



## Nano chitosan–zinc complex improves the growth performance and antioxidant capacity of the small intestine in weaned piglets

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### Abstract

The present study was conducted to test the hypothesis that dietary supplementation with a nano chitosan–zinc complex (CP–Zn, 100 mg/kg Zn) could alleviate weaning stress in piglets challenged with enterotoxigenic *Escherichia coli* K88 by improving growth performance and intestinal antioxidant capacity. The *in vivo* effects of CP–Zn on growth performance variables (including gastrointestinal digestion and absorption functions and the levels of key proteins related to muscle growth) and the antioxidant capacity of the small intestine (SI) were evaluated in seventy-two weaned piglets. The porcine jejunal epithelial cell line IPEC-J2 was used to further investigate the antioxidant mechanism of CP–Zn *in vitro*. The results showed that CP–Zn supplementation increased the jejunal villus height and decreased the diarrhoea rate in weaned piglets. CP–Zn supplementation also improved growth performance (average daily gain and average daily feed intake), increased the activity of carbohydrate digestion-related enzymes (amylase, maltase, sucrase and lactase) and the mRNA expression levels of nutrient transporters (Na<sup>+</sup>-dependent glucose transporter 1, glucose transporter type 2, peptide transporter 1 and excitatory amino acid carrier 1) in the jejunum and up-regulated the expression levels of mammalian target of rapamycin (mTOR) pathway-related proteins (insulin receptor substrate 1, phospho-mTOR and phospho-p70S6K) in muscle. In addition, CP–Zn supplementation increased glutathione content, enhanced total superoxide dismutase (T-SOD) and glutathione peroxidase (GSH-px) activity, and reduced malondialdehyde (MDA) content in the jejunum. Furthermore, CP–Zn decreased the content of MDA and reactive oxygen species, enhanced the activity of T-SOD and GSH-px and up-regulated the expression levels of nuclear factor erythroid 2-related factor 2 (Nrf2) pathway-related proteins (Nrf2, NAD(P)H:quinone oxidoreductase 1 and haeme oxygenase 1) in lipopolysaccharide-stimulated IPEC-J2 cells. Collectively, these findings indicate that CP–Zn supplementation can improve growth performance and the antioxidant capacity of the SI in piglets, thus alleviating weaning stress.

**Key words:** Weaned piglets; Chitosan–zinc nanoparticles; Growth performance; Antioxidant capacity; Small intestine

The small intestine (SI) is an important nutrient digestion and absorption site for mammals (including pigs and humans), and its health is closely related to the growth of young animals. In pig production, early-weaned piglets are susceptible to infection by enteropathogenic bacteria because the immune function of the gastrointestinal tract has not yet matured<sup>(1–3)</sup>. In particular, severe diarrhoea after weaning due to infection with

enterotoxigenic *Escherichia coli* (ETEC) is a main cause of piglet growth performance decline and even death<sup>(4,5)</sup>. ETEC and its derived enterotoxins can stimulate the secretion of pro-inflammatory factors and induce oxidative stress, which further cause epithelial cell apoptosis and villous atrophy in the SI<sup>(5–7)</sup>. Atrophy of the villi in the SI (especially in the jejunum) directly disturbs the transport of nutrients and ultimately affects the growth

**Abbreviations:** ADG, average daily gain; CON, basal diet; CP–Zn, basal diet supplemented with 100 mg/kg Zn from CP–Zn; CS + ZnSO<sub>4</sub>, basal diet supplemented with 100 mg/kg Zn from ZnSO<sub>4</sub> and 766 mg/kg of CS; CS, chitosan; basal diet supplemented with 766 mg/kg of CS but not supplemented with Zn; EAAC1, excitatory amino acid carrier 1; ETEC, enterotoxigenic *Escherichia coli*; GLUT2, glucose transporter type 2; GSH-px, glutathione peroxidase; HO1, haeme oxygenase 1; H–ZnO, basal diet supplemented with 1600 mg/kg Zn from ZnO; IGF1, insulin-like growth factor 1; IRS1, insulin receptor substrate 1; LPS, lipopolysaccharide; MDA, malondialdehyde; mTOR, mammalian target of rapamycin; NQO1, NAD(P)H:quinone oxidoreductase 1; Nrf2, nuclear factor erythroid 2-related factor 2; PEPT1, peptide transporter 1; ROS, reactive oxygen species; SGLT1, Na<sup>+</sup>-dependent glucose transporter 1; SI, small intestine; SLC6A19, neutral amino acid transporter; T-SOD, total superoxide dismutase; VH, villus height; ZnSO<sub>4</sub>, basal diet supplemented with 100 mg/kg Zn from ZnSO<sub>4</sub>.

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performance and feed conversion efficiency of piglets<sup>(1,2)</sup>. Dietary supplementation with pharmacological concentrations of inorganic Zn (1000–3000 mg/kg Zn<sup>2+</sup>) is an effective nutrition strategy and can improve intestinal health and alleviate weaning stress in weaned piglets<sup>(8,9)</sup>. However, high dietary Zn supplementation also increases the concentration of Zn in faeces, thereby polluting the environment<sup>(10)</sup>. As a result, many countries have issued policies to limit high-Zn diets in weaned piglets. At present, the Ministry of Agriculture and Rural Affairs of China has stipulated that the maximum Zn content in the feed of weaned piglets should be 1600 mg/kg<sup>(8)</sup>. Therefore, it is necessary to find an effective and environmentally safe alternative.

Recently, the potential applications of nanotechnology in feed additives have been investigated<sup>(11)</sup>. The use of nano-sized ZnO to alleviate weaning stress in piglets can reduce the final Zn concentration in the diet<sup>(8)</sup>. However, the paradox is that nano-sized ZnO causes cytotoxicity in the SI and can also disrupt liver metabolism<sup>(12,13)</sup>. Chitosan (CS) is a natural product obtained from partial *N*-deacetylation of chitin, which is the second most abundant carbohydrate polymer in nature<sup>(14)</sup>. The structure of CS is well suited for Zn ion binding via ion exchange, complexation, physical sorption and inter- and intra-molecular entrapment<sup>(15)</sup>. Some studies have shown that CS–Zn chelate (in non-nanoparticle form) has a positive effect on the intestinal health of weaned piglets<sup>(16,17)</sup>. However, the regulatory mechanism of the CS–Zn complex on piglet growth performance and intestinal antioxidant activity needs to be further clarified. In mammals, nanoparticles may have a higher bioavailability due to easier diffusion into the intestinal submucosa<sup>(18,19)</sup>. Thus far, some synthetic methods for loading Zn ions into CS-based nanoparticles have been widely studied in the field of agricultural science<sup>(20,21)</sup>. However, little is known about the roles of CS–Zn nanoparticles in mammals. CS–Zn nanoparticles exhibit good antibacterial activity and have lower cytotoxicity than inorganic Zn *in vitro*<sup>(22,23)</sup>, suggesting that they may protect the intestine against ETEC infection *in vivo*. In this study, a nano chitosan–Zn complex (CP–Zn; particle diameter 50–150 nm) was synthesised by an ionotropic gelation method. Considering the similarities in anatomy and physiology of the gastrointestinal tract between pigs and humans<sup>(24)</sup>, studying the intestinal health and growth mechanisms of weaned piglets could provide insights for future studies on infants and young children. Here, we hypothesised that dietary supplementation with a low concentration of Zn from CP–Zn could alleviate weaning stress in piglets. We combined an *in vivo* experiment on weaned piglets challenged with ETEC K88 and an *in vitro* experiment on IPEC-J2 cells to study the effects of CP–Zn on the growth performance and antioxidant capacity of the SI in weaned piglets and elucidate the underlying mechanisms.

## Materials and methods

### Ethics approval

All experimental protocols and procedures involving piglets were conducted in accordance with the guidelines established by the Institutional Animal Care and Use Committee of Nanjing Agricultural University. The study was approved by

the Animal Care and Use Committee of Nanjing Agricultural University (approval no. SYXK 2019-0771).

### Diets, experimental design and sample collection

Piglets received diets supplemented with different feed additives based on an antibiotic-free maize/soyabean meal that were formulated according to the recommendations of the National Research Council (NRC, 2012). The composition and nutrient content of the basal diet are listed in online Supplementary Table S1. CS with a deacetylation degree >95% and an average molecular weight of 80 000–90 000 Da was purchased from Haidebei Marine Biological Engineering Co. Ltd. Zinc sulphate heptahydrate (ZnSO<sub>4</sub>) and ZnO, analytical reagents, were purchased from Sinopharm. CP–Zn (diameter 50–150 nm), a CS–Zn complex containing 5–10% Zn and 50–60% CS, was made in our laboratory at the College of Animal Science and Technology of Nanjing Agricultural University (Chinese patent: 201910039700.4).

A total of seventy-two piglets (Duroc × Landrace × Large White, male) weaned at day 21 with an average body weight of 6.21 (SD 0.49) kg were randomly divided into six treatment groups, twelve piglets in each treatment, and three piglets in one pen. The six dietary treatment groups are as follows: (1) basal diet (CON group); (2) the basal diet supplemented with 1600 mg/kg Zn from ZnO (H-ZnO group); (3) the basal diet supplemented with 100 mg/kg Zn from ZnSO<sub>4</sub> (ZnSO<sub>4</sub> group); (4) the basal diet supplemented with 100 mg/kg Zn from CP–Zn (CP–Zn group); (5) the basal diet supplemented with 100 mg/kg Zn from ZnSO<sub>4</sub> and the same concentration of CS (766 mg/kg) as the CP–Zn group (CS + ZnSO<sub>4</sub> group) and (6) the basal diet supplemented with the same concentration of CS as that in the CP–Zn group but not supplemented with Zn (CS group). All piglets had free access to feed and water. The pig pens were cleaned twice a day to maintain cleanliness, the ambient temperature of the pig house was maintained at 22–25°C, and the living environment of the piglets was in accordance with the animal welfare guidelines.

A 14-d feeding trial was performed, the diarrhoea incidence, average daily gain (ADG), average daily feed intake and feed:gain ratio of weaned piglets were determined according to the methods described by Long *et al.*<sup>(25)</sup> and the pen was used as the experimental unit. The piglet faeces were visually assessed every day by observers blinded to the treatments. Fresh excreta were graded according to the following scale: 1 = hard faeces, 2 = slightly soft faeces, 3 = soft faeces (partially formed), 4 = loose (semi-liquid) faeces and 5 = watery (mucous-like) faeces. Piglets with stool scores of 4 and 5 were identified as having diarrhoea. The diarrhoea incidence was calculated according to the following formula: diarrhoea rate (%) = 100 × (number of piglets with diarrhoea × number of diarrhoea days)/(total number of piglets × number of experiment days). After the feeding trial, at 08.00 hours on the 15th day, six piglets were randomly selected from each treatment group for oral ingestion of ETEC K88 at a concentration of approximately 1 × 10<sup>9</sup> colony-forming units/ml in 10 ml of PBS per piglet according to the methods of Huang *et al.*<sup>(5)</sup>. After 48 h of ETEC K88 challenge, all challenged piglets were slaughtered and sampled. The piglets were



anaesthetised with intravenous sodium pentobarbital (50 mg/kg body weight), and the piglets were euthanised by a competent person in a separate room out of sight of the other piglets. Blood was sampled from the anterior vena cava and centrifuged at 860 *g* for 15 min to obtain serum. Then, the serum samples were stored at  $-80^{\circ}\text{C}$  for subsequent analysis. The contents of the gastrointestinal tract (stomach, duodenum and jejunum), the mucosa of the SI (duodenum and jejunum) and the longissimus dorsi muscle were collected and immediately stored in liquid  $\text{N}_2$  for subsequent analysis. In addition, fresh faeces at the end of the rectum of each piglet were collected and immediately stored at  $4^{\circ}\text{C}$ . The live *E. coli* in faeces were quantitated using MacConkey agar (Beijing AOBIO Biotechnology) within 24 h according to the methods of a previous study<sup>(25)</sup>.

#### Digestive organ index assessment and haematoxylin–eosin staining

After piglets were slaughtered, the stomach and SI of each piglet were removed. The wet weights of these organs were determined after gently removing the mesentery and fat on the organ surface. Then, the length of the SI was measured with a ruler. Next, samples of duodenal and jejunal tissue were lightly cut and fixed in a 4% paraformaldehyde solution for 24 h. The fixed samples were dehydrated with alcohol, cleared with xylene and finally embedded in paraffin. The embedded samples were sectioned into 5  $\mu\text{m}$  thick sections and stained with haematoxylin–eosin. The stained sections were observed under a light microscope (Nikon YS100; Nikon Corporation). The villus height (VH) and crypt depth were measured using ImageJ software.

#### ELISA assay

The levels of growth hormone, insulin, insulin-like growth factor 1 (IGF1) and epidermal growth factor in serum were measured with ELISA kits purchased from CUSABIO Biotechnology according to the manufacturer's instructions. In addition, the levels of inflammatory cytokines, including TNF- $\alpha$ , IL-1 $\beta$ , interferon- $\gamma$  and myeloperoxidase in serum were determined with ELISA kits purchased from Enzyme-linked Biotechnology according to the manufacturer's instructions. For each type of assay, the intra-assay CV (CV%) was  $<5\%$  and the inter-assay CV% was  $<8\%$ .

#### Cell culture and treatment

Cells of the porcine jejunal epithelial cell line (IPEC-J2) were cultured in an antibiotic-free DMEM-F12 medium (Life Technologies) containing 10% fetal bovine serum (Life Technologies). The IPEC-J2 cells were maintained at  $37^{\circ}\text{C}$  in a humidified atmosphere of 5%  $\text{CO}_2$ . To evaluate the oxidative status and the expression levels of nuclear factor erythroid 2-related factor 2 (Nrf2) pathway-related proteins, the IPEC-J2 cells were pretreated with  $\text{ZnSO}_4$ , CP-Zn, CS +  $\text{ZnSO}_4$  or CS for 24 h and then co-treated with lipopolysaccharide (LPS) for another 6 h. The appropriate treatment concentrations of  $\text{ZnSO}_4$ , CP-Zn, CS +  $\text{ZnSO}_4$  and LPS were chosen based on the results of a cell viability assay conducted using an MTT kit (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instructions.

#### Analysis of digestive enzymes and antioxidant parameters

To determine the levels of digestive enzymes and antioxidant parameters, tissue and cell samples were homogenised and then centrifuged at 9500 *g* at  $4^{\circ}\text{C}$  for 15 min to obtain the supernatant. The protein concentrations of jejunal mucosal samples from weaned piglets and *in vitro* cell samples were determined with bicinchoninic acid kits (Solarbio Life Sciences). The activity levels of the digestive enzymes pepsin, trypsin, amylase, lipase, maltase, sucrase and lactase in the jejunums of weaned piglets were determined with commercial reagent kits (Nanjing Jiancheng Bioengineering Institute). Antioxidant parameters, namely, the levels of total superoxide dismutase (T-SOD), glutathione peroxidase (GSH-px), catalase, malondialdehyde (MDA), GSSG and GSH in the jejunal mucosa and serum of weaned piglets and in IPEC-J2 cells, were assayed with commercial reagent kits (Nanjing Jiancheng Bioengineering Institute). In addition, the intracellular reactive oxygen species (ROS) levels in IPEC-J2 cells were determined by the DCFH-DA method (Sigma) according to the manufacturer's instructions.

#### Quantitative real-time PCR analysis

Quantitative real-time PCR analysis was performed according to the methods in a previous study<sup>(26)</sup>. Briefly, total RNA was extracted from the jejunal mucosae of piglets using an RNAiso Plus Kit (TaKaRa) according to the instructions. The concentration and purity of RNA were determined with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific), and the quality and integrity of the PCR products were evaluated by electrophoresis on 1% agarose gels. Total RNA was reverse-transcribed into cDNA using a PrimeScript™ RT Reagent Kit (TaKaRa). The cDNA samples were twofold-diluted to determine the amplification efficiency. Quantitative real-time PCR was conducted with a QuantStudio® 5 Real-Time PCR Instrument (Applied Biosystems) using a TB Green® Premix Ex Taq™ II Kit (TaKaRa) according to the manufacturer's instructions. The relative mRNA expression levels were calculated using the  $2^{-\Delta\Delta\text{CT}}$  method with  $\beta$ -actin serving as the housekeeping gene<sup>(27)</sup>. The relative mRNA expression of each target gene was normalised to the expression in the control group. The primers are listed in online Supplementary Table S2.

#### Western blot analysis

Western blot analysis was performed according to Tang's work<sup>(26)</sup>. Briefly, all samples (longissimus dorsi muscle, jejunal mucosa and IPEC-J2 cells) were lysed using radio-immunoprecipitation assay (RIPA) buffer with protease inhibitors (Solarbio Life Sciences). Then, the samples were homogenised and centrifuged at 9500 *g* for 15 min at  $4^{\circ}\text{C}$ , and the supernatant was collected. For standardisation, the total protein concentrations of all samples were determined with a bicinchoninic acid kit. The extracted protein samples were electrophoresed on an 8% SDS-PAGE gel (GenScript) at 140 V for 50 min and then electrotransferred onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad) at 200 mA for 40–200 min. Primary antibodies against insulin receptor substrate 1 (IRS1), ribosomal S6 kinase (p70S6K), mammalian target of rapamycin (mTOR), Nrf2, NAD(P)H:quinone oxidoreductase 1 (NQO1), haeme oxygenase





1 (HO1) and  $\beta$ -actin (1:500–5000) were purchased from Proteintech. Primary antibodies against phospho-p70S6K and phospho-mTOR (1:1000) were purchased from Cell Signaling Technology. The PVDF membranes with migrated proteins were incubated with the primary antibodies overnight at 4°C. After washing with TRIS-buffered saline with Tween, the immunoblots were incubated with horseradish peroxidase-conjugated anti-rabbit (or anti-mouse) IgG (1:2000; Cell Signaling Technology) for 1 h at room temperature. Subsequently, the Western blot bands were visualised using a Tanon™ High-sig ECL Western Blotting Substrate (Tanon) with a Tanon 4200SF Gel Imaging System (Tanon), and the band densities were quantitated by densitometry using ImageJ software (NIH). The levels of all phosphorylated proteins were normalised to those of their corresponding non-phosphorylated proteins, and  $\beta$ -actin served as an internal reference.

### Statistical analysis

Statistical analyses were performed using GraphPad Prism version 7.01 (GraphPad Software Inc.) and SPSS 22.0 software. Significant differences among treatment groups were evaluated by one-way ANOVA (with Tukey's multiple comparisons test) if the data exhibited a Gaussian distribution and had equal variance or by the Kruskal–Wallis test (with Dunn's multiple comparisons test) if the data were not normally distributed. The data are presented as mean values with their standard errors. *P* values <0.05 were considered to indicate significant differences.

## Results

### Growth performance and diarrhoea rate

There were no adverse events in piglets during the whole experimental period. As shown in Table 1, there were no significant differences in the body weight of piglets on day 15 among all treatment groups (*P* > 0.05). However, the ADG in the H-ZnO group was significantly higher than that in the CON group (H-ZnO, 293.33 g; CON, 236.76 g; *P* < 0.05), and the ADG in the CP-Zn group was significantly higher than that in the CON and ZnSO<sub>4</sub> groups (ZnSO<sub>4</sub>, 247.86 g; CP-Zn, 312.02 g; *P* < 0.05). Compared with the values in the CON group, the

average daily feed intake values in the H-ZnO and CP-Zn groups were significantly higher (H-ZnO, 465.36 g; CON, 384.82 g; CP-Zn, 471.49 g; *P* < 0.05), while the feed:gain ratio in the CP-Zn group was significantly lower (CON, 1.63; CP-Zn, 1.51; *P* < 0.05). In addition, compared with the values in the CON group, the diarrhoea rate (H-ZnO, 7.14%; CON, 18.45%; CP-Zn, 5.95%; day 1–15) and the count of *E. coli* in faeces (log<sub>10</sub> colony-forming units/g wet faeces) (H-ZnO, 7.37; CON, 8.06; CP-Zn, 7.34; day 17) in the H-ZnO and CP-Zn groups were significantly lower (*P* < 0.05).

### Digestive organ indexes and small intestine morphology

The digestive organ indexes of weaned piglets are presented in online Supplementary Table S3, and the results showed that the SI weight in the CP-Zn group was significantly higher than that in the CON group (CON, 827.93 g; CP-Zn, 991.77 g; *P* < 0.05). In addition, as shown in Fig. 1, in the duodenum, there were no significant differences in the VH, villus width, crypt depth or VH:crypt depth ratio among all treatment groups (*P* > 0.05). However, the VH in the jejunum was significantly higher in the CP-Zn group than in the CON and ZnSO<sub>4</sub> groups (CON, 359.03  $\mu$ m; ZnSO<sub>4</sub>, 383.17  $\mu$ m; CP-Zn, 483.91  $\mu$ m; *P* < 0.05) and was significantly higher in the CS + ZnSO<sub>4</sub> group than in the CON group (CS + ZnSO<sub>4</sub>, 440.72  $\mu$ m; *P* < 0.05). Compared with the CON group, the CP-Zn group had a significantly higher VH:crypt depth ratio (CON, 1.52; CP-Zn, 2.15; *P* < 0.05).

### Digestive enzymes and nutrient transporters in the gastrointestinal tract

The digestive enzyme results are presented in Table 2. In the stomach, no significant differences were observed in the pepsin activity of the mucosa or the mucosal contents among all treatment groups (*P* > 0.05). In the SI, no significant differences were observed in the activity levels of trypsin and lipase in the duodenum and jejunum among all treatment groups (*P* > 0.05). Compared with the CON group, the CP-Zn, CS + ZnSO<sub>4</sub> and CS groups exhibited significantly higher amylase activity in the duodenum (CON, 327.10 U/mg protein; CP-Zn, 466.57 U/mg protein; CS + ZnSO<sub>4</sub>, 460.89 U/mg protein; CS, 451.95 U/mg protein; *P* < 0.05) and jejunum (CON, 175.37 U/mg protein;

**Table 1.** Effects of nano chitosan–zinc complex (CP-Zn) on growth performance and the diarrhoea rate in weaned piglets (Mean values and standard errors)

Variable*	H-ZnO	CON	ZnSO <sub>4</sub>	CP-Zn	CS + ZnSO <sub>4</sub>	CS	SEM	<i>P</i>
BW (day 1) (kg)	6.20	6.21	6.22	6.20	6.21	6.23	0.21	0.999
BW (day 15) (kg)	10.31	9.58	9.69	10.57	10.20	9.95	0.40	0.122
ADG (days 1–15) (g)	293.33 <sup>a,c</sup>	236.76 <sup>b</sup>	247.86 <sup>c</sup>	312.02 <sup>a</sup>	284.35 <sup>a,b,c</sup>	265.95 <sup>a,b,c</sup>	17.24	0.003
ADFI (days 1–15) (g)	465.36 <sup>a</sup>	384.82 <sup>b</sup>	397.62 <sup>a,b</sup>	471.49 <sup>a</sup>	442.2 <sup>a,b</sup>	416.67 <sup>a,b</sup>	23.33	0.006
F:G ratio (days 1–15)	1.59 <sup>a,b</sup>	1.63 <sup>a</sup>	1.60 <sup>a,b</sup>	1.51 <sup>b</sup>	1.56 <sup>a,b</sup>	1.57 <sup>a,b</sup>	0.03	0.030
Diarrhoea rate (days 1–15) (%), §†	7.14 <sup>a</sup>	18.45 <sup>b</sup>	16.67 <sup>a,b</sup>	5.95 <sup>a</sup>	9.52 <sup>a,b</sup>	13.10 <sup>a,b</sup>	1.89	0.002
Log <sub>10</sub> (CFU/g wet faeces)‡	7.37 <sup>a</sup>	8.06 <sup>b</sup>	7.93 <sup>a,b</sup>	7.34 <sup>a</sup>	7.69 <sup>a,b</sup>	7.76 <sup>a,b</sup>	0.20	0.005

H-ZnO, basal diet supplemented with 1600 mg/kg Zn from ZnO; CON, basal diet; ZnSO<sub>4</sub>, basal diet supplemented with 100 mg/kg Zn from ZnSO<sub>4</sub>; CP-Zn, basal diet supplemented with 100 mg/kg Zn from CP-Zn; CS + ZnSO<sub>4</sub>, basal diet supplemented with 100 mg/kg Zn from ZnSO<sub>4</sub> and 766 mg/kg of chitosan; CS, basal diet supplemented with 766 mg/kg of chitosan but not supplemented with Zn; BW, body weight; ADG, average daily gain; ADFI, average daily feed intake; F:G ratio, feed:gain ratio; CFU, colony-forming units.

<sup>a,b,c</sup> Mean values within a row with unlike superscript letters were significantly different (*P* < 0.05).

\*BW (*n* 12); ADG (*n* 4); ADFI (*n* 4); F:G ratio (*n* 4); diarrhoea rate (*n* 4).

† With §: Kruskal–Wallis test; without §: ordinary one-way ANOVA.

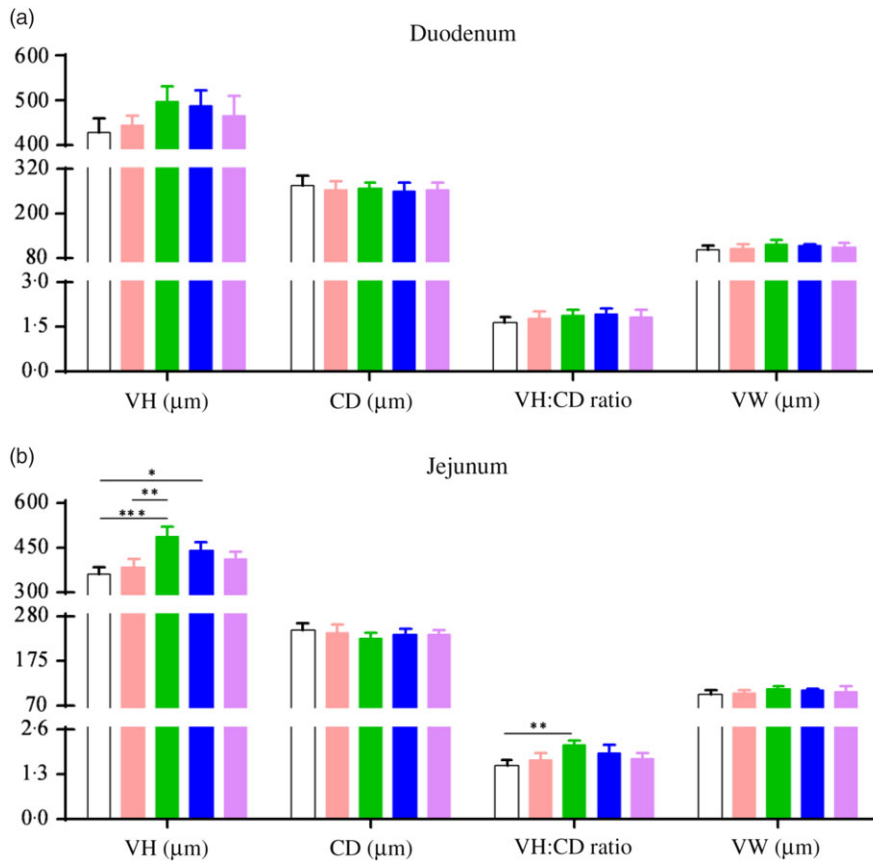
‡ CFU = count of *Escherichia coli* (log<sub>10</sub> CFU/g wet faeces) in faeces was determined on day 17 (*n* 6).

**Table 2.** Effects of nano chitosan–zinc complex (CP–Zn) on digestive enzymes of weaned piglets (*n* 6) (Mean values and standard errors)

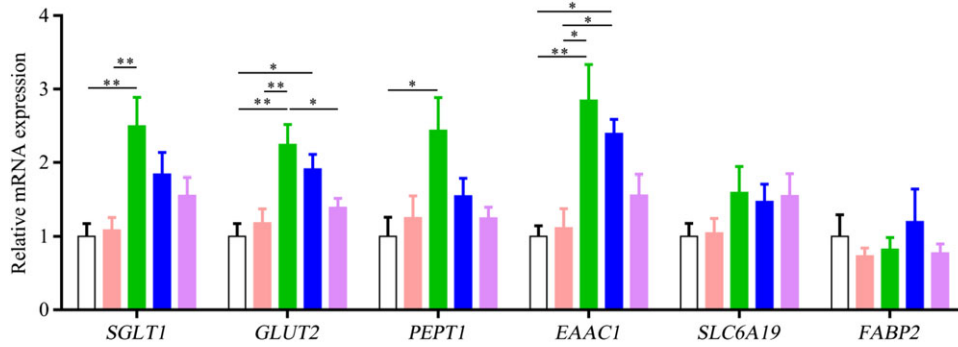
Variable	CON	ZnSO <sub>4</sub>	CP–Zn	CS + ZnSO <sub>4</sub>	CS	SEM	<i>P</i>
<b>Stomach</b>							
Pepsin (U/mg protein) contents	41.66	46.38	51.04	54.82	51.71	8.68	0.597
Pepsin (U/mg protein) mucosa	173.56	179.61	188.63	195.24	189.27	12.16	0.426
<b>Duodenum</b>							
Trypsin (U/mg protein) contents	56.55	61.53	67.31	66.11	61.94	5.566	0.347
Amylase (U/mg protein) contents	327.10 <sup>a</sup>	357.62 <sup>a,b</sup>	466.57 <sup>b</sup>	460.89 <sup>b</sup>	451.95 <sup>b</sup>	40.03	0.003
Lipase (U/mg protein) contents	30.00	32.70	36.60	35.40	34.94	3.99	0.503
<b>Jejunum</b>							
Trypsin (U/mg protein) contents	23.63	25.32	31.29	30.87	27.79	4.12	0.286
Amylase (U/mg protein) contents	175.37 <sup>a</sup>	185.42 <sup>a,b</sup>	269.49 <sup>c</sup>	256.29 <sup>c</sup>	251.17 <sup>b,c</sup>	23.97	0.001
Lipase (U/mg protein) contents, §*	15.25	17.53	14.35	15.08	11.34	4.50	0.283
Maltase (U/mg protein) mucosa	50.74 <sup>a</sup>	58.94 <sup>a,b</sup>	81.54 <sup>b,c</sup>	84.44 <sup>c</sup>	80.81 <sup>b,c</sup>	8.043	0.001
Sucrase (U/mg protein) mucosa	21.97 <sup>a</sup>	24.63 <sup>a,b</sup>	34.57 <sup>c</sup>	31.62 <sup>b,c</sup>	32.16 <sup>b,c</sup>	3.18	0.002
Lactase (U/mg protein) mucosa	5.70 <sup>a</sup>	6.63 <sup>a,b</sup>	9.26 <sup>b</sup>	9.09 <sup>b</sup>	7.90 <sup>a,b</sup>	1.13	0.016

CON, basal diet; ZnSO<sub>4</sub>, basal diet supplemented with 100 mg/kg Zn from ZnSO<sub>4</sub>; CP–Zn, basal diet supplemented with 100 mg/kg Zn from CP–Zn; CS + ZnSO<sub>4</sub>, basal diet supplemented with 100 mg/kg Zn from ZnSO<sub>4</sub> and 766 mg/kg of chitosan; CS, basal diet supplemented with 766 mg/kg of chitosan but not supplemented with Zn.

<sup>a,b,c</sup> Mean values within a row with unlike superscript letters were significantly different (*P* < 0.05).  
 \*With §: Kruskal–Wallis test; without §: ordinary one-way ANOVA.



**Fig. 1.** Effects of nano chitosan–zinc complex (CP–Zn) on intestinal morphology in weaned piglets. (a) Villus height (VH), crypt depth (CD), VH:CD ratio and villus width (VW) in the duodenum; (b) VH, CD, VH:CD ratio and VW in the jejunum. The VH:CD ratio in the duodenum and the CD in the jejunum were analysed with the Kruskal–Wallis test, and the other variables were analysed by ordinary one-way ANOVA. The data are mean values with their standard errors, *n* 6. \* *P* < 0.05, \*\* *P* < 0.01, \*\*\* *P* < 0.001. □, Basal diet; ■, basal diet supplemented with 100 mg/kg Zn from ZnSO<sub>4</sub>; ■, basal diet supplemented with 100 mg/kg Zn from CP–Zn; ■, basal diet supplemented with 100 mg/kg Zn from ZnSO<sub>4</sub> and 766 mg/kg of chitosan; ■, basal diet supplemented with 766 mg/kg of chitosan but not supplemented with Zn.



**Fig. 2.** Effects of nano chitosan–zinc complex (CP–Zn) on the mRNA expression levels of nutrient transporters in the jejunums of weaned piglets. *SGLT1*, Na<sup>+</sup>-dependent glucose transporter 1; *GLUT2*, glucose transporter type 2; *PEPT1*, peptide transporter 1; *EAAC1*, excitatory amino acid carrier 1; *SLC6A19*, neutral amino acid transporter; *FABP2*, fatty acid binding protein 2. The data for *PEPT1* and *FABP2* were analysed with the Kruskal–Wallis test, and the other data were analysed by ordinary one-way ANOVA. The data are mean values with their standard errors,  $n$  6. \*  $P < 0.05$ , \*\*  $P < 0.01$ . □, Basal diet; ■, basal diet supplemented with 100 mg/kg Zn from ZnSO<sub>4</sub>; ■, basal diet supplemented with 100 mg/kg Zn from CP–Zn; ■, basal diet supplemented with 100 mg/kg Zn from ZnSO<sub>4</sub> and 766 mg/kg of chitosan; ■, basal diet supplemented with 766 mg/kg of chitosan but not supplemented with Zn.

ZnSO<sub>4</sub>, 185.42 U/mg protein; CP–Zn, 269.49 U/mg protein; CS + ZnSO<sub>4</sub>, 256.29 U/mg protein; CS, 251.17 U/mg protein;  $P < 0.05$ ). The amylase activity of the jejunum was also significantly higher in the CP–Zn and CS + ZnSO<sub>4</sub> groups than in the ZnSO<sub>4</sub> group ( $P < 0.05$ ). With regard to disaccharide-splitting enzymes in the jejunum, the activity levels of maltase (CON, 50.74 U/mg protein; ZnSO<sub>4</sub>, 58.94 U/mg protein; CP–Zn, 81.54 U/mg protein; CS + ZnSO<sub>4</sub>, 84.44 U/mg protein; CS, 80.81 U/mg protein) and sucrase (CON, 21.97 U/mg protein; ZnSO<sub>4</sub>, 24.63 U/mg protein; CP–Zn, 34.57 U/mg protein; CS + ZnSO<sub>4</sub>, 31.62 U/mg protein; CS, 32.16 U/mg protein) were significantly higher in the CP–Zn, CS + ZnSO<sub>4</sub> and CS groups than in the CON group ( $P < 0.05$ ). In addition, the activity of maltase in the CS + ZnSO<sub>4</sub> group and sucrase in the CP–Zn group was significantly higher than those in the ZnSO<sub>4</sub> group ( $P < 0.05$ ). Compared with the CON group, the CP–Zn and CS + ZnSO<sub>4</sub> groups exhibited significantly higher lactase activity (CON, 5.70 U/mg protein; CP–Zn, 9.26 U/mg protein; CS + ZnSO<sub>4</sub>, 9.09 U/mg protein;  $P < 0.05$ ).

The mRNA expression levels of nutrient transporters in the jejunum are shown in Fig. 2. The relative mRNA expression levels of Na<sup>+</sup>-dependent glucose transporter 1 (*SGLT1*), glucose transporter type 2 (*GLUT2*) and excitatory amino acid carrier 1 (*EAAC1*) in the CP–Zn group were significantly higher than those in the CON and ZnSO<sub>4</sub> groups ( $P < 0.05$ ), and the relative mRNA expression level of peptide transporter 1 (*PEPT1*) in the CP–Zn group was significantly higher than that in the CON group ( $P < 0.05$ ). In addition, the relative mRNA expression level of *GLUT2* in the CS + ZnSO<sub>4</sub> group was significantly higher than that in the CON group ( $P < 0.05$ ), and the relative mRNA expression level of *EAAC1* in the CS + ZnSO<sub>4</sub> group was significantly higher than those in the CON and ZnSO<sub>4</sub> groups ( $P < 0.05$ ).

#### Growth factors and inflammatory cytokines in serum

The concentrations of hormones in serum are shown in Fig. 3(a)–(d). The results showed that there were no significant differences in the levels of growth hormone and epidermal growth factor among all treatment groups ( $P > 0.05$ ). The insulin content in the CP–Zn group was significantly higher than that in

the CON group (CON, 10.09  $\mu$ IU/ml; CP–Zn, 15.47  $\mu$ IU/ml;  $P < 0.05$ ), and the IGF1 content in the CP–Zn group was significantly higher than that in the CON and ZnSO<sub>4</sub> groups (CON, 65.22 ng/ml; ZnSO<sub>4</sub>, 68.48 ng/ml; CP–Zn, 93.74 ng/ml;  $P < 0.05$ ).

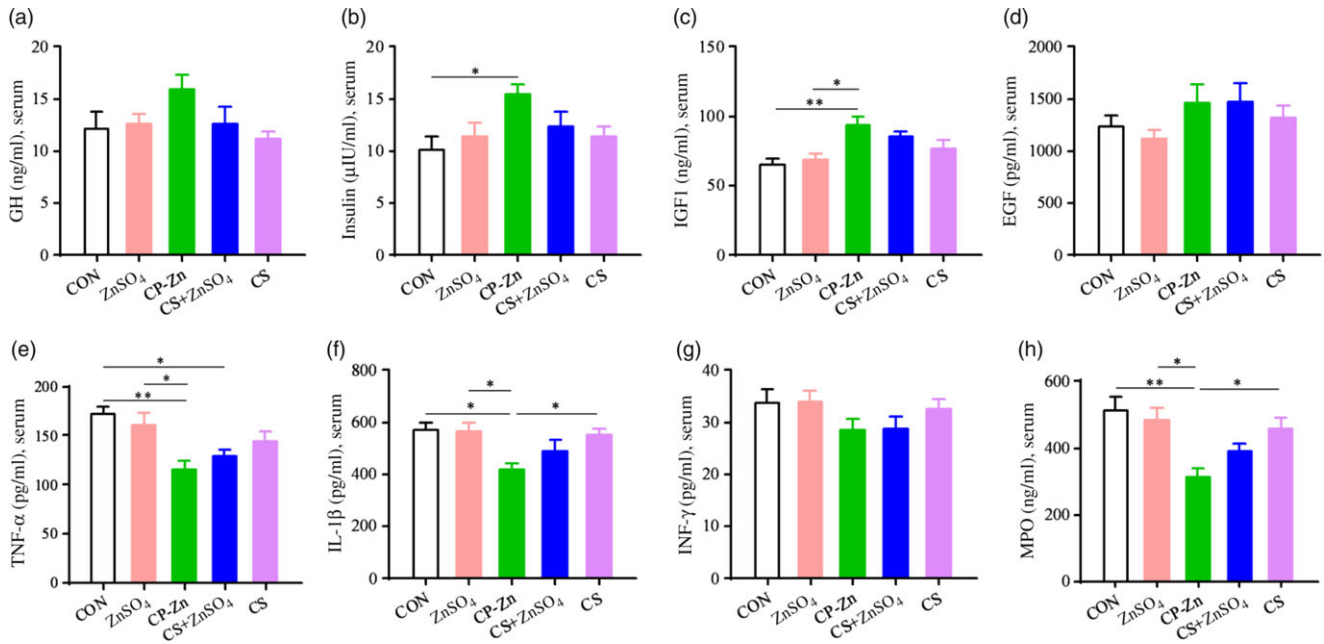
The inflammatory cytokine content of serum is shown in Fig. 3(e)–(h). The content of TNF- $\alpha$  in the CP–Zn group was significantly lower than that in the CON and ZnSO<sub>4</sub> groups (CON, 171.19 pg/ml; ZnSO<sub>4</sub>, 160.55 pg/ml; CP–Zn, 115.29 pg/ml;  $P < 0.05$ ), and the content of TNF- $\alpha$  in the CS + ZnSO<sub>4</sub> group was also significantly lower than that in the CON group ( $P < 0.05$ ). The levels of IL-1 $\beta$  (CON, 568.44 pg/ml; ZnSO<sub>4</sub>, 565.84 pg/ml; CP–Zn, 421.25 pg/ml; CS, 553.60 pg/ml) and myeloperoxidase (CON, 512.39 ng/ml; ZnSO<sub>4</sub>, 483.69 ng/ml; CP–Zn, 311.91 ng/ml; CS, 456.04 ng/ml) in the CP–Zn group were significantly lower than those in the CON, ZnSO<sub>4</sub> and CS groups ( $P < 0.05$ ).

#### Mammalian target of rapamycin pathway-related proteins in muscle

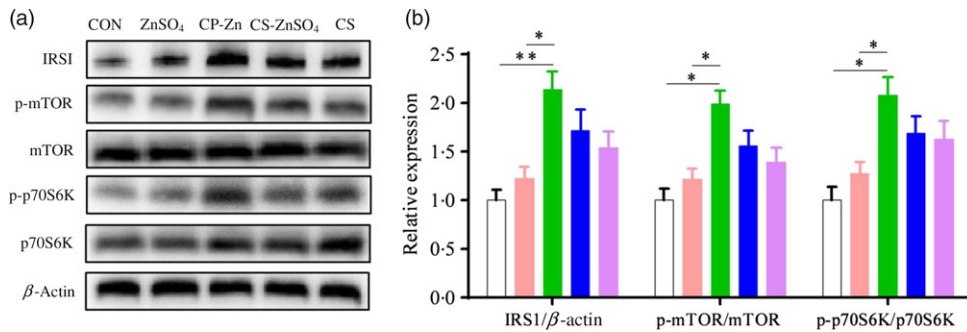
As shown in Fig. 4, Western blot analysis showed that the relative protein expression levels of IRS1 (IRS1/ $\beta$ -actin), phosphorylated mTOR/mTOR and phosphorylated p70S6K (p-p70S6K/p70S6K) in muscle were significantly higher in the CP–Zn group than in the CON and ZnSO<sub>4</sub> groups ( $P < 0.05$ ).

#### Antioxidant parameters and nuclear factor erythroid 2-related factor 2 pathway-related proteins in the jejunum

The antioxidant parameters in the serum and jejunums of weaned piglets are presented in Table 3. In serum, the MDA content in the CP–Zn group was significantly lower than that in the CON and ZnSO<sub>4</sub> groups (CON, 10.34 nmol/ml; ZnSO<sub>4</sub>, 9.88 nmol/ml; CP–Zn, 6.42 nmol/ml;  $P < 0.05$ ) and the GSH content in the CP–Zn group was significantly higher than that in the CON group (CON, 23.17 nmol/ml; CP–Zn, 36.74 nmol/ml;  $P < 0.05$ ). In the jejunum, the activity of T-SOD (CON, 52.88 U/mg protein; ZnSO<sub>4</sub>, 60.62 U/mg protein; CP–Zn, 83.36 U/mg protein) and GSH-px (CON, 94.44 U/mg protein; ZnSO<sub>4</sub>, 100.86 U/mg protein; CP–Zn, 155.01 U/mg protein; CS + ZnSO<sub>4</sub>, 137.51 U/mg protein) and the content of GSH (CON, 16.93 nmol/mg protein; ZnSO<sub>4</sub>, 19.30 nmol/mg protein; CP–Zn, 31.49 nmol/mg protein;



**Fig. 3.** Effects of nano chitosan–zinc complex (CP–Zn) on the concentrations of growth factors and inflammatory cytokines in the serum of weaned piglets. (a) Growth hormone (GH); (b) insulin; (c) insulin-like growth factor 1 (IGF1); (d) epidermal growth factor (EGF); (e) TNF- $\alpha$ ; (f) IL-1 $\beta$ ; (g) interferon- $\gamma$  (INF- $\gamma$ ); (h) myeloperoxidase (MPO). The EGF data were analysed with the Kruskal–Wallis test, and the other data were analysed by ordinary one-way ANOVA. The data are mean values with their standard errors,  $n$  6. \*  $P < 0.05$ , \*\*  $P < 0.01$ .



**Fig. 4.** Effects of nano chitosan–zinc complex (CP–Zn) on the mammalian target of rapamycin (mTOR) pathway in muscle in weaned piglets. (a, b) The protein expression levels of insulin receptor substrate 1 (IRS1), phosphorylated mammalian target of rapamycin (p-mTOR) and phosphorylated p70S6K (p-p70S6K) in muscle were detected by Western blot analysis. The data were analysed by ordinary one-way ANOVA. The data are mean values with their standard errors,  $n$  6. \*  $P < 0.05$ , \*\*  $P < 0.01$ .  $\square$ , Basal diet;  $\square$ , basal diet supplemented with 100 mg/kg Zn from ZnSO<sub>4</sub>;  $\square$ , basal diet supplemented with 100 mg/kg Zn from CP–Zn;  $\square$ , basal diet supplemented with 100 mg/kg Zn from ZnSO<sub>4</sub> and 766 mg/kg of chitosan;  $\square$ , basal diet supplemented with 766 mg/kg of chitosan but not supplemented with Zn.

CS + ZnSO<sub>4</sub>, 27.70 nmol/mg protein) in the CP–Zn group were significantly higher than those in the CON and ZnSO<sub>4</sub> groups ( $P < 0.05$ ). The activity of GSH-px and the content of GSH in the CS + ZnSO<sub>4</sub> group were also significantly higher than those in the CON group ( $P < 0.05$ ). The MDA content in the jejunum was significantly lower in the CP–Zn group than in the CON, ZnSO<sub>4</sub> and CS groups (CON, 2.07 nmol/mg protein; ZnSO<sub>4</sub>, 1.83 nmol/mg protein; CP–Zn, 1.10 nmol/mg protein; CS + ZnSO<sub>4</sub>, 1.46 nmol/mg protein; CS, 1.70 nmol/mg protein;  $P < 0.05$ ) and was significantly lower in the CS + ZnSO<sub>4</sub> group than in the CON group ( $P < 0.05$ ).

In addition, the results of Western blot analysis of Nrf2 pathway-related proteins in the jejunum are shown in Fig. 5. The results showed that the relative protein expression level

of Nrf2 was significantly higher in the CP–Zn group than in the CON, ZnSO<sub>4</sub> and CS + ZnSO<sub>4</sub> groups ( $P < 0.05$ ) and also significantly higher in the CS + ZnSO<sub>4</sub> group than in the CON group ( $P < 0.05$ ). The relative protein expression level of NQO1 in the CP–Zn group was significantly higher than those in the CON and ZnSO<sub>4</sub> groups ( $P < 0.05$ ), and the relative protein expression level of HO1 in the CP–Zn group was significantly higher than those in the CON, ZnSO<sub>4</sub> and CS groups ( $P < 0.05$ ).

#### Antioxidant parameters and nuclear factor erythroid 2-related factor 2 pathway-related proteins in IPEC-J2 cells

The cell viability of IPEC-J2 cells is shown in online Supplementary Fig. S1. The results showed that treatment with 0.5–2 mM Zn from



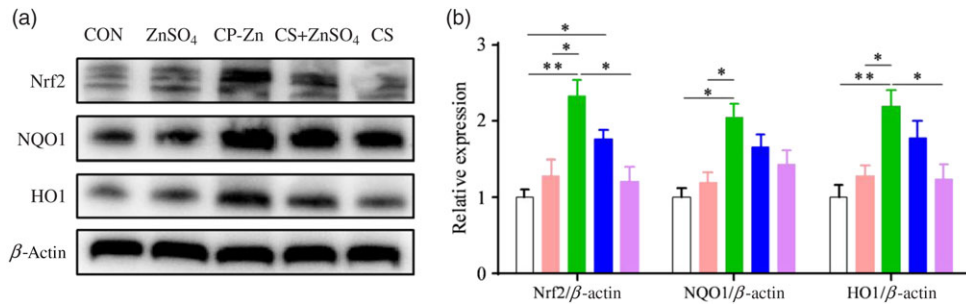
**Table 3.** Effects of nano chitosan–zinc complex (CP–Zn) on the antioxidant parameters of weaned piglets (*n* 6) (Mean values and standard errors)

Variable*	CON	ZnSO <sub>4</sub>	CP–Zn	CS + ZnSO <sub>4</sub>	CS	SEM	<i>P</i>
<b>Serum</b>							
T-SOD (U/ml)	106.62	108.65	123.53	114.56	108.91	7.13	0.151
GSH-px (U/ml)	139.74	139.11	169.88	163.12	149.54	12.37	0.068
CAT (U/m), §*	60.62	57.64	46.23	47.63	50.43	5.43	0.115
MDA (nmol/ml), §*	10.34 <sup>a</sup>	9.88 <sup>a</sup>	6.42 <sup>b</sup>	7.74 <sup>a,b</sup>	8.60 <sup>a,b</sup>	0.96	0.003
GSSG (nmol/ml)	3.13	3.31	3.75	3.48	3.05	0.53	0.687
GSH (nmol/ml)	23.17 <sup>a</sup>	25.67 <sup>a,b</sup>	36.74 <sup>b</sup>	32.45 <sup>a,b</sup>	25.38 <sup>a,b</sup>	4.35	0.023
<b>Jejunum</b>							
T-SOD (U/mg protein)	52.88 <sup>a</sup>	60.62 <sup>a</sup>	83.36 <sup>b</sup>	72.34 <sup>a,b</sup>	67.47 <sup>a,b</sup>	7.29	0.004
GSH-px (U/mg protein)	94.44 <sup>a</sup>	100.86 <sup>a,b</sup>	155.01 <sup>c</sup>	137.51 <sup>b,c</sup>	108.49 <sup>a,b</sup>	13.08	0.000
CAT (U/mg protein)	4.54	5.60	6.22	5.72	5.85	1.05	0.585
MDA (nmol/mg protein)	2.07 <sup>a</sup>	1.83 <sup>a,b</sup>	1.10 <sup>c</sup>	1.46 <sup>b,c</sup>	1.70 <sup>a,b</sup>	0.20	0.001
GSSG (nmol/mg protein)	4.39	4.39	5.51	5.04	4.66	0.66	0.403
GSH (nmol/mg protein)	16.93 <sup>a</sup>	19.30 <sup>a,b</sup>	31.49 <sup>c</sup>	27.70 <sup>b,c</sup>	22.25 <sup>a,b,c</sup>	3.54	0.002

CON, basal diet; ZnSO<sub>4</sub>, basal diet supplemented with 100 mg/kg Zn from ZnSO<sub>4</sub>; CP–Zn, basal diet supplemented with 100 mg/kg Zn from CP–Zn; CS + ZnSO<sub>4</sub>, basal diet supplemented with 100 mg/kg Zn from ZnSO<sub>4</sub> and 766 mg/kg of chitosan; CS, basal diet supplemented with 766 mg/kg of chitosan but not supplemented with Zn; T-SOD, total superoxide dismutase; GSH-px, glutathione peroxidase; CAT catalase; MDA, malondialdehyde.

<sup>a,b,c</sup> Mean values within a row with unlike superscript letters were significantly different (*P* < 0.05).

\*With §: Kruskal–Wallis test; without §: ordinary one-way ANOVA.



**Fig. 5.** Effects of nano chitosan–zinc complex (CP–Zn) on the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway of the jejunum in weaned piglets. (a, b) The protein expression levels of Nrf2, NAD(P)H:quinone oxidoreductase 1 (NQO1) and haem oxygenase 1 (HO1) in the jejunum were detected by Western blot analysis. The data were analysed by ordinary one-way ANOVA. The data are mean values with their standard errors, *n* 6. \* *P* < 0.05, \*\* *P* < 0.01. □, Basal diet; ■, basal diet supplemented with 100 mg/kg Zn from ZnSO<sub>4</sub>; ■, basal diet supplemented with 100 mg/kg Zn from CP–Zn; ■, basal diet supplemented with 100 mg/kg Zn from ZnSO<sub>4</sub> and 766 mg/kg of chitosan; ■, basal diet supplemented with the 766 mg/kg of chitosan but not supplemented with Zn.

ZnSO<sub>4</sub>, 1–2 mM Zn from CP–Zn and 1–2 mM Zn from CS + ZnSO<sub>4</sub> significantly decreased the viability of IPEC-J2 cells (*P* < 0.05) (online Supplementary Fig. S1A). Treatment with 5–20 µg/ml LPS also significantly decreased the viability of IPEC-J2 cells (*P* < 0.05) (online Supplementary Fig. S1B). In addition, the protective effect of CP–Zn on IPEC-J2 cells challenged with LPS is presented in online Supplementary Fig. S1C. Compared with the LPS-treated group, the groups treated with CP–Zn containing 0.05 mM, 0.1 mM and 0.2 mM Zn exhibited significantly higher cell viability (*P* < 0.05). Based on these results, we chose 0.05 mM Zn for the ZnSO<sub>4</sub>, CP–Zn and CS + ZnSO<sub>4</sub> groups and 1 µg/ml LPS as the further treatment concentrations.

The antioxidant parameters of IPEC-J2 cells are shown in Fig. 6(a)–(e). Compared with the values in the LPS group, the MDA content (CON, 4.37 nmol/mg protein; LPS, 7.86 nmol/mg protein; CP–Zn, 5.59 nmol/mg protein; CS + ZnSO<sub>4</sub>, 5.66 nmol/mg protein) and ROS intensity (CON, 100%; LPS, 208%; CP–Zn, 144%; CS + ZnSO<sub>4</sub>, 148%) in the CON, CP–Zn and CS + ZnSO<sub>4</sub> groups were significantly lower (*P* < 0.05). Compared with the LPS group, the ZnSO<sub>4</sub>, CP–Zn and CS + ZnSO<sub>4</sub> groups exhibited significantly higher T-SOD activity

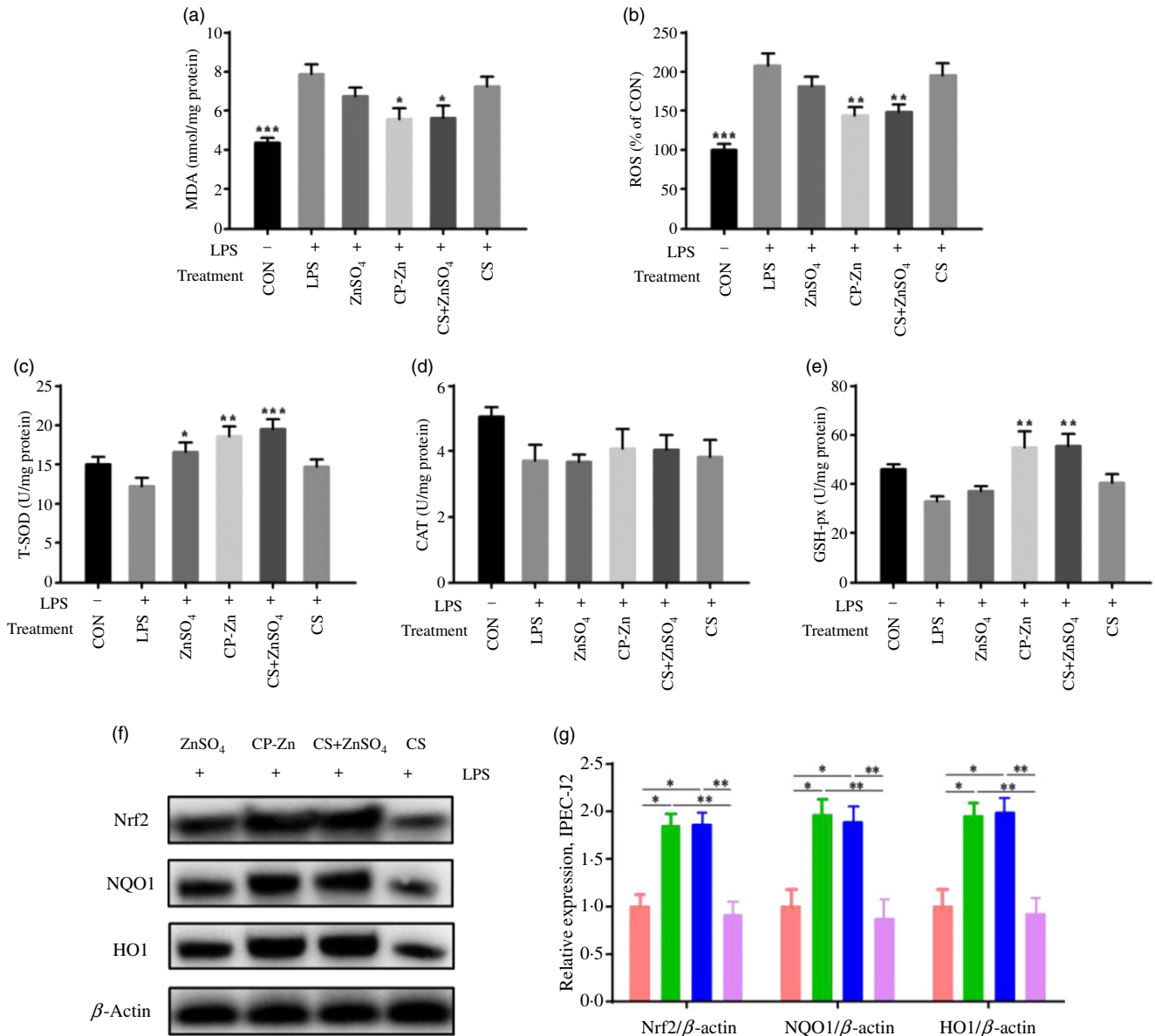
(LPS, 12.29 U/mg protein; ZnSO<sub>4</sub>, 16.64 U/mg protein; CP–Zn, 18.65 U/mg protein; CS + ZnSO<sub>4</sub>, 19.48 U/mg protein; *P* < 0.05), and the CP–Zn and CS + ZnSO<sub>4</sub> groups exhibited significantly higher GSH-px activity (LPS, 33.02 U/mg protein; CP–Zn, 54.90 U/mg protein; CS + ZnSO<sub>4</sub>, 55.72 U/mg protein; *P* < 0.05). In addition, as shown in Fig. 6(f) and (g), Western blot analysis showed that the relative protein expression levels of Nrf2, NQO1 and HO1 in the CS + ZnSO<sub>4</sub> and CP–Zn groups were significantly higher than those in the ZnSO<sub>4</sub> and CS groups (*P* < 0.05).

## Discussion

Body weight, ADG and average daily feed intake are considered to be key indicators of the growth performance of weaned piglets<sup>(5,28)</sup>. Previous studies have shown that dietary supplementation with pharmacological doses of ZnO can improve the growth performance of weaned piglets<sup>(8,29,30)</sup>. In the present study, we found that both H-ZnO treatment (1600 mg/kg Zn) and CP–Zn treatment (100 mg/kg Zn) significantly increased



Nano chitosan–zinc complex



**Fig. 6.** Effects of nano chitosan–zinc complex (CP–Zn) on antioxidant parameters and the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway in IPEC-J2 cells. IPEC-J2 cells were pretreated with ZnSO<sub>4</sub>, CP–Zn, chitosan (CS) + ZnSO<sub>4</sub> and CS for 24 h (the zinc concentration was 0.05 mM in the ZnSO<sub>4</sub>, CP–Zn, CS + ZnSO<sub>4</sub> groups, and the content of CS (25 µg/ml) was the same in the CP–Zn, CS + ZnSO<sub>4</sub> and CS groups) and then co-treated with lipopolysaccharide (LPS) (1 µg/ml) for another 6 h. (a) Malondialdehyde (MDA); (b) reactive oxygen species (ROS); (c) total superoxide dismutase (T-SOD); (d) catalase (CAT); (e) glutathione peroxidase (GSH-px); (f, g) protein expression levels of Nrf2, NAD(P)H:quinone oxidoreductase 1 (NQO1) and haem oxygenase 1 (HO1) in muscle as detected by Western blot analysis. (g) +LPS: ■ ZnSO<sub>4</sub>; ■ CP–Zn; ■ CS + ZnSO<sub>4</sub>; ■ CS. The data were analysed by ordinary one-way ANOVA. The data are mean values with their standard errors, n 6. (a–e) Compared with the LPS group. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .

ADG and average daily feed intake, indicating that dietary CP–Zn supplementation can improve the growth performance of weaned piglets. Diarrhoea is usually an important cause of growth retardation in young piglets. Generally, fluids containing digestive factors are secreted in the SI to promote the digestion of nutrients, and the fluids are later reabsorbed in the large bowel (through a process that uses SCFA for energy)<sup>(31,32)</sup>. However, ETEC adhesion can lead to fluid hypersecretion in the SI such that the fluid cannot be fully recovered by the large bowel, resulting in diarrhoea<sup>(4,5,33)</sup>. In the present study, CP–Zn treatment reduced the diarrhoea rate and the faecal *E. coli* count,

suggesting that CP–Zn treatment inhibited adhesion of *E. coli* to the intestine. Similar findings were also observed in the H-ZnO treatment group. Improved growth performance and reduced diarrhoea rates are considered important signs of weaning stress alleviation in piglets. Therefore, the above results indicate that CP–Zn supplementation could be used as a substitute for high-Zn supplementation to relieve piglet weaning stress.

We further studied the mechanism by which dietary supplementation with CP–Zn improved the growth performance of weaned piglets. Growth performance is affected by digestive



and absorptive functions. The endogenous enzymes of the gastrointestinal tract, including proteases, amylase and lipase, are critical for the digestion of nutrients<sup>(34,35)</sup>. In this study, dietary supplementation with CP-Zn did not change trypsin or lipase activity but significantly increased amylase activity in the duodenum and jejunum. Furthermore, dietary CP-Zn supplementation significantly increased maltase, sucrase and lactase activity in the jejunum. These results suggest that CP-Zn improves the digestibility of dietary carbohydrates in the SI of weaned piglets. Interestingly, our results also showed that amylase, sucrase and lactase activity was significantly increased by CS treatment and CS + ZnSO<sub>4</sub> treatment. Therefore, we speculate that dietary supplementation with CP-Zn improves the digestibility of carbohydrates in the SI related to CS. Consistent with this hypothesis, previous reports have shown that CS can increase the activity of enzymes related to carbohydrate digestion in the SI of weaned piglets<sup>(36,37)</sup>. One possible explanation for this effect is that CS, as a natural polysaccharide, can act as a substrate similar to disaccharidases and thus promote the activity of disaccharidases<sup>(38,39)</sup>.

Generally, after the diet is hydrolysed by digestive enzymes, nutrients are absorbed mainly in the jejunum<sup>(34)</sup>. Some key nutrient transporters of intestinal epithelial cells are responsible for transporting nutrients into the bloodstream<sup>(35)</sup>. Glucose uptake in epithelial cells depends primarily on two transporters, *SGLT1* and *GLUT2*<sup>(28)</sup>. *PEPT1*, which is highly expressed in the jejunal parietal membrane, plays a key role in absorbing dietary di- and tripeptides<sup>(28)</sup>. *EAAC1* and neutral amino acid transporter (*SLC6A19*) are the major transporters of glutamate and neutral amino acids in the intestine, respectively<sup>(40)</sup>. In the present study, dietary CP-Zn supplementation increased the mRNA expression levels of *SGLT1*, *GLUT2*, *PEPT1* and *EAAC1* in the jejunum, suggesting that CP-Zn improved the nutrient absorption capacity of the SI. Notably, absorption of nutrients depends on the healthy development of the SI, which can be directly reflected by the VH and SI weight<sup>(28)</sup>. ETEC K88 gavage can disrupt the intestinal morphological integrity of piglets by reducing their VH<sup>(4,5)</sup>. In this study, CP-Zn supplementation enhanced the jejunal VH. In addition, CP-Zn supplementation increased SI weight, indicating that CP-Zn promoted the development of the SI; however, the mechanism needs to be further studied. We also found that dietary supplementation with CP-Zn reduced the feed:gain ratio, which might have been attributable to the improvements in digestion and absorption in the SI.

Muscle growth is an important part of body development in young animals. *E. coli* LPS challenge impairs muscle growth in weaned piglets<sup>(41)</sup>. During muscle growth, IGF1, insulin, growth hormone and epidermal growth factor have pleiotropic effects that regulate cell proliferation, differentiation, metabolism, and myofibrillar growth and regeneration<sup>(42)</sup>. The results of the present study showed that dietary supplementation with CP-Zn enhanced the levels of insulin and IGF1 in serum. The binding of insulin and IGF1 to their receptors on the cell surface in muscle tissue can activate the downstream mTOR signalling pathway through upstream IRS1 to mediate the PI3K-AKT pathway<sup>(43,44)</sup>. In the mTOR signalling pathway, phosphorylated mTOR protein further phosphorylates p70S6K through a protein signalling cascade to promote cellular protein synthesis<sup>(44)</sup>. In this study, the protein expression levels of IRS1, phosphorylated

mTOR and phosphorylated p70S6K in the CP-Zn treatment were significantly higher than those in the CON and ZnSO<sub>4</sub> treatments, suggesting that CP-Zn supplementation promoted muscle development by activating the mTOR signalling pathway. On the other hand, previous studies have shown that weaned piglets subjected to immunological challenge (with ETEC or LPS) have increased expression levels of pro-inflammatory factors, which inhibit activation of the mTOR signalling pathway in muscle<sup>(5,42,45)</sup>. Notably, our results showed that dietary CP-Zn supplementation reduced the levels of the pro-inflammatory factors TNF- $\alpha$ , IL-1 $\beta$  and myeloperoxidase in the serum, suggesting that CP-Zn attenuated the inhibitory effects of overexpressed inflammatory factors on the mTOR signalling pathway in muscle.

Oxidative stress is an important factor that aggravates the intestinal inflammatory response, induces intestinal epithelial cell apoptosis and even destroys intestinal morphology<sup>(7,46)</sup>. MDA is produced by lipid peroxidation and is often regarded as a key indicator of oxidative injury<sup>(47)</sup>. The results of the present study showed that dietary CP-Zn supplementation reduced the MDA content of the jejunum, indicating that CP-Zn alleviated oxidative stress injury in the jejunum of piglets. In animals, non-enzymatic antioxidants (e.g. GSH) and some key antioxidative enzymes (e.g. SOD, GSH-px and catalase) protect cells from oxidative stress injury<sup>(47,48)</sup>. In the present study, the GSH content of the jejunum was significantly increased in the CP-Zn and CS + ZnSO<sub>4</sub> groups, consistent with the mRNA expression levels of *EAAC1*. *EAAC1* is abundantly expressed on the apical and brush border membranes of the SI in pigs and is responsible for glutamate transport<sup>(49)</sup>. Glutamate is an important precursor substance for the synthesis of GSH<sup>(49)</sup>. On the other hand, glutamine production via biotransformation of glutamate, the main energy substrate of enterocytes, can reduce oxidative stress and alleviate apoptosis of the intestinal epithelium, thereby preventing intestinal dysfunction<sup>(50)</sup>. In addition, studies have shown that immunological challenge (with LPS and ETEC K88) can impair the expression of antioxidant enzymes in weaned piglets<sup>(6,7,46)</sup>. In this study, our results showed that dietary CP-Zn supplementation enhanced the activity of T-SOD and GSH-px in the jejunum, indicating that dietary CP-Zn supplementation relieved oxidative stress in the jejunums of weaned piglets by promoting the expression of antioxidant enzymes.

The expression of antioxidant enzymes in cells is closely related to the Nrf2 signalling pathway<sup>(51)</sup>. Under normal physiological conditions, Nrf2, a basic leucine zipper transcription factor, binds to its inhibitor protein Keap1 to form an inactive complex in the cytoplasm. Upon stimulation, Nrf2 dissociates from the Nrf2/Keap-1 complex and moves to the nucleus, where it binds to the antioxidant response element<sup>(51)</sup>. As a result, HO1 and NQO1 are abundantly expressed and further promote the production of antioxidant enzymes<sup>(46,51)</sup>. In the present study, Western blot analysis revealed that dietary CP-Zn supplementation significantly increased the protein expression levels of Nrf2, HO1 and NQO1 in the jejunum, suggesting that CP-Zn regulated oxidative stress by activating the Nrf2 signalling pathway. Interestingly, we also found that CS + ZnSO<sub>4</sub> increased the protein expression level of Nrf2 and the activity of GSH-px and reduced the content of MDA in the jejunum. To further clarify the mechanism by which CP-Zn improved antioxidant capacity,



an *in vitro* experiment was performed on the porcine jejunal epithelial cell line IPEC-J2.

ROS are other key indicators of oxidative stress at the cellular level<sup>(46)</sup>. In the present study, the levels of ROS and MDA were significantly higher in the LPS group than in the CON group, indicating that LPS induced oxidative stress in IPEC-J2 cells, consistent with the findings of a previous study<sup>(46)</sup>. Interestingly, we found that CS + ZnSO<sub>4</sub> and CP–Zn significantly decreased the levels of ROS and MDA in IPEC-J2 cells. In addition, in terms of antioxidant capacity, both CS + ZnSO<sub>4</sub> and CP–Zn significantly increased T-SOD and GSH-px activity and activated the Nrf2 signalling pathway by increasing the protein expression levels of Nrf2, HO1 and NQO1 in IPEC-J2 cells. These results demonstrated that CS + ZnSO<sub>4</sub> and CP–Zn alleviated oxidative stress by activating the Nrf2 signalling pathway *in vitro*. The results also suggested that CS and Zn had a synergistic beneficial effect on the antioxidant capacity of intestinal epithelial cells. However, CS + ZnSO<sub>4</sub> did not significantly affect the protein expression levels of HO1 and NQO1 or the activity of T-SOD in the jejunums of weaned piglets, indicating that CS + ZnSO<sub>4</sub> was less effective than CP–Zn in improving antioxidant capacity *in vivo*. We speculate that the effectiveness of CP–Zn may be attributable to its small particle size. Nano-sized CP–Zn may diffuse into the submucosal layers of the intestine easily and thus be able to interact efficiently with intestinal epithelial cells<sup>(48,19)</sup>.

In conclusion, the present study demonstrates that dietary supplementation with nano CP–Zn (100 mg/kg Zn) can improve growth performance and the antioxidant capacity of the SI, thus alleviating weaning stress in piglets. CP–Zn supplementation increases the activity of carbohydrate digestion-related enzymes and the mRNA expression levels of nutrient transporters in the jejunum and up-regulates the mTOR signalling pathway in muscle. In addition, CP–Zn improves the antioxidant capacity of the jejunum by activating the Nrf2 signalling pathway. These findings provide a valuable reference for research on the mechanisms by which nano Zn improves the intestinal health and growth of piglets and infants.

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The authors declare that they have no competing interests.

### Supplementary material

For supplementary materials referred to in this article, please visit <https://doi.org/10.1017/S0007114520004766>

### References

- Campbell JM, Crenshaw JD & Polo J (2013) The biological stress of early weaned piglets. *J Anim Sci Biotechnol* **4**, 19.
- Wijten PJA, Der Meulen JV & Verstegen MWA (2011) Intestinal barrier function and absorption in pigs after weaning: a review. *Br J Nutr* **105**, 967–981.
- Den Hil PJR, Litjens R, Oudshoorn A, *et al.* (2017) New perspectives to the enterotoxigenic *E. coli* F4 porcine infection model: susceptibility genotypes in relation to performance, diarrhoea and bacterial shedding. *Vet Microbiol* **202**, 58–63.
- Pan L, Zhao P, Ma X, *et al.* (2017) Probiotic supplementation protects weaned pigs against enterotoxigenic *Escherichia coli* K88 challenge and improves performance similar to antibiotics. *J Anim Sci* **95**, 2627–2639.
- Huang C, Wang Y, He X, *et al.* (2019) The involvement of NF-κB/P38 pathways in *Scutellaria baicalensis* extracts attenuating of *Escherichia coli* K88-induced acute intestinal injury in weaned piglets. *Br J Nutr* **122**, 152–161.
- Jiang X, Li X, Awati A, *et al.* (2017) Effect of an essential oils blend on growth performance, and selected parameters of oxidative stress and antioxidant defence of *Escherichia coli* challenged piglets. *J Anim Feed Sci* **26**, 38–43.
- Xu J, Xu C, Chen X, *et al.* (2014) Regulation of an antioxidant blend on intestinal redox status and major microbiota in early weaned piglets. *Nutrition* **30**, 584–589.
- Sun Y, Xia T, Wu H, *et al.* (2019) Effects of nano zinc oxide as an alternative to pharmacological dose of zinc oxide on growth performance, diarrhea, immune responses, and intestinal microflora profile in weaned piglets. *Anim Feed Sci Technol* **258**, 114312.
- Lei XJ & Kim IH (2018) Low dose of coated zinc oxide is as effective as pharmacological zinc oxide in promoting growth performance, reducing fecal scores, and improving nutrient digestibility and intestinal morphology in weaned pigs. *Anim Feed Sci Technol* **245**, 117–125.
- Jensen J, Kyvsgaard NC, Battisti A, *et al.* (2018) Environmental and public health related risk of veterinary zinc in pig production—using Denmark as an example. *Environ Int* **114**, 181–190.
- Swain PS, Rao SB, Rajendran D, *et al.* (2016) Nano zinc, an alternative to conventional zinc as animal feed supplement: a review. *Anim Nutr* **2**, 134–141.
- Moreno-Olivas F, Tako E & Mahler GJ (2019) ZnO nanoparticles affect nutrient transport in an *in vitro* model of the small intestine. *Food Chem Toxicol* **124**, 112–127.
- Zhang W, Zhao Y, Li F, *et al.* (2018) Zinc oxide nanoparticle caused plasma metabolomic perturbations correlate with hepatic steatosis. *Front Pharmacol* **9**, 57.
- Hamed I, Ozogul F & Regenstein JM (2016) Industrial applications of crustacean by-products (chitin, chitosan, and chitooligosaccharides): a review. *Trends Food Sci Tech* **48**, 40–50.
- Qi L, Xu Z, Jiang X, *et al.* (2004) Preparation and antibacterial activity of chitosan nanoparticles. *Carbohydr Res* **339**, 2693–2700.
- Han X, Ma Y, Lv M, *et al.* (2014) Chitosan–zinc chelate improves intestinal structure and mucosal function and decreases apoptosis in ileal mucosal epithelial cells in weaned pigs. *Br J Nutr* **111**, 1405–1411.
- Hu L, Cheng S, Li Y, *et al.* (2018) Chitosan–Zn chelate down-regulates TLR4-NF-κB signal pathway of inflammatory response and cell death-associated proteins compared to inorganic zinc. *Biol Trace Elem Res* **184**, 92–98.
- Desai MP, Labhasetwar V, Amidon GL, *et al.* (1996) Gastrointestinal uptake of biodegradable microparticles: effect of particle size. *Pharm Res* **13**, 1838–1845.

19. Tian Y, Xu J, Li Y, *et al.* (2019) MicroRNA-31 reduces inflammatory signaling and promotes regeneration in colon epithelium, and delivery of mimics in microspheres reduces colitis in mice. *Gastroenterology* **156**, 2281–2296.e6.
20. Rajasekaran P & Santra S (2015) Hydrothermally treated chitosan hydrogel loaded with copper and zinc particles as a potential micronutrient-based antimicrobial feed additive. *Front Vet Sci* **2**, 62.
21. Deshpande P, Dapkekar A, Oak MD, *et al.* (2017) Zinc complexed chitosan/TPP nanoparticles: a promising micronutrient nanocarrier suited for foliar application. *Carbohydr Polym* **165**, 394–401.
22. AbdElhady MM (2012) Preparation and characterization of chitosan/zinc oxide nanoparticles for imparting antimicrobial and UV protection to cotton fabric. *J Carbohydr Chem* **2012**, 840591.
23. Kaur P, Thakur R, Barnela M, *et al.* (2015) Synthesis, characterization and *in vitro* evaluation of cytotoxicity and antimicrobial activity of chitosan–metal nanocomposites. *J Chem Technol Biotechnol* **90**, 867–873.
24. Gonzalez LM, Moeser AJ & Blikslager AT (2015) Porcine models of digestive disease: the future of large animal translational research. *Transl Res* **166**, 12–27.
25. Long S, Xu Y, Pan L, *et al.* (2018) Mixed organic acids as antibiotic substitutes improve performance, serum immunity, intestinal morphology and microbiota for weaned piglets. *Anim Feed Sci Technol* **235**, 23–32.
26. Tang Q, Huang K, Liu J, *et al.* (2019) Fine particulate matter from pig house induced immune response by activating TLR4/MAPK/NF- $\kappa$ B pathway and NLRP3 inflammasome in alveolar macrophages. *Chemosphere* **236**, 124373.
27. Livak KJ & Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the  $2^{-\Delta\Delta CT}$  method. *Methods* **25**, 402–408.
28. Zong E, Huang P, Zhang W, *et al.* (2018) The effects of dietary sulfur amino acids on growth performance, intestinal morphology, enzyme activity, and nutrient transporters in weaning piglets. *J Anim Sci* **96**, 1130–1139.
29. Sales J (2013) Effects of pharmacological concentrations of dietary zinc oxide on growth of post-weaning pigs: a meta-analysis. *Biol Trace Elem Res* **152**, 343–349.
30. Park B, Jung D, Kang S, *et al.* (2015) Effects of dietary supplementation of a zinc oxide product encapsulated with lipid on growth performance, intestinal morphology, and digestive enzyme activities in weanling pigs. *Anim Feed Sci Technol* **200**, 112–117.
31. Topping DL & Clifton PM (2001) Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch. *Physiol Rev* **81**, 1031–1064.
32. Ramakrishna BS, Venkataraman S, Srinivasan P, *et al.* (2000) Amylase-resistant starch plus oral rehydration solution for cholera. *N Engl J Med* **342**, 308–313.
33. Jin LZ & Zhao X (2000) Intestinal receptors for adhesive fimbriae of enterotoxigenic *Escherichia coli* (ETEC) K88 in swine—a review. *Appl Microbiol Biotechnol* **54**, 311–318.
34. Fan C, Han X, Xu Z, *et al.* (2009) Effects of  $\beta$ -glucanase and xylanase supplementation on gastrointestinal digestive enzyme activities of weaned piglets fed a barley-based diet. *J Anim Physiol Anim Nutr* **93**, 271–276.
35. Patra AK, Amasheh S & Aschenbach JR (2019) Modulation of gastrointestinal barrier and nutrient transport function in farm animals by natural plant bioactive compounds—a comprehensive review. *Crit Rev Food Sci Nutr* **59**, 3237–3266.
36. Guo X (2014) Effects of chitosan supplementation on the growth performance, nutrient digestibility, and digestive enzyme activity in weaned pigs. *Czech J Anim Sci* **59**, 156–163.
37. Zhang J, Wan J, Wu G, *et al.* (2020) Low-molecular-weight chitosan relieves enterotoxigenic *Escherichia coli*-induced growth retardation in weaned pigs. *Int Immunopharmacol* **78**, 105798.
38. Collins AJ, James PS & Smith MW (1989) Sugar-dependent selective induction of mouse jejunal disaccharidase activities. *J Physiol* **419**, 157–167.
39. Toshinao G & Sachiko T (1994) Dietary carbohydrate and fat independently modulate disaccharidase activities in rat jejunum. *J Nutr* **11**, 2233–2239.
40. Yang H, Xiong X, Wang X, *et al.* (2016) Effects of weaning on intestinal upper villus epithelial cells of piglets. *PLOS ONE* **11**, e0150216.
41. Liu Y, Li D, Gong L, *et al.* (2003) Effects of fish oil supplementation on the performance and the immunological, adrenal, and somatotrophic responses of weaned pigs after an *Escherichia coli* lipopolysaccharide challenge. *J Anim Sci* **81**, 2758–2765.
42. Wang L, Yi D, Hou Y, *et al.* (2016) Dietary supplementation with  $\alpha$ -ketoglutarate activates mTOR signaling and enhances energy status in skeletal muscle of lipopolysaccharide-challenged piglets. *J Nutr* **146**, 1514–1520.
43. Glass DJ (2003) Molecular mechanisms modulating muscle mass. *Trends Mol Med* **9**, 344–350.
44. Hsieh C-T, Chuang J-H, Yang W-C, *et al.* (2014) Ceramide inhibits insulin-stimulated Akt phosphorylation through activation of Rheb/mTORC1/S6K signaling in skeletal muscle. *Cell Signal* **26**, 1400–1408.
45. Kang P, Wang X, Wu H, *et al.* (2017) Glutamate alleviates muscle protein loss by modulating TLR4, NODs, Akt/FOXO and mTOR signaling pathways in LPS-challenged piglets. *PLOS ONE* **12**, e0182246.
46. Wu W, Wang S, Liu Q, *et al.* (2018) Metformin protects against LPS-induced intestinal barrier dysfunction by activating AMPK pathway. *Mol Pharm* **15**, 3272–3284.
47. Goethals S, Vossen E, Michiels J, *et al.* (2019) Impact of red versus white meat consumption in a prudent or Western dietary pattern on the oxidative status in a pig model. *J Agric Food Chem* **67**, 5661–5671.
48. Young D, Fan MZ & Mine Y (2010) Egg yolk peptides up-regulate glutathione synthesis and antioxidant enzyme activities in a porcine model of intestinal oxidative stress. *J Agric Food Chem* **58**, 7624–7633.
49. Wang QJ, Cui YZ, Zhang XY, *et al.* (2017) Effect of early weaning on the expression of excitatory amino acid transporter 1 in the jejunum and ileum of piglets. *Mol Med Rep* **16**, 6518–6525.
50. Wang J, Chen L, Li P, *et al.* (2008) Gene expression is altered in piglet small intestine by weaning and dietary glutamine supplementation. *J Nutr* **138**, 1025.
51. Kobayashi M & Yamamoto M (2005) Molecular mechanisms activating the Nrf2-Keap1 pathway of antioxidant gene regulation. *Antioxid Redox Signal* **7**, 385–394.

