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# A novel distinctive form of identification for differential diagnosis of irritable bowel syndrome, inflammatory bowel disease, and healthy controls

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## Abstract

**Background:** Irritable bowel syndrome (IBS) is a functional disorder affecting around 11% of the world population, which diagnostic is mainly based on clinical parameters. IBS shares many symptoms with other gastrointestinal disorders such as inflammatory bowel disease (IBD), which makes positive diagnosis a difficult task.

**Aim:** This work presents the design of a new test, called RAID-Dx, which is a mathematical algorithm based on the combination of nine faecal microbial markers capable of diagnosing IBS.

Sara Ramió-Pujol and Joan Amoedo contributed equally to the manuscript.

**Methods:** A cohort consisting of 165 subjects (52 IBS and 52 IBD patients, and 61 healthy controls) was recruited from the Gastroenterology Department of six hospitals. Each patient provided a stool sample from which DNA was extracted, and microbial markers composing RAID-Dx were analysed by qPCR. The results obtained were used to define and validate the RAID-Dx algorithm.

**Results:** The abundance of the biomarkers included in the algorithm differed according to the diagnosis. RAID-Dx shows a high capacity to diagnose IBS with a sensitivity of 82.4% and a specificity of 85.7%. RAID-Dx also reports higher sensitivity and specificity values than faecal calprotectin for IBS and IBD differentiation.

**Conclusions:** RAID-Dx is a noninvasive tool aimed to diagnose IBS with high sensitivity and specificity. The use of this new tool for IBS diagnosis could significantly improve disease management, minimise its misdiagnosis and increase patients' quality of life.

## 1 | INTRODUCTION

Irritable bowel syndrome (IBS) is a functional disorder in which recurrent abdominal pain is associated with defecation or changes in bowel habits.[1] IBS affects around 11.0% of the population[2] and only a minority of IBS patients (30%-50%) seek healthcare, accounting for 25% of the visits to a gastroenterologist and up to 12% of the visits to primary care doctors.[3] Thus, it results in the generation of a substantial workload in both primary and secondary care being a significant socioeconomic burden.

IBS pathophysiology is exceptionally complex since it involves different factors such as abnormal intestinal motility, visceral hyperalgesia, increased intestinal permeability, immune activation, altered intestinal microbiota, and disturbance in brain-gut function.[2] Amongst people who meet clinical criteria for IBS diagnosis, symptoms severity varies over a broad spectrum, ranging from very mild to incapacitating,[4] which makes IBS patients have a worse quality of life when compared to the healthy population.[5]

Despite the fact that IBS is a bothersome disorder with high prevalence and its pathophysiology is quite well known, to date, no positive test diagnosis exists. Several clinical diagnostic criteria (ie, Kruis, Manning, Rome) have been traditionally used to distinguish IBS patients from those with organic bowel disease in daily clinical practice,[6] being the most recent and commonly used the Rome IV criteria.[2] Nonetheless, there is still considerable overlap between IBS symptoms and those shown by some organic diseases such as Inflammatory Bowel Disease (IBD). Therefore, the current diagnose of IBS consists of conducting a series of tests (including laboratory tests, imaging tests, and endoscopies) in order to rule out some other diseases that may mimic IBS. Currently, it is estimated that up to 45% of patients wait for more than one year for the diagnosis of these conditions and up to 17% wait for more than 5 years.

Nowadays, one of the biomarkers most extensively used in clinical practice to differentiate IBS from IBD is Faecal Calprotectin (FC). Its abundance in faeces highly correlates with the inflammation activity found in the bowel mucosa,[7] with a high sensitivity to discriminate IBD. This biomarker is especially suited for screening IBD since the gold standard method to diagnose it is the colonoscopy. However, colonoscopy procedures are not considered the best option due to their associated costs, ever-increasing waiting lists, risks, and patients' inconvenience. Unfortunately, a considerable number of IBS are diagnosed as false positive, and all these patients must undergo an unnecessary colonoscopy.

Although the aetiology of this disorder has not been determined to date, research findings have revealed that IBS patients feature metabolomic changes and alterations in colonic fermentation, and that gut microbiota may be relevant for the disease pathogenesis.[8-10] Some studies report that the change in intestinal microbiota caused by acute gastroenteritis is associated with an increased risk of subsequent development of IBS.[11-13] Besides, antibiotic therapy, even when given systemically, has also been significantly associated with IBS.[14] A recent systematic review has reported an exhaustive analysis of the literature, demonstrating the presence of pro-inflammatory species in the gut microbiota of patients with IBS, including phylum Proteobacteria, family Enterobacteriaceae, and genus *Bacteroides* (phylum Bacteroidetes).[15] Additionally, potentially beneficial bacterial species such as *Faecalibacterium prausnitzii* were also found in the microbiota associated with IBS patients.[15] The variety of techniques and samples used in the different studies may hamper reaching a consensus on the IBS-dysbiosis signature.[10,16,17]

A preliminary, prospective study performed in our laboratory with intestinal mucosal samples of 111 individuals (10 IBS, 45 Crohn's Disease (CD), 25 Ulcerative Colitis (UC), and 31 healthy volunteers) showed differences in the relative abundance of *F prausnitzii* and *Escherichia coli* (ECO) when IBS patients were compared with IBD patients or with healthy controls.[18] Based on these data, a noninvasive test capable of diagnosing IBS and differentiating it from IBD was designed. The resulting tool, called RAID-Dx, is based on a mathematical combination of the abundances of eight faecal microbial biomarkers. The aim of this study was to evaluate the capacity of RAID-Dx to diagnose IBS and differentiate it from IBD, comparing the results with those obtained with FC.

## 2 | MATERIALS AND METHODS

### 2.1 | Study population

A cohort consisting of 52 IBS patients, 52 IBD patients (25 diagnosed of CD and 27 diagnosed of UC), and 61 healthy individuals were recruited by the Gastroenterology Departments of six medical centres: Hospital Universitari Doctor Josep Trueta [Girona, Spain], Hospital Santa Caterina [Salt, Spain], Hospital Universitari de Bellvitge [l'Hospitalet de Llobregat, Spain], Hospital Universitari Germans Trias i Pujol [Badalona, Spain], Centro Médico Teknon [Barcelona, Spain], and Beth Israel Medical Center [Boston, USA].

The diagnosis of IBS was performed according to established clinical criteria and, lately, confirmed by a colonoscopy performed within the last three years, in which no valuable macroscopic lesions were found. The diagnosis of CD and UC was performed according to established clinical, endoscopy, and histological criteria. The control group consisted of two different populations of healthy controls, 52.5% of them were certified by a gastroenterologist as being digestively healthy according to established clinical criteria. The other half of the healthy population (47.5%) underwent a colonoscopy for CRC screening based on familial studies or because of hemorrhoidal bleeding.

The inclusion criteria to participate in the study differed depending on the diagnosis. IBS patient's inclusion criteria were: (1) subjects aged over 17 years, (2) IBS diagnosis according to Rome IV criteria, and (3) colonoscopy performed or scheduled without value macroscopic lesions. The inclusion criteria for CD and UC patients were: (1) subjects aged over 17 years old, (2) diagnosed of CD or UC, (3) with active disease defined by colonoscopy (SES-CD > 0 and MES > 0, endoscopic scores for CD and UC, respectively). Finally, inclusion criteria for healthy controls were: (1) aged over 17 years old, (2) certified by a gastroenterologist as digestively healthy or with a routine colonoscopy undergone for familial studies or hemorrhoidal bleeding. Exclusion criteria were the same for all recruited subjects: (1) individuals who received antibiotic treatment, prebiotic or probiotic drugs within last month prior to inclusion, (2) pregnancy, (3) severe morbidity, and (4) previous intestinal surgery or any significant intestinal condition that may alter the results according to investigators criteria. The baseline clinical characteristics of the recruited subjects are shown in Table 1.

### 2.2 | Ethical considerations

The study protocols (clinical investigation code: RAIDCD2016_2, RAIDCD2016_4, and GG-IBS-1001) were approved by the Clinical Research Ethics Committee from all the participating centres between 2016 and 2018.

### 2.3 | Faecal sample collection

Patients collected faecal samples from a single bowel movement at home in a sterile container of faeces. Samples were immediately frozen after deposition in a domestic freezer. Patients collected the samples the week prior to their scheduled colonoscopy and before undergoing bowel cleansing. Those patients without a scheduled colonoscopy had no restrictions on sample collection. Then, subjects brought samples to the hospital, where they were kept frozen at −20°C for short-term storage, and at −80°C upon arrival at GoodGut SL facilities in Girona (Spain).

### 2.4 | Faecal calprotectin determination

The concentration of FC was measured at LABCO (SynLab – Barcelona), using a quantitative enzyme-linked immunosorbent assay (ELISA, Buhlman Test). Sensitivity and specificity values obtained using those predetermined cut-off values established for the IBS and IBD discrimination were examined (50 and 150 µg FC/g of faeces).[19]

### 2.5 | DNA extraction from stool samples

Genomic DNA was extracted from faecal samples after homogenisation using NucleoSpin® Soil (Macherey-Nagel GMbH & Co., Duren, Germany) by following the manufacturer's instructions. DNA was finally eluted in a 100 µL of SE Elution Buffer and stored at −20 °C until its use.

### 2.6 | qPCR assay for IBS biomarkers

The specific microbial sequences targeted different groups according to their characteristics: Eubacteria (EUB) as the total bacterial load; *Faecalibacterium prausnitzii* (FPR), *F prausnitzii* phylogroup I (PHGI), *F prausnitzii* phylogroup II (PHGII), *Akkermansia muciniphila* (AKK), *Ruminococcus* sp (RUM), *E coli*, Bacteroidetes (BAC), and *Methanobrevibacter smithii* (MSM). The sequences of the corresponding forward and reverse primers and probes (when it applied) are described in Table 2. These bacterial markers were firstly defined in biopsy samples (unpublished results) and later tested on stool samples from patients suffering from IBD or IBS, and healthy controls, in order to check their capability of being used as noninvasive indicators for IBS diagnosis. The definition of microbial markers in biopsy samples, which are homogenous, is highly representative of the mucosa status. The later optimisation of these markers in stool samples, which present a high variability content, indicates that the selected species are not subject to changes caused by external factors and remain stable throughout different samples and individuals. Subsequently, quantitative polymerase chain reaction systems (qPCR) were designed to target the microbial markers composing the microbial signature specifically.

Quantification standards were used as positive controls of each microbial marker and were prepared in a genetic construction by

**TABLE 1** Sample size and clinical characteristics of recruited subjects

| | IBS | IBD Crohn's Diseases | Ulcerative Colitis | Control |
|---|---|---|---|---|
| N | 52 | 25 | 27 | 61 |
| Gender [M/F] | 15/37 | 14/13 | 11/14 | 22/39 |
| Age [years ± SD], yr [a] | 48.2 ± 13.6 | 51.0 ± 17.7 | 53.9 ± 15.4 | 44.8 ± 14.0 |
| Smoker [Y/N][b] | 11/36 | 2/5 | 4/11 | 13/48 |
| Treatment [N, %][c] | | | | |
|   No treatment | 52 (100) | 1 (4.0) | 2 (7.0) | 61 (100) |
|   Mesalazina | na | 2 (8.0) | 8 (29.6) | na |
|   Moderate immunosuppressant | na | 12 (48.0) | 6 (22.2) | na |
|   Anti- TNFα | na | 14 (56.0) | 4 (14.8) | na |
| Healthy control [N] | na | na | na | Certified as digestively healthy by a gastroenterologist [32] and underwent a colonoscopy for familial studies or hemorrhoidal bleeding [29] |
| IBS subtype [N][d] | Diarrhoeal predominant type [35], Mixed type [5], Constipation predominant type [3] | na | na | na |
| Activity [N, %] | na | 25 (100)[e] | 27 (100)[f] | na |
| Disease distribution[g] | na | Representation of ileal [1], ileocolonic [2] and colonic [21] distribution | Representation of proctitis [3], distal UC [4], extensive or pancolitis [5] | na |

na, not applicable.

[a]IBD population is significantly older than control population ($P = 0.008$).

[b]Smoker condition at the time of sampling was available in 47/52 patients with IBS, 7/25 patients with CD, 15/27 patients with UC, and 61/61 healthy controls.

[c]Medical treatment at the time of sampling was available in 22/25 patients with CD and 15/27 patients with UC.

[d]IBS subtype at the time of sampling was available in 43/52 patients with IBS.

[e]Harvey-Bradshaw-Index ≥ 5 and SES-CD ≥ 1.

[f]Partial Mayo Scoring Index ≥ 2 and Endoscopic partial mayo scoring index ≥ 1.

[g]Disease distribution at the time of sampling was available in 24/25 patients with CD and 12/27 patients with UC.

inserting the targeted genetic sequence. The selected sequences of the target species were amplified by qPCR and further introduced in a pGEM-T-Easy cloning Vector by the pGEM-T and pGEM-T-Easy Vector System and following the manufacturer's guidelines. Plasmids were extracted using the NucleoSpin® Plasmid kit (Macherey-Nagel GmbH&Co., Germany). Initial target concentration was inferred, considering the theoretical molecular weight and the size of the construction. Standard curves were obtained from 10-fold serial dilutions of the titrated suspension of plasmids, and ranged from $10^8$ to $10^3$ copies/reaction, which corresponds to the linear range for all the reactions.

Quantification of EUB, AKK, RUM, MSM, and BAC was performed by preparing single reactions of each biomarker using GoTaq qPCR Bryt Master mix (GoTaq® qPCR Master Mix, Promega. Madison, USA). Reactions consisted of 10 μL containing 1 X GoTaq® qPCR Master Mix (Promega), between 200 and 300 nmol/L of each primer (specified in Table 2), and between 12 and 20 ng of genomic DNA template. Quantification of FPR, PHGI, PHGII, and ECO was performed by preparing a single reaction for each biomarker using GoTaq qPCR Probe Master Mix (GoTaq® qPCR Master Mix, Promega. Madison, USA). Reactions consisted of 10 μL containing 1 X GoTaq® qPCR Master Mix (Promega), 300 nmol/L of each primer, between 100 and 250 nmol/L of each probe (specified in Table 2), and between 12 and 20 ng of genomic DNA template. Thermal profiles were different depending on the biomarker analysed (Table 3).

Primers used in this study were purchased from Macrogen (Macrogen, Seoul, South Korea). All quantitative PCRs were run on an AriaMx Real-time PCR System (Agilent Technologies, Santa Clara, USA). A melting curve step was added at the end of each qPCR when

**TABLE 2** Probes, forward and reverse primers used in this study

| Target | Primers | Sequence 5'-3' | Final Conc. (nM) | References |
|---|---|---|---|---|
| EUB | EUB_F | ACTCCTACGGGAGGCAGCAGT | 200 | Modified from 39 |
|  | EUB_R | GTATTACCGCGGCTGCTGGCAC | 200 |  |
| FPR | Fpra_F | TGTAAACTCCTGTTGTTGAGGAAGATAA | 300 | 25 |
|  | Fpra_R | GCGCTCCCTTTACACCCA | 300 |  |
|  | Fpra_PR | FAM-CAAGGAAGTGACGGCTAACTACGTGCCAG-TAMRA | 250 |  |
| PHGs | PGH_F | CTCAAAGAGGGGGACAACAGTT | 900 | 18 |
|  | PHG_R | GCCATCTCAAAGCGGATTG | 900 |  |
|  | PHGI_PR | TAAGCCCACGACCCGGCATCG | 300 |  |
|  | PHGII_PR | HEX-TAAGCCCACRGCTCGGCATC-BHQ1 | 300 |  |
| ECO | Eco_F | CATGCCGCGTGTATGAAGAA | 300 | 40 |
|  | Eco_R | CGGGTAACGTCAATGAGCAAA | 300 |  |
|  | Eco_PR | FAM-TATTAACTTTACTCCCTTCCTCCCCGCTGAA-TAMRA | 100 |  |
| AKK | Akk_F | CAGCACGTGAAGGTGGGGAC | 250 | 41 |
|  | Akk_R | CCTTGCGGTTGGCTTCAGAT | 250 |  |
| RUM | Rum_F | GGCGGCYTRCTGGGCTTT | 250 | 42 |
|  | Rum_R | CCAGGTGGATWACTTATTGTGTTAA | 250 |  |
| MSM | Msm_F | ACGCAGCTTAAACCACAGTC | 200 | This study |
|  | Msm_R | AAAGACATTGACCCRCGCAT | 200 |  |
| BAC | Bac_F | CRAACAGGATTAGATACCCT | 300 | 43 |
|  | Bac_R | GGTAAGGTTCCTCGCGTAT | 300 |  |

Abbreviations: EUB, Eubacteria; FPR, *F prausnitzii*; PHGI, *F prausnitzii* phylogroup I; PHGII, *F prausnitzii* phylogroup II; ECO, *E coli*; AKK, *Amuciniphila*; RUM, *Ruminococcus* sp, MSM, *M smithii*; BAC, Bacteroidetes.

**TABLE 3** Thermal profiles used for the amplification according to the biomarkers

| Microbial markers | Total cycles | Denaturing Tª (°C) | Time (min) | Annealing and Extension Tª (°C) | Time (min) |
|---|---|---|---|---|---|
| EUB | 40 | 95 | 10:00 | 95 54 | 00:15 01:00 |
| FPR, ECO | 40 | 95 | 10:00 | 95 60 | 00:15 01:00 |
| PHGI, PHGII | 40 | 95 | 10:00 | 95 64 | 00:15 01:00 |
| AKK, RUM, MSM, BAC | 40 | 95 | 10:00 | 95 60 | 00:15 01:00 |

Abbreviations: EUB, Eubacteria; FPR, *F prausnitzii*; PHGI, *F prausnitzii* phylogroup I; PHGII, *F prausnitzii* phylogroup II; ECO, *E coli*; AKK, *Amuciniphila*; RUM, *Ruminococcus* sp; MSM, *M smithii*; BAC, Bacteroidetes.

GoTaq qPCR Bryt Master Mix was used to verify the presence of the expected amplicon size as well as to control primer dimer formation. Data were collected and analysed with the Aria Software version 1.5 (Agilent Technologies, Santa Clara, USA). All samples were amplified in duplicates, which were considered valid when the difference between threshold cycles (*Ct*) was less than 0.6 or than 1.0 at Ct lower or higher than 28, respectively. Moreover, a nontemplate control reaction was included in each qPCR run.

## 2.7 | Statistical analysis

Data normality was assessed through the Kolmogorov-Smirnov test. The nonparametric Kruskal-Wallis and Mann-Whitney tests were used to analyse differences amongst groups or pairwise comparisons, respectively. All comparisons using microbial markers were performed between the relative abundances, which were normalised by the total bacterial load abundances.

The receiver operating characteristic (ROC) curve analysis was applied to determine the usefulness of each biomarker to distinguish between IBS and no IBS (IBD or healthy controls). The accuracy of discrimination was measured by the area under the ROC curve (AUC). Statistical analyses were carried out with the SPSS 23.0 statistical package (IBM, NY, NY). MANOVA (Wilks test) was performed with RStudio after data conversion to the geometrical mean of all the variables for each diagnosis using CoDaPack 2.02.21. Significance levels were established for *P* values ≤ .05.

In this proof-of-concept study, analysis to determine which combination of microbial markers was capable of distinguishing IBS patients from those healthy controls and IBD patients was performed. The methodology used consisted of initial training with the 70% of a

random partition of the data set and further validation with the 30% left of the data set. RAID-Dx was eventually designed by the combination of eight bacterial markers and one archaeal marker. The final algorithm is based on a Decision Abundance (DA) calculated using the following equation:

$$DA = \frac{\frac{Ct_{ind} - b_{ind}}{m_{ind}}}{\frac{Ct_{EUB} - b_{EUB}}{m_{EUB}}} \qquad (1)$$

where $Ct$ is the threshold cycle; $b$ is the intercept point; $m$ is the slope; $ind$, is the microbial marker; and EUB are eubacteria (total bacterial load). The values for each biomarker are listed in Table 4.

The sample size effects have been calculated using G*Power 3.1.9.2 program, module: Many groups: ANOVA: One way (one independent variable), F tests. The effect size set as large ($f = 0.4$) according to criteria described by Cohen,[20] the level of significance set to 5%, and the N sample of the cohort (165) were used in order to compute the achieved power by selecting post hoc analysis. The statistical power of this proof-of-concept is 99.7%.

Sensitivity, specificity, positive predictive values (PPV), and negative predictive values (NPV) of the designed algorithm were calculated using the software Epidat 3.1 (SERGAS, Xunta de Galicia, Spain).

## 3 | RESULTS

### 3.1 | Faecal microbial markers in irritable bowel syndrome patients

Eubacteria have been used to normalise the abundance of all the microbial markers as the total bacterial load in order to avoid data bias. The relative abundance of each microbial marker has been analysed, and significant differences amongst diagnoses were found (Figure 1). PHGII showed significant differences amongst all the diagnoses ($P < .001$) being higher in healthy controls and lower in IBD patients. Patients diagnosed with IBS showed a lower relative abundance of BAC than healthy controls and IBD patients ($P = .005$ and $P = .001$, respectively), indicating its potential as an IBS biomarker. Besides, two different microbial markers have been shown to be less abundant in IBD patients when compared to healthy controls and IBS patients, AKK ($P < .001$ and $P = .009$, respectively) and

MSM ($P < .001$ and $P = .002$, respectively). RUM has been shown to be a good biomarker to differentiate healthy controls, since it presented higher relative abundance than in IBS ($P = .023$) or IBD ($P < .001$). Also, PHGI presented significant differences between healthy controls and IBD ($P = .018$). Finally, the total bacterial load presented no significant differences between any of the compared populations ($P = .434$).

A more in-depth exploratory data analysis was performed comparing the relative abundance of all microbial markers amongst the studied diagnosis. The geometrical mean of all the variables was calculated and compared to the overall mean for each diagnosis (Figure 2). Significant differences are found amongst all diagnoses (MANOVA—Wilks test: $P$ value $< .001$). As shown in the figure, healthy controls and IBD patients presented an inverse relationship amongst the analysed microbial markers. In healthy controls, beneficial species such as *F prausnitzii* and its phylogroups, *A muciniphila* and *Ruminococcus* sp, are more predominant compared to pro-inflammatory microbial marker (*E coli*) and Bacteroidetes. This pattern is found to be opposite to that observed in IBD. Observing IBS pattern, it is found between that of healthy controls and the one of the IBD, showing less variation than the other two populations, except for Bacteroidetes, which could be a good IBS indicator.

Receiver operating characteristic curves analysis was performed to test the accuracy of the different biomarkers presenting abundances significantly different amongst the studied populations, more specifically to differentiate IBS patients from the others. In line with the results observed in Figure 2, the best AUC was obtained by BAC with a value of 0.670, producing a sensitivity and specificity of 65.4% and 59.3%, respectively.

ROC curves analysis confirmed that the reduction of a single species load in IBS patients is insufficient for diagnostics purposes.

### 3.2 | Microbial markers vs faecal calprotectin for the differential diagnosis of IBS and IBD

Receiver operating characteristics curves analysis was performed to test the accuracy of those biomarkers, which showed abundances significantly different between the two populations (Figure 3). The best results of AUC were obtained with BAC with a value of 0.689, followed by MSM with a value of 0.680. However, faecal calprotectin obtained the highest AUC, with a value of 0.874 (Table 5). Despite the highest AUC

**TABLE 4** Slope and intercept point (m and b from Equation 1) with efficiency and $r^2$ for each microbial marker

|  | EUB | FPR | PHGI | PHGII | ECO | AKK | RUM | MSM | BAC |
|---|---|---|---|---|---|---|---|---|---|
| Slope (m) | −3.25 | −3.27 | −3.62 | −3.48 | −3.25 | −3.48 | −3.27 | −3.41 | −3.25 |
| Intercept point (b) | 38.29 | 41.15 | 42.63 | 43.32 | 41.52 | 39.55 | 35.96 | 38.53 | 37.58 |
| Efficiency | 103.12 | 102.09 | 88.91 | 93.89 | 103.12 | 93.97 | 102.49 | 97.18 | 103.28 |
| $R^2$ | 0.999 | 0.998 | 0.999 | 0.998 | 0.999 | 0.999 | 0.999 | 0.998 | 0.997 |

Abbreviations: EUB, Eubacteria; FPR, *F prausnitzii*; PHGI, *F prausnitzii* phylogroup I; PHGII, *F prausnitzii* phylogroup II; ECO, *E coli*; AKK, *Amuciniphila*; RUM, *Ruminococcus* sp; MSM, *M smithii*; BAC, Bacteroidetes.
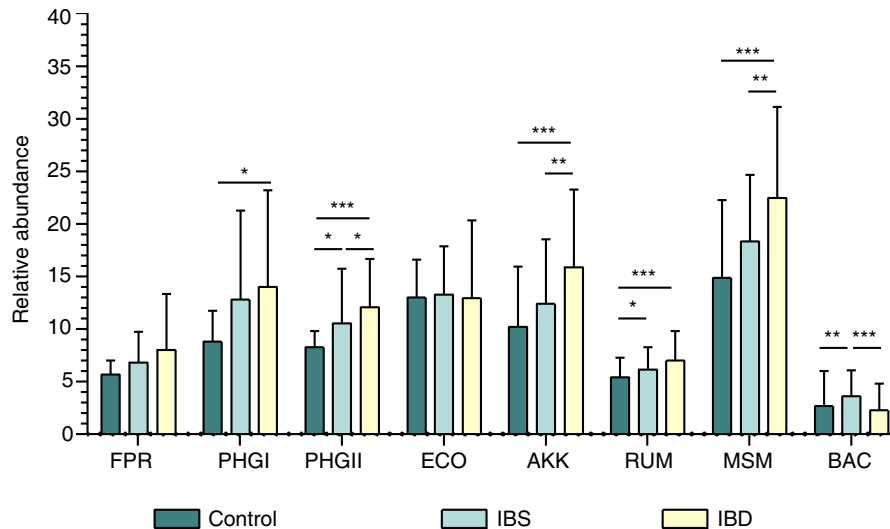
**FIGURE 1** Relative abundances ($Ct_{marker} - Ct_{EUB}$) of the analysed microbial markers (*F prausnitzii* (FPR), *F prausnitzii* phylogroup I (PHGI), *F prausnitzii* phylogroup II (PHGII), *E coli* (ECO), *Amuciniphila* (AKK), *Ruminococcus* sp (RUM), *M smithii* (MSM), and Bacteroidetes (BAC)); for healthy controls (H), patients with Irritable Bowel Syndrome (IBS) and patients with Inflammatory Bowel Disease (IBD). The relative abundance is read inversely, as higher the value, lower the abundance, and vice versa. Significance levels are described as *P value < .05; **P value < .01; and ***P value < .001
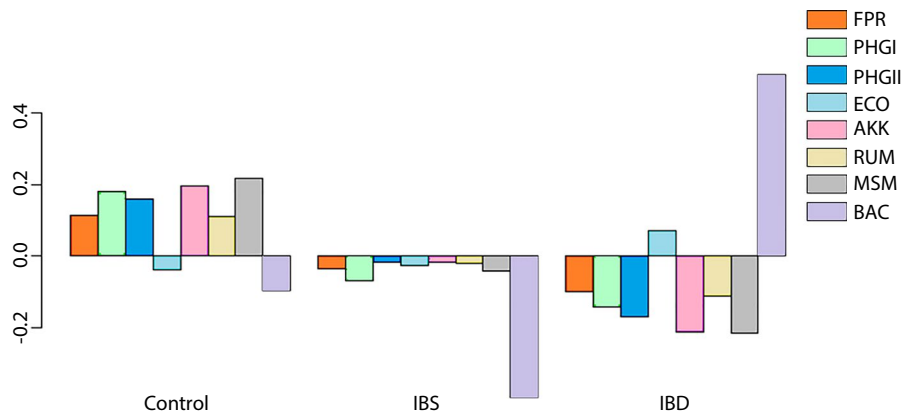


**FIGURE 2** Geometrical mean for each of the three diagnoses (healthy controls – Control, Irritable Bowel Syndrome – IBS, Inflammatory Bowel Disease – IBD) compared with the overall mean. *F prausnitzii* (FPR), *F prausnitzii* phylogroup I (PHGI), *F prausnitzii* phylogroup II (PHGII), *E coli* (ECO), *A muciniphila* (AKK), *Ruminococcus* sp (RUM), *M smithii* (MSM), and Bacteroidetes (BAC)

value, the sensitivity obtained for IBS diagnosis at the pre-determined cut-off of 50 µg/g of faeces was lower than that obtained with the analysed microbial markers. The specificity value calculated for the IBD diagnosis confirms that FC is an excellent biomarker for IBD. Sometimes, medical doctors hesitate about the diagnosis when FC values are between 50 and 150 µg/g of faeces; thus, frequently, the test is repeated when this situation appears. Therefore, 150 µg/g of faeces is also considered another pre-determined cut-off. In our cohort, this cut-off obtained a sensitivity and specificity of 76.0% and 83.3%, respectively.

ROC curves analysis showed that a single species relative load in IBS could present high values of sensitivity; however, the specificity is highly reduced when compared to FC, the currently used methodology.

## 3.3 | RAID-Dx algorithm development and validation

An algorithm combining the previously analysed biomarkers was developed in order to differentiate IBS patients from IBD patients and healthy controls, and lately validated. As commented above, in this proof-of-concept, initial training with 70% of a random partition of the data set was used for the definition of the algorithm, and the remaining 30% of the data was used for its validation (Table 6).

The development of the RAID-Dx algorithm was focused on obtaining higher sensitivity and specificity values to diagnose IBS. The combination of the relative abundance of the above described eight functional species led to the achievement of an algorithm with
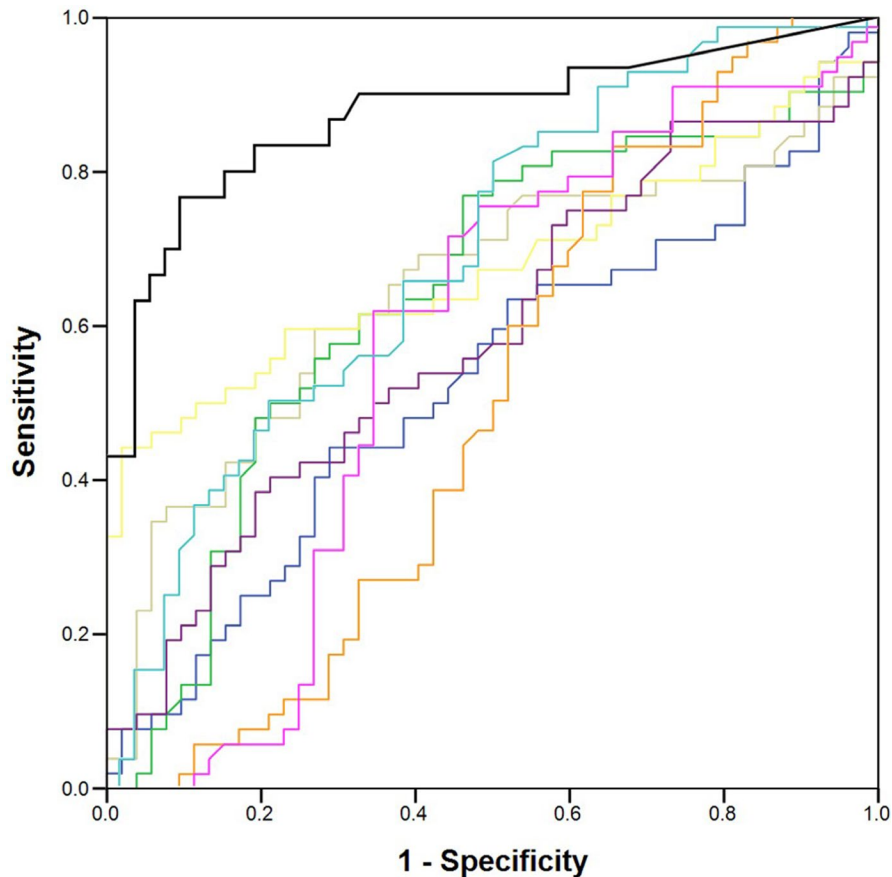
**FIGURE 3** Receiver operator curve (ROC) comparing relative abundance of *F prausnitzii* (orange curve), *F prausnitzii* phylogroup I (dark blue curve), *F prausnitzii* phylogroup II (green curve), *E coli* (pink curve), *A muciniphila* (grey curve), *Ruminococcus* sp (purple curve), *M smithii* (yellow curve), Bacteroidetes (light blue curve), and Faecal Calprotectin (black curve) for IBS and IBD patient's discrimination

**TABLE 5** Diagnostic performance of *F prausnitzii* (FPR), *F prausnitzii* phylogroup I (PHGI), *F prausnitzii* phylogroup II (PHGII), *E coli* (ECO), *A muciniphila* (AKK), *Ruminococcus* sp (RUM), *Msmithii* (MSM), Bacteroidetes (BAC), and faecal calprotectin (FC) at a predetermined cut-off of 50 and 150 µg/g of faeces

|  | AUC | Sensitivity (%) | Specificity (%) |
|---|---|---|---|
| FPR | 0.501 | 50.0 | 50.0 |
| PHGI | 0.530 | 51.9 | 57.7 |
| PHGII | 0.641 | 67.3 | 61.5 |
| ECO | 0.570 | 61.5 | 65.4 |
| AKK | 0.648 | 67.3 | 61.5 |
| RUM | 0.585 | 59.6 | 53.8 |
| MSM | 0.680 | 67.3 | 61.5 |
| BAC | 0.689 | 65.4 | 61.5 |
| FC at predetermined cut-off 50 µg/g faeces | 0.874 | 50.0 | 90.0 |
| FC at predetermined cut-off 150 µg/g faeces | 0.874 | 76.0 | 83.3 |

a sensitivity of 91.4% and specificity of 83.3 (Table 7), with which 83.5% of the subjects were classified correctly. When RAID-Dx was applied to the validation subcohort, high values of sensitivity and specificity were maintained; in this case, 73.2% of the subjects were correctly classified. More specifically, RAID-Dx consists of

nine ratios: AKK/EUB, FPR/EUB, PHGI/EUB, PHGII/EUB, ECO/ EUB, RUM/EUB, BAC/EUB, MSM/EUB, and (FPR/EUB x ECO/EUB).

When the defined algorithm is only applied to IBS and IBD population, it resulted to a substantial increase of the sensitivity value when compared to FC pre-determined cut-off (training partition of 94.3% and validation partition of 88.2%); whereas specificity, in this case for IBD diagnosis, was maintained.

## 4 | DISCUSSION

Functional gastrointestinal disorders most likely exist on a continuum rather than in isolation as separate and discrete disorders, with significant symptoms overlap amongst these conditions. The symptoms of IBS patients can mimic those associated with IBD, posing a challenge for diagnosis.[21] The recently updated Rome IV criteria were designed to facilitate making a positive diagnosis of IBS based on the presence of characteristic symptoms and the absence of objective findings from body imaging or endoscopy.[21]

All IBS symptoms are focused on the intestinal tract causing a modification in the intestinal habit of the patients. Intense metabolic microbial activity, including fermentation, occurs mainly in the proximal colon, where substrate availability is higher than in the distal colon. Also, the availability of substrate declines and the high extraction of free water reduces the diffusion of substrates and microbial products. Thus, the majority of nonabsorbable carbohydrates

**TABLE 6** Baseline clinical characteristics of the data set partition: training (70% of the database) and validation (30% of the database)

| Partition | Group | N | Gender [M/F] | Age [years ± SD]* | Subtype [N] |
|---|---|---|---|---|---|
| Training | IBS | 35 | 10/25 | 47.3 ± 13.5 | NA |
| | IBD | 35 | 19/16 | 54.1 ± 16.3 | Crohn's Disease [17] Ulcerative Colitis [18] |
| | Control | 43 | 14/29 | 45.3 ± 13.6 | Certified by a gastroenterologist as digestively healthy [22] Underwent a colonoscopy for familial studies or hemorrhoidal bleeding [21] |
| Validation | IBS | 17 | 5/12 | 50.2 ± 14.2 | NA |
| | IBD | 17 | 6/11 | 49.4 ± 16.8 | Crohn's Disease [8] Ulcerative Colitis [9] |
| | Control | 18 | 8/10 | 43.6 ± 15.4 | Certified by a gastroenterologist as digestively healthy [10] Underwent a colonoscopy for familial studies or hemorrhoidal bleeding [8] |

Abbreviation: NA, not apply.

*IBD population is significantly older than control population in training partition ($P = 0.013$).

**TABLE 7** Diagnostic performance of RAID-Dx in the proof-of-concept study (N = 165)

| | IBS diagnosis | | IBS and IBD discrimination | |
|---|---|---|---|---|
| | Algorithm definition (training – 70%) | Algorithm validation (validation – 30%) | Algorithm definition (training – 70%) | Algorithm validation (validation – 30%) |
| Sensitivity (%) | 91.4 | 82.4 | 94.3 | 88.2 |
| Specificity (%) | 83.3 | 85.7 | 88.6 | 94.1 |
| PPV (%) | 71.1 | 73.7 | 89.2 | 93.8 |
| NPV (%) | 95.6 | 90.9 | 93.9 | 89.0 |

Abbreviations: Sensitivities, specificities, Positive Predictive Value (PPV), and Negative Predictive Value (NPV) of the algorithm in its definition and validation phases for IBS diagnosis and IBS vs IBD population discrimination.

are fermented in the proximal colon by saccharolytic bacteria, mainly by primary fermenters like Bacteroidetes.[22] The bacteria belonging to this phylum are a cornerstone of the homeostasis in a healthy gut since they are linked to specific metabolic functions regarding nutrient digestion and calorie absorption,[22] which may influence human health, making them good candidates to be part of RAID-Dx. Besides, three representatives of the mucolytic group have been selected: *A muciniphila, F prausnitzii, F prausnitzii* phylogroup I, *F prausnitzii* phylogroup II, and *Ruminococcus* sp The presence of a conspicuous layer of intestinal mucus may help the ecosystem in two different ways: harbouring protective and mucus promoter bacteria, while keeping the transient harmful bacteria away from the inflammatory triggers of the mucosa membrane, and facilitating the intestinal transit.[23] Therefore, the presence of these five bacterial markers is associated with a healthy microbial profile which, in turn, exerts a major effect on physiological functions and homeostasis of

the intestine. A decrease in their abundance has been repeatedly reported to be associated with functional bowel disorders.[10,24-30]

On the other side, *E coli* has been found in the mucus layer, close to the intestinal epithelial cells and in ulcers of both CD and UC patients, with a higher proportion when compared to healthy controls or IBS patients.[25,31-33] These studies indicate that *E coli* is one of the main modulators of intestinal inflammation, and its use as a bacterial marker for diagnosis is, therefore, required. Last, *M smithii* was selected because it is the predominant methanogen (methane producer archaea) of the human intestine. It plays an important role in the efficiency of polysaccharide digestion by the consumption of the final products of bacterial fermentation.[34] Likewise, observational studies show a strong association between delayed intestinal transit and the production of methane, and an association between low abundances of methanogens and diarrheic IBS has been also reported.[35,36] The intestinal microbiota, like any other ecosystem, is defined by a community of living organisms

in conjunction with the nonliving components of their environment. Changes or effects that may occur either due to environment modification or in the balance of the intestinal microbiota will not only affect a single species but the whole system as it was observed in IBD.[37] Therefore, the combination of different species provides more robust and reliable results in the diagnosis of different diseases.

Currently, FC is the most widely used faecal marker, both in primary and secondary care, for differentiating IBS from IBD due to its high accuracy in ruling out intestinal inflammation.[19] As commented, a value of 50 μg FC/g of faeces has been the most commonly adopted cut-off. In the review by *Mumolo* et al,[19] which evaluates FC testing for distinguishing between inflammatory and noninflammatory bowel disease at the predetermined cut-off, FC showed a pooled sensitivity and specificity for noninflammatory disease diagnosis of 78.8% and 85.0%, respectively. Although the reported specificity value is similar to the one obtained in the present study with FC, the analysed cohort presented a lower sensitivity value than the reported in the literature. Another biomarker capable of differentiating IBS from IBD, although not with such good results as those obtained with FC, is the *IBS test* developed by *Pimentel* et al,[38] who proved the utility of anti-vinculin and anti-CdtB (Cytolethal distending toxin B) levels to differentiate IBS from IBD patients. As RAID-Dx, *IBS test* favour IBD diagnosis (specificity) obtaining high values for both markers (83.8% and 91.6% for anti-vinculin and anti-CdtB, respectively). However, sensitivity values were lower than 50.0% (32.6% and 43.7%, respectively). RAID-Dx obtained higher sensitivities and specificities when compared to FC and *IBS test* (pooled sensitivity and specificity from training and validation cohort 91.3% and 92.8%, respectively). The proper and reliable performance of RAID-Dx is based on the intestinal microbiota response to the complex multidirectional communication system amongst the different factors involved in IBS pathophysiology.

Therefore, the use of the defined microbial signature as a first-line test for IBS diagnosis could significantly improve disease management and minimise its misdiagnosis, which could result in a reduction of those laboratory and imaging tests that expect a negative result for organic lesions. In a post-pandemic Covid-19 world, it will be of major importance to rely on marker allowing stricter decision-making on colonoscopy performance. Thus, RAID-Dx may become a very promising tool for IBS diagnosis and management.

## ETHICAL CONSIDERATIONS

The study protocols (clinical investigation code: RAIDCD2016_2, RAIDCD2016_4, and GG-IBS-1001) were approved by the Clinical Research Ethics Committee from all the participating centres between 2016 and 2018. All authors had access to the study data and reviewed and approved the final manuscript.

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