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Gut microbiota alterations are associated with phenotype and genotype in familial Mediterranean fever

Marion Delplanque[1,2,3,†], Nicolas Benech[2,3,†], Nathalie Rolhion[2,3], Cyriane Oeuvray[2,3], Marjolène Straube[2,3], Chloé Galbert[2,3], Loic Brot[2,3], Thomas Henry [4], Yvan Jamilloux[4], Léa Savey[1], Gilles Grateau[1], Harry Sokol [2,3,5], Sophie Georgin-Lavialle[1,2,3,*]

[1]Sorbonne Université, Service Médecine Interne, Centre de Référence des Maladies Autoinflammatoires et des Amyloses (CEREMAIA), APHP, Hôpital Tenon, Paris, France
[2]Gastroenterology Department, Sorbonne Université, INSERM, Centre de Recherche Saint-Antoine, CRSA, AP-HP, Saint Antoine Hospital, French Group of Faecal Microbiota Transplantation (GFTF), Paris, France
[3]Paris Center for Microbiome Medicine, Fédération Hospitalo-Universitaire, Paris, France
[4]CIRI, Centre International de Recherche en Infectiologie, Inserm U1111, Université Claude Bernard Lyon 1, CNRS, UMR5308, ENS de Lyon, University Lyon, Lyon, Rhônes, France
[5]INRAE, UMR1319 Micalis & AgroParisTech, Jouy en Josas, Yvelines, France

*Correspondence to: Sophie Georgin-Lavialle, Service de Médecine Interne, Centre de référence des Maladies Auto-Inflammatoires et des Amyloses Inflammatoires (CEREMAIA), Sorbonne Université, Assistance Publique des Hôpitaux de Paris, Hôpital Tenon, Paris, France.
E-mail: sophie.georgin-lavialle@aphp.fr

†M.D. and N.B. contributed equally.

Abstract

Objective: FMF is the most common monogenic autoinflammatory disease associated with *MEFV* mutations. Disease phenotype and response to treatment vary from one patient to another, despite similar genotype, suggesting the role of environmental factors. The objective of this study was to analyse the gut microbiota of a large cohort of FMF patients in relation to disease characteristics.

Methods: The gut microbiotas of 119 FMF patients and 61 healthy controls were analysed using 16s rRNA gene sequencing. Associations between bacterial taxa, clinical characteristics, and genotypes were evaluated using multivariable association with linear models (MaAslin2), adjusting on age, sex, genotype, presence of AA amyloidosis ($n=17$), hepatopathy ($n=5$), colchicine intake, colchicine resistance ($n=27$), use of biotherapy ($n=10$), CRP levels, and number of daily faeces. Bacterial network structures were also analysed.

Results: The gut microbiotas of FMF patients differ from those of controls in having increased pro-inflammatory bacteria, such as the *Enterobacter*, *Klebsiella* and *Ruminococcus gnavus* group. Disease characteristics and resistance to colchicine correlated with homozygous mutations and were associated with specific microbiota alteration. Colchicine treatment was associated with the expansion of anti-inflammatory taxa such as *Faecalibacterium* and *Roseburia*, while FMF severity was associated with expansion of the *Ruminococcus gnavus* group and *Paracoccus*. Colchicine-resistant patients exhibited an alteration of the bacterial network structure, with decreased intertaxa connectivity.

Conclusion: The gut microbiota of FMF patients correlates with disease characteristics and severity, with an increase in pro-inflammatory taxa in the most severe patients. This suggests a specific role for the gut microbiota in shaping FMF outcomes and response to treatment.

Keywords: FMF, gut microbiota, dysbiosis, colchicine

Rheumatology key messages

- FMF patients with severe phenotype, resistance to colchicine, and homozygous mutations had a specific microbial signature, with an increase in known pro-inflammatory taxa.
- Colchicine treatment was associated with an increase in anti-inflammatory bacteria.
- Severe FMF was associated with an altered structure of the gut microbiota.

Introduction

FMF is the most common monogenic auto-inflammatory disease and is associated with *MEFV* mutations. These mutations induce a hyperactivation of the pyrin inflammasome, an overproduction of pro-inflammatory IL, and a marked inflammatory response [1]. Patients have recurrent febrile attacks with abdominal pain, sometimes associated with cutaneous and/or rheumatological symptoms [2]. The treatment is based on colchicine, which prevents attack recurrence and complications due to chronic inflammation, such as AA

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amyloidosis (AAA) [3]. In case of colchicine resistance or severe FMF, biotherapies targeting IL-1 can be used [4].

The gut microbiota is a key player in host physiology. Among its functions, it has a fundamental role in the development and regulation of the immune system [5]. Specific species with pro-inflammatory properties (pathobionts) or anti-inflammatory properties (symbionts) have been described, with the capacity to, respectively, promote or prevent inflammatory processes in various diseases [6–15]. Among pathobionts, the Enterobacteriaceae family has been associated with pro-inflammatory properties in various models of colitis and systemic inflammation [6, 7, 9, 10, 13]. Similarly, *Ruminococcus gnavus,* a bacteria of the Lachnospiraceae family (formerly belonging to the Ruminococceae family), promotes gut inflammation depending on the composition of its capsular polysaccharide [11] and is associated with IBDs and SpA [12, 13]. In contrast, *Faecalibacterium prausnitzii*, a bacterium of the Ruminococceae family, has been extensively studied for its anti-inflammatory properties. In Crohn's disease (CD), the absence of *F. prausnitzii* in the ileal mucosa is associated with active mucosal inflammation and a higher risk of recurrence after surgery [14, 15]. Production of short-chain fatty acids (SCFAs) and of an anti-inflammatory protein (Microbial Anti-inflammatory Molecule—MAM) has been described to mediate *F. prausnitzii* anti-inflammatory effects [16, 17]. Moreover, *F. prausnitzii* also promotes the production of the anti-inflammatory cytokine IL-10 by dendritic cells (DCs) and Tregs [18, 19].

In FMF, recent data suggest a potential role for the intestinal microbiota in disease pathogenesis and severity [20–25]. *Clostridioides difficile* toxin B activates the pyrin inflammasome, leading to IL-1 production, and has been suggested as a trigger for FMF attacks [20, 21]. Furthermore, bacterial overgrowth in the small intestine is associated with a lack of response to colchicine, and decontamination with the antibiotic rifaximin reduces the frequency of FMF attacks [22]. In addition, concurrent *Helicobacter pylori* infection may increase the severity and frequency of FMF attacks, while fever and inflammatory cytokine levels were lower following its eradication [23, 24]. Finally, our team has previously shown that FMF was associated with specific modification of the gut microbiota, with a decrease in its diversity and a significant change in its composition being observed in a small cohort of patient with AAA [25].

Based on these findings, we hypothesized that gut microbiota may play a central role in the pathophysiology of FMF, particularly in mediating the onset of flares or colchicine resistance/disease severity.

Methods

Patient recruitment and data retrieval

This prospective study included patients followed for FMF at the adult National Reference Centre for Autoinflammatory Diseases and Inflammatory Amyloidosis (CEREMAIA) at Tenon Hospital in Paris, France. All patients were residents of France. Controls were recruited from spouses of participating patients and from the general population. Patients were recruited by phone call or during hospitalization or consultation with their referring physician at Tenon Hospital. All patients and controls enrolled received a stool sampling kit. In addition, patients performed a blood test assessing the complete blood count, CRP level, serum creatinine, and proteinuria/creatininuria ratio. The inclusion period ranged from April 2016 to October 2020. The blood count was performed with a Sysmex XN 3000 Module 2 machine (Kobe, Japan), and the platelets count was measured using an impedance-based method. For the serum creatinine and CRP measurements, an Atellica® Solution Siemens machine (Munich, Germany) was used with, respectively, a latex-enhanced immunoturbidimetric assay (reagent: Atellica CH C-Reactive Protein_2 (Munich, Germany)) and an enzymatic method (reagent: Atellica CH Enzymatic Creatinine_2 (Munich, Germany)). The measurement range was 45–97 µmol/l for creatininemia, <5.00 mg/l for CRP and $4–10 \times 10^9$/l for leucocytes. Demographic and clinical data were retrieved from the patients' clinical records.

Patient and control inclusions

All patients met the Tel HaShomer criteria for the diagnosis of FMF (confirmed by a physician of the referral centre) and had at least one confirmed *MEFV* mutation. They had to have been seen at least once in consultation at Tenon Hospital, to have an accessible medical record, to be >18 years old and to have had their diagnosis more than 1 year ago. Patients and controls with chronic IBDs were excluded, as these conditions have been described as statistically [1] associated with FMF and intestinal dysbiosis [25]. Patients were excluded if they had history of a major intestinal surgery such as stomas, sleeves or a bypass, or if they had been treated by antibiotics less than 1 month preceding stool sampling. Stool and blood samples were collected at the same time (for the study flow charts, see Supplementary Fig. S1, available at *Rheumatology* online).

Resistance to colchicine was defined by one or more attacks each month despite receiving the maximally tolerated dose of colchicine for at least 6 months in accordance with the European Alliance and Associations for Rheumatology guidelines [4].

Gut microbiota analysis

The samples were taken at home, collected in RNAlater solution R0901-500ML (Saint Louis, USA)/Sigma, and sent and packaged in aliquots within 7 days of release. The samples were then stored at –80°C until DNA extraction. Following a previously described method [26], the stool samples were resuspended in 250 µl of 4 M guanidine thiocyanate G9277-100G (Saint Louis, USA)/Sigma and 40 µl of 10% N-lauroyl sarcosine L9150-50g (Saint Louis, USA)/Sigma; 500 µl of 5% N-lauroyl sarcosine L9150-50g (Saint Louis, USA)/Sigma was then added. DNA was extracted by mechanical disruption of the microbial cells in a FastPrep instrument (MP Biomedicals, Waltham, USA) after the addition of 500 mg of 0.1-mm diameter glass beads (10526332/Fisher). The nucleic acids were recovered from the clear lysate by alcohol precipitation. The DNA was stored at −80°C until 16S ribosomal RNA (rRNA) gene sequencing. In order to determine gut diversity, each V3 and V4 hypervariable region of the 16S rRNA gene [5′-CTT TCCCTACACGACGCTCTTCCGATCTACGGRAGGCAG CAG-3′ (sense) and 5′-GGAGTTCAGACGTGTGCTCTTC CGATCTTACCAGGGTATCTAATCC-3′ (antisense)] of each sample was amplified, and sequencing was performed using a 250–base pair sequencing protocol on an Illumina MiSeq sequencer (Illumina, San Diego, CA, USA) at the @BRIDGe Platform of INRAE Jouy en Josas, France.

Statistical analysis

Following DNA extraction and sequencing, raw paired-end reads were processed in a data curation pipeline, including a step in which low-quality reads were removed (Qiime2 2021.4). The remaining sequences were assigned to samples based on barcode matches, and barcode and primer sequences were then trimmed. The sequences were denoised using the DADA2 method, and reads were classified using the Silva reference database (version 138). Alpha and beta diversity were computed using Qiime2 2020.8 with rarefied data (depth = 15 352 sequences/sample). Principal coordinate analyses of the Bray–Curtis distance and the Jaccard index were performed to assess beta diversity. The statistical significance of sample grouping for beta diversity analysis was performed using the Permanova method (9999 permutations). Chao1 and Shannon indices were calculated and used to characterize the alpha diversity. A Mann–Whitney *U* test when comparing two groups, using GraphPad Prism V.6.0 (San Diego, California, USA) and to obtain Shannon indices, the connectivity index, and Chao1. Differential analysis was performed using R 4.1.0 (R Core Team, 2021), with the MaAsLin2 package [27], using rarefaction as the normalization method and the Compound Poisson Analysis method. For MaAslin2 multivariate analysis comparing FMF patients with healthy controls, analysis was adjusted on disease status, age, sex, and sampling centre, defined as fixed effects. For MaAslin2 analyses assessing FMF characteristics, analysis was adjusted on genetic (homozygous or composite heterozygous *vs* heterozygous), AAA, hepatopathy, resistance to colchicine, biotherapy, CRP > 5 mg/l (normal < 5mg/l), colchicine intake, age, sex, and number of faeces per day, defined as fixed effects. Only differences with a q value of <0.25 were considered significant after correction for the false discovery rate, using the Benjamini–Hochberg procedure.

A Spearman correlation matrix of the clinical and biological features was determined using the R package Hmisc and corrplot (v0.92). Correlations were considered significant when P values were <0.05, with $q < 0.2$ after correction for the false discovery rate using the Benjamini–Hochberg procedure.

The Spearman's correlations between bacterial taxa, and between bacterial taxa and patients' characteristics, were analysed. Correlations were considered significant when P values were <0.05, with $q < 0.25$ after correction for the false discovery rate, using the Benjamini–Hochberg procedure. Network analysis comparing colchicine-resistant patients with colchicine-sensitive patients was performed using Cytoscape version 3.9.1, after randomly selecting the same number of samples from each group ($n = 27$ for each condition). The neighbourhood connectivity of each taxon of a network was calculated as the average of its connectivity with its connected neighbours.

Ethics

All patients provided written informed consent. The study was approved by the ethics committee of 'Ile de France' with the reference A92 DC-2015–2586.

Results

Population characteristics

Faecal microbiota was analysed in 119 patients with FMF and 61 healthy controls. The patients' characteristics are shown in Table 1 (and Supplementary Fig. S1, Supplementary Tables S1 and S2, available at *Rheumatology* online). Women accounted for 55.5% of all FMF patients ($n = 66$) and 47.5% of healthy controls ($n = 29$). The median disease duration at sampling was 22 years [interquartile range (IQR) = 26.5 years]. One hundred and twelve patients (94.1%) received colchicine, and 27 were resistant to colchicine (22.7%). Seventeen patients had AAA (14.2%), and five patients had chronic hepatopathy related to FMF (4.2%). As expected, FMF severity and resistance to colchicine were correlated with homozygous mutations status for the *MEFV* gene (Fig. 1).

The gut microbiota composition was distinct in FMF patients

The gut microbiota of FMF patients and healthy subjects was dominated by bacteria from the Firmicutes, Bacteroidota, Proteobacteria and Actinobacteriota phyla (Fig. 2A). Some differences between FMF and healthy microbiota were visible at the phylum and family levels (Fig. 2A and B). A decrease in alpha diversity was observed in FMF patients, as assessed by the Chao1 ($P = 0.0038$) and Shannon indices (Fig. 2C, $P = 0.019$). Beta-diversity analyses confirmed a distinct taxonomic composition difference between FMF patients and control subjects, using both the Bray–Curtis distance and the Jaccard Index (Fig. 2D, $P = 0.001$ and $P = 0.001$, respectively). Multivariate analysis with MaAsLin2 identified bacterial taxa associated with FMF. There was an increase in several pro-inflammatory taxa, such as members of the Proteobacteria phylum and the Enterobacteriaceae family, including *Enterobacter* and *Klebsiella*, or taxa from the *Ruminococcus gnavus* group (Fig. 2E). Interestingly, among FMF patients, and after adjusting for other disease characteristics, colchicine treatment was associated with an increase in the genera with known anti-inflammatory properties, such as *Faecalibacterium* or *Roseburia*, and a decrease in the genera with known pro-inflammatory properties, such as the *Ruminococcus gnavus* group or *Paracoccus* (Fig. 2F).

Altogether, these data showed that the gut microbiota of FMF patients was distinct from that of healthy controls and was enriched in pro-inflammatory taxa, which were reduced in patients under colchicine.

The gut microbiota composition was correlated with disease characteristics in FMF patients

As a homozygous *MEFV* mutation status was associated with resistance to colchicine, which is associated with the use of biotherapy or with long-term complications of FMF (i.e. hepathopathy/AAA) (Fig. 1), we specifically explored this aspect at the microbiota level. Both at the phylum and family levels, some taxonomic differences were observed between homozygous and heterozygous patients (Supplementary Fig. S2A and B, available at *Rheumatology* online), and the differences were confirmed by beta-diversity analysis (Supplementary Fig. S2C, available at *Rheumatology* online). Moreover, homozygous patients had a decrease in richness as assessed by the Chao1 index (Supplementary Fig. S2D, available at *Rheumatology* online, $P = 0.0007$). Maaslin2 analysis identified several taxa as associated with the genetic status (Supplementary Fig. S2E, available at *Rheumatology* online). In particular, pro-inflammatory bacteria such as the genera *Escherichia* and *Shigella* were increased in homozygous patients, whereas anti-inflammatory species such as

Table 1. Population characteristics

Characteristics	Patients ($n = 119$)	Controls ($n = 61$)
Sex ratio	1.25	0.91
Age (years)	45 (32–59)	35 (32–55)
FMF features		
Age (in years) at:		–
First symptoms	7.5 (3.8–12)	–
Diagnosis	16 (10–28)	–
Beginning of treatment	21 (10–32)	–
Genetics	Mutation of *MEFV* M694V/M694I/M726A/M680I	–
Homozygous, no. (%)	65 (55) 52/22/11/7	–
Compound heterozygous, no. (%)	23 (19) 17/5/16/7	–
Heterozygous, no. (%)	31 (26) 28/3/1/1	–
Disease complication		
AA Amyloidosis, no. (%)	17 (14)	–
Hepatopathy, no. (%)	5 (4.2)	–
Flare at the time of sampling, no. (%)	13 (10.9)	–
Treatment		
Colchicine, no. (%)	112 (94.1)	–
Colchicine dosage/day, (mg)	1 (1–1.7)	–
Time since colchicine onset, (years)	18 (11–34.3)	–
Resistance to colchicine, no. (%)	27 (23)	–
Biotherapy (anakinra), no. (%)	10 (8)	–
Time since anakinra onset (years)	3 (2.8–3.5)	–
Laboratory findings		
Haemoglobin (g/dl)	13.4 (12.5–14.6)	–
Leucocytes (G/l)	6.6 (5.2–7.3)	–
Neutrophils (G/l)	3.7 (2.8–4.6)	–
CRP (mg/l)	3 (1.5–8)	–
Creatinemia (µmol/l)	65 (55–78)	–
Proteinuria/creatinuria (mg/g)	54 (11–99)	–
Gastrointestinal features		
Bristol stool scale	4 (3–4)	3 (3–4)
Abdominal pain, no. (%)	13 (10.9)	3 (4.9)
Number of stools/day	1 (1–2)	1 (1–2)

Unless specified, data are presented as interquartile ranges (Q1–Q3).

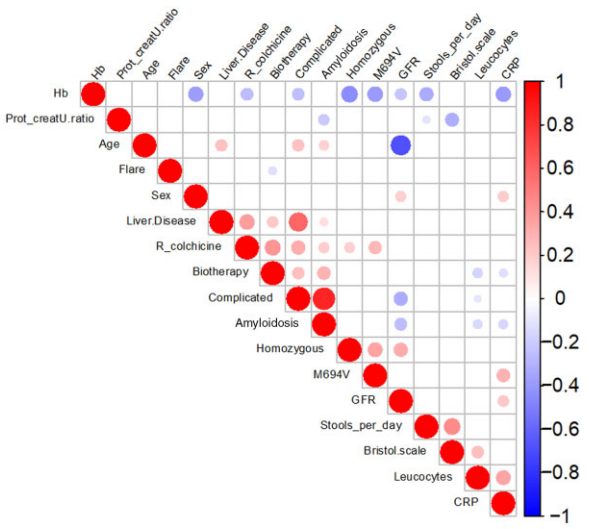

Figure 1. Clinical features and severity were correlated with homozygous genotype in FMF. Spearman correlation matrix of clinical and biological features. Correlations were considered significant when P values were <0.05, with $q < 0.2$ after correction for the false discovery rate, using the Benjamini–Hochberg procedure. GFR: glomerular filtration rate; Hb: haemoglobin; Prot_creatU.ratio: proteinuria/creatinine ratio; R_colchicine: resistant to colchicine

Butyrivibrio were decreased. Other taxa independently associated with AAA and hepatopathies as well as CRP levels were also identified (Fig. 3), such as *Paracoccus*, an aerobic bacterium with nitrate reductase activity—a metabolic function shared with Enterobacteriaceae and known to be a selective advantage for overgrowth in a proinflammatory environment [26]. Regarding disease activity at the time of stool sampling, after adjusting on other confounding factors, *Catenibacter* and *Oxalobacter* were associated with a flare at the time of collection, and Erysipelotrichaceae_UCG.003 with remission.

Colchicine resistance is a prognostic shift in FMF that cannot be predicted from patient mutations, nor from the initial presentation, suggesting the role of environmental factors. We evaluated whether the gut microbiota of colchicine-resistant patients was different from that of colchicine responders (Fig. 4A–F). Despite observing no difference in the alpha diversity (Fig. 4C), beta-diversity analysis using the Bray–Curtis distance showed a distinct gut microbiota composition according to colchicine response (Fig. 4D, $P = 0.022$). Maaslin2 analysis identified specific taxa associated with colchicine resistance, such as the potential pathobionts *Enterobacter*, *Eggerthella* and *Haemophilus* (Fig. 4E). Strikingly, when considering the network structure of the gut

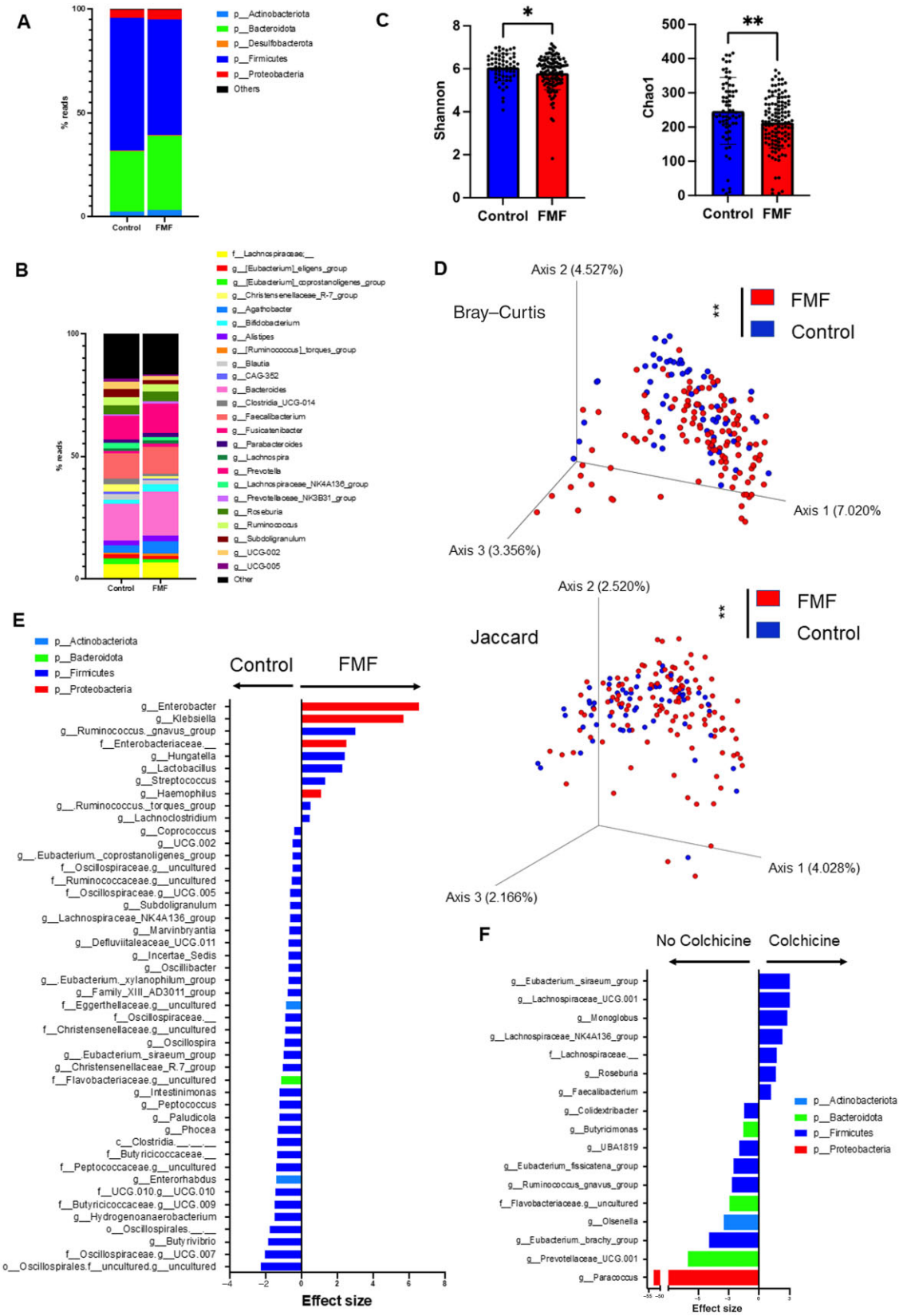

Figure 2. The gut microbiota differs between healthy control and FMF patients. The global composition of bacterial microbiota at the (**A**) phylum and (**B**) genus levels in healthy controls and patients with FMF. Taxa are represented with an average relative abundance. (**C**) The Shannon index and the Chao1 index, describing the α diversity of the bacterial microbiota in the various groups studied. Significant differences were determined using the Mann–Whitney test. (**D**) Principal coordinate analysis of the Bray–Curtis distance and the Jaccard index . The fraction of diversity captured by the coordinate is given as a percentage. Groups were compared using the Permanova method (999 permutations). (**E**) Multivariate analysis using Maaslin2 at the genus level of faecal bacteria associated with disease/control condition, adjusted on disease status, age, sex, and sampling centre. (**F**) Among FMF patients, multivariate analysis using Maaslin2 at the genus level of faecal bacteria associated with colchicine intake, adjusted on genotype, AA amyloidoisis, hepatopathy, resistance to colchicine intake, biotherapy, CRP > 5 mg/l ($N < 5$ mg/l), age, sex, and number of faeces per day. $^{*}P < 0.05$, $^{**}P < 0.01$

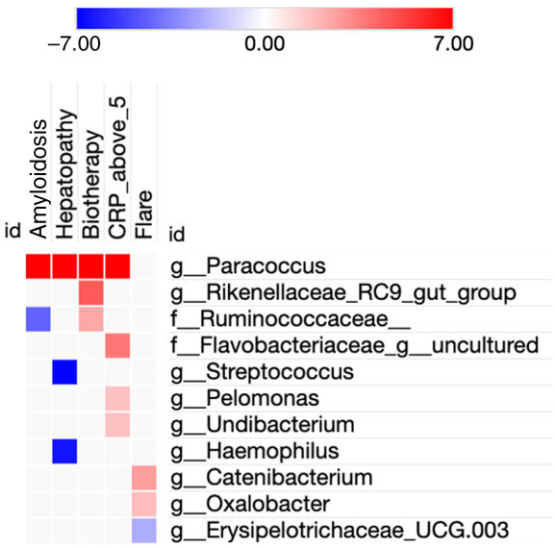

Figure 3. Disease complications and activity are associated with specific taxa. Heatmap of effector size from the multivariate analysis using Maaslin2, showing significant association between taxa and disease activity and complication after adjustment on genotype, colchicine intake, resistance to colchicine, age, sex, and number of faeces per day. Only differences with a *q* value of <0.25 were considered significant, after correction for the false discovery rate, using the Benjamini–Hochberg procedure

microbiota, colchicine-resistant patients presented a decreased intertaxa connectivity (Fig. 5A), as quantified by the lower neighbourhood connectivity (Fig. 5B, $P = 0.0095$). This suggests that, in addition to differences in bacterial composition, colchicine-resistant patients have an altered gut microbiota structure.

Discussion

In this large study, we compared the gut microbiota of FMF patients with that of healthy controls in the largest cohort to date, and data between subcategories of FMF severity. We found an alteration of the gut microbiota associated with FMF, both in terms of diversity and composition, and specific associations with the disease genotype, phenotype, and response to colchicine. Interestingly, an overrepresentation of pro-inflammatory bacteria of the Enterobacteriaceae family was associated with the disease and with the most severe phenotype. In addition, the decreased alpha diversity observed in FMF patients was stronger in homozygous patients than in heterozygous patients. Finally, we observed a decrease in bacterial network connectivity in colchicine-resistant patients.

Among the pro-inflammatory bacteria that were increased in FMF patients, Enterobacteriaceae have been associated with mucosal inflammation in various diseases and animal models [6, 8, 9, 10, 28, 29]. Interestingly, these bacteria favour innate and adaptive immune responses at the mucosal interface. They express potent pathogen-associated molecular patterns (PAMPs) such as the bacterial lipopolysaccharide or specific unmethylated immunostimulatory motifs recognized by Toll-like receptors (TLR) 4 and TLR 9, respectively [30, 31]. Transfer of altered gut microbiota enriched in Enterobacteriaceae favours colitis in mice [9] and has been associated with an increased risk of relapse after surgery in CD [32], suggesting that these bacteria promote an early pro-inflammatory response in genetically susceptible individuals. In the same line, the *Ruminococcus gnavus* group, increased in FMF patients, is associated with CD and promotes gut inflammation [11]. The hypothesis of an immunogenic microbiota in FMF is supported by the increased immune response directed against commensal gut microbiota in FMF patients, as illustrated by the increased levels of IgG antibodies directed against commensal species (i.e. *Bacteroides*, *Parabacteroides*, *Escherichia* and *Enteroccocus* antigens) [32]. Altogether, these pro-inflammatory taxa may favour immune system activation, fuelling disease activity.

Apart from direct immune modulation, alterations of the gut microbiota can potentially induce FMF attacks through impaired microbial metabolism and pathogen expansion [20–24]. Indeed, the loss of gut microbiota diversity observed in FMF patients is a known risk factor for *C. difficile* expansion and infection, through toxin production that can, subsequently, activate the pyrin inflammasome [21].

More generally, alteration of bile acid metabolism by the microbiota may affect activation of the pyrin inflammasome directly and/or indirectly. In patients with CD, in which similar expansion of the Enterobacteriaceae family is observed, secondary bile acid biosynthesis from conjugated primary bile acid is decreased [33]. Interestingly, this decrease favours *C. difficile* germination and pathogenicity [34]. Bile acids analogs (BAA485 and BAA473) directly activate the pyrin inflammasome, and colchicine can counteract this effect [35]. Exploration of bile acid metabolism in FMF patients may thus constitute a promising approach when aiming to target the host–microbial cross-talk that could impact disease evolution.

FMF patients with severe phenotypes, colchicine resistance, and/or homozygous mutations had a specific microbial signature. Whether these changes were simply a consequence of inflammatory changes induced by the disease within the intestine is questionable. Indeed, an inflammatory environment favours the expansion of the Enterobacteriacea family through various mechanisms, including increased oxidative stress and limited iron availability [6]. However, unlike IBD patients, most FMF patients do not have macroscopic inflammatory lesions, although low-grade inflammation may be present, as suggested by a moderate increase in faecal calprotectin, a biomarker of mucosal inflammation, in some FMF patients [36]. Furthermore, in our cohort, the majority of FMF patients were in clinical remission at the time of sampling, without systemic inflammation, as indicated by normal CRP levels. However, to completely eliminate potential biases induced by disease activity, the results were confirmed in a multivariate analysis adjusted for clinical activity and CRP level, suggesting that the microbiota alterations were genuinely associated with FMF status independently of the occurrence of an attack. This pro-inflammatory microbiota might fuel a vicious cycle that can induce pyrin activation in genetically susceptible individuals.

Synergistic approaches targeting both pyrin activation and the pro-inflammatory imbalance of the microbiota may be of interest in severe patients, particularly those resistant to colchicine who present profound alterations in gut microbiota composition and structure. There is already evidence that therapeutic manipulation of the gut microbiota can be effective in FMF. For example, patients treated by antibiotics for *Helicobacter pylori* infection or small intestinal bacterial overgrowth have a lower rate of FMF flare [22–24]. Specific

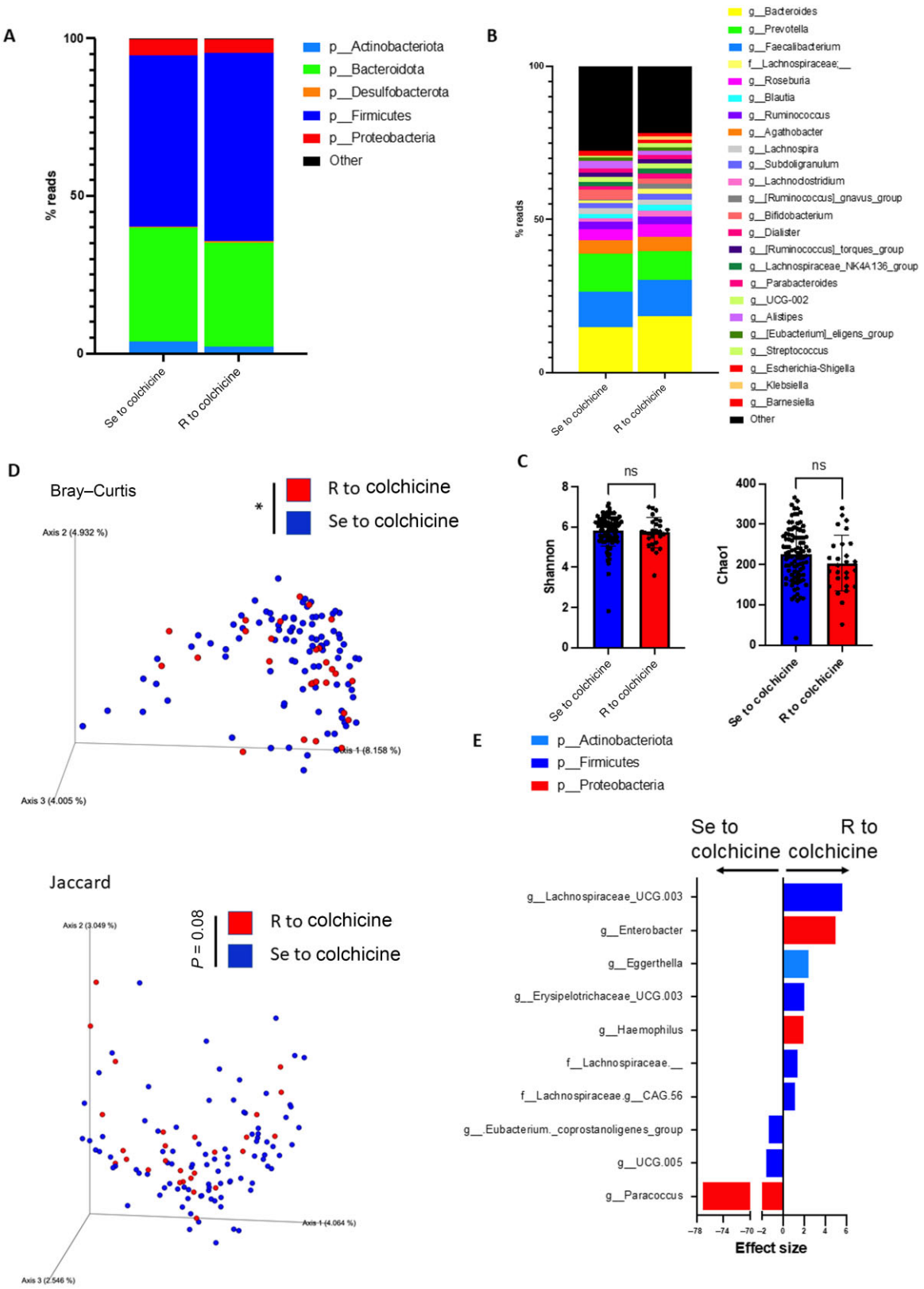

Figure 4. Gut microbiota structure and composition differed between colchicine-resistant and -sensitive patients. Global composition of bacterial microbiota at the (**A**) phylum and (**B**) genus levels in colchicine-resistant and -sensitive patients. Coloured blocks indicate taxa with an average relative abundance. (**C**) The Shannon index and the Chao1 index, describing the α diversity of the bacterial microbiota in the two groups studied. Significant differences were determined using the Mann–Whitney test. (**D**) Principal coordinate analysis of the Bray–Curtis distance and the Jaccard index, with each sample coloured according to disease status. The fraction of diversity captured by the coordinate is given as a percentage. Groups were compared using the Permanova method (999 permutations). (**F**) Multivariate analysis using Maaslin2 at the genus level of faecal bacteria associated with resistance to colchicine adjusted on genotype, AA amyloidoisis, hepatopathy, biotherapy, CRP of >5 mg/l (N < 5mg/l), colchicine intake, age, sex, number of faeces per day. The colour represents the phylum. $*P < 0.05$, $**P < 0.01$. R: resistance; Se: sensitivity

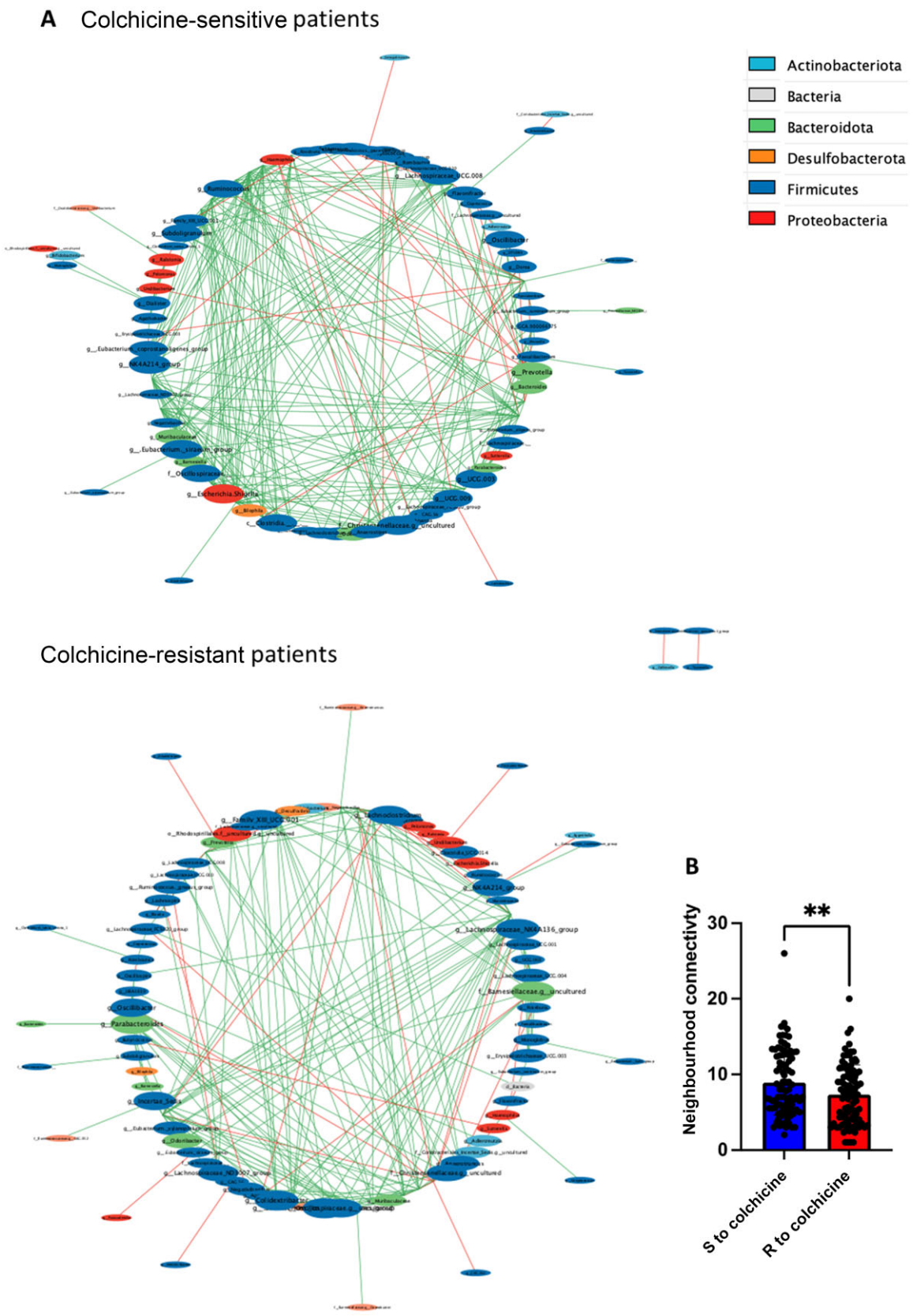

Figure 5. The intestinal microbiota of colchicine-resistant patients shows an alteration of the bacteria–bacteria network. (**A**) Genus-level correlation networks for bacterial abundance in colchicine-sensitive and -resistant patients. Correlation networks were built using Spearman's correlation. Each circle (node) represents a genus, the colour represents the phylum, and the size increases with the number of direct edges. The colour of the edges indicates the direction of the correlation (green for positive and red for negative). Only significant correlations ($P < 0.05$ and $q < 0.25$ after correction for the false discovery rate using the Benjamini–Hochberg procedure) are shown. (**B**) The neighbourhood connectivity of each correlated taxon in colchicine-sensitive and -resistant patients. $^{**}P < 0.01$. R: resistance; Se: sensitivity

probiotic therapy can induce a normalization of serum CRP in FMF patients with high CRP levels during clinical remission [37], and treatment with *Lactobacillus acidophilus* was able to shift the microbiota composition in a small controlled trial in patients with FMF [38]. Thus, novel therapeutic strategies in FMF patients could emerge in the coming years, using innovative tools, such as next-generation probiotics or faecal microbiota transplantation.

To date, no clear predictive factors for patients not responding to colchicine have been identified. Interestingly, we were able to identify specific taxa associated with colchicine resistance, such as the potentially pathogenic bacteria *Enterobacter* and *Haemophilus*. In addition, exposure to colchicine was also associated with specific gut microbiota changes, with expansion of anti-inflammatory bacteria such as *Faecalibacterium* or *Roseburia* and a decrease in genera with known pro-inflammatory properties such as the *Ruminococcus gnavus* group or *Paracoccus*. This suggests that the therapeutic effects of colchicine may be partly related to the modulation of the gut microbiota.

In addition, we show that colchicine-sensitive patients have increased intertaxa connectivity. This suggests that, in addition to being associated with anti-inflammatory taxa, colchicine treatment efficacy is associated with a profound restructuring of the bacterial ecosystem. Alteration of the bacterial correlation network with decreased intertaxa connectivity has been associated with mucosal inflammation and systemic inflammation in various diseases [28, 39]. Furthermore, these community structures are associated with ecosystem robustness and protection against pathogens [40].

Based on these data, the structure of the bacterial ecosystem could be a relevant therapeutic and diagnostic target for the control of FMF and the risk of gastrointestinal infection, especially by *C. difficile*.

This work has several limitations. Although it is tempting to postulate an inciting role for the microbiota in the natural history of FMF in addition to the genetic background, the study only describes associations and not causality. Interventional studies attempting to determine whether dysbiosis is truly causal in FMF pathophysiology are needed. Although bacteria are the most abundant component of the gut microbiota, other kingdoms, such as the fungi, have been shown to be altered in FMF patients [38], and it would have been interesting to evaluate them.

This study showed that FMF patients had specific alterations in their gut microbiota, both in structure and composition, which were correlated with disease characteristics and treatment (severe phenotype, colchicine resistance/exposure, and homozygous mutations). This dysbiosis, causal and/or consequential to FMF inflammation through a vicious circle, may be a good candidate for combined approaches targeting both inflammatory mediators and the gut microbiota in FMF patients with severe disease or colchicine resistance.

Supplementary material

Supplementary material is available at *Rheumatology* online.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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References

1. Jamilloux Y, Lefeuvre L, Magnotti F *et al.* Familial Mediterranean fever mutations are hypermorphic mutations that specifically decrease the activation threshold of the Pyrin inflammasome. Rheumatology (Oxford) 2018;57:100–11.
2. Ben-Chetrit E, Touitou I. Familial Mediterranean Fever in the world. Arthritis Rheum 2009;61:1447–53.
3. Zemer D, Pras M, Sohar E *et al.* Colchicine in the prevention and treatment of the amyloidosis of familial Mediterranean fever. N Engl J Med 1986;314:1001–5.
4. Ozen S, Demirkaya E, Erer B *et al.* EULAR recommendations for the management of familial Mediterranean fever. Ann Rheum Dis 2016;75:644–51.
5. Belkaid Y, Harrison OJ. Homeostatic immunity and the microbiota. Immunity 2017;46:562–76.
6. Lupp C, Robertson ML, Wickham ME *et al.* Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of Enterobacteriaceae. Cell Host Microb 2007;2:119–29.
7. Levast B, Benech N, Gasc C *et al.* Impact on the gut microbiota of intensive and prolonged antimicrobial therapy in patients with bone and joint infection. Front Med (Lausanne) 2021;8:586875.
8. Sokol H, Leducq V, Aschard H *et al.* Fungal microbiota dysbiosis in IBD. Gut 2017;66:1039–48.
9. Carvalho FA, Koren O, Goodrich JK *et al.* Transient inability to manage proteobacteria promotes chronic gut inflammation in TLR5-deficient mice. Cell Host Microbe 2012;12:139–52.
10. Garrett WS, Gallini CA, Yatsunenko T *et al.* Enterobacteriaceae act in concert with the gut microbiota to induce spontaneous and maternally transmitted colitis. Cell Host Microbe 2010;8:292–300.
11. Henke MT, Brown EM, Cassilly CD *et al.* Capsular polysaccharide correlates with immune response to the human gut microbe *Ruminococcus gnavus*. Proc Natl Acad Sci USA 2021;118:e2007595118.
12. Hall AB, Yassour M, Sauk J *et al.* A novel *Ruminococcus gnavus* clade enriched in inflammatory bowel disease patients. Genome Med 2017;9:103.
13. Breban M, Tap J, Leboime A *et al.* Faecal microbiota study reveals specific dysbiosis in spondyloarthritis. Ann Rheum Dis 2017;76:1614–22.
14. Sokol H, Pigneur B, Watterlot L *et al. Faecalibacterium prausnitzii* is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. Proc Natl Acad Sci USA 2008;105:16731–6.
15. Wright EK, Kamm MA, Wagner J *et al.* Microbial factors associated with postoperative Crohn's disease recurrence. J Crohns Colitis 2017;11:191–203.
16. Louis P, Flint HJ. Formation of propionate and butyrate by the human colonic microbiota. Environ Microbiol 2017;19:29–41.
17. Quévrain E, Maubert MA, Michon C *et al.* Identification of an anti-inflammatory protein from *Faecalibacterium prausnitzii*, a commensal bacterium deficient in Crohn's disease. Gut 2016;65:415–25.

18. Rossi O, van Berkel LA, Chain F *et al.* *Faecalibacterium prausnitzii* A2-165 has a high capacity to induce IL-10 in human and murine dendritic cells and modulates T cell responses. Sci Rep 2016;6: 18507.
19. Touch S, Godefroy E, Rolhion N *et al.* Human $CD4^{+}CD8\alpha^{+}$ Tregs induced by *Faecalibacterium prausnitzii* protect against intestinal inflammation. JCI Insight 2022;7:e154722.
20. Deshayes S, Fellahi S, Bastard JP *et al.* La fièvre méditerranéenne familiale et l'amylose AA s'accompagnent d'une dysbiose: étude transversale sur 60 patients. La Rev Méd Interne 2018;39:A97–8.
21. Xu H, Yang J, Gao W *et al.* Innate immune sensing of bacterial modifications of Rho GTPases by the Pyrin inflammasome. Nature 2014;513:237–41.
22. Verrecchia E, Sicignano LL, La Regina M *et al.* Small intestinal bacterial overgrowth affects the responsiveness to colchicine in familial Mediterranean fever. Mediators Inflamm 2017;2017:7461426.
23. Ozel AM, Demirturk L, Aydogdu A *et al.* Effect of *Helicobacter pylori* infection and eradication therapy on interleukin-6 levels in patients with Familial Mediterranean Fever. Int J Clin Pract 2008; 62:754–61.
24. Demirtürk L, Ozel AM, Cekem K, Yazgan Y, Gultepe M. Co-existence of *Helicobacter pylori* infection in patients with Familial Mediterranean Fever (FMF) and the effect of *Helicobacter pylori* on the frequency and severity of FMF attacks. Dig Liver Dis 2005; 37:153–8.
25. Deshayes S, Fellahi S, Bastard JP *et al.* Specific changes in faecal microbiota are associated with familial Mediterranean fever. Ann Rheum Dis 2019;78:1398–404.
26. Zeng MY, Inohara N, Nuñez G. Mechanisms of inflammation-driven bacterial dysbiosis in the gut. Mucosal Immunol 2017;10: 18–26.
27. Mallick H, Rahnavard A, McIver LJ *et al.* Multivariable association discovery in population-scale meta-omics studies. PLoS Comput Biol 2021;17:e1009442.
28. Mondot S, Lepage P, Seksik P *et al.*; GETAID. Structural robustness of the gut mucosal microbiota is associated with Crohn's disease remission after surgery. Gut 2016;65:954–62.
29. Salosensaari A, Laitinen V, Havulinna AS *et al.* Taxonomic signatures of cause-specific mortality risk in human gut microbiome. Nat Commun 2021;12:2671.
30. Kant R, de Vos WM, Palva A, Satokari R. Immunostimulatory CpG motifs in the genomes of gut bacteria and their role in human health and disease. J Med Microbiol 2014;63:293–308.
31. Baldelli V, Scaldaferri F, Putignani L, Del Chierico F. The role of enterobacteriaceae in gut microbiota dysbiosis in inflammatory bowel diseases. Microorganisms 2021;9:697.
32. Manukyan GP, Ghazaryan KA, Ktsoyan ZA *et al.* Elevated systemic antibodies towards commensal gut microbiota in autoinflammatory condition. PLoS One 2008;3:e3172.
33. Heinken A, Ravcheev DA, Baldini F *et al.* Systematic assessment of secondary bile acid metabolism in gut microbes reveals distinct metabolic capabilities in inflammatory bowel disease. Microbiome 2019;7:75.
34. Schnizlein MK, Young VB. Capturing the environment of the *Clostridioides difficile* infection cycle. Nat Rev Gastroenterol Hepatol 2022;19:508–20.
35. Alimov I, Menon S, Cochran N *et al.* Bile acid analogues are activators of pyrin inflammasome. J Biol Chem 2019;294:3359–66.
36. Gucenmez OA, Kume T, Makay B *et al.* Role of fecal calprotectin in the assessment of intestinal inflammation in children with familial Mediterranean fever. Int J Rheum Dis 2018;21:1844–8.
37. Di Ciaula A, Stella A, Bonfrate L, Wang DQH, Portincasa P. Gut microbiota between environment and genetic background in Familial Mediterranean Fever (FMF). Genes (Basel) 2020;11:1041.
38. Pepoyan A, Balayan M, Manvelyan A *et al.* Probiotic *Lactobacillus acidophilus* strain INMIA 9602 Er 317/402 administration reduces the numbers of *Candida albicans* and abundance of enterobacteria in the gut microbiota of familial Mediterranean fever patients. Front Immunol 2018;9:1426.
39. Sokol H, Contreras V, Maisonnasse P *et al.* SARS-CoV-2 infection in nonhuman primates alters the composition and functional activity of the gut microbiota. Gut Microbes 2021;13:1–19.
40. Bäumler AJ, Sperandio V. Interactions between the microbiota and pathogenic bacteria in the gut. Nature 2016;535:85–93.