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Characterization of Intestinal Microbiota and Serum Metabolites in Patients with Mild Hepatic Encephalopathy

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Authors' contributions:

GY, JZ, ZP and FF carried out the experiments. MW and FL designed and conceived the study. YL wrote the paper. All authors read and approved the final manuscript.

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Keywords:

Mild micro-hepatic encephalopathy; Intestinal microbiota; Serum metabolites; Variable characteristics

1. Abstract

1.1. Introduction and Objectives: Mild Micro-Hepatic Encephalopathy (MHE) is a high complication of cirrhosis. At present, there are differences in the consistency of detection strategies and treatment directions for the MHE. The characteristic changes in intestinal microbiota and serum metabolites in the MHE patients and the possible relevant interaction mechanism would inevitably affect the developmental direction of MHE. The changes in the characteristics of intestinal microbiota and serum metabolites of MHE patients were determined, and the possible interactions between them were analyzed.

1.2. Materials and Methods: The stool and serum tests were performed on both the MHE patients and healthy individuals. 16S rRNA gene high-throughput sequencing and bioinformatics analyses were used to analyze the differences in intestinal microbiota among the MHE patients. The serum metabolites were detected using liquid LC-MS/MS (Liquid Chromatograph Mass Spectrometer) technology and the differences in the metabolic networks of blood metabolites among the MHE patients were analyzed. A comprehensive bioinformatics analysis approach was adopted to identify the composition and characteristic of microbiota and serum metabolites, and the possible correlation between them.

1.3. Results: The main characteristics of the structural imbalance in the intestinal microbiota of MHE patients included decrease in the beneficial bacteria at the level of Phylum, Class, Order, Family and Genus, increase in the pathogenic bacteria, and the imbalance in the abundance of bacteria in the intestinal microbiota. The main predicted functions that showed significant differences included chromosome, amino acid-related enzymes, methane metabolism, and arginine and proline metabolism. The detection of serum metabolites resulted in 10 different metabolites, including taurocholic acid, citrulline, D-phenyl-lactic acid, L-tyrosine, benzoate, phenylalanine, linoleic acid, eicosapedienic acid, A-dicarboxylic acid and dehydroepiandrosterone. The subsequent metabolite pathways analysis showed that there were differences in the metabolism of linoleic acid, phenyl-propane, caffeine, arginine, proline, glycine, serine, threonine, tyrosine and pyrimidine as compared to the control group. Finally, the characteristic analysis showed that the intestinal microbiota and serum metabolites might have interactive correlation at the levels of amino acids, fatty acids, bile acids and inflammation.

1.4. Conclusions: There were characteristic changes in the intestinal microbiota and serum metabolites in the MHE patients. There might be a related interaction mechanism between the two, which would provide evidence and direction for the detection and treatment strategies of MHE.

2. Introduction

MHE is a special type of Hepatic Encephalopathy (HE), with usually no obvious signs and symptoms. Its mechanism of pathogenesis is highly similar to that of the HE, but differ in progression and severity of the disease [1,2]. Recent studies have found that cirrhosis is associated with the changes in composition and function of intestinal microbiota, which may accelerate the progression of multiple complications, including infections, such as MHE, Spontaneous Bacterial Seritonitis (SBP), and renal dysfunction [3,4]. The effective intervention and control of the imbalance of intestinal microbiota is conducive to delay and prevent the occurrence and development of liver diseases [5]. Similarly, the serum metabolites and related inflammatory factors are also reported to affect the occurrence and development of MHE [6,7]. Therefore, the studies on MHE cognitive impairment point towards the changes in enterohepatic and cerebral axis, including changes in the intestinal microbiota, composition of serum metabolites, impaired immune response, and increase in the local and systemic inflammation [8,9]. This study aimed to provide relevant evidences and strategies for the clinical detection and treatment of MHE by analyzing the characteristic changes in the composition and function of intestinal microbiota and serum metabolites in MHE patients and studying their possible interactions.

3. Methods

3.1 Research Objects and Criteria

In this population-based study, the stool and blood samples were collected from the MHE patients for the characterization of intestinal microbiota and serum metabolites, respectively. After obtaining the informed consent, 16 MHE outpatients and 10 social volunteers were recruited from the liver disease clinic of the First Affiliated Hospital of Guangxi University of Traditional Chinese Medicine. The inclusion criteria included the following: (1) The patients were diagnosed with post-hepatitis B cirrhosis using any of the following diagnostic method: liver biopsy, transient elastography, evidence of varicose vein, and hepatic morphology or thrombocytopenia in patients with chronic liver disease or decompensated cirrhosis. (2) Diagnostic criteria for MHE patients conformed to the diagnostic criteria for post-hepatitis B cirrhosis: The child-pugh of cirrhosis was classified into Class A and B, where both the Number Connection Test (NCT) and Symbol Digit Test (SDT) or one of them was abnormal. (3) Healthy individuals (control group) were included in the study after complete medical history, physical examination, chest X-ray, routine hematuria, blood glucose, liver and renal function and other physical and chemical examinations if they had no disease of heart, brain, liver, kidney, and lung and in other major organ systems. (4) The ages of the patients included in this study ranged between 18-65 years, who signed the informed consent from individuals without prior TIPS (Transjugular intrahepatic portosystemic shunt). Patients with a history of cirrhosis, including those having unclear history of cirrhosis and current alcohol or drug users, including those taking antipsychotics, painkillers, older antidepressants, or benzodiazepines, were excluded. The patients having trans-jugular intrahepatic portosystem shunt recently (within the last 3 months), those treated with opioid drugs for changes recently (within the last 3 months), and those hospitalized most recently (within 1 month).

3.2 Research Methods

The diet plan of the 26 included subjects for the first three days of specimen collection was uniformly managed by emphasizing on the intake of calorie and proteins, and meat and vegetables. A 200-300 mg of fresh stool and 5 mL of fasting venous blood were collected from the subjects in the morning. Invitrogen DNA Mini Kit was used for the extraction of DNA from stool samples, which was quantified using fluorescence meter, while its integrity was tested using E-Gel electrophoresis system. The composition of intestinal microbiota was analyzed using 16S rRNA gene sequencing in two steps according to Gillevet and Hamady [10,11]. The Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathways spectrum analysis was used to select OUT sequence at 97% similarity level using QIIME software. The output script was analyzed using PICRUSt software to analyze the abundance of metabolic functions at KEGG pathway levels. The analysis of serum metabolomics was performed using the published technique, LC-MS/MS [12].

3.3 Statistical Analysis

All the data was expressed as mean±standard deviation. Levene's test, Analysis of Variance (ANOVA) and Students-News-Keuls test were used for the homogeneity of variance test, comparison between groups, and the pair-wise comparison between groups, respectively (Kruskal-Wallis H test was used for the comparison between groups with uneven variances, and Wilcoxon Rank Sum test was used for the pair-wise comparison between groups). The multi-factor non-bar logistic regression analysis and Backward stepwise selection were adopted for the simultaneous calculation of the Odds Ratio (OR) and 95% Confidence Interval (95%CI) of all the factors. The spearman's rank correlation coefficient was used for the correlation analysis of normal distribution data (SPSS22.0 statistical software was used for the correlation analysis of non-normal distribution data). $P<0.05$ was considered to be statistically significant.

4. Results

4.1 Analysis of the Intestinal Microbiota Using 16S Rrna Gene Sequencing

4.1.1 Alpha and Beta Diversity Analyses of the Intestinal Microbiota Differences in MHE Patients

The Alpha and Beta diversity analyses were used to analyze the significance of differences in microbial composition between the two sample groups. Alpha index was used to analyze the richness and diversity of microbial communities. As shown in (Figure 1 and Figure 2), the Alpha curve flattened with the increase in sample size, indicating that the sample size was sufficient to reflect the richness of the species community and other changes. The sequencing depth required for the curve of control group to reflect the richness of the species community was higher than that of the MHE group, indicating lower species richness in the MHE group. Similarly, in Table 1, both the Alpha diversities, including Chao1 and Shannon indices were reduced (P<0.01), indicating that the richness and diversity of intestinal microbiota in the MHE group decreased significantly with significant differences in the structure of individual's intestinal microbiota. The subsequent box diagram (Figure 3) and corrected P values (Table 2) also confirm these conclusions.

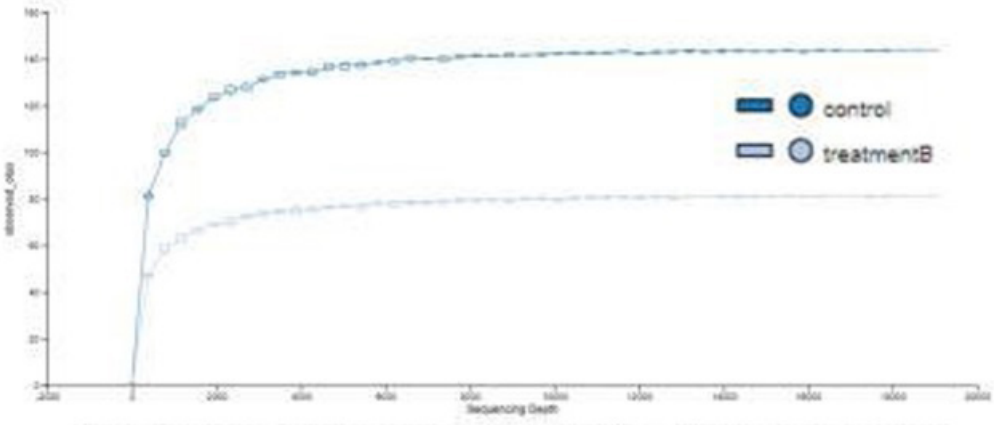

Figure 1: X-axis represents the extracted sequencing quality; Y-axis represents the corresponding Alpha diversity index value.

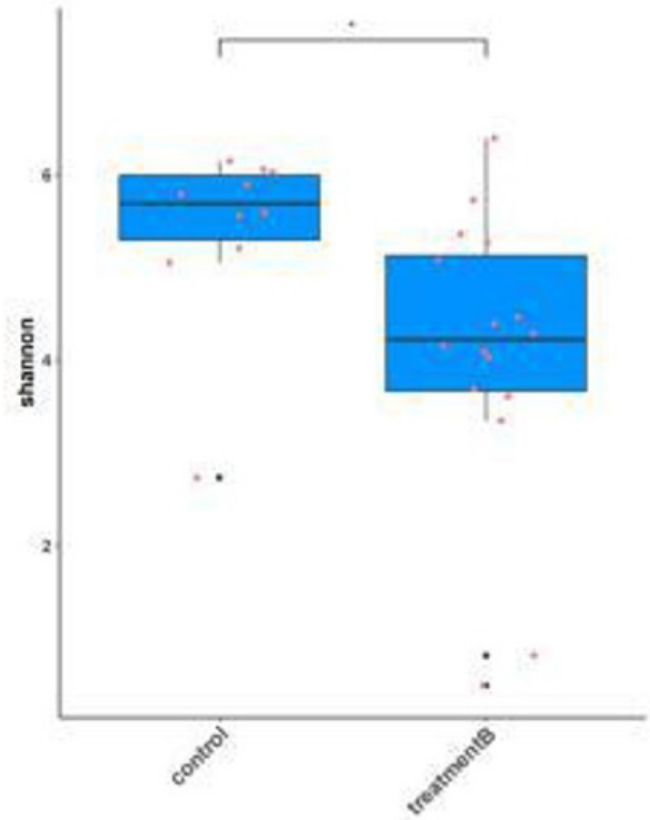

Figure 2: Multiple comparisons of Shannon index between groups
*represents p<0.05

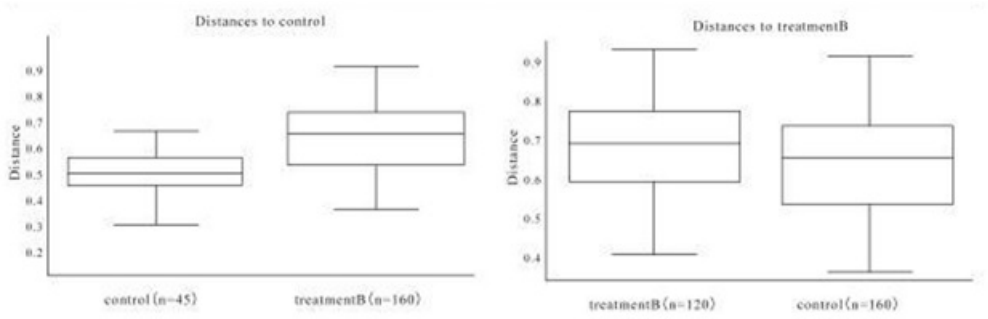

Figure 3: The boxplot shows the grouping information horizontally. The n value in parenthesis after each grouping name represents the number of comparisons between the groups, and the height of the groups between comparison samples vertically.

Table 1: Aloha index of diversification(X±S)

Group	Chao 1	Shannon
Control	149.00±44.32	5.41±1.01
Treatment B	84.53±4.01**	3.80±1.52**

Note: *P<0.05 was compared with the blank group; **p<0.01 was compared with the blank group

Table 2: PERMANOVA results of the two groups

Group 1	Group 2	Sample size	Permutations	p-value	p-value
Control	Treatment B	26	999	0.002	0.002

4.1.2 Analysis of the Differences in Taxonomic Composition of Intestinal Microbiota in the MHE

Patients at the Level of Phylum, Class, Order, Family and Genus

According to the results of species (as shown in Fig. 4), at the level of Phylum, Class, Order, Family and Genus, Firmicutes and Proteobacteria; Clostrida, Coriobacteriia and Bacilli; Lactobacillales, Clostridiales, Actinomycetales, and Coriobacteriales; *Lachnospiraceae and Coriobacteriaceae; Roseburia, Lachnospiraceae, Coprococcus, and Veillonella*, respectively showed statistically significant differences in their relative abundances between the two groups (Table 3, $P < 0.05$). In order to further explore the intestinal microbiota in the MHE patients at genus level, the top 20 OTUs were ranked in horizontal abundance, which showed the abundance of *Bacteroides, Enterococcus, Faecalibacterium, Blautia, Veillonella, Megamonas, Unspecified_Enterobacteriaceae, Unspecified_Lachnospiraceae, Roseburia, Prevotella, Lactobacillus, Ruminococcus, Gemmiger, Coprococcus, Streptococcus, Unspecified_Clostridiales, Ruminococcus, Megasphaera, Klebsiella, and Parabacteroides*.

4.1.3 Correlation Statistical Analysis of the Differences in Intestinal Microbiota

Using the above-mentioned analysis, the differences in the composition of intestinal microbiota between the MHE and healthy group were analyzed, but the correlation of the interaction among the microbiota in same individual was still unclear. Therefore, the spearman's rank analysis was conducted on the basis of the relative abundances of genus in the same sample to construct the antagonistic or cooperative species information through the network (Figure 5).

It was found that *Rothia* was significantly negatively correlated with Oscillospira, Collinsella, Parabacteroides, and Roseburia. Similarly, *Enterococcus, Ruminococcus, Ralstonia showed significant negative correlation with Blautia, Phascolarctobacterium and Actinomyces*.

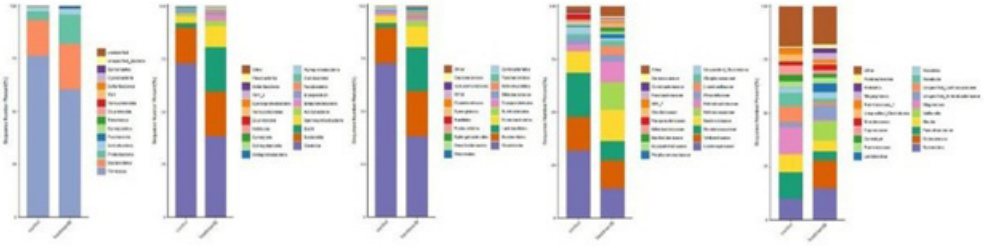

Figure 4: Show the taxonomic composition and abundance distribution of the communities at the levels of phylum, class, order, family and genu

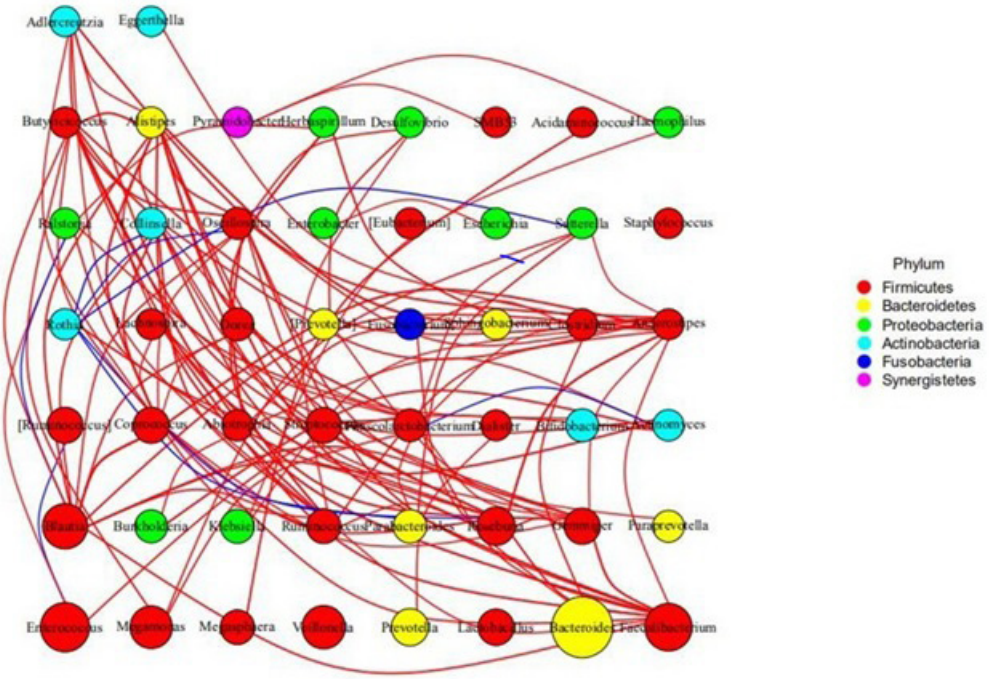

Figure 5: Each circle represents a species, the size represents the abundance, the lines are positively correlated in red, negatively correlated in blue and the thickness represents the correlation coefficient.

4.1.4 Analysis of the Function of Microbial Communities of Different Intestinal Microbiota

(Figure 6) shows the Greengenes database-based PICRUSt analysis in which the spectrum of gene functions in the species was inferred, and then the functions of the whole spectrum genes were predicted. Finally, the composition of intestinal microbiota was mapped to the KEGG database L3 bar charts. (Table 4) is based on the statistical data at the level of L3 function prediction. It can be seen that there were significant differences in the predicted function between MHE and control groups, including the differences in chromosomes, amino acids, methane metabolism related enzyme, arginine and proline metabolism ($P < 0.05$).

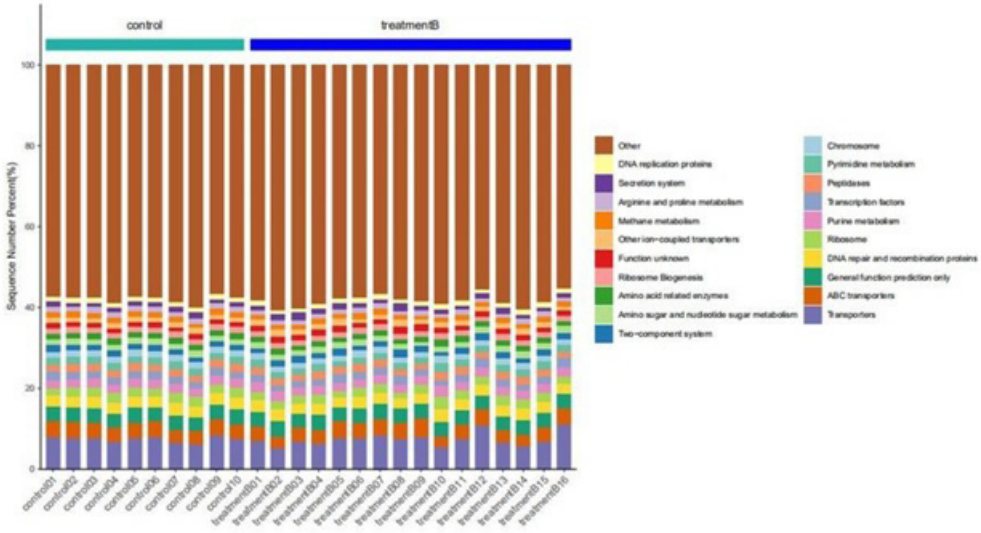

Figure 6: Bar chart composed of KEGG L3 level functional prediction

Table 3: Comparison of MHE group and control group at the level of phylum, class, order, family, and genus

Phylum	Class	Order	Family	Genus	P-value	Control		TreatmentB	
						Mean	Standard deviation	Mean	Standard deviation
Fimicutes					0.026	0.7650	0.0975	0.6051	0.2383
Proteobacteria					0.039	0.0398	0.0633	0.1365	0.1563
	Clostridia				0.00006	0.7290	0.0909	0.3837	0.2500
	Bacilli				0.03	0.0207	0.0340	0.2095	0.3160
	Coriobacteriia				0.036	0.0010	0.0086	0.0034	0.0065
		Clostridiales			0.00006	0.729	0.0909	0.3837	0.2500
		Lactobacillales			0.03	0.0205	0.0339	0.2078	0.3166
		Coriobacteriales			0.036	0.0010	0.0086	0.0034	0.0065
		Actinomyeetales			0.048	0.0002	0.0004	0.0012	0.0269
			Coriobacteriaeeae		0.036	0.0010	0.0086	0.0034	0.0065
			Lachnospiraeeae		0.009	0.3195	0.1496	0.1401	0.1640
				Veillonella	0.022	0.0005	0.0011	0.0906	0.1416
				Unspecified_ Lachnospiraeeae	0.017	0.0722	0.0642	0.0124	0.0184
				Roseburia	0.026	0.0617	0.0505	0.0172	0.0297
				Coprpepecus	0.026	0.0434	0.0398	0.0141	0.0236

Table 4: Function prediction of KEGG L3 microbial community (X±S)

Group	Chromosome	Amino acid related enzymes	Methane matabolism	Arginine and proline metabolism
Control	0.0157±0.0006	0.0148±0.0006	0.0134±0.00 11	0.012±0.0004
Treatment B	0.0148±0.0013*	0.0139±0.0016*	0.0121±0.0017*	0.0119±0.0012**

4.2 Analysis of Serum Metabolites Based on LC-MS/MS

4.2.1 Data Preprocessing

In order to ensure the reliability of data and reduce the error in measurement system, the Quality Assessment (QA), Quality Control (QC) and standardized processing were conducted on the sample data (Figure 7a and 7b). (Figure 7c) shows that the Principal Components Analysis (PCA) scores of both the groups were with 95% CI, which validated the standardization. Figure 7 shows that the distribution of the metabolite contents of the samples after standardization was close to the normal distribution, which was suitable for the subsequent PCA, Partial Least Squares Discrimination Analysis (PLS-DA), Orthogonal PLS-DA (OPLS-DA) analyses, T-test and ANOVA tests.

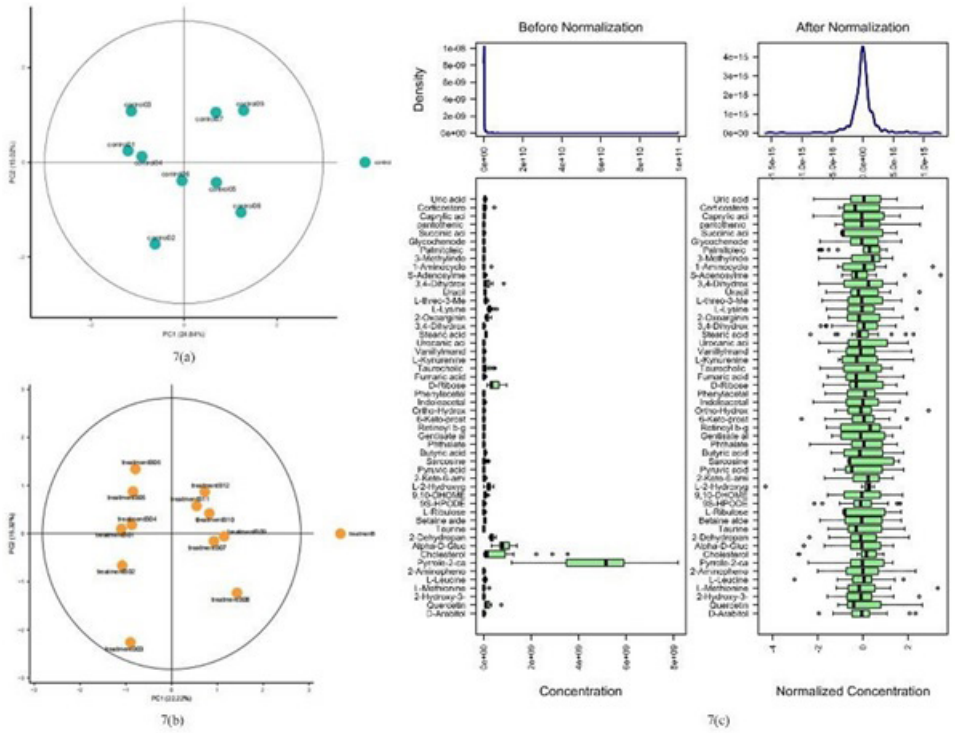

Figure 7: Shows the PCA scores of the two groups and the content distribution of each metabolite before and after standardization.

4.2.2 Differential Analysis Between MHE and Control Groups

Significant differences can be seen from the heat-maps of the clustering of metabolites between the MHE and control groups (Figure 8), among which, the clustering of metabolites, such as Pyroglutamic acid, D-ribose, Phenol and Gentamicin C1a in the MHE group were more obvious than that in the control group. The thermal metabolites, including Protoporphyrinogen IX, O-Phosphoethanolamine, Myo-Inositol and L-Carnitine, etc. were more concentrated in the control group. As the heat-maps of the clustering results could show only the rough differences in the metabolites between groups, the subsequent accurate PCA, PLSDA and OPLSDA analysis were conducted. The PCA and PLSDA point cloud map showed significant separation between the two groups, and significant differences in metabolites between the two groups (Figure 9a and 9b). In (Figure 9c), OPLS-DA permutation test Q2 was 0.87, and the actual observed Q2 indicated by the arrow is on the right side of the random distribution. The predictive ability of the model was significant, indicating significant differences in metabolites between the two groups.

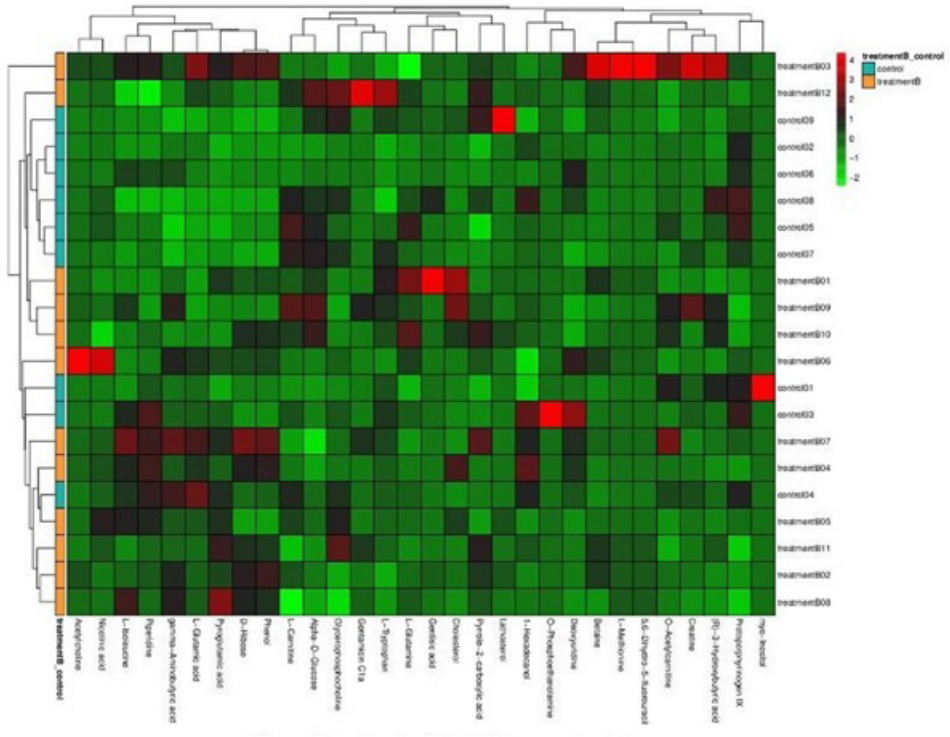

Figure 8: Results of metabolite heat map clustering

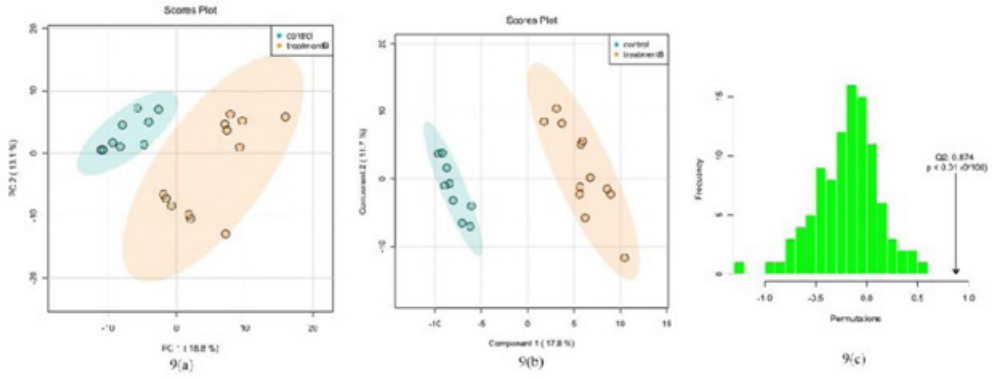

Figure 9: Shows the Q2 distribution and p value of PCA and PLSDA point cloud graph and OPLS-DA test statistics, respectively

4.2.3 Screening and Identification of Intergroup Characteristic Metabolites

In order to reduce the influence of different experimental methods and increase the persuadability of the results, three different ways were chosen to compare the characteristic metabolites between groups and the same differential metabolites were screened out in two group. (Figure 10) shows the yellow area of the volcanic chart shows the metabolites with $P < 0.05$ and the absolute value of variation multiple greater than 2 (Figure 10a). The box chart with univariate analysis and the highest ranking of metabolites among groups (the top 25 with small P values, Figure 10b). These metabolites have significant differences between the two groups. (Figure 11a and 11b) show the random forest and Support Vector Machine (SVM) analyses, in which 15 metabolites with the highest differences were selected in each of the analysis.

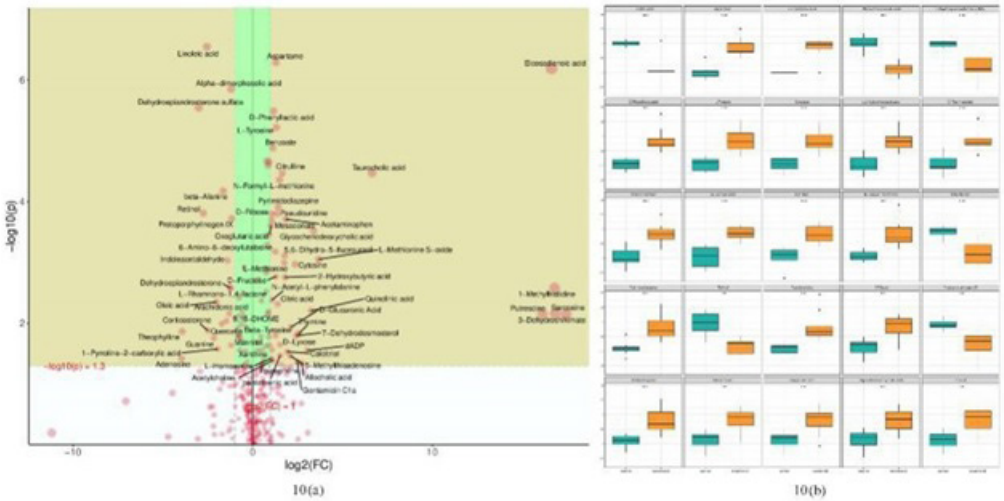

Figure 10: Volcanic diagram with multiple changes and box diagram of the differences of the first 25 metabolites (*,**,***, respectively correspond p<0.05, p<0.01, p<0.001).

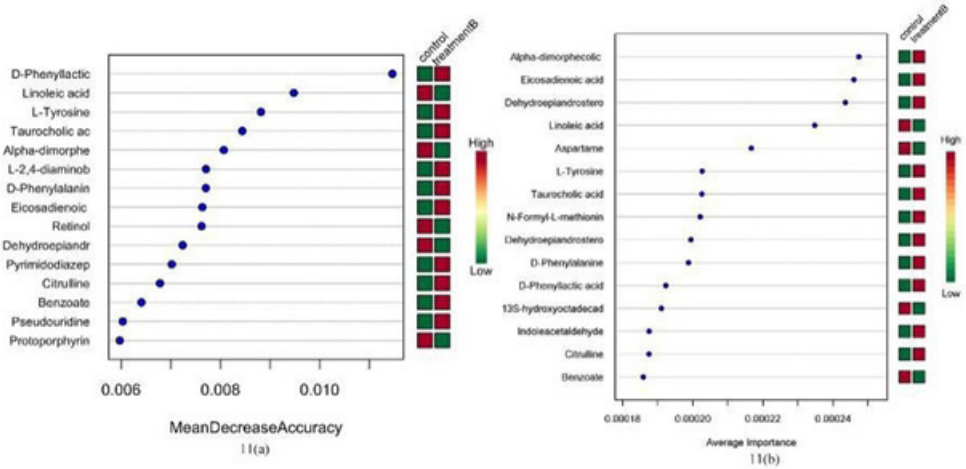

Figure 11: Shows the metabolites in the random forest and 15 metabolites in the SVM

4.2.4 Identification of the Characteristic Metabolites

Ten metabolites having the same characteristic metabolic markers were statistically identified in the three analysis methods, which included Taurocholic acid, Citrulline, D.P henyllactic acid, L.T yrosine, Benzoate, D-Phenylalanine, Linoleic acid, Eicosadienoic acid, alpha-Dimorphecolic acid, and Dehydroepiandrosterone sulfate. These metabolites mainly belonged to the metabolism of fatty acids, amino acids, bile acids and lactic acid (Table 5).

4.2.5 Analysis of the Metabolic Pathways of Characteristic Metabolites Between the Two Groups

By comparing the spectra of metabolite correlations of each group, the changes in metabolites correlation between the two groups were observed. The pathological conditions might change the correlation between certain metabolites from positive to irrelevant or negative correlation, suggesting the importance of metabolite pathways. Therefore, the metabolic pathways of the intergroup

characteristic metabolites were analyzed. (Figure 12) shows that the metabolite pathways in the clustering correlation of the two groups using Pearson correlation analysis had a significant positive and negative correlation (red means positive correlation and green means negative correlation.) between the two groups. The enrichment analysis was subsequently used to identify the biological pathways that play a key role in a biological process, and to reveal and understand the basic molecular mechanisms of biological processes. (Figure 12) shows that the metabolic pathways significantly enriched with differential metabolites, but it was still unclear whether these metabolites played a key role in the metabolic pathways. Therefore, the Over-Representation Analysis (ORA) enrichment analysis and topological analysis (Figure 12) were also performed. It was observed that Glycine had significant enrichment and key roles in the metabolism of Linoleic acid, Phenylalanine, Caffeine, Arginine and Proline, Serine and threonine metabolism, Tyrosine metabolism, and Pyrimidine metabolism pathways.

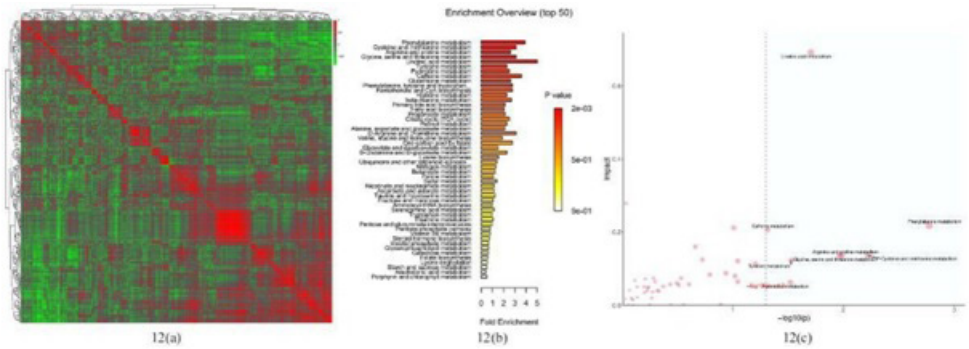

Figure 12: Show Pearson correlation heat map, ORA enrichment analysis, ORA enrichment analysis and topology analysis respectively.

Table 5: Identification of different metabolites between groups

Metabolic	Metabolic Pathways	Comparison of MHE and control
Citrulline	Amino acid metabolism	↑*
L. Tyrosine	Amino acid metabolism	↑*
D-Phenylalanine	Amino acid metabolism	↑*
Taurocholic.acid	Bile acid metabolism	↑*
Benzoate	Fatty acid metabolism	↑*
Lenoleic.acid	Fatty acid metabolism	↓*
Eicosadienoic.acid	Fatty acid metabolism	↑*
Alpha.dimorphecolic.acid	Fatty acid metabolism	↓*
Dehydroepiandrosterone.sulfate	Fatty acid metabolism	↓*
D.phenyllactic.acid	Lactic acid metabolism	↑*

↑: Compared with the corresponding group, the content is relatively high; ↓: Compared with the corresponding group, the content is relatively low; *: It's statistically significant(P<0.05).

4.2.6 Interaction Between Intestinal Microbiota and Serum Metabolites

The analysis of the intestinal microbiota and serum metabolites of the MHE patients showed that there might exist mutual correlation, such as citrulline and arginine are involved in the body an important components of urea cycle; and increase in the phenylalanine and tyrosine nitration is closely associated with the ammonia levels (Table 6), which conformed to the MHE "theory of plasma amino acid imbalance". The production of intestinal ammonia is the main source of blood ammonia. In the analysis of intestinal microbiota, the increase in the abundance of Veillonella and

the decrease in the abundance of *Lachnospiraceae*, Roseburia and *Coprococcus* were closely correlated to the level of intestinal ammonia. Similarly, there were statistically insignificant but obvious variations in the abundance of *Faecalibacterium, Blautia, Ruminococcus* and other genera. The abnormal metabolism of substances, such as benzoate, linoleic acid, alpha-Dimorphecolic acid, and caffeine was associated with the oxidation of fats in body. The intestinal microbiota, including *Lachnospiraceae, Roseburia* and *Coprococcus* participated in the synthesis of butyrate, which involved in the main pathway responsible for the breakdown of carbohydrates into SCFAs (Short-Chain Fatty Acids), inhibiting liver cholesterol and low density lipoprotein cholesterol (hdl-c) biosynthesis. In addition, *Collinsella, Coriobacteriaceae* and *Actinomycetaceae* belonging to Actinobacteria were also closely related to the metabolism of polyunsaturated fatty acids, serum cholesterol and triglycerides, and participated in the metabolism of endogenous lipids. Similarly, the taurocholic acid is a primary bile acid, plays an important role in the subsequent secondary and tertiary

anabolism of bile acid, while the increase in its metabolism, along with the abnormal metabolism of pyrimidine, is associated with the metabolism of bile acid, as shown by the increase of the genera *Parabacteroides* and *Veillonella* in the intestines. As another example, the increase in the metabolism of d-phenyl lactic acid in serum corresponds to the increase in the abundance of *Enterococcus, Enterobacteriaceae* and other genera in the intestinal microbiota, as well as that of *Ralstonia, Actinomyces*, *Blautia* and *Phascolarctobacterium* in the mutual analysis of genera. This all indicated an increased level of pathogenic bacteria in inflammatory infections and that the fluctuation in bacterial genera were correlated to the butyrate metabolism in vivo.

Table 6. Interaction between intestinal flora and serum metabolites

Intestinal flora (Genus)	Metabolic pathway	Metabolites in serum
Collinsella*	Fatty acid metabolism	Benzoate*
Faecalibacterium*		Linoleic acid*
Blautia*		Alpha.dimorphecolic.acid
Ruminococcus*		Caffeine*
Parabacteroides*	Inflammatory response	D-Phenylalanine*
Enterococcus*		
Enterobacteriaceae*	Bile acid metabolism	Pyrimidine*
Veillonella**		Taurocholic.acid*
Lachnospiraceae**	Amino acid metabolism	Citrulline*
Roseburia**		Arginine*
Coprococcus**		
Faecalibacterium*		
Blautia*		
Ruminococcus*		

Note:*:difference between the control group, but there was no statistical significance;**:statistically significant(P<0.05).

5. Discussion

The pathogenesis of MHE has not been clarified yet. The ammonia poisoning theory is the earliest hypothesis with the most evidences. The ammonia poisoning, caused by the disordered metabolism of ammonia, can trigger the cascade reaction of neuron apoptosis [13]. In depth, both the MHE and dominant HE have different degrees of cognitive dysfunction, but their pathologic basis is quite different (results in different diseases). MHE is mostly followed by cirrhosis, while the dominant HE is mostly followed by liver failure. It can be speculated that there might be certain differences in the pathogenesis of HE evolved on different pathological basis. Recent studies have gradually revealed the complex interaction between intestinal microbiota and the development of brain and neurophysiological functions [14]. Literature evidence shows that the intestinal microbiota can directly affect the blood-brain barrier, myelin sheath, neurogenesis, maturation of microglial cell and other basic neural development processes, and can regulate a variety of neurophysiological activities [15]. It also forms tryptamine, a neurotransmitter arylamine, which affects the acquisition of amino acids that are used in the synthesis of neuroactive peptides. It also regulates the exchange of peptide signals between the peripheral nervous system and brain through the blood-brain barrier, and participates in the brain's response to the signal generated from intestinal microbiota [16,17]. In addition, the serum metabolites have also been proved to be closely associated with the incidence of MHE, and may have an interactive correlation with intestinal microbiota [6]. Therefore, starting from intestinal microbiota and serum metabolites, this

study conducted the correlation analyses of the structure, function and metabolism of different bacteria with MHE, as well as with the characteristic serum metabolites and metabolite pathways.

Veillonella is a potential pathogenic bacterium for MHE in general, which is one of the human oral colonizing bacteria. It can play a synergistic role after being trans-located along the gastrointestinal tract into the intestine, by inducing the aggravation of HE [18]. It was found that the pathogenic process was mainly inhibited by the reduction in pH caused by the fermentation of lactic acid, which indirectly promoted the expression of pro-inflammatory cytokines in intestinal mucosa and produced endotoxin to affect the intestinal immunity [19]. At the same time, the increase in the production

of ammonia led to subtle or obvious HE, resulting in cognitive decline and increase in the MELD (Model for End-Stage Liver Disease) score [20]. Moreover, *Veillonella* increases the production of ammonia, which has been found to be associated with endothelial activation and bile metabolism in vivo [21,22]. Among the top 20 strains, *Enterococcus* and *Klebsiella* were also included in the potential pathogenic genera of MHE. The increased contents of *Enterococcus* and *Klebsiella* in the intestinal mucosa of MHE patients and fecal samples of patients with cirrhosis, respectively, have been experimentally confirmed [23,24]. Meanwhile, the *Enterococcus* and *Enterobacteriaceae* are pathogenic bacteria that are associated with inflammation and infection in vivo. It is speculated that the inflammatory response might open the Blood-Brain Barrier (BBB) and cause the toxin to enter brain, which is related to most of the MHE [25]. *Lachnospiraceae, Roseburia* and *Coprococcus*, on the other hand, play opposite roles to that of *Veillonella*. They are called beneficial bacteria and mainly participate in the production of butyrate [26]. A study has confirmed that the production of butyrate by the genera *Lachnospiraceae, Roseburia*, and *Coprococcus* was mainly caused by their ability to decompose carbohydrates into SCFAs, thereby inhibiting the synthesis of cholesterol and low-density lipoproteins in liver [19]. The butyrate, as the main energy source of epithelial cells, can enhance the function of intestinal barrier by the regulation of intestinal PH, inhibition of nuclear factor –κB signaling pathway, promotion of the production of mucin and antimicrobial peptides, and reduction of the expression of pro-inflammatory factors and cell adhesion molecules by enhancing the integrity of intestinal epithelial cells [27,28]. Relevant evidences [21,28] show that the Lachnospiraceae, Roseburia, Coprococcus are negatively correlated with the HE, related inflammation and endothelial activation caused by the increased level of intestinal ammonia, and significantly positively correlated with the good cognitive ability, level of intestinal immunoglobulin A and immunity, exhibiting a good inhibitory effect on the development of HE. Similarly, the top 20 bacteria having differences in their abundances between the MHE and control groups, including *Faecalibacterium, Blautia, Ruminococcus* and *Ruminococcaceae*, are all correlated to the normal production of butyrate. In addition, the increase in the abundances of families *Coriobacteriia, Coriobacteriales* and *Coriobacteriaceae* that belonged to the *Actinomyceae* was statistically significant, and the same was for the *Actinomycetales* that belong to *Actinomyceae*. *Collinsella* (*Coriobacteriaceae*) and *Actinomycetaceae* are closely associated with the metabolism of polyunsaturated fatty acids, serum cholesterol and triglycerides, indicating that they may be involved in the metabolism of endogenous lipids [29]. Interestingly, the subsequent analysis of species correlations found that there was reciprocal inhibition in the genus, confirming the previous discussion. *Roseburia* and *Collinsella* are butyrate producing bacterial genera, and *Oscillospira* was found to have significantly decreased-abundance in the patients having inflammation, which might be related to the production of butyrate, and the changes in its level could lead to decrease in the cognitive behavior and alteration in the brain neurotransmitter level [30]. *Parabacteroides* have been shown to improve liver injury, regulate liver inflammation and expression of oxidative stress without causing significant steatosis [31]. They have also been found to improve the glucose and lipid metabolism in mice by affecting the metabolism of intestinal bile acid and production of succinic acid [32]. In this study, the above-mentioned four genera were significantly negatively correlated with *Rothia* in the subjects, suggesting the mutual inhibition among genera. Subsequently, the negative correlation between *Enterococcus* and *Ruminococcus* conformed to the previous discussion, with inverse effects on the production of butyrate. In addition, *Ralstonia* and *Actinomyces* were prone to cause infection in intestine and promote inflammation, while *Blautia* and *Phascolarctobacterium*, which are negatively correlated with them, played an anti-inflammatory role and were significantly associated with the systemic inflammatory cytokines [33]. The subsequent prediction of community function using KEGG showed significant differences in the MHE and control groups, which included the differences in chromosomes, amino acids, methane metabolism related enzyme, arginine and proline metabolism differences ($P < 0.05$). This indicated that the occurrence and development of MHE might be correlated with the possible changes in function of intestinal microbiota. Therefore, it was speculated that the intestinal microbiota and serology might have a correlation, as the results were analyzed using serum metabolomics.

The analysis of the serum metabolites in subjects was carried out using LC-MS/MS. After verifying significant differences in the metabolites between the MHE and control groups, 10 metabolic markers having the same differences were compared, which included taurocholic acid, citrulline, D-phenyl-lactic acid, L-tyrosine, benzoate, phenylalanine, linoleic acid, eicosapedienic acid, A-diformic acid, and dehydroepiandrosterone. As for the synthesis of blood ammonia, the citrulline is mainly catalyzed by ornithine carbamoyl transferase after entering to the mitochondria,

and transferred to the cytoplasm during the next step of arginine synthesis, known as ureas cycle while ornithine is produced by hydrolysis [34]. In this study, as compared to the control group, a significant increase in the citrulline in MHE group indicated an increase in blood ammonia content resulted from the increase in ornithine cycle, which was consistent with the pathogenic characteristics of MHE. The increase in the tyrosine metabolites in the blood in MHE group was considered to increase the acute ammonia poisoning, which induced the oxidative stress in the brain. This was in accordance with the previous results of Reinehr and Murthy. The rise of ammonia levels in rat astrocyte culture and brain slices induced oxidation/nitrosation stress, which led to the

formation of a Protein Tyrosine Nitration (PTN) and 8-hydroxy guanosine oxidation caused by RNA, which were closely related to the patients having HE [35,36]. Tyrosine is produced by the hydroxylation of phenylalanine in human body. The phenylalanine also increased significantly in the MHE group as compared to the control group, in accordance with the MHE "plasma amino acid imbalance theory". In addition, the bile acids are synthesized by cholesterol in liver, and the taurocholic acid, which was increased in the MHE group, belongs to primary bile acids. The taurocholic acid combines with taurine under the action of micro-mitochondrial bile acid-N-transacylase and sulfonate transferase in the cytoplasm, and plays an important role in the subsequent synthesis of secondary and tertiary bile acids [37]. In chronic liver disease (cirrhosis),

the synthesis of liver bile acids are reduced and the portal vena cava bypass is opened, the bile acids are no longer confined to the enterohepatic circulation, which results in the abnormal distribution of bile acids and increased level of bile acids in blood. At the same time, the intestinal microbiota and metabolism of bile acids are interdependent and competitive. The abnormal metabolism of bile acids led to the disturbances in the structure of intestinal microbiota, deficiency of beneficial bacteria, inflammation and abnormal increase in ammonia level [38,39]. Moreover, some studies have found that the abnormal bile acid signals were involved in the HE caused by acute liver function injury, including the neuronal dysfunction, neuro-inflammation and BBB permeability [40]. In the serum of the MHE group, the concentration of benzoate, linoleic acid, alpha-Dimorphecolic, and acid , dehydroepiandrosterone (dhea) in comparison with the control group, showed that the levels of fatty acids were abnormal, which might be due to the metabolic abnormalities of bile acids (bilirubin) caused by the changes in intestinal microbiota [41], or due to the damage of mitochondria caused by the decrease in the oxidation of fatty acid, thereby posing difficulty in the tricarboxylic acid cycle. The D-phenyl-lactic acid, catalyzed by D-lactate dehydrogenase to produce phenylpyruvate, is a broad-spectrum antibacterial compound with antibacterial and fungal activities. The increase in the concentration of D-phenyl-lactic acid in the serum of MHE group was considered to be correlated to the increase in the inflammation level in MHE patients. Finally, the topology analysis for the enrichment of group differences among the metabolites of metabolic pathways showed that the metabolism of linoleic acid, benzene propane, caffeine, arginine, proline, glycine, serine, threonine and tyrosine metabolism had remarkable significant effects on the enrichment of metabolic pathways, where the metabolites analysis in the metabolism of linoleic acid, benzene propane, tyrosine was consistent with the previous results. In the other four metabolic pathways, the arginine metabolism is the third step of ornithine cycle in the metabolism of ammonia in MHE, where the citrulline and aspartic acid are catalyzed to arginine, which then undergoes the hydrolysis of arginine to form urea. The metabolism of serine and threonine could promote the phospholipid synthesis and fatty acid oxidation, both of which are correlated to the metabolism of fatty acids in the body [42]. The proline and glycine are commonly used as raw materials for the synthesis of essential amino acids. The metabolism of pyrimidine is considered to be correlated to accumulation of lactic acid due to the imbalance of lactic acid metabolism in the body and reduction of carbon and nitrogen sources caused by the abnormal metabolism of bile acids [43]. The caffeine metabolism occurs in the liver, and three different dimethylxanthine form after oxidation by the cytochrome oxidase P450 system [44]. The parxanthine accounts for 84% of the total xanthine, which mainly accelerates lipolysis in the body and increases the contents of fatty acids in the plasma, which was consistent with the regulation of fatty acid metabolism in the MHE group. To sum up, the structural imbalance in the composition of intestinal microbiota in MHE patients was mainly characterized by the decrease in the beneficial bacteria at the level of phylum, class, order, family and genus, increase in the pathogenic bacteria, and the imbalance in the relative abundances of all bacteria and genera in the intestine. The main predicted functions that showed significant differences included chromosome, amino acid-related enzymes, methane metabolism, and arginine and proline metabolism. In the LC-MS /MS studies, the serum metabolites characteristic of MHE are mainly involved in metabolism of fatty acids, amino acids, bile acids and lactic acid, and are also correlated with the metabolism of caffeine and pyrimidine

in the enrichment pathway analysis. In this study, the MHE-specific intestinal microbiota and serum metabolites were analyzed, and their mutual correlation was investigated. The interaction among bacteria due to the imbalance in the intestinal microbiota and its effects on the changes in serum metabolites, including increased inflammatory response and endotoxin level were studied, which promoted the pathological progress of MHE. This understanding can be used for subsequent studies and can provide basis for designing the effective therapeutic strategies against MHE.

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