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Original Research Article

The impact of combined thymol and rosmarinic acid on the intestinal microbiota and barrier function of the piglets challenged by *Escherichia coli* K88

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ABSTRACT

It has been found that thymol (Thy) and rosmarinic acid (Ros-A) improve the growth performance of piglets and relieve intestinal inflammation in animals. The effects of Thy and Ros-A separately or in combination (Thy × Ros-A) on the intestinal function and health of piglets challenged with *Escherichia coli* K88 (*E. coli* K88) were investigated. A total of 30 piglets aged 21 d were assigned to 5 groups ($n = 6$). The control (Con) and K88 groups piglets received a basal diet, while the Thy, Ros-A, and Thy × Ros-A groups were fed a basal diet supplemented with 500 mg/kg Thy, 500 mg/kg Ros-A, and 250 mg/kg Thy + 250 mg/kg Ros-A, respectively. On the 19th and 20th day, piglets in the K88, Thy, Ros-A, and Thy × Ros-A groups were orally administered 10 mL of phosphate-buffered saline (PBS) containing approximately 1×10^9 CFU/mL of *E. coli* K88, while the Con group received an equal volume of PBS. The results showed that the Thy × Ros-A treatment reduced the damage to ileal villi induced by the *E. coli* K88 challenge, leading to longer villi in the ileum ($P < 0.05$). Thy and Ros-A modulated the composition of the ileal microbiota. Compared to the K88 group, the Thy × Ros-A group had a higher abundance of *Lactobacillus* and *Romboutsia*, while *Escherichia-Shigella* and *Desulforvibrio* were lower ($P < 0.05$). Additionally, the Thy × Ros-A group showed elevated levels of gene and protein expressions for zonula occludens-1, occludin, and claudin-1 compared to the K88 group ($P < 0.05$). In conclusion, combining Thy and Ros-A reduced ileal damage and relieved the inflammation in weaned piglets challenged with *E. coli* K88 by regulating intestinal microflora and improving barrier function.

1. Introduction

The intestine contains a variety of microbes that are crucial for enhancing host nutrient utilization, regulating immunity, and promoting intestinal health (Kabat et al., 2014). Maintaining intestinal health ensures the integrity of intestinal epithelial function and retain normal digestion-absorption function (Svihus, 2014). However, the weaned piglets have an immature gastrointestinal tract which is susceptible to multiple stress and pathogens in the environment, causing intestinal dysfunction, intestinal inflammation and barrier damage. Among these pathogens, *Escherichia coli* causes intestinal barrier dysfunction, immune disorders, and nutrient digestibility decline, which leads to growth restriction and diarrhea (Fl et al., 2020). Therefore, there is a need for safe, efficient and environmentally-friendly feed additives, with plant extracts gaining significant attention for their potential in this regard (Nour et al., 2017).

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Thymol (Thy), a small-molecule monoterpenoid, has a broad-spectrum antibacterial effect (Barbosa et al., 2019). Studies have found that Thy improved nutrient bioavailability in the intestine by directly acting on the intestinal epithelial cells to enhance nutrient absorption (Omonijo et al., 2019; Zargar et al., 2019). Thy has also shown promise in modulating microbiota composition and enhancing the intestinal barrier in piglets (Van Noten et al., 2020). Moreover, combining Thy with other phenolic acids has been found to enhance efficacy in mitigating the impacts of *E. coli* infection on growth performance and intestinal health of weaned piglets (Chang et al., 2022; Rodrigues et al., 2020; Yang et al., 2019). Rosmarinic acid (Ros-A), predominantly found in *Labiatae* plants, exhibits potent inhibitory effects against *E. coli* and *Staphylococcus aureus* (Sun et al., 2005). Previous studies found that Ros-A relieved inflammatory bowel disease and promoted intestinal health (Quan et al., 2021). Although no studies have shown that Ros-A could promote the growth and development of weaning piglets, the research has confirmed that Ros-A could improve the barrier function of IPEC-J2 cells and has anti-inflammatory and antioxidant effects (Pomothy et al., 2020), which may contribute to the intestinal health. Furthermore, in our previous study, we found that the combination of Thy and Ros-A enhanced the antibacterial susceptibility and improved free radical scavenging rate (Fig. S1). Based on these findings, we hypothesized that a diet supplemented with a combination of Thy and Ros-A could modulate the intestinal microbiota, improve barrier function, and relieve inflammation in piglets challenged with *E. coli* K88. To verify our hypothesis, an *E. coli* K88 challenged piglet model was established, and intestinal morphology, intestinal microbiota, and intestinal barrier function were evaluated.

2. Materials and methods

2.1. Animal ethics statement

All procedures conducted in animal experiments strictly adhered to the Guidelines for the Care and Use of Laboratory Animals issued by the People's Republic of China's National Science and Technology Committee in 1988, and in accordance with the ARRIVE. Furthermore, the experiment was approved by the Animal Care and Use Committee of Nanjing Agricultural University (SYXK 2019-0771).

2.2. Animal trial

A total of 30 piglets aged 21 d (Duroc × Landrace × Large White, 6.85 ± 0.75 kg) were randomly divided into five groups with six replicates of one pig each, including a control group (Con), *E. coli* K88 group (K88), thymol (Thy) group, rosmarinic acid (Ros-A) group, and combination of thymol and rosmarinic acid group (Thy × Ros-A, 1:1). The dietary interventions in this study were as follows: 1) a basal diet (Con and K88); 2) the basal diet with the addition of 500 mg/kg Thy; 3) the basal diet with the addition of 500 mg/kg (Ros-A); 4) the basal diet with the addition of 250 mg/kg Thy + 250 mg/kg Ros-A (Thy × Ros-A). Thy (>99%) and Ros-A (>95%) were purchased from J&K Scientific Biotechnology Co., Ltd. (Beijing, China). The basal diet was formulated in accordance with NRC (2012), as detailed in Table 1. Feed samples were crushed (GJ100-2, Tongyong, Nanchang) and sieved to a 0.42-mm standard, analyzed for dry matter (method 930.15) and crude protein (method 984.13) according to AOAC (2005), and amino acids were determined according to the method described in China National Standards (GB/T 18246-2019). During the 3-d adaptation period, all piglets were exclusively fed the basic diet daily.

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

Item	Content
Ingredients	
Corn	42.30
Soybean meal	19.60
Extruded full-fat soybean	10.00
Soy protein concentrate	4.00
Fish meal	10.00
High protein whey powder	5.00
Soybean oil	4.00
Salt	0.25
L-Lysine HCl, 78%	0.65
DL-Methionine, 98%	0.05
L-Threonine, 98%	0.14
L-Tryptophan, 98%	0.01
Premix[2]	4.00
Total	100.00
Nutrient levels[1]	
Dry matter	87.05
CP	20.62
DE, Mcal/kg	35.61
Crude fat	7.90
Lys	1.60
Met + Cys	0.85
Thr	0.84
Try	0.25

[1] Digestible energy was calculated based on the Chinese Feed Database (2012), and the nutrient levels such as crude protein and crude fat were determined values.
[2] One kilogram premix contained the following: vitamin A 200 kIU, vitamin D_3 50 kIU, vitamin E 500 mg, vitamin K_3 30 mg, vitamin B_1 25 mg, vitamin B_2 100 mg, vitamin B_6 35 mg, vitamin B_{12} 0.25 mg, niacin 400 mg, pantothenic acid 300 mg, folic acid 14 mg, D-biotin 1.0 mg, choline chloride 5000 mg, Cu 50 mg, Fe 100 mg, Zn 100 mg, Mn 50 mg, I 3 mg, Se 2.5 mg, Ca 10%, total P 1.5%, sodium chloride 5%, arginine 7%, phytase 20,000 U.

On the 19th and 20th d, piglets in the K88 group, Thy group, Ros-A group, and Thy × Ros-A group were orally given 10 mL of phosphate-buffered saline (PBS) containing approximately 1×10^9 CFU/mL of *E. coli* K88 in accordance with the specific protocol conducted by Huang et al. (2020). Meanwhile, Con group piglets were given equal amounts of PBS, and all piglets had free access to feed and water throughout the experiment. On the 21st d of the experiment, all piglets were anesthetized and sampled.

We followed the research methods outlined by Hu et al. (2019), and recorded the weights of piglets on d 1, 7, 14, 19, and 21, as well as their daily feed intake. For the piglets, we calculated their average daily gain (ADG), average daily feed intake (ADFI), and feed-to-gain ratio (F:G). Additionally, the diarrhea rate and fecal score were calculated using the daily observed piglet fecal morphology (Zhang et al., 2021). The feces were visually assessed daily, and fresh excrement was graded as follows: 1 = hard feces, 2 = slightly soft feces, 3 = soft feces (partially formed), 4 = loose (semi-liquid) feces, and 5 = watery (mucous) feces. Piglets with fecal scores of 4 and 5 were classified as having diarrhea.

Fecal score = sum of fecal scores per piglet/(number of experiment days × number of piglets).
Diarrhea rate (%) = 100 × (number of piglets with diarrhea × number of diarrhea days)/(total number of piglets × number of experiment days).

Piglets (n = 6) were anesthetized by intravenous injection of 90 mg/kg BW pentobarbital sodium following the procedure outlined by Moeser et al. (2022) and then euthanized. Subsequently, ileal chyme was preserved at −80 °C. Mid-ileum was isolated and 1 to 2 cm ileum samples were collected and gently rinsed with 0.9%

normal saline. To assess intestinal morphology, 2 cm mid-ileum specimens were fixed in a 4% paraformaldehyde solution after rinsing. Another set of mid-ileum tissue was used to obtain ileal mucosa by glass slides and preserved at −80 °C.

2.3. 16S rRNA analysis of ileal chyme

A quantity of 0.3 g thawed ileal chyme was weighed for each group ($n = 6$), and the microbial cDNA was exacted from the ileal chyme according to the cetyltrimethyl ammonium bromide (CTAB) method (Hu et al., 2019). OD 260/280 absorbance ratio was used to evaluate the quality and concentration of cDNA using a micro-spectrophotometer (Thermo Fisher Scientific, Waltham, U.S.). This ratio is commonly used to gauge the purity of nucleic acid samples, with an optimal value typically being around 1.8 to 2.0 for high-quality cDNA.

Afterward, the 16S rRNA gene is amplified using the primer pair of 338F (5′-ACTCCTACGGGAGGAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) and specific amplification conditions via PCR (Hu et al., 2019). Subsequently, these PCR products were purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, USA). This purification step is essential to remove any unwanted contaminants and obtain high-quality DNA samples for downstream applications. To generate sequencing libraries, the purified amplicons were processed using Illumina's Ion Plus Fragment Library Kit 48 reactions (Thermo Fisher Scientific). Following standard protocols, the prepared libraries were sequenced on the MiSeq platform by Illumina. 16S sequencing raw data have been deposited in the NCBI Sequence Read Archive Database and are accessible via Accession Number: SUB12204336.

The paired-end reads generated by MiSeq sequencing were initially processed through a series of steps: demultiplexing and quality filtering by using QIIME (version 1.70), comparative OTU clustering, representative sequence analysis, and taxonomic analysis with Silva 16S rRNA database (http://www.arb-silva.de). To identify and classify microbial taxa in samples, this pipeline analyzes 16S rRNA gene sequencing data. The analysis of microbiota diversity was conducted using MOTHUR (version 1.30.2, http://www.mothur.org/wiki/Download_mothur), which included the calculation of various diversity indices. Additionally, to calculate dissimilarities, a Bray-Curtis-based distance algorithm was applied using the vegan package in R (version 3.5.3).

2.4. Measurements of microbial metabolites

Lactate in the chyme was determined by the Lactate assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Pre-processing of ileal chyme ($n = 6$) was performed following the procedure described by Hu et al. (2019). The concentrations of short-chain fatty acids (SCFA) in the ileal chyme were determined using a gas chromatograph (Shimadzu, GC-14A with FID detector) with a capillary GC column (30 m × 0.25 μm × 0.32 mm). Column temperature was 140 °C, inlet temperature was 180 °C, and detector temperature was 180 °C.

2.5. Measurements of mucosal morphology in the ileum

Following fixation in 4% paraformaldehyde solution for a duration of 48 h, middle ileal specimens ($n = 6$) were randomly selected for each treatment, and embedded with a standard paraffin technique, and then sectioned into slices with a thickness of 6 μm. The tissue slices, which were subjected to hematoxylin and eosin staining, were placed in the virtual microscope, and 10 random fields of view were selected to be observed and taken pictures. Finally, the quantitative measurements for the key parameters of the ileum were conducted by Image-Pro-Plus software.

2.6. Measurements of cytokines and diamine oxidase (DAO) level in the ileum mucosa

Ileal mucosa samples (0.1 g) were weighed and homogenized using an ultrasonic homogenizer in 0.9 mL of PBS (4 °C, precooling). The homogenate was subjected to centrifugation (3000 × g, 15 min at 4 °C). The protein concentration of the supernatant was initially assessed for calibration purposes using a commercial BCA kit (Solarbio, Beijing, China).

The levels of cytokines in the ileal mucosa, including interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-12 (IL-12), tumor necrosis factor-α (TNF-α), secretory immunoglobulin A (sIgA) and the levels of malondialdehyde (MDA), total superoxide dismutase (T-SOD), and total antioxidant capacity (T-AOC), glutathione peroxidase (GSH-PX) were evaluated by Elisa assay kits (Enzyme-linked Biotechnology Co., Ltd., Shanghai, China) in accordance with manufacturer's instructions.

2.7. RNA extraction and RT- PCR

Total RNA was extracted from ileal mucosal tissue (0.1 g) using TRIzol reagent (Takara Bio, Otsu, Japan) in accordance with manufacturer's instructions. A real-time PCR System was conducted using a sequence detector system (ABI 7300 SDS; Foster City, U.S.). A 20-μL reaction mixture (Takara Biotechnology, Dalian, China) was composed following the guidelines provided by the manufacturer, and each sample was subjected to three replications. PCR amplification conditions were incubated at 95 °C for 30 s, followed by 45 cycles of 5 s at 95 °C, incubated at 62 °C for 30 s. All primers and their sequences are detailed in Table S1. The expression levels of target mRNA were normalized to β-actin and calculated using the $2^{-\Delta\Delta Ct}$ method.

2.8. Western blot

Protein extraction and immunoblotting techniques employed in this experiment were performed following the methodology outlined by Hu et al. (2012). 100 mg ileal tissue was weighed for each group, and 1 mL of a pre-cooling mixture radioimmunoprecipitation assay lysis buffer and protease inhibitors (Beyotime Institute of Biotechnology) were added into the samples, and the mixture was ground to extract protein from ileal tissue. The supernatant was acquired through centrifugation (1000 × g, 10 min at 4 °C). Afterward, each sample was normalized based on the detected protein levels.

Equal protein samples were boiled and then subjected to isolation through a 4% to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-Page) process. Following electrophoresis, the proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Merck Millipore). The membranes were first blocked in a solution containing 1 × Tris-buffered saline-Tween (TBST), which consisted of 0.05% Tween-20, 100 mmol/L Tris-HCl, and 150 mmol/L NaCl, with a pH of 7.5. To this blocking solution, 5% defatted milk powder was added, and the membranes were allowed to incubate in this blocking buffer for 60 min. The membranes were then incubated with antibodies (anti-rabbit) for occludin (1:1500; Proteintech), Toll-like receptor-4 (TLR-4; 1:1800; Proteintech), zonula occludens-1 (ZO-1; 1:1800; Proteintech), myeloid differentiation primary response 88 (MyD88; 1:1800; Proteintech), claudin-1 (1:1800; Proteintech), nuclear factor-kappa-B (NF-κB; 1:1800; Proteintech), β-actin (1:1800; Proteintech) and p-NF-κB (1:1800; Proteintech) for 10 h at 4 °C.

Subsequently, the membranes were subjected to three 10 min rinse in 1 × TBST. Then the membranes were incubated with a secondary antibody (1:2000, Abcam) for a duration of 1 h. Finally, the target bands on PVDF membranes were quantified by using an electrochemiluminescence system (Shanghai Tanon Technology Co., Ltd, Shanghai, China) and Image-Pro Plus software.

2.9. Statistical analysis

Data analysis was conducted using SPSS 26.0 (IBM, USA). For normally distributed data, a one-way analysis of variance (ANOVA) model was used, described as

$$Y_{ij} = \mu + \alpha_i + \epsilon_{ij},$$

where Y_{ij} is the observed value, μ is the overall mean, α_i represents the group effect, and ϵ_{ij} is the error term. Between-group differences were evaluated using Tukey's post-hoc test, calculating the standardized mean difference as

$$q = \frac{|\overline{Y}_i - \overline{Y}_j|}{\sqrt{\frac{\mathrm{MSE}}{n}}},$$

where $\overline{Y}_i$ and $\overline{Y}_j$ are the group means, MSE is the mean square error, and n is the sample size per group. For non-normally distributed data, the Kruskal–Wallis test was applied, based on rank distribution, with the test statistic

$$H = \frac{12}{N(N+1)} \sum_{i=1}^{k} \frac{R_i^2}{n_i} - 3(N+1),$$

where N is the total number of samples, R_i is the sum of ranks for group i, n_i is the number of samples in the i-th group. If significant differences were found, Dunn's post-hoc test was conducted to compare mean ranks between groups, calculating the standardized value as

$$Z = \frac{|\overline{R}_i - \overline{R}_j|}{\sqrt{\frac{N(N+1)}{12}\left(\frac{1}{n_i} + \frac{1}{n_j}\right)}},$$

where N is the total number of samples, $\overline{R}_i$ and $\overline{R}_j$ are the mean ranks for groups i and j, n_i and n_j are the number of samples in the i-th and j-th group, respectively. A significance level of $P < 0.05$ was set to determine statistical significance.

3. Results

3.1. Growth performance of piglets

During 14 to 21 d, the K88 group had the highest feed-to-gain ratio among the five treatments ($P < 0.05$, Table 2). During d 19 and 21, the K88 group exhibited significantly lower ADFI and ADG than the Con group with a significantly higher F:G ($P < 0.05$). In contrast, Thy × Ros-A group had these indicators significantly lower than the K88 group ($P < 0.05$). The Thy × Ros-A group had significantly lower diarrhea rates and fecal score than the Con and K88 groups during d 1 to 7 and d 7 to 14, and during d 19 to 21, their diarrhea rates and fecal score were lower than the K88 group ($P < 0.05$).

3.2. Morphological structure of ileum

The K88 group exhibited a major reduction in villus height and an increase in crypt depth compared to the Con group ($P < 0.05$). In contrast, the Thy × Ros-A group demonstrated a significant rise in both villus height and the ratio of villus-to-crypt, whereas crypt depth exhibited a decline ($P < 0.05$) compared to the K88 group. Similarly, a decrease in crypt depth and an increase in villus height was observed in the K88 group, Thy group and Ros-A group ($P < 0.05$, Fig. 1).

3.3. Antioxidant enzyme in ileal mucosa

The K88 group showed a decreased antioxidant capacity in ileal mucosa (Table 3). The MDA concentration in the K88 group significantly exceeded that in the Con group, while it was reduced in the Thy group and notably decreased in the Thy × Ros-A group ($P < 0.05$). Conversely, the concentrations of GSH-PX and T-AOC in the Thy × Ros-A group showed a significant increase compared to the K88 group ($P < 0.05$).

3.4. Nutrient transporters in ileal mucosa

The nutrient transport function in the ileum was decreased after the piglets were challenged by *E. coli* K88 (Fig. 2). The relative gene expressions of *EACC-1*, *SLC6A19* and *SGLT1* in the ileum of the K88 group were down-regulated than those in the Con group ($P < 0.05$). However, Thy and Ros-A and their combination treatments improved the nutrient transport ability. Specifically, *EACC-1*, *SLC6A19*, and *GLUT2* relative expression in the Thy group, Ros-A group, and Thy × Ros-A group were greatly increased compared to the K88 group ($P < 0.05$).

3.5. Microbiota and metabolites in ileal chyme

In the K88 group, the Shannon index of the ileal microbiota was substantially decreased ($P < 0.05$, Fig. 3A). PCoA analysis was used to evaluate the β-diversity of piglets' ileal microbiota, revealing noteworthy changes among the five groups ($P < 0.05$, Fig. 3B). Firmicutes were substantially decreased, and Proteobacteria was greatly increased after the *E. coli* K88 challenge ($P < 0.05$, Fig. 4A–B). However, the relative abundance of Firmicutes was greatly increased in the Ros-A group and Thy × Ros-A group ($P < 0.05$), while Proteobacteria were significantly decreased in the Thy group, Ros-A group, and Thy × Ros-A group ($P < 0.05$) in comparison to the K88 group.

Furthermore, we examined distinctions among the various groups at the genus level, as illustrated in Fig. 4C and D. The *E. coli* K88 challenge had a major impact on the microbiota within the K88 group, notably leading to a reduction in the relative abundances of *Lactobacillus* and *Romboutsia* ($P < 0.05$), and an increase in the relative abundances of *Escherichia-Shigella*, *Actinobacillus*, *Terrisporobacter*, and *Desulforvibrio* ($P < 0.05$). However, Thy, Ros-A and their combination in piglet diets appeared to mitigate these changes in the ileal microbiota of *E. coli* K88-challenged piglets. For instance, the relative abundances of *Lactobacillus* and *Romboutsia* in the Thy × Ros-A group were greatly increased, while the *Escherichia-Shigella*, *Actinobacillus*, *Terrisporobacter* and *Desulforvibrio* were greatly decreased ($P < 0.05$) compared to that in the K88 group. Importantly, these changes in the Thy × Ros-A group were slightly different from those observed in the Con group.

Table 2
Effects of thymol, rosmarinic acid, and their combination on the growth performance of weaned piglets.

Item	Con	K88	Thy	Ros-A	Thy × Ros-A	SEM	*P*-value
1–7 d							
ADFI, g	336.83	348.67	322.92	308.21	318.91	9.392	0.710
ADG, g	0.27	0.27	0.26	0.26	0.29	0.005	0.475
F:G	1.31	1.30	1.24	1.25	1.11	0.038	0.510
Fecal score	2.11^{a}	2.19^{a}	1.83^{ab}	2.04^{ab}	1.62^{b}	0.056	0.002
Diarrhea rate, %	50.00^{a}	47.62^{a}	33.33^{ab}	35.17^{ab}	23.81^{b}	3.379	0.043
7–14 d							
ADFI, g	592.45	582.89	566.94	554.76	563.58	12.079	0.881
ADG, g	0.27^{b}	0.27^{b}	0.28^{ab}	0.28^{ab}	0.31^{a}	0.006	0.027
F:G	1.93^{a}	1.92^{a}	1.85^{ab}	1.76^{ab}	1.58^{b}	0.049	0.045
Fecal score	1.83	1.86	1.79	1.79	1.64	0.030	0.174
Diarrhea rate, %	23.81	21.43	16.67	16.67	9.52	1.459	0.075
14–21 d							
ADFI, g	529.64	507.65	536.23	571.67	562.74	24.334	0.934
ADG, g	0.27	0.26	0.28	0.28	0.30	0.007	0.321
F:G	1.97^{b}	2.33^{a}	1.90^{b}	1.99^{b}	1.92^{b}	0.047	0.017
Fecal score	1.83^{b}	2.42^{a}	2.17^{b}	2.20^{b}	1.89^{b}	0.050	0.012
Diarrhea rate, %	13.89^{b}	47.22^{a}	25.00^{b}	25.00^{b}	16.67^{b}	2.852	0.012
19–21 d							
ADFI, g	620.44^{a}	521.98^{b}	581.13^{ab}	579.09^{ab}	611.33^{a}	11.693	0.048
ADG, g	0.35^{a}	0.27^{b}	0.31^{ab}	0.31^{ab}	0.33^{a}	0.007	0.012
F:G	1.78^{b}	1.97^{a}	1.90^{ab}	1.87^{ab}	1.82^{b}	0.019	0.010
Fecal score	1.67^{c}	2.72^{a}	2.50^{b}	2.44^{b}	2.00^{bc}	0.090	0.010
Diarrhea rate, %	22.22^{b}	66.67^{a}	33.33^{b}	41.27^{ab}	27.78^{b}	4.883	0.014

ADG = average daily gain; ADFI = average daily feed intake; F:G = feed-to-gain ratio. K88 = *Escherichia coli* K88 group.
Con and K88, basal diets; Thy, basal diets supplemented with 500 mg/kg thymol; Ros-A, basal diets supplemented with 500 mg/kg rosmarinic acid; Thy × Ros-A, basal diets supplemented with 250 mg/kg thymol and 250 mg/kg rosmarinic acid.
Piglets were orally gavaged with 10 mL PBS, containing *E. coli* K88 at 10^{9} CFU/mL, except that piglets in the Con group were administered 10 mL of PBS.
Noteworthy differences ($P < 0.05$) among the five groups are indicated by different lowercase superscript letters, $n = 6$.

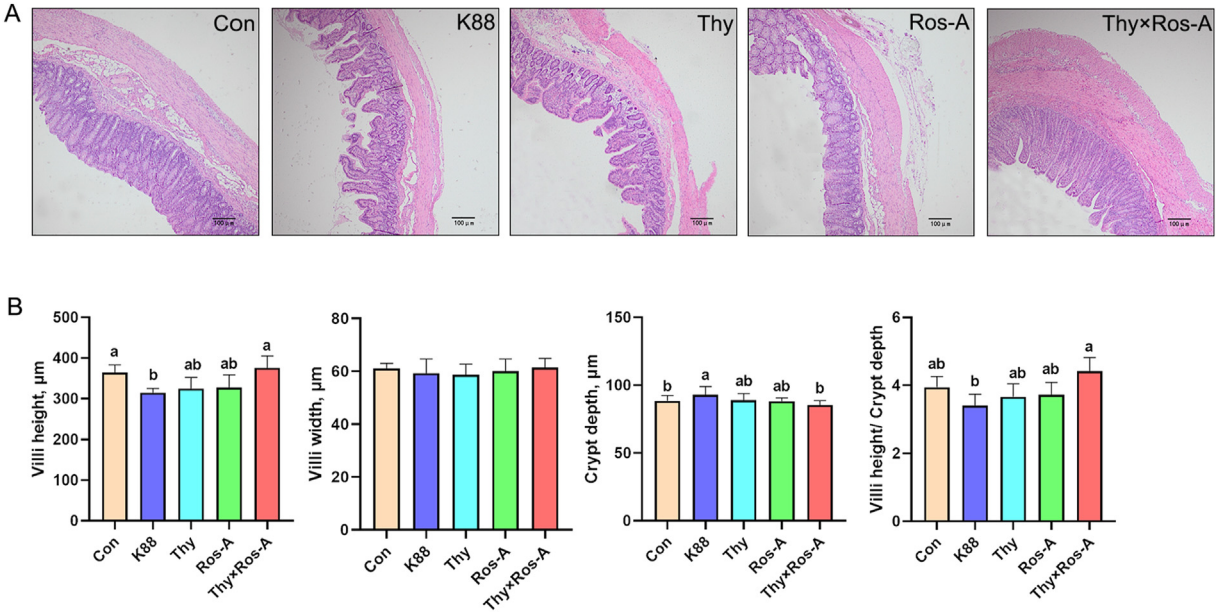

Fig. 1. Effects of thymol, rosmarinic acid and their combination on morphology in the ileum of weaned piglets challenged by *E. coli* K88. (A) Representative histological micrographs of ileum in weaned piglets (hematoxylin and eosin staining; magnification 50×). (B) Histological parameters of ileum. Con and K88, basal diets; Thy, basal diets supplemented with 500 mg/kg thymol; Ros-A, basal diets supplemented with 500 mg/kg rosmarinic acid; Thy × Ros-A, basal diets supplemented with 250 mg/kg thymol and 250 mg/kg rosmarinic acid. Piglets were orally gavaged with 10 mL PBS containing *E. coli* K88 at 10^{9} CFU/mL, except that piglets in the Con group were administered 10 mL of PBS. Noteworthy differences ($P < 0.05$) among the five groups are indicated by different lowercase letters, $n = 6$.

3.6. PICRUSt function analysis and correlation analysis

The PICRUSt function analysis revealed large changes in the ileal microbiota function of piglets challenged with *E. coli* K88 (Fig. 5A), with most metabolic functions being downregulated. However, Thy × Ros-A treatment could significantly change this trend, as indicated by the functional analysis heat map resembling that of the Con group. Quantitative analysis of three metabolic pathways (Fig. 5B) revealed significant decreases ($P < 0.05$) in the ileal microbiota involved in carbohydrate metabolism, amino acid metabolism, and energy metabolism in the K88 group compared to the Con group. Conversely, the functions related to amino acid metabolism in the Thy × Ros-A group were substantially up-regulated ($P < 0.05$). Importantly, there were no major differences observed between the Thy × Ros-A group and the Con group in terms of these functions.

Table 3
Effects of thymol, rosmarinic acid and their combination on the concentrations of antioxidant enzymes in the ileum of weaned piglets challenged by *E. coli* K88.

Item	Con	K88	Thy	Ros-A	Thy × Ros-A	SEM	*P*-value
T-AOC, U/mg protein	102.08^{ab}	93.76^{b}	119.82^{a}	116.13^{ab}	123.98^{a}	3.769	0.044
T-SOD, U/mg protein	0.63^{ab}	0.41^{b}	0.48^{b}	0.34^{b}	0.91^{a}	0.051	0.011
MDA, nmol/mg protein	1.15^{b}	1.90^{a}	1.46^{ab}	1.66^{ab}	1.21^{b}	0.068	0.010
GSH-Px, U/mg protein	31.88^{ab}	25.07^{b}	28.80^{ab}	25.07^{b}	34.11^{a}	1.178	0.037

MDA = malondialdehyde; T-SOD = total superoxide dismutase; T-AOC = total antioxidant capacity; GSH-Px = glutathione peroxidase.
Con and K88; basal diets; Thy; basal diets supplemented with 500 mg/kg thymol; Ros-A, basal diets supplemented with 500 mg/kg rosmarinic acid; Thy × Ros-A, basal diets supplemented with 250 mg/kg thymol and 250 mg/kg rosmarinic acid.
Piglets were orally gavaged with 10 mL of PBS, containing *E. coli* K88 at 10^9 CFU/mL, except that piglets in the Con group were administered 10 mL of PBS.
Noteworthy differences ($P < 0.05$) among the five groups are indicated by different superscript letters, $n = 6$.

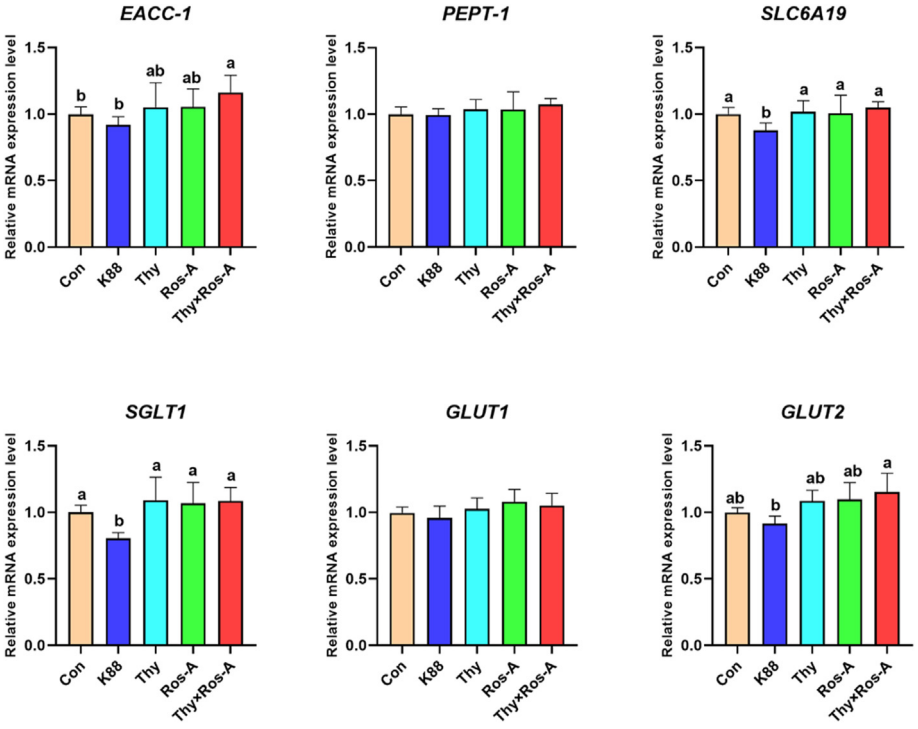

Fig. 2. Effects of thymol, rosmarinic acid and their combination on messenger RNA (mRNA) expression of nutritional transport in the ileum of weaned piglets challenged by *E. coli* K88. *EAAC1* = excitatory amino acid carrier-1; *PEPT1* = peptide transporter 1; *SLC6A19* = solute carrier family 6 member 19; *SGLT1* = sodium glucose co-transporter 1; *GLUT1* = glucose transporter type 1; *GLUT2* = glucose transporter type 2. Con and K88, basal diets; Thy, basal diets supplemented with 500 mg/kg thymol; Ros-A, basal diets supplemented with 500 mg/kg rosmarinic acid; Thy × Ros-A, basal diets supplemented with 250 mg/kg thymol and 250 mg/kg rosmarinic acid. Piglets were orally gavaged with 10 mL phosphate-buffered saline (PBS) containing *E. coli* K88 at 10^9 CFU/mL, except that piglets in the Con group were administered 10 mL of PBS. Noteworthy differences ($P < 0.05$) among the five groups are indicated by different lowercase letters, $n = 6$.

3.7. Microbial metabolites in ileal chyme

After being challenged by *E. coli* K88, concentrations of SCFA and lactate in ileal chyme decreased remarkably, with acetate, butyrate, total SCFA and lactate concentrations in the K88 group notably lower than those in the Con group ($P < 0.05$, Table 4). However, in the Thy group, Ros-A group and Thy × Ros-A group, the concentrations of SCFA and lactate in the ileum increased. Remarkably, in the Thy × Ros-A group, these metabolites were much higher than those in the K88 group ($P < 0.05$), with minor differences observed between the Thy × Ros-A group and the Con group.

3.8. Intestinal barrier integrity

Compared to the Con group, DAO levels in the mucosa of the K88 group decreased significantly ($P < 0.05$), whereas DAO concentrations in the serum increased substantially ($P < 0.05$, Fig. 6A). Furthermore, when comparing the Thy × Ros-A group to the K88 group, there was a significant increase in DAO concentrations in the ileal mucosa ($P < 0.05$), whereas, DAO levels in the serum were substantially decreased ($P < 0.05$). *E. coli* K88 challenge greatly reduced the gene and protein expression of ZO-1, occludin, and claudin-1 in the K88 group ($P < 0.05$, Fig. 6B–D). However, the protein expression of occludin and claudin-1 in the Thy × Ros-A group was also higher than those in the Thy group ($P < 0.05$). In addition, the expression of these proteins was significantly increased in the Thy group, Ros-A group, and Thy × Ros-A group ($P < 0.05$).

3.9. The level of cytokines in the ileal mucosa

In the K88 group, the concentrations of IL-1β, IL-6, IL-8, IL-12, and TNF-α were substantially increased ($P < 0.05$, Table 5) compared to those in the Con group, while IL-10 and sIgA exhibited a significant decrease in concentrations than Con group ($P < 0.05$). However, in Thy group, Ros-A group, and Thy × Ros-A group, the concentrations of IL-1β, IL-6, and IL-12 were significantly decreased ($P < 0.05$), while IL-8 and TNF-α in the Thy group and Thy × Ros-A group also exhibited substantial decrease ($P < 0.05$) compared to

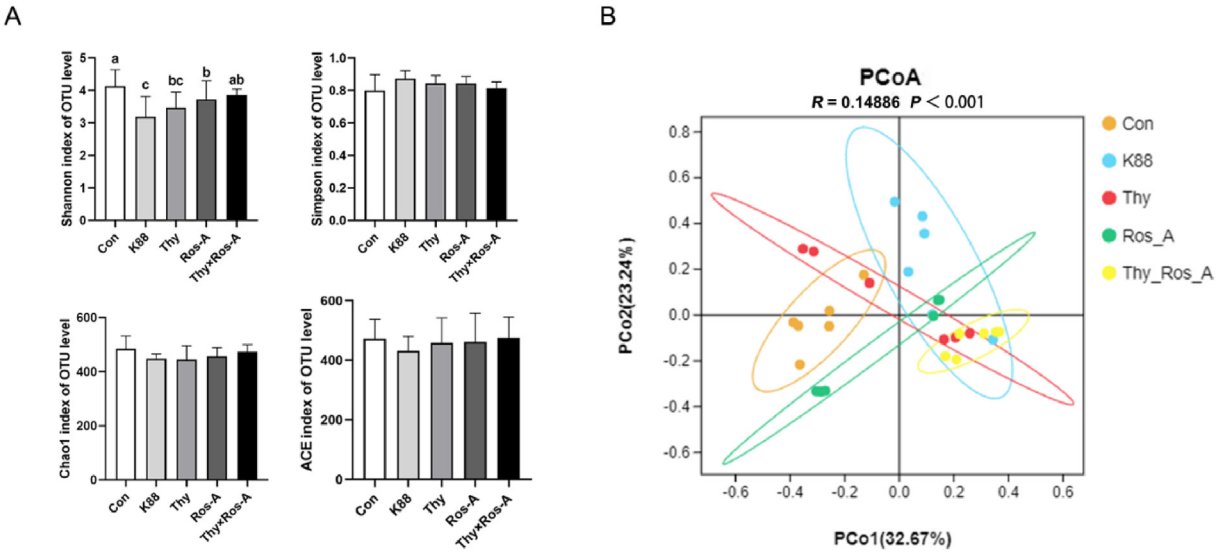

Fig. 3. Effects of thymol, rosmarinic acid and their combination on microbial diversity in the ileum of weaned piglets challenged by *E. coli* K88. (A) Alpha-diversity analysis of ileal microbiota. (B) Beta-diversity analysis of ileal microbiota. Con and K88, basal diets; Thy, basal diets supplemented with 500 mg/kg thymol; Ros-A, basal diets supplemented with 500 mg/kg rosmarinic acid; Thy × Ros-A, basal diets supplemented with 250 mg/kg thymol and 250 mg/kg rosmarinic acid. Piglets were orally gavaged with 10 mL phosphate buffered solution (PBS) containing *E. coli* K88 at 10^9 CFU/mL, except that piglets in the Con group were administered 10 mL of PBS. Noteworthy differences ($P < 0.05$) among the five groups are indicated by different lowercase letters, $n = 6$. OTU = operational taxonomic unit; PCoA = principal co-ordinate analysis.

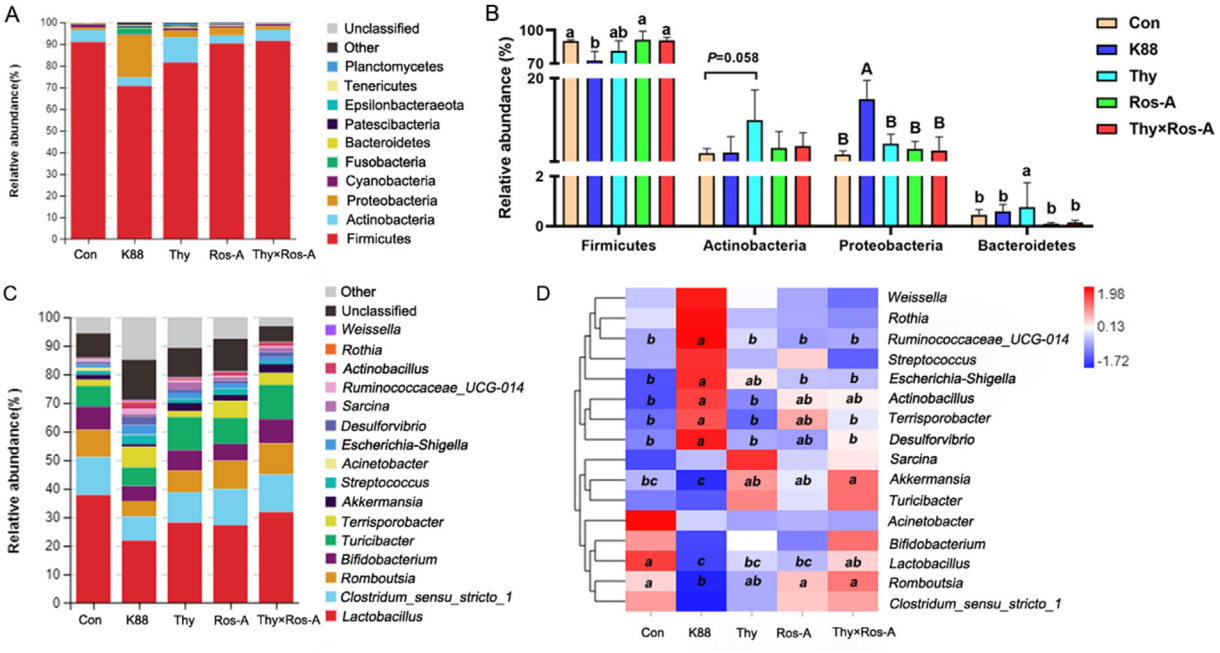

Fig. 4. Effects of thymol, rosmarinic acid, and their combination on the microbiota in the ileum of weaned piglets challenged by *E. coli* K88. (A) The phylum-level taxonomic composition of the ileal microbiota. (B) The differentially enriched phyla found in the ileum. (C) The genus-level taxonomic composition of the ileal microbiota. (D) The differentially abundant genera found in the ileum. Con and K88, basal diets; Thy, basal diets supplemented with 500 mg/kg thymol; Ros-A, basal diets supplemented with 500 mg/kg rosmarinic acid; Thy × Ros-A, basal diets supplemented with 250 mg/kg thymol and 250 mg/kg rosmarinic acid. Piglets were orally gavaged with 10 mL PBS containing *E. coli* K88 at 10^9 CFU/mL, except that piglets in the Con group were administered 10 mL of PBS. Noteworthy differences ($P < 0.05$) among the five groups are indicated by different lowercase letters, $n = 6$.

the K88 group. Additionally, the concentrations of IL-10 and sIgA in the Thy × Ros-A group piglets were much higher than those in the K88 group ($P < 0.05$).

3.9. The protein expression of MyD88/NF-κB pathway in the ileal mucosa

The relative gene expressions of *TLR-2*, *TLR-4*, *TLR-5*, *MyD88* and *NF-κB*, as well as the relative protein expressions of TLR-4, MyD88, and NF-κB in the K88 group were greatly increased ($P < 0.05$, Fig. 7A and C) compared with those in the Con group. In the Thy × Ros-A group, Thy group and Ros-A group, the gene expression levels of *TLR-2*, *TLR-4*, and *NF-κB* were substantially decreased ($P < 0.05$). The protein expression of TLR4, MyD88, NF-κB and the level of p-NF-κB/NF-κB in the Thy × Ros-A group were much lower than that in the K88 group ($P < 0.05$). Furthermore, these indicators did not show a significant difference from the Con group ($P > 0.05$).

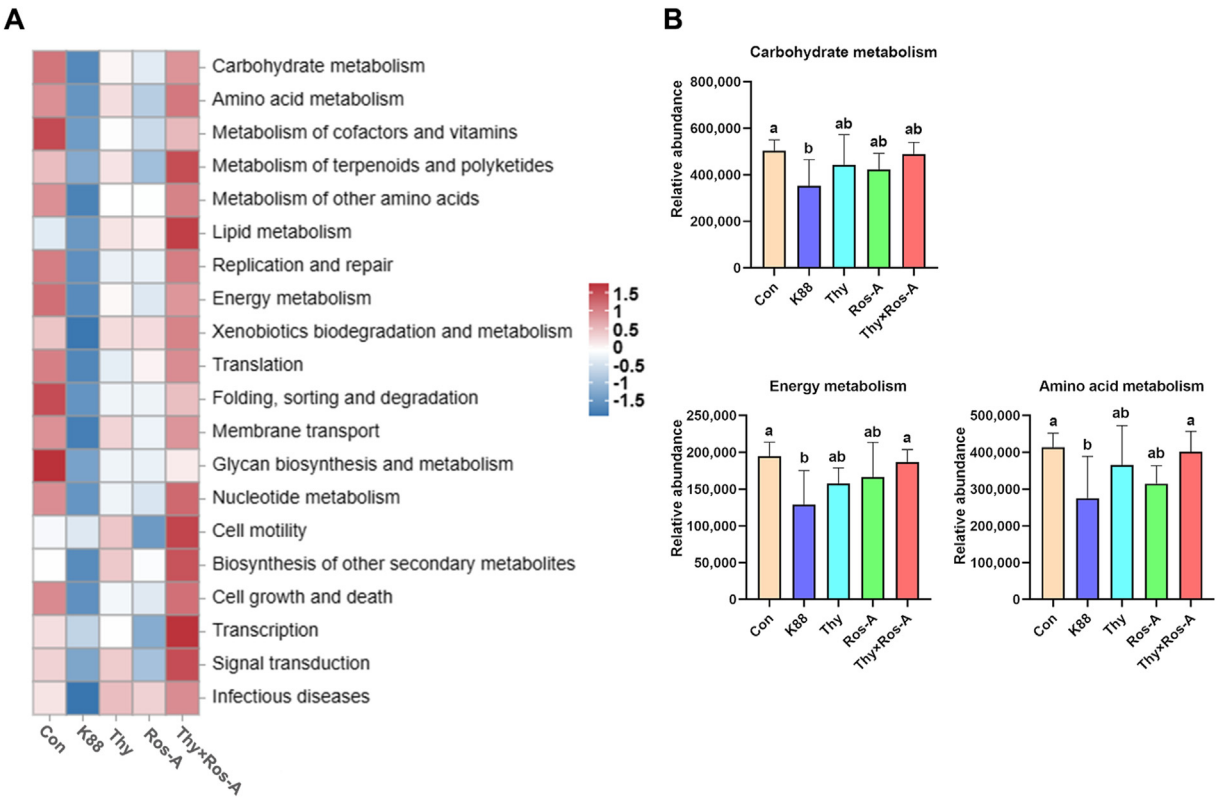

Fig. 5. PICRUSt function analysis. (A) PICRUSt function analysis. (B) Carbohydrate metabolism, amino acid metabolism and energy metabolism. Con and K88, basal diets; Thy, basal diets supplemented with 500 mg/kg thymol; Ros-A, basal diets supplemented with 500 mg/kg rosmarinic acid; Thy × Ros-A, basal diets supplemented with 250 mg/kg thymol and 250 mg/kg rosmarinic acid. Piglets were orally gavaged with 10 mL phosphate buffered solution containing *E. coli* K88 at 10^9 CFU/mL, except that piglets in the Con group were administered 10 mL of PBS. Noteworthy differences ($P < 0.05$) among the five groups are indicated by different lowercase letters, $n = 6$. PICRUSt = Phylogenetic Investigation of Communities by Reconstruction of Unobserved States.

Table 4
Effects of thymol, rosmarinic acid and their combination on the concentrations of short-chain fatty acids (SCFA) in the ileum of weaned piglets challenged by *E. coli* K88.

Item	Con	K88	Thy	Ros_A	Thy_Ros_A	SEM	*P*-value
Acetate, μmol/g chyme	3.47^{a}	2.59^{b}	3.04^{a}	3.12^{a}	3.38^{a}	0.084	0.003
Propionate, μmol/g chyme	0.67	0.47	0.64	0.60	0.66	0.026	0.094
Butyrate, μmol/g chyme	0.49^{a}	0.35^{b}	0.46^{a}	0.43^{ab}	0.49^{a}	0.017	0.049
Total SCFA, μmol/g chyme	4.87^{a}	4.03^{b}	4.31^{ab}	4.49^{ab}	4.82^{a}	0.085	0.002
Lactate, mmol/mL	6.95^{a}	6.05^{b}	6.56^{ab}	6.41^{ab}	6.83^{a}	0.096	0.015

Con and K88, basal diets; Thy, basal diets supplemented with 500 mg/kg thymol; Ros-A, basal diets supplemented with 500 mg/kg rosmarinic acid; Thy × Ros-A, basal diets supplemented with 250 mg/kg thymol and 250 mg/kg rosmarinic acid.
Piglets were orally gavaged with 10 mL of PBS, containing *E. coli* K88 at 10^9 CFU/mL, except that piglets in the Con group were administered 10 mL of PBS.
Noteworthy differences ($P < 0.05$) among the five groups are indicated by different superscript letters, $n = 6$.

4. Discussion

Promoting the growth of weaned piglets is an important goal during the piglet nursing stage, with intestinal health serving as the cornerstone for effective digestion and nutrient absorption (Che et al., 2017). Weaned piglets have immature digestive organs, compromised immune systems, and high susceptibility to environmental stressors and pathogenic bacteria, resulting in weaning stress syndrome (Yang et al., 2016). Among the various stressors, *E. coli* infection stands out as the primary cause of diarrhea and potential mortality among weaned piglets, frequently employed to establish stress models in this population (Yu et al., 2020). Animal experiments have revealed that challenges with *E. coli* K88 lead to intestinal villus atrophy, morphological damage in the intestines, and provoke diarrheal episodes alongside inflammatory responses in weaned piglets (Jin et al., 2021). This study corroborates these findings, demonstrating that a 10-mL (10^9 CFU/mL) *E. coli* K88 challenge caused intestinal mucosal damage and weakened the barrier function in piglets.

The structural integrity of the intestine is essential in maintaining optimal intestinal absorption function in piglets (Cheng et al., 2023). Key parameters such as intestinal villus height and crypt depth serve as crucial indicators, reflecting intestinal mucosal morphology and directly influencing the digestion and absorption of nutrients, fluids, and electrolytes in the intestine (Waititu et al., 2016). Oral administration of 3 mL (10^{10} CFU/mL) *E. coli* significantly disrupted the intestinal morphology of weaned piglets and greatly reduced the villus height of the intestine (Wong et al., 2022). However, the combination of plant extracts, such as garlic extract and chili oleoresin, exhibited significant mitigation of the adverse effects of *E. coli* on intestinal morphology (Wong et al., 2022). The studies reported that thymol, in conjunction with other substances

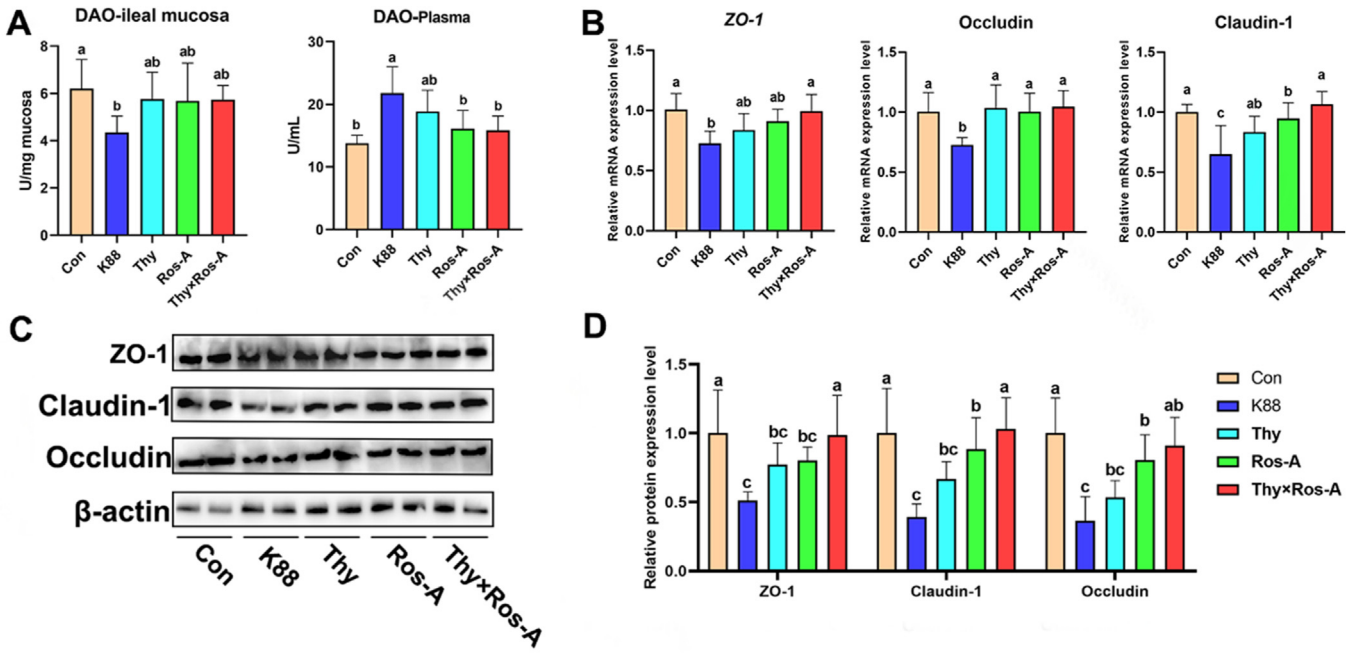

Fig. 6. Effects of thymol, rosmarinic acid and their combination on the intestinal barrier function in ileal mucosa of weaned piglets challenged by *E. coli* K88. (A) Diamine oxidase (DAO) concentrations. (B) Gene expression of tight junction proteins in ileal mucosa. (C) The Western blot of zonula occludens-1 (ZO-1), claudin-1, occludin, and β-actin of the ileal mucosa. (D) Protein expression of tight junction proteins in ileal mucosa. Con and K88, basal diets; Thy, basal diets supplemented with 500 mg/kg thymol; Ros-A, basal diets supplemented with 500 mg/kg rosmarinic acid; Thy × Ros-A, basal diets supplemented with 250 mg/kg thymol and 250 mg/kg rosmarinic acid. Piglets were orally gavaged with 10 mL phosphate buffered solution (PBS) containing *E. coli* K88 at 10^9 CFU/mL, except that piglets in the Con group were administered 10 mL of PBS. Noteworthy differences ($P < 0.05$) among the five groups are indicated by different lowercase letters, $n = 6$.

Table 5
Effects of thymol, rosmarinic acid and their combination on the inflammatory factor concentrations in ileal mucosa of weaned piglets challenged by *E. coli* K88.

Item	Con	K88	Thy	Ros-A	Thy × Ros-A	SEM	*P*-value
IL-1β, pg/mg protein	50.45^{c}	75.85^{a}	63.18^{bc}	67.15^{ab}	57.87^{bc}	2.194	0.011
IL-6, pg/mg protein	5.03^{b}	7.34^{a}	5.17^{b}	5.19^{b}	5.02^{b}	0.219	0.023
IL-8, pg/mg protein	3.41^{c}	5.79^{a}	4.07^{bc}	4.95^{ab}	4.26^{bc}	0.193	0.013
IL-10, pg/mg protein	4.84^{a}	3.97^{b}	4.55^{ab}	4.43^{ab}	4.61^{a}	0.081	0.040
IL-12, pg/mg protein	6.36^{b}	9.14^{a}	7.17^{b}	7.05^{b}	6.82^{b}	0.228	0.010
TNF-α, pg/mg protein	6.36^{c}	9.45^{a}	7.15^{bc}	7.86^{b}	6.58^{bc}	0.277	0.011
sIgA, μg/mg protein	2.64^{a}	2.24^{b}	2.64^{a}	2.37^{ab}	2.88^{a}	0.067	0.012

IL-1β = interleukin-1β; IL-6 = interleukin-6; IL-8 = interleukin-8; IL-12 = interleukin-12; TNF-α = tumor necrosis factor-α; sIgA = secretory immunoglobulin A.
Con and K88, basal diets; Thy, basal diets supplemented with 500 mg/kg thymol; Ros-A, basal diets supplemented with 500 mg/kg rosmarinic acid; Thy × Ros-A, basal diets supplemented with 250 mg/kg thymol and 250 mg/kg rosmarinic acid.
Piglets were orally gavaged with 10 mL PBS, containing *E. coli* K88 at 10^9 CFU/mL, except that piglets in the Con group were administered 10 mL of PBS.
Noteworthy differences ($P < 0.05$) among the five groups are indicated by different superscript letters, $n = 6$.

like benzoic acid and carvacrol, enhanced the intestinal morphological parameters of piglets (Silva et al., 2020). In this study, the villi in the ileum of the K88 group displayed signs of damage, exhibiting noticeable differences in morphological parameters compared to the Con group. However, the piglets in the Thy × Ros-A group showed a remarkable improvement in both villus height and the villus-to-crypt ratio, alongside a notable reduction in crypt depth. These observations indicated that the dietary supplementation of Thy and Ros-A combination positively affected the intestinal morphological parameters in piglets challenged by *E. coli* K88. The improvement of intestinal morphological parameters in the Thy × Ros-A group correlated with the alleviation of oxidative stress, which caused intestinal villus rupture (Hong et al., 2022; Qiu et al., 2020; Zou et al., 2016). In this study, MDA concentrations in the K88 group were much higher than that in the Con group, while GSH-PX concentrations were much lower. Conversely, the Thy × Ros-A group exhibited an opposite pattern, with these markers demonstrating significant increases compared to the K88 group and minor variances compared to the Con group. Thy and Ros-A both possess strong antioxidant properties, suggesting that their combination mitigates the impact of *E. coli* K88 challenge on intestinal villus morphology by regulating the redox state of the intestinal epithelium.

The improvement of the morphology of intestinal villi may be related to the enhancement of intestinal function. Longer intestinal villi enhance the contact area between villi and nutrients, potentially boosting the efficacy of nutrient absorption (Martin et al., 2003). Furthermore, amino acid transport vectors such as *EAAC1* and *SLC6A19* are expressed along the intestinal villus, thus damage to these intestinal villi can impair the amino acid transport function (Thomson et al., 1994). Therefore, we measured the expression level of intestinal transport vectors of small peptides and amino acids, as well as glucose transport vectors such as *SGLT1*, *GLUT1* and *GLUT2* (Chen et al., 2019). The gene expression of *SLC6A19* and *SGLT1* in the Thy group, Ros-A group and Thy × Ros-A group were much higher than that in the K88 group, and the

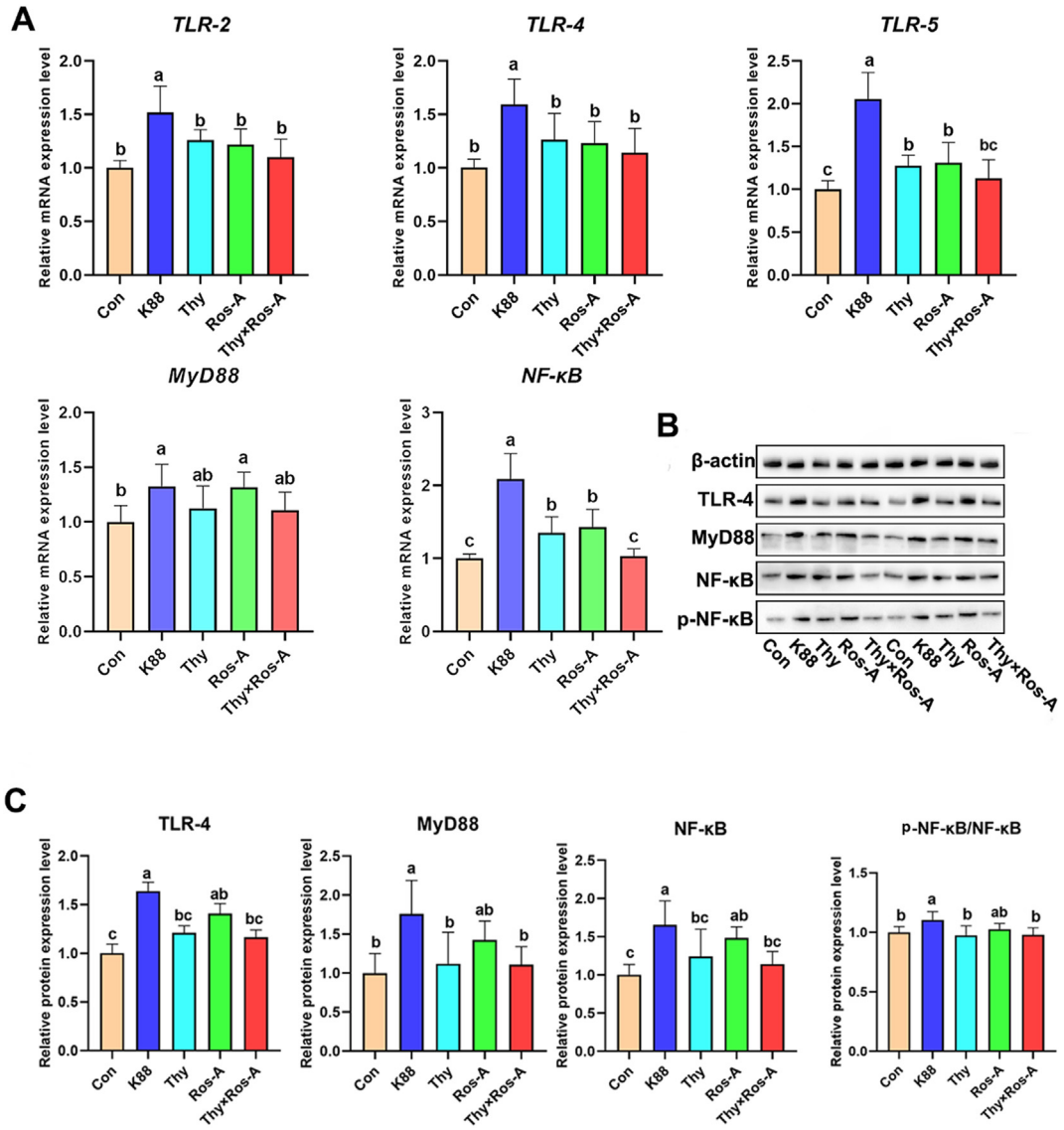

Fig. 7. Effects of thymol, rosmarinic acid and their combination on the gene and protein expression of TLR-4/MyD88/NF-κB pathway in ileal mucosa of weaned piglets challenged by *E. coli* K88. (A) Gene expression of TLR-4/MyD88/NF-κB pathway in ileal mucosa. (B) The Western blot of Toll-like receptor-4 (TLR-4), myeloid differentiation primary response 88 (MyD88), nuclear factor-kappa-B (NF-κB) and p-NF-κB of the ileal mucosa. (C) Protein expression of TLR-4/MyD88/NF-κB pathway in ileal mucosa. TLR-2 = Toll-like receptor 2; TLR-4 = Toll-like receptor 4; TLR-5 = Toll-like receptor 5; MyD88 = myeloid differentiation primary response 88; NF-κB = nuclear factor kappa-B. Con and K88, basal diets; Thy, basal diets supplemented with 500 mg/kg thymol; Ros-A, basal diets supplemented with 500 mg/kg rosmarinic acid; Thy × Ros-A, basal diets supplemented with 250 mg/kg thymol and 250 mg/kg rosmarinic acid. Piglets were orally gavaged with 10 mL PBS containing *E. coli* K88 at 10^9 CFU/mL, except that piglets in the Con group were administered 10 mL of PBS. Noteworthy differences ($P < 0.05$) among the five groups are indicated by different lowercase letters, $n = 6$.

expression of *EAAC1* and *GLUT2* in the Thy × Ros-A group also exhibited substantial increases. Studies have found that thymol could significantly increase the expression of *SGLT1*, *EAAC1*, and *PepT1* in LPS-stimulated IPEC-J2 cells (Omonijo et al., 2019). Rosmarinic acid has also been reported to influence glucose transport in the intestine by regulating the expression of *SGLT1* in rats (Azevedo et al., 2011). Additionally, the combination of Thy and organic acids can have a synergistic effect, alleviating the damage to intestinal villus morphology caused by ETEC-F4 challenge and increasing the expression of *GLUT1* and *SLC6A19* genes in the jejunum (Choi et al., 2020). These studies suggest that Thy or Ros-A may directly act on the intestine, enhancing the transport function of nutrients in the gut. The enhancement of amino acid and glucose transport capacity provides a greater supply of nutrients to the body, boosting the growth performance of weaned piglets. Consistent with the aforementioned findings, we found that piglets in the Thy × Ros-A group had the highest daily average weight gain (Thy × Ros-A group, 0.31 kg/d) and the lowest feed-to-gain ratio (Thy × Ros-A group, 1.76) between 7 and 14 d of age, compared to the Con and K88 groups in this study. Conversely, the average feed-to-gain ratio of piglets in the K88 group was higher than that of the other four groups between 14 and 21 d of age (2.17). Additionally, during d 19 to 21, piglets in the K88 group exhibited the lowest daily average weight gain (0.27 kg/d) and the highest feed-to-gain ratio (1.97). These observations suggest that the combination of Thy and Ros-A could mitigate intestinal damage resulting from *E. coli* K88 challenge, preserve intestinal mucosal morphology integrity, and enhance the activity of nutrient transport carriers, thereby fostering piglet growth.

The microbes influence the intestinal health of piglets (Wang et al., 2018). Alpha diversity analysis primarily assesses the degree of diversity of the gut microbiota. In our study, *E. coli* challenge substantially reduced the Shannon index of the K88 group, consistent with previous research findings (Xu et al., 2020). However, we observed a substantial increase in the Shannon index in the Thy × Ros-A group compared to the K88 group, indicating that the combination of Thy and Ros-A increased the diversity and richness of the ileal mucosal microbial community in *E. coli* K88-challenged piglets. The PCoA results revealed significant dissimilarities in the ileal microbiota among the piglets across the five distinct groups. Firmicutes and Bacteroidetes directly affect the host's energy intake and fat metabolism, and an increased ratio of Firmicutes to Bacteroidetes promotes the production of microbial metabolites such as acetate and butyrate (Turnbaugh et al., 2006).

Proteobacteria comprise a diverse range of pathogenic microorganisms, including *E. coli* and *Shigella*, etc. which can cause a variety of inflammatory bowel diseases (Rizzatti et al., 2017). In our study, the K88 group had a significant reduction in the relative abundance of Firmicutes and a major increase of Proteobacteria. In contrast, the Thy × Ros-A group displayed the opposite pattern, with a notable increase of Firmicutes and a significant decrease of Proteobacteria. Following the *E. coli* K88 challenge, there was a considerable decrease in the relative abundances of *Akkermansia*, *Lactobacillus* and *Romboutsia*, accompanied by a significant increase in the relative abundances of *Escherichia-Shigella*, *Actinobacillus*, *Terrisporobacter and Desulforvibrio.* This indicates that the plant extracts, especially the combination of Thy and Ros-A could counteract the impact of *E. coli* K88 challenge on the ileal microbiota. Moreover, the ileal microbiota at the genus level in the Thy × Ros-A group closely resembled that in the Con group. Similar findings have been reported in other research studies. For instance, the *E. coli* K88 challenge substantially decreased the relative abundance of *Lactobacillus* and greatly increased *Escherichia Shigella* and Proteobacteria in the ileum and cecum of piglets (Xu et al., 2020). In contrast, dietary supplementation with Thy and benzoic acid resulted in a major increase in *Lactobacillus* and a substantial decrease in *Escherichia Shigella* in the ileum of piglets (Diao et al., 2015). Indeed, the microbes residing in the gut play a pivotal role in regulating the host's metabolism and health regulation. For example, *Lactobacillus* in piglets' intestines metabolizes nutrients to produce a variety of organic acids (Krieger-Weber et al., 2020), and directly influences various immune responses in the body, such as reducing inflammation (Zhang et al., 2018). *Akkermansia*, which belongs to the phylum Verrucomicrobia, is known to enhance gut health in the host (Johansson et al., 2011). *Escherichia-Shigella* is a potential pathogen that reduces intestinal absorption of intestinal nutrients, impairs intestinal barrier function and causes host diarrhea (Laohachai et al., 2003). *Desulforvibrio*, the main genus of Proteobacteria, may proliferate and result in epithelial cell damage, and mucosal inflammation (Brown et al., 2011). The findings suggested that the combination of Thy and Ros-A mitigates the impact of the *E. coli* K88 challenge on potential beneficial bacteria in the gut, thereby restoring the growth of beneficial bacteria. According to PICRUSt, these bacteria mainly function in energy metabolism, carbohydrate metabolism, and amino acid metabolism. The composition of the microbiota can also be modified by improving *Lactobacilli* and *Akkermansia* and reducing the level of *Escherichia-Shigella*. The modification can improve the intestinal morphology and integrity of weaned piglets, increase the height of intestinal villi, and enhance the protein digestion and absorption function of piglets (Ma et al., 2021). This indicates that the regulatory effect of the combination of Thy and Ros-A on intestinal microbiota influenced the morphology and intestinal integrity of the ileum in weaned piglets, ultimately improving digestion and absorption function.

Changes in the microbiota in the intestine also lead to the alteration in metabolites, such as the production of SCFA. The *E. coli* K88 challenge resulted in a substantial reduction in SCFA concentrations, accompanied by a decrease in *Lactobacillus* and *Romboutsia*. In contrast, piglets in the plant extract group, particularly those in the Thy × Ros-A group, had a noteworthy increase in the concentrations of SCFA in their ileal chyme. SCFA have been found to promote intestinal health in animals across multiple studies; for example, feeding SCFA to germ-free weaned piglets increased the villus height of the ileum and reduced intestinal inflammatory reactions (Zhou et al., 2020). Intestinal epithelial cells can directly use butyrate, facilitating the morphological advancement of the intestinal mucosa (Dong et al., 2016). In addition, the presence of SCFA has been associated with a decrease in intestinal inflammation (Zeng et al., 2021) and an enhancement of intestinal barrier function (Korsten et al., 2023), which may also contribute to improved intestinal health. These findings suggest that the combination of Thy and Ros-A regulates the intestinal microbiota and their metabolites in weaned piglets, thereby mitigating the damage to the intestinal morphology caused by *E. coli* K88 and improving intestinal health.

Intestinal inflammation disrupts the morphology of intestinal mucosa and increases intestinal permeability, adversely affecting intestinal health (Peuhkuri et al., 2010; Tappenden, 2008). The TLR4-mediated MyD88/NF-κB pathway triggers intestinal inflammation in response to *E. coli* infection, resulting in elevated levels of IL-1β and IL-6, while decreasing the levels of IL-10 (Boeckman et al., 2022). In our study, the *E. coli* K88 challenge resulted in a significant increase of IL-1β, IL-6, IL-8, IL-12, and TNF-α within the ileal mucosa, alongside a substantial decrease in concentrations of IL-10 and sIgA. However, in the Thy × Ros-A group, there was a notable reduction in the concentrations of IL-1β, IL-6, IL-8, IL-12, and TNF-α compared to the K88 group, while IL-10 and sIgA were markedly increased. Previous studies have also shown that Thy or Ros-A, when used individually, could inhibit inflammatory responses in animals and cells (Al-Khrashi et al., 2022; Omonijo et al., 2019; Villalva et al., 2018). TLR-4/NF-κB is a classic inflammatory pathway in animals, regulating the inflammatory process of the body. Our study demonstrated that the *E. coli* K88 challenge significantly upregulated gene and protein expression for TLR-4, MyD88, and NF-κB in the ileal mucosa of piglets, which has similar results to those found in previous studies (Wang et al., 2016). However, the inclusion of Thy, Ros-A, and their combined dietary supplementation significantly down-regulated the gene and protein expression levels of TLR-4, MyD88 and NF-κB in the ileum compared to the Con group. Previous research has consistently demonstrated that Thy exerts anti-inflammatory effects by suppressing the activation of the NF-κB and TLR pathways, while Ros-A inhibited the activation of the NF-κB pathway to reduce cellular inflammatory responses (Mo et al., 2022; Wei et al., 2018). Moreover, microbes and their metabolites play important roles in reducing intestinal inflammation. For example, *Lactobacillus* inhibited the proliferation of harmful pathogens and directly reduced the body's inflammatory response (Sherman et al., 2004). Butyric acid was associated with a reduction in the production of proinflammatory cytokines and chemokines, exhibiting anti-inflammatory properties (Li et al., 2021). Acetate and propionate have demonstrated their ability to diminish the inflammatory response by reducing the activation of the NF-κB signaling pathway (Zhang et al., 2019). These findings indicate that SCFA may alleviate intestinal inflammation in piglets by exhibiting anti-inflammatory properties.

The intestinal barrier function serves as a vital defense mechanism for the intestinal tract, protecting the body against invading pathogenic bacteria and toxic substances (Wijtten et al., 2011). Diamine oxidase (DAO) levels are commonly used to reflect intestinal

barrier permeability, with decreased levels in the intestine and increased levels in the plasma indicating heightened intestinal permeability (Alizadeh et al., 2016). In our study, the *E. coli* K88 challenge led to an increase in plasma DAO concentration and a decrease in ileal mucosa DAO concentration. However, the Thy × Ros-A group showed the opposite trend, indicating that the combination of thymol and rosmarinic acid alleviated the effect of the *E. coli* K88 challenge on the permeability of ileum in weaned piglets.

Intestinal tight junction proteins, such as occludin, claudin-1, and ZO-1, contribute to normal intestinal function (Zeisel et al., 2019). *E. coli* K88 infection significantly reduced the protein expression of occludin and ZO-1 in piglets' intestinal tract (Jin et al., 2021). Our results suggest that the expression of Occludin and Claudin-1 was significantly reduced in the K88 group. Conversely, the expression of ZO-1, Occludin, and Claudin-1 in the Thy × Ros-A group was markedly higher compared to the K88 group. Similar patterns of improvement were also observed in both the Thy group and the Ros-A group. Previous studies have found that adding 3.7 mmol/kg Thy to the diet enhanced the intestinal barrier function in weaned piglets (Metzler-Zebeli et al., 2012), and Ros-A increased the expression of Claudin-1 and Occludin in IPEC-J2 (Pomothy et al., 2020). Moreover, the increase of SCFA levels in the Thy × Ros-A groups was associated with the improved intestinal barrier, as SCFA participated in the phosphorylation of NF-κB and AMPK pathways and promoted gene expression of barrier proteins (Boshuai et al., 2018). The results suggest that the addition of both Thy and Ros-A to the diet alleviated the damage of *E. coli* K88 challenge on the intestinal barrier function. Furthermore, several studies have demonstrated that enhancing the intestinal barrier protects against harmful bacteria, endotoxins, and other noxious substances from infiltrating the mucosa of the small intestine, thus reducing intestinal inflammation (Jiao et al., 2023). Therefore, the combination of Thy and Ros-A regulates the intestinal microbiota and its metabolites, improves the intestinal barrier, and reduces intestinal inflammatory response, ultimately contributing to nutrient absorption and the growth performance of weaned piglets.

5. Conclusion

In summary, the findings from this study suggest that the combination of Thy and Ros-A enhanced the intestinal barrier function and reduced intestinal inflammation and modulated the intestinal microbiota and its metabolites in weaned piglets challenged by *E. coli* K88.

CRediT authorship contribution statement

Runlin Li: Writing – original draft, Investigation, Formal analysis. **Xuedong Ding:** Writing – review & editing. **Mingkang Lei:** Formal analysis. **Panpan Li:** Formal analysis. **Ilias Giannenas:** Writing – review & editing. **Jing Wang:** Writing – original draft, Investigation, Formal analysis. **Weiyun Zhu:** Writing – review & editing.

Availability of data and materials

In this study, all data generated or analyzed can be obtained from the corresponding author upon reasonable request. The 16S sequencing data mentioned in this article are stored in the NCBI sequence database (accession number: SUB12204336).

Declaration of competing interest

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.

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Appendix supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2024.11.008.

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