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ORIGINAL ARTICLE

Faecal microbiome in new-onset juvenile idiopathic arthritis

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Abstract Alterations in the intestinal microbial flora have been linked with autoimmune diseases. Our objective was to analyse the composition of the faecal microbiome of children with new-onset juvenile idiopathic arthritis (JIA) compared to healthy controls, and to identify specific gut bacteria associated with JIA. Stool samples from patients were taken at the time of diagnosis of JIA. The microbiome profiles of samples of 30 children with JIA (mean age 6.2 years, 22 girls) were analysed with 16S region-based sequencing profiling and compared to the stool samples of healthy controls ($n = 27$, mean age 5.4 years, 18 girls). The proportion of bacteria belonging to the phylum Firmicutes was significantly lower in children with JIA [21 % (95 % confident interval [CI]: 17–25 %)] compared to controls [33 % (95 % CI: 26–41 %), $p = 0.009$]. Bacteria belonging to Bacteroidetes were significantly more abundant in JIA [78 % (95 % CI: 74–82 %)] than in control samples [65 % (95 % CI: 57–73 %), $p = 0.008$]. Shared operational taxonomic units (OTUs) between the groups revealed that genera *Actinobacteria* and *Fusobacteria* were present only in JIA patients and *Lentisphaerae* only in controls. In summary, faecal flora in JIA is characterised by a low level of Firmicutes and an abundance of Bacteroidetes, resembling the aberration reported in type 1 diabetes. We suggest that alterations in the intestinal microbial flora may challenge the mucosal immune system of genetically susceptible subjects predisposing to local proinflammatory cascades, thus contributing to the development of JIA.

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Introduction

The concept of intestinal dysbiosis encompasses alterations in the microbiome, leading to changes in the gut immune system, such as increased permeability and impaired tolerance to environmental and host antigens. There is emerging data that support the presence of intestinal microbial alterations in the pathogenesis of autoimmune disorders, such as inflammatory bowel disease, type 1 diabetes mellitus and rheumatoid arthritis [1–4]. Faecal microbiota in children with type 1 diabetes shows an abundance of Bacteroidetes, loss of Firmicutes and a lack of butyrate-producing bacteria [2, 5]. The onset of rheumatoid arthritis has been associated with increased abundance of *Prevotella copri* [6] and reduced abundance of *Bifidobacterium* spp. in faecal flora [7]. Earlier, in the context of intestinal bypass surgery, colonisation of the proximal part of the gut with bacterial flora from the distal gut was shown to lead to the development of seronegative polyarthritis [8].

While the pathogenic mechanism linking the faecal microbiome to the development of autoimmune diseases is

still mostly unknown, some animal studies [9, 10] highlight the importance of microbial flora in the maturation of the immune system to identify self and non-self antigens. For example, mice raised in germ-free conditions have a vulnerable mucosal immune system. Introduction of segmented filamentous bacteria to these animals and colonisation in close proximity to the intestinal epithelium, especially in the terminal ileum, leads to an increased proportion of Th17 cells compared to regulatory T cells and, finally, to the autoimmune manifestation of arthritis [11, 12].

So far, there are only a few studies indicating the presence of intestinal mucosal dysbiosis in juvenile idiopathic arthritis (JIA). Stoll et al. found low representation of *Faecalibacterium prausnitzii* in faecal flora in children with enthesitis-related arthritis [13]. Malin et al. reported increased urease activity in faecal samples in JIA, and they interpreted this finding as indirect evidence of disturbances in intestinal anaerobic bacterial flora [14]. However, there is clear evidence of functional aberration of the gut mucosa in JIA. Picco et al. found enhanced leakiness of the gut epithelial barrier in children with JIA [15]. In JIA patients suffering from gastrointestinal symptoms [16–18], signs of altered mucosal immunity were observed, such as inflammatory lesions in the gut [18], ileal lymphonodular hyperplasia [16, 17] and HLA-DR expression in abnormal mucosal sites [16]. We also reported an inverse correlation of mucosal anti-inflammatory mediators (IL10, TGFβ, FoxP3) and correlation of massive lymphonodular hyperplasia with JIA disease activity [16]. These studies favour the idea that alterations of both the intestinal immune system and the intestinal microbial flora are involved in the pathogenesis of JIA. Therefore, we characterised the intestinal microbiomes from faecal samples in children with JIA and in healthy control children.

Materials and methods

Study cohort and sample collection

Children with JIA at the Paediatric Rheumatology Outpatient Department of Oulu University Hospital, Finland, were recruited for the study at diagnosis and before initiation of any disease-modifying anti-rheumatic drug (DMARD) or corticosteroid treatment. Stool samples were collected from the JIA patients and the control children between June 2011 and January 2014. The JIA group included 30 children aged from 1 to 15 years, and the control group consisted of 27 children without any autoimmune disease aged from 0 to 14 years. The clinical characteristics of the patients (including the JIA category) and of the controls are presented in Table 1.

The stool samples were collected in vials and stored for a maximum of 2 days at +4 °C. Parents delivered the samples to the outpatient clinic, where they were stored at −80 °C in cryotubes until microbiome analysis. The samples were collected at a time when the study subjects had not recently had any infection or received any antibiotic treatment.

Ethical considerations

Subjects and their parents signed a written informed consent and the protocol was approved by the Ethical Committee for Clinical Science of Oulu University Hospital.

DNA extraction

DNA was extracted from each faecal sample of patients with JIA and healthy children by using the QIAamp DNA Stool Mini Kit, according to the manufacturer's protocol (Qiagen, USA) and stored at −80 °C until use. The DNA was quantified using a NanoDrop spectrophotometer.

Amplification of bacterial rRNA genes

The hypervariable region V4–V5 of the 16S rRNA gene was amplified using primers F519 and R926. The F519 primer contained an Ion Torrent pyrosequencing adapter sequence A (Life Science Technologies, USA), a 9-bp unique barcode sequence and a nucleotide linker. The R926 primer contained an Ion Torrent adapter trP1 sequence. Polymerase chain reactions (PCRs) were performed in triplicate, each containing 1× Phusion GC buffer, 0.4 μM of forward and reverse primers, 200 μM dNTPs, 0.5 U Phusion enzyme (Thermo Scientific, Finland) and 10 ng genomic community DNA as the template, and molecular-grade water in a total reaction volume of 25 μl. The following cycling conditions were used: 30 cycles of 98 °C, 10 s; 64 °C, 10 s; 72 °C, 20 s, after an initial denaturation of 98 °C, 3 min. After the PCR amplification, pooled triplicate reactions were sequenced using a 316 Chip Kit v2 and Ion Torrent 400 bp chemistry (Life Technologies, USA).

Bioinformatics analysis

The sequences were processed and analysed using state-of-the-art procedures with Quantitative Insights Into Microbial Ecology (QIIME) [19]. Chimeric sequences were removed with UCHIME [20] using the rRNA16S.gold.fasta reference database. The final dataset consisted of 1,574,860 reads after filtering out low-quality and chimeric reads from the 57 samples, with a median of 23,457 reads per sample. The sequences were clustered into operational taxonomic units (OTUs) using a similarity threshold of 97 % in the Ribosomal Database Project (RDP) Naive Bayesian Classifier [20], with a score filtering threshold of 0.5. The OTU table was constructed in a Biological Observation Matrix (BIOM)-formatted table in QIIME, and reads of <5 were removed across the dataset before further analysis. All samples were rarefied to 10,139 sequences prior to the

Table 1 Demographic and clinical characteristics of the patients with juvenile idiopathic arthritis (JIA) and control children

	JIA	Controls
Number of children in analysis	30	27
Gender, female, *n* (%)	22 (71)	18 (63)
Age at sample date in years, mean	6.2	5.4
JIA subtype, *n* (%)		
Oligoarthritis	9 (30)	
Oligoarthritis, extended	4 (13.3)	
Polyarthritis, seronegative	15 (50)	
Polyarthritis, seropositive	1 (3.3)	
Enthesitis-related arthritis	1 (3.3)	
Systemic arthritis	0	
HLA-B27 positivity, *n* (%)	6 (20)	N/A
Anti-nuclear antibody positivity, *n* (%)[a]	14 (47)	N/A
NSAID use more than 2 days before sample date (%)	80	N/A
Duration of NSAID use in days, mean	19	N/A
Duration of arthritis symptoms in days, mean	166	N/A
Active joints in sample date, mean (range)	7 (0–41)	N/A
Physicians VAS, mean (range)[b]	24.4 (2–65)	N/A

[a] Positive if titre ≥160

[b] Scale 0–100

OTU-based analysis, as this was the number of the lowest observed reads in the community. The rarefaction, relative abundance, alpha diversity indices and core microbiome analyses were done with QIIME using a rarefied OTU table. Pie charts were drawn using the Krona software package version 2.4 [21]. Relative abundances of <0.02 % are not shown in the pie charts. The Venn diagram was drawn by the Euler Venn Applet (http://www.cs.kent.ac.uk/people/staff/pjr/EulerVennCircles/EulerVennApplet.html). We have deposited the Ion Torrent raw data in NCBI-SRA with the accession number SRP057688.

Statistical analysis

Statistical analyses were performed with SPSS 20 software (SPSS Inc., Chicago, IL, USA). The Student's *t*-test for independent samples or the Mann–Whitney *U*-test were used, depending on the normality of the distribution, and a p-value < 0.05 was considered to indicate statistical significance. For testing the association of the diversity indexes and clinical characteristics, Pearson's or Spearman's correlation tests were used, depending on the type of clinical variable.

Results

Composition of the faecal bacterial community

Among 57 stool samples analysed, there were seven phyla and 55 genera in the full community, and the majority (99 %) of the sequences belonged to the three most abundant phyla: Bacteroidetes (72 %), Firmicutes (27 %) and Proteobacteria (1 %) (Fig. 1). The most noteworthy result was that the abundance of the Bacteroidetes phylum was higher in children with JIA [78 % (95 % confident interval [CI]: 74–82 %)] than in controls [65 % (95 % CI: 57–73 %), $p=0.008$]. In contrast, the abundance of Firmicutes was higher in controls [33 % (95 % CI: 26–41 %)] than in JIA children [21 % (95 % CI: 17–25 %), $p=0.009$] (Fig. 1). At the family level, Bacteroidaceae (57 % of the Bacteroidetes), Veillonellaceae (7.6 % of the Firmicutes), Ruminococcaceae (7 % of the Firmicutes), Lachnospiraceae (6.3 % of the Firmicutes) and the Rikenellaceae (5.1 % of the Bacteroidetes) were the most common in the whole series of both JIA children and controls.

At the genus level, *Bacteroides* (39 %) was the most dominant, followed by *B. uniformis* (8 %), *B. fragilis* (5 %), *Dialister* (4 %), *Prevotella copri* (4 %), *B. ovatus* (3 %) and *Phascolarctobacterium* (2 %). When the JIA patients were compared to the controls, the proportions of the genera *Bacteroides* (44 vs. 34 %, $p=0.04$) were found to differ significantly, but *B. uniformis* (9 vs. 7 %), *B. fragilis* (4 vs. 6 %), *P. copri* (4 vs. 3 %), *B. ovatus* (2 vs. 4 %) and *Dialister* (4 vs. 6 %) were found to differ non-significantly between groups (Fig. 1, Table 2).

The OTUs within the two most dominant phyla, Bacteroidetes and Firmicutes, were analysed for low relative abundance of genera among JIA patients and control children (Table 3). Many of the genera in the phylum Bacteroidetes, such as *Dysgonomonas* and *CF231*, were present only in

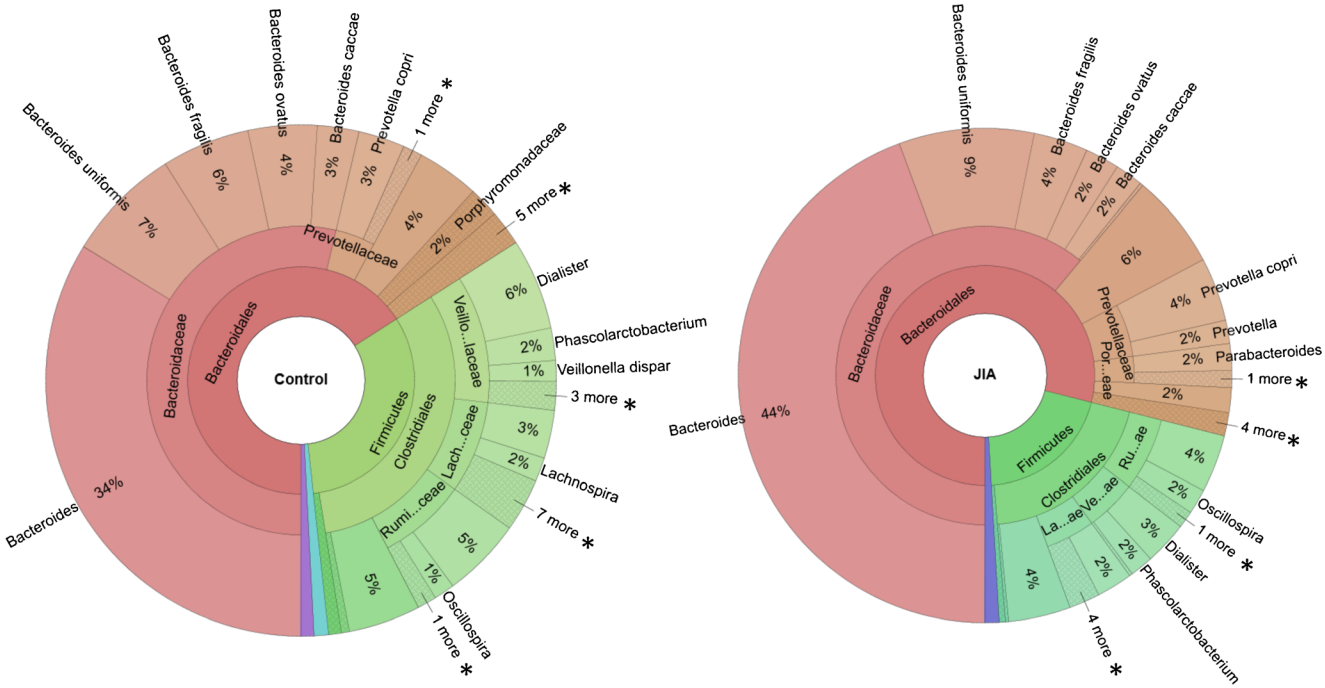

Fig. 1 The relative abundance of gut bacterial flora is shown in control ($n=27$) and juvenile idiopathic arthritis (JIA) samples ($n=30$). Different taxonomic ranks are represented as circles and distinct taxa as columns, while minor taxa are not shown in the figures. *Less abundant (<1 %) genera are not shown or are combined

control samples. Furthermore, *Prevotella* and *Porphyromonas* were present in all samples, irrespective of origin. In the phylum Firmicutes, OTUs of Veillonellaceae, *Sarcina*, *Clostridium butyricum* and *C. difficile* were present only in control samples, whereas members of *Megasphaera* and *Eubacterium biforme* were present only in JIA samples. Genera *Veillonella*, *Streptococcus*, *Coprococcus*, *Lachnobacterium* and *Anaerostipes* tended to be more abundant in controls than in JIA samples (Table 2).

Comparison of OTUs and the core microbiome between control and JIA samples

To further understand the bacterial distribution within the microbiota, the shared OTUs between the control and JIA samples were analysed (Fig. 2). The control and JIA samples were separated and unique microbiomes were identified for both control and JIA patients, and a Venn diagram was drawn for the shared OTUs. Out of the total 1659 OTUs, 86 and 76 OTUs were unique in control and JIA patients, respectively, and 1497 were shared between them. The bacterial phyla Bacteroidetes, Firmicutes, Proteobacteria and Tenericutes were present in both the controls and the JIA patients, whereas members of Actinobacteria and Fusobacteria were present only in the JIA patients and Lentisphaerae only in the control samples.

The core microbiome analysis for the controls and JIA patients revealed a different view, as there were six and 35 OTUs in the control and JIA samples, respectively, and there were only eight OTUs shared between the groups at the 80 % confidence level (Fig. 3). There was no core microbiome remaining at confidence levels higher than 85 %. The core microbiome of the control samples was dominated by *Bacteroides* (three OTUs), Lachnospiraceae (two OTUs) and *Veillonella* (one OTU), and in JIA samples, 30 OTUs of *Bacteroides*, followed by two OTUs of *Oscillospira* and one OTU each of *Ruminococcus*, *Veillonella* and *Blautia*, were found. The shared community was dominated by genera *Bacteroides* (four OTUs), followed by *Oscillospira* (two OTUs) and one each of *Streptococcus* and family Rikenellaceae. The rarefaction curves of the faecal microbial diversity of the whole community were constructed and compared between the control and the JIA samples. The diversity of the microbial community tended to be slightly but not significantly higher in the JIA samples compared to the controls (data not shown).

Diversity indexes

We did not find any differences between JIA patients and control children in the diversity index analyses of Chao1 (median in JIA 29.0 vs. controls 28.1, NS), Simpson (median 29.5 vs. 28.4, NS) or Shannon (30.3 vs. 27.5, NS). In the entire study population, there was a correlation between age and the diversity indexes of Chao1 (Pearson's rho 0.43; $p=0.001$) and Shannon (Pearson's rho 0.34; $p=0.009$), but

Table 2 Comparison of the relative abundance (%) of bacterial flora present in the samples of patients with juvenile idiopathic arthritis (JIA) and control children. The increase or decrease in abundance are indicated by upward/downward arrows

Phylum	Genera	JIA	Control	Up/down
Bacteroidetes	Bacteroides[a]	43.60	33.29	↑
	Bacteroides uniformis	8.68	7.23	↑
	Rikenellaceae[b]	6.13	3.91	↑
	Prevotella copri	4.08	2.91	↑
	Bacteroides fragilis	3.49	5.49	↓
	Bacteroides ovatus	2.16	4.29	↓
	Bacteroides caccae	1.85	2.61	↓
	Parabacteroides	1.75	1.11	↓
	Prevotella	1.49	1.18	↓
Firmicutes	Clostridiales[b]	3.99	4.54	↓
	Ruminococcaceae[b]	3.70	5.01	↓
	Dialister	2.79	5.74	↓
	Lachnospiraceae[b]	2.31	2.96	↓
	Oscillospira	1.61	1.29	↑
	Phascolarctobacterium	1.50	2.03	↓
	Ruminococcus	1.07	1.21	↓
	Lachnospira	0.84	1.46	↓
	Veillonella dispar	0.18	1.31	↓
	Roseburia faecis	0.13	1.02	↓
Total abundance		91.33	88.60	

Increased in JIA vs. control ↑, decreased in JIA vs. control ↓

[a] $p = 0.04$

[b] Family

Table 3 Comparison of the relative abundance (%) of less abundant (<1 %) bacterial genera in the samples of patients with juvenile idiopathic arthritis (JIA) and control children. The presence/absence of particular genera in control/JIA samples are highlighted in bold

Phylum	Genera	JIA	Controls
Bacteroidetes	***Dysgonomonas***	0	0.004
	CF231	0	0.005
	Bacteroides plebeius	0.246	0
	Paraprevotella	0.471	0.154
	Odoribacter	0.229	0.246
	S24-7	0.506	0.247
	Bacteroidales[a]	0.256	0.709
	Parabacteroides distasonis	1.108	0.824
	Barnesiellaceae[b]	1.619	0.934
Firmicutes	***Clostridium butyricum***	0	0.001
	Clostridium difficile	0	0.005
	Veillonellaceae[b]	0	0.618
	Sarcina	0	0.002
	Megasphaera	0.002	0
	Eubacterium biforme	0.060	0
	Lachnobacterium	0.029	0.217
	Anaerostipes	0.049	0.256
	Blautia	0.235	0.276
	Coprococcus	0.234	0.315
	Streptococcus	0.201	0.376
	Erysipelotrichaceae[b]	0.192	0.426
	Ruminococcus gnavus	0.054	0.430
	Veillonella parvula	0.048	0.438
	Christensenellaceae[b]	0.079	0.544
	Roseburia	0.797	0.790
	Veillonella	0.104	0.815
Fusobacteria	***Fusobacterium***	0.009	0
Proteobacteria	*Sutterella*	0.901	0.767
Tenericutes	*RF39*	0.062	0.874

[a] Order

[b] Family

not in the Simpson index (0.18, NS). The correlation was not seen when only the JIA patient group was analysed. In JIA patients, the anti-nuclear antibody titre level correlated inversely with diversity indexes analysed by Spearman's rho (Simpson −0.43, $p = 0.02$, Shannon −0.42, $p = 0.02$ and Chao1 −0.38, $p = 0.04$).

Discussion

To the best of our knowledge, this is the first report showing that the intestinal microbiome is altered in JIA at the time of diagnosis and before the initiation of DMARDs or corticosteroids. At the phylum level, our JIA patients showed a significantly lower abundance of faecal Firmicutes, but a higher abundance of members of Bacteroidetes, than the control children. The high ratio of Bacteroidetes compared to the Firmicutes phylum in JIA is in accordance with findings in type 1 diabetes mellitus patients [2, 5]. At the genus level, a higher abundance of *Bacteroides* was found in patients with JIA than in controls, which is also similar to previous findings in type 1 diabetes [2, 3]. Analysis of shared OTUs between the groups revealed that *Actinobacteria* and *Fusobacteria* were present only in JIA patients, and *Lentisphaerae* was present only in control samples. A high abundance of *Actinobacteria* has also been found in faecal samples of Crohn's disease patients compared to healthy controls [22]. Finally, in parallel with a recent report from Costello et al. on patients with ankylosing spondylitis [23], OTUs of Veillonellaceae were present only in control samples. Our results indicate that a specific microbial alteration is present in JIA, and that the altered microbiome shares features reported for other autoimmune diseases, including rheumatic diseases.

Our patients with JIA had a low abundance of faecal Firmicutes and a high abundance of members of Bacteroidetes, which was in contrast to the controls. Although the possible

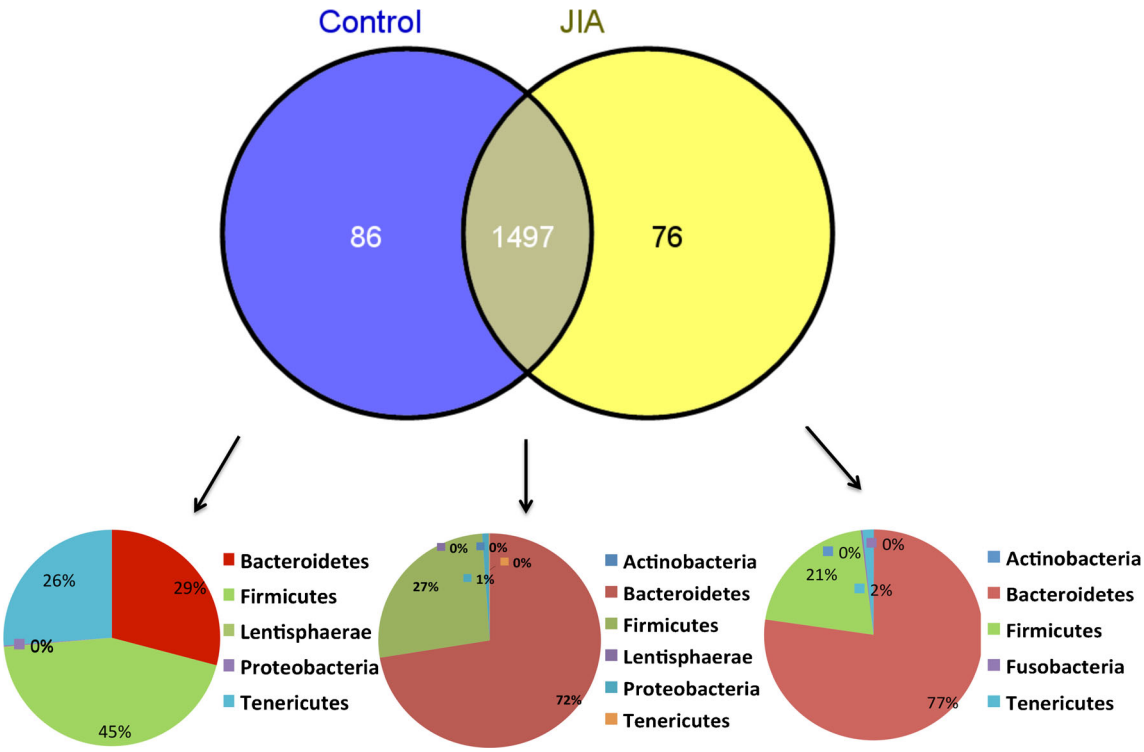

Fig. 2 Venn diagram showing unique and shared operational taxonomic units (OTUs) between control and juvenile idiopathic arthritis (JIA) samples. A pie chart of the respective proportions of phyla is shown

mechanism linking this shift with the pathogenesis of JIA remains speculative, the occurrence of a similar microbial shift in type 1 diabetes [2, 5] favours the idea that abnormal immune responses are involved [2]. Several environmental factors may contribute to such a microbial shift [24]. Of the possible dietary factors, consumption of red meat containing haem could modify the Firmicutes/Bacteroidetes ratio in the observed direction [25]. Recently, an association has been found between early exposure to antibiotics, especially clindamycin, and the development of JIA [26, 27]. Of the antimicrobial agents, exposure to clindamycin may lead to loss of Firmicutes and an abundance of the Bacteroidetes phylum in humans [24] and in animal models [28]. In spite of the association of antibiotics with JIA, we did not find an association between low bacterial diversity and JIA. However, the low diversity of Firmicutes has earlier been associated with polyarticular JIA, as presented in a congress abstract [29].

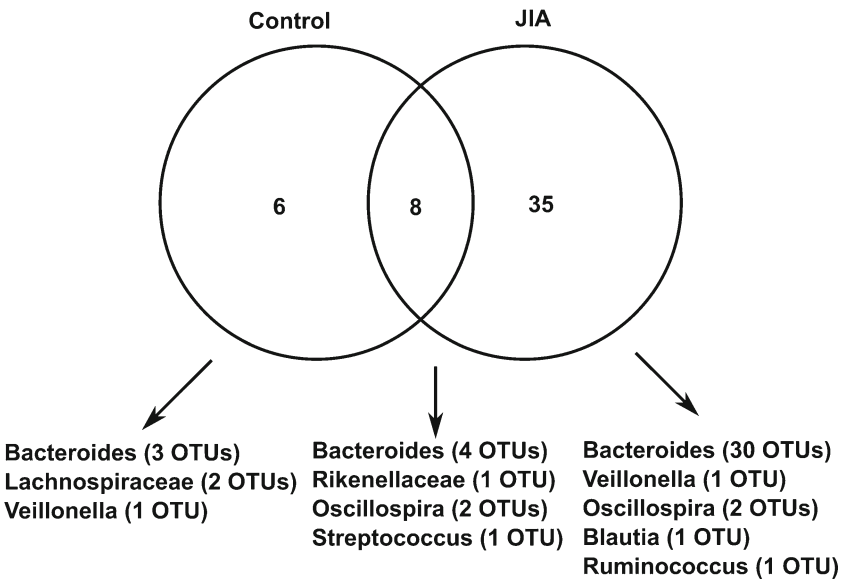

Fig. 3 Venn diagram showing the core microbiome of the control and juvenile idiopathic arthritis (JIA) samples at the 80 % confidence level. The numbers in brackets are operational taxonomic units (OTUs) present in the genera

It could be asked whether specific genetic factors of JIA are causally linked to the observed alterations in the intestinal microbial flora. Indeed, HLA-B27-positive rats have shown an increase in abundance of faecal *Prevotella* spp. and *Bacteroides* spp. and a decrease in Rikenellaceae compared with wild-type rats [30], which resembles the results in our patients with JIA. However, the observed phylogenic differences between JIA cases and controls could not be explained by HLA-B27, and the effect of HLA-B27 should be tested in a larger series because the rate of HLA-B27 positivity in our JIA patient group (20 %) was almost as low as in the general Finnish population (14 %) [31]. Other HLA classes were not analysed in our JIA series, and, so, the effect of genetic factors on microbial flora [32] cannot be excluded and further studies are needed. There are also reports of geographical differences in faecal microbial composition, such as a higher Firmicutes/Bacteroidetes ratio in children in the USA compared with children in Bangladesh [33]. Therefore, current findings in JIA should be confirmed in other populations.

A healthy mucosal commensal flora induces the mucosal barrier function and prevents excessive antigen-induced activation of the mucosal lymphoid tissues in several ways [34, 35]. Experimentally, these reactions are achieved via optimal stimuli from the luminal commensal bacterial antigens of the toll-like receptors (TLRs), which mediate induction of the synthesis of tight-junction proteins [36] and regulatory T cells [37]. Observations in children with JIA are in line with experimental evidence. In JIA, Picco et al. [15] found intestinal barrier leakiness by using the lactulose-mannitol test. More recently, in JIA patients with gastrointestinal symptoms, we observed evidence of antigen-induced activation of the intestinal mucosa, and in active JIA patients, there was low ileal mRNA levels of anti-inflammatory mediators, such as IL10, TGF-β and FoxP3, simultaneously with low TLR2 [16]. Therefore, we expected to see an alteration in the mucosal flora linked with mucosal barrier function and regulatory T cell function.

In our study, there was a higher abundance of *Bacteroides* in JIA, but a trend towards low representation of lactic acid-producing bacteria, such as *Veillonella*, *Streptococcus* and Aerococcaceae, in JIA patients compared with the controls. This finding is interesting since, in stools from diabetes-prone rats, there was a higher proportion of genera *Bacteroides* but lower proportions of *Lactobacillus* than in diabetes-resistant rats [38]. *Lactobacillus* spp. has been demonstrated to reinforce gut barrier function simultaneously with the induction of intestinal anti-inflammatory cytokine production [39]. The administration of *Lactobacillus* subspecies was also reported to inhibit the development of experimentally induced arthritis [40, 41] and to alleviate disease activity of rheumatoid arthritis [42].

In conclusion, we found alterations in the faecal microbiome in children with new-onset JIA compared to healthy children. The patterns of the microbiome in JIA show features previously reported in type 1 diabetes, Crohn's disease and ankylosing spondylitis. We suggest that the observed alterations in the microbiome play a role in the pathogenesis of JIA, as they potentially explain increased mucosal permeability and altered mucosal immunity previously demonstrated in JIA [15–18]. However, more studies are needed in order to confirm these findings in different populations and to dissect the biological mechanisms.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

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