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ORIGINAL ARTICLE

Alterations of biliary and gut microbiota in relation to gallstone formation

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ABSTRACT

Background: The gut and biliary microbiota are important components of the complex microecology system in the human body. However, it is often difficult to obtain bile in clinical practice to manage gallstone diseases, warranting further microbiota research to evaluate the relationship between biliary microbiota and gallstone formation.
Aims: We aimed to characterize the diversity and alterations of biliary and gut microbiota in patients with gallstones and analyze their possible correlations to gallstone formation.
Methods: We collected gallstones, bile, gallbladder mucosa, and feces from 21 patients with gallstone disease during operation and fecal samples from 20 healthy subjects without gallstones. We performed high-throughput sequencing of the V3-V4 regions of the 16S rRNA gene in the gallstone and control groups and analyzed the final optimization sequence.
Results: We identified a total of 23,427 operational taxonomic units. *Achromobacter* ($P = 0.010$), *Faecalibacterium* ($P = 0.042$), and *Lachnospira* ($P = 0.011$) were significantly reduced, while *Enterococcus* ($P = 0.001$) was increased in the gallstone group. The diversity and composition between the biliary and gut microbiota in gallstone patients had statistical differences. The diversity of gut microbiota was significantly higher than that of biliary microbiota ($P < 0.05$). In addition, linear discriminant analysis (LDA) >4 indicated that the characteristic flora was specific to five samples. *Prevotella* and Proteobacteria had LDA values >4 in the feces and both bile and gallbladder mucosa, respectively, of patients with gallstones.
Conclusion: The biliary and gut microbiota of patients with gallstones displayed bacterial heterogeneity. *Prevotella* and Proteobacteria may serve as biomarkers for dysbacteriosis in patients with gallstones, suggesting that alterations of biliary and gut microbiota are involved in the formation of gallstones. This study highlights the potential application of fecal microbiota transplantation technology in the treatment of gallstone diseases.
Relevance for Patients: Microecology of the digestive tract is closely related to the formation of gallstones, providing new ideas for the prevention and treatment of patients with gallstones.

1. Introduction

Gallstones are crystal deposits in the biliary system, including the gallbladder and bile duct. Gallstones commonly affect 10 – 20% of the global adult population [1], and the resultant cost of gallbladder disease constitutes a major health burden [2]. With changes in dietary patterns and the aging population, the incidence of gallstones is increasing. Approximately 5% of patients with gallstones develop acute cholecystitis, suppurative cholangitis, severe acute pancreatitis, biliary fistula, and other serious complications [3,4]. In addition, gallstones have been associated with an increased risk of chronic diseases, such as diabetes and cardiovascular disease [5,6]. There is compelling evidence

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supporting the idea that long-term gallstone stimulation can lead to the transformation of benign hyperplasia into malignant gallbladder mucosal epithelial cells, leading to gallbladder cancer [7,8]. Recent studies have also reported that patients with asymptomatic gallstones have a significantly increased risk of the right-sided colon cancer after 15 years [9,10]. Therefore, more emphasis should be placed on new prevention strategies against the formation of gallstones.

The current understanding of the pathogenesis of gallstones is very complex, mainly involving local and systemic factors. The local factors include gallbladder wall motility disorder, local persistent immune-mediated inflammation, mucin secretion and accumulation, cholesterol supersaturation, and solid crystal precipitation [11,12]. Likewise, the systemic factors generally include gene polymorphism, epigenetic factors, expression and activity of nuclear receptors, insulin resistance, slow intestinal peristalsis, and increased cholesterol absorption [11,13]. The activity of gut microbiota can dysregulate the lipids in bile and increase the excretion of bile acids, leading to the development of gallstones [14]. Some patients with gallstones suffer from discomforts such as belching, abdominal pain, abdominal distension, and constipation for a long time. In recent years, much emphasis has been placed on understanding whether the formation of gallstones is related to gut microbiota disorders. The screening of biliary and intestinal microbiota has evolved from microscopic characterization and identification to various culture technologies (e.g., bacterial smear and culture methods) and molecular biology techniques. Nonetheless, the bacterial smear method has limitations in identifying bacteria, while the bacterial culture method has a low positive rate and limited efficacy in studying biliary tract microorganisms. Conversely, molecular biology techniques (e.g., polymerase chain reaction [PCR] amplification, fluorescence *in situ* hybridization, gene chip technology, 16S rRNA gene sequencing, and whole genome sequencing) have displayed advantages in accuracy, reliability, and repeatability in studying the diversity and subtle changes of microbiota. Among them, 16S rRNA gene sequencing is the most suitable method for bacterial phylogeny and species classification.

In this study, the V3-V4 variable regions of the 16S rRNA gene were sequenced to reveal the diversity of biliary and gut microbiota in patients with biliary stones. We aimed to investigate the alterations of biliary and gut microbiota and their possible correlation to gallstone formation.

2. Methods

2.1. Study design

We recruited patients who underwent a physical examination at the Beijing Tiantan Hospital affiliated with the Capital Medical University from November 2019 to November 2020 based on the following inclusion and exclusion criteria:

i. Inclusion criterion: Patients with gallstones that were confirmed by abdominal B-mode ultrasound or computed tomography.
ii. Exclusion criteria: Patients who received antibiotics within the past 3 months; the presence of other serious metabolic diseases, such as severe obesity, uncontrollable hyperlipidemia, and diabetes; patients who took a large dose of probiotics 3 months before the study; patients who took somatostatin or other drugs affecting gallstone formation, such as oral biliary acid therapy and proton-pump inhibitors; pregnant women or long-term contraceptive users; patients who underwent endoscopic retrograde cholangiopancreatography or intestinal surgery; and patients who have primary sclerosis cholangitis, primary biliary cholangitis, or Gilbert diseases.

Based on the above selection criteria, we included 21 patients who underwent gallstone surgery (gallstone group) and 20 healthy patients without gallstones (control group). Clinical information on all patients was obtained, including gender, age, body mass index (BMI), and cholesterol level. Gallstone, bile, gallbladder mucosa, and feces specimens were collected from the gallstone group, and feces were collected from the control group. This study was performed in accordance with the ethical standards of the responsible committee on human experimentation (International Council for Medical Sciences) and with the Helsinki Declaration. This study was approved by the Institutional Research Ethics Committee of the Beijing Tiantan Hospital, Capital Medical University (KY 2020-032-02). All experimental personnel involved in specimen collection and transportation received professional training.

2.2. Sample collection and processing

During laparoscopic cholecystectomy, general surgeons collected the gallstones, bile (2 mL), and gallbladder mucosa (about 1 × 1 cm in size). The assistant cleaned the surface of the gallstones and mucosa specimens with sterile normal saline and stored them in sterile and labeled cryopreservation tubes. Fresh feces from the gallstone group were collected before surgery, while feces from the control group were collected after admission. Approximately 5 g of fresh feces were collected and placed in sterile and labeled cryopreservation tubes for bacterial community detection. All specimens from the study participants were placed in liquid nitrogen for at least 3 min for rapid freezing and then stored in a freezer at −80°X.

2.3. DNA extraction and bacterial 16S rRNA amplification and sequencing

DNA was extracted from samples (0.5 g) using the QIAamp PowerFecal DNA Kit (QIAGEN, Germany) according to the manufacturer's protocols. Subsequently, the V3-V4 regions of the bacterial 16S rDNA gene were amplified by PCR using primers 16s-336F (5'-GTACTCCTACGGGAGGCAGCA-3') and 16s-806R (5'-GTGGACTACHVGGGTWTCTAAT-3'). The first PCR reaction was performed in a 25 μL mixture containing 5 μL 5× GC Buffer, 0.5 μL KAPA dNTP Mix, 0.5 μL KAPA HiFi HotStart DNA polymerase (KAPA Biosystems, America), 0.5 μL forward primer (10 pM), 0.5 μL reverse primer (10 pM), and 50 – 100 ng of template DNA. PCR cycling included 95°C denaturation for 3 min, followed by 25 cycles at 95°C for 30 s, 55°C annealing for 30 s, and 72°C elongation

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for 30 s, with a final extension at 72°C for 5 min. The amplified products from gallstones, bile, gallbladder mucosa, and feces samples were verified by gel electrophoresis with a 1.5% agarose gel, a mixture of 3 μL PCR product and 3 μL 3× loading buffer, and 3 μL 100 bp ladder marker (Yingwei Jieji Trading, China) at 100V voltage over 35 – 40 min.

Agencourt AMPure XP (Beijing Huaruikang Technology, China) was used to purify the 16S V3-V4 amplicons to be free of primers and primer-dimer species. The second PCR reaction was performed in a 25 μL mixture containing 5 μL 5× GC buffer, 0.75 μL KAPA dNTP mix, 0.5 μL KAPA HiFi HotStart DNA polymerase, 1.5 μL barcode F (10 pM), 1.5 μL barcode R (10 pM), 5 μL purified product, and 10.75 μL retinoblastoma. The purified product was amplified by PCR using primers, where the barcode is an eight-base sequence unique to each sample. Denaturation, annealing, elongation, and cycling were the same as the first PCR amplification. The amplicons were subsequently purified by AMPure XP beads to clean up the final library before quantification. Finally, purified amplicons were pooled in equimolar and paired-end sequences (2 × 250) on an Illumina MiSeq platform according to the standard protocols.

2.4. Bioinformatics analysis of sequencing data

Fast length adjustment of short reads was used to merge paired-end reads from next-generation sequencing [15]. Low-quality reads were filtered by fastq_quality_filter (−p 90 −q 25 −Q 33) in FASTX Toolkit 0.0.14, and chimera reads were removed by USEARCH 64-bit version 8.0.1517. The number of reads for each sample was normalized based on the smallest size of samples by random subtraction. The final optimized sequence was obtained to ensure the reliability of the effective sequence used as operational taxonomic units (OTUs). OTUs were aligned by the Uclust algorithm with a 97% identity and taxonomically classified using the Silva16S rRNA database (https://www.arbsilva.de/documentation/release-128/). From the levels of phylum and genus, the dominant bacteria obtained by sequencing in each group were statistically analyzed. The α-diversity reflects a comprehensive indicator of microbial evenness and abundance in a single sample and mainly includes the abundance index Chao1, Shannon's index, and Simpson's index. In contrast, β-diversity is a comparative analysis of microbial community composition among different groups. Both α- and β-diversities were generated in the Quantitative Insights Into Microbial Ecology (QIIME) software and calculated based on weighted and unweighted Unifrac distance matrices [16]. Venn diagram selects OTUs with a similarity level of 97% and displays the number of OTUs shared by multiple groups, reflecting the similarity and overlap of environmental samples. The linear discriminant analysis (LDA) coupled with effect size measurement (LefSe) method was used to identify metagenomic biomarkers that exhibited statistically significant differential abundances among groups [17].

2.5. Statistical analysis

SPSS 22, GraphPad Prism7, and QIIME were used for statistical analysis. The Chi-square test was used for categorical data. Quantitative values are expressed as the mean ± standard deviation ($M \pm SD$). Two-sample independent t-test and the Wilcoxon rank-sum test were used between the two groups. For multi-group comparison, one-way analysis of variance and the Kruskal–Wallis rank-sum test were used. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Study population characteristics

This study investigated the relationship between gallstone formation and bacteria in the bile, gallbladder mucosa, and feces of 21 gallstone patients (eight males and 11 females; age range: 32 – 73 years old). From the gallstone group, we obtained 13 gallstone specimens (S1 – S13), nine bile specimens (Z1 – Z9), 13 gallbladder mucosa specimens (N1 – N13), and 17 feces specimens (F1 – F17). Meantime, we collected 20 feces (HF1 – HF17) samples from the control group. We rejected three samples due to amplification failure; one from the gallstone specimens, one from the gallstone patients' feces specimens, and one from the healthy subjects' feces specimens. The average age and BMI of the patients in the gallstone group were higher than that of the control group ($P = 0.004$). There were no statistically significant differences in gender and cholesterol levels between the gallstone and control groups (Table 1).

3.2. Bacterial diversity of sample species under different sequencing quantities and OTUs dilution curve

In this study, we identified a total of 23427 OTUs (340 ± 93) based on the conventional criterion of 97% sequence similarity, with 4095 OTUs in gallstones, 3065 OTUs in bile, 4687 in gallbladder mucosa, 5203 OTUs in patients' feces, and 6377 OTUs in normal feces. There was no significant difference in the intestinal microbiota diversity based on the feces of the gallstone and control groups. There was also no statistical difference in the bacterial diversity between gallstones, bile, and gallbladder mucosa in the gallstone group ($P > 0.05$). The gut microbiota was reportedly diverse in gallstones ($P = 0.004$), bile ($P = 0.045$), and gallbladder mucosa ($P = 0.008$). In addition, the gut microbiota was more diverse in the gallstone group than the control group (Table 2).

When the number of sequences increased, the diversity index did not increase significantly, indicating that the number of sequences was sufficient to reflect the overall community structure (Figure 1). In addition, the increase in the number of sequences did not generate new OTUs.

Table 1. Clinical data of the gallstone and control groups

Clinical parameter	Group		*P*-value
	Gallstone	Control	
Gender (males/females)	8/13	11/9	0.278
Age (years)	52.8±14.4	40.1±12.4	0.004
BMI (kg/cm^2)	24.4±2.4	22.8±2.1	0.032
Cholesterol (mmol/L)	1.6±0.7	1.3±0.5	0.126

Notes: Gender and cholesterol were analyzed with a Chi-square test; age and BMI were analyzed with a two-sample independent t-test.
Abbreviation: BMI: body mass index

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Table 2. Microflora sequencing results of each sample type

Statistical parameters	Sample type				
	Gallstone	Bile	Gallbladder mucosa	Patients' feces	Normal feces
Total number of sequences	1818953^{+}	1324611^{+}	2089573^{+}	2163367$^{\#}$	3033379$^{\#}$
Mean number of sequences	151579±80025	147179±62622	160736±58717	135210±56672	159651±40231
Total OTUs	4095^{+}	3065^{+}	4687^{+}	5203$^{\#}$	6377$^{\#}$
Mean OTUs	341±136	340±124	361±118	325±59	335±39
Chao1	434±131^{+}	416±119^{+}	445±120^{+}	418.15±51.50$^{\#}$	411.18±49.15$^{\#}$
Shannon's index	3.77±1.66^{+}	4.08±1.91^{+}	3.75±1.68^{+}	4.87±0.93$^{\#}$	5.17±0.64$^{\#}$
Simpson's index	0.71±0.19*$^{+}$	0.74±0.23*$^{+}$	0.69±0.18*$^{+}$	0.89±0.08$^{\#}$ *	0.92±0.06$^{\#}$

Notes: $^{\#}P > 0.05$ between gallstone, bile, and gallbladder mucosa; $^{+}P > 0.05$ between patients' feces and normal feces; $*P < 0.05$.
Abbreviation: OTU: Operational taxonomic unit

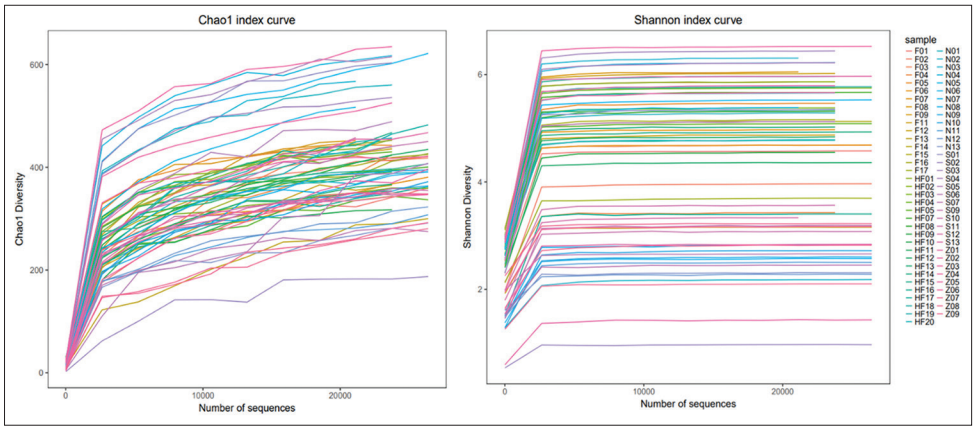

Figure 1. Operational taxonomic unit dilution curves.

3.3. Variable regions (V3-V4) of the bacterial 16S rRNA gene and bacterial community sequencing

Sequencing of V3-V4 fragments of the 16s rRNA gene yielded a total of 10,429,883 sequences with a mean ± standard deviation of 151 158 ± 57 813 from the gallstone, bile, gallbladder mucosa, and fecal samples (Table 2). The raw sequence reads were deposited in National Center for Biotechnology Information (NCBI) under Bioproject (accession no.: PRJNA 929661).

3.4. Comparative metagenomic analysis between gut and biliary tract at the phylum and genus level

The composition and diversity of bacteria at the genus level are often used to reflect changes in the microenvironment of specific human body parts [18]. Accordingly, we structurally analyzed each group of bacteria at the phylum and genus levels (Tables 3 and 4). At the phylum level (Table 3), Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, Verrucomicrobia, Fusobacteria, Gemmatimonadetes, and Cyanobacteria reported no statistical difference in gut microbiota diversity between the gallstone and control groups (i.e., in the fecal samples) ($P > 0.05$). However, Proteobacteria, Firmicutes, and Bacteroidetes reported statistical differences among gallstone, bile, gallbladder mucosa, and fecal samples in the gallstone group.

At the genus level (Table 4), in comparison with the control group, the gallstone group displayed a decreased abundance of *Achromobacter*, *Faecalibacterium*, and *Lachnospira* ($P < 0.05$) and an increased abundance of *Enterococcus* ($P < 0.05$), while the other genera reported no statistical differences ($P > 0.05$). In the gallstone group, the abundance of *Achromobacter* was significantly higher in the biliary tract (including gallstones, bile, and gallbladder mucosa) than in the intestinal tract. In addition, the abundance of *Bacteroides*, *Faecalibacterium*, *Lachnoclostridium*, and *Subdoligranulum* in the gallstone group was significantly lower in the biliary tract (including gallstones, bile, and gallbladder mucosa) than in the intestinal tract. The abundance of *Enterococcus* was significantly higher and the abundance of *Parabacteroides* was significantly lower in the gallstone and bile specimens than in the fecal specimens of the gallstone group.

Pairwise comparison of the gallstone, bile, gallbladder mucosa, and fecal samples in the gallstone group revealed a significantly higher abundance of *Acinetobacter* in the biliary tract (including gallstones, bile, and gallbladder mucosa) and a significantly lower abundance of *Bacteroides*, *Faecalibacterium*, *Lachnoclostridium*, and *Subdoligranulum* in the intestinal tract (Figure 2).

In the gallstone group, the abundance of Proteobacteria was significantly higher and the abundance of *Firmicutes* and

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Table 3. Bacterial composition and diversity among sample types at the phylum level.

Phylum	Sample type (%)				
	Gallstone	Bile	Gallbladder mucosa	Patients' feces	Normal feces
Proteobacteria	83.68^{y}	89.53^{y}	90.78^{y}	6.51x^{y}	3.74^{x}
Firmicutes	16.75±21.55^{y}	13.33±15.56^{y}	9.85±13.37^{y}	36.20±16.52^{xy}	44.21±11.79^{x}
Bacteroidetes	6.11^{y}	3.76^{y}	2.04^{y}	44.13^{xy}	50.56^{x}
Actinobacteria	2.27	1.85	2.12	0.98^{x}	1.10^{x}
Verrucomicrobia	0.15	0.11	0.17	0.01^{x}	0.00^{x}
Fusobacteria	0.01	0.03	0.02	0.00^{x}	0.00^{x}
Gemmatimonadetes	0.05	0.00	0.12	0.00^{x}	0.00^{x}
Cyanobacteria	0.00	0.00	0.00	0.00^{x}	0.00^{x}

Notes: $^{x}P > 0.05$ between gallstone, bile, gallbladder mucosa, and patients' feces; $^{y}P < 0.05$ between patients' feces and normal feces

Table 4. Bacterial composition and diversity among sample types at the genus level.

Genus	Sample type (%)				
	Gallstone	Bile	Gallbladder mucosa	Patients' feces	Normal feces
Achromobacter	34.97^{m}	0.00^{m}	78.77^{m}	0.00z^{m}	0.29^{z}
Bacteroides	4.84^{n}	2.58^{n}	1.43^{n}	32.40^{n}	32.86
Escherichia/Shigella	0.76	0.28	0.24	0.86	0.18
Faecalibacterium	1.26^{n}	0.72^{n}	0.46^{n}	6.52z^{n}	10.15^{z}
Prevotella	0.11	0.21	0.08	0.84	0.45
Acinetobacter	0.12	0.12	0.09	0.00	0.01
Lachnospira	0.08	0.17	0.15	0.43^{z}	3.24^{z}
Lachnoclostridium	0.20^{n}	0.16^{n}	0.08^{n}	1.10^{n}	1.80
Blautia	0.16	0.27	0.14	1.62	1.03
Megamonas	0.14	0.07	0.09	0.05	0.02
Subdoligranulum	0.13^{n}	0.08^{n}	0.11^{n}	0.74^{n}	1.61
Enterococcus	0.19^{M}	0.04^{M}	0.14	0.02z^{M}	0.00^{z}
Bifidobacterium	0.16	0.28	0.13	0.30	0.57
Parabacteroides	0.40^{N}	0.10^{N}	0.10	0.82^{N}	0.76
Eubacterium	0.25	0.48	0.24	0.80	0.40

Notes: $^{z}P < 0.05$ between patients' feces and normal feces; $^{m}P < 0.05$ denote significantly higher % than in the intestinal tract; $^{n}P < 0.05$ denote significantly lower % than in the intestinal tract; $^{M}P < 0.05$ denote significantly higher % than in patients' feces; NP < 0.05 denote significantly lower % than in patients' feces

Bacteroidetes was significantly lower in the biliary tract than in the intestinal tract (Figure 3A). Likewise, the abundance of *Enterococcus* was significantly higher and the abundance of *Parabacteroides* was significantly lower in the gallstone and gallbladder mucosa samples than in the intestinal tract (Figure 3B). There was no statistical difference between bile and gut microbiota, and the abundance of *Prevotella* was significantly lower in the gallbladder mucosa of patients with gallstones than in the gut microbiota. We found no statistical difference in the other microflora structures between the biliary and intestinal tracts in patients with gallstones.

3.5. Beta diversity analysis and Venn plots

Principal coordinate analysis (PcoA analysis) was conducted based on the Bray-Curtis algorithm to validate the above findings (Figure 4). Between principal coordinate (PC) 1 and PC2, the diversity of the gut and biliary microbiota of patients in the gallstone and control groups was relatively similar. However, the diversity between PC2 and PC3 was relatively similar for some gut and biliary microbiota. The bile and gallbladder mucosa shared 757 OTUs, accounting for more than 90% (i.e., 90.1%, 93.9%, and 91.6%) of each group (i.e., between PC1 and PC2; PC1 and PC3; and PC2 and PC3). The gut microbiota of patients in the gallstone and control groups shared 607 OTUs, accounting for more than 85% of each group (87.8% and 95.0%, respectively). The five sample types shared 541 OTUs, accounting for more than 60% in each group (i.e., 65.6% in gallstone, 64.4% in bile, 67.1% in gallbladder mucosa, 78.3% in feces of the gallstone group, and 84.7% in feces of the control group) (Figure 5). The Venn plot selected OTUs with a similarity of 97% (or higher) and displayed the mutually shared number of OTUs between multiple groups, reflecting the similarity and overlap of the environmental samples.

3.6. Characteristic bacteria of the biliary and intestinal tract in gallstone patients and healthy subjects

LDA was used to reduce data dimensionality and evaluate the different abundance of each bacteria species [17]. Species with LDA values greater than the set threshold were regarded as biomarkers with statistical differences. It is generally believed

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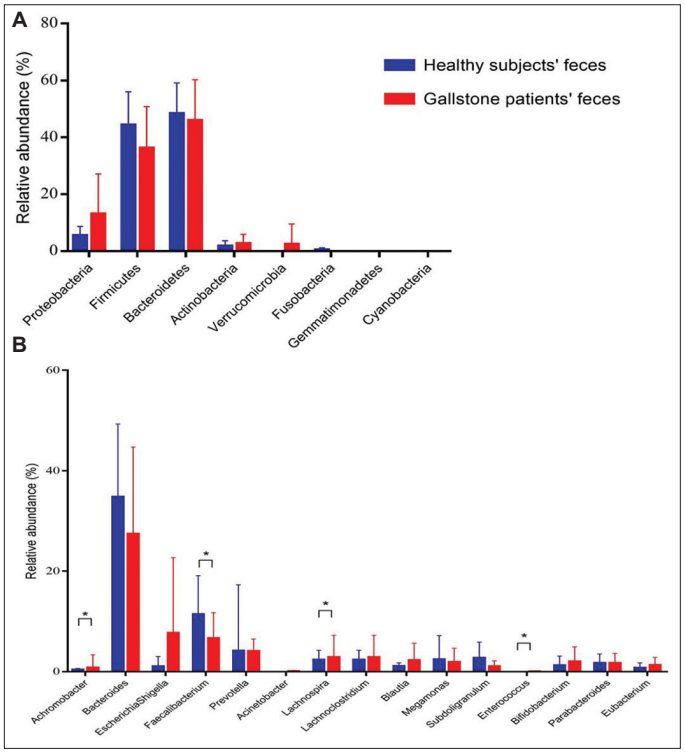

Figure 2. Relative abundance of bacterial flora at the (A) phylum and (B) genus levels between gallstone patients' feces and healthy subjects' feces specimens. Blue: healthy subjects' feces; red: gallstone patients' feces; *$P < 0.05$.

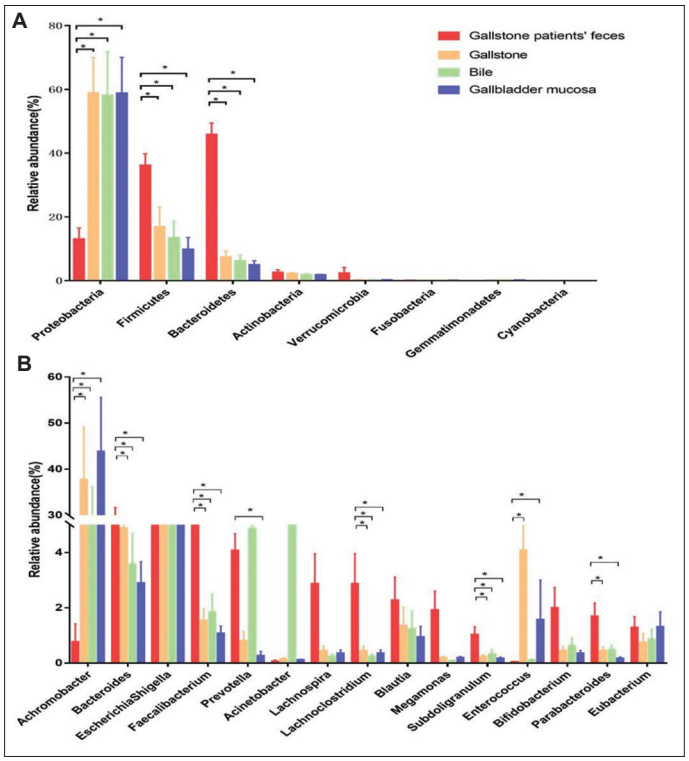

Figure 3. Relative abundance of bacterial flora at the (A) phylum and (B) genus levels between the biliary and intestinal tracts of gallstone patients. Blue: gallbladder mucosa; green: bile; orange: gallstone; red: gallstone patients' feces; *$P < 0.05$.

that LDA >3 indicates a significant difference. However, given the large variety of bacteria in the bile, gallbladder mucosa, and fecal samples from the gallstone and control groups, LDA >4 was adopted as the threshold value for screening characteristic bacteria (Figure 6). We found that in the gallstone group, *Prevotella* had LDA >4 in the fecal samples; Gamma proteobacteria, Pseudomonadales, *Moraxellaceae*, and *Acinetobacter* had LDA >4 in the bile sample; Proteobacteria, *Betaproteobacteria*, and *Burkholderiales* had LDA value >4 in the gallbladder mucosa sample. None of the bacteria had LDA >4 in gallstone samples, while the representative *Bacilli*, *Lactobacillales*, *Enterococcus*, and *Enterococcaceae* had LDA >3. Bacteria species with LDA >4 in the feces of the control group included *Bacteroidia*, *Bacteroidales*, *Bacteroidaceae*, *Bacteroides*, *Clostridia*, *Clostridiales*, *Firmicutes*, and *Ruminococcaceae* (Figure 6).

4. Discussion

Gut microbiota studies have gained momentum in recent years. The human intestine is colonized by over 100 trillion bacteria, involved in many body activities. Intestinal dysbiosis has been associated with various human diseases, such as kidney stones, obesity, diabetes, osteoporosis, and polycystic ovary syndrome [19]. The relationship between gallstones and gut microbiota has gradually become a research hotspot. Given that the gut microbiota is subject to many potential influencing factors, strict inclusion and exclusion criteria were established for this study. For instance, we excluded patients with severe bacteremia, sepsis, and a history of antibiotic or probiotic use in the past 3 months [20]. Moreover, we excluded patients with serious comorbidities (e.g., metabolic diseases) [21], prior use of somatostatin and other drugs affecting gallstone formation [22], history of intestinal surgery [23], and pregnant women or long-term contraceptive users. Due to the strict exclusion criteria, the number of samples enrolled in this study was limited. In this study, we studied the composition of bacterial communities in gallstones, bile, gallbladder mucosa, and intestinal samples from 21 gallstone patients, as well as the gut of 20 normal individuals. High-throughput sequencing was used to sequence V3-V4 fragments of the bacterial 16S rRNA gene. The total number of sequences obtained was 10 429 883 from 72 samples for subsequent statistical analysis.

This study found no significant difference in the abundance and diversity of gut microbiota between patients in the gallstone and control groups, consistent with previous literature [18]. However, studies have reported a decrease in intestinal microbial diversity and the abundance of the genus *Roseburia* [24]. This discrepancy in findings warrants further multicenter studies with larger samples to validate and evaluate the robustness of our findings. In comparison to the control group, the gallstone group reported a decreased abundance of *Achromobacter*, *Faecalibacterium,* and *Lachnospira* and an increased abundance of *Enterococcus*. Research has found that *Enterococcus* can shorten the nucleation time of cholesterol crystals in simulated bile and promote nuclear activity [25],

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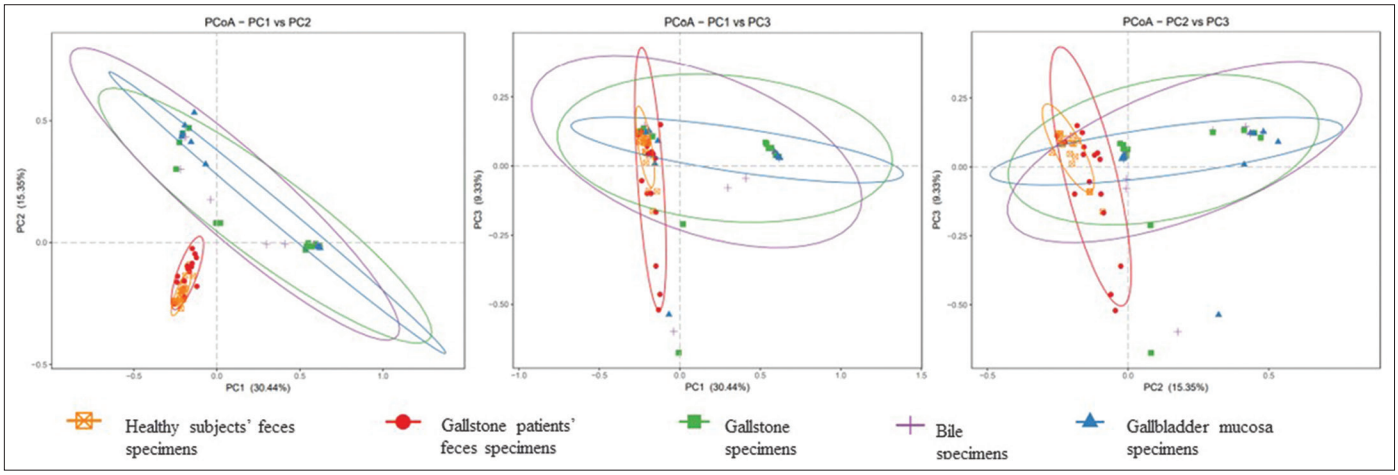

Figure 4. Principal coordinate analysis (PcoA) based on the Bray-Curtis algorithm between principal coordinate (PC) 1 and PC2, PC1 and PC3, and PC2 and PC3.

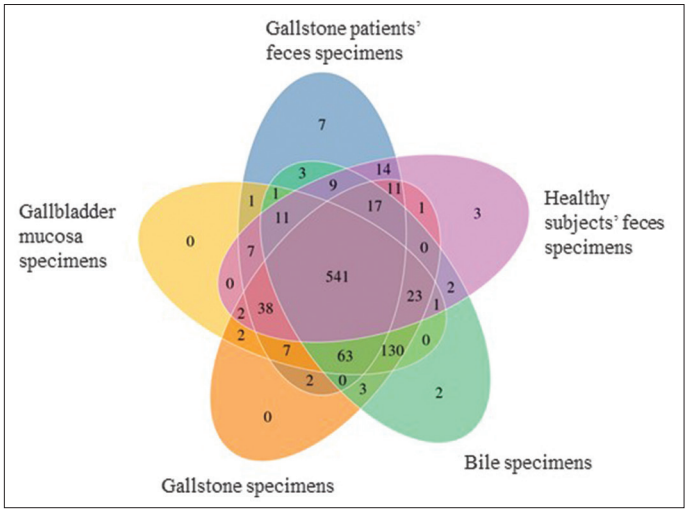

Figure 5. Venn plot of operational taxonomic units between the sample types.

representing a potential mechanism for the increased abundance of *Enterococcus* in patients with gallstones. Studies have also demonstrated that Proteobacteria in the intestine of patients with gallstones were prone to bacterial overgrowth, which was also observed in a wide range of pathogenic microorganisms, such as *Escherichia coli*, *Salmonella*, *Vibrio,* and *Helicobacter* [18]. Animal experiments found that cholesterol stones were formed in mice fed with a lithogenic diet, and the gut microbiota of *Firmicutes* and *Bacteroidetes* was significantly reduced [26]. Taken together, the above findings suggested that gut microbiota disorders are common in patients with gallstones.

We found that the bacterial diversities of gallstones, bile, and gallbladder mucosa were significantly different from the gut microbiota (based on Simpson's index), indicating greater diversity in the gut microbiota than in the biliary microbiota of patients with gallstones. Several studies have concluded that the average biodiversity of bile microorganisms decreases in patients with recurrent choledocholithiasis [27], suggesting that the decreased biodiversity may weaken the elasticity of natural ecosystems and increase the possibility of serious ecosystem degradation [28]. Nonetheless, it has been observed that the bacterial diversity of the biliary tract was significantly higher than that of the intestinal tract [18]. This discrepancy may be caused by the significant differences in biliary microbiota between individuals [29].

At the phylum level, the abundance of Proteobacteria was significantly higher and the abundance of *Firmicutes* and *Bacteroidetes* was significantly lower in the biliary tract than in the intestinal tract, similar to existing research. Proteobacteria can participate in oxidative stress and are a potential microbial diagnostic marker of epithelial dysfunction [30]. The formation of gallstones is also related to epithelial dysfunction [31], which can explain the high abundance of Proteobacteria in patients with gallstones to a certain extent. Increasing evidence corroborates that the number of Proteobacteria in bile from patients with recurrent choledocholithiasis is significantly higher than in patients without cholelithiasis [32].

In this study, LefSe analysis identified many types of Proteobacteria with LDA >4 in the biliary tract and *Firmicutes* and *Bacteroidetes* with LDA value >4 in the intestine of the control group. This finding further validated the differences in bacterial community structure at the phylum level. At the genus level, the abundance of *Acinetobacter* (belonging to Proteobacteria), *Bacteroides*, *Faecalibacterium*, and *Lachnoclostridium* was significantly higher in the biliary tract than in the intestinal tract. The abundance of *Subdoligranulum* was significantly lower than in the gut. Several studies have revealed that *Acinetobacter* can produce *β*-glucuronidase, which hydrolyzes bilirubin glucuronic acid to produce free bilirubin that combines with free calcium ions to form gallstones [25,33].

This study found a significant increase in *Prevotella* in the intestines of patients with gallstones compared to the biliary microbiota. In LefSe analysis, *Prevotella* had LDA >4 in the feces of patients with gallstones, suggesting that *Prevotella* can be used as a biomarker for bacterial dysregulation in patients with gallstones. In this regard, a meta-analysis of 1791 patients demonstrated that *Prevotella* is involved in atherosclerosis [34],

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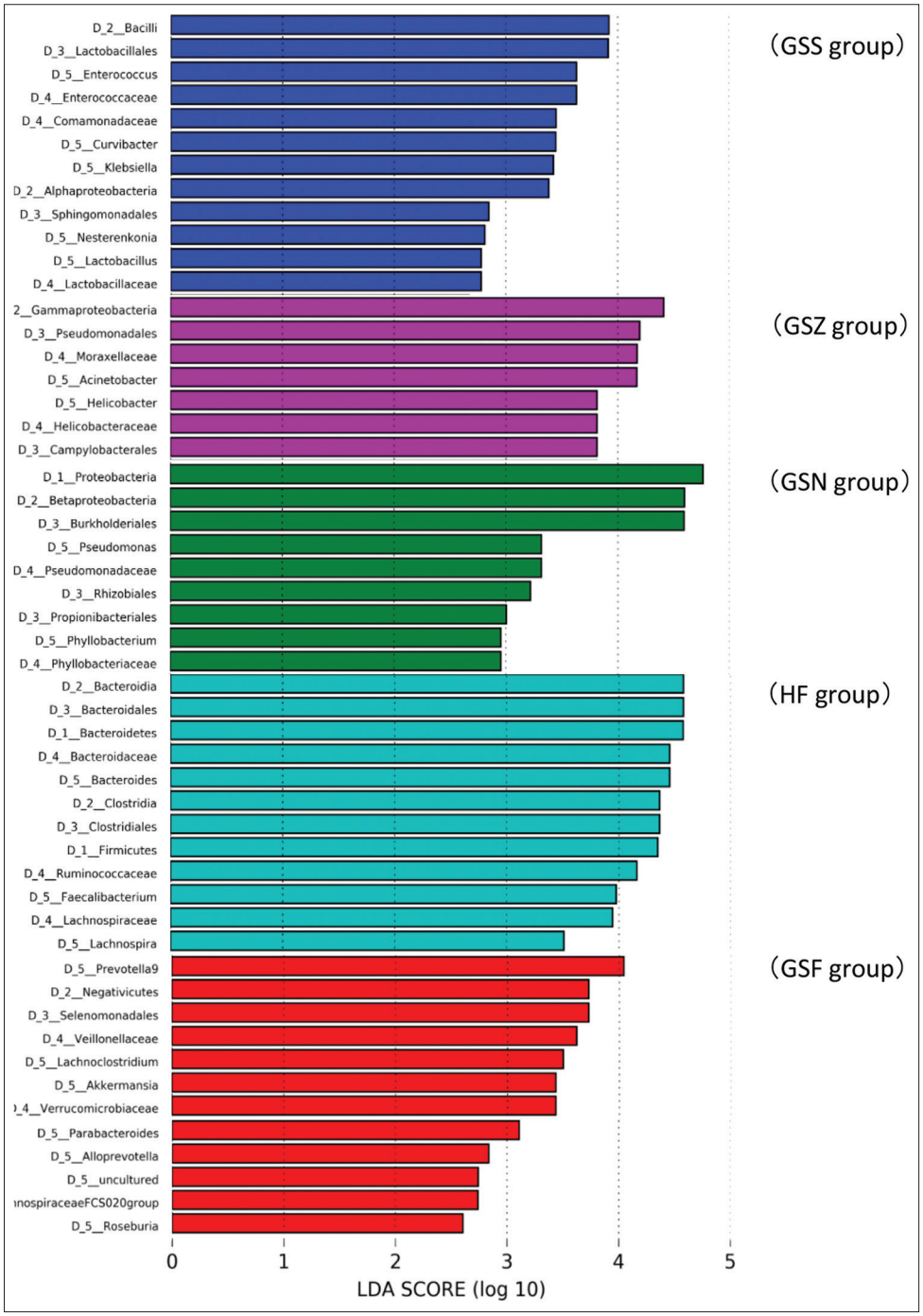

Figure 6. Histogram of LDA scores for differentially abundant genera in the gallstone, bile, gallbladder mucosa, gallstone patients' feces specimens, and healthy subjects' feces specimens. The cladogram was calculated by LDA and displayed according to effect size.
Abbreviations: GSF: Feces from the gallstone group; GSN: Gallbladder mucosa; GSS: Gallstone; GSZ: Bile; HF: Feces from the control group; LDA: Linear discriminant analysis.

and it is known that metabolic factors related to atherosclerosis are closely associated with gallstone formation [12].

In this study, the PC1 versus PC2 plots displayed relatively similar gut and biliary microbiota between the gallstone and control groups, suggesting the specificity of the biliary tract and gut microbiota. However, the PC2 versus PC3 plot revealed some diversity between the gut and biliary microbiota, indicating a certain degree of overlap between the intestinal and biliary microbiota. The OTUs of the biliary and intestinal tract were visualized with a Venn plot, and the results reflected the overlapping OTUs among the samples, indicating that the biliary microbiota may be partly derived from a retrograde intestinal infection and providing novel insights into the source of biliary microbiota. Subsequent PCoA analysis validated this finding,

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and elucidating the diversity of biliary and gut microbiota in gallstone diseases may facilitate the prognosis and management of gallstones.

Nonetheless, there were several limitations in this study, such as the small sample size. Cholecystectomy and endoscopic operations are invasive procedures, and this study did not include bile samples from the control group for comparisons. This study selectively excluded the research subjects, resulting in self-selection and sample-selection biases, thereby limiting an accurate representation of the population.

5. Conclusion

The biliary tract (e.g., gallstones, bile, and gallbladder mucosa) and gut microbiota of patients with gallstones exhibited specific changes compared to healthy individuals, providing novel insights into the source of the biliary tract flora and the formation of gallstones. Patients with gallstones had an obvious microbiota imbalance at the genus level. The diversity of gut microbiota was higher than that of biliary microbiota among gallstone patients. *Enterococcus* abundance was higher in the intestines of patients with gallstones than in healthy individuals. *Prevotella* and Proteobacteria may serve as biomarkers for dysbacteriosis in patients with gallstones as alterations of the biliary and gut microbiota may be related to the formation of gallstones. The identification of characteristic microbiota warrants further studies on gene function annotation and metabolic pathways to explore the pathogenesis of gallstones and its significance in gallstone prevention and treatment. In addition, our research provided new ideas for exploring the feasibility of fecal microbiota transplantation for the treatment of gallstone disease. Based on the specific microbiota of gallstone patients identified in this study, we will conduct further studies on bile acid metabolomics to (i) explore the correlation between gut microbiota imbalance and bile acid metabolism, (ii) identify potential metabolic pathways and targets, and (iii) establish a network relationship between gut microbiota imbalance and gallstones formation. In summation, fecal microbiota transplantation could reconstruct new gut microbiota and evaluate the treatment of extraintestinal diseases.

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Conflict of Interest

The authors have no relevant financial or non-financial interests to disclose.

Ethical Approval and Consent to Participate

Ethical approval was obtained from the Institutional Research Ethics Committee of Beijing Tiantan Hospital, Capital Medical University (KY2020-032-02). We obtained informed consent from human patients before their participation by signing an informed consent form.

Consent for Publication

We obtained informed consent from human patients before their participation by signing an informed consent form.

Availability of Data

The datasets generated during the present study are available in the NCBI database. The raw sequence reads of this study were deposited at NCBI under Bioproject with the accession number PRJNA 929661.

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