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Research article

Antitumor potentials of onco-microbial in Chinese patients with pancreatic cancer

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ABSTRACT

Recent studies have revealed that intratumoral microbiota is implicated in pancreatic cancer (PC), yet the spectra of intratumoral microbiota and their relationship with PC in Chinese patients remained to be clarified. In this study, tumor and paired paracancerous tissue from 53 patients were profiled by bacterial 16S rRNA gene sequencing. Both α- and β-diversity displayed significant differences between tumors and adjacent tissues, with higher diversity in tumors. Three bacteria phyla (Proteobacteria, Firmicutes, and Actinobacteria) were prevalent in both cancers and adjacent normal tissues. A high prevalence of *Pseudomonas* has been identified in the PC tumor microenvironment and was associated with prolonged overall survival. Furthermore, the results of *in vitro* experiments suggested that *Pseudomonas fluorescens* (*P. fluorescens*) could inhibit the proliferation and induce apoptosis of pancreatic cancer cells. These findings revealed distinctive microbial features of the PC tumors and normal tissues in Chinese populations and exhibited the antitumor potential of *P. fluorescens* in PC.

1. Introduction

Pancreatic cancer (PC) is a common lethal cancer worldwide with a five-year survival rate of around 12 % [1,2], and its global burden has at least doubled over the past two decades [3]. PC usually develops over the years without obvious symptoms, with the pancreatic microenvironment changing along with the disease progression to form a highly fibrotic immunosuppressive environment that leads to the pancreatic tumor microenvironment (TME) being physically complex, dense, and hypoxic [4,5]. The constitution of TME has been extensively recognized as the hub determinant of multiple solid tumors, influencing their occurrence, development, and

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therapeutic efficacy [6,7]. Clarifying the relationship between TME and cancer could enhance understanding for identifying new biomarkers and developing therapeutic strategies for PC.

Recently, numerous studies have indicated that human gut commensal bacteria were tightly associated with pancreatic health and canceration [8,9], thus manipulating specific microorganisms has shown high potential in tumor therapy [10]. Since tissue-enriched microbes were detected in the TME, more studies have focused on the relationship between intratumoral microbiome and the progression and treatment of malignant tumors [11–14]. Cancer type-specific microbial composition has been identified, and interestingly most of the intratumoral microbes are commonly located within both tumor cells and immune cells [11,12]. The tumor microbiota has been documented to influence cancer pathology by activating oncogenic signals [15], inducing DNA damage [16], and forming immunosuppressive TME [17–19]. However, the interplay between intratumoral microbiota and TME differs in different organs and has not been fully explored. Elucidating the complex interaction between intratumoral microbes, cancer cells, and TME could offer invaluable insights into tumor diagnosis, treatment, and prognosis [20,21].

Preclinical studies have found that intestinal symbiotic bacteria *Enterococcus faecalis* and oral anaerobic bacteria *Porphyromonas gingivalis* could translocate into the pancreas, suggesting that endogenous bacteria could directly influence the local TME of pancreatic ductal adenocarcinoma (PDAC) and promote cancer development [22,23]. Gammaproteobacteria was found to be enriched in PDAC, which could produce cytidine deaminase to meditate chemo-resistance by metabolizing gemcitabine into an inactive form, whereas microorganisms ablation with antibiotics reversed chemosistance [24]. Meanwhile, significant variations in the diversity and composition of the intratumoral microbiome in patients with long-term survival (LTS) or short-term survival (STS) were associated with cross-talking between the gut microbiome and the host's immunity [25]. Furthermore, specific intratumoral microbiome have been proved to have close relationship with the regulation of antitumor immunity in preclinical studies [25–27]. Despite these advances, the clinical significance of the intratumoral microbiota in PC remained to be poorly understood. While the above-mentioned researches indicated an association between microbiome in PC and patients' clinical characteristics, it was likely that their results were compromised due to inadequate quality control or limited sample size. Therefore, it's urgently needed to further explore the pattern of the intratumoral microbiome and elucidate its clinical significance in PC.

Table 1
Clinical characteristics of PC patients enrolled in the study.

Characters	Count (n, %)
Sex	
Male	31 (58.5 %)
Age (year)[a]	59.0 (52.5–68.0)
Smoking status	(n = 52)
Never smoker	36 (67.9 %)
Former smoker	4 (7.5 %)
Current smoker	12 (22.6 %)
Alcohol consumption	
Never or occasional	50 (94.3 %)
Regular or heavy	2 (3.8 %)
Missing	1 (1.9 %)
Diabetes	
Yes	15 (28.3 %)
No	38 (71.7 %)
Hypertension	
Yes	19 (35.8 %)
No	34 (64.2 %)
Tumor location	
Head	42 (79.2 %)
Body/Tail	11 (20.8 %)
Biliary obstruction	28 (52.8 %)
PDAC	
Yes	39 (73.6 %)
No	12 (22.6 %)
NA	2 (3.8 %)
Tumor differentiation	
Well	4 (7.5 %)
Medium	34 (64.2 %)
Poor	12 (22.6 %)
NA	3 (5.7 %)
Tumor stage	
IA	2 (3.8 %)
IB	14 (26.4 %)
IIA	7 (13.2 %)
IIB	13 (24.5 %)
III	11 (20.8 %)
IV	2 (3.8 %)
NA	4 (7.5 %)

[a] Data are median (IQR), IQR, interquartile range.

Herein, we explored the microbial spectra of paired PC tumor and adjacent normal tissue in the Chinese cohort by 16S rRNA sequencing and compared the intratumoral heterogeneity between them. Meanwhile, we uncovered the potentially relationships between the microbiome in the PC TME and clinical characters of PC. We found that the relative abundance of *Pseudomonas* had a positive association with overall survival (OS), and confirmed the growth inhibition effects of *Pseudomonas fluorescens* (*P. fluorescens*) on PC cell lines via *in vitro* experiments. In general, our study suggested the critical role of the intratumoral microbiome in PC with confirmation of the tumor inhibitory effects of bacteria, which may provide new insights for PC treatment.

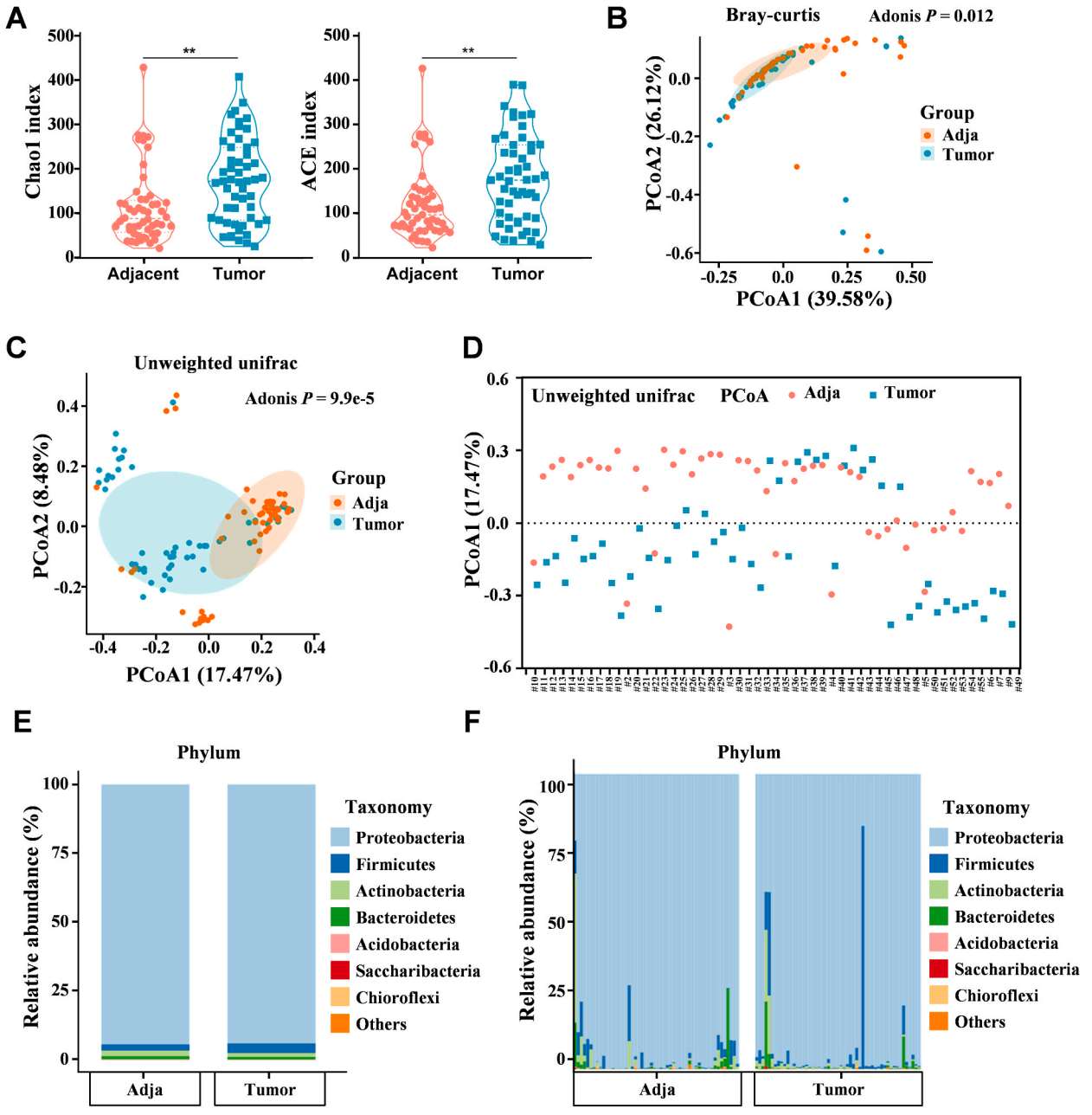

Fig. 1. Intratumoral microbial community differs between pancreatic cancer and adjacent normal tissues. (A) Alpha diversity boxplot (Chao1 and ACE indices) in the tumor and adjacent tissues (Adja). Principal coordinates analysis (PCoA) using Bray-Curtis metric distance (B) and Unweighted-Unifrac distance (C) of the tumor and adjacent tissues. (D) PCoA of Unweighted-Unifrac distances among the 16S rRNA profiles of tumor and adjacent tissues. The X-axis represented the sample ID of different pancreatic cancer patients. (E&F) Bar plots showed the phylum level of microbiota in the tumor and adjacent tissues. Each bar represents the mean abundance (E) and different patients of bacteria at the phylum level in the indicated tissues. $^{**}p < 0.01$.

2. Results

2.1. Basic characteristics of the study cohort

To investigate the intra-bacterial characteristics of PDAC, samples of tumor tissues and paired para-tumor normal tissues were collected from 53 patients who had undergone surgical resection in Xiangya Hospital between 2021 and 2022. Table 1 showed the clinical details of the included cases. The median age was 59.0 (52.5–68.0), and 31 patients (58.5 %) were male. Biliary obstruction was reported in 28 (52.8 %) patients, type 2 diabetes mellitus was diagnosed in 15 (28.3 %) patients, and hypertension was identified in 19 (35.8 %) patients. Only two patients (3.8 %) were documented with regular or heavy alcohol intake. The percentage of patients with TNM stages of I, II, and III was 30.2 %, 37.7 %, and 20.8 %, respectively.

2.2. The bacterial diversity of pancreatic tumors was significantly different from that of the normal tissues

Microbial DNAs were isolated from 52 paired samples and 16S rDNA sequencing was performed. An average sequencing depth of 80,322 with a minimal sequencing depth of 54,483 was obtained. ASVs (amplicon sequencing variants) were identified with DADA2 at a 97 % sequence similarity using the RDP database. These reads corresponded to 1132 ASVs sequences, producing 24 phyla-, 47 class-, 93 order-, 161 family-, and 344 genera-level assignments (Table S1). The rarefraction curves for all samples approached the plateau, indicating that taxon richness approached saturation (Fig. S1). After decontamination, 1079 ASVs were finally identified, including 23 phyla, 46 classes, 91 orders, 157 families, and 328 genera (Table S2).

Although the Shannon index of α-diversity showed no significant difference between the tumors and adjacent tissues (Fig. S2), the Chao1 index and ACE index in tumors were higher than those in paired normal tissues ($P < 0.01$, Fig. 1A). The β-diversity, which was calculated by PCoA using Bray-Curtis distance, revealed a significant difference between the tumors and normal samples ($P < 0.05$, Fig. 1B). Additionally, PCoA by the Unweighted-Unifrac distance also confirmed the variability in microbial profiles between the two groups ($P = 9.9e\text{-}5$, Fig. 1C and D). On average, three bacteria phyla (Proteobacteria, Firmicutes, and Actinobacteria) accounted for almost 99 % of the sequences in both carcinoma and adjacent normal samples, indicating an overall similar microbial composition pattern of tumor and adjacent tissues (Fig. 1E). This result was consistent with a previous report [11]. The intratumor microbial composition of each patient was plotted at the phylum level. As shown in Fig. 1F, the microbial features of the individual with pancreatic cancer showed notable differences.

2.3. Differential microbial features between tumors and adjacent normal tissues

To better clarify the difference of microbiota between the tumor and normal tissues, differential analysis was conducted using edgeR. As shown in Fig. 2A, 6 ASVs were upregulated and 9 ASVs were downregulated in the tumor tissues. Additionally, we compared the bacteria taxa that differed in abundance between tumor and normal samples at various taxonomy levels. The results showed that the order, Pseudomonadales (Wilcoxon's test, adjusted $P = 0.0263$), the family of Pseudomonadaceae ($P = 0.0259$), and the genera of *Pseudomonas* ($P = 0.0259$) and *Proteus* ($P = 0.00297$) were significantly enriched in tumors compared with precancerous tissues (Fig. 2B-D, Fig. S3). Among them, increased *Pseudomonas* has been reported as highly enriched and consistently found in human PDAC samples [22,25]. Herein, the family of Burkholderiaceae ($P = 0.025$) and the genera of *Ralstonia* ($P = 0.025$) were significantly decreased in pancreatic cancers (Fig. 2C and D, Fig. S3B). The LEfSe method was used to discover taxa that could help distinguish tumors from normal tissues. Taxa of Burkholderiaceae, Enterobacteriaceae, Hyphomonadaceae, Methylobacteriaceae, Sphingobacteriaceae, Bacillaceae, Enterococcaceae, and Pseudomonadaceae possibly explained the differences between the two groups (Fig. 2E). A random classifier was established based on genus abundance, which achieved a moderate performance in distinguishing tumors from adjacent tissues with an accuracy of 86.67 % (Fig. S4). Genera belonging to the above-mentioned family made top contribution to the distinguishment of tumor versus adjacent tissues (Fig. 2F).

Based on the 16S data combined with the KEGG database, the function of pancreatic tumor microbiota was inferred by PICRUSt. Analysis of KEGG pathways at level 2 revealed that 6 functional pathways were significantly changed between tumor and normal groups (Fig. S5A). Compared with adjacent tissues, two KEGG pathways were upregulated in the tumor, including signaling molecules and interaction ($P_{adj} = 0.014$), transport and catabolism ($P_{adj} = 0.035$), while four pathways were downregulated in tumor, including folding, sorting and degradation ($P_{adj} = 8.45e\text{-}3$), excretory system ($P_{adj} = 9.06e\text{-}3$), metabolic diseases ($P_{adj} = 9.88e\text{-}3$), and metabolism of cofactors and vitamins ($P_{adj} = 0.031$). As shown in KEGG data at level three, a deeper level, more metabolic changes were identified between tumor and normal groups, including the upregulation of linoleic acid metabolism and primary bile acid biosynthesis (Fig. S5B). Altogether, these functional changes of different pancreatic microbiota may be closely related to pancreatic cancer.

2.4. Relationship between intratumoral microbial profiles and clinical characteristics

The correlation between key clinical characteristics (such as smoking status, diabetes, hypertension, tumor location, tumor differentiation, and TNM stage) and intratumorally microbial signatures were further studied. The results revealed no notable differences in α- and β-diversities among different TNM stages (Fig. 3A), tumor location (Fig. 3B), or tumor differentiation (Fig. 3C). Thirty-five (73.6 %) tumor samples of PDAC (Table S3), the main type of pancreatic cancer [28], were employed in this study. However, PDAC patients showed comparable intratumoral microbial diversity as other patients (Fig. S6A). Although cigarette exposure, diabetes, and hypertension were essential risk factors for pancreatic cancer [29], variation in the diversity of the tumoral microbiome was barely

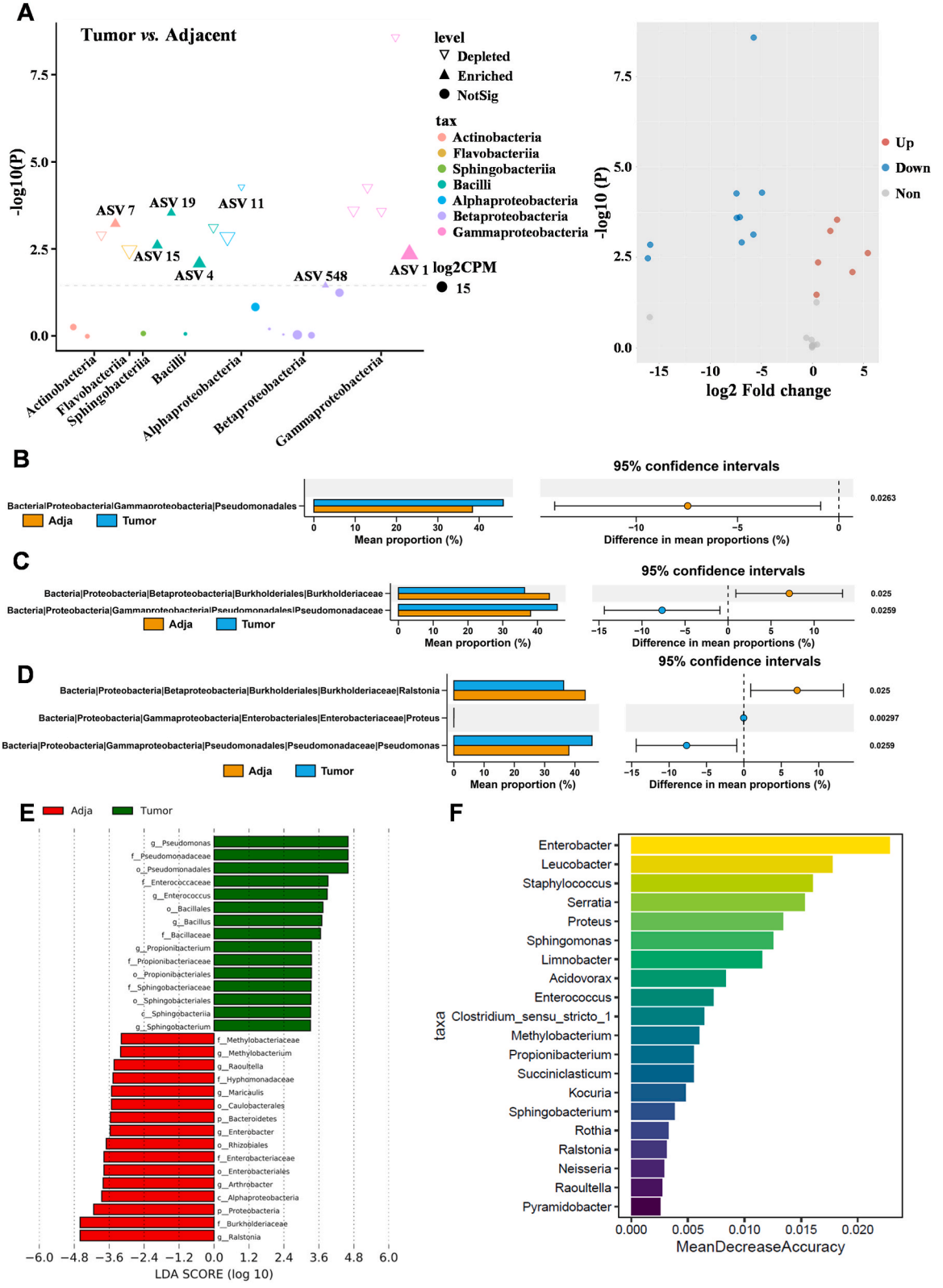

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Fig. 2. Distinct microbial community between pancreatic cancer and adjacent normal tissues. (A) Manhattan plot (Left panel) and a volcano plot (Right panel) of differential amplicon sequence variants (ASVs) between tumor and normal groups. Differential ASVs were selected based on | log2 fold-change| > 1 and $P < 0.05$. The bacterial taxa showed a significant difference between tumor and adjacent tissues at Order (B), Family (C), and Genus (D) levels. The two-tailed *P*-value was calculated using the Wilcoxon sum-rank test, and the *P*-value was adjusted using the FDR method. The 95 % confidence intervals (CIs) were calculated by the bootstrap method. (E) Lefse analysis was applied to compute the linear discriminant analysis (LDA) score of bacterial abundance among tumor and adjacent tissues. The bacteria with values of LDA score >3 were shown. (F) Bar plot of the top 20 genera with the highest discrimination power of tumors from adjacent tissues calculated from a random forest model.

identified between the groups (Figs. S6B–6D).

The correlation between the Top 100 ASVs enriched in tumors and clinical biochemical parameters in PC patients was further analyzed. As shown in Fig. 3D, Bacteroidetes were associated with a reduction in the level of albumin (ALB), which might indicate decreased survival in patients with PC [30]. Proteobacteria were correlated with the reduction of neutrophil-lymphocyte ratio (NLR), while Firmicutes were associated with the elevation of NLR. Increased NLR values have been reported to be associated with poor survival in patients with PDAC after surgery [31].

2.5. Identification of intratumoral community clusters

Next, we investigated whether microbiome changes in PC TME might classify tumors into distinct community subtypes. K-means clustering of the 45 PC samples from our cohort using tumor enriched species at ASV level, identified four major groupings (Fig. 4A and B). Cluster 1, 20.0 % (9/45) of tumor samples, was characterized by retention of ASV_4 (*Enterococcus faecalis*), which was depletion in the other clusters. In addition, increased ASV_13 (*Staphylococcus epidermidis*) and decreased ASV_3 (*Delftia*) were identified in Cluster 1. Cluster 3, 13.3 % (6/45) of tumor samples, was marked by tumor-enriched ASV_1 (*Pseudomonas fluorescens*) and downregulated ASV_13 (*Staphylococcus epidermidis*). Cluster 4, 20 % (9/45) of tumor samples, was characterized by enriched ASV_3 (*Delftia*) and downregulated ASV_13 (*Propionibacterium acnes*). Cluster 2 contained the largest number of tumor samples (21/45), without specific changes in ASV compared with other clusters. Considering all detected ASVs in tumors, Cluster 3 and Cluster 4 showed higher α-diversity compared with Cluster 1 and Cluster 2 (Fig. 4C), while Cluster 1 displayed the lowest α-diversity. The result of β-diversity analysis based on the Bray-Curtis distance revealed significant distinction among these clusters (Fig. 4D). However, its clinical value needed to be further analyzed.

2.6. Higher Pseudomonas biomass was correlated with increased overall survival

Microbes with anticancer and cancer-promoting effects might coexist in the TME. Riquelme et al. [25] found that the enrichment of several intratumoral bacterial species in PDAC with LTS is predictive of improved survival. To further understand the relationship between intratumoral microbiota and patient survival, we investigated the correlation between the relative microbial abundance and overall survival. As shown in Fig. 2B and C, *Pseudomonas* and *Ralstonia* were selected as hub genera between tumor and adjacent samples, both of which were identified by Wilcoxon's test and LEfSe method. Subsequently, we analyzed the tumor microbiome 16S rRNA sequencing data of PDAC patients with long or short survival time. Analyzing the ASV level bacterial counts, *Pseudomonas* was identified in 94.1 % of patients (64/68), while *Ralstonia* only be identified in one patient. Examining the bacterial counts of *Pseudomonas* at the genus level, we stratified the patients into two groups based on median value. Interestingly, we found that patients with relatively high abundance of *Pseudomonas* had more individuals with LTS compared to patients with low *Pseudomonas* abundance (66.7 % *vs.* 40.0 %, $P = 0.028$, Fig. 5A). However, there was no significant difference in the relative abundance of *Pseudomonas* between LTS and STS patients (Fig. 5B). Two cohorts (MDACC cohort and JHH cohort) were subjected to the above-mentioned study, in which we separately examined the correlation between the relative abundance of *Pseudomonas* and survival. As expected, the relative abundance of *Pseudomonas* was significantly higher in LTS compared with STS patients in the MDACC cohort (Fig. 5C). Moreover, more individuals with patients with LTS were identified in patients with high relative abundance of *Pseudomonas* (71.4 % *vs.* 27.3 %, $P = 0.004$, Fig. 5D), and higher biomass of *Pseudomonas* significantly associated with prolonged OS ($P = 0.00012$, Fig. 5E). Nevertheless, no statistical difference was found in the JHH cohort (Fig. S7). Taken together, these results suggested that the presence of *Pseudomonas* was correlated with improved survival in patients with pancreatic cancers.

2.7. P. fluorescens inhibited the proliferation and induces apoptosis of pancreatic cancer cells

Previous studies have indicated that *Pseudomonas* species could play a therapeutic role in managing human cancers [32,33]. To investigate the effects of *Pseudomonas* species in pancreatic cancer, the detailed information of ASVs was confirmed to belong to *Pseudomonas* in tumor samples, and it was found that more than 99.9 % of related ASVs were classified as *P. fluorescens*. Therefore, *P. fluorescens* ATCC 13525 was selected to study the effects on pancreatic cancer cells, with *Faecalibacterium prausnitzii* (*F. prausnitzii* ATCC 27768), a gut commensal but not been detected in normal pancreas and pancreatic cancer tissues, as control. The cell viability was examined in Panc02 and MIApaca-2 cell lines. The results demonstrated that *P. fluorescens* exhibited obvious growth inhibition effects under MOI 1:1000, in a MOI- and time-dependent manner (Fig. 6A and B). However, *F. prausnitzii* didn't inhibit the growth of pancreatic cancer cells. The subsequent apoptosis assay showed that *P. fluorescens* induced an apoptosis rate of 4.97 % on Panc02 cells at a low MOI (1:1000), and the apoptosis rate increased to 25.86 % at a high MOI (1:5000) (Fig. 6C). Similar results were observed in MIApaca-2 cells (Fig. 6D). Overall, the above findings indicated that *P. fluorescens* displayed significant antitumor activity, and the

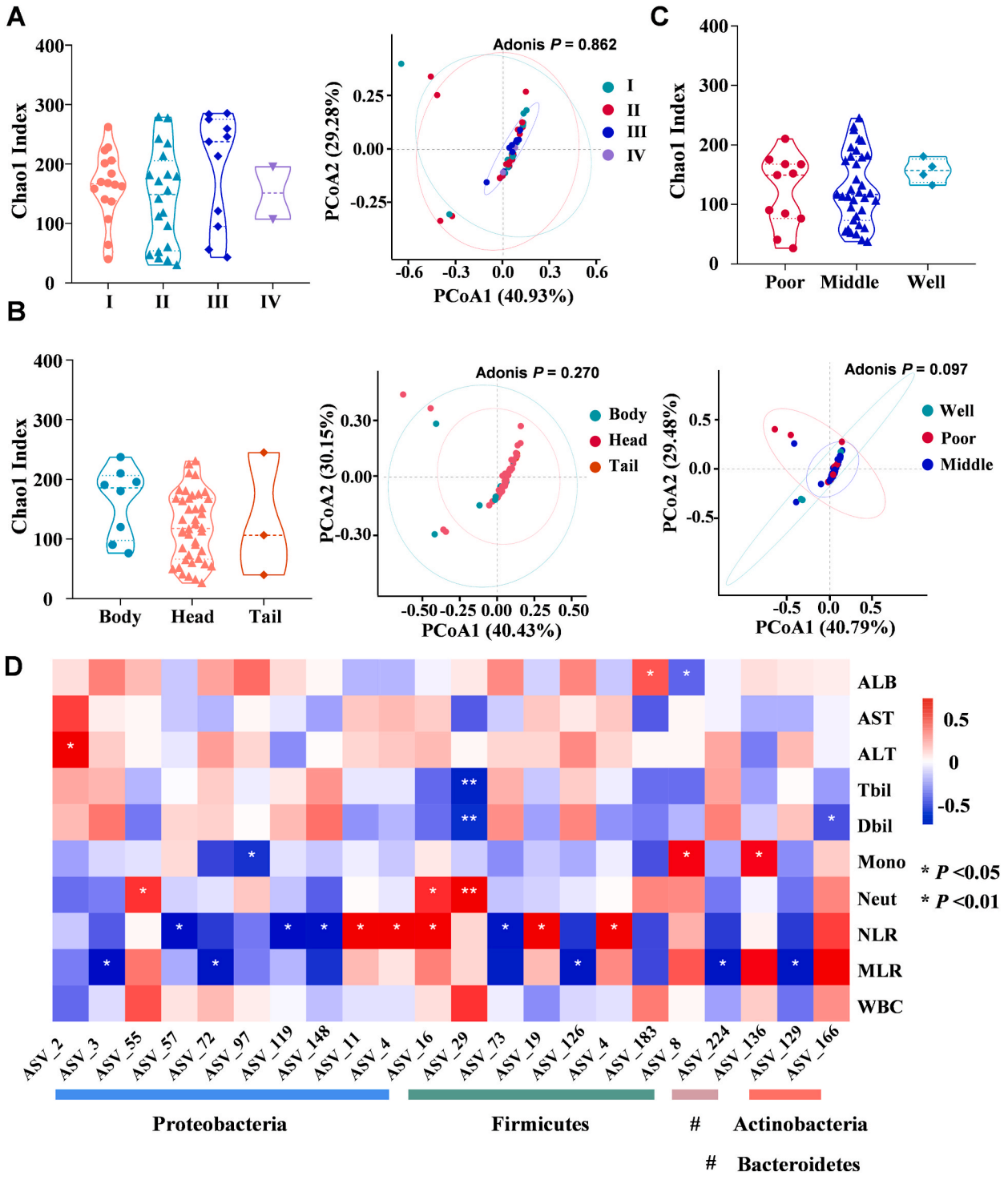

Fig. 3. Associations between the tumoral microbiome of pancreatic cancer with clinical characteristics. (A–C) The α-diversity (Chao1 index) and β-diversity (Bray-Curtis distance) of tumor tissue classification by TNM stage, tumor location, and tumor differentiation. (D) Heatmap showing the relationships between the ASVs of tumors and clinical indices, including systemic inflammatory markers and liver function tests. ALB: albumin; AST: aspartate aminotransferase; ALT: alanine aminotransferase; Tbil: total bilirubin; Dbil: direct bilirubin; Mono: monocytes; Neut: neutrophil; NLR: neutrophil-lymphocyte ratio; MLR: monocyte-lymphocyte ratio; WBC: white blood cell.

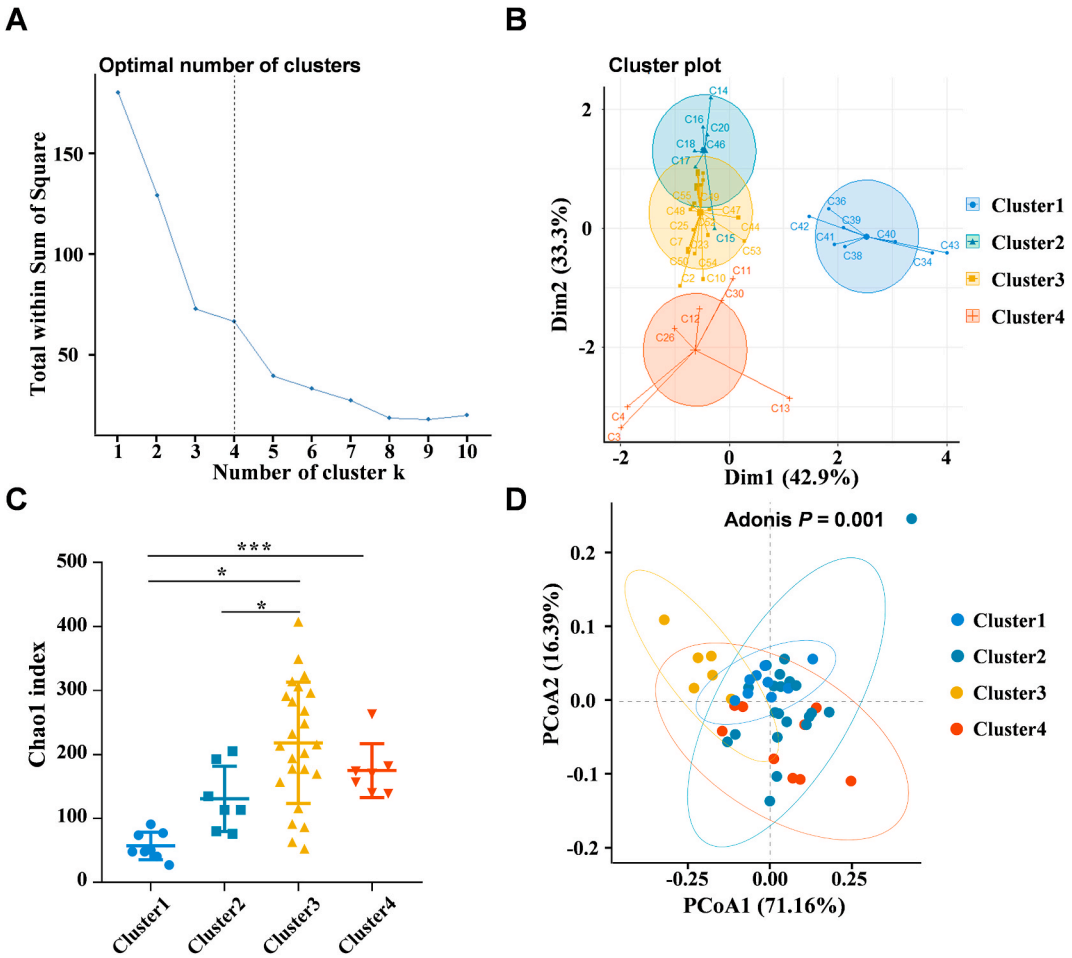

Fig. 4. Intratumoral microbial community clusters in pancreatic samples from our cohort. (A) Elbow plot for K-means clustering of all PC samples (n = 45) using tumor enriched species at ASV level, indicating optimal classification into four community clusters. (B) Principal component analysis showing the four identified clusters. (C) Bar plot showed ASV-level α-diversity of tumor samples belonging to different Clusters. (D) PCoA of Bray-Curtis distance among the 16S rRNA profiles of tumor tissues belonging to the corresponding cluster. Data with error bars represent mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

effect would be further enhanced with the increase of MOI.

3. Discussion

The history of microbes in human tumors dates back to 1550 BC [34]. Accumulating evidence has confirmed the presence of specific intratumoral microbiota in a variety of human cancers, which may be translocated from the gut or others selectively. However, a clear and systematic understanding of the clinical significance of the tumor microbiome is still lacking. Recent studies have characterized the feature of microbiome in PDAC TME, but its clinical value in PC needs to be further interpreted in different cohorts.

Previous reports have verified the dysbiotic pancreas environment following tumorigenesis, characterized by an increased abundance of microbiota, including fungi and pathobionts [35]. The pancreatic microbiome and dysbiosis are known to influence carcinogenesis, patient survival, and the efficacy of treatment [35]. In this study, we explored tumor microbiota in PC based on 16S rRNA sequencing data of tumor samples and paired normal tissues of 53 patients from our native Chinese cohort. Consistent with previous studies on PDAC, we proved the significant distinct microbial feature between tumors and adjacent normal tissues of Chinese populations with PC. The finding suggested that the intratumoral microbiome of PC was mainly composed of Proteobacteria, Firmicutes, and Actinobacteria, with notable variations in abundance across different specimens. Therefore, the uniqueness of the intratumoral microbiome may act as a potential biomarker for the discrimination of tumor and paracancerous tissues, as claimed by some researchers [36]. A random forest classifier established in our study could discriminate tumors from paracancerous tissues with an accuracy of 86.67 %. However, no significant difference in tumor microbial patterns was found between PDAC and non-PDAC, despite their differing locations and triggers. Additionally, a higher level of *Pseudomonas*, a dominant bacterium in PC, was correlated with favorable OS.

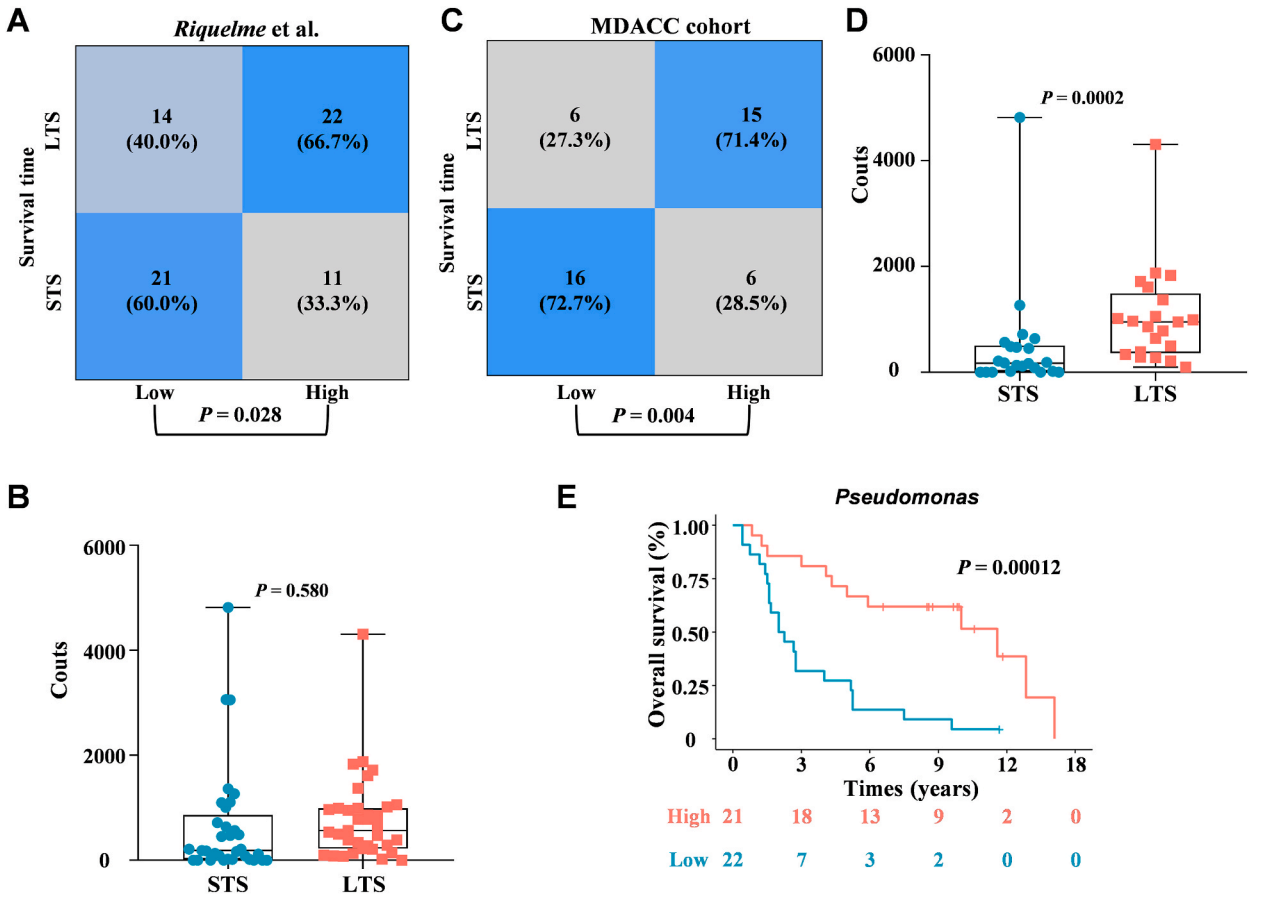

Fig. 5. Abundance of *Pseudomonas* correlate with prolonged overall survival in pancreatic cancer patients. (A) Statistical analysis was conducted on the basis of the abundance of the genus *Pseudomonas* and survival rate in the study conducted by *Riquelme* et al. (B) Box plot showed relative abundance of genus *Pseudomonas* within tumor microbiome of long-term (LTS) and short-term (STS) survival patients with PDAC (related to Fig. 5A). (C) Statistical analysis was conducted on the basis of the abundance of genus *Pseudomonas* and the survival rate in the MDACC cohort. (D) Box plot presented relative abundance of genus *Pseudomonas* of LTS and STS with PDAC in the MDACC cohort (related to Fig. 5C). (E) Kaplan-Meier estimates for overall survival of patients with high abundance versus low abundance of genus *Pseudomonas* in the MDACC cohort. In (A) and (C), data were analyzed using chi-square (and Fisher's exact) test. In (B) and (D), a Mann-Whitney nonparametric test was applied to the comparison of relative abundance values of *Pseudomonas* between STS and LTS. In (E), the *P* value was calculated by the log-rank test.

Compared with a recent national study on PDAC [26], our research examined the tumor microbial characteristics of PC using frozen tissues and compared the bacterial features with matched normal samples, which provided more reliable and valuable insights into the microbial component of PC TME in Chinese individuals. Our findings revealed that the α-diversity of the tumor was higher than that of the adjacent tissues. Similar to the reports microbial community diversity increased as pancreatic cancer progresses [35]. In PCoA, samples in the tumor group tended to spread from their corresponding normal tissues. This was the case for breast cancer, which showed higher abundance and richness in the tumor samples than in healthy breast tissues and adjacent tissues [11]. However, this trend was not observed in the gastric mucosal microbiome, which displayed a decreased bacterial diversity in cancer [37]. Furthermore, we conducted a microbiota-based cluster system for PC, which allowed for classifying tumors into one of four distinct clusters, and found different clusters exhibited discriminate α-diversity. The positive association between high microbial diversity in pancreatic cancer TME and better outcomes has been documented [25]. These findings suggested that tumor progression and outcome may be associated with the diversification or simplification of microbiota within the TME.

Consistent with the PDAC cohort from Caucasians [25], we disclosed that phyla Proteobacteria, Firmicutes, and Actinobacteria were the most enriched taxa in pancreatic tumors. Proteobacteria has been widely observed in the gastrointestinal microbiota of patients with pancreatic cancer. According to previous studies, an increased enrichment of Proteobacteria in the gut may shorten survival and increase the risk of cachexia in cancer patients [38]. Meanwhile, Proteobacteria has been confirmed to participate in the regulation of inflammation [39] and the onset of endotoxemia [40]. Given that the gut-pancreas axis is essential for regulating pancreatic homeostasis [41,42]. It has been preliminarily confirmed that bacteria can translocate from the gut to the pancreas [22], as many intestinal bacteria can be detected in the TME [11]. Similar to Proteobacteria, Actinobacteria has also been demonstrated to be enriched in the TME of patients with poor prognosis [43].

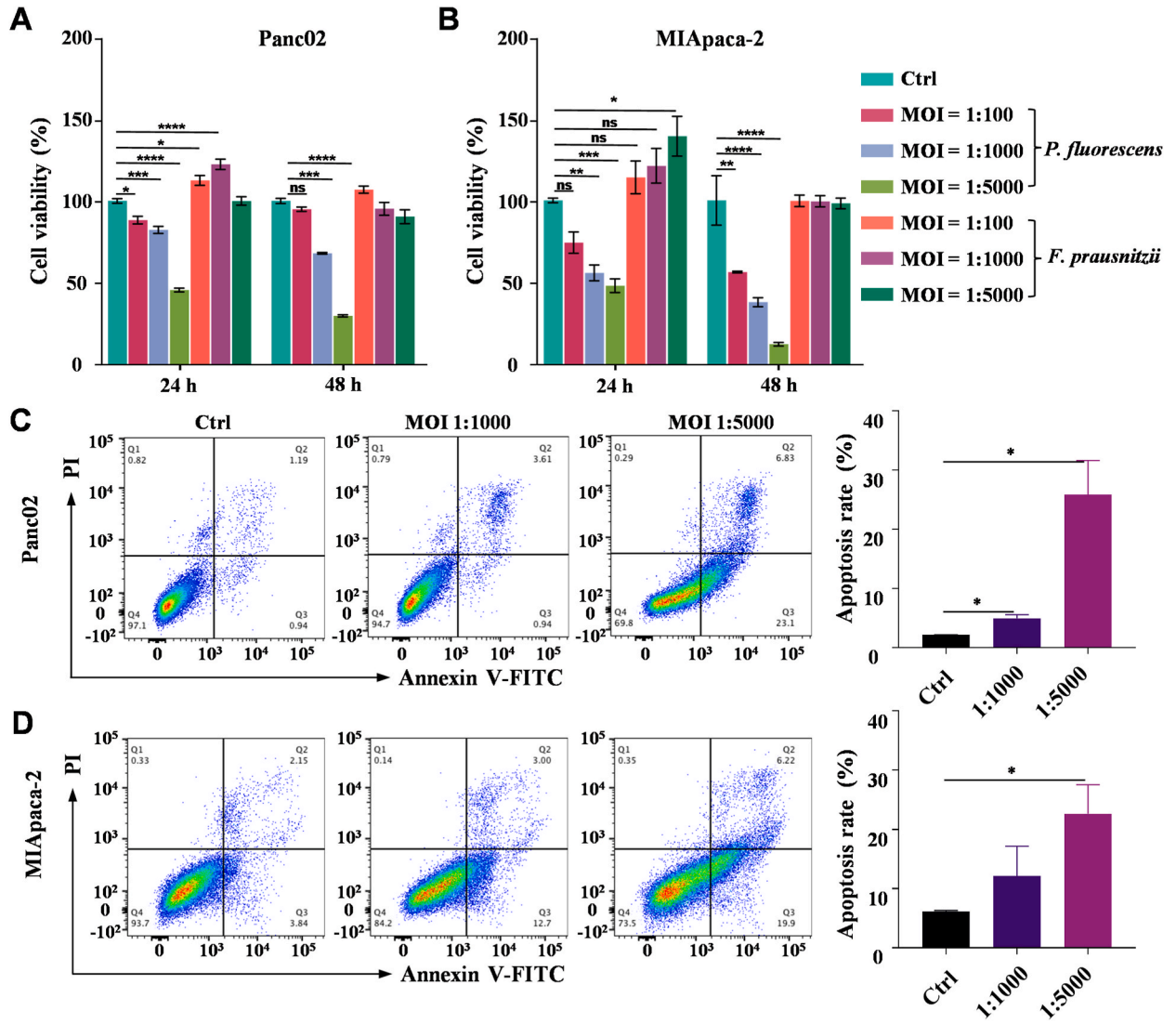

Fig. 6. *P. fluorescens* inhibits the growth of pancreatic cancer cell lines. The cell viability of Panc02 (A) and MIApaca-2 (B) cells co-cultured with different multiplicity of infection (MOI) of *P. fluorescens* and *F. prausnitzii* at 24 h and 48 h. Data are represented as mean ± SEM. Representative FCM images and the apoptosis rates of Panc02 (C) and MIApaca-2 (D) cells after being treated with different biomass of *P. fluorescens*. Data are represented as mean ± SD. In (A) and (B), statistical differences were determined by the two-way ANOVA analysis. In (C) and (D), statistical differences were determined using a two-tailed Student's *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

We analyzed the functional changes inferred from different pancreatic microbiota by PICRUSt and disclosed a distinct metabolic function of bacteria in pancreatic tumor tissues. Compared with paracancerous tissues, linoleic acid metabolism and primary bile acid biosynthesis pathways were increased in pancreatic tumors. Linoleic acid levels were found to be positively associated with PC incidence [44] and played an important role in PC development and progression [45]. Enriched primary bile acid biosynthesis pathways and its various metabolites were identified to be associated with malignant transformation and liver metastasis of PC [46]. However, it is worth noting that the functional prediction was based on indirect inference from the 16S rRNA gene sequencing of the pancreatic microbiome. Integrating further multi-omics analysis, including metabolomics and metaproteomics, may provide a deeper insight into the function of the microbial community in the TME of PC.

In our study, the dominant bacteria in pancreatic cancer, specifically *Pseudomonas*, were identified with significantly higher abundance in cancerous tissues compared to paracancerous tissues. Previous studies have indicated the potential antitumor effects of *Pseudomonas* spp [32,33]. Our findings disclosed that higher levels of *Pseudomonas* in PC tissues were correlated with a prolonged OS, suggesting the potential benefits of *Pseudomonas* spp. in regulating PC TME. We further confirmed the growth inhibitory effects of *Pseudomonas fluorescens* (*P. fluorescens*) in pancreatic cancer cell lines via *in vitro* experiments. *Sindhu* et al. [47] found that L-asparaginase derived from *P. fluorescens* has a deglycosylation activity, which could exhibit an additional barrier for proliferating cancer cells. In addition, *Sato* et al. [48–50] confirmed that high mannose-binding lection from *P. fluorescens* can decrease the protein

abundance of various cancer-related integrins and thereby promote cell death. However, a recent study suggested that *Pseudomonas* within the tumor was highly related to carcinogenesis in the basal-like subtype of pancreatic cancer [51]. Their results were inconsistent with our study, possibly due to the differences in sample types. *Ralstonia*, a Gram-positive bacteria, has previously been recognized as the most dominant bacteria in breast cancer [52]. In our present study, *Ralstonia* failed to show any association with survival status in the published validation cohort, as it was only detected in a single sample.

There are some limitations in the current study. Firstly, the major deficiencies of our work were its retrospective property and the lack of normal pancreatic tissues derived from healthy volunteers. Secondly, although *Pseudomonas* and *Ralstonia* were identified as the major discriminant genus between pancreatic tumors and adjacent normal tissues, we examined the relationship between *Pseudomonas* and patient survival in another independent cohort from Caucasian populations. Thirdly, although four distinct intratumoral community subtypes were identified in our present study, their clinical value remains to be explored. Therefore, further investigations are needed to disclose the clinical effects of the intratumoral microbiome in PC and identify key points for clinical intervention treatment to prolong patient survival.

In conclusion, our investigation provided new insights into the microbial profiles in the PC TME and adjacent normal tissues in the Chinese cohort and suggested the association of specific bacteria biomass with patient survival. The specific taxa can even used as an intervention target for the clinical treatment of PC. However, the molecular mechanisms of intratumoral microbiota that participate in regulating PC progression and how to use them as therapeutic targets remain to be deeply clarified.

CRediT authorship contribution statement

Yong-Chao Gao: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Ding-Ding Zhou:** Writing – original draft, Investigation. **Ye-Bin Lu:** Formal analysis, Data curation. **Li Yang:** Data curation. **Xue-Jun Gong:** Data curation. **Man-Yun Chen:** Data curation. **Shuai Liang:** Writing – review & editing, Supervision, Conceptualization. **Wei-Hua Huang:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Wei Zhang:** Writing – review & editing, Supervision, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e40890.

STAR★Methods

KEY RESOURCE TABLE

REAGENT OR RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
IBM→SPSS→Statistics Version 20.0	IBM, Chicago, IL, USA	https://www.ibm.com/products/spss-statistics
GraphPad Prism 9.0.0	GraphPad Software	https://www.graphpad.com/
RStudio Version 3.5.2	RStudio Inc	N/A
FlowJo V10	TreeStar, Ashland, OR, USA	https://www.flowjo.com/
Cell lines		
MIApaca-2	ATCC, Manassas, VA, USA	https://www.atcc.org/
Panc02	ATCC, Manassas, VA, USA	https://www.atcc.org/
Bacterial strains		
Pseudomonas fluorescens	BNCC	https://www.bncc.com/
Faecalibacterium prausnitzii	ATCC	https://www.atcc.org/
Biological samples		
Tumor tissues from pancreatic cancer patients	Xiangya Hospital affiliated Central South University	N/A
Paracancerous tissues from pancreatic cancer patients	Xiangya Hospital affiliated Central South University	N/A
Critical commercial assays		
Annexin V-FITC/PI	Beyotime	C1062S

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Wei Zhang (csuzhangwei@csu.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

For human studies

In total, 53 patients with PC who underwent surgery at Xiangya Hospital of Central South University were enrolled. None of the patients has a history of other malignancies. Fresh tumor and paracancerous tissues were obtained immediately after surgery. All tissues were collected in the operating room to avoid contamination and placed in sterile tubes after washed twice with cold sterile saline. Samples were frozen in liquid nitrogen and stored at −80 °C freezer until DNA extraction. The adjacent normal tissue was missing in one patient. Written informed consent was gained from all participants.

For cell lines

The human pancreatic cell line MIApaca-2 and mouse pancreatic cancer cell line Panc02 were acquired from the American Type Culture Collection (ATCC, Manassas, VA, USA), cultured in DMEM supplemented with 10 % FBS (Cellmax) (v/v), and incubated at 37 °C in a 5 % CO_2 atmosphere. *Pseudomonas fluorescens* ATCC 13525 was purchased from the BeNa Culture Collection (BNCC), and was routinely cultured aerobically in lysogeny broth medium. *Faecalibacterium prausnitzii* ATCC 27768 was obtained from ATCC and cultured in brain heart infusion medium supplemented with 0.5 % yeast extract, 1 mg/mL D-(+)-Cellobiose, 1 mg/mL D-(+)-Maltose monohydrate, 0.5 mg/mL cysteine, and 0.2 % Porcine Hemin solution in an anaerobic condition using N_2:CO_2:H_2 (80 %:10 %:10 %) as the gas phase.

METHOD DETAILS

DNA extraction and 16S rRNA sequencing study

The total genome DNA was extracted by the CTAB method as previously described [53]. 16S genes in 16S V3-V4 hypervariable region were amplified with the primer (forward primer, 5′-ACTCCTACGGGAGGCAGCA-3′; and reverse primer, 5′-GGACTACHVGGGTWTCTAAT-3′) and barcodes. Sequencing was performed on an Illumina NovaSeq platform following the 2 × 250 bp paired-end protocol.

Raw data processing

After obtaining the raw data, the reads were demultiplexed based on the distinct barcodes, and the barcodes and primers sequences were removed. After quality control and filtering, the EasyAmplicon [54] pipeline was applied to analyze the data on vsearch and usearch. The high-quality sequences after chimaera removal and denoising were assigned to amplicon sequence variants (ASV) at 97 % similarity. Species were annotated using the Ribosomal Database Project (RDP) database (performed in DADA2). To minimize the potential effect of contaminants, a list of contaminants generated by *Poore* and colleagues using "Decontam" software was applied [20]. The resulting contaminant-free 16S rRNA reads were applied to calculate bacterial abundance and the following comparison.

The α-diversity was computed by the R package 'vegan', and the β-diversity was calculated by principal coordinate analysis (PCoA) based on Bray-Curtis distance and Unweighted-Unifrac distance at the ASV level. Adonis algorithm was used to check the difference between groups, and the replacement number is 999. Different abundant taxa between groups were analyzed with the R package 'edgeR' and the linear discriminant analysis effect size (LEfSe) method, with a linear discriminant analysis (LDA) score greater than 3 and a *P* value less than 0.05. To identify intratumoral community clusters, we applied k-means for all tumor-enriched ASVs with 0.1 % or higher relative abundance. PICRUSt2 was applied to microbiome function prediction based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Generated metagenomes were input for the reconstruction of metabolic function, using level2 and level3 KEGG pathways to predicted the function of the intratumoral microbiome. Differential taxa and pathways between cancerous and paracancerous tissues were presented by STAMP software. Multiple testing correction was performed using a two-tailed Wilcoxon test.

Random forest model construction

To identify intratumoral signatures capable of discriminating tumors from paracancerous tissues, a random forest model by using R package 'randomForest' was established in this study [36]. Bacterial taxa were ranked in the model by 100 iterations. A 10-fold cross-validation with the rcf(.) function was performed to determine the representative genera and obtain the minimum cross-validation error.

Survival analysis in an independent cohort

We downloaded 16S sequencing data for long- and short-term survival patients with PDAC [25] from the National Center for Biotechnology Information (NCBI) BioProject (accession number: PRJNA542615). Bacteria counts at the genus level were generated, and the relative abundance values were computed via normalizing raw counts to the total number of reads in the corresponding samples. Statistical analysis was conducted on the basis of the level of *Pseudomonas* and LTS rate using Chi-square or Fisher's exact. MDACC cohort in PRJNA542615, for which patients with available survival information, was additionally applied to compare overall survival between patients with high versus low abundance of *Pseudomonas*, using Kaplan-Meier survival curves and Log-rank test. A $P < 0.05$ was regarded as statistically significant.

Bacterial co-culture assay

For bacterial co-culture assays, MIApaca-2 and Panc02 cells were seeded in 6-well plates (3×10^5 cells/well) and incubated at 37 °C in 5 % CO_2 overnight. On the day of the experiments, the medium was removed, and the wells were washed twice by cold phosphate-buffered saline (PBS). To obtain the pellet of each bacterial strain, logarithmic phase bacterial cultures were centrifuged at 3000×*g* for 15 min at 4 °C, and the bacteria were washed once in PBS. Subsequently, bacteria were resuspended in DMEM and supplemented to the pancreatic cells at various final multiplicity of infection (MOI) ranging from 1:100 to 1:5000 (for cell death assay) or at specific MOI of 1:1000 and 1:5000 (for apoptosis analysis) for 2 h. Then, the medium was aspirated from the wells and the cells were washed twice with 1 mL PBS before the addition of 1 mL of DMEM medium supplemented with 400 U/mL gentamicin, to kill the bacteria. After 2 h incubation, the medium containing gentamicin was aspirated and the cells were washed as described above. Fresh DMEM supplemented with 10 % FBS and 1 % penicillin and streptomycin (Thermo Fisher Scientific) were added, and the wells were continually cultured at 37 °C in 5 % CO_2 for 24 h (for cell death assay and apoptosis analysis) or 48 h (for cell death assay). After incubation, cells were detached by trypsin-EDTA (Thermo Fisher Scientific) and washed with cold PBS. Cells were stained with 0.2 % Trypan Blue Solution, and viable cell number was determinged by cell counting. The ratio of cell viability was calculated as follow: Cell viability = Viable cell number in specific bacteria treated group/Viable cell number in control group. For apoptosis assay, the cells were incubated with Annexin V-FITC reagent (Beyotime, C1062S) in the dark for 15 min at room temperature. Thereafter, the cells were immediately measured with flow cytometry (FCM). FCM data were analyzed by FlowJo software (TreeStar; Ashland, OR).

Data analysis and statistics

All statistical analyses were conducted by GraphPad Prism 9.0.0 or the statistical environment R 3.5.2. For *in vitro* experiments, the two-way analysis of variance (ANOVA) was used to determine whether there were any statistically significant differences between the different treatment and control groups. Statistical evaluations of clinical characters were carried out using IBM SPSS Statistics (version 20.0). Wilcoxon's rank sum test was applied to check the α- and β-diversity between different groups. Differences were considered statistical significantly when $P < 0.05$.

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