

Review

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Gastrointestinal microbiota in irritable bowel syndrome: present state and perspectives

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Irritable bowel syndrome (IBS) is a functional gastrointestinal disorder that has been associated with aberrant microbiota. This review focuses on the recent molecular insights generated by analysing the intestinal microbiota in subjects suffering from IBS. Special emphasis is given to studies that compare and contrast the microbiota of healthy subjects with that of IBS patients classified into different subgroups based on their predominant bowel pattern as defined by the Rome criteria. The current data available from a limited number of patients do not reveal pronounced and reproducible IBS-related deviations of entire phylogenetic or functional microbial groups, but rather support the concept that IBS patients have alterations in the proportions of commensals with interrelated changes in the metabolic output and overall microbial ecology. The lack of apparent similarities in the taxonomy of microbiota in IBS patients may partially arise from the fact that the applied molecular methods, the nature and location of IBS subjects, and the statistical power of the studies have varied considerably. Most recent advances, especially the finding that several uncharacterized phylotypes show non-random segregation between healthy and IBS subjects, indicate the possibility of discovering bacteria specific for IBS. Moreover, tools are being developed for the functional analysis of the relationship between the intestinal microbiota and IBS. These approaches may be instrumental in the evaluation of the ecological dysbiosis hypothesis in the gut ecosystem. Finally, we discuss the future outlook for research avenues and candidate microbial biomarkers that may eventually be used in IBS diagnosis.

Introduction

We are colonized from birth by a complex microbiota that affects health and disease. By far the greater part of this microbiota resides in the gastrointestinal (GI) tract, and can be considered as an organ within an organ (Bocci, 1992). As many of the GI tract microbes have not yet been cultured and are only recognized based on their 16S rDNA sequences, molecular high-throughput approaches have been developed to study the diversity and functionality of the thousands of phylotypes that have been predicted to be present in the GI tract (for a full review, see Zoetendal *et al.*, 2008). These studies have revealed that the composition of the microbiota in healthy adults is highly subject-specific and stable, indicating an individual core. Comparative microbiota analyses between multiple healthy subjects indicate that a limited proportion of phylotypes are shared between individuals, forming a common core microbiota that is assumed to be functionally redundant (Qin *et al.*, 2010; Tap *et al.*, 2009; Turnbaugh *et al.*, 2009). Recent metagenomic sequence analysis of the GI tract microbiota has confirmed this and defined a reference set of over three million unique genes that vastly exceeds the

coding capacity of the human genome (Qin *et al.*, 2010). Insight into the microbiota of healthy subjects allows comparisons with that of compromised subjects. In the case of inflammatory bowel diseases (IBDs), such an approach has uncovered significant differences in overall diversity as well as in specific bacteria (Qin *et al.*, 2010; Sokol *et al.*, 2009).

Irritable bowel syndrome (IBS) is a very common disorder with a worldwide prevalence of 10–20% (Longstreth *et al.*, 2006). Even though IBS does not predispose patients to severe illness, it profoundly affects the quality of life of its sufferers and incurs significant economic costs due to the need for medical consultations and work absenteeism. The symptoms of IBS vary with the individual affected, and include abdominal pain or discomfort, irregular bowel movements, flatulence, and constipation or diarrhoea. According to the Rome II criteria, IBS sufferers can be grouped into three symptom subtypes based on the stool form, stool frequency and defaecatory symptoms: diarrhoea predominant (IBS-D), constipation predominant (IBS-C), and mixed subtype (IBS-M) with alternating episodes of both diarrhoea and constipation (Thompson

et al., 1999; Drossman, 2000). More recently, the Rome III criteria, which focus on the stool form over the defaecation frequency, have been issued (Longstreth *et al.*, 2006). The aetiology and pathophysiology of IBS are complex and not well-described. The most important aberrations include visceral hypersensitivity, abnormal gut motility and autonomous nervous system dysfunction, the interactions of which are suggested to make the bowel function susceptible to a number of exogenous and endogenous factors, such as the GI microbiota, diet and psychosocial factors (recently reviewed by Karantanos *et al.*, 2010). In addition, the presence of low-level inflammation in the GI mucosa of IBS patients has also been observed in several studies, including those reported by Aerssens *et al.* (2008), Chadwick *et al.* (2002) and Macsharry *et al.* (2008).

Current conceptions of the intestinal microbiota in IBS

This review focuses on recent GI microbiota research in IBS patients, with a special emphasis on mining the subtype-specific findings obtained using culture-independent methods, as the initial culture-based studies had various limitations and have already been thoroughly reviewed in the existing literature (Lee & Tack, 2010; Parkes *et al.*, 2008; Quigley, 2009; Ringel & Carroll, 2009). Evidence for gut microbes playing a role in the pathogenesis of IBS is convincing and is supported by three main lines of reasoning. (1) A cause and effect relationship has been documented between the GI microbiota and a specific form of IBS, post-infectious IBS (PI-IBS). Due to the lack of any published studies addressing the microbiota in PI-IBS patients, this IBS subtype is not discussed here but is reviewed elsewhere (Spiller & Garsed, 2009). (2) The GI microbiota is altered in IBS patients, as will be discussed in detail below. (3) IBS symptoms can be improved by treatments that target the microbiota (antibiotics, probiotics, prebiotics). This aspect has also been recently reviewed (Moayyedi *et al.*, 2010; Parkes *et al.*, 2010; Pimentel & Lezcano, 2007) and will not be further discussed in this review.

The differences in the intestinal microbiota between IBS patients and healthy controls (HCs) have mostly been studied using faecal material, as this is the most accessible source of the GI microbiota. For clarity and easier comparability of the results, we first discuss the results in which IBS patients were not subdivided according to Rome II criteria but were compared as a single group with the HCs. The lack of segregation occurred either because the subtypes were not specified during the recruitment of subjects or because all IBS samples were intentionally analysed as one group to get an overall picture and to avoid problems of statistical power. The temporal variation of the GI microbiota in IBS subjects was addressed in 2005 in IBS patients by denaturing gradient gel electrophoresis (DGGE) complemented with sequencing of 45 partial 16S rDNA amplicons (Mättö *et al.*, 2005). The results suggested

temporal instability in IBS subjects, although this was based on qualitative inspection of the profiles and the patients included many that were using antibiotics. Moreover, a trend was noted whereby some *Clostridium* spp. were increased and *Eubacterium* spp. decreased in the IBS patients. In addressing the degree of inter-individual variation of the gut microbiota in IBS, a recent DGGE-based study reported a highly significant loss of variation in IBS patients (Codling *et al.*, 2010). In contrast, another DGGE study, as well as comprehensive microbiota profiling with the Human Intestinal Tract Chip (HITChip), suggested that the microbiota of IBS subjects is more heterogeneous than that of HCs (Rajilić-Stojanović, 2007). In conclusion, both an increase and a decrease of variation have been proposed to characterize the GI microbiota in IBS. From an ecological perspective, the abnormal variation likely reflects a loss of homeostasis, in which the community is unable to maintain its normal structure. A high degree of variation is typical for disturbed and re-establishing communities undergoing succession, while the loss of variation is usually associated with a loss of diversity and outgrowth of certain taxa.

Microbiota in different IBS subtypes

The statistically significant microbiota differences from peer-reviewed molecular studies that have analysed IBS subtypes separately are summarized in Table 1. Of the eight studies listed in Table 1, five analysed samples from the same Finnish IBS cohort with complementary methodologies: qualitative microbiota profiling (Maukonen *et al.*, 2006; Mättö *et al.*, 2005) was followed by targeted analyses using quantitative PCR (qPCR) assays that were specific to dominant genera and species (Malinen *et al.*, 2005) and phylotypes with a putative association with IBS (Lyra *et al.*, 2009). To obtain a holistic assessment of the entire microbiota, 16S rDNA sequencing of clone libraries was performed on the selected fractions of all IBS subtypes (Kassinen *et al.*, 2007) as well as on the entire GI microbiota of IBS-D patients and HCs (Krogius-Kurikka *et al.*, 2009).

An important basis for this field was provided by the first study that addressed the putative quantitative microbiota differences in IBS patients by the application of qPCR assays that covered the predominant bacteria as well as taxa with a potential association with IBS based on culturing (Malinen *et al.*, 2005). To obtain an overall impression of the GI microbiota in the patients, all IBS samples were combined, and were found to contain fewer *Clostridium* *coccoides* and *Bifidobacterium* *catenulatum* groups than the HCs. Upon comparison of the three IBS subgroups with the HCs, lactobacilli were significantly decreased in the IBS-D compared with the IBS-C patients, who also had significantly more *Veillonella* spp. than the HCs. Other group-level assays did not show significant differences between the subject groups; at the species level, *Ruminococcus* *productus*-*C. coccoides* was significantly increased in all IBS

subtypes compared with the HCs. A single IBS subject harboured *Campylobacter jejuni*, while no other indications of intestinal pathogens previously linked to IBS were found (Malinen *et al.*, 2005).

The same IBS subjects that had previously shown temporal instability (Mättö *et al.*, 2005) were readdressed by excluding those patients who had recently received antibiotics (Maukonen *et al.*, 2006). The temporal variation of the GI microbiota was not higher in IBS patients according to the DNA-based DGGE profiles, while faecal RNA amplicons were more stable in the HCs. The study focused on the dominant clostridial populations due to their possible link to gas-related problems in IBS, and detected a relative decrease of *Clostridium* cluster XIVa in IBS-C and IBS-A samples, in line with the qPCR results of Malinen *et al.* (2005).

The first study to apply extensive 16S rDNA gene cloning and sequencing to compare the faecal microbiota of IBS patients with that of HCs was published in 2007 (Kassinen *et al.*, 2007). In that study, faecal community DNA was pooled within IBS subgroups and the HCs in order to focus on the IBS status of subjects rather than on individual variation. The community DNA was fractionated according to the %G+C content to facilitate the recovery of less abundant species and of sequences with a high G+C content. The three most variable %G+C fractions between the groups, representing bacterial genomes with %G+C values of 25–30, 40–45 and 55–60, were then subjected to cloning and sequencing. Within-fraction comparison of the sequences showed that the libraries derived from the three IBS subtypes and HCs were clearly distinguishable in Bayesian population structure analysis, and multiple statistically significant differences were found at the genus level (Table 1; Kassinen *et al.*, 2007). In brief, IBS-D patients were depleted in several Firmicutes and *Bacteroides* spp. in the middle %G+C region, while IBS-C patients had elevated amounts of many of the lower G+C content Firmicutes. Actinobacteria in the highest %G+C fraction were depleted in all IBS subtypes compared with the HCs. Finally, to complement and verify the sequencing data from the pooled samples, individual samples were analysed using a set of qPCR assays designed to target the phylotypes that differed most between the libraries. *Collinsella aerofaciens*, an abundant actinobacterium, as well as two uncharacterized Firmicutes, *Clostridium cocleatum*-related and *Coproccoccus eutactus*-related, were significantly decreased in all IBS subtypes compared with the HCs. Essentially similar differences were obtained when samples from around 30 additional IBS subjects were analysed, suggesting a true negative association between these phylotypes and IBS (Kassinen *et al.*, 2007).

The same diarrhoea-predominant IBS patients were further analysed by extending the analysis coverage from the selected %G+C regions, which comprised one-third of the total community DNA, to the entire microbiota. Comparison of the 16S rDNA clone libraries between the IBS-D

group and HCs pointed out several significant differences (Table 1; Krogius-Kurikka *et al.*, 2009), confirming and extending the findings of Kassinen *et al.* (2007). In brief, *Lachnospira* and family- or genus-level representatives of the gammaproteobacteria and bacilli were enriched in IBS-D, while *Bacteroides* and *Ruminococcus* spp., as well as three genera assigned to the Actinobacteria, were depleted. Interestingly, these taxa also stood out from clone libraries derived from IBD patients (Frank *et al.*, 2007), and from an individual suffering from *Clostridium difficile*-associated disease (CDAD) with severe diarrhoea and colitis (Khoruts *et al.*, 2010). IBD, CDAD and IBS-D patients were all significantly depleted in sequences affiliated to Bacteroidetes and had a concomitant enrichment of other commensals. More specifically, proteobacteria and bacilli were enriched in IBS-D and IBD samples, while streptococci, Erysipelotrichi and *Lachnospiraceae* Incertae Sedis were increased in IBS-D and CDAD patients. The Erysipelotrichi are a Firmicute class containing only a few named species, including the long-known animal and human pathogen *Erysipelothrix rhusiopathiae*. Recently, the increase of Erysipelotrichi has been associated with obesity (Zhang *et al.*, 2009) as well as high fat intake (Fleissner *et al.*, 2010). Another emerging taxon of interest is the abundant and typically butyrate-producing family *Lachnospiraceae*, and especially the genus *Lachnospiraceae* Incertae Sedis, which has been shown to be significantly enriched in IBS-D and CDAD but depleted in IBD patients (Frank *et al.*, 2007). Representatives of the *Lachnospiraceae* express highly antigenic flagellins (Duck *et al.*, 2007), which have been recognized as triggers of elevated immune reactivity in Crohn's disease patients and a subset of IBS patients (Schoepfer *et al.*, 2008). In summary, these findings suggest new candidate bacteria with potential associations with the health status of the carriers, and underline the importance of studying them in more detail.

The relevance of the diverging phylotypes identified by sequence analysis (Kassinen *et al.*, 2007) was addressed by the design of phylotype-specific qPCR assays, which were applied to IBS and HC samples with 6 months' follow-up (Lyra *et al.*, 2009). Multivariate analysis of the collective data from 14 qPCR assays showed that the IBS-D samples differed significantly from the other sample groups, confirming that a panel of selected bacteria can be used to distinguish IBS subtypes from each other and from the HCs. When the individual assays were analysed, four uncharacterized Firmicutes phylotypes diverged significantly between the different IBS subtypes and the HCs persistently over time (Table 1). Two of the phylotypes showed moderate similarity to *Ruminococcus torques*, a mucin-degrading *Clostridium*.

Phylogenetic microarrays offer high-resolution and -throughput tools to study complex microbial ecosystems (reviewed by Zoetendal *et al.*, 2008). Application of the HITChip has allowed simultaneous detection and identification of over one thousand intestinal phylotypes in IBS patients and HCs (Rajilić-Stojanović, 2007). In an overall

Table 1. Alterations in the GI microbiota composition of subtyped IBS patients

Comparisons were made among all studied IBS groups and healthy controls; statistically significant differences between at least one pair wise comparison are listed. All subjects were recruited according to Rome II criteria. Studies marked with □ have used samples from the same cohort.

IBS-D	IBS-C	IBS-A	HCs	Common in IBS	Method	Taxonomic coverage and resolution	Reference
Lactobacilli ↓ <i>n</i> =12	Lactobacilli ↑ <i>Veillonella</i> ↑ <i>n</i> =9	<i>n</i> =6	<i>n</i> =22	<i>B. catenulatum</i> ↓ <i>C. coccoides</i> groups ↓ <i>R. productus</i> ↑ <i>C. coccoides</i> species ↑	qPCR	12 genera/groups, 8 species	Malinen <i>et al.</i> (2005)□
<i>n</i> =7	<i>C. coccoides</i> – <i>Eubacterium rectale</i> group ↓ <i>n</i> =6	Temporal stability of <i>Clostridium histolyticum</i> group ↑ <i>n</i> =3	<i>n</i> =16	Temporal stability of predominant bacteria ↓	DGGE, affinity capture	Total bacteria and 4 clostridial groups	Maukonen <i>et al.</i> (2006)□
10 genera* ↓ Streptococci ↑ <i>n</i> =10	5 genera† ↓ 6 genera‡ ↑ <i>n</i> =8	5 genera§ ↓ 7 genera \$ ↑ <i>n</i> =6	<i>n</i> =23	<i>Collinsella</i> ↓ <i>Collinsella aerofaciens</i> ↓ <i>C. cocleatum</i> 88 % ↓ <i>Coprococcus eutactus</i> 97 % ↓	GC profiling + sequencing of 16S rDNA library qPCR	Whole community; phylotypes	Kassinen <i>et al.</i> (2007)□
<i>Clostridium symbiosum</i> -like ↓ 5 genus-level taxa¶ ↑ <i>n</i> =7	5 genus-level taxa# ↓ 6 genus-level taxa** ↑ <i>n</i> =8	<i>C. symbiosum</i> ↓ <i>Prevotella oralis</i> ↓ 4 genus-level taxa†† ↑ <i>n</i> =5	<i>n</i> =20	<i>Bacteroides</i> spp. ↓ <i>Bacillaceae</i> ↑	Microarray	Whole community; 131 genus-level groups	Rajilić-Stojanović (2007)
<i>n</i> =14	<i>n</i> =11	<i>n</i> =16	<i>n</i> =24	Bifidobacteria ↓ <i>Clostridium lituseburense</i> group ↓	FISH, qPCR	8 genera/groups; 4 species	Kerckhoffs <i>et al.</i> (2009)
Higher richness of Firmicutes 6 genera‡‡ ↓ 6 genera\$\$ ↑ <i>n</i> =10			<i>n</i> =23		GC profiling + sequencing of 16S rDNA library	Whole community; phylotypes	Krogus-Kurikka <i>et al.</i> (2009)□
IBS-D most different in multivariate analysis <i>R. torques</i> 94 % ↑ <i>Clostridium thermosuccinogenes</i> 85 % ↑ <i>n</i> =8	<i>Ruminococcus bromii</i> - like ↑ <i>n</i> =8	<i>C. thermosuccinogenes</i> 85 % ↑ <i>R. torques</i> 93 % ↓ <i>n</i> =4	<i>n</i> =15		qPCR	14 phylotypes	Lyra <i>et al.</i> (2009)□
<i>n</i> =8	<i>n</i> =11	<i>Veillonella</i> ↑ <i>n</i> =7	<i>n</i> =26	Lactobacilli ↑ <i>Veillonella</i> ↑	qPCR, culture	10 genera/groups	Tana <i>et al.</i> (2010)

Table 1. cont

* <i>Butyrivibrio</i> , <i>Lactobacillus</i> , <i>Allisonella</i> , <i>Bacteroides</i> , <i>Eubacterium</i> , <i>Roseburia</i> , <i>Ruminococcus</i> , <i>Streptococcus</i> , <i>Bifidobacterium</i> , <i>Collinsella</i> .
† <i>Lactobacillus</i> , <i>Streptococcus</i> , <i>Allisonella</i> , <i>Bacteroides</i> , <i>Collinsella</i> .
‡ <i>Dorea</i> , <i>Lactococcus</i> , <i>Roseburia</i> , <i>Ruminococcus</i> , <i>Roseburia</i> , <i>Bifidobacterium</i> .
§ <i>Lactobacillus</i> , <i>Roseburia</i> , <i>Eubacterium</i> , <i>Ruminococcus</i> , <i>Collinsella</i> .
<i>Butyrivibrio</i> , <i>Ruminococcus</i> , <i>Allisonella</i> , <i>Bacteroides</i> , <i>Lactobacillus</i> , <i>Streptococcus</i> , <i>Bifidobacterium</i> .
¶ <i>Aneurinibacillus</i> spp., <i>Streptococcus</i> <i>intermedius</i> -like, <i>Staphylococcus</i> <i>colinum</i> -like, <i>Roseburia</i> <i>intestinalis</i> -like.
<i>Bacteroides</i> <i>ovatus</i> -like, <i>Bacteroides</i> <i>uniformis</i> -like, <i>Bacteroides</i> <i>vulgatus</i> -like, <i>Clostridium</i> <i>stercorarium</i> -like, <i>Anaerostipes</i> <i>caccae</i> -like.
** <i>Bacillus</i> spp., <i>Anaerotruncus</i> <i>colihominis</i> -like, <i>Clostridium</i> <i>cellulosi</i> -like, <i>Oscillospira</i> <i>guilliermondii</i> -like, <i>Sporobacter</i> <i>termitidis</i> -like, <i>Subdoligranulum</i> <i>variabile</i> -like.
†† <i>S. intermedius</i> -like, <i>Anaerostipes</i> <i>caccae</i> -like, <i>C. colinum</i> -like, <i>Ruminococcus</i> <i>gnavus</i> -like.
‡‡ <i>Slackia</i> , <i>Collinsella</i> , <i>Bifidobacterium</i> , <i>Bacteroides</i> , <i>Lactobacillus</i> , <i>Coprococcus</i> , <i>Ruminococcus</i> .
§§ <i>Enterobacter</i> , <i>Eggerthella</i> , <i>Streptococcus</i> , <i>Coprobaecillus</i> , <i>Megasphaera</i> , <i>Lachnospira</i> <i>Incertae Sedis</i> .

comparison, the amount of *Bacteroides* spp. was significantly decreased and the amount of bacilli increased in the IBS patients. Separate analysis of each of the IBS subgroups showed that IBS-D patients differed the most and IBS-C patients the least from the HCs, although none of the differences reached statistical significance. A compositional analysis of samples representing all three subtypes and the HCs revealed that a total of 19 genus-level taxa, representing Bacteroidetes, bacilli and *Clostridium* clusters III, IV and XIVa, differed significantly between the groups (Rajilić-Stojanović, 2007). The amount of *Bacteroides* spp. was the lowest in IBS-C patients, while IBS-D patients had elevated amounts of streptococci. Most of the significant differences occurred between a single IBS subtype and the HC group, while five genera from *Clostridium* cluster IV differed most between the IBS-C and IBS-D groups, the first group having higher levels of these organisms.

Bifidobacteria and lactobacilli have been studied in many IBS trials due to their potential association with health. Decreased counts of bifidobacteria have been reported in IBS patients (Kerckhoffs *et al.*, 2009; Malinen *et al.*, 2005), especially of the *B. catenulatum* group (Kerckhoffs *et al.*, 2009; Lyra *et al.*, 2009; Malinen *et al.*, 2005). A decrease of lactobacilli has been detected in IBS-D (Malinen *et al.*, 2005; Krogius-Kurikka *et al.*, 2009) and IBS-A patients (Kerckhoffs *et al.*, 2009). On the other hand, untypically high faecal amounts of lactobacilli and/or streptococci have been reported for IBS-D patients (Kassinen *et al.*, 2007; Krogius-Kurikka *et al.*, 2009; Carroll *et al.*, 2008) as well as IBS patients independent of the subtype (Tana *et al.*, 2010). This trend suggests that at least some IBS patients, similarly to ileostomy patients, have an outgrowth of aerobic bacteria that may be linked to the overproduction of organic acids, as will be discussed below.

Few studies have addressed the variability and composition of mucosa-associated GI microbiota in IBS patients. A fluorescence *in situ* hybridization (FISH) study showed untyped IBS patients to harbour slightly more total bacteria and significantly more members of *Clostridium* cluster XIVa than the HCs (Swidsinski *et al.*, 2005). Otherwise, the amount and distribution of mucosal bacteria were comparable with that of healthy subjects, in contrast to the drastic quantitative and qualitative differences detected in the IBD samples. Fingerprinting analysis to qualitatively characterize the mucosal microbiota has been employed in a small number of untyped IBS patients (Codling *et al.*, 2010) as well as in IBS-D patients in parallel to HCs (Carroll *et al.*, 2010). Codling and co-workers found no significant differences in the variability of faecal and mucosal microbiota. The study of Carroll and co-workers found significant differences in the mucosal but not in the faecal communities between the patients and HCs, while the mucosal and faecal communities differed significantly within a subject independently of the health status. Not only the composition but also the degree of diversity of the microbiota was changed in a more pronounced manner in the mucosal samples. A

qPCR analysis of subtyped IBS patients has revealed a statistically significant decrease of *B. catenulatum* in the duodenal mucosa of IBS patients independently of the subgroup (Kerckhoffs *et al.*, 2009). This study was limited to selected bifidobacterial species and did not investigate the other genera in the duodenal microbiota at all. In summary, it is too early to conclude that the mucosal microbiota in IBS patients differs from that in healthy subjects. In any case, integrated studies of both mucosal and luminal communities are relevant, because the former directly communicates with the host while the latter vastly dominates the metabolic output of the GI microbiota, providing two complementary aspects to address the role of gut bacteria in IBS.

How should the present data on IBS-related microbiota be interpreted?

The existing literature on the GI microbiota in IBS, as summarized in Table 1 and discussed above, does not reveal uniform alterations in microbiota composition shared among all patients. The in-depth analysis of a single IBS cohort using different methodologies supports the notion that studies that apply different analysis methods to target bacteria with variable taxonomic resolution are difficult to compare. Nevertheless, the results complement each other, and together with other trials reveal some subtype-specific microbiota features. The GI microbiota of IBS-D patients seems to deviate the most and that of IBS-C patients the least from that of HCs (Lyra *et al.*, 2009; Rajilić-Stojanović, 2007). Most of the observed differences could be assigned to the Firmicutes, as they dominate the significant alterations in the comparison of clone libraries (Kassinen *et al.*, 2007; Krogius-Kurikka *et al.*, 2009), microarray-derived microbiota profiles (Rajilić-Stojanović, 2007) and qPCR assays (Lyra *et al.*, 2009; Malinen *et al.*, 2005; Tana *et al.*, 2010) between IBS patients and HCs. More specifically, the genera *Streptococcus*, *Lactobacillus* and *Veillonella*, as well as members of the families *Lachnospiraceae* and *Ruminococcaceae*, stand out from the comparisons, although with a somewhat inconsistent association with IBS status. In particular, IBS-D patients are frequently enriched with streptococci and show a distinctive set of dominant *Clostridia*. Members of the Bacteroidetes and Proteobacteria show some differences, although this was not the case in several studies. Actinobacteria, including bifidobacteria and coriobacteria (mainly of the genus *Collinsella*), contribute to the differences in several datasets as well. The repeated appearance of the above-mentioned taxa as significantly different between IBS patients and HCs suggests a highly probable association of these organisms with IBS, and warrants their further investigation in this area of research.

In addition to the limited number of phylum- and group-level differences, several genus- and species-level alterations in abundance have been identified in IBS patients in studies that achieved this taxonomic resolution (Table 1; Kassinen *et al.*, 2007; Krogius-Kurikka *et al.*, 2009; Lyra *et al.*, 2009;

Rajilić-Stojanović, 2007). Most of these differences would have remained undetected by conventional approaches that study the higher taxonomic levels. As the evolutionary distance of different bacterial phyla is comparable with that of animals and plants in the rRNA-based tree of life (Woese, 1987), the vast genetic and phenotypic heterogeneity of the taxa classified into a phylum makes the phylum-wide differences unlikely. Thus, microbial analysis on the genus or lower taxonomic level facilitates the identification of differences that more probably reflect the phenotypic alterations of the microbiota. However, the phylogenetic similarity does not necessarily reflect the functional similarity and vice versa. To exemplify, the abundance of two uncultured phylotypes having 93 and 94 % similarity to the 16S gene of *R. torques* differed significantly between the clone libraries derived from healthy and IBS-D patients, so that the former phylotype was significantly enriched in the HCs and the latter in IBS-D (Lyra *et al.*, 2009). Such a polarized segregation of related phylotypes explains the lack of uniformity at the higher taxonomic levels and highlights the need to verify the compositional differences with additional genetic information whenever available. Currently, we cannot functionally interpret most of the findings; for many of the taxa that differ significantly there is a complete lack of prior information or at best only moderate similarity to a characterized member of the gut ecosystem. The limited characterization of the GI microbiota not only challenges the understanding of its role in health but also highlights the diagnostic potential of its undiscovered taxonomic and functional attributes. After all, despite the missing information about the functional impact of the emerging phylotypes, they still can function as valuable (bio)markers for a particular health status.

Functional perspective on IBS-related microbiota

Beyond the above-discussed compositional differences, some data are available on the functional impact of the GI microbiota and its dysbiosis on the aetiology of IBS. The investigation of IBS-related microbial functions is currently limited to organic acids and intestinal gas, as will be discussed below. A significant increase of bacterially produced short chain fatty acids (SCFAs) was recently recorded in the faeces of Japanese IBS patients who also harboured significantly more lactobacilli and *Veillonella* spp. compared with HCs (Tana *et al.*, 2010). The increase in total SCFAs was due to the increase of acetic and propionic, but not butyric, acid. Conceptually, this study adds a new perspective to the IBS field, as it utilized multiple datasets in the same cohort by simultaneously addressing the microbiota, their metabolites and the amount of gas produced, as well as making a subjective evaluation of GI symptoms and quality of life. Except for colonic gas, the parameters were found to vary non-randomly between the patients and the HCs. In brief, the authors hypothesize that the higher numbers of lactobacilli and *Veillonella* spp. in IBS patients result in a high level of

organic acids, which correlates with abdominal pain, bloating, anxiety and poor quality of life. The increase in acids was suggested to affect visceral sensation and manifest as a low somatic and emotional score. Although the work of Tana and co-workers is of great interest, their conclusions should be considered with some caution, because the central hypothesis is based solely on culture-based enumeration of lactobacilli that resulted in untypically low values both in the patients and the HCs (mean \log_{10} bacterial counts of 4.6 and 5.6 per gram of faeces, respectively). Moreover, qPCR was used to quantify 10 bacterial groups excluding lactobacilli, despite their high relevance, further emphasizing the tentative nature of the link between lactobacilli, SFCAs and GI symptoms.

Nevertheless, the suggested link between the SFA profile and GI symptoms can be discussed in the light of the contrasting biological activities of the SFCAs. Acetate is a known chemical irritant, and at high concentrations is used to induce mucosal lesions and abdominal cramps in experimental animals, while butyrate is considered as protective and able to dose-dependently reduce abdominal pain in humans *in vivo* (Vanhoutvin *et al.*, 2009). While IBS patients as a single group differed significantly from the HCs with respect to organic acids and microbiota, none of the three subtypes alone showed a significant difference in these aspects (Tana *et al.*, 2010). Similarly, in the study of Malinen *et al.* (2005), all IBS subgroups had elevated levels of *Veillonella* spp., *C. coccoides* and *R. productus*, all able to produce acetate and succinate (Liu *et al.*, 2008). However, as IBS-D patients have also been documented to have more butyrate but less total SCFAs than HCs (Treem *et al.*, 1996), and as butyrate induces visceral hypersensitivity in a rat model (Bourdu *et al.*, 2005), the clinical effects of organic acids in IBS remain to be verified.

In addition to the acid producers, GI microbes associated with the control of colonic gas either through its production or disposal may be implicated in IBS through flatulence and bloating. While all intestinal hydrogen (H_2) results from microbial fermentation, the bacteria involved and their role in IBS are difficult to study. First, the conventional quantification of H_2 is based on breath tests of controversial reliability, and second, the majority of gut bacteria can produce H_2 . An initial study that suggested the role of H_2 in IBS analysed the total excretion of colonic gas in six IBS patients, who showed elevated production of H_2 on a fibre-rich diet, although the total volume of gas did not differ when compared with the HCs (King *et al.*, 1998). Similarly, Tana *et al.* (2010) found no difference in the amount of X-ray-quantified colonic gas between the IBS patients and the HCs. Recently, Serra *et al.* (2010) demonstrated that IBS patients have sensory dysfunction and poor tolerance to moderate gas loads, in line with the hypothesis that the IBS symptoms arise more from the visceral hypersensitivity than from the elevated amount of intestinal gas.

Three alternative and potentially competing microbial groups dispose of most of the colonic H_2 : acetogens,

methanogenic Archaea and sulfate-reducing bacteria (SRB) (extensively reviewed by Nakamura *et al.*, 2010). The removal of H_2 not only effectively reduces the volume of gas and the pressure in the gut but also regulates the amount of acetate, a potential chemical trigger of IBS (discussed above). The phylogenetically diverse and numerically abundant acetogenic bacteria have not yet been specifically addressed in any IBS cohort. Despite the fact that methane (CH_4), produced by methanogenic Archaea, has a proven causative and mechanistically described link to constipation (Pimentel *et al.*, 2006), only a few studies have studied the methanogenic Archaea in IBS patients. Based on breath testing, both the prevalence and the rate of CH_4 production were lower in unsubtyped IBS patients than in HCs (Rana *et al.*, 2009). However, direct PCR-based enumeration of methanogens found no difference in their prevalence between the IBS and HC groups, in contrast to the significantly lowered incidence of these organisms in IBD patients (Scanlan *et al.*, 2008). H_2 disposal via sulfate reduction generates toxic hydrogen sulphide (H_2S), and the impact of SRB is considered detrimental to the host. Two studies so far have studied the amount of SRB in IBS patients. In the qPCR analysis of Malinen *et al.* (2005), IBS subjects tended to harbour fewer *Desulfovibrio* spp., the predominant SRB genus in the gut, while culture- and FISH-based analysis of IBS-C subjects showed significantly more SRB compared with HCs (Chassard *et al.*, 2009). While the current studies do not provide conclusive results about the role of H_2 -disposing bacteria in IBS, their involvement in the regulation of colonic gas, transit rate and chemical stimuli associates them strongly with GI symptomology and thereby warrants their further research.

From an ecological perspective, any species with a relevant trait can contribute to the disease process, assuming its metabolic activity in a given environment. Currently, we lack knowledge of bacterial genes associated with specific GI symptoms. Even after identification of the relevant microbial activities, their examination remains challenging, as most of the ecological niches in the colonic ecosystem are occupied by phylogenetically diverse and functionally redundant bacteria that require targeting of key enzymes in a given pathway instead of the conventional analyses relying on 16S rDNA. So far, the only described gene-specific assays putatively relevant to IBS are the ones that target butyrate biogenesis (Louis *et al.*, 2010) and the different H_2 disposal routes discussed above (Nakamura *et al.*, 2010).

Suggestions for future research

In addition to the identification of bacteria potentially affiliated with IBS, the employment of recent high-throughput methods has also emphasized that several conceptual challenges prevail in the current attempts to scrutinize the GI microbiota in IBS or indeed any other disease. Despite the intensive attempts to define the

parameters for a normal GI microbiota in the absence of any detectable disease, currently we must content ourselves with the conception that healthy subjects carry an immense selection of different phylotypes that vary greatly in their abundance and show little overlap between individuals. Even at the phylum level, substantial variation can exist in relative abundance not only between individuals but also within individuals over time (Turnbaugh *et al.*, 2009). Moreover, the abundance of many bacteria varies remarkably, by as much as 2000-fold or more (Qin *et al.*, 2010). Therefore, the depth of analysis largely determines which bacteria will be considered as present and consequently the extent of shared bacteria (J. Jalanka-Tuovinen & W. M. de Vos, unpublished results; Qin *et al.*, 2010). If the susceptibility of the host has a decisive role in IBS manifestation, we may expect a subset of patients to carry a microbiota that is not substantially disturbed, further adding to the high subject-to-subject variation among IBS patients. Due to the low number of IBS patients analysed so far and the high individuality of the GI microbiota, future cohorts of sufficient size will be essential. They will be imperative both for validating the taxa with a recently established association with IBS and for discovering new diagnostic species.

Focused analyses can remove some of the heterogeneity in IBS patients. The relevance of IBS subtyping to the microbiological analyses is highlighted by the recognition of the large microbiota variation between the subtypes and the consequent difficulty in extrapolating microbial characteristics from one IBS subtype to another. For some bacterial groups, a trend whereby the abundance gradually descends from the IBS-C samples to the HCs and further to the IBS-D samples (Kerckhoffs *et al.*, 2009; Malinen *et al.*, 2005; Rajilić-Stojanović, 2007) can be observed. This finding suggests that addressing luminal conditions such as transit time and pH, known to critically influence the composition of the GI microbial community (Duncan *et al.*, 2009; Louis *et al.*, 2007), would be highly relevant in IBS and HCs, as we need to learn more about how the gut environment and related ecological selection forces relate to intestinal function and gut health.

Self-reported symptom diaries, routinely used to measure possible symptom alleviation in clinical trials, should also be employed in basic research because they allow coupling of the microbiological parameters to the subjective feeling of intestinal health on the day of faecal sampling. Diaries should be collected not only from patients but also from the HCs, as occasional digestive complaints such as bloating and flatulence are frequent in healthy subjects as well. Using this approach, the research findings have the potential to expand from microbiota traits that differ between the IBS patients and the HCs to symptom-specific traits that are independent of the carrier's diagnosis. Pilot studies correlating GI symptoms with the microbiota have recently been published (Malinen *et al.*, 2010; Tana *et al.*, 2010). As discussed earlier, the work of Tana and co-workers suggests that lactobacilli and *Veillonella* spp. correlate positively with GI symptoms and impaired quality of life. Malinen and co-workers correlated symptom data with 13 different qPCR assays targeting predominant genus-/group-level taxa as well as distinct phylotypes that were previously associated with IBS patients in sequence analyses (Lyra *et al.*, 2009). The major finding was that *R. torques* 94 %, a phylotype originally identified as being overrepresented in an IBS-D-derived clone library (Lyra *et al.*, 2009), showed a strong and significant positive correlation with various bowel symptoms in another set of untyped IBS patients. Such a finding is encouraging in the search for putative symptom-associated bacteria that may eventually be employed in mechanistic studies and for diagnostic purposes.

Finally, to improve the comparability and generalization of the outcome of different trials, the plethora of variables arising from the non-standardized recruitment criteria, sample processing and analysis techniques, as well as the bioinformatic and statistical methods used for the data analysis, should be controlled.

Outlook for microbes as biomarkers for IBS

Table 2 lists the different aspects of the GI microbiota that are considered relevant to future research with regard to

Table 2. Future GI health biomarker candidates arising from the microbiota

The list does not aim to be exhaustive but rather gives examples of how to compare GI microbiota in patients and healthy subjects.

Conceptual target	Variable to measure	Reasoning
Composition	Presence, absence, relative over- or underrepresentation of specific bacteria (see text for potential candidate taxa)	Specific measurement of compositional differences
Ecology	Diversity, richness, evenness, resilience	Overall assessment of the ecosystem
Activity and/or functional dysbiosis	Metabolites, proteins, gases/acidosis, aerobiosis, accumulation of gas, toxemia	Measurement of the functional attributes, which can contribute to symptoms either directly or by interfering with normal host-microbiota mutualism
Stability	Temporal variation	Measurement of how the composition, ecological parameters and activity vary within and between individuals

intestinal health, and thus have the potential to be employed as biomarkers for IBS and other functional intestinal aberrations. As we currently lack the criteria to define when a microbiota is aberrant, we believe that the simultaneous examination of multiple aspects listed in Table 2 will be the most fruitful and reliable approach in determining the extent to which particular microbiota profiles overlap between patients and differ from those of HCs.

Due to the paucity of molecular data currently available from IBS patients, it remains possible that specific IBS biomarker bacteria will be discovered in future studies. More probably, an over- or underrepresentation of certain bacteria features in the microbiota in IBS, reflecting compositional shifts that either precede or follow the pathophysiological changes in the host. This field of research is expected to capitalize on the current availability of high-throughput sequencing and microarray technologies so that community-level surveys between IBS patients and HCs become a standard approach. The application of diversity and stability indexes together with other ecological measures such as the degree of complexity and evenness may provide informative measures to evaluate overall differences between communities. At present, stability and diversity indexes are used as descriptive measures, as their optima and overall relevance with respect to GI health are unclear.

As discussed above, we have limited knowledge of the functionality of the GI microbiota in health and disease. The first publications that utilize whole-community metagenomics or functional genomics (metatranscriptomics, metaproteomics and meta-metabolomics) on IBS cohorts are still to come. They will allow a holistic assessment of putative IBS-specific functions in the gut microbiota and reveal possible gene dosage differences. A recent metagenomic study revealed a 25% gene loss in IBD patients compared with HCs (Qin *et al.*, 2010), establishing a basis for the identification of microbiota-derived functions that are decisive for the manifestation of GI symptoms. Finally, the initial finding of the temporal instability of IBS patient microbiota (Mättö *et al.*, 2005) should be readdressed by repeated sampling of patients to provide a longitudinal aspect for the structural and functional attributes of their microbiota. A subset of healthy individuals without any intestinal complaints appear to carry unstable GI microbiota (Engelbrektson *et al.*, 2009). While this may be anecdotal, it emphasizes the need for further research to confirm the relevance and extent of the temporal instability that possibly characterizes the microbiota in IBS.

Current research has uncovered subtle changes rather than dramatic alterations in the GI microbiota of IBS patients, with considerable structural overlap with HCs. This synopsis strongly suggests homeostasis of the microbiota to be a critical element in the pathophysiology of IBS, in a manner analogous to the development of dental caries according to the proposed ecological plaque hypothesis

(Marsh, 2003). This hypothesis is based on the concept that the presence or abundance of an individual taxon has little clinical significance, and that the development of disease is instead associated with community-level alterations that reflect the breakdown of homeostasis. The existing data strongly support the existence of the intestinal ecological dysbiosis hypothesis, which states that multispecies signatures specify the GI microbiota in a given health status such as IBS. Consequently, community-level analyses and an ecological perspective are needed to identify and interpret these signatures. Understanding the metabolic co-dependence between different bacteria will provide the biological basis for understanding how a certain set of species can contribute to disease, and consequently how to manipulate the GI environment to restore a healthy microbiota state.

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