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ORIGINAL ARTICLE

Tonsillar microbiota in children with PFAPA (periodic fever, aphthous stomatitis, pharyngitis, and adenitis) syndrome

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Abstract Periodic fever, aphthous stomatitis, pharyngitis, and adenitis (PFAPA) is a childhood febrile syndrome of unknown origin that is often cured with tonsillectomy. We aimed to compare the bacterial microbiota of the tonsils removed from PFAPA patients with those of controls. We used next-generation sequencing technology to investigate the bacterial microbiota of the tonsils of 30 PFAPA patients and 24 controls. We found significant differences in the presence and relative abundance of many bacteria between PFAPA cases and controls. For example, cyanobacteria, potential producers of microcystins and other toxins, were more common in the case samples (14/30, 47 %) than in the controls (4/24, 17 %, $p = 0.02$), and the mean relative abundance of cyanobacteria was higher in the case samples (0.2 %) than in the controls (0.01 %, $p = 0.01$). Streptococci were present in all samples in both groups, but their mean relative abundance was lower in the case samples (3.7 %) than in the controls (9.6 %, $p = 0.01$). Typical nasopharyngeal microbes such as fusobacteria, *Prevotella*, *Tannerella*, *Porphyromonas*, and *Parvimonas* dominated the microbiota of the tonsils in both groups. The microbiota of the tonsils removed from PFAPA patients differed significantly from those of the controls. Tonsillar microbiota may play a role in triggering the inflammatory processes that lead to symptoms of PFAPA.

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Introduction

Periodic fever, aphthous stomatitis, pharyngitis, and adenitis (PFAPA) is a childhood febrile syndrome in which the fever episodes occur in regular three- to five-week cycles. Between febrile episodes, patients are asymptomatic [1, 2]. The etiology and pathogenesis of PFAPA are unknown. Acute phase parameters such as leukocyte counts, the erythrocyte sedimentation rate, C-reactive protein, and the excretion of proinflammatory cytokines increase during the fever episodes [3–5]. A single dose of corticosteroid rapidly reduces the fever, but does not prevent subsequent episodes [2, 6]. Calculations of the cumulative incidence of PFAPA estimate that the illness affects about 2 in 10,000 children up to 5 years of age [7], making PFAPA the most common periodic childhood febrile syndrome.

Randomized controlled studies indicate that tonsillectomy may be a curative treatment for PFAPA syndrome [8, 9], but the mechanism of this effect remains unclear. Recent studies support the hypothesis that PFAPA syndrome is a disease involving dysregulated IL-1β production [10–12]. The regulation of IL-1β production is closely related to a molecular complex known as inflammasome, which may be activated by environmental stimuli, such as the presence of microbes [13, 14]. No single infectious agent has been identified as a cause of PFAPA. Changes in the entire microbiota and interactions between the microbiome and the underlying lymphoid tissue in the tonsils may be important in the activation of inflammatory reactions.

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With conventional microbiological methods, we have previously shown that the microbiology of the tonsil samples from the PFAPA cases differed from those of the controls [15]. To investigate this issue further, we used next-generation sequencing technology to compare the bacterial microbiotas of the tonsils of PFAPA patients to those of the controls.

Methods

Patients and controls

Between March 2006 and April 2010, we recruited 31 consecutive children who underwent tonsillectomy due to PFAPA and 24 control children undergoing tonsillectomy due to clinical suspicion of obstructive sleep apnea and hypertrophied tonsils in order to study the microbiology and biofilm formation on their tonsils [15]. We stored 30 tonsil samples from PFAPA patients and 24 control samples at −80 °C and later used them for microbiota analyses. A questionnaire served to collect data on the children's symptoms prior to surgery. The median age of the PFAPA patients at the onset of the fever periods was 2.4 years (Table 1). The average duration of the PFAPA symptoms prior to tonsillectomy was 12 months. The mean of the maximum fevers was 39.7 °C, and the mean duration of the febrile episodes was 3.9 days. The mean time interval between the first febrile days of two subsequent febrile episodes was 26 days. At the time of surgery, the median age in the PFAPA group was 3.4 years and that in the control group 5.8 years. The symptoms of all PFAPA patients resolved after tonsillectomy.

From the Finnish national Drug Purchase Register, maintained by the Social Insurance Institution of Finland (Kela), we obtained information on the children's previous use of any antimicrobials in the 12 months prior to tonsillectomy. Exposure to antimicrobials in the 12 months prior to tonsillectomy was higher in the PFAPA group (mean number of antimicrobial courses 2.4) than in the control group (1.4), but the difference was not statistically significant [95 % confidence interval (CI) for the difference −0.25 to 2.2, $p = 0.09$] (Table 1). Descriptions of the other patient details and surgeries appear in a previous publication [15]. The parents provided their written informed consent, and the Ethics Committee of the Northern Ostrobothnia Hospital District, Oulu, Finland, approved the study plan.

DNA extraction

We used Ion Torrent high-throughput sequencing technology to study the bacterial microbiota of the 54 tonsil samples. We extracted DNA from the samples with a DNeasy Blood & Tissue Kit (Qiagen, USA). After thawing the tissues, we added 180 μl of ATL buffer and 20 μl of proteinase K, and incubated the tissue samples at 56 °C until they were completely lysed. We then added 200 μl of AL buffer and 200 μl of absolute ethanol and vortexed the samples thoroughly. The mixture was pipetted into a DNeasy Mini spin column and centrifuged at 8000 rpm for 1 min. We then washed the column with AW1 and AW2 buffers, eluted the pure DNA in 200 μl of molecular-grade water, and stored it at −80 °C for later use. NanoDrop served to quantify the concentration of DNA.

Amplification of bacterial rRNA genes

A portion of the 16S small-subunit ribosomal gene was amplified with primers F519 (5-CAGCMGCCGCGGTAATWC-3)

Table 1 Demographic characteristics of 30 children with periodic fever, aphthous stomatitis, pharyngitis, and adenitis (PFAPA) syndrome and 24 controls

	PFAPA ($N = 30$)	Controls ($N = 24$)
Age when symptoms began, median (range), years	2.4 (0.1–16.5)	
Age at the time of surgery, median (range), years	3.4 (1.7–18.2)	5.8 (2.7–15.1)
Gender, boys, *n* (%)	18 (58 %)	8 (33 %)
Antimicrobial courses, mean (SD)		
Within 12 months prior to TE	2.4 (2.5)	1.4 (1.9)
Within 3 months prior to TE[a]	0.6 (0.9)	0.3 (0.9)
Amoxicillin	0.17 (0.46)	0.21 (0.51)
Penicillin	0.13 (0.43)	0.0 (0.0)
Cephalexin	0.13 (0.43)	0.0 (0.0)
Azithromycin	0.13 (0.57)	0.0 (0.0)

SD standard deviation; *TE* tonsillectomy

[a] In addition, one child received amoxicillin–clavulanate in the control group, one child trimethoprim–sulfamethoxazole and clarithromycin in both the PFAPA and control groups, and one child clarithromycin in the PFAPA group

and R926 (5-CCGTCAATTCCTTTRAGTTT-3). The F519 primer contained an Ion Torrent pyrosequencing adapter sequence A (Lifescience Technologies, USA), a 9-bp unique barcode sequence, and one nucleotide linker. R926 primer contained an Ion Torrent adapter trP1 sequence. Polymerase chain reaction (PCR) assays were performed in 25-μl reactions in three replicates, each containing 1 × Phusion GC buffer, 0.4 μM of forward and reverse primers, 200 μM dNTPs, 0.5 U of Phusion enzyme (Thermo Scientific, Espoo, Finland), and 10 ng of genomic community DNA as the template in a total reaction volume of 25 μl with molecular-grade water. After an initial 3-min denaturation at 98 °C, the following cycling conditions were used: 30 cycles of 98 °C, 10 s; 64 °C, 10 s; 72 °C, 20 s. After PCR amplification, pooled triplicate reactions were purified using the AMPure XP PCR clean-up kit (Agencourt Bioscience, CA, USA) and DNA concentration was measured with a Bioanalyzer DNA chip (Agilent Technologies, CA, USA). For sequencing, individual samples were pooled in equivalent amounts, samples were size-selected with a BluePippin automated electrophoresis system (Sage Science, MA, USA) on 1.5 % agarose gel, and purified twice with an AMPure XP kit; final DNA concentrations were measured on a Bioanalyzer DNA chip. Sequencing samples were diluted to 15 pM and sequenced on a 316 Chip v2 with Ion Torrent 400 bp chemistry (Life Technologies, USA).

Bioinformatics analysis

We used an Ion Torrent Sequencer to sequence the hypervariable regions V4-V5 of the 16S rRNA gene to characterize the microbiotas of the tonsillar tissue samples. The Ion Torrent sequences were processed and analyzed using state-of-the-art procedures Quantitative Insights Into Microbial Ecology (QIIME) [16]. Sequences were preprocessed by binning samples to sample specific barcodes with a sequence quality threshold of 20 and unassigned sequences were removed. Primer, barcode sequences, and sequences less than 200 bp were removed from the dataset. The average length of the sequences was 362 bp. Chimeric sequences were removed with a usearch quality filter using the rRNA16S.gold.fasta reference database. The sequences were clustered into operational taxonomic units (OTUs) with a similarity threshold of 97 % and in the taxonomy was assigned using the Ribosomal Database Project (RDP) Naive Bayesian Classifier [17]. The OTU table was constructed in a Biological Observation Matrix (BIOM)-formatted table in QIIME, and reads of<100 were removed across the dataset before further analysis. Using the QIIME summarize_taxa command, genera and species information was retrieved from OTU table. All samples were rarefied to 26,000 sequences prior to the OTU-based diversity analysis, as it was the number of the lowest observed reads in the community.

After filtering out low-quality and chimeric reads from the 54 samples, the final dataset consisted of 2.63 million reads with a median of 47,542 reads per sample. The reads were clustered into species-level OTUs to assess the shared diversity among the samples. Each representative OTU was assigned to a taxonomic lineage using the RDP Bayesian classifier. The rarefaction analysis served to compare the richness of bacterial species between the samples against the sequencing effort, as well as between the cases and controls.

We performed the rarefaction, relative abundance, alpha diversity indices, and core microbiome analyses with QIIME using a rarefied OTU table. We defined the core microbiome as the presence of the 16 s OTU in all (100 %) subjects sampled. We drew the Venn diagram using the Euler Venn Applet (http://www.cs.kent.ac.uk/people/staff/pjr/EulerVennCircles/EulerVennApplet.html).

The detailed taxonomic composition of bacteria from phylum to species levels was visualized with Krona [18]; a relative abundance of less than 0.002 of the genera or species does not appear in the Krona pie charts.

Principal coordinate analysis (PCoA) served to compare the PFAPA and control samples and to study the relationships among all the samples. PCoA was analyzed based on the phylogenetic distance calculations between samples using weighted UniFrac metrics. The OTUs found in the 30 PFAPA samples were compared with each other and with the 24 control samples. We have deposited the Ion Torrent raw data in the NCBI Sequence Read Archive (SRA) with the accession number SRP059223.

Statistical analyses

We used the Student's *t*-test for independent samples to compare the mean number of observed OTUs and the means of the indices describing the diversity of the microbiota between patients and controls. The proportions of samples positive for different phyla, genera, or species were calculated in cases and controls, and the statistical differences of the proportions between the groups were tested with the standard normal deviate test. The mean and median relative abundances of the bacteria with their standard deviation (SD) and range, respectively, were calculated in each group, and the statistical significances of the differences were tested with the Mann–Whitney *U*-test. Since this is an explorative study and a large number of tests were made, the *p*-values for statistically significant differences between the groups are given together with the results after Bonferroni correction. All analyses were carried out blinded to indications for tonsillectomy. The analyses were performed with SPSS 20 software (SPSS Inc., Chicago, IL, USA).

Results

In the rarefaction analysis for the majority of the samples, the curves were in the upward phase, indicating that the sampling was good, and all samples showed relatively high species diversity. The species richness of PFAPA and control samples was considerably high, with a total of 1097 OTUs identified in the complete dataset. The number of OTUs observed in the community varied from 346 to 740, signifying a highly complex community structure. Upon examination of the taxonomic assignment at the phylum level, the community comprised ten phyla and 106 genera.

PCoA showed that the OTUs found in the PFAPA samples are poorly separated from the controls along the principal coordinate 1, which accounts for 47 % of the observed variation. This indicates that the microbial populations found in the PFAPA were indistinct from those found in the control samples, and this finding confirms the results of the taxonomic classification and relative abundance of the taxa present (Fig. 1).

The Shannon and Simpson indices in the samples varied from 3.30 to 7.1 and from 0.68 to 0.98, respectively, indicating the overall high bacterial diversity in the community (Table 2). We found no statistically significant differences between the two groups in the bacterial diversity measured with the Shannon, Simpson, and Chao1 indices (Table 2).

We found 47 core OTUs in the patients and 84 core OTUs in the controls and plotted them in a Venn diagram (Fig. 2). The percentage of core OTUs shared between the controls and cases is 81 % of the OTUs in the patients and 45 % of the OTUs in the control samples. In the core microbiome, the control and case samples shared 11 genera (38 OTUs), and the genus *Fusobacterium*, which was present in both cases and controls, dominated (Fig. 2). Furthermore, the cases and controls also shared Comamonadaceae, *Fusobacterium*, *Gemella*, Gemellaceae, Mogibacteriaceae, *Parvimonas*, *Peptostreptococcus*, *Porphyromonas*, *Prevotella tannerae*, and *Streptococcus*. Because OTUs are picked at 97 % similarity and differ by 3 %, many OTUs of the same genera are present. Thus, the nine OTUs found only in the cases belonged to genera present in all samples in both groups.

Table 2 Mean number (SD) of observed OTUs and mean indices (SD) describing the diversity of the microbiota in the tonsils of 30 PFAPA children and 24 controls

	PFAPA ($N=30$)	Controls ($N=24$)	p-Value for the difference
Observed OTUs	588 (88)	564 (85)	0.243
Simpson index	0.95 (0.03)	0.93 (0.08)	0.508
Shannon index	6.16 (0.68)	6.10 (1.02)	0.531
Chao1 index	712 (107)	686 (775)	0.243

We found no phylum, genera, or species that tested positive in all cases and none of the controls or vice versa. However, the proportions of the samples that tested positive for some bacteria differed significantly between the cases and controls, and the relative abundances of some microbes also showed significant differences between the groups (Tables 3, 4, and 5). At the phylum level, *Actinobacteria* were found in all cases and in 92 % of the controls (Fig. 3). *Synergistetes* were present in only seven of the cases (23 %) and one of the controls (4 %, $p=0.049$ for the difference, Table 3). Cyanobacteria were more commonly found in the case samples (14/30, 47 %) than in the controls (4/24, 17 %, $p=0.02$). The mean relative abundance of cyanobacteria was also statistically significantly higher in the cases (0.2 %) than in the controls (0.01 %, $p=0.01$). At the genera level, streptococci were present in all samples in both groups, but the mean relative abundance was lower in the cases (3.7 %) than in the controls (9.6 %, $p=0.01$). *Prevotella* were also present in all the samples, but the mean relative abundance in the cases was 9 % and that in

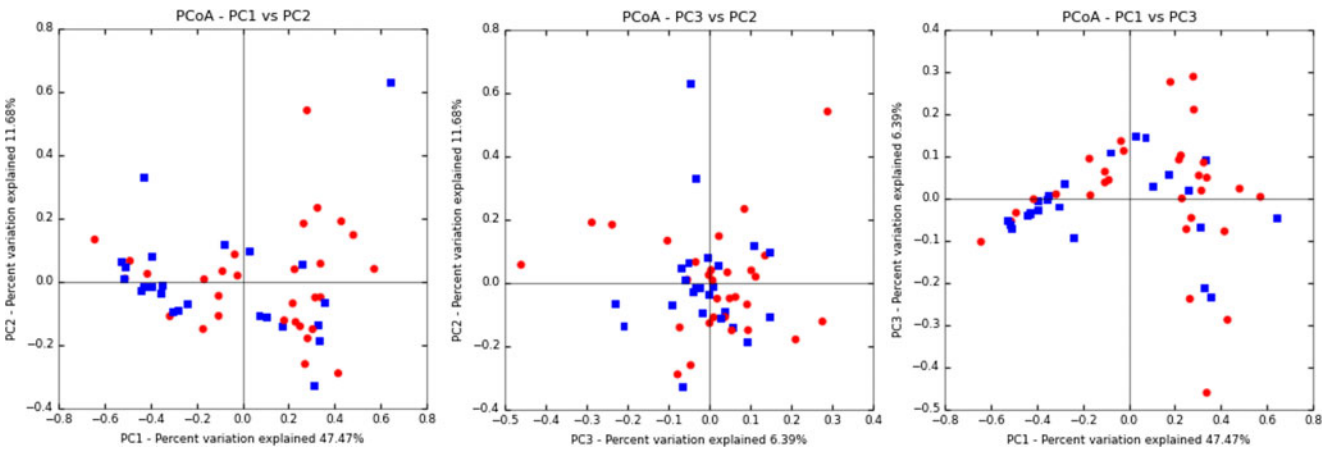

Fig. 1 Principal coordinate analysis (PCoA) plots showing the weighted (B) UniFrac analysis of the periodic fever, aphthous stomatitis, pharyngitis, and adenitis (PFAPA) (*red*) and control (*blue*) samples

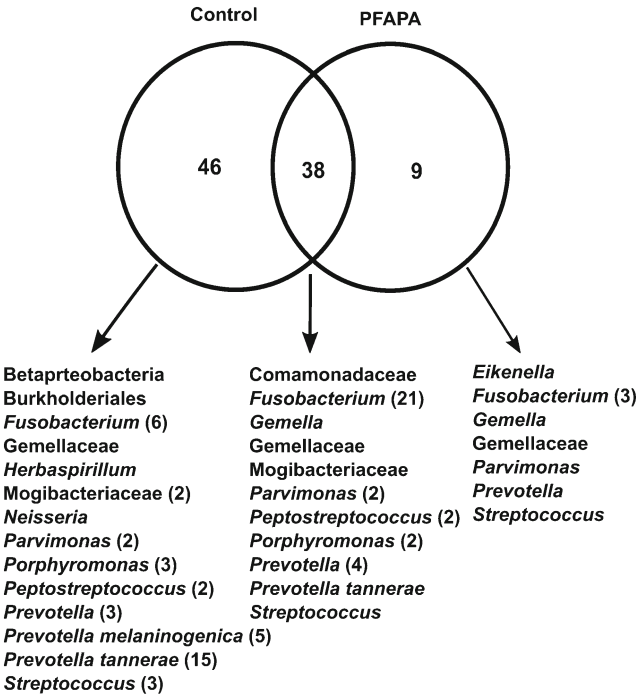

Fig. 2 Venn diagram showing the core microbiome of the control and PFAPA samples at a 97 % confidence level. The numbers in the brackets represent the numbers of operational taxonomic units (OTUs) in each genera. The OTUs are picked at 97 % similarity and there is a difference of 3 % among each OTU, so many OTUs of the same genera are present

the controls was 6 % ($p = 0.02$). Genera or species present only in some of the cases but not at all in the controls were: *Schwartzia* (present in 27 % of the cases), *Cloacibacterium* (17 %), *Vestibaculum illigatum* (13 %), and *Lactobacillus iners* (3 %); 17 genera or species tested positive in the cases statistically significantly more often than in the controls or vice versa (Table 4). Typical nasopharyngeal pathogens, such as *Haemophilus* and *Mycoplasma*, as well as *Staphylococcus aureus* (90 % vs. 88 %), were evenly distributed in cases and controls.

Table 3 Proportions of the samples with bacterial phyla present in the tonsils of 30 PFAPA children and 24 controls

Bacterial phyla	PFAPA ($N = 30$)	Controls ($N = 24$)	p-Value for the difference
Actinobacteria	30/30 (100 %)	22/24 (92 %)	0.1
Bacteroidetes	30/30 (100 %)	24/24 (100 %)	
Cyanobacteria	14/30 (47 %)	4/24 (17 %)	0.02
Firmicutes	30/30 (100 %)	24/24 (100 %)	
Fusobacteria	30/30 (100 %)	24/24 (100 %)	
Proteobacteria	30/30 (100 %)	24/24 (100 %)	
Spirochaetes	25/39 (83 %)	23/24 (96 %)	0.15
Synergistetes	7/30 (23 %)	1/24 (4 %)	0.049
Tenericutes	14/30 (47 %)	11/24 (46 %)	0.951

Table 4 Proportions of the tonsil samples with bacterial genera or species that tested positive in the tonsils of 30 PFAPA children and 24 controls. Only the microbes that showed statistically significant differences between the groups are presented

	PFAPA ($N = 30$)	Controls ($N = 24$)	p-Value for the difference
Genera			
Cloacibacterium	5/30 (17 %)	0	0.036
Clostridiales	21/30 (70 %)	8/24 (33 %)	0.007
Delftia	10/30 (33 %)	17/24 (71 %)	0.006
Mogibacterium	17/30 (57 %)	4/24 (17 %)	0.003*
Oxalobacteraceae	14/30 (47 %)	19/24 (79 %)	0.015
Paludibacter	17/30 (57 %)	7/24 (29 %)	0.043
Peptostreptococcaceae	26/30 (87 %)	15/24 (63 %)	0.039
Phyllobacterium	7/30 (23 %)	14/24 (58 %)	0.009
Ruminococcaceae	15/30 (50 %)	4/24 (17 %)	0.011
Schwartzia	8/30 (27 %)	0	0.006
Slackia	12/30 (40 %)	1/24 (4 %)	0.002*
Tannerella	25/30 (83 %)	14/24 (58 %)	0.042
Species			
Kingella denitrificans	29/30 (97 %)	19/24 (79 %)	0.042
Prevotella nanceiensis	29/30 (97 %)	18/24 (75 %)	0.019
Porphyromonas endodontalis	5/39 (17 %)	10/14 (42 %)	0.042

*Statistically significant after Bonferroni correction

Discussion

The tonsil samples from the PFAPA patients revealed significant differences in the presence and relative abundance of many bacterial phyla, genera, and species from those of the controls. Even though the sequencing technique can also detect dormant bacteria in biofilms, we found no single bacterial genus or species, whether present or absent, in the samples from PFAPA cases only, so our results support previous findings that PFAPA is not an infectious disease. Rather, our findings imply that differences in the tonsillar microbiota could trigger or protect from the dysregulated cytokine production in the inflammasomes of the tonsils [10–12].

The microbiomes studied for several diseases have revealed differences in the microbial communities of the oral cavity, tonsils, gastrointestinal, and urogenital tract from those of healthy controls [19–24]. Various factors, such as diet, environment, and host genetics, associate with the microbiome. A shift in the microbial community can have clinical implications in diseases such as cancer [25], cardiovascular disease [26], diabetes [27], and obesity [28, 29]. PFAPA could be another example of the diseases where interaction between the microbiome of the mucous membrane and the underlining inflammatorily active tissue plays an important role.

In the present study, cyanobacteria were more prevalent and had a higher relative abundance in the cases than in the

Table 5 Mean relative abundances as a percentage (SD) of the bacterial genera or species in the tonsils of 30 PFAPA children and 24 controls. Only the microbes that showed statistically significant differences between the groups are presented

	PFAPA ($N=30$)	Controls ($N=24$)	p-Value for the difference
Phylum			
Cyanobacteria	0.2 (1.0)	0.02 (0.07)	0.01
Genera			
Aerococcaceae	0.06 (0.1)	0.2 (0.4)	0.048
Dialister	0.1 (0.3)	0.1 (0.2)	0.012
Gemellaceae	0.5 (0.5)	0.7 (0.5)	0.03
Granulicatella	0.1 (0.2)	0.6 (1.7)	0.003*
Prevotella	3.9 (11.2)	4.0 (4.7)	0.017
Streptococcus	3.7 (4.0)	9.6 (10.3)	0.01
Species			
Prevotella melaninogenica	2.2 (2.2)	3.3 (2.1)	0.045
Prevotella nanceiencis	1.1 (4.3)	2.2 (3.4)	0.001*
Selenomonas noxia	0.04 (0.1)	0.02 (0.05)	0.05

*Statistically significant after Bonferroni correction

controls. Even if this difference may be a consequence of differences in food intake, the finding is interesting as cyanobacteria represent bacteria that possess many peculiar features. They can obtain energy through photosynthesis and are potential producers of cyanotoxins, such as microcystins. The effects of microcystins and other cyanotoxins are not well understood, but animal and human studies have shown the ingestion of microcystins to induce gastrointestinal, hepatic, and renal damage and symptoms [30]. Cyanobacteria are found largely in natural waters [31, 32]. Also, *Cloacibacterium*, which were more prevalent in the PFAPA cases than in the controls, are commonly found in marine and freshwater [33, 34].

Previous attempts to detect possible infectious agents on the tonsils removed from PFAPA patients have shown negative results. Pignataro et al. was the first to use molecular methods to analyze removed tonsil tissue with an aim to find some atypical bacteria, but the results were negative [35]. Cultures for *Streptococcus pyogenes* and serum antibodies against herpes simplex virus type 1, cytomegalovirus, and Epstein–Barr virus served mainly to exclude infectious diseases, and each tested positive in about 20 % of their series of 18 patients [35]. Recently, Freeman et al. found no differences in the overall composition and diversity of the tonsillar microbiota in a small study of six PFAPA patients and eight age-matched controls [36]. In the present study, the number of OTUs from both PFAPA cases and controls was much higher than the number recently reported by Jensen et al. in their study of the tonsillar crypts of children and adults [24]. These differences in the results may be due to different DNA extraction kits, PCR amplification conditions, different sequencing technologies, and processing of reads using different methods. In their study, Jensen et al. collected the samples from tonsillar crypts, whereas we used pieces of removed tonsillar tissue, which may have affected the results. The most abundant phyla were similar in both studies.

Fig. 3 Pie chart showing the relative abundance of the structure of the entire bacterial community in 54 tonsil tissue samples (30 PFAPA and 24 controls). Less abundant (<1 %) genera are not shown or are combined

Previously, scanning electron microscopy and culture-based methods showed that biofilm formation and the presence of *Candida albicans* were more common, and the presence of *S. aureus* rarer in the tonsils of PFAPA patients than in those of controls [15]. With sequencing, *S. aureus* tested positive in almost all samples in both groups. We did not have the opportunity to analyze fungal microbiota. Thus, this study could not reassess our previous finding of increased positivity for *C. albicans* in PFAPA patients.

Sequencing-based techniques involve the possibility of contaminations from DNA extraction kits and other laboratory reagents, especially when dealing with samples with small amounts of bacterial material [37]. We used negative controls and the samples contained large amounts of DNA, so contamination seems unlikely to have confounded our results.

One limitation of our study was that the control samples were not taken from healthy children, which would be impossible in practice. We chose to use children with hypertrophic tonsils as controls. The study by Jensen et al., which used pyrosequencing in children and adults to study the bacterial microbiota of the tonsillar crypts, found some differences between samples from hyperplastic tonsils and samples from patients with recurrent tonsillitis [24]. Different exposures to antimicrobials before tonsillectomy may have affected the microbiological findings. In our series, the cases had more often received courses of antimicrobials 12 months prior to tonsillectomy, but the difference was not statistically significant.

In conclusion, we found significant differences in the presence and relative abundance of the phylum cyanobacteria, as well as some other bacterial genera and species, between the tonsils removed from PFAPA patients and controls. Tonsillar microbiota may act as a trigger of inflammatory processes in PFAPA or protect from inflammation in healthy children.

Compliance with ethical standards The Ethics Committee of the Northern Ostrobothnia Hospital District, Oulu, Finland, approved the study plan.

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Conflict of interest The authors declare that they have no conflict of interest.

Informed consent The parents provided their written informed consent.

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