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

Qing Gao , Daiwen Chen , Xuemei Ding , Zhiwen Xu , Aimin Wu , Keying Zhang *

Posted Date: 30 October 2023

doi: 10.20944/preprints202310.1919.v1

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

Article

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

Qing Gao [1,2], Daiwen Chen [1], Xuemei Ding [1], Zhiwen Xu [3], Aimin Wu [1] and Keying Zhang [1,*]

[1] Animal Nutrition Institute, Key Laboratory for Animal Disease-Resistance Nutrition，Ministry of Education, Ministry of Agriculture and Rural Affairs, Key Laboratory of Sichuan Province, Sichuan Agricultural University, No.211. Huimin Road, Wenjiang District, Chengdu city, Sichuan, 611130, P. R. China.

[2] College of Animal Science, Xichang University, No.24. Xuefu Road, Xichang city, Sichuan, 615013, P. R. China.

[3] Animal Biotechnology Centre, Sichuan Agricultural University, No.211. Huimin Road, Wenjiang District, Chengdu city, Sichuan, 611130, P. R. China.

* Correspondence: Prof. Keying Zhang. Address: Animal Nutrition Institute,, Key Laboratory for Animal Disease-Resistance Nutrition，Ministry of Education, Ministry of Agriculture and Rural Affairs, Key Laboratory of Sichuan Province, Sichuan Agricultural University, No.211. Huimin Road, Wenjiang District, Chengdu city, Sichuan, 611130, P.R. China. Tel: +86-13981605686; Fax: +86-028-86290920; E-mail: zkeying@sicau.edu.cn

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 with 6 dietary FA levels at 0.35, 0.66, 2.19, 5.18, 7.51 or 10.81 mg/kg was conducted. 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 6 dietary groups (6 cages/group). Piglets were fed *ad libitum* for 24 days. Blood samples were collected on the 24th day. The growth performance and the 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 d to 45 d of age ($p < 0.01$), and with significant effects at 21 d to 45 d of age with FA 2.19 or 7.51 mg/kg ($p < 0.05$). Dietary FA at 0.66 or 2.19 mg/kg increased the serum $CD3^+CD8^+$ subset ($p < 0.05$), decreased the $CD3^+CD4^+$ / $CD3^+CD8^+$ ratio ($p < 0.05$); Moreover, FA addition increased the serum IFN-γ level ($p < 0.05$). In conclusion, the adequate dietary FA is necessary to improve the immunity and growth performance of weaned piglets at 21 d of age. The suitable dietary FA level for immune function from 21 d to 45 d of age was 0.66 mg/kg, while were 6.82 mg/kg from 21 d to 28 d of age and 5.42 mg/kg from 29 d to 45 d of age respectively for feed efficiency.

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

1. Introduction

Folates play a crucial role in various biological processes, including biosynthesis and epigenetic regulation, by providing one-carbon moieties (Sesay et al., 2017). As mammals, including pigs, are unable to synthesize folates, their metabolic needs must be primarily met through dietary sources (Wang et al. 2021). 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 (Pluske et al., 1997). Weaning can also affect the absorption of dietary folate, and research has shown that the folate status of piglets tends to decline after weaning (Liu et al., 2013).

Folate is generally believed to be essential for optimal immune function in pigs (Wang et al., 2020). However, it is currently unclear whether different levels of folate intake during weaning,

 doi:10.20944/preprints202310.1919.v1

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 (Grieshop et al., 1998). Although the metabolic demand for vitamins B12, folate, and niacin may be reduced in high-health animals, it is possible that rapidly growing animals may require greater amounts of these vitamins due to increased nucleotide synthesis in proliferating satellite muscle cells (Stahly et al., 2007). Therefore, we propose that dietary supplementation with folic acid 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 fatty acid (FA) supplementation at varying levels 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 Institutional Animal Care and Use 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 with an average initial body weight of 6.93 ± 0.07 kg. The sows had been fed the same diets containing recommended folate levels in gestation and lactation (NRC, 2012). The piglets were equally assigned into 36 cages with a similar initial body weight of 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 0.3 mg/kg was the level recommended by NRC (2012). FA supplied by Bayer Sichuan Animal Health Co., Ltd. The basal diet was prepared on the basis of corn-soybean meal to meet or exceed nutrient requirements for 5-10 kg piglets recommended by NRC (Table 1) with all other vitamins at 200% level of the NRC requirements.

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

Item	Basal diet
Ingredients	
Corn	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 [1]	0.30
Total	100.00

 doi:10.20944/preprints202310.1919.v1

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
DE [2], kcal/kg	3440
ME [3], kcal/kg	3285
Folate [4], mg/kg	0.33

[1] Supplied diets with (per kg diet): retinol acetate 8.8mg; cholecalciferol 880μg; dl-R-tocopheryl acetate 64mg; 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 ($FeSO4.7H_2O$); Zn 100 mg ($ZnSO4.7H_2O$); Cu 6 mg ($CuSO_4.5H_2O$); Mn 4 mg ($MnSO4.H_2O$); Se 0.3 mg (Na_2SeO_3); I 0.15 mg (KI); [2] DE = digestible energy; [3] ME = metabolic energy; [4] The measured value was 0.35 mg/kg. In the diets supplemented with folic acid, 0.3, 3.0, 6.0, 9.0 or 15.0 mg/kg folic acid were added at the expense of corn, and 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 ℃) and a cycle of 16 h light / 8 h dark were maintained in the mechanically ventilated room. Piglets were weighted on the beginning, the 7th and the 24th day of the study. The feed disappearance per cage and body weight (BW) was measured at 29 d and 45 d of age, and 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. Diarrhea index (DI) was defined as the total grades for fecal consistency of 3 piglets in each cage during different feeding period.

2.3. Sample collection

After fasting for 8 hours, blood sample was obtained from one piglet per cage by vena cava puncture in the morning on the 24th day (at 45 d of age of piglets). Samples of 5ml blood per piglet were centrifuged at 1000 × g for 15 min to obtain serum, which were stored at -80 ℃ until analysis 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 3ml heparinized blood per sample was centrifuged (4 ℃; 1000 × g, 5 min) to obtain precipitated cells, which were treated by 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 litre of distilled water) 1 min and centrifuged (4 ℃; 1000 × g, 5 min) again. After washing twice using cold RPMI-1640 (Sigma, USA), cells were resuspended with cold RPMI-1640 (10% FBS; Sigma, USA) and immediately dispersed mechanically by vortex mixing.

The phenotypes of lymphocyte subpopulations from peripheral blood were monitored by a standard FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA, USA) operated by CELLQuest software, using a panel of fluorescein (FITC), spectral red (SPRD) or phycoerythrin (PE)-labeled monoclonal antibodies (MABs) (South. Biotech. Assoc., Inc., Birmingham, AL, USA). The MABs includes mouse anti-porcine CD3ε-FITC (Lot F7207-N598), mouse anti-porcine CD4α-PE (Lot E4510-02), mouse anti-porcine CD8α-SPRD (Lot K0602-NB08z). The cells as $5*10^5$ per tube were incubated for 30 min with 10 μl of each MAB and after three times washes, the percentage of single-positive cells (such as $CD3^+$ subset is the percentage of cells single-positive to mouse anti-porcine

 doi:10.20944/preprints202310.1919.v1

CD3ε-FITC) or double-positive cells (such as $CD3^+CD4^+$ subset is the percentage of cells double-positive with both mouse anti-porcine CD3ε-FITC and mouse anti-porcine CD4α-PE, and $CD3^+CD8^+$ subset is the percentage of cells double-positive with both mouse anti-porcine CD3ε-FITC and mouse anti-porcine CD8α-SPRD in the peripheral lymphocyte) 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

Concentration of interleukin-4 (IL-4), tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) in serum were determined using swine ELISA kits (Rapid Bio. Lab, USA) for IL-4 (Lot 09150901), TNF-α (Lot 02060904) and IFN-γ (Lot 03080607). Following the instructions of the manufacturer, optical density 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) in were determined by a nitrite detection kit (Beyotime Biotech Inc., Jiangsu, PRC) 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, optical density was read in a microplate reader at 540 nm.

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

2.6. Microbiological assay for folic acid

The content of FA in diet or serum were measured by 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) with minor modification. Homogenized diet or serum sample at 1 ml was measured into a 50 ml sterile centrifuge vial, filled up to 40 ml with phosphate buffer (0.05 mol/l; 0.1% ascorbate; pH 7.2) for an extracting 30 min at 90-100 ℃ in a water bath shaking well, and chilled down quickly to room temperature.

To isolate the folate from serum protein, the extract of serum was added 10 mg of dry Pancreatin (P1750; Sigma, USA) and incubated for 24 hours at 37 ℃, and heated for 30 min in a water bath at 90-100 ℃, thereafter chilled down quickly to below 30 ℃. The sample extraction as 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 which 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 bacteria growth stopped until the vitamin was consumed (Molloy and Scott, 1997). 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 ℃ 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). 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. On the data, the correlation analysis and the regression analysis were also carried out using the procedure of SPSS 20.0 software. Quadratic effects on growth performance due to dietary FA levels were determined, and dietary FA levels needed to optimize feed efficiency or ADG were calculated respectively. 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.

 doi:10.20944/preprints202310.1919.v1

3. Results

3.1. Growth performance and diarrhoea 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 containing FA 2.19 mg/kg or 7.51 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 containing FA 2.19 mg/kg or 7.51 mg/kg had bigger 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 diarrhoea in weaned piglets [1] (n=6).

Item [2]	Feeding period [4]	Dietary folic acid level, mg/kg						SEM.	*p*-value
		0.35	0.66	2.19	5.18	7.51	10.81		
BW, kg	21 d	6.928	6.928	6.931	6.928	6.928	6.931	0.004	0.999
	28 d	7.017	7.078	7.061	7.072	7.319	7.156	0.039	0.275
	45 d	10.350	10.947	11.103	10.560	11.228	10.878	0.102	0.097
ADG, g	21 d to 28 d	12.7	21.7	18.8	20.7	56.0	32.0	5.53	0.256
	29 d to 45 d	196.1	227.6	236.3	200.0	229.9	219.0	4.99	0.097
	21 d to 45 d	142.6	167. 5	174.0	151.2	179.2	164.5	4.26	0.098
ADFI, g	21 d to 28 d	132.9	131.5	147.2	129.8	149.5	147.7	4.68	0.691
	29 d to 45 d	329.6	364.1	360.8	336.8	356.0	360.9	6.48	0.549
	21 d to 45 d	272.2	296.2	301.5	278.3	295.8	298.7	5.54	0.585
G/F, g/g	21 d to 28 d	0.068	0.106	0.102	0.104	0.370	0.217	0.040	0.245
	29 d to 45 d	0.595^{ab}	0.626^{bc}	0.654^{c}	0.591^{a}	0.645^{c}	0.607^{ab}	0.006	0.002
	21 d to 45 d	0.523^{a}	0.565^{b}	0.576^{bc}	0.540^{ab}	0.605^{c}	0.551^{ab}	0.006	0.000
DI [3]	21 d to 28 d	6.5	13.2	11.4	8.4	3.8	8.3	1.25	0.293
	29 d to 45 d	18.2	10.3	15.2	17.0	8.3	11.5	1.67	0.471
	21 d to 45 d	24.7	23.5	26.6	25.4	12.2	19.8	1.96	0.283

[1] Different superscript letters indicate significant difference at $p < 0.05$; [2] BW = Body weight, ADG = Average daily gain, ADFI = Average daily feed intake, G/F = Feed efficiency, and DI = Diarrhoea index; [3] Diarrhoea index (DI) was defined as the total grades for faecal consistency of 3 piglets per cage during different feeding period; [4] Feeding period is expressed by the days of age in weaned piglets.

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 containing FA 2.19 mg/kg or 7.51 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 diet containing FA 0.66 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 DI were found.

As showed in Table 3, the quadratic effects of dietary FA levels on the growth performance (including ADG from 21 d to 28 d of age, ADG from 21 d to 45 d of age, G/F from 21 d to 28 d of age, G/F from 29 d to 45 d of age, and G/F from 21 d to 45 d of age) were significant ($p < 0.05$). The dietary FA levels needed to optimize ADG or G/F were 6.58 or 6.82 mg/kg from 21 d to 28 d of age and 5.78 or 5.42 mg/kg from 29 d to 45 d of age.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 30 October 2023 doi:10.20944/preprints202310.1919.v1

Table 3. Quadratic regressions of performance index (Y) on dietary folic acid level as independent variables (X) (n=6).

Performance index [1]	Quadratic regression equation	R^2	P-value	Optimization [2]	
				X	Y
$ADG_{21-28\ d}$	$y = -1.021x^2 + 13.435x + 8.040$	0.48	0.035	6.58	52.2
$G/F_{21-28\ d}$	$y = -0.00679x^2 + 0.0926x + 0.0239$	0.49	0.029	6.82	0.340
$ADG_{29-45\ d}$	$y = -0.918x^2 + 10.619x + 208.540$	0.33	0.088	5.78	239.2
$G/F_{29-45\ d}$	$y = -0.00204x^2 + 0.0221x + 0.603$	0.61	0.002	5.42	0.663
$ADG_{21-45\ d}$	$y = -0.947x^2 + 11.441x + 150.027$	0.43	0.048	6.04	184.6
$G/F_{21-45\ d}$	$y = -0.00247x^2 + 0.0288x + 0.529$	0.72	0.000	5.83	0.613

[1] $ADG_{21-28\ d}$ = ADG from 21 d to 28 d of age, $ADG_{29-45\ d}$ = ADG from 29 d to 45 d of age, $ADG_{21-45\ d}$ = ADG from 21 d to 45 d of age, $G/F_{21-28\ d}$ = G/F from 21 d to 28 d of age, $G/F_{29-45\ d}$ = G/F from 29 d to 45 d of age, and $G/F_{21-45\ d}$ = G/F from 21 d to 45 d of age. [2] Dietary FA levels (X) needed to optimize the values of Y (ADG or G/F) were calculated respectively.

3.2. Folate status

As expected, FA supplementation increased the serum FA concentration ($p < 0.01$) in weanling piglets (Table 4). At 45 d of age, the control group had a lower serum FA concentration than other groups ($p < 0.01$); and the group fed diet containing FA 7.51 mg/kg had a higher serum FA concentration than that of the group fed diet containing FA 0.66 mg/kg ($p < 0.05$).

Table 4. Effects of dietary folic acid levels on serum levels of folic acid, cytokines, nitric oxide and immunoglobulins G [1, 2] (n=6).

Item	Dietary folic acid level, mg/kg						S.E.M.	*P*-value
	0.35	0.66	2.19	5.18	7.51	10.81		
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, $\mu mol.l^{-1}$	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 the blood samples at 45 d of age in piglets. [2] Different superscript small letters indicate significant difference 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.

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 piglet at 45 d of age, and the control group had a lower one than those of other groups ($p < 0.05$) (Table 4). The serum levels of IL-4, TNF-α, and NO were not significantly different among each group. 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 diet containing FA 7.51 mg/kg ($p < 0.05$) or 10. 81 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 $CD3^+CD8^+$ ($p < 0.1$) in weaned piglet at 45 d of age (Table 5). The group fed diet containing FA 0.66 mg/kg had a higher $CD3^+CD8^+$ subset than the control group or the group diet containing FA 5.18 mg/kg ($p < 0.05$), and the group fed diet containing FA 2.19 mg/kg had a higher $CD3^+CD8^+$ subset than the control group ($p < 0.05$) too. The control group had a higher ratio of $CD3^+CD4^+$ to $CD3^+CD8^+$

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 30 October 2023 doi:10.20944/preprints202310.1919.v1

than the groups diet containing FA 0.66 mg/kg ($p < 0.05$), or 2.19 mg/kg ($p < 0.05$), or 10.81 mg/kg ($p < 0.05$) at 45 d of age respectively.

Table 5. Effects of dietary folic acid levels on the peripheral lymphocyte subpopulation (n=6).

Item [1]	Dietary folic acid level，mg/kg						S.E.M.	*P*-value
	0.35	0.66	2.19	5.18	7.51	10.81		
$CD3^+$，%	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] $CD3^+$ subset is the percentage of cells single-positive to mouse anti-porcine CD3ε-FITC in the peripheral lymphocyte assessed by flow cytometry. $CD3^+CD4^+$ subset is the percentage of cells double-positive with both mouse anti-porcine CD3ε-FITC and mouse anti-porcine CD4*α*-PE in the peripheral lymphocyte assessed by flow cytometry. $CD3^+CD8^+$ subset is the percentage of cells double-positive with both mouse anti-porcine CD3ε-FITC and mouse anti-porcine CD8*α*-SPRD in the peripheral lymphocyte assessed by flow cytometry. .

4. Discussion

4.1. The effects of dietary supplementation with FA on the growth performance in weanling piglets

The observed values of dietary FA that 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 (Molloy and Scott, 1997; Guay et al., 2002). 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.

Easter et al. (1983) conducted a study on pigs fed a corn-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) reported improved daily weight gain in weanling piglets fed a corn-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, ADG of piglets from 21 to 45 days of age fed diets with FA at 2.19 or 7.51 mg/kg were significantly 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 status of intestinal microbiota (Wang et al., 2021).

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 decreased incidence of diarrhoea may have played a partial role in the improvement. Previous research conducted in South Africa showed that oral folate shortened the duration of diarrhoea in children (Haffejee, 1988). However, this result was not supported by another study, which found that folic acid was ineffective in the treatment of acute watery diarrhoea in children (Ashraf et al., 1998). In a study by Shoda et al. (2007), large-dose FA supplementation 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 diarrhoea and malnutrition induced by a lectin-based diet.

 doi:10.20944/preprints202310.1919.v1

Enterocytes are known to have a high rate of turnover, with their proliferation and apoptosis being sensitive to the status of FA (Bressenot et al., 2013). 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 diarrhoea and postweaning villus atrophy. The present study showed that the group fed a diet containing 10.81 mg/kg of FA had a lower incidence of diarrhoea than the group fed a diet containing 2.19 mg/kg of FA, indicating that dietary FA levels may play a role in the health of the intestinal tract. These findings further support the potential benefit of dietary FA supplementation for piglets in improving gut health and reducing the risk of intestinal diseases.

The present study revealed significant quadratic effects of dietary FA levels on the growth performance of weanling piglets. The results indicated that to optimize average daily gain (ADG) or feed efficiency, dietary FA levels of 6.58 or 6.82 mg/kg were needed from 21 d to 28 d of age, and 5.78 or 5.42 mg/kg were needed from 29 d to 45 d of age, respectively. Notably, these levels were higher than the current recommendation of 0.3 mg FA/kg diet for 5-10 kg piglets by NRC (2012). Results of our successive trials (data were not given) indicated that the level of FA supplemented into diet would be declined following with the growth of piglet.

4.2. The effects of dietary supplementation with FA on the 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 (Stepanova et al., 2007). Folate is essential for DNA synthesis and is involved in several reactions of amino acid metabolism (Guo et al., 2017). 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 (Hwang et al., 2018; Konrad et al., 2018). Courtemanche et al. (2004) 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 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 containing 0.66 mg/kg or 2.19 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 active proliferative cells such as lymphocytes may increase the FA requirements of weaning piglets (Elmadfa & Meyer, 2019). Therefore, it is necessary to supplement sufficient FA in 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 (Fangman et al., 1996; Williams et al., 1997). In the present study, the control group had a higher $CD3^+CD4^+$ and $CD3^+CD8^+$ ratio than those of the groups fed diets containing 0.66 mg/kg, 2.19 mg/kg, or 10.81 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 by Field et al. (2006) in 24-month-old rats that showed an increase in tissular IFN-γ levels of the spleen with dietary FA supplementation. Moreover, Field et al. (2006) also found that the folate-supplemented diet had an impact on the T-helper (Th)1/Th2-type response, as evidenced by the ratio of TNF-α to IL-4. 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 containing FA at 7.51 mg/kg or 10.81mg/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

 doi:10.20944/preprints202310.1919.v1

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 demonstrates that sufficient dietary FA is essential for both growth performance and immune function of weaned piglets. The results indicate that a dietary FA level of 0.66 mg/kg or more is needed from 21 to 45 days of age to ensure immune function. Moreover, to optimize feed efficiency, a dietary FA level of 6.82 mg/kg from 21 to 28 days of age and 5.42 mg/kg from 29 to 45 days of age is recommended. These findings have important implications for the development of optimal nutritional strategies for weaned piglets, as well as for the promotion of their health and well-being.

Author Contributions: Qing Gao designed and performed the trial, and wrote the original manuscript. **Daiwen Chen** contributed to methodology and reviewing. **Xuemei Ding** assisted with the data curation and analyses. **Aimin Wu** helped in modifying the manuscript. **Zhiwen Xu** helped in the sample collection and detection. **Keying Zhang** obtained funding and contributed to experimental design. All authors read and approved the submission of this manuscript.

Acknowledgments: This work was supported by the Program for Changjiang Scholars and Innovative Research Team in University (Project No. IRTO555-5) and the specific research supporting program for academic sustentation research team in Sichuan Agricultural University. We wish to acknowledge the kind assistance of **Ziqiang Zhang, Qiaobo Lei** and **Xiangqi Zhang** in the team.

Conflict 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.

References

Ashraf H, Rahman MM, Fuchs GJ, Mahalanabis D. 1998. Folic acid in the treatment of acute watery diarrhea in children: a double-blind, randomized, controlled trial. Acta Paediatrica. 87:1113-5. doi: 10.1080/080352598750031077

Bressenot A, Pooya S, Bossenmeyer-Pourie C, Gauchotte G, Germain A, Chevaux JB, et al. (2013). Methyl donor deficiency affects small-intestinal differentiation and barrier function in rats. Br J Nutr, 109(4), 667-77. doi:10.1017/S0007114512001869.

Courtemanche C, Elson-Schwab I, Mashiyama ST, Kerry N, Ames BN. 2004. Folate deficiency inhibits the proliferation of primary human $CD8^+$ T lymphocytes in vitro. J Immunol. 173: 3186-92. doi: 10.4049/jimmunol.173.5.3186

Easter RA, Anderson PA, Michel EJ, Corley JR. 1983. Response of gestating gilts and starter, grower and finisher swine to biotin, pyridoxine, folacin and thiamine additions to a corn-soybean meal diet. Nutr Rep Int. 28: 945–53.

Elmadfa I., & Meyer AL. 2019. The Role of the Status of Selected Micronutrients in Shaping the Immune Function. Endocr Metab Immune Disord Drug Targets, 19(8), 1100-15. doi:10.2174/1871530319666190529101816.

Fangman TJ, Tubbs RC, Becker BA, Allee GL, Misfeldt ML, Henningsen-Dyer K. 1996. Evaluation of segregated early weaning investigating performance, immunologic indicators (CD4, CD8), and herd health status. J Swine Health Prod. 4: 217-22.

Field CJ, Van Aerde A, Drager KL, Goruk S, Basu T. 2006. Dietary folate improves age-related decreases in lymphocyte function. J Nutr Bioch. 17: 37-44. doi: 10.1016/j.jnutbio.2005.04.010.

Grieshop CM, Stahly TS, Ewan RC, Nonnecke BJ, Cunnick JE. 1998. Effect of gestational folic acid supplementation on offspring immune organ development and postnatal immune response. ISU Swine Research Report, ASLR1562. pp. 35-37.

Guay F, Matte JJ, Girard CL, Palin MF, Giguère A andLaforest JP. 2002. Effect of folic acid and glycine supplementation on embryo development and folate metabolism during early pregnancy in pigs. J Anim Sci. 80: 2134-43. doi: 10.1093/ansci/80.8.2134

Guo X, Ni J, Zhu Y, Zhou T, Ma X, Xue J et al. (2017). Folate deficiency induces mitotic aberrations and chromosomal instability by compromising the spindle assembly checkpoint in cultured human colon cells. Mutagenesis, 32(6), 547-60. doi:10.1093/mutage/gex030.

Haffejee IE. 1988. Effect of oral folate on duration of acute infantile diarrhoea. Lancet. 2: 334-5. doi: 10.1016/s0140-6736(88)92384-7.

Huang RF, Ho YH, Lin HL, Wei JS, Liu TZ. 1999. Folate deficiency induces a cell cycle-specific apoptosis in HepG2 cells. J Nutr. 129: 25-31. doi: 10.1038/sj.ijo. 0800766

Hwang SY, Kang YJ, Sung B, Jang JY, Hwang NL, Oh HJ, et al. (2018). Folic acid is necessary for proliferation and differentiation of C2C12 myoblasts. J Cell Physiol, 233(2), 736-47. doi: 10.1002/jcp.25989

Konrad KD, Campbell RA, Thiel V, Sullivan-Brown J. 2018. The folic acid metabolism gene mel-32/Shmt is required for normal cell cycle lengths in Caenorhabditis elegans. Int J Dev Biol, 62(9-10), 641-5. doi:10.1387/ijdb.180023js

Lindemann MD, Kornegay ET. 1986. Folic acid additions to weanling pig diets. J Anim Sci. 63(Suppl.1): 35 (Abstr.).

Liu J, Yao Y, Yu B, Mao X, Huang Z, Chen DW. (2013). Effect of maternal folic acid supplementation on hepatic proteome in newborn piglets. Nutrition, 29(1), 230-4. doi:10.1016/j.nut.2012.08.001.

Molloy AM, Scott JM. 1997. Microbiological assay for serum, plasma, and red cell folate using cryopreserved, microtiter plate method. Methods Enzymol. 281: 43-53. doi: 10.1016/s0076-6879(97)81007-5.

NRC (National Research Council). 2012. Nutrient Requirements of Swine, Eleventh Revised Edition; National Academic Press: Washington, DC. 20418 USA.

Pluske JR, Hampson DJ, Williams IH. 1997. Factors influencing the structure and function of the small intestine in the weaned pig: a review. Livest Prod Sci. 51: 215-36. doi: 10.1016/S0301-6226(97)00057-2

Sesay DF, Habte-Tsion HM, Zhou Q, Ren M, Xie J, Liu B. et al. 2017. The effect of dietary folic acid on biochemical parameters and gene expression of three heat shock proteins (HSPs) of blunt snout bream (Megalobrama amblycephala) fingerling under acute high temperature stress. Fish Physiol Biochem, 43(4), 923-40. doi: 10.1007/s10695-016-0311-6.

Shoda R, Mahalanabis D, Islam KN, Wahed MA, Albert MJ. 2007. Folic acid supplementation on red kidney bean-induced diarrhoea and enteric bacterial translocation into mesenteric lymph nodes in rats: a pilot study. Acta Pædiatrica. 91: 51-4. doi: 10.1111/j.1651-2227.2002.tb01639.x

Stahly TS, Williams NH, Lutz TR, Ewan RC, Swenson SG. 2007. Dietary B vitamin needs of strains of pigs with high and moderate lean growth. J Anim Sci. 85: 188-95. doi:10.2527/jas.2006-086

Stepanova H, Samankova P, Leva L, Sinkora J, Faldyna M. 2007. Early postnatal development of the immune system in piglets: The redistribution of T lymphocyte subsets. Cell Immunol. 249: 73–9. doi:10.1016/j.cellimm.2007.11.007

Wang D, Lindemann MD, Estienne MJ. 2020. Effect of Folic Acid Supplementation and Dietary Protein Level on Growth Performance, Serum Chemistry and Immune Response in Weanling Piglets Fed Differing Concentrations of Aflatoxin. Toxins (Basel), 12(10): 651. doi:10.3390/toxins12100651.

Wang L, Zou L, Li J, Yang H, Yin Y. 2021. Effect of dietary folate level on organ weight, digesta pH, short-chain fatty acid concentration, and intestinal microbiota of weaned piglets. J Anim Sci, 99(1):1-9. doi:10.1093/jas/skab015.

Williams NH, Stahly TS, Zimmerman DR. 1997. Effect of chronic immune system activation on rate, efficiency and composition of growth and lysine needs of pigs fed from 6 to 27 kg. J Anim Sci. 75: 2463–71.