Prospective Cohort Study

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The bile microbiome is a surrogate for the intratumoral microbiome in pancreatic ductal adenocarcinoma and is associated with oncological outcomes: a cohort study

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Background: The bile microbiome has been shown to be associated with the development of pancreatic ductal adenocarcinoma (PDAC). However, the utility of bile fluid as a potential source of microbial biomarkers remains unknown. We aimed to characterize the bile microbial composition in PDAC compared to benign and malignant pancreatico–biliary disease, as well as correlate our findings with the pancreatic intratumoral and neighboring adjacent tissue (NAT) microbiome.

Methods: Prospective matched pancreatic tumor, NAT, and bile samples were obtained from 54 patients who underwent surgery for a head of pancreas mass at Royal Surrey NHS Hospital Trust. Full-length 16S rRNA (V1-V9) gene sequencing was performed on the Oxford Nanopore MinION™ platform. The cohort consisted of 30 PDAC, 14 biliary tract cancers, and 10 benign cases.

Results: We identified biliary microbial biomarkers *Streptococcus* (false discovery rate [FDR] = 0.0047), *Klebsiella* (FDR = 0.0095), *Enterobacter* (FDR = 9.68×10^{-7}), and *Veillonella* (FDR = 0.0140) that were found both in the bile and tumor in patients having surgery for PDAC. These bacterial genera were significantly more abundant in PDAC tumors compared to matched NAT and benign disease. We detected a negligible number of microbial reads in the NAT samples. Our microbial signature was highly predictive of PDAC within tissue (AUC = 0.9233) and bile (AUC = 0.8101). Positive bile cultures in the PDAC cohort increased the risk of deep-seated surgical site infections (SSIs), delayed gastric emptying, and post-operative pancreatic fistula. Biliary stenting did not affect microbial composition, and the abundance of specific genera significantly correlated with overall survival and disease-free survival in PDAC.

Conclusion: We have shown that the normal pancreas is a relatively sterile organ, whilst the PDAC tumor and bile are colonized with specific genera. In fact, 71% of the tumor microbiome is shared with the bile microbiome in PDAC, suggesting that bile may serve as a potential future diagnostic and prognostic microbial biomarker as well as a surrogate readout of the tumor microbiome. The function of these microbes during pancreatic tumorigenesis remains to be determined. Furthermore, intra-operative biliary culture may serve as a low-cost predictor of post-operative outcomes.

Keywords: 16S rRNA gene, diagnostic biomarker, microbiome, pancreatic cancer, post-operative outcome, surgery

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a silent tumor that evades early detection, and patients are often diagnosed with locally advanced or metastatic disease, ruling out potentially curative surgical resection. Even for those patients who undergo surgery, the 5-year survival remains low globally, in the range of 5–15%[1]. The poor outcomes in the PDAC treatment are partly due to the complex and highly heterogeneous immunosuppressive tumor microenvironment[2–5], which induces resistance to current systemic treatment options. The presence of intratumoral microorganisms in PDAC has recently gathered attention, as these may influence the immune response to the tumor, and the efficacy of chemotherapy[6]. Microbes not only thrive in environmentally exposed human niches like the gut, but also colonize tumors, where they can influence tumor progression, response to treatment, and patient outcomes across various cancers[7]. Indeed, it has been demonstrated that both the microbiota[8] and mycobiota[9] play a role in the development of PDAC. Our previous work has shown that patients presenting with obstructive jaundice caused by PDAC have an altered microbiome composition in the bile, compared to those with benign disease[10]. In our analysis of 16S rRNA gene sequencing to assess the microbial composition of PDAC, we identified common microbes associated with the disease and observed the influence of the microbial metabolites on tumorigenesis[11]. Recent studies have investigated the microbiota present in PDAC biopsies and identified a higher diversity of bacteria in long-term survivors[9]. Bacterial and fungal species present within the patient's gastrointestinal (GI) tract migrate via the pancreatic duct and infect the tumor[12]. However, a recent study by Eckhoff *et al* discovered that the pancreatic microbiome demonstrates a low intrinsic biomass that may not drive malignant transformation[13]. The precise mechanisms by which microorganisms can promote or inhibit pancreatic tumor growth are not fully understood. However, chemotherapy efficacy has been shown to depend on specific species present within the intratumoral microbial community[8,9]. Bile consists mainly of water, electrolytes, bile salts, cholesterol, phospholipids, and proteins. The bacteria in GI tract play a role in determining the composition of bile acids (BAs)[14]. The microbiota heavily rely on BAs for their abundance, diversity, and metabolic activity[15]. Certain bacterial species can interrupt this pathway, creating a toxic bile environment to GI tract cells, and accelerating cancer growth by inducing pro-tumorigenic activities[16]. Indeed, it has been shown that alteration of the bile microbiome after biliary stenting increases the expression of inflammatory markers, bacterial metabolites, and toxins in PDAC cells, which directly influenced PDAC cell survival and immune evasion[17]. There is a growing body of studies investigating the intrapancreatic microbiota in the development of PDAC[11,18,19]. Yet, it remains unknown if the bile fluid acts as the source of bacterial translocation and influences clinical outcome. We aimed to characterize the microbes present in matched fresh bile, pancreatic tumor, and neighboring adjacent tissue (NAT) taken from PDAC patients at the time of surgery. In this prospective observational study, we sought to discover specific microbes in bile and tumor tissue that could be used as potential biomarkers for the detection and prognostication of PDAC. This study was conducted without using artificial intelligence (AI) tools in accordance with the TITAN Guidelines 2025[20] (Supplemental Digital Content Table 1, available at: http://links.lww.com/JS9/H277).

HIGHLIGHTS

- Bile microbiome reflects the intratumoral microbiome, offering a minimally-invasive biomarker source for pancreatic ductal adenocarcinoma (PDAC) diagnosis and prognosis.
- A microbial signature (*Streptococcus, Klebsiella, Enterobacter*, and *Veillonella*) accurately differentiates PDAC from benign conditions (AUC = 0.9233 in tissue, 0.8101 in bile).
- Specific genera (*Plesiomonas, Streptococcus*, and *Enterobacter*) correlate with improved overall survival and disease-free survival, alluding to their potential role in tumor biology as well as prognostic biomarkers.
- PDAC tumors harbor unique and diverse microbiomes compared to nearly sterile adjacent tissue, suggesting site-specific microbial colonization.
- Positive bile cultures link to surgical complications (e.g., SSIs, DGE, and POPF) and worse oncological outcomes, emphasizing the clinical significance of microbiome profiling.

Methods

Patients and study design

Patients scheduled to undergo pancreaticoduodenectomy (PD) for known or suspected cancer in the head of the pancreas were included in this study. All patients planned for surgical resection were identified through the hepato–pancreato–biliary (HPB) multidisciplinary team meeting and by the clinical team. All patients received information about the trial in written form and provided informed consent before enrolment. These patients were approached either in the clinic or at the preoperative assessment. Patients included were those with a resectable PDAC or other biliary-tract cancers (BTCs), as well as those having surgery for benign pancreaticobiliary disease. Patients were excluded if deemed unsuitable for resection (locally advanced, borderline, or metastatic disease), had received neo-adjuvant chemotherapy, were unwilling or unable to provide written informed consent, were known to be pregnant, or were aged <16 years. The patient's control groups consisted of those who had undergone surgery for benign pathology (e.g., adenoma, cysts, and chronic pancreatitis), or other malignant pancreaticobiliary disease (e.g., duodenal carcinoma, ampullary carcinoma, neuroendocrine tumors, and cholangiocarcinoma). The findings are reported in accordance with established guidelines, including the Strengthening the Organization and Reporting of Microbiome Studies checklist[21] (Supplemental Digital Content Table 2, available at: http://links.lww.com/JS9/H278), and the "RIDE" minimum standards for low microbial biomass microbiome studies[22]. This work has been reported in line with the STROCSS criteria[23] (Supplemental Digital Content Table 3, available at: http://links.lww.com/JS9/H279).

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Study population and sample collection

For analysis, multiple confounding factors were considered, including antibiotic use, age, sex, medication, biliary stenting, alcohol, and tobacco consumption. Most patients who presented to their local hospital deeply jaundiced and requiring urgent biliary stenting/decompression had a course of broad-spectrum antibiotics within the month before surgery. The patients did not receive any bowel preparation regimen before surgery, and all patients had standard perioperative broad-spectrum prophylactic antibiotics at the start of surgery.

Biospecimen collection

The specimens were kept sterile for immediate tissue procurement. The time between the completion of surgical resection and tissue procurement was kept to a minimum, at approximately <5 minutes. Preoperative CT images were utilized to determine the position of the lesion and assist in guiding the needle for extracting a core biopsy that would yield optimal tumor samples. Core (Tru-cut) biopsies of the tumor were obtained from the surgical specimen, and areas of central necrosis were avoided. A core tumor biopsy was sent to the pancreatic histopathologist (I.B.), who confirmed the percentage of tumor obtained within the sample. NAT biopsies were taken from the pancreatic resection margin site (pathologically confirmed as no evidence of tumor). Samples without histologic quality control assessment were excluded. Bile was obtained at the time of the common hepatic duct transection. Approximately, 500–1000 µl of bile were aspirated with a sterile syringe and transferred into aliquots. These were immediately frozen at −80 °C. A standard bile microbial microscopy, culture, and sensitivity swab was collected from all PDAC patients using a charcoal transport medium. Species identification was performed in the hospital laboratory through Gram staining, biochemical tests, and antibiotic susceptibility testing. The study flow chart and overview of the sample collection steps are shown in Figure 1.

Contamination control

To minimize the risk of contamination and ensure the integrity of microbial profiling, all sample processing and sequencing were conducted in accordance with the RIDE (Reporting of In Vitro Diagnostics Experiments) checklist. Samples were processed in sequencing batches, each incorporating positive and negative PCR controls to detect and account for potential contaminants, filter out low-abundance artefacts, and standardize microbial profiling. Negative PCR controls were included in each batch to monitor for contamination introduced during DNA extraction and amplification. These consisted of DNase/RNase-free UltraPure water and empty Eppendorf tubes, processed in parallel with biological samples. These controls were essential for subsequent bioinformatic decontamination, ensuring that only genuine microbial signals were retained in the final dataset. Positive PCR controls were also incorporated to assess amplification efficiency and consistency across batches. These included previously sequenced bile, fecal, and nasal samples with validated reproducible microbial profiles, and ZymoBIOMICS Microbial Community Standard (Zymo Research), a commercially available mock community comprising a defined mixture of bacterial DNA. This control allowed for benchmarking of taxonomic resolution and pipeline accuracy. DNA extracted from all negative controls was below the limit of detection of the Qubit fluorometer, and no full-length 16S rRNA gene amplicons were generated from these controls during Oxford Nanopore MinION sequencing. Following basecalling and quality filtering, zero reads from negative controls were retained for downstream analysis. As negative controls yielded no retained sequencing reads, prevalence- or frequency-based statistical decontamination methods were not applicable, as such approaches require a detectable background signal to identify contaminant taxa. Consequently, no taxa were identified or removed as contaminants, and contamination mitigation relied on the use of stringent laboratory controls and conservative downstream filtering criteria.

Contamination mitigation, therefore, depended on negative controls, minimum read-count thresholds, and relative-abundance cut-offs for taxonomic reporting, rather than statistical contaminant-identification algorithms, due to the absence of a detectable background signal in controls.

DNA extraction

DNA for microbiome analyses of the pancreatic tissue was extracted using the ZymoBIOMICS DNA/RNA Miniprep tissue kit with bead beating and column-based extraction of samples according to the manufacturer's protocol. DNA for microbiome analysis of the matched intraoperative bile was extracted using the MasterPure™ Complete DNA and RNA Purification Kit, LGC, Biosearch Technologies.

Nanopore 16S rRNA (V1-V9) gene sequencing on the MinION™ platform

After extraction, DNA concentrations were measured using a Qubit 3.0 fluorometer with a Qubit™ dsDNA HS Assay Kit (Life Technologies, Grand Island, NY, USA, Cat. No Q33230). Quality of the genomic DNA (gDNA) extracted from tissue and bile was assessed by the electrophoresis Agilent 4200 TapeStation system before sequencing. For amplification of the V1–V9 region of the 16S rRNA gene, the 16S Barcoding Kit 1-24, (SQK-16S024; Oxford Nanopore Technologies, Oxford, UK) was used. The DNA was amplified by PCR using 16S-specific primers (27F and 1492R) that contain 5' tags which facilitate the ligase-free attachment of Rapid Sequencing Adapters and span the full length of the 16S rRNA gene. To reduce the risk of contamination between samples, a new R9.4.1 Flow Cell was used for each sequencing run. Sequencing was performed for up to 4 hours with a minimum read length set at 200bp. Thirteen patients were excluded from further analysis due to low read numbers within the tissue cohort, leaving 41 out of 54 patients with optimal sequencing results. Similarly, one patient was excluded from the bile cohort, resulting in optimal sequencing results for 53 out of 54 patients.

Nanopore 16S rRNA sequencing, base-calling and bioinformatics analysis

The Basecalling was performed by Guppy basecaller (Oxford Nanopore Technologies, R9.4.1)[24] on an NVIDIA GPU machine. Correctly basecalled sequences (Q > 10) were searched for adaptors and barcodes and were trimmed with Porechop (v0.2.4) (https://github.com/rrwick/Porechop). The quality and status of trimmed sequences were also assessed with Nanoplot v1.42.0[25]. To further reduce the influence of spurious low-level

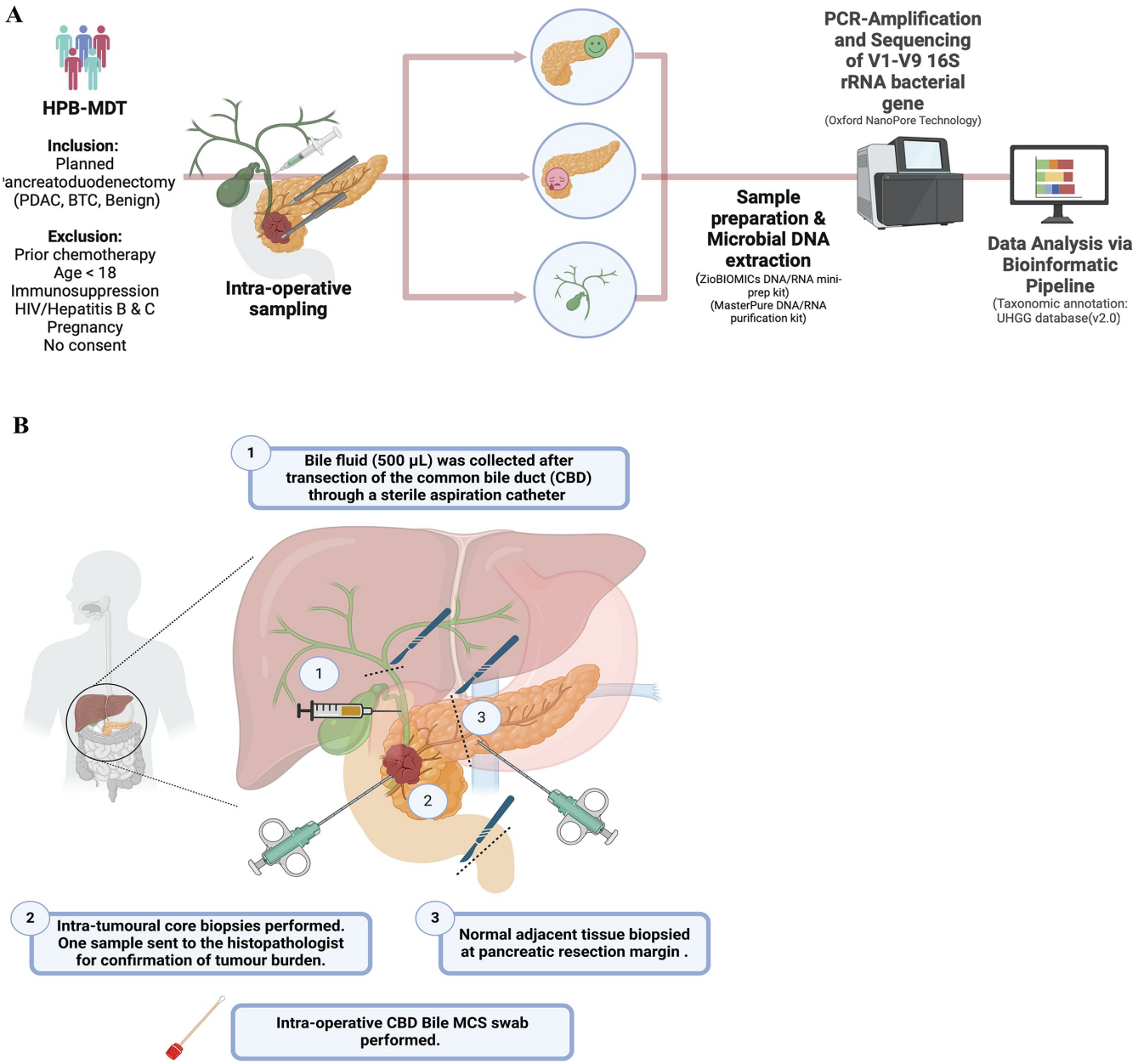


Figure 1. Schematic of study design. (A) Overview of the study workflow for intraoperative microbiome analysis in pancreatic surgery using full-length Nanopore 16S rRNA gene sequencing. Patients undergoing surgery for PDAC (n = 30), BTC (n = 14), and benign biliary conditions (n = 10) were prospectively selected by the HPB multidisciplinary team based on defined inclusion and exclusion criteria. During surgery, sterile sampling was performed to obtain matched tumor tissue, adjacent neighboring pancreatic tissue (NAT), and bile. Taxonomic classification was conducted through a standardized bioinformatic pipeline. (B) Intra-operative sampling: (1) complete transection of the common hepatic duct was performed, and a sterile swab was taken within the bile duct and bile fluid drawn by a sterile aspiration catheter; (2) Tru-cut biopsies were taken from the site of the tumor; and (3) the pancreatic resection margin (tumor-free site). A sample was sent to the histopathologist to confirm tumor burden, and the samples were immediately frozen at −80 °C.

signals, samples with fewer than 10 and 3 classified reads were excluded from the PDAC and BTC groups, respectively, before the downstream analyses. This threshold was selected a priori to minimize artefactual classifications arising from low read depth in low-biomass samples. Sensitivity analyses using higher read-count thresholds yielded qualitatively similar taxonomic profiles and did not alter the main findings. The reads of bacterial 16S rRNA were classified by the Kraken v2.1.2[26] and Bracken v2.6.2[27] with the human gut-microbiome specific Unified Human Gastrointestinal Genome (UHGG) catalogue database (v.2.0)[28]. Although Kraken–Bracken is commonly applied in shotgun metagenomic studies, the method is k-mer–based and independent of sequencing strategy and can be applied to full-length 16S rRNA gene sequences when appropriate reference databases are used. The primary reference database was UHGG, selected for its genome-resolved coverage of human-associated microbiota relevant to PDAC-derived bile and tissue samples. The read numbers and their relative abundances were aggregated

with Pavian (version 1.2.0)[29]. Diversity matrices and principal coordinates analysis (PCoA) were calculated at the Genus level. The Shannon diversity index was normalized, and the PCoA was analyzed using the vegdist function in the vegan package in R v3.6.0 R Core Team (2021; https://www.R-project.org/). Differential abundance analysis (DAA) was conducted to identify potential microbial biomarkers in PDAC. The *P*-values were calculated using the Wilcoxon rank–sum test and corrected with false discovery rate (FDR) using Benjamini–Hochberg. The significance level is $P < 0.05$. A minimum cut-off of 5 reads per taxon (genus) was used to construct the Venn diagrams. Data visualization was performed in R with ggplot2 and the pheatmap package. PERMANOVA testing was performed to assess whether biliary stenting or recent antibiotic use acted as confounding factors in microbial composition analyses.

Microbial feature selection

To assess the prognostic value of the tumor-associated microbiome in PDAC, we stratified patients based on the relative abundance of seven bacterial genera identified by 16S rRNA gene sequencing combined with DAA using the Wilcoxon rank–sum test with Benjamini–Hochberg FDR correction. The seven genera *(Lactobacillus, Streptococcus, Veillonella, Enterococcus, Enterobacter, Klebsiella*, and *Plesiomonas*) were selected based on their significant enrichment in PDAC tissue and/or bile relative to benign or non-PDAC disease cohorts. Stratification thresholds were defined using the median relative abundance of each genus within the PDAC cohort, allowing patients to be categorized as having either a high or low abundance of each genus. Shared taxa between tumor tissue and bile were defined using a presence/absence–based criterion, whereby a taxon was considered shared if detected in both sample types within the same patient. Percentages of shared taxa, therefore, reflect overlap in taxonomic presence rather than relative abundance. Abundance-weighted metrics were used only for compositional analyses and are explicitly indicated where applicable. This approach was applied independently to each taxon. Kaplan–Meier analysis and log-rank tests were then used to explore associations with overall and disease-free survival (DFS). Genera with a relative abundance of ≥1% of total reads for PDAC (tissue and bile), BTC bile, and benign bile, and ≥2% of total reads for benign tissue were included in the graphical representation of the relative abundance of taxa. For the heatmap, genera that had a minimum relative abundance of ≥5% in at least one sample were included in the plot. Samples with less than 10 reads were excluded from the PDAC group, and samples with less than 3 reads were excluded from BTC and benign groups for overall analyses. For downstream modelling, a composite microbial signature score was created by calculating the mean relative abundance of all seven genera for each patient. To evaluate the discriminatory power of this composite signature in differentiating PDAC from non-PDAC conditions (benign and BTCs), we conducted Receiver Operating Characteristic (ROC) curve analysis and calculated the Area Under the Curve (AUC) using GraphPad Prism software v 9.4.1. ROC analysis was performed separately for both tumor tissue and bile samples. An AUC value of >0.8 was considered to represent good diagnostic accuracy. To adjust for potential clinical confounders, we used a multivariable Cox proportional hazards regression model to evaluate their association with overall survival (OS). In addition to the low or high abundance of the respective genus tested, the multivariable model included other prognostic covariates. In order to select variables for the multivariate analysis, a univariate analysis of prognostic variables was carried out. Variables with a significance of $P < 0.25$ were considered for inclusion as covariates in the final model. Variables considered included age at diagnosis, gender, Eastern Cooperative Oncology Group (ECOG) performance status (0, 1, or 2), preoperative CA19-9 levels, tumor size in mm, nodal status (negative vs positive), perineural invasion (PNI) or lymphovascular invasion (LVI) (absent vs present), and received adjuvant chemotherapy (no vs yes). These are all clinically relevant variables that are well regarded as being associated with survival in PDAC. There were no missing fields for the covariates. The proportional hazards were assessed using Schoenfield residuals. SPSS was used for the analysis. In the multivariate analysis, a $P \leq 0.05$ was considered significant.

Results

Study cohort and clinical characteristics

In this study, we prospectively recruited 54 patients having a pancreaticoduodenectomy (PD or Whipple's procedure). Matched tumor and bile samples were obtained from PDAC ($n = 30$), BTCs ($n = 14$; positive controls), and benign non-cancer cases ($n = 10$; negative controls). All the PDAC cases had tumors in the head of the pancreas, and 70% of cases had biliary stents. There were no cholangitic patients in either group, with similar median C-Reactive Protein (CRP) values of 4 mg/l at the time of surgery, and no significant differences between the groups. For OS and DFS analysis, there were 18 and 25 events, respectively. Additional demographic, clinical, and outcome variables are listed in Tables 1 and 2.

PDAC is characterized by a distinct intratumoral and biliary microbiome compared to other benign and malignant conditions

To investigate the microbial composition within the tumor and bile samples across different disease states, we performed full-length 16S rRNA sequencing. The kit-reagent negative control did not yield detectable DNA by fluorometric quantification and failed to generate full-length 16S rRNA gene sequences on the MinION platform. No reads from the negative control passed quality filtering, and therefore, no contaminant taxa were identified or removed. These results indicate minimal reagent- or laboratory-derived bacterial background under the experimental conditions used. To evaluate whether the use of a genome-resolved human-associated reference influenced classification outcomes, a subset of PDAC bile samples was reclassified using the SILVA v138 16S database with identical Kraken-Bracken parameters. UHGG consistently yielded higher classification rates across all taxonomic ranks, with pronounced improvements at lower levels (family, genus, and species) (Supplemental Digital Content Figure 1, available at: http://links.lww.com/JS9/H275). These findings indicate that the observed performance differences reflect reference database composition rather than incompatibility of the classifier with 16S data.

Our analysis revealed that the PDAC tumor tissue (Fig. 2A) and associated bile samples (Fig. 2B) had significantly higher abundance of bacterial genera when compared to samples obtained from patients with BTCs and those with benign biliary conditions

Table 1

Patient clinicopathological factors.

Patient characteristics	PDAC (n = 30)	Biliary tract and other cancers (n = 14)[a]	Benign cases (n = 10)[b]	P-Value
Age at surgery, median (range), years	70 (53–81)	72 (56–84)	67 (44–81)	0.8344
Sex, (male; female), n (%)	17 (57%); 13 (43%)	11 (79%); 3 (21%)	5 (50%); 5 (50%)	0.735
BMI, median (range), kg/m^2	24.1 (19–34)	23.2 (17.9–30.1)	20.1 (17.2–29)	0.018
ASA 2	20 (67%)	11 (78%)	7 (70%)	0.723
ASA 3	10 (33%)	3 (22%)	3 (30%)	
Smoking status, n (%)				
Never-smoked	16 (53%)	10 (71%)	7 (70%)	0.423
Smoked	14 (%)	4 (%)	3 (%)	
History of alcohol, n (%)				
Yes	19 (63%)	1 (7%)	4 (40%)	0.002
No	11 (37%)	13 (93%)	6 (60%)	
T2DM, presence, n (%)	9 (30%)	0	0	0.013
Regular laxative use, n (%)	12 (40%)	1 (7%)	2 (20%)	0.063
Regular PPI use, n (%)	11 (37%)	5 (36%)	5 (50%)	0.610
Antibiotics used pre-surgery (Within < 1-month)				
Yes	10 (33%)	5 (36%)	0	0.093
No	20 (67%)	9 (64%)	10 (100%)	
Preoperative biliary stenting, n (%)				
Stent	21 (70%)	8 (58%)	2 (20%)	0.022
No stent	9 (30%)	6 (42%)	8 (80%)	
Positive CBD MC&S culture swab, n (%)				
Yes	20 (67%)	5 (36%)	0	0.017
No	10 (33%)	9 (64%)	10 (100%)	

[a]Ampullary carcinoma (n = 2), cholangiocarcinoma (n = 3), squamous cell carcinoma (n = 1), cohesive carcinoma (n = 2), duodenal adenocarcinoma (n = 4), neuroendocrine tumor (n = 2).
[b]Chronic pancreatitis with stone disease (n = 3), IgG4 pancreatitis (n = 1), benign epithelial cyst (n = 2), intraductal papillary mucinous neoplasms (n = 1), tubular-villous adenoma (n = 2), fibroinflammatory periductal fibrosis lesion (n = 1).
Statistical program used was GraphPad Prism 9.4.1 version.
Age and BMI variables were compared by using the Kruskal–Wallis non-parametric test.
Contingency categorial variable data outcomes were measured using the chi square test.

(Fig. 2). However, in general, bile samples had more microbial abundance than the tissue samples. Within the PDAC tumor tissue, the five most abundant bacterial genera were *Klebsiella*, *Streptococcus*, *Enterobacter*, *Staphylococcus*, and *Pseudomonas* (Fig. 3). In contrast, the dominant genera identified in PDAC-associated bile samples were *Enterobacter*, *Plesiomonas*, *Streptococcus*, *Klebsiella*, and *Citrobacter*. The five benign tissue samples demonstrated a lower number of genera per sample compared to both the PDAC and BTCs cohorts. Six genera, *Streptococcus*, *Enterobacter*, *Pseudomonas*, *Vibrio*, *Plesiomonas*, and *Ligilactobacillus*, were consistently present in both PDAC tumor tissues and benign samples. However, the remaining predominant genera differed notably between the PDAC and benign tissue groups, highlighting distinct microbial profiles associated with

Table 2

Tumor characteristics.

Characteristics	PDAC (n = 30)	Biliary tract and other cancers (n = 14)[a]	Benign cases (n = 10)[b]	P-Value
Bilirubin, median (range), µmol/l	42 (5–540)	13 (7–173)	10 (3–37)	0.0055
CRP, median (range), µmol/l	4 (1–129)	4 (2–22)	4 (4–21)	0.925
CA-199, median (range)	306 (13–17 805)	26 (2–5699)	14.5 (2–1045)	0.0023
Tumor size, median (range), mm	30 (17–50)	20 (1–45)		0.0027
Location of tumor in head of pancreas				
Peri-ampullary	3 (10%)	2 (14%)		
Peri-hilar	0	2 (14%)		
Intrapancreatic portion of CBD	9 (30%)	2 (14%)		
Infiltrates the duodenal wall	18 (60%)	6 (44%)		
Stage, n (%)				
IA	0	0		
IB	9 (30%)	0		
IIA	3 (10%)	9 (64%)		
IIB	18 (60%)	5 (36%)		

[a]Ampullary carcinoma (n = 2), cholangiocarcinoma (n = 3), squamous cell carcinoma (n = 1), cohesive carcinoma (n = 2), duodenal adenocarcinoma (n = 4), and neuroendocrine tumor (n = 2).
[b]Chronic pancreatitis with stone disease (n = 3), IgG4 pancreatitis (n = 1), benign epithelial cyst (n = 2), intraductal papillary mucinous neoplasms (n = 1), tubular-villous adenoma (n = 2), and fibroinflammatory periductal fibrosis lesion (n = 1).
All variables were compared using Kruskal–Wallis non-parametric test. Statistical program used was GraphPad Prism 9.4.1 version.

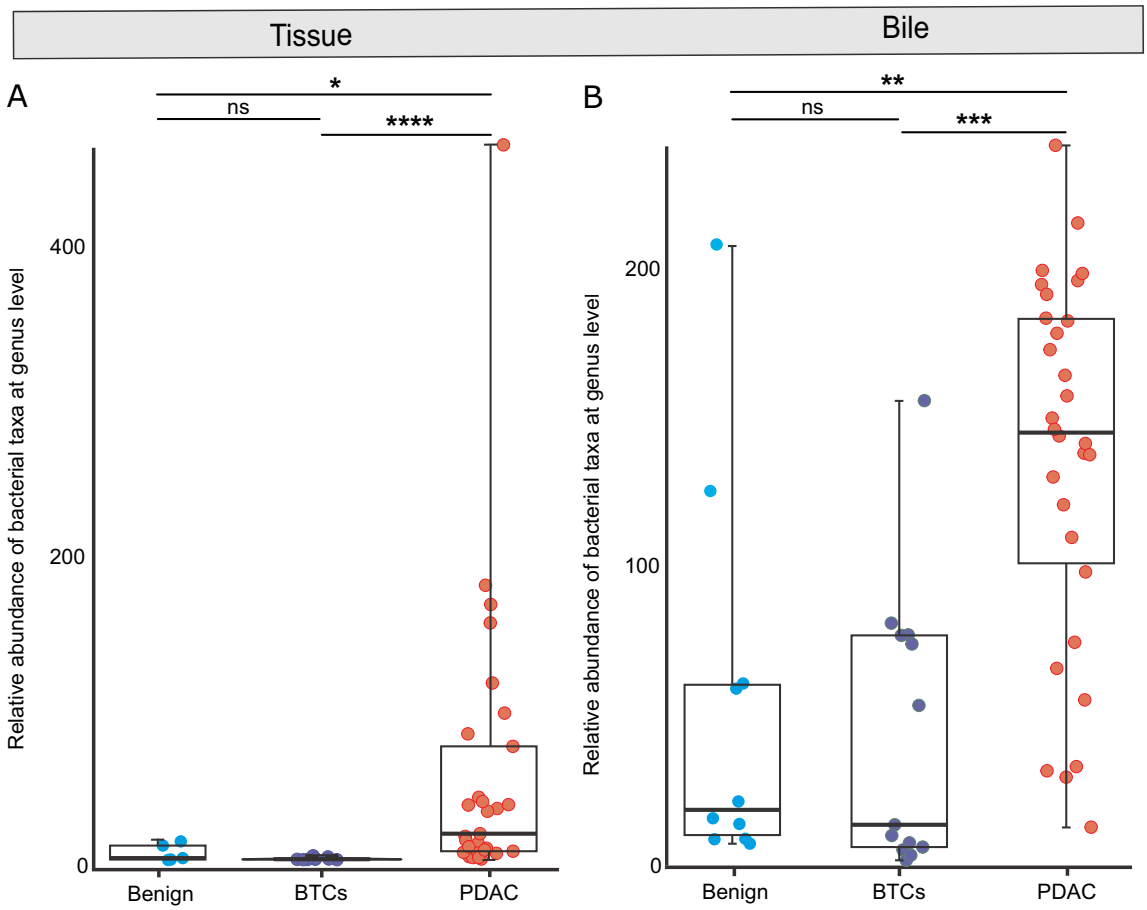


Figure 2. PDAC samples exhibited elevated bacterial abundance. Log_2-transformed relative abundance of microbial genera in (A) tissue samples benign (n = 5), BTCs (n = 9), and PDAC (n = 27) patient types and (B) bile samples benign (n = 10), BTCs (n = 13), and PDAC (n = 30) patient types. Each point represents an individual patient sample. Statistical differences in genus-level abundance across disease groups were assessed using the Kruskal–Wallis with *post hoc* Dunn test and Benjamini–Hochberg for false discovery rate correction. Data presented as boxes from the 25th to 75th percentiles, bars at the median and whiskers from minimum to maximum. Significance is denoted as follows: ns = not significant; *P < 0.05; **P < 0.005; ***P < 0.0005).

malignant versus non-malignant conditions (Fig. 3). The PDAC tumor and bile samples displayed a markedly higher abundance of microbial taxa, in contrast to the comparatively fewer microbes observed in the BTC and benign cohorts (Fig. 4). Notably, bile samples generally harbored greater microbial abundance than their corresponding tissue samples across all patient groups. Alpha diversity of microbial composition, as measured by the Shannon index, was significantly higher in PDAC tumors compared to both BTCs and benign tissues (Fig. 5A), suggesting a more complex and heterogeneous intratumoral microbial community associated with PDAC. No significant differences in Shannon diversity were observed among bile samples across the three patient groups (Fig. 5B), suggesting similar microbial richness and evenness in bile, despite compositional differences at the taxonomic level (Fig. 4).

The intratumoral microbiome harbors a unique and diverse microbiome relative to neighboring adjacent pancreatic tissue

To investigate whether the PDAC microbiome is distinct to neighboring pancreatic tissue, we examined the abundance of microbial genera across the different matched compartments (i.e., tumor, NAT, and bile) in 30 PDAC patients (Fig. 6A). Microbial richness in pancreatic NAT was limited, and nearly all genera identified in NAT were also present in tumoral tissue and/or bile, suggesting a lack of unique microbial signatures in adjacent non-tumor tissue. Notably, Shannon diversity was also significantly higher in PDAC tumoral tissues compared to matched neighboring adjacent tissue (NAT) (Fig. 5C), indicating that the tumor microenvironment harbors a distinct microbial community relative to adjacent non-malignant regions. This suggests a site-specific microbial colonization of tumors over normal pancreatic parenchyma.

To further characterize these differences, differential abundance analysis (DAA) was performed and identified several genera, *Enterobacter*, *Streptococcus*, *Klebsiella*, *Veillonella*, and *Plesiomonas*, that were significantly enriched in PDAC tumoral tissue relative to matched NAT samples (Table 3), highlighting potential microbial biomarkers associated with the tumor microenvironment.

PDAC tumors and bile microbiomes are colonized with shared and compartment-specific genera

Previous work has postulated that microbes from the duodenum or upper gastrointestinal tract may translocate to the biliary system and colonize the pancreas during tumor development[9,12]. Given

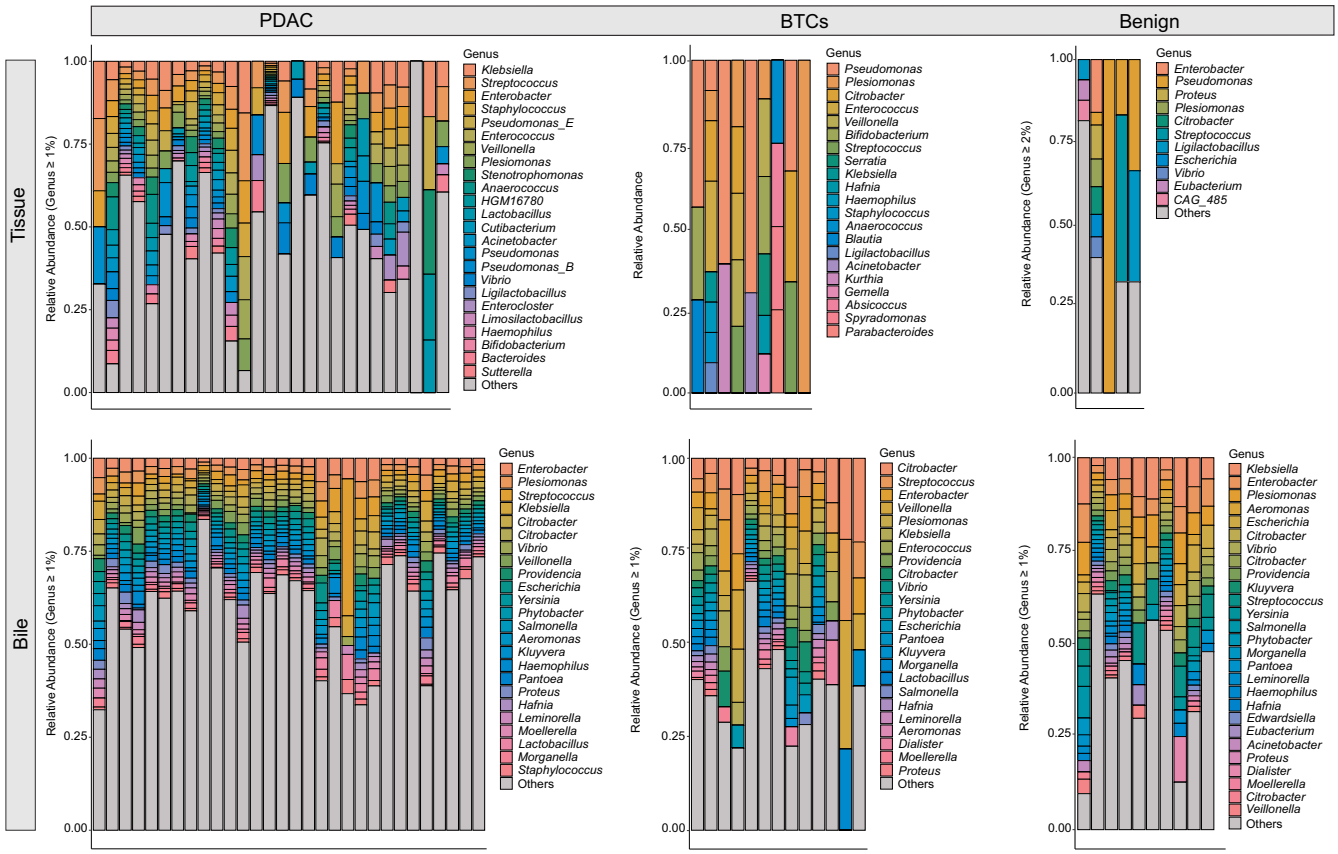


Figure 3. Log_2-transformed relative abundance of bacterial genera in tissue and bile samples from PDAC, BTCs, and benign patients. Genera are shown for PDAC tissue (n = 27), BTC tissue (n = 9), and benign tissue (n = 5), as well as PDAC bile (n = 30), BTC bile (n = 13), and benign bile (n = 10). Each bar represents the microbial profile of an individual patient, with y-axis indicating the proportional abundance and colors denoting bacterial genera. Genera not classified or below the threshold are grouped under "Others."

this hypothesis, we investigated whether the PDAC bile and tumor microbiomes are distinct or shared. If indeed shared, bile may serve as a surrogate for characterizing the tumor microbiome.

Beta diversity analysis using Bray-Curtis dissimilarity and visualized through PCoA plots showed that a partial overlap was observed between PDAC tumoral tissue and bile (Supplemental Digital Content Figure 2D, available at: http://links.lww.com/JS9/H276), suggesting some shared microbial features within the PDAC microenvironment. In keeping with this finding of shared microbial features in PDAC tumor and bile, we examined the abundance of microbial genera across different matched compartments (i.e., tumor, NAT and bile) in 30 PDAC patients (Fig. 6A). Although distinct bacterial taxa were identified within the tumor and bile compartments, a substantial proportion of genera were shared between the two, supporting the potential use of bile as a surrogate for profiling the intratumoral microbiome in PDAC. Specifically, 268 genera were common to both tumoral tissue and bile (87.9% of tumor genera were shared with bile), indicating a strong microbial overlap between these compartments. Additionally, comparison with the benign cohort (Fig. 6B) revealed a notably higher number of microbial genera in both tumoral tissue and bile samples from PDAC patients, further supporting the disease-specific microbial landscape of PDAC. Among the identified genera, 123 were unique to PDAC bile and 63 to PDAC tumor tissue, with 112 genera shared between these two compartments but absent in the benign group. The number of bile-specific genera in PDAC (123) was significantly higher than in the benign bile group (5), emphasizing a distinct bile microbial profile associated with malignancy. Shared genera between PDAC tumor tissue, bile and NATs included *Staphylococcus, Enterococcus, Haemophilus, Lactobacillus, Plesiomonas, Klebsiella, Streptococcus*, and *Enterobacter*. Although it remains unclear whether the shared or compartment-specific microbes play a more critical role in PDAC pathogenesis, these findings highlight their potential relevance as diagnostic or prognostic microbial biomarkers. As well as the bile's value as a potential surrogate readout of the intratumoral microbiome. Furthermore, our findings suggest that PDAC tumors may indeed be colonized via the biliary tree; however clearly there are alternative routes of colonization involved e.g., via the mesenteric vasculature.

A distinct 16S rRNA-based microbial signature predicts PDAC across bile and tumor samples

To investigate possible microbial biomarkers for PDAC we sought to identify genera that were unique to PDAC. Differential abundance analysis (DAA) revealed enrichment of six genera, *Klebsiella, Streptococcus, Brachyspira, Plesiomonas, Enterococcus*, and *Enterobacter* in PDAC tumoral tissue compared to benign tissue samples (Table 3). However, statistical significance was not retained after correction for multiple tests. In contrast, genus-level analysis of bile samples identified several

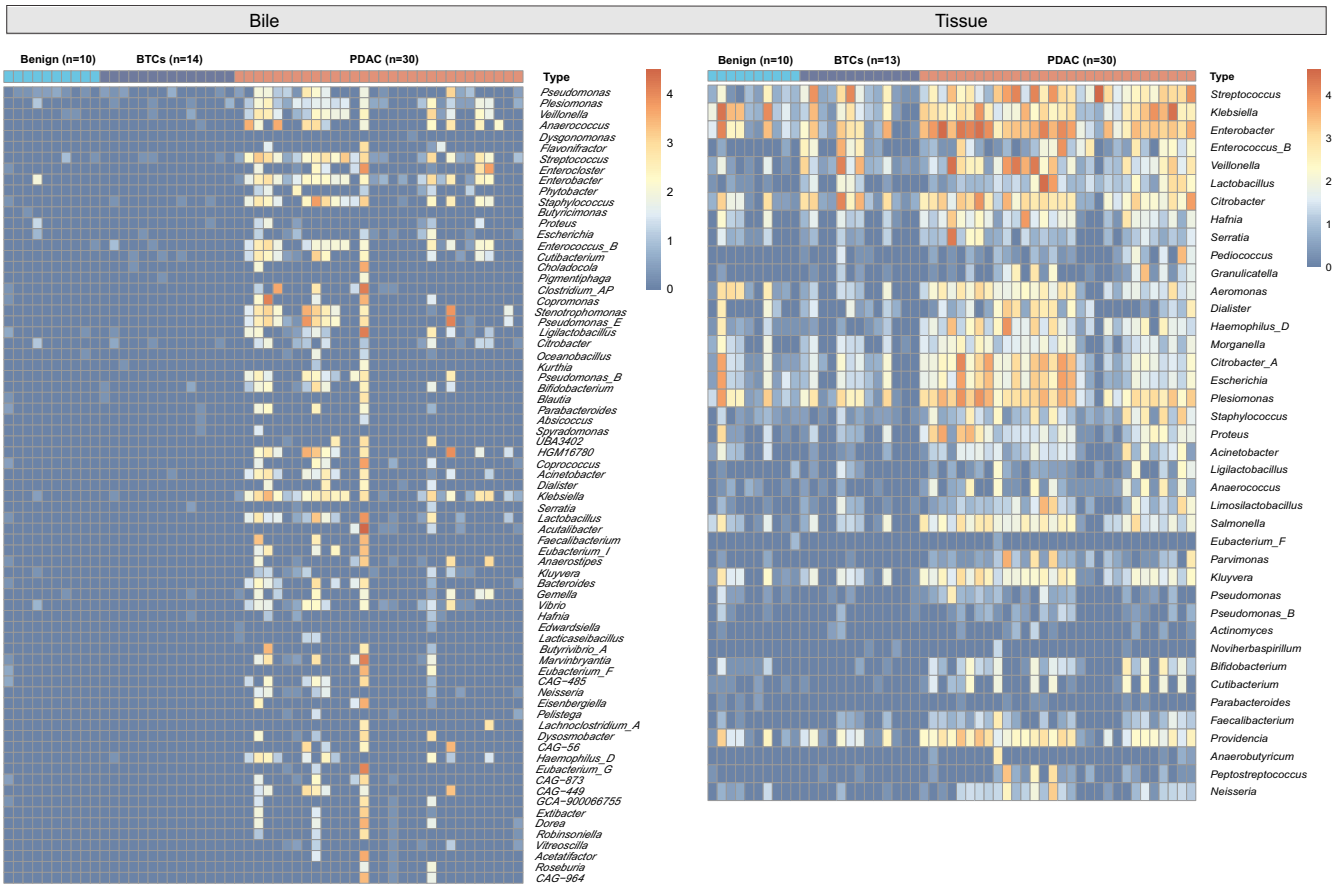


Figure 4. Relative abundance of bacterial genera in matched tissue (right) and bile samples (left) across patient groups. The tissue group consisted of benign (n = 10), BTCs (n = 13) and PDAC (n = 30). The bile group consisted of benign (n = 10), BTCs (n = 14), and PDAC (n = 30). Each row represents a bacterial genus, and each column represents an individual sample. Colours indicate the scaled abundance, with blue signifying low, yellow signifying intermediate, and red signifying high relative abundance. Sample classifications are annotated at the top: benign (green), BTC (red), and PDAC (blue). Raw read counts were log-transformed ($\log_2 + 1$) and z-scaled for the heat map.

taxa with statistically significant enrichment in PDAC bile compared to benign bile, including *Lactobacillus* (FDR = 0.007), *Streptococcus* (FDR = 0.021), *Enterococcus* (FDR = 0.047), *Limosilactobacillus* (FDR = 0.050), and *Veillonella* (FDR = 0.047) (Table 3). These findings suggest that specific bacterial genera found in PDAC bile not only differ from those in benign bile, but are also observed in the PDAC tumor-associated microbiome. A combined microbial signature comprising *Lactobacillus*, *Streptococcus*, *Veillonella*, *Enterococcus*, *Enterobacter*, *Klebsiella*, and *Plesiomonas*, all uniquely enriched in PDAC tumor and bile, demonstrated strong discriminatory power in distinguishing PDAC from benign and BTC cases. ROC curve analysis showed high predictive performance of this microbial signature, with an AUC of 0.9233 for tissue and 0.8101 for bile (P = 0.0001, Fig. 6C–D), highlighting the potential of microbial profiling as a diagnostic tool in PDAC.

Antibiotic use within 1 month or preoperative biliary stenting are not confounding factors in the microbial analysis of this study cohort

We included patients who had antibiotics within 1 month and/or pre-operative biliary stenting. This provided a unique opportunity to investigate if these factors influence the microbiome in our study cohort. Beta diversity analysis using Bray-Curtis dissimilarity and visualized through PCoA plots showed no distinct clustering of microbial composition between stented and non-stented PDAC bile samples (Supplemental Digital Content Figure 2E, available at: http://links.lww.com/JS9/H276). PERMANOVA analysis revealed no significant differences in microbial beta diversity based on biliary stenting status (P = 0.314) or antibiotic use within 1 month (P = 0.903), suggesting that neither factor acted as a confounder in the microbial composition analysis (Supplemental Digital Content Table 4, available at: http://links.lww.com/JS9/H280).

The abundance of specific biliary and intratumoral genera correlates with oncological outcomes (OS & DFS) in PDAC

All significantly enriched genera found in PDAC bile and tumor were correlated with oncological outcomes. Four taxa, *Plesiomonas*, *Enterobacter*, *Streptococcus*, and *Enterococcus*, demonstrated significant associations with clinical outcomes. Our data illustrates that a high abundance of *Plesiomonas* found within PDAC tumoral tissues was associated with a longer DFS (17 vs 11 months; P = 0.0388; Fig. 7A) and

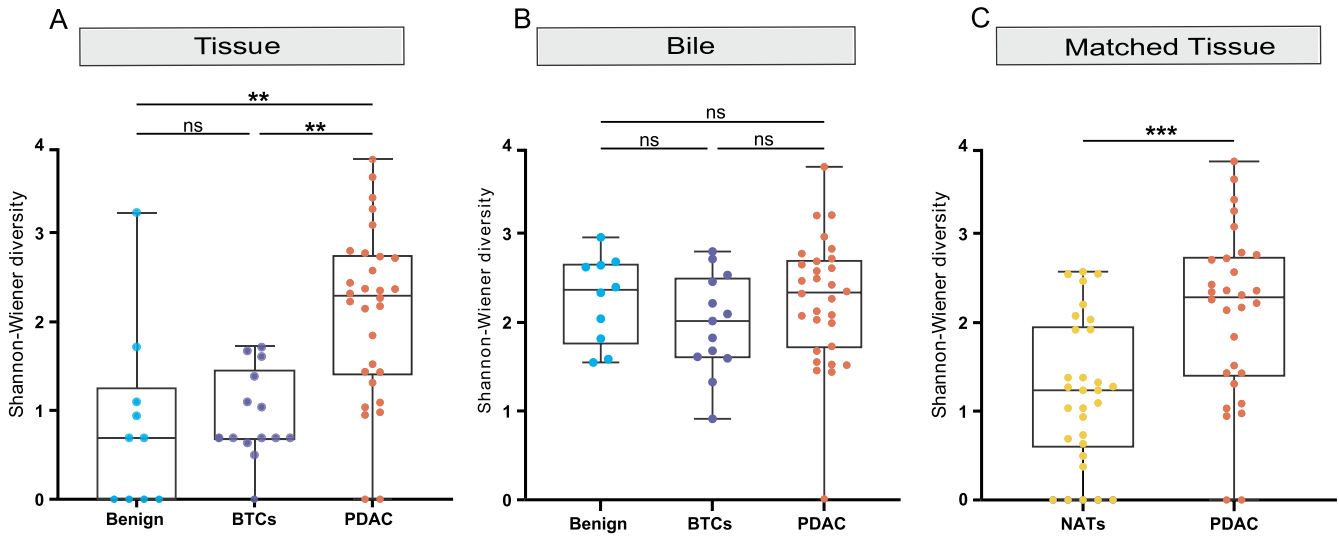


Figure 5. Microbial alpha diversity measured using Shannon diversity index. Comparison of alpha diversity among (A) tissue and (B) bile samples that consisted of benign (n = 10), BTCs (n = 14), and PDAC (n = 30). (C) Paired comparison of alpha diversity between matched PDAC tumor tissue (n = 30) and neighboring adjacent tissue (NAT). Each point represents an individual patient sample. Statistical comparisons were performed using the Kruskal–Wallis rank test. Data presented as boxes from the 25th to 75th percentiles, bars at the median and whiskers from minimum to maximum. ns, not significant; *P < 0.05; **P < 0.005; ***P < 0.0005.

OS (34 vs 18 months; P = 0.0020; Fig. 7B). Similarly, a high abundance of *Enterobacter* (31 vs 21 months; P = 0.0289; Fig. 7C) and *Streptococcus* (18 vs 34 months; P = 0.0304; Fig. 7D) found within PDAC tumoral tissues was associated with a significantly longer OS. In contrast, high levels of *Enterococcus* in PDAC bile were associated with significantly shorter OS (18 vs 34 months; P = 0.0060; Fig. 7E). These findings were further explored with multivariate analysis. Clinicopathological data used for the PDAC cohort employed in the multivariate analysis are available in Supplemental Digital Content Table 5, available at: http://links.lww.com/JS9/H281 and Supplemental Digital Content Table 6, available at: http://links.lww.com/JS9/H282). The high abundance of intratumoral *Streptococcus* was independently associated with lower overall mortality risk (multivariate HR: 0.35; 95% CI: 0.13–0.95, P = 0.05). High abundance of intratumoral *Enterobacter* was also independently associated with lower overall mortality risk (multivariate HR: 0.126; 95% CI: 0.09–0.80, P = 0.02). Similar findings were observed for high intratumoral *Plesiomonas* (multivariate HR: 0.27; 95% CI: 0.10–0.73, P = 0.01). High abundance of bile *Enterococcus* was independently associated with increased overall mortality risk (multivariate HR: 1.64; 95% CI: 0.60–4.52, P = 0.34).

Intra-operative biliary cultures demonstrating bacterobilia correlate with post-operative outcomes in PDAC patients undergoing a pancreatoduodenectomy

Intra-operative biliary MC&S swabs provide an accessible clinical tool for investigating microbes associated with post-operative outcomes and guiding antimicrobial therapy. We postulated that bacterobilia identified intra-operatively may be associated with post-operative outcomes, in keeping with previous work[30,31]. Microbiological cultures were performed on intraoperative bile duct swab samples from 30 PDAC patients. Of these, 21 (70%) yielded positive bacterial growth, while 9 (30%) showed no growth after 48 hours of incubation. *Enterococcus faecium* (30%), *Escherichia coli* (20%), *Enterobacter cloacae* (20%), and *Klebsiella pneumoniae* (17%) were frequently cultured from the bile duct swabs. Patients with positive bile cultures were associated with increased postoperative morbidity. SSI occurred in 57% of bile-positive patients compared with 11% of bile-negative patients [relative risk (RR): 5.7, 95% CI: 1.30–32.74, P = 0.020]. Similarly, delayed gastric emptying was more frequent in bile-positive patients (52% versus 11%; RR: 5.5, 95% CI: 1.24–31.62, P = 0.223). Higher rates of clinically relevant postoperative pancreatic fistula (ISGPS grade B/C) were also observed in the bile-positive group (81% versus 44%; RR: 2.0, 95% CI: 1.06–4.85, P = 0.045). These findings suggest that the presence of biliary microbial colonization at the time of resection is associated with an increased postoperative complication burden in PDAC (Table 4).

Discussion

In this study, we identified that patients with PDAC have a distinct biliary microbiome, compared to those with benign disease. Consistent with previous studies[10,11,18,32–34], we have identified significant differences in the relative abundance of bacterial genera between PDAC and benign cohorts. Analyses of relative abundance revealed an active microbial environment within PDAC bile and tumor tissues, in contrast to the comparatively lower microbial profiles of benign and BTC samples. Although low-biomass microbiome studies are inherently sensitive to contamination, the absence of detectable signal in negative controls and the robustness of results to read-depth filtering support the validity of the observed microbial profiles. DAA

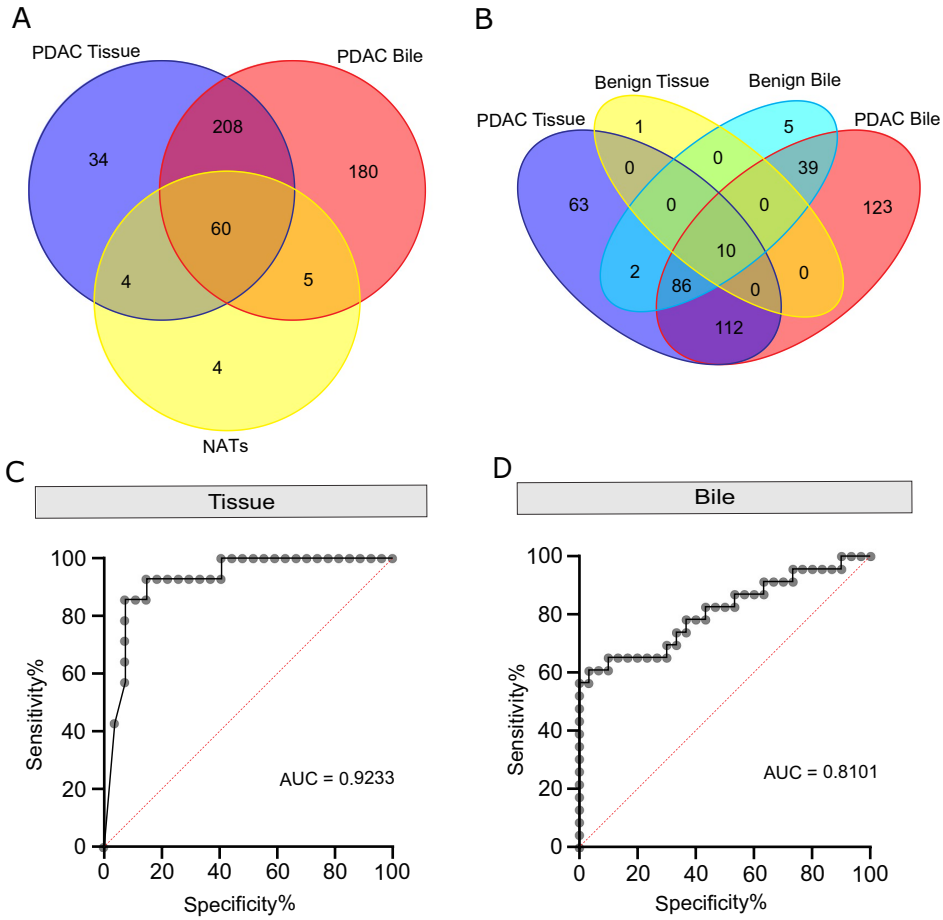


Figure 6. Shared and unique bacterial genera in from different tissue samples. (A) Venn diagram showing the bacterial genera shared among matched PDAC tumor tissue, PDAC bile, and neighboring adjacent tissue (NAT) (n = 30). (B) Venn diagram comparing genera found in matched PDAC tumor and bile samples (n = 30) with those in benign tissue (n = 5) and bile (n = 10). Presence–absence data were filtered to include genera with a minimum of 3 reads. Each circle represents a sample group, and overlapping regions indicate shared genera. (C and D) Receiver operating characteristic (ROC) curves evaluating the performance of a combined PDAC microbial signature for distinguishing PDAC from other disease types (BTCs and benign) in (C) tissue and (D) bile samples. The microbial signature included *Lactobacillus, Streptococcus, Veillonella, Enterococcus, Enterobacter, Klebsiella*, and *Plesiomonas*. The area under the curve (AUC) values reflect model accuracy, which is assessed using independent test datasets. (C) AUC = 0.9233; $P < 0.0001$; 27 PDAC vs 14 other. (D) AUC = 0.8101; $P < 0.0001$; 30 PDAC vs 23 other.

identified significant enrichment of *Lactobacillus, Streptococcus, Veillonella, Enterococcus, and Limosilactobacillus* in PDAC bile compared to benign bile. Although there were some overlapping genus-level microbes across cohorts, certain taxa were unique to the PDAC group, suggesting these microbes may serve as potential diagnostic biomarkers. Our previous work using hypervariable V3–V4 region of 16S rRNA sequencing of pre-operative endoscopic retrograde cholangiopancreatography (ERCP) bile samples identified *Streptococcus* (FDR = 0.033) as significantly enriched in PDAC[10], a finding replicated in this study. This consistency supports the potential utility of bile microbial profiling for both diagnostic settings such as during ERCP, and in therapeutic decision-making following resection.

The alpha diversity matrices of the bile fluid between the PDAC, benign, and BTCs groups showed no significant differences in microbial richness or overall community structure. While some studies align with this finding[33,35], others report contrasting results[10,34]. PDAC tumor tissue has previously been shown to harbor significant differences in the microbiome in stented vs non-stented patients, for example, an increased relative abundance of the *Enterobacteriaceae* family in those with biliary stents[36]. Although *Enterobacter* was significantly abundant in our PDAC bile samples, and the majority of these patients had undergone preoperative stenting, our subgroup analysis revealed no significant genus-level differences between stented and non-stented PDAC patients. Importantly, preoperative CRP and bilirubin levels did not differ significantly, suggesting that cholangitis did not confound the bile microbiome composition across the study cohorts. Moreover, both PERMANOVA and PCA analyses demonstrated no significant impact of biliary stenting or prior antibiotic usage on the bile microbial composition, further confirming that these variables did not confound our primary findings. Our approach, employing full-length 16S rRNA gene sequencing using Nanopore technology, provides deeper taxonomic resolution than commonly used short-read platforms[32,37,38]. Standardizing microbiome research protocols in PDAC remains essential to facilitate reproducibility and cross-study comparisons. While universal 16S reference databases remain valuable for broad community profiling, genome-resolved human-associated

Table 3

Differential abundance analysis of the significant bacterial genera in study comparisons.

Comparison	Genus	Unadjusted *P*-value	Adjusted *P*-value (false discovery rate)
PDAC tumor tissue ($n = 30$) vs matched NAT ($n = 30$)	*Enterobacter*	3.04E−05	0.006
	Streptococcus	3.98E−05	0.006
	Klebsiella	6.17E−05	0.006
	Veillonella	3.66E−04	0.028
	Plesiomonas	6.74E−04	0.041
PDAC tumor ($n = 30$) vs benign tumor tissue ($n = 10$)	*Klebsiella*	0.00971273	0.779
	Streptococcus	0.01406327	0.779
	Brachyspira	0.02005734	0.779
	Plesiomonas	0.02340242	0.779
	Enterococcus_B	0.03743783	0.779
	Enterobacter	0.0385425	0.779
PDAC bile ($n = 30$) vs benign bile ($n = 10$)	*Lactobacillus*	1.64E−05	0.007
	Streptococcus	9.43E−05	0.021
	Veillonella	0.00040906	0.047
	Enterococcus_B	0.00036442	0.047
	Limosilactobacillus	0.00055023	0.050

The *P*-values were calculated using the Wilcoxon rank–sum test and corrected with FDR using Benjamini–Hochberg. The significance level is $P < 0.05$ and the values are highlighted in red. Statistical programme used was GraphPad Prism 9.4.1 version.

references can provide improved taxonomic resolution when analyzing full-length 16S data from clinically relevant, host-derived niches.

In this study, we also demonstrated that the PDAC tumoral tissue harbors distinct microbiome both in diversity and composition, when compared to matched neighboring adjacent tissue (NAT). Tumor tissues contained a higher number of genera, whereas NAT samples had notably fewer microbial taxa. Importantly, nearly the majority of genera identified in NAT were also found in tumor tissues and bile, suggesting a baseline microbiome that may undergo a site-specific colonization during the development and progression of PDAC. This finding is supported by previously published data in mouse models[8,9] showing that intrapancreatic bacteria and fungi expand during the development of PDAC. Using GFP-tagged microbes administered via oral gavage, these studies have shown that microbes from the gut can migrate and colonize the tumor-bearing pancreas in significantly greater numbers than controls. Furthermore, we previously carried out a systematic review investigating the microbiome of different bodily compartments in PDAC and found that very few studies included a normal tissue control[11]. The relative sterility of NAT in our study compared to PDAC tissues supports the conclusion that microbial signatures are not attributable to intraoperative nor lab-based contamination. We observed both shared and unique genera between PDAC tumor tissue and bile. This adds support

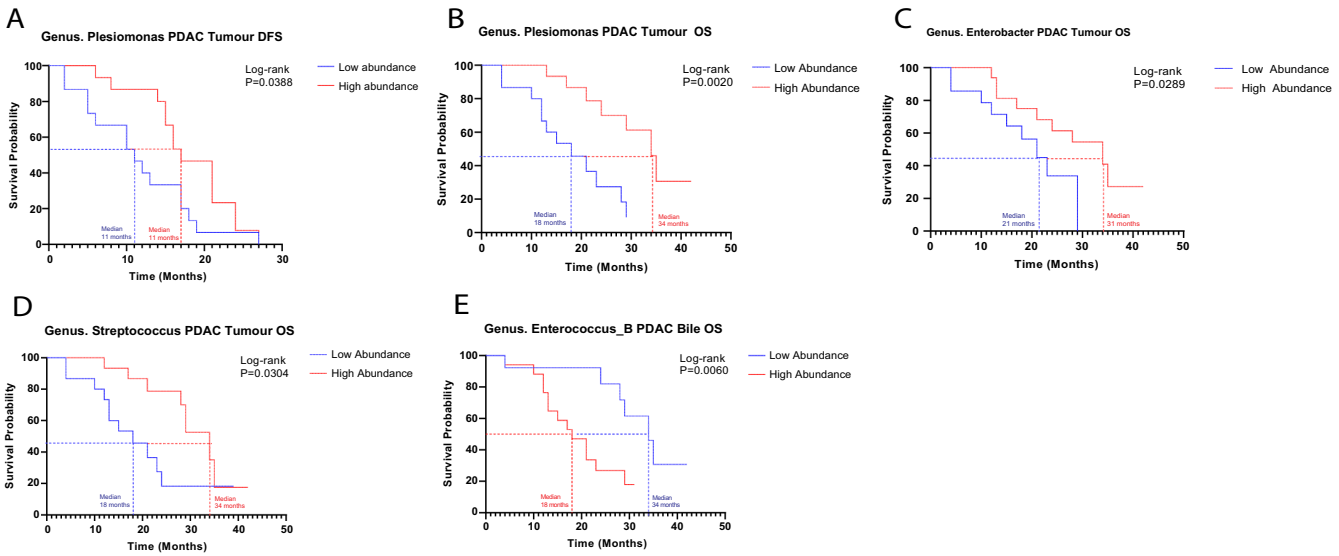


Figure 7. Kaplan–Meier curves stratified by high and low normalized relative abundance counts of significant bacteria genera identified with 16s RNA gene sequencing. (A) Disease-free survival stratified by *Plesiomonas*; overall survival stratified by (B) *Plesiomonas*, (C) *Enterobacter*, and (D) *Streptococcus* in PDAC tumors; and (E) *Enterococcus* in PDAC Bile. Each graph compares patients with a low abundance of a specific bacteria (blue line) to patients with a high abundance of that bacteria (red line). The *P*-values generated from the log–rank test between the two groups are shown on each plot.

Table 4

Clinical outcomes and association between bile culture positivity and postoperative complications in PDAC (*n* = 30).

Outcome	Total PDAC cohort (*n* = 30)	Positive bile culture (*n* = 21)	Negative bile culture (*n* = 9)	Effect size (relative risk ratio, 95% confidence interval)	*P*-value
Overall survival, months (median, range)	21.0 (4–42)	20.0 (4–42)	23.0 (15–31)	–	–
Disease-free survival, months (median, range)	15.5 (2–27)	14.0 (2–27)	16.0 (10–21)	–	–
Surgical site infection, n (%)	13 (43%)	12 (57%)	1 (11%)	RR: 5.7, 95% CI: 1.299–32.74	0.020
Delayed gastric emptying, n (%)	12 (40%)	11 (52%)	1 (11%)	RR: 5.5, 95% CI: 1.236–31.62	0.223
Postoperative pancreatic fistula (ISGPS B/C), *n* (%)	20 (67%)	17 (81%)	4 (44%)	RR: 2, 95% CI: 1.06–4.85	0.045

Overall survival and disease-free survival are reported in months from the date of surgery. Survival times were summarized using medians and ranges. Bile culture positivity refers to the presence of bacterial growth on Intra-operative common bile duct cultures. Risk ratios (RRs) represent the ratio of complication risk among bile culture–positive versus negative patients. Confidence intervals were calculated using the Katz log method with continuity correction. p-values were obtained from Fisher's exact test. . The significance level is $P < 0.05$ and the values are highlighted in red. Statistical programme used was GraphPad Prism 9.4.1 version.

to the hypothesis that microbes from the duodenum or upper gastrointestinal tract may translocate to the biliary system and colonize the pancreas during tumor development[9,12]. DAA revealed that *Enterobacter, Streptococcus, Klebsiella, Veillonella*, and *Plesiomonas* were more abundant in tumor tissue compared to NAT, although whether these taxa are at all involved in PDAC pathogenesis remains an open question.

We found significant differences in microbial diversity and composition between PDAC tumoral tissue and bile samples from benign and BTC cases. Enriched genera such as *Streptococcus, Klebsiella, Enterobacter*, and *Veillonella* in PDAC confirm previous studies[33,36,39–43], many of which also report their presence in PDAC bile. This overlap suggests that bile may serve as a surrogate for assessing the intratumoral microbiome. Our data show that a combined microbial signature comprising *Lactobacillus, Streptococcus, Veillonella, Enterococcus, Enterobacter, Klebsiella*, and *Plesiomonas* was highly predictive of PDAC across both tissue and bile samples, offering strong classification performance. This shared signature may serve as a useful biomarker for early diagnosis and enable the design of targeted microbial modulation strategies that do not impair anticancer treatment efficacy.

We also discovered that high levels of *Enterobacter, Streptococcus*, and *Plesiomonas* in PDAC tumor tissues were associated with significantly improved OS and DFS, independent of other prognostic factors (Fig. 7A–D). In support of our findings, Henderson *et al* discovered that Group A *Streptococcus* secretes Scl1, a collagen-like protein with direct anti-tumor effects on PDAC in mice[44]. This may help explain the survival benefit associated with high *Streptococcus* abundance in our cohort. *Plesiomonas* is a gram-negative anaerobic bacillus that was more abundant in PDAC tumoral tissue compared to NAT. Its association with improved DFS and OS is a novel finding in 16S rRNA gene sequencing studies of PDAC and warrants further exploration. Conversely, high levels of *Enterococcus* in PDAC bile were associated with a significant reduction in OS (Fig. 7E), consistent with Stein-Thoeringer *et al*, who demonstrated that *Enterococcus* in bile increased the risk of SSI and decreased OS following PDAC resection[45].

Bile cultures obtained at surgery revealed frequent colonization with *Enterococcus faecium, Enterobacter cloacae*, and *Klebsiella pneumonia* (Supplemental Digital Content Table 7, available at: http://links.lww.com/JS9/H283), consistent with our 16S rRNA sequencing data and validating our sequencing-based approach. Positive bile cultures were associated with increased risk of deep/organ-space SSIs, DGE, and POPF (Table 4). Fong *et al* reported similar findings in a multicenter study of 1623 pancreatic surgery patients, identifying *Streptococcus pneumoniae, Enterococcus faecalis, Staphylococcus aureus*, and *Escherichia* coli in both bile and SSI cultures[46]. Our results align with these studies[30,46–49] reinforcing the recommendation that patients undergoing ERCP, percutaneous transhepatic cholangiography (PTC), or surgery should have bile sampled for culture and sensitivity to guide targeted antimicrobial therapy and reduce postoperative morbidity.

Limitations and considerations

This study has several limitations. It was single-center, non-randomized, and involved a relatively small sample size. Low microbial biomass prevented us from performing shotgun metagenomic sequencing, limiting our ability to assess functional microbial genes and metabolic pathways. Additionally, we included patients who had received antibiotics and/or undergone biliary stenting, which can influence the microbiome. However, this choice reflects the real-world clinical context, where PDAC patients commonly present with obstructive jaundice requiring urgent intervention. Notably, the benign cohort did not receive antibiotics yet exhibited lower microbial diversity and abundance than PDAC patients. Additionally, both PERMANOVA and PCA tests did not detect significant confounding effects of either stenting or prior antibiotic exposure on microbial diversity or composition, reinforcing the robustness of our findings. A key challenge is the lack of a universally accepted "healthy" pancreatic or bile control. In this study, benign samples and NATs served as comparators. We chose to individually compare the PDAC microbiome with each cohort to minimize any overgeneralization or bias.

It should be noted that several well-established prognostic factors in PDAC (lymphovascular invasion, perineural invasion, nodal status) do not reach significance in the univariate Cox analysis. This might suggest limited statistical power due to the small sample size and number of events. As a result, the multivariable model may be under-adjusted for key clinical confounders, making the associations between specific bacterial genera and survival vulnerable to overinterpretation. That being said, we have included variables that are prognostically relevant (e.g., age and received adjuvant chemotherapy) and did reach significance in univariate Cox analysis.

The models used lack a temporal/external validation, which limits their clinical utility. A temporal validation approach is not possible in this cohort, given the fact that resectable PDAC patients who have an upfront resection rarely have a tissue diagnosis before surgery and thus there is limited opportunity for serial assessment of tissue samples. Our findings are considered exploratory and validation of these studies' findings in an external cohort is most certainly required.

Furthermore, although bile sampling was performed intraoperatively, limiting its utility as an early diagnostic tool, our findings support its potential as a dynamic biomarker throughout disease progression. This study's strength lies in its comprehensive approach: matched PDAC tumor, NAT, and bile samples were analyzed using long-read Nanopore 16S rRNA sequencing. Rigorous decontamination protocols were followed, including RIDE checklist adherence and histopathological verification to exclude samples with necrosis or low tumor content.

Conclusion

In conclusion, we identified significantly enriched microbial genera in both bile and matched tumor tissues of PDAC patients, as compared to NATs and benign disease. A shared microbial signature was highly predictive of PDAC in both tissue and bile, suggesting that bile microbial profiling could serve as a surrogate for tumor microbiome assessment and aid in disease discrimination. Future work should focus on validating these findings in larger, multi-center cohorts and integrating metagenomic sequencing where possible to identify strain-specific contributions and microbial functions. Such insights could be applied at the time of ERCP or PTC in obstructive jaundice cases, allowing microbial signatures to inform treatment pathways (e.g., surgery vs chemotherapy). This novel theranostic approach has the potential to personalize PDAC treatment and support more informed clinical decision-making.

Ethical approval

All study-related procedures and protocols were in accordance with the ethical guidelines of the Declaration of Helsinki. Ethical approval was obtained as part of the CIRcular and Non-coding RNAs as Clinically USeful Biomarkers in Pancreaticobiliary Cancers (CIRCUS) study. Patient samples were obtained with Research Ethics Committee (REC) approval (IRAS Project ID: 277406).

Consent

Written informed consent was obtained from the patient for publication of this case report and accompanying images. A copy of the written consent is available for review from the Editor-in-Chief of this journal on request.

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Author contributions

I.B., N.D.K., R.K., R.P.L., T.D.P., E.P., A.R., T.R.W., and A.E.F.: sample provision; N.M. and A.E.F.: clinical data collection; N.M., T.C., P.S., S.S., and V.A.P.: bioinformatics and statistical analysis; N.M., T.C., P.S., V.A.P., N.E.A., and A.E.F.: original draft preparation; N.M., E.V., N.E.A., and A.E.F.: funding acquisition. All authors: concept, design, statistical analysis, interpretation, and critical revision and editing of the manuscript. All authors read and approved the final manuscript.

Conflicts of interest disclosure

The authors have no disclosures for this work.

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Guarantor

All authors accept full responsibility for the work and/or the conduct of the study. N.E.A., V.P., and A.E.F. controlled the decision to publish.

Provenance and peer review

Not applicable.

Data availability statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. N.M. and A.E.F. had access to the data. The sequencing data have been deposited and are available under NCBI BioProject PRJNA1284364, and the metadata can be found in Supplemental Digital Content Table 8, available at: http://links.lww.com/JS9/H458. A read-only reviewer-link can be accessed via: https://dataview.ncbi.nlm.nih.gov/object/PRJNA1284364?reviewer=tuhbkuonilkadd4utn8okvt91. The codes used for statistical analyses and plots were deposited on GitHub and are available at https://github.com/PuspenduSardar/PDAC-Microbiota.

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Not applicable.

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