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Dietary supplementation of *Lactobacillus zeae* regulated the gut microbiome in piglets infected with enterotoxigenic *Escherichia coli*

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Abstract: This study was conducted to investigate the effect of *Lactobacillus zeae* LB2 on the gut microbiota in piglets infected with enterotoxigenic *Escherichia coli* (ETEC). Thirty-two healthy 7-day-old piglets were randomly divided into four treatment groups: control group (basal diet), LB2 group (supplemented with 1×10^8 CFU/pig/day *L. zeae* LB2), ETEC group (infected with 1×10^{10} CFU/pig/day ETEC) and ETEC+LB2 group (LB2 supplementation + ETEC infection). Intestinal contents were collected for DNA extraction and Illumina sequencing. Significant result was observed for alpha diversity in the four intestinal sections, and both ETEC infection and LB2 supplementation showed a higher Chao1 alpha diversity. At the phylum level, Firmicutes and Bacteroidetes were dominant in the healthy piglets, while Proteobacteria were dominant in the ETEC-infected piglets. At the genus level, ETEC infection decreased the abundance of *Prevotella*, *Ruminococcaceae*, *Lactobacillus*, *Alloprevotella*, *Flavobacterium*, and *Sutterella* and increased the abundance of *Actinobacillus*. The LB2 supplementation reduced the abundance of *Ruminococcaceae*, *Actinobacillus*, *Porphyromonas*, and *Alloprevotella*, and increased the abundance of *Prevotella* and *Lactobacillus*. Both ETEC infection and LB2 supplementation affected several functional pathways associated with cellular processes, environmental information processing, genetic information processing, diseases, metabolism, and organismal systems. In summary, ETEC infection induced dysbiosis of the gut microbiome in piglets, while *L. zeae* supplementation could positively regulate the gut microbiome during ETEC infection. Therefore, *L. zeae* LB2 may be an ideal probiotic for the prevention or treatment of ETEC infection.

Keywords: alpha diversity; ETEC; gut microbiota; *L. zeae*; pig

Microbial community is an essential determinant of the host health (Leser and Molbak 2009). The balanced gut microbiota provides metabolic and nutritional benefits to the host. It could regulate various signalling molecules involved in the immune system, protect the intestine against pathogen infection, and promote a healthy intestinal structure and an optimal intestinal function (Leser and Molbak 2009; Lyu et al. 2020a). On the other hand, the unbalanced microbiome could lead

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to gastrointestinal diseases such as inflammatory bowel disease and colorectal cancer (Harris et al. 2012; Mondot et al. 2013). The gut microbiome also plays a vital role in the pathologies that affect the gut (Mondot et al. 2013; Wu et al. 2021a). Many investigations have unravelled the shifts in the gut microbiome during infection of several pathogens in pigs, such as *Salmonella enterica*, *Clostridium difficile* and *Escherichia coli* (Mon et al. 2015; Bin et al. 2018). However, the relationship between gut microbiota and diseases remains to be elucidated.

Enterotoxigenic *Escherichia coli* (ETEC) is an important pathogen causing diarrhoea in both humans and young animals (new-born piglets, calves, lambs, and weaned piglets) (Lv et al. 2018; Wu et al. 2018a). After being infected by ETEC, young animals often die from severe watery diarrhoea and rapid dehydration, with high morbidity and mortality (Wu et al. 2018a). About 35% of piglet diarrhoeas in China is caused by ETEC, which brings serious economic losses to the pig breeding industry (Wu et al. 2018a). The mechanism of ETEC-induced diarrhoea has been widely revealed, which was associated with its adhesion, enterotoxins, edema disease principle (EDP), endotoxin and haemolysin, etc., particularly the produced heat-stable and heat-labile enterotoxins (Nagy and Fekete 2005; Lv et al. 2018). Antibiotics have long been used to treat ETEC and other bacterial pathogens. However, due to their inappropriate use in animals, antibiotic resistant pathogens are rising sharply, and the ecology of animal microbiome gets heavily disrupted (Heo et al. 2013; Hu and Cowling 2020). Herein, it is urgent to explore possible alternatives to antibiotics against ETEC infection.

Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Hill et al. 2014). They have been considered as promising alternatives to antibiotics, due to their natural activities against pathogenic bacteria (Silva et al. 2020). Among them, *Lactobacilli* have been frequently used as probiotic bacteria in humans and animals (Lyu et al. 2020b). Their beneficial effects include a reduction in the colonization of pathogenic bacteria in animal intestines, improvement of animal performance, and enhancement of the animal immune system (Sornplang and Piyadeatsoontorn 2016). Several studies also reported that *Lactobacilli* supplementation could positively regulate the microbiota composition in mice (Park et al. 2017; Ni et al. 2019; Yan et al. 2019). Our previous study has demonstrated that the supplementation of *Lactobacillus zeae* could alleviate diarrhoea, oxidative stress and death induced by ETEC infection, modulate immune system and biochemical indicators of blood, and restore intestinal integrity (Wu et al. 2021b). This makes *L. zeae* an ideal candidate for piglets to alleviate the intestinal disorder during ETEC infection, and we hypothesised that *L. zeae* could positively regulate the porcine gut microbiome and alleviate dysbiosis induced by ETEC. Therefore, this study was conducted to evaluate the effect of *L. zeae* supplementation on the gut microbiome in healthy and ETEC-infected piglets. Findings of this research can contribute to unravel the role of *Lactobacillus* in the microbiome and provide vital clues for the treatment or prevention of ETEC infection.

MATERIAL AND METHODS

Animal experiments and sample collection

The animal use protocol for this research was approved by the Animal Care and Use Committee of Wuhan Polytechnic University (1126-2018-0053). Thirty-two 7-day-old crossbred (Duroc × Landrace × Large White) healthy piglets with similar body weight (2.541 ± 0.23 kg) were randomly allocated into four treatment groups (eight pigs per group): (1) control group, piglets fed basal diet; (2) LB2 group, piglets supplemented with 1×10^8 CFU/pig/day *L. zeae* LB2; (3) ETEC group, piglets orally inoculated with 1×10^{10} CFU/pig/day *E. coli* K88; (4) ETEC+LB2 group, piglets orally inoculated with 1×10^{10} CFU/pig/day *E. coli* K88 and supplemented with 1×10^8 CFU/pig/day *L. zeae* LB2. All piglets in each group were from different sows.

The trial lasted 10 days (day 0–9), inclusive a three-day adaptation (day 0–2). A commercial milk replacer (D80 Poly Milk; Anyou Feed Co., Ltd, Shanghai, China) was used as the basal diet to meet the nutrition requirement for suckling piglets. The nutrient components of the milk replacer are listed in Table 1. Prior to feeding, the milk replacer (powder) was dissolved in warm water (45–55 °C) to form a liquid feed (dry matter content of 20%), as described by Wang et al. (2017). *L. zeae* LB2 was obtained from the Guelph Research and

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Table 1. Nutrient components of the milk replacer (as fed basis), 100%

Items	Crude protein	Crude ash	Crude fiber	Moisture	Lysine	NaCl	Calcium	Total phosphorus
Milk replacer	≥ 20.0	≤ 9.0	≤ 1.0	≤ 10.0	≥ 1.4	0.3–1.5	0.4–1.1	≥ 0.3

Development Centre, Agriculture and Agri-Food Network, Canada. *L. zeae* LB2 was anaerobically cultured in de Man, Rogosa and Sharpe liquid medium at 37 °C for 16 hours. From day 3 to day 7 of the trial, *L. zeae* LB2 was mixed with 1 ml phosphate-buffered saline and then orally fed to piglets at 15:00 each day. The ETEC (*E. coli* K88) was from China Institute of Veterinary Drug Control, Beijing, China (Xu et al. 2020), and prepared as described previously (Wu et al. 2021a). From day 7 to 9 of the trial, 2 ml ETEC was orally delivered to piglets at 1×10^{10} CFU/pig each time at 8:00 and 20:00, the same volume of sterile saline was inoculated to the piglets in the control and LB2 groups. On day 9 of the trial, all piglets were sacrificed under diethyl ether anaesthesia to collect the intestinal content from jejunum, ileum, colon, and caecum. All samples were rapidly frozen in liquid nitrogen and then stored at −80 °C until analysis.

DNA extraction

Total microbial DNA from 2.5 g intestinal content samples was extracted using the QIAampDNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The extracted DNA was purified using the QIAamp spin column (Qiagen, Hilden, Germany), and the quantity was determined with PicoGreen assay. The quality of DNA was evaluated by 1% denatured agarose gel electrophoresis.

Amplification and sequencing

The V3–V4 region of the 16S rRNA gene was amplified as described previously (Wu et al. 2021a). Basically, it was amplified by two-stage polymerase chain reaction (PCR). The first stage of PCR was a setup using the HiFi HotStart ReadyMix Kit (Kapa Biosystems, Wilmington, MA, USA). The second stage attached dual index and Illumina sequencing adapters using the Nextera XT Index Kit (Illumina, San Diego, CA, USA) according to the manufacturer's protocol. The PCR products were purified using the Agencourt AMPure XP purification system (Beckman, Brea, CA, USA) and the concentrations were measured by the SpectraMax i3X Multi-Mode Detection Platform (Molecular Devices, San Jose, CA, USA). The purified DNA samples were sequenced using Illumina MiSeq Platform (Illumina, San Diego, CA, USA).

Data processing and statistical analysis

The raw reads were outputs for quality control after base calling, then they were subjected to the pipeline Quantitative Insights into Microbial Ecology (QIIME v1.8.0; https://qiime2.org/) for operational taxonomic units (OTU) picking and taxonomy as described by Huang et al. (2018). Briefly, the repetitive sequences were removed using UPARSE software (http://drive5.com/uparse/) to acquire representative sequences, which were subsequently ranked in order of size and annotated using RDP classifier v2.2 (http://rdp.cme.msu.edu/) and Greengenes database v13.5 (http://greengenes.secondgenome.com/. All the representative sequences were aligned by using Multiple Protein Sequence Alignment (MUSCLE) software v3.8.31 (https://www.ebi.ac.uk/Tools/msa/muscle/) to determine the phylogenetic relationships, and all data were then normalized and subjected to alpha and beta diversity analysis.

The alpha diversity (Shannon, Simpson, and Chao1) and beta diversity (Bray-Curtis index) were calculated using QIIME as described by Fu et al. (2019). The comparisons between different groups were determined using the one-way analysis of variance (ANOVA) with Turkey's test as post hoc and analysis of similarities (ANOSIM), respectively. Dendrograms and collinear relation diagrams were generated using Majorbio Cloud Platform (Shanghai Majorbio Bio-pharm Technology, Shanghai, China). Relative abundance of OTUs was compared by ANOVA using Majorbio Cloud Platform, with Turkey's test as post hoc and *P*-values adjusted by false discovery rate (FDR). Functional potentials of OTUs were evaluated by Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway enrichment using PICRUSt

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(http://huttenhower.sph.harvard.edu/galaxy/), and the heatmap was generated using TBtools (https://github.com/CJ-Chen/TBtools). Significance was set at $P < 0.05$ for all comparisons.

RESULTS

Sequencing data and extraneous clustering

A total of 9 291 475 reads were obtained with an average sequence length of 457.89 bp. Among them, 1 940 881 reads were from the jejunum, 246 332 reads from the ileum, 2 874 028 reads from the colon, and 2 012 234 reads from the caecum.

Evenness, diversity, and richness were calculated by Shannon, Simpson, and Chao1 indices, respectively. Results are presented in Table 2. ETEC infection increased Chao1 index in the colon ($P = 0.001$) compared to the control group. Compared to the ETEC group, the ETEC+LB2 group increased Chao1 index in the jejunum ($P = 0.030$), and decreased Shannon ($P = 0.017$) and Chao1 ($P = 0.001$) indices in the colon.

Based on the Bray-Curtis index calculated by PCoA, beta diversity was generated and it is shown in Figure 1. Among the four treatments, distinct bacterial communities were observed in the jejunum ($P < 0.001$), ileum ($P = 0.049$), colon ($P < 0.001$), and caecum ($P = 0.003$).

The structure of intestinal flora

The microbiota in all samples was composed of four predominant bacterial phyla: Firmicutes, Bacteroidetes, Proteobacteria, and Epsilonbacteraeota, accounting for more than 98% of the total sequence (Figure 2). In the jejunum and ileum, Firmicutes were dominant in the control and LB2 groups, while Proteobacteria were dominant in the ETEC and ETEC+LB2 group. In the colon, the LB2 group displayed a higher proportion of Firmicutes and a lower proportion of Epsilonbacteraeota compared to the other three treatments. In the caecum, the ETEC group had a higher proportion of Proteobacteria than the other three groups.

A total of 12 genera were identified as the dominant bacterial populations (> 3%) in all groups. In the jejunum, *Lactobacillus*, *Actinobacillus*, *Eschericha-Shigella*, and *Chloroplast* were the most abundant genera (Figure 3A). In the ileum, *Actinobacillus*, *Lactobacillus*, *Escherichia-Shigella*, and *Prevotella* were the most abundant genera (Figure 3B). In the colon, *Prevotella*, *Alloprevotella*, *Campylobacter*, *Prevotellaceae*,

Table 2. Alpha diversity of gut microbiota in piglets

Item	Control	LB2	ETEC	ETEC+LB2	*P*-value
Jejunum					
Shannon	3.30 ± 0.64	3.25 ± 0.40	2.67 ± 0.74	3.50 ± 0.56	0.061
Simpson	0.10 ± 0.06	0.09 ± 0.02	0.21 ± 0.19	0.08 ± 0.03	0.081
Chao1	555.2 ± 127.8^{ab}	507.5 ± 128.1^{ab}	400.4 ± 155.3^{a}	617.1 ± 141.2^{b}	0.030
Ileum					
Shannon	3.67 ± 0.88	3.40 ± 0.61	3.52 ± 0.65	3.12 ± 0.63	0.506
Simpson	0.08 ± 0.06	0.11 ± 0.07	0.12 ± 0.11	0.10 ± 0.05	0.751
Chao1	530.0 ± 226.2	672.0 ± 115.4	746.0 ± 148.9	505.5 ± 234.2	0.103
Colon					
Shannon	3.92 ± 0.32^{ab}	4.06 ± 0.13^{ab}	4.13 ± 0.37^{b}	3.61 ± 0.37^{a}	0.017
Simpson	0.04 ± 0.02	0.03 ± 0.01	0.04 ± 0.03	0.06 ± 0.02	0.163
Chao1	465.7 ± 78.2^{a}	490.4 ± 57.5^{ab}	638.3 ± 90.7^{b}	377.2 ± 161.5^{a}	0.001
Caecum					
Shannon	3.66 ± 0.58	4.02 ± 0.23	3.84 ± 0.45	3.72 ± 0.31	0.384
Simpson	0.06 ± 0.05	0.04 ± 0.01	0.06 ± 0.05	0.05 ± 0.02	0.589
Chao1	377.5 ± 106.4	470.5 ± 100.2	514.7 ± 88.0	396.8 ± 119.6	0.049

ETEC = enterotoxigenic *Escherichia coli*; LB2 = *Lactobacillus zeae* LB2

^{a,b}Values with different letters are significantly different ($P < 0.05$)

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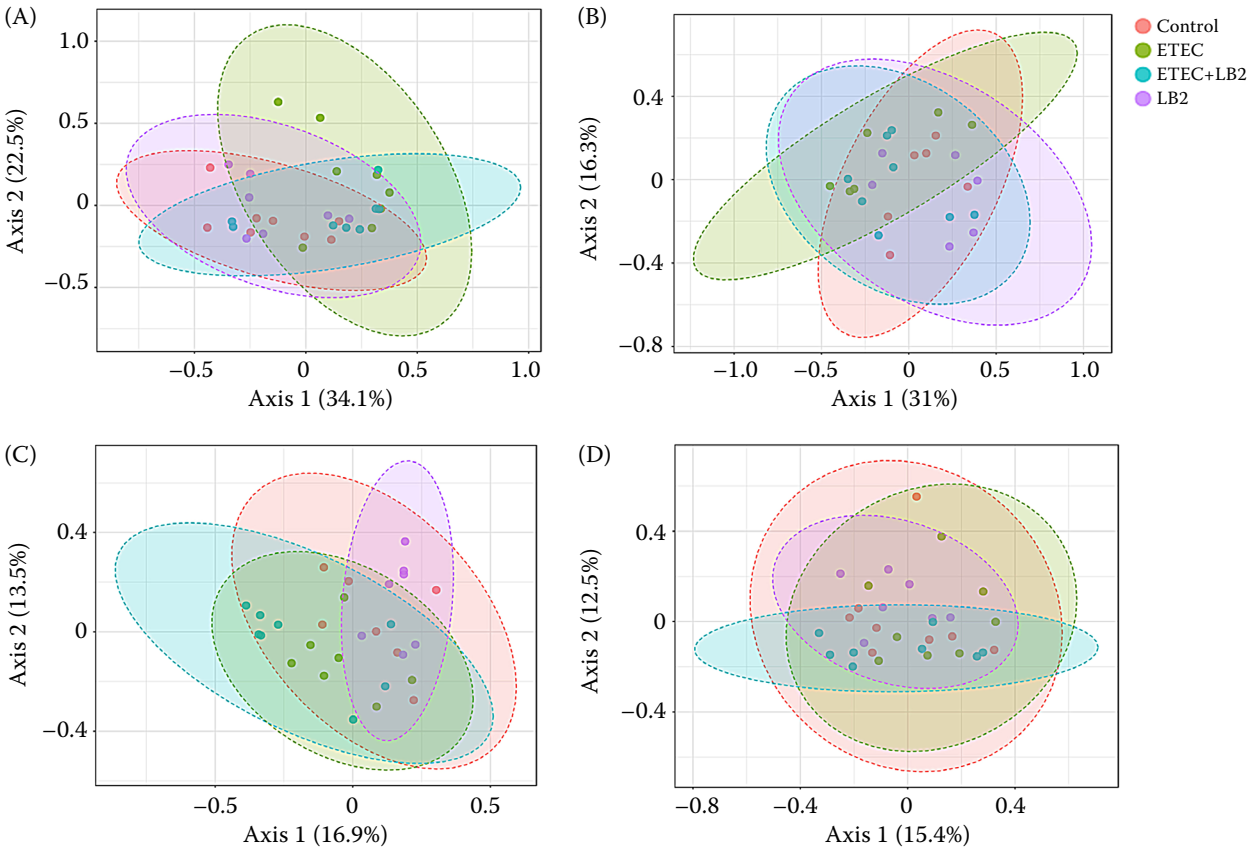

Figure 1. Beta diversity of gut microbiota in piglets
(A) Jejunum, $P < 0.001$; (B) ileum, $P = 0.049$; (C) colon, $P < 0.001$; (D) caecum, $P = 0.003$

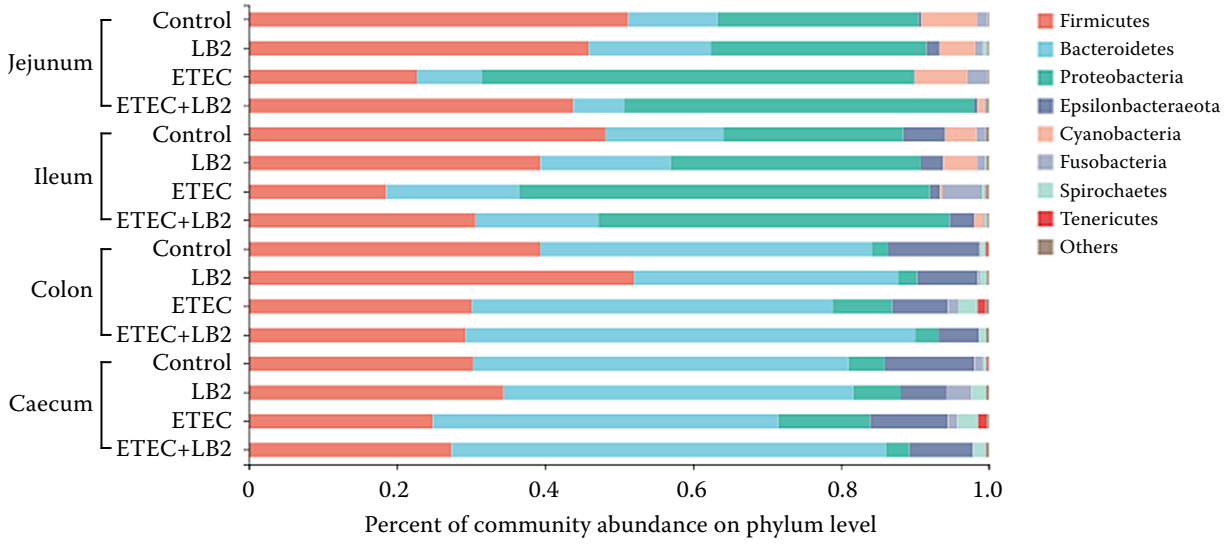

Figure 2. Relative abundance of sequences at the phylum level (%)
ETEC = enterotoxigenic *Escherichia coli*; LB2 = *Lactobacillus zeae* LB2
Only sequences with relative abundance over 0.1% are presented

Lactobacillus, *Roseburia*, were the dominant genera (Figure 3C). In the caecum, *Prevotella*, *Campylobacter*, *Alloprevotella*, *Prevotellaceae*, *Eschericha-Shigella*, *Prevotella*-9, *Roseburia*, *Prevotellaceae* NK3B31-group, and *Lachnospiraceae*, were the main genera (Figure 3D). Compared with the control group, ETEC

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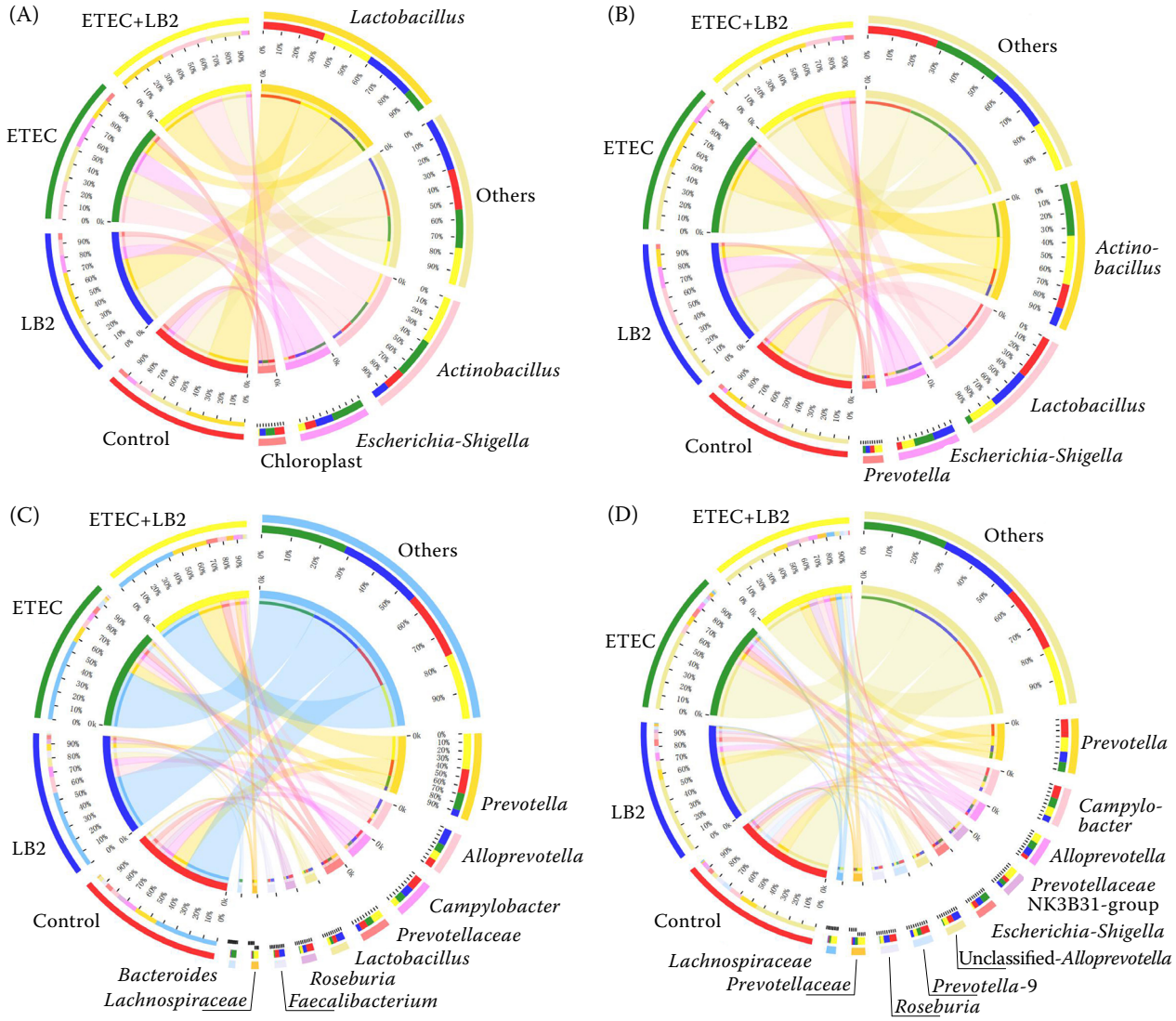

Figure 3. Relative abundance of operational taxonomic units at the genus level (%)
ETEC = enterotoxigenic *Escherichia coli*; LB2 = *Lactobacillus zeae* LB2
(A) Jejunum, (B) ileum, (C) colon, (D) caecum. Only operational taxonomic units with relative abundance over 3% are presented

infection decreased the abundance of *Lactobacillus*, while it was increased with LB2 supplementation in the jejunum and ileum. Likewise, LB2 supplementation increased the abundance of *Prevotella* in the ileum, colon and caecum, which was decreased by ETEC infection. Compared with the control group, the abundance of *Escherichia-Shigella* was increased in the ETEC group, while it was decreased in the ETEC+LB2 group.

Comparison of OTU abundance

The comparisons of OTU abundance between all groups are presented in Table 3. In the jejunum, compared with the control group, the ETEC group significantly decreased the abundance of *Lactobacillus*, *Porphyromonas*, and *Prevotella* ($P < 0.05$), the amounts of *Flavobacterium*, *Alloprevotella*, and *Sutterella* were decreased as well with no statistical difference. Compared with the ETEC group, the ETEC+LB2 group decreased the abundance of *Porphyromonas* and *Alloprevotella* ($P < 0.05$). In the ileum, compared with the control group, the ETEC group decreased the abundance of *Prevotella* and increased the abundance of *Porphyromonas* ($P < 0.05$); compared with the ETEC group, the ETEC+LB2 group decreased the abundance of *Prevotella* and *Porphyromonas* ($P < 0.05$). In the colon, compared with the control group, the LB2 group decreased

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Table 3. Comparison of operational taxonomic unit abundance (%)

Taxonomy	Control	LB2	ETEC	ETEC+LB2	*P*-value
Jejunum					
o_Lactobacillales; f_*Lactobacillaceae*; g_*Lactobacillus*	2.59 ± 1.59^{a}	2.81 ± 1.63^{a}	0.28 ± 0.42^{b}	1.32 ± 1.74^{ab}	0.002
o_Bacteroidales; f_*Porphyromonadaceae*; g_*Porphyromonas*	0.48 ± 0.40^{a}	0.43 ± 0.33^{a}	0.11 ± 0.18^{b}	0.01 ± 0.02^{c}	0.005
o_Flavobacteriales; f_*Flavobacteriaceae*; g_*Flavobacterium*	0.14 ± 0.12^{ab}	0.37 ± 0.32^{a}	0.01 ± 0.02^{b}	0.05 ± 0.11^{b}	0.017
o_Bacteroidales; f_*Prevotellaceae*; g_*Alloprevotella*	1.76 ± 1.66^{ab}	2.31 ± 2.40^{a}	0.69 ± 1.27^{b}	0.03 ± 0.07^{c}	0.019
o_Bacteroidales; f_*Prevotellaceae*; g_*Prevotella*	0.50 ± 0.44^{a}	0.43 ± 0.46^{a}	0.05 ± 0.12^{b}	0.05 ± 0.08^{b}	0.035
o_Betaproteobacteriales; f_*Burkholderiaceae*; g_*Sutterella*	0.02 ± 0.03^{ab}	0.03 ± 0.03^{a}	0.003 ± 0.004^{b}	0.006 ± 0.01^{b}	0.039
Ileum					
o_Bacteroidales; f_*Prevotellaceae*; g_*Prevotella*	0.95 ± 1.00^{a}	0.69 ± 0.46^{ab}	0.38 ± 0.45^{b}	0.05 ± 0.04^{c}	0.021
o_Bacteroidales; f_*Porphyromonadaceae*; g_*Porphyromonas*	0.20 ± 0.13^{bc}	0.49 ± 0.45^{b}	1.01 ± 1.12^{a}	0.04 ± 0.05^{c}	0.023
Colon					
o_Bacteroidales; f_*Prevotellaceae*; g_*Prevotella*	3.95 ± 3.41^{b}	1.11 ± 0.69^{c}	3.29 ± 2.75^{b}	13.82 ± 8.23^{a}	0.004
o_Pasteurellales; f_*Pasteurellaceae*; g_*Actinobacillus*	0.01 ± 0.01^{b}	0.01 ± 0.01^{b}	0.08 ± 0.05^{a}	0.01 ± 0.01^{b}	0.019
o_Clostridiales; f_*Ruminococcaceae*; g_*Ruminococcaceae*	0.41 ± 0.44^{a}	0.07 ± 0.07^{c}	0.17 ± 0.11^{b}	0.02 ± 0.06^{c}	0.025
Caecum					
o_Bacteroidales; f_*Prevotellaceae*; g_*Prevotella*	6.16 ± 5.95^{ab}	3.27 ± 2.67^{b}	2.76 ± 1.82^{b}	10.73 ± 5.52^{a}	0.014
o_Bacteroidales; f_*Prevotellaceae*; g_unclassified	1.01 ± 0.95^{a}	1.26 ± 0.55^{a}	0.36 ± 0.38^{b}	0.57 ± 0.75^{b}	0.022
o_Clostridiales; f_unclassified;	0.35 ± 0.42^{ab}	0.64 ± 0.44^{a}	0.43 ± 0.39^{ab}	0.09 ± 0.23^{b}	0.048

ETEC = enterotoxigenic *Escherichia coli*; LB2 = *Lactobacillus zeae* LB2

^{a,b,c}Values with different letters are significantly different ($P < 0.05$)

the abundance of *Prevotella* and *Ruminococcaceae*, and the ETEC group increased the abundance of *Actinobacillus* and decreased the abundance of *Ruminococcaceae* ($P < 0.05$); compared with the ETEC group, the ETEC+LB2 group increased the abundance of *Prevotella* and decreased the abundance of *Actinobacillus* and *Ruminococcaceae* ($P < 0.05$). In the caecum, compared with the control group, the ETEC and the ETEC+LB2 groups decreased the abundance of f_*Prevotellaceae*; g_unclassified (an unclassified genus that belongs to the family *Prevotellaceae*) ($P < 0.05$).

Functional potential of OTUs

Functional potential of the microbiome was evaluated by KEGG pathway enrichment, significant differences were observed for a total of 40 functional pathways between groups, inclusive of functional sets associated with cellular processes, environmental information processing, genetic information processing, diseases, metabolism, and organismal systems (Figure 4). Profound changes were observed for the functional pathways in the ileum and colon. Briefly, compared to the control group, the ETEC group increased the abundance of 38 pathways in the ileum; compared to the LB2 group, the ETEC group increased the abundance of 39 pathways in the colon; compared to the ETEC group, the ETEC+LB2 group increased the abundance of 32 pathways in the ileum and decreased the abundance of 39 pathways in the colon ($P < 0.05$).

DISCUSSION

Diarrhoea is associated with dysbiosis of the gut microbiota (Pop et al. 2014). As one of the most common causes of diarrhoea in piglets, ETEC thereby plays an important role in the dysbiosis of porcine microbiota (Bin et al. 2018). Several studies have revealed the dysbiosis of porcine gut microbiome induced by ETEC infection (Pop et al. 2016; Bin et al. 2018; Chen et al. 2020; Zhao et al. 2020), which was associated with the higher proportion of pathogenic bacteria (e.g., *Shigella*, *Clostridium*, and *Enterococcus*) and the lower proportion of short-chain fatty acid (SCFA)-

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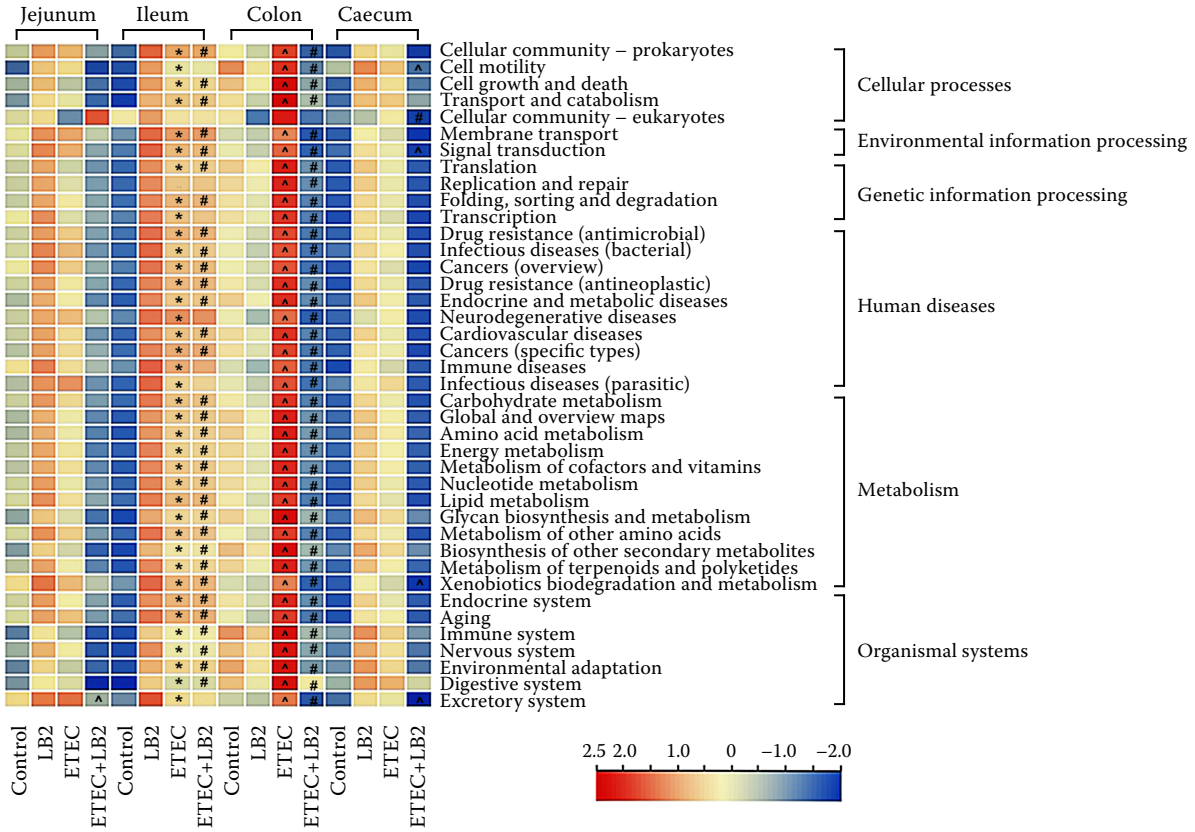

Figure 4. KEGG pathway enrichment of the gut microbiome in piglets

ETEC = enterotoxigenic *Escherichia coli*; LB2 = *Lactobacillus zeae* LB2

*Significant difference compared to the control group ($P < 0.05$); ^significant difference compared to the LB2 group ($P < 0.05$); #significant difference compared to the ETEC group ($P < 0.05$)

Different colours represent the values of log-transformed abundances of KEGG pathways

producing bacteria (e.g., *Bacteroides*, *Prevotella*, and *Butyricicoccus*), as well as a lower microbiota diversity. However, few studies investigated the impact of the potential anti-ETEC substance on the gut microbiota in piglets. *L. zeae* has been proven to be one possible candidate for the prevention and treatment of (ETEC-induced) post-weaning diarrhoea as well as related intestinal disorder (Wu et al. 2021b). The present study evaluated the effect of *L. zeae* on the gut microbiome in healthy and ETEC-infected piglets.

Dissimilar bacterial community was observed in this study, as illustrated by the significant results in the alpha diversity, indicating that the microbiota community was remarkably influenced by the ETEC infection and LB2 supplementation. Interestingly, an increased Chao1 alpha diversity was observed in the colon in the ETEC-infected piglets, which is contrary to the previous studies (Bin et al. 2018; Zhao et al. 2020), when a lower microbiota diversity was observed in the jejunum and faeces. Moreover, the ETEC+LB2 group decreased Shannon and Chao1 indices in the colon. In fact, a lower diversity of the bacterial community is often associated with microbiota dysbiosis (Mosca et al. 2016). These results might be due to the massive invasion of ETEC in the intestine, future study is warranted to explain the mechanism and the implication of this increased alpha diversity during ETEC infection. Nevertheless, the ETEC+LB2 group also increased the Chao1 index in the jejunum, implying a positive effect of *L. zeae* on the jejunal microbiota.

The present study found remarkable shifts in the structure of the gut microbiota by ETEC infection and LB2 supplementation. The ETEC-infected piglets displayed a higher proportion of Proteobacteria in the jejunum, ileum, and colon than the healthy piglets, while the healthy piglets showed a higher proportion of Firmicutes and Bacteroidetes in the jejunum and higher Firmicutes in the ileum.

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The phylum Bacteroidetes is important for carbohydrate fermentation, polysaccharide catabolism, and amino acid utilisation (Sheridan et al. 2016). The phylum Firmicutes, which ferments plant polysaccharides to produce SCFAs, has been shown to be involved in energy resorption (Ley et al. 2006). Many studies suggested that Proteobacteria are associated with imbalance of the microbiota, inflammation, and various clinical anaerobic infections (Swidsinski et al. 2011). These results demonstrated that ETEC infection induced severe variations in the microbiota composition in the piglets, which could lead to the microbiota dysbiosis and affect the host metabolism. However, the LB2 group showed a higher proportion of Firmicutes and a lower proportion of Epsilonbacteraeota in the colon. Firmicutes are very important for metabolism as described above. Epsilonbacteraeota have been reported to be increased in mice with dextran sulphate sodium-induced colitis, which may be associated with inflammatory bowel disease and related intestinal disorder (Wang et al. 2020). These results suggested that LB2 supplementation plays a positive role in regulating metabolism and microbiome dysbiosis.

Several distinct markers of the microbiome profile were observed between the healthy and the ETEC-infected piglets. Particularly, ETEC infection was associated with the increase in the abundance of *Actinobacillus*, and the decrease in the abundance of *Lactobacillus*, *Flavobacterium*, *Alloprevotella*, *Prevotella*, *Sutterella*, and *Ruminococcaceae*. Previous studies have demonstrated that increased *Actinobacillus* was found in human patients with primary biliary cirrhosis and intestinal inflammation (Lv et al. 2016; Bercier et al. 2019). The increased abundance of *Actinobacillus* implied that ETEC infection could affect the microbiota composition and may be involved in the intestinal inflammation or even cancer. *Lactobacillus* has been highlighted as a beneficial gut bacterium, playing a very important role in nutrition and immune system (Lyu et al. 2020b). *Flavobacterium* is a well-known pathogen in fish, while its role in mammals is rather unexplored (Bernardet and Bowman 2006). *Alloprevotella*, *Prevotella*, *Sutterella*, and *Ruminococcaceae* help the host degrade diverse plant polysaccharides and produce SCFAs (Flint et al. 2012; Boutard et al. 2014; Wu et al. 2018b; Smith et al. 2020), can provide a higher energy harvesting and an adequate prevention strategy for pathogen infection (Dou et al. 2017). The decreased abundance in these bacteria indicated that ETEC infection induced microbiota dysbiosis and affected immune and metabolic functions.

In this study, minimal change in the microbiome profile was observed for the LB2 supplementation in the healthy piglets. However, marked shifts by LB2 were found in the ETEC-infected piglets. Interestingly, LB2 supplementation in ETEC-infected piglets was associated with the decrease in the abundance of *Porphyromonas*, *Alloprevotella*, and *Ruminococcacea*. Elevated *Porphyromonas* was significantly associated with intestinal inflammation and colorectal cancer (Rezasoltani et al. 2018), while the decrease of *Porphyromonas* in the ETEC+LB2 group implied that LB2 supplementation may play a positive role in the dysbiosis during ETEC infection. Nevertheless, as described above, decreased *Alloprevotella*, and *Ruminococcaceae* are associated with decline in carbohydrate metabolism. Future study is needed to further investigate the structural and functional changes in the microbiome under *L. zeae* supplementation and ETEC infection.

One previous study has revealed changes in the functional potential of microbiome in relation to ETEC infection (Bin et al. 2018); the six most abundant changed functional categories were cell motility, biosynthesis of other secondary metabolites, excretory system, immune system diseases, immune system and circulatory system. Interestingly, the present study demonstrated a profound impact of LB2 supplementation in the piglets during ETEC infection. Particularly, in the ETEC-infected piglets, LB2 supplementation increased the abundance of 32 pathways in the ileum and decreased the abundance of 39 pathways in the colon, associated with cellular processes, environmental information processing, genetic information processing, diseases, metabolism, and organismal systems. These results reflected a remarkable anti-ETEC effect of *L. zeae*, and the ileum and colon might be the most active site of *L. zeae* against ETEC infection. Nevertheless, PICRUSt inferences depend on microbial gene annotations which are occasionally inaccurate (Langille 2018), and these PICRUSt derived conclusions should be treated as hypotheses. Future studies are needed to in-depth unravel the functional changes

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by ETEC infection and *L. zeae* supplementation using combinatorial tools like metabolomics and metatranscriptomics.

In conclusion, this study evaluated the effects of dietary *L. zeae* LB2 supplementation on the gut microbiota in healthy and ETEC-infected piglets. ETEC infection caused severe dysbiosis of the gut microbiome, as indicated by the dissimilar bacterial community (alpha diversity), the altered composition at the phylum level (higher Proteobacteria, and lower Firmicutes and Bacteroidetes) and at the genus level (increased *Actinobacillus*; decreased *Prevotellaceae*, *Ruminococcaceae*, *Lactobacillus*, *Flavobacterium*, *Alloprevotella*, and *Sutterella*). The supplementation of LB2 played a positive effect on regulating the gut microbiome during ETEC infection, as demonstrated by the community variation (alpha diversity), composition shifts at the phylum level (higher Firmicutes and Bacteroidetes and lower Proteobacteria) and at the genus level (decreased *Porphyromonas*, *Alloprevotella*, *Ruminococcaceae*, and *Actinobacillus*, increased *Prevotella* and *Lactobacillus*). Moreover, both ETEC infection and LB2 supplementation remarkably influenced the functional potentials of the microbiome, which was associated with several functional pathways in relation to cellular processes, environmental information processing, genetic information processing, diseases, metabolism, and organismal systems. Therefore, regulating the gut microbiome by probiotics could be taken as a promising candidate for the prevention or treatment of ETEC infection, and *L. zeae* could be used as such.

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Conflict of interest

The authors declare no conflict of interest.

REFERENCES

Bercier P, Gottschalk M, Grenier D. Effects of Actinobacillus pleuropneumoniae on barrier function and inflammatory response of pig tracheal epithelial cells. Pathog Dis. 2019 Feb;77(1): 7 p.

Bernardet JF, Bowman JP. The genus flavobacterium. Prokaryotes. 2006;7:481-531.

Bin P, Tang Z, Liu S, Chen S, Xia Y, Liu J, Wu H, Zhu G. Intestinal microbiota mediates Enterotoxigenic Escherichia coli-induced diarrhea in piglets. BMC Vet Res. 2018 Dec 5;14(1): 13 p.

Boutard M, Cerisy T, Nogue PY, Alberti A, Weissenbach J, Salanoubat M, Tolonen AC. Functional diversity of carbohydrate-active enzymes enabling a bacterium to ferment plant biomass. PLoS Genet. 2014 Nov 13;10(11): 12 p.

Chen S, Wu X, Xia Y, Wang M, Liao S, Li F, Yin J, Ren W, Tan B, Yin Y. Effects of dietary gamma-aminobutyric acid supplementation on amino acid profile, intestinal immunity, and microbiota in ETEC-challenged piglets. Food Funct. 2020;11(10):9067-74.

Dou S, Gadonna-Widehem P, Rome V, Hamoudi D, Rhazi L, Lakhal L, Larcher T, Bahi-Jaber N, Pinon-Quintana A, Guyonvarch A, Huerou-Luron IL. Characterisation of early-life fecal microbiota in susceptible and healthy pigs to post-weaning diarrhoea. PLoS One. 2017 Jan 10;12(1): 20 p.

Flint HJ, Scott KP, Duncan SH, Louis P, Forano E. Microbial degradation of complex carbohydrates in the gut. Gut Microbes. 2012 Jul 14;3(4):289-306.

Fu S, Zhuang F, Guo L, Qiu Y, Xiong J, Ye C, Liu Y, Wu Z, Hou Y, Hu CA. Effect of baicalin-aluminum complexes on fecal microbiome in piglets. Int J Mol Sci. 2019 May 14;20(10): 15 p.

Harris K, Kassis A, Major G, Chou CJ. Is the gut microbiota a new factor contributing to obesity and its metabolic disorders? J Obes. 2012 Jan 24;2012: 14 p.

Heo JM, Opapeju FO, Pluske JR, Kim JC, Hampson DJ, Nyachoti CM. Gastrointestinal health and function in weaned pigs: A review of feeding strategies to control post-weaning diarrhoea without using in-feed antimicrobial compounds. J Anim Phys Anim Nutr. 2013 Apr; 97(2):207-37.

Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, Morelli L, Canani RB, Flint HJ, Salminen S, Calder PC. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat Rev Gastroenterol Hepatol. 2014 Jun 10;11(8):506-14.

Hu YJ, Cowling BJ. Reducing antibiotic use in livestock, China. Bull World Health Organ. 2020 May 1;98(5):360-1.

Huang MZ, Wang SY, Wang H, Cui DA, Yang YJ, Liu XW, Kong XJ, Li JY. Differences in the intestinal microbiota between uninfected piglets and piglets infected with porcine epidemic diarrhea virus. PLoS One. 2018 Feb 15; 13(2): 13 p.

https://doi.org/10.17221/136/2021-CJAS

Langille MGI. Exploring linkages between taxonomic and functional profiles of the human microbiome. mSystems. 2018 Mar 27;3(2): 4 p.

Leser TD, Molbak L. Better living through microbial action: The benefits of the mammalian gastrointestinal microbiota on the host. Environ Microbiol. 2009 Sep;11 (9):2194-206.

Ley RE, Turnbaugh PJ, Klein S, Gordon JI. Human gut microbes associated with obesity. Nature. 2006 Dec;444 (7122):1022-3.

Lv LX, Fang DQ, Shi D, Chen DY, Yan R, Zhu YX, Chen YF, Shao L, Guo FF, Wu WR, Li A. Alterations and correlations of the gut microbiome, metabolism and immunity in patients with primary biliary cirrhosis. Environ Microbiol. 2016 Jul;18(7):2272-86.

Lv Y, Li X, Zhang L, Shi Y, Du L, Ding B, Hou Y, Gong J, Wu T. Injury and mechanism of recombinant E. coli expressing STa on piglets colon. J Vet Med Sci. 2018; 80(2):205-12.

Lyu Y, Debevere S, Bourgeois H, Ran M, Broeckx BJ, Vanhaecke L, Hesta M. Dose-dependent effects of dietary xylooligosaccharides supplementation on microbiota, fermentation and metabolism in healthy adult cats. Molecules. 2020a Oct 29;25(21): 14 p.

Lyu Y, Su C, Verbrugghe A, Van de Wiele T, Martinez-Caja AM, Hesta M. Past, present, and future of gastrointestinal microbiota research in cats. Front Microbiol. 2020b Jul 24;11: 15 p.

Mon KK, Saelao P, Halstead MM, Chanthavixay G, Chang HC, Garas L, Maga EA, Zhou H. Salmonella enterica serovars Enteritidis infection alters the indigenous microbiota diversity in young layer chicks. Front Vet Sci. 2015 Nov 23;2: 16 p.

Mondot S, de Wouters T, Dore J, Lepage P. The human gut microbiome and its dysfunctions. Dig Dis. 2013;31 (3-4):278-85.

Mosca A, Leclerc M, Hugot JP. Gut microbiota diversity and human diseases: Should we reintroduce key predators in our ecosystem? Front Microbiol. 2016 Mar 31;7: 12 p.

Nagy B, Fekete PZ. Enterotoxigenic Escherichia coli in veterinary medicine. Int J Med Microbiol. 2005 Oct 5;295 (6-7):443-54.

Ni Y, Yang X, Zheng L, Wang Z, Wu L, Jiang J, Yang T, Ma L, Fu Z. Lactobacillus and Bifidobacterium improves physiological function and cognitive ability in aged mice by the regulation of gut microbiota. Mol Nutr Food Res. 2019 Aug 21;63(22).

Park S, Ji Y, Jung HY, Park H, Kang J, Choi SH, Shin H, Hyun CK, Kim KT, Holzapfel WH. Lactobacillus plantarum HAC01 regulates gut microbiota and adipose tissue accumulation in a diet-induced obesity murine model. Appl Microbiol Biotechnol. 2017 Feb 1;101(4):1605-14.

Pop M, Walker AW, Paulson J, Lindsay B, Antonio M, Hossain MA, Oundo J, Tamboura B, Mai V, Astrovskaya I, Bravo HC. Diarrhea in young children from low-income countries leads to large-scale alterations in intestinal microbiota composition. Genome Biol. 2014 Jun 27;15(6): 12 p.

Pop M, Paulson JN, Chakraborty S, Astrovskaya I, Lindsay BR, Li S, Bravo HC, Harro C, Parkhill J, Walker AW, Walker RI. Individual-specific changes in the human gut microbiota after challenge with enterotoxigenic Escherichia coli and subsequent ciprofloxacin treatment. BMC Genom. 2016 Jun 8;17(1): 11 p.

Rezasoltani S, Aghdaei HA, Dabiri H, Sepahi AA, Modarressi MH, Mojarad EN. The association between fecal microbiota and different types of colorectal polyp as precursors of colorectal cancer. Microb Pathog. 2018 Nov 1; 124:244-9.

Sheridan PO, Martin JC, Lawley TD, Browne HP, Harris HM, Bernalier-Donadille A, Duncan SH, O'Toole PW, Scott KP, Flint HJ. Polysaccharide utilization loci and nutritional specialization in a dominant group of butyrate-producing human colonic Firmicutes. Microb Genom. 2016 Feb;2(2): 16 p.

Silva DR, Sardi JDCO, de Souza Pitangui N, Roque SM, da Silva ACB, Rosalen PL. Probiotics as an alternative antimicrobial therapy: Current reality and future directions. J Funct Foods. 2020 Oct 1;73: 12 p.

Smith BJ, Miller RA, Schmidt TM. Muribaculaceae genomes assembled from metagenomes suggest genetic drivers of differential response to acarbose treatment in mice. BioRxiv. 2020 Jan 1: 28 p.

Sornplang P, Piyadeatsoontorn S. Probiotic isolates from unconventional sources: A review. J Anim Sci Technol. 2016 Dec;58(1): 11 p.

Swidsinski A, Dorffel Y, Loening-Baucke V, Theissig F, Ruckert JC, Ismail MV, Rau WA, Gaschler D, Weizenegger M, Kuhn S, Schilling J. Acute appendicitis is characterised by local invasion with Fusobacterium nucleatum/necrophorum. Gut. 2011 Jan 1;60(1):34-40.

Wang L, Zhou J, Hou Y, Yi D, Ding B, Xie J, Zhang Y, Chen H, Wu T, Zhao D, Hu CA. N-Acetylcysteine supplementation alleviates intestinal injury in piglets infected by porcine epidemic diarrhea virus. Amino Acids. 2017 Mar 3; 49(12):1931-43.

Wang J, Zhu G, Sun C, Xiong K, Yao T, Su Y, Fang H. TAK-242 ameliorates DSS-induced colitis by regulating the gut microbiota and the JAK2/STAT3 signaling pathway. Microb Cell Factories. 2020 Aug 6;19(1): 17 p.

Wu T, Lv Y, Li X, Zhao D, Yi D, Wang L, Ding B, Chen H, Hou Y. Establishment of a recombinant Escherichia coli-

https://doi.org/10.17221/136/2021-CJAS

induced piglet diarrhea model. J Anim Sci. 2018a Dec 7; 96(Suppl 3):1517-34.

Wu T, Zhang Y, Lv Y, Li P, Yi D, Wang L, Zhao D, Chen H, Gong J, Hou Y. Beneficial impact and molecular mechanism of Bacillus coagulans on piglets' intestine. Int J Mol Sci. 2018b Jul 18;19(7): 17 p.

Wu T, Lyu Y, Li X, Wu M, Yu K, Li S, Ji C, Zhang Q, Zhang Y, Zhao D, Yi D. Impact of N-acetylcysteine on the gut microbiota in the piglets infected with porcine epidemic diarrhea virus. Front Vet Sci. 2021a Jan 12;7: 10 p.

Wu T, Shi Y, Zhang Y, Zhang M, Zhang L, Ma Z, Zhao D, Wang L, Yu H, Hou Y, Gong J. Lactobacillus rhamnosus LB1 alleviates Enterotoxigenic Escherichia coli-induced adverse effects in piglets by improving host immune response and anti-oxidation stress and restoring intestinal integrity. Front Cell Infect Microbiol. 2021b Nov 2;11: 13 p.

Xu X, Pan Y, Xu B, Yan Y, Yin B, Wang Y, Hu S, Ma L. Effects of Cortex phellodendri extract on post-weaning piglets diarrhoea. Vet Med Sci. 2020 Jun 25;6(4):901-9.

Yan F, Li N, Shi J, Li H, Yue Y, Jiao W, Wang N, Song Y, Huo G, Li B. Lactobacillus acidophilus alleviates type 2 diabetes by regulating hepatic glucose, lipid metabolism and gut microbiota in mice. Food Funct. 2019;10(9):5804-15.

Zhao Y, Wang J, Wang H, Huang Y, Qi M, Liao S, Bin P, Y. Effects of GABA supplementation on intestinal SIgA secretion and gut microbiota in the healthy and ETEC-infected weanling piglets. Mediat Inflamm. 2020 May 26;2020: 17 p.

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