

Intestinal flora and inflammation in acute coronary syndromes

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Abstract

Background: Acute coronary syndromes (ACS) is closely associated with chronic low-grade inflammation and gut microbiome composition. However, the composition and functional capacity of the gut microbiome in relation to ACS have not been systematically examined.

Results: we perform a metagenome-wide association study on stools and plasma from 66 individuals with ACS and 46 healthy controls (HC). We then compared gut microbial composition using 16S ribosomal RNA gene sequencing in fecal samples to detect species with differential abundance between 2 groups. We reported that the altered composition of gut microbiota was associated with ACS and exacerbated inflammatory status. Moreover, parameters in ACS including body weights (BWs), low-density lipoprotein (LDL), triglyceride (TG), total cholesterol (TC), C-reactive protein (CRP) and high homocysteine (HCY) were elevated; whereas high-density lipoprotein (HDL) was decreased. pro-inflammatory interleukin (IL)-1 β , IL-6, IL-10, tumor necrosis factor (TNF)- α , monocyte chemoattractant protein-1 (MCP-1) and lipopolysaccharide (LPS) in ACS were increased respectively. The results of 16S rRNA sequencing and analysis displayed that the overall community of gut microbiota in ACS was notably changed mainly through increasing the abundance of *Bacteroidetes*, *Verrucomicrobia*, *Proteobacteria Parabacteroide*, *Unidentified_Enterobacteriaceae*, *Subdoligranulum*, *Akkermansia*, *Alistipes*, *Streptococcus*, *Paraprevotella* as well as decreasing *Subdoligranulum*, *Roseburia*, *Faecalibacterium*, *Blautia*, *Agathobacter*, *Anaerostipes*, *Bifidobacterium*. Further analysis showed that there was a significant correlation between the above differences in gut microbiota and inflammatory factors.

Conclusions: Our data represent a comprehensive resource for further investigations on the role of the gut microbiome in promoting or preventing ACS.

Background

Acute Coronary Syndromes (ACS) are a group of disorders that can be caused by a significant reduction of blood flow in coronary arteries due to narrowing or blockage of the vessels, including unstable angina pectoris (UAP) and acute myocardial infarction (AMI)[1]. The most common reason of the vessel dysfunction is the development of atherosclerotic lesions and a blood clot formed in place of plaque rupture[2]. Cardiovascular disease is the leading cause of death worldwide. Atherosclerosis(AS), the most common cause of cardiovascular disease, is the result of a complex series of events occurring within the arterial wall involving rheology, lipid metabolism, and inflammation[3]. Growing evidence has implicated gut microbiota alterations in the development of cardiovascular disease[4]. Next generation sequencing techniques and multi-omics approaches have dramatically expanded our knowledge of the intestinal microbial world[5].

The gut microbiota, comprising the trillions of bugs inhabiting the gastrointestinal tract, may be considered a complex bioreactor with several metabolic and immunological effects that extend beyond

the gut itself[6]. Previous studies found that intestinal flora disturbance may lead to the occurrence or aggravation of coronary heart disease (CHD) to a certain extent[7]. Atherosclerotic heart disease is not only a lipid disorder but also a chronic inflammatory disease [8]. The formation and rupture of atherosclerotic plaque are related to high levels of gut microbiota-derived lipopolysaccharide (LPS) and inflammatory cytokines in the circulation[9]. Moreover, Trimethylamine (TMA) and TMA N-oxide, which are gut microbiota metabolites of dietary phosphatidylcholine, are known to be associated with cardiovascular disease and the atherosclerosis process in particular[10, 11].

At present, a large number of studies have found that there is a serious systemic or local inflammatory response in ACS, and the degree of inflammatory reaction is related to the occurrence of coronary events. With the aggravation of inflammatory reaction, it may increase the occurrence of myocardial infarction and sudden death caused by coronary plaque rupture and thrombosis.

The changes of intestinal flora and inflammatory factors in ACS and their relationship are not clear in the north-western parts of China, so this study was to explore the changes of intestinal flora and immune inflammatory factors in ACS such as acute myocardial infarction (AMI) and unstable angina pectoris (UAP).

To address this, in this study, fecal samples from ACS patients and healthy controls were collected, variable regions of gut bacterial 16S rRNA were amplified, and DNA library was constructed. Then, high-throughput sequencing was used to assess the taxonomic composition of the gut microbiota in ACS patients. The data of this study may provide a theoretical basis of the development and progress of ACS and intestinal flora

Results And Discussion

Study population.

Comparison of general data between the HC and ACS group. There were no statistically significant differences in age, sex, previous history of basic diabetes between the HC and the ACS groups ($P>0.05$), and the data were comparable (Table 1). A total of 66 consecutive cardiology patients were enrolled according to the exclusion criteria, the remaining 66 were divided in three groups for analysis: an ACS group, including 31AMI patients, 29 UAP patients and 6AS patients; and a control group comprising 46 healthy volunteers, no evidence of enrollment bias was found.

Table 1

Comparisons of clinical characteristics between ACS patients and controls. BMI, Body mass index; AST, Aspartate aminotransferase; ALT, alanine aminotransferase; CHOL, cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; CRP, C reactive protein; Cre, creatinine; WBC, white blood cell; Glu, serum glucose. Data were shown as mean \pm SD. Differences between two groups (AMI vs. HC, UAP vs. HC, AS vs. HC) were compared using Fisher's exact test for sex;

two-sample t-test for numerical data with normal distribution; Mann-Whitney U test for numerical data without normal distribution. A value of $P < 0.05$ was considered statistically significant. * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$.

Variables	HC (n=46)	AMI (n=31)	UAP (n=29)	AS (n=6)
Age, y	35.3±11.1	55±11.6	58.1±4.8	56.8±12.4
Sex, male	27(46)	25(66)	22(66)	6(66)
BMI, kg/m ²	21.43±2.5	25.2±2.6***	24.9±3.5***	25.7±2.9**
AST, U/L	18.64±4	116.2±105***	31.7±23.9	26.8±10.8
ALT, U/L	17.9±8.6	46.5±22.1***	45.4±33.7***	42.6±18.5**
CHOL, mg/dL	4.3±0.7	4.1±0.9	3.8±0.2	1±0.2
TG, mg/dL	1.2±0.6	1.8±1.1*	2.2±1.4***	1.7±1.2
HDL-C, mg/dL	1.4±0.3	0.9±0.2***	1.8±0.2***	1±0.2***
LDL-C, mg/dL	2.3±0.6	2.2±0.5	1.9±0.6*	2.3±0.5
CRP, mg/dL	0.9±0.8	5.3±3.2	1.5±1.3	2.1±1.9
Cre, μmol/L	54.4±7.3	64.5±15.7**	71±14***	72±14**
WBC, 10 ⁹ /L	6.6±1.4	9.8±2.7***	7.1±2.4	6.6±1.5
Glu, mmol/L	4.7±0.4	5.8±1.4***	5.3±1.2*	4.9±0.5

Characteristics of study participants.

The baseline characteristics of UAP patients, AMI patients, AS patients and healthy controls were shown in Table 1. Compared to healthy controls, parameters in ACS including body weights (BWs), low-density lipoprotein (LDL)(Fig1), triglyceride (TG), total cholesterol (TC), C-reactive protein (CRP) and high homocysteine (HCY) were elevated; whereas high-density lipoprotein (HDL) were decreased.

Gut microbial profile in patients with ACS and HC

diversity index analysis

Shown in Figure 2.A and B, with the increase of sample sequencing depth, the observed species index curve tended to be flat, indicating that the current sequencing depth is sufficient to detect predominant species contained in each sample. The abundance was reflected by the length of the curve on the horizontal axis and the evenness was reflected by the shape of the curve. After analyzing the rank-

abundance curve about OTU of the samples, we found a smooth curve, indicating high evenness among samples. The gut microbiota of HCs and patients with ACS showed consistent values in the analysis of observed species and Shannon diversity index. Figure 2C represented species diversity and indicated significant differences in the number of species among AS-HC ($p=0.0000$), HC-UAP ($p=0.0000$) and AMI-HC ($p=0.0000$). There was no significant difference in the number of species among AMI-AS ($p=0.7204$), AMI-UAP ($p=0.792$) and AS-UAP ($p=0.8776$). It was suggested that there were significant differences between the disease group (AMI, UAP, AS) and the healthy group. There was no difference in comparative analysis between disease groups. Figure 2D representing the Shannon index found the significant differences in HC-UAP ($p=0.0000$), AMI-HC ($p=0.0001$) and AS-HC ($p=0.0291$). There was no significant difference in Shannon index between AS-UAP ($p=0.5457$), AMI-AS ($p=0.7100$) and AMI-UAP ($p=0.7232$). Principle Coordinate Analysis (PCoA) (weighted UniFrac distance) between the ACS and control groups showed distinguished in total (Fig. 2C), top 40 abundant stool microbial taxa, and overlapping clustering (Fig. 2D) was observed in AMI, UAP and AS groups.

β diversity analysis

Based on unweighted UniFrac distance and Bray-Curtis distance matrices of the 16S rRNA sequence, samples contribution rates of the first pCoA(PC1), second pCoA(PC2) were 21.45% and 9.25%, respectively, which highlighted a clear clustering of the microbial populations of the ACS patients away from that of the healthy controls. (A) NMDS (B) showed that the ACS and control groups were distinguished by total (Fig. 3A) or top 40 (Fig. 3B) abundant stool microbial taxa (stress=0.16, Stress<0.2), and overlapping clustering was observed in AMI, UAP and AS groups.

OUT analysis

A Venn diagram (Fig. 4) showed that 789 OTUs were commonly detected between HC and AMI, common OTUs in the two groups of HC and UAP were 788, 632 OTUs were generally recognized between HC and AS, in addition, 596 OTUs were commonly detected in the four groups, while 450, 91, 144 and 20 OTUs were unique in the HC, AMI, UAP, or AS, respectively.

The ACS and HC samples were significantly different in multivariate analyses. At the phylum level (Fig. 5), compared to healthy controls, the dominant stool microbes in the ACS group were *Firmicutes*, *Bacteroidetes*, *Proteobacteria*. The relative abundances of *Bacteroidetes*, *Proteobacteria*, and *Verrucomicrobia* were increased, while *Firmicutes* was decreased in the ACS group compared to the HC group ($p<0.05$). At the genus level (Fig. 6), compared to HC group, the levels of *Bacteroides*, *Parabacteroides*, *Unidentified Enterobacteriaceae*, *Subdoligranulum*, *Akkermansia*, *Alistipes*, *Streptococcus*, *Paraprevotella* and *Paraprevotella* were significantly increased, whereas *Subdoligranulum*, *Roseburia*, *Faecalibacterium*, *Blautia*, *Agathobacter*, *Bifidobacterium* and *Anaerostipes* were significantly reduced.

Further analysis of the vegetation of the AMI, UAP, AS and HC healthy groups using the network diagram showed that there are significant differences in four groups (Fig.7). Compared with the entire HC group, the dominant ACS bacteria (AMI, UAP) in *Bacteroides*, *Parabacteroides*, *Unidentified_Enterobacteriaceae* and *Subdoligranulum* were increased significantly. *Akkermansia*, *Anaerostipes*, *Blautia*, *Agathobacter* were decreased significantly. The abundances of *Alistipes*, *Streptococcus* and *Paraprevotella* were also higher in ACS than those in control samples.

Analysis of inflammatory factors

Compared with the HC healthy group (Fig. 8), the inflammatory factors including TNF- α ($P=0.049$), MCP-1 ($P=0.046$), IL-6 ($P=0.047$), IL-1 β ($P=0.035$), IL-10 ($P=0.049$) and LPS ($P=0.0001$) in ACS group (AMI group and UAP group) were significantly increased, suggesting that there were significant changes in inflammatory factors in ACS.

Correlations of changes in clinical indexes, inflammatory factors with alterations in intestinal flora

According to the correlation between clinical acute coronary syndrome and clinical correlation index and flora inflammation, we further analyzed the correlation between clinical related indexes such as age (Age), body mass index (BMI), alanine aminotransferase (ALT), hyperhomocysteine (HCY) and differential bacteria (Fig. 9A). The analysis of the relationship between age and differential bacteria found that age was negatively correlated with *Firmicutes*, but positively correlated with *Bacteroidetes*, *Proteobacteria* and *Verrucomicrobia*, suggesting that under disease conditions, the increase of age had obvious influence on the disorder of microflora. BMI was negatively correlated with *Firmicutes* and positively correlated with *Bacteroidetes*. In the disease condition, changes in body weight affected microbial changes. ALT was negatively correlated with *Firmicutes* and positively correlated with *Bacteroidetes*, suggesting that liver function damage was closely related to the bacterial community. HCY was positively correlated with *Proteobacteria* and *Bacteroidetes*, and negatively correlated with *Firmicutes*. There was a correlation between HDL-C and different bacteria. HDL-C is positively correlated with *Firmicutes*, negatively correlated with *Bacteroidetes*, and positively correlated with F/B. There was no statistical difference in the correlation analysis between LDL-C and bacteria. It may be caused by the small number of cases, drug intervention and other related interfering factors. TG was negatively correlated with *Firmicutes* and positively correlated with *Bacteroidetes*. It was suggested that age, weight, liver function damage and HCY sepsis in AMI and UAP patients can affect the distribution of microflora.

The relative abundance of *Firmicutes* was positively correlated with plasma inflammatory factors IL-1 β , TNF- α , MCP-1 and LPS (Fig. 9B). The abundance of *Bacteroides* was negatively correlated with IL-1 β , TNF- α , MCP-1 and LPS; *Akkermansia* was positively correlated with IL-10, and there was no significant

difference in the correlation between IL-6 and *Firmicutes* and *Streptococcus*, suggesting that intestinal flora and inflammatory indicators interfered with each other and were closely related in ACS.

Awareness increases markedly regarding the involvement of gut microbes in the development of numerous cardiometabolic disease[12-14]. Growing evidences have shown that intestinal microflora plays an important role in the development of coronary heart disease.[15] The intestinal flora of healthy people was dominated by beneficial bacteria, with fewer harmful bacteria ,these two kinds of bacteria were dynamically balanced to maintain the health of the host. However, in abnormal conditions, the intestinal microbiota could be significantly changed by reducing beneficial bacteria and increasing harmful flora. [16] Unbalanced intestinal microflora could further worsen the disease, leading to a vicious circle. Studies have shown that intestinal microflora imbalance was closely related to infectious diseases, inflammation and metabolic diseases. The imbalance of intestinal microflora can lead to the disorder of bacterial structure and destroy the basic metabolic process of the host, which may be closely related to the occurrence of cardiovascular diseases such as coronary heart disease, hypertension and heart failure[17-20].

A large number of studies have shown that intestinal flora was involved in the process of atherosclerosis, but the specific regulatory role was unknown. Recent macroeconomic studies displayed the expression profile of intestinal microorganisms in patients with coronary heart disease, but the efficacy of specific intestinal flora in the prevention and treatment of coronary heart disease were still unclear [21].The purpose of this study was to explore the changes of bacterial diversity among ACS(AMI, UAP), AS patients and healthy controls. The results showed that compared with healthy group, there were significant flora changes in acute myocardial infarction and unstable angina pectoris, and there were also flora changes between AS and HC healthy controls, which were consistent with previous studies[22]. It was also supported that there were significant differences in intestinal flora in patients with atherosclerosis. At the same time, there was no difference between AMI and UAP, AMI and AS, UAP and AS, which may be related to clinical complexity, such as multiple unknown risk factors, drugs and so on.

In this study, dilution curve, rank-abundance, species richness, shannon index, PCoA and NMDS were separately used to analyze the diversity of different samples and species. The results showed that there were obvious changes in AS vs. HC, HC vs. UAP and AMI vs. HC flora, supporting that the flora distribution of coronary atherosclerotic heart disease and AS patients was significantly different from that of healthy controls[23].

In this study, the common and unique OUTs among diverse groups were obtained from the Wayne diagram (VennGraph). There were 789 species of common bacteria in AMI and HC healthy groups, 368 species of endemic bacteria in AMI, 788 species of endemic bacteria in UAP and HC healthy groups, 414 species of endemic bacteria in UAP, 632 species in healthy groups of AS and HC, and 182 species of endemic bacteria in AS. There were 596 species of healthy bacteria in AMI, UAP, AS and HC. It was further suggested that there were significant differences in flora between the disease group and the healthy group.

Further analysis on the flora composition at the phylum and genus level, compared with the healthy group, at the phylum level: *Firmicutes*, *Bacteroidetes* and *Proteobacteria* were the main dominant flora, the relative abundance of *Firmicutes* was decreased significantly, the relative abundance of *Bacteroidetes* was increased significantly, the F/B ratio was decreased, and the relative abundance of *Verrucomicrobia* was increased significantly. At the genus level, there were also flora differences among the four groups. Compared with the HC healthy group, ACS (AMI, UAP) analysis showed that *Bacteroides*, *Unidentified_Enterobacteriaceae*, *Subdoligranulum*, *Alistipes*, *Streptococcus*, *Akkermansia* and *Parabacteroides* were increased significantly, *Subdoligranulum*, *Roseburia*, *Faecalibacterium*, *Blautia*, *Agathobacter*, *Anaerostipes*, *Bifidobacterium* were decreased significantly. Butyrate-producing bacteria played a key role in human health, and these bacteria, including *Roseburia*, *Subdoligranulum* and *Faecalibacterium*, were relatively depleted in atherosclerotic cardiovascular disease (ACVD) and type 2 diabetes (T2D) samples[24]. The butyrate-producing bacterium *Roseburia* was inversely correlated with atherosclerotic lesion development in mice, and the addition of *Roseburia* in combination with a high-fiber diet reduced the sizes of atherosclerotic plaques in the aorta. *Faecalibacterium* was an anti-inflammation-associated bacterium that produced butyrate. Treatment with atorvastatin increased the abundance of *Faecalibacterium* in 27 hypercholesterolemic patients compared with that found in 15 untreated hypercholesterolemic patients[25]. The results of this study were consistent with the results of Emoto et al.[26] using terminal restriction fragment length polymorphism (T-RFLP) and 16s rRNA to study the differences of intestinal microorganisms between patients with coronary heart disease and healthy volunteers in 2016. The results showed that the number of mature *Lactobacilli* increased significantly, but *Bacteroides* (*Bifidobacterium* and *Proteus*) were notably decreased in patients with coronary heart disease. In addition, the ratio of *Firmicutes* to *Bacteroides* was increased significantly. The results were consistent with the results reported by Karlsson et al in 2012 [21] using genome-wide sequencing to determine the possible link between changes in intestinal microflora and atherosclerotic heart disease. Compared with healthy people, the number of *Escherichia coli* was increased, while numbers of *Rosella* and *Eubacterium* were decreased.

Inflammation was an important process of myocardial infarction, which was caused by the release of cytokines and activation of the immune system in the injured myocardium[27-29]. In the first 24-72 hours after myocardial infarction, cardiomyocyte injury triggered the activation of macrophages mediated by damage-associated molecular patterns (DAMPs), which secreted pro-inflammatory cytokines including IL-1 β , IL-6 and TNF- α . The cascade release of inflammatory factors aggravated fibrosis, microvascular and myocardial dysfunction. In this study, when intestinal flora disturbance occurred in ACS patients, the intestinal mucosa would be damaged to lead to excessive inflammatory response with elevated serum levels of CRP, IL-6, MCP-1 and TNF- α , which may contribute to the pathogenesis of atherosclerotic diseases. At the same time, we analyzed the presence of intestinal flora disorder in ACS. At present, a large number of studies have shown that inflammatory factors were closely related to intestinal flora disorder. Therefore, this study analyzed the relationship between inflammatory factors and intestinal flora, especially the correlation between differential bacteria and inflammatory factors including IL-1 β , IL-6, TNF- α , MCP-1, IL-10, LPS. The results showed that the relative abundance of *Firmicutes* was positively

correlated with plasma IL-1 β , TNF- α , IL-6, MCP-1 and LPS, while the relative abundance of *Bacteroidetes* was negatively correlated with inflammatory factors IL-1 β and TNF- α . The abundance of *Bacteroides* was negatively correlated with IL-1 β , TNF- α , MCP-1 and LPS, while *Akkermansia* was positively correlated with IL-10, revealing that the disturbance of intestinal flora and inflammatory indexes were closely correlated with each other in myocardial infarction. Interestingly, increases in both pro-inflammatory and anti-inflammatory factors suggested that there were dynamic changes of inflammation in AMI and UAP.

Conclusions

Changes of intestinal bacterial microbiota in ACS patients might be important factors affecting the degree of metabolic disorder, which might be one of the important reasons for exacerbating clinical outcome and disease progression in ACS patients. The treatments with the rectification of intestinal flora disturbance and the reduction of inflammatory response may potentially contribute to the control of the disease.

Methods

Study population

A cohort composed of 66 patients with ACS was recruited to the Department of Internal Medicine of the Heart Center in Hospital of Ningxia Medical University from April 2019 to October 2019 consecutively, and 30 healthy volunteers were recruited from the hospital health examination center during the same period. The subjects were Chinese residents aged 18-70 years, convenience sample is defined as a non-probability/non-random sample of subjects nearest and most available to participate in this study. Inclusion criteria were diagnosed of UAP or STEMI, defined as follows: TEMI diagnostic criteria. cardiac troponin (cTn) I/T > the upper limit of the normal reference value or creatine kinase isoenzyme > the upper limit of the normal reference value; electrocardiogram (ECG) showed ST segment elevation on 2 or more adjacent leads; and one or more of the following: persistent ischemic chest pain, abnormal segmental wall motion upon ECG, and abnormal coronary angiography. UAP diagnostic criteria. cTnI/T negative; ischemic chest pain; and either transient ST-segment depression / low-level T-wave or inverted, rare ST-segment elevation upon ECG. Exclusion criteria: history of organic digestive system or digestive tract surgery; history of stroke, diabetes, kidney disease or respiratory diseases; history of alcohol abuse; infection within one month of the study or the use of a probiotic, antacid, antibiotic, or antibiotic preparation.

Ethical considerations

The study protocol was approved by the Ethics Committee of Hospital of Ningxia Medical University (2020-527) and all participated subjects provided signed informed consent. All procedures performed in

studies relating to human participants were in accordance with the ethical standards of the Helsinki declaration and its later amendments or comparable ethical standards.

Specimens

Fasting blood specimens and fresh stool specimens were collected and centrifuged at 4 °C, 3000xg for 10 min, and the supernatant (serum, plasma) was frozen and stored at -80 °C for testing. The morning fresh fecal specimens (>300 mg) were collected from the AMI group, UAP group, AS group and the healthy control group, sealed and transported to the sample bank at 4 °C. Fecal specimens (300 mg) were placed into a sterile externally-circulated cryotube, and then sealed and placed in a refrigerator at -80 °C for storage.

Determination of Plasma Inflammatory Indicators

Plasma and ovarian inflammatory cytokines including IL-1 β , IL-6, IL-10, monocyte chemoattractant protein-1 (MCP-1) and TNF- α were respectively measured by ELISA kits according to the manufacturer's instructions (Shanghai Jianglai Biotech, Shanghai, China). The sensitivities of the assays were 0.1, 1.0, 0.1, 1.0, and 0.1 pg/mL for IL-1 β , IL-6, IL-10, TNF- α , and MCP-1, respectively. Each sample was tested in triplicate. The plasma LPS levels in each group were examined using a limulus amoebocyte lysate kit (Xiamen Bioendo Technology Co, Ltd, Xiamen, China) according to the manufacturer's instruction. Briefly, 50 μ l of diluted plasma (1:4 dilutions with endotoxin-free water) was dispensed to each well in a 96-well plate. At the initial time point, 50 μ l of the limulus amoebocyte lysate reagent was added, respectively. The plate was incubated at 37°C for 30 min. Then, 100 μ l of the chromogenic substrate warmed to 37°C was added to each well, and incubation was extended for an additional 6 min at 37°C. The reaction was stopped by adding 100 μ l of 25% solution of glacial acetic acid. Optical density at 545 nm was measured with a microplate reader (Thermo Scientific, USA).

Gut microbiota analysis

Extraction of bacterial DNA by cetyltrimethylammonium bromide (CTAB) was performed by adding the appropriate amount of lysozyme and sample to 1,000 μ l CTAB lysate. The mixture was placed in a 65°C water bath and mixed by inversion several times in order to facilitate the complete lysis of the sample. Next, phenol (pH 8.0), chloroform, and isoamyl alcohol were added to the supernatant after centrifugation so that the ratio of the three was 25:24:1, with mixing by inversion and centrifugation at 12,000 \times g for 10 min. In the same way, chloroform and isoamyl alcohol (24:1) were added to the obtained supernatant, followed by centrifugation. The collected supernatant was added with isopropanol. The mixture was precipitated at -20°C after shaking up and down. Then, the mixture was centrifuged again according to the previous centrifugation conditions. The obtained precipitate was washed twice with 1 ml 75% ethanol. Then, the precipitate was blown dry on a clean bench or air-dried at room

temperature. The DNA samples were dissolved in ddH₂O. If the sample was difficult to dissolve, it needed to be incubated at 55–60°C for 10 min. Finally, 1 µl of RNase A was added to the dissolved DNA sample, which was allowed to be kept at 37°C for 15 min to obtain bacterial DNA. The extracted DNA was stored at –20°C until application. The DNA sequences involving the V3 and the V4 regions of the 16S rDNA hypervariable regions were amplified by PhusionR, High-Fidelity PCR Master Mix with GC Buffer (New England Biolab, USA) using the following primers (5' to 3'): 341F-CCTAYGGGRBGCASCAG, 806R-GGACTACNNGGGTATCTAAT. The PCR product was analyzed and separated on 2% agarose gel, which was purified using the GeneJE Gel Recovery Kit (Thermo Scientific, USA). The library was constructed using the TruSeqR DNA PCR-Free Sample Preparation Kit in order to carry out Qubit quantitation and library detection. After passing the test, the library was sequenced using the illumina HiSeq 2500 platform by Beijing Novogene Technology Co., Ltd., China.

Statistical analysis.

Demographic and clinical characteristics, prognostic markers, and microbial taxa are presented as n (%) for sex and mean ± standard. Differences between groups were compared using Fisher's exact test for sex; two-sample comparison was used t-test while numerical data with normal distribution; Mann-Whitney U test was used for numerical data without normal distribution; one-way ANOVA for numerical data after adjusting for variables that varied significantly by demographic or clinical characteristics. All statistical assessments were two-tailed and considered significantly at $P < 0.05$. All statistical analyses were carried out using IBM SPSS statistical software version 26 (IBM Corp., Armonk, NY, USA) and Graphpad 8.0.

Declarations

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Not applicable.

Authors' contributions

JSB, WH, and MHY designed and wrote the paper. MHY, YLB, YN, ZH, ZXX performed research. All authors have read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

The study protocol was approved by the Ethics Committee of Hospital of Ningxia Medical University (2020-527) and all participated subjects provided signed informed consent. All procedures performed in studies relating to human participants were in accordance with the ethical standards of the Helsinki declaration and its later amendments or comparable ethical standards.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

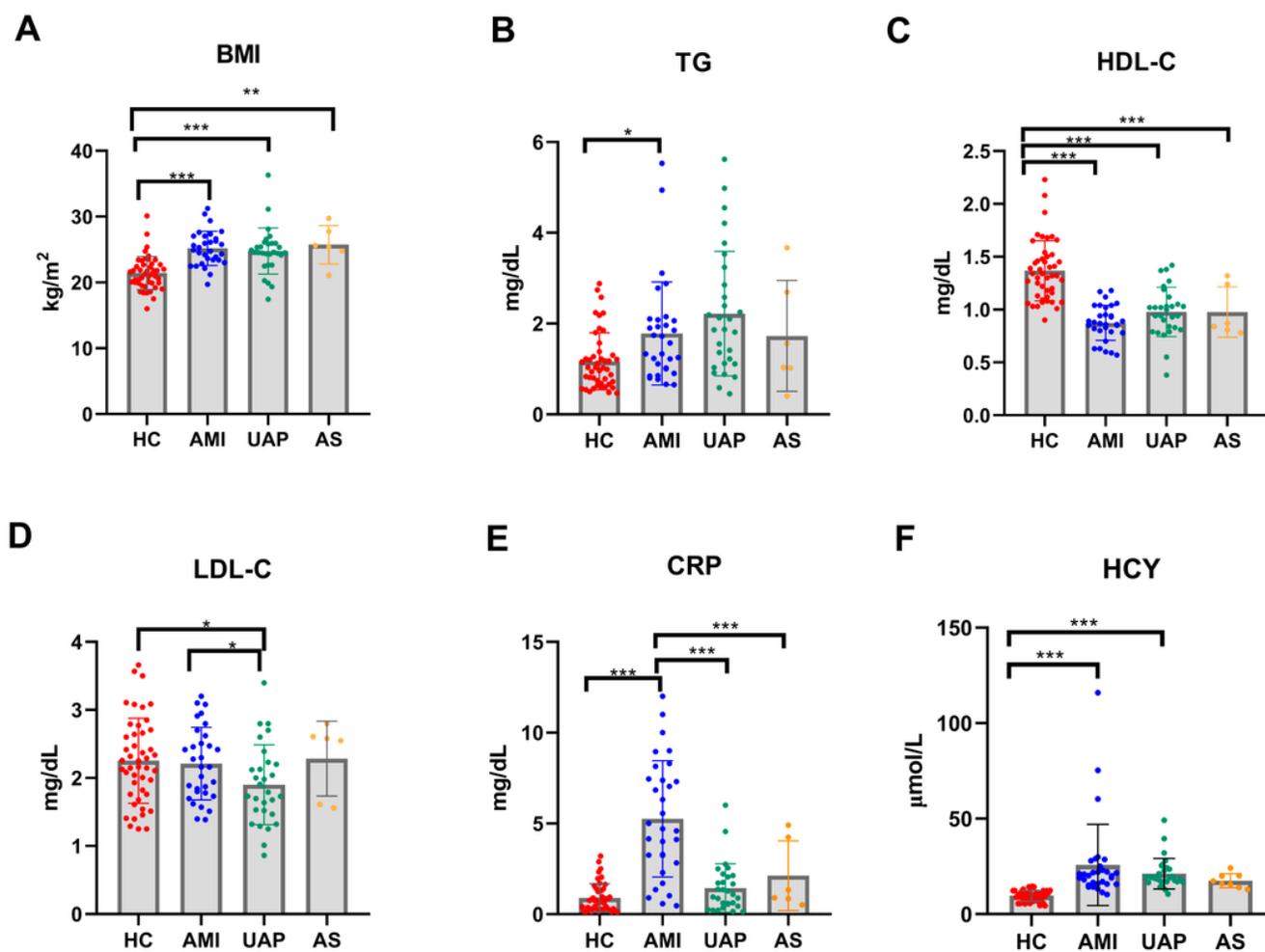


Figure 1

Clinical indexes in different groups. * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$.

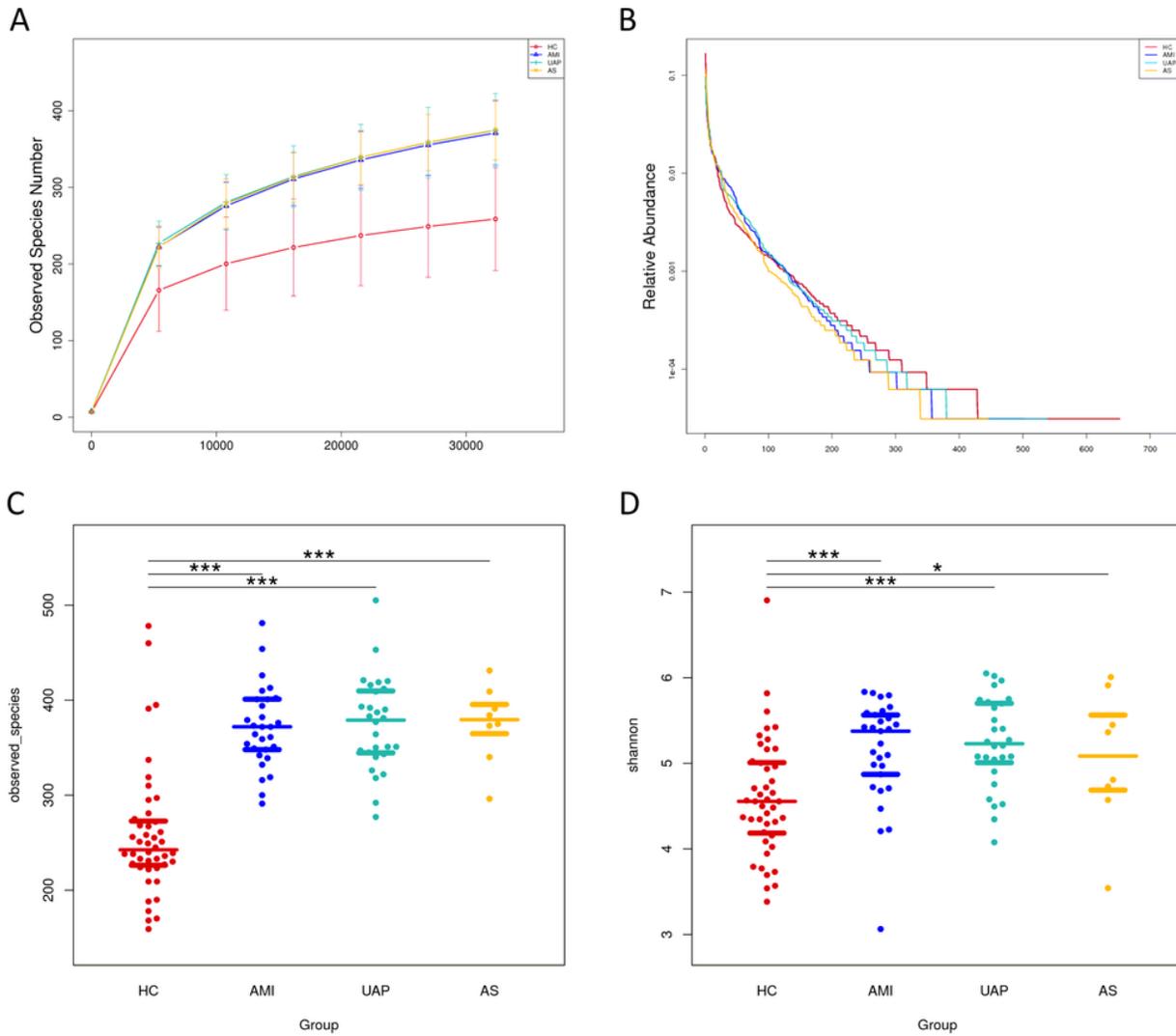


Figure 2

Gut microbial composition in stool specimens of human adults with or without ACS. (A) and (B), with the increase of sample sequencing depth. The microbial abundance and α -diversity (Shannon index) based on the profile of family and genera between (C) control and ACS groups and (D) control, UAP, AMI and AS groups. * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$.

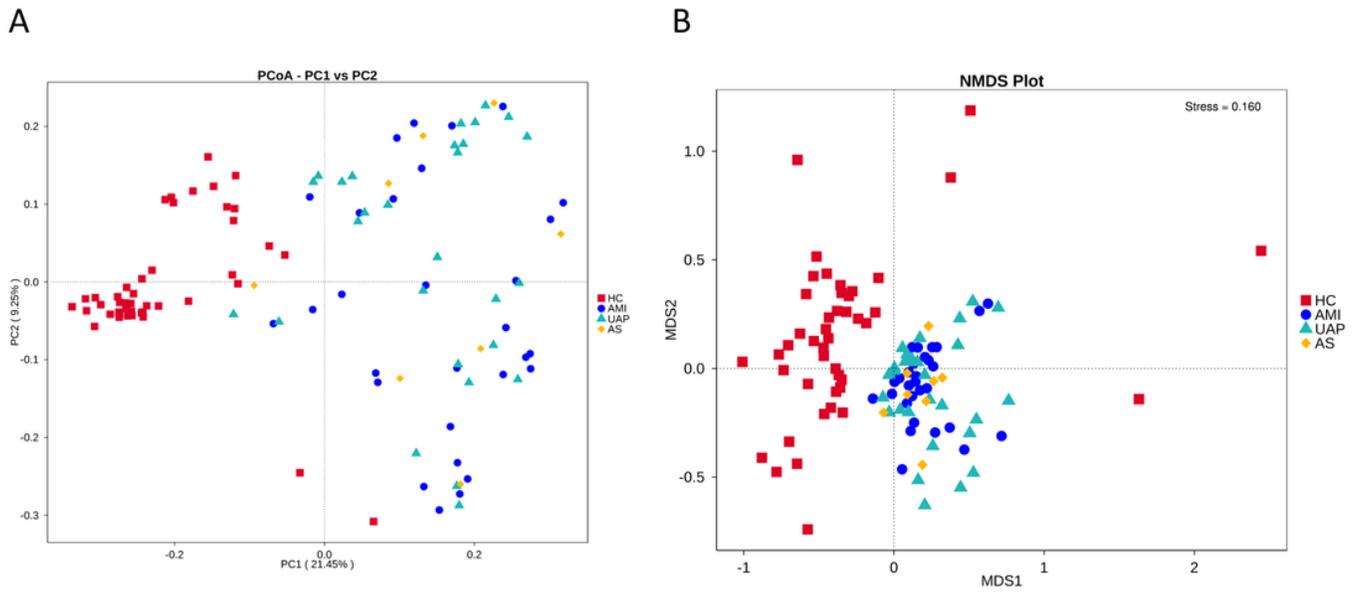


Figure 3

β diversity analysis. Principle Coordinate Analysis (PCoA) and NMDS showed that ACS and control groups were distinguished by total (A) or top 40 (B) abundant stool microbial taxa, yet overlapping clustering was observed among UAP AMI and AS groups.

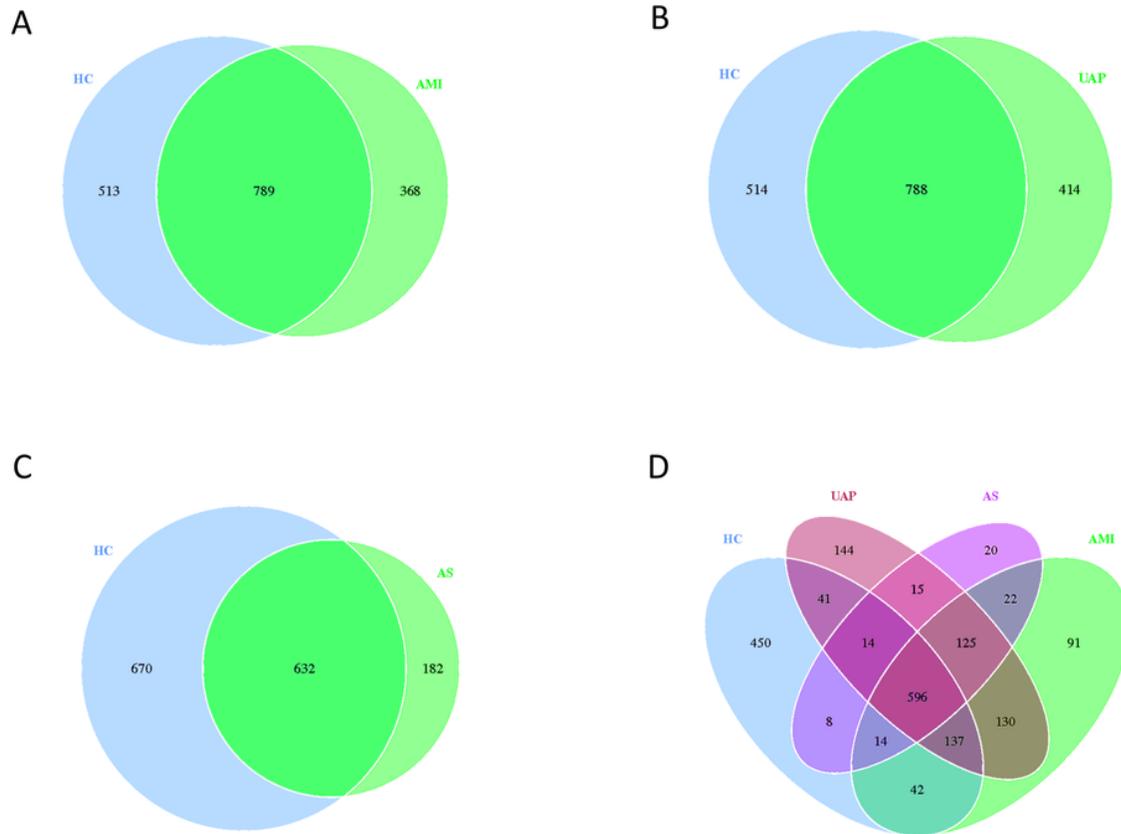


Figure 4

OUT analysis. Venn Graph analysis of common and unique OUTs between groups. (A)HC vs. AMI; (B) HC vs. UAP; (C) HCvs.AS; (D) Comparison of diverse groups.

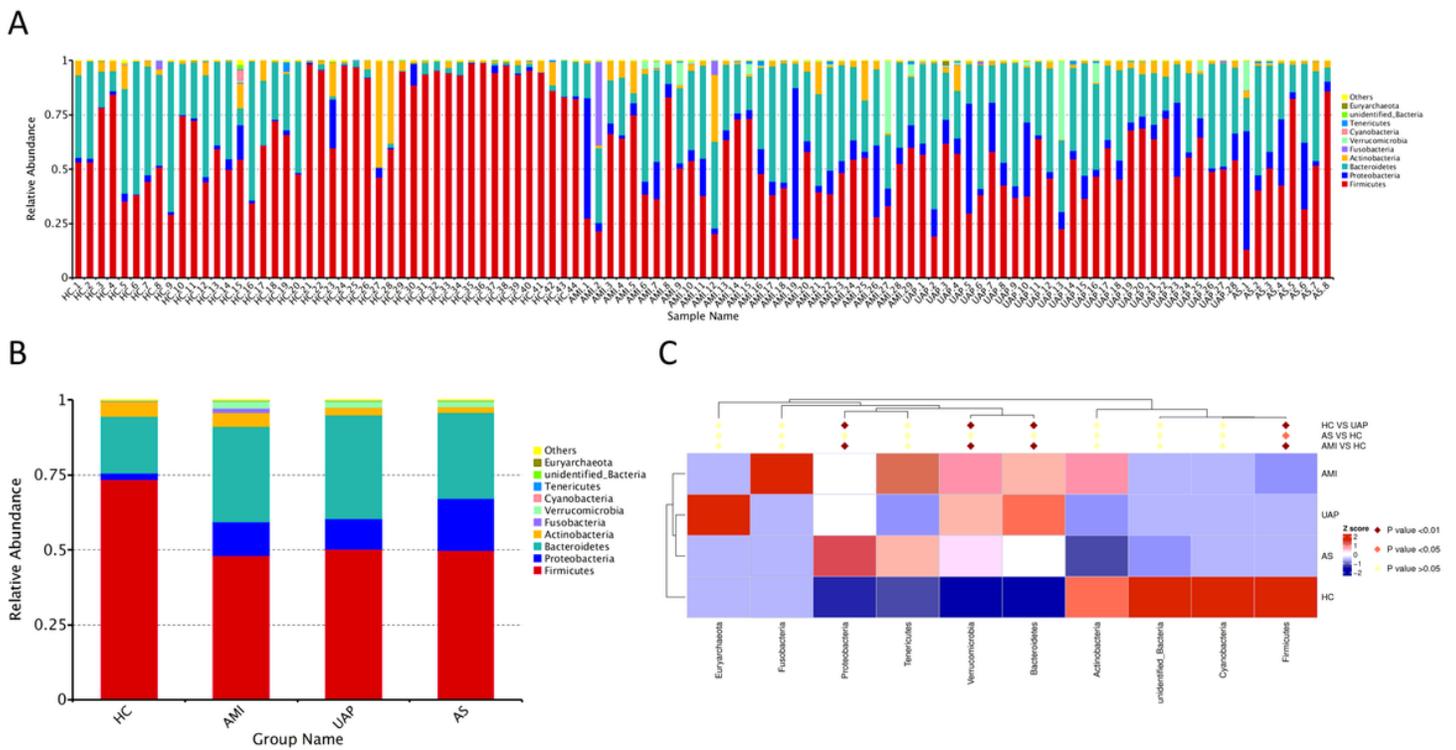


Figure 5

Relative abundance of microbial species at the phylum levels in the feces of different groups. (A) and (B) Community abundance at the phylum level. (C) A box plot of the abundance distribution of different among diverse groups at the phylum level. * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$.

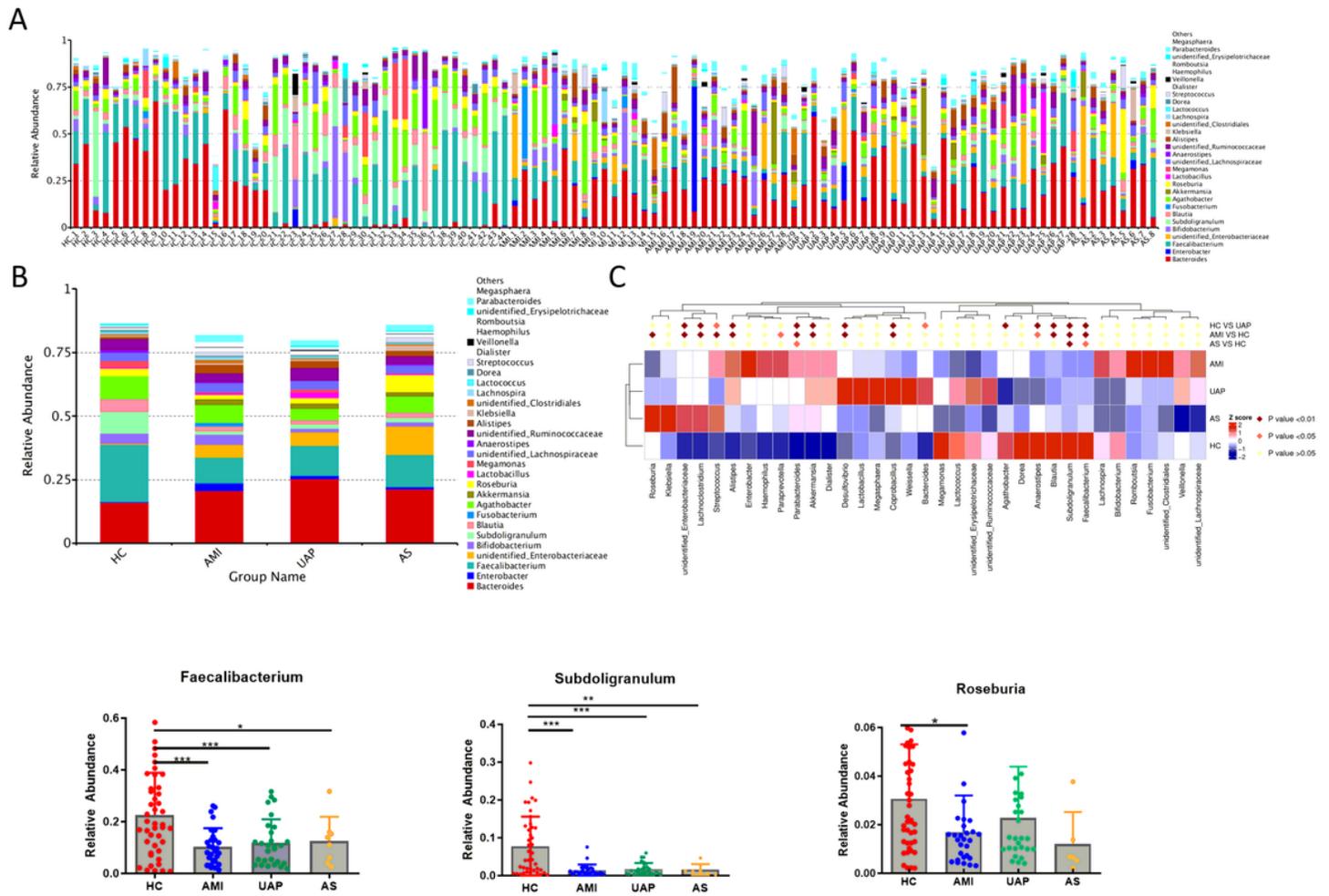


Figure 6

Relative abundance of microbial species at the genus levels in the feces of different groups. (A) and (B) Community abundance at the genus level. (C) A box plot of the abundance distribution of different species among diverse groups at the genus level. * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$.

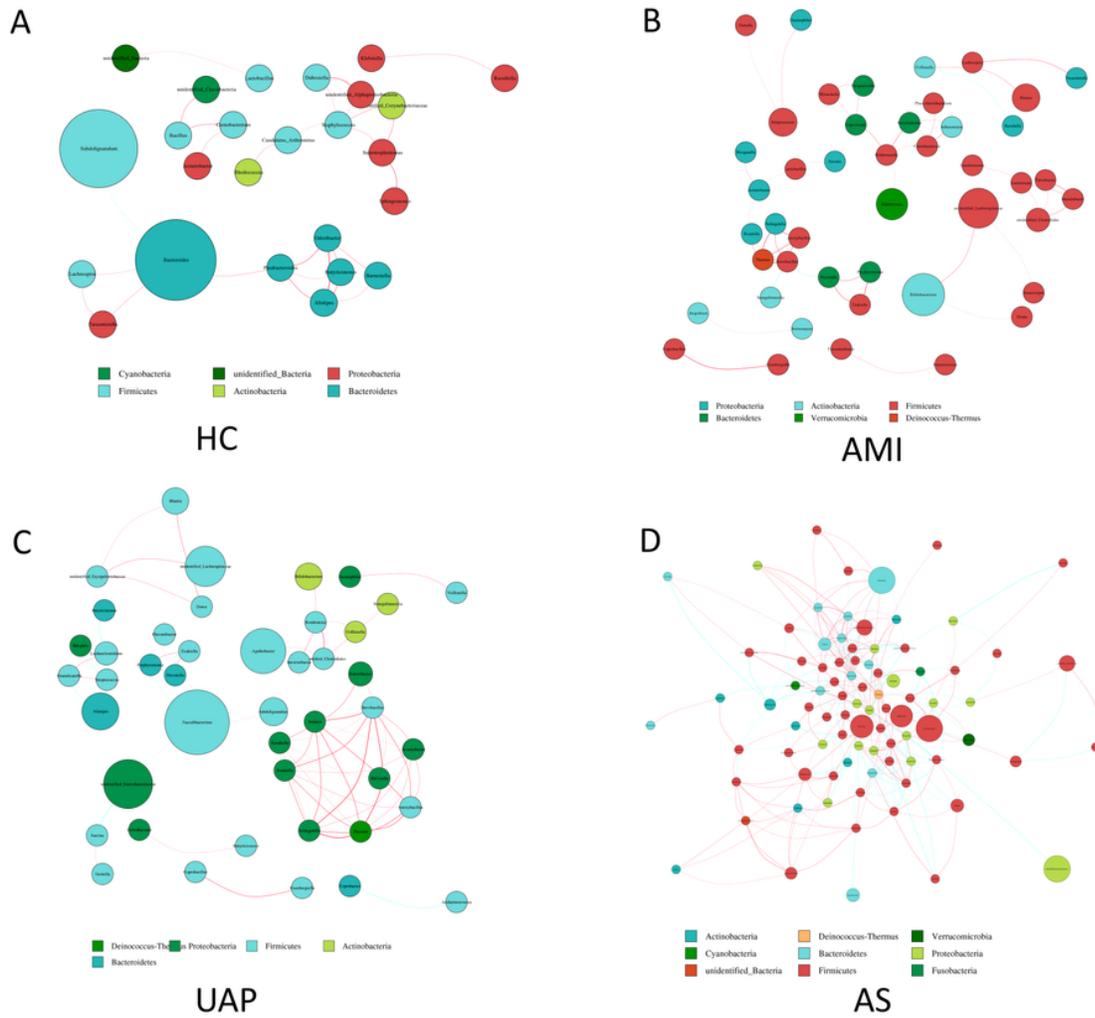


Figure 7

Network diagram at the genus level. (A) The dominant bacteria of the HC group; (B) The dominant bacteria of AMI; (C) The dominant bacteria of UAP; (D) represents the dominant bacteria of AS.

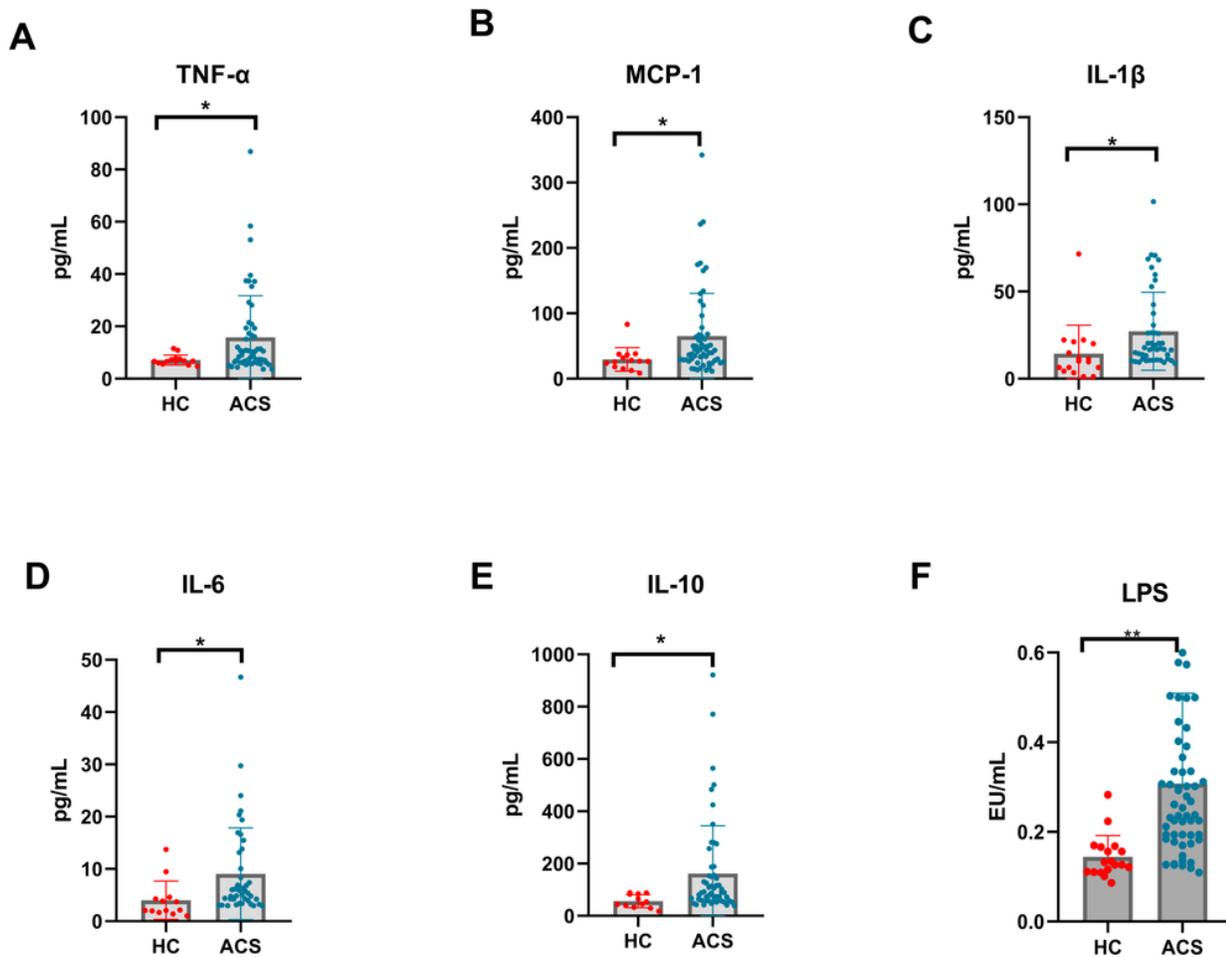


Figure 8

Determination of plasma inflammatory cytokine levels and lipopolysaccharide (LPS) in different groups. Plasma from 4 groups were respectively collected for the determination of tumor necrosis factor (TNF)- α ; monocyte chemoattractant protein-1(MCP-1); interleukin (IL)-6; IL-1 β and IL-10 concentrations using ELISA kit. Plasma lipopolysaccharide (LPS) levels in diverse groups were determined using a Limulus ameobocyte lysate kit. * $P < 0.05$.

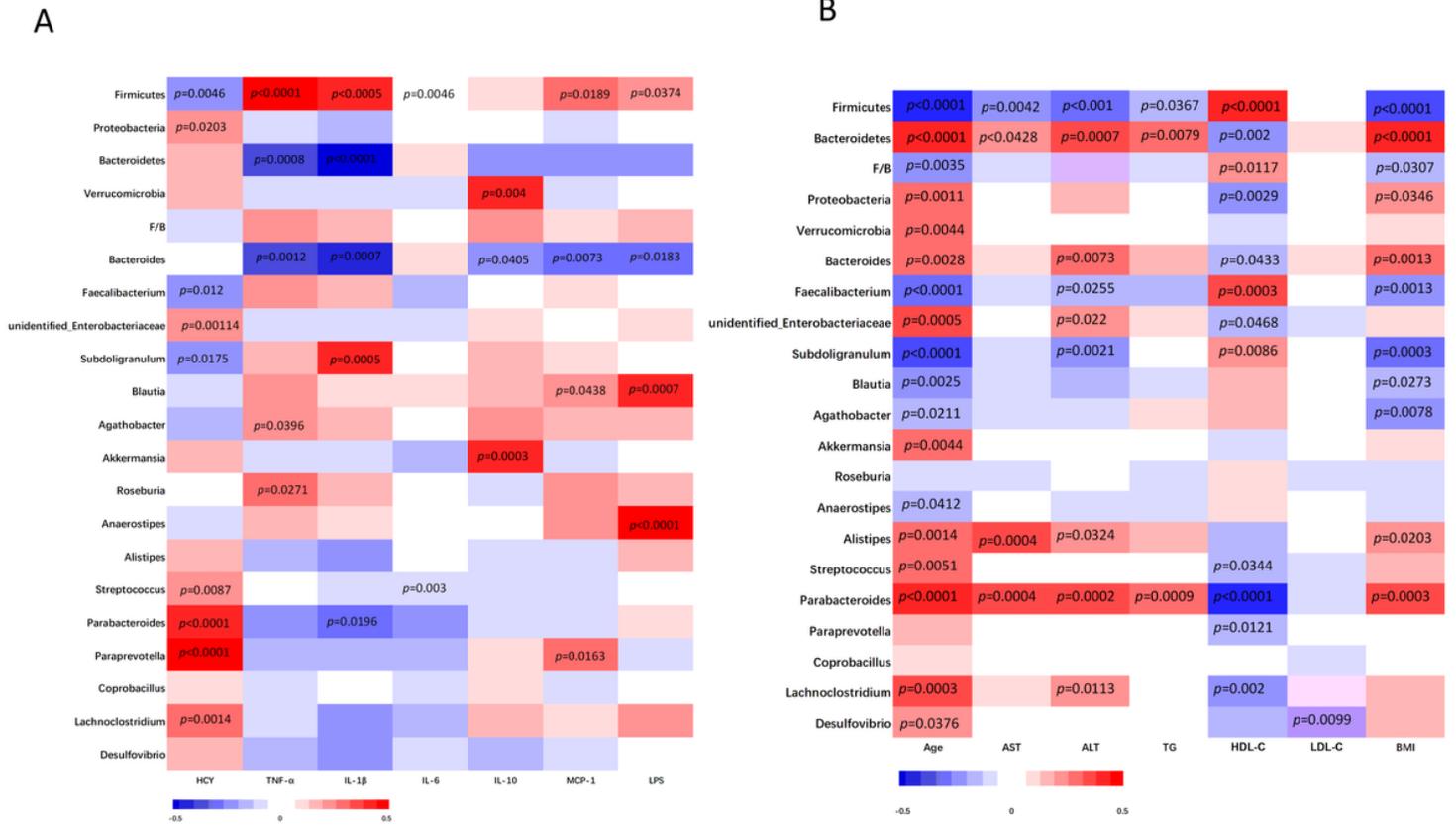


Figure 9

Correlation analysis. (A) Correlations between clinical indexes and alterations in intestinal flora. (B) Correlations of plasma inflammatory indicators with relative abundance (%) of gut microbiota. * $P<0.05$ ** $P<0.01$ *** $P<0.001$.