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Functional dysbiosis within the gut microbiota of patients with constipated-irritable bowel syndrome

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SUMMARY

Background
The role of the gut microbiota in patho-physiology of irritable bowel syndrome (IBS) is suggested by several studies. However, standard cultural and molecular methods used to date have not revealed specific and consistent IBS-related groups of microbes.

Aim
To explore the constipated-IBS (C-IBS) gut microbiota using a function-based approach.

Methods
The faecal microbiota from 14 C-IBS women and 12 sex-match healthy subjects were examined through a combined strictly anaerobic cultural evaluation of functional groups of microbes and fluorescent *in situ* hybridisation (16S rDNA gene targeting probes) to quantify main groups of bacteria. Starch fermentation by C-IBS and healthy faecal samples was evaluated *in vitro*.

Results
In C-IBS, the numbers of lactate-producing and lactate-utilising bacteria and the number of H_2-consuming populations, methanogens and reductive acetogens, were at least 10-fold lower ($P < 0.05$) compared with control subjects. Concomitantly, the number of lactate- and H_2-utilising sulphate-reducing population was 10 to 100 fold increased in C-IBS compared with healthy subjects. The butyrate-producing *Roseburia – E. rectale* group was in lower number ($0.01 < P < 0.05$) in C-IBS than in control. C-IBS faecal microbiota produced more sulphides and H_2 and less butyrate from starch fermentation than healthy ones.

Conclusions
A major functional dysbiosis was observed in constipated-irritable bowel syndrome gut microbiota, reflecting altered intestinal fermentation. Sulphate-reducing population increased in the gut of C-IBS and were accompanied by alterations in other microbial groups. This could be responsible for changes in the metabolic output and enhancement in toxic sulphide production which could in turn influence gut physiology and contribute to IBS pathogenesis.

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INTRODUCTION

Irritable bowel syndrome (IBS) is a frequent functional gastrointestinal (GI) disorder defined by abdominal pain or discomfort and modifications in bowel habits in the absence of organic cause.[1] Although morbidity of IBS remains very low, its detrimental impact on quality of life together with its worldwide prevalence (10–20%) and the absence of curative therapy explains the considerable economic impact of this disorder.[2] The symptoms of IBS vary between affected individuals, but are better defined from a clinical point of view by the Rome criteria.[1, 3] The aetiology and pathophysiology of IBS remains poorly understood and is most likely multifactorial. Multiple interacting mechanisms may contribute to the development of IBS symptoms. Dysregulation of brain-gut interactions, generating gut dysmotility and visceral hypersensitivity, are considered as important factors in the pathology, although the causes of these features have not yet been determined.[4] Other factors include psychological stress, low-grade inflammation potentially following GI infections and alteration within the gut microbiota.[5, 6]

The microbiota of the normal human intestine represents a complex mostly anaerobic ecosystem that plays a key role in maintenance of health and physiological functions of the host. This microbiota acts as a barrier against pathogens, stimulates the host immune system and produces a great variety of compounds from the metabolism of dietary and endogenous substrates that could affect the host. Disruption of the microbial ecosystem has been reported in different pathologies including inflammatory bowel disease and type-2 diabetes.[7, 8] Such microbial alteration may also be involved in the onset and maintenance of IBS. Indeed, IBS frequently follows antibiotic therapies or gastroenteritis. Furthermore, disturbances in the composition and stability of the gut microbiota were reported in IBS individuals compared with healthy ones.[9–14] Using standard cultural methods and culture-independent approaches, these studies showed abnormal variations within the faecal IBS microbiota affecting different bacterial groups, the most reproducible results concerning alterations in the *Bifidobacterium* and *Clostridium coccoides – E. rectale* subgroup. Specific IBS-related groups of microbes were not revealed from these studies. However, these approaches that quantified phylogenetic groups of bacteria could not assess the functional groups of microbes, i.e. all the bacterial species sharing the same metabolic activity.

Carbohydrate metabolism by gut microorganisms is a central process allowing supply of nutrients and energy to the host. This fermentative process is complex and involves several functional groups of bacteria with complementary metabolic activities that interact to ensure the biotransformation of polymers (resistant starch, nonstarch polysaccharides, proteins, mucins…) into end-products (mainly short chain fatty acids and gases). Hydrolytic communities transform complex substrates into smaller fragments that can also be used by other bacterial groups unable to hydrolyse polymers. Other microbial cross-feeding interactions are related to the utilisation of fermentative products such as succinate, lactate[15, 16] or hydrogen[17] and involve specific groups of microorganisms. Elimination of hydrogen, the main gas produced from organic matter fermentation, is essential to maintain efficient fermentation in the gut. Its main route is utilisation by H_2-consuming micro-organisms which comprise methanogenic archaea, sulphate-reducing and/or reductive acetogenic bacteria.[18] Abnormality in microbial fermentation has already been suggested in IBS patients.[19, 20] In keeping with this, a range of fermentable dietary carbohydrates can exacerbate or provoke gastrointestinal symptoms through their fermentation by the gut microbiota.[21]

We hypothesised that a functional dysbiosis might exist within IBS intestinal microbiota, inducing alteration in carbohydrate metabolism. We thus used a function-based approach of the ecosystem to compare the gut microbiota of IBS patients to that of healthy controls. This approach, which has been validated in healthy subjects,[22] combines cultural evaluation of functional groups of microbes and fluorescent *in situ* hybridisation (FISH). Functional groups of microbes can only be quantified using specific cultural methods. By contrast, most of the phylogenetic groups composing the gut microbiota cannot be selectively cultivated and are quantified using molecular approaches based on 16S rDNA gene sequence. The metabolic capability of the IBS and healthy microbiota was further evaluated *in vitro*. Our work was focused on one IBS subtype, the constipated-IBS (C-IBS), to reduce heterogeneity between the IBS subjects studied, especially the variation in gut microbial composition due to different modifications of transit time. Only women were recruited for this study as they are more affected by IBS than men.

MATERIALS AND METHODS

Subjects

We characterised the faecal microbiota from 14 patients with C-IBS and 12 sex-matched healthy subjects (age range 20–59 years). All participants have undergone clinical investigation by experienced physicians. The 14 women IBS patients (age range: 36–59, mean age: 48)

included in this study fulfilled the Rome II criteria for IBS.[3] They were classified as IBS with constipation (C-IBS) by a questionnaire following the Rome II subgrouping criteria[3] which have been recognised as valid also in the Rome III criteria.[1] The C-IBS subjects included in the study showed moderated bowel trouble (stool frequency of 2 to 3 days). Twelve healthy women (age range: 20–53, mean age: 30), without gastro-intestinal symptom and with a normal stool frequency (one to two stools per day) were recruited to form the control group.

Exclusion criteria included organic intestinal disease, other systemic disease, previous abdominal surgery, lactation, pregnancy, dementia or inadequate cooperative capability and antibiotic therapy during the previous 2 months. IBS patients were advised not to take laxatives or antidiarrhoeals as well as antispasmodic and/or analgesic during the week prior to the faecal sampling.

All control and IBS volunteers were instructed to follow their ordinary western diet. The daily ingestion of 15 g to 30 g of dietary fibres represented a selective inclusion criterion for both healthy and IBS subjects that was evaluated by a dietician through dietary questionnaires. In the 2 weeks prior to faecal sampling, subjects were requested not to consume yogurt and all others probiotic containing products. All volunteers had a normal body mass index (BMI between 18 and 25 kg/m^2).

Ethical considerations

All participants gave their written informed consent to the protocol and were permitted to withdraw from the study at any time. The study was approved by the local Human Ethics Committee (CPP Sud-Est VI, France).

Faecal samples

Freshly voided faeces were obtained from all volunteers, stored under anaerobic conditions and processed within 8 h. One gram of collected faecal sample was diluted 10-fold (wet w/v) in an anaerobic mineral solution. Serial 10-fold dilutions down to 10^{-12} were then carried out. Separate aliquots of the faeces samples were immediately prepared for subsequent FISH analysis. The fresh samples (0.5 g) were thoroughly mixed with 4.5 mL phosphate buffered saline and fixed by storing 1:3 (v/v) in 4% (w/v) paraformaldehyde at 4 °C for 16 h. Aliquots of the fixed samples (0.8 mL) were then stored at −20 °C.

Media and enumeration procedures of functional groups of microorganisms

Functional groups of microorganisms were enumerated in faecal samples by the Most Probable Number (MPN) method as previously described.[22] Briefly, all liquid media were prepared, dispensed and inoculated using strictly anaerobic techniques, with 100% O_2-free CO_2 gas.[23] Total anaerobes, hydrolytic, H_2-consuming and lactate-utilising communities were enumerated in selective liquid medium. A series of three liquid culture tubes were inoculated per faecal dilution (10^{-3} to 10^{-11}) for each selective medium. After incubation at 37 °C, the number of positive or negative culture tubes for MPN estimation was based on the detection of specific activity (production of enzymes, production of metabolite… etc.). Estimations of MPN were thus made according to Clarke and Owens.[24]

Total anaerobes were enumerated in a clarified rumen fluid containing medium.[25] Hydrolytic bacteria were enumerated in the basal medium previously described,[26] containing specific carbon sources added to the medium. The substrates used for enumeration of hydrolytic communities were: Sigmacell 101 cellulose (Sigma chemicals Co, St Louis, MO, USA) for cellulolytic population, oat spelts xylan (Sigma) for xylanolytic community, dietary-fibre derived substrates enriched in different cell-wall polysaccharides (spinach and wheat) for total fibre-degrading bacteria; potatoes starch (Sigma) for starch-degrading bacteria; porcine stomach mucin (Sigma) for mucin-fermenting bacteria and casein (Sigma) for proteolytic bacteria. The substrate was added to the basal medium at a final concentration of 7 g/L. The cellulolytic, xylanolytic and total fibre-degrading populations were enumerated as previously described[26, 27] by detecting specific hydrolases (carboxymethylcellulase or xylanase) in each culture obtained from MPN determinations. Starch-degrading bacteria, mucin-fermenting bacteria and proteolytic bacteria were enumerated by recording bacterial growth in culture tubes obtained from each dilution inoculated (10^{-7} to 10^{-12}) and by measuring the production of short chain fatty acid by H^1 NMR.[28]

H_2-utilising methanogens, sulphate-reducing bacteria and reductive acetogens were enumerated following the method previously described.[29] H_2-metabolism was evaluated in each culture obtained from these MPN determinations. Methane production was analysed by gas phase chromatography in methanogens MPN determination whereas acetate production was determined by enzymatic kit (enzymatic kit, Enzytec, Scil, Viernheim, Germany) in vials from acetogens MPN. Formation of black precipitate of FeS was recorded for MPN determination of sulphate-reducing bacteria.

Lactate-utilising bacteria were enumerated in L-lactate (35 mM) containing basal medium.[26] After incubation at

37 °C for 5 days, the concentration of the remaining lactate was determined (enzymatic kit, Enzytec, Scil, Viernheim, Germany). Vials with lactate concentration below 20 mM (i.e. lactate consumption of at least 10 mM) were scored positive. Lactate-utilising sulphate-reducing bacteria were enumerated in the Postgate E medium.[30]

In addition, total facultative anaerobes, bifidobacteria, lactobacilli and *Enterobacteriaceae* were enumerated in selective agar media. Colonies developed on these solid media were counted after incubation at 37 °C (CFU/g faeces). Facultative anaerobes were cultivated on G20 agar medium containing peptone (15 g/L), tryptone (10 g/L), yeast extract (5 g/L), glucose (20 g/L) and bacteriological agar type A (10 g/L), with pH adjusted to 7.7. Bifidobacteria were cultivated on Man Rogosa Sharp (MRS) agar medium (Oxoid) adjusted to pH 7.0, and incubated for 3 days in an anaerobic cabinet. Lactobacilli were cultivated on MRS agar medium adjusted to pH 5.5 and incubated aerobically. *Enterobacteriaceae* were cultivated on MacConkey agar medium (Roth) and incubated aerobically for 2 days at 37 °C.

Fluorescent *In situ* hybridization analysis

FISH analysis of the faecal bacterial composition was performed on the paraformaldehyde-fixed samples that have been stored at −20 °C as described above.[31, 32] Briefly, appropriate dilutions of cell suspensions were applied to gelatine-coated slides and allowed to dry. These slides were fixed in 96% ethanol for 10 min, dried and stored for up to 3 months. Slides were then hybridised overnight with 10 μL of the respective oligonucleotide probe (50 ng/μL stock solution) at the appropriate incubation temperature. After further incubation in washing buffer and rinsing in water, slides were dried and each well overlaid with 10 μL Vectashield (Vector Laboratories, Inc., Peterborough, CA, USA) and sealed using a cover slip.[32] Cells were counted manually using a DMRXA epifluorescence microscope (Leica, Milton Keynes, UK). Total bacterial numbers were estimated with Eub338 probe whereas other main bacterial groups were assessed using one of a panel of oligonucleotide probes as previously described.[22, 32] This set of probes has been previously validated to detect most of the bacterial groups present in human faeces.[32]

In vitro starch fermentation by faecal microbiota

Faecal samples from four healthy (2 methanogen- and 2 nonmethanogen harbouring subjects) and eight C-IBS subjects (3 methanogen- and 5 nonmethanogen harbouring subjects) were used for *in vitro* fermentation assays. Each faecal sample was 10-fold diluted in N_2-saturated phosphate buffer (100 mM, pH 6.8). The suspension was mixed in a stomacher for 1 min and further filtered through a surgical gaze. A bacterial pellet was obtained from the filtered liquid by centrifugation at 27 000 *g* for 30 min at 4 °C and three successive washes with phosphate buffer. It was then half diluted in buffer (w/v) to constitute the inoculum. Four mL of this bacterial suspension were inoculated to 120 mL-penicillin flasks containing 30 mL of anaerobic basal medium[26] with potatoes starch (4 g/L) as carbohydrate source. Three flasks were inoculated per faecal samples. After 24 h incubation at 37 °C, the composition of the gas phase was analysed by gas chromatography.[22] Short chain fatty acids production was analysed by ^{1}H NMR[28] in suspension after centrifugation at 15 000 *g* for 30 min. Sulphides concentration was determined in these suspensions by photometric kit (Sulfid-test, Merck, Germany).

Statistical analysis

Microbial counts were expressed as $\log_{10}$ micro-organisms per gram of wet weight faeces. Data are expressed as mean ± s.d. Statistical analyses were performed with the GraphPad Instat statistical software (La Jolla, CA, USA). Student's *t*-test was used to compare means. All tests were two-tailed paired and the level used to establish significance was $P < 0.05$.

RESULTS

Enumeration of functional groups of microorganisms in faecal samples from C-IBS vs. healthy subjects

Total strict and facultative anaerobes. The total viable counts of strict and facultative anaerobes in faecal samples of the C-IBS patients were not significantly different from those of the healthy control subjects (Table 1). The population of facultative anaerobes represented less than 1% of total anaerobes in faecal samples from both patients and healthy subjects. The population level of *Enterobacteriaceae,* however, showed a significant increase of 10-fold in C-IBS compared with healthy individuals.

Hydrolytic bacterial communities. The hydrolytic bacterial communities, which play key roles in the gut ecosystem by transforming polymers into smaller fragments through the synthesis of specific degradative enzymes, were found at rather similar levels in C-IBS and in healthy control subjects (Table 1). All were present at high levels in all faecal samples, indicating that these bacterial groups predominate in both the C-IBS and

Table 1 | Comparison of the population levels of microbial communities (expressed as $\log_{10}$ microorganisms per gram faeces) involved in carbohydrate metabolism in faecal samples from healthy and C-IBS individuals

$\log_{10}$ N/g faeces	Healthy subjects (n = 12)	C-IBS subjects (n = 14)	P
Total strict anaerobes	11.3 ± 0.3	11.4 ± 0.4	N.S.
Total facultative anaerobes	8.5 ± 0.9	8.6 ± 0.8	N.S.
Enterobacteriaceae	6.4 ± 0.9	7.4 ± 0.8	0.0107
Hydrolytic populations			
Starch-degrading bacteria	10.8 ± 0.8	11.0 ± 1.2	N.S.
Cellulose-degrading bacteria	9.2 ± 0.6	8.6 ± 0.9	N.S.
Xylan-degrading bacteria	10.1 ± 0.4	9.7 ± 0.8	N.S.
Spinach-degrading bacteria	8.4 ± 0.6	8.4 ± 0.7	N.S.
Wheat-degrading bacteria	9.0 ± 0.8	8.6 ± 0.9	N.S.
Proteolytic population	9.8 ± 0.9	9.8 ± 0.5	N.S.
Mucin-degrading population	10.4 ± 0.8	10.7 ± 1.2	N.S.
Lactic acid producing bacteria			
Bifidobacteria	7.8 ± 0.5	6.8 ± 0.7	<0.0001
Lactobacilli	6.9 ± 0.7	5.5 ± 0.9	0.0007
Lactate utilising bacteria			
Lactate-utilising bacteria	9.3 ± 0.4	7.9 ± 1.2	0.0046
Suphate-reducing bacteria	5.9 ± 0.4	8.4 ± 0.3	0.0002

N.S., not significantly different ($P > 0.05$).

healthy gut ecosystem. Starch and xylan-degrading bacteria, as well as the mucin-degrading bacteria, were in larger numbers than the other microbial sub-populations in both subject groups.

Bacterial populations involved in lactate metabolism. The bacterial populations involved in the metabolism of lactate, an important intermediate metabolite in the gut, showed significant differences in their distribution in C-IBS patients compared with healthy ones (Table 1). The lactic acid bacteria, mainly represented by bifidobacteria and lactobacilli, were present in significantly lower numbers ($P < 0.01$) in C-IBS patients than in healthy individuals. The number of bifidobacteria was more than 10-fold lower in C-IBS compared with control subjects, as further confirmed by FISH analysis of the microbiota (Figure 1b). Lactobacilli, found in lower numbers than bifidobacteria in faecal samples of healthy subjects, were also found to be significantly decreased by 10-fold in C-IBS patients by cultural methods. The lactate-utilising bacterial population that had an average count of 10^9 bacteria per g faeces in healthy subjects, was also significantly decreased ($P < 0.01$) by 10-fold in C-IBS patients (Table 1). In contrast, the population level of the lactate-utilising sulphate-reducers was highly significantly increased by 100-fold in C-IBS patients compared with healthy ones (Table 1).

H_2-utilising microbial communities. Methanogen- and nonmethanogen harbouring subjects were found in both C-IBS and healthy groups (Table 2). However, the number of methanogen-harbouring subjects (more than 10^7 methanogens/g wet faeces) was higher in the C-IBS group compared with the healthy one. In methanogen-harbouring subjects, the population level of methanogens was found to be quite significantly decreased ($0.01 < P > 0.05$) in C-IBS compared with healthy subjects (Table 2). Similarly, the population level of reductive acetogens was 10-fold decreased ($P < 0.01$) in nonmethanogen-harbouring C-IBS compared with healthy ones (Table 2). By contrast, a strongly significant higher number of H_2-consuming sulphate-reducing bacteria (SRB) was found in all C-IBS patients compared with healthy ones ($P < 0.01$), whatever their methanogen-harbouring status. Analysis of the distribution of this SRB group in faecal samples of healthy and C-IBS subjects showed that most of the C-IBS patients (10 out of 14 subjects) harboured more than 10^7 SRB per g faeces whereas the majority of control subjects had less than 10^6 SRB per g faeces.

FISH analyses of the faecal microbiota composition in C-IBS patients vs. healthy subjects

The composition of the faecal microbiota from the 12 healthy and 14 C-IBS volunteers was analysed by FISH using group-specific probes (Figure 1). The most

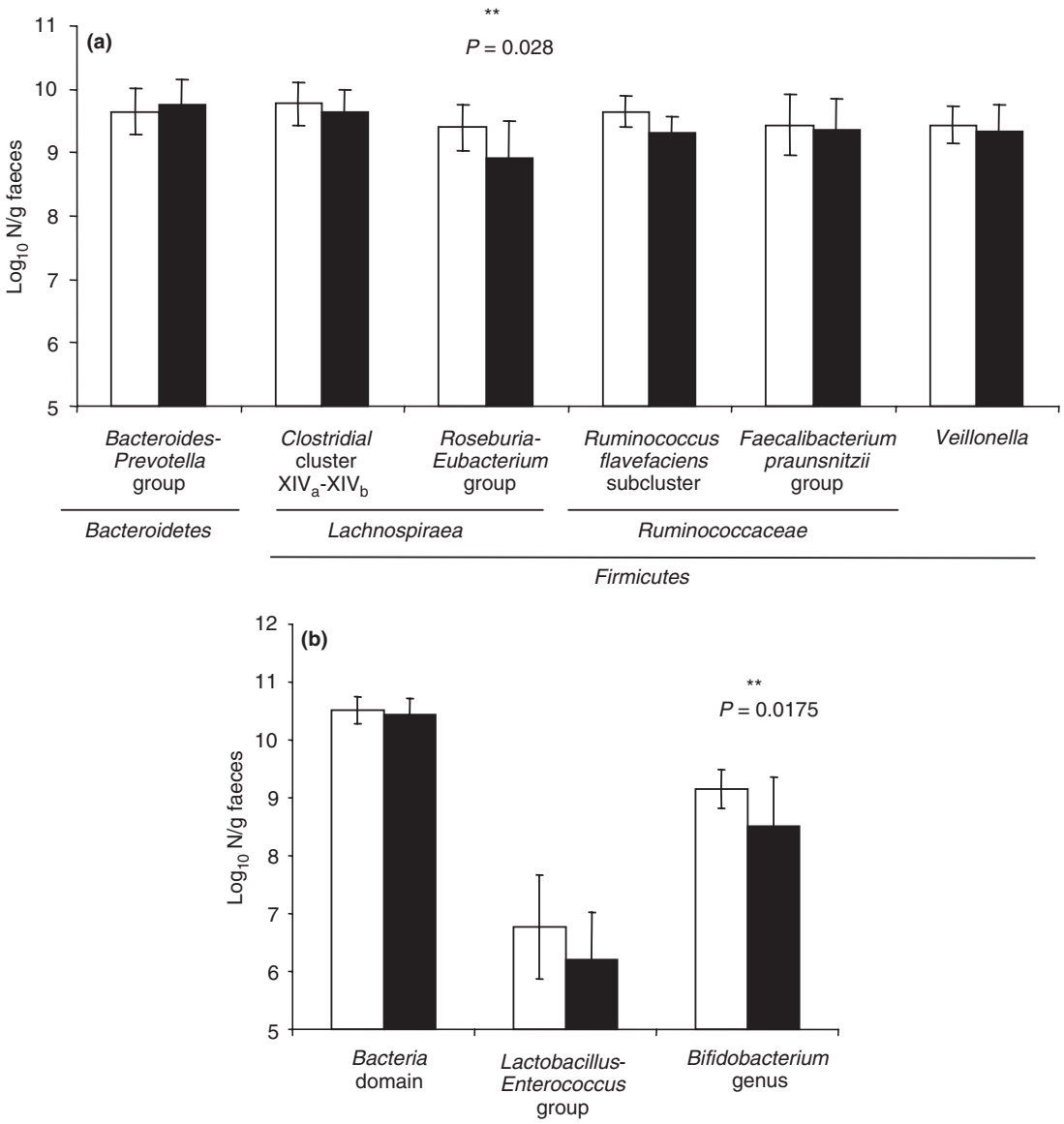

Figure 1 | Total counts of main bacterial taxonomic groups as obtained by FISH analysis in faeces of healthy (n = 12) (□) and IBS (n = 14) (■) individuals. (a) Main *Bacteroidetes* and *Firmicute* groups, (b) Total bacteria and main lactic acid bacteria.

abundant groups, detected at similar levels in both healthy and C-IBS faecal samples, were the predominant *Bacteroides-Prevotella* group and the *Lachnospiraceae* (Clostridial cluster XIV). The *Ruminococcaceae* (Clostridial cluster IV) and the *Veillonellaceae* (Clostridial cluster IX) accounted for about 10 to 12% of bacterial cells in healthy individuals as well as in C-IBS patients.

The numbers of *Roseburia – E. rectale* group (Figure 1a), a predominant butyrate-producing bacterial group of *Lachnospiraceae* in the human gut,[33] and of bifidobacteria (Figure 1b) were detected at quite significantly lower levels in C-IBS patients compared with healthy subjects ($0.01 < P > 0.05$). By contrast, there was no significant difference in the number of lactobacilli and of *F. prausnitzii*, another important butyrate-producing bacterial species, between C-IBS and healthy subjects.

Starch fermentation by faecal microbiota from C-IBS and healthy subjects

No differences were observed in fermentative pattern between methane- and nonmethane producing faecal samples in both subjects groups (healthy and C-IBS),

Table 2 | Distribution of H_2-utilising communities (expressed as log_{10} microorganisms per gram faeces) in faecal samples of healthy and C-IBS volunteers

Log_{10} N/g faeces	Healthy subjects	C-IBS subjects	*P*
Methanogens			
CH_4^{+*}	9.71 ± 0.24 (*n* = 3)	8.25 ± 0.77 (*n* = 8)	0.0344
CH_4^-	5.78 ± 0.69 (*n* = 9)	5.41 ± 0.98 (*n* = 6)	N.S.
Reductive acetogens			
CH_4^+	5.18 ± 0.20 (*n* = 3)	4.11 ± 1.02 (*n* = 8)	N.S.
CH_4^-	6.1 ± 0.62 (*n* = 9)	4.71 ± 0.98 (*n* = 6)	0.0049
Suphate-reducing bacteria	6.09 ± 1.2 (*n* = 12)	7.65 ± 1.00 (*n* = 14)	0.0029

n, number of subjects; N.S., not significantly different ($P > 0.05$).

* CH_4^+: subjects harbouring more than 10^7 archaea methanogens/g faeces CH_4^-: subjects harbouring less than 10^7 archaea methanogens/g faeces.

Table 3 | Main end-products of *in vitro* starch fermentation by faecal microbiota from healthy (*n* = 4) and C- IBS (*n* = 8) subjects

	Healthy (*n* = 4)	C- IBS (*n* = 8)	*P*
Sulphides (μg/mL)	493 ± 160	1150 ± 120	<0.0001
Hydrogen (μmol/mL)	0.3 ± 0.3	1.4 ± 0.6	0.0087
Methane (μmol/mL)	5.5 (*n* = 2)	0.8 ± 0.8 (*n* = 3)	
Total SCFA (mM)	71.4 ± 12.1	60.7 ± 12.8	N.S.
Acetate(mM)	46.1 ± 6.5	41.4 ± 9.8	N.S.
Propionate (mM)	13.1 ± 4.4	9.7 ± 2.1	N.S.
Butyrate(mM)	12.3 ± 1.7	9.6 ± 1.9	0.0377
Lactate (mM)	1.1 ± 0.5	1.2 ± 0.7	N.S.

n = number of faecal samples producing methane (methane was only detected in faecal incubations from two healthy and three C-IBS subjects); N.S., not significantly different ($P > 0.05$).

except methane production. By contrast, starch fermentation by C-IBS faecal microbiota showed major differences compared with healthy ones (Table 3), especially in gaseous metabolite production. Sulphide concentration increased 2-fold in C-IBS faecal incubations compared with healthy ones, whatever the methane-status of the samples. Similarly, the amount of hydrogen produced increased 4-fold in C-IBS compared with control incubations. Methane, produced by two of the four healthy and three of the eight IBS subjects studied, was 2-fold decreased in C-IBS faecal incubation compared with healthy samples. The total amount of SCFA produced by C-IBS microbiota was not different than that of healthy ones. The quantities of acetate and propionate produced by C-IBS microbiota was rather similar to that formed by healthy ones. Lactate was found in very low concentration in both C-IBS and healthy faecal samples. Butyrate was the only SCFA found to be in quite significantly lower concentration in C-IBS faecal incubation compared with healthy ones ($0.01 < P > 0.05$).

DISCUSSION

Using a function-based approach to analyse the intestinal microbial ecosystem, we have demonstrated, in the present study, a critical functional dysbiosis in C-IBS gut microbiota, which can ultimately alter intestinal fermentative processes and host physiology. Microbial alterations identified here in C-IBS may be involved in genesis of different IBS symptoms, suggesting that these findings might be applicable to other IBS subtypes.

Previous studies have suggested that abnormalities of the intestinal microbiota occur in IBS.[9–14, 34, 35] However, pronounced deviations within taxonomic groups of bacteria were not identified. As previously reported,[13] we found no difference between healthy and C-IBS subjects in either the total bacterial number or the major bacterial groups (*Bacteroides*, *Lachnospiraceae* and *Ruminococcaceae*) that compose the gut microbiota. A decrease in the lactic acid bacteria population (bifidobacteria and to a lesser extent, lactobacilli) was observed in faecal microbiota of our C-IBS patients, as also reported in several studies.[9, 10, 13, 14, 34, 35] The number of *Enterobacteriaceae* was shown to be increased in C-IBS compared with healthy individuals also as previously shown.[9] Bifidobacteria are considered beneficial for the host[13] in particular as they can inhibit growth of potential pathogenic bacteria. In C-IBS, the decrease in the bifidobacteria population may thus potentially affect gut heath by promoting growth of *Enterobacteriaceae*.

The function-based approach used in our study, has allowed demonstrating that the C-IBS gut microbiota is characterised by an important functional imbalance that was not detected using molecular approaches. Although molecular approaches mostly target one specific bacterial gene (16S ribosomal DNA gene), the functional approach is based on the detection of specific metabolic activity expressed by a group of bacterial species. This

cultural method allows detecting and enumerating all viable microorganisms present in the gut, whatever their population level, whereas molecular approaches detected both dead and alive microbes with a detection limit closed to 10^6 to 10^7/g faeces for most of the methods.

Using the function-based approach, we did not observe significant differences in the distribution of predominant hydrolytic microorganisms involved in degradation of macromolecules such as fibre, protein or mucin, and belonging to the main bacterial groups of the gut microbiota, between C-IBS patients and controls. By contrast, we were able to identify important alterations in the population levels of major microbial groups involved in lactate and H_2 metabolism as well as in butyrate synthesis. In particular, the C-IBS microbiota was characterised by a high number of lactate- and H_2-utilising sulphate-reducing bacteria (SRB) compared with healthy subjects. Lactate and H_2 are two of the main intermediate metabolites in the gut that support growth of various lactate-utilising and H_2-consuming microorganisms. Among these microbial communities, SRB represent a group of bacteria that is able to use sulphate as terminal electron acceptor to form H_2S with a wide range of substrates as electron donors, including lactate and H_2. SRB are known to compete efficiently for utilisation of these two substrates in the human gut.[36, 37]

Lactate is quickly metabolised by specific bacterial species in the healthy gut microbiota into butyrate or propionate.[15, 22, 38] The number of these lactate-utilising bacteria was decreased 10-fold in the faecal microbiota of C-IBS patients compared with healthy ones. Concomitantly, the number of lactate-utilising SRB was increased by a 2 log-order in IBS compared with healthy subjects. This represents a major shift in the composition of the lactate-utilising community which is likely to be accompanied by a major shift in fermentation products. Lactate utilisation by SRB rather than by the non-SRB lactate-utilising community could explain the enhancement in sulphides production at the expense of butyrate formation observed *in vitro* in faecal sample incubations.

The slight decrease in butyrate production by C-IBS microbiota could further be due to the decrease in the number of certain butyrate-producing bacteria. FISH analysis showed that the population level of the *Roseburia – E. rectale* group (belonging to *Lachnospiraceae*), was lower in C-IBS subjects compared with control individuals, as previously reported.[11] The reduction in butyrate production in C-IBS gut may reduce the potential health benefit from this metabolite, including anti-inflammatory effects, colonic defence barrier and decrease in oxidative stress.[39] Butyrate oxidation by colonocytes was further shown to be altered by increasing H_2S concentration.[40]

Hydrogen is another important fermentative metabolite that is mostly removed from the ecosystem by H_2-consuming microorganisms (methanogenic archaea, reductive acetogens or sulphate-reducing bacteria). In C-IBS, H_2-utilising SRB were found in higher numbers than in healthy subjects, this increase coinciding with a decrease in the other H_2-utilising microbial groups (i.e. acetogenic bacteria or methanogenic archaea). A shift in H_2 metabolism may thus also exist in C-IBS subjects, contributing to increased sulphide production.

The predominance of the SRB population in the C-IBS gut microbiota should thus generate important shifts in fermentative pathways through alteration of inter-species transfers of lactate and H_2. Results from *in vitro* fermentation of starch, one of the main polysaccharides available for gut microbes, further suggest that alterations in carbohydrate metabolism could exist in C-IBS gut microbiota, less butyrate and especially, more hydrogen and sulphide (H_2S) being produced.

The functional dysbiosis observed in C-IBS microbiota may have important clinical implications, due to changes in metabolism output, and plays a major role in genesis and/or maintenance of different IBS symptoms including abdominal pain, modulation of gut transit and gas-related symptoms. In this context, the enhancement in SRB population and the consequent over-production of deleterious sulphides should have an important impact on IBS patho-physiology.

Abdominal pain is a prevalent symptom in IBS that is mainly related to enhancement in visceral sensitivity.[41] More than 90% of IBS patients were shown to suffer from visceral hypersensitivity as measured by rectal distension.[41] H_2S was recently shown to have a major role in visceral nociception.[42] Matsunami *et al.*[42] reported that colonic luminal H_2S could cause visceral pain-like nociceptive behaviour in mice through sensitisation/activation of T-type Ca^{2+} channels probably present in primary afferents. It is well known that colonic luminal H_2S is mainly produced by SRB, with colonic tissues also forming some H_2S from L-cysteine metabolism. Potential roles for colonic luminal H_2S and/or SRB in inflammatory bowel diseases and colon cancer have been reported in several studies.[37, 43, 44] Our results support the hypothesis that H_2S produced from SRB metabolism could play key role in human colonic pain.

H_2S produced by SRB could also be involved in colonic transit regulation. Exogenous H_2S was shown to inhibit *in vitro* motor patterns in human and rodent colon mainly through an action on multiple potassium channels.[37] This is consistent with our finding suggesting higher H_2S production in C-IBS subjects. Results from previous studies, [45] however, showed that the number of SRB was lower under conditions of slower gut transit in healthy volunteers, the transit time being, in this case, reduced artificially.

The stimulation of sulphate-reduction in C-IBS was also shown to alter H_2 metabolism. This could further contribute to generate gas-related symptoms, i.e. bloating and flatus, which are frequently reported by IBS patients.[37] An over-excretion of H_2 in IBS patients was already reported by King *et al.*[20] Similarly, *in vitro* starch fermentation by C-IBS faecal microbiota led to accumulation of H_2 in the gas phase. Gas-related symptoms may thus be associated with H_2 accumulation in the gut. Our results further suggest that this alteration could be due to a decreased capacity of the gut microbiota to re-utilise fermentative H_2.

Some of the microbial changes observed in C-IBS may be consequences of the slower gut transit. *In vitro* continuous culture models have shown that dilution rate has an important impact on the composition of the human colonic microbial community.[46] *In vivo*, a slower gut transit was shown to be related with higher methane-excretion in IBS.[37] Similarly, the number of methane-producing subjects detected in our study was higher in C-IBS (8 over 14 subjects) than in healthy group (3 over 12 subjects). However, other important microbial alterations could not be explained simply by modification of gut transit and factors other than transit time may thus contribute to the altered microbial ecology observed in C-IBS. This suggests that certain of our findings on C-IBS gut microbiota might be applicable to other IBS subtypes.

In conclusion, we showed here, with a function-based approach, a major functional dysbiosis within gut microbiota of C-IBS. This cultural approach has allowed identifying variations within different functional groups of microorganisms that could have important physiological impacts for the host. This dysbiosis could indeed change the metabolic output and especially enhance production of toxic sulphides which could in turn influence motility and visceral sensitivity and generate IBS symptoms. The SRB community may thus have a central role in the microbial dysbiosis and in IBS patho-physiology. The contribution of SRB to IBS pathogenesis deserves further investigation and is currently under studies in our laboratory.

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