGGAGAAG		

by jugular puncture in conventional blood collection tubes. Afterwards, these piglets were euthanized using the previously recommended method on the 14th day (Yan et al., 2018). The weight of the liver, spleen, 2 kidneys, and the total length of the small intestine of each piglet was recorded. The organ indexes (ratio of total weight or total length to BW) were calculated based on the 14th-day BW of the weaned piglets. Jejunum and ileum samples (at least 2 cm)—from which intestinal contents had been removed—were immediately collected and stored in a 4% formalin solution at room temperature. The jejunum and ileum segments of the piglets were cut longitudinally along the intestine; the contents of the intestine were then rinsed with PBS solution. The upper intestinal mucosa was carefully collected with a glass slide, wrapped in tin foil, frozen in liquid nitrogen, and brought back to the laboratory for −80 °C frozen storage until it was required for analysis.

Intestinal morphology analysis

The jejunum and ileum samples soaked in the 4% formalin solution were embedded in paraffin in accordance with standard paraffin embedding procedures (Barea et al., 2011) and then cut into 4-μm-thick sections and stained with hematoxylin and eosin. The villi and crypts of the intestine were observed under a microscope in a 40× combined magnification and image processing and analysis system (Version 4.12, Leica Imaging Systems Ltd., Cambridge, UK). The villus height (**VH**), crypt depth (**CD**), and villus width (**VW**) of the jejunum and ileum were measured using Image-pro Plus 6.0 software. The ratio of VH to CD (VH:CD) and the villus surface area (**VSA**) were calculated. VSA (= π × VH × VW) was calculated according to the method recommended by Kisielinski et al. (2002). At least 20 intact villi and their associated crypts were selected from the intestinal section of each piglet. The corresponding mean value of VH:CD and VSA in each piglet was calculated and used for further analysis (Chen et al., 2019).

Analysis of intestinal enzyme activities

The piglets' jejunum and ileum mucosa tissue samples were homogenized in liquid nitrogen, immediately collected in PBS solution, allowed to stand for 3 h, and centrifuged (3,000 × *g*, 4 °C, 10 min). The supernatants were taken for analysis of intestinal maltase, sucrase, lactase, and alkaline phosphatase activities using the purchased kits according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, China).

Real-time quantitative PCR

The total RNA of the jejunum and ileum samples were isolated from the liquid nitrogen frozen and ground tissues using RNAiso Plus (Code No. 9109, TaKaRa, Dalian, China) and then treated with the PrimeScript RT reagent Kit with gDNA Eraser (Code No. RR047A, TaKaRa, Dalian, China) to remove the genomic DNA (42 °C, 2 min). Reverse transcription was conducted at 37 °C for 15 min and at 85 °C for 5 s, as per the instructions in the product manual. The methods of total RNA extraction, reverse transcription, and RT-qPCR of organoid samples are the same as those of tissue samples. Primers used in this study were designed via Primer design software 6.0 for leucine-rich-repeat-containing G-protein-coupled receptor 5 (*Lgr5*⁺), solute carrier family 15 member 1 (*Slc15a1/PepT1*), solute carrier family 7 member 1 (*Slc7a1/CAT1*), solute carrier family 6 member 19 (*Slc6a19/B⁰AT1*), solute carrier family 1 member 1 (*Slc1a1/EAAT3*), solute carrier family 2 member 2 (*Slc2a2/GLUT2*), solute carrier family 27 member 4 (*Slc27a4/FATP4*), *Chromogranin A*, *Muc2*, *Spp1*, *Trop2*, and the housekeeping gene (*β-actin*) according to pig gene sequence (Table 2). The cDNA was obtained by reverse transcription and diluted 5 times with RNase-free water for real-time quantitative PCR (ABI 7900HT Fast Real-Time PCR System; Applied Biosystems, Carlsbad, CA) analysis using a final volume of 10 μL. The first step was pre-denaturation (95 °C, 30 s); the second step was PCR reaction (95 °C, 5 s and 60 °C, 34 s at 40 cycles). There were 3 repetitions per gene, with each sample

corresponding to β-actin as a reference. The target gene mRNA relative expression (RE) was calculated as RE = $2^{-\Delta\Delta Ct(treat-control)}$; $-\Delta\Delta Ct$ (treat − control) = $(Ct_{target\ gene} - Ct_{\beta\text{-}actin})_{treat} - (Ct_{target\ gene} - Ct_{\beta\text{-}actin})_{control}$. The target mRNA and β-actin mRNA were amplified with comparable efficiencies. The double distilled water (ddH_2O) was used instead of cDNA for the negative control, as described in previous research (Yang et al., 2016b,c).

Three-dimensional culture of piglet intestinal organoids

We first need to prepare precool PBS, thaw Matrigel (BD Biosciences, SanJose, CA) at 4 °C, and prewarm 24-well plate (LabServ, Thermo Fisher Scientific) at 37 °C. Seven-day-old piglets were euthanized, small intestine (jejunum) was taken out and stored in PBS on ice; proximal part of small intestine was cut longitudinally, washed in PBS, scraped off villi using a cover slips slowly and lightly, and removed the fat as completed as possible. Then, the small intestine was washed 2 times in PBS, cut into small pieces (0.2 to 0.3 cm) to make resuspend easier, transferred to a 50-mL tube, and washed 3 to 4 times with ice-cold PBS. Then, the tissue was incubated with 2 mM ethylenediaminetetraacetic acid (Sigma–Aldrich) at 4 °C for 30 min for epithelial isolation. The tube was swayed for 5 min until high crypt purity was obtained and then were filtered through a 70-μm cell strainer. Crypt suspension was added with 10% (vol/vol) FCS and spin down at 300 × *g* for 5 min. The supernatant was discarded. The crypts were resuspended in 15-mL advanced DMEM-GF (Gibco, Grand Island, NY) and spin at 150 × *g* for 2 min. The supernatant was discarded. Crypts were suspended in phenol-red free Matrigel. Then, there was a 50-μl droplet of Matrigel-crypts mix in the each well center of a 24-well plate, and was incubated at 37 °C with 5% CO_2 for 15 min, subsequently. Five hundred microliters of culture medium, which consisted of Wnt3a, Noggin, and R-Spondin 1-conditioned medium, B27 supplement (Invitrogen), N2 supplement (Invitrogen, Carlsbad, CA), glutamine (Sigma-Aldrich), N-acetyl cysteine (Sigma-Aldrich), recombinant murine epidermal growth factor (PeproTech, Rocky Hill, NJ), nicotinamide (Sigma-Aldrich), and SB202190 (Sigma-Aldrich), was added per well after Matrigel got solidification. The culture medium was maintained until passaging organoids. The passaging was performed every 5 d with a 1:3 split ratio. Each well contains at least 10 organoids. The concentrations of retinol and retinoic acid for application to the organoids were confirmed according to the calculated concentrations of VA in Table 1 and previous studies (Matsumoto et al., 2016). The organoids were treated with 0, 5, 10, and 15 μM retinol (Aladdin, V111674, Shanghai, China) and retinoic acid (Aladdin, R106320, Shanghai, China) for 5 d and observed under a microscope in a 20× combined magnification and image processing and analysis system (Version 4.12, Leica Imaging Systems Ltd., Cambridge, UK) on the third and fifth days, respectively. The number of budded and nonbudded organoids, as well as the number of budding on each budding organoid, was counted. We calculated the ratio of budded organoids to all organoids and the average budded numbers per budding organoid. RT-qPCR was used to detect the mRNA abundance of cell differentiation markers (*Chromogranin A* and *Muc2*) and embryonic stem cell markers (*Spp1 and Trop2*) in organoids.

Statistical Analysis

All data were analyzed in accordance with the mixed model procedure (PROC MIXED) of the SAS software (Version 9.4; SAS Institute, Inc., Cary, NC). Linear and quadratic effects of treatment were investigated. Before analysis, all data were tested for the normality using the univariate procedure. Data were presented as means ± SEM. Differences were considered significant at $P < 0.05$.

Results

Growth performance and diarrhea rate

There were no significant differences in the BW, ADFI, and diarrhea rates of piglets among the 4 VA diets throughout the trial (Table 3). However, dietary VA supplementation significantly increased both ADG (linear, $P = 0.020$) and G:F (linear, $P = 0.005$) of piglets from days 8 to 14.

Table 3. Growth performance of weaned piglets with different VA concentrations[1]

	Dietary VA, mg/kg						Contrast, P <	
Items	2	4	8	16	SEM	Model P value	Linear	Quadratic
BW, kg								
0 d	8.33	8.34	8.34	8.34	0.127	0.976	0.979	0.991
7 d	9.18	9.25	9.33	9.08	0.171	0.793	0.901	0.653
14 d	10.38	10.50	10.80	10.66	0.182	0.535	0.505	0.738
ADFI, g/d								
0 to 7 d	237.30	255.45	252.60	239.06	15.277	0.666	0.986	0.624
8 to 14 d	388.67	381.89	407.58	400.78	9.689	0.476	0.495	0.999
0 to 14 d	312.98	318.67	330.09	319.92	10.995	0.646	0.757	0.733
ADG, g/d								
0 to 7 d	120.54	129.46	141.97	105.36	14.784	0.666	0.812	0.464
8 to 14 d	172.32	178.57	209.82	225.89	9.201	0.039	0.020	0.781
0 to 14 d	146.43	154.02	175.89	165.62	9.525	0.404	0.372	0.652
G:F, g/d:g/d								
0 to 7 d	0.49	0.50	0.45	0.51	0.038	0.779	0.981	0.805
8 to 14 d	0.44	0.47	0.51	0.56	0.017	0.025	0.005	0.825
0 to 14 d	0.46	0.48	0.51	0.51	0.016	0.229	0.203	0.696
Diarrhea rate, %								
0 to 14 d	20.54	15.18	10.71	19.64	2.730	0.208	0.776	0.209

[1]Values are expressed as mean ± SEM, $n = 8$.

Organ index analysis and *Lgr5*+ mRNA abundance

There was no significant difference in the total weight of the liver, spleen, kidneys, and small intestinal length and the relative weight based on the day 14 BW of the weaned piglets (Table 4). The mRNA abundance of *Lgr5*+ gene was significantly increased in the 16 mg/kg VA group compared with that in the control group (P = 0.012, Fig. 1).

Intestinal morphology histology

This study found that the jejunum VH (P = 0.027) and VSA (P = 0.035) were significantly increased in the 4 mg/kg VA treatment group (Table 5). The CD in ileum was significantly increased in the 4 and 8 mg/kg VA treatment groups (quadratic, P = 0.043), and the ileum VH:CD ratio was significantly increased in the 16 mg/kg VA group (quadratic, P = 0.015). Other indicators showed no significant difference among the 4 groups.

Small intestinal brush border enzyme activity and nutrient transporter mRNA abundance

Changes in dietary VA content did not significantly affect the jejunal mucosal digestive enzyme activity in weaned piglets (Table 6). However, the maltase (P = 0.032), sucrose (P = 0.041), and alkaline phosphatase activity (linear, P = 0.024) in the ileum mucosa were significantly reduced when supplemented with 8 and 16 mg/kg VA. The mRNA abundance of the jejunal and ileal nutrient transporters was presented in Table 7. *Slc2a2* mRNA abundance was significantly increased in the 2 mg/kg VA group (linear, P = 0.024). However, there was no significant difference in the abundance of other nutrient transporters.

Growth of piglet intestinal organoids and mRNA abundance of markers

Figures 2 and 3 show that the growth of intestinal organoids of piglets treated with 0 (control), 5, 10, and 15 μM retinol and

Table 4. Organ index of weaned piglets with different VA concentrations[1]

	Dietary VA, mg/kg						Contrast, P <	
Items[2]	2	4	8	16	SEM	Model P value	Linear	Quadratic
Liver								
Total weight, g	211.00	216.44	224.00	225.81	3.917	0.609	0.154	0.821
Relative weight, g/kg	20.33	20.63	20.78	21.20	0.191	0.216	0.121	0.887
Spleen								
Total weight, g	23.88	26.81	25.81	23.94	0.792	0.140	0.910	0.143
Relative weight, g/kg	2.31	2.55	2.38	2.25	0.065	0.144	0.530	0.154
Both kidneys								
Total weight, g	55.94	58.31	59.00	56.86	0.958	0.165	0.702	0.261
Relative weight, g/kg	5.42	5.56	5.53	5.55	0.097	0.237	0.706	0.763
Small intestine								
Total length, m	9.24	9.23	9.61	9.68	0.112	0.177	0.099	0.846
Relative length, m/kg	0.90	0.88	0.90	0.91	0.015	0.539	0.773	0.645

[1]Values are expressed as mean ± SEM, n = 8.
[2]Relative weight = the ratio of total weight to BW of 14 d old; relative length = the ratio of total length to BW of 14 d old.

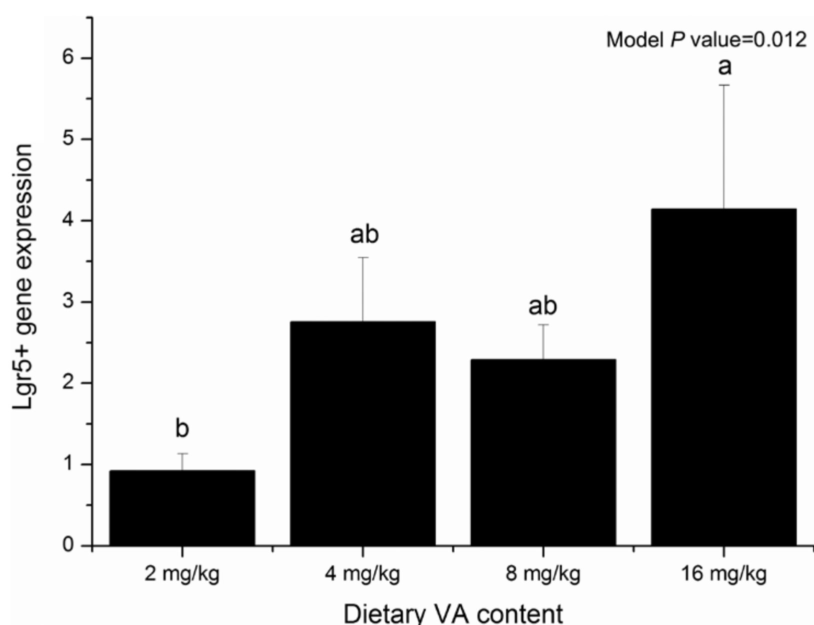

Figure 1. The expression of *Lgr5*+ gene within weaning piglets' jejunum mucosa at different VA concentrations. Different lowercase letters indicate statistical significance (P < 0.05). Values are expressed as mean ± SEM, n = 8.

Table 5. Intestinal morphology histology of weaned piglets with different VA concentrations[1]

	Dietary VA, mg/kg						Contrast, $P <$	
Items[2]	2	4	8	16	SEM	Model P value	Linear	Quadratic
VH, µm								
Jejunum	343.83	357.08	311.39	327.14	7.262	0.027	0.133	0.062
Ileum	321.08	300.98	319.27	316.19	6.156	0.312	0.949	0.505
CD, µm								
Jejunum	257.05	262.00	245.63	240.20	5.664	0.335	0.202	0.654
Ileum	222.36	241.61	240.19	219.78	4.786	0.185	0.828	0.043
VW, µm								
Jejunum	136.74	140.72	138.49	138.58	1.928	0.522	0.855	0.633
Ileum	130.54	132.74	134.83	130.41	1.620	0.299	0.911	0.330
VH:CD, µm:µm								
Jejunum	1.36	1.38	1.28	1.38	0.036	0.366	0.870	0.586
Ileum	1.45	1.26	1.34	1.44	0.031	0.025	0.828	0.015
VSA, µm								
Jejunum	147484.54	157421.48	135795.17	142154.11	3568.284	0.035	0.232	0.063
Ileum	131501.77	125264.26	135438.62	129994.09	3085.549	0.469	0.844	0.951

[1]Values are expressed as mean ± SEM, n = 8.

Table 6. Enzyme activity of weaned piglets with different VA concentrations in intestinal mucosa[1]

	Dietary VA, mg/kg						Contrast, $P <$	
Items	2	4	8	16	SEM	Model P value	Linear	Quadratic
Jejunal mucosa								
Maltase	196.36	244.60	164.73	212.61	20.640	0.604	0.870	0.997
Sucrase	59.69	98.36	68.37	49.74	7.631	0.124	0.365	0.058
Lactase	18.76	31.95	31.40	29.74	3.762	0.243	0.361	0.343
Alkaline phosphatase	467.34	417.14	389.76	424.68	41.149	0.523	0.686	0.625
Ileal mucosa								
Maltase	136.08	231.58	104.94	262.62	22.734	0.032	0.179	0.455
Sucrase	35.84	55.47	16.73	34.55	4.843	0.041	0.282	0.919
Alkaline phosphatase	284.43	293.01	174.86	123.21	29.394	0.028	0.024	0.593

[1]Values are expressed as mean ± SEM, n = 8.

Table 7. The mRNA abundance of nutrient transporters in intestinal mucosa of weaning piglets with different VA concentrations[1]

	Dietary VA, mg/kg						Contrast, $P <$	
Items[2]	2	4	8	16	SEM	Model P value	Linear	Quadratic
Jejunal mucosa								
Slc15a1	1.17	1.03	1.28	1.17	0.068	0.468	0.694	0.919
Slc7a1	0.90	0.95	0.94	1.03	0.056	0.450	0.470	0.987
Slc6a19	1.10	1.02	1.12	0.91	0.065	0.382	0.406	0.630
Slc1a1	1.38	0.99	1.15	1.37	0.122	0.297	0.896	0.234
Slc2a2	1.05	1.04	1.23	1.08	0.076	0.452	0.680	0.662
Slc27a4	1.00	1.01	1.00	1.02	0.027	0.841	0.850	0.830
Ileal mucosa								
Slc15a1	1.04	0.70	0.84	0.81	0.068	0.363	0.390	0.260
Slc7a1	0.88	0.75	0.84	0.74	0.081	0.528	0.658	0.944
Slc6a19	1.04	0.77	0.77	0.86	0.061	0.352	0.316	0.144
Slc1a1	1.24	0.97	0.94	1.05	0.066	0.137	0.340	0.163
Slc2a2	1.06	0.65	0.71	0.69	0.063	0.024	0.053	0.118
Slc27a4	1.18	1.08	0.85	1.07	0.072	0.153	0.406	0.285

[1]Values are expressed as mean ± SEM, n = 8.
[2]*Slc15a1*, solute carrier family 15 member 1, *Slc7a1*, solute carrier family 7 member 1, *Slc6a19*, solute carrier family 6 member 19, *Slc1a1*, solute carrier family 1 member 1, *Slc2a2*, solute carrier family 2 member 2, *Slc27a4*, solute carrier family 27 member 4.

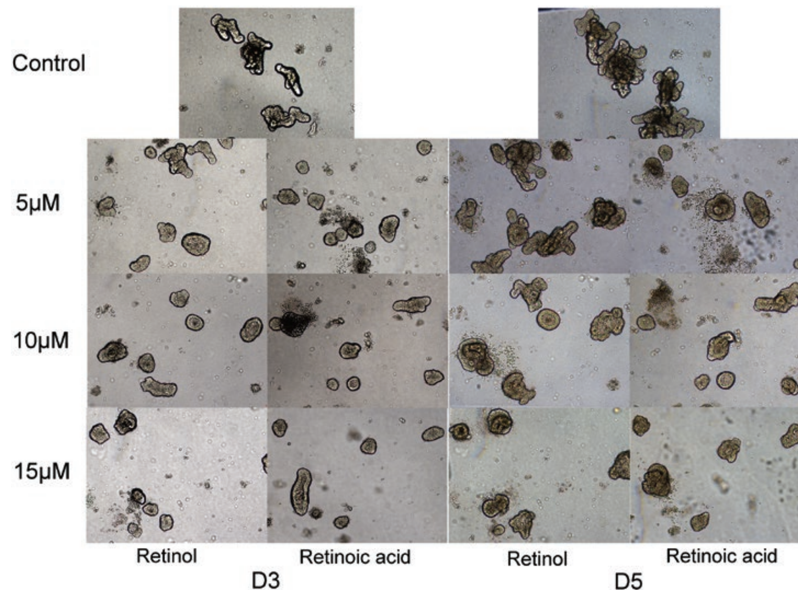

Figure 2. The organoid images observed under a microscope in a 20× combined magnification and image processing and analysis system.

retinoic acids. Compared with the control group, budding rates and the number of buddings per organoid in retinol acid- and retinoic acid-treated organoids were significantly reduced ($P < 0.05$). Moreover, the effects of retinol and retinoic acid on budding rates and the number of buddings per organoid were similar on days 3 and 5. Figures 4 and 5 are the mRNA abundance of the cell differentiation markers and embryonic stem cell markers in the organoids collected on day 5, respectively. This study showed that the expression of *Chromogranin A* and *Muc2* (cell differentiation markers) in retinol- and retinoic acid-treated organoids was suppressed ($P < 0.05$). Meanwhile, the mRNA abundance of *Spp1* and *Trop2* (embryonic stem cell markers) increased significantly ($P < 0.05$).

Discussion

The trial focused on the effects of dietary VA supplementation on the growth and intestinal function in weaned piglets. VA is an important nutrient for regulating piglet growth. Previous studies have shown that retinol can protect intestinal epithelium from the intestinal toxicity of *C. difficile* TxA (Maciel et al., 2007). It can be seen that VA dietary supplementation affects the intestinal epithelial structure, and thus the intestinal function, of piglets. The post-weaning diet is a major factor in regulating the growth and maturation of the small intestine in weaned piglets (Cera et al., 1988). Weaning stress causes a sharp decrease in feed intake and growth rate in piglets. It has been reported that pigs fed on VA-restricted diets had lower ADG and feed efficiency during the growing period (Ayuso et al., 2015). VA deficiency has also been associated with low growth in children (West et al., 1997). The NRC had similar reports on VA deficiency in piglets (NRC, 2012), but other authors have not found any evidence that VA deficiency has any effect on pig growth performance (Ching et al., 2002; D'Souza et al., 2003; Olivares et al., 2009a, 2009b, 2011). In our current work, the supplement of 16 mg/kg VA in the diet significantly increased the piglets' ADG and feed remuneration in the second week after weaning. This was a little different from the outcomes in previous studies. It must be taken into account that the experimental subjects in this study were 21-d-old weaned pigs with an initial BW of about 8.3 kg. The piglets at this stage were more susceptible to weaning stress than the experimental animals used by previous researchers. Therefore, our work verifies that VA supplementation helped relieve weaning stress at this stage, which led to a significant improvement in piglet growth performance.

Disaccharidases are located on the brush border of the small intestine mucosa. Disaccharidase activity determines carbohydrate digestion and transport capacity in weaned piglets. It is an important marker for assessing intestinal development in animals (Huygelen et al., 2015; Pieper et al., 2016). In this study, the digestive enzymes activity of ileum mucosa in the 4 mg/kg VA treatment group increased significantly. This shows that the VA supplement alleviates intestinal damage. Intestinal function is closely related to intestinal morphological structure (Pluske et al., 1996). Weaning usually causes damage to the intestinal structure of piglets, which is characterized by a decrease in VH and increase in CD (Hampson, 1986). Weaned piglets absorb nutrients primarily through the villus on the inside of the intestines. The increased VSA allows the weaned piglets to absorb more nutrients into the body. As the VH decreases, the area of contact between the nutrients and intestinal villus decreases, which limits the absorption of nutrients. VH and VSA of jejunum in the 4 mg/kg VA treatment group were significantly increased. Four mg/kg VA may be the optimal dose for weaning piglets among 4 treatment groups. The VH:CD ratio in the 2 mg/kg VA group significantly increased, caused by the gradual migration of stem cells from the base of the crypt to the top of the villi. This also has a positive effect on intestinal repair.

The intestinal epithelium can achieve a process of self-renewal in adult mammals (Schepers et al., 2011). *Lgr5*⁺ (leucine-rich-repeat-containing G-protein-coupled receptor 5) is a stem cell located above the Paneth cells in the small intestine expressed only at the base of the crypt. It has been reported to be a marker of intestinal stem cells (Barker et al., 2007; Yan et al., 2012). The jejunum is the main site of digestion and absorption of nutrients. The intestinal stem cells can be detected via a RT-qPCR detection of *Lgr5*⁺ gene expression in the jejunum. In this study, we found that the *Lgr5*⁺ gene in the 16 mg/kg VA treatment group

Figure 3. The growth of intestinal organoids in piglets treated with different retinol and retinoic acid concentrations. (A) The organoid budding rates on the third and fifth days treated with 0 (control), 5, 10, and 15 μM retinol. (B) The organoid budding rates on the third and fifth days treated with 0 (control), 5, 10, and 15 μM retinoic acid. (C) The average budding numbers of per organoid on the third and fifth days treated with 0 (control), 5, 10, and 15 μM retinol. (D) The average budding numbers of per organoid on the third and fifth days treated with 0 (control), 5, 10, and 15 μM retinoic acid. Different lowercase letters indicate statistical significance ($P < 0.05$). Values are expressed as mean ± SEM, $n = 8$.

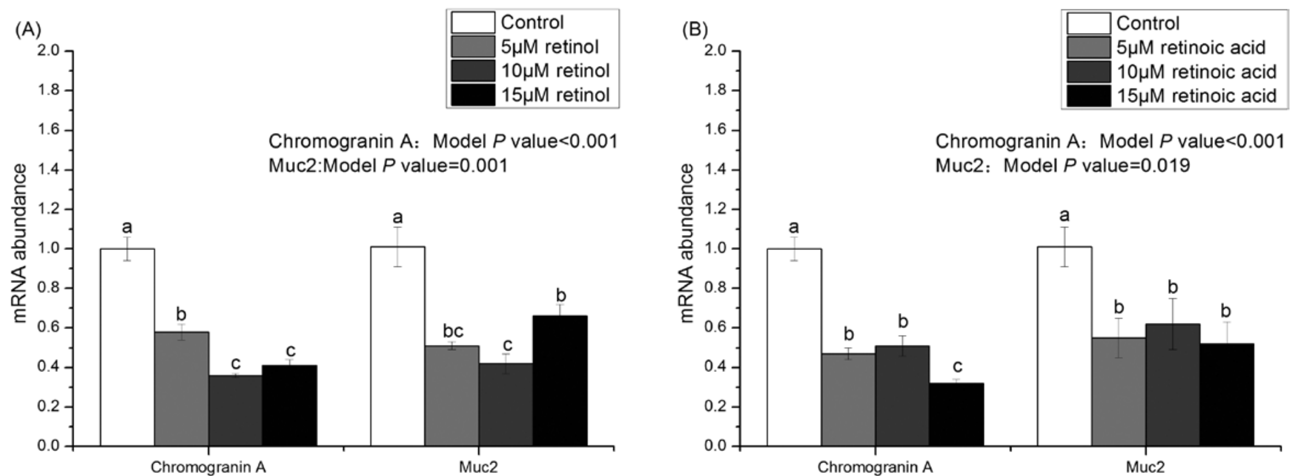

Figure 4. The mRNA abundance of *Chromogranin A* and *Muc2* in organoids treated with different concentrations of retinol and retinoic acid. Different lowercase letters indicate statistical significance ($P < 0.05$). Values are expressed as mean ± SEM, $n = 8$.

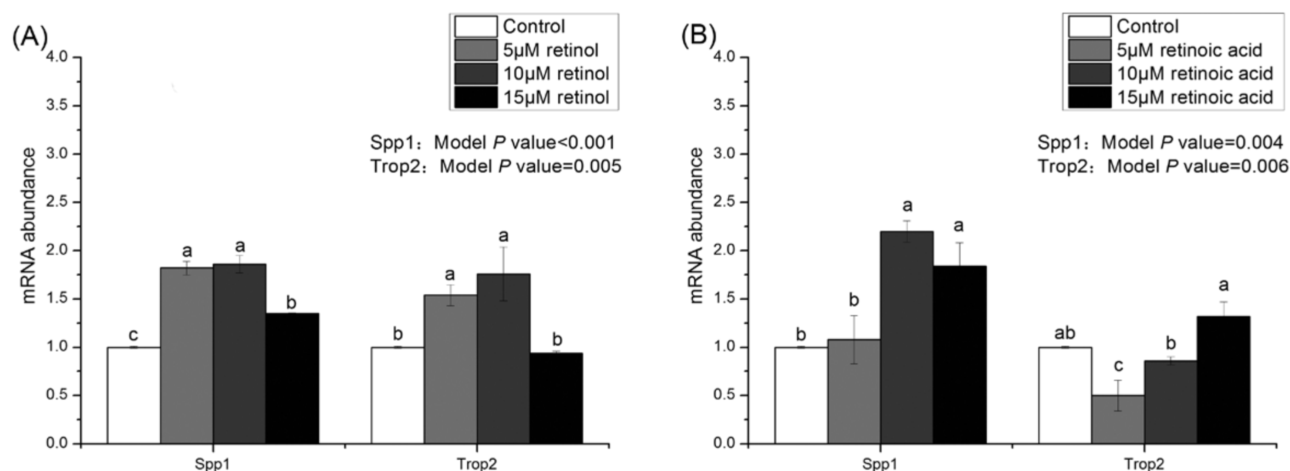

Figure 5. The mRNA abundance of *Spp1* and *Trop2* in organoids treated with different concentrations of retinol and retinoic acid. Different lowercase letters indicate statistical significance ($P < 0.05$). Values are expressed as mean ± SEM, $n = 8$.

was highly expressed in the jejunum mucosa compared with the control group. The small intestinal alkaline phosphatase is a tissue-specific phosphatase (**TSAP**). Its expression is associated with cell differentiation and is also commonly considered a marker of cell differentiation (Barnard and Warwick, 1993; Hodin et al., 1996; Kovaňková et al., 2000; Hýžd'alová et al., 2008). It maintains the intestinal barrier's integrity by limiting bacterial transepithelial channels and dephosphorylation of bacterial lipopolysaccharides. Additionally, alkaline phosphatase can also increase the intestinal digestion and absorption functions by catalyzing the hydrolysis of various phosphorylated compounds and lysing phosphate esters that do not readily penetrate cell membranes (Grant et al., 2015). In this study, the alkaline phosphatase activity in the ileal mucosa of the 8 and 16 mg/kg VA treatment groups was found to be significantly reduced. A high concentration of dietary VA has been found to inhibit intestinal epithelial cell differentiation.

Previous studies have shown that VA absence resulted in an uncontrolled proliferation of epithelial stem cells that fail to differentiate into the normal phenotype in many lining epithelia during development (Rexer et al., 2001). Retinol is a form of VA. Retinoic acid is the major active metabolite of VA. The anabolic process of retinoic acid is sequentially carried out in the small intestine, liver and target cells. The small intestine is the first gateway for contact with dietary VA as well as the unique organ for its absorption and metabolism. Dietary VA enters the small intestine and is metabolized to produce retinoic acid. The un-metabolized retinol (which may contain retinol acetate) forms a mixture with the metabolically produced retinoic acid, which is absorbed by the intestinal epithelial cells and absorbed by the piglets. The self-renewing epithelium of the small intestine includes crypts and villi. Cells are newly created in the crypts and are lost due to apoptosis at the tip of the villi. Most crypts can be cultured in a 3D model of the intestinal organoids, comprising several crypt domains surrounding a central lumen lined by a villus-like epithelium ("villus domain"). This is a good in vitro model for studying intestinal stem cells (Sato et al., 2009; Sato et al., 2011). Our study found that both retinol and retinoic acid inhibited the budding of organoids compared to the control group. The expression of *chromogranin A* and *Muc2* (intestinal cell differentiation marker) in organoids treated with retinol and retinoic acid was suppressed. The expression of differentiation markers was significantly reduced. There were a large number of spherical organoids in the medium. These spherical organoids are similar to embryonic small intestine organoids that lack differentiated cells. Embryonic stem cells are highly undifferentiated cells (Mustata et al., 2013). Studies have shown that the expression of *Spp1* and *Trop2* (embryonic stem cell markers) in organoids treated with retinol and retinoic acid increased significantly. The number of differentiated cells decreased and the number of undifferentiated embryonic stem cells increased. The differentiation of organoids treated by VA and its metabolites was inhibited. In vivo, the results of alkaline phosphatase and *Lgr5*$^+$ gene abundance in the 16 mg/kg VA group showed inhibition of cell differentiation and increased crypt stem cells. The reason may be that high-dose supplementation of VA may limit the differentiation of intestinal stem cells, leading to an increase in stem cell stemness. Thus, our finding that VA is an important regulator of stemness and differentiation in the intestinal crypt provides important insight for the cellular mechanism that maintains homeostasis.

Proteins, sugars, and fats are broken down into free AA, monosaccharides, and fatty acids after entering the small intestine, respectively. They are absorbed into the cells by animals via the corresponding transporter or other forms. *Slc2a2* is a transport carrier associated with glucose. Our results only observed significant differences in the *Slc2a2* gene expression, whereas other indicators did not differ significantly. When VA was added to the piglet diet, ADG was higher in weaned piglets as the concentration of VA increased. However, from the perspective of the intestinal lumen development, the addition of high concentration of VA seems to reduce the expression of GLUT2 and reduce the activity of TSAP. The sample we collect was the terminal ileum, which was connected to the large intestine. Studies have shown that high concentrations of VA have longer small intestinal lengths and may have the potential to better digest and absorb nutrients. Fewer nutrients may be available to reach the terminal ileum, and related enzyme activities and expression of nutrient transporters are reduced. In the future, I will verify this conjecture based on more experiments.

In conclusion, this study suggests that VA supplementation affects the growth in weaned piglets. Moreover, VA can affect intestinal function by regulating the intestinal stem cells within the jejunum, which provides guidance for feed formula of weaned piglets.

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Conflict of interest

The authors declare that they have no competing interests.

LITERATURE CITED

Ayuso, M., C. Ovilo, A. Fernández, Y. Nuñez, B. Isabel, A. Daza, C. J. López-Bote, and A. I. Rey. 2015. Effects of dietary vitamin A supplementation or restriction and its timing on retinol and α-tocopherol accumulation and gene expression in heavy pigs. *Anim. Feed Sci. Tech.* **202**:62–74. doi:10.1016/j.anifeedsci.2015.01.014

Balmer, J. E., and R. Blomhoff. 2002. Gene expression regulation by retinoic acid. *J. Lipid Res.* **43**:1773–1808. doi:10.1194/jlr.r100015-jlr200

Barea, R., R. Nieto, F. Vitari, C. Domeneghini, and J. F. Aguilera. 2011. Effects of pig genotype (Iberian v. Landrace × Large White) on nutrient digestibility, relative organ weight and small intestine structure at two stages of growth. *Animal* **5**:547–557. doi:10.1017/S1751731110002181

Barker, N., J. H. van Es, J. Kuipers, P. Kujala, M. van den Born, M. Cozijnsen, A. Haegebarth, J. Korving, H. Begthel, P. J. Peters, *et al.* 2007. Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature* **449**:1003–1007. doi:10.1038/nature06196

Barnard, J. A., and G. Warwick. 1993. Butyrate rapidly induces growth inhibition and differentiation in HT-29 cells. *Cell Growth Differ.* **4**:495–501.

Bhatia, A. L. 1998. The anti-aging role of vitamin A and β-carotene. *Indian J. Gerontol.* **12**:70–79.

Blomhoff, R. 1994. Vitamin A in health and disease. Boca Raton (FL): CRC Press, Taylor & Francis Group.

Blomhoff, R., and H. K. Blomhoff. 2006. Overview of retinoid metabolism and function. *J. Neurobiol.* **66**:606–630. doi:10.1002/neu.20242

Cera, K. R., D. C. Mahan, R. F. Cross, G. A. Reinhart, and R. E. Whitmoyer. 1988. Effect of age, weaning and postweaning diet on small intestinal growth and jejunal morphology in young swine. *J. Anim. Sci.* **66**:574–584. doi:10.2527/jas1988.662574x

Chen, C. C., Z. B. Wang, J. Z. Li, Y. L. Li, P. F. Huang, X. Q. Ding, J. Yin, S. P. He, H. S. Yang, and Y. L. Yin. 2019. Dietary vitamin E affect small intestinal histomorphology, digestive enzyme activity and the expression of nutrient transporters by inhibiting proliferation of intestinal epithelial cells within jejunum in weaned piglets. *J. Anim. Sci.* **97**:1212–1221. doi:10.1093/jas/skz023

Ching, S., D. C. Mahan, T. G. Wiseman, and N. D. Fastinger. 2002. Evaluating the antioxidant status of weanling pigs fed dietary vitamins A and E. *J. Anim. Sci.* **80**:2396–2401. doi:10.2527/2002.8092396x

D'Souza, D. N., D. W. Pethick, F. R. Dunshea, J. R. Pluske, and B. P. Mullan. 2003. Nutritional manipulation increases intramuscular fat levels in the Longissimus muscle of female finisher pigs. *Austral. J. Agric. Res.* **54**:745–749. doi:10.1071/AR03009

Grant, C. N., S. G. Mojica, F. G. Sala, J. R. Hill, D. E. Levin, A. L. Speer, E. R. Barthel, H. Shimada, N. C. Zachos, and T. C. Grikscheit. 2015. Human and mouse tissue-engineered small intestine both demonstrate digestive and absorptive function. *Am. J. Physiol. Gastrointest. Liver Physiol.* **308**:G664–G677. doi:10.1152/ajpgi.00111.2014

Hampson, D. J. 1986. Alterations in piglet small intestinal structure at weaning. *Res. Vet. Sci.* **40**:32–40. doi:10.1016/S0034-5288(18)30482-X

Hodin, R. A., S. Meng, S. Archer, and R. Tang. 1996. Cellular growth state differentially regulates enterocyte gene expression in butyrate-treated HT-29 cells. *Cell Growth Differ.* **7**:647–653.

Huygelen, V., M. De Vos, S. Prims, H. Vergauwen, E. Fransen, C. Casteleyn, S. Van Cruchten, and C. Van Ginneken. 2015. Birth weight has no influence on the morphology, digestive capacity and motility of the small intestine in suckling pigs. *Livest Sci.* **182**:129–136. doi:10.1016/j.livsci.2015.11.003

Hýžd'alová, M., J. Hofmanová, J. Pacherník, A. Vaculová, and A. Kozubík. 2008. The interaction of butyrate with TNF-α during differentiation and apoptosis of colon epithelial cells: Role of NF-κB activation. *Cytokine* **44**:33–43. doi:10.1016/j.cyto.2008.06.003

Kisielinski, K., S. Willis, A. Prescher, B. Klosterhalfen, and V. Schumpelick. 2002. A simple new method to calculate small intestine absorptive surface in the rat. *Clin. Exp. Med.* **2**:131–135. doi:10.1007/s102380200018

Kovařiková, M., J. Pacherník, J. Hofmanová, Z. Zadák, and A. Kozubík. 2000. TNF-α modulates the differentiation induced by butyrate in the HT-29 human colon adenocarcinoma cell line. *Eur. J. Cancer* **36**:1844–1852. doi:10.1016/S0959-8049(00)00178-7

Lima, A. A., A. M. Soares, N. L. Lima, R. M. Mota, B. L. Maciel, M. P. Kvalsund, L. J. Barrett, R. P. Fitzgerald, W. S. Blaner, and R. L. Guerrant. 2010. Effects of vitamin A supplementation on intestinal barrier function, growth, total parasitic, and specific *Giardia* spp infections in Brazilian children: A prospective randomized, double-blind, placebo-controlled trial. *J. Pediatr. Gastroenterol. Nutr.* **50**:309–315. doi:10.1097/MPG.0b013e3181a96489

Maciel, A. A., R. B. Oriá, M. B. Braga-Neto, A. B. Braga, E. B. Carvalho, H. B. Lucena, G. A. Brito, R. L. Guerrant, and A. A. Lima. 2007. Role of retinol in protecting epithelial cell damage induced by *Clostridium difficile* toxin A. *Toxicon* **50**:1027–1040. doi:10.1016/j.toxicon.2007.07.010

Matsumoto, T., W. Mochizuki, Y. Nibe, S. Akiyama, Y. Matsumoto, K. Nozaki, M. Fukuda, A. Hayashi, T. Mizutani, S. Oshima, *et al.* 2016. Retinol promotes in vitro growth of proximal colon organoids through a retinoic acid-independent mechanism. *PLoS One* **11**:e0162049. doi:10.1371/journal.pone.0162049

Montagne, L., G. Boudry, C. Favier, I. Le Huërou-Luron, J. P. Lallès, and B. Sève. 2007. Main intestinal markers associated with the changes in gut architecture and function in piglets after weaning. *Br. J. Nutr.* **97**:45–57. doi:10.1017/S000711450720580X

Mustata, R. C., G. Vasile, V. Fernandez-Vallone, S. Strollo, A. Lefort, F. Libert, D. Monteyne, D. Pérez-Morga, G. Vassart, and M. I. Garcia. 2013. Identification of Lgr5-independent spheroid-generating progenitors of the mouse fetal intestinal epithelium. *Cell Rep.* **5**:421–432. doi:10.1016/j.celrep.2013.09.005

NRC. 2012. *Nutrient requirements of swine*. 11th rev. ed. Natl. Acad. Press, Washington, DC.

Olivares, A., A. Daza, A. I. Rey, and C. J. López-Bote. 2009a. Dietary vitamin A concentration alters fatty acid composition in pigs. *Meat Sci.* **81**:295–299. doi:10.1016/j.meatsci.2008.07.029

Olivares, A., A. I. Rey, A. Daza, and C. J. Lopez-Bote. 2009b. High dietary vitamin A interferes with tissue α-tocopherol concentrations in fattening pigs: A study that examines administration and withdrawal times. *Animal* **3**:1264–1270. doi:10.1017/S175173110900487X

Olivares, A., A. I. Rey, A. Daza, and C. J. López-Bote. 2011. Low levels of dietary vitamin A increase intramuscular fat content and polyunsaturated fatty acid proportion in liver from lean pigs. *Livest Sci.* **137**:31–36. doi:10.1016/j.livsci.2010.09.023

Pieper, R., L. Scharek-Tedin, A. Zetzsche, I. Rohe, S. Kroger, W. Vahjen, and J. Zentek. 2016. Bovine milk-based formula leads to early maturation-like morphological, immunological,

and functional changes in the jejunum of neonatal piglets. *J. Anim. Sci.* **94**:989–999. doi:10.2527/jas.2015–9942

Pluske, J. R., D. J. Hampson, and I. H. Williams. 1997. Factors influencing the structure and function of the small intestine in the weaned pig: A review. *Livest. Prod. Sci.* **51**:215–236. doi:10.1016/S0301-6226(97)00057-2

Pluske, J., I. Williams, and F. Aherne. 1996. Maintenance of villous height and crypt depth in piglets by providing continuous nutrition after weaning. *Anim. Sci.* **62**:131–144. doi:10.1017/S1357729800014417

Rexer, B. N., W. L. Zheng, and D. E. Ong. 2001. Retinoic acid biosynthesis by normal human breast epithelium is via aldehyde dehydrogenase 6, absent in MCF-7 cells. *Cancer Res.* **61**:7065–7070.

Sarkar, A., A. Bishayee, and M. Chatterjee. 1995. Beta-carotene prevents lipid peroxidation and red blood cell membrane protein damage in experimental hepatocarcinogenesis. *Cancer Biochem. Biophys.* **15**:111–125.

Sato, T., J. H. van Es, H. J. Snippert, D. E. Stange, R. G. Vries, M. van den Born, N. Barker, N. F. Shroyer, M. van de Wetering, and H. Clevers. 2011. Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts. *Nature* **469**:415–418. doi:10.1038/nature09637

Sato, T., R. G. Vries, H. J. Snippert, M. van de Wetering, N. Barker, D. E. Stange, J. H. van Es, A. Abo, P. Kujala, P. J. Peters, *et al.* 2009. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature* **459**:262–265. doi:10.1038/nature07935

Schepers, A. G., R. Vries, M. van den Born, M. van de Wetering, and H. Clevers. 2011. Lgr5 intestinal stem cells have high telomerase activity and randomly segregate their chromosomes. *Embo J.* **30**:1104–1109. doi:10.1038/emboj.2011.26

van Beers-Schreurs, H., and E. Bruininx. 2002. *Nutritional management to prevent disorders in post-weaning pig health. Nutrition health of the gastrointestinal tract*. Wageningen Acad. Publ., Wageningen, The Netherlands. p. 135–158.

West, K. P., S. C. LeClerq, S. R. Shrestha, L. S. Wu, E. K. Pradhan, S. K. Khatry, J. Katz, R. Adhikari, and A. Sommer. 1997. Effects of vitamin A on growth of vitamin A-deficient children: Field studies in Nepal. *J. Nutr.* **127**:1957–1965. doi:10.1093/jn/127.10.1957

Wolf, G., T. C. Kiorpes, S. Masushige, J. B. Schreiber, M. J. Smith, and R. S. Anderson. 1979. Recent evidence for the participation of vitamin A in glycoprotein synthesis. *Fed. Proc.* **38**:2540–2543.

Yan, K. S., L. A. Chia, X. Li, A. Ootani, J. Su, J. Y. Lee, N. Su, Y. Luo, S. C. Heilshorn, M. R. Amieva, *et al.* 2012. The intestinal stem cell markers Bmi1 and Lgr5 identify two functionally distinct populations. *Proc. Natl. Acad. Sci. USA* **109**:466–471. doi:10.1073/pnas.1118857109

Yan, S. L., L. N. Long, E. Y. Zong, P. F. Huang, J. Z. Li, Y. L. Li, X. Q. Ding, X. Xiong, Y. L. Yin, and H. S. Yang. 2018. Dietary sulfur amino acids affect jejunal cell proliferation and functions by affecting antioxidant capacity, Wnt/beta-catenin, and the mechanistic target of rapamycin signaling pathways in weaning piglets. *J. Anim. Sci.* **96**:5124–5133. doi:10.1093/jas/sky349

Yang, H. S., X. Xiong, X. C. Wang, T. J. Li, and Y. L. Yin. 2016a. Effects of weaning on intestinal crypt epithelial cells in piglets. *Sci. Rep. UK* **6**:36939. doi:10.1038/srep36939

Yang, H., X. Xiong, X. Wang, and Y. Yin. 2016b. Mammalian target of rapamycin signaling pathway changes with intestinal epithelial cells renewal along crypt-villus axis. *Cell. Physiol. Biochem.* **39**:751–759. doi:10.1159/000445665

Yang, H. S., X. Xiong, and Y. L. Yin. 2016c. Metabolomic analysis of intestinal epithelial cell maturation along the crypt–villus axis. *RSC Adv.* **6**:27566–27574. doi:10.1039/C5RA27722A

Yin, Y. L., S. K. Baidoo, H. Schulze, and P. H. Simmins. 2001. Effects of supplementing diets containing hulless barley varieties having different levels of non-starch polysaccharides with β-glucanase and xylanase on the physiological status of the gastrointestinal tract and nutrient digestibility of weaned pigs. *Livest. Prod. Sci.* **71**:97–107. doi:10.1016/S0301-6226(01)00214-7