Distinct composition and metabolic potential of biliary microbiota in patients with malignant bile duct obstruction

Lijuan Qian[a,*], Limei Wang[a,*], Zigui Zou[b,*], Fujuan Luan[a], Xiaodi Cai[a], Jikai Zhou[a], Dongming Zhu[c], Jianxin Ye[a] and Chenguang Dai[a]

Background Emerging evidence highlights the role of biliary microbiota in hepato-biliary-pancreatic diseases. The characteristics of biliary microbiota in malignant bile duct obstruction remain poorly understood. This study aims to investigate the composition and metabolic functions of biliary microbiota in patients with malignant obstruction.

Methods Eligible patients were enrolled in this prospective study at First Affiliated Hospital of Soochow University between December 2022 and October 2023, including distal cholangiocarcinoma, hilar cholangiocarcinoma, pancreatic ductal adenocarcinoma, periampullary carcinoma, and gallbladder carcinoma. The patients with choledocholithiasis served as controls. Bile samples were collected via endoscopic retrograde cholangiopancreatography. Microbiota identification was performed using *16S rRNA* sequencing, while bile acids were analyzed using mass spectrometry.

Results A total of 56 patients were successfully enrolled in this study, 25 in the tumor group and 31 in the stone group. A distinct biliary microbial community was observed in patients with malignant bile duct obstruction, consisting of *Prevotella*, *Uruburuella*, *Atopostipes*, *Clostridium IV*, *Halomonas*, *Tannerella*, *Porphyromonas*, *Achromobacter*, *Rouxiella*, *Campylobacter*, *Corynebacterium*, *Turicibacter*, *Muribaculum*, *Selenomonas*, and *Alloprevotella* at genus level. Notably, *Clostridium IV*, *Halomonas*, *Rouxiella*, and *Turicibacter* were exclusively present in the tumor group. Bile acid levels were significantly lower in the tumor group ($P < 0.05$), except for ursodeoxycholic acid and taurocholic acid. Additionally, 22 metabolic pathways were enriched in the tumor group.

Conclusions This study elucidates the community and metabolic potential of biliary microbiota in malignant bile duct obstruction. The findings offer valuable insights for disease assessment and provide a foundation for further research into the role of biliary microbiota in malignancy. Eur J Gastroenterol Hepatol 37: 585–593

Introduction

Bile duct obstruction usually attacks patients with hepato-biliary-pancreatic diseases [1]. Benign bile duct obstruction is commonly caused by choledocholithiasis, while hepato-biliary-pancreatic tumors contribute to the most of malignant obstruction. Due to the difficulty in early diagnosis and treatment, the prognosis of malignant bile duct obstruction is poor. It is meaningful to develop a new strategy that enables accurate and individualized management.

The microbiota in the human body is now considered an irreplaceable component that interacts with a variety of organs. Different microbial niches have been identified in multiple organs, including the alimentary canal, vagina, urinary tract, skin, and oral cavity [2]. The occurrence and development of several diseases were demonstrated to be affected by microorganisms and their metabolites [3]. The biliary tract was traditionally recognized as a sterile environment due to the protective effect of bile acids, antimicrobial peptides, and Oddi's sphincter [4]. With the development of next-generation sequencing technology, the existence of microbiota was confirmed in the biliary system, both in healthy and ill populations [5]. The relationship between gut microbiota and hepato-biliary-pancreatic diseases has been revealed in previous studies [6,7]. It is unclear whether the biliary microbiota is correlated with the occurrence of malignant bile duct obstruction. To investigate the potential impact from biliary flora, this study was designed to compare the characteristics of the biliary microbial community between patients with malignant and benign bile duct obstruction.

European Journal of Gastroenterology & Hepatology 2025, 37:585–593

Keywords: *16S rRNA* sequencing, biliary microbial community, malignant biliary obstruction, metabolic function

Departments of [a]Gastroenterology, [b]Pathology and [c]General Surgery, First Affiliated Hospital of Soochow University, Suzhou, China

Correspondence to Chenguang Dai, MD, Department of Gastroenterology, First Affiliated Hospital of Soochow University, Pinghai Road 899, Suzhou, Jiangsu Province 215006, China
Tel: +86 0512 67781055; fax: +86 0512 67780374; e-mail: daicg_soochow@163.com

*Lijuan Qian, Limei Wang, and Zigui Zou contributed equally to the writing of this article.

Received 11 January 2025 **Accepted** 3 February 2025.

Supplemental Digital Content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's website, www.eurojgh.com.

In the present study, *16S rRNA* sequencing technology and bioinformatics methods were employed to analyze the species structure and distribution in bile from patients with malignant tumors and common bile duct stones (CBDS). Metabolic functions were predicted through phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSTs). Additionally, ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) system was introduced to analyze the bile samples and identify the 15 bile acids quantitatively.

Methods

Study design and patient enrollment

This study was designed as a prospective case–control study. Eligible patients were consecutively enrolled in the study between December 2022 and October 2023 at the First Affiliated Hospital of Soochow University. This study was reviewed and approved by the ethics committee. All the patients provided their written informed consent to participate in this study.

The inclusion criteria were as follows: (1) patients who suffered from bile duct obstruction; (2) all the diagnoses confirmed by imaging examinations or histopathological tests, including pancreatic ductal adenocarcinoma (PDAC), distal cholangiocarcinoma (DCCA), hilar cholangiocarcinoma (HCCA), periampullary carcinoma (PAC), gallbladder cancer (GBC), and CBDS; (3) no antibiotics usage within the preceding 3 months. The exclusion criteria were as follows: (1) patients who were attacked by acute obstructive suppurative cholangitis or acute hepatic failure at the same time; (2) without gallbladder; (3) surgical history concerning upper gastrointestinal tract or common bile duct (CBD); (4) history of gastrointestinal or hepato-biliary-pancreatic neoplastic diseases. Depending on the causes of bile duct obstruction, the patients were divided into two groups: the tumor group and the stone group.

Sample collection

Endoscopic retrograde cholangiopancreatography (ERCP) was performed on the eligible patients once the informed consents were acquired. All bile samples were collected during ERCP procedures. To ensure the sterility of the working channel, the duodenoscope underwent rigorous disinfection before the operation. All instruments used in ERCP, including aspiration catheter, guide wire, and bile storage tube, were sterile. In brief, the guide wire was first inserted into CBD under fluoroscopic guidance, followed by the insertion of the aspiration catheter. Bile was then aspirated through the catheter before the injection of contrast medium. The samples were immediately stored in a freezer at –80 °C for preservation.

16S rRNA amplicon sequencing

Based on the manufacturer's protocols, microbial DNA was extracted from the obtained bile samples using QIAamp DNA Mini Kit (250) (QIAGEN, Hilden, Germany). Briefly, the bile samples were homogenized with InhibitEX Buffer. The mixture was beaten at 60 Hz for 1 min twice by a Homogeneous instrument (FASTPREP-24, Aosheng Biotech, Handan, China). Thermo Nano Drop 2000 UV microspectrophotometer and agarose gel electrophoresis were introduced to evaluate the total DNA quality.

The amplification of V3-V4 region of *16S rRNA* genes was performed by PCR using primers 341F (5'-CCTACGGGRSGCAGCAG-3') and 806R (5'-GGACTACVVGGGTATCTAATC-3') [8]. The PCR reactions were carried out in a 25 µl mixture, consisting of 12.5 µl of HiScript III RT SuperMix (Vazyme Biotech, Nanjing, China), 1 µl of each primer (10 µM), 50 ng of template DNA, and ddH2O. Amplicons were subsequently extracted from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, California, USA). Quantified analysis of the products was performed by Qubit2.0 (Invitrogen, Carlsbad, California, USA). Following library preparation, purified amplicons were subjected to 250 bp paired-end sequencing on the Illumina NovaSeq platform (Illumina, San Diego, California, USA). *16S rRNA* sequencing data are available from the BioProject database of the National Center for Biotechnology Information under accession no. PRJNA1121599.

Identification of bile acids

An aliquot of 50 µl bile sample was mixed with 100 µl isotope-labeled internal standard and 50 µl methanol. The mixture was vortexed for 10 min to precipitate proteins, followed by centrifugation at 14 000 rpm for 10 min. Subsequently, 50 µl of the supernatant was subjected to dilution at varying ratios. Finally, a 20 µl aliquot of the diluted sample was injected into the AB SCIEX Triple Quad 6500+ LC/MS/MS system (Waters, Milford, Massachusetts, USA) for the detection and identification of bile acids.

An electric spray ion source in negative ion mode served as the ion source. The ion source parameters were as follows: 60 psi for desolvation gas, 55 psi for heating gas, 500 °C for desolvation gas, 30 psi for curtain gas, 12 psi for collision gas, and a spray voltage of –4500 V. Analyst 1.6.3 software and MultiQuant software (AB SCIEX, Concord, Ontario, Canada) were applied for data acquisition and quantitative analysis. A multiple-reaction monitoring mode was employed for scanning. Liquid phase separation was conducted using a C18 chromatographic column. The mobile phase was water and methanol combined with 5 mM ammonium acetate. Elution of the gradient took 9 min. Blank samples were injected before data acquisition to balance the instrument. The testing sequences were blanks, calibration curves, blanks, quality control (QC) samples, samples, and QC samples.

Data analysis

Demographic characteristics were recorded, including age, gender distribution, BMI, laboratory examination results, and medical history. The data were analyzed using SPSS statistics 27.0 (IBM, Amunk, New York State, USA). Continuous variables were expressed as mean with SD (mean ± SD) or median, and compared using Student's *t* test or Mann–Whitney test. Categorical variables were described as count and percentage and analyzed using chi-square test. Statistical significance was set at $P < 0.05$.

Table 1. Demographics and preoperative characteristics

	Stone group $n = 31$	Tumor group $n = 25$	P value
Age, years, mean ± SD	68.97 ± 15.88	70.08 ± 13.40	0.781
Gender, male/female	22/9	19/6	0.672
BMI, kg/m², mean ± SD	21.96 ± 2.57	21.37 ± 2.76	0.415
Past medical history, *n* (%)			
Hypertension	9 (29.0%)	9 (36.0%)	0.579
Diabetes	8 (25.8%)	6 (24.0%)	0.877
Others[a]	17 (54.8%)	13 (52.0%)	0.832
Hb, g/L, mean ± SD	129.75 ± 20.06	107.75 ± 18.07	<0.001
WBC, ×10⁹/L, mean ± SD	6.44 ± 2.80	6.98 ± 3.75	0.553
PLT, ×10⁹/L, mean ± SD	191.57 ± 77.33	187.88 ± 76.93	0.864
TBIL, μmol/L, median	21.20	263.60	<0.001
DBIL, μmol/L, median	10.15	199.40	<0.001
ALT, U/L, median	79.10	84.90	0.391
AST, U/L, median	38.10	102.60	0.002
γ-GGT, U/L, median	316.70	277.90	0.691
ALP, U/L, median	136.10	409.40	<0.001
ALB, g/L, mean ± SD	36.59 ± 6.56	30.87 ± 6.30	0.002
CA19-9, U/ml, median	11.12	585.04	<0.001

γ-GGT, γ-glutamyl transpeptidase; ALB, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DBIL, direct bilirubin; Hb, hemoglobin; PLT, platelet; TBIL, total bilirubin; WBC, white blood cell.

[a]Other coexisting disorders included cerebral infarction, Parkinson's disease, lung cancer, bronchiectasis, bronchiectasis, gout, rheumatoid arthritis, syphilis, hepatitis B and C, liver cirrhosis, chronic kidney disease, myoma of uterus, coronary heart disease, and atrial fibrillation.

Once the bile samples were successfully sequenced, tags, trimmed of barcodes, and the primers were meticulously examined and filtered based on the quality. The tags whose lengths were shorter than 250 bp or longer than 500 bp would be screened out. The Phred score of bases was better than 30 (Q30) and less than 1 ambiguous N. Redundant duplicate tags were eliminated after enumerating the copy number. Only the sequences whose frequency was greater than 1 would be clustered into operational taxonomic units (OTUs). UPARSE (http://drive5.com/uparse/) was employed to cluster OTUs by a 97% similarity threshold. Usearch (version 7.0) was used to identify and subsequently remove chimeric sequences. The taxonomy of each representative sequence was identified via RDP Classifer (http://rdp.cme.msu.edu/) against the RDP database (http://rdp.cme.msu.edu/) with a confidence threshold of 80%.

OTU profiling tables and alpha/beta diversity analyses were conducted through QIIME (version 1.9.1). Sequencing depth was evaluated by Good's coverage index. Community richness and diversity were assessed through observed species, Shannon, Simpson, and Chao1 indices. The microbial composition and distribution between the two groups were assessed by analysis of similarities (ANOSIM) analysis, multi response permutation procedure (MRPP) analysis, and heatmap based on weighted UniFrac distance. A Venn diagram was generated using R/Perl SVG to show the core microbiome. Linear discriminant analysis (LDA) effect size (LEfSe) was utilized to assess the impact of each component. The LDA threshold was set at 2.0. Wilcoxon analysis was utilized to identify the distinct flora between the two groups, and $P < 0.05$ was considered statistically significant. Both analyses were applied to find the genera that significantly influenced sample categorization. With the help of the corrplot package in R, the Spearman correlation heatmap was figured out according to the top 30 different genera.

To predict the metabolic functions, metabolic pathways were calculated by PICRUSTs 2.0 based on MetaCyc database. Principal component analysis was used to illustrate the difference in metabolic functions between the two groups. The contents of bile acids were recorded through Analyst 1.6.2 software and carried out quantitatively via MultiQuant software (AB SCIEX, Concord, Ontario, Canada).

Results

Demographics and preoperative characteristics

A total of 56 patients were eventually eligible for the study, 25 in the tumor group and 31 in the stone group, respectively. In detail, the cohort in the tumor group included 3 DCCA, 3 HCCA, 6 PAC, 11 PDAC, and 2 GBC patients. No significant differences were observed in terms of age (70.08 ± 13.40 vs. 68.97 ± 15.88, $P = 0.781$), sex distribution ($P = 0.672$), and BMI (21.37 ± 2.76 vs. 21.96 ± 2.57 kg/m², $P = 0.415$) (Table 1). The two groups had comparable medical histories.

The results of several preoperative laboratory examinations were comparable between the two groups, including white blood cell (6.98 ± 3.75 vs. 6.44 ± 2.80 ×10⁹/L, $P = 0.553$), platelet (187.88 ± 76.93 vs. 191.57 ± 77.33 ×10⁹/L, $P = 0.864$), alanine aminotransferase (84.90 vs. 79.10 U/L, $P = 0.391$), and γ-glutamyl transpeptidase (277.90 vs. 316.70 U/L, $P = 0.691$) (Table 1). The patients with malignant bile duct obstruction experienced higher level of total bilirubin (263.60 vs. 21.20 μmol/L, $P < 0.001$), direct bilirubin (199.40 vs. 10.15 μmol/L, $P < 0.001$), aspartate aminotransferase (102.60 vs. 38.10 U/L, $P = 0.002$), alkaline phosphatase (409.40 vs. 136.10 U/L, $P < 0.001$), and CA19-9 (585.04 vs. 11.12 U/ml, $P < 0.001$), while these patients had lower level of hemoglobin (107.75 ± 18.07 vs. 129.75 ± 20.06 g/L, $P < 0.001$) and albumin (30.87 ± 6.30 vs. 36.59 ± 6.56 g/L, $P = 0.002$).

Diversity and annotation analyses of biliary microbiota

The average sequence length ranged from 420 to 440 bp. Both the Good's coverage indices approached 1.00, and they were comparable between the two groups (Supplementary Figure S1A, Supplemental digital content 1, *http://links.lww.com/EJGH/B137* and Supplementary Table S1, Supplemental digital content 2, *http://links.lww.com/EJGH/B138*, $P = 0.817$). The sequencing was at enough depth in this study. No significant difference was found in the richness of biliary microbiota according to the results of alpha diversity analysis, including Chao1, observed species, Shannon and Simpson indices (Fig. 1 and Supplementary Table S1, Supplemental digital content 2, *http://links.lww.com/EJGH/B138*, all $P > 0.05$). Beta diversity analysis was further carried out to explore the differences in the composition of biliary microbial community. The difference between groups was significantly greater than the difference within groups based on ANOSIM analysis employing weighted Unifrac distances (Fig. 2a, $P = 0.004$). Similar results were also figured out from MRPP analysis using weighted Unifrac distances ($P = 0.026$, no figure) and the heatmap (Fig. 2b). Different from CBDS, there were unique microbial composition

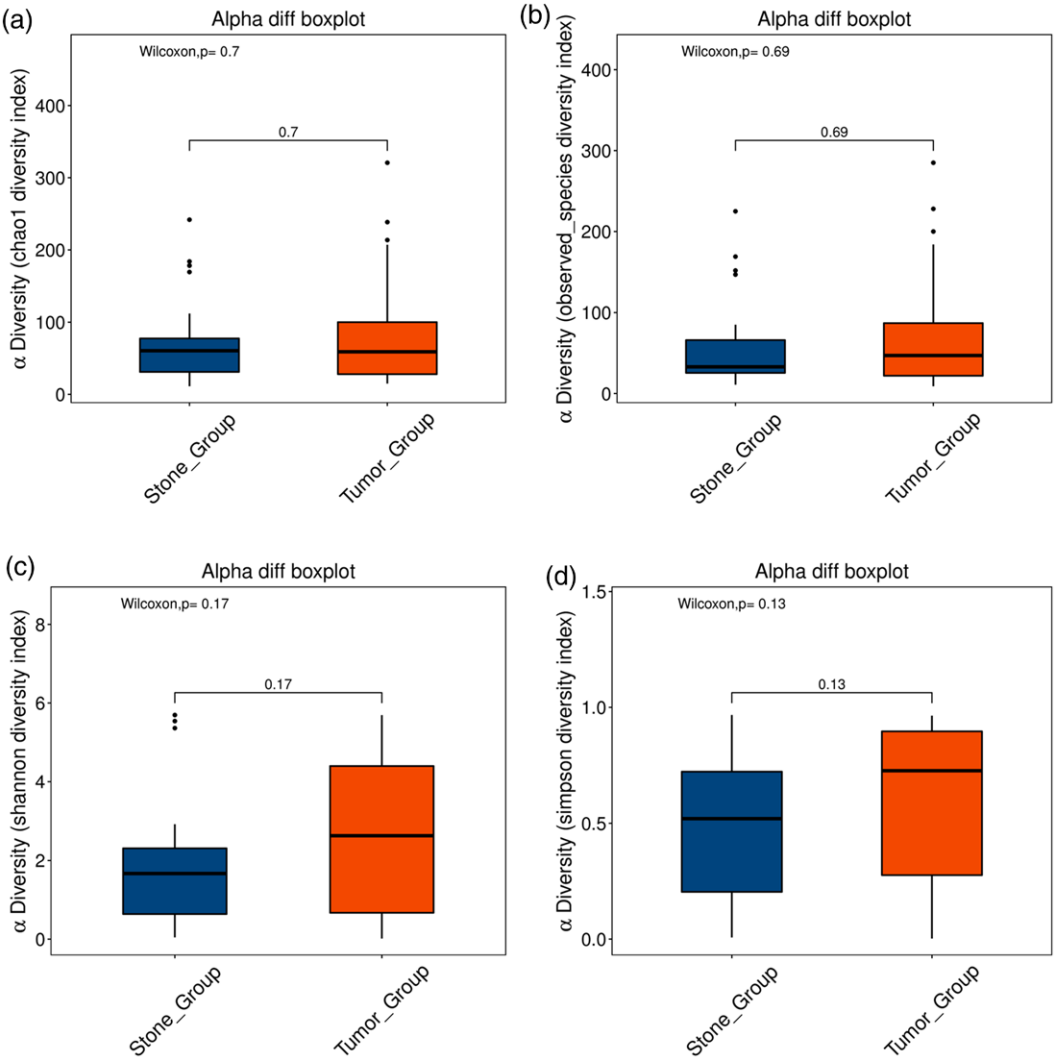

Fig. 1. Alpha diversity analysis showed that the richness of biliary microbiota was comparable between the two groups: (a) Chao1 index (P = 0.699); (b) observed species index (P = 0.686); (c) Shannon index (P = 0.168); (d) Simpson index (P = 0.134).

and distribution in bile from patients with malignant biliary obstruction, even though they shared similar flora richness.

There were 711 OTUs and 609 OTUs identified in the tumor group and the stone group, respectively, of which 432 OTUs were shared between the two groups (Supplementary Figure S1B, Supplemental digital content 1, *http://links.lww.com/EJGH/B137*). The numbers of OTUs assigned to different taxonomic levels are shown in Supplementary Table S2, Supplemental digital content 2, *http://links.lww.com/EJGH/B138*. To acquire detailed information about species distribution, the relative abundance of detected bacteria was calculated and compared. The composition of biliary microbiota varied widely at each taxonomic level (Fig. 3 and Supplementary Figure S2, Supplemental digital content 3, *http://links.lww.com/EJGH/B139*). *Firmicutes* and *Proteobacteria* were the predominant phyla among all the individuals (Fig. 3c). *Firmicutes* accounted for 40.67% at the phylum level in the tumor group, followed by *Proteobacteria* (36.44%), *Bacteroidetes* (11.33%), *Actinobacteria* (6.81%), and *Fusobacteria* (3.71%). At the genus level, the top five genera in patients with malignant bile duct obstruction were *Streptococcus* (14.44%), *Klebsiella* (12.27%), *Enterococcus* (9.99%), *Prevotella* (6.80%), and *Acinetobacter* (4.51%). On the contrary, *Escherichia/Shigella* (23.95%) played a dominant role in the stone group, followed by *Enterococcus* (23.59%), *Klebsiella* (16.26%), *Streptococcus* (8.62%), and *Veillonella* (3.62%) (Fig. 3b and Table 2).

Characteristics of biliary microbiota in patients with malignant bile duct obstruction

LEfSe analysis was performed to unveil the distinct composition of biliary microbiota and identify the dominant genera in the two groups (Fig. 4a). The relative abundance of 43 genera exhibited significant differences between the two groups, with 33 and 10 genera enriched in the tumor group and the stone group, respectively (Fig. 4b). A total of 15 genera were significantly abundant in the tumor group, including *Prevotella*, *Uruburuella*, *Atopostipes*, *Clostridium IV*, *Halomonas*, *Tannerella*, *Porphyromonas*, *Achromobacter*, *Rouxiella*, *Campylobacter*, *Corynebacterium*, *Turicibacter*, *Muribaculum*, *Selenomonas*, and *Alloprevotella*, which contributed

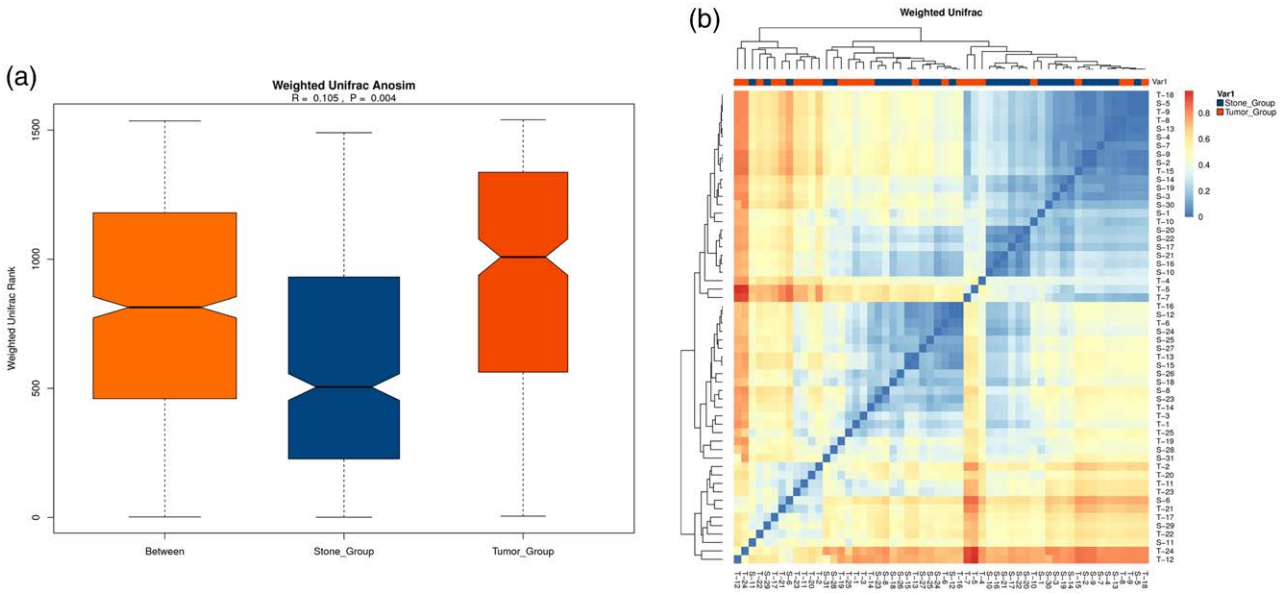

Fig. 2. Beta diversity analysis indicated the distinct community structure of biliary microbiota in patients with malignant bile duct obstruction: (a) ANOSIM analysis using weighted UniFrac distance (P = 0.004); (b) heatmap using weighted UniFrac distance ('T' for tumor group, 'S' for stone group).

to the distinct biliary microbial composition in patients with malignant obstruction (Fig. 4a). Escherichia/*Shigella*, *Enterococcus*, *Klebsiella*, *Clostridium* sensu stricto, *Citrobacter,* and *Rhodobacter*, however, dominate the biliary microbial community in the stone group.

Wilcoxon test was further performed to verify the difference between the two groups, based on the bacterial abundance at the genus level (Supplementary Table S3, Supplemental digital content 2, *http://links.lww.com/EJGH/B138* and Supplementary Figure S3, Supplemental digital content 4, *http://links.lww.com/EJGH/B140*). Consistent with the aforementioned findings, a lot of genera exhibited significant enrichment in the tumor group, including *Alloprevotella*, *Campylobacter*, *Clostridium IV*, *Corynebacterium*, *Halomonas*, *Porphyromonas*, *Prevotella*, *Rouxiella*, *Selenomonas*, *Tannerella*, and *Turicibacter*. Notably, *Clostridium IV*, *Halomonas*, *Rouxiella*, and *Turicibacter* were exclusively present in the tumor group. The genera abundant in the tumor group showed positive correlations with each other, however, negative correlations with the genera enriched in the stone group (Fig. 4c).

Alterations in biliary metabolism

Bile acids were successfully extracted from all the bile samples. The levels of several bile acids significantly decreased in the tumor group, including cholic acid (CA), deoxycholic acid, chenodeoxycholic acid (CDCA), lithocholic acid, glycocholic acid, glycodeoxycholic acid, glycochenodeoxycholic acid, glycoursodeoxycholic acid, glycolithocholic acid, taurodeoxycholic acid, taurochenodeoxycholic acid, tauroursodeoxycholic acid, and taurolithocholic acid (Table 3, all $P < 0.05$), while no significant differences were identified in ursodeoxycholic acid (UDCA) and taurocholic acid (TCA).

To explore the potential functions that might influence the progression of malignant bile duct obstruction, PICRUSt 2.0 calculation was carried out, based on the abundance of biliary microbiota. There were 22 and 39 metabolic pathways enriched in the tumor group and the stone group, respectively (Fig. 5a). The metabolic functions of the biliary microbiota in the tumor group were significantly distinct from those in the stone group (Fig. 5b).

Discussion

It was challenging to investigate biliary microbiota due to the invasive nature of bile sample collection. The advancements in ERCP techniques and high-throughput sequencing, however, have enabled a more comprehensive understanding of the relationship between hepato-biliary-pancreatic diseases and the microbiota [9].

Since the colonization of bacteria in gallstones was confirmed by Maki *et al.* [10] in 1996, it has been debated on the existence and influence of biliary microbiota for decades. The bacteria could originate from various systems, including the duodenum, colon, oral, and portal vein. Compared with healthy stool samples, microbial diversity decreased in bile samples from patients with gallstone [11]. Molinero *et al.* [12] innovatively utilized liver transplant donors as controls to demonstrate the presence of biliary microbiota in healthy individuals. *Firmicutes, Bacteroidetes, Proteobacteria,* and *Actinobacteria* dominate the biliary microbial community, both in the healthy population and the patients with cholelithiasis. A series of studies have illustrated the unique composition of biliary microbiota in various diseases, including primary sclerosing cholangitis [13], primary biliary cirrhosis [14], giant CBDS [15], and recurrent CBDS [16]. A special microbial cluster exists in the bile of all the individuals, different from gut flora. The relative abundance of the core microbiome influences the biological and clinical characteristics of hepato-biliary-pancreatic diseases.

Emerging studies have recently focused on the potential role of biliary microbiota in the development of malignant

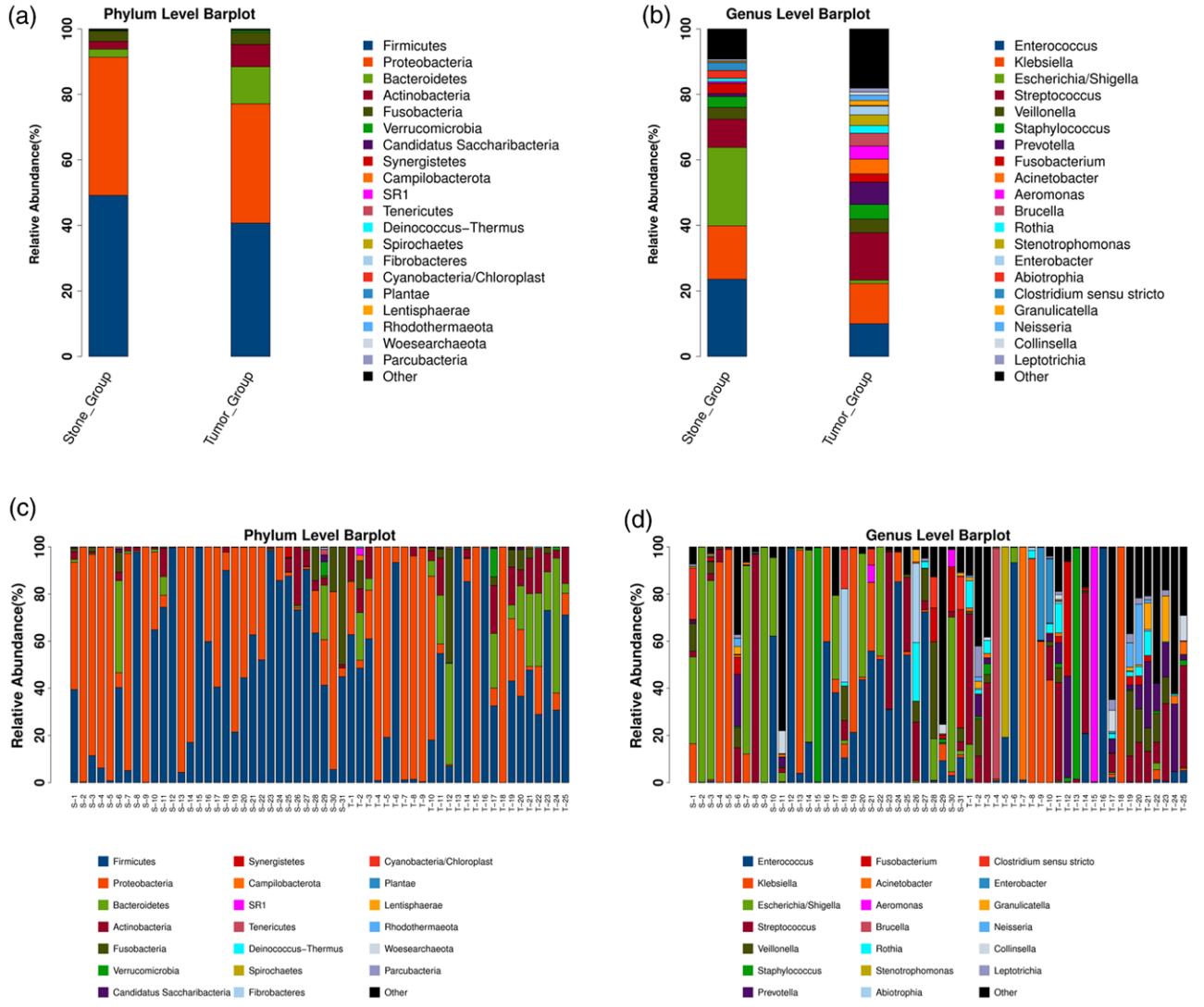

Fig. 3. OTU and annotation analysis: the relative abundance and distribution of biliary microbiota at different taxonomic levels ('T' for tumor group, 'S' for stone group): (a) comparison at the phylum level; (b) comparison at the genus level; (c) microbial community at the phylum level in each sample; (d) microbial community at the genus level in each sample. OTU, operational taxonomic unit.

bile duct obstruction [1,17]. Chen *et al.* [17] revealed the different microbial communities in the biliary tract from cancerous and noncancerous patients. A significant increase in the phyla Gemmatimonadetes, Nitrospirae, Chloroflexi, Latescibacteria, and Planctomycetes was identified in the bile from patients with DCCA. The shift of microbial niches could potentially be a crucial factor in the development of DCCA, but no more information about biliary microbiota in other malignant tumors could be acquired from this study. According to the description by Li *et al.* [1], the distinct composition of biliary microbiota is valuable for differential diagnosis between CBDS and malignant diseases, including HCCA, DCCA, and PDAC. Different from benign bile duct obstruction, both species abundance and community diversity decreased in the bile of malignant obstruction, along with the alteration of the core microbiome. Furthermore, the composition of biliary microbiota varies from disease to disease. The selection bias, however, is inevitable in this retrospective study with only a small number of cases.

The present study was designed to investigate the characteristics of biliary microbiota for patients who suffered from malignant bile duct obstruction. The focus of this study was bile. Presently, all the methods for obtaining bile involve invasive procedures, which are not ethically justifiable in healthy individuals as they lack the necessary medical indications for such interventions. The previous studies concerning biliary microbiota usually utilized patients with CBDS as control. Therefore, the control group in the present study consisted of patients with CBDS. The difference between the tumor group and the stone group was greater than the difference within groups based on beta diversity analysis, even though alpha diversity was comparable between the two groups. The composition and distribution of the biliary microbial community in the tumor group were significantly different from the stone group, even though the individuals shared similar flora richness. *Firmicutes* and *Proteobacteria* were the dominant phyla, no matter in the tumor group or the stone group, which was consistent with the previous

studies [12]. It is different from the alimentary tract, where *Bacteroidetes* govern the microenvironment. The combined relative abundance of *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, and *Fusobacteria* constituted 99% of the biliary microbiota. Both the decrease of *Firmicutes* and *Proteobacteria* and the increase of *Bacteroidetes* and *Actinobacteria* were identified in patients with malignant bile duct obstruction.

Table 2. Relative abundance of the top 20 genera

Genera names	Stone group $n = 31$	Tumor group $n = 25$
Enterococcus	23.58591409	9.990818971
Klebsiella	16.26370808	12.2667525
Escherichia/Shigella	23.94769501	1.035640952
Streptococcus	8.618828155	14.44262246
Staphylococcus	3.30391698	4.450586526
Veillonella	3.618161006	4.187956983
Prevotella	0.9226333	6.804519792
Fusobacterium	3.06192565	2.540274313
Aeromonas	0.473679786	4.006549734
Acinetobacter	0.107419084	4.51221042
Brucella	0.000144778	3.91223187
Rothia	0.994973201	2.344638154
Stenotrophomonas	0.002898514	3.253261575
Abiotrophia	2.388878078	0.151234112
Clostridium sensu stricto	2.403944329	0.028801356
Enterobacter	0.008473651	2.67276948
Granulicatella	0.384135836	1.533743153
Neisseria	0.135155492	1.644753015
Collinsella	0.459244027	0.930224808
Leptotrichia	0.149115619	1.213707703
Unclassified genera	9.169155331	18.07670212

Distinct microbial composition at different taxonomic levels was illustrated in patients with malignant bile duct obstruction (Fig. 3 and Supplementary Figure S2, Supplemental digital content 3, *http://links.lww.com/EJGH/B139*). According to the LEfSe analysis (Fig. 4a, b), *Escherichia/Shigella*, *Enterococcus*, *Klebsiella*, *Clostridium sensu stricto*, *Citrobacter*, *and Rhodobacter* served as biomarkers for patients in the stone group, along with our previous study [15]. The unique microbial community structure in the bile from malignant bile duct obstruction comprised a distinct set of genera (Fig. 4a, b), including *Prevotella*, *Uruburuella*, *Atopostipes*, *Clostridium IV*, *Halomonas*, *Tannerella*, *Porphyromonas*, *Achromobacter*, *Rouxiella*, *Campylobacter*, *Corynebacterium*, *Turicibacter*, *Muribaculum*, *Selenomonas*, and *Alloprevotella*. A significant shift was confirmed in biliary microbial niches from benign diseases to malignant obstruction. The pathophysiological properties of the biliary microenvironment may be altered by bile duct damage and obstruction, resulting in changes to microbial composition. The changes in biliary microbiota have the potential to alter the relationship between the colonizing bacteria and the biliary epithelial cells. It may contribute to cell transformation or DNA damage, thereby increasing the risk of precancerous lesions and cancer.

The levels of bile acids in the tumor group were significantly lower than those in the stone group, with the exception of UDCA and TCA. The finding is consistent with the results reported by Park *et al.* [18], who observed that the patients with cholangiocarcinoma

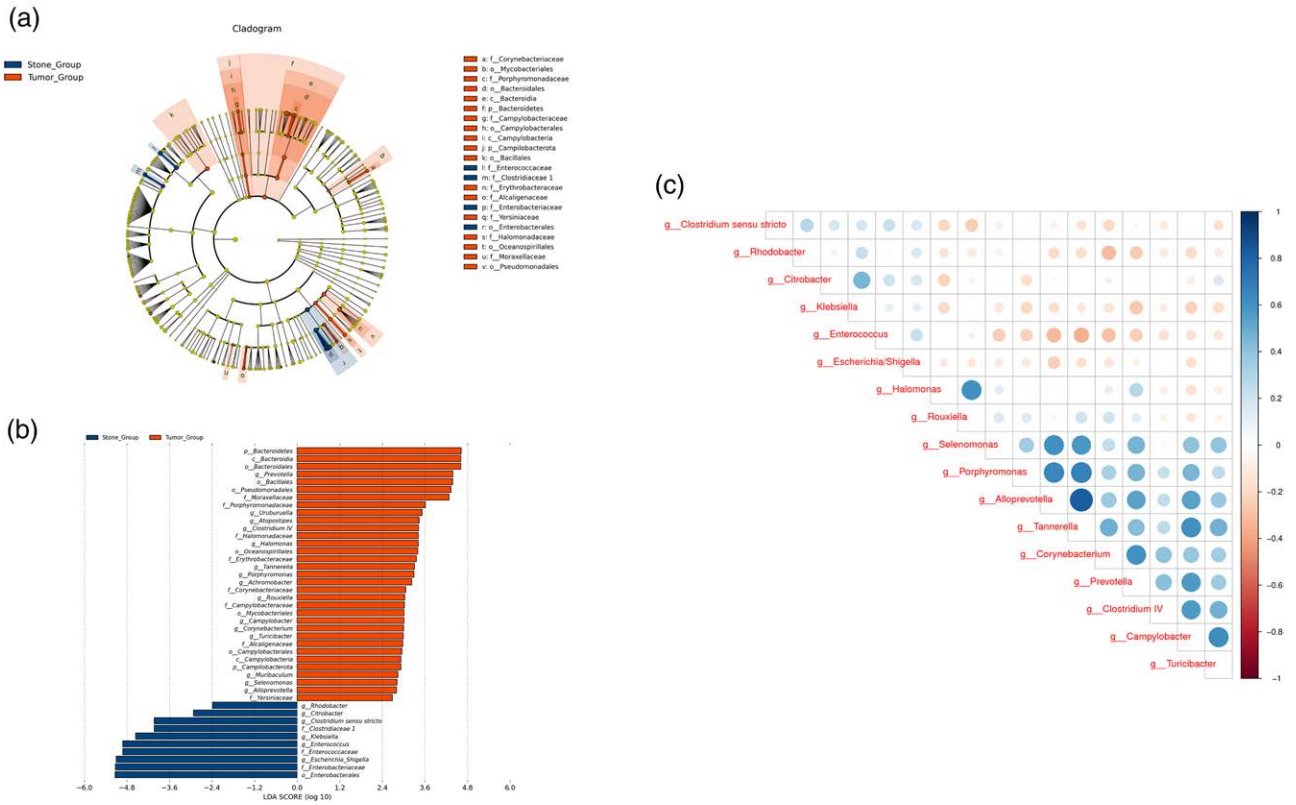

Fig. 4. Characteristics of biliary microbiota between the two groups: (a) LEfSe analysis revealed the different composition and the unique predominant bacteria; (b) the relative abundance of the 43 significantly different bacteria (LDA score threshold was 2.0); (c) Spearman correlation analysis showed that the genera abundant in the tumor group had positive correlations with each other, however, negative correlations with the genera enriched in the stone group. LDA, linear discriminant analysis; LEfSe, linear discriminant analysis effect size.

exhibited similar concentrations of UDCA to those with benign diseases. UDCA has been shown to inhibit the proliferation and invasion of cholangiocarcinoma cells [19]. In contrast, the genera abundant in the tumor group did not exhibit significant correlations with bile acids, while the genera enriched in the stone group generally showed positive correlations, particularly with CA and CDCA. As a typical hydrophobic bile acid, the accumulation of CDCA can increase the risk of cholestasis, thereby contributing to the formation of choledocholithiasis [20]. Further researches are warranted to elucidate the relationship between biliary microbiota and bile acids, as well as the potential mechanisms underlying carcinogenesis.

It is recognized that cellular metabolism is involved in tumorigenesis, such as carbohydrate metabolism, lipid metabolism [21,22], and nitric oxide (NO) metabolism [23]. The unique niche of biliary microbiota in the tumor group has the potential to modulate the bile metabolism, leading to the development of malignant bile duct obstruction. A total of 22 metabolic pathways were enriched in the tumor group, of which thiazole biosynthesis II (PWY-6891) has been proved to be corelated with the genus *Bacillus*. The growth of several malignant tumors could be regulated by the metabolites produced by the genus *Bacillus*, including breast cancer, colorectal cancer, lung cancer, and kidney cancer [24,25]. In the present study, the order Bacillales to which the genus *Bacillus* belongs was significantly abundant in the tumor group (Fig. 4b). Further study is imperative to validate the underlying pathogenesis.

The collection of bile samples could not be acquired from healthy individuals in consideration of ethical principles and requirements. With reference to the literature, the patients with CBDS were included in this study as controls. A large number of patients were initially referred to primary clinics due to acute cholangitis, followed by antibiotic treatment. Therefore, the patients did not meet the inclusion criteria when they were transferred to the research hospital for ERCP, resulting in the small sample size in this study. It is vital to improve the referral process and conduct further clinical studies in multiple centers to enhance the generalizability of the findings.

Table 3. Ultra-performance liquid chromatography-tandem mass spectrometry analysis of bile acids

Bile acids[a] μmol/ml	Stone group *n* = 31	Tumor group *n* = 25	*P* value
CA	27.12 (0.17–1051.84)	1.42 (0.03–17.86)	<0.001
DCA	1.96 (0.08–266.99)	0.09 (0.02–0.65)	<0.001
CDCA	9.52 (0.18–2366.36)	0.55 (0.02–20.90)	<0.001
UDCA	0.77 (0.02–1460.43)	0.39 (0.02–38.28)	0.580
LCA	0.19 (0.01–40.63)	0.02 (0.01–0.15)	0.019
GCA	7521.54 (400.25–16–094.48)	2832.03 (1.31–19–565.34)	<0.001
GDCA	404.39 (3.52–7326.02)	7.48 (0.16–925.35)	<0.001
GCDCA	5707.87 (167.72–19–993.29)	1413.96 (0.37–8325.47)	<0.001
GUDCA	265.81 (4.63–12–365.51)	35.04 (0.30–3748.96)	<0.001
GLCA	7.97 (0.04–330.94)	1.27 (0.03–17.80)	0.001
TCA	1526.75 (240.22–4421.31)	1229.57 (0.48–7254.18)	0.239
TDCA	160.86 (279.42–2216.99)	8.46 (0.06–352.09)	<0.001
TCDCA	1645.07 (279.42–7218.59)	502.29 (0.15–6903.05)	0.005
TUDCA	62.59 (1.85–1479.22)	7.49 (0.01–261.28)	0.001
TLCA	1.43 (0.07–134.40)	0.34 (0.01–12.61)	0.023

CA, cholic acid; CDCA, chenodesoxycholic acid; DCA, deoxycholic acid; GCA, glycocholic acid; GCDCA, glycochenodeoxycholic acid; GDCA, glycodeoxycholic acid; GLCA, glycolithocholic acid; GUDCA, glycoursodeoxycholic acid; LCA, lithocholic acid; TCA, taurocholic acid; TCDCA, taurochenodeoxycholic acid; TDCA, taurodeoxycholic acid; TLCA, taurolithocholic acid; TUDCA, tauroursodeoxycholic acid; UDCA, ursodeoxycholic acid.

[a]The concentration of bile acids is described as median (minimum, maximum).

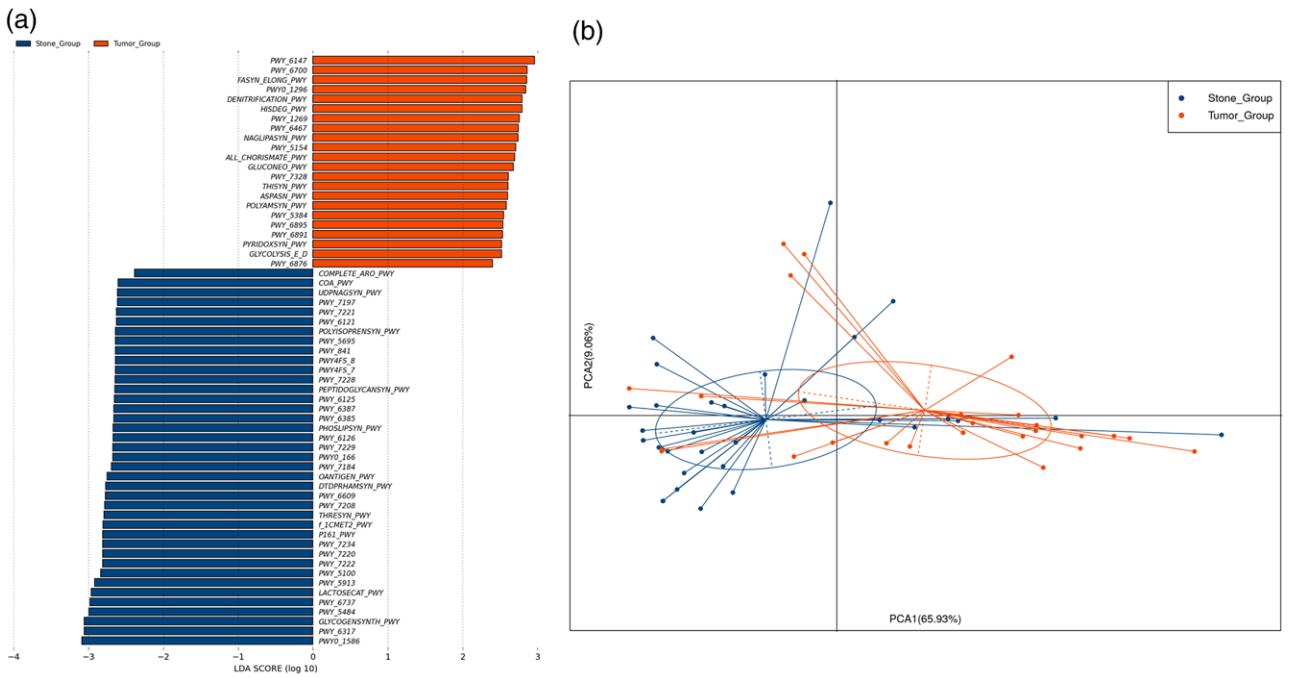

Fig. 5. Metabolic pathways were calculated by PICRUSTs 2.0 based on the abundance of biliary microbiota to predict the metabolic functions: (a) there were 22 and 39 metabolic pathways enriched in the tumor group and the stone group, respectively; (b) PCA indicated that metabolic functions of biliary microbiota in the tumor group differed from the stone group. PCA, principal component analysis; PICRUSTs, phylogenetic investigation of communities by reconstruction of unobserved states.

Conclusions

This prospective study revealed the characteristics of biliary microbiota in patients with malignant bile duct obstruction using *16s rRNA* sequencing technology. The distinct composition and distribution of biliary microbiota are valuable for the assessment of diseases. The metabolic functions enriched in the tumor group imply the underlying mechanism of malignant bile duct obstruction. These findings provide new insights about biliary microbiota into the etiology of malignant bile duct obstruction.

Acknowledgement

This study was supported by the Science and Technology Program of Suzhou (SKY2022139).

Conflicts of interest

There are no conflicts of interest.

References

1 Li Z, Chu J, Su F, Ding X, Zhang Y, Dou L, *et al*. Characteristics of bile microbiota in cholelithiasis, perihilar cholangiocarcinoma, distal cholangiocarcinoma, and pancreatic cancer. *Am J Transl Res* 2022; 14:2962–2971.
2 Cho I, Blaser MJ. The human microbiome: at the interface of health and disease. *Nat Rev Genet* 2012; 13:260–270.
3 Xue C, Chu Q, Zheng Q, Yuan X, Su Y, Bao Z, *et al*. Current understanding of the intratumoral microbiome in various tumors. *Cell Rep Med* 2023; 4:100884.
4 Nicoletti A, Ponziani FR, Nardella E, Ianiro G, Gasbarrini A, Zileri Dal Verme L. Biliary tract microbiota: a new kid on the block of liver diseases? *Eur Rev Med Pharmacol Sci* 2020; 24:2750–2775.
5 Nascimento FSD, Suzuki MO, Taba JV, de Mattos VC, Pipek LZ, D'Albuquerque EMC, *et al*. Analysis of biliary MICRObiota in hepatoBILIOpancreatic diseases compared to healthy people [MICROBILIO]: study protocol. *PLoS One* 2020; 15:e0242553.
6 Thomas RM, Gharaibeh RZ, Gauthier J, Beveridge M, Pope JL, Guijarro MV, *et al*. Intestinal microbiota enhances pancreatic carcinogenesis in preclinical models. *Carcinogenesis* 2018; 39:1068–1078.
7 Zhang T, Zhang S, Jin C, Lin Z, Deng T, Xie X, *et al*. A predictive model based on the gut microbiota improves the diagnostic effect in patients with cholangiocarcinoma. *Front Cell Infect Microbiol* 2021; 11:751795.
8 Wang Y, Qian PY. Conservative fragments in bacterial 16S rRNA genes and primer design for 16S ribosomal DNA amplicons in metagenomic studies. *PLoS One* 2009; 4:e7401.
9 Helmink BA, Khan MAW, Hermann A, Gopalakrishnan V, Wargo JA. The microbiome, cancer, and cancer therapy. *Nat Med* 2019; 25:377–388.
10 Maki T. Pathogenesis of calcium bilirubinate gallstone: role of *E. coli*, beta-glucuronidase and coagulation by inorganic ions, polyelectrolytes and agitation. *Ann Surg* 1966; 164:90–100.
11 Shen H, Ye F, Xie L, Yang J, Li Z, Xu P, *et al*. Metagenomic sequencing of bile from gallstone patients to identify different microbial community patterns and novel biliary bacteria. *Sci Rep* 2015; 5:17450.
12 Molinero N, Ruiz L, Milani C, Gutierrez-Diaz I, Sanchez B, Mangifesta M, *et al*. The human gallbladder microbiome is related to the physiological state and the biliary metabolic profile. *Microbiome* 2019; 7:100.
13 Pereira P, Aho V, Arola J, Boyd S, Jokelainen K, Paulin L, *et al*. Bile microbiota in primary sclerosing cholangitis: impact on disease progression and development of biliary dysplasia. *PLoS One* 2017; 12:e0182924.
14 Hiramatsu K, Harada K, Tsuneyama K, Sasaki M, Fujita S, Hashimoto T, *et al*. Amplification and sequence analysis of partial bacterial 16S ribosomal RNA gene in gallbladder bile from patients with primary biliary cirrhosis. *J Hepatol* 2000; 33:9–18.
15 Dai C, Xu C, Zheng L, Wang M, Fan Z, Ye J, Su D. Characteristics and metabolic potential of biliary microbiota in patients with giant common bile duct stones. *Front Cell Infect Microbiol* 2023; 13:1259761.
16 Tan W, Chen R, Song J, He D, Wu J, Chen X, *et al*. Microbiota analysis with next-generation 16S rDNA gene sequencing in recurrent common bile duct stones. *Ann Transl Med* 2022; 10:576.
17 Chen B, Fu SW, Lu L, Zhao H. A preliminary study of biliary microbiota in patients with bile duct stones or distal cholangiocarcinoma. *Biomed Res Int* 2019; 2019:1092563.
18 Park JY, Park BK, Ko JS, Bang S, Song SY, Chung JB. Bile acid analysis in biliary tract cancer. *Yonsei Med J* 2006; 47:817–825.
19 Lee J, Hong EM, Kim JH, Kim JH, Jung JH, Park SW, Koh DH. Ursodeoxycholic acid inhibits epithelial-mesenchymal transition, suppressing invasiveness of bile duct cancer cells: an in vitro study. *Oncology Letters* 2022; 24:448.
20 Petrescu AD, DeMorrow S. Farnesoid X receptor as target for therapies to treat cholestasis-induced liver injury. *Cells* 2021; 10:1846.
21 Tesfay L, Paul BT, Konstorum A, Deng Z, Cox AO, Lee J, *et al*. Stearoyl-CoA desaturase 1 protects ovarian cancer cells from ferroptotic cell death. *Cancer Res* 2019; 79:5355–5366.
22 Poulose N, Amoroso F, Steele RE, Singh R, Ong CW, Mills IG. Genetics of lipid metabolism in prostate cancer. *Nat Genet* 2018; 50:169–171.
23 Gallo O, Masini E, Morbidelli L, Franchi A, Fini-Storchi I, Vergari WA, Ziche M. Role of nitric oxide in angiogenesis and tumor progression in head and neck cancer. *J Natl Cancer Inst* 1998; 90:587–596.
24 Hajare SN, Subramanian M, Gautam S, Sharma A. Induction of apoptosis in human cancer cells by a Bacillus lipopeptide bacillomycin D. *Biochimie* 2013; 95:1722–1731.
25 Park SY, Kim JH, Lee YJ, Lee SJ, Kim Y. Surfactin suppresses TPA-induced breast cancer cell invasion through the inhibition of MMP-9 expression. *Int J Oncol* 2013; 42:287–296.