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## Overgrowth of the indigenous gut microbiome and irritable bowel syndrome

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**Core tip:** The majority of the gut microbiota is uncultivable. Use of culture-independent molecular methods, without reliance on traditional microbiological culture techniques, has the potential to determine microbial composition in the small intestine of patients with irritable bowel syndrome. Current data concerning culture-based and culture-independent analyses of the small intestinal microbiome in patients with irritable bowel syndrome are considered here.

### Abstract

Culture-independent molecular techniques have demonstrated that the majority of the gut microbiota is uncultivable. Application of these molecular techniques to more accurately identify the indigenous gut microbiome has moved with great pace over recent years, leading to a substantial increase in understanding of gut microbial communities in both health and a number of disorders, including irritable bowel syndrome (IBS). Use of culture-independent molecular techniques already employed to characterise faecal and, to a lesser extent, colonic mucosal microbial populations in IBS, without reliance on insensitive, traditional microbiological culture techniques, has the potential to more accurately determine microbial composition in the small intestine of patients with this disorder, at least that occurring proximally and within reach of sampling. Current data concerning culture-based and culture-independent analyses of the small intestinal microbiome in IBS are considered here.

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### INTRODUCTION

Culture-independent molecular techniques have demonstrated that the majority of the gut microbiota is uncultivable<sup>[1,2]</sup>. Application of these molecular techniques to more accurately identify the indigenous gut microbiome has moved with great pace over recent years, leading to a substantial increase in understanding of gut microbial communities in both health and a number of disorders, including irritable bowel syndrome (IBS). Most studies of the gut microbiome in this highly prevalent disorder, characterised by abdominal pain, abdominal distension and altered bowel habit, have to date focussed on analyses of faecal samples and have demonstrated disturbances in a range of bacterial populations in both adults and children with IBS<sup>[2-10]</sup>. In adults, disturbances in

faecal *Clostridium cocleatum*, *Clostridium coccoides*, *Clostridium thermosuccinogenes*, *Collinsella aerofaciens*, *Coprococcus eutactus*, *Staphylococcus aureus* (*S. aureus*), *Bifidobacterium catenulatum* (*B. catenulatum*), *Ruminococcus torques*, *Ruminococcus bromii*-like, bifidobacteria, Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, *Lactobacillus* spp and *Veillonella* spp have been demonstrated in IBS<sup>[2,3,5-10]</sup>. In children, a faecal microbiome characterised by a significantly increased percentage of Gammaproteobacteria, including *Haemophilus parainfluenzae*, a novel *Ruminococcus*-like microbe and an increased number of several bacterial taxa from the genus *Alistipes* has been reported in the IBS setting<sup>[4]</sup>. Analyses of colonic mucosa-associated microbial populations, as determined from mucosal biopsies, suggest compositional differences compared to faecal microbiota may occur in IBS<sup>[11]</sup> and it has been hypothesised that disturbances at this mucosa-associated level may be more important than those occurring luminally in the pathogenesis of IBS symptoms<sup>[12]</sup>. In further support of the notion that the gut microbiome participates in the pathogenesis of IBS are the findings of systematic reviews and a meta-analysis, which suggest that probiotics may be of therapeutic value, although results of individual studies are inconsistent and trial designs variable, such that it remains uncertain as to which bacterial species or strains may be of most benefit for which particular symptom component of the IBS complex<sup>[13-15]</sup>.

As opposed to faecal and colonic mucosa-based analyses, possible disturbances in the microbial ecology of the small intestine in patients with IBS have been less well studied. In particular, the prevalence of small intestinal bacterial overgrowth (SIBO) has long remained a matter of conjecture, with concern over the accuracy of diagnostic tests for SIBO one factor clouding this issue. Notably, reported prevalence rates of SIBO in patients with IBS are lower when the diagnosis of SIBO has been based on culture of proximal small intestinal luminal secretions compared to when based on indirect breath hydrogen tests, performed following the ingestion of a fermentable substrate such as lactulose<sup>[16]</sup>. False-negative culture results have been hypothesised as an explanation for this discrepancy, as a result of SIBO possibly occurring distal to the region of sampling<sup>[17]</sup>. Conversely, a high false-positive rate of the lactulose breath hydrogen test (LBHT) for SIBO is recognised, based on an initial study performed to investigate the diagnostic accuracy of the LBHT in patients with predisposition to SIBO in which breath testing was combined with scintigraphy<sup>[18]</sup>, recently replicated in the IBS setting<sup>[19]</sup>, that demonstrated that a “positive” result for SIBO may, in fact, result from the test substrate being metabolised by colonic, rather than small intestinal, microbial flora. Sensitivity for culture-proven SIBO has also been shown to be lacking, even with combined scintigraphic assessment<sup>[18]</sup>, such that the LBHT has fallen out of favour as a diagnostic test for SIBO, including in patients with IBS<sup>[20]</sup>.

Another possibility is that disturbances of the small intestinal microbial ecology - either overgrowth or re-

duced levels of various bacterial species - may, indeed, be present in the region of sampling in patients with IBS but simply not be represented by standard bacteriological culture results, due to the inherent inability to properly demonstrate the gut microbiota in this way. Use of culture-independent molecular techniques already employed to characterise faecal and, to a lesser extent, colonic mucosa-associated microbial populations in IBS, without reliance on insensitive, traditional microbiological culture techniques, has the potential to more accurately determine microbial composition in the small intestine of patients with this disorder, at least that occurring proximally and within reach of sampling. Current data concerning culture-based and culture-independent analyses of the small intestinal microbiome in IBS are considered here.

## PROXIMAL SMALL INTESTINAL MICROBIOTA IN IBS

A total of six published studies have investigated the proximal small intestinal microbiota in well-categorised cohorts of IBS patients and reported findings in relation to IBS-status<sup>[16-21]</sup>. Four of these studies analysed microbiota in luminal secretions, using standard culture techniques, with one also employing culture-independent molecular methods<sup>[21-24]</sup>. An additional two studies analysed mucosa-associated microbiota, with both of these using culture-independent molecular techniques<sup>[25,26]</sup>. Whether there exist compositional differences between small intestinal luminal and mucosa-associated microbial populations in IBS is currently unknown, as no study performed to date has contemporaneously analysed luminal and mucosa-associated microbiota in the same cohort of IBS patients.

### Assessments of luminal secretions

Posserud *et al.*<sup>[21]</sup> prospectively investigated 162 consecutive patients in Sweden with a clinical diagnosis of IBS based on Rome II criteria, including 49 (30%) with diarrhoea-predominant IBS (IBS-D), 37 (23%) with constipation-predominant IBS (IBS-C) and 76 (47%) with alternating-type IBS (IBS-A), with culture of a jejunal aspirate obtained *via* the central lumen of a water-perfused manometry catheter after a meal. The mean age of IBS patients was 38 years. Twenty-six healthy subjects (mean age 40 years) served as controls. No subject had been treated with antibiotics within 2 wk prior to the study or had received medications that might affect the gastrointestinal tract within 48 h of assessment. SIBO, defined by viable counts of colonic-type bacteria  $\geq 10^5$  colony forming units/mL (CFU/mL), was found in 7 patients (4%) (mean age 49 years), including 2/49 (4%) with IBS-D, 3/37 (8%) with IBS-C and 2/76 (3%) with IBS-A. Bacterial isolates in IBS subjects with SIBO variously included *Escherichia coli*, *Enterococcus* species, *Clostridium* species, *Enterobacter* species, *S. aureus* and *Klebsiella* species. The prevalence of SIBO in patients with IBS was comparable to that in asymptomatic controls (1/26;

**Table 1** Studies investigating the prevalence of small intestinal bacterial overgrowth in patients with irritable bowel syndrome, using culture-based assessments of proximal small intestinal luminal secretions

Country	IBS patients	Controls	Mean age (yr)	Aspirate details	Definition and prevalence of SIBO in patients and controls
Sweden <sup>[21]</sup>	<i>n</i> = 162	<i>n</i> = 26, healthy	IBS: 38	Non-fasting; <i>via</i> water-perfused manometry catheter from jejunum	$\geq 10^5$ CFU/mL colonic-type bacteria: IBS patients 7/162 (4%)
	IBS-D, <i>n</i> = 49		Controls: 40		IBS-D 2/49 (4%)
	IBS-C, <i>n</i> = 37				IBS-C 3/37 (8%)
	IBS-A, <i>n</i> = 76				IBS-A 2/76 (3%)
					Controls 1/26 (4%)
The Netherlands <sup>[22]</sup>	<i>n</i> = 8 (out of a cohort of 12 symptomatic patients)	<i>n</i> = 9, healthy	Symptomatic: 39 Control: 26	Fasting; <i>via</i> weighted catheter from jejunum	$\geq 5 \times 10^3$ CFU/mL colonic-type bacteria: IBS patients 17/162 (11%)
					Controls 1/26 (4%)
					$\geq 5 \times 10^3$ CFU/mL any bacteria: IBS patients 70/162 (43%) <sup>1</sup>
					Controls 3/26 (12%)
					Symptomatic patients 1/12 (8%)
United States <sup>[23]</sup>	<i>n</i> = 148	<i>n</i> = 527, symptomatic	Overall: 53	Fasting; <i>via</i> endoscopy from duodenum	Colonic-type bacteria: Symptomatic patients 0/9 (0%)
					Controls 0/9 (0%)
					Controls 0/9 (0%)
Greece <sup>[24]</sup>	<i>n</i> = 112	<i>n</i> = 208, symptomatic	SIBO: 63.6	Fasting; <i>via</i> endoscopy from duodenum	$\geq 10^5$ CFU/mL colonic-type aerobic bacteria OR $\geq 10^4$ CFU/mL anaerobic bacteria: IBS patients: 2%
	IBD-D, <i>n</i> = 35		No SIBO: 69.5		Controls 10%
	IBD-C, <i>n</i> = 19				
	IBD-A, <i>n</i> = 58				
					IBS patients 24/112 (21%) <sup>2</sup>
					Controls 11/208 (5%)
					IBS patients 42/112 (38%) <sup>2</sup>
					IBS-D 21/35 (60%) <sup>2,5</sup>
					IBS-C 6/19 (32%) <sup>3</sup>
					IBS-A 15/58 (26%) <sup>4</sup>
					Controls 20/208 (10%)

<sup>1</sup>*P* = 0.002 compared to controls; <sup>2</sup>*P* < 0.0005 compared to controls; <sup>3</sup>*P* = 0.012 compared to controls; <sup>4</sup>*P* = 0.003 compared to controls; <sup>5</sup>*P* = 0.001 compared to controls. IBS-D: Diarrhoea predominant-type irritable bowel syndrome; IBS-C: Constipation predominant-type irritable bowel syndrome; IBS-A: Alternating-type irritable bowel syndrome; CFU: Colony forming units; SIBO: Small intestinal bacterial overgrowth; IBS: Irritable bowel syndrome.

4%). Neither did prevalences of SIBO differ significantly between IBS patients and controls when alternative definitions of SIBO were employed (viable counts of any bacteria  $\geq 10^5$  CFU/mL, 6% and 4%, respectively; viable counts of colonic-type bacteria  $\geq 5 \times 10^3$  CFU/mL, 11% and 4%, respectively). Conversely, viable counts of any bacteria  $\geq 5 \times 10^3$  CFU/mL were significantly more common in the IBS cohort than in healthy controls (43% *vs* 12%). While water perfusion through the manometry catheter may have diluted the absolute values of viable bacterial counts obtained, and the ingestion of a test meal prior to sampling for bacteriological analysis may, alternatively, have increased these values compared to those that may have been recovered under fasting conditions, any differences between IBS patients and controls were unlikely explained on these bases, as subjects were studied under identical conditions (Table 1).

As expected since small intestinal dysmotility typically promotes SIBO with colonic-type bacteria<sup>[27]</sup>, this increased prevalence of mildly elevated non-colonic-type bacterial counts in IBS patients reported by Posserud *et al*<sup>[21]</sup> could not reliably be accounted for by small intestinal dysmotility, as assessed by manometry. Notably, the use of proton pump inhibitors (PPIs) and other drugs that reduce gastric acidity was not controlled for prior to 48 h of study and, given that IBS patients are often

treated with such drugs, it is possible that the mildly elevated, non-colonic-type viable bacterial counts found in the IBS cohort may have occurred as a consequence of treatment of symptoms rather than as an initial cause of symptoms. Such a possibility could not be assessed by this study design.

Kerckhoffs *et al*<sup>[22]</sup> in The Netherlands subsequently reported on 12 symptomatic patients, including 8 with IBS, and 9 healthy subjects, from whom a fasting jejunal aspirate could be obtained using a weighted catheter after infusion of 10 mL of normal saline. Studied IBS patients within the symptomatic group and controls came from initial cohorts of 10 IBS patients (mean age 39 years) and 11 controls (mean age 26 years), respectively, with two IBS patients and two controls ultimately excluded as a jejunal aspirate could not be obtained. Aspirates were subjected to both standard culture and molecular-based analyses, the latter following deoxynucleic acid (DNA) extraction and quantitative polymerase chain reaction (PCR) amplification. No antibiotics were permitted in the two weeks prior to study, although PPIs were permitted until the day before study. With regard to culture results and notwithstanding the possibility of dilution by the saline infusion, SIBO, as defined by a viable colonic-type bacterial count  $> 10^5$  CFU/mL, was present in 1/12 (8%) of the symptomatic group (the 8 IBS patients within the

symptomatic group were not separately reported) and none of the 9 controls. Using an alternative definition still based on colonic-type bacteria (*Enterobacteriaceae*  $\geq 10^3$  CFU/mL or *Bacteroides* species  $\geq 10^2$  CFU/mL or *Clostridium* species  $\geq 10^2$  CFU/mL), the prevalence of SIBO remained 1/12 (8%) in symptomatic patients and 0/9 (0%) in controls. Moreover, no significant difference in median total viable bacterial counts between symptomatic patients and healthy controls was apparent. Similarly, no significant difference in the total bacterial DNA count between symptomatic patients and healthy controls was evident, while PCR analysis demonstrated that levels of the colonic-type flora, *Enterobacteriaceae*, *Faecalibacterium prausnitzii*, *Bacteroides fragilis* and *Clostridium coccoides*, were also comparable in the symptomatic and healthy groups. Sub-analyses in relation to IBS-D, IBD-C and IBD-A status were not included.

In another analysis, Choung *et al.*<sup>[23]</sup> undertook a retrospective assessment of 675 symptomatic patients in the United States who had undergone culture of a duodenal aspirate, obtained endoscopically under fasting conditions, to assess for possible SIBO, including 148 (22%) patients with a clinical diagnosis of IBS. By comparison to the studies from Sweden and The Netherlands<sup>[13,14]</sup>, the mean age of study subjects in this analysis was older (53 years) and no asymptomatic controls were included. The IBS patients included did not represent a consecutive cohort, but rather a select group attending an academic institution whose physicians deemed symptoms troublesome enough to warrant microbiological assessment. SIBO, defined by a viable colonic-type aerobic bacterial count  $\geq 10^5$  CFU/mL or an anaerobic viable count  $\geq 10^4$  CFU/mL, was present in only 2% of the IBS group. The species of the overgrowth bacteria isolated from patients with SIBO were not reported. Placed in context, a diagnosis of IBS was associated with an odds ratio for an abnormal aspirate result in keeping of SIBO of only 0.2 (95%CI: 0.1-0.7) compared to the likelihood of SIBO in patients with non-IBS diagnoses, including inflammatory bowel disease, pancreatitis and small intestinal diverticula, which were associated with three-fold, nearly five-fold and over seven-fold increases in odds for SIBO, respectively. Overall, the likelihood of SIBO was significantly related to older age, with the mean age of those with SIBO found to be 66 years. A substantial number of studied IBS patients were taking a PPI at the time of assessment and the proportion of this subgroup that was found to have SIBO remained low (2%). Conversely, the proportion of IBS patients with detectable viable bacterial counts in duodenal secretions, although not sufficient to fulfil criteria for SIBO, was five-fold higher in the setting of PPI use (15%) than in the absence of PPI use (3%), in keeping with the concept that proximal small intestinal microbial ecology may be disturbed by such therapy, even if not to a degree to constitute SIBO as commonly defined. Data in relation to IBS-D, IBD-C and IBD-A sub-categories of IBS were not provided.

A fourth culture-based study investigated the prevalence of SIBO in a consecutive cohort of 320 symptomatic patients undergoing outpatient upper gastrointestinal endoscopy in Greece, including 112 (35%) with a diagnosis of IBS according to Rome II criteria (IBS-D:  $n = 35$ , 31.2%; IBD-C:  $n = 19$ , 16.9%; IBD-A:  $n = 58$ , 51.8%)<sup>[24]</sup>. Most common indications for endoscopy in IBS patients were dyspepsia ( $n = 75$ , 66.9%), anaemia ( $n = 24$ , 21.4%) and change in bowel habit ( $n = 9$ , 8.0%). Aspirates for microbiological assessment were obtained endoscopically under fasting conditions from the third part of duodenum. The prevalence of SIBO, defined by  $> 10^3$  CFU/mL of colonic-type aerobic bacteria, was significantly higher in IBS patients than non-IBS patients (42/112, 38% *vs* 20/208, 10%). Among the IBS cohort, SIBO was present in 21/35 (60%) with IBS-D, 6/19 (32%) with IBS-C and 15/58 (26%) with IBS-A. *Escherichia coli* was the colonic-type bacterial species most commonly isolated in IBS patients with SIBO. Using a more restrictive definition of SIBO of  $> 10^5$  CFU/mL of colonic-type aerobic bacteria, the prevalence of SIBO remained significantly higher in patients with IBS (24/112, 21%) than in those without IBS symptoms (11/208, 5%). The mean age of patients enrolled in this study was higher than that in the other three above-mentioned reports, with values of 63.6 years and 69.5 years in the SIBO and non-SIBO groups, respectively. That the highest prevalence of SIBO in IBS patients in the four studies discussed here should be found in the oldest of the four study cohorts, especially in those older patients with IBS-D, is in keeping with a previous report demonstrating a high prevalence of SIBO with colonic-type bacteria, including *Escherichia coli*, in the symptomatic elderly, including those with otherwise unexplained chronic diarrhoea<sup>[28]</sup>. Notably, distinct age-related disturbances in faecal microbiota, including increased levels of *Escherichia coli*, have also recently been demonstrated in the elderly<sup>[29]</sup>.

### Assessments of mucosa-associated microbiota

Kerckhoffs *et al.*<sup>[25]</sup> investigated 41 patients with IBS fulfilling Rome II criteria, including 14 (34%) with IBS-D, 11 (27%) with IBS-C and 16 (39%) with IBS-A, and 26 healthy controls. The mean age of IBS subjects was significantly older than that of controls (42 years and 31 years, respectively). Duodenal brushings were obtained and samples were subjected to DNA extraction and PCR amplification. Based on detection of significantly lower levels of *B. catenulatum* in faecal samples of the IBS cohort, the authors focussed on whether contemporaneous duodenal mucosal levels of *Bifidobacterium* species were similarly disturbed. A significant reduction in duodenal mucosa-associated *B. catenulatum* levels as a percentage of total duodenal mucosa-associated bifidobacterial loads was found in the IBS group ( $4.85\% \pm 0.5\%$ ) compared to healthy controls ( $17.04\% \pm 2.3\%$ ), with this relationship consistent across all three IBS subgroups. By contrast, levels of *B. adolescentis*, *B. bifidum* and *B. longum* did not dif-



fer significantly between healthy subjects, IBS patients or IBS subgroups.

In a subsequent case-control analysis, the same authors collected duodenal mucosal brush and faecal samples from 37 IBS patients (mean age  $42 \pm 2.3$  years), including 13 (35%) IBS-D, 11 (29%) IBS-C and 13 IBS-A (35%), and 20 healthy controls (mean age  $32 \pm 2.6$  years)<sup>[26]</sup>. Bacterial 16S rRNA gene was amplified and analysed using PCR denaturing gradient gel electrophoresis (DGGE). Pooled average DGGE profiles were generated and fingerprints compared. DGGE band fragments confined to healthy or IBS patient groups were further characterised by sequence analysis. Significantly higher levels of *Pseudomonas aeruginosa* were evident in duodenal brushings of the IBS patients than in healthy subjects ( $8.3\% \pm 0.95\%$  of clones *vs*  $0.1\% \pm 0.069\%$  of clones, respectively), a trend replicated in paired faecal samples and across all IBS-subtypes. While antibiotic pre-treatment has been shown to increase the colonisation potential of *Pseudomonas* species<sup>[30]</sup>, it is notable that no antibiotic therapy was permitted within one month of study in this analysis. Nonetheless, it remains to be determined whether the elevated levels of *Pseudomonas aeruginosa* reported by the authors are of pathophysiological relevance or merely epiphenomenal, perhaps related to the reduced expression of *B. catenulatum* previously reported or other factors yet to be defined.

#### Effect of antibiotic therapy on small intestinal microbiota and symptoms in IBS

Randomised trials of the orally administered antibiotics, neomycin and rifaximin, have separately demonstrated a reduction in IBS symptoms in non-IBS-C patients following antibiotic treatment<sup>[31-34]</sup>. Nonetheless, whether or not treated patients had SIBO and whether antibiotic use was associated with a reduction in viable small intestinal bacterial counts or microbial compositional change that correlated with symptom improvement was not assessed.

To date, only one study has investigated the impact on antibiotic therapy on SIBO and symptom improvement in patients with IBS<sup>[21]</sup>. In that analysis, seven patients with culture proven SIBO in jejunal secretions (mean age 49 years) were treated with oral ciprofloxacin, 500 mg twice daily for 10 d. Follow-up cultures following antibiotic treatment showed decreased viable bacterial counts in five patients (71%), although four (57%) still fulfilled criteria for SIBO. Three patients (43%) reported at least a 25% improvement in IBS symptoms following the course of ciprofloxacin, but IBS symptom responder status was not consistently related to reduction in small intestinal luminal viable bacterial counts. Whether symptom responder status may have correlated more closely with any antibiotic-related changes in faecal or colonic microbiota was not assessed.

No data are currently available with regard to the possible impact of antibiotic therapy on duodenal mucosa-associated composition and whether any antibiotic-related compositional change in the duodenal mucosa-

associated microbial community correlates with symptom improvement in patients with IBS.

#### Efficacy of probiotic regimens that include microbiota shown by molecular techniques to be deficient in IBS

The health benefits of *B. catenulatum* for the host, if any, are currently unknown. However, members of the bifidobacteria group are often included in probiotic regimens used for the treatment of IBS<sup>[35]</sup>. Trials of probiotics that specifically include *B. catenulatum* and any other small intestinal mucosa-associated bacterial species that may be shown in future to be reduced in patients with IBS will be of considerable interest, from both therapeutic and disease mechanism perspectives.

## CONCLUSION

Current microbial data, although relatively limited and based predominantly on culture-based assessments of luminal secretions, suggest that only a minority of patients with IBS have luminal SIBO, irrespective of the definition employed, with the exception of older subjects with the diarrhoea-predominant form. Available data obtained from a relatively young cohort demonstrating that symptom improvement following antibiotic therapy in IBS patients with SIBO does not necessarily depend upon reversal of the SIBO, as assessed in luminal secretions, cast doubt as to the importance of luminal SIBO in the pathophysiology of IBS symptoms, at least in younger subjects. Comparable studies have not been performed in elderly IBS patients with luminal SIBO. Similarly, the pathophysiological relevance of any disturbances of duodenal mucosa-associated microbiota in patients with IBS, including the reduced levels of *B. catenulatum* and increased levels of *Pseudomonas aeruginosa* levels so far demonstrated by culture-independent means, remains to be determined.

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