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Metagenomic sequencing of bile from gallstone patients to identify different microbial community patterns and novel biliary bacteria

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Despite the high worldwide prevalence of gallstone disease, the role of the biliary microbiota in gallstone pathogenesis remains obscure. Next-generation sequencing offers advantages for systematically understanding the human microbiota; however, there have been few such investigations of the biliary microbiome. Here, we performed whole-metagenome shotgun (WMS) sequencing and 16S rRNA sequencing on bile samples from 15 Chinese patients with gallstone disease. Microbial communities of most individuals were clustered into two types, according to the relative enrichment of different intestinal bacterial species. In the bile samples, oral cavity/respiratory tract inhabitants were more prevalent than intestinal inhabitants and existed in both community types. Unexpectedly, the two types were not associated with fever status or surgical history, and many bacteria were patient-specific. We identified 13 novel biliary bacteria based on WMS sequencing, as well as genes encoding putative proteins related to gallstone formation and bile resistance (e.g., β -glucuronidase and multidrug efflux pumps). Bile samples from gallstone patients had reduced microbial diversity compared to healthy faecal samples. Patient samples were enriched in pathways related to oxidative stress and flagellar assembly, whereas carbohydrate metabolic pathways showed varying behaviours. As the first biliary WMS survey, our study reveals the complexity and specificity of biliary microecology.

Cholelithiasis (gallstone disease) is prevalent in many regions of the world, and can occur at any place of the human biliary tract, including the gallbladder, extrahepatic duct or intrahepatic duct¹. Ethnicity, gender, age, obesity, diet and lifestyle factors impact cholelithiasis morbidity, which varies between countries and regions². In Western countries, cholesterol gallstones are highly prevalent (~75%), followed by black pigment stones (~20%)³. In Asia, the overwhelming majority of gallstones are brown pigment stones (BPSs), comprised of calcium bilirubinate, fatty acid soaps (e.g., calcium palmitate and calcium stearate) and mucin³.

Many studies have suggested a role of bacterial infection in gallstone pathogenesis^{4–8}; however, a definitive causal relationship between biliary bacteria and gallstone formation has never been established.

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De novo formation of BPSs in bile ducts is generally thought to be related to biliary bacteria/parasite infection and partial biliary tract obstruction^{2,9}. Many bacteria, such as *Escherichia coli*, *Klebsiella pneumoniae*, *Enterococcus faecium*, *Enterobacter cloacae* and *Pseudomonas aeruginosa*, have been identified in bile or gallstone samples through cultivation or polymerase chain reaction (PCR). Bacterially produced β -glucuronidase was suggested to be associated with the formation of pigment gallstones^{4,10}. Other bacterial products, such as phospholipase and slime, might also participate in gallstone formation⁶.

Antimicrobial therapies are commonly used for biliary infections caused by gallstone disease, with bile cultures being used to select the appropriate antibiotic agent^{11,12}. Furthermore, the Tokyo Guidelines for antimicrobial therapy of Acute Cholangitis, a common gallstone complication, proposed the use of bile cultures to aid in patient treatment¹³. However, as multiple bacteria coexist in the biliary tract, culture-dependent methods are somewhat insensitive and biased for bacterial identification and are inadequate to study the entire microbial community^{9,14}.

Therefore, improved understanding of the biliary tract microbiota would be helpful for studies of bacteria-related gallstone pathogenesis and antibiotic therapies. Unlike conventional methods for identifying microorganisms, such as cultivation and bacteria-specific PCR, next-generation sequencing (NGS) provides a comprehensive picture of the microbial flora^{15,16}. NGS has shown advantages for microbiome studies across multiple human body sites, including the gastrointestinal tract, oral cavity, nasal cavity, skin, vagina and placenta^{17–19}. However, there have been few extensive NGS-based researches of the biliary tract. Wu *et al.* performed bacterial 16S rRNA amplicon sequencing (referred to as ‘16S sequencing’) on gallbladder bile, gallstones and faeces from cholesterol gallstone patients¹⁶. They detected many gut bacterial operational taxonomic units (OTUs) in the biliary tract and found that faecal samples from gallstone patients exhibited microbiota dysbiosis.

To the best of our knowledge, no study has used whole-metagenome shotgun (WMS) sequencing on biliary samples. Compared to 16S sequencing which employs bacterial universal primers, WMS sequencing has increased taxonomic resolution and provides insights into microbial functionality and biological processes¹⁵. Here, we used WMS and 16S sequencing to analyse bile samples of 15 Chinese patients with choledocholithiasis. Bile was collected from the common bile duct, where the gallstones *de novo* formed. To obtain an accurate and comprehensive picture of the biliary microbiota, we filtered host components within WMS data with stringent criteria, and constructed a large reference dataset including 9,671 bacterial genomes for bioinformatics analysis. We characterized the biliary microbial communities and compared them to those of gut flora. Thirteen novel biliary bacterial species with reliable WMS genome coverage were identified. Finally, we investigated genes possibly related to gallstone formation and bile resistance, and the metabolic network of the biliary microbiota was reconstructed.

Results

Microbial community heterogeneity among patients. We performed unbiased WMS sequencing of total DNA extracted from 15 bile samples, obtaining an average of 7.29 Gbp per sample (range: 6.81–7.81 Gbp). WMS reads were filtered multiple times to remove host components, and then aligned to datasets containing 9,671 bacterial genomes from 3,608 species. Among these individuals, bacterial read percentages ranged from <0.001% to 1.66% (Fig. 1a). We did not find apparent between-group bias of bacterial ratios; that is, whether patients were febrile and/or had a history of endoscopic retrograde cholangiopancreatography (ERCP) was not correlated with the bacterial fraction in bile samples.

Bile microbial community analysis revealed heterogeneity among individuals. A total of 173 bacterial species were identified, and hierarchical clustering at species level divided these individuals into two major clusters (I and II) and one minor cluster (Fig. 1b; Supplementary Table S1). Clustering was not consistent with patient grouping. Interestingly, we found that oral cavity and respiratory tract inhabitants were more prevalent than intestinal inhabitants in the bile samples. Through literature mining, we defined 25 oral cavity/respiratory tract inhabitants, 11 intestinal inhabitants and 14 inhabitants of both sites among the 54 microbes observed in at least three patients with an abundance $\geq 0.1\%$ (Supplementary Table S2). The 11 intestinal inhabitants were basically distributed in Cluster I (containing individuals A1, B1, B5, C1 and C3), which was highly enriched in *E. coli* (abundance range: 52.3–85.7%). Cluster I contained most of the intestinal microbes, whereas Cluster II (containing individuals A2–A5, B2, B6, C4 and C5) only contained two intestinal microbes. In particular, intestinal microbes such as *Shigella spp.* and *Salmonella enterica* were only detected in Cluster I and not found in Cluster II. Both major clusters contained 17 of the 25 oral cavity/respiratory tract inhabitants and 11 of the 14 microbes reported in both body sites. Samples in Cluster I consistently had higher bacterial read ratios (0.62–1.66%) than samples in Cluster II (0.0008–0.056%). Overall, the heterogeneity of the microbial communities can be attributed to the distribution of intestinal inhabitants, despite the presence of oral cavity/respiratory tract inhabitants.

Microbial species were frequently identified in only one ($n = 77$) or two individuals ($n = 43$) (Supplementary Table S1). High within-sample abundance was observed for some species, such as *Raoultella ornithinolytica* (15%) in A1, *Pyramidobacter piscicola* (3.39%) in C3 and *Proteus mirabilis* (0.6%) in C1. *K. pneumoniae* was the only species identified in almost all individuals (14/15).

Bile microbial diversity revealed by WMS and 16S sequencing. Microbial communities determined by parallel 16S sequencing of 14 bile samples (Supplementary Table S3; DNA was unavailable for C5) generally agreed with communities determined by WMS sequencing, with 41 overlapping genera.

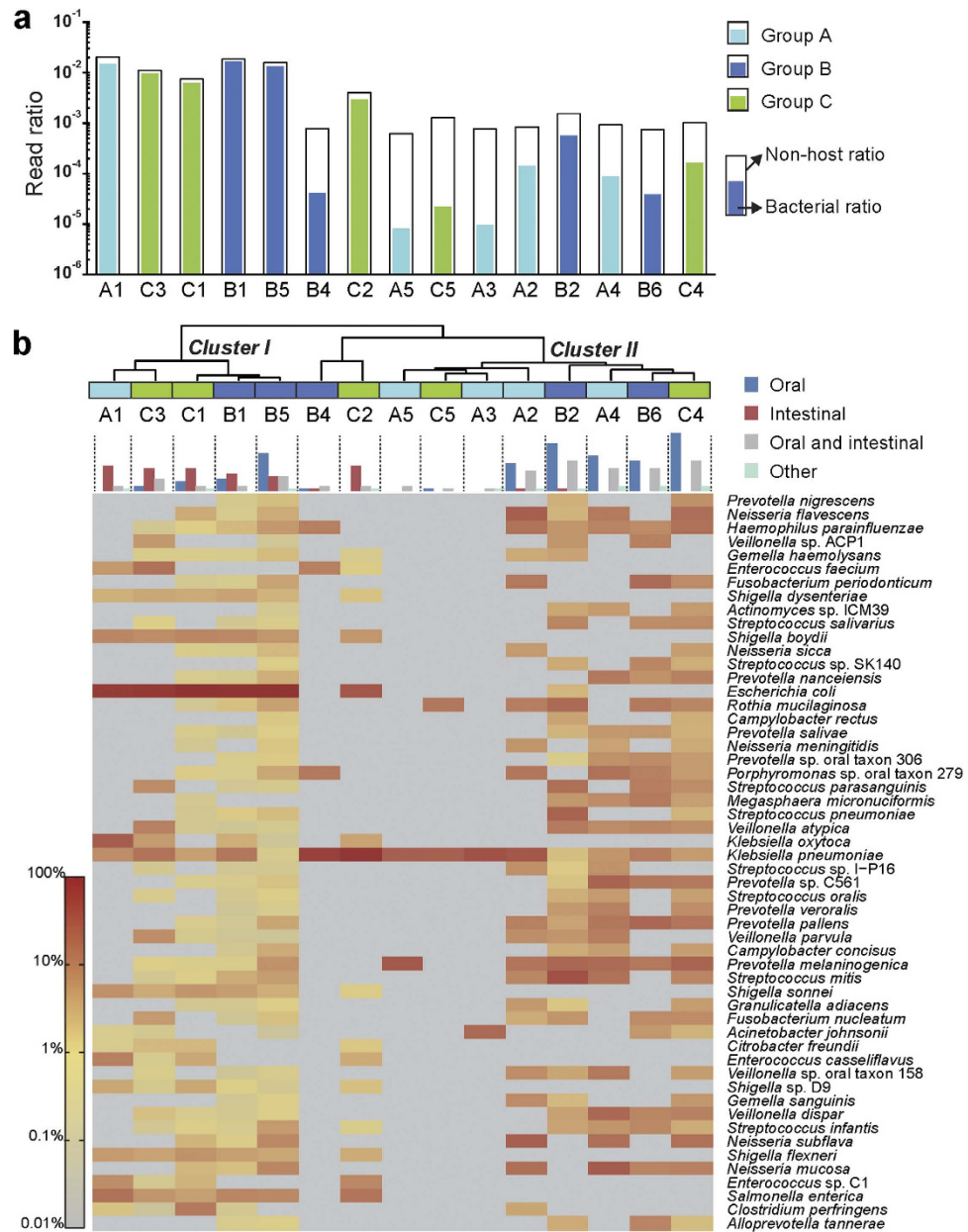


Figure 1. Distributions of WMS sequencing reads and microbial communities within bile samples.

(a) Ratios of WMS sequencing reads after removing host background (hollow black-edged bars) and ratios of reads aligned to bacterial database (inner bars). All ratios were calculated by dividing by the total clean read number after quality control. Sample IDs are labelled on the x-axis, and ranked by their clustering relationships. Bacteria-aligned read ratios of samples from groups A, B and C are indicated by light blue, dark blue and green bars, respectively. (b) Hierarchical clustering of samples based on microbial community, distribution of bacterial origin and heatmap of species abundances. Individuals are denoted by coloured blocks, as in (a). Bacterial origins were classified as oral (referring to the oral cavity/respiratory tract), intestinal, both oral and intestinal, and other (environmental or unknown). For bacteria with $\geq 0.1\%$ abundance in at least three individuals, the distribution of their origin in each individual is reflected by the histogram above the heatmap. The heatmap colour scale quantifies the \log_{10} relative abundance of species, from grey (none or low abundance) to dark red (high abundance).

Abundances of the genera exhibited a high correlation (Pearson correlation coefficient = 0.91) (Fig. 2a,b). For samples with insufficient bacterially aligned WMS reads (i.e., A5 and B4), 16S sequencing had greater sensitivity and could identify more genera. Although hierarchical clustering was not performed for 16S sequencing, the distributions of overlapping genera were somewhat similar (Fig. 2a,b), especially for highly abundant genera. For example, Cluster I was still enriched with intestinal genera (e.g., *Escherichia* and *Enterococcus*). However, 16S sequencing exhibited greater intra-sample differences in abundance

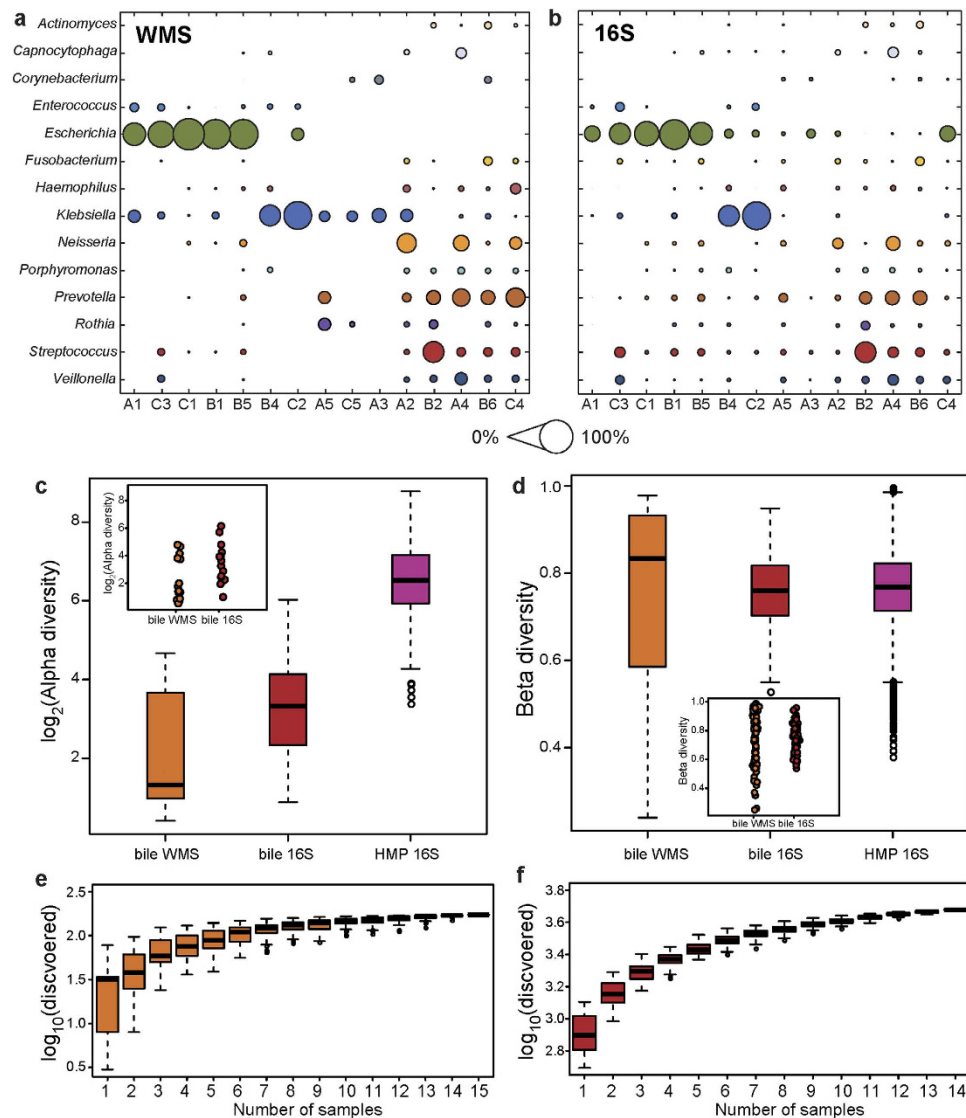


Figure 2. Microbial community characteristics generated by WMS and 16S sequencing. (a–b) Relative abundances at the genus level by WMS (a) and 16S sequencing (b). Circle sizes represent abundance, and circles are coloured by genus. Sample IDs are shown on the x-axis and are ranked as in Fig. 1. Common genera were those with a relative abundance $\geq 1\%$ in at least three samples. The genera names for (a,b) are shown on the y-axis of (a). (c–d) Distributions of \log_2 alpha (c) and beta diversities (d). Orange and red boxplots denote distributions of WMS and 16S sequencing of bile samples respectively, accompanied with the distributions of faecal samples from HMP (purple boxplots). \log_2 alpha and beta diversities are presented as scatter plots in insets (orange, bile WMS; red, bile 16S), with scales identical to the corresponding boxplot y-axis. (e–f) Species and OTU counts by WMS (e) and 16S sequencing (f) with increasing sample number. At a given sample number, a maximum 100-time random sampling of these bile samples was performed to calculate species or OTU counts. Boxes represent the interquartile range (IQR) between first and third quartiles (25th and 75th percentiles, respectively). Lines inside denote the median, and whiskers denote the most extreme values within 1.5 times IQR from the first and third quartiles, respectively. Outlier values are represented as circles.

(i.e., A5 and C4) than WMS sequencing, which could be attributed to the experimental biases of 16S sequencing.

Next, we compared the alpha and beta diversities of bile samples with those of healthy faecal samples ($n = 319$) from Human Microbiome Project (HMP)^{17,18} (Fig. 2c,d). As measured by inverse Simpson index, alpha diversities of bile samples based on WMS and 16S sequencing were significantly lower than those of faecal samples (Wilcoxon rank-sum test, $P < 10^{-8}$). The difference in alpha diversity between bile WMS and 16S sequencing data ($P = 0.029$) might be due to differences in the sequencing strategy (Fig. 2c). On the contrary, there was no significant difference between the Bray–Curtis beta diversities

Species	Sample No.	Genus in 16S	Species in 16S ^a	WGS Genome Coverage ^b
<i>Pyramidobacter piscolens</i>	1	Yes	Yes	33.13%
<i>Cellulosimicrobium cellulans</i>	1	Yes	No	10.33%
<i>Lactobacillus mucosae</i>	1	Yes	Yes	4.88%
<i>Prevotella pallens</i>	6	Yes	Yes	4.30%
<i>Bifidobacterium dentium</i>	1	Yes	No	4.05%
<i>Gordonia sputi</i>	1	Yes	No	2.33%
<i>Gordonia bronchialis</i>	1	Yes	No	2.04%
<i>Eubacterium infirmum</i>	1	No	No	1.80%
<i>Porphyromonas endodontalis</i>	2	Yes	Yes	1.75%
<i>Streptococcus infantis</i>	5	Yes	No	1.55%
<i>Aggregatibacter segnis</i>	2	Yes	Yes	1.53%
<i>Raoultella ornithinolytica</i>	1	No	No	1.46%
<i>Olsenella uli</i>	1	No	No	1.37%

Table 1. Bacteria newly identified within bile samples by WMS/16S sequencing, ranked by WMS genome coverage. ^aSpecies-level OTUs required $\geq 97\%$ sequence identity with Greengenes references. ^bFor species in multiple bile samples, the highest coverage value is shown.

of bile by 16S sequencing and faecal samples ($P = 0.1753$). Beta diversities of bile by WMS sequencing were borderline-significantly higher than those of bile by 16S sequencing ($P = 0.044$) and of faecal samples ($P = 0.021$). This result may be due, at least in part, to the varied sensitivity of WMS sequencing for bacterial identification. We also employed other alpha (Simpson index and Shannon index) and beta (Jaccard index) diversity indices to compare these samples, and obtained qualitatively consistent results (Supplementary Figs S1 and S2).

Accumulation curves of species and OTU counts are shown in Fig. 2e,f. Although bile samples exhibited less microbial diversity than faecal samples, the curves were not fully saturated. More samples and a more sensitive experimental design could be useful for enabling a more comprehensive analysis of the biliary microbiota.

Newly identified microbes in bile. Through WMS sequencing, we identified microbes that have not been reported previously in the human biliary tract. To ensure the reliability of taxon identification, we selected strain-specific WMS sequencing reads having $\geq 99\%$ identity and full-length (100-bp) alignment with their reference genomes. We obtained 35 reference genomes with $\geq 1\%$ coverage by these reads. Among these genomes were 13 microbe species that, to the best of our knowledge, have not previously been reported in bile samples (Table 1; Fig. 3). Nine of these species were individual-specific. In particular, *P. piscolens* and *Cellulosimicrobium cellulans*, identified in C3 and A1, respectively, exhibited $\geq 10\%$ reference genome coverage. Five of the 13 novel species were also identified by 16S sequencing with $\geq 97\%$ identity to species-level OTUs in Greengenes.

According to the Human Oral Microbiome Database²⁰ and our literature investigation, eight of the 13 novel species were human oral microbial taxa; the rest were possible environmental taxa. We focused on the opportunistic pathogens. *P. piscolens* has been found in odontogenic abscesses, inflamed gingival crevices and infected canals^{21,22}. *Olsenella uli* and *Porphyromonas endodontalis* are associated with human endodontic infections^{23,24}. *C. cellulans* (strain LMG 16121), identified in A1 with a coverage of 10.32%, is an inhabitant of soil, water, decaying plant material and brewery sewage²⁵. It rarely causes human infections, but may be involved in human septic arthritis²⁶ and bacteraemia²⁷. *R. ornithinolytica*, *Gordonia sputi* and *Gordonia bronchialis* are environmental bacteria that can cause bacteraemia^{28–30}.

Other potentially novel microbe species in bile with $< 1\%$ reference genome coverage included opportunistic pathogens, such as *Streptococcus spp.*, *Prevotella spp.*, *Veillonella spp.*, *Neisseria spp.*, *Megasphaera micronuciformis* and *Gemella haemolysans*^{31–35}, which often colonize the human oral cavity and upper respiratory tract. *Prevotella spp.* and *Veillonella spp.* are also inhabitants of the human gut. Many previously reported bacteria were also identified (Supplementary Table S4).

Gallstone formation and bile resistance. We further investigated genes that might be related to gallstone formation or bile resistance. Previous studies have demonstrated potential associations of gallstone formation with bacterial products β -glucuronidase, phospholipase and urease^{6,7,36–39}. Among the 173 bacterial species identified in the 15 bile samples, we extracted 34 species harbouring genes encoding these enzymes by searching the KEGG (Kyoto Encyclopedia of Genes and Genomes) GENOME

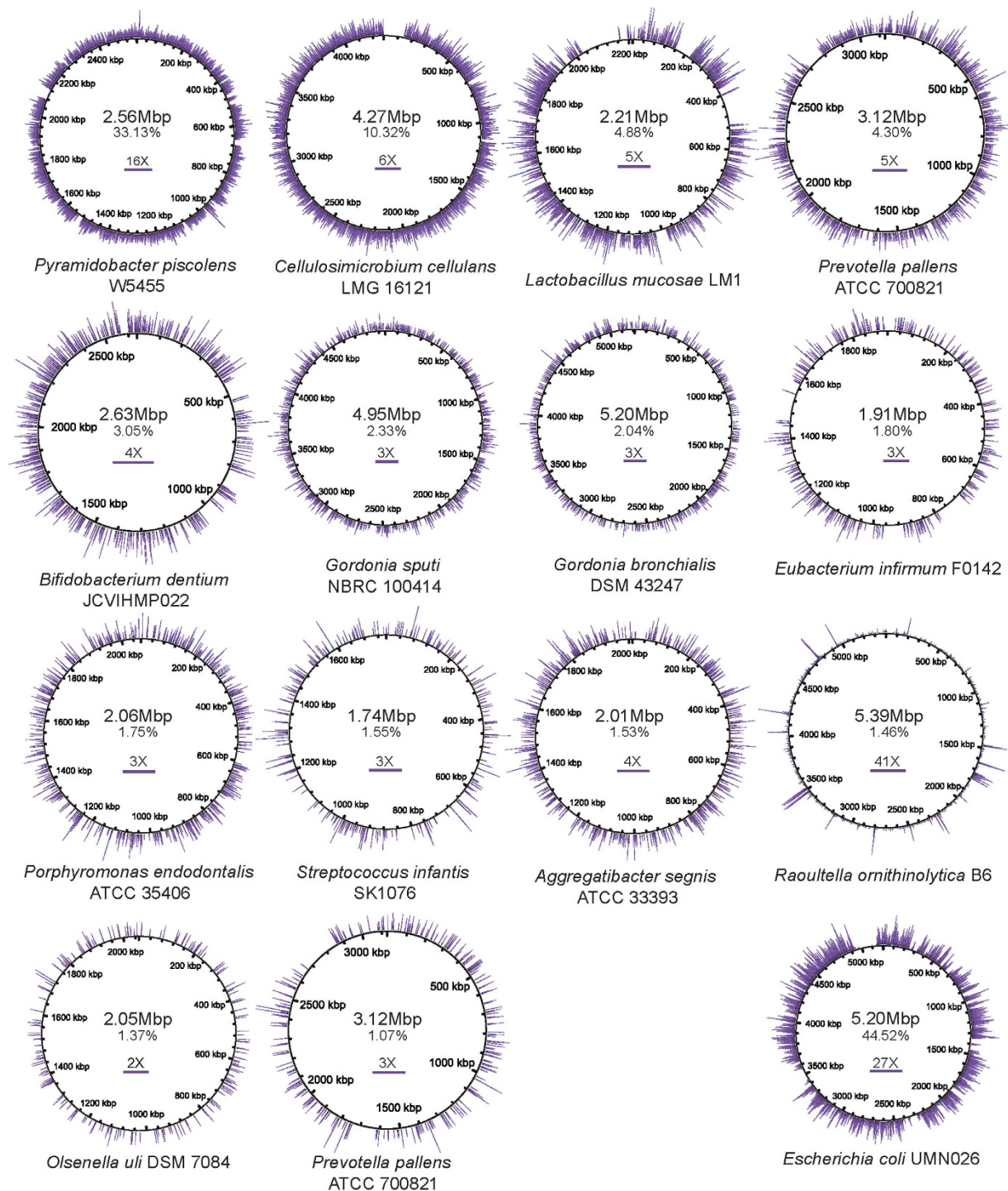


Figure 3. Genome coverage ($\geq 1\%$) of bacteria newly identified in bile samples by WMS. Reference genomes of 13 newly identified bacteria are represented by circles generated by BLAST Ring Image Generator v0.95 (BRIG), with genome sizes labelled inside. Highly confident WMS read alignments (unique mapping and $\geq 99\%$ identity) to these genome are illustrated by purple bars around circles. Bar height represents site coverage depth, with the corresponding scale indicated inside the circles. Overall coverage of the reference genome is labelled below the genome size. Newly identified bacteria are sorted by genome coverage. *P. pallens* ATCC 700821 appears twice because it was observed in two samples with $\geq 1\%$ coverage (B5 and C4). *E. coli* UMN026, which has the highest WMS coverage (44%, in sample B5), is also shown at the end for reference.

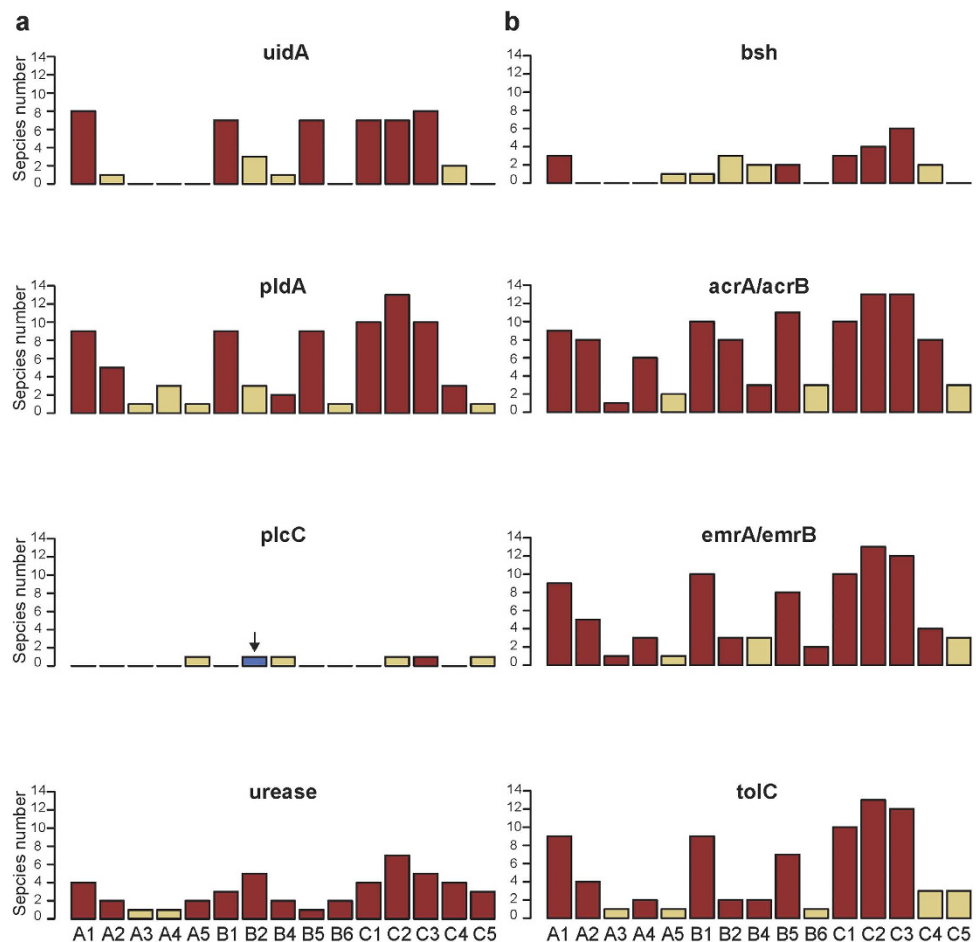


Figure 4. Distributions of bacterial species harbouring genes involved in gallstone formation (a) or bile resistance (b). Numbers of species within individuals are indicated by bars. Red bars denote genes that were also identified in metagenomic assemblies by gene prediction, or existed within metabolic reconstruction results. The gene encoding phospholipase C within individual B2 (blue bar with an arrow on top) was identified by metabolic reconstruction, but not by genome annotation. The term ‘urease’ on the left panel stands for genes encoding urease, which include ureA, ureB, ureC, ureD, ureE, ureF and ureG. The term ‘acrA/acrB’ stands for genes *acrA* and *acrB*, and ‘emrA/emrB’ stands for genes *emrA* and *emrB*.

Database. Using WMS sequencing, we also validated gene existence through metagenomic assemblies and metabolic reconstruction.

β -glucuronidase and phospholipase hydrolyse biliary bilirubin and phosphatidylcholine, respectively, leading to precipitation of calcium bilirubinate and calcium palmitate and thereby facilitating pigment solid formation⁶. Seven and ten individuals had samples containing ≥ 3 species harbouring genes *uidA* (encoding β -glucuronidase) and *pldA* (encoding phospholipase A1), respectively (Fig. 4a; Supplementary Table S5). Samples from some individuals (i.e., A3, A5, B6 and C5) had one or no unvalidated related species. As the species numbers were correlated with bacterial WMS sequencing read ratios (Fig. 1a), failure to identify genes encoding β -glucuronidase or phospholipase might be due to issues related to DNA extraction or sequencing. For most individuals, the gene predictions did not identify the gene *plcC* encoding phospholipase C, which promotes cholesterol nucleation in human gallbladder bile³⁷. *Helicobacter*-produced urease was reported to promote calcium precipitation, which might initiate gallstone formation³⁸. Although the genes encoding urease were prevalent in the bile samples, *Helicobacter* spp. were not found among the urease-containing bacteria.

We also investigated genes involved in bile resistance (Fig. 4b). Bile salt deconjugation and multidrug efflux pump proteins are two mechanisms for bacterial bile resistance^{8,40}. Within bile microbial communities in this study, we found 13 species with the gene encoding bile salt hydrolase (*bsh*) and 30 species with genes encoding efflux pump proteins (*acrA*, $n = 29$; *acrB*, $n = 29$; *emrA*, $n = 23$; *emrB*, $n = 23$; *tolC*, $n = 22$). Species harbouring *bsh* were less prevalent among individuals than species with efflux pump proteins, and the gene predictions were consistent with these observations.

Moreover, bacterial slime (i.e., glycocalyx) and biofilm play important roles in gallstone formation and bile resistance^{6,41–43}. Strong slime producers, including *Prevotella intermedia*, *Clostridium perfringens*,

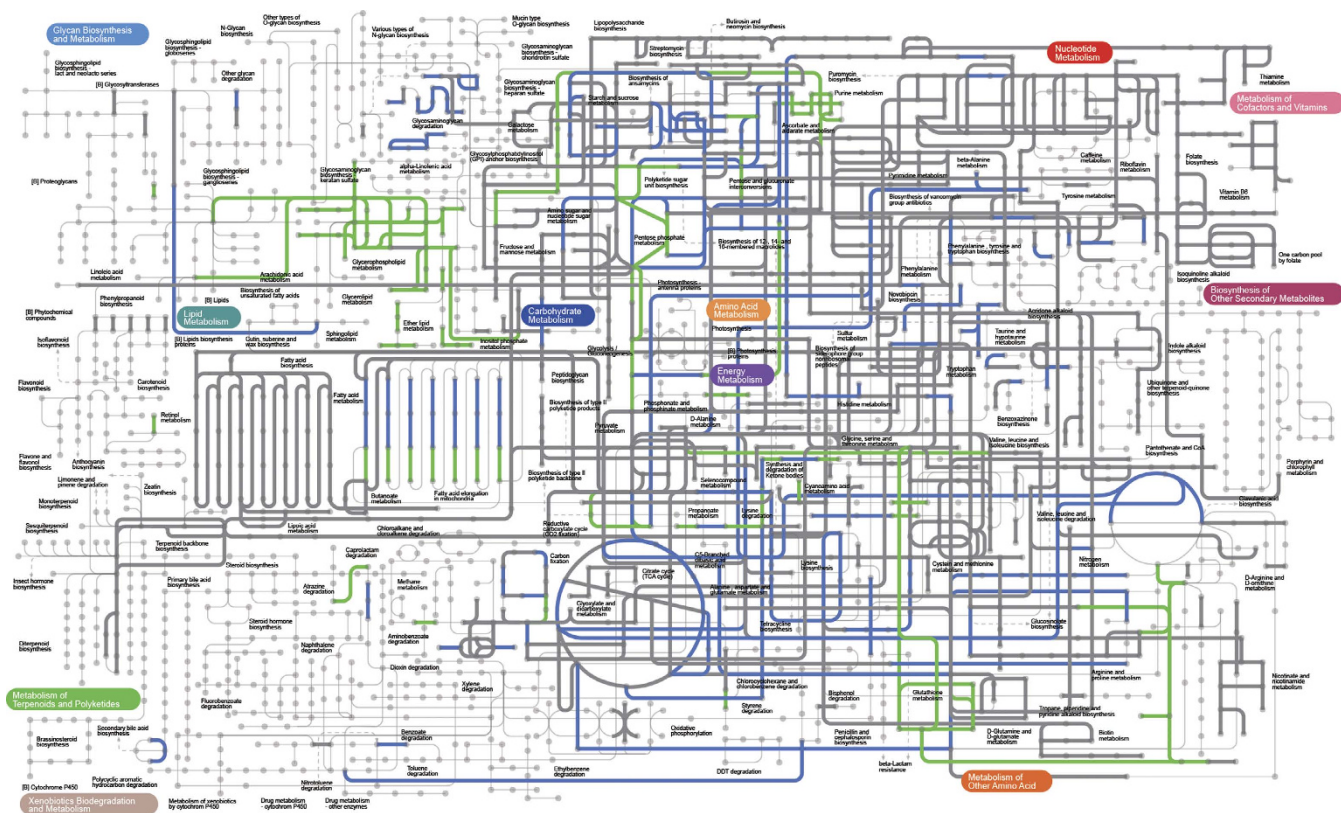


Figure 5. Metabolic reconstruction of the biliary microbiota. Bold grey lines represent abundant pathways that existed in at least five bile samples with $\geq 1\%$ abundance. Pathways that are enriched or depleted in the biliary microbiota compared to healthy faecal microbiota from HMP are highlighted by green and blue lines, respectively.

Enterococcus faecalis and *E. faecium*, were identified in nine individuals at abundances of 0.1–7.99%³⁹. High abundances ($>0.5\%$) of biofilm producers, such as *E. coli*⁴⁴, *P. aeruginosa*⁴⁵, *P. intermedia*³⁹ and *S. enterica*⁴³, were also identified.

Metabolic reconstruction of the biliary microbiota. Metabolic reconstruction was performed on the basis of the host-removed WMS reads (Fig. 5; Supplementary Table S6). Pathways involved in basic bacterial life dominated the bile microbiota metabolic network. Using predicted gene sets, we identified core eggNOG (evolutionary genealogy of genes: Non-supervised Orthologous Groups) functional categories, including carbohydrate transport and metabolism, amino acid transport and metabolism, transcription as well as energy production and conversion (Supplementary Table S7; Supplementary Fig. S3). When we projected eggNOG orthologous groups onto the KEGG pathways with iPath v2.0⁴⁶ (Supplementary Fig. S4), we found that the overall distribution of pathways was similar to that of pathways identified by HUMAnN⁴⁷ as shown in Fig. 5.

Next, we compared the metabolic networks of bile microbiota with those of faecal microbiota from HMP faecal samples ($n = 136$), focusing on differentially enriched pathways (Fig. 5; Supplementary Table S8). Bile samples were significantly enriched in pathways related to glycerophospholipid and glutathione metabolism (Wilcoxon rank-sum test, both adjusted $P < 1.5 \times 10^{-4}$), which are involved in inflammation and oxidative stress responses, respectively^{48,49}. Upon investigating KEGG orthologues associated with resistance to oxidative stress (Supplementary Table S9), we observed an enrichment of glutathione reductase (NADPH, K00383, adjusted $P < 2.1 \times 10^{-4}$) and putative iron-dependent peroxidase (K07223, adjusted $P < 0.042$), consistent with the pathway analysis results. There was also an enrichment in the flagellar assembly pathway (adjusted $P = 0.00022$), which might be involved in biofilm development⁴⁵ and bacterial sensory systems⁵⁰. Glycolysis/gluconeogenesis, propanoate metabolism, ascorbate/aldarate metabolism, ABC transporter and phosphotransferase system pathways were also enriched (all adjusted $P < 4 \times 10^{-5}$).

There were many depleted pathways in bile samples compared to faecal samples (Fig. 5; Supplementary Table S8). For instance, the bile microbiota had decreased abundances of glycosaminoglycan and other glycan degradation pathways (both adjusted $P < 1.3 \times 10^{-6}$); this finding implies a decreased ability to

employ glycan as a bacterial nutrition source⁵¹. Other depleted pathways included carbon fixation pathways in prokaryotes (adjusted $P < 5.3 \times 10^{-7}$) and amino acid metabolic pathways involving lysine, histidine, taurine and hypotaurine (all adjusted $P < 2.9 \times 10^{-6}$). Pentose phosphate pathway and starch/sucrose metabolism, which were related to carbohydrate metabolism, were also among the depleted pathways (both adjusted $P < 2.4 \times 10^{-3}$).

Discussion

Asian countries, including China, have a high prevalence of BPS disease, which is the gallstone disease that is most closely associated with microbial infection. Thus, BPS disease could serve as a good model for gallstone-related bacterial studies^{2,3}. Unexpectedly, we did not observe an apparent relationship between patient groups and microbial community features, as individuals from the same group had different patterns of biliary microbiota. Moreover, the bacterial read ratios of bile samples by WMS sequencing, which might reflect the bacterial load in the biliary environment, were not correlated with patient grouping. These results imply that the biliary microbiota might be shaped by more complicated factors, such as diet, lifestyle, host immune responses and/or specific anatomy structures, rather than by microbiota alone.

On the other hand, the lack of a correlation between patient grouping and microbial community may provide some insight into the clinical debate about ERCP procedures. Whether to perform endoscopic sphincterotomy (EST) or endoscopic papillary balloon dilation during ERCP remains controversial^{52,53}, given concerns that EST could increase the risk of biliary tract infection⁵⁴. We found that the microbial distribution of samples from choledocholithiasis patients who had no history of EST (groups A and B) did not differ greatly from the distribution of samples from afebrile patients who had a history of EST (group C). This observation implies that EST might not be a determining factor for the biliary microbial composition of BPS patients.

Our results were basically consistent between the WMS and 16S sequencing experiments, but had some discrepancies with the results of the investigation of Wu *et al.*¹⁶. Using 16S sequencing to study bile samples, these authors did not observe different biliary microbial community patterns among their subjects, and found that the bile samples had higher alpha diversities than faecal samples from healthy individuals and patients. The disagreement between their study and ours might be attributed to differences in gallstone type (cholesterol gallstones vs. BPSs), bile collection site (gallbladder vs. common bile duct), host and/or sequencing details (i.e., different 16S rRNA variable regions, V1–V2 vs. V3–V4) between the studies.

Compared to bacteria-specific 16S sequencing, biliary WMS reads were dominated by host components (>98%). Thus, the proportion of effective bacterial reads was relatively small, similar to those of the human vaginal introitus and mid-vagina¹⁸. Consequently, WMS sequencing exhibited less sensitivity to uncover less-abundant bacterial species, especially for samples with low bacterial ratios. Nonetheless, our recruited patients had distinct dominant species when either WMS or 16S sequencing was used. Moreover, even with limited data, WMS sequencing provided higher resolution for bacterial species identification and gene-level analysis. Finally, we reliably identified novel biliary bacteria by using a WMS sequencing analysis workflow with strict alignment thresholds.

WMS sequencing can be employed to obtain insights into microbial functionality. For example, information on gene abundance can be obtained by metagenomic assembly or direct alignment with protein databases. As this study is the first to apply WMS sequencing to bile samples, we paid special attention to genes encoding bacterial proteins involved in gallstone formation (e.g., β -glucuronidase, phospholipase and urease), which were identified by metagenomic assembly, metabolic reconstruction and/or KEGG GENOME annotation. The results agreed with previous reports and could be indicative of the roles of these genes in gallstone pathogenesis. Bile resistance-related genes, which could be crucial for bacterial survival, were also identified by the same methods. For example, although beneficial for microbial life in the biliary microenvironment, the role of bsh is not yet fully understood⁸. Genes encoding multidrug efflux pump proteins were more prevalent than bsh in our bile samples, suggesting that the former proteins might be more favourable for bacterial colonization and overgrowth in these patients.

To explore the biliary microbial functionality further, we studied the microbiota-host interplay at the pathway level. Bile acid and inflammation in the bile duct could both induce bacterial oxidative stress responses. Bacteria employ multiple pathways (e.g., cysteine/methionine, glutathione and riboflavin metabolism) to address oxidative stress⁴⁹. Compared to HMP stool samples, patient bile samples were enriched in pathways related to glutathione metabolism and genes related to oxidative stress resistance (i.e., glutathione reductase). Another enriched gene, the putative iron-dependent peroxidase, is also involved in the oxidative stress response. These findings might reflect a mechanism by which the biliary microbiome maintains bacterial homeostasis and redox metabolism. Other oxidative stress-related pathways were not significantly enriched, implying that glutathione metabolism might be more crucial for microbial life in the biliary microenvironment. The observed increase in flagellar assembly suggests the possible enhanced motility of biliary inhabitants. Bile samples showed enrichment of pathways related to glycolysis/gluconeogenesis, propanoate metabolism and ascorbate/aldarate metabolism, but depletion of pentose phosphate and starch/sucrose metabolism pathways. These carbohydrate metabolic pathway results suggest a specific energy source and nutrition intake mode for the biliary microbiota.

Our study elucidates aspects of the biliary microbiota for patients with BPS disease. Nevertheless, the biliary microenvironment, microbial activity and their interplay with the host require further investigation. Specifically, to obtain a comprehensive picture of the biliary microbial composition and function, more ‘metaomic’ (e.g., metagenomic, metatranscriptomic, metametabolomic and metaproteomic) datasets are required. Metaomic studies could offer enormous potential for choledocholithiasis research and clinical practice.

Conclusions

We applied WMS and parallel 16S sequencing methods to samples collected from the common bile ducts of Chinese choledocholithiasis patients. Microbial communities of these individuals were clustered into two categories: Cluster I, featuring species from the gut and oral cavity/respiratory tract, and Cluster II, featuring species from the oral cavity/respiratory tract. Given the direct connection between the bile duct and small intestine, biliary tract infections are generally believed to originate from retrograde infection of gut bacteria^{55,56}. Surprisingly, we observed that oral cavity and respiratory tract inhabitants were more prevalent in bile samples than intestinal inhabitants. Thus, in addition to gut species, bacteria from the oral cavity/respiratory tract might be relevant to human biliary infection.

Using WMS sequencing, we identified 13 novel biliary bacterial species, which were of oral cavity (primarily) or environmental origin. Many patient-specific bacteria were found, implying the strong individuality of the biliary microbial community. We also identified putative genes related to gallstone formation and bile resistance. On the other hand, compared to HMP faecal samples from healthy individuals, the within-sample diversity of the bile microbial community was reduced, whereas the abundance levels of pathways involved in inflammation, oxidative stress response, flagellar assembly and membrane transport were enriched. Taken together, our results provide new insights into the biliary microbiota with respect to microbial composition and function, which could be valuable for clinical applications, such as the diagnosis and treatment of bile duct diseases.

Materials and Methods

Patients and sample collection. Bile samples were collected from the common bile ducts of 15 patients (mean age: 70.7 ± 16.8 years; 6 men, 9 women) who had been diagnosed with choledocholithiasis by computed tomography, magnetic resonance imaging, and B-mode ultrasonography at Hangzhou First People’s Hospital. All patients had BPSs in the common bile duct. There were no occurrences of gallbladder gallstones or hepatolithiasis. Patients were divided into three groups ($n = 5$ each), according to their fever status and/or history of ERCP: group A, febrile with no ERCP history (A1, A2, A3, A4 and A5); group B, afebrile with no ERCP history (B1, B2, B4, B5 and B6); and group C, afebrile with a history of ERCP with EST of Oddi’s sphincter (C1, C2, C3, C4 and C5).

A bile sample (2–5 mL) was collected from each patient during ERCP at Hangzhou First People’s Hospital, and the procedure of sample collection was sterilized (for details, see Supplementary Methods). Samples were immediately placed in germ-free sputum cups and stored at -80°C until further processing. All patients provided written informed consent upon enrolment. The study conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Institutional Review Board of Hangzhou First People’s Hospital.

DNA extraction from bile samples. For each patient, the total DNA was extracted from 400 μL of noncentrifuged bile sample by using the Invitrogen Purelink Genomic DNA Mini Kit (Life Technologies, Carlsbad, CA, USA), following manufacturer’s blood DNA extraction protocol. The DNA concentration was quantified with a Qubit 2.0 Fluorometer (Life Technologies), and quality was examined with the E-Gel electrophoresis system (Life Technologies).

WMS sequencing. Total DNA from bile samples was sheared into ~ 350 -bp fragments by the Covaris M220 Focused-ultrasonicator (Covaris, Woburn, MA, USA). Multiplexed libraries were prepared by using TruSeq Nano DNA Sample Preparation Kits (Illumina, San Diego, CA, USA) according to manufacturer’s instructions. Illumina HiSeq2500 platform was used to generate 2×100 -bp pair-end sequencing reads.

16S sequencing. A two-step PCR protocol for 16S rRNA gene amplification and library preparation was used to perform 16S sequencing on Illumina MiSeq platform. The detailed protocol is listed in Supplementary Methods.

Removing host reads from WMS sequencing data. Low-quality WMS reads (having $>50\%$ of bases with a quality $<Q20$ or having $>10\text{N}$ bases) were removed. Then, clean reads were aligned to the human genome (version 38) and to human sequences deposited in the National Center for Biotechnology Information (NCBI) Nucleotide Database (770,558 sequences) by BWA v0.7.5a⁵⁷ and BMTagger (<ftp://ftp.ncbi.nlm.nih.gov/pub/agarwala/bmtagger>), both with default parameters. Unaligned reads were aligned again to human sequences in the NCBI Nucleotide Database with BLASTN v2.2.28+ (word size = 20, identity $\geq 80\%$, alignment length ≥ 80 bp, other parameters as default). Reads that failed these alignments were used for further analysis.

Taxonomic analysis of host-removed WMS sequencing data. We downloaded 2,773 complete and 6,898 draft bacterial genomes from NCBI (Jul–Aug 2014). Host-removed WMS reads were aligned to these genomes by BLASTN (parameters: perc identity = 85, word size = 16, max target seqs = 30, outfmt = 6, evalue = 0.0000001, dust = no). Alignments were further filtered by using the following criteria: (1) sequence identity $\geq 90\%$, (2) alignment length ≥ 80 bp, (3) paired-end reads align to the same reference genomes, and (4) one reference genome should have reads that uniquely mapped to it. Abundances of reference genomes were calculated as described⁵⁸ and normalized by genome size. For each individual, the abundances of the reference genomes were scaled to sum to one (i.e., to calculate the corresponding relative abundance). Relative abundances of species, genus and phylum were calculated by adding the relative abundances of all their taxonomic members deposited in the NCBI Taxonomy Database. Species with $\geq 0.1\%$ relative abundance in at least one individual was considered for further analysis.

Taxonomic analysis of 16S sequencing data. For details on quality control, chimera removal, OTU clustering and taxonomy assignment of 16S sequencing data, see Supplementary Methods.

Hierarchical clustering of microbial communities. Microbial communities of samples were hierarchically clustered and visualized by the R language (version 3.1.2) gplots package (heatmap.2 function). Z-scores for microbes were calculated based on the microbial abundance distributions. Then, hierarchical clustering was performed with the complete linkage method based on the Euclidian distance between Z-scores for all possible sample pairs.

HMP data retrieval. The OTU table generated by 16S sequencing and the corresponding metadata were downloaded from <http://hmpdacc.org/HMQCP>. Metabolic profiles by WMS sequencing, including metadata, were downloaded from <http://hmpdacc.org/HMMRC>. The procedure to analyse these 16S and WMS sequencing datasets was described previously^{17,18}. Datasets of stool samples from healthy individuals were extracted for downstream analysis.

Microbial diversity index. Inverse Simpson and Bray–Curtis dissimilarity indices were used to calculate the within-sample alpha diversity and the between-sample beta diversity, respectively. Both indices were measured by 16S OTU counts or species-aligned WMS reads. The R language vegan package was employed for these calculations.

Metabolic reconstruction and functional annotation. For details on metabolic reconstruction, gene prediction and annotation, see Supplementary Methods.

Statistical analysis. Correlations between the overlapping genera of the WMS and 16S sequencing were identified by using Pearson's correlation in the R language. Wilcoxon rank-sum test (R v3.1.2) was employed to detect differences between the bile and HMP faecal samples with respect to microbial diversities and KEGG profiles. Under the condition of multiple comparisons, *P*-values were corrected to control the false-discovery rate by using the method described previously⁵⁹. Differences with *P* < 0.05 or adjusted *P* < 0.05 were considered to be statistically significant.

References

- Shaffer, E. A. Epidemiology and risk factors for gallstone disease: has the paradigm changed in the 21st century? *Curr. Gastroenterol. Rep.* **7**, 132–140 (2005).
- Stinton, L. & Shaffer, E. Epidemiology of gallbladder disease: cholelithiasis and cancer. *Gut Liver* **6**, 172–187 (2012).
- Stinton, L. M., Myers, R. P. & Shaffer, E. A. Epidemiology of gallstones. *Gastroenterol. Clin. North Am.* **39**, 157–169 (2010).
- Maki, T. Pathogenesis of calcium bilirubinate gallstone: role of *E. coli*, beta-glucuronidase and coagulation by inorganic ions, polyelectrolytes and agitation. *Ann. Surg.* **164**, 90–100 (1966).
- Swidsinski, A. & Lee, S. P. The role of bacteria in gallstone pathogenesis. *Front. Biosci.* **6**, 93–103 (2001).
- Stewart, L., Oesterle, A. L., Erdan, I., Griffiss, J. M. & Way, L. W. Pathogenesis of pigment gallstones in Western societies: the central role of bacteria. *J. Gastrointest. Surg.* **6**, 891–904 (2002).
- Stewart, L., Griffiss, J. M., Jarvis, G. A. & Way, L. W. Biliary bacterial factors determine the path of gallstone formation. *Am. J. Surg.* **192**, 598–603 (2006).
- Begley, M., Gahan, C. G. & Hill, C. The interaction between bacteria and bile. *Fems Microbiol. Rev.* **29**, 625–651 (2005).
- Kaufman, H. S., Magnuson, T. H., Lillemo, K. D., Frasca, P. & Pitt, H. A. The role of bacteria in gallbladder and common duct stone formation. *Ann. Surg.* **209**, 584–592 (1989).
- Tabata, M. Bacteria and gallstones. *Dig. Dis. Sci.* **26**, 218–224 (1981).
- Salvador, V. B. D., Lozada, M. C. H. & Consunji, R. J. Microbiology and antibiotic susceptibility of organisms in bile cultures from patients with and without cholangitis at an Asian academic medical center. *Surg. Infect.* **12**, 105–111 (2011).
- Chang, W.-T. *et al.* Bacteriology and antimicrobial susceptibility in biliary tract disease: an audit of 10-year's experience. *Kaohsiung J. Med. Sci.* **18**, 221–228 (2002).
- Tanaka, A. *et al.* Antimicrobial therapy for acute cholangitis: Tokyo Guidelines. *J. Hepatobiliary Pancreat. Surg.* **14**, 59–67 (2007).
- Theron, J. & Cloete, T. Molecular techniques for determining microbial diversity and community structure in natural environments. *Crit. Rev. Microbiol.* **26**, 37–57 (2000).
- Segata, N. *et al.* Computational metaomics for microbial community studies. *Mol. Syst. Biol.* **9**, 666 (2013).
- Wu, T. *et al.* Gut microbiota dysbiosis and bacterial community assembly associated with cholesterol gallstones in large-scale study. *BMC Genomics* **14**, 669 (2013).
- Consortium, H. M. P. Structure, function and diversity of the healthy human microbiome. *Nature* **486**, 207–214 (2012).
- Consortium, H. M. P. A framework for human microbiome research. *Nature* **486**, 215–221 (2012).

19. Aagaard, K. *et al.* The placenta harbors a unique microbiome. *Sci. Transl. Med.* **6**, 237ra265–237ra265 (2014).
20. Chen, T. *et al.* The Human Oral Microbiome Database: a web accessible resource for investigating oral microbe taxonomic and genomic information. *Database* **2010**, baq013 (2010).
21. Downes, J. *et al.* *Pyramidobacter piscicolens* gen. nov., sp. nov., a member of the phylum ‘Synergistetes’ isolated from the human oral cavity. *Int. J. Syst. Evol. Microbiol.* **59**, 972–980 (2009).
22. Rôças, I. N. & Siqueira, J. F. Identification of bacteria enduring endodontic treatment procedures by a combined Reverse Transcriptase–Polymerase Chain reaction and Reverse-Capture Checkerboard approach. *J. Endod.* **36**, 45–52 (2010).
23. Tomazinho, L. F. & Avila-Campos, M. J. Detection of *Porphyromonas gingivalis*, *Porphyromonas endodontalis*, *Prevotella intermedia*, and *Prevotella nigrescens* in chronic endodontic infection. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* **103**, 285–288 (2007).
24. Göker, M. *et al.* Complete genome sequence of *Olsenella uli* type strain (VPI D76D-27C T). *Stand. Genomic Sci.* **3**, 76–84 (2010).
25. Brown, J. M. *et al.* Characterization of clinical isolates previously identified as *Oerskovia turbata*: proposal of *Cellulosimicrobium funkei* sp. nov. and emended description of the genus *Cellulosimicrobium*. *Int. J. Syst. Evol. Microbiol.* **56**, 801–804 (2006).
26. Magro-Checa, C. *et al.* Septic Arthritis due to *Cellulosimicrobium cellulans*. *J. Clin. Microbiol.* **49**, 4391–4393 (2011).
27. Rowlinson, M.-C., Bruckner, D. A., Hinnebusch, C., Nielsen, K. & Deville, J. G. Clearance of *Cellulosimicrobium cellulans* bacteremia in a child without central venous catheter removal. *J. Clin. Microbiol.* **44**, 2650–2654 (2006).
28. Hadano, Y. *et al.* *Raoultella ornithinolytica* bacteremia in cancer patients: report of three cases. *Intern. Med.* **51**, 3193–3195 (2012).
29. Sng, L.-H. *et al.* Bacteremia caused by *Gordonia bronchialis* in a patient with sequestered lung. *J. Clin. Microbiol.* **42**, 2870–2871 (2004).
30. Renvoise, A., Harle, J., Raoult, D. & Roux, V. *Gordonia sputi* bacteremia. *Emerg. Infect. Dis.* **15**, 1535–1537 (2009).
31. Rajagopal, T. *et al.* *Gemella haemolysans* spondylodiscitis A report of two cases. *J. Bone Joint Surg.-Br.* Vol. **94**, 825–828 (2012).
32. Rapeport, K. B., Giron, J. A. & Rosner, F. *Streptococcus mitis* endocarditis: report of 17 cases. *Arch. Intern. Med.* **146**, 2361–2363 (1986).
33. Bhatti, M. A. & Frank, M. O. *Veillonella parvula* meningitis: case report and review of *Veillonella* infections. *Clin. Infect. Dis.* **31**, 839–840 (2000).
34. Odeh, M., Oliven, A., Potasman, I., Solomon, H. & Srugo, I. Pyomyositis of the thigh due to *Prevotella melaninogenica*. *Infection* **28**, 49–50 (2000).
35. Janda, W. *et al.* *Neisseria* in *Manual of clinical microbiology* Volume 1 (eds Murray, P. R. *et al.*) 601–620 (ASM Press, 2006).
36. Maurer, K. J., Carey, M. C. & Fox, J. G. Roles of infection, inflammation, and the immune system in cholesterol gallstone formation. *Gastroenterology* **136**, 425–440 (2009).
37. Pattinson, N. & Willis, K. Effect of phospholipase C on cholesterol solubilization in model bile. A concanavalin A-binding nucleation-promoting factor from human gallbladder bile. *Gastroenterology* **101**, 1339–1344 (1991).
38. Belzer, C., Kusters, J., Kuipers, E. & Van Vliet, A. Urease induced calcium precipitation by *Helicobacter* species may initiate gallstone formation. *Gut* **55**, 1678–1679 (2006).
39. Guaglianone, E. *et al.* Microbial biofilms associated with biliary stent clogging. *FEMS Immunol. Med. Microbiol.* **59**, 410–420 (2010).
40. Ruiz, L., Margolles, A. & Sánchez, B. Bile resistance mechanisms in *Lactobacillus* and *Bifidobacterium*. *Front. Microbiol.* **4**, 396 (2013).
41. Stewart, L., Ponce, R., Oesterk, A. L., Griffiss, J. M. & Way, L. W. Pigment gallstone pathogenesis: slime production by biliary bacteria is more important than beta-glucuronidase production. *J. Gastrointest. Surg.* **4**, 547–553 (2000).
42. Sung, J., Leung, J., Shaffer, E., Lam, K. & Costerton, J. Bacterial biofilm, brown pigment stone and blockage of biliary stents. *J. Gastroenterol. Hepatol.* **8**, 28–34 (1993).
43. Prouty, A., Schwesinger, W. & Gunn, J. Biofilm Formation and Interaction with the Surfaces of Gallstones by *Salmonella* spp. *Infect. Immun.* **70**, 2640–2649 (2002).
44. Pratt, L. A. & Kolter, R. Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili. *Mol. Microbiol.* **30**, 285–293 (1998).
45. O’Toole, G. A. & Kolter, R. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Mol. Microbiol.* **30**, 295–304 (1998).
46. Yamada, T., Letunic, I., Okuda, S., Kanehisa, M. & Bork, P. iPath2.0: interactive pathway explorer. *Nucleic Acids Res.* **39**, W412–W415 (2011).
47. Abubucker, S. *et al.* Metabolic reconstruction for metagenomic data and its application to the human microbiome. *PLoS Comput. Biol.* **8**, e1002358 (2012).
48. Morita, H., Nakanishi, K., Dohi, T., Yasugi, E. & Oshima, M. Phospholipid turnover in the inflamed intestinal mucosa: arachidonic acid-rich phosphatidyl/plasmenyl-ethanolamine in the mucosa in inflammatory bowel disease. *J. Gastroenterol.* **34**, 46–53 (1999).
49. Morgan, X. C. *et al.* Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment. *Genome Biol.* **13**, R79 (2012).
50. Wadhams, G. H. & Armitage, J. P. Making sense of it all: bacterial chemotaxis. *Nat. Rev. Mol. Cell Biol.* **5**, 1024–1037 (2004).
51. Martens, E. C., Chiang, H. C. & Gordon, J. I. Mucosal glycan foraging enhances fitness and transmission of a saccharolytic human gut bacterial symbiont. *Cell Host Microbe* **4**, 447–457 (2008).
52. Ding, J., Li, F., Zhu, H. & Zhang, X. Endoscopic treatment of difficult extrahepatic bile duct stones, EPBD or EST: An anatomic view. *World J. Gastrointest. Endosc.* **7**, 274–277 (2015).
53. Xu, L., Kyaw, M., Tse, Y. & Lau, J. Endoscopic Sphincterotomy with Large Balloon Dilation versus Endoscopic Sphincterotomy for Bile Duct Stones: A Systematic Review and Meta-Analysis. *Biomed Res. Int.* **2015**, 673103 (2015).
54. Natsui, M., Honma, T., Genda, T. & Nakadaira, H. Effects of endoscopic papillary balloon dilation and endoscopic sphincterotomy on bacterial contamination of the biliary tract. *Eur. J. Gastroenterol. Hepatol.* **23**, 818–824 (2011).
55. dos Santos, J. S., Júnior, W. S., Modena, J., Brunaldi, J. E. & Ceneviva, R. Effect of preoperative endoscopic decompression on malignant biliary obstruction and postoperative infection. *Hepatogastroenterology* **52**, 45–47 (2005).
56. Ipek, S. *et al.* Evaluation of the effectiveness of endoscopic retrograde cholangiopancreatography in patients with perihilar cholangiocarcinoma and its effect on development of cholangitis. *Gastroenterol. Res. Pract.* **2014**, 508286 (2014).
57. Li, H. & Durbin, R. Fast and accurate long-read alignment with Burrows–Wheeler transform. *Bioinformatics* **26**, 589–595 (2010).
58. Qin, N. *et al.* Alterations of the human gut microbiome in liver cirrhosis. *Nature* **513**, 59–64 (2014).
59. Benjamini, Y. & Hochberg, Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. Roy. Stat. Soc. B* **57**, 289–300 (1995).

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Author Contributions

X.Z., M.N. and X.B. conceived and designed the study. X.Z., H.S., L.X. and J.Y. collected samples and patient information. Z.L., P.X., F.M. and L.L. generated sequencing data. Y.C. performed statistical analysis. F.Y., H.S. and M.N. analysed and interpreted the data. F.Y. and M.N. wrote the manuscript with contributions from all other authors. All authors reviewed and approved the final manuscript.

Additional Information

Accession codes: All WMS and 16S sequence data were deposited in the NCBI database under BioProject PRJNA278310. All bile samples were registered in the NCBI database under BioSample numbers SAMN03417491–SAMN03417505.

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