



Bacteroides in colonic mucosa-associated microbiota affects the development of minimal hepatic encephalopathy in patients with cirrhosis

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Abstract

Background/purpose Gut microbiota has been associated with liver cirrhosis and, possibly, hepatic encephalopathy. However, only a few studies have examined the link between mucosa-associated microbiota (MAM) and minimal hepatic encephalopathy (MHE). Our aim was to investigate this relationship.

Methods Twenty-four patients with cirrhosis underwent colon biopsies at our institution, between January 2014 and April 2015. Patterns of microbial colonization were examined using 16S rRNA gene sequences. MHE was diagnosed using the Neuropsychological Test.

Results Ten (41.7%) of the 24 patients were diagnosed as having MHE. There was no significant difference in the diversity of gut microbiota by sampling locations between those with and without MHE. However, the diversity of the gut microbiota and the proportion of the genus *Bacteroides* decreased as a function of declining liver function. We divided patients into those with the highest proportion of the genus *Bacteroides* (*Bacteroides*-dominant group; $n = 9$) and into a *Bacteroides* non-dominant group ($n = 15$). In the *Bacteroides*-dominant group, only 1 patient (11.1%) was diagnosed as having MHE, with the incidence rate of MHE being significantly lower in the *Bacteroides*-dominant group than in the non-dominant group ($p = 0.019$). The Child–Pugh score ($p = 0.05$) and use of proton-pump inhibitors ($p = 0.015$) were negatively correlated to the proportion of *Bacteroides*. Furthermore, the proportion of the family *Clostridiaceae* was significantly higher in the *Bacteroides*-dominant group than in the non-dominant group ($p = 0.078$).

Conclusions The decrease in microbial diversity and genus *Bacteroides* in MAM is a risk factor for MHE in patients with liver cirrhosis.

Keywords Microbiota · Cirrhosis · Minimal hepatic encephalopathy · Next-generation sequencing

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Introduction

Hepatic encephalopathy (HE) is one of the severe complications of liver cirrhosis and is associated with poor prognosis and a reduced quality of life [1]. The diagnosis of overt HE is based on symptoms and clinical examination. On the other hand, minimal HE (MHE) might only be apparent on specific neuropsychological tests, as patients with MHE do not exhibit overt neurological symptoms. However, diagnosis of MHE among patients with cirrhosis is important as MHE is a risk factor for overt HE [2]. MHE is diagnosed in 30.1% of patients with cirrhosis in Japan [3] and, thus, MHE is not a rare condition among patients with liver cirrhosis. Therefore, an effective screening method is urgently needed to identify patients with MHE.

Gut microbiota is closely associated with a series of chronic diseases, such as obesity [4], non-alcoholic fatty liver disease [5, 6], and type 2 diabetes [7]. Recent studies have demonstrated that gut microbiota is also different in patients with cirrhosis, *with* or *without* HE [8, 9]. Small-bowel bacterial overgrowth (SIBO) could also be associated with MHE occurrence [10]. Therefore, understanding gut microbiota is important as current treatments for HE, such as the use of lactulose or antibiotics, act through their influence on the gut microbiota [11, 12].

With regard to the analysis of gut microbiota, most previous studies have focused on fecal-associated microbiota (FAM), although mucosal-associated microbiota (MAM) has also been considered to be important in recent years. A difference in the MAM of the sigmoid colon has been identified between patients with overt and non-overt HE [13]. Moreover, a significant difference has been reported between the MAM and FAM in cirrhotic patients, *with* and *without* overt HE, and controls. Teltschik et al. also reported on the possibility of several processes of HE pathogenesis at the level of the intestinal mucosa rather than the lumen [14]. Hepatic encephalopathy (HE) may be associated with bacterial translocation (BT) [15], with Wang et al. suggesting that controlling MAM may be useful for the prevention of BT [16]. Therefore, it is important to investigate the MAM in patients with cirrhosis to clarify the relationship between HE and gut microbiota. Thus, although the collection of MAM is more invasive than the collection of FAM, there is the possibility that investigation of MAM may lead to the discovery of novel drug therapies for HE.

Various reports have examined the relationship between HE in liver cirrhosis and gut microbiota. These studies have evaluated the relationship between fecal microbiota and HE, with the relationship between colonic mucosal microbiota and HE remaining to be defined. Additionally, there remains several unclear points regarding the relationship between gut microbiota and MHE. Therefore, the aim of this study was to investigate the relationship between the gut microbiota in patients with liver cirrhosis and MHE.

Methods

Patients

Our study group consisted of 24 patients with liver cirrhosis who were admitted to the Department of Gastroenterology and Hepatology at Nagasaki University Hospital, between January 2014 and April 2015. We excluded patients with the following: a current infection; variceal bleeding within the last 4 weeks; renal dysfunction, defined by a creatinine concentration > 2.0 mg/dL (reference interval, 0.40–1.10 mg/dL); psychiatric disorder; severe anemia, defined by a

hemoglobin concentration < 7.0 g/dL (reference interval, 11.3–15.2 g/dL); current overt HE; and major portosystemic shunt, such as a gastro-renal shunt.

The etiology of the liver disease was determined through a combination of clinical, laboratory, radiological, and histological variables. Liver function was evaluated using the Child–Pugh score and the Model for End-Stage Liver Disease (MELD) score.

Diagnosis of MHE

The Neuropsychological Test (NPT) was designed to evaluate psychomotor, attention, memory, and special brain functions, using the following eight cognitive tests: number connection tests A and B; figure position test; digit symbol test; block design test; and reaction time tests A, B, and C. The NPT is used in clinical practice to screen for MHE. Although the individual score is a continuous variable, the threshold for normal score or abnormal score is age-dependent. Software for the NPT was developed by Otsuka Pharmaceutical Co, Ltd, Kokuyo Co, Ltd, and ISB Co, Ltd. In the present study, MHE was diagnosed based on methods previously described by our group [17]: an abnormal score ≥ 2 on the number connection test A, number connection test B, digit symbol test, and block design test.

Collection of mucosal samples and DNA extraction

All the patients underwent a total colonoscopy in hospital. Normal mucosal tissues were obtained from the ileocecum, ascending colon, and sigmoid colon (total 72 samples) under endoscope observation. Samples were snap-frozen and stored at -80 °C until analysis. DNA was extracted from mucosal tissue, using a Fast-DNA Spin kit for soil (MP Biomedicals, LLC, Solon, OH, USA), according to the manufacturer's recommendations.

PCR amplification, Miseq sequencing, and Sequence data process

After quantification of DNA (dsDNA HS Assay kit, Thermo Fisher Scientific), libraries were generated from V1–V2 region of the 16S rRNA gene using 2-step tailed PCR methods. In the first step, samples were incubated in 10X Ex buffer, 200 μ M of dNTP, 0.05 U/ μ l of Ex Taq polymerase (Takara Bio, Inc., Shiga), and 500 nM of each of the following primers (1st-forward primer (27F): 5'-ACA CTCTTCCCTACACGACGCTCTTCCGATCTAGRGTT TGATYMTGGCTCAG-3', and 1st-reverse primer (338R): 5'-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC TTGCTGCCTCCCGTAGGAGT-3'), and 1 ng of extracted DNA, under the following conditions: 2 min at 94 °C; 25 cycles of 94 °C for 30 s; 55 °C for 30 s, and 72 °C for 30 s,

and a final 5-min extension at 72 °C. The second PCR step was performed in 10X Ex buffer, 200 μ M of dNTP, 0.05 U/μl of Ex Taq polymerase, 500 nM of each primers, containing each index sequences (2nd-forward primer (2ndF); 5'-AATGATACGGCGACCACCGAGATCTACAC-Index2-ACACTCTTTCCCTACACGACGC-3', and 2nd-reverse primer (2ndR); 5'-CAAGCAGAAGACGGCATAACGAGAT-Index1-GTGACTGGAGTTCAGACGTGTG-3'), and 2.0 μl of 1st-PCR products under the following conditions: 2 min at 94 °C; 8 cycles at 94 °C for 30 s; 55 °C for 30 s; and 72 °C for 30 s; and a final 5-min extension at 72 °C.

After quantification, using the Qubit dsDNA HS Assay kit (Thermo Fisher Scientific, Waltham, MA, USA) and the High Sensitivity NGS Fragment Analysis Kit (Advanced Analytical Technologies), the generated libraries were sequenced, using the MiSeq system (2 × 250 bp), according to the manufacturer's manual (Illumina, San Diego, CA). Adapters and low-quality reads were removed from the primary sequences, using the Fastq Barcode Splitter (Fastx toolkit) and sickle tools. Multiple bioinformatics analyses were then performed, including operational taxonomic unit (OTU), α, and β diversity.

OTU analysis and taxonomic levels classification

The obtained usable pair-end reads were subjected to the uchime algorithm (Usearch; <https://www.drive5.com/usearch/>) to choose chimera-free sequences, based on a 97% OTU in the Greengene database. The OTU analysis and taxonomic classification were performed using the workflow script of the QIIME software (version 2; Scikit-Bio open source; <http://qiime.org/>).

Bacterial diversity analysis

α-diversity was used to describe the abundance of various species in each sample. Based on the OTU analysis, the diversity index was calculated using a PD whole tree analysis (PD whole). β-diversity, represented by the principal coordinate analysis (PcoA), reveals the magnitude of community composition and describes the alterations in species distribution. The weighted Unifrac distance from the samples was used to calculate the values of the three principal components—PC1, PC2, and PC3, analyzed using the PcoA. Each sample was then compared against the three principal coordinates to identify the relative similarities and abundances between the samples.

Statistical analysis

Statistical analyses were performed using Stat Flex (ver. 6.0; Artec, Osaka, Japan). Data were presented as a mean and standard deviation (SD). Correlations were evaluated using

Spearman's correlation test. Pairwise comparisons were evaluated using the Mann–Whitney *U* test. A *p* value < 0.05 was considered statistically significant for all tests.

Results

Characteristics of the study group

Relevant patient characteristics are reported in Table 1. The mean age of the study sample was 54.8 ± 15.9 years and included 11 (45.8%) males and 13 (54.2%) females. The cause of cirrhosis in our study group was as follows: alcohol-related in 8 (33.3%) patients; hepatitis C virus in 7 (25.0%); and hepatitis B virus in 1 (4.2%). The distribution of Child–Pugh grade was as follows: grade A, 3 (12.5%) patients; grade B, 8 (33.3%) patients; and grade C, 13 (54.1%) patients. Proton pump inhibitors (PPI) were used by 13 (52%) patients. There were three patients with Child–Pugh grade C and one patient with Child–Pugh grade B cirrhosis who had developed overt HE, controlled using lactulose or kanamycin. The distribution of abnormal test scores was as follows: NCT A test, 3/24 (12.5%); NCT B test, 7/24 (29.2%); Figure Position test, 3/24 (12.5%); and Digit Symbol test, 13/24 (53/1%). MHE was diagnosed in 10/24 (41.7%) patients, with the characteristics of these

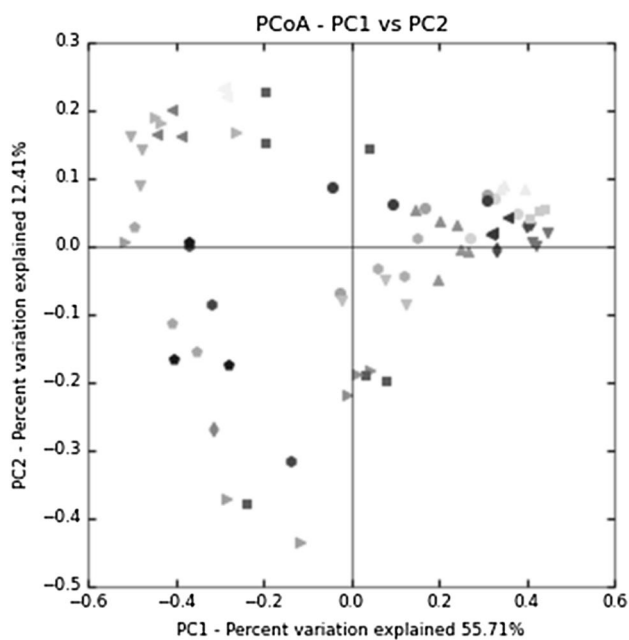
Table 1 Clinical characteristics of the study group

Male: female	11: 13
Age (years)	54.8 ± 15.90
BMI (kg/m ²)	23.92 ± 3.92
Disease	
Alcohol-related (%)	8 (33.30)
HCV-related (%)	7 (25.0)
HBV-related (%)	1 (4.2)
PSC (%)	3 (12.5)
PBC (%)	2 (8.3)
AIH (%)	2 (8.3)
Others (%)	1 (4.0)
Child–Pugh classification (A/B/C)	3/8/13
Child–Pugh score	9.08 ± 2.12
NH ₃ (μg/dL)	83.61 ± 41.38
liver VTQ (m/s)	2.65 ± 0.78
Type 2 diabetes (%)	4 (16)
Use of PPI (%)	13 (52%)
Use of lactulose (%)	7 (28)
Use of kanamycin (%)	4 (16)

BMI body mass index (kg/m²), *HCV* hepatitis C virus, *HBV* hepatitis B virus, *PSC* primary sclerosing cholangitis, *PBC* primary biliary cholangitis, *AIH* autoimmune hepatitis, *NH₃* ammonia, *VTQ* virtual touch tissue quantification, *PPI* proton pump inhibitor. The 'other' category includes one case of Wilson's disease

Table 2 Clinical characteristics of MHE patients

Covariate	MHE (-) (n=14)	MHE (+) (n=10)	P value
Female: male	8: 6	5: 5	0.73
Age (years)	50.93 ± 18.74	60.3 ± 9.40	0.28
Child–Pugh score	8.29 ± 2.16	10.20 ± 1.55	0.026
NH3 (µg/dL)	77.92 ± 36.56	91.00 ± 45.34	0.51
liver VTQ (m/s)	2.65 ± 0.85	2.66 ± 0.57	0.97
Use of PPI (%)	6 (42%)	7 (70%)	0.19
Use of lactulose (%)	5 (35.7%)	2 (20%)	0.40
Use of kanamycin (%)	2 (14.3%)	2 (20%)	0.71
Proportion of genus <i>Bacteroides</i> (%)	24.6 ± 0.21	11.2 ± 0.14	0.10

**Fig. 1** Difference in the diversity of gut microbiota by sampling location. Symbols distinguish between each patients. The proximity of the same symbols indicates that there is not a large difference between biopsy sites

patients summarized in Table 2. The Child–Pugh score was significantly higher among patients with than without MHE ($p=0.026$).

Absence of a large difference in gut microbiota by sampling locations

We investigated differences in gut microbiota as a function of sampling location using β -diversity analysis. The clustering of same shaped and colored symbols in Fig. 1 indicates the absence of a large difference in gut microbiota between sampling locations.

Correlation between the Child–Pugh classifications and gut microbiota

The genus *Bacteroides* formed the highest proportion of gut microbiota (Fig. 2a), but with a large variation in proportion among patients (Fig. 2b). With regard to the diversity of gut microbiota and clinical characteristics, the α -diversity analysis indicated a decrease in the diversity of the gut microbiota with declining liver function, although this change was not significant (Supplementary Fig. 1a). The specific proportion of the genus *Bacteroides* also decreased as a function of declining liver function (Supplementary Fig. 1b), with no association between other microbiota and liver function. Additionally, the proportion of genus *Bacteroides* tended to be lower among patients with MHE, compared to those without MHE (Table 2). Of note, there was no difference in the proportion of *Bacteroides* between patients treated using lactulose or kanamycin than those not treated using these drugs, as well as no difference in the proportion of *Bacteroides* and the ratio of patients classified into the *Bacteroides*-dominant group between patients with normal or abnormal scores on each test of the NPT (Supplementary Table 1).

Correlation between MHE and the bacteroides-dominant group

We divided patients into those with the highest composite proportion of the genus *Bacteroides* (*Bacteroides*-dominant group, $n=9$) and those in which the proportion was not dominant (*Bacteroides* non-dominant group, $n=15$). In the dominant group, the proportion of patients with a Child–Pugh grade A score was significantly greater than that of those with a Child–Pugh grade C score ($p<0.05$; Fig. 3a). With regard to the relationship between MHE and dominance of the genus *Bacteroides*, MHE was diagnosed in only one patient (11.1%) in the dominant group, compared to 9 (60.0%) in the non-dominant group. The incidence rate of MHE was significantly lower in the dominant than non-dominant *Bacteroides* group ($p=0.019$; Fig. 3b). On multivariate analysis (including age, sex, *Bacteroides*-dominant group, Child–Pugh score, and PPI use), the *Bacteroides*-dominant group was significantly correlated with MHE (odds ratio = 20.73, $p=0.043$) (supplementary Table 2).

Analysis of the factor contributing to *Bacteroides*-dominant group

Finally, we evaluated factors associated with the proportion of genus *Bacteroides* (Supplementary Table 3.), identifying the Child–Pugh score ($p=0.05$) and PPI use ($p=0.015$) as being negatively correlated with a dominance of the genus *Bacteroides* in the gut microbiota. Furthermore,

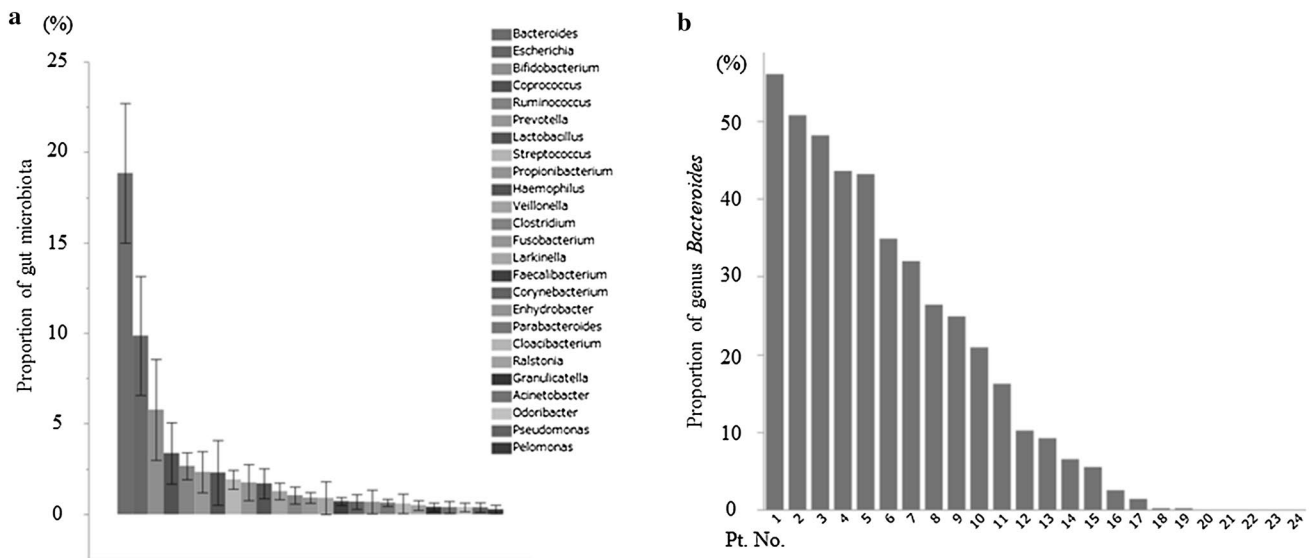
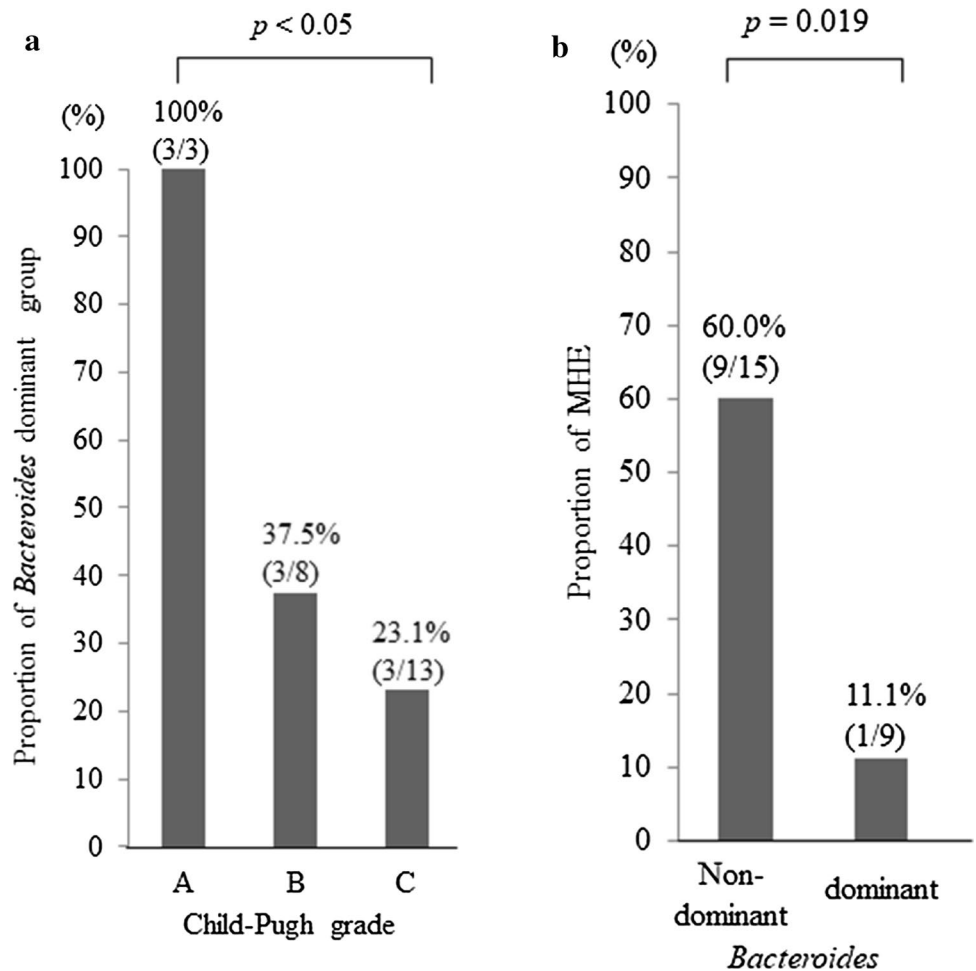


Fig. 2 Proportion of gut microbiota. Among all gut microbiota, the proportion of genus *Bacteroides* was the highest (a), but with a large variation in the proportion of genus *Bacteroides* between patients (b)

Fig. 3 Relationship between *Bacteroides* groups and MHE. The proportion of patients with a Child–Pugh grade A score was significantly higher than the proportion of patients with a Child–Pugh grade C score in the *Bacteroides* dominant group (a). The incidence rate of MHE was significantly lower in the *Bacteroides* dominant group (11.1%) than in the *Bacteroides* non-dominant (60.0%) group (b)



the proportion of genus *Bacteroides* tended to be lower in patients treated with PPTs than those without ($p=0.055$). We also investigated the relationship between *Bacteroides* and other gut microbiotas comprehensively. The proportion of the genus *Bacteroides* positively correlated with the proportion of the family *Clostridiaceae* ($r=0.5518$, $p<0.01$; Fig. 4a) and genus *Ruminococcus*, which is a member of the *Clostridiaceae* family ($r=0.5452$, $p<0.01$; Fig. 4b). Additionally, the proportion of the family *Clostridiaceae* was significantly higher in the *Bacteroides*-dominant than non-dominant group ($p<0.01$; Fig. 4c.). The family *Micrococcaceae* ($r=-0.49$, $p=0.013$) and family *Erysipelotrichaceae* ($r=0.43$, $p=0.034$) were also correlated to the proportion of genus *Bacteroides*, although their median proportion was very small overall (*Micrococcaceae*, 0.20%, *Erysipelotrichaceae*, 0.30%).

Discussion

We examined the relationship between gut microbiota and MHE, identifying a positive association between the proportion of genus *Bacteroides* and the onset of MHE. We focused on MAM to elucidate the role of colonic microbiome on MHE development. Although the investigation of MAM is invasive and time consuming, requires expertise, and may not be easily useful as a clinical biomarker, our results do contribute to understanding of the pathomechanism of MHE, which could inform about the development of novel treatment drugs.

To date, most studies have focused on FAM, and not MAM, in their evaluation of gut microbiota, although the

role of MAM has attracted research attention in recent years. Bajaj et al. reported the possibility that MAM and FAM were different among patients with cirrhosis, with a greater association identified between MAM and HE, than between FAM and HE [18]. Furthermore, the composition of MAM differed from that of FAM among patients with cirrhosis, with a lower diversity of the gut microbiota measured with MAM than FAM among patients with irritable bowel syndrome [19]. These reports underline the importance of considering MAM, and not only FAM.

In this study, we collected tissue from three different sites to address the possibility of intestinal microbial species being different depending on the biopsy site. However, the β -diversity analysis confirmed that there was little difference in the diversity of microbiota in the large intestine by site of biopsy. To our knowledge, this is the first study to have evaluated differences in MAM by sampling location, and to have evaluated the association of MAM, rather than FAM, with MHE.

Our α -diversity analysis revealed that the diversity of gut microbiota decreased with deteriorating liver function. Specifically considering the decline in the proportion of genus *Bacteroides* with deteriorating liver function, it is possible that genus *Bacteroides* is associated with MHE. This possibility is supported by our finding of a significantly smaller proportion of patients with MHE among those with a higher proportion of genus *Bacteroides*.

Gut microbiota can impact HE through multiple pathways, with the production of ammonia by gut microbiome possibly being a principal factor. Vince et al. reported a lower production of ammonia from *Bacteroides* than Gram negative anaerobes [20]. As such, the accumulation and

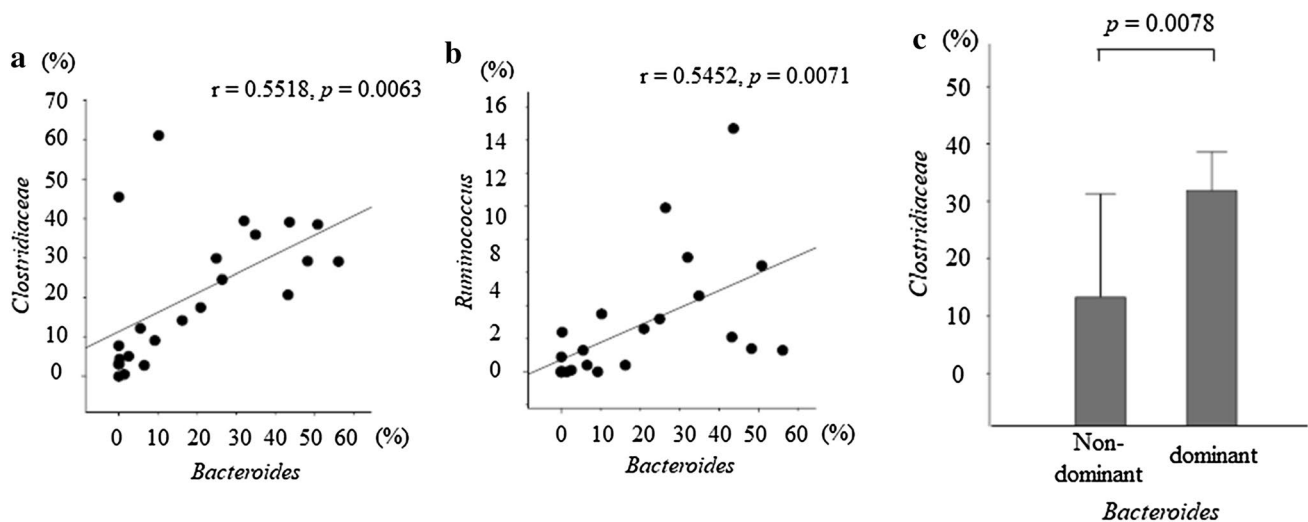


Fig. 4 Correlation between *Bacteroides* and *Clostridium*. The proportion of the genus *Bacteroides* was positively correlated with the proportion of the family *Clostridiaceae* (a) and genus *Ruminococcus* (b).

Furthermore, the proportion of the family *Clostridiaceae* was significantly higher in the *Bacteroides* dominant group than in the *Bacteroides* non-dominant group (c)

intestinal absorption of ammonia might be lower in the MAM *Bacteroides*-dominant group than in the non-dominant group. Further, it has been reported that *Bacteroides* have immunomodulatory functions and promote IgA production in the large intestine [21]. It has also been reported that the family *Bacteroidaceae* is negatively correlated with systemic and neural inflammation in cirrhotic mice, suggesting that *Bacteroides* might inhibit the development of HE [22]. Our findings are consistent with these findings.

We compared background factors between patients with a dominance of genus *Bacteroides* and those with a non-dominance. The proportion of patients who did not take PPI was significantly higher in the *Bacteroides*-dominant than non-dominant group. Yamamoto et al. reported that PPI use increased oral bacterial flora and decreased autochthonous flora, increasing the risk of HE [23]. Tsai et al. reported that use of PPIs in patients with cirrhosis increased the risk of HE [24], and Jackson et al. [25] reported that the use of PPIs decreased microbial diversity, due to the removal of the low pH barrier. PPI use also decreases the abundance of *Bacteroides* in mammals [26]. Based on these previous reports, the use of PPIs was thought to be associated with the development of encephalopathy in our study.

In addition, we found that the proportion of the genus *Bacteroides* was positively correlated to the proportion of the order *Clostridiales* and genus *Ruminococcus*. It has been reported that *Clostridium* induces the activity of regulatory T cell, which mitigates inflammation [27, 28]. Therefore, it is possible that HE might have been suppressed not only by *Bacteroides* but also by *Clostridium*. Further study on the association between *Bacteroides* and *Clostridium* is warranted.

The limitations of our study should be acknowledged in the interpretation of the results. Foremost, our study sample was small. Moreover, our study did not include an analysis of FAM. It would be desirable if future studies compared MAM with FAM. Our data also lacked control samples obtained from patients without liver cirrhosis, due to the difficulty in setting up a control group because of the invasiveness of total colonoscopy and biopsy. Additionally, it is possible that lactulose and kanamycin exerted effects on the measured gut microbiota. To consider this possibility, we investigated the relationship between MHE and gut microbiota in the 13 patients, in our study group, with neither lactulose nor kanamycin. Among these 13 patients, MHE was identified in six, with *Bacteroides* dominance identified in one (16.7%) of these six patients. Among the seven patients without MHE, four (57.1%) were classified in the *Bacteroides* dominant-group. The number of *Bacteroides* dominant groups tended to be small in those with MHE ($p=0.13$). Further investigation on the effect of gut microbiota on MHE is warranted. We also note that an effect of selection bias cannot be denied in our study due to the exclusion of patients with

overt HE. CP score of encephalopathy was 1 for all patients, then the patients with a Child–Pugh grade C in our study are somewhat different from those in the general population. This exclusion was based on the inability of these patients to provide informed consent for colonoscopy. Our evaluation also lacked objective assessment of MHE, such as electroencephalography or magnetic resonance imaging. Future studies should confirm the correlation between the NPT and neurophysiological tests. Moreover, correlation between MHE and clinical outcomes, through long-term observation, will be also desired.

In conclusion, a decrease of microbial diversity, and genus *Bacteroides* more specifically, in MAM was associated with MHE among patients with liver cirrhosis. We believe that MHE could be improved by controlling gut microbiota.

Compliance with ethical standards

Conflict of interest Authors Dr. Haraguchi, Dr. Miuma, Dr. Masumoto, Dr. Ichikawa, Dr. Kanda, Dr. Sasaki, Dr. Fukushima, Dr. Miyaaki, Dr. Taura, and Dr. Nakao declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All patients provided informed consent, and our study protocol conformed to the guidelines of the Declaration of Helsinki and was approved by the Nagasaki University Ethics Committee (approval no. 13120201).

Informed consent Informed consent was obtained from all individual participants included in the study.

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