RESEARCH ARTICLE

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Microbial Composition Study in Cholangiocarcinoma: Stage-Specific Diversity Analysis in Bile and Tumor Microbiome

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Abstract

Objective: Cholangiocarcinoma (CCA) is an aggressive malignancy of the biliary tract with poor prognosis and limited early diagnostic tools. Microbial dysbiosis has been implicated in carcinogenesis, yet microbial signatures across disease stages and anatomical sites in CCA remain poorly defined. This study aimed to characterize the microbial diversity in bile and tumor tissue of CCA patients and evaluate association with disease stage. **Methods:** Microbiome profiles were analyzed from 36 subjects using 16Sr RNA gene sequencing data from the Sequence Read Archive (SRA). Alpha diversity (Chao1 index) and beta diversity (Bray-Curtis dissimilarity) were assessed across bile and intratumoral samples. Linear discriminant analysis effect size (LEfSe) was employed to identify stage-specific microbial taxa. **Result:** Alpha diversity did not differ significantly between disease stages (ANOVA, $F = 0.385$, $p = 0.816$), suggesting stable microbial richness throughout progression. However, beta diversity analysis (PERMANOVA, $R^2 = 0.279$, $p = 0.013$) revealed distinct clustering by sample type and tumor stage. LEfSe identified enrichment of Fusobacterium, Escherichia-Shigella, and Enterococcus in advanced-stage tumor samples, while Lactobacillus and Streptococcus were more abundant in early-stage bile samples. **Conclusion:** This study demonstrates distinct microbial composition patterns across disease stages and anatomical sites in CCA. Enrichment of specific pathogenic taxa in advanced stages supports a role for microbiome alterations in CCA pathogenesis. Microbial markers may serve as potential tools for staging and prognosis, warranting further functional and prospective validation studies.

Keywords: Cholangiocarcinoma- microbiome- alpha- beta diversity

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Introduction

Cholangiocarcinoma (CCA) is a highly fatal adenocarcinoma of the biliary epithelium and the second most common primary hepatic malignancy after hepatocellular carcinoma (HCC) [1, 2]. It is an aggressive adenocarcinoma that contributes significantly to the high mortality rate within the hepatobiliary system. Based on anatomical location, CCA is classified into intrahepatic, perihilar, and distal types [1]. The global prevalence of CCA rose from 0.67 per 100.000 in 2007 to 1.40 in 2016, with incidence significantly higher in Asia than Europe [2-4]. Regional differences likely reflect varying risk factors, including chronic liver injury from primary sclerosing cholangitis (PSC), viral hepatitis, cirrhosis, alcohol use, or parasitic infections from Opisthorchis viverrini or Clonorchis sinensis [5-7]. In lower income Asian regions, hepatitis B prevalence exceeds 10%, often limited treatment access [8].

The etiology and pathogenesis of CCA remain incompletely understood. However, accumulating evidence suggests that CCA develops through the progressive accumulation of genetic and epigenetic alterations, which may be influenced by the human microbiome [9, 10]. The human microbiome refers to the collective genetic material of microorganisms residing in the human body, including those in the intestinal tract, bile ducts, oral cavity, vagina, nasal cavity, and skin [11]. The gut microbiome plays a critical role in regulating digestion, nutrient absorption, immune responses, and host metabolism [12]. Similarly, the bile microbiome is essential for maintaining hepatobiliary health disturbances or dysbiosis within this microbiome have been associated with an increased risk of various hepatobiliary, pancreatic, and intestinal diseases [13].

Several studies have investigated the role of gut microbiota in the development and progression of CCA. Some findings suggest the presence of inhomogeneous

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microbial alterations in patients with CCA compared to healthy individuals [5, 14, 15]. However, these results remain inconclusive due to methodological limitations, such as insufficient information regarding prior antibiotic use and inconsistencies in study design and analysis [5]. In contrast, a study by Zhang et al. demonstrated significant differences in gut microbiota composition between patients with advanced-stage CCA (stages III and IV) and those with early-stage disease (stages I and II). Notably, Candida albicans was identified as potentially associated with advanced disease [16]. Building on these findings, the aim of this study to further investigate the role of gut microbiota as a diagnostic and prognostic biomarker in CCA, with the aim of improving therapeutic response assessment and contributing to personalized patient management.

Materials and Methods

Study Population and Sample Collection

The samples in this study were collected from the Sequence Read Archive (SRA), "A public database by the National Center for Biotechnology Information (NCBI)". A total of 36 subjects were used, divided into five groups based on their staging, which includes 26 bile samples from patients with CCA, 10 of intratumor tissue samples from patients with CCA. The bile samples were extracted from patients undergoing Endoscopic Retrograde Cholangiopancreatography (ERCP) at Mayo Clinic for known or suspected CCA. The diagnosis of CCA was established either by pathological confirmation from bile duct specimens obtained endoscopically or percutaneously or based on a progressive clinical course with radiologic findings consistent with malignancy. None of the 26 bile samples analyzed were from patients with a history of PSC. All the patients had varying histories of antibiotic exposure within the past week. For intratumoral samples, tissues were collected from 10 patients with intrahepatic cholangiocarcinoma (ICC) who had previously undergone curative surgical resection without subsequent adjuvant therapy. Clinical and demographic information, including age, gender, body mass index (BMI), past medical history, antibiotic exposure (categorized as none, temporary use, or continuous use), and the presence or history of cholangitis, were obtained from the database. According to the TNM staging system, among the bile-derived CCA samples, 14 patients were classified as stage II, 11 patients as stage IIIA, 3 patients as stage IIIB, 4 patients as stage IIIC, and 4 patients as stage IV.

DNA Isolation and Amplicon-Based Sequencing

In the primary analysis, bile and intratumoral samples were collected for genetic amplification using 16S rRNA sequencing. DNA extraction was performed on 26 bile samples and 10 intratumoral CCA samples to identify and characterize the microbial community. The amplicons (DNA fragments) were then sequenced using the Illumina paired-end platform, which provides high accuracy at both ends of the DNA fragments. The Illumina platform generates 250 base-pair (bp) paired-end raw reads, producing two sequences from each DNA fragment. These raw paired-end reads are subsequently filtered to remove low-quality sequences, resulting in high-quality "Clean Tags" for further analysis.

Taxonomic Classification and Profiling

Taxonomic classification and profiling were conducted on high-quality sequencing reads using Kraken 2 and Concatenate. Kraken 2 was employed to assign metagenomic DNA sequences to microbial taxa by aligning them with reference genome databases, while non-target sequences were filtered out. Concatenate further refined taxonomic resolution by enhancing classification depth and providing functional insights into the detected microbial taxa. Taxonomic profiles were generated at both genus and species levels, facilitating downstream comparative analyses. These profiles were subsequently utilized to explore microbial community compositions and their potential associations with intratumoral bile microbiota in colorectal and hepatobiliary diseases.

Analysis of Microbial Community Structure

This study analyzed bile and tumor tissue samples from included patients to investigate microbial diversity at both phylum and genus levels. Within the analysis, the data obtained from paired-end reads were merged utilizing FLASH (V1.2.7) and filtered using Qiime (V1.7.0) to obtain high-quality tags. The UCHIME algorithm was used in the detection of chimeric sequences. OTU abundance was normalized according to a standard sequence number. Then, Kraken 2 and CAT were used to obtain precise and accurate taxonomic profiling and functional annotation in the metagenomic data. Discrepancies in microbial composition at the phylum level among patient groups were established by OTU analysis. Sankey diagram was utilized to visualize species abundance distribution. Eventually, alpha diversity analysis was carried out to determine gut microbiota richness and diversity.

Phylogenetic Tree Construction

A phylogenetic tree was constructed to represent the hierarchical structure and relative proportions of microbial taxa within the samples. Visualization techniques, such as Sankey diagrams, were employed to depict the distribution of microbial communities across different taxonomic levels, from phylum to genus. Pavian, a taxonomic profiling visualization tool, was also utilized to enhance the clarity of classification patterns and relationships within the microbiome data. These tools facilitated the interpretation of microbial diversity and composition within the analyzed samples.

Further statistical analysis was conducted using MaAsLin2 within MicrobiomeAnalyst to identify key microbial taxa and potential biomarkers. MaAsLin2 applies a generalized linear model (GLM) framework to assess associations between microbial communities and various clinical or environmental factors. This approach enabled the identification of specific microbial signatures linked to intratumoral bile microbiota, potentially serving as diagnostic markers for colorectal and hepatobiliary diseases based on their phylogenetic placement within the taxonomy tree.

Results

Participant Characteristics, Bile and Intratumoral Sample Collection, and Clinical Data

This study involved patients diagnosed with CCA who had already undergone staging. All participants were adults aged ≥18 years with a range of 38-85 years of age, with a male and female proportion of (65.3% vs. 34.6%). A total of 36 CCA patients were included, of whom 26 provided bile samples, while 10 had intratumoral samples collected.

Microbial Composition Profiling

Figure 1, illustrates the Sankey phylogenetic diagram depicting microbial composition across four stages of CCA. In bile samples from stage 2, Firmicutes is the most abundant phylum and followed by Proteobacteria. Meanwhile, Streptococcus and Vellonella were the most abundant at genus level (Figure 1a). In stage 3a, Firmicutes and Proteobacteria remained dominant, while Streptococcus and Enterococcus were most prevalent genera (Figure 1b). On the other hand, at stage 3b, Proteobacteria had become the most abundant phylum, followed by Firmicutes. At genus level, Haemophilus and Enterococcus were dominant (Figure 1c). A similar phylum-level composition was observed in stage 3c and 4. However at genus level, Pseudomonas and Enterococcus were more prominent at stage 3c, whereas Veillonella and Haemophilus were highly abundant at stage 4 of CCA bile sample (Figure 1d-e). Interestingly, Actinobacteria emerge as the most abundant phylum of intratumoral sample of CCA following the Proteobacteria. At genus level, Rhodococcus and Ralstonia were the most prominent (Figure 2).

Diversity and Relative Abundance of the Gut Microbiome

Alpha diversity assessment provided information on the richness and evenness of microbiome diversity across five groups, as well as insights into common and unique OTUs among the different groups. This approach enabled us to capture broader changes or differences in microbial composition, offering a comprehensive view

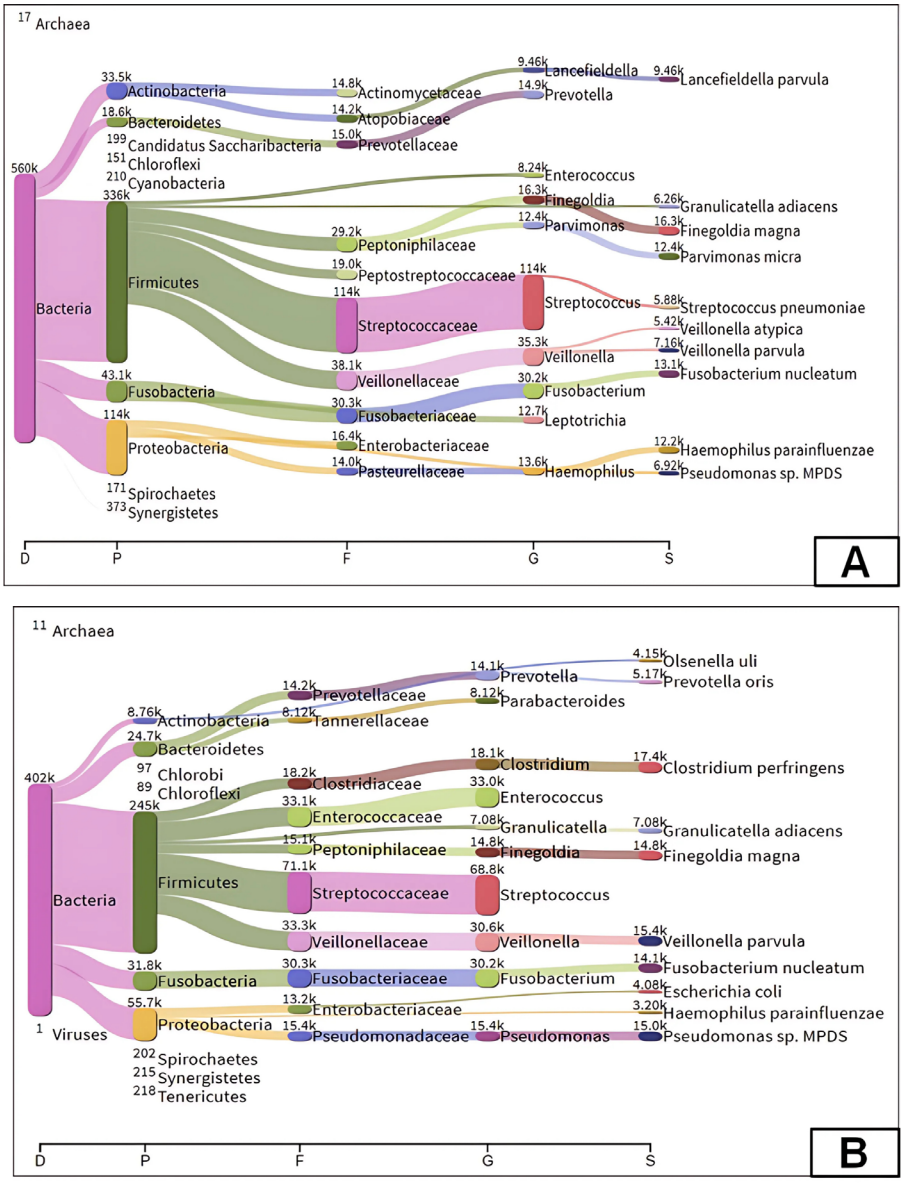
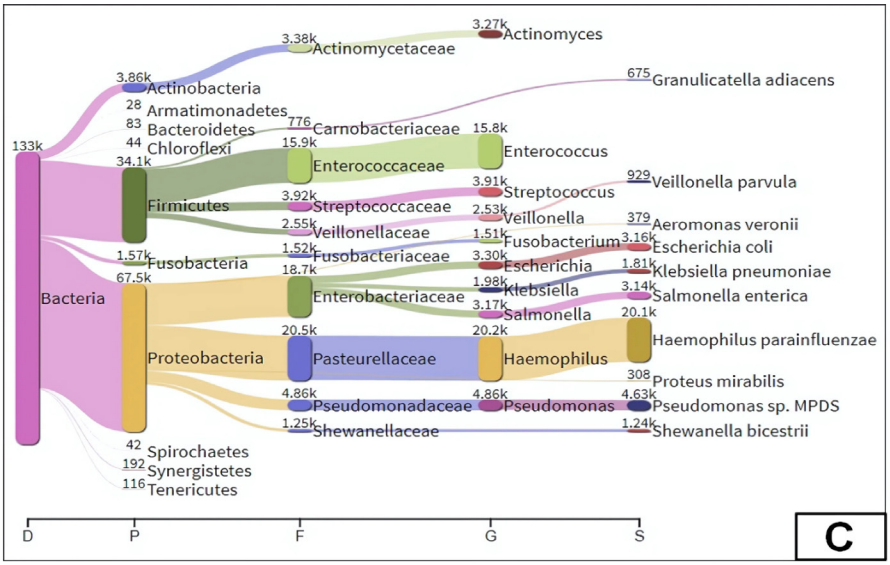
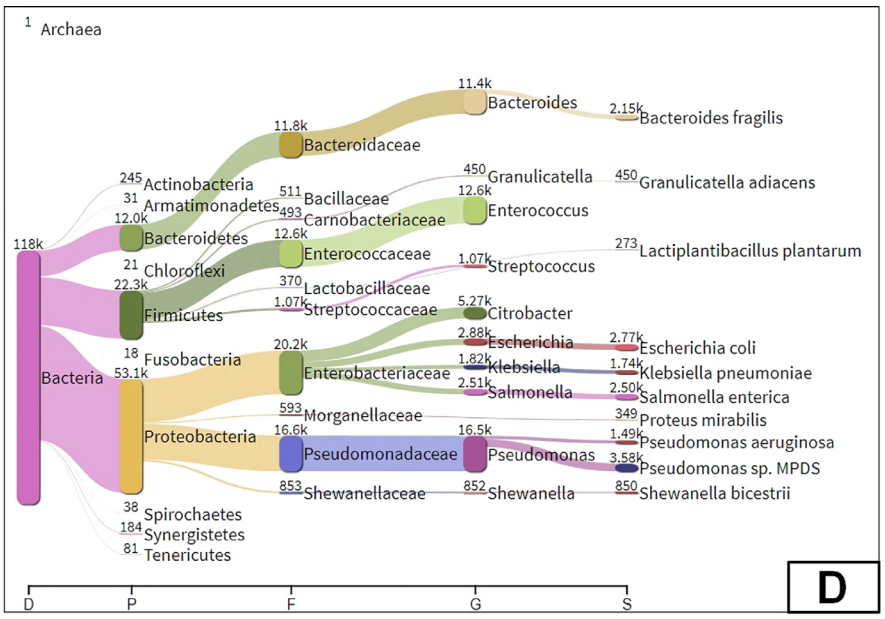


Figure 1. Sankey Diagram of CCA Bile Sample: (A) Stage 2, (B) Stage 3a





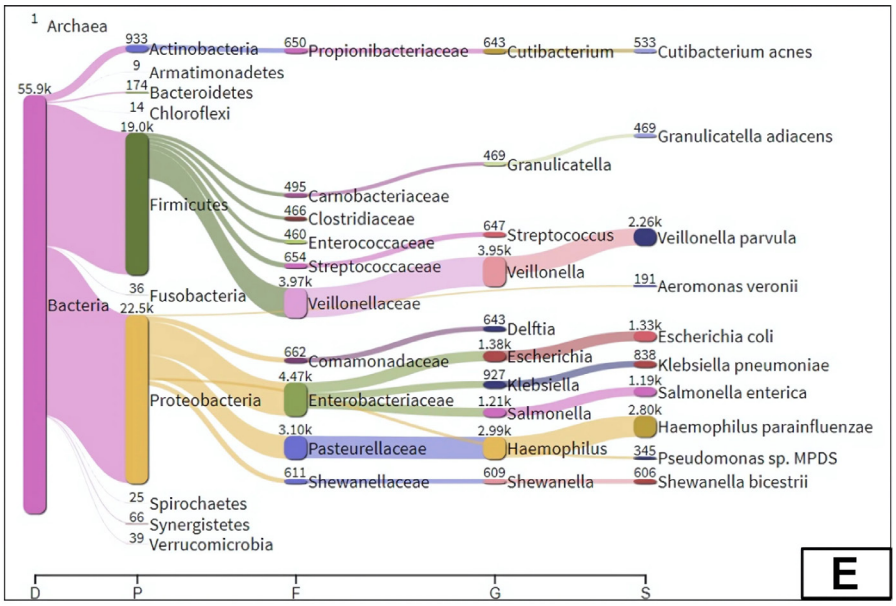


Figure 1. Sankey Diagram of CCA Bile Sample: (C) stage 3b, (D) stage 3c, (E) stage 4

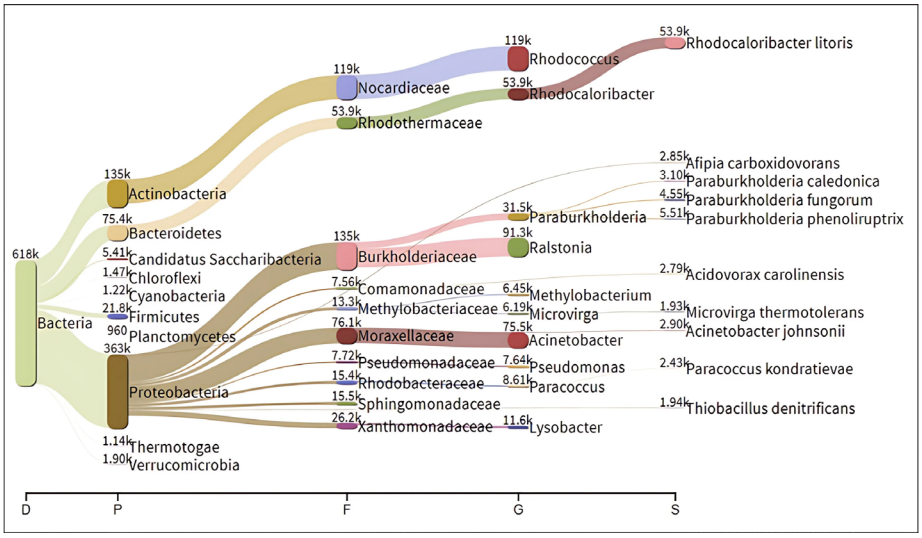


Figure 2. Sankey Diagram of CCA Intratumoral Sample

of the microbiome structure. To observe the OTUs, the Shannon index (Figure 3a) and Chao1 index (Figure 3b) were generated from the alpha diversity assessment via box plot visualization. Alpha diversity assessment can highlight changes or differences in microbiota components based on metrics like Shannon diversity, Chao1 diversity, and observed species in between three groups as shown in Figure 3a-b, there are no differences in microorganism components between the three groups (F=0.385, P=0.816). These findings indicate that there are no differences in the developmental patterns of microorganisms between the five groups that play a role in each stage specific CCA.

Additionally, to assess other variables that might contribute to differences between samples, we conducted a permutational multivariate analysis of variance (PERMANOVA) (Figure 4a). This analysis quantified the strength of associations among the gut microbiome compositions of the five groups, revealing significant differences in microbial composition, ($F = 1.94$, $p = 0.013$).

Alterations in Microbiome Composition

The abundance of Alphaproteobacteria increases significantly in later stages ($p < 0.001$). It shows that Alphaproteobacteria is enriched in the advanced to end stage of CCA and may serve as disease progression biomarker (Figure 5). The same thing also appears on Cutibacterium which exhibit extremely low abundance in early stages and increase sharply in later stages. This could as well be a potential biomarker for advanced-stage CCA, as well as a shift in the tumor microbiome as the disease progresses. Furthermore, Betaproteobacteria abundance is more variable across stages, but its composition is still relevant in disease progression ($p = 0.00177$). Meanwhile, Propionibacteriaceae abundance is low in the early stage, but increases sharply in the end stage. The statistical significance ($p = 0.00129$) indicates that it may be a

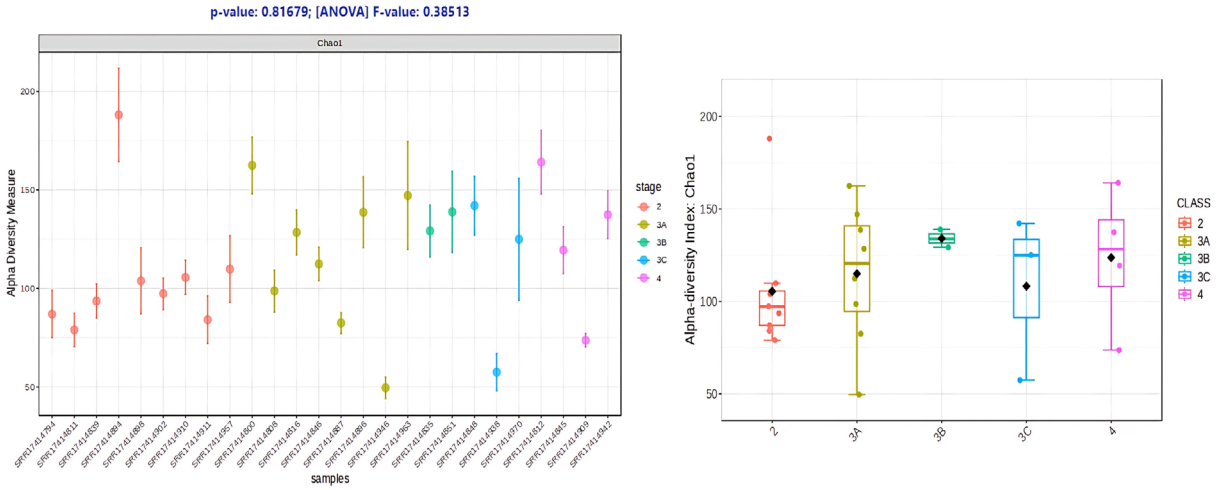


Figure 3. Alpha Diversity from Five Difference Staging. (a) Shannon diversity for individual samples, stratified by disease stage. Each point represents a sample, color-coded by stage. The vertical bars represent standard deviations. ANOVA analysis showed no significant difference in Shannon diversity among the different stages (p=0.81676; F=0.38513). (b) Boxplot showing the distribution of Chao1 Index Alpha diversity for stages (2A,2B, 3C, and 4). The bottom and top edges of each box represent the 25th and 75th percentiles (interquartile range), the horizontal line within the box indicates the median, and the diamond shaped marker is the mean

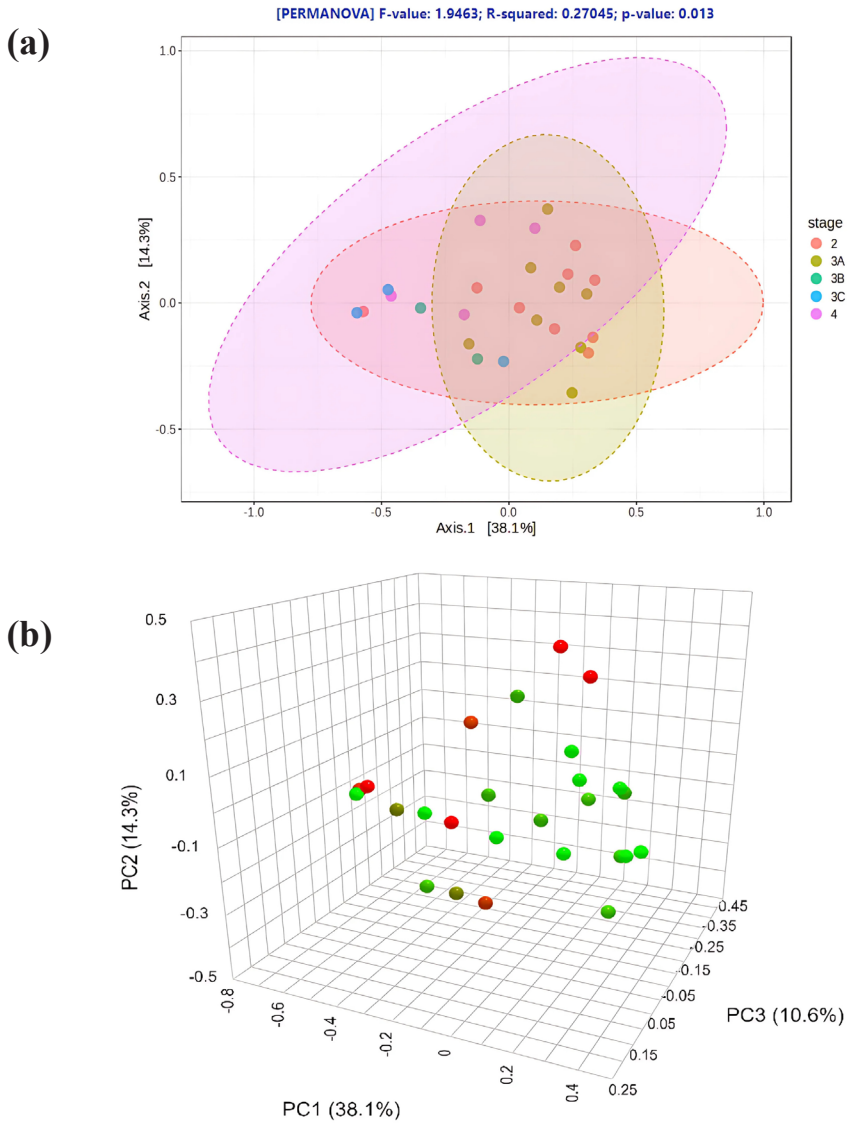


Figure 4. Beta Diversity from Five Different Staging CCA (a) Principal Coordinates Analysis (PCoA plot based on beta diversity analysis across disease stages. Each dot represents an individual sample, colored by stages (2A, 2B, 3A, 3B, 4). Ellipses represent 95% CI. and (b) Interactive 3D plot illustrating the clustering of samples based on microbial community structure.

possible microbial marker. Overall, five microbes have statistically significant values, which indicate that the microbial taxa are differentially abundant between stages of CCA. The five microbes also show higher abundance at advanced stages, which implies its potential as microbial biomarkers for disease progression.

Discussion

In this study, we investigated the microbial diversity across different stages of CCA in bile and tumor tissue samples. Our analysis revealed that Firmicutes and Proteobacteria is the most abundant phylum in bile samples from stage 2 CCA. Meanwhile, Actinobacteria is a dominant phylum of intratumoral samples in CCA. Additionally, we analyzed the specific abundance in different stages of CCA to identify the potential microbial biomarker. Our findings suggested that Alphaproteobacteria and Cutibacterium significantly increase in advanced stages of CCA. Another microbiome, Propionibacteriaceae, also shows a significant increase in the later stages of CCA, which is low in the early stage of CCA. These findings suggest that dysbiosis of the biliary and intratumoral microbiome may contribute to the disease progression of CCA and may serve as a potential microbial marker.

Microbiome plays a crucial role in regulating metabolism which has been implicated in cancer development. The dysregulation of gut microbiome has been associated with alterations in amino acid metabolism and inflammatory pathways implicated in carcinogenesis [17, 18]. Study by Lee at al, reveals that bile samples of CCA patients decrease in microbial diversity than non CCA [19]. Analysis of the CCA microbiome shows that Escherichia coli is the predominant species which can synthesize amino acids. Changes in the abundance can lead to imbalance in amino acid metabolism, which have been implicated in various diseases [19]. The human

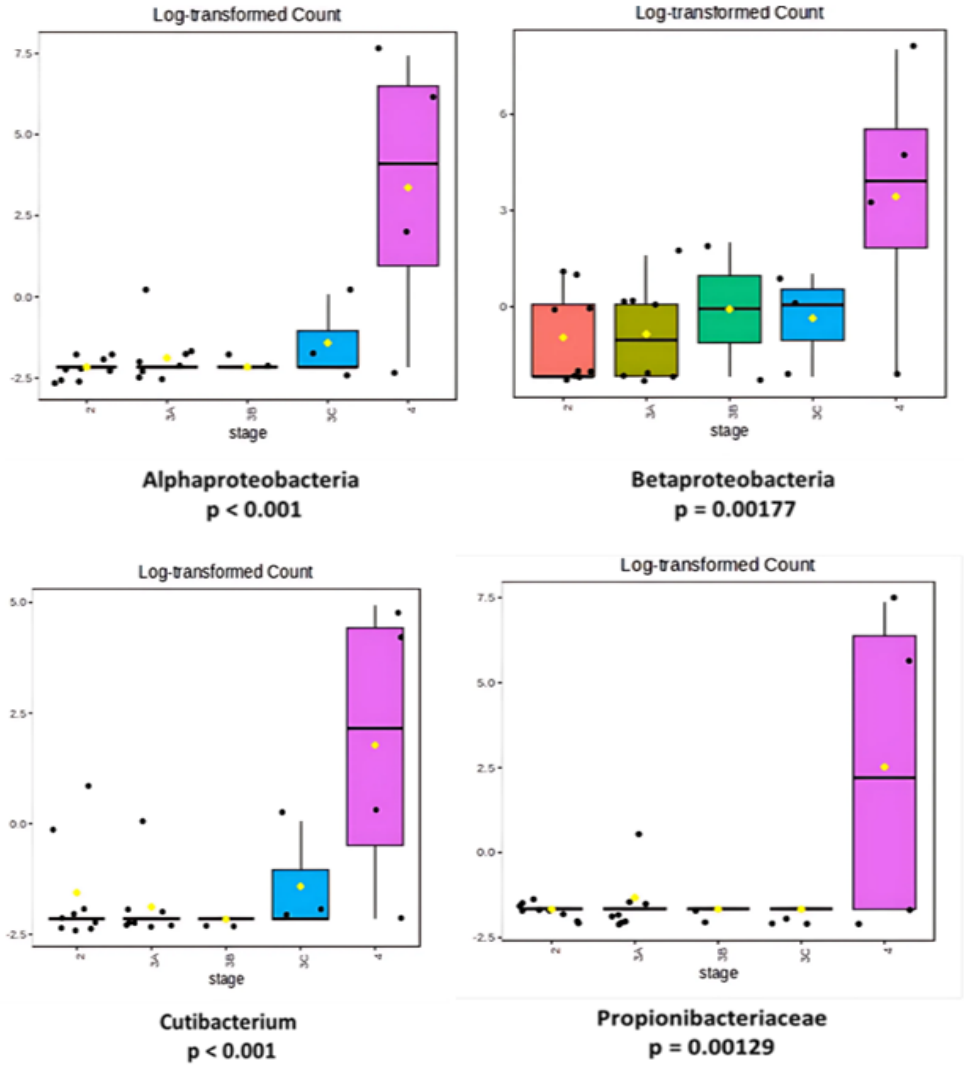


Figure 5. Log-transformed Counts of Stage-Specific Microbial Taxa Differences

microbiome, including gut and bile-associated microbiota, has been increasingly linked to the carcinogenesis of CCA. In the study by Jia et al., 28 patients with intrahepatic CCA showed that the gut microbiome of the patient with iCCA had the highest diversity. Compared with the other group at the genus level, Alloscardovia, Peptostreptococcaceae, Actinomyces, and Lactobacillus were significantly high in patients with ICC [20]. The bile samples analysis in patient with CCA in the other study showed that Pyramidobacter, Klebsiella, Bacteroides, and Enterococcus were the most dominant bacteria in the bile patients with perihilar cholangiocarcinoma (PCC) [21]. In line with our study, the blood microbiome analysis of the CCA patients also identified the most abundant bacteria were, Bacteroidetes, Actinobacteria, and Proteobacteria. But analysis of microbiomes using blood was poorly understood, and more studies are needed to understand the mechanism and changes [10].

The low abundance of microbes in tumor tissues poses a challenge in establishing suitable biological models for systematic validation. A previous study examining microbiota in iCCA tissue found that many bacteria within tumor tissues reside intracellularly, being present in both cancer and immune cells. This finding suggests that there is no clear physical barrier between bacteria and tumor cells, raising of close spatial association rather than direct interaction. Another study showed that adipose tissue is associated with markers of immune cell infiltration, inflammation, and metabolism. In our findings, the microbiome data revealed that the most dominant bacterial species were less abundant in iCCA tissues. Research by Chai et al, reported a weak negative correlation between bacterial presence and CA-199 levels, suggesting a possible tumor-suppressive effect. Certain bacteria have been hypothesized to be associated with tumor-suppressive immune profiles. However, as mechanistic validation was not performed, these findings should be interpreted as indicating an association between intratumoral microbial diversity and tumor behavior [22].

Microorganisms can colonize the biliary ducts. Studies have identified microbiota such as Escherichia coli and Enterobacter genera in healthy individuals [23, 24]. Furthermore, microbes commonly found in the gastrointestinal tract such as Enterococcus spp., Enterobacteriaceae spp., and Candida spp. have also been identified in the biliary tract [25]. Microbial colonization varies across different diseases. For instance, in primary sclerosing cholangitis, colonization by Enterococcus

spp. or Candida spp. has been associated with disease progression [26]. In pancreatic cancer or distal bile duct carcinoma, colonization by Escherichia coli and Pseudomonas spp. has been reported [27]. Notably, Paraburkholderia fungorum has been detected in iCCA and has been hypothesized to be associated with immune-related pathways [22]. Several studies have reported microbial differences across CCA subtypes and progression. Jia et al, [28] found that CCA with venous inflitration had more Oscillospiraceae and fewer Eubacteriaceae (e.g., Allobaculum, Pediococcus, Pseudoramibacter, Peptostreptococcus). Zhang et al. [16] observed gut microbiota shifts between early and advanced stages. Bacteroides, Geobacillus, and Meiothermus were more abundant in eCCA, while ICC showed increased Lactobacillus, Actinomyces, Peptostreptococcaceae, Alloscardovia, and Bifidobacteriacease [28, 29]. Chng et al. [29] reported distinct taxa in O. viverrini associated and non-asscociated CCA, with Dietziaceae, Pseudomonadaceae, and Oxalobacteraceae predominating in the former, and Enterobacteraceae, Lachnospiraceae, Sphingomonadaceae, and Bifidobacteraceae in the latter.

Our study identified five bile bacteria Alphaproteobacteria, Cutibacteria, Betaproteobacteria, Limasilactobacillus, and Propionibacteriaceae that increase with CCA progression. Prior research showed higher Escherichia coli levels in CCA, which can dysregulate amino acids metabolism, notably lowering isoleucine, a metabolite with anti-proliferative effects highlighting its potential as a biomarker for early CCA detection [19]. Study by Saab et al, found domination genera Clostridium, Fusobacteria, Granulicatella, Pseudomonas, Bacillus, Actinomyces, Citrobacter, and Campylobacter in CCA patient [30]. Moreover, study by Jia et al, also found Actinomyces as the significant genera in CCA, along with Lactobacillus, Peptostreptococcacease, and Alloscardovia, compare HCC or liver cirrhosis and healthy sample [20]. Additionally, compared to benign biliary disease, a significant level of Bifidobacterium (p=0.002) and Lactobacillus (p=0.002) also reported [31]. Genera Lactobacillus and Alloscardovia were positively correlated with plasma tools ratio of glycoursodioxycholic acid and tauroursodeoxycholic acid (TUDCA). These bile acid metabolites were profoundly increased in CCA compared to other three diseases. In advance of CCA or vascular invasion, a greater abundance of Ruminococcus was noted, along with increase in IL-4 and six conjugated bile acids. Therefore, Actinomyces, Lactobacillus, and Alloscardovia could be potential diagnostic biomarkers, while Ruminococcus could mark an advanced stage of CCA [20]. Recently Park et al, [31] also discover four potential biomarkers for differentiate CCA with benign biliary disease i.e. Enterobacteriaceae (LDA score 5.34), Pseudomonadota (LDA score 5.31), Escherichia (LDA score 5.12) and Enterobacter (LDA 4.01). However, there is no previous study linked bile microbiota involving Alphaprobacteria, Cutibacteria, Betaproteobacteria, Limasilactobacillus, and Propionibacteriaceae to CCA.

In terms of therapeutic response, specific abundance of bacteria leads to a better outcome. Bacteria that belong to Firmicutes phylum including Facelibacterium prauznitzii, Ruminococcus callidus were found di higher in clinical benefit of anti PD-1, and significantly correlated with better overall survival. In contrast, higher abundance of Bacteroidetes phylum i.e Parabacteroides distasonis were found in non-clinical benefit of anti PD-1 and correlated with poor overall survival in hepatobiliary tract cancer [32]. Additionally, most abundance of Bacillus was found in non-durable clinical benefit of CCA, while Alistipes was found dominantly durable clinical benefit in anti PD-1 therapy. Furthermore, Bacilli, Alistipes, and Lactobacillales were potential candidates as biomarkers for predicting CCA survival with anti PD-1 therapy. High abundance of Bacilli showed a significantly worsened PFS (4.2 vs 6.43 months) and OS (9.07 vs 13.53 months). Patients with Alistipes enriched showed a better survival of both PFS (8.13 vs 5.23 months) and OS (19.3 vs 10.2 months). The finding highlights a potential association between microbiome composition and the efficacy of anti-PD-1 immunotherapy [32]. However, there is no significant difference in microbiota abundance in terms of chemotherapy vs radiotherapy [33]. Relative abundance of gut microbiomes also correlated with treatment adverse effects. The patients with lower richness tend to have severe diarrhea following anti-PD-1 therapy. Specific bacteria also found significantly higher in severe diarrhea patients compared to mild diarrhea, including Negativicutes class, Veillonellaceae family, Dialister genus, and Prevotellamassilia timonensis [32].

Microbiome-based markers show promising potential as diagnostic and prognostic tools for CCA, which remains a challenging hepatic malignancy [34, 35]. Microbial profiling can distinguish healthy from malignant tissue and may support early detection and treatment stratification [36, 37]. One study identified Burkholderia-Caballeronia-Paraburkholderia, Faecalibacteriam, and Ruminococcus early-stage CCA markers with an AUC of 0.973 [38]. A nomogram incorporating Blautia, bilirubin, and fecal metabolites also showed predictive value for surgical outcomes in extrahepatic CCA (C-index=0.718) [39]. Microbiome also holds a potential therapeutic strategy, such as probiotics or targeted antibiotics, has shown potential in cancer therapy. Ongoing clinical trials exploring microbiome-based interventions (e.g., probiotics, fecal microbial transplant (FMT), and modulators) highlight their promise as adjunct strategies in hepatobiliary malignancies, including CCA [40, 41].

This study has several limitations, including a relatively small sample size and the use of secondary SRA data, which may introduce variability. Lack of clinical metadata (e.g., diet, comorbidities, lifestyle) may confound microbiome interpretation. The retrospective nature of the data and lack of prospective sampling may introduce selection bias and limit causal inference. Despite these limitations, consistent analytical methods applied across multiple datasets support the reliability of the observed results. Although microbial comparison was performed across disease stages and basic demographic characteristics were reported descriptively, formal multivariable adjustment for additional host clinical covariate was limited by sample size. Additionally, functional analysis is needed to validate microbiome-

metabolome interactions and assess their biological relevance in CCA progression.

In conclusion, this study reveals stage-specific shifts in the bile microbiome of CCA patients, marked by a transition from Firmicutes to Proteobacteria and changes in key genera. Intratumoral tissue displayed a distinct microbial profile, with Actinobacteria as the dominant phylum. The divergence between bile and tumor microbiota suggests a localized, tumor-specific micro-environment. These findings support the potential of microbial profiling for disease staging and personalized therapeutic strategies in CCA.

Author Contribution Statement

All authors contributed equally in this study.

Acknowledgements

General

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Approval

There is no clinical trial number since this study is not classified as a clinical trial or experimental study.

Ethical Declaration

This study data provided by the SRA database which the sample collected from another study research that available as open-access data.

Data Availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Conflict of Interest

There was no conflict of interest

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