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Paired Duodenal and Salivary Microbiome Analysis in Pancreatic Cancer Without Duct Obstruction

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ABSTRACT

Background: Bacterial migration from the oral cavity to the upper gastrointestinal tract has been proposed as a contributor to pancreatic ductal adenocarcinoma (PDAC) onset and prognosis. Whether PDAC is associated with alterations of the oral–duodenal microbiome continuum remains unclear.

Methods: In this prospective study, we profiled matched saliva and duodenal brushings from 24 treatment-naïve PDAC patients without ducts obstruction and 24 age- and sex-matched healthy controls (HC). Microbial composition was assessed by 16S rRNA gene sequencing. α-Diversity was evaluated using Faith's phylogenetic diversity (PD), observed ASVs, and Pielou's evenness; β-diversity using UniFrac, Bray–Curtis, and distance-based redundancy analysis (db-RDA). Associations with overall survival were examined using Cox models and ROC-derived cut-offs, with leave-one-out cross-validation for robustness.

Results: Duodenal Faith's PD was significantly lower in PDAC than HC ($q = 0.034$), whereas richness and evenness did not differ; no α-diversity differences were observed in saliva. After adjustment for diabetes and periodontitis, lower duodenal Faith's PD ($q = 0.048$) and ASV richness ($q = 0.030$) in PDAC remained significant. β-Diversity was primarily driven by body site, but adjusted db-RDA revealed a small yet significant PDAC–HC difference in duodenal community composition (pseudo-F = 2.16, $p = 0.002$). Several genera showed differential abundance between groups. Higher salivary phylogenetic diversity predicted longer survival (aHR = 0.19, $p = 0.001$), along with specific genera associated with favourable prognosis.

Discussion: PDAC is associated with reduced duodenal phylogenetic diversity and subtle disease-related shifts in duodenal microbiota, independent of major confounders and in the absence of duct obstruction. Both α-diversity and selected genera may hold prognostic relevance, supporting further validation in larger, stage-stratified cohorts.

1 | Introduction

Pancreatic ductal adenocarcinoma (PDAC) incidence and mortality are increasing, especially in younger individuals [1], due to late diagnosis and limited treatment efficacy [2]. Understanding factors promoting pancreatic tumourigenesis and identifying reliable diagnostic or prognostic biomarkers are critical [3]. The association between microbiome and PDAC occurrence and progression has been investigated [4, 5], especially regarding faecal [6] and oral microbiome [7]. While faecal microbiota screening may serve as a diagnostic tool, changes in the oral microbiome associated with periodontal disease [8, 9]

Key Summary

- Summarize the established knowledge on this subject:
  - The oro–duodenal route has been proposed as a pathway for microbial transfer towards the pancreas, but no previous study has profiled paired oral and duodenal microbiota in PDAC.
- What are the significant and/or new findings of this study:
  - PDAC was associated with reduced duodenal phylogenetic diversity and distinct microbiota profiles, identified through paired oral–duodenal sampling.
  - These alterations were independent of biliary or pancreatic duct obstruction and remained significant after adjustment for major microbial confounders.
  - Higher salivary α-diversity and specific genera were associated with overall survival, suggesting potential prognostic biomarkers and targets for microbiome-based interventions in PDAC.

have been linked to increased PDAC risk. Although initially considered an epiphenomenon, accumulating evidence supports a potential causal role, suggesting that oral bacteria may reach the pancreas and promote tumourigenesis [10]. Indeed, PDAC lesions display bacteria, and the tumour microbiome is more abundant than that in paired healthy pancreatic tissue [11], with a microbial profile closely resembling that of the duodenum [12]. This supports the hypothesis that the oral microbiome could migrate to the duodenum and pancreas via the major papilla [13, 14]. Nonetheless, few studies on the duodenal microbiome in humans included mainly patients with pancreatic head lesions, often treated with biliary stents [15, 16], in whom microbiome alterations may be secondary to duct obstruction rather than to the disease itself, considering that pancreatic juice and bile profoundly affect microbiome composition [17, 18]. Moreover, most studies did not analyse matched oral and duodenal samples or perform a priori power calculations. Also, established modifiers of the oral and upper gastrointestinal microbiome, including proton-pump inhibitor use, diabetes, and periodontitis, may act as relevant confounders in PDAC microbiome studies and have not been considered extensively.

The primary aim of the present study was to investigate matched oral and duodenal brushing microbiomes in PDAC patients with lesions not obstructing the biliary or pancreatic duct compared with matched healthy controls. The secondary aim was to explore possible associations of the oral and duodenal microbiomes with patient survival.

2 | Materials and Methods

2.1 | Study Design and Population

We conducted a prospective observational study upon approval of the Internal Review Board (BIO-PANCREAS version 3.2021, NCT06552078). Patients were enroled during initial diagnostic Endoscopic Ultrasound (EUS) performed for a clinico-radiological suspicion of PDAC, naïve to any treatment. We excluded patients with a biliary or pancreatic stent, lesions obstructing the pancreatic and/or biliary duct as assessed during EUS, or with a final cytological report indicating a tumour other than PDAC. Healthy controls (HC) were patients undergoing gastroscopy for any reason, excluding those with oesophageal or gastric resection, known pancreatic diseases (ongoing or previous), atrophic gastritis, coeliac disease, or neoplastic conditions.

All participants underwent blood tests prior to endoscopy and sedation as part of the study protocol. Patients with abnormal liver function tests (transaminases, GGT, bilirubin) or frank jaundice were excluded from both the PDAC and healthy control groups to avoid confounding from biliary obstruction or liver disease. Healthy controls with abnormal blood tests, including anaemia or elevated tumour markers, underwent further evaluation with abdominal imaging and colonoscopy to rule out significant disorders. HC were enroled based on matching (gender and age) at a 1:1 ratio to PDAC; for each case, the first eligible control of the same sex and age ($\pm$ 2 years) was enroled. All subjects provided informed consent. A trained physician conducted a face-to-face interview using a structured questionnaire to register demographics, clinical variables associated with microbiome alteration, indication for gastroscopy in HC, and tumour features in PDAC. All variables were reported in a Case Report Form (CRF). Patients with PDAC were treated according to the standard of care and followed up for updates on overall survival (OS).

2.2 | EUS, Gastroscopy and Specimen Processing

EUS procedures were performed using a Pentax (Tokyo, Japan) therapeutic linear echoendoscope (EG3870UTK, EG38J10UT) and Hitachi ultrasound platforms (Arietta 850, Arietta V70) by expert endoscopists. Gastroscopies were performed using a Pentax gastroscope (EG-2901). All endoscopic procedures were performed using high-level–disinfected instruments following manufacturer protocols. As part of institutional quality processes, periodic surveillance cultures and biofilm assessments are routinely conducted, with no evidence of persistent contamination during the study period. Eligible patients, based on clinical history, were approached for enrolment and, upon giving informed consent, unstimulated saliva samples were collected from participants prior to endoscopy. Participants were asked to refrain from eating, drinking, smoking or chewing gum or performing oral hygiene for at least 1 h before collection. Saliva (4 mL) was collected in the stabilizing solution Omnigen-Oral DNA—Microbial DNA (Voden Medical, Italy). Duodenal brushing samples were collected during EUS or gastroscopy using a gastrointestinal cytology brush (Cook Medical ECB-5-180-3-S) to brush the duodenal wall within 3 cm of the major papilla, and then inserted into a vial with OMNIgene Gut stabilizing solution (Voden Medical, Italy). A standardized distance from the major papilla was chosen to sample the mucosa anatomically closest to the pancreatic duct while avoiding direct contact with the papilla,

thereby reducing bleeding risk and minimizing potential contamination from biliary or pancreatic secretions. To avoid contamination, brushing was always performed before any biopsy or other use of the endoscope's operating channel. Vials were handled by the same operator and stored at 4°C for ≤ 3 months before analysis, following manufacturer instructions. Duodenal and saliva samples were analysed by BMR Genomics s.r.l. (Padua, Italy). Details on DNA extraction, library preparation, sequencing, and microbial data preprocessing are provided in Supporting Information S1.

2.3 | Statistics for Clinical, Ecological and Differential Abundance Analysis

A descriptive analysis was conducted to show PDAC patient characteristics at diagnosis. Case-control comparisons used Fisher's exact tests for categorical variables and Student's t-test for continuous variables. Overall survival (OS) was analysed using Kaplan–Meier estimates and Cox proportional hazards models. Alpha-diversity metrics (Faith's Phylogenetic Diversity and ASV richness) were dichotomized using optimal cut-offs derived from ROC analyses (Youden index). Multivariable Cox models were adjusted for age and disease stage. Given the small cohort size, model robustness was assessed using leave-one-out cross-validation (LOOCV). For each iteration, one patient was excluded, and the Cox model was refitted on the remaining 20 patients. The regression coefficients obtained from each of the 21 iterations were collected to evaluate parameter stability and the influence of individual observations. Stability was quantified by the mean, standard deviation, range, and coefficient of variation of the β estimates.

All reported p values are 2-sided, with values < 0.05 considered significant. MedCalc v.13 (MedCalc Software, Belgium) was employed. For each rarefied sample, α-diversity was calculated using Faith Phylogenetic Diversity (PD, richness), observed features (ASVs, richness), and Pielou's evenness. A sample size calculation after enrolment of the first 10 patients (7 PDAC, 3 HC) used the method of Casals-Pascual et al. [19]; 24 PDAC and 24 HC were required to achieve 90% power. Kruskal–Wallis test evaluated pairwise differences among groups. Beta-diversity distance matrices (Bray–Curtis, Jaccard, Weighted and Unweighted UniFrac) and corresponding principal coordinate analyses (PCoA) were computed to assess bacterial community dissimilarity between conditions and sites. Beta-diversity analyses employed PERMANOVA with 999 permutations and p-values were corrected for multiple testing using Benjamini-Hochberg FDR to obtain q values. PCoAs were projected onto 3D plots using skbio and matplotlib. The same alpha- and beta-diversity metrics were used for longitudinal analyses on paired samples (Qiime2 longitudinal plugin) to detect intra-individual variations between PDAC and HC. Differentially abundant taxa were evaluated at multiple levels (family, genus, species, ASV) using the ANCOM plugin in Qiime2, with pseudocounts added during normalization using 'Qiime composition add-pseudocount'. The most discriminative genera for each group were identified using sparse Partial Least Square–Discriminant Analysis (sPLS-DA) in mixOmics-R. Additional details are provided in Supporting Information S1.

3 | Results

3.1 | Demographics and Clinical Features of Pancreatic Cancer Cases and Controls

In total, 126 samples were collected: 67 salivary samples and 59 from duodenal brushing. Following exclusion criteria (see Figure S1), the analysis was conducted on a final population of 24 PDACs not obstructing the ducts and 24 HCs matched for sex and age, totalling 96 samples.

Demographic data and environmental factors potentially associated with microbiome modifications in the two groups (PDAC and HC) are reported in Table 1.

As expected, there was a higher rate of diabetics and patients with periodontitis in the PDAC group. Other factors that may influence microbial composition, such as smoking, use of proton pump inhibitors (PPI), antibiotics, probiotics, and mouthwash, were not significantly differently distributed between PDAC and HC. Among the 24 PDAC patients, according to NCCN classification, 2 (8%) were resectable, 4 (17%) borderline-resectable, 12 (50%) locally advanced, and 6 (25%) metastatic at diagnosis. One patient was lost to follow-up, and during a median follow-up of 15.5 months (95% CI 11–19), 13 were deceased (56.6%). The most common indications for upper endoscopy in the HC group were Gastroesophageal Reflux Disease ($n = 9$, 37.5%) and dyspepsia ($n = 7$, 29.2%). Among them, 2/24 (8.3%) subjects tested positive for *Helicobacter pylori*, and 3/24 (12.5%) reported a previous infection, but tested negative at gastroscopy. In the PDAC group, 2/24 (8.3%) patients were also *H. pylori*–positive based on tests performed near the time of diagnosis. The similar prevalence of *H. pylori* infection across the two groups suggests a limited potential impact on between-group microbial comparisons.

3.2 | Microbiome Sequencing Data

A total of 8,461,005 raw paired-end sequences were obtained from the 96 samples analysed in this study, with an average of 88,135 reads per sample (range 624–144164). After denoising, 6,047,122 paired end reads were retained with an average of 62,991 reads per sample (range 234 to 103,681). A total of 3868 ASVs were assembled, of which 614 were further selected after length (> 380 nt) and frequency filtering (> 0.01%). Rarefaction analysis with a cut-off of 16,037 reads led to discarding 2 samples, one brushing sample of a HC and one salivary sample from a PDAC (technical success 97.9%). In addition, rarefaction curves generated after collapsing ASVs to the genus level (L6) showed a clear plateau for Observed features, confirming that sequencing depth was sufficient to capture the underlying microbial diversity (Figure S2).

3.3 | Alpha-Diversity of Duodenal and Salivary Microbiota in PDAC and Controls

Faith's PD was significantly lower in duodenal brushings from PDAC patients compared with HC ($q = 0.034$; Figure 1A), indicating reduced phylogenetic breadth. In contrast, richness (ASVs; Figure 1B) and evenness (Figure 1C) did not differ

TABLE 1 | Demographic data and environmental factors potentially associated with microbiome modifications in the two subgroups, pancreatic cancer (PDAC) and healthy controls (HC), with *p*-value of their comparison. Diabetes and periodontitis were both more frequent in PDAC patients.

	PDAC (n = 24)	HC (n = 24)	p-value
Age, mean (± SD)	68.5 (± 9.9)	67.8 (± 15.5)	0.86
Gender, male	11 (45.8%)	11 (45.8%)	1
Tumour stage (NCCN)	Resectable = 2 Borderline resectable = 4 Locally advanced = 12 Metastatic = 6	NA	—
BMI, mean (± SD)	24.6 (± 4.6)	25.1 (± 3.6)	0.64
BMI ≥ 25	8 (33.3%)	10 (41.6%)	0.47
Ever smoker	14 (58.3%)	11 (55%)	0.82
Active smoker	6 (26.1%)	2 (10%)	0.19
Diabetes	7 (29.2%)	0	0.0094
Periodontitis	10 (45.5%)	3 (15%)	0.0407
Allergies	2 (8.3%)	4 (17.4%)	0.36
Mouthwash	7 (31.8%)	8 (38.1%)	0.66
Mouthwash in the morning	0	4 (19%)	0.11
PPI in the last month	12 (54.5%)	15 (68.2%)	0.35
Antibiotics in the last month	5 (20.8%)	2 (9.1%)	0.28
Probiotics in the last month	8 (38.1%)	3 (15%)	0.10

Abbreviations: BMI = body mass index; NCCN = national comprehensive cancer network; PPI = proton pump inhibitors; SD = standard deviation.

significantly (q = 0.084 and q = 0.492). None of the alpha-diversity metrics showed significant differences in saliva between PDAC and HC. In PDAC samples, alpha-diversity was lower in duodenal brushings compared with saliva, measured by Faith's PD (q = 0.030) and ASVs (q = 0.016; Figure 1A–B), whereas no differences were observed between duodenal and salivary samples in HC (q = 0.113 and q = 0.133). Evenness did not differ between sample types in either group (Figure 1C).

To further explore differences, pairwise comparisons of salivary and duodenal samples from the same subjects using Wilcoxon rank-sum test showed saliva had higher ASV richness than duodenal brushings in both PDAC (p = 0.0009) and HC (p = 0.001), with a larger difference in PDAC (W = 35) than HC (W = 21.5). The pairwise difference (saliva minus duodenum) between PDAC and HC was not statistically significant (p = 0.20; Figure S3).

Collectively, these results suggest a narrower phylogenetic breadth in the duodenum of PDAC patients.

To investigate whether factors known to affect the microbiome could account for the differences, we evaluated smoking, diabetes, and PPI use. Among 47 duodenal brushings, PPI users exhibited reduced alpha-diversity measured by Evenness (q = 0.01), with no differences in ASVs or Faith's PD. As PPI use was more prevalent among HCs, the lower alpha-diversity in PDAC cannot be explained by PPI. Similarly, brushing samples from patients with diabetes showed higher Evenness (q = 0.02), with no differences in ASVs or Faith's PD. As diabetes was more prevalent in PDAC, the lower alpha-diversity observed cannot be explained by diabetes.

To further evaluate the potential impact of diabetes and periodontitis, we fitted multivariable generalized models including these variables as covariates. In mixed-effects models pooling duodenal and salivary samples, neither diabetes nor periodontitis showed significant associations with Faith's PD or ASVs, and the difference between PDAC and HC remained directionally unchanged. When analysing duodenal brushings alone, PDAC samples continued to show lower Faith's PD (q = 0.048) and lower ASV richness (q = 0.029) after adjustment, whereas no associations were observed in saliva. These findings indicate that the reduced duodenal alpha-diversity observed in PDAC is not explained by diabetes or periodontitis.

Regarding smoking, gender, alcohol, antibiotics, or probiotics, no significant differences were observed in duodenal samples (data not shown). Similarly, in saliva, none of the factors, including PPI, smoking, diabetes, or periodontitis, significantly affected alpha-diversity. In detail, regarding antibiotics, among participants reporting their use, three PDAC patients had courses within 13–29 days prior to sampling, and two controls had rifaximin 12–22 days before sampling, with timing and type broadly comparable between groups.

3.4 | Beta-Diversity Differences Between Saliva and Duodenal Brushings in PDAC and Controls

Unweighted UniFrac revealed significant differences between duodenal brushings and saliva in both PDAC patients (q = 0.003) and HC (q = 0.01). No significant differences were observed between PDAC and HC within the same sample type. Pairwise comparisons did not reveal any significant differences

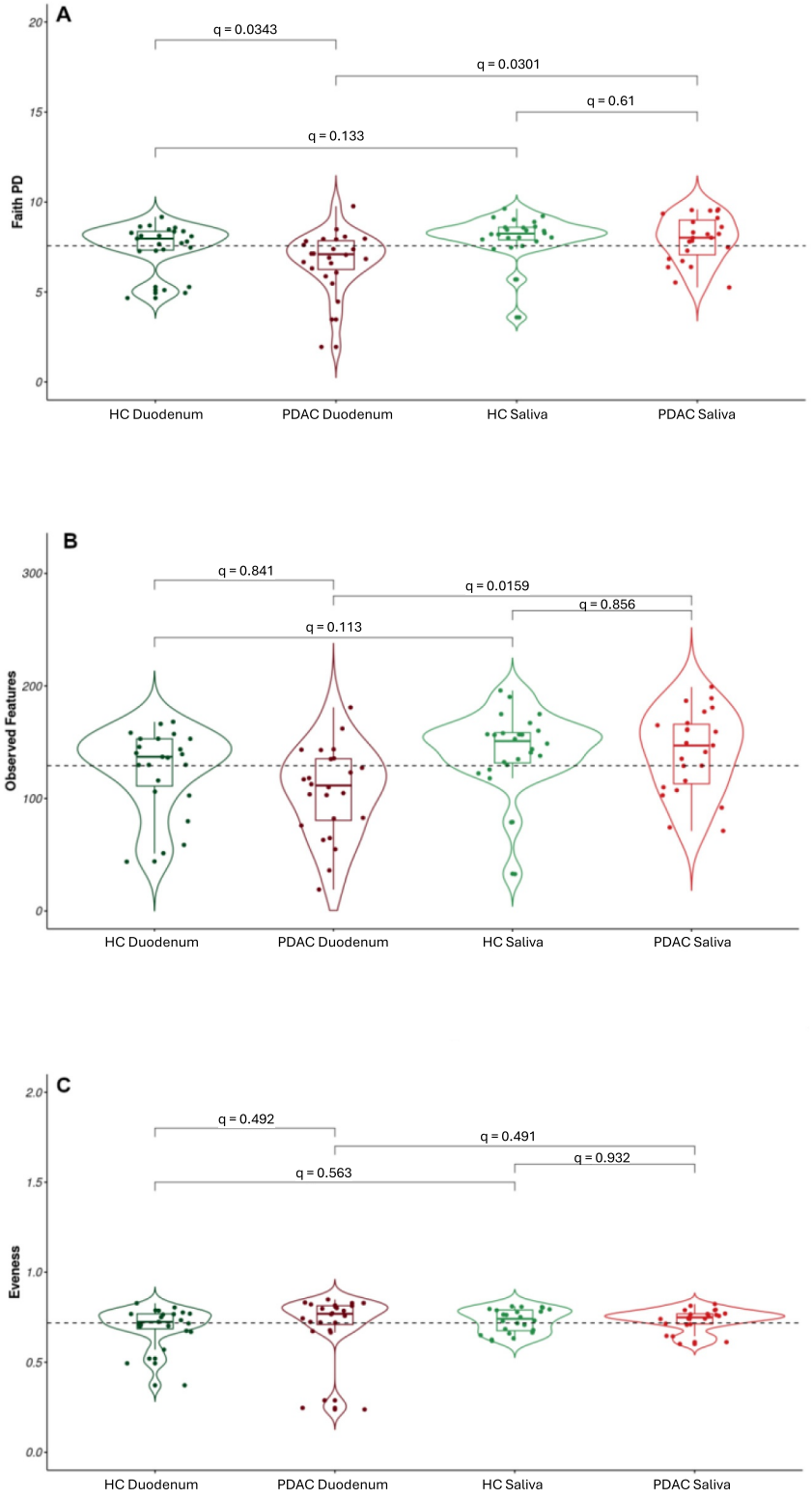

FIGURE 1 | Violin plots showing group significance analysis of different alpha-diversity metrics: faith's PD (Panel A), observed features (Panel B), evenness (Panel C).

(data not shown). Weighted UniFrac, which accounts for taxa abundance, also showed significant differences between saliva and duodenal brushings in both HC ($q = 0.04$) and PDAC ($q = 0.009$) (Figure 2A–D). Bray–Curtis dissimilarity showed similar results, with significant differences between brushing and saliva for HC ($q = 0.004$) and PDAC ($q = 0.003$). None of the tested covariates, including PPI use, smoking, or history of diabetes or periodontitis, significantly affected beta-diversity (data not shown). These results indicate that beta-diversity differences are mainly driven by sample site.

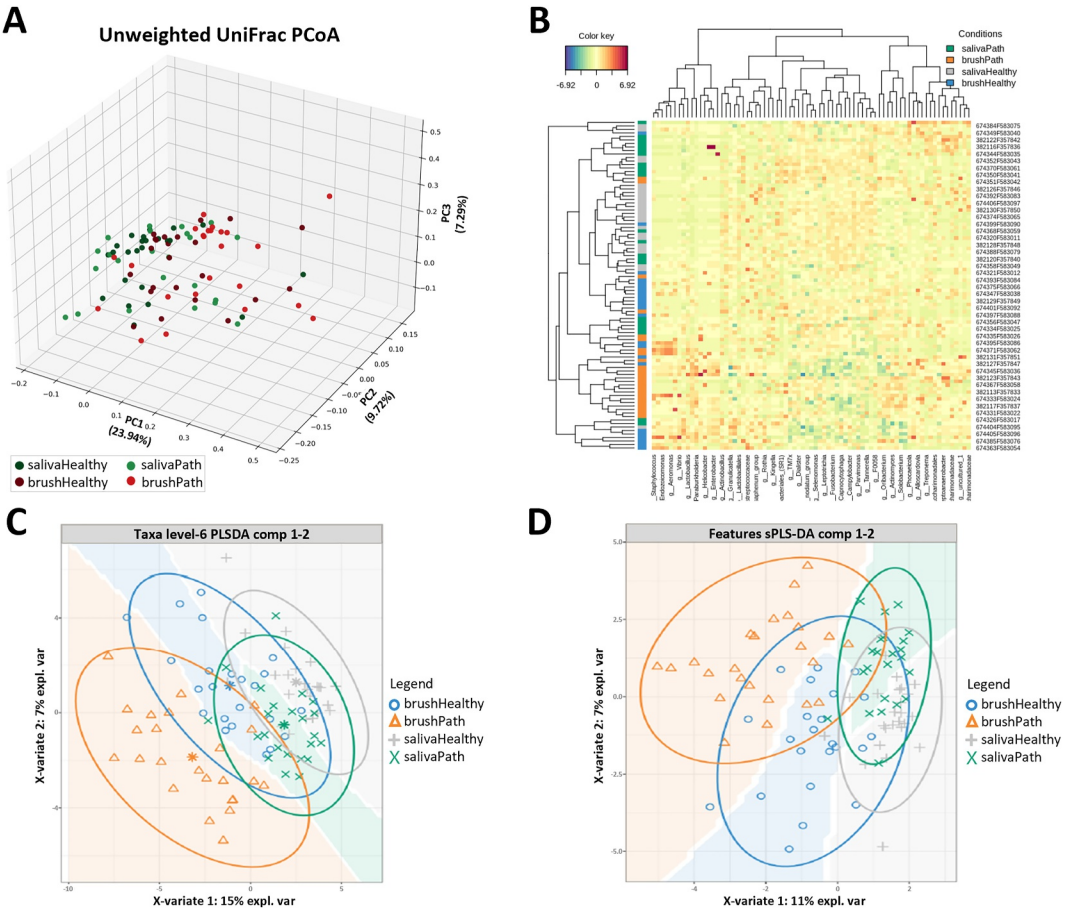

FIGURE 2 | Beta-diversity measures of the microbiome of pancreatic tumour saliva and brushing and healthy control saliva and brushing. (Panel A): unweighted unifrac principal component analysis (PCA) of beta-diversity of the four cohort of patients, where each point represents a single sample coloured by group, indicated by the three main principal components' axes; panel B: heatmap of the whole cohort of samples, with abundance of different genus; panels C and D: partial least squares discriminant analysis (PLS-DA) and sparse partial least squares discriminant analysis (sPLS-DA) for the four different subgroups and their relative overlap determined more on the site of the sample than on the type of patient.

To assess whether potential confounders influenced overall community structure, we performed a distance-based redundancy analysis (db-RDA) including diabetes, periodontitis, and body site (saliva or duodenum) as covariates. In this multivariable model, PDAC versus HC showed a small but statistically significant association with β-diversity (pseudo-F = 2.16, $p = 0.002$), while periodontitis contributed modestly (pseudo-F = 1.36, $p = 0.042$) and diabetes was not significant (pseudo-F = 1.27, $p = 0.079$). The body site remained the dominant source of variation (pseudo-F = 4.82, $p = 0.001$). These adjusted analyses suggest that although unadjusted pairwise comparisons revealed no PDAC–HC differences within the same sample type, disease status accounts for a small proportion of β-diversity variation when evaluated in a multivariable framework.

3.5 | Microbial Taxa Distinguishing PDAC Patients From HC

Differential abundances were evaluated using PLS-DA and sPLS-DA across the four subgroups: saliva and duodenal brushing from PDAC patients and HC. Analysis of the PLS-DA loadings identified genera contributing to the separation between groups (Figure 3). The Proteobacteria genus *Aggregatibacter* was more abundant in both saliva and duodenal brushing of HC compared with PDAC samples. In contrast, the Bacillota genera *Solobacterium* and *Stomatobaculum*, and the Firmicutes genus *Selenomonas* were more abundant in duodenal brushings of PDAC patients than in HC. The Firmicutes genus *Megasphaera* was more abundant in both saliva and brushings of PDAC patients.

3.6 | Alpha-Diversity and Genus-Level Signatures Are Associated With PDAC Survival

Among PDAC patients, we investigated associations between microbiome signatures and overall survival in 23 patients with available follow-up data. Receiver operating characteristic (ROC) curves were used to determine the optimal alpha-diversity cut-off for α-diversity using Faith PD and observed species (ASVs) and associated with overall survival. The ROC analysis indicated limited predictive capacity for both Faith PD (best cut-off 68123604, AUC = 0.58, sensitivity = 0.69, specificity = 0.70, Youden index = 0.39) and ASVs (best cut-off = 81, AUC = 0.52, $p = 0.85$, sensitivity = 0.62, specificity = 0.70,

Youden index = 0.31) in duodenal brushing of PDAC patients. Accordingly, alpha-diversity in the duodenal samples was not associated with OS at the univariable analysis (data not shown).

The ROC analysis on duodenal samples again indicated only a slightly better predictive capacity of Faith PD (best cut-off = 5850590726, AUC = 0.62, p = 0.37, sensitivity = 0.50, specificity = 0.91, Youden index = 0.41) and ASVs (best cut-off = 88, AUC = 0.61, p = 0.85, sensitivity = 0.50, specificity = 0.82, Youden index = 0.32). However, when stratifying patients by saliva alpha-diversity, higher diversity was associated with longer overall survival at a multivariable analysis corrected for age and stage of disease with both metrics: Faith's PD (aHR = 0.18, 95% CI 0.03–0.82, p = 0.02, median of patients with low diversity = 2 months vs not reached for high diversity) (Figure 4A) and ASVs (aHR = 0.17, 95% CI 0.04–0.81, p = 0.03, median of patients with low diversity = 8 months vs not reached for high diversity) (Figure 4B).

Given the small sample size, we evaluated the stability of the survival model using leave-one-out cross-validation (LOOCV). Each iteration involved removing one patient at a time and refitting the Cox proportional hazards model on the remaining dataset (n = 20). The regression coefficient for the covariate demonstrated good stability, with a mean value of −1.49 (SD 0.18), ranging from −2.11 to −1.32, and a coefficient of variation of approximately 12%. These findings support the robustness of the estimated effect and suggest that no individual patient exerted an undue influence on the survival association. Moreover, in a subgroup analysis restricted to the 16 patients with borderline resectable or locally advanced disease, higher oral microbial diversity remained associated with improved overall

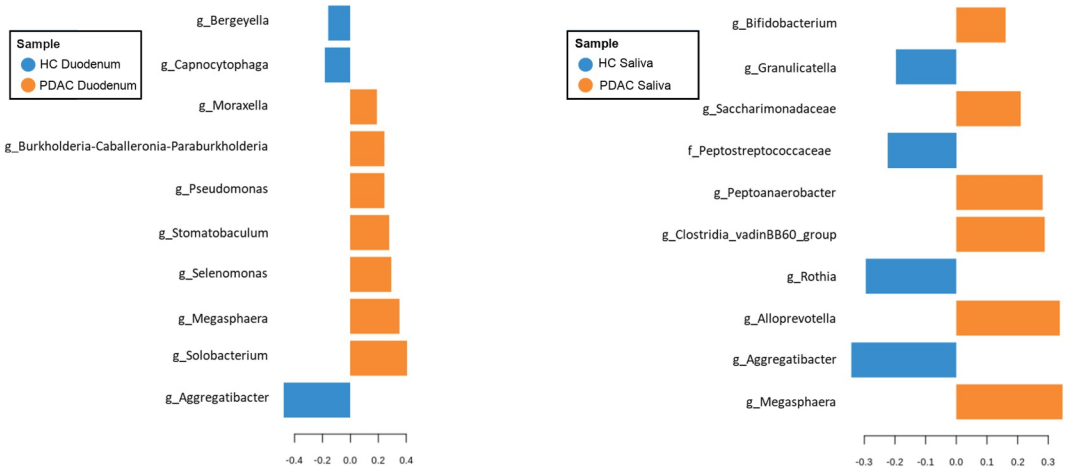

FIGURE 3 | Analysis of the partial least squares discriminant analysis (PLS-DA) loadings identified genera contributing to the separation between PDAC and HC in duodenal (Panel A) and saliva (Panel B) samples.

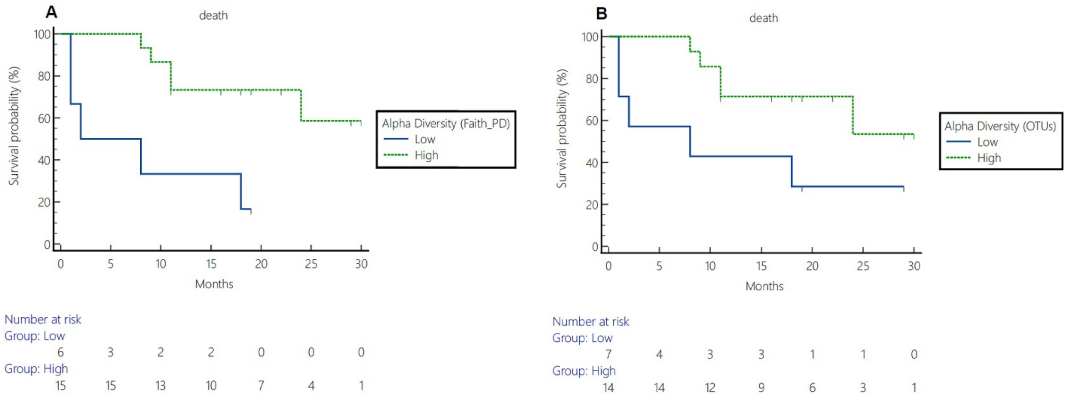

FIGURE 4 | Association between alpha diversity in the saliva samples and overall survival in 23 PDAC patients. Kaplan–Meier survival curves stratified by alpha diversity metrics. Panel A: Faith's phylogenetic diversity was significantly associated with overall survival, with higher diversity being associated with better prognosis at a multivariable analysis corrected for age and stage of disease (HR = 0.18, 95% CI 0.03–0.82, p = 0.02, median of patients with low diversity = 2 months vs not reached for high diversity). Panel B: observed species also showed worse survival in patients with lower observed richness (HR = 0.17, 95% CI 0.04–0.81, p = 0.03, median of patients with low diversity = 8 months vs not reached for high diversity). In both panels, green lines represent patients with high alpha diversity and blue lines represent patients with low alpha diversity. With both metrics, stage of disease according to NCCN was also associated with overall survival (HR = 7.8, 95% CI 1.49–41.2, p = 0.01 for panel A and HR = 9.8, 95% CI = 1.94–50.2, p = 0.005 for panel B per increasing disease stage). Numbers at risk at each time point are shown below the plot.

survival. Using Faith's Phylogenetic Diversity, and after adjustment for age and disease stage in a multivariable model, higher oral diversity significantly predicted longer survival (best cut-off = 572,333.99999; AUC = 0.59; $p = 0.56$; sensitivity = 0.50; specificity = 0.88; Youden index = 0.38; aHR = 0.19, 95% CI 0.05–0.71; $p = 0.001$). Median survival was 8 months in patients with low diversity and 36 months in those with high diversity (Figure S4). In contrast, ASV richness did not show a comparable predictive performance (best cut-off = 55; AUC = 0.51; $p = 0.96$; sensitivity = 0.13; specificity = 1.00; Youden index = 0.13), and the association with survival was not statistically significant (aHR = 0.17, 95% CI 0.01–2.20; $p = 0.18$; median survival 2 vs. 36 months). The lack of significance for ASV richness likely reflects the limited sample size and its lower discriminatory ability in this specific subgroup.

Furthermore, several genera were independently associated with overall survival after adjustment for age and disease stage (Table 2). In saliva, higher abundances of *Fusobacterium* and *Tannerella* were associated with better prognosis, whereas *Streptococcus* and *Actinobacillus* were associated with worse survival. In duodenal brushings, *Catonella* remained significantly associated with improved survival in multivariable models. These findings suggest that specific oral and duodenal taxa may hold prognostic relevance in PDAC, although these results remain exploratory given the cohort size.

4 | Discussion

This study, to our knowledge the first of its kind, evaluated paired oral and duodenal microbiota in patients with PDAC without biliary or pancreatic duct obstruction compared with matched controls.

As hypothesized in our sample size calculation, a lower alpha-diversity was observed in the duodenum of PDAC patients compared with HC, indicating a narrower ecological niche, independent of bile or pancreatic juice flow. In contrast, alpha-diversity in saliva did not differ between PDAC and HC. In paired analyses of saliva and duodenal samples from the same subjects, saliva consistently harboured higher ASV richness than matched duodenal brushings, both in PDAC and HC, with the difference slightly wider in PDAC patients, though not significantly. Because diabetes and periodontitis were more common in PDAC patients and may influence the upper GI microbiome, we performed multivariable models to account for these potential confounders. Neither condition showed a significant association with alpha-diversity, and the difference between PDAC and controls remained directionally unchanged. In duodenal samples, the reduction in Faith's PD and ASV richness persisted after adjustment. These findings suggest that the observed decrease in duodenal alpha-diversity reflects disease-related changes rather than the influence of comorbidities.

The higher richness observed in saliva compared with duodenal samples likely reflects the intrinsically more diverse oral ecosystem, whereas the reduced duodenal diversity in PDAC may relate to disease-associated changes in motility, pH, or subclinical exocrine dysfunction; importantly, this pattern is unlikely to be driven by PPI use, which was more common among controls. Complete exclusion of all microbiome-modifying factors (e.g., PPI use, diabetes, smoking) was not feasible and would have limited recruitment and generalizability; instead, these variables were recorded and considered in comparative and stratified analyses. Indeed, evaluation of potential confounders revealed that PPI use was associated with lower, while a history of diabetes with higher alpha-diversity. However, as PPI use was more prevalent in HC and diabetes in PDAC, these results support the robustness of our findings. Kohi et al. [16] also reported that PDAC patients have decreased duodenal microbial alpha diversity compared with age-matched controls; however, in their cohort, most tumours were in the pancreatic head, and 17% had biliary obstruction, possibly contributing to altered duodenal microbiome.

In unadjusted analyses, β-diversity differed markedly between saliva and duodenal samples in both PDAC and HC, whereas no differences were observed between groups within the same site, indicating that sample type was the dominant determinant of community structure. The peculiarity of the duodenal microbiome has already been reported by others, with features of greater diversity but lower abundance compared with the rectum [20]. Also, in PDAC patients, a clear separation between bacterial communities in the oral cavity and those in intestinal and pancreatic tissue samples has been demonstrated [21]. To further evaluate potential confounding factors, we conducted a db-RDA including diabetes, periodontitis, and body site. In this multivariable analysis, PDAC status accounted for a small but statistically significant proportion of variance in β-diversity, whereas periodontitis contributed modestly and diabetes was not significant. The body site remained the primary source of variation. Taken together, these analyses suggest that although the ecological separation between oral and duodenal niches is the main driver of β-diversity, disease status exerts an independent, albeit limited, effect when evaluated alongside major microbial-impacting variables.

Differential abundance analyses identified specific genera distinguishing PDAC and HC. Notably, *Aggregatibacter* was enriched in HC, whereas *Solobacterium, Stomatobaculum, Selenomonas,* and *Megasphaera* were more abundant in PDAC duodenal brushings. These results are consistent with prior studies showing site-specific microbial alterations in PDAC, as *Megasphaera* has been reported to be enriched in long-term survivor PDAC patients, suggesting a potential role in prognosis [22]. They are also compatible with, though not conclusive for, a potential oro-duodenal microbial route [10].

Interestingly, microbiome characteristics were also associated with OS in PDAC patients. Higher alpha-diversity in the saliva, but not in the duodenum, was associated with prolonged OS. Why the oral but not the duodenal alpha diversity is associated with OS in our cohort is uncertain. It is possible that oral diversity better reflects systemic host–microbe interactions and long-term environmental exposures influencing tumour biology, whereas duodenal samples may be more prone to sampling bias during endoscopic collection and largely reflect local, transient microbial communities. Riquelme et al. [22] showed that higher

TABLE 2 | Bacteria were significantly associated with prognosis (overall survival) in the 24 patients with pancreatic cancer at the univariable and multivariable analysis.

Phylum	Family	Genus	Site	Prognosis	Univariable HR (95% CI)	*p* value	Multivariable* HR (95% CI)	*p* value
Firmicutes	Lachnospiraceae	*Catonella*	Saliva	Better	0.65 (0.48–0.67)	0.001	Best ROC cut-off: 0.29 (0.03–3) Continuous: 0.99 (0.97–1.003)	0.3 0.13
Firmicutes	***Lachnospiraceae***	***Catonella***	**Duodenum**	**Better**	**0.64 (0.46–0.88)**	**0.01**	**Best ROC cut-off: 0.22 (0.05–0.92)** **Continuous: 0.98 (0.97–1)**	**0.03** **0.06**
Firmicutes	***Streptococcaceae***	***Streptococcus***	**Saliva**	**Worse**	**1.74 (1.09–2.77)**	**0.02**	**Best ROC cut-off: 4.2 × 10e + 6 (6.2e − 248 – 286e + 258)** **Continuous: 1.001 (1.00001–1.0002)**	**0.95** **0.007**
Fusobacteriota	*Fusobacteriacae*	*Fusobacterium*	Duodenum	Better	0.67 (0.48–0.94)	0.02	Best ROC cut-off: 2.79 (0.71–10.8) Continuous: 1.0001 (0.99–1.0003)	0.14 0.61
Fusobacteriota	***Fusobacteriacae***	***Fusobacterium***	**Saliva**	**Better**	**0.56 (0.34–0.94)**	**0.03**	**Best ROC cut-off: 0.15 (0.03–0.73)** **Continuous: 0.99 (0.99–1.002)**	**0.0185** **0.27**
Firmicutes	Peptostreptococcaceae	*Filifactor*	Saliva	Better	0.76 (0.6–0.97)	0.03	Best ROC cut-off: 0.19 (0.03–1) Continuous: 0.99 (0.98–1.01)	0.052 0.95
Firmicutes	Peptostreptococcaceae	*Filifactor*	Duodenum	Better	0.72 (0.54–0.97)	0.03	Best ROC cut-off: 0.32 (0.07–1.37) Continuous: 1.003 (0.99–1.008)	0.12 0.34
Actinobacteriota	Bifidobacteriaceae	*Bifidobacterium*	Duodenum	Worse	1.2 (1.01–1.43)	0.03	Best ROC cut-off: 2.2 (0.63–7.83) Continuous: 1.001 (0.99–1.003)	0.21 0.15
Firmicutes	Carnobacteriaceae	*Granulicatella*	Duodenum	Better	0.72 (0.53–0.98)	0.04	Best ROC cut-off: 1.5 (0.44–5.59) Continuous: 1.001 (0.99–1.005)	0.49 0.28
Proteobacteria	***Pasteurellaceae***	***Actinobacillus***	**Saliva**	**Worse**	**1.24 (1–1.53)**	**0.05**	**Best ROC cut-off: 2.9 (0.49–16.7)** **Continuous: 1.002 (1.0002–1.003)**	**0.24** **0.03**
Bacteroidota	***Tannerellaceae***	***Tannerella***	**Saliva**	**Better**	**0.77 (0.6–1)**	**0.05**	**Best ROC cut-off: 0.16 (0.03–0.7)** **Continuous: 0.99 (0.98–1.003)**	**0.015** **0.18**

Note: The genus that retained statistical significance in the multivariable analysis is highlighted in bold. Values in the multivariable column refer to Cox models adjusted for age and stage. No multiple-testing correction was applied because only a limited number of taxa with prior biological relevance were evaluated and the analysis is exploratory.

tumour microbial diversity is associated with better outcomes in resected PDAC patients and that the microbiome can shape the immune response promoting T-cell activation.

At the genus level, a small subset of taxa remained associated with overall survival after adjustment for age and disease stage. In saliva, higher abundance of *Streptococcus* and *Actinobacillus* was associated with increased risk of death, whereas *Fusobacterium* and *Tannerella* were linked to better outcomes. In duodenal brushings, *Catonella* remained associated with improved survival. Although these associations are exploratory and derived from a small, clinically heterogeneous cohort, they suggest that, beyond alpha-diversity, specific genera may carry prognostic relevance in PDAC.

Several mechanisms could explain microbial influences on prognosis, including modulation of antitumour immunity, alterations of host metabolic pathways, and microbial inactivation of chemotherapeutic agents. The association of higher salivary *Fusobacterium* with improved survival contrasts with prior work reporting adverse effects of intratumoral *Fusobacterium* [22, 23]. This discrepancy likely reflects the distinct ecological roles of the same taxon across niches (oral cavity vs. tumour tissue) and methodological differences between studies. Conversely, higher salivary *Streptococcus* abundance was associated with poorer survival in our cohort, consistent with reports linking both intratumoral *Streptococcus* to immunosuppressive tumour phenotypes [22, 23] and oral *Streptococcus* to increased PDAC risk in prospective cohorts [24].

We also observed that the γ-Proteobacteria genus *Actinobacillus* was associated with worse survival. Although *Actinobacillus* itself has not been implicated in chemoresistance, other γ-Proteobacteria, particularly Enterobacteriaceae, can metabolize gemcitabine via long-isoform cytidine deaminase [22, 23, 25–27]. Whether *Actinobacillus* contributes to similar pathways is unknown, but its association with poorer prognosis may reflect oral dysbiosis, inflammatory signalling, or microbial translocation.

Because the survival analysis involved multiple genera and the study was not powered for extensive multiple-comparison testing, these findings should be interpreted cautiously and considered hypothesis-generating until validated in larger cohorts.

Collectively, these findings support the microbiome as a prognostic biomarker and highlight its potential as a target for therapeutic strategies, including antibiotics, dietary modulation, probiotics, or metabolite-directed interventions, pending further validation.

Strengths of the present study include the paired analysis of oral and duodenal samples allowing intra-individual comparisons, the comprehensive evaluation of alpha- and beta-diversity metrics alongside differential abundance analyses, and consideration of potential confounders, strengthening the validity of the observations. Limitations include the small sample size, although based on sample size calculation, the cross-sectional design, and the 16S-based profiling, limiting species-level resolution and functional insights. In particular, survival analysis is limited by the small sample size, which reduces statistical power and may affect the precision of estimates. Nevertheless, the consistency of the findings across multiple diversity metrics and the validation through leave-one-out cross-validation strengthen the reliability of the survival associations observed. The LOOCV analysis demonstrated good stability of the effect estimates (coefficient variation ~12%) and indicated that no single observation disproportionately influenced the results. Although larger cohorts are required to confirm these associations, the present analysis provides robust preliminary evidence supporting a link between oral microbial diversity and overall survival in PDAC.

While duodenal microbiome sampling is not standardized, prior studies have used biopsies or fluid aspirates [15, 16]. Mucosal brushing may provide a more representative profile, with broader coverage, higher bacterial yield, and lower host DNA contamination [28–31]. On the other hand, as the duodenal brush, like biopsies, is extracted through the operating channel of the endoscope, we cannot exclude contamination from aspirated saliva or gastric fluid. *H. pylori* infection has been reported to influence oro-gastric microbial communities [32]. However, given the low and comparable prevalence of *H. pylori* infection in our HC (8.3%) and PDAC (8.3%) groups, a major confounding effect is unlikely in this cohort. In addition, a small proportion of participants had a recent antibiotic exposure (5 PDAC patients, 2 controls). Timing and type of antibiotic use were similar between cases and controls, suggesting that any impact on microbiota diversity is unlikely to have biased our main findings. Also, we cannot exclude the contribution of unmeasured factors such as subtle exocrine pancreatic dysfunction, although all PDAC patients had non-obstructing lesions and no clinical signs of malabsorption. Importantly, adjusted models showed that neither diabetes nor periodontitis explained the lower duodenal alpha-diversity in PDAC, showing that these differences likely reflect disease-related rather than comorbidity-driven alterations.

Another limitation of our study is the absence of negative controls for contamination assessment, which would have been particularly valuable for EUS-derived duodenal samples. Although consistent handling by a single clinical and a single laboratory operator reduces the likelihood of systematic bias between groups, low-biomass contamination cannot be fully excluded.

In conclusion, our data highlight a narrowed duodenal phylogenetic landscape in PDAC patients without duct obstruction. While no strong diagnostic role emerges, exploratory findings suggest possible prognostic associations of oral microbiome and compatibility with an oro-duodenal microbial route. Larger, longitudinal, and multi-omic studies are needed to validate these associations and explore their mechanistic and therapeutic implications.

Author Contributions

L.A., and G.C.: conception and design, analysis and interpretation of the data, drafting of the article, critical revision of the article for important intellectual content, final approval of the article. L.B., G.B., E.S., and

G.V. analysis and interpretation of the data, drafting of the article, critical revision of the article for important intellectual content, final approval of the article. R.P.D.L.P., C.F., A.M., G.R., M.C.P., M.R., M.F., and P.G.A. critical revision of the article for important intellectual content, final approval of the article.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

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

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting Information S1: ueg270179-sup-0001-suppl-data.docx. **Supporting Information S2:** ueg270179-sup-0002-suppl-data.docx. **Figure S1**: Flow diagram depicting enrolled patients with excluded samples and reasons for exclusion. **Figure S2**: *Genus-level rarefaction curves demonstrating sequencing depth sufficiency.* ASVs were collapsed to the genus level (L6), and rarefaction was performed using the Observed Features metric. All samples reach a clear saturation plateau, indicating that the sequencing depth was sufficient to capture the underlying microbial diversity and that the microbial profiles reported are unlikely to be affected by undersampling. **Figure S3**: Distribution of pairwise differences in ASV richness (saliva minus duodenal brushing) between Pancreatic Cancer (PDAC, red, right) and Healthy Controls (HC, green, left). While the pairwise difference tended to be larger in PDAC than in HC, the difference between groups was not statistically significant ($p = 0.20$). **Figure S4**: Overall survival according to salivary alpha diversity (Faith's Phylogenetic Diversity) in the subgroup of patients with borderline-resectable or locally advanced PDAC ($n = 16$). Patients were stratified into high (green lines)- and low (blue lines)-diversity groups, using the ROC-derived optimal cut-off. Kaplan–Meier estimates show significantly longer overall survival in the high-diversity group compared with the low-diversity group (multivariable aHR = 0.19, 95% CI 0.05–0.71; $p = 0.001$). Numbers at risk at each time point are shown below the plot.