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TOPIC HIGHLIGHT

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Methodological issues in the study of intestinal microbiota in irritable bowel syndrome

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Abstract

Irritable bowel syndrome (IBS) is an intestinal functional disorder with the highest prevalence in the industrialized world. The intestinal microbiota (IM) plays a role in the pathogenesis of IBS and is not merely a consequence of this disorder. Previous research efforts have not revealed unequivocal microbiological signatures of IBS, and the experimental results are contradictory. The experimental methodologies adopted to investigate the complex intestinal ecosystem drastically impact the quality and significance of the results. Therefore, to consider the methodological aspects of the research on IM in IBS, we reviewed 29 relevant original research articles identified through a PubMed search using three combinations of keywords: "irritable bowel syndrome + microflora", "irritable bowel syndrome + microbiota" and "irritable bowel syndrome + microbiome". For each study, we reviewed the quality and significance of the scientific evidence obtained with respect to the experimental method adopted. The data obtained from each study were compared with all considered publications to identify potential inconsistencies and explain contradictory results. The analytical revision of the studies referenced in the present review has contributed to the identification of microbial groups whose relative abundance significantly alters IBS, suggesting that these microbial groups could be IM signatures for this syndrome. The identification of microbial biomarkers in the IM can be advantageous for the development of new diagnostic tools and novel therapeutic strategies for the treatment of different subtypes of IBS.

Key words: Intestinal dysfunction; Irritable bowel syndrome; Intestinal microbiota; Bifidobacteria; New generation DNA sequencing

Core tip: Irritable bowel syndrome (IBS) is the intestinal functional disorder with the highest prevalence in the industrialized world. The intestinal microbiota (IM) plays a role in its pathogenesis. Since the methodological aspects of the research on IM in IBS have never been considered in detail before, we carried out a revision of 29 original research articles. We reviewed the scientific microbiological message in light of the experimental method adopted. The analytical revision of the studies referenced in our review leaded to the identification of microbial groups whose relative abundance resulted significantly altered in IBS. Such microbial groups are potential IM signatures of IBS.

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INTRODUCTION

Irritable bowel syndrome (IBS) is a functional intestinal

disorder with the highest prevalence in the industrialized world[1,2]. Due to the absence of an evident pathogenesis, IBS is exclusively diagnosed based on the absence of mucosal, structural and biochemical diseases and the evaluation of specific symptoms according to Rome Ⅲ criteria[3,4]. The main symptoms that characterize IBS include abdominal pain and discomfort, accompanied by diarrhea (IBS-D), constipation (IBS-C), or a combination of the two (alternating IBS, IBS-A). The frequency and intensity of these symptoms largely varies, thereby affecting the quality of life of the patients[5].

The etiopathogenesis and pathophysiology of IBS are ambiguous and likely include many different factors, such as improper immune activation, visceral hypersensitivity, colon dysmotility, history of gastrointestinal infections, and psychological conditions[6-9]. In addition, many studies have also investigated a potential role for intestinal microbiota (IμB) in IBS.

Experimental observations showed that in IBS (1) toll-like receptor genes are upregulated[10]; (2) fecal levels of defensins are increased[11]; and (3) short chain fatty acid concentrations are frequently augmented[12-16]. Furthermore, it was shown that probiotics and antibiotics treatments could reduce IBS symptoms[17-19]. These data suggest that changes in the IμB are not only a consequence of IBS, but could also be a plausible causative factor. Nonetheless, current research efforts have not identified any definitive microbiological signatures of IBS and the experimental results are occasionally contradictory. The heterogeneity of the results on the role of IμB in IBS primarily reflects the high variability among various manifestations of IBS and marked differences in the IμB composition among subjects[20]. Moreover, the experimental methodologies employed and the specific protocols adopted to investigate complex ecosystems, such as the IμB, drastically impact the quality and significance of the results. To examine the methodological aspects of the research on the role of IμB in IBS, we reviewed 29 relevant original research articles obtained through a PubMed search using three combinations of keywords: "irritable bowel syndrome + microflora", "irritable bowel syndrome + microbiota" and "irritable bowel syndrome + microbiome". For each study, we reviewed the scientific evidence obtained with respect to the experimental technology adopted. The collected data from each study were compared among all considered studies to detect potential inconsistencies and explain contradictory results.

METHODOLOGIES EMPLOYED TO INVESTIGATE THE INTESTINAL MICROBIOTA IN IBS SUBJECTS

The 29 original research studies considered in the present review address the microbial community structure in the intestine of IBS subjects using several different experimental techniques. Only a few of these studies used classical (culture-based) strategies, which have extensively been replaced with molecular techniques (*i.e.*, strategies based on the analysis of nucleic acids).

The molecular methods employed in the selected studies primarily included (1) fluorescence *in situ* hybridization (FISH); (2) DNA microarrays; and (3) polymerase chain reaction (PCR)-based methods. The PCR-based methods can be further divided into three main groups: Real-time quantitative PCR (qPCR); Genetic fingerprinting [denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP)]; PCR fragment sequencing.

In the following paragraphs, the advantages and limitations of the technologies employed to correlate IμB to IBS are discussed. In addition, the experimental results obtained using each methodological strategy are presented and compared.

Culture-based methods

The classical strategies of microbial ecology, based on the cultivation of microorganisms, have been demonstrated as inappropriate for the analysis of complex microbial ecosystems, such as the intestinal environment, because the vast majority of the microorganisms (between 80% and 99%) in any environment are not cultivable using standard culturing techniques[21,22]. A few studies in the last 10 years, however, have adopted culture-dependent approaches to characterize the IμB of subjects with IBS (Table 1). For example, Mättö *et al*[23] found a moderate increase in the coliform bacteria concentration and aerobe/anaerobe ratio in fecal samples obtained from IBS patients (26 subjects: 12 IBS-D, 9 IBS-C and 5 IBS-A) compared with healthy controls (HCs, 25 subjects), whereas the bifidobacterial concentrations did not differ. More recently, Enck *et al*[24] applied culture-based analyses to examine fecal samples from more than 34000 subjects, including 7784 people with IBS. In contrast to Mättö[23], among the few bacterial groups considered, only bifidobacteria were significantly decreased in IBS samples. The differences in these results, however, are plausible, considering that Mattö used the Beerens medium[25], containing propionic acid, as a selective agent for bifidobacteria, whereas Enck *et al*[24] used DIC agar (Heipha GmbH, Germany), a commercial medium containing gentamycin and vancomycin as selective agents. Although bifidobacteria are considered resistant to these antibiotics, sensitivity has been reported for stressed cells belonging, for example, to the species *Bifidobacterium longum* (*B. longum*)[26]; therefore, the use of antibiotics as selective agents compromises the cultivation of viable bifidobacterial cells in a fecal sample. Furthermore, the bifidobacteria concentration was not significantly different in 10 IBS-D subjects compared with 10 healthy controls in another study[27] in which a medium similar to Beerens agar was used for the isolation. On the contrary, Chassard *et al*[28] detected reduced bifidobacteria concentrations in the fecal samples of 14 IBS-C women compared with 12 sex-matched HCs. However, in this study, bifidobacteria were isolated using de Man Rogosa Sharp (MRS) agar medium (pH 7.0),

Table 1 Outcomes of the selected original research studies (see text for details), which have been carried out to characterize the intestinal microbiota composition in irritable bowel syndrome

Participants	Rome criteria	Results	Sample	Technique	Ref.
27 IBS (20 F/7 M) 12 IBS-D 9 IBS-C 6 IBS-A Age: 20-65 22 HCs (15 F/7 M) Age: 25-64	II	↑ *Ruminococcus productus - Clostridium coccoides* ↓ *Lactobacillus* (IBS-D *vs* IBS-C) ↓ *Bifidobacterium* (IBS-D *vs* HC, IBD-C, IBS-A) ↓ *Desulfovibrio* (IBS-D *vs* HC, IBD-C, IBS-A) ↑ *Veillonella* (IBS-C *vs* HC)	Fecal	qPCR (SYBR Green)	[16]
26 IBS (19 F/7 M) 12 IBS-D 9 IBS-C 5 IBS-A Age: 20-65 25 HCs (18 F/7 M) Age: 23-63	II	More temporal instability in predominant bacterial population in IBS subjects Slight increase of coliforms in IBS and higher aerobe/anaerobe ratio in IBS ↑ *Clostridium* spp. ↓ *Eubacterium* spp.	Feces	DGGE Cloning and sequencing of 5 IBS and 4 HC samples (29 IBS and 16 HC clones); ABI PRISM BigDye terminator Cycle sequencing kit v. 3.0 Culture method	[23]
20 IBS (14 F/6 M) Mean age: 47.8 20 HCs (13 F/7 M) Mean age: 46.2	II	Mucosal bacteria concentration higher than 10^9 cells/mL in 65% of IBS subjects (35% in HC) Prevalence of *Eubacterium rectale-Clostridium coccoides* in IBS biofilm	Ileal and colonic biopsies	FISH	[33]
16 IBS (11 F/5 M) 7 IBS-D 6 IBS-C 3 IBS-A Age: 24-64 16 HCs (12 F/4 M) Age: 26-63	II	More temporal instability of predominant microbiota only in RNA-DGGE profiles in IBS *vs* HCs (not in DNA-DGGE) ↓ *C. coccoides* - *E. rectale* in IBS-C *vs* HC No differences in *Clostridium* population stability between IBS and HC	Feces	DGGE Multiplexed quantification of clostridial 16S rRNA through MultipleTranscript analysis with the aid of affinity capture (TRAC)	[49]
24 IBS 10 IBS-D 8 IBS-C 6 IBS-A Age: 21-65 23 HCs (16 F/7 M) Age: 26-64	II	Significant differences in microbiota composition in different IBS subcategories pooled in 3 groups on the basis of %GC (7-10-13 fractions) In fraction group 7: ↓ *Lactobacillus* in all IBS subgroups *vs* HC ↑ *Ruminococcus* in IBS-C and IBS-A ↑ *Streptococcus* in IBS-D In fraction group 13: ↓ *Collinsella* in IBS-C and IBS-D	Feces	16S rRNA gene cloning and sequencing of 3753 clones	[37]
20 IBS 8 IBS-D 8 IBS-C 4 IBS-A Age: 24-64 15 HCs Age: 25-64	II	↑ *Clostridium thermosuccinogenes* (IBS-A *vs* IBS-D) ↑ *Ruminococcus torques* 94% phylotype (IBS-D *vs* HCs and IBS-A) ↑ *Ruminococcus bromii*-like phylotype (IBS-C *vs* HCs) ↑ *Bacteroides intestinalis*-like and *C. cocleatum* (IBS-A and HCs *vs* IBS-D) ↓ *Clostridium aerofaciens*-like (IBS-D *vs* other groups)	Feces	qPCR (SYBR Green)	[46]
41 IBS (29 F/12 M) 14 IBS-D 11 IBS-C 16 IBS-A Mean age: 42 26 HCs (18 F/8 M) Mean age:32		↓ *Bifidobacterium* ↓ *B. catenulatum*	Feces Feces and duodenal brushes	FISH qPCR (Taqman)	[31]
10 IBS-D (6 F/4 M) Age average: 46.5 23 HCs Age average: 45 12 IBS-D (7 F/5 M) Age average: 46.5 22 HCs Age average: 45	II	Decreased diversity in the intestinal microbiota of IBS-D *vs* HCs ↑ Proteobacteria and Firmicutes ↑ Lachnospiraceae ↓ Actinobacteria and Bacteroidetes No significant differences in Enterobacteriaceae and *Eggerthella lenta*-type (*Atopobium*) phylotype between IBS-D and HCs	Feces	Genomic DNA fractioning on the basis of %GC (35%-40%/40%-45%/50%-55%/ 55%-60%/ 60-65/65%-70%/70%-75%); amplification of 16S rRNA gene; sequencing of 3267 clones for IBS subjects qPCR (SYBR Green)	[47]

47 IBS (47 F) Age: 24-66 33 HCs Age: 21-38	II	Significant difference in DGGE profile between IBS and HC, less microbial variation in IBS No significant intra and inter-differences in IBS subjects between luminal and mucosal microbiota. IBS impacts equally on both communities	Feces Feces and colonic biopsies	DGGE of V1-V3 region of the 16S rRNA DGGE of V6-V8 Region of the16S rRNA	[70]
26 IBS (13 F/13 M) 8 IBS-D 11 IBS-C 7 IBS-A Age: 21.7 ± 2.0 26 HCs Age: 21.9 ± 2.9		↑ *Veillonella* ↑ *Lactobacillus* spp.	Feces	qPCR (SYBR Green) Culture method	[12]
10 IBS-D (8 F/2 M) Age: 23-50 10 HCs (6 F/4 M) Age: 21-54	III	↓ Aerobic counts in fecal samples of IBS-D No difference in mucosal samples between IBS-D and HC ↑ *Lactobacillus* spp. in fecal samples of IBS-D *vs* HC No difference in mucosal samples between IBS-D and HC	Feces samples and colonic biopsy	Culture method qPCR (SYBR Green)	[27]
11 IBS (7 F/4 M) Age: 25-64 22 HCs (17 F/5 M) Age: 21-61	II	Reduced biodiversity in IBS subjects Significant differences in profiles between IBS and HC subjects ↓ *B. vulgatus, B. ovatus, B. uniformis*, *Parabacteroides sp.* in IBS *vs* HC	Feces	DGGE on universal and specific primers for *Bacteroides* Sequencing of V3 region of the 16S rRNA genes	[69]
22 IBS (8 F/14 M) 1 IBS-D 13 IBS-C 8 IBS-A Age: 7-12 22 HCs (11 F/11 M)	Pediatric Rome III	No differences in total bacterial load between IBS and HCs Profile differences in IBS subtypes among each other, and between IBS and HCs In IBS: ↑ Proteobacteria ↑ γ-Proteobacteria ↑ *Haemophilus parainfluenzae* ↑ *Veillonella* ↑ *Dorea* ↓ *Eubacterium* ↓ *Anaerovorax* ↓ *Bacteroides vulgatus*	Feces	16S Metagenomics 454 Pyrosequencing (V1-V3 and V3-V5 regions of 16S rRNA) PhyloChip Microarray Hybridization on purified 27F and 1492R regions of 16S rRNA (on 28 IBS and 27 HC)	[35]
62 IBS (57 F/5 M) 25 IBS-D 19 IBS-C 19 IBS-A Age: 22-66 46 HCs (34 F/12 M) Age: 23-58	II	↑ Firmicutes/Bacteroidetes ratio ↑ *Bacillus* ↑ *Steptococcus* ↑ *Dorea* ↑ *Ruminococcus* ↑ *R. gnavus* ↑ *Blautia* ↑ *Clostridium* ↓ *Faecalibacterium* ↓ *Bacteroides* ↓ *B. vulgatus* ↓ *Prevotella* ↓ *Bifidobacterium* ↓ *B. gallicum* ↓ *B. pseudocatenulatum* ↓ *Methanobrevibacter* in IBS *vs* HC, particularly in IBS-C subgroup	Feces	HITChip phylogenetic microarray	[36]
11 IBS (5F/6M) 8 HCs (2 F/6 M) Age: 18-74	II	Greater biological variability of predominant bacteria among IBS subjects *vs* HC and higher microbial diversity (especially Bacteroides and lactobacilli) in IBS *vs* HC In IBS, Exclusive detection of *Eubacterium biforme* (absent in HC) ↑ *Bacteroidetes* ↑ *Lactobacillus* ↓ *Bifidobacterium* ↓ *C. coccoides*	Feces	qPCR (SYBR Green) DGGE on V3-V5 region of 16S rRNA gene qPCR (SYBR Green)	[63]
37 IBS (26 F/11 M) 13 IBS-D 13 IBS-C 13 IBS-A Age: 21.7 ± 2.0 20 HC (15 F/5 M) Age: 21.7 ± 2.0	II	No evident difference in predominant microbiota from profiles of both sample sites between IBS and HC ↑ *P. aeruginosa* in all subgroups if IBS and in both body niche samples	Duodenal brushes and feces	DGGE on V6-V8 region of 16S rRNA gene, generation of 51 clones and sequencing qPCR (Taqman)	[57]

16 IBS-D (11 F/5 M) Age: 23-52 21 HCs (17 F/4 M) Age: 21-60	Ⅲ	Lower biodiversity in IBS-D *vs* HCs in fecal samples, no biodiversity differences in mucosal samples ↓ Clostridiales ↓ Planctomycetaceae	Feces samples and colonic biopsy	T-RFLP	[71] [62]
81 IBS (69 F/27 M) 15 IBS-C Age: 20-73 23 HCs (16 F/7 M) Age: 26-64	Ⅰ and Ⅱ	*Staphylococcus aureus* detected only in IBS subjects, with higher prevalence in IBS-C Enterotoxin-encoding gene of *C. perfringens* detected only in IBS subjects *Helicobacter pylori* detected in 3 IBS subjects, none in HCs	Feces	 qPCR (SYBR Green) Sequencing of *S. aureus* amplicons	
23IBS-D (17 F/6 M) Age: 23-70 23 HCs (18/5 M) Age: 21-58	Ⅲ	Lower microbial richness in IBS-D Structural changes in IBS-D *vs* HC, from phylum to genus ↑ Proteobacteria ↑ γ-proteobacteria ↑ *Enterobacteriales* ↑ *Enterobacteriaceae* ↓ *Faecalibacterium* ↓ *F. prausnitzii*	Feces	454 Pyrosequencing of the V1-V3 and V6 regions of 16S rRNA gene qPCR (SYBR Green)	[54]
37 IBS (26 F/11 M) 15 IBS-D 10 IBS-C 12 IBS-A Age: 37 ± 12 20 HCs (13 F/7 M) Age: 39 ± 9	Ⅱ	Clustering by microbiota composition revealed subgroups of IBS patients: (1) a group (n = 15) with normal-like microbiota composition compared with HCs; and (2) a group (n = 22) with large microbiota-wide changes characterized by an increase of Firmicutes (mainly clostridia/Clostridiales) and a depletion of Bacteroidetes ↓ *Bacteroides* ↓ *Alistipes* ↑ *Lachnospiraceae incertae sedis* ↑ Butyrate-producing *Eubacterium halli* and *desmolans* ↑ *B. adolescentis*	Feces	Pyrosequencing of the V4 region of 16S rRNA gene	[77]
47 IBS 27 IBS-D 20 IBS-C Age average: 34.3 26 HCs Age average: 46.1	Ⅲ	Higher number of mucosa-associated bacteria in IBS ↑ *Bacteroides* ↑ *Eubacterium rectale-C. coccoides* ↓ *Bifidobacterium* in IBS-D than in IBS-C	Rectal biopsies	FISH	[32]
75 rural IBS Age: 4-18 20 Hurban HCs Age: 5-15	Ⅲ	↓ *Enterobacter* ↓ *Enterococcus* ↓ *Lactobacillus* ↓ *Bifidobacterium*	Feces	Culture-based analysis	[29]
22 IBS-D Age: 8-18	Ⅱ	Higher variability among IBS subjects No difference between IBS-D and HCs at phylum level. No difference for *Clostridium* and *Faecalibacterium*	Feces	Microbiota array	[40]
22 HCs Age: 11-18		↑ *Veillonella* ↑ *Prevotella* ↑ *Enterobacter* ↑ *Lactobacillus* ↓ *Bifidobacterium* ↓ *Verrucomicrobium* Difference at species level in the genus *Bacteroides*: ↓ *B. fragilis* ↓ *B. thetaiotaomicron* ↑ *B. ovatus* ↑ *B. salyersiae* Positive abundance correlation between *Veillonella-Haemophilus* and *Streptococcus*; negative for *Ruminococcus* Confermation of data on Clostridia, Bacteroidetes, *Bifidobacterium* Confermation of data on *Bifidobacterium*, *Prevotella*, *Faecalibacterium*		Pyrosequencing (V1-V2-V3 region of 16S rRNA) FISH qPCR	

14 IBS-C (14 F) 12 HCs (14 F) Age: 20-59	II	No differences in total strict and facultative anaerobes between IBS-C and HCs No difference in hydrolytic bacterial communities ↑ Lactate utilizing sulphate-reducing bacteria (SRB) ↓ Lactate non SRB (butyrate-producing) ↑ H_2-utilizing SRB ↓ H_2-utilizing non SRB (acetogenic, methanogens) ↑ *Enterobacteriaceae* ↓ *Bifidobacterium* ↓ *Lactobacillus* ↓ *Bifidobacterium* ↓ *Roseburia-E. rectale*	Feces	Culture-based analysis FISH	[28]
19 IBS 24 HCs Age: 33.6 ± 9.1	III	↑ Bifidobacteriaceae ↑ Lactobacillaceae ↑ *Clostridium* cluster IX ↑ *Eubacterium rectale* ↑ *Enterococcus faecium* ↑ *Clostridium difficile* ↑ *Bacillus cereus* and *B. clausii* ↑ *Campilobacter* spp. ↓ *Bacteroides/Prevotella* ↓ *Veillonella*	Feces	Microbiota Array	[42]
14 IBS-D(3 F/11 M) 18 HCs (7 F/11 M) Age: 18-65	III	↑ *E. coli* ↓ *Clostridium leptum* ↓ *Bifidobacterium*	Feces	qPCR (SYBR Green)	[58]
16 IBS 9 HCs		Reduced microbial diversity in IBS In mucosal samples: ↑ Bacteroidaceae In fecal samples: ↑ Rikenellaceae ↑ Porphyromonadaceae ↓ Ruminococcaceae IBS-D: ↑ *Acinetobacter*, *Leuconostoc*, *Butyricimonas*, *Odoribacter* (fecal) ↓ *Desulfovibrio*, *Oribacterium* (biopsies) IBS-C: ↑ *Alistipes*, *Butyricimonas* (feces) and *Bacteroides* (biopsies) ↓ *Fusobacterium*, *Eubacterium*, *Coprococcus*, *Eubacterium*, *Haemophilus*, *Neisseria*, *Streptococcus*, *Veillonella*	Colonic biopsies and feces	Pyrosequencing (V1-V2 regions of 16S rRNA)	[48]
2 IBS-D 1 HCs Several sampling over 6-8 wk	III	↑ *Alphaproteobacteria* ↑ Facultative anaerobe (Proteobacteria, *Streptococcus*) in days of acute diarrhea	Feces	Pyrosequencing (16S rRNA gene)	[89]

qPCR: Real time quantitative polymerase chain reaction; DGGE: Denaturing gradient gel electrophoresis; T-RFLP: Terminal restriction fragment length polymorphism; FISH: Fluorescence in situ hybridization; Ref.: Reference; IBS: Irritable bowel syndrome; IBS-D: Diarrhea-associated IBS; IBS-C: Constipation-associated IBS; IBS-A: Alternating symptoms IBS; HCs: Healthy controls. ↑: Increased presence in IBS; ↓: Reduced presence in IBS.

which is actually not a suitable selection medium for the isolation of these bacteria from feces.

In addition, Carroll *et al*[27] demonstrated a significant reduction in the concentration of aerobic bacteria in fecal samples from D-IBS patients compared with healthy controls. This result is not consistent with the results obtained by Mättö *et al*[23]. However, to determine the number of aerobes, Mättö *et al*[23] used nutrient agar, which is a particularly poor medium compared with the brain heart infusion agar, containing L-cysteine (0.05%) and hemin, adopted by Carroll *et al*[27]. Thus, the aerobic plate counts obtained from these two studies cannot be compared.

The inconsistencies in bacterial counts reflect the primary intrinsic flaw in culture-based methods: obtaining an appropriate selection medium for all members of a genus (or superior taxa).

The genus *Lactobacillus* is another microbial group often examined in microbiology. Tana *et al*[12] reported an increase in lactobacilli in fecal samples obtained from IBS patients (26 IBS subjects compared with 26 healthy controls). However, Chassard *et al*[28] reported that this same microbial group was reduced in IBS samples using MRS agar medium adjusted to pH 5.5 and incubation in aerobic conditions, whereas Mättö *et al*[23] and Enck *et al*[24] reported that the lactobacilli concentrations were not significantly different using the same selective medium as Tana *et al*[12] (Rogosa agar) for the cultivation of these microbes. Therefore, the use of different culture media cannot explain the contradictory results concerning lactobacilli.

Moreover, culture-based analyses were used to characterize the fecal samples from 75 IBS children and adoles-

cents living in rural areas in chernobyl compared with 20 healthy controls living in urban areas[29]. In this study, the researchers reported a lower abundance of all bacterial groups investigated, *i.e.*, the genera *Enterobacter*, *Enterococcus*, *Lactobacillus*, *Bifidobacterium*, in the IBS group. Thus, the choice of the selection medium profoundly affects the significance of the results obtained from analyses of the microbial ecology of a biological sample. The results of studies based on culture-dependent strategies suggest that changes in bifidobacteria, lactobacilli and the total aerobic count are typically associated with IBS. However, the intrinsic limitations of culture-based techniques, which do not examine a large majority of intestinal microorganisms, severely reduce the significance of these experimental data.

FISH

FISH in microbial ecology involves the detection of whole-microbial cells through the labeling of cellular rRNA using an oligonucleotide probe containing a fluorescent dye at the 5' end[30]. FISH probes, which commonly target 16S rRNA, are designed at various taxonomic levels, facilitating the *in situ* phylogenetic identification and enumeration of individual microbial cells. The FISH technique does not require PCR amplification; therefore, FISH does not have the potential problems associated with the nonspecific amplification of DNA during the PCR reaction.

Limitations: Similar to qPCR, FISH requires the preliminary selection of a target microbial taxonomic group (ribotype); therefore, only a limited number of previously known microbial groups can be analyzed. More importantly, FISH involves a labor-intensive protocol that includes intricate steps, such as the *in situ* acquisition of the target. Consequently, low signal intensity and background fluorescence are common problems.

In FISH experiments, reduced bifidobacteria concentrations have been detected in the fecal samples obtained from 41 IBS patients compared with 26 HCs[31], and in 14 IBS-C subjects compared with 12 HCs[28]. In addition, Parkes *et al*[32] showed reduced bifidobacteria concentrations in IBS-D patients compared with HCs and IBS-C patients.

FISH was also applied for the analysis of ileal and colonic biopsies, revealing a higher number of mucosa-associated bacteria in IBS patients (n = 20) compared with HCs (n = 20)[33]. This same study revealed the prevalence of *Eubacterium rectal* (*E. rectale*)-*Clostridium coccoides* (*C. coccoides*) in IBS. Similarly, higher mucosa-associated bacteria and increased numbers of *E. rectale-C. coccoides* were detected in rectal biopsies from IBS patients (n = 47) compared with those from HCs (n = 26)[32]. Furthermore, FISH analyses revealed increased *Bacteroides*[32] and reduced butyrate-producing bacteria, such as *Roseburia-E. rectale* (belonging to the family Lachnospiraceae), in IBS[28].

DNA microarrays (PhyloChip)

DNA microarray methods are based on the direct hybridization of PCR products amplified from total environmental DNA[34]. Therefore, the PCR amplicons are initially fluorescently labeled, and after hybridization, the signal intensity, which is directly proportional to the abundance of hybridization (*i.e.*, the amount of a specific sequence in the sample), is monitored through confocal laser scanning microscopy. The DNA microarrays used in microbial ecology are commonly based on the analysis of a pool of 16S rRNA gene fragments amplified through PCR from the total environmental DNA (PhyloChip). This technology facilitates the rapid high-throughput analysis of hundreds of microbial species in an environmental sample.

Limitations: Similarly to the binding of a primer to a nonspecific DNA target in PCR, cross hybridization is a major limitation of microarray technology. In addition, only those taxa included in the microarray can be analyzed; therefore, similar to qPCR and FISH, the ecological importance of a taxon that has not been previously selected could be erroneously omitted. Moreover, the results obtained solely through microarray are not considered sufficiently reliable, unless confirmation of these data is provided through other techniques, particularly qPCR.

Saulnier *et al*[35] did not detect a difference in the microbial richness between groups using high-resolution Phylochip Microarray on 28 IBS children and 27 HCs and the majority of taxa in IBS belonged to γ-Proteobacteria, particularly *Haemophylus parainfluenzae*. The results of the Phylochip Microarray analysis also showed the prevalence of the genera *Dorea* and *Veillonella* in IBS, similar to the results obtained for the same samples using 454 Pyrosequencing (see paragraph 2.4.3 for more details). Moreover, IBS children harbored lower levels of *Bacteroides*, including *B. vulgatus*. A previous study based on HITChip Phylogenetic Microarray showed reduced *Bacteroides* spp., including *B. vulgatus*, in IBS patients[36]. In this study, significant differences in the microbiota composition between 62 IBS patients and 46 HCs based on 129 phylotypes were revealed; specifically, IBS subjects presented a higher Firmicutes/Bacteroidetes ratio and increased numbers of *Bacillus*, *Streptococcus*, *Dorea*, *Blautia*, *Clostridium* and *Ruminococcus*. A significant abundance in the phylotype *Ruminococcus gnavus* (*R. gnavus*), including the species *Ruminococcus torques* (*R. torques*) (now reclassified as *Blautia torques*), was also detected. These findings suggested that *R. torques* and *R. gnavus* are potential IBS biomarkers. In addition, other phylotypes related to the genus *Ruminococcus* (*e.g.*, *R. productus*) were increased in IBS. However, IBS patients presented reduced levels of *Faecalibacterium*, *Prevotella* and *Bifidobacterium*, with high significant differences in *B. gallicum* and *B. pseudocatenolatum*. Interestingly, the authors also showed a positive correlation with IBS symptoms, thus confirming the results of previous data[37-39].

To characterize the IμB of young IBS-D patients, Rigsbee *et al*[40] used the Microbiota Array Affymetrix, a platform containing sets of phylogenetic 16S rRNA gene probes, for the detection of 775 bacterial phylotypes in the human IμB[41]. In this study, IBS-D samples contained lower levels of the genus *Bifidobacterium* and higher levels of the genera *Veillonella*, *Prevotella* and *Lactobacillus*. Although there was no difference in the abundance of the complete *Bacteroides* genus between IBS patients and HCs, significant differences were observed for certain species, such as reduced *B. fragilis* and *B. thetaiotaomicron* and increased *B. ovatus* and *B. salyersiae*.

Similarly, Maccaferri *et al*[42] detected higher amounts of Lactobacillaceae in 19 IBS subjects compared with HCs using a fully validated high taxonomic fingerprint microbiota array. In the same study, a higher Bifidobacteriaceae concentration and a lower *Veillonella* concentration were detected in the IBS samples. Notably, the enrichment of several pathobiont bacterial species[43], such as *E. rectale*, *Enterococcus faecium*, *Campilobacter* spp. and *C. difficile*, was also reported in this study.

Culture-independent, PCR-based methods

Most culture-independent methods include PCR for the amplification of a specific DNA region from the total (metagenomic) DNA isolated from an environmental sample (*e.g.*, feces or intestinal biopsies). Although alternative genes are available, nearly all of the molecular methods used in these studies include an analysis of the gene encoding the ribosomal RNA subunit 16S (16S rRNA). The 16S rRNA gene is a conserved region of the bacterial chromosome that has been extensively used in microbial ecology research, as this gene is present in all bacterial genomes and contains both highly conserved and variable regions[22]. The highly conserved sequences, therefore, can be used as target regions for universal oligonucleotide probes (named universal primers) in the PCR amplification of the 16S rRNA gene from virtually all bacteria. Except for FISH, which is based on the direct *in situ* hybridization of an oligonucleotide probe onto rRNA targets, all the molecular methodologies reported here include the initial PCR amplification of the 16S rRNA gene using specific or universal primers. Consequently, all molecular biology protocols described herein inevitably require the extraction of nucleic acids from an environmental sample, which are subsequently used as templates for the characterization of microorganisms.

The protocol employed for DNA extraction affects the results of the downstream reactions. An efficient DNA extraction, producing high-quality genomic DNA, is essential to properly reflect the actual microbial diversity of a complex ecosystem and detect less represented microbial populations[44,45]. In the studies reviewed herein, different DNA extraction protocols have been adopted, including home-made methods[46,47] and commercial kits, such as the QIAamp DNA Stool Mini Kit (Qiagen)[48], the Fast DNAII spin kit (BIO 101)[23], the FastDNA Spin Kit (*QBIO* gene)[49], the ZR Fecal DNA Isolation kit (Zymo Research Corporation)[40], and the AccuPrep Genomic DNA Extraction Kit (Bioneer)[50]. Different kits generate diverse results in terms of DNA yield, purity and integrity, significantly affecting the microbial profiles[51] and differently impacting microbial diversity scores detected on the basis of the downstream techniques employed[52]. Understanding how an extraction protocol affects an analysis is difficult and outside of the scope of this review. However, other studies have addressed this technical issue[53,54].

qPCR: Specific oligonucleotides for the quantification of particular taxa *via* PCR (qPCR method) have been extensively used to overcome the problems of microbial cultivation.

The qPCR technique has clear advantages, such as the high sensitivity (*i.e.*, also limited concentrations of bacteria can be detected). Furthermore, qPCR facilitates the analysis of a large number of samples in a short time. Another important feature of qPCR is the design of primers that potentially target genes at any taxonomic level; thus, the identification of unique genetic signatures also facilitates quantification at the strain level, which is important when analyzing particular microbial behaviors, such as the fate of a probiotic strain in the gastrointestinal tract[50,55].

Limitations: However, the specificity of primers, particularly those targeting conserved ubiquitous genes, such as 16S rRNA, significantly varies depending on the experimental conditions of the assay. In other words, the protocol for a pair of primers targeting a specific group of microorganisms could lose specificity when using a different thermocycler[56] because even small changes in the reaction conditions could lead to the amplification of the genes from related taxa. Specificity problems can be drastically reduced using TaqMan fluorophore-quencher probes. However, with only two exceptions[31,57], the studies considered in this review exclusively used intercalating fluorescent dyes, such as SYBR Green, to measure the accumulation of amplicons in real time during each PCR cycle for the analysis of the IμB in IBS. The main limitation of qPCR is that this technique can only analyze one microbial group per reaction. Furthermore, the microbial groups are selected in advance, thereby limiting the potential identification of microbial groups that were not initially considered but might play an important role.

We selected 13 manuscripts published in the last 10 years that employed qPCR to characterize the IμB associated with IBS. Malinen *et al*[16] considered 20 different microbial groups ranging from the species and genus levels to supra-generic groups. This study showed several significant differences among IBS and HCs. Particularly, these authors showed a higher concentration of *Ruminococcus productus*/*C. coccoides* in IBS patients (n = 27) than in the controls (n = 26). Several other differences were exclusively observed for diarrhea-predominant IBS patients (IBS-D, n = 12), including a reduced concentration of

Lactobacillus spp., compared with IBS-C subjects (n = 9), and diminished *Bifidobacterium* spp. and *Desulfuvibrio* ssp., compared with controls and IBS-A subjects. Moreover, the Clostridiales genus *Veillonella* was more abundantly represented in IBS-C patients than in controls[16]. The qPCR analysis also showed a significant increase in the *Veillonella* spp. concentration in 26 young IBS patients (Age: 21.7 ± 2.0; 8 IBS-D, 11 IBS-C, 7 IBS-A) compared with age-matched HCs (n = 26)[12]. A significant decrease of bifidobacteria in diarrhea-predominant IBS patients was also observed in other studies using qPCR (22 IBS-D *vs* 22 HCs[40]; 14 IBS-D *vs* 18 HCs[58]). In another study, qPCR with Taqman technology was used to detect differences in the abundance of four different *Bifidobacterium* species in adult IBS patients (n = 19) and age-matched HCs (n = 19)[31]. These analyses revealed a significant reduction in the abundance of *B. catenulatum* in fecal specimens and duodenal mucosa brush samples obtained from IBS subjects. Although differences among the bifidobacterial species have been shown[59,60], the study of Kerckhoffs *et al*[57] is one of the very few that investigated bifidobacteria at intra-genus level in IBS (another example is[36]). Bifidobacteria are frequently analyzed in qPCR experiments, as these microbes are univocally recognized as health-promoting bacteria[61]. Thus, the available data obtained from bifidobacterial research, and reported herein, support the idea that a reduction of bifidobacteria is associated with IBS.

Interestingly, based on a previous study[37], Lyra *et al*[46] used qPCR to quantify 14 phylotypes in the fecal samples obtained from 20 IBS patients (8 IBS-D, 8 IBS-C, 4 IBS-A) and 15 healthy controls. Specifically, the abundance of several phylotypes, including the Clostridiales genera *Clostridium* and *Ruminococcus*, significantly differed among these subjects (Table 1). Moreover, in this study, the authors proposed *C. thermosuccinogenes* and *R. torques*-like phylotypes as potential biomarkers for IBS[38].

Rinttilä *et al*[62] used qPCR on samples obtained from IBS subjects (81 patients) to detect the presence of pathogens, such as *S. aureus* (with higher prevalence in IBS-C), *C. perfringens* and *H. pylori*, which were not identified in any of the control subjects (23 HCs).

Lactobacilli have often been included in qPCR analyses for the characterization of the IμB associated with IBS. In contrast to data concerning bifidobacteria, studies concerning lactobacilli have generated less convincing results, as previously shown for the culture-dependent studies described above. Malinen *et al*[16] reported reduced concentrations of *Lactobacillus* spp. in IBS-D patients (n = 12) compared with IBS-C patients (n = 9) but no differences were observed when compared with HCs (n = 22). In contrast, more recent studies have shown that lactobacilli were increased in the fecal samples of IBS-D patients (n = 10[27]) and IBS (n = 11[63]) compared with HCs (n = 10 and 8, respectively). Notably, in these studies, the same qPCR chemistry (SYBR Green) and primers[64] were used for the quantification of lactobacilli. Therefore, the observed differences might more accurately reflect actual differences in microbiota composition rather than methodological biases. The limited number of recruited subjects should also be considered to analyze these results.

Most studies have exclusively considered microbial groups belonging to the Bacteria superkingdom (also called "Eubacteria"). Experiments based on qPCR, however, have also revealed potential differences in the IμB associated with IBS in Archeabacteria. For instance, the reduced abundance of the genus *Methanobrevibacter* was reported in IBS subjects[36], particularly the IBS-C subgroup, consistent with the results of a previous study[65].

DGGE/T-RFLP: DGGE and T-RFLP are molecular techniques that produce an electrophoretic profile of microbial communities. Specifically, in DGGE, PCR products are obtained from environmental DNA using primers for a specific molecular marker (most commonly the 16S rRNA gene) and subsequently electrophoresed on a polyacrylamide gel under denaturing conditions using a chemical denaturant (*e.g.*, urea and formamide[66,67]).

In T-RFLP, the DNA fragments are obtained through PCR using a fluorescently labeled primer, followed by digestion with one or more restriction enzymes, and separated on an automated DNA sequencer[68] that only detects terminal fluorescently labeled restriction fragments, thereby simplifying the banding pattern and facilitating the analysis of complex microbial communities.

DGGE and TGGE are rapid and inexpensive techniques. These methods facilitate the simultaneous analysis and comparison of multiple samples. Different from qPCR, DGGE and TGGE facilitate the examination of different microbial groups in the same analysis.

Limitations: DGGE and T-RFLP are based on the PCR amplification of a specific genetic target; therefore, these methods have the same limitations concerning primer specificity as described for qPCR. Furthermore, DGGE does not provide direct taxonomic identification and involves the separation of DNA bands (excision from electrophoretic gel), cloning and sequencing. The separation of all DNA amplicons, however, is practically impossible because the PCR amplification of a target gene, such like the 16S rRNA gene from DNA isolated from an environmental sample, such as human feces, generates numerous DNA fragments. Consequently, only the most represented amplicons can be visualized in electrophoresis, and several DNA fragments might have similar melting points. Finally, the abundance of a specific microbial group can be exclusively estimated on the basis of the band intensity in electrophoresis. Thus, only those microbial groups represented with dominant bands in electrophoresis and showing markedly different abundance between the two conditions investigated can be identified as significant in DGGE. In T-RFLP, the separation of DNA amplicons through the amplification of the 16S rRNA gene is facilitated using an automated DNA sequencer; however, no more than approximately 100 fragments can be resolved per analysis, and more

importantly, the taxonomic identification and quantification of the detected ribotypes can be deeply distorted by the fact that different bacterial species can share the same terminal restriction fragment length.

Concerning the characterization of the IμB associated with IBS using DGGE, an increase in *Clostridium* spp. and *Eubacterium* spp. and a decrease *Parabacteroides* spp. and several *Bacteroides* species in IBS samples was reported[69]. Furthermore, Kerckhoffs *et al*[57] showed the augmented presence of *Pseudomonas* spp. in duodenal mucosal brush and fecal samples from 37 IBS patients compared to 20 healthy subjects. Subsequent qPCR experiments confirmed the increased abundance of *Pseudomonas aeruginosa* in the same samples. In addition, DGGE technique displayed reduced biodiversity in IBS subjects, consistent with the results obtained by Noor *et al*[69]. In contrast, a Korean study showed that IBS subjects (n = 11) had a significantly higher diversity of total bacteria than HCs (n = 8)[64]. Maukonen *et al*[49] and Kerckhoffs *et al*[57] detected no significant differences in the microbiota variability between IBS patients and HCs. However, Codling *et al*[70] showed higher variability in HC subjects compared with IBS patients. The results of the DGGE analysis concerning microbial biodiversity in IBS are contradictory. In these studies, however, the general biodiversity was calculated according to the numbers and relative intensities of the bands detected among individual samples. Thus, this analysis has intrinsic technical limitations. Indeed, many taxa could be present at low levels and could be therefore only marginally amplified, generating bands that cannot be easily visualized on the electrophoretic gel. Therefore, DDGE profiles are not adequate for the determination of the biodiversity of a complex microbial ecosystem. Thus, the use of primers for the amplification of a specific group of bacteria (*e.g.*, genus-specific primers), generating a reduced number of taxa, could improve the significance of the evaluation of microbial diversity using DGGE. Indeed, Ponnusamy *et al*[63] used group-specific and detected the increased diversity of Bacteroidetes and lactobacilli and the decreased diversity of bifidobacteria and *C. coccoides* in IBS samples.

T-RFLP fingerprinting of the bacterial 16S rRNA gene was used to analyze the microbiota in fecal and mucosal samples from 16 IBS-D patients and 21 HCs, revealing lower biodiversity and the reduced abundance of Gram-positive Clostridiales and Gram-negative Planctomycetaceae in the IBS-D fecal samples[71]. These data are partially inconsistent with the results of the studies cited above, which showed an increase in certain taxa belonging to Clostridiales in IBS using qPCR. This inconsistency might reflect the fact that T-RFLP potentially included all taxa belonging to the Clostridiales, whereas qPCR analyses only quantified selected genera. Furthermore, the intrinsic limitations of T-RFLP fingerprinting distort the results.

16S rRNA gene library (clone library method): The preparation of a clone library containing microbial DNA fragments derived from an environmental sample is the "gold standard" for microbial community analyses. The most widely used methods include the PCR amplification of the 16S rRNA genes from an environmental sample, followed by cloning and sequencing of the individual DNA fragments[72]. The obtained sequences are subsequently compared with known sequences database, such as GenBank or the Ribosomal Database Project. For the data analysis, each clone sequence is assigned to a taxonomic lineage according to sequence similarity cut-off values (*e.g.*, cut-off values of 80%, 85%, 90%, 92%, 94%, and 97% for phylum, class, order, family, subfamily, and species, respectively)[72].

16S rRNA clone libraries facilitate the initial survey of the microbial diversity in an environmental sample, and differently from the methodologies described above, these libraries contribute to the identification of novel taxa.

Limitations: Environments characterized by complex microbial ecosystems, such as soil or feces, might require more than 40000 clones to document 50% of the richness[73]. However, until recently, 16S rRNA clone libraries rarely contained numbers of sequences of this magnitude. Therefore, these studies only revealed a small portion of the microbial biodiversity present in an environmental sample. This problem directly reflects the fact that the clone library method was, until recently, a time-consuming, labor-intensive and particularly expensive microbial ecology strategy.

Consistent with the limitations described above, the quality of the first studies employing clone libraries to characterize the IμB in IBS was drastically affected by the limited number of sequenced clones. Indeed, Mättö *et al*[23] sequenced the partial 16S rRNA gene from only 45 amplicons (29 amplicons from 5 IBS patients and 16 amplicons from 4 HCs), revealing the increased prevalence of *Clostridium* spp. and reduced prevalence of *Eubacterium* in IBS patients. Kerckhoffs *et al*[57] also evaluated a limited number of clones (n = 51) and did not detect significant differences between in the microbiota composition of both duodenal biopsies and fecal samples from IBS patients and HCs, except for an increase of *Pseudomonas aeruginosa* in IBS.

Kassinen *et al*[37] made an important contribution to the field of microbial ecology in IBS through 16S rRNA cloning and sequencing using a conventional sequencer (ABI PRISM® BigDye™ Terminator Cycle Sequencing, Applied Biosystems), generating 3753 sequences from the analysis of the fecal samples obtained from 24 IBS patients (10 IBS-D, 8 IBS-and 6 IBS-A patients) and 23 HCs. This study overcame the intrinsic problem inherent in most experimental approaches using PCR with universal primers, such as the 16S rRNA amplification, for the preparation of a clone library. Indeed, biases in favor targets with low guanine and cytosine (%GC) contents are observed in PCR amplification from a pool of 16 rRNA gene targets containing different se-

quences[74]. Therefore, the numbers of bacteria characterized by higher %GC in the 16S rRNA gene, such as bifidobacteria, might be underestimated. To overcome this problem, Kassinen *et al*[37] used cesium chloride gradient centrifugation to separate the genomic DNA from IBS and HC samples into three fractions based on %GC: fraction 7 (with a %GC between 25% and 30%), fraction 10 (%GC: 40%-45%), and fraction 13 (%GC: 55%-60%). Using this strategy, significant differences in the microbiota composition were detected among different IBS subcategories. In fraction 7, the members of the genus *Lactobacillus* were reduced in all IBS subgroups, whereas the *Ruminococcus* was higher in IBS-C and IBS-A patients, and *Streptococcus* was higher in IBS-D patients. Furthermore, in fraction 13, the high %GC bacterium *Collinsella*, phylum Actinobacteria (similar to bifidobacteria), was less abundant in IBS-C and IBS-D patients. This research group used a similar strategy to separate the genomic DNA obtained from 10 IBS-D subjects into 7 fractions based on %GC[47]. The sequences of 3267 clones were subsequently compared with an analogous HC library of 23 subjects, revealing an increase in Proteobacteria and *Firmicutes* (in particular, the family *Lachnospiraceae*) and a decrease in *Actinobacteria* and *Bacteroidetes* in IBS-D patients; decreased diversity in IBS-D was also observed.

Despite these efforts, studies based on the use of the clone library method have not completely overcome the problem of the limited bacterial diversity observed in intestinal samples, as only a limited number of clone sequences are observed. Thus, next-generation DNA sequencing technologies, such as the pyrosequencing, have made significant advancements.

Pyrosequencing: Pyrosequencing is a sequencing strategy based on the production of light from luciferase for the detection of individual nucleotides added to the nascent DNA; the resulting data are subsequently used to generate sequence read-outs. The rapid technological development of this strategy facilitates massive parallel high-throughput sequencing, which is applied to microbial ecology to sequence the hypervariable regions of 16S rRNA genes in large numbers. The use of pyrosequencing technology generates at least 100 times higher coverage of microbial diversity in a sample compared with typical Sanger sequencing. With this technology, the sequences of the hypervariable regions are generally short (100-350 bases) but provide sufficient phylogenetic information to determine the taxonomic level of genus.

In recent years, 454 Pyrosequencing has been used to study the microbial ecology of IBS. Carroll *et al*[75] used this technology to characterize the fecal DNA isolated from 23 IBS-D patients and 23 HCs. To this aim, the variable regions V1-V3 (an average of 8232 reads per sample) and V6 (an average of 6591 reads per sample) of the 16S rRNA gene were sequenced, revealing less microbial richness and a higher presence of the phylum Proteobacteria (particularly the class γ-Proteobacteria and the family Enterobacteriaceae) in the IBS-D population. Furthermore, the genus *Faecalibacterium* was less abundant in IBS-D samples, consistent with a significant reduction of the anti-inflammatory species *Faecalibacterium prausnitzii*[76], determined through qPCR. Saulnier *et al*[35] obtained analogous results concerning increased γ-Proteobacteria[35]. In this study, the 16S rRNA gene fragments from the fecal samples of 22 pediatric IBS patients and 22 HCs were sequenced through pyrosequencing, generating an average of 54287 reads per sample. The data analysis showed an abundance of γ-Proteobacteria and particularly, the species *Haemophilus parainfluenzae*. In addition, the Firmicutes genera *Dorea* and *Veillonella* were significantly represented in IBS patients. Similarly, Rigsbee *et al*[40] showed that the genus *Veillonella* was increased in pediatric IBS-D patients.

Moreover, Durbán *et al*[48] used pyrosequencing to study the microbiota population in feces and colon mucosa samples obtained from 16 IBS patients and 9 HCs. In this study, DNA was extracted from three types of samples per subject: biopsies of the ascending and the descending colon mucosa, and feces. Prior to pyrosequencing, the 16S rRNA genes were amplified from the extracted DNA, and equal amounts of the PCR products from different samples were pooled. The analysis of approximately 268000 reads showed reduced microbial diversity in the IBS samples and significant differences in the representation of several microbial taxa between IBS patients and HCs. Particularly, the families *Rikenellaceae* and *Porphyromonadaceae* were increased and *Ruminococcaceae* spp. were decreased in the fecal samples of IBS subjects. Furthermore, the family *Bacteroidaceae* was more abundant in mucosal samples. Several other taxa were diversely represented in IBS-D and IBS-C samples compared with HCs. This study, therefore, indicated several potential microbial signatures for IBS and IBS subtypes. However, these results were based on a limited number of sequence reads per subject (approximately 3500).

CONCLUSION

Intestinal microbiota plays a role in the pathogenesis of IBS and is not merely a consequence of the disorder[77]. A number of factors profoundly influence the identification of specific microbial modifications etiologically associated with IBS: The etiology of this disorder is heterogeneous and might profoundly vary among individuals. There is great variability among different subgroups of IBS (diarrhea, constipation-predominant and alternating IBS). The technologies adopted to characterize the IμB have intrinsic pitfalls associated with particular biases.

Despite these limitations, the analytical revision of the studies referenced in the present review resulted in the identification of microbial groups whose relative abundance, consistent with different studies using diverse methodological approaches, significantly altered IBS. These results suggest that the following microbial groups are potential IμB signatures of IBS, as briefly summarized below.

Bifidobacterium

Lower levels of members of the genus *Bifidobacterium*

have predominantly been identified in studies on IμB in IBS. Indeed, almost all of the studies analyzed in the present review (with only one exception[42]) suggest that bifidobacteria are underrepresented in IBS, particularly in the diarrhea-predominant type. Interestingly, most probiotic preparations shown as effective in managing IBS symptoms contain bifidobacteria (particularly, the species *B. animalis* subsp. *lactis*, *B. bifidum*, *B. breve* and *B. longum* subsp. *B. infantis*)[17,78], suggesting a preventing role for these microorganisms in IBS.

A mechanism underlying the beneficial role of bifidobacteria in IBS might depend on the presence of serine protease inhibitors (SERPINs) in these bacteria[79]. Indeed, supernatants obtained from IBS biopsy samples have high levels of these proteases (derived from the host or potentially produced by certain members of the phylum Firmicutes[80]). Such proteases have been implicated in the observed over-stimulation of sub-mucosal neurons in IBS subjects[81]. Therefore, the SERPINs from bifidobacteria might act on extra-cellular proteases to suppress the activity of these enzymes.

Veillonella

Different studies have shown an increase in the Firmicutes genus *Veillonella* in IBS patients[16,12,35,40] using different techniques (qPCR, Pyrosequencing and Microbiota Array Hybridization). Particularly, Tana *et al*[12] showed higher levels of *Veillonella* in IBS-C patients and demonstrated a correlation with severity of pain and increased levels of acetate and propionate in the feces of subjects. Interestingly, it has been demonstrated that *Veillonella* is abundant in jejunal samples of IBS patients and this bacteria might be involved in small-intestine bacterial overgrowth (SIBO)[82]. SIBO is defined as a malabsorption syndrome resulting from the presence of abnormal bacterial load in the small intestine (greater than 10^5 CFU per mL of intestinal aspirate and/or colonic-type species). Several studies have reported the prevalence of SIBO in IBS patients, although conflicting data have also been reported[83-85].

Furthermore, Rigsbee *et al*[40] showed a positive correlation among *Veillonella*, *Haemophilus* and *Streptococcus*, suggesting that *Veillonella* forms co-aggregation complexes with other bacteria present in the small intestine, such as *Streptococcus* and *Haemophilus*[12,86,87]. Higher proportions of *Haemophilus* and *Veillonella* have also been observed in microbiomes associated with esophagitis[88]. Thus, these data suggest that *Veillonella* might play a role in the onset of gastro-intestinal disorders, such as IBS.

γ-Proteobacteria

The studies described in the present review have presented non-controversial data concerning the increased prevalence of members the phylum Proteobacteria in IBS subjects[35,47,54,89]. Some studies have reported a significant increase in the abundance of the class γ-Proteobacteria in IBS[35]. Notably, *Haemophilus* was most represented among γ-Proteobacteria, and *Haemophilus parainfluenzae* was the predominant species.

The class γ-Proteobacteria comprises several families that include pathogenic bacteria (*e.g.*, Enterobacteriaceae, Legionellaceae, Aeromonadaceae, Vibrionaceae). Particularly, Enterobacteriaceae were increased in IBS[54]. Thus, it is likely that these bacteria are among those (potential) pathogens (also known as pathobionts) that contribute to the onset and maintenance of IBS.

Clostridiales/Blautia

Clostridiales is a wide and heterogenic Firmicutes order that includes several bacterial groups differently represented in IBS. Clostridiales also include the family Lachnospiraceae, a group of microorganisms that normally occur in the gut of humans and animals. This family comprises the genus *Blautia*, which comprises several misclassified species belonging to the *Clostridium* cluster XIVa, including *C. coccoides* and several *Ruminococcus* species related to *R. gnavus* (that also include *R. torques*)[90]. In several studies described herein, the increased presence of these bacteria has been demonstrated in IBS patients[16,32,33,36,46].

Clostridia abundantly colonize mucin[91], and it was proposed that an increase in these bacteria might reflect the increased production of rectal mucus in both IBS-C and IBS-D patients[92]. Particularly, *Clostridium* cluster XIVa has previously been associated with IBS[93]. More specifically, Jeffery *et al*[77] showed that the butyrate-producing clostridia of cluster XIVa are associated with IBS. Butyrate has been shown to cause visceral hypersensitivity[94]; thus, it is likely that an increase in butyrate-producing bacteria might promote sensory dysfunctions typical of IBS[77].

Faecalibacterium

Reduced levels of *Faecalibacterium* spp. has been shown in two studies reported in this review. Rajilić-Stojanović *et al*[36] showed that *Faecalibacterium* was the only microbial group within the phylum Firmicutes that was significantly underrepresented in both IBS-C and IBS-A subjects. Interestingly, *Faecalibacterium prausnitzii* possess anti-inflammatory properties[77], suggesting that the presence of this bacterium might modulate inflammatory conditions associated with IBS.

The available experimental data indicate modifications in the IBS IμB composition at the phylum level. Specifically, a general increase in *Firmicutes* and Proteobacteria with a concomitant reduction of *Bacteroidetes* and *Actinobacteria* has been associated with IBS.

Concluding remarks

The progress in DNA sequencing technologies offers promise to microbial ecology studies, facilitating the adequate detection and quantification of less represented microorganisms within the large microbial biodiversity in the intestinal ecosystem. Thus, sufficient research studies for the investigation of the IμB should include the following basic elements: New generation DNA sequencing technologies, such as 454 Pyrosequencing and Ion Tor-

rent[95], to obtain a high number of reads to satisfy the biodiversity requirements specified through rarefaction curves. Confirmation of the results using other methods, preferentially qPCR. An investigation of the microbiota components other than eubacteria, such as archaebacteria, fungi, yeasts and viruses.

The identification of microbial biomarkers in the IμB will contribute to the development of new diagnostic tools and novel therapeutic strategies for the treatment of different subtypes of IBS.

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