Characterization of the Gut Microbiota in Patients with Primary Sclerosing Cholangitis Compared to Inflammatory Bowel Disease and Healthy Controls

Samaneh Ostadmohammadi
Shahid Beheshti University of Medical Sciences: Shaheed Beheshti University of Medical Sciences
Masoumeh Azimirad
Shahid Beheshti University of Medical Sciences Research Institute for Gastroenterology and Liver Diseases
Hamidreza Houri
Shahid Beheshti University of Medical Sciences Research Institute for Gastroenterology and Liver Diseases
Kaveh Naseri
Shahid Beheshti University of Medical Sciences Research Institute for Gastroenterology and Liver Diseases
Ehsan Javanmard
Shahid Beheshti University of Medical Sciences Research Institute for Gastroenterology and Liver Diseases
Hamed Mirjalali
Shahid Beheshti University of Medical Sciences Research Institute for Gastroenterology and Liver Diseases
Abbas Yadegar (a.yadegar@sbmu.ac.ir)
Shahid Beheshti University of Medical Sciences Research Institute for Gastroenterology and Liver Diseases
Amir Sadeghi
Shahid Beheshti University of Medical Sciences Research Institute for Gastroenterology and Liver Diseases
Hamid Asadzadeh Aghdaei
Shahid Beheshti University of Medical Sciences Research Institute for Gastroenterology and Liver Diseases
Mohammad Reza Zali
Shahid Beheshti University of Medical Sciences Research Institute for Gastroenterology and Liver Diseases

Research Article

Keywords: Gut microbiota, Primary sclerosing cholangitis, Inflammatory bowel disease, Ulcerative colitis, Real-time PCR

Posted Date: April 27th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-431119/v1

Version of Record: A version of this preprint was published at Molecular Biology Reports on July 25th, 2021. See the published version at https://doi.org/10.1007/s11033-021-06567-8.

Abstract

Primary sclerosing cholangitis (PSC) is a chronic cholestatic liver disease. Its aetiology remains largely unknown, although frequent concomitant inflammatory bowel disease (IBD) hints towards common factors underlying intestinal and bile duct inflammation. Herein, we aimed to explore the relative abundance of fecal microbiota in PSC-IBD patients compared to IBD-only subjects and healthy controls.

We included 14 PSC-IBD patients, 12 IBD patients, and 8 healthy controls (HCs). A quantitative real-time PCR (qPCR) assay was to determine a selection of bacterial phyla, families, and genera.

Relative abundance of taxa showed that *Bacteroidetes* was the most abundant phylum among the patients with PSC-IBD (29.46%) and also HCs (39.34%), whereas the bacterial species belonging to the phylum *Firmicutes* was the most frequent group in IBD-only subjects (37.61%). The relative abundance of *Enterobacteriaceae* family was higher among PSC-IBD (3%) than HCs (0.5%), and thus, could be used as a PSC-associated microbial signature.

Our findings showed that intestinal microbiota composition in PSC-IBD patients was completely different from that of IBD-only patients. Further studies using large-scale cohorts should be performed to better describe the contribution of the gut microbiota to PSC pathogenesis with underlying IBD.

Introduction

Primary sclerosing cholangitis (PSC) is an immune-mediated, chronic cholestatic liver disease of unknown aetiology, characterized by inflammation and fibrosis of the intra- and extrahepatic bile ducts [1]. It is a progressive disorder without effective medical treatment, which eventually leads to cirrhotic end-stage liver disease and represents a major risk factor for cholangiocarcinoma, which indicates the need for a liver transplantation. However, PSC recurrence occurs in up to 20% of patients after liver transplantation and the median survival time until death is estimated about 20 years [2, 3]. The pathogenesis of PSC has long served as a controversial point of debate, with current evidence suggesting that the exposure of genetically predisposed individuals to environmental antigens elicits an aberrant immune response, leading to development of the disease [4].

PSC is strongly associated with inflammatory bowel disease (IBD), in which 60–80% of the subjects with PSC have concomitant IBD, predominantly ulcerative colitis (UC) [5, 6]. PSC patients with underlying IBD display a distinct phenotype as compared to IBD alone, with a predominance of pancolitis in PSC-UC patients and invariably colonic involvement in PSC-Crohn's disease (CD) patients [7, 8]. Hence, these data suggest that an inflamed colon, but not small bowel, is of importance in PSC development and the disease is mainly associated with right-sided colonic involvement [7].

The association between PSC and IBD could in part be explained by shared immune-related genetic susceptibility [9]. Accordingly, immune-related gene polymorphisms, particularly genetic variations in inflammatory pathways, associated with both PSC and IBD, may increase the abundance of the

deleterious intestinal microbiota capable to translocate from the gut to the liver. Moreover, some immune-related variants enhance the sensitivity of the biliary epithelium to bacterial antigens, leading to chronic inflammation and fibrosis [10]. However, genetic predisposition has only clarified a fraction of disease risk, signifying that environmental factors could also play a major role in disease development. Among potential contributing environmental factors, alternations in the gut microbiota composition, i.e., intestinal dysbiosis, are hypothesized to have important role in the disease process as they mediate intestinal inflammatory responses by local effects and hepatic inflammation through the entero-hepatic circulation of bacterial antigens [11]. In addition, a few small clinical trials of antibiotics on PSC have indicated that both vancomycin and metronidazole can reduce alkaline phosphatase in patients with PSC, however, so far not proven clinically effective [12, 13]. Moreover, some evidence previously revealed that biliary histological and cholangiographical abnormalities which found in animal models of small intestinal bacterial overgrowth (SIBO) resemble features of PSC [11]. Collectively, these data suggest that manipulation of the gut microbes could be directly involved in the pathogenesis of PSC as well as PSC-IBD.

Alterations in the diversity and composition of the gut microbiota have lately been implicated in the pathogenesis of several inflammatory and metabolic gut disorders, particularly IBD and PSC. However, to the best of our knowledge, no data are available on the gut microbiota composition among Iranian patients with PSC-IBD so far. Therefore, the aim of the current study was to compare the structure of the gut microbiota of Iranian patients with PSC-IBD compared to patients with IBD-only without liver disease as well as healthy controls (HCs).

Materials And Methods

Study population

PSC patients with concomitant IBD, IBD-only patients, and non-IBD HCs were involved from February 2018 to July 2019. The study protocol was approved by the Institutional Ethical Review Committee of Research Institute for Gastroenterology and Liver Diseases (RIGLD) at Shahid Beheshti University of Medical Sciences (Project No. IR.SBMU.RIGLD.REC.1395.140). All experiments were performed in accordance with relevant guidelines and regulations recommended by the institution and informed consents were obtained from all subjects and/or their legal guardians prior to sample collection. Patients with non-transplanted PSC referred to the Research Institute for Gastroenterology and Liver Diseases at Taleghani Hospital in Tehran, Iran, were eligible for the current study. The diagnosis of PSC was made according to accepted clinical, radiological and pathological criteria including: chronically elevated alkaline phosphatase (ALP) and γ-glutamyl transferase (GGT); standard radiological evidence of PSC on endoscopic retrograde cholangiopancreatography (ERCP); and/or typical findings on liver histology consistent with PSC; as well as no evidence of secondary cholangitis [14]. All patients with PSC had also undergone screening for IBD. The IBD diagnosis was made by clinical, endoscopic and pathological findings compatible with IBD based on the Lennard Jones criteria [15]. Patients who had been receiving antibiotics, ursodeoxycholic acid, corticosteroid, or immunosuppressant medications were excluded.

Moreover, subjects with a previous orthotopic liver transplant, bowel resection, or on specific diets (e.g., vegan, vegetarian, gluten-free and milk-free diets) were also excluded from this study. Additionally, patients with UC without a medical history of liver disorders, and in clinical remission, were recruited. HC subjects were randomly selected from individuals who were scheduled for surveillance colonoscopy for diagnostic purposes. Demographic data, medical history, clinical presentation, and medication of subjects fulfilling inclusion criteria were assessed by using a validated questionnaire. Laboratory biochemical parameters were retrieved from hospital databases, including total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), ALP, GGT, albumin, creatinine, platelets, international normalized ratio, as well as, perinuclear anti-neutrophil cytoplasmic antibody (P-ANCA) status. Mayo risk scores were calculated using the algorithm for the revised Mayo risk score [16]. A flowchart of inclusion of subjects and study design is shown in Fig. 1.

Sample collection, handling and DNA extraction

Fresh stool samples were collected using Stool Collection Tubes via a standardized collection device from all participants after voiding. All samples were homogenized thorough agitation using a vortex and divided into aliquots within 3 h of defecation. The aliquots were immediately frozen and stored at -80°C in screw-capped cryovial tubes until used for DNA extraction. Total DNA content was extracted from stool samples using the QIAamp DNA Stool Mini Kit (Qiagen Retsch GmbH, Hannover, Germany) according to the manufacturer's instructions with some minor modifications. The concentration and purity of extracted DNA were assessed by NanoDrop ND-2000 Spectrophotometer (NanoDrop products, Wilmington, DE, USA). Extracted DNA samples were stored at -20°C until further analysis.

Taxonomic Profiling Of Gut Microbiota By Quantitative Real-time Pcr

In the current study, a quantitative real-time PCR (qPCR) assay was performed for determining a selection of bacterial taxa (phyla, families, and genera) including *Eubacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Gamma-proteobacteria*, *Epsilon-proteobacteria*, *Verrucomicrobia*, *Enterobacteriaceae*, *Clostridium coccoides* group, *Clostridium* Cluster I, *Bifidobacterium*, *Lactobacillus*, *Enterococcus*, and *Staphylococcus*. The qPCR was performed using universal and group-specific primers based on the bacterial 16S rRNA sequences and reaction conditions as described previously with minor modifications (Table 1). Each PCR reaction was performed in a final volume of 25 µL, comprising of 12.5 µL of SYBR green PCR master mix (Ampliqon, Odense, Denmark), 1 µL of 10 pmol of forward and reverse primers, and 100 ng of the DNA template. The temperature profile for the amplification was 10 min at 95°C, followed by 40 cycles of 20s at 95°C, 30s of annealing at optimal temperature for each primer pair as indicated in Table 1, and 20s at 72°C. All PCR amplifications were carried out in triplicate by using a Rotor-Gene® Q (Qiagen, Germany) real-time PCR system. The specificity of amplification was determined by the melting curve analysis with increasing temperature from 60 to 95°C (at regular increment of 0.5°C for 5s). The relative abundance of each taxon among PSC-IBD patients as well as IBD-only and HC subjects was calculated according to the method described previously by Bacchetti et al. [17].

Accordingly, the average Ct value obtained from each primer pair was transformed into a percentage using the following formula:

Table 1
The gut microbiota taxon-specific primers used in this study.

Target taxon	Primer name	Primer sequence (5′-3′)	Amplicon length (bp)	Reference
Eubacteria	UniF340	ACTCCTACGGGAGGCAGCAGT	~ 200	[37]
	UniR514	ATTACCGCGGCTGCTGGC		
Firmicutes	Firm934-F	GGAG**Y**ATGTGGTTTAATTCGAAGCA	~ 129	[38]
	Firm1060-R	AGCTGACGACAACCATGCAC		
Bacteroidetes	Bac960-F	GTTTAATTCGATGATACGCG	~ 137	[38]
	Bac1100-R	TTAAGCCGACACCTCACG		
Actinobacteria	Actino-F	GCG**K**CCTATCAGCTTGTTGGTG	~ 333	[39]
	Actino-R	CCGCCTACGAGC**Y**CTTTACGC		
Gamma-proteobacteria	Gamma395-F	C**M**ATGCCGCGTGTGTGAA	~ 498	[17]
	Gamma871-R	ACTCCCCAGGCGGTC**D**ACTTA		
Epsilon-proteobacteria	Epsilon-F	TGGTGTAGGGGTAAAATCCG	~ 286	[39]
	Epsilon-R	AGGTAAGGTTCTTCG**Y**GTATC		
Verrucomicrobia	Ver1165-F	TCA**K**GTCAGTATGGCCCTTA	~ 123	[38]
	Verru-R	GCAGCCTACAGTCCGAAC		This study
Enterobacteriaceae	Enterob-F	CGTCGCAAGM**M**CAAAGAG	~ 351	[39]
	Enterob-R	TTACCGCGGCTGCTGGCAC		
Clostridium coccoides group	g-Ccoc-F	AAATGACGGTACCTGACTA	~ 438	[40]
	g-Ccoc-R	CTTTGAGTTTCATTCTTGCGA		
Clostridium cluster I	Chis150-F	AAAGGAAGATTAATACCGCATA	~ 538	[41]
	ClostI-R	TTCTTCCTAATCTCTACGCA		
Bifidobacterium spp.	Bifid-F	GGGATGCTGGTGTGGAAGAG	~ 200	[42]
	Bifid-R	TGCTCGCGTCCACTATCCAG		
Lactobacillus spp.	Lacto-F	TGGATGCCTTGGCACTAG	~ 89	[42]
	Lacto-R	AAATCTCCGGATCAAAGCTTAC		

The nucleotides in bold type represent: Y, C or T; K, G or T; M, A or C; D, A or G or T; R, A or G.

Target taxon	Primer name	Primer sequence (5′-3′)	Amplicon length (bp)	Reference
Enterococcus spp.	Str1-F	GTACAGTTGCTTCAGGACGT	~ 195	[42]
	Str2-R	GTTCGATTTCRTCACGTTG		
Staphylococcus spp.	Staph-F	GAACGTGGTCAAATCAAAG	~ 328	[43]
	Staph-R	CAACACCAGTTACGTCAGTAG		
The nucleotides in bold type represent: Y, C or T; K, G or T; M, A or C; D, A or G or T; R, A or G.				

$$X = \frac{(\mathbf{Eff.Univ})^{\mathrm{Ct\,univ}}}{(\mathbf{Eff.Spec})^{\mathrm{Ct\,spec}}} \times 100$$

where, the Eff.Univ refers to the calculated efficiency of the universal primers for *Eubacteria* (2 = 100% and 1 = 0%) and Eff.Spec indicates the efficiency of the taxon-specific primers. Ct univ and Ct spec represent the threshold cycles registered by the thermocycler. “X” represents the percentage (%) of 16S taxon-specific copy number existing in a sample.

Statistical analysis

Mann-Whitney and Student t tests were used for analysis of parametric and Spearman's correlation was used for non-parametric data. The principal component analysis (PCA) was conducted to determine if there were large patters of variability in the data using the FactoMineR package from the open-source statistical program R version 3.6.1 (R Core Team, Vienna, Austria). All graphs were prepared using GraphPad Prism software version 5.04 (GraphPad software, San Diego, CA, USA). Differences were considered to be statistically significant when p value < 0.05.

Results

Baseline demographics and clinical characteristics of the subjects

In total, 14 PSC patients with concomitant IBD, 12 age, gender and BMI-matched IBD-only (UC) patients without PSC and 8 age, gender and BMI-matched non-IBD HC subjects were included in this study after exclusions and data quality control. PSC-IBD group in our study classified as UC in 12 patients (85.7%) and as CD in two patients (14.3%). None of the included PSC-IBD patients had overt cirrhosis. All PSC-UC patients had a pancolitis, one PSC-CD patients had colonic disease localization, and one had localization of disease in both the terminal ileum and colon. Ten of UC patients (83.3%) had pancolitis and two patients had left-sided colitis (16.7%). Non-IBD HC subjects had a blank medical history and none of them used immunosuppressive therapy. PSC-IBD patients, UC patients and non-IBD HCs were not

exposed to therapeutic antibiotic treatment during the last 4 weeks before fecal sample collection. Baseline demographics and clinical characteristics of three cohorts are summarized in Table 2.

Table 2
Baseline demographic and clinical characteristics of subjects enrolled in this study

Patients' characteristics	**PSC-IBD (*n* = 14)**	**IBD (UC) (*n* = 12)**	**HC (*n* = 8)**
Gender			
Female (%)	5 (35.7)	3 (25)	3 (37.5)
Male (%)	9 (64.3)	9 (75)	5 (62.5)
Age (years)			
Median age ± SD	41.07 ± 12.3	43 ± 11.9	35.8 ± 7.53
Median age at diagnosis of PSC	34 ± 11.9	NA	NA
Median BMI (kg/m^2)	23.45	24.1	21
Smoker status (%)			
Never	13 (92.9)	12 (100)	7(87.5)
Ever	1 (7.1)	0	1 (12.5)
Unknown	0	0	0
Type of IBD			
UC/IBD	12 (85.7)	12 (100)	0
CD/IBD	2 (14.3)	0	0
Disease-specific variables			
IBD duration, median years	15.5 (4–31)	8 (2–12)	NA
PSC disease duration (years ± SD)	7 (1–10)	NA	NA
PSC mayo score, median years (IQR)	0.5 (1.6–2.1)	NA	NA
Medication at the time of colonoscopy			
Unknown	0	0	
5-ASA (%)	2 (14.3)	3 (25)	NA
UDCA (%)	4 (28.6)	0	NA
Anti-TNF (%)	1 (7.1)	0	NA

PSC: primary sclerosing cholangitis; IBD: Inflammatory bowel disease; BMI: body mass index; UC: ulcerative colitis; CD: Crohn's disease; 5-ASA: 5-aminosalicylic acid; UDCA: ursodeoxycholic acid; PPI: proton pump inhibitor; CA-19-9: carbohydrate antigen 19 – 9; AST: aspartate aminotransferase; ALT: alanine aminotransferase; NA: not applicable.

Patients' characteristics	PSC-IBD (*n* = 14)	IBD (UC) (*n* = 12)	HC (*n* = 8)
Corticosteroids (%)	2 (14.3)	4 (33.3)	NA
Immunosuppression (%)	2 (14.3)	1 (8.3)	NA
PPI	0	3 (25)	
Underlying conditions			
Cirrhosis	0	0	NA
Pouch	0	0	NA
Liver transplantation	0	0	NA
Laboratory parameters			
Platelet count, 10^9/L, median (min–max)	220 (57–590)	242 (189–472)	250 (195–320)
Creatinine, mmol/L, median (min–max)	0.8 (0.7–1.1)	0.7 (0.5–1.8)	0.67 (0.4–0.8)
Albumin, g/L, median (min–max)	3.9 (3.2–4.3)		NA
CA 19–9	4.9 (0.6–35.7)	1.1 (0.9–4.2)	NA
AST (UI/L) median (min–max)	24 (16–74)	17 (10–46)	NA
ALP (UI/L) median (min–max)	23 (12–227)	20 (8-171)	NA
Alkaline phosphatase (UI/L)	208 (132–363)	177 (61–335)	NA
Total Bilirubin, mmol/L, median (min–max)	0.1 (0.1–13)	0.5 (0.2–1.8)	NA
Direct Bilirubin, mmol/L, median (min–max)	0.3 (0.1–1.1)	0.2 (0.1-7)	NA

PSC: primary sclerosing cholangitis; IBD: Inflammatory bowel disease; BMI: body mass index; UC: ulcerative colitis; CD: Crohn's disease; 5-ASA: 5-aminosalicylic acid; UDCA: ursodeoxycholic acid; PPI: proton pump inhibitor; CA-19-9: carbohydrate antigen 19 – 9; AST: aspartate aminotransferase; ALT: alanine aminotransferase; NA: not applicable.

Relative Abundance Of Analyzed Microbiota

Analysis of the relative abundance of taxa using the 16S rRNA qPCR assay showed that *Bacteroidetes* was the most abundant phylum among the patients with PSC-IBD (29.46%) and HCs (39.34%), whereas the bacterial strains belonging to the phylum *Firmicutes* was the most frequent group detected in IBD-only patients (37.61%). Our findings also showed that the relative abundance of *Enterobacteriaceae* family in fecal samples of PSC-IBD patients was similar to those with IBD-only, which was significantly higher than HCs (*p value* = 0.031). Interestingly, when performing a relative comparison of the investigated taxa between PSC-IBD and IBD-only cohorts, three genera including *Enterococcus*,

Lactobacillus, and *Bifidobacterium* had different abundances in PSC-IBD and IBD-only, and were observed to be enriched in IBD-only patients.

The relative abundance and mean percentage of each bacterial group among PSC-IBD and IBD patients, as well as HCs are shown in Fig. 2 and Fig. S1. Distribution of the explored bacterial phyla and genera among PSC-IBD and IBD-only patients, as well as HCs is illustrated in Fig. 3. The PCA also revealed that taxonomic profiles were notably different in the microbial communities of IBD patients compared to PSC-IBD patients and HCs, as schematically described in Fig. 4.

Determination of Firmicutes**/**Bacteroidetes **ratio**

Our measurements of the *Firmicutes/ Bacteroidetes* ratio achieved by qPCR indicated that this ratio was significantly higher in IBD-only subjects compared to both PSC-IBD patients (*p value* = 0.0013) and HCs (*p value* = 0.01). Moreover, this ratio was lower, albeit not significantly, in PSC-IBDs compared to HCs (*p value* = 0.56). Our experiments also showed that the *Firmicutes/ Bacteroidetes* ratio was significantly higher among IBD patients than both PSC-IBDs and HCs (Fig. 5).

Discussion

In this study, fecal samples of 14 patients with PSC-IBD, 12 subjects with IBD-only and 8 HCs from an academic medical center in Tehran, Iran, were analyzed to investigate the relative abundance of the gut microbiota. As main results, we were not able to demonstrate a consistently significant difference in microbial abundance, at the phylum level, in PSC-IBD cohorts compared to HCs. However, our results showed that family *Enterobacteriaceae* was significantly more abundant in patients with PSC-IBD and IBD-only compared to HCs.

One of the important observations in this report was that the estimated relative abundances of fecal microbiota found to be similar between the PSC-IBD cohorts and HCs, which were significantly different from the IBD-only patients. These results are in contrast with earlier findings showing a significant difference in the gut microbiota of patients with PSC compared with controls [10, 18], however, are in accordance with those indicating gut microbiota differences between PSC-IBD and IBD-only patients [18–20]. These data suggest a strong effect of other factors such as host genetic determinants, ethnic background, and diet on the fecal microbiome composition. Moreover, as the recruited patients in our study were from various geographical regions and ethnicities, the geographical distance of samples could remarkably affect the gut microbiota composition [21, 22].

In our study, altered abundances of few bacterial groups contributed to the unique gut microbial signature found in PSC-IBD patients compared to HCs, including a marked enrichment of the *Enterobacteriaceae* family and a decrease in phylum *Verrucomicrobia* and *Clostridium coccoides* group. Recently, Nakamoto et al. have described that the pathobiont community of *Enterobacteriaceae* family are associated with intestinal barrier dysfunction, liver inflammation and progressive fibrotic conditions in PSC patients [23]. Several authors previously described that the members of the *Enterobacteriaceae* family are often

observed in the microbiota of individuals with hepatobiliary diseases, such as primary biliary cholangitis (PBC) and liver cirrhosis [24, 25]. Recently, Nakamoto et al. identified *Klebsiella pneumoniae* and *Proteus mirabilis* in the microbiota of patients with PSC and demonstrated that these bacterial strains disrupt the intestinal epithelial barrier to increase the permeability and bacterial translocation, inducing strong hepatobiliary inflammatory responses [26]. Moreover, it has been long demonstrated that the most biliary infection in patients with obstructive biliary disorders are caused by the members of *Enterobacteriaceae* such as *Escherichia coli*, *K. pneumoniae* and *P. mirabilis* [27–29]. In a study conducted by Pohl et al., it was found that *Enterobacteriaceae* members were detected in the bile specimens of 40% of PSC patients with dominant stenosis, suggesting an important role of these bacteria in the progression of PSC [30]. Although, such data suggest that the human gut *Enterobacteriaceae* pathobionts have a significant role in the IBD pathogenesis [31], the evident association between the overgrowth of *Enterobacteriaceae* pathobionts and PSC warrant further investigations.

Our qPCR analysis revealed that while the relative abundance of fecal microbiota of patients with IBD without PSC was different from HCs in our study, as reported by others [32], IBD-only cohort also showed a different microbial abundance from PSC patients with concomitant IBD. Kummen et al. previously described that the occurrence of IBD could not influence the gut microbiota composition in PSC-IBD patients, signifying that patients with PSC exhibit a gut microbial signature distinct from IBD disease [18].

In our study, the *Firmicutes/ Bacteroidetes* ratio was analyzed among cohorts and it was significantly higher among IBD-only patients than both PSC-IBD and HC cohorts. Interestingly, there was no significant differences in the *Firmicutes/ Bacteroidetes* ratio between PSC-IBD patients and HCs. In contrary some previous studies indicated a relative depletion of *Firmicutes*, such as *Faecalibacterium* and *Coprococcus* in PSC patients [33]. Moreover, the *Firmicutes/ Bacteroidetes* ratio could describe the degree of dysbiosis in IBD. Some authors previously reported that this ratio is significantly reduced in both forms of IBD, in both inflamed and normal mucosa, and regardless of treatment [34, 35]. In contrary, our analysis revealed that the *Firmicutes/ Bacteroidetes* ratio was significantly increased among IBD patients compared to both PSC-IBD and HC cohorts. These controversial findings might be explained by differences in study participant characteristics (especially host genetic determinants, ethnic background and diet), employing of varied microbiome assessment approaches, and the small number of patients enrolled in this study resulting in reduced statistical power.

The close association between PSC and IBD could propose a common microbial landscape or inflammatory pathway that initiates and develops intestinal and hepatic inflammation. Accumulating evidence generated from animal model studies has suggested that alteration of gut microbiota is associated with the development of PSC [36]. However, our study indicated no significant microbial differences between PSC-IBD patients and HCs. The data is in accordance with those reported by Kevans et al., in which the absence of a strong microbial association with PSC-IBD was found [10]. Although it might reflect a true lack of differential microbial contributions in PSC-IBD, however, could also be due to the limited sample size and technical factors. Recruiting an adequate number of homogeneous PSC and control cohorts is commonly a challenging issue due to the low-prevalence of PSC worldwide. In contrary,

Sabino et al. reported a unique microbial signature of three bacterial genera in patients with PSC, independent from that of healthy controls and IBD patients, suggesting the intestinal microbiota could be a contributing factor in PSC pathogenesis [20]. Taken together, these data suggest the need for further investigations to assess the potential role of the gut microbiota in PSC-IBD.

Conclusion

In conclusion, this study characterized fecal microbiota in PSC-IBD patients compared to IBD-only patients and also HCs using by a qPCR-based approach. Overall, our study showed no significant alterations in the gut microbiota composition of PSC-IBD compared to the HC cohort. However, the relative abundance of *Enterobacteriaceae* family was higher than HCs and thus, could be used as a PSC-associated microbial signal. Furthermore, our microbial analysis indicated that the fecal microbiota composition in IBD patients was completely different from that of PSC-IBD patients and HCs. Importantly, some limitations could be noted in our study. For instance, this study was limited by a small sample size. Another important limitation in our study is that we used qPCR analysis to investigate the relative abundance of the gut microbiota, in which several microbial taxa were not explored and also, we were not able to identify diversity in the gut microbiota of cohorts. Further large-scale metagenome prospective studies with more refined characterization of the gut microbiota should be performed to describe the contribution of the gut microbiota to PSC pathogenesis.

Declarations

Funding

This study was supported financially by a grant (no. RIGLD 930) from Foodborne and Waterborne Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

Compliance with ethical standards

Conflict of interest: None to declare

Author contributions: SO, MA participated in experimental work, qPCR assays and data collection. EJ extracted the DNA from fecal samples. KN, HH and AY participated in data analysis and interpretation. AY and AS designed the study and worked on conception. HH drafted the manuscript. AY, HH, HM critically revised the manuscript. AS, HAA and MRZ participated in intellectual input and clinical consultation. All authors approved the final version of the manuscript and the authorship list.

Acknowledgments

The authors wish to thank the laboratory staff of Foodborne and Waterborne Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical

Sciences, Tehran, Iran.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article.

ORCID

A. Yadegar https://orcid.org/0000-0002-2135-7581

References

1. Hirschfield GM, Karlsen TH, Lindor KD, Adams DH (2013) Primary sclerosing cholangitis. Lancet 382(9904):1587–1599
2. de Vries EM, Wang J, Williamson KD, Leeflang MM, Boonstra K, Weersma RK, Beuers U, Chapman RW, Geskus RB, Ponsioen CY (2018) A novel prognostic model for transplant-free survival in primary sclerosing cholangitis. 67(10):1864–1869
3. Boonstra K, Weersma RK, van Erpecum KJ, Rauws EA, Spanier BW, Poen AC, van Nieuwkerk KM, Drenth JP, Witteman BJ, Tuynman HA et al (2013) Population-based epidemiology, malignancy risk, and outcome of primary sclerosing cholangitis. Hepatol (Baltimore Md) 58(6):2045–2055
4. Eaton JE, Talwalkar JA, Lazaridis KN, Gores GJ, Lindor KD (2013) Pathogenesis of primary sclerosing cholangitis and advances in diagnosis and management. Gastroenterol 145(3):521–536
5. Olsson R, Danielsson A, Jarnerot G, Lindstrom E, Loof L, Rolny P, Ryden BO, Tysk C, Wallerstedt S (1991) Prevalence of primary sclerosing cholangitis in patients with ulcerative colitis. Gastroenterol 100(5 Pt 1):1319–1323
6. Boonstra K, Beuers U, Ponsioen CY (2012) Epidemiology of primary sclerosing cholangitis and primary biliary cirrhosis: a systematic review. J hepatol 56(5):1181–1188
7. O'Toole A, Alakkari A, Keegan D, Doherty G, Mulcahy H, O'Donoghue D (2012) Primary sclerosing cholangitis and disease distribution in inflammatory bowel disease. Clin gastroenterol hepatol: the official clinical practice journal of the American Gastroenterological Association 10(4):439–441
8. Boonstra K, van Erpecum KJ, van Nieuwkerk KM, Drenth JP, Poen AC, Witteman BJ, Tuynman HA, Beuers U, Ponsioen CY (2012) Primary sclerosing cholangitis is associated with a distinct phenotype of inflammatory bowel disease. IBD 18(12):2270–2276
9. Liu JZ, Hov JR, Folseraas T, Ellinghaus E, Rushbrook SM, Doncheva NT, Andreassen OA, Weersma RK, Weismüller TJ, Eksteen B et al (2013) Dense genotyping of immune-related disease regions identifies nine new risk loci for primary sclerosing cholangitis. Nat Genet 45(6):670–675
10. Kevans D, Tyler AD, Holm K, Jorgensen KK, Vatn MH, Karlsen TH, Kaplan GG, Eksteen B, Gevers D, Hov JR et al (2016) Characterization of Intestinal Microbiota in Ulcerative Colitis Patients with and without Primary Sclerosing Cholangitis. *JCC* 10(3):330–337

11. Lichtman SN, Keku J, Clark RL, Schwab JH, Sartor RB (1991) Biliary tract disease in rats with experimental small bowel bacterial overgrowth. Hepatol (Baltimore Md) 13(4):766–772
12. Tabibian JH, Weeding E, Jorgensen RA, Petz JL, Keach JC, Talwalkar JA, Lindor KD (2013) Randomised clinical trial: vancomycin or metronidazole in patients with primary sclerosing cholangitis - a pilot study. Aliment pharmacol therapeut 37(6):604–612
13. Farkkila M, Karvonen AL, Nurmi H, Nuutinen H, Taavitsainen M, Pikkarainen P, Karkkainen P (2004) Metronidazole and ursodeoxycholic acid for primary sclerosing cholangitis: a randomized placebo-controlled trial. Hepatol (Baltimore Md) 40(6):1379–1386
14. Chapman R, Fevery J, Kalloo A, Nagorney DM, Boberg KM, Shneider B, Gores GJ (2010) Diagnosis and management of primary sclerosing cholangitis. Hepatol (Baltimore Md) 51(2):660–678
15. Lennard-Jones JE (1989) Classification of inflammatory bowel disease. Scand J Gastroenterol Supplement 170:2–6; discussion 16–19
16. Kim WR, Therneau TM, Wiesner RH, Poterucha JJ, Benson JT, Malinchoc M, LaRusso NF, Lindor KD, Dickson ER (2000) A revised natural history model for primary sclerosing cholangitis. Mayo Clin proceed 75(7):688–694
17. Bacchetti De Gregoris T, Aldred N, Clare AS, Burgess JG (2011) Improvement of phylum- and class-specific primers for real-time PCR quantification of bacterial taxa. JMM 86(3):351–356
18. Kummen M, Holm K, Anmarkrud JA, Nygård S, Vesterhus M, Høivik ML, Trøseid M, Marschall HU, Schrumpf E, Moum B et al (2017) The gut microbial profile in patients with primary sclerosing cholangitis is distinct from patients with ulcerative colitis without biliary disease and healthy controls. Gut 66(4):611–619
19. Rossen NG, Fuentes S, Boonstra K, D'Haens GR, Heilig HG, Zoetendal EG, de Vos WM, Ponsioen CY (2015) The mucosa-associated microbiota of PSC patients is characterized by low diversity and low abundance of uncultured Clostridiales II. JCC 9(4):342–348
20. Sabino J, Vieira-Silva S, Machiels K, Joossens M, Falony G, Ballet V, Ferrante M, Van Assche G, Van der Merwe S, Vermeire S et al (2016) Primary sclerosing cholangitis is characterised by intestinal dysbiosis independent from IBD. Gut 65(10):1681–1689
21. Kolde R, Franzosa EA, Rahnavard G, Hall AB, Vlamakis H, Stevens C, Daly MJ, Xavier RJ, Huttenhower C (2018) Host genetic variation and its microbiome interactions within the Human Microbiome Project. Genome Med 10(1):6–6
22. Gupta VK, Paul S, Dutta C (2017) Geography, Ethnicity or Subsistence-Specific Variations in Human Microbiome Composition and Diversity. *Front Microbiol* 8(1162)
23. Nakamoto N, Sasaki N, Aoki R, Miyamoto K, Suda W, Teratani T, Suzuki T, Koda Y, Chu PS, Taniki N et al (2019) Gut pathobionts underlie intestinal barrier dysfunction and liver T helper 17 cell immune response in primary sclerosing cholangitis. Nat microbiol 4(3):492–503
24. Tang R, Wei Y, Li Y, Chen W, Chen H, Wang Q, Yang F, Miao Q, Xiao X, Zhang H et al (2018) Gut microbial profile is altered in primary biliary cholangitis and partially restored after UDCA therapy. Gut 67(3):534–541

25. Steffen EK, Berg RD, Deitch EA (1988) Comparison of translocation rates of various indigenous bacteria from the gastrointestinal tract to the mesenteric lymph node. J of infect di 157(5):1032–1038
26. Nakamoto N, Sasaki N, Aoki R, Miyamoto K, Suda W, Teratani T, Suzuki T, Koda Y, Chu P-S, Taniki N et al (2019)Gut pathobionts underlie intestinal barrier dysfunction and liver T helper 17 cell immune response in primary sclerosing cholangitis. *Nat microbio* 4(3):492–503
27. Jackaman FR, Hilson GR, Smith L (1980) Bile bacteria in patients with benign bile duct stricture. Br J Surg 67(5):329–332
28. Maddocks AC, Hilson GR, Taylor R (1973) The bacteriology of the obstructed biliary tree. Ann R Coll Surg Engl 52(5):316–319
29. Rerknimitr R, Fogel EL, Kalayci C, Esber E, Lehman GA, Sherman S (2002) Microbiology of bile in patients with cholangitis or cholestasis with and without plastic biliary endoprosthesis. Gastrointest endos 56(6):885–889
30. Pohl J, Ring A, Stremmel W, Stiehl A (2006) The role of dominant stenoses in bacterial infections of bile ducts in primary sclerosing cholangitis. Europ j gastroenterol hepatol 18(1):69–74
31. Nadalian B, Yadegar A, Houri H, Olfatifar M, Shahrokh S, Asadzadeh Aghdaei H, Suzuki H, Zali MR (2020) Prevalence of the pathobiont adherent-invasive Escherichia coli and inflammatory bowel disease: a systematic review and meta-analysis. J *Gastroenterol hepatol*, n/a(n/a)
32. Machiels K, Joossens M, Sabino J, De Preter V, Arijs I, Eeckhaut V, Ballet V, Claes K, Van Immerseel F, Verbeke K et al (2014) A decrease of the butyrate-producing species Roseburia hominis and Faecalibacterium prausnitzii defines dysbiosis in patients with ulcerative colitis. Gut 63(8):1275–1283
33. Little R, Wine E, Kamath BM, Griffiths AM, Ricciuto A (2020) Gut microbiome in primary sclerosing cholangitis: A review. World journal of gastroenterology 26(21):2768–2780
34. Kabeerdoss J, Jayakanthan P, Pugazhendhi S, Ramakrishna BS (2015) Alterations of mucosal microbiota in the colon of patients with inflammatory bowel disease revealed by real time polymerase chain reaction amplification of 16S ribosomal ribonucleic acid. Indian J Med Res 142(1):23–32
35. Sokol H, Seksik P, Furet J, Firmesse O, Nion-Larmurier I, Beaugerie L, Cosnes J, Corthier G, Marteau P, Doré J (2009) Low counts of Faecalibacterium prausnitzii in colitis microbiota. IBS 15(8):1183–1189
36. Hirschfield GM, Karlsen TH, Lindor KD, Adams DH (2013) Primary sclerosing cholangitis. The Lancet 382(9904):1587–1599
37. de Moraes JG, Motta MEFdA, Beltrão MFdS, Salviano TL, da Silva GAP (2016) Fecal Microbiota and Diet of Children with Chronic Constipation. *Int J Pediatr* 2016:6787269–6787269
38. Yang YW, Chen MK, Yang BY, Huang XJ, Zhang XR, He LQ, Zhang J, Hua ZC (2015) Use of 16S rRNA Gene-Targeted Group-Specific Primers for Real-Time PCR Analysis of Predominant Bacteria in Mouse Feces. Appl Environ Microbiol 81(19):6749–6756

39. Hermann-Bank ML, Skovgaard K, Stockmarr A, Larsen N, Mølbak L (2013) The Gut Microbiotassay: a high-throughput qPCR approach combinable with next generation sequencing to study gut microbial diversity. BMC genomic 14:788
40. Matsuki T, Watanabe K, Fujimoto J, Miyamoto Y, Takada T, Matsumoto K, Oyaizu H, Tanaka R (2002) Development of 16S rRNA-gene-targeted group-specific primers for the detection and identification of predominant bacteria in human feces. Appl Environ Microbiol 68(11):5445–5451
41. Hung C-H, Cheng C-H, Cheng L-H, Liang C-M, Lin C-Y (2008) Application of Clostridium-specific PCR primers on the analysis of dark fermentation hydrogen-producing bacterial community. Int JHydro Energy 33(5):1586–1592
42. Wang IK, Lai H-C, Yu C-J, Liang C-C, Chang C-T, Kuo H-L, Yang Y-F, Lin C-C, Lin H-H, Liu Y-L et al (2012) Real-Time PCR Analysis of the Intestinal Microbiotas in Peritoneal Dialysis Patients. Appl Environ Microbiol 78(4):1107
43. Martineau F, Picard FJ, Ke D, Paradis S, Roy PH, Ouellette M, Bergeron MG (2001) Development of a PCR Assay for Identification of Staphylococci at Genus and Species Levels. *JCM*39(7):2541

Figures

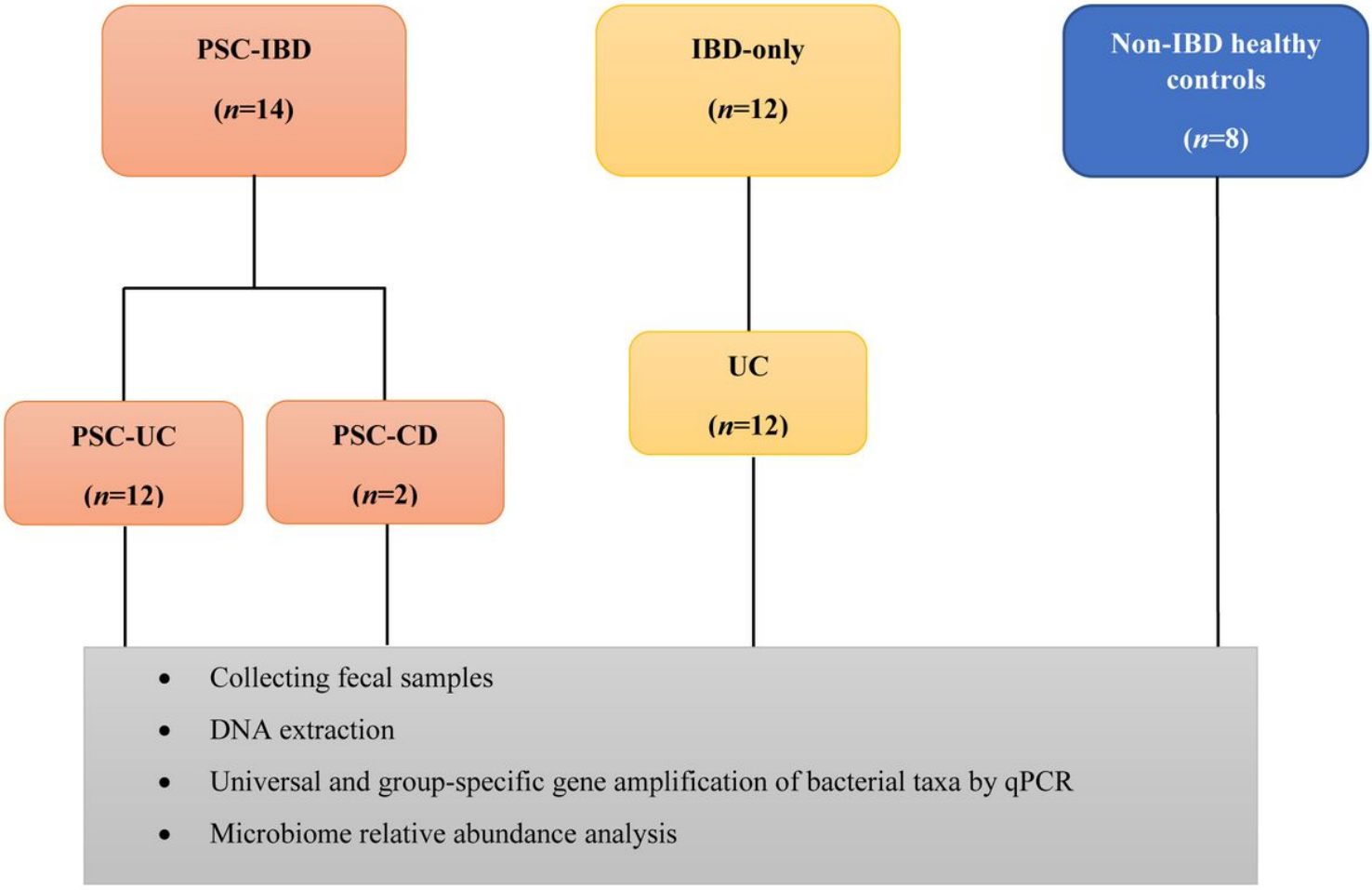

Figure 1

Flowchart of the recruitment process and study design.

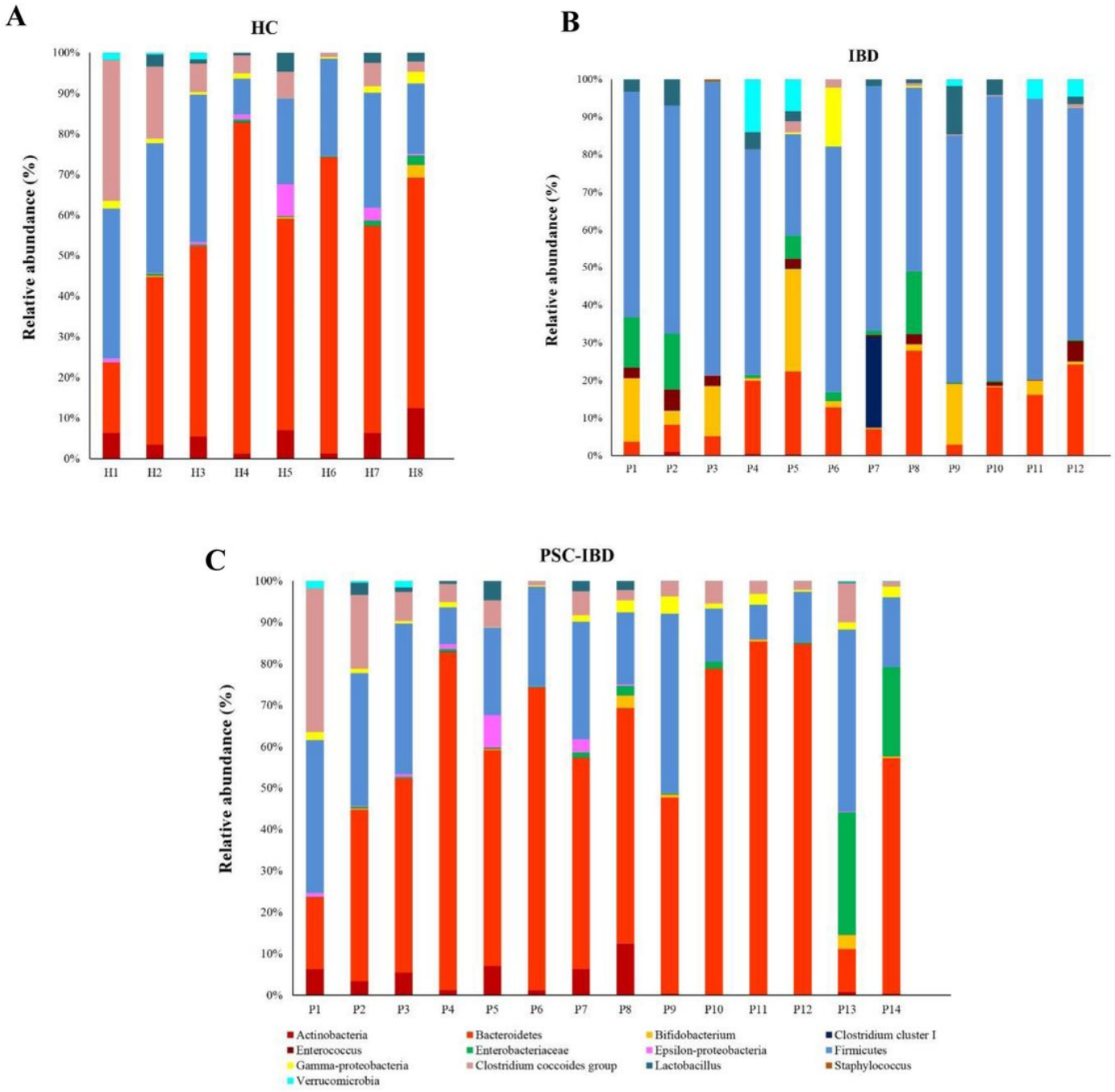

Figure 2

The relative abundance and diversity of intestinal microbiota composition in healthy controls (n=8) (A), patients with IBD-only (n=12) (B), and patients with PSC-IBD (n=14) (C). Each color corresponds to a type of microbiota assessed in this study.

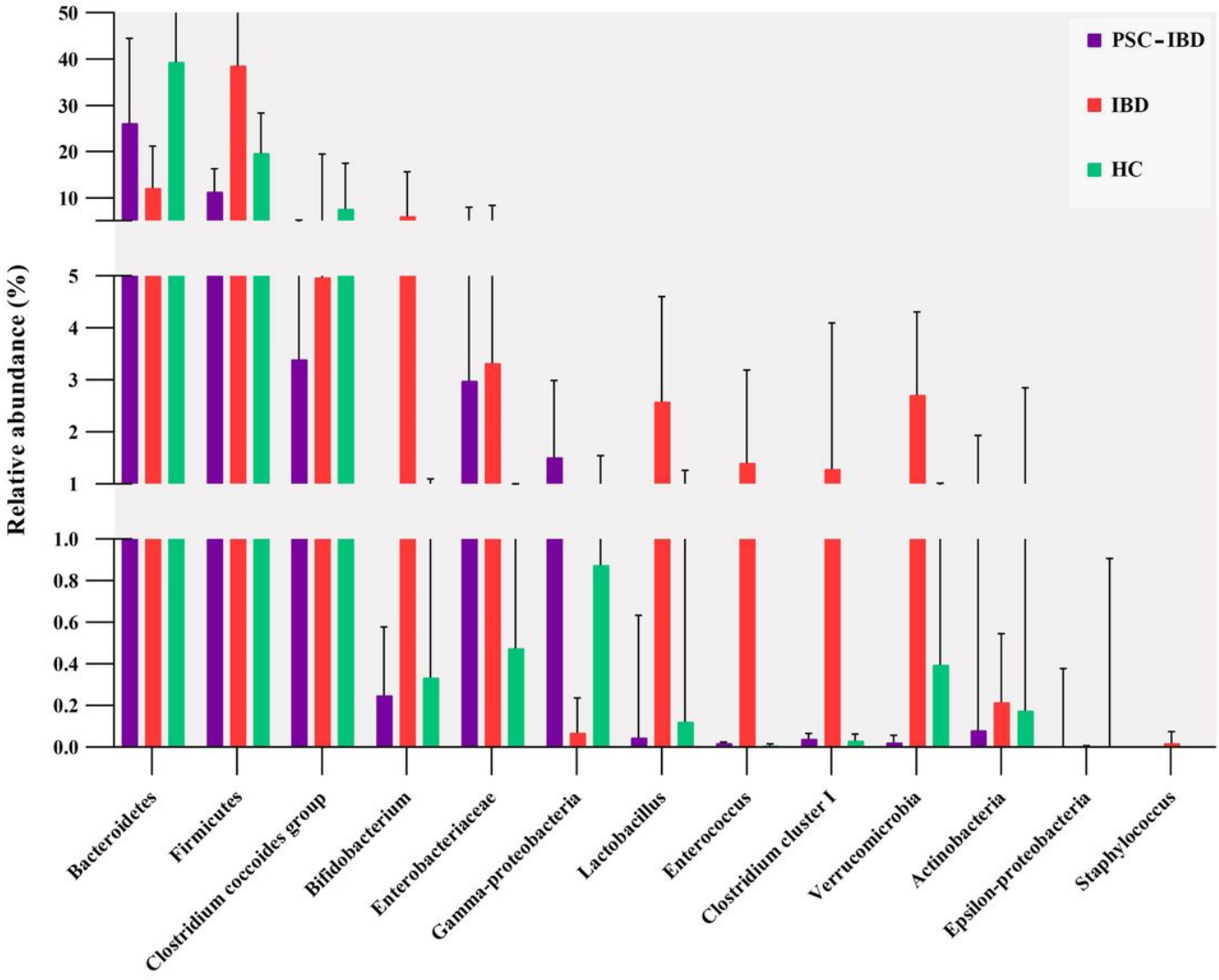

Figure 3

The mean percentage and distribution of the selected microbial taxa in fecal samples of patients with PSC-IBD, IBD-only patients, and healthy controls.

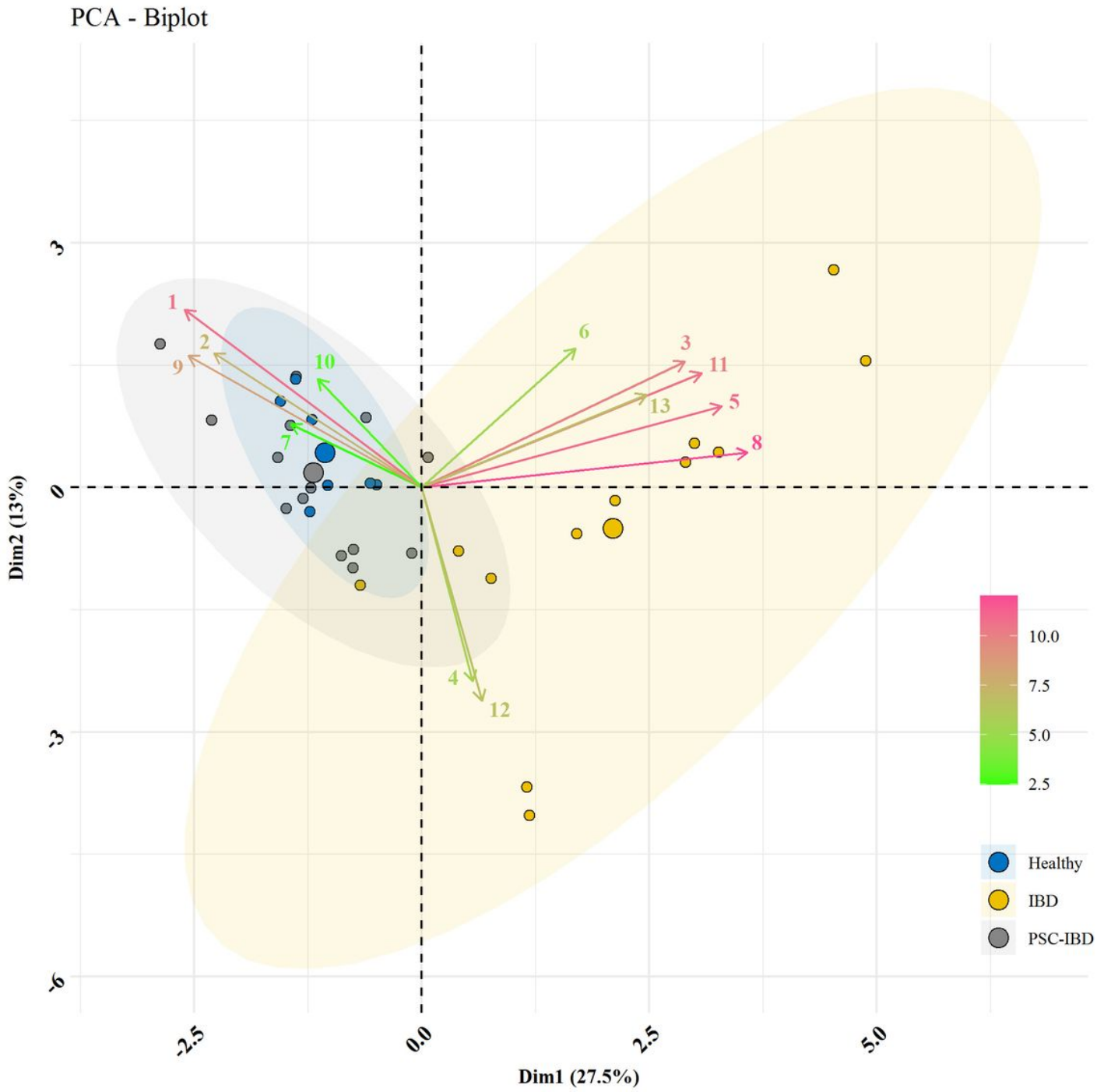

Figure 4

Bacterial community clustering and variations in microbial community composition represented in a principal component analysis (PCoA). Patients with PSC-IBD and IBD are significantly different, while PSC-IBD and healthy control subjects are almost similar. Percentage values in parentheses next to Dim1 and Dim2 represents percentage of variance explained by each component. Arrows show the contribution of each type of microbiota on the Dim1 and Dim2. Each data point denotes an individual patient, colored based on their group.

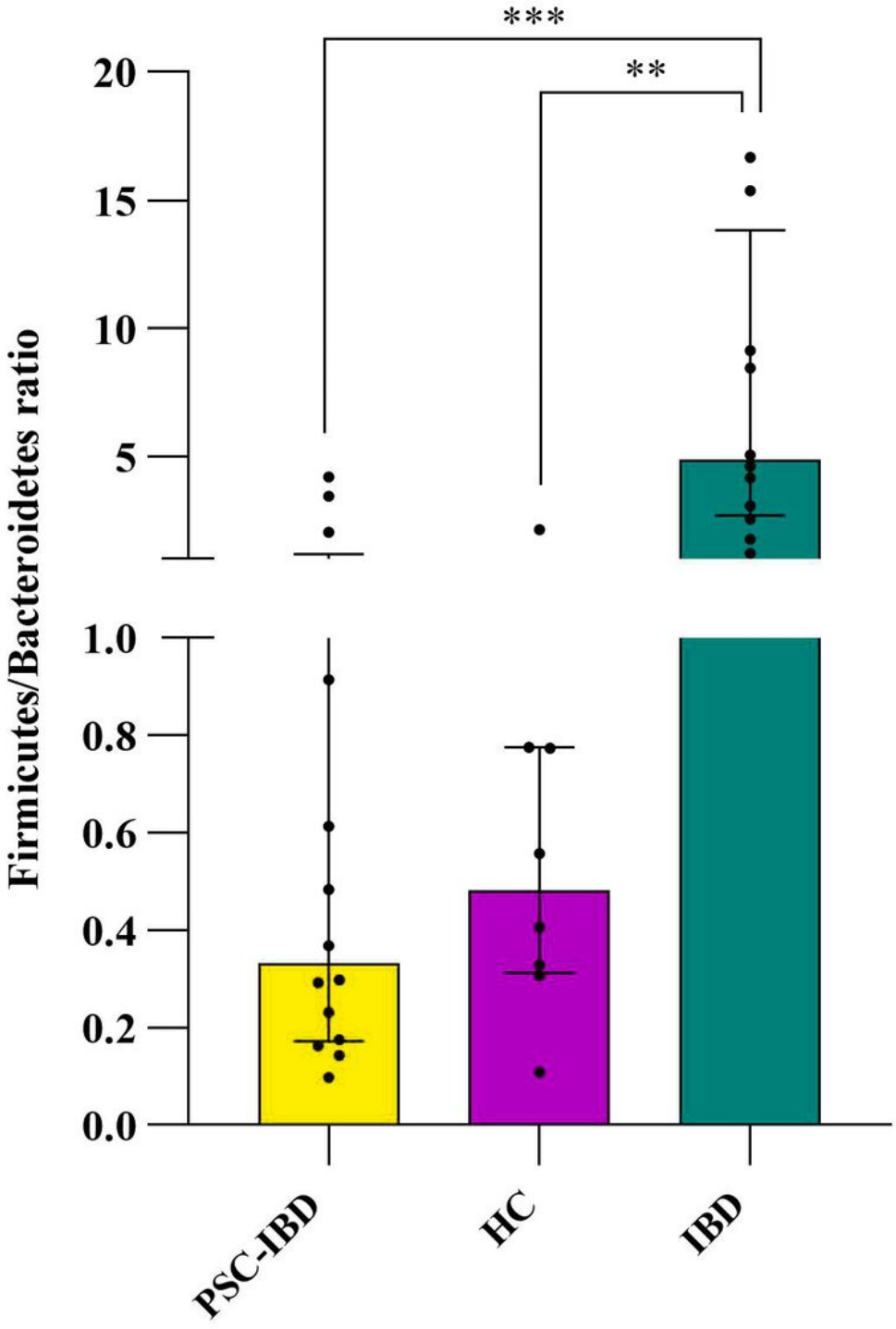

Figure 5

Bar graph illustrating the Firmicutes/Bacteroidetes (F/B) ratio in fecal samples from patients with PSC-IBD and IBD-only patients compared to healthy controls. This ratio was significantly higher in IBD-only subjects compared to both PSC-IBD patients (p value = 0.0013) and HCs (p value = 0.01). Moreover, this ratio was non-significantly lower in PSC-IBDs compared to HCs (p value = 0.56).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- FigureS1.jpg