Animal Nutrition 17 (2024) 110–122

Contents lists available at ScienceDirect

Animal Nutrition

journal homepage: http://www.keaipublishing.com/en/journals/aninu/

Original Research Article

Next-generation probiotic candidates targeting intestinal health in weaned piglets: Both live and heat-killed *Akkermansia muciniphila* prevent pathological changes induced by enterotoxigenic *Escherichia coli* in the gut

Cong Lan [†], Hua Li [†], Yuqing Shen, Yang Liu, Aimin Wu, Jun He, Jingyi Cai, Gang Tian, Xiangbing Mao, Zhiqing Huang, Bing Yu, Ping Zheng, Jie Yu, Junqiu Luo, Hui Yan, Yuheng Luo [*]

Key Laboratory for Animal Disease-Resistance Nutrition of Ministry of Education of China, Key Laboratory for Animal Disease-Resistance Nutrition and Feed of Ministry of Agriculture of China, Key Laboratory of Animal Disease-Resistant Nutrition of Sichuan Province, Engineering Research Center of Animal Disease-Resistance Nutrition Biotechnology of Ministry of Education of China, Animal Nutrition Institute, Sichuan Agricultural University, Chengdu 611100, China

ARTICLE INFO

Article history:
Received 25 September 2023
Received in revised form
3 January 2024
Accepted 23 January 2024
Available online 6 March 2024

Keywords:
Akkermansia muciniphila
Weaned piglet
Enterotoxigenic *Escherichia coli* challenge
Intestinal health

ABSTRACT

The use of next-generation probiotics (NGP) in pigs for combating diseases has been subject to limited research. Here we explored the potential of a well-known NGP candidate *Akkermansia muciniphila* targeting pig gut health. In the first screening experiment, we found that the abundance of *A. muciniphila* peaked at 14 d old but decreased at weaning (21 d old; $P < 0.05$), suggesting the weaning period may be an effective window for *A. muciniphila* intervention. Following that, 48 crossbred weaned pigs at 28 d old were randomly assigned to five groups: control (CON), high/low live *A. muciniphila* (HA/LA), and high/low heat-killed *A. muciniphila* (HIA/LIA). From 1 to 28 d old, the CON group received gastric infusion of anaerobic sterile saline every other day; the HA and LA groups were gavaged every other day with 1×10^{10} CFU/5 mL and 5×10^{8} CFU/5 mL live *A. muciniphila*, respectively; and the HIA and LIA groups were gavaged every other day with 1×10^{10} CFU/5 mL and 5×10^{8} CFU/5 mL heat-killed *A. muciniphila*, respectively. At d 29, pigs in the CON group were randomly and equally divided into two groups, one of which was named the enterotoxigenic *Escherichia coli* (ETEC) group, and all groups except CON received a 5-d ETEC challenge. The supplementation of *A. muciniphila* numerically reduced the diarrhea rate of weaned pigs compared to the pigs that only received the ETEC challenge ($P = 0.57$), but the LIA group had a higher diarrhea rate than the CON group ($P < 0.05$). Consistent with this, the supplementation of *A. muciniphila* improved the small intestinal morphology and structure, proportion of $CD4^+$ T lymphocytes in the blood, as well as the expression of genes related to intestinal barrier and antioxidant indices of pigs with ETEC challenge, especially for the LA group ($P < 0.05$). Meanwhile, *A. muciniphila* supplementation reduced the expression of ETEC virulence factor genes in the ileum and colon of pigs challenged by ETEC ($P < 0.05$). Therefore, *A. muciniphila* may protect the intestinal health of weaned piglets from damage caused by ETEC infection, but the effect may vary depending on the concentration and activity of *A. muciniphila*.
© 2024 The Authors. Publishing services by Elsevier B.V. on behalf of KeAi Communications Co. Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

* Corresponding author.
E-mail address: yhluo@sicau.edu.cn (Y. Luo).
[†] Both authors contributed equally to this work.
Peer review under responsibility of Chinese Association of Animal Science and Veterinary Medicine.

Production and Hosting by Elsevier on behalf of KeAi

1. Introduction

In the piglet rearing process after weaning, diarrhea has long been a prominent health challenge. This condition not only impairs the economic performance of the livestock industry but also exerts significant impacts on animal welfare and food safety. As a result, the search for effective diarrhea management strategies has

https://doi.org/10.1016/j.aninu.2024.01.007
2405-6545/© 2024 The Authors. Publishing services by Elsevier B.V. on behalf of KeAi Communications Co. Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

remained a crucial imperative within the livestock sector (Guevarra et al., 2019; Heo et al., 2013). In this context, our research has delved into the realm of a microorganism that has been subject to intense scrutiny—*Akkermansia muciniphila*—to explore its potential utility in alleviating the issue of diarrhea in piglets.

In the domain of mucin-utilizing microorganisms (Derrien et al., 2004), *A. muciniphila* has been heralded as a "next-generation probiotic" that plays a crucial role in maintaining intestinal homeostasis (Luo et al., 2022a). In patients suffering from intestinal inflammation, such as those with inflammatory bowel disease (IBD), the population of *A. muciniphila* in the colon has been observed to be significantly lower compared to healthy individuals (Earley et al., 2019; Vigsnæs et al., 2012; Zhang et al., 2020), potentially serving as a diagnostic biomarker for intestinal inflammation. Consequently, numerous studies have incorporated *A. muciniphila* into animal models with gut health compromise, including mice with dextran sodium sulfate (DSS)-induced colitis, obese mice, and *Salmonella pullorum*-infected chickens, to explore its impact on intestinal health. It has been shown that oral administration of live *A. muciniphila* enhances the expression of tight junction proteins such as zonula occludens-1 (ZO-1) and occludin in mice with DSS-induced colitis (Bian et al., 2019). Furthermore, *A. muciniphila* has been found to increase the number of goblet cells and upregulate the expression of mucin 2 (*MUC*2) and trefoil factor 2 in *S. pullorum*-infected chickens (Zhu et al., 2020). Additionally, it has been shown that both the membrane proteins and secreted extracellular vesicles of *A. muciniphila* can improve intestinal barrier function in obese mice (Plovier et al., 2017), as well as enhance the expression of tight junction proteins in lipopolysaccharide (LPS)-induced Caco-2 cells (Chelakkot et al., 2018). Interestingly, these beneficial effects persist even in the presence of non-viable *A. muciniphila* (Wang et al., 2020), indicating the potential application of *A. muciniphila* as a postbiotic. Furthermore, research has revealed that *A. muciniphila* can enhance the host's antioxidant capacity, thereby improving overall metabolic processes (Ma et al., 2023; Zhao et al., 2017). However, research and understanding of *A. muciniphila* in post-weaning piglets remain notably limited, and its role as a mucin-degrading microorganism in relation to piglet diarrhea demands in-depth investigation.

This study began by initially quantifying the population of *A. muciniphila* in fecal samples from post-weaning piglets at different ages, hypothesizing that its abundance may undergo significant changes during this period. This can provide evidence for whether the weaning period can serve as a window for *A. muciniphila* treatment. Subsequently, we gavaged post-weaning piglets with varying doses of both live and heat-killed *A. muciniphila*, aiming to explore its impact on piglet growth performance and incidence of diarrhea. Additionally, we employed an enterotoxigenic *Escherichia coli* (ETEC) infection model to investigate whether oral administration of *A. muciniphila* protects against piglet diarrhea and to elucidate the potential underlying mechanisms involved. This research effort aims to provide novel nutritional strategies and establish a solid theoretical foundation for addressing piglet diarrhea, thereby contributing to the advancement of swine husbandry practices.

2. Materials and methods

2.1. Animal ethics statement

The present research was conducted in accordance with the rules for the care of laboratory animals (2017 Revision) formulated by the State Council of China, and the protocol was approved by the Animal Care and Use Committee of Sichuan Agricultural University, Chengdu, China (approval no. SYXK-Chuan-2014-184). All animal experiments were carried out in accordance with the ARRIVE guidelines.

2.2. Collection of fecal samples from piglets of different ages

Fecal samples were collected from pigs at 7, 14, 21, 39 and 57 d old in a commercial pig farm (Kunming, Yunnan, China). The piglets were weaned at 21 d old. After collection, the samples were promptly snap-frozen in liquid nitrogen and stored at −80 °C for subsequent determination of the abundance of *A. muciniphila*. Information regarding the number and gender of pigs at different ages is provided in Table S1.

2.3. Animals and experimental design

A total of 48 crossbred (Duroc × Landrace × Yorkshire) weaned piglets at 28 d old with an average initial body weight of 6.41 ± 0.16 kg were randomly assigned to five groups, each consisting of 16 or 8 replicates with 1 piglet per replicate. The groups were named as control (CON, $n = 16$), high level of *A. muciniphila* (HA, $n = 8$), low level of *A. muciniphila* (LA, $n = 8$), high level of inactivated *A. muciniphila* (HIA, $n = 8$), and low level of inactivated *A. muciniphila* (LIA, $n = 8$) groups. In the first phase spanning 28 d, the CON group received gastric infusion of 5 mL of anaerobic sterile saline every other day at 08:00, and the HA and LA groups were gavaged every other day with 1×10^{10} CFU/5 mL and 5×10^{8} CFU/5 mL live *A. muciniphila* (DSM 22959), respectively, and the HIA and LIA groups were gavaged every other day with 1×10^{10} CFU/5 mL and 5×10^{8} CFU/5 mL heat-killed *A. muciniphila*, respectively. To prepare a bacterial solution, a brain heart infusion (BHI) bacterial culture that had been cultivated for 20 to 24 h was centrifuged at 9000 × *g* for 5 min. Following that, the supernatant was carefully discarded, and the resultant pellet was resuspended in anaerobic physiological saline to achieve the desired bacterial concentration. All operations were carried out in an anaerobic glove box. Our research group modified the previous method (Luo et al., 2021b) to obtain heat-killed *A. muciniphila* by heating it in boiling water for 20 min. On the 29th day, pigs in the CON group were randomly and equally divided into two groups, one of which was named the ETEC group, and the other group was still named the CON group. The ETEC, HA, LA, HIA, and LIA groups received a daily gastric infusion of ETEC at 10 mL/kg BW at a concentration of 6×10^{9} CFU/mL (Serotype O149:K91:K88ac, China Institute of Veterinary Drug Control, China) for five consecutive days. Simultaneously, the CON group received a gastric infusion of sterile saline using the same method. The experimental design diagram is shown in Fig. 1.

2.4. Experimental diets and management

The basal diet (Table 1) was formulated following the recommendations by NRC (2012). Each piglet was individually housed in a metabolism cage measuring 1.5 m × 0.7 m × 1.0 m with free access to both water and feed. The housing conditions maintained a controlled temperature ranging from 26 to 28 °C. The individual piglet weights were recorded on d 0, 29 and 34 to calculate the average daily gain (ADG). Daily feed intake was carefully recorded to determine the average daily feed intake (ADFI). The feed-to-gain ratio (F/G) was calculated as the ADFI divided by the ADG. During the entire experimental period, fecal consistency was evaluated and categorized as follows: 0, normal; 1, soft; 2, semiliquid; and 3,

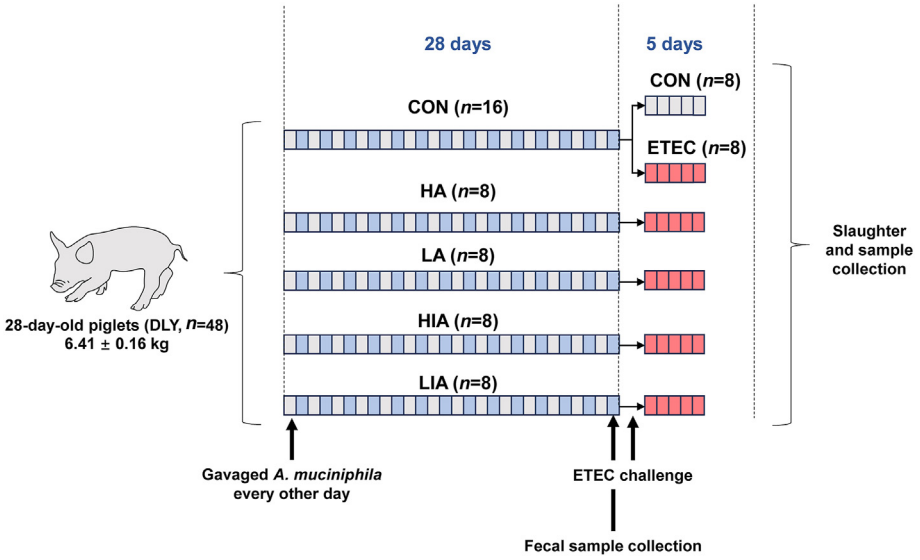

Fig. 1. Experimental design diagram. From 0 to 28 d, CON group received a standard diet and daily gavage of 5 mL of sterile saline; HA group received a standard diet and gavage of 1×10^{10} CFU/5 mL of live *A. muciniphila* every other day, LA group received a standard diet and gavage of 5×10^{8} CFU/5 mL of live *A. muciniphila* every other day, HIA group received a standard diet and gavage of 1×10^{10} CFU/5 mL of heat-killed *A. muciniphila* every other day, LIA group received a standard diet and gavage of 5×10^{8} CFU/5 mL of heat-killed *A. muciniphila* every other day. From 29 to 33 d, all groups except the CON group received a daily gastric infusion of ETEC (6×10^{9} CFU/mL) at 10 mL/kg of body weight, and the CON group received a gastric infusion of sterile saline using the same method. CON = control; HA = high level of *A. muciniphila*; LA = low level of *A.* muciniphila; HIA = high level of inactivated *A. muciniphila*; LIA = low level of inactivated *A. muciniphila*; ETEC = enterotoxigenic *Escherichia coli*.

liquid. Fecal consistency with a score greater or equal to 2 was considered indicative of diarrhea. The diarrhea rate was calculated as follows:

Diarrhea rate (%) = (total number of pigs per pen with diarrhea/ number of pigs per pen × experimental duration in days) × 100.

2.5. Chemical analyses

Analytical procedures for dry matter (DM), crude protein (CP), ether extract (EE), crude fiber (CF), total phosphorus (TP) and ash in feed were analyzed according to AOAC (2007). The determination of gross energy (GE) in feed was conducted using an automatic oxygen bomb calorimeter (Parr Instrument Co., Moline, IL). Amino acid concentrations of each dietary sample were analyzed using an AA analyser (L8800; Hitachi, Tokyo, Japan).

2.6. Sample collection and processing

On d 28 of the experiment, fecal samples were collected from piglets in each group. At the end of the experiment, the body weights of all piglets were measured accurately, and 20 mL of anterior vena cava blood were collected from each piglet. Half of the collected blood was used for serum separation, while the other half was utilized for the enumeration of T lymphocyte subsets (8 mL with 2 mL heparin sodium solution). Subsequently, each pig was anesthetized with pentobarbital sodium at a dose of 200 mg/kg BW and euthanized by exsanguination. Immediately thereafter, a 2-cm long segment from the middle portion of the jejunum, ileum, and colon was fixed in a 4% paraformaldehyde solution for histopathological analysis. The mucosa was scraped from a 20-cm long segment of each intestinal segment after rinsing with 0.9% cold physiological saline and was stored at −80 °C for gene expression and ELISA analysis. The digesta in the ileum and colon were collected and stored at −80 °C for subsequent microbial flora and short chain fatty acids (SCFA) analysis. Approximately 5 g of spleen tissue were also collected for the determination of T lymphocyte subsets.

2.7. Intestinal pathological observation

Each intestinal segment collected and preserved in a formaldehyde solution was prepared using a standard paraffin embedding protocol. The paraffin-embedded jejunum, ileum, and colon samples were sliced into 5 μm sections and stained with hematoxylin-eosin for histopathological evaluation. Digital images of intestinal pathological photomicrographs were captured using a digital camera coupled to a light microscope (Olympus CKX 53 microscope, Japan) at a magnification of 40×. To measure villus height (VH) and crypt depth (CD), a total of 10 intact fields were selected from each image (representing one section), and VH and CD were recorded for each of these fields. The average values for the 10 fields were calculated as the final data. The VH/CD ratio is referred to as the villus height to crypt depth ratio (VCR).

2.8. Serum and mucosal-related parameters assessment

The LPS concentration, diamine oxidase (DAO) activity and levels of cytokines including tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), interleukin-4 (IL-4), interleukin-10 (IL-10), and secretory immunoglobulin A (sIgA) in serum, as well as the concentration of sIgA in the jejunal and ileal mucosa were measured using the corresponding porcine enzyme-linked immunosorbent assay (ELISA) kits (Jiangsu Meimian Industrial Co., Ltd, Yancheng, Jiangsu, China) following the manufacturer's instructions. The activity of glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), catalase (CAT) and level of malondialdehyde (MDA) were measured using assay kits (Jiancheng, Nanjing, Jiangsu, China) following the manufacturer's instructions.

Table 1
Ingredients and nutrient levels of the basal diet (%, as-fed basis).

Item	Content
Ingredients	
Corn, CP 7.8%	32.30
Extruded corn, CP 7.8%	30.53
Soybean meal, CP 46.0%	7.35
Extruded soybean, CP 35.5%	8.60
Fish meal, CP 62.5%	4.00
Whey powder, CP 3.8%	5.00
Soybean protein concentrate, CP 65.0%	6.40
Soybean oil	1.62
Sucrose	2.00
Limestone	0.62
Dicalcium phosphate	0.46
NaCl	0.20
L-Lys·HCl (78%)	0.32
DL-Met (99%)	0.07
L-Thr (98.5%)	0.02
L-Trp (98.5%)	0.01
Chloride choline (50%)	0.15
Vitamin premix[1]	0.05
Mineral premix[2]	0.30
Total	100.00
Nutrients level[3]	
GE, Mcal/kg	4.05
EE	5.87
CP	18.59
CF	2.45
Ash	4.55
Lys	1.48
Met	0.45
Met + Cys	0.86
Thr	0.93
Trp	0.26
Ca	0.76
TP	0.62

GE = gross energy; EE = ether extract; CP = crude protein; CF = crude fiber; Lys = lysine, Met = methionine; Cys = cysteine; Thr = threonine; Trp = tryptophane; Ca = calcium; TP = total phosphorus.
[1] Vitamin premix supplied per kilogram of diets: vitamin A 15,000 IU, vitamin D_3 5,000 IU, vitamin E 40 IU, vitamin K_3 5.0 mg, vitamin B_1 5.0 mg, vitamin B_2 12.5 mg, vitamin B_6 6.0 mg, vitamin B_{12} 0.06 mg, niacin 50.0 mg, pantothenic 25.0 mg, folic acid 2.5 mg, biotin 0.25 mg.
[2] Mineral premix supplied per kilogram of diets: Fe ($FeSO_4·H_2O$), 100.0 mg; Cu ($CuSO_4·5H_2O$), 6.0 mg; Zn ($ZnSO_4·H_2O$), 100.0 mg; Mn ($MnSO_4·H_2O$), 10.0 mg; I (KI), 0.3 mg; Se (Na_2SeO_3), 0.3 mg.
[3] Nutrient levels are measured values.

2.9. RNA extraction and gene expression

Total RNA was extracted from approximately 0.1 g of mucosal samples obtained from each piglet using Trizol (TAKARA, Japan). Subsequently, genomic DNA was eliminated using a HiScript III RT reagent kit and cDNA for each sample was produced using a reverse transcription kit (Vazyme, Nanjing, Jiangsu, China). The real-time PCR reaction mixture, with a total volume of 10 μL, comprised 1.0 μL of cDNA template, 5 μL of SYBR Green mix (Vazyme, Nanjing, Jiangsu, China), 0.4 μL each of forward and reverse primers, 0.2 μL of ROX reference dye (Vazyme, Nanjing, Jiangsu, China), and 3 μL of double-distilled water. The PCR reaction for each gene was performed using the ABI 7900 instrument (Applied Biosystems, California, USA) under the following conditions: initial denaturation at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 5 s, primer annealing at 60 °C for 34 s, and extension at 72 °C for 15 s. Three housekeeping genes, β-actin, *18S rRNA*, and *GAPDH*, were selected as internal references, and the relative expression of each target gene was calculated using the $2^{-\Delta\Delta Ct}$ method. The sequences of primers, the length of the PCR products, and corresponding references are provided in Table S2.

2.10. DNA extraction, quantification of bacteria and related genes and measurement of SCFA in the colonic digesta samples

The methods for the isolation of genomic DNA from digesta samples and the determination of SCFA including acetate, propionate, butyrate, isobutyrate, valerate, and isovalerate have been comprehensively detailed in our previous work (Luo et al., 2017). Additionally, the bacterial cell numbers of total bacteria, *Bifidobacterium*, *Lactobacillus*, *Clostridium* cluster IV, *Clostridium* cluster XIVa, *E. coli*, *A. muciniphila* and the five virulence genes encoding ETEC (heat-stable toxin a [*STa*], heat-stable toxin b [*STb*], enteroaggregative *E. coli* heat-stable enterotoxin-1 [*astA*] and heat-labile enterotoxin [*LT*]) were quantified using real-time PCR on a Bio-RadCFX96 real-time system (Bio-Rad, California, USA) with SYBR Green as the fluorescent dye (Vazyme, Nanjing, Jiangsu, China). Primer details were provided in Table S2.

2.11. Determination of T lymphocyte subsets in the blood and spleen

The enumeration of T lymphocyte subsets in jugular vein blood and spleen has been comprehensively detailed in our previous work (Luo et al., 2021a). In the current study, four controls were applied for each sample: a blank control without any fluorescent antibodies, and three positive controls with fluorescent monoclonal antibodies against cluster of differentiation 3 (CD3, FITC-mouse anti-pig CD3, Cat. No. 559582), cluster of differentiation 4 (CD4, PerCp-Cy5.5-mouse anti-pig CD4a, 561474), and cluster of differentiation 8 (CD8, Alexa FluorR 647-mouse anti-pig CD8a, 561475) (BD Biosciences, Franklin Lakes, NJ, USA). Subsequently, cells were resuspended in PBS buffer, and a minimum of 10,000 events (lymphocytes or peripheral blood mononuclear cells) were collected for each sample using a BD Verse flow cytometer (BD Biosciences, San Jose, CA, USA). Data analysis was performed using FlowJo software version 8.7 (Tree Star Inc., Ashland, OR, USA).

2.12. Statistical analysis

All data was firstly tested for normal distribution using the descriptive statistic (Explore) module in the SPSS 22.0 software. In the first phase, spanning 28 d, the samples from the CON group and the ETEC group were combined into one group for statistical analysis. For the continuous variables with a normal distribution, one-way ANOVA was used to analyze the difference among groups, and the homogeneity of variance among groups was further tested using LSD multiple-range tests. The data are presented as the mean with the standard error of the mean (SEM). For categorical variables, specifically in the context of diarrhea data, the Kruskal–Wallis nonparametric test was used to compare differences among groups, and the values were represented as medians. Differences were considered significant when $P < 0.05$.

3. Results

3.1. Colonization pattern of A. muciniphila in pigs of different ages

From 7 to 57 d old, the population of *A. muciniphila* in pigs showed an initial increase followed by a decrease (Table 2). As pigs were born just before this period, the population of *A. muciniphila* was the lowest at 7 d old. However, at 14 d old, there was a significant increase in the abundance of *A. muciniphila*, surpassing that of other age groups ($P < 0.05$). Subsequently, after reaching 21 d old, the population of *A. muciniphila* in pig feces gradually decreased, being significantly lower than that at d 14 ($P < 0.05$).

Table 2
Abundance of *A. muciniphila* in feces of piglets at different ages.

Item	Day 7	Day 14	Day 21	Day 39	Day 57	Pooled SEM	*P*-value
Number, $\log_{10}$(copies/g)	4.54[b]	5.23[a]	4.78[b]	4.52[b]	4.43[b]	0.073	0.002

[a,b] Within a row, means that do not share the same superscripts are significantly different ($P < 0.05$).

3.2. Impact of A. muciniphila on the growth performance of weaned piglets

We did not observe any significant differences in growth performance among the groups during the 0 to 28 d interval ($P > 0.05$, Table 3). During the challenge phase, there were no differences in growth performance among the groups ($P > 0.05$, Table 4). However, it was evident that the ETEC group had numerically lower ADG and ADFI than the CON group. Additionally, the HA and LA groups had numerically lower ADG than the ETEC group, while the HIA and LIA groups had higher ADG than both the ETEC and CON groups ($P = 0.218$). Furthermore, the ADFI was numerically higher in the HA, HIA, and LIA groups than in the ETEC and CON groups, with the LA group having lower ADFI than the ETEC group ($P = 0.277$).

3.3. Impact of A. muciniphila on the diarrhea rate of healthy and ETEC-challenged piglets

In the first phase, there were no differences in the weekly diarrhea rates and overall diarrheal rate (Table 5). During the challenge phase, analysis using the Kruskal–Wallis test indicated that there were differences in diarrhea rates among the groups after ETEC challenge (Table 6). Pairwise comparison analysis revealed that the diarrhea rate in the LIA group was higher than that in the CON, HA, LA, and HIA groups ($P < 0.05$). Although the diarrhea incidence in the ETEC group did not differ from that in the CON group, numerically it was higher than that in the CON group ($P > 0.05$). However, the HA, LA, and HIA groups all exhibited lower diarrhea rates compared to the ETEC group ($P > 0.05$), with the LA group having the lowest diarrhea rate (decrease of 13%).

3.4. Impact of A. muciniphila on the intestinal barrier function of healthy and ETEC-challenged piglets

ETEC challenge damaged the integrity of intestinal villi in the jejunum and ileum of piglets (Table 7) and the VH and VCR in both the jejunum and ileum of the ETEC group were significantly lower compared to the CON group ($P < 0.05$, Table 7). However, CD among all groups showed no differences ($P > 0.05$, Table 7). Compared with the ETEC group, gavage with either live or heat-killed *A. muciniphila* numerically increased the VH of the jejunum ($P > 0.05$, Table 7), and the VCR in the HA, LA, and LIA groups also increased ($P < 0.05$, Table 7). In the ileum, the VH in the HA, LA, and HIA groups increased compared to the ETEC group ($P < 0.05$, Table 7), with only the LA group showing an increase in VCR ($P < 0.05$, Table 7). The changes in intestinal morphology are usually closely related to permeability. We found that compared to the CON group, the level of serum LPS increased in the ETEC group ($P < 0.05$, Table 7), and the LIA group showed significantly higher LPS level compared to the other groups ($P < 0.05$, Table 7). The DAO activity in serum increased in both the ETEC and LIA groups compared to the CON group ($P < 0.05$, Table 7). When comparing to the ETEC group, the DAO activity in serum of the HA and LA groups decreased ($P < 0.05$, Table 7). Secretory immunoglobulin A, as a component of the immune defense barrier, plays a role in resisting infections and protecting the intestinal tract from harm (Mantis et al., 2011). Although the sIgA content in the jejunum did not statistically increase in all groups following ETEC challenge compared to the CON group ($P > 0.05$, Table 7), both the ETEC and LA groups exhibited higher sIgA level compared to the CON group in the ileum ($P < 0.05$, Table 7), with the LA group having significantly higher sIgA level than the HA, HIA, and LIA groups ($P < 0.05$, Table 7).

3.5. Cytokine concentration, antioxidant incidence and peripheral T lymphocyte subsets in serum of pigs in each group

Regarding pro-inflammatory factors, there was no difference in serum TNF-α levels among the groups ($P > 0.05$, Table 8). However, the ETEC group exhibited higher levels of IL-6 compared to the CON, HA, and LIA groups ($P < 0.05$), with no significant difference from the LA and HIA groups ($P > 0.05$, Table 8). In terms of anti-inflammatory factors, IL-4 levels were lower in the HA, LA, HIA groups compared to the ETEC group ($P < 0.05$), with no significant difference from the CON group ($P > 0.05$). Additionally, serum IL-4 level in the HIA and LIA groups were higher than that in the HA and LA groups ($P < 0.05$, Table 8). The ETEC group showed significantly lower levels of IL-10 compared to the other groups ($P < 0.05$, Table 8).

In the ETEC group, both GSH-PX and SOD enzyme activities were significantly lower than in the CON group ($P < 0.05$, Table 8). Furthermore, both active and heat-killed *A. muciniphila* gavage increased serum GSH-Px enzyme activity compared to the ETEC group ($P < 0.05$, Table 8). However, only the HA and LIA groups exhibited an increase of SOD enzyme activity ($P < 0.05$, Table 8). There were no differences in serum CAT enzyme activity among the groups ($P > 0.05$, Table 8). Although no difference in serum MDA level was observed between the CON group and the ETEC, HA, LA, and LIA groups ($P > 0.05$), it was reduced in the HIA group compared to the CON group ($P < 0.05$, Table 8).

Bacterial infections in the intestine can lead to changes in peripheral immunity (Belkaid and Hand, 2014). Thus, we determined the proportions of T lymphocyte subpopulations in blood and

Table 3
Growth performance of piglets in each group during d 0 to 28.

Item	CON	HA	LA	HIA	LIA	Pooled SEM	*P*-value
Initial weight, kg	6.59	6.58	6.75	6.70	6.62	0.164	0.997
Final weight, kg	16.78	17.39	16.54	16.81	16.66	0.482	0.988
ADG, g/d	363.80	386.40	349.70	360.70	358.70	12.901	0.947
ADFI g/d	614.53	662.88	585.11	646.97	621.09	21.069	0.860
F/G	1.70	1.72	1.67	1.83	1.74	0.024	0.412

CON = control; HA = high level of *A. muciniphila*; LA = low level of *A.* muciniphila; HIA = high level of inactivated *A. muciniphila*; LIA = low level of inactivated *A. muciniphila*; ADG = average daily gain; ADFI = average daily feed intake; F/G = feed-to-gain ratio; SEM = standard error of the mean.

Table 4
Growth performance of piglets in each group during ETEC challenge.

Item	CON	ETEC	HA	LA	HIA	LIA	Pooled SEM	*P*-value
Initial weight, kg	17.15	16.36	17.39	16.54	16.81	16.66	0.475	0.991
Final weight, kg	21.43	20.44	21.38	20.22	21.29	21.21	0.532	0.984
ADG, g/d	855.71	817.33	798.75	735.00	895.71	908.75	22.108	0.218
ADFI, g/d	1320.59	1256.70	1357.80	1138.30	1350.94	1416.50	33.963	0.277
F/G	1.54	1.57	1.71	1.56	1.55	1.57	0.027	0.453

CON = control; HA = high level of *A. muciniphila*; LA = low level of *A.* muciniphila; HIA = high level of inactivated *A. muciniphila*; LIA = low level of inactivated *A. muciniphila*; ETEC = enterotoxigenic *Escherichia coli*; ADG = average daily gain; ADFI = average daily feed intake; F/G = feed-to-gain ratio; SEM = standard error of the mean.

Table 5
Diarrhea rates changes for the entire study period.[1]

Item	CON ($n = 16$)	HA ($n = 8$)	LA ($n = 8$)	HIA ($n = 8$)	LIA ($n = 8$)	H statistic	*P*-value
Diarrhea rate, %	7.14	5.36	7.14	10.71	10.71	2.94	0.568

CON = control; HA = high level of *A. muciniphila*; LA = low level of *A.* muciniphila; HIA = high level of inactivated *A. muciniphila*; LIA = low level of inactivated *A. muciniphila*.
[1] The data are presented as statistical descriptions including the median, and it was analyzed using the Kruskal–Wallis test statistic.

Table 6
Diarrhea rate changes during the challenge period.[1]

Item	CON ($n = 8$)	ETEC ($n = 8$)	HA ($n = 8$)	LA ($n = 8$)	HIA ($n = 8$)	LIA ($n = 8$)	H statistic	*P*-value
Diarrhea rate, %	10.00^{b}	20.00^{ab}	10.00^{b}	10.00^{b}	10.00^{b}	40.00^{a}	11.30	0.046

CON = control; HA = high level of *A. muciniphila*; LA = low level of *A.* muciniphila; HIA = high level of inactivated *A. muciniphila*; LIA = low level of inactivated *A. muciniphila*; ETEC = enterotoxigenic *Escherichia coli*.
[a,b] Within a row, means that do not share the same superscripts are significantly different ($P < 0.05$).
[1] The data is presented as statistical descriptions including the median, and it was analyzed using the Kruskal–Wallis test.

Table 7
Intestinal morphology and intestinal permeability related incidence in weaned pigs.

Item	CON	ETEC	HA	LA	HIA	LIA	Pooled SEM	*P*-value
Jejunum								
VH, μm	430.12^{a}	342.2^{b}	433.61^{a}	482.59^{a}	467.41^{a}	454.64^{a}	10.039	<0.001
CD, μm	315.79	353.77	316.01	341.76	380.32	296.36	10.343	0.207
VCR	1.40^{a}	1.08^{b}	1.39^{a}	1.45^{a}	1.24^{b}	1.55^{a}	0.045	0.036
sIgA, μg/mL	20.50	25.82	25.92	26.44	25.87	26.84	0.745	0.131
Ileum								
VH, μm	412.51^{a}	273.89^{c}	372.67^{ab}	396.04^{a}	348.31^{ab}	326.87^{bc}	10.985	0.001
CD, μm	278.97	315.41	305.68	295.51	333.13	294.37	5.949	0.138
VCR	1.51^{a}	0.90^{c}	1.17^{bc}	1.41^{a,b}	1.06^{c}	1.17^{bc}	0.050	0.001
sIgA, μg/mL	25.79^{c}	29.30^{ab}	27.43^{bc}	32.29^{a}	26.41^{bc}	27.01^{bc}	0.513	0.002
Serum								
LPS, ng/L	61.15^{c}	65.81^{b}	63.40^{bc}	64.81^{b}	64.57^{b}	69.66^{a}	0.509	<0.001
DAO, IU/L	206.31^{bc}	225.51^{a}	198.42^{c}	207.73^{bc}	216.79^{ab}	222.83^{a}	2.245	0.001

CON = control; HA = high level of *A. muciniphila*; LA = low level of *A.* muciniphila; HIA = high level of inactivated *A. muciniphila*; LIA = low level of inactivated *A. muciniphila*; ETEC = enterotoxigenic *Escherichia coli*; VH = villus height; CD = crypt depth; VCR = villus height to crypt depth ratio; sIgA = secretory immunoglobulin A; LPS = lipopolysaccharide; DAO = diamine oxidase; ETEC = enterotoxigenic *Escherichia coli*.
[a-c] Within a row, means that do not share the same superscripts are significantly different ($P < 0.05$).

Table 8
Serum cytokine, antioxidant incidence in weaned pigs.

Item	CON	ETEC	HA	LA	HIA	LIA	Pooled SEM	*P*-value
TNF-α, pg/mL	272.65	261.22	263.29	262.53	260.72	284.18	3.339	0.257
IL-6, ng/L	50.00^{b}	98.82^{a}	45.56^{b}	70.52^{ab}	66.62^{ab}	58.56^{b}	4.845	0.044
IL-4, ng/L	63.11^{de}	62.40^{e}	66.49^{cd}	68.12^{c}	71.92^{b}	76.70^{a}	0.875	<0.001
IL-10, ng/L	141.16^{a}	129.40^{b}	143.68^{a}	144.05^{a}	145.33^{a}	152.66^{a}	1.668	0.008
GSH-Px, U/mL	537.56^{c}	499.70^{d}	548.08^{bc}	575.17^{abc}	591.12^{ab}	620.43^{a}	8.138	<0.001
SOD, U/mL	10.44^{a}	9.25^{b}	10.79^{a}	9.71^{b}	10.08^{ab}	10.79^{a}	0.169	0.038
CAT, U/mL	6.94	7.32	7.09	6.13	7.95	7.58	0.486	0.936
MDA, nmol/mL	3.42^{a}	3.83^{a}	3.70^{a}	2.96^{ab}	2.52^{b}	3.71^{a}	0.133	0.020

CON = control; HA = high level of *A. muciniphila*; LA = low level of *A.* muciniphila; HIA = high level of inactivated *A. muciniphila*; LIA = low level of inactivated *A. muciniphila*; ETEC = enterotoxigenic *Escherichia coli*; TNF-α = tumor necrosis factor-alpha; IL-6 = interleukin-6; IL-4 = interleukin-4; IL-10 = interleukin-10; GSH-Px = glutathione peroxidase; SOD = superoxide dismutase; CAT = catalase; MDA = malondialdehyde.
[a-e] Within a row, means that do not share the same superscripts are significantly different ($P < 0.05$).

spleen using flow cytometry (Table 9). The results showed that the proportion of $CD4^+$ T lymphocytes in the blood of ETEC-infected pigs decreased compared to CON group($P < 0.05$, Table 9), but it was restored in LA and LIA groups ($P < 0.05$, Table 9).

3.6. Relative expression of genes related to intestinal barrier and antioxidant function in healthy and ETEC-challenged piglets

We assessed the impact of *A. muciniphila* on intestinal health by measuring the expression of genes related to intestinal barrier and antioxidant function across mucosa of different segments using real-time PCR. The results revealed that in the duodenal mucosa, there were no differences in the expression of *ZO-1*, occludin, and claudin-1 among the groups ($P > 0.05$, Fig. 2A, 2B and 2C).

In the jejunum, there were no differences in the expression of *ZO-1* among the groups ($P > 0.05$, Fig. 2D). Compared to the CON group, the LIA group exhibited an increase in the expression of occludin ($P < 0.05$, Fig. 2E). Moreover, the expression of occludin in the LA, HIA, and LIA groups was higher than that in the ETEC group ($P < 0.05$, Fig. 2E). Both the HIA and LIA groups displayed a higher expression of claudin-1 compared to the HA group ($P < 0.05$, Fig. 2F).

Table 9
Peripheral T lymphocyte subpopulations in blood and spleen of weaned pigs.

Item	CON	ETEC	HA	LA	HIA	LIA	Pooled SEM	*P*-value
Blood								
$CD3^+$, %	40.55	39.69	42.94	43.81	41.79	49.82	1.738	0.715
$CD4^+$, %	24.81^{a}	15.74^{c}	19.02^{bc}	23.39^{ab}	22.01^{abc}	26.90^{a}	1.844	0.019
$CD8^+$, %	25.65	27.24	28.63	24.76	27.14	27.90	0.796	0.726
$CD4^+/CD8^+$	1.02	0.62	0.73	0.95	0.84	1.00	0.051	0.162
Spleen								
$CD3^+$, %	51.23	51.33	49.92	49.75	48.29	47.41	0.927	0.821
$CD4^+$, %	35.94	29.86	34.71	36.66	33.67	33.73	0.958	0.503
$CD8^+$, %	21.09	26.36	22.55	22.37	25.36	23.84	1.107	0.787
$CD4^+/CD8^+$	1.95	1.52	1.57	1.67	1.41	1.46	0.094	0.607

CON = control; HA = high level of *A. muciniphila*; LA = low level of *A.* muciniphila; HIA = high level of inactivated *A. muciniphila*; LIA = low level of inactivated *A. muciniphila*; ETEC = enterotoxigenic *Escherichia coli*; CD3 = cluster of differentiation 3; CD4 = cluster of differentiation 4; CD8 = cluster of differentiation 8.
$^{a-c}$ Within a row, means that do not share the same superscripts are significantly different ($P < 0.05$).

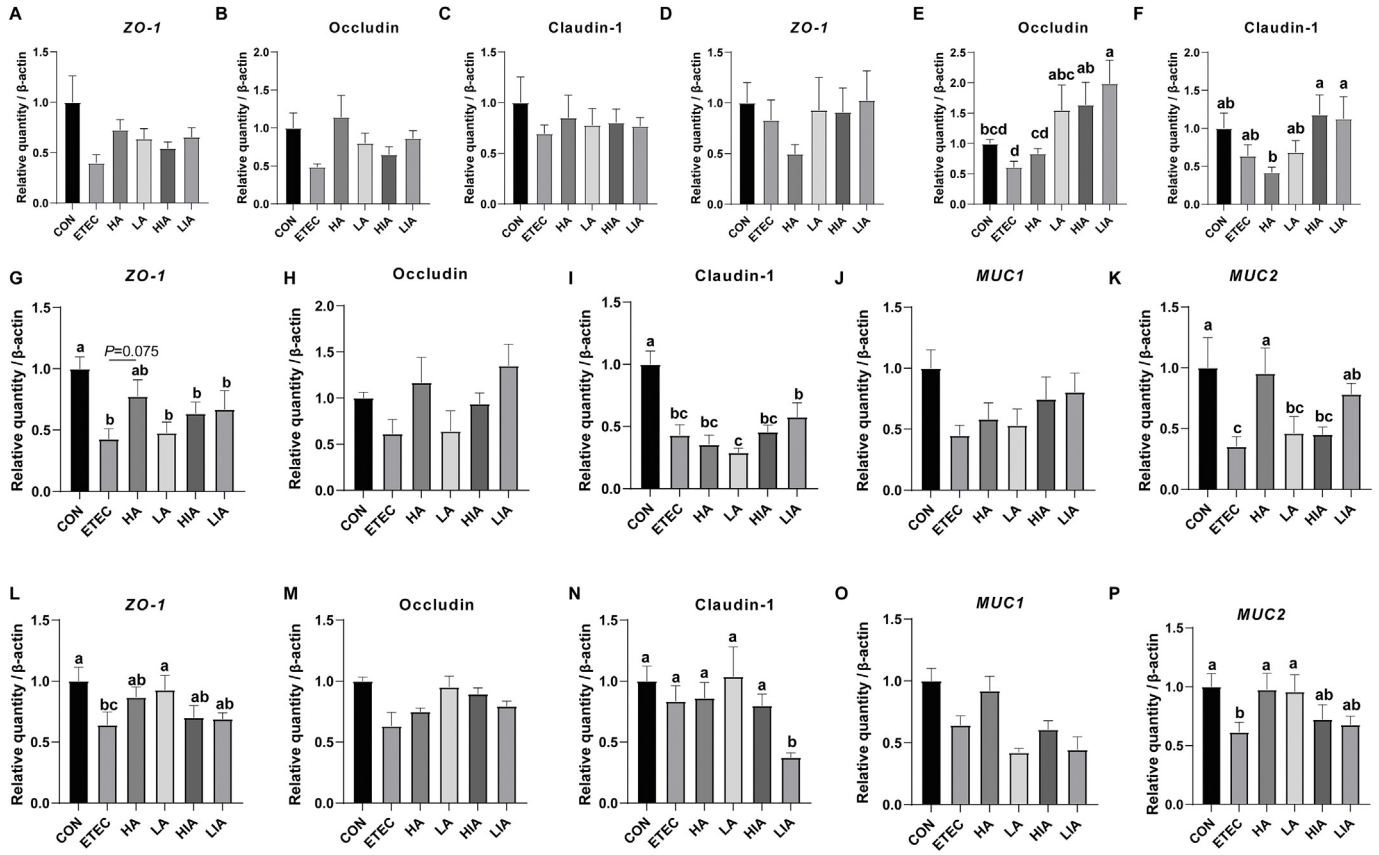

Fig. 2. Expression of intestinal barrier-related genes in the intestinal mucosa of weaned piglets. (A-C) Relative expression of tight junction protein genes in the duodenum. (D-F) Relative expression of tight junction protein genes in the jejunum. (G-K) Relative expression of tight junction protein and mucin genes in the ileum. (L-P) Relative expression of tight junction protein and mucin genes in the colon. Bars that do not share the same superscripts are significantly different ($P < 0.05$). CON = control; HA = high level of *A. muciniphila*; LA = low level of *A.* muciniphila; HIA = high level of inactivated *A. muciniphila*; LIA = low level of inactivated *A. muciniphila*; ETEC = enterotoxigenic *Escherichia coli*; *ZO-1* = zonula occludens-1; *MUC1* = mucin 1; *MUC2* = mucin 2.

In the ileum, compared to the CON group, the ETEC group exhibited decreased expression of *ZO-1*, claudin-1, and *MUC2* ($P < 0.05$, Fig. 2G, 2I and 2K), while the expression of occludin and *MUC1* showed no change ($P > 0.05$, Fig. 2H and 2J). The expression of *ZO-1* in the HA group tended to increase compared to the ETEC group ($P < 0.01$, Fig. 2G). Meanwhile, both the HA and LIA groups showed an increase of *MUC2* expression compared to the ETEC group ($P < 0.05$, Fig. 2K).

In the colon, compared to the CON group, the ETEC group exhibited decreased expression of *ZO-1* and *MUC2* ($P < 0.05$, Fig. 2L and 2P), with no changes in the expression of occludin, claudin-1, and *MUC1* ($P > 0.05$, Fig. 2M, 2N, and 2O). When compared to the ETEC group, the expression of claudin-1 in the LIA group showed a decrease ($P < 0.05$, Fig. 2N), and the expression of *ZO-1* in the LA group was increased ($P < 0.05$, Fig. 2L). In addition, the expression of *MUC2* in both the HA and LA groups showed an increase compared to the ETEC group ($P < 0.05$, Fig. 2P).

The integrity of the intestinal mucosal barrier is closely linked to oxidative stress at the mucosal level (Wang et al., 2020). In the duodenal mucosa, the expression of *CAT* was significantly decreased in the ETEC, LA, and HIA groups compared to the CON group ($P < 0.05$, Fig. 3A). However, the HA group displayed significantly higher *CAT* expression compared to the ETEC group ($P < 0.05$, Fig. 3A). In the jejunal mucosa, there were no differences in the expression of heme oxygenase 1 (*HO1*) between the CON and ETEC groups ($P > 0.05$, Fig. 3B), but both the HIA and LIA groups exceeded the CON, ETEC, and LA groups ($P < 0.05$, Fig. 3B), with the LIA group showing higher expression than the HA group ($P < 0.05$, Fig. 3B). While not statistically significant, the expression of Kelch-like ECH-associated protein 1 (*KEAP1*) was lower in the ETEC, HA, and LA groups compared to the CON group ($P > 0.05$, Fig. 3B). The LIA group exhibited higher *KEAP1* level than other groups except HIA ($P < 0.05$, Fig. 3B). Compared to the CON group, nuclear factor erythroid 2-related factor 2 (*Nrf2*) expression was decreased in the ETEC, HA, and LA groups ($P < 0.05$, Fig. 3B). Both the HIA and LIA groups displayed higher *Nrf2* expression than the HA group ($P < 0.05$, Fig. 3B).

In the ileal mucosa, compared to the CON group, the ETEC group showed reduced expression of *SOD2*, *KEAP1*, and *Nrf2* ($P < 0.05$, Fig. 3C). The expression of *SOD2* in all *A. muciniphila*-treated groups did not differ from the ETEC group ($P > 0.05$, Fig. 3C). The expression of *KEAP1* in the HA group was higher than that in the ETEC group ($P < 0.05$, Fig. 3C).

In the colonic mucosa, compared to the CON group, the ETEC group had reduced expression of glutathione peroxidase 1 (*GPX1*), *SOD1*, *SOD2*, and *KEAP1* ($P < 0.05$, Fig. 3D). For *GPX1* expression, the HA group exceeded the ETEC, LA, HIA, and LIA groups ($P < 0.05$, Fig. 3D), with no difference compared to the CON group (Fig. 3D). Regarding the expression of NAD(P)H quinone dehydrogenase 1 (*NQO1*), the LA group exhibited higher expression than the LIA group ($P < 0.05$, Fig. 3D), with the LA group having numerically higher expression compared to the other groups, while the LIA group had the lowest numerical expression. For the expression of *SOD2*, the HA group showed higher expression than the ETEC and LIA groups ($P < 0.05$, Fig. 3D). Meanwhile, the HA group had higher expression of *KEAP1* than the ETEC, LA, HIA, and LIA groups ($P < 0.05$, Fig. 3D), while the LIA group exhibited lower expression than the other groups ($P < 0.05$, Fig. 3D). The LIA group had lower expression of *Nrf2* than the HA, LA, and HIA groups ($P < 0.05$, Fig. 3D).

3.7. Number of specific microbial groups, expression of genes encoding ETEC virulence factors and the concentration of SCFA in digesta samples

During the 28-d experimental period, the abundance of *A. muciniphila* in the fecal samples of the HA group was significantly higher than that in the CON, HIA and LIA groups ($P < 0.05$, Table 10). Following ETEC challenge, the quantity of *E. coli* in the ileum and colon was higher than that in the CON group ($P < 0.05$, Table 11), while gavage with *A. muciniphila* showed no impact on the number of *E. coli* compared with ETEC group ($P > 0.05$, Table 11). No differences were found in other specific microbial populations among the groups ($P > 0.05$, Table 11).

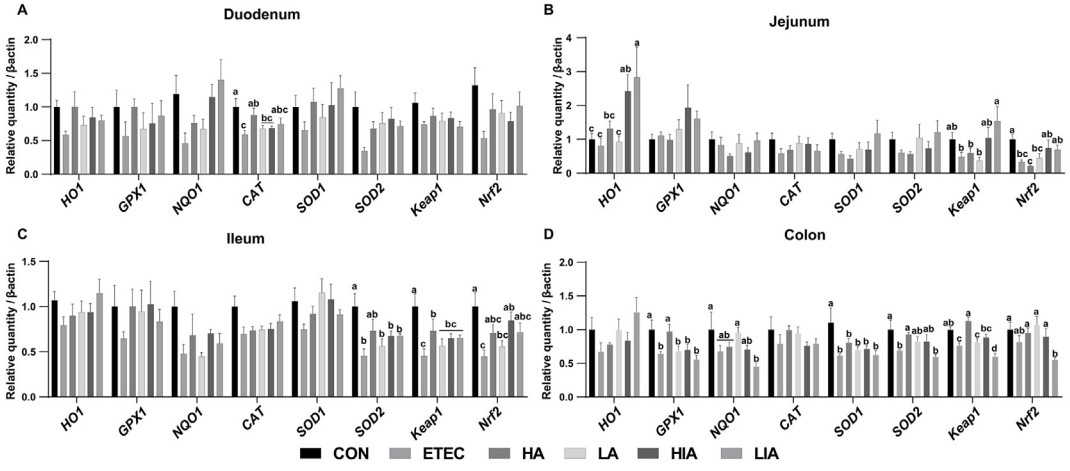

Fig. 3. Expression of intestinal antioxidant-related genes in the intestinal mucosa of weaned piglets. (A) Relative expression of antioxidant-related genes in the duodenum. (B) Relative expression of antioxidant-related genes in the jejunum. (C) Relative expression of antioxidant-related genes in the ileum. (D) Relative expression levels of antioxidant-related genes in the colon. Bars that do not share the same superscripts are significantly different ($P < 0.05$). CON = control; HA = high level of *A. muciniphila*; LA = low level of *A.* muciniphila; HIA = high level of inactivated *A. muciniphila*; LIA = low level of inactivated *A. muciniphila*; ETEC = enterotoxigenic *Escherichia coli*; *HO1* = heme oxygenase 1; *GPX1* = glutathione peroxidase 1; *NQO1* = NAD(P)H quinone dehydrogenase 1; *CAT* = catalase; *SOD1* = superoxide dismutase 1; *SOD2* = superoxide dismutase 2; *KEAP1* = Kelch-like ECH-associated protein 1; *Nrf2* = nuclear factor erythroid 2-related factor 2.

Table 10
Abundance of *A. muciniphila* in the feces of each group at d 28.

Item	CON	HA	LA	HIA	LIA	Pooled SEM	*P*-value
Number, log_{10}(copies/g)	4.54[b]	5.23[a]	4.78[ab]	4.53[b]	4.43[b]	0.073	0.002

CON = control; HA = high level of *A. muciniphila*; LA = low level of *A.* muciniphila; HIA = high level of inactivated *A. muciniphila*; LIA = low level of inactivated *A. muciniphila*.
[a,b] Within a row, means that do not share the same superscripts are significantly different ($P < 0.05$).

Regarding the expression of ETEC virulence factor genes, the ETEC group showed numerically higher *STb* level in ileal and colonic digesta than the CON group ($P = 0.617$, $P = 0.158$, Table 11). Compared with the ETEC group, the expression of *STa* in the HA and LA groups was lower in the ileum ($P < 0.05$, Table 11). After ETEC challenge, *STa* expression in the LA and LIA groups was lower than that in all other groups in colon ($P < 0.05$, Table 11), with no differences compared to the CON group ($P > 0.05$, Table 11). The expression of *astA* in the LA group was lower than in the other groups in the colon ($P < 0.05$, Table 11).

Moreover, we did not find any difference in the concentration of SCFA in the colon among the groups ($P > 0.05$, Table 12). Nevertheless, it can be observed numerically that the LA group had higher levels of various SCFA compared to the other inoculated groups.

4. Discussion

After weaning, the ability to find alternative products and strategies in the post-antibiotic era is of paramount importance for animal cultivation to address diarrhea issues. Microbial modulation of intestinal homeostasis to improve gut health is a widely adopted approach. Among these, mucin-cohabiting bacteria, which closely interact with the intestinal epithelium, play a more significant role in microbe-host communication (Duncan et al., 2021; Paone and Cani, 2020). However, research and application regarding these bacteria in pigs are scarce. Hence, this experiment selected *A. muciniphila* to investigate its potential in addressing intestinal health issues related to infectious diarrhea in piglets.

During the weaning transition period, there is a drastic shift in the composition of the gut microbiota in piglets, which is

Table 11
Specific microbial communities in the ileal and colonic digesta of each group and their correlation with ETEC virulence factor-related genes, log_{10}(copies/g).

Item	CON	ETEC	HA	LA	HIA	LIA	Pooled SEM	*P*-value
Ileum								
Total bacteria	13.91	14.56	14.30	14.58	14.48	14.61	0.101	0.425
Escherichia coli	10.83[b]	12.71[a]	12.39[ab]	12.46[a]	11.95[ab]	11.83[ab]	0.269	0.007
Lactobacillus group	12.01	12.44	11.89	12.10	12.71	11.82	0.150	0.506
Bifidobacterium	10.70	10.90	10.92	11.12	10.93	10.44	0.116	0.602
Clostridium cluster XIV	9.55	10.06	9.69	9.82	10.18	9.97	0.184	0.949
Clostridium cluster IV	10.49	10.96	10.05	10.18	10.85	10.49	0.105	0.072
STa	10.06[abc]	10.41[a]	9.43[bc]	9.42[c]	10.21[ab]	10.00[abc]	0.120	0.027
STb	9.83	10.55	9.80	10.40	10.40	10.19	0.150	0.617
astA	10.32	11.06	10.66	11.15	11.10	10.61	0.140	0.508
LT	4.64	4.70	5.32	4.54	5.00	4.12	0.252	0.820
Colon								
Total bacteria	15.20	15.16	15.21	15.16	15.15	14.94	0.049	0.697
Escherichia coli	11.94[b]	12.59[a]	12.63[a]	12.39[ab]	12.70[a]	12.54[a]	0.076	0.024
Lactobacillus group	12.53	12.27	12.74	12.24	12.56	12.82	0.109	0.589
Bifidobacterium	11.34	11.28	11.48	11.07	11.27	10.76	0.098	0.303
Clostridium cluster XIV	12.83	12.74	12.82	12.71	12.73	12.37	0.185	0.982
Clostridium cluster IV	13.26	13.18	13.22	13.20	13.24	12.57	0.116	0.469
STa	9.90[bc]	11.17[ab]	9.64[bc]	9.17[c]	12.29[a]	8.94[c]	0.300	0.002
STb	9.86	10.93	12.09	8.98	11.10	10.20	0.371	0.158
astA	13.01[a]	13.54[a]	13.36[a]	12.01[b]	13.14[a]	13.26[a]	0.144	0.017
LT	6.12	6.97	6.83	7.45	5.61	7.07	0.341	0.671

CON = control; HA = high level of *A. muciniphila*; LA = low level of *A.* muciniphila; HIA = high level of inactivated *A. muciniphila*; LIA = low level of inactivated *A. muciniphila*; ETEC = enterotoxigenic *Escherichia coli*; *STa* = heatstable toxin a; *STb* = heat-stable toxin b; *astA* = enteroaggregative *E. coli* heat-stable enterotoxin-1; *LT* = heat-labile enterotoxin.
[a-c] Within a row, means that do not share the same superscripts are significantly different ($P < 0.05$).

Table 12
Concentrations of SCFA in the colonic digesta of each group (mmol/g).

Item	CON	ETEC	HA	LA	HIA	LIA	Pooled SEM	*P*-value
Acetic acid	2.53	2.81	2.12	2.62	2.02	2.18	0.091	0.118
Propanoic acid	1.83	1.92	1.79	2.03	1.58	1.81	0.077	0.826
Isobutyric acid	0.18	0.14	0.15	0.24	0.18	0.19	0.011	0.430
Butyric acid	1.11	1.01	1.00	1.42	1.04	1.19	0.055	0.535
Isovaleric acid	0.34	0.30	0.31	0.42	0.39	0.41	0.014	0.125
Valeric acid	0.37	0.37	0.30	0.37	0.32	0.32	0.017	0.838

SCFA = short chain fatty acid; CON = control; HA = high level of *A. muciniphila*; LA = low level of *A.* muciniphila; HIA = high level of inactivated *A. muciniphila*; LIA = low level of inactivated *A. muciniphila*; ETEC = enterotoxigenic *Escherichia coli*.

accompanied by alterations in gut morphology, leading to a potential imbalance in intestinal homeostasis (Frese et al., 2015; Luo et al., 2022b). To the best of our knowledge, for the first time, this study evaluated the abundance dynamics of *A. muciniphila* in feces from piglets and observed a decrease in its abundance starting from 21 d old. This observation may suggest that supplementation with *A. muciniphila* could alleviate gut microbiota imbalance and subsequently improve intestinal health in piglets. *A. muciniphila* is commonly used in the treatment of metabolic disorders such as overweight and obesity, and current research has demonstrated the safety of *A. muciniphila* intervention (Depommier et al., 2019; Turck et al., 2021). Feeding healthy rats with *A. muciniphila* for 14 d had no impact on their food intake and body weight (Turck et al., 2021), and similar results were observed in mice (Bian et al., 2019). During the initial growth phase in weaned piglets, *A. muciniphila* did not affect growth performance and diarrhea rate, but a notable increase in food intake was observed with high-dose live and heat-killed strains. In a randomized, double-blind, placebo-controlled trial involving 40 individuals, pasteurized *A. muciniphila* supplementation led to weight reduction in obese patients after three months, while live *A. muciniphila* had no effect (Depommier et al., 2019). However, in this experiment, it was found that high-dose heat-killed *A. muciniphila* in the final week of the initial trial phase increased the F/G ratio, potentially associated with its role in lipid metabolism regulation (Plovier et al., 2017).

In the ETEC challenge phase, the diarrhea rate in the ETEC group was twice that of the CON group, and the increase in the pro-inflammatory cytokine IL-6 and decrease in the anti-inflammatory cytokine IL-10 indicated the successful establishment of the diarrhea model (Dahmer et al., 2023). However, the impact of live *A. muciniphila* and heat-killed *A. muciniphila* on ETEC-challenged piglets differed. Firstly, there was no significant effect on growth performance and diarrhea rate in all groups. Among them, the low-dose live bacteria group had the lowest diarrhea rate, while the low-dose heat-killed *A. muciniphila* remarkably increased piglet feed intake. Increased feed intake has been proved to lead to an increase in undigested protein in the hindgut, increasing the risk of nutritional diarrhea (Opapeju et al., 2009; Pluske et al., 1997). This may explain why the diarrhea rate in the low-dose heat-killed *A. muciniphila* group was higher than in the other groups without affecting their growth performance. However, there is limited research on how *A. muciniphila* affects central regulation of feeding, and it has been found that *A. muciniphila* mainly increases reward alterations in obesity, which may be related to reducing systemic inflammation (Huwart et al., 2022). Therefore, further investigations are needed to determine how *A. muciniphila* can reduce IL-6 level in serum and increase the levels of anti-inflammatory cytokines such as IL-10 and IL-4. IL-6 plays a crucial role in the body's immune response to bacterial infections, with neutrophils playing a vital role in this response (Dalrymple et al., 1996). IL-4 can induce the production of IL-10 in macrophages and T cells through signaling, possibly by stimulating the differentiation of IL-10-producing Treg cells (Zhou et al., 2021), a specific type of $CD4^+$ T cell with critical regulatory functions in the immune system (Okeke and Uzonna, 2019). Previous studies have shown that IL-4 can inhibit neutrophil function under inflammatory conditions (Lewkowicz et al., 2006; Okeke et al., 2017). Flow cytometry results showing an upregulation of $CD4^+$ cell proportions support this notion. In addition, *E. coli* has a higher propensity for colonization in the ileum and proximal colon (Sauvaitre et al., 2022). Peyer's patches (PP) within the ileum are capable of mounting a response to bacterial infections, resulting in the production of sIgA to counteract pathogen invasion (Kadaoui and Corthésy, 2007). Therefore, our study revealed a significant increase in the concentration of sIgA in the ileal mucosa of ETEC-challenged piglets. Interestingly, low-dose live *A. muciniphila* administration caused even higher levels of sIgA, suggesting that low-dose live *A. muciniphila* is more effective at enhancing the mucosal immune function of the ileum.

The organism's intestinal antioxidant status is closely associated with systemic inflammation and intestinal barrier integrity. Enhanced production of reactive oxygen species (ROS) by immune cells at inflammatory sites can result in oxidative stress and tissue damage (Dziubla and Butterfield, 2016; Rao, 2008). Several polyphenolic compounds have been found to alleviate oxidative stress and improve metabolic disorders. In addition, *A. muciniphila* has been observed to be enriched in the intestinal tract (Zhang et al., 2017, 2018). *A. muciniphila* has been shown to significantly mitigate oxidative stress and inflammatory responses induced by acetaminophen (APAP). This includes restoring the balance of reduced glutathione/oxidized glutathione (GSH/GSSG) and enhancing the activity of superoxide dismutase (SOD) (Xia et al., 2022). In this study, similar results were obtained, suggesting that both live and heat-killed *A. muciniphila* have an effect in alleviating oxidative stress after ETEC challenge. Nevertheless, no clear dose-dependent benefit was observed. Additionally, the observations of intestinal morphology and serum levels of LPS and DAO indicated that *A. muciniphila* could improve intestinal morphology and barrier function. Interestingly, live bacteria performed better in improving the morphology of the ileum than dead bacteria. This could be attributed to the physiological structure of the intestine itself, which also results in *A. muciniphila* being more likely to colonize in the posterior intestinal segment and exert its function (Luo et al., 2022a). Furthermore, the findings from genes related to intestinal mucosal barriers and antioxidant activities support this hypothesis. Notably, heat-killed *A. muciniphila* enhanced the expression of jejunal tight junction proteins. However, the enhancements in colonic *ZO-1*, claudin-1, and *MUC2* were less significant compared to live bacteria. Specifically, low-dose heat-killed *A. muciniphila* adversely affected colonic claudin-1 expression. As the colon is responsible for water reabsorption, a reduction in claudin-1 is closely linked to diarrhea (Cheng et al., 2015). This could explain the higher incidence of diarrhea in piglets receiving low-dose heat-killed *A. muciniphila*. On the other hand, live bacteria showed dose-dependent variations in phenotypes. High-dose live *A. muciniphila* downregulated the expression of mucosal barrier protein genes in the jejunum, but significantly enhanced the expression of these genes and *MUC2* in the ileum and colon. In contrast, low-dose live *A. muciniphila* improved the expression of the above genes in the jejunum and colon, with a more pronounced effect in the colon than high-dose live *A. muciniphila*. This may also explain the lower diarrhea rate in piglets receiving low-dose live *A. muciniphila*. Additionally, regarding antioxidant gene expression, it was observed that heat-killed *A. muciniphila* had a more significant impact on antioxidant capacity in the jejunum compared to live *A. muciniphila*, with enhancements also noted in the ileum. However, in piglets that received low-dose heat-killed *A. muciniphila*, colonic antioxidant capacity was significantly reduced, consistent with the results of the expression of tight junction protein encoding genes. In high-fat diet-induced obese mice, the improvement in colonic mucus layer thickness with 2×10^8 CFU inactivated *A. muciniphila* was less pronounced compared to live bacteria (Everard et al., 2013). Since the small intestine is the primary site for digestion and absorption, low-dose heat-killed *A. muciniphila* were already depleted in the small intestine. In contrast, live *A. muciniphila* demonstrated a dose-dependent advantage in terms of antioxidant capacity, with high-dose live *A. muciniphila* exhibiting more substantial improvements in genes related to antioxidant function in the duodenum and jejunum and a similar effect in the colon. These findings were

in accordance with the levels of inflammatory cytokines and antioxidant markers in serum. The mechanism of *A. muciniphila* inactivation remains to be further elucidated.

In this experiment, the successful gastric inoculation of pigs with 1×10^{10} CFU/5 mL of *A. muciniphila* for 28 d resulted in an increase in the abundance of *A. muciniphila* in the gut, indicating the successful establishment of the inoculated piglet model. Oral administration of *A. muciniphila* can influence the gut microbiota and improve host health in specific diseases (Bian et al., 2019; Hänninen et al., 2018). However, in aging Ercc1$^{-/\Delta 7}$ mice, the abundance of *A. muciniphila* and the composition of gut microbiota remained unchanged (van der Lugt et al., 2019). Consistent with this, we did not observe any changes in the numbers of *E. coli*, *Lactobacillus*, *Bifidobacterium*, *Clostridium* cluster XIV, and *Clostridium* cluster IV in ileal and colonic digesta among the groups. However, oral treatment with live *A. muciniphila* can downregulate the expression of *STa* in the ileum, and low-dose live and heat-killed *A. muciniphila* supplementation can also decrease the quantities of *STa* and *astA* in the colon. ETEC adheres to the small intestine villi through fimbriae, produces enterotoxins that act locally on intestinal cells, and competes with mucin-degrading bacteria for mucin-binding sites (Sauvaitre et al., 2022). Multiple factors can regulate the expression of virulence genes encoded by bacteria (Nagy and Fekete, 2005). Live *A. muciniphila* supplementation significantly reduces the expression of *STa* in the ileum, and low-dose *A. muciniphila* supplementation significantly decreases the expression of virulence factors in the colon, indicating a competition between *A. muciniphila* and ETEC for mucosal niche occupation, which reduces the virulence of ETEC. SCFA play a crucial role in maintaining gut homeostasis (Parada Venegas et al., 2019). In this experiment, *A. muciniphila* did not affect the concentration of SCFA in colonic digesta, but the concentration of SCFA in pigs receiving low-dose live *A. muciniphila* was remarkably increased compared to other groups, which may also contribute to lower diarrhea rate. At the same time, extensive experiments will be needed to substantiate the efficacy of *A. muciniphila* (live or inactivated) in preventing diarrhea in weaned piglets.

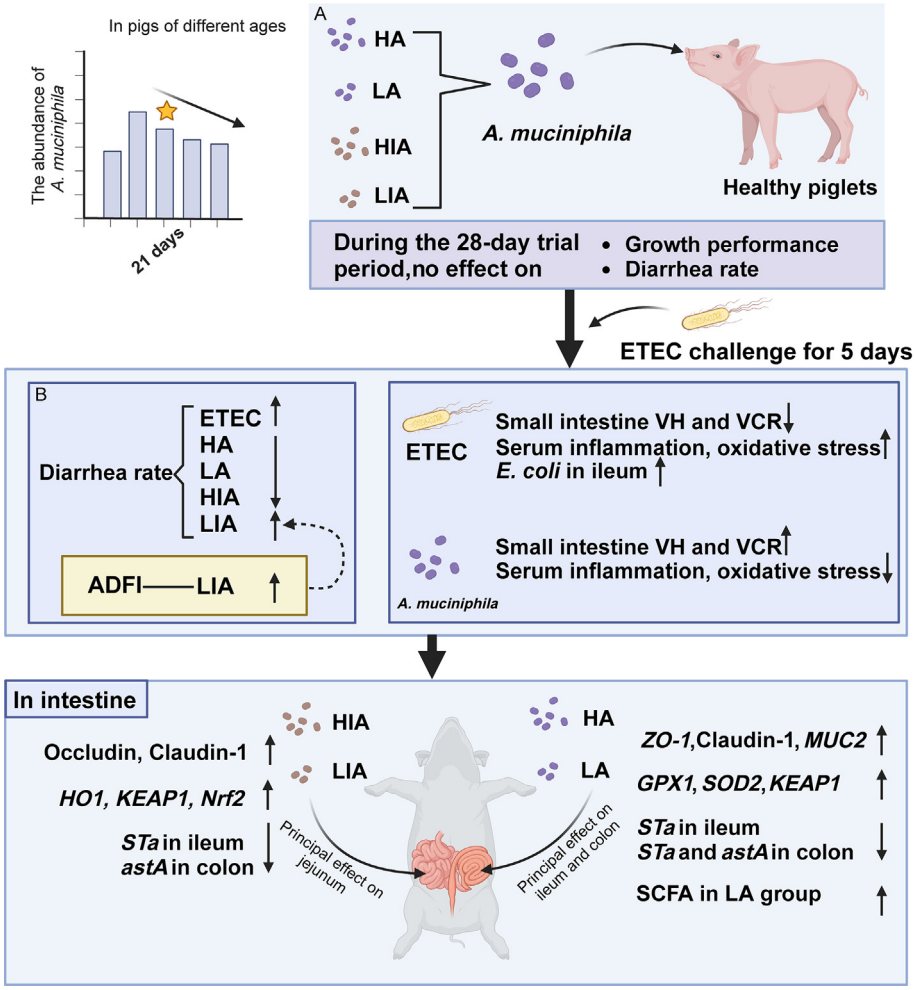

Fig. 4. Summary chart drawn based on the results of this study. Figure is created with Biorender.com. (A) The first experiment lasted for 28 days. (B) Changes in growth performance and diarrhea rates of piglets in each group during the ETEC challenge phase. Dashed lines indicate potential mechanism. HA = high level of *A. muciniphila*; LA = low level of *A.* muciniphila; HIA = high level of inactivated *A. muciniphila*; LIA = low level of inactivated *A.* muciniphila; ETEC = enterotoxigenic *E. coli*; ADFI = average daily feed intake; VH = villus height; VCR = villus height to crypt depth ratio; *ZO-1* = zonula occludens-1; *MUC2* = mucin 2; *HO1* = heme oxygenase 1; *GPX1* = glutathione peroxidase 1; *SOD2* = superoxide dismutase 2; *KEAP1* = Kelch-like ECH-associated protein *1*; *Nrf2* = nuclear factor erythroid 2-related factor 2; *STa* = heatstable toxin a; *astA* = enteroaggregative *E. coli* heat-stable enterotoxin-1; SCFA = short-chain fatty acids.

5. Conclusion

As summarized in Fig. 4, in the current study, we observed a decrease in the abundance of *A. muciniphila* in feces of piglets at 21 d old (the first day of weaning). From this, we surmise that weaning is the optimal window period for *A. muciniphila* intervention. Logically, when live and heat-killed *A. muciniphila* were supplemented, we found that *A. muciniphila* had no significant impact on the growth performance and diarrhea rate of healthy weaned piglets, but it did demonstrate the ability to alleviate the inflammatory response and enhance antioxidant levels. Additionally, it improved the morphology of the small intestine and reduced intestinal permeability in piglets challenged with ETEC, but not affect their growth performance. *A. muciniphila* also effectively reduced the incidence of diarrhea in ETEC-challenged piglets, with the specific outcomes being influenced by both dosage and activity. It is worth noting that the low-dose heat-killed *A. muciniphila* group exhibited a higher diarrhea rate, which may be associated with increased feed intake. Furthermore, we discovered differences in the impact of live *A. muciniphila* and heat-killed *A. muciniphila* on gut barrier and antioxidant-related genes. Live *A. muciniphila* appears to exert its effects primarily in the ileum and colon, while heat-killed *A. muciniphila* acts primarily in the small intestine. Additionally, we found that live *A. muciniphila* can downregulate the expression of ETEC virulence factors in the ileum, while low-dose *A. muciniphila* can downregulate the expression of ETEC virulence factors in the colon. To the best of our knowledge, our study is the first investigation to explore the potential of mucosal symbiont *A. muciniphila* to alleviate the impact of ETEC challenge in piglets. It demonstrates that both live and heat-killed *A. muciniphila* may have specific potential for alleviating pathological changes related to diarrhea, with different sites of action. Nevertheless, further evaluation of the effects of *A. muciniphila* in larger animal populations is warranted in future research.

Author contributions

Cong Lan: Visualization, Writing – original draft, Methodology, Software, Data curation. **Hua Li:** Methodology, Software, Data curation. **Yuqing Shen:** Investigation, Methodology. **Yang Liu:** Investigation, Methodology. **Aimin Wu:** Investigation, Methodology. **Jun He:** Conceptualization, Methodology. **Jingyi Cai:** Conceptualization, Methodology. **Gang Tian:** Conceptualization, Methodology. **Xiangbing Mao:** Conceptualization, Methodology. **Zhiqing Huang:** Conceptualization, Methodology. **Bing Yu:** Conceptualization, Investigation. **Ping Zheng:** Conceptualization, Investigation. **Jie Yu:** Conceptualization, Investigation. **Junqiu Luo:** Writing – review editing. **Hui Yan:** Writing – review editing. **Yuheng Luo:** Conceptualization, Methodology, Writing – review editing.

Declaration of conflict of interests

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

Acknowledgments

The work presented in this manuscript was supported by Sichuan Provincial Natural Science Foundation (2023NSFSC0237) and National Natural Science Foundation of China (NSFC, grant number 32372900 and 32072743). Special thanks to Professor Li Liu from Nanjing Agricultural University for donating *A. muciniphila* (DSM 22959).

Appendix Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2024.01.007.

References

AOAC. Official method of analysis. 18th ed. Gaithersburg, MD: AOAC International; 2007.
Belkaid Y, Hand TW. Role of the microbiota in immunity and inflammation. Cell 2014. https://doi.org/10.1016/j.cell.2014.03.011.
Bian X, Wu W, Yang L, Lv L, Wang Q, Li Y, et al. Administration of Akkermansia muciniphila ameliorates dextran sulfate sodium-induced ulcerative colitis in mice. Front Microbiol 2019. https://doi.org/10.3389/fmicb.2019.02259.
Chelakkot C, Choi Y, Kim D-K, Park HT, Ghim J, Kwon Y, et al. Akkermansia muciniphila-derived extracellular vesicles influence gut permeability through the regulation of tight junctions. Exp Mol Med 2018. https://doi.org/10.1038/emm.2017.282.
Cheng P, Yao J, Wang C, Zhang L, Kong W. Molecular and cellular mechanisms of tight junction dysfunction in the irritable bowel syndrome. Mol Med Rep 2015. https://doi.org/10.3892/mmr.2015.3808.
Dahmer PL, DeRouchey JM, Gebhardt JT, Paulk CB, Jones CK. Summary of methodology used in enterotoxigenic Escherichia coli (ETEC) challenge experiments in weanling pigs and quantitative assessment of observed variability. Transl Anim Sci 2023. https://doi.org/10.1093/tas/txad083.
Dalrymple SA, Slattery R, Aud DM, Krishna M, Lucian LA, Murray R. Interleukin-6 is required for a protective immune response to systemic Escherichia coli infection. Infect Immun 1996. https://doi.org/10.1128/iai.64.8.3231-3235.1996.
Depommier C, Everard A, Druart C, Plovier H, van Hul M, Vieira-Silva S, et al. Supplementation with Akkermansia muciniphila in overweight and obese human volunteers: a proof-of-concept exploratory study. Nat Med 2019. https://doi.org/10.1038/s41591-019-0495-2.
Derrien M, Vaughan EE, Plugge CM, de Vos WM. Akkermansia muciniphila gen. nov., sp. nov., a human intestinal mucin-degrading bacterium. Int J Syst Evol Microbiol 2004. https://doi.org/10.1099/ijs.0.02873-0.
Duncan K, Carey-Ewend K, Vaishnava S. Spatial analysis of gut microbiome reveals a distinct ecological niche associated with the mucus layer. Gut Microb 2021. https://doi.org/10.1080/19490976.2021.1874815.
Dziubla T, Butterfield DA. Oxidative stress and biomaterials. London, UK: Elsevier; 2016.
Earley H, Lennon G, Balfe Á, Coffey JC, Winter DC, O'Connell PR. The abundance of Akkermansia muciniphila and its relationship with sulphated colonic mucins in health and ulcerative colitis. Sci Rep 2019. https://doi.org/10.1038/s41598-019-51878-3.
Everard A, Belzer C, Geurts L, Ouwerkerk JP, Druart C, Bindels LB, et al. Cross-talk between Akkermansia muciniphila and intestinal epithelium controls diet-induced obesity. Proc Natl Acad Sci USA 2013. https://doi.org/10.1073/pnas.1219451110.
Frese SA, Parker K, Calvert CC, Mills DA. Diet shapes the gut microbiome of pigs during nursing and weaning. Microbiome 2015. https://doi.org/10.1186/s40168-015-0091-8.
Guevarra RB, Lee JH, Lee SH, Seok M-J, Kim DW, Kang BN, et al. Piglet gut microbial shifts early in life: causes and effects. J Anim Sci Biotechnol 2019. https://doi.org/10.1186/s40104-018-0308-3.
Hänninen A, Toivonen R, Pöysti S, Belzer C, Plovier H, Ouwerkerk JP, et al. Akkermansia muciniphila induces gut microbiota remodelling and controls islet autoimmunity in NOD mice. Gut 2018. https://doi.org/10.1136/gutjnl-2017-314508.
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 Physiol Anim Nutr 2013. https://doi.org/10.1111/j.1439-0396.2012.01284.x.
Huwart SJP, de Wouters d'Oplinter A, Rastelli M, van Hul M, de Vos WM, Luquet S, et al. Food reward alterations during obesity are associated with inflammation in the striatum in mice: beneficial effects of Akkermansia muciniphila. Cells 2022. https://doi.org/10.3390/cells11162534.
Kadaoui KA, Corthésy B. Secretory IgA mediates bacterial translocation to dendritic cells in mouse Peyer's patches with restriction to mucosal compartment. J Immunol 2007. https://doi.org/10.4049/jimmunol.179.11.7751.
Lewkowicz P, Lewkowicz N, Sasiak A, Tchórzewski H. Lipopolysaccharide-activated CD4+CD25+ T regulatory cells inhibit neutrophil function and promote their apoptosis and death. J Immunol 2006. https://doi.org/10.4049/jimmunol.177.10.7155.
Luo Y, Zhang L, Li H, Smidt H, Wright A-DG, Zhang K, et al. Different types of dietary fibers trigger specific alterations in composition and predicted functions of colonic bacterial communities in BALB/c mice. Front Microbiol 2017. https://doi.org/10.3389/fmicb.2017.00966.

Luo Y, He J, Li H, Lan C, Cai J, Chen H, et al. Wheat bran fermented by mixed fungal strains improves the digestibility of crude fiber and may benefit the gut health without impacting the growth performance in weaned pigs. Food Funct 2021. https://doi.org/10.1039/d1fo00273b.

Luo Y, Lan C, Xie K, Li H, Devillard E, He J, et al. Active or autoclaved Akkermansia muciniphila relieves TNF-α-induced inflammation in intestinal epithelial cells through distinct pathways. Front Immunol 2021. https://doi.org/10.3389/fimmu.2021.788638.

Luo Y, Lan C, Li H, Ouyang Q, Kong F, Wu A, et al. Rational consideration of Akkermansia muciniphila targeting intestinal health: advantages and challenges. NPJ Biofilms Microbiomes 2022. https://doi.org/10.1038/s41522-022-00338-4.

Luo Y, Ren W, Smidt H, Wright A-DG, Yu B, Schyns G, et al. Dynamic distribution of gut microbiota in pigs at different growth stages: composition and contribution. Microbiol Spectr 2022. https://doi.org/10.1128/spectrum.00688-21.

Ma J, Liu Z, Gao X, Bao Y, Hong Y, He X, et al. Gut microbiota remodeling improves natural aging-related disorders through Akkermansia muciniphila and its derived acetic acid. Pharmacol Res 2023. https://doi.org/10.1016/j.phrs.2023.106687.

Mantis NJ, Rol N, Corthésy B. Secretory IgA's complex roles in immunity and mucosal homeostasis in the gut. Mucosal Immunol 2011. https://doi.org/10.1038/mi.2011.41.

Nagy B, Fekete PZ. Enterotoxigenic Escherichia coli in veterinary medicine. Int J Med Microbiol 2005. https://doi.org/10.1016/j.ijmm.2005.07.003.

NRC (National Research Council). Nutrient requirements of swine. 11th ed. Washington, DC, USA: National Academies Press; 2012.

Okeke EB, Uzonna JE. The pivotal role of regulatory T cells in the regulation of innate immune cells. Front Immunol 2019. https://doi.org/10.3389/fimmu.2019.00680.

Okeke EB, Mou Z, Onyilagha N, Jia P, Gounni AS, Uzonna JE. Deficiency of phosphatidylinositol 3-kinase δ signaling leads to diminished numbers of regulatory T cells and increased neutrophil activity resulting in mortality due to endotoxic shock. J Immunol 2017. https://doi.org/10.4049/jimmunol.1600954.

Opapeju FO, Krause DO, Payne RL, Rademacher M, Nyachoti CM. Effect of dietary protein level on growth performance, indicators of enteric health, and gastrointestinal microbial ecology of weaned pigs induced with postweaning colibacillosis. J Anim Sci 2009. https://doi.org/10.2527/jas.2008-1310.

Paone P, Cani PD. Mucus barrier, mucins and gut microbiota: the expected slimy partners? Gut 2020. https://doi.org/10.1136/gutjnl-2020-322260.

Parada Venegas D, La Fuente MK, Landskron G, González MJ, Quera R, Dijkstra G, et al. Short chain fatty acids (SCFAs)-Mediated gut epithelial and immune regulation and its relevance for inflammatory bowel diseases. Front Immunol 2019. https://doi.org/10.3389/fimmu.2019.00277.

Plovier H, Everard A, Druart C, Depommier C, van Hul M, Geurts L, et al. A purified membrane protein from Akkermansia muciniphila or the pasteurized bacterium improves metabolism in obese and diabetic mice. Nat Med 2017. https://doi.org/10.1038/nm.4236.

Pluske JR, Hampson DJ, Williams IH. Factors influencing the structure and function of the small intestine in the weaned pig: a review. Livest Prod Sci 1997. https://doi.org/10.1016/S0301-6226(97)00057-2.

Rao R. Oxidative stress-induced disruption of epithelial and endothelial tight junctions. Front Biosci 2008. https://doi.org/10.2741/3223.

Sauvaitre T, van Landuyt J, Durif C, Roussel C, Sivignon A, Chalancon S, et al. Role of mucus-bacteria interactions in Enterotoxigenic Escherichia coli (ETEC) H10407 virulence and interplay with human microbiome. NPJ Biofilms Microbiomes 2022. https://doi.org/10.1038/s41522-022-00344-6.

Turck D, Bohn T, Castenmiller J, de Henauw S, Hirsch-Ernst KI, Maciuk A, et al. Safety of pasteurised Akkermansia muciniphila as a novel food pursuant to Regulation (EU) 2015/2283. EFSA J 2021. https://doi.org/10.2903/j.efsa.2021.6780.

van der Lugt B, van Beek AA, Aalvink S, Meijer B, Sovran B, Vermeij WP, et al. Akkermansia muciniphila ameliorates the age-related decline in colonic mucus thickness and attenuates immune activation in accelerated aging Ercc1-/Δ7 mice. Immun Ageing 2019. https://doi.org/10.1186/s12979-019-0145-z.

Vigsnæs LK, Brynskov J, Steenholdt C, Wilcks A, Licht TR. Gram-negative bacteria account for main differences between faecal microbiota from patients with ulcerative colitis and healthy controls. Benef Microbes 2012. https://doi.org/10.3920/BM2012.0018.

Wang Y, Chen Y, Zhang X, Lu Y, Chen H. New insights in intestinal oxidative stress damage and the health intervention effects of nutrients: a review. J Funct Foods 2020. https://doi.org/10.1016/j.jff.2020.104248.

Xia J, Lv L, Liu B, Wang S, Zhang S, Wu Z, et al. Akkermansia muciniphila ameliorates acetaminophen-induced liver injury by regulating gut microbial composition and metabolism. Microbiol Spectr; 2022. https://doi.org/10.1128/spectrum.01596-21.

Zhang T, Li P, Wu X, Lu G, Marcella C, Ji X, et al. Alterations of Akkermansia muciniphila in the inflammatory bowel disease patients with washed microbiota transplantation. Appl Microbiol Biot 2020. https://doi.org/10.1007/s00253-020-10948-7.

Zhang Z, Wu X, Cao S, Cromie M, Shen Y, Feng Y, et al. Chlorogenic acid ameliorates experimental colitis by promoting growth of Akkermansia in mice. Nutrients 2017. https://doi.org/10.3390/nu9070677.

Zhang L, Carmody RN, Kalariya HM, Duran RM, Moskal K, Poulev A, et al. Grape proanthocyanidin-induced intestinal bloom of Akkermansia muciniphila is dependent on its baseline abundance and precedes activation of host genes related to metabolic health. J Nutr Biochem 2018. https://doi.org/10.1016/j.jnutbio.2018.02.009.

Zhao S, Liu W, Wang J, Shi J, Sun Y, Wang W, et al. Akkermansia muciniphila improves metabolic profiles by reducing inflammation in chow diet-fed mice. J Mol Endocrinol 2017. https://doi.org/10.1530/JME-16-0054.

Zhou JY, Alvarez CA, Cobb BA. Integration of IL-2 and IL-4 signals coordinates divergent regulatory T cell responses and drives therapeutic efficacy. Elife 2021. https://doi.org/10.7554/eLife.57417.

Zhu L, Lu X, Liu L, Voglmeir J, Zhong X, Yu Q. Akkermansia muciniphila protects intestinal mucosa from damage caused by S. pullorum by initiating proliferation of intestinal epithelium. Vet Res 2020. https://doi.org/10.1186/s13567-020-00755-3.