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Molecular Characterisation of Blood Microbiome in Patients with Ankylosing Spondylitis and Healthy Controls

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Abstract

Background: In human and animal studies, ankylosing spondylitis (AS) has been increasingly linked to changes in the microbial inhabitants in the human body (microbiome). These studies have primarily now concentrated on the microbial communities that live in the gastrointestinal tract. However, evidence suggests that various molecular techniques can be used to detect microbial DNA in blood circulation. This DNA might be an unknown reservoir of biomarkers with the potential to track alterations in the microbiomes of remote locations, such as the gut. To this end, we compared the presence and identity of microbial DNA in blood samples taken from ankylosing spondylitis patients to healthy control subjects by amplifying and sequencing the bacterial 16S rRNA variable region four.

Methods: The study's design is a case study based on the presence and identity of bacterial DNA in the blood of Ankylosing spondylitis (AS) patients (n = 10) and healthy control subjects (n = 10) was investigated by amplifying and sequencing the bacterial 16S rRNA gene. Blood concentrations of the cytokines TNF alpha, IL-17A, and IL-23 were determined by the Human Magnetic Luminex Screening, and data were analysed using an Unpaired T-test.

Results: Using PCR amplification, 8 of 10 AS patients (80%) and 8 of 10 healthy control samples (80%) had microbial 16S rRNA in their blood. At the phylum level, Proteobacteria (Control = 48.5%, AS = 52%), Firmicutes (Control = 27.8%, AS = 26.1%), Actinobacteria (Control = 15.4%, AS = 10.7%), and Bacteroidetes (Control = 6.5%, AS = 10%) dominated the blood microbiome. A two-tailed Mann-Whitney test found that Ankylosing Spondylitis was associated with significantly elevated Bacteroides ($P < 0.05$), Prevotella ($P < 0.001$), and Micrococcus ($P < 0.01$), and significantly reduced levels of Corynebacterium 1 ($P < 0.001$), Gemella ($P < 0.01$), and Alloprevotella ($P < 0.05$), compared to healthy controls. Additionally, it was shown that the presence of the Prevotella genus was highly positively correlated with higher levels of TNF-alpha ($P < 0.05$; $r = 0.8$) in AS patients' blood.

Conclusion: This article reveals that a blood microbiome exists in healthy individuals and identifies particular taxa modulated in disease. These blood-derived signatures indicate that this field needs more research and may be helpful as disease biomarkers.

Keywords: Blood Microbiome, Ankylosing Spondylitis, 16S Rrna Gene, Dysbiosis, Biomarker

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Introduction

Ankylosing spondylitis (AS) is a long-term, excruciating, and worsening axial skeleton inflammation that pri-

↑What is "already known" in this topic:
Ankylosing spondylitis has been associated with shifts in the microbial communities that live in and on the body (the microbiome) in human and animal research. Until now, such research has focused chiefly on the microbial populations that live in the gut. Growing evidence reveals that microbial DNA may be found in the bloodstream using various molecular techniques.

→What this article adds:
Here, we show that a blood microbiome exists in healthy individuals and show that it, like other classical microbiome niches, is influenced by disease status (AS). The data gathered from blood might be highly beneficial as disease biomarkers.

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marily affects the backbone and sacroiliac joints (1, 2).

The symptom of AS is often lower back pain, sometimes accompanied by morning stiffness eased by movement. The joints where the spine joins the pelvis are usually afflicted (3). Other joints, such as the shoulders or hips, might also be affected (3). There is also a chance of eye and intestinal issues. Back pain is a frequent symptom of AS, and it occurs and disappears. The incidence varies from 0.5 to 14 per 100,000 per year, depending on the nation (4). It is more prevalent among males than women, according to the male-to-female ratio, roughly 3:1 (5).

While we know that AS results from a chronic inflammatory response, we do not know the initial trigger for this inflammation; various susceptibility genes have been identified in AS, such as the B27 gene; however, these genes are neither necessary nor sufficient to explain the presence of the disease (6). Increasingly, the microbiome's role in initiating and evolving AS disease is being considered.

Changes in the microbiome emerge as one of the most promising opportunities. The gut contains the most extensive microbial ecosystem, and alterations in gut populations (dysbiosis) have been associated with a variety of illnesses (6). Several studies have found that patients with AS are distinguished by gut dysbiosis. Patients with AS exhibit a reduction in the entire Veillonellaceae and Prevotellaceae (7). However, Lachnospiraceae, Ruminococcaceae, Porphyromonadaceae, Bacteroidaceae, and Rikenellaceae are enriched (8). Recently, research by Zhou and his colleagues found AS-enriched species such as Acidaminococcus fermentans, Prevotella copri, Eubacterium siraeum, Parabacteroides distasonis, and Bacteroides coprophilus (9).

Even though the gastrointestinal population has the greatest microbiota, certain symbiotic microbes have been shown to coexist in other organs and systems (10). For example, the blood microbiome is a recently developed notion (11, 12). Long thought to be a sterile environment, it has recently been demonstrated that the blood contains a variety of latent, non-immediately cultivatable bacteria (13) that have transferred into the bloodstream, mainly from the gut and the mouth cavity (11).

Several illnesses have been associated with aberrant blood microbiota (14-20), and it has been proposed that this may impact the progression of these conditions. According to the newly available information, the blood microbiome's dysbiosis may be a factor in the occurrence or progression of several rheumatic disorders.

In this study, we sought to describe the blood microbiomes of AS patients and compared them with those of healthy participants. This made evaluating any changes in the bacterial population possible based on important blood inflammatory indicators (TNF-alpha, IL-6, IL-17A, and IL-23).

The blood microbiome composition of healthy donors and AS patients was assessed using 16s rDNA-based next-generation sequencing. We show the structure and diversity of the blood microbiomes of AS patients and healthy control participants. We hypothesise that alterations to a circulation microbiome may eventually participate in the development of AS.

Methods

Study participants

In this prospective investigation, ten Ankylosing Spondylitis patients and ten healthy control volunteers had their whole blood analysed for bacterial 16S rRNA (free from illness). In addition, they provided a sample of their blood for investigation purposes. The samples of participants were obtained from different hospitals in Najaf province, including AL-Sadder teaching hospital, Al-Hakim, and Al-Farat hospitals.

Sample collection and DNA extraction

After cleaning the skin with an alcohol swab, blood samples were taken utilising a peripheral vein punch in a sterile environment. In addition, each subject's whole blood was obtained in a purple-top (EDTA) vacutainer and stored at –4°C immediately.

QIAamp DNA Blood Mini Kit from Qiagen Company, Germany, was utilised to extract DNA from 300 µl of whole blood samples.

Microbiome characterisation

The bacterial 16S rRNA gene's V4 region was amplified and sequenced to determine the microbial population in the sample provided. A 50µl reaction containing 4 µl of extracted DNA, 5 µl of 10X High Fidelity PCR Buffer, 1 µl of each of the barcoded primers that target the 16SV4 XT F and 16SV4 XT R described in (Table 1), 2 µl of 50 mM MgSO4, 1 µl of 10mM dNTP mixture, 0.2 µl of Platinum Taq High Fidelity polymerase, and 35.8 µl of molecular biology grade water.

The tests also included a negative control reaction, where whole blood DNA was replaced with an equal amount of molecular biology-grade water to guarantee that no reagents were contaminated with target DNA.

Steps in the PCR procedure were as follows: 33 cycles of initial denaturation at 94°C for 15 seconds, annealing at 55°C for 30 seconds, and extension at 68°C for 45 seconds were performed after the first denaturation at 94°C for 2 minutes.

Agarose gel electrophoresis and ethidium bromide staining were utilized to visualize all PCR products after they had been purified utilizing AMPure XP magnetic beads (Agencourt) at a ratio of 0.8 beads to the sample (v/v). The elute was then diluted in 20 µl of molecular biology-grade water. Moreover, high-sensitivity DNA quantification was performed utilizing the Qubit 3.0 hsDNA kit

Table 1. Primers utilised in this investigation

Primer Name	Primer Sequence (5’ – 3’)	Length
16SV4_XT_F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA	52
16SV4_XT_R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACHVGGGTWTCTAAT	54

from Invitrogen to quantify DNA in all PCR-negative data.

The samples were barcoded utilizing the Nextera DNA library kit, then multiplexed for efficiency, and sequenced using the Illumina MiSeq system with a 250bp paired-end read metric. Consequently, bioinformatic analysis was performed using QIIME implemented as part of the Nephele 16S paired-end QIIME pipeline, employing open reference clustering on the SILVA database for bacteria with a sequence identity of 99 percent. All other parameters remained at their default values.

Measuring blood cytokines

In accordance with the manufacturer's instructions (R&D Systems, Minneapolis, USA), the Human Magnetic Luminex Screening Assay was utilised to assess inflammatory markers in the blood. In addition, the Luminex kit LXSAHM-04 was used to measure TNF alpha, IL-6, IL-17A, and IL-23. After four minutes of 16,000 x-g centrifugation, blood samples were diluted in a 1:2 ratio by mixing 25 µl of the blood sample with 25 µl of the assay buffer.

Statistical Analysis

A two-tailed Mann-Whitney test was employed to define the statistical significance of variances in individual bacteria abundance between AS and control participants.

Additionally, an unpaired T-test examined whether there were statistically significant variations in cytokine levels between AS blood and control donations. Consequently, Spearman's test was used to assess the relationship between the microbial community and the levels of cytokine profiles. We utilised GraphPad 8 software, and P 0.05 was considered statistically significant in all situations.

Results

Clinical characteristics of the donors and the outcomes of the 16S rRNA PCR amplification

Twenty human donors provided whole blood samples. Ten patients, all of whom were males, were diagnosed with AS. The patients with AS ranged in age from 33 to 45 years, with a mean (SD) age of 38.4 (3.6) years. From ten men, ten control blood samples were taken. Their ages varied from 32 to 44 years old, with a mean (SD) of 38.1 (3.7). The two cohorts' age differences were statistically insignificant (Unpaired T-test, $P > 0.05$) (Table 2).

Table 2. Characteristics of the studied patients

Sample	Sex	Age	Diagnosis	PCR for 16S RNA
AS-1	Male	38	AS	+
AS-2	Male	38	AS	+
AS-3	Male	39	AS	−
AS-4	Male	40	AS	+
AS-5	Male	45	AS	+
AS-6	Male	33	AS	−
AS-7	Male	34	AS	+
AS-8	Male	43	AS	+
AS-9	Male	37	AS	+
AS-10	Male	37	AS	+
Control-1	Male	32	Healthy individual	−
Control-2	Male	41	Healthy individual	+
Control-3	Male	38	Healthy individual	+
Control-4	Male	37	Healthy individual	+
Control-5	Male	39	Healthy individual	+
Control-6	Male	41	Healthy individual	−
Control-7	Male	44	Healthy individual	+
Control-8	Male	36	Healthy individual	+
Control-9	Male	33	Healthy individual	+
Control-10	Male	40	Healthy individual	+

Microbial 16S rRNA was found in the blood of 8 out of 10 patients with AS (80%) and 8 out of 10 healthy control samples (80%) utilising PCR amplification (Figure 1). Our different experimental controls (accessible template/kit control) failed to generate a visible band following PCR amplification and gel electrophoresis (Figure 1). Afterward, the QuBit high-sensitivity DNA analysis tool confirmed the absence of amplified products. As an added precaution, we examined additional negative control reaction reads during the same sequencing run and concurrently with the samples shown here.

One of these samples (sample NEGF) yielded mappable sequencing data and had a small number of reads that mapped to Neisseria (15), Staphylococcus (38), and Serratia (1643) but was primarily made up of reads that matched to the Lachnospiraceae NK4A136 group (2434). To account for the probable origin of contamination, we emphasize that any taxa found in sample NEGF at a level over 25% of the mean experimental sample level, i.e., a suspected contaminant, should be determined at a level four times the negative control to be considered persuasive. Using this approach, the Lachnospiraceae NK4A136 group and Serratia were identified as possible contaminants, and this information will be incorporated in discussions of these taxa.

Bacterial population characterisation utilising blood 16S rRNA sequencing

PCR amplification and sequencing of the microbial 16S rRNA gene, V region 4, was used to determine bacterial DNA's existence in blood. Each sample provided an average of 69,000 reads, with 64,742 reads in the AS samples and 73,741 reads in the healthy control samples. Even though the control samples produced more reads on average, the variation was insignificant statistically (Unpaired T-test; $P \geq 0.05$). Nonetheless, rarefaction was utilised before differential abundance analysis to account for differences in sequencing depth. After the above-mentioned taxonomic categorisation using the Nephele platform, we performed Principal Coordinates Analysis (PCoA) to decrease the complexity of the data and visualise any evident distinction between the various experimental samples (Figure 2).

It became apparent after ordination that the samples from our AS cohort clustered differently from the control subjects. The AS samples and control samples were distinguished substantially. Our results suggest that our AS patients' blood had a bacterial population significantly different from healthy subjects.

Proteobacteria (Control = 48.5%, AS = 52%) and Firmicutes (Control = 27.8%, AS =

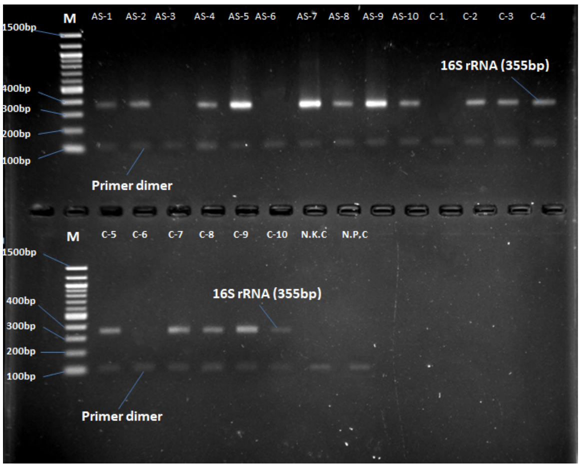

Figure 1. PCR amplified the product of 16s rRNA genes from diseased and healthy samples using 16SV4_XT F/R primers to amplify approximately 355 bp of the 16S rRNA gene. Lane M indicates that a ladder of 100 bp was utilised as a standard-size maker. Lanes AS-1 to AS-10 represent AS samples; in contrast, lanes C-1 to C-10 represent control samples. Lanes NKC and NPC represent negative controls (sterile water instead of template for DNA extraction kit and PCR reaction). Our agarose gel electrophoresis results of PCR showed that 16S rRNA V4 was successfully amplified from AS and control samples, with an 80% success rate for both.

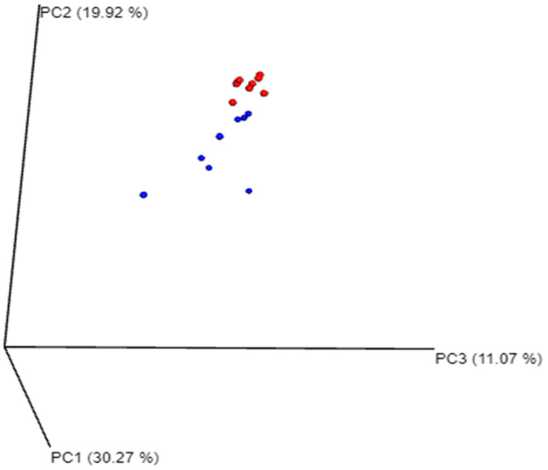

Figure 2. A weighted UniFrac distance matrix of the V4 region of the 16S rRNA's blood microbial community structure for ankylosing spondylitis patients (red) and control participants (blue). The axes display the percentages of variance that primary coordinates can account for. According to PCoA, the highest variance was 30.2% for PC1, 19.9% for PC2, and 11% for PC3. It is fantastic to see that the AS blood samples clustered distinct from the control blood samples after ordination. This is because the microbiome makeup of samples near one another is more similar.

26.1%), Actinobacteria (Control = 15.4%, AS = 10.7%) and Bacteroidetes (Control = 6.5%, AS = 10%) dominated the blood microbiome at the phylum level.

Our blood samples were predominated via the genera Pseudomonas (Control = 26.7%, AS = 25.7%), Corynebacterium 1 (Control = 12.6%, AS = 7.4%), Methylobacterium (Control = 7.8%, AS = 7.4%), Anaerococcus (Control = 6.7%, AS = 6.6%) Streptococcus (Control = 3.9%, AS = 3.7%), Achromobacter (Control = 3.7%, AS = 5.2%), Staphylococcus (Control = 3.1%, AS = 6.8%), followed by Serratia* (Control = 2.9%, AS = 3%), to a lesser extent, blood samples contained the Bacteroidales S24-7 group (Control = 2.8%, AS = 1.8%), and Lachnospiraceae NK4A136 group* (Control = 2.1%, AS = 2.1%) (Figure 3).*A single negative control reaction had a possible contamination level greater than what we observed in our experimental samples.

A two-tailed Mann-Whitney test with a $P \leq 0.05$ was used to conduct a statistical analysis of those genera representing at least 1% of each experimental group. According to statistical analysis, six genera were significantly changed via illness status. At presentation, Ankylosing Spondylitis was associated with significantly elevated Bacteroides ($P < 0.05$), Prevotella ($P < 0.001$), and Micrococcus ($P < 0.01$), and significantly decreased levels of Corynebacterium 1 ($P < 0.001$), Gemella ($P < 0.01$), and Alloprevotella ($P < 0.05$), compared to healthy controls (Figure 4).

Detection of inflammatory markers in blood

As reported, TNF-alpha, IL-6, IL-17A, and IL-23 serum concentrations were evaluated utilising the Luminex system. Median (SD) cytokine levels in AS patients and control blood were substantially different, with cytokines existing at greater levels in AS patients in all instances (Unpaired T-test) (Table 3).

Alterations in the taxonomic diversity of the blood microbiome were correlated with changes in cytokine responses in individuals with AS.

We assessed whether dramatically shifting genera in the blood of AS patients are correlated with an increased particular proinflammatory cytokine response (TNF-alpha, IL-6, IL-17A, and IL-23). The taxa include Bacteroides, Prevotella, Micrococcus, Corynebacterium 1, Gemella, and Alloprevotella.

According to Spearman's rank correlation, the genus Prevotella was also found to be strongly positively correlated with an increased level of TNF-alpha in the blood of AS patients ($r = 0.8$, $P < 0.05$) (Figure 5).

Discussion

This research aimed to compare the circulating bacterial DNA profiles and the concentrations of proinflammatory cytokines in various blood compartments of patients with AS besides healthy control volunteers.

Our research showed the existence of complex serum microbiome populations in both disease and healthy subjects.

The four significant phyla that composed blood samples at the phylum level were Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes. These findings agree with other research (21-24), strengthening the concept that four important phyla dominate the core blood microbiome.

Several genera discussed in this research were previously described in healthy adult blood in a separate study, albeit in various ratios (11, 24), and by others in both diseased and healthy participants.

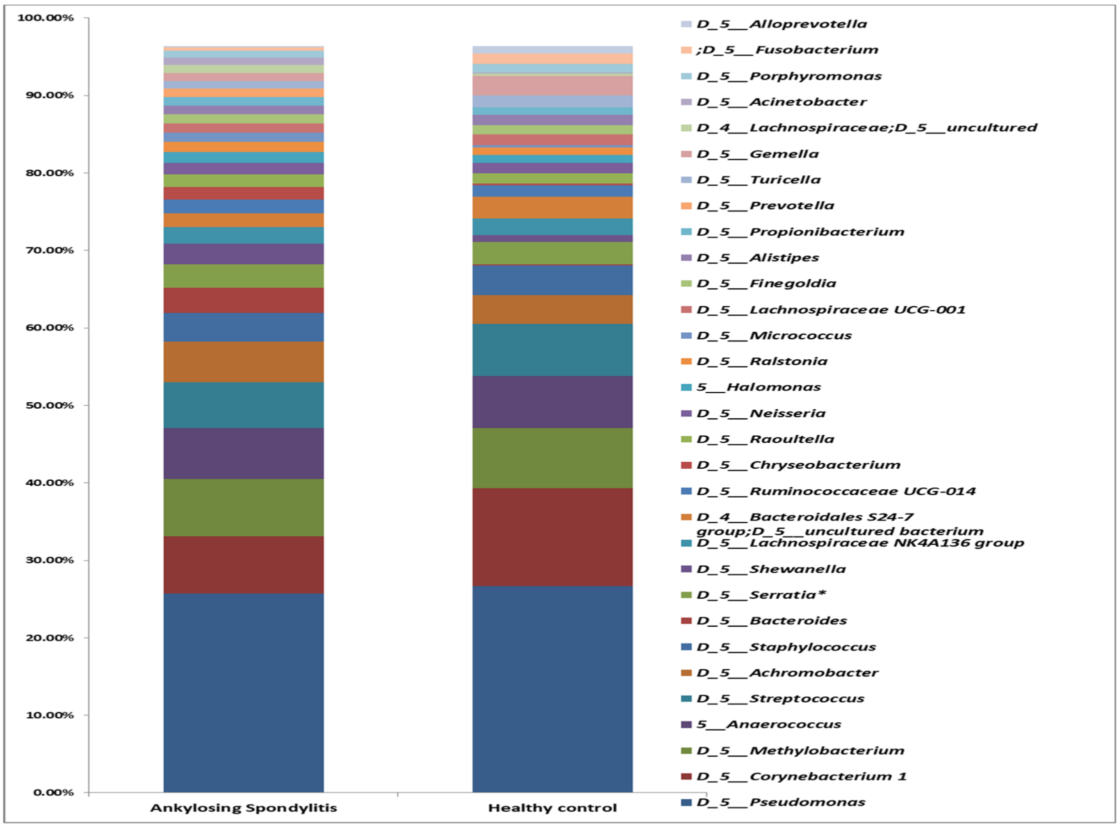

Figure 3. Depicts the relative abundance of the numerous bacterial taxa observed in the blood of controls and AS patients. By using amplification and sequencing of the V4 region of the 16S rRNA in blood samples from patients with ankylosing spondylitis (AS, n = 8), and healthy controls (Control, n = 8), it was possible to determine the abundance of the major bacterial taxonomic groups, which were recognized as having a mean abundance of more than 1% of the total bacteria content in the two cohorts. The data shows a mean abundance as a percentage based on the total number of bacterial sequences.

We found a substantial clustering of bacterial blood patterns between health and disease. The disparities could be attributed to the different relative abundances of various bacterial genera, which propose an alteration in the circulating bacterial populations in patients with AS.

When comparing AS patients to healthy controls, we found the relative prevalence of Bacteroides, Prevotella, Chryseobacterium, and Micrococcus as markers enriched explicitly in the serum of patients with AS.

Bacteroides genera are among the bacteria identified as being involved in AS aetiology (25). Nonetheless, microorganisms' presence is usually associated with a causative immunological response rather than infection (25). According to a mouse model study, Bacteroides contribute to inflammation in peripheral joint conditions (26). In line with previous investigations (25, 27, 28), this study found that Bacteroides abundance was greater in AS patients in comparison to the healthy group. Investigations have shown that combined inflammatory bowel disease and arthritis occurred due to increased HLA-B27 in transgenic mouse guts, indicating the presence of Bacteroides (29-31).

IFN- production can be induced by a Bacteroides peptide that mimics type II collagen. Prior theories have suggested that autoimmune triggers may be caused by an interaction between autoantibodies and microbiological elements (32). It has been shown that multiple Bacteroides peptides enriched in AS group are closely related to known AS auto-epitopes using bioinformatic alignment. A specific bacterial peptide, "HIGQPGVIG," produced via Bacteroides, was found to induce the secretion of IFN-alpha by peripheral blood mononuclear cells from AS patients (32). At the same time, when PBMCs from healthy people were used, the same inflammatory reaction was not observed (32). Bacteroides genera have already been linked to molecular mimicry. Bacteroides protein BfUbb, which resembles human ubiquitin, may bind to autoimmune patients' blood IgG (33). Bacteroides peptide mimics the human protein type II collagen, the basic structure of articular cartilage (33). As a result, autoantibodies generated by the microbial peptide may aggravate AS development by destroying the cartilage of inflamed joints.

Prevotella genera were noticed to have proinflammatory properties through increased inflammatory mediator's production of immune cells and different stromal cells (34), implying that specific Prevotella strains may be critical clinical pathobionts and may promote human disorders through inducing systemic inflammation.

Rheumatoid arthritis & ulcerative colitis are two inflammatory disorders associated with Micrococcus taxa. (35, 36). More research is needed to establish an associa-

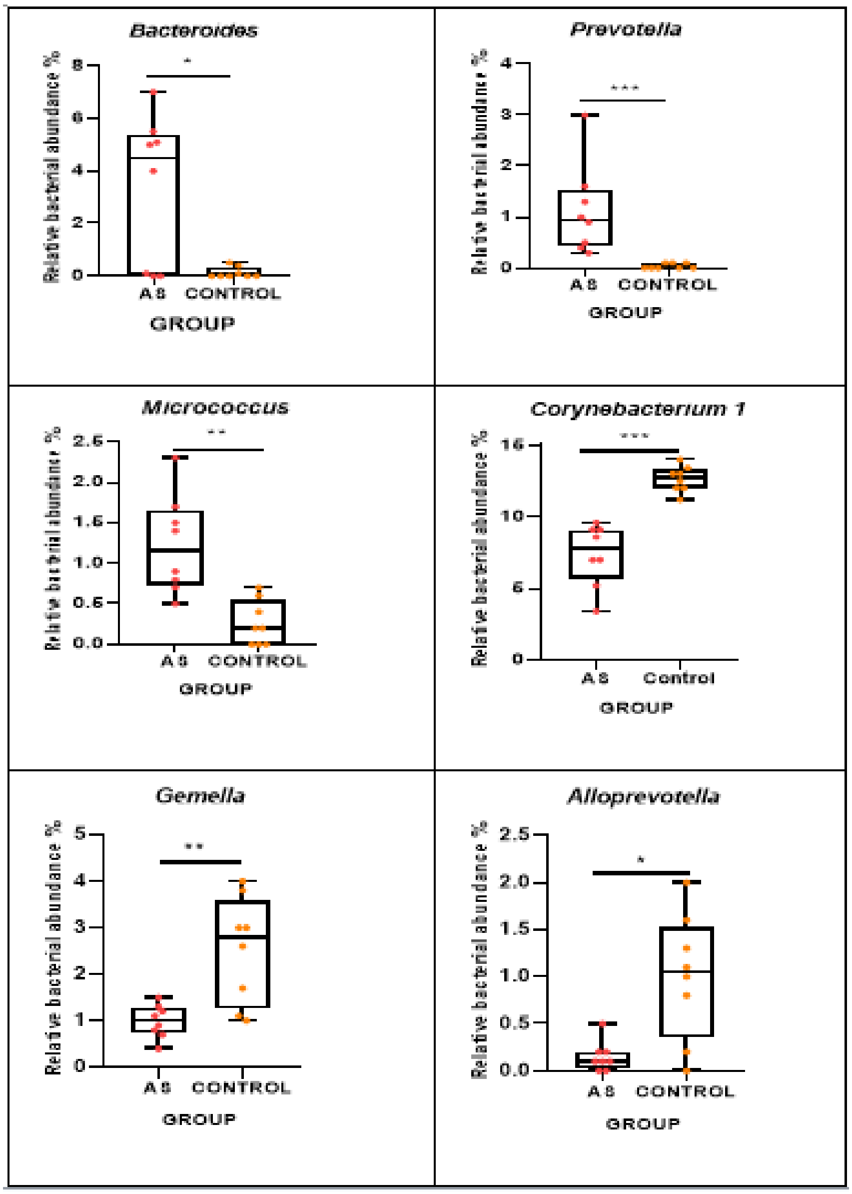

Figure 4. The relative abundance of substantially changed bacterial genera was seen in ankylosing spondylitis (AS) blood compared to control blood. The 16S rRNA gene was amplified and sequenced to obtain the data. The data represents a median abundance proportion of the entire bacterial sequence.

Table 3. Alterations in the levels of cytokines across groups.

Cytokine	AS median (SD) (pg/ml)	Control median (SD) (pg/ml)	*P* value
TNF-alpha	46 (41.5)	6.6 (4.7)	< 0.01
IL-6	55.7 (13.1)	24.3 (9.1)	< 0.001
IL-17A	22.1 (6.8)	10.4 (4.7)	< 0.001
IL-23	74.5 (46.5)	30 (12.1)	< 0.05

tion between Micrococcus and AS illness.

The taxa Gemella, Corynebacterium-1, and Alloprevotella had considerably lower abundance in the serum of AS patients than control participants.

Gemella taxa are most commonly found on the mucosal surface of humans, particularly those of the upper gastrointestinal organs (37). Interestingly, Gemella is less abundant in the periodontal microbiome of inflammatory diseases such as rheumatoid arthritis (38, 39); therefore, Gemella genus in the serum declines of AS patients may be a sign of a proinflammatory state.

Corynebacterium and Alloprevotella are components of the typical human gut, skin, and mouth microbiome. In addition, healthy people's blood has newly been found to include these genera (23).

As a result, their decline, which we have seen in the peripheral blood microbiome of patients with AS, combined with the other changes noted above, suggests the presence of dysbiosis in these remote places, mirrored in the abundance of DNA that reaches the circulation and supports

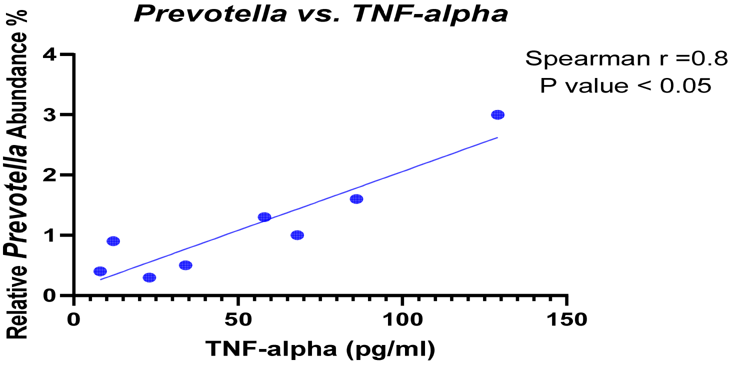

Figure 5. Correlation between the abundance of Prevotella with the increase of proinflammatory cytokine levels found in the AS patient's blood. The strongest positive correlation was identified between genus Prevotella and an increase in TNF-alpha levels (r = 0.8, $P \leq 0.5$). The Spearman correlation test was utilised to analyse the data statistically. The Prism 8.0 program was used to conduct the statistical analysis, and the 95 percent confidence level (p 0.05) was chosen as the statistical significance threshold. Significant data were defined as $P < 0.05$.

the idea that the circulating microbiome may recreate a significant role in AS disease.

Numerous cytokines have been associated with the occurrence of AS. For instance, AS patients' blood had considerably greater levels of IL-17-A, TNF-alpha, IL23, and IL-6 than those of healthy people (39-42).

Furthermore, Prevotella taxa and TNF-alpha have a positive correlation. TNF- can trigger multiple signaling pathways, stimulating the production of inflammatory mediators, such as IL-6 and IL-1, and stimulating macrophages, T cells, or B cells, among other immune mechanisms (43-46). It is associated with the pathophysiology of a number of autoimmune disorders. In the joints of AS patients, TNF- is found in significant amounts close to the sites of new bone formation (47). Additionally, the fact that this is found in the initial stage of the AS illness raises the possibility that TNF- directly contributes to AS pathogenesis (48). Prevotella lipopolysaccharide is known to induce the production of tumor necrosis factor-alpha in monocyte-derived macrophages through mitogen-activated protein kinase signaling pathways (49, 50).

One of the limitations of this study that could be addressed in future research is the study used a small number of samples because our sources provided a small number of cohorts size.

Conclusion

We characterise the blood microbiome's existence in AS patients as well as healthy control individuals by utilising 16S rRNA-seq from bacterial populations. We determine taxa that seem to be associated with AS condition.

These findings are consistent with our emerging theory that bacterial DNA in human blood moves from more classical microbiome places disturbed by disease. Therefore, it may be a unique biomarker in the AS pathogenesis and its therapeutic response. It will take more research to explore these preliminary results fully.

Acknowledgments

Our profound gratitude goes out to everyone who voluntarily participated in this research.

Ethics Approval and Consent

The research process and its objectives were explained to the participants then the subject /legally authorized representative signed the informed consent before the beginning of the project. The questionnaires were anonymous, and the subjects were reassured about the confidentiality of the data.

Authors contributions

DH and OA conducted the original research. DH created the final statistics after analysis of the sequencing data. The data were analyzed, and DH, OA, AK, and FA assisted in this manuscript's preparation. All writers accepted the last draft.

Conflict of Interests

The authors declare that they have no competing interests.

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