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Both Disease Activity and HLA–B27 Status Are Associated With Gut Microbiome Dysbiosis in Spondyloarthritis Patients

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Objective. Gut microbiome dysbiosis has previously been reported in spondyloarthritis (SpA) patients and could be critically involved in the pathogenesis of this disorder. The objectives of this study were to further characterize the microbiota structure in SpA patients and to investigate the relationship between dysbiosis and disease activity in light of the putative influence of the genetic background.

Methods. Shotgun sequencing was performed on fecal DNA isolated from stool samples from 2 groups of adult volunteers: SpA patients (n = 102) and healthy controls (n = 63). A subset of the healthy controls comprised the age-matched siblings of patients whose HLA–B27 status was known. Changes in gut microbiota composition were assessed based on species diversity, enterotypes, and taxonomic and functional differences.

Results. Dysbiosis was confirmed in SpA patients as compared to healthy controls. The restriction of microbiota diversity was detected in patients with the most active disease, and the abundance of several bacterial species was correlated with Bath Ankylosing Spondylitis Disease Activity Index score. Among healthy controls, significant differences in microbiota composition were also detected between the HLA–B27–positive and the HLA–B27–negative siblings of SpA patients. We highlighted a decreased abundance of several species of bacteria in SpA patients, especially those bacteria belonging to the Clostridiales order. Among the few species of bacteria showing increased abundance, *Ruminococcus gnavus* was one of the top differentiating species.

Conclusion. These findings reveal that genetic background and level of disease activity are likely to influence the composition of the gut microbiota of patients with SpA. It may be appropriate for further research on chronic arthritis to focus on these key parameters.

INTRODUCTION

Spondyloarthritis (SpA) is a group of inflammatory rheumatic disorders with a population prevalence ranging between 0.2% and 1.6% (1). During the disease course, osteoarticular inflammation predominantly affects the axial skeleton (i.e., the sacroiliac, spinal, and thoracic

joints), but may also involve the peripheral joints and entheses. Moreover, patients with SpA frequently experience extraarticular manifestations, including anterior uveitis, psoriasis, and inflammatory bowel diseases (IBDs) such as Crohn's disease or ulcerative colitis (2).

This multifaceted disease is thought to result from complex interactions between the genetic background and environmental

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factors. Several genetic polymorphisms have been shown to be associated with an increased risk of SpA (3). The genetic factor most strongly associated with the development of SpA, which was discovered nearly 50 years ago, is the presence of the major histocompatibility complex class I allele HLA-B27. More recently, genome-wide studies have demonstrated that other polymorphisms which predispose to SpA also predispose to IBD, reinforcing the possibility of shared mechanisms between both diseases (3). Of note, a large subset of responsible genes code for proteins involved in the immune response, and in particular for the interleukin-23/Th17 pathway of T cell differentiation, which is primarily implicated in the response against extracellular pathogens including bacteria and yeasts and/or in microbial sensing (2,3). Given that deregulated gut microbial composition, i.e., dysbiosis, is a hallmark of IBD (4,5), the foregoing observations lend support to the hypothesis that microbial dysbiosis could also contribute to the pathogenesis of SpA. Observations from animal models of SpA also support such a hypothesis (6). For example, dysbiosis is present in HLA-B27/human β_2 -microglobulin-transgenic rats that develop both arthritis and IBD, but breeding under germ-free conditions has been shown to prevent the development of both joint and gut inflammation.

In several recent studies, the composition of the microbiota in gut mucosal biopsy samples or stool samples from SpA patients was investigated using high-throughput sequencing technologies. In most of these studies, including our own previous work, targeted amplicon sequencing of the 16S ribosomal RNA (16S rRNA) gene (6,7) was used, whereas a whole-metagenome shotgun sequencing approach was used on stool samples (8–10) in only 3 of these previous studies. As highlighted by the recent literature, shotgun sequencing is superior for all aspects of metagenomic analysis. Indeed, although the 16S rRNA gene is widely used for the taxonomic profiling of microbial communities, the methodology suffers from low taxonomic resolution and poor taxa quantification, which may lead to erroneous conclusions (11–13). Shotgun sequencing is a much preferred method to accurately detect and quantify microbial species, estimate species richness, and perform functional analysis up to the strain level (14–17). Whatever the methodology, dysbiosis was shown in all of the studies described, but there were differences in their specific findings. In a study by Costello et al and a study by Tito et al, increased taxa diversity was described in the mucosal-associated microbiota, although one limitation of those studies was the relatively few number of samples examined (9–27 patients versus 9–15 healthy controls) (18,19). In contrast, reduced diversity of the microbiota has been observed in studies of fecal samples from SpA and ankylosing spondylitis patients, which is a finding similar to those usually reported in the context of IBD (7,8,10). Despite the larger number of analyzed samples, results from the latter studies were quite divergent with respect to the contrasted bacterial genera/species between SpA patients and controls, with one

of the major differences being the sequencing technology that was used.

In the present study, we sequenced DNA from the fecal samples of SpA patients and healthy controls (including a subset of HLA-B27-positive individuals and HLA-B27-negative individuals who were the spouses or siblings of SpA patients) using whole-metagenome shotgun sequencing. Our findings will help to refine the analysis of SpA dysbiosis.

PATIENTS AND METHODS

Participant recruitment and study design. This study was conducted according to the Declaration of Helsinki and the French legislation. Before study inclusion, each participant gave written informed consent for research use and publication of their data. Stool samples were collected from 197 volunteers, including 102 SpA patients fulfilling the Assessment of SpondyloArthritis international Society classification criteria for SpA (20) and 63 healthy controls. The healthy controls included 23 genetically independent controls and 40 controls who were recruited as siblings of SpA patients enrolled in the study (21 HLA-B27-positive and 19 HLA-B27-negative siblings) (Table 1). Of those, 82 samples from SpA patients and 62 samples from healthy controls had been included in our previous analysis of 16S rDNA targeted sequencing (7). Notably, the cohort comprised 11 sibling trios, each consisting of an HLA-B27-positive SpA patient matched with an HLA-B27-positive healthy control and an HLA-B27-negative healthy control.

Study samples were obtained from patients with established SpA who were seen in a tertiary care center (i.e., the Rheumatology clinic of the Ambroise Paré Hospital, Boulogne, France), or from SpA patients belonging to French families whose samples had been collected by Groupe Français d'Etude Génétique des Spondyloarthrites for genetic purposes as previously described (21,22). The healthy control group consisted of hospital staff members and the age-matched healthy spouses or siblings of SpA patients. Additional details on the methods used in this study are available on the *Arthritis & Rheumatology* website at <http://onlinelibrary.wiley.com/doi/10.1002/art.42289>.

All participants were required not to have received antibiotics during the preceding month, nor to have undergone gut preparation for colonoscopy during the preceding 6 months. Individuals affected by medical conditions other than the diseases under investigation, which we thought could potentially affect the results of the study, were excluded. Notably, this included other osteoarticular inflammatory disorders and/or autoimmune disorders, obesity, diabetes, cancer, and any chronic organ failure. Clinicobiologic data relevant to disease classification or disease activity evaluation (i.e., Bath Ankylosing Spondylitis Disease Activity Index [BASDAI]) and current treatments were collected.

Table 1. Characteristics of spondyloarthritis (SpA) patients and healthy controls included in this study on the microbiota structure of the gut in SpA patients and the relationship between dysbiosis and disease activity*

Characteristic	SpA patients (n = 102)	Healthy controls (n = 63)	P†
Age, mean ± SD years	50 ± 11.3	48.7 ± 11.7	0.8
Disease duration, mean ± SD years	25.1 ± 11.9	NA	
Sex ratio, % of men	49	39.7	0.3
HLA-B27 positivity‡	82.5	43.1	1.8 ⁻⁰⁶
BASDAI, mean ± SD score	3.78 ± 2.29	NA	
Axial/peripheral SpA	88.2/11.8	NA	
Radiographic sacroiliitis§	56	ND	
Extraarticular manifestations			
Uveitis	28.4	ND	–
Psoriasis	30.4	ND	–
Inflammatory bowel disease	12.7	0	0.03
Treatments of interest			
Any treatment	67.3	6.5	1 ⁻¹³
NSAID	53.5	4.8	7.7 ⁻¹⁰
Glucocorticoid	5.0	0	0.1
DMARD	4.0	0	0.2
Anti-TNF biotherapy	28.4	0	8.5 ⁻⁰⁶
Antacid	32.7	1.6	5.62 ⁻⁰⁶

* Except where indicated otherwise, values are the percent of participants. The registered manifestations correspond to those present at the time of examination or retrieved from medical history. The only disease-modifying antirheumatic drug (DMARD) received by patients in the study was sulfasalazine. The anti-tumor necrosis factor (anti-TNF) medications received by patients in the study included etanercept (n = 12 patients), infliximab (n = 11 patients), and adalimumab (n = 5 patients). BASDAI = Bath Ankylosing Spondylitis Disease Activity Index; NSAID = nonsteroidal antiinflammatory drug; NA = not applicable; ND = not determined.

† P values were determined by chi-square test, except for the variables of age, sex ratio, HLA-B27 status, and inflammatory bowel disease, which were determined by Wilcoxon's test. P values of ≤0.05 were considered significant.

‡ HLA-B27 status was determined in all SpA patients and in 51 of 63 healthy controls.

§ Radiographic sacroiliitis diagnoses fulfilling the modified New York criteria (50) were determined in 100 of 102 SpA patients.

Fecal DNA extraction, metagenomic sequencing, and mapping procedures.

Details of the fecal DNA extraction procedure used in this study have been described previously (7). Whole-metagenome shotgun sequencing was performed using 5500 SOLiD Wildfire (Life Technologies) to generate single-end reads 35–50 bases long. Read quality control was performed with an in-house pipeline using a quality score cutoff of >20. In addition, reads aligned to the human genome (GRCh38.p13) or to the SOLiD adapter sequences were discarded. The resulting filtered, high-quality reads (mean ± SEM 47.8 ± 24.7 million reads per sample) were mapped onto the 10.4 million-gene reference catalog IGC2 (8) using the in-house Meteor software suite (software available upon request). Read alignments were performed in colorspace with Bowtie software version 1.2.3 in a 2-step procedure (using the options -f -C -integer-quals -v 2 -k 10000 -sam -sam-nohead -sam-nosq -no-unal and an identity threshold of 95% to account for gene variability). In the first step, unique mapped reads (reads mapped to a unique gene in the catalog) were attributed to their corresponding genes. In the second step, shared reads (reads that mapped with the same alignment identity to multiple genes in the catalog) were weighted according to the ratio of unique mapped counts. The subsequent steps to generate the gene abundance profiling table included first a rarefaction procedure, which involved drawing out 10 million mapped reads per sample without replacement (to account for

difference in sequencing depth between samples), followed by the normalization of fragments per kilobase million (normalization by gene size and total number of mapped reads per sample, reported in relative frequency), which was completed using the MetaOMineR (momr) R package (23).

Metagenomic species pangenome (MSP) determination and assessment of the gut microbiome functional potential.

Metagenomic species pangenomes (MSPs) were used to identify and quantify the microbial species associated with the IGC2 reference catalog (8). MSPs are clusters of coabundant genes (minimum size >100 genes) reconstructed from 1,601 metagenomes into 1,990 MSP species and used as proxies for microbial species (14,24). MSP abundances were estimated as the mean abundance of their 100 marker genes, as far as at least 20% of these genes are detected. An MSP abundance table was then normalized for each sample by dividing the abundance of the MSP by the sum of MSP abundances detected in the sample. MSP taxonomical annotation was processed as previously described (6). MSP species richness was determined by counting the number of MSPs detected in the corresponding sample.

Assessment of the gut microbiome functional potential.

The metabolic potential of the intestinal microbiome

was assessed using an in-house pipeline. Genes of the 10.4 million-gene reference catalog were annotated using the KEGG version 8.9 database (25), the eggNOG database (26), and the TIGRFAMs database (27). Using DIAMOND (28), each gene from the IGC2 catalog was mapped onto KEGG orthologs from the KEGG database and was assigned to the best-ranked KEGG ortholog among hits with E-values of $<10^{-5}$ and a bit score of >60 . The same procedure was used with eggNOG version 3.0. The gene catalog was searched against TIGRFAM version 15.0 profiles using HMMER version 3.2.1 (29). Next, KEGG modules, gut metabolic modules (30), and gut-brain modules (31) were reconstructed for each metagenomic species using their pathway structures (and potential alternative pathways), based on their detected annotated KEGG orthologs (or NOGs, or TIGRFAMs). Since MSPs are pangenomes, their genes are divided into “core” genes, which are present in all samples harboring the MSP, and “accessory” genes, which are specific to certain strains of the MSP and might be absent from a sample even if the MSP is detected. Functional modules were considered to be present in a given species if at least 90% of its components were present in the core genes of the corresponding MSP. Core modules were then considered to be present whenever the MSP was present in a given sample. Other modules (accessory modules) were identified sample-by-sample according to all of the MSP genes (core and accessory) in a given sample. Finally, we estimated the abundance of a module in a sample by summing the abundances of all MSPs found to carry this module in the sample.

Enterotype determination. Enterotyping was performed using the species abundance table for a Dirichlet Multinomial Mixtures approach (32) by means of the Dirichlet Multinomial R package version 1.24.1 (33). The optimal number of clusters was determined based on the stability of the clustering; enterotypes were considered stable if samples were affected into the same cluster in at least 15 out of 20 tests performed. Three enterotypes were obtained corresponding to Bacteroides, Firmicutes, and Prevotella.

Data analysis and statistical methods. Prior to the analyses, the normalized MSP abundance table was filtered out by using a 10% occurrence threshold across samples, which resulted in a total of 662 MSPs. The nonparametric Wilcoxon test was used to compare 2 conditions, and the Kruskal-Wallis test was used to compare more than 2 conditions. The Benjamini-Hochberg adjustment was used to control for the false discovery rate and provided q values that gave the expected proportion of false positives among all positive results. Unless stated otherwise, q values of ≤ 0.1 were considered significant, and q values between 0.1 and 0.3 were considered to show a tendency toward significance. All statistical analyses and visualizations were performed in R environment version 3.6.0 (<https://www.r-project.org>). Box plots and bar plots were computed using the ggpubr

R package (34), and the metagenomic analyses were performed using the momr R package (23). Heatmaps were created with the ComplexHeatmap package (35). Observed richness, the Shannon diversity index, the inverse Simpson index, and the Fisher index were used for the α -diversity analysis and all were computed with the R package VEGAN (36). For the β -diversity analysis, Bray–Curtis distances were computed with the R package VEGAN (36), the principal coordinates analysis was computed with the R package ade4, and graphical displays were produced with the R package factoextra. To assess the differences in β -diversity, we performed permutational multivariate analysis of variance (PERMANOVA) using distance matrices with the function Adonis ($n = 10,000$ permutations).

Data availability. Metagenomic reads generated in this study are available on the INRAE data portal (dataset X9BHTZ), a public, open access repository, at <https://doi.org/10.15454/X9BHTZ>.

RESULTS

Alteration of the SpA gut microbiome in proportion to disease activity. We compared the composition of the gut microbiota between patients with SpA and healthy controls, taking into account clinical parameters which included disease activity index. MSP richness was not significantly different between SpA patients and healthy controls ($P = 0.12$ by nonparametric Wilcoxon test), although it was lower in SpA patients (Figure 1A). However, a significant negative correlation was observed between MSP richness and BASDAI score in SpA patients (Pearson's $r = -0.34$; $P = 6.6 \times 10^{-4}$) (Figure 1D). By partitioning patients into tertiles based on their BASDAI score (BASDAI score ≤ 2.3 for mild SpA [$n = 34$], BASDAI score >2.3 and <4.8 for moderate SpA [$n = 33$], and BASDAI score ≥ 4.8 for severe SpA [$n = 33$]), we consistently found a progressive loss of richness concomitant with increasing disease activity (Figure 1B), which was strongly significant in patients with the most active SpA compared to healthy controls (for details on the characteristics of the SpA patients according to BASDAI score group, see Supplementary Information 1, available at <http://onlinelibrary.wiley.com/doi/10.1002/art.42289>). For further comparisons with other studies, we also performed the comparisons using the traditional BASDAI cutoff score of 4 (Supplementary Figures 2–4, <http://onlinelibrary.wiley.com/doi/10.1002/art.42289>), which led to similar results.

We also determined the gut microbiome enterotypes of the SpA patients and the healthy controls (Figure 1C). Three enterotypes were observed in both groups and were similarly dominated by the Firmicutes and Bacteroides. Interestingly, disease activity was associated with enterotype prevalence, and the most dramatic differences in enterotype prevalence occurred in the severe SpA patient group compared to healthy controls

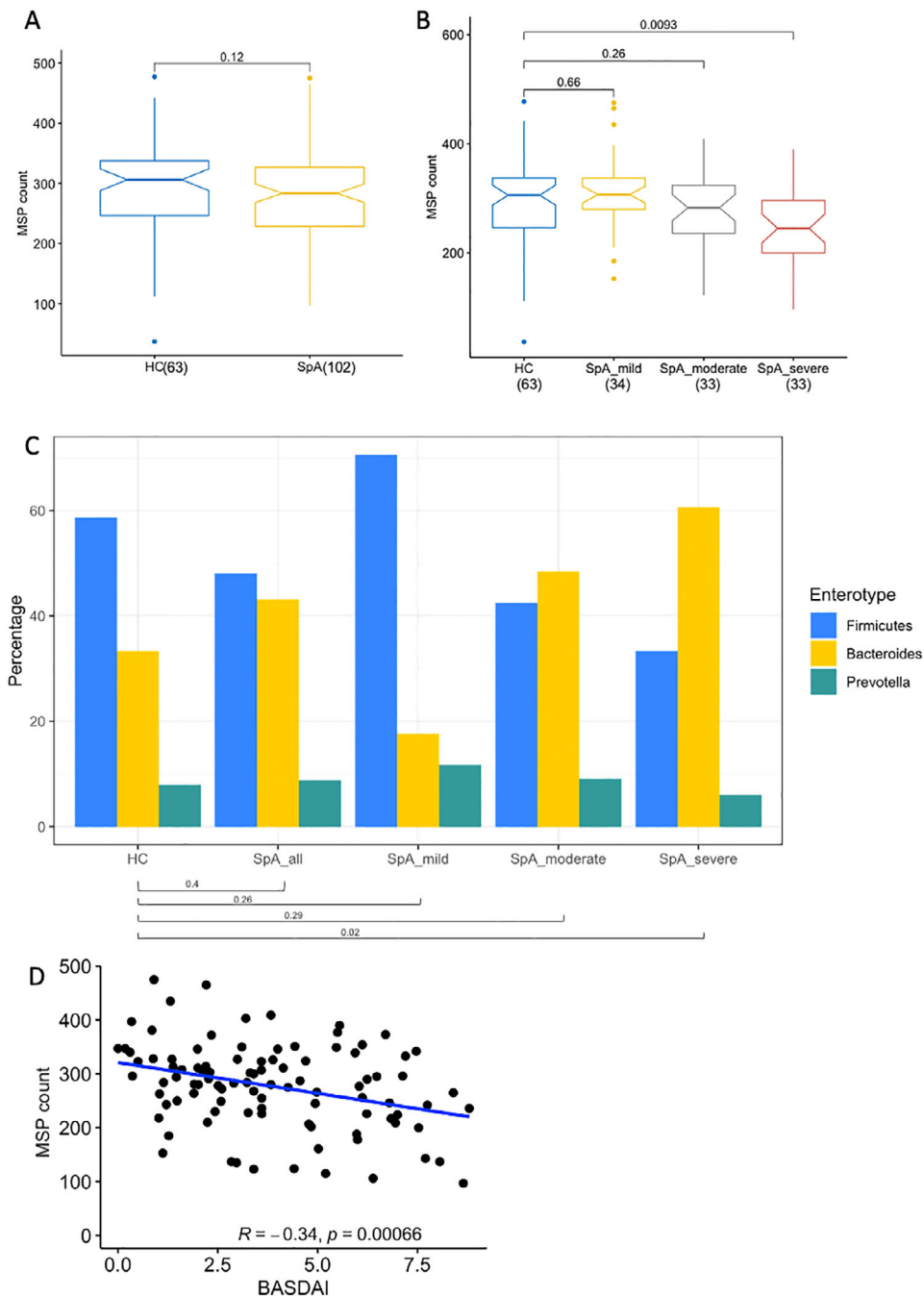


Figure 1. Gut microbiome dysbiosis in patients with spondyloarthritis (SpA) and the relation to disease activity. **A** and **B**, Metagenomic species pangenome (MSP) richness in healthy controls (HC) and SpA patients in total (**A**) and in SpA patients according to severity of disease based on the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) score tertile (**B**). Results are shown as box plots, in which lines inside the box represent the mean, boxes show the upper and lower interquartile range (IQR), and whiskers show 1.5 times the upper and lower IQRs. Circles indicate outliers. **C**, Distribution of the 3 main enterotypes among healthy controls and SpA patients, in total and according to BASDAI score tertiles. Values over horizontal lines in **A–C** indicate *P* values, determined by Wilcoxon's test in **A** and **B** and by chi-square test in **C** and **D**. **D**, Pearson's correlations between BASDAI score and MSP richness in SpA patients.

($P = 0.02$ by chi-square test) (Figure 1C). Of note, compared to healthy controls, the Bacteroides enterotype was increased in patients with severe SpA and decreased in patients with mild SpA (adjusted $P = 0.028$ by chi-square test with post hoc correction).

Taxonomic analysis of the gut microbiome dysbiosis in SpA patients. Finer analysis was performed at the MSP level (i.e., species level), resulting in 16 differentially abundant MSPs between SpA patients and healthy controls ($q \leq 0.3$) (Supplementary Table 1, <http://onlinelibrary.wiley.com/doi/10.>

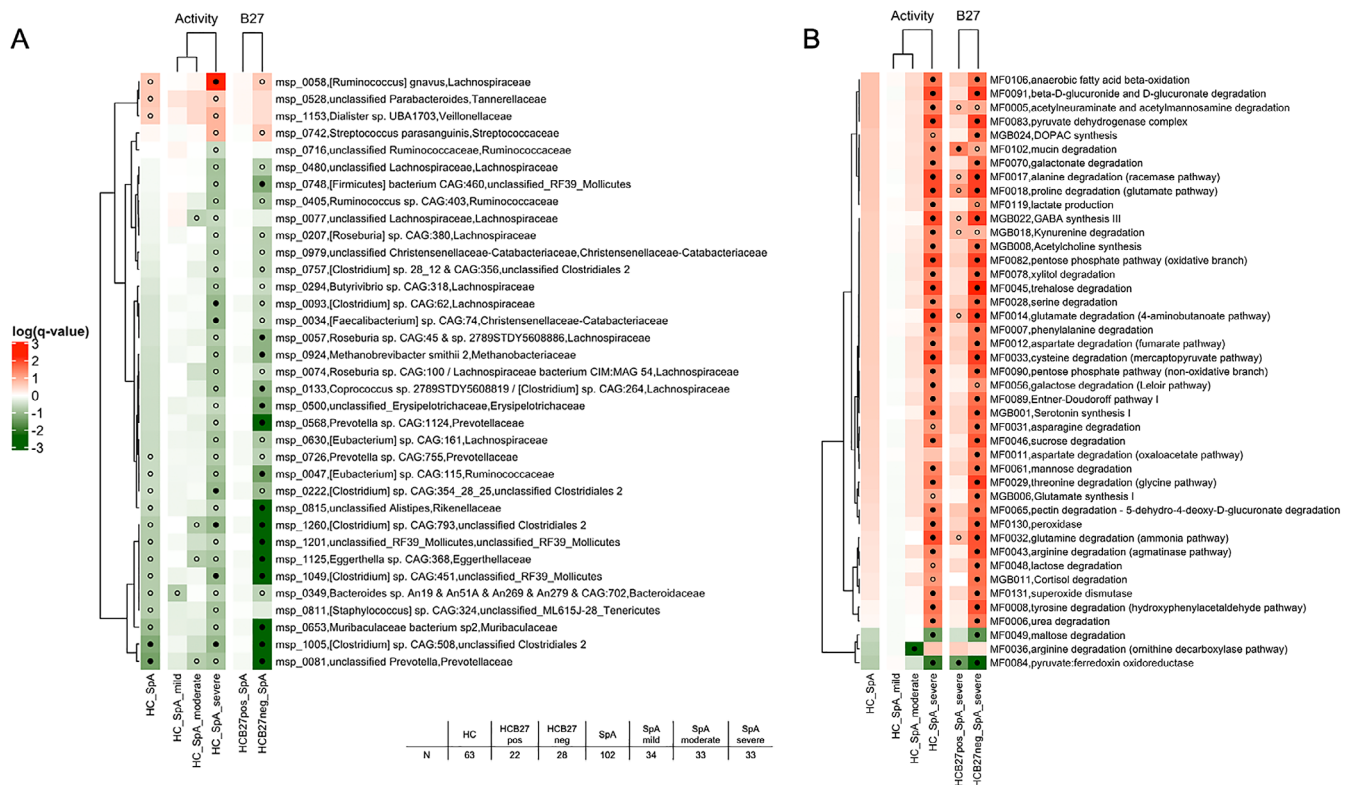


Figure 2. Summary heatmaps for comparisons of MSPs (A) and MSP functional modules (MF) (B) between SpA patients as a whole or by disease activity level and healthy controls according to HLA-B27 status. Wilcoxon's tests were performed for the following comparisons: healthy controls vs. all SpA; healthy controls vs. mild SpA; healthy controls vs. moderate SpA; healthy controls vs. severe SpA; HLA-B27-positive or negative healthy controls vs. all SpA; and HLA-B27-positive or negative healthy controls vs. severe SpA. All q-values were log-transformed, signed, and color coded according to their respective status: green = negative and enriched in the healthier condition; red = positive and enriched in the diseased condition. Those MSPs or MFs in which comparison resulted in a q-value of ≤ 0.3 are indicated with open circles; those in which comparison resulted in a q-value of ≤ 0.1 are indicated with solid circles. See Figure 1 for other definitions.

1002/art.42289). Most of these contrasted MSPs were found to be enriched in healthy controls (81%), and corresponded to species not yet isolated. The 3 MSPs highly enriched in SpA patients were *Ruminococcus gnavus*, unclassified *Dialister*, and *Parabacteroides* species (Figure 2A).

We observed that alterations in the gut microbiota composition at the species level increased in parallel with SpA activity index scores. We detected 182 differentially abundant MSPs between the BASDAI tertiles ($q \leq 0.3$ by Kruskal-Wallis test) (Supplementary Table 2, <http://onlinelibrary.wiley.com/doi/10.1002/art.42289>), representing a variation of almost 30% of the SpA microbiome composition related to BASDAI stratification. Among these MSPs, 22% were found to be differentially abundant between the mild and moderate SpA groups, and 88% were found to be differentially abundant between the mild and severe SpA groups. These progressive changes were not related to clinical characteristics other than the BASDAI scores. A set of 30 MSPs was found to be consistently enriched in patients with moderately active SpA and patients with highly active SpA and all were correlated to BASDAI score ($q \leq 0.3$ by Kruskal-Wallis test, Spearman's $\rho > 0$ for BASDAI score, and $q \leq 0.3$ by Spearman's correlation test) (Supplementary Table 2).

Within the most contrasted MSP ($q \leq 0.1$; Figure 3), most of the species enriched in the mild SpA group corresponded to unknown species from different Firmicutes families, whereas the 4 MSPs enriched in the severe SpA group were of the following known species: *Ruminococcus gnavus*, *Erysipelatoclostridium ramosum*, *Clostridiumymbiosum*, and *Clostridium bolteae*. Comparisons of each SpA patient group with healthy controls reinforced the evidence of a progressive alteration of the SpA microbiome structure corresponding to increasing disease activity, by showing a much larger difference in MSPs between patients with highly active SpA and healthy controls than between patients with mild or moderate SpA and healthy controls (Figure 2A and Supplementary Table 3, <http://onlinelibrary.wiley.com/doi/10.1002/art.42289>). We also performed a β -diversity analysis, and the difference in species-level microbial composition between the mild, moderate, and severe SpA patient groups was nearly significant ($P = 0.072$ by PERMANOVA; $R^2 = 0.025$) (Supplementary Figure 1, <http://onlinelibrary.wiley.com/doi/10.1002/art.42289>).

Functional overview of the gut microbiome dysbiosis in SpA patients. Similar to what was observed at the

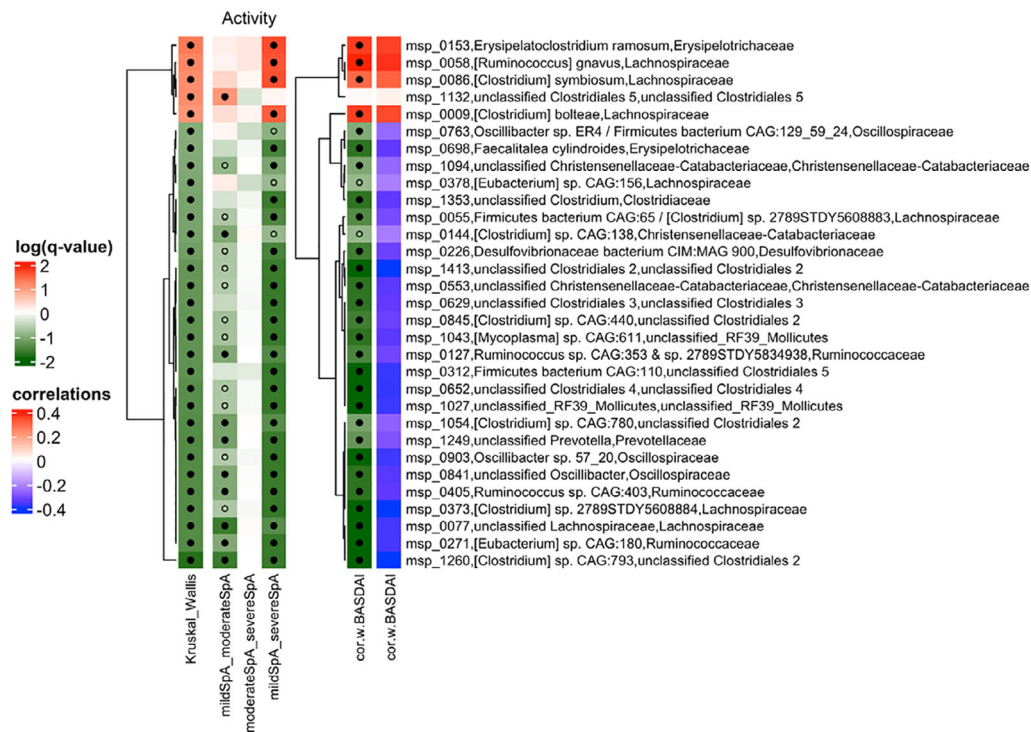


Figure 3. Summary heatmaps for comparisons of MSPs that were differentially abundant in at least 1 of the disease activity groups among SpA patients (q -values of ≤ 0.1 by Kruskal-Wallis test), and MSPs that showed a positive or negative correlation with the BASDAI score (by Pearson's correlation test). All log-transformed q -values and ρ values were signed and color coded according to their respective status: green or blue = negative and enriched in the healthier condition; red = positive and enriched in the diseased condition. Solid dots indicate q -values of ≤ 0.1 ; open dots indicate q -values of ≤ 0.3 . See Figure 1 for definitions.

species level, there was a progressive shift of contrasted microbial functional modules from mild to severe SpA (Figure 2B). There were no significant differences in microbial functions between patients with mildly active SpA and healthy controls. In patients with moderately active SpA, the module for arginine degradation via the ornithine decarboxylase pathway (MF0036) was depleted compared to healthy controls. Of note, 18 metabolic pathways differed between healthy controls and patients with severely active SpA ($q \leq 0.1$). Among these, 17 metabolic pathways were enriched in patients with highly active SpA. In particular, pathways related to the following 8 functions involved in amino acid degradation were enriched: alanine, proline, glutamate, phenylalanine, cysteine, threonine, glutamine, and tyrosine degradation. Interestingly, 2 metabolic pathways involved in energy metabolism were conversely contrasted between patients with highly active SpA and healthy controls. The pathway for pyruvate synthesis with the ferredoxin oxidoreductase enzyme was enriched in healthy controls, whereas the pyruvate dehydrogenase complex module was increased in patients with SpA. The degradation of several glycans constituents of mucin 2, including sialic acid (*N*-acetylneuraminic acid) and its *N*-acetylmannosamine precursor, *N*-acetylglucosamine as well as fucose, was increased in patients with highly active SpA ($q \leq 0.3$) (Supplementary Table 4, <http://onlinelibrary.wiley.com/doi/10.1002/art.42289>).

Influence of HLA-B27 status. We further investigated the potential influence of HLA-B27 status on the microbiome structure by comparing SpA patients and healthy controls stratified based on their HLA-B27 status. Interestingly, MSP richness was significantly decreased in SpA patients compared to HLA-B27-negative healthy controls ($n = 28$), whereas there was no significant difference between SpA patients and HLA-B27-positive healthy controls ($n = 22$) (Figure 4A). Moreover, a comparison of the microbiome compositions at the species level between SpA patients and healthy controls stratified by HLA-B27 status revealed several contrasting MSPs that were not detected in the whole-group comparisons (Figure 2A). Strikingly, the composition of the gut microbiome of SpA patients was found to be more divergent from that of HLA-B27-negative healthy controls than from that of HLA-B27-positive healthy controls (Figure 2 and Supplementary Table 5, <http://onlinelibrary.wiley.com/doi/10.1002/art.42289>), suggesting early microbiome alterations in HLA-B27-positive individuals who had not developed any SpA symptoms. Similar results were found when analyzing the samples of the 11 sibling trios. The HLA-B27-negative healthy controls had a significantly greater MSP richness than both their HLA-B27-positive healthy siblings and their siblings with SpA (Figure 4B).

We also performed a β -diversity analysis, and the differences in MSP composition between patients with SpA

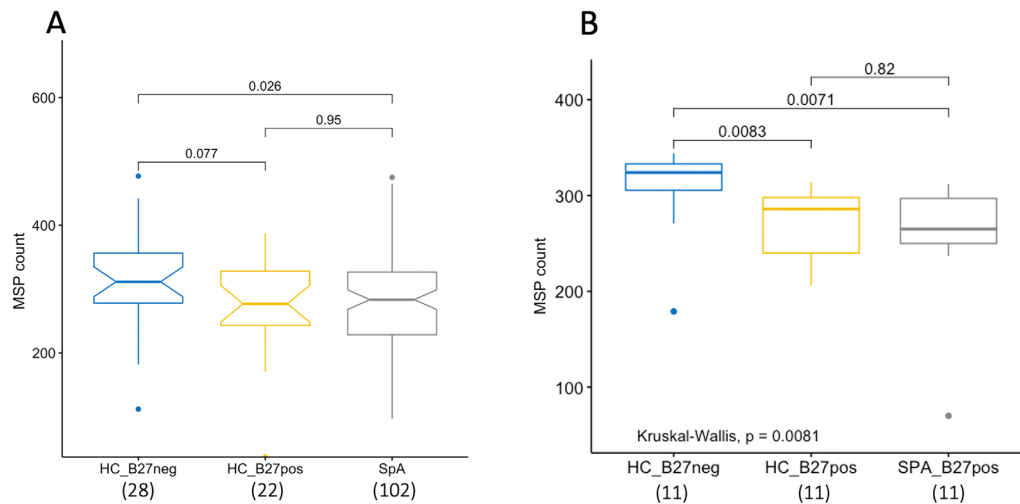


Figure 4. Impact of the HLA-B27 allele on gut microbiome dysbiosis. MSP counts in HLA-B27-negative (neg) healthy controls, HLA-B27-positive (pos) healthy controls, and SpA patients (A) and in the subcohort of family trios comprising healthy control siblings (HLA-B27-negative and HLA-B27-positive) of the HLA-B27-positive SpA patients (B). Results are shown as box plots, in which lines inside the box represent the mean, each box shows the upper and lower IQR, and whiskers show 1.5 times the upper and lower IQRs. Circles indicate outliers. *P* values shown over the horizontal lines were determined by Wilcoxon's test. See Figure 1 for other definitions. Color figure can be viewed in the online issue, which is available at <http://onlinelibrary.wiley.com/doi/10.1002/art.42289/abstract>.

and HLA-B27-positive and HLA-B27-negative healthy controls were nearly significant ($P = 0.0625$ by PERMANOVA; $R^2 = 0.017$) (Supplementary Figure 1, <http://onlinelibrary.wiley.com/doi/10.1002/art.42289>). This was not the case for the sibling trios ($P = 0.39$; data not shown), which was probably due to the smaller size of the groups.

The metabolic functions of patients with severe SpA were also more highly divergent from the metabolic functions of the HLA-B27-negative healthy controls than from the HLA-B27-positive healthy controls (Figure 2B). Similar but more striking contrasts in metabolic pathways (i.e., more statistically significant differences) were seen in comparing patients with severe SpA to HLA-B27-negative healthy controls than in comparing severe SpA patients to the healthy control group as a whole. Of note, several suggestive modules with a *q* value of ≤ 0.3 in comparison with the HLA-B27-positive healthy controls reached a *q* value of ≤ 0.1 in comparison with the HLA-B27-negative healthy controls, including those modules involved in the degradation of several amino acids (aspartate, serine, asparagine, arginine [Figure 2B]).

Impact of treatments on gut microbiota in patients

with SpA. We investigated whether our observations of changes in the microbiota could be confounded by the effects of various drugs taken by the individuals participating in the study. The vast majority of healthy controls did not take any medication ($n = 58$ patients [94%]), whereas 4 individuals were taking nonsteroidal antiinflammatory drugs (NSAIDs) ($n = 3$) or antacids ($n = 1$) and information was missing for 1 individual. For the SpA patients, 32% did not take any medication, 18% received only NSAIDs, 8% received treatment with only biologic agents, 1% received only

glucocorticoids, and 1% received only antacids. The other SpA patients (40%) received treatment with a combination of drugs.

There was no significant difference in MSP richness between SpA patients who did not receive treatment and healthy controls ($n = 280$ MSPs and $n = 293$ MSPs, respectively; $P = 0.296$ by nonparametric Wilcoxon test). This is consistent with the fact that the mean BASDAI score of SpA patients who did not receive treatment was lower than the BASDAI score of SpA patients receiving at least 1 drug (3.2 versus 4.0; $P = 0.0498$ by nonparametric Wilcoxon test).

Similar to what we observed previously, the gap between healthy controls and SpA patients not receiving treatment was wider when considering only patients with highly active disease ($n = 10$ patients with severe SpA, $n = 253$ MSPs; $P = 0.086$ by nonparametric Wilcoxon test) or when compared to HLA-B27-negative healthy controls ($n = 28$ healthy controls, $n = 313$ MSPs; $P = 0.037$ by nonparametric Wilcoxon test). There was no significantly contrasted MSP between these groups, probably because of their small size. A negative correlation between BASDAI score and MSP richness was observed (Spearman's $\rho = -0.32$; $P = 0.007$) for patients receiving treatment with at least 1 drug, and this negative correlation remained significant for patients not receiving treatment (Spearman's $\rho = -0.35$; $P = 0.049$). Overall, these results suggest that the SpA microbiome signature exists independently of the treatment patients receive.

To strengthen our conclusion, we performed a β -diversity analysis (based on the Bray-Curtis distance) that did not show any significant differences between SpA patients receiving no treatment compared to SpA patients receiving treatment with at least 1 drug ($P = 0.781$ by PERMANOVA) (Supplementary

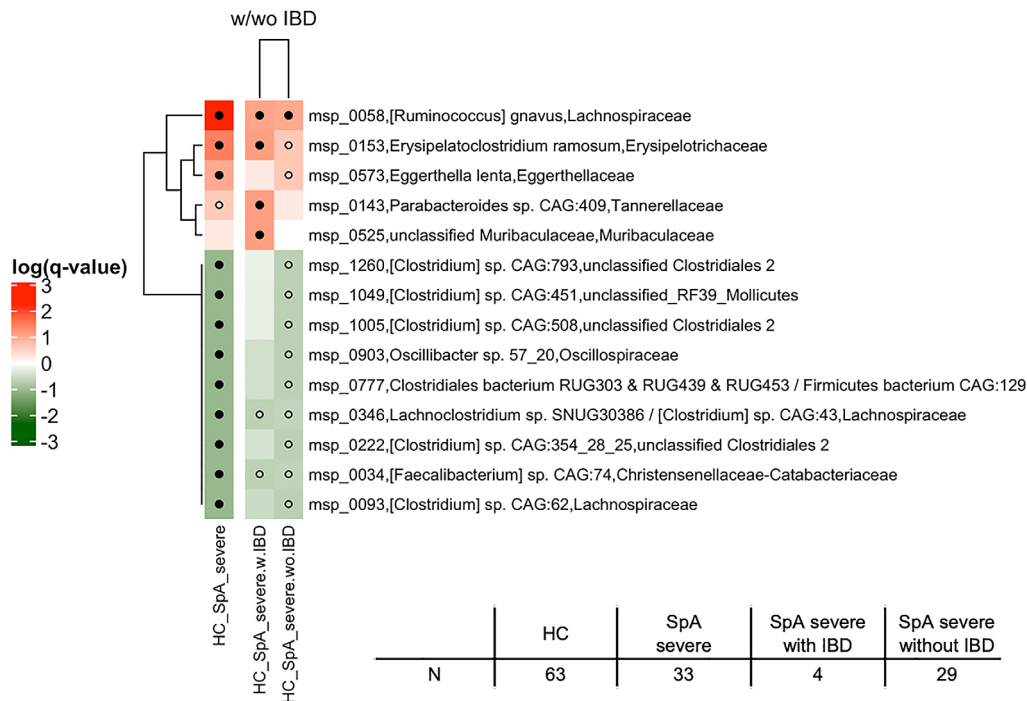


Figure 5. Gut microbiome dysbiosis in severe SpA patients with inflammatory bowel disease (w. IBD) compared to severe SpA patients without IBD (wo. IBD) and compared to healthy controls. Summary heatmaps show the MSPs ordered by q-value (determined by Wilcoxon's test) resulting from comparative analysis of the groups. All q-values of ≤ 0.1 in at least 1 of the comparisons are indicated (solid circles). Open circles indicate q-values of ≤ 0.3 . All q-values were log-transformed, signed, and color coded according to their respective status: green = negative and enriched in the healthier condition; red = positive and enriched in the diseased condition. See Figure 1 for other definitions.

Figure 1, <http://onlinelibrary.wiley.com/doi/10.1002/art.42289>. Regarding antacid usage, the β -diversity analysis showed a weak but not statistically significant difference between the microbiota of patients taking antacids compared to others ($P = 0.082$). There was no such trend for patients receiving treatment with biologic agents ($P = 0.996$).

Association of extraarticular manifestations of disease with SpA microbiome structure. We evaluated the potential impact of extraarticular SpA manifestations on the microbiome structure by comparing patients with and without histories of uveitis, psoriasis, or IBD. A history of uveitis and psoriasis did not significantly affect the abundance of any MSPs ($q > 0.97$), nor did it affect global MSP richness (Supplementary Information 2, <http://onlinelibrary.wiley.com/doi/10.1002/art.42289>). In contrast, we found 25 differentially abundant MSPs ($q \leq 0.1$) (Supplementary Table 6, <http://onlinelibrary.wiley.com/doi/10.1002/art.42289>) between SpA patients with and without a history of IBD, despite the low number of SpA patients with IBD. Members of the Ruminococcaceae family (such as *Faecalibacterium prausnitzii* and *Ruthenibacterium lactatiformans*) were decreased in SpA patients with IBD. In contrast, *R. gnavus* was enriched with a moderate level of significance ($q = 0.17$). Figure 5 shows the discriminant MSPs

between healthy controls and patients with severe SpA with or without IBD, showing that a history of IBD did not seem to significantly impact the SpA-associated variations in microbiota.

DISCUSSION

Using targeted sequencing of fecal samples, we previously reported gut dysbiosis in SpA patients which was characterized by decreased microbial diversity and increased levels of the *R. gnavus* species correlated to disease activity in a subset of patients with a history of IBD (7). One limitation of this sequencing approach is that it rarely allows taxonomic characterization at the species level. Here, using a more thorough DNA shotgun sequencing method that allows precise detection and quantification of microbial species, we could refine SpA-associated fecal dysbiosis. This resulted in 2 new major findings. First, the extent of dysbiosis appeared to correlate strikingly with disease activity. Second, the HLA-B27 genotype, which strongly predisposes to SpA, was associated with significant dysbiosis in the healthy siblings of SpA patients, so that inclusion of this group of healthy controls in the primary whole analysis resulted in somewhat blunted differences between SpA patients and healthy controls.

Dysbiosis was first characterized by a reduction of microbial diversity, a consistent finding across several microbiota studies

in SpA and other inflammatory disorders that appeared here to predominate in the most active SpA cases (7,8,10,37,38). Moreover, the proportion of patients whose enterotype was dominated by the *Bacteroides* genus was increased among those with the most active disease, a finding consistent with this enterotype being associated with low-diversity microbiota (15). At the taxonomic level, the most striking changes also concerned patients with the most active disease and consisted predominantly of a decreased abundance of several species, the most numerous and significant belonging to the Clostridiales order.

In contrast, fewer species were found to be increased in SpA patients compared to controls, among which *R. gnavus* was one of the top differentiating species, a finding consistent with our previous 16S rRNA study (7). Here, we showed that the abundance of *R. gnavus* was positively correlated with disease activity in the SpA patient group as a whole, even if its abundance appeared to be greater in those with a history of IBD. Moreover, increased levels of *R. gnavus* remained significantly associated with SpA in the comparison restricted to HLA-B27-negative participants, showing that it was associated with disease independently of HLA-B27 status. This highlights the potential role of *R. gnavus* in the pathogenesis of SpA, which is known to produce proinflammatory metabolites and which has also been associated with IBD and lupus activity (39–41). *R. gnavus* has the faculty to initiate the degradation of mucin 2 by removing sialic acid, a function that may weaken the mucus barrier (42). This is consistent with increased mucus degradation modules in the setting of active SpA.

The abundance of 6 other species was positively associated with SpA ($q < 0.3$) and/or positively correlated with BASDAI scores in our study ($q < 0.1$). Two of these species, *C. bolteae* and *C. symbiosum*, were found to be positively associated with ankylosing spondylitis in a previous shotgun sequencing study, in the whole SpA group (*C. bolteae*) or in untreated patients compared to anti-tumor necrosis factor (TNF)-treated patients and healthy controls (*C. symbiosum*), suggesting that the latter species was preferentially increased in the patients with the most active disease (9). In that same study, an unclassified *Eggerthella* (a genus that contains only a few species and has been associated with other inflammatory disorders including IBD and rheumatoid arthritis [43]) was increased in SpA patients. This observation could be consistent with an increase of *E. lenta* in the patients with the most severe disease in our present study. An increase in *Dialister* species is also consistent with the reportedly increased abundance of the *Dialister* genus in the inflamed gut mucosa-associated microbiota, in correlation with disease activity in another study (19). It is noteworthy that the 4 species that were positively correlated with BASDAI scores in our study (i.e., *R. gnavus*, *C. bolteae*, *C. symbiosum*, and *E. ramosum*) have been associated with low-richness microbiota and autoimmune diseases (15,44). It must be mentioned that *Clostridium bolteae* has been recently reclassified as *Enterocloster bolteae* (45).

Altogether, the most striking changes associated with SpA activity may reflect a shift of the gut lumen to a more oxidative milieu in the context of inflammation, conferring a survival advantage to bacteria such as the *Bacteroides* or *R. gnavus* over strict anaerobes Clostridiales (46,47). This is consistent with increased aerobic pyruvate synthesis via pyruvate dehydrogenase in the setting of SpA and the opposite change for the anaerobic ferredoxin oxidoreductase module. It is noteworthy that several of the microbiota functional changes associated with severe SpA could have detrimental consequences on the host, such as the degradation of an array of amino acids, including the 2 essential amino acids phenylalanine and threonine.

Similar to SpA patients, the HLA-B27 genotype was associated with reduced microbiota diversity in healthy controls. This was particularly significant in the analysis restricted to paired trios, in which SpA patients were matched with an HLA-B27-negative and an HLA-B27-positive sibling. Since reduced diversity likely reflects some degree of inflammation, this indicated that HLA-B27 itself might pave the way for dysbiosis, even in asymptomatic healthy controls. Consistent with this interpretation, dysbiosis was more pronounced in SpA patients with active disease than in HLA-B27-positive healthy controls. However, the observation that HLA-B27-positive healthy controls had a microbial diversity profile that was in close proximity to that of SpA patients may explain why we could not find evidence of significant taxonomic differences between these groups. A previous study demonstrated that the composition of microbiota was influenced by the carriage of the HLA-B27 allele in healthy individuals but did not indicate whether this corresponded to dysbiosis (48).

SpA patients have long been divided into subgroups based on clinical findings that mostly include histories of extraarticular manifestations. This was the reason for separately analyzing patients who had a history of psoriasis, uveitis, or IBD. The former 2 groups did not differ, whereas the third group (even if IBD was inactive in a majority of the patients at the time of sampling [8 of 13 patients]) exhibited striking differences as compared to patients without these manifestations. A majority of the bacteria species were enriched in SpA patients who had a history of IBD, whereas fewer species were decreased, including *Faecalibacterium prausnitzii*, an antiinflammatory bacteria that is known to be negatively associated with IBD (49). Interestingly, despite a noticeable impact on microbiota structure, among the species that were significantly different between patients with and those without IBD, only *R. gnavus* was found to be associated with disease activity, reinforcing the hypothesis that this species could provide a link between IBD and SpA.

The analysis of a possible confounder (especially medication) is always an interesting point as it may impact the gut microbiota composition. In our analysis, we did not observe a strong effect of the medication on the dysbiosis (Supplementary Figure 1, <http://onlinelibrary.wiley.com/doi/10.1002/art.42289>), but that

does not mean that the medication taken by the patients had no effect at all. We just may not have enough data to explore this question thoroughly. In summary, our findings in patients with SpA reveal that both genetic background and level of disease activity are likely to account for dysbiotic gut microbiota composition. Further elucidating the causal relationship between dysbiotic microbiota and SpA development may allow us to propose new therapeutic strategies.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Breban had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Acquisition of data. Leboime, Said-Nahal, Levenez, Galleron, Quinquis.

Analysis and interpretation of data. Berland, Meslier, Berreira Ibrahim, Le Chatelier, Pons, Maziers, Thirion, Gauthier, Oñate, Ehrlich, Breban.

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