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In vitro fermentation mediated by *Akkermansia muciniphila* caused lactate accumulation and blocked butyrate production *via* shaping functional bacterial community

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ABSTRACT

To investigate the effect of *Akkermansia muciniphila* on hypertension, the study performed an *in vitro* fermentation on the feces of hypertensive patients. The experimental groups were hypertensive elderly (E), hypertensive elderly + *A. muciniphila* (EA), and hypertensive long-lived elderly (L). Results showed that the pH value decreased among the 3 groups and produced numerous lactate at 9 h. Then, the pH value increased and lactate was almost exhausted at 48 h in group E. However, compared to groups E and L, group EA showed lower pH value, lower butyrate level, and higher lactate level. Compared to group E in 9 h, EA significantly increased the relative abundance of *Akkermansia* and *Lactococcus* and decreased *Clostridium_sensu_stricto*_1. The relative abundances of *Akkermansia* and *Bifidobacterium* increased in the group EA compared to group E, whereas the relative abundances of *Clostridium_sensu_stricto*_1 decreased at 48 h. Metabolomics showed that *A. muciniphila* altered the levels of 9 metabolites with reduced trimethylamine (TMA) and trimethyl-5-aminovaleric acid (TMAVA) related to hypertension. The lactate concentration showed positive correlation with *Enterococcus* and *Bifidobacterium*, and the butyrate concentration showed positive correlation with *Clostridium_sensu_stricto*_1, indicating that metabolites shifts by those functional bacterial communities. These results present a potential perspective for *A. muciniphila* mediated microbial micro-environment change to increase the relative abundance of *Bifidobacterium* and lactate production.

1. Introduction

Over 100 trillion microorganisms inhabiting the human gastrointestinal tract form an intricate ecosystem with host health, wherein Firmicutes and Bacteroidetes represent the dominant phyla, followed by Proteobacteria, Actinobacteria, Verrucomicrobia, and Fusobacteria[1-2]. Gut microbes ferment in the colon to produce various products, such as short-chain fatty acids (SCFAs), lactate, bile acids, gases, bioactive substance, and others[3-4]. As the most abundant microbial-derived metabolites, SCFAs, derived mainly from dietary fiber and to a lesser extent from dietary protein and blood circulation[5]. As the main SCFAs, acetate, propionate, and butyrate can provide energy for intestinal epithelium, maintain mucosal immune homeostasis, and inhibit intestinal pathogens[6]. As a pivotal endogenous metabolite, lactate is produced through mammalian muscle tissue metabolism and microbial fermentation in the gut[3]. Lactate is produced mainly in the small intestine enriched with lactate-producing bacteria and utilized in the colon enriched with lactate-utilization bacteria[7]. Lactate exemplifies cross-feeding, crucial for the survival of specific gut microbes and the generation of beneficial metabolites. For example, *Eubacterium hallii*, *Anaerostipes caccae*, and species from *Clostridial* cluster XIVa have been demonstrated to

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utilize lactate and produce butyrate[7]. In addition, lactate is abundant in the infant's gut with several enriched lactate-producing bacteria and beneficial for nutrient chain transfer during fermentation. On the contrary, butyrate is absent with low abundance of butyrate-producing bacteria in the infant's gut[8]. The cross-feeding phenomenon of lactate utilization and butyrate production is of particular interest because it may explain the specific role of gut microbes.

Akkermansia muciniphila is commonly recognized as a gut commensal microbe with probiotic effects, including maintaining mucosal homeostasis, increasing SCFA production, and promoting beneficial gut microbes[9]. The decreased level of *A. muciniphila* was correlated with various diseases, such as obesity, metabolic syndrome, diabetes, and gut inflammation[10]. The ecological niche of *A. muciniphila* is defined by mucin degradation, leading to its preferential colonization of the large intestine (thick mucus layer) over the small intestine (thin mucus layer)[11]. Similarly, *A. muciniphila* is more abundant in the lumen (0.57%) than in the colonic mucosa (0.21%)[12]. However, the abundance of *A. muciniphila* present in the colon is subject-specific[13]. The colonization of *A. muciniphila* depends on pH and mucin concentration in the intestinal environment. The abundance of *A. muciniphila* peaks in the distal colon (pH 6.6–6.9)[14]. This distribution pattern confirms its strong preference for the large intestinal environment. From the nutrient preference, *A. muciniphila* most avidly use circulating lactate in addition to mucin[5]. The colonization of *A. muciniphila* in the mucus layer (near the intestinal epithelial cells) coincides with an increase in their access to circulating lactate. From the production of the metabolites, *A. muciniphila* produces 1,2-propanediol, propionate, acetate, and lactate and liberates mucin sugars for cross-feeding of other bacteria[15]. *A. muciniphila* shows strong potential for various diseases treatment, but its role in mediating gut microbe-metabolites remains to be determined.

As the main cause of cardiovascular disease, the prevalence of hypertension increased worldwide[16]. The stability of blood pressure is controlled by the kidneys and regulated by genetic and environmental factors. Recent reports showed that dysbiosis of gut microbiota affects gene pathways related to the host's microbiota, leading to hypertension[17]. Specifically, the enrichment of gut microbiota in *Klebsiella pneumoniae* directly leads to the occurrence of hypertension[18]. Microbial metabolites are key mediators of intestinal host communication, which independently affecting blood pressure from the host immune system[19]. SCFAs and indole-3-lactic acid are beneficial for blood pressure, while trimethylamine (TMA) and TMA oxide are harmful[17,20]. The precise regulation of intestinal bacteria and supplementation of beneficial microbial metabolites represent promising strategies for lowering blood pressure[21]. However, it is difficult to understand the microbiota shifts and the cross-feeding of gut microbes solely from fecal samples. *In vitro* fecal fermentation modeling is effective in exploring fermentation characteristics and short-term gut microbe-metabolite changes mediated by specific prebiotic or probiotic strains[22]. *In vitro* fermentation results of human milk oligosaccharides with different conformations showed that their fermentation characteristics depend on the initial intestinal microbiota composition[23]. This indicates that while prebiotics serve as essential fermentation substrates, the initial gut microbiota plays a more dominant role in shaping the *in vitro* fermentation outcomes. Above all, *in vitro* fecal fermentation model is a simple and effective method to evaluate the effects of prebiotics or probiotics on gut microbiota composition and metabolites production. To better understand the relationship between *A. muciniphila* and hypertension, more studies are expected to focus on the effects of *A. muciniphila* mediated fermentation on fecal inoculum with hypertension.

Based on the previous research[24], this study hypothesis that *A. muciniphila* mediated fermentation could play a potential role by promoting a functional bacterial community assembly and changing the metabolites. This study aims to reveal the effect of *A. muciniphila* mediated *in vitro* fecal fermentation profiles in elderly patients with hypertension, particularly on gut microbiota composition and metabolites change.

2. Materials and methods

2.1 Materials

Mucin was obtained from Sigma Chemical Co., Ltd. (St. Louis, USA). Fructooligosaccharides (FOS) were purchased from Quantum Hi-Tech Biology Co., Ltd. (Jiangmen, China). Brain heart infusion broth (BHI), yeast extract, and *L*-cysteine hydrochloride were purchased from Qingdao Hi-Tech Industrial Park Hope Bio-Technology Co., Ltd. (Qingdao, China). All other chemicals are of analytical grade.

2.2 Fecal samples selection and gut microbiota analysis

The previous study revealed that *K. pneumoniae*'s microbial pathways had a similar trend with *Escherichia coli*, while *A. muciniphila* was contrary to them[24]. It showed that *A. muciniphila* and their related microbial pathways may play a critical role in treating high blood pressure caused by *E. coli* and *K. pneumoniae.* Therefore, we hypothesize that inoculating *A. muciniphila* into fecal samples rich in *E. coli* and *K. pneumoniae* can improve the microbial microenvironment and play a beneficial role. Thus, the fecal samples and their 16S rRNA gene sequences were selected from a larger former subgroup. The selection criteria were based on the significant differences in the relative abundance of *K. pneumoniae*, *E. coli*, and *A. muciniphila*. This study was approved by the Ethics Committee of Guangxi University (gxdxyxll05). Inclusion of donors with no experience of gastrointestinal disease or antibiotics in the past 6 months. Finally, 3 hypertensive elderly donors (aged 70–80, E) and 3 hypertensive long-lived elderly donors (aged 95–105, L) with normal body mass index were recruited at Hechi (Guangxi, China). The blood pressure characteristics of 6 donors were showed in Table S1. All donors signed the informed consent and cooperated with sample collection. Stool samples were collected in sterile tubes and stored in a –20 °C car refrigerator until they were transported back to the laboratory, where were then mixed with 15% glycerol and stored in a –80 °C ultra-low temperature refrigerator until use. The full-length 16S rRNA gene sequencing data of 6 subjects were obtained from the NCBI SRA database (BioProject: PRJNA888351). The sequences were analyzed using the BMKCloud (www.biocloud.net). After each operational taxonomic unit (OTU) was assigned to bacterial species, principal coordinate analysis (PCoA) with weighted-unifrac distance and subsequent clustering was performed to differentiate the 2 groups.

2.3 Isolation and preparation of *A. muciniphila* strain

Fecal samples used for isolation bacteria were selected from the centenarians who enriched *A. muciniphila*. Briefly, 1 g fecal sample was dissolved in 9 mL of sterile phosphate buffered saline (PBS) containing *L*-cysteine hydrochloride and diluted to an appropriate gradient. Then, 100 μL dilution was coated on the BHI agar medium containing 0.5% purified mucin and vancomycin to obtain colonies at 37 °C in anaerobic workstation (AW200SG MINI MICRO) for about 72 h. Different colonies on each plate were selected and identified using *A. muciniphila* specific primers F (5′-CAGCACGTGAAGGTGGGGAC-3′) and R (5′-CCTTGCGGTTGGCTTCAGAT-3′). Then, agarose gel electrophoresis to determine whether there is a target band in 327 bp and identify specific species by sequencing. *A. muciniphila* strain LTA21F2 was isolated and used for this experiment. *A. muciniphila* (LTA21F2) was cultured with BHI liquid medium under anaerobic conditions at 37 °C to get pure culture of live bacteria. *A. muciniphila* fermentation cultures were centrifuged (4 000 r/min, 15 min, 4 °C), washed, and resuspended with sterile PBS to a final concentration of 1.0×10^8 CFU/mL[25].

2.4 In vitro fecal fermentation

The method of *in vitro* fermentation was slightly modified based on previous research[4,26]. Preparation for sterilized media: 1 L distilled water, 37.8 g BHI, 5 g yeast extract, 2 g FOS, 0.5 g mucin (mucin from porcine stomach, type II), 0.3 g $NaHCO_3$, 0.02 g hemin, 0.5 g *L*-cysteine, 0.5 g bile salt, 10 mL vitamin K_1, 2.0 mL Tween-80, 1.0 mL 1% resazurin. PBS buffer and liquid medium were prepared and sterilized (121 °C, 20 min). In addition, hemin, vitamin K_1, and *L*-cysteine were sterilized by 0.22 μm membrane filtration and added to the medium cooled to room temperature. The oxygen in PBS was removed by carbon dioxide bubbling and *L*-cysteine hydrochloride was added as a reducing agent. The thawed fecal samples was mixing with PBS in a ratio of 1:3 (*m*/*V*) and then filtered with 4 layers of gauze to obtain fecal slurry. The pH meter (F20, METTLER TOLEDO) measured the pH value. The bacterial growth density was measured at 600 nm absorbance using the medium as a blank.

The amount of fecal slurry added in this experiment was 10%[4] and the concentration of *A. muciniphila* resuspension added was 1.0×10^8 CFU/mL[25]. Based on the characteristics of this experiment (the relative abundance of *A. muciniphila* in group E and group L was 0.0% and 46.8%). We designed to simulate the relative abundance of *A. muciniphila* in the bacterial intervention group (50.0%) to reach the abundance of *A. muciniphila* in the group L (46.8%). Therefore, the specific grouping information was as follows: hypertensive elderly group (E) concluded 45 mL medium and 5 mL fecal slurry of elderly; hypertensive elderly + *A. muciniphila* group (EA) concluded 45 mL medium, 2.5 mL fecal slurry of elderly and 2.5 mL *A. muciniphila* PBS re-suspension (1×10^8 CFU/mL); hypertensive longevity elderly group (L) concluded 45 mL of medium, 5 mL fecal slurry of long-lived elderly. Each group has 3 biological replicates and 2 experimental replicates. A 150 mL conical flask was used as a fermentation container and placed in an anaerobic workstation for *in vitro* fermentation. The sampling throughout the entire fermentation process was carried out in an anaerobic workstation to ensure anaerobic conditions (90% N_2, 5% CO_2, 5% H_2) and continuous fermentation. At 0, 3, 6, 9, 12, 24, and 48 h of fermentation, assigned fermentation broth were taken out for measurement pH and $OD_{600\ nm}$. The samples from different fermentation times were collected and stored at –80 °C until metabolite determination and 16S rRNA gene sequencing.

2.5 Extraction of metabolites from in vitro fermentation broth

The method of extracting metabolites from *in vitro* fermentation broth was borrowed from Wu et al.[27] with minor modifications. Briefly, 1 mL sample was mixed thoroughly with 1 mL extract solution. The extraction solution was a combination of NaH_2PO_4-K_2HPO_4 buffer and acetonitrile in a ratio of 1:1 (*V*/*V*). In order to break the cells, the mixture was treated with an ultrasonic ice bath (25 cycles with 5 s ultrasonication followed by a 10 s break). Then, it was placed at –20 °C for 2 h to maximize the extraction of metabolites. After that, the thawed sample was centrifuged (12 000 × *g*, 15 min, 4 °C) and the metabolite-containing supernatant was collected into a clean test tube. For the precipitated residue, 1 mL of the extraction solution was added again and reprocessed as described above to obtain a secondary supernatant. The 2 supernatants were combined and water and acetonitrile were removed by rotary evaporation. After rotary evaporation, the sample was dissolved in 700 μL deuterated heavy water containing 0.01% TSP, centrifuged, and 600 μL of the supernatant was pipetted to a nuclear magnetic resonance (NMR) tube for test.

2.6 Metabolites detection by ^{1}H-NMR

^{1}H-NMR spectra at 298 K were performed by a BrukerAvance 500.13 MHz NMR spectrometer (Bruker, Germany). One-dimensional spectra were collected using a standard NOESYPR1D pulse sequence (RD-90°-(t-180°-t)n-ACQ) while suppressing the water peaks using presaturation. The acquisition parameters were: number of scans (64), size of fid (65 536), acquisition time (3.276 8 s), pulse (9.95 μs), spectral width (10.00 kHz), relaxation delay (2 s), collection period (1.36 s), mixing time (100 ms).

2.7 ^{1}H-NMR data spectroscopy processing and multivariate statistical analysis

MestReNova 14.2 (Mestrelab Research, Spain) was used to perform phase, baseline, and TSP (δ 0.0) corrections on ^{1}H-NMR spectra. The metabolites were identified and quantified according to the chemical shifts and type of peak spectrum in combination with previous metabolomic references and the website of Human Metabolome Database. The chemical shift was calibrated and the water region (δ 4.70–5.10) was excluded to eliminate the effect of hydrogen spectral signals in water. The chemical shifts were divided into δ 0.01 and normalized the sum of peak intensities of δ 0.0–10.0. The normalized data were collected for subsequent analysis. The multivariate statistical analysis of metabolites normalized data analyzed by MetaboAnalyst 5.0 (http://www.metaboanalyst.ca/). For example, the principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were used to separate groups. The validity of the PLS-DA model was assessed through R_Y^2 and Q^2 values, which reflect the explained variables and the model's predictability. In addition, *P*-values were calculated among the 3 groups to obtain significantly different metabolites.

2.8 Fermentation broth bacterial DNA Extraction and 16S rRNA (V3-V4) sequencing

The fermentation broth DNA was extracted according to instructions of the TGuide S96 magnetic stool DNA kit (TianGen Biotech, Beijing, China). Determination of concentration and purity of extracted DNA by NanoDrop 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific, Waltham, USA). The quality of DNA was checked by 1.8% agarose gel electrophoresis. The 16S rRNA V3–V4 region for each DNA sample was amplified with primer 338F: 5′-ACTCCTACGGGAGGCAGCA-3′ and 806R: 5′-GGACTACHVGGGTWTCTAAT-3′. The PCR amplified product was tested for success using agarose gel electrophoresis and DNA was purified using a SanPrep column PCR product purification kit (Sangon Biotech, Shanghai, China). Then, 16S rRNA (V3–V4) sequencing of purified products using the Illumina Novaseq 6000 platform (Illumina, San Diego, USA).

2.9 Bioinformatics analysis

USEARCH (version 10.0) was conducted to assign eligible sequences (97% similarity) to one OTU. Based on the Naive Bayes classifier in QIIME2[28], the OTU was annotated by SILVA database (release 138.1) with a confidence threshold of 70%[29]. One-way analysis of variance (ANOVA) was applied to compare the relative abundance of taxa and diversity among groups. PCoA was used to assess differences. Redundancy analysis (RDA) was analyzed to calculate correlations between predominant taxa and environmental factors. All the sequencing data were analyzed on the BMKCloud online (https://www.biocloud.net).

2.10 Statistical analysis

SPSS version 26.0 was used for statistical analysis of experimental data, and the data analysis results were presented as mean ± standard deviation (SD). Due to the two independent variables of time and treatment, a Two-ways analysis of variance was conducted for data analysis, with a significance level of $P < 0.05$. GraphPad Prism version 9.5 was used to generate figures. The combination of figures and the drawing of the summary schematic were performed by Adobe Illustrator version 2022. Microbiological analyses were performed using the platform of BMKClound (Biomarker Technologies Co., Ltd., Beijing, China).

3. Results

3.1 Distinguishing the dominant structure of gut microbiota among the 6 donors

The 6 donors were divided into group E and group L according to significant differences in relative abundance of *K. pneumoniae*, *E. coli*, and *A. muciniphila* (Fig. 1D). PCoA in *β*-diversity was used to evaluate the initial gut microbiota characteristics between group E and L. Biaxials of the PCoA plot explained 95.07% total inter-sample variance (PC1: 84.58% and PC2: 10.49%) with weighted-unifrac distance (Fig. 1A). The microbiota could be differentiate to 2 groups according to the PCoA plot. The *α*-diversity of ACE, Shannon, and Simpson indices showed no significant difference between the 2 groups, but the Chao1 index in group E was significantly higher than that of group L (Fig. 1B). The microbiota composition at species level of all 6 selected donors are showed in Fig. 1C. From the species level, *E. coli* accounted for 61%–81% and *K. pneumoniae* accounted for 4.7%–9.4% of the E group. In comparison, *A. muciniphila* accounted for 27%–62% in group L (Fig. 1D). It indicated that group E dominated by *E. coli* (mean relative abundance, 70.5%) and *K. pneumoniae* (mean relative abundance, 6.51%), while group L dominated by *A. muciniphila* (mean relative abundance, 46.8%).

3.2 Determination of pH and bacterial growth density during in vitro fermentation

The pH value changes after 0, 3, 6, 9, 12, 24, 36, and 48 h of fermentation are demonstrated in Fig. 2A. The initial pH values

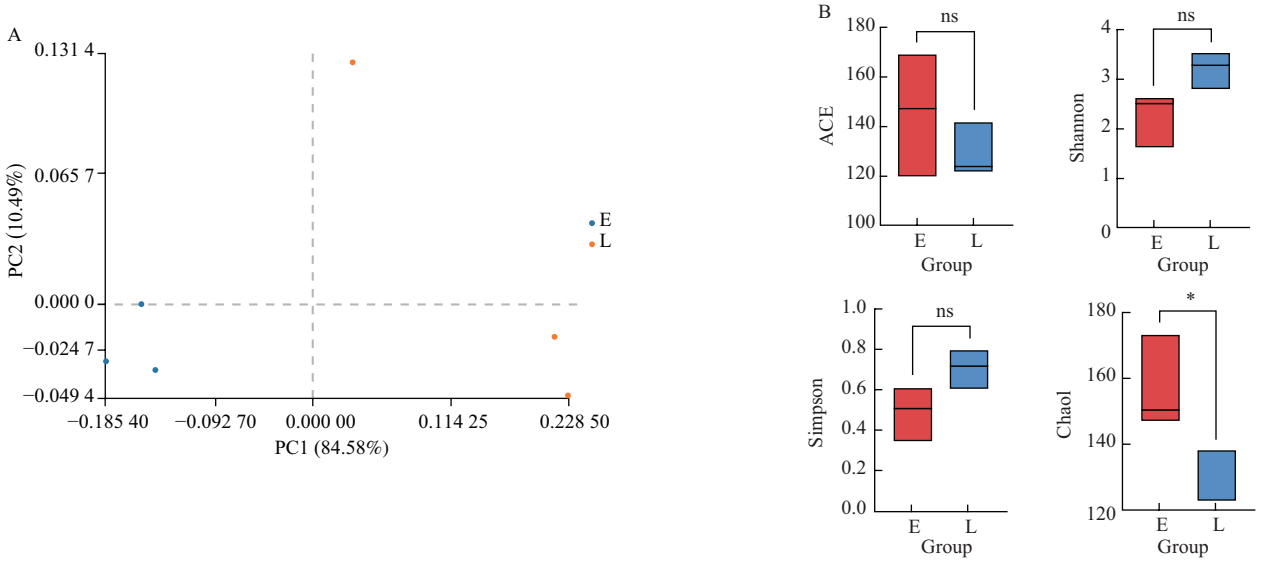

Fig. 1 Microbiota profiles of elderly and long-lived elderly fecal samples with hypertension. (A) PCoA plots of gut microbiota profiles with the weighted-unifrac distance. (B) Comparison of *α*-diversity between groups E and L in ACE, Shannon, Simpson, and Chao1 indices. (C) Gut microbiota composition of the top 10 species in 6 fecal samples. (D) The comparison of dominant species between groups E and L. ns. not significant ($P > 0.05$); *$P < 0.05$, **$P < 0.01$.

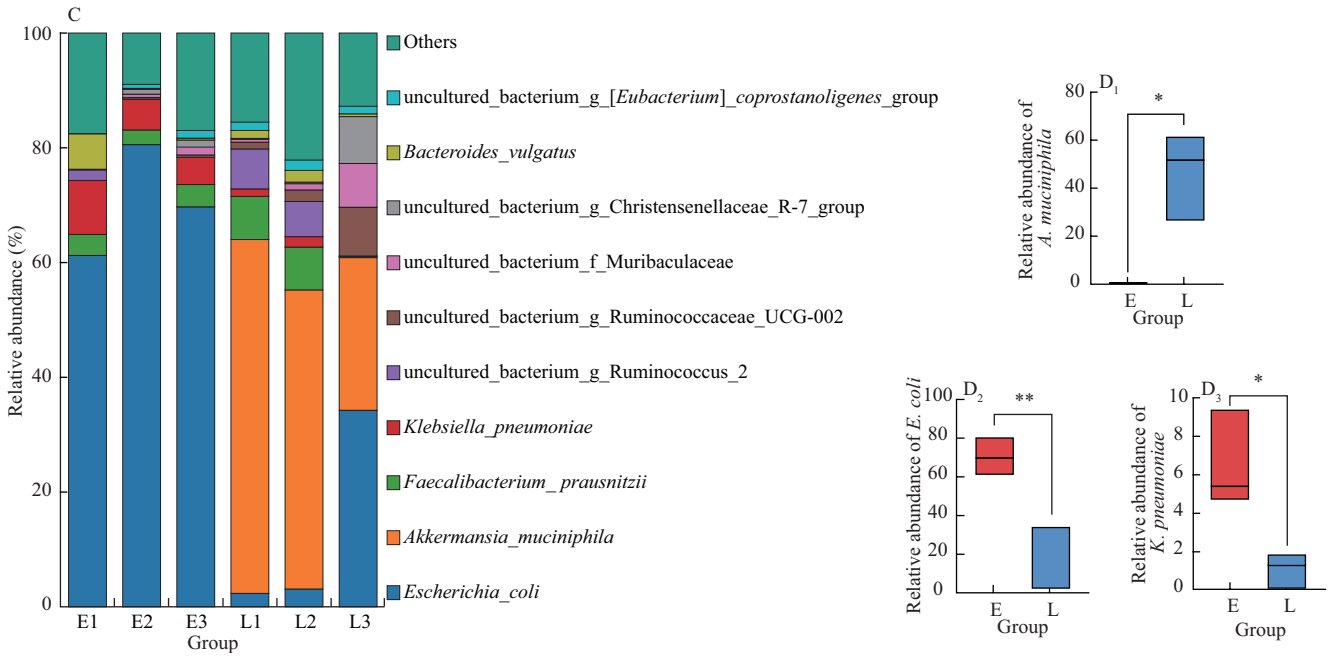

Fig. 1 (*Continued*)

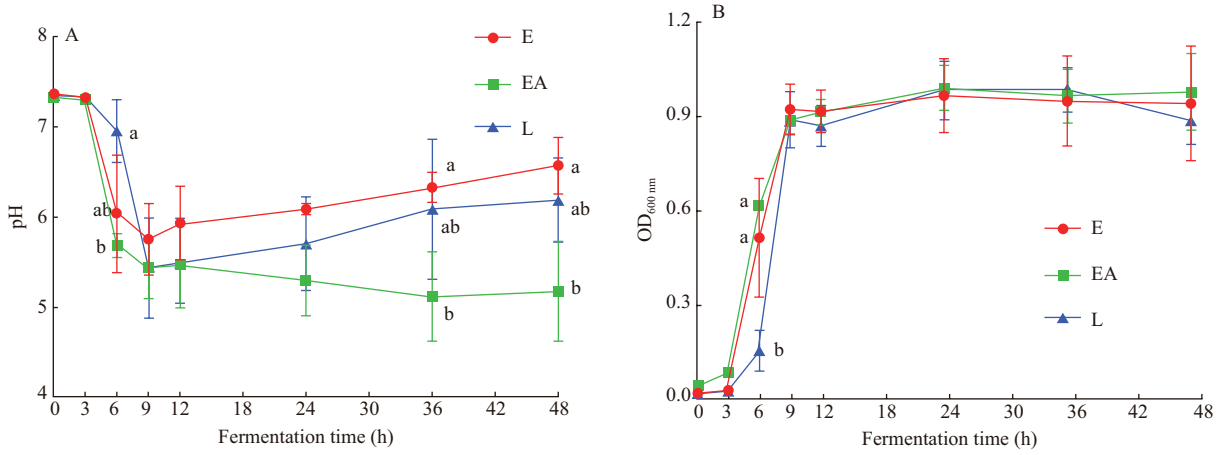

Fig. 2 (A) pH and (B) $OD_{600\ nm}$ values during fermentation process acid production and bacterial growth density changed rapidly in the first 12 h. So it is measured every 3 h at the first 12 h in order to better monitor the initial fermentation process. After 12 h, measurements were made every 12 h. Different lowercase letters indicate significant differences in the indicators measured during that time ($P < 0.05$). One-way ANOVA was used for statistical analysis among 3 groups.

of E, EA, and L were not significantly different. Moreover, at 6 h after fermentation, the pH value of E ($P > 0.05$) and EA ($P < 0.05$) was lower than that of group L. It suggests that the initial fermentation activity of EA is better than that of group L. Then, at 9 h of fermentation, the pH value of the groups E, EA, and L decreased with time and reached the average value of 5.76, 5.44, and 5.43, respectively. Interestingly, among all sampling points, E and L groups reached the lowest pH value at 9 h. Then, the pH value of E and L groups increased with time at 12 h after fermentation and finally reached the average value of 6.58 and 6.18 at 48 h. In contrast, the pH value of EA did not increase over time but remained consistently low. Noticeably, the pH value of group E ($P < 0.05$) and group L ($P > 0.05$) were higher than the EA at 48 h, which showed 1.28 and 1.20 times higher. It means that the acid produced by the E and L groups was consumed after 9 h of fermentation, while the EA group continued to produce acid. These phenomena will be explained in the subsequent results on metabolic products.

Fig. 2B shows the production of total bacteria in groups of group E, EA, and L during the fermentation process, represented by the $OD_{600\ nm}$. During the first 6 h, the $OD_{600\ nm}$ value increased rapidly in the groups E (0.02–0.52) and EA (0.04–0.62) ($P < 0.05$), respectively. In contrast, $OD_{600\ nm}$ value in group L at 6 h was significantly lower than the group E and EA, just from 0.03 to 0.16. The total amount of bacteria in the 3 groups reached the same level at 12 h (approximately 0.91), and the follow-up changed little. It showed that the rapid growth period of E and EA is 0–6 h, while L was 6–12 h, which was in line with the result of the pH change.

3.3 *In vitro fermentation metabolic profiling analysis based on ^{1}H-NMR*

The metabolite production was determined *via* the ^{1}H-NMR at 9 and 48 h. Representative one-dimensional ^{1}H-NMR spectra of fermentation at 48 h from 3 groups are depicted in Fig. 3. A total of 27 metabolites were identified; the attribution of metabolite peak profiles from fermentation broth samples are shown in Table S2. These identified metabolites belong to amino acids, fatty acids, organic acids, sugar, ketone bodies, microbiota-derived metabolites, and other substances. At the end of fermentation, acetate, propionate, butyrate, lactate, leucine, alanine, and lysine were the primary metabolites. Among all sampling points, the pH of the E and L reached the lowest value at 9 h, and their lactate content at 9 h was significantly higher than at 48 h (Fig. S1). After that, the lactate-utilizing species metabolized the lactate to produce the butyrate, which decreased with fermentation time and increased pH value. After fermentation for 48 h, the SCFAs concentration of acetate, propionate, and butyrate in groups E and L increased noticeably compared to 9 h, while the lactate in group E was almost exhausted (Fig. S1). On the contrary, the group EA had significantly higher lactate levels than the groups E and L and produced almost no butyrate at 48 h, accompanied by lower pH values. It indicates that pH change during *in vitro* fermentation is largely determined by lactate concentration rather than butyrate. The results also showed that *A. muciniphila* mediated *in vitro* fermentation can maintain the acidification of the fermentation microenvironment.

3.4 *Multivariate analysis of the fermentation broth metabolites*

PCA and PLS-DA analysis was conducted to determine whether it could differentiate 3 groups based on the NMR spectra. The PCA score plot showed that group E can be slightly distinguished from groups EA and L (Fig. 4A). Furthermore, the PLS-DA model showed a significant differentiation among groups E, EA, and L (Fig. 4B). At the same time, the PLS-DA model was obtained with an accuracy of 0.900 (R^2 = 0.81 and Q^2 = 0.71) by cross-validation. It indicated model was well fitted and can be used for subsequent analysis. Furthermore, 9 metabolites were identified to be significantly altered among the 3 groups. A box plot of the differential metabolite levels among the 3 groups is presented in Fig. 4C. Compared to the group EA, the levels of butyrate, TMA, and putrescine were increased significantly in the E and L groups ($P < 0.05$). Lysine, TMAVA, and 3-hydroxybutyrate in group E were significantly higher than the groups EA and L ($P < 0.05$). The results showed that lactate and formate contents were higher in the EA and L ($P < 0.05$) groups compared with the contents in group E. In addition, group L had significantly lower levels of tyrosine ($P < 0.05$) compared with group EA.

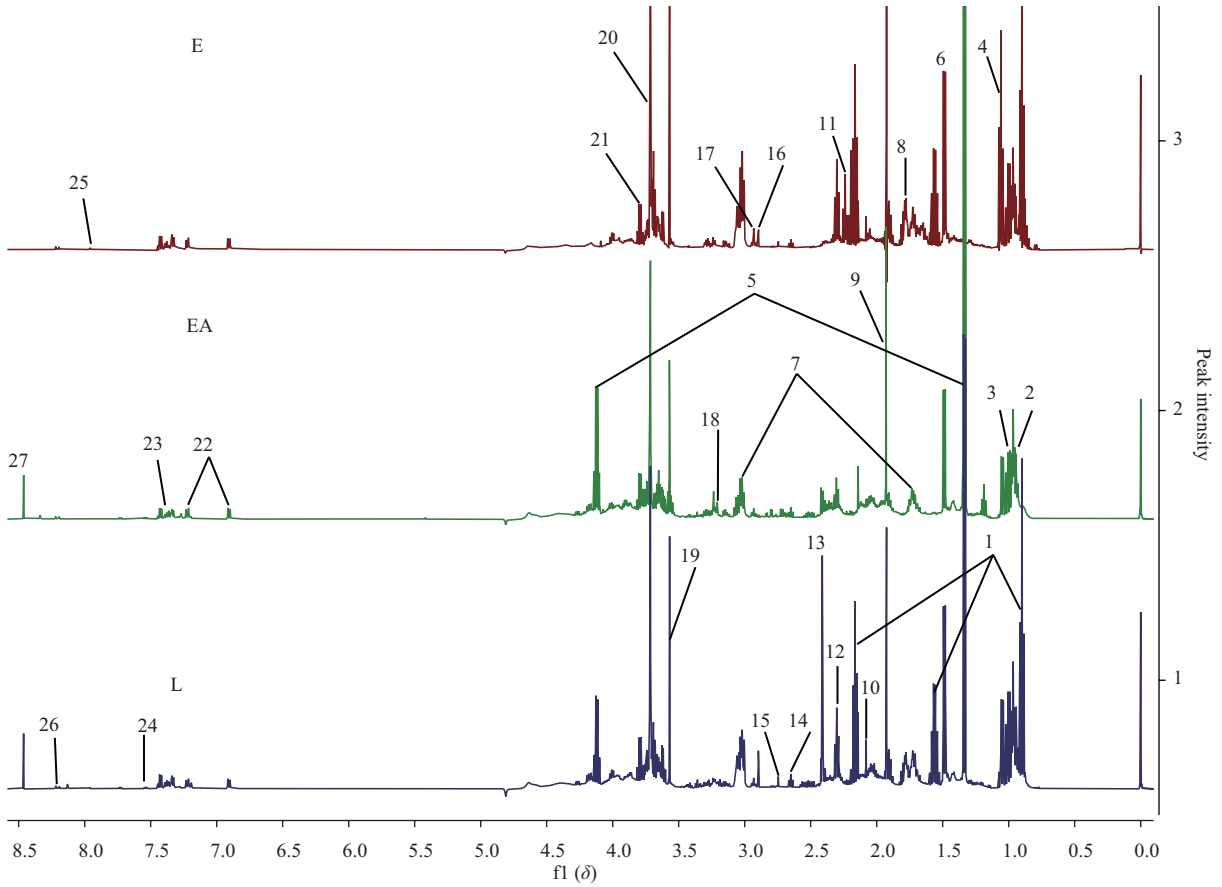

Fig. 3 Representative ^{1}H-NMR spectra (500 MHz) of fermentation broth from 3 groups. Assignments and marks of metabolites: 1. Butyrate; 2. Leucine; 3. Valine; 4. Propionate; 5. Lactate; 6. Alanine; 7. Lysine; 8. Putrescine; 9. Acetate; 10. Methionine; 11. TMAVA; 12. 3-Hydroxybutyrate; 13. Succinate; 14. Malic acid; 15. Creatine; 16. 3-Methyl-2-oxovalerate ; 17. TMA; 18. Choline; 19. Glycine; 20. *α*-Glucose; 21. Arginine; 22. Tyrosine; 23. Phenylalanine; 24. Uracil; 25. Histidine; 26. Hypoxanthine; 27. Formate.

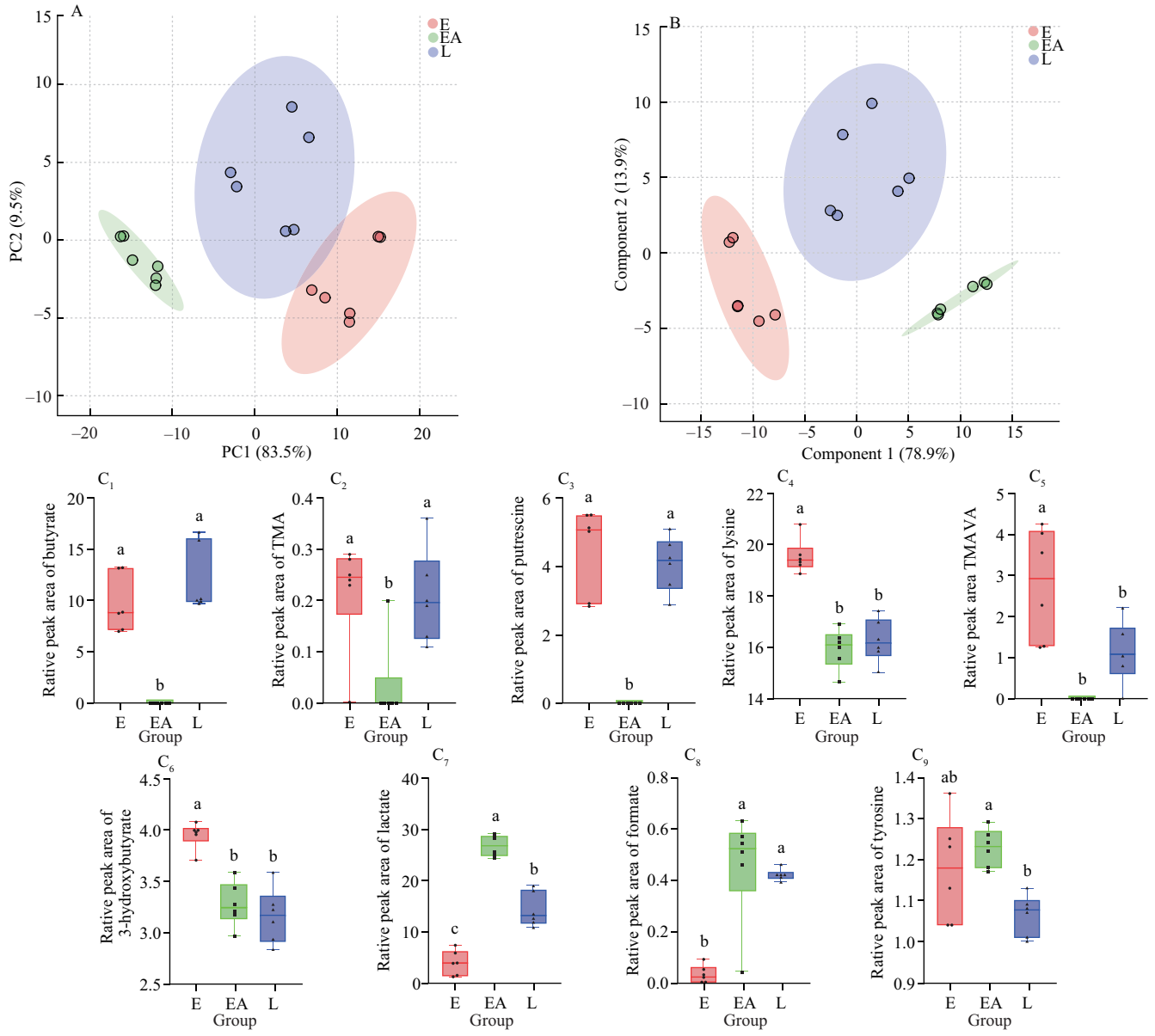

Fig. 4 Changes in metabolites in fermentation broth. (A) PCA and (B) PLS-DA score plots based on metabolite data obtained from fermentation broths of E (red), EA (green), and L (blue). (C) Three groups of metabolites with significant differences after 48 h of fermentation. Each dot means a individual sample. Different lowercase letters indicate significant differences ($P < 0.05$).

3.5 Changes in gut microbiota composition after in vitro fermentation

16S rRNA gene sequencing was used on 18 samples after 9 and 48 h of fermentation to analysis *A. muciniphila* mediated change in gut microbiota. After quality control of dual end reads and splicing, a total of 1 252 352 clean reads were generated, with an average of 69 575 ± 8 210 clean reads produced per sample. For α-diversity, there is no significant difference in ACE and Chao1 indices, while the Shannon and Simpson indices showed significant differences (Fig. S2). The Shannon and Simpson index of group E_48h was significantly higher than that of group E_9h. Moreover, the Shannon index in group E_9h was significantly lower than that of EA_9h, EA_48h, and L_48h. The common characteristic of α-diversity was that the later fermentation stage (48 h) of all 3 groups were higher than the early fermentation stage (9 h).

Although the initial gut microbiota compositions of the 6 donors differed, they still shared some common responses to microbial changes regarding *in vitro* fermentation mediated by *A. muciniphila*. *A. muciniphila* mediated fermentation showed direct microbial community assembly changes at 9 and 48 h (Fig. 5A). The top 15 genera are shown in Table 1 and significant differences among 6 groups were calculated based on two-ways analysis of variance. Compared to group E_9h, EA_9h significantly increased the relative abundance of *Lactococcus* and *Akkermansia* and decreased the relative abundance of *Clostridium_sensu_stricto_*1. Compared to groups E_48h and L_48h, the relative abundance of *Akkermansia* and *Bifidobacterium* significantly increased in group EA_48h. Meanwhile, the EA_48h showed significantly lower relative abundance of *Clostridium_sensu_stricto_*1 than the E_48h. Along with the fermentation process, the group EA maintained a low pH value while not in the E. It suggested that *A. muciniphila* could promote the growth of *Bifidobacterium* during

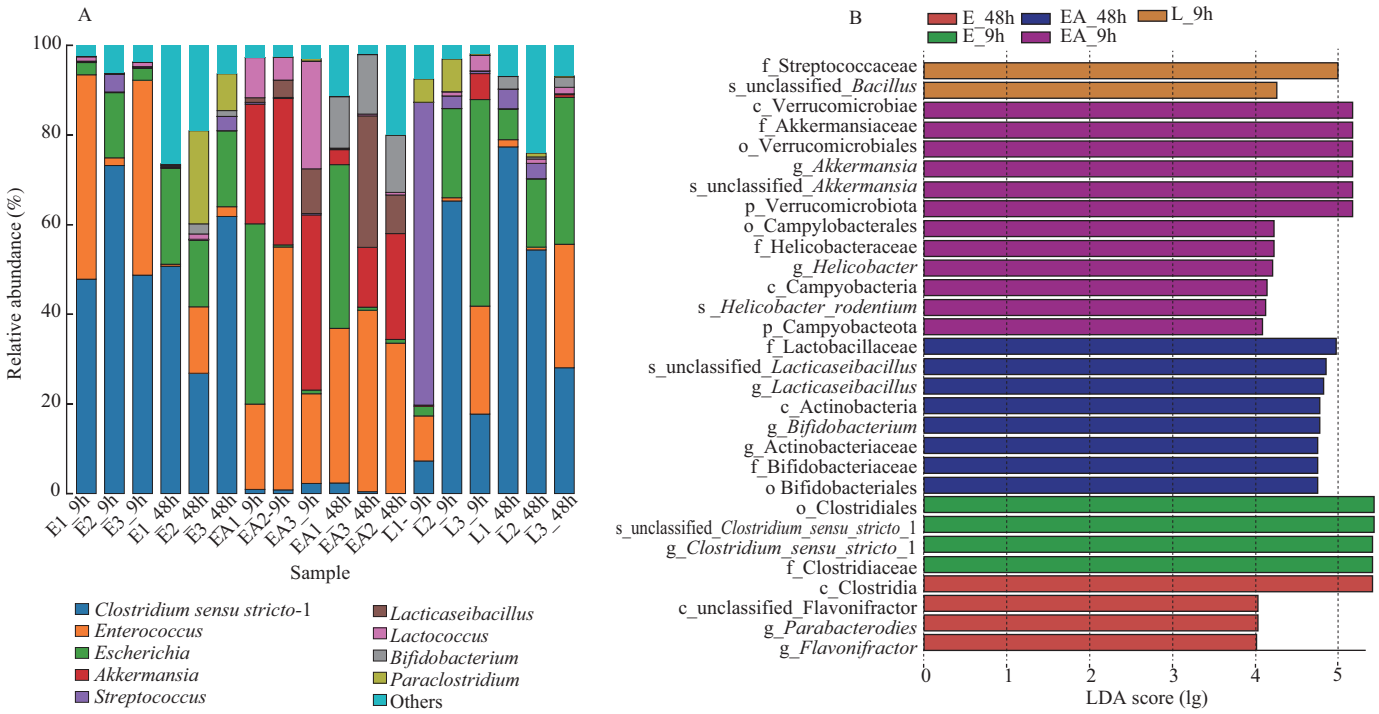

Fig. 5 Analysis of composition and differences in microbial community. (A) Microbial community composition at the genus level after 9 and 48 h of *in vitro* fecal fermentation of each sample. E1_9h, E2_9h, and E3_9h means 3 samples after 9 h of *in vitro* fermentation of group E. Similarly, E1_48h, E2_48h, and E3_48h means 3 samples after 48 h of *in vitro* fermentation of group E. (B) Differences in composition of microbial community based on LDA effect sizes. The score criterion for LDA is higher than 4.0.

Table 1
Relative abundance (%) among different groups at genus levels.

Genus	E_9h	E_48h	EA_9h	EA_48h	L_9h	L_48h
Clostridium_sensu_stricto_1	56.59 ± 14.39^{a}	46.47 ± 17.88^{a}	1.33 ± 0.80^{b}	0.93 ± 1.25^{b}	30.10 ± 30.10^{ab}	53.27 ± 24.66^{a}
Enterococcus	30.27 ± 24.75^{a}	5.81 ± 7.85^{a}	31.08 ± 20.02^{a}	36.18 ± 3.75^{a}	11.62 ± 11.76^{a}	9.92 ± 15.27^{a}
Escherichia	6.65 ± 6.84^{a}	17.75 ± 3.33^{a}	13.84 ± 22.85^{a}	12.68 ± 20.65^{a}	22.71 ± 22.10^{a}	18.28 ± 13.24^{a}
Akkermansia	0.12 ± 0.02^{c}	0.09 ± 0.08^{c}	32.82 ± 6.19^{a}	13.43 ± 10.14^{b}	2.03 ± 3.27^{c}	0.25 ± 0.31^{c}
Streptococcus	1.45 ± 2.16^{a}	1.20 ± 1.71^{a}	0.32 ± 0.08^{a}	0.04 ± 0.05^{a}	23.63 ± 38.06^{a}	2.62 ± 2.18^{a}
Lacticaseibacillus	0.02 ± 0.02^{a}	0.03 ± 0.02^{a}	4.96 ± 4.56^{a}	12.69 ± 15.03^{a}	0.02 ± 0.03^{a}	0.03 ± 0.01^{a}
Lactococcus	0.65 ± 0.52^{b}	0.47 ± 0.62^{b}	12.7 ± 10.00^{a}	0.36 ± 0.23^{b}	1.46 ± 1.78^{b}	0.81 ± 0.72^{b}
Bifidobacterium	0.04 ± 0.03^{b}	1.27 ± 1.01^{b}	0.02 ± 0.00^{b}	12.5 ± 0.92^{a}	0.04 ± 0.03^{b}	1.84 ± 1.21^{b}
Paraclostridium	0.13 ± 0.06^{a}	9.66 ± 10.46^{a}	0.18 ± 0.31^{a}	0.07 ± 0.07^{a}	4.28 ± 3.56^{a}	0.41 ± 0.40^{a}
Dorea	0.03 ± 0.02^{a}	3.78 ± 6.54^{a}	0.02 ± 0.02^{a}	0.02 ± 0.02^{a}	0.09 ± 0.15^{a}	1.01 ± 1.69^{a}
Ligilactobacillus	0.11 ± 0.03^{a}	0.20 ± 0.18^{a}	0.08 ± 0.03^{a}	3.80 ± 5.66^{a}	0.08 ± 0.06^{a}	0.05 ± 0.02^{a}
Klebsiella	1.13 ± 0.30^{a}	1.60 ± 1.61^{a}	0.28 ± 0.31^{a}	0.08 ± 0.09^{a}	0.11 ± 0.16^{a}	0.96 ± 1.66^{a}
Fusobacterium	0.01 ± 0.01^{a}	2.64 ± 4.58^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.02 ± 0.04^{a}	1.19 ± 2.04^{a}
Bacteroides	0.03 ± 0.03^{a}	1.11 ± 1.33^{a}	0.03 ± 0.01^{a}	0.06 ± 0.09^{a}	0.06 ± 0.05^{a}	2.41 ± 2.97^{a}
Lactobacillus	0.10 ± 0.06^{a}	0.23 ± 0.21^{a}	0.12 ± 0.07^{a}	2.32 ± 3.71^{a}	0.05 ± 0.03^{a}	0.09 ± 0.02^{a}

Note: The value represents the mean ± SD. Due to the 2 independent variables of treatment and time, a Two-way analysis of variance was used for statistical analysis. Different lowercase letters in each row indicate significant differences among different groups ($P < 0.05$).

the fermentation and continuously produce lactate. Furthermore, linear discriminant analysis (LDA) with score > 4.0 was used to screen microbial community biomarker (Fig. 5B). A total of 30 species were screened as potential biomarkers. From the perspective of family level, the highest scores were Streptococcaceae, Akkermansiaceae, Lactobacillaceae, and Clostridiaceae in the group L_9h, EA_9h, EA_48h, and E_9h, respectively.

3.6 Relationships of structural variation of microbial communities and its environmental variables

A PCoA plot with the Bray-Curtis distance was applied to further analyze the variability of microbial community assembly change after 9 and 48 h fermentation (Fig. 6A). PC1 and PC2 explained 56.62% of the total variation. The results displayed that groups E and L cannot be distinguished at 9 or 48 h. However, groups E and L

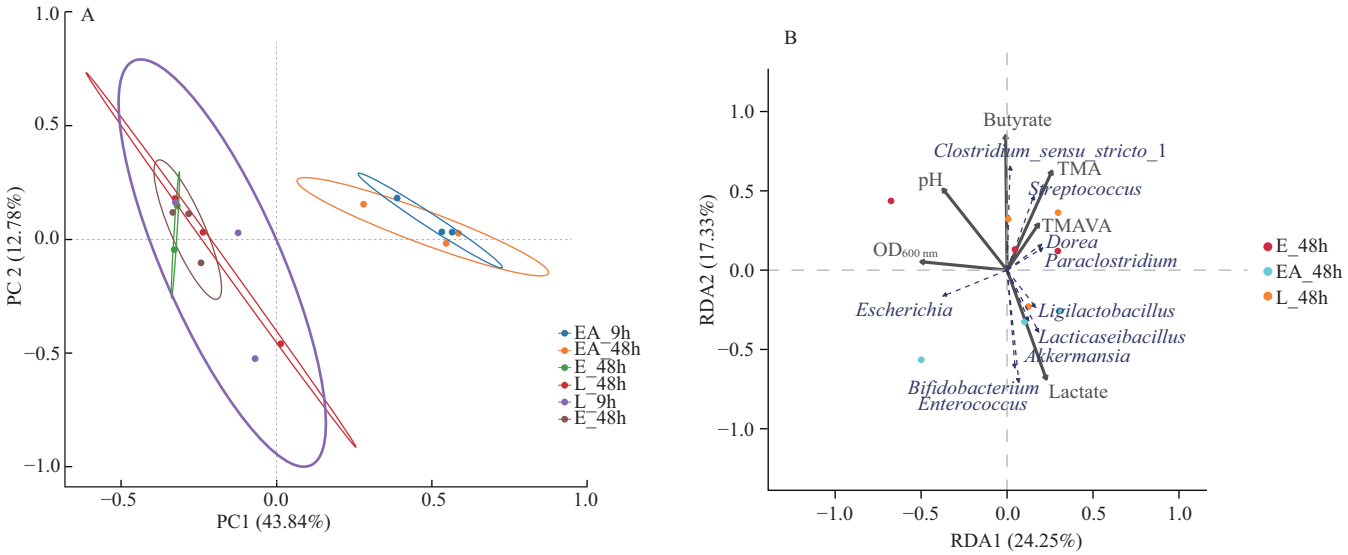

Fig. 6 The variability of microbial community assembly and its correlation with environmental factors. (A) PCoA plots and (B) RDA analysis of gut microbiota and environmental variables.

showed a significant separation from group EA and a significant aggregation at 9 and 48 h within the same group. To analysis the impact of *A. muciniphila* mediated *in vitro* fermentation on the microbial community assembly, acid production, pH, and cell density at 48 h, RDA analysis was conducted (Fig. 6B). RDA analysis showed all samples accounted for 41.58% of the total variation. The pH was negatively related to the concentration of lactate, which explains EA group maintains a lower pH due to its high lactate concentration. The lactate showed positive correlation with the abundance of *Enterococcus*, *Bifidobacterium*, and *Akkermansia*, while the butyrate showed the opposite direction. The butyrate showed positive correlation with the abundance of *Clostridium_sensu_stricto*_1 and negative correlation with the lactate. Meanwhile, the typical hypertension-related risk markers TMA and TMAVA were positively correlated with *Streptococcus.* The RDA plot indicated that lactate was positively correlated with lactate producing bacteria (*Enterococcus* and *Bifidobacterium*) and butyrate was positively correlated with butyrate producing bacteria *Clostridium_sensu_stricto*_1, which means the metabolites shifts by those functional bacterial community assembly.

4. Discussion

A. muciniphila has been reported to exert multiple benefits for the human body by acting on gut homeostasis[9]. Abnormal *A. muciniphila* abundance was associated with multiple diseases. The relative abundance of *A. muciniphila* decreases in metabolic diseases, such as diabetes, obesity and cardiovascular diseases[10]. In addition, the decline in *A. muciniphila* abundance is associated with various disease, such as colitis[30], colorectal cancer[31], autism spectrum disorder[32], alcoholic liver disease[33], and psoriasis[34], etc. On the other hand, the relative abundance of *A. muciniphila* increases in Parkinson's disease[35], multiple sclerosis[36], and irritable bowel syndrome[37]. However, few studies have focused on the relationship between *A. muciniphila* and hypertension. Only correlation analysis showed that increased *A. muciniphila* abundance was associated with reduced blood pressure[38]. There is a distinguished difference in bacterial composition of feces between elderly and long-lived elderly donors with hypertension. Moreover, the microbial pathways associated with *K. pneumoniae* and *E. coli* may promote hypertension, while *A. muciniphila* may play a role in reversing the development of hypertension in long-lived elderly[24]. Therefore, we designed an *in vitro* fermentation experiment of *A. muciniphila* to explore its potential role.

During *in vitro* fermentation, gut bacteria metabolize and produce a variety of metabolites that affect pH[39]. *In vitro*, the decrease in pH may depend primarily on the concentration of organic acids produced, such as SCFAs, lactate, and succinic acid, etc.[26,39]. During the fermentation period of 0–9 h, the pH of the 3 groups decreased significantly. It indicates that 3 groups of intestinal bacteria rapidly produce organic acids using the culture medium, leading to a rapid decrease in pH value. It is worth noting that there is an increase in pH during the fermentation period of 9–48 h in groups E and L. Then, the study focused on the cross-feeding of gut microbiota to explain this phenomenon. In the human colon, lactic acid is metabolized into butyrate by a species called lactate-utilizing bacteria[3]. This explains the metabolic transformation of lactate to butyrate by cross-feeding between intestinal bacteria[40]. Compared with the EA group, the pH in groups E and L increased during 9–48 h, producing a high concentration of butyrate. In contrast, EA always maintained a low pH value and produced a high lactate concentration but almost no butyrate. Thus, this study strongly suggests that pH change in fermentation is more determined by the lactate concentration than butyrate.

TMA is a metabolite of gut bacteria and is metabolized to TMA oxide by the liver, which shows a high risk of cardiovascular disease or hypertension[17]. TMAVA is a recently reported cardiovascular risk associated metabolite of gut microbiota[41]. *Enterococcus faecalis* can metabolize trimethyl lysine to TMAVA and inhibit carnitine synthesis, which plays a key role in developing cardiomegaly[42]. The TMA and TMAVA in the EA group were significantly reduced, indicating that *A. muciniphila* mediated fermentation could reduce hypertension-related metabolites. Putrescine is a polyamine that is abundant in the human

gut, and a new production of putrescine was identified recently[43]. After acidogenic bacteria such as *Bifidobacterium* spp. acidify the environment, acid-resistant *E. coli* and *Enterococcus* with guanidine butylamine deaminase system can cooperate to produce putrescine[44]. Putrescine exacerbated colitis in mice by disrupting the integrity of tight junction proteins[45]. The EA group showed a low level of putrescine, indicating that under *A. muciniphila* mediated acidic environment, *E. coli* is almost inhibited, making it almost completely impossible to cooperate with *Enterococcus* to produce putrescine.

Bifidobacterium, as a strict anaerobic bacterium, often requires 48 h to reach the logarithmic growth phase[46]. This explains the phenomenon that *A. muciniphila* mediated fermentation significantly increased *Bifidobacterium* at 48 h but not at 9 h. *Bifidobacterium* is a well-known probiotic and a common lactate producing bacteria that benefit the host by regulating the immune system[47]. Depletion of *Bifidobacterium* has also been reported to be a feature of gut dysbiosis in a variety of disease conditions[48]. A certain concentration of lactate and acetate produced by *Bifidobacterium* can inhibit the growth of gut microbes of Bacteroides and Firmicutes[3]. Therefore, the proliferation of *Bifidobacterium* can continuously produce lactate to ensure fermentation acidification in the later stage of fermentation and further inhibit potential pathogenic bacteria, such as *Streptococcus*. Consequently, the proliferation of *Bifidobacterium* promoted by *A. muciniphila* could maintain the continuous lactate production of fermentation broth and maintain the stability of the immune microenvironment.

The observation of changes in functional microbial communities in the present study showed interesting results, which have yet to be found in other *in vitro* fermentation and gut microbiota studies. Only a few studies on the fermentation of wastewater under high salinity stress that show related interesting results. High salinity fermentation decreased *Clostridium_sensu_stricto*_1 and increased *Enterococcus* and *Bifidobacterium* when compared with low salinity. Meanwhile, high salinity delayed butyrate production with the lower abundance of butyrate-producing bacteria[49]. *Enterococcus* (phylum Firmicutes) is commonly considered a lactate producing bacteria genera. Therefore, high salinity enriched *Enterococcus* and decreased *Clostridium_sensu_stricto*_1, thus changing the common conversion of lactate to butyrate[50]. During the fermentation process, while lactate accumulates, the production of butyrate is also greatly reduced, accompanied by changes in microbial community assembly, such as *Actinobacteria* and *Lactobacillus* replacing Bacteroidetes and Firmicutes[3]. Our study further revealed from the genus level that *Bifidobacterium* (phylum Actinobacteria) played a crucial role in lactate accumulation. This phenomenon revealed the key role of *A. muciniphila* mediated enrichment of functional microbiota, manifested by an increase in lactate producing bacteria and a decrease in butyrate producing bacteria.

The available data on the relationship between *Clostridium_sensu_stricto*_1 and gut homeostasis are inconsistent. The results about *Clostridium_sensu_stricto*_1 are inconsistent due to differences in sample sources, types, and doses, as well as experimental type in animal model and population survey research. *Clostridium_sensu_stricto*_1 showed a positive correlation with blood pressure in hypertension non-treated patients[51]. In 2 *in vitro* fermentation experiments, the high proportion of soluble dietary fiber produced significantly higher level of *Clostridium_sensu_stricto*_1[52]. Similarly, the chitosan oligosaccharides fermentation by the gut microbes to produce a large amount of SCFAs by increasing *Clostridium_sensu_stricto*_1[53]. *Clostridium_sensu_stricto*_1, as a butyrate-producing bacteria[54], can grow as a dominant bacteria *in vitro* fermentation and produce numerous butyrate. *Clostridium_sensu_stricto*_1 showed positive correlation with butyrate concentration, which also confirmed it in this study. However, the fermentation broth maintaining acidification characterized by high concentration of lactate and low abundance of *Clostridium_sensu_stricto*_1, which indicated a potential relationship between *A. muciniphila* mediated fermentation acidification and the lower relative abundance of *Clostridium_sensu_stricto*_1. C*lostridium* spp. is an opportunistic pathogen that has been reported to induce intestinal diseases in goats[55]. Mechanically, the high relative abundance of *Clostridium_sensu_stricto*_1 promoted high expression of IL-1β and TNF-α in colon tissue, thus leading to intestinal epithelial damage[56]. Meanwhile, an increase in the abundance of *Clostridium_sensu_stricto*_1 was also observed in patients with colitis[57]. However, *A. muciniphila* is considered a new generation of probiotics that functions as a therapeutic role in intestinal diseases[10]. For example, *A. muciniphila* can effectively alleviate *Salmonella typhimurium*-induced[58] and DSS-induced gut inflammation disease[30]. This may explain the antagonistic relationship between *Clostridium_sensu_stricto*_1 and *A. muciniphila*. In total, *Clostridium_sensu_stricto*_1 can produce butyrate, but it is also closely related to intestinal diseases. *A. muciniphila* mediated acidification microenvironment that has an antagonistic effect on *Clostridium_sensu_stricto*_1 to protect intestinal health.

Our research has some limitations. The *in vitro* fermentation effect mediated by different doses of *A. muciniphila* or different *A. muciniphila* strains has not been explored. In addition, the effects of *A. muciniphila* in ameliorating gut diseases associated with hypertension needs further exploration *in vivo*. Nevertheless, we have provided some reference significance for *A. muciniphila* mediated fecal fermentation experiments in this study, that leads to increased growth of beneficial bacteria and lactate production *in vitro*.

In conclusion, *A. muciniphila* mediated fermentation can regulate the microbial microenvironment by increasing the relative abundance of *Bifidobacterium* and lactate concentration, and reducing the pH value of the fermentation broth.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://doi.org/10.26599/FSHW.2025.9250490.

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