Effects of *Lactobacillus acidophilus* and zinc oxide on the growth performance, jejunal morphology and immune function of weaned piglet following an *Escherichia coli* K88 challenge

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ABSTRACT

This study evaluated the effects of *Lactobacillus acidophilus* (LA) and zinc oxide (ZnO) supplementation on the growth performance, jejunal morphology and immune function of weaned piglets challenged with *Escherichia coli* K88. A total of 96 weaned piglets were randomly allocated to four treatments in a 28-day experiment with two levels of *L. acidophilus* (0 and 5 × 10\textsuperscript{10} CFU/kg feed), and two levels of ZnO (0 or 3000 mg/kg feed). One piglet per pen was challenged with *Escherichia coli* K88 on day 28 and slaughtered at 6 hours post-infection. The results showed that LA supplementation increased the average daily gain from day 0 to day 28 (\(p < .05\)). Piglets fed with the diets containing LA or ZnO showed lower feed conversion ratio than the unsupplemented group (\(p < .05\)). In addition, LA supplementation increased villus height (VH), villus width and the ratio of VH and crypt depth (CD) compared to the unsupplemented group (\(p < .001\)). Administration of ZnO increased VH (\(p = .001\)) and VH:CD ratio (\(p = .039\)). Moreover, dietary LA and ZnO upregulated the mucin 2 and \(\beta\)-defensin 3 expression in the jejunum (\(p < .001\)), and LA supplementation increased immunoglobulin A concentration in the jejunal tissue (\(p = .019\)). In conclusion, the administration of LA or ZnO was effective in improving growth performance of weaned piglets, and both supplements may benefit the jejunal development and immune function of piglets following *E. coli* K88 challenge.

Introduction

At weaning, young piglets suffer abrupt changes that can result in morphological and functional damages in the intestine, such as villus shorting, crypt elongation, reduction of activities of digestive enzymes and nutritional transports (Montagne et al. 2007; Hu et al. 2013). Enterotoxigenic *Escherichia coli* (ETEC) is a major issue in the swine industry and results in scouring, increased mortality and poor performance during the post-weaning period (Bhandari et al. 2008). Antibiotics are routinely used in an attempt to control pathogenic infection. However, the development of antibiotic resistance in a number of important pathogenic bacterial species becomes the most pressing issue in public health (Witte 1998), and thus, safe antibiotic alternatives for piglets are highly required.

Studies have shown that *Lactobacillus* strains (Lee et al. 2012; Yang et al. 2014) and ZnO (Hill et al. 2001) were beneficial to growth performance and health status of weaning piglet. However, some studies indicated that *L. brevis*-supplementation feeding induced a non-significant increase in piglet body weight and caused no change in the morphology of the intestinal mucosa (Lähteinen et al. 2014). The small intestine morphology of weaned piglet, measured by villus height and crypt depth, was not modified by feeding the diet containing *L. sobrius* when the piglets were challenged with *E. coli* F4 (Konstantinov et al. 2008). The growth performance of weaned piglet was not improved with ZnO supplementation (Schell and Kornegay 1996). These non-positive results might be correlated with multiple parameters, such as *Lactobacillus* strains, addition amount and structure of ZnO. However, ZnO was...
banned and (or) restricted in the European Union because zinc remains a heavy metal tends to accumulate in soil and as such toxic to animals (Mavromichalis 2011), thus the limited ZnO intake (≤3000 ppm) in the short time after weaning should be applied.

To the best of our knowledge, the effect of the combination of ZnO and probiotics supplementation on the growth performance, intestinal microbiota and immune status of weaned piglets is rarely reported. Therefore, the aim of the present study was to evaluate the impact of a new probiotic *L. acidophilus* (Qiao et al. 2015) and ZnO on the growth performance, jejunal morphology and immune function of weaned piglet orally challenged with *E. coli* K88.

**Materials and methods**

All animals used in this study were humanely managed according to the Chinese Guidelines for Animal Welfare. The experimental protocol was approved by the Animal Care and Use Committee of the Tianjin Academy of Agricultural Sciences.

**Animals and experimental design**

A total of 96 crossbred piglets (Duroc × Landrace × Yorkshire) with an average weight of 7.33 ± 0.12 kg, weaned at 28 ± 2 d age and randomly allotted to four treatments in a completely randomised design, were used in a 28-d growth experiment. Pigs were allotted to four treatments, in which had six pens of four piglets. Each treatment was randomly allocated to a 2 × 2 factorial experiment involving two levels of *L. acidophilus* supplementation (0 or 5 × 10^10 CFU/kg feed) and two levels of ZnO supplementation (0 or 3000 mg ZnO/kg feed). The compositions of the basal diets are shown in Table 1. The basal diet was formulated and manufactured before starting the trial, without the inclusion of any antibiotic growth promoters or antibiotic growth promoter alternatives. Pigs had free access to experimental diets and drinking water. A combination of daylight and artificial light was used, with a 12-h light/dark cycle. Ventilation was achieved by using variable-speed fans. The starting temperature of 28°C was adjusted weekly to reach a final temperature of 24°C. The weight and feed disappearance were measured on day 0 and 28 post-weaning for the calculation of average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR).

At 08:00 on day 28, one piglet per pen with the body weights were close to the pen average was chosen after all piglets were weighed and was then orally given a single dose of 10 mL 0.9% NaCl solution containing approximately 1 × 10^11 CFU of *E. coli* K88 using a disposable syringe. At 6 h after the *E. coli* K88 challenge, piglets were humanely sacrificed with sodium pentobarbital (50 mg/kg, intravenously injected) for sampling the jejunum.

**Histology on the jejunum**

At necropsy, a 3-cm long segment of ascending jejunum was longitudinally cut along mesenteric attachment and rinsed with 0.9% NaCl solution, and then the tissue was fixed immediately in 10% neutral buffered formalin until processing. Paraffin-embedded intestinal samples were sectioned (5 μm) and stained with haematoxylin and eosin for histological analysis. Ten villi and corresponding crypts were randomly chosen from different well-orientated parts of the sections, where most of the villi and crypts were cut longitudinally from the tip of villi to the bottom of the crypts in the stained sections. The villus height (VH), villus width (VW) and crypt depth (CD) were measured with computer-assisted microscopy (Micrometrics TM; NikonECLIPSE E200, Tokyo, Japan).

**RNA extraction, gene expression and intestinal IgA analysis**

At necropsy, another 10 cm segment of jejunal tissue was snap-frozen in liquid nitrogen and stored at

| Table 1. Ingredients and calculated composition of the basal diet (percentage as-fed basis). |
| --- |
| Item | Amount |
| Ingredients, % | |
| Corn, yellow | 63.20 |
| Soybean meal, 43% CP (crude protein) | 19.00 |
| Whey powder | 4.80 |
| Fish meal, 65% CP | 8.60 |
| Glucose | 1.00 |
| Acidifier | 0.30 |
| Calcium hydrogen phosphate | 0.60 |
| Limestone | 0.70 |
| Salt | 0.30 |
| L-Lys·HCL, 78% Lys | 0.30 |
| DL-Met, 99% Met | 0.10 |
| L-Thr, 98% Thr | 0.10 |
| Vitamin and mineral premix | 1.00 |
| Calculated composition | |
| DE (digestible energy), Mcal/kg | 3.25 |
| Lys, % | 1.39 |
| Met, % | 0.53 |
| Analyzed composition | |
| Crude protein, % | 18.60 |
| Calcium, % | 0.82 |
| Total phosphorus, % | 0.65 |

aSupplying a minimum per kilogram complete diet of: 12,500 U Vitamin A; 1,250 U Vitamin D; 125 U Vitamin E; 90 μg Vitamin B12; 10 mg riboflavin; 48 mg pantothenic acid; 35 mg niacin; 4.5 mg folic acid; 0.25 mg biotin; 130 mg Fe; 180 mg Zn; 15 mg Cu; 30 mg Mn; 0.60 mg I and 0.25 mg Se.
−80 °C until mRNA extraction, gene expression and IgA analysis performed. Total RNA was extracted from tissue samples using the Trizol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s guidelines, and 1 μg of RNA was used for cDNA synthesis using All-in-one™ First-Strand cDNA Synthesis Kit (GeneCopoeia). RNA was spectrophotometrically quantified (A260) and its integrity was verified by agarose gel electrophoresis. Real-time PCR was performed using specific primers for MUC2 (Liu et al. 2014) (sense 5′-CTGCTCCGGGTCTGTGGGA-3′, antisense 5′-CCCCTGGCTGGTGCTGATA-3′) with accession number AK231524, DEFB3 (Liu et al. 2014) (sense 5′-GTGCAGAAGGGCAATGGTCG-3′, antisense 5′-GTGTCAGCAGGATGAAGCA-3′) with accession number AY460576 or GAPDH (Yu et al. 2007) (sense 5′-GAAGG TCGGAATGAACGGAT-3′, antisense 5′-CATGGGTAGATGAAGCA-3′) and All-in-one™ qPCR mix kit (GeneCopoeia) in accordance with the manufacturer’s protocol for the ABI7500 Real-time PCR system (Applied Biosystems, Life Technologies, Carlsbad, CA). The amplification was performed with the following conditions: one cycle of denaturation at 95 °C for 10 min, followed by 40 PCR cycles, each consisting of 10 s of denaturation at 95 °C, 20 s of annealing to 60 °C, 15 s of extension at 72 °C. The PCR efficiency and melting curves were checked to ensure consistent amplification of a single PCR product. Gene expression was normalised to GAPDH (internal reference) and presented as relative fold change compared with control (−LA−ZnO). All samples were tested in triplicate. The amplification efficiency of each primer pair was determined by using twofold serial dilutions of cDNA as previously described (Liu et al. 2012). The amplification efficiencies of all target genes and the reference housekeeping gene GAPDH were close to 100%. Agarose gel electrophoresis and sequencing were conducted to verify the amplification products. The relative expression of the target gene mRNA were analysed by using the 2^−ΔΔCT method as previously described (Zhu et al. 2014).

A sandwich IgA ELISA (Nanjing Jiancheng Bioengineering Institue, Nanjing, China) was used to quantify the IgA concentration of the jejunal tissue homogenate supernatant. Briefly, an ELISA plate was coated with goat anti-pig IgA antibody and incubated at room temperature (25 °C). After washing, standards diluted samples were added to designated wells. Following further washings, goat anti-pig IgA HRP (horseradish peroxidase) conjugated antibody was added to each well as a detection antibody. Following a further incubation period, TMB enzyme substrate (3,3′-5,5′-tetramethyl benzidine) was added to each well. After 20 min, the reaction was stopped with 2 M H2SO4 and individual well absorbencies were determined at 450 nm. The standards were used to construct a standard curve from which the IgA concentration of the samples could be determined. The results were expressed as μg IgA per g jejunal tissue.

**Statistical analysis**

The data were analysed as a completely randomised design with a 2 × 2 factorial treatment arrangement by ANOVA using the GLM procedure in SAS v. 9.2 (SAS Inst. Inc., Cary, NC). The statistical model included the effects of L. acidophilus (− or +), ZnO (− or +) and their interactions. The pen represented the experimental unit for growth performance, and the piglet was the experimental unit for other parameters. Treatment comparisons were performed using a Tukey’s honestly significant difference test for multiple testing. Probability values of ≤.05 were considered to be significant, whereas a treatment effect trend was noted for p ≤ .10.

**Results**

**Growth performance**

Table 2 shows the effects of LA and ZnO supplementation on the growth performance of weaned piglets. The administration of LA increased ADG of piglets

![Table 2. Effects of LA and ZnO supplementation on the growth performance of weaned piglets.](https://example.com/table2.png)

**Note:** ADG: average daily gain; ADFI: average daily feed intake; BW: body weight; FCR: feed conversion ratio; LA: Lactobacillus acidophilus; ZnO: zinc oxide.

^n= 6 replicates/treatment.
compared to the control group from day 0 to 28 ($p = .025$). Addition of both LA and ZnO to the diet lowered FCR significantly ($p = .026$ and $.011$, respectively). In addition, dietary LA tended to increase BW on day 28 ($p = .090$) and dietary ZnO had the tendency to increase ADG compared to the unsupplemented group ($p = .064$). However, there was no significant interaction between LA and ZnO on the growth performance ($p > .05$).

**Morphometric characteristics**

The effects of LA and ZnO supplementation on the morphometric characteristics of the jejunal mucosa are presented in Table 3. Compared to the control group, LA supplementation enhanced the VH, VW and VH:CD and lowered the CD ($p < .001$), and ZnO administration significantly increased the VW ($p = .001$) and VH:CD ($p = .039$). The results also indicated that the VH in the jejunal mucosa of the piglets tended to be affected by LA and ZnO (LA = ZnO, $p = .059$).

**Gene expression and IgA concentration**

The relative gene expression of MUC2, DEFB3 and IgA concentration in the jejunum of weaned piglets are summarised in Table 4. Both LA and ZnO administrations upregulated the MUC2 and DEFB3 expression ($p < .001$). Supplementation of LA increased the IgA concentration in jejunal tissue ($p = .019$). However, there was no significant interaction between LA and ZnO on the gene expression and IgA concentration ($p > .05$).

**Discussion**

The objective of the present study was to determine both independent and interactive effects of supplementation with $5 \times 10^{10}$ CFU/kg *Lactobacillus acidophilus* and 3000 mg/kg ZnO on the growth performance, jejunal morphology and immune function of weaned piglet in an *E. coli* K88 infection model. Weaning stress impairs the development of mucosal barrier in the porcine intestine (Smith et al. 2010) characterised by increased intestinal permeability, which results in the translocation of luminal bacteria, toxins and antigens into subepithelial tissues, inciting mucosal and systemic inflammatory responses that are central cause to many gastro-intestinal disorders (Blikslager et al. 2007). In the present study, the results indicated that piglets fed $5 \times 10^{10}$ CFU *L. acidophilus* or 3000 mg ZnO/kg feed had greater ADG, lower F:G than the unsupplemented group. Consistently, previous studies have established improved ADFI and ADG of piglets fed *Lactobacillus* strains (Lee et al. 2012; Yang et al. 2014; Qiao et al. 2015) or ZnO (Hill et al. 2001). Thus, we can speculate that both LA and ZnO could improve the growth and might partly attenuate the effects of the stress of piglets during the post-weaning period.

The oral challenge of pigs with ETEC has been widely used as a model of post-weaning diarrhoea. ETEC colonises the small intestine by fimbriae that attach to intestinal epithelial glycoprotein receptors (Kulkarni et al. 2010), and the subsequent secretion of heat-stable and/or heat-labile enterotoxins leads to a disruption of electrolyte balance and diarrhoea (Ondrackova et al. 2012; Zhou et al. 2012). In the weaning period, the most promising effects of

### Table 3. Effects of LA and ZnO supplementation on the morphometric characteristics of the jejunal mucosa of weaned piglets challenged with *E. coli* K88*

| Item   | –ZnO  | +ZnO  | –LA  | +LA  | SEM  | LA p value | ZnO p value | LA × ZnO p value |
|--------|-------|-------|------|------|------|------------|-------------|------------------|
| VH, µm | 397   | 412   | 430  | 429  | 4    | <.001      | .091         | .059             |
| VW, µm | 140   | 160   | 160  | 170  | 4    | <.001      | .001         | .182             |
| CD, µm | 164   | 155   | 146  | 143  | 3    | <.001      | .088         | .369             |
| VH:CD  | 2.42  | 2.66  | 2.95 | 3.01 | 0.07 | <.001      | .039         | .175             |

CD: crypt depth; LA: Lactobacillus acidophilus; VH: villus height; VW: villus width; VH:CD: villus height to crypt depth ratio; ZnO: zinc oxide.

*n = 6 replicates/treatment.

### Table 4. Effects of LA and ZnO supplementation the relative gene expression of MUC2, DEFB3 and IgA concentration in the jejunum of weaned piglets*

| Item   | –ZnO | +ZnO | –LA | +LA | SEM | LA p value | ZnO p value | LA × ZnO p value |
|--------|------|------|-----|-----|-----|------------|-------------|------------------|
| MUC2   | 1    | 1.80 | 2.43| 3.82| 0.28| <.001      | .091         | .059             |
| DEFB3  | 1    | 1.80 | 4.24| 5.74| 0.50| <.001      | .001         | .153             |
| IgA, µg/g | 21.25| 21.36| 22.18| 22.32| 0.48| .019       | .517         | .943             |

DEFB3: β-defensin 3; IgA: immunoglobulin A; LA: Lactobacillus acidophilus; MUC2: mucin 2; ZnO: zinc oxide.

*n = 6 replicates/treatment.
probiotics are related to their competitive exclusion of pathogenic bacteria (Lee et al. 2012). The way in which Lactobacillus species inhibit pathogens growth has been attributed to steric hindrance of binding sites (Ouwehand and Conway 1996), pH values (Lehto and Salminen 1997) and certain components of the lysed cell wall (Lehto and Salminen 1997). Moreover, Lactobacillus strains would synthesise exopolysaccharides in their growth phase (Lebeer et al. 2008). In our previous study, we have proved that L. acidophilus could modify intestinal microflora (Qiao et al. 2015), which may be important to enhance health state of piglets. Previous studies reported that the breakdown of intestinal barrier function was the deleterious effect of weaning (Spreeuwenberg et al. 2001; Boudry et al. 2004). The intestinal barrier is composed of the single layer of columnar epithelial cells that line the intestinal tract and serves as the body’s first line of defence against potentially harmful microorganisms and antigens residing within the intestinal lumen (Gewirtz et al. 2002; Smith et al. 2010; Niguez-Palomares et al. 2011). In the present study, the short villus height and long crypt depth observed in the control treatment was in agreement with the established literature, which reported a drastic deterioration of intestinal morphology after weaning and consumption of dry diets (Kim et al. 2012). Probiotics have been widely used to promote gut microbiota balance, intestinal epithelial integrity and appropriate maturation of the gut-associated tissue (Metzler et al. 2005). In our study, L. acidophilus supplementation improved the morphometric characteristics in the jejunal mucosa of piglets via enhancing the villus height, villus width and villus height-to-crypt depth ratio and decreasing the crypt depth. Zinc plays a major role in the regulation and differentiation of the intestinal tissues, likely through molecular regulators that include Zn finger proteins such as Krüppel-like factor 9 (Simmen et al. 2007). Our present results indicated that ZnO administration increased the villus width and the villus height-to-crypt depth ratio, which was in agreement with the previous findings; other authors observed, indeed, that administration of high concentration of dietary Zn moderately increased the villus height and tended to decrease the crypt depth in the lower small intestine in piglets (Li et al. 2001).

The development of the digestive tract, as well as the associated immune defence mechanisms, is very important for optimised feeding concepts in pigs (Pluske 2013). To the best of our knowledge, data of the influence of nutritional factors on mucin gene expression in the intestinal tract of pigs are limited (Sargeant et al. 2010; Liu et al. 2014). The mucus layer is organised by the highly glycosylated MUC2 mucin (Johansson et al. 2011), and MUC2 synthesis rate might be a potential factor for intestinal barrier (Schaart et al. 2009). No influence of age or Zn supplementation was observed on the expression of MUC2 in the distal jejunum of weaned piglets (Liu et al. 2014), however, MUC2 concentration was improved with increased Zn supplementation. In this study, piglets fed diets containing 3000 mg ZnO/kg had increased MUC2 level compared to the control group. Zinc supplementation was associated with a marked decrease in the expression of immune response genes concerned with inflammation and possibly related to the stage of infection. A reduced level of MUC4 (a proposed ETEC K88 receptor) was associated with zinc supplementation, which suggests how zinc supplementation might restrict ETEC infection (Sargeant et al. 2010). In this study, L. acidophilus supplementation enhanced MUC2 expression in the jejunum, which is consistent with the findings from the previous in vitro studies that several Lactobacillus species increased mucin expression in the human intestinal cell lines Caco-2 (MUC2) and HT29 (MUC2 and 3), thus blocking pathogenic E. coli adherence invasion (Mack et al. 2003) and is consistent with the findings from the previous in vivo studies that dietary Lactobacillus fermentation IS007 modulated immunity along with promoting the expression of MUC2 and MUC3 (Yu et al. 2008). As a major family of antimicrobial peptides, defensins are widely expressed in a variety of epithelial cells and sometimes in leukocytes, playing an important role in the innate immune system due to their antimicrobial, chemotactic and regulatory activities, and the β-defensins play a critical role in the mammalian innate immunity (Liu et al. 2014). In this study, we evidenced that L. acidophilus and ZnO could up-regulate the expression of DEFB3 in the jejunal tissue. Vinderola et al. (2006) observed that oral administration of the exopolysaccharide produced by Lactobacillus kefiranofaciens enhanced the IgA production at both the small and large intestine level. Yoshida et al. (2009) also reported that oral administration of Lactobacillus plantarum Lq80 tended to increase intestinal IgA of weaned piglets. In agreement with the previous studies, our results showed the increased levels of jejunal tissue IgA in the piglets fed with LA compared to the control group, which might modify immune response of piglet and attenuate the influence by E. coli K88 infection, and consequently explicate the enhanced growth performance of piglet with L. acidophilus administration.
Conclusions

Our observations suggest that both Lactobacillus acidophilus and zinc oxide supplementation may improve the growth performance of weaned piglets and also beneficially modulate the jejunal development and immune function in piglets after an E. coli K88 challenge. However, there is no significant interaction between LA and ZnO in this study.

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Disclosure statement

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