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Structural alterations of faecal and mucosa-associated bacterial communities in irritable bowel syndrome

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Summary

Irritable bowel syndrome (IBS) is the most common functional gastrointestinal disorder in western countries. Previous studies on IBS, mostly based on faecal samples, suggest alterations in the intestinal microbiota. However, no consensus has been reached regarding the association between specific bacteria and IBS. We explore the alterations of intestinal bacterial communities in IBS using massive sequencing of amplified 16S rRNA genes. Mucosal biopsies of the ascending and descending colon and faeces from 16 IBS patients and 9 healthy controls were analysed. Strong inter-individual variation was observed in the composition of the bacterial communities in both patients and controls. These communities showed less diversity in IBS cases. There were larger differences in the microbiota composition between biopsies and faeces than between patients and controls. We found a few over-represented and under-represented taxa in IBS cases with respect to controls. The detected alterations varied by site, with no changes being consistent across sample types.

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Introduction

Irritable bowel syndrome (IBS) is the most common functional disorder of the gastrointestinal tract (Quigley *et al.*, 2006). Symptoms include chronic abdominal pain, discomfort or bloating that is relieved with defecation and/or is associated with altered bowel habits (Longstreth *et al.*, 2006). IBS has a profound impact on patients' quality of life and high economic costs (Pace *et al.*, 2003; Quigley *et al.*, 2006). The cause of the disorder is thought to be multifactorial but remains poorly understood. Recent studies indicate that the main factors include visceral hypersensitivity, abnormal gut motility and autonomous nervous system dysfunction (Karantanos *et al.*, 2010). Psychosocial factors are also known to play an important role in the development and persistence of IBS symptoms (Drossman, 1994; Bennet *et al.*, 1998).

Evidence for the involvement of the gastrointestinal (GI) microbiota in IBS is supported by several observations. First, the onset of IBS frequently follows an acute episode of infectious gastroenteritis, which is in turn the strongest risk factor for IBS (Spiller and Garsed, 2009). Second, clinical trials targeting the microbiota (antibiotics, probiotics) seem to alleviate IBS symptoms (Moayyedi *et al.*, 2010; Parkes *et al.*, 2010). Finally, some studies have suggested an altered GI microbiota in IBS patients, with specific features associated to the three IBS subtypes (diarrhoea predominant, constipation predominant or alternating between both). Some of the findings are large temporal instability and inter-subject variation of the GI microbiota, altered abundance of specific taxa and altered bacterial metabolism in the colon of IBS patients compared with controls. To date, molecular studies have not revealed pronounced IBS-related deviations of the microbiota composition and the results have been inconsistent (Salonen *et al.*, 2010).

Most studies on IBS carried out so far have used faecal samples because they are easily collected in a non-invasive manner. However, it is known that faecal communities are not necessarily representative of those found in other parts of the GI tract (Zoetendal *et al.*, 2002;

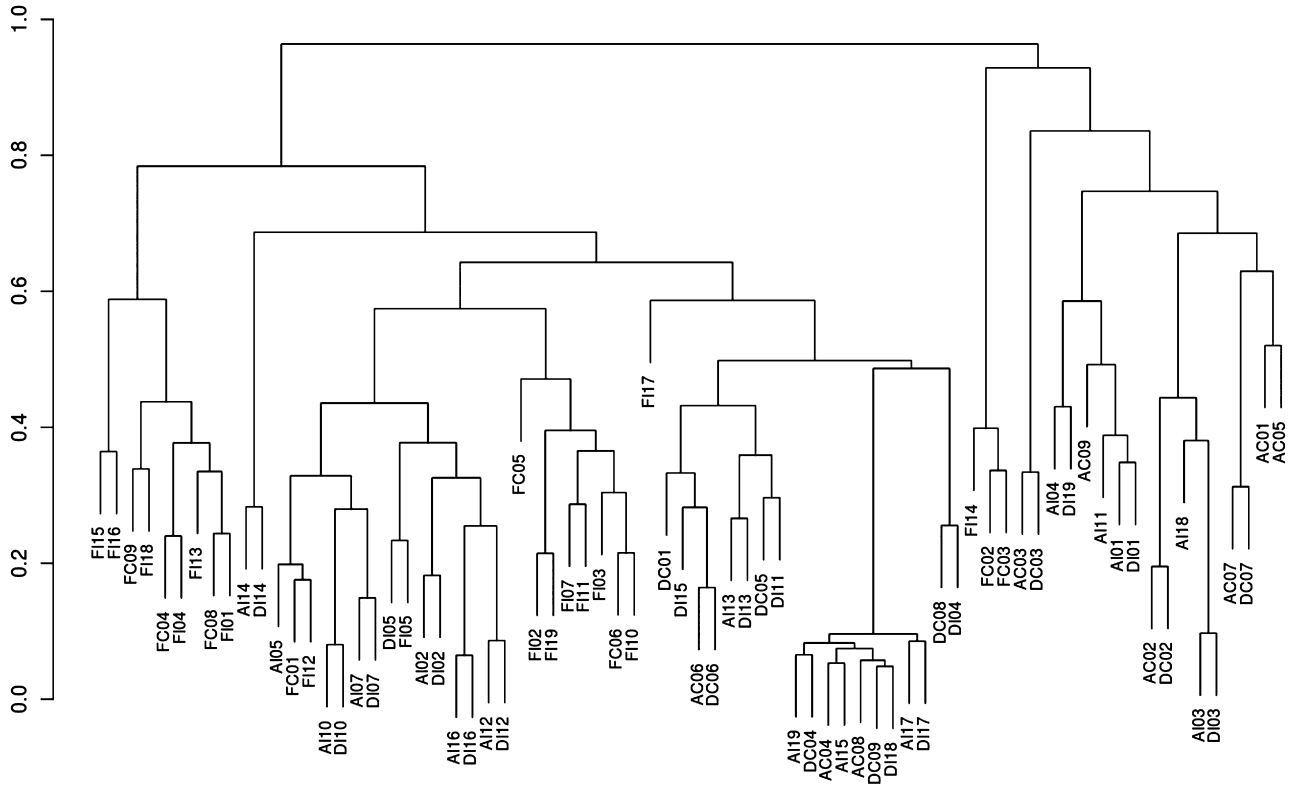

Fig. 1. Hierarchical cluster of the individual samples based on Bray–Curtis distances between the observed distributions of genera. AC, DC and FC: ascending colon, descending colon and faeces of healthy controls; AI, DI and FI: ascending colon, descending colon and faeces of IBS patients. Patients with constipation-predominant IBS: I10, I13, I15; patients with diarrhoea-predominant IBS: the rest.

Eckburg *et al*., 2005; Lepage *et al*., 2005; Willing *et al*., 2010; Durbán *et al*., 2011). Therefore, it is important to assess the different intestinal habitats when studying the role of the gut microbiota in the aetiology of a disease such as IBS because each intestinal habitat may provide a distinct and complementary picture of the microbiota.

This is the first study that explores the potential alterations of the gut microbiota in IBS patients simultaneously in intestinal mucosa (ascending and descending colon) and faeces through massive sequencing, though this approach has been widely used in recent times to study microbiomes in both faeces and intestinal mucosa in relation to other intestinal pathologies (Willing *et al*., 2010). The study included 16 IBS patients and 9 healthy controls. Patients were classified into diarrhoea subtype (IBS-D, 13 patients) and constipation subtype (IBS-C, 3 patients), according to the Rome II criteria. The entire bacterial communities were analysed through massive sequencing of PCR-amplified V1 and V2 variable regions of the 16S ribosomal RNA genes. See Appendix S1 for further details on sample collection, DNA extraction, PCR amplification and sequencing, sequence analysis and statistical methods.

Results and discussion

To assess differences in the microbiota composition between groups (IBS-D/IBS-C and controls), we used taxa abundances in the individual samples and also in the pooled samples obtained by pooling the individual ones by sample type (ascending colon, descending colon or faeces) within each group.

Cluster analyses based on community composition at genus level (Fig. 1) reveal larger differences in the microbiota composition between biopsies and faeces than between IBS patients and controls. Controls and IBS patients appeared mixed within clusters. Samples from the same IBS-subtype did not cluster either. Both mucosal samples from the same individual clustered together at short distances in many cases. However, faecal samples formed several clusters far from the respective biopsies. The analysis of similarities (ANOSIM) and permutational multivariate ANOVA (PMANOVA) tests found significant differences in the global composition between sampling sites (ANOSIM $R = 0.158$, $P = 0.001$; PMANOVA $R^2 = 0.117$, $P = 0.001$) but not between IBS patients and controls (ANOSIM $R = -0.005$, $P = 0.483$; PMANOVA $R^2 = 0.077$, $P = 0.401$).

Diarrhoea-predominant IBS (IBS-D)/constipation-predominant IBS (IBS-C)/controls

The Shannon diversity indices (H) and the Chao1 and ACE estimators of richness were on average lower in IBS cases than in healthy controls in the three intestinal compartments (Table 1). The richness estimators indicated substantial numbers of unrecovered phylotypes (defined at 97% of sequence identity), while the estimated numbers of genera were closer to the observed ones.

The community structure of the pooled samples of IBS patients and controls looked overall quite similar to each other (Fig. S1). However, mucosal samples of patients presented higher counts of *Bacteroidaceae* (IBS-D and ascending colon of IBS-C ≈ 38%, descending colon of IBS-C 54%, controls ≈ 28%). In addition, faeces of patients had more *Rikenellaceae* than controls (IBS-D 12%, IBS-C 29%, controls 7%) as well as more *Porphyromonadaceae* (IBS-D 11%, IBS-C 16%, controls 7%) and less *Ruminococcaceae* (IBS-D 8%, IBS-C 11%, controls 15%). Some other characteristics of the IBS-C subtype were higher counts of *Enterobacteriaceae* in mucosal samples and of *Rikenellaceae* in faecal samples when compared with the rest. Interestingly, Willing and colleagues (2010) also found an increase in *Enterobacteriaceae* and a decrease in *Ruminococcaceae* in faecal and ileal samples of ileal Crohn's disease patients, as we have observed in IBS patients.

We assessed the homogeneity in the prevalence of each bacterial taxon within each group (IBS-D, IBS-C and control) using the Gini coefficient (that measures the evenness of a distribution) and found large inter-individual variability in the abundance of nearly all bacterial taxa for both cohorts (Table S1), as previously reported in healthy subjects (Zoetendal *et al*., 1998; Eckburg *et al*., 2005).

Univariate chi-squared tests were applied to assess the homogeneity in the relative abundance of each bacterial taxon in the IBS and control pooled samples. Odds ratios (ORs) were calculated to measure the magnitude of the over- or under-representation of taxa between samples. Previous studies on IBS using 16S rRNA gene sequencing based their conclusions on pooled faecal samples of patients and healthy controls without accounting for the within-group variability (Kassinen *et al*., 2007; Krogius-Kurikka *et al*., 2009). This may create spurious differences due to just a few individuals dominating the composition of these bacterial taxa in the pooled samples instead of a general trend of the IBS and control cohorts. To avoid this problem, we randomly labelled the individual samples as IBS patient or control and then aggregated them to build new pooled samples (Appendix S1). By repeating this process many times, we obtained the distribution of the OR under the null hypothesis of no difference between cases and controls that accounts for the

Table 1. Average values (and standard deviations) of the Shannon diversity index (H) and the Chao1 and ACE richness estimators of the individual samples within the control group and the IBS-diarrhoea and IBS-constipation groups at each sampling site at the levels of genus and phylotype defined at 97% of sequence identity.

			Ascending colon			Descending colon			Faeces		
				IBS patients			IBS patients			IBS patients	
			Controls	Diarrhoea	Constipation	Controls	Diarrhoea	Constipation	Controls	Diarrhoea	Constipation
No. of subjects			9	13	3	9	13	3	8	13	3
No. of sequences			27 277	36 672	7494	26 750	43 896	15 338	49 743	47 652	13 010
Genus	Average (SD)	Shannon H	2.87 (0.38)	2.43 (0.64)	2.21 (0.74)	2.71 (0.45)	2.38 (0.56)	1.95 (0.61)	2.24 (0.46)	2.14 (0.48)	2.18 (0.14)
		Chao1	96 (23.54)	85 (44.51)	81 (35.85)	100 (29.13)	89 (30.70)	58 (4.69)	58 (17.00)	51 (18.78)	41 (13.16)
		ACE	96 (20.48)	81 (24.63)	88 (47.25)	98 (29.15)	85 (25.37)	60 (4.53)	57 (12.52)	54 (21.23)	43 (13.33)
Phylotype (97% identity)	Average (SD)	Shannon H	4.74 (0.45)	4.47 (0.66)	4.61 (1.05)	4.84 (0.64)	4.59 (0.62)	4.23 (0.77)	4.49 (0.44)	4.25 (0.44)	4.36 (0.27)
		Chao1	671 (509.14)	597 (348.03)	658 (510.44)	765 (367.16)	687 (396.40)	565 (72.88)	639 (124.13)	469 (190.05)	403 (128.70)
		ACE	684 (488.13)	615 (332.74)	669 (513.33)	785 (374.13)	699 (377.17)	554 (89.37)	636 (116.05)	474 (196.23)	425 (151.11)

variability between individuals. Extreme OR values for the actual pooled samples were indicative of true association (Table S1, Fig. S2). After accounting for the large inter-individual variation, only a few of the genera showed statistically significant differences between IBS cases and controls. Specifically, we found in the IBS-D subtype cases compared with controls an over-representation of *Acinetobacter* (OR = 16.71, $P = 0.02$), *Butyricimonas* (OR = 2.29, $P = 0.042$), *Leuconostoc* (OR = 21.42, $P = 0.018$) and *Odoribacter* (OR = 6.11, $P = 0.003$) in faeces, and an under-representation of *Desulfovibrio* (OR = 0.03, $P = 0.037$) and *Oribacterium* (OR = 0.17, $P = 0.041$) in the ascending colon, and of *Brevundimonas* (OR = 0.09, $P = 0.009$) and *Butyricicoccus* (OR = 0.38, $P = 0.026$) in the descending colon. We found evidence for the following changes in IBS-C subtype cases compared with controls: an increase in *Alistipes* (OR = 5.82, $P = 0.01$) and *Butyricimonas* (OR = 3.27, $P = 0.004$) in faeces, as well as an increase in *Bacteroides* (OR = 3.15, $P = 0.039$) and a decrease in *Coprococcus* (OR = 0.03, $P = 0.007$), *Eubacterium* (OR = 0.08, $P = 0.044$), *Fusobacterium* (OR = 0.02, $P = 0.036$), *Haemophilus* (OR = 0, $P = 0.019$), *Neisseria* (OR = 0.02, $P = 0.037$), *Odoribacter* (OR = 0.14, $P = 0.02$), *Streptococcus* (OR = 0.06, $P = 0.007$) and *Veillonella* (OR = 0.03, $P = 0.044$) in the descending colon. Of these altered genera, *Alistipes, Bacteroides, Butyricimonas, Eubacterium*, *Fusobacterium, Odoribacter* and *Streptococcus* had a relative abundance greater than 1%. These results are in agreement with recent research on IBS that points to subtle rather than pronounced compositional deviations in the gut microbiota of IBS patients (Salonen *et al*., 2010).

To establish whether the changes seen at the genus level were due to one, a few or many bacterial species/strains within them, we also analysed the compositional differences between patients and controls for each phylotype defined at 97% of sequence identity (Table S2). We found several phylotypes within the same genus with an altered abundance. Differences of opposite sign were found for phylotypes belonging to the same genus (e.g. within *Bacteroides* and *Alistipes*). Only a few differences between IBS cases and controls were shared between sample types or IBS subtypes. One phylotype was consistently over-represented in the colon mucosa and faeces of IBS-D subtype patients. It accounted for ≈ 2% of the sequences in these samples and was closely related to *Bacteroides vulgatus* (96.52% identity).

None of the statistically significant changes in the relative abundance of genera were common to all or even two of the sampling sites, and only a few were consistent at the phylotype level. In faeces, we found more significantly over-represented taxa in IBS patients with respect to controls. In contrast, mucosal sites showed more under-represented taxa in IBS cases compared with controls. The fact that bacterial communities within the colon and in faeces are affected differently by the disorder must serve to caution researchers about the risk of inferring the role of endogenous microbiota in IBS from that found in faeces, especially considering that the mucosa-associated microbiota may have a more relevant pathogenic role than the faecal one because it is closer to host epithelial and immune cells.

Three precedent studies compared mucosal and faecal bacterial communities of IBS patients and controls, though not via high-throughput sequencing. In contrast to our study, Kerckhoffs and colleagues (2009) found a decrease in *Bifidobacterium* in both sample types in the IBS patients, whereas Carroll and colleagues (2010a) found an increase in *Lactobacillus* in faeces, both using group-specific probes. Carroll and colleagues (2010b) characterized the faecal and sigmoid mucosal microbiota of IBS-D patients and controls by T-RLFP fingerprinting. They found significant differences in the bacterial composition between patients and healthy controls and a higher diversity in the former only in the mucosal communities. However, we found a lower diversity and alterations in composition in IBS patients compared with controls in both colon mucosal and faecal communities.

Ascending colon/descending colon/faeces

Faeces showed lower richness and diversity than biopsies in IBS patients and in healthy controls, as measured by the Shannon diversity indices (H) and the Chao1 and ACE richness estimators (Table 1), with few differences between ascending and descending colon (except for the descending colon of the IBS-C subtype). The ascending and the descending colon harboured similar communities. The *Bacteroidetes* phylum accounted for 58% of the sequences in the colon mucosa and 72% in faeces. In contrast, 29% of the sequences in mucosa belonged to the *Firmicutes* phylum compared with 21% in faeces, and 9.5% of the sequences in mucosa and 0.9% in faeces belonged to *Proteobacteria*. At family level, faeces had more members of *Rikenellaceae* (11.3% in faeces versus 2.4% in mucosa) and less of *Lachnospiraceae* (2.1% versus 7.6%) and *Streptococcaceae* (0.1% versus 2.3%) (Fig. S1). These differences between mucosal sites and faeces were found statistically significant in at least two thirds of the individuals. Comparisons between the bacterial composition of the communities found in the ascending colon, descending colon and faeces at the genus level are shown in Table S3. We found a statistically significant under-representation in colon mucosa with respect to faeces of *Barnesiella* (OR ≈ 0.20) and *Alistipes* (OR ≈ 0.19), and an over-representation of *Streptococcus* (OR ≈ 39.50) and *Dorea* (OR ≈ 7.75). Results were consistent within the IBS and the control

cohorts, further confirming the shifts detected. These patterns could be due to differences in the ecological conditions between the two environments, to the susceptibility of species in the intestine to ending up in faeces and to the possibility that faeces better represent luminal than mucosal-associated microbiota. The results of this study are overall in agreement with previous work by Durbán and colleagues (2011) comparing faeces and rectal mucosa in healthy individuals, despite biopsies there were collected without any previous colon preparation and the entire 16S rRNA gene, instead of the V1-V2 region, was targeted. Eckburg and colleagues (2005), Ott and colleagues (2004) and Willing and colleagues (2010) also found differences in the composition of bacterial communities in intestinal mucosa compared with faeces. In addition, they found lower diversity in colon biopsies than in faecal samples, in contrast to our study. This could be partly explained by the different experimental methods used for sample processing and DNA extraction as well as the different target regions of the 16S rRNA considered.

There are several explanations for the lack of reproducibility in the transversal studies carried out so far on the role of gut microbiota in IBS. Dysbiosis in IBS seems to be characterized by subtle alterations instead of the high-level phylogenetic alterations that occur in other pathologies such as obesity (Ley *et al*., 2006) or inflammatory bowel diseases (Qin *et al*., 2010). The use of relatively small cohorts and the high level of inter-subject variability in microbiota composition unrelated to the pathologic state make difficult the detection of such subtle changes. In this study, no patterns relating bacterial composition and clinical and lifestyle factors (Table S4) were detected in canonical correspondence analyses. Also, IBS patients with heterogeneous aetiology and symptoms at the moment of sampling could have been included in these studies, making the detection of patterns further difficult.

Recently, Arumugam and colleagues (2011) found three main profiles in the microbiota composition in faeces. These enterotypes are another source of variation in the composition of the microbiota between individuals that could affect the comparison between IBS patients and controls if individuals from a single enterotype dominated each group and the enterotype of the cases was different from that of the controls. However, the differences we found between IBS patients and controls do not point towards the differences reported between enterotypes by Arumugam *et al*. Longitudinal studies with sampling at moments with different symptoms would help to mitigate the confusion caused by inter-subject variability and heterogeneity within IBS that is problematic in cross-sectional studies. Further research is also needed to assess the implication of the gut microbiota in IBS from a functional perspective.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Percentage of sequences (grouped into six intervals) at phylum, class, order and family taxonomic levels belonging to the pooled samples of healthy controls (HC), diarrhoea-predominant IBS patients (IBS-D) and constipation-predominant IBS patients (IBS-C).
Fig. S2. Distribution of the odds ratio values calculated for the IBS-diarrhoea subtype and HC pooled samples and 999 replicas obtained after random labelling the individual ones as IBS-diarrhoea subtype or control and then aggregating them in new pooled samples. Red lines indicate the value for the comparison of the actual pooled samples.
A and B. examples of genera for which the change detected in the pooled samples is well supported by the individual samples.
C and D. examples of genera for which the change is not supported.
Table S1. Comparison between controls (HC) and IBS-diarrhoea subtype (IBS-D) (a), and between controls and IBS-constipation subtype (IBS-C) (b). It is shown the odds ratios (OR) for the comparison between IBS and control pooled samples at genus level for genera in which significant differences were found; the Gini coefficients for the abundances of each genus in the individual samples within the IBS and control groups; the 'OR rank', which is the rank of the OR value for the IBS and control pooled samples when compared with the values of 999 simulated pooled samples obtained after random labelling the individual ones as IBS or control (extreme values are indicative of true association).
Table S2. Odds ratios for the comparisons between sampling site-pooled samples (ascending colon, descending colon and faeces) at phylotype level (clusters at 97% of sequence identity). In the comparison X versus Y, phylotypes over-represented in X compared to Y are in red, those under-represented are in green. Only phylotypes for which significant differences between the two pooled samples under comparison were found in a chi-squared test that were also supported by a permutation test and with a relative abundance greater than 0.1% are shown. The closest isolates are the species of best blast hits with more than 96% of sequence identity and more than 95% of query coverage.
Table S3. Odds ratios for the comparisons between sampling site-pooled samples (ascending colon, descending colon and faeces) at genus level. Genera for which significant differences between two pooled samples were found in the chi-squared test (corrected P-value < 0.05) are coloured. In the comparison X versus Y, genera over-represented in X compared with Y are in red, those under-represented are in green. The support for the trends between pooled samples is shown as the number of individuals for which significant over- and under-representations were found, distinguishing between the IBS and control cohorts. In the comparison X versus Y, upward arrows mean over-representation in X compared to Y; downward arrows mean under-representation. 'n', number of individuals included in each group.
Table S4. Summary of physical, lifestyle and clinically relevant characteristics of the subjects included in this study.
Appendix S1. Experimental procedure.

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