



A disease-specific decline of the relative abundance of *Bifidobacterium* in patients with autoimmune hepatitis

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Summary

Background: The pathogenesis of autoimmune hepatitis (AIH) is poorly understood and little is known about enteric microbiota in AIH.

Aim: To investigate disease-specific microbiome alterations in AIH.

Methods: The V1-V2 variable regions of the 16S rRNA gene were sequenced in faecal samples from 347 patients with AIH and controls (AIH n = 72, healthy controls (HC) n = 95, primary biliary cholangitis (PBC) n = 99 and ulcerative colitis (UC) n = 81).

Results: Biodiversity (Shannon entropy) was decreased in AIH patients compared to HC ($P = 0.016$), which was partially reversed by azathioprine ($P = 0.011$). Regarding between-sample diversity, AIH patients separated from HC, PBC and UC individuals (all $P = 0.001$). Compared to HC, decreased relative abundance of anaerobic genera such as *Faecalibacterium* and an increase of *Veillonella* and the facultative anaerobic genera *Streptococcus* and *Lactobacillus* were detected. Importantly, a disease-specific decline of relative abundance of *Bifidobacterium* was observed in AIH patients. Lack of *Bifidobacterium* was associated with failure to achieve remission of AIH ($P < 0.001$). Of potential therapeutic implication, *Bifidobacterium* abundance correlated with average protein intake ($P < 0.001$). Random forests classification between AIH and PBC on the microbiome signature yielded an area under receiver operating characteristic curve (AUC) of 0.787 in the training cohort, and an AUC of 0.849 in an external validation cohort.

Conclusion: Disease-specific faecal microbial alterations were identified in patients with AIH. Intestinal dysbiosis in AIH was characterised by a decline of *Bifidobacterium*, which was associated with increased disease activity. These results point to the contribution of intestinal microbiota to AIH pathogenesis and to novel therapeutic targets.

AF and CS contributed equally to this work.

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1 | INTRODUCTION

Autoimmune hepatitis (AIH) is an autoimmune liver disease of unknown origin.¹ If untreated, the disease can rapidly progress to cirrhosis and liver failure. Since a causal therapy is lacking, AIH is treated with unspecific immunosuppression. Corticosteroids typically serve for induction therapy and azathioprine as a maintenance treatment.^{1,2} The breach of self-tolerance in AIH is poorly understood. Unknown environmental triggers may lower the threshold for autoimmune responses in vulnerable individuals.¹ Dietary ingredients and microorganisms represent potential sources of such triggers. The human intestine is colonised by a complex community of predominant bacterial microorganisms ('microbiota', their genomes termed 'microbiome'). The enteric microbiota is a key regulator of the host's physiology in health and disease.³ The liver is engaged in a cross-talk with the intestine (gut-liver axis), and is constantly exposed to gut-derived immune cells and microbial products. The 'strategic' position of the liver between the portal and the systemic circulation highlights its role in translating microbial and immune signals originating from the intestine into systemic outcomes.⁴ Recently, intestinal microbiome alterations ('dysbiosis') were reported in patients with AIH and primary biliary cholangitis (PBC), pointing towards a possible link between microbiome alterations and autoimmune liver diseases.^{5,6} In a Chinese cohort, disease-associated dysbiosis in untreated patients with AIH was characterised by reduced biodiversity, decreased abundance of anaerobes and increase of the genera *Veillonella*, *Klebsiella*, *Streptococcus* and *Lactobacillus*.⁵ However, it remains unclear whether this microbial signature is specific compared to other autoimmune liver diseases or other immune-mediated diseases, and whether it is reproducible across geographic borders. In the present study, a comprehensive analysis of the luminal microbiota was performed in patients with AIH in comparison to healthy controls (HC), patients with PBC and ulcerative colitis (UC), an inflammatory bowel disease with well-defined dysbiosis.

2 | METHODS

2.1 | Study cohort

AIH and PBC were diagnosed based on clinical, biochemical, serological and histological criteria (the latter are not generally required for PBC) according to the most recent guidelines.^{2,7} All AIH patients received a liver biopsy at initial diagnosis. All patients with AIH and PBC underwent treatment and regular follow-up at the University Medical Center Hamburg-Eppendorf, Germany. Patients were followed-up for at least 12 months after stool collection. Therefore, the diagnosis of AIH or PBC was unequivocal in all cases. In order to assess disease-specific microbial alterations, patients with variant syndromes (formerly termed 'overlap-syndromes', such as AIH/PBC or AIH/primary sclerosing cholangitis) were excluded from the analysis. HC and UC patients were recruited by the PopGen Biobank (Institute of Epidemiology, Kiel, Germany). The diagnosis of UC was established based on medical history, exclusion of infectious colitis and colonoscopy with biopsy

according to the most recent guidelines.⁸ UC patients with concurrent liver disease were excluded. Eligible samples from 347 participants (AIH *n* = 72, PBC *n* = 99, UC *n* = 81, HC *n* = 95) were available (detailed description provided in Table 1). All study participants in the discovery cohort lived in Northern Germany for several years. Patients with AIH (*n* = 10) or PBC (*n* = 15) from an independent cohort were enrolled at the University Hospital Munich, Germany (clinical patient characteristics given in Table 2). Comprehensive biometric, lifestyle and medical data were retrieved from a questionnaire and medical records and validated by a physician. Blood parameters and transient elastography, as a surrogate for liver fibrosis, were measured at the time of stool sampling. Liver stiffness measurement by transient elastography was performed using FibroScan (EchoSens, Paris, France). All HC individuals underwent screening for factors disqualifying their eligibility as controls. Any symptoms and routine laboratory parameters deviating from healthy reference ranges, that is CRP ≤ 5 mg/L, glucose between 55 and 115 mg/dL and a wide range of diseases covered by a comprehensive questionnaire (including diabetes, cancer, respiratory, liver, coronary heart disease, heart attack, neuropathy, IBD, IBS, chronic diarrhoea, asthma, organ transplantation, phlebitis, varices, and venous insufficiency) were criteria for study exclusion. All study participants were unrelated individuals. Exclusion criteria for both healthy and diseased participants were age <18 years, decompensated cirrhosis or acute liver failure at time of stool collection, concurrent liver disease, other severe medical comorbidity, pregnancy and use of antibiotics within 6 weeks before stool collection. In AIH patients, full biochemical remission was defined as normalisation of both transaminase levels and serum IgG levels, according to recent guidelines.² The study protocol was approved by the respective institutional review boards (A148/14 and MC-111/15). Written informed consent was obtained from all participants.

2.2 | Assessment of dietary data

Dietary data were assessed using a self-administered food frequency questionnaire, which is validated for the German population.⁹ Information on average supply with macro- and micronutrients was retrieved using the German Food Code and Nutrient Database,¹⁰ and provided by the German Institute of Human Nutrition, Potsdam-Rehbrücke, Germany. Complete dietary information was available for 257 patients (HC *n* = 85, AIH *n* = 39, PBC *n* = 57, UC *n* = 76).

2.3 | Faecal sample collection and sequencing

A cross-sectional collection of stool samples was performed. Standard tubes with DNA stabiliser were used (Stratec Molecular, Berlin, Germany). The samples were collected by the participants at home and sent to the sequencing facility within 24 hours, where they were stored at -80°C until further processing. All faecal samples were processed and sequenced according to the same standard operating procedure at the Institute of Clinical Molecular Biology

TABLE 1 Clinical patient characteristics of the main patient population

	AIH	HC	PBC	UC	P-value
Patients (n)	72	95	99	81	
Age, years (median [IQR])	62.50 [53, 73]	48.00 [37, 55]	61.50 [56, 68.8]	45.00 [35, 54]	< 0.001
Body mass index, kg/m ² (median [IQR])	24.93 [22.4, 30.1]	22.66 [21.8, 23.8]	26.53 [22.9, 30.6]	24.94 [22.2, 26.7]	< 0.001
Sex, female, n (%)	55 (76.4)	49 (51.6)	90 (90.9)	49 (60.5)	< 0.001
Disease duration, years (median [IQR])	8.00 [5, 11.3]	0	7.00 [3.3, 10]	11.00 [5, 20]	0.003
Smoking, n (%)	7 (9.7)	0	22 (22.2)	0	< 0.001
Medication at time of stool collection, n (%)					
Antibiotics use in last 12 mo	5 (6.9)	0	7 (7.1)	10 (12.3)	—
Mesalazine	0	0	0	60 (75.0)	—
Sulfasalazine	0	0	0	10 (14.5)	—
Budesonide	13 (18.6)	0	3 (3.2)	0	—
Prednisolone	40 (58.0)	0	14 (14.4)	34 (47.2)	—
Azathioprine	51 (72.9)	0	6 (6.2)	27 (33.3)	—
Mercaptopurine	10 (14.5)	0	0 (0.0)	2 (2.8)	—
Infliximab	1 (1.4)	0	3 (3.2)	13 (17.8)	—
Mycophenolate	6 (9.7)	0	0	0	—
Ursodeoxycholic acid	2 (3.2)	0	80 (93.0)	0	—
Varices on last gastroscopy, n (%)	9 (12.5)	—	3 (3.0)	0	—
Splenomegaly, n (%)	8 (11.1)	—	2 (2.0)	—	—
History of ascites, n (%)	2 (2.8)	0	0	0	—
Fibrosis stage on last biopsy, n (%)	(available in n = 72)		(available in n = 34)		
0/4	15 (25.0)	—	10 (29.4)	—	—
¼	8 (13.3)	—	14 (41.2)	—	—
2/4	8 (13.3)	—	7 (20.6)	—	—
3/4	10 (16.7)	—	0	—	—
4/4	19 (31.7)	—	3 (8.8)	—	—
Transient elastography, kPa (median [IQR])	6.00 [4.9, 8.4]	—	5.85 [4.8, 6.8]	—	—
Serum Immunoglobulin G, g/L (median [IQR])	12.70 [10.6, 15.1]	—	10.90 [9.2, 13.1]	—	—
Serum bilirubin, mg/dl (median [IQR])	0.50 [0.4, 0.7]	—	0.50 [0.4, 0.5]	—	—
Serum alanine aminotransferase, U/L (median [IQR])	24.00 [20, 30.5]	—	25.00 [20, 33]	—	—
Serum alkaline phosphatase, U/L (median [IQR])	68.50 [54.5, 87.8]	—	110.00 [86, 135]	—	—
Serum aspartate aminotransferase, U/L (median [IQR])	26.00 [22, 29.5]	—	25.00 [21, 28.3]	—	—
Platelets count, 10 ⁵ /μL (median [IQR])	265.00 [220, 303.5]	—	275.00 [224, 306.3]	—	—
Serum albumin, mg/dL (median [IQR])	37.00 [36, 38.8]	—	38.25 [37, 39.4]	—	—
Complete biochemical remission of AIH, n (%)	56 (77.8%)	—	-	—	—
Presence of serum autoantibodies, n (%)					
Anti-nuclear antibodies	69 (95.8)	—	53 (57.0)	—	—
Anti-mitochondrial M2 antibodies	0 (0.0)	—	73 (83.0)	—	—
Smooth muscle antibodies	44 (62.9)	—	6 (6.5)	—	—
Soluble liver antigen antibodies	8 (11.3)	—	0	—	—

(Continues)

TABLE 1 (Continued)

	AIH	HC	PBC	UC	P-value
Sp100 nuclear antigen antibodies	0 (0.0)	—	19 (21.1)	—	—
Glycoprotein-210 antibodies	0 (0.0)	—	12 (13.3)	—	—
History of abdominal surgery, n (%)	0	0	0	14 (17.3)	—
Dietary variables (median [IQR])					
Total energy, kJ/d	8350.40 [6374.50, 10117.85]	9025.20 [7758.5, 11001]	7003.40 [6210.1, 8792.1]	9304.00 [7870.3, 12254.5]	< 0.001
Fibre, g/d	17.81 [14.8, 24.8]	20.08 [16.8, 25.5]	17.94 [13.5, 22.5]	21.66 [17.4, 25.5]	0.010
Protein, g/d	73.76 [55.4, 83.1]	77.32 [63.6, 93.8]	64.88 [54, 75.7]	78.82 [62.5, 97.4]	< 0.001
Fat, g/d	86.36 [69.3, 108.4]	98.03 [79, 122.4]	82.14 [70.3, 95.8]	95.78 [82.5, 125.1]	0.001
Carbohydrates, g/d	205.92 [151.5, 268.9]	215.90 [173.7, 263.2]	176.30 [132, 220]	242.76 [197.8, 291.6]	< 0.001
Water, g/d	2605.30 [2228.5, 3123]	3145.10 [2604.8, 3911.3]	2590.00 [2309, 3130]	2786.50 [2206.9, 3337.6]	0.001

Differences were assessed for statistical significance using Kruskal-Wallis (unadjusted) nonparametric test.

(Kiel, Germany) as described previously.¹¹ In brief, DNA was extracted using QIAamp Stool Mini Kit (Qiagen) on a QIAcube system with an additional bead-beating step. The variable regions V1-V2 of the 16S ribosomal RNA gene were sequenced in a dual-barcode approach using the primer pair 27F-338R. Sequencing was carried out using Illumina MiSeq (Illumina Inc.).

2.4 | Bioinformatic analysis

The 'dada2' R package (v1.9.1) was used to infer the ribosomal sequence variants (RSVs),¹² following the DADA2 workflow for big data (benjjneb.github.io/dada2/bigdata_paired.html). Forward and reverse reads were trimmed and filtered. Forward reads were truncated to 240 nucleotides and reverse reads to 200 nucleotides. Reads with ambiguous bases and more than two expected errors were discarded. Chimeric RSVs were removed. Singletons, that is RSVs with a singular count, were pruned to reduce bias by rare or spurious sequences. Taxonomic annotation was carried out against the SILVA database (v132) using the implementation of the naïve Bayesian classifier available in DADA2.

2.5 | Statistical analysis

All statistical analyses were carried out with R (v3.5.1). To measure within-sample diversity (α -diversity), Shannon entropy was calculated on Hellinger transformed RSVs abundance matrix using the 'vegan' package (v2.5.6) functions 'decostand' and 'diversity'.¹³ Chao1 estimated richness was calculated using 'chao1' function in the 'fossil' package (v0.3.7). Biodiversity was modelled using least-squares regression ('stats' function 'lm'). To measure between-sample diversity (β -diversity), both Bray-Curtis dissimilarity and Canberra distance were calculated using the vegan

function 'vegdist' on RSV abundance matrix transformed according to the geometric mean of pairwise ratios ('GMPR' package, v0.1.3).¹⁴ Both unconstrained ordination by multidimensional scaling and constrained ordination were performed using the vegan function 'capscale'. Statistical significance of separation among groups was assessed by permutational multivariate analyses of variance (PERMANOVA, 'vegan' function 'adonis'). Pairwise differences were tested using 'pairwise.perm.manova' function in the 'RVAideMemoire' package (v0.9-73). Overall differences in questionnaire-derived dietary variables were analysed using PERMANOVA on Euclidean distance. The 'envfit' function in 'vegan' was used to correlate dietary data to microbiome community composition. Hurdle models with negative binomial distribution ('hurdle' function in the 'countreg' package, v0.2.1) were used to model single-taxon relative abundances.¹⁵ A GMPR size-factor was included as an offset to account for differences in library size. Statistical significance of model predictors was assessed jointly for the count- and zero-parts using likelihood ratio test ('lrtest' function in the 'lrmtest' package, v0.9.37). Dietary predictor selection to model single-taxon count abundance was carried out using the 'be.zeroinfl' function in the 'mpath' package (v0.3.18). Linear regression, multivariate permutational analysis, environmental vector fitting, hurdle models and zero-inflated count regression were de-confounded by including body mass index, sex, smoking status and use of antibiotics during the past 12 months as covariables. Predictive modelling with random forests and classifier evaluation were performed using the packages 'Boruta' (v6.0.0) and 'randomForest' (4.6.14). Modelling was performed on abundance tables clustered on the genus level and rarefied to an even sequencing depth of 10 005 counts. The area under the receiver operating characteristic curve (AUC) was calculated and tested for statistical significance according to Mason and Graham using the 'roc.area' function in the 'verification' package (v1.42).¹⁶ A $P < 0.05$ was accepted as statistically

TABLE 2 Clinical patient characteristics of the validation cohort

	AIH	PBC	P-value
Patients (n)	10	15	
Age, years (median [IQR])	62.0 [43, 67]	64.0 [60, 71]	0.36
Sex, female, n (%)	10 (100)	13 (86.7)	0.652
Disease duration, years (median [IQR])	4.50 [1, 10]	8.0 [4, 13]	0.2
Smoking, n (%)	1 (10)	2 (13.3)	1.0
Medication at time of stool collection, n (%)			
Antibiotics use in last 12 months	1 (10)	1 (6.7)	1.0
Mesalazine	0	0	—
Sulfasalazine	0	0	—
Budesonide	2 (20)	3 (20)	1.0
Prednisolone	9 (90)	1 (6.7)	< 0.001
Azathioprine	7 (70)	1 (6.7)	0.004
Mercaptopurine	0	0	—
Infliximab	0	0	—
Mycophenolate	0	0	—
History of abdominal surgery, n (%)	2 (20)	0	0.292
Dietary variables (median [IQR])			
Total energy, kJ/d	7610.60 [7034.18, 8375.07]	6924.00 [6477.40, 8622.23]	0.572
Fibre, g/d	19.92 [16.48, 22.70]	16.98 [15.06, 20.38]	0.480
Protein, g/d	61.36 [59.13, 64.39]	63.62 [59.12, 73.23]	0.671
Fat, g/d	83.66 [75.66, 91.13]	77.71 [76.06, 99.13]	0.777
Carbohydrates, g/d	199.94 [184.67, 226.55]	168.01 [136.05, 212.89]	0.322
Water, g/d	3016.25 [2655.58, 3187.17]	3095.55 [2207.25, 3394.03]	0.888

Clinical information available in the PopGen database is given. Differences were assessed for statistical significance using Wilcoxon rank-sum test (unadjusted).

significant. Multiple testing was controlled by correcting for the false-discovery rate (FDR).

3 | RESULTS

3.1 | Reduced enteric microbiota diversity in AIH

We first investigated measures of within-sample biodiversity (α -diversity), which can be indicative for healthy ecosystem

functioning and resilience. The average Shannon entropy was reduced in AIH patients as compared to HC (least-squares regression, $P = 0.016$). Whereas patients with PBC had an average Shannon entropy comparable to AIH patients ($P = 0.727$), patients with UC showed a further reduction in enteric microbial diversity ($P < 0.001$; Figure 1A). Compared to HC, AIH patients had a reduced average estimated richness (Chao1 richness, $P < 0.001$), which was similar to PBC and UC patients (both $P > 0.1$; Figure 1B).

3.2 | Disease-specific microbiota alterations in AIH

Between-sample diversity (β -diversity) varied significantly according to disease state in the overall analysis (PERMANOVA, $R^2 = 1.76\%$, $P = 0.001$) and regarding all pairwise comparisons (pairwise PERMANOVA, all FDR = 0.001) on both unconstrained (Figure 1C) and constrained ordination (Figure 1D). As expected, the microbial communities were mainly composed of three phyla, Firmicutes, Bacteroidetes and Proteobacteria. Firmicutes were decreased in all diseased groups (median relative abundance, HC: 48.7%, AIH: 41.9%, PBC: 40.4%, UC: 40.6%; Kruskal-Wallis test, $P < 0.001$). While Bacteroidetes showed no significant difference between the groups ($P = 0.051$), Proteobacteria (a phylum comprising numerous pathogens) was increased in all diseased states (HC: 4.3%, AIH: 6.7%, PBC: 14.1%, UC: 11.4%; $P < 0.001$; Figure 1E).

3.3 | Disease-specific decline of *Bifidobacterium* in AIH

Pairwise differential abundances between the diagnosis groups were analysed using zero-truncated negative-binomial hurdle models (Figure 2A-C). Compared to HC, patients with AIH showed a decline of several genera, including *Faecalibacterium* (log 2FC = -1.34, FDR = 0.021) and *Bifidobacterium* (log 2FC = -1.89, FDR = 0.021), and an increase of *Streptococcus* (log 2FC = 1.77, FDR = 0.004). Compared to PBC, AIH patients showed a greater abundance of *Faecalibacterium* (log 2FC = 2.53, FDR < 0.001), whereas *Bifidobacterium* showed the strongest decrease in AIH (log 2FC = -2.57, FDR < 0.001). In addition, we performed a targeted analysis for genera previously reported to be relatively enriched in patients with AIH compared to HC⁵. *Klebsiella* was not found to be significantly enriched in patients with AIH (log 2FC = 0.25, $P = 0.490$). However, *Veillonella* (log 2FC = 1.33; $P = 0.010$, FDR = 0.067) and *Lactobacillus* (log 2FC = 2.43, $P = 0.007$, FDR = 0.060) were validated to be increased in AIH patients but did not withstand multiple testing correction. The results for the targeted testing based on prior knowledge are shown in Figure S1 in Results S1. Comparing AIH vs UC patients, *Bifidobacterium* showed the strongest decline (log 2FC = -1.73, FDR < 0.001). As *Bifidobacterium* was the only genus significantly reduced in AIH patients across all pairwise comparisons (Figure 2D), we performed a

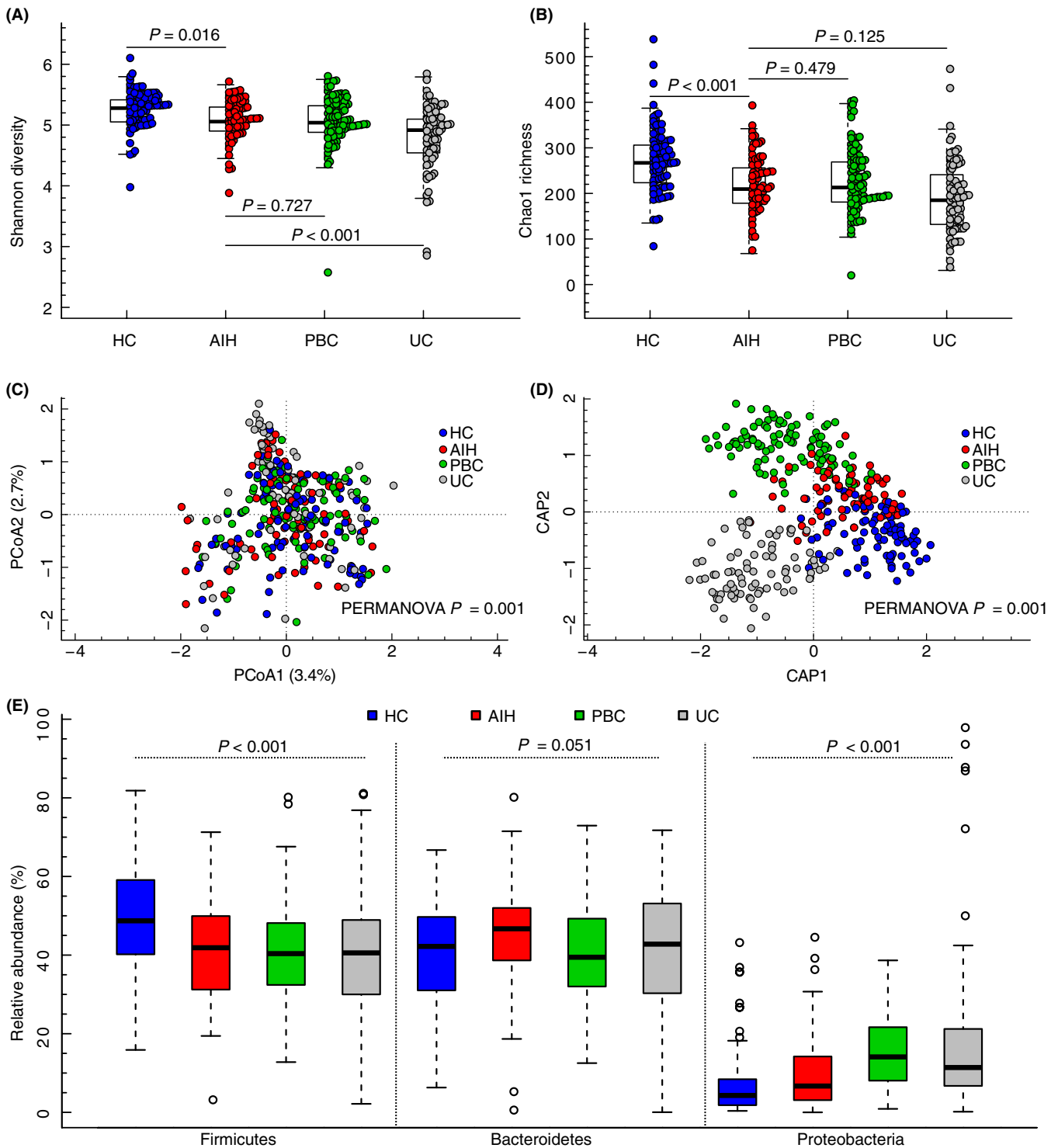


FIGURE 1 Altered faecal microbiome diversity and composition in AIH. (A) Significantly reduced Shannon entropy and (B) Chao1 estimated richness in AIH patients. Microbiome community composition was significantly different between AIH patients and both healthy and diseased controls regarding (C) multidimensional scaling on Bray-Curtis dissimilarity and (D) constrained ordination on Canberra distance. (E) AIH patients and diseased controls showed relatively reduced Firmicutes and increased Proteobacteria levels. AIH: autoimmune hepatitis; HC: healthy controls; PBC: primary biliary cholangitis; UC: ulcerative colitis

targeted analysis on the *Bifidobacterium* species level. Among the bifidobacterial species identified, three species showed a reduced abundance in AIH across all group comparisons, *Bifidobacterium*

adolescentis ($P = 0.007$, FDR = 0.051), *Bifidobacterium longum* ($P < 0.001$, FDR = 0.001) and *Bifidobacterium pseudocatenulatum* ($P = 0.014$, FDR = 0.084; Figure 2E).

3.4 | The enteric microbiome accurately discriminates AIH

We next investigated whether the faecal microbiota profile can be used to discriminate the different autoimmune liver diseases. For this purpose, we used random forests. For feature selection, we employed an *all-relevant* feature selection approach termed 'Boruta', an algorithm which is based on random forests and iteratively removes features which are proved by a statistical test to be less relevant than random probes. Using this approach, six features were selected (Figure 3A). Subsequently, a random forest classifier was built including these six features on AIH and PBC patients from the Hamburg cohort using a number of 9,999 trees, a node size of 1 and a number of variables at each node (*mtry*) corresponding to the

square root of included model features. In the training cohort from Hamburg, an AUC of 0.787 (95% CI 0.719–0.855, Mason-Graham test, $P < 0.001$) for the discrimination between AIH and PBC was achieved. *Bifidobacterium* and *Faecalibacterium* were among the most important discriminating features (Figure 3A). The taxonomic signature discriminating AIH from PBC was validated using random forests in an independent cohort recruited at the University Hospital Munich. Validation of the classifier in this cohort yielded an AUC of 0.849 (95% CI 0.679–1, $P = 0.002$; Figure 3A–C), indicating robust differences between the two autoimmune liver diseases AIH and PBC. Moreover, upon classifying between AIH and HC (Figure S2 in Results S1), we achieved a comparably accurate classification performance with an average area under the ROC curve of 89.8% upon repeated cross-validation.

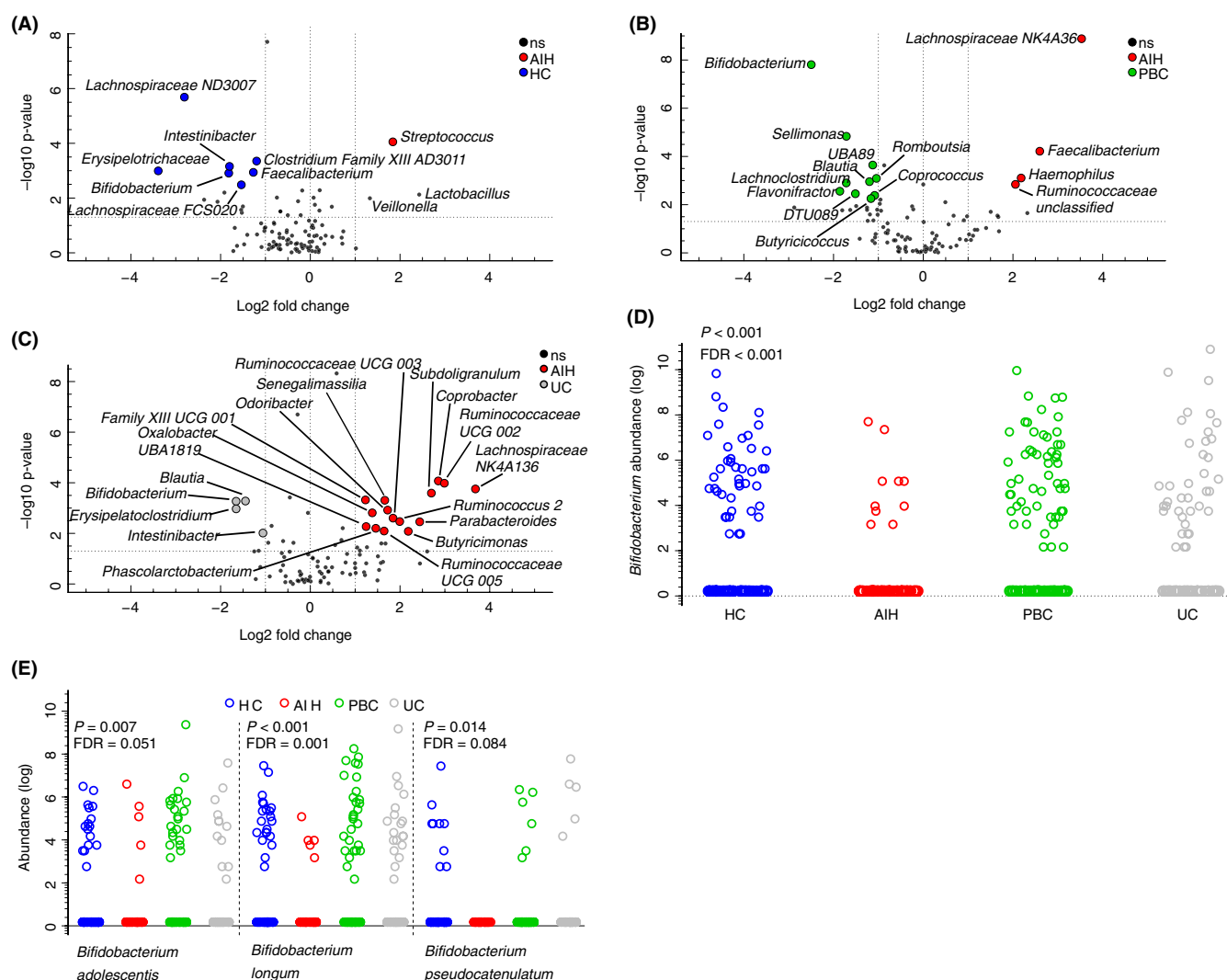


FIGURE 2 Differential relative abundance. Genera with significantly different relative distribution comparing (A) AIH vs HC, (B) AIH vs PBC and (C) AIH vs UC are displayed by volcano plots. The x-axis shows the log2 fold change, while the y-axis displays the -log 10 transformed P-value. Genera were considered differentially abundant when reaching an FDR < 0.05 and absolute log 2 fold change > 1. (D) Strip chart showing differential abundance of *Bifidobacterium* across all groups. (E) Log-transformed count abundance of individual *Bifidobacterium* species compared across diagnosis groups. AIH, autoimmune hepatitis; FDR, false discovery rate; HC, healthy controls; PBC, primary biliary cholangitis; UC, ulcerative colitis; ns: not significant

3.5 | Associations between diet, *Bifidobacterium* abundance and biochemical remission in AIH

We interrogated a wide array of clinical metadata (Table 1), with a particular focus on AIH, for association with microbial features,

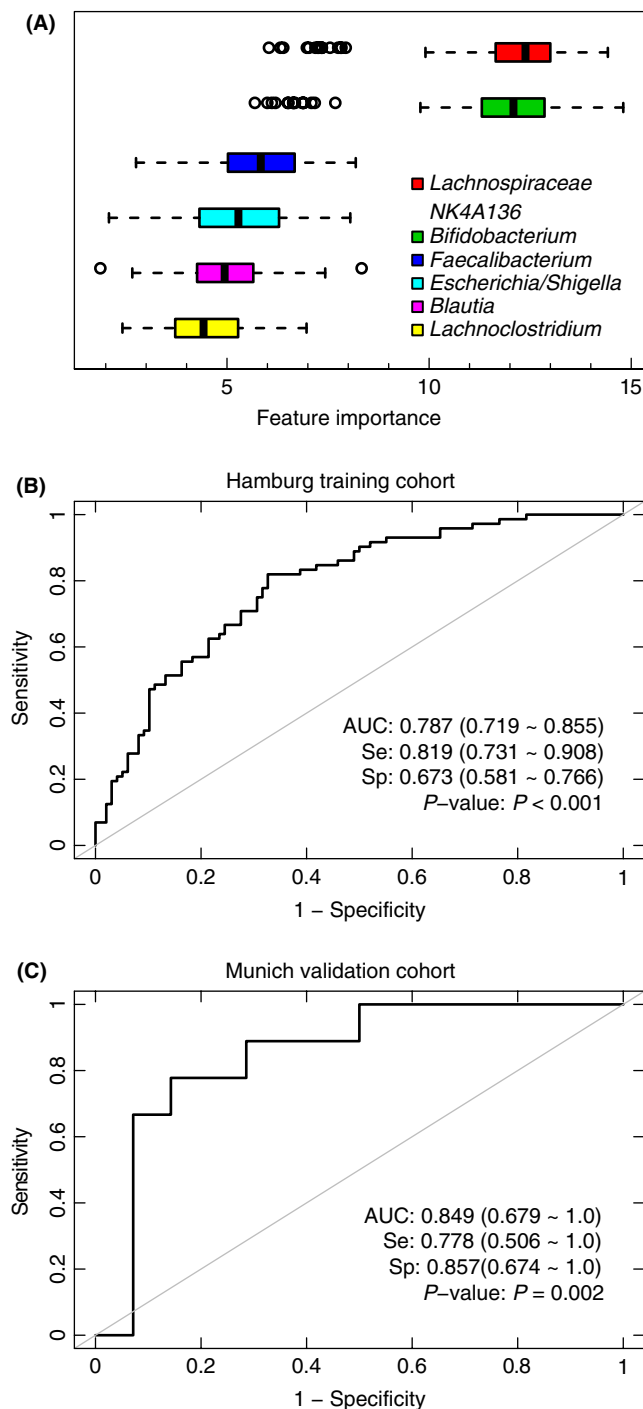


FIGURE 3 The enteric microbiome discriminates between AIH and PBC. (A) Feature importance of bacterial genera selected by Boruta feature selection algorithm. (B) Random forest classifier performance in the training cohort. (C) Random forest classifier performance in the Munich validation cohort. The values in brackets indicate the 95% confidence interval. AUC, area under the receiver operating characteristic curve; Se, sensitivity; Sp, specificity

both on the level of overall ecologic indices and relative abundance of individual taxa. Questionnaire-derived average consumption of macro- and micronutrients did not show statistically significant differences between the diagnosis groups adjusting for sex and age (PERMANOVA, $R^2 = 1.82$, $P = 0.070$). We observed no significant association between α -diversity and dietary variables (least-squares regression, all $P > 0.1$). Adjusting for the diagnosis, average protein consumption was identified as a strong positive predictor of *Bifidobacterium* abundance in the entire cohort ($P < 0.001$; Figure 4A). The strong disease-specific decline of *Bifidobacterium* in AIH was unaffected by adjusting for questionnaire-derived dietary items ($P < 0.001$). On pairwise comparisons, average protein consumption did not differ in AIH patients compared to other groups (Wilcoxon test, all $P > 0.1$). At the time of stool collection, complete biochemical remission was achieved by 56 AIH patients (77.8%). Biochemical remission status showed no statistically significant association with either α -diversity or β -diversity in the AIH cohort (both $P > 0.1$). No significant associations were detected between the fibrosis stage on the last liver biopsy and microbial features (all $P > 0.1$). An exploratory analysis was performed to detect associations between *Bifidobacterium* and markers of disease activity in AIH using zero-inflated count regression. Decreased *Bifidobacterium* levels were associated with an increase of serum IgG ($P = 0.024$). Importantly, *Bifidobacterium* showed a more pronounced depletion in AIH patients who failed to achieve complete biochemical remission according to recent guidelines ($P < 0.001$; Figure 4B). As expected, several dietary variables showed significant association with microbiome composition ('envfit', Figure 4C). However, this did not interfere with the highly significant disease-associated shift in microbiome composition (PERMANOVA, $R^2 = 3.17\%$, $P = 0.001$).

Of interest, in AIH patients, treatment with azathioprine was an independent predictor of increased α -diversity ($P = 0.011$; Figure 4D). This was independent from the presence of biochemical remission, pointing towards direct impact of azathioprine on intestinal microbiota or intestinal homeostasis. Such an association was not observed in the UC cohort, indicating an AIH-specific effect of azathioprine. Budesonide was received by 13 (18.6%) AIH patients, and 40 (58.0%) of AIH patients were treated with oral prednisolone. However, for both medications, a statistically significant association was neither found regarding overall diversity indices nor abundance of individual taxonomic markers of AIH (all $P > 0.1$).

In agreement with a previous report,⁵ *Veillonella* abundance was positively associated with alanine aminotransferase (ALT) levels in AIH patients ($P = 0.029$; Figure 4E). However, it was not associated with overall remission status ($P = 0.270$), and association with disease severity was not observed in the PBC cohort. An association with dietary variables was not detected for *Veillonella*.

4 | DISCUSSION

In the present study, a comprehensive analysis of faecal microbial alterations was performed in large cohorts of patients with AIH

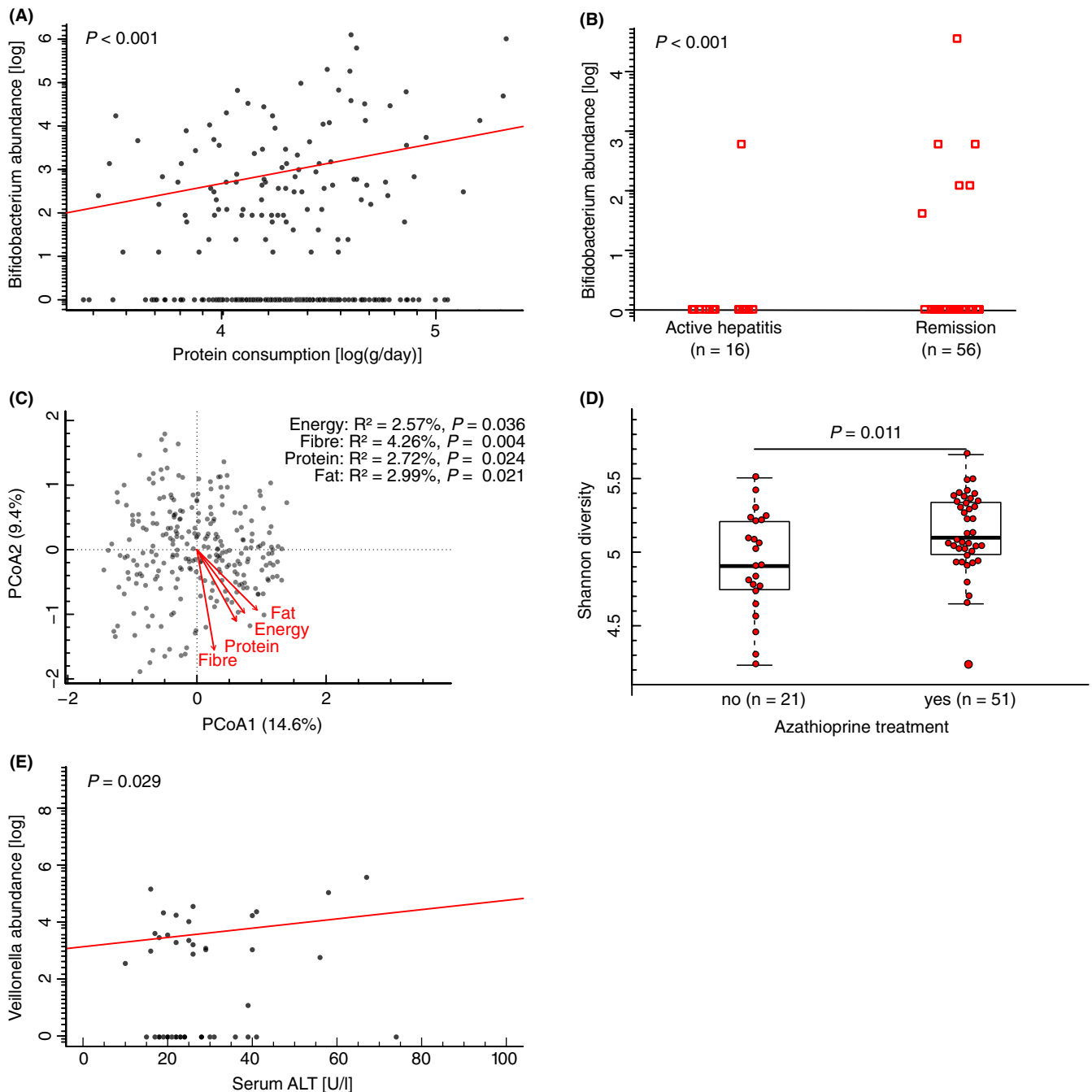


FIGURE 4 Associations between microbiome, clinical and dietary metadata. (A) *Bifidobacterium* abundance was associated with average protein consumption in the entire cohort. (B) Association between biochemical remission status and *Bifidobacterium* abundance in the AIH cohort. Complete biochemical remission was achieved by 56 AIH patients (77.8%), this association was independent from steroid treatment. (C) Correlation between questionnaire-derived dietary variables and gut microbiota configuration in the entire cohort. (D) In the AIH cohort, average within-sample (α) diversity was increased in patients treated with azathioprine. (E) Correlation between alanine aminotransferase levels and *Veillonella* abundance in AIH patients. ALT, alanine aminotransferase; R^2 , percentage of variance in between-sample diversity explained by the denoted variable

and both healthy and diseased controls. We hereby demonstrated that enteric microbiota is distinctly altered in AIH, largely independent from multiple clinical and dietary confounders. Moreover, the disease-specific decline of *Bifidobacterium* in AIH was linked with failure to achieve remission and may be amenable to dietary intervention.

We observed a decline of gut microbial diversity in patients compared to HC, which may be associated with loss of beneficial functions and ecosystem resilience.¹⁷ A bloom of Proteobacteria was observed in AIH akin to PBC and UC. Overabundance of this phylum is considered an indicator of disturbed host-microbiota homeostasis, inflammation and epithelial dysfunction.¹⁸ In

agreement with a previous study,⁵ the shift in the enteric microbiota of patients with AIH towards a more aerotolerant milieu was accompanied by a decline of obligate anaerobic taxa producing short-chain fatty acids (SCFAs). SCFA serve as an energy source for colonic epithelia and regulate multiple immune responses including regulatory T cells.¹⁹ In our study, we observed a decrease of *Faecalibacterium* (only known species *Faecalibacterium prausnitzii*) in AIH patients compared to HC. There is an increasing interest in *F. prausnitzii* due to its well-established anti-inflammatory properties, which render the bacterium a promising candidate for new-generation probiotic treatment in inflammatory diseases.²⁰ Interestingly, similar to a previous report, we detected a relative increase of *Veillonella* and the facultative anaerobes *Streptococcus* and *Lactobacillus*.⁵ Although the increase of *Veillonella* and *Lactobacillus* was statistically weaker compared to the aforementioned study with treatment-naïve patients, our finding highlights the stability of AIH-associated dysbiosis across geographic areas and after several years of immunosuppressive treatment. However, we also clearly demonstrate that these microbial alterations were not disease-specific. Nevertheless, *Veillonella* showed a reproducible association with increased ALT levels in AIH patients. Animal studies have demonstrated that specific pathobionts may increasingly colonise the gut in the context of dysbiosis and drive intestinal or liver inflammation.^{21,22} As *Veillonella* was previously found increased in patients with PBC⁶ and patients with primary sclerosing cholangitis,^{23,24} it may drive hepatic inflammation in autoimmune and cholestatic liver diseases.

The bacterial alterations in AIH discussed so far seem to be rather unspecific corollaries of chronic inflammation or liver disease. Nevertheless, we detected a disease-specific decrease of *Bifidobacterium* in AIH patients. The decline of *Bifidobacterium* was associated with failure to achieve biochemical remission. Although *Bifidobacterium* was depleted in AIH independently of diet, higher average protein intake may favour its growth, as has been recently demonstrated for an amino acid mixture in an animal model.²⁵ *Bifidobacterium* species are considered key regulators of gut homeostasis and may exert several health-promoting effects.²⁶ In infants, lower intestinal levels of bifidobacteria were prospectively linked to the incidence of immunologic disorders, pointing towards a critical role of enteric bifidobacteria for immune regulation in humans.^{27,28} Recent evidence also suggests that *B. longum*, *B. adolescentis* and *B. pseudocatenulatum* (all of which were specifically decreased in AIH) attenuate immunologic liver injury in animal models by modulation of intestinal homeostasis and enteric microbiota.^{29–31} It is tempting to speculate that *Bifidobacterium* supplementation or *Bifidobacterium*-targeted diet may raise the threshold for autoimmune responses in patients with AIH. Since the standard therapy of AIH is burdened by complications of long-term immunosuppression and corticosteroid use, such novel therapeutic avenues would represent an attractive alternative. However, more data on the potential functional role of *Bifidobacterium* and strain-specific characteristics in AIH are clearly required.

AIH patients receiving azathioprine, a thiopurine class drug, had on average a higher microbial diversity compared to patients without azathioprine treatment—independently of parameters of disease activity or stage. Although azathioprine is the mainstay for maintenance of remission in AIH, the precise mechanism of action in AIH is still unclear. Thiopurines can modulate gut microbial composition and ameliorate colitis without entering the systemic circulation.³² Therefore, the beneficial effects of azathioprine in AIH may in part be related to the modulation of gut microbiota and intestinal homeostasis. We could not detect significant associations between microbial features and histological parameters. However, the major limitation here is the time gap of months to several years between liver biopsy and the time point of faecal sample collection in our cohort, as both features of inflammation and fibrosis tend to regress in AIH patients under immunosuppressive treatment.²

Our study has strengths and limitations that should be addressed by future studies. First, 16S-based analyses represent an important first step to detect microbial alterations in human diseases. However, shotgun sequencing for metagenomics facilitates more accurate taxonomic information and functional insights. Second, the enteric mucosal microbiota was not analysed in the present study. Since stool samples cannot fully reflect the diversity of mucosal communities, future studies should investigate mucosal microbiota in order to complete the landscape of enteric microbiota alterations in AIH. Third, untreated patients were not included in our study. Whereas this may introduce confounding by medication, unspecific inflammation-induced changes in untreated AIH patients were thus circumvented. A notable strength of our study is the comprehensive control for potential confounding by diet and other important lifestyle and clinical variables, and thorough assessment of disease specificity using another autoimmune liver disease and a nonhepatic immune-mediated disease. Our results show significant accordance with the previous work published by Wei et al⁵ To which extent the observed discrepancies, especially with regard to alterations of *Bifidobacterium* abundance, are related to amplicon-sequencing inherent bias, discordance in sequencing protocols, differences in disease duration, treatment or even dietary differences between Germany and China currently cannot be answered. Direct comparisons of larger international cohorts are required to validate disease-specific microbial signatures in AIH independent from varying dietary and geodemographic circumstances.

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AUTHORSHIP

Guarantor of the article: Christoph Schramm is the guarantor of the present article.

Author contributions: TL validated the clinical metadata, performed all statistical analyses, interpreted the results, and wrote the manuscript. CS and AF conceptualized the study design, and supervised the study, and critically revised the manuscript. CB led the sequencing of the microbiome samples. CC and MCR gave advice in data analysis and interpretation. TL, CS, MS and AWL participated in patient management and study enrolment. SH and GD were responsible for management and enrollment of the validation cohort. WL provided the infrastructure for data acquisition, storage and management. All authors critically read the manuscript, and constructively commented on the intellectual content.

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SUPPORTING INFORMATION

Additional supporting information will be found online in the Supporting Information section.

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