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Alimentary Tract

Gut mucosal-associated microbiota better discloses inflammatory bowel disease differential patterns than faecal microbiota

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ABSTRACT

Background: Growing evidence supports the potential role of intestinal microbiota in the pathophysiology of inflammatory bowel diseases (IBD) even if the literature does not reveal uniform alterations. The aim of the study was to evaluate the mucosal (MM) and faecal microbiota (FM) composition in a cohort of IBD patients compared to healthy controls (CTRLs).

Methods: Faecal and mucosal samples were collected from 14 IBD patients and 11 CTRLs. The V1-V3 region of 16S rRNA locus was amplified on a 454-Junior Genome Sequencer. Reads were grouped into operational taxonomic units (OTUs) at a sequence similarity level of 97% for taxonomic assignment, and aligned for OTUs matching against Greengenes database.

Results: Irrespective of disease localization and activity, in the MM of IBD patients a statistically significant increase of Proteobacteria (especially Enterobacteriaceae, *Acidaminococcus*, *Veillonella dispar*) and decrease of Firmicutes (especially *Roseburia* and *Faecalibacterium prausnitzii*) and Actinobacteria was found compared to CTRLs. In the colon district some specific bacterial biomarkers were identified: Enterobacteriaceae for IBD stools, *Bacteroides* for IBD biopsies, Mogibacteriaceae, Ruminococcaceae and *Prevotella* for CTRL stools, Ruminococcaceae for CTRL biopsies.

Conclusions: The profiles of FM were more similar to CTRLs, suggesting that microbiota adhering to the gut mucosa better discriminates patients from controls, with the identification of some interesting biomarkers.

1. Introduction

There is an emerging literature on the role of microbiota in the pathogenesis of several disorders, such as obesity, type 1 diabetes and rheumatoid arthritis [1], which have been linked to deviations from normal microbial gut balance, known as "dysbiosis" [2]. Dysbiosis is closely related to onset and flare up of gastrointestinal disorders, including Crohn's disease (CD) [3,4], ulcerative colitis (UC) [5] irritable bowel syndrome (IBS) [4] and diverticular disease [6].

The altered composition of gut microbiota is thought to play an important role also in the initiation and perpetuation of inflammatory bowel disease [3,7,8]. Indeed, CD and UC reflect the cooperative influence of numerous host and environmental factors [4,5], including elements of intestinal immune system, gut microbiota, and dietary habits [9,10].

The current hypothesis on the pathogenesis of IBD is that the mucosal immune system shows an aberrant response towards luminal antigens in genetically susceptible individuals [10–13]. One of the most predominant link to the onset of CD is a genetic mutation in the innate immune receptor nucleotide-binding oligomerization domain-containing 2 (NOD2) that is associated with innate immune responses [14,15]. Moreover, IBD is mainly localized to areas of the intestines where most of the bacteria are plentiful (distal small intestine and colon) and the presence

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of commensal microbiota has been found to be essential for the development of experimental colitis in several IBD animal models [16].

The "dysbiosis hypothesis" emphasizes that the altered diversity and composition of the gut microbiota, rather than the presence of specific pathogens, are relevant to the pathogenesis of IBD [16–18]. In 1988 Van de Merwe et al. showed that the faecal "flora" of CD patients contained more cultivable anaerobic Gram positive coccoid rods and Gram negative rods than healthy subjects [19]. Similarly, faecal samples from CD patients were found to be enriched with *Escherichia coli* but depleted of *Lactobacillus* and Bifidobacteria species compared with healthy controls [20], and a specific reduction in Bifidobacteria has also been described in UC patients by quantitative culture together with a substantial increase in group D Streptococci [21]. Moreover, Casén et al. have demonstrated that characterisation of microbiota profiling in IBD may improve new therapeutic approaches by designing diagnostic tools for dysbiosis assignment [4]. Various microbial-based strategies for treating IBD such as probiotics, prebiotics and antibiotics, have been demonstrated to be potential options for patients non-responding to conventional treatment [22–24]. Most studies suggest that the common feature of the intestinal microbiota of UC and CD patients is the decrease in bacterial species richness and diversity, evaluated in faecal samples [25]. Particularly, a decrease in members of the phylum Firmicutes and Bacteroidetes and concomitant increase in Proteobacteria and Actinobacteria has been demonstrated [26]. A recent deep comparative analysis of the FM in paediatric IBD patients showed the decrease of microbial richness: in that study, the main difference related to the low abundance of bacterial groups was related to *Clostridium* clusters IV and XIVa, which also are the two most abundant bacterial groups found in the healthy adult microbiota [13]. Nevertheless, very few studies have fully analyzed the altered microbiota in mucosal samples of IBD patients and the available data are often conflicting. Some researchers have found similar microbiota alterations in biopsy and faecal samples from patients with IBD [27], others have documented differences related to sources of samples [18], while more recent studies have observed a comparable pattern in total mucosa-associated bacteria in patients with CD and UC compared to CTRLs in absence of significant differences [2].

Furthermore, the genera *Bacteroides*, *Bifidobacterium*, *Streptococcus*, *Enterococcus*, *Clostridium*, *Lactobacillus* and *Ruminococcus* were all found in the faeces, making the composition representative of the luminal community, whereas *Clostridium*, *Lactobacillus*, and *Enterococcus* were only detected in the mucus layer and epithelial crypts of the small intestine [28].

Therefore, much remains to be done in order to explore the difference between mucosal microbiota (MM) and FM composition in IBD patients and the role of microbiota taxa acting as IBD dysbiosis players.

In the present investigation, the MM and FM composition of IBD patients and healthy controls (CTRLs) are assessed by a targeted-metagenomics (MG) approach to propose potential disease microbial markers within the context of the microbiota ecology.

2. Materials and methods

2.1. Study population

Fourteen consecutive IBD patients [10 with Crohn's disease (CD), 4 with ulcerative colitis (UC)] were recruited at the Campus Bio-Medico Hospital in Rome (Rome, Italy). The diagnosis of IBD was based on clinical, endoscopic and pathological criteria [11,29]: active CD and UC were defined by a CD activity index of >150 and a UC activity index of >3 [30,31], respectively. Eleven CTRLs were recruited from asymptomatic patients undergoing colonoscopy for colorectal cancer screening or follow-up of polyposis with the following inclusion criteria: absence of chronic pain conditions, infectious or inflammatory disorders, psychiatric illness, or administration of systemic-acting pharmaceuticals. The study participants had not assumed corticosteroids or antibiotics agents during the past year, and did not have a current history of alcohol abuse. All patients filled out a standardized questionnaire for the GI symptoms and the quality of life. All the patients were enrolled after fulfilling the informed consent. The study was performed in accordance with the principles of the declaration of Helsinki and was approved by the Medical Ethics Committee of Campus Bio-medico University (Prot. 21/15 ComEt CBM).

For MM sampling, colonic cleansing was performed using a 50% magnesium sulfate solution. Subsequently, colonoscopy was performed using a videoendoscope, and biopsies were obtained from 10 CD patients, 4 UC patients, and 11 CTRLs. The biopsies were obtained from terminal ileum and descending colon of all CD, UC patients and CTRLs, using disposable forceps for each subject, regardless of presence of inflammation. In both patients and healthy controls biopsies were collected in the same site (terminal ileum and sigmoid colon) in order to better compare the microbiome composition in each district. Fresh FM samples were collected from 8 IBD patients (5 CD and 3 UC) and 5 CTRLs. Unwashed biopsies and faecal samples were sent to the Human Microbiome Unit of Bambino Gesù Children's Hospital and Research Institute of Rome in dry ice within one hour and immediately stored at −80 °C, until processing to strictly prevent anaerobic bacteria from being exposed to oxygen and to avoid bacterial overgrowth before DNA extraction.

Sample sets were defined as follows: (i) biopsies_IBD; (ii) biopsies_CTRL; (iii) stool_IBD, (iv) stool_CTRL.

2.2. Targeted-MG of mucosal microbiota and faecal microbiota

The DNA extraction, amplification and sequencing steps were performed in spatially and temporally distant steps, using dedicated cabinets and disposable reagent aliquots to minimize environmental and procedural cross contaminations. All experimental procedures were certified by quality controls (QC) check points for each pre-analytical and analytical step, accordingly to ISO UNI EN ISO 9001 for microbiome analyses.

Mucosal DNA was isolated by Bio Robot EZ-1 (Qiagen, Germany) following manufacturer's procedures, while faecal DNA was isolated using the QIAamp DNA Stool Mini Kit (Qiagen, Germany), The V1–V3 region of the 16S ribosomal RNA (rRNA) locus was amplified for pyrosequencing utilizing a 454-Junior Genome Sequencer (Roche 454 Life Sciences, Branford, USA), according to the pipeline described in Ercolini et al. [32].

Primers (FW 5′-GAGTTTGATCNTGGCTCAG-3′, RV 5′-GTNTTACNGCGGCKGCTG-3′) were barcoded by 8 unique nucleotide sequences (Roche 454 Life Sciences, Branford, USA). The polymerase chain reactions were performed using a Hi-Fi PCR Taq polymerase (FastStart™ High Fidelity PCR System, dNTPack, Roche Diagnostics, Mannheim, Germany) reducing per base PCR error rates and chimeric sequences [33].

Reads were analyzed by Quantitative Insights into Microbial Ecology (QIIME, v.1.8.0) [34]. Reads with an average quality score lower than 25, shorter than 300 bp and with an ambiguous base calling were excluded from the analysis. The denoised sequences were chimera-checked by *identify_chimeric_seqs.py* using either Blast_fragments and ChimeraSlayer (http://qiime.org/scripts/identify_chimeric_seqs.html) approaches. Reads were aligned by PyNAST [33], and clustered by UCLUST [34] for OTUs matching against Greengenes database (v. 13.8) (similarity level

Table 1
Clinical characteristics of enrolled patients and healthy subjects.

		Crohn's disease	Ulcerative colitis	Controls
Sex	M	6	3	7
	F	4	1	4
Mean age		51,3	45	49,9
BMI		23	27	24.5
Disease status	Active	2	1	–
	Remission	8	3	–
Therapies	Immunosoppressant	3	0	–
	Biologic therapies	6	2	–

of 97%). The α and β diversity and the Kruskal–Wallis test were performed by QIIME software, using "alpha_rarefaction.py, beta_diversity_through_py, group_significance.py" scripts [35].

2.3. Statistics

Statistical tests (Shapiro-Wilk test, ANOVA, Least Significant Difference [LSD] test, Mann–Whitney *U* test, Receiver Operating Characteristic [ROC], Discriminant analysis, Spearman's correlations) were performed by IBM SPSS statistic software version 21. Shannon Index was used to discriminate the four groups of samples and the average area under ROC curve was applied to all sample set.

3. Result

3.1. Clinical characteristics

The demographic and clinical characteristics of the IBD patients are shown in Table 1. Biopsies were obtained from different intestinal sites in all IBD patients and CTRLs (Table 1**S**).

3.2. Metagenomics, MM and FM sampling

A total of 166,633.00 sequencing reads were obtained from 58 biological samples, with a mean value of 2872.98 ± 1657.22 sequences per sample. From 45 biopsies samples a total of 108.796 sequences were obtained, with a mean value of 2417.69 ± 1398.56 sequences per sample, while for stool samples were obtained a total of 57,837 sequences, with a mean value of 4449.00 ± 1550.93 sequences per sample.

3.2.1. Alpha and beta diversity analysis

The Shapiro–Wilk test, performed on alpha diversity Shannon indices computed for IBD stools, CTRL stools, IBD biopsies, CTRL biopsies, assigned a normal distribution for each patient and CTRLs dataset (P = >0.05) (Table S2).

The Fisher's Least Significant Difference (LSD) Test performed on Shannon Index values amongst the 4 groups revealed a statistically significant difference between the median values of stools_IBD and biopsies_IBD, stools_CTRL and stools_IBD, stools_CTRL and biopsies_IBD, and finally, biopsies_CTRL and biopsies_IBD (Table 2, and Fig. 1).

To define if Shannon Index was able to discriminate the 4 groups of samples, the average area under the Relative Operating Characteristic (ROC) was applied to all sample sets. The results confirmed that the Shannon index was able to determinate all normal distribution only in CTRL stool group (AUROC = 0.992) respect to other groups of samples (Fig. S1, Table S3).

The beta diversity, visualized by Principal Coordinate Analysis (PCA), showed clear separation between biopsy and stool groups. Moreover, stratifying samples into stool IBD, stool CTRL, biopsies IBD and biopsies CTRL, a clearly defined cluster, composed by stool groups, and a less defined cluster, composed by biopsy groups, were identified (Fig. 2).

3.2.2. Percent variation of bacteria in mucosal specimens

Grouping only biopsies samples from IBD and CTRL subjects, Kruskal–Wallis test showed a statistically significant increase of Proteobacteria and decrease of Firmicutes and Actinobacteria, compared to CTRLs (P [FDR] < 0.05) (Fig. 3, Panel A; Table S4).

At family/species level the two sets showed a completely different microbiota phenotype. Particularly, a statistical increment of *Bacteroides fragilis*, *Roseburia*, *Faecalibacterium prausnitzii*, Lachnospiraceae, *Ruminococcus* in CTRL compared to IBD sample set (P [FDR] < 0.05) (Fig. 3, Panel B; Table S4).

3.2.3. Percent variation of bacteria in stool

The microbiota analysis of IBD stool samples showed an increment of Proteobacteria (P < 0.05) and a decrease of Bacteroidetes although the difference was not significant compared to CTRLs (Fig. 3, Panel C; Table S5).

At family/species level, a predominant presence of Enterobacteriaceae in IBD and a predominant presence of Ruminococcaceae, Rikenellaceae Clostridiales and *Prevotella* in CTRLs (P < 0.05) were prevalent (Fig. 3, Panel D; Table S5).

3.2.4. Percent variation of bacteria in different gut locations

Describing IBD patient biopsies according to intestinal sampling site, no statistical difference between ileum and colon sites was observed at phylum level (Fig. S2, Panel A; Table S6).

Analysis at Family/species level revealed that only Ruminococcaceae resulted statistically increased in the colon district (P < 0.05) (data not shown).

By comparing IBD colon biopsies *versus* CTRL biopsies, a significant increment of Proteobacteria (P (FDR) < 0.05) and a decrement of Firmicutes were observed in IBD (P < 0.05) (Fig. S2, Panel B; Table S7).

At family/species level a significant reduction of *Coprococcus*, *Faecalibacterium prausnitzii*, Lachnospiraceae, Rikenellaceae, *Roseburia*, Ruminococcaceae *and* an increment of Enterobacteriaceae were observed in IBD respect to CTRL (Fig. S2, Panel C; Table S7).

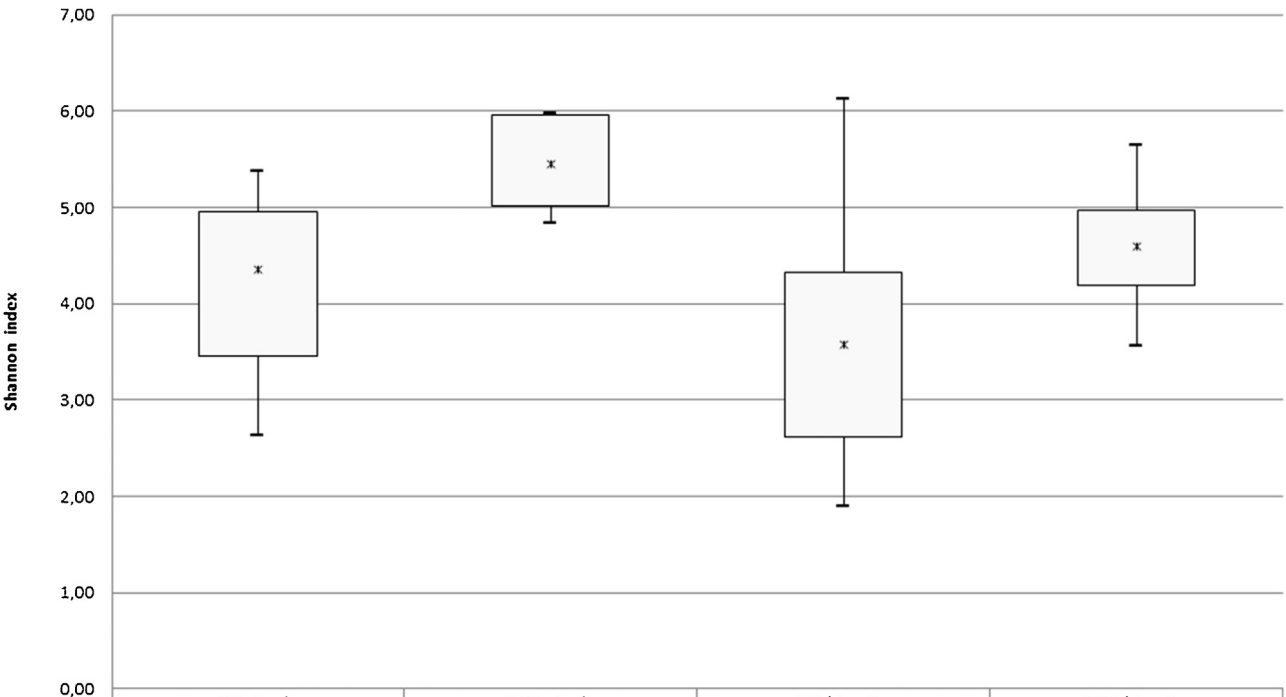
Finally, looking at disease activity, no significant difference in OTUs distribution at phylum level, as expected, was observed between active and non-active disease groups (Fig. S3, Panel A; Table S8), even if the small number of patients limits this evaluation. However, at Family/species level, a decrease of *Ruminococcus,* Peptostreptococcaceae *and Paraprevotella* and an increase in *Enterococcus* were associated to active disease status (P < 0.05) (Fig. S3, Panel B; Table S8).

3.2.5. Comparison of faecal microbiota versus mucosal microbiota composition

The Kruskal–Wallis test on OTU distribution at phylum level for biopsy and faecal CTRL samples showed an increase of Proteobacteria (P[FDR] < 0.05) and Bacteroidetes (P < 0.05) and a reduction of Firmicutes (P[FDR] < 0.05) in mucosal specimens compared to faecal samples (Fig. S4, Panel A; Table S9). At species level Clostridiales, *Prevotella*, Ruminococcaceae were over-expressed in faecal specimens compared to biopsies while *Bacteroides* appeared higher in biopsies rather than in to the faeces (Fig. S4, Panel B; Table S9).

Comparing IBD patient's biopsies versus faeces, Proteobacteria and Firmicutes appeared reduced in the biopsies, while Bacteroidetes resulted increased (Fig. S4, Panel C; Table S10). At species level Bacteroides, Peptostreptococcaceae appeared overexpressed in biopsies while *Lactobacillus reuteri*, *Granulicatella*, *Streptococcus* were over-expressed in faeces (Fig. S4, Panel D; Table S10).

The Mann–Whitney test, applied to the OTUs distribution in stool samples, revealed that the relative abundances of Mogibacte-



	IBD stools	CTRL stools	IBD biopsies	CTRL biopsies
Q1	3,46	5,02	2,61	4,19
– Minimum	2,63	4,84	1,89	3,56
x Mean	4,35	5,46	3,57	4,59
– Maximum	5,38	5,97	6,13	5,65
Q3	4,95	5,96	4,33	4,97

Fig. 1. Box plot of mean values of Shannon index for the 4 groups. The plot reports the mean, minimum (min), maximum (max) values, first (q1) and third (q3) quartiles calculated for IBD stools, CTRL stools, IBD biopsies and CTRL biopsies groups. The summary table shows detailed values.

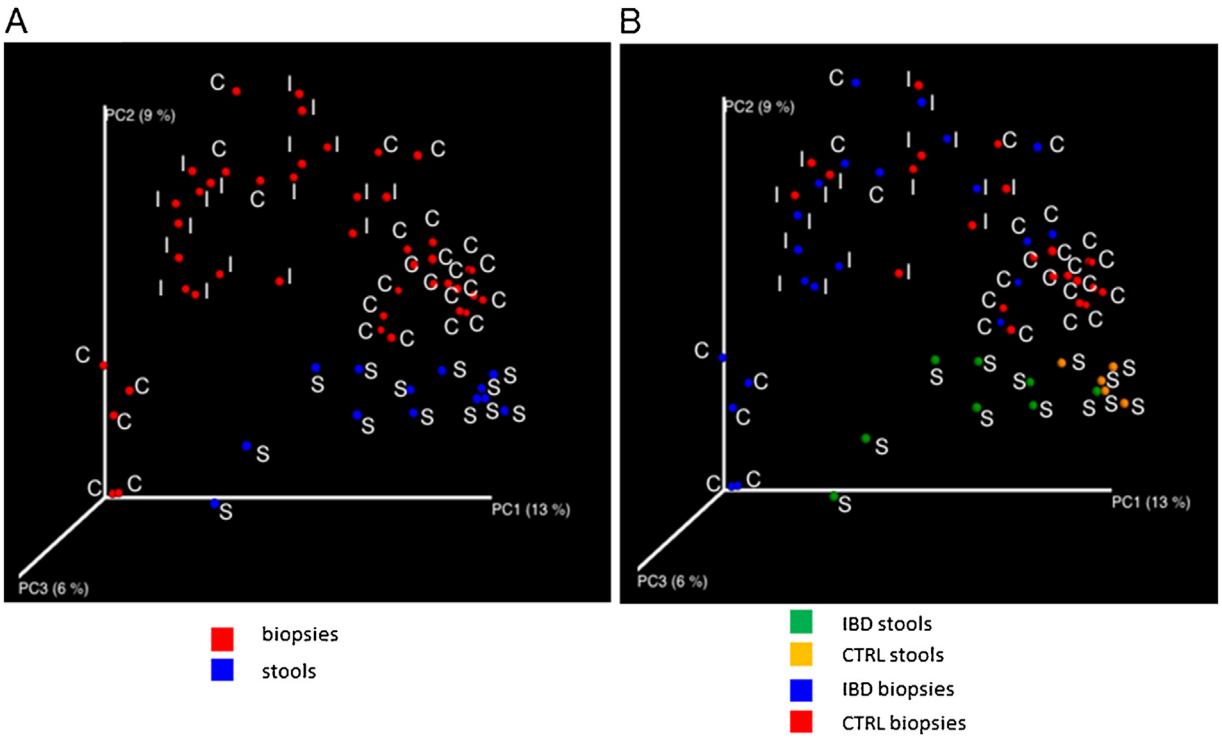


Fig. 2. Principal coordinate analysis plot of biopsy and stool samples (A) and of IBD stools, CTRL stools, IBD biopsies and CTRL biopsies. The plots show the first two principal coordinates for principal component analysis (PCoA) using unweighted UniFrac algorithm.

Table 2
Post Hoc analysis on alpha diversity Shannon index values.

Least significant difference (LSD)	VAR (I)	VAR (J)	Mean difference (I – J)	SD	P value	95% confidence interval	
						Lower Bound	Upper Bound
Shannon index	**stools_IBD**	**stools_CTRL**	**−1.11**	**0.50**	**0.03**	**−2.11**	**−0.11**
		biopsies_IBD	**0.78**	**0.35**	**0.03**	**0.07**	**1.49**
		biopsies_CTRL	−0.24	0.37	0.52	−1.00	0.51
	stools_CTRL	**stools_IBD**	**1.11**	**0.50**	**0.03**	**0.11**	**2.11**
		biopsy_IBD	**1.89**	**0.43**	**0.00**	**1.03**	**2.74**
		biopsies_CTRL	0.86	0.44	0.06	−0.03	1.76
	biopsies_IBD	**stools_IBD**	**−0.78**	**0.35**	**0.03**	**−1.49**	**−0.07**
		stools_CTRL	**−1.89**	**0.43**	**0.00**	**−2.74**	**−1.03**
		biopsies_CTRL	**−1.02**	**0.27**	**0.00**	**−1.57**	**−0.47**
	biopsies_CTRL	stools_IBD	0.24	0.37	0.52	−0.51	1.00
		stools_CTRL	−0.86	0.44	0.06	−1.76	0.03
		biopsies_IBD	**1.02**	**0.27**	**0.00**	**0.47**	**1.57**

The p values < 0.05 are reported in bold.

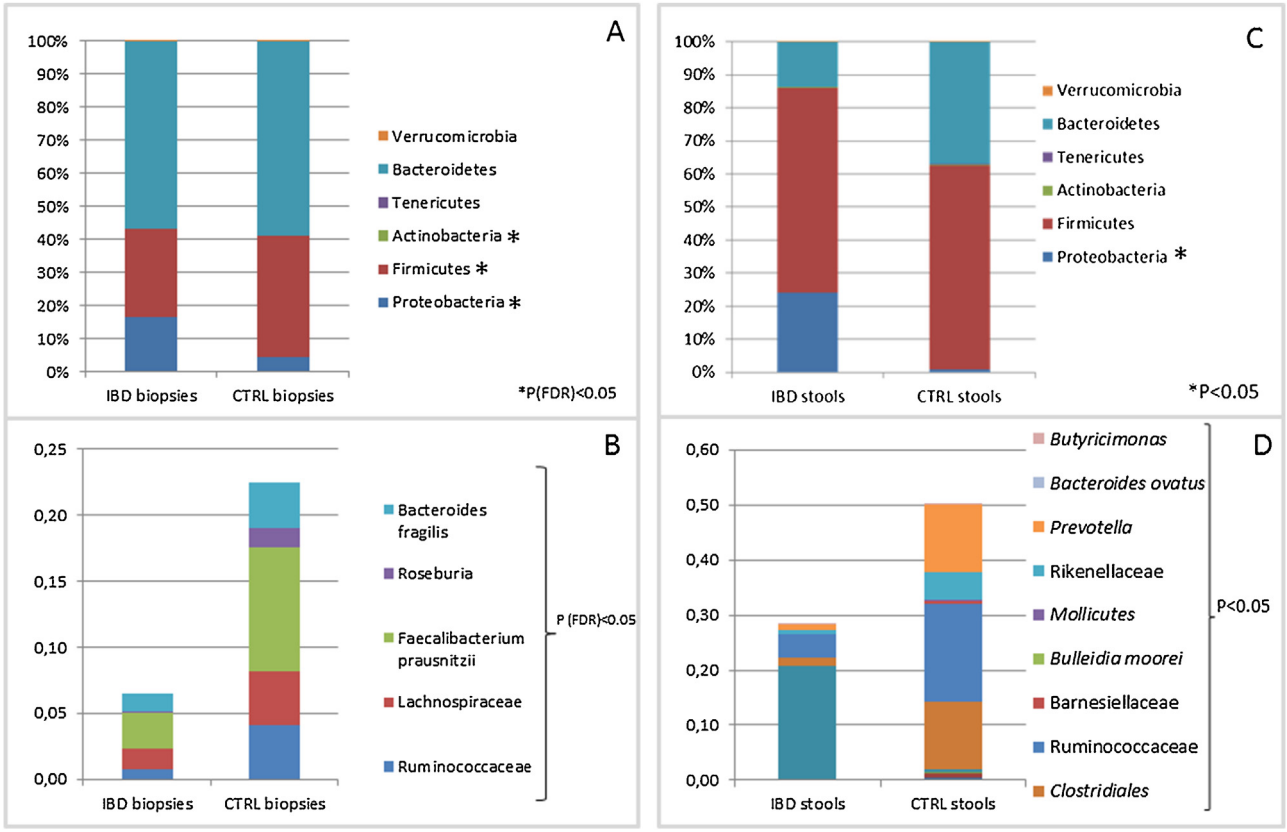


Fig. 3. Bar chart reporting Kruskal–Wallis test results on OTUs at the phylum (Panel A) and species/family (panel B) levels of biopsy samples and at the phylum (Panel C) and species/family (panel D) levels of stool samples in IBD compared to CTRL. Each column in the plot represents a group, and each color in the column represents: A and C, the percentage of relative abundance for each OTU; B and D, the values of relative abundance for each OTU.

riaceae, *Prevotella*, *Butyricimonas*, *Lachnospira*, *Bacteroides caccae*, Rikenellaceae, Ruminococcaceae were lower, while Barnesiellaceae and Enterobacteriaceae were higher in IBD stools respect to CTRL stools (P values <0.05) (Table S11). Comparing IBD stools to IBD biopsies, a lower relative abundance of *Acidovorax delafieldii, Fusobacterium, Bacteroides caccae, Odoribacter, Sutterella, Bacteroides* and a higher abundance of Peptostreptococcaceae and *Streptococcus* were observed in stools compared with biopsies (P < 0.05). In the comparison between IBD stool and CTRL biopsies the relative abundances of *Fusobacterium*, *Bacteroides caccae*, *Odoribacter*, Rikenellaceae, *Roseburia*, *F. prausnitzii*, *Parabacteroides distasonis*, *Sutterella*, *Bacteroides* were lower in patient stools respect to CTRL biopsies, while the contrary was observed for Peptostreptococcaceae, *Veillonella dispar* (*V. dispar*), *Streptococcus* and Enterobacteriaceae (P < 0.05).

Analyzing the OTUs relative abundances of CTRL stools respect IBD biopsies the comparison revealed that only for Enterobacteriaceae and Bacteroides the abundances resulted higher in IBD biopsies compared to CTRL stools, while for Mogibacteriaceae, *Anaerostipes*, *Prevotella*, *Lachnospira*, *Streptococcus*, *Coprococcus*, Rikenellaceae, Bacteroidaceae, *Ruminococcus*, *Blautia*, *Faecalibacterium prausnitzii*, Ruminococcaceae we observed the opposite (P > 0.05).

Also for the OTUs Mogibacteriaceae, *Anaerostipes*, *Prevotella*, *Lachnospira*, *V. dispar*, *Streptococcus*, Bacteroidaceae, *Ruminococcus*, *Sutterella*, *Bacteroides* the relative abundances resulted higher in

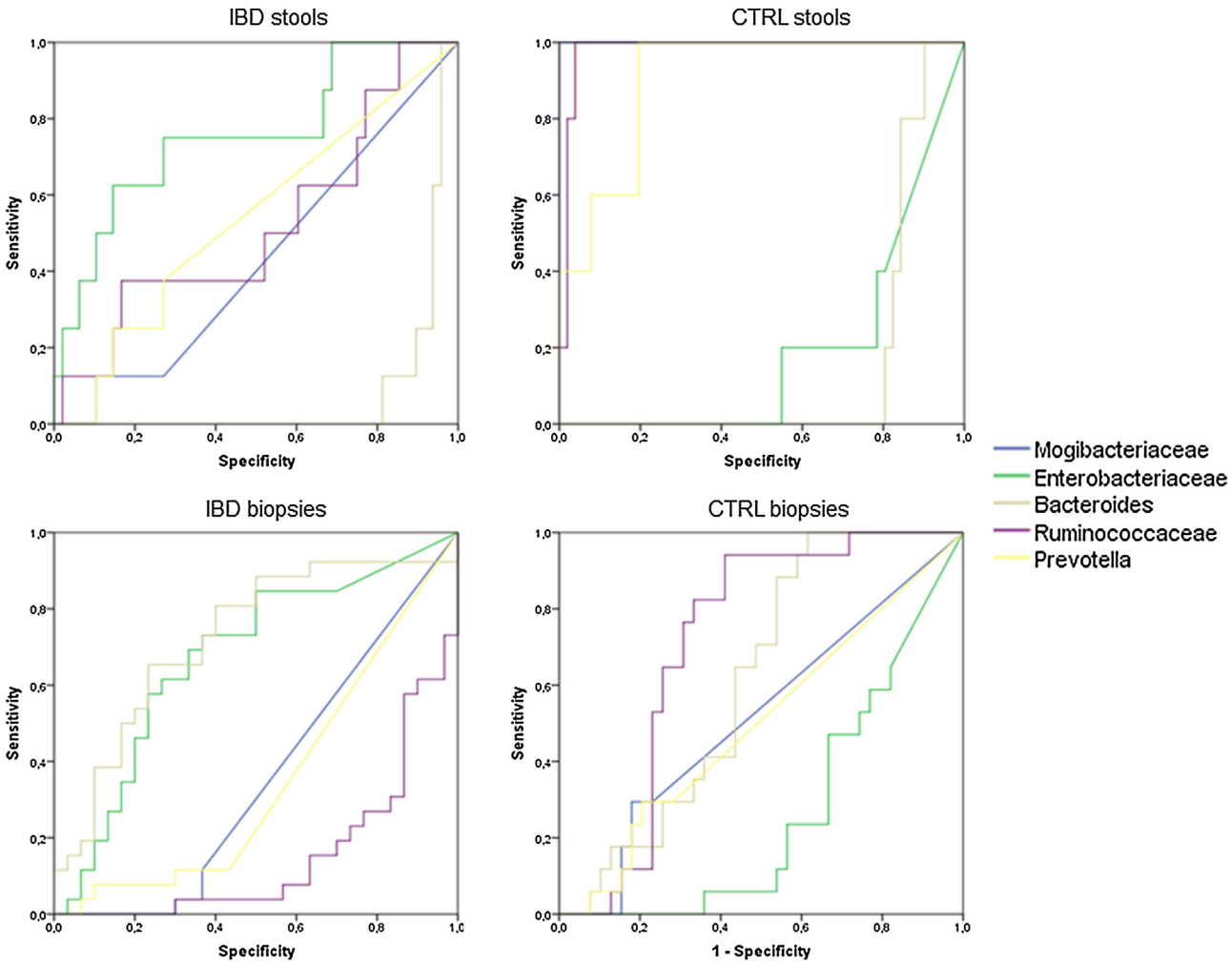


Fig. 4. Receiver Operating Characteristic (ROC) curve plots. The areas under the ROC curves (AUROC) represent the specificity and sensitivity of the 5 selected OTUs (AUROC > 0.7) able to discriminate IBD stools, CTRL stools, IBD biopsies, CTRL biopsies groups.

CTRL stools respect to CTRL biopsies, while only Ruminococcaceae were higher in CTRL biopsies respect to CTRL stools ($P > 0.05$).

Finally, comparing IBD versus CTRL biopsies, only *V. dispar* and Enterobacteriaceae resulted higher in IBD, while Lachnospira, *Coprococcus*, Rikenellaceae, *Roseburia*, *F. prausnitzii*, Ruminococcaceae, Lachnospiraceae resulted lower respect to CTRL biopsies ($P < 0.05$) (Table S12).

Considering only OTUs for which the relative abundance comparison was statistically significant by Mann–Whitney *U* test, OTUs variables on the bases of mean values >0.1 in almost one comparison were filtered: Mogibacteriaceae, Enterobacteriaceae, *Bacteroides*, Ruminococcaceae, *Prevotella* were selected as variables for ROC curve analysis applied on each separated group of samples. As shown in Fig. 4 (Table S11) the analysis resulted accurate for Enterobacteriaceae in IBD stools, Mogibacteriaceae, Ruminococcaceae and *Prevotella* for CTRL stools, *Bacteroides* for IBD biopsies, and Ruminococcaceae for CTRL biopsies on the basis of Swets classification model [36].

3.3. Validation analysis

Appling the Fisher's linear discriminant functions for classification analysis on the above selected OTUs (Table S11), we obtained that the 78.6% of original grouped cases correctly classified, and by Leave one-out test for cross-validation (CV) analysis we obtain that 73.2% of cross-validated grouped cases correctly classified (Table S13).

4. Discussion

Growing evidence demonstrate that modifications in intestinal microbiota are common in patients with IBD [37–39]. Most of the studies have analyzed FM [36,37] or MM [38,39] composition, while few investigations compare the two bacterial populations [28,40]. In the present study both FM and MM were analyzed by a targeted-metagenomics (MG) approach.

The bacterial richness analysis highlighted a high microbial diversity in CTRL stool and biopsy samples, and a decrease of microbial richness in IBD samples. As expected, the stools had a Shannon Index higher than biopsies, probably due to a low number of bacteria cleaved to the mucosal surface after enema administration for colonoscopy [41]. Applying ROC curve test on Shannon Index values, this alpha diversity index revealed the capability to distinguish only CTRL stool samples from other samples. Focusing on biopsies a less defined cluster composition was identified between IBD and CTRLs samples, suggesting that rare taxa between the two sample types differ [41].

Despite previous investigations were focused mostly on faecal samples for their easy collection [42,43], the degree to which composition and function of the FM differ from MM remains unclear.

In a recent study on IBS patients, few differences in the mucosal-associated microbiota between healthy individuals and IBS patients were found compared to faecal microbiota, suggesting that microbial aberrations are more pronounced in the faeces [44]. On the contrary, in the present study profiles of FM only partially replicate those of the MM and were more similar to controls. This could suggest that gut-mucosa associated microbiota better discriminates patients from controls, especially at the family level.

In IBD patients, the most represented phylum of the MM was Proteobacteria with a predominant presence of Enterobacteriaceae, *Acidaminococcus*, *V. dispar* while, Ruminococcaceae, Rikenellaceae, *Prevotella stercorea*, Lachnospiraceae, *F. prausnitzii*, *Coprococcus* and *Bacteroides fragilis* were prevalent in controls.

These data confirm previous observations, in mucosal biopsies of IBD patients, in which a significant reduction of *F. prausnitzii* was observed [28,45,46]. Growing evidence suggest a beneficial role of *F. prausnitzii*; recently, *Quèvrain* et al. detected a 15 kDa protein with anti-inflammatory properties produced by *F. prausnitzii*, able to inhibit the NF-κB pathway in intestinal epithelial cells and to prevent colitis in an animal model [47]. These data suggest a potential beneficial role from *F. prausnitzii* enrichment. This promising potential had been confirmed also by other authors [48].

In the present study, IBD patients appear to have a statistically significant reduction of *Roseburia*, a well-known butyrate-producing bacterium of the Firmicutes phylum, as well as of *F. prausnitzii*. In a recent paper, a lower abundance of *Roseburia hominis* and *F. prausnitzii* only in UC patients compared to controls was observed [49]. Therefore, it seems that the reduced abundance of Firmicutes in the group of IBD patients could be correlated with the presence or the persistence of inflammation [49].

Previous studies have shown that the fermented products of these bacteria provides energy for colonic epithelial cells and play an important role in epithelial barrier integrity and immune modulation [45,50]. Interestingly, in a recent paper, in which the geographical patterns of gut microbiome in IBD patients was investigated, decreasing of *Ruminococcus*, *Roseburia* Lachnospiracee and *Coprococcus* seems to be specific of European patients [45].

At our knowledge, the present study is the first to detect a statistically significant decrease, at mucosal level, of bacteria belonging to the Actinobacteria phylum, whose role in gastrointestinal diseases, and especially in IBD, remains to be clarified. Recent studies show a significant association between the Actinobacteria increase in faeces and the onset of different autoimmune diseases, including juvenile idiopathic arthritis, type 1 diabetes and Behçet's syndrome [1,51].

In MM of IBD patients, a statistically significant increase in bacteria belonging to the Proteobacteria phylum has also been detected. This appears to be driven by the relative abundance of Enterobacteriaceae, including *V. dispar* and *Bacteroides egghertii*. Despite *V. dispar* has been always considered a non-pathogenic bacteria, recent studies have shown that is able to actively ferment organic acids and produce a highly endotoxic lipopolysaccharide [52]. Furthermore an increased concentration of *V. dispar* in the microbiota of patients with colorectal cancer [52] and IBS patients [53] has been observed, suggesting a possible pathogenic role in gastrointestinal diseases. In agreement with our results, in IBD patients a role of *V. dispar* in the dysbiosis of salivary microbiota has been reported [54], possibly suggesting a swalling-mediated microbiota transit. However, the clinical relevance of these findings has to be further investigated.

Concerning *B. egghertii*, there are still few studies in the literature because it is also considered a normal commensal of the human gut microbiota. Only a recent study on animal models of colitis has however shown that *B. egghertii* together to *Parabacteroides distasonis* showed its ability to worsen the dextran sodium sulfate induced colitis [55]. Upon the results obtained in the present study, a possible specific role of both *Bacteroides* and *Veillonella* in the pathogenesis of IBD could be hint.

In IBD patients the diversity of the FM and MM was significantly decreased compared to healthy individuals. This decrease was also evident between mucosal and faecal samples, as also reported in the Chinese population [46].

Our data showed that ileum less defines the IBD enterophenotype, as shown by colon plus ileum description of MM. Although the number of biopsies was limited, these findings are similar to previous reports where in which microbiota associated with ileal, cecal and rectal mucosa were not significantly different [45,50,56]. The relationship between specific intestinal commensal bacteria and disease activity and complications is controversial [22,57–60]. In our series, a statistically significant correlation between active disease and decrease of *Ruminococcus*, *Peptostreptococcus* and *Paraprevotella* and increase of *Enterococcus* was found only at family level.

In conclusion, data from the present study suggest that the evaluation of MM better discriminate IBD patients from healthy individuals than FM, especially when considering at L6 taxonomic level [16]. Interestingly, a recent study has demonstrated similar results in patients with chronic constipation, for whom the MM better discriminate patients with constipation than FM [61]. In this context, while trying to keep going onto FM composition of our patients' set, the focus onto MM composition may bring some possible added values to the current literature. Indeed, so far, very few studies have fully analyzed the altered microbiota in mucosal samples of IBD patients, and the available data are also conflicting.

Finally, the present study may suggest new FM- and MM-associated OTUs intended as specific IBD bacterial biomarkers or predictors, as demonstrated by ROC analyses and CV classifications: Enterobacteriaceae were associated to IBD stools; *Bacteroides* to IBD biopsies; Mogibacteriaceae, Ruminococcaceae and *Prevotella* to CTRL stools; Ruminococcaceae to CTRL biopsies. Interestingly, *Prevotella* appeared in the FM profile filtered by ROC curve more abundant in CTRLs than in IBD stools, similarly with quantitative microbiota profiling (QMP) described for CD patients [62]. *Bacteroides* resulted not predictor of patients' FM, this also in agreement with Vandeputte et al. [62], while it was associated as microbial biomarker to patients' MM.

These data, even if preliminary due to a small number of patients included, anyway might suggest to keep going into the direction of diagnostic pipeline to improve microbiota profiling with special reference to mucosal biosystem: disease bacterial biomarker searching and characterization. Therefore, new definition of IBD-associated dysbiosis could be revised in term of MM rather than in term of FM, suggesting other disease diagnostic targets to be tested in scale-up patients' cohorts.

Conflict of interest

LP has served as a consultant and has received research funding from Alfawassermann. All of the other authors have not conflict of interests.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.dld.2018.11.021.

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