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The impact of *Opisthorchis felineus* infection and praziquantel treatment on the intestinal microbiota in children

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ABSTRACT

The presence of some species of helminths is associated with changes in host microbiota composition and diversity, which varies widely depending on the infecting helminth species and other factors. We conducted a prospective case-control study to evaluate the gut microbiota in children with *Opisthorchis felineus* infection (n=50) before and after anthelmintic treatment and in uninfected children (n=49) in the endemic region. A total of 99 children and adolescents aged between 7 and 18 years were enrolled to the study. Helminth infection was assessed before and at 3 months after treatment with praziquantel. A complex examination for each participant was performed in the study, including an assessment of the clinical symptoms and an intestinal microbiota survey by 16S rRNA gene sequencing of stool samples. There was no change in alpha diversity between *O. felineus*-infected and control groups. We found significant changes in the abundances of bacterial taxa at different taxonomic levels between the infected and uninfected individuals. Enterobacteriaceae family was more abundant in infected participants compared to uninfected children. On the genus level, *O. felineus*-infected participants' microbiota showed higher levels of *Lachnospira, Escherichia-Shigella, Bacteroides, Eubacterium eligens* group, *Ruminiclostridium 6, Barnesiella, Oscillibacter, Faecalitalea* and *Anaerosporobacter* and reduction of *Blautia,* Lachnospiraceae FCS020 and *Eubacterium hallii* group in comparison with the uninfected individuals. Following praziquantel therapy, there were significant differences in abundances of some microorganisms, including an increase of *Faecalibacterium* and decrease of *Megasphaera, Roseburia*. Enterobacteriaceae and *Escherichia* abundances were decreased up to the control group values. Our results highlight the importance of the host-parasite-microbiota interactions for the community health in the endemic regions.

1. Introduction

There is an increase in the prevalence of chronic, non-communicable diseases, including allergic and autoimmune diseases in developed countries (Burisch et al., 2014; Lai et al., 2009). According to the "old friends hypothesis", insufficient infectious stimulation and decrease of helminthiasis prevalence are associated with chronic inflammatory disease development (Cooper et al., 2003; Wendel-Haga and Celius, 2017; Yazdanbakhsh et al., 2001). Helminth infection can also be detrimental in reducing vaccine responses, increasing susceptibility to coinfection and potentially reducing tumor immunosurveillance (Maizels and McSorley, 2016). Helminths establish long-term chronic infections associated with the suppression of the host immune response (Maizels et al., 2018; Ramanan et al., 2016; Wammes et al., 2016). This effect potentiates their long-term survival within their host, but can additionally affect the immune response to other infections and foreign

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antigens (Hsieh et al., 2014; Reynolds et al., 2017; Su et al., 2018). Multiple mechanisms contribute to the modulation of host immunity during helminth infection (Maizels et al., 2018). It is suggested that the impact on composition and function of the intestinal microbiome is one of the mechanisms by which helminths affect host immunity (Reynolds et al., 2014; Walk et al., 2010). Altered microbial composition is associated with allergic diseases, inflammatory bowel diseases, cancer and autoimmune diseases (Imhann et al., 2018; Repass et al., 2016; Tyakht et al., 2018; Wendel-Haga and Celius, 2017).

The study of the relationship between helminth infection and gut microbiota is becoming more important because they both contribute to the modulation of the immune system in various inflammatory diseases (Schachter et al., 2020; Zaiss et al., 2015). However, findings of previous studies of the effect of helminths on the microbiome are inconsistent and depend on the type, duration and intensity of helminth infection (Rosa et al., 2018; Xu et al., 2018). Previous study showed that the presence of *Opisthorchis viverrini* infection changed the composition of the gut and bile microbiome in experimental animals (Plieskatt et al., 2013). Supposedly, liver flukes and helminth-modified microbiota together lead to chronic inflammation that promotes the fibrosis and malignancies (Chng et al., 2016; Sripa et al., 2012).

The liver fluke *Opisthorchis felineus* is endemic in Europe and in large parts of the Russian Federation, particularly in Siberia, where the prevalence may exceed 50% in adults (Fedorova et al., 2018). The infection occurs through the consumption of raw or undercooked cyprinoid fish. Chronic *O. felineus* infection may lead to several severe liver and bile duct diseases, such as cholecystitis, cholangitis and periductal fibrosis and might increase the risk for cholangiocarcinoma (Gouveia et al., 2017). Recent studies indicate that a chronic *O. felineus* infection modifies the immune reactivity by increasing of the synthesis of suppressors of the immune response IL10 and TGF-β and reducing the level of proinflammatory markers IL-4, IL-5, and might have a protective effect against allergies (Fedorova et al., 2017).

There is increasing evidence that helminth-induced changes in gut microbial composition affect the host immune response (Gause and Maizels, 2016; Zaiss et al., 2015). In this regard, it is important to study the mutual influence of the intestinal microbiota and helminth infections on the development of chronic non-communicable diseases. The aim of this study was to identify potential interactions between *O. felineus* infection, praziquantel treatment and intestinal microbiota.

2. Materials and methods

2.1. Ethics statement

Study procedures were followed in accordance with the ethical standards of the Helsinki Declaration of the World Medical Association. The study protocol was approved by the Ethical Committee of Siberian State Medical University (SibMed), Tomsk (N 4981, 24.10.2016). Written informed consents were obtained from adolescents 15 years and older, and parental consents were obtained for children under 15 years old.

2.2. Study design and sampling

A prospective case-control study was conducted in Tomsk Region (Western Siberia, Russian Federation) between November 1, 2016 and June 30, 2018. We recruited two groups of children aged 7-18 years consisting of subjects infected with the helminth *O. felineus* (n=50) and uninfected by *O. felineus* and other helminths (n=50) (Fig.S1 in the supplemental material). The recruitment of the infected patients was conducted at the Regional Children's Hospital, uninfected controls were enrolled in the Center of Health for Children. Prior to the study, all subjects were asked to collect two stool samples collected on two consecutive days. Patients with *O. felineus* infection received standard treatment by praziquantel with a dose of 60 mg/kg body weight divided into three doses (at intervals of 4 hours) for one day. Follow-up period after the treatment was 3 months and included the assessment of clinical symptoms and efficacy of anthelmintic treatment via microscopy of stool samples.

Inclusion criteria for patients with *O. felineus* infection were participants who provided two stool samples for examination (microscopy) and at least one stool sample positive for the *O. felineus* infection. Inclusion criteria for uninfected controls and patients after treatment were: two stool sample negative for the *O. felineus* infection and other helminthic/protozoa infections. Exclusion criteria for patients with *O. felineus* infection, patients after treatment and uninfected children were: anthelmintic and/or antimicrobial treatment 3 months prior to the study, probiotic treatment within the 4 weeks prior to the start of the study, were co-infection with other helminths/protozoa and/or had comorbidities, less than 5,000 reads per sample according to sequencing. In total, 50 infected and 49 uninfected children and adolescents were enrolled and completed assessments (two stool samples, completed examination, sequencing results); of those, 48 infected patients finished follow-up period after the treatment. Samples from two subjects, those collected after treatment, were taken out of the analysis.

2.2.1. Interviews and physical examination

Study participants were interviewed using a pre-tested questionnaire (Zvonareva et al., 2018). Data collected was about demographic status (age, gender), risk factors for *O. felineus* infection (habitual practices of raw fish consumption, personal hygiene) and comorbidities. Study participants passed clinical examination by a physician. We assessed complaints, skin, respiratory, cardiovascular, digestive and hepatobiliary systems, height and weight. Particular attention was paid to symptoms associated with *O. felineus* infection: pain in right subcostal area, nausea, skin rash before and after treatment. The biochemical tests of the blood serum samples were carried out using DiaSys Diagnostic Systems GmbH kits (Table S1). The following indicators were measured: total protein, glucose, bilirubin total, the bound fraction bilirubin, enzymatic activity of alanine aminotransferase, aspartate aminotransferase, gamma-glutamyltransferase, and alkaline phosphatase. Ultrasonography of the liver and bile ducts was performed by a mobile, high resolution ultrasound device (Shenzhen Mindray Bio-Medical Electronics, Co, Ltd).

2.2.2. Stool examination and sample storage

Prior to the study, all subjects were asked to collect two stool samples on two consecutive days. The participants were provided with sterile plastic containers to store the samples. The stool samples were transported in cooled containers (4°C) no more than one hour to the laboratory. We used PARASEP (DiaSys Ltd, UK) for the examination of the parasite eggs. The microscopy analysis for the presence of intestinal parasites/eggs was carried out in the diagnostic laboratory of SibMed by a certified specialist on the day of sampling. The number of eggs per gram (EPG) of feces was recorded for *O. felineus* and the infection intensity was calculated. The intensity *O. felineus* infection was grouped in low (0-999 EPG), moderate (1000-9999 EPG), and severe intensities (10,000 and above EPG) (Elkins et al., 1991). Three months post-treatment the microscopy were carried out using the same procedure as described above.

For the DNA extraction, there were stool samples collected from 50 uninfected children and from patients with *O. felineus* infection before (n=50) and 3 months after treatment (n=48). All post-treatment samples sequenced for this study were helminth-negative by microscopy. The collected stool samples were frozen and stored at -80°C at the "Biobank Sibmed" department until used for DNA extraction. DNA from all stool samples was isolated less than 2 months following collection.

2.3. DNA extraction and sequencing of 16S rRNA gene

The DNA samples were isolated from each sample (150 mg) by using

FastDNA SPIN Kit for Soil (MP Biomedicals, USA) and according to the manufacturer's recommendations. The DNA samples were diluted 500 times. DNase and pyrogen free water (DES) from the FastDNA SPIN Kit for Soil was used for dilution of the purified DNA. Isolated DNA was stored at -80°C. Blank extractions were included as negative controls to monitor for bacterial contamination. The V4 hypervariable region of the 16S rRNA gene was amplified by PCR in 1 round using a modification of F515 and R806 barcoded primers (GTGBCAGCMGCCGCGGTAA and GACTACNVGGGTMTCTAATCC, respectively) with the double-index sample barcoding primer system described in (Fadrosh et al., 2014). Amplification was performed using C1000 Touch Thermal Cycler (Bio-Rad, USA) under the following cycling conditions: initial denaturation 60 s at 98°C, followed by 30 cycles of 15 s at 98°C, 15 s at 58°C and 25 s at 72°C, final extension of 5 min at 72°C. Purification of the PCR products was performed using the Cleanup Mini kit for the extraction of DNA for reagent mixtures (Evrogen, Russia). The concentration of the obtained 16S rRNA gene libraries in the solution was measured with Qubit fluorometer (Invitrogen, USA) using Quant-iT dsDNA High-Sensitivity Assay Kit. The purified amplicons were mixed equimolarly in accordance with the assessed concentrations. The quality of the library prepared for sequencing was assessed using the electrophoresis in 2% agarose gel. The final concentration of the pooled library prepared for sequencing on MiSeq platform was 12.5 pM (measured with Qubit fluorometer as described above for the individual libraries).

Sequencing of the pooled library was performed using MiSeq Reagent Kit v2 (2*250 cycles) on MiSeq sequencer (Illumina, USA) following the manufacturer's instructions. Each sample was sequenced in duplicate to even the depth of coverage. In addition to patient samples, the sequencing of isolation, amplification and sequencing of control samples also was carried out.

2.4. Bioinformatic analysis

The pooled reads were demultiplexed using deML (Renaud et al., 2015). After the quality trimming, the concatenation of the paired reads was performed using SeqPrep tool. Prior the analysis, the replicates were concatenated into one FASTQ file. Sequence processing and diversity analyses were performed with QIIME 2 version 2019.1.0 (Bolyen et al., 2019).

After being imported into the QIIME 2 artifact, all sequences were quality-checked in sliding window via quality-filter q-score-joined script, filtered with a threshold of Phred score = 30 and then dereplicated. For chimeric reads identification and removing, a de novo approach was used with vsearch uchime-denovo script. Closed-reference clustering strategy was used for operational taxonomic units (OTUs) selection with 99% similarity SILVA 132 release (Yilmaz et al., 2014) as a reference database and q2-vsearch as picking algorithm. OTUs taxonomic classification was performed using q2-feature-classifier plugin with naive Bayes classifier trained on 515F/806R region of 16S rRNA gene sequences from SILVA database. Technical control samples were manually checked and the reads identified as contaminants were subtracted from the microbiome samples. Samples containing less than 5000 reads were excluded from further analysis.

Prior to the taxonomic richness and α-diversity computation, the samples were rarefied to the depth of 5000 classified reads. Faith index, Shannon index, and the number of observed OTUs were calculated for each of the samples at the level of OTUs. The DEICODE plugin of QIIME 2 with default parameters was used to calculate pairwise samples dissimilarity (β-diversity)(Martino et al., 2019). Beta-diversity was calculated for all samples and separately for the group of *O. felineus*-infected before and after treatment, and uninfected individuals.

2.5. Statistical analysis

Statistical analysis was conducted in R language, version 3.6.1(R Core Team, 2019). Differences in measured clinical symptoms, biochemical and ultrasonography parameters of *O. felineus*-infected and uninfected individuals were assessed with Mann-Whitney-Wilcoxon test (for quantitative variables) and with chi-square test (for qualitative variables).

The three-groups (*O. felineus*-infected, after treatment, and uninfected) alpha-diversity comparison was implemented using a linear mixed model with *O. felineus* infection status as a fixed effect and patient's identifier as a random effect (Bates et al., 2015). For *post hoc* analysis, the difflsmeans function of lmerTest package was used (Kuznetsova et al., 2017). Associations between infection intensity and alpha-diversity indices were examined with negative binomial regression on *O. felineus* infected group (Venables and Ripley, 2002). The *O. felineus* EPG counts value was used as a dependent variable.

Beta-diversity analysis was performed for all samples and the groups of infected patients and uninfected controls. On whole sample, pairwise permutational analysis of variance (PERMANOVA) for distance matrices with 9999 permutations was performed on all samples to check the separation between *O. felineus*-infected, after treatment, and uninfected (R Core Team, 2019). For the *O. felineus*-infected and uninfected individuals, the impact of different clinical factors, including complaints, biochemistry, results of physical and ultrasound examination along with helminthic infection was tested in multivariate adonis model (Oksanen et al., 2019).

Prior to the statistical analysis of taxa abundance, OTU table was exported to R with import_biom function, rarefied to the depth of 5000 reads per sample and aggregated on the levels of phyla, families, genera, and species with tax_glom function (McMurdie and Holmes, 2013). All phylogenetic levels were inspected separately.

-Analyses of taxa abundance were performed using a generalized linear mixed model with negative binomial distribution for taxa abundance (Brooks et al., 2017). Infection status was selected as a fixed effect and patient's identifier as a random effect. All models were checked with II-type ANOVA (Therneau, n.d.); for the significant models, the estimated marginal means were calculated in each group, and Tukey *post hoc* analysis was performed (Lenth, 2019). Associations of infection intensity and microbial phylotypes abundance were identified similarly to α-diversity.

In case of multiple hypotheses testing, the p-values were adjusted with FDR method and referred as q-values. Visualization was performed with ggplot2 package (Wickham, 2006). Additional exploratory analysis of the microbiome data was performed using Knomics-Biota platform (Efimova et al., 2018)

3. Results

3.1. Characteristics of the study cohort

The study groups included 99 children (50 patients with *O. felineus* infection and 49 uninfected subjects). Two samples collected at post-treatment were excluded: one subject used antibiotics, another one refused to perform stool sample collection.

Participants' age ranged from 7 to 18 years old, with a median age of 11 (8-14) for *O. felineus* infected patients and 10 (8-13) for the control group. Age and gender distribution were similar between the two groups. Table 1 summarizes the participants' characteristics: clinical symptoms, biochemical tests, abdominal ultrasound. Among the most common complaint in *O. felineus* infected patients was pain in abdomen ($q<0.01$), in particular, pain in the right subcostal area. According to the results of the biochemical test of the blood serum, the level of glucose and the activities of alkaline phosphatase, alanine aminotransferase, and gamma-glutamyltransferase were significantly higher in *O. felineus* infected participants than in uninfected ones. The levels of protein, total and conjugated bilirubin were statistically significantly lower in infected patients (Table 1). After anthelmintic treatment, the frequency of abdominal pain (94% vs 40%, $q<0.01$) and other clinical symptoms

Table 1
The clinical and demographic characteristics of participants of the study.

Characteristics	uninfected (n=49)	*O. felineus*-infected (n=50)	adjusted *p*-value
Male, %*	42.86	30	0.401
Female, %*	57.14	70	0.401
Years**	10 (8;13)	11 (8.25;13.75)	0.463
Body Mass Index**	16.64 (15.11;18.67)	17.19 (15.46;20.67)	0.463
Clinical symptoms, %*			
Abdominal pain	22.45	94	<0.001
Pain in right subcostal area	12.24	56	<0.001
Abdominal pain in other sites	14.29	82	<0.001
Nausea	32.65	52	0.141
Pyrosis	14.29	8	0.590
Diarrhea	14.29	6	0.435
Constipation	22.45	22	0.956
Weakness	34.69	18	0.158
Skin rash	18.37	24	0.715
Biochemistry**			
Protein, g/L	76 (72.75;78)	73 (70;75.925)	0.011
Glucose, mmol/L	4.05 (3.6;4.6)	4.55 (4.4;4.7)	2.00E-004
Bilirubin total, μmol/L	14.65 (11.73;16.8)	9.35 (6.85;13.95)	<0.001
Bilirubin conjugated, μmol/L	2.8 (2.53;3)	1.35 (1;2.1)	<0.001
Alanine aminotransferase, U/L	9 (7;11.75)	12 (10;14.75)	9.00E-004
Aspartate aminotransferase, U/L	19 (14.25;24)	22.5 (18;27)	0.054
Gamma glutamyl transferase, U/L	14 (11;16)	17.5 (15;21)	<0.001
Alkaline phosphatase, U/L	187.5 (148;240)	431.5 (280.75;582.5)	<0.001
Abdominal ultrasound			
Gallbladder wall thickened, %*	14.29	20	0.708
Oblique vertical size, mm **	114 (106;126)	113 (106.25;123.25)	0.590
Gallbladder length, mm **	59 (55;66)	65 (60;72.75)	0.055

* - Chi-square test
** Median (Q1;Q3), Mann-Whitney-Wilcoxon test
P < 0.05 indicates significant different.

tended to decrease (Table S2).

According to results of the ultrasound examination, we found a trend towards gallbladder length increase and wall thickening in infected subjects; however, these differences were not statistically significant (Table 1).

Infection intensity varied between 2 and 3800 eggs/gram, and the mean rate count was 363±1004 EPG of feces, with 92.8% of participants having low intensity infection (2-500 EPG).

3.2. Sequencing results

There were 153 samples (148 fecal samples and 5 technical blank controls) prepared for sequencing. The Illumina sequencing produced 3,736,220 reads in total. Quality check and chimeric reads exclusion resulted in 3,549,659 reads retained. After filtering of contaminant sequences according to negative controls, there were 3,300,456 reads left for which 13,415 OTUs were identified. One sample in the uninfected group was excluded from further analysis due to low coverage (less than 5,000 reads per sample). A total of 147 samples were included in the further analysis. The number of reads per sample was 21,576 [16,621; 25,594] (Median [Q2; Q4]).

3.3. Microbial diversity, helminthic infection and treatment

Taxonomic richness and α-diversity of subjects' gut microbiota was calculated using Faith and Shannon indices as well as the number of observed OTUs at a depth of 5000 sequences per sample. There were no differences in α-diversity between *O. felineus*-infected, uninfected, and treated patients for any of these indices (Fig. 1A to C; Table S3; Fig. S2). However, we identified significant positive correlation between infection intensity pretreatment and richness and α-diversity for each of the 3 metrics (Fig. 1D to F). One patient with an outlying infection intensity of 3800 EPG of feces was excluded only from the EPG and bacterial abundance associations testing for the purpose to control false positives associations.

PERMANOVA applied to all samples showed separation between *O. felineus*-infected and uninfected microbiomes (R2=0.053, p=0.006), as well as between *O. felineus*-infected and treated groups (R2=0.072, p=0.002) (Fig. 2). There was no significant differences in gut microbiota between samples collected from infected children before and after anthelmintic treatment (R2=0.018, p=0.168). Significant associations were observed for *O. felineus* infection (R2=0.065, *p*=0.002), age (R2=0.038, *p*=0.017), and skin rash presence (R2=0.033, *p*=0.028) (Table S4).

3.4. Gut microbiota composition and O. felineus infection

On the level of phyla, patients with *O. felineus* infection had depletion in Firmicutes and increase of Proteobacteria abundance in gut microbiome compared to healthy controls (Table 2).

On the family level, *O. felineus* infection was linked to an increase of Enterobacteriaceae in the gut microbiota of children (Table 2).

Among the genera positively associated with the liver fluke infection, we identified *Lachnospira, Escherichia-Shigella, Bacteroides, Eubacterium eligens* group, *Ruminiclostridium* 6, *Barnesiella, Oscillibacter, Faecalitalea,* and *Anaerosporobacter*. Healthy volunteers showed higher levels of *Blautia, Lachnospiraceae* FCS020 group and *Eubacterium hallii* group in comparison with the infected individuals (Table 2).The observations were generally consistent with the results of a complementary pipeline available online as interactive reports at https://biota.knomics.ru/opisthorchis-and-gut-microbiome-2019.

3.5. Infection intensity and gut microbiota

For the *O. felineus*-infected patients, negative binomial regression was performed to identify associations between the infection intensity and the levels of microbial taxa. On the level of phyla, positive associations were identified for Firmicutes and Tenericutes, and negative associations were identified for Verrucomicrobia and Proteobacteria (Fig. S3A). On the family level, Desulfovibrionaceae and Erysipelotrichaceae showed direct associations with infection intensity, and inverse correlations were found for Bifidobacteriaceae (Fig. S3B). Among the genera positively associated with the infection intensity we identified *Catenibacterium, Desulfovibrio, Mitsuokella, Ruminococcaceae* UCG-003, *Enterorhabdus, Howardella, Olsenella, Moryella, Rikenellaceae* RC9 gut group, *Candidatus Soleaferrea, Senegalimassilia, Eubacterium coprostanoligenes* group. *Erysipelatoclostridium, Anaerostipes, Bifidobacterium, Eubacterium hallii* group, *Ruminococcus gnavus* group, *Gordonibacter* and *Akkermansia* abundances demonstrated negative associations with the number of *O. felineus* EPG of faeces (Fig. S3C).

3.6. Liver fluke treatment affects gut microbiota structure

After anthelmintic treatment Firmicutes abundance was increased whereas Proteobacteria level was decreased to the uninfected participants' proportions (Table 2). Enterobacteriaceae content also was decreased to the levels observed in uninfected children. Drug administration led to a decrease of Synergistetes and Atopobiaceae levels compared with *O. felineus*-infected and control groups (Table 2).

Atthe genus level, in gut microbiota of praziquantel-treated patients we identified the reduction of *Lachnospira, Ruminiclostridium* 6,

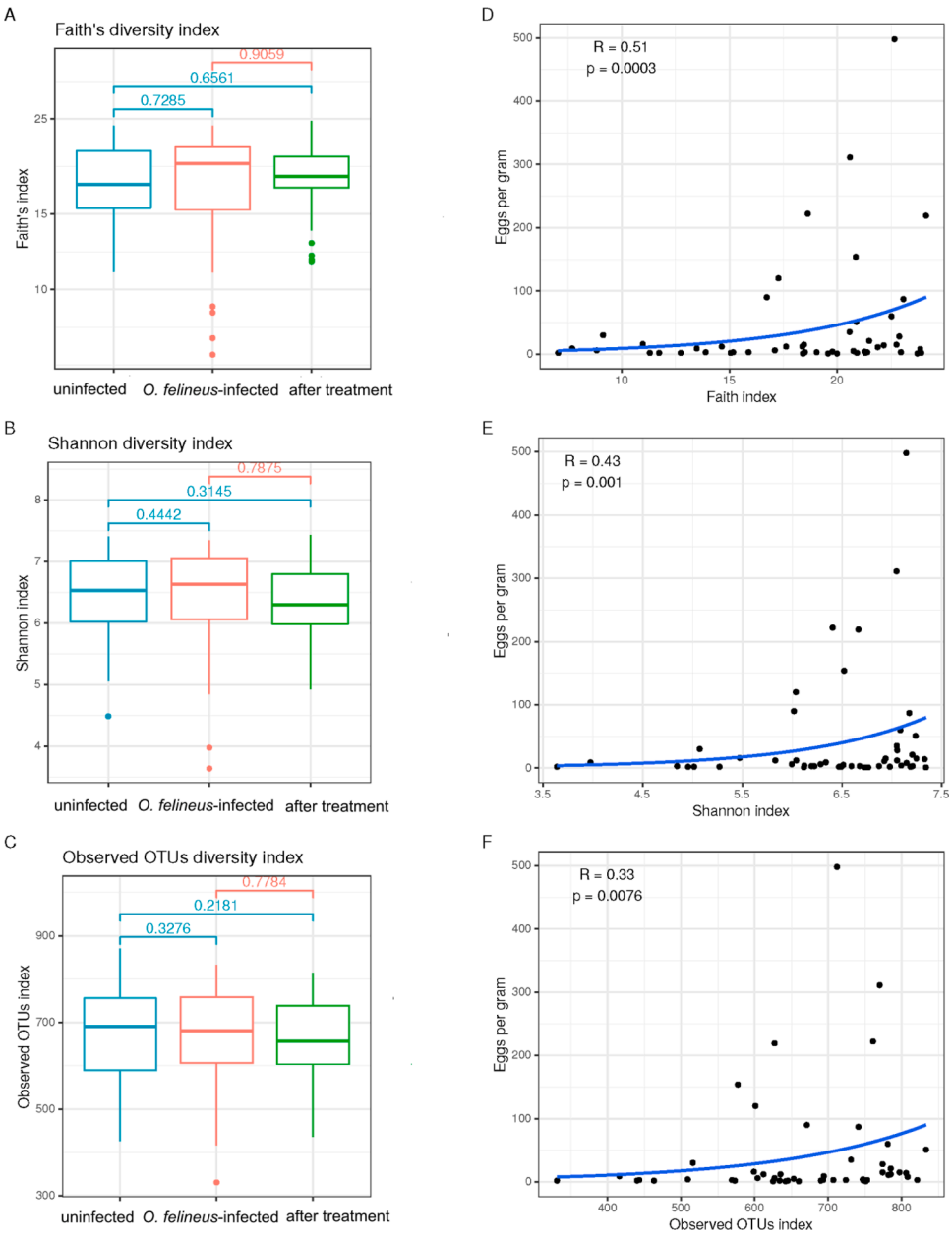

Fig. 1. Alpha diversity plots.
On the figure, plots A, B, and C represents Faith and Shannon diversity indices along with the number of observed OTUs respectively measured for gut microbiota of studied cohort. Boxes represent the interquartile range (IQR) between the first and third quartiles, and the line inside represents the median. Whiskers denote the lowest and the highest values within 1.5 IQR from the first and third quartiles, respectively. Individual points represent outliers beyond the whiskers. Plots D, E, and F represents associations between infection intensity measured as the number of *O. felineus* eggs per gram of feces and the Faith index, Shannon index and the number of observed OTUs for infected children.

Anaerosporobacter and *Escherichia-Shigella* abundances along with increase in *Blautia, Eubacterium hallii* group and *Lachnospiraceae* FCS020 group levels up to the control group values (Table 2). Additionally, we observed the depletion of *Roseburia, Megasphaera* levels and expansion of *Faecalibacterium, Eisenbergiella, Sellimonas, Olsenella, Varibaculum, Rikenellaceae* RC9 gut group, *Erysipelatoclostridium* in gut microbiota after treatment compared with *O. felineus*-infected and control groups. *Flavonifractor* and *Leuconostoc* had higher proportions in gut microbiota after treatment compared with controls (Table 2). In addition, praziquantel treatment results in reduction of *Ruminococcus gauvreauii* group, *Howardella* and *Slackia* abundances in gut microbiome compared with controls and, for *Lachnospiraceae* NK4A136 group and ND3007 group, *Tyzzerella* 4, *Prevotella* 2, in comparison with *O. felineus*-infected patients (Table 2). Praziquantel-treatment did not reduce levels of *Bacteroides, Oscillibacter, Faecalitalea, Eubacterium eligens* group and *Barnesiella* augmented in the gut microbiota of *O. felineus*-infected individuals (Table 2).

4. Discussion

In this study, we described for the first time the changes in the gut microbiota of *O. felineus*-infected children before and after anthelmintic treatment based on oral praziquantel application in an endemic rural area. Neither the presence of *O. felineus* infection nor the praziquantel treatment were associated with differences in the alpha-diversity, richness, or clinical symptoms compared with the control group. In other studies the analysis of the microbiota by high-throughput sequencing revealed that infection with the liver flukes *O. viverrini* and *C. sinensis* lead to increases in alpha-diversity in microbial communities in the colon (Plieskatt et al., 2013; Xu et al., 2018). Analysis of the composition of the bile microbiota in the presence of *O. felineus* infection was carried out in two studies (in experimental animals and patients with cholelithiasis) using the sequencing of the 16S rRNA gene (Saltykova et al., 2016, 2017). In an experimental study, the *O. felineus* infection was associated with increased bacterial diversity in the bile ducts as assessed by the Chao index (Saltykova et al., 2017). The study of patients with cholelithiasis showed that the presence of *O. felineus* infection did not

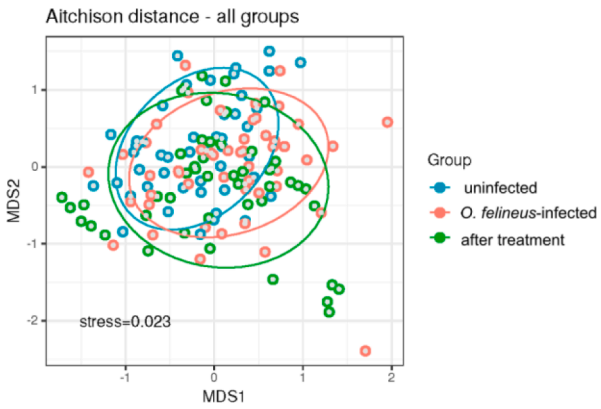

Fig. 2. Beta-diversity analysis.
Non-metric multidimensional scaling in Aitchison metric for gut microbiota of all study cohort. Each point represents a sample and points with the same color belong to the same group.

lead to increased diversity of bile microbiota (Saltykova et al., 2016). However, we found significant positive associations between the *O. felineus* infection intensity and gut community structure in children. We did not detect increased alpha-diversity of the microbiota, probably due to the low intensity of invasion in the study group. Additionally, the *O. felineus* infection is endemic and hence may have already left its imprint on the gut microbiota composition of all individuals in the study region (Easton et al., 2019).

Many microbial taxa were differentially abundant between the infected and non-infected individuals. In the main, significant microbiota changes were observed within the Firmicutes phylum, including the Clostridales order. This result was consistent with some previous studies of the gut microbiota in subjects infected by soil-transmitted helminths. (Cooper et al., 2013; Rosa et al., 2018).

Our data revealed that *O. felineus* infection is associated with an increase in the relative abundance of pro-inflammatory Proteobacteria, mainly Enterobacteriaceae, and a decrease in the relative abundance of commensal Firmicutes (*Blautia, Eubacterium hallii* group, *Lachnospiraceae* FCS020 group). At the same time, at the genus level we observed an increase in the abundance of some bacteria from the Firmicutes compared with the control group. In contrast, a positive correlation was identified for Firmicutes and a negative association was found for Proteobacteria with the infection intensity. The decrease in Firmicutes and an increase in Proteobacteria taxa is one of the signatures of the microbial dysbiosis in inflammatory bowel diseases (IBD) (Gophna et al., 2006). Enterobacteriaceae, including *Escherichia*, were also increased in *O. felineus* infected samples. This family is associated with the liver fluke *O. viverrini* infection in hamsters (Plieskatt et al., 2013). Helminth-induced changes in gut microbial diversity can participate in the development of hepatobiliary diseases (Carpenter, 1998; Itthitaetrakool et al., 2016; Sanduzzi Zamparelli et al., 2017; Scholte et al., 2018). A number of studies demonstrated that patients with liver cirrhosis and primary biliary cholangitis showed a significant increase in the abundance of the Enterobacteriaceae family compared with the healthy group (Liu et al., 2012; Lu et al., 2011). There is growing evidence that the numbers of the Enterobacteriaceae are linked to IBD (Nishida et al., 2018; Swidsinski et al., 2002; Tyakht et al., 2018; Varela et al., 2013). A number of studies have shown that patients with celiac disease, ulcerative colitis, and Crohn's disease have an increased proportion of *E. coli* in the gut microbiota compared to healthy individuals (Collado et al., 2009; Mondot et al., 2011; Nadal et al., 2007; Swidsinski et al., 2002).

Compared to healthy participants, *O. felineus*-infected patients manifested a significant decrease of *Blautia, Eubacterium hallii* group, and an increase of *Lachnospira* abundances, that may produce anti-inflammatory short-chain fatty acids (SCFAs) (Park et al., 2015, 2012). One of the potential biomarkers of a healthy gut often considered in microbiome surveys is the relative abundance of butyrate-producing microbes (Chang et al., 2014; Machiels et al., 2014). Bacteria that ferment dietary fibers to produce SCFAs are typically reduced in mucosa and feces of patients with IBD, as compared to healthy individuals (Kumari et al., 2013; Morgan et al., 2012; Takahashi et al., 2016). Infection with intestinal helminthes probably increases bacterially produced SCFAs concentrations, and, as a result, allergic airway inflammation is attenuated in the host (Zaiss et al., 2015). On the other hand, the ability of SCFAs to promote host regulatory T-cell responses may contribute to limited immuno-pathology and promotes chronic parasite infection (Arpaia et al., 2013; Furusawa et al., 2013). However, we did not directly measure intestinal SCFAs levels. More studies are needed to dissect this effect of *O. felineus* infection.

At the genus level, the abundance of *Eubacterium eligens* group was significant increased in the *O. felineus*-infected children. *E. eligens* is a pectin-degrading Firmicutes species that has the anti-inflammatory potential by promoting the production of IL-10 by epithelial cells (Chung et al., 2017). Two taxa belonging to Bacteroidetes were significantly increased in *O. felineus* infected patients. Consistent with previous reports, several Bacteroides species can produce enterotoxin previously associated with ulcerative colitis, stimulate interleukin-8 (IL-8) and tumor necrosis factor alpha (TNF-α) secretion in intestinal epithelial cells and intensify colitis symptoms in a murine model of ulcerative colitis (Ohkusa et al., 2009), while some strains of this genus have anti-inflammatory effects (Gauffin Cano et al., 2012; Hiippala et al., 2020; Ihekweazu et al., 2019). In previous studies, the lower abundance of Bacteroides was observed in the *C. sinensis* infected patients (Xu et al., 2018). Since changes were observed in populations of several bacteria with putative pro-/anti-inflammatory properties, whether the gut microbiota affected by the *O. felineus* infection could either contribute to or protect from inflammation is difficult to ascertain and, therefore, further analyses are required.

We also evaluated the association between intensity of helminth infection and gut microbiota composition. Bifidobacteriaceae, including genus *Bifidobacterium*, and *Akkermansia* were negatively correlated with the number of *O. felineus* EPG of feces. *Bifidobacterium* is an important probiotic bacterium, considered as potentially anti-inflammatory taxa (Khokhlova et al., 2012). As it shown in a previous study, the abundance of this genus also has a negative correlation with EPG of feces in *C. sinensis*-infected individuals (Xu et al., 2018). *Akkermansia* is a mucin-degrading bacteria, reduction in their abundance has been associated with inflammation and alterations of metabolic processes (Verhoog et al., 2019). On the family level, Desulfovibrionaceae and Erysipelotrichaceae showed direct associations with infection intensity. These taxa were implicated in the development of obesity-related metabolic disorders (Zhang et al., 2010). Apparently, the gut microbiome signature of *O. felineus* combines features known to be both directly and inversely associated with different disorders highlighting the complex nature of the helminth's interactions with the host and commensal microbiome.

Our results show that deworming was not associated with changes in microbial alpha diversity, however, some significant differences were seen in the abundances of some microorganisms in the gut microbiota of the infected children before and after anthelmintic treatment. Specifically, the abundance of bacteria belonging to the Enterobacteriaceae family, *Escherichia, Lachnospira, Anaerosporobacter, Ruminiclostridium 6*, and *Blautia* genera (altered by *O. felineus* infection) after treatment resembled the levels typical for the control group. Conversely, the disease-specific shifts for some taxa persisted as long as 3 months after treatment (*Barnesiellaceae, Bacteroides*). At the phylum level, praziquantel treatment was associated with a decreased relative abundance of Synergistetes. This phylum is associated with periodontal diseases (Belibasakis et al., 2013; McCracken and Nathalia Garcia, 2020). In the other study, a higher proportion Synergistetes was evident in the group of participants infected with *O. felineus* in comparison with the

Table 2
Significant differences in the abundances of gut bacteria (at several taxonomic levels) between comparison groups.

Phylotype	Estimated marginal means			ANOVA *p*-value		Between group *p*-value		
	uninfected (SE)	*O. felineus*-infected (SE)	after treatment (SE)	P	adjusted p-value	uninfected vs *O. felineus*-infected	uninfected vs after treatment	*O. felineus*-infected vs after treatment
Proteobacteria	71.64 (14.31)	257.19 (54.49)	96.67 (17.96)	2.58E-06	2.32E-05	1.57E-05	0.520	0.001
Firmicutes	3757.84 (118.74)	3287.10 (102.87)	3717.67 (118.70)	0.004	0.017	0.009	0.969	0.018
Synergistetes	0.37 (0.33)	0.59 (0.51)	0.01 (0.01)	0.014	0.041	0.916	0.039	0.012
Enterobacteriaceae	31.63 (9.94)	150.61 (48.97)	21.03 (6.76)	1.11E-06	2.51E-05	0.001	0.616	4.80E-06
Atopobiaceae	12.44 (4.90)	6.69 (3.24)	0.57 (0.24)	1.23E-07	5.53E-06	0.508	5.36E-07	0.0002
Escherichia-Shigella	27.71 (9.21)	131.61 (45.48)	18.66 (6.21)	6.00E-06	0.0004	0.002	0.662	1.92E-05
Lachnospira	2.67 (0.78)	9.70 (2.83)	4.04 (1.27)	0.003	0.026	0.004	0.570	0.048
Anaerosporobacter	0.18 (0.10)	1.50 (0.70)	0.02 (0.02)	0.0002	0.004	0.013	0.187	0.001
Ruminiclostridium 6	1.72 (0.73)	6.24 (2.33)	1.69 (0.66)	0.006	0.035	0.022	0.999	0.017
Eubacterium eligens group	3.42 (1.13)	13.00 (4.53)	5.63 (1.73)	0.007	0.035	0.006	0.476	0.117
Faecalitalea	0.02 (0.02)	0.52 (0.29)	1.21 (0.66)	0.005	0.035	0.030	0.004	0.531
Bacteroides	105.37 (24.28)	238.04 (50.03)	235.06 (51.92)	0.006	0.035	0.014	0.017	0.999
Barnesiella	2.54 (0.81)	7.81 (2.80)	8.97 (3.19)	0.007	0.035	0.026	0.011	0.937
Blautia	789.02 (90.70)	444.76 (50.65)	685.46 (79.62)	0.001	0.015	0.002	0.666	0.024
Eubacterium hallii group	182.82 (22.52)	110.84 (13.55)	123.77 (15.43)	0.010	0.048	0.013	0.070	0.803
Lachnospiraceae FCS020 group	13.90 (2.13)	6.96 (1.09)	10.02 (1.57)	0.007	0.035	0.005	0.296	0.229
Sellimonas	1.49 (0.76)	1.08 (0.55)	19.40 (9.71)	5.03E-05	0.001	0.895	0.001	0.0002
Eisenbergiella	1.18 (0.58)	0.88 (0.43)	9.40 (4.51)	0.001	0.013	0.905	0.008	0.002
Faecalibacterium	0.90 (0.32)	2.07 (0.61)	5.94 (1.79)	9.19E-06	0.0004	0.124	3.47E-05	0.005
Erysipelatoclostridium	1.13 (0.45)	1.10 (0.37)	3.40 (1.38)	0.010	0.046	0.998	0.033	0.024
Varibaculum	0.02 (0.02)	0.18 (0.10)	2.48 (1.22)	1.80E-05	0.001	0.194	0.0004	0.002
Megasphaera	16.96 (11.86)	18.52 (12.82)	0.04 (0.04)	3.79E-07	5.24E-05	0.996	6.78E-06	4.59E-06
Roseburia	54.37 (9.86)	53.60 (9.38)	27.80 (4.97)	0.003	0.031	0.998	0.018	0.009
Olsenella	3.51 (1.42)	2.26 (0.91)	0.25 (0.12)	9.30E-05	0.002	0.722	0.0002	0.002
Rikenellaceae RC9 gut group	2.31 (1.36)	2.70 (1.57)	0.13 (0.09)	0.001	0.017	0.980	0.006	0.003
Leuconostoc	0.12 (0.10)	0.52 (0.37)	4.60 (3.24)	0.003	0.026	0.374	0.003	0.079
Flavonifractor	0.36 (0.16)	0.95 (0.33)	1.68 (0.57)	0.003	0.026	0.091	0.003	0.170
Oscillibacter	0.99 (0.26)	2.19 (0.53)	3.05 (0.88)	0.005	0.035	0.050	0.005	0.554
Howardella	3.39 (1.10)	1.14 (0.39)	0.79 (0.28)	0.006	0.035	0.055	0.008	0.738
Ruminococcus gauvreauii group	45.35 (9.55)	26.35 (5.86)	18.38 (4.13)	0.007	0.035	0.139	0.006	0.398
Slackia	3.04 (0.87)	1.96 (0.57)	0.79 (0.25)	0.007	0.036	0.531	0.006	0.096
Lachnospiraceae NK4A136 group	11.35 (2.13)	18.14 (3.34)	6.23 (1.21)	0.0003	0.006	0.179	0.071	0.0003
Lachnospiraceae ND3007 group	6.15 (1.53)	7.86 (1.97)	3.04 (0.93)	0.009	0.044	0.703	0.084	0.009
Prevotella 2	2.14 (1.28)	11.62 (6.78)	0.75 (0.46)	0.005	0.035	0.109	0.441	0.004
Tyzzerella 4	1.45 (0.74)	3.10 (1.54)	0.25 (0.14)	0.004	0.032	0.534	0.061	0.003

SE, Standard Error. $P < 0.05$ indicates significant differen

uninfected participants (Saltykova et al., 2016). We found a significant decrease in the proportion of bacteria *Megasphaera, Roseburia*, and *Olsenella* in praziquantel-treated patients compared to infected and control participants. It has been reported that patients with cirrhosis and primary biliary cholangitis have a higher level of *Megasphaera* compared to healthy controls (Chen et al., 2016; Lv et al., 2016). *Olsenella* also is positively associated with soil-transmitted helminths infections across Liberia and Indonesia (Rosa et al., 2018). We observed that abundance of *Olsenella* directly correlates with the *O. felineus* infection intensity. Species of *Roseburia* genus are known to be important butyrate-producing bacteria (Louis and Flint, 2009). The abundance of other butyrate-producing bacteria, namely *Faecalibacterium*, was increased in praziquantel-treated patients in comparison with infected or control participants.

The data presented here have some limitations. In our study, participants were examined one time posttreatment. The 3-month time point allowed us to examine the longer-term impact of helminth clearance. This study was not aimed to estimate the data regarding the effect of short term removal of helminths on microbiota and the impact of praziquantel treatment on uninfected children. The V4 region of 16S rRNA was analyzed. Although sequencing a longer region would result in a more precise taxonomic classification of each read, the V4 format allows a more accurate estimate of the gut archaea levels than V3-4 (due to variability in amplicon length for these prokaryotes). Moreover, V4 region is widely used in human gut microbiome surveys (Klimenko et al., 2018; McDonald et al., 2018; Wei et al., 2020). As each gene region produces a certain bias, an alternative would be to use a mix of primers covering multiple gene regions (Barb et al., 2016; Tremblay et al., 2015).

5. Conclusion

We obtained new data regarding the impact of *O. felineus* infection and praziquantel treatment on the intestinal microbiome, and some of these effects were associated with the infection intensity. These results could be used to the prevention, and treatment of *O. felineus* infection, and the potential development of new strategies for the control of chronic non-communicable diseases. This requires further studies.

Data availability

The data sets supporting the conclusions of this article are available in the European Nucleotide Archive (accession number PRJEB35995).

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actatropica.2021.105835.

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