Effects of pyrroloquinoline quinone supplementation on growth performance and small intestine characteristics in weaned pigs^{1,2}

Xindi Yin,[†] Dongxu Ming,[†] Lili Bai,[†] Fei Wu,[†] Hu Liu,[†] Yifan Chen,[†] Linlin Sun,[‡] Yidong Wan,[†]
Philip Alfred Thacker,[§] Guoyao Wu,^{†,¶,•} and Fenglai Wang^{†,3}

[†]State Key Laboratory of Animal Nutrition, College of Animal Science and Technology, China Agricultural University, Beijing, 100193, China; [‡]Shanghai Medical Life Science Research Center Company, Shanghai, 200032, China; [§]Changmao Biochemical Engineering Company, Changzhou, 213000, China; [¶]Department of Animal Science, University of Saskatchewan, Saskatoon, SK S7N 5A2, Canada; and [•]Department of Animal Science, Texas A&M University, College Station, TX 77843

ABSTRACT: This study was conducted to explore the effect of graded levels of pyrroloquinoline quinone disodium (PQQ·Na₂) on the performance and intestinal development of weaned pigs. A total of 216 pigs weaned at 28 d were assigned in a randomized complete block design to 6 diets containing 0, 1.5, 3.0, 4.5, 6.0, or 7.5 mg/kg PQQ·Na₂ for 28 d. Performance, diarrhea incidence, intestinal morphology, redox status, cytokines, and the expression of tight junction proteins were determined. Pigs had increased ADG (linear, $P < 0.01$), G:F (quadratic, $P < 0.01$), and lower diarrhea incidence ($P < 0.01$) with the increase of PQQ·Na₂ supplementation. Villus height increased (quadratic, $P < 0.01$) in all segments of the small intestine, and crypt depth in the duodenum and jejunum was decreased (linear, $P < 0.05$) in pigs with the increase of PQQ·Na₂ supplementation. Pigs fed PQQ·Na₂-supplemented diets had higher ($P < 0.05$) activities of antioxidant enzymes including total superoxide dismutase

in duodenum, jejunum, and ileum; glutathione peroxidase (GSH-Px) in jejunum and ileum; catalase (CAT) in duodenum and ileum; and lower ($P < 0.05$) malondialdehyde concentrations in the intestinal mucosa of all segments. In the intestinal mucosa, cytokines including interleukin (IL)-1 β , IL-2, and interferon- γ were significantly decreased ($P < 0.05$) in pigs fed PQQ·Na₂-supplemented diets. The protein expression of zonula occludens protein-1 (ZO-1) and occludin in the jejunum was significantly increased ($P < 0.05$) in pigs fed diets containing PQQ·Na₂. In conclusion, these results have indicated that dietary PQQ·Na₂ supplementation improves growth performance and gut health in weaned pigs. Moreover, pigs fed diet with as low as 1.5-mg/kg PQQ·Na₂ have better performance compared with pigs fed no PQQ·Na₂-supplemented diet; pigs fed diet with 4.5-mg/kg PQQ·Na₂ have highest G:F among treatments during the whole period.

Key words: growth performance, intestinal morphology, pyrroloquinoline quinone, redox status, weaned pigs

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³Corresponding author: wangfl@cau.edu.cn

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INTRODUCTION

Weaning is a critical stage affecting pig growth and development due to alterations in the gastrointestinal tract including changes in intestinal morphology, the immune system, and barrier function (Pluske et al., 1997; Hu et al., 2013). Pigs are often challenged by postweaning stresses, which can result in poor performance and an increased incidence of diarrhea (Madec and Josse, 1983; Madec et al., 1998; Heo et al., 2013). Therefore, it is necessary to identify possible nutritional mitigation strategies to control the negative effects of postweaning stress.

Pyrroloquinoline quinone (PQQ) was initially recognized as a redox cofactor of dehydrogenases in bacteria (Hauge, 1964). It was first identified as a possible growth factor in mice (Killgore et al., 1989). It improved the reproduction performance and stimulated neonatal growth when more than 0.3-mg PQQ/kg diet was added to purified diets in rodent models (Steinberg et al., 1994, 2003). Its deprivation results in defects in the immune function of mice (Steinberg et al., 1994). Dietary supplementation of 0.2-mg PQQ disodium (PQQ·Na₂)/kg diet also enhanced performance and carcass yield of broiler chicks (Samuel et al., 2015; Wang et al., 2015a). Studies showed that PQQ can serve as a powerful antioxidant in vivo and in vitro in many models through modulating cellular redox status and cell signaling pathways (He et al., 2003; Tao et al., 2007; Harris et al., 2013). PQQ-containing products have been authorized as a dietary supplement for human health benefit in some countries (Health Canada, 2012). The optimal dosage of PQQ to be taken daily is currently not known. Studies suggested that actual daily dietary consumption amount of PQQ and its derivatives is 1 to 2 mg for human and this amount is in the range that clearly influences optimization of growth and health in animal models (Stites et al., 2000; Rucker et al., 2009).

The important roles of PQQ in mammalian growth, development, reproduction, and immune function have attracted considerable attention. However, little is known about the effects of dietary PQQ supplementation on the growth performance and health in pigs. The characteristics of PQQ in growth promotion and redox status regulation can make it a potential feed supplement in swine industry. Therefore, our objectives were to explore the influence of PQQ·Na₂ on the performance, intestinal morphology, and redox status of weaned pigs and to determine the recommended level of supplementation in the diet.

MATERIALS AND METHODS

The protocol employed in this trial was approved by the China Agricultural University Animal Care and Use Committee (Beijing, China).

Pigs and Experimental Protocol

The current study with weaned pigs included 6 treatments standing for 6 experimental diets. A total of 216 pigs (Duroc × Landrace × Yorkshire; BW = 8.01 ± 1.14 kg) weaned at 28 d were blocked based on initial body weight and sex and then distributed to pens and treatments in a completely randomized block design. Each one of 6 treatments in this study consisted of 6 replicate pens with 6 pigs per pen. The proportion of barrows to gilts was equal in each pen. Pigs were fed isocaloric and isonitrogenous diets formulated to meet or exceed NRC (2012) nutrient requirements and did not contain any in-diet antibiotics. Six experimental diets were formulated based on corn and soybean meal supplemented with 0, 1.5, 3.0, 4.5, 6.0, or 7.5 mg/kg PQQ·Na₂, which replaced corn in the diet (Table 1). The PQQ·Na₂ (purity, ≥ 98%) was synthesized by chemical reactions and donated by the Changmao Biochemical Engineering Company (Changzhou, China). It was diluted with corn starch to a concentration of 1 g/kg mixture before being mixed into the diet. Based on the known range of PQQ in foods (Noji et al., 2007), we infer that the concentration of PQQ in the basal diet is less than 0.01 mg/kg.

Pigs were housed in a temperature-controlled nursery room and had ad libitum access to diet and water for 28 d. Health status, diarrhea incidence, and mortality of pigs were recorded every day. The severity of diarrhea was measured using a fecal consistency scoring system. Fecal consistency was visually assessed each day by observers who were blind to the treatments during 0900 to 1000 h. Fresh excreta were graded using the following scale: 0 = solid, 1 = semisolid, 2 = semiliquid, and 3 = liquid. The occurrence of diarrhea was defined as production of grade 2 or 3 feces for 2 continuous days. Growth performance was measured based on ADG, ADFI, and G:F on 0, 14, and 28 d.

Sample Collection and Processing

At the end of this trial, 4 pens were randomly selected from each treatment. Two barrows and 2 gilts with an average BW in each pen were selected for sacrifice. Pigs were euthanized for tissue sampling by intracardiac administration of sodium

Table 1. Ingredient and formulated dietary nutrient levels of the experimental diets (as-fed basis)

Item	PQQ·Na ₂ ¹ , mg/kg diet					
	0	1.5	3.0	4.5	6.0	7.5
Ingredients, %						
Corn	59.83	59.68	59.53	59.38	59.23	59.08
Soybean meal	14.00	14.00	14.00	14.00	14.00	14.00
Extruded soybean	14.30	14.30	14.30	14.30	14.30	14.30
Fish meal	3.00	3.00	3.00	3.00	3.00	3.00
Dried whey	4.00	4.00	4.00	4.00	4.00	4.00
Limestone	0.35	0.35	0.35	0.35	0.35	0.35
Dicalcium phosphate	1.80	1.80	1.80	1.80	1.80	1.80
Salt	0.34	0.34	0.34	0.34	0.34	0.34
L-Lysine·HCl	0.50	0.50	0.50	0.50	0.50	0.50
L-Threonine	0.18	0.18	0.18	0.18	0.18	0.18
L-Tryptophan	0.05	0.05	0.05	0.05	0.05	0.05
L-Methionine ²	0.15	0.15	0.15	0.15	0.15	0.15
Glucose	0.50	0.50	0.50	0.50	0.50	0.50
PQQ·Na ₂ premix ³	0.00	0.15	0.30	0.45	0.60	0.75
Vitamin and mineral premix ⁴	1.00	1.00	1.00	1.00	1.00	1.00
Nutrient levels ⁵ , %						
Digestible energy, MJ/kg	14.30	14.30	14.30	14.30	14.30	14.30
Metabolizable energy, MJ/kg	13.13	13.13	13.13	13.13	13.13	13.13
Crude protein	18.19	19.09	19.20	19.14	19.18	18.47
Lysine	1.31	1.39	1.39	1.42	1.39	1.39
Methionine	0.42	0.42	0.44	0.44	0.44	0.42
Threonine	0.82	0.87	0.87	0.86	0.84	0.84
Tryptophan	0.24	0.24	0.25	0.24	0.24	0.24
Calcium	0.71	0.71	0.68	0.68	0.68	0.67
Total phosphorus	0.64	0.64	0.64	0.63	0.63	0.63

¹PQQ·Na₂ = pyrroloquinoline quinone disodium.

²Methionine was provided by Novus International (St. Louis, USA).

³PQQ·Na₂ was diluted with corn starch to a concentration of 1.0 g/kg mixture.

⁴Provided the following vitamins and minerals per kg of diet: vitamin A, 11,000 IU as retinyl acetate; vitamin D₃, 1,500 IU as cholecalciferol; vitamin E, 44.1 IU as DL- α -tocopherol acetate; vitamin K₃, 4 mg as menadione; vitamin B₁, 1.4 mg; vitamin B₂, 5.2 mg; vitamin B₆, 20 mg; vitamin B₁₂, 10 μ g; niacin, 26 mg; pantothenic acid, 14 mg; folic acid, 0.8 mg; biotin, 44 μ g; Fe, 100 mg from FeSO₄; Cu, 16.5 mg from CuSO₄·5H₂O; Zn, 90 mg from ZnO; Mn, 35 mg from MnSO₄; I, 0.3 mg from KI; Se, 0.3 mg from Na₂SeO₃.

⁵Digestible and metabolizable energy are calculated values. Other nutrient levels in the table are analyzed values.

pentobarbital (50-mg/kg BW) and jugular exsanguination. Tissue samples from the middle of duodenum, jejunum, and ileum were harvested as follows: tissues were rinsed several times with ice-cold phosphate-buffered saline containing 0.86% NaCl and divided into 2 sections. One segment (approximately 1 to 2 cm) was fixed with 4% formaldehyde-phosphate buffer and kept at 4 °C for a microscopic assessment of mucosal morphology, whereas the other was used for collecting mucosa. The mucosa cell layer was scraped off to fill up the 1.5-mL Eppendorf tubes and rapidly frozen in liquid N₂. For sample processing, briefly, tissues were minced and homogenized (10% wt/vol) in ice-cold sodium-potassium phosphate buffer (0.01 M, pH 7.4) containing 0.86% NaCl. The homogenate was centrifuged at 3,000 $\times$ g for

10 min at 4 °C, and the resultant supernatants were collected for later measurements of intestinal mucosal redox status, cytokines concentration, and protein abundance.

Measurement of Intestinal Morphology

Villus height and crypt depth in the duodenum, jejunum, and ileum were determined. Cross sections of intestinal samples were fixed in 4% paraformaldehyde for 24 h and then embedded in paraffin wax. Sections of 4 μ m were cut and stained with hematoxylin and eosin. In each cross section of tissue, at least 6 complete villous-crypt structures were examined under a microscope, and villous height and crypt depth were measured and analyzed using the Image Pro-Plus 6.0 software (Media Cybernetics, Rockville, MD).

Assay of Intestinal Mucosal Redox Status and Cytokines

Antioxidant capacities, including total superoxide dismutase (**T-SOD**), glutathione peroxidase (**GSH-Px**), catalase (**CAT**), and malondialdehyde (**MDA**) in the small intestinal mucosa, were determined using assay kits according to the manufacturer instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). SOD activity was detected by monitoring the inhibition of nitro blue tetrazolium reduction, whereas GSH-Px activity was measured with 5,5'-dithiobis (*p*-nitrobenzoic acid), and the change in absorbance at 412 nm was recorded. CAT activity was measured using ammonium molybdate methods by incubating samples with H_2O_2 . The MDA level was analyzed with 2-thiobarbituric acid, and the change in absorbance was read at 532 nm. A bicinchoninic acid (**BCA**) protein assay kit was used to determine the total protein concentration of all samples (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Cytokines including interleukin (IL)-1 β , IL-2, tumor necrosis factor (**TNF**)- α , and interferon (**INF**)- γ were determined in the small intestinal mucosa using assay kits according to the manufacturer's instructions. The assay kits for IL-1 β , IL-2, and TNF- α were purchased from the Tianjin Jiuding Medical and Biological Engineering Company (Tianjin, China). The assay kit for INF- γ was purchased from the eBioscience Company (California, USA).

Immunoblot Analysis of Intestinal Mucosal Tight Junction Proteins

The total protein contained in the jejunal mucosa samples of pigs fed 0, 1.5, 4.5, or 7.5 mg/kg PQQ·Na₂ was extracted according to the method described by a ProteoJET Total Protein Extraction Kit (Fermentas, Glen Burnie, MD). A BCA protein assay kit was used to determine the total protein concentration. Proteins in the supernatant fluids were separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to polyvinylidene fluoride membranes at 4 °C. The blots were blocked at room temperature for 1 h in 5% nonfat milk and tris buffered saline-tween and then incubated with rabbit anti-claudin-1, anti-occludin, anti-ZO-1, and anti- β -actin (Santa Cruz Biotechnology Inc., CA) over night at 4 °C. Membranes were washed 3 times in TBS-T and then incubated with the corresponding IRDyeTM 800-conjugated secondary antibodies

(LI-COR Bioscience, Lincoln, NE) in the dark for 1 h at room temperature. Following another wash with TBS-T and PBS, the electro-chemi-luminescence plus Western Blotting Detection System Kit (Amersham, Piscataway, NJ) was used to visualize the protein bands according to the manufacturer's instructions. X-ray films were developed using an SRX-101A Film Processor (Konica, Japan), and images of the resulting X-ray films were analyzed using Image Pro-Plus 6.0 software (Media Cybernetics, Rockville, MD).

Statistical Analysis

All data except for diarrhea incidence were subjected to MIXED procedure and polynomial contrasts of SAS 9.2 (SAS Inst. Inc., Cary, NC). Differences in diarrhea incidence among treatments were tested by the procedure GLIMMIX. Initial BW and sex were considered random effects, and the dietary PQQ·Na₂ level was considered a fixed effect. The pen or an individual pig was experimental units for the analysis of performance data and other parameters, respectively. Statistical differences among mean values were assessed using Duncan's multiple range test. Data are presented as means $\pm$ SEM and considered statistically significant if $P \leq 0.05$.

RESULTS

Performance and Diarrhea Incidence

During days 0 to 14, pigs had increased ADG (linear, $P < 0.01$) and G:F (quadratic, $P < 0.01$) with the increase of PQQ·Na₂ supplementation from 1.5 mg/kg (Table 2). No significant differences were observed for ADFI among each treatment groups ($P = 0.174$). During days 15 to 28, pigs fed diets supplemented with increasing PQQ·Na₂ had greater ADG (linear, $P < 0.01$) and G:F (linear, $P < 0.01$), but ADFI did not differ ($P > 0.05$) among the 6 groups of pigs. During days 0 to 28, pigs had increased ADG (linear, $P < 0.01$), ADFI (quadratic, $P = 0.046$), and G:F (quadratic, $P < 0.01$) with the increasing PQQ·Na₂ supplementation (Table 2).

As shown in Table 3, pigs fed diets supplemented with 1.5, 3.0, 4.5, 6.0, or 7.5 mg/kg PQQ·Na₂ had decreased diarrhea incidence ($P < 0.01$) than pigs fed diets supplemented with 0-mg/kg PQQ·Na₂ during days 0 to 14. During days 15 to 28, diarrhea incidence did not differ ($P > 0.05$) among the 6 groups of pigs. For the whole period, pigs fed diets supplemented with 1.5, 3.0, 4.5, 6.0, or 7.5 mg/kg PQQ·Na₂ had decreased diarrhea

Table 2. Performance of weaned pigs fed graded levels of PQQ·Na₂¹ for 28 d²

Item	PQQ·Na ₂ , mg/kg diet						SEM	P value		
	0	1.5	3.0	4.5	6.0	7.5		ANOVA	Linear	Quadratic
days 0 to 14										
ADG, ³ g/d	252 ^c	284 ^{ab}	262 ^{bc}	289 ^a	285 ^{ab}	290 ^a	8	0.013	0.005	0.453
ADFI, ³ g/d	393	403	382	400	407	434	13	0.174	0.054	0.134
G:F ³	0.64 ^b	0.71 ^a	0.69 ^{ab}	0.72 ^a	0.70 ^a	0.67 ^{ab}	0.02	0.017	0.223	0.002
days 15 to 28										
ADG, g/d	424 ^b	429 ^b	442 ^b	482 ^a	481 ^a	481 ^{ab}	9	<0.001	<0.001	0.329
ADFI, g/d	780	728	739	765	793	770	24	0.316	0.364	0.302
G:F	0.54 ^a	0.59 ^b	0.60 ^b	0.63 ^b	0.61 ^b	0.63 ^b	0.01	<0.001	<0.001	0.015
days 0 to 28										
ADG, g/d	336 ^c	355 ^b	350 ^{bc}	383 ^a	380 ^a	383 ^a	5	<0.001	<0.001	0.111
ADFI, g/d	582	561	557	578	594	598	12	0.055	0.032	0.046
G:F	0.58 ^c	0.63 ^b	0.63 ^b	0.67 ^a	0.64 ^{ab}	0.64 ^{ab}	0.01	<0.001	<0.001	<0.001

¹PQQ·Na₂ = pyrroloquinoline quinone disodium.

²Values are means of 6 pens (6 pigs/pen, experiment unit was the pen) and pooled SEM, *n* = 6. Means within a row without common superscripts differ significantly (*P* < 0.05).

³ADG = average daily gain; ADFI = average daily intake; G:F = feed efficiency (gain:feed).

Table 3. Diarrhea incidence of weaned pigs fed graded levels of PQQ·Na₂¹ for 28 d²

Item	PQQ·Na ₂ , mg/kg diet						SEM	P value
	0	1.5	3	4.5	6	7.5		
Diarrhea incidence, ³ %								
days 0 to 14	23.21 ^a	11.51 ^c	14.88 ^{bc}	13.69 ^{bc}	16.27 ^b	17.06 ^b	0.74	0.001
days 15 to 28	7.54	3.57	5.36	3.57	5.36	4.37	0.52	0.073
days 0 to 28	15.38 ^a	7.54 ^c	10.12 ^{bc}	8.63 ^{bc}	10.81 ^b	10.71 ^b	0.5	<0.001

¹PQQ·Na₂ = pyrroloquinoline quinone disodium.

²Values are means of 6 pens (6 pigs/pen, experiment unit was the pen) and pooled SEM, *n* = 6. Means within a row without common superscripts differ significantly (*P* < 0.05).

³Number of pigs with diarrhea in each pen × diarrhea days/(total number of pigs × 28 d) × 100.

incidence (*P* < 0.01) than pigs fed diets supplemented with 0-mg/kg PQQ·Na₂.

For the estimation of optimal level of dietary PQQ·Na₂, the comparison among treatments showed that pigs fed diet with as low as 1.5 mg/kg PQQ·Na₂ have significantly better performance compared with pigs fed no PQQ·Na₂ diet; the optimal level of dietary PQQ·Na₂ was 4.5 mg/kg for highest G:F during the whole period.

Villous Morphology in the Small Intestine

With the increase of PQQ·Na₂ supplementation, villus height and villus height:crypt depth increased (quadratic, *P* < 0.01) in all segments of the small intestine, and the crypt depth in the duodenum and jejunum was decreased (linear, *P* < 0.05) in pigs (Table 4).

Redox Status and Cytokines

Pigs had increased activities of T-SOD (quadratic, *P* < 0.05) and CAT (quadratic, *P* < 0.01),

and decreased concentration of MDA (quadratic, *P* < 0.01) in the duodenum with the increase of PQQ·Na₂ supplementation (Table 5). The activities of T-SOD and GSH-Px were increased (quadratic, *P* < 0.05) and the concentration of MDA was decreased (quadratic, *P* < 0.01) in the jejunum in response to the increase of PQQ·Na₂ supplementation. In the ileum, pigs had increased activities of T-SOD (quadratic, *P* < 0.05), GSH-Px (linear, *P* < 0.01), and CAT (quadratic, *P* < 0.01), and the concentration of MDA was decreased (quadratic, *P* < 0.01) with the increasing PQQ·Na₂ supplementation.

In the duodenum, the concentration of IFN-γ was decreased (quadratic, *P* < 0.05) in pigs fed with increasing PQQ·Na₂ supplementation (Table 6), and no difference was observed in the concentration of TNF-α, IL-1β, and IL-2. In the jejunum, the concentrations of IFN-γ, IL-1β, and IL-2 were decreased (quadratic, *P* < 0.01) in pigs as the PQQ·Na₂ supplementation was increased while no significant change in TNF-α concentration. No

Table 4. Small intestinal morphology of weaned pigs fed graded levels of PQQ·Na₂¹ for 28 d²

Item	PQQ·Na ₂ , mg/kg diet						SEM	P value		
	0	1.5	3.0	4.5	6.0	7.5		ANOVA	Linear	Quadratic
Duodenum										
Villus height, μm	305 ^b	381 ^a	388 ^a	396 ^a	373 ^a	389 ^a	11	<0.001	<0.001	0.001
Crypt depth, μm	103 ^a	102 ^a	90 ^b	92 ^b	91 ^b	93 ^b	2	0.004	0.002	0.021
Villus height: crypt depth	2.96 ^c	3.75 ^b	4.31 ^a	4.33 ^a	4.08 ^{ab}	4.20 ^{ab}	0.14	<0.001	<0.001	<0.001
Jejunum										
Villus height, μm	300 ^c	345 ^b	357 ^{ab}	364 ^a	348 ^b	349 ^b	5	<0.001	<0.001	<0.001
Crypt depth, μm	106 ^a	101 ^{ab}	106 ^a	97 ^b	97 ^b	99 ^{ab}	2	0.045	0.013	0.589
Villus height: crypt depth	2.85 ^c	3.40 ^b	3.36 ^b	3.76 ^a	3.61 ^{ab}	3.55 ^{ab}	0.10	<0.001	<0.001	0.002
Ileum										
Villus height, μm	308 ^b	365 ^a	366 ^a	376 ^a	363 ^a	375 ^a	8	<0.001	<0.001	0.001
Crypt depth, μm	100	103	98	96	91	99	2	0.054	0.055	0.387
Villus height: crypt depth	3.11 ^c	3.53 ^b	3.73 ^{ab}	3.91 ^a	4.01 ^a	3.80 ^{ab}	0.10	<0.001	<0.001	0.001

¹PQQ·Na₂ = pyrroloquinoline quinone disodium.²Values are means of 4 pigs in each treatment and pooled SEM, *n* = 4. Means within a row without common superscripts differ significantly (*P* < 0.05).**Table 5.** Antioxidant indexes in the small intestinal mucosa of weaned pigs fed graded levels of PQQ·Na₂¹ for 28 d²

Item	PQQ·Na ₂ , mg/kg diet						SEM	P value		
	0	1.5	3.0	4.5	6.0	7.5		ANOVA	Linear	Quadratic
Duodenum										
T-SOD ³ , U/mg protein	73.5 ^b	92.9 ^a	89.2 ^a	94.0 ^a	92.1 ^a	92.3 ^a	3.5	0.007	0.005	0.014
GSH-Px ⁴ , U/mg protein	362	414	410	410	421	425	14	0.076	0.013	0.197
CAT ⁵ , U/mg protein	16.1 ^b	20.4 ^a	21.6 ^a	20.9 ^a	21.9 ^a	20.9 ^a	0.8	<0.001	<0.001	<0.001
MDA ⁶ , nmol/mg protein	8.25 ^a	5.30 ^b	5.60 ^b	5.37 ^b	5.91 ^b	6.12 ^b	0.41	0.002	0.023	<0.001
Jejunum										
T-SOD, U/mg protein	73.7 ^b	97.9 ^a	90.4 ^a	100.3 ^a	96.6 ^a	99.3 ^a	3.3	<0.001	<0.001	0.013
GSH-Px, U/mg protein	373 ^b	429 ^a	462 ^a	421 ^a	459 ^a	433 ^a	17	0.013	0.018	0.015
CAT, U/mg protein	19.5	20.6	20.8	21.0	20.4	21.0	0.5	0.238	0.080	0.199
MDA, nmol/mg protein	6.05 ^a	4.42 ^b	4.43 ^b	4.81 ^b	4.91 ^b	4.92 ^b	0.25	0.003	0.107	0.003
Ileum										
T-SOD, U/mg protein	85.5 ^b	95.2 ^a	101.4 ^a	101.7 ^a	96.0 ^a	101.0 ^a	3.0	0.007	0.006	0.020
GSH-Px, U/mg protein	352 ^b	408 ^a	402 ^a	417 ^a	426 ^a	429 ^a	12	0.005	<0.001	0.082
CAT, U/mg protein	15.1 ^c	18.0 ^b	16.4 ^{bc}	21.6 ^a	17.9 ^b	18.0 ^b	0.7	<0.001	0.002	0.001
MDA, nmol/mg protein	6.38 ^a	4.87 ^b	4.70 ^b	4.48 ^b	5.02 ^b	5.15 ^b	0.29	0.002	0.015	<0.001

¹PQQ·Na₂ = pyrroloquinoline quinone disodium.²Values are means of 4 pigs in each treatment and pooled SEM, *n* = 4. Means within a row without common superscripts differ significantly (*P* < 0.05).³T-SOD = total superoxide dismutase.⁴GSH-Px = glutathione peroxidase.⁵CAT = catalase.⁶MDA = malondialdehyde.

difference was observed in the concentration of cytokines in the ileum among the treatments.

of PQQ·Na₂. No difference was observed in the expression of claudin-1 among the treatments.

Intestinal Tight Junction Proteins

As shown in Table 7 and Figure 1, the expression of the jejunal tight junction protein ZO-1 (quadratic, *P* < 0.05) and occludin (quadratic, *P* < 0.01) was significantly higher in pigs with the increase

DISCUSSION

Pyrroloquinoline quinone has been proved to be a novel growth factor in animals (Killgore et al., 1989). In rodent models, dietary PQQ deprivation can impair normal development, immune

Table 6. Inflammation indexes in the small intestinal mucosa of weaned pigs fed graded levels of PQQ·Na₂¹ for 28 d²

Item	PQQ·Na ₂ , mg/kg diet							P value		
	0	1.5	3.0	4.5	6.0	7.5	SEM	ANOVA	Linear	Quadratic
Duodenum										
TNF-α ³ , ng/mg protein	2.56	2.28	2.38	2.26	2.41	2.21	0.26	0.940	0.521	0.818
IFN-γ ⁴ , pg/mg protein	51.2 ^a	41.8 ^b	41.4 ^b	44.3 ^b	42.9 ^b	44.1 ^b	1.9	0.024	0.091	0.014
IL-1β ⁵ , ng/mg protein	0.23	0.22	0.25	0.25	0.20	0.22	0.02	0.411	0.491	0.361
IL-2 ⁶ , ng/mg protein	4.89	4.00	4.53	3.36	3.78	4.02	0.42	0.213	0.116	0.222
Jejunum										
TNF-α, ng/mg protein	1.94	2.24	1.76	2.00	2.12	1.87	0.23	0.703	0.823	0.850
IFN-γ, pg/mg protein	46.0 ^a	34.5 ^d	41.3 ^b	36.5 ^{cd}	35.2 ^{cd}	38.4 ^{bc}	1.2	<0.001	<0.001	0.001
IL-1β, ng/mg protein	0.23 ^a	0.17 ^{bc}	0.17 ^{bc}	0.17 ^{bc}	0.15 ^c	0.18 ^b	0.01	<0.001	0.002	<0.001
IL-2, ng/mg protein	7.99 ^a	4.43 ^b	4.08 ^b	3.14 ^c	3.10 ^c	3.14 ^c	0.29	<0.001	<0.001	<0.001
Ileum										
TNF-α, ng/mg protein	2.69	2.59	2.65	2.62	2.71	2.64	0.11	0.960	0.927	0.805
IFN-γ, pg/mg protein	39.3	35.4	35.0	39.2	39.0	39.9	3.3	0.818	0.550	0.463
IL-1β, ng/mg protein	0.22	0.20	0.19	0.21	0.20	0.19	0.01	0.608	0.224	0.860
IL-2, ng/mg protein	3.73	3.56	3.64	3.40	3.67	3.50	0.28	0.959	0.636	0.775

¹PQQ·Na₂ = pyrroloquinoline quinone disodium.²Values are means of 4 pigs in each treatment and pooled SEM, *n* = 4. Means within a row without common superscripts differ significantly (*P* < 0.05).³TNF-α = tumor necrosis factor-α.⁴IFN-γ = interferon-γ.⁵IL-1β = interleukin-1β.⁶IL-2 = interleukin-2.**Table 7.** Tight junction protein expression in the small intestinal mucosa of weaned pigs fed graded levels of PQQ·Na₂¹ for 28 d²

Item	PQQ·Na ₂ , mg/kg diet				SEM	P value		
	0	1.5	4.5	7.5		ANOVA	Linear	Quadratic
ZO-1	0.34 ^b	0.61 ^a	0.62 ^a	0.60 ^a	0.05	0.005	0.009	0.012
Occludin	0.34 ^b	0.49 ^a	0.50 ^a	0.33 ^b	0.06	0.020	0.502	0.003
Claudin-1	0.39	0.38	0.38	0.32	0.09	0.865	0.490	0.697

¹PQQ·Na₂ = pyrroloquinoline quinone disodium.²Values are means of 4 pigs in each treatment and pooled SEM, *n* = 4. Means within a row without common superscripts differ significantly (*P* < 0.05). **Figure 1.** Immunoblot analysis of ZO-1, occludin, and claudin-1 protein expression in the jejunal mucosa of weaned pigs fed diets containing 0, 1.5, 4.5, or 7.5 mg/kg pyrroloquinoline quinone disodium (PQQ·Na₂) for 28 d. Representative western blots are shown.

function, and reproductive performance (Steinberg et al., 1994, 2003). Dietary PQQ·Na₂ is an effective dietary additive for promoting nutrient utilization, stimulating breast muscle development, and maintaining redox status in broiler chicks (Samuel et al., 2015). In this study, we demonstrated that dietary PQQ can improve growth and feed efficiency and decrease the occurrence of diarrhea in weaned pigs. These improvements were accompanied by improved intestinal morphology, increased expression of mucosal tight junction proteins and cytokines, and enhanced antioxidant status. Moreover, the results of the current study provide support for the use of PQQ·Na₂ as a feed supplement in commercial swine production. The results indicate that dietary supplementation started with

1.5-mg/kg PQQ·Na₂ improved growth performance and health in weaned pigs.

The weaning period is one of the most stressful stages in pig production. During this period, young pigs must rapidly adapt to a large number of psychosocial and environmental stressors, such as maternal and litter mate separation, mixing stress, transport, changes in diet, and increased pathogen exposure (Madec and Josse, 1983; Madec et al., 1998). Significant changes in the histology and biochemistry of the small intestine that occur after weaning include villus atrophy and crypt hyperplasia, which cause decreased digestive and absorptive capacity that contribute to postweaning diarrhea (O'Loughlin et al., 1991; Pluske et al., 1997). Decreased villus height in the intestine is due to either an increased rate of cell loss or a reduced rate of cell renewal (Pluske et al., 1997). The villus height to crypt depth ratio is one of the potential criteria to assess nutrient digestion and the absorption capacity of the small intestine (Montagne et al., 2003). Increased crypt depth can be used as a predictor of increased crypt cell production rate and decreased overall stimulation of cell turnover in the small intestine (Montagne et al., 2007). In this study, the increased villus height, villus height to crypt depth ratio, and decreased crypt depth in pigs fed dietary PQQ·Na₂ may enhance the nutrient digestion and absorption capacity which can partly explain the improvement in pig performance and diarrhea incidence.

Cytokines play a critical role in the immune response and inflammation and can be important mediators for the protection against or susceptibility to infection and some gastrointestinal dysfunctions (Ye et al., 2006; Praveena et al., 2010). Both in vitro and in vivo studies showed that the uncontrolled synthesis of proinflammatory cytokines has a negative impact on gut integrity and epithelial function, including permeability to macromolecules and transport of nutrients and ions (McKay and Baird, 1999). Weaning in pigs is associated with an early and transient response in gene expression of inflammatory cytokines in the gut (Pié et al., 2004). Proinflammatory cytokines such as IL-1 β and IL-2 have been shown to mediate the host inflammatory response to prevent infection (Al-Sadi and Ma, 2007); IL-1 β can also increase the tight junction permeability (Al-Sadi et al., 2008). The present study has shown that dietary PQQ·Na₂ supplementation downregulated the expression of the proinflammatory cytokines IL-2 and IL-1 β in the small intestine of pigs. Dietary PQQ·Na₂ supplementation also resulted in a significant decrease in the level of the

proinflammatory cytokine IL-6 in humans (Harris et al., 2013). Dietary PQQ·Na₂ supplementation had a beneficial role in ameliorating inflammation which was consistent with previous study.

Some proinflammatory cytokines including TNF- α and IFN- γ have been shown to exert negative effects on intestinal epithelial tight junction permeability (Madara and Stafford, 1989; Youakim and Ahdieh, 1999; Grotjohann et al., 2000; Mazzon and Cuzzocrea, 2008). It is reported that TNF- α induces intestinal epithelial cell hyper-permeability by disrupting tight junctions, in part through myosin light-chain kinase (MLCK) up-regulation, in which nuclear factor kappa B (NF- κ B) is the positive upstream regulator for MLCK (He et al., 2012). A leaky epithelial barrier inducing macro-pinocytosis of tight junction proteins can be produced by IFN- γ (Bruewer et al., 2005). Similarly, IFN- γ and TNF- α can also disrupt epithelial barrier function in several ways including altering lipid composition in the membrane microdomains of the tight junction (Li et al., 2008; Capaldo and Nusrat, 2009). In the present experiment, dietary PQQ·Na₂ decreased the concentration of IFN- γ in the small intestinal mucosa but had no effect on the concentration of TNF- α in the small intestine of pigs.

Previous study showed that PQQ suppressed the production of proinflammatory mediators such as TNF- α and IL-6 in IL-1 β -treated SW982 cells via modulating the nuclear translocation of NF- κ B and the phosphorylation level of mitogen-activated protein kinase (MAPK) pathways (Yang et al., 2014; Liu et al., 2016). In addition, cytokines have different expression levels in different sites in the intestine. Further research about the mechanism of interaction among PQQ·Na₂, cytokines, and intestinal barrier function still need to be conducted.

It has been proved that oxidative stress can induce diarrhea (Duine et al., 1990; Sies, 1993; Wu et al., 2004; Wang et al., 2009). Pyrroloquinoline quinone is a redox active cofactor for bacterial quinoproteins that can be reduced to pyrroloquinolinequinol (PQQH₂) (Paz et al., 1996; Hara et al., 2007; Ouchi et al., 2009). Moreover, the aroxyl radical-scavenging activity of PQQH₂ was 7.4-fold higher than that of vitamin C (Ouchi et al., 2009). Studies have showed that PQQ played an important role in the promotion of plant growth (Choi et al., 2008), protection of mitochondria (Nunome et al., 2008; Ohwada et al., 2008), neuroprotection (Nunome et al., 2008; Tchapanian et al., 2010), and cardioprotection (Tao et al., 2007) via its antioxidant characteristics. The present study has shown that dietary PQQ·Na₂

increased the activities of T-SOD, GSH-Px, and CAT, but reduced the concentration of MDA in the small intestine. Superoxide dismutase catalyzes the efficient dismutation of O_2^- to H_2O_2 which is scavenged by GSH-Px and CAT (Yin et al., 2013). Malondialdehyde concentration in tissue and blood is generally implicated in endogenous lipid peroxidation and free radical-induced damage (Yousef et al., 2009). Decreased oxidative stress may result in reduced damage to the intestinal mucosal barrier, which subsequently leads to reduced intestinal permeability. These findings suggest that dietary supplementation with PQQ·Na₂ may influence performance and diarrhea in weaned pigs by modulating the redox status.

The tight junction is an intracellular structure that mediates adhesion between epithelial cells and is essential for epithelial cell function (Shin et al., 2006). Tight junction proteins are made up of a complex of integral membrane proteins, which inhibit the translocation of intraluminal toxins, antigens, and enteric flora from the lumen into sub-epithelial tissues and systemic blood circulation (Wang et al., 2015b). In the present study, we determined the expression of intestinal tight junction protein ZO-1, occludin, and claudin-1. The results have shown that the expression of the ZO-1 and occludin was higher for the pigs fed diets supplemented with PQQ·Na₂ than for pigs fed the control diet. This suggests that dietary supplementation with PQQ·Na₂ promotes intestinal barrier integrity for pigs which was consistent with the enhanced performance and reduced diarrhea incidence.

Pyrroloquinoline quinone has been applied in animal models such as rodents (Steinberg et al., 1994) and broiler chickens (Samuel et al., 2015; Wang et al., 2015a) for multiple purposes and been approved to use in food and drug in humans (Health Canada, 2012), indicating that PQQ has great potential in market. In our trial, we observed that supplementation of 1.5 mg/kg PQQ·Na₂ started to increase the G:F significantly during days 0 to 28 compared with the basal diet group; thus, we recommended a PQQ·Na₂ dose of 1.5 mg/kg for weaned pigs in practical use.

In conclusion, our results indicate that PQQ can improve growth performance and ameliorate diarrhea by enhancing the intestinal morphology, tight junction function, and improving the antioxidant status in pigs. The dietary supplementation of 1.50-mg/kg PQQ·Na₂ is the lowest functional dose to improve the growth performance for weaned pigs based on the results from the current study. The specific optimal PQQ supplementation level in

diets and the mechanisms of how PQQ can improve performance and health in weaned pigs still need further research.

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