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Basic Study

Distinct gut microbiota profiles in patients with primary sclerosing cholangitis and ulcerative colitis

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Abstract**AIM**

To characterize the gut bacterial microbiota of patients with primary sclerosing cholangitis (PSC) and ulcerative colitis (UC).

METHODS

Stool samples were collected and relevant clinical data obtained from 106 study participants, 43 PSC patients with ($n = 32$) or without ($n = 11$) concomitant inflammatory bowel disease, 32 UC patients, and 31 healthy controls. The V3 and V4 regions of the 16S ribosomal RNA gene were sequenced on Illumina MiSeq platform to cover low taxonomic levels. Data were further processed in QIIME employing MaAsLin and LEfSe tools for analysis of the output data.

RESULTS

Microbial profiles in both PSC and UC were characterized by low bacterial diversity and significant change in global microbial composition. *Rothia*, *Enterococcus*, *Streptococcus*, *Veillonella*, and three other genera were markedly overrepresented in PSC regardless of concomitant inflammatory bowel disease (IBD). *Rothia*, *Veillonella* and *Streptococcus* were tracked to the species level to identify *Rothia mucilaginosa*, *Streptococcus infantus*, *S. alactolyticus*, and *S. equi* along with *Veillonella parvula* and *V. dispar*. PSC was further characterized by decreased abundance of *Adlercreutzia equolifaciens* and *Prevotella copri*. Decrease in genus *Phascolarctobacterium* was linked to presence of colonic inflammation regardless of IBD phenotype. *Akkermansia muciniphila*, *Butyrivibrio pullicaecorum* and *Clostridium colinum* were decreased in UC along with genus *Roseburia*. Low levels of serum albumin were significantly correlated with enrichment of order Actinomycetales.

CONCLUSION

PSC is associated with specific gut microbes independently of concomitant IBD and several bacterial taxa clearly distinguish IBD phenotypes (PSC-IBD and UC).

Key words: Dysbiosis; Inflammatory bowel disease; Ulcerative colitis; Gut microbiota; Primary sclerosing cholangitis

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Core tip: This study demonstrates specific microbial patterns associated with PSC and/or concomitant inflammatory bowel disease (PSC-IBD). Several bacterial taxa convincingly distinguish PSC-IBD from ulcerative colitis. Gut microbiota composition also differs in patients with PSC overlap with autoimmune hepatitis. Disease-specific microbial features traceable down to the species level may lead to establishing suitable biomarkers or outlining new research directions in the field of PSC and IBD pathogenesis.

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INTRODUCTION

Primary sclerosing cholangitis (PSC) is a chronic liver disorder of unknown etiology, characterized by inflammation and stenosis of the bile ducts^[1,2]. The disease may progress to severe liver fibrosis and subsequent liver cirrhosis, and may eventually lead to liver failure and death^[3]. Orthotopic liver transplantation (OLT) is the only currently available effective treatment for end-stage liver disease^[4]. Inflammatory bowel disease (IBD) is present in 60% to 80% of patients with PSC^[5]. Concomitant colonic disease is often classified as ulcerative colitis (UC)^[6], but is also considered to be a distinct phenotype and referred to as "PSC-IBD"^[7].

The involvement of gut bacteria in IBD is widely accepted, but the etiology and pathogenesis of IBD is still not fully understood. It is generally assumed that the inflammation results from an aberrant immune response to antigens of gut microbiota resident in genetically susceptible individuals^[8]. It has been proposed that either an imbalance of the intestinal microbiota (dysbiosis), or presence of commensal bacteria with increased virulence could promote exaggerated local and systemic immune responses by disrupting microbiota-mucosa interactions^[9,10].

Evidence from animal models^[11] and success of antibiotic treatment in PSC patients^[12] suggest that disruption of gut microbiota may play a significant role in pathogenesis of PSC. Recently, several studies described specific changes in the gut microbiota of PSC patients that were not related to dysbiosis with concomitant IBD^[13-20]. However, those studies differed in sampling, experimental methods and design, in addition to outcome.

The aim of this study was to identify microbial features specific to patients with PSC (with and without concomitant IBD), in an independent, well-characterized cohort, to compare them with healthy controls (HC) and patients with UC, and to verify previous findings.

MATERIALS AND METHODS

Study participants

PSC, PSC-IBD, and UC patients for this single center, cross-sectional study were recruited at the out-patient department of the Institute for Clinical and Experimental Medicine, Prague. Healthy controls (HCs) were age and sex matched volunteers with insignificant medical history that were randomly selected from the hospital patient database. HCs were eligible if they had no history of malignant or autoimmune disease, major abdominal surgery, gastrointestinal disease or chronic gastrointestinal symptoms. Anyone, healthy or

diseased, with antibiotic use within the previous 3 mo, a history of colorectal surgery, or ongoing or recent infectious colitis were excluded. Patients who received liver transplants were excluded because we assumed that OLT and related medications, immunosuppressive therapy in particular, might significantly influence the microbiota composition, and therefore cause substantial bias. To exclude the potential impact of obesity or malnutrition, only patients and controls with a normal BMI were recruited.

Sample collection, DNA extraction and amplification

Stool samples were freshly collected in standardized, sterile collection tubes by participants and brought to the clinic when they made their routine clinic visits. All samples were delivered within 6 h of collection, immediately frozen at -20 °C and within 2 wk transferred to a -80 °C freezer for long-term storage. Care was taken to prevent thawing until samples were suspended in extraction buffer^[21,22]. A MasterPure™ Complete DNA and RNA Purification Kit (Epicentre) was used for DNA extraction using a FastPrep®-24 Instrument homogenizer and Lysing Matrix Y zirconium oxide spheres (both MP Biomedicals). DNA concentration was equalized in each sample after quantitation with a Qubit™ 2.0 Fluorometer and a dsDNA BR Assay Kit (Life Technologies). KAPA 2G Robust Hot Start DNA Polymerase (Kapa Biosystems) was used to amplify segments of the 16S rRNA gene including the V3 and V4 regions using 341F and 806R primers^[23]. The PCR protocol was initialized with 94 °C for 3 min, followed by 25 cycles of denaturation (94 °C, 30 s), annealing (54.2 °C, 1 min) and extension (72 °C, 1 min and 15 s), with the final extension (72 °C) for 10 min. The PCR products were purified and normalized for concentration with SequalPrep™ Normalization Plate Kit (ThermoFisher Scientific).

Library preparation and sequencing

After pooling, sample ligation was performed with a TruSeq DNA PCR-free LT Sample Preparation Kit (Illumina) following the standard protocol provided by the manufacturer. The libraries were validated by a KAPA Library Quantification Kit (Kapa Biosystems) prior to submission to The Genomics Core Facility, Central European Institute of Technology (CEITEC; Brno, Czech Republic) for sequencing on a MiSeq Platform (Illumina) using a Miseq Reagent Kit v3 (Illumina).

Data processing and quality control

Sequencing data were processed using a QIIME package^[24]. Briefly, raw reads were demultiplexed and quality filtered, allowing no N characters, a maximum of three consecutive low-quality base calls, a maximum unacceptable Phred quality of Q20, and a maximum of 1.5 barcode errors. Chimeric reads were detected and discarded using USEARCH algorithms^[25]. In the final dataset, the median of number of reads per sample reached 42459 sequences. Community statistics and

sample comparisons were done on resampled datasets at the level of 16400 sequences. This led to the exclusion of two samples, one from the PSC and one from the HC group because of an insufficient number of reads.

Statistical analysis and bioinformatics

The appropriateness of sequencing depth was checked in rarefaction plots. Alpha diversity statistics were calculated in QIIME^[24] using Shannon, Simpson, and observed species diversity indices, and the *P* value for group comparisons was determined by analysis of variance (ANOVA). Principal coordinate analysis plots were constructed to illustrate the beta diversity of samples based on phylogenetically informed weighted and unweighted Unifrac distance matrices^[26]. Significance of clustering patterns was tested by PERMANOVA, Adonis and ANOSIM in the R-vegan package. The multivariate homogeneity of group dispersions was assessed by Permdisp methods. Multivariate association with linear models (MaAsLin) for multivariate analysis^[27] applying an additive general linear model was used to assess the association between microbial abundance and patient metadata: Age, sex, gender, presence of PSC/AIH overlap, IBD activity, probiotic use (*E. coli* Nissle 1917), and use of anti-TNF treatment. A *P* value < 0.05 was considered statistically significant after adjusting for false discovery rate (FDR). Raw demultiplexed sequencing data, with sample annotations, were submitted to the Short Read Archive (<http://www.ncbi.nlm.nih.gov/bioproject/368966>).

Ethical approval

This study was approved by The Ethics Committee with multicenter competence of the Institute for Clinical and Experimental Medicine and Thomayer Hospital. All patients and healthy controls signed the informed consent form at the time of sample collection.

RESULTS

Characteristics of the study population

A total of 106 individuals were enrolled, including 32 patients with PSC-IBD, 11 with PSC (without IBD), 32 with UC and 31 HCs. The diagnosis of PSC or PSC overlap with autoimmune hepatitis (PSC/AIH) syndrome was established following recommended biochemical, immunological, histological, and cholangiographic evaluation^[28-30]. The extent of UC was categorized endoscopically^[31] and Mayo classification^[32] was used to describe disease severity. All other patient characteristics were obtained on the day of sample collection (Table 1).

Decrease of microbial diversity is tightly linked to the presence of IBD

A decrease of alpha diversity in all patients compared

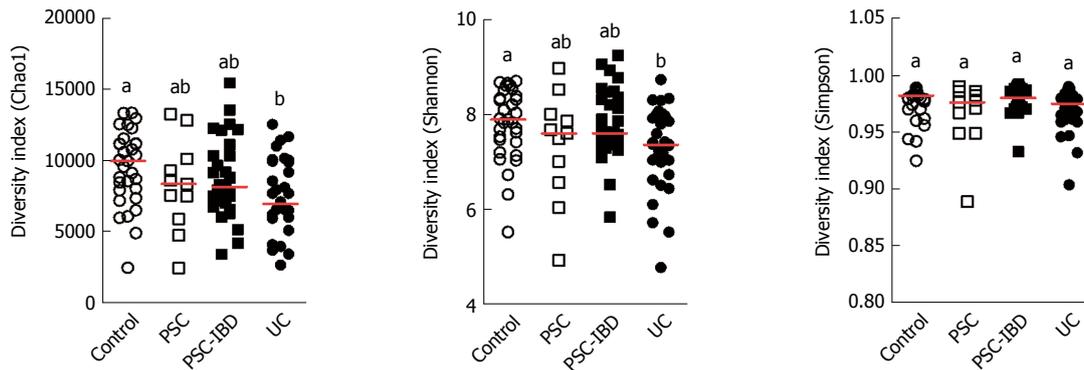


Figure 1 Alpha diversity was consistently reduced in patients with ulcerative colitis as determined by Chao1 index, Shannon index and Simpson index. Groups labeled by the same letter (a, b) on the graph are not significantly different from each other ($P < 0.05$) as analyzed by ANOVA with Tukey's post-hoc test.

Table 1 Demographical characteristics of the study population categorized by disease phenotype (and healthy controls) n (%)

	PSC-IBD ($n = 32$)	PSC ($n = 11$)	UC ($n = 32$)	Healthy controls ($n = 31$)
Gender; male/female	24/8 (75%/25%)	10/1 (90.9%/9.1%)	17/15 (53.1%/46.9%)	13/18 (41.9%/58.1%)
Median age (range), yr	35 (18-60)	45 (18-69)	40 (20-71)	44 (22-72)
Overlap syndrome PSC/AIH	6 (18.8)	2 (18.2)	N/A	N/A
Total bilirubin ($\mu\text{mol/L}$; mean \pm SD)	47 \pm 56.8	34.4 \pm 32.8	13.1 \pm 6.7	12.8 \pm 6.2
AST ($\mu\text{kat/L}$; mean \pm SD)	1.5 \pm 0.8	1.1 \pm 0.8	0.4 \pm 0.1	0.5 \pm 0.3
ALT ($\mu\text{kat/L}$; mean \pm SD)	1.8 \pm 0.9	1.3 \pm 1.1	0.5 \pm 0.2	0.6 \pm 0.4
ALP ($\mu\text{kat/L}$; mean \pm SD)	7.4 \pm 5.7	4.3 \pm 4.9	1.1 \pm 0.4	1 \pm 0.3
GGT ($\mu\text{kat/L}$; mean \pm SD)	7.5 \pm 7.4	12.2 \pm 25.5	0.5 \pm 0.5	0.7 \pm 1.6
IBD extent:				
Pancolitis	28 (87.5)	N/A	25 (78.1)	N/A
Left-sided	0 (0)	N/A	7 (21.9)	N/A
Right sided	4 (12.5)	N/A	0 (0)	N/A
IBD activity:				
Mild or remission	24 (75)	N/A	20 (62.5)	N/A
Moderate	4 (12.5)	N/A	3 (9.4)	N/A
Severe	4 (12.5)	N/A	9 (28.1)	N/A
Medication during last month:				
UDCA	33 (100)	11 (100)	0 (0)	0 (0)
5-ASA	26 (81.3)	0 (0)	31 (96.9)	0 (0)
Corticosteroids	12 (37.5)	2 (18.2)	3 (9.4)	0 (0)
Azathioprine	13 (40.6)	1 (9.1)	14 (43.8)	0 (0)
Anti-TNF α	0 (0)	0 (0)	10 (31.3)	0 (0)
Probiotics, <i>E. coli</i> Nissle 1917	4 (12.5)	1 (9.1)	9 (28.1)	0 (0)

PSC: Primary sclerosing cholangitis; IBD: Inflammatory bowel disease; UC: Ulcerative colitis; AIH: Autoimmune hepatitis; UDCA: Ursodeoxycholic acid; ASA: Aminosalicic acid

with HCs was apparent from differences in the Chao1 and Shannon indices, the decrease was particularly clear in patients with IBD, especially in the UC group. No evident differences in the Simpson indices of the groups were observed (Figure 1).

The global microbiota composition was clearly shifted by the presence of liver disease in PSC patients compared with HCs (Adonis $R^2 = 0.021$, $P = 0.001$; Figure 2A). Similarly, samples from patients with IBD clustered separately, depending significantly on the UC factor (Adonis $R^2 = 0.021$, $P < 0.001$) (Figure 2B). However, the homogeneity of group dispersion was significantly different when measured with Permdisp ($P > 0.1$). No such significant shifts were detected when comparing PSC and PSC-IBD groups. The disruption of bacterial beta diversity is shown in stack plots (Figure 3).

***Rothia* and five other genera were increased in PSC regardless of concomitant IBD**

We compared relative abundance corrected for FDR, and identified 12 genera that were significantly increased in patients with PSC compared with HCs (Table 2). Seven of these remained relatively more abundant regardless of concomitant IBD. These were *Rothia*, *Enterococcus*, *Streptococcus*, *Clostridium*, *Veillonella* and *Haemophilus*. On the other hand, *Coprobacillus*, *Escherichia*, *Corynebacterium* and *Lactobacillus* genera were associated with PSC-IBD, but not with isolated PSC. A distinct increase in the relative abundance of family *Micrococcaceae* ($P < 0.001$) in PSC and PSC-IBD ($P < 0.05$) was driven by the strong overrepresentation of *Rothia*. Similarly, enrichment of genus *Lactobacillus* and *Streptococcus*

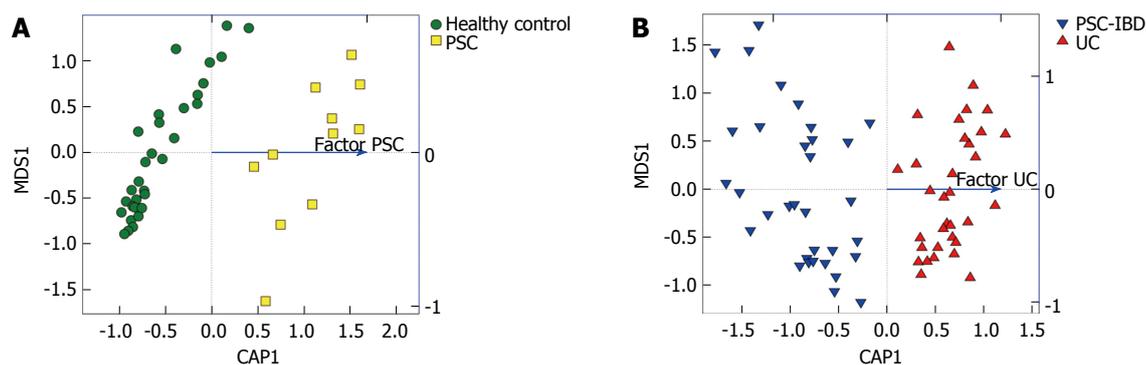


Figure 2 Ordination plots shows a distinct clustering pattern of sampled bacterial communities, explained either by the liver damage (primary sclerosing cholangitis vs Healthy controls) (A) and by the intestinal inflammation (primary sclerosing cholangitis-inflammatory bowel disease vs ulcerative colitis) (B). Both graphs are based on unweighted Unifrac distance matrix and constructed by PERMANOVA. PSC: Primary sclerosing cholangitis; UC: Ulcerative colitis; IBD: Inflammatory bowel disease.

Table 2 Statistical expression of relative abundances disruption in the study groups at genus level

Genus	PSC	PSC-IBD	UC
<i>Rothia</i>	↑↑↑	↑↑	NS
<i>Enterococcus</i>	↑↑↑	↑↑	NS
<i>Streptococcus</i>	↑↑	↑↑	NS
<i>Clostridium</i>	↑↑	↑	NS
<i>Veillonella</i>	↑	↑	NS
<i>Haemophilus</i>	↑	↑	NS
<i>Staphylococcus</i>	NS	↑	NS
<i>Coprobacillus</i>	NS	↑	NS
<i>Escherichia</i>	NS	↑	NS
<i>Corynebacterium</i>	NS	↑	NS
<i>Lactobacillus</i>	NS	↑	NS
<i>Coprococcus</i>	↓↓	↓↓	↓↓
<i>Phascolarctobacterium</i>	NS	↓↓	↓↓
<i>Akkermansia</i>	NS	NS	↓↓↓
<i>Roseburia</i>	NS	NS	↓

Only taxa with $P < 0.05$ in at least one group are presented. ↑↑↑, ↓↓↓: Increased, decreased with $P < 0.001$; ↑↑, ↓↓: $P < 0.01$; ↑, ↓: $P < 0.05$; NS: Non-significant; PSC: Primary sclerosing cholangitis; IBD: Inflammatory bowel disease; UC: Ulcerative colitis.

along with increase of family *Carnobacteriaceae* ($P < 0.01$) was responsible for a significant increase of order *Lactobacillales* ($P < 0.001$) and class *Bacilli* ($P < 0.001$) in patients with PSC-IBD. Additionally, genus *Coprococcus* was significantly reduced ($P < 0.01$) in all diseased individuals, including those with PSC and UC, along with several other unidentified genera and species belonging to family *Lachnospiraceae*. A low abundance of genus *Phascolarctobacterium* positively correlated ($P < 0.01$) with the presence of IBD, both PSC-IBD and UC.

At the species level, seven taxa belonging to genera *Rothia*, *Lactobacillus*, *Streptococcus* and *Veillonella*, were overrepresented, specifically in PSC patients, compared with both HCs and patients with UC (Table 3). Conversely, abundance of *Prevotella copri* was negatively associated with PSC, particularly in patients with concomitant IBD ($P < 0.001$). A highly significant decrease of *Adlercreutzia equolifaciens* ($P < 0.001$)

was detected only in PSC patients without concomitant IBD. *Faecalibacterium prausnitzii*, *Coprococcus catus* and *Ruminococcus gnavus* were decreased in all patients compared with HCs.

Several species clearly distinguish PSC-IBD from UC patients

A distinct dysbiotic pattern characterized by the significant underrepresentation of phylum *Verrucomicrobia* ($P < 0.001$) distinguished patients in the UC group from HCs. The low signal of this group of bacteria was also evident at the family (*Verrucomicrobiaceae*, $P < 0.001$) and genus (*Akkermansia*, $P < 0.001$) levels and with the extremely low abundance of *Akkermansia muciniphila* ($P < 0.001$), reduction of *Butyrivibrio pullicaecorum* ($P < 0.05$) and genus *Roseburia* ($P < 0.05$). The decrease of genus *Akkermansia* ($P < 0.01$) and *Akkermansia muciniphila* ($P < 0.01$) remained significant (along with the respective family and phylum signals) when compared with microbiota from PSC-IBD patients. Furthermore, *Clostridium colinum* was significantly underrepresented when compared with both HCs ($P < 0.001$) and PSC-IBD patients ($P < 0.01$). On the other hand, three genera that distinguished PSC patients from HCs (*Rothia* $P < 0.05$; *Streptococcus*, $P < 0.05$; *Veillonella*, $P < 0.05$) were increased in PSC-IBD compared to UC. The pattern continued at the species level with *R. mucilaginoso* ($P < 0.05$), *V. parvula* ($P < 0.05$), *V. dispar* ($P < 0.05$), and unclassified species of genus *Streptococcus* ($P < 0.01$) and genus *Blautia* ($P < 0.05$). Order *Fusobacteriales* ($P < 0.01$) and family *Fusobacteriaceae* ($P < 0.05$) were more abundant in UC than in PSC-IBD, but we were not able to track this tendency to lower taxonomic levels (Tables 2 and 3).

Disturbance in order Actinomycetales correlates with serum albumin levels in the overall population and has a distinct pattern in patients with PSC/AIH overlap

In the multivariate analysis, an OTU (operational taxonomic unit) belonging to genus *Actinomycetes*

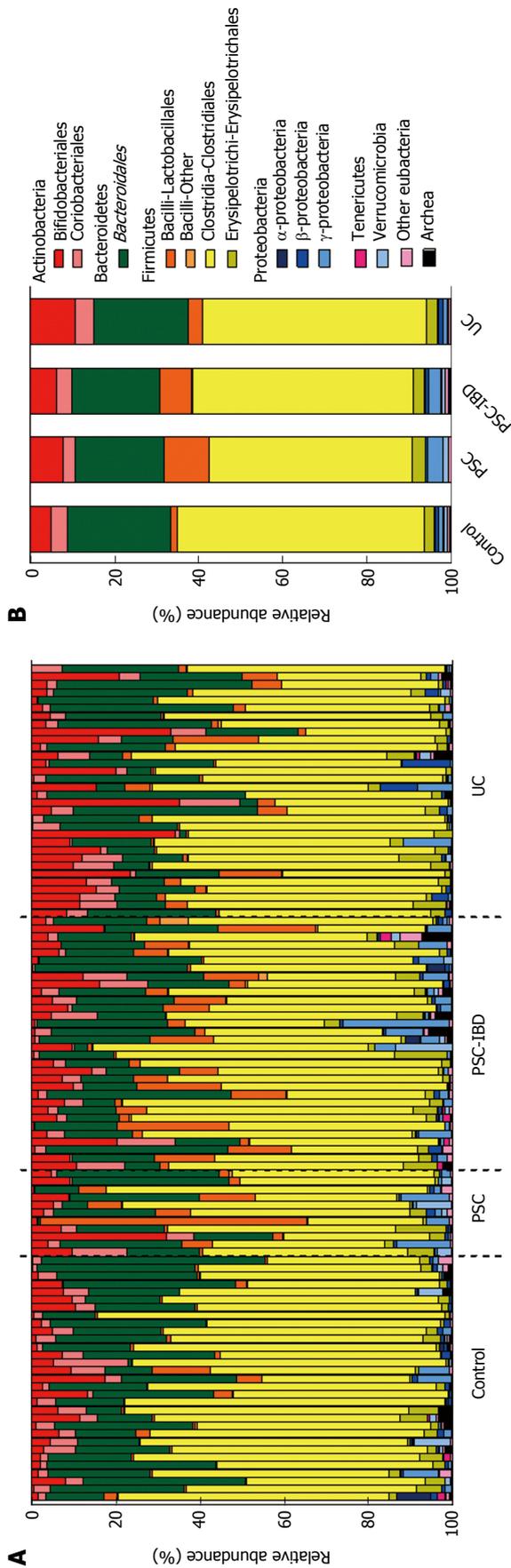


Figure 3 Median relative abundances of microbiota at order - level in all study subjects (A) and averaged for each study groups (B). We identified (mean \pm SD) 40.83% \pm 10.00% of sequences on the species level and 84.18% \pm 6.83% of sequences on the genus level. PSC: Primary sclerosing cholangitis; UC: Ulcerative colitis; IBD: Inflammatory bowel disease.

was significantly ($P < 0.01$) enriched, when overlapping AIH was present in patients with PSC (including those with PSC-IBD). Multivariate analysis using MaASLin found a significant tendency of order Actinomycetales to be increased in study subjects with low serum albumin level ($P < 0.01$). This finding was no longer significant when adjusting for the specific patient groups and HCs (Figure 4).

DISCUSSION

The intestinal microbiota is involved in the pathogenesis of various diseases and is, therefore, a potential biomarker or even a therapeutic target^[33]. We identified specific signatures of the fecal microbiota that distinguished patients with PSC from those with UC and from HCs. This is the first study of its kind from central Europe, a region characterized by specific dietary habits. Moreover, since recruitment centre may have a serious impact on microbial composition in multi-centric studies^[18], we decided to perform this study in a single-centre design with meticulous care for consistency of sample collection, processing and analysis.

We found disruption of gut bacteria homeostasis in patients with UC, which was characterized by decreased microbial diversity. This is in line with previous studies in which low bacterial diversity was associated with IBD^[34]. Moreover, significant disease-specific shifts of global microbiota composition were apparent when comparing PSC patients with healthy controls and PSC-IBD with UC patients.

In complex diseases, several distinct environmental factors may influence the composition of the gut microbial community. In our series of PSC and PSC-IBD patients, it was apparent that liver disease was primary factor associated with disease-specific dysbiosis, independent of dysbiotic influences of IBD, as the changes in gut microbiota did not significantly differ in PSC patients with and without established, concomitant IBD. However, stratifying PSC patients into "with or without IBD" subphenotypes may be controversial because PSC-IBD may have a subclinical course that might be easily missed during endoscopic and histological assessment. In addition, there is no agreement on the recommended interval for endoscopic evaluation in patients with PSC and no clinical suspicion of IBD. Furthermore, previous

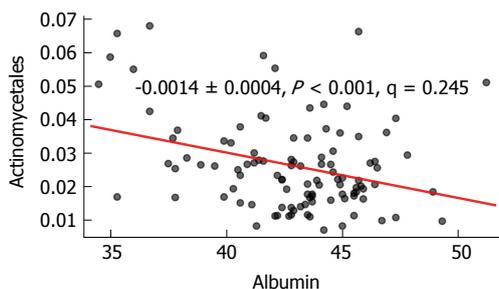


Figure 4 Abundance of order Actinomycetales negatively correlates with serum albumin levels in the total study population.

Table 3 Statistical expression of relative abundances disruption in the study groups at species level

Species	PSC	PSC-IBD	UC
<i>Rothia mucilaginosa</i>	↑↑↑	↑↑	NS
<i>Lactobacillus salivarius</i>	NS	↑↑↑	NS
<i>Streptococcus infantis</i>	↑↑	↑↑	NS
<i>Streptococcus alactolyticus</i>	↑↑	↑↑	NS
<i>Streptococcus equi</i>	↑↑	↑↑	NS
<i>Veillonella dispar</i>	↑↑	↑↑	NS
<i>Veillonella parvula</i>	↑	↑↑	NS
<i>Prevotella copri</i>	↓	↓↓↓	NS
<i>Adlercreutzia equolifaciens</i>	↓↓↓	NS	NS
<i>Faecalibacterium prausnitzii</i>	↓	↓↓↓	↓↓
<i>Coprococcus catus</i>	↓	↓↓	↓
<i>Ruminococcus gnavus</i>	↓↓	↓↓	↓↓↓
<i>Akkermansia muciniphila</i>	NS	NS	↓↓↓
<i>Butyricicoccus pullicaecorum</i>	NS	NS	↓↓↓
<i>Clostridium colinum</i>	NS	NS	↓↓↓

Only taxa with $P < 0.01$ in at least one group are presented. ↑↑↑, ↓↓↓: Increased, decreased with $P < 0.001$; ↑↑, ↓↓: $P < 0.01$; ↑, ↓: $P < 0.05$; NS: Non-significant; PSC: Primary sclerosing cholangitis; IBD: Inflammatory bowel disease; UC: Ulcerative colitis.

studies evaluated PSC-UC and PSC-CD as subgroups of their study cohorts^[13,16]. However, we assumed, that IBD in PSC rarely has macroscopic features typical for CD and that histological findings might be often misinterpreted and the diagnosis is often eventually changed to UC. Therefore, we included only patients with typical features of IBD associated with PSC and universally categorized the phenotype as PSC-IBD^[7].

We found a close association of the relative abundance of several genera with PSC, including *Rothia*, *Streptococcus*, *Enterococcus*, *Veillonella*, *Clostridium*, and *Haemophilus* regardless of the presence or absence of concomitant IBD. The association of *Rothia* with PSC was the most striking, and to our knowledge, this is the first study to describe such a significant relation with PSC.

The increased abundance of genus *Rothia*, and *R. mucilaginosa* in particular, in patients with PSC suggests that oral microbiota may be overrepresented in the lower GI populations of patients with advanced liver disease, which is in line with previous reports^[35]. In previous studies, infections caused by *Rothia* species were predominantly found in immunocompromised

individuals and patients with indwelling vascular devices^[36]. Since *Rothia* is sensitive to gastric fluid^[37], we speculate, that our findings may reflect contamination of the intestinal microbial community by previous endoscopic retrograde cholangio-pancreatography (ERCP), with repeated stenting in particular. Our data alone are insufficient to indicate a direct link to disease pathogenesis. However, recent reports of successful treatment of PSC and recurrent PSC with vancomycin^[12,38], suggest that *Rothia* or other vancomycin-sensitive microbes^[39] are reasonable targets of further research in this field.

The increased abundance of genera *Enterococcus* and *Lactobacillus* confirms the results of a recent complex study from Belgium^[16]. Even though we did not find a significant increase of genus *Fusobacterium* (one of three significantly increased genera in the Belgian study), we observed the clear enrichment of family *Fusobacteriaceae* in PSC-IBD patients ($P < 0.001$).

We could replicate previous results of enrichment of *Veillonella*, notably *V. parvula* and *V. dispar*, as a key feature of PSC-associated dysbiosis^[13]. Previously, this genus was associated with several other progressive fibrotic disorders^[40]. As in our study, Kummel *et al.*^[13] did not evaluate the influence of liver cirrhosis, which might be the condition that predisposes towards *Veillonella* increase, not necessarily PSC itself. This is further supported by Sabino *et al.*^[16] who found that *Veillonella* abundance in PSC was no longer significantly changed when patients with confirmed liver cirrhosis were excluded from the analysis. Sabino *et al.*^[16] diagnosed liver cirrhosis diagnosis biopsy and/or MRI imaging and/or elastography. Confirming cirrhosis in advanced cholestatic liver disease by elastography may be challenging because of imaging distortion, and biopsy can miss focally expressed morphological features^[41]. Therefore, it is not certain, whether the alteration in microbiota composition was a PSC-specific characteristic, or more likely reflects homeostatic disruption in advanced chronic liver disease in general.

In our dataset, genus *Adlercreutzia* was significantly decreased in PSC compared with HCs. *Adlercreutzia* is a genus that includes only one species (*A. equolifaciens*) capable of converting ingested isoflavones, which are abundant in legumes and soya beans, into equol^[42]. Equol has a high affinity for estrogen receptors (ERs) and may be a selective estrogen receptor modulator^[43]. ER expression on cholangiocytes is increased in cholestatic liver diseases but is absent in healthy individuals^[44]. Taking into account the fact that the prevalence of PSC is much decreased in women and Asian populations^[45] (and presumably in populations with higher than average consumption of isoflavones), we could speculate upon potential pathogenetic pathways with significant impact on disease development that are influenced by

dietary habits and endocrine signaling.

We observed a significant decrease of *Ruminococcus gnavus* in all diseased individuals, Png *et al.*^[46] described a manifold increase of this mucin-degrading species in the colonic mucosa of both CD and UC patients. This discrepancy could be, however, caused by the fact that the luminal and mucosal microbiota are significantly different^[47]. The low abundance of another mucolytic bacteria, *Akkermansia muciniphila* (and therefore genus *Akkermansia*), in UC is consistent with Png *et al.*^[46]. The absence of this bacterium from the microbial community could affect the use of mucins as a carbon sources by other bacteria that are important members of microbial community in healthy individuals^[46]. In our study, the UC phenotype was characterized by a substantial decrease of *Butyricoccus pullicaecorum* sp., which is in line with Eeckhaut *et al.*^[48] who found that *B. pullicaecorum* attenuated trinitrobenzene sulfonic acid (TNBS)-induced colitis in rats, and that supernatants of cultures of *B. pullicaecorum* increased transepithelial resistance. Decreased abundance of *Faecalibacterium prausnitzii* has recently been reported in IBD-associated dysbiosis^[49,50], which also occurred in this study in both UC and with PSC. The decrease of *F. prausnitzii* was particularly significant in patients with an inflamed colon, regardless of IBD phenotype. The decreased abundance of *B. pullicaecorum*, *F. prausnitzii*, and genus *Roseburia* demonstrates disruption in butyrate-producing bacteria, which probably have anti-inflammatory activity. Furthermore, it has been proposed recently that the anti-inflammatory properties of *F. prausnitzii* is associated with the production of 15 kDa protein capable of inhibiting the NF- κ B pathway in intestinal epithelial cells^[51].

Bile acids and changes in production, circulation, and conversion may be associated with changes of microbiota composition^[52]. Microbiota might thus be influenced by long-term use of ursodeoxycholic acid (UDCA) leading to substantial changes in bile composition. Sabino *et al.*^[16] reported that only 66.7% of a series of 147 patients with PSC-UC used UDCA. In our series, all patients with an established diagnosis of PSC received UDCA as routine clinical practice at our center follows the recommendations of EASL^[53] and ECCO^[54], which are contradictory to the AASLD guidelines advocating against routine use of UDCA in PSC^[5]. We were thus not able to evaluate the influence of UDCA on microbiota composition. However, recent reports have not reported substantial differences in the microbiota of PSC patients treated or not treated with UDCA^[13,16].

The impact of antibiotics on gut microbiota composition is clear, but there is no consensus on how to adjust for the antibiotic use, we included only subjects who had not used antibiotics within the previous 3 mo. Sabino *et al.*^[16] enforced only 1 mo cutoff, and Kummen *et al.*^[13] observed no substantial differences related to antibiotic use within the previous 12 mo.

The role of biologics for PSC treatment is currently

not sufficiently clear. α 4 β 7 integrin antagonists have demonstrated a positive effect on PSC course^[55], and the effect of anti-TNF treatment is dubious^[56] with potential risk of serious adverse events^[57]. In our series, no PSC patients had been treated with biologics. Nearly one-third of the UC patients had received infliximab, adalimumab, or golimumab, but these agents did not result in microbial shifts that distinguished the UC group.

Multivariate analysis revealed a significant negative correlation of serum albumin level and the relative contribution of order Actinomycetales in the total study population. This might reflect a decrease in this microbial subgroup in patients with advanced liver disease and subsequent alteration of proteosynthesis. As the relation could not be assigned to any specific subgroup of patients, the relevance of this observation is not clear.

Due to the advances in sequencing and bio-informatics, we were able to found some of the most striking changes at the low taxonomic level (species). These disease-specific bacteria could be the cornerstone in search for suitable biomarkers for PSC development or in non-invasive distinguishing of different IBD phenotypes. The fact that most of our findings were consistent with those reported in previous studies validates the study design and methodology. The current evidence supports a key role of microbiota in PSC pathogenesis which, however, needs to be further elucidated by future mechanistic studies.

In conclusion, PSC- and UC-associated dysbiosis was characterized by reduced bacterial diversity, significant changes in global bacterial composition and relative abundance of distinct taxa, primarily at the genus and species levels. The most prominent changes related to PSC were in the genera *Rothia*, *Streptococcus*, *Veillonella*, *Enterococcus*, *Clostridium*, *Haemophilus*, and *Adlercreutzia*. Several microbes including *Akkermansia muciniphila*, *Butyricoccus pullicaecorum* and *Clostridium colinum* clearly distinguished the UC and PSC-IBD phenotypes. Specific changes in occurring with PSC/AIH overlap involved enrichment of *Actinomyces* spp.

COMMENTS

Background

Primary sclerosing cholangitis (PSC) is a chronic liver disease with particularly high incidence in northern Europe and North America. Concomitant inflammatory bowel disease (IBD) is present in majority of patients with PSC. This IBD phenotype is distinct from Crohn's disease and ulcerative colitis and is often referred to as PSC-IBD. Gut microbiota composition is most likely involved in pathogenesis of this complex and often unfavorable clinical entity that involves the liver and intestine.

Research frontiers

Disruption of microbial ecology has previously been described in several clinical conditions including IBD. The few reports on gut microbiota composition in PSC and PSC-IBD have used diverse methods and yielded inconsistent results.

Innovations and breakthroughs

This study describes specific gut microbial patterns associated with PSC and differ in individual IBD phenotypes (UC and PSC-IBD).

Applications

Identifying disease-specific microbial features could be the cornerstone of further research in the field of PSC and IBD pathogenesis as well as the search for suitable biomarkers.

Terminology

PSC is a severe liver disease characterized by fibrosis and stenosis of both intra- and extrahepatic bile ducts; PSC-IBD is a specific phenotype of inflammatory bowel disease associated with PSC; gut microbiota comprises the ecological community of microorganisms residing in the (human) intestine.

Peer-review

Interesting and complex article, a very good work and a beautiful writing. It was difficult to read, because includes so many data, but also very useful in the end.

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