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MAJOR ARTICLE

Gut Dysbiosis Associated With Hepatitis C Virus Infection

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(See the Editorial Commentary by Meissner on pages 878–80.)

Background. Little is known about the effect of hepatitis C virus (HCV) infection on gut microbiota and the relationship between alteration of gut microbiota and chronic hepatitis C (CHC) progression. We performed a comparative study of gut microbiota composition between CHC patients and healthy individuals.

Methods. Fecal samples from 166 CHC patients were compared with those from 23 healthy individuals; the gut microbiota community was analyzed using 16S ribosomal RNA gene sequencing. CHC patients were diagnosed with persistently normal serum alanine aminotransferase without evidence of liver cirrhosis (LC) (PNALT, n = 18), chronic hepatitis (CH, n = 84), LC (n = 40), and hepatocellular carcinoma in LC (n = 24).

Results. Compared with healthy individuals, bacterial diversity was lower in persons with HCV infection, with a decrease in the order Clostridiales and an increase in *Streptococcus* and *Lactobacillus*. Microbiota dysbiosis already appeared in the PNALT stage with the transient increase in *Bacteroides* and Enterobacteriaceae. Predicted metagenomics of microbial communities showed an increase in the urease gene mainly encoded by viridans streptococci during CHC progression, consistent with a significantly higher fecal pH in CH and LC patients than in healthy individuals or those in the PNALT stage.

Conclusions. HCV infection is associated with gut dysbiosis, even in patients with mild liver disease. Additionally, overgrowth of viridans streptococci can account for hyperammonemia in CH and LC. Further studies would help to propose a novel treatment strategy because the gut microbiome can be therapeutically altered, potentially reducing the complications of chronic liver disease.

Keywords. chronic hepatitis C; gut dysbiosis; viridans streptococci; hyperammonemia; fecal microbiota transplantation.

Hepatitis C virus (HCV) affects 130–210 million people worldwide, and HCV infection is a major risk factor for liver cirrhosis (LC) and hepatocellular carcinoma (HCC) [1]. HCV infection progresses chronically through several clinical stages determined by liver damage: persistently normal serum alanine aminotransferase (PNALT), chronic hepatitis (CH), LC, and HCC.

Gut microbiota is regarded as "a hidden organ" that carries 100 trillion bacteria cells comprising several hundred species, totally encoding 100-fold more genes than the human genome [2]. With its collective activities and cooperative metabolisms, gut microbiota influences a host's susceptibility to various diseases, including chronic liver diseases [3]. Due to its anatomical location, the liver is exposed to gut-derived bacterial components [4]. The gut microbial community is closely associated with the progression of liver diseases because of gut–liver circulation via the gut–microbiota–liver axis [3]. Previous studies indicate that alteration of gut microbiota, called dysbiosis, affects chronic liver disease, including nonalcoholic fatty liver [5], nonalcoholic steatohepatitis [6], alcoholic liver disease [7], and primary biliary cholangitis [8]. Recent evidence supports the association of gut microbiota with the most severe forms of liver disease (ie, cirrhosis [9] and HCC [4]). Petersen et al previously categorized 3 types of dysbiosis: loss of beneficial microbial organisms, expansion of pathobionts or potentially harmful microorganisms, and loss of overall microbial diversity [10].

Gut microbiota may be associated with the progression of CHC and with related complications. Recently, Aly et al compared the gut microbiota from 6 LC patients infected with HCV with that from 8 healthy individuals, suggesting possible microbiome remodeling in CHC [11]. Additionally, Heidrich et al analyzed the gut microbiota of 95 CHC patients, including 38 cirrhosis and 50 healthy controls, in a cross-sectional approach. They showed that not only the stage of liver disease but also HCV infection were associated with a reduced alpha diversity and different microbial community patterns [12]. However, to the best of our knowledge, detailed investigations of the characteristics of gut microbiota associated with HCV infection and dysbiosis have never been performed in a large population of clinically classified CHC patients covering each clinical stage (PNALT, CH, LC, and HCC).

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To elucidate the relationship between gut microbiota and CHC, we characterized gut microbiota from a large number of CHC patients with PNALT, CH, LC, and HCC using high-throughput 16S rRNA gene sequencing. Our findings can help in the development of novel and advanced approaches to control gut microbiota, such as the use of pre- and probiotics or fecal microbiota transplantation (FMT) as novel treatments to prevent CHC progression.

METHODS

Materials and methods are fully described in the Supplementary Materials. Briefly, 166 CHC patients and 23 healthy individuals were enrolled. The definition of clinical stages is provided in the Supplementary Materials. Each CHC patient provided fecal samples once, and each healthy individual provided samples twice, with a 1-month interval.

Total bacterial DNA was isolated from stool samples using the bead-beating method followed by phenol extraction. The variable V1-V2 region of the 16S rRNA gene was amplified using polymerase chain reaction and then subjected to high-throughput sequencing using the MiSeq paired-end sequencing system (Illumina Inc., San Diego, California). The obtained pairs of sequences were processed using the Uparse pipeline in Usearch v9.2 [13]. They were merged and classified into 1431 taxonomically annotated operational taxonomic units (OTUs). The diversity index was calculated using the Shannon effective species count [14], based on the OTU composition in each sample. The read counts of each OTU in each sample were tabulated, subsampled for a sequence depth equal to 5000 (Supplementary Table 2), and applied to UniFrac-nonmetric multidimensional scaling (NMDS), partial least square discriminant analysis (PLS-DA), and phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) analyses. The OTU table was further summarized at different taxonomic levels from phylum to genus (Supplementary Table 3a, b, c, d, and e) and applied to Bray-Curtis NMDS and the linear discriminant analysis effect size.

Measurement of fecal pH and statistical analysis are fully described in the Supplementary Materials.

RESULTS

Clinical Characteristics of the Recruited CHC Patients and Healthy Individuals

The numbers of CHC patients diagnosed with PNALT, CH, LC, and HCC were 18, 84, 40, and 24, respectively (Table 1). Twelve (30%) LC patients and 14 (58.3%) LC with HCC patients were diagnosed with decompensated cirrhosis. All LC patients received advanced methods in addition to ultrasonography as follows: biopsy alone (n = 4), magnetic resonance imaging (MRI) alone (n = 11), elastography plus MRI (n = 4), and MRI plus biopsy (n = 21).

There were significant differences in the age between PNALT, LC, and HCC patients and healthy individuals (P = .017, .026, and .0051, respectively), whereas there was no significant difference in age between CH patients and healthy individuals. Other significant differences are shown in Table 1.

Gut Microbial Community Shift in CHC Patients

The paired samples obtained from the same individuals were rather consistent compared to those across individuals (Supplementary Figure 2). Moreover, the variance among CHC patients was greater than among healthy individuals. The

Table 1. Demographics and Clinical Characteristics of Chronic Hepatitis C Patients and Healthy Individuals

Characteristics at Time of Fecal Sampling	Category (Number of Candidates)				
	Persistently Normal Serum Alanine Aminotransferase (n = 18)	Chronic Hepatitis (n = 84)	Liver Cirrhosis (n = 40)	Hepatocellular Carcinoma in Liver Cirrhosis (n = 24)	Healthy (n = 23)
Gender (M/F)	8/10	34/50	19/21	13/11	14/9
Age (years)	71.4 ± 12.5[a]	66.1 ± 12.8	69.2 ± 9.8[b]	71.8 ± 8.2[c]	60.5 ± 8.1[a, b, c]
Platelet count (×10^4/mm^3)	18.3 ± 4.7[d, e]	15.7 ± 5.0[f, g]	9.7 ± 4.8[d, f]	10.4 ± 4.5[e, g]	n.d.
Prothrombin time (%)	97.2 ± 13.6[h, i]	89.6 ± 11.8[j, k]	77.5 ± 15.1[h, j]	77.1 ± 13.1[i, k]	n.d.
Serum albumin (g/dL)	4.1 ± 0.4[l]	4.1 ± 0.4[m]	3.8 ± 0.6	3.5 ± 0.6[l, m]	n.d.
Asparate-2-oxoglutarate aminotransferase (IU/L)	24.1 ± 6.2[n]	49.0 ± 43.0	48.2 ± 28.2	67.5 ± 67.3[n]	n.d.
Alanine-2-oxoglutarate aminotransferase (IU/L)	16.9 ± 7.7	55.5 ± 113.2	32.2 ± 17.5	51.0 ± 64.6	n.d.
γ-Glutamyl transpeptidase (IU/L)	17.3 ± 5.5[o]	39.1 ± 35.2	40.2 ± 42.8	51.0 ± 46.9[o]	n.d.
Total bilirubin (mg/dL)	0.9 ± 0.4[p]	1.0 ± 0.7[q]	1.1 ± 0.5	1.7 ± 1.7[p, q]	n.d.
Alpha fetoprotein (ng/mL)	2.7 ± 0.9	7.5 ± 15.7[r]	10.3 ± 9.3[s]	348.9 ± 1261.1[r, s]	n.d.
Protein induced by vitamin K absence or antagonist-II (mAU/mL)	18.6 ± 5.5	20.9 ± 15.0	41.2 ± 58.2	6785.2 ± 31036.5	n.d.
Fibrosis-4 index	2.2 ± 0.8[t, u]	3.6 ± 2.2[v, w]	7.5 ± 4.2[t, v]	7.6 ± 4.0[u, w]	n.d.

Continuous data are expressed as means ± standard deviation. Superscript letters indicate a significant difference in 1-way analysis of variance followed by Tukey–Kramer post-analysis (P < .0001: d, e, f, g, h, l, j, m, t, u, v, and w; P < .001: k and l; P < .01: c, n, and q; P < .05: a, b, o, p, r, and s).

Abbreviation: n.d., not determined.

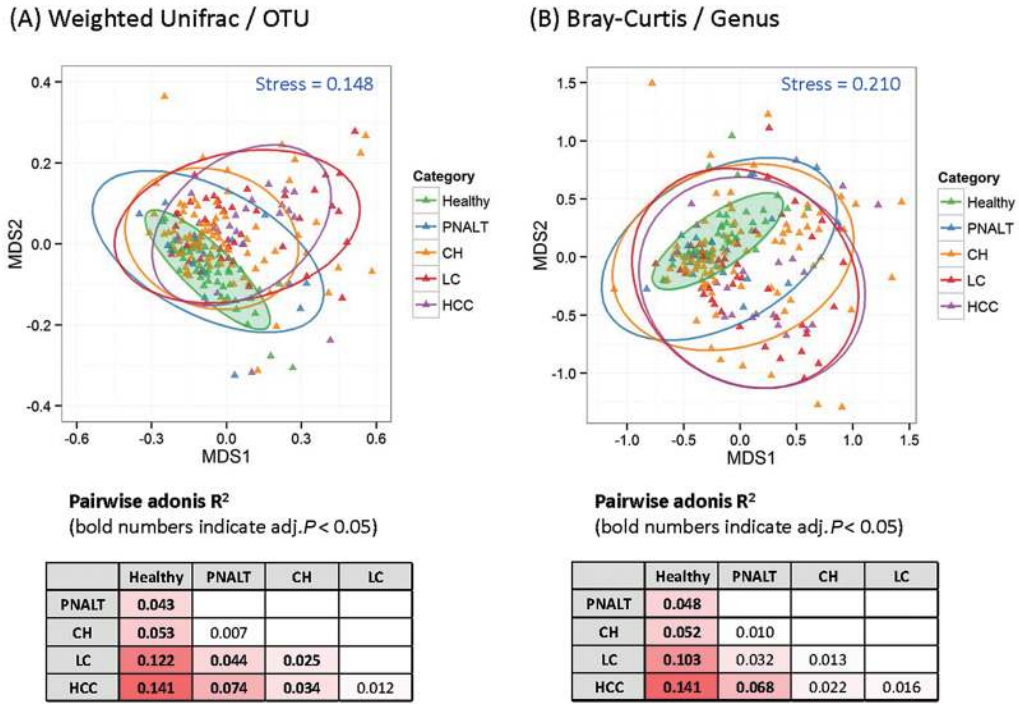
compositional differences of individual fecal microbiota was displayed on the NMDS plot (Figure 1A), suggesting that gut microbiotas of CHC patients varied substantially, whereas those of healthy patients were more consistent. Further, the Bray-Curtis distance based on genus composition showed a similar trend as the weighted Unifrac distance (Figure 1B). These results indicate that the gut microbial community was altered in most of the CHC patients, notably in LC or HCC patients.

Subsequently, we performed the PLS-DA, a supervised classification with a selected set of 50 OTUs that characterized the community structure of each group. CH and LC + HCC samples were clustered separately from the healthy group with the quality assessment (Q^2) statistics higher than 0.5, a generally acceptable threshold, while PNALT samples were placed between these groups (Figure 2A). Healthy and CHC groups diverged in the positive and negative regions of component 1; HCV infection highly correlated with component 1 with adjusted R^2 = 0.53 and p = 2.2e-16. The component 2 score of the PLS-DA plot slightly separated PNALT and CH groups from the LC + HCC group. The component 2 score significantly correlated with some clinical parameters associated with the progression of CHC, notably the fibrosis-4 (FIB-4) index, which represents liver fibrosis (R^2 = 0.164 and p = 4.03e-8 in the envfit permutation test).

Furthermore, the effect of possible confounding factors on the microbiota was evaluated by multilinear regression in the PLS-DA model (Figure 2B and Supplementary Table 4). Age significantly correlated with component 1 scores, since healthy individuals were significantly younger than CHC patients. The proportion of variance (R^2) explained by HCV infection for component 1 and the FIB-4 index for component 2 remained dominant after adjustment by these confounding factors (standardized coefficient = −0.690 and −0.326 with $P < .001$, respectively), whereas that by age for component 1 and 2 were −0.139 ($P = .006$) and −0.156 ($P = .045$), respectively. The administration of proton pump inhibitors (PPIs) marginally but not significantly correlated with the component 2 score.

Decrease of Gut Microbial Diversity in Association With CHC Progression

The Shannon effective number showed a decreasing gradient from the healthy individuals cluster with high scores (Figure 3A). Indeed, gut microbial diversity in PNALT, CH, LC,



Pairwise adonis R^2
(bold numbers indicate adj.P< 0.05)

	Healthy	PNALT	CH	LC
PNALT	**0.043**			
CH	**0.053**	0.007		
LC	**0.122**	**0.044**	**0.025**	
HCC	**0.141**	**0.074**	**0.034**	0.012

Pairwise adonis R^2
(bold numbers indicate adj.P< 0.05)

	Healthy	PNALT	CH	LC
PNALT	**0.048**			
CH	**0.052**	0.010		
LC	**0.103**	0.032	0.013	
HCC	**0.141**	**0.068**	0.022	0.016

Figure 1. Nonsupervised multivariate analyses of gut bacterial community in healthy individuals and chronic hepatitis C (CHC) patients. *A,* Nonmetric multidimensional scaling (NMDS) plot based on weighted UniFrac distances calculated using the operational taxonomic unit (OTU) compositions and phylogeny. *B,* NMDS plot based on Bray-Curtis distances calculated using genus compositions. A total of 212 fecal samples (2 sets from 23 healthy individuals and 1 set from 166 CHC patients diagnosed as persistently normal serum alanine aminotransferase, chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma) were subjected to 16S rRNA gene sequencing. Weighted UniFrac distance was calculated using OTU compositions in (*A*), and Bray-Curtis distance was calculated using genus compositions in (*B*). The NMDS plots were constructed based on these distance matrices. Ellipses indicate 95% confidence intervals around samples from each category. Adonis R^2 (effect size) between groups of different category subjects was calculated with 999 permutations and shown in the lower part of the figure. Abbreviations: CH, chronic hepatitis; HCC, hepatocellular carcinoma; LC, liver cirrhosis; MDS, multidimensional scaling; OTU, operational taxonomic unit; PNALT, persistently normal serum alanine aminotransferase.

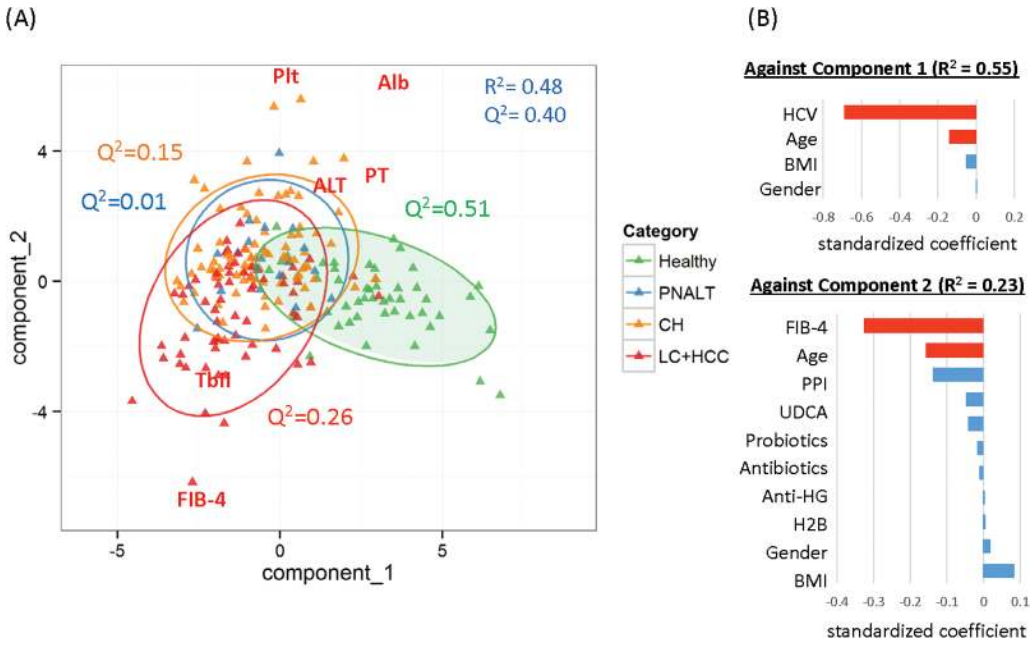


Figure 2. Partial least square discriminant analysis (PLS-DA) of gut bacterial community associated with chronic hepatitis C (CHC). *A,* PLS-DA plot of gut bacterial community in healthy and CHC groups. PLS-DA was analyzed using the operational taxonomic unit compositions of 212 samples from healthy, persistently normal serum alanine aminotransferase (PNALT), chronic hepatitis (CH), and liver cirrhosis + hepatocellular carcinoma (LC+HCC) groups. Q^2 for 4 groups was 0.40, whereas that for healthy, PNALT, CH, an LC+HCC groups against the others was 0.51, 0.01, 0.15, and 0.26, respectively. Regression of samples distribution in the PLS-DA plot to clinical parameters were calculated using the Envfit R program, and clinical parameters whose R^2 was higher than 0.1 were plotted. The clinical parameters were regressed within the groups except for the healthy group. *B,* Relative weight (standardized coefficient) of clinical parameters, physical factors, and clinical factors in multilinear regression against PLS-DA scores. For component 1 of PLS-DA, hepatitis C virus infection was used as an independent variable to represent clinical parameters. For component 2, the fibrosis-4 index represents the parameter for liver fibrosis. Factors showing *P* value < .05 are depicted in red (See Supplementary Table 4 for details of the multilinear regression analysis). Abbreviations: Alb, serum albumin; ALT, alanine-2-oxoglutarate aminotransferase; BMI, body mass index; CH, chronic hepatitis; FIB-4, fibrosis-4; H2B, histamine H2-receptor antagonist; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HG, hyperglycemic agents; LC, liver cirrhosis; Plt, platelet count; PNALT, persistently normal serum alanine aminotransferase; PPI, proton pump inhibitor; PT, prothrombin time; TBil, total bilirubin; UDCA, ursodeoxycholic acid. Bold characters: factors that significantly relate to the category.

and HCC patients significantly decreased compared with that in healthy individuals ($P < .05$; Figure 3B) and was associated with the severity of the clinical stage (Figure 3B and C). This was mainly due to the loss of a number of OTUs belonging to gut commensal families Lachnospiraceae and Ruminococcaceae. On the other hand, the number of OTUs belonging to the families Streptococcaceae, Lactobacillaceae, and Enterobacteriaceae significantly increased in CHC patients (Figure 3C).

Phylogenetic Skew in Each Clinical Stage of CHC

The relative abundance of all taxonomic groups was statistically compared at each taxonomic level among healthy, PNALT, CH, and LC+HCC groups using linear discriminant analysis (LDA). Taxa statistically abundant in each group were represented by the corresponding group color in a cladogram (Figure 4A). The healthy group was highly abundant in class Clostridia, notably families Lachnospiraceae and Ruminococcaceae, a highly diversified taxonomic class and common gut commensals. Interestingly, the PNALT group showed unique features, with increased family Enterobacteriaceae and genus *Bacteroides*. Meanwhile, class Bacilli, mainly including genus *Streptococcus* and *Lactobacillus*, was significantly abundant in the LC group (Figure 4A), suggesting that the bacterial community established in healthy individuals was replaced by subdominant groups in CHC patients. Next, the OTUs with significantly higher LDA scores for either the PNALT or the HCC group were shown with the closest species and relative abundance in each group (Figure 4B). *Streptococcus salivarius* drastically increased in association with CHC progression. Additionally, some other minor species belonging to viridans streptococci or genus *Lactobacillus* gradually increased with CHC progression.

Streptococcus overgrew in the gut microbiota of a large number of CH, LC, and HCC patients (Figure 5). This trend was more evident in patients who received PPI or histamine H2-receptor antagonists (H2Bs), suggesting that these treatments may affect gut microbiota. However, the overgrowth of *Streptococcus* was observed even in patients without PPI or H2B, particularly in LC and HCC patients, indicating that alteration of gut microbiota could occur independently of PPI or H2B. We further examined the influence of the other possible confounders on the gut microbiota (antibiotics, ursodeoxycholic acid [UDCA], and probiotics). As a result, no statistical changes in *Streptococcus* were found in the groups with the treatment including antibiotics or probiotics ($P > .05$ in Wilcoxon rank-sum test), suggesting

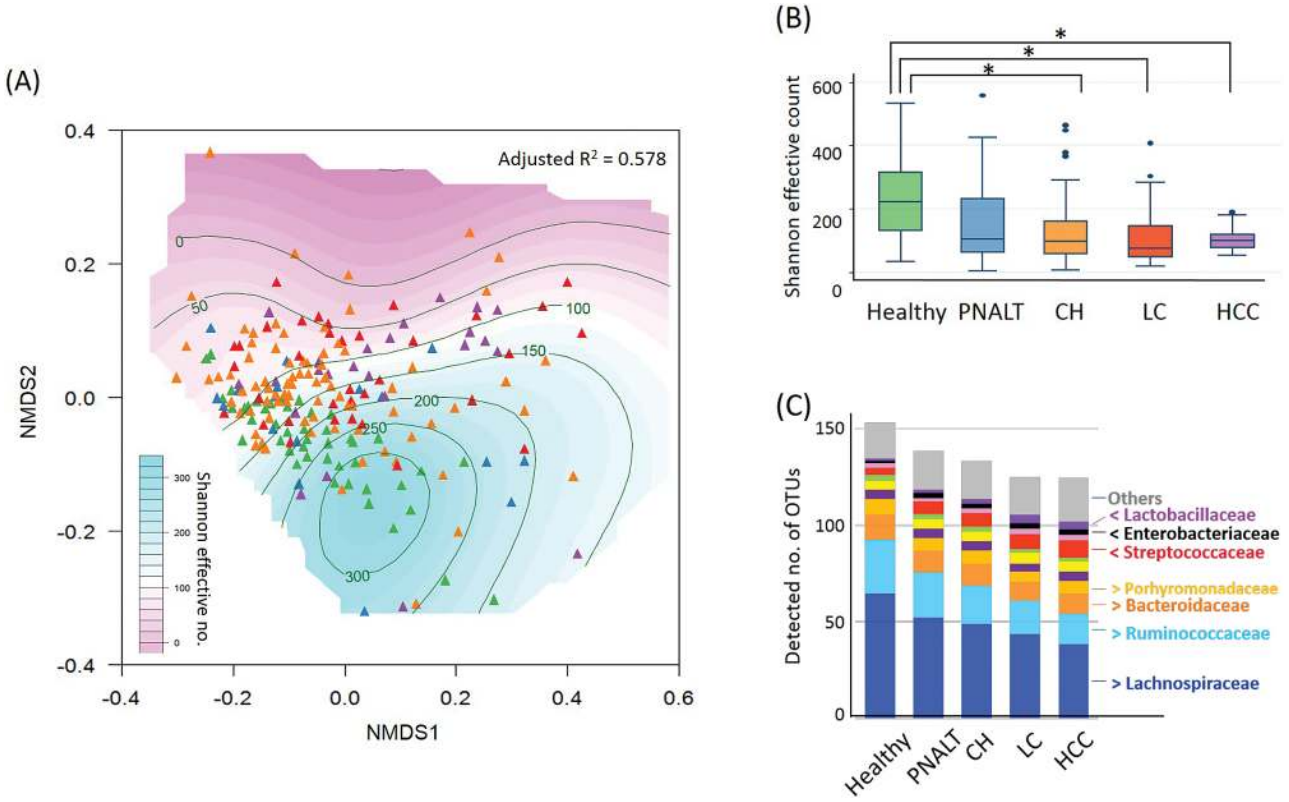


Figure 3. Decrease in gut bacterial diversity in association with chronic hepatitis C (CHC) progression. *A,* Contour plot of Shannon effective species count on the weighted UniFrac NMDS plot prepared in Figure 1A. *B,* Box plot of Shannon effective species count in each group. The box plots show the smallest and largest values, 25% and 75% quartiles, the median, and outliers. Statistical difference between groups was examined by generalized estimating equation test with 2 measurements for healthy controls. Significant difference ($P < .05$) is indicated by an asterisk. *C,* Detected number of operational taxonomic units (OTUs) of each genus in each group. Detected number of OTUs of each genus in each sample was averaged among each group and graphed. ">" and "<" before the genus name indicate significantly higher or lower ($q < 0.001$ in generalized estimating equation test with 2 measurements for healthy control) in a healthy group against CHC groups, including chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. Abbreviations: CH, chronic hepatitis; HCC, hepatocellular carcinoma; LC, liver cirrhosis; NMDS, nonmetric multidimensional scaling; PNALT, persistently normal serum alanine aminotransferase. Green lines, Shannon effective numbers; purple triangles, HCC; red triangles, LC; yellow triangles, CH; blue triangles, PNALT; green triangles, healthy.

that these prescriptions did not affect the gut microbiota in CHC patients. Meanwhile, between the compensated and decompensated cirrhosis groups, there were no significant differences in the relative abundance of *Streptococcus* (compensated cirrhosis, 0.11 ± 0.12; decompensated cirrhosis, 0.15 ± 0.16; $P = .31$). In addition to *Streptococcus*, some bacteria overgrew in gut microbiota of CHC patients (Figure 5), as described below.

Gut Microbial Hyperammonemia in CHC

To predict bacterial functions coded by gut microbiome, PICRUSt analysis was performed. We found significant alteration in the balance of functionality encoded by the predicted metagenomes of CHC patients, as shown in Supplementary Table 5. Notably, genes involved in "energy metabolism" decreased in CHC patients and those involved in "environmental information processing" increased in LC + HCC patients. This shift may reflect a worsening environment in the intestine. Interestingly, KO1429 annotated as the urease gene significantly increased with the progression of clinical stages (Figure 6A). The urease gene in the predicted metagenome was mainly from genus *Streptococcus* followed by unclassified Moraxellaceae (Figure 6B). Although samples available for pH measurement were limited, fecal pH significantly correlated with the abundance of the urease gene in both individuals with and without PPI administration (Figure 6C), and fecal pH in the CH or LC group was significantly higher than that in the healthy or PNALT group (Figure 6D).

DISCUSSION

In this study, we present for the first time the association of HCV infection with the alteration of gut microbiota (dysbiosis) in a large number of clinically classified CHC patients covering each clinical stage (PNALT, CH, LC, and HCC). Considering the effects caused by HCV infection, it is important to trace the changes in gut microbiota associated with the progression of CHC. Our data show that gut bacterial diversity significantly decreases in CHC patients compared with healthy individuals and that this decrease is associated with the severity of the clinical stage. With the decrease in commensal bacteria, including

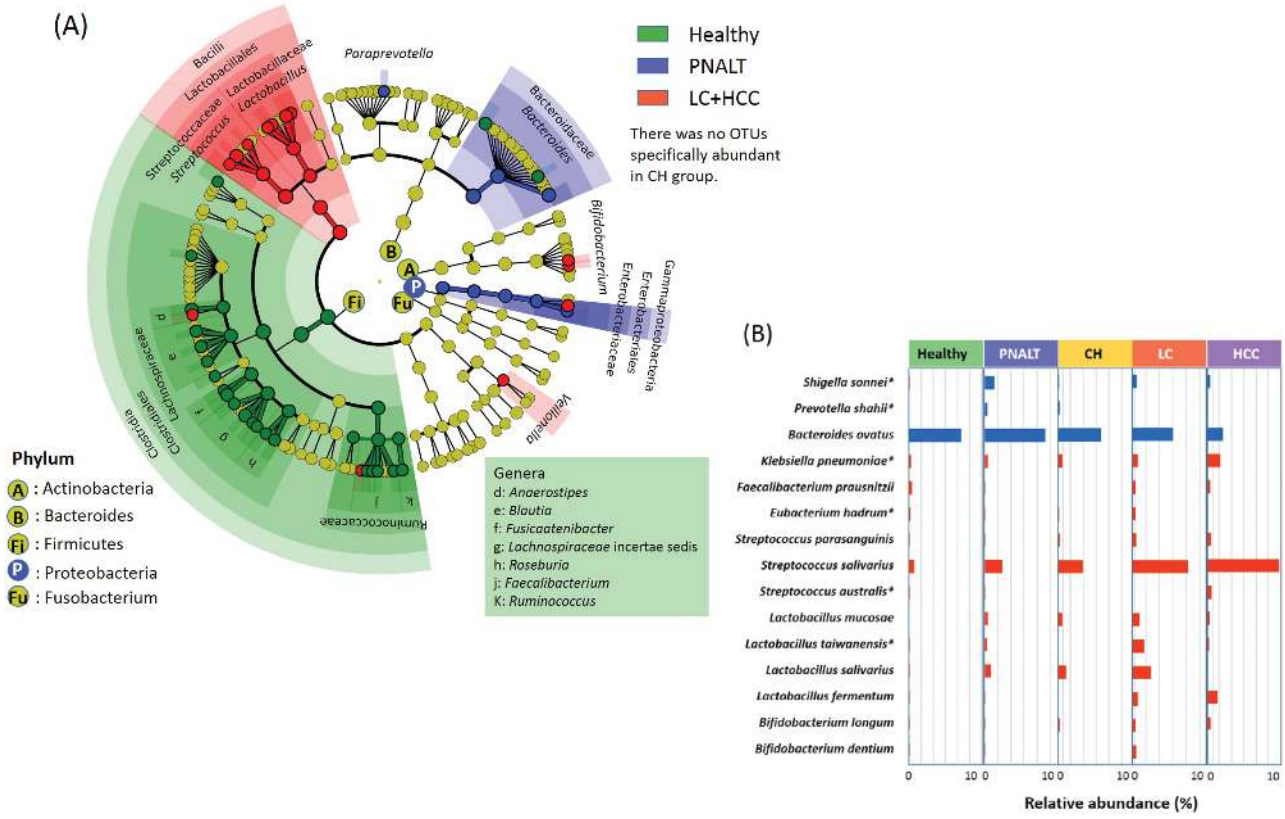


Figure 4. Phylogenetic skew in gut bacterial community associated with chronic hepatitis C (CHC). *A,* Bacterial taxa showing positive association with either healthy, persistently normal serum alanine aminotransferase (PNALT), or liver cirrhosis + hepatocellular carcinoma (LC + HCC) group (linear discriminant analysis [LDA] score >3.2) are highlighted. Operational taxonomic units whose average abundance among all samples were higher than 0.1% were subjected to LDA. LDA score was analyzed by using the one-against-all strategy. In the circle graph, the objects from the center of the circumference show phylum, class, order, family, genus, and OTU, respectively. *B,* The relative abundance of the overrepresented OTUs (LDA score > 3.2) was averaged by each CHC stage group and graphed. OTUs overrepresented in the healthy group were omitted. Red and blue bars indicate taxa associated with LC + HCC and PNALT groups, respectively. The closest species were assigned to these OTUs and shown. Asterisks at the end of species name indicate that the assignment scores are lower than the cutoff value of 0.8. Abbreviations: CH, chronic hepatitis; HCC, hepatocellular carcinoma; LC, liver cirrhosis; OTU, operational taxonomic unit; PNALT, persistently normal serum alanine aminotransferase. Green, healthy; blue, PNALT; red, LC + HCC.

families Lachnospiraceae and Ruminococcaceae, class Bacilli (viridans streptococci and lactobacilli) increased mainly in CH, LC, and HCC patients, and family Enterobacteriaceae and genus *Bacteroides* increased in PNALT patients transiently with significant differences. Interestingly, our data demonstrate that *S. salivarius* increased in HCC patients with LC, implying that *S. salivarius* might promote LC as well as HCC development.

We performed this study paying close attention to confounding factors. Fecal samples were collected from independent CHC patients living in different areas in Japan in order to minimize influences from domestic practices. Furthermore, the effects caused by lactulose, β2B, UDCA, and probiotics, which can affect gut microbiota, were confirmed to have no significant influence. In contrast, PPI increased *Streptococcus*. However, the alteration of gut microbiota significantly correlated with CHC even after removal of the effect by PPI administration. In this study, we show all 3 categories of dysbiosis described in the Introduction.

The alteration of microbial diversity has been associated with CHC [12]. The major effect on gut microbiota is associated with the decrease in bile production, leading to overgrowth of pathogenic and proinflammatory members including Enterobacteriaceae and Porphyromonadaceae, in association with the decrease in Firmicutes (*Clostridium* cluster XIVa), including secondary bile acid producers [15]. Accordingly, our findings suggest that bile acid production by the host and modulation of bile acid profile by the gut microbiota are keys for maintenance of an unfavorable gut microenvironment induced by HCV infection. Indeed, Clostridia, dominant commensal and butyrate-producing bacteria, play an important role in the induction of colonic regulatory T (Treg) cells, which suppress inflammation [16, 17]. Additionally, Lachnospiraceae [18] and Ruminococcaceae [19] participate in carbohydrate fermentation into short-chain fatty acids (SCFAs) in human intestines [18]. Among SCFAs, microbial-derived butyrate regulates the differentiation of Treg cells [16], indicating that the decrease in these bacteria causes a failure in the differentiation of Treg cells. Since SCFAs are nutrients for colonic epithelium and modulate colonic pH [20], a decrease in SCFAs would result in increased

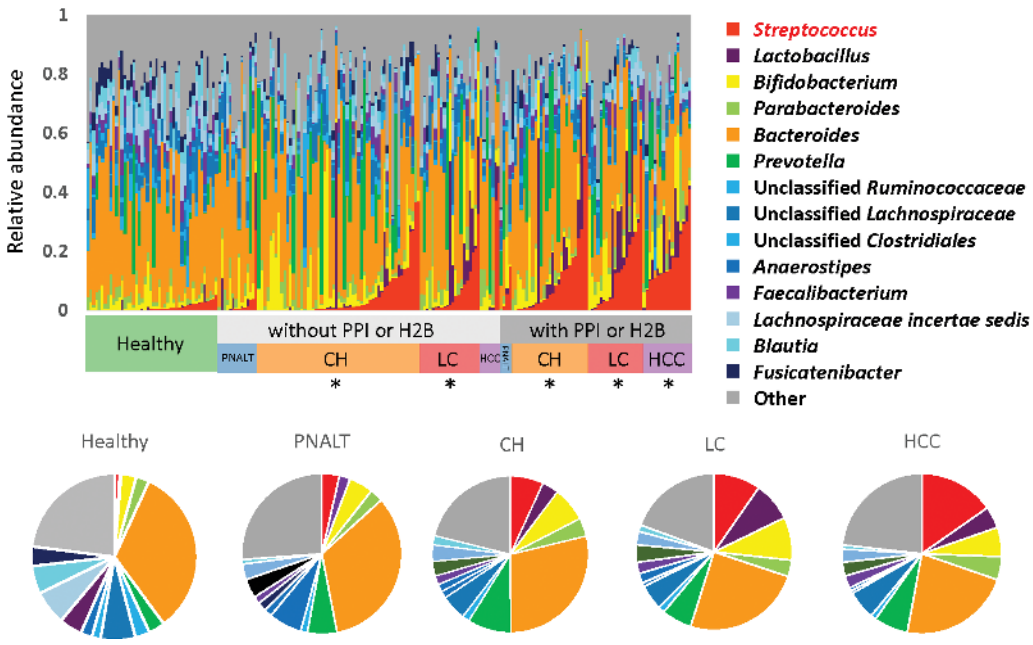


Figure 5. Genus composition of fecal samples from healthy individuals and chronic hepatitis C (CHC) patients. Fecal samples from healthy individuals (n = 23, 2 sets) and CHC patients diagnosed as persistently normal serum alanine aminotransferase (n = 18), chronic hepatitis (n = 84), liver cirrhosis (n = 40), and hepatocellular carcinoma (n = 24) were grouped according to the clinical stage of CHC and ordered according to *Streptococcus* abundance. Asterisks indicate significant differences in the relative abundance of *Streptococcus* against the healthy group ($P < .05$ in generalized estimating equation test with 2 measurements for the healthy group). The circle graphs show the bacterial balance of gut microbiome in each group. Abbreviations: CH, chronic hepatitis; H2B, histamine H2-receptor antagonist; HCC, hepatocellular carcinoma; LC, liver cirrhosis; PNALT, persistently normal serum alanine aminotransferase; PPI, proton pump inhibitor.

fecal pH, ammonia production, and absorption in the gut [21], causing hyperammonemia. As a result, the decrease in Lachnospiraceae and Ruminococcaceae worsens the condition of CHC patients.

It is interesting to note that altered gut microbiota were already observed in PNALT patients, suggesting a "predysbiosis." PNALT patients show a significant increase in Enterobacteriaceae and genus *Bacteroides* compared with healthy individuals, a characteristic observed only in PNALT, not in advanced stages. This increase was reported in previous studies [22–24] in the LC stage, but not in the PNALT stage. In these reports, LC decreased conversion of primary to secondary fecal bile acids, which is related to the abundance of major gut microbiome taxa [23, 24]. Furthermore, it is known that patients with hepatic encephalopathy show proinflammation and endotoxemia [17] and that specific microbial families, including Enterobacteriacae, are associated with inflammation [25]. The transient increase in Enterobacteriaceae and also of *Bacteroides* may be a sign of proinflammation leading to endotoxemia.

Most (perhaps 90%) HCV-related HCC arise in a background of advanced fibrosis or cirrhosis [26]. In addition to the previous data that showed the increase in *S. salivarius* in patients with cirrhosis [15], our data demonstrated that *S. salivarius* increased in HCC patients in LC, implying that *S. salivarius* might support LC from CH as well as HCC development. It was reported that *S. salivarius* downregulates the innate immune responses of human epithelial cells [27], probably accelerating HCC progression.

Zhang et al found worse gut microbial community, which means the continuous overrepresentation of the 2 bacterial families Streptococcaceae and Veillonellaceae, in all cirrhotic patients compared with healthy individuals and revealed that patients with minimal hepatic encephalopathy (MHE) had specific changes, such as an increase in *S. salivarius* [28]. They concluded that gut dysbiosis may be associated with MHE in LC patients, in particular, with ammonia-increasing bacteria [28]. We also suggest that urease encoded by *S. salivarius*, and perhaps by some other bacterial groups, released ammonia and increased pH in the gut of our CHC patients. According to the previous finding that acid production in the stomach is reduced in LC patients [29], the viridans streptococci in the oral cavity may pass through the stomach and colonize the gut, and PPI may accelerate this event by neutralizing gastric acid.

Taken together, gut dysbiosis is associated with CHC progression, possibly through endotoxemia and hyperammonemia. Therefore, targeting gut dysbiosis with pre- and probiotics or FMT can be an innovative treatment. Previous reports have shown several promising treatments against bacterial translocation in LC (any origin), such as obeticholic acid [30, 31] and microRNAs [32], which are still being evaluated [33]. In active Crohn's disease, FMT could treat the dysbiosis characterized by reduced bacterial diversity [34]. In chronic liver diseases,

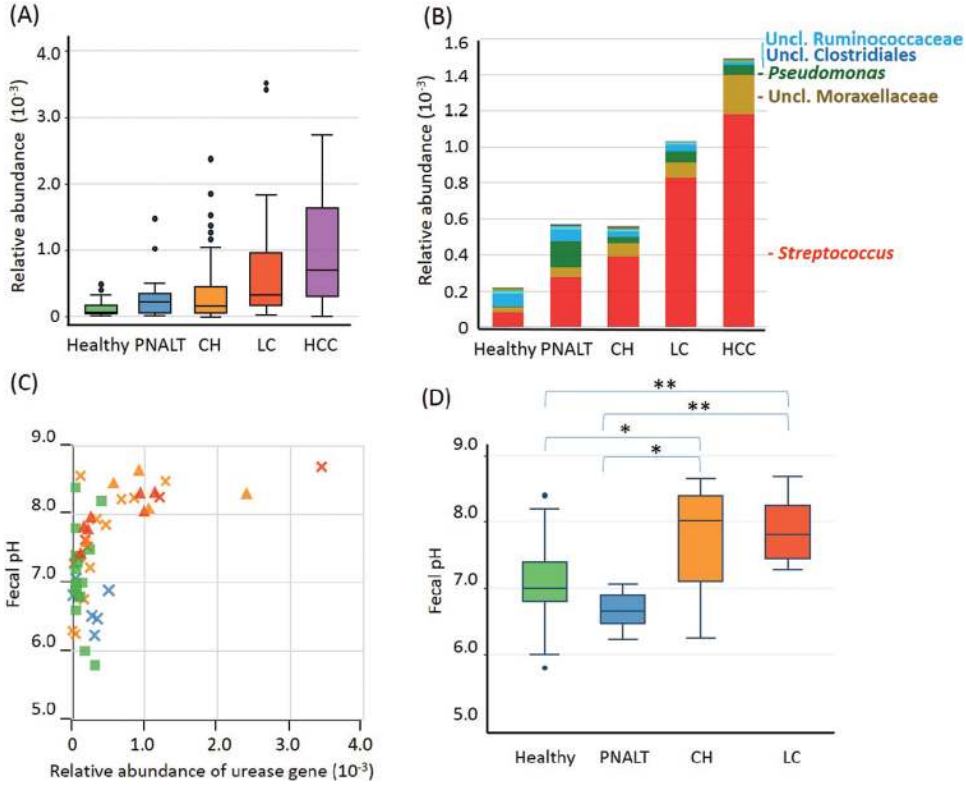


Figure 6. Increase in urease gene and fecal pH in association with chronic hepatitis C (CHC) progression. *A,* Box plot of urease gene in healthy individuals and CHC patients. The abundance of urease gene (K01429) was estimated by predicted metagenomic analysis (PICRUSt) based on 16S rRNA profile of each sample. *B,* Abundance of genera that predicted the encoded urease gene in each group. The composition was calculated for each sample and averaged by the subject groups. *C,* Correlation between the predicted abundance of the urease gene and the actual fecal pH. Spearman rank correlation showed $\rho = 0.55$ with $P < -.0001$ for whole dataset, $\rho = 0.53$ with $P < -.0074$ for patients without proton pump inhibitor (PPI) administration (cross), and $\rho = 0.68$ with $P < .015$ for patients with PPI administration (triangle). The colors correspond to those used in (D). *D,* Box plot showing distribution of fecal pH in each group. Pairwise Wilcoxon rank-sum test was performed with the Benjamini-Hochberg adjustment, and pairs showing $P < .05$ or $P < .005$ were marked by single and double asterisks, respectively. Abbreviations: CH, chronic hepatitis; HCC, hepatocellular carcinoma; LC, liver cirrhosis; PNALT, persistently normal serum alanine aminotransferase; Uncl, unclassified.

several reports have suggested the possibility of FMT for primary sclerosing cholangitis [35], alcoholic and nonalcoholic fatty liver disease, LC, and HCC [36, 37]. A broad spectrum of the antibiotic rifaximin reduced endotoxemia [38, 39] reduced levels of secondary bile acids involved in barrier dysfunction [23], and reduced harmful metabolites, which were positively correlated with Bacteroiaceae, Enterobacteriaceae, and Porphyromonadaceae [38]. In HCV infection, FMT following rifaximin treatment may be curative for patients in broad clinical stages by remodeling altered microbiota (removal of *S. salivarius* followed by replacement with a healthy microbiome).

Our study has some limitations. First, the real urease activity should be analyzed and fecal ammonia produced by bacteria should be quantified. Second, with regards to CHC, a prospective cohort study that can predict disease progression (eg, the onset of HCC from LC) based on the result of the gut microbiome is needed. Furthermore, the alteration of the gut microbiota after HCV eradication should be examined prospectively. Third, whether the difference in HCV genotype affects the gut microbiota should be analyzed. Fourth, we estimated fibrosis stages of CHC patients using liver biopsy/Fibroscan and/or FIB-4 index, but no cutoff of the FIB-4 index is given for the CH group. Because liver biopsy was not performed for all recruited CHC patients, some early LC patients might be included in the CH group. Fifth, because our PNALT patients were old, the FIB-4 index was relatively high. Sixth, the reason why some bacteria, including *L. salivarius*, increase in CHC patients is not completely understood and should be examined and discussed in further studies.

In summary, we carefully investigated the alteration of gut microbiota in HCV patients encompassing PNALT, CH, LC, and HCC and found that Enterobacteriaceae and *Bacteroides* transiently increased in mild liver disease, with HCV and viridans streptococci and lactobacilli increased in association with the progression of CHC. Microbiome transition could be a biological indicator of CHC progression, although further investigation into the mechanism of this shift will help us to understand the etiology of CHC progression. Because the gut microbiome can be therapeutically altered and potentially reduce the complications of chronic liver disease, further studies would help to propose a novel treatment strategy.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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