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Biliary Tract Microbiota Similarities in Pancreatic Ductal Adenocarcinoma

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Abstract

Objective: to analyze microbiota profiles in the biliary tract, of pancreatic ductal adenocarcinoma (PDAC) patients and gallstones patients, in order to identify differences, which may contribute to a better understanding of PDAC carcinogenesis.
Methods: using microbiota analysis, a total of 25 samples from 14 patients were collected during surgery and compared. Samples were divided into three groups; one GS group (N = 3), and two PDAC groups; PDAC gallbladder group (N = 11) and PDAC brush group (N = 11).
Results: upon comparison of bacterial communities' alpha and beta diversity indices and relative abundances by group (anatomic site) and condition (GS vs PDAC), we found no statistically significant results. However, we can highlight the high similarity of the compared parameters among the two different anatomic locations over the biliary tract in PDAC patients.
Conclusion: to the best of our knowledge, this is the first study comparing two different anatomic locations over the biliary tract in PDAC patients. Among PDAC groups microbiota along the semi-closed duct system of the biliary tract showed substantial similarity, reflected in the alpha and beta diversity indices and relative abundances.

Keywords: pancreatic cancer$_1$, microbiota$_2$, bile$_3$, biliary tract microbiota$_4$

Similitudes en la Microbiota de Tracto Biliar en Adenocarcinoma Ductal de Páncreas

Resumen

Objetivo: analizar los perfiles de microbiota en el tracto biliar de pacientes con adenocarcinoma ductal pancreático (PDAC) y pacientes con cálculos biliares (GS), con el fin de identificar diferencias, lo que puede contribuir a una mejor comprensión de la carcinogénesis de PDAC.
Métodos: mediante análisis de microbiota, se recolectaron durante la cirugía un total de 25 muestras de 14 pacientes y se compararon. Las muestras se dividieron en tres grupos;
Grupo GS (N = 3) y dos grupos PDAC; Grupo de vesícula biliar PDAC (N = 11) y grupo de cepillado PDAC (N = 11).
Resultados: al comparar los índices de diversidad alfa y beta de las comunidades bacterianas y las abundancias relativas por grupo (sitio anatómico) y condición (GS vs PDAC), no encontramos diferencias estadísticamente significativas. Sin embargo, podemos destacar la gran similitud de los parámetros comparados entre las dos ubicaciones anatómicas diferentes en el tracto biliar en pacientes con PDAC.
Conclusión: hasta donde sabemos, este es el primer estudio que compara dos ubicaciones anatómicas diferentes sobre el tracto biliar en pacientes con PDAC. Entre los dos grupos de PDAC, la microbiota del sistema de conductos semicerrados del tracto biliar, se encontró una similitud sustancial, reflejada en los índices de diversidad alfa y beta y en abundancias.

Palabras clave:

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a lethal malignant neoplasm of unknown etiology. High age-standardized incidence rates of PDAC are present in first world countries, partially attributed to risk factors associated with specific lifestyles, ageing population and environmental conditions[1,2]. Classical PDAC risk factors are absent in most patients, and modifiable and non-modifiable risk factors for PDAC have an unconvincing molecular association with the disease[3]. Over the next 5 years PDAC is expected to ascend from the fourth to the second leading cause of cancer-related deaths, due both to the stagnation in outcome improvement for PDAC over the past 20 years and the improved outcome of other malignancies[4–6]. Additionally, the lack of screening methods for premalignant conditions, and the hitherto undeciphered PDAC carcinogenesis process, hinders progress in PDAC prevention and treatment.

As in other gastrointestinal tract malignancies, bacteria have been associated with PDAC carcinogenesis, but the strength of this association is weak. The three most frequent bacteria associated with PDAC are *Helicobacter pylori, Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*,

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but each has insufficient empirical support for their role in PDAC carcinogenesis and no translation in clinical practice. Science has shown a pivotal role of *Helicobacter pylori* in intestinal-type gastric adenocarcinoma carcinogenesis[7], but in PDAC patients the isolation of *Helicobacter pylori* has had contradictory results[8,9]. On the other hand, *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* are well-known oral bacteria associated with periodontal disease, but it is improbable that they exert biological activity in PDAC carcinogenesis from their oral location[10,11]. While the epidemiological association of *Helicobacter pylori* with intestinal-type gastric cancer has strong support, clarification of bacterial association with PDAC will be needed before any clinical interventions can be proposed.

Bacteria can reach the biliary tract and the gallbladder from the duodenum and by the entero-hepatic circulation[12]. The biliary tract, including gallbladder and intra-pancreatic bile ducts, is a semi-closed duct system possessing its own microbiota[13,14], shaped by local conditions including exposure to high bile concentrations. Indeed, it has been shown that these conditions inform, at least at the phylum level, differences in gastrointestinal tract microbiota[15]. Many surveys have tried to characterize the microbiota in PDAC patients, comparing their results with normal or gallstone patients. To date there has been no success in ascribing a bacterial signature to the PDAC carcinogenesis process. It is possible that microbiota-modifying factors such as age, gender and diet, among others, representing a major obstacle in experimental control, have obscured the detection of such a bacterial signature[16]. To overcome microbiota-modifying factors, we compare the microbiota in PDAC patients in two different anatomic locations over the biliary system, and GS patients, with the aim to examine any differences. We found no statistically significant differences either in alpha or beta diversity between groups, or as other surveys have found[18], in the relative abundances of bacteria. However, the high similarity in PDAC patients between the two sites sampled in the microbiota analysis (gallbladder bile and bile duct brush over the tumor), upon comparison in alpha and beta diversity, is very interesting. This finding shows that microbiota can be the same along the extrahepatic biliary tract despite the presence of a PDAC in the head of the pancreas. As a result, a more accessible anatomic structure such as the gallbladder can act as a surrogate anatomic site for evaluation of the biliary tract microenvironment.

Materials and Methods

Ethics and sample acquisition

The Institutional Human Ethics Committee of CES University and Clinic approved this study (minute number No 115/2017), and written, informed consent was given by all patients. Samples were de-identified before performing microbiota analysis. A surgical pathologist collected a total of 25 samples, a gallbladder bile sample from 3 patients with gallstones (GS), and 22 samples from patients with PDAC in the head of the pancreas (11 gallbladder bile samples: PC gallbladder group, and 11 intrapancreatic bile duct brush samples: PC duct brush group). All patients were Colombian, and residents of Medellín (Colombia). For GS patients, bile was obtained in the operating room immediately after laparoscopic extraction of the gallbladder, puncturing the gallbladder fundus with a syringe, and aspirating at least 5 mL of bile. For PDAC patients, bile was similarly collected by aspirating bile with a syringe from the gallbladder pancreatoduodenectomy specimen and sent to the pathology lab for a frozen section margin report. The cyto-brush was also obtained from the pancreatoduodenectomy specimen sent to the pathology lab for a frozen section margin report, cutting with sterile scissors the common bile duct margin and carefully introducing and moving the cyto-brush inside the duct, without reaching the duodenum. Immediately after collection, bile samples and the brush were transported on ice, aliquoted, and stored at −80 °C until further analysis. Patients with a clinical history of previous malignant neoplasms, chemotherapy, prior biliary tract surgery or biliary stent placement, HIV, pregnancy, chronic pancreatitis, choledocholithiasis, cystic fibrosis, hepatolithiasis, primary biliary cholangitis, liver cirrhosis, primary sclerosing cholangitis, or acute cholecystitis were excluded from this study.

DNA extraction

Total DNA was extracted from gallbladder bile and brush, using the high pure PCR template preparation Kit (Roche Diagnostics), following the manufacturer's recommendations[17]. Briefly, 200 µL of bile were used, in the case of the cyto-brush, the brush was washed with 1 mL of PBS, vortexing for bacteria removal, and extracting 200 µL of the solution. Then, 200 µL of binding buffer plus 40 µL of proteinase K was added to each sample, regardless of sample origin. The mix was incubated at 70 °C for 10 min. Then, 100 µL of isopropanol was added, mixed and centrifuged for 1 minute at 8.000 × g. After centrifugation, 500 µL of inhibitor removal buffer was added and centrifuged for 1 minute at 8.000 × g. 500 µL of Wash Buffer was added and centrifuged for 1 minute at 8.000 × g. For DNA elution, 200 µL of prewarmed elution buffer was added and centrifuged for 1 minute at 8.000 × g. The DNA was stored at −80 °C until further analysis. Negative controls using ultra-pure water alongside the kit reagents were performed, without bacterial DNA retrieval, using the amplification primers verified by gel analysis.

16S rRNA Sequencing and Microbiota Analysis

Amplicon libraries for pair-end (2 x 300 bp) sequencing on the Illumina MiSeq platform (Illumina Inc., San Diego CA, USA) were constructed using universal primers targeted across the V3 and V4 hypervariable regions of the 16S rRNA gene. The 16S rRNA gene was amplified using primers 341F 5′ -CCTACGGGNGGCWGCAG-3′ and 805R 5′-GACTACNVGGGTATCTAATCC-3′ which include overhang adapter sequences at the 5′ end to add multiplexing indices. Fastq files were analyzed using Qiime2-2019.4[18]. The analysis pipeline includes Dada2 for sequence quality control[19] and an in-house trained clas-

sifier based on the Greengenes 13_8 database for taxonomic analysis using a Qiime2 feature-classifier[20]. Thereafter, the feature table [Frequency] was filtered to exclude archaea and bacteria without at least an identified phylum. All the sequences were used to construct a phylogenetic tree with SEPP[21]. Using Qiime2 diversity core-metrics with and without phylogeny, we analyzed the alpha and beta diversity indices comparing them using Kruskal-Wallis or PERMANOVA (permutational analysis of variance) test. A significance threshold of < 0.01was used for p and q values.

We looked for statistically significant differences in relative abundance between the groups, using the command line ANCOM (Analysis of Composition of Microbiomes) (22), comparing condition, anatomic site and taxonomic level. Additionally, a metagenomic inference analysis was performed using Picrust2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States, v2.1.4 beta) (23,24), and the output tables for KEGG (KO), enzyme nomenclature (EC) and pathways were further analyzed with ANCOM to highlight statistically significant frequencies between the groups and among conditions.

Results

A total of 14 patients and 25 samples were included in the study. Samples were divided into three groups; one GS group (N = 3), and two PDAC groups; the PDAC gallbladder group (N = 11) and PDAC brush group (N = 11). In the GS group there were 2 females and 1 male, mean age 47 years, and in the PDAC groups there were 7 males and 4 females, mean age 58 years. We identified a total of 2277953 sequences. The mean sequences per group were 63051 in the GS group, 86972 in the PDAC gallbladder group and 102918 in the PDAC brush group, with a minimum of 48001 (PC brush 8) and a maximum of 194153 (PC brush 11).

After filtering unassigned sequences and archaea, 488 OTUs (operational taxonomic units) were identified; 197 in the GS group and 325 in the PDAC brush group. There were 53 OTUs present in all three groups (shown in Fig. 1). Around 85-95 % of all identified OTUs in each group were comprised of only 3 % of phyla in each group. In the GS group the predominant phyla were Proteobacteria (40 %) followed by Bacteroidetes (30 %) and Firmicutes (16 %). Conversely, in both PDAC groups the predominant phylum was Proteobacteria (64-76 %) followed by Firmicutes (14-25%) and Bacteroidetes (5-6 %). In the same way, through different taxonomic levels such as class, order and family, there are differences in bacterial community structure regarding percentage. At the class taxonomic level, *Gammaproteobacteria* represents 21 %, 73 % and 64 % in the GS, PDAC brush and PDAC gallbladder groups, respectively. For the order taxonomic level, the percentages of *Enterobacteriales* in the PDAC brush (71 %) and PDAC gallbladder (57 %) groups were considerably higher than in the GS group (12 %). In the case of the *Enterobacteriaceae* family, we observed the same trend (12 % in GS, 71 % in PDAC brush and 57% in PDAC gallbladder) (shown in Fig. 2). Nevertheless, the pairwise Kruskal-Wallis analysis for the aforementioned differences compared by group and condition, showed no statistically significant results.

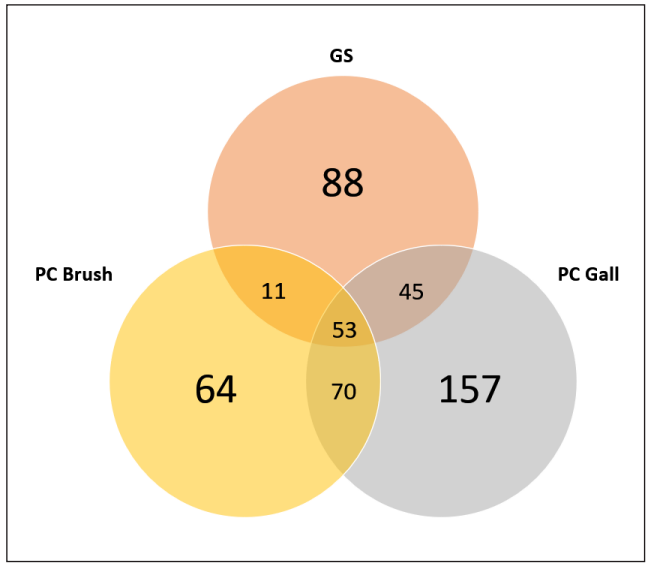

Figura 1. Venn diagram depicting OTUs number per group and shared OTUs. GS: gallstones, PC gall: pancreatic ductal adenocarcinoma gallbladder bile sample group, PC duct: pancreatic ductal adenocarcinoma duct brush sample group

We compared the alpha and beta diversity of the bacterial communities per group (anatomic site) and condition (GS vs PC). Alpha diversity was initially analyzed using Chao1, Shannon and Pielou's evenness index. There were no statistically significant differences upon comparison by group (GS = 3 vs PDAC gallbladder = 11 vs PDAC brush = 11) or condition, (GS = 3 vs PDAC = 22) in Kruskal-Wallis pairwise analysis. Although the Kruskal-Wallis pairwise analysis was not statistically significant, the GS group had higher values than the PDAC groups (shown in Fig. 3), and the PDAC groups have very similar values. Regarding beta diversity, we obtained statistically significant results in the PERMANOVA pairwise analysis (shown in Table 1) for Bray-Curtis and Jaccard indices upon comparison by condition (p and q < 0.01). The remaining pairwise analysis by group and condition for Bray-Curtis, Jaccard, unweighted and weighted unifrac indices were not statistically significant (shown in Fig. 4).

The ANCOM compositional analysis did not reveal any statistically significant differences in relative abundances regarding partially observed species among the groups. Picrust2-ANCOM metagenomic inference pipeline revealed three over-represented enzymes, two over-represented KO and two over-represented pathways (shown in Table 2) that were statistically significant, coinciding in condition and group comparison. All over-represented features were over-represented in the GS group regardless of the comparison made by group or condition.

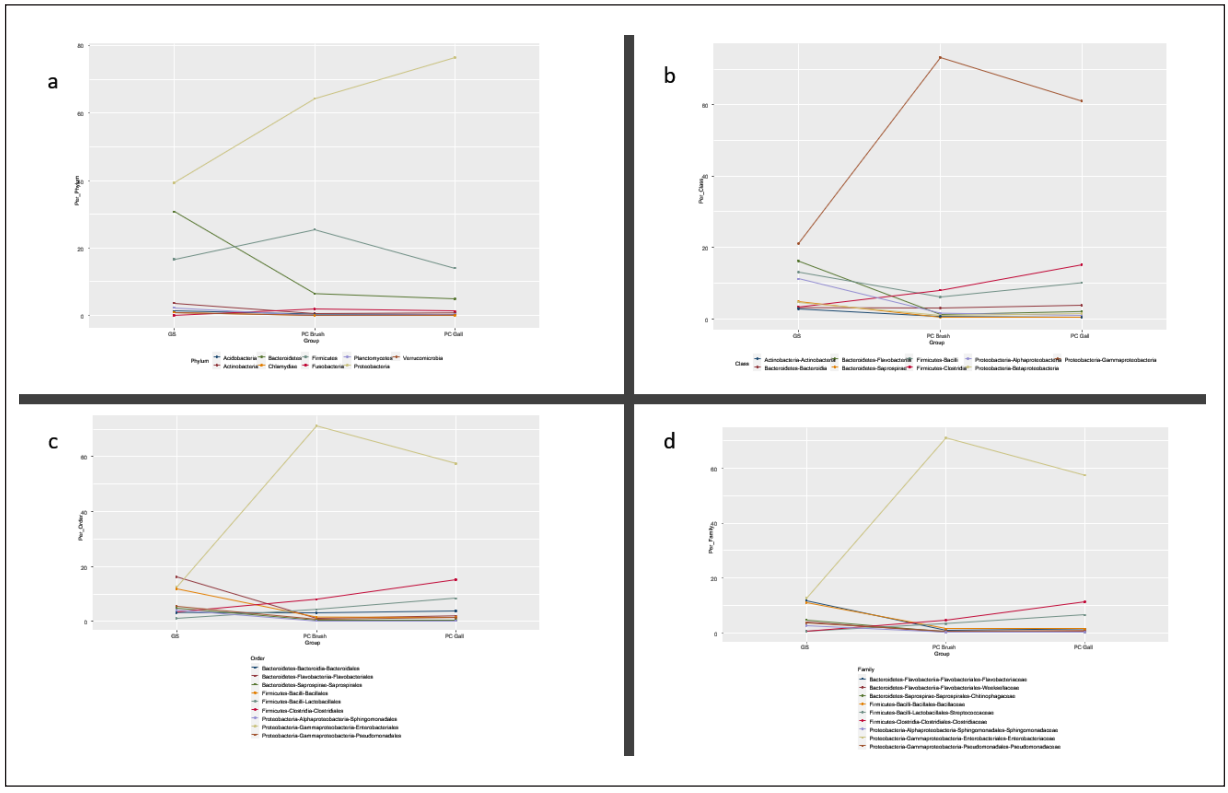

Figura 2. Graphical representation of the 9 % percent higher bacterial community composition for different taxonomic levels (a) phylum, b) class, c) order, d) family). GS: gallstones, PC gall: pancreatic ductal adenocarcinoma gallbladder bile sample group, PD duct: pancreatic ductal adenocarcinoma duct brush sample group

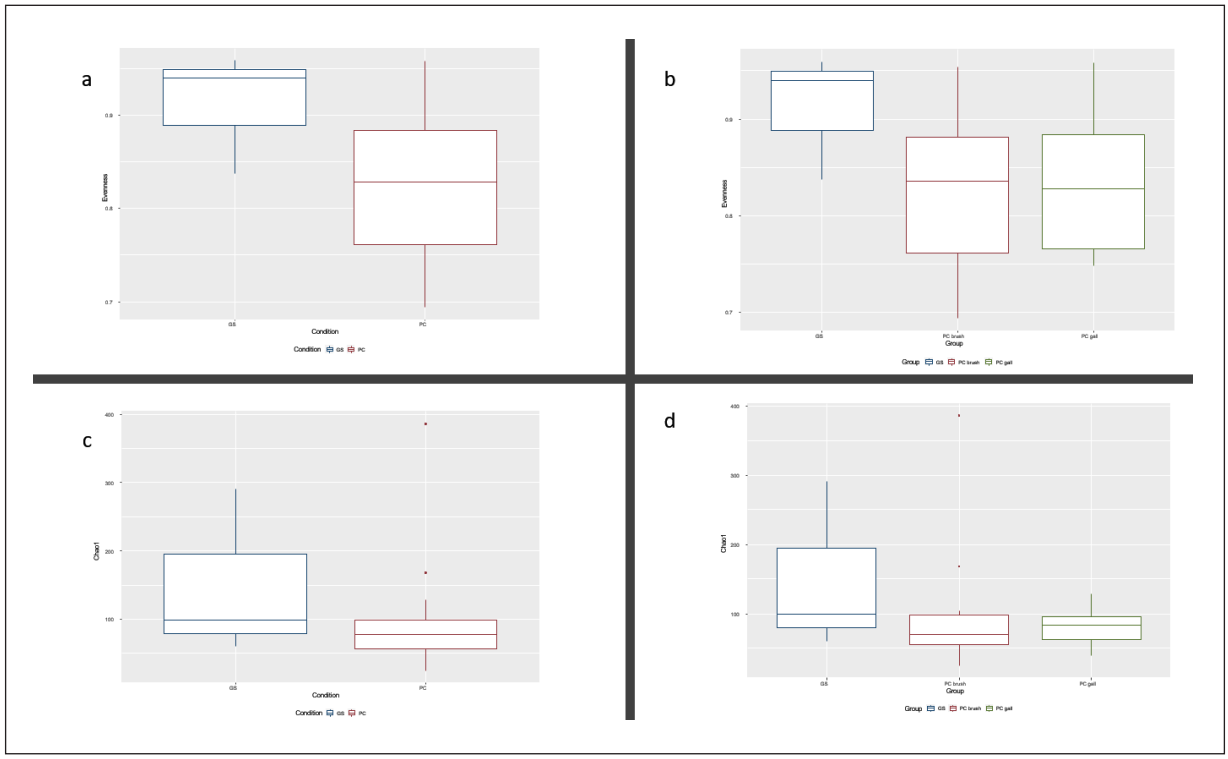

Figura 3. Boxplot of evenness (a) and b)) and chao1 (c) and d)) alpha diversity indices for group and condition. GS: gallstones, PC gall: pancreatic ductal adenocarcinoma gallbladder bile sample group, PD duct: pancreatic ductal adenocarcinoma duct brush sample group

Table 1. Beta diversity indices comparison by group and condition

Bray Curtis					
Condition	**Condition**	**Sample size**	**pseudo-F**	**p-value**	**q-value**
GS	PDAC	25	2,93155559	0,004	0,004
Group 1	**Group 2**	**Sample size**	**pseudo-F**	**p-value**	**q-value**
GS	PC brush	14	2,67848469	0,008	0,012
GS	PC gall	14	2,3880233	0,007	0,012
PC brush	PC gall	22	0,47773149	0,964	0,964
Jaccard					
Condition	**Condition**	**Sample size**	**pseudo-F**	**p-value**	**q-value**
GS	PDAC	25	1,82410608	0,003	0,003
Group 1	**Group 2**	**Sample size**	**pseudo-F**	**p-value**	**q-value**
GS	PC brush	14	1,65153525	0,01	0,015
GS	PC gall	14	1,64696108	0,01	0,015
PC brush	PC gall	22	0,65904797	0,999	0,999
Unweighted Unifrac					
Condition	**Condition**	**Sample size**	**pseudo-F**	**p-value**	**q-value**
GS	PDAC	25	2,01048541	0,018	0,018
Group 1	**Group 2**	**Sample size**	**pseudo-F**	**p-value**	**q-value**
GS	PC brush	14	1,69027063	0,036	0,054
GS	PC gall	14	1,99074787	0,013	0,039
PC brush	PC gall	22	0,71076354	0,954	0,954
Weighted Unifrac					
Condition	**Condition**	**Sample size**	**pseudo-F**	**p-value**	**q-value**
GS	PDAC	25	3,46406551	0,032	0,032
Group 1	**Group 2**	**Sample size**	**pseudo-F**	**p-value**	**q-value**
GS	PC brush	14	3,62315922	0,041	0,061
GS	PC gall	14	2,64263612	0,028	0,061
PC brush	PC gall	22	0,29186192	0,878	0,878

GS: gallstones, PC brush: pancreatic ductal adenocarcinoma patients' brush sample, PC gall: pancreatic ductal adenocarcinoma patients' bile sample.

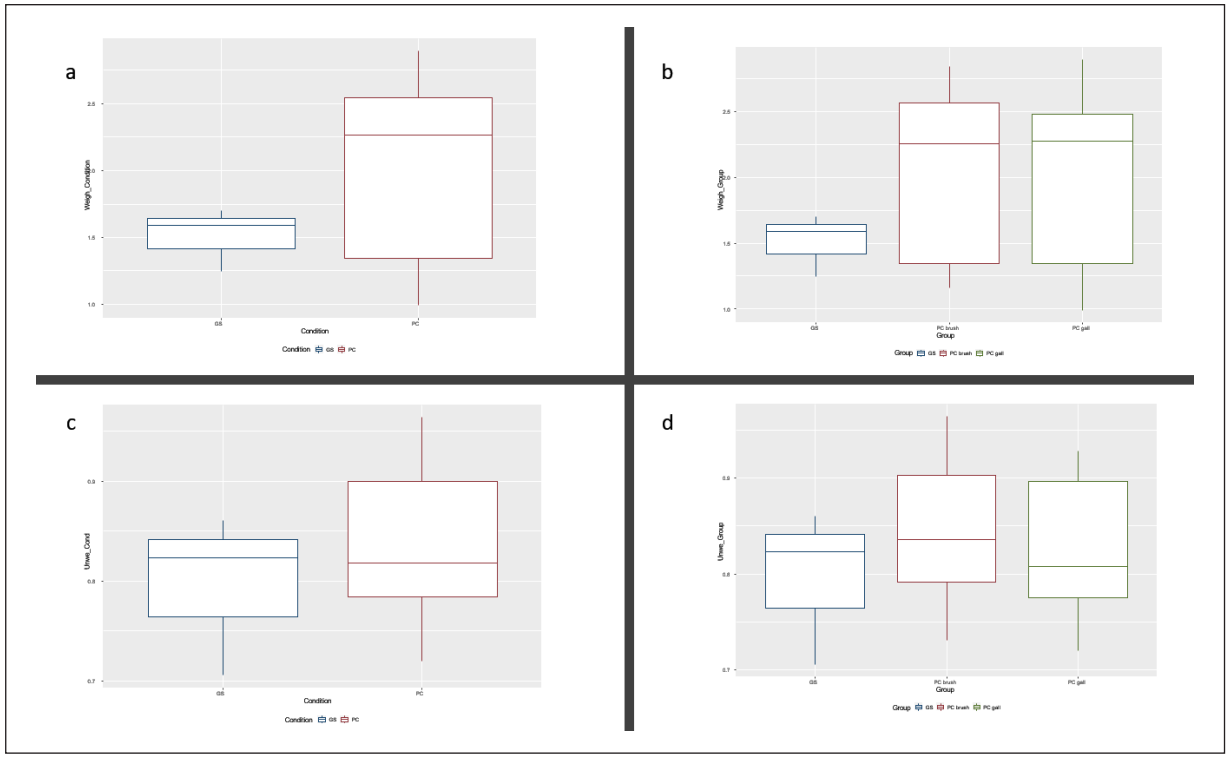

Figura 4. Boxplot of weighted (a) and b)) and unweighted (c) and d)) unifrac beta diversity indices for group and condition. GS: gallstones, PC gall: pancreatic ductal adenocarcinoma gallbladder bile sample group, PD duct: pancreatic ductal adenocarcinoma duct brush sample group

Table 2. Statistically significant, over-represented signaling pathways in metagenomic inference analysis GS group.

Metagenomic Inference	Condition		Group	
Enzyme	**EC**	**W**	**EC**	**W**
Glutaminyl-peptide cyclotransferase	EC:2.3.2.5	1131	EC:2.3.2.5	980
L-arabinose 1-dehydrogenase	EC:1.1.1.376	1074	EC:1.1.1.376	962
Corrinoid/iron-sulfur protein Co-methyltransferase	EC:2.1.1.245	1019	EC:2.1.1.245	802
Staphylopain	EC:3.4.22.48	952		
KEGG Orthology	**KO**	**W**	**KO**	**W**
L-arabinose 1-dehydrogenase	K13873	3099	K13873	2009
Glutaminyl-peptide cyclotransferase	K00683	3261	K00683	1624
Pathway	**Nomenclature**	**W**	**Nomenclature**	**W**
Pyrimidine deoxyribonucleotides biosynthesis	PWY-7210	245	PWY-7210	230
Isoprene biosynthesis	PWY-7391	243	PWY-7391	223
Pyrimidine deoxyribonucleotides de novo biosynthesis	PWY-7198	240		

GS: gallstone group, EC: enzyme nomenclature, KEEG: Kyoto encyclopedia of genes and genomes, KO: KEEG identifier

Discussion

Alpha and beta diversity analysis suggest a competitive microenvironment and phylogenetic similarities. The analyzed alpha diversity indices, while not statistically significant, point out a possible, more equitable distribution of species regarding richness and relative abundance in the GS group, and similarities in the PDAC groups. The more equitable distribution in the GS vs PDAC groups can be interpreted as an indirect sign of competence in the PC groups where just a few species dominate the bacterial community composition at the different taxonomic levels[25]. The beta diversity results for Bray-Curtis and Jaccard indices were statistically significant upon comparison by condition, but these results cannot be extended to other indices such as unweighted and weighted unifrac. However, PERMANOVA pairwise results for beta diversity comparing PDAC groups were almost identical, highlighting that perhaps the biliary tract, a semi-closed system, has similar beta diversity distributions among different anatomic locations. Interestingly, this beta diversity similarity remains unchanged along the biliary tract despite PDAC in the head of the pancreas.

There are just a few studies comparing the biliary tract microbiota in PDAC patients. Thomas et al., investigated the impact of microbiota in pancreatic carcinogenesis using tissue samples, amplifying and sequencing the 16S rRNA V1-V3 regions. The study enrolled 26 patients with pancreatic disease, six normal (no disease), four with pancreatitis and sixteen with PDAC. There were no significant differences in bacterial associations between pathological stage, beta diversity and bacterial taxa. Differences in Chao1 and Shannon indices among normal and pancreatic cancer, after false discovery rate correction[26] gave similar results. Del Castillo and colleagues characterized microbiota in PDAC patients using normal surrounding fresh or neoplastic frozen tissue. They obtained swabs and tissue samples of normal pancreas, pancreatic tumor, normal bile duct, stomach, duodenum, jejunum, ileum and stools. Microbiome analysis was carried out, amplifying and sequencing the 16S rRNA V3-V4 gene regions. They found that bacterial profiles in duodenal and pancreatic tissue were very similar in the same patient, and that upon comparison of PDAC vs non-PDAC patients, the relative abundance of genus *Lactobacillus* was significantly increased in non PDAC patients compared to *Fusobacterium spp*, which was significantly increased in PDAC patients[27]. Finally, Riquelme et al., designed a multicenter approach that focused on describing PC microbiota in long-term PDAC survivors (> 5 years, N = 22, MD Anderson patients), regular PDAC survivors (< 5 years, N = 21 MD Anderson patients, N = 10 Johns Hopkins patients) and very long-term PDAC survivors (> 10 years, N = 15 Johns Hopkins patients), matched by age, gender and prior neo or adjuvant therapies. They used formalin-fixed paraffin-embedded tissue (FFPE), amplifying the V4 region. They found that alpha diversity measured by Shannon and Simpson indices in long-term and very long-term PDAC survivor groups was higher than the regular PDAC survivor groups. Additionally, long-term PDAC survivors showed a differential abundance of Proteobacteria (*Pseudoxanthomonas*) and Actinobacteria (*Saccharopolyspora and Streptomyces)*, proposing this microbiota profile as a signature for better PDAC outcomes[28].

To the best of our knowledge this is the first study comparing microbiota using 16S rRNA in two anatomic locations of the biliary tract in PDAC patients (bile and biliary tract brush over pancreatic tumor). Like the research of Thomas et al, differences in alpha diversity indices were not statistically significant, but in the present study it was considered that the lower values of the alpha diversity indices in the PDAC groups may be related to competitive bacterial exclusion. In the relative abundance analysis using ANCOM pipeline in Qiime2, a specific bacterial signature associated with PDAC was not identified. There are many studies with relevant findings of specific bacterial signatures in PDAC patients, both locally and in different anatomic locations. Despite this, specific bacterial signatures cannot be translated to clinical practice, cannot be included in a carcinogenesis model or even be considered a risk factor amenable to detection and control. For these reasons, a paradigm shift approach in PDAC research is required. The results of the metagenomic inference analysis, while sta-

tistically significant, are very complex for inferring meaningful biological information. Further studies are needed from an integrated, complementary analysis standpoint, such as metaproteomics, to correlate the microbiota profile, diversities, metagenomic inference, and differential relative abundance with human and bacterial protein profiles. We hypothesize that through this integrative model, we may find the elusive biological information that will allow the natural history of PDAC to be improved.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this investigation. The Institutional Human Ethics Committee of CES University and Clinic approved this study, and written, informed consent was given by all patients. Complying with the guidelines for human studies in accordance with the World Medical Association Declaration of Helsinki

Right to privacy and informed consent. The authors declare that no data that enables identification of the patients appears in this article.

Conflict of interest. The authors declare that the revision was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

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Author Contribution Statement. AAA, MSJ and NCC designed the study. AAA participated in sample acquisition. MSJ, NCC, and AAA participated in the laboratory procedures, microbiota analysis, and biological pathway interpretation. AAA and NCC wrote the manuscript. All the authors participated in manuscript revision.

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