Characteristics of the intestinal microbiome in ankylosing spondylitis

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Abstract. The importance of intestinal microbiota in the development of various systemic diseases has been highlighted over time. Ankylosing spondylitis (AS) is a systemic disease with a complex pathogenesis involving a particular genetic marker and distinctive environmental triggers such as a specific gut dysbiosis. We conducted a prospective case-control study which included 60 subjects from Iasi Rehabilitation Hospital: 28 AS cases and 32 healthy controls. Intestinal microbiota analysis was performed by real-time polymerase chain reaction (qPCR) in stool samples. We performed the quantitative analysis of gut microbiome, focusing both on anti-inflammatory (*Bifidobacterium, Lactobacillus, Faecalibacterium prausnitzii*) and pro-inflammatory (*Bacteroides, Escherichia coli*) species. Overall, intestinal bacterial diversity in the AS group was decreased compared to that noted in the control. A significantly decreased level of *Clostridium leptum* was observed, associated with an increased level of *Escherichia coli*. We showed correlations between laboratory tests (liver and kidney functional tests, inflammatory syndrome), the presence of HLA-B27, smoker status, the forms of AS with peripheral arthritis vs. pure axial forms and bacterial structures. No significant correlations were shown for disease activity scores, radiological stage of sacroiliitis or for body mass index. Our findings support that the intestinal microbiome in AS patients has a special signature characterized by an inflammatory status. Numerous environmental, genetical, clinical and paraclinical factors can lead to changes in gut bacterial diversity in these cases.

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Introduction

Ankylosing spondylitis (AS) is a chronic immune-mediated disease from the larger group of spondyloarthritis (SpA) characterized mainly by axial damage (1). The pathogenesis of AS includes the presence of a particular genetic marker, human leukocyte antigen (HLA)-B27, that interacts with environmental factors capable of initiating development of the disease (2-4). Many clinical studies based on animal models have attempted to explain the complex pathogenesis of AS, but to date, the pathogenic mechanism of the disease remains partially unknown (5,6).

AS can be considered a systemic condition, presenting both musculoskeletal (axial and peripheral involvement) and extraarticular manifestations, the most common being ocular, digestive, cardiovascular and pulmonary ones. The close link between the gut and SpA is well known; inflammatory bowel disease (IBD) and AS having etiopathogenic similarities and being considered distinct phenotypes of the same immuno-mediated disease (7,8). A high percentage, up to 70%, of patients with AS have subclinical intestinal inflammation and up to 10% of them will develop a clinically manifested IBD (9,10).

The importance of the intestinal microbiota in the development of various systemic diseases has been highlighted over time. It has been shown that under germ-free conditions, HLA-B27/β2-microglobulin-transgenic rats do not present any manifestation of the disease; by introducing commensal intestinal bacteria in this sterile environment, the development of both intestinal and joint inflammation was observed (11). Furthermore, in this rat model there was evidence of an impaired mucosal immunity (12) and a specific intestinal dysbiosis characterized by decreased species of *Rikenellaceae* and increased *Prevotella* species (13).

A recently published article attempts to explain the role of intestinal microbiota in the development of AS based on a literature review (14). Thus, in the first place, is the interaction between the antigen HLA-B27 and the intestinal bacterial structures that can lead to a misfolding of HLA-B27 and to an unfolded protein response of the endoplasmic reticulum (14,15). This unfolded response is responsible for the induction of pro-inflammatory proteins (16) as well as for the

phenomenon of autophagy (17). Moreover, there is a molecular mimicry between bacterial peptides presented by HLA-B27 and various self-peptides which may induce cross-immune responses (14,18).

On the other hand, changes were observed in the intestinal mucosa characterized by an increased intestinal permeability (14,19,20), by an increased secretion of A immunoglobulins (IgA) (21) and proinflammatory cytokines mediated by the activation of Th17 lymphocytes (22-25).

Taking all this into consideration, our study aimed to analyze intestinal dysbiosis in patients with AS in terms of composition, highlighting the correlations with different demographic, clinical and paraclinical features.

Patients and methods

We conducted a prospective, case-control study in Northeastern Romania which included 60 subjects. The enrolled cases were distributed as follows: 28 cases in the AS group and 32 healthy controls. The individuals included in the study were enrolled at the 1st Rheumatology Clinic of the Rehabilitation Hospital Iasi from April 2016 to March 2017. All the included cases expressed their informed consent to participate in the study. Approval was obtained from the Ethics Committees of the Grigore T Popa University of Medicine and Pharmacy and Rehabilitation Hospital Iasi from which the cases were selected.

The inclusion criteria were: Age over 18 years; signed consent by the participant to be included in the study; definite diagnosis of AS. Patients diagnosed with AS met the 1984 modified New York Diagnostic Criteria (26). All subjects included in the analysis completed a food questionnaire regarding the food components on which their diet was based during the last month. The selected group of patients with AS was as homogeneous as possible in terms of diet, excluding cases in which probiotic medication were prescribed.

Exclusion criteria consisted of: Patient refusal to participate in this study, uncertain diagnosis of AS, serious infections in the last 3 months (tuberculosis, *Clostridium difficile*), colorectal cancer, antibiotic therapy during the last 3 months.

For each case, a monitoring form was completed which included demographic data [name, age, area of origin, ethnicity, occupation, smoking status, body mass index (BMI)], year of diagnosis, family and personal pathological history, and current treatment. The group of patients with AS was divided in two clinical subgroups representing a pure axial form and a form associated with peripheral manifestations. For evaluating disease activity, the BASDAI (Bath Ankylosing Spondylitis Disease Activity Index) and BASFI (Bath Ankylosing Spondylitis Functional Index) scores were used (1,22).

Intestinal microbiota analysis was performed by real-time polymerase chain reaction (qPCR) in stool samples. From each case included in the study, 20 g of feces was obtained. The stool samples were transported (within a maximum of 4 h from sampling) to the microbiology laboratory and were frozen at a temperature of -80˚C for one week maximum until deoxyribonucleic acid (DNA) extraction.

DNA extraction from fecal samples. For DNA extraction from feces, the GenElute™ Stool DNA Isolation Kit (Sigma Aldrich; Merck KGaA) was used. DNA extraction included the following steps: i) From the 20 g of feces collected from patients, 200 mg was isolated and added to a special extraction tube (Bead Tube) along with 1 ml of Lysis Buffer L; ii) 100 μl of another special lysis solution (Lysis Additive A) was added; iii) mixed for 3 min, then centrifuged for 2 min at 14,000 rpm; iv) from the obtained supernatant, 600 μl was transferred to another DNA tube (DNAase-free microcentrifuge tube) over which 100 μl of Binding Buffer I was added and the mixture was incubated for 10 min on ice, then centrifuged for 2 min; v) from the newly obtained supernatant, 700 μl was separated in a 2-ml tube (DNAase-free microcentrifuge tube) over which 700 μl of ethanol was added and centrifuged; vi) from the ethanol clarification supernatant, 600 μl was separated, introduced into a specific DNA binding tube and centrifuged for 1 min at 6,000 rpm; vii) the DNA-binding column was mixed with 500 μl of wash buffer (SK buffer) and centrifuged for 1 min; viii) the washed DNA binding column was introduced into an Elution tube and 50 μl of Elution Buffer (E) was added, centrifuged for 2 min at 2,000 rpm, and then 1 min at 14,000 rpm.

After DNA extraction from the feces, DNA quantity and purity were checked using a NanoDrop spectrophotometer. The purity of the samples was checked using 2 wavelengths: OD $260/280_{nm}$, respectively OD $260/230_{nm}$. The entire phase which consisted in the extraction of pure DNA from feces was carried out in the microbiology laboratory under the Thermo DNA extraction hood.

qPCR. Through this study, we tried to highlight the characteristics of different populations of the gut microbiota: Certain genera, species and possibly a phylum. The qPCR reaction targeted different populations of the microbiota: Total bacteria, *Bacteroides, Bifidobacterium, Clostridium coccoides* (*XIVa*) (*C. Coccoides*), *Clostridium leptum* (*IV*) (*C. Leptum*), *Faecalibacterium prausnitzii* (*F. Prausnitzii*), *Lactobacillus, Escherichia coli* (*E. Coli*) and β-globin gene used as an internal control. The primer structures were taken from an article published by Wang *et al* (27) and verified using OligoAnalyzer 3.1 (https://eu.idtdna.com). In addiion, the primer annealing temperature was checked. Table I shows the primer structures and the annealing temperatures.

The qPCR reaction was performed using SYBR Green intercalary fluorochromes method that only bind to double-stranded DNA molecules and included the following steps: i) The amplification reaction was carried out at a final volume of 25 μl containing: 9.8 μl SYBR Mix, 0.5 μl of each primer at a final concentration of 0.2 μM, 0.5 μl fluorochrome ROX (5-carboxy-X-rhodamine), 5 μl of bacterial DNA and ultra-pure water to a volume of 20 μl (9.8 μl); ii) 1 cycle at 95˚C for 10 min; iii) 40 cycles at 95˚C for 10 sec; iv) 30 sec at normalization temperature; and v) 30 sec at 72˚C, the annealing temperature.

In order to reduce the quantitative error of detected bacteria and to characterize changes in bacterial copies, abundance of 16S rRNA gene was calculated from standard curves. Specific bacterial groups are expressed as a percentage of total bacteria determined by universal primers. The standard curve was constructed from decimal dilutions of the 16S rRNA amplicon using reference strains for each bacterial target. Based on

Table I. Bacterial-specific 16S rRNA primers and the annealing temperatures.

Bacterial species	Primer direction	Sequence (5' to 3')	Annealing temp. (˚C)
All bacteria	F	ACTCCTACGGGAGGCAGCAGT	61
	R	GTATTACCGCGGCTGCTGGCAC	
Bacteroides	F	GTCAGTTGTGAAAGTTTGC	61.5
	R	CAATCGGGAGTTCTTCGTG	
Bifidobacterium	F	AGGGTTCGATTCTGCTCAG	62
	R	CATCCGGCATTACCACCC	
C. coccoides (XIVa)	F	AAATGACGGTACCTGACTAA	60.7
	R	CTTTGAGTTTCATTCTTGCGAA	
C. leptum (IV)	F	GTTGACAAAACGGAGGAAGG	60
	R	GACGGGCGGTGTGTACAA	
F. prausnitzii	F	AGATGGCCTCGCGTCCGA	61.5
	R	CCGAAGACCTTCTTCCTCC	
Lactobacillus	F	GCAGCAGTAGGGAATCTTCCA	61.5
	R	GCATTYCACCGCTACACATG	
E. coli	F	GTTAATACCTTTGCTCATTGA	61
	R	ACCAGGGTATCTAATCCTGTT	
β-globin	F	CAACTTCATCCACGTTCACC	-
	R	GAAGAGCCAAGGACAGGTAC	

F, forward; R, reverse.

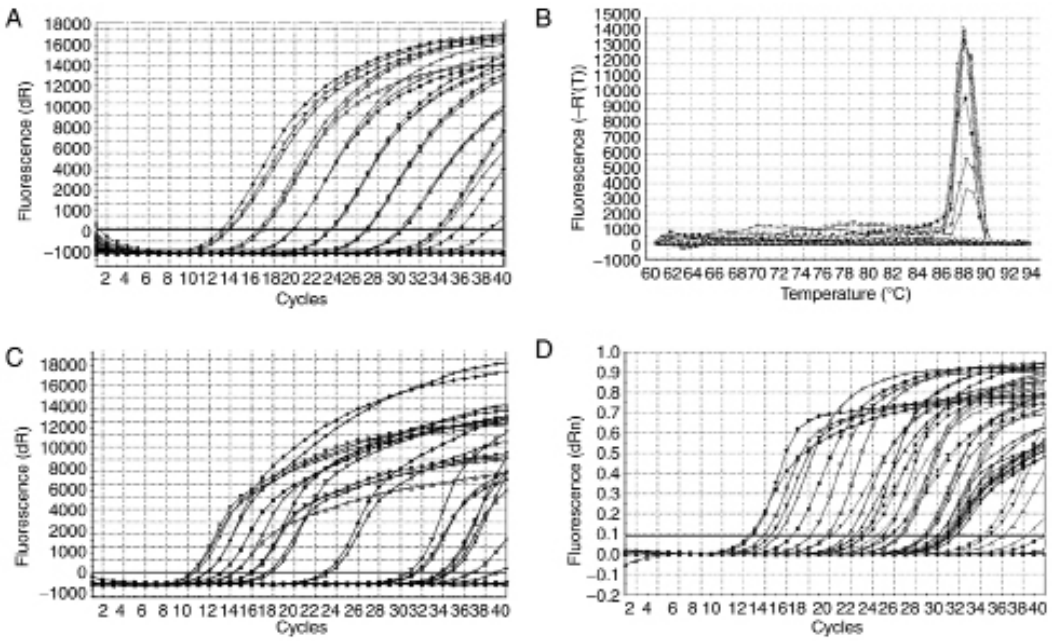

Figure 1. (A) Standard curve of the amplification (triplicates). (B) Dissociation curve. (C) Amplification curve for *E. coli*. (D) Amplification curve for *Bacteroides*.

this standard curve, all sample amplification was performed (Fig. 1A). The dissociation and amplification curves confirmed that DNA amplification occurred specifically (Fig. 1B-D).

Following extraction, pure DNA was obtained by spectrophotometer analysis (OD 260/280 nm, OD 260/230 nm). Data on DNA concentration, quantity and purity are documented in Table II.

Statistical analysis. The obtained data were centralized in the SPSS 22.0 database (IBM Corp.). Statistical analysis used both descriptive and analytical methods at 95% significance (CI 95% CI). The statistical tests used included ANOVA and Chi-square tests, linear regression, and odds ratio. For comparisons between groups having a non-linear distribution, Mann-Whitney U test and Kruskal-Wallis method were used.

Table II. Concentration, quantity and purity of the extracted DNA.

DNA	Cases	N	Median	Standard deviation	95% CI	
					Min	Max
Concentration (ng/μl)	AS	28	48.61	24.81	38.98	58.23
	Control	32	36.86	26.34	27.36	46.36
Quantity (μg)	AS	28	2.91	1.48	2.33	3.49
	Control	32	2.21	1.58	1.64	2.78
Purity 260/280 nm	AS	28	1.97	0.083	1.94	2.008
	Control	32	2.03	0.17	1.96	2.09
Purity 260/230 nm	AS	28	0.48	0.35	0.34	0.62
	Control	32	0.44	0.33	0.32	0.56

AS, ankylosing spondylitis; DNA, deoxyribonucleic acid; CI, confidence interval.

A P-value less than 0.05 (P<0.05) was considered statistically significant.

Results

Characteristics of the study group. The demographic, clinical and paraclinical characteristics of the cases included in this study are presented in Table III. The compared groups were homogeneous in terms of area of origin, smoking, social status and BMI. We observed a higher percentage of male sex and a younger age in the AS group. All cases were overweight.

Characteristics of the intestinal dysbiosis in the study groups. The microbiota analysis was performed for each of the two groups, being calculated quantitatively. The data in Table IV show the quantitative results expressed logarithmically.

In order to be able to compare the bacterial groups according to each arm, the non-parametric Kruskal Wallis test was used. Thus, applying the test for all combinations of 2 groups, the following results were found. Statistically significant data were found only for *C. leptum* (P=0.019) and *E. coli* (P=0.013). In cases with AS, a significantly decreased level of *C. leptum* was observed, associated with an increased level of *E. coli*. The other analyzed microbial populations did not show significant statistical differences with the control arm. The group of cases with AS also showed a decreased microbial diversity than the control group, but without any statistical value (Fig. 2).

Using the Spearman correlation coefficient, significant correlations were found between paraclinical tests (liver and kidney function, inflammatory syndrome) and bacterial species (Table V). Thus, ESR and CRP were inversely correlated with the level of *Bacteroides* and directly proportional to *C. coccoides* and *C. leptum*. Serum transaminases levels were directly proportional to total bacteria (P=0.001). Only the ALT level was inversely correlated with *Bifidobacterium* (P=0.006). Creatinine was inversely correlated with *Bacteroides* (P=0.045) and *E. coli* (P=0.027) and directly proportional to *C. coccoides* (P<0.001) and *C. leptum* (P=0.005).

No correlations were found between the degree of radiological sacroiliitis and bacterial groups (P=0.053, Kruskal-Wallis test) or between BMI and bacterial populations (P=0.366).

Of the 28 cases with AS, 22 patients tested positive for the antigen HLA-B27 (human leucocyte antigen B27). Following the statistical analysis (Mann-Whitney test), significant correlations were highlighted between HLA-B27 and *Lactobacillus* (P=0.027) and *E. coli* (P=0.004) (Table VI).

Other significant data were recorded in relation to smoker status. Thus, correlations between *C. coccoides* (P=0.033) and *Bifidobacterium* (P=0.006) were found in the arm of smokers diagnosed with AS. The level of *C. coccoides* was decreased, while *Bifidobacterium* was increased. On the other hand, correlations with a decreased level of *F. prausnitzii* (P=0.027) were found for active smokers in the control group (Table VII).

Using the Spearman correlations, we aimed to ascertain whether there were correlations between the disease activity quantified by BASDAI and BASFI scores and the bacterial populations. We did not find statistically significant data between these scores and the bacteria species (Table VIII). It should be mentioned that the patients included in the analysis had a moderate disease activity, with an average BASDAI of 4.83 (3.87-5.79 95% CI) and an average BASFI of 9.11 (4.44-13.78 95% CI).

According to the form of the disease, patients with AS were divided into an axial and a form with peripheral arthritis. Using the Mann-Whitney statistical test, significant data were found only for the *Bifidobacterium* bacterial group (P=0.035). Thus, in the peripheral form of AS there was a significant decrease of *Bifidobacterium* compared to the axial form (Table IX).

Regarding the treatment followed by patients with AS, all of them were receiving drug therapy at the time of enrollment in the study. Thus, most cases (n=19; 67.85%) were receiving anti-TNFα treatment, 5 cases (17.85%) were receiving sulfasalazine (SSZ) therapy, and 4 patients (14.28%) were receiving only symptomatic treatment with nonsteroidal anti-inflammatory drugs (NSAIDs). Because the number of cases was relatively small for each therapy, the statistical analysis did not have consistency. Thus, we grouped the

Table III. Demographic characteristics of the AS and control groups.

Characteristic	Subcategory	AS (n=28)	Control (n=32)
Sex, n (%)	Female	11 (39.3)	20 (62.5)
	Male	17 (60.7)	12 (37.5)
Age, years	Mean (SD)	52.1 (13.6)	61.5 (10)
	Range	46-57	57-65
Area of origin, n (%)	Urban	20 (71.4)	16 (50)
	Rural	8 (28.6)	16 (50)
Smoking status, n (%)	Smokers	10 (35.7)	12 (37.5)
	Ex-smokers	2 (7.1)	6 (18.7)
	Non-smokers	16 (57.2)	14 (43.8)
BMI (kg/m^2)	Mean (SD)	28.08 (5.2)	26.9 (3.7)
	Range	26.04-30.1	25.6-28.3
Form of disease (n,%)	Axial	17 (60.7)	NA
	Peripheral	11 (39.3)	NA
BASDAI	Mean	4.83	NA
	CI 95%	3.87-5.79	NA
BASFI	Mean	9.11	NA
	CI 95%	4.44-13.78	NA
Hemoglobin (g/dl)	Mean (SD)	13.01 (0.9)	13.5 (1.6)
	Range	12.6-13.3	12.9-14.1
Iron (μg/dl)	Mean (SD)	93.8 (31.1)	77.7 (22.6)
	Range	81.7-105.8	69.6-85.9
Leukocytes ($/mm^3x10^3$)	Mean (SD)	7.1 (1.8)	6.5 (1.03)
	Range	6.4-7.8	6.1-6.8
Thrombocytes ($/mm^3x10^3$)	Mean (SD)	293.6 (75.6)	250.1 (35.5)
	Range	264.2-322.9	237.2-262.9
ESR (mm/h)	Mean (SD)	23.4 (12.7)	16.4 (10.5)
	Range	18.5-28.3	12.6-20.2
CRP (mg/dl)	Mean (SD)	0.9 (1.3)	0.8 (0.9)
	Range	0.4-1.4	0.4-1.1
AST (IU/l)	Mean (SD)	28.6 (16.4)	24.3 (15.9)
	Range	22.2-35.07	18.5-30.05
ALT (IU/l)	Mean (SD)	24.9 (12.3)	27.5 (15.8)
	Range	20.1-29.7	21.8-33.2
GGT (U/l)	Mean (SD)	42.9 (23.7)	32.8 (19.01)
	Range	33.7-52.1	26.02-39.7
Total serum proteins (g/dl)	Mean (SD)	7.2 (0.4)	7.2 (0.4)
	Range	7.07-7.4	7.06-7.4
Creatinine (mg/dl)	Mean (SD)	0.9 (0.2)	0.9 (0.1)
	Range	0.8-1	0.8-0.9

AS, ankylosing spondylitis; SD, standard deviation; BMI, body mass index; ESR, erythrocyte sedimentation rate; CRP, C reactive protein; AST, aspartate transaminase; ALT, alanine transaminase; GGT, γ-glutamyltransferase; NA, non-applicable; CI, confidence interval; BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; BASFI, Bath Ankylosing Spondylitis Functional Index.

patients as follows: the group that had NSAID treatment (n=4) and those on immunosuppressive treatment (SSZ + antiTNFα; n=24). It was observed that patients who were treated only with NSAIDs showed a decrease in bacterial diversity (P=0.560), in *Bifidobacterium* (P<0.001) and *Lactobacillus* (P=0.382) followed by an increase in *Bacteroides* (P<0.001), *C. coccoides* (P=0.005), *C. leptum* (P<0.001), *F. prausnitzii* (P=0.001) and *E. coli* (P=0.001). All these data are presented in Table X and Fig. 3.

Discussion

The present study analyzed different populations of the gut microbiome in patients with ankylosing spondylitis (AS):

Table IV. Quantitative characteristics of the microbial populations analyzed in the AS and control groups.

Bacterial population	Study group	Mean	Standard deviation	95% CI	
				Min	Max
All bacteria	AS	2.18E+10	2.27E+10	1.3E+10	3.06E+10
	Control	4.13E+10	3.77E+10	2.77E+10	5.49E+10
Bacteroides	AS	3.57E+09	4.37E+09	1.88E+09	5.26E+09
	Control	7.5E+09	6.96E+09	4.99E+09	1.00E+10
C. coccoides	AS	5.13E+09	5.47E+09	3.01E+09	7.25E+09
	Control	1.28E+10	1.49E+10	7.44E+09	1.82E+10
C. leptum	AS	2.72E+09	2.57E+09	1.72E+09	3.72E+09
	Control	9.64E+09	1.19E+10	5.36E+09	1.39E+10
F. prausnitzii	AS	2.16E+09	2.73E+09	1.1E+09	3.22E+09
	Control	5.51E+09	5.13E+09	3.66E+09	7.36E+09
Bifidobacterium	AS	3.72E+08	3.93E+08	2.2E+08	5.25E+08
	Control	4.25E+08	3.11E+08	3.13E+08	5.38E+08
Lactobacillus	AS	5.21E+08	5.63E+08	3.02E+08	7.39E+08
	Control	8.07E+08	7.72E+08	5.29E+08	1.09E+09
E. coli	AS	1.06E+09	1.09E+09	6.41E+08	1.48E+09
	Control	8.72E+08	9.43E+08	5.32E+08	1.21E+09

CI, confidence interval; AS, ankylosing spondylitis.

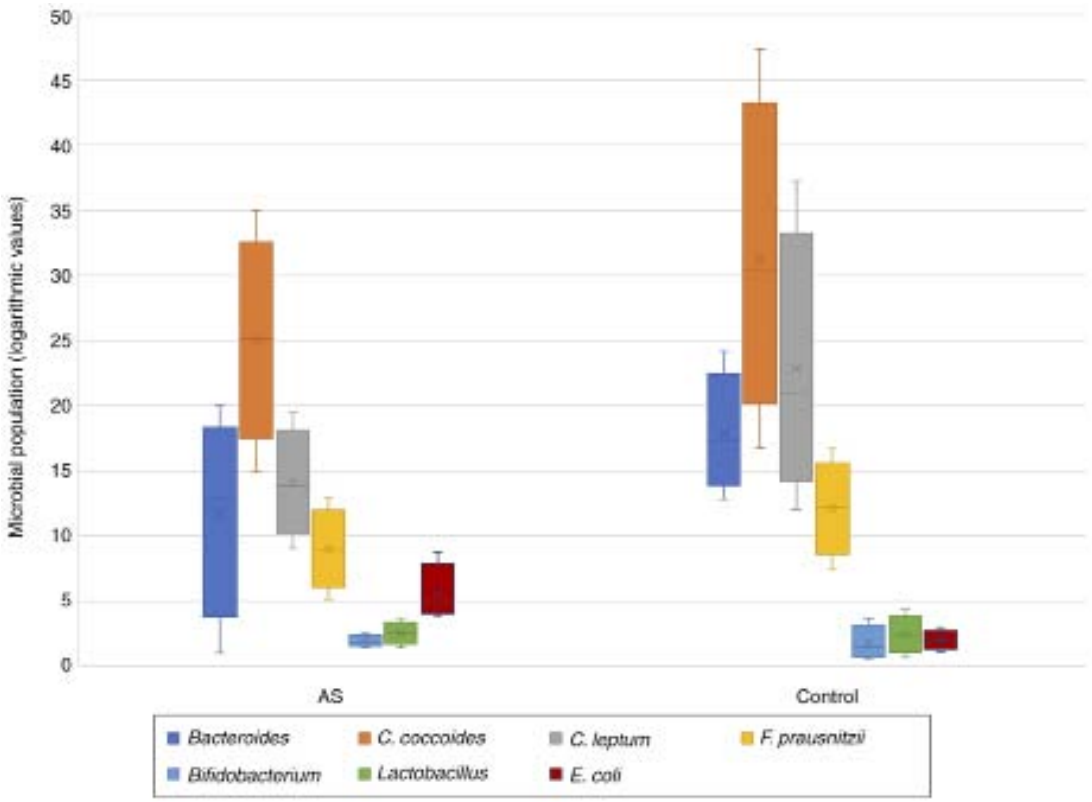

Figure 2. Microbial populations highlighted in the study cases. AS, ankylosing spondylitis.

Certain species, genera and possibly a phylum compared to a control group formed by healthy individuals. The two study arms were carefully selected to be as homogeneous as possible. Analysis of the intestinal microbiome was performed in the feces using the qPCR technique. We took into consideration the quantitative analysis of the microbiome, focusing

Table V. Correlations between bacterial species and paraclinical tests.

Test	Correlation	All bacteria	*Bacteroides %*	*C. coccoides %*	*C. leptum %*	*F. prausnitzii %*	*Bifidobacterium %*	*Lactobacillus %*	*E. coli %*
Hb	ρ	0.172	0.128	-0.077	-0.062	-0.028	0.048	0.008	-0.020
	P-value	0.056	0.158	0.397	0.493	0.759	0.594	0.928	0.762
Leukocytes	ρ	-0.061	0.200	-0.239	-0.225	-0.152	0.182	0.139	0.156
	P-value	0.498	**0.026**	**0.008**	**0.012**	0.091	**0.043**	0.123	0.083
Thrombocytes	ρ	-0.07	0.063	-0.157	-0.101	-0.125	0.129	0.092	0.132
	P-value	0.441	0.486	0.082	0.265	0.166	0.154	0.311	0.144
Iron	P	0.133	-0.192	-0.039	0.075	0.168	-0.067	-0.018	-0.210
	P-value	0.139	**0.033**	0.665	0.405	0.063	0.463	0.841	**0.015**
ESR	ρ	0.081	-0.479	0.380	0.482	0.507	-0.162	-0.25	-0.44
	P-value	0.369	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.073	**0.005**	**<0.001**
CRP	ρ	-0.103	-0.18	0.199	0.325	0.095	-0.02	-0.015	-0.090
	P-value	0.253	**0.045**	**0.026**	**<0.001**	0.292	0.828	0.868	0.300
AST	ρ	0.329	0.032	0.026	0.057	0.120	-0.120	-0.169	-0.170
	P-value	**<0.001**	0.723	0.776	0.529	0.183	0.183	0.061	0.052
ALT	ρ	0.307	-0.009	0.082	0.101	0.171	-0.247	-0.159	-0.210
	P-value	**0.001**	0.921	0.364	0.262	0.057	**0.006**	0.077	**0.017**
GGT	ρ	0.07	-0.151	0.081	0.161	0.228	-0.157	-0.226	-0.160
	P-value	0.437	0.094	0.372	0.074	**0.011**	0.081	**0.012**	0.076
Total proteins	ρ	-0.018	0.275	-0.154	-0.227	-0.238	0.071	-0.046	0.219
	P-value	0.842	**0.002**	0.088	**0.011**	**0.008**	0.435	0.611	**0.015**
Creatinine	ρ	0.159	-0.18	0.342	0.251	0.132	-0.136	-0.137	-0.190
	P-value	0.077	**0.045**	**<0.001**	**0.005**	0.144	0.132	0.130	**0.027**

ESR, erythrocytes sedimentation rate; CRP, C reactive protein; AST, aspartate transaminase; ALT, alanine transaminase; GGT, γ-glutamyltransferase; ϱ, Spearman correlation. P-values indicating significant results are shown in bold print.

Table VI. Correlations between the antigen HLA B27 and microbial populations in the AS group.

Test	Total bacteria	*Bacteroides %*	*C. coccoides %*	*C. leptum %*	*F. prausnitzii %*	*Bifidobacterium %*	*Lactobacillus %*	*E. coli %*
Mann-Whitney U	46.0	50.0	59.0	58.0	41.0	56.0	**26.5**	**15.0**
Wilcoxon W	299.0	71.0	80.0	79.0	294.0	309.0	**279.5**	**268.0**
Z	-1.123	-0.897	-0.392	-0.448	-1.401	-0.561	**-2.216**	**-2.86**
P-value	0.262	0.370	0.695	0.654	0.161	0.575	**0.027**	**0.004**

AS, ankylosing spondylitis. P-values indicating significant results are shown in bold print.

Table VII. Correlations between smoking status and microbial populations.

Arm	Test	Total bacteria	*Bacteroides %*	*C. coccoides %*	*C. leptum %*	*F. prausnitzii %*	*Bifidobacterium %*	*Lactobacillus %*	*E coli %*
Control	Z	-1.066	-1.598	-0.228	-0.228	-2.206	-1.598	-1.446	-1.293
	P-value	0.286	0.110	0.819	0.819	**0.027**	0.110	0.148	0.196
AS	Z	-0.768	-1.394	-2.138	-1.208	-0.232	-2.744	-1.465	-1.488
	P-value	0.443	0.163	**0.033**	0.227	0.816	**0.006**	0.143	0.137

AS, ankylosing spondylitis. P-values indicating significant results are shown in bold print.

Table VIII. Correlations between disease activity and microbial populations in the AS group.

Score	Microbial population	Spearman coefficient	P-value
BASDAI	All bacteria	-0.336	0.080
	Bacteroides %	-0.371	0.052
	C. coccoides %	0.111	0.573
	C. leptum %	0.340	0.077
	F. prausnitzii %	-0.371	0.052
	Bifidobacterium %	0.183	0.351
	Lactobacillus %	0.331	0.085
	E. coli %	0.309	0.110
BASFI	All bacteria	-0.018	0.927
	Bacteroides %	-0.061	0.758
	C. coccoides %	0.053	0.788
	C. leptum %	0.010	0.960
	F. prausnitzii %	0.104	0.597
	Bifidobacterium %	0.048	0.809
	Lactobacillus %	0.019	0.924
	E. coli %	-0.015	0.941

AS, ankylosing spondylitis; BASFI, Bath Ankylosing Spondylitis Functional Index; BASDAI, Bath Ankylosing Spondylitis Disease Activity Index.

Table IX. Correlations between microbial populations and the form of AS.

Microbial population	Form of AS	Mean rank	Sum of ranks	P-value
Total bacteria	Axial	23.94	766.00	0.451
	Peripheral	20.69	269.00	
Bacteroides %	Axial	22.44	718.00	0.652
	Peripheral	24.38	317.00	
C. coccoides %	Axial	23.41	749.00	0.744
	Peripheral	22.00	286.00	
C. leptum %	Axial	23.19	742.00	0.880
	Peripheral	22.54	293.00	
F. prausnitzii %	Axial	23.09	739.00	0.940
	Peripheral	22.77	296.00	
Bifidobacterium %	Axial	**25.63**	820.00	**0.035**
	Peripheral	**16.54**	215.00	
Lactobacillus %	Axial	23.80	761.50	0.522
	Peripheral	21.04	273.50	
E. coli %	Axial	23.66	757.00	0.598
	Peripheral	21.38	278.00	

AS, ankylosing spondylitis. P-values indicating significant results are shown in bold print.

both on bacterial structures having an anti-inflammatory role (*Bifidobacterium, Lactobacillus, F. prausnitzii*), and on some with pro-inflammatory actions that favor intestinal and systemic inflammation *(Bacteroides, E. coli*).

Following the data analysis, intestinal bacterial diversity in the AS group was decreased compared to the control. Significant data were highlighted only for 2 bacterial species. A significant numerical increase was observed for *E. coli* associated with a decrease in *C. leptum*.

The data in the literature regarding gut dysbiosis in AS are contradictory. Our results are in agreement with other studies. Zhang *et al* (28) investigated fecal microbiota in 103 cases of AS and concluded that alpha diversity in these cases was no different from the control group. Similar to our results, they

Table X. Quantitative analysis of the microbiome according to the treatment followed by patients with AS.

Microbial population	Treatment	Mean	95% CI		Median	Standard deviation	P-value
			Min	Max			
Total bacteria	IS	2.31E+10	7.36E+09	3.88E+10	1.31E+10	2.48E+10	0.560
	NSAIDs	1.82E+10	1.44E+10	2.2E+10	1.66E+10	5.95E+09	
Bacteroides %	IS	26.90	24.51	29.29	28.06	3.76	**<0.001**
	NSAIDs	40.91	40.41	41.42	41.00	0.79	
C. coccoides %	IS	19.51	17.18	21.84	18.06	3.66	**0.005**
	NSAIDs	24.09	23.66	24.53	23.94	0.69	
C. leptum %	IS	7.79	7.12	8.47	7.42	1.06	**<0.001**
	NSAIDs	12.37	11.48	13.26	12.20	1.40	
F. prausnitzii %	IS	3.09	2.54	3.63	3.03	0.85	**0.001**
	NSAIDs	4.46	4.11	4.81	4.19	0.55	
Bifidobacterium %	IS	3.33	2.87	3.79	2.97	0.72	**<0.001**
	NSAIDs	1.48	1.28	1.68	1.50	0.31	
Lactobacillus %	IS	3.10	2.64	3.57	3.32	0.72	0.382
	NSAIDs	2.95	2.66	3.24	3.00	0.45	
E. coli %	IS	14.37	13.48	15.26	14.20	1.40	**0.001**
	NSAIDs	20.52	18.40	22.64	21.81	3.33	

AS, ankylosing spondylitis; NSAIDs, nonsteroidal anti-inflammatory drugs; CI, confidence interval; IS, immunosuppressive. P-values indicating significant results are shown in bold print.

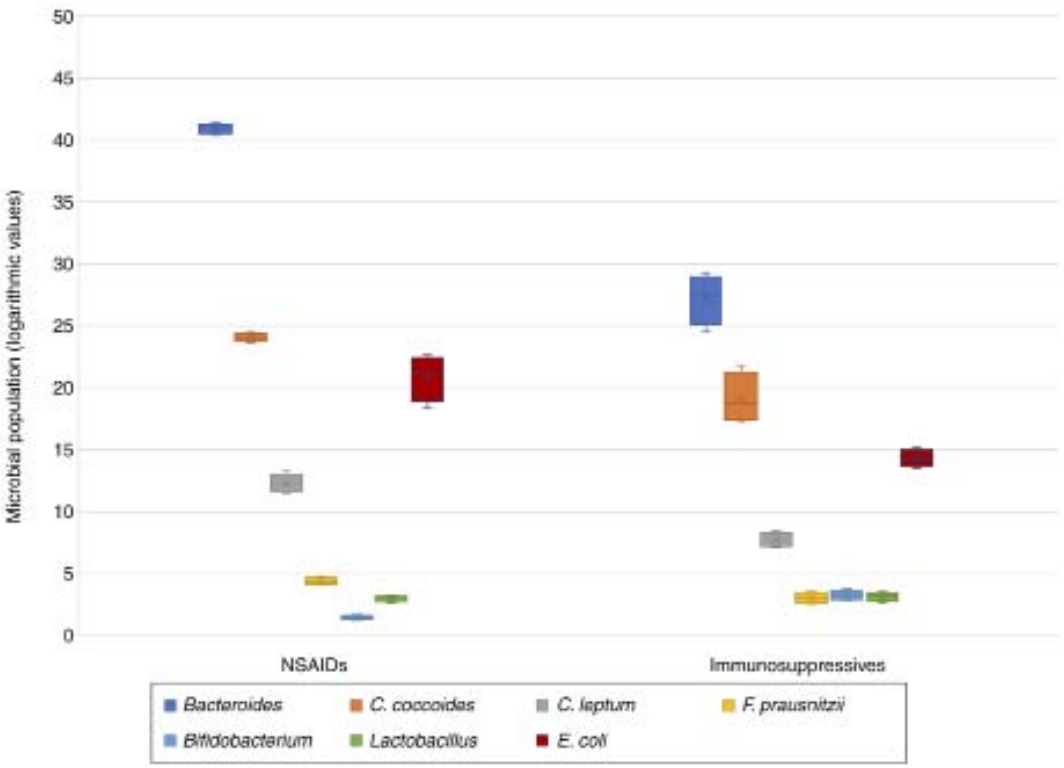

Figure 3. Quantitative analysis of the microbiome according to the treatment followed by the patients with AS. AS, ankylosing spondylitis; NSAIDs, nonsteroidal anti-inflammatory drugs.

showed a decrease in *Clostridium_XIVb*, but an increase in *Bacteroides*. Breban *et al* (29) analyzed the intestinal microbiota in 2 cohorts: rheumatoid arthritis and SA compared to a control group. They showed an overall decreased microbial diversity in both groups, followed by a significant increase in *Ruminococcus gnavus* in SA.

Numerous data in the literature support this reduction in intestinal microbial diversity in patients with chronic inflammatory autoimmune diseases (30,31). However, there are several studies that have shown an increase in intestinal bacteria in patients with SA, especially in those having associated intestinal inflammation (10,32).

We demonstrated a close link between liver and kidney function and the analyzed bacteria. Serum transaminases levels were directly proportional to total bacteria. Only the ALT level was inversely correlated with *Bifidobacterium*. Serum creatinine was inversely correlated with *Bacteroides* and *E. coli* and directly proportional with *C. coccoides* and *C. leptum*.

The close connection between the intestine and the liver is a certainty, with many authors reporting about the presence of a liver-intestine axis (33-36). The composition of the intestinal microbiota can modulate chronic liver diseases by i) the production of metabolites, by altering the integrity of the mucosal barrier, ii) by the portal system or iii) by the liver-intestinal immune link (37).

On the other hand, important studies have highlighted the existence of a 'colo-renal system' that can influence each other. The intestinal microbiota can alter renal function, contributing to the development of many pathologies. There is a communication between various intestinal bacterial groups and the cells of the renal parenchyma, which causes a disturbance of the normal renal molecular processes, leading to the appearance of kidney diseases (38-40).

The presence of systemic inflammation was quantified by measuring the acute phase reactants: ESR and CRP. Most often, the normal values of these biological constants are associated with low activity or remission of inflammatory joint diseases. Moreover, they are directly proportional to the disease activity quantified in our study by BASDAI and BASFI scores. Following analysis, ESR and CRP were inversely correlated with the level of *Bacteroides* and directly proportional to *C. coccoides* and *C. leptum*.

Two studies that are part of the RISTOMED project (Impact of personalized diet and probiotic supplementation on inflammation, nutritional parameters and intestinal microbiota) (41,42) analyzed the correlations between the composition of the intestinal microbiota and various clinical parameters, including inflammatory markers. The study included 125 cases divided into a group with low inflammation and a group with moderate-high inflammation. At the level of intestinal microbiota, the *Bifidobacterium* and *Clostridium group IV* species were analyzed. The results showed, in the arm with low inflammation, a decrease in *Bifidobacterium* followed by an increase in *Clostridium IV* and the strong correlation with the level of acute phase reactants.

Regarding disease activity, we did not find significant correlations between BASDAI and BASFI scores and the bacteria species. Our results are consistent with other studies that have not shown an association between disease activity or function and intestinal dysbiosis (43). Moreover, these studies showed similarities regarding the highlighted bacterial species, namely an increase in *E. coli* and a decrease in *Clostridium, F. prausnitzii* and *Bacteroides* (4,44-46). On the other hand, many data support the close link between the activity of AS and the composition of the intestinal microbiota (29,32) and between gut inflammation and disease activity in AS (47,48).

Considering the genetic predisposition, significant correlations were found between the antigen HLA-B27 and *Lactobacillus*, respectively *E. coli*. The levels of *Lactobacillus* and *E. coli* were decreased in patients tested positive for the HLA B27 antigen. The ability of this antigen to modulate the intestinal microbiota in transgenic laboratory animals has been demonstrated and published recently (13). Intestinal dysbiosis in HLA-B27-positive laboratory animals was characterized by a decrease in *Firmicutes* followed by a significant increase in *Proteobacteria* group, of which *E. coli* takes part (49). Moreover, Breban *et al* (29) showed a different intestinal dysbiosis in members of the same family depending on the presence or absence of the HLA-B27 antigen.

Further correlations between gut microbiota composition and clinical parameters included the following data: The BMI, radiological sacroiliitis, smoking status, disease phenotype and the treatment. No correlations were found between the degree of radiological sacroiliitis and bacterial groups or between BMI and gut microbiome. A study published in 2018 highlighted the analysis of the intestinal microbiota in 61 cases of obese people. Intestinal dysbiosis was characterized by a decrease in microbial diversity which correlated with various metabolic parameters (50). Many of the published results are contradictory. Some support a decrease in diversity in the phyla group, others do not show significant differences between normal and obese people. Many authors have shown an increase in *Firmicutes* followed by a decrease in *Bacteroides* in cases of obesity (51,52). Kasai *et al* showed an increase in the diversity of intestinal microbiota in cases of obesity (53). Angelakis *et al* observed a numerical increase in anaerobic bacteria in obese patients (54). Hu and colleagues found no significant differences between normal and obese individuals in the *Bacteroidetes, Firmicutes*, and *Proteobacteria* groups (55).

In the present study, significant data were recorded in relation to smoker status. Correlations between *C. coccoides* and *Bifidobacterium* were found in the arm of smokers diagnosed with AS. The level of *C. coccoides* was decreased, while *Bifidobacterium* was increased. On the other hand, correlations with a decreased level of *F. prausnitzii* were found for active smokers in the control group. The relationship between smoking and IBD is well known. This connection is not fully understood, but the alteration of the intestinal microbiota, as well as of the innate and adaptative immune system, has been incriminated (56).

In a recently published meta-analysis (57), the intestinal microbiota was analyzed in healthy smokers. The authors noted a decrease in the diversity of the intestinal microbiome characterized by a decrease in *Bifidobacterium* and *Lactobacillus* species and an increase in *Bacteroides, Clostridium* and *Prevotella*. Intestinal dysbiosis caused by smoking was similar to IBD dysbiosis.

In the present study, according to the form of the disease, significant data were found only for the *Bifidobacterium* bacterial group. In the peripheral form there was a significant decrease in *Bifidobacterium* compared to the axial form of AS.

Differences in the intestinal microbiome according to disease phenotype were also observed in a study by Chen *et al* (58). They noted an increase in *Prevotella* in axial disease and increased *Collinsella, Streptococcus* and *Comamonas* in the peripheral form of AS.

In the present study, the final data focused on the correlations between the composition of the intestinal microbiome and treatment. Significant data were recorded only for NSAID therapy. Thus, these patients showed a decrease in bacterial diversity, in *Bifidobacterium* and *Lactobacillus* and an increase in *Bacteroides, C. coccoides, C. leptum, F. prausnitzii* and *E. coli*.

Studies have shown that the intestinal microbiome can predict the therapeutic response and can be considered a 'biomarker' for inflammation (59). NSAID treatment may cause enteropathy (60,61) or may aggravate intestinal inflammation in patients with IBD. Chronic administration of NSAIDs (celecoxib) had the ability to modulate intestinal microbiome, leading to a decrease in *Bifidobacterium* and *Lactobacillus* followed by an increase in *Coriobacteriaceae*, having also a chemoprotective role by decreasing fecal metabolites (62). Montenegro *et al* (63) support the harmful effect of NSAIDs, characterized by a marked reduction in *Lactobacillus* and by modulation of local motility and immunity through the *Bifidobacteria* group.

In the present study, in contrary, synthetic and biological immunosuppressive drugs had a positive effect on the intestinal microbiome, improving dysbiosis and decreasing systemic inflammation. Anti-TNFα agents can act on the intestinal microbiome by inhibiting the onset of vascular inflammation and by inducing T cell apoptosis (64). Other authors state that this improvement of the intestinal microbiome after TNFα therapy is due to a decrease in bacterial arthritogenic peptides (65).

A study which included proteoglycan-induced mice treated with etanercept for 4 weeks demonstrated the efficacy of anti-TNF therapy on articular manifestations and on gut microbiome composition, leading even to a microbial composition similar to that of the control group (66). Another study (67) highlighted the changes in the intestinal microbiome of patients with AS at 1, 3 and 6 months after initiating anti-TNFα therapy. Initially, a decreased biodiversity was observed, which improved almost to normal after the first month of treatment.

In conclusion, our findings indicate that the intestinal microbiome in patients with AS has a special signature characterized by an inflammatory status induced by the increase in some bacterial species associated with the decrease in other species. We demonstrated that the composition of the intestinal microbiome is influenced by numerous factors, among which genetic background, smoking status, inflammatory markers, disease phenotype and treatment play the most important roles. Our results are similar with those already published and participate in the enrichment of knowledge in the field, bringing new data on intestinal dysbiosis in patients with AS.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

AC, ER and AMB conceived the study, the methodology and drafted the manuscript. ER supervised and designed the study. AC, SC, FP, AMB and CR contributed to the literature resources; and AC, AMB, FP and ER validated the data and data analysis. AC, SC, FP, AMB, CR and ER contributed to the review and editing; AC, AMB, SC, CR, FP and ER contributed to the approval of the final version of the manuscript. All authors have read and agreed to the published version of the manuscript.

Ethics approval and consent to participate

Approval was obtained from the Ethics Committees of the Grigore T Popa University of Medicine and Pharmacy and Rehabilitation Hospital Iasi from which the cases were selected. All the included cases expressed their informed consent to participate in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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