Diarrhea Accompanies Intestinal Inflammation and Intestinal Mucosal Microbiota Dysbiosis during Fatigue Combined with a High-Fat Diet

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Abstract

Objective: It was reported fatigue or a high-fat diet triggers gastrointestinal (GI) disorders, and intestinal microbiota may play central roles in GI disorders. Therefore, we investigated the association between the intestinal mucosal microbiota and the intestinal mucosal barrier from fatigue combined with a high-fat diet.

Method: This study divided the Specific pathogen-free (SPF) male into the normal group (MCN) and the standing united lard group (MSLD). After establishing the model for 14 days, interleukin-6 (IL-6), interleukin-17 (IL-17), immunoglobulin A (sIgA), mucin 2 (Muc2), and intestinal mucosal microbiota were analyzed. Furthermore, the correlations between bacterial genera, inflammation, and immune function were measured.

Result: The pathological analysis proved obvious damage to the small intestinal structure after fatigue combined with a high-fat diet. MSLD mice presented an increased trend of IL-6 and IL-17 and a decreased tendency of sIgA and Muc2, aggravating the injury of the intestinal mucus barrier and GI symptoms. Fatigue combined with a high-fat diet considerably decreased *Limosilactobacillus reuteri*, positively associated with Muc2 and negatively with IL-6. **Conclusion:** The interactions between *Limosilactobacillus reuteri*and intestinal inflammation might be involved in the process of intestinal mucosal barrier impairment in fatigue combined with high-fat diet-induced diarrhea.

Introduction

Diarrhea is defined by the World Health Organization as excretion three or more times a day, with no fecal shape, and as a thin/watery stool (1, 2). With the change in people's lifestyles and diets, the number of diarrheal diseases has been increasing year by year and has become a major health problem worldwide (3). There is no consensus on the specific pathogenesis of diarrhea, which may be related to genetic susceptibility, epithelial barrier defects, immune response disorders, and environmental factors (4, 5). The dietary composition was found to influence the incidence and progression of diarrhea (6, 7). Protein and a high-fat diet were associated with diarrhea, significantly reduced *Lactobacillus* and *Bifidobacterium*, and decreased digestive enzyme activity and microbial activity in mice (8, 9). Lard is a common edible oil used by Chinese residents and decreased intestinal microbial diversity in mice fed lard (10). Decreased intestinal digestive enzyme activity, decreased the number of *Bifidobacterium* and *Lactobacillus* in the intestines, and disrupted glycolipid metabolism in mice fed lard for a long time (11, 12). Physical activity regulates intestinal microbiota and affects health. The body is unable to provide or maintain the energy load required for prolonged or intense exercise, resulting in performance degradation and fatigue (13). Excessive exercise reduces microbial diversity and intestinal permeability, damages the intestinal mucosal barrier, increases inflammation, and occurs abdominal pain, and diarrhea, while probiotic therapy can reduce the incidence and severity of GI symptoms (14).

Diarrhea is closely related to intestinal microbiota disorder and intestinal mucosa barrier injury. Diarrheal mice had decreased intestinal microbiota diversity, increased inflammatory factors, decreased secretive immunoglobulin A (sIgA), abnormal energy metabolism, increased harmful intestinal bacteria, and decreased beneficial bacteria (15, 16). Impairment of intestinal mucosa integrity increased inflammatory factors and destruction of the intestinal mucosa barrier in diarrhea patients (17). The intestinal mucosa barrier consists of biological, chemical, mechanical, and immune barriers. Among them, intestinal microbiota forms a biological barrier to the intestinal mucosa, intestinal mucosa tissue forms a mechanical barrier, mucus secreted by intestinal mucosa cells forms a chemical barrier, and intestinal mucosa lymphatic tissue forms an immune barrier with immune cells and secretions (18, 19). Changes in intestinal mucosal permeability and damage to the intestinal mucosal mechanical barrier were found to promote intestinal inflammation leading to diarrhea (20).

Influenced by diet, environment, genetics, drugs, age, etc., human intestinal microbiota has nutritional functions, participates in energy metabolism, maintains the integrity of intestinal mucosa, and regulates immune response, which are important factors in maintaining human health (21, 22); Related to GI diseases, immune and metabolic diseases, neurological and psychiatric disorders (23). sIgA is the most secreted immunoglobulin in the intestine and the primary line of defense against pathogen adhesion and colonization in the intestinal mucosa. Goblet cells secrete mucus that forms the intestinal mucus layer, of which Mucin 2 (Muc2) is the core mucin and a major component of the intestinal mucus barrier (24, 25). Cytokines are small molecule proteins secreted by cells that control cell proliferation and differentiation, regulate angiogenesis, and immune and inflammatory responses, and primarily play a role in the differentiation and activation of immune cells (26). Interleukin-17 (IL-17) and interleukin-6 (IL-6) are cytokines with many activities. IL-17 stimulates the production of multiple cytokines, such as IL-1β, IL-6, tumor necrosis factor (TNF) -α, and TGF-β, which cause and exacerbate inflammation and play an important role in inflammation, immunity, and autoimmunity (27). The intestinal mucosa mechanical barrier is the most important part of the intestinal mucosa barrier. The intestinal mucosa acts as a mechanical barrier to protect the intestinal tissue while facilitating the transport of nutrients, water, and waste, and regulating the interaction between the intestinal microbiota and the immune system (28). The intestinal microbiota has a protective effect on host intestinal epithelial cells and can strengthen the intestinal mechanical barrier. Conversely, intestinal microbiota disruption leads to increased intestinal permeability and damage to the intestinal mechanical barrier (24, 29, 30).

Therefore, we established a diarrhea model in mice induced by fatigue combined with a high-fat diet to detect Muc2, sIgA, IL-6, and IL-17, analyze intestinal mucosa microbiota, and observe small intestinal pathology. This study aims to analyze the characteristics of intestinal mucosal microbiota in diarrhea, investigate the relationship between characteristic microbiota and mucosal barrier index, and investigate the role of the mucosal barrier in diarrhea caused by fatigue combined with a high-fat diet.

Materials And Methods

1.1 Animal

To rule out the effect of sex on intestinal microbiota (31), specific pathogen-free (SPF) male Kunming mice (male, 20 ± 2, license: SCXK (Hunan) 2019-0004) were selected and purchased from Hunan Slx Jingda Experimental Animal Co., Ltd. (license: SYXK (Hunan) 2019-0009). Breed at the laboratory animal center of the Hunan University of Chinese Medicine, temperature 23-25℃, humidity 47-53%, free diet, and drinking water during adaptive feeding.

1.2 Diet

General feed, the nutritional composition is detailed in Table 1, by the Hunan University of Chinese Medicine Laboratory Animal Center, Jiangsu Madison Biomedical Co. Ltd. Jinluo Refined Lard, Main Ingredient: Energy 44%, Fat 167%, Manufacturer: XinCheng Jinluo Meat Products Co., Ltd (Linyi City, Shandong Province). Production license: SC10337130200099, Production Lot No: GB 10146, Heat until the lard melts in the first 37℃ water bath.

1.3 Animal grouping and intervention

The 20 male Kunming mice were divided into control group (MCN) and standing united lard group (MSLD) according to a randomized numerical method after 3 days of adaptive feeding. 5 mice with obvious and consistent model characteristics are selected for analysis. The MCN group was not treated with intervention for 7 days and was given 0.4 mL of sterile water twice daily for 7 consecutive days from day 8. MSLD group interventions based on the literature (32-36) and pre-experimental results improved the modeling method and induced diarrhea in mice by fatigue combined with a high-fat diet. Stand on a small homemade water environment platform box for 4 h/day for 14 days, giving 0.4 ml lard to the stomach from day 8, twice daily for 7 days. Mice were quickly executed by an experienced experimenter using cervical dislocation at the end of the experiment. All the animal experiments were carried out by the animal control and use committee approved by the Hunan University of Chinese Medicine.

1.4 General features

The animals were observed daily in the morning, observing their body size, fecal shape, eyes, hair, and activity, recording their initial weight, and then weighing them every other day. The mice's initial feces were recorded and then collected daily from 9: 00 a.m. to 9: 30 a.m., the number of feces in each group was recorded to observe the texture of the feces, and photos were taken of the feces.

1.5 Organ index

After treatment, each mouse was weighed and dissected to remove the spleen, thymus, and liver before blood was taken. After the filter paper was drained and weighed, the spleen index, thymus index, and liver index were calculated: Organ index = organ weight (mg)/body weight (g).

1.6 Detection of sIgA in serum

After 14 days of intervention, blood was collected via eyeball under aseptic conditions, blood samples were left at 4℃ for 1-2 h, the supernatant solution was absorbed and as for centrifuges in high-speed centrifuges at 3000 r/min centrifuge, upper serum was taken for backup. Enzyme-linked immunosorbent assays (ELISA) were performed according to the kit instructions, followed by an enzyme labeling analyzer to detect sIgA levels in serum samples (the kit was provided by Quanzhou Konodi Biotech Ltd.).

1.7 Detection of Muc2, sIgA, IL-6 and IL-17 in small intestinal tissue

Under sterile conditions, the small intestine of the mice was dissected, the contents of the small intestine were flushed with saline, and 1-2 cm of small intestine tissue was cut with a scalpel. According to the ELISA assay instructions, a certain amount of small intestine was mixed with saline in a ratio of 1: 9 and steel beads were added. Tissue-homogenized pulp was milled at 3 min in a high-speed centrifuge at 4℃, while tissue-homogenized pulp was centrifuged for 15 minutes and the supernatant was absorbed and then sampled. Finally, the contents of Muc2, sIgA, IL-6, and IL-17 in small intestine tissue were measured using an enzyme labeling analyzer (the kit was provided by Quanzhou Konodi Biotech Ltd.).

1.8 Histopathology of the small intestine

Under sterile conditions, the small intestine of the mice was dissected, the contents of the small intestine were flushed with saline, and 1-2 cm of small intestine tissue was cut with a scalpel and fixed in a 4% paraformaldehyde solution at room temperature. According to dehydration of gradient ethanol, xylene transparent paraffin was embedded in four um sections, and the small intestine tissue of mice was sectioned for routine dewaxing, then stained with hematoxylin and eosin-methylene blue solution (hematoxylin-eosin staining), sealed with neutral gum, and photographed under the microscope.

1.9 Collection of intestinal mucosa samples

Intestinal mucosa samples were collected concerning previous methods (37). In sterile conditions, intestinal tissue from the pyloric to the ileocecal region was cut lengthwise with sterile scissors, the contents of the intestine were flushed with sterile saline, and the intestinal mucosa of each mouse was individually scraped with sterile lids. The mucosa was collected in an EP tube and stored in a -80℃ refrigerator.

1.10 DNA extraction16S rRNA gene amplicon sequencing and sequence analysis

All samples were sent to Shanghai Paceno Biotech Co., Ltd. (Shanghai, China) for processing. The total microbial genomic DNA of each tube of samples was extracted following the steps of the OMEGA Soil DNA Kit (M5635-02) kit to extract nucleic acid instructions. The quantity and quality of extracted DNAs were measured using a NanoDrop NC2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis, respectively. Forward primer 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and reverse primer 1492R (5'-GGACTACHVGGGTWTCTAAT-3') were used for PCR amplification of bacterial 16S rRNA near the full-length gene. The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using Q5 high-fidelity DNA polymerase. PCR products were detected by 2% agarose gel

electrophoresis and purified by a DNA gel extraction kit. The recovered PCR amplification products were quantified by fluorescence intensity using the dsDNA Assay Kit. Based on the fluorescence quantification results, the samples were mixed proportionally according to the sequencing requirements of each sample.

1.11 Bioinformatics

The intestinal mucosal microbiota was analyzed by high-throughput sequencing of 16S rRNA, and sequences with similarity higher than 97% were assigned to an OUT. Species accumulation curves were used to test the sequencing depth and evaluate sequence data quality. Chao1 and Observed species indices reflect the abundance of the community. The larger the index, the higher the abundance of the community. Simpson and Shannon indexes indices reflect the diversity of the community, and higher index values indicate higher community diversity. Principal coordinate analysis (PCoA) and non-metric multidimensional scaling (NMDS) examined similarities in community structure between samples. LEfSe and random forest analysis detected groups that differed significantly in intestinal mucosal abundance.

1.12 Statistical analysis

Statistical analysis was performed using SPSS 25.00 software, and each group of data was expressed as mean ± standard deviation. If the two sets of data conform to normal distribution and homoscedasticity, the independent sample t-test is used, and the non-homoscedasticity T-test is used. Mann-Whitney U assays were used if the data did not match the normal distribution and the non-homoscedasticity ($p < 0.05$ indicated statistical difference).

Results

2.1 General characteristics of mice with fatigue combined with a high-fat diet

During adaptive feeding, the mice were responsive, with flexible eyes, glossy hair, and ruddy skin mucosa. Mice with long strips of fecal matter, clean anus area, weight, and the number of feces movements close. After 7 days of molding, mice in the MSLD group showed reduced activity, sleepiness, squinting, pale skin mucosa, increased number of feces, loss of luster, and slow weight gain, significantly lower than those in the MCN group ($p < 0.05$, Figure 1E-F); After 14 days of molding, MSLD mice showed a marked decrease in activity, frequent squinting, matte and slightly yellow skin (Figure 1B), pale mucosa, soft and shapeless feces (Figure 1D), significantly increased frequency, some mice had anal filth and lost weight compared to MCN mice ($p < 0.05$, Figure 1E-F). MCN group mice had the same status as before (Figure 1A, Figure 1C).

2.2 Organ index of mice with fatigue combined with a high-fat diet

Organ index can initially reflect organ function. As shown in Table 2, the spleen index was lower in the MSLD group than in the MCN group ($p > 0.05$); Thymus and liver indices were higher than in the MCN group ($p > 0.05$). fatigue combined with a high-fat diet had little effect on organ function in mice.

2.3 Muc2 and sIgA of mice with fatigue combined with a high-fat diet

Muc2 in small intestine tissue forms a chemical barrier to the intestinal mucosa, sIgA forms an immune barrier to the intestinal mucosa, and sIgA in serum reflects overall immune levels. As shown in Figure 2A-C, compared to the MCN group, the MSLD group presented an increased trend of IL-6 and IL-17 and a decreased tendency of sIgA and Muc2 ($p > 0.05$).

2.4 Small intestine tissue morphology of mice with fatigue combined with a high-fat diet

In the MCN group, the mucosa of the small intestine is clear, the layer is complete, the muscularis mucosae is intact, there is no obvious edema, inflammation, or lymphocyte infiltration, and it is a normal tissue structure (Figure 3A). The MSLD group had a clear mucosal structure, disrupted intestinal villi continuity, thinning muscularis mucosae, atrophy of the small intestine gland, and infiltration of lymphocytes (Figure 3B). IL-6 and IL-17 were higher in small intestine tissue in the MSLD group than in the MCN group ($p > 0.05$, Figure 3C-D).

2.5 Intestinal mucosa microbiota of mice with fatigue combined with a high-fat diet

2.5.1 Effects of fatigue combined with a high-fat diet on the number and diversity of ASV in mouse intestinal microbiota

Dilution curves and species accumulation curves can be used to determine whether sample sequencing data is reasonable. As shown in Figures 4A, and 4B, the increase in the number of ASV decreased with the increase in the number of sequenced data, and the curve flattened, suggesting that the amount of sequenced data was sufficient for this analysis. As shown in Figure 4C, the depth of sequencing in this experiment is sufficient to reflect the microbial diversity contained in the community sample, the reasonableness of the experimental design, and the reliability of the data. Combining sequences with similarities of more than 97% into one ASV cluster, the analysis showed 105 ASV in the MCN group, 459 ASV in the MSLD group, and 49 ASV in the same number in the four experimental groups, as shown in Figure 4D. These results suggest that fatigue combined with a high-fat diet may increase the number of ASV.

Alpha diversity analysis reflects the abundance and diversity of the microbiota. The Chao1, Observed species indices measure the number of species in a community, and the larger the index, the more species there are. Shannon and Simpson's indices are used to measure species diversity, primarily the number and uniformity of species. The higher the Shannon index, the more diverse Alpha is, and the higher the Simpson index, the lower the diversity of Alpha. Chao1 (Figure 4E) and observed species indices (Figure 4F) were increased in the MSLD group, with no significant difference ($p > 0.05$); Shannon index (Figure 4G) and Simpson index (Figure 4H) were elevated with no significant difference ($p > 0.05$).

NMDS reflects the information of the distance matrix between samples. As shown in Figure 4I, the MCN group is significantly different from the MSLD group and can be distinguished well. The MCN group was concentrated and the MSLD group was widely distributed. PCoA is used to study similarities or

differences in the composition of a sample community, where two samples are closer together, representing a more similar composition of the two species. As shown in Figure 4J, Pco1 was 54.5%, Pco2 was 23.7%, and samples from the MCN and MSLD groups were significantly different. The results showed that there were some differences in intestinal mucosal microbiota between the MCN group and the MSLD group. Figure 4K clustering analysis showed that the distance between samples in the MCN group was relatively small and the variation within the group was small, while the variation within the MLD group was larger than in the MCN group. To sum up, fatigue combined with a high-fat diet affected the diversity and microbiota structure of the mice.

2.5.2 Effects of fatigue combined with a high-fat diet on intestinal mucosal microbiota composition in mice

Classification of intestinal mucosal flora at phylum, genus, and species levels. Figure 5A shows the composition and distribution of intestinal mucosa microbiota at the phylum level in mice, showing that the MCN group had the largest proportion of Firmicutes, followed by Bacteroidetes, and Proteobacteria. However, in the MSLD group, Firmicute decreased, Bacteroidetes, and Proteobacteria increased with no significant difference ($p > 0.05$) and Firmicute/Bacteroidetes decreased with no significant difference ($p > 0.05$). Figure 5B shows the abundance of intestinal mucosa microbiota at the genus level in each group, *Candidatus arthromitus* was the first dominant genus. *Candidatus arthromitus* had 75.23% of intestinal mucosa in the MCN group and 30.94% in the MLD group, lower than in the MCN group ($p > 0.05$), suggesting inhibition of *Candidatus arthromitus* by fatigue combined with a high-fat diet. By counting the top 20 genera of abundance, *Limosilactobacillus* was significantly reduced ($p < 0.05$), and *Anaerotruncus* was significantly increased ($p < 0.05$) in the MSLD group compared to the MCN group.

Figure 5C shows the abundance of intestinal mucosa microbiota at the species level in each group, with *Ligilactobacillus murinus* (4.57%), *Lactobacillus johnsonii* (13.48%), and *Limosilactobacillus reuteri* (10.8%) dominating the MCN group. The relative abundance of *Lactobacillus johnsonii* (30.63%) and *Lactobacillus johnsonii* (18.12%) in the MSLD group increased compared to the MCN group, with no significant difference ($p > 0.05$). The abundance of *Limosilactobacillus reuteri*, and *Limosilactobacillus vaginalis* decreased significantly ($p < 0.05$, Figure 5D-G).

2.5.3 Effects of fatigue combined with a high-fat diet on intestinal mucosa characteristic microbiota in mice

As shown in Figure 6, the LEfSe analysis identified differentially altered characteristic microbiota, with LDA scores greater than 4, and differences in population abundance in the MCN and MSLD groups, of which 6 bacteria were identified as key differentiators. *Tannerellaceae*, *Oscillospiraceae*, *Anaerotruncus* significantly enriched in MCN group, *Limosilacillus vaginalis*, *Limosilacillus reutrei*, *Limosilactobacillus* significantly enriched in MCN group.

Combined with a randomized forest diagnostic model (Figure 6C), the MCN and MSLD groups were distinguished using 20 different species levels of bacteria. ROC results (Figure 6D and 6E),

Limosilactobacillus reuteri, Ligilactobacillus murinus, Limosilactobacillus vaginalis, Mus musculus, Akkermansia muciniphila, Roseburia inulinivorans, Phocaeicola coprocola, Parabacteroides distasonis and *Fusobacterium mortiferum* used as markers for the diagnosis of diarrhea at least 80% of the intestinal tract. *Limosilactobacillus vaginalis* (AUC = 0.9) showed the highest AUC, suggesting that fatigue combined with a high-fat diet resulted in characteristic enrichment of *Limosilactobacillus vaginalis*, which can be identified as a key bacterium for diarrhea.

2.5.4 Effects of fatigue combined with a high-fat diet on intestinal mucosa microbiota function in mice

To determine the metabolic and functional effects of fatigue combined with a high-fat diet on intestinal mucosa microbiota in mice, PICRUSt2 analysis based on the KEGG database predicted microbiota-related metabolic pathways. Figure 7A shows seven major functional types (Cellular Processes, Environmental Information Processing, Genetic Information Processing, Human Diseases, Glycan Pathways, and Metabolism) consisting of 29 functional pathways, with the greatest abundance of Metabolism pathways.

The median metabolic function of the Metabolism Level 3 pathway > 342.8465 was selected (27 classes). As shown in Figure 7B, mainly Amino acid metabolism, Carbohydrate metabolism, Metabolism of cofactors and vitamins, Metabolism of terpenoids and polyketides, and Lipid metabolism. As shown in figure7-C, compared to the MCN group, MSLD group Lysine biosynthesis, Pentose phosphate pathway, Peptidoglycan biosynthesis, Fatty acid biosynthesis, Secondary bile acid biosynthesis, D-Glutamate metabolism, and Terpenoid backpack biosynthesis was significantly reduced ($p < 0.05$) and One carbon pool by folate was significantly increased ($p < 0.05$).

2.6 Correlation Analysis of sIgA, Muc2, Metabolic Pathways and Characteristic microbiota

To investigate the relationship between intestinal mucosa microbiota, metabolic pathway, and intestinal mucosa barrier, we performed Spearman correlation analysis of sIgA, Muc2, IL-6, and IL-17 by selecting nine signature enrichment diagnostic differentially enriched bacteria at the species level and the metabolic pathways with abundance in the top 27. The aim is to determine the key role of intestinal mucosa microbiota in maintaining the stability of the intestinal microenvironment. Correlation heat maps (Figure 8A and 8B) show that *Limosilactobacillus reuteri*, and *Limosilactobacillus vaginalis* are significantly associated with Pentose photosynthesis pathway, Peptidoglycan biosynthesis, Lysine biosynthesis, Terpenoid backbone biosynthesis, Thiamine metabolism. *Limosilactobacillus reuteri* was significantly positively correlated with Muc2 levels in the small intestine and negatively correlated with IL-6.

Discussion

In recent years, it has become a hot topic to study the effects of diet and exercise on the body based on intestinal microbiota. The study found that a high-fat diet (HFD) leads to obesity and inflammation, leads to energy metabolism disorders, promotes inflammatory markers such as IL-6, and TNF-α, and increases

inflammatory lesions (38). Increased risk of liver toxicity after HFD consumption, leading to diabetes, and metabolic dysfunction associated with fatty liver disease (39, 40). Chronic physical fatigue can impair normal bodily functions, lead to endocrine disruption, immune decline, and then organic diseases, affecting health (41). The study found that fatigue combined with a high-fat diet led to an increased number of feces, shapeless feces, losing weight, decreased spleen index, and increased liver index and thymus index in mice. It showed that fatigue combined with a high-fat diet could affect liver and thymus function, decrease spleen immune function and cause diarrhea in mice.

The intestinal barrier may be compromised by severe structural damage to mucous membranes or by changes in the barrier's regulatory composition. Damage to the intestinal barrier was found to be associated with low inflammation of the small intestinal mucosa in celiac disease, inflammatory bowel disease, and irritable bowel syndrome (IBS) (42). Relevant cytokines such as IL-6 and IL-17 are involved in colonic mucosal inflammation in patients with ulcerative colitis (UC). Forced treadmill training increased inflammation and symptoms, and significantly increased the expression of IL-6 and IL-17 colon genes in a mouse model of colitis (43). Muc2-deficient mice develop colonic inflammation, mucosal thickening, increased proliferation, and superficiality, and Muc2 is critical to a functional mucus barrier (44). In patients with UC, sIgA expression was reduced, intestinal barrier function was impaired, and damage to intestinal mechanical and chemical barriers was proportional to disease severity (45). Our results showed that fatigue combined with a high-fat diet in mice had impaired small intestine structure, decreased sIgA and Muc2, and increased IL-6 and IL-17. These results suggest that fatigue combined with a high-fat diet can lead to inflammation of the small intestine, damage the intestinal mucosa barrier and cause diarrhea in mice.

HFD alters microbial diversity, leading to intestinal microecological disorders that promote local inflammation and increase intestinal wall permeability (46). In this study, fatigue combined with a high-fat diet was associated with increased ASV count and Alpha diversity index of intestinal mucosal microbiota and dispersion of community structure. The results showed that fatigue combined with a high-fat diet increased the abundance, diversity, and structure of intestinal mucosa microbiota. A growing body of data shows that the intestinal microbiota mediates the relationship between diet and health, with the intestinal microbiota influencing the development and progression of many diseases. The abundance of Firmicute, Bacteroidetes, and Actinobacteria was associated with host obesity and increased Firmicute/Bacteroidetes ratios and changes in bacterial species were associated with obesity progression (47). The reduction of Firmicute/Bacteroidetes in this study suggests that fatigue combined with a high-fat diet, although high in fat, does not increase body weight and may be associated with fatigue status in mice. HFD increases *Anaerotruncus*, promotes inflammation, and damages the intestinal barrier (48, 49). HFD reduces the abundance of dominant bacteria and reduces the abundance of beneficial bacteria such as *Lactobacillus johnsonii* and *Lactobacillus reuteri* (50). This is consistent with our results that showed a significant increase in *Anaerotruncus* and a significant decrease in *Limosilactobacillus*, *Limosilactobacillus reuteri*, *Limosilactobacillus vaginalis* in mice following overexertion combined with lard dietary intervention.

Anaerotruncus promotes inflammation and tumorigenesis, undermines the integrity of the epithelial barrier, has pro-inflammatory properties, and has been identified as a potential biomarker for colorectal cancer recurrence and patient prognosis (51). *Anaerotruncus colihominis* DSM 17241 was significantly increased in diabetic mice and could serve as a key bacterium for the identification of diabetic-induced intestinal inflammation (52). In this study, fatigue combined with a high-fat diet intervention significantly increased *Anaerotruncus* in mice, which may promote inflammation, damage the intestinal mucosa barrier, and lead to diarrhea in mice. Lactobacilli is a key member of the endogenous microbiota of mucous membranes in the oral, GI, respiratory, and urinary genitalia systems. It plays a key role in regulating local microbiota, restoring barrier function, preventing inflammation associated with GI disorders, improving growth performance, and preventing infectious diseases (53). Lactobacillus attenuates pro-inflammatory cytokines, regulates intestinal microbiota and metabolic and immune parameters in obese mice on HFD, and serves as a complementary probiotic strain for nutritional treatment of obesity and overweight (54). *Limosilactobacillus reuteri* reduces inflammatory response, repairs epithelial tissue structure, protects barrier function, and prevents colitis (55). Combining the LefSe analysis with a randomized forest diagnostic model, we showed significant concentrations of *Limosilacillus vaginalis*, *Limosilacillus reutrei*, and a large AUC value of *Limosilactobacillus vaginalis* at the species level. Correlation analysis showed that *Limosilactobacillus reuteri* was significantly positively correlated with Muc2 levels in the small intestine and negatively correlated with IL-6. Therefore, fatigue combined with a high-fat diet intervention in mice harmful bacteria, and beneficial bacteria decreased, intestinal inflammation and infection prevention role decreased, and the intestinal mucosa barrier lost its protective effect, which may be an important cause of diarrhea. *Limosilactobacillus vaginalis*, whose abundance is associated with Muc2 and IL-6 levels, can be used as a characteristic bacterium for diarrhea diagnosis.

Intestinal homeostasis is determined by complex interactions between the intestinal microbiota, epithelial barrier, and host immune system. Diet can induce changes in the composition of intestinal microbiota that affect host metabolism. Intestinal microbiota participates in the synthesis and metabolism of proteins, carbohydrates, lipids, vitamins, and minerals, balances salt and water intake, increases energy in intestinal epithelial cells, and breaks down lipids and cholesterol (56). By predicting the metagenomic function of the microbiota, we found significant changes in the Metabolism pathway in mice following overexertion combined with lard dietary intervention.

Micronutrient deficiency increases the incidence of bowel diseases such as UC and IBS (57). Folic acid deficiency can lead to severe carbon metabolism abnormalities and lead to chronic disease and developmental disorders (58). Patients with IBS showed lower levels of lysine (59). Lysine is one of the essential amino acids in the human body. It can synthesize proteins, regulate fat metabolism, promote the release of endocrine hormones, promote human development, and strengthen immunity (60). Lysine is an important precursor to the synthesis of glutamate, the most important excitable neurotransmitter in the mammalian central nervous system. Excessive lysine is metabolized as a source of energy, and a lack of lysine in the diet will impair animal immunity and increase animal susceptibility to infectious diseases (61). Bile acids (BA), fatty acids, are common metabolites of the microbiota. BA affects intestinal

function through fluid absorption and secretion, intestinal mucosal permeability, intestinal peristalsis, and intestinal microbiota. IBS significantly increased primary BA and decreased secondary BA (62). Short-chain fatty acids (SCFAs) are key fermentation products of the intestinal microbiota, affecting intestinal mucosa integrity, glucose and lipid metabolism, immune system, and inflammatory response, and are associated with the pathogenesis of various GI diseases (63). Most SCFAs play an active role in regulating related diseases by regulating inflammation, the immune system, and related G-protein-coupled receptors to lower blood pressure, prevent atherosclerosis, and improve heart function after cardiac arrest (64). According to Spearman correlation analysis, *Limosilactobacillus reuteri*, and *Limosilactobacillus vaginalis* were significantly positively associated with the Pentose photosynthesis pathway, Peptidoglycan biosynthesis, Lysine biosynthesis, Terpenoid backbone biosynthesis, and Thiamine metabolism. *Limosilactobacillus reuteri* in this study, *Limosilactobacillus vaginalis* significantly decreased. Therefore, we hypothesized that metabolic function may be associated with changes in characteristic bacterial interactions following fatigue combined with a high-fat diet, suggesting that microbiota influences metabolic function leading to diarrhea in mice.

Conclusion

The interactions between *Limosilactobacillus reuteri* and intestinal inflammation might be involved in the process of intestinal mucosal barrier impairment in fatigue combined with high-fat diet-induced diarrhea.

Declarations

Ethics approval and consent to participate

All methods are reported in accordance with ARRIVE guidelines (https://arriveguidelines.org) for the reporting of animal experiments, and were carried out in accordance with relevant guidelines and regulations. This study was approved by the Animal Ethics and Welfare Committee of Hunan University of Chinese Medicine with ethical review number LLBH202206200003. All authors were aware of and agreed to this animal experiment.

Consent for publication

Not applicable

Availability of data and materials

The data underlying this study was available within the manuscript. The gut content microbiota sequencing data has been uploaded to the NCBI database: PRJNA903506.

Competing interests

We declare that there is no conflict of interest regarding the publication of this paper.

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Author Contributions

Jing Liu: performed the experiments, analyzed the data, and wrote the original manuscript. Bo Qiao: performed the experiments and analyzed the data. Ying Cai: revised the manuscript. Na Deng and Zhoujin Tan: reviewed the manuscript and funded the acquisition. All authors contributed to the article and approved the submitted version.

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References

1. Schiller LR, Pardi DS, Sellin JH. Chronic diarrhea: diagnosis and management. Clin Gastroenterol Hepatol. 2017 Feb;15(2):182-193.e3. doi: 10.1016/j.cgh.2016.07.028.
2. Farthing M, Salam MA, Lindberg G, Dite P, Khalif I, Salazar-Lindo E, Ramakrishna BS, Goh KL, Thomson A, Khan AG, Krabshuis J, LeMair A; WGO. Acute diarrhea in adults and children: a global perspective. J Clin Gastroenterol. 2013 Jan;47(1):12-20. doi: 10.1097/MCG.0b013e31826df662.
3. GBD 2019 Diseases and Injuries Collaborators. Global burden of 369 diseases and injuries in 204 countries and territories, 1990-2019: a systematic analysis for the Global Burden of Disease Study 2019. Lancet. 2020 Oct 17;396(10258):1204-1222. doi: 10.1016/S0140-6736(20)30925-9.
4. Dahlgren D, Agréus L, Stålhammar J, Hellström PM. Ulcerative colitis progression: a retrospective analysis of disease burden using electronic medical records. Ups J Med Sci. 2022 Oct 18;127. doi: 10.48101/ujms. v127.8833.
5. Behera DK, Mishra S. The burden of diarrhea, etiologies, and risk factors in India from 1990 to 2019: evidence from the global burden of disease study. BMC Public Health. 2022 Jan 13;22(1):92. doi: 10.1186/s12889-022-12515-3.
6. Ibrahim F, Stribling P. A 5Ad dietary protocol for functional bowel disorders. Nutrients. 2019 Aug 17;11(8):1938. doi: 10.3390/nu11081938.
7. Gibson PR, Shepherd SJ. Food choice as a key management strategy for functional gastrointestinal symptoms. Am J Gastroenterol. 2012 May;107(5):657-66; quiz 667. doi: 10.1038/ajg.2012.49.

8. Guo KX, Yan YW, Zeng CY, Shen L, He YS, Tan ZJ. Study on Baohe Pills regulating intestinal microecology and treating diarrhea of high-fat and high-protein diet mice. Biomed Res Int. 2022 May 17; 2022:6891179. doi: 10.1155/2022/6891179.
9. Zhou K, Deng N, Yi X, Cai Y, Peng MJ, Xiao NQ. Baohe pill decoction for diarrhea induced by high-fat and high-protein diet is associated with the structure of lactase-producing bacterial community. Front Cell Infect Microbiol. 2022 Aug 26; 12:1004845. doi: 10.3389/fcimb.2022.1004845.
10. Xu Y, Zhu WZ, Ge QF, Zhou XY. Effect of different types of oil intake on the blood index and the intestinal flora of rats. AMB Express. 2022 May 5;12(1):49. doi: 10.1186/s13568-022-01387-w.
11. Qiao B, Li XY, Zheng T, Tan ZJ. Different effects of lard and vegetable blend oil on intestinal microorganisms, enzyme activity and blood routine in mice. J Oleo Sci. 2022 Feb 3;71(2):301-310. doi: 10.5650/jos. ess21247.
12. Qiao B, Li XY, Wu Y, Guo T, Tan ZJ. Comparative analysis of the gut microbiota in mice under lard or vegetable blend oil diet. J Oleo Sci. 2022 Oct 5. doi: 10.5650/jos. ess22056.
13. Lee MC, Hsu YJ, Ho HH, Kuo YW, Lin WY, Tsai SY, Chen WL, Lin CL, Huang CC. Effectiveness of human-origin Lactobacillus plantarum PL-02 in improving muscle mass, exercise performance and anti-fatigue. Sci Rep. 2021 Sep 30;11(1):19469. doi: 10.1038/s41598-021-98958-x.
14. Smarkusz-Zarzecka J, Ostrowska L, Leszczyńska J, Cwalina U. Effect of a multi-strain probiotic supplement on gastrointestinal symptoms and serum biochemical parameters of long-distance runners: a randomized controlled trial. Int J Environ Res Public Health. 2022 Jul 30;19(15):9363. doi: 10.3390/ijerph19159363.
15. Zhu JY, Li XY, Deng N, Peng XX, Tan ZJ. Diarrhea with deficiency kidney-yang syndrome caused by adenine combined with Folium senna was associated with gut mucosal microbiota. Front Microbiol. 2022 Oct 11; 13:1007609. doi: 10.3389/fmicb.2022.1007609.
16. Li XY, Peng XX, Qiao B, Peng MJ, Deng N, Yu R, Tan ZJ. Gut-kidney impairment process of adenine combined with folium sennae-induced diarrhea: association with interactions between Lactobacillus intestinalis, Bacteroides acidifaciens and acetic acid, inflammation, and kidney function. Cells. 2022 Oct 17;11(20):3261. doi: 10.3390/cells11203261.
17. Camilleri M, Lasch K, Zhou W. Irritable bowel syndrome: methods, mechanisms, and pathophysiology. The confluence of increased permeability, inflammation, and pain in irritable bowel syndrome. Am J Physiol Gastrointest Liver Physiol. 2012 Oct;303(7): G775-85. doi: 10.1152/ajpgi.00155.2012.
18. Wu YQ, Jha R, Li A, Liu HW, Zhang Z, Zhang CC, Zhai QX, Zhang JC. Probiotics (Lactobacillus plantarum HNU082) supplementation relieves ulcerative colitis by affecting intestinal barrier functions, immunity-related gene expression, gut microbiota, and metabolic pathways in mice. Microbiol Spectr. 2022 Nov 2: e0165122. doi: 10.1128/spectrum.01651-22.
19. Turner JR. Intestinal mucosal barrier function in health and disease. Nat Rev Immunol. 2009 Nov;9(11):799-809. doi: 10.1038/nri2653.

20. Chen SS, Zhang C, He BH, He RN, Xu L, Zhang S. The role of lncRNAs in regulating the intestinal mucosal mechanical barrier. Biomed Res Int. 2021 Nov 15; 2021:2294942. doi: 10.1155/2021/2294942.
21. Becattini S, Taur Y, Pamer EG. Antibiotic-induced changes in the intestinal microbiota and disease. Trends Mol Med. 2016 Jun;22(6):458-478. doi: 10.1016/j.molmed.2016.04.003.
22. Sebastián Domingo JJ, Sánchez Sánchez C. From the intestinal flora to the microbiome. Rev Esp Enferm Dig. 2018 Jan;110(1):51-56. doi: 10.17235/reed.2017.4947/2017.
23. Chang CT, Yuan XZ, Zhang XX, Chen XR, Li K. Gastrointestinal microbiome and multiple health outcomes: umbrella review. Nutrients. 2022 Sep 9;14(18):3726. doi: 10.3390/nu14183726.
24. Caballero S, Pamer EG. Microbiota-mediated inflammation and antimicrobial defense in the intestine. Annu Rev Immunol. 2015; 33:227-56. doi: 10.1146/annurev-immunol-032713-120238.
25. Fu QH, Song TY, Ma XQ, Cui J. Research progress on the relationship between intestinal microecology and intestinal bowel disease. Animal Model Exp Med. 2022 Dec;5(4):297-310. doi: 10.1002/ame2.12262.
26. Chen Q, Xiao Z, He QY, Zhang RR, Chen SX, Dong JW, Zhang H, Chen XF. Effect of Shenling Baizhu powder on immunity to diarrheal disease: A systematic review and meta-analysis. Front Pharmacol. 2022 Sep 14; 13:938932. doi: 10.3389/fphar.2022.938932.
27. Chen XW, Zhou SF. Inflammation, cytokines, the IL-17/IL-6/STAT3/NF-κB axis, and tumorigenesis. Drug Des Devel Ther. 2015 Jun 8; 9:2941-6. doi: 10.2147/DDDT.S86396.
28. Odenwald MA, Turner JR. The intestinal epithelial barrier: a therapeutic target? Nat Rev Gastroenterol Hepatol. 2017 Jan;14(1):9-21. doi: 10.1038/nrgastro.2016.169.
29. Llewellyn SR, Britton GJ, Contijoch EJ, Vennaro OH, Mortha A, Colombel JF, Grinspan A, Clemente JC, Merad M, Faith JJ. Interactions between diet and the intestinal microbiota alter intestinal permeability and colitis severity in mice. gastroenterology. 2018 Mar;154(4):1037-1046.e2. doi: 10.1053/j.gastro.2017.11.030.
30. Abdalqadir N, Adeli K. GLP-1 and GLP-2 orchestrate intestine integrity, gut microbiota, and immune system crosstalk. Microorganisms. 2022 Oct 19;10(10):2061. doi: 10.3390/microorganisms10102061.
31. Wu Y, Peng XX, Li XY, Li DD, Tan ZJ, Yu R. Sex hormones influence the intestinal microbiota composition in mice. Front Microbiol. 2022 Oct 31; 13:964847. doi: 10.3389/fmicb.2022.964847.
32. Yang X, Li F, Ma J, Mao M, Wu FZ, Du Q, Zhang ZL, Yin ZT, Li FY. Establishment and evaluation of a Spleen-Deficiency rat model by a small platform with water beneath. Shandong Journal of Traditional Chinese Medicine 2016 May 35(5): 452-457. doi: 10.16295/j.cnki.0257-358x.2016.05.030.
33. Ma J, Liu J, Liu Y, Li F, S YH, Han CX, Gua SY, Yang X. Based on behavioral experiments to explore the weary change of small platform station water environment model. Acta Chinses Medicine 2015 Jun 30(06): 843-845. doi: 10.16368/j.issn.1674-8999.2015.06.291.

34. Li YM, Han YM, Zhu KJ. Comprehensive evaluation of symptoms of spleen deficiency model mice by eating disorder method. Hunan Journal of Traditional Chinese Medicine 2011 Jul 27(04): 126-127. doi: 10.16808/j.cnki.issn1003-7705.2011.04.077.
35. Lu C, Zhou LF, Cao Dong, Wang SG, Yang XJ. Experimental study on the effect of Shenling Baizhu granule on splenic strength of mice with spleen deficiency syndrome Chinese. Journal of Experimental Traditional Medical Formulae 1999 (04):41-43.
36. Gao YH, Li H, Song L, Wu Y, Chen TF, Hou HP, Peng B, Ye ZG, Li JX, Chen GP. Pharmacodynamic study of Shenling Jianpiwei Keii on gastrointestinal regulation in spleen deficiency model rats. Drug Evaluation Research 2022 Jun 45(10): 1992-2001. doi: 10.19347/j.cnki.2096-1413.202217041.
37. Li XY, Zhang CY, Hui HY, Tan ZJ. Effect of Gegenqinlian decoction on intestinal mucosal flora in mice with diarrhea induced by high temperature and humidity treatment. 3 Biotech. 2021 Feb;11(2):83. doi: 10.1007/s13205-020-02628-0.
38. Tavares MEA, Veras ASC, Thorpe HHA, Baptista DB, Teixeira GR. Physical exercise regulates apoptosis and prostatic inflammatory effects induced by high-fat diet in PPAR-alpha deleted mice. Prostaglandins Other Lipid Mediat. 2022 Dec; 163:106669. doi: 10.1016/j.prostaglandins.2022.106669.
39. Achterbergh R, Lammers LA, Klümpen HJ, Mathôt RAA, Romijn JA. Short-term high-fat diet alters acetaminophen metabolism in healthy individuals. Ther Drug Monit. 2022 Dec 1;44(6):797-804. doi: 10.1097/FTD.0000000000000993.
40. da Cruz LL, Vesentini G, Sinzato YK, Villaverde AISB, Volpato GT, Damasceno DC. Effects of high-fat diet-induced diabetes on autophagy in the murine liver: A systematic review and meta-analysis. Life Sci. 2022 Nov 15; 309:121012. doi: 10.1016/j.lfs.2022.121012.
41. Zhao YQ, Zeng L, Yang ZS, Huang FF, Ding GF, Wang B. Anti-fatigue effect by peptide fraction from protein hydrolysate of croceine croaker (Pseudosciaena crocea) swim bladder through inhibiting the oxidative reactions including DNA damage. Mar Drugs. 2016 Dec 13;14(12):221. doi: 10.3390/md14120221.
42. Linsalata M, Riezzo G, Clemente C, D'Attoma B, Russo F. Noninvasive biomarkers of gut barrier function in patients suffering from diarrhea predominant-IBS: an update. Dis Markers. 2020 Oct 13; 2020:2886268. doi: 10.1155/2020/2886268.
43. Cook MD, Martin SA, Williams C, Whitlock K, Wallig MA, Pence BD, Woods JA. Forced treadmill exercise training exacerbates inflammation and causes mortality while voluntary wheel training is protective in a mouse model of colitis. Brain Behav Immun. 2013 Oct;33:46-56. doi: 10.1016/j.bbi.2013.05.005.
44. Van der Sluis M, De Koning BA, De Bruijn AC, Velcich A, Meijerink JP, Van Goudoever JB, Büller HA, Dekker J, Van Seuningen I, Renes IB, Einerhand AW. Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection. Gastroenterology. 2006 Jul;131(1):117-29. doi: 10.1053/j.gastro.2006.04.020.

45. Shi T, Wei JP, Liu G, Han M, Liu T. Researches on the change of intestinal barrier function in patients with ulcerative colitis. Zhonghua Yi Xue Za Zhi. 2015 Jun 23;95(24):1941-3. Chinese.
46. Barber TM, Valsamakis G, Mastorakos G, Hanson P, Kyrou I, Randeva HS, Weickert MO. Dietary influences on the microbiota-gut-brain Axis. Int J Mol Sci. 2021 Mar 28;22(7):3502. doi: 10.3390/ijms22073502.
47. Chang CJ, Lin CS, Lu CC, Martel J, Ko YF, Ojcius DM, Tseng SF, Wu TR, Chen YY, Young JD, Lai HC. Ganoderma lucidum reduces obesity in mice by modulating the composition of the gut microbiota. Nat Commun. 2015 Jun 23; 6:7489. doi: 10.1038/ncomms8489.
48. Bailén M, Bressa C, Martínez-López S, González-Soltero R, Montalvo Lominchar MG, San Juan C, Larrosa M. Microbiota features associated with a high-fat/low-fiber diet in healthy adults. Front Nutr. 2020 Dec 18; 7:583608. doi: 10.3389/fnut.2020.583608.
49. Zhang X, Coker OO, Chu ES, Fu K, Lau HCH, Wang YX, Chan AWH, Wei H, Yang X, Sung JJY, Yu J. Dietary cholesterol drives fatty liver-associated liver cancer by modulating gut microbiota and metabolites. Gut. 2021 Apr;70(4):761-774. doi: 10.1136/gutjnl-2019-319664.
50. Huang LL, Zheng T, Liu YW, Guo T, Tan Z J. Diarrheal mice induced by a high-fat and high-protein diet could be cured by bohe pill regulating intestinal mucosal bacteria. J. Food Nutr. Res. 2022 Oct; 10:459-466. doi: 10.12691/jfnr-10-7-3
51. Huo RX, Wang YJ, Hou SB, Wang W, Zhang CZ, Wan XH. Gut mucosal microbiota profiles linked to colorectal cancer recurrence. World J Gastroenterol. 2022 May 14;28(18):1946-1964. doi: 10.3748/wjg. v28.i18.1946.
52. Liu L, Chen Y, Wu Q, Shu A, Sun J. Sodium butyrate attenuated diabetes-induced intestinal inflammation by modulating gut microbiota. Evid Based Complement Alternat Med. 2022 Aug 22; 2022:4646245. doi: 10.1155/2022/4646245.
53. Gangaiah D, Ryan V, Van Hoesel D, Mane SP, Mckinley ET, Lakshmanan N, Reddy ND, Dolk E, Kumar A. Recombinant Limosilactobacillus (Lactobacillus) delivering nanobodies against Clostridium perfringens NetB and alpha toxin confers potential protection from necrotic enteritis. Microbiologyopen. 2022 Apr;11(2): e1270. doi: 10.1002/mbo3.1270.
54. Fabersani E, Marquez A, Russo M, Ross R, Torres S, Fontana C, Puglisi E, Medina R, Gauffin-Cano P. Lactic acid bacteria strains differently modulate gut microbiota and metabolic and immunological parameters in Hhigh-fat diet-fed mice. Front Nutr. 2021 Sep 9; 8:718564. doi: 10.3389/fnut.2021.718564.
55. Liu HY, Gu F, Zhu C, Yuan L, Zhu C, Zhu M, Yao J, Hu P, Zhang Y, Dicksved J, Bao W, Cai D. Epithelial heat shock proteins mediate the protective effects of Limosilactobacillus reuteri in dextran sulfate sodium-induced colitis. Front Immunol. 2022 Mar 7; 13:865982. doi: 10.3389/fimmu.2022.865982.
56. Resta SC. Effects of probiotics and commensals on intestinal epithelial physiology: implications for nutrient handling. J Physiol. 2009 Sep 1;587(Pt 17):4169-74. doi: 10.1113/jphysiol.2009.176370.
57. Park YE, Park SJ, Park JJ, Cheon JH, Kim T, Kim WH. Incidence and risk factors of micronutrient deficiency in patients with IBD and intestinal Behçet's disease: folate, vitamin B12, 25-OH-vitamin D,

and ferritin. BMC Gastroenterol. 2021 Jan 21;21(1):32. doi: 10.1186/s12876-021-01609-8.

58. Schalinske KL, Steele RD. Methotrexate alters carbon flow through the hepatic folate-dependent one-carbon pool in rats. Carcinogenesis. 1996 Aug;17(8):1695-700. doi: 10.1093/carcin/17.8.1695.
59. Zhang M, Zheng YJ, Li X, Wu HM, Liu P, Zhang KL, Shi ZF, Lv M, Wang FY, Tang XD. Tong-Xie-Yao-Fang alleviates diarrhea-predominant irritable bowel syndrome in rats via the GCN2/PERK-eIF2α-ATF4 signaling pathway. Phytomedicine. 2022 Dec; 107:154350. doi: 10.1016/j.phymed.2022.154350.
60. Hu XC, Huo B, Yang JM, Wang K, Huang LJ, Che LQ, Feng B, Lin Y, Xu SY, Zhuo Y, Wu CM, Wu D, Fang ZM. Effects of dietary lysine levels on growth performance, nutrient digestibility, serum metabolites, and meat quality of Baqing pigs. Animals (Basel). 2022 Jul 23;12(15):1884. doi: 10.3390/ani12151884.
61. Liao SF, Wang T, Regmi N. Lysine nutrition in swine and the related monogastric animals: muscle protein biosynthesis and beyond. Springerplus. 2015 Mar 27; 4:147. doi: 10.1186/s40064-015-0927-5.
62. Min YW, Rezaie A, Pimentel M. Bile acid and gut microbiota in irritable bowel syndrome. J Neurogastroenterol Motil. 2022 Oct 30;28(4):549-561. doi: 10.5056/jnm22129.
63. Xiao L, Liu Q, Luo M, Xiong LS. Gut microbiota-derived metabolites in irritable bowel syndrome. Front Cell Infect Microbiol. 2021 Sep 23; 11:729346. doi: 10.3389/fcimb.2021.729346.
64. Hu TT, Wu QQ, Yao Q, Jiang KB, Yu JB, Tang QZ. Short-chain fatty acid metabolism and multiple effects on cardiovascular diseases. Ageing Res Rev. 2022 Nov; 81:101706. doi: 10.1016/j.arr.2022.101706.

Tables

Table 1 Common feed (per kg of feed)

component	content
water (g)	≤100
crude protein (g)	≥200
crude fiber (g)	≥40
crude fat (g)	≤50
crude ash (g)	≤80
calcium (g)	10-18
phosphorus (g)	6-12
calcium: phosphorus	1.2: 1 - 1.7: 1
lysine (g)	≥13.2
methionine and cysteine (g)	≥7.8

Table 2 Organ indices (Organ indices = Organ weight/Mouse weight, mean ± standard deviation)

organ index	MCN	MSLD
spleen(mg/g)	2.98±0.45	2.86±0.25
thymus(mg/g)	2.53±0.77	3.24±0.56
liver(mg/g)	53.7±8.28	58.09±5.99

Figures

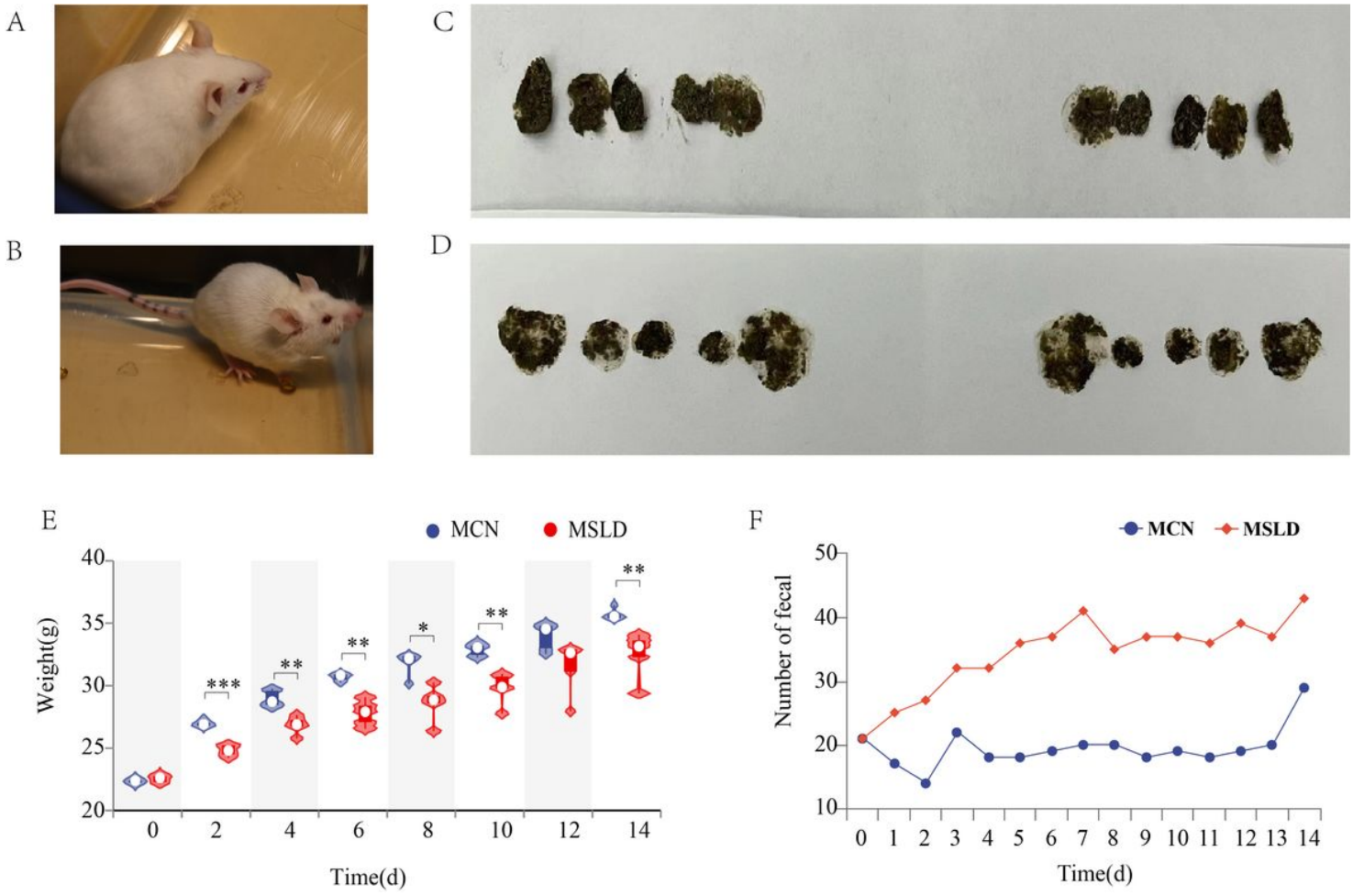


Figure 1

(A) General characteristics of MCN group mice after 14 days. (B) General characteristics of MSLD mice after 14 days of molding. (C) Fecal images of MCN group mice after 14 days; (D) Fecal images of MSLD mice after 14 days of molding; (E) Violin chart of the weight difference in each group of mice (n = 5); (F) A fold diagram of the number of feces per group of mice for half an hour (n = 5 ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$)).

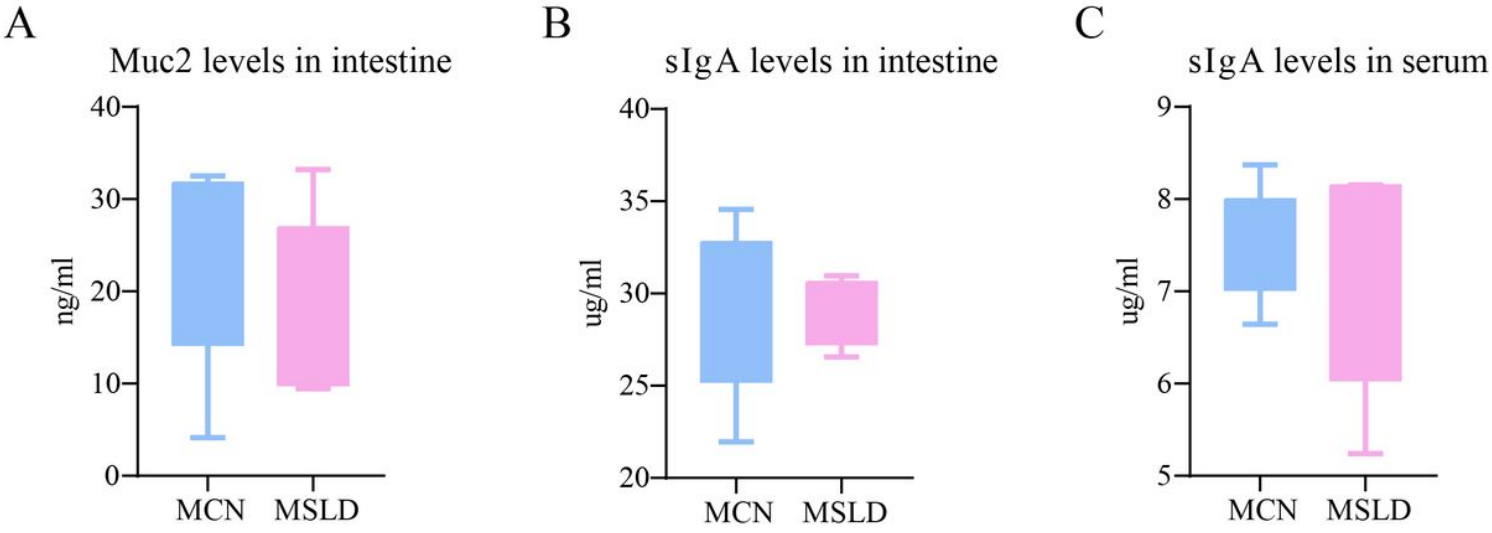
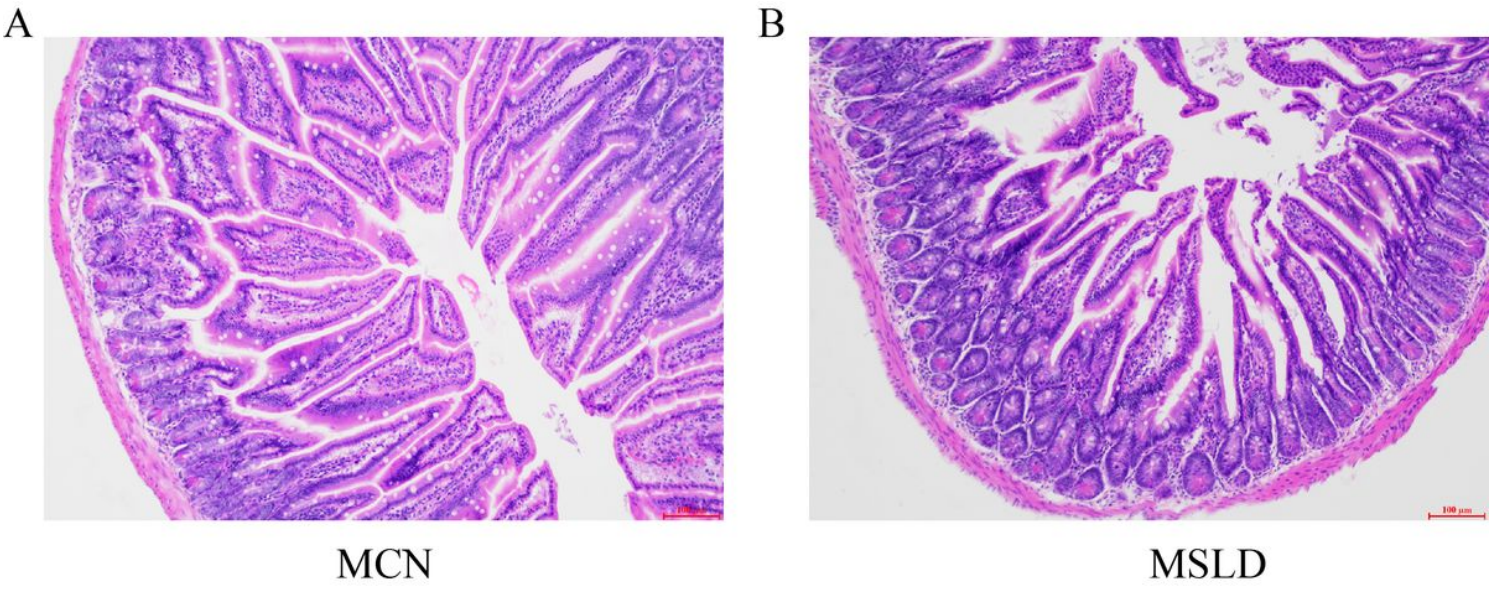


Figure 2

(A) Muc2 levels in intestinal tissue; (B) sIgA levels in intestinal tissue; (B) sIgA levels in serum.



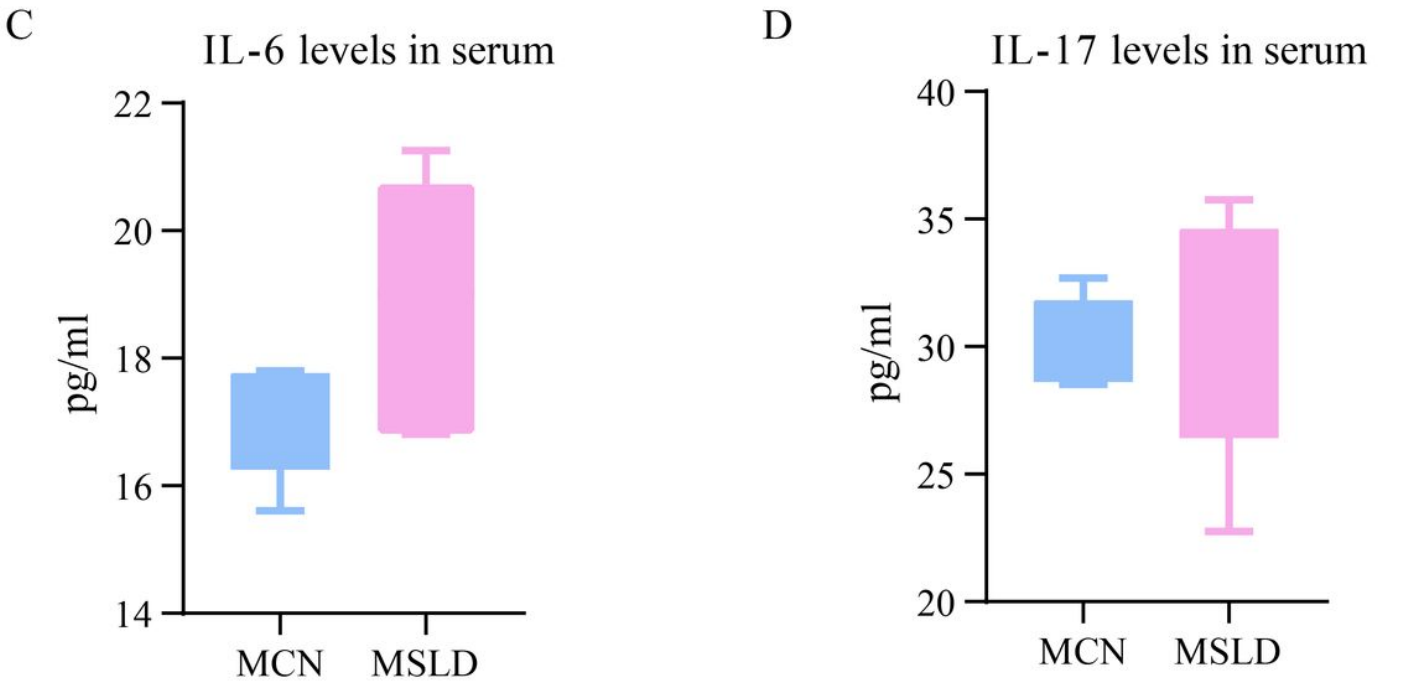


Figure 3

(A) The HE dye of small intestine tissue from MCN group; (B) The HE dye of small intestine tissue from MSLD group; (C) IL-6 levels in intestinal tissue; (D) IL-17 levels in intestinal tissue;

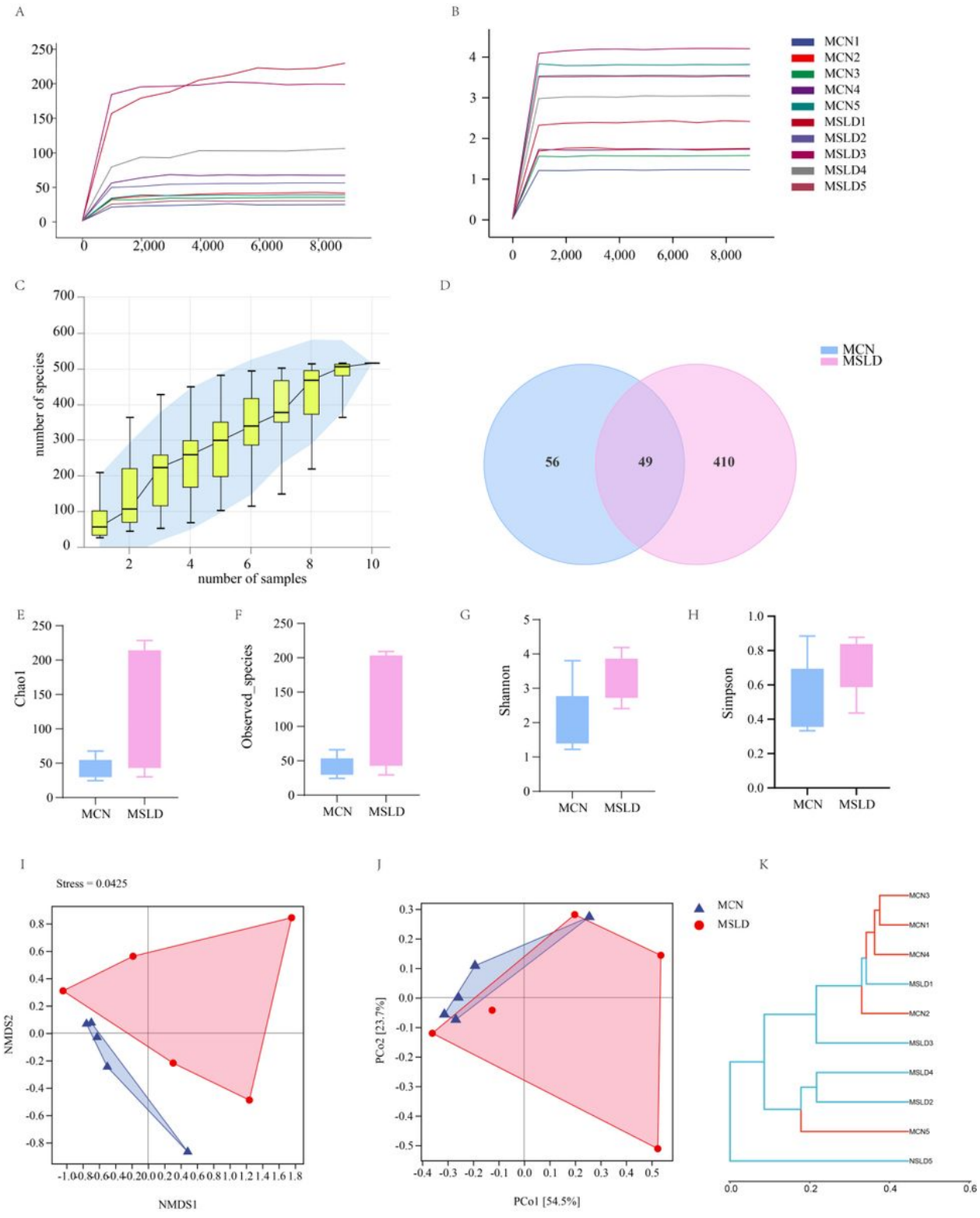


Figure 4

(A)Chao1 Wiener curves of intestinal mucosal microflora; (B)Shannon Wiener curves of intestinal mucosal bacteria; (C)Species accumulation curves; (D)Venn diagram: distribution of the number of ASV of intestinal mucosal microflora; (E)Chao1 index; (F)Observed species index; (G)Shannon index; (H)Simpson index; (I)PCoA analysis; (J)NMDS analysis; (K)Clustering analysis.

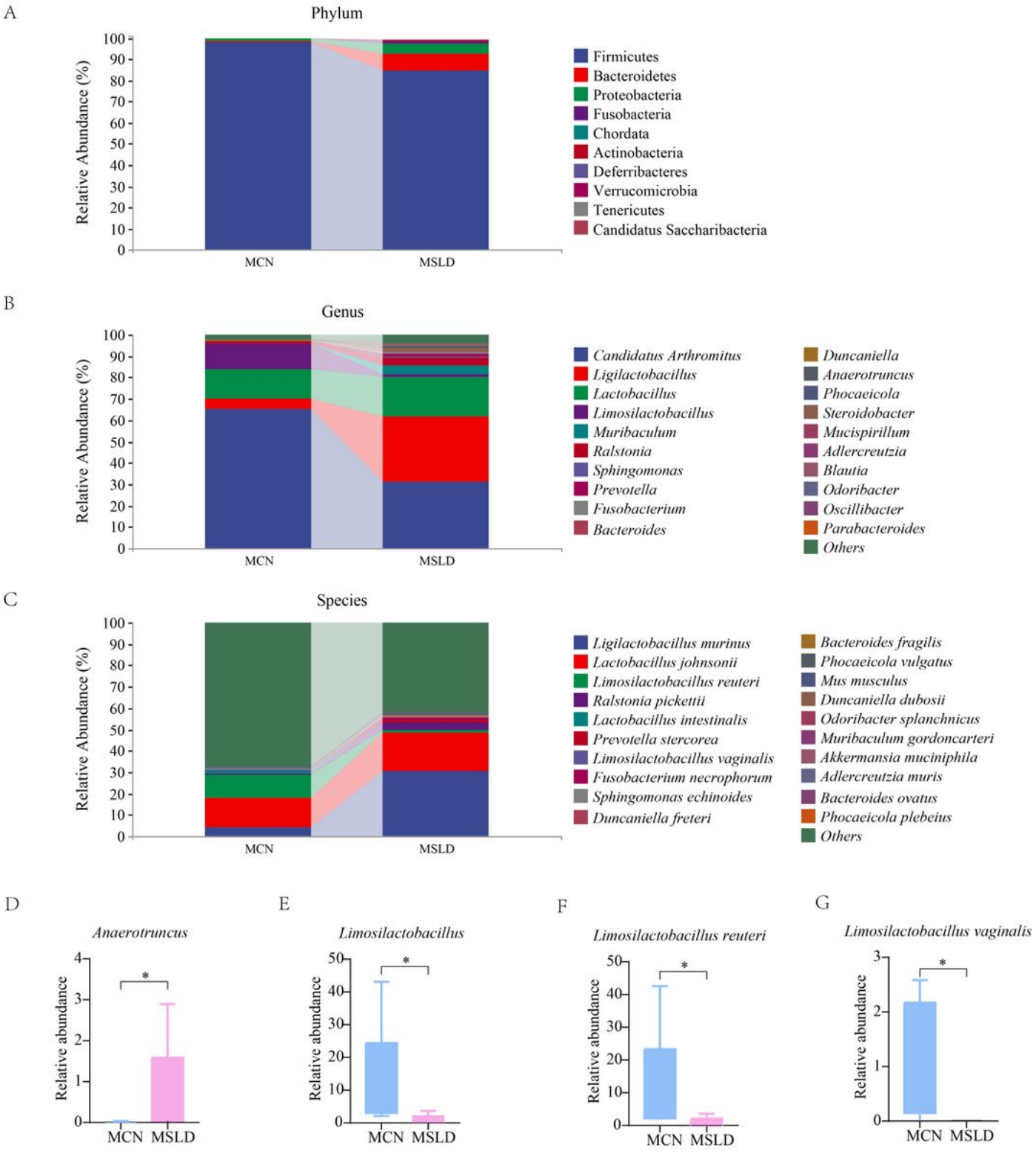


Figure 5

(A) phylum level intestinal mucosa microbiota; (B) genus level intestinal mucosa microbiota; (C) species level intestinal mucosa microbiota; (D-G) Genus and species of intestinal mucosa dominant bacteria in mice ($^{*}p < 0.05$).

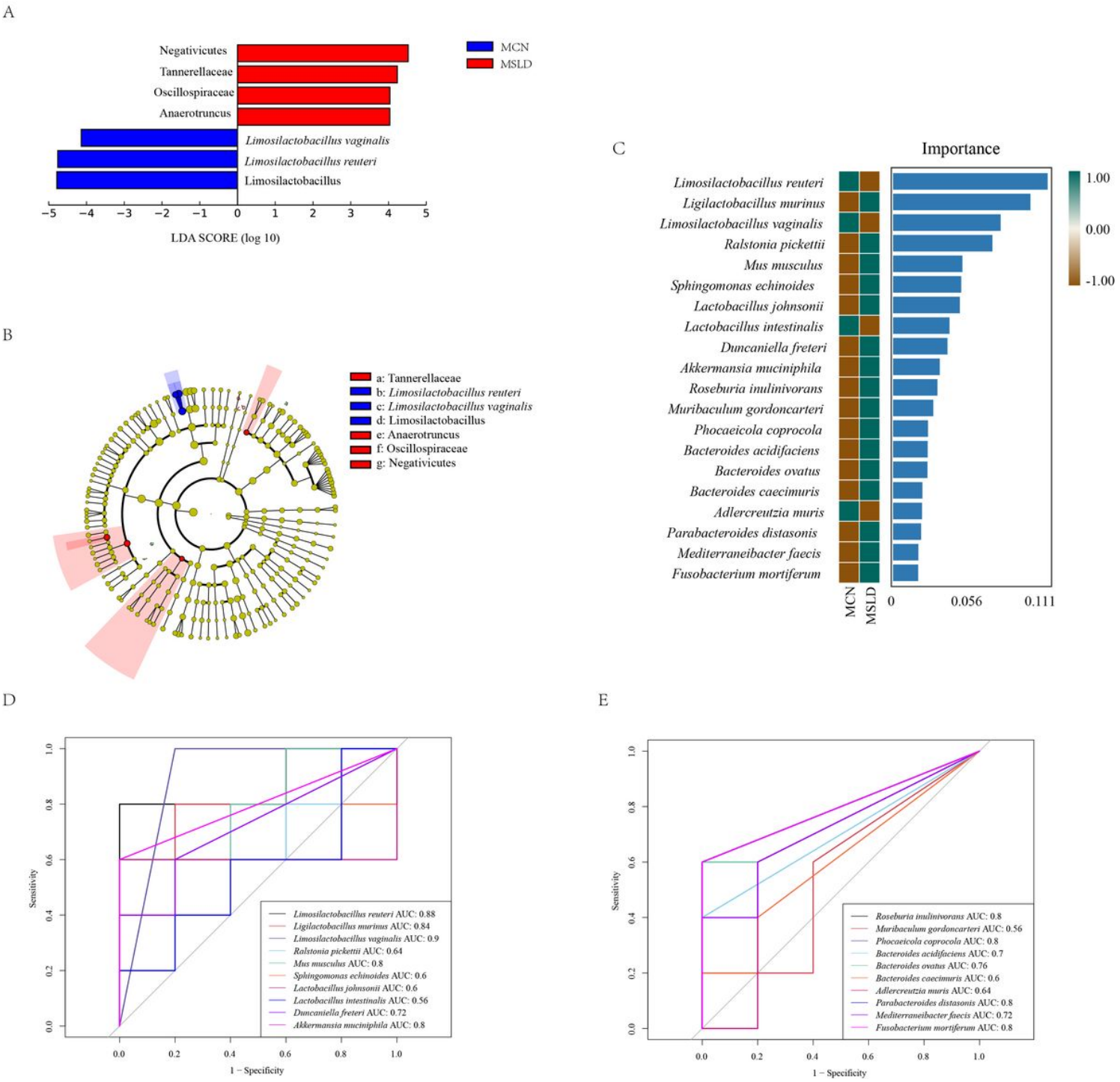


Figure 6

Core characteristic bacterial analysis of intestinal mucosal microbiota. (A)LDA diagram; (B)Cladogram diagram; (C)Random Forest diagram of species level; (D-E) ROC curve of species.

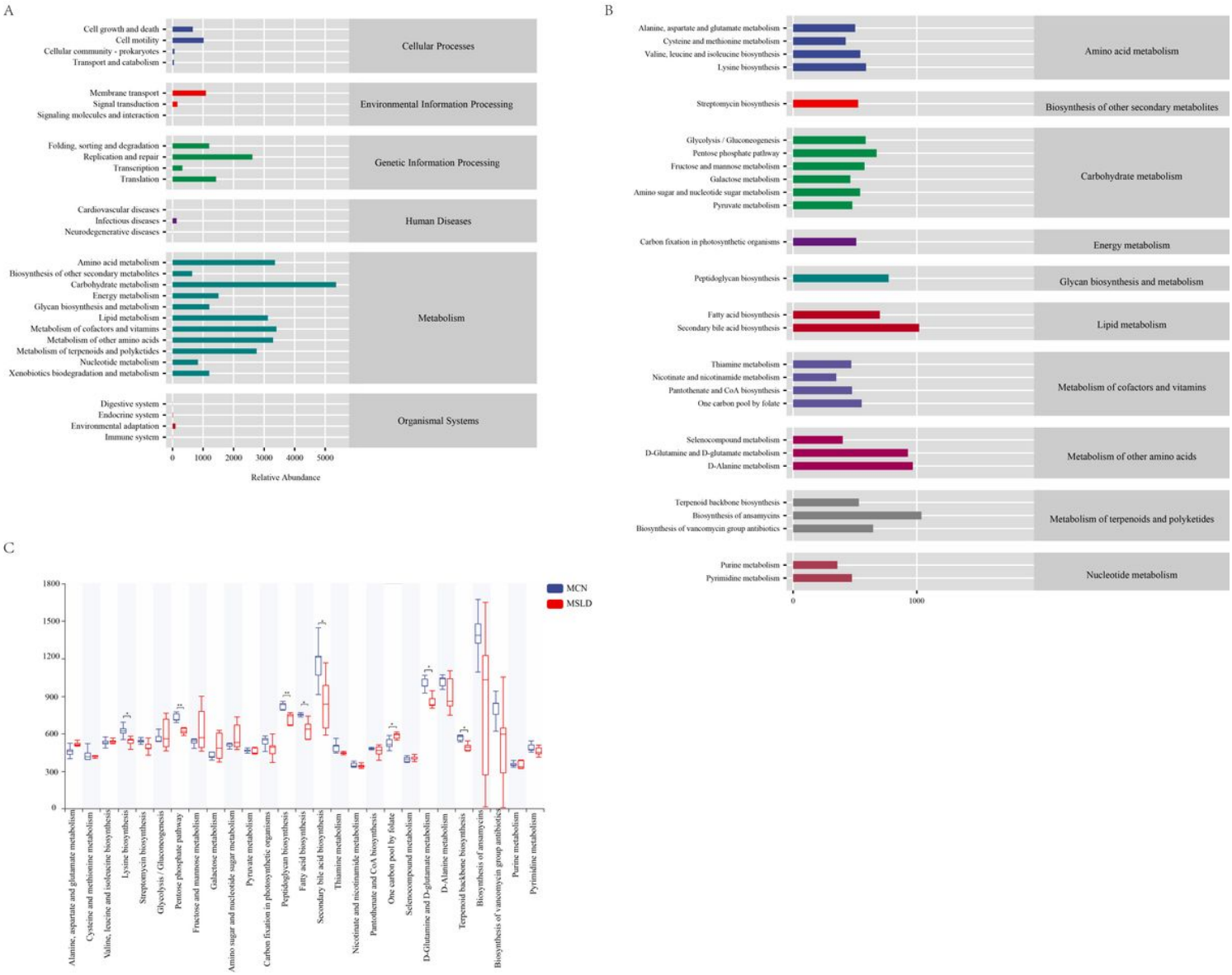


Figure 7

Prediction of intestinal mucosal microbiota metabolism based on PICRUSt2. (A-B) Predicted abundance of KEGG function with horizontal coordinates of KEGG functional pathway and longitudinal coordinates of KEGG functional pathway classification; (C) Comparison between groups of metabolic functional groups ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

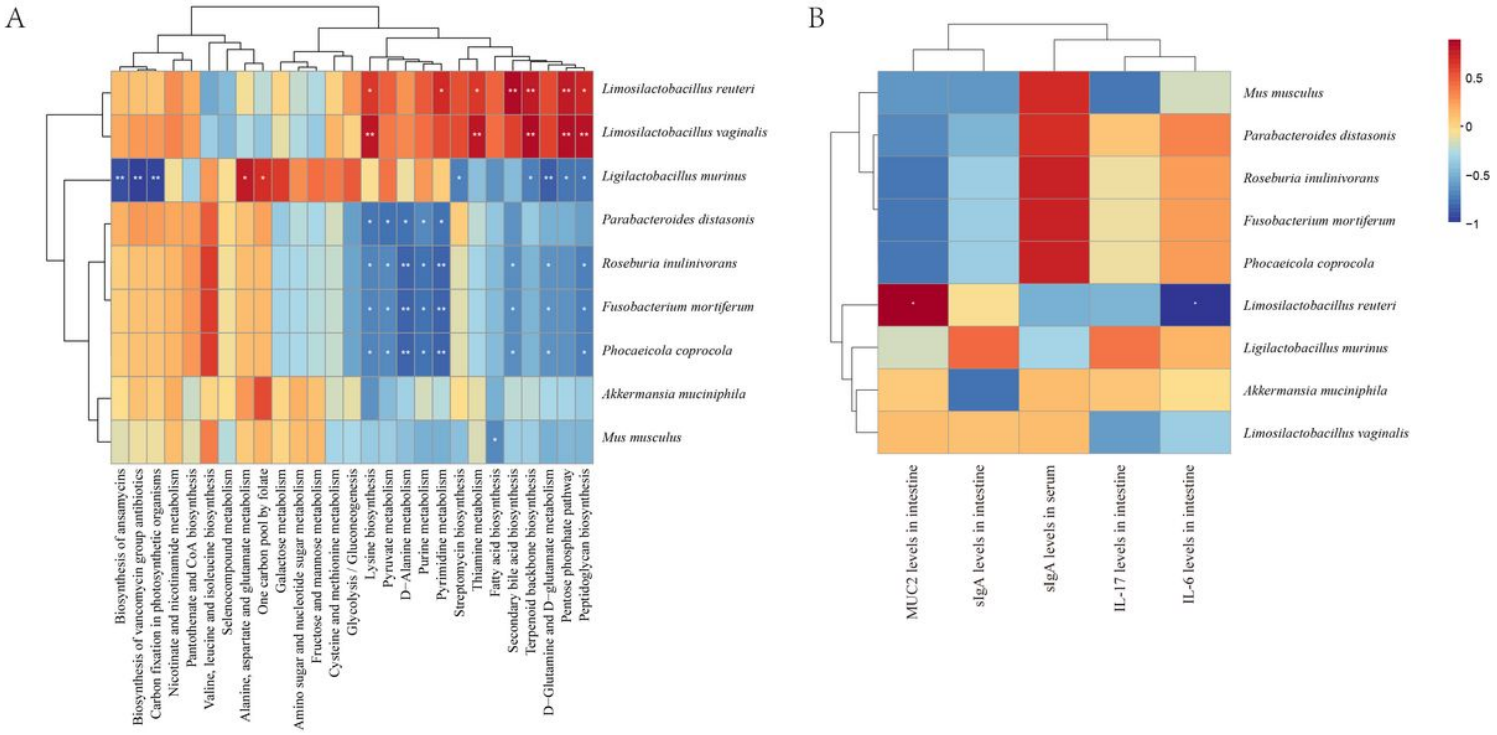


Figure 8

Spearman correlation analysis heatmap: blue represents negative correlation, red represents positive correlation, and the closer the color is to blue, the stronger the negative correlation between the two parameters, and the closer the color is to red, the stronger the positive correlation between the two parameters. (A) Correlation heatmap of intestinal mucosa microbiota and metabolic pathways; (B) Correlation heatmap of intestinal mucosa microbiota with sIgA, Muc2, IL-6, and IL-17.