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Mucosa-associated *Faecalibacterium prausnitzii* and *Escherichia coli* co-abundance can distinguish Irritable Bowel Syndrome and Inflammatory Bowel Disease phenotypes.

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RUNNING TITLE:

Bacterial indicators to distinguish between IBD entities

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ABSTRACT

Background:

Crohn's disease (CD) and ulcerative colitis (UC) diagnosis requires comprehensive examination of the patient. *Faecalibacterium prausnitzii* and *Escherichia coli* have been reported as representatives of Inflammatory Bowel Disease (IBD) dysbiosis. The aim was to determine whether or not quantification of these species can be used as a complementary tool either for diagnostic or prognostic purposes.

Methods:

Mucosa-associated *F. prausnitzii* and *E. coli* abundance was determined in 28 controls (H), 45 CD, 28 UC patients and 10 irritable bowel syndrome (IBS) subjects by quantitative polymerase chain reaction (qPCR) and the *F. prausnitzii-E. coli* index (F-E index) was calculated. Species abundances were normalized to total bacteria and human cells. Data was analyzed taking into account patients' phenotype and most relevant clinical characteristics.

Results:

IBD patients had lower *F. prausnitzii* abundance than H and IBS ($P<0.001$). CD patients showed higher *E. coli* counts than H and UC patients ($P<0.001$). The F-E index discriminated between H, CD and UC patients, and even between disease phenotypes that are usually difficult to distinguish as ileal-CD (I-CD) from ileocolonic-CD and colonic-CD from extensive colitis. *E. coli* increased in active CD patients, and remission in I-CD patients was compromised by high abundance of this species. Treatment with anti-tumor necrosis factor (TNF) ! diminished *E. coli* abundance in I-CD whereas none of the treatments counterbalanced *F. prausnitzii* depletion.

Conclusion:

F. prausnitzii and *E. coli* are useful indicators to assist in IBD phenotype classification. The abundance of these species could also be used as a supporting prognostic tool in I-CD patients. Our data indicates that current medication does not restore these two species levels to those found in a healthy gut.

KEYWORDS

Faecalibacterium prausnitzii, *Escherichia coli*, Inflammatory Bowel Disease, Irritable Bowel Syndrome, diagnostics, prognostics

INTRODUCTION

Inflammatory bowel disease (IBD) comprises a group of idiopathic, chronic, inflammatory intestinal disorders. Its two most important disease categories are Crohn's disease (CD) and ulcerative colitis (UC) (Baumgart and Carding, 2007; Baumgart and Sandborn, 2007; Xavier and Podolsky, 2007). Although both intestinal diseases differ in terms of their location, the distribution of inflamed areas and their histology, classification of these disease states can be difficult given their overlapping clinical and pathological characteristics (Yantiss and Odze, 2006). To clearly discriminate both diseases is essential to establish an appropriate treatment strategy. In addition, other digestive disorders such as irritable bowel syndrome (IBS) can mimic IBD clinically, particularly in the early stages, increasing its likelihood of misdiagnosis (Bernstein et al., 2010; Nikolaus and Schreiber, 2007).

Given the absence of pathognomonic features, the diagnosis for IBD currently requires a comprehensive examination of the patient that includes clinical, endoscopic, radiologic, and histological criteria (Bernstein and Shanahan, 2008). IBD is an intermittent disease, whose clinical manifestations are hardly predictable and unstable during its course. Symptoms range from mild to severe during relapses and may disappear or decrease during episodes of remission. Thus, careful consideration of a patient's clinical data and a long monitoring period are necessary to accurately classify the disease phenotype (Bernstein et al., 2010; Louis et al., 2001).

Although the pathogenesis of IBD is incompletely understood, it is known that it is a complex disease in which many factors determine who develops IBD, the age of presentation, and the specific manifestations of disease (Bernstein et al., 2010; Kaser et al., 2010; Manichanh et al., 2012). Currently, the most generally accepted hypothesis is that genetic and environmental factors such as altered luminal bacteria and enhanced

intestinal permeability play a role in the deregulation of intestinal immunity, which in turn may lead to gastrointestinal injury (Sartor, 2006; Xavier and Podolsky, 2007).

The role of the gut microbiota in the onset and perpetuation of intestinal inflammation in IBD has been a topic systematically studied during the last 10 years (for review see (De Cruz et al., 2012; Elson and Cong, 2012; Manichanh et al., 2012) and references therein). It is well established by studies performed both in fecal or mucosa-associated communities, either by culture-dependent or molecular methods that CD patients have an altered microbiota, which differs from that found in patients with UC and as well as of that in healthy controls (Manichanh et al., 2012). This dysbiosis is characteristic of the disease as it is not shared with unaffected monozygotic twins or relatives despite the common genetic background and the shared environment (Joossens et al., 2011; Willing et al., 2009). Although the reported changes are not always consistent, most studies agree that numbers of *Firmicutes,* particularly the species *Faecalibacterium prausnitzii,* are depleted in patients with CD (Frank et al., 2007; Martinez-Medina et al., 2006; Miquel et al., 2013; Sokol et al., 2009; Swidsinski et al., 2008; Willing et al., 2009) whereas *Proteobacteria*, especially *Escherichia coli*, are increased predominantly in CD patients with ileal involvement (Martinez-Medina et al., 2009; Mondot et al., 2011; Seksik et al., 2003; Willing et al., 2009). Taken together these findings indicate that the abundance of these two bacterial groups might be a reliable indicator of dysbiosis in CD patients.

Application of molecular methods to specifically monitor changes of key microorganisms in the gut is of particular interest, since it may provide an innovative source of additional information to assist clinicians in disease diagnosis and management. To our knowledge, few studies have been conducted with this aim in respect of IBD. Interestingly, a reduction in *F. prausnitzii* abundance has however been

correlated with IBD patients' activity, flare ups and remission state (Sokol et al., 2009), but few studies have addressed the question of whether this bacterium or other key dysbiosis representatives could be useful to assist IBD diagnostics or to monitor disease progression. Swidsinski and colleagues have reported that CD and UC could be diagnosed through monitoring *F. prausnitzii* abundance in conjunction with fecal leucocyte counts (Swidsinski et al., 2008). Recently new phylogenetic specificities of CD microbiota have been highlighted by identifying a set of six species discriminatory for CD patients with ileal involvement, which also provides a preliminary diagnostic tool (Mondot et al., 2011). However, further analysis including all CD and UC phenotypes should be performed in order to determine the extent of dysbiosis within all disease categories. In addition, comprehensive studies are lacking to show how patients' clinical data correlates with changes in the abundance of these bacterial indicators, and how the different therapies may affect the abundance of these species.

This work aims at testing whether or not mucosa-associated *F. prausnitzii* and *E. coli* abundances could be used to differentially diagnose IBD patients and monitor the evolution of the disease. To achieve this objective, the abundance of both bacterial species was determined in CD, UC and IBS patients and in healthy controls. A novel multiplex qPCR assay was developed for *F. prausnitzii*, valid for the quantification of the two known phylogroups within this species. Furthermore, data were analyzed taking into account patients' most relevant clinical characteristics, in order to determine its usefulness to predict disease progression. Medication at sampling was also considered in order to determine whether any of the current therapies are effective in correcting this dysbiosis.

MATERIALS AND METHODS

Patients, clinical data and sampling.

A Spanish cohort consisting of 73 IBD patients, including 45 CD and 28 UC has been compared with those from ten IBS patients and 28 healthy control subjects (H). Subjects were recruited by the Gastroenterology Services of the Hospital Universitari Dr. Josep Trueta (Girona, Spain) and the Hospital Santa Caterina (Salt, Spain). Patients were sex- and age-matched, except CD patients who were younger than those in the H and IBS groups (Table 1). IBD patients were diagnosed according to standard clinical, pathological and endoscopic criteria, were categorized according to the Montreal classification (Silverberg et al., 2005), and clinically relevant data was collected. IBS patients were diagnosed according to Rome III criteria (available at <http://www.romecriteria.org/criteria/>). The control group consisted of subjects with normal colonoscopy who underwent this procedure for different reasons as rectorrhagia (N=9), colorectal cancer familial history (N=10), and abdominal pain (N=9). None of the subjects received antimicrobial treatment for at least two months before colonoscopy.

Prior to colonoscopy, patients were subjected to cleansing of the gastrointestinal tract using Casenglicol® following manufacturer's guidelines. During routine endoscopy, up to three biopsy samples per patient were taken from different locations along the gut (distal ileum, colon, and rectum) following standard procedures. For IBD patients, additional samples from ulcerated and non ulcerated mucosa according to macroscopic criteria were taken when technically possible. All biopsies were immediately placed in sterile tubes without any buffer and stored at -20 ºC following completion of the whole endoscopic procedure, for each patient. DNA extraction was then performed on these samples within the following 6 months.

A subgroup of 10 CD patients who started adalimumab therapy (HUMIRA; Abbott Laboratories, Chicago, IL) was enrolled on a follow-up study and rectal samples were also collected one and three months after the first colonoscopy.

Ethical considerations

This work was approved by the Ethics Committee of Clinical Research of the Hospital Universitari Dr. Josep Trueta (Girona, Spain) and the Institut d'Assistència Sanitària of Girona (Salt, Spain) on 24th February 2009 and 21st April 2009, respectively. Informed consent from the subjects was obtained before enrollment.

Sample treatment and DNA extraction.

Prior to DNA extraction, biopsies were subjected to two mild ultrasound wash cycles to discard transient and loosely attached bacteria as previously reported (Martinez-Medina et al., 2006). DNA was extracted using the NucleoSpin® Tissue Kit (Macherey-Nagel GmbH &Co., Germany). The support protocol for Gram positive bacteria and the RNAse treatment step were carried out. Genomic DNA was stored at -80 ºC until use. DNA concentration and optical density ratios at 260/280 nm and 230/260 nm to check the purity of the extracts were determined with a NanoDrop ND-100 spectrophotometer (NanoDrop Technologies, USA).

Bacterial strains, growth conditions and DNA extraction from pure cultures.

F. prausnitzii strains were from stocks held by the authors (Rowett Institute of Nutrition and Health, Aberdeen, United Kingdom) and several came from previous studies (Barcenilla et al., 2000; Cato, 1974; Duncan et al., 2002; Lopez-Siles et al., 2012; Louis et al., 2004). Additional bacterial strains were either available in our laboratory collection or were otherwise obtained from several biological resource centers specified in Table S2. When possible, bacteria were cultured aerobically or anaerobically on the recommended medium. DNA was extracted and purified by using the Wizard™

Genomic Purification Kit (Promega Corporation, USA) following the manufacturer's guidelines.

Quantification of standards for quantitative PCR (qPCR).

Quantification standards of the *F. prausnitzii* DSM 17677 and *E. coli* CECT 105 16S rRNA genes were prepared in a genetic construct. The whole 16S rRNA gene of the target species were amplified by conventional PCR as previously reported (Lane, 1991 ; Weisburg et al., 1991) and further introduced in a pCR®4-TOPO® cloning plasmid by using the TOPO TA Cloning® Kit for sequencing (Invitrogen, CA, USA) following the manufacturer's guidelines. Plasmids were extracted using the NucleoSpin® Plasmid (Macherey-Nagel GmbH&Co., Germany). Inserts were further confirmed by sequencing using the Big Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on an ABI Prism 3130 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). Purified plasmids were linearized with *SpeI* (*F. prausnitzii*) or *PstI* (*E. coli*), and DNA quantified as detailed above. Initial target concentration was inferred considering the theoretical molecular weight (3.58×10^6 Da) and size (5421 bp) of the construct. Standard curves were obtained from 10-fold serial dilutions of the titrated suspension of linearized plasmids, and ranged from 100 to 10^7 copies/reaction, which correspond to the linear range span for all the reactions. As it is recommended to use the same standard for species-specific and group-specific primers and probe sets (Suzuki et al., 2000), the standard curve built for *F. prausnitzii* quantification was used for the total bacterial 16S rRNA gene quantification. Total bacteria 16S rRNA gene quantification and the *F. prausnitzii* standard curve were used to check the *E. coli* standard curve quantification in order to make sure that results obtained with both standard curves were comparable. For human

205 cells, ten-fold serial dilutions of the human Xsomal DNA (Eurogentec, Belgium) were
206 used to obtain the standard curve.

207 **Quantitative PCR conditions.**

208 The species-specific 16S rRNA gene-targeted primers and probes used in this study are
209 shown in Table 2. The abundance of *F. prausnitzii* was determined by using a novel
210 assay, designed following the guidelines set by Applied Biosystems (Foster City, CA,
211 USA) for the design of primers and probes, and taking into account the inclusion of
212 both *F. prausnitzii* phylogroups (see details described in the supplemental material,
213 according to the MIQE guidelines (Bustin et al., 2009)). The amplification reactions
214 were carried out in a total volume of 20 µl containing: 1× TaqMan® Universal PCR
215 Master Mix 2× (Applied Biosystems, Foster City, CA, USA), 300 nM of each primer
216 and 200 nM of each probe, 10^3 copies of an internal amplification control (IAC)
217 template and up to 50 ng of genomic DNA template.

218 Previously reported 16S rDNA-targeting primers and probe were used for *E. coli*
219 (Huijsdens et al., 2002) and total bacteria (Furet et al., 2009) quantifications, and
220 amplification reactions were carried out as previously described (Martinez-Medina et
221 al., 2009; Furet et al., 2009). Human cell numbers were determined with the control kit
222 RT-CKFT-18S (Eurogentec, Belgium) according to manufacturer's instructions. All
223 primers and hydrolysis probes were purchased from Applied Biosystems (Foster City,
224 CA, USA). The IAC's DNA was synthesized by Bonsai technologies group
225 (Alcobendas, Spain).

226 Samples were quantified in duplicate. For data analysis, the mean of the duplicate
227 quantifications was used. Duplicates were considered valid if the standard deviation
228 between quantification cycles (C_q) was <0.34 (*i.e.* a difference of <10% of the quantity
229 was tolerated). Quantification controls to assess inter-run reproducibility were

performed consisting of at least five reactions with a known number of target genes. Inhibition was tested by addition of an IAC in each reaction. It was considered that there was no inhibition if the obtained C_q was <0.34 different from those obtained when quantifying the IAC alone for any of the replicates. A no-template control consisting of a reaction without target (*F. prausnitzii, E. coli* or human) DNA template as well as a non-amplification control which did not contain any DNA template (either bacterial, human or IAC) were also included in each run. Negative controls resulted in undetectable C_q values in all cases.

All quantitative PCR were performed using a 7500 Real Time PCR system (Applied Biosystems, Foster City, CA, USA). The thermal profile was: a first step at 50 ºC during 2 min for amperase treatment, followed by a 95 ºC hold for 10 min to denature DNA and activate Ampli-Taq Gold polymerase, and a further 40 cycles consisting of a denaturation step at 95 ºC for 15 seconds followed by an annealing and extension step at 60 ºC for 1 min. Data was collected and analyzed with the 7500 SDS system software version 1.4 (Applied Biosystems, Foster City, CA, USA). The PCR efficiency ranged between 80 and 100% in all the reactions.

Sample size, data normalization, *F. prausnitzii*-*E. coli* index and statistical analysis.

Sample size was defined taking into account the number of patients analysed in similar studies of bacterial abundance in patients suffering of these conditions (Frank et al., 2007; Martinez-Medina et al., 2006; Sokol et al., 2009; Swidsinski et al., 2008; Willing et al., 2009).

F. prausnitzii and *E. coli* 16S rRNA gene copy numbers were normalized to the total bacteria 16S rRNA gene. Data is given as $\log_{10}$ 16S rRNA gene copies of the target microorganism per million of bacterial 16S rRNA genes detected in the same sample. The *F. prausnitzii-E. coli* index (F-E index) was calculated as (F/Hc) –

255 (E/Hc)/ (TB/Hc), being F the $\log_{10}$16S rRNA gene copies of *F. prausnitzii*, E the $\log_{10}$
256 16S rRNA gene copies of *E. coli*, Hc a million of human cells, and TB a millon of
257 16S rRNA gene copies of total bacteria. This index allows the normalization of the
258 biopsy size by quantifying human cells and includes total bacteria as an additional
259 parameter, as it has been reported that it can vary between groups of patients (Kleessen
260 et al., 2002; Schultsz et al., 1999; Swidsinski et al., 2002).
261 The variation coefficient was calculated as a measure of dispersion between samples
262 from the same patient. As within a patient there were high differences between samples
263 from different zones along the intestinal tract, analyses pooling all the biopsies together
264 and separated by location were performed. The non-parametric Kruskal-Wallis test was
265 used to test differences in variables with more than two categories (*i.e.* diagnostics, CD
266 and UC phenotypes, and current medication). Pairwise comparisons of subcategories of
267 these variables were further analyzed using a Mann-Whitney U test. This test was also
268 used to compare, within a subgroup of patients variables with two categories as activity
269 (active CD and UC patients when CDAI>150 (Best et al., 1976) and a Mayo score >3,
270 respectively), and intestinal resection.
271 Spearman correlation coefficient and significance between the two species quantities
272 was calculated. The same statistics were used to analyze the correlation between each
273 one of the species and the F-E index with respect to simple endoscopic score for CD
274 (SES-CD), Mayo endoscopic score for UC (Pineton de Chambrun et al., 2010), C-
275 reactive protein, and months to flare up in inactive IBD patients.
276 The receiver operating characteristic (ROC) curve analysis, a plot of the true positive
277 rate (sensitivity) versus false positive rate (1-specificity), was applied to establish the
278 usefulness of *F. prausnitzii*, *E. coli* and the F-E index to distinguish amongst different
279 intestinal disorders. The accuracy of discrimination was measured by the area under the

ROC curve (AUC). An AUC approaching 1 indicates that the test is highly sensitive as well as highly specific whereas an AUC approaching 0.5 indicates that the test is neither sensitive nor specific.

All the statistical analyses were conducted via the SPSS 15.0 statistical package for Windows (LEAD Technologies, Inc.). Significance levels were established for P values ≤ 0.05.

RESULTS

Features of the novel multiplex qPCR assay for *F. prausnitzii* (both phylogroups).

In this study, a novel primer set and probe to quantify *F. prausnitzii* has been developed (Table 2, supplemental material), taking into account that it should equally detect and quantify the two recently described phylogroups of this species (Lopez-Siles et al., 2012). Additionally, an IAC has been included in order to report quantitative errors or false negative reactions due to inhibition, thus ensuring accurate quantification when using the assay for the analysis of clinical samples. The assay is totally specific, as assessed both *in silico* and *in vitro* with an average efficiency of 86%. The theoretical detection limit is of 106.6 16S rRNA genes of *F. prausnitzii* per reaction and allows quantification over a linear range span of at least 7 logarithms, starting at 10^3 target genes per reaction. The tool hereby developed is suitable to be applied for determinations of *F. prausnitzii* in human biopsy samples, considering that healthy persons harbor around 1.7×10^5 *F. prausnitzii*·mg tissue^{-1} (Ahmed et al., 2007).

Abundance of mucosa-associated *F. prausnitzii* and *E. coli* in healthy subjects, IBS and IBD patients by disease phenotype.

The abundance of *F. prausnitzii* and *E. coli* from all the biopsies pooled together (Table 3) and by sample location (Table 4) was compared amongst patients with different intestinal disorders and healthy controls in order to determine whether or not their relative abundance could be employed as a useful indicator to distinguish between IBS and IBD patients, and within IBD phenotypes.

F. prausnitzii abundance.

F. prausnitzii abundance decreased in IBD patients, especially CD patients ($P<0.001$), whereas IBS patients more closely resembled the H group (Table 3). Within UC patients, those with proctitis and extensive UC presented intermediate *F. prausnitzii*

levels between CD patients and H subjects. In CD patients, those with the lowest levels of this bacterium were CD patients with ileal involvement (either I-CD or IC-CD), and CD patients with stricturing disease behavior, whereas C-CD patients resembled UC. ROC curve analysis, applied to test the accuracy of the indicators to differentiate between two groups of patients, confirmed that the reduction of *F. prausnitzii* abundance is a good discriminator for IBD patients, when compared to the H subjects and, more interestingly, with IBS patients (Table 5). The specificity was also improved when proctitis patients were removed from the analysis. Moreover, this indicator accurately distinguished I-CD patients from UC patients and, more interestingly also from C-CD patients. Precisely, when comparing I-CD patients with C-CD, the AUC values were greater than 0.772, corresponding to 82.5% sensitivity and above 57.14% specificity at a set threshold (Table 5).

When analyzing data by sample location, the trend to distinguish these disease phenotypes was observed at rectum and colon level, although only statistical significance was reached for the latter (Table 4). In contrast, *F. prausnitzii* abundance in ileal samples was not a suitable indicator to distinguish between IBD phenotypes.

E. coli abundance.

E. coli abundance varied differently in IBD subjects (Table 3). UC patients presented a reduced abundance of this species (P=0.002), with the exception of those with extensive UC, which harbored similar abundances to the CD group. By comparison, CD patients showed increased levels of *E. coli* when compared to H and IBS patients. Within CD phenotypes, all reached statistical significance except for the IC-CD group, probably due to the high variability of the data. As regards IBS patients, this parameter only allowed their discrimination from CD patients as confirmed by ROC curve analysis (Table 5). Interestingly, ROC curve analysis also showed that *E. coli* might differentiate

extensive UC and C-CD (Table 5), which are two different pathological entities that feature overlapping clinical manifestations and are therefore difficult to diagnose

Analysis by location of the sample indicated that colonic *E. coli* quantification can be a good marker to differentiate these two IBD phenotypes (Table 4). It is of note that *E. coli* was approximately ten times more abundant in ulcerated biopsies of CD patients than in those taken from non-ulcerated zones [median values of $\log_{10}$ (16S rRNA gene copies/ million bacterial 16S rRNA gene copies) from ulcerated (N=17) 5.02±0.88 and non-ulcerated zones (N=71) 4.13±1.07; P=0.009].

F. prausnitzii-E. coli index.

Although both bacterial species were confirmed to be good indicators of IBD dysbiosis, we further investigated if the discriminatory power was enhanced when analyzing both species together. Thus, an index was calculated subtracting *E. coli* numbers from *F. prausnitzii* abundance and this data was further normalized to total bacterial 16S rRNA gene copies and to human cell numbers to correct for variations due to sample size (as detailed in Materials and Methods section).

When all the biopsies from different locations were pooled for analysis, a positive F-E index, indicating a predominance of *F. prausnitzii* over *E. coli*, was observed in H, IBS and UC patients, suggesting that these three groups of patients were undistinguishable from each other (Table 3). Nevertheless, the differentiation of IBS patients from all the CD subjects, irrespective of their disease location and behavior, improved when the F-E index was used in spite of the bacterial indicators alone. Noteworthy, using the F–E index we gained sensitivity (80%) and specificity (60.71%) to differentiate extensive UC from C-CD patients, which was not possible when considering *F. prausnitzii* alone and was achieved with low specificity (35.71%) when only taking *E. coli* into account (Table 5). Interestingly, negative values of the index were mainly reached for those CD

patients with ileal involvement, indicating that in this subgroup of patients, *E. coli* populations numerically dominate that of *F. prausnitzii*.

When data was analyzed by sample location, all these features were observed in both rectum and colon samples, however the latter was shown to be the most discriminatory sample (Table 4). Conversely, from the ileum samples, the F-E index of IBS patients also reached negative values that hampered the differentiation with CD patients. Moreover, C-CD patients showed higher values of the F-E index, resembling UC patients. Thus, our data suggests that ileal samples alone are not suitable for a correct diagnosis. However, the higher F-E index for C-CD patients for ileal samples in comparison to that in IC-CD patients provides an additional discrimination point as the two disease phenotypes had similar F-E index from colon samples.

The usefulness of a ratio F/E (16S rRNA *F. prausnitzii* genes /16S rRNA *E. coli* genes) and $\log_{10}$ ratio F/E was also evaluated. All the indexes achieved similar scores concerning discrimination between disorders and disease phenotypes, but the accuracy of discrimination measured by the area under the ROC curve was better with the F-E index (data not shown).

Correlation between *F. prausnitzii* and *E. coli* abundances in healthy subjects, IBS and IBD patients by disease phenotype.

F. prausnitzii and *E. coli* numbers were analyzed in order to determine if they were positively or negatively correlated, and whether this could provide supporting evidence about a putative common factor affecting negatively/positively both bacterial populations in a given patient or about a direct/indirect effect of one population over the other (Fig. 1).

In H subjects, *E. coli* abundance fluctuated over a 5-$\log_{10}$ span irrespective of *F. prausnitzii* quantity, which in turn was reasonably stable (2-$\log_{10}$ span) within this

group of subjects. No correlation between these two species was found. Similar results were observed for IBS patients irrespective of disease phenotype. Interestingly, in UC patients there was a positive correlation between *F. prausnitzii* and *E. coli*. This could not be associated with an increase in total bacteria, as the abundance of both species was normalized to total bacterial 16S rRNA gene copies. Although this trend was observed for all UC phenotypes (data not shown), statistical significance was achieved only when patients with extensive UC were considered (Fig. 1). In CD patients *F. prausnitzii* quantity was extremely variable and was spread over a 6-$\log_{10}$ span, whereas *E. coli* abundances were as disperse as in H subjects, but reaching higher values. Whereas no correlation was found when all the CD phenotypes were grouped, C-CD patients considered alone showed positive correlation resembling that observed in UC patients, although these did not reach statistical significance (Fig. 1). Moreover, a tendency that suggests a possible negative correlation between *F. prausnitzii* and *E. coli* was observed when analyzing those patients with ileal involvement (Fig. 1) with 21% of the patients with I-CD and 15% of those with IC-CD exhibiting an increase in *E. coli* abundance with a concomitant decrease in *F. prausnitzii* numbers in comparison to H subjects. This suggests that the microbial imbalance is not homogeneously distributed among all the patients and that some feature a more severe dysbiosis. It is of note that the trend of a negative correlation between *F. prausnitzii* and *E. coli* was stronger when patients under anti-tumor necrosis factor (TNF) α therapy were removed from the analysis (r=-0.237, P=0.105).

***F. prausnitzii* and *E. coli* abundance in relation to patients clinical data.**

F. prausnitzii and *E. coli* abundances were compared between active and inactive patients (active CD and UC were defined by a CDAI of >150 (Best et al., 1976) and a Mayo score >3, respectively) in order to determine if these indicators vary according to

411 the activity status of the patient. *F. prausnitzii* abundance did not differ between active
412 and inactive IBD patients (Fig. 2A). Conversely, *E. coli* load was increased in active
413 IBD patients, although only statistically significant differences were found for CD
414 patients (Fig. 2B), and particularly in those with ileal involvement (Table S3). However,
415 no correlation was found between *E. coli* abundance with the SES-CD, nor with the
416 levels of blood C-reactive protein (data not shown).
417 We also investigated whether or not the abundance of *F. prausnitzii* and *E. coli* at the
418 time of sampling could be correlated with time to recurrence of disease in five inactive
419 CD patients (three with I-CD, one with IC-CD and one with C-CD) of whom we had
420 available information on disease relapse (Fig. 3). Interestingly, *F. prausnitzii* abundance
421 correlated positively with months to next flare-up (ρ=0.660, p<0.001) indicating that the
422 higher the *F. prausnitzii* abundance, the longer the remission. In contrast, *E. coli* was
423 negatively correlated with months to next flare-up (ρ=-0.129, p=0.030), suggesting that
424 when *E. coli* numbers are higher, the period of remission is shortened. These results
425 suggest that the abundance of both species might be applicable as predictors of disease
426 recurrence.
427 *F. prausnitzii* and *E. coli* quantities were also analyzed taking into account whether or
428 not the patients required intestinal resection during the course of the disease.
429 *F. prausnitzii* abundance was reduced in those CD patients that underwent intestinal
430 resection [median values of $\log_{10}$ (16S rRNA gene copies/ million bacterial 16S rRNA
431 gene copies) from non-resected (N=30) 4.57±1.40, and resected (N=11) 3.95±0.78;
432 P=0.009], whereas *E. coli* numbers were similar between resected and non-resected
433 patients.
434 **_F. prausnitzii_ and _E. coli_ abundances by treatment**

435 In order to establish which therapy might have an effect in correcting dysbiosis, the
436 abundance of both species was analyzed by current medication of the patients at the
437 time of sampling.

438 All IBD patients regardless of their medication showed decreased *F. prausnitzii* loads
439 when compared with the H group, indicating that this species abundance was not
440 restored by any of the therapies considered in this study (Table 6). No differences in
441 *F. prausnitzii* abundance were observed between medications within any disease
442 phenotype. Conversely, *E. coli* numbers were lower in I-CD patients under anti- TNFα
443 treatment, suggesting that this treatment has a direct effect on modulating the abundance
444 of this pro-inflammatory bacterium in the gut of patients with this disease phenotype
445 (Table 6).

446 As we observed that *E. coli* numbers were lower in CD patients under anti-TNFα
447 treatment, we enrolled a subgroup of 10 CD patients (4 C-CD, 2 IC-CD and 4 I-CD)
448 who started TNFα inhibition therapy with adalimumab in a follow-up study, who were
449 monitored before starting the treatment and at months one and three after initiation.
450 Although *F. prausnitzii* abundance did not increase substantially after adalimumab
451 treatment, in agreement with the previous results *E. coli* numbers markedly decreased
452 when adalimumab was given at a dose of 80 mg every two weeks during the first month
453 (induction dose) and were maintained slightly lower than before treatment when the
454 dose was decreased to 40 mg fortnightly (maintenance dose) (Fig. 4). This result was
455 not statistically supported probably due to the low number of patients enrolled in this
456 trial and the high variability between subjects.

457

DISCUSSION

In the present study we have analyzed the abundance of mucosa associated *F. prausnitzii* and *E. coli* in H, IBS and IBD subjects, paying careful attention to the diversity of disease phenotypes and clinical features of the patients. We show that these two bacterial species can be good indicators to assist in IBD diagnostics and, for some disease phenotypes, in disease prognosis. Moreover, new information about which current therapies in IBD might correct dysbiosis towards "normobiosis" (Roberfroid et al., 2010) is also revealed.

Our data showed that *F. prausnitzii* and *E. coli* abundances behave differently among intestinal disorders and IBD phenotypes and confirmed quantitatively that *F. prausnitzii* is a specific IBD dysbiosis indicator that has allowed us to distinguish UC and CD patients from those with IBS. This is in agreement with previous work based on fecal samples (Swidsinski et al., 2008) although this study did not determine to what extent the bacterial imbalance found was a common feature of all the disease phenotypes. Our study confirmed that the depletion in *F. prausnitzii* abundance is a feature of all the IBD patients with the exception of those UC patients with proctitis and extensive UC, and therefore additional information is required in order to distinguish these disease phenotypes. Using *E. coli* as a second indicator in combination with *F. prausnitzii* we gained discrimination power and UC proctitis patients were distinguishable from H subjects.

When using *F. prausnitzii* or *E. coli* as single indicators it was not possible to fully distinguish within all the UC and CD phenotypes. In contrast, the F-E index allowed a neat differentiation of I-CD patients with respect to other IBD subgroups, that could be useful to assist differential diagnosis between I-CD and IC-CD. More interestingly, the F–E index allowed for good differentiation of C-CD patients from those patients with

extensive UC, as well as for the other UC disease phenotypes, which is of relevance for diagnostic purposes as these two disorders may present similar clinical manifestations (Bernstein et al., 2010; Nikolaus and Schreiber, 2007). The quantification of these two species could therefore be implemented as a reliable marker to aid diagnosis of these intestinal disorders. Unfortunately, it was not possible to distinguish distal UC from extensive UC, or C-CD patients from those with IC-CD by using these indicators or the F-E index, therefore, additional bacterial indicators are needed to properly distinguish all the IBD phenotypes. Further investigations to test the usefulness of the indicators to assign disease phenotype at early disease stages would be also of interest.

Our results suggest that the colon is the location that allows us to distinguish most of the phenotypes and therefore should be the location of choice to sample. Nevertheless, ileal samples could provide an additional discrimination point to support differentiation between certain disease phenotypes such as IC-CD and C-CD. Although dysbiosis observed in the rectum resembled that found in the colon, additional studies with larger number of rectal samples should be performed in order to corroborate this observation. Besides, testing the usefulness of the microbiological biomarkers presented here in non-invasive fecal samples would be of interest in order to assist in early diagnosis.

F. prausnitzii abundance was similar between active and inactive patients with the same IBD phenotype, which indicates that this species can be a reliable marker to screen IBD patients even in remission. Although our results do not concur with previous studies based on fecal samples (Duboc et al., 2013; Sokol et al., 2009), a reduction in *F. prausnitzii* numbers in CD patients in remission has already been reported in studies based on biopsies (Willing et al., 2009). We hypothesize that the depletion in *F. prausnitzii* at the mucosal level (which is the site of microbial recognition by the host

and where the inflammatory process is developing) may be more evident than in feces of patients in remission.

In contrast, *E. coli* abundance was higher in active CD patients by comparison with those in remission at sampling, which supports the hypothesis that *E. coli* is involved in CD pathogenesis (Darfeuille-Michaud et al., 2004; Martin et al., 2004; Martinez-Medina et al., 2009; Sasaki et al., 2007). It is of note that indices of endoscopic activity (SES-CD) and general inflammation (C-reactive protein) did not correlate with imbalances in these indicators and, reinforces the necessity of using several parameters to define a real deep remission. It may therefore be worth considering to assess "microbiological remission" as a new parameter in the future.

In agreement with a previous study (Sokol et al., 2008) lower numbers of *F. prausnitzii* were observed in resected CD patients although our study did not allow us to decipher whether this depletion could be associated with the need for surgical intervention. Thus there should be further investigation to assess the usefulness of this biomarker to precisely predict when such intervention might be needed.

Concerning the applicability of these two indicators for prognostic purposes, we observed that increased levels of *E. coli* were associated with a relapse in a short period of time in CD patients, whereas high levels of *F. prausnitzii* and low levels of *E. coli* were associated with longer remission periods. Our data is in agreement with the previous work of Sokol et al. (2008) reporting that a reduction in *F. prausnitzii* abundance was associated with endoscopic recurrence of the disease (Sokol et al., 2008; Sokol et al., 2009). Nevertheless, we observed that high *F. prausnitzii* abundance without a decrease in *E. coli* numbers did not ensure a long remission period, therefore the subgroup of patients analyzed, predominated by I-CD patients, showed that an imbalance in *E. coli* abundance plays a greater role in inducing inflammation than the

depletion of the *F. prausnitzii* load. This suggests that *F. prausnitzii* and *E. coli* are potentially useful for prognostics in I-CD. However further prospective studies in a larger cohort of patients are needed to confirm this hypothesis.

Interestingly, in this study we observed that a correlation exists between the abundance of these two species in IBD patients, a feature that, to our knowledge, has not been described to date. In UC patients, the relative abundance of *F. prausnitzii* and *E. coli* were positively correlated, suggesting that under this intestinal disorder populations of both species might be affected similarly by gut environment or host factors. Conversely, a negative correlation trend was observed in CD patients with ileal disease location, with *E. coli* being more abundant than *F. prausnitzii*. This negative correlation between species specially associated to I-CD patients leads us to hypothesize that both species are directly linked to the disease pathogenesis by playing different roles. This hypothesis is sustained by several reports that implicate the adherent-invasive *E. coli* (AIEC) pathovar in CD pathogenesis (Darfeuille-Michaud et al., 2004; Martin et al., 2004; Martinez-Medina et al., 2009; Sasaki et al., 2007) and those that postulate that a reduction of *F. prausnitzii* might be a crucial factor to enhance disease recurrence (Sokol et al., 2008; Sokol et al., 2009). However, we could not confirm whether or not the observed increase in *E. coli* was due to the AIEC pathovar since to date, no molecular tool for its specific quantification is available. Another possibility to explain the negative correlation between the two species is that changes in gut or host environmental factors may be implicated. For instance, bile salts, whose composition has been recently demonstrated to be altered in IBD patients (Duboc et al., 2013), can negatively affect *F. prausnitzii* growth (Lopez-Siles et al., 2012) and also induce the expression of virulence factors in *E. coli* (Chassaing et al., 2013). Moreover, a direct or indirect effect of one population on the other also cannot be ruled out, and further co-

556 culture experiments would be helpful to fully elucidate the interactions between these
557 two species.
558 Our results give valuable insight as to how current therapies applied in IBD treatment
559 might be leading to a correction of dysbiosis by modulating the populations of these two
560 species. We observed that *E. coli* numbers were lower in I-CD patients under anti-TNFα
561 treatment when compared with other therapies, and it was further corroborated in a
562 prospective study in which CD patients were treated with adalimumab. It has been
563 previously reported that TNFα promotes the expression of carcinoembryonic antigen–
564 related cell adhesion molecule 6, which is a molecule used by *E. coli* to adhere to
565 enterocytes via the interaction with type 1 pili (Barnich et al., 2007). Besides, AIEC
566 strains have been reported to be more efficient than non-AIEC strains isolated from the
567 intestinal mucosa of IBD patients and controls, at colonizing the gut due to special
568 mutations in the FimH adhesion of type 1 pili (Dreux et al., 2013). We hypothesize that
569 the blockage of TNFα can lead to lower expression of carcinoembryonic antigen–related
570 cell adhesion molecule 6 which in turn might imply lower AIEC colonization. However,
571 to prove this hypothesis specific quantification of this pathovar is needed. In contrast,
572 none of the current medication regimes analyzed in the present study was shown to be
573 effective in restoring the *F. prausnitzii* populations. Therefore, it is probable that to
574 restore this species it might be necessary to re-establish the overall ecological
575 conditions in the gut environment.
576
577 **CONCLUSIONS**
578 Our study confirms that *F. prausnitzii* and *E. coli* are good indicators of IBD dysbiosis
579 and provides evidence for the applicability for disease diagnostics allowing the
580 differentiation of IBD from IBS and also between some IBD subtypes as C-CD from

extensive UC. We further investigated the potential applicability for prognostics and, our data, although preliminary, allows us to conclude that this tool could be used as a supporting prognostic tool in CD patients since the remission in I-CD patients was associated with the abundance of these two species. The present study shows that current therapies are not sufficient to counterbalance dysbiosis and further investigations are required to show which other factors, other than medication, might help to revert bacterial populations back to a typical structure.

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767

FIGURE LEGENDS

Fig. 1. Spearman correlation between mucosa-associated *F. prausnitzii* and *E. coli* in control (H), Irritable Bowel Syndrome (IBS), Ulcerative Colitis (UC), and Crohn's disease (CD) patients (16S rRNA gene copies/ million bacterial 16S rRNA gene copies). Correlations in extensive ulcerative colitis (E3) and CD patients with ileal involvement (I-CD and IC-CD) are specified.

Fig. 2. *F. prausnitzii* (A) and *E. coli* (B) abundances categorized by activity status of Ulcerative Colitis (UC) and Crohn's disease (CD) patients (grey, active; white, inactive). The number of patients and biopsies (*values in italics*) in each group has been indicated. Homogeneous subgroups (P>0.05) within each panel are indicated with the same superscript.

Fig. 3. Retrospective study to determine the usefulness of *F. prausnitzii* (A) and *E. coli* (B) abundances to predict time to flare-ups (black triangles) in CD patients. Disease phenotypes of the patients has been indicated (I-CD, ileal CD; IC-CD, ileocolonic CD; C-CD, colonic CD).

Fig. 4. *F. prausnitzii* (white squares) and *E. coli* (grey diamonds) abundances over a three months period in rectal biopsies of a group of patients who started adalimumab therapy.

TABLES

Table 1. Sample size and clinical characteristics of subjects.

	Healthy controls*	IBD		Irritable bowel syndrome (IBS)
		Crohn's disease	Ulcerative colitis	
N (patients)	28	45	28	10
Age (mean years ± SD)	47.1±16.0	34.4±11.2	40.5±15.2	43.8±10.8
Male (N, %)	16 (57.1%)	23 (50.0%)	17 (60.7%)	2 (20.0%)
Active (N, %)	na	28 (60.9%)	21 (75.0%)	nd
Previous surgery(N, %)	0	10 (21.7%)	1 (3.6%)	nd
Smokers (N, %)	nd	12 (26.1%)	2 (7.1%)	0
Treatment (N, %) **				
No treatment or mesalazine	na	17 (37.0%)	17 (60.7%)	nd
Moderate immunosuppressant	na	17 (37.0%)	4 (14.3%)	nd
Anti-TNFα (infliximab, adalimumab)	na	11 (23.9%)	5 (17.9%)	nd
CD Montreal classification				
Age of diagnosis (N, %) **				
diag < 16y (A1)	na	5 (10.9%)	1 (3.6%)	nd
diag 17-40y (A2)	na	33 (71.7%)	12 (42.9%)	nd
diag >41y (A3)	na	6 (13.0%)	11 (39.3%)	nd
Location (N, %)				
Ileal-CD (L1)	na	19 (41.3%)	na	na
Colonic-CD (L2)	na	13 (28.3%)	na	na
Ileocolonic-CD (L3)	na	13 (28.3%)	na	na
Behavior (N, %) **				
Non-stricturing, non-penetrating (B1)	na	31 (67.4%)	na	na
Stricturing (B2)	na	7 (15.2%)	na	na
UC classification (N, %) **				
Ulcerative proctitis (E1)	na	na	6 (21.4%)	na
Distal UC (E2)	na	na	13 (46.4%)	na
Extensive UC or pancolitis (E3)	na	na	7 (25.0%)	na
IBS subtype (N, %) **				
Diarrhea predominant type	na	na	na	2 (20.0%)
Constipation predominant type	na	na	na	2 (20.0%)

IBD, Inflammatory bowel disease; IBS, Irritable bowel syndrome; TNF, tumour necrosis factor; nd, not determined; na, not applicable

*Controls consisted of subjects who underwent colonoscopy for different reasons: 9/28 rectorrhagia, 10/28 colorectal cancer familial history and 9/28 abdominal pain.

** Medical treatment at the time of sampling was available in 26/28 UC patients; Age of disease onset was available for 44/45 CD patients, and 24/28 UC patients; Disease behavior at last follow-up before the time of sampling was available in 38/45 CD patients, and none had penetrating CD (B3); Maximal disease extent at the time of sampling was available in 26/28 UC patients; disease subtype was available in 4/10 Irritable bowel syndrome patients, and none had alternating predominant type.

Table 2. 16S rRNA-targeted primers and probes used in this study.

Target	Primer and Probe*	Sequence 5'-3'	Reference
Bacteria	F_Bact 1369	CGGTGAATACGTTCCCGG	(26)
	R_Prok_1492	TACGGCTACCTTGTTACGACTT	
	P_TM_1389F	**6FAM-CTTGTACACACCGCCCGTC-TAMRA**	
E. coli	E.coli 395 F	CATGCCGCGTGTATGAAGAA	(25)
	E.coli 490 R	CGGGTAACGTCAATGAGCAAA	
	E.coli 437 PR	**6FAM-TATTAACTTTACTCCCTTCCTCCCCGCTGAA-TAMRA**	
F. prausnitzii	Fpra 428 F	TGTAAACTCCTGTTGTTGAGGAAGATAA	this study
	Fpra 583 R	GCGCTCCCTTTACACCCA	
	Fpra 493 PR	**6FAM-CAAGGAAGTGACGGCTAACTACGTGCCAG-TAMRA**	
DNA IAC‡	IAC F	TACGGATGAGGAGGACAAAGGA	this study
	IAC R	CACTTCGCTCTGATCCATTGG	
	IAC PR	**VIC®-CGCCGCTATGGGCATCGCA-TAMRA**	

* Probe sequences are in bold. P_TM1389F, E.coli 437 PR and Fpra493PR probes were 5'-labelled with FAM™ (6-carboxyfluorescin) as the reporter dye, whereas the IAC probe was 5' labeled with VIC® (6-carboxyrhodamine) as reporter dye to allow multiplex detection. TAMRA™ was used as quencher dye at the 3'end for all the probes.

‡ IAC, Internal Amplification Control; DNA IAC sequence (5'-3'):
TACGGATGAGGAGGACAAAGGACGCCGCTATGGGCATCGCACCAATGGATCAGAGCGAAGTG

Table 3. Abundances of mucosa-associated *F. prausnitzii*, *E. coli* and F-E index in controls (H), Irritable Bowel Syndrome (IBS), Ulcerative Colitis (UC), and Crohn's disease (CD) patients. Disease phenotypes of UC and CD patients are analyzed as independent groups.

	n patients ***(n biopsies)***	***F. prausnitzii**§*	***E. coli**§*	***F-E index**†*
H	**28** ***(59)***	**5.41±0.55** ^{a}	**4.05±1.18** ^{a}	**0.22±0.21** ^{a}
IBS	**10** ***(26)***	**5.34±0.57** ^{a}	**3.30±1.13** ^{a,b}	**0.29±0.17** ^{a,b}
UC	**28** ***(66)***	**4.95±0.63** ^{b}	**3.04±1.22** ^{b}	**0.30±0.19** ^{a}
Location				
Ulcerative proctitis (E1)	6 *(18)*	5.12±0.31 ^{a,b}	3.04±0.75 ^{b}	0.33±0.09 ^{b}
Distal UC (E2)	13 *(35)*	4.44±0.62 ^{c}	2.92±1.31 ^{b}	0.33±0.22 ^{a,b}
Extensive UC or pancolitis (E3)	7 *(13)*	5.24±0.68 ^{a,b}	4.57±1.43 ^{a,b,c}	0.18±0.15 ^{a}
CD	**46** ***(91)***	**4.30±1.28** ^{c}	**4.51±1.08** ^{c}	**-0.02±0.28** ^{c}
Location				
Ileal-CD (L1)	19 *(39)*	3.84±1.38 ^{d}	4.58±1.11 ^{c,d}	-0.19±0.29 ^{d}
Colonic-CD (L2)	13 *(28)*	5.08±0.93 ^{b,c}	4.58±0.91 ^{c}	0.01±0.20 ^{c}
Ileocolonic-CD (L3)	13 *(24)*	4.44±1.01 ^{c,d}	3.85±1.23 ^{a,b,c,d}	-0.02±0.29 ^{c}
*Behavior**				
Non-stricturing, non-penetrating (B1)	31 *(64)*	4.26±1.30 ^{c}	4.35±1.00 ^{a,c}	0.00±0.26 ^{c}
Stricturing (B2)	7 *(17)*	3.52±0.97 ^{d}	5.25±1.25 ^{d}	-0.24±0.25 ^{d}

* Homogeneous subgroups (P>0.05) within each variable (column) are indicated with the same superscript.

§ Median $\log_{10}$ 16S rRNA gene copies/ million bacterial 16S rRNA gene copies ± standard deviations

†Median F-E index ± standard deviations. F-E index has been calculated as [(*F. prausnitzii* $\log_{10}$ 16S rRNA gene copies/ million human cells)-(*E. coli* $\log_{10}$ 16S rRNA gene copies/ million human cells)]/ (total bacteria $\log_{10}$ 16S rRNA gene copies/ million human cells).

Table 4. Abundances of mucosa-associated *F. prausnitzii*, *E. coli* and F-E index by zone of the gastrointestinal tract (ileum, colon and rectum) in controls (H), Irritable Bowel Syndrome (IBS), Ulcerative Colitis (UC), and Crohn's disease (CD) patients. Disease phenotypes of UC and CD patients are analyzed as independent groups.

Ileum	**N biopsies**	***F. prausnitzii**§**	***E. coli**§**	***F-E index**†**
H	**15**	**5.51±0.53** [a]	**4.07±1.23** [a,b]	**0.22±0.15** [a]
IBS	**6**	**4.86±1.43** [ab]	**4.92±1.39** [a,b,c]	**-0.01±0.16** [b,c]
UC				
Ulcerative proctitis (E1)	6	5.18±0.23 [a,b]	3.18±0.87 [a]	0.31±0.09 [a,b]
Distal UC (E2)	9	5.36±0.54 [a,b]	2.97±1.15 [a]	0.37±0.09 [a]
Extensive UC or pancolitis (E3)	5	5.24±0.69 [a,b]	4.89±1.47 [a,b,c]	0.14±0.14 [b,c]
CD				
Ileal-CD (L1)	11	3.96±1.35 [b]	4.96±0.87 [b,c]	-0.16±0.25 [c]
Colonic-CD (L2)	7	5.03±1.00 [a,b]	4.32±0.85 [a]	0.11±0.23 [a,b,c]
Ileocolonic-CD (L3)	5	3.33±1.36 [a, b]	5.26±0.89 [b,c]	-0.23±0.26 [c]

Colon	**N biopsies**	***F. prausnitzii**§**	***E. coli**§**	***F-E index**†**
H	**33**	**5.46±0.63** [a]	**4.08±1.24** [a,d]	**0.21±0.24** [a,b]
IBS	**10**	**5.46±0.19** [a]	**3.19±1.31** [a,b]	**0.33±0.16** [a,b]
UC				
Ulcerative proctitis (E1)	6	5.11±0.17 [b]	3.04±0.77 [b]	0.32±0.10 [a]
Distal UC (E2)	13	4.42±0.61 [b, c]	2.97±1.51 [b]	0.28±0.25 [a,b]
Extensive UC or pancolitis (E3)	7	5.12±0.71 [a,b,c]	3.45±1.48 [a,b,d]	0.20±0.16 [a,b]
CD				
Ileal-CD (L1)	19	2.74±1.30 [d]	4.55±1.03 [c,d]	-0.26±0.29 [d]
Colonic-CD (L2)	13	4.84±0.85 [c]	4.93±0.68 [c]	-0.01±0.19 [c]
Ileocolonic-CD (L3)	13	4.49±1.07 [a,b,c]	3.85±1.30 [d]	0.13±0.32 [c]

Rectum	**N biopsies**	***F. prausnitzii**§**	***E. coli**§**	***F-E index**†**
H	**11**	**5.28±0.33** [a]	**3.86±1.00** [a]	**0.22±0.17** [a,b]
IBS	**10**	**5.31±0.31** [a]	**3.32±1.70** [a,b]	**0.28±0.25** [a,b]
UC				
Ulcerative proctitis (E1)	6	5.13±0.49 [a]	3.19±0.66 [a,b]	0.33±0.05 [a]
Distal UC (E2)	13	4.49±0.68 [a,c]	2.55±0.86 [b]	0.33±0.14 [a]
Extensive UC or pancolitis (E3)	1	5.76 [a,b,c]	4.76 [a,b]	0.18 [a,b,c]
CD				
Ileal-CD (L1)	9	4.25±1.51 [c]	4.01±1.38 [a,b]	0.01±0.32 [c]
Colonic-CD (L2)	8	5.09±1.12 [a,b,c]	4.53±1.14 [a]	0.04±0.16 [b,c]
Ileocolonic-CD (L3)	6	4.05±0.32 [b,c]	3.36±0.50 [a,b]	0.13±0.10 [c]

* Homogeneous subgroups (P>0.05) within each variable (column) are indicated with the same superscript.
§ Median $\log_{10}$ 16S rRNA gene copies/ million bacterial 16S rRNA gene copies ± standard deviations
†Median F-E index ± standard deviations. F-E index has been calculated as [(*F. prausnitzii* $\log_{10}$ 16S rRNA gene copies/ million human cells)-(*E. coli* $\log_{10}$ 16S rRNA gene copies/ million human cells)]/ (total bacteria $\log_{10}$ 16S rRNA gene copies/ million human cells).

Table 5. Area under the curve (AUC) obtained by receiver operating characteristic analysis (ROC curve) to establish the usefulness of *F. prausnitzii*, *E. coli* and the F-E index to distinguish amongst different intestinal disorders (H, controls; IBD, inflammatory Bowel Disease; IBS, Irritable Bowel Syndrome; UC, ulcerative colitis; CD, Crohn's disease; I-CD, ileal CD; IC-CD, ileocolonic CD, C-CD, colonic CD). Sensitivity and specificity values at a set threshold have been included for comparative purposes. Only analysis with AUC values above 0.6 are shown as a test is considered to be suitable if the AUC range from 0.6 to 0.75, and to have good sensitivity and specificity if the AUC range from 0.75 to 0.9.

	AUC	**Sensitivity (%)**	**Specificity (%)**
F. prausnitzii			
H vs IBD	0.765	81.35	55.17
H vs IBD (without proctitis patients)	0.778	81.35	61.44
IBS vs IBD	0.696	80.77	54.60
IBS vs IBD (without proctitis patients)	0.710	80.76	61.44
I-CD vs C-CD	0.772	82.50	57.14
I-CD vs UC	0.793	82.50	53.84
E. coli			
IBS vs CD	0.693	82.29	57.69
C-CD vs extensive UC	0.636	86.67	35.71
F-E index			
IBS vs CD	0.797	80.21	61.54
IBS vs I-CD	0.868	80.77	72.50
IBS vs IC-CD	0.746	80.76	52.00
IBS vs C-CD	0.784	80.76	57.14
C-CD vs extensive UC	0.767	80.00	60.71

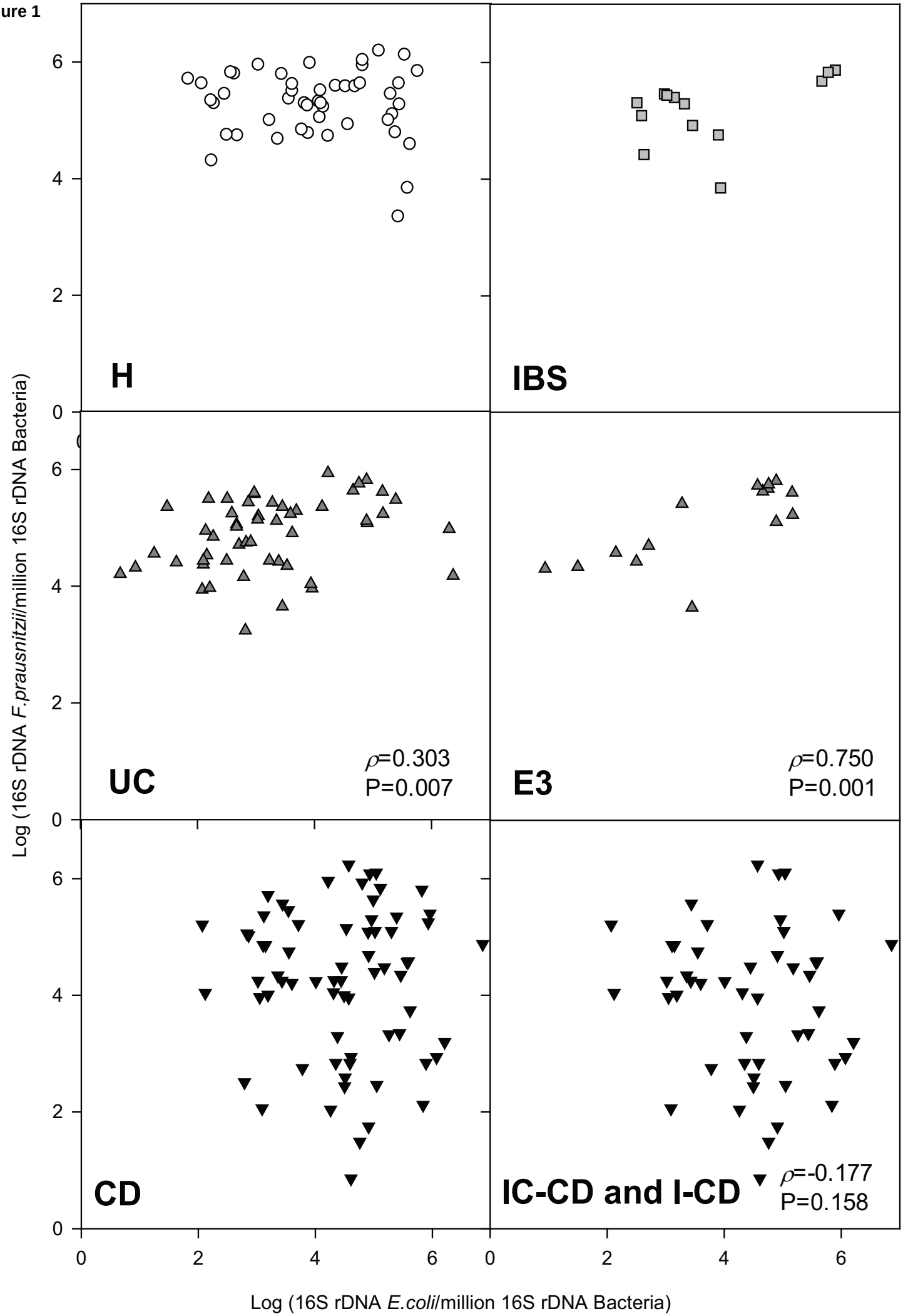
Table 6. *F. prausnitzii* and *E. coli* abundances in different Inflammatory Bowel Disease phenotypes by medication at sampling.

	***F. prausnitzii**§*							***E. coli**§*						
	N patients *(n biopsies)*	No treatment or mesalazine	N patients *(n biopsies)*	moderate immunosuppresants	N patients *(n biopsies)*	Anti-TNF	P value	N patients *(n biopsies)*	No treatment or mesalazine	N patients *(n biopsies)*	moderate immunosuppresants	N patients *(n biopsies)*	Anti-TNF	P value
UC	**17*(38)***	**4.98±0.59**	**4*(14)***	**4.42±0.56**	**5*(14)***	**4.94±0.80**	**ns**	**17*(47)***	**3.20±1.28**	**4*(14)***	**2.43±0.92**	**5*(14)***	**3.21±0.91**	**ns**
E1	6(*18*)	5.12±0.31						6 (*18*)	3.04±0.75					
E2	5*(9)*	4.42±0.41	4*(14)*	4.42±0.56	3*(12)*	5.44±0.77	ns	5 *(9)*	3.30±2.06	4*(14)*	2.43±0.92	3*(12)*	3.39±0.71	ns
E3	4*(11)*	5.62±0.59			2*(2)*	3.99±0.47	ns	4 *(11)*	4.57±1.31			2*(2)*	2.20±1.77	ns
CD	**17 *(40)***	**4.57±1.45**	**17 *(30)***	**4.07±0.96**	**11*(21)***	**4.04±1.33**	**ns**	**17 *(40)***	**4.32±0.88**	**17 *(30)***	**4.96±1.18**	**11*(21)***	**4.50±1.03**	**0.021**
C-CD	6*(13)*	5.15±0.67	4 *(10)*	4.27±0.89	3*(5)*	5.72±1.46	ns	6*(13)*	4.71±0.71	4 *(10)*	4.73±0.93	3*(5)*	3.19±0.90	ns
IC-CD	4*(10)*	4.53±0.96	6 *(8)*	4.30±0.73	3*(6)*	3.78±1.57	ns	4*(10)*	3.75±0.69	6 *(8)*	4.14±1.55	3*(6)*	4.99±0.98	ns
I-CD	6 *(17)*	4.21±1.76	7*(12)*	3.44±1.02	5*(10)*	3.97±1.06	ns	6 *(17)*	4.26±1.04	7*(12)*	5.45±0.75	5*(10)*	4.51±0.96	0.002

* Homogeneous subgroups (P>0.05) within each variable (column) are indicated with the same superscript; TNF, tumour necrosis factor; ns, not significant .

§ Median $\log_{10}$ 16S rRNA gene copies/ million bacterial 16S rRNA gene copies ± standard deviations

Figure 1


H
IBS
UC
ρ=0.303
P=0.007
E3
ρ=0.750
P=0.001
CD
IC-CD and I-CD
ρ=-0.177
P=0.158
0
2
4
6
Log (16S rDNA F.prausnitzii/million 16S rDNA Bacteria)
Log (16S rDNA E.coli/million 16S rDNA Bacteria)