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Gut microbiome in children with enthesitis-related arthritis in a developing country and the effect of probiotic administration

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Summary

In Asia, enthesitis-related arthritis (ERA) is the most frequent category of juvenile idiopathic arthritis. ERA has a strong association with human leucocyte antigen (HLA)-B27 and subclinical gut inflammation. In an HLA-B27 transgenic rat model, the presence of *Bacteroides* bacteria in the gut appears to cause spondyloarthropathy (SpA). Thus, we studied gut microbiota in children with ERA. Stool specimens from 33 patients with ERA and 14 age-matched healthy controls were studied; none had any gastrointestinal symptom, or had received a drug known to affect gut motility or microbiota in the preceding 6 weeks. From each specimen, a cDNA library for the V3 region of bacterial 16S rRNA was subjected to high-throughput, massively parallel sequencing. Relationship of the specimens was studied using principal co-ordinate analysis (PCoA), and abundances of various bacterial taxa and alpha diversity were compared between groups. In eight patients, a repeat faecal specimen was studied after 12 weeks of probiotic therapy. The 55 specimens yielded a median (range) of 397 315 (102 093–1 502 380) high-quality reads each. In PCoA, gut microbiota from ERA showed a wider dispersion than those from controls. In patients, families Bacteroidaceae and Enterobacteriaceae were more abundant and Prevotellaceae were less abundant than in controls. Also, genera *Bacteroides*, *Entercoccus* and *Klebsiella* were over-represented and genus *Prevotella* was under-represented in ERA patients. Probiotic therapy led to a non-significant increase in Prevotellaceae. Patients with ERA have a dysbiosis in the gut, with increased abundance of *Bacteroides* and reduction of *Prevotella*. Probiotic supplementation in a subset of patients did not reverse these changes significantly.

Keywords: gut, juvenile arthritis, microbiome

Introduction

Commensal micribiota of the gut, comprised of anaerobic and aerobic bacteria, and acquired at or shortly after birth, play a crucial role in body homeostasis. These microbiota harvest energy by fermenting the substances that reach the colon undigested, suppress the growth of pathogenic organisms and help in the development of intestinal microvasculature [1]. In addition, they help in the development and maturation of gut-mucosal and systemic immune systems, including the development and organization of tertiary lymphoid structures, such as Peyer's patches [2,3].

A change in gut micribiota appears to alter host immune responses, thereby influencing the course of systemic immune-inflammatory diseases [3,4]. In the K/B×N mouse model of arthritis, the disease is attenuated if the animals are raised in a germ-free environment [5]. Similar attenuation of disease has also been observed in germ-free mice in the experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis [6]. In both these models, mono-colonization of the intestine with segmented filamentous bacteria belonging to genus *Clostridium* has been shown to enhance the T helper type 17 (Th17) immune response and to result in flare of autoimmune features [6,7].

Interestingly, a particular group of bacteria can have varying effects in different diseases. For instance, in a

human leucocyte antigen (HLA)-B27 transgenic rat model of spondyloarthropathy (SpA), in which arthritis develops in rats raised under conventional conditions but not in those raised in germ-free conditions [8], experimental colonization with *Bacteroides* spp. in the latter led to the induction of arthritis [8]. By contrast, in the EAE mouse model, introduction of polysaccharide-A positive *B. fragilis* attenuates the manifestations through induction of tolerogenic dendritic cells and interleukin (IL)−10 producing a regulatory T cell response [9].

Data on the role of gut microbiota in human arthritis have begun to emerge in recent years. Enthesitis-related arthritis (ERA), a category of juvenile idiopathic arthritis (JIA), is a disease of children and young adults, which is characterized by arthritis and enthesitis and resembles the ankylosing spondylitis (AS) of adults. Several clinical and laboratory findings suggest that joint inflammation in ERA is related to perturbations in the intestinal tract. We have shown previously that children with ERA have (i) a demonstrable lymphoproliferative response to gut pathogens; (ii) an increased expression on peripheral blood and synovial fluid mononuclear cells of Toll-like receptors which can sense bacterial products; and (iii) a Th1/Th17 predominant immune response [10–13]. In addition, these patients have a subclinical inflammation in the gut [14]. ERA is also associated with the presence of the HLA-B27 allele, similar to other spondyloarthropathies which are related to gut inflammation and gut micribiota. Further, this subset of JIA is most prevalent in geographical regions where gastrointestinal infections are common, comprising 36% of JIA in India in contrast to 10% in North America [15,16]. All these findings strongly suggest a pathogenic role for gut micribiota in ERA.

Some recent studies on stool specimens from patients with AS and ERA using bacterial 16S ribosomal-DNA sequencing have revealed differences in the composition of gut micribiota compared to healthy controls [17–19]. However, most of these studies have been performed in the developed world. Because ERA is particularly common in developing countries, where diet and intestinal micribiota are different than in the developed world, we compared gut microbiota in children with ERA and healthy controls in India.

Probiotics are preparations that contain live bacteria that have a beneficial effect on the host by multiple mechanisms, such as preventing growth of pathogens by altering gut micribiota or by altering the metabolites in the gut lumen. In addition, they also modulate the immune response. They have shown efficacy in animal models of immune-inflammatory disorders such as rheumatoid arthritis, inflammatory bowel disease and also in small human studies [20]. Thus, in order to determine if probiotics affect faecal microbiota in ERA, we also analysed stool samples from a small number of patients enrolled into a clinical trial of probiotics [21].

Methods

Patients and controls

We studied 33 patients fulfilling the International League of Associations for Rheumatology (ILAR) classification criteria for ERA [22], and 14 healthy children and young adults as controls. From each subject, a morning stool specimen was collected, transported immediately to the laboratory at 4°C and then frozen at −80°C until analysis. Children exposed to antibiotics, intra-articular or systemic steroids, any other immunosuppressive drugs or symptomatic gastrointestinal infection during the last 6 weeks were excluded from the study. No patient had ever received biological therapy.

Repeat stool samples from eight patients who had received one capsule of a probiotic (VSL#3; Sun Pharmaceuticals, Mumbai, India) twice daily orally, with each capsule containing 112·5 billion bacterial cells belonging to eight species, namely *Streptococcus thermophilus*, *Bifidobacterium breve*, *B. longum*, *B. infantis*, *Lactobacillus acidophilus*, *L. plantarum*, *L. paracasei* and *L. delbrueckii* as part of a clinical trial [21], were collected after 12 weeks of probiotic administration.

Stool processing and 16S rRNA sequencing

One gram of stool sample was taken for DNA isolation. Cells were lysed using sodium dodecyl sulphate (SDS) lysis buffer and the phenol-chloroform method was used for DNA extraction. After DNA extraction from stool, Illumina sequencing libraries were prepared using a one-step polymerase chain reaction (PCR) in a 50-μl reaction mixture that contained 200 ng input DNA, 6·25 pmol each of forward and reverse primers (Supporting information, Table S1) and KAPA Hi-Fi PCR master mix (Kapa Biosystems, Boston, MA, USA). The PCR conditions were: an initial denaturation at 95°C for 5 min, followed by 20 cycles of 95°C, 65°C and 72°C for 1 min each and a final extension at 72°C for 5 min. The amplification products were purified using 2% agarose gel electrophoresis, followed by recovery of amplicons of the desired length (GenElute Gel extraction kit; Sigma-Aldrich, St Louis, MO, USA). The purified libraries were checked for size distribution, quantitated (Agilent Bioanalyser DNA1000; Agilent, Santa Clara, CA, USA) and normalized to 10 nM. The normalized libraries were pooled in sets of eight to 12 specimens each and sequenced in one lane of an IlluminaHiScan SQ sequencing flow cell using a standard 2 × 151-cycle paired-end multiplex sequencing format. The library pool was spiked with 30% Illumina PhiX control library to enhance sequence diversity for efficient base calling. Data were then demultiplexed using Illumina CASAVA software.

Processing of sequence data

The raw paired-end reads were trimmed to remove the primer sequences and merged using PANDAseq software [23]. During this step, any sequences with an overlap of fewer than 20 nucleotides in opposing reads, merged length of < 100 nucleotides or containing any ambiguous nucleotide were purged. The merged reads were subjected to quality control using an NGSQC Toolkit (National Institute of Plant Genome Research, Aruna Asaf Ali Marg, New Delhi, India) [24], and any reads with an average Phred score below 30 were excluded. Any chimeric sequences, identified using Usearch61 [25], were also purged. The remaining high-quality, non-chimeric merged reads were assigned to operational taxonomic units (OTUs) using the UCLUST-based subsampled open-reference OTU picking protocol of QIIME version 1.8 [26]. A representative sequence for each OTU was then aligned with the Greengenes core set alignment using the PyNAST tool [27] and a phylogenetic tree was constructed using the FastTree tool [28]. Taxonomy was assigned to each OTU using the QIIME's UCLUST Consensus Taxonomy Assigner against the Greengenes version 13.8 reference OTUs pre-clustered at the 97% threshold (ftp://greengenes.microbio.me/greengenes_release/gg_13_5/gg_13_8_otus. tar. gz), using the software's default parameters. Any sequences that failed to align, and singleton OTUs (those with only one sequence in a specimen), unassigned OTUs and eukaryotic (chloroplast and mitochondrial) OTUs were removed. Further, to reduce noise, OTUs that were observed in fewer than 10% of stool specimens or accounted for fewer than 0·002% of reads in all the specimens taken together were purged. A specimen-wise observation count of each OTU was tabulated as an OTU table in the 'biom' format (referred to hereafter as 'filtered OTU table').

Beta-diversity analysis (comparison of specimens from controls *versus* ERA patients)

The filtered OTU table was assembled into a classic table format, where each row represented an OTU and each column represented a faecal specimen. The cells contained observation counts for a particular OTU in a particular specimen, normalized using a log-frequency transformation, as follows:normalized value $= \log_{10}\left(\frac{OC}{n} \times \frac{\Sigma x}{N} + 1\right)$, where OC represents the actual observed count of a particular OTU in a specimen, n is the sum of observed counts for all OTUs in a particular specimen (column total), Σx is the sum of n across all specimens (sum of column totals) and N is the total number of specimens in the table.

Beta diversity was then assessed using principal coordinate analysis (PCoA) based on weighted UniFrac distance matrices.

Alpha-diversity analysis

As species richness is affected by the depth of sequencing, the OTU table for each specimen was rarefied using PhyloSeq (version 1.12.2) to the same depth, i.e. the number of reads in the specimen with the fewest reads (specimen SE030 with 99 583 reads) [29]. Measures of alpha diversity (observed, Chao 1 and abundance-based coverage estimate (ACE) indices which measure species richness, and Shannon and Simpson indices which represent richness and evenness of taxa) were estimated [30] using PhyloSeq (version 1.12.2), and compared between groups using compare_alpha_diversity.py script of QIIME 1.8, using a non-parametric test with Bonferroni's correction for multiple comparisons.

Comparison of composition of faecal microbiota between groups

For identification of differentially abundant bacterial OTUs between controls and patients, we first undertook linear discriminant analysis effect size (LefSe) (linear discriminant analysis (LDA) coupled with effect size measurement) analysis [30]; OTUs with $\log_{10}$ LDA score ≥ 2 (in either direction) and *P*-values below 0·05 were considered as discriminating markers. As this analysis does not include correction for multiple hypothesis testing, we also compared the abundances of various bacterial taxa at different taxonomic levels in patients and controls using the Mann–Whitney *U*-test, followed by the Benjamini–Hochberg false discovery rate (FDR) correction; FDR values below 0·10 were considered significant.

Analysis of paired data (before and after probiotic)

Data processing for this comparison was similar, except that filtering for low-abundance OTUs removed OTUs with sequences that accounted for less than 0·001% of all the reads. For comparison of data before and after probiotic, abundances of individual taxa were compared using Wilcoxon's signed-rank test with FDR correction. *P*- and FDR-value cut-offs used were similar to those for unpaired data.

Ethical considerations

The study protocol was approved by our institution's Ethics Committee and written informed consent was taken from either the patient or a parent.

Results

Study subjects

The median age of 33 children (32 male) with ERA was 15 (range = 5–20) years and the median duration of disease was 24 (1·5–120) months (Table 1). All the patients had

Table 1. Clinical details of patients with enthesitis-related arthritis (ERA) studied ($n = 33$)

Characteristic	Value
Age (years), median (range)	15 (5–10)
Duration of disease (months), median (range)	24 (1·5–120)
Active arthritis, *n* (%)	33 (100%)
Enthesitis, *n* (%)	22 (66%)
Sacroiliitis, *n* (%)	15 (45%)
Uveitis, *n* (%)	0
Inflammatory back pain, *n* (%)	7 (21%)
HLA B27 positivity, *n* (%)	31 (93%)
Erythrocyte sedimentation rate (Westergren method) (mm), median (range)	80 (20–130)

HLA = human leucocyte antigen.

active arthritis and were receiving non-steroidal anti-inflammatory drug (NSAID) therapy. No patients were receiving any immunosuppressive drugs, including disease modifying anti-rheumatic drugs or biologicals. Median erythrocyte sedimentation rate was 80 (20–130) mm. The median age of 14 healthy controls (13 male) was similar [13 (5–22) years].

Gut microbiota in patients with ERA *versus* controls

Median (range) number of high-quality reads in specimens from 14 control subjects and 33 ERA were 272 327(136 563–761 951; total reads 4 728 631) and 397 315 (102 093–1 502 380; total reads 15 871 719), respectively. There was a total of 20 600 350 high-quality reads (ERA: 15 871 719, healthy controls: 4 728 631), of which 586 852 reads were removed by filtering rare OTUs. These reads belonged to 17 539 non-singleton OTUs. Of these, 533 OTUs, distributed in 10 phyla, were identified in at least five specimens each and accounted for $> 0{\cdot}002\%$ of the total reads, and were analysed further.

On PCoA of weighted UniFrac OTU profile distances, the distributions of data points for faecal microbiota from patients with ERA overlapped with those from healthy controls (Fig. 1a). However, visual inspection of the graph reveals that data points for faecal microbiota from patients showed a wider dispersion than those from controls. There was no significant difference in measures of alpha diversity between healthy controls and patients with ERA (Fig. 1b).

The abundance of various bacterial phyla, classes, orders, families and genera in the specimens in the two groups are shown in Supporting information, Table S2. On LefSe analysis, several phyla, classes, orders, families and genera showed high LDA scores (values $> 2{\cdot}0$ and with uncorrected *P*-values $< 0{\cdot}05$; Supporting information, Table S3; however, the corrected *P*-values, after applying FDR correction for multiple comparisons, were not significant for any taxon.

Figure 2a,b shows the results of comparison of abundances of various families and genera between patients with ERA and controls. In this analysis, bacteria belonging to families Bacteroidaceae, Enterobacteriaceae and Enterococcaceae had significantly higher abundances and those for family Prevotellaceae had a lower abundance in faeces from patients with ERA than in those from controls, even after correction for multiple comparisons (Fig. 2a). Similarly, bacteria belonging to genera *Bacteroides*, *Enterococcus* and *Klebisella* were more abundant, and those belonging to genus *Prevotella* were less abundant in the faeces of patients with ERA than in those from controls (Fig. 2b).

Table 2 shows a comparison of relative abundance of some selected species belonging to the genus *Bacteroides* and *Prevotella*. This showed that some species belonging to genus *Bacteroides*, namely *B. fragilis*, *B. plebeius* and *B. eggerthii*, had higher abundance and some species belonging to genus *Prevotella*, i.e. *P. copri* and *P. stercorea*, had reduced abundance in the faecal microbiota of patients with ERA than in controls.

Gut microbiota before and after probiotic administration

There were 5 834 497 high-quality reads from 16 specimens from eight patients with paired data; 85 289 reads were removed because of filtering rare OTUs. Data for 875 OTUs across 11 phyla that accounted for at least 0·001% of all reads were analysed further. On PCoA analysis, no separation was noticed between the specimens collected before and after probiotic intake (Supporting information, Fig. S1a); also, the two types of specimens showed no difference in measures of alpha diversity (Supporting information, Fig. S1b).

Abundances of various bacterial taxa in the faeces collected at baseline and after probiotic administration (Supporting information, Table S4) showed no differences at phylum, class, order, family (Supporting information, Fig. S2a) and genus (Supporting information, Fig. S2b) levels.

Discussion

The intestinal tract plays a major role in the pathogenesis of HLA-B27-related diseases, and recent data on gut microbiome in animal models of these diseases and in patients with SpA suggest the presence of an intestinal dysbiosis. In our study, children with ERA in India showed an increased abundance in their faecal microbiota of bacterial families Bacteroideaceae, Enterobacteriaceae and Enterococcaceae, and genera *Bacteroides and Enterococcus*. Further, these children also had a larger proportion of *Bacteroides fragilis* and *B. plebius* and *B. eggerthii* in their faeces. By contrast, family Prevetollaceae and genus *Prevotella* were less abundant in the stool specimens from these patients. In a subset of our patients who received a probiotic, there was no

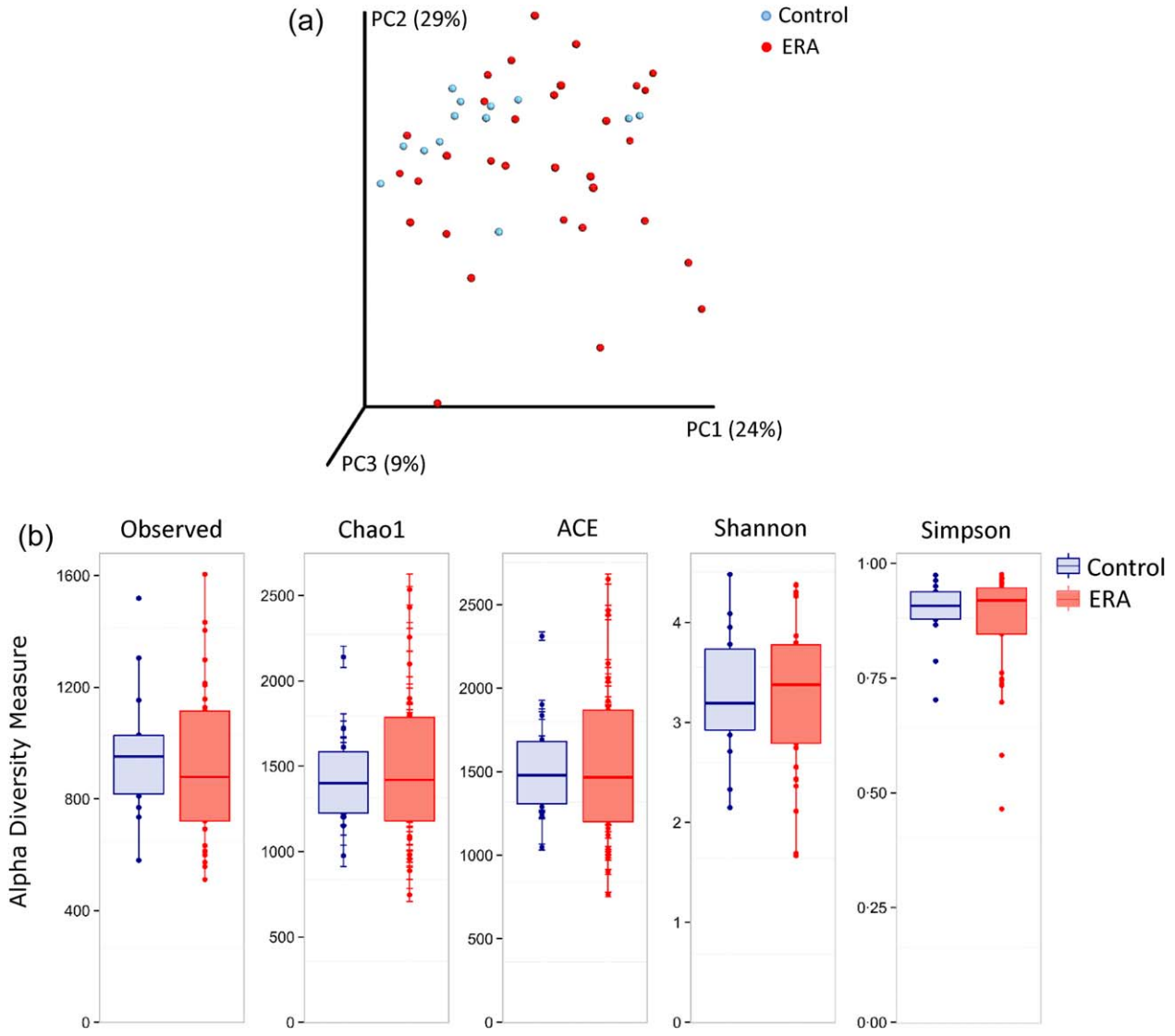

Fig. 1. (a) Principal co-ordinate analysis (beta diversity) of weighted UniFrac distances between specimens collected from patients with enthesitis-related arthritis (ERA; in red) and healthy controls (in blue). (b) Comparison of measures of alpha diversity between specimens from patients with ERA (red) and healthy controls (blue) showed no significant difference. Median and interquartile range are shown.

significant change in the faecal microbiota during a 12-week period.

Our primary finding was an increase in the abundance of genus *Bacteroides* in patients with ERA. Data on the abundance of genus *Bacteriodes* in such patients are also available from other studies. First, in a study from Alabama, USA, abundance of *Bacteriodes* in 25 patients with ERA was somewhat higher than in 13 controls, although the difference was not statistically significant. However, on further analysis, the authors found two distinct clusters among their patients – one of eight patients who had an increased abundance of *Bacteroides* and another of 17 patients who lacked this finding [18]. This suggested that increased abundance of *Bacteroides* could be responsible for at least a subset of patients with ERA in that study. The more marked change in the abundance of these bacteria in our patients could suggest that these may be responsible for a larger proportion of cases of ERA in developing countries. Another recent study in children with other categories of JIA (mainly oligoarticular or polyarticular) has also reported an increase in Bacteroidetes and a reduction in Firmicutes [31]. Thus, it appears that an increased abundance of *Bacteroides*, observed by us, is a common theme in patients with JIA.

In HLA-B27 transgenic rats, intestinal colonization with *Bacteroides* spp. led to the development of arthritis and colitis, whereas the rats raised in a germ-free environment did not develop the disease [8]. A recent study comparing gut microbiota in HLA-B27 transgenic rats to wild-type rats showed an increased abundance of bacteria belonging to phylum Bacteroidetes in the caecal lumen and tissue. At

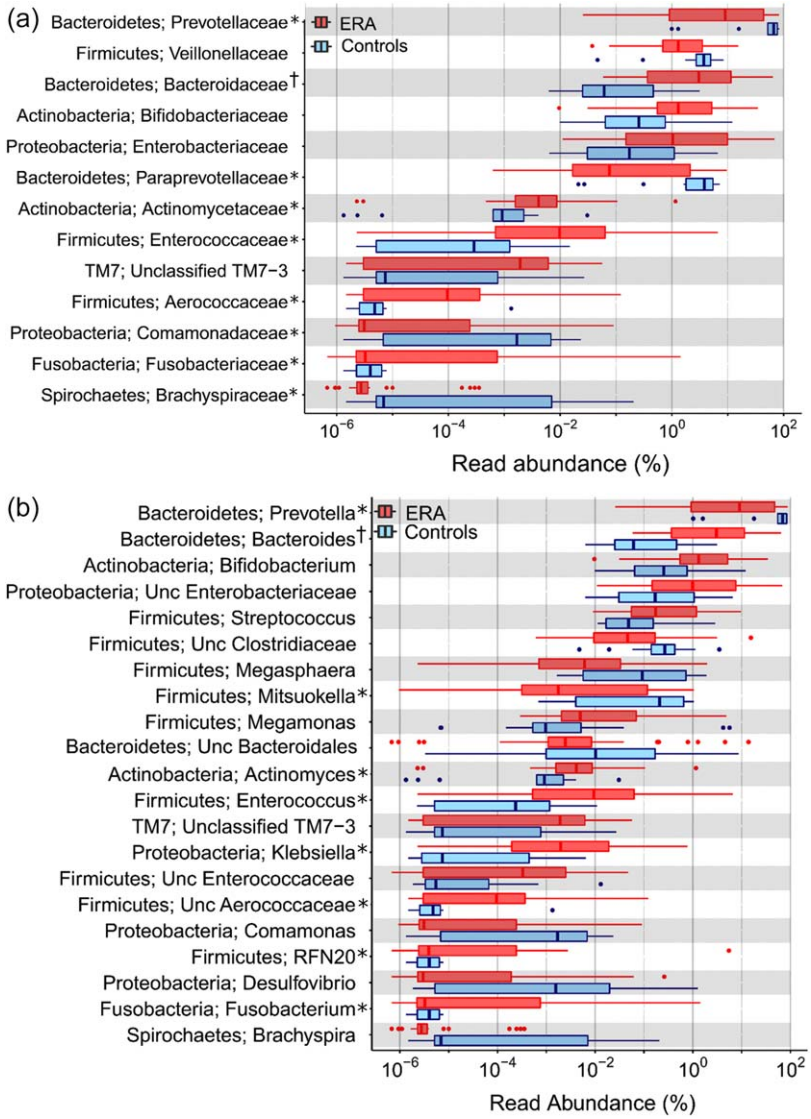

Fig. 2. Differences in abundances of various bacterial families (a) and genera (b) in fecal microbiota from patients with enthesitis-related arthritis (ERA; red) and healthy controls (blue). The data are shown using box-plots and percentage values on a $\log_{10}$ scale. The ends of the boxes represent 25th–75th percentiles, and dots to their left or right indicate outliers. Data are shown for taxa where there was a significant intergroup difference using the Mann–Whitney *U*-test. *Indicates taxa with corrected *P*-values (following correction for multiple testing using the Benjamini–Hotchberg false discovery rate procedure) of < 0.10; †indicates those with corrected *P*-values of $< 0{\cdot}05$.

species level, *Bacteroides vulgatus* and *B. fragilis* were found to be more abundant in transgenic rats [32]. In a model of ankylosing enthesopathy, arthritis developed in germ-free mice after colonization with a mixture of anaerobic bacteria; further, the gut microbiota of these mice showed an increased abundance of *B. fragilis, B. ovatus* and *Enterococcus faecalis* [33].

Studies on microbiota in human subjects with SpA are limited. In patients with AS, analysis of tissue from the terminal ileum revealed a higher abundance of bacteria belonging to the Bacteroidaceae family [34]. Similarly, colonic biopsies of patients with Crohn's disease and ulcerative colitis also showed an increased abundance of *Bacteroides* [35]. Faeces from patients with psoriatic arthritis have also shown an increased abundance of Bacteroidetes than those with psoriasis, but no arthritis [36].

Bacteroides in the gut are mainly commensal organisms. However, they contain virulence factors that can generate inflammatory responses. Agglutinins and histolytic enzymes of *B. fragilis* help these bacteria to adhere to the host mucosa and to cause tissue destruction, respectively [37]. These bacteria are also known to evade the host immune response by decreasing the production of inducible nitric oxide synthase and inhibiting the phagocytosis by macrophages [38]. In addition, the *B. fragilis* enterotoxin is known to disrupt the tight junctions in intestinal epithelium, leading to increased gut permeability in patients with inflammatory bowel disease (IBD) [39].

Another observation in our study was an increased abundance of members of the family Enterobacteriaceae, particularly those belonging to genus *Klebsiella*, in the stools of patients with ERA. This suggests a role for these bacteria in the causation of ERA. Previous studies on gut microbiome have not shown an increase in Enterobacteriaceae in patients with AS and JIA [18,31,34], and thus our finding is somewhat novel. However, it is supported by

Table 2. Comparison of relative abundance (%) of bacterial species of phylum bacteroidetes in fecal specimens from healthy controls and patients with enthesitis-related arthritis (ERA)

	Control		Patients		*P*	FDR *P*
Species	Median	(Range)	Median	(Range)		
Bacteroides plebeius	2.6^{E-04}	(0–0·99)	0·01	(0–52·88)	0·0001	0·01
Prevotella copri	58.17457	(0·79–77·16)	8·92	(0·02–67·53)	0·0016	0·07
B. fragilis	1.2^{E-03}	(0–0·07)	0·01	(0–17·96)	0·0036	0·09
Bacteroides eggerthii	0	(0–1·0E-03)	$2·3^{E-04}$	(0–2·52)	0·01	0·09
P. stercorea	3.855227	(0·03-15·70)	0·08	(1·2E-03-17·93)	0·01	0·09
B. caccae	3.1^{E-03}	(0–0·45)	$3·1^{E-03}$	(0–0·45)	0·16	0·35

P-values were calculated using the Mann–Whitney *U*-test, and were corrected for multiple comparisons using the Benjamini–Hochberg procedure for false discovery rate (FDR).

several additional pieces of evidence. First, members of Enterobacteriaceae, such as *Salmonella*, *Yersenia* and *Shigella*, have been identified as triggers for enetrically acquired reactive arthritis [40]. Secondly, demonstrable lymphoproliferative T cell responses against enteric bacteria are observed more often in patients with ERA than in healthy people [10]. Furthermore, using conventional stool culture techniques, faecal carriage of *Klebsiella* has been reported to be more common in patients with AS than in healthy people [41]. In addition, in a collated data set from 1556 patients with AS from 16 countries, serum titres of antibodies to *Klebsiella* were higher than in healthy people [42]. Also, the nitrogenase enzyme of *Klebsiella pneumoniae* has a sequence homology with HLA-B27 and could play a role in the induction of joint inflammation via molecular mimicry [43]. Another starch-digesting enzyme, pullulanase, produced by *Klebsiella*, has sequence homology with collagens type I, III and IV, which are key components of spinal and synovial tissue, the primary site of involvement in AS [41]. Interestingly, a high carbohydrate and low protein diet has been shown to be associated with increased faecal concentration of *Klebsiella* [44]; whether or not this can increase the risk of ERA needs further study.

Patients with ERA in our study also had an increased abundance of bacteria belonging to the Enterococcaceae family as well as *Enterococcus* genus in their faecal microbiota. Several studies in HLA-B27 transgenic and other animal models of IBD have shown a higher abundance of these bacteria, and linked it to the severity of colitis [33,35,45]. However, data on abundances of these groups have not been reported in previous studies in AS or in JIA. Bacteria belonging to the *Bifidobacteriaceae* family were also more abundant in patients with ERA than in healthy controls – a finding similar to that reported previously in ERA and AS [17,18]. Members of *Bifidobacterium* genus are believed to influence the immune system through increased expression of IL-12 in the gut lymphoid tissue and the generation of regulatory T cells [46].

Bacteria belonging to the *Prevotella* group were less abundant in faeces of patients with ERA, both at family and genus levels. A similar low abundance of Prevotellaceae has been reported previously in ileal biopsies from patients with AS [34]. However, this finding has not been reported previously in patients with JIA. Expansion of *P. copri* has been reported in rheumatoid arthritis [46], although *P. histicola* has been shown to attenuate arthritis in animal models of RA [47]. However, RA has very different pathogenesis to spondyloarthropathies, including ERA. In our population, healthy controls showed a high abundance of *Prevotella*; similar data have been shown in another study from India [48]. In this context, it may be interesting to know that low fibre and high protein diets are associated with a reduction of *Prevotella* in the gut [49]. In India, the diet has high fibre and low protein content, and this may account for high *Prevotella* in healthy subjects.

Another interesting component of our study was the comparison of faecal microbiota before and after administration of a probiotic preparation. This treatment led to a slight increase in the abundance of *Prevotella* and a decrease in the abundance of *Bifidobacterium*. A recent study using genus/species-specific real time PCR had shown an increase in *Lactobacillus* and *Bifidobacterium* after consumption of probiotic yoghurt in patients with inflammatory bowel disease as well as healthy people [50]. However, a recent systematic review [51] concluded that probiotic supplementation did not alter faecal microbiota in healthy adults, except in one study in which the probiotic group had increased beta diversity compared to placebo [52].

We believe that our results are fairly robust, as we studied a fairly large number of patients as well as controls. Furthermore, our study was performed in a developing world setting, in which ERA is the predominant category of JIA. The main limitation of our study was that our patients were receiving treatment for arthritis and this, by itself, could have had an effect on the gut microbiota. However, it is difficult to obviate this limitation in this disease, particularly in tertiary-care settings in developing countries, as patients often present late and have marked symptoms. However, by contrast, our study was also helped by the fact

that none of our patients were receiving biologicals or disease-modifying anti-rheumatic drugs – thus obviating a limitation with studies from developed countries. Also, the effect of probiotics was studied in only a small number of subjects.

In conclusion, our data suggests the existence of a dysbiosis in the guts in children with ERA with increased abundance of bacterial groups that may promote a proinflammatory state. This, together with previous data from various experimental models of arthritides, suggests that this alteration of gut microbiota can change the gut and the systemic immune response, and thus play a role in the causation of this type of JIA. Hence, restoration of gut microbiota towards normal may be helpful in ameliorating this form of arthritis. In this respect, our data on gut microbiota following administration of a probiotic in the usual dose, albeit limited by a small sample size, were not encouraging, and suggest that this intervention may not be adequate for achieving either a major change in gut microbiota or a clinical response [21]. However, it may be worthwhile trying a higher than usual dose of a probiotic, different formulation of a probiotic or faecal microbial transplantation in the treatment of this condition.

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Disclosure

The authors have no disclosutres to declare.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1. Results of beta and alpha diversity analysis for patients with enthesitis-related arthritis (ERA) before and after probiotic administration. (a) Principal co-ordinate analysis (beta diversity) of weighted UniFrac distances between specimens collected from patients with ERA at baseline (red) and after administration of a probiotic preparation for 12 weeks (blue). (b) Comparison of measures of alpha diversity between specimens from patients with ERA at baseline (red) and after probiotic (blue).

Fig. S2. Abundances of bacterial families (a) and genera (b) in faecal microbiota from patients with enthesitis-related arthritis (ERA) at baseline (red) and after probiotic administration (blue). Data are shown using boxplots and percentage values on a $\log_{10}$ scale. The ends of the boxes represent 25th to 75th centiles, and any dots to the left or right of the boxes indicate outliers.

Table S1. Sequences of primers used for 16S rRNA sequencing

Table S2. Relative percentage abundance of each taxonomic group in faecal specimens from healthy controls and patients with enthesitis-related arthritis (ERA). The data at each taxonomic level are shown in the ascending order of *P*-values. Column 'P' shows uncorrected *P*-values using the Mann–Whitney *U*-test for comparison of controls *versus* patients, and column 'FDR_P' shows values for false discovery rates using the Benjamini–Hochberg procedure to control for multiple comparisons. Cells with FDR values < 0.10 are highlighted in grey

Table S3. Linear discriminant analysis effect size (LefSe) linear discriminant analysis (LDA) scores for abundances of different taxonomic groups in patients with enthesitis-related arthritis (ERA) *versus* healthy controls

Table S4. Relative percentage abundance of each taxonomic group in faecal specimens from patients with enthesitis-related arthritis (ERA) before and after 12 weeks of probiotic treatment. The data at each taxonomic level are shown in the ascending order of *P*-values. Column 'P' shows uncorrected *P*-values using Wilcoxon's signed-rank test for comparison of controls *versus* patients and column 'FDR_*P*' shows values for false discovery rates using the Benjamini–Hochberg procedure to control for multiple comparisons

