Beneficial Effects of Lactobacillus Zeae on the Gut Health of Piglets Challenged with Enterotoxigenic Escherichia Coli

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Abstract

Background Enterotoxigenic *Escherichia coli* (ETEC) is a common enteric pathogen that causes diarrhoea in humans and animals. *Lactobacillus zeae* LB1 has been shown to reduce ETEC infection to *Caenorhabditis elegans* and *Salmonella* burden in pigs. The present study was to evaluate the effects of *L. zeae* LB1 on the gut health of lactating piglets that were challenged with ETEC.

Results Six-four 7-day-old piglets were assigned into 8 groups: 1) control group (basal diet, phosphate buffer saline); 2) CT group (basal diet + 40 mg/kg colistin); 3) LL group (basal diet + 1×10^7 CFU/pig/day LB1); 4) HL group (basal diet + 1×10^8 CFU/pig/day LB1); 5) ETEC group: (basal diet + ETEC challenged); 6) CT + ETEC group (basal diet + CT + ETEC); 7) LL + ETEC group (basal diet + 1×10^7 CFU/pig/day LB1 + ETEC); 8) HL + ETEC group (basal diet + 1×10^8 CFU/pig/day LB1 + ETEC). The trial lasted ten days including 3 days of adaptation. Several significant interactions were found on blood parameters, intestinal morphology, gene, and protein expression. ETEC infection disrupted the cell structure and biochemical indicators of blood, undermined the integrity of the intestinal tract, and induced oxidative stress, diarrhoea, intestinal damage, and death of piglets. The supplementation of *L. zeae* LB1 alleviated ETEC's adverse effects by reducing pig diarrhoea, oxidative stress, and death, modulating cell structure and biochemical indicators of blood, improving the capacity of immunity and anti-oxidation of pigs, and restoring their intestinal integrity. At the molecular level, the beneficial effects of *L. zeae* LB1 appeared to be mediated by regulating functional related proteins (including HSP70, Caspase-3, NLRP3, AQP3, and AQP4) and genes (including RPL4, IL-8, HP, HSP70, Mx1, Mx2, S100A12, Nrf2, GPX2 and ARG1).

Conclusions These results suggest that dietary supplementation of *L. zeae* LB1 improved the intestinal functions and health of piglets.

Background

Enterotoxigenic *Escherichia coli* (ETEC) is a common enteric pathogen causing diarrhoea, which is one of the most critical diseases in young farm animals, also the second leading cause of death in children under five years old [1–3]. The ETEC induced diarrhoea in post-weaning piglets mainly occurs within the first week after weaning, resulting in considerable economic losses to the swine industry due to reduced growth performance as well as increased morbidity and mortality [4–5]. The swine industry has largely relied on the prophylactic use of antibiotics in the past to control ETEC and its associated diarrhoea [6]. However, there is a growing public concern over the practice due to the widespread of antibiotic resistance in zoonotic bacterial pathogens, which poses a threat to public health. Thus, non-antibiotic strategies and technologies to control the pathogen using a natural alternative to antibiotics are urgently required to reduce the prophylactic use of antibiotics in swine production [7].

Lactobacilli are often used as probiotic bacteria. Their beneficial effects include a reduction in the colonization of animal intestines by pathogenic bacteria, improvement of animal performance, and enhancement of the animal immune system [8, 9]. Our previous studies have shown that a *Lactobacillus* isolate (*L. zeae* LB1) was effective in reducing either ETEC or *Salmonella* infection in *Caenorhabditis elegans* [6, 10]. In addition, it could lessen *Salmonella* infection in both chickens and post-weaning pigs [11, 12]. We hypothesized that *L. zeae* LB1 could also protect piglets from ETEC infection. Therefore, this research was to investigate the beneficial effects of *L. zeae* LB1 on the gut health of piglets in lactation that had been challenged with ETEC via the analyses of intestinal morphology, blood parameters, and redox status at both animal and molecular levels.

Material And Methods

Animals and Design. The animal use protocol for the present study was approved by the Animal Care and Use Committee at Wuhan Polytechnic University (2018 – 0625, 25th Jun 2018). Sixty-four 7-day-old healthy crossbred lactating piglets (Duroc × Landrace × Yorkshire) reared by sows were chosen and the commercially available milk replacer (D80 Poly milk, Shanghai, China) was used as a basic diet. After 3 days adaptation, the piglets were assigned randomly, on the basis of body weight (BW) and litter origin, to 8 groups: (8 piglets/group): 1) control group - piglets fed the basal diet and treated with phosphate buffered saline (PBS, pH = 7.4); 2) CT group - piglets fed the basal diet supplemented with 40 mg/kg BW of colistin (CT; Zhongnong Pharmaceutical, Xinxiang, China); 3) LL group - piglets fed the basal diet supplemented with 1×10^7 LB1/pig/day (relative low concentration of LB1, LL); 4) HL group - piglets fed the basal diet supplemented with 1×10^8 CFU/pig/day LB1 (relative high concentration of LB1, HL); 5) ETEC group: piglets fed the basal diet, and challenged with 1×10^{10} CFU/pig/day ETEC; 6) CT + ETEC group - piglets fed the basal diet supplemented with 4 mg/kg BW of CT, and challenged with 1×10^{10} CFU/pig/day ETEC; 7) LL + ETEC group - piglets fed the basal diet supplemented with 1×10^7 CFU/pig/day LB1, and challenged with 1×10^{10} CFU/pig/day ETEC; 8) HL + ETEC group - piglets fed the basal diet supplemented with 1×10^8 CFU/pig/day LB1, and challenged with 1×10^{10} CFU/pig/day ETEC.

Sixty-four 7-day-old healthy crossbred piglets were housed individually in pens that were placed in a temperature controlled nursery barn (28–30°C). All diets were isocaloric. The trial lasted 10 days (D0–D9), including three days of adaptation (D0–D2). The treatment with PBS, colistin, or LB1 was from D3 to D7. ETEC challenge was carried out on D7. The trial ended on D9. *L. zeae* LB1 was from the Guelph Research and Development Centre, Agriculture, and Agri-Food Canada. The bacterium was anaerobically cultured in MRS (de Man, Rogosa and Sharpe) liquid medium at 37°C for 16 hours. From day 3, the colistin and *L. zeae* LB1 were mixed with the PBS and then orally fed to piglets at 15:00 each day during the trial. The ETEC (*E. coli* K88) was from Hubei Key Laboratory of Animal Nutrition and Feed Science, Wuhan Polytechnic University. On the day 7 of the trial, ETEC was orally delivered to piglets at 5×10^9 CFU/pig each time at 8:00 and 20:00. From day 7 to day 8 observe the performance of each group of pigs such as diarrhea, feeding. ETEC was cultured in LB (Lysogeny broth) liquid medium at 37°C for 12 hours with agitation at 120 r/min and then used as an inoculum to piglets.

Samples Collection. The D-xylose absorptive test was performed to measure intestinal absorption capacity and mucosal integrity prior to scarification of piglets, which was conducted on D9 of the trial with 50% piglets per groups each day. At 1 h after the oral administration of 10% D-xylose at the dose of 1 ml/kg BW, blood samples were collected from the anterior vena cava into heparinized vacuum tubes (Becton-Dickinson Vacutainer System, Franklin Lake, NJ, USA). Blood samples were centrifuged at 1300 g/min for 15 min at 4 °C to obtain plasma, which was stored at – 80°C until analysis.

After blood sampling, all pigs were killed under sodium pentobarbital anaesthesia (50 mg/kg BW, iv) in order to obtain the small intestine mucosal samples. The pig abdomen was opened immediately from sternum to pubis, and the whole gastrointestinal tract was immediately exposed, the small intestine was immediately dissected free of the mesentery on a chilled stainless-steel tray, then the 5-cm segments were respectively cut at each distal duodenum, mid-jejunum and mid-ileum. The 5-cm segments were gently flushed with ice-cold PBS and placed in chilled formalin solution (10%), then processed by embedding and staining for the observation of intestinal morphology. All the samples were collected within 10 min. Intestinal tissue and digesta samples were stored at - 80 °C until analysis.

Blood Parameters. Plasma biochemical indicators were measured with corresponding kits using a Hitachi 7060 Automatic Biochemical Analyzer (Hitachi, Tokyo, Japan), and blood cell counts were estimated using ADVIA®2120i Automatic Haematology System (Siemens Healthcare, Erlangen, Germany). The activities of diamine oxidase (DAO), total superoxide dismutase(T-SOD), glutathione peroxidase (GSH-PX), and catalase(CAT), and the content of D-xylose, malondialdehyde (MDA), and hydrogen peroxide (H₂O₂) were determined using commercially available kits (Jiancheng Bioengineering Institute, Nanjing, China).

Intestinal Morphology. Intestinal tissue samples used for the morphometric study were dehydrated and embedded in paraffin, sectioned at a thickness of 4 mm, and stained with haematoxylin and eosin. Morphological measurements were carried out with a light microscope (Leica microsystems, Wetzlar, Germany) with the Leica Application Suite image analysis software (Leica microsystems, Wetzlar, Germany). Intestinal villus height and width, as well as crypt depth, were measured to calculate both the villus crypt ratio and villous surface area.

Expression Levels of Genes. The gene expression levels were quantitated using the method of quantitative PCR (qPCR). The qPCR was carried out with primers (Table 1) for the target genes as well as for reference genes including ribosomal protein L 4 (RPL4), and the SYBR® Premix Ex Taq™ (Takara, Dalian, China) on 7,500 Fast Real-Time PCR system (Foster City, USA). Data were analysed using the 2^{-ΔΔCt} method as described [13].

Table 1
Primers for qPCR analysis

Genes	Forward	Reverse
RPL4	F:5'-CAGAAATGGGAATGGAAAGTTG-3'	R: 5'CCATTGGTCAGGTCATTCAATACA-3'
IL-8	F:5'-ACAGCAGTAACAACAACAAG-3'	R:5'-GACCAGCACAGGAATGAG-3'
HP	F:5'-TGAATGTGAAGCAGTGTGCG-3'	R:5'-CGAGGTGAGGTTATGGTGGG-3'
HSP70	F:5'-GACGGAAGCACAGGAAGGA-3'	R:5'-GAAGACAGGGTGCGTTTGG-3'
S100A12	F:5'-TTGAAGGGTGAACGCAAGG-3'	R:5'-AATGCCCCAACCGAACTG-3'
Mx1	F:5'-AGTGC GGCTGTTTACCAAG-3'	R:5'-TTCACAAACCCTGGCAACTC-3'
Mx2	F:5'-CGCATTCTTCTACTCGCATC-3'	R:5'-CCTCAACCCACCAACTCACA-3'
Nrf2	F:5'-ATCACCTCTTCTGCACCGAA-3'	R:5'-GCTTTCTCCGCTCTTTCTG-3'
GPX2	F:5'-CAGGGCAGTGCTGATTGAGA-3'	R:5'-GCAAGGGAAGCCAAGAACC-3'
ARG1	F:5'-GGCTGGTCTGCTTGAGAAAC-3'	R:5'-ATCGCCATACTGTGGTCTCC-3'

Expression Levels of Proteins. The expression levels of proteins were determined by Western blot analysis. The primary antibodies included: aquaporins 3 (AQP3, Santa Cruz Biotechnology, Shanghai, China), aquaporins 4 (AQP4, Santa Cruz Biotechnology, Shanghai, China), NLR family pyrin domain containing 3 (NLRP3, Invitrogen, Carlsbad, USA), Heat Shock Protein 70 (HSP70, Assay Designs, Inc., Michigan, USA), Caspase-3 (Cell Signalling Technology, Inc., Danvers, USA), β-actin (Cell Signalling Technology, Inc., Danvers, USA). The secondary antibodies were: anti-rabbit (1: 10000) and anti-mouse (1:5000). Blots were carried out by utilizing a chemiluminescence kit (Amersham Biosciences, Uppsala, Sweden) and forming image system (Alpha Innotech, New York, USA).

Statistical Analysis. Data were analysed using a two-way analysis of variance (ANOVA), expressed as mean values. Tukey–Kramer range test was processed as post hoc test to present the significant difference. All experimental data was analysed using SPSS version 23.0 (SPSS Inc., Chicago, IL, USA). A p-value of < 0.05 was considered statistically significant, and A p-value of < 0.10 was considered as a significant trend.

Results

Growth Performance. During the experimental period (D3-D7), the average daily gain (ADG), average daily feed intake (ADFI), diarrhoea rate (DR) and mortality rate (MR) were recorded and calculated (Table 2). The ETEC infection significantly reduced the ADG (*p* < 0.05). Significant increases of ADG and ADFI were observed in the group of HL (*p* < 0.05), while the LL group had a tendency to increase ADG and ADFI (*p* < 0.10). The supplementation of CT significantly increased ADG (*p* < 0.05) and exhibited a tendency to increase ADFI (*p* < 0.10). Compared with the control group, the DR of the ETEC group was significantly increased (*p* < 0.05). An extremely significant ETEC × probiotic interaction was found on the diarrhoea rate (*p* < 0.001). Compared with the ETEC group, the groups of CT + ETEC, LL + ETEC and HL + ETEC had significantly reduced DR (*p* < 0.05). Additionally, a significant trend of ETEC × probiotics interaction was observed on ADFI (*p* < 0. 10).

Table 2
Effects of *Lactobacillus zeae* LB1 on the growth performance of piglets.

Item	- ETEC				+ ETEC				SEM	ETEC		Pro				P-value	
	-	CT	LL	HL	-	CT	LL	HL		+	-	-	CT	LL	HL	E	Pro
ADG g	10.00	41.70	54.00	78.00	-184	84	-114	33	16.60	-45	46	-87 ^a	63 ^b	-30 ^{ab}	55 ^b	0.030	0.032
ADFI g	89.7	87.3	90.5	93.0	75.8	91.7	86.9	95.7	9.23	87.5	90.1	82.8 ^a	89.5 ^{ab}	88.7 ^{ab}	94.3 ^b	0.310	0.027
DR %	0.09 ^a	0.17 ^a	0.14 ^a	0.13 ^a	0.78 ^b	0.31 ^a	0.23 ^a	0.20 ^a	0.25	0.38	0.13	0.44	0.24	0.19	0.16	< 0.001	< 0.001
MR %	0.00	0.00	0.00	0.00	0.25	0.00	0.13	0.00	0.21	0.09	0.00	0.13	0.00	0.06	0.00	0.074	0.267
Pro: probiotics (same as below). ADG: average daily gain. ADFI: average daily feed intake. DR: diarrhoea rate. MR: mortality rate. ^{a, b} Values within a row with letters differ ($p < 0.05$).																	

Blood Parameters. The concentration of various blood cells, such as red blood cells and white blood cells, often presents the health status of human and animals [14]. The effects of *L. zeae* LB1 on blood cell counts of piglets are summarised in Table 3. Briefly, a significant ETEC × probiotic interaction was observed on eosinophilic granulocyte proportion (EOS%) ($p < 0.05$), in addition to a significant trend on monocytes proportion (MONO%) ($p < 0.10$). Oral administration of CT, LL, and HL in piglets significantly reduced the content of Hemoglobin (HGB) in plasma ($p < 0.05$). Among them, oral administration of LL and HL significantly reduced MONO% ($p < 0.05$), while CT had a tendency to reduce MONO% ($p < 0.10$). Compared with the control group, the EOS% in the CT and HL groups decreased significantly ($p < 0.05$), and the EOS% in the LL group tended to decrease ($p < 0.10$).

Table 3
Effects of *Lactobacillus zeae* LB1 on blood cell counts of piglets.

Item	- ETEC				+ ETEC				SEM	ETEC		Pro		
	-	CT	LL	HL	-	CT	LL	HL		+	-	-	CT	LL
HGB	125.5	117.9	121.6	127.7	152.5	118.4	130.2	124.0	19.62	131.3	123.2	139.0 ^b	118.1 ^a	125.9 ^a
MONO %	1.98	2.06	2.03	1.77	2.63	1.96	1.59	1.58	0.63	1.94	1.96	2.31 ^b	2.01 ^{ab}	1.81 ^a
EOS %	5.80 ^b	3.86 ^a	4.69 ^{ab}	3.60 ^a	3.58 ^a	4.08 ^a	4.02 ^a	3.42 ^a	1.17	3.76	4.49	4.69	3.93	4.36
TP g/L	52.24	49.64	53.86	58.53	58.48	54.18	60.09	59.64	5.52	58.10	53.57	55.36 ^b	51.91 ^a	56.97 ^{bc}
ALT U/L	44.13 ^{bc}	42.0 ^{bc}	44.75 ^{bc}	46.57 ^c	30.63 ^a	35.69 ^{ab}	45.71 ^c	37.88 ^{abc}	7.92	37.48	44.36	37.38	38.84	45.23
ALP U/L	490.4 ^{ab}	492.1 ^{ab}	451.9 ^{ab}	437.6 ^{ab}	375.8 ^a	419.1 ^{ab}	575.6 ^b	502.9 ^{ab}	110.7	468.4	468.0	433.1	455.6	513.7
TC mg/dL	68.78	75.98	81.25	87.14	79.24	79.01	86.33	88.78	11.91	83.34	78.29	74.01 ^a	77.49 ^{ab}	83.79 ^{bc}
GLU mg/dL	83.84 ^{ab}	81.65 ^{ab}	82.20 ^{ab}	85.04 ^{ab}	72.32 ^a	75.36 ^a	110.90 ^b	73.41 ^a	24.15	83.00	83.18	78.08	78.51	96.55
P mg/dL	6.35	6.09	6.86	6.51	6.06	6.30	8.55	6.46	1.49	6.84	6.45	6.20 ^a	6.20 ^a	7.70 ^b
CREA mg/dL	75.33 ^{ab}	70.63 ^a	71.58 ^a	74.66 ^a	68.44 ^a	73.63 ^a	105.8 ^b	77.0 ^{ab}	21.51	81.22	73.05	71.88	72.13	88.70

HGB: haemoglobin; MONO: monocyte; EOS: eosinophil granulocyte; BASO: basophil granulocyte; TP: total protein; ALT: alanine; transaminase; TC: total cholesterol; CREA: creatinine. ^{a, b, c} Values within a row with different letters differ ($p < 0.05$).

Similarly, the blood biochemistry can give further information on what have potentially happened behind the physiology of human and animals [15]. The effects of *L. zeae* LB1 on plasma biochemical indicators are shown in Table 3. Significant ETEC × probiotic interactions were observed on the activity of alanine amino transferase (ALT) and alkaline phosphatase (ALP) as well as the content of glucose (GLU) and creatinine (CREA) ($p < 0.05$). The ETEC infection markedly increased total protein (TP) content and reduced ALT activity compared with none-challenged piglets ($p < 0.05$). The HL supplementations increased the content of total cholesterol (TC) and TP compared with those in none-supplemented piglets ($p < 0.05$). The LL supplementations increased the content of total cholesterol (TC) and phosphorus compared with those in none-supplemented piglets ($p < 0.05$). Among the ETEC infected groups, the LL supplementation significantly increased the activity of ALT and ALP, GLU and CREA content ($p < 0.05$). The HL supplementation had a tendency to increase the activity of ALT and ALP and CREA content ($p < 0.10$). The CT supplementation had a tendency to increase the activity of ALT and ALP ($p < 0.10$).

The results of redox status are summarised in Table 4. DAO activity and the content of H₂O₂, D-xylose and MDA were found to have significant ETEC × probiotic interactions in this study ($p < 0.05$). The ETEC infection significantly increased H₂O₂ content and the activity of DAO compared with those in none-

infected piglets ($p < 0.05$). Both the HL and LL supplementation had a tendency to increase the activity of GSH-PX compared with that in none-supplemented piglets ($p < 0.10$). Among the piglets unchallenged with ETEC, the CT and HL supplementations had a tendency to decrease MDA content ($p < 0.10$). The CT supplementation significantly decreased H_2O_2 content ($p < 0.05$), while the LL and HL supplementations had a tendency to decrease H_2O_2 content ($p < 0.10$). Among the piglets challenged with ETEC, the CT supplementation significantly decreased the content of H_2O_2 and D-xylose ($p < 0.05$) and had a tendency to decrease the activity of DAO and MDA content ($p < 0.10$). The supplementation of LL significantly decreased the activity of DAO and MDA content ($p < 0.05$) and had a tendency to decrease the content of H_2O_2 ($p < 0.10$). The supplementation of HL significantly decreased the activity of DAO ($p < 0.05$) and had a tendency to decrease the content of MDA ($p < 0.10$).

Table 4
Effects of *Lactobacillus zeae* LB1 on plasma enzymes and related products of piglets.

Item	- ETEC				+ ETEC				SEM	ETEC		Pro	
	-	CT	LL	HL	-	CT	LL	HL		+	-	-	CT
CAT(U/ml)	13.04	7.39	11.05	8.57	11.07	10.74	15.38	10.63	4.20	11.95	10.01	12.06 ^{bc}	9.06 ^a
GSH-Px(μ mol/L)	645.8	1209	1007	830.4	705.1	826.9	720.6	942.9	336.9	798.9	923.1	675.5 ^a	1018 ^b
DAO(U/ml)	15.18 ^a	16.65 ^a	17.43 ^{ab}	23.13 ^{abc}	46.23 ^d	32.94 ^{cd}	21.60 ^{abc}	30.96 ^{bc}	12.93	32.93	18.10	30.71	24.79
MDA(nmol/ml)	12.33 ^{ab}	8.20 ^a	12.21 ^{ab}	8.84 ^a	14.47 ^b	9.89 ^{ab}	8.29 ^a	12.76 ^{ab}	3.63	11.35	10.39	13.40	9.04
SOD(U/ml)	53.02	53.94	51.24	46.61	49.69	59.85	63.65	49.96	13.86	55.79	51.20	51.36	56.89
H_2O_2 (mmol/ml)	209.9 ^{bc}	88.85 ^a	197.1 ^{abc}	110.4 ^{ab}	258.7 ^c	103.0 ^{ab}	184.6 ^{abc}	254.6 ^c	93.0	200.2	151.6	234.3	95.93
D-xylose(mmol/L)	0.63 ^{bc}	0.61 ^{bc}	0.41 ^{ab}	0.67 ^c	0.54 ^{bc}	0.28 ^a	0.76 ^c	0.61 ^{bc}	0.19	0.55	0.58	0.59	0.45
IL-1 β (pg/ml)	121.8 ^{ab}	116.9 ^a	124.6 ^{ab}	122.4 ^{ab}	151.8 ^c	129.9 ^{ab}	134.9 ^b	133.6 ^b	13.0	137.6	121.4	136.8	123.4
IL-8(pg/ml)	82.2 ^b	70.4 ^a	68.2 ^a	68.2 ^a	59.3 ^a	59.2 ^a	61.5 ^a	64.9 ^a	9.72	61.23	72.21	70.73	64.81
TNF- α (pg/ml)	117.1 ^{ab}	103.3 ^a	128.4 ^{ab}	112.9 ^{ab}	136.5 ^b	99.9 ^a	109.4 ^{abc}	122.1 ^{ab}	19.79	117.00	115.40	126.80	101.56

CAT: catalase; GSH-Px: glutathione peroxidase; DAO: diamine oxidase; MDA: malondialdehyde; IL: interleukin; TNF: tumour necrosis factor. ^{a, b, c} Values within the same row with different superscripts are significantly different ($p < 0.05$). The results of inflammatory parameters are also summarised in Table 4. There was a significant interaction of ETEC $\times$ probiotic on the content of interleukin-1 β (IL-1 β), IL-8 and TNF- α ($p < 0.05$). Compared with the control group, the CT group showed a tendency in the reduction of IL-1 β and TNF- α ($p < 0.10$) and a significant decrease of IL-8 ($p < 0.05$); IL-8 in the LL and HL groups were also significantly reduced ($p < 0.05$). The ETEC group exhibited a significant increase of IL-1 β ($p < 0.05$), and a tendency in the reduction of IL-8 ($p < 0.1$). Compared with the ETEC group, IL-1 β and TNF- α in the CT + ETEC group were significantly reduced ($p < 0.05$). While IL-1 β in the LL + ETEC group and HL + ETEC group was significantly reduced ($p < 0.05$), TNF- α had a tendency to decrease ($p < 0.10$).

Intestinal Morphology. The microscopic pictures of intestinal mucosal are shown in Fig. 1. The ETEC infection obviously injured intestinal integrity and villus structure, whereas the supplementation of LB1 remarkably restored the damage caused by ETEC ($p < 0.05$). Results of intestinal mucosal morphology are summarised in Table 5. There were significant interactions of ETEC $\times$ probiotic on VH and VH/CD in the jejunum and ileum and CD in the colon ($p < 0.05$). Compared with none-challenged piglets, the ETEC infection significantly increased CD in the duodenum, ileum and colon, decreased VH, surface area and ratio of VH/CD in the duodenum, jejunum and ileum ($p < 0.05$). Compared with LB1-none-supplemented piglets, the LL supplementation significantly decreased CD in the duodenum ($p < 0.05$), and the LL and HL supplementations had a tendency to increase villus surface area in the ileum ($p < 0.10$). The CT supplementation significantly increased villus surface area in the ileum ($p < 0.05$) and had a tendency to decrease CD in the duodenum ($p < 0.10$). Compared with the control group, the CT group had significantly increased ileal VH ($p < 0.05$), and the LL and HL groups tended to show increased ileal VH ($p < 0.10$). The ETEC group demonstrated significantly reduced jejunal VH and VH/CD, reduced ileal VH, and significantly increased colon CD ($p < 0.05$). Compared with the ETEC group, the CT + ETEC and LL + ETEC groups had significantly reduced colon CD ($p < 0.05$), which also exhibited a tendency to increase jejunal and ileal VH. The HL + ETEC group showed significantly reduced colon CD, significantly increased ileal VH ($p < 0.05$) and an increased jejunal VH trend ($p < 0.10$).

Table 5
Effects of *Lactobacillus zeae* LB1 on the intestinal morphology of piglets.

Item	- ETEC				+ ETEC				SEM	ETEC		Pro		
	-	CT	LL	HL	-	CT	LL	HL		+	-	-	CT	LL
Duodenum														
VH (µm)	404.4	372.4	354.0	381.1	296.5	341.3	291.8	310.5	67.62	310.0	378.0	350.4	356.9	322.9
VW (µm)	106.1	107.3	101.1	104.8	95.32	100.7	105.9	99.19	12.02	100.3	104.8	100.7	104.0	103.1
CD (µm)	117.4	109.0	96.01	117.7	116.9	121.3	113.9	129.4	17.71	120.4	110.1	117.2 ^b	115.2 ^{ab}	104.9
VSA	10461	9873	8801	9607	6727	8387	7067	7079	2278	7315	9685	8594	9130	7934
VH/CD	3.48	3.33	3.72	3.24	2.48	2.66	2.56	2.57	0.67	2.57	3.44	2.98	2.99	3.14
Jejunum														
VH (µm)	354.5 ^c	274.8 ^{abc}	266.2 ^{abc}	287.3 ^{bc}	228.2 ^a	264.8 ^{abc}	284.7 ^{abc}	238.5 ^{ab}	41.55	254.1	283.2	266.4	269.8	275.1
VW (µm)	91.49	90.92	94.56	93.56	87.18	86.08	84.85	77.74	9.10	83.96	92.63	89.33	88.50	89.70
CD (µm)	94.60	106.9	105.5	103.6	102.5	107.1	109.7	110.5	12.01	107.5	102.7	98.55	107.0	107.0
VSA	6849	6648	6644	6532	4649	5641	4868	4740	1319	4975	6668	5749	6144	5756
VH/CD	3.34 ^c	2.75 ^{abc}	2.53 ^{ab}	2.95 ^{bc}	2.24 ^{ab}	2.55 ^{ab}	2.60 ^{abc}	2.18 ^a	0.57	2.39	2.89	2.79	2.65	2.56
Ileum														
VH (µm)	301.6 ^{bc}	352.4 ^d	315.3 ^{cd}	314.5 ^{cd}	260.1 ^a	293.8 ^{abc}	268.4 ^{ab}	316.2 ^{cd}	36.56	284.6	321.0	280.9	323.1	291.9
VW (µm)	89.01	99.30	93.37	88.70	86.57	93.93	88.01	95.66	12.17	91.04	92.59	87.79	96.61	90.61
CD (µm)	92.0	99.48	88.19	92.40	94.95	99.35	93.70	110.0	10.99	99.50	93.02	93.47 ^{ab}	99.41 ^{bc}	90.94
VSA	6609	8151	7071	6687	5205	6307	5453	6742	1508	5927	7130	5907 ^a	7229 ^b	6262
VH/CD	3.25	3.55	3.61	3.48	2.68	2.97	2.91	2.76	0.51	2.83	3.47	2.96	3.26	3.26
Colon														
CD (µm)	117.5 ^a	126.7 ^{ab}	124.7 ^{ab}	128.9 ^{ab}	264.1 ^e	175.7 ^d	145.2 ^{bc}	158.9 ^{cd}	47.63	186.0	124.5	190.8	151.2	135.0
VH: villus height, CD: crypt depth, VSA: villus surface area. ^{a, b, c} Values within a row with different letters differ (<i>p</i> <0.05).														

Regulation of Gene Expression. The profile of relative expression levels of genes is summarised in Table 6. Significant interactions of ETEC × Probiotic were observed on expressions of genes IL-8), HSP70, S100 calcium binding protein A12 (S100A12), myxovirus resistance 1 (Mx1), myxovirus resistance 2 (Mx2), nuclear factor erythrocyte two related factors-2 (Nrf2), glutathione peroxidase 2 (GPX2), and Arginase1 (ARG1) in the jejunum and ileum, as well as haptoglobin (HP) expression in the jejunum ($p < 0.05$). Compared with none-challenged piglets, the ETEC infection significantly increased expression levels of IL-8, HP and HSP70 ($p < 0.05$), decreased expression levels of Mx1, Mx2 and GPX2 in the jejunum ($p < 0.05$). In the ileum, the ETEC infection significantly increased expression levels of HP, HSP70, S100A12, Nrf2 and ARG1, and decreased expression levels of Mx2 and GPX2 ($p < 0.05$).

Table 6
Effects of *Lactobacillus zeae* (LB1) on gene expression in Jejunum and ileum of piglets.

Item	- ETEC				+ ETEC				SEM	ETEC		Pro	
	-	CT	LL	HL	-	CT	LL	HL		+	-	-	CT
Jejunum													
IL-8	1.000 ^{abc}	0.757 ^a	0.986 ^{abc}	1.260 ^{cd}	1.448 ^d	1.101 ^{abcd}	1.141 ^{bcd}	0.891 ^{ab}	0.080	1.145	1.001	1.224	0.929
HP	1.000 ^a	0.814 ^a	1.079 ^a	1.694 ^b	1.809 ^b	0.953 ^a	1.697 ^b	0.782 ^a	0.097	1.310	1.147	1.405	0.883
HSP70	1.000 ^a	1.674 ^{bc}	1.243 ^{ab}	1.323 ^{abc}	1.072 ^a	1.168 ^a	1.775 ^{cd}	2.179 ^d	0.111	1.548	1.310	1.036	1.421
S100A12	1.000 ^e	0.446 ^{abc}	0.542 ^{bcd}	0.723 ^d	1.528 ^f	0.352 ^{ab}	0.697 ^{cd}	0.259 ^a	0.059	0.709	0.678	1.264	0.399
Mx1	1.000 ^{bcd}	1.023 ^{bcd}	1.113 ^{cd}	1.174 ^d	0.446 ^a	0.845 ^{bc}	0.298 ^a	0.772 ^b	0.070	0.590	1.078	0.723	0.934
Mx2	1.000 ^{bc}	0.985 ^{bc}	1.272 ^c	0.809 ^b	0.350 ^a	1.249 ^c	0.684 ^b	1.288 ^c	0.073	0.893	1.017	0.675	1.117
Nrf2	1.000 ^c	0.467 ^a	0.578 ^{ab}	0.637 ^{ab}	0.994 ^c	0.604 ^{ab}	0.705 ^b	0.496 ^{ab}	0.053	0.700	0.670	0.997	0.536
GPX2	1.000 ^c	0.735 ^b	1.310 ^d	1.546 ^d	0.584 ^{ab}	0.431 ^a	0.693 ^b	0.377 ^a	0.058	0.521	1.148	0.792	0.583
ARG1	1.000 ^c	1.187 ^c	0.653 ^{ab}	0.444 ^a	1.698 ^d	0.876 ^{bc}	0.384 ^a	0.486 ^a	0.073	0.861	0.821	1.349	1.031
Ileum													
IL-8	1.000 ^{bcd}	0.634 ^a	1.041 ^{bcd}	1.224 ^d	0.930 ^{abcd}	0.805 ^{ab}	1.115 ^{cd}	0.897 ^{abc}	0.069	0.937	0.975	0.965	0.719
HP	1.000	0.743	0.798	0.883	1.226	1.170	0.972	1.002	0.071	1.093	0.856	1.113 ^b	0.957 ^a
HSP70	1.000 ^{bc}	0.778 ^{ab}	0.774 ^{ab}	0.587 ^a	1.041 ^c	0.786 ^{ab}	0.879 ^{bc}	0.930 ^{bc}	0.057	0.909	0.785	1.021	0.782
S100A12	1.000 ^c	0.259 ^a	0.531 ^{ab}	1.055 ^c	2.352 ^d	0.535 ^{ab}	0.806 ^{bc}	0.397 ^a	0.088	1.023	0.712	1.676	0.397
Mx1	1.000 ^c	0.550 ^a	0.680 ^{ab}	0.896 ^{bc}	0.707 ^{ab}	0.883 ^{bc}	0.554 ^a	1.051 ^c	0.060	0.799	0.782	0.854	0.716
Mx2	1.000 ^d	0.581 ^{bc}	0.634 ^c	0.453 ^b	0.057 ^a	0.090 ^a	0.070 ^a	0.136 ^a	0.037	0.090	0.667	0.529	0.335
Nrf2	1.000 ^{bc}	0.645 ^a	0.742 ^{ab}	0.826 ^{ab}	1.635 ^d	1.184 ^c	1.003 ^{bc}	0.974 ^{abc}	0.077	1.199	0.803	1.318	0.915
GPX2	1.000 ^b	0.509 ^a	1.094 ^b	0.964 ^b	0.692 ^a	0.592 ^a	0.620 ^a	0.612 ^a	0.058	0.629	0.892	0.846	0.551
ARG1	1.000 ^b	0.630 ^a	0.743 ^{ab}	0.836 ^{ab}	1.515 ^c	1.409 ^c	1.593 ^c	0.767 ^{ab}	0.081	1.321	0.802	1.257	1.020

Compared with the control group, the CT group had significantly reduced expression of Nrf2 and S100A12 ($p < 0.05$) and a tendency to reduce the expression of IL-8 in the jejunum ($p < 0.10$), and significantly reduced expression levels of IL-8, S100A12, Nrf2 and ARG1 in the ileum ($p < 0.05$). There was also a tendency in reducing the expression of HSP70 in the ileum ($p < 0.10$). In the LL group compared with the control group of piglets, there were significantly reduced expression of Nrf2, S100A12 and ARG1 and an increased expression of GPX2 in the ileum ($p < 0.05$) with a tendency in increasing the expression of Mx1, Mx2 ($p < 0.10$) in the jejunum. A significant reduction in the expression of S100A12 ($p < 0.05$) and a tendency to reduce the expression of HSP70, Nrf2 and ARG1 ($p < 0.10$) were also detected in the ileum. In the HL group, significantly reduced expression levels of S100A12, Nrf2 and ARG1 and increased expression of GPX2 were observed in the jejunum. In the ileum, the expression of HSP70 was significantly reduced ($p < 0.05$), and the expression levels of Nrf2 and ARG1 tended to decrease ($p < 0.10$). The ETEC group demonstrated significantly increased expression levels of IL-8, HP, S100A12 and ARG1, and reduced expression of Mx1, Mx2 and GPX2 in the jejunum ($p < 0.05$). In the ileum, the expression levels of S100A12, Nrf2 and ARG1 were significantly increased, and the expression levels of Mx1, Mx2 and GPX2 were significantly reduced ($p < 0.05$).

Compared with the ETEC group, the CT + ETEC group had significantly reduced expression levels of HP, S100A12, ARG1, and Nrf2, and increased expression of Mx1 and Mx2 in the jejunum ($p < 0.05$). In the ileum, the CT + ETEC group exhibited significantly reduced expression of HSP70, S100A12, and Nrf2 ($p < 0.05$), and a tendency to reduce the expression of IL-8 ($p < 0.10$). In the LL + ETEC group, there were significantly reduced expression levels of S100A12, ARG1 and Nrf2 and increased expression of Mx2 ($p < 0.05$), and a decreased trend in the expression of IL-8 in the jejunum ($p < 0.10$). In the ileum, the expression of S100A12 and Nrf2 was significantly reduced ($p < 0.05$), while the expression HSP70 showed a tendency to decrease ($p < 0.10$). In the HL + ETEC group, while the expression levels of IL-8, HP, S100A12, Nrf2 and ARG1 were significantly reduced in the jejunum ($p < 0.05$), the expression levels of Mx1 and Mx2 were significantly increased ($p < 0.05$). In the ileum, the expression levels of S100A12, Nrf2 and ARG1 were significantly reduced and the expression of Mx1 was significantly increased ($p < 0.05$). The expression of HP and HSP70 exhibited a tendency to decrease ($p < 0.10$).

Regulation of Protein Production. The profile of relative levels of protein production in the jejunum and ileum is summarised in Fig. 2. Compared with the control group (basal diet), the ETEC infection significantly increased the production of NLRP3 and HSP70 ($p < 0.05$), and the HL treatment significantly increased the production of HSP70 ($p < 0.05$), which was much lower than that in the ETEC group ($p < 0.05$). The production of AQP3 in the HL group showed a tendency to increase ($p < 0.10$). Compared with the ETEC group, the HL + ETEC group exhibited significantly reduced production of NLRP3 and HSP70 and a tendency to increase the production of AQP3.

Discussion

Piglet diarrhoea is one of the most challenging problems in swine production, which causes a substantial economic loss [16]. ETEC is the primary pathogenic bacteria of piglet diarrhoea, whose pathogenicity depends on the co-effect of adhesion and enterotoxins. A marked finding from the present study is the significant reduction of diarrhoea and mortality rate induced by ETEC infection by LB1 supplementation, indicating that *L. zeae* could effectively relieve diarrhoea in lactating piglets.

The intestine is not only the terminal organ for digestion and absorption of dietary nutrients, but is also crucial for preventing the entry of exogenous pathogens into the systemic circulation. Thus, intestinal integrity is vital to the survival, growth, and health of both animals and humans [17]. Intestinal morphology indexes such as villus height, surface area, crypt depth, and the ratio of villus height to crypt depth are the common indicators of intestinal morphologic development and intestinal morphological integrity [18]. Usually, the increases in villus height, villus surface area, as well as the ratio villus/crypt reflect the improvement of intestinal absorption capacity and intestinal health [19]. In the present study, ETEC infection undermined the integrity of the intestinal tract, leading to severe intestinal damage of piglets, while the supplementation of *L. zeae* LB1 alleviated the intestinal injury caused by ETEC, demonstrating beneficial effects on intestinal integrity.

DAO activity is frequently used as a non-invasive biomarker of alterations in the function and structure of the intestinal mucosa [20]. Under certain conditions, cells in the intestinal mucosa experience necrosis, 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 [21]. In the present study, LB supplementation decreased blood DAO, indicating that *L. zeae* could restore the damage of intestinal barrier caused by ETEC.

Gut ion transporters and AQP may be potential targets for the prevention and treatment of diarrhoea in humans and animals [22]. The gene knockout mice of AQP3 have a mucosal innate immune disorder [23]. NLRP3 is a subfamily of NLRs, called inflammatory bodies, which can respond to a variety of microorganisms, environmental stimuli, and host cell damage, and regulate intestinal homeostasis and inflammatory responses through these pathways [24, 25]. HSP70 is the first protein that responds to heat stress in the liver and is accompanied by haemorrhagic shock [26]. By increasing the expression level of HSP70, the animal body promotes protein folding and prevents the deformation of protein aggregation. In the present study, *L. zeae* supplementation decreased the gene expression of NLRP3 and HSP70, increased AQP expression, which may be one of the primary mechanisms to alleviate intestinal mucosal injury and diarrhoea caused by ETEC.

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 injury that frequently occurs after piglets' weaning [27], whereas, cells protect themselves from hydroxyl radicals and other oxygenates by antioxidant enzymes, including SOD, GSH-Px and CAT [28, 29]. MDA can induce noxious stress in cells and constitute homopolar protein adducts, known as advanced epoxidation end-products (ALEs), which are usually utilized as a marker to evaluate the oxidant stress levels [30]. H_2O_2 is the main product of oxidative stress in the body [31]. Results in the present study implied that the supplementation of *L. zeae* could alleviate oxidative stress induced by ETEC and improve anti-oxidative capacity.

IL-1 β , IL-8, and TNF- α have a synergistic and inducing relationship, which are responsible for regulating and mediating the body's immune function. With the activation of macrophages, the body first secretes a large amount of TNF- α , then promotes the production of IL-1 β , increases cell permeability, promotes the synthesis of adhesion molecules, and releases inflammatory mediators to white blood cells [32]. The primary function of IL-8 is to induce neutrophil chemotaxis, and also induce another granulocyte chemotaxis, causing them to migrate to the infection site, thereby playing a phagocytic effect [33, 34]. The expression of IL-8 promotes the aggregation of neutrophils and activates neutrophils to release various active substances. Although this physiological activity can cause cell damage, it can also play a bactericidal role [35]. Some related *in vitro* studies have shown that lactic acid bacteria can alleviate the inflammation caused by pathogenic bacteria, *e.g.* *L. plantarum*, and *L. rhamnosus* can inhibit the expression of pro-inflammatory cytokines IL-1 β and TNF- α induced by *E. coli* [36–37]. In the present study, ETEC infection significantly increased the content of IL-1 β and IL-8, whereas *L. zeae* supplementation decreased the content of IL-1 β and TNF- α , suggesting its effects on alleviation of the inflammation and enhancement of host immunity.

Many cytokines are essential for intestinal function [38]. IL-8 can bind to CXCR1 and CXCR2 receptors with high affinity, and as the primary chemokine of neutrophils, it plays a vital role in the initiation and maintenance of inflammatory response [39]. S100A12 found in mammalian tissues is considered to be an indicator for the diagnosis of inflammatory responses [40]. S100A12 is associated with innate immune responses and is mainly expressed in neutrophils and monocytes. Some recent studies have shown that S100A12 is increased in certain human inflammatory diseases [41, 42]. IFN- α/β can induce the expression of genes encoding antiviral proteins, especially myxovirus (MX) and 2'-5' oligoadenylate synthetase (OAS) [43, 44]. Nuclear factor-erythrocyte-associated factor 2 (Nrf2) is a transcriptional regulatory element that maintains the cell's redox state by promoting antioxidant response participants (glutathione peroxidase et al.) to the cell protection and antioxidant gene promoters. Under normal circumstances, the concentration of Nrf2 in the cytoplasm is low. When the animal body is subjected to oxidative stress, Nrf2 will transfer to the nucleus and activate related enzymes in the nucleus, such as the combination of antioxidant response elements, thereby activating the transcription of many protective genes. These protective genes play a role in maintaining the redox balance and exert anti-inflammatory and anti-oxidant effects [45]. In addition, a recent study has shown that the concentration of Nrf2 is steadily increased in the cancerous tissues and organs detected [46]. Among the antioxidants, GPX, CAT, and SOD are the first protections in antioxidant stress [47]. GPX is responsible for catalyzing the reduction of hydrogen peroxide and protecting tissues from damage by ROS. Previous studies have shown that polyphenols (hypericin, rotenone) and other plant extracts containing quercetin and rutin can significantly induce the expression of GPX, SOD, and CAT genes [48–50]. In the present study, the supplementation of *L. zeae* had opposite effects on regulating expressions of genes related to inflammation and anti-oxidation, which may be one of the primary mechanisms to alleviate the inflammatory response and oxidative stress caused by ETEC and improve intestinal health.

Conclusions

Supplementation of *L. zeae* LB1 relieved diarrhoea, intestinal mucosal damage, inflammatory and oxidative stress, and improved intestinal integrity, immune and anti-oxidative capacity. The beneficial effects appeared to be regulated by changing the gene expression and protein production of the functionally related molecules.

Declarations

Ethics approval and consent to participate

All animal procedures were reviewed and approved by the Wuhan Polytechnic University Committee on Animal Care.

Consent for publication

All authors provide their consent to this publication.

Availability of data and material

The datasets used and/or analyzed during this study are available from the corresponding author on reasonable request.

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Authors' contributions

TW, YH and JG conceived and designed the experiments. YS, MZ, LZ and ZM performed the experiments. TW, YS, DZ, LW, BD and YZ analysed the data. TW, YH, YS and YZ wrote the paper. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1
Effects of *Lactobacillus zeae* LB1 on the intestinal morphology of piglets. (A) duodenum; (B) jejunum; (C) ileum; (D) colon; (1) control group; (2) CT group; (3) LL group; (4) HL group; (5) ETEC group; (6) CT + ETEC group; (7) LL + ETEC group; (8) HL + ETEC group.

Figure 2

Effects of *Lactobacillus zeae* LB1 on protein production in the jejunum of piglets.