

**Intestinal dysbiosis in Inflammatory Bowel Disease associated with primary immunodeficiency.**

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59 **DECLARATION OF INTERESTS**

60 The authors declare no competing financial interests.

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**CAPSULE SUMMARY:** The gut microbiota plays a key role in host physiology and is an actor in inflammatory bowel disease pathogenesis. Patients with primary immunodeficiency causing intestinal inflammation have disease-specific dysbiosis.

**Keywords:** gut microbiota; inflammatory bowel disease; primary immunodeficiency

*To the Editor:*

The gut microbiota is composed of a huge amount and diversity of microorganisms playing major roles in physiological and pathological settings such as inflammatory bowel disease (IBD). The triggering role of the gut microbiota on intestinal inflammation is known and an intestinal dysbiosis (i.e. an imbalance in the microbiota composition and function) has been pointed out in IBD 15 years ago. Moreover, a positive efficacy signal has been observed in studies evaluating fecal microbiota transplantation in IBD demonstrating that the gut microbiota alterations is an actor in the inflammatory process and not simple consequence<sup>1</sup>.

Environmental factors have an important effect on gut microbiota composition, but host genetic has an impact too. This effect has been demonstrated in knockout mouse for innate immunity genes. Microbiome genome-wide association studies have identified several associations between genetic polymorphisms and the gut microbiota composition offering some clues for the effects of genes on microbiota composition in humans<sup>2</sup>. However, the effect of major specific disease-causing genetic defects on gut microbiota has never been studied in humans yet.

Here we characterized the fecal microbiota composition of patients with three types of rare primary immunodeficiency (PID) causing IBD conditions, chronic granulomatous disease (CGD, 11 samples), X-linked inhibitor of apoptosis (XIAP, 7 samples) deficiency and partial Tetratricopeptide Repeat Domain 7A (TTC7A, 7 samples) deficiency, in comparison to patients with non-genetic determined IBD (18 samples) and healthy subjects (HS, 23 samples, Suppl Table 1).

The microbiota composition was assessed by 16S sequencing. As seen previously, gut microbiota composition was influenced by age, antifungal and antibiotic treatment<sup>3</sup> (Suppl

Figure 1). Beta diversity analysis showed a remarkable clustering of samples according to disease phenotypes (Figure 1A). The difference with HS was statistically significant for CGD and TTC7A groups while it did not reach significance for XIAP (Figure 1 A-D). Interestingly the difference with IBD patients was statistically significant for all three PID patients groups (Figure 1A). Age was very heterogeneous in patients from TTC7A and XIAP groups (0.62 to 18 and 1.3 to 34 years respectively). The gut microbiota composition changes constantly between birth and the age of 3 years when it reaches an adult-like configuration<sup>4</sup>. We thus performed the same analysis after segregating the patients according to their age (younger or older than 3 years). The difference with HS was maintained for TTC7A patients in both age groups and reached significance in XIAP patients older than 3 (only 2 XIAP patients younger than 3) (Suppl Figure 2).

Patients with CGD, XIAP and TTC7A deficiency can experience intestinal inflammation mimicking IBD<sup>5</sup>. In our study population, samples were taken from patients with (PID<sub>IBD</sub>) or without (PID<sub>no-IBD</sub>) IBD involvement. In addition, PID patients could be in active phase (flare) or remission of IBD (PID, IBD flare/inactive). Microbiota from patients with active intestinal inflammation (PID<sub>IBD (flare)</sub>) was significantly different from the one of PID<sub>no-IBD</sub> patients (Figure 1E). We observed a significant diversity reduction in patients with PID<sub>IBD</sub> compared to HS (Figure 1F, Suppl Figure 3A). Moreover, this reduction was even stronger in patients with active intestinal inflammation (Figure 1G, Suppl Figure 3B). These effects were seen in the three-studied PID although the numbers were too low to reach statistical significance when analyzing each PID independently (Suppl Figure 4).

In accordance with the published literature, the bacterial microbiota of all groups was dominated by bacteria from Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria phyla. Differences were noticed between PID patients and HS with notably a dramatic increase

118 in Proteobacteria from the Enterobacteriaceae family in the TTC7A group and an increase  
119 proportion in bacteria from the Bacteroidetes phylum and from the Clostridiaceae family in the  
120 CGD and XIAP groups respectively (Figure 1 H, Suppl Figure 3C). These alterations were  
121 found both in patients younger and older than 3 years (Suppl Figure 4 B, D) and were clearly  
122 stronger in patients with IBD and PID<sub>IBD</sub> with active inflammation (Figure 1I; Suppl Figure 4  
123 C, E). We then looked for the microbial features associated with the three studied PID and used  
124 a multivariate association test (MaAsLin) to control for potential confounding factors such as  
125 age, gender, smoking, intestinal inflammation and treatment. We observed several alterations  
126 specific to each PID compared to HS. Patients with TTC7A deficiency exhibited an increased  
127 abundance of Proteobacteria involving several components of the Gammaproteobacteria and  
128 Epsilonproteobacteria classes (Figure 2A). On the other hand, bacteria from the  
129 Ruminococcaceae family and notably the *Oscillospira* genera were decreased. Patients with  
130 XIAP deficiency exhibited an increased abundance of several bacterial taxa including members  
131 of the Proteobacteria, Firmicutes, Actinobacteria and Fusobacteria phyla (Figure 2B). Four of  
132 these taxa (*Scardovia*, *Fusobacterium*, *Rothia dentocariosa*, *Veillonella*) are not usually found  
133 in the gut but are known members of the oral microbiota that are implicated in dental caries,  
134 but also IBD, colorectal cancer, and liver diseases<sup>6</sup>. Interestingly, an increased abundance of  
135 oral bacteria in the gut has been observed in several intestinal and extra-intestinal diseases<sup>7</sup>.  
136 Moreover, a recent study showed that some members of the oral microbiota can have pro-  
137 inflammatory effects when colonizing the gut<sup>7</sup> suggesting a possible direct effect of these oral  
138 bacteria in the intestinal inflammatory phenotype observed in patients with XIAP deficiency.  
139 Another important specificity in XIAP patients was the presence of *Lactococcus garvieae*  
140 which is a highly virulent pathogen affecting saltwater fish<sup>8</sup> and is rarely involved in human  
141 infection. *L. garvieae* was found at a high level (0.23-0.5% of all reads) in XIAP patients with  
142 active intestinal inflammation but not in all other patients in this study. Patients with CGD

exhibited an increased abundance of *Ruminococcus gnavus* that has also been associated with ileal Crohn's disease<sup>9</sup> (Figure 2C).

In summary, we showed that the gut microbiota of patients with CGD, TTC7A and XIAP deficiency have distinct alterations suggesting a primary defect in host immune system as a basis of dysbiosis. Although it remains to be experimentally documented, the microbial alterations induced by the host genetic defect might play a role in some aspects of the PID phenotype and particularly intestinal involvement. The number of subjects studied was low because of the extreme rarity of the studied diseases. However, independent and ideally larger and longitudinal studies are required to confirm our findings. Controlling for potential confounding factors such as diet, treatment, age, delivery mode, socio-economic features, gastrointestinal symptoms, is particularly difficult in studies aiming at discriminating genetic factors from environmental and inflammation influences. Similarly, identification of ideal controls is not trivial. Although non-affected healthy siblings sharing similar lifestyles would be attractive, there are obvious limitations in term of feasibility.

Finally, if these observations are confirmed, the alteration in gut microbiota composition might have clinical interest as diagnosis biomarkers.

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208    Recruited patients with IBD: Harry Sokol, Laurent Beaugerie, Philippe Seksik, Olivier Goulet,  
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210    Recruited controls: Stéphanie Pannier, Perrine Bach, François Danion

211    Genetic diagnosis of patients with PID: Geneviève de Saint Basile, Capucine Picard, Sylvain  
212    Latour, Christelle Lenoir

213    Original study developed by Sylvain Latour, Claire Aguilar and Alain Fischer

214    Analysis of the data and manuscript writing: Harry Sokol, Alain Fischer, Nizar Mahlaoui.

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## FIGURE LEGENDS

**Figure 1: Abnormal bacterial microbiota composition and diversity in PID.** Principal coordinate analysis of Bray–Curtis distance with each sample colored according to the studied group. PC1, PC2 and PC3 represent the top three principal coordinates that captured most of the diversity. The fraction of diversity captured by the coordinate is given as a percentage. Groups were compared using ANOSIM method (9999 permutations). **(A)** All the studied groups plotted together. **(B)** CGD group compared to healthy subjects. **(C)** TTC7A group compared to healthy subjects. **(D)** XIAP group compared to healthy subjects. **(E)** Patients with active IBD involvement ( $PID_{IBD(flare)}$ ) compared to non-active IBD involvement ( $PID_{IBD(non-active)}$ ) and without IBD involvement ( $PID_{no-IBD}$ ). **(F,G)** Bacterial diversity based on the Shannon index in the fecal samples of indicated groups. Statistical significance was assessed using ANOVA with a post hoc Dunn’s test. **(H,I)** Global composition of bacterial microbiota at the phylum levels for the indicated groups \*:  $p<0.05$ ; \*\*:  $p<0.01$ ; \*\*\*:  $p<0.001$ ; \*\*\*\*:  $p<0.0001$ .

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256 **Figure 2: Bacterial taxa associated with TTC7A, XIAP and CGD.** Bacterial taxa associated  
257 with TTC7A deficiency (**A**), XIAP deficiency (**B**) and CGD (**C**). Differences in abundance are  
258 shown for the bacterial taxa detected using a multivariate statistical approach (see ‘Material and  
259 Methods’). All the indicated differences are statistically significant with  $p < 0.05$  after correction  
260 for multiple testing. The fold change for each taxon was calculated by dividing the mean  
261 abundance in the cases by that of the controls.

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Figure 1

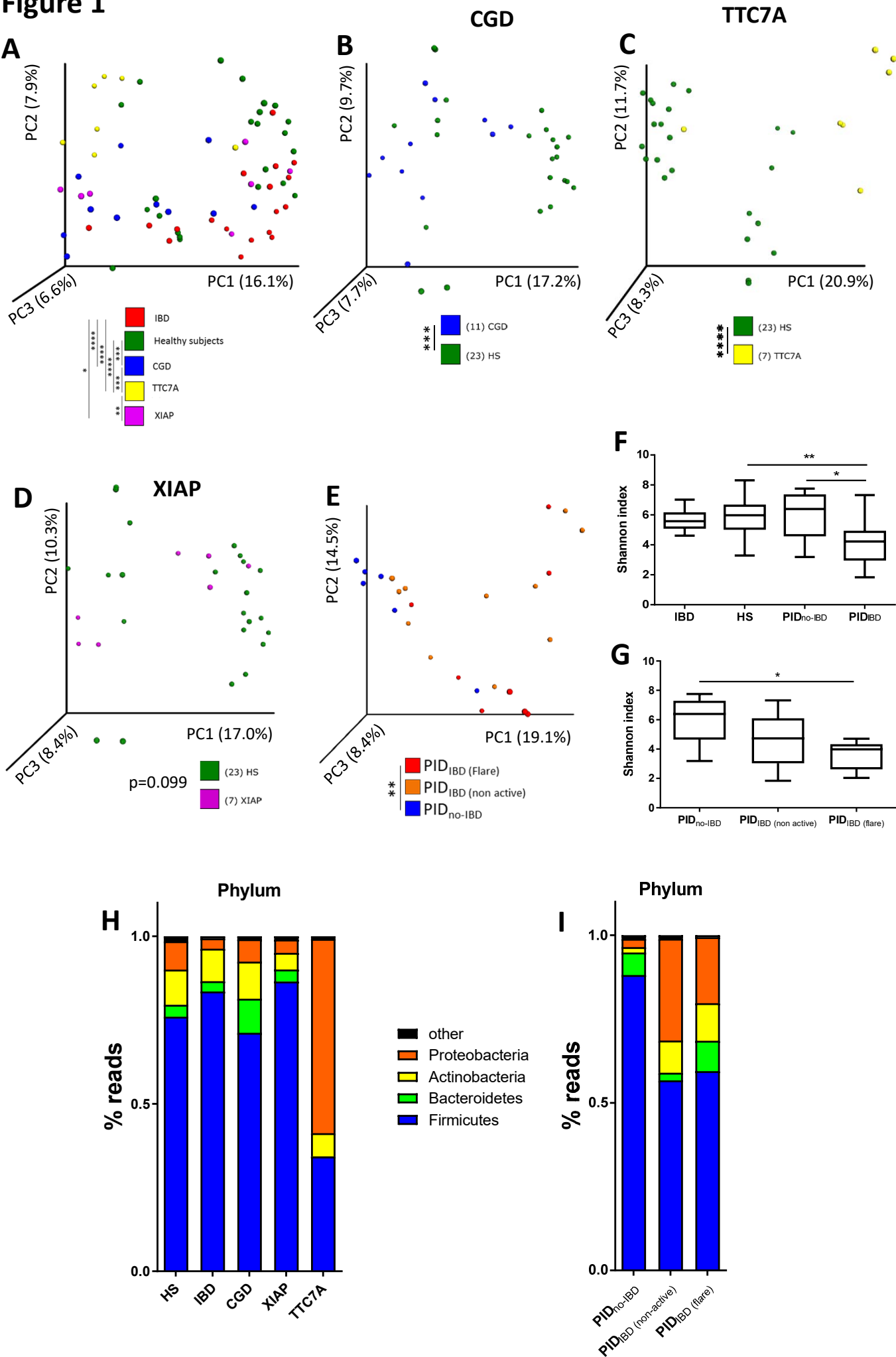
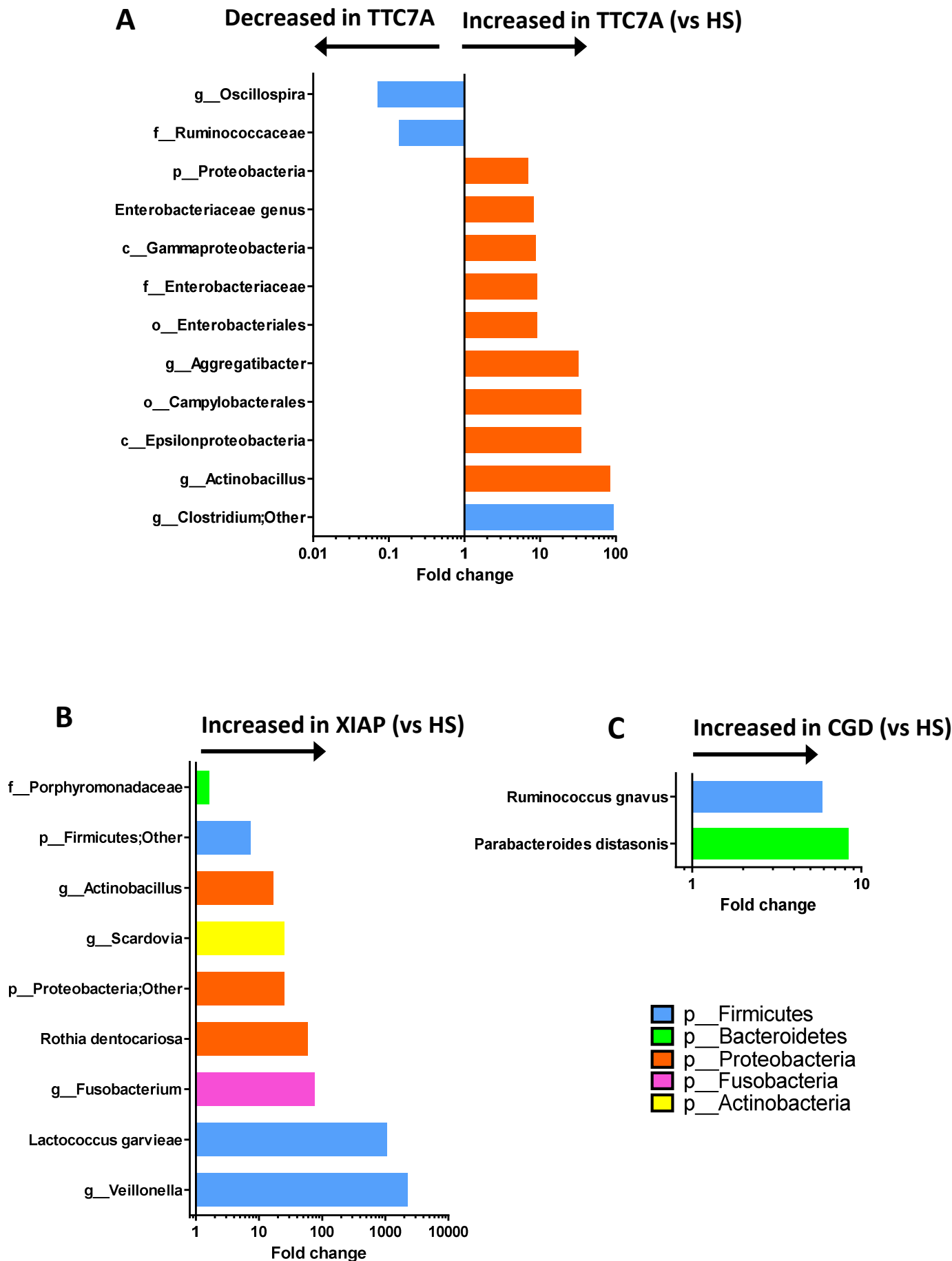
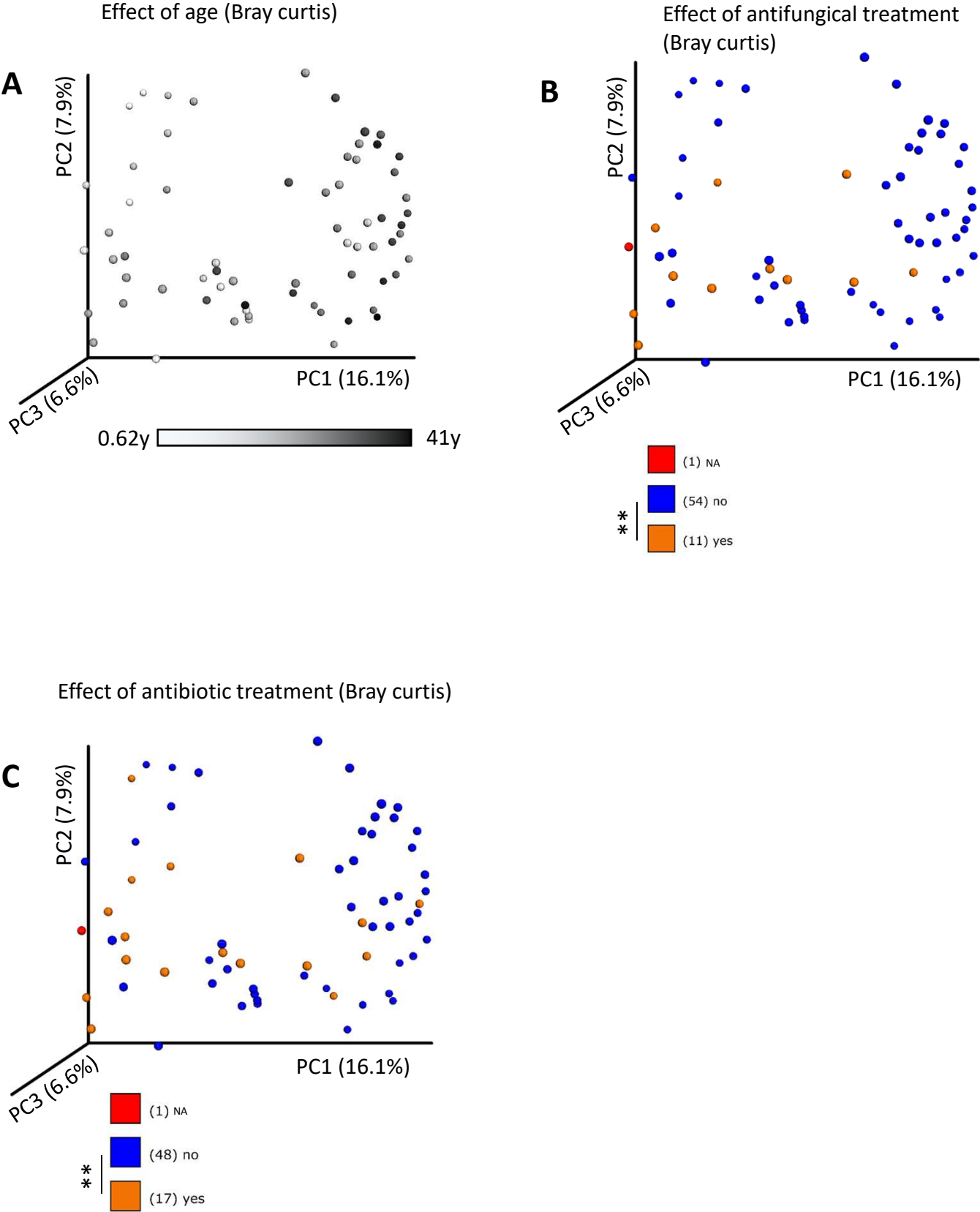


Figure 2

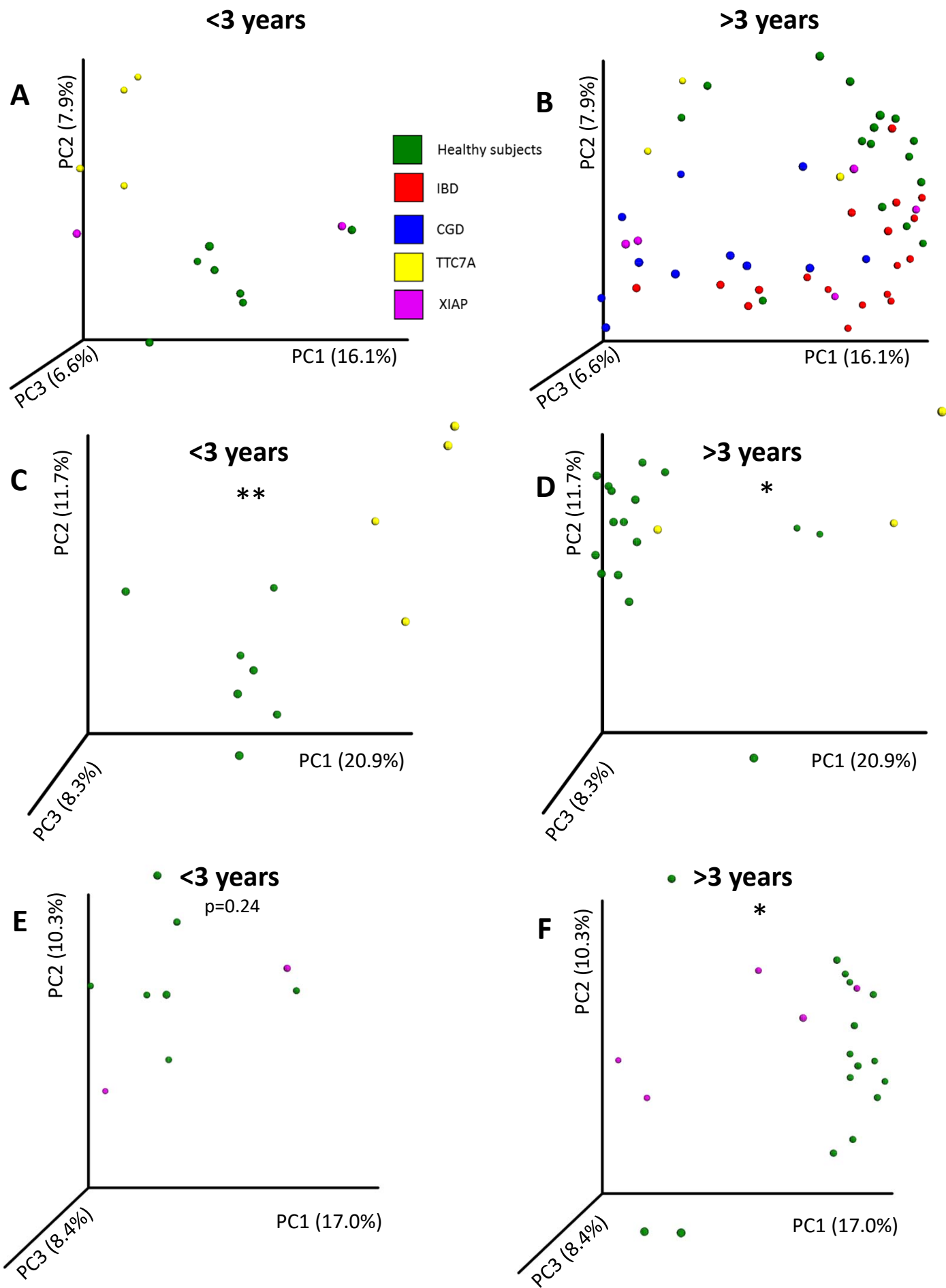


Supplementary Figure 1

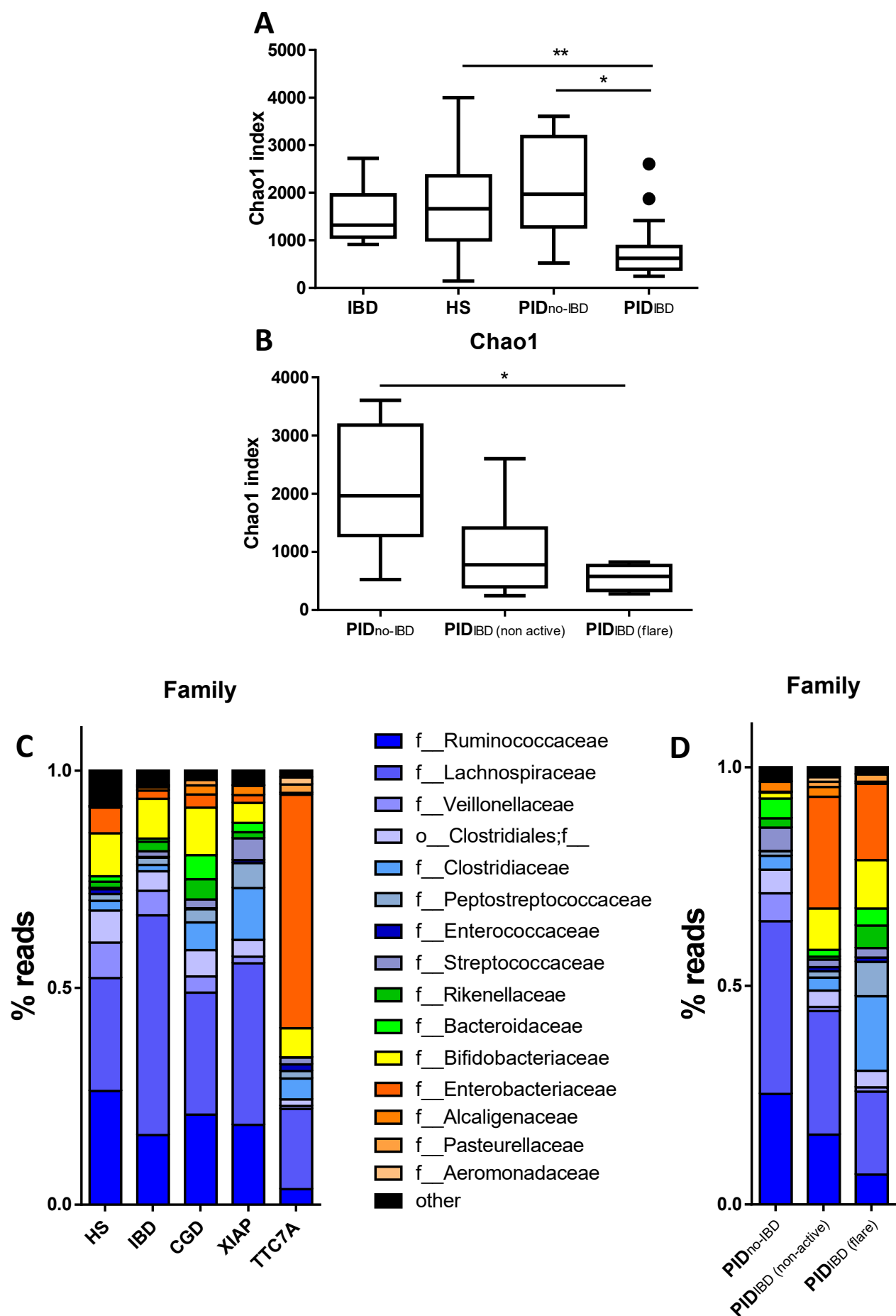




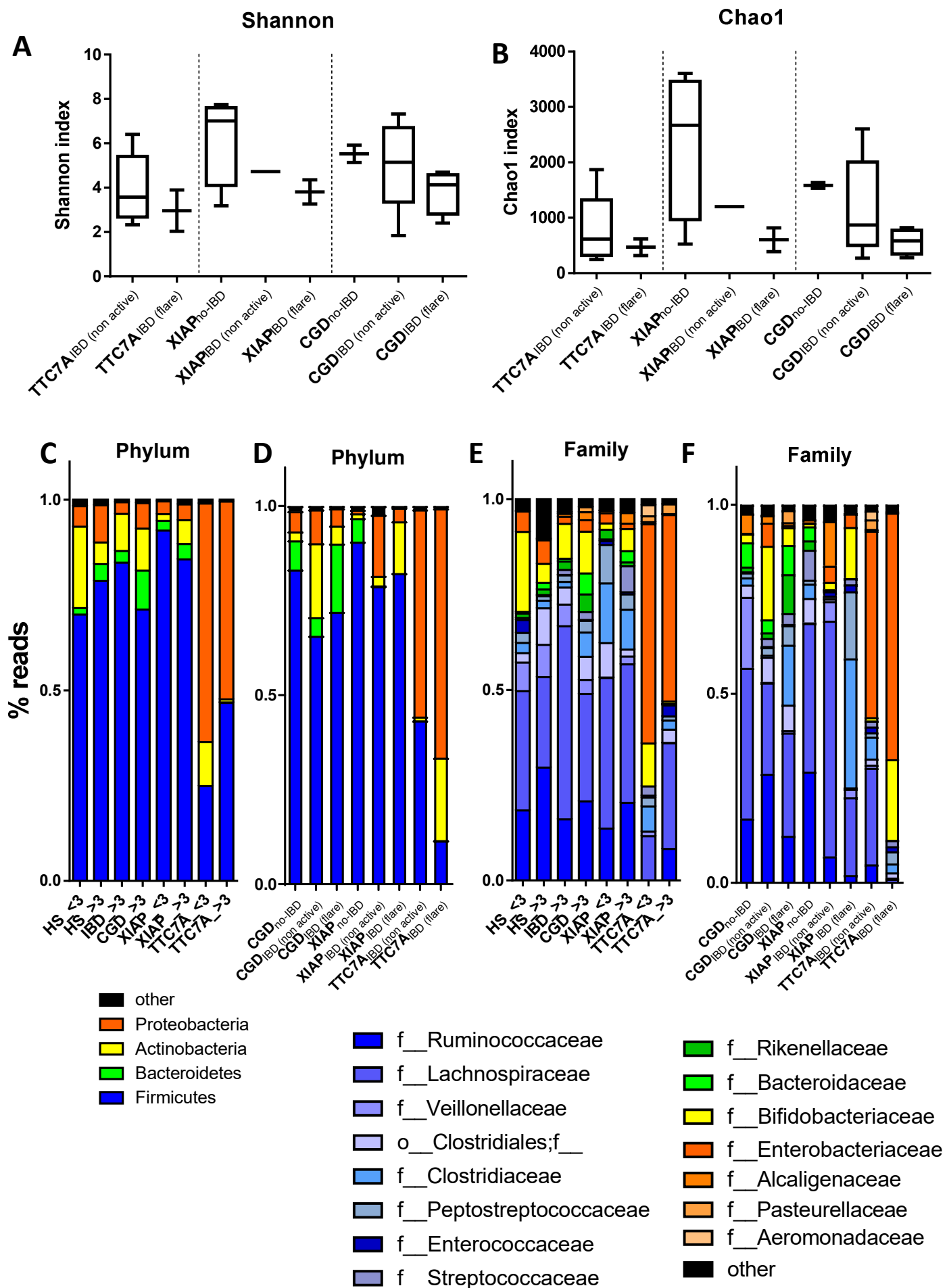
Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4



**Supplementary Table 1 : Characteristics of patients**

Patients n	<b>HS 23</b>	<b>IBD 18</b>	<b>CGD 10</b>	<b>TTC7A 5</b>	<b>XIAP 6</b>
Sample n	23	18	11	7	7
Age: median (min-max)	9 (0.83-32)	28.5 (10-41)	10 (6-31)	1.8 (0.62-18)	8 (1.3-34)
Male Gender % ( n)	61% (14)	89% (16)	100% (11)	43% (3)	100% (7)
Gut inflammation history	0	100% (18)	82% (9)	100% (7)	43% (3)
Active gut inflammation	0	0	64% (7)	71% (5)	71% (5)
Smoking	4% (1)	22% (4)	0	0	43% (3)
Sulfamethoxazole / trimethoprim	0	0	100% (11)	29% (2)	50% (3)
Other antibiotics	0	0	27% (3)	0	29% (2)
Itraconazole	0	0	91% (10)	0	0
Posaconazole	0	0	9% (1)	0	0
5-ASA	0	33% (6)	18.2% (2)	0	0
Corticosteroid	0	0	0	0	33% (2)
Azathioprin	0	50% (9)	0	0	0
Methotrexate	0	0	9% (1)	0	17% (1)
Anti-TNF $\alpha$	0	61% (11)	0	0	0
Anti IL1 therapy	0	0	0	0	17% (1)
Natalizumab (anti $\alpha$ 4) therapy	0	0	0	0	17% (1)
Any immunosuppressant	0	61% (11)	9% (1)	0	50% (3)
PPI	0	0	0	14% (1)	0
Ig substitution	0	0	0	100% (7)	17% (1)
Enteral nutrition	0	0	0	43% (3)	0
Hydroxychloroquine	0	0	9% (1)	0	0

5-ASA: 5 aminosaclicylate ; PPI: proton pump inhibitor ; Ig: Immunoglobulin substitution

1 CGD patient, 1 XIAP patient and 2 TTC7A patients experienced flare during the follow up and were then sampled twice

## **SUPPLEMENTARY INFORMATION**

### **MATERIAL AND METHODS**

#### **Patients and sample collection**

Patients with primary immunodeficiency were recruited at Necker-Enfants malades University Hospital, Paris, France (Pediatric Immunology-Hematology-Rheumatology Unit, Adult Hematology Unit, Adult infectious Disease Unit), Saint-Louis Hospital, Paris, France and Lille Regional University Hospital, Lille, France and provided informed consent. Pediatric healthy subjects were recruited from the pediatric orthopedic surgery unit, Necker-Enfants malades University Hospital. Approval was obtained from the local ethics committee (Comité de Protection des Personnes III (Ref.: 3149, 2014, June 10<sup>th</sup>, Dipobiota study, ClinicalTrials.gov Identifier: NCT02909244). Patients with IBD in remission were recruited at the Gastroenterology Department of the Saint Antoine Hospital (Paris, France) and provided informed consent (local ethics committee: Comité de Protection des Personnes Ile-de-France IV, Suivitheque study). None of the IBD patients and the healthy subjects reported having taken antibiotics, probiotics or using colon-cleansing products for at least 1 months prior to enrollment. Patient characteristics are presented in Table 1. Whole stools were collected in sterile boxes and immediately homogenized, and 0.2 g aliquots were frozen at  $-80^{\circ}\text{C}$  for further analysis. Patients' data were collected, including clinical, treatment, immunological and genetic diagnosis. Enrolled patients with PID had damaging causal mutation respectively in either the TTC7A gene "ELA syndrome"<sup>26</sup>, BIRC4 (XIAP deficiency) and CYBBNox2 (X-linked CGD). None of the subjects received probiotics.

#### **Fecal DNA extraction**

Genomic DNA was extracted from 200 mg of feces as previously described.<sup>27</sup> . Following microbial lysis with both mechanical and chemical steps, nucleic acids were precipitated in isopropanol for 10 minutes at room temperature, incubated for 15 minutes on ice and

centrifuged for 30 minutes at 15,000 *g* and 4°C. Pellets were suspended in 112 µL of phosphate buffer and 12 µL of potassium acetate. After RNase treatment and DNA precipitation, nucleic acids were recovered via centrifugation at 15,000 *g* and 4°C for 30 minutes. The DNA pellet was suspended in 100 µL of TE buffer.

### **16S rRNA gene sequencing**

Fecal DNA was extracted from the weighted feces before and during the infection as previously described<sup>27</sup>. Microbial diversity was determined for each sample by targeting a portion of the ribosomal genes. A 16S rRNA gene fragment comprising V3 and V4 hypervariable regions (16S; 5'-TACGGRAGGCAGCAG-3' and 5'-CTACCNGGGTATCTAAT-3') was amplified using an optimized and standardized 16S-amplicon-library preparation protocol (Metabiotec, GenoScreen). Briefly, 16S rRNA gene PCR was performed using 5 ng genomic DNA according to the manufacturer's protocol (Metabiotec) using 192 bar-coded primers (Metabiotec MiSeq Primers, GenoScreen) at final concentrations of 0.2 µM and an annealing temperature of 50°C for 30 cycles. The PCR products were purified using an Agencourt AMPure XP-PCR Purification system (Beckman Coulter), quantified according to the manufacturer's protocol, and multiplexed at equal concentrations. Sequencing was performed using a 250-bp paired-end sequencing protocol on an Illumina MiSeq platform (Illumina) at GenoScreen. Raw paired-end reads were subjected to the following process: (1) quality filtering using the PRINSEQ-lite PERL script<sup>5</sup> by truncating the bases from the 3' end that did not exhibit a quality < 30 based on the Phred algorithm; (2) paired-end read assembly using FLASH (fast length adjustment of short reads to improve genome assemblies) with a minimum overlap of 30 bases and a 97% overlap identity; and (3) searching and removing both forward and reverse primer sequences using CutAdapt, with no mismatches allowed in the primers sequences. Assembled sequences for which perfect forward and reverse primers were not found were eliminated.

### **16S rRNA gene sequence analysis**

The sequences were demultiplexed and quality filtered using the Quantitative Insights Into Microbial Ecology (QIIME, version 1.9.1) software package <sup>28</sup>, and the forward and reverse Illumina reads were joined using the fastq-join method (<http://code.google.com/p/ea-utils>). The sequences were assigned to OTUs using the UCLUST algorithm <sup>29</sup> with a 97% threshold of pairwise identity and classified taxonomically using the Greengenes reference database <sup>30</sup>. Principal component analyses of the Bray Curtis distance were built and used to assess the variation between experimental groups (beta diversity). Significance was assessed using ANOSIM (9999 permutations). The Shannon and Chao1 diversity index were calculated using rarefied data (depth = 17,000 sequences/sample) and used to characterize species diversity in a community.

### **Statistical analysis**

GraphPad Prism version 6.0 (San Diego, CA) was used for all analyses and graph preparation. For all graph data, the results are expressed as the mean  $\pm$  SEM, and statistical analyses were performed using the 2-tailed nonparametric Mann–Whitney *U*-test or Kruskal-Wallis test with Dunn’s Multiple Comparison Test. Statistical significance of sample grouping for beta diversity analysis was performed using Anosim method (9999 permutations). Differences with a *p* value less than 0.05 were considered significant.

Multivariate Analysis by Linear Models (MaAsLin), a multivariate statistical framework, was used to find associations between clinical metadata and microbial community abundance <sup>31</sup>. We took into account as much factor as possible in the analysis to control for the effects of potential confounding factors including: age, gender, smoking, intestinal inflammation and treatment and enteral nutrition.

## **SUPPLEMENTARY FIGURE LEGEND**

**Supplementary Figure 1: Beta diversity analysis reveals effect of age, antibiotics and antifungics on the microbiota composition.** Principal coordinate analysis of Bray–Curtis distance with each sample colored according to the studied group. PC1, PC2 and PC3 represent the top three principal coordinates that captured most of the diversity. The fraction of diversity captured by the coordinate is given as a percentage. Groups were compared using ANOSIM method (9999 permutations). All the studied samples are plotted together and colored according to age (**A**), antifungic treatment (**B**), and antibiotic treatment (**C**). \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ ; \*\*\*\*:  $p < 0.0001$ .

**Supplementary Figure 2: Beta diversity analysis according to age.** Principal coordinate analysis of Bray–Curtis distance with each sample colored according to the studied group and stratified by age (< or > 3 years). PC1, PC2 and PC3 represent the top three principal coordinates that captured most of the diversity. The fraction of diversity captured by the coordinate is given as a percentage. Groups were compared using ANOSIM method (9999 permutations). (**A**, **B**) all the studied groups plotted together. (**C**, **D**) TTC7A group compared to healthy subjects. (**E**, **F**) XIAP group compared to healthy subjects. \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ ; \*\*\*\*:  $p < 0.0001$ . In brackets, number of samples.

## **Supplementary Figure 3: Abnormal microbiota composition at Family level in PID.**

Bacterial diversity based on Chao1 index (**A**, **B**) in the fecal samples of indicated groups. Statistical significance was assessed using ANOVA with a post hoc Dunn's test. \*:  $p < 0.05$ ; \*\*:



p<0.01; \*\*\*: p<0.001; \*\*\*\*: p<0.0001. Global composition of bacterial microbiota at the levels for the indicated groups (**C, D**).

**Supplementary Figure 4: Alpha diversity and microbiota composition according to age and IBD intestinal inflammation.** Bacterial diversity based on the Shannon index (**A**) and Chao1 index (**B**) in the fecal samples of indicated groups. Global composition of bacterial microbiota at the phylum (**C, D**) and family (**E, F**) levels for the indicated groups.