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Fecal Microbiota in Untreated Children With Juvenile Idiopathic Arthritis: A Comparison With Healthy Children and Healthy Siblings

Anders Öman[1], Johan Dicksved[2], Lars Engstrand[3], and Lillemor Berntson[1]

ABSTRACT. ***Objective.*** Changes in the composition of gut microbiota have been suggested to be associated with juvenile idiopathic arthritis (JIA). The objective in this study was to investigate if the diversity and composition of the fecal microbiota differed between children with JIA and healthy controls (HCs), and if the microbiota differed between children with JIA and their healthy siblings.

Methods. In this multicenter, case-control study, fecal samples were collected from 75 children with JIA and 32 HCs. Eight of the HCs were siblings to 8 children with JIA, and they were compared only pairwise with their siblings. The microbiota was determined using sequencing amplicons from the V3 and V4 regions of the 16S rRNA gene. Alpha diversity, community composition of microbiota, and relative abundances of taxa were compared between children with JIA and healthy unrelated controls as well as between children with JIA and healthy siblings.

Results. Our data revealed no significant differences in α-diversity or community composition of microbiota between children with JIA, healthy unrelated controls, or healthy siblings. Analyses of relative abundances of phyla, families, and genera identified trends of differing abundances of some taxa in children with JIA, in comparison with both HCs and healthy siblings, but none of these findings were significant after adjustment for multiple comparisons.

Conclusion. There were no significant differences in the composition of fecal microbiota in children with JIA compared with HCs. The composition of microbiota in children with JIA did not differ significantly from that in their healthy siblings.

Key Indexing Terms: case-control studies, child, gastrointestinal microbiome, humans, juvenile arthritis, siblings

Juvenile idiopathic arthritis (JIA) is a heterogeneous condition that is divided into 7 categories based on the International League of Associations for Rheumatology (ILAR) criteria.[1] Although much research has been performed, there is still insufficient knowledge on the pathogenesis of the disease and the factors that influence the disease course. The etiology of JIA is considered to be multifactorial and influenced by several different environmental factors. There is also a genetic contribution to the risk of JIA, but familial factors can only partly explain the etiology.[2,3]

There has been increased attention on disturbances in the intestinal canal and changes in the microbiota as possible environmental factors involved in autoimmune diseases.[4] The gut microbiota is involved in the development and regulation of the innate and adaptive immune system in the mucosa,[5] and it also influences the permeability of the intestinal barrier.[6] A few studies have identified associations between environmental factors that affect the development of the normal gut microbiota and an increased risk of developing JIA. Early-life treatment with antibiotics,[7,8] delivery by cesarean section,[9] and a short duration of breastfeeding[10] are potential risk factors for developing JIA. This could be due to changes in the microbiota or decreased early colonization with favorable bacteria from the mother.

Children with JIA have also been shown to have an increased gut permeability,[11,12] which could contribute to chronic inflammation as the immune system is confronted with an increased number of macromolecules from the intestine. Colonization of the intestine with commensal bacteria promotes normal intestinal barrier function,[6] but a disrupted microbial composition could increase gut permeability.[13] A disturbed composition of

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[1]A. Öman, MD, L. Berntson, MD, Associate Professor, Department of Women's and Children's Health, Uppsala University, Uppsala; [2]J. Dicksved, PhD, Associate Professor, Department of Animal Nutrition and Management, Swedish University of Agricultural Sciences, Uppsala; [3]L. Engstrand, MD, Professor, Center for Translational Microbiome Research, CTMR, Department of Microbiology, Tumor and Cell Biology (MTC), Karolinska Institutet, Science for Life Laboratory, Solna, Sweden.

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Address correspondence to Dr. A. Öman, Unit for Pediatric Rheumatology, Department of Pediatrics, Uppsala University Hospital, S-75185 Uppsala, Sweden. Email: anders.oman@kbh.uu.se.

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the microbiota has thus been suggested to have a potential role in the development of JIA.

Earlier studies have shown some alterations in the diversity and composition of the fecal microbiota in children with JIA, compared with healthy controls (HCs).[14–20] In 2 of the studies, a decreased bacterial α-diversity in children with JIA compared with HCs was found, but this has not been seen in the other studies where α-diversity was evaluated.[14,15,19,20] The most common finding in previous studies comparing children with JIA and HCs is an increased relative abundance of *Bacteroides* in children with JIA.[14,15,18,19,20]

The composition of gut microbiota is influenced by several other factors, such as diet,[21,22] genetic factors,[23] and environment,[24] which are difficult to correct for in statistical comparisons. Siblings living in the same household and environment usually have similar diets and genetics. It has been shown that the gut microbiota in healthy siblings are very similar[25,26]; thus, comparisons of gut microbiota between healthy siblings and siblings with JIA could eliminate some confounding factors.

Our aim was to study whether the composition and diversity of fecal microbiota in untreated children with JIA differed from those of fecal microbiota in healthy children. To further control for different dietary and environmental exposures, we also compared the fecal microbiota in children with JIA with the fecal microbiota in their healthy siblings. To our knowledge, no such study has been published previously.

METHODS

Study design. For this case-control study, children with newly diagnosed JIA were included from 4 Swedish counties (Uppsala, Dalarna, Gävleborg, Västmanland). Fecal samples were collected prior to treatment with disease-modifying antirheumatic drugs (DMARDs), except in 5 children with JIA who were previously treated with DMARDs but had been without treatment for at least 6 months. Children with previously diagnosed JIA but without any ongoing medication with DMARDs were also included. The collection of fecal samples was carried out between 2012 and 2017.

A total of 75 children with JIA and 32 HCs were included in the study. Eight of the HCs were siblings to children with JIA, living in the same household. Since earlier studies have shown that siblings living in the same household have a similar microbiota, these controls were analyzed separately and compared only pairwise with their siblings with JIA. Clinical features for the children with JIA and the HCs are presented in Table 1 and Table 2.

The children with JIA were categorized based on the criteria established by the ILAR.[1] One child diagnosed with systemic-onset JIA was excluded, since that category is considered an autoinflammatory disease and differs from the other categories in many ways.

Healthy children were recruited among children of employees at the pediatric ward (n = 11), children undergoing minor surgery for orthopedic reasons (n = 13), and healthy siblings to children with JIA (n = 8).

Information regarding other autoimmune diseases and recent use of antibiotics was collected from all children. None of the children with JIA were diagnosed with inflammatory bowel disease or other autoimmune diseases. An exclusion criterion for both children with JIA and HCs was use of antibiotics within the 6 months preceding sampling. Exclusion criteria for HCs were presence of any inflammatory disease, diabetes, any atopic disease with continuous medication, or special diet because of intolerance.

Fecal samples. Fecal samples were collected by the participants and parents at home and stored at 4°C until delivered to the hospital, within a maximum of 60 hours after sampling. Upon arrival at the hospital, the fecal samples were placed in –25°C freezers and then moved to and stored in –70°C freezers.

Table 1. Clinical features for 75 children with JIA receiving no medical treatment and 24 healthy controls.

	Children With JIA, n = 75	Controls, n = 24
Female sex, n (%)	44 (59)	10 (42)
Age at sampling, yrs, median (IQR)	10.9 (5.7–14.3)	6.7 (3.5–9.0)
Time from JIA onset to sampling, yrs, median (IQR)	0.45 (0.20–3.05)	–
Treatment-naïve	70	–
Not treatment-naïve (n = 5)		
Time from end of treatment to sampling, yrs, mean (min–max)	0.9 (0.5–1.7)	–
JIA category at onset, n (% female)		
Oligoarticular	42 (69)	–
Polyarticular RF–	15 (53)	–
Polyarticular RF+	1 (100)	–
Enthesitis-related	11 (27)	–
Undifferentiated	5 (60)	–
Psoriatic	1 (0)	–

JIA: juvenile idiopathic arthritis; RF: rheumatoid factor.

Table 2. Clinical features for 8 children with JIA receiving no medical treatment and their 8 healthy siblings.

	Children With JIA, n = 8	Siblings, n = 8
Female sex, n (%)	6 (75)	2 (25)
Age at sampling, yrs, median (IQR)	5.4 (3.4–9.1)	9.0 (6.0–9.9)
Time from JIA onset to sampling, yrs, median (IQR)	0.6 (0.2–2.6)	–
Treatment-naïve	7	–
Not treatment-naïve (n = 1)		
Time from end of treatment to sampling, years	0.5	–
JIA category at onset, n (% female)		–
Oligoarticular	4 (50)	–
Polyarticular RF–	3 (38)	–
Undifferentiated	1 (13)	–

JIA: juvenile idiopathic arthritis; RF: rheumatoid factor

The microbiota was analyzed with sequencing amplicons from the V3 and V4 regions of the 16S rRNA gene using Illumina MiSeq (Illumina Inc.). The sequencing was performed using the National Genomics Infrastructure, hosted by Scilifelab, Solna, Sweden, and the sample preparation and bioinformatics processing was carried out in accordance with the procedure described by Hugerth and colleagues.[27]

The final operational taxonomic unit (OTU) table was further processed to remove all OTUs that were represented in < 4 samples and also had < 4 sequences in the dataset.

Statistics. Demographics and disease characteristics were described using median and IQR, or total number and percent of the study cohort. The data on microbiota did not follow a normal distribution and nonparametric tests were therefore employed.

The α-diversity of the fecal samples was measured using the Chao-1 index and the Shannon diversity index, calculated on the entire OTU matrix. For a comparison of these indices between children with JIA and HCs, we used a logistic regression model with age at sampling as a covariate.

To analyze the community composition of the microbiota, principal coordinate analysis (PCoA) plots, based on Bray-Curtis dissimilarity, were generated for visual comparisons, and analyses of similarity (ANOSIM) were used to test for differences. To estimate the variation in species composition at different ages—as age is a factor that might influence the results — the children with JIA and the HCs were categorized into 4 groups based on age at sampling. The groups included children aged 0.0 to < 3 years (Group A, n = 17), 3.0 to < 8 years (Group B, n = 22), 8.0 to < 13 years (Group C, n = 33), and 13.0 to < 18 years (Group D, n = 27). Comparisons were made between different age groups, between children with JIA and controls, and between different categories of JIA.

Univariate analyses were used to compare relative abundances of taxa between children with JIA and HCs, and were performed at 3 taxonomic levels: phyla, families, and genera. For analyses of phyla and families, all taxa were used, but for analyses of bacterial genera, only genera present in more than 25% of all fecal samples and with a medium relative abundance < 0.1% were included. A logistic regression model, with age at sampling as a covariate, was used to compare children with JIA and HCs.

For the 8 pairs of siblings, Wilcoxon signed-rank test was used to compare α-diversity between children with JIA and their healthy siblings, and PCoA plots were generated to visually compare the community composition of microbiota between the pairs of siblings. Wilcoxon signed-rank test was used to reveal if the relative abundances of phyla, families, and genera differed between children with JIA and their healthy siblings. Moreover, the community compositions of microbiota for the 8 healthy siblings were also compared with those of the other 24 HCs using ANOSIM.

The calculations of the Chao-1 index, the Shannon diversity index, PCoA, and ANOSIM were carried out using the PAST software, version 3.22.[28] The logistic regression models and the Wilcoxon signed-rank test were calculated using the SPSS version 26 (SPSS Inc.). $P < 0.05$ was considered significant and the Benjamini-Hochberg procedure was used to adjust for multiple comparisons. The false discovery rate was set to 0.05. We performed univariate tests at 3 phylogenetic levels and the numbers of tests corrected for with the Benjamini-Hochberg method were 13 for phyla, 39 for families, and 50 for genera.

Ethics. The study was approved by the local ethics committee in Uppsala County, Sweden (Dnr 2006/327, 2006/327/1, 2006/327/3, and 2014/335) and was conducted in accordance with the Declaration of Helsinki. Informed consent was obtained from participating children and parents, and written informed consent was obtained from all parents.

RESULTS

The most abundant phyla in all samples were *Firmicutes* (50% for JIA and 51% for controls), *Bacteroidetes* (27% for JIA and 29% for controls), and *Proteobacteria* (4% for JIA and 3% for controls), but there was also a large number of taxa only categorized as "bacteria" (13% for JIA and 15% for controls; data not shown). The number of sequences obtained from the fecal samples ranged from 9417 to 100,032, with a mean of 44,225 sequences per sample. There were no differences in numbers of sequences between the groups, with the mean number of sequences obtained from samples being 44,163 in children with JIA and 44,421 in HCs. The relative abundances of taxa were used for univariate calculations, with any differences in the number of sequences adjusted.

Alpha diversity. The logistic regression models, with age as covariate, did not show any significant differences in α-diversity between children with JIA and healthy children, neither with the Chao-1 index ($P = 0.16$), which gives more weight to rare species, nor with the Shannon diversity index ($P = 0.08$; Figure 1), which encompasses both OTU richness and evenness. We also compared the children with enthesitis-related arthritis (ERA; n = 11) with the children belonging to all the other categories of JIA (n = 64; Table 1) and found no significant differences in α-diversity, neither with the Chao-1 index ($P = 0.15$) nor with the Shannon diversity index ($P = 0.88$).

Community composition of the microbiota. A PCoA plot for the different age groups (Figure 2) did not show any distinct clustering, but samples from the age groups 8.0 to < 13 years (Group C) and 13.0 to < 18 years (Group D) were located in the left part of the plot, whereas samples from the youngest age group of 0.0 to < 3 years (Group A) were located in the right part of the plot. Samples from the age group 3.0 to < 8 years (Group B) were the most evenly distributed samples along the X-axis. The youngest age group was significantly different from all the other age groups when analyzed with ANOSIM (Group A compared with Group B, $P = 0.01$; Group A compared with Group C, $P < 0.01$; Group A compared with Group D, $P = 0.04$), but these analyses had low R values (R = 0.16, R = 0.23, and R = 0.16, respectively), indicating weak correlations. Although the correlations were weak, we did the comparisons of community composition between children with JIA and HCs, both with and without Group A, to confirm that the age factor did not affect the other results.

A PCoA plot for all the children with JIA and all the HCs did not show any separate clustering (Figure 2), nor did the PCoA plot where the youngest age group was excluded. However, analysis with ANOSIM showed a significant difference between groups for all children ($P = 0.03$), but with a very low R value (R = 0.13). The ANOSIM analysis without the youngest age group did not show any significant differences between JIA and controls ($P = 0.16$; R = 0.08).

In the PCoA plot for the different categories of JIA (Figure 2), most of the samples from children with polyarticular rheumatoid factor (RF)-negative JIA and ERA clustered in the left part of the plot, whereas samples from oligoarticular JIA were more evenly spread out. However, analyses with ANOSIM did not reveal any significant differences between any categories and did not show any significant differences between controls and any JIA categories, and these results were not affected when the youngest age group was excluded from the analyses.

A PCoA plot was also made for the 8 pairs of siblings to compare each child with JIA with their healthy sibling (Figure 2). According to this analysis, the siblings in each pair had similar community compositions of microbiota, whether they were diagnosed with JIA or healthy. Analyses with ANOSIM showed no differences between children with JIA and their healthy siblings ($P = 0.93$, R = –0.10).

Last, we compared the healthy siblings (n = 8) with the other HCs (n = 24) using ANOSIM, and the analysis showed no differences between the groups ($P = 0.48$, R = –0.008; data not shown).

Univariate analyses. To test for differences in relative abundances of specific taxa between children with JIA and controls, logistic regression analysis was used, with age as covariable. This analysis was applied at 3 taxonomic depths (phylum, family, and

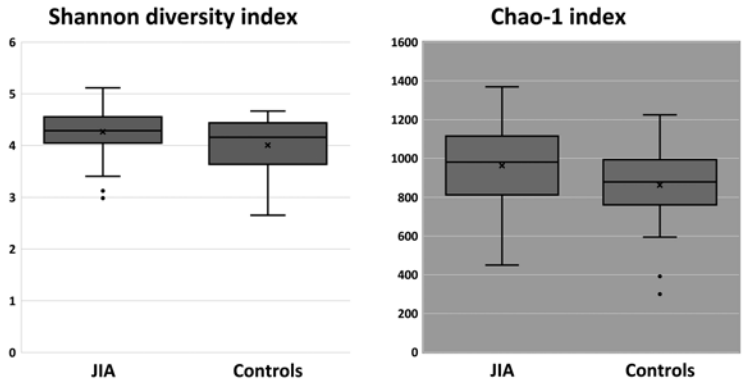

Figure 1. Box plots of the Chao-1 index and the Shannon diversity index for α-diversity in fecal samples from 75 children with JIA and 24 healthy controls. JIA: juvenile idiopathic arthritis.

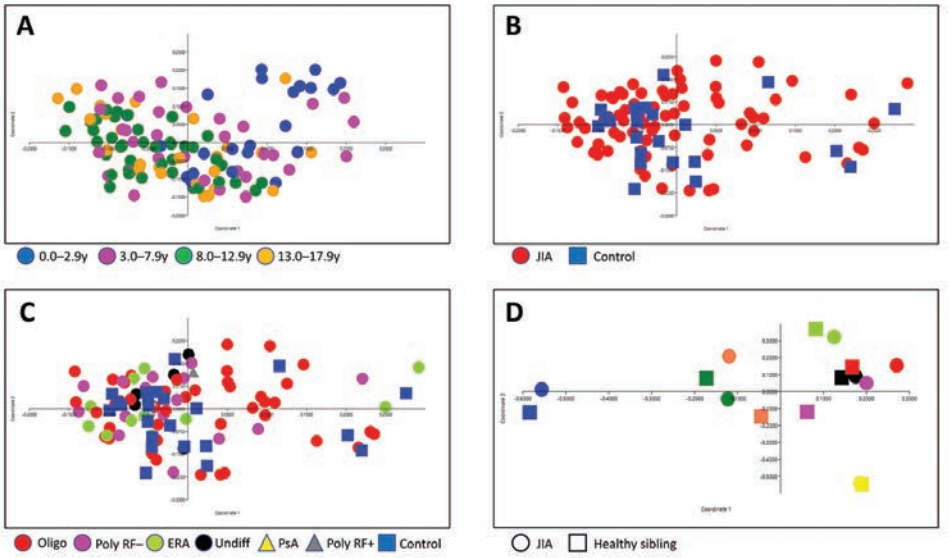

Figure 2. PCoA of microbial community composition in fecal samples. The PCoA plots illustrate how samples clustered based on the composition of bacteria. (A) PCoA for 99 children (75 children with JIA and 24 HCs), divided into 4 groups based on age at sampling. (B) PCoA for 75 children with JIA and 24 HCs. (C) PCoA for 75 children with JIA and 24 HCs. The children with JIA are divided into categories based on the International League of Associations for Rheumatology criteria[1]. (D) PCoA for 8 children with JIA and their healthy siblings. Different colors represent different pairs of siblings. Circles represent children with JIA and squares represent healthy siblings. ERA: enthesitis-related arthritis; HC: healthy control; JIA: juvenile idiopathic arthritis; oligo: oligoarticular; PCoA: principal coordinate analysis; PsA: psoriatic arthritis; poly RF+: positive for polyarticular rheumatoid factor; poly RF–: negative for polyarticular rheumatoid factor; undiff: undifferentiated.

genus) and revealed some trends, including higher abundances of Ruminococcaceae, *Bacteroides*, and *Akkermansia* and lower abundance of Lachnospiraceae in children with JIA. However, these results were not significant after adjustment for multiple analyses (Table 3). We also compared the relative abundance of taxa between children with ERA and the children belonging to other categories of JIA, using logistic regression with age as a covariate, and these analyses did not show any significant differences (data not shown).

The relative abundances of bacterial taxa in fecal samples from the 8 siblings were analyzed for phyla, families, and genera, but comparisons did not show any statistically significant differences between the siblings with JIA and the healthy siblings, after adjustment for multiple analyses (Table 4).

DISCUSSION

In this study, the fecal microbiota of children with JIA and unrelated HCs was compared. The microbiota was also compared

Table 3. Relative abundance of phyla, families, and genera in 75 children with JIA compared with 24 healthy controls. Selected list of taxa with crude *P* values < 0.05.

OTU[a]	Relative Abundance		Crude	Adjusted
	JIA, Mean (SE)	Controls, Mean (SE)	*P**	*P***
Actinobacteria (P)	0.029 (0.004)	0.015 (0.002)	0.035	0.455
Ruminococcaceae (F)	0.228 (0.010)	0.186 (0.020)	0.029	1.131
Lachnospiraceae (F)	0.120 (0.006)	0.170 (0.028)	0.033	0.644
Prevotella_9 (G)	0.045 (0.013)	0.121 (0.040)	0.020	1.000
Bacteroides (G)	0.112 (0.010)	0.071 (0.012)	0.024	0.600
Bifidobacterium (G)	0.020 (0.003)	0.010 (0.002)	0.042	0.700
Akkermansia (G)	0.019 (0.003)	0.006 (0.002)	0.050	0.625

[a] (P) = phylum, (F) = family, (G) = genus. * *P* values calculated using logistic regression with age at sampling as covariate. ** *P* values adjusted using the Benjamini-Hochberg procedure. JIA: juvenile idiopathic arthritis; OTU: operational taxonomic unit; SE: standard error.

Table 4. Relative abundance of phyla, families, and genera in 8 children with JIA and their 8 healthy siblings. Selected list of taxa with crude *P* values < 0.05.

OTU[a]	Relative Abundance		Crude	Adjusted
	JIA, Mean (SD)	Controls, Mean (SD)	*P**	*P***
Actinobacteria (P)	0.010 (0.029)	0.030 (0.060)	0.025	0.325
Bifidobacteriaceae (F)	0.011 (0.015)	0.026 (0.026)	0.025	0.975
Rikenellaceae (F)	0.033 (0.013)	0.016 (0.018)	0.025	0.488
Ruminococcaceae_UCG-005 (G)	0.0012 (0.0017)	0.0004 (0.0007)	0.018	0.900
Alistipes (G)	0.033 (0.013)	0.016 (0.018)	0.025	0.625
Bifidobacterium (G)	0.011 (0.015)	0.026 (0.026)	0.025	0.417
Ruminococcus_gauvreauii_group (G)	0.006 (0.008)	0.018 (0.016)	0.036	0.450

[a] (P) = phylum, (F) = family, (G) = genus. * *P* values calculated using Wilcoxon matched pair signed-rank test. ** *P* values adjusted using the Benjamini-Hochberg procedure. JIA: juvenile idiopathic arthritis; OTU: operational taxonomic unit.

between children with JIA and healthy siblings. All categories of JIA, except systemic-onset JIA, were included in the study, which has been done in only 2 previous studies.[14,17] To our knowledge, this is the first study on microbiota in JIA in which siblings were compared.

We did not find any significant differences in diversity, community composition, or relative abundances of taxa between children with JIA and controls after correction for multiple analyses. Nor did we find any significant differences in fecal microbiota composition when comparing children with JIA and their healthy siblings. Two earlier studies have suggested a reduced microbial α-diversity in children with JIA compared with healthy children,[16,17] but the results from our study did not show any significant differences between the groups, neither with the Chao-1 index nor with the Shannon diversity index. Rather, the trend was toward a higher α-diversity in children with JIA.

The composition and diversity of fecal microbiota have been shown to be age-dependent as the gut microbiota undergoes major changes during the first 3 years of life before becoming more stable.[29,30] Thus, we were not surprised that the youngest age group (Group A, 0.0 to < 3 yrs) in our study differed significantly from all the other age groups, although with low R values. This aspect has not been studied in other studies exploring microbiota in children with JIA, but highlights that age correction may be necessary in the statistical analyses when including microbiota from very young subjects. To correct for age in our study, we analyzed α-diversity and relative abundances of taxa with logistic regression analyses with age as a covariable and performed analyses on community composition both with and without the youngest age group.

When comparing the community composition of the microbiota between children with JIA and HCs, the ANOSIM analysis revealed a significant difference between groups ($P = 0.03$) but with a low R value (0.13), indicating a very poor effect of the factor. The ANOSIM analysis without the youngest age group (Group A) did not differ significantly between JIA and controls ($P = 0.16$) and since none of the PCoA plots comparing children with JIA and controls showed any clustering of groups, our study does not support that there are significant differences in the community composition of microbiota between the groups.

Previous studies by Stoll, *et al* have demonstrated differences in community composition of microbiota between children with ERA and healthy children,[15,18] but no earlier studies have compared the community composition in other categories of JIA with HCs. In our present study, the categories ERA,

oligoarticular JIA, RF-negative polyarticular JIA, and undifferentiated JIA were compared with HCs and with each other, but analyses with ANOSIM did not reveal any significant differences between any of the categories and healthy children, nor did the categories differ significantly from each other.

Earlier studies on fecal microbiota in children with JIA have shown divergent results on relative abundance for many bacterial taxa, but some patterns have been noted. Increased abundance of *Bacteroides* has been reported in 5 studies,[14,15,18,19,20] increased abundance of the family Ruminococcaceae in 2 studies,[16,17] and decreased abundance of *Faecalibacterium* or *F. prausnitzii* in 3 studies.[15,16,18] However, only 2 of the previous studies included all categories of JIA, except systemic-onset JIA,[14,17] as our present study does.

Arvonen, *et al*[31] recently published a review of the microbiota in children with JIA and used machine learning to reanalyze 143 samples. The machine learning model identified one of the OTUs belonging to the Lachnospiraceae family (unclassified Lachnospiraceae) as the most important OTU in distinguishing children with JIA from HCs, but unclassified *Bacteroides* and *Faecalibacterium* were also among the most important OTUs.

In this study, we found similar trends as in previous studies, with higher abundances of *Bacteroides*, Ruminococcaceae, and *Akkermansia*, and lower abundance of Lachnospiraceae in children with JIA, but these findings were not significant after adjustment for multiple comparisons.

To further investigate whether JIA was linked to alterations of the gut microbiota, we compared fecal microbiota from children with JIA with the microbiota of their healthy siblings, living in the same household. We had only 8 pairs of siblings and did not find any differences in community composition or relative abundances of taxa between children with JIA and their healthy siblings after adjustment for multiple comparisons. This could indicate that both siblings in each pair had an unfavorable microbiota, increasing the risk of contracting JIA, but that other environmental factors are also necessary for developing the disease. It could also indicate that the composition of fecal microbiota has a very small or no correlation with JIA. Considering that the children with JIA did not differ significantly from HCs in the other analyses in this study, it seems unlikely that the sibling pairs would have a microbiota significantly different from that in other children. To further examine this, we compared the compositions of the microbiota in fecal samples from the 8 healthy siblings with those in the other 24 HC samples, and found no significant differences between those groups either.

One limitation of this study was the differing age distribution between the children with JIA and the HCs, where the median age was higher in the children with JIA. However, we corrected for the skewed age distribution by performing logistic regression analyses of α-diversity and relative abundances with age as a covariate. To correct for a different community composition in children under 3 years of age, we did ANOSIM calculations with and without the youngest age group (Group A) for comparisons. However, removing the youngest age group also reduces the number of participants and thereby makes it less likely to get statistically significant differences. Another limitation of the study was that the fecal samples were collected at home. While instructions were given, we cannot be sure of the exact time for sampling or how the fecal samples were stored before delivery to the hospital. Last, obtaining statistically significant results from small differences between groups requires large sample sizes. Our study had a large number of participants compared to many other studies in this field, but it is still possible that the sample size was not large enough. This is particularly conceivable regarding the comparisons of microbiota between siblings, since we had only 8 pairs of siblings and subtle differences between groups are difficult to detect with so few subjects.

In conclusion, our data do not support that there are any significant differences in microbiota between children with JIA and HCs. Also, we could not find any significant differences in community composition of microbiota or relative abundances of taxa in microbiota between children with JIA and their healthy siblings.

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