Original Research Article

Effects of varying dietary folic acid during weaning stress of piglets

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A B S T R A C T

The present study was conducted to evaluate the effect of dietary folic acid on the growth performance, intestinal morphology, and intestinal epithelial cells renewal in post-weaning piglets. Twenty-eight piglets (weaned at day 21, initial body weight of 6.73 ± 0.62 kg) were randomly allotted to 4 treatments with 7 pens per diet and 1 piglet per pen. The piglets were fed the same antibiotic-free and zinc oxide-free basal diets supplemented with folic acid at 0, 3, 9, and 18 mg/kg for 14 days. The results showed that dietary supplementation with folic acid increased villus height (VH) (P = 0.003; linear, P = 0.001), VH-to-crypt depth (VH:CD) ratio (P = 0.002; linear, P = 0.001), villus surface area (VSA) (P = 0.026; linear, P = 0.010). The analyzed parameters ADG, serum urea nitrogen (BUN) content, VH, VSA, and serum folate (SF) concentration responded linearly to the dietary folic acid concentration when the dietary folic acid concentration was below 4.42, 5.26, 4.79, 3.47, and 3.53 mg/kg respectively (R² = 0.995, 0.999, 0.999, 0.999, 0.872, P = 0.09, 0.07, 0.09, 0.09, 0.36, respectively), as assessed by a two-linear broken-line regression. Above these breakpoints, the response of ADG, VH, VSA, and SF plateaued in response to changes in dietary folic acid concentration. Moreover, dietary supplementation with folic acid significantly increased the lactase (P = 0.001; linear, P = 0.001) and sucrase activities (P = 0.021; linear, P = 0.010) in the jejunal mucosa of weaned piglets. The mRNA expression of solute carrier family 6 member 19 (SLC6a19), solute carrier family 1 member 1 (SLC7a1), tumor necrosis factor-a (TNF-a), the number of Ki67 positive cells, and cell shedding rate had a significant linear contrast (P = 0.023, 0.021, 0.038, 0.049, and 0.008, respectively) in dietary folic acid groups. In conclusion, our results indicate that folic acid supplementation can improve the growth performance and intestinal morphology of weaned piglets by maintaining the balance of epithelial cell renewal.

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1. Introduction

Weaning of piglets is a stressful process associated with gastrointestinal function disorders, and often accompanied by severe growth retardation and diarrhea. It is well established that this process is multi-factorial, and that post-weaning anorexia and under-nutrition are major aetiological factors [Pluske et al., 1995]. In order to ensure the healthy growth of weaning piglets, the antibiotics and zinc oxide at pharmacological levels have been widely used as feed additives to improve growth performance of piglets in their transition to the nursery period.
However, the abuse of antibiotics increases the antibiotic resistance of bacteria, thereby threatening human health and raising environmental concerns. According to a series of studies, folic acid deficiency can lead to symptoms such as anemia, diarrhea and can reduce weight gain and litter size (Davidson and Townley, 1977; Ashraf et al., 2008). Although the supplementation of folic acid can alleviate chronic diarrhea, folic acid was thought to have no effect on severe cases in children and piglets (Carruthers, 1946; Ashraf et al., 1998).

Folic acid refers to the essential water-soluble vitamin B9. Mammals cannot synthesize folic acid, which is indispensable for one-carbon transfer reactions, including nucleic acid synthesis, amino acid metabolism, and methylation (Hayashi et al., 2007). It, therefore, plays a crucial role in protein deposition and tissue synthesis. Folic acid prevents the occurrence of cardiovascular disease, anemia, and fetal development (Matte et al., 1992). Rapidly growing tissues or cells with a high turnover rate are sensitive to folic acid levels; therefore, supplemental folic acid promotes the growth of young animals.

From 1998 to 2012, the National Research Council (NRC) recommended dietary supplementation of folic acid at 0.3 mg/kg of diet for post-weaning piglets (NRC, 1998; NRC, 2012), and the best estimate of the minimum amount required per day for a piglet from 7 to 11 kg BW is 0.14 mg. However, this means the ADFI of piglets is at least 467 g per day, which seems difficult for post-weaning piglets. Folic acid deficiency is caused due to the instability of folic acid and insufficient daily feed intake. Therefore, often, 6 to 10 times of the normal dosage is administered to ensure a safe margin. There are substantial studies on the effects of maternal supplementation of folic acid on reproductive performance and offspring (Rerat, 1978; Matte et al., 1984; Lindemann and Kornegay, 1989; Joshi et al., 2004; Liu et al., 2011a). Yu et al. (2010) showed that dietary supplementation with a high level of folic acid (2.5 mg/kg) improved the growth performance of weaned piglets that were fed a diet with 0.3% zinc oxide. The intestines of post-weaning piglets grow faster compared to their other body parts. Therefore, the proliferation rate of intestinal cells, which is more sensitive to folic acid concentration, is higher than that of other body parts. Moreover, weaning stress has negative effects on the intestinal morphology and functions, resulting in villus atrophy, crypt hyperplasia, barrier function impairment, and cell renewal and change in metabolism, which requires folic acid for intestinal epithelial repair (Yang et al., 2016a, 2016b; Montagne et al. 2017). However, in a post-weaning diet lacking antibiotics and zinc oxide, the effect of folic acid on the growth performance and underlying mechanism on the intestinal morphology and functions in weaned piglets are still unclear. In the present study, we hypothesized that the phenotype of weaning-stress associated pathologies in diets without antibiotics and pharmacological zinc oxide is to some extent folic acid deficiency, which can be alleviated by dietary supplementation with folic acid levels above the current NRC recommendation. For piglets without antibiotics and zinc oxide in feed, we added pharmacological levels (10, 30, 60 times) of folic acid to ensure the dietary supplementation with a relative high level of folic acid, which is particularly important for ensuring the normal growth and integrity of intestine in piglets.

Therefore, the present study was conducted to evaluate the effect of dietary folic acid levels on the growth performance, intestinal morphology and functions, and intestinal epithelial cell renewal in post-weaning piglets fed an antibiotic-free and zinc oxide-free diet.

## 2. Materials and methods

The experiment design and procedures were reviewed and approved (approval number 2017-099) by the Animal Care and Use Committee of the Hunan Normal University, Changsha city, Hunan, China.

### 2.1. Animals and treatments

Twenty-eight weaned piglets (Duroc × Landrace × Yorkshire; barrow; initial average body weight of 6.73 ± 0.62 kg) at 21 days of age were selected from 10 litters based on body weight (BW) and randomly assigned to 4 treatments, with 7 pens and 1 piglet per pen and the ambient temperature was maintained at 29 ± 1 °C in the same room with the same feeding management. This study followed a fully randomized block design, where the block was based on average body weight. Twenty-eight boar piglets were selected from 10 litters with weight from 6 to 8 kg. Piglets were weighed, labelled from 1 to 28, then Excel was used to sort the weights and allot the piglets into 4 groups: A, B, C, D. Following this, adjustments were made to the average weight and standard deviation of each group and to ensure that the positions of each group in the room were scattered. The piglets were fed the same antibiotic-free and zinc oxide-free basal diets supplemented with folic acid at 0, 3, 9, and 18 mg/kg, respectively. The feed formulation (Table 1) met the nutrient specifications for piglets from 7 to 11 kg BW, the analyzed and calculated values of diets were shown in Table 1. The folic acid used in this study is a spray-dried micro-granular commercial product (Beijing, Blooming Biotechnology Co., Ltd.) containing 80% folic acid. The product uses dextrin as the carrier, which has good fluidity and is easy to mix and dilute at different ratios with the vitamin premix. A vitamin premix was prepared using a step-by-step dilution process. Then, the vitamin premixes with different folic acid contents were used for producing compound feeds. The piglets were given a high-quality diet ad

### Table 1

*Ingredients of piglet diets (%) as fed basis.*

| Ingredients               | Content |
|---------------------------|---------|
| Corn                      | 39.7    |
| Extruded corn             | 20.0    |
| Soybean meal, 44% CP      | 9.0     |
| Fish meal, 63% CP         | 7.0     |
| Spray dried porcine plasma| 5.0     |
| Whey powder               | 9.0     |
| Glucose                   | 3.0     |
| Soybean oil               | 3.8     |
| Limestone                 | 1.05    |
| Choline chloride          | 0.1     |
| Antioxidant premix        | 0.05    |
| Citric acid               | 0.5     |
| NaCl                      | 0.1     |
| Mineral premix            | 0.15    |
| t-Lys HCl                 | 0.45    |
| t-Met                     | 0.20    |
| t-Thr                     | 0.14    |
| t-Trp                     | 0.02    |
| Vitamin premix            | 0.74    |
| Total                     | 100     |

1. Supplied 50 mg ethoxyquin per kilogram of feed.
2. Supplied the following per kilogram of feed: 150 mg Fe (FeSO₄), 100 mg Zn (ZnSO₄), 30 mg Mn (MnSO₄), 25 mg Cu (CuSO₄), 0.5 mg I (KI), 0.3 mg Co (CoSO₄), and 0.3 mg Se (Na₂SeO₃).
3. Supplied the following per kilogram of feed: 2,200 IU vitamin A, 220 IU vitamin D₃, 0.5 mg vitamin K₃, 0.0175 mg vitamin B₁₂, 3.5 mg riboflavin, 30 mg niacin, 10 mg d-pantothenic acid, 0.05 mg biotin, 0.3 mg thiamine, and 7 mg pyridoxine.
libitum and free access to water throughout the study. Initial body and final body weight were calculated to evaluate growth performance.

2.2. Sample treatment and collection

At the end of the 14-day treatment, a 10-mL blood sample was harvested from the anterior vena cava and serum samples separated after centrifugation at 3,000 × g for 10 min under 4 °C. Serum amino acids were determined by the high-speed Amino Acid Analyzer L-8900 (Hitachi Co., Tokyo, Japan). Blood biochemical analysis was conducted by commercial kits following the manufacturer’s instructions (Jiancheng Bioengineering Institute, Nanjing, China) and identified using the TBA-120FR Automatic Biochemistry Radiometer (Hitachi Co., Tokyo, Japan). The program was performed according to the previous study (Wang et al., 2019).

2.3. Fecal score

Fecal consistency was monitored, and the scores were recorded every day during this experiment and quantified using a scale ranging from 0 to 3 with 0 = hard feces, 1 = normally shaped feces, 2 = shapeless (loose) with liquid (soft) feces and 3 = severe diarrhea with watery feces (Liu et al., 2008).

2.4. Intestinal histomorphology analysis

The intestines were removed and approximately 2 cm of the jejunum was collected from the middle part of the small intestine, then placed in a 4% neutral buffer formalin solution. Specimens of the cross-sections of the intestinal segments identified above were embedded in low-melt paraffin wax and cut into 4-μm thick histological sections for hematoxylin and eosin (H&E) staining. The tissue sections were measured under a microscope using a 40× combined magnification and an image processing and analysis system (Leica Imaging Systems Ltd., Cambridge, UK). The program Image-pro Plus 6.0 (Media Cybernetics, Inc., Georgia, USA) was used to determine the villus height (VH), crypt depth (CD) and villus height-to-crypt depth (VH:CD) ratio. At least 25 villous samples with intact lamina propria were blindly selected and examined for measurement.

2.5. Assay for intestinal disaccharidase activities

The disaccharidase activity of mucosal scrapings was assessed using a modified glucose oxidase peroxidase enzyme system. Jejunal mucosal tissue samples were homogenized in saline and centrifuged (2,500 × g, 4 °C, 10 min) to produce the supernatant. The enzyme sacrose, lactase and maltase activities were analyzed using commercial kits (Jiancheng Bioengineering Institute, Nanjing, China) and Synergy HTX Multi-Mode Microplate Reader (BioTek, Vermont, USA) following the manufacturer’s instructions. The concentration of protein was also determined also using commercial bicinchoninic acid assay kits (Jiancheng Bioengineering Institute, Nanjing, China) and Synergy HTX Multi-Mode Microplate Reader (BioTek, Vermont, USA) according to the manufacturer’s instructions. Throughout this paper, disaccharidase activity has been expressed as units per milligram protein of the mucosal sample.

2.6. Quantitative real-time PCR (RT-PCR) analysis

RNA isolation and RT-PCR analysis of jejunal mucosal samples was performed according to the previous study (Wang et al., 2019). Briefly, total RNA was isolated from liquid nitrogen frozen jejunal mucosal samples using RNAiso Plus (Takara, Dalian, China). The RNA quality was then checked using 1% agarose gel electrophoresis, and the concentration and purity were measured using a Synergy HTX Multi-Mode Microplate Reader (BioTek, Vermont, USA). After removing trace DNA by incubation with DNase (Takara, Dalian, China), 1 μg of RNA was reverse transcribed to cDNA using PrimeScript RT Master Mix (Perfect Real Time; Takara, Dalian, China). 1 μg of cDNA was used for the RT-PCR analysis.

Table 2

Chemical composition of piglet diets (% as fed basis).

| Chemical composition | Supplement folic acid level, mg/kg |
|----------------------|----------------------------------|
|                      | 0                                | 3         | 9         | 18        |
| DM                   | 89.62 ± 0.05                     | 90.22 ± 0.20 | 89.33 ± 0.13 | 89.88 ± 0.15 |
| CP                   | 18.50 ± 1.24                     | 18.25 ± 1.28 | 18.36 ± 1.10 | 18.20 ± 0.91 |
| Calcium              | 0.80 ± 0.02                      | 0.78 ± 0.03 | 0.81 ± 0.02 | 0.82 ± 0.03 |
| Phosphorus           | 0.38 ± 0.02                      | 0.37 ± 0.02 | 0.36 ± 0.02 | 0.36 ± 0.02 |
| Folic acid, mg/kg    | 0.20 ± 0.02                      | 3.21 ± 0.03 | 9.23 ± 0.02 | 18.20 ± 0.03 |

1 The values are means ± SD. 

2 The concentrations of metabolizable energy, and amino acids were calculated according to the composition of feed ingredients used in swine in NRC 2012. Vitamin and trace element concentrations met the requirements according to NRC 2012.

1 The concentration of protein was also analyzed also using commercial bicinchoninic acid assay kits (Jiancheng Bioengineering Institute, Nanjing, China) and Synergy HTX Multi-Mode Microplate Reader (BioTek, Vermont, USA) according to the manufacturer’s instructions. Throughout this paper, disaccharidase activity has been expressed as units per milligram protein of the mucosal sample.
sample to normalize the expression of the target gene (Table 3). Due to the wide distribution of β-actin in various tissues, the amino acid sequence is highly conservative, the expression level little among different tissues, and the expression level is high. Relative expression of target genes was calculated according to the formula $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (Ct_{Target} - Ct_{\beta-actin})_{treatment} - (Ct_{Target} - Ct_{\beta-actin})_{control}$. The ideal internal reference has the following conditions: 1) Large expression; 2) Stable expression in different types of cells and tissues; 3) The expression level has nothing to do with the cell cycle and cell activation; 4) Not subject to any endogenous or the influence of exogenous factors; 5) The absence of pseudogenes to avoid the contamination and amplification of genomic DNA. Housekeeping gene beta-actin was selected and applied in our study. Previous studies have reported that β-actin must be used in isolation as an endogenous control (Darwish et al., 2010; Radonić et al., 2004). Beta-actin is widely distributed in various tissues and cells of mammalian animals, where the amino acid sequence is highly conservative (Nicot et al., 2005; Cornejo et al., 2010). The many intestine-related experiments we have previously conducted revealed that results are reliable when β-actin was used alone (Zou et al., 2019). Additionally, pretests were done that showed β-actin alone can be used. Therefore, the housekeeping gene β-actin was selected and employed in our study.

2.7. Immunohistochemistry for Ki67 and shedding rate

Four-μm thick histological section of each jejunal specimen were obtained as described above and placed on poly-l-lysine-coated glass slides. The slides were dewaxed in xylene twice for 10 min and rehydrated in a descending ethanol series, from 100% descending to 95% and 85%, and finally to 70% ethanol with 5 min intervals. The sections were washed 3 times in PBS buffer (5 min each) and then heated twice (5 min each) in an aqueous sodium citrate solution (0.01 mol/L, pH 6.0) to retrieve antigen epitopes, and then washed another 3 times (10 min each) in PBS buffer. Endogenous peroxidase was inhibited with 3% hydrogen peroxide in methanol for 10 min and then the sections were washed for 15 min in PBS buffer. During incubation for 30 min at 37 °C, 5% bovine serum albumin (BSA; Boster Biological Technology Co. Ltd., Wuhan, China) was used at 1:10 dilution to block non-specific bindings. After incubation with the Ki-67 antibody (ab15580, Abcam, Cambridge, UK; 1:600 dilution) overnight, the sections were thrice washed in the PBS buffer and treated with a goat anti-rabbit IgG secondary antibody (ZSGB-BIO, Beijing, China) for 45 min at 37 °C. Three 5-min washes were performed in PBS buffer after each step save the blocking step. Positive cells were visualized using a diaminobenzidine (DAB) kit (ZSGB-BIO, Beijing, China) according to the manufacturer’s instructions. Fifteen microscope fields of each sample were captured using an optical microscope at magnification 20× (Leica DM3000, Leica Microsystems, Wetzlar, Germany). The number of Ki-67 positive cells in each field was calculated using the Image-Pro Plus 6.0 software (Media Cybernetics, Inc. Georgia, USA; Wang et al., 2018; Chen et al., 2019). The number of villi with shedding cells were counted using the above H&E-stained section and at least 100 villi of each sample were blindly selected. The shedding rate was termed as the number of villi with shedding cells per 100 villi according to the method of a previous study (Bullen et al., 2006).

2.8. Statistical analysis

All results except fecal scores were performed using SPSS software (version 22.0; IBM Corp., Chicago, IL, USA). Statistical analysis among the groups was performed using one-way ANOVA analysis followed by Duncan’s multiple-range test for comparing the differences among treatments. Linear and quadratic contrasts were used to examine the effects of dietary treatment (dietary supplement with 0, 3, 9, 18 mg/kg of folic acid in the basal diet). The results are presented as the mean and the standard error of the mean (SEM) in the tables ($n = 7$). Diarrhea rate was analyzed using the Fisher’s Exact Test procedure of SAS (version 6.12; SAS Inst. Inc., Cary, NC). P-value < 0.05 was considered statistically significant and 0.05 < P < 0.1 was considered as tending towards significance.

Two-linear broken-line regression model was estimated based on group means relative to dietary folic acid concentration ($n = 7$). It can be written as $y = L + U \times (R - x)$ for $x < R$, $(R - x)$ is defined as zero when $x > R$, where the optimal demand is the breakpoint, and $L$ represents the Y-intercept of the breakpoint, minus $U$ represents the slope, $R$ represents the X-intercept of the breakpoint (Robbins et al., 2006). Parameter estimates are presented as means ± SE to indicate the precision of estimation, and breakpoint ($X$-intercept [folic acid mg/kg diet] ± SE, $Y$-intercept ± SE) of the two-linear broken-line regression model as determined by Proc NLIN (SAS 9.3). P < 0.05 was considered statistically significant (Brugger et al., 2014, 2019). Using the following code (Robbins et al., 2006):

Data one;
Input x y;
Datalines;
Treatment Mean-value;
Data fill; *Generates multiple x values to facilitate graph of predicted values;
Do x = 0.22 to 18.22 by 0.22; y = ; output; end;
Run;
Data one; set one fill; run;
Proc sort data = one; by x;
Proc nlin data = one; *straight broken-line;
Parameters L = 0.14 U = -1600 to 1600 by 1 R = 4;
z1 = (x < R) * (R - x);
model y = L + U * (z1);
output out = ppp p = predy; run;
proc gplot;
title2 ‘Two linear broken lines’;
options lpos = 35 vpos = 35 ftext = swiss;
symbol1 v = dot c = black;
symbol2 i = join v = none c = black;
plot y*x predy*x/overlay;
run;

Notes for this code: “Treatment mean-value” were according to the different nutrient levels and the mean of indexes. Parameters L, U, and R were predicted according to the mean of the indexes of trial.

The effect size was calculated as 0.8 for the present experiment based on plasma folate status parameters (Yu et al., 2010). Based on these assumptions, with α = 0.05, a power of 0.90, it has been calculated that total sample size was 28. The G*Power 3.1.9.2 was used to run the power analysis.

3. Results

3.1. Growth performance and fecal score in piglets

The effects of folic acid on the growth performance and fecal score are presented in Table 4. The initial body weight (IBW) of piglets showed no difference. The ADG (P = 0.018) and ADFI (P = 0.032) of weaned piglets expressed a significant linear contrast. However, no significant difference was found in the final body weight and G: F among the 4 treatments.

3.2. Serum biochemical parameters and free amino acids

Serum alanine aminotransferase activity (P = 0.015), as well as urea nitrogen (BUN) content (P = 0.001; linear, P < 0.001) and the serum folate (SF) concentrations (P = 0.001; linear, P < 0.001) expressed a significant linear contrast. However, there were no significant differences in the serum total protein, albumin, creatinine, glucose, cholesterol contents, aspartate transaminase, and hepatic lipase (Table 5). Dietary supplementation with folic acid increased the serum free Asp (P = 0.034; linear, P = 0.021) and Hyp (P = 0.010; linear, P = 0.030; quadratic, P = 0.011) concentrations, and decreased the serum free Glu (P = 0.030; linear, P = 0.009), Val (P = 0.010; linear, P = 0.002), Met (P = 0.002; linear, P = 0.001), Ile (P = 0.016; linear, P = 0.024), Leu (P = 0.009; linear, P = 0.001), Tyr (P = 0.001; linear, P < 0.001), and Lys (P = 0.049; linear, P = 0.007) concentrations (Table 6). There were no significant differences among the serum free Thr, Sar, Gly, Ala, Phe, Orn, His, and Arg concentrations among the 4 dietary treatments (Table 6).

3.3. Intestinal morphology

The VH, CD, VH:CD ratio, and villus surface area (VSA) in the jejunum of piglets are shown in Table 7. Dietary supplementation with folic acid increased the VH (P = 0.003; linear, P = 0.001), VH:CD ratio (P = 0.002; linear, P = 0.001), and VSA (P = 0.026; linear, P = 0.010). However, no significant difference was observed in jejunal CD among the 4 groups.

3.4. Jejunal mucosal disaccharide enzyme activity and mRNA expression

Dietary supplementation with folic acid significantly increased the activities of lactase (P = 0.001; linear, P = 0.001) and sucrase (P = 0.021; linear, P = 0.010) in the jejunal mucosa of weaned piglets (Table 8). The mRNA expression of SLC6a19 was a significant linear contrast (P = 0.023) in the dietary folic acid groups. However, the expression of SLC7a1 (P = 0.021) and TNF-α (P = 0.038) was a significant linear contrast in dietary folic acid groups (Table 9). No differences were observed in the mucosal maltase activity, and the SLC1a1, IFN-γ, and IL-1β mRNA abundance among these groups (Tables 8 and 9).

3.5. Jejunal epithelial cells renewal

The effects of dietary folic acid on the proliferation of jejunal epithelial cells and cell shedding are presented in Fig. 1. The number of Ki67 positive cells, and the shedding rate of epithelial cells had a significant linear contrast in dietary folic acid groups (Table 9). No differences were observed in the jejunal CD antigen 63 and 4 molecules among the dietary folic acid groups.

3.6. Result of two linear broken line regression

As shown in Fig. 2, the breakpoints were based on significant estimates for the X and Y intercepts of respective thresholds ([X-intercept (folic acid mg/kg diet) ± SE, Y-intercept ± SE); (4.42 ± 0.51, 89.97 ± 1.87 g/d), (5.26 ± 0.56, 2.90 ± 0.07 mmol/L), (4.79 ± 0.22, 312.50 ± 127 μm), (3.47 ± 0.31, 119,307 ± 1407 μm²); and (3.53 ± 1.40, 54.86 ± 6.94 μg/L) for ADG, BUN, VH, VSA and SF, respectively).

Above these breakpoints, the response of ADG, VH, VSA, and SF plateaued in response to the changes in dietary folic acid
Table 5
Effects of different folic acid levels on serum biochemical parameters in post-weaning piglets.

| Item         | Supplement folic acid level, mg/kg | SEM  | P-value | Contrast, P-value |
|--------------|-----------------------------------|------|---------|-------------------|
| TP, g/L      | 51.49, 49.46, 49.96, 51.31        | 0.66 | 0.659   | 0.998, 0.225      |
| ALB, g/L     | 32.16, 31.64, 32.36, 31.23        | 0.68 | 0.943   | 0.749, 0.832      |
| ALT, U/L     | 24.41, 25.51, 28.80, 31.79        | 1.16 | 0.095   | 0.015, 0.667      |
| AST, U/L     | 36.71, 40.29, 48.86, 43.43        | 2.53 | 0.395   | 0.217, 0.383      |
| BUN, mmol/L  | 4.63, 3.60b, 2.97b, 2.83b         | 0.20 | 0.001   | <0.001, 0.159     |
| CRE, mg/L    | 71.29, 60.71, 61.29, 62.86        | 2.11 | 0.259   | 0.192, 0.865      |
| GLU, mmol/L  | 4.63a, 3.60b, 2.97b, 2.83b        | 0.20 | 0.001   | <0.001, 0.221     |
| TG, mmol/L   | 0.48, 0.44, 0.43, 0.37            | 0.02 | 0.312   | 0.075, 0.849      |
| CHOL, mmol/L | 3.57, 3.49, 3.43, 3.29            | 0.08 | 0.646   | 0.220, 0.825      |
| Folate, mg/L | 24.41b, 52.03a, 47.92a, 61.79a    | 3.69 | 0.001   | <0.001, 0.221     |

**TP** – total protein; **ALB** – albumin; **ALT** – alanine aminotransferase; **AST** – aspartate transaminase; **BUN** – urea nitrogen; **CREA** – creatinine; **GLU** – glucose; **TG** – triglyceride; **CHOL** – cholesterol; **LIPC** – hepatic lipase.

* Means in the same row followed by different superscript letters indicate significant differences (P < 0.05).

Table 6
Effects of different folic acid levels on serum free amino acids (mg/mL) in post-weaning piglets.

| Item   | Supplement folic acid level, mg/kg | SEM  | P-value | Contrast, P-value |
|--------|-----------------------------------|------|---------|-------------------|
| Tau    | 42.48, 44.38, 44.73, 43.02       | 1.00 | 0.848   | 0.838, 0.399      |
| Urea   | 178.92a, 88.62b, 119.23b, 113.68b| 9.67 | 0.002   | 0.024, 0.010      |
| Asp    | 4.34a, 3.72a, 4.49a, 6.27a       | 0.34 | 0.034   | 0.021, 0.644      |
| Thr    | 16.43, 19.42, 16.31, 17.21        | 1.07 | 0.735   | 0.940, 0.644      |
| Glu    | 42.57a, 45.50a, 34.19ab, 30.40b  | 2.12 | 0.030   | 0.009, 0.379      |
| Sar    | 0.56, 0.73, 0.50, 0.70           | 0.07 | 0.591   | 0.735, 0.885      |
| Gly    | 77.41, 69.86, 93.09, 81.06       | 4.19 | 0.254   | 0.368, 0.787      |
| Ala    | 45.52, 49.85, 48.63, 47.13       | 1.64 | 0.833   | 0.819, 0.404      |
| Val    | 25.58a, 23.27ab, 19.30b, 19.56b  | 0.84 | 0.010   | 0.002, 0.369      |
| Met    | 9.13ab, 10.83a, 6.30b, 2.64b     | 0.88 | 0.002   | 0.001, 0.064      |
| Ile    | 13.50ab, 15.73a, 12.50b, 11.15b  | 0.56 | 0.016   | 0.024, 0.082      |
| Leu    | 26.13a, 23.06ab, 21.02b, 20.54b  | 0.71 | 0.009   | 0.001, 0.283      |
| Tyr    | 13.67a, 13.08a, 10.69b, 9.23b    | 0.47 | <0.001  | <0.001, 0.534     |
| Phe    | 0.25, 0.23, 0.11, 0.25           | 0.04 | 0.438   | 0.705, 0.271      |
| Orn    | 8.11, 8.31, 9.77, 7.80           | 0.52 | 0.558   | 0.911, 0.318      |
| Lys    | 37.14a, 29.24ab, 24.31b, 21.21b  | 2.21 | 0.049   | 0.007, 0.553      |
| His    | 13.20, 12.53, 10.66, 11.10        | 0.60 | 0.412   | 0.150, 0.650      |
| Hyp    | 12.24a, 14.60ab, 16.90b, 14.39ab | 0.52 | 0.010   | 0.030, 0.011      |
| Arg    | 26.16, 31.10, 24.38, 26.15        | 1.18 | 0.293   | 0.497, 0.508      |

* Means in the same row followed by different superscript letters indicate significant differences (P < 0.05).

Table 7
Effects of different folic acid levels on jejunal mucosal histomorphology in post-weaning piglets.

| Items       | Supplement folic acid level, mg/kg | SEM  | P-value | Contrast, P-value |
|-------------|-----------------------------------|------|---------|-------------------|
| Villus height, μm | 247b, 290a, 314a, 311a           | 8    | 0.003   | 0.001, 0.077      |
| Crypt depth, μm  | 305, 299, 277, 293               | 5    | 0.196   | 0.182, 0.249      |
| VH:CD ratio, μm/μm | 0.811, 0.955, 1.14a, 1.07ab   | 5.04 | 0.002   | 0.001, 0.082      |
| Surface area, mm² | 91,868b, 117,210a, 117,908a, 120,723a | 4,001 | 0.026   | 0.010, 0.122      |

VH:CD ratio = the villus height-to-crypt depth ratio.

* Means in the same row followed by different superscript letters indicate significant differences (P < 0.05).

Table 8
Effects of different folic acid levels on disaccharide enzyme activity of jejunal mucosal in post-weaning piglets (U/mg protein).

| Items      | Supplement folic acid level, mg/kg | SEM  | P-value | Contrast, P-value |
|------------|-----------------------------------|------|---------|-------------------|
| Lactase    | 6.6a, 8.8b, 19.5b, 26.8b          | 3.08 | 0.001   | 0.001, 0.458      |
| Sucrase    | 15.1a, 24.6b, 38.2b, 31.3b         | 2.9  | 0.021   | 0.010, 0.115      |
| Maltase    | 11.9, 9.5, 9.9, 15.3             | 1.6  | 0.571   | 0.465, 0.238      |

* Means in the same row followed by different superscript letters indicate significant differences (P < 0.05).
serum alanine aminotransferase activity that is significantly high in the 18 mg/kg folic acid group may affect liver metabolism. Serum folate concentration in the control group was significantly lower than folate acid treatment groups (3, 9, and 18 mg/kg), and in the broken-line model, the SF responded positively to changes below the dietary folate acid 3.53 mg/kg concentration. There were no significant differences in other serum biochemical parameters, such as total protein, glucose, and cholesterol, which indicated that additional supplementation with folic acid may not affect other nutrients or organs metabolism. Additionally, dietary supplementation with 18 mg/kg folic acid down-regulated in the mRNA expression of TNF-α, which suggests that folic acid can improve the intestinal integrity of weaned piglets by reducing the inflammatory response.

During the process of weaning, the piglets were subjected to a lot of stressors (e.g., change in nutrition and new environment), leading to post-weaning ‘growth check’, mainly because feed intake had decreased remarkably, and there was an acute deficiency of nutrients (Pluske et al., 1997). Weaning led to intestinal villus atrophy and crypts hyperplasia, and a lower VH:CD ratio within 3 to 5 days, resulting in a decrease in the digestive and absorptive capacity which contributed to post-weaning diarrhea (Hampson, 1986; Miller et al., 1986). The previous study showed that the addition of folic acid can revert the changes in the structural and functional abnormalities of the small intestine in infants, caused by nutritional folic acid deficiency (Davidson and Townley, 1977). The present study revealed that folic acid treatment (3, 9, and 18 mg/kg) can significantly increase jejunal VH and VA when compared to the control group, and using the broken-line model, VH, VA positively responded to changes below the dietary folic acid 4.79, and 3.47 mg/kg concentration, respectively. However, this finding was inconsistent with previous studies, in which Liu et al. (2011b) reported that although maternal folic acid supplement modulated apoptosis-related gene expressions in the jejunum of newborn piglets, there was no significant change in the intestinal morphology. The reason for the deviation in our results may be because the weaned piglets had a low feed intake and a relative lack of folic acid, while the suckling piglets had enough folic acid from milk to maintain the intestinal morphology. Taken together, our results indicated that folic acid supplementation could improve the intestinal morphology in weaned piglets.

Intestinal morphology is correlated with gut capacity to digest and absorb the nutrients (Wang et al., 2018). Weaning stress not only affects the intestinal morphology but also impairs the intestinal digestive and absorptive functions (Montagne et al., 2007). Small intestinal disaccharidases are key enzymes in the digestion and absorption of carbohydrates in piglets, while the disaccharidases activity and the digestive capacity decrease after weaning, resulting in the occurrence of diarrhea (Miller et al., 1986; Kelly et al., 1991a; Kelly et al., 1991b). Consistent with the improvement in intestinal morphology, dietary supplementation with 9 and 18 mg/kg folic acid can significantly increase lactase and sucrase activity in the jejunal mucosa of weaned piglets. Our results are consistent with previous studies where the increase of folic acid supplementation enhanced the activities of intestinal enzymes and reversed the ethanol-induced inhibition of intestinal enzyme activities (Greene et al., 1974; Sesay et al., 2016). In addition, dietary supplementation with 18 mg/kg can up-regulate the mRNA expression of SLC6a19, a neutral amino acid transporter. Our results also indicate that additional supplementation with folic acid could improve intestinal digestive and absorptive functions. However, dietary supplementation with folic acid downregulated the mRNA expression of SLC7a1, a cationic amino acid transporter, in the jejunal mucosa of weaned piglets. Therefore, clarification will be needed on the mechanism of the action of folic acid on jejunal amino acid transporters expression and the effects of folic acid on nutrient digestibility. As a proinflammatory cytokine, TNF-α high

### Table 9

| Item          | Supplement folic acid level, mg/kg | SEM | P-value | Contrast, P-value |
|---------------|-----------------------------------|-----|---------|-------------------|
| SLCA1         | 3.1                              | 0.06| 0.995   | 0.999             |
| SLCA2         | 2.0                              | 0.06| 0.999   | 0.999             |
| SLCA6a19      | 1.1                              | 0.06| 0.999   | 0.999             |
| TNF-α         | 1.1                              | 0.06| 0.999   | 0.999             |
| IFN-γ         | 1.1                              | 0.06| 0.999   | 0.999             |
| IL-1β         | 1.1                              | 0.06| 0.999   | 0.999             |
expression has negative effects on intestinal integrity (Pié et al., 2004). Our results showed that dietary supplementation with 18 mg/kg folic acid can significantly downregulate the expression of TNF-α, suggesting that additional supplementation with folic acid may also decrease the signs of intestinal inflammation of weaned piglets. However, the expression of another two proinflammatory cytokines, IFN-γ and IL-1β, were not significantly affected by dietary folic acids, which may be because these proinflammatory cytokines have different expression patterns in the intestine of piglets during the post-weaning period (Pié et al., 2004).

It is well known that the normal morphology of the intestine is determined by the balance between cell proliferation and cell shedding (Miguel et al., 2017). However, weaning stress can promote the apoptosis of intestinal epithelial cells and proliferation of intestinal stem cells in the crypts (Zhu et al., 2014; Yang et al., 2016a), thereby accelerating villus atrophy and intestinal epithelial cell renewal, which results in a dysfunction of the small intestine (Pluske et al., 1997; Boudry et al., 2004; Yang et al., 2013). To evaluate the effects of folic acid on the proliferation of intestinal epithelial cells, we measured the number of Ki-67 positive cells, a reliable marker of cell proliferation (Scholzen and Gerdes, 2000; Wang et al., 2018), in the jejunum of weaned piglets. Our results showed that dietary supplementation with 9 mg/kg folic acid can significantly decrease the number of Ki-67 positive cells and the shedding rate of intestinal epithelial cells, which indicates that high doses of folic acid can alleviate the cell shedding induced by weaning stress and thus maintain the function of the intestinal epithelial cells. Therefore, additional supplementation with folic...
acid may maintain the balance of intestinal epithelial cell renewal to keep the intestinal morphology and function of weaned piglets intact.

5. Conclusion

The present study indicates that piglets supplemented with folic acid had positive growth performance and intestinal morphology during the post-weaning period. In addition, dietary supplementation with folic acid can decrease the number of Ki-67 positive cells and the shedding rate of intestinal epithelial cells. Further studies need to be conducted to find the optimal concentration of folic acid for the weaned piglets through the final estimation of the requirement threshold. In general, the present study will provide valuable guidance for adding a suitable concentration of folic acid in weaning piglets.

Author contributions

Huansheng Yang: conceptualization, methodology, software, validation. Yulong Yin: supervision, funding acquisition, writing—review and editing.

Conflict of interest

We declare that we have no financial or personal relationships with other people or organizations that might inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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