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Effects of Dietary Folic Acid Supplementation on Growth Performance and Immune Parameters in Weanling Piglets

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Abstract: In order to study the effects of dietary folic acid (FA) supplementation on growth performance and immune status in weanling piglets, a single factorial randomized block design trial was conducted with six diets supplemented with FA at 0, 0.30, 3.00, 6.00, 9.00 or 15.00 mg/kg. A total of 108 crossbred (Landrace × Yorkshire) castrated weanling piglets (at 21 d of age) were allocated by body weight into 36 feeding cages (3 piglets/cage), which were allotted randomly into six dietary groups (six cages/group). Piglets were fed ad libitum for 24 days. Blood samples were collected on the 24th day. The growth performance and immune parameters were measured. Results showed that FA supplementation increased the serum FA level of weaned piglets ($p < 0.01$) and tended to increase the body weight (BW) at 45 d of age ($p < 0.1$) and the average daily gain (ADG) from 29 d to 45 d of age ($p < 0.1$). FA addition improved the feed efficiency (G/F) from 21 to 45 d of age ($p < 0.01$) with supplementary FA levels of 0.3, 3.0, and 9.0 mg/kg compared with the control group with no FA supplementation. FA supplementation showed a trend ($p < 0.1$) to increase the peripheral blood $CD3^+CD8^+$ lymphocyte subpopulation and a tendency ($p < 0.1$) to decrease the $CD3^+CD4^+/CD3^+CD8^+$ ratio; in particular FA supplementation of 0.3 and 3.0 mg/kg showed significant differences in comparison to the non-supplemented control group. Moreover, FA addition increased the serum interferon-γ (IFN-γ) level ($p < 0.05$) and tended to reduce the ratio of tumor necrosis factor-α to interleukin-4 (TNF-α/IL-4, $p < 0.1$) and immunoglobulin G (IgG, $p < 0.1$) in serum, but had no significant effect on serum IL-4, TNF-α, and nitric oxide. In conclusion, FA supplementation up to 3 mg/kg to the diet showed a tendency to improve immune function, while FA supplementation of up to 9 mg/kg improved feed efficiency, which resulted in a trend for higher growth in weaned piglets between 7 to 11 kg BW.

Keywords: weaning piglet; folic acid; feed efficiency; immune parameter

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1. Introduction

Folates play a crucial role in various biological processes, including biosynthesis and epigenetic regulation, by providing one-carbon moieties [1]. As mammals, including pigs, are unable to synthesize folates, their metabolic needs must be primarily met through dietary sources [2]. Weaning pigs often experience a decrease in digestive and absorptive capacity, as well as a significant reduction in food intake, which can result in undernutrition, growth suppression, and increased susceptibility to infections [3]. Weaning can also affect the absorption of dietary folate and research has shown that the folate status of piglets tends to decline after weaning [4].

Folate is generally believed to be essential for optimal immune function in pigs [5]. However, it is currently unclear whether different levels of folate intake during weaning,

particularly in fast-growing piglets, could have an impact on immune status. Recent research has suggested that the folate status of the gravid sow may influence the serum folate concentrations of both the dam and her offspring and affect postnatal immune responses [6]. The rapidly growing animals, especially with high lean growth, might require greater amounts of vitamins including folate due to increased nucleotide synthesis in proliferating satellite muscle cells [7]. After weaning, the supply of milk-derived folate stops and feed intake decreases, resulting in insufficient folate in piglets, which may aggravate the immune stress of weaned piglets [2]. It also reported that folic acid improved intestinal morphology, which makes sense as these cells have high turn over (rapid growth, like immune cells) [8]. Depending on the magnitude of the reduction in feed intake, and given the importance of folate, high doses of folic acid (FA) at 3.0–9.0 mg/kg were often added to piglet diets in production. However, few studies have reported the exact effect of dietary folic acid supplementation on promoting growth and improving immunity in weaned piglets. Therefore, we propose that dietary supplementation with FA at different levels may have a positive impact on the immune status and growth performance of rapidly growing weanling pigs, particularly under conditions of low feed intake. Further research in this area may help to elucidate the potential role of folate in immune function and growth regulation in pigs.

The objective of this study was to investigate the effects of dietary FA supplementation on the performance and immune parameters of newly weaned piglets. Additionally, the study aimed to optimize the level of FA supplementation in the diet of weanling piglets weighing between 7 to 11 kg.

2. Materials and Methods

2.1. Animal Ethics

All experiments involving swine were carried out in strict accordance with the Guide for the Care and Use of Laboratory Animals Monitoring Committee of Sichuan Province, China, and the protocols approved by the Sichuan Agricultural University Animal Ethical and Welfare Committee (Approval No. 20180021).

2.2. Experimental Animals and Diets

A total of 108 crossbred (Landrace × Yorkshire) castrated piglets were used in this study, which were newly weaned at 21 days of age. The sows had been fed the same diets containing folate levels of 2.5 mg/kg in gestation and 3.0 mg/kg in lactation, respectively, which were above the NRC (2012) recommended level [9]. The piglets (with an average initial body weight of 6.93 ± 0.07 kg) were equally assigned into 36 cages with a similar initial body weight with 3 piglets per cage. Six cages were allotted randomly into 6 dietary groups with FA supplemented at 0, 0.3, 3.0, 6.0, 9.0 or 15.0 mg/kg, respectively. The corresponding measured values of dietary FA were 0.35, 0.66, 2.19, 5.18, 7.51, or 10.81 mg/kg. FA at 0.3 mg/kg is the level recommended by NRC (2012) [9]. In production, based on the different understanding of the possible important functions of folic acid supplementation, the dosage of folic acid supplementation in the diet of weaned piglets is often much higher than the NRC recommended amount, usually between 3.0 and 9.0 mg/kg. Therefore, in order to fully determine the possible effects of FA supplementation, we set the above higher folate addition levels and increased the high level of folic acid addition to 15.0 mg/kg. Folic acid was supplemented in the basal diet as pteroylglutamic acid ($C_{19}H_{19}N_7O_6$), which was provided by a product composed of 80% pteroylglutamic acid and 20% carrier (Bayer Sichuan Animal Health Co., Ltd., Chengdu, China). The basal diet was prepared on the basis of maize-soybean meal to meet or exceed nutrient requirements for 5–10 kg piglets as recommended by NRC (Table 1) with all other vitamins at 200% of the NRC [9] requirements to avoid other vitamin dosage restrictions becoming contributing factors.

Table 1. Ingredient composition and nutrient concentrations of basal diet [1] (%, as-fed basis).

Item	Percentage
Ingredients	
Maize	63.40
Soybean meal	25.60
Fish meal	4.00
Whey, dried	3.00
Soy oil	1.70
Sodium chloride	0.30
Dicalcium Phosphate	0.86
Limestone	0.66
DL-Methionine	0.05
L-Lysine · HCL	0.11
L-Threonine	0.02
Vitamin and mineral premix [2]	0.30
Total	100.00
Calculated composition	
Crude protein	20.00
Lysine	1.16
Methionine	0.36
Methionine + Cysteine	0.66
Tryptophan	0.24
Threonine	0.80
Calcium	0.75
Available phosphorus	0.38
Digestible energy, MJ/kg	14.39
Metabolic energy, MJ/kg	13.74
Folate [3], mg/kg	0.33

[1] The China Feed Composition and Nutritional Value Table (19th Edition, 2008) was used for calculation of nutrient concentrations. [2] Supplied diets with (per kg diet): retinol acetate 8.8 mg; cholecalciferol 880 μg; dl-R-tocopheryl acetate 64 mg; menadione 1 mg; riboflavin 7 mg; pantothenic acid 20 mg; nicotinic acid 30 mg; biotin 0.1 mg; thiamin 2 mg; Vitamin B_{12} 35 μg; choline 500 mg; Fe 100 mg ($FeSO_4 \cdot 7H_2O$); Zn 100 mg ($ZnSO_4 \cdot 7H_2O$); Cu 6 mg ($CuSO_4 \cdot 5H_2O$); Mn 4 mg ($MnSO_4 \cdot H_2O$); Se 0.3 mg (Na_2SeO_3); I 0.15 mg (KI). [3] The measured value was 0.35 mg/kg. In the experimental diets supplemented with folic acid at 0.3, 3.0, 6.0, 9.0, or 15.0 mg/kg, folic acid was added at the expense of maize, and the corresponding measured values of the total dietary folic acid were 0.66, 2.19, 5.18, 7.51, or 10.81 mg/kg.

All piglets were housed in the same 2.5 × 1 × 1 m stainless steel feeding cages, equipped with a feeder and nipple water. Piglets were fed ad libitum and free access to water. Temperature (23–27 °C) and a cycle of 16 h light/8 h dark were maintained in the mechanically ventilated room. Piglets were weighed on the first, the 7th and the 24th day of the study. The feed intake per cage and body weight (BW) was measured at 29 d and 45 d of age, and the average daily gain (ADG), average daily feed intake (ADFI), and feed efficiency (G/F) were calculated. Feces were visually assessed after feeding three times daily (08:00, 12:00 and 20:00) and assigned a consistency score by a single person blinded to dietary treatments. A score of 0, 1, 2, or 3 was recorded to indicate firm, soft but formed, runny, or severe watery diarrhea, respectively. The Diarrhea index (DI) was defined as the total grades for fecal consistency of 3 piglets in each cage during different feeding periods.

2.3. Sample Collection

After fasting for 8 h, a blood sample was obtained from one piglet per cage by vena cava puncture in the morning of the 24th day (at 45 d of age of piglets). Samples of 5 mL blood per piglet were centrifuged at 1000× *g* for 15 min to obtain serum, which were stored at −80 °C for future analyses for FA, antibodies, and cytokines. Samples of 3 mL blood per piglet at 45 d of age were immediately heparinized (20 i.u./mL), and prepared for flow cytometric analysis (FACS).

2.4. Cell Preparation and Flow Cytometric Analysis

About 3 mL of heparinized blood per sample was centrifuged (4 °C; 1000× *g*, 5 min) to obtain precipitated cells, which were treated with 2 mL red blood cell lysis buffer (8.26 g of NH_4Cl, 1 g of $KHCO_3$ and 0.037 g of Na_4EDTA per liter of distilled water) for 1 min and centrifuged (4 °C; 1000× *g*, 5 min) again. After washing twice using cold Roswell Park Memorial Institute-1640 (RPMI-1640) (Sigma, Burlington, MA, USA), cells were resuspended with cold RPMI-1640 (10% fetal bovine serum (FBS); Sigma, Burlington, MA, USA) and immediately dispersed mechanically by vortex mixing.

The phenotypes of lymphocyte subpopulations from peripheral blood were monitored by a standard flow cytometry fluorescence sorting technology (FACS) Calibur flow cytometer (Becton-Dickinson, San Jose, CA, USA) operated by CELLQuest software v3.0.1, using a panel of flourescein isothiocyanate (FITC), spectral red (SPRD) or phycoerythrin (PE)-labeled monoclonal antibodies (MABs) (South. Biotech. Assoc., Inc., Birmingham, AL, USA). The MABs included mouse anti-porcine CD3ε-FITC (Lot F7207-N598), mouse anti-porcine CD4α-PE (Lot E4510-02), and mouse anti-porcine CD8α-SPRD (Lot K0602-NB08z). The cells, aliquoted at 5×10^5 per tube, were incubated for 30 min with 10 µL of each MAB and after three washes, the percentage of single-positive cells (such as the $CD3^+$ subset, which is the percentage of cells single-positive to mouse anti-porcine CD3ε-FITC) or double-positive cells (such as the $CD3^+CD4^+$ subset, which is the percentage of cells double-positive with both mouse anti-porcine CD3ε-FITC and mouse anti-porcine CD4α-PE, and the $CD3^+CD8^+$ subset, which is the percentage of cells double-positive with both mouse anti-porcine CD3ε-FITC and mouse anti-porcine CD8α-SPRD in the peripheral lymphocytes) were then assessed by flow cytometry, and the relative number of CD cell subpopulations was determined in each sample.

2.5. Quantification of Cytokines and Immunoglobulins in Serum

Concentrations of interleukin-4 (IL-4), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) in serum were determined using swine enzyme-linked immunosorbent assay (ELISA) kits (Rapid Bio. Lab, South Miami, FL, USA) for IL-4 (Lot 09150901), TNF-α (Lot 02060904) and IFN-γ (Lot 03080607). Following the instructions of the manufacturer, values were read in a microplate reader at 450 nm, and the levels of serum cytokines were calculated by interpolation using standard curves.

The levels of serum nitric oxide (NO) were determined by a nitrite detection kit (Beyotime Biotech Inc., Nantong, China) according to instructions provided by the manufacturer. Briefly, 50 µL of serum or standard $NaNO_2$ was mixed with 100 µL of Griess reagent in a 96-well plate. After 15 min, values were read in a microplate reader at 540 nm.

The levels of serum immunoglobulin G (IgG) were determined by the immunoturbidimetry method provided in an IgG assay kit (Sichuan Maker Science & Technology Co., Ltd., Chengdu, China; Lot 1007051). Briefly, 3 µL of serum sample or standard serum was incubated with 300 µL goat serum reagent (containing goat anti-pig IgG, and removed lipid) in a 96-well plate. After 10 min, values were read in a microplate reader at 340 nm.

2.6. Microbiological Assay for Folic Acid

The FA content in diet or serum was measured by the microbiological plate method using a VitaFast® FA kit (R-Biopharm AG, Darmstadt, Germany; Lot KF40011). Following the instructions of the manufacturer, procedures were used as described by Molloy and Scott (1997) [10] with minor modification. Homogenized diet or serum samples of 1 mL were measured into 50 mL sterile centrifuge vials, filled up to 40 mL with phosphate buffer (0.05 mol/L; 0.1% ascorbate; pH 7.2) for an extraction for 30 min at 90–100 °C in a water bath shaking well, and then chilled down quickly to room temperature.

To isolate the folate from serum protein, the extract of serum was added to 10 mg of dry Pancreatin (P1750; Sigma, Burlington, MA USA) and incubated for 24 h at 37 °C, and heated for 30 min in a water bath at 90–100 °C, thereafter chilled down quickly to below 30 °C. The sample extraction of 1.0–1.5 mL was filtered with a sterile filter (0.2 µm) into a

sterile reaction vial. The diluted extract and the FA assay-medium were pipetted into the wells of a microtiter plate that had been coated with *Lactobacillus rhamnosus* (ATCC Nr.7469) and sealed airtight. Fully responding to both oxidized and reduced folates with 3 or fewer glutamate residues, the bacterial growth stopped when the vitamin was consumed [10]. The intensity of metabolism or growth in relation to the extracted folates was measured as turbidity and compared to a standard curve. After incubating in the dark at 37 °C for 44–48 h, the measurement was done using an ELISA reader at 620 nm.

2.7. Statistical Analysis

The data were analyzed statistically by single factorial variance analysis using the general linear model procedure of SPSS 20.0 software (SPSS Inc., Chicago, IL, USA). Data sets were further analyzed using post hoc tests (least significant difference, LSD) for multiple comparisons to determine the statistical differences between groups, which were denoted by different letter superscripts. Each feeding cage with 3 piglets was used as the experimental unit for growth performance. Data were expressed as mean ± SEM. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Growth Performance and Diarrhea Index

As shown in Table 2, all groups had a similar initial average bodyweight ($p = 0.999$). Dietary FA addition tended to influence the BW at 45 d of age ($p < 0.1$). Compared with the control group, the groups fed diets supplemented with FA at 3.00 mg/kg or 9.00 mg/kg had larger BW at 45 d of age, respectively ($p < 0.05$). In addition, dietary FA levels tended to influence the ADG from 29 d to 45 d of age ($p < 0.1$) and the ADG from 21 d to 45 d of age ($p < 0.1$). The groups fed diets supplemented with FA at 3.00 mg/kg or 9.00 mg/kg had greater ADG from 29 d to 45 d of age and ADG from 21 d to 45 d of age than those of the control group, respectively ($p < 0.05$). However, no significant effects of dietary FA levels on ADFI were found.

Table 2. Effects of dietary folic acid levels on performance and diarrhea in weaned piglets [1] (n = 6).

Feeding Period [2]	Item [3]	FA Supplementation [5], mg/kg						SEM	*p*-Value
		0	0.30	3.00	6.00	9.00	15.00		
21 d	BW, kg	6.928	6.928	6.931	6.928	6.928	6.931	0.004	0.999
28 d	BW, kg	7.017	7.078	7.061	7.072	7.319	7.156	0.039	0.275
45 d	BW, kg	10.350	10.947	11.103	10.560	11.228	10.878	0.102	0.097
21 d to 28 d									
	ADG, g	12.7	21.7	18.8	20.7	56.0	32.0	5.53	0.256
	ADFI, g	132.9	131.5	147.2	129.8	149.5	147.7	4.68	0.691
	G/F, g/g	0.068	0.106	0.102	0.104	0.370	0.217	0.040	0.245
	DI [3]	6.5	13.2	11.4	8.4	3.8	8.3	1.25	0.293
29 d to 45 d									
	ADG, g	196.1	227.6	236.3	200.0	229.9	219.0	4.99	0.097
	ADFI, g	329.6	364.1	360.8	336.8	356.0	360.9	6.48	0.549
	G/F, g/g	0.595 ^{ab}	0.626 ^{bc}	0.654 ^{c}	0.591 ^{a}	0.645 ^{c}	0.607 ^{ab}	0.006	0.002
	DI [4]	18.2	10.3	15.2	17.0	8.3	11.5	1.67	0.471
21 d to 45 d									
	ADG, g	142.6	167.5	174.0	151.2	179.2	164.5	4.26	0.098
	ADFI, g	272.2	296.2	301.5	278.3	295.8	298.7	5.54	0.585
	G/F, g/g	0.523 ^{a}	0.565 ^{b}	0.576 ^{bc}	0.540 ^{ab}	0.605 ^{c}	0.551 ^{ab}	0.006	0.000
	DI [3]	24.7	23.5	26.6	25.4	12.2	19.8	1.96	0.283

[1] Different superscript letters indicate significant differences at $p < 0.05$. [2] Feeding period is expressed by the days of age in weaned piglets. [3] BW = Body weight, ADG = Average daily gain, ADFI = Average daily feed intake, G/F = Feed efficiency, and DI = Diarrhea index. [4] Diarrhea index (DI) was defined as the total grades for fecal consistency of 3 piglets per cage during different feeding periods. [5] The corresponding measured values of the total dietary folic acid were 0.66, 2.19, 5.18, 7.51, or 10.81 mg/kg, respectively.

There were significant differences of G/F from 29 d to 45 d of age ($p < 0.01$) and from 21 d to 45 d of age ($p < 0.01$) among the groups. Compared with the control group, the weanling piglets fed diets supplemented with FA at 3.00 mg/kg or 9.00 mg/kg had better G/F from 29 d to 45 d of age and G/F from 21 d to 45 d of age, respectively ($p < 0.05$), and the group fed a diet supplemented with FA at 0.30 mg/kg had a better G/F from 21 d to 45 d of age ($p < 0.05$). No significant effects of dietary FA levels on the DI were found.

3.2. Folate Status

As expected, FA supplementation increased the serum FA concentration ($p < 0.01$) in weanling piglets (Table 3). At 45 d of age, the control group had a lower serum FA concentration than other groups ($p < 0.01$); and the group fed a diet supplemented with FA at 9.00 mg/kg had a higher serum FA concentration than that of the group fed a diet supplemented with FA at 0.30 mg/kg ($p < 0.05$).

Table 3. Effects of dietary folic acid levels on serum levels of folic acid, cytokines, nitric oxide, and immunoglobulin G [1,2] (n = 6).

Item.	FA Supplementation [4], mg/kg						SEM	*p*-Value
	0	0.30	3.00	6.00	9.00	15.00		
Folic acid, ng·mL^{-1}	64.7 [a]	77.2 [b]	83.7 [bc]	84.7 [bc]	91.4 [c]	88.0 [bc]	5.48	0.000
IgG, g·L^{-1}	5.85	5.66	5.50	5.57	5.26	5.44	0.198	0.097
IL-4, pg·mL^{-1}	41.3	47.5	46.4	45.8	42.5	44.0	3.05	0.325
TNF-α, pg·mL^{-1}	528.2	573.7	562.9	582.2	536.1	556.8	36.1	0.644
IFN-γ, pg·mL^{-1}	132.8 [a]	152.7 [b]	155.1 [b]	149.4 [b]	150.4 [b]	148.4 [b]	6.99	0.048
NO, μmol·L^{-1}	20.0	23.6	24.0	35.9	28.9	29.7	12.04	0.818
TNF-α/IL-4 (Th1/Th2) [3]	12.8	12.1	12.2	12.7	12.6	12.7	0.29	0.091

[1] Sera were obtained from blood samples of piglets at 45 d of age. [2] Different superscript letters indicate significant differences at $p < 0.05$. [3] The ratio of TNF-α to IL-4 is commonly used to examine the balance of the T-helper (Th)1/Th2-type response. [4] The corresponding measured values of the total dietary folic acid were 0.66, 2.19, 5.18, 7.51, or 10.81 mg/kg, respectively.

3.3. The Levels of Serum Cytokines, Nitric Oxide, and Immunoglobulin G

FA supplementation increased the serum IFN-γ concentration ($p < 0.05$) in weaned piglets at 45 d of age and the control group had a lower concentration than those of other groups ($p < 0.05$) (Table 3). The serum levels of IL-4, TNF-α, and NO were not significantly different among the groups. Dietary FA addition tended to reduce the serum TNF-α/IL-4 ($p < 0.1$).

Moreover, dietary FA levels tended to decrease the serum IgG ($p < 0.1$) and the control group had a higher serum IgG concentration than the groups fed diets supplemented with FA at 9.00 mg/kg ($p < 0.05$) or 15.00 mg/kg ($p < 0.05$) at 45 d of age.

3.4. The Peripheral Lymphocyte Subpopulation

Dietary FA addition tended to increase the $CD3^+CD8^+$ subset ($p < 0.1$) and the ratio of $CD3^+CD4^+$ to $CD3^+CD8^+$ cells ($p < 0.1$) in weaned piglets at 45 d of age (Table 4). The group fed a diet supplemented with FA at 0.30 mg/kg had a higher $CD3^+CD8^+$ subset than the control group or the group fed a diet supplemented with FA at 6.00 mg/kg ($p < 0.05$). The group fed a diet supplemented with FA at 3.00 mg/kg had a higher $CD3^+CD8^+$ subset than the control group ($p < 0.05$) as well. The control group had a higher ratio of $CD3^+CD4^+$ to $CD3^+CD8^+$ cells than the groups fed diets supplemented with FA at 0.30 mg/kg ($p < 0.05$), 3.00 mg/kg ($p < 0.05$), or 15.00 mg/kg ($p < 0.05$) at 45 d of age, respectively.

Table 4. Effects of dietary folic acid levels on peripheral lymphocyte subpopulations (n = 6).

Item [1]	FA Supplementation [2], mg/kg						SEM	*p*-Value
	0	0.30	3.00	6.00	9.00	15.00		
$CD3^+$, %	65.2	62.0	69.3	64.2	63.3	67.6	1.12	0.400
$CD3^+CD4^+$, %	34.4	32.9	34.5	33.6	31.5	34.7	0.82	0.881
$CD3^+CD8^+$, %	22.0	32.2	30.1	27.5	24.7	28.5	1.07	0.065
$CD3^+CD4^+/CD3^+CD8^+$	1.57	1.07	1.20	1.24	1.31	1.22	0.050	0.090

[1] The $CD3^+$ subset is the percentage of cells single-positive to mouse anti-porcine CD3ε-FITC in the peripheral lymphocytes assessed by flow cytometry. The $CD3^+CD4^+$ subset is the percentage of cells double-positive with both mouse anti-porcine CD3ε-FITC and mouse anti-porcine CD4α-PE in the peripheral lymphocytes assessed by flow cytometry. The $CD3^+CD8^+$ subset is the percentage of cells double-positive with both mouse anti-porcine CD3ε-FITC and mouse anti-porcine CD8α-SPRD in the peripheral lymphocytes assessed by flow cytometry. [2] The corresponding measured values of the total dietary folic acid were 0.66, 2.19, 5.18, 7.51, or 10.81 mg/kg, respectively.

4. Discussion

4.1. The Effects of Dietary Supplementation with FA on the Growth Performance in Weanling Piglets

The observed values of dietary FA, which were lower than the calculated values, could potentially be attributed to the expenses associated with FA during feed processing, as well as the determination method utilized [10,11]. Although the feeds were powdered and blended without extrusion and granulation in this study, dietary folic acid may also be affected by contact with other components, especially if exposed to light and pH below 5.0, and the content may decrease over time [12,13]. However, given the similar gradient and high correlation between the observed and calculated values of dietary FA, it can be concluded that the experimental treatments employed in this study were meaningful and valid.

The data obtained from the present study indicated that the changes in serum FA levels were consistent with the level of FA supplementation in the diet of weanling piglets. This finding confirmed that the FA supplemented in the diet was effectively absorbed by the piglets, leading to an improvement in the folate status of the weanling piglets. The group fed a diet supplemented with FA at 9.00 mg/kg had the highest serum FA concentration, but the addition of 15 mg/kg did not lead to a further increase in folic acid status in serum. The probable reason is that the absorption of folic acid decreases with increasing the dose too high.

Easter et al. (1983) [14] conducted a study on pigs fed a maize-soybean meal diet during the starting phases, with an initial weight of 9.0 kg. They found that the pigs gained weight and used their feed efficiently, with no significant difference observed between those supplemented with 0.5 or 1.5 mg of FA/kg of diet. However, a study by Lindemann & Kornegay (1986) [15] reported improved daily weight gain in weanling piglets fed a maize-soybean meal diet supplemented with FA at 0.5 mg/kg. In a recent study, dietary supplementation with FA was found to improve the growth performance of piglets. Specifically, the ADG of piglets from 21 to 45 days of age fed diets supplemented with FA at 3.00 or 9.00 mg/kg was increased. However, it is important to note that various factors can impact the effects of FA supplementation on growth performance in piglets, including genotypes, feeding phases, body weight, feed ingredients, levels of other nutrients (including other vitamins) in feed, immune states, use of sulfa drugs, and the status of intestinal microbiota [4,8].

The results of the present study indicate that dietary supplementation with a suitable level of FA significantly improves feed efficiency in piglets, but has no obvious effects on feed intake. These findings suggest that the improved growth performance observed in the study was primarily due to increased feed efficiency resulting from FA supplementation, while the beneficial effects on gut health may have played a partial role in the improvement [2]. The present study revealed significant effects of dietary FA levels on the feed efficiency of weanling piglets at 29 d to 45 d of age. It indicated that the group

supplemented with folic acid at 9.00 mg/kg achieved the greatest ADG and the best feed efficiency(G/F) in weaned piglets weighing between 7 to 11 kg from 21 d to 45 d of age in this study. Notably, this FA level was higher than the current NRC (2012) recommendation of 0.3 mg FA/kg diet for 5–10 kg piglets [9]. It was also reported that piglets fed with 9 mg/kg FA improved the villus height to crypt ratio and the intestinal microbiota, which may improve absorption, and probably growth and even decrease diarrhea [2].

Enterocytes are known to have a high rate of turnover, with their proliferation and apoptosis being sensitive to the status of FA [16]. This sensitivity to FA suggests that one potential beneficial effect of folate for piglets could be in accelerating the regeneration of damaged cells associated with diarrhea and postweaning villus atrophy. Previous research conducted in South Africa showed that oral folate shortened the duration of diarrhea in children [17]. However, this result was not supported by another study in the treatment of acute watery diarrhea in children [18]. Similar results were also found in weaning piglets in the present study, but the variability was too high to show differences. In a study by Shoda et al. (2007) [19], large-dose (160 μg/g feed) FA supplementation in rats was found to significantly reduce the count of enteric bacteria translocated into the mesenteric lymph nodes and showed a trend towards a reduction in indigenous bacteria adhering to the jejunal mucosa, although it did not prevent diarrhea and malnutrition induced by a lectin-based diet.

4.2. The Effects of Dietary Supplementation with FA on Immune Parameters in Piglets

The porcine immune system is not fully developed at birth, and the period around weaning (3–4 weeks of age) is critical for its development, particularly for the cellular component [20]. Folate is essential for DNA synthesis and is involved in several reactions of amino acid metabolism [21]. Cells lacking folate accumulate in the S phase, leading to increased uracil misincorporation and DNA damage. When folate is reintroduced to folate-deficient cells, S phase accumulation is reversed and proliferation is restored [22,23]. Courtemanche et al. (2004) [24] found that folate deficiency was associated with reduced T-cell proliferation, increased apoptosis, a marked decrease of $CD8^+$ cells, an increase of the $CD4^+/CD8^+$ ratio, and that the proliferation of activated $CD8^+$ cells was more sensitive to the lack of folate than $CD4^+$ cells in vitro. The results of the present study showed that groups fed diets supplemented with 0.30 mg/kg or 3.00 mg/kg of FA had higher $CD3^+CD8^+$ subsets at 45 days of age than the control group. These results suggest that $CD8^+$ cell proliferation is sensitive to dietary supplementation with FA, even at lower levels, and that actively proliferating cells such as lymphocytes may increase the FA requirements of weaning piglets [25]. Therefore, it is necessary to supplement sufficient FA into the diet of weanling piglets to ensure $CD8^+$ cell proliferation.

Immature T lymphocytes ($CD4^+CD8^+$) undergo processing in the thymus and are released into the peripheral blood in two subpopulations: Th cells ($CD4^+$ cells), which activate other immune cells, and CTLs ($CD8^+$ cells), which recognize and destroy infected cells, foreign tissue, and tumor cells. The ratio between these two subpopulations is crucial for proper immune function, and changes in the $CD4^+/CD8^+$ ratio have been observed in various immune status conditions [26,27]. In the present study, the control group had a higher $CD3^+CD4^+$ to $CD3^+CD8^+$ ratio than those of the groups fed diets supplemented with 0.30 mg/kg, 3.00 mg/kg, or 15.00 mg/kg of FA, which indicates that the immune system of weanling piglets in the control group may be vulnerable to activation. This finding confirms that dietary supplementation with folic acid can affect the immune status of weanling piglets. Based on the data, dietary FA levels of at least 0.66 mg/kg are required for weaned piglets from 21 d to 45 d of age.

The results of the present study demonstrated that serum IFN-γ levels increased in weanling piglets with FA supplementation, which is consistent with a previous study in 23-month-old rats, which showed an increase in tissular IFN-γ levels in the spleen, when fed diets supplemented with FA at 35.7 mg/kg [28]. Moreover, Field et al. (2006) [28] also found that the folate-supplemented diets had an impact on the T-helper (Th)1/Th2-type response,

as evidenced by the ratio of TNF-α to IL-4. Th1 cells primarily mediate the body's cellular immune response and play an important role in the induction of anti-infection, acute rejection, organ transplant rejection, and autoimmune diseases. Th2 cells primarily mediate the humoral immune response, assist in antibody production, and play a leading role in allergic reactions, enabling antigen-specific B cells to secrete IgG and IgE antibodies [29]. In the present study, dietary FA levels tended to impact the balance of the Th1/Th2-type response, as indicated by the serum IgG level of the control group, which was higher than those of the groups fed diets supplemented with FA at 9.00 mg/kg or 15.00 mg/kg. These findings suggest that sufficient FA is essential for the immune system, and dietary FA levels might be involved in immune regulation in weanling piglets, necessitating dietary FA at 0.66 mg/kg or more from 21 d to 45 d of age. While no dose response was observed, these immune parameters were impacted by dietary FA levels, highlighting the importance of proper FA supplementation for optimal immune function.

5. Conclusions

In summary, our study indicates that under the stressful conditions of weaning, FA supplementation levels of up to 9 mg/kg may have a beneficial impact on immunity and growth performance in pigs.

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Institutional Review Board Statement: The study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals Monitoring Committee of Sichuan Province, China, and approved by the Sichuan Agricultural University Animal Ethical and Welfare Committee (Approval No. 20180021).

Data Availability Statement: The data is unavailable due to ethical restrictions.

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Conflicts of Interest: We declare that we have no financial and personal relationships with other people or organizations that can 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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