Case-Control Study

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Alteration of the fecal microbiome in patients with cholecystectomy: potential relationship with postcholecystectomy diarrhea – before and after study

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Background: Bile acid (BA) is a crucial determinant of the gut microbiome, and cholecystectomy can alter the physiology of BA. Physiological changes in BA resulting from cholecystectomy can also influence the gut microbiome. We aimed to identify the specific taxa associated with perioperative symptoms, including postcholecystectomy diarrhea (PCD), and to evaluate the effect of cholecystectomy on the microbiome by investigating the fecal microbiome of patients with gallstones.

Methods: We analyzed the fecal samples of 39 patients with gallstones (GS group) and 26 healthy controls (HC group) to evaluate their gut microbiome. We also collected fecal samples from GS group 3 months postcholecystectomy. Symptoms of patients were evaluated before and after cholecystectomy. Further, 16S ribosomal RNA amplification and sequencing were performed to determine the metagenomic profile of fecal samples.

Results: The microbiome composition of GS differed from that of HC; however, the alpha diversity was not different. No significant microbiome alterations were observed before and after cholecystectomy. Moreover, GS group showed a significantly lower *Firmicutes* to *Bacteroidetes* ratio before and after cholecystectomy than the HC group (6.2, $P < 0.05$). The inter-microbiome relationship was lower in GS than in HC and tended to recover 3 months after surgery. Furthermore, ~28.1% ($n = 9$) of patients developed PCD after surgery. The most prominent species among PCD (+) patients was *Phocaeicola vulgatus*. Compared with the preoperative state, *Sutterellaceae*, *Phocaeicola*, and *Bacteroidals* were the most dominant taxa among PCD (+) patients.

Conclusion: GS group showed a different microbiome from that of HC; however, their microbiomes were not different 3 months after cholecystectomy. Our data revealed taxa-associated PCD, highlighting the possibility of symptom relief by restoring the gut microbiome.

Keywords: 16S RNA sequencing, cholecystectomy, gallstone, microbiome, postcholecystectomy diarrhea

Introduction

The prevalence of gallstones ranges from 10 to 15% in the United States and Europe. Although 75% of patients with gallstones are asymptomatic, treatment is required if symptoms develop[1]. Cholecystectomy, the surgical removal of gallbladder (GB), is the standard surgical intervention for treating gallstone diseases[2]. The GB stores and concentrates the bile which is released into the gastrointestinal tract via the ampulla. Therefore, the secretory function of GB may influence bile composition in the small intestine[3].

Bile acid (BA) is a major determinant of the gut microbiome[4]. Gut bacteria have been linked to gallstone formation, and patients with gallstone-related diseases show a less diverse gut microbiome than healthy individuals[5]. GB, the BA reservoir, is removed during cholecystectomy. Therefore, BA flows directly into the duodenum after production, regardless of food intake. The physiological changes in BA, resulting from cholecystectomy,

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can also influence the gut microbiome[5]. A case–control study reported that patients who underwent cholecystectomy showed alterations in the beta diversity and abundance of *Blautia obeum* and *Veillonella parvula* in fecal samples, as compared with the healthy group[6].

Furthermore, BA released directly into the intestines stimulates bowel motility, thereby decreasing the gut transit time by 20%[7]. In the mice model, elevated serotonin levels following cholecystectomy enhance colon motility[8]. This can result in diarrhea following cholecystectomy[9–12]. Postcholecystectomy diarrhea (PCD) is a symptom of postcholecystectomy syndrome, and its prevalence is 35.6%[13]. Whether such symptoms are related to an altered gut microbiome is unknown, and a recent study proposed that dysbiosis of the gut microbiome can play a role in the onset of PCD[14].

Cholecystectomy is a surgical intervention that could alter bile physiology, which may have an impact on the gut microbiome and contribute to the onset of PCD. Therefore, we hypothesized that an association existed between PCD and gut microbiome alteration. Furthermore, our knowledge of the role of the gut microbiome in patients with gallstones is limited, and research on postcholecystectomy alterations in the gut microbiome is currently in its nascent stages. Therefore, we aimed to explore the relationship between cholecystectomy-related symptoms and the gut microbiome. Accordingly, we investigated the gut microbiome in patients with gallstones and evaluated the extent of microbiome restoration after cholecystectomy.

HIGHLIGHTS

- Bile acid (BA) is a crucial determinant of the gut microbiome, and cholecystectomy can alter the physiology of BA.
- Physiological changes in BA resulting from cholecystectomy can also influence the gut microbiome.
- Patients with gallstones showed a different microbiome from that of healthy controls; however, their microbiomes were not different 3 months after cholecystectomy.
- Our data revealed taxa-associated PCD, highlighting the possibility of symptom relief by restoring the gut microbiome.

Materials and methods

Patients

This prospective, single-center study included patients with gallstones and abdominal symptoms scheduled for cholecystectomy between 1 June 2018 and 31 May 2020. The exclusion criteria were as follows: age less than 20 years, suspected GB malignancy in the preoperative workup, use of antibiotics or probiotics within 3 months before study enrollment, anatomical changes due to prior gastrointestinal surgery, history of inflammatory bowel disease, and history of irritable bowel syndrome (IBS). Furthermore, we enrolled healthy controls (HC group) who visited our center for regular health screening during the study period. Among them, we selected HC confirmed to have no known medical history, normal laboratory test results, and no abnormal findings on imaging workup (computed tomography or ultrasonography). This study was conducted following the reporting guideline of the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE)[15] and Strengthening The Reporting Of Cohort Studies in Surgery (STROCSS) criteria[16] (Supplemental Digital Content 1, http://links.lww.com/JS9/A607).

Sample collection

Fecal samples from the patients with gallstones (GS group) were collected twice before (1 week before the scheduled operation date) and 3 months after cholecystectomy. The HC group submitted fecal samples only once. All patients were educated on the standard guidelines for fecal sample collection. On the morning of the scheduled visit date to the center, the patients collected ~3 g of their fecal sample at home using an aseptic exclusive stool collector (Stool Nucleic Acid Collection and Preservation Tubes, Catalog number 45660; Norgen Biotek Corp., Ontario, Canada), and stored the obtained sample in a freezer at – 20°C immediately after collection[17,18]. Upon arrival at the center, samples were immediately stored in a – 80°C laboratory freezer. The patients completed a questionnaire on abdominal pain and other abdominal symptoms at the time of fecal sample submission. The patients submitted another fecal sample 3 months after surgery by visiting the center and completed a questionnaire about any changes or newly developed symptoms after surgery. The common bile duct reportedly undergoes a physiologic dilatation within a certain period (4–6 months) after cholecystectomy, resulting in the restoration of the bile physiology to its preoperative state[19,20]. However, only minimal alterations in the microbiome may be observed if sampling was performed at an early time point after the surgery. Therefore, we decided to perform the fecal sampling 3 months after surgery. The collected fecal samples were sent to Macrogen Inc. (Gwangmyung, Korea) for gut microbiome analyses. In the GS group, patients were administered a single dose of preoperative intravenous antibiotic (2 g cefoxitin) 30 mins before the operation.

Study outcome

The primary outcome of this study was to identify the predominant bacterial species in patients with PCD and examine whether these species can predict the occurrence of the associated symptoms. Secondary outcomes included comparing the gut microbiomes of fecal samples before and after cholecystectomy in the GS group and between the GS group (before and after cholecystectomy) and the HC group (Fig. 1). The concept behind this scientific exploration has been illustrated in Supplementary Figure 1 (Supplemental Digital Content 2, http://links.lww.com/JS9/A608). Furthermore, we assessed the variations in the gut microbiome of GS based on typical preoperative biliary colic symptoms. Preoperative biliary colic is defined as acute severe abdominal pain in the right upper quadrant or epigastrium lasting 15–30 min or longer[21]. PCD is defined as the development of diarrhea more than three times a day for more than 4 weeks in patients with cholecystectomy status.

The detailed materials and methods, including sequencing profile and statistical analysis, are provided in Supplementary Methods, Supplemental Digital Content 2, http://links.lww.com/JS9/A608.

Figure 1. Flow diagram of enrolled patients and schematic study design. PCD, postcholecystectomy diarrhea.

Results

Baseline characteristics of the participants

Overall, 39 patients were enrolled, and their preoperative fecal samples were submitted. Seven patients were lost to follow-up, and 32 submitted their fecal samples after surgery (Fig. 1). HC ($n=26$) included in the study also submitted fecal samples. Their baseline demographics are shown in Table 1. The median age was 47 years in both groups, and 56.5 and 57.7% of the subjects were males in GS and HC groups, respectively (P for all >0.05). All patients were confirmed to have gallstones in the preoperative radiologic evaluation (computed tomography or ultrasonography), and GB polyps and adenomyomatosis were confirmed in 12.8% ($n=3$) and 23.1% ($n=9$) of the patients, respectively. The most common surgical approach was laparoscopic cholecystectomy (82.1%, $n=32$). The postoperative pathology reports confirmed chronic cholecystitis in 87.2% ($n=34$) and GB wall thickening in 20.5% ($n=8$) of the patients. Further, 53.8% ($n=21$) of patients had typical preoperative biliary colic, and 28.1% ($n=9$) of patients had PCD after GB removal.

Comparison of gut microbiome between GS (before cholecystectomy) and HC group

In our study, the sequencing depth was sufficient to analyze all samples because all samples reached a plateau in the rarefaction curve (Supplementary Figure 2, Supplemental Digital Content 2, http://links.lww.com/JS9/A608). In alpha diversity, there was no significant difference between the two groups, that is GS (before

Table 1

Characteristics of patients with cholecystectomy ($n = 39$).

Variables	Patients with GB stone ($n = 39$)	Healthy controls ($n = 26$)
Sex, *n* (%)		
Males	22 (56.4)	15 (57.7)
Females	19 (48.7)	11 (42.3)
Age, year, median (range)	47 (28–71)	47 (32–70)
Body mass index, mean, kg/m^2	24.7 ± 3.0	23.8 ± 2.4
Medical history, *n* (%)		
Hypertension	10 (25.6)	
Diabetes	4 (10.3)	
Dyslipidemia	8 (20.5)	
Alcohol history, *n* (%)		
None	28 (71.8)	26 (100.0)
Social	7 (17.9)	
More than twice a week	4 (10.3)	
Presence of typical biliary colic, *n* (%)	21 (53.8)	
Radiologic evaluation[a] (preoperation), *n* (%)		
GB stone	39 (100)	
GB polyp	5 (12.8)	
GB adenomyomatosis	9 (23.1)	
Chronic cholecystitis	36 (92.3)	
Operation type, *n* (%)		
Laparoscopic cholecystectomy	32 (82.1)	
Robotic-assisted laparoscopic cholecystectomy	7 (17.9)	
Surgical complication, *n* (%)		
None	39 (100.0)	
Hospital stay, mean, days	2.8 ± 0.6	
Pathologic evaluation, *n* (%)		
Chronic cholecystitis	34 (87.2)	
GB polyp	6 (15.4)	
GB adenomyomatosis	7 (17.9)	
GB wall thickening	9 (23.1)	
GB surface erosion	32 (82.1)	
GB exudate	2 (5.1)	
Presence of postcholecystectomy diarrhea, *n* (%)	9 (23.1)	
Laboratory parameters (preoperation)		
WBC, median (range)	6600 (3900–9300)	
Total bilirubin, median (range), mg/dL	0.5 (0.2–1.4)	
ALP, median (range), U/L	66 (32–91)	

Values are presented as medians (interquartile ranges) for continuous variables or numbers (percentages) for categorical variables.

[a]Radiological evaluation included in abdominal ultrasonography and computed tomography.

ALP, alkaline phosphatase; GB, gall bladder; WBC, white blood cell.

cholecystectomy) and HC (Fig. 2A). However, unweighted UniFrac PCoA demonstrated a clear separation between GS and HC [PC1 = 14.21%, analysis of similarities (ANOSIM) $R = 0.240$, $P = 0.001$; Fig. 2B]. Hierarchical clustering heatmaps of Pearson's correlation coefficients also revealed that the fecal microbiome composition of GS differed from that of HC (Fig. 2C).

Changes in the relative abundance of bacteria between GS and HC are presented in the Krona chart (Fig. 2D) and bar plot (Fig. 3A). At the phylum level, *Bacteroidetes* [HC vs. GS, mean (standard error): 35.7 (3.7)% vs. 47.8 (2.1)%, $P = 0.009$] and *Proteobacteria* [2.5 (0.5)% vs. 5.2 (1.0)%, $P = 0.008$] were less abundant in the HC group than in the GS group. In contrast, *Firmicutes* [55.3 (3.3)% vs. 43.9 (2.1)%, $P = 0.008$] and *Actinobacteria* [4.3 (1.0)% vs. 0.7 (0.2)%, $P < 0.001$] were more abundant in the HC group than in the GS group (Supplementary Table 1, Supplemental Digital Content 3, http://links.lww.com/JS9/A609). At the species level, *Prevotella copri* was the most prominent species in GS [18.1 (3.3)%]. The F/B ratio was significantly higher in the HC group than in the GS group before cholecystectomy (6.2 vs. 1.1, $P = 0.012$; Fig. 4).

We identified the specific microbial taxa that differed between HC and GS via linear discriminant analysis effect size (LEfSe) and visualized them through a cladogram (Fig. 2E; Supplementary Figure 3, Supplemental Digital Content 2, http://links.lww.com/JS9/A608). We found significant differences in bacterial composition between the two groups. Among the taxa with dominant abundance in the fecal samples (GS group), *Phocaeicola* (genus), *Bacteroidales* (order), and *Bacteroidia* (class) showed the highest AUROC (area under the receiver operating characteristic curve) values (0.701, 0.692, and 0.692, respectively) (Fig. 2F). The detailed linear discriminant analysis (LDA) scores between the two groups are shown in Supplementary Table 2, Supplemental Digital Content 4, http://links.lww.com/JS9/A610.

Changes in gut microbiome before and after cholecystectomy

Alpha diversity, based on the Shannon ($P = 0.510$) and Gini–Simpson index ($P = 0.560$), did not change after surgery compared with the baseline (Fig. 5A). There was also no significant difference in beta diversity between the two statuses (PC1 = 21.1%, ANOSIM $R = -0.007$, $P = 0.304$; Fig. 5B). The hierarchical clustering of the two groups was visualized using a heat map (Fig. 5C). We illustrated the differential abundance of bacteria using a Krona chart; however, the intergroup difference before and after cholecystectomy was less remarkable than that between patients with gallstones before cholecystectomy and HC (Fig. 5D).

The relative abundance of bacteria through their levels and distribution is shown in Figure 3A. In addition, the relative changes in microbial abundance before and after cholecystectomy were evaluated using LEfSe analysis. Only four taxa showed significantly different abundances in patients with gallstones who underwent cholecystectomy. At the species level, *Lachnospir pectinoschiza* and *Roseburia hominis* were dominant in patients with gallstones before cholecystectomy. In contrast, *Blautia luti* was the most abundant species in patients who underwent cholecystectomy (Fig. 5E, F). In other words, the relative abundances of most species did not change markedly after cholecystectomy.

The three bacterial species with the highest relative abundance were *P. copri* [before vs. after: 18.1 (3.3)% vs. 20.6 (3.8)%], *Fecalibacterium prausnitzii* [13.7 (1.6)% vs. 11.2 (1.7)%], and *Phocaeicola vulgatus* [5.4 (1.4)% vs. 4.9 (1.4)%], but the difference was not statistically significant (P for all > 0.05). However, *F. prausnitzii*, which accounted for a higher proportion in GS than in HC [13.7 (1.6)% vs. 10.1 (2.4)%, respectively; $P = 0.017$], decreased after surgery ($P = 0.236$). The detailed LDA score data for the cholecystectomy and HC groups are shown in Supplementary Table 3 (Supplemental Digital Content 5, http://links.lww.com/JS9/A611). The bubble chart shows the changes in the four major phyla in all patients after surgery compared with baseline (Fig. 6). Additionally, the F/B ratio of patients who

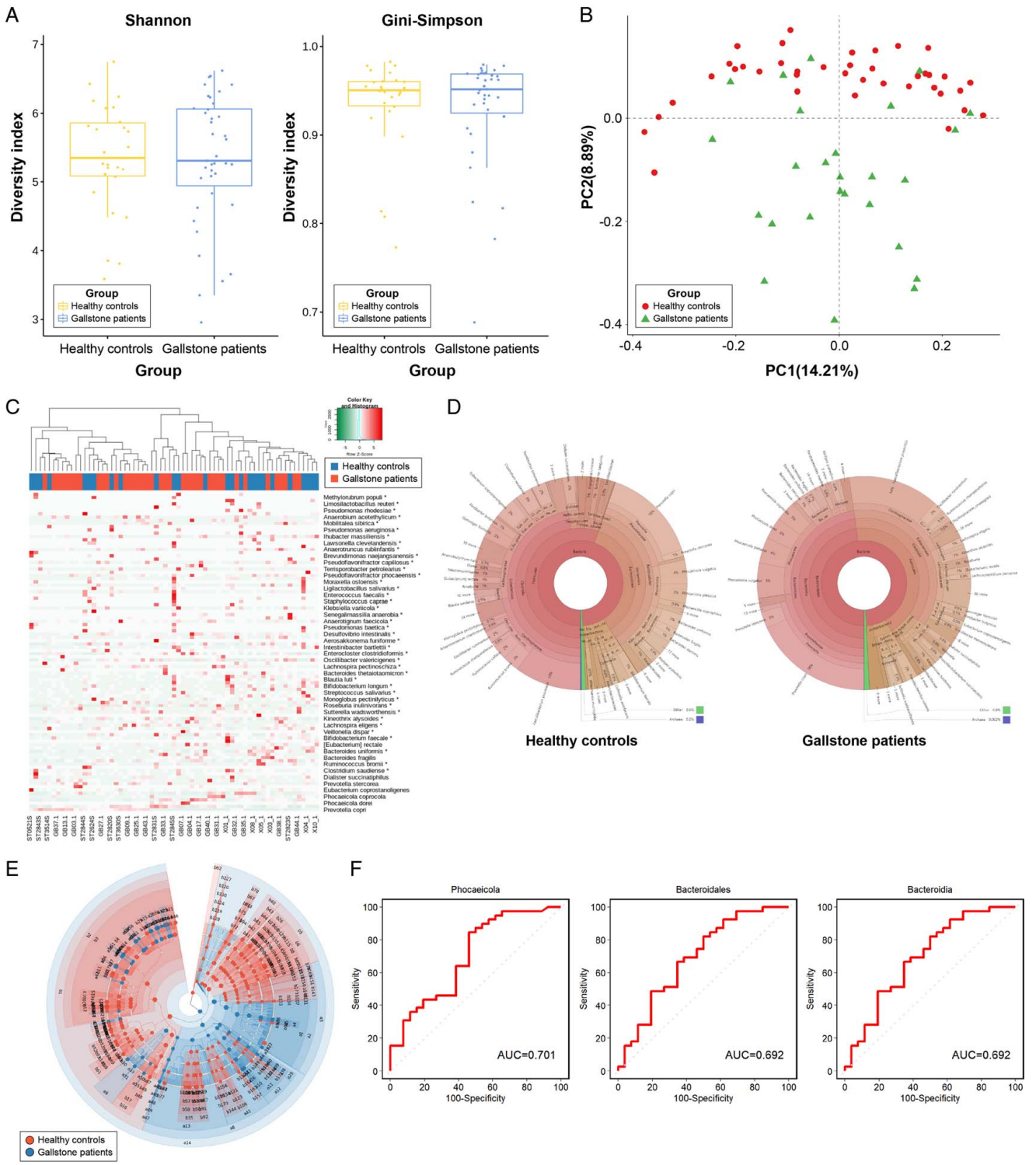

Figure 2. Comparison of the fecal microbiome in patients with gallstones (GS) and healthy controls (HC). (A) Comparison of alpha diversity between the two groups (Shannon and Gini–Simpson indices). (B) Unweighted UniFrac principal coordinate analysis. GS (red dot) versus HC (green dot). (C) Heat map of taxonomic assignment of fecal samples. The colored columns in the upper part of the heat map indicate GS and HC, and those in the lower part of the heat map indicate each participant. Taxonomic abundance is proportional to color intensity (color scale in the upper-left panel of the figure). (D) Krona chart illustrating the differential abundance of bacteria in GS and HC. (E) Cladogram highlighting the distribution of the fecal microbiome with differential abundance. (F) Receiver operating characteristic curves of genera with dominant abundance in the fecal microbiome of GS compared with HC.

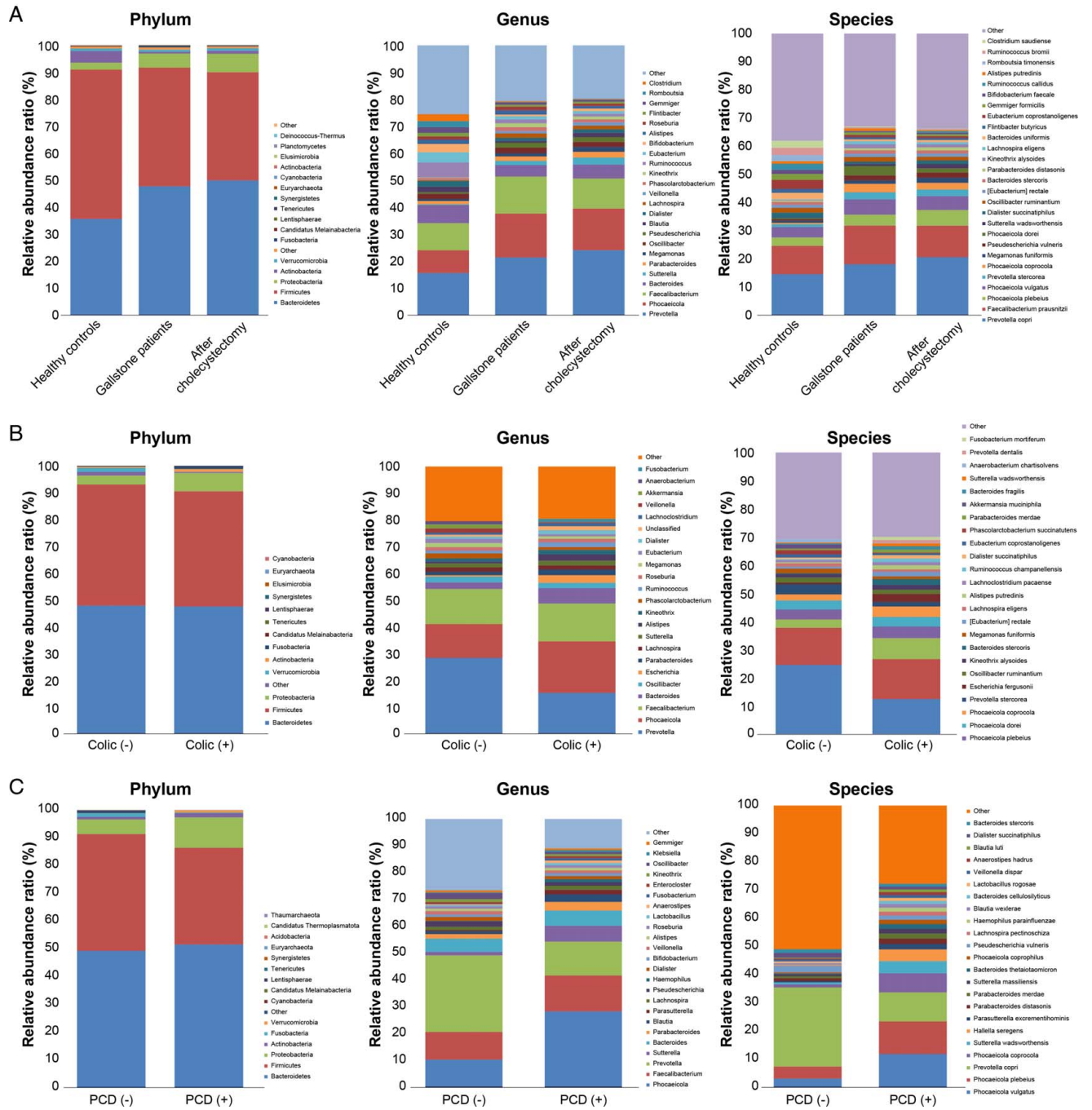

Figure 3. Relative abundance of bacteria at the phylum, genus, and species level. (A) Healthy controls versus patients with gallstones versus after cholecystectomy. (B) Presence of typical biliary colic symptoms before cholecystectomy. (C) Presence of postcholecystectomy diarrhea 3 months after cholecystectomy. The top 15 bacteria detected at the three levels (phylum, genus, and species) are indicated. PCD, postcholecystectomy diarrhea.

underwent cholecystectomy was not different between the two conditions (Fig. 4).

The HC and GS after cholecystectomy groups showed no statistically significant difference in alpha diversity (Shannon and Gini–Simpson indices, $P = 0.409$ and $P = 0.550$, respectively); however, the difference in beta diversity was statistically significant (PC1 = 14.7%, ANOSIM $R = 0.205$, $P = 0.001$). This is similar to the comparison between the HC and pre-cholecystectomy groups. The relative abundance, assessed using a bar plot for the two groups, was also similar before and after surgery (Fig. 3A). Detailed information is provided in Supplementary Figure 4 (Supplemental Digital Content 2, http://links.lww.com/JS9/A608) and Supplementary Table 4 (Supplemental Digital Content 6, http://links.lww.com/JS9/A612).

Network analysis revealed microbial relationships among operational taxonomic units (OTU) (Fig. 7). GS group showed reduced edge density compared with the HC group

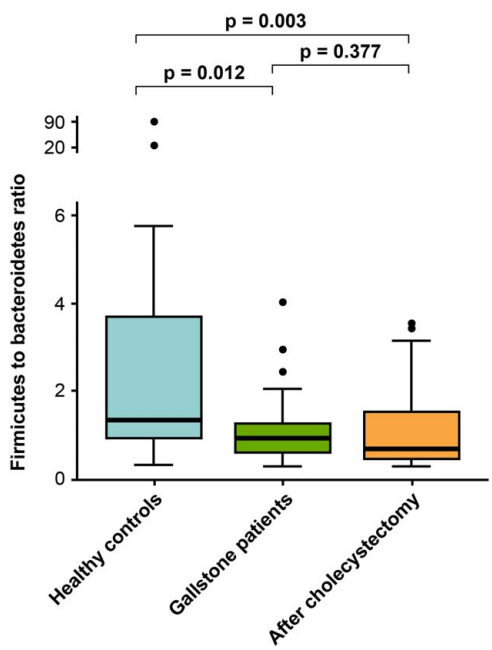

Figure 4. The ratio of *Firmicutes* to *Bacteroidetes* in healthy controls, patients with gallstones, and after cholecystectomy status.

(estimated density of the network, $d = 0.010$ vs. $d = 0.012$). However, edge density increased in GS after cholecystectomy ($d = 0.012$), suggesting a stronger microbial relationship than before surgery. Postoperative edge density was similar to that of HC, suggesting that the relationship among OTU was relatively more restored 3 months after surgery than before surgery.

Symptoms-related analysis

Gut microbiome analysis associated with typical biliary colic symptoms before cholecystectomy

Typical preoperative biliary colic was significantly reduced after surgery [21 (65.6)% vs. 7 (21.9)%, $P < 0.001$]. We divided the patients into two groups depending on the presence of preoperative symptoms [colic (−) and colic (+)] and analyzed the differences in the microbiome between the two groups. The two groups were not different in alpha and beta diversities but differed in the proportion of bacterial species (Fig. 3B; Supplementary Figure 5, Supplemental Digital Content 2, http://links.lww.com/JS9/A608). At the species level, 19 taxa were different between the two groups (Supplementary Table 5, Supplemental Digital Content 7, http://links.lww.com/JS9/A613), and the species that accounted for the highest proportion were *P. copri*, *Prevotella stercorea*, and *Bacteroides stercoris*. *P. copri* [24.7 (5.3)% vs. 12.5 (3.9)%, $P = 0.049$] and *P. stercorea* [3.6 (0.9)% vs. 1.6 (1.0)%, $P = 0.005$] were higher in the colic (−) group, and *B. stercoris* [0.2 (0.1)% vs. 2.1 (0.8)%, $P = 0.012$] was higher in the colic (+) group.

Gut microbiome analysis associated with PCD after cholecystectomy

Results showed that 28.1% ($n = 9$) of the patients did not have IBS symptoms (e.g. loose stool, frequent defecation, and abdominal discomfort relief after defecation) before surgery but newly developed these symptoms after surgery. We divided the patients into two subgroups based on the occurrence of PCD [PCD (−) and PCD (+)] and analyzed the differences in their microbiomes (Fig. 8A–E; Supplementary Figure 6, Supplemental Digital Content 2, http://links.lww.com/JS9/A608). The two groups did not differ in alpha diversity, and although the two groups seemed to differ in PCoA, the difference was not statistically significant (PC1 = 22.5%, ANOSIM $R = 0.135$, $P = 0.075$). However, the proportion of bacterial species differed between the two groups (Fig. 3C). LEfSe confirmed that *P. copri* was the most abundant species (LDA score: 4.923) in the PCD (−) group [PCD (−) vs. PCD (+), 25.2 (4.6)% vs. 8.9 (5.1)%, $P = 0.024$] and *P. vulgatus* was the most abundant species (LDA score: 4.466) in the PCD (+) group [2.8 (0.8)% vs. 10.3 (4.3)%, $P = 0.009$] (Supplementary Table 6, Supplemental Digital Content 8, http://links.lww.com/JS9/A614). We examined the AUROC to determine whether preoperative LEfSe results can be used to predict PCD (Fig. 8F). *Sutterellaceae* (family), *Phocaeicola* (genus), and *Bacteroidales* (order) showed the highest AUROC values in the taxa with PCD (+) patients compared with those before cholecystectomy (0.863, 0.735, and 0.735, respectively).

Discussion

Bile is a crucial factor influencing the gut microbiome[4,22]. Most bile flowing into the duodenum is reabsorbed in the small bowel via enterohepatic circulation and transported to the liver, and is partly excreted through feces[23]. BA accounts for ~50% of the bile and can directly influence the gut microbiome composition. As the gut microbiome plays a key role in BA metabolism, bile and gut microbiome are said to be in a complex relationship[4,24]. Cholecystectomy alters the physiology of BA. This is because the removal of the GB, which serves as a BA reservoir, causes the direct flow of bile into the duodenum after production[6]. The flow of BA into the duodenum without being stored in the GB alters the gut microbiome composition, and this may be linked to postoperative symptoms such as PCD[14,25]. In the present study, we obtained fecal microbiome samples from patients with gallstones before and after cholecystectomy to examine the changes in the microbiome after GB removal. In addition, we compared these microbiome compositions with those of fecal samples from HC to identify the bacterial species that are predominant in the GS group. We also analyzed the association between PCD symptoms and the microbiome 3 months after cholecystectomy, and to the best of our knowledge, this study is the first to analyze this relationship. According to our results, GS and HC showed no difference in alpha diversity but showed a difference in beta diversity. The microbiome of GS 3 months after cholecystectomy was similar to those before surgery. After cholecystectomy, the typical biliary colic significantly improved, but 28.1% of patients developed PCD. PCD symptoms were not associated with diversity. However, *P. vulgatus* was the most abundant species in the PCD (+) group. The taxa of both *Phocaeicola* and *Sutterellaceae* were significantly more abundant in PCD (+) patients. Network analysis showed that patients with gallstones had reduced microbial relationships compared with HC, but their microbial relationship increased to levels similar to those of HC after surgery, suggesting that it was restored to similar levels observed among HC.

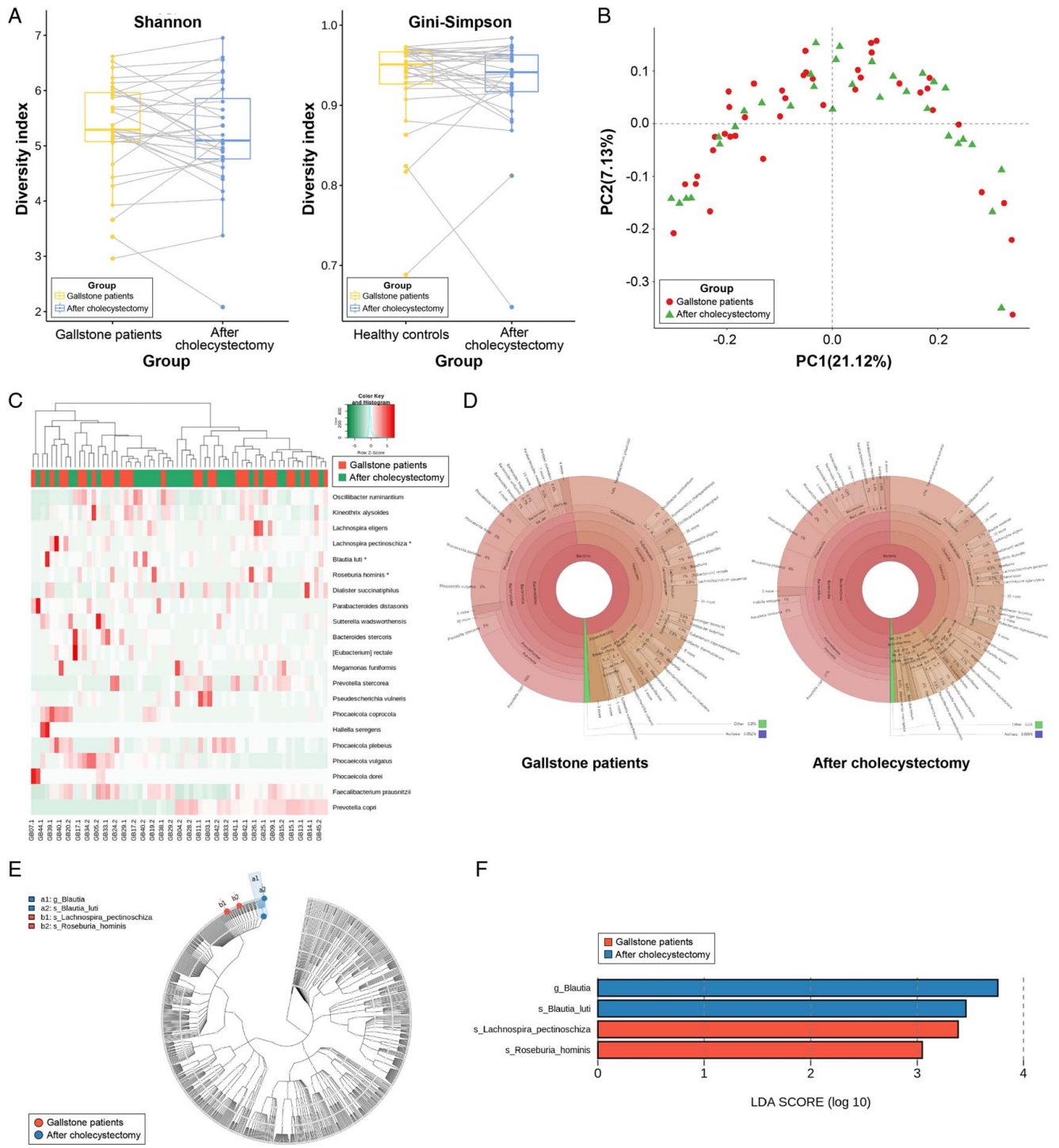

Figure 5. Comparison of the fecal microbiome in patients with gallstones (before cholecystectomy) and after cholecystectomy ($n = 32$). (A) Comparison of overall diversity (Shannon and Gini–Simpson indices) between patients with gallstone and after cholecystectomy. (B) Unweighted UniFrac principal coordinate analysis [patients with gallstones (red dot) vs. after cholecystectomy (green dot)]. (C) Heat map of taxonomic assignment of fecal samples. The colored columns in the upper part of the heat map indicate patients with gallstones and after cholecystectomy, and those in the lower part of the heat map indicate each participant. Taxonomic abundance is proportional to the color intensity (color scale in the upper-left panel of the figure). (D) Krona chart illustrating the differential abundance of bacteria in patients with gallstones and after cholecystectomy. (E) Cladogram highlighting the distribution of the fecal microbiome with differential abundance between the two conditions. (F) Linear discriminant analysis coupled with effect size measurements illustrating the most differentially abundant taxa between the two groups.

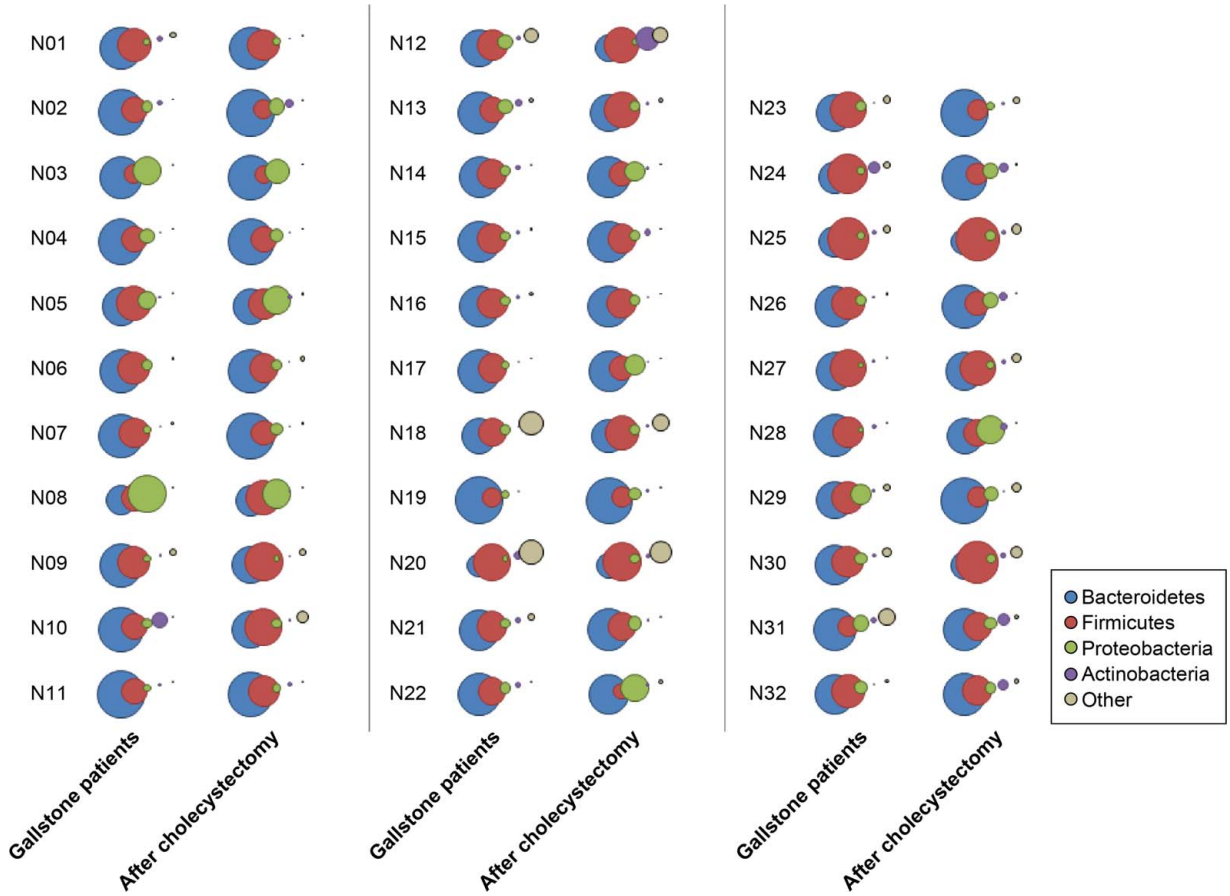

Figure 6. Bubble chart illustrating the differential abundance of bacteria at the phylum level between patients with gallstones and those 3 months after cholecystectomy.

Some factors contributing to gallstone formation include GB motility, BA metabolism, cholesterol metabolism, BA secretion, and the gut microbiome. Emerging evidence supports the role of the gut microbiome in BA metabolism[26]. BA and host metabolism can modulate the composition of the gut microbiome. At the same time, changes in microbiome composition can influence BA metabolism[27,28]. Little research has been conducted on the gut microbiome of patients with gallstones. According to previous studies, HC and patients with gallstones display different microbiome compositions. However, each study has reported

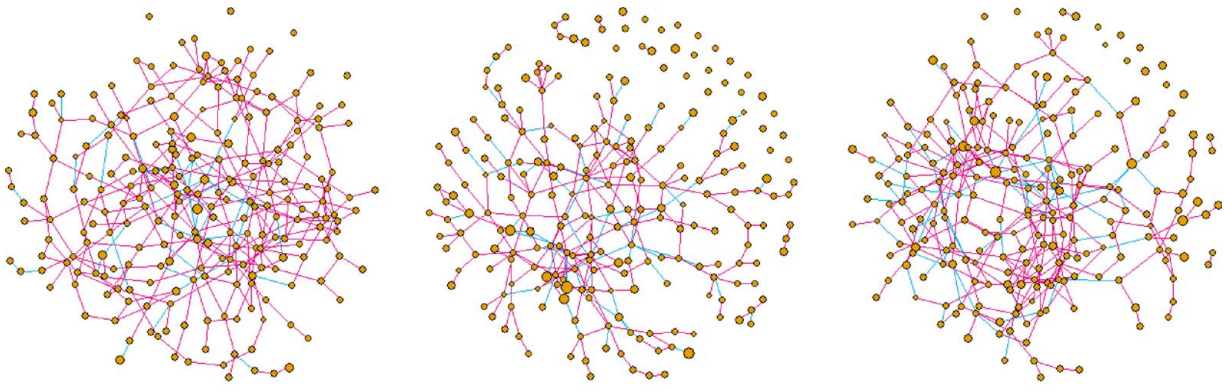

Figure 7. Microbial network analysis. Each node indicates an amplicon sequence variant and node size indicates relative abundance. Each edge indicates the correlation between nodes. Pink and blue lines reflect positive and negative relationships between nodes, respectively.

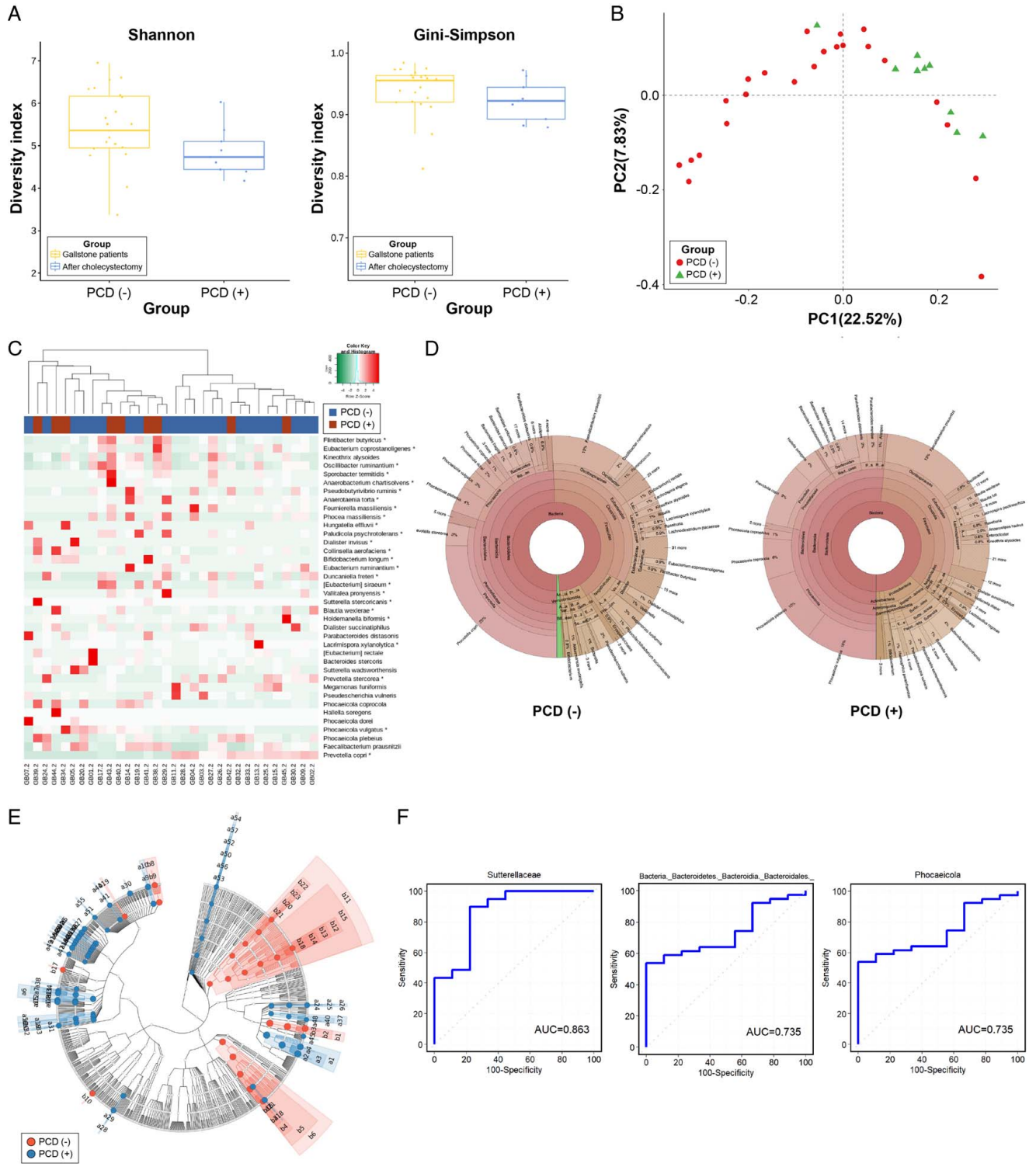

Figure 8. Comparison of the gut microbiome in patients with gallstones with postcholecystectomy diarrhea (PCD) (−) and PCD (+). (A) Comparison of alpha diversity (Shannon and Gini–Simpson indices). (B) Unweighted UniFrac principal coordinate analysis. PCD (−) (red dot) versus PCD (+) (green dot). (C) Heat map of taxonomic assignment of fecal samples. The colored columns in the upper part of the heat map indicate patients with PCD (−) and PCD (+), and those in the lower part of the heat map indicate each participant. Taxonomic abundance is proportional to color intensity (color scale in the upper-left panel of the figure). (D) Krona chart illustrating the differential abundance of bacteria in PCD (−) and PCD (+). (E) Cladogram highlighting the distribution of the fecal microbiome with differential abundance. (F) The prediction of PCD 3 months after cholecystectomy with the relative abundance of fecal microbiome and receiver operating curve of top three genera with dominant abundance in the fecal samples. PCD, postcholecystectomy diarrhea.

varying results. Keren *et al.*[5] reported that *Ruminococcaceae* (family) and *Oscillospira* (genus) were more abundant in patients with gallstones before cholecystectomy than in controls. In contrast, *Roseburia* (genus) and *Bacteroides uniformis* were decreased in these patients. Wu *et al.*[29] reported that *Proteobacteria* (phylum) was increased in patients with gallstones, but *Fecalibacterium* (genus), *Lachnospira* (genus), and *Roseburia* (genus) were decreased compared with the control. *B. uniformis* was also reduced in GS group in our study [2.3 (0.6)% vs. 0.7 (0.2)%, $P = 0.043$]. However, *Fecalibacterium* [10.1 (2.4)% vs. 13.7 (1.6)%, $P = 0.017$] and *Lachnospira* [0.3 (0.1)% vs. 1.6 (0.3)%, $P < 0.001$] were higher in GS than in the HC group. There may be a few reasons underlying the inconsistencies in bacterial compositions reported in the literature. First, the studies, including ours, had small sample sizes. Second, many factors may influence the gut microbiome even in HC; therefore, whether HC is an appropriate comparison group should be examined. Third, the bacterial species may vary depending on the type of gallstone[30]. Thus, validation studies using a larger study population are needed, and for these reasons, the composition of the gut microbiome of patients with gallstones must be interpreted with caution.

Cholecystectomy is a representative treatment option for symptomatic cholelithiasis, and GB removal may be the most potent factor that alters bile physiology[31,32]. Because GB functions as a reservoir for bile produced in the liver, removal of GB results in the direct flow of bile into the duodenum, which in turn may alter the composition of the gut microbiome[3,33]. Studies on the effect of cholecystectomy on the microbiome are scarce. In the mice model, GB-driven surfactant protein D is reportedly synthesized in the GB, delivered to the intestinal lumen, and bound selectively to gut commensal bacterial species[34]. A deficiency of this protein is linked to gut microbial dysbiosis, which can alter the commensal intestinal bacteria following cholecystectomy, leading to the onset of diarrhea. A case–control study reported that alpha diversity was lower in the cholecystectomy group than in the control group and that beta diversity also differed between the two groups[6]. Another study reported that the postcholecystectomy group showed altered microbiome composition and abundance compared with the control group[31]. Studies comparing the preoperative and postoperative states are lacking. A study comparing the preoperative and immediate postoperative (1–3 days after the operation) states in a Russian female cohort reported that there were no changes in alpha diversity, but the microbiome compositions differed[35]. In contrast, our data showed no changes in alpha and beta diversity. The discrepancy between our results and previous studies may be attributable to the following reasons; however, few studies have been conducted. First, we collected fecal samples 3 months after surgery. Three months may be too early to assess the effect of cholecystectomy on the microbiome. Second, the changes observed immediately after surgery in previous studies may be transient changes caused by general anesthesia and antibiotics. Since the bacterial composition differed according to the presence of PCD in our study, long-term follow-up data are required. However, our network analysis showed that the GS group had an increase in the microbial relationship after surgery to levels similar to that of the HC group. The results of previous studies and our study suggest that cholecystectomy can influence the gut microbiome; however, more studies should be performed, including a large number of participants with reasonable environmental control.

The postcholecystectomy syndrome is a new onset of abdominal symptoms, such as diarrhea, following laparoscopic cholecystectomy. Diarrhea occurring following cholecystectomy is referred to as PCD[36]. The prevalence of PCD is up to 57%[24,37]. The importance of PCD is increasing, as it is a delayed complication of cholecystectomy and is pertinent to the quality of life. However, its pathogenesis remains unclear. Owing to the removal of GB, which reserves and concentrates bile, the enterohepatic circulation of BA is elevated, resulting in increased BA concentration in the colon[3]. Primary BA synthesis occurs in the liver, and secondary BA synthesis in the intestine via the gut microbiome[38]. Not all microbial species are involved in BA synthesis, and because BA has bactericidal effects, the amount and concentration of bile influence microbial species, which in turn affects BA metabolism[39]. An increased proportion of primary BA in feces as a result of microbiome alteration has been observed in patients with IBS, suggesting that the alteration of BA proportion may be linked to the symptoms[40,41]. In the mice model transplanted with the fecal microbiome of PCD patients, tryptophan metabolism was increased, and abundant serotonin levels were observed in their serum and colon[8]. In other words, elevated BA in the colon after cholecystectomy stimulates colonic 5-hydroxytryptamine and increased colon motility, which can cause diarrhea. Thus, given that bile flows directly into the duodenum after cholecystectomy, the BA is predicted to increase, resulting in IBS-like symptoms, such as PCD.

Recent reports have suggested a link between PCD and microbiome. Xu *et al.*[25] reported that bacterial composition was altered in patients with PCD compared with the controls, and the co-abundance network was decreased in patients with PCD. In our study, PCD occurred in 28.1% of the patients. While the two groups (PCD [+] and PCD [−]) did not differ in microbiome diversity, their bacterial proportions were different. The two characteristic species were *P. copri* and *P. vulgatus*. *P. copri* is an anaerobic gram-negative bacterium that induces inflammation via the T-helper 17 cell-related immune response and is associated with chronic inflammation[42–44]. While the abundance of *P. copri* was higher in patients with PCD than in those without PCD, *P. copri* was the predominant bacterial species before and after surgery in our study. Thus, it is difficult to conclude that *P. copri* is associated with PCD. On the other hand, *P. vulgatus* is one of the most common species of the *Bacteroidaceae* family present in the colon[45]. The genus *Bacteroides* is known to contribute to the maintenance of a healthy human gut ecosystem[46]. *Phocaeicola* contributes to the breakdown of complex heteropolysaccharides into small-chain fatty acids and is thus known to play an essential role in the human colon[47]. Its roles are not well known, but it has been reported to influence the dominance of *Bacteroidales* species by producing antibacterial toxins[48]. However, whether this species and toxin are linked to gastrointestinal symptoms remains unknown. In a mouse model, it is suggested that, while the mechanism of *P. vulgatus* is unclear, it could be related to the pathogenesis of bowel inflammation and thus explains the development of inflammatory bowel disease[49]. A recent study suggested that *P. vulgatus* could inhibit the production of colon microbial lipopolysaccharide, which is related to the immune response, in a mouse model[50]. In our study, *P. vulgatus* was 3.7-fold more abundant in PCD (+) patients. Because its abundance was significantly elevated, we suspected it was associated with these symptoms. We believe that the observed discrepancy in the proportion of a gut microbiome ecosystem, where there is an

interaction with many different strains, is very meaningful. However, future biological or functional analysis studies are needed to understand the exact mechanism of the association with PCD because whether the increased proportion of *P. vulgatus* has a protective role due to a defense mechanism or has a causative role in PCD remains unclear. We identified bacterial species that may predict PCD using LEfSe and AUROC, and *Phocaeicola* and *Sutterellaceae* had the highest AUROC. Data on *Sutterellaceae* are also scarce; however, they have been proposed to be linked to IBS. PCoA showed that patients with IBS were clustered and distinguished from the control group, and these patients had an increased abundance of *Sutterellaceae*[40]. *Parasutterella* is a gram-negative anaerobe in the *Sutterellaceae* family and has been linked to chronic inflammation and IBS development[51]. Therefore, these two species may be associated with IBS symptoms and are suspected to be linked to the onset of PCD in patients with gallstones after cholecystectomy. Functional analysis profile with shotgun metagenome sequencing should be performed in future studies to explain the role of cholecystectomy and its influence on the gut microbiome in patients with gallstones.

This study has some limitations. First, we did not include patients with acute cholecystitis who required emergency cholecystectomy. Therefore, the microbial alterations observed in patients with elevated inflammation due to gallstones remain uncertain. Second, we did not analyze the BA component in fecal samples. The relationship between the microbiome and BA concentration needs to be analyzed because BA concentration can trigger PCD. Not all patients who undergo cholecystectomy develop the symptoms; hence, the degree of BA release may influence the gut microbiome. Third, we could not perform long-term postoperative follow-up. Collecting fecal samples 6 months after surgery might have been appropriate for a more accurate analysis of microbial alterations. Fourth, diet is a known factor affecting the gut microbiome composition[52]. The lack of dietary information in this study may have disregarded the effects of diet on the analysis. Fifth, obesity is known to have an impact on the gut microbiome[53,54]. The GS group has a slightly higher BMI than the HC group; however, no statistical difference was found ($P = 0.558$), and we believe this has a negligible impact on the results. Sixth, due to the small number of participants, further studies with a larger cohort number will be required to validate the results. Finally, we could not assess the bile secretion function of the GB before surgery in the GS group.

In conclusion, using fecal samples from GS before and after cholecystectomy, we confirmed that the gut microbiome in GS differed from that of HC in beta diversity. Furthermore, cholecystectomy did not influence the gut microbiome 3 months after surgery in our study. However, patients' symptoms that had been present before surgery were significantly reduced, and network analysis confirmed an elevated inter-microbial relationship after surgery in the GS group. Thus, long-term follow-up data are required to determine the recovery of the gut microbiome. Moreover, we propose that PCD, a delayed postoperative complication, may be associated with the gut microbiome, suggesting that the gut microbiome may play a crucial role in predicting and modulating symptoms. Finally, in the future, we expect to obtain further evidence through clinical trials to collect long-term, large-scale serial data on post-cholecystectomy gut microbial alteration in humans and to further validate our findings using animal models. Functional analysis and further validation with a larger study population are needed to clarify the roles of bacterial species linked to the onset of PCD.

Ethical approval

The study protocol was approved by the Ajou University Hospital Institutional Review Board and Ethics Committee (approval no. AJIRB-MED-OBS-18-153).

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Author contribution

C.-K.N. and J.C.H.: conception and design; W.J., M.J.Y., and W.H.K.: administrative support; W.J. and J.C.H.: provision of study materials or patients; C.-K.N., W.J., and J.C.H.: collection and assembly of data; C.-K.N. and J.C.H.: data analysis and interpretation; C.-K.N. and J.C.H.: manuscript writing. All authors were involved in the final approval of the manuscript.

Conflicts of interest disclosure

The authors have no conflicts of interest.

Research registration unique identifying number (UIN)

1. Name of the registry: The Clinical Research Information Service (cris.nih.go.kr), Korea Centers for Disease Control and Prevention, Ministry of Health and Welfare, Osong, Republic of Korea).
2. Unique identifying number or registration ID: KCT0003033.
3. Hyperlink to your specific registration (must be publicly accessible and will be checked): https://cris.nih.go.kr/cris/search/detailSearch.do?seq=12114&search_page=L.

Guarantor

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Data availability statement

The data that support the findings of this study are available from the corresponding author, [J.C.H.], upon reasonable request. But, all sequencing data is available at DOI: 10.17632/hyndhh7xgn.1

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