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ORIGINAL ARTICLE

Influence of proton pump inhibitors on microbiota in chronic liver disease patients

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Abstract

Background Current knowledge suggests that proton pump inhibitors (PPIs) are associated with an increased risk of hepatic encephalopathy (HE) and spontaneous bacterial peritonitis (SBP). These conditions and PPI use are related to gut microbiota. The aim of this study is to research the changes in gut microbiota caused by PPI in patients with chronic liver disease.

Methods From 198 Japanese patients, 31 patients in the PPI and non-PPI groups were matched using propensity score matching (PSM) based on age, sex, and Child–Turcotte–Pugh class. We investigated the gut microbial composition of stool samples using the Illumina MiSeq sequencing platform and compared them using linear discriminant analysis effect size and phylogenetic investigation of communities by reconstruction of unobserved states.

Results Before PSM, Child–Turcotte–Pugh score ($p=0.038$), ascites ($p=0.049$), encephalopathy ($p=0.023$), and esophageal varices ($p<0.01$) were significantly higher in the PPI group than in the non-PPI group. After PSM, six genera, consisting of *Lactobacillus, Streptococcus, Selenomonas, Veillonella, Campylobacter*, and *Haemophilus* were enriched in the PPI group. *Eggerthella, Paraprevotella, Turicibacter*, *Dorea*, *Anaerotruncus*, and *Ruminococcus* were less abundant in the PPI group. We identified five types of level 3 KEGG pathways predicted to be significantly different.

Conclusions Part of microbial changes caused by PPI use was common to the changes by progression of liver cirrhosis. Increases in oral bacterial flora and decreases in autochthonous flora may produce the intestinal environment which tends to make the risk factor for HE or SBP.

Keywords Microbiota · Proton pump inhibitor · Liver · Next generation sequencing · Propensity score matching methods

Introduction

There have been recent reports on the risks associated with the use of proton pump inhibitors (PPIs) in patients with cirrhosis. These reports commonly show an increased risk of nutritional deficiency, bone fracture, *Clostridium difficile* infection, and other infections. Recently, the possibility of an increased risk of hepatic encephalopathy (HE) [1] and spontaneous bacterial peritonitis (SBP) [2] have also been noted in patients with cirrhosis.

The cause of these increased risks is suspected to be related to changes in gut microbiota. Many reports have already described changes in gut microbiota, such as increased relative abundance of *Streptococcus* and other resident oral flora [3]. The reasons of changes caused by PPI in gut microbiota are speculated to include altered gut immunity, increased gastric pH, and breakdown of the gastric acid barrier [4]. Chronic hepatitis and liver cirrhosis also change the gut flora [5]. The presence of HE or SBP is reported to cause further changes in gut microbiota. Conversely, HE and SBP are treated with laxatives and antibiotics. Few reports have investigated the combined effect of liver cirrhosis and PPIs on gut microbiota.

Bajaj et al. [6] reported in detail on the association between use of PPI and liver cirrhosis based on four cohort studies. They reviewed that PPI increases oral-origin

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microbial taxa in decompensated cirrhosis patients. But in these studies, PPI group has worse MELD score or higher rate of rifaximin. As a further study, we believe that the comparison with equal patient background is necessary to clarify the relationship between PPI use and cirrhosis with HE or SBP because it is known that some factors, age, obesity, severe cirrhosis, and diabetes mellitus, affect gut microbiota. Furthermore, this is the first study in Japanese patients. Microbiota varies depending on race and diet. If the same tendency is proved in Japanese as well, the reproducibility of that will be sufficient. This study investigated to elucidate changes in gut microbiota caused by PPI in chronic liver disease patients and research the relationship with risk of PPI about HE and SBP.

Patients and methods

Study design

In this single-center, Japanese patients with chronic hepatitis or cirrhosis were matched by age, sex, and Child–Turcotte–Pugh (CP) class to evaluate the effect of PPI on gut microbiota. Of 198 patients, 31 patients in each group were selected using propensity score matching (PSM) with careful exclusion criterion. The gut microbiome, which was analyzed using a next generation sequencer, was compared between PPI and non-PPI groups.

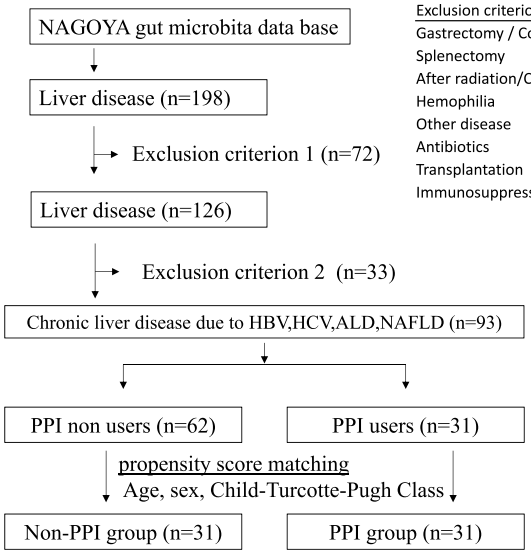
Our database is anonymously constructed with the clinical information and sample information. All patients whose blood samples were collected in our department are listed in the NAGOYA gut microbiota database.

Patient selection

The NAGOYA gut microbiota database includes 198 patients with liver disease. All patients were diagnosed by 2 hepatologists. We excluded 72 patients with some factor that might affect the gut microbiota (Fig. 1). We focused on patients with chronic liver disease due to hepatitis C, hepatitis B, alcoholic, or nonalcoholic fatty liver disease. And patients with CP class C were excluded because severe cirrhosis might affect on their gut microbiota themselves. The remaining 93 patients with chronic hepatitis or cirrhosis were followed with blood tests and imaging studies including ultrasound, computed tomography, and magnetic resonance imaging for more than 3 years before stool sample collection. They were followed every 3–6 months with liver function tests after sample collection.

Age, sex, and CP class matching using PSM

To minimize possible bias based on the study design, we used PSM [7] to adjust for age, sex, and CP class using IBM SPSS Statistics, version 24 (IBM Corp., Armonk, NY, USA). Patients taking PPIs ($n = 31$) were propensity score–matched in a 1:1 ratio with patients who did not take



Exclusion criterion 1	72
Gastrectomy / Cololectomy	25
Splenectomy	4
After radiation/Chemotherapy	17
Hemophilia	2
Other disease	5
Antibiotics	8
Transplantation	3
Immunosuppressive drugs	8

Exclusion criterion 2	33
Acute hepatitis	5
AIH/PBC	14
Rare liver disease	5
Child-Pugh C	3
Inappropriate samples	6

Fig. 1 Study design for age, sex, and Child–Turcotte–Pugh class matching. The table labeled "Exclusion criterion 1" shows the details of the excluded cases; these factors might change the gut microbiota. Selection criteria were used to include patients with chronic liver disease due to HCV, HBV, ALD, or NAFLD. Although the total number of patients was small, PSM was successful because there were no significant differences in age and gender between PPI users and non-users before matching. *HBV* hepatitis B, *HCV* hepatitis C, *ALD* alcoholic liver disease, *NAFLD* nonalcoholic fatty liver disease, *PPI* proton pump inhibitor, *AIH* autoimmune hepatitis, *PBC* primary biliary cholangitis, *OUT* operational taxonomic unit

PPIs. All 31 patients in the PPI group and 50% of the PPI non-users ($n=31$, non-PPI group) were selected. All patients in PPI group continuously used PPIs for more than 1 year and patients in non-PPI group never used PPI at least 1 year before fecal samples. Although the total number of patients was small, statistical matching was successful because there were no significant differences in age and sex between PPI users and non-users before matching.

Sample collection and 16S rRNA gene sequencing

Fecal samples were collected at home or in the hospital. DNA was isolated using DNeasy PowerSoil Kit (Qiagen, Hilden, Germany). Isolated DNA was amplified to target the V3–4 regions of bacterial 16S rRNA. PCR products were pooled to construct the sequencing library, which was then sequenced using an Illumina MiSeq sequencer to generate pair-ended reads using the MiSeq Reagent Kit v3 with 2×300 reads and 600 cycles (Illumina, San Diego, CA, USA). Quantitative Insights into Microbial Ecology (QIIME 1.9.1) software and USEACH 6.1 software with Greengenes version 13_8 were used for basically analysis of 16S rRNA gene sequencing data. Closed-reference OTU picking for Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) was performed in QIIME with Greengenes version 13_5 and PICRUSt predicted functional genes categorized into Kyoto Encyclopedia of Genes and Genome (KEGG) orthology (http://www.kegg.jp/) (Supplementary material 1: Materials and methods).

Statistical analysis

The two groups were compared using the Chi square test or Fisher's exact test for categorical variables and Student's *t* test or Mann–Whitney test for continuous variables, where appropriate. The log-rank test was used to evaluate prognosis. Data were analyzed using IBM SPSS Statistics version 24. A p value <0.05 was considered to indicate significance.

The microbial composition of the PPI and non-PPI groups was compared using Linear discriminant analysis Effect Size (LEfSe) on relative taxonomic abundances at various phylogenetic levels from phylum to genus. Predicted KEGG functional classes were compared by functional gene category using STAMP. Statistical differences in the KEGG pathway were determined using White's non-parametric *t* test. (Supplementary material 2: Statistical analysis).

Results

Study population

Among 93 patients, 31 patients used PPIs (PPI group). The types of PPI were: esomeprazole 20 mg/day, daily ($n=10$), rabeprazole 10 mg/day, daily ($n=10$), lansoprazole 15 mg/day, daily ($n=9$), and vonoprazan 20 mg/day, daily ($n=2$). Before matching, the PPI users had worse CP scores and liver function. The proportion of patients with ascites ($p=0.049$), encephalopathy ($p=0.023$), and esophageal varices ($p<0.01$) was significantly higher in the PPI group than in the non-PPI group (Table 1). After PSM adjusted for age, gender, and CP class, 31 of the non-PPI users were selected into the non-PPI group. There were no significant differences in patients' background characteristics after PSM (Table 1).

Most of the samples are collected while the patient is in the hospital. A total of 4 patients, 2 for each group of PPI group and non-PPI group, collected samples at home, it is unlikely that there will be a bias in statistical difference. Some patients are hospitalized for treatment of HCC in our hospital. Therefore, most HCCs are active excluded one patient was inactive HCC in the PPI group and two in the non-PPI group. However, samples are collected prior to treatment such as RFA or TACE. At least 3 months have passed since the previous treatment. Moreover, there is no significant difference between BCLC classification in both groups.

Prognosis

The PPI group was followed for 353.6 ± 155.6 days and the non-PPI group was followed for 411.0 ± 154.0 days ($p=0.254$). Eleven patients died of hepatocellular carcinoma and one patient died due to pneumonia. There was no significant difference in mortality rate between the two groups (log rank test $p=0.285$). Regarding conditions that may relate to PPI use or non-use, two bone fractures in each group, one pneumonia in the PPI group, one duodenum ulcer in the non-PPI group, and one SBP in the PPI group were observed.

Microbiological taxonomy of the two groups at the phylum and genus levels

Figure 2a shows the comparison of relative abundance at the phylum level from stool: 88.9% of all sequence reads was classified into the *Firmicutes* (PPI vs. non-PPI group: 31.9% vs. 36.7%) or *Bacteroidetes* (58.4% vs. 50.7%) phylum. According to the other major phylum, total relative abundance of *Actinobacteria* (1.7% vs. 1.9%), *Fusobacteria*

Table 1 Patients background before and after propensity score matching

	Before propensity score matching				After propensity score matching			
	N	Non-PPI	PPI	*p*	*n*	Non-PPI	PPI	*p*
Gender (female/male)	93	28/34	8/23	0.071	62	12/19	8/23	0.277
Age (years old)	93	68.1 ± 11.5	68.4 ± 10.3	0.924	62	69.1 ± 10.7	68.4 ± 10.3	0.666
BMI	93	23.6 ± 3.8	24.8 ± 4	0.158	62	23.2 ± 3.5	24.8 ± 4	0.792
Etiology (ALD/HBV/HCV/NAFLD)	93	6/12/32/12	8/4/10/9	0.084	62	4/8/14/5	8/4/10/9	0.214
Alcohol (0/< 60 g/< 60 mg/day)	93	41/17/4	20/5/6	0.115	62	20/9/2	20/5/6	0.208
Child–Pugh score (5/6/7/8/9)	93	43/12/4/0/3	15/6/7/2/1	0.038	62	17/7/4/0/3	15/6/7/2/1	0.403
Ascites (absent/slight/moderate)	93	57/5/0	24/7/0	0.049	62	26/5	24/7	0.520
Encephalopathy (None/1–2/3–4)	93	61/0/1	28/3/0	0.023	62	30/0/1	28/3/0	0.060
History of HCC (no/yes)	93	22/40	9/22	0.534	62	7/24	9/22	0.562
Number of HCC (≤ 3/> 3)	60	25/13	14/8	0.916	44	13/9	14/8	0.939
BCLC stage (A/B/C)	62	19/16/5	8/10/4	0.745	46	10/12/2	8/10/4	0.724
statin-based medicine (yes/no)	93	10/52	4/27	0.681	62	2/29	4/27	0.390
beta-blocker (yes/no)	93	5/57	4/27	0.457	62	1/29	4/27	0.161
Smoker (never/ex-smoker/smoker)	64	25/12/7	9/6/5	0.712	43	11/9/3	9/6/5	0.621
Weekly exercise (yes/no)	64	20/25	6/13	0.352	43	13/11	6/13	0.131
Frequency of bowel movement per week (> 8/≤7)	63	13/31	5/14	0.622	42	5/18	5/14	0.522
Hardness of stool (hard/normal soft)	63	28/10/5	10/8/2	0.559	42	15/4/3	10/8/2	0.435
Varix (no/yes)	62	20/14	10/18	0.001	50	12/10	10/18	0.065
Platelet count (× 10^3cells/ul)	93	147.2 ± 66.8	118.7 ± 76.5	0.084	62	129.8 ± 56.3	118.7 ± 76.5	0.862
AST (IU/l)	93	64.4 ± 166.8	40.9 ± 18.6	0.277	62	48.5 ± 52.8	40.9 ± 18.6	0.274
ALT (IU/l)	93	93.1 ± 384.4	30.7 ± 17.9	0.207	62	50.5 ± 96.9	30.7 ± 17.9	0.549
Albumin (g/dl)	93	3.9 ± 0.5	3.6 ± 0.4	0.005	62	3.7 ± 0.5	3.6 ± 0.4	0.627
Total bilirubin (mg/dl)	93	1.0 ± 0.5	1.1 ± 0.7	0.389	62	1.0 ± 0.6	1.1 ± 0.7	0.862
Prothrombin time (INR)	93	1.0 ± 0.1	1.1 ± 0.1	0.335	62	1.1 ± 0.1	1.1 ± 0.1	0.351
CRP (mg/dl)	76	0.2 ± 0.4	0.2 ± 0.2	0.571	56	0.2 ± 0.2	0.2 ± 0.2	0.734
HbA1c (%)	52	5.9 ± 0.9	6.8 ± 1.7	0.056	36	5.7 ± 0.8	6.8 ± 1.7	0.815
Creatinine (mg/dl)	93	0.8 ± 0.2	0.8 ± 0.2	0.699	62	0.8 ± 0.3	0.8 ± 0.2	0.893
eGFR (mL/min/1.73 m^2)	93	72.2 ± 19.6	74.6 ± 22	0.606	62	73.8 ± 23.1	74.6 ± 22	0.450
Fib4-index	93	4.4 ± 3.3	5.5 ± 3	0.121	62	5.3 ± 4	5.5 ± 3	0.429

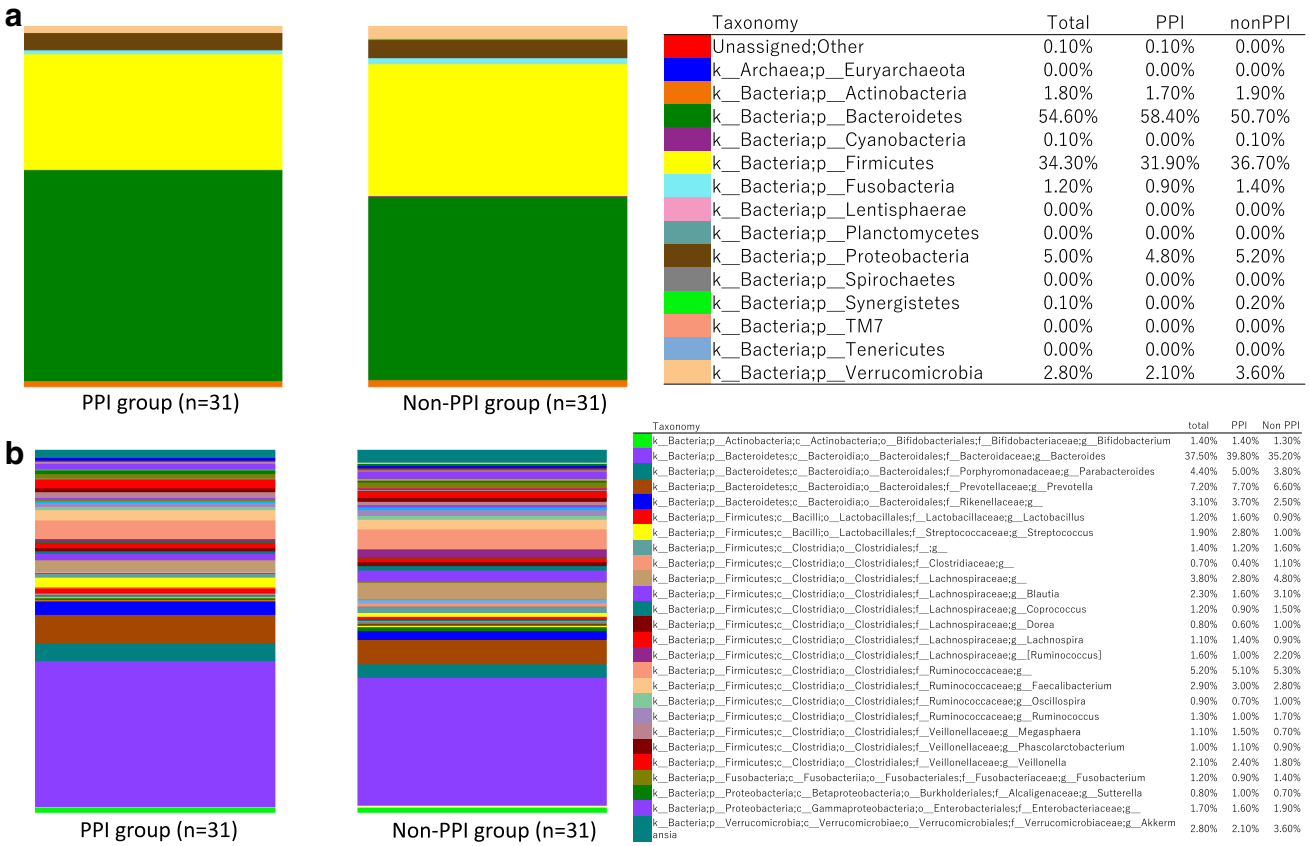
ALD alcoholic liver disease, *NAFLD* nonalcoholic fatty liver disease, encephalopathy: Grade 1: altered mood/confusion, Grade 2: inappropriate behavior, impending stupor, somnolence, Grade 3: markedly confused, stuporous, but arousable, Grade 4: comatose/unresponsive. Weekly exercise: exercise habit more than 1 time a week. *eGFR* estimate glomerular filtration rate, *Fib4-index* fibrosis index based on the four factors

(0.9% vs. 1.4%), *Proteobacteria* (4.8% vs. 5.2%), and *Verrucomicrobia* (2.1% vs. 3.6%) were over 1%. But there were no significant differences between the two groups. At the genus level (Fig. 2b) OTUs were classified into 21 different types of major genera, which were defined as having relative abundance > 1%. There was some variety between the two groups (Supplementary table). Significant differences were not shown in α and β diversity between two groups (Supplementary Figure).

Relative abundance of the two groups by LEfSe

To detect differences in relative abundance between the PPI and non-PPI groups, we compared the microbiota from the phylum to genus levels with the LEfSe program (Fig. 3a). Across the bacterial genera with significant differences in relative abundance, six genera (*Lactobacillus, Streptococcus, Selenomonas, Veillonella, Campylobacter,* and *Haemophilus*) were enriched in the PPI group. These genera belong to the *Firmicutes* or *Proteobacteria* phylum. In the PPI group, six genera (*Eggerthella, Paraprevotella, Turicibacter, Dorea, Anaerotruncus,* and *Ruminococcus*) were relatively less abundant than in the non-PPI group.

The important results are part of these microbial changes caused by PPI was shared with the changes in microbiota caused by liver cirrhosis, HE and SBP. We showed these similarities in Table 2. The second to 4th columns of Table 2 are the bacterial family level and genus level detected by LEfSe. The 5th column shows changes in the microbiota by PPI in healthy volunteers based on the reference. Changes



Taxonomy	Total	PPI	nonPPI
Unassigned;Other	0.10%	0.10%	0.00%
k__Archaea;p__Euryarchaeota	0.00%	0.00%	0.00%
k__Bacteria;p__Actinobacteria	1.80%	1.70%	1.90%
k__Bacteria;p__Bacteroidetes	54.60%	58.40%	50.70%
k__Bacteria;p__Cyanobacteria	0.10%	0.00%	0.10%
k__Bacteria;p__Firmicutes	34.30%	31.90%	36.70%
k__Bacteria;p__Fusobacteria	1.20%	0.90%	1.40%
k__Bacteria;p__Lentisphaerae	0.00%	0.00%	0.00%
k__Bacteria;p__Planctomycetes	0.00%	0.00%	0.00%
k__Bacteria;p__Proteobacteria	5.00%	4.80%	5.20%
k__Bacteria;p__Spirochaetes	0.00%	0.00%	0.00%
k__Bacteria;p__Synergistetes	0.10%	0.00%	0.20%
k__Bacteria;p__TM7	0.00%	0.00%	0.00%
k__Bacteria;p__Tenericutes	0.00%	0.00%	0.00%
k__Bacteria;p__Verrucomicrobia	2.80%	2.10%	3.60%

Taxonomy	total	PPI	Non PPI
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Bifidobacteriales;f__Bifidobacteriaceae;g__Bifidobacterium	1.40%	1.40%	1.30%
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides	37.50%	39.80%	35.20%
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;g__Parabacteroides	4.40%	5.00%	3.80%
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Prevotella	7.20%	7.70%	6.60%
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Rikenellaceae;g__	3.10%	3.70%	2.50%
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__Lactobacillus	1.20%	1.60%	0.90%
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae;g__Streptococcus	1.90%	2.80%	1.00%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__;g__	1.40%	1.20%	1.60%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__	0.70%	0.40%	1.10%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__	3.80%	2.80%	4.80%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Blautia	2.30%	1.60%	3.10%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Coprococcus	1.20%	0.90%	1.50%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Dorea	0.80%	0.60%	1.00%
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k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__[Ruminococcus]	1.60%	1.00%	2.20%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__	5.20%	5.10%	5.30%
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k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Oscillospira	0.90%	0.70%	1.00%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Ruminococcus	1.30%	1.00%	1.70%
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k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Veillonella	2.10%	2.40%	1.80%
k__Bacteria;p__Fusobacteria;c__Fusobacteriia;o__Fusobacteriales;f__Fusobacteriaceae;g__Fusobacterium	1.20%	0.90%	1.40%
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Alcaligenaceae;g__Sutterella	0.80%	1.00%	0.70%
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__	1.70%	1.60%	1.90%
k__Bacteria;p__Verrucomicrobia;c__Verrucomicrobiae;o__Verrucomicrobiales;f__Verrucomicrobiaceae;g__Akkermansia	2.80%	2.10%	3.60%

Fig. 2 Taxonomy of bacteria in the two groups at the phylum and genus levels. **a** Relative abundance of bacteria in the PPI and non-PPI groups at the phylum level. **b** Relative abundance of bacteria in the two groups at the genus level. The table shows only the major genera (relative abundance > 1%). At the phylum level, *Firmicutes* and *Bacteroidetes* were the predominant groups. The abundance of each phylum was similar between two groups. At the genus level, OTUs were classified and color-labeled by type of major genera. The list shows the only the type of major genera, defined as those with a relative abundance over 1%, in the PPI and non-PPI groups. Data on other types of genera are shown in the Supplemental Data

caused by the progression of cirrhosis irrespective of PPI are described in columns 6th to 8th. As a result of comparing these results, *Streptococcus, Veillonella, Lactobacillus, Dorea* and *Ruminococcus* are changed in each row among our research results. Some oral-origin microbiota (*Streptococcaceae,* and *Veillonellaceae*) was detected as rich taxes abundance in PPI group and some autochthonous (*Lachnospiraceae* and *Ruminococcaceae*) was also detected as rich taxes abundance in non-PPI group. In other words, it is suspected to be common change of bacteria. It shows the possibility that it is a more important bacteria among changes caused by PPI.

Figure 3b shows the differences in microbiota between PPI users ($n = 31$) and non-users ($n = 62$) before PSM. When the PPI group was compared to PPI non-users, who had better liver function, some common changes at the genus level, such as increased relative abundance of *Lactobacillus, Streptococcus, Haemophilus,* and *Campylobacter* and decreased relative abundance of *Anaerotruncus* and *Ruminococcus,* were observed.

Subgroup analysis for results by LEfSe about the factors of "etiology", "habitual drinking" "mild cirrhosis", "HCC" and "CP class"

Several factors can affect the relationship between liver disease and microbiome. Therefore, subgroup analysis was performed on factors of "etiology", "habitual drinking", "mild cirrhosis", "HCC" and "CP class" respectively (Supplementary material 3 subgroup analysis). In our study, we showed that 6 types of bacteria detected by LEfSe at the genus level have a higher relative abundance each in the PPI group or the non-PPI group (Table 2). We compared 12 genus for each factor of which relative abundance is higher. Even though it was classified into groups for each factor, most of bacteria belonging to the rich relative abundance in PPI group increased in the PPI group. The same result showed about

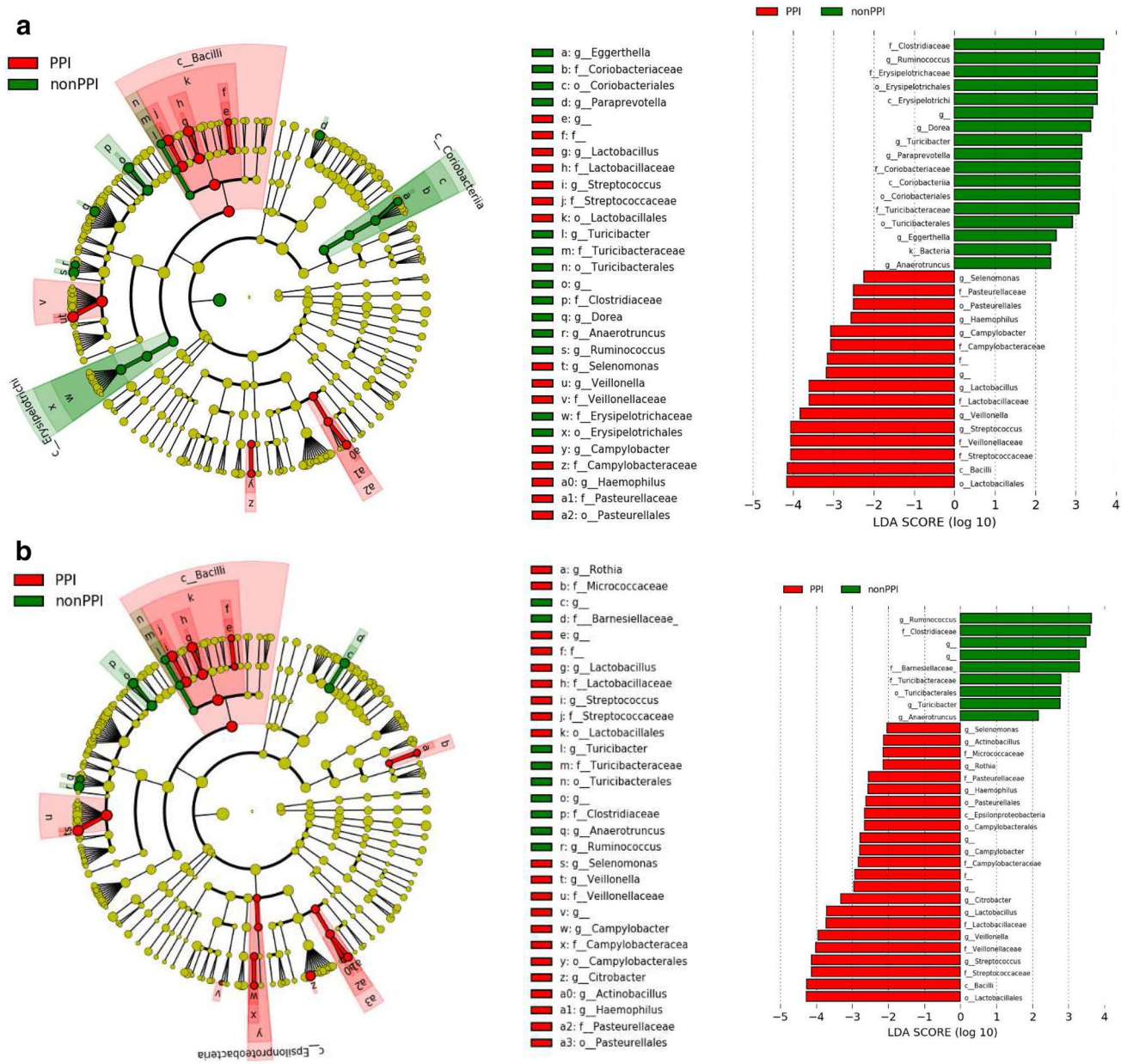

Fig. 3 Significant differences in microbiota between the two groups by LEfSe before and after matching. **a** Comparison of microbiota between the PPI and non-PPI groups after LEfSe matching. In the PPI group, six genera, consisting of *Lactobacillus*, *Streptococcus*, *Selenomonas*, *Veillonella*, *Campylobacter*, and *Haemophilus* were enriched and six genera, consisting of *Eggerthella*, *Paraprevotella*, *Turicibacter*, *Dorea*, *Anaerotruncus*, and *Ruminococcus* were less abundant. **b** Comparison of microbiota between PPI users ($n=31$) and PPI non-users ($n=62$) before matching. An increased relative abundance of *Lactobacillus, Streptococcus, Haemophilus,* and *Campylobacter* and decreased abundance of *Anaerotruncus* and *Ruminococcus* were found; findings were similar before and after matching

the non-PPI group. It is thought that most changes in microbiota were consistently observed independently regardless of these factors.

Differences in the functional metagenome based on PICRUSt

Lastly, we used PICRUSt to identify the difference of KEGG pathways in microbiota between the two groups at levels 1–3 (Fig. 4). In addition, differences in one type of KEGG pathways at level 1 (Fig. 4a) and six type at

Table 2 The relationship between the gut microbiological change by PPI use and the reference which reported about the effects by cirrhosis or its complication

	Our study			Reference			
	Family	Genus	CP A/B with PPI v.s. CP A/B without PPI	HC with PPI v.s. HC without PPI	LC v.s. HC	HE	SBP***
Rich relative abundance in PPI group	Lactobacillaceae		↑		↑ [25]	↑ [26] [25]	
	Lactobacillaceae	Lactobacillus	↑	↑ [17]			
	Streptococcaceae		↑	↑ [9] [10]	↑ [19]	↑ [27] [24]	
	Streptococcaceae	Streptococcus	↑	↑ [10] [9] [28]	↑ [29] [30]		
	Veillonellaceae		↑		↑ [19] [23]	↑ [24]	↓ [31]
	Veillonellaceae	Selenomonas	↑				
	Veillonellaceae	Veillonella	↑	↑ [17]	↑ [23] [30]		
	Campylobacteraceae		↑				
	Campylobacteraceae	Campylobacter	↑				
	Pasteurellaceae		↑				
	Pasteurellaceae	Haemophilus	↑				
Rich relative abundance in non-PPI group	Erysipelotrichaceae		↓	↓ [9]			
	Coriobacteriaceae		↓				
	Coriobacteriaceae-	Eggerthella	↓				
	Paraprevotellaceae	Paraprevotella	↓				
	Turicibacteraceae		↓				
	Turicibacteraceae	Turicibacter	↓	↓ [28]			
	Clostridiaceae		↓	↓ [28]		↓ [25]****	↓ [31]****
	Clostridiaceae	Unclassified	↓				
	Lachnospiraceae	Dorea	↓	↓ [9]*	↓ [23]*	↓ [26]*	↓ [31]*
	Ruminococcaceae	Anaerotruncus	↓				
	Ruminococcaceae	Ruminococcus	↓	↓ [9]** [10] [17]↑ [28]	↓ [27] [32] [23]**	↓ [25] [26] [27]**	↓ [31]**

CP Child–Turcotte–Pugh class, *HC* healthy control, *PPI* proton pump inhibitors, *LC* liver cirrhosis, *HE* encephalopathy, *SBP* spontaneous bacterial peritonitis

*Decrease Lachnospiriaceae

**Decrease Ruminococcaceae

***Compared cirrhosis patients between with/without infection. Twelve SBP patients is included to 38 cirrhosis patients with infection

****Decrease *Firmicutes_Clostridialies_XIV*

level 2 (Fig. 4b) were found. We also identified five types of KEGG pathways predicted to be significantly different at level 3. These included pathways associated with drug metabolism—other enzymes, bacterial motility protein, apoptosis, bacterial chemotaxis, and chloroalkane and chloroalkane degradation (Fig. 4c).

PICRUSt is a predictor function gene, not all genes are expressed. The genes shown are genes of bacteria. Among them, important factors related as microbiome are the following factors. Metabolism of other amino acids include beta-alanine, D-glutamine, D-arginine, D-ornithine and D-alanine metabolism. Lipid metabolism is not only related to Fatty acid biosynthesis and degradation including short chain fatty acids but also steroid synthesis involved in bile acid synthesis. Bacterial motility proteins and bacterial chemotaxis are both related to the movement of bacteria. Loss of motility presumably is associated with an adaptive strategy to escape detection by the host immune system. Although some of these factors could be detected, it is necessary to clarify more pathways to systematically explain these factors.

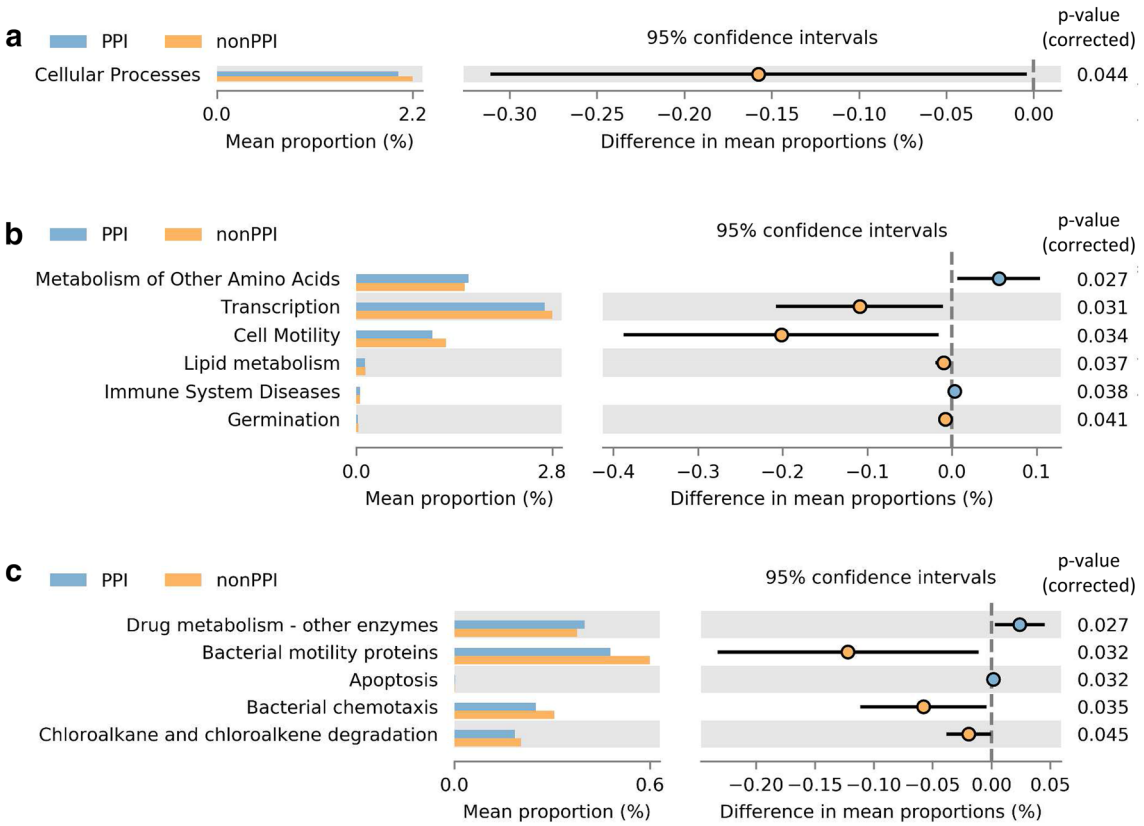

Fig. 4 Differences in predicted functional gene content of bacterial communities at levels 1–3 of the KEGG pathway analyzed using PICRUSt. **a** level 1, **b** level 2, **c** level 3. Functional gene content was predicted based on 16S rRNA data using the PICRUSt algorism. These predicted genes were classified by KEGG pathway. The figure identifies the categories with significant differences between the PPI and non-PPI groups. Orange and blue bars show the abundance of genes in each category. At level 1, the abundance of genes related to cellular processes was enriched in the non-PPI group. Five categories at level 2 and six categories at level 3 were predicted as being functionally different. The function and role of the gut microbiota of the two groups might differ

Discussion

A recent increase in the number of patients with gastroesophageal reflex disease and the use of antiplatelet drugs has been associated with an increase in PPI use. As a long-known adverse reaction, bone fracture, *Clostridium difficile* infection and intestinal infection are reported. These adverse reactions are speculated to be caused by the breakdown of the gastric acid barrier due to PPIs lowering the concentration of gastric acid, [4, 8] which results in an increase in resident oral bacteria normally broken down by gastric acid and alteration of the microbiota [9, 10].

Risks associated with PPI use in patients with liver cirrhosis have also been discussed. An increased risk of SBP and HE has been identified in patients with liver cirrhosis who use PPIs. Tsai et al. [1] noted that among 1166 patients with liver cirrhosis who developed HE, 38% used PPIs and the risk of HE increased was PPI dose-dependent. Dam et al. [11] and Bian et al. [12] showed that PPI use increases the risk of HE in case–control studies. Nevertheless, there is still some debate over the risk posed by PPIs due to an insufficient number of cases and lack of prospective studies [11].

Some meta-analyses have also been published on the relationship between PPIs and SBP [13]. Yu et al. [2] analyzed 10 case–control studies and six cohort studies involving 8145 people. The overall analysis showed that PPIs are associated with SBP (OR = 2.11, 95% CI 1.46–3.06). However, no association between PPIs and SBP was apparent in many prospective cohort studies included in the analysis, which demonstrates a need for studies that investigate the cause of this association as well as epidemiological studies.

HE and SBP are associated with gut microbiota. Clinically, antibiotics such as rifaximin and laxatives such as lactulose and polyethylene glycol can prevent HE. SBP is also presumed to be caused by bacterial infection from the gastrointestinal tract. This has led to speculation that PPI-induced changes in gut microbiota may cause HE and SBP. This issue would normally be explained by an evaluation of small intestinal bacterial overgrowth (SIBO) and

an exhaustive analysis of microbiota using next-generation sequencing.

There is still some debate on whether there is a relationship between PPIs and SIBO. Su et al. performed a meta-analysis of 19 studies and reported that PPIs cause a moderate increase in the risk of SIBO, although a positive correlation was only identified in 7 of the 19 studies [14]. Some recent reports have also found no association between PPIs and SIBO [15] [16], indicating a need for further studies.

On the other hand, a comparison of gut microbiota between healthy individuals with versus without PPI use has identified consistent results. We reviewed the changes in microbiota as a healthy control group in the 5th column of Table 2. There have already been many reports on the effect of PPI on microbiome in patients without liver disease. The two papers published in Gut are very carefully considered, and therefore, highly credible [9, 17]. In addition, a review by Naito et al. [3] has summarized the specific increase and decrease of bacteria. It reviewed eight studies, found many common features, including the increased presence of the *Streptococcaceae* family or *Streptococcus* genus in all studies and the decreased presence of the *Ruminococcaceae* family in four studies. Similar changes are reported in Japanese as well [18]. In other words, it is shown that healthy control use PPI has increased oral bacteria such as *Streptococcus* and decrease Luminococcus. We believe that our results are acceptable because our study also identified similar changes, including the increased presence of the *Streptococcus* genus and decreased presence of the *Ruminococcaceae* family. Especially our findings were similar to the study by Imhann et al. [17]. It showed the increased presence of the *Bacilli* class, *Lactobacillales* order, *Pasteurellales* order, *Veillonella* genus, and the decreased presence of the *Ruminococcus* genus in PPI users without liver disease. In other words, PPIs will have almost identical effects in the patients with or without chronic liver disease.

Some reports have shown that this increase in *Streptococcacus* and decrease in *Ruminococcus* can also occur with progression of liver cirrhosis. In 2011, Chen et al. [26] reported an association between CP class and increased *Streptococcusae, Veillonellaceae* and decreased *Ruminococcus*. Figure 3a and Table 2 shows the result of microbiota extracted by LEfSe. Microbial changes due to cirrhosis, HE and SBP based on past reports. Increased presence of the *Lactobacillaceae, Streptococcaceae,* and *Veillonellaceae* families and decreased presence of the *Lachnospiraceae* and *Ruminococcaceae* families appear common to both PPI use and liver cirrhosis. In clinical practice, PPI user tends to have worse liver function and MELD score. Figure 3b shows the microbial change could not be counterbalanced by the effect of worse liver function. And the similar change is recognized. This change may be more similar to the change that occurs in the actual patient.

There are several factors that affect microbiota on liver disease. We did subgroup analysis on etiology, daily alcohol intake and thrombocytopenia among them. Thrombocytopenia was used as an indicator of mild cirrhosis. Our study has a mixture of etiologies for chronic liver disease in the 62 patients included for final analysis (ALD, HBV, HCV, NAFLD). Even though it was classified into groups for each etiology, most of bacteria belonging to the rich relative abundance in PPI group increased in the PPI group. The same result was obtained for the non-PPI group. It is thought that our research results were consistent regardless of etiology. And various stages of liver diseases may affect the microbiota; we do not include patients with severe liver cirrhosis because we are analyzing CP A or B patients in our study. Additionally, many patients are classified as CP A. Therefore, the stage of progression of liver disease is almost similar. But even the patients belong to the CP A, mild cirrhosis may affect microbiota. In patients with thrombocytopenia, mild cirrhosis, completely matched the result and most of results are consistent in the patients without thrombocytopenia in text. About the daily alcohol intake, the patient's microbiota is affected by PPI as well as those who are not drinking. So we think that the results of this study are likely to have similar results on the various stages of liver disease. The impact of PPI was a stronger factor than these factors.

This is the first clinical study that compares the gut microbiota of Japanese patients with chronic liver disease based on statistically matched backgrounds who regularly take oral PPIs. The common microbial changes we reported between PPI use and progression of liver cirrhosis have possibility to be associated with increased risks of HE and SBP. If the patients with liver disease use PPIs, the microbiota will show a change similar to the cirrhotic change (Table 2). Its intestinal environment caused by PPI may have relationship to increase the risk factor for HE or SBP. The result in our study is similar to the report by Bajaj. They focused mainly on patients with severe liver cirrhosis. Some trend of microbial change, higher oral-origin microbiota (*Streptococcaceae,* and *Veillonellaceae*) and lower autochthonous (*Lachnospiraceae* and *Ruminococcaceae*), are common to our study. But severe cirrhosis might change the gut microbiota. Just only one report about the patients with CP class A cirrhosis was published. It compared the intra-individual changes by short-term PPI use. They took 40 mg/day of omeprazole for 14 days, which was associated with an increased relative abundance of *Streptococcaceae* and reduced abundance of autochthonous bacteria, including *Ruminococcaceae*. It is also necessary to evaluate this relationship in studies of inter-individual differences. Regarding gut microbiota, inter-individual differences are larger than

intra-individual changes. The number of reports about the patients without CP class C is not enough. And this will be helpful to clarify the relationship.

We hypothesized that the cause of this change is interrelation of the intestinal environment. Although not yet proven in the human body, many bacteria in the *Bacilli* class, to which bacteria in the *Streptococcaceae* family belong, commonly synthesize lactate via fermentation in nature. Meanwhile, members of the *Veillonellaceae* family that use lactate will tend to increase in conjunction with lactate synthesis. PPIs reportedly cause an increase in hydrochloride salts, and the growth rate of butyric acid–producing bacteria such as members of the *Ruminococcaceae* and *Lachnospiraceae* families is suppressed in the reduced pH environment caused by lactic acid. There is a relationship between butyric acid and other short-chain fatty acids that arise from these changes and IgA levels and gut immunity.

Inoue et al. [20] reported that *Lachnospiraceae* [21] and *Ruminococcaceae* [22] are involved in carbohydrate fermentation to short chain fatty acids (SCFAs) in the human intestine. SCFA is a nutrient of the colonic epithelium, which regulates the pH of the colon, indicating that a decrease in SCFA results in an increase in fecal pH, an increase in ammonia production, absorption in the intestinal tract, and hyperammonemia. It has also been shown that these bacteria decrease and that *Veillonellaceae* increases, which is related to bile acid metabolism [23]. Zhang et al. [24] showed that *Streptococcaceae* and *Veillonellaceae* are related to minimal encephalopathy and overt encephalopathy via ammonia production increase.

To prove these intestinal environments, functional profiling via PICRUSt was performed. Although a number of factors yielded a significant difference, we were unable to identify reasons that could be related to increased risk of HE and SBP. As in few previous reports on the topic, further research is needed to fully understand the significance of these results.

This study has some limitations. We showed the relationship between PPI and microbiota and its risk of HE and SBP. But clear mechanism could not be elucidated. We evaluated microbiota with fecal sample only. But fecal sample does not show all of intestinal microbiota. It is necessary to further investigate various samples such as SIBO, small intestine or colon mucosal bacteria. And a large prospective study is necessary to evaluate whether PPI really increases adverse events. But reports published in Europe, the US, Taiwan, etc. point out the possibility that PPI may increase the risk of encephalopathy and SBP. Therefore, there is a high possibility that risk increases in Japan as well. Our clinical incidence was low because our patients were belonging to CP class A or B and the observation period is short. These factors make the incidence of adverse events low. The further research with long-term and large number of patients is necessary to evaluate the real incidence of PPI side effect in Japan. Since we use PSM to equalize age, we could not independently consider volunteer group of the same age. Because people in this age often had a history of disease and surgery. However, we reviewed changes due to PPI based on high quality reference.

What we wanted to show is the possibility of side effects caused by PPI via microbiota. In other words, we do not think that PPI directly causes these complications; we think that microbiota affected by PPI may be related as a reason for risk of encephalopathy or SBP. This hypothesis is clinically important. Because when encephalopathy or SBP is suspected as a side effect of PPI, it can be treated with antibiotics or laxatives.

In conclusion, we showed that a part of microbial changes caused by PPI was common to the changes by progression of liver cirrhosis. And some of these changes may have potential to be the risk factor of HE and SBP. And PPI increases oral-origin microbial taxa and decreases autochthonous taxa in Japanese liver disease patients. PPI-induced changes are thought to be present from early to advanced stages of hepatitis. Such long-term changes may increase the risk for a variety of events. Our findings may have a potential to be used as a biomarker for the risk patients who may develop HE or SBP. And some prebiotic or probiotic treatments witch can control these microbiota may be useful to prevent the risk.

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Compliance with ethical standards

Ethical approval This study was approved by the Research Ethics Committee of Nagoya University Hospital on August 30, 2016 (Protocol number 2015-0420). In accordance with the Declaration of Helsinki, written informed consent was obtained from all patients before registration. This study is registered in University Hospital Medical Information Network Clinical Trials Registry (UMIN ID: 000020269).

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