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Original Research Article

Dietary supplementation with pyrroloquinoline quinone promotes growth, relieves weaning stress, and regulates metabolism of piglets compared with adding zinc oxide

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ABSTRACT

Hindered growth often occurs because of psychological and environmental stress during the weaning period of piglets. This study aimed to compare the effects of growth performance, diarrhea indices, digestibility of nutrients, antioxidant capacity, neurotransmitters levels and metabolism of weaned pigs fed diets supplemented with pyrroloquinoline quinone (PQQ) and zinc oxide (ZnO). Pigs weaned at d 28 (n = 108) were fed with three different diets including: the basal diet (CTRL group), the basal diet supplemented with 3.0 mg/kg PQQ (PQQ group) and the basal diet containing 1,600 mg/kg ZnO (ZNO group). During the first 14 d, weaned pigs fed the diet supplemented with PQQ and ZnO decreased feed to gain ratio and diarrhea rate ($P < 0.01$). Compared with the CTRL group, average daily gain was increased in weaned pigs in the PQQ group from d 15 to 28 ($P = 0.03$). Compared with the CTRL group, pigs fed PQQ and ZnO supplemented diets showed improved apparent total tract digestibility (ATTD) of nutrients ($P \leq 0.05$). During the overall experimental period, the concentration of malondialdehyde was decreased in plasma of pigs in the PQQ and ZNO groups compared with the CTRL group ($P < 0.05$). At d 28, the concentration of vasoactive intestinal peptide (VIP) and calcitonin gene-related peptide (CGRP) was lower in plasma of weaned pigs in the PQQ and ZNO groups compared with the CTRL group ($P < 0.05$). There was no difference between the PQQ and ZNO group in growth performance, ATTD of nutrition, antioxidant capacity and neurotransmitters levels. PQQ increased 3-methoxy-4-hydroxymandelate ($P < 0.05$) compared with the CTRL group. According to metabolomic analysis, erucamide, formononetin and 3-methyl-L-histidine were up-regulated in the PQQ group ($P < 0.05$). Compared with the CTRL group, aloesin and dibutyl adipate were down-regulated in the PQQ group ($P < 0.05$). In conclusion, similar to ZnO, PQQ improves growth performance, digestibility of nutrients, antioxidant capacity, neuromodulation and metabolism of weaned pigs. Thus, like ZnO, PQQ can be effectively applied in weaned pigs.

1. Introduction

Pyrroloquinoline quinone (PQQ) was originally discovered as the coenzyme for methanol dehydrogenase (Salisbury et al., 1979; Westerling et al., 1979). PQQ is widespread in nature but uncommon in food materials (Noji et al., 2007). Previous researchers have shown that PQQ is an outstanding antioxidant and acts as a growth factor (Rucker et al., 2005). As a natural antioxidant, PQQ can catalyze a variety of redox reactions such as the oxidation of methanol to formaldehyde (Bergethon, 1990; Ma et al., 2017;

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Vemuluri et al., 2017). PQQ has neuroprotective effects, can reduce nerve damage by inhibiting neuronal degeneration, and promotes secretion of neurotrophic factors (Liu et al., 2005; Zhang et al., 2009). PQQ inhibits fibril formation of alpha-synuclein to alleviate neuronal damage in neurodegenerative diseases (Cheng et al., 2021; Kim et al., 2010; Lu et al., 2018). Our previous study demonstrated that dietary supplementation with around 1.5 to 7.5 mg/kg PQQ improved growth performance and decreased diarrhea ratio of weaned pigs (Yin et al., 2019). PQQ supplementation attenuated oxidative stress in weaned pigs via regulated redox factors and activation of the NF-E2-related factor 2 (Nrf2)/heme oxygenase 1 (HO-1) pathway (Huang et al., 2021). PQQ demonstrated regulation of enteric neurochemical plasticity of enteritis rats in our previous research (Shi et al., 2022).

As an essential trace element for all organisms, Zn can recover function of testicles, reduce Parkinsons disease symptoms, increase lymphocyte function, and alleviate skin lesions (Brignola et al., 1993; Gearhart et al., 1997; Prasad, 1995; Prasad et al., 1999). Inappropriate nutrition with Zn deficiency may result in immunological diseases. Zn can modulate immunostimulants and function of cytokines (Rink and Gabriel, 2000). Prophylactic administration of Zn can reduce the inflammatory response in a porcine sepsis model by inducing stress proteins (Klosterhalfen et al., 1996). Zinc oxide (ZnO) is a trace mineral that alleviates diarrhea, improves growth performance, protects intestinal morphology, and increases digestibility of dietary crude protein (CP) and gross energy (GE) in weaned pigs (Peng et al., 2019; Zhang et al., 2022). Microencapsulated ZnO can increase average daily gain (ADG) and gain to feed ratio of weaned pigs (Grilli et al., 2015). Effect of ZnO in intestinal morphology has received a lot of attention in studies of weaned pigs, with results showing that villus height to crypt depth ratio and the levels of occludin and zonula occludens-1 proteins were increased by high treatment dosages of ZnO (Guan et al., 2021; Zhu et al., 2017). Dietary Zn is metabolized and deposited in a dose-dependent manner in blood and feces of weaned pigs (Case and Carlson, 2002; Hill et al., 2001).

Weaning stress, which includes psychological, nutritional, and environmental stresses, induces decreased digestive and absorptive function, diarrhea, and hindered growth of piglets (Pluske et al., 1997). The intestinal barrier, including the epithelial layer, immune system, enteric nervous system, and endocrine system, is a critical factor that influences weaning stress in piglets (Modina et al., 2019; Yin et al., 2014). The nervous system of piglets responds to changes in the social environment and daily rhythm during weaning by releasing neurotransmitters which regulate the endocrine and immune systems (Abot et al., 2018; Furness et al., 2013; Jakob et al., 2020; Rytel et al., 2021). The levels of neurotransmitters and redox factors are both important indicators of weaning stress in piglets. Dietary supplementation with ZnO and PQQ promotes growth and intestinal health in weaned pigs. ZnO is a common feed additive in weaned pigs because of its role in improving intestinal morphology and alleviating diarrhea. Due to concerns about antimicrobial resistance and long-term deposition of Zn in soils resulting from high dietary Zn supplementation, dietary Zn in compound feed for piglets has been limited to 1,600 mg/kg by the Ministry of Agriculture of the People's Republic of China since 2018. Dietary supplementation with 3.0 mg/kg PQQ affects the intestinal barrier in various ways, including the morphology, oxidation, and the microbiome (Huang et al., 2020, 2021). Because of its ability to improve the performance and function of weaned pigs, PQQ may be a new feed additive in weaned pigs, like ZnO.

With the aim of establishing a theoretical framework for PQQ as a feed additive, we hypothesized that both dietary supplementation with 3.0 mg/kg PQQ and 1,600 mg/kg ZnO could positively affect growth performance, diarrhea, digestibility of nutrients, and metabolism of weaned pigs. Our study compared growth performance, diarrhea index, and apparent total tract digestibility (ATTD) of nutrients of weaned pigs fed diets supplemented with PQQ or ZnO. We also used metabolomics to measure the concentration of redox factors and neurotransmitters in plasma.

2. Materials and methods

2.1. Animal ethics

All experimental protocols were approved by the Animal Subjects Committee of China Agricultural University (Beijing, China). The research was based on the National Research Council's Guide for the Care and Use of Laboratory Animals (AW82302202-1-2).

2.2. Animals and experimental procedures

Pigs (Duroc × Landrace × Yorkshire; $n = 108$; half barrows and half gilts) were weaned at 28 ± 5 d with an average initial body weight (BW) of 9.66 ± 0.34 kg. Pigs were fed with three different diets including the basal diet (CTRL group), the basal diet supplemented with 3.0 mg/kg PQQ (PQQ group) and the basal diet containing 1,600 mg/kg ZnO (ZNO group). Each treatment had 6 replicated pens with 6 pigs per pen (3 barrows and 3 gilts) which were housed in a well-ventilated and environmentally controlled nursery. Room temperature was maintained at 27 ± 2 °C throughout the experiment. The basal diet was formulated to the nutrient requirements of weaned pigs described by the NRC (NRC, 2012) (Table 1). The diets in the three groups contained no antibiotics throughout the experiment. The PQQ·Na_2 (≥98% purity) chemically synthesized by Changmao Biochemical Engineering Company (Changzhou, China) was premixed with corn starch to a concentration of 1.0 g/kg mixture before addition to the diet.

Diarrhea scores were recorded daily by the same person based on the following scale: 1 = well-formed feces, 2 = sloppy feces, 3 = diarrhea (Marquardt et al., 1999). The incidence of diarrhea (%) for weaned pigs in each pen was calculated as follows: [(number of weaned pigs with diarrhea × number of days of diarrhea)/(total number of weaned pigs × number of days of experiment)] × 100. Pigs were weighed on d 14 and 28, and feed consumption for each pen was recorded, which were then used to calculate ADG, average daily feed intake (ADFI) and feed-to-gain ratio (FCR).

2.3. Sample collection

Blood was collected from the anterior vena cava of 1 pig per pen into a vacuum tube containing K2-EDTA (ethylenediaminetetraacetic acid disodium salt) on d 14 and 28. After centrifuging for 10 min at 3,000 × *g*, plasma was separated. From 26 to 28 d, feed in each group and fresh fecal excrement of pigs in each pen were collected in clean sample bags, respectively, each day. These samples were stored at −20 °C until analysis.

2.4. Chemical analysis of nutrients in feed and feces

After thawing at 4 °C and drying at 65 °C for 72 h, feed and feces were ground through 40-mesh sieves. All chemical analyses were performed in duplicate. GE was determined in an automatic isoperiodic oxygen bomb calorimeter (Parr 1281, Automatic Energy Analyzer, Moline, IL, USA). Concentrations of dry matter (DM), ash, organic matter (OM), CP, ether extract (EE) and acid-

Table 1
Ingredient composition and analyzed nutrient concentration of experimental diets (%, as-fed basis).[1]

Item	CTRL	PQQ	ZNO
Ingredients			
Corn	58.14	58.04	58.18
Soybean meal	16.20	16.00	16.00
Extruded soybean	5.00	5.00	5.00
Soy protein concentrate	2.00	2.00	2.00
Fish meal	3.00	3.00	3.00
Dried whey	6.00	6.00	6.00
Spray-dried porcine plasma	2.00	2.00	2.00
Soybean oil	2.20	2.20	2.20
Sucrose	2.00	2.00	2.00
limestone	0.90	0.90	0.90
Dicalcium phosphate	1.00	1.00	1.00
Salt	0.25	0.25	0.25
L-Lysine·HCl	0.48	0.48	0.48
L-Threonine	0.16	0.16	0.16
L-Tryptophan	0.05	0.05	0.05
L-Methionine	0.12	0.12	0.12
Vitamin and mineral premix[2]	0.50	0.50	0.50
PQQ·Na_2 premix[3]	0.00	0.30	0.00
Inorganic ZnO	0.00	0.00	0.15
Nutrient composition[4]			
Gross energy, MJ/kg	16.90	16.97	16.76
Crude protein	19.91	18.90	18.32
Ether extract	4.81	5.35	4.98
Crude fiber	4.29	4.25	4.21
Neutral detergent fiber	12.71	12.12	11.91
Acid detergent fiber	4.47	4.58	4.43
Organic matter	83.33	83.32	82.74
Lysine	1.78	1.77	1.77
Methionine	0.43	0.43	0.43
Threonine	1.05	1.05	1.05
Tryptophan	0.30	0.30	0.30
Calcium	0.77	0.77	0.77
Total phosphorus	0.62	0.62	0.62

[1] CTRL, the basal diet; PQQ, basal diet supplemented with 3.0 mg/kg pyrroloquinoline quinone disodium (PQQ·Na_2); ZNO, basal diet containing 1,600 mg/kg of ZnO.
[2] Provided the vitamin and minerals as following per kilogram of diet: 11,000 IU vitamin A as retinyl acetate; 1,500 IU vitamin D_3 as cholecalciferol; 44.1 IU vitamin E as DL-α-tocopherol acetate; 4 mg vitamin K_3 as menadione; 1.4 mg vitamin B_1; 5.2 mg vitamin B_2; 20 mg vitamin B_5; 10 μg vitamin B_{12}; 26 mg niacin; 14 mg pantothenic acid; 0.8 mg folic acid; 44 μg biotin; 100 mg Fe; 16.5 mg Cu; 90 mg Zn; 35 mg Mn; 0.3 mg KI; 0.3 mg Na.
[3] PQQ·Na_2 was diluted as 1.0 g/kg mixture with corn starch.
[4] Gross energy, crude protein, ether extract, crude fiber, neutral detergent fiber, acid detergent fiber and organic matter are the results of chemical analysis. Other nutrient levels are calculated values.

insoluble ash (AIA) were analyzed using methods of the Association of Official Agricultural Chemists (AOAC, 2007; Thiex et al., 2019). Crude fiber (CF), neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined using fiber analyzer equipment (Ankom Technology, Macedon, NY, USA) following a modified procedure (Van Soest et al., 1991). Apparent total tract digestibility of nutrients was calculated using the following equation (Mccarthy et al., 1974).

$$\text{ATTD}\ (\%) = 100 - (\text{AIA}_{\text{diet}} \times \text{Nutrient}_{\text{feces}})/(\text{AIA}_{\text{feces}} \times \text{Nutrient}_{\text{diet}}) \times 100.$$

2.5. Redox factor expression

Activity of redox factors including glutathione peroxidase (GSH-Px), malondialdehyde (MDA) and total-superoxide dismutase (T-SOD) in plasma collected on d 14 and 28 were detected according to the manufacturer's instructions using ELISA (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Six samples were tested for each treatment group.

2.6. Determination of the concentration of neuropeptides

Neuropeptides including vasoactive intestinal peptide (VIP), calcitonin gene-related peptide (CGRP), and substance P (SP) in plasma collected on d 14 and 28 were determined using commercial kits according to the manufacturer's instructions (Beijing Kangjia Hongyuan Biotechnology Co., Beijing, China). Six samples were tested for each treatment group.

2.7. Targeted detection of neurotransmitters in plasma

Plasma samples (CTRL group, $n = 6$; PQQ group, $n = 5$; ZNO group, $n = 6$) were thawed at 4 °C. Plasma (50 μL) was pipetted and placed in a clean tube. Precooled methanol–acetonitrile solution (1:1, vol/vol) (1499230-935, Merck, USA) was added into sample. The homogenate was vortexed for 60 s, stored at −20 °C for 1 h and centrifuged for 20 min (14,000 × *g*, 4 °C). The supernatant was collected and freeze dried. Samples were reconstituted with 1% formic acid (FA) in acetonitrile:H_2O solution (1:1, vol/vol) and separated using the Agilent 1290 Infinity LC ultra performance liquid chromatography system (Agilent Technologies, USA). Two kinds of liquid were used as the mobile phase: an aqueous solution containing 25 mM ammonium formate and 0.1% FA as liquid A; acetonitrile containing 0.1% FA as liquid B. The 2-μL samples were then put in 4 °C automatic sampler (column temperature, 45 °C; flow velocity, 300 μL/min). The relevant liquid phase gradient of liquid B was as follows: liquid B changes linearly from 90% to 40% during the first 18 min; then liquid B changes linearly from 40% to 90% within 6 s; at last, liquid B was maintained at 90% until 24 min. After three experimental samples, a quality control sample was included to detect and evaluate stability and repeatability. The standard mixture was included for chromatographic retention time correction in the sample cohort. The QTRAP 5500 mass spectrometer (AB SCIEX, Thermo, USA) was used for mass spectrometry in positive ion mode. Conditions for the mass spectrometer were: source temperature, 450 °C; ion source gas1 (Gas1), 60; ion source gas2 (Gas2), 60; curtain gas (CUR), 30; ion sapary voltage floating (ISVF), 5,000 V. The ion pair was detected using MRM mode. The chromatographic peak area and retention time was extracted by using MultiQuant software. Neurotransmitter standards were used to identify the metabolites and accomplish retention time correction.

2.8. Untargeted metabolomics analysis of plasma

Untargeted metabolomics analysis of plasma samples (CTRL group, $n = 6$; PQQ group, $n = 6$; ZNO group, $n = 6$) was conducted following the procedures described by Gu et al. (2018) using the database reported by Blazenovic et al. (2018). Plasma samples were thawed at 4 °C and aliquots (100 μL) were mixed with cold methanol–acetonitrile solution (1:1, vol/vol; 400 μL). The supernatant was collected after centrifuging for 15 min (14,000 × *g*, 4 °C) and dried in a vacuum centrifuge (5430R, Eppendorf, Germany). Samples were re-dissolved in acetonitrile-water (1:1, vol/vol; 100 μL) solvent for liquid chromatograph mass spectrometer (LC–MS) analysis.

LC–MS analysis was performed using ultra high performance liquid chromatography (UHPLC 1290 Infinity LC, Agilent Technologies, USA) coupled to a quadrupole time-of-flight (AB Sciex TripleTOF 6600). Samples were analyzed using an ACQUIY UPLC BEH column (1.7 μm, 2.1 × 100 mm; Waters, Ireland) for HILIC separation. Two kinds of liquid were used in ESI positive and negative modes: an aqueous solution containing 25 mM ammonium formate and 0.1% FA as liquid A; acetonitrile solution containing 0.1% FA as liquid B. The gradient was 85% B for 1 min, linearly decreasing to

65% within 11 min, then decreasing to 40% within 0.1 min and holding for 4 min, then increasing to 85% within 0.1 min, using a 5 min rebalancing period.

Using ProteoWizard MSConvert to convert raw mass spectrometer (MS) data (wiff.scan files) to MzXML files before importing into freely available XCMS software, the following parameters were used: centWave m/z = 10 ppm, peakwidth = c (10, 60), prefilter = c (10, 100) for peak picking. For peak grouping, bw = 5, mzwid = 0.025, minfrac = 0.5 were used. Using CAMERA (Collection of Algorithms of MEtabolite pRofile Annotation) to annotate isotopes and adducts, only the variables having more than 50% of the nonzero measurement values in at least one group were recorded in the extracted ion features. By comparing the accuracy of the m/z value (<10 ppm), and verifying MS/MS spectra with an in-house database established with available authentic standards, compound identification of metabolites was performed.

2.9. Statistical analysis

Data were analyzed using the general linear model (GLM) procedures in SAS (version 9.2; SAS Inst. Inc., Cary, NC, USA). Growth performance and apparent digestibility of piglets were analyzed with a pen as the experimental unit. The chi-square test was used to discern effects of dietary treatment on diarrhea rate of piglets. Data are expressed as least squares mean and standard error of the mean (SEM). Differences were deemed significant at $P \leq 0.05$ and $0.05 < P < 0.10$ was considered a statistical trend. Figs. were created with GraphPad Prism 9 (version 5; GraphPad Software Inc., San Diego, CA, USA).

The processed data of metabolites were analyzed by R package (ropls) after normalization to total peak intensity. The data were subjected to multivariate data analysis, including Pareto-scaled principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA). The variable importance in the projection value of each variable in the OPLS-DA model was calculated to indicate its contribution to the classification. The significance of each metabolite was measured by using Student's *t*-test at univariate level when variable importance in the projection value > 1.

3. Results

3.1. Growth performance

During the first 14 d, weaned pigs fed the diet supplemented with PQQ and ZnO decreased FCR and diarrhea rate ($P < 0.01$; Table 2). There were no differences in FCR and diarrhea during the first 14 d between the PQQ and ZNO group. Compared with the CTRL group, ADG was increased in the PQQ group from d 15 to 28 ($P = 0.03$). There were no differences in ADG and FCR from d 15 to 28 between the PQQ and ZNO group. During the total experiment, FCR showed a decreasing trend in the PQQ and ZNO groups ($P = 0.07$). There were no differences in FCR from d 0 to 28 between the PQQ and ZNO group.

3.2. Apparent total tract digestibility of nutrients

Compared with the CTRL group, pigs fed diets supplemented with PQQ improved the ATTD of DM, OM, EE, GE, CF, NDF and ADF ($P < 0.05$; Table 3). ATTD of DM, OM, EE, GE, CF and ADF were higher in pigs fed dietary supplementation with ZnO than pigs in the CTRL group ($P < 0.05$). There were no differences in ATTD of DM, OM, CP, EE, GE, CF, NDF and ADF between the PQQ and ZNO group.

Table 2
Effects of PQQ and ZnO supplementation on growth performance of weaned pigs ($n = 6$).[1]

Item	CTRL	PQQ	ZNO	SEM	*P*-value
Initial BW, kg	9.64	9.70	9.66	0.344	0.99
Final BW, kg	22.76	24.66	24.44	0.690	0.51
Days 0 to 14					
ADG, kg	0.48	0.53	0.54	0.020	0.37
ADFI, kg	0.64	0.64	0.67	0.025	0.86
FCR	1.40[a]	1.25[b]	1.26[b]	0.024	<0.01
Diarrhea, %	15.28[a]	13.09[b]	11.71[b]	0.515	<0.01
Days 15 to 28					
ADG, kg	0.46[b]	0.56[a]	0.51[ab]	0.015	0.03
ADFI, kg	0.96	0.92	0.98	0.045	0.87
FCR	2.14	1.67	1.95	0.098	0.16
Diarrhea, %	5.56	5.34	5.77	0.253	0.81
Days 0 to 28					
ADG, kg	0.47	0.54	0.53	0.014	0.13
ADFI, kg	0.80	0.78	0.82	0.033	0.88
FCR	1.73	1.47	1.57	0.046	0.07
Diarrhea, %	10.60	9.36	8.85	0.320	0.16

BW = body weight; ADFI = average daily feed intake; ADG = average daily gain; FCR = feed-to-gain ratio; SEM = standard error of the mean.
[a,b]Mean values within a row with different superscript letters are significantly different ($P \leq 0.05$).
[1] CTRL, the basal diet; PQQ, basal diet supplemented with 3.0 mg/kg of pyrroloquinoline quinone disodium (PQQ·Na_2); ZNO, basal diet containing 1,600 mg/kg of ZnO.

Table 3
Effect of PQQ and ZnO supplementation on apparent total tract digestibility of nutrients in weaned pigs ($n = 6$, %).[1]

Item	CTRL	PQQ	ZNO	SEM	*P*-value
Dry matter	78.07[b]	82.18[a]	81.39[a]	0.589	<0.01
Organic matter	80.95[b]	84.47[a]	83.80[a]	0.540	<0.01
Crude protein	69.78	74.10	72.40	0.841	0.08
Ether extract	58.83[b]	67.90[a]	66.40[a]	1.264	<0.01
Gross energy	77.70[b]	82.20[a]	81.10[a]	0.600	<0.01
Crude fiber	9.59[b]	30.60[a]	23.60[a]	2.632	<0.01
Neutral detergent fiber	45.37[b]	52.80[a]	50.40[ab]	1.316	0.05
Acid detergent fiber	42.12[b]	53.70[a]	49.00[a]	1.609	<0.01

SEM = standard error of the mean.
[a,b] Mean values within a row with different superscript letters are significantly different ($P \leq 0.05$).
[1] CTRL, the basal diet; PQQ, basal diet supplemented with 3.0 mg/kg of pyrroloquinoline quinone disodium (PQQ·Na_2); ZNO, diet containing 1,600 mg/kg of ZnO.

3.3. Concentration of redox factors in plasma

At d 14, plasma of weaned pigs showed a higher concentration of T-SOD in the PQQ and ZNO groups compared with the CTRL group ($P < 0.05$; Fig. 1A). Concentration of MDA was decreased in the PQQ and ZNO groups compared with the CTRL group ($P < 0.05$). There were no differences in T-SOD and MDA at d 14 between PQQ and ZNO group. At d 28, plasma of weaned pigs showed a higher concentration of T-SOD and GSH-Px in the PQQ and ZNO groups compared with the CTRL group ($P < 0.05$; Fig. 1B). The concentration of MDA was decreased in the PQQ and ZNO groups compared with the CTRL group ($P < 0.05$). There were no differences in T-SOD, GSH-Px and MDA at d 28 between PQQ and ZNO group.

3.4. Concentration of neuropeptides in plasma

At d 14, PQQ supplementation decreased the concentration of SP in plasma of weaned pigs compared with the CTRL and ZNO groups ($P < 0.05$; Fig. 2A). VIP was decreased in the PQQ and ZNO groups compared with the CTRL group ($P < 0.05$; Fig. 2B). There were no differences in VIP between PQQ and ZNO group at d 14. At d 28, the concentrations of VIP and CGRP in plasma were decreased in the

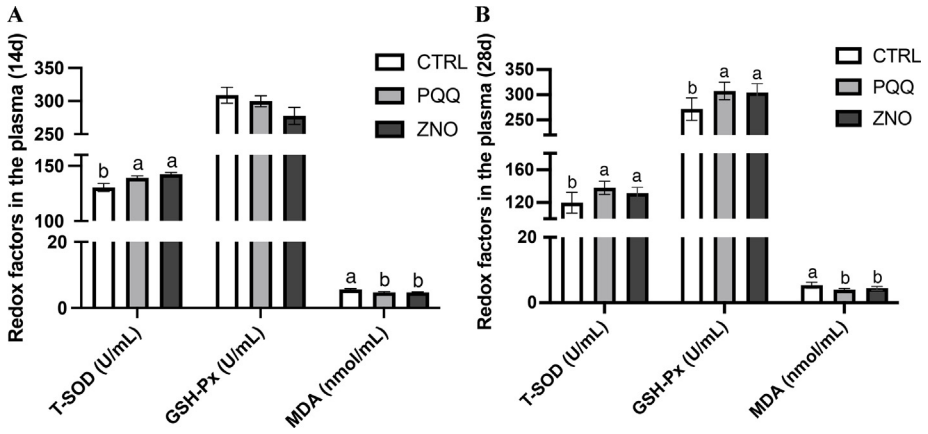

Fig. 1. Concentration of redox factors in plasma (n = 6). (A) The concentration of redox factors in plasma on d 14. (B) The concentration of redox factors in plasma on d 28. T-SOD = total-superoxide dismutase; GSH-Px = glutathione peroxidase; MDA = malondialdehyde. CTRL, the basal diet; PQQ, basal diet supplemented with 3.0 mg/kg of pyrroloquinoline quinone disodium (PQQ·Na_2); ZNO, basal diet containing 1,600 mg/kg of ZnO. [a,b]Bars with different superscript letters are significantly different ($P \leq 0.05$).

PQQ and ZNO groups compared with the CTRL group ($P < 0.05$, Fig. 2E and F). There were no differences in VIP and CGRP between PQQ and ZNO group.

3.5. Quantitative detection of neurotransmitters in plasma

Neurotransmitters were detected quantitatively after stability evaluation was completed (RSD < 30%). Compared with the CTRL group, PQQ increased 3-methoxy-4-hydroxymandelate (also known as vanillylmandelic acid [VMA]) (P = 0.04; Fig. 2H). Compared with the ZNO group, PQQ treatment tended to increase VMA (P = 0.06). Other neurotransmitters in the PQQ and ZNO groups showed no differences with the CTRL group.

3.6. Quantitative analysis of metabolomics in plasma

The evaluation parameters showed that the positive mode was more stable and reliable compared with the negative mode. Plasma yielded 783 metabolites when positive modes were merged. Altered metabolites were demonstrated by univariate statistical analysis across the three groups (Fig. 3A–C). The difference between the three groups was analyzed by the OPLS-DA model (Fig. 3D–F). Compared with the CTRL group, PQQ supplementation up-regulated 9 metabolites and down-regulated 2 metabolites, using P-value and OPLS-DA variable importance in the projection to show the impact strength and explanatory power of each metabolite (Table 4). Erucamide, 1-palmitoyl-sn-glycero-3-phosphocholine, formononetin, 3-methyl-L-histidine, N-alpha-(tert-butoxycarbonyl)-L-histidine, 2-oleoyl-1-palmitoyl-sn-glycero-3-phosphocholine, hexaethylene glycol, 1,2-dipalmitoleoyl-sn-glycero-3-phosphocholine and beta-homoproline were up-regulated in the PQQ group. Dibutyl adipate and aloesin were down-regulated in the PQQ group. The correlation heatmap shows the relationship between metabolites and neuropeptides, redox factors and ATTD of nutrients (Fig. 4). Compared with the CTRL group, ZnO supplementation up-regulated 9 metabolites and down-regulated 9 metabolites (Table S1). Compared with the ZNO group, PQQ supplementation up-regulated 7 metabolites and down-regulated 7 metabolites (Table S2).

4. Discussion

PQQ was identified as a novel coenzyme in bacteria and is widely found in common foods (Noji et al., 2007; Salisbury et al., 1979). As a natural antioxidant, PQQ improves the oxidative state, reduces apoptosis, and promotes mitochondrial function which ultimately improves growth (Huang et al., 2021; Killgore et al., 1989; Saihara et al., 2017). In PQQ-deprived mice, reproductive performance was compromised, and PQQ improved survival and BW of offspring (Steinberg et al., 2003). Diets supplemented with PQQ improved feed efficiency and stimulated breast muscle development in broiler chicks (Wang et al., 2015). PQQ is approved for use as a natural antioxidant for human consumption in America, Canada, the European Union, and China (Canada, 2012; European Commission, 2018; FDA, 2008; NHC, 2022). According to our previous studies, dietary supplementation with 1.5 to 7.5 mg/kg PQQ increased ADG and gain:feed compared with a basal diet in weaned pigs (Ming et al., 2021; Yin et al., 2019). As a component of several digestive enzymes, Zn plays an important role in nutrient metabolism (Wang et al., 2010, 2018). Pharmacological doses of ZnO in pig diets serve as a growth promoter, similar to in-feed antibiotics (Pluske, 2013; Zhu et al., 2017) and high doses of ZnO have been shown to improve growth performance of weaned pigs (Carlson et al., 1999; Hill et al., 2001; Wei et al., 2020). In our study, the diets supplemented with PQQ or ZnO decreased FCR of weaned pigs during the first 14 d postweaning. PQQ increased ADG of weaned pigs during 15 to 28 d postweaning. The results showed no differences in ADG, ADFI and FCR between the PQQ and ZNO groups. The results suggested to us that both PQQ and ZnO have growth promoting effects as previously reported (Carlson et al., 1999; Hill et al., 2001; Ming et al., 2021; Wei et al., 2020; Yin et al., 2019). During the late feeding stages, PQQ showed more improvement in growth performance of weaned pigs compared with ZnO.

Weaning stress in piglets disrupts redox equilibrium and intestinal barrier function which induces diarrhea and reduces digestibility of nutrients. Previous studies have shown that dietary supplementation with PQQ reduces incidence of diarrhea and protects the intestinal barrier. Intestinal protection provided by PQQ includes regulation of intestinal morphology, oxidation, inflammation, glycolipid metabolism, and gut microbiota in weaned pigs (Huang et al., 2020, 2022; Ming et al., 2021; Yin et al., 2019). Dietary supplementation with Zn reduces diarrhea, improves intestinal morphology, and regulates anti-inflammatory effects and the intestinal microbiome in weaned pigs (Peng et al., 2019; Wang et al., 2018; Wei et al., 2020). Furthermore, several researchers demonstrated that pharmacological ZnO increased the ATTD of nutrients (Christensen et al., 2022; Oh et al., 2021). In the present study, ATTD of nutrients was increased and incidence of diarrhea was decreased in weaned pigs that were supplemented with dietary PQQ and ZnO. Elevated ATTD might explain how PQQ and ZnO improved FCR. The results showed no differences in ATTD

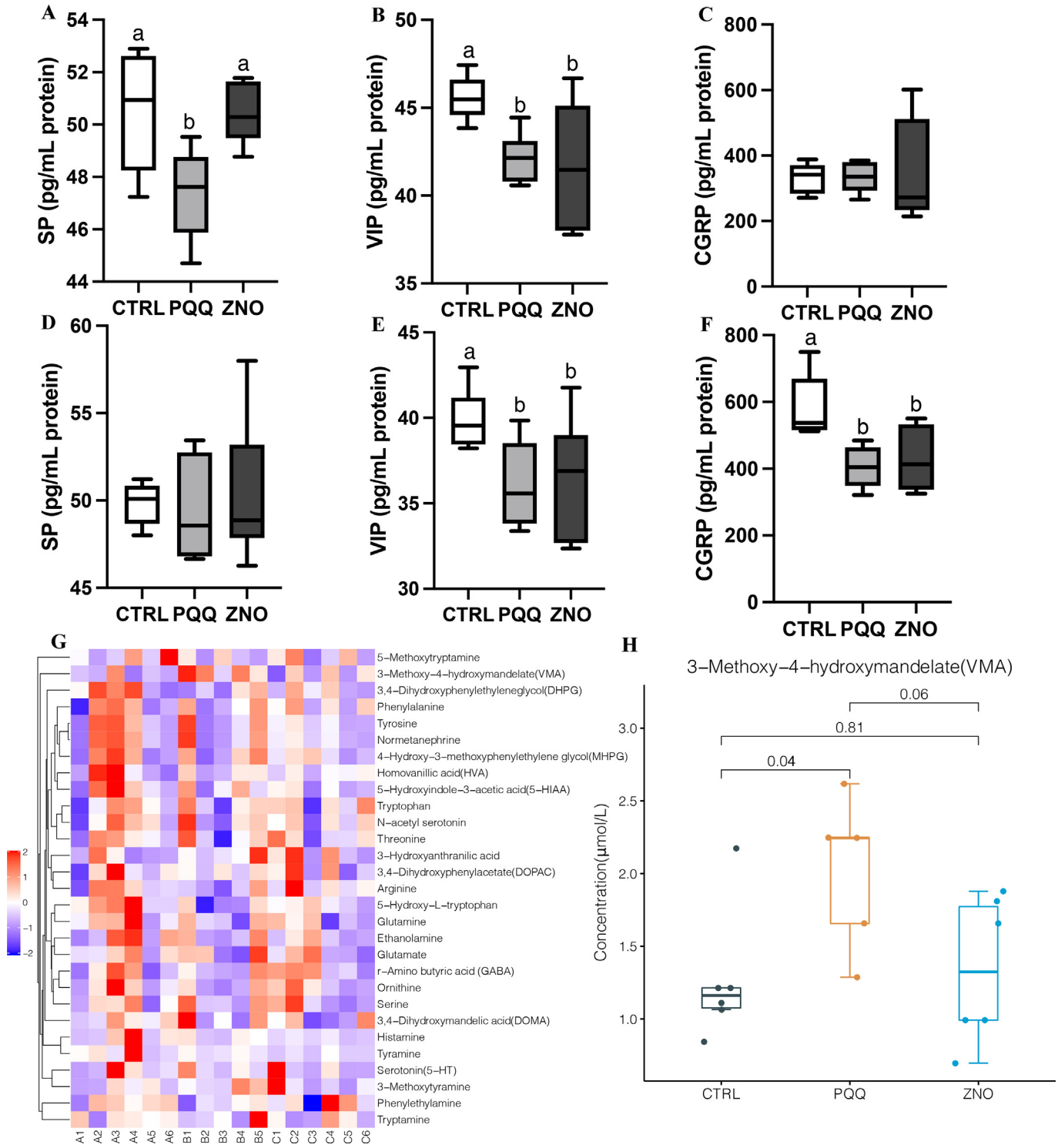

Fig. 2. Concentration of neuropeptides in plasma. (A–C) The concentration of neurotransmitters in plasma on d 14 ($n = 6$). (D–F) The concentration of neurotransmitters in plasma on d 28 ($n = 6$). (G) Cluster heat map of differentially expressed neurotransmitters. Red represents highly expressed neurotransmitters and blue represents lowly expressed neurotransmitters. (H) Concentration of 3-methoxy-4-hydroxymandelate (VMA) in plasma. SP = substance P; VIP = vasoactive intestinal peptide; CGRP = calcitonin gene-related peptide. CTRL, the basal diet; PQQ, basal diet supplemented with 3.0 mg/kg of pyrroloquinoline quinone disodium (PQQ·Na_2); ZNO, basal diet containing 1,600 mg/kg of ZnO. [a,b]Bars with different superscript letters are significantly different ($P \leq 0.05$). The *P*-values are above the horizontal line (Fig. 2H).

of nutrients and diarrhea between the PQQ and ZNO groups, which suggested to us that PQQ has similar effects on the promotion of digestion of nutrients in weaned pigs as ZnO.

Dietary PQQ supplementation has been found to increase antioxidant capacity in the placenta of gestating rats and in the intestine of their offspring (Zhang et al., 2019). T-AOC was increased in the plasma of broiler chicks fed diets supplemented with PQQ (Wang et al., 2015). PQQ treatment improved mitochondrial biogenesis and maintenance of redox homeostasis in the liver in chickens (Qiu et al., 2021). Previous research showed that PQQ

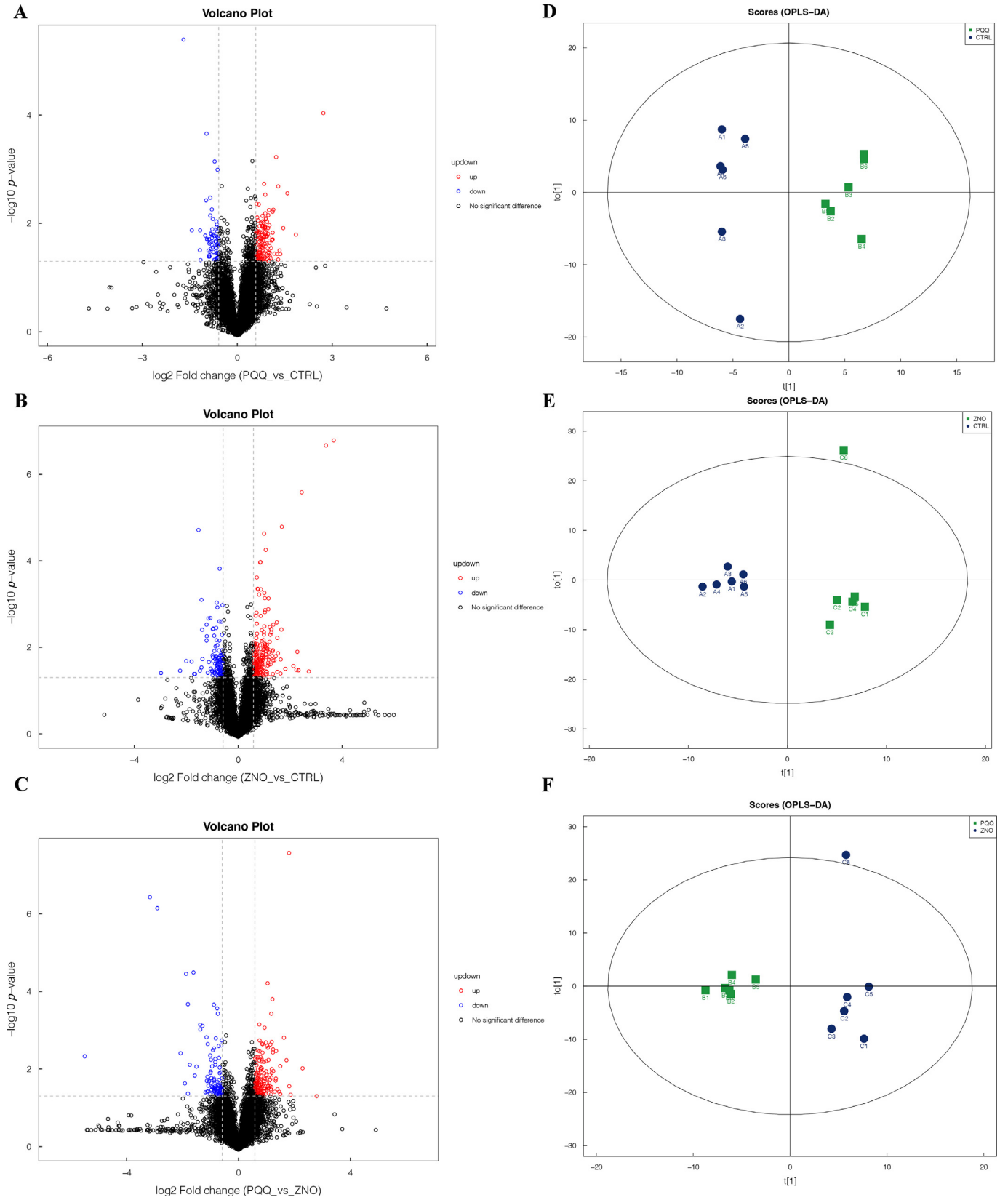

Fig. 3. Analysis of metabolites in plasma of weaned pigs in three groups. Volcano map and orthogonal partial least squares discriminant analysis (OPLS-DA) of metabolites ($n = 6$). (A–C) Volcano map of altered metabolites analyzed by positive mode. The abscissa is the logarithm of $\log_2$ of the fold change multiple. The ordinate is the logarithm of $-\log_{10}$ of the *P*-value. The up-regulated metabolites were defined as $P < 0.05$ and FC > 1.5. One little circle denotes one metabolite, the red circles and blue circles represent up- and down-regulated metabolites, respectively, and the black circles represent metabolites that were not differentially expressed between groups. (D–F) OPLS-DA of metabolites. The abscissa and ordinate represent principal component 1 and principal component 2, respectively. The ellipse represents the 95% confidence interval. CTRL, the basal diet; PQQ, basal diet supplemented with 3.0 mg/kg of pyrroloquinoline quinone disodium (PQQ·Na_2); ZNO, basal diet supplemented with 1,600 mg/kg of ZnO.

Table 4
Differentially expressed metabolites analyzed by metabolomics ($n = 6$).

Name	Variable importance in the projection	Fold change	*P*-value
Up-regulated			
Erucamide	13.97	1.82	0.01
1-Palmitoyl-sn-glycero-3-phosphocholine	22.47	1.28	0.01
Formononetin	2.84	2.39	0.04
3-Methyl-L-histidine	1.20	1.45	0.04
N-alpha-(tert-Butoxycarbonyl)-L-histidine	2.95	1.94	0.04
2-Oleoyl-1-palmitoyl-sn-glycero-3-phosphocholine	8.46	1.22	0.04
Hexaethylene glycol	1.01	1.58	0.04
1,2-Dipalmitoleoyl-sn-glycero-3-phosphocholine	1.74	1.41	0.04
beta-Homoproline	1.20	1.40	0.05
Down-regulated			
Dibutyl adipate	1.26	0.70	0.04
Aloesin	1.02	0.73	0.05

supplementation improved activities of T-SOD, GSH-Px, and catalase and reduced concentration of MDA in plasma of weaned pigs (Ming et al., 2021; Yin et al., 2019). According to experiments in vitro and in vivo, PQQ attenuated oxidative stress via regulation of the Nrf2/HO-1 pathway (Huang et al., 2021). Diets supplemented with Zn improved antioxidant capacity in plasma of weaned pigs (Zhu et al., 2017). ZnO treatment regulated redox factors in plasma at 14 and 28 d in the current study. Furthermore, dietary supplementation with PQQ and ZnO increased T-SOD, GSH-Px and decreased MDA in plasma of weaned pigs which indicated the antioxidant capacity of PQQ and ZnO. The results demonstrated that PQQ and ZnO treatments both had effective measures to reduce oxidative stress in weaned pigs.

In addition to oxidative stress, piglets suffer mental stress because of the changes in social environment at weaning. Psychological stressors are important factors in development of ulcerative colitis (Levenstein et al., 2000). Stress signals are transmitted by neurotransmitters from the central nervous system to the enteric nervous system through parasympathetic innervation (Furness et al., 2013). PQQ inhibits the damage in brain neurons and treats Parkinson's disease, schizophrenia and cognitive deficits in rats (Scanlon et al., 1997; Zhang et al., 2016; Zhou et al., 2020). The neuroprotective effects of PQQ include development of nerve cells and regeneration of synapses via regulating the phosphatidylinositol-3 kinases (PI3K)/protein kinase B (Akt) pathway and modulating the N-methyl-D-aspartic acid receptor (NMDA) (Falcon et al., 2019; Iqbal et al., 2016; Majewska and Szeliga, 2017). Supplementation with high doses of ZnO regulates stimulation of neurotransmitters, such as serotonin (5-HT), in the ileum of weaned pigs (Feng et al., 2006). Previous research demonstrated that PQQ supplementation regulated enteric neurochemical plasticity of weaned rats by stimulating the expression of neuropeptides in damaged intestine (Shi et al., 2022). In the current study, the concentrations of SP, VIP and CGRP in plasma were decreased in PQQ- and ZnO-fed pigs compared with the CTRL group at d 14 and 28. Furthermore, PQQ decreased SP in plasma of weaned pigs at d 14 compared with the CTRL and ZNO groups. SP affects pain, inflammation, sensory processing depressive disorder, gut function, and hormone regulation (Nässel et al., 2019). The level of SP in plasma of rats was increased when high levels of reactive oxygen species were present (Ping-Chia et al., 2009). In mouse models of colitis, nociceptors release CGRP which modulates M cell density and affects immune cell function (Lai et al., 2020; Russell et al., 2014). CGRP can relax muscle via direct action on smooth muscle cells to contract the gut (Holzer, 1998). VIP is also a neuroprotective and immunomodulator in neural injurious situations (Gonzalez-Rey and Delgado, 2005). Toll-like receptor 4 mediates myenteric neurons and sensory neurons to recognize lipopolysaccharides which release CGRP and VIP to alleviate neurodegeneration (Arciszewski et al., 2008; Meseguer et al., 2014). VMA is the end-product of catecholamine metabolism (Soler Arias et al., 2021). Catecholamines including dopamine, epinephrine and norepinephrine are secreted by chromaffin cells in postganglionic fibers in the autonomic nervous system (Eisenhofer et al., 2004). VMA scavenges free radicals and increases vagal tone to decrease heart rate (Kolentinis et al., 2013). In this

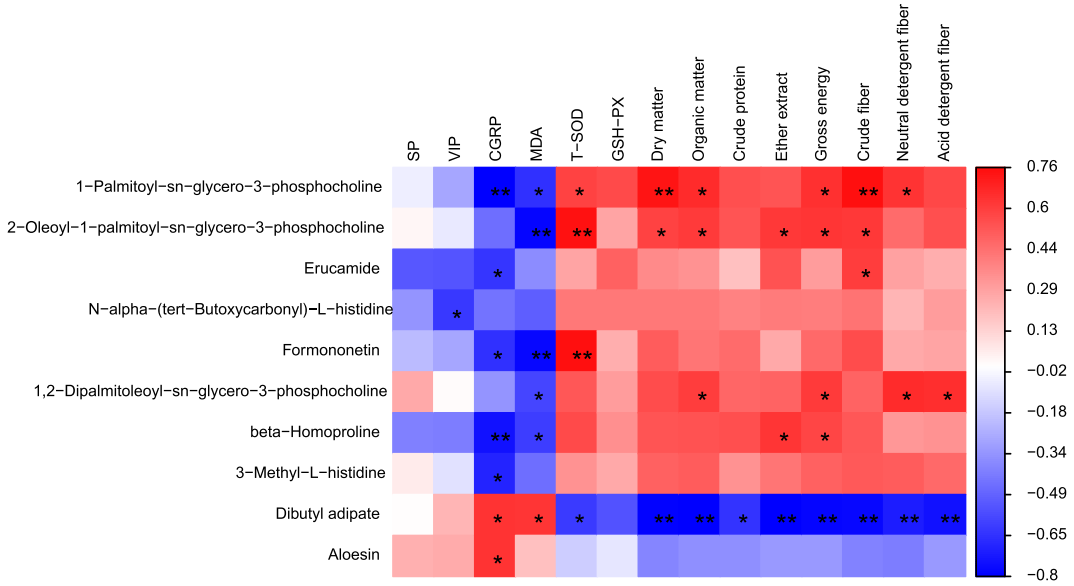

Fig. 4. Association analysis of metabolites with individual detection indicators between CTRL and PQQ group. The X-axis presents neuropeptides, redox factors and apparent total tract digestibility (ATTD) of nutrients. The Y-axis presents metabolites. The correlation coefficients are shown in different colors on the right side of the legend. SP = substance P; VIP = vasoactive intestinal peptide; CGRP = calcitonin gene-related peptide; MDA = malondialdehyde; T-SOD = total-superoxide dismutase; GSH-Px = glutathione peroxidase. *$0.01 < P \leq 0.05$, **$0.001 < P \leq 0.01$.

study, we hypothesized that piglets were suffering weaning stress. The results suggested that piglets in the CTRL group exhibited increased expression of neuropeptides, including SP, VIP and CGRP, to adapt to inflammation and oxidation because of weaning stress. PQQ supplementation increased the concentration of VMA compared with the CTRL group according to the quantitative detection of neurotransmitters in plasma. PQQ treatment tended to increase VMA compared with the ZNO group. The results demonstrated that PQQ influences neuromodulation to relieve weaning stress. The different concentrations of neurotransmitters in plasma demonstrate physiological adaptions and neuroplasticity in piglets. PQQ and ZnO both modulated the levels of neurotransmitters which might be one of the mechanisms responsible for the growth promoting effects of these additives. The mechanism of neuroprotection of PQQ in weaned pigs needs further study.

Plasma of PQQ-fed pigs displayed a metabolic signature distinct from the CTRL group on the OPLS-DA plot. The metabolomics showed that PQQ treatment up-regulated 139 and down-regulated 66 metabolites. PQQ up-regulated the expression of erucamide, formononetin and 3-methyl-L-histidine, and down-regulated aloesin and dibutyl adipate in plasma compared with the CTRL group. Erucamide is a primary fatty acid amide and accumulates in lung, kidney, liver, brain and intestine, and modulates other physiological functions in a receptor-mediated fashion (Hamberger and Stenhagen, 2003). Endogenous bioactive lipids can change the activation of neurotransmitters and redox factors during stress (Cravatt et al., 1995; Li et al., 2017).

The correlation heatmap showed that erucamide had a positive correlation with ATTD of CF, and a negative correlation with CGRP. Formononetin can lower blood sugar, improve insulin resistance (Oza and Kulkarni, 2019), reduce oxidative stress, relieve alloxan-induced diabetes (Qiu et al., 2017), and decrease the concentration of inflammatory cytokines in damaged portions of the brain (Li et al., 2018). Formononetin reduces mechanical allodynia and improves conduction velocity in sciatic nerve tissue (Oza and Kulkarni, 2020). Formononetin affects the PI3K/Akt signaling pathway to recover injured nerve functions after ischemic stroke and Alzheimer's disease (Ma and Wang, 2022; Wu et al., 2020). The correlation heatmap showed formononetin had a positive correlation with T-SOD, and a negative correlation with CGRP and MDA. 3-Methyl-L-histidine influences neurovascular units to improve memory functions in AD-model mice that exhibit the antidepressant phenotype (Ding et al., 2021; Kaneko et al., 2017). The correlation heatmap showed 3-methyl-L-histidine had a negative correlation with CGRP. PQQ has been shown to regulate glycolipid metabolism in the jejunum of weaned pigs via the AMP-activated protein kinase (AMPK) signaling pathway (Huang et al., 2022). Similarly, the Akt signaling pathway is regulated by PQQ with neuromodulation demonstrated in previous studies (Shi et al., 2022; Zhou et al., 2018), however the mechanism of action is not known and needs further study. Aloesin is a competitive inhibitor of tyrosinase and tyrosine hydroxylase, regulating hyperpigmentation (Hollinger et al., 2018; Jones et al., 2002). Dibutyl adipate is an opacifying agent and a surfactant-foam booster in cosmetic formulations (Andersen, 2006). The correlation heatmap showed a highly significant negative correlation among dibutyl adipate and ATTD. The results suggest that PQQ has a role in pigmentation.

5. Conclusion

Diets supplemented with PQQ or ZnO increased growth performance, regulated antioxidant capacity and decreased incidence of diarrhea in weaned pigs. Similarly, dietary supplementation with PQQ or ZnO could improve digestibility of dietary nutrients and regulate neurotransmitters in weaned pigs. PQQ showed a stronger effect on promotion of growth performance and function in neurotransmitters compared with ZnO. Metabolites regulated by PQQ suggested to us the mechanism of action for PQQ in weaned pigs. This study provides a new theoretical basis to explain the action of PQQ as an alternative to in-feed antibiotic in weaned pigs.

Author contributions

Chenyu Shi: conceptualization, data curation, formal analysis, investigation, methodology, writing – original draft. **Zirou Yu:** conceptualization, data curation, formal analysis, investigation, methodology, writing – original draft. **Zijie Wang:** data curation, methodology. **Ran Ning:** data curation, methodology. **Caiyun Huang:** data curation, methodology. **Youjun Gao:** methodology, funding acquisition. **Fenglai Wang:** conceptualization, writing – review, supervision, funding acquisition. All authors have read and agreed to the published version of the manuscript.

Declaration of competing 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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Appendix supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2023.06.015.

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