

Research Note: Effects of organic zinc on broiler intestinal permeability and integrity in *Clostridium perfringens*–challenged condition

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ABSTRACT This study evaluates the effects of a zinc–amino acid complex on broiler’s intestinal permeability and integrity challenged with *Clostridium perfringens*. A total of 180 Arbor Acres 1-day-old male broilers were assigned to 6 treatments in a completely randomized 2 × 3 factorial design. The experiment investigated the comparative effects of inorganic and organic zinc supplements, that is (ZnSO₄ treatment: 80 mg zinc/kg from ZnSO₄; iso-dose replacement group. (ISO) treatment: 40 mg zinc/kg from ZnSO₄ plus 40 mg zinc/kg from a zinc–amino acid complex; and organic treatment: 40 mg zinc/kg from a zinc–amino acid complex) on *C. perfringens*–challenged broilers. *C. perfringens*, on the one hand, compromised intestinal mucosal barrier function by increasing the intestinal permeability of fluorescein isothiocyanate–labeled dextran ($P < 0.05$) and plasma endotoxin level, on the

other hand, decreased both the transepithelial electrical resistance and the relative expression of occludin levels in the ileum at day 21 ($P < 0.05$). However, zinc supplement alleviates *C. perfringens*–induced pathologic changes in the intestinal permeability. ISO treatment, in particular, enhanced the villus height–to–crypt depth ratio and transepithelial electrical resistance and also reduced plasma endotoxin levels. In addition, zinc supplement relatively enhanced the expression of occludin levels in the ileum compared with the *C. perfringens*–challenged group. ISO treatment had the highest relative expression of occludin levels in the ileum. Thereby, the results indicated that partial replacement of ZnSO₄ with a zinc–amino acid complex in the broiler diet could promote intestinal mucosal barrier function during *C. perfringens* infection via increased expression of occludin in the ileum.

Key words: zinc–amino acid complex, *Clostridium perfringens*, ileum, permeability and integrity

2020 Poultry Science 99:6653–6656

<https://doi.org/10.1016/j.psj.2020.09.032>

INTRODUCTION

The intestinal mucosa serves as a link between the external environment and the intestinal immune system, thus functioning as an immune barrier. The integrity of mucosal barrier functions is critical to maximizing nutrient absorption and preventing the development of inflammatory diseases in the intestine (Groschwitz and Hogan, 2009; Bischoff et al., 2014). *Clostridium perfringens* is present in the broilers’ intestine under normal physiological conditions, but its pathogenic consequence on the intestine is of particular interest to researchers. *C.*

perfringens can cause animal cell necrosis, tissue edema, and necrotizing enteritis.

Zinc, an essential trace element for animals, can relieve intestinal inflammation by regulating the permeability of the intestine barrier layers. Inorganic zinc is widely used in animal production owing to its low cost. However, organic zinc potentially has a higher bioavailability level. In comparison, organic zinc poses to be more effective either in its complete or partial replacement for inorganic zinc by promoting intestinal health and tissue morphology—including the intestinal villi height, the goblet cells count, and the villus height–to–crypt depth ratio. Nevertheless, the underlying mechanism of the organic zinc effect to improve intestinal integrity is undefined at the moment (Hu et al., 2013; Bortoluzzi et al., 2019). Thus, the purpose of this study was to evaluate the effect and possible mechanisms of a zinc–amino acid complex on intestinal permeability and integrity of broilers during a *C. perfringens* challenge.

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Received February 26, 2020.

Accepted September 11, 2020.

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MATERIALS AND METHODS

Birds, Experimental Design, and Diets

This experiment was carried out with the approval by the Chinese Agricultural University Laboratory Animal Welfare and Experimental Ethical Committee. The study adopted a completely randomized design with a 2×3 factorial pattern. Herein, the factors include 3 patterns of Zn supplements (ZnSO₄ treatment: 80 mg zinc/kg from ZnSO₄; iso-dose replacement group. (ISO) treatment: 40 mg zinc/kg from ZnSO₄ and 40 mg zinc/kg from a zinc–amino acid complex; and organic treatment: 40 mg zinc/kg from a zinc–amino acid complex) with or without *C. perfringens* challenge. A total of 180 1-day-old male Arbor Acres broilers were obtained from a commercial hatchery and randomly divided into 6 treatments with 30 birds each. The birds were raised for 21 d in a temperature-controlled insulator with a continuous 24-h light supply. All birds had ad libitum access to feed and water throughout the experiment period. A corn–soybean basal diet was formulated to meet the NRC requirements for broilers. A zinc–amino acid complex containing zinc lysine and zinc glutamic acid was purchased from Zinpro (Wuxi Additives Biotechnology Co., Ltd.

C. perfringens Challenge

Field strain type A chicken *C. perfringens* isolated from a clinical case of necrotic enteritis was obtained from the China Veterinary Culture Collection Center. The bacteria were cultured anaerobically on tryptose-sulfite-cycloserine agar for 8 h at 37°C, and then aseptically inoculated into cooked meat medium and incubated anaerobically overnight at 37°C. All the challenged birds were orally inoculated with 0.5 mL (1.0×10^8 cfu/mL) suspension on day 7, and on day 14–20, the birds were further orally inoculated with 1.0 mL daily. Nonchallenged birds were orally inoculated with the same volume of buffer solution during the same periods.

Sample Collection

On day 14 and day 21, 8 birds were selected from each treatment, and the blood was collected aseptically from the wing vein into heparinized vacutainers, then plasma was obtained by centrifuging blood at $2,000 \times g$ for 15 min at 4°C and stored at –20°C for plasma endotoxin assay. In addition, the ileum was collected for histologic examination, intestinal permeability, and tight junction protein mRNA expression measurement. Samples for mRNA determination were frozen in liquid nitrogen immediately and stored at –80°C.

Sample Analysis

Plasma endotoxin assay: Plasma endotoxin was detected using Pyrochrome Chromogenic Endotoxin

Testing (Associates of Cape Cod. Inc., East Falmouth, MA), and the endotoxin standard (*Escherichia coli* O113: H10) was used as a standard curve.

Small intestinal histomorphology: The small intestine segment at the same position was dissected, immediately fixed in 4% paraformaldehyde, and then embedded in paraffin sectioned (5 µm). The samples were stained and observed with hematoxylin and eosin and an Olympus microscope (CX23, Tokyo, Japan) consecutively. The stained samples were analyzed using ProgRes CapturePro software (Jenoptik, Jena, Germany) to measure the intestinal villi height (from the tip of the villi to the crypt opening) and the crypt depth (from the base of the crypt to the level of the crypt opening). A total of 15 villi and corresponding crypts were randomly selected for each tissue to calculate the villi height-to-the crypt depth ratio.

Intestinal permeability: Ileal segments of each bird were mounted into chambers (Physiological Instruments, San Diego, CA) for the determination of intestinal integrity. Briefly, intestinal samples were placed into chambers, connected to dual-channel current and voltage electrodes. Both the mucosal and serosal sides of the tissue were bathed in Krebs-Henseleit bicar-bonate and provided with a constant O₂–CO₂ mixture. Individual segments were voltage-clamped at 0 mv, and transepithelial electrical resistance (TER) was determined. After stabilization, intestinal segments were tested for permeability to the macromolecule fluorescein isothiocyanate-labeled dextran (Sigma, St. Louis, MO).

Tight junction protein mRNA expression: Total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA), and cDNA was synthesized using the PrimeScript RT reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa, Tokyo, Japan) as per the manufacturer's instructions. Gene expression was detected by the ABI 7500 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA). The claudin-1, occludin, mucin 2, and β-actin gene-specific primer sequences are as follows: claudin-1: forward 5'-CATACTCCTGGGTCTGGTTGGT-3', reverse 5'-GACAGCCATCCGCATCTTCT-3' (GenBank accession number: AY750897.1); occludin: forward 5'-ACGG-CAGCACCTACCTCAA-3', reverse 5'-GGGCGAAG AAGCAGATGAG-3' (GenBank accession number: D21837.1); mucin 2: forward 5'-TTCATGATGCCTG CTCTTGTG-3', reverse 5'-CCTGAGCCTTGGTACATTCTTGT-3' (GenBank accession number: NM_001318434); and β-actin: forward 5'-ACTCTGGTGAT GGTGTTAC-3', reverse 5'-GGCTGTGATCTCCTT CTG-3' (GenBank accession number: NM_205518). The relative gene expression level was calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2000).

Statistical Analyses

All data were analyzed using SPSS 25.0 software (IBM, Armonk, NY). The treatment effects, *C. perfringens* challenge, and their interactions were analyzed using repeated-measures 2-way ANOVA. The *C.*

perfringens challenge was added to the model as a repeated factor. For zinc levels, it was analyzed using the software's GLM procedure. Comparisons of means for each significant effect were analyzed by Duncan's test using the least-squares mean statement. Final data are presented as means and are considered statistically significant at P -value < 0.05 .

RESULTS AND DISCUSSION

C. perfringens is an important zoonotic pathogen that is widely distributed in nature. Previous studies have shown that oral administration of *C. perfringens* can successfully infect and establish an intestinal injury model (Liu et al., 2012). In this study, we observed that *C. perfringens* caused pathologic changes in the intestines of broilers—such as visible thinning of the intestinal wall, congestion, and a large number of bleeding points. The pathologic changes could be because of the *C. perfringens* proliferation in the small intestine as well as the secretion of exotoxin that results in intestinal damages. *C. perfringens* (a gram-positive bacterium), on the other hand, can promote the proliferation of several gram-negative bacteria in the ileum (such as *E. coli*), resulting in the translocation of endotoxin to the blood and increasing the level of endotoxin in the blood (Liu et al., 2012). In agreement, our findings revealed that *C. perfringens* infection in 21-day-old broilers contributed to elevated plasma endotoxin and impaired intestinal permeability. On the contrary, dietary zinc supplement reduced the plasma endotoxin. Compared with the inorganic zinc treatment, partial replacement of inorganic zinc by organic zinc in the diet (ISO treatment) had the lowest plasma endotoxin level (Table 1). Likewise, dietary zinc treatments significantly affect ($P < 0.05$) the ileum villus height and villus

height/crypt depth ratio of 14-day-old broilers. The ISO treatment group had the highest ($P < 0.05$) ileum villus height values and villus height/crypt depth ratio than the $ZnSO_4$ group (Table 1). However, both *C. perfringens* challenge and Zn addition did not have any significant effects ($P > 0.05$) on ileum villus height, crypt depth, and villus height/crypt depth ratio of 21-day-old broilers (data not shown). Endotoxin is a good bacterial marker for the measurement of intestinal permeability and assessment of epithelial integrity. It has been previously used to evaluate the intestinal barrier impairment and permeability gradient in vivo (Bischoff et al., 2014), whereas the villus height/crypt depth ratio could be used to assess the integrity of the intestinal mucosa. Altogether, this study showed that organic zinc alleviates the intestinal tract damage caused by *C. perfringens* better than inorganic zinc.

Impairment of the epithelial layers permeability, microbial dysfunction, as well as the immune defense system could potentially compromise the intestinal integrity. As a result, the level of harmful compounds will increase in the mucosa because of epithelial cell wall damage. In this study, our results confirmed that *C. perfringens* infection significantly increased ($P < 0.05$) intestinal fluorescein isothiocyanate-labeled dextran permeability and decreased ($P < 0.05$) TER on day 21 (Table 1); thus, *C. perfringens* could undermine intestinal permeability and integrity. Zinc has been shown to protect against *Salmonella enterica* in broiler chickens and enhanced occludin and zonula occludens proteins-1 expressions in weaning piglets in our previous studies (Zhang and Guo, 2009; Zhang et al., 2012). In accordance with the previous data, dietary zinc treatment increased ileal TER on day 21 ($P = 0.074$) and the ileal TER in ISO treatment was the highest (Table 1) in this study. The results are

Table 1. Effects of dietary zinc supplements on ileum histomorphologic parameters, plasma endotoxin, and intestinal permeability of broilers challenged with *Clostridium perfringens*.¹

Treatments	<i>C. perfringens</i>	21-d FITC-Dextran permeability ($\mu\text{g}/\text{mL}$)	21-d TER (Ω/cm^2)	21-d plasma endotoxin	14-d villus height (μm)	14-d villus height/crypt depth ratio
$ZnSO_4$ ²	–	4.73	114.97	0.0581	277.1	2.60
	+	5.94	81.91	0.1016	326.0	3.27
ISO ²	–	4.76	136.62	0.0413	435.8	4.00
	+	5.84	93.07	0.0773	344.4	3.51
Organic ²	–	4.05	94.80	0.0544	333.0	3.34
	+	5.96	76.31	0.1050	300.1	3.21
SEM		0.279	12.476	0.00526	13.62	0.096
Zn treatment	$ZnSO_4$	5.29	98.44	0.0798	301.6 ^b	2.96 ^b
	ISO	5.34	114.85	0.0593	376.5 ^a	3.74 ^a
	Organic	5.00	85.56	0.0797	316.6 ^{ab}	3.27 ^{ab}
Challenge	–	4.50 ^b	115.46 ^a	0.0513 ^b	348.6	3.31
	+	5.91 ^a	83.77 ^b	0.0946 ^a	323.5	3.33
<i>P</i> -value						
Zn treatment		0.604	0.074	0.080	0.024	0.005
Challenge		0.033	0.003	< 0.001	0.361	0.928
Zn \times Challenge		0.795	0.605	0.776	0.128	0.056

^{a,b}The same index but the difference is significant ($P < 0.05$).

Abbreviations: –, without *C. perfringens* challenge; +, with *C. perfringens* challenge; FITC-Dextran, fluorescein isothiocyanate-labeled dextran; TER, transepithelial electrical resistance.

¹Each value represents the mean of 8 replicate ($n = 8$).

² $ZnSO_4$ treatment: 80 mg zinc/kg from $ZnSO_4$; ISO treatment: 40 mg zinc/kg plus $ZnSO_4$ plus 40 mg zinc/kg from a zinc–amino acid complex; Organic treatment: 40 mg zinc/kg from a zinc–amino acid complex.

Table 2. Effects of dietary zinc supplements on relative occludin expressions in the ileum of broilers challenged with *Clostridium perfringens*.¹

Treatments	<i>C. perfringens</i>	14-d occludin	21-d occludin
ZnSO ₄ ²	–	1.00	1.00
	+	0.75	0.56
ISO ²	–	2.02	1.61
	+	1.54	0.83
Organic ²	–	1.50	1.19
	+	1.11	0.59
SEM		0.156	0.089
Zn source	ZnSO ₄	0.88	0.78
	ISO	1.78	1.22
	Organic	1.30	0.89
Challenge	–	1.51	1.27 ^a
	+	1.13	0.66 ^b
<i>P</i> -value			
Zn		0.065	0.056
Challenge		0.228	<0.001
Zn × Challenge		0.953	0.671

Abbreviations: –, without *C. perfringens* challenge; +, with *C. perfringens* challenge.

¹Each value represents the mean of 8 replicate (n = 8).

²ZnSO₄ treatment: 80 mg zinc/kg from ZnSO₄; ISO treatment: 40 mg zinc/kg plus ZnSO₄ plus 40 mg zinc/kg from a zinc–amino acid complex; Organic treatment: 40 mg zinc/kg from a zinc–amino acid complex.

consistent with the plasma endotoxin and intestinal villus height/crypt depth ratio. Therefore, partial replacement of inorganic zinc by organic zinc deduced to reduce the increased intestinal damage caused by *C. perfringens*.

The intestinal barrier comprises the unicellular layers of the intestinal epithelium that allows selective absorption of nutrients and water while avoiding the entry of pathogenic materials. The epithelium maintains its selective barrier functions via complex protein networks that form 3 adhesive complexes, including desmosomes, adhesion junctions, and tight junctions (Groschwitz and Hogan, 2009). These complexes help to regulate epithelial cell permeability in the intestine by expressions of cadherin, claudin, occluding, and junctional adhesion molecule proteins (McCarthy et al., 1996; Sonoda et al., 1999). The results in this study showed that zinc alleviated the intestinal epithelial damage caused by the *C. perfringens* infection. To further elucidate the mechanism involved in the role zinc played to promote intestinal integrity, we investigated the relative expression of occludin, claudin-1, and mucin-2 in the ileum samples. The results showed no significant difference ($P > 0.05$) between *C. perfringens* challenge and Zn treatment in the relative expression of claudin-1 and mucin-2 genes (data not shown). However, *C. perfringens* infection significantly decreased ($P < 0.05$) the expression of occludin genes, whereas, zinc treatments upregulated the relative expression of occludin in the

ileum both at day 14 ($P = 0.065$) and day 21 ($P = 0.056$) with the highest expression recorded from ISO treatment (Table 2). In conclusion, we infer that a dietary zinc–amino acid complex could potentially alleviate *C. perfringens*–induced intestinal mucosal damage by promoting intestinal integrity and upregulating expression of occludin in the ileum.

ACKNOWLEDGMENTS

The authors are grateful to GaoHe and Hamed Ayansola for their assistance, authors also wish to appreciate the support from the National Natural Science Foundation of China (No. 31672443).

DISCLOSURES

The authors declare that there are no conflicts of interest.

REFERENCES

- Bischoff, S. C., G. Barbara, W. Buurman, T. Ockhuizen, J. D. Schulzke, M. Serino, H. Tilg, A. Waston, and J. M. Wells. 2014. Intestinal permeability - a new target for disease prevention and therapy. *BMC Gastroenterol.* 14:189.
- Bortoluzzi, C., B. Lumpkins, G. F. Mathis, M. Franca, W. D. King, D. E. Graunard, K. A. Dawson, and T. J. Applegate. 2019. Zinc source modulates intestinal inflammation and intestinal integrity of broiler chickens challenged with coccidia and *Clostridium perfringens*. *Poult. Sci.* 98:2211–2219.
- Groschwitz, K. R., and S. P. Hogan. 2009. Intestinal barrier function: molecular regulation and disease pathogenesis. *J. Allergy Clin. Immunol.* 124:3–20.
- Hu, C. H., Z. C. Qian, J. Song, Z. S. Luan, and A. Y. Zhuo. 2013. Effects of zinc oxide-montmorillonite hybrid on growth performance, intestinal structure, and function of broiler chicken. *Poult. Sci.* 92:143–150.
- Liu, D., S. Guo, and Y. Guo. 2012. Xylanase supplementation to a wheat-based diet alleviated the intestinal mucosal barrier impairment of broiler chickens challenged by *Clostridium perfringens*. *Avian Pathol.* 41:291–298.
- Livak, K., and T. Schmittgen. 2000. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods* 25:402–408.
- McCarthy, K. M., I. B. Skare, M. C. Stankewich, M. Furuse, S. Tsukita, R. A. Rogers, R. D. Lynch, and E. E. Schneeberger. 1996. Occludin is functional component of the tight junction. *J. Cell Sci.* 109:2287–2298.
- Sonoda, N., M. Furuse, H. Sasaki, S. Yonemura, J. Katahira, Y. Horiguchi, and S. Tsukita. 1999. Clostridium perfringens enterotoxin fragment removes specific claudins from tight junction strands: evidence for direct involvement of claudins in tight junction barrier. *J. Cell Biol.* 147:195–204.
- Zhang, B., and Y. Guo. 2009. Supplemental zinc reduced intestinal permeability by enhancing occludin and zonula occludens protein-1 (ZO-1) expression in weaning piglets. *Br. J. Nutr.* 102:687–693.
- Zhang, B., Y. Shao, D. Liu, P. Yin, Y. Guo, and J. Yuan. 2012. Zinc prevents *Salmonella enterica serovar Typhimurium*–induced loss of intestinal mucosal barrier function in broiler chickens. *Avian Pathol.* 41:361–367.