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A shared mucosal gut microbiota signature in primary sclerosing cholangitis before and after liver transplantation

Mikal Jacob Hole[1,2,3] | **Kristin Kaasen Jørgensen**[1,4] | **Kristian Holm**[1,2,3] | **Peder R. Braadland**[1,2,3] | **Malin Holm Meyer-Myklestad**[2,5,6] | **Asle Wilhelm Medhus**[7] | **Dag Henrik Reikvam**[2,5] | **Alexandra Götz**[1,2,3] | **Krzysztof Grzyb**[8] | **Kirsten Muri Boberg**[1,2,9] | **Tom Hemming Karlsen**[1,2,3,9] | **Martin Kummen**[1,2,10] | **Johannes R. Hov**[1,2,3,9]

[1]Norwegian PSC Research Centre, Department of Transplantation Medicine, Oslo University Hospital Rikshospitalet, Oslo, Norway

[2]Institute of Clinical Medicine, University of Oslo, Oslo, Norway

[3]Research Institute of Internal Medicine, Oslo University Hospital, Rikshospitalet, Oslo, Norway

[4]Department of Gastroenterology, Akershus University Hospital, Lørenskog, Norway

[5]Department of Infectious Diseases, Oslo University Hospital, Ullevål, Oslo, Norway

[6]Department of Microbiology, Oslo University Hospital, Ullevål, Oslo, Norway

[7]Department of Gastroenterology, Oslo University Hospital, Ullevål, Oslo, Norway

[8]Division of Pathology, Oslo University Hospital, Rikshospitalet, Oslo, Norway

[9]Section of Gastroenterology, Department of Transplantation Medicine, Oslo University Hospital, Rikshospitalet, Oslo, Norway

[10]Department of Oncology, Oslo University Hospital, Oslo, Norway

Correspondence
Johannes R. Hov, Norwegian PSC Research Center, Department of Transplantation Medicine, Division of Surgery, Inflammatory Medicine and Transplantation, Oslo University Hospital, Rikshospitalet, Postboks 4950 Nydalen, N-0424 Oslo, Norway.
Email: j.e.r.hov@medisin.uio.no

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Abstract

Background and Aims: Several characteristic features of the fecal microbiota have been described in primary sclerosing cholangitis (PSC), whereas data on mucosal microbiota are less consistent. We aimed to use a large colonoscopy cohort to investigate key knowledge gaps, including the role of gut microbiota in PSC with inflammatory bowel disease (IBD), the effect of liver transplantation (LT), and whether recurrent PSC (rPSC) may be used to define consistent microbiota features in PSC irrespective of LT.

Approach and Results: We included 84 PSC and 51 liver transplanted PSC patients (PSC-LT) and 40 healthy controls (HCs) and performed sequencing of the 16S ribosomal RNA gene (V3–V4) from ileocolonic biopsies.

Abbreviations: AC, ascending colon; ALP, alkaline phosphatase; AOM score, Amsterdam–Oxford model score for PSC; APRI, AST to platelet ratio index; AST, aspartate aminotransferase; ASVs, amplicon sequence variants; DC, descending colon; FDR, false discovery rate; FIB-4, Fibrosis-4 score; HCs, healthy controls; IBD, inflammatory bowel disease; LT, liver transplantation; no-rPSC, PSC-LT without rPSC; PERMANOVA, permutational ANOVA; PERMDISP, permutational analysis of multivariate dispersions; PSC, primary sclerosing cholangitis; RMST, restricted mean survival time; rPSC, recurrent primary sclerosing cholangitis; SC, sigmoid colon; SCFAs, short-chain fatty acids; TI, terminal ileum

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Intraindividual microbial diversity was reduced in both PSC and PSC-LT versus HCs. An expansion of Proteobacteria was more pronounced in PSC-LT (up to 19% relative abundance) than in PSC (up to 11%) and HCs (up to 8%; $Q_{FDR} < 0.05$). When investigating PSC before (PSC vs. HC) and after LT (rPSC vs. no-rPSC), increased variability (dispersion) in the PSC group was found. Five genera were associated with PSC before and after LT. A dysbiosis index calculated from the five genera, and the presence of the potential pathobiont, *Klebsiella*, were associated with reduced LT-free survival. Concomitant IBD was associated with reduced *Akkermansia*.

Conclusions: Consistent mucosal microbiota features associated with PSC, PSC-IBD, and disease severity, irrespective of LT status, highlight the usefulness of investigating PSC and rPSC in parallel, and suggest that the impact of gut microbiota on posttransplant liver health should be investigated further.

INTRODUCTION

Primary sclerosing cholangitis (PSC) is characterized by progressive inflammation and fibrosis of the bile ducts and development of cirrhosis.[1] Up to 80% of PSC patients have concomitant inflammatory bowel disease (IBD), which was early suggested to be implicated in the etiopathogenesis.[2,3] Liver transplantation (LT)-free survival among PSC patients is typically 13–21 years,[4] and even though outcome after LT is excellent, the literature suggests that up to 30% of patients develop recurrent PSC (rPSC) in the new liver. The occurrence of rPSC is clinically challenging and increases the risk of death or retransplantation 4-fold.[5]

Gut microbiota composition has been extensively studied in fecal samples from patients with PSC compared with controls. The most consistent finding has been loss of intraindividual microbial diversity (alpha diversity),[6–10] which is observed in many autoimmune diseases.[11] Several bacterial taxa have also been associated with PSC, including *Veillonella*, which, in multiple studies, has been shown to have increased relative abundance in patients with PSC compared with controls.[7–9] The first evidence of functional changes of the microbiome in PSC has also been published, suggesting altered metabolism of essential nutrients like B vitamins and branched-chain amino acids, which could have impact on disease severity.[12] Furthermore, in mouse models of bile duct disease, the presence of specific bacteria like *Klebsiella pneumoniae* has been shown to aggravate the disease.[13]

Although the above observations strongly link fecal gut microbiota alterations to PSC, possibly also involving disease-modifying effects, fundamental questions are left unanswered by the current literature. Only a few and small studies of the mucosal microbiota have been published in PSC.[10,14–17] In brief, previous studies have observed global compositional changes in PSC-IBD compared with controls and nonreplicated alterations of taxa such as reduced Clostridiales,[10] increased *Streptococcus* and *Pseudomonas*,[16] and increased *Blautia* and Barnesiellaceae.[15] With its closer proximity to human cells and the immune system, mucosa could potentially be more relevant than fecal samples. Surprisingly, there is also little evidence of any effect of concomitant IBD on the microbiota in PSC patients, in contrast to the large effect of IBD on the microbiota observed in patients without liver disease.[18,19] Data on gut microbiota and posttransplantation liver health are also scarce. Is the PSC microbiota “normalized” after LT, as suggested by studies in other liver diseases,[20,21] or could a posttransplant dysbiosis contribute to disease recurrence? And, consequently, is rPSC microbiota similar to PSC pretransplantation microbiota?

Based on the above, we aimed to make a comprehensive assessment of the ileocolonic mucosal microbiota in nontransplanted and transplanted PSC patients to investigate the effect of LT, concomitant IBD, and the relationship to disease severity. Specifically, we here perform a parallel analysis of PSC and rPSC to define consistent LT-independent features associated with sclerosing cholangitis.

PATIENTS AND METHODS

Participants

One hundred eighty-four consecutive nontransplanted PSC (PSC) and transplanted PSC patients (PSC-LT)

TABLE 1 Patient characteristics

Variables	PSC ($n = 84$)	PSC-LT ($n = 51$)
Male, *n* (%)	65 (77.4)	36 (70.6)
Age, years, median (min-max)	40 (17–77)	48.5 (23–70)
Years from diagnosis/LT to inclusion, median (min-max)	3.3 (0.0–27.8)	3.1 (0.5–18.9)
Small duct PSC, *n* (%)	3 (3.6)	2 (3.9)
Endpoint, *n* (%)	52 (61.9)	N/A
Recurrent PSC at inclusion, *n* (%)	N/A	13 (25.5)
IBD, *n* (%)	66 (78.6)	42 (82.4)
Crohn's disease, *n* (%)	7 (8.3)	2 (3.9)
Ulcerative colitis, *n* (%)	57 (67.9)	37 (72.5)
Indeterminate colitis, *n* (%)	2 (2.4)	3 (5.9)
Remission of IBD (SCCAI < 3)	68 (81.0)	44 (86.3)
Mayo PSC score, median (min-max)	0.29 (−2.2 to 3.5)	N/A
AOM PSC score, median (min-max)	1.95 (0.5–3.8)	N/A
APRI score, median (min-max)	0.68 (0.05–5.50)	0.53 (0.18–3.55)
FIB-4 score, median (min-max)	1.22 (0.08–8.60)	1.74 (0.46–5.75)
Medication		
Ursodeoxycholic acid, *n* (%)	41 (48.8)	21 (41.2)
5-Aminosalicylate, *n* (%)	42 (51.2)	25 (49.0)
Budesonide, *n* (%)	1 (1.2)	0
Prednisolone, *n* (%)	48 (57.1)	51 (100)
Azathioprine, *n* (%)	14 (16.7)	12 (23.5)
Salazopyrine, *n* (%)	32 (38.1)	13 (35.5)
Tacrolimus, *n* (%)	0	43 (84.3)
Mycophenolic acid, *n* (%)	0	31 (60.8)
Cyclosporine, *n* (%)	1 (1.2)	12 (23.5)
Biochemistry		
Hemoglobin, g/dl, median (min-max)	13.9 (9.0–17.1)	14.0 (8.5–16.5)
Platelet count 10^9/l, median (min-max)	278 (37–712)	201 (69–434)
Creatinine µmol/l, median (min-max)	65.5 (39–232)	82 (59–159)
Bilirubin mg/dl, median (min-max)	24 (3–319)	21 (11–52)
Albumin, g/l, median (min-max)	41.0 (26–49)	42.0 (24–50)
INR, median (min-max)	1.1 (0.9–1.7)	1.1 (0.9–2.4)
AST U/l, median (min-max)	67 (13–514)	39 (12–239)
ALT U/l, median (min-max)	96.5 (15–515)	45 (6–267)
ALP U/l, median (min-max)	253.5 (53–1040)	87 (27–836)
Fecal calprotectin mg/kg, median (min-max)	56.5 (1–2844)	35 (1–1945)
Leukocytes 10^9/l, median (min-max)	6.3 (1.0–17.2)	6.1 (3.0–18.5)
CRP, median (min-max)	4.0 (1–37)	2.5 (1–47)

Abbreviations: INR, international normalized ratio; ALT, alanine aminotransferase; CRP, C-reactive protein.

underwent ileocolonoscopy between 2005 and 2008 at Rikshospitalet, Oslo University Hospital.[22] The cause of referral was mainly confirmation of diagnosis, management of PSC, or follow-up after LT. Patients with previous colectomy were excluded, leaving 135 (75% male) patients included in the study (84 PSC and 51 PSC-LT; Table 1). In addition, mucosal microbiota profiles from 40 healthy subjects (80% male) were included as healthy controls (HCs).[23] HCs were recruited among subjects followed at the Department of Gastroenterology, Ullevål for regular control of polyps by colonoscopy in 2016–2017 and in 2022.

Diagnoses of PSC and IBD were made according to clinical guidelines.[1,24] Remission of ulcerative colitis was defined as a Simple Clinical Colitis Activity Index <3.[25] Biopsies were available from at least two of the four segments (terminal ileum [TI], ascending colon [AC], descending colon [DC], and sigmoid colon [SC])

for all but 5 patients who only had biopsies from one segment. For HCs, biopsies were available from three segments (terminal ileum, ascending colon, and sigmoid colon). The diagnosis of rPSC was made when cholangiography (routine MRI at 3 months, 1, 3, and 5 years, and then every 5 years after transplantation or on indication) or histology findings consistent with PSC were present in the absence of defined causes of secondary sclerosing cholangitis (hepatic artery thrombosis/stenosis, established ductopenic rejection, anastomotic biliary strictures alone, nonanastomotic strictures occurring before day 90 after LT, or ABO incompatibility between donor and recipient).[5,26]

LT-free survival was defined as the time from sampling until death or LT (ascertained in our database from The National Population Registry [death] and from the Nordic Liver Transplant Registry) or otherwise until the last day of follow-up whereby subjects who had not experienced an event were censored (December 31, 2018). Recurrence-free survival was defined among posttransplant patients not diagnosed with rPSC at the time of sampling, and was calculated as the time from inclusion until a diagnosis of rPSC or the final date of follow-up of a previous study on recurrent disease in this population (December 31, 2014).[5] Revised Mayo PSC risk score, Amsterdam–Oxford model (AOM) score for PSC, the aspartate aminotransferase (AST) to platelet ratio index (APRI), and the Fibrosis-4 score (FIB-4) were calculated according to earlier works.[27–30]

Written informed consent was obtained from all patients. The study was performed in accordance with the Declaration of Helsinki, and was approved by the Regional Committee for Medical and Health Research Ethics (projects 2015/2140 and 2016/1690).

Sample collection

Mucosal biopsies were sampled using standard forceps and were snap-frozen in dry tubes without preservatives at the time of endoscopy and stored at −80°C until time of analysis. Biopsies from HCs were preserved in RNAlater (ThermoFisherScientific, Waltham, MA), which has been shown to yield similar results to snap-freezing in dry tubes.[14] Demographic data, medication, and medical history were available from a previous study, where a questionnaire and patient charts were used.[22]

DNA isolation, library preparation, and sequencing

DNA from epithelial biopsies was extracted using the Qiagen All Prep DNA/RNA mini kit (Qiagen, Hilden, Germany).[31] DNA libraries were generated from PCR amplicons targeting the hypervariable regions, V3 and V4, of the 16S ribosomal RNA (rRNA) gene, according to a described protocol.[32] Using a dual-indexing approach, universal primers 319F (forward) and 806R (reverse) were applied for amplification, along with Phusion High-Fidelity PCR Master mix m/HF buffer (ThermoFisherScientific). Cleaning and normalization of PCR products were performed using the SequalPrep Normalization Plate Kit (ThermoFisherScientific). Quality control and quantification of pooled libraries were performed using Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA) and the Kapa Library Quantification Kit (Kapa Biosystems, London, UK). Sequencing was performed at the Norwegian Sequencing Centre (Oslo, Norway), applying the Illumina MiSeq platform and v3 kit (Illumina, San Diego, CA), allowing for 300-bp paired-end reads.

Sequence processing and bioinformatics

Paired-end reads containing Illumina Universal Adapters or PhiX were discarded using bbduk version 38.86 (BBTools; https://jgi.doe.gov/data-and-tools/bbtools/).[33] The merged contigs were trimmed to 400 bp and denoised to amplicon sequence variants (ASVs) with deblur in QIIME2 version 2020.8.[34,35] Taxonomic classification of ASVs was done based on RESCRIPt in QIIME2 using a naïve Bayes classifier trained on the V3–V4 region of a preclustered version (99% sequence similarity) of the Silva database version 138.[36–38]

Filtering of contaminants was done with the R package, microDecon, based on a negative control sample, and ASVs from mitochondria, chloroplast, or lacking taxonomy on order level were manually removed.[39] A *de novo* phylogenetic tree was built in QIIME2 based on the remaining ASVs. Samples were rarefied (subsampled without replacement) to an even level of 6321 counts per sample, and all further analyses were performed on this data set.[40,41] Only genera present in at least 25% of samples were included in the primary statistical analyses of differences in relative abundances. Alpha- and beta-diversity metrics were calculated in QIIME2. Because permutational ANOVA (PERMANOVA) is sensitive to both centroid location and within-group variability (dispersion), analysis of multivariate homogeneity of group dispersions (permutational analysis of multivariate dispersions; PERMDISP) was calculated.

Statistical analysis

The Mann–Whitney U test was applied for comparison of continuous variables. False discovery rate (FDR) was calculated using the Benjamini–Hochberg method, and FDR-corrected p values are denoted as Q_{FDR}. For visualization in case–control analyses, fold-changes of mean relative abundances were $\log_2$-transformed for

generation of taxonomic heatmaps. The PSC dysbiosis index was calculated as $\log_e$ ((sum relative abundance of increased PSC genera) / (sum relative abundance of decreased PSC genera)), as described by Gevers et al.[19]

Discrimination was assessed by visual inspection of Kaplan–Meier survival curves, the log-rank test and uni- and multivariable Cox proportional hazards modeling. Restricted mean survival times were calculated using the *survival* package in R with time horizons corresponding to the maximum time for all curves. Patients who underwent LT within 3 months from inclusion were excluded from time-to-event analyses to avoid confounding from differences in graft access ($N = 7$).

Unless otherwise specified, all other statistical analyses were performed in SPSS (version 27.0; IBM Corp., Armonk, NY). Figures were made in QIIME2 and GraphPad Prism software (version 9.0.2; GraphPad Software Inc., La Jolla, CA).

RESULTS

Major mucosal microbial alterations associated with PSC before and after LT compared with HCs

Mucosal microbiota composition was investigated in 84 patients with PSC (median age, 40 years; range, 17–77), 51 PSC-LT patients (median, 48.5 years; range, 23–70), and 40 HCs (median, 62 years; range, 33–94; 80% male; Table 1 and Figure 1).

In the first step of this study, a beta-diversity analysis (based on Bray–Curtis dissimilarity) was performed to investigate the global microbiota composition of all samples (Figure 2A). There was a large overlap between HC samples and patient samples, but also many patient samples outside the HC cluster. There was a statistically significant difference between both PSC and PSC-LT versus HC in all segments (PERMANOVA: PSC vs. HCs: pseudo-F range, 4.69–5.31; R^2 range, 0.043–0.054; $p = 0.001$), which was, in part, driven by a larger within-group variability (dispersion) among PSC samples (PERMDISP, $p = 0.200$–0.002). As previously found using fecal samples,[8] intraindividual (alpha) diversity was the main determinant of this nonoverlap (Figure 2B). In line with this, alpha diversity was reduced in all segments in PSC and PSC-LT compared with HCs, but was similar in PSC and PSC-LT (Figure 2C,D). Alpha diversity was also similar in all investigated gut segments within each group (Figure 2C,D).

When considering the taxonomic composition, segment-wise analyses at the phylum level showed major differences between HCs and both PSC and PSC-LT (Figure 3A), although the most prominent changes were in the PSC-LT cohort. The main characteristic was

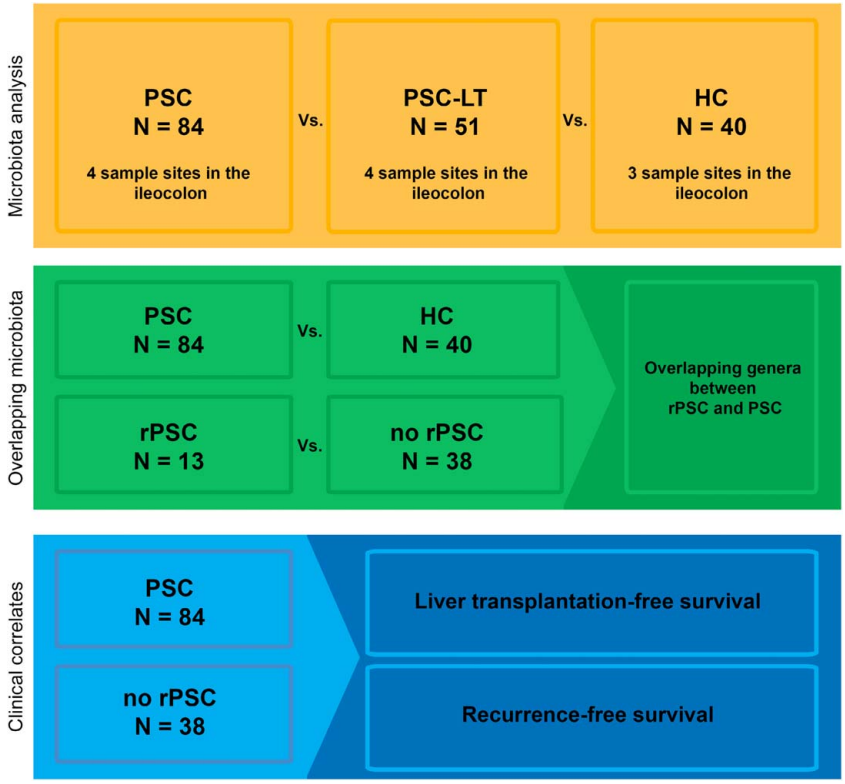

FIGURE 1 Study flowchart showing main groups and analyses performed. In a first step, microbiota analysis of the main study cohorts was performed. Next, identification of overlapping microbiota features between PSC and rPSC was performed. Last, identified microbiota features were used to investigate clinical correlates, among them LT-free survival and time to diagnosis of recurrent PSC (recurrence-free survival).

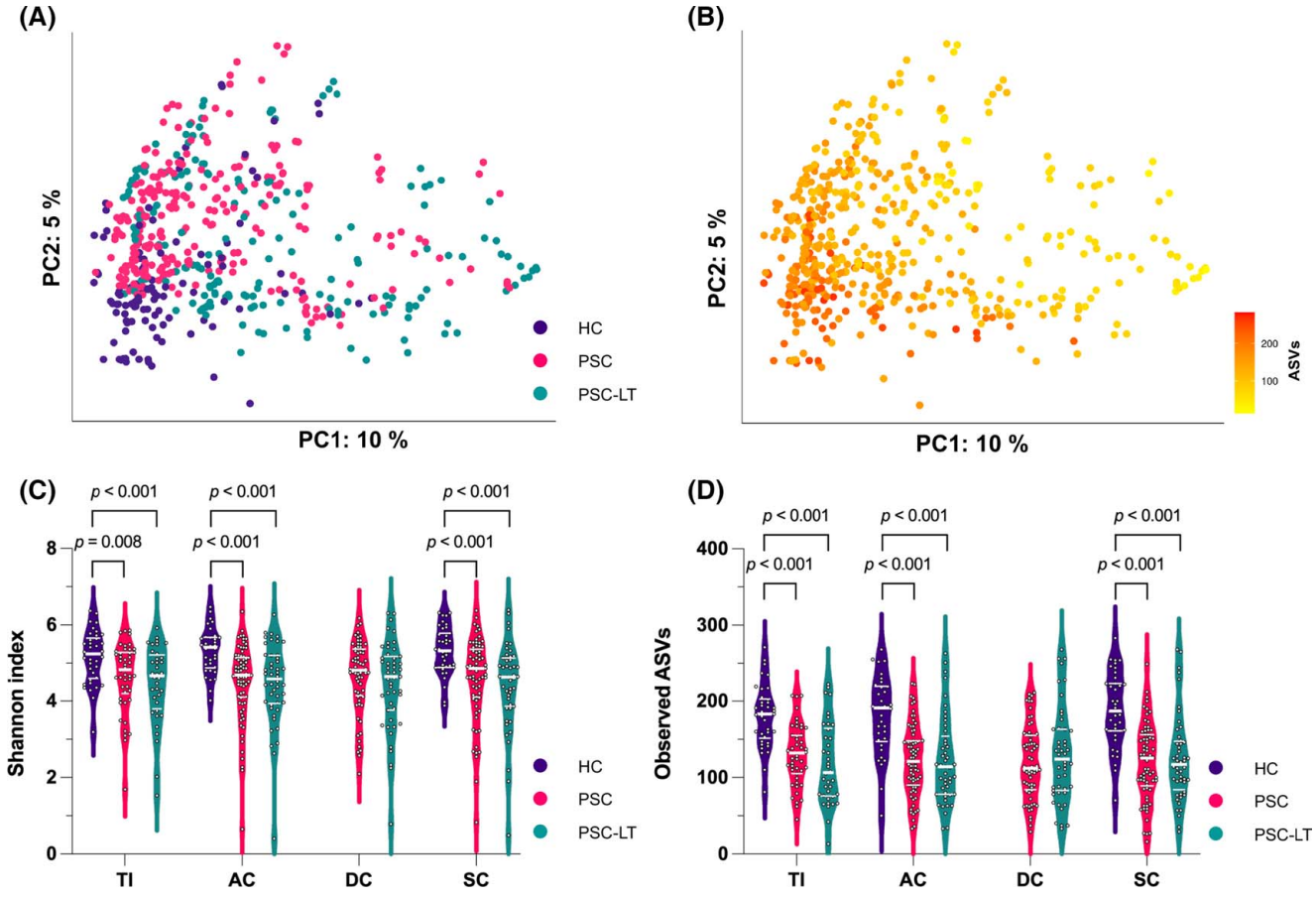

FIGURE 2 Diversity metrics for PSC, PSC-LT, and HCs. (A) Principal coordinate analysis plot (Bray–Curtis dissimilarity) showing that PSC and PSC-LT have unequal microbiota composition (PERMANOVA: R^2: 0.017, pseudo-F: 7.38, $p = 0.001$; PERMDISP: $p = 0.74$), and that both are unequal compared with HCs, which, in part, is explained by higher dispersion in PSC and PSC-LT (PERMANOVA: R^2: 0.041, pseudo-F: 15.60, $p = 0.001$ and R^2: 0.064, pseudo-F: 19.72, $p = 0.001$, respectively, and PERMDISP: $p = 0.001$ and $p = 0.001$, respectively). (B) Principal coordinate analysis in panel A colored according to alpha diversity (observed ASVs), showing that the pattern along the PCA1-axis is driven by alpha diversity, Spearman correlation coefficient: −0.65, $p < 2.2 \times 10^{-16}$. (C) Reduced alpha diversity (Shannon diversity index) in PSC and PSC-LT compared with HC in all segments and similar alpha diversity between segments in all groups. (D) Reduced alpha diversity (observed ASVs) in all available segments in PSC and PSC-LT compared with HCs. PC, principal coordinate; PSC-LT, transplanted PSC.

an expansion of Proteobacteria, which was statistically significant (at a Q_{FDR} threshold of 0.05) in PSC-LT, with mean relative abundances of 17.5%–18.5% in the different segments compared with 6.7%–8.1% in HCs (Q_{FDR}[TI] = 0.037, Q_{FDR}[AC] = 0.007, Q_{FDR}[SC] = 0.056). Actinobacteria was reduced in the ascending colon of PSC (1.1%) and PSC-LT (1.2%) compared with 1.8% in HCs (Q_{FDR} = 0.046 and Q_{FDR} = 0.018, respectively). Also, Firmicutes was reduced in both the ascending (56.3%) and sigmoid colon (53.6%) of PSC-LT compared with 66.4% and 65.1% in HCs (Q_{FDR}[AC] = 0.009 and Q_{FDR}[SC] = 0.003), respectively.

At the genus level and using a Q_{FDR} threshold of 0.05, 61 and 59 genera were significantly different in at least one segment between HCs and both PSC and PSC-LT, respectively (Figure S1). Among these, eight genera were increased in both PSC and PSC-LT as compared with HCs. This included *Haemophilus*, *Veillonella*, and *Roseburia*, which also were significantly increased in all three ileocolonic segments. Expansion of Proteobacteria in PSC-LT was primarily driven by increased relative abundances of the *Escherichia-Shigella* genus and genera of the *Enterobacteriaceae* family (Figure 3B). In contrast, short-chain fatty acid (SCFA) producers, such as *Faecalibacterium* and *Odoribacter*, were reduced in both PSC and PSC-LT compared with HC (Figure S1).

Mucosal *Akkermansia* was depleted in PSC with IBD irrespective of transplantation status

Because intestinal inflammation may impact the mucosal microbiota,[19] we compared the microbiota profiles of patients with or without concomitant IBD. Prevalence of IBD was similar in the pre-LT and post-LT PSC groups (108 [80%] and 66 [77%], respectively; Table 1). Groups were also similar in terms of their

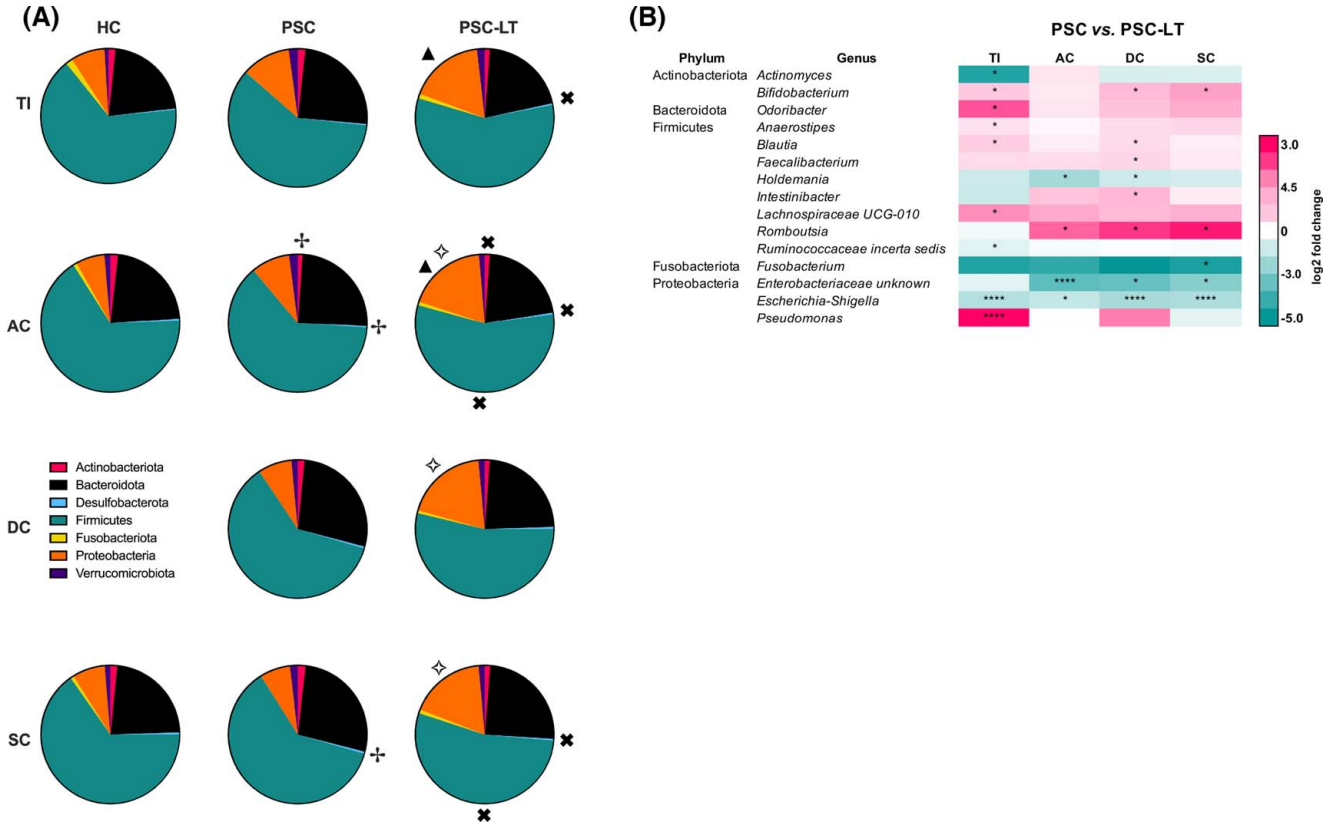

FIGURE 3 Phylum distribution in study cohorts and taxonomic heatmaps in PSC versus PSC-LT. (A) Mean relative abundance of major phyla in HC, PSC, and PSC-LT. ✧ Increased in PSC-LT versus PSC. ✢ Reduced in PSC versus HC. ▲ Increased in PSC-LT versus HC. ✖ Reduced in PSC-LT versus HC. Significance-level $Q_{FDR} < 0.05$. (B) Log_2-fold change of significantly increased or decreased genera (Mann–Whitney U test) in PSC compared with PSC-LT. Log_2-fold change >0 indicates increased relative abundance in PSC. *$p < 0.05$; ****$Q_{FDR} < 0.05$. PSC-LT, transplanted PSC.

fecal calprotectin level and fractions of patients in clinical IBD remission (ulcerative colitis; data not shown).

In the analysis of global microbiota composition (beta diversity), PSC-IBD was significantly different from PSC without IBD irrespective of transplantation status (PERMANOVA: pseudo-F: 3.6, $p = 0.001$; PERMDISP: $p = 0.09$; Figure 4A). There were no overall significant differences in alpha diversity between patients with IBD and those without (Figure 4B). However, when we investigated patients before and after transplantation separately, PSC-LT with IBD had lower alpha diversity than those without IBD in the ascending colon ($p = 0.02$; Figure 4C).

On a taxonomic level, *Akkermansia* was identified as reduced in patients with IBD in the sigmoid colon ($Q_{FDR} = 0.024$), and there was also evidence of it being reduced in the other three segments (unadjusted $p < 0.05$; Figure 4D). When subgrouped by transplantation status, the reduction of *Akkermansia* was most evident in the nontransplanted group with concomitant IBD, with 26.6% being carriers of *Akkermansia* compared with 72.2% in non-IBD (χ^2 test, $p = 0.001$). In PSC-LT, 35.7% were *Akkermansia* carriers in IBD compared to 55.6% in non-IBD (χ^2 test, $p = 0.3$).

Overlapping microbial signature in PSC and recurrent PSC

To identify a PSC-specific microbiota irrespective of LT, we investigated the overlap between features altered in PSC versus HC and in rPSC versus PSC-LT without recurrence (no-rPSC). Of 51 transplanted PSC patients, 13 (25.5%) had or were diagnosed with rPSC at inclusion (Table 1). There were no significant differences in alpha diversity between patients with rPSC and no-rPSC (Figure 5A). Analysis of global microbiota composition (beta diversity) showed that rPSC had more extensive within-group variability (dispersion) compared with no-rPSC (PERMDISP: $p = 0.006$; Figure 5B). To identify overlapping features of PSC and rPSC at the genus level, all genera with different relative abundance in PSC versus HC at $Q_{FDR} < 0.05$ in at least one segment were compared between rPSC and no-rPSC and selected if a similar difference ($p < 0.05$) was observed in at least one segment (Figure 5C). With this approach, five genera were found to be uniformly associated within any segment both between PSC and HC and between rPSC and no-rPSC. Three of these showed a reduced abundance in both PSC and rPSC: *Lachnospiraceae ND3007 group*, *Lachnospiraceae CAG-56*, and *Bilophila*;

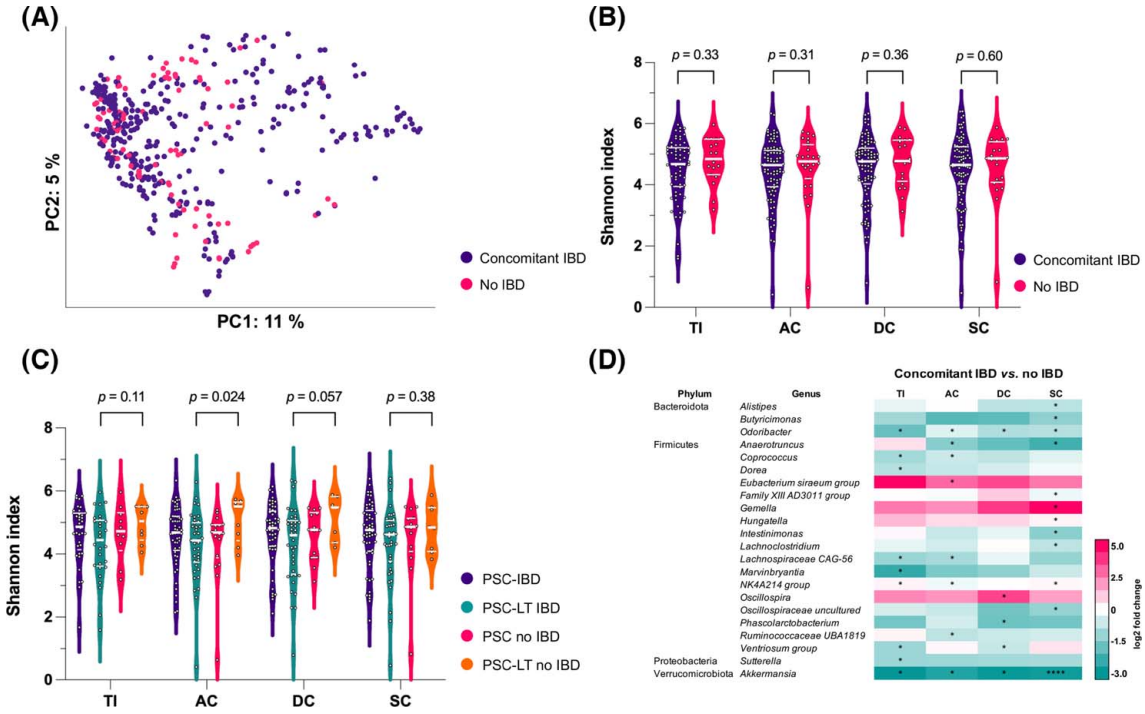

FIGURE 4 Diversity metrics and taxonomic heatmap in PSC with concomitant IBD. (A) Principal coordinate analysis plot (Bray–Curtis dissimilarity) showing that PSC patients (irrespective of LT status) with IBD have significantly different microbiota composition compared with PSC without IBD (PERMANOVA: R^2: 0.0082, pseudo-F: 3.61, $p = 0.001$; PERMDISP: $p = 0.092$). (B) No significant differences in alpha diversity (Shannon diversity index) between PSC with IBD and PSC without IBD (irrespective of LT status). (C) Alpha diversity (Shannon diversity index) in PSC-LT with IBD compared to PSC-LT without IBD. (D) Taxonomic heatmap of significantly increased or decreased genera (Mann–Whitney U test) in PSC-LT and PSC with IBD compared to those without. Log_2-fold change >0 indicates increased genus in PSC with concomitant IBD. *$p < 0.05$; ****$Q_{FDR} < 0.05$. PC, principal coordinate; PSC-LT, transplanted PSC.

and two were uniformly increased: *Streptococcus* and *Hungatella*. There was no evidence of an increased abundance or difference in carriership of *Veillonella* in rPSC compared to no-rPSC (data not shown).

Clinical impact of mucosal gut microbiota associations in PSC and rPSC

We hypothesized that gut microbiota alterations associated with PSC and rPSC could associate with disease severity, liver biochemistry, and survival. A total of 77 PSC patients were eligible for LT-free survival analysis, of which 45 either died or underwent an LT within the follow-up (median transplantation-free survival, 8.4 years; 95% CI, 4.5–12.4). In PSC-LT, 38 patients were rPSC free at the time of inclusion. Twelve of these developed rPSC within a median time of 8.1 years.

First, we assessed the relationship between the five PSC- and rPSC-associated genera and LT-free survival. Patients with relative abundance of *Streptococcus* in the highest quartile had the poorest survival across all segments, although the overall log-rank tests were not statistically significant for the ascending and sigmoid colon (Figure S2). None of the genera were associated with time to rPSC after transplantation.

Next, we calculated a dysbiosis index (Patients and Methods) per segment based on the five overlapping genera associated with both PSC and rPSC. Samples with undetectable levels of all the increased or decreased genera were excluded. Considering all PSC patients together, the dysbiosis index in the sigmoid colon correlated positively with Mayo PSC score ($r_s = 0.25$, $p = 0.03$), alkaline phosphatase (ALP; $r_s = 0.27$, $p = 0.01$), and AST ($r_s = 0.22$, $p = 0.04$). Modeling the sigmoid dysbiosis index as a continuous variable in a univariable Cox regression, an increase in the index was associated with an elevated risk of LT or death (HR, 1.35; 95% CI, 1.08–1.68; $p = 0.01$), with similar results in the remaining colonic segments and the terminal ileum (Table S1). Adjusting for Mayo PSC risk score had no discernible impact on the estimates (Table 2). The risk estimate of the dysbiosis index was similar with recurrence-free survival, but this was not statistically significant (HR = 1.47; 95% CI, 0.97–2.22; $p = 0.07$; Table S1). When modeled as a dichotomous marker, a high sigmoid dysbiosis index corresponded to a restricted mean transplantation-free survival time (RMST) of 5.52 versus 8.99 years (log-rank, $p = 0.05$; Figure 6A) and reduced time to diagnosis of rPSC (RMST, 6.56 vs. 8.53 years; log-rank, $p = 0.02$; Figure 6B and Figure S3).

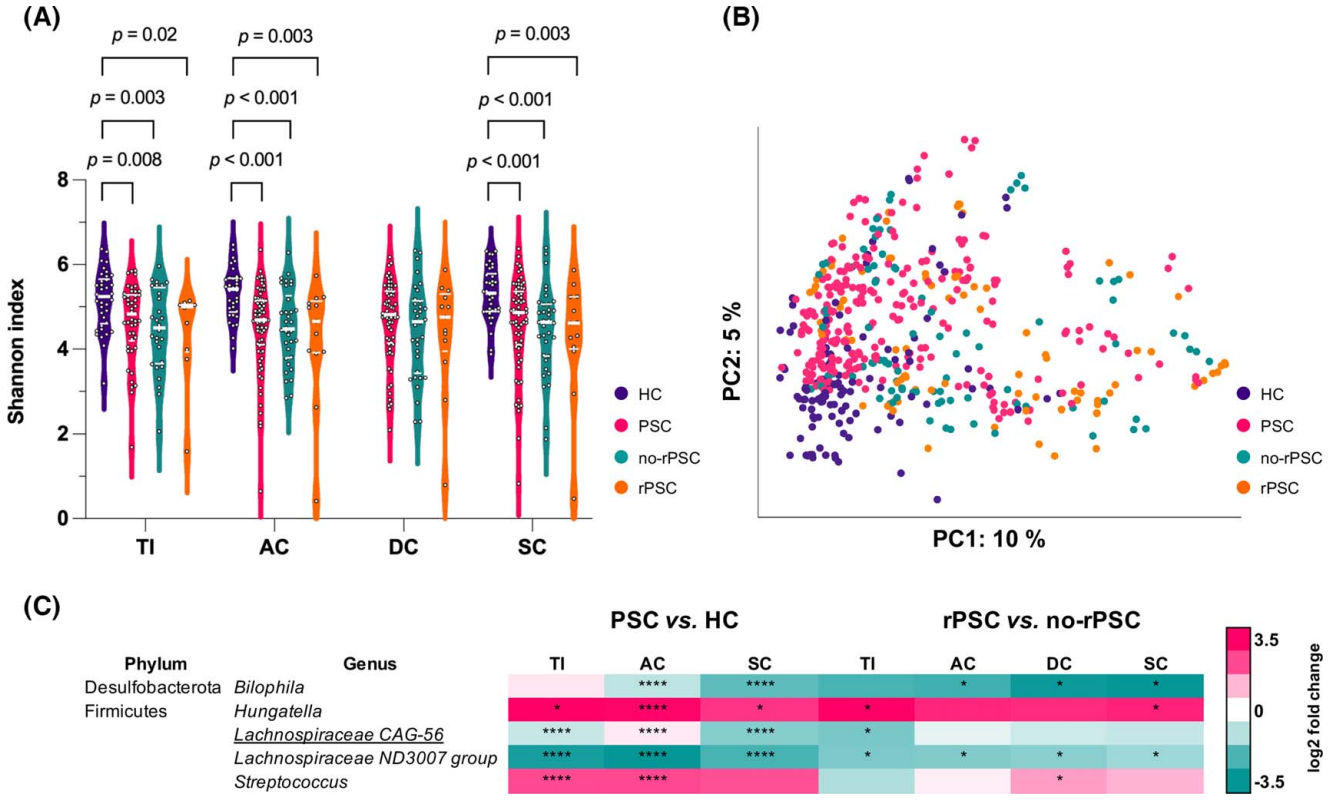

FIGURE 5 Diversity and overlapping genera in PSC and rPSC. (A) Reduced alpha diversity (Shannon diversity index) in rPSC (at inclusion) compared with HC. (B) Principal coordinate analysis plot (Bray–Curtis dissimilarity) showing that rPSC have significantly different dispersion compared to no-rPSC (PERMANOVA: R^2: 0.02 pseudo-F: 3.4, $p = 0.001$; PERMDISP: $p = 0.006$). (C) Five significantly increased or decreased genera associated with PSC or rPSC compared with HCs and no-rPSC, respectively (Mann–Whitney U test). Log_2-fold change >0 indicates increased genus in PSC/rPSC. *Lachnospiraceae CAG-56* (underscored) was, on average, increased in the ascending colon in PSC relative to HCs, although the median in PSC was lower than that of HCs. *$p < 0.05$; ****$Q_{FDR} < 0.05$. no-rPSC, transplanted PSC without rPSC; PC, principal coordinate.

Given that *K. pneumoniae* has been shown to worsen experimental biliary disease,[13] we specifically investigated its impact on PSC and rPSC despite its overall low prevalence. *Klebsiella* was observed in at least one segment in 14 of 84 PSC patients and in 21 of 51 PSC-LT patients (16.7% vs. 41.2%, respectively; χ^2 test, $p = 0.002$; Figure S4). *Klebsiella* was only detected in three mucosal biopsies from 2 HCs (5%). Regardless of transplantation status, PSC patients who were carriers of *Klebsiella* in any segment had a higher AOM PSC score (Mann–Whitney U test, $p = 0.002$), Mayo PSC risk score ($p = 0.001$), APRI ($p = 0.02$), and FIB-4 score ($p = 0.001$; Figure 6C and Figure S4). Furthermore, *Klebsiella* in any segment was associated with shorter LT-free survival (RMST 4.28 vs. 7.87 years; log-rank, $p = 0.01$; Figure 6D), an effect that was similar when investigating carriership in the individual segments (Figure S5). After LT, *Klebsiella* was detected in 7 of 13 (53.8%) of patients with rPSC at inclusion compared with 14 of 38 (36.8%) in no-rPSC (χ^2 test, $p = 0.3$). *Klebsiella* carriers in the ascending colon had reduced time to diagnosis of rPSC (RMST 6.51 vs. 8.35 years; log-rank, $p = 0.04$), whereas no such association was observed in the remaining segments (Figure S5).

Neither measures of alpha diversity nor the relative abundance of *Veillonella* or the IBD-associated genus, *Akkermansia*, showed associations with PSC risk scores, biochemical markers, or survival in PSC or rPSC (data not shown).

TABLE 2 Multivariable Cox regression for dysbiosis index and transplantation-free survival in PSC and time to diagnosis of recurrent PSC from inclusion, in PSC-LT

Variable	HR	95% CI	*p* value
Transplantation-free survival			
Dysbiosis index sigmoid colon	1.28	1.02–1.61	0.03
Mayo PSC risk score	1.52	1.09–2.12	0.02
Recurrence-free survival			
Dysbiosis index sigmoid colon	1.42	0.93–2.15	0.10
FIB-4 score	0.53	0.15–1.89	0.30

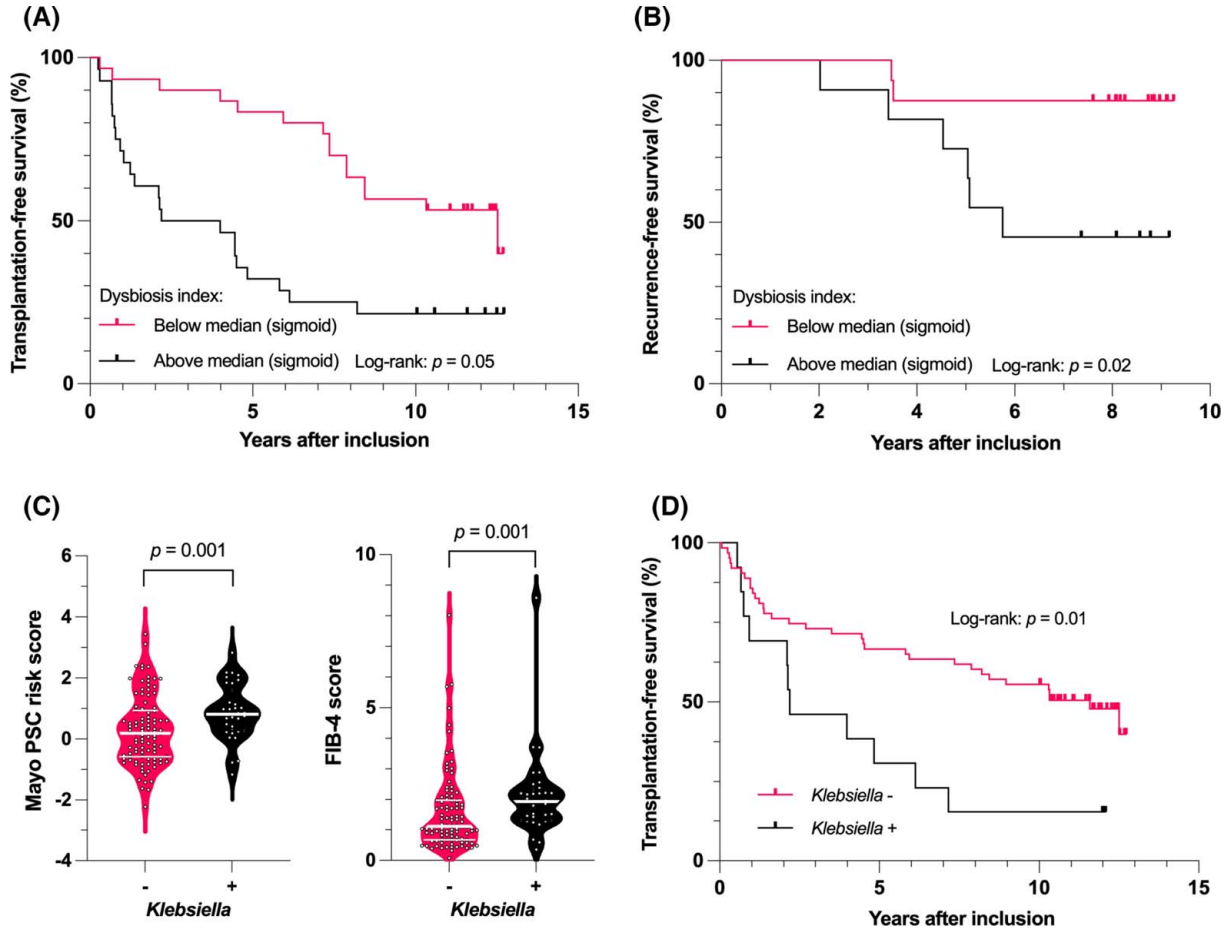

FIGURE 6 Microbiota associations with disease severity and survival. (A) LT-free survival was reduced in those with a dysbiosis index (sigmoid colon) above median. RMST 5.52 versus 8.99 years, log-rank: $p = 0.05$, Kaplan–Meier plot. (B) Recurrence-free survival from date of inclusion was reduced in those with dysbiosis index (sigmoid colon) above median. RMST 6.56 vs. 8.53 years, $p = 0.02$, Kaplan–Meier plot. (C) Mayo PSC risk score and FIB-4 score were increased in PSC patients (regardless of transplantation status) positive for *Klebsiella* in gut mucosal samples. (D) LT-free survival was reduced in patients positive for mucosal *Klebsiella*, in any segment, RMST 4.28 versus 7.87 years, $p = 0.01$, Kaplan–Meier plot. no-rPSC, transplanted PSC without rPSC.

DISCUSSION

In this cross-sectional study of the gut mucosal microbiota of the ileocolon of 135 nontransplanted and transplanted PSC patients, we made the following observations: (1) Microbial alterations in patients with PSC, such as increased Proteobacteria, were more pronounced in PSC-LT compared with HCs, suggesting that transplantation for PSC does not normalize the microbiota. (2) In contrast to findings in fecal microbiota, concomitant IBD was associated with altered mucosal microbiota, including reduced abundance of *Akkermansia*. (3) By considering PSC and rPSC in parallel, five genera were found to associate with PSC both before and after LT. (4) Presence of the potential pathobiont, *Klebsiella*, as well as an index of the PSC- and rPSC-associated genera were associated with worse outcome in PSC. Taken together, our data suggest that mucosal microbiota composition provides information beyond what is observed in fecal samples, and open the possibility that microbial alterations may contribute to disease and disease severity both before and after LT. We also propose that rPSC is a possible human model system to study PSC.

Major overlapping differences were observed between both PSC and PSC-LT compared with HCs, including overall reduced alpha diversity and expansion of known PSC-associated genera. The possibility that liver disease is the primary driver of gut microbiota alterations in PSC suggests that LT could normalize the microbiota composition, which is supported by some studies.[20] However, our transplanted PSC patients had extensive microbiota alterations compared to HCs and PSC, including expansion of known pathogens and PSC-associated genera such as *Escherichia*, *Streptococcus*, and *Veillonella*. Previous studies on the posttransplant microbiota are few, and none have involved autoimmune liver disease. A recent study found that patients after LT with abnormal liver function tests had reduced alpha diversity and enrichment of opportunistic pathogens, such as *Klebsiella* and *Escherichia/Shigella*,[42] whereas another study found

no such differences between pretransplant or posttransplant patients and controls.[21] It is therefore an open question whether the gut microbial environment observed after LT in PSC may contribute to posttransplant health or illness. Future attempts to clarify this should be a priority, given that gut-targeted preventive measures could possibly reduce the risk of posttransplant liver disease.

Recurrent disease after LT represents an opportunity to study PSC development from its earliest stages. We did not find a reduction in alpha diversity in rPSC compared with no-rPSC as hypothesized, but we did observe an increased within-group variability (dispersion) in both PSC compared with HCs and rPSC versus no-rPSC, perhaps suggesting an increased instability of microbiota composition in patients that have acquired sclerosing cholangitis. Still, when considering the parallel case–control groups PSC versus HC and rPSC versus no-rPSC, we identified five genera uniformly increased or decreased, specific to PSC and rPSC (Figure 4C); for example, *Streptococcus* has been found in several former studies in PSC and could be of particular relevance.[6,7,9,16] Two of the three reduced genera are known SCFA producers, and alterations in SCFA function could be speculated to influence the mucosal barrier, immune cell regulation, and inflammation.[43] Identification of overlapping features in PSC and rPSC and the observations that these may associate with transplantation- and recurrence-free survival, suggest that this is a useful strategy in future studies of PSC. Notably, independent validation in larger cohorts, in particular posttransplant, would be a prerequisite in such studies, and it would be advisable to define the important features using unsupervised statistical methods.

K. pneumoniae has been of special interest after recent experimental findings suggest that it is able (together with other bacteria) to impair the intestinal barrier and promote liver inflammation.[13] *Klebsiella* was not part of our primary analysis because of low prevalence in the nontransplanted cohort (detected in <25% of samples). However, when performing a dedicated hypothesis-driven assessment, the presence of *Klebsiella* was associated with reduced LT-free survival and increased AOM and Mayo PSC risk score. The similar relationship between *Klebsiella* and rPSC was not statistically significant, but these observations represent a potential human parallel to the previous experimental animal data by Nakamoto et al.[13] The *Klebsiella* genus reported here would include potentially pathogenic *K. pneumoniae* strains, but the 16S rRNA-based data do not allow analysis at species resolution, and it is not possible to assess whether our findings are driven by, for example, *K. pneumoniae* or substrains thereof or other *Klebsiella* species. Further studies are therefore needed to establish the concept of *Klebsiella* species as clinically relevant modifier microbes in human PSC, which could be speculated to represent a parallel to, for example, the cytolysin-producing *Enterococcus* found in alcohol-associated acute liver injury.[44]

Previous studies of the fecal microbiota have not found consistent differences between PSC patients with and without IBD, perhaps suggesting that liver disease is the driver of microbiota alterations and not IBD.[6–9] However, when studying the mucosal microbiota, we consistently found reduced relative abundance of *Akkermansia* in all segments of the colon of PSC patients with concomitant IBD, irrespective of transplantation status. This observation clearly contrasts the data from fecal microbiota studies in PSC,[6,8,12,45] but is in line with several studies observing reduced *Akkermansia* in IBD patients (without PSC) compared with HCs.[46–48] Several important points can be discussed. First, *A. muciniphila* uses mucins as its primary energy source and mucins are reduced in IBD,[49] which suggests that the reduction could be a secondary phenomenon. On the other hand, extracellular vesicles from *A. muciniphila* have been shown to alleviate experimentally induced colitis and protect epithelial integrity.[50] It has also been shown to exhibit protective properties against metabolic disease,[51] overall suggesting that *A. muciniphila* may be a disease modifier. Second, there is evidence that IBD status impacts prognosis of PSC and development of rPSC,[2,5] but there is limited knowledge of whether gut microbiota and intestinal disease contribute directly to hepatobiliary disease. Third, different observations in the microbiota of the mucosa and the lumen (stool) suggest not only differences in biomarker abilities, but also the functional role of these two compartments. This means that choice of study design and materials are critical in the further delineation of the relationship between microbiota alterations, mucosal inflammation, and the potential impact on liver disease, and perhaps mucosal biopsies should be prioritized in further studies focusing on PSC-IBD.

This is, so far, the largest study of the mucosal microbiota in PSC, where the parallel assessment of PSC and rPSC made it possible to identify overlapping microbiota associations in PSC before and after LT. Long follow-up time allowed us to identify microbiota features predicting death or LT and diagnosis of rPSC. However, the final number of participants in the individual segments in the individual groups, in particular in the PSC-LT group, limited the power of the analysis. A control group of IBD patients without PSC would have been of interest, in particular to identify what features of the PSC microbiota are typical in IBD irrespective of liver disease. Also, given that patients with other liver diseases are not routinely investigated by colonoscopy before or after transplantation, there is a lack of non-PSC controls making it possible to determine whether the findings are PSC specific. Future

assessments should therefore preferably include both control liver diseases and external cohorts to show generalizability, the latter also because the present cohort is from a tertiary care center in Norway with a fairly homogeneous population. Furthermore, our study is cross-sectional with inherent limitations in establishing causality, and we also did not have information about diet or previous antibiotic or probiotic use.

In conclusion, the parallel investigation of patients both before and after LT highlights a limited set of microbes that could be particularly relevant for the presence and severity of PSC, with an unhealthy gut microbial environment after LT potentially contributing to recurrent disease. The findings provide a further rationale for targeting the gut microbiota and possibly modulate liver disease in PSC.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to the intellectual content of the article and approved the final manuscript. Mikal Jacob Hole: conceptualization; acquisition of data; data analysis; drafting of the manuscript; manuscript review and editing, approval of the final draft. Kristin Kaasen Jørgensen: acquisition of data; data analysis; drafting of the manuscript; manuscript review and editing, approval of the final draft. Kristian Holm: acquisition of data; data analysis; drafting of the manuscript; manuscript review and editing, approval of the final draft. Peder R. Braadland: data analysis; drafting of the manuscript; manuscript review and editing, approval of the final draft. Malin H. Meyer-Myklestad: participant recruitment; manuscript review and editing, approval of the final draft. Asle W. Medhus: participant recruitment; manuscript review and editing, approval of the final draft. Dag Henrik Reikvam: participant recruitment; manuscript review and editing, approval of the final draft. Alexandra Götz: acquisition of data; manuscript review and editing, approval of the final draft. Krzysztof Grzyb: acquisition of data; data analysis; manuscript review and editing, approval of the final draft. Kirsten Muri Boberg: acquisition of data; data analysis; manuscript review and editing, approval of the final draft. Tom Hemming Karlsen: acquisition of data; data analysis; manuscript review and editing, approval of the final draft. Martin Kummen: conceptualization; data analysis; drafting of the manuscript; manuscript review and editing, approval of the final draft. Johannes R. Hov: conceptualization; project supervision; acquisition of data; data analysis; drafting of the manuscript; manuscript review and editing, approval of the final draft.

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CONFLICT OF INTEREST

Tom Hemming Karlsen owns stock in Ultimovacs. He advises for Intercept, Gilead, and Albireo. Kristin Kaasen Jørgensen advises for Celltrion and Norgine. She is on the speakers' bureau for BMS and Roche.

DATA AVAILABILITY STATEMENT

The datasets of the current study are not publicly available due to Norwegian legislation regarding general data protection regulation. Data are available from the corresponding author (JRH), on reasonable request, pending a material and data transfer agreement and an amendment application to the committee for medical research ethics.

ORCID

Peder R. Braadland https://orcid.org/0000–0002–0794–7737
Malin Holm Meyer-Myklestad https://orcid.org/0000–0003–3162–5196
Dag Henrik Reikvam https://orcid.org/0000–0002–4499–0320
Krzysztof Grzyb https://orcid.org/0000–0002–2718–8125
Martin Kummen https://orcid.org/0000–0001–9660–6290
Johannes R. Hov https://orcid.org/0000–0002–5900–8096

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