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Effects of chitosan nanoparticle supplementation on growth performance, humoral immunity, gut microbiota and immune responses after lipopolysaccharide challenge in weaned pigs

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Abstract

In this study, we aimed to determine the effects of dietary supplementation with chitosan nanoparticles (CNP) on growth performance, immune status, gut microbiota and immune responses after lipopolysaccharide challenge in weaned pigs. A total of 144 piglets were assigned to four groups receiving different dietary treatments, including basal diets supplemented with 0, 100, 200 and 400 mg/kg CNP fed for 28 days. Each treatment group included six pens (six piglets per pen). The increase in supplemental CNP concentration improved the average daily gain (ADG) and decreased the feed and gain (F/G) and diarrhoea rate ($p < .05$). However, significant differences in the average daily feed intake (ADFI) among different CNP concentrations were not observed. CNP also increased plasma immunoglobulin (Ig)A and IgG, and C3 and C4 concentrations in piglets in a dose-dependent manner on day 28, whereas IgM concentration was not affected by CNP. A total of 24 piglets in the control diet and control diet with 400 mg/kg CNP supplementation groups were randomly selected for the experiment of immunological stress. Half of the pigs in each group ($n = 6$) were injected *i.p.* with *Escherichia coli* lipopolysaccharide (LPS) at a concentration of 100 μg/kg. The other pigs in each group were injected with sterile saline solution at the same volume. Plasma concentrations of cortisol, prostaglandin E2 (PEG2), interleukin (IL)-6, tumour necrosis factor (TNF)-α and IL-1β dramatically increased after LPS challenge. However, CNP inhibited the increase in cortisol, PEG2, IL-6 and IL-1β levels in plasma, whereas TNF-α level slightly increased. Moreover, the effects of CNP on the gut microbiota were also evaluated. Our results showed that dietary supplementation with CNP modified the composition of colonic microbiota, where it increased the amounts of some presumably beneficial intestinal bacteria and suppressed the growth of potential bacterial pathogens. These findings suggested CNP supplementation improved the growth performance and immune status, alleviated immunological stress and regulated intestinal ecology in weaned piglets. Based on these beneficial effects, CNP could be applied as a functional feed additives supplemented in piglets diet.

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KEYWORDS
chitosan nanoparticles, growth performance, immune status, immunological stress, intestine ecology, weaned piglets

1 | INTRODUCTION

Weaning is a major critical period of pig rearing because of its association with environmental and dietary stress, which can result in immunological responses and an increased susceptibility to infections. In pigs, bacterial infections are responsible for considerable economic loss and reduced animal welfare owing to the decreased growth rate, morbidity, mortality and medication costs (Lalle et al., 2004).

Lipopolysaccharide (LPS), a structural part of the outer membrane of Gram-negative bacteria, is one of the most effective stimulators of the immune system and has been widely used in pigs as an experimental model for bacterial infections (Wyns, Plessers, De Backer, Meyer, & Croubels, 2015).

Chitosan, the most important derivative of chitin, has gained increasing attention in colon targeting because of its specific biodegradability by the colonic bacteria (Zhang & Neau, 2002), well-documented biocompatibility, low toxicity and mucoadhesive properties (Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004; Muzzarelli & Muzzarelli, 2009). Because of their small size and high zeta potential, chitosan-based particulate systems, particularly chitosan nanoparticles (CNP), exhibit higher oral absorption than that of chitosan and have been exploited as carriers for oral delivery of peptides, proteins and nucleotides (Elgadir et al., 2015). We have focused on the preparation and biological activities of various chitosan microspheres (chitosan nanoparticles and different metal ion-loaded nanoparticles) for more than 15 years. Our previous studies showed that CNP possessed diverse biological activities, including antibacterial (Du, Xu, Xu, & Fan, 2008), antitumour (Qi, Xu, & Chen, 2007), anti-angiogenesis (Xu, Wen, & Xu, 2010) and immunological adjuvant activities (Wen, Xu, Zou, & Xu, 2011). Recently, we found that CNP could protect the Caco-2 cells, a model of human enterocytes, from LPS-induced cell membrane damage (Tu, Xu, Xu, Ling, & Cai, 2016), indicating that CNP might exhibit anti-inflammatory effects and could be used as a feed additive for weaning piglets to reduce the weaning stress.

In this study, we aimed to evaluate the efficacy of CNP supplementation on growth performance, humoral immunity, gut microbiota and immune responses under inflammatory challenge conditions.

2 | MATERIALS AND METHODS

2.1 | Materials

Chitosan nanoparticles with a mean particle size of approximately 50 nm (Figure 1) were prepared in our laboratory using cationic chitosan (Chitosan Company of Pan'an) with an average molecular weight of 220 kDa and a degree of deacetylation of 95%. LPS (*E. coli* serotype O111:B4, Sigma Chemical) was dissolved in sterile saline (0.9%).

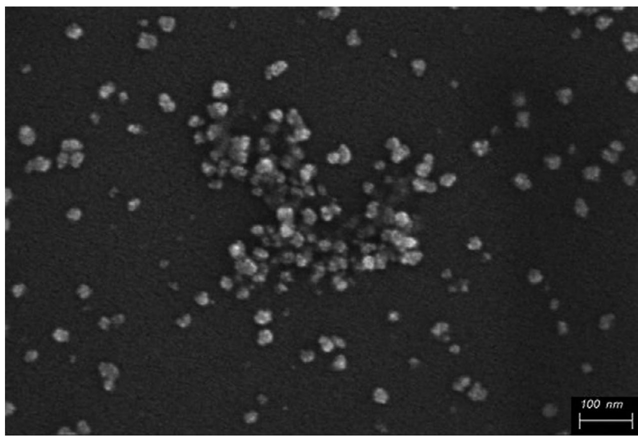

FIGURE 1 Morphology of chitosan nanoparticles (CNP) by scanning electron microscope (*SEM*)

2.2 | Animals, housing and treatments

A total of 144 piglets (21 ± 2 days, Duroc × Landrace ×Yorkshire) were assigned to four dietary treatment groups, taking into consideration the principle of equal numbers of males and females and similar body weight in all groups. Each treatment group included six pens (six piglets per pen). The piglets were acclimatized for 1 week before the study. The dietary treatments included a corn–soybean meal-based control diet with NRC (1998) requirements (Table 1) and control diet supplemented with 100, 200 or 400 mg/kg CNP. This experiment lasted for 28 days.

A total of 24 piglets in the groups of control diet and control diet with 400 mg/kg CNP supplementation were randomly selected in the experiment of immunological stress (12 piglets per group, male: female = 1:1). Half of the pigs (six piglets per group, male: female = 1:1) in each treatment group were injected *i.p.* with LPS at 100 μg/kg. The other piglets were injected with sterile saline solution. Therefore, LPS challenge (with or without) was considered as the second factor. No antibiotics were administered to the pigs prior to or during the experimental period. The dosage of LPS was selected based on the results of previous studies (Tuchscherer, Kanitz, Puppe, Tuchscherer, & Stabenow, 2004). Blood samples were collected, and at 4 hr after injection of LPS, pigs were sacrificed by bleeding.

2.3 | Growth performance and diarrhoea rate determination

Individual pig body weight (BW) was measured initially and on day 28 of the experiment. Feed disappearance per pen was assessed. The diarrhoea status was recorded daily. The data of average daily

TABLE 1 Compositions of experimental diets (as-fed basis)

Ingredients (g/kg)		Nutrient levels[a]	
Corn	600	DE(MJ/kg)	14.3
Soybean meal	160	Crude protein (g/kg)	180.7
Fermented soybean meal	28	Lysine (g/kg)	15.0
Fish meal	20	Methionine (g/kg)	5.9
Soy protein concentrate	20	Calcium (g/kg)	7.8
brewer's yeast	10	Phosphorus (g/kg)	8.8
Sucrose	20		
Glucose	20		
Whey powder	20		
Calcium hydrophosphate	10		
Mineral meal	6.4		
NaCl	3.6		
Wheat flour	48		
Soybean oil	14.4		
Lysine 98	4.8		
Methionine 98	0.8		
Threonine	1.2		
Tryptophan	0.24		
Zinc oxide	2.4		
Acidifier	2.4		
Santoquin	0.2		
Rovabio AP10%[b]	0.24		
Phytase 5,000 IU	0.2		
Choline chloride 50%	1		
Premix (0.5%)[c]	5		
Peptide iron	0.52		
Sweeter	0.6		

[a]Calculated values.
[b]Endo-1,4-β-xylanase 5,500 visco units/g (equivalent to 350 AXC units/g); Endo-1,3(4)-β-glucanase 500 AGL units/g; Endo-1,4-β-glucanase (cellulase) 1,600 DNS units/g.
[c]Provided per kg of complete diet: vitamin A, 11,025 IU; vitamin D3, 1,103 IU; vitamin E, 44 IU; vitamin K, 4.4 mg; riboflavin, 8.3 mg; niacin, 50 mg; thiamine, D-pantothenic acid, 29 mg; choline, 166 mg; and vitamin B12, 33 μg; Fe, 200 mg; Cu, 12 mg; Zn, 200 mg; Mn, 8 mg; I, 0.28 mg; Se, 0.15 mg.

gain (ADG), feed and gain (F/G), and diarrhoea rate (%) were calculated as follows: ADG = (Total weight at the deadline − total weight at the start)/(experiment days × amounts); F/G = Average feed intake/average daily gain; Diarrhoea rate (%) = (Diarrhoea amounts of each group × diarrhoea days)/(experiment days × amounts).

2.4 | Sample collection and analysis

On day 28, blood samples were collected before LPS challenge (0 hr) and at 1.5 and 3 hr after LPS challenge via jugular venipuncture into 3-ml heparinized vacuum tubes (Becton Dickinson vacutainer Systems). Blood was centrifuged (3,500 × *g* for 10 min) to collect plasma. Immunoglobulin (Ig)G, IgA, and IgM, and complement (C)3 and C4 concentrations in the plasma from blood samples collected before LPS challenge were analysed by an immunoturbidimetric method using commercially available kits (Jiancheng Bioengineering). Plasma interleukin (IL)-6, tumour necrosis factor (TNF)-α, IL-1β, prostaglandin G2 (PEG2) and cortisol concentrations were also determined using commercially available ELISA kits (Jiancheng Bioengineering).

Immediately after the piglets were killed, fresh colonic content samples from piglets in the control and 400 mg/kg CNP supplementation groups without LPS challenge were collected, frozen in liquid nitrogen and then stored at −80°C. Total microbial DNA was extracted from samples using the FastDNA SPIN kit for soil (MP Biomedicals), according to the manufacturer's instructions. The final DNA concentration and purity were determined by a NanoDrop 2,000 UV–vis spectrophotometer, and DNA quality was further verified and monitored by 1% agarose gel electrophoresis. 16S rDNA V4 region was amplified by using the 515f/806r primers (515f: 5′-GTGCCA GCM GCC GCG GTA/A3′, 806r: 5′-XXX XXX GGA CTA CHV GGG TWT CTA AT-3′). A unique six base pair error-correcting barcode sequence was attached to each sample. The PCRs were conducted using the following programme: 3 min of denaturation at 95°C, 30 cycles of 10 s at 98°C, 30 s for annealing at 50°C, 30 s for elongation at 72°C and a final extension at 72°C for 5 min. The resulting PCR products were extracted from 2% agarose gel and further purified by using the Qiagen gel extraction kit (Qiagen). The PCR products were quantified using QuantiFluor™ ST (Promega). Sequencing libraries were generated using Ion Plus Fragment Library Kit 48 rxns (Thermo Scientific) according to the manufacturer's protocol and assessed using a Qubit 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2,100 system. Finally, the library was sequenced on Ion S5™XL (Thermo Scientific), and 250 bp paired-end reads were generated.

Paired-end reads were trimmed by cutting off the barcode and primer sequence using Cutadapt software (Martin M, 2011) to generate the raw reads. Then, chimeric sequences of the raw reads were removed using the USEARCH software based on UCHIME algorithm, and the clean reads were obtained (Edgar, Haas, Clemente, Quince, & Knight, 2011). The clean reads were clustered into operational taxonomic units (OTUs) based on a 97% similarity threshold using Uparse software. Taxonomy assignment of OTUs was performed by RDP Classifier against the Silva (SSU123) Database using a confidence threshold of 80% (Quast et al., 2013; Wang, Garrity, Tiedje, & Cole, 2007). OTU abundance data were normalized using a standard sequence number corresponding to the sample with the fewest sequences. Subsequent statistical analyses of alpha diversity and beta diversity were all performed based on these normalized output data. Alpha diversity was used to analyse the complexity of species diversity through observed species Chao1, Shannon and Simpson diversity indices. Weighted UniFrac was calculated using QIIME software (Version 1.9.1). Statistical analyses of bacterial taxa were carried out using Student's *t* test. Beta diversity was evaluated by principal coordinate analysis (PCoA) based on

weighted UniFrac analysis and unweighted pair group method with arithmetic mean (UPGMA) clustering. Linear discriminant analysis coupled with effect size (LEfSe) was performed to identify the bacterial taxa that were differentially represented among groups at the genus or higher taxonomy level.

2.5 | Statistical analysis

All data were expressed as the means ± standard error. Statistical significance was analysed by one-way analysis of variance (ANOVA) followed by Tukey's HSD *post hoc* test using SPSS 18.0 software. p values <.05 were considered statistically.

3 | RESULTS

3.1 | Growth performance and diarrhoea frequency

The preliminary feeding period for all piglets was 1 week, and the initial weight of each piglet was measured at the beginning of the experimental period. Results of ADG, average daily feed intake (ADFI), F/G and diarrhoea rate (%) are shown in Table 2. CNP supplementation significantly increased the ADG and decreased the F/G and diarrhoea rate in a dose-dependent manner, compared with those of the control group ($p < .05$). Notably, the ADFI of 400 mg/kg CNP supplementation group was much higher than that of the other groups ($p < .05$), indicating that CNP might act as an appetite stimulant at concentrations up to 400 mg/kg. Diarrhoea was observed in the control and 100 mg/kg CNP groups. On days 14 and 15 of the trial period, six piglets in the control group developed diarrhoea, four healed after 3 days and two healed after 4 days respectively. On day 15, four piglets in the 100 mg/kg CNP group developed diarrhoea, three resolved spontaneously 3 days later and one healed after 4 days. There were no diarrhoea found in 200 mg/kg CNP group and 400 mg/kg CNP group.

3.2 | Humoral immunity

Table 3 shows the effects of CNP supplementation on immunoglobulin (Ig)A, IgG and IgM, and complement (C)3 and C4 levels in the plasma. Results showed that CNP improved plasma IgA, IgG, C3 and C4 concentrations in a dose-dependent manner on day 28, whereas the concentration of IgM was not affected by CNP supplementation.

These results implied that dietary supplementation with CNP improved the growth performance and immune status in weaned piglets in a dose-dependent manner. Additionally, the appropriate CNP dose ranged from 200 to 400 mg/kg.

3.3 | Inflammatory response

As shown in Table 4, before LPS challenge, PEG2 ($p > .05$) and TNF-α ($p < .05$) levels were high in piglets fed with CNP-supplemented diet. Then, LPS administration resulted in a dramatic increase in IL-1β, IL-6, TNF-α, cortisol and PEG2 plasma levels ($p < .05$). In the LPS-challenged piglets, CNP-supplemented diet decreased plasma cortisol, PEG2, IL-6 and IL-1β concentrations, compared with those in piglets fed with basal diet at 1.5 hr ($p < .05$) and 3 hr ($p < .05$) post-LPS challenge. However, TNF-α concentration was higher in the piglets fed with CNP-supplemented diet after LPS challenge ($p < .05$) than that in the piglets fed with basal diet. Collectively, CNP inhibited LPS-induced increase in cortisol, PEG2, IL-6 and IL-1β levels, whereas TNF-α level slightly increased.

3.4 | Gut microbiota

To investigate the effects of CNP on the gut microbiota, we profiled the overall composition of the gut microbiota in colonic contents by barcoded pyrosequencing of the V4 region of 16S rDNA genes. The 16S rRNA gene survey data indicated that CNP induced some changes in gut microbiota composition in the piglets fed with CNP-supplemented diet, compared to that of the piglets fed with basal diet. UniFrac-based PCoA and UPGMA showed the individual variations between the CNP and control groups (Figure 2a, b). The PCoA plot showed that the control and CNP groups were separated by 57.54% principal component 1 (PC1) and 16.6% PC2 variability. In consistence with the results of PCoA, UPGMA showed that piglets fed with CNP-supplemented diet tended to be clustered in their own group.

TABLE 2 Effects of CNP supplementation on growth performance in weaned piglets

Items	CNP, mg/kg 0	100	200	400
ADG/g	251.69 ± 10.09[a]	255.49 ± 11.70[a]	280.7 ± 11.10[ab]	290.51 ± 11.030[b]
ADFI/g	497.32 ± 18.53	494.65 ± 20.83	519.75 ± 17.55	521.40 ± 15.44
F/G	1.98 ± 0.05[a]	1.94 ± 0.02[a]	1.85 ± 0.01[b]	1.79 ± 0.01[b]
Diarrhoea rate (%)	1.98 ± 0.90[a]	1.29 ± 0.62[a]	0.00 ± 0.00[b]	0.00 ± 0.00[b]

Note: Different superscripts within a row indicate a significant difference ($p < .05$).
Abbreviations: ADFI, average daily feed intake; ADG, average daily gain; F/G, feed and gain.

TABLE 3 Effects of CNP supplementation on humoral immunity in weaned piglets

Items	CNP, mg/kg 0	100	200	400
IgA(g/L)	0.34 ± 0.04[c]	0.35 ± 0.06[c]	0.45 ± 0.07[b]	0.58 ± 0.06[a]
IgG(g/L)	2.55 ± 0.60[c]	2.63 ± 0.68[c]	3.13 ± 0.28[b]	3.38 ± 0.54[a]
IgM(g/L)	0.58 ± 0.08	0.58 ± 0.07	0.58 ± 0.08	0.58 ± 0.08
C3(mg/L)	68.57 ± 4.14[c]	73.83 ± 2.33[c]	84.57 ± 4.32[b]	99.09 ± 2.95[a]
C4(mg/L)	32.63 ± 1.70[c]	34.57 ± 1.18[bc]	37.15 ± 1.57[ab]	39.65 ± 1.01[a]

Note: Different superscripts within a row indicate a significant difference ($p < .05$).

TABLE 4 Effects of CNP supplementation on inflammatory response of weaned piglets challenged with LPS

LPS(100 μg/kg BW)	−LPS		+LPS	
CNP(mg/kg)	0	400	0	400
Cortisol (ng/ml)				
0 hr	253.48 ± 13.05[a]	248.86 ± 9.55[a]	225.48 ± 6.66[b]	254.94 ± 12.05[a]
1.5 hr	282.44 ± 10.24[c]	269.98 ± 14.79[c]	330.29 ± 11.38[a]	314.08 ± 10.14[b]
3 hr	290.96 ± 10.58[c]	264.98 ± 9.93[d]	335.25 ± 7.43[a]	320.94 ± 9.20[b]
PEG2(ng/ml)				
0 hr	873.93 ± 18.50[b]	923.41 ± 30.32[a]	904.74 ± 18.47[a]	925.66 ± 22.34[a]
1.5 hr	972.66 ± 26.93[c]	975.05 ± 29.46[c]	1,253.81 ± 97.34[a]	1,055 ± 30.04[b]
3 hr	979.91 ± 19.78[c]	1,003.21 ± 32.09[bc]	1,219.57 ± 98.58[a]	1,066.47 ± 46.85[ab]
IL−6(ng/L)				
0 hr	61.00 ± 3.89[b]	66.26 ± 5.25[a]	67.70 ± 3.85[a]	62.15 ± 3.49[ab]
1.5 hr	66.82 ± 4.24[b]	70.82 ± 4.81[b]	80.48 ± 4.49[a]	70.74 ± 2.98[b]
3 hr	72.82 ± 3.64[c]	71.05 ± 3.19[c]	115.69 ± 5.83[a]	86.57 ± 8.79[b]
IL−1ß(ng/L)				
0 hr	25.19 ± 1.58[a]	23.49 ± 1.42[b]	24.12 ± 1.54[ab]	24.68 ± 1.56[a]
1.5 hr	30.40 ± 2.64[c]	27.94 ± 0.94[c]	43.30 ± 2.71[a]	33.28 ± 3.35[b]
3 hr	31.83 ± 2.80[c]	28.30 ± 1.90[c]	48.21 ± 1.61[a]	41.96 ± 1.62[b]
TNF-α(ng/L)				
0 hr	26.46 ± 1.17[b]	29.21 ± 0.83[a]	27.26 ± 0.80[b]	28.23 ± 1.01[a]
1.5 hr	28.66 ± 1.00[c]	30.28 ± 1.00[c]	36.92 ± 1.19[b]	39.07 ± 2.37[a]
3 hr	26.33 ± 1.82[d]	28.14 ± 1.29[c]	33.14 ± 1.42[b]	35.53 ± 1.38[a]

Note: Different superscripts within a row indicate a significant difference.

We evaluated the ecological features of the bacterial communities in the CNP and control groups using various indices based on the OTU level. As shown in Table 5, bacterial diversity was significantly higher in the CNP group than that in the control group, as indicated by the Shannon index (*t* test $p < .05$). The average Shannon's diversity index was 6.82 ± 0.53 in the CNP group, which was approximately 10% higher than that in the control group, whereas Simpson's index showed 18.5% more diversity in the CNP group (*t* test $p > .05$). The CNP group also showed high values of richness estimators (Chao1 and ACE; both *t* test $p > .05$), whereas the good coverage of all the samples was 0.999.

The relative abundance of the gut microbiota at the phylum level is shown in Figure 2c. Consistent with previous findings in the ileal, caecal and faecal microbiota of weaning and finishing pigs (Buzoianu et al., 2012; Kim et al., 2011), *Firmicutes* and *Bacteroidetes phyla* accounted for more than 90% of the total sequences. In the CNP group, the phylum *Bacteroidetes* increased from 15.8% (in the control group) to 22.4%, and *Firmicutes* decreased from 77.2% (in the control group) to 69.1%. These results indicated that long-term consumption of CNP could cause a slight decrease in the F/B ratio.

The metagenome analysis, LEfSe was used to identify the key phylotypes responsible for the differences between the CNP and control groups. As shown in Figure 2d, CNP feeding resulted in decreased abundance of *Clostridiaceae* and increased abundance of *Prevotellaceae* and *Ruminococcaceae* families.

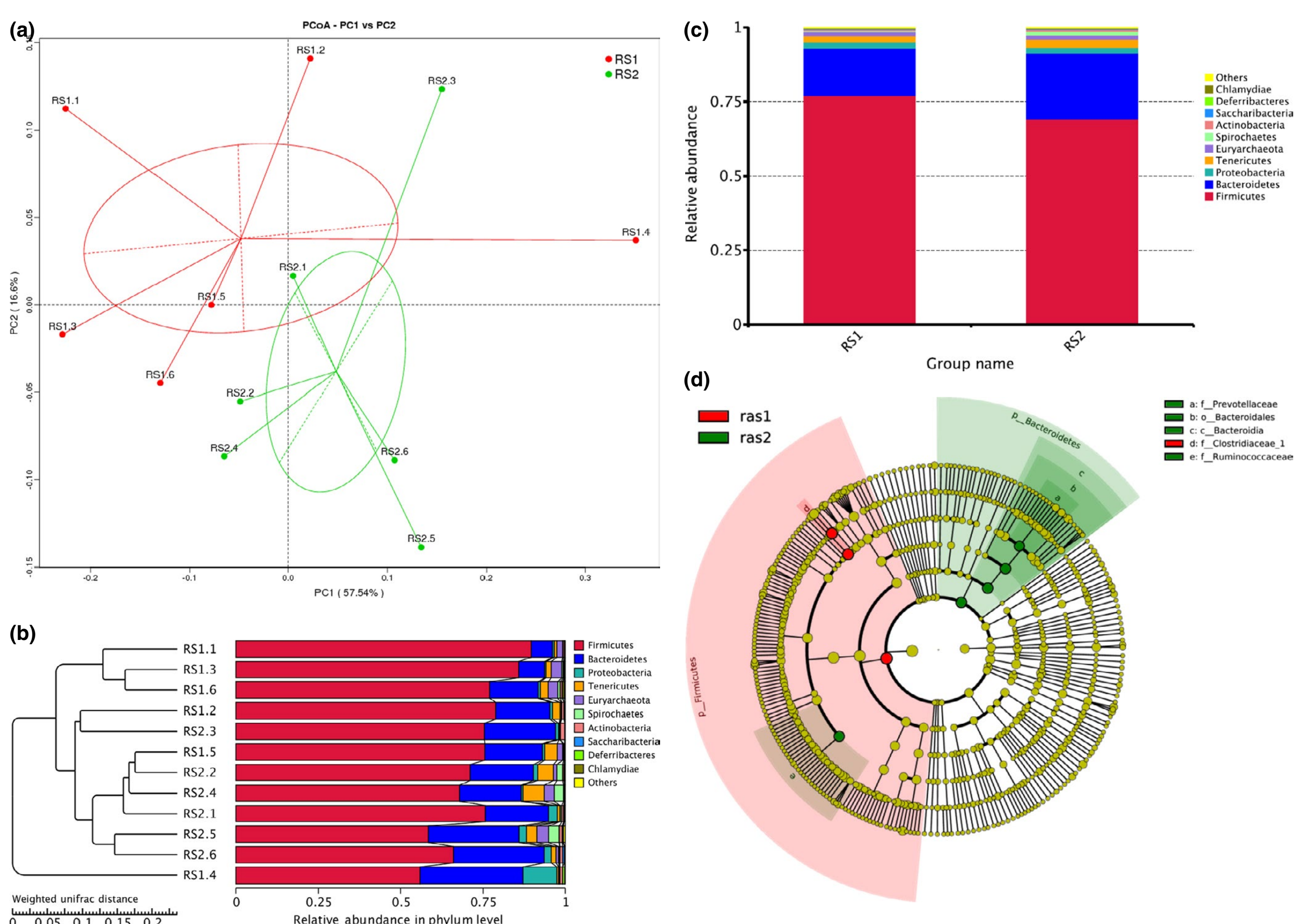

FIGURE 2 Modulation effect of CNP on the gut microbiota (RS1: control group; RS2: CNP group). Plots shown were generated using the weighted version of the UniFrac-based PCoA (a) UPGMA (b). The relative abundance of gut microbiota composition at the phylum level (n = 6). (c) Specific phylotype responding to HFD and AGSP was indicated by LEfSe (log = 10) (d) [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 5 Effect of CNP on Diversity of gut microbiota in weaned piglets

Diversity index	CNP(mg/kg) 0	400	*p* value
observed_species	711 ± 45.143	798 ± 116.72	0.1385
shannon	6.147 ± 0.32	6.824 ± 0.533	0.02783
simpson	0.953 ± 0.014	0.971 ± 0.013	0.05258
chao1	757.529 ± 54.958	842.052 ± 115.548	0.1488
ACE	759.4 ± 48.635	847.496 ± 113.411	0.1252
PD_whole_tree	48.945 ± 3.062	54.089 ± 6.047	0.1031
Goods_coverage	0.999	0.999	NA

4 | DISCUSSION

Chitosan and its derivatives have been previously classified as pre-biotics. Their beneficial effects on growth performance, immunity, blood profile, and gut microflora of pigs and poultry have been reported (Kong et al., 2014; Li et al., 2007; Liu et al., 2008). However, previous studies (Chae, Jang, & Nah, 2005; Lee, Senevirathne, Ahn, Kim, & Je, 2009; Thanou, Nihot, Jansen, Verhoef, & Junginger, 2001) showed that *N*-deacetylation and the molecular weight were the main factors affecting the efficacy of chitosan as a modulator of the immune responses. Therefore, CNP with nanosize and high density amino groups might exhibit high efficacy in domestic animals. In this study, the effects of dietary CNP supplementation on growth performance, immunity and gut microflora were evaluated for the first time to determine the application potential of CNP as a feed additive.

Diarrhoea, low feed intake and nutrient digestibility were always challenging for weaning pigs. Tang et al. (2005) reported that chitosan could improve the growth performance and feed efficiency of piglets, where the increase in growth performance might be principally attributed to the increase in growth hormone or insulin growth factor 1 (IGF-1) concentration. However, Yang et al. (2012) showed that chito-oligosaccharide supplementation increased the growth rates of piglets owing to the increased feed intake. Results of the current study showed that CNP supplementation increased ADG; however, ADFI tended to increase only at CNP concentration of 400 mg/kg. Therefore, further studies are needed to confirm whether CNP exhibits growth promoter effects and if these effects are related to digestibility or hormonal regulation.

The humoral immune response is very important for animals since it can protect against most bacterial, as well as certain viral infections (McKee, Munks, & Marrack, 2007). Our results showed that CNP supplementation improved the immune status of piglets after weaning, as indicated by the increase in IgG, IgA, C3 and C4 levels in the plasma. Kobayashi et al. (2013) showed that administration of ovalbumin (OVA) with CNP or cationic chitosan induced a high OVA-specific IgA response in mice, suggesting that CNP could improve the animal-specific immune defences. Results of our previous study (Wen et al., 2011) showed that the use of CNP as an adjuvant significantly increased serum IgG titres in mice. CNP-induced increase in serum IgG was particularly important because it might countervail the decrease in antibodies owing to weaning. The complement system is a major effector of innate immunity and an adjuvant of adaptive immunity. Complements comprise approximately 30 plasma and cell-surface proteins that interact with one another to induce a series of inflammatory responses involved in the defences against infections (Ricklin, Hajishengallis, Yang, & Lambris, 2010). Minamia, Suzuki, Okamoto, Fujinagab, and Shigemasa (1998) found that chitin and chitosan activated the complement components, C3 and C5, via the alternative pathway. Additionally, they found that the number of amino groups and trapping of C3b represented important evidence of complement activation via the alternative pathway by chitosan and non-water-soluble chito-oligosaccharides (Suzuki, Miyatake, Okamoto, Muraki, & Minami, 2003). Our results also showed that CNP significantly improved plasma C3 concentrations in piglets in a dose-dependent manner on day 28, most probably because of the high density of amino groups on CNP surface.

Chitosan nanoparticles supplementation at 400 mg/kg resulted in better growth performance effects than those of lower concentrations. Therefore, this concentration was used in the subsequent challenge experiment. Social and handling stress can provoke activation of the hypothalamic-pituitary-adrenal axis in pigs, which in turn affects the production of proinflammatory cytokines (Tuchscherer, Kanitz, Puppe, & Tuchscherer, 2010). The results of our study verified that LPS evoked dramatic inflammatory responses since IL-1β, IL-6, TNF-α, cortisol and PEG2 plasma levels dramatically increased after LPS administration. Piglets fed with CNP-supplemented diet exhibited less disease-associated stress than those fed with basal diet. CNP treatment decreased IL-1β, IL-6, cortisol and PEG2 concentrations during part of the challenge period, compared with those in the basal diet-fed group. However, TNF-α level increased in the CNP supplementation group. Symptoms, such as intermittent coughing, salivation, retching and vomiting, were also observed after LPS administration. Ninety minutes after LPS challenge, all piglets fed with basal diet became depressed, as manifested by lethargy and respiratory difficulties ranging from panting to severe dyspnoea, whereas only 2 piglets fed with CNP-supplemented diet showed these symptoms. Cortisol is a glucocorticoid with potential immunosuppressant and proinflammatory cytokine regulatory effects. PGE2 exerts its pyrogenic action by binding to receptors on thermoregulatory neurons in the hypothalamus. It is responsible for the increase in body temperature in most species (Blatteis, Li, Li, Feleder, & Perlik, 2005). TNF-α has been considered an excellent marker for endotoxin tolerance since its levels increase both rapidly and dramatically following LPS administration to reduce the responsiveness to LPS challenge (Biswas & Lopez-Collazo, 2009). Based on the current understanding, the less severe symptoms of CNS depression in the CNP group could be associated with the decreased PEG2 level and increased TNF-α level in the plasma.

The intestinal microbiota and its metabolic activities are considered key factors for animal health and performance. The microbiota composition largely affects many aspects of the host health, including digestion of feed to breakdown products, stimulation of the immune system and competition with pathogens. Microbial diversity has been associated with better health outcomes (Roselli et al., 2017). Low bacterial diversity has been linked to obesity and inflammatory bowel disease. Deep 16S rDNA sequencing showed differences in the gut microbial communities between piglets fed with CNP-supplemented diet and those fed with basal diet. CNP significantly increased community diversity, as shown by Shannon's diversity index. Furthermore, the ratio of the predominant microbial *phyla*, *Firmicutes* to *Bacteroidetes*, in the gut is significantly relevant to the overall health status of the animal. Elevated proportions of *Firmicutes* have been associated with increased susceptibility to inflammation, infections, oxidative stress and insulin resistance (Hakansson & Molin, 2011; Mafra et al., 2014). In the current study, the gut microbiota of CNP-supplemented piglets showed a shift from *Firmicutes* to *Bacteroidetes*, compared with that in the control piglets. Mach et al. (2015) found that the microbiota composition coevolved with piglets comprised two different clusters after weaning, primarily distinguished by unclassified *Ruminococcaceae* and *Prevotellaceae* abundance. Moreover, specific changes in microbial ecology and presumably in its functional activity that can occur early in life are associated with subsequent susceptibility of pigs to post-weaning diarrhoea. A recent study conducted by Dou et al. (2017) emphasized the potential of early microbiota diversity and composition as indicators of the susceptibility to post-weaning diarrhoea. Data showed that healthy piglets exhibited a higher abundance of *Prevotellaceae*, *Lachnospiraceae*, *Ruminocacaceae* and *Lactobacillaceae* than that in piglets with diarrhoea during their early lives. Therefore, our results suggested that CNP might increase the abundance of the beneficial bacterial species (belonging to *Prevotellaceae* and *Ruminococcaceae*

families) and decrease that of several potential pathogens (belonging to *clostridiaceae* family) in the intestinal luminal content.

Recent breakthroughs in CNP preparation technology developed in our laboratory have made it possible to supplement it into piglet food. To the best of our knowledge, this is the first study to evaluate the effects of CNP in weaning piglets. Results of the present study indicated that dietary supplementation with CNP could improve the growth performance and immune status, alleviate the immunological stress and regulate intestine ecology in weaning piglets. Therefore, CNP could be used as a potential supplement in piglet feed. Moreover, further studies are needed to investigate the potential mechanisms underlying these beneficial effects.

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CONFLICT OF INTEREST

There is no conflict of interest.

ANIMAL WELFARE STATEMENT

We have confirmed all the experiment involving animals were conducted followed EU standards for the protection of animals used for scientific purposes and approved by Animal Ethics Committee of Zhejiang A&F University. The piglets were fed in the farm of Hangzhou Zhengxing animal husbandry Co. LTD and maintained in an appropriate animal care facility with free access to feed and water.

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