



Original Research Article

Enteromorpha polysaccharide-zinc replacing prophylactic antibiotics contributes to improving gut health of weaned piglets



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ABSTRACT

This research aimed to study whether *Enteromorpha* polysaccharide-zinc (EP-Zn) can act as an alternative to antibiotics in weaned piglet feeds. Two hundred and twenty-four weaned piglets from 14 pens were randomly assigned into 1 of 2 groups according to their body weight and litter size (7 pens/group). The piglets in the antibiotics group were fed with olaquinox at 400 mg/kg and enduracidin at 800 mg/kg basal diet, and piglets in the EP-Zn group were fed with EP-Zn at 800 mg/kg basal diet. One piglet per pen was selected to collect samples after 14 d of feeding. Results showed that EP-Zn supplementation significantly increased the plasma anti-oxidants level compared with the antibiotics group. However, a nonsignificant difference was observed in growth performance between treatment groups. Additionally, the intestinal tight junction (TJ) protein expression and the histopathologic evaluation data showed that EP-Zn contributed to improving intestinal development. Further, piglets in the EP-Zn group had a lower level of intestinal inflammation-related cytokines including IL-6 ($P < 0.001$), IL-8 ($P < 0.05$), IL-12 ($P < 0.05$) and tumor necrosis factor- α (TNF- α) ($P < 0.001$), and showed an inhibition of the phosphorylation nuclear transcription factor-kappa B (p-NF- κ B) ($P < 0.05$) and total NF- κ B ($P < 0.001$) level in the jejunal mucosa. Taken together, it is supposed that EP-Zn, to some extent, would be a potent alternative to prophylactic antibiotics in improving the health status of weaned piglets.

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1. Introduction

In modern pig husbandry systems, piglets in their weaning period experience a variety of environmental factors that change in time or intensity, such as mixing of the piglets from other pens,

feedstuffs, temperatures, medicines usage (antibiotics or vaccines), and so on (Qin et al., 2019). Indeed, antibiotics have been used in feed to improve pig health and growth performance (Diana et al., 2017). It has been reported that antibiotics administration via feed or water to pigs can cause more serious antibiotic resistance bacteria than parenteral treatment (Burow et al., 2019). Thus, prophylactic antibiotics have been frequently administered at the subtherapeutic level to prevent intestinal tract infections or improve the growth rate of piglets during the early weaning stage (Schokker et al., 2015), which has contributed to an increase in antibiotics resistant microbes for human as well as animals.

The prophylactic use of antibiotics in feed was banned in Europe and Australia in 2006. Following the Europe-wide ban of antimicrobial growth promoters, the use of zinc as a feed supplement

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increased in the livestock industry. Studies have shown that supplementation of piglets' diet with zinc could increase the proportion of multi-resistant *Escherichia coli* in vivo (Bednorz et al., 2013). With the ban of prophylactic antibiotics in China, there is a growing drive to search for a novel potential alternative to prophylactic antibiotics in weaned piglets.

Most natural polysaccharides possess multiple functions, such as antimicrobial (Ghazala et al., 2015), antioxidant (Salvia-Trujillo et al., 2016) and immune activities (Huang et al., 2006). As one of the common fouling green algae, *Enteromorpha* is a large medicinal and food algae, which is rich in active ingredients of polysaccharides, but it has also been associated with a number of environmental problems in past years, for example, unpleasant odors and adverse public reactions, etc (Zhao et al., 2014). Polysaccharides extracted from *Enteromorpha prolifera* have a potent antimicrobial, antioxidant (Shao et al., 2017), and immunomodulatory effect in vitro and in vivo (Kim et al., 2011). Algae polysaccharides are one of the best metal-ion chelating agents for their digestive availability and good stability of the polysaccharide-iron complexes (Chi et al., 2018). In addition, it is well established that the metal-chelating complex shows a high antioxidant capacity (Borrelli et al., 2003). Organic zinc has a higher bioavailability and fewer toxic side effects in the human body compared to inorganic zinc (Liao et al., 2015). Compared with polysaccharides or inorganic zinc alone, organic zinc polysaccharides have higher antioxidant and immune function (Wang et al., 2015). These reports confirmed that *Enteromorpha* polysaccharide-zinc (EP-Zn) might be a good candidate for exploration as a natural alternative to prophylactic antibiotics for its function in triggering immune responses.

Despite extensive studies on the biological activities of EP-Zn or organic zinc alone, few studies have been conducted about EP-Zn as a substitute for prophylactic antibiotics in weaned piglet diets. Based on the assumption that the immune function and antioxidant capacity of *Enteromorpha* polysaccharide and Zn might be strengthened via their synergic effect, we hypothesized that dietary supplementation with EP-Zn in weaned piglets could result in similar outcomes as antibiotics but with fewer side effects. When compared with antibiotics supplement under physiological conditions, for example, xylan-chitooligomer-zinc complex, possessing antioxidant and antimicrobial activity, has been explored for its use as a novel food preservative (Wu et al., 2013a). Thus, the objective of this study was to determine the efficacy of EP-Zn in replacing antibiotics in terms of growth performance and health status in weaned piglets.

2. Materials and methods

All animal care and experimental protocols in this study were approved by the Animal Care and Use Committee of the Institute of Subtropical Agriculture, Chinese Academy of Sciences (IACUC # 201302).

2.1. Animals and experimental treatments

Two hundred and twenty-four weaned piglets from 14 pens (16 piglets/pen) (Duroc × Landrace × Large Yorkshire) (Guangan Co., Zhengzhou, China) with average initial body weight (5.92 ± 0.19 kg) were used for this study. All the piglets in a pen were born by the same sow. The sex of the piglets were ignored except for balancing the number of piglets per pen. Piglets from 14 pens were then randomly assigned into 2 groups ($n = 7$ pens/group): (1) the antibiotics group in which piglets were fed the basal diet with olaquinox (400 mg/kg diet) and enduracidin (800 mg/kg diet), (2) the EP-Zn group in which piglets were fed the basal diet supplemented with the EP-Zn (800 mg/kg diet). The basal diet (Table 1) met or exceeded the nutritional requirements of weaned piglets as

recommended by the NRC (1998) (Council, 1999). The experiment was carried out from the end of July to early August in Henan province, China. The ambient temperature ranged from 24 to 31 °C, and the humidity was 60%. The whole experiment lasted 14 d. All piglets had free access to drink water and diets throughout the whole experimental period and were weighed at d 0 and 15. The feed consumption of piglets was measured daily, then the average daily weight gain (ADG; g/d) and feed-to-gain ratio of piglets were calculated. In addition, diarrhea incidence of piglets was also recorded and expressed as follows:

Diarrhea rate per pen (%) throughout the experiment = Number of diarrhea piglet × 100/16.

2.2. Sample collection

At the end of the experiment, all piglets fasted overnight, and one piglet of near average body weight per pen was chosen for blood sample collection (7 piglets/diet, $n = 7$) from the jugular vein as our previous study (Wu et al., 2018). The plasma sample was obtained by centrifugation at $3,000 \times g$ for 20 min at 4 °C. Then, the piglets were sacrificed by a jugular puncture after being anesthetized with sodium pentobarbital. The middle section of the jejunum and ileum tissue samples were collected and immediately fixed in formaldehyde solution for morphological analysis. The intestinal mucosa from the jejunum and ileum was obtained and immediately frozen in liquid nitrogen. All blood and intestinal mucosa samples were stored at -80 °C until analysis (Xie et al., 2015).

2.3. Biochemical assays

Plasma glucose, triglyceride (TG), cholesterol (CHO), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), immunoglobulin G (IgG), immunoglobulin M (IgM) and diamine oxidase (DAO) were measured using commercial kits (Sino-German Beijing Leadman Biotech Ltd., Beijing, China) and analyzed by a biochemical analytical instrument (Beckman CX4, Beckman Coulter Inc., Brea, CA). In addition, the plasma total antioxidant capacity (T-AOC), total superoxide dismutase activity (SOD), catalase activity (CAT) and malondialdehyde (MDA) were performed with commercially available kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.4. Determination of intestinal morphology

The jejunum and ileum segments were mounted in paraffin blocks with a routine histological method. Five-micrometer-thick sections were stained with the Masson's trichrome solution. Then, villus height and crypt depth were measured using an image-analysis system under a light microscope, and villus height: crypt depth ratio was calculated (Nikon, Japan).

2.5. RNA isolation and real-time quantitative PCR (RT-qPCR)

Approximately 0.1 g of jejunal and ileal mucosa samples were pulverized in liquid nitrogen, and total RNA was isolated with the TRIzol reagents (0.1 g tissue per 1 mL TRIzol; Invitrogen, Carlsbad, CA, USA). The RNA purity and yield were evaluated using a Nano-Drop 1000 (Thermo Fisher Scientific, New York, NY, USA) at an absorbance of 260/280. We considered the RNA for further analysis whose OD value at 260/280 was 1.8 to 2.0. Then, 1 µg of RNA sample was used to generate cDNA in a volume of 20 µL using the Prime Script RT reagent Kit (Takara, Tokyo, Japan).

Primers used in this study were designed with Primer 5.0, according to the pig sequence (Table 2). Real time-qPCR was

Table 1
Ingredients and nutrient composition of the piglet diets (as-fed basis, %).

Item	Contents
Major ingredients	
Corn	37.9
Rice (broken)	10.0
Wheat flour	12.8
Soy protein concentrate	4.0
Soybean meal (CP, 46%)	13.48
Soy oil	1.5
Fish meal (CP, 65%)	4.0
FFSB	4.0
Whey power (CP, 3.8%)	3.76
Glucose	2
CaHPO ₄ (23/17)	0.83
Calcium lactate	0.85
Salt	0.14
Sugar	2
Lys (78.8%)	0.39
Met (99%)	0.08
L-Thr (98.5%)	0.12
Lycine	0.1
Choline (60%)	0.07
Nucleotides	1
Flavoring agent	0.13
Vitamin-mineral premix ¹	0.4
Nutrient composition	
CP	19.37
ME, kcal/kg	3,434.6
Crude fat	4.52
Crude fibre	1.92
Crude ash	3.89
Salt	0.235
Ca	0.722
Available P	0.445
Lys	1.60
Met	0.67
Cys	0.465
Thr	1.07
Try	0.30

FFSB = full-fat soybean; ME = metabolizable energy.

¹ Premix provided the following per kilogram of diet: Fe (FeS-O₄·H₂O), 80 mg; Mn (MnSO₄·5H₂O), 45 mg; Zn (ZnO), 100 mg; Cu (CuSO₄·5H₂O), 20 mg; I (KI), 0.70 mg; Se (Na₂SeO₃·H₂O), 0.25 mg; vitamin A, 10,000 IU; vitamin D₃, 2,500 IU; vitamin E, 100 IU; vitamin K, 10 IU; vitamin B₂, 10 mg; vitamin B₆, 1 mg; vitamin B₁₂, 50 µg; biotin, 80 µg; folic acid, 5 mg; nicotinic acid, 15 mg; choline chloride, 1,500 mg.

performed as described previously (Xie et al., 2015). Briefly, the PCR reaction was run in an ABI Prism 7900 HT sequence detection system (Applied Biosystems, Foster, CA, USA) with a total volume of 10 µL, containing 5 µL of SYBR Premix Ex Taq (2 ×) mix, 0.2 µL ROX (50 ×), 1 µL cDNA, 3 µL doubled-distilled water and 0.4 µL forward and reverse pairs (10 mmol/L). The thermocycler protocol consisted of 30 s at 95 °C, followed by 40 cycles of 5 s at 95 °C, and 20 s at 60 °C. The β-actin was used as a housekeeping gene to normalize target gene levels, and relative mRNA expression of the target genes was expressed as a ratio to the mRNA level of the β-actin gene using the 2^{-ΔΔCt} method.

2.6. Western blotting

The jejunal and ileal mucosa samples were extracted using radio immunoprecipitation assay (RIPA) lysis buffer (Beyotime Biotechnology, China) containing 1 mmol/L phenylmethanesulfonyl fluoride (PMSF) and phosphatase inhibitors. After centrifugation at 12,000 × g for 10 min at 4 °C, the supernatants were collected as protein samples for the assay. Then, protein concentration was determined using a bicinchoninic acid (BCA) assay (Pierce,

ThermoFisher, USA), and 40 µg protein per lane were separated by SDS-PAGE gel and transferred onto nitrocellulose membranes. Then primary antibodies against claudin-1 (CST Inc., USA), occludin (Proteintech, USA), ZO-1 (Proteintech, USA), phosphorylation nuclear transcription factor-kappa B (p-NF-κB) (CST Inc., USA), total NF-κB (CST Inc., USA), nuclear factor erythroid-2 related factor 2 (Nrf2) (Abcam Inc., USA) and β-actin (Santa Cruz Inc., USA) were incubated at 4 °C for 12 h. The membranes were then incubated with the corresponding secondary antibody for 2 h, and detected by the EZ-ECL kit (Biological Industries, Cromwell, CT, USA). The intensities of protein bands were quantified using Alpha Imager 2200 software (Alpha Innotech Corporation, CA, USA). Western blot images were quantified by measuring the resultant signals and normalizing the data to β-actin abundance.

2.7. Statistical analysis

Statistical analyses were performed using the independent samples *t*-test (SPSS Statistics 17; SPSS Inc., Chicago, IL). All the results were expressed as means with the SEM. Mean differences were determined as statistically significant when probability values were less than 0.05, and probability less than 0.10 and equal or greater than 0.05 was considered as a tendency.

3. Results

3.1. Growth performance and diarrhea incidence of piglets

All piglets were weaned on d 21 and had the identical raising environment and nursery treatment. During the experimental period, all piglets consuming antibiotics or EP-Zn grew at a comparable growth rate, such as average daily gain ($P > 0.10$), feed intake ($P > 0.10$) and feed-to-gain ratio ($P > 0.10$) (Table 3). Moreover, the diarrhea rate of piglets in the EP-Zn group was less than that in the antibiotics group, especially at the 7th d of the experiment period ($P < 0.01$), which is one of the important health indexes for weaned piglets (Fig. 1).

3.2. Plasma biochemical parameters

The plasma biochemical parameters are listed in Table 4. Compared with prophylactic antibiotics, feeds supplemented with EP-Zn significantly increased the plasma IgG ($P < 0.05$) and IgM ($P < 0.001$). Moreover, piglets in the EP-Zn group had greater plasma glucose content ($P < 0.05$) than that in the antibiotics group, which was important to the survival of weaned piglets. In comparison, the plasma lipids and diamine oxidase levels did not differ between 2 groups ($P > 0.05$).

3.3. Intestinal morphology

The intestinal morphology results showed that EP-Zn or antibiotics supplementation had similar villus height ($P > 0.10$) and villus width ($P > 0.10$) in the jejunum and ileum. The ileal crypt depth in the EP-Zn group was significantly higher ($P < 0.05$) than the antibiotics group. Furthermore, piglets in the EP-Zn group exhibited a significantly lower ileum villus height: crypt depth ratio than the antibiotics group. No significant difference in crypt depth ($P > 0.10$) and villus height: crypt depth ratio ($P > 0.10$) in the jejunum were observed between EP-Zn and the antibiotics group (Fig. 2).

Table 2
Primers used for RT-qPCR.

Genes	Sequences (5' to 3')	Size, bp	Accession no.
<i>IL-1β</i>	F: GCTAACTACGGTGACAACAA R: TCITCATCGGCTTCTCCACT	196	XM_021085847.1
<i>IL-6</i>	F: TCAGTCCAGTCGCCTTCTCC R: GGCATTTGTGGTGGGGTTAG	494	NM_214399.1
<i>IL-8</i>	F: CTGGCTGTTGCCTTCTTG R: TCGTGGAATGCGTATTATG	115	NM_213867.1
<i>IL-10</i>	F: ATGGGGCGACTTGTGTGCTGAC R: CACAGGGCAGAAATGATGACA	217	NM_214041.1
<i>IL-12</i>	F: GCCAAGGTTACATGCCACAA R: TAGAACCTAATTGCAGGACACAGATG	108	NM_214013.1
<i>IL-17</i>	F: CTCTCGTGAAGCGGGAATC R: GTAATCTGAGGGCCGTCTGG	137	NM_001005729.1
<i>TNF-α</i>	F: ACTGCACTTCGAGGTTATCGG R: GGGACCGGCTTATCTGA	118	NM_214022.1
<i>IFN-γ</i>	F: TGGTAGCTCTGGGAAACTGAATG R: GGCTTTGCGCTGGATCTG	79	NM_213948.1
<i>GPX-1</i>	F: TGGGGAGATCCTGAATTG R: GATAAACTGGGGTCGGT	184	NM_214201.1
<i>GPX-4</i>	F: GATTCTGGCCTTCCCTTGC R: TCCCTTGGGCTGGACTTT	173	NM_214407.1
<i>CAT</i>	F: CGAAGCGAAGGTGTTT R: AGTGTGCGATCCATATCC	374	XM_021081498.1
<i>Cu/Zn-SOD</i>	F: CAGGTCTCACTCAATCC R: CCAACGACTTCCAGCAT	255	NM_001190422.1
<i>HSP70</i>	F: GCCCTGAATCCGAGAATA R: TCCCCACGGTAGGAAACG	152	NM_001123127.1
<i>HSP90</i>	F: GAGGAAACCCAGACCCAA R: AGAAGCCGACCCGAACT	409	NM_213973.2
<i>Claudin-1</i>	F: AAGGACAAAACCGTGTGGGA R: CTCTCCCACATTCGAGATGATT	247	NM_001244539.1
<i>ZO-1</i>	F: CCAACCATGTCTGAAGCAGC R: TGCAGGAGTGTGGTCTTAC	215	XM_021098896.1
<i>Occludin</i>	F: TCAGGTGCACCCTCCAGATT R: TATGTCTGCTGGGTGCAT	169	NM_001163647.2
<i>E-cadherin</i>	F: CAAACGGCCATTTCCAGCTTCA R: GTCACCTTGGTGACAGCTT	90	NM_001163060.1
<i>β-actin</i>	F: CTGCGCATCCACGAAACT R: AGGGCCGTGATCTCTTCTG	147	XM_021086047.1

IL-1β = interleukin-1 beta; *IL-6* = interleukin-6; *IL-8* = interleukin-8; *IL-10* = interleukin-10; *IL-12* = interleukin-12; *IL-17* = interleukin-17; *TNF-α* = tumor necrosis factor-alpha; *IFN-γ* = interferon-gamma; *GPX-1* = glutathione peroxidase-1; *GPX-4* = glutathione peroxidase-4; *CAT* = catalase activity; *Cu/Zn-SOD* = Cu/Zn superoxide dismutase; *HSP70* = heat shock protein 70; *HSP90* = heat shock protein 90; *ZO-1* = zonula occluden-1.

3.4. The relative level of oxidative stress related factors in the intestine and plasma

As shown in Table 5, the EP-Zn supplementation group had higher plasma glutathione peroxidase (GSH-Px) ($P < 0.01$), T-AOC ($P < 0.01$), and CAT activity ($P = 0.073$) than those in the antibiotics group. Also, piglets from the EP-Zn group had a higher MDA level ($P < 0.001$) than that in the antibiotics group. To further evaluate the antioxidant status of piglets, we studied the mRNA expression level of some key oxidative stress-related genes (Fig. 3A and B). RT-qPCR results showed that *GPX-4*, *CAT*, *Cu/Zn-SOD* and *HSP90* in the jejunal and ileal mucosa had no big difference between the 2 groups ($P > 0.10$). However, *GPX-1* level in the ileum from the EP-Zn group was less ($P < 0.01$) than that in the antibiotics group.

Table 3
The growth performance of weaning piglets¹.

Item	Antibiotics	EP-Zn	P-value
Initial body weight, kg	5.91 ± 0.25	5.92 ± 0.31	0.998
Final body weight, kg	9.24 ± 0.27	9.44 ± 0.47	0.719
Average daily gain, g	237.6 ± 15.54	251.8 ± 15.2	0.525
Average feed intake, g	379.4 ± 11.7	398.9 ± 16.7	0.357
Feed-to-gain ratio	1.64 ± 0.11	1.61 ± 0.09	0.841

EP-Zn = *Enteromorpha* polysaccharide-zinc.

¹ Values are expressed by means ± SEM, $n = 7$.

Interestingly, *HSP70* in the jejunal ($P = 0.07$) and ileal mucosa ($P < 0.05$) from the EP-Zn group were greater than those in the

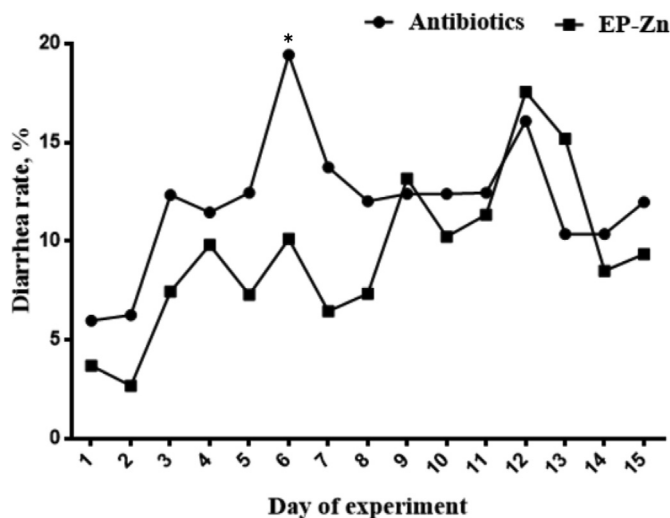


Fig. 1. The diarrhea rate of weaning piglets per pen through experiment. Diarrhea rate per pen (%) = Number of diarrhea piglet × 100/16. * indicates a statistically significant difference between antibiotics and EP-Zn groups ($P < 0.05$), $n = 7$. EP-Zn = *Enteromorpha* polysaccharide-zinc.

antibiotics group, which may have contributed to protecting the weaned piglets from hyperthermia in the summer.

Based on the above results, we also determined the relative expression of Nrf2 in the jejunal and ileal mucosa tissues (Fig. 3C and D), which is important for maintaining cellular redox homeostasis in weakening the oxidative stress-associated tissue destruction (Sima et al., 2016). Our results showed that compared with the antibiotics group, EP-Zn supplementation could upregulate the protein expression of Nrf2 ($P < 0.05$) in the jejunal and ileal mucosa tissues.

3.5. Intestinal NF- κ B and inflammatory cytokines expression

The mRNA expression of *IL-1 β* , *IL-6*, *IL-8*, *IL-10*, *IL-12*, *IL-17*, *TNF- α* and *IFN- γ* in the jejunal and ileal mucosa of piglets were also detected in this study (Fig. 4). We found that the EP-Zn group had significantly lower mRNA expression of *IL-6* ($P < 0.001$), *IL-8* ($P < 0.05$), *IL-10* ($P = 0.08$), *IL-12* ($P < 0.05$) and *TNF- α* ($P < 0.001$) in the jejunal mucosa (Fig. 4A). However, there was no significant difference in the mRNA expression of inflammatory cytokines ($P > 0.10$) in the ileal mucosa between the 2 groups, except for *IL-12* ($P < 0.05$) (Fig. 4B), suggesting the improvement of intestinal inflammation by EP-Zn supplement.

The NF- κ B transcription factor has been considered to be the key participant in innate and adaptive immune responses and the central mediator of the inflammatory process (Fan et al., 2013). Western blot results showed that EP-Zn supplementation inhibited the p-NF- κ B ($P < 0.05$) and total NF- κ B ($P < 0.001$) expression in the jejunal mucosa compared to those in the antibiotics group (Fig. 4C and D). Similar results were obtained in the ileal mucosa, although it was not as statistically significant ($P = 0.079$) as that in the jejunal mucosa (Fig. 4E and F).

3.6. EP-Zn substitution regulate the intestinal tight junction (TJ) protein of occludin expression

In addition to its effect on the antioxidative stress capacity and inflammatory responses in the intestine, the effect of EP-Zn supplementation on intestinal barrier function was evaluated in terms of relative mRNA expression and protein expression of TJ molecules. The RT-qPCR and WB results of TJ proteins in the jejunal mucosa are presented in Fig. 5 and the ileum mucosa results are shown in Fig. 6. As shown in Figs. 5A and 6A, no significant difference was observed in the relative mRNA expression levels of claudin-1, *ZO-1*, and E-cadherin ($P > 0.10$), except for the mRNA expression of occludin in the jejunal ($P < 0.05$) and ileal mucosa ($P < 0.05$), which was higher in the EP-Zn group than those in the antibiotics group. The Western blot results were consistent with

the mRNA results, which showed that EP-Zn supplementation improved the relative protein expression of Occludin in the jejunum and ileum, and Claudin-1 in the ileum (Fig. 5B and C, Fig. 6B and C).

4. Discussion

In this study, our results showed that dietary supplementation with EP-Zn could reach a similar effect as antibiotics on the growth performance of weaned piglets, which confirms that replacing antibiotics with EP-Zn will not affect the growth rate of weaned piglets.

Plasma immunoglobulin levels can provide essential information on the immune status, which are thought to provide protection against infectious diseases by the opsonization of pathogens or neutralizing toxins (Svendesen and Larsen 1977). Therefore, the greater plasma IgG and IgM in the EP-Zn group are thought to contribute to the defense against infectious disease by assisting the piglet's innate immune response. Moreover, it is important to the weaned piglets to get adequate plasma glucose, which helps to reduce hypoglycemia via maintaining energy supply (Xie et al., 2016). In contrast to the present finding, a previous study showed that *Enteromorpha* polysaccharide supplementation could improve glucose metabolism via reducing plasma glucose in diabetic rats (Lin et al., 2015), which may be due to the different material and animal model, but the exact reason for the difference needs further study. Hence, it is plausible that EP-Zn supplement, instead of prophylactic antibiotics, may help to improve the survival rate of weaned piglets by increasing the plasma immunoglobulin and glucose level.

Antioxidant enzymes are the first line of defense against reactive oxygen species (ROS), particularly against superoxide anion radicals (Cheng et al., 2014). It is well known that zinc is an inducer of the generation of metallothionein and co-factor of SOD; meanwhile, *Enteromorpha* polysaccharide could improve the enzymatic activities of GSH-Px and SOD (Prasad 2014; Wei et al., 2014; Lin et al., 2015). Moreover, it has been demonstrated that zinc polysaccharide complex could eliminate the increased oxidative stress through stimulating the activities of antioxidants or reducing the lipid peroxidative product level after being fed to the mice (Wang et al., 2015). In this study, EP-Zn supplemented in feeds not only increased the activities of plasma T-AOC, GSH-Px and SOD, but also increased plasma MDA of piglets, which reflects the lipid peroxidation level in the body or the degree of cellular damage (Wallace 2002), although these observations are not completely consistent with the previous study (Lin et al., 2015). It is known that both zinc deficiency and excessive zinc consumption can cause cellular oxidative stress. Hiller et al. (1995) also found that higher serum Zn levels were associated with higher levels of fat, which could increase superoxide production and the ROS-induced risk of diseases, and attenuate the antioxidant capacity (Moreno et al., 2005). We speculated that the higher MDA might be produced by the higher Zn concentration in the feeds, which lead to higher activities of plasma T-AOC, GSH-Px and SOD to achieve internal redox homeostasis in weaned piglets. However, the potential mechanism needs further investigation.

To further evaluate the redox state of weaned piglets, we determined the antioxidant related genes level in the jejunal and ileal mucosa. The overexpression of HSP70 can relieve oxidative stress, cell injury, and even has cytoprotective effects (Hirata et al., 2009; Wu et al. 2013b, 2013c). Interestingly, EP-Zn treatment increased or tended to increase the mRNA level of HSP70 in the jejunal and ileal mucosa. This result was in accordance with the previous study that zinc chelate could suppress small intestinal apoptosis by increasing HSP70 expression (Wu et al., 2010; Qin

Table 4

Plasma biochemical parameters of weaning piglets (mmol/L)¹.

Item	Antibiotics	EP-Zn	P-value
Glucose	5.38 ± 0.12	5.86 ± 0.18	0.041
TG	0.61 ± 0.07	0.49 ± 0.05	0.170
CHO	2.46 ± 0.14	2.40 ± 0.16	0.778
HDL-C	0.90 ± 0.06	0.87 ± 0.08	0.758
LDL-C	1.31 ± 0.10	1.30 ± 0.13	0.979
IgG, g/L	1.08 ± 0.05	1.28 ± 0.07	0.032
IgM, g/L	0.52 ± 0.03	0.91 ± 0.07	0.000
DAO	1.70 ± 0.11	1.62 ± 0.07	0.561

EP-Zn = *Enteromorpha* polysaccharide-zinc; TG = triglyceride; CHO = cholesterol; HDL-C = high-density lipoprotein cholesterol; LDL-C = low-density lipoprotein cholesterol; IgG = immunoglobulin G; IgM = immunoglobulin M; DAO = diamine oxidase.

¹ Values are expressed by means ± SEM, $n = 7$.

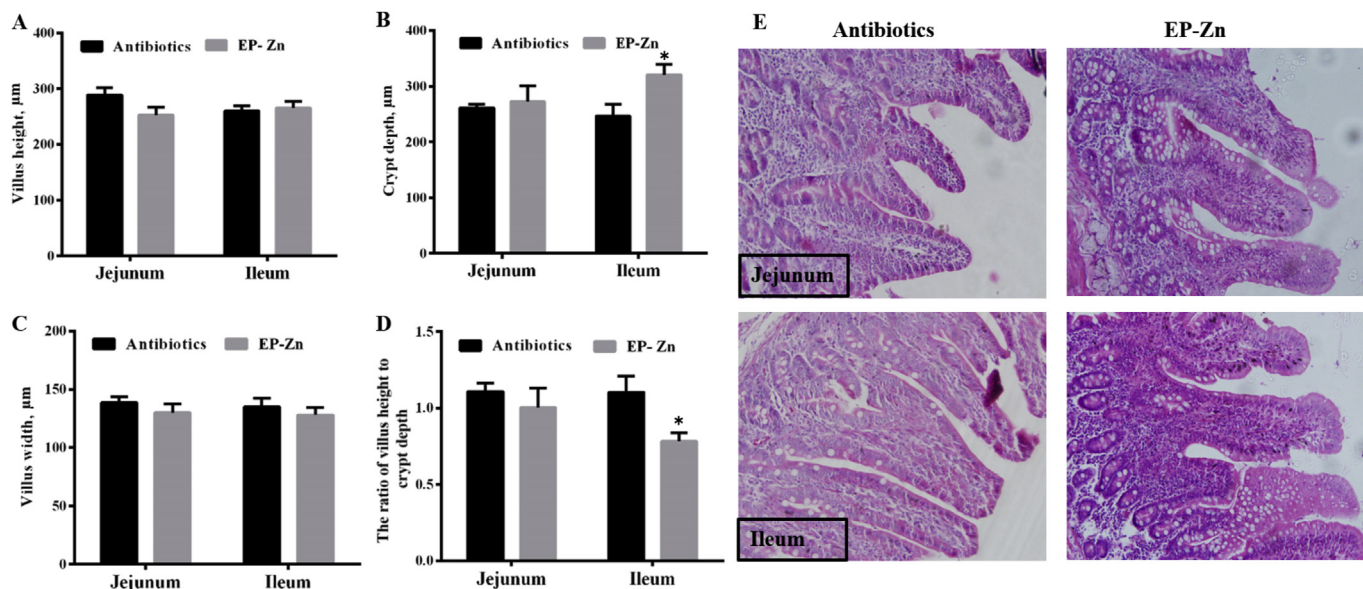


Fig. 2. Intestinal morphology of weaning piglets in the jejunum and ileum. (A) Villus height. (B) Crypt depth. (C) Villus width. (D) The ratio of villus height to crypt depth. (E) Representative image (200 × magnification) of intestinal morphology. Values are expressed as means ± SEM, $n = 7$. * indicates a statistically significant difference between antibiotics and EP-Zn groups ($P < 0.05$). EP-Zn = *Enteromorpha* polysaccharide-zinc.

Table 5

Plasma oxidative stress related enzyme activities and malondialdehyde (MDA) of weaning piglets (units/mL)¹.

Item	Antibiotics	EP-Zn	P-value
GSH-Px	477.1 ± 17.7	593.4 ± 30.0	0.006
T-AOC	1.45 ± 0.08	2.60 ± 0.27	0.005
CAT	199.1 ± 4.58	232.6 ± 16.4	0.073
T-SOD	136.8 ± 8.23	143.1 ± 5.09	0.529
MDA, nmol/mL	2.92 ± 0.11	9.55 ± 0.36	0.000

EP-Zn = *Enteromorpha* polysaccharide-zinc; GSH-Px = glutathione peroxidase; T-AOC = total antioxidant capacity; CAT = catalase activity; T-SOD = superoxide dismutase.

¹ Values are expressed by means ± SEM, $n = 7$.

et al., 2011). Furthermore, we also found EP-Zn supplement up-regulated the protein level of Nrf2, which was consistent with the results of plasma antioxidant enzymes in the weaned piglets. Nuclear factor erythroid-2 related factor 2 is not only crucial for the adaptive response under oxidative stress but also a key factor in maintaining redox homeostasis under normal conditions (Wu et al., 2012). It has been shown that knockout of Nrf2 in mice substantially increased the susceptibility of mice to a broad range of chemical toxicity and disease conditions associated with oxidative pathology (Kensler et al., 2007). Our results also showed that EP-Zn supplementation could protect weaned piglets from oxidative stress via increasing the Nrf2 expression in the small intestine. Collectively, it is inferred that EP-Zn supplementation is more advantageous than antibiotic supplementation in improving the antioxidant capacity of weaned piglets.

Weaning is often coupled with intestinal morphology damage, which has been widely applied to evaluate intestinal health or functions of piglets. Crypt depth and villus height: crypt depth ratio are critical factors to reflect the number and maturation rate of enterocytes, which affect the intestinal nutrients transport and absorption (Ma and He 2012; Szeffel et al., 2015). Our study showed that the jejunum morphology wasn't affected by EP-Zn or antibiotics supplement in feeds. In contrast, the crypt depth and villus height: crypt depth ratio in the ileum were improved in the piglets from the EP-Zn group, suggesting that EP-Zn could

improve ileum development without affecting the intestinal morphology of jejunum. In addition, the TJ proteins play an essential role in regulating intestinal epithelial permeability and the maintenance of barrier function (Suzuki 2013). Under physiological conditions, diet components play a vital role in intestinal TJ regulation, and some of these could be regarded as preventive or therapeutic tools in defective barrier-associated diseases (Ruemmele 2016). Interestingly, no big difference in the expression of intestinal TJ proteins was found. However, the occludin level increased in the jejunal and ileal mucosa, which is consistent with the observations in the small intestinal morphology and the diarrhea rate of weaned piglets. The epithelial TJ maintain the intestinal barrier and regulate the permeability of ions and nutrients (Lee 2015). These findings further confirmed that EP-Zn supplement, instead of antibiotics in weaned piglets feed, would have positive effects on the intestinal morphology, permeability and function of weaned piglets.

Several lines of evidence have shown that there is a relationship between epithelial TJ barrier disruption and the permeation of luminal pro-inflammatory molecules, which stimulates the mucosal immune system, generating sustained inflammation, and even tissue damage (Turner 2009; Lee 2015). It has been established that antibiotics have significant anti-inflammatory effects, including the alteration of neutrophil function by modulating the NF-κB pathway and pro-inflammatory cytokines (Buret 2010). A previous study showed *Enteromorpha* polysaccharide has a marked immunomodulatory effect through activating T cells by increasing IFN-γ and IL-2 secretions (J.K. Kim et al., 2011), and zinc supplementation suppressed the stimulation of NF-κB, inducing a lower level of inflammatory cytokines (Prasad 2014). Our results showed that EP-Zn supplementation significantly inhibited the activation of NF-κB in the jejunal mucosa, and attenuated the inflammatory cytokines release when compared with antibiotics supplemented in weaned piglet feeds, suggesting EP-Zn can be added to the feed of weaned piglets as an alternative to antibiotics to alleviate chronic immune adjustment (W. T. Oliver et al., 2014). Hence, it is plausible that we could withdraw prophylactic antibiotics from the weaned piglet diet and supplement EP-Zn as a substitute.

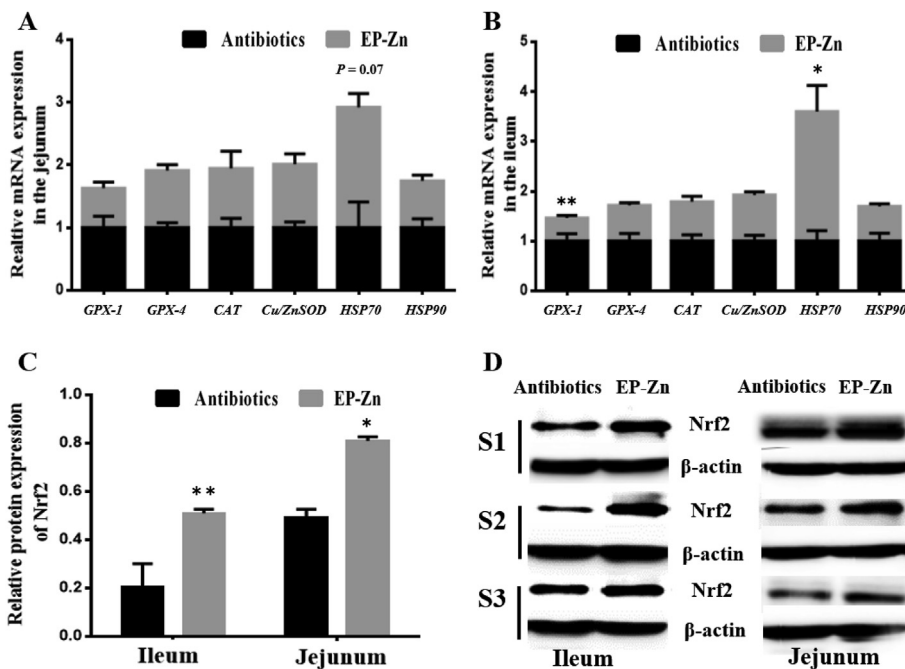


Fig. 3. The relative levels of nuclear factor erythroid-2 related factor 2 (Nrf2) and antioxidant related genes in the jejunum and ileum. (A) Antioxidant related genes in the jejunum. (B) Antioxidant related genes in the ileum. (C and D) Relative protein expressions of Nrf2 in the jejunum and ileum. S1, S2 and S3 denote different samples. Values are expressed as means \pm SEM, $n = 7$; * indicates a statistically significant difference between antibiotics and EP-Zn group ($P < 0.05$); ** indicates a statistically significant difference between antibiotics and EP-Zn group ($P < 0.01$). EP-Zn = *Enteromorpha* polysaccharide-zinc.

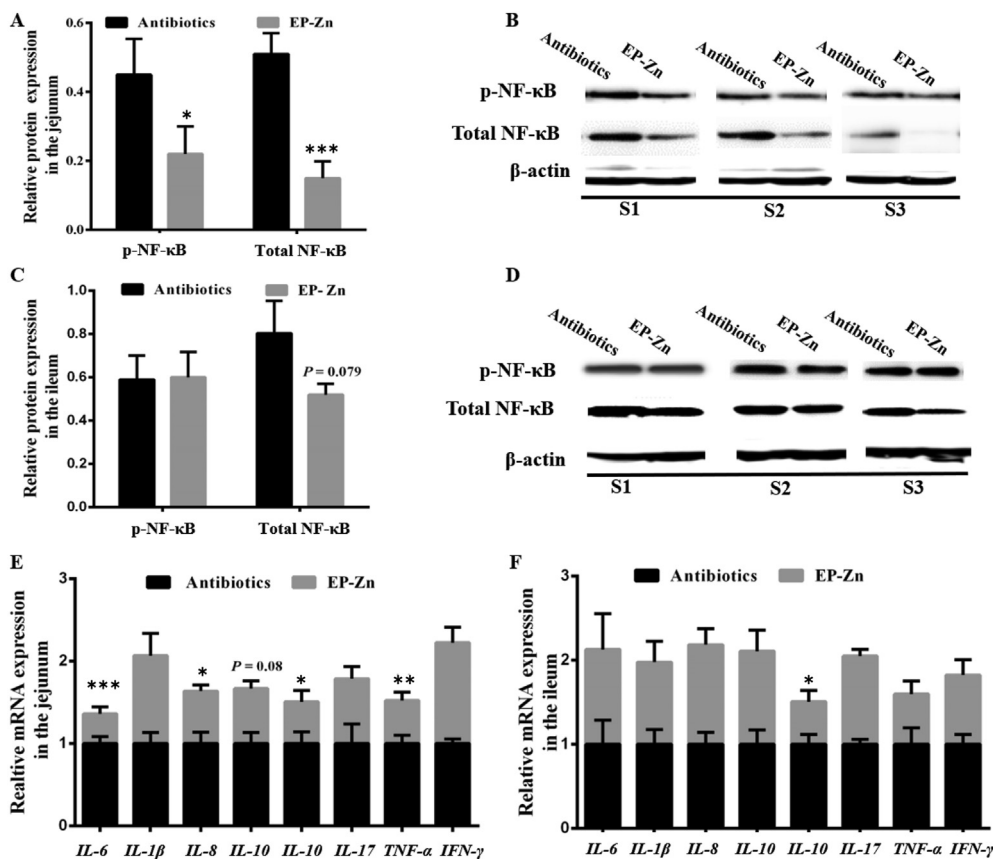


Fig. 4. The relative expression of phosphorylation nuclear transcription factor-kappa B (NF-κB) and inflammatory cytokines in the jejunum and ileum. (A and B) are the relative mRNA expressions of inflammatory cytokines in the jejunum and ileum, respectively; (C, D, E and F) are the relative protein expressions of p-NF-κB and total NF-κB in the jejunum and ileum, respectively. S1, S2 and S3 denote different samples. Values are expressed as means \pm SEM, $n = 7$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. p-NF-κB = phosphorylation nuclear transcription factor-kappa B; EP-Zn = *Enteromorpha* polysaccharide-zinc; IL = interleukin; TNF = tumor necrosis factor; IFN = interferon.

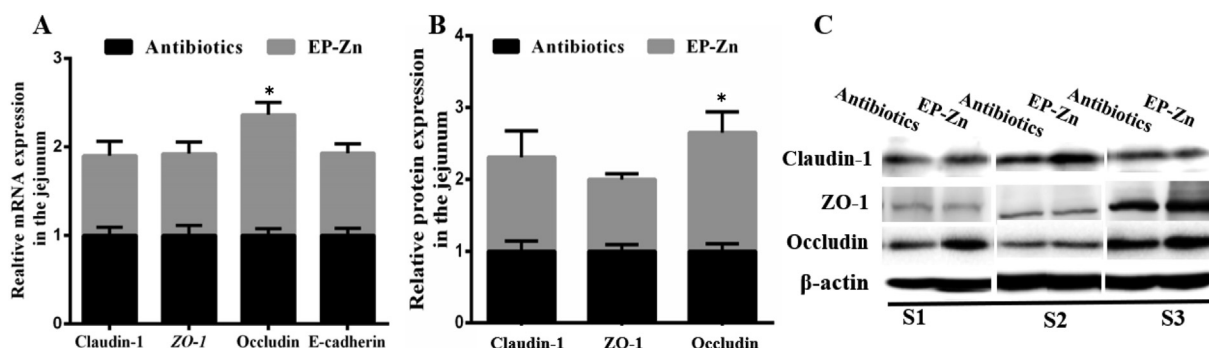


Fig. 5. The relative mRNA and protein expression of tight junction (TJ) proteins in the jejunum. (A) Relative mRNA expression. (B and C) Relative protein expression. S1, S2 and S3 denote different samples. Values are expressed as means \pm SEM, $n = 5$. * $P < 0.05$. EP-Zn = *Enteromorpha* polysaccharide-zinc; ZO-1 = zonula occluden-1.

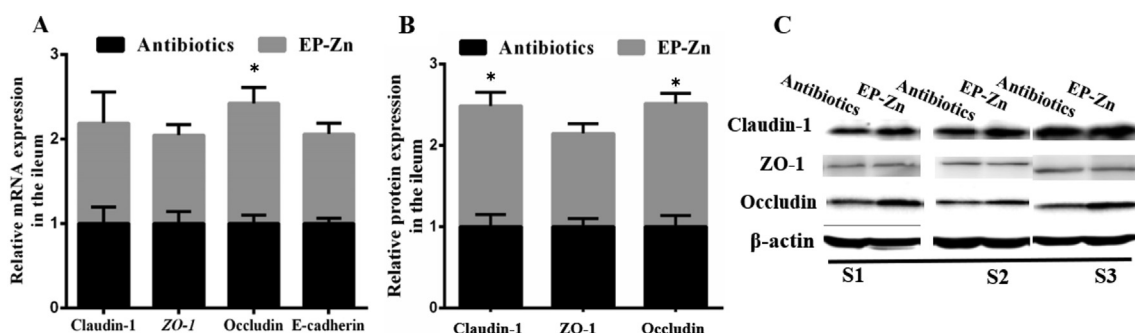


Fig. 6. The relative mRNA and protein expression of tight junction (TJ) proteins in the ileum. (A) Relative mRNA expression. (B and C) Relative protein expression. S1, S2 and S3 denote different samples. Data are presented as means \pm SEM, $n = 5$. * $P < 0.05$. EP-Zn = *Enteromorpha* polysaccharide-zinc; ZO-1 = zonula occluden-1.

However, a limitation must be acknowledged in the animal experiment. We did not set one group without antibiotics and EP-Zn supplements as a normal control in this study for some reasons. First, weaning piglets on most livestock farms in China without prophylactic antibiotic supplementation would pose an acceptable threat to the life of the piglets before 2020, although it is illegal to add prophylactic antibiotics to piglets feed now; Second, we should not completely disturb the management system of the pig farm during the experiment. Thus, further researches are still necessary to be done for evaluating the effect of withdrawal of prophylactic antibiotics in weaned piglets.

5. Conclusions and prospects

Concerning the potent threat that weaned piglets encounter now, the breeding industry has growing pressure to lower or remove antibiotics from feeds. In this study, we found that EP-Zn could be a potential substitution for prophylactic antibiotics in feeds through assessing the growth performance, small intestine development, and antioxidant/inflammatory capacities of weaned piglets under physiological conditions. Ultimately, our findings may supply a new strategy for reducing or eliminating prophylactic antibiotics in weaned piglet feeds, together with efficient management systems.

Author contributions

Chunyan Xie: Conceptualization, methodology, investigation, formal analysis, data curation, writing original draft. **Yumei Zhang:** Conceptualization, methodology. **Kaimin Niu:** Writing original draft. **Xiaoxiao Liang:** conceptualization, methodology. **Haihua**

Wang: Conceptualization, methodology. **Junwei Shan:** Methodology. **Xin Wu:** Supervision, writing - review & editing.

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.

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