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Dietary supplementation of natural tannin relieved intestinal injury and oxidative stress in piglets challenged with enterotoxigenic *Escherichia coli*

*Qian Zhang[1], Lin Zhang[2], Linxiao Du[1], Yanyan Zhang[1], Dan Yi[1], Di Zhao[1], Binying Ding[1], Yongqing Hou[1], Tao Wu[1]**

[1]*Hubei Key Laboratory of Animal Nutrition and Feed Science, Engineering Research Centre of Feed Protein Resources on Agricultural By-products, Ministry of Education, Wuhan Polytechnic University, Wuhan, P.R. China*

[2]*Yangtze River Fisheries Research Institute, Chinese Academy Of Fishery Sciences, Wuhan, P.R. China*

Qian Zhang and Lin Zhang contributed equally to this work

**Corresponding author: wtao05@163.com*

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Abstract: This study is to explore the effects of natural tannin (NBT) on intestinal injury in piglets challenged with enterotoxigenic *Escherichia coli* (ETEC). Thirty-two 7-day-old piglets were divided into four groups: I) control group, piglets were fed a basic diet without challenge; II) ETEC group, piglets were fed a basic diet and challenged with enterotoxigenic *E. coli*; III) NBT + ETEC group, piglets were fed a basic diet with supplementation of 2 g/kg NBT and challenged with enterotoxigenic *E. coli*; and IV) ZnO + ETEC group, piglets were fed a basic diet with supplementation of 3 g/kg ZnO and challenged with enterotoxigenic *E. coli*. Results showed that diarrhoea rate was significantly increased in ETEC group, whereas it was decreased after NBT supplementation ($P < 0.05$); NBT supplementation significantly increased the average daily gain of ETEC-infected piglets. ETEC group showed damaged intestinal morphology (as indicated by decreased villus height, surface area and increased crypt depth) and barrier function (as indicated by decreased D-xylose content and increased diamine oxidase activity), induced oxidative stress (as indicated by increased myeloperoxidase activity) while supplementation of NBT had an obvious impact on the recovery of intestinal function and alleviated the oxidative damage. Further analysis showed that NBT could decrease the expression of genes related to intestinal injury (matrix metalloproteinase-3), and specifically upregulate the expression of oxidative stress-related genes (nuclear factor erythroid 2-related factor 2 and hypoxia inducible factor-1). Overall, dietary supplementation of NBT relieved intestinal injury and oxidative stress in piglets challenged with ETEC. NBT could be an alternative to ZnO as a feed additive in piglet diet.

Keywords: tannins; intestine; oxidative stress; ETEC; piglets

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Pig diarrhoea causes serious damage in suckling and weaned piglets, and it is responsible for considerable economic losses of the pig industry. In some outbreaks, it causes high morbidity and mortality in suckling and weaned piglets, and survivors exhibit retarded growth (Xia et al. 2018). Moreover, pig diarrhoea also increases the risks for public health due to the production of pathogenic bacteria-infected pork, which has troubled the global pig industry for many years (Lv et al. 2018). Therefore, it is one of the most heated topics in the pig industry to develop safe, effective and economical prevention or treatment of piglet diarrhoea (Wu et al. 2018a). For which, many methods have been established, attempting to relieve diarrhoea by means of vaccines, drugs or feed additives. Unfortunately, despite such enthusiasm and dedication of researchers, threat of diarrhoea has never been declining.

In medicine, astringents cause constriction of mucous membranes and exposed tissues, and are often used to reduce discharge of blood serum and mucous secretions (Goel et al. 2005). Zinc oxide (ZnO) is a mild astringent and has been shown great therapeutic efficiency for diarrhoea. However, its potential risk of environmental pollution limits its application in large-scale production. Tannins have traditionally been considered antinutritional, nevertheless, as an astringent, it is now known that their beneficial or antinutritional properties depend on their chemical structure and dosage (Das et al. 2020). In traditional Chinese medicine, the application of astringents like natural tannins on human diarrhoea can be traced back to 5 000 years ago (Xie et al. 2013). In Europe, pigs fattened on acorns or chestnuts in traditional animal breeding are known to consume tannin-rich feedstuffs, as they can quickly adapt to high-tannin diets by increasing salivary secretion (Das et al. 2020). Tannins have been shown to affect gut microflora and protein digestibility (Goel et al. 2005). Recent studies have demonstrated that the chicks fed a diet containing tannin had significantly better growth performance than the control group (Schiavone et al. 2008). Some tannins, at appropriate concentrations in the intestines, are known to exert anthelmintic, antibacterial and antiviral effects and are used as a supportive treatment for diarrhoea (Mueller-Harvey 2006; Das et al. 2020). Tannins also exert an antioxidant and hepatoprotective potential (Pithayanukul et al. 2009; Frankic and Salobir 2011).

Enterotoxigenic *Escherichia coli* (ETEC) is a primary pathogenic bacterium of diarrhoea in piglets, over two-thirds of piglet diarrhoeas caused by ETEC resulted from STa- (heat-stable enterotoxin A) producing ETEC strains (Lv et al. 2018; Wu et al. 2018b). Our previous study has demonstrated that ETEC induced diarrhoea, morphological and functional injuries in piglet intestines, by regulating expression of proteins related to the transportation function and genes associated with inflammatory cytokines (Lv et al. 2018; Wu et al. 2018b). The objective of the present study was to evaluate the effect of natural-based tannin (NBT) on growth performance and intestinal function in piglets challenged with ETEC, attempting to search an alternative to ZnO as a feed additive to treat diarrhoea and expand the application of astringents in the pig industry.

MATERIAL AND METHODS

Experimental animals and design

All experimental protocols were approved by and carried out in accordance with the Animal Care and Use Committee at Wuhan Polytechnic University (approval number: 2017-0912). All methods were approved by the Animal Care and Use Committee at Wuhan Polytechnic University. Thirty-two 7-day-old healthy crossbred piglets (Duroc × Landrace × Yorkshire) were divided into four groups: I) control group; II) ETEC group, piglets were challenged with ETEC; III) NBT + ETEC group, piglets were supplemented with 2 g/kg NBT, and challenged with ETEC; and IV) ZnO + ETEC group, piglets were supplemented with 3 g/kg zinc oxide (ZnO), and challenged with ETEC. The experiment lasted for nine days. All piglets were fed milk substitutes (Nouriz Fine Food Ltd., Shanghai, China), containing 2 131 KJ energy, 24.2 g protein, 28.6 g fat, 28.9 g carbohydrate, 0.264 g sodium and 0.875 g calcium per 100 g milk. ZnO (Aladdin Bio-Chem Technology Ltd., Shanghai, China) was used as a positive control which was also an astringent. NBT was purchased from Wuhan Yuancheng Gongchuang Technology Ltd (Shanghai, China). After a 3-day adaptation period, piglets in the NBT + ETEC group and ZnO

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+ ETEC group were fed milk substitutes supplemented with NBT and ZnO, respectively, for seven days. On the 8th and 9th day of the trial, ETEC strains K88 (State Key Laboratory of Agricultural Microbiology, Wuhan, China) were orally inoculated at 5×10^9 CFU. The dosage of NBT, ZnO and ETEC strains was chosen according to results of a preliminary experiment. On day 10 of the trial, blood samples were collected from the *anterior vena cava* of piglets, and then all piglets were euthanized under anaesthesia with an intravenous injection of pentobarbital sodium (50 mg/kg BW). Intestinal samples were collected and processed as described previously (Wu et al. 2018c).

Blood index

The activities of diamine oxidase (DAO), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT) and myeloperoxidase (MPO), and the content of D-xylose and malondialdehyde (MDA) were determined using commercially available kits (Jiancheng Bioengineering Institute, Nanjing, China). Assays were performed in triplicate.

Intestinal morphology

To determine the intestinal morphology, paraformaldehyde-fixed jejunum and ileum samples were dehydrated and embedded in paraffin. The 5 cm sections were cut and then stained with haematoxylin and eosin. Intestinal morphology including the villus height and width, as well as crypt depth was determined using a light microscope (Leica, Solms, Germany), with the Leica Application Suite image analysis software v4.0 (Leica, Solms, Germany). Then the villus crypt ratio and villous surface area were calculated.

Gene expression

Total RNA of 100 mg of each frozen intestinal sample was extracted by using the Trizol Reagent protocol (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed by using a Prime Script® RT reagent kit with gDNA Eraser (Takara, Dalian, China), and the synthesized cDNA was stored at –20 °C. Primers used in this study are listed in Table 1. qPCR was performed using the SYBR® Premix Ex Taq™ (Takara, Shiga, Japan) with the 50 µl reaction system on an Applied Biosystems 7500 Fast Real-Time PCR instrument (Foster City, CA, USA). Ribosomal protein L4 (RPL4) was used as the reference genes. Results were analysed by using the $2^{-\Delta\Delta Ct}$ method. Each biological sample was run in triplicate.

Statistical analysis

Data were analysed using one-way analysis of variance (ANOVA), expressed as mean values ± SEM. Duncan's multiple range test (DMRT) was processed as post hoc test to present the significant difference. All experimental data was analysed using SPSS v23.0 (SPSS Inc., Chicago, IL, USA). A P-value < 0.05 was considered statistically significant.

RESULTS

Growth performance

During the experimental period, average daily gain (ADG), average daily feed intake (ADFI), and diarrhoea rate (DR) were observed and calculated (Table 2). From day 0 to day 7 of the trial, a significant decrease of ADFI was observed in ZnO + ETEC group (P < 0.05), no other significant difference was found. During day 8 to day 9, diarrhoea

Table 1. Primer sequences for real-time PCR analysis

Genes	Forward	Reverse
MMP3	GATGTTGGTTACTTCAGCAC	ATCATTATGTCAGCCTCTCC
NRF2	ATCACCTCTTCTGCACCGAA	GCTTTCTCCCGCTCTTTCTG
HIF-1	TTACTCATCCGTGCGACCAT	CTCCGCTGTGTATTTTGCTCTTT
RPL4	CAGAAATGGGAATGGAAAGTTG	CCATTGGTCAGGTCATTCAATACA

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Table 2. Growth performance of piglets

Item	Control	ETEC	NBT + ETEC	ZnO + ETEC
Day 1–7				
ADG (g)	117.86 ± 33.49	117.55 ± 26.73	122.45 ± 31.05	117.14 ± 26.80
ADFI (g)	100.00 ± 0.00^{b}	99.92 ± 0.22^{b}	99.67 ± 0.09^{b}	98.01 ± 2.51^{a}
DR (%)	0.36	1.14	0	0.75
Day 8–9				
ADG (g)	148.31 ± 42.41^{c}	–20.70 ± 2.74^{a}	31.35 ± 7.82^{b}	104.72 ± 19.59^{c}
ADFI (g)	99.37 ± 0.89^{b}	91.71 ± 2.82^{b}	57.65 ± 10.51^{a}	70.74 ± 4.02^{a}
DR (%)	0.36^{a}	67.5^{b}	12.5^{a}	26.63^{a}

ADFI = average daily feed intake; ADG = average daily gain; DR = diarrhoea rate; ETEC = enterotoxigenic *Escherichia coli;* NBT = natural-based tannin

$^{a–c}$Values within a row with different letters differ ($P < 0.05$)

rate was significantly increased in ETEC group, whereas it decreased after NBT or ZnO supplementation ($P < 0.05$). Additionally, ADG in ETEC group was significantly reduced, while it was increased compared with NBT+ETEC or ZnO + ETEC groups ($P < 0.05$). No significant difference in ADFI was observed between the control group and ETEC group. However, ADFI in the ZnO + ETEC and NBT + ETEC groups was significantly lower than that in the control group.

Intestinal absorbability and permeability

D-xylose content and DAO activity in blood were determined to evaluate the intestinal absorbability and permeability; the results are presented in Figure 1. D-xylose content in blood was significantly decreased in ETEC and ZnO + ETEC groups ($P < 0.05$), whereas it was increased in NBT + ETEC group compared with either ETEC or control group ($P < 0.05$). Additionally, DAO activity in blood was significantly increased after ETEC infection ($P < 0.05$), while it was decreased in NBT + ETEC and ZnO + ETEC groups.

Intestinal morphology

The mucosal micrographs showed that ETEC infection caused severe villous atrophy in the small intestine, whereas dietary supplementation of NBT obviously improved the intestinal morphology (Figure 2). As shown in Table 3, ETEC significantly decreased villus height, surface area and villus height/crypt depth ratios in duodenum, jejunum and ileum, and it increased crypt depth in all intestinal sections ($P < 0.05$). However, dietary supplementation of NBT significantly increased villus height and surface area in duodenum and ileum, increased villus height/crypt depth ratios in duodenum, jejunum and ileum, and it decreased crypt depth in all intestinal sections ($P < 0.05$) compared to the ETEC group. ZnO supplementation altered these parameters as well, while NBT supplemen-

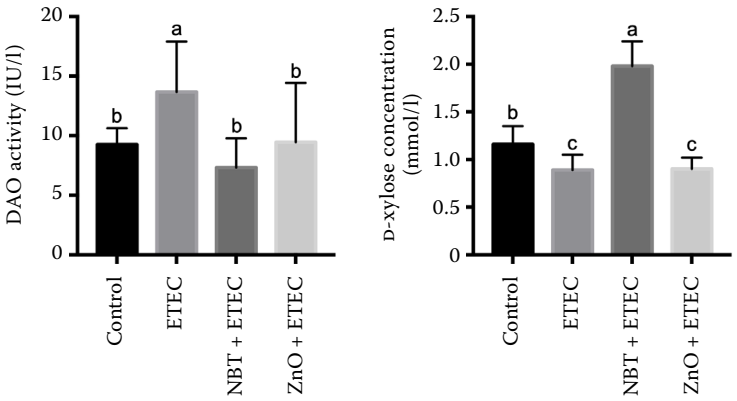

Figure 1. D-xylose content and diamine oxidase (DAO) activity in blood

ETEC = enterotoxigenic *Escherichia coli*; NBT = natural-based tannin

$^{a–c}$Values within a row with different letters differ ($P < 0.05$)

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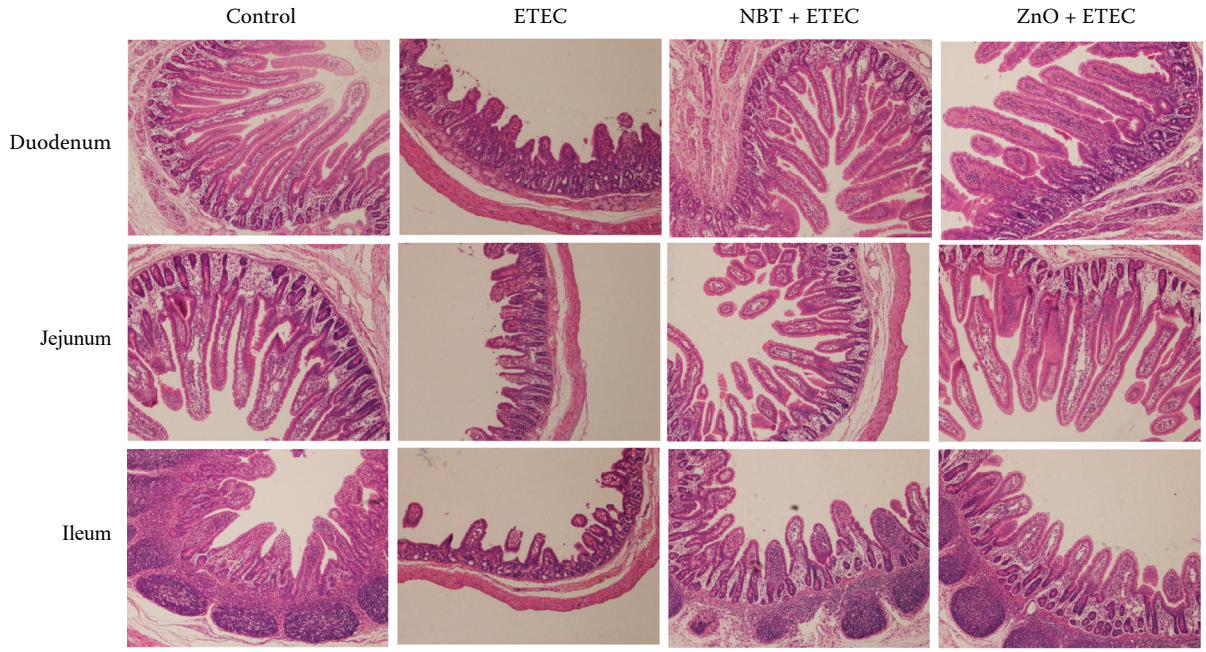

Figure 2. Intestinal morphology of piglets
ETEC = enterotoxigenic *Escherichia coli;* NBT = natural-based tannin

tation achieved better restoration compared with ZnO ($P < 0.05$).

Redox status

The activity of SOD, GSH-Px, CAT and MPO, and the content of the oxidative end product MDA in blood were determined to evaluate the intestinal anti-oxidative capacity. As shown in Figure 3, compared with the control group, SOD activity was significantly decreased in ETEC group, the activity of MPO and CAT in blood ($P < 0.05$) was significantly increased in ETEC group. Compared with ETEC group, MPO activity and MDA content were significantly decreased ($P < 0.05$) in the blood of NBT + ETEC group.

Table 3. Intestinal morphology of piglets

Item	Control	ETEC	NBT + ETEC	ZnO + ETEC
Duodenum				
Villus height (μm)	650.3 ± 45.88^{c}	280.2 ± 78.94^{a}	499.1 ± 37.40^{b}	475.1 ± 78.61^{b}
Crypt depth (μm)	98.34 ± 8.10^{a}	128.4 ± 26.79^{c}	105.2 ± 11.6^{ab}	118.2 ± 14.89^{bc}
Villus surface area (μm^2)	17 159 ± 2 290^{c}	6 661 ± 2 241^{a}	15 516 ± 3 427^{bc}	12 939 ± 2 410^{b}
Villus height/crypt depth	6.78 ± 0.54^{d}	1.93 ± 0.33^{a}	5.01 ± 0.31^{c}	4.10 ± 0.40^{b}
Jejunum				
Villus height (μm)	582.8 ± 248.9^{c}	226.7 ± 69.77^{a}	356.9 ± 43.37^{ab}	426.7 ± 122.2^{b}
Crypt depth (μm)	84.00 ± 11.93^{a}	121.9 ± 10.25^{c}	81.0 ± 3.49^{a}	97.81 ± 10.78^{b}
Villus surface area (μm^2)	15 984 ± 7 787^{c}	4 782 ± 1 258^{a}	8 869 ± 1 788^{ab}	10 093 ± 3 272^{b}
Villus height/crypt depth	6.99 ± 2.36^{c}	1.69 ± 0.37^{a}	4.49 ± 0.43^{b}	4.54 ± 1.32^{b}
Ileum				
Villus height (μm)	301.6 ± 18.98^{c}	172.6 ± 27.33^{a}	255.1 ± 14.22^{b}	272.5 ± 47.57^{bc}
Crypt depth (μm)	70.10 ± 5.64^{ab}	110.2 ± 15.38^{c}	62.82 ± 1.61^{a}	78.88 ± 11.69^{b}
Villus surface area (μm^2)	7 858 ± 1 054^{c}	3 185 ± 526.6^{a}	5 589 ± 488.3^{b}	6 352 ± 1 357^{b}
Villus height/crypt depth	4.42 ± 0.52^{c}	1.64 ± 0.18^{a}	4.2 ± 0.15^{c}	3.63 ± 0.77^{b}

$^{a–d}$Values within a row with different letters differ ($P < 0.05$)
ETEC = enterotoxigenic *Escherichia coli;* NBT = natural-based tannin

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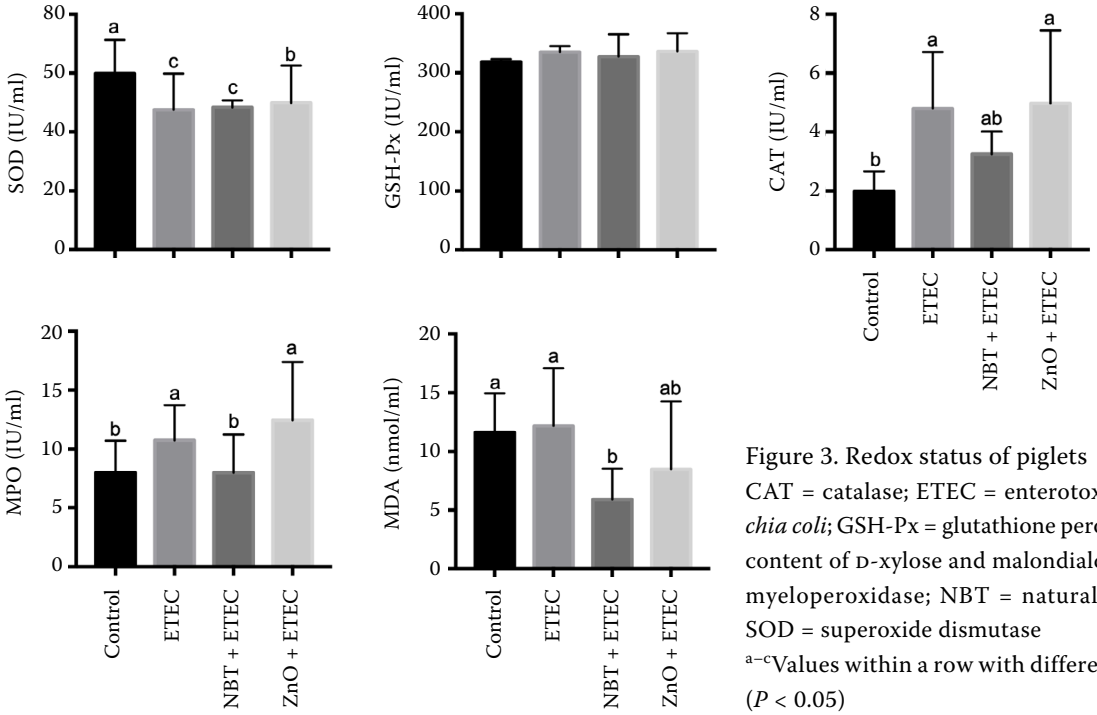

Figure 3. Redox status of piglets

CAT = catalase; ETEC = enterotoxigenic *Escherichia coli*; GSH-Px = glutathione peroxidase; MDA = content of D-xylose and malondialdehyde; MPO = myeloperoxidase; NBT = natural-based tannin; SOD = superoxide dismutase

[a–c]Values within a row with different letters differ ($P < 0.05$)

Gene expression profile

Relative expression levels of genes involved in intestinal injury and oxidative stress in jejunum and ileum were determined (Figure 4). Compared with the control group, ETEC infection significantly increased expression levels of MMP3 in jejunum and ileum while it decreased expression levels of HIF-1 and NRF2 in ileum ($P < 0.05$). Compared with ETEC group, NBT supplementation significantly decreased expression levels of matrix metalloproteinase-3 (MMP3) in ileum ($P < 0.05$), and it increased expression levels of hypoxia inducible factor-1 (HIF-1) and nuclear factor erythroid 2-related factor 2 (NRF2) in jejunum and ileum; ZnO supplementation significantly decreased expression levels of MMP3 in jejunum and ileum when it had no effect on the expression of HIF-1 and NRF2 ($P < 0.05$).

DISCUSSION

ETEC-induced diarrhoea is a major problem in the pig industry, causing substantial economic losses each year. In this study, we evaluated the potential of NBT as a treatment for intestinal injury in piglets challenged with ETEC. Our results demonstrate that NBT can effectively alleviate the symptoms of ETEC-induced diarrhoea and improve the intestinal health of the piglets, indicating that NBT could be used as an alternative to ZnO. Therefore, our study provides important insights into the potential use of NBT as an effective and sustainable solution to the problem of ETEC-induced diarrhoea in pig farming.

In the present study, NBT supplementation significantly alleviated diarrhoea caused by ETEC infection and significantly increased the ADG of ETEC-infected piglets, which indicated that administration of 2 g/kg NBT was safe and effective. Similar findings were observed in other studies conducted in both ruminant and monogastric animals (Huang et al. 2018). Upon ETEC infection, tannins could contribute to the gut health by inhibition of bacterial growth or bacterial adhesion to the intestinal epithelium and biofilm formation. It could also reduce damage to the intestine by inhibiting enterotoxin production and activities (Girard and Bee 2020). Therefore, supplementation of NBT could effectively alleviate diarrhoea caused by ETEC infection. There is a report showing that tannins are anti-nutritive substances and could decrease protein digestibility in pigs fed tannin-rich diets (Jansman et al. 1995). This may explain the lower ADG and

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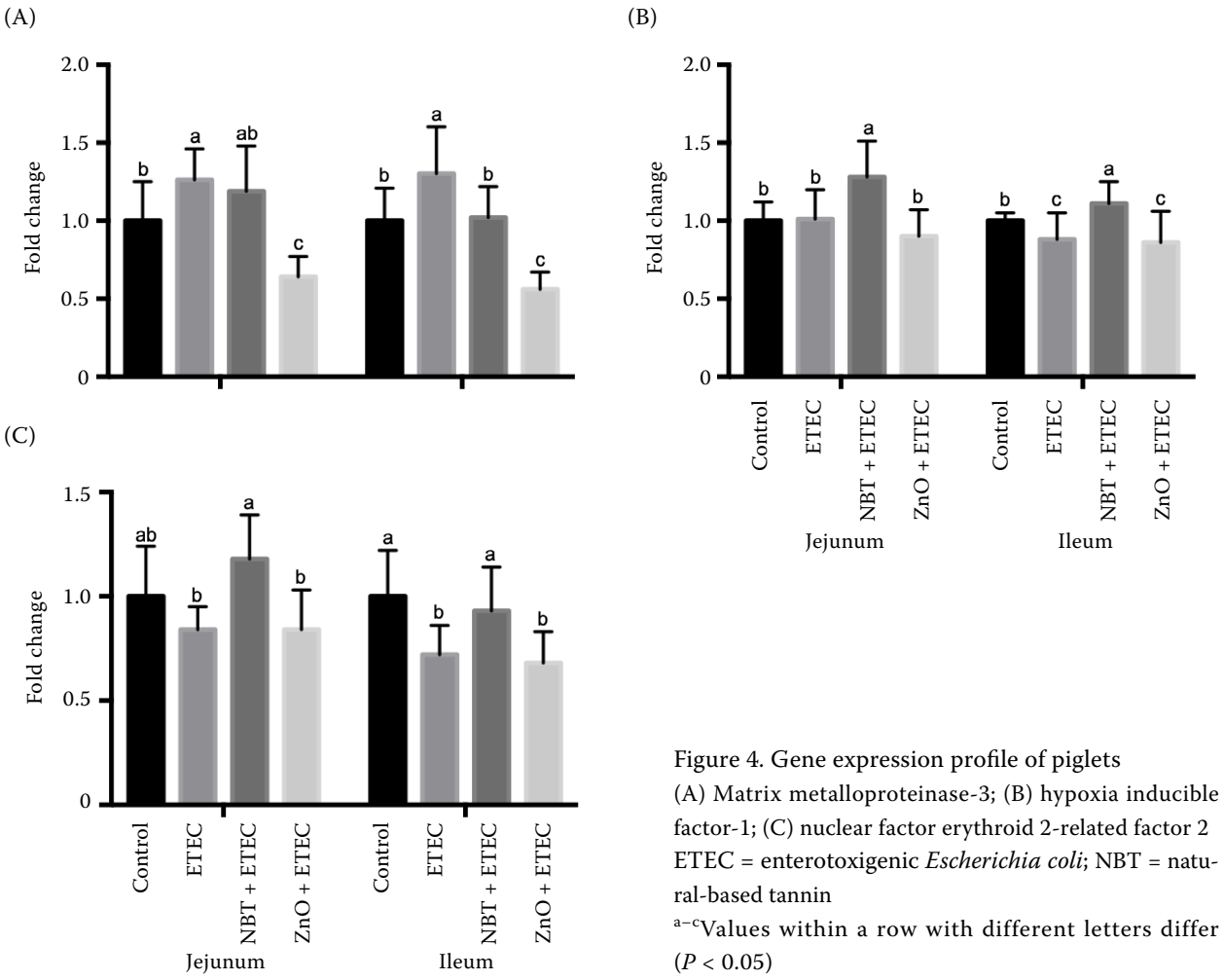

Figure 4. Gene expression profile of piglets
(A) Matrix metalloproteinase-3; (B) hypoxia inducible factor-1; (C) nuclear factor erythroid 2-related factor 2
ETEC = enterotoxigenic *Escherichia coli*; NBT = natural-based tannin
[a–c]Values within a row with different letters differ ($P < 0.05$)

ADFI in NBT + ETEC group compared with ZnO + ETEC group. However, due to their structural diversity, the amount ingested, and differences in how they are taken, the nutritional effects vary (Mueller-Harvey 2006). Studies have suggested that using low concentrations of tannins can minimize their anti-nutritive effects (Huang et al. 2018). Thus, a more elaborate investigation is warranted to determine the optimal condition for the action of NBT.

The intestinal barrier is fundamental to the proper functioning of the epithelial cells and to prevent the entry of pathogenic bacteria (Vancamelbeke and Vermeire 2017). The injured intestinal barrier increased the epithelial permeability and decreased the epithelial absorbability. Diarrhoea associated with ETEC infection leads to the impaired mucosal barrier function, and influences intestinal absorbability and permeability (Lv et al. 2018; Wu et al. 2018b). Plasma DAO and D-xylose have been proposed as non-invasive biomarkers of alterations in the function and structure of intestinal mucosa (Lutgens and Lambin 2007). Under certain conditions, cells in the intestinal mucosa experience necroses, and slough off into the enteric enterocele, resulting in a decline of DAO levels in the intestinal mucosa and an increase of DAO levels in circulation (Kitanaka et al. 2002). D-xylose is readily absorbed by the small intestine in healthy pigs, however, the entry of D-xylose from the intestinal lumen to the portal vein is impaired under conditions of malabsorption, resulting in a reduction of D-xylose in the blood (Huntley and Patience 2018). In this study, the activity of DAO was significantly increased, and the concentration of D-xylose was significantly decreased after ETEC infection, demonstrating that ETEC infection injured the intestinal barrier and absorption capacity of piglets. However, DAO activity was significantly decreased and D-xylose concentration was significantly increased in NBT + ETEC group, indicating that supplementation

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of 2 g/kg NBT could enhance epithelial absorbability and relieve the barrier damage caused by ETEC infection.

Intestinal morphology indices are the common indicators of intestinal morphological development. Typically, the increases in villus height, villus surface area, and the villus/crypt ratio indicate the improvement of intestinal absorption capacity (Wu et al. 2018d). While a shortening of the villus decreases the surface area for nutrient absorption (Walton et al. 2016). The crypt is the area where stem cells divide to permit the renewal of the villus, and a large crypt indicates the fast tissue turnover and high demand for a new tissue (Yang and Liao 2019). Previous study revealed that ETEC infection injured the integrity of intestinal mucosal morphology (Lv et al. 2018; Wu et al. 2018b), like similarly presented in this study. Nevertheless, the effect of NBT supplementation on repairing the intestinal morphology damaged by ETEC was extremely remarkable compared to ETEC group, and even ZnO + ETEC group, which demonstrated that supplementation of 2 g/kg NBT could alleviate the intestinal structural damage caused by ETEC infection, and suggested that NBT would be a potential alternative to ZnO.

Oxidative stress reflects the unbalance between the systematic phenomenon of reactive oxygen species and the capacity of the biosystem to readily detoxify the reactive intermediaries or to renovate the resulting damage (Cabello-Verrugio et al. 2016). Whereas cells protect themselves from hydroxyl radicals and by antioxidant enzymes, such as GSH-Px, SOD and CAT (Munteanu and Apetrei 2021). MDA can induce noxious stress in cells and constitute homopolar protein adducts, known as advanced lipoxidation end products (ALEs), which are usually utilized as a marker to evaluate the oxidant stress levels in a biome (Del Rio et al. 2005). Results in a previous study showed that ETEC infection induced oxidative stress, which is similar to the present study (Lv et al. 2018; Wu et al. 2018b). NBT supplementation showed a marked effect against oxidative stress, by regulating the activity of MPO and decreasing MDA product, which suggested that supplementation of 2 g/kg NBT could alleviate the oxidative damage. A similar effect to ZnO was suggested by NBT as a potential ZnO alternative as well.

MMP3 plays a crucial role in the reparations of extracorpuscular matrix homeostasis, which is expressed at high levels in the intestinal bowel diseases and celiac diseases (Raleigh et al. 2009). In this study, ETEC infection increased the expression levels of MMP3, whereas supplementation of NBT and ZnO decreased expression levels of MMP3, which implied the body returning to homeostasis. NRF2 is a transcription factor that could bind to the antioxidant response element present in the promoter region of many cytoprotective genes (Robledinos-Anton et al. 2019). In this study, NBT, but not ZnO, could obviously reverse the reduction of NRF2 caused by ETEC infection. A similar change was observed for HIF-1, which is also a transcription factor that mediates adaptive responses to oxidative stress and regulates genes important for the cell survival (Choudhry and Harris 2018). Studies showed that HIF-1 could regulate multiple intestinal barrier protecting factors and favour the epithelial restitution by inducing epithelial integrin expression (Shao et al. 2018). Therefore, the upregulation of NRF2 and HIF-1 by NBT would contribute to alleviating the intestinal injury induced by ETEC. Besides, the difference between the effects of ZnO and NBT on the expression of NRF2 and HIF-1 suggested that NBT and ZnO may utilize a different mechanism to cope with the intestinal injury.

CONCLUSION

ETEC infection induced diarrhoea, intestinal injury and oxidative stress in piglets by damaging mucosal morphology, absorbability and permeability. However, supplementation of 2 g/kg NBT had a beneficial impact on recovery of the intestinal function, including improving growth performance, lowering diarrhoea rate, improving intestinal morphology (as indicated by increased villus height and surface area and villus height/crypt depth ratios, decreased crypt depth), absorbability (as indicated by increased D-xylose activity) and permeability (as indicated by decreased DAO activity), alleviating oxidative stress (as indicated by decreased MPO activity and MDA product). Further research of molecular mechanisms showed that these beneficial impacts might be achieved by changing expres-

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sion levels of HIF-1 and NRF2 in the intestine. Overall, the results in this study suggest that natural tannins could protect against the intestinal injury and oxidative stress in piglets challenged with ETEC, and that they have the potential to be an alternative to ZnO.

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Conflict of interest

The authors declare no conflict of interest.

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