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Original Article

Inflammatory Bowel Disease Associated with Primary Sclerosing Cholangitis is Associated with an Altered Gut Microbiome and Bile Acid Profile

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Abstract

Background: Primary sclerosing cholangitis associated with inflammatory bowel disease [IBD-PSC] carries significant morbidity compared to IBD without PSC. Alterations in microbial composition and bile acid [BA] profiles have been shown to modulate chronic inflammation in IBD, but data in IBD-PSC are scarce. We aimed to assess the differences in gut microbiome composition as well as in the BAs profile and BA-related microbial functions between IBD-PSC and IBD-only.

Methods: In total, 54 IBD-PSC and 62 IBD-only subjects were enrolled from 2012 to 2021. Baseline samples were collected for faecal DNA shotgun metagenomic sequencing, faecal and serum BA quantification using mass spectrometry, and faecal calprotectin. Liver fibrosis measured by transient elastography was assessed in the IBD-PSC group. Data were analysed using general linear regression models and Spearman rank correlation tests.

Results: Patients with IBD-PSC had reduced microbial gene richness [$p = 0.004$] and significant compositional shifts [PERMANOVA: $R^2 = 0.01$, $p = 0.03$] compared to IBD-only. IBD-PSC was associated with altered microbial composition and function, including decreased abundance of *Blautia obeum*, increased abundance of *Veillonella atypica*, *Veillonella dispar*, and *Clostridium scindens* [$q < 0.05$ for all], and increased abundance of microbial genes involved in secondary BA metabolism. Decreased serum sulphated and increased serum conjugated secondary BAs were associated with IBD-PSC and increased liver fibrosis.

Conclusion: We identified differences in microbial species, functional capacity, and serum BA profiles in IBD-PSC compared with IBD-only. Our findings provide insight into the pathophysiology of IBD associated with PSC and suggest possible targets for modulating the risk and course of IBD in subjects with PSC.

Key Words: Inflammatory bowel disease; primary sclerosing cholangitis; gut microbiome; bile acids

1. Introduction

Primary sclerosing cholangitis [PSC] is an immune-mediated, chronic liver disease characterized by inflammation and fibrosis of the bile ducts and is closely associated with inflammatory bowel disease [IBD].[1] The IBD phenotype associated with PSC [IBD-PSC] differs from that of IBD without PSC [IBD-only] and is more frequently characterized by a quiescent disease course, right-sided colonic predominance, and higher prevalence of colonic neoplasia.[2]

The aetiology of PSC and the factors that lead to IBD-PSC and its unique phenotype are unknown; however, one hypothesis is that IBD-PSC compared to IBD-only is characterized by differences in gut microbiome structure and function. However, this has mainly been demonstrated in comparison with healthy controls.[3–6] Additionally, it is well recognized that the gut microbiome and bile acids [BAs] have a close bidirectional relationship, resulting in modulation of microbiome composition by BAs and formation of secondary BAs by BA-metabolizing microbiota,[4,5,7–9] which are involved in host immunity and barrier function.[10–12] However, although the role of BAs is well described in IBD,[10,13,14] it is not well characterized in IBD-PSC.[9]

BA synthesis takes place in the liver resulting in the formation of primary BAs which are then conjugated and secreted via bile into the small intestine. The majority of conjugated BAs will then be recirculated to the liver via the portal vein, whereas the remainder will be subject to microbial transformation in the colon including deconjugation by bile salt

hydrolase [BSH], dehydrogenation, and dihydroxylation via enzymes, such as those encoded on the microbial BA-inducible [bai] operon, resulting in the formation of secondary BAs.[15] However, under cholestatic conditions, such as PSC, in which the biliary outflow and enterohepatic circulation are impaired, hepatic sinusoidal transporters are upregulated, resulting in BA efflux towards the peripheral circulation.[9,16]

In this study, we used shotgun metagenomic sequencing and a large array of serum and stool BA metabolites to assess the PSC-driven differences in stool microbial composition and BA-related microbial functions in IBD with and without PSC comorbidity.

2. Methods

2.1. Subject recruitment

In total, 116 subjects were recruited between 2012 and 2021 from two centres in Toronto, Canada [Mount Sinai Hospital and the Toronto Centre for Liver Disease at University Health Network]. Eligible patients were identified by review of existing databases and outpatient booking records at recruitment sites. Eligible subjects were approached by telephone or in-person during outpatient clinic attendance to ascertain their willingness to participate in the study and to provide written informed consent in accordance with the protocol approved by the local institutional Research Ethics Board at each institution. Subjects with IBD, with and without PSC [IBD-PSC and IBD-only, respectively], were recruited. PSC diagnosis was based on clinical guidelines and typical findings on liver biopsy or cholangiography. IBD diagnosis was based on clinical guidelines and typical findings on endoscopy. Patients in the IBD group were screened to rule out chronic liver disease based on history, liver enzymes, and, where available, imaging studies. Patients in the IBD-only group had to have colon-predominant IBD [ulcerative colitis or IBD-unclassified].

All subjects had to be on stable medication treatment for at least 3 months prior to enrolment and without antibiotic treatment for at least 6 weeks prior to sample collection. Subjects with prior bowel resection, gastrointestinal stoma, or pregnancy were excluded. Subjects with PSC-related orthotopic liver transplantation prior to recruitment were allowed to be enrolled to the study if they had clear evidence of PSC recurrence in the graft.

Baseline demographic information [biological sex, age at recruitment, age at IBD, and PSC diagnosis] and clinical parameters (duration of IBD and PSC, clinical activity based on the partial Mayo score, smoking status, body mass index [BMI], current medications, blood work, and faecal calprotectin) were recorded for each subject. Median imputation was applied for features with less than 10% missing data [BMI and faecal calprotectin, 6% and 6.7% respectively]. In the IBD-PSC group, degree of liver fibrosis was measured by transient elastography [TE] [Fibroscan®]. Acceptable cutoff values were utilized [F0/F1 0–8.5 kPa; F2 8.6–9.5 kPa; F3 9.6–14.3 kPa; F4 ≥ 14.4 kPa] and the score was dichotomized to F0–3 [none-to-moderate] and F4 [severe] fibrosis.[17] For each subject, the scan with the highest degree of liver fibrosis measured during the follow-up period was selected for analysis while taking into account the time difference from recruitment to TE measurements [Table 1].

2.2. Stool sampling and DNA extraction

In total, 115 out of 116 subjects had available stool samples. Subjects collected their baseline stool samples using a provided stool commode [Fisher Scientific]. A polypropylene specimen collection container [Starplex Scientific] was used to take an aliquot of stool from the commode. The stool sample was then immediately placed into the subject's home freezer. To prevent freeze thawing, the home frozen stool sample was transferred frozen using icepacks to the recruitment centre where it was stored in monitored −80°C freezers. Stool DNA extraction was performed manually by the DNeasy Powersoil Pro kit [47014], following the company's protocol.

2.3. Metagenome library preparation, sequencing, and post-sequencing processing

The libraries were prepared at the Centre for the Analysis of Genome Evolution and Function [CAGEF], using the Illumina DNA Prep kit [Illumina] according to the manufacturer's guidelines. To avoid batch effects, all samples were pooled together and sequenced together on an Illumina NextSeq high-output device using V2 chemistry with 150 × 2 paired-end reads to a sequence depth of 5 million reads per sample. The sequences were trimmed to remove adapters and low-quality sequences using Trimmomatic, following default parameters, an average quality minimum of 20, and a minimum sequence length of 125 bp.[18] PCR duplicates were identified and removed using PrinSeq.[19] Human sequences were identified using Bowtie2.[20] One sample from the IBD-PSC group failed quality control and was excluded. HUMAnN3.6 [v0.11.2] software was used to identify the taxonomic and functional profiles of each community, using MetaPhlAn3.0 [v2.7.8] for taxonomy and the UniRef90 database for function, following all default parameters.[21,22] Resulting functional annotations were mapped to the MetaCyc gene family ontology.[23]

2.4. Stool and serum BA metabolites

Baseline stool and serum samples from 108 and 102 subjects, respectively, were used for metabolomic analysis. In total, 100 µL of serum was aliquoted, and 50 mg of stool was aliquoted using Integra™ Miltex™ Standard Biopsy Punches and immediately frozen at −80°C until ready to ship on dry ice to the Metabolomics Innovation Centre [TMIC, Alberta, Canada]. Eighty-three serum BA [µM] and 84 stool BA [nmol/g] samples were quantified by ultra-performance liquid chromatography [UPLC]-multiple reaction monitoring mass spectrometry [MRM/MS]. Measurements below the limit of detection [LOD] were set to half of the LOD value for each metabolite in the dataset (MetaboAnalystR [v3.1.0] R package).

2.5. Assessment of faecal calprotectin

The faecal calprotectin concentration was measured by the BÜHLMANN fCAL® ELISA test following the manufacturer's protocol. A faecal calprotectin working range of 30–1800 µg/g was used.

2.6. Statistical analysis of microbial data

To assess alpha diversity, the sum of observed gene families [microbial gene richness], and species-level Chao1 index and Shannon index were compared between IBD-PSC and IBD-only using the Wilcoxon rank sum test. Beta diversity was assessed with principal coordinates analysis plots at the species

Table 1. Cohort characteristics.

	IBD-PSC [*N* = 54]	IBD-only [*N* = 62]	*p*-Value
Sex, male [%]	38 [70.4%]	39 [62.9%]	0.51
Age, median years [IQR]	36.0 [30.0; 49.0]	41.0 [33.0; 54.0]	0.19
IBD diagnosis age, median years [IQR]	28.0 [20.0; 36.0]	29.0 [22.0; 35.0]	0.78
PSC diagnosis age, median years [IQR]	30.5 [25.0; 43.7]	N/A	
IBD duration, median years [IQR]	7.5 [3.0; 17.0]	10.0 [5.0; 19.0]	0.25
PSC duration, median years [IQR]	5.0 [1.0; 9.0]	N/A	
Partial Mayo score, *n* [%]			0.15
Remission [<2]	40 [74.1%]	51 [82.3%]	
Mild activity [2–4]	11 [20.4%]	11 [17.7%]	
Moderate activity [5–7]	3 [5.5%]	0 [0.0%]	
Severe [>7]	0 [0.0%]	0 [0.0%]	
Smoking, *n* [%]			0.32
Current	7 [12.9%]	3 [5.1%]	
Past	5 [9.3%]	5 [8.5%]	
Never	42 [77.8%]	51 [86.4%]	
BMI, median kg/m^2 [IQR]	24.6 [22.4; 26.2]	24.6 [23.5; 28.0]	0.21
Medications current, yes [%]			
5-ASA	28 [52.8%]	26 [41.9%]	0.33
Sulfasalazine	2 [3.8%]	1 [1.6%]	0.89
Infliximab/adalimumab	4 [7.4%]	18 [29.0%]	0.01
Vedolizumab	5 [9.3%]	11 [17.7%]	0.29
Prednisone	1 [1.9%]	1 [1.6%]	1.00
Ursodeoxycholic acid	18 [33.3%]	N/A	N/A
Blood work, median [IQR]			
ALT [U/L] [missing 15.5%]	59.0 [34.0; 106.0]	20.0 [12.0; 24.0]	0.001
ALP [U/L] [missing 18.9%]	175.5 [112.5; 315.5]	61.5 [55.0; 75.0]	0.001
Bilirubin [mmol/L] [missing 22.4%]	11.0 [7.0; 23.0]	8.5 [6.0; 11.5]	0.012
Albumin [g/L] [missing 14.6%]	41.0 [39.0; 44.0]	45.8 [43.0; 47.0]	0.001
Faecal calprotectin [µg/g], median [IQR]	262.7 [148.0; 756.8]	120.0 [54.2; 509.6]	0.006
Highest TE measurement, *n* [%]			
F0–F3	35 [64.8%]	N/A	N/A
F4	15 [27.7%]	N/A	N/A
Missing	4 [7.5%]	N/A	N/A
Time between recruitment and TE, median months [IQR]	18.5 [8.2; 44.0]	N/A	N/A

Demographic and clinical data of subjects with IBD associated with PSC [IBD-PSC, *n* = 54] and IBD without PSC [IBD-only, *n* = 62]. Comparison of categorical variables was performed using the χ^2 test. For continuous variables, Kruskal–Wallis or Wilcoxon signed-rank tests were applied to compare the medians. IBD = inflammatory bowel disease, PSC = primary sclerosing cholangitis, IQR = interquartile range, BMI = body mass index, 5-ASA = 5-aminosalicylic acid, ALT = alanine aminotransferase, ALP = alkaline phosphatase, TE = transient elastography.

level based on Bray–Curtis dissimilarity index to assess microbial community shifts between the two groups. Permutation analysis of variance [PERMANOVA] was applied using the adonis function on distance matrices with 1000 permutations (vegan [v2.5-7] R package).

For the microbial compositional data, after removal of singletons, data were converted to relative abundance. Next, agglomeration to the species level was performed leaving 426 species-level taxa available for analysis. Only features with minimum prevalence of 10% and minimum abundance higher than 0.001% were included in the analysis. To assess associations between IBD-PSC and IBD-only with microbial species, a multivariable model (multivariate analysis by linear models [MaAsLin2, v1.4.0] R package[24]) adjusted for age, sex, and BMI was used. The model parameters were set to compound Poisson linear model method and total-sum scaling normalization.[24] To adjust for multiple comparisons, the *q*-values were calculated using the Benjamini–Hochberg method with *q*-values <0.05 considered significant.[25]

A subgroup analysis was performed in subjects with normal alkaline phosphatase [ALP] defined as lower than the upper limit of normal [ULN] [ALP < ULN; *n* = 22 IBD-PSC, *n* = 42 IBD-only] to control for the potential impact of cholestasis on identified associations with two-sided *p*-value <0.05 considered significant.

A targeted analysis using a multivariable linear regression model adjusted for age, sex and BMI was performed to assess associations between IBD-PSC and IBD-only with previously reported BA-related microbial genes [*N* = 28], enzyme commission [EC] categories [*N* = 7], and metabolic pathways [*N* = 3] [stats [v4.0.2] R package], with two-sided *p*-value <0.05 considered significant.

2.7. Statistical analysis of metabolite data

Serum and stool metabolite data were normalized using $\log_2$ transformation and row-wise normalization (MetaboAnalystR [v3.1.0] R package). BA metabolites were analysed at both the individual level and family level. Family level was generated by clustering of the individual BAs based on common chemical properties into six clusters [Supplementary Table 1].

Multivariable generalized linear models (stats [v4.0.2] R package) adjusted for age, sex, BMI, and ursodeoxycholic acid [UDCA] were used to assess association between IBD-PSC and IBD-only with either serum or stool BA metabolites. To assess the effect of cholestasis on the BA profile we performed a subgroup analysis in subjects with normal ALP < ULN [$n = 21$ IBD-PSC, $n = 34$ IBD-only], and additionally in subjects with normal ALP < ULN defined as 'non-cirrhosis' [F0-3 fibrosis degree], 'non-UDCA' [not on UDCA at time of recruitment] [$n = 16$ IBD-PSC, $n = 34$ IBD-only]. After adjusting for multiple comparisons, q-values <0.05 were considered significant.[25]

A Spearman's rank-order correlation test was used to explore the correlation between BA metabolites and microbial species and BA-related microbial genes in subjects with complete data overlap [$n = 48$ IBD-PSC and $n = 52$ IBD-only] (stats [v4.0.2] R package).

2.8. Statistical analysis for PSC-related outcomes

Fifty subjects [92.6%] in the IBD-PSC group had available TE data [measured in kPa] allowing for assessment of degree of liver fibrosis. We performed an exploratory analysis to assess associations between degree of liver fibrosis and microbial composition and serum and stool BA metabolites. Multivariable linear regression models adjusted for age, sex, UDCA, ALP level, and time difference from recruitment to TE measurement were used to assess associations between degree of liver fibrosis and microbial species and BA composition [with BMI added as a covariate only for the microbial taxa association analysis]. In this sub-group analysis, q-values <0.1 were considered significant considering the smaller sample size.

Additionally, to explore the correlation between BA concentrations and ALP, a Spearman's rank-order correlation test was used in the IBD-PSC group with available ALP and serum [$n = 48$] and stool [$n = 44$] BA metabolite data.

3. Results

3.1. Cohort description

The cohort comprised 54 participants with IBD-PSC and 62 participants with IBD-only. There were no differences in age, sex, and BMI between the groups. The majority were in clinical remission at time of recruitment with partial Mayo score <2 [74.1% and 82.3%, respectively]. Subjects in the IBD-PSC group had higher baseline levels of liver enzymes and faecal calprotectin. More subjects in the IBD-only group were on anti-tumour necrosis factor [anti-TNF] therapy and one-third of the IBD-PSC group were on UDCA treatment [Table 1].

3.2. Subjects with IBD-PSC have altered microbial composition compared to subjects with IBD-only

Subjects with IBD-PSC had reduced alpha diversity based on microbial gene richness [observed genes] [$p = 0.004$] compared to IBD-only [Figure 1A]. A consistent direction of effect, indicating diminished alpha diversity in the IBD-PSC group, was also observed utilizing other indices such as the Chao1 index [$p = 0.057$] and Shannon index [$p = 0.12$], but these differences did not reach statistical significance [Supplementary Figure 1]. Beta diversity, measured by the Bray–Curtis index, showed significant community compositional shifts between the IBD-PSC and IBD-only groups [PERMANOVA: $R^2 = 0.01$, $p = 0.03$] [Figure 1B].

After applying filtering steps, 145 bacterial species were available for analysis. In the multivariable model adjusted for age, sex, and BMI, 11 species that were differentially abundant in IBD-PSC compared to IBD-only were identified [$q < 0.05$ for all] [Figure 1C, Supplementary Table 2]. Among those species, IBD-PSC was associated with increased abundance of two species belonging to the genus *Veillonella*, i.e. *V. atypica* (beta coefficient 3.76, 95% confidence interval [CI] 2.5–5.0) and *V. dispar* [beta coefficient 2.56, 95% CI 1.1–4.0]. Also, in the IBD-PSC population, two key BA-metabolizing species were also significantly different, i.e. there was increased abundance of *Clostridium scindens* [beta coefficient 2.02, 95% CI 0.7–3.3] and decreased abundance of *Blautia obeum* [beta coefficient −0.92, 95% CI −1.5 to −0.3] compared with IBD-only.

Next, to account for the potential confounding effect of faecal calprotectin or UDCA, the model was adjusted for each of these variables separately. Even after adjustment, the majority of species remained significant [except for *C. scindens* after adjusting for faecal calprotectin and *B. obeum*, *Haemophilus parainfluenzae*, and *Fusicatenibacter saccharivorans* after adjusting for UDCA], all with consistent direction of effect [Supplementary Tables 3 and 4].

Additionally, to control for the potential impact of cholestasis on species composition, we performed a subgroup analysis in subjects with normal ALP < ULN. Both *C. scindens* and *B. obeum* remained significantly associated with IBD-PSC even in this subgroup of patients, but both *V. atypica* and *V. dispar* were no longer significantly associated with IBD-PSC [Supplementary Table 5].

3.3. Subjects with IBD-PSC have higher abundance of microbial genes involved in secondary BA metabolism

We next explored the microbial genetic landscape involved in BA metabolism by performing a targeted analysis assessing for associations between BA-related microbial genes and IBD-PSC compared to IBD-only. Subjects with IBD-PSC had significantly higher abundance of microbial genes involved in the formation of secondary BAs belonging to the microbial bai operon, namely *UniRef90_B4YST2:baiA* [$p = 0.037$], *UniRef90_P19337:baiA2* [$p = 0.048$], and *UniRef90_P19412:baiE* [$p = 0.045$]. Microbial *UniRef90_P19410:BaiCD* was also more highly expressed in IBD-PSC but this difference did not reach statistical significance [$p = 0.064$]. *C. scindens* was the main species encoding these genes [Figure 2]. No significant differences in microbial EC3.5.1.24:BSH [$p = 0.07$] or 7α- and 7β-dehydroxylating enzymatic pathways (PWY7554 [$p = 0.06$] and PWY8134 [$p = 0.09$]) were observed between IBD-PSC and IBD-only.

Interestingly, although *C. scindens* was no longer significantly associated with IBD-PSC after adjusting for faecal calprotectin, this was not the case with respect to the bai operon genes, with *UniRef90_B4YST2:baiA* remaining significant [$p = 0.047$] and *UniRef90_P19337:baiA2* [$p = 0.061$]

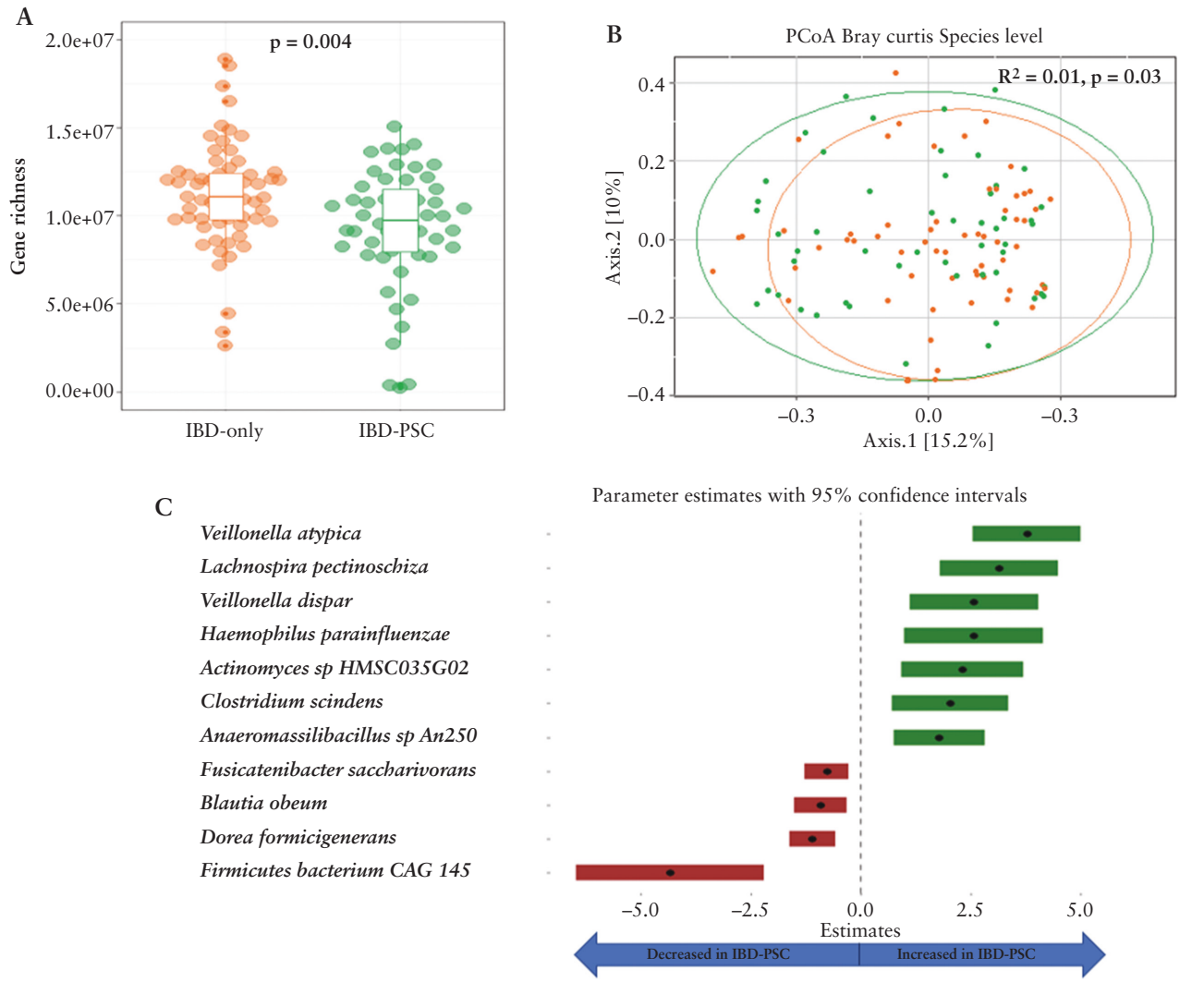

Figure 1. Diversity and species differential abundance in IBD-PSC compared to IBD-only. [A] Alpha diversity expressed by microbial gene richness [observed genes]; *p*-value calculated using the Wilcoxon test. [B] Beta diversity was assessed with principal coordinates analysis plots at the species level based on Bray–Curtis dissimilarity index. Ellipsoids represent a 95% confidence interval surrounding each group [orange = IBD-only; green = IBD-PSC]. Permutation analysis of variance [PERMANOVA] was applied on distance matrices with 1000 permutations. [C] Coefficient plot showing the effect size and 95% confidence interval [*x*-axis] for the 11 taxa found to be associated with IBD-PSC [*y*-axis], based on MaAsLin2 adjusted for age, sex, and body mass index. Green colour denotes taxa associated with increased risk of IBD-PSC and red colour denotes taxa associated with decreased risk of IBD-PSC. IBD = inflammatory bowel disease, PSC = primary sclerosing cholangitis.

and *UniRef90_P19412:baiE* [$p = 0.055$] showing a trend in the same direction even after adjusting for faecal calprotectin.

3.4. IBD-PSC is associated with decreased serum concentrations of sulphated BAs and increased serum concentrations of conjugated secondary BAs

We next assessed the associations between serum [$n = 71$] and stool [$n = 76$] BA composition and disease phenotype. The total BA pool was significantly increased in serum [$p = 8.9e\text{-}3$] and depleted in stool [$p = 2.8e\text{-}3$] in IBD-PSC compared to IBD-only. In the multivariable linear regression model adjusted for age, sex, BMI, and current UDCA treatment, we identified 24 individual serum BAs that were differentially abundant in IBD-PSC compared to IBD-only [13 positively and 11 negatively associated] [$q < 0.05$ for all] [Figure 3A, Supplementary Table 6]. IBD-PSC was significantly associated with increased serum concentrations of conjugated secondary BAs [beta coefficient 34.8, 95% CI 18.8–50.9] and glucuronidated BAs [beta coefficient 11.3, 95% CI 4.6–17.9], and decreased serum concentrations of sulphated BAs [beta coefficient –28.9, 95% CI –41.8 to –15.9] and conjugated primary BAs [beta coefficient –23.9, 95% CI –38.0 to –9.8] [$q < 0.05$ for all] [Supplementary Figure 2A, Supplementary Table 7]. No associations were found with stool BAs [Supplementary Figure 2B, Supplementary Tables 8 and 9]. Additionally, we found that the secondary to primary BA ratio was increased in the serum of subjects with IBD-only and decreased in those with IBD-PSC, while an inverse direction was observed in the stool, with decreased secondary to primary BA ratio in subjects with IBD-only and increased in those with IBD-PSC.

We again performed a subgroup analysis in subjects with normal ALP < ULN to explore the potential influence of cholestasis on BA associations. We found that the decrease in sulphated BAs [beta coefficient –17.9, 95% CI –30.9 to –4.9,

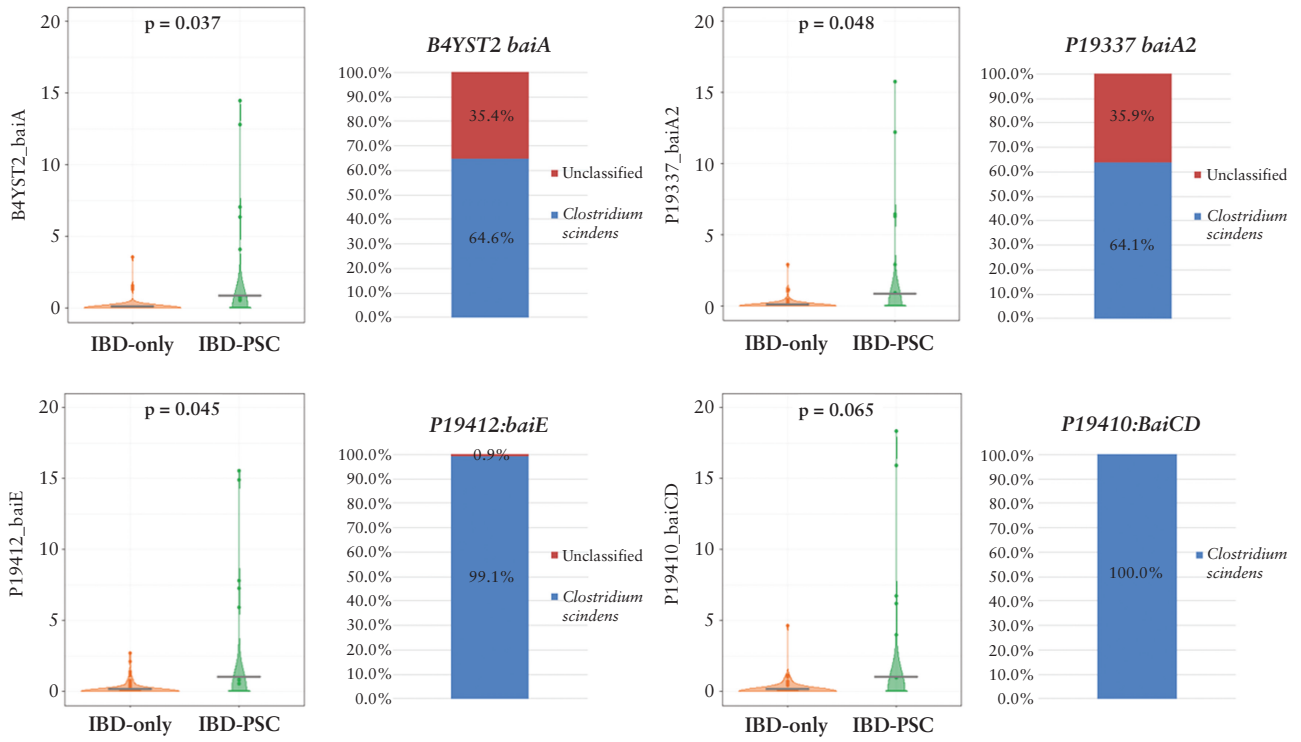

Figure 2. Microbial bile acid inducible [bai] operon genes in IBD-PSC compared to IBD-only. Association between microbial bai operon genes and IBD-PSC [green] compared to IBD-only [orange]; *p*-values calculated using a multivariable linear regression model adjusted for age, sex, and body mass index. Grey lines represent the means. The stacked bar-plot next to each gene shows the relative abundance of the bacterial species contributing to each microbial gene. IBD = inflammatory bowel disease, PSC = primary sclerosing cholangitis.

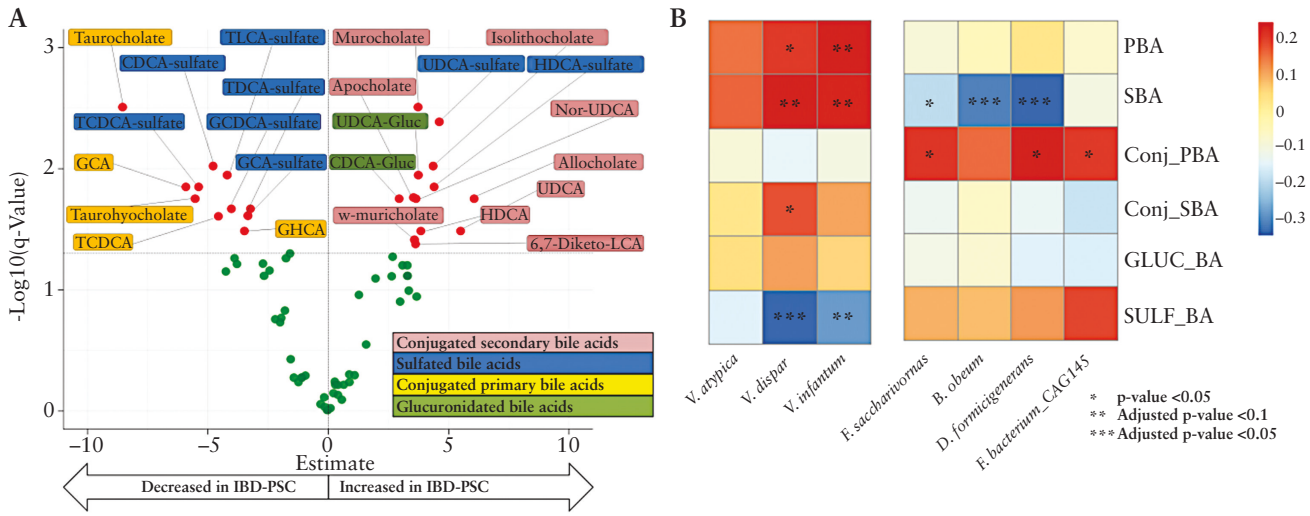

Figure 3. Serum bile acid associations with IBD-PSC phenotype and microbial species. [A] Volcano plot showing the associations of serum bile acids [BAs] with IBD-PSC; *x*-axis: effect size [estimate], *y*-axis: $\log_{10}$ of the *q*-value [log transformation is for visualization purposes]. Red dots represent BAs with $q < 0.05$, whereas green dots represent BA with $q \geq 0.05$. Red dots right of the dotted vertical line are positively associated with IBD-PSC, whereas those to the left of the dotted vertical line are negatively associated with IBD-PSC. Each BA with $q < 0.05$ is coloured based on the BA family it belongs to [red—conjugated secondary BAs, blue—sulphated BAs, yellow—conjugated primary BAs, green—glucuronidated BAs]. [B] Heatmap of BAs [*y*-axis] and microbial species [*x*-axis] associated with IBD-PSC based on the Spearman rank correlation test. Species in the left panel are those belonging to the genus *Veillonella* whose relative abundance was significantly increased in IBD-PSC, whereas those in the right panel are those whose relative abundance was significantly decreased in IBD-PSC. Inverse correlation is seen between these species and sulphated as well as secondary BAs. IBD = inflammatory bowel disease, PSC = primary sclerosing cholangitis, PBA = primary BAs, SBA = secondary BAs, conj_PBA = conjugated primary BAs, conj_SBA = conjugated secondary BAs, GLUC_BA = glucuronidated BAs, SULF_BA = sulphated BAs. Asterisks: *$p < 0.05$, **adjusted $p < 0.1$, ***adjusted $p < 0.05$.

$p = 0.042$] and the increase in conjugated secondary BAs [beta coefficient 18.4, 95% CI 4.2–32.6, $p = 0.042$] remained significantly associated with IBD-PSC with a consistent direction of effect [Supplementary Table 10]. The results remained consistent even in the subgroup of subjects with normal ALP < ULN, F0–3 fibrosis degree, and not on UDCA,

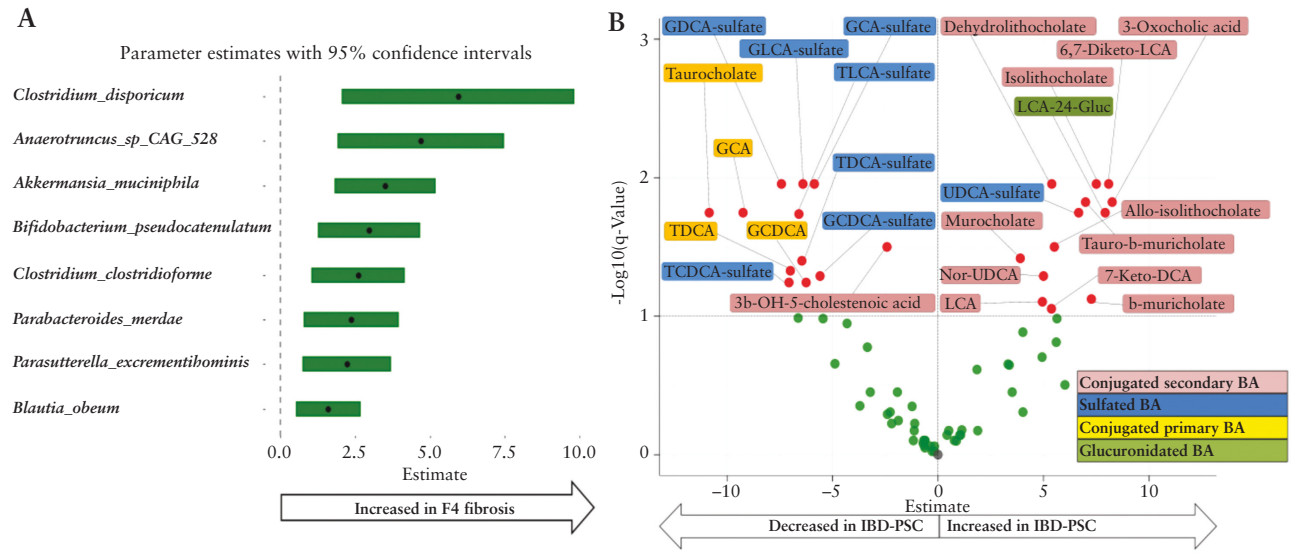

Figure 4. Associations of microbial species and serum bile acid composition with degree of liver fibrosis. [A] Coefficient plot showing the effect size and 95% confidence interval [*x*-axis] for the eight taxa found to be associated with degree of liver fibrosis [F4 vs F0–3] measured by transient elastography [TE] in the subgroup of patients with IBD-PSC and available TE data [$n = 50$] [*y*-axis], based on MaAsLin2 adjusted for age, sex, ursodeoxycholic acid treatment at recruitment, body mass index, alkaline phosphatase level, and time difference from recruitment to TE measurements. [B] Volcano plot showing the associations of serum BAs with degree of liver fibrosis [F4 vs F0–3] measured by TE in the subgroup of patients with IBD-PSC and available TE data [$n = 50$]; *x*-axis: effect size [beta coefficient], *y*-axis: $\log_{10}$ of the *q*-value [log transformation is for visualization purposes]. Red dots represent BAs with $q < 0.1$, whereas green dots represent BAs with $q \geq 0.1$. Red dots right of the dotted vertical line are positively associated with F4 fibrosis degree, whereas those to the left of the dotted vertical line are negatively associated with F4 fibrosis degree. Each BA with $q < 0.1$ is coloured based on the BA family it belongs to [red—conjugated secondary BAs, blue—sulphated BAs, yellow—conjugated primary BAs, green—glucuronidated BAs]. IBD = inflammatory bowel disease, PSC = primary sclerosing cholangitis.

suggesting that these associations were minimally impacted by cholestasis [Supplementary Table 11].

3.5. Serum concentrations of sulphated BAs were inversely correlated with *Veillonella* abundance in IBD-PSC

We next performed a correlation analysis to explore the relationship between BAs and microbial species identified to be associated with IBD-PSC. Among the species with abundance significantly increased in IBD-PSC compared to IBD-only, we found that species belonging to *Veillonella* genera [*V. atypica*, *V. dispar*, and *V. infantum*] were inversely correlated with serum sulphated BA concentrations and positively correlated with secondary BA concentrations [Figure 3B]. Among the species with abundance significantly decreased in IBD-PSC compared to IBD-only, we found an inverse correlation between *B. obeum* and *Dorea formicigenerans* and serum secondary BA concentrations [Figure 3B]. No statistically significant correlations were found between either serum or stool BAs and the BA-related microbial genes. However, an inverse correlation pattern was noted between stool and serum conjugated BAs with the bai operon-related genes and some of the genes coding for 7α-dehydroxylase [Supplementary Figure 3].

3.6. BAs and microbial species association with specific PSC-related prognostic factors and outcomes

Lastly, in the subgroup of patients with IBD-PSC, we assessed for associations between BAs and microbial species with PSC-related prognostic factors and outcomes. We found that an increase in ALP, a known prognostic factor in PSC, was positively correlated with the serum concentrations of secondary BAs [rho = 0.35, $p = 0.01$], conjugated secondary BAs [rho = 0.39, $p = 0.006$], and glucuronidated BAs [rho = 0.4, $p = 0.005$] and showed a strong inverse correlation with the serum concentrations of conjugated primary BAs [rho = –0.66, $p = 4.7e\text{-}7$]. No correlation was found between ALP and serum concentrations of sulphated BAs [rho = -0.18, $p = 0.21$] [Supplementary Figure 4]. A positive correlation was also identified between ALP and stool glucuronidated BAs [rho = 0.32, $p = 0.03$]. In contrast to the positive correlation with serum secondary BAs, ALP was inversely correlated with stool secondary BAs [rho = –0.34, $p = 0.027$] [Supplementary Figure 5].

Next, we assessed microbial taxa and BA associations with degree of liver fibrosis and we identified eight microbial species associated with F4 compared to F0–3 fibrosis [$q < 0.1$ for all] [Figure 4A, Supplementary Table 12]. Additionally, 25 serum BAs were associated with F4 compared to F0–3 fibrosis degree [$q < 0.1$ for all] [Figure 4B, Supplementary Table 13]. No associations were identified between stool BAs and degree of liver fibrosis. Interestingly, both decreased concentrations of serum sulphated BAs [beta coefficient –48.8, 95% CI –71.2 to –26.5, $q = 3.7e\text{-}4$] and increased concentrations of serum conjugated secondary BAs [beta coefficient 66.3, 95% CI 40.9–91.8, $q = 5.6e\text{-}5$], which were both associated with IBD-PSC, were also significantly associated with F4 compared to F0–3 fibrosis [Supplementary Table 14].

4. Discussion

In this study, we aimed to identify microbial and BA alterations in subjects with IBD-PSC compared to those with IBD-only, thereby highlighting the microbial and BA signatures differentiating IBD with and without PSC. We identified several bacterial species and BA-related microbial genes

associated with IBD-PSC and showed that these associations were not influenced by the level of gut inflammation. Moreover, we identified that alterations of specific groups of BAs, namely serum sulphated and conjugated secondary BAs, were associated with IBD-PSC compared to IBD-only as well as with a higher degree of liver fibrosis.

The genus *Veillonella* has been previously reported in several studies to be found in increased abundance in PSC [irrespective of IBD status] compared to healthy controls and ulcerative colitis.[3,26,27] Here we also found *Veillonella* abundance to be significantly increased in IBD-PSC compared to IBD-only, suggesting that *Veillonella* abundance is commonly elevated in subjects with PSC regardless of IBD status. Moreover, due to the higher taxonomic resolution of shotgun metagenomics sequencing we were able to identify two species belonging to this genus, *V. atypica* and *V. dispar*, as associated with IBD-PSC. Notably, these associations were independent of faecal calprotectin levels suggesting that the abundance of these species in IBD-PSC is not confounded by gut inflammation. Moreover, as a higher abundance of *Veillonella* has also been reported in other liver diseases,[28–30] we hypothesized that the increased abundance of this taxon might be related to degree of liver cholestasis. Indeed, we found that in the subgroup of patients with normal ALP levels, both *V. atypica* and *V. dispar* were no longer significantly associated with IBD-PSC, implying that these taxon abundances are probably dependent on the degree of cholestasis.

C. scindens is a well-known 7-dehydroxylating bacterium involved in the transformation of BAs to their secondary form mediated through its bai operon encoding genes.[31,32] Here, we identified *C. scindens* as well as several bai operon encoding genes to be significantly increased in IBD-PSC compared to IBD-only. It has been shown that secondary BA depletion is associated with increased inflammation in IBD via modulation of Th17 and Treg host immune responses.[7,10,33] Moreover, supplementation of secondary BAs in murine colitis models reduced intestinal inflammation.[34] Here we found that compared to IBD-only, subjects with IBD-PSC had an increased secondary to primary BA ratio in stool which may be one potential explanation for the more quiescent inflammatory burden associated with this phenotype.[35] Moreover, it has been shown that increased concentrations of stool and serum secondary and conjugated secondary BAs are associated with increased risk for colon cancer.[36,37] Therefore, based on our findings that IBD-PSC is associated with an increase in these BAs as well as in species and genes involved in their metabolism, such as *C. scindens*, it would be tempting to speculate that these alterations may be related to the higher prevalence of colon cancer in subjects with IBD-PSC compared to IBD-only.

We further assessed the BA profile in IBD-PSC and we identified, for the first time, two groups of serum BAs, specifically depletion of sulphated BAs and enrichment of conjugated secondary BAs, that were significantly altered in IBD-PSC compared to IBD-only, even in subjects with normal ALP. In a subgroup analysis, we found that these BAs were also significantly associated with a more advanced liver fibrosis and that serum secondary and conjugated secondary BAs were positively correlated with ALP levels. One potential explanation for the decreased capacity of BA sulphation in IBD-PSC may be related to the expression of the gene encoding the liver enzyme sulphotransferases-2A1 responsible for BA sulphation, which has been shown to be downregulated in PSC.[38,39] Moreover, sulphation of BAs serves as an important detoxification pathway of BAs by increasing their water solubility, decreasing intestinal absorption, and enhancing urinary excretion.[40] Depletion of sulphated BAs may then lead to impaired elimination of cytotoxic BAs leading to bile duct epithelium damage as well as hepatocyte inflammation and apoptosis resulting in enhanced fibrosis.[38,41] Based on these findings, we may speculate that depletion of sulphated BAs may trigger PSC among susceptible IBD patients and serve as a marker for a more aggressive PSC course.

As noted, although we identified associations between IBD-PSC and serum BAs, no significant associations were found with stool BAs. In line with our results, Torres and colleagues found that the total stool BA pool was reduced in IBD-PSC compared to IBD-only, but they also did not find major differences in the individual stool BAs.[9] One potential explanation might be related to shifts in the serum and stool BA pools in PSC, which may result in more pronounced serum alterations.[15] Under normal conditions, most BAs are contained within the enterohepatic system, with minimal spillover into blood. However, under cholestatic conditions, BAs efflux through the sinusoidal membrane, resulting in a higher proportion in the peripheral blood.[42]

Lastly, we further explored the interplay between altered BAs and microbial species identified in our study and found an inverse correlation between *Veillonella* species and serum sulphated BAs. A previous study showed that suppression of BA synthesis by aldafermin, an analogue of the gut hormone FGF19, led to enrichment of *Veillonella* in subjects with non-alcoholic steatohepatitis and fibrosis, demonstrating that this taxon is BA sensitive.[43] Therefore, the enrichment of *Veillonella* species in IBD-PSC may be driven by BA alternations, potentially through a primary liver-dependent process resulting in depletion of sulphated BAs.

Our study has several strengths. First, the use of shotgun metagenomic sequencing allowed us to get better resolution for microbial species level and microbial genes related to BA metabolism. Second, as gut inflammation is known to influence gut microbiome composition,[3,44] we included measures of faecal calprotectin to assess its confounding effect. Third, the use of a wide panel of BAs provided us with the opportunity to discover and better characterize previously underreported important BA groups such as sulphated BAs. Our study also has several limitations. First, we acknowledge the lack of an external validation cohort, which is difficult to acquire, especially when studying a rare entity such as IBD-PSC. However, in addition to novel findings in our study, some of our associated taxa and functions such as enrichment of *Veillonella* have been previously reported in PSC, thus providing external validation of these results.[3,4] Second, we did not include a non-PSC cholestatic control group. However, IBD comorbidity with cholestatic liver diseases other than PSC is much less frequent,[45] and the focus of this study was the unique IBD phenotype associated with PSC. Third, there may be potential unmeasured confounders such as endoscopic activity, presence of backwash ileitis, and biliary interventions such as endoscopic retrograde cholangiopancreatography that were not captured in this study. Fourth, although our functional microbial and microbiome–BA interaction analyses provide some mechanistic insight, we cannot claim causality. Further mechanistic studies are needed to better assess the impact of

the BAs and gut microbiome relationship in IBD-PSC and PSC-related outcomes such as liver fibrosis.

In summary, in this study we showed differences in gut microbial and BA profiles associated with IBD-PSC compared to IBD without PSC. Our findings provide insight into the pathogenesis of IBD-PSC and suggest possible targets for modulating the risk and course of IBD in subjects with PSC.

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Conflict of Interest

All authors disclose no potential conflicts [financial, professional, or personal] that are relevant to the manuscript.

Author Contributions

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Data Availability

The data that support the findings of this study are available on request from the corresponding author [M.S.S.].

Supplementary Data

Supplementary data are available online at *ECCO-JCC* online.

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