



## Original Research Article

# *Macleaya cordata* extract exhibits some potential as a surrogate antibiotic by improving gastrointestinal epithelial status and humoral response in goats

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## ABSTRACT

*Macleaya cordata* extract (MCE) is a potential replacement for antibiotics. In the current study, effects of MCE on the gastrointestinal health and humoral responses of host animals were explored. A total of 30 weanling goats with similar body weight of  $9.15 \pm 1.36$  kg were randomly allocated into three groups ( $n = 10$  per group): control group (CON group, fed with a basal diet), antibiotic group (Abx group, fed with the basal diet supplemented with 0.18 g/d vancomycin and 0.36 g/d neomycin), and MCE group (fed with the basal diet supplemented with 5 g/d MCE), for three weeks. Results showed that antibiotic addition decreased the height and area of rumen papillae, ruminal mucosa Toll-like receptor 8 (TLR8), interleukin-8 (IL-8) and interleukin-1 $\beta$  (IL-1 $\beta$ ) gene relative expression levels and microbial diversity, altered the volatile fatty acid (VFA) profile in the rumen, and increased monocytes amount and CD4<sup>+</sup> T cells percentage in the peripheral blood ( $P < 0.05$ ) compared to CON group. MCE addition increased the average daily gain, ileal villus height, villus height/crypt depth, and immunoglobulin M (IgM) content in the peripheral blood ( $P < 0.05$ ) compared to the CON. Additionally, MCE addition decreased the proportion of isobutyric acid in the chyme of the ileum ( $P = 0.005$ ) compared to the CON group. These results suggest that antibiotic supplementation may suppress the epithelial state and microbial diversity and fermentation in goats, but stimulate cellular response to maintain the growth performance of goats. MCE administration improved the epithelial state and humoral response to promote the growth performance in goats.

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## 1. Introduction

Antibiotics have been acknowledged as efficient agents for protecting hosts against infection by killing various pathogenic microbes. However, the problems of bacterial resistance and drug residues in livestock and poultry caused by the overuse of antibiotics have been plaguing the development of the animal husbandry industry (Cantas et al., 2013). Antibiotic residues in meat or milk products also threaten the health of human beings. Neomycin is an

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aminoglycoside antibiotic that protects the host against streptomycin-resistant bacteria and inhibits the initial process of protein synthesis in both Gram-negative and Gram-positive bacteria (Waksman and Lechevalier, 1949). The administration of neomycin reduced the gene expression of zonula protein-1 and claudin-1 in colon tissue, causing damage to the intestine (Bose et al., 2013). Vancomycin, a glycopeptide antibiotic that mainly targets Gram-positive bacteria, is poorly absorbed by the human intestinal tract when administered orally (Rao et al., 2011). Oral administration of vancomycin has been demonstrated to markedly disrupt the intestinal microbiota (Lewis et al., 2015), and to influence the development of the intestinal tract and immune system in mice (Cheng et al., 2017). Intake of a neomycin, vancomycin, and metronidazole cocktail leads to a profound reduction in gut bacterial load and persistent changes in bacterial diversity, further altering the antibody response to influenza vaccination in humans (Hagan et al., 2019).

The traditional herb *Macleaya cordata*, which is primarily distributed in China, North America, and Europe, has been used as a medicinal plant for more than 1000 years (Lin et al., 2018). *M. cordata* extract (MCE) is a natural growth promoter based on its composition of isoquinoline alkaloids including sanguinarine and chelerythrine. Isoquinoline alkaloid inclusion in steers fed a high-energy diet increases acetate production without changes in total volatile fatty acid (VFA) molar concentration (Aguilar-Hernandez et al., 2016), ameliorates the negative effects of severe heat on growth performance of feedlot ewes and improves rumen pathomorphism (Estrada-Angulo et al., 2016). However, the application of MCE or sanguinarine in early weaned goats, dairy cows, and calves does not exert beneficial effects on their growth performance (Chen et al., 2020; Wang et al., 2018; Zhang et al., 2018). Interestingly, sanguinarine supplementation meliorates humoral immunity by enhancing serum immunoglobulin G (IgG) and immunoglobulin A (IgA) contents or increasing the white blood cells and neutrophil numbers in calves and dairy goats (Ling et al., 2021; Zhang et al., 2018). Intake of MCE in early weaned goats affects the gene expression pattern of Toll-like receptors (TLR) and downstream signal pathways of the lower gut (Yang et al., 2021). Additionally, dietary MCE or sanguinarine inclusion has been proven to increase the beneficial microorganisms and resist harmful microorganisms in the intestine of piglets and chickens (Bavarsadi et al., 2017; Chen et al., 2019; Pickler et al., 2013). Due to the difference in colonized microflora in the gastrointestinal tract and immune systems between monogastric and ruminant animals, the gastrointestinal microbiota and the host immune response affected by MCE remains understudied.

The hypothesis of this study was whether and how MCE affected the gastrointestinal health and humoral responses of host animals. Therefore, the potential that MCE should replace antibiotics was evaluated in the present study by investigating gastrointestinal morphology, colonized microbiota community, fermentation pattern, TLR-myeloid differentiation factor 88 (MyD88) signal and humoral immunity of kid goats. Neomycin and vancomycin were chosen based on their broad antibacterial spectrums to establish a microbiota perturbation model.

## 2. Materials and methods

### 2.1. Animal ethics statement

All animal-related procedures were conducted in accordance with the Animal Care and Use Guidelines of the Animal Care Committee, Institute of Subtropical Agriculture, the Chinese Academy of Sciences, Changsha, China (ISACAS-02-2018-06), and

followed the National Research Council's Guide for the Care and Use of Laboratory Animals.

### 2.2. Experimental design and animal management

The animal feeding of this study was carried out on a farm in Yueyang (Hunan, China) from May to July 2019. Thirty female Chuanzhong Black one-month-old growing goats with similar body weight (BW,  $9.15 \pm 1.36$  kg) were selected. During the pre-feeding period from day 31 to day 45, kid goats were housed individually, fed the same basal diet, and provided goat milk at night to adapt to the basal diet. From day 46, the goats were randomly divided into three groups, control group (CON group,  $n = 10$ ), antibiotic group (Abx group,  $n = 10$ ), and MCE group ( $n = 10$ ). Goats in the CON group were fed the basal diet. Each goat in the Abx group was fed the basal diet supplemented with vancomycin (0.18 g/d) and neomycin (0.36 g/d) as per a previous study (Mao et al., 2018). At the same time, goats in the MCE group were fed the basal diet supplemented with a MCE premix (5 g/d; Sangrovit, Micolta Bio-resource Inc., Changsha, China; containing 0.375 g MCE and 99.625 g starch per 100 g premix). Sanguinarine and chelerythrine contents were 0.15 and 0.075 g, respectively, in MCE. The dosage of MCE premix was increased because our laboratory demonstrated that the inclusion of 3 g MCE per goat did not affect the growth performance in weaned goats (Chen et al., 2020). Vancomycin, neomycin, and MCE were added to the basal diet as a dry powder and mixed evenly.

All the goats were fed a concentrate and dry peanut vine at a ratio of 40:60 at 08:00 and 17:00 twice daily, and had ad libitum access to water. The formal feeding period lasted three weeks. During the entire feeding period, goats were reared in the same pen and confined individually in metabolic cages (140 cm  $\times$  100 cm  $\times$  124 cm) with no forage bedding. The pen was cleaned every day and disinfected once a week.

The composition and nutrient levels of the basal diet is presented in Table 1. Metabolizable energy was calculated according to the Nutritional Parameters and Feeding Standard for Goats (Zhang, 2010). Contents of dry matter (method No. 934.01, AOAC, 2005),

**Table 1**

Composition and nutrient levels of the basal diet (DM basis, %).

| Item                               | Content |
|------------------------------------|---------|
| <b>Ingredients</b>                 |         |
| Dried peanut vine                  | 60.00   |
| Maize flour                        | 6.67    |
| Extruded soybean                   | 12.84   |
| Whey powder                        | 4.58    |
| Fat powder                         | 7.28    |
| Soybean meal                       | 5.33    |
| Calcium hydrophosphate             | 1.01    |
| Calcium carbonate                  | 0.42    |
| Sodium chloride                    | 0.53    |
| Premix <sup>1</sup>                | 1.34    |
| Total                              | 100.00  |
| <b>Nutrient levels<sup>2</sup></b> |         |
| Metabolizable energy, MJ/kg        | 10.05   |
| Dry matter                         | 87.56   |
| Organic matter                     | 91.96   |
| Crude protein                      | 10.19   |
| Ether extract                      | 10.54   |
| Neutral detergent fiber            | 43.42   |
| Acid detergent fiber               | 27.92   |

<sup>1</sup> One kilogram of premix contained the following:  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  2.5 g,  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  0.8 g,  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$  3 g,  $\text{ZnSO}_4 \cdot \text{H}_2\text{O}$  5 g,  $\text{Na}_2\text{SeO}_3$  10 mg, KI 40 mg,  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  30 mg, vitamin A 95,000 IU, vitamin D 17,500 IU, vitamin E 18,000 IU.

<sup>2</sup> Metabolizable energy and organic matter were calculated values, while the others were measured values.

crude protein (method No. 2001.11, AOAC, 2005), ether extract (method No. 2003.05, AOAC, 2005), and ash (method No. 942.05, AOAC, 2000) were analyzed. Organic matter content was calculated as 100 minus the ether extract content (AOAC, 2005). Neutral detergent fiber and acid detergent fiber contents were measured according to Van Soest et al. (1991) by a Fibretherm Fiber Analyzer (Gerhardt, Bonn, Germany). Goats were weighed on the first and end days of the feeding period. The feed intake of each goat was recorded every day.

### 2.3. Samples collection

Blood samples were collected aseptically into tubes with different anticoagulation (heparin sodium, ethylene diamine tetraacetic acid dipotassium salt [EDTA-K<sub>2</sub>], or sodium citrate) from the jugular vein after overnight fasting on the last day of the feeding period. Plasma was separated by centrifugation at 1000 × g for 15 min at 4 °C and stored at –20 °C. Whole blood samples with EDTA-K<sub>2</sub> or sodium citrate were transferred into a lab for further blood routine examination, immunometabolic biomarker determination and flow cytometry analysis.

All the experimental goats were euthanized after intravenous injection of sodium pentobarbital (50 mg/kg BW). Thymus and spleen tissues were collected and weighed immediately. Thymus and spleen organ indexes were calculated by dividing the weight of the thymus or spleen by the body weight. About 10 g chyme each separately from the rumen and ileum was taken into a 10 mL sterile centrifuge tube, frozen in liquid nitrogen, and stored at –80 °C for intestinal microbial DNA extraction and diversity analysis. Rumen chyme of the abdominal sac was filtrated with four layers of gauze and the ruminal fluid was then centrifuged at 13,523 × g, 4 °C for 10 min. The supernatant (about 1.5 mL) and ileal chyme collected from the middle segment were immediately mixed with 0.15 mL 25% (wt/vol) metaphosphoric acid, stored the mixture at –20 °C to ensure sufficient fixation for the determination of VFA proportion. Mucosa samples were collected from the sites with rumen and ileum chyme, frozen in liquid nitrogen and stored at –80 °C. Samples of the rumen and ileum tissues were immediately fixed in 4% polyformaldehyde for morphological determination.

### 2.4. Gastrointestinal morphology

Rumen and ileum tissues were dehydrated, embedded, and stained with hematoxylin and eosin following the procedure reported in the literature (Jiao et al., 2015; Wang et al., 2020).

### 2.5. Determination of VFA proportion

The pre-treatment and gas chromatograph determination of VFA in the rumen and ileum chyme were performed according to standard methods (Jiao et al., 2016; Wang et al., 2014).

### 2.6. Real-time quantitative PCR

The isolation of total RNA extraction, cDNA synthesis and PCR procedure were performed according to the method of our previous study (Yan et al., 2018). The primer information was presented in Table 2. Quantification of the target genes was calculated according to the 2<sup>–ΔΔCt</sup> method (Livak and Schmittgen, 2001), and β-actin was used as a housekeeping gene.

### 2.7. 16S rRNA sequencing

The total DNA of microbes in the rumen and ileum chyme samples was extracted using the QIAamp DNA Stool Mini kit

**Table 2**

Sequences of primers used for real-time quantitative PCR.

| Genes          | Primer sequence (5' to 3')                                            | Product size, bp |
|----------------|-----------------------------------------------------------------------|------------------|
| <i>TLR1</i>    | Forward: AGAGCGAGTGGTGCATTATGAAC<br>Reverse: GCTTGTGGTAGCTGCTAGGAATGG | 129              |
| <i>TLR2</i>    | Forward: ACGACGCCTTTGTGCTCTAC<br>Reverse: GGGACGAAGTCTCGTTATG         | 121              |
| <i>TLR4</i>    | Forward: GGGACGAAGTCTCGTTATG<br>Reverse: GCCGTGATACGGTTGAAACT         | 124              |
| <i>TLR6</i>    | Forward: CTGCCATCCAATCACCACGAGTC<br>Reverse: AGCTTAGGTGCAAGTGAGCAACAG | 173              |
| <i>TLR7</i>    | Forward: GTTCCATTTCCTTGACACC<br>Reverse: GGGCACATGCTGAAGAGAGT         | 123              |
| <i>TLR8</i>    | Forward: TGAGGCTGCGGCAGAGGATC<br>Reverse: ACCGTGAATCGTGGCTGTAGG       | 120              |
| <i>TLR9</i>    | Forward: AGCTTAACCTCAGCGCCAAC<br>Reverse: TCCAGCAGGAAGTCCACGAA        | 142              |
| <i>MyD88</i>   | Forward: GAGGACGTGCTGATGGAAT<br>Reverse: CGAGGGATGCTGCTGTCTAT         | 125              |
| <i>IRAK1</i>   | Forward: GACACCGACACCTTCAGCTT<br>Reverse: TGCCTCTCTTCAACCAAGT         | 117              |
| <i>IL-1β</i>   | Forward: AAGCCTCTCCACCTCTCTC<br>Reverse: TTGTCCCTGATACCCAAAG          | 114              |
| <i>IL-6</i>    | Forward: TGACTTCTGCTTCCCTACCC<br>Reverse: GCCAGTGTCTCTTGCTGTT         | 193              |
| <i>IL-8</i>    | Forward: GTGGGCCACACTGCGAAAAT<br>Reverse: TGCACCCACTTTCTCTGGG         | 88               |
| <i>IFN-β</i>   | Forward: CCATATTGAGACCTCCTT<br>Reverse: AGGTGAAGATCGGTCGTGTC          | 118              |
| <i>β-actin</i> | Forward: ATGGCTACTGCTGCGTCGT<br>Reverse: TTGAAGGTGCTCTCGTGGAT         | 161              |

*TLR* = Toll like receptor; *MyD88* = myeloid differentiation factor 88; *IRAK1* = interleukin 1 receptor associated kinase 1; *IL* = interleukin; *IFN-β* = interferon-beta.

(Qiagen, Hilden, Germany). The extracted DNA samples were evaluated by 1% agarose gel electrophoresis and stored at –20 °C until further PCR analysis. Primers of 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATC-TAATCC-3') were used to amplify the V3 to V4 region of the 16S rRNA gene of prokaryotic bacteria. The PCR amplification system contained 25 ng of template DNA, 12.5 μL PCR premix, 2.5 μL of each primer, and the total reaction system was adjusted to 25 μL with PCR-grade water. The amplification conditions, setting of negative control, and confirmation of PCR product were the same as previous reported (Zhang et al., 2022). After purification and quantification of the qualified PCR product, thirty amplicon libraries (ten samples from each group) were used for sequenced on NovaSeq PE 250 platform.

### 2.8. Bioinformatic analysis

The process of bioinformatic analysis was performed according to the standard procedure (Sun et al., 2022). Briefly, paired-end reads were firstly assigned, truncated, and merged based on their individual barcode and primer sequence. High-quality clean tags were then obtained by filtering on the raw reads and chimeric sequences using fqtrim (v0.94) and Vsearch (v2.3.4), respectively. Feature table and feature sequence were obtained after dereplication using DADA2 (Callahan et al., 2016). Alpha and beta diversities were performed according to SILVA database (release 138) and calculated with QIIME2 (Bolyen et al., 2019). Alpha diversity indexes including Chao1 index, observed operational taxonomic units (Observed\_OTU), coverage, Shannon index, Simpson index and beta diversity graphs drawn by R package were presented. The differences in alpha diversity and genus-level abundance among three groups were analyzed by the Wilcoxon test with false discovery rate correction. Microbiome phenotypes analysis were implemented using the Bugbase tool (<https://bugbase.cs.umn.edu/>).

### 2.9. Immunometabolic biomarkers analysis

Plasma samples were completely thawed at 4 °C, then centrifuged at 1900 × g at 4 °C for 10 min, and the supernatant was used for further analysis. Activities of aspartate transaminase (AST), alanine transaminase (ALT) and contents of albumin, creatinine, and urea nitrogen were detected by automated biochemistry analyzer (Cobas c311, Roche, Auckland, Switzerland). Cytokines (interleukin [IL]-1 $\beta$ , IL-4, IL-12, IL-17, granulocyte-macrophage colony-stimulating factor [GM-CSF] and interferon- $\beta$  [IFN- $\beta$ ]), complements (complement 2 and complement 4) and immunoglobulins (IgM, IgG, and IgA) contents were determined by ELISA kits (Jiangsu Meimian Industrial Co., Ltd., Yancheng, China).

### 2.10. Blood routine examination

Lymphocytes and monocytes were immediately counted by automated hematology analyzer (BC-5000Vet, Mindray, Shenzhen Mindray Biomedical Electronics Co., LTD., Shenzhen, China).

### 2.11. Flow cytometer analysis

Fresh whole blood was first centrifuged at 500 × g for 5 min and the plasma was abandoned. Ten-fold volume of lysis buffer (C3702, Beyotime Biotechnology Corporation, Shanghai, China) was added into the tube and allowed to react for 5 min to promote hemocytes lysing. The hemocytes were centrifuged and lysing for two or three times until no red color remained in the supernatants. After that, a five-fold volume of phosphate buffer (PBS) was added to remove the lysis buffer. CD4 phycoerythrin (PE)-conjugated antibody (MA1-81450, Thermo Fisher Scientific Inc., USA) and CD8 FITC-conjugated antibody (MA1-80900 Thermo Fisher Scientific Inc., USA) diluted at 1:10 with PBS were incubated with the hemocytes for 30 min at 4 °C in darkness. CD4 or CD8 single-stained tubes were also prepared. Tubes with no antibody in the incubation were set as negative control. All the test tubes were centrifuged at 200 × g for 5 min, washed twice with PBS and centrifuged again. A volume of 300  $\mu$ L PBS was added and the solution was filtrated through a piece of 48  $\mu$ m nylon membrane. Finally, the filter solution was analyzed by flow cytometer (Moflo XDP, Beckman Coulter, Fullerton, CA). The CD4 positive T cells and CD8 positive T cells populations were gated based on their forward and side scatter characteristics. The CD4<sup>+</sup>T and CD8<sup>+</sup>T lymphocytes were identified by fluorochrome detection (FL1 and FL2). The results were analyzed using the software Summit 6.2 and expressed as a percentage of the total lymphocytes (at least 10,000 cells) from peripheral blood.

### 2.12. Statistical analysis

Data for growth performance, immune organ indexes, immunometabolic biomarkers, immune cells, morphology, gene expression and VFA profile were detected for normality and outliers by using the Levene and Grubbs's tests, respectively. SPSS 19.0 statistical software (SPSS Inc., Chicago, IL, USA) was used to determine the difference between the control and experimental groups by one-way ANOVA, with treatment as the fixed factor and the goat as a random factor. Duncan's new multiple range test was used for multiple group comparisons. All results are expressed as the mean and standard error of the mean (SEM). Statistical significance was declared at  $P < 0.05$ .

## 3. Results

### 3.1. Growth performance and immune organ indexes

MCE addition increased the average daily gain by 49.1% and 65.3% compared to the CON group and the Abx group, respectively ( $P = 0.044$ , Table 3). Meanwhile, MCE addition also increased the dry matter intake by 22.3% compared to the Abx group ( $P = 0.045$ ). Additionally, MCE addition did not affect the final body weight, thymus and spleen indexes compared to the CON group and the Abx group ( $P > 0.05$ ).

### 3.2. Gastrointestinal morphology

A healthy gut contributes to improved growth performance, so we detected the anatomic structure of the rumen and ileum. It is worth mentioning that at the rumen (Table 4), antibiotics decreased both the height and area of papillae ( $P < 0.05$ ) compared to the CON and MCE groups, but did not affect the width of papillae ( $P = 0.689$ ). At the ileum, MCE addition increased villus height and villus height/crypt depth ( $P < 0.05$ ) compared to the CON group or the Abx group. Additionally, antibiotics decreased the villus height/crypt depth compared to the CON group ( $P < 0.001$ ).

### 3.3. VFA profile in the gastrointestinal chyme

Compared with the CON group (Table 5), the addition of antibiotics increased the percentage of propionic acid ( $P < 0.001$ ), but decreased the percentages of acetic acid, butyric acid, valeric acid, total VFA and the acetic acid/propionic acid in the rumen chyme ( $P < 0.05$ ). MCE addition did not affect the total VFA and individual VFA percentage compared to the CON group ( $P > 0.05$ ). In the ileum, MCE addition increased acetic acid percentage ( $P = 0.044$ ), but decreased the percentages of isobutyric acid and valeric acid compared to CON group ( $P < 0.05$ ). Antibiotics addition decreased isobutyric acid and valeric acid percentages compared to the CON group ( $P < 0.05$ ).

**Table 3**  
Growth performance and immune organ indexes of goats.

| Item                    | CON                  | Abx                 | MCE                 | SEM   | P-value |
|-------------------------|----------------------|---------------------|---------------------|-------|---------|
| Initial body weight, kg | 9.02                 | 9.26                | 9.19                | 0.249 | 0.927   |
| Final body weight, kg   | 10.09                | 10.23               | 10.59               | 0.291 | 0.784   |
| Average daily gain, g/d | 38.39 <sup>b</sup>   | 34.62 <sup>b</sup>  | 57.23 <sup>a</sup>  | 4.041 | 0.044   |
| Dry matter intake, g/d  | 288.87 <sup>ab</sup> | 255.65 <sup>b</sup> | 312.67 <sup>a</sup> | 9.596 | 0.045   |
| Thymus index, %         | 0.07                 | 0.06                | 0.06                | 0.007 | 0.986   |
| Spleen index, %         | 0.17                 | 0.15                | 0.17                | 0.006 | 0.141   |

CON = control group; Abx = antibiotics addition group; MCE = *Macleaya cordata* extract addition group; SEM = standard error of the mean.

<sup>a,b</sup>Means within a row with no common superscripts differ ( $P < 0.05$ ).

**Table 4**  
Gastrointestinal morphology of goats.

| Item                      | CON                  | Abx                 | MCE                  | SEM    | P-value |
|---------------------------|----------------------|---------------------|----------------------|--------|---------|
| Rumen papillae            |                      |                     |                      |        |         |
| Height, $\mu$ m           | 1307.50 <sup>a</sup> | 877.91 <sup>b</sup> | 1452.61 <sup>a</sup> | 74.436 | 0.001   |
| Width, $\mu$ m            | 277.97               | 270.59              | 252.59               | 11.964 | 0.689   |
| Area, mm <sup>2</sup>     | 0.38 <sup>a</sup>    | 0.27 <sup>b</sup>   | 0.40 <sup>a</sup>    | 0.024  | 0.042   |
| Ileum                     |                      |                     |                      |        |         |
| Villus height, $\mu$ m    | 329.88 <sup>b</sup>  | 339.47 <sup>b</sup> | 398.62 <sup>a</sup>  | 12.273 | 0.040   |
| Villus crypt, $\mu$ m     | 234.07               | 282.07              | 265.43               | 8.571  | 0.062   |
| Villus height/crypt depth | 1.40 <sup>b</sup>    | 1.24 <sup>c</sup>   | 1.56 <sup>a</sup>    | 0.037  | <0.001  |

CON = control group; Abx = antibiotics addition group; MCE = *Macleaya cordata* extract addition group; SEM = standard error of the mean.

<sup>a-c</sup>Means within a row with no common superscripts differ ( $P < 0.05$ ).

**Table 5**  
Volatile fatty acid profile in the chyme of the rumen and ileum in goats.

| Item                                         | CON                | Abx                 | MCE                | SEM   | P-value |
|----------------------------------------------|--------------------|---------------------|--------------------|-------|---------|
| <b>Rumen</b>                                 |                    |                     |                    |       |         |
| Total VFA, mmol/L                            | 65.11 <sup>a</sup> | 49.56 <sup>b</sup>  | 70.26 <sup>a</sup> | 2.851 | 0.004   |
| Individual VFA molar percentage, mol/100 mol |                    |                     |                    |       |         |
| Acetic acid                                  | 66.65 <sup>a</sup> | 55.55 <sup>b</sup>  | 66.28 <sup>a</sup> | 1.292 | <0.001  |
| Propionic acid                               | 15.56 <sup>b</sup> | 34.07 <sup>a</sup>  | 16.15 <sup>b</sup> | 1.844 | <0.001  |
| Isobutyric acid                              | 1.49               | 1.52                | 1.66               | 0.144 | 0.887   |
| Butyric acid                                 | 12.67 <sup>a</sup> | 6.55 <sup>b</sup>   | 11.36 <sup>a</sup> | 0.673 | <0.001  |
| Isovaleric acid                              | 2.09               | 2.09                | 2.37               | 0.214 | 0.837   |
| Valeric acid                                 | 1.03 <sup>a</sup>  | 0.12 <sup>b</sup>   | 1.09 <sup>a</sup>  | 0.097 | <0.001  |
| A/P                                          | 4.49 <sup>a</sup>  | 1.79 <sup>b</sup>   | 4.28 <sup>a</sup>  | 0.271 | <0.001  |
| <b>Ileum</b>                                 |                    |                     |                    |       |         |
| Total VFA, mmol/L                            | 6.94               | 5.58                | 8.22               | 0.611 | 0.204   |
| Individual VFA molar percentage, mol/100 mol |                    |                     |                    |       |         |
| Acetic acid                                  | 79.99 <sup>b</sup> | 85.98 <sup>ab</sup> | 88.32 <sup>a</sup> | 1.432 | 0.044   |
| Propionic acid                               | 8.37               | 6.94                | 5.48               | 0.531 | 0.080   |
| Isobutyric acid                              | 1.48 <sup>a</sup>  | 0.54 <sup>b</sup>   | 0.67 <sup>b</sup>  | 0.140 | 0.005   |
| Butyric acid                                 | 5.38               | 4.16                | 3.44               | 0.418 | 0.168   |
| Isovaleric acid                              | 1.78               | 1.14                | 1.17               | 0.129 | 0.065   |
| Valeric acid                                 | 1.90 <sup>a</sup>  | 0.96 <sup>b</sup>   | 0.86 <sup>b</sup>  | 0.187 | 0.033   |
| A/P                                          | 12.59              | 13.26               | 15.87              | 1.002 | 0.395   |

CON = control group; Abx = antibiotics addition group; MCE = *Macleaya cordata* extract addition group; SEM = standard error of the mean; VFA = volatile fatty acid; A/P = the ratio of acetic acid/propionic acid.

<sup>a,b</sup>Means within a row with no common superscripts differ ( $P < 0.05$ ).

### 3.4. TLR-MyD88 signal pathway in the gastrointestinal mucosa

Mucosal immunity plays a key role in the host defence against pathogen invasion. Hence, we detected the alterations in the TLR signal pathway at the mRNA level. At the rumen mucosa (Table 6), MCE addition stimulated *TLR8* and *IL-8* mRNA relative expression

**Table 6**  
Expression of genes involved in TLR-MyD88 pathway and effector molecules in the gastrointestinal mucosa of goats.

| Item         | CON               | Abx               | MCE               | SEM   | P-value |
|--------------|-------------------|-------------------|-------------------|-------|---------|
| <b>Rumen</b> |                   |                   |                   |       |         |
| <i>TLR1</i>  | 1.09              | 0.77              | 1.00              | 0.104 | 0.447   |
| <i>TLR2</i>  | 1.09              | 1.05              | 0.95              | 0.068 | 0.716   |
| <i>TLR4</i>  | 1.04              | 0.87              | 0.93              | 0.060 | 0.505   |
| <i>TLR6</i>  | 1.04              | 1.00              | 0.97              | 0.057 | 0.894   |
| <i>TLR7</i>  | 1.12              | 0.98              | 0.83              | 0.105 | 0.540   |
| <i>TLR8</i>  | 0.98 <sup>b</sup> | 0.56 <sup>c</sup> | 1.43 <sup>a</sup> | 0.095 | <0.001  |
| <i>TLR9</i>  | 1.08              | 1.10              | 0.90              | 0.097 | 0.675   |
| <i>MyD88</i> | 1.02              | 1.08              | 0.99              | 0.038 | 0.211   |
| <i>IRAK1</i> | 1.03              | 1.08              | 0.99              | 0.038 | 0.646   |
| <i>IL-1β</i> | 1.08 <sup>a</sup> | 0.41 <sup>b</sup> | 0.86 <sup>a</sup> | 0.087 | 0.003   |
| <i>IL-6</i>  | 1.55              | 0.56              | 1.22              | 0.229 | 0.205   |
| <i>IL-8</i>  | 1.03 <sup>b</sup> | 0.32 <sup>c</sup> | 1.45 <sup>a</sup> | 0.149 | 0.002   |
| <i>IFN-β</i> | 1.06              | 1.11              | 1.11              | 0.089 | 0.963   |
| <b>Ileum</b> |                   |                   |                   |       |         |
| <i>TLR1</i>  | 1.15              | 1.47              | 1.29              | 0.146 | 0.675   |
| <i>TLR2</i>  | 0.90              | 1.21              | 1.11              | 0.069 | 0.180   |
| <i>TLR4</i>  | 1.06              | 1.34              | 1.23              | 0.058 | 0.150   |
| <i>TLR6</i>  | 1.06              | 1.59              | 1.31              | 0.110 | 0.138   |
| <i>TLR7</i>  | 1.07 <sup>b</sup> | 2.00 <sup>a</sup> | 1.00 <sup>b</sup> | 0.182 | 0.038   |
| <i>TLR8</i>  | 1.05              | 1.18              | 1.16              | 0.043 | 0.429   |
| <i>TLR9</i>  | 1.25              | 1.69              | 1.83              | 0.248 | 0.631   |
| <i>MyD88</i> | 1.03              | 1.20              | 1.20              | 0.045 | 0.206   |
| <i>IRAK1</i> | 1.04              | 1.00              | 1.03              | 0.047 | 0.926   |
| <i>IL-1β</i> | 1.11              | 1.50              | 1.18              | 0.079 | 0.093   |
| <i>IL-6</i>  | 1.10              | 1.53              | 1.38              | 0.099 | 0.212   |
| <i>IL-8</i>  | 1.13              | 0.80              | 0.84              | 0.098 | 0.342   |
| <i>IFN-β</i> | 1.10              | 1.51              | 1.10              | 0.093 | 0.108   |

CON = control group; Abx = antibiotics addition group; MCE = *Macleaya cordata* extract addition group; SEM = standard error of the mean; TLR = Toll like receptor; *MyD88* = myeloid differentiation factor 88; *IRAK1* = interleukin 1 receptor associated kinase 1; *IL* = interleukin; *IFN-β* = interferon-beta.

<sup>a-c</sup>Means within a row with no common superscripts differ ( $P < 0.05$ ).

levels ( $P < 0.05$ ) compared to the CON group. However, antibiotic addition suppressed *TLR8*, *IL-8* and *IL-1β* mRNA relative expression levels ( $P < 0.05$ ) compared to CON group. Neither MCE nor antibiotics affect other TLR, including *TLR1*, *TLR2*, *TLR4*, *TLR6*, *TLR7* and *TLR9*, *MyD88*, interleukin 1 receptor associated kinase 1 (*IRAK1*), *IL-6* and *IFN-β* mRNA relative expression levels ( $P > 0.05$ ). At the ileum mucosa, antibiotics stimulated *TLR7* mRNA relative expression levels compared to CON and the MCE groups ( $P = 0.038$ ). MCE or antibiotics did not change the expression of other genes ( $P > 0.05$ ).

### 3.5. Diversity and composition of gastrointestinal bacterium

Due to the importance of microbiota to the gastrointestinal function, we further investigated the bacterial community in the rumen and ileum chyme by employing 16S rRNA sequencing. The results showed that antibiotics decreased the observed\_operational taxonomic units (OTU), Chao1 index, Shannon index and Simpson index ( $P < 0.05$ ), but increased the coverage of bacteria both in the rumen and ileum, compared to the CON group ( $P < 0.05$ , Table 7). MCE addition did not affect the alpha diversity of bacteria in the rumen and ileum compared to the CON group ( $P > 0.05$ ).

Furthermore, detection of the bacterial phyla in the rumen showed that antibiotics increased the relative abundance of Bacteroidetes ( $P < 0.001$ ) and Proteobacteria ( $P < 0.05$ ) and decreased the relative abundance of Firmicutes ( $P < 0.001$ ) and Fibrobacteres ( $P < 0.001$ , Fig. 1A and B). However, MCE addition exerted an opposite effect on these bacteria compared to the Abx group. At the genus level, the relative abundance of *Prevotella\_1* ( $P < 0.01$ ), *Prevotella* ( $P < 0.001$ ) and *Prevotellaceae\_UCG-001* ( $P < 0.01$ ) was increased by antibiotics addition compared with those in the CON group, but the relative abundance of *Christensenellaceae\_R-7\_group*, *Muribaculaceae\_unclassified*, *Ruminococcaceae\_NK4A214\_group* and *Butyrivibrio\_2* was decreased by antibiotics ( $P < 0.001$ , Fig. 1C and D). Similarly, MCE addition exerted the opposite effect on these bacteria except *Prevotellaceae\_UCG-001* compared to the Abx group.

Results of beta-diversity of the ileal bacteria phyla showed that the relative abundance of Firmicutes ( $P < 0.001$ ) and Tenericutes was decreased by antibiotics ( $P < 0.01$ ) compared to the CON group, however, the relative abundance of Actinobacteria ( $P < 0.05$ ) and Cyanobacteria was increased by antibiotics ( $P < 0.01$ , Fig. 2A and B). More significantly, MCE addition exerted the opposite effect on these bacteria compared to the Abx group. At the genus level, the relative abundance of *Eubacterium\_coprostanoligenes* ( $P < 0.01$ ), *Firmicutes\_unclassified* ( $P < 0.01$ ), *Christensenellaceae\_R-7* ( $P < 0.01$ ), *Ruminococcaceae\_UCG-014* ( $P < 0.01$ ), and *Ruminococcaceae\_NK4A214* ( $P < 0.001$ ) was decreased by antibiotic addition compared with that in the CON group, while the relative abundance of *Olsenella* was increased by antibiotics ( $P < 0.01$ ). Similarly, the relative abundance of these genera was opposite in the MCE group compared with those in the Abx group (Fig. 2C and D).

The microbiome phenotypes in the rumen and ileum were further analyzed. Compared to the CON and MCE groups, the proportion of bacteria with pathogenic potential in the rumen rather than the ileum was increased by antibiotics ( $P < 0.001$ ). The proportion of Gram-positive bacteria both in the rumen ( $P < 0.001$ ) and ileum ( $P < 0.05$ ) was decreased by antibiotic addition, while the population of Gram-negative bacteria in the rumen ( $P < 0.001$ ) and ileum ( $P < 0.05$ ) was increased by antibiotic addition compared with that of the CON and MCE groups (Fig. 3).

### 3.6. Immunometabolic biomarkers in the blood

To figure out which immuno-metabolites contributed to improve growth performance, we further determined their

**Table 7**  
Rumen and ileum bacterial alpha diversity of goats.

| Item          | CON   | Abx   | MCE   | SEM    | P-value    |            |            |
|---------------|-------|-------|-------|--------|------------|------------|------------|
|               |       |       |       |        | Abx vs CON | MCE vs CON | MCE vs Abx |
| Rumen         |       |       |       |        |            |            |            |
| Coverage, %   | 99.50 | 99.80 | 99.58 | 0.001  | 0.014      | 0.829      | 0.067      |
| Observed_OTU  | 1157  | 643   | 1133  | 63.0   | 0.001      | 0.879      | 0.002      |
| Chao1 index   | 1212  | 664   | 1176  | 67.5   | 0.001      | 0.859      | <0.001     |
| Shannon index | 8.76  | 7.05  | 8.80  | 0.184  | <0.001     | 0.839      | 0.001      |
| Simpson index | 0.994 | 0.963 | 0.993 | 0.0048 | <0.001     | 0.842      | 0.001      |
| Ileum         |       |       |       |        |            |            |            |
| Coverage, %   | 99.68 | 99.94 | 99.66 | 0.001  | 0.001      | 0.902      | <0.001     |
| Observed_OTU  | 776   | 243   | 827   | 56.1   | <0.001     | 0.611      | <0.001     |
| Chao1 index   | 812   | 249   | 861   | 59.1   | <0.001     | 0.648      | <0.001     |
| Shannon index | 7.07  | 4.65  | 7.27  | 0.282  | 0.001      | 0.666      | <0.001     |
| Simpson index | 0.964 | 0.857 | 0.971 | 0.021  | 0.012      | 0.582      | 0.002      |

CON = control group; Abx = antibiotics addition group; MCE = *Macleaya cordata* extract addition group; SEM = standard error of the mean; OTU = operational taxonomic unit.

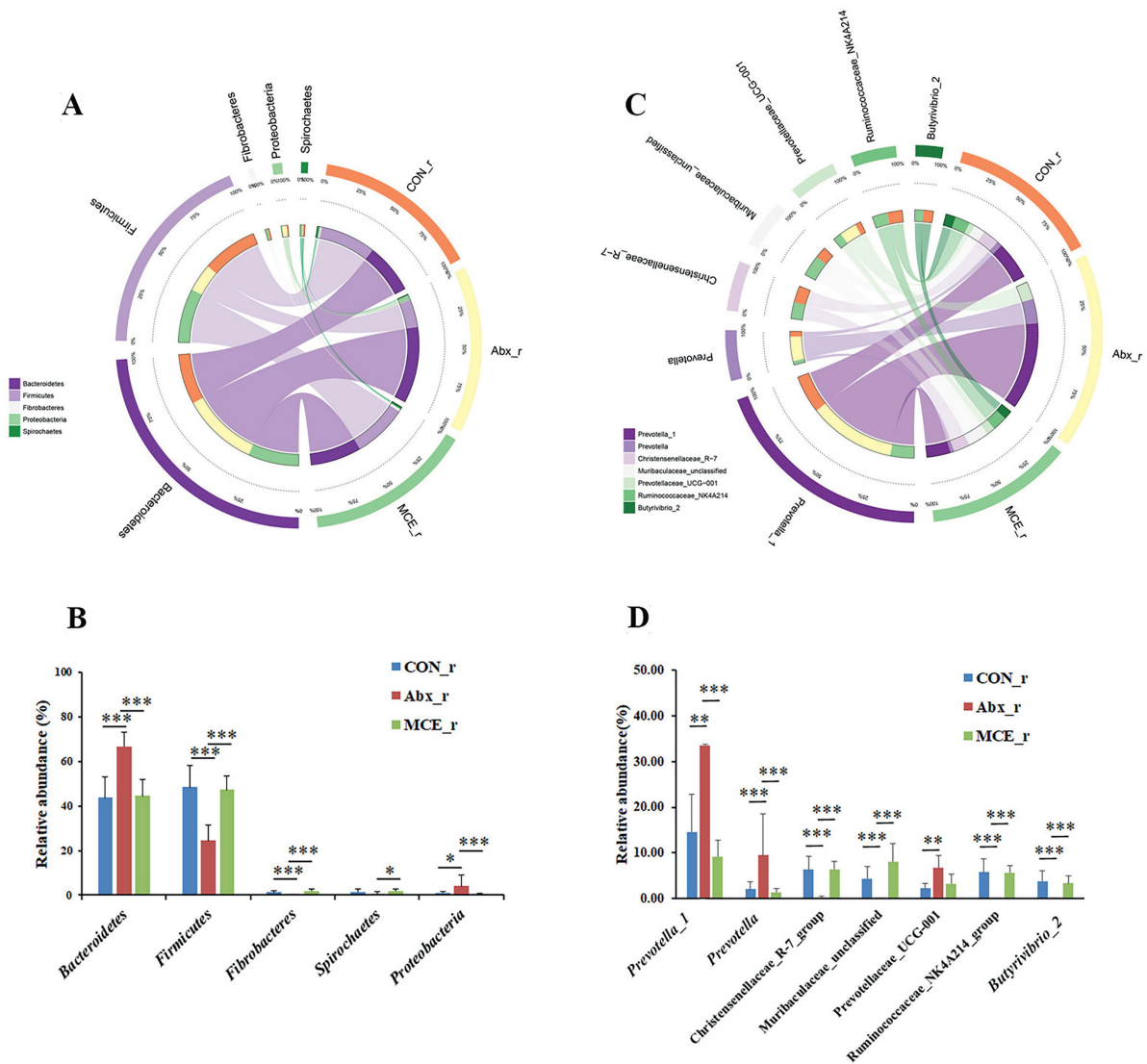


**Fig. 1.** Beta-diversity of rumen bacteria in goats. (A) Relative abundance of the top 20 phyla. (B) Relative abundance of the differential phyla. (C) Relative abundance of the top 20 genera. (D) Relative abundance of the differential genera. CON\_r = rumen of control group; Abx\_r = rumen of antibiotics addition group; MCE\_r = rumen of *Macleaya cordata* extract addition group. Results are presented as the mean  $\pm$  SEM of ten independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  are significantly different from each group.

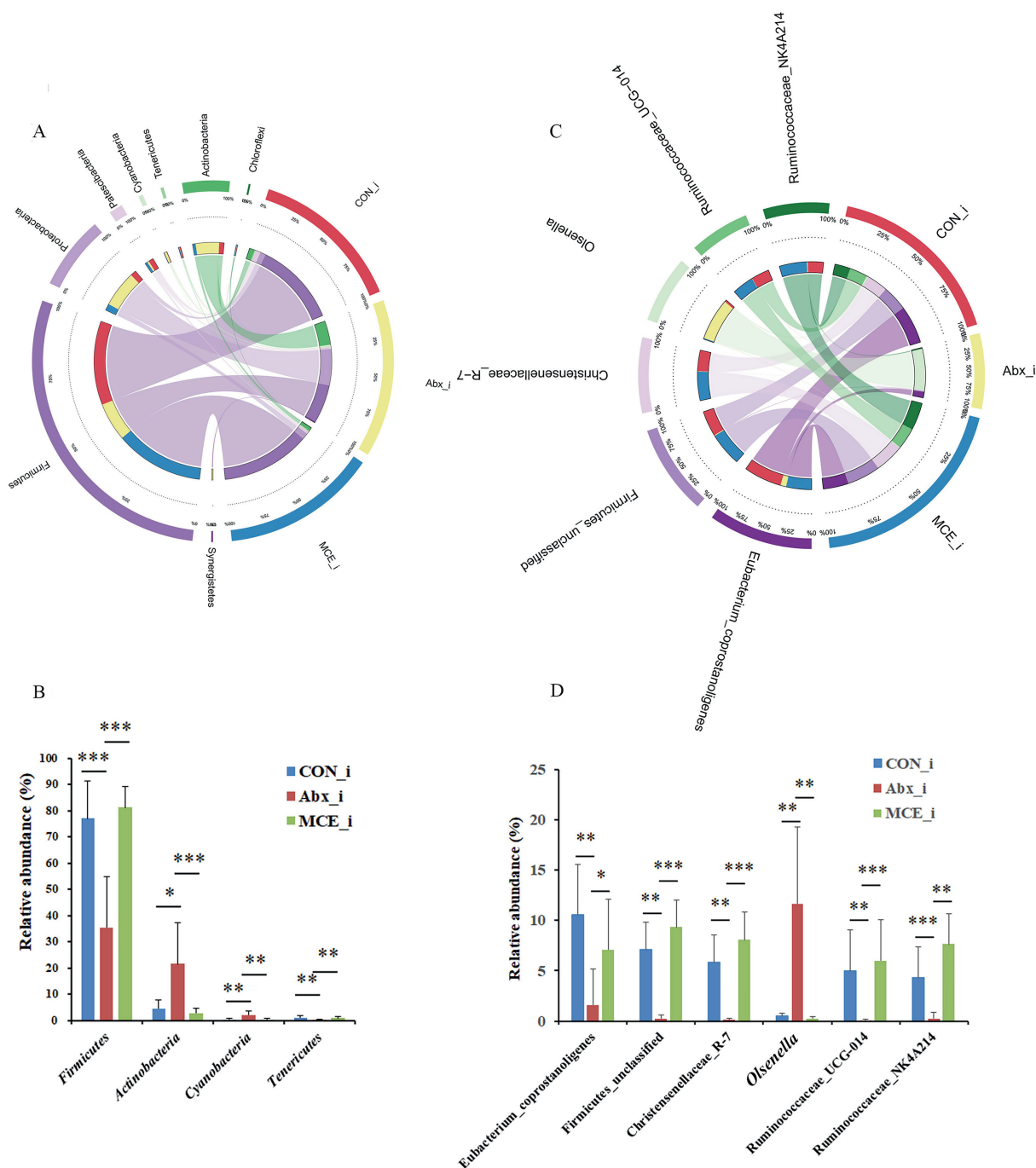


**Fig. 2.** Beta-diversity of ileum bacteria in goats. (A) Relative abundance of the top 20 phyla. (B) Relative abundance of the differential phyla. (C) Relative abundance of the top 20 genera. (D) Relative abundance of the differential genera. CON\_i = ileum of control group; Abx\_i = ileum of antibiotics addition group; MCE\_i = ileum of *Macleaya cordata* extract addition group. Results are presented as the mean  $\pm$  SEM of ten independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  is significantly different from each group.

concentrations at the end of the experiment (Table 8). Compared to the CON group, antibiotic addition increased the creatinine content ( $P = 0.036$ ), but MCE addition did not alter its content. Neither MCE nor antibiotics changed the activities of AST, ALT and the contents of albumin, and urea nitrogen ( $P > 0.05$ ). For the cytokines, antibiotics decreased the IL-1 $\beta$  content compared to the CON group ( $P = 0.033$ ). However, there were no difference in the IL-1 $\beta$  content between the MCE and CON groups ( $P > 0.05$ ). Furthermore, MCE or antibiotics did not affect the contents of IL-4, IL-17, GM-CSF, IFN- $\gamma$  and IFN- $\gamma$ /IL-4 ( $P > 0.05$ ). For the complements and immunoglobulins, MCE addition markedly increased the content of IgM by 19.9%

and 26.0% compared to the CON and Abx groups ( $P < 0.05$ ). Additionally, MCE or antibiotic addition did not influence the contents of complement 2, complement 4, IgG and IgA ( $P > 0.05$ ).

### 3.7. Lymphocytes, monocytes, and T lymphocytes subset in the blood

Compared with the CON group, MCE addition decreased the amount of lymphocytes ( $P = 0.018$ ), but did not alter their proportion ( $P > 0.05$ , Table 9). Antibiotic addition also decreased the amount of lymphocytes but increased that of monocytes ( $P < 0.05$ ).

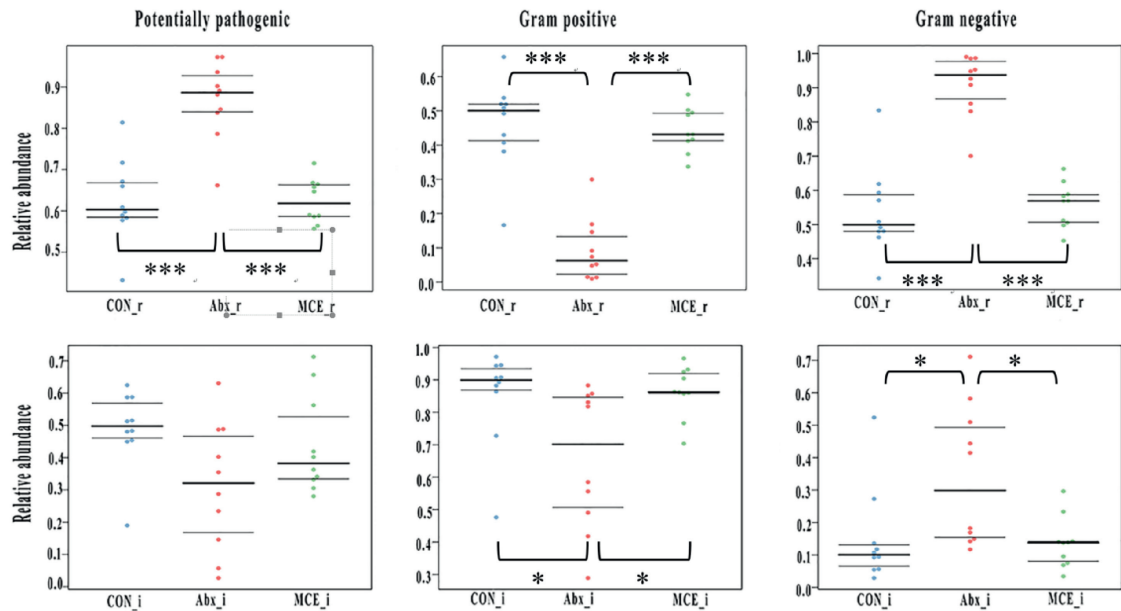


**Fig. 3.** Proportion of microbiome phenotypes in rumen and ileum of goats. CON\_r = rumen of control group; Abx\_r = rumen of antibiotics addition group; MCE\_r = rumen of *Macleaya cordata* extract addition group; CON\_i = ileum of control group; Abx\_i = ileum of antibiotics addition group; MCE\_i = ileum of *Macleaya cordata* extract addition group. \* $P < 0.05$ , \*\*\* $P < 0.001$  is significantly different from each group.

**Table 8**  
Immunometabolic biomarkers in the blood of goats.

| Item                              | CON                  | Abx                  | MCE                  | SEM    | P-value |
|-----------------------------------|----------------------|----------------------|----------------------|--------|---------|
| Biochemistry parameters           |                      |                      |                      |        |         |
| AST, U/L                          | 97.89                | 105.80               | 99.33                | 3.283  | 0.585   |
| ALT, U/L                          | 22.41                | 23.86                | 21.05                | 0.934  | 0.483   |
| Albumin, g/L                      | 28.99                | 25.50                | 27.14                | 0.751  | 0.173   |
| Creatinine, $\mu\text{mol/L}$     | 29.56 <sup>b</sup>   | 39.00 <sup>a</sup>   | 30.00 <sup>b</sup>   | 1.744  | 0.036   |
| Urea nitrogen, mmol/L             | 5.51                 | 5.12                 | 5.27                 | 0.223  | 0.789   |
| Cytokines, pg/mL                  |                      |                      |                      |        |         |
| IL-1 $\beta$                      | 135.20 <sup>a</sup>  | 106.53 <sup>b</sup>  | 115.55 <sup>ab</sup> | 4.737  | 0.033   |
| IL-4                              | 51.58                | 49.87                | 57.16                | 2.087  | 0.343   |
| IL-12                             | 739.24               | 600.46               | 728.00               | 27.808 | 0.071   |
| IL-17                             | 39.66                | 37.75                | 42.21                | 1.559  | 0.523   |
| GM-CSF                            | 1104.54              | 1103.86              | 1167.75              | 44.572 | 0.812   |
| IFN- $\gamma$                     | 542.15               | 542.35               | 564.11               | 17.998 | 0.860   |
| IFN- $\gamma$ /IL-4               | 10.63                | 10.90                | 10.34                | 0.400  | 0.861   |
| Complements, ng/mL                |                      |                      |                      |        |         |
| Complement 2                      | 119.38               | 96.85                | 115.09               | 4.726  | 0.115   |
| Complement 4                      | 16.01                | 14.37                | 14.15                | 0.484  | 0.235   |
| Immunoglobulins, $\mu\text{g/mL}$ |                      |                      |                      |        |         |
| IgM                               | 1794.24 <sup>b</sup> | 1707.64 <sup>b</sup> | 2151.98 <sup>a</sup> | 70.250 | 0.016   |
| IgG                               | 544.24               | 540.21               | 567.64               | 27.287 | 0.914   |
| IgA                               | 220.18               | 181.82               | 209.57               | 0.990  | 0.456   |

CON = control group; Abx = antibiotics addition group; MCE = *Macleaya cordata* extract addition group; SEM = standard error of the mean; AST = aspartate aminotransferase; ALT = alanine transaminase; IL-1 $\beta$  = interleukin-1 $\beta$ ; IL-4 = interleukin 4; IL-12 = interleukin-12; IL-17 = interleukin-17; GM-CSF = granulocyte-macrophage colony-stimulating factor; IFN- $\gamma$  = interferon- $\gamma$ ; IFN- $\gamma$ /IL-4 = the ratio of interferon- $\gamma$  to interleukin-4; IgM = immunoglobulin M; IgG = immunoglobulin G, IgA = immunoglobulin A.

<sup>a,b</sup>Means within a row with no common superscripts differ ( $P < 0.05$ ).

Neither MCE or antibiotics influenced the proportion of monocytes ( $P > 0.05$ ). Considering the alteration in the level of lymphocytes by MCE or antibiotics, we further analyzed their subset by flow cytometry. Unexpectedly, antibiotics rather than MCE addition increased the proportion of CD4<sup>+</sup>T cells ( $P = 0.038$ ). However,

**Table 9**  
Amount and proportion of blood cells in goats.

| Item                                  | CON                | Abx                | MCE                 | SEM   | P-value |
|---------------------------------------|--------------------|--------------------|---------------------|-------|---------|
| Lymphocytes, $\times 10^9/\text{L}$   | 10.82 <sup>a</sup> | 7.58 <sup>b</sup>  | 6.21 <sup>b</sup>   | 0.697 | 0.018   |
| Lymphocytes, %                        | 55.60              | 42.62              | 57.51               | 3.274 | 0.140   |
| Monocytes, $\times 10^9/\text{L}$     | 0.97 <sup>b</sup>  | 2.71 <sup>a</sup>  | 1.33 <sup>b</sup>   | 0.266 | 0.010   |
| Monocytes, %                          | 14.70              | 16.03              | 10.14               | 2.014 | 0.486   |
| CD4 <sup>+</sup> T, %                 | 5.30 <sup>b</sup>  | 18.79 <sup>a</sup> | 10.24 <sup>ab</sup> | 2.151 | 0.038   |
| CD8 <sup>+</sup> T, %                 | 7.57               | 10.39              | 7.16                | 1.246 | 0.553   |
| CD4 <sup>+</sup> T/CD8 <sup>+</sup> T | 0.98               | 2.03               | 1.57                | 0.203 | 0.132   |

CON = control group; Abx = antibiotics addition group; MCE = *Macleaya cordata* extract addition group; SEM = standard error of the mean.

<sup>a,b</sup>Means within a row with no common superscripts differ ( $P < 0.05$ ).

neither MCE or antibiotics changed the CD8<sup>+</sup>T proportion and CD4<sup>+</sup>T/CD8<sup>+</sup>T ( $P > 0.05$ ).

#### 4. Discussion

The gastrointestinal epithelium is not only the central site of nutrient absorption but also is a physical and functional barrier between the host and the outside world. As the largest compartment of forestomach in ruminants, the rumen wall comprises five different layers including the epithelial layer, lamina propria, submucosa, muscular layer, and serous layer. In addition to absorptive functions, the rumen epithelium performs many other essential physiological functions, such as transportation, VFA metabolism, and protection (Connor et al., 2013). The ileal epithelium consists of a single layer of intestinal epithelial cells and it is a major site for the development of the gut-associated lymphoid tissues during early life, which is not found in the rumen (Mebius, 2003). In the present study, the crypt and villus morphology of the rumen and ileum was impaired by the addition of antibiotics, however, ileum morphology was improved by MCE addition. This result indicated

that MCE rather than antibiotics could enhance the digestion and absorption functions of the ileum and maintain the homeostatic of the rumen epithelium. Literature findings indicate that neomycin treatment decreases fecal population of *Lactobacillus* spp. and changes the histology of intestine without inducing inflammation (Bose et al., 2013).

In the forestomach, VFA originate from microbial digestion of carbohydrates and are largely absorbed across the epithelium of the rumen and omasum, providing up to 70% to 80% of daily energy requirements (Flint and Bayer, 2008). Indeed, metabolites produced by bacterial populations either adhere to the gut epithelium or in the lumen can pass into the host circulation and exert beneficial or harmful effects (Canfora et al., 2019), such as branch-chain fatty acids (BCFA) like isobutyric acid and isovaleric acid. Unlike the well-known major VFA (acetic acid, butyric acid, and propionic acid), data on the physiological function of BCFA is sparse. Some in vivo studies suggest that BCFA might play a pathological role in cardiometabolic diseases, such as obesity (Tiihonen et al., 2010), nonalcohol fatty liver (Da Silva et al., 2018), and hypercholesterolemias (Granado-Serrano et al., 2019). Other in vitro studies revealed their gluconeogenic potential (Choi et al., 2021) in mice hepatocytes and lipolysis blocking abilities in rat and human adipocytes (Heimann et al., 2016). The decreased total rumen VFA and ileal BCFA in goats supplemented with antibiotics demonstrated the damaging effects of antibiotics on rumen carbohydrate fermentation. The decline of BCFA generation in the ileum of goats supplemented with antibiotics and MCE suggested that both antibiotics and MCE had the abilities to block the detrimental effects of BCFA on small intestinal health.

Among the digestive organs, the rumen is inhabited by the largest number and variety of microorganisms, including bacteria, protozoa, and anaerobic fungi. These microorganisms are able to digest 50% to 55% of the total carbohydrate intake every day. Therefore, rumen microorganisms play an important role in the nutrient digestion process. Except for the rumen, the ileum also functions as an indispensable fermentation site in goats (Jiao et al., 2014a,b). In the current study, the observed\_OTU, Chao1, Shannon and Simpson indexes of both the rumen and ileum chyme were decreased by antibiotic addition, demonstrating that neomycin and vancomycin application could disrupt the ecological balance of the microorganisms in the rumen and ileum. This observation confirmed the combined abilities of neomycin and vancomycin to destroy the gastrointestinal microbial community (Cheng et al., 2017; Lewis et al., 2015) in weaner goats.

At the phylum level, Firmicutes and Synergistetes primarily colonize in the rumen of goats aged between 80 and 100 days, and Firmicutes and Bacteroidetes become dominant in the rumen of 110-day-old goats (Han et al., 2015). Our data showed that Bacteroidetes and Firmicutes were the predominant phyla and accounted for more than 90% of the ruminal chyme of goats in each treatment. Notably, Bacteroidetes became the dominant phylum after antibiotic inclusion, suggesting that Bacteroidetes, to an extent, were resistant to neomycin and vancomycin. Our findings were inconsistent with a previous report (Han et al., 2015) and breed (Chuanzhong Black goat in this study vs. Shaanbei white-cashmere) was potential a reason for the conflicting results. It is reported that Firmicutes can degrade fiber such as cellulose, which is closely related to the body's energy metabolism and plays an essential role in the process of polysaccharide fermentation. Moreover, Firmicutes and Bacteroidetes have interactive effects on energy metabolism, which can cooperatively promote the absorption, utilization, and storage of energy (Koliada et al., 2017; Qu et al., 2017; Salyers, 1984). The phylum Fibrobacteres are prolific cellulolytic bacteria that produce lesser amounts of acetic acids (Jewell et al., 2013). The decrease in the abundances of Firmicutes and

Fibrobacteres in the rumen chyme of goats treated with antibiotics instead of MCE was observed in our study, revealing that antibiotics may weaken the ability of microorganisms in the rumen of goats to degrade cellulose. It is worth mentioning that MCE administration could restore Firmicutes and Fibrobacteres abundances to normal levels, reflecting its protective effect on rumen microbial ecosystem.

In the current study, the microbial community structure in the ileum and the rumen was different. At the phylum level, Firmicutes and Actinobacteria were the predominant bacteria in the ileum, whereas Actinobacteria and their subordinate genus *Olsenella* were increased after antibiotic inclusion. The phylum Actinobacteria is a probiotic bacterium, which can generate large amounts of bioactive compounds, including antimicrobial substances (Gomes et al., 2017). Therefore, we speculated that neomycin and vancomycin might regulate the synthesis of antimicrobial substances in the small intestine of goats.

Humoral immune response involves high titers of different antibody classes in the serum of infected host. Generally, there is an initial increase of IgM, followed by IgG and other specific antibody classes, such as IgA (Hughes et al., 1985). Under the condition of broad-spectrum antibiotics, including neomycin, vancomycin and metronidazole, antibody responses are not altered in healthy adults. However, a significant impairment in H1N1-specific binding IgG1 and IgA responses were observed in adults subjected with seasonal influenza vaccination (Hagan et al., 2019). An increase in blood IgM of goats after MCE inclusion was observed in this study, proving the role of MCE in improving humoral immunity (Zhang et al., 2018; Ling et al., 2021).

Cellular immune responses are also important in the host defence against pathogenic bacteria. Monocytes produced by the bone marrow play critical roles in immune response against infections and injuries. Three monocytes subsets have been identified in bovine peripheral blood (Corripio-Miyar et al., 2015; Hussen et al., 2013) and showed functional differences in phagocytic capability, reactive oxygen species production and inflammasome activation (Hussen and Schuberth, 2017). It is reported that vancomycin administration characteristically influences differentiation of splenic Foxp3<sup>+</sup> Treg cells in neonatal mice (Cheng et al., 2017). A study in vitro reported the rapid proliferation of CD4<sup>+</sup>T and CD8<sup>+</sup>T cells when vancomycin was administered (Ogese et al., 2021). An elevate in monocytes and CD4<sup>+</sup>T/CD8<sup>+</sup>T were found in the blood of goats treated with antibiotics. The elevation in CD4<sup>+</sup>T/CD8<sup>+</sup>T was induced mainly by the expansive CD4<sup>+</sup>T cells, indicating the prominent role of antibiotics on the proliferation capacity of CD4<sup>+</sup>T cells than CD8<sup>+</sup>T cells. This proliferation of CD4<sup>+</sup>T cells in the peripheral blood after antibiotic treatment could enhance the goat defence by modulating its immune response toward humoral (Th2) and cell-mediated (Th1) immunity. Increased circulating monocytes and CD4<sup>+</sup>T cells in the goats could indicate an important immune regulatory and homeostatic mechanism for controlling excessive inflammation, which was consistent with the reduction of inflammatory cytokines such as IL-1 $\beta$  in the blood.

Correlations between peripheral blood mononuclear leukocyte (PBML) subsets and growth performance were investigated, which stated that several of these PBML including the number of monocytes and CD8 $\alpha^+$  cells, were strongly and negatively correlated with daily gain (Clapperton et al., 2009, 2008). Therefore, the increased number of monocytes and CD4<sup>+</sup>T/CD8<sup>+</sup>T in the blood may hinder average daily gain of goats administrated with antibiotics. Serum immunoglobulin levels are particularly important to newborn kids. Earlier research reported that there was no discernible correlation between initial serum immunoglobulin content and average daily gain in goat kids that remained healthy through weaning (Obrien and Sherman, 1993). However, the study with pre-weaned dairy

calves stated a significant positive association between serum IgG content and average daily gain (Elsohaby et al., 2019). As a pentameric and polyreactive antibody, IgM has a key function in protecting against a range of viral, bacterial, fungal, and parasitic infections and works as a scavenger, protector, and regulator (Ehrenstein and Notley, 2010). An increase in serum IgM content and ileal villus height, villus height/crypt depth, and average daily gain were observed in goats fed with MCE rather than antibiotics in our study. Whether increased serum IgM content contributed to the elevated average daily gain in goats requires more investigation. The increase of villus height can increase the area available for nutrient absorption in the ileum. The villus height/crypt depth comprehensively reflects the functional state of the ileum. Therefore, MCE addition could enhance nutrient absorption and improve the functional state of the small intestine.

## 5. Conclusions

Supplementation of antibiotics containing vancomycin and neomycin suppressed the epithelial state and microbial diversity and fermentation in goats by decreasing the height and area of rumen papillae, ruminal mucosa *TLR8*, *IL-8* and *IL-1 $\beta$*  expression and microbial diversity, and altering the VFA profile in the rumen. But supplementation of antibiotics stimulated the cellular response to maintain the growth performance of goats by increasing the amounts of monocytes and CD4<sup>+</sup>T cells in the peripheral blood. Administration of MCE with 5 g/d improved epithelial state and humoral response to promote the growth performance in goats by increasing the average daily gain, ameliorating ileal microvilli morphology, and promoting IgM secretion in the peripheral blood.

## Author contributions

**Qiongxin Yan:** Methodology, Data curation, Funding acquisition. **Xinlin Li:** Investigation, Writing-original draft, Funding acquisition. **Wenxun Chen:** Investigation, Funding acquisition. **Xiaoling Zhou** and **Pramote Paengkoum:** Writing-review & editing. **Xinzhou Tian** and **Suntorn Wittayakun:** Visualization. **Zhiliang Tan:** Conceptualization, Funding acquisition.

## 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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