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Association between *Brachyspira* and irritable bowel syndrome with diarrhoea

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ABSTRACT

Objective The incidence of IBS increases following enteric infections, suggesting a causative role for microbial imbalance. However, analyses of faecal microbiota have not demonstrated consistent alterations. Here, we used metaproteomics to investigate potential associations between mucus-resident microbiota and IBS symptoms.

Design Mucus samples were prospectively collected from sigmoid colon biopsies from patients with IBS and healthy volunteers, and their microbial protein composition analysed by mass spectrometry. Observations were verified by immunofluorescence, electron microscopy and real-time PCR, further confirmed in a second cohort, and correlated with comprehensive profiling of clinical characteristics and mucosal immune responses.

Results Metaproteomic analysis of colon mucus samples identified peptides from potentially pathogenic *Brachyspira* species in a subset of patients with IBS. Using multiple diagnostic methods, mucosal *Brachyspira* colonisation was detected in a total of 19/62 (31%) patients with IBS from two prospective cohorts, versus 0/31 healthy volunteers ($p<0.001$). The prevalence of *Brachyspira* colonisation in IBS with diarrhoea (IBS-D) was 40% in both cohorts ($p=0.02$ and $p=0.006$ vs controls). *Brachyspira* attachment to the colonocyte apical membrane was observed in 20% of patients with IBS and associated with accelerated oro-anal transit, mild mucosal inflammation, mast cell activation and alterations of molecular pathways linked to bacterial uptake and ion–fluid homeostasis. Metronidazole treatment paradoxically promoted *Brachyspira* relocation into goblet cell secretory granules—possibly representing a novel bacterial strategy to evade antibiotics.

Conclusion Mucosal *Brachyspira* colonisation was significantly more common in IBS and associated with distinctive clinical, histological and molecular characteristics. Our observations suggest a role for *Brachyspira* in the pathogenesis of IBS, particularly IBS-D.

Significance of this study

What is already known on this subject?

- IBS incidence increases after enteric infections, suggesting a possible causative role for microbial perturbation.
- Studies of the faecal microbiota have failed to demonstrate consistent alterations associated with IBS symptoms.

What are the new findings?

- This study represents the first comparison of the microbial community of the colonic inner mucus layer of patients with IBS and healthy volunteers.
- Colonisation of the colonic epithelial surface or mucus layers by pathogenic *Brachyspira* species was detected in 40% of patients with IBS with diarrhoea but not in any healthy individual.
- *Brachyspira*-associated IBS was linked to distinctive clinical, histological and molecular characteristics, suggesting that it should be considered a separate diagnostic entity.
- Treatment paradoxically resulted in relocation of the *Brachyspira* into goblet cell secretory granules.

How might it impact on clinical practice in the foreseeable future?

- The presence of *Brachyspira* may be used to identify a distinct subset of patients with IBS, who could potentially be responsive to eradication therapy.
- The relocation of the *Brachyspira* into goblet cell mucus granules likely represents a novel bacterial strategy to evade antibiotics, which could inform our understanding of other persistent or recurrent mucosal infections.

INTRODUCTION

The incidence of IBS steeply increases following a gastroenteritis episode, suggesting a possible causative role for microbial perturbation.[1] Still, previous investigations have not demonstrated consistent associations between intestinal microbiota and IBS symptoms.[2 3]

Gut microbiota composition studies overwhelmingly rely on faecal material. However, these samples largely reflect the luminal microbial community, which is spatially separated from the colonic epithelium and underlying immune cells through a two-tiered mucus barrier.[4] By contrast, certain species have found a niche in the outer mucus layer, feeding on the abundant mucin *O*-glycans.[5] Thus, faecal and mucus-associated bacteria represent distinctive populations, with the latter

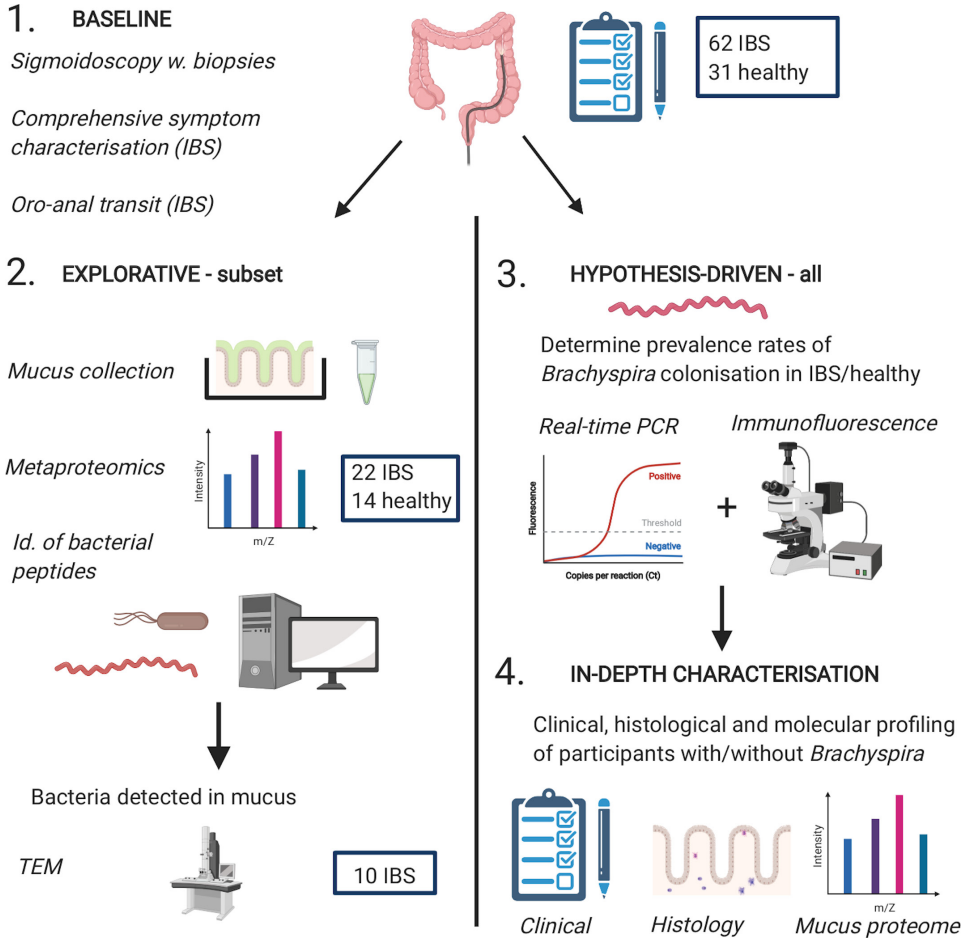

Figure 1 Graphical summary of the study design. Schematic representation of the different phases of the study. In-depth profiling of clinical, histological and molecular characteristics (phase 4) was performed in participants where *Brachyspira* colonisation could be confirmed/rejected with high confidence, typically based on consistent results from two different methods. Quantification of mucosal immune cells by histology was performed in a representative subset of participants with good-quality biopsy sections, whereas analysis of the human mucus proteome was largely restricted to participants from the explorative cohort. The figure was created with BioRender.com.

more likely to influence the epithelium. In particular, bacterial presence in the inner mucus layer might result in epithelial stress and immune activation. Here, we performed a metaproteomic analysis of inner mucus layer samples from patients with IBS and healthy individuals, to investigate potential associations between mucus-resident microbiota and IBS symptoms.

MATERIALS AND METHODS

Study design

Patients aged 18–65 years fulfilling the Rome III criteria for IBS were prospectively included at a secondary/tertiary care unit (Sahlgrenska Hospital, Gothenburg, Sweden) June 2012 through April 2018. Healthy volunteers were recruited through advertisements and screened by questionnaires and interviews to rule out gastrointestinal complaints and comorbidities. Exclusion criteria are listed in the online supplemental file. Patients with IBS were subtyped based on the Rome III criteria and the Bristol Stool Form scale, as follows: IBS with diarrhoea (IBS-D), IBS with constipation (IBS-C), IBS with mixed bowel habits (IBS-M) and unsubtyped IBS without diarrhoea or constipation (IBS-U). All participants provided written informed consent.

All study participants (IBS n=62, healthy n=31) underwent sigmoidoscopy with sampling of biopsies in methanol-Carnoy for future histology/immunohistochemistry and real-time PCR analysis (figure 1). Carnoy's fixative enables optimal preservation of both the mucus layers and the tissue nucleic acids.[6] Comprehensive clinical characterisation, including assessment of oro-anal transit time (OATT) and rectal sensitivity, was undertaken in patients with IBS.

In a randomly selected subset of participants (the *first/explorative* cohort, IBS n=22, healthy n=14), mucus was collected from ex vivo sigmoid colon biopsies and analysed by mass spectrometry (MS). The objectives of this explorative phase were twofold: to characterise the metaproteomic composition of the inner mucus layer of patients with IBS and healthy individuals, and to generate hypotheses to pursue in the entire study population. Patients in whose mucus/tissue samples bacteria were detected by MS (n=9), or, in certain cases, other methods (n=3), underwent repeat sigmoidoscopy, with biopsy sampling for electron microscopy, mucus collection and/or routine clinical histopathology.

The metaproteomic analysis identified a putative link between the *Brachyspira* genus and IBS. This association was validated in the *explorative cohort*, as well in the remainder of the study population—the second cohort, comprising 40 patients with IBS and 17 healthy individuals—using real-time PCR and immunofluorescence. Sampling and analysis were, whenever possible, performed with blinding to participant identity. Missing data

due to technical failures or suboptimal biopsy specimens are tabulated in online supplemental table S1.

Patient and public involvement statement

Patients were not involved in the planning, design or evaluation of this study.

Patient-reported symptoms

Participants recorded the frequency and Bristol scale consistency of their bowel movements in a 2-week structured diary. Patients with IBS completed the IBS Severity Scoring System (IBS-SSS) questionnaire as well as an Extracolonic Symptom Severity Score (online supplemental file).[7 8]

Oro-anal transit time

OATTs were assessed for 60/62 (97%) patients with IBS (online supplemental file).

Rectal sensitivity

Rectal sensitivity was evaluated in 45/62 (73%) patients with IBS, using an electronic barostat (online supplemental file).

Mucus collection

Sigmoidoscopy was performed without prior bowel preparation. Two biopsies per participant (explorative cohort n=36, second cohort n=4) were transported in oxygenated, ice-cold Krebs buffer, mounted in our in-house developed ex vivo mucus measurement chambers and allowed to secrete mucus for 1 hour.[9] Mucus was then collected by gentle scraping (online supplemental figure S1). Since the outer, easily removable, mucus layer is lost during sampling and transport, sample microbial protein content was considered to reflect that of the inner mucus layer.

MS and data processing

Mucus samples were prepared for MS according to a modified version of the Filter-Aided Sample Preparation (FASP) protocol.[10] Nano-liquid chromatography-tandem MS was performed on a Q-Exactive instrument (Thermo). Peptides were identified using the Andromeda search engine integrated into MaxQuant (V.1.3.0.5) and the MASCOT software (V.2.2).[11] Searches were performed against all reviewed human and eubacteria sequences of the SwissProt-UniProt database (February 2016). Minimum one unique peptide at a threshold of 1% was required for protein identification. The identification threshold for a bacterial family/genus was generally defined as ≥3 proteins, each identified by at least one unique, family/genus-specific peptide. Detailed information is provided in the online supplemental file.

Histology and immunohistochemistry

One biopsy per participant (both cohorts) was fixed in methanol-Carnoy, paraffin-embedded and sectioned. Tissue sections were stained with either H&E, Alcian blue/periodic acid-Schiff (AB-PAS), toluidine blue, Gram stain or with antibodies for fluorescent microscopy (online supplemental file). Lamina propria and subepithelial/intraepithelial immune cell populations were counted in five high-power fields in H&E or toluidine blue-stained sections by two to three independent observers.

Transmission electron microscopy

Transmission electron microscopy analysis of biopsies from 10 participants was performed as detailed in the online supplemental file.

DNA isolation and real-time PCR analysis

DNA was isolated from methanol-Carnoy-fixed, paraffin-embedded tissue as outlined in the online supplemental file. In certain participants, DNA was also isolated from fresh frozen biopsies and/or faecal material (online supplemental file). *Brachyspira* species were identified using a melting curve-based method.[12] To further validate our observations, a multiplex hydrolysis probe assay specific for *Brachyspira aalborgi*/*hominis* and *Brachyspira pilosicoli* was designed (online supplemental file).

16S rDNA sequencing

16S rDNA sequencing of faecal samples was performed as described in the online supplemental file.

Mucus penetrability analysis

Mucus penetrability was assessed by confocal microscopy, using fluorescent beads as surrogate markers for bacteria (online supplemental file).[9]

Antibiotic treatment—pilot study

The first four patients with IBS in the study to be diagnosed with epithelial *Brachyspira* colonisation/infection were treated with 500 mg metronidazole three times a day for 14 days.[13] As the rationale for the treatment was clinical, the intervention was open-label and uncontrolled. Patients completed IBS-SSS questionnaires before treatment, and 2, 4, 6 and 8 weeks, as well as 6, 12 and 15 months after commencing antibiotics. They kept a diary of their bowel habits from 2 weeks before, until 8 weeks after, treatment, and also for 2 weeks at 6 and 12 months after metronidazole therapy, respectively. Patients underwent sigmoidoscopy with biopsy sampling for histology, immunohistochemistry and PCR 6 weeks post-treatment and collected faecal samples for probe-based PCR analysis 6 weeks, 6 months and 1 year after antibiotic therapy.

Statistics

Fisher's exact test was used for categorical data. For continuous data, Welch's t-test or the Mann-Whitney U test was used for two-group comparisons, depending on data distribution as verified by the Kolmogorov-Smirnov test. Kruskal-Wallis test was used for multigroup comparisons. P values are two-sided. The significance threshold (0.05) was adjusted according to the Bonferroni/Holm-Bonferroni methods in the event of multiple (>2) comparisons. Data was analysed with GraphPad Prism (V. 8).

RESULTS

Study population

A graphical summary of the study design is provided in figure 1. In an explorative cohort of 22 patients with IBS and 14 healthy controls, inner mucus layer samples were collected from sigmoid colon biopsies and analysed by metaproteomics, revealing a tentative link between *Brachyspira* and IBS. This association was confirmed through histology, electron microscopy, immunofluorescence and targeted real-time PCR analysis, and further investigated in a second cohort of 40 patients with IBS and 17 healthy individuals, using immunofluorescence and PCR. Clinical and

Table 1 The study cohorts

	First (explorative) cohort		Second cohort		Study population*	
	IBS patients	Controls	IBS patients	Controls	IBS patients	Controls
Participants	22	14	40	17	62	31
Females	15 (68%)	8 (57%)	33 (83%)	10 (59%)	48 (77%)	18 (58%)
Median age (range)	29 (22–55)	29 (23–49)	28 (20–62)	27 (19–55)	28 (20–62)	29 (19–55)
IBS-D	10 (45%)	NA	15 (38%)	NA	25 (40%)	NA
IBS-C	5 (23%)	NA	8 (20%)	NA	13 (21%)	NA
IBS-M	5 (23%)	NA	14 (35%)	NA	19 (31%)	NA
IBS-U	2 (9%)	NA	3 (8%)	NA	5 (8%)	NA
Mucus samples analysed by proteomics	22 (100%)	14 (100%)	3 (8%)	1 (6%)	25 (40%)	15 (48%)
Tissue sections analysed by immunofluorescence	19 (86%)	12 (86%)	37 (93%)	16 (94%)	56 (90%)	28 (90%)
Biopsies analysed by real-time PCR	18 (82%)	9 (64%)	36 (90%)	16 (94%)	54 (87%)	25 (81%)
Patients treated with metronidazole	3 (14%)	0	1 (3%)	0	4 (6%)	0

*Entire study population (both cohorts).
IBS-C, IBS with constipation; IBS-D, IBS with diarrhoea; IBS-M, IBS with mixed bowel habits; IBS-U, unsubtyped IBS without diarrhoea or constipation; NA, not applicable.

demographic characteristics of both cohorts are compiled in table 1. The number/proportion of individuals analysed by each method are summarised in table 1; participant-level data are provided in online supplemental data file S1. Missing data are tabulated in online supplemental table S1, stratified by IBS diagnosis and *Brachyspira* colonisation status. Demographic factors did not differ between patients with IBS and healthy individuals (table 1).

Bacterial proteins were more frequently detected in mucus from patients with IBS

Using stringent criteria, bacteria were identified in mucus from 9/22 (41%) patients with IBS and 1/14 (7%) healthy controls (p=0.05; online supplemental figure S2A). The most frequently identified bacterial family was Pseudomonadaceae (online supplemental figure S2B–D). Proteins from the Brachyspiraceae family, genus *Brachyspira* were detected in 3/22 (14%) patients with IBS but not in any controls (online supplemental figure S2C,D).

The *Brachyspira* genus includes putative human pathogens associated with intestinal spirochetosis. In this condition, tentatively linked to diarrhoea and abdominal pain, *Brachyspira* penetrates the mucus layers, frequently colonising the colonocyte apical membrane.[12–15] Therefore, we focused our investigation on the potential link between *Brachyspira* and IBS. Numbers of identified peptides and proteins per family are compiled in online supplemental table S2 and *Brachyspira* peptide/protein identifications in online supplemental table S3.

Histology and immunohistochemistry confirmed proteomic *Brachyspira* identifications

In the three patients with proteomic *Brachyspira* identifications, spirochetes could be visualised at the apical membrane by AB-PAS staining (figure 2A,B). Transmission electron microscopy showed *Brachyspira* densely colonising the epithelial surface, attaching between the microvilli (figure 2C,D). These observations were further confirmed by immunofluorescent staining with *Brachyspira* antiserum (figure 2E).[14] When the immunofluorescence analysis was extended to the entire explorative cohort, *Brachyspira* colonisation of the colonocyte membrane (membrane-associated spirochetosis) was detected in three additional patients with IBS. In other cases, *Brachyspira* was observed in the mucus, occasionally invading the inner layer (figure 2F). By contrast, all healthy volunteer samples were negative for *Brachyspira*.

Remarkably, routine histological assessment of six individuals with membrane-associated spirochetosis failed to identify this condition in any patient. To further investigate why an association between *Brachyspira* and IBS has not previously been observed, 16S rDNA sequencing of faecal samples was undertaken in four patients with spirochetosis diagnosed by multiple methods (online supplemental data file S2); this approach also failed to detect *Brachyspira* in all cases.

Targeted analyses of colonic biopsies verified high prevalence of spirochetosis in IBS

Two different methods for targeted real-time PCR analysis of biopsy material were used in conjunction with immunofluorescence to establish spirochetosis prevalence rates in both cohorts.[12 14] In the entire study population, the prevalence of *Brachyspira* colonisation in IBS was 31% (19/62 patients), with no cases identified among the 31 healthy controls (p<0.001). To minimise the risk of false positive results, subsequent analyses were focused on cases that were either positive, or consistently negative for *Brachyspira* according to ≥2 methods. These criteria were fulfilled for 80/93 (86%) participants; 50 patients with IBS and 30 healthy individuals. With this restriction, the overall prevalence of *Brachyspira* colonisation in IBS was 28% (14/50; p<0.001; figure 3A). *Brachyspira* prevalence in IBS-D was 42%, with highly congruent results in the two cohorts (44% and 40%; p=0.01 for both comparisons with healthy volunteers; online supplemental table S4).

A total of 43 patients with IBS and 27 controls had conclusive results from immunofluorescence analysis, which could be verified by at least one additional method. Membrane-associated spirochetosis was detected in 21% (9/43) of patients with IBS but not in any control (p=0.01; figure 3B). The prevalence of membrane-associated spirochetosis was 38% for IBS-D versus 0% for IBS-C (p=0.05). Prevalence rates stratified by cohort and IBS subtype are compiled in online supplemental table S4A–D. Individual and combined results for the different methods for *Brachyspira* identification in patients with confirmed spirochetosis are provided in online supplemental table S5.

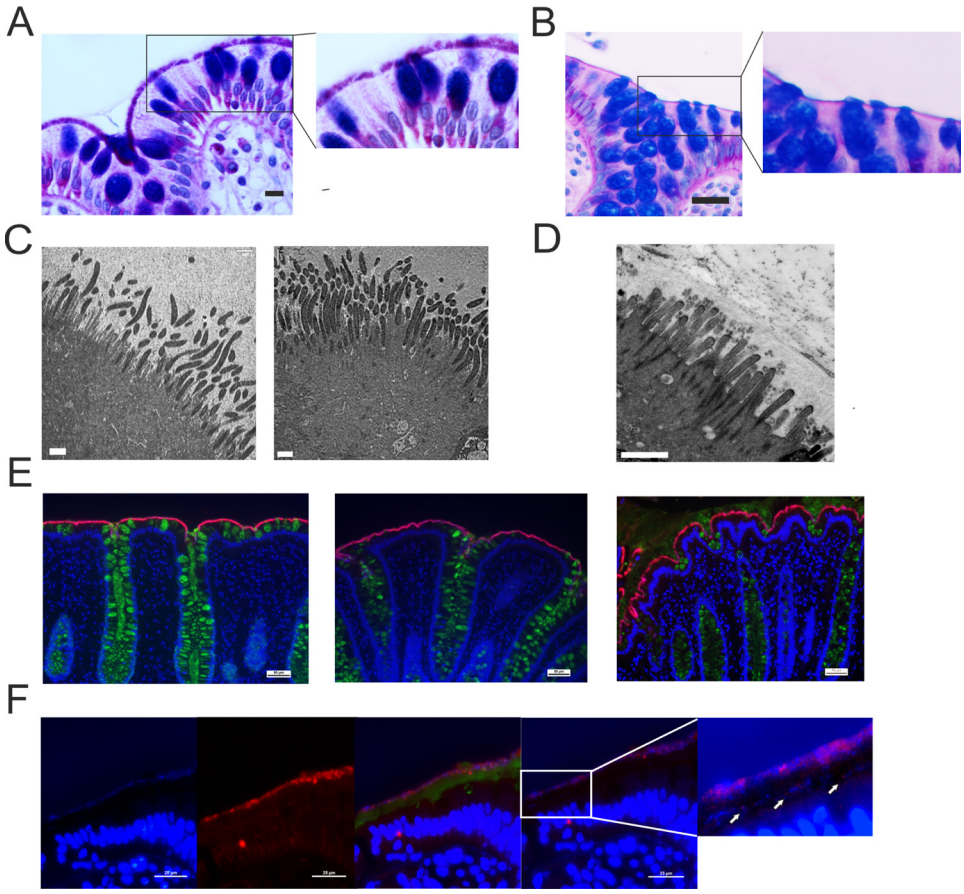

Figure 2 Histology, immunohistochemistry and transmission electron microscopy demonstrated the presence of *Brachyspira* at the colonic epithelial surface. (A) Alcian blue-periodic acid-Schiff (PAS) stain showing *Brachyspira* bacteria at the apical membrane. Scale bar: 10 µm. (B) Representative Alcian blue-PAS stain from a patient with IBS without *Brachyspira*. Scale bar: 25 µm. The right-hand images represent zoomed sections from the left panel. (C) Transmission electron micrographs showing *Brachyspira* attaching to the epithelial surface between the (shorter and less electron-dense) microvilli. Scale bar: 1 µm. (D) Transmission electron micrograph from a patient without *Brachyspira*. Scale bar: 1 µm. (E) Sections from three patients with IBS where *Brachyspira* species were detected by proteomics, stained with *Brachyspira* antiserum (red) and co-stained for mucus with anti-CLCA1 (green). DNA was counterstained with Hoechst (blue). Scale bars: 50 µm. (F) In certain patients, *Brachyspira* colonisation was restricted to mucus. From left to right: (1) bacteria at a distance from the epithelium, visualised by DNA stain (Hoechst); (2) immunostaining for *Brachyspira*; (3) merge of images showing *Brachyspira* (red), mucus (anti-CLCA1, green) and DNA (Hoechst, blue) staining of nuclei and bacteria, demonstrating co-localisation of *Brachyspira* and DNA staining for bacteria in the outer mucus layer. (4) *Brachyspira* (red) and other bacteria (blue) could also be observed in the inner mucus layer, as indicated by arrows. Scale bars: 25 µm.

Different niche preferences for *B. aalborgi/hominis* and *B. pilosicoli*

According to species discrimination by real-time PCR, 50% of patients with spirochetosis were colonised by *B. pilosicoli*; others had either *B. aalborgi* or the closely related, unconfirmed, species *B. hominis*.[12] Membrane-associated spirochetosis was linked to *B. aalborgi/hominis* in 78% of cases, whereas *B. pilosicoli* accounted for 83% of identifications restricted to mucus (p=0.04; figure 3C), suggesting that these species typically occupy different niches.

Membrane-associated spirochetosis defined a clinically distinctive subset of patients with IBS

For the analysis of clinical characteristics and immune responses, the two cohorts were merged. The analysis was restricted to participants with consistent results from ≥2 methods for *Brachyspira* identification (n=80) or with negative results from real-time PCR with no other tests performed (n=3). The demographic distribution of patients with IBS with versus without spirochetosis did not differ significantly, nor did total and component IBS-SSS scores or rectal sensitivity (table 2, figure 4A, online supplemental figure S3A,B).[7] By contrast, Extracolonic Symptom Severity Scores were significantly lower in membrane-associated spirochetosis (figure 4A).[8] To quantify diarrhoeal symptoms, a 'stool score', representing the sum of average stool frequency and Bristol scale consistency, was constructed for each participant. This score was higher in membrane-associated spirochetosis than in patients with IBS without *Brachyspira* and controls (p=0.02 and <0.001, respectively; figure 4B). Separate comparisons of stool frequency and consistency are shown in figure 4C. Oro-anal transit was also accelerated in membrane-associated spirochetosis as compared with other patients with IBS (p=0.03; figure 4D).

Brachyspira colonisation was linked to alterations of molecular pathways associated with membrane remodelling and ion–fluid homeostasis

To delineate the underlying mechanisms behind the symptoms observed in spirochetosis, the host mucus proteome was analysed (online supplemental data file S3). A global increase

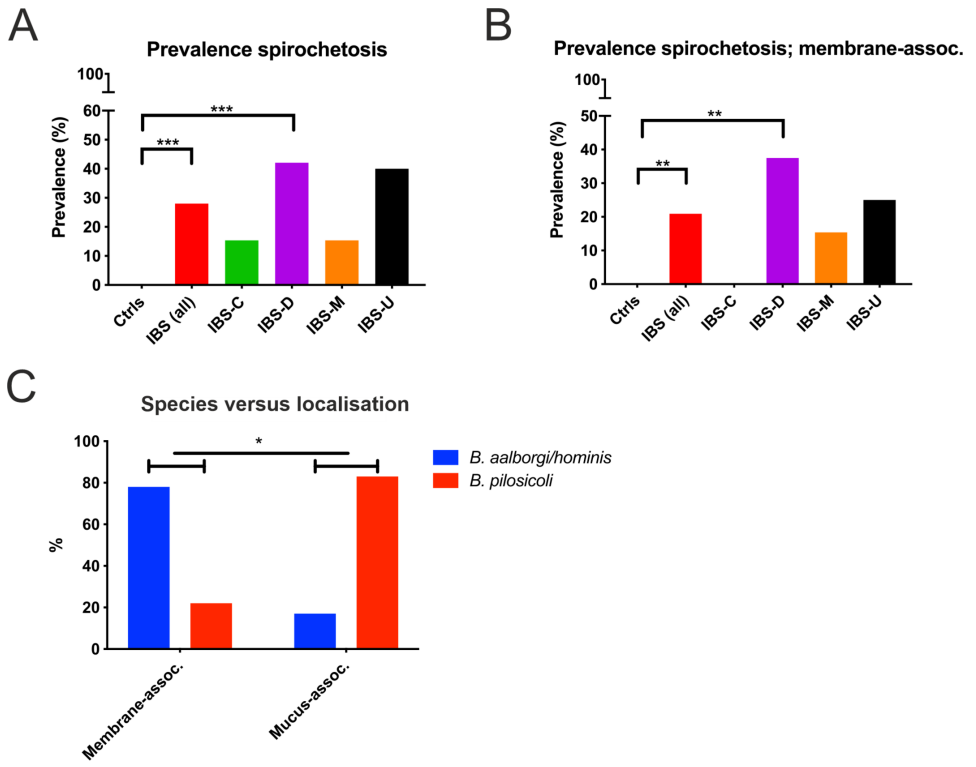

Figure 3 Prevalence of intestinal spirochetosis in IBS, and association between species and colonisation pattern. (A) Overall prevalence rates of *Brachyspira* colonisation in healthy individuals and in different IBS subtypes. (B) Prevalence of membrane-associated spirochetosis. In (A) and (B) data from the entire study population (both cohorts) are shown; these are based on agreement between at least two independent diagnostic methods. Membrane-associated spirochetosis was diagnosed by immunohistochemistry, with *Brachyspira* colonisation confirmed by at least one additional method. Patients with mucus-associated spirochetosis are not included in the analysis in (B). (C) Comparison of the distribution of *Brachyspira* species among patients with membrane-associated and mucus-associated spirochetosis. Groups were compared by Fisher's exact test: *p≤0.05 **p≤0.01 ***p≤0.001. IBS-C, IBS with constipation; IBS-D, IBS with diarrhoea; IBS-M, IBS with mixed bowel habits; IBS-U,unsubtyped IBS without diarrhoea or constipation.

in proteins associated with bacterial adhesion and invasion, notably the Arp2/3 complex, was observed in patients with proteomic *Brachyspira* identifications (figure 5A). Furthermore, inflammatory mediators—complement factors, inflammasome and immunoproteasome components and particularly immunoglobulins—were strongly induced. Consequently, the most enriched gene ontology biological pathways were associated with antibody-mediated phagocytosis (online supplemental figure S4A). An overlay with the Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway library revealed distinct

Table 2 Demographic and clinical characteristics of patients with IBS with and without intestinal spirochetosis

	No spirochetosis*	Spirochetosis; all†	Spirochetosis; mucus-associated‡	Spirochetosis; membrane-associated‡
No of participants	36	14	5	9
No of females (%)	27 (75)	10 (71)	4 (80)	6 (67)
Median age (p25–p75)§	31 (24–42)	28 (24–31)	29 (28–45)	26 (23–30)
Median duration, years (p25–p75)§	15 (7–24)	10 (4–13)	10 (7–18)	10 (1–10)
No of IBS-D (%)	11/36 (31)	8/14 (57)	2/5 (40)	6/9 (67)
No of IBS-C (%)	11/36 (31)	2/14 (14)	2/5 (40)	0/9 (0)
No of IBS-M (%)	11/36 (31)	2/14 (14)	0/5 (0)	2/9 (22)
No of IBS-U (%)	3/36 (8)	2/14 (14)	1/5 (20)	1/9 (11)
Median IBS-SSS (p25–p75)§	318 (231–365)	280 (220–354)	277 (203–282)	336 (233–355)
Median extracolonic SSS (p25–p75)§	189 (123–271)	125 (75–175)	202 (174–244)	75 (73–106)
Median OATT5 (p25–p75)§	1.3 (0.8–2.4)	1.1 (0.8–1.8)	1.9 (1.1–2.6)	1.0 (0.8–1.4)
Median rectal pain threshold, mm Hg (p25–p75)§	28 (20–28)	28 (20–32)	32 (27–37)	20 (20–32)

*Patients with IBS negative for *Brachyspira* according to at least two different methods, with no positive results.
†Patients with IBS positive for *Brachyspira* according to at least two methods.
‡The stratification of mucus-associated and membrane-associated spirochetosis was based on immunofluorescence analysis of biopsy sections.
§p25, 25th percentile; p75, 75th percentile.
IBS-C, IBS with constipation; IBS-D, IBS with diarrhoea ; IBS-M, IBS with mixed bowel habits; IBS-U, unsubtyped IBS without diarrhoea or constipation; OATT, oro-anal transit time (days); SSS, Severity Scoring System.

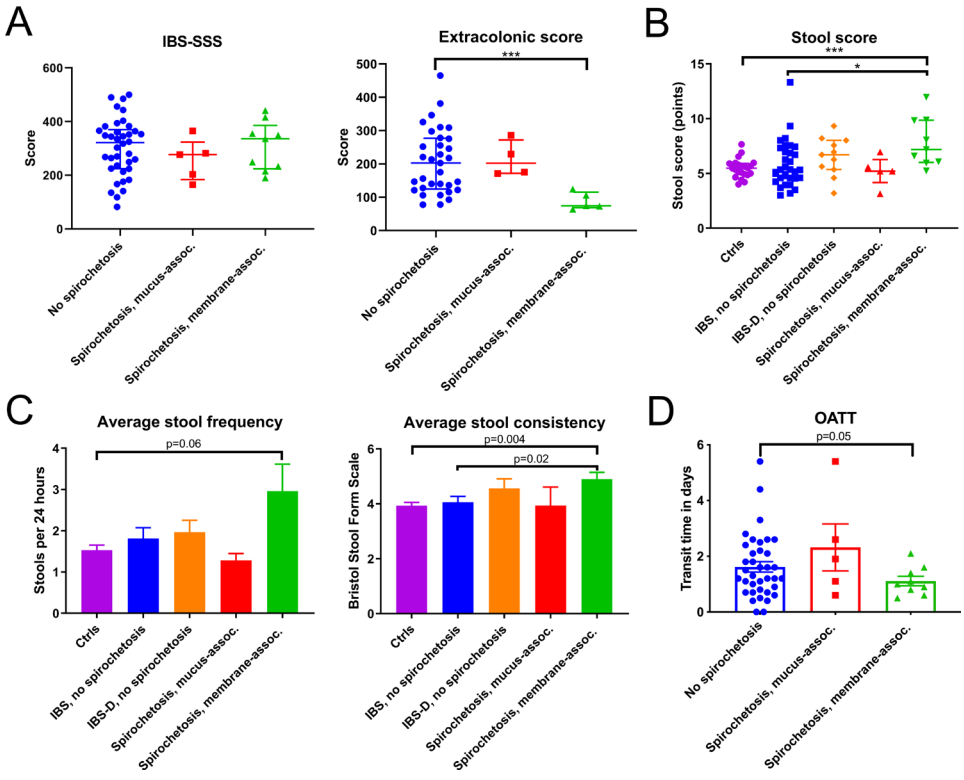

Figure 4 Individuals with membrane-associated spirochetosis constitute a clinically distinctive subgroup of patients with IBS. (A) Patient-reported IBS symptom severity according to the IBS Severity Scoring System (IBS-SSS) did not differ between patients with and without spirochetosis. However, Extra-colonic Symptom Scores were lower in membrane-associated spirochetosis (Mann-Whitney U=11.5; p<0.001). (B) Stool scores (summed average stool frequency and average Bristol scale consistency) were higher in membrane-associated spirochetosis than in patients with IBS without *Brachyspira*, indicating more diarrhoeal symptoms (U=68.0; p=0.02). In (A) and (B) individual observations are overlaid by the median and interquartile range. The significance threshold was adjusted by the Bonferroni method. *p≤0.025, ***p≤0.001. (C) Average stool frequency and consistency for controls and the different patient groups. Results for patients with IBS with diarrhoea (IBS-D) without *Brachyspira* (n=11) are separately displayed, as well as included in the category of all patients with IBS without spirochetosis. (D) Oro-anal transit times (OATT) for patients with IBS with versus without *Brachyspira*. OATT were shorter in membrane-associated spirochetosis as compared with IBS without spirochetosis (p=0.05) and patients with IBS without *Brachyspira* colonisation of the epithelial membrane (p=0.03; Welch-corrected t=2.3). For (C) and (D) bars represent the mean and error bars the SE of the mean (SEM); groups were compared by unpaired Welch's t-tests. For all analyses of clinical characteristics, the two study cohorts were merged. The analysis was restricted to participants where *Brachyspira* colonisation could be confirmed or rejected by at least two independent methods or cases where real-time PCR was negative for *Brachyspira* with no conclusive results from other methods.

resemblances with enteric infections, such as shigellosis and salmonellosis (figure 5B).

Other significantly dysregulated proteins were linked to guanylate cyclase/cGMP (guanylin, online supplemental figure S4B) and somatostatin (SST, PCSK1N; online supplemental figure S4C) signalling, as well as to methylglyoxal detoxification (GLO1, PARK7; online supplemental figure S4D). Guanylin is an endogenous ligand of guanylate cyclase-C (GC-C), also known as the heat-stable enterotoxin receptor.[16] GC-C signalling attenuates, whereas somatostatin promotes, fluid reabsorption.[17] Methylglyoxal is a minor by-product of human glycolysis, as well as a toxic metabolite of several intestinal bacterial species.[18] While guanylin and proteins related to methylglyoxal toxicity were increased in spirochetosis, somatostatin levels were decreased (online supplemental figure S4B–D).

In patients with proteomic *Brachyspira* identifications, indicating dense membrane colonisation, glycocalyx and desmosomal proteins were reduced, suggesting multilevel mucosal barrier weakening (figure 5A). Indications of membrane remodelling were less conspicuous in individuals with no, or patchy, epithelial colonisation (figure 5C, online supplemental figure S4E). By contrast, inflammatory mediators did not substantially differ depending on colonisation pattern.

Spirochetosis was associated with mild mucosal inflammation and mast cell activation

Histology revealed a modest increase in lamina propria immune cells (p<0.001), particularly plasma cells (p<0.001), in membrane-associated spirochetosis compared with IBS without *Brachyspira* (figure 5D–F). Intraepithelial/subepithelial eosinophils were also augmented (p=0.002; online supplemental figure S5A,B). Full results of the differential counting of mucosal immune cells are provided in online supplemental tables S6A,B.

Strikingly, total and activated mast cells were significantly more abundant in patients with IBS with *Brachyspira*, based on toluidine blue staining (figure 5G, online supplemental figure S5C,D). Subepithelial mast cell counts correlated closely with abdominal pain in spirochetosis (figure 5H, online supplemental figure S5E) but not in patients with IBS without *Brachyspira*. In the proteomic analysis, mast cell proteins tryptase, chymase and/or beta-hexosaminidase were more prevalent in mucus from

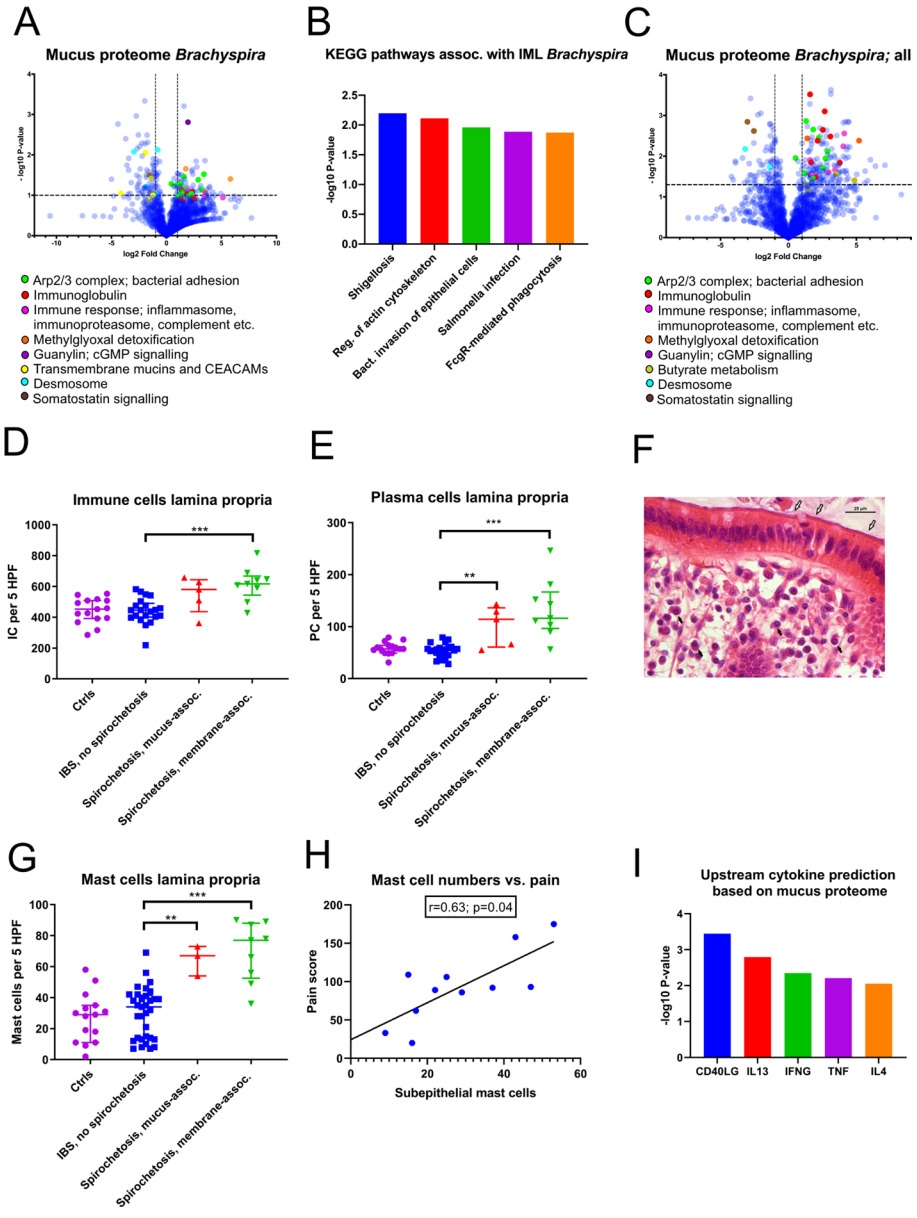

Figure 5 Spirochetosis was linked to distinct mucosal responses at the molecular and cellular level. (A) Volcano plot showing host mucus proteome alterations in samples where *Brachyspira* was detected by metaproteomics (n=6, vs n=18 samples from individuals without *Brachyspira*). The transformed p values were obtained by Welch's t-tests. (B) Top five predicted Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways based on mucus protein enrichment in individuals with metaproteomic *Brachyspira* identifications. Proteins at least twofold upregulated with a p value <0.10 were included in the analysis. Pathway p values were obtained through a modified version of Fisher's exact test (EASE score), using DAVID bioinformatics tool. IML, inner mucus layer. (C) Host mucus proteome alterations in samples (n=17) from patients where *Brachyspira* could be detected by two independent methods (some of whom were negative for *Brachyspira* in the mass spectrometry analysis). (D) Increased immune cell infiltration of the lamina propria of patients with membrane-associated spirochetosis compared with patients with IBS without *Brachyspira* (Mann-Whitney U=17.0; p<0.001). The graph shows results from patients with membrane-associated spirochetosis (n=9), mucus-associated spirochetosis (n=5), patients with IBS without *Brachyspira* (n=21) and healthy volunteers (n=15). (E) Plasma cell counts were increased in spirochetosis (membrane-associated spirochetosis vs IBS without *Brachyspira*: U=9.0; p<0.001). (F) Plasma cells (black arrows) in an H&E-stained sigmoid colon section from a patient with membrane-associated *Brachyspira* (white arrows). Scale bar: 25 µm. (G) Mast cell expansion in the lamina propria of patients with spirochetosis (membrane-associated spirochetosis vs IBS without *Brachyspira*: U=19.5; p<0.001). The graph shows results from 9 patients with IBS with membrane-associated spirochetosis, 3 patients with mucus-associated spirochetosis, 33 patients with IBS without *Brachyspira* and 15 healthy volunteers. (H) Correlation between subepithelial mast cell numbers and pain (summed pain frequency and intensity scores from the IBS Severity Scoring System questionnaire). The line was fitted using linear regression analysis. Spearman's rho: 0.63 (95% CI: 0.02 to 0.90). Immune cells were counted in five high-power fields (MAG ×600) in H&E or toluidine blue (mast cells) stained sigmoid colon sections; individual observations are overlaid by the median and IQR. Groups were compared by the Mann-Whitney U test: **p≤0.01, ***p≤0.001. (I) Upstream activation of cytokines in patients with proteomic *Brachyspira* identifications, predicted from Ingenuity Pathway Analysis of the host mucus proteome. Data were analysed through the use of IPA (Qiagen, https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis). Minimum twofold upregulated proteins with a p value <0.15 were included in the analysis. P values for cytokine prediction were obtained by Fisher's exact test. CEACAMs, carcinoembryonic antigen-related cell adhesion molecule; IC, immune cell; PC, plasma cell.

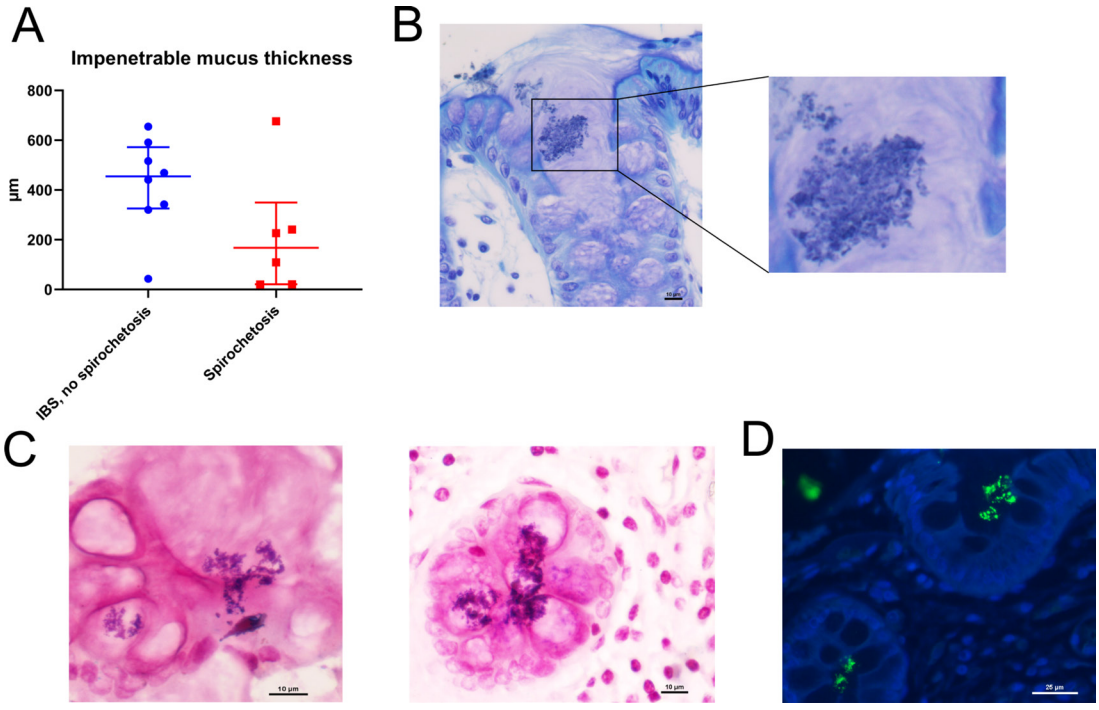

Figure 6 *Brachyspira* colonisation was associated with mucus barrier failure. (A) Impenetrable mucus thickness tended to be reduced in patients with spirochetosis (p=0.11; Mann-Whitney U test). Toluidine blue (B) and Gram (C) staining of sigmoid colon sections from patients with spirochetosis showed bacterial invasion of crypt lumina and goblet cells. Some of the invading bacteria appeared Gram positive. Scale bars: 10 µm. (D) Section from a patient with spirochetosis stained with anti-lipoteichoic acid (green) and counterstained for DNA with Hoechst (blue), showing Gram-positive bacteria inside crypts. Scale bar: 25 µm.

patients with spirochetosis (p=0.09), whereas enzymes involved in histamine metabolism were more abundant (p=0.04 for the comparison with patients with IBS without *Brachyspira*; online supplemental figure S5F, online supplemental data file S3). Based on the overall mucus proteome alterations, cytokines CD40LG, IL13 and IL4 were predicted as major upstream regulators (figure 5I). Taken together, our observations suggest the immune response in *Brachyspira* infection to be predominantly Th2-driven.

Brachyspira colonisation was linked to mucus barrier failure and crypt invasion by other bacteria

While the main component of the mucus, the MUC2 mucin, tended to be more abundant in spirochetosis, the thickness of the impenetrable mucus was reduced (figure 6A, online supplemental figure S6A). Moreover, bacterial invasion into crypts and goblet cells was occasionally detected (figure 6B,C, online supplemental figure S6B–D). These observations did not match *Brachyspira* staining (online supplemental figure S6E). Instead, at least some of the invading bacteria appeared Gram positive, which was verified through staining with anti-lipoteichoic acid (figure 6C,D).

Antibiotic treatment in *Brachyspira*-associated IBS might be linked to partial clinical improvement over time

Based on current recommendations, four patients with membrane-associated spirochetosis received metronidazole treatment (online supplemental table S7).[13] Although initial responses were variable, three patients could potentially be classified as long-term responders, with a reduction in overall IBS-SSS scores >50 points 1 year post-treatment (figure 7A,B).[7] In particular, pain, bloating and IBS interference with quality of life were reduced (figure 7C, online supplemental figure S7A,B). Bowel habit dissatisfaction and stool consistency did not conclusively improve (online supplemental figure S7C,D). However, in two responders, stool frequency was significantly reduced post-treatment, while the third responder had a normal frequency at baseline (figure 7D, online supplemental figure S7E).

Spirochaete relocation into crypts and goblet cells post-antibiotics

In all four cases, AB-PAS staining of tissue sections demonstrated clearance of the *Brachyspira* from the epithelium 6 weeks post-treatment (figure 8A). Real-time PCR of colonic biopsies and faecal samples corroborated a drastic reduction in *Brachyspira* (online supplemental table S8A,B).

However, results from immunostaining for *Brachyspira* in biopsies obtained 6 weeks post-metronidazole were less encouraging. Although the spirochetes had largely disappeared from the epithelial surface, *Brachyspira* invasion into crypts and goblet cell mucus granules was observed in all four patients (figure 8B–G, online supplemental figure S8A). Gram staining did not indicate a similar relocation of Gram-positive bacteria post-treatment (online supplemental figure S8B), suggesting a specific rather than general response.

While overall mucosal immune cell numbers remained unchanged after treatment, there was a reduction in plasma cell, mast cell and eosinophil counts (online supplemental figure S8C–F, figure 8H). Taken together, the cellular composition appeared to deviate from a Th2-like to a Th1-like pattern—possibly reflecting the *Brachyspira* shift to an intracellular lifestyle.

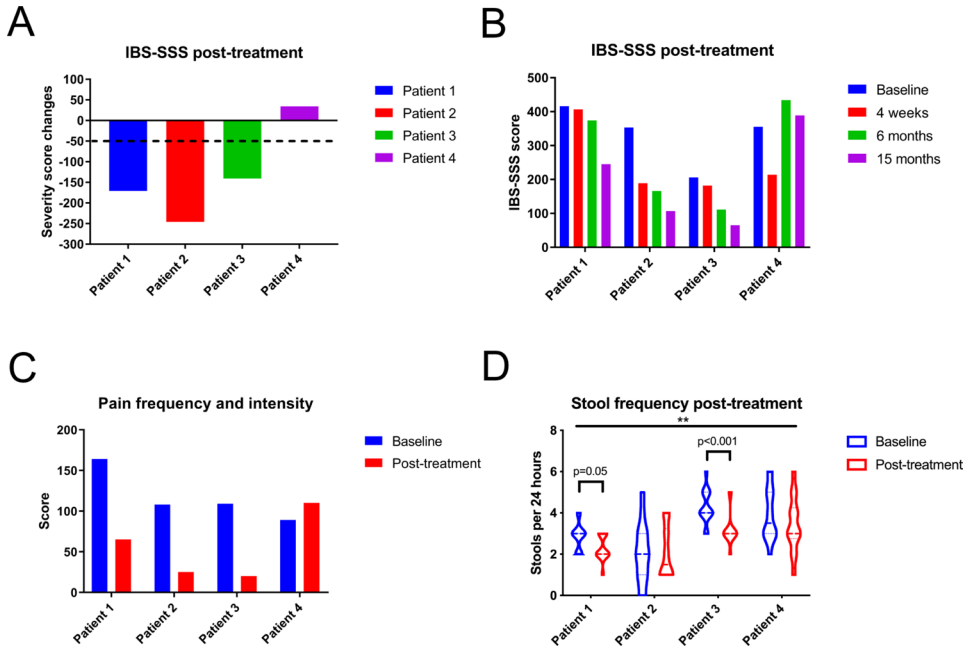

Figure 7 Three in four patients with spirochetosis experienced partial clinical improvement following antibiotic treatment. (A) Three patients had a sustained clinical response, defined as a reduction of their overall scores from the IBS Severity Scoring System (IBS-SSS) of at least 50 points, after 15 months. (B) Overall IBS-SSS scores at baseline and at 1, 6 and 15 months post-treatment. (C) Summed IBS-SSS pain frequency and intensity values at baseline and at 15 months post-treatment. (D) Violin plot of stool frequency at baseline and 1 year post-treatment. The midline represents the median and the upper and lower lines the interquartile range (25th and 75th percentile). Time points were compared through a two-way analysis of variance, with the horizontal bar on top referring to the overall comparison of stool frequency before and after treatment (F=7.0; p=0.01). Subsequent intraindividual comparisons were performed by Mann-Whitney U tests, with an adjusted significance threshold of 0.0125.

DISCUSSION

The increased incidence of IBS after a gastroenteritis episode suggests microbial perturbation as an underlying factor.[1] However, studies of faecal microbiota in IBS have not demonstrated reproducible alterations.[2] In this investigation, potentially pathogenic *Brachyspira* species were identified in the colonic mucosa of 31% of patients with IBS but not in any healthy individual. *Brachyspira* attachment to the epithelial brush border was observed in every fifth patient with IBS and associated with diarrhoea, accelerated oro-anal transit, mild mucosal inflammation and mast cell activation. Hence, *Brachyspira* colonisation defines a sizeable and distinctive subgroup of patients with IBS that might be responsive to antibiotic therapy. However, metronidazole treatment paradoxically resulted in spirochaete invasion into crypts and goblet cells. Thus, our observations suggest a role for *Brachyspira* in IBS-D pathogenesis but also urge caution with regard to antibiotic therapy in IBS.

While *Brachyspira* species are well-established pathogens in veterinary medicine, reports on the relationship between spirochetosis and symptoms in humans are contradictory.[15 19–23] Variable, frequently partial, responses to treatment have added to the controversy.[13 15 20] Potentially human-pathogenic *Brachyspira* species are notoriously difficult to culture. Therefore, the diagnosis typically relies on histology, where perpendicular attachment of the *Brachyspira* to the apical membrane may appear as a distinctive 'fringe'.[14 15 19–21 24] Reported prevalence rates of intestinal spirochetosis range from 0.5% to 3% in industrialised countries.[13 15 21–23] Here, based on results from two independent prospective cohorts, *Brachyspira* were observed in contact with the epithelium in 20%–40% of patients with IBS-D but not in patients with predominant constipation or healthy individuals.

Although one previous publication has suggested a link between IBS and intestinal spirochetosis, this is the first report of increased prevalence of *Brachyspira* colonisation in IBS.[21] Patients with IBS-D routinely undergo recto-/colonoscopy with histology. Moreover, 16S rDNA sequencing of faecal samples has been performed in several large-scale studies.[2 3] Thus, it may be surprising that this association should have gone undetected. Here instead, the initial observations were made through unbiased metaproteomic analysis of mucus samples and corroborated by immunostaining and targeted PCR analysis. By contrast, routine histology and faecal 16S rDNA sequencing failed to detect *Brachyspira* in these patients. The ability of standard 16S amplicon sequencing to identify *Brachyspira* species was recently shown to be hampered by primer incompatibility.[25] This underlines the importance of innovative and complementary methods to identify novel associations between gut microbiota and human disease.

The effects of *Brachyspira* attachment on the epithelium and underlying tissue have traditionally been regarded as minimal.[15 24] Here, in-depth analysis of host mucosal responses revealed a striking increase in inflammatory mediators and proteins associated with membrane remodelling. Unbiased functional annotation analysis mapped these alterations to pathways associated with bacterial adhesion and phagocytosis, detecting mechanistic resemblances to enteric infections such as salmonellosis and shigellosis. Histology confirmed a modest but distinctive inflammatory response, with expansion of plasma cells, eosinophils and mast cells. The cellular composition, in conjunction with upstream cytokine prediction based on the proteomic analysis, indicated that the inflammation may be predominantly Th2-driven. Further studies are required to establish a causal relationship between spirochetosis and concomitant mucosal inflammation. Still, our observations suggest *Brachyspira* as a potential human pathogen rather than merely a harmless commensal.

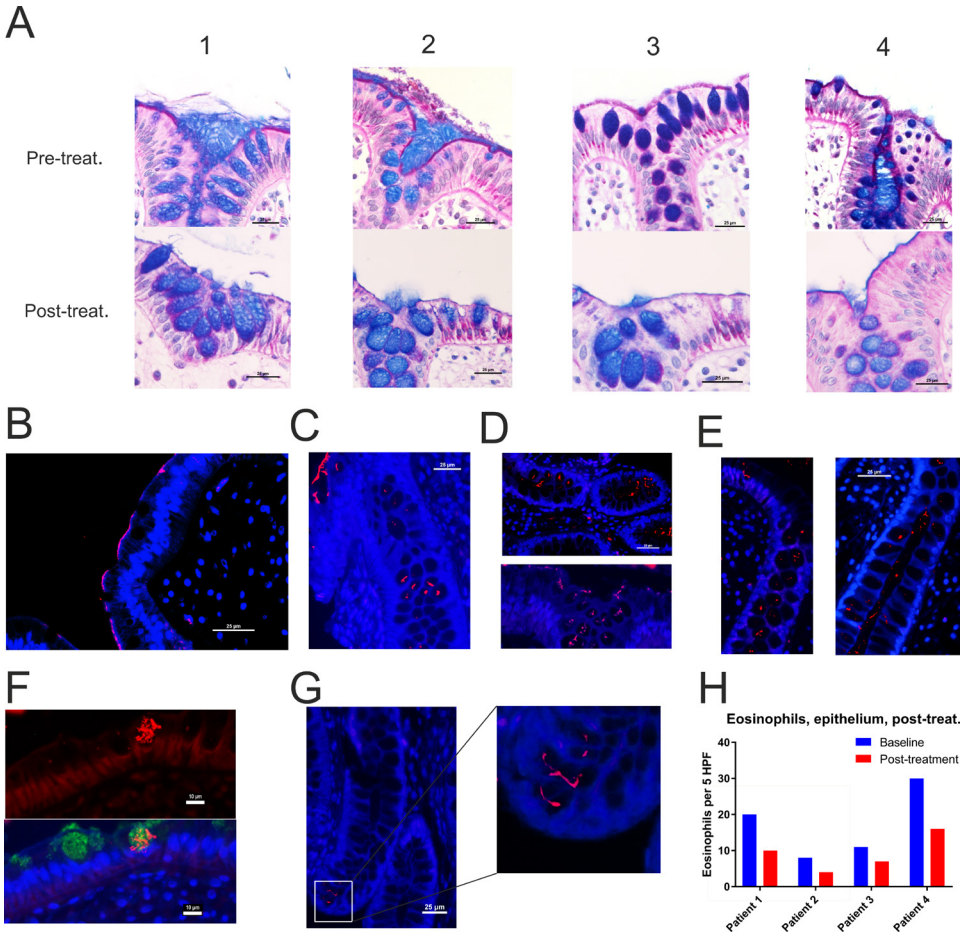

Figure 8 Invasion of spirochetes into crypts and goblet cells post-treatment. (A) Alcian blue-periodic acid-Schiff stain of (from left to right) patients 1–4 before and 6 weeks after the completion of treatment. Staining with *Brachyspira* antiserum (red) revealed a persistent, faint/patchy staining of the apical membrane after treatment in the non-responder ((B), patient 4) and in patient 1 (C). Furthermore, *Brachyspira* (red) was observed in crypt lumina and goblet cells in all cases, as shown for patients 1 (C), 2 (D), 3 (E) and 4 (see online supplemental figure S8). (F) *Brachyspira* (red) co-localised with anti-MUC2 staining (green) inside goblet cells. The figure shows images of the same area that were obtained from adjacent sections, manually fitted and superimposed. (G) *Brachyspira* (red) penetrating into the crypt base. In all immunofluorescent images, nucleic acids were counterstained with Hoechst (blue). Scale bars in (F) are 10 µm, all other scale bars 25 µm. (H) There was a reduction of subepithelial/intraepithelial eosinophils post-treatment in all cases.

While diarrhoea has previously been related to spirochetosis, the mechanistic link has not been explored.[15 19] Here, based on analysis of host responses, several molecular shifts that may synergistically induce diarrhoea were identified. First, remodelling of the apical membrane might reduce the surface available for fluid re-uptake. Second, a pronounced increase in the mucus abundance of guanylin was observed in membrane-associated spirochetosis. Guanylin is a ligand of the apical GC-C receptor, which is also the target of heat-stable *Escherichia coli* enterotoxin.[16] GC-C signalling attenuates sodium uptake by NHE3 (SLC9A3) and promotes chloride secretion by cystic fibrosis transmembrane conductance regulator (CFTR), resulting in impaired fluid absorption.[16] Accordingly, activating mutations of GC-C is a rare cause of IBS-D.[16] Moreover, endogenous GC-C signalling is induced in certain enteric infections and may contribute to pathogen clearance.[26] Another potential cause of diarrhoea could be decreased somatostatin signalling, as suggested by the proteomic analysis. Somatostatin promotes intestinal fluid absorption, possibly via the NHE8 (SLC9A8) transporter, and is used to treat refractory diarrhoea.[17] Somatostatin-producing cells are highly susceptible to *Helicobacter pylori*-related inflammation and might conceivably also be particularly affected by spirochetosis. Finally, histamine secretion from activated mast cells may promote secretomotor diarrhoea.[27] In conclusion, this study identified several mechanistic clues to the association between spirochetosis and IBS-D, supporting the biological relevance of this unexpected observation.

Antibiotic treatment of patients with spirochetosis was associated with alleviated abdominal pain and bloating, suggesting that *Brachyspira* may contribute to IBS symptoms other than diarrhoea. Mast cell activation has previously been linked to IBS-related abdominal pain, although reports are conflicting.[28 29] In this study, mucosal mast cell counts were selectively increased in *Brachyspira*-associated IBS and correlated closely with abdominal pain scores in patients with spirochetosis but not in IBS without *Brachyspira*. Thus, the controversial link between mast cells and IBS symptomatology might in part depend on *Brachyspira* colonisation status.

Spirochetosis was associated with mucus barrier failure, decreased glycocalyx components and sporadic crypt invasion by other genera. Furthermore, there were indications of enhanced mucosal responses to bacterial metabolites—including methylglyoxal that is likely not produced by *Brachyspira*. Methylglyoxal has been linked to visceral hypersensitivity, for example,

through the induction of nociceptor ion channel TRPA1.[18 30] Hence, inappropriate host contact with commensal microbiota could aggravate symptoms in patients with spirochetosis and may partly explain variations of the clinical picture.

Several studies have reported positive effects of non-absorbable antibiotics in a subset of patients with IBS.[2 31] In our investigation, three in four patients with spirochetosis experienced partial, but sustained, symptom relief following metronidazole treatment. The response correlated with improvement of objective parameters, such as stool frequency and mast cell counts. Results from this small-scale, open-label intervention must obviously be interpreted with caution. The effects of *Brachyspira* eradication in IBS will hopefully be the topic of future, appropriately powered, placebo-controlled trials.

Our observations tentatively suggest that stratification of patients with IBS with spirochetosis for antibiotic treatment might result in improved response rates. Nonetheless, there are obvious caveats, including growing resistance problems reported from veterinary medicine.[32] In this study, the therapeutic intervention had to be discontinued due to spirochaete relocation into goblet cells. Concerningly, *Brachyspira* were also detected in the crypt base post-antibiotics, with the potential to directly influence the stem cell reservoir. Thus, therapeutic strategies other than antibiotics may have to be considered to treat IBS-associated spirochetosis. These might include short-term laxative treatment, bismuth subsalicylate or probiotics/prebiotics, used alone or as adjuvants to antibiotics.

While bacterial invasion of goblet cell mucus granules post-antibiotics is a novel phenomenon, it is partly reminiscent of goblet cell-associated antigen passages (GAPs).[33] In mice, GAPs mediate translocation of live bacteria through the colonic epithelium in response to dysbiosis, for instance during metronidazole treatment.[33] Nevertheless, the time lapse since antibiotic therapy and the massive scale of *Brachyspira* relocation rather support an adaptation of the spirochetes. Thus, certain *Brachyspira* presumably have a tropism for colonising mucus granules—a trait that may have been favoured by antibiotic treatment. Although metronidazole easily diffuses into cells, the densely packed granule contents might restrict its access to the *Brachyspira*. Future studies should investigate whether antibiotics promote similar adaptations in other pathogenic or commensal bacterial species.

The study has some limitations. Suboptimal biopsy specimens and technical failures resulted in a small proportion of missing data for certain methods, although with no systematic bias between participant categories. Most importantly, the observational nature of this study did not allow us to establish a causal relationship between *Brachyspira* and IBS symptoms. Other unanswered questions concern the potential contribution of epidemiological factors to *Brachyspira* colonisation—including antibiotic treatment, which may be more frequent in patients with IBS and has been linked to increased risk of post-infectious IBS.[1] The exact molecular mechanisms of the *Brachyspira*-host interaction also require further investigation. However, as the main species associated with intestinal spirochetosis, *B. aalborgi*, is exceptionally difficult to culture and infects only primates, experimental models to address this question are lacking.[14 24] Hence, there are major challenges to overcome in order to fully elucidate the role of *Brachyspira* in IBS-D pathogenesis.

In conclusion, we report a novel, strong association between the *Brachyspira* genus and IBS. Based on results from two independent prospective cohorts, mucosal *Brachyspira* colonisation was observed in 40% of patients with IBS-D but not in any healthy individual. *Brachyspira*-associated IBS was linked to distinctive clinical, histological and molecular characteristics, suggesting that it should be considered a separate diagnostic entity. Thus, *Brachyspira* eradication therapy could conceivably have substantial effects on the overall IBS morbidity burden. However, the increased invasiveness of the spirochetes post-treatment urges caution with regard to antibiotic therapy in IBS. Strikingly, the invasion of goblet cell mucus granules could represent a novel bacterial strategy to survive antibiotics, which may have bearings on other recurrent and chronic infections originating at mucosal surfaces.

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Contributors KSJ, MS and GCH conceived the original idea; KSJ, BD, LE, CW, AE and ÅJ performed experimental procedures and data analysis; HT and MS supervised subject enrolment; KSJ, LE, HT and MS analysed clinical data, KSJ drafted the manuscript with input from all authors.

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Supplement to

Association between *Brachyspira* and irritable bowel syndrome with diarrhoea

Jabbar KS, et al.

Contents

Methods

Study population- exclusion criteria and sample size considerations

General *a priori* established exclusion criteria for study participation were inability to provide informed consent, gastrointestinal disease (diagnosed or suspected) apart from IBS, severe comorbidities, pregnancy, breastfeeding, medication with immunomodulators, and the use of probiotics, antibiotics and NSAIDs one month before, and for the duration of the study. Exclusion criteria did not differ between study cohorts.

The sample size could not be calculated *a priori*, given the explorative nature of the study. The required sample size for the second cohort was determined as follows: Prevalence rates of intestinal spirochetosis in the first cohort were 41% among IBS patients (based on at least one positive test) and 0% among healthy volunteers. The required confidence level was set to 95%, and power to 80% (alpha 0.05 and beta 0.2). Based on these considerations, a sample size of 15 IBS patients and an equal number of healthy volunteers was calculated to be sufficient to verify a difference in the prevalence of spirochetosis between participant groups (IBS vs. healthy) in a second cohort. Still, to be able to verify a potentially clinically meaningful difference, we aimed to confirm a prevalence of Brachyspira colonization >20% for IBS patients. The minimum sample size for the IBS group that would ensure that the lower limit of the 95% confidence interval for the prevalence rate would exceed 20% was determined to be 35, based on results from the first cohort. To allow for the assessment of prevalence differences between IBS subtypes, a slightly higher number of participants with IBS (n=40) was enrolled. By contrast, the number of healthy volunteers in the study was kept to a minimum, based on the above estimations. Sample size calculations were performed using the normal approximation to the binomial distribution.

Patient symptom severity scoring systems

Participants with IBS completed a two-part questionnaire in order to assess the severity of their IBS-related and extra-colonic symptoms. Results are reported for patients where Brachyspira colonization

could be confirmed/rejected by at least two independent methods (n=50), and for patients where real-time PCR was negative for Brachyspira, and where other tests were not performed (n=3). The IBS-specific questionnaire includes the following components: intensity and frequency of abdominal pain, severity of bloating/abdominal distension, dissatisfaction with bowel habits, and symptom interference with daily life. The score range is 0-500, with higher scores indicating more severe symptoms.[1] The extra-colonic score measures the severity of ten symptoms (including nausea, early satiety, flatulence, heartburn, headache, back pain, fatigue, pain involving thighs, muscles and joints, and urinary urgency), again with a score range of 0-500.[2]

Oro-anal transit time

In order to assess the oro-anal transit time (OATT), IBS patients (n=60) ingested 10 radiopaque rings daily for six days. Results are reported for 51 of these patients, where Brachyspira colonization could confirmed/rejected with high confidence (see previous section). The last day of the protocol, IBS patients ingested five radiopaque rings in the morning and five radiopaque rings in the evening in order to better define patients with accelerated transit time. On day 7, fluoroscopic visualization and counting of the retained markers was performed, using an Exposcop 7000 compact instrument (Ziehm, Nüremberg, Germany). The oro-anal transit time in days was obtained by dividing the number of retained rings by 10 (corresponding to the number of markers ingested each day).[3] Patients were requested to discontinue laxatives and anti-diarrhoeals, as well as other medications known to affect gastrointestinal motility, two days prior to the start of the examination protocol.

Rectal sensitivity

Rectal sensitivity was assessed using an electronic barostat (Dual Drive Barostat, Distender Series II; G & J Electronics, Toronto, Canada) in a total of 45 IBS patients from both cohorts. Results are reported for 37 patients, where Brachyspira colonization could be confirmed/rejected with high confidence. After a habituation sequence, the rectal balloon distension protocol started from 0 mmHg with 4 mmHg increments every minute. Patients were instructed to report first sensation, desire/urge to defecate, discomfort and pain, to enable determination of individual pressure thresholds (mmHg) for these sensations.[4]

Ex vivo mucus collection from sigmoid colon biopsies

Two sigmoid colon biopsies per participant (n=40; first cohort n=36, second cohort n=4) were transported in oxygenated Krebs buffer on ice and mounted in our *ex vivo* mucus measurement chambers with an inner diameter of 1.5 mm.[5] Immediately after mounting, a thin mucus layer (corresponding to the thickness of the adherent inner mucus layer) could be visualized through the addition of a charcoal suspension in oxygenated Krebs-mannitol buffer to the apical side of the biopsy. The biopsy was then allowed to secrete mucus for one hour, with continuous basolateral perfusion with oxygenated Krebs-glucose. Following a stepwise increase over 10 min., the temperature of the chamber was kept at 37°C for the remainder of the experiment. The mucus typically grew to approximately 1.5 times the initial thickness during the observation time (median thickness 398 µm immediately after the mounting of the biopsy in the *ex vivo* system, vs. 644 µm at the end of the experiment). After one hour, the mucus was collected by gentle scraping and stored together with protease inhibitors (1 x cOmplete™ Mini, EDTA free; Roche) at -80°C. The mucus sampling procedure was performed under clean, but not fully aseptic, conditions.

Preparation of mucus samples for mass spectrometry

Mucus samples were prepared for MS according to a modified version of the Filter-Aided Sample Preparation (FASP) protocol.[6] Briefly, samples were solubilized and reduced overnight, using 0.1M dithiothreitol in 6M guanidium chloride. They were then transferred to 10 kDa mass cut-off spin filter units (Nanosep®, Pall, Ann Arbor, MI) and centrifuged at 7,500 x g for 10 min. Following alkylation with 0.05 M iodoacetamide (20 min.), proteins were digested with trypsin (Promega, Madison, WI) overnight. Tryptic digests were acidified and cleaned using micro-scale C18 stage tips as previously described, dried under vacuum and reconstituted in 0.2% formic acid.[7]

Mass spectrometry and data processing

Nano-liquid chromatography-tandem MS was performed using an EASY-nLC system (Thermo Scientific, Odense, Denmark) connected to a Q-Exactive (Thermo Scientific, Bremen, Germany) through a nanoelectrospray ion source. Peptides were loaded onto a reverse-phase column (150 mm x

75 µm inner diameter, New Objective, Woburn, MA), packed with Reprosil-Pur 3 µm C18-AQ particles (Dr. Maisch, Ammerbuch, Germany). After loading in 0.1% formic acid, peptides were separated with a 60-min gradient from 5 to 35% of 80% acetonitrile in 0.1% formic acid. Full mass spectra were then acquired in the Orbitrap analyzer, and fragmentation performed for the 12 most intense ions per scan. Peptides were identified using the Andromeda search engine integrated into the MaxQuant environment (version 1.3.0.5), as well as the MASCOT software (version 2.2, Matrix Sciences, London, UK).[8] Searches were performed against all reviewed human and eubacteria sequences of the Swissprot-Uniprot database, as of February 2016.[9] Settings for the searches were as follows: one missed cleavage allowed, precursor tolerance 7 ppm, fragment ion tolerance 0.5 Da, carbamidomethylated cysteine as a fixed modification, and oxidized methionine as a variable modification. Minimum one unique peptide at a false discovery rate threshold of 1% was required for protein identification. For the identification of a bacterial family/genus, the following criteria were set: 1) at least three proteins, each identified by a minimum of one unique, family/genus-specific, non-modified peptide, with an ion score >20, or 2) at least one protein identified by a minimum of one unique, family/genus-specific, non-modified peptide with an ion score >45.

Analysis of the human mucus proteome

For the analyses of the host mucus proteome, the following exclusion criteria were established: 1) identification of other bacteria in the inner mucus layer, in the absence of *Brachyspira*, 2) contradictory results or borderline identifications from other analyses with regard to *Brachyspira* identification (such as detection after 40 cycles in the real-time PCR analysis), and 3) less than 200 proteins identifications in the sample. In total, 18 samples from an equal number of individuals without *Brachyspira* (11 healthy individuals and 7 IBS patients) and 17 samples from 10 patients with spirochetosis could be analysed. To account for differences in sample amounts, individual protein intensities were normalized against the total intensity of all proteins in the sample. Since the overall protein composition of the mucus samples from patients with and without spirochetosis tended to differ substantially, proteins that could not be detected were generally considered as absent for the analysis. The DAVID bioinformatics tool (v. 6.8) and Ingenuity Pathway Analysis (QIAGEN Inc.,

https://www.qiagenbio-informatics.com/products/ingenuity-pathway-analysis; Redwood City, CA) were used for functional annotation analysis, and for the prediction of upstream regulators, respectively.[10-12] Only proteins that were at least twofold up- or downregulated were considered for these analyses; the significance threshold was set to 0.05, 0.10 or 0.15, depending on the number of samples, as stated in the figures/legends.

Histology and immunohistochemistry

One sigmoid colon biopsy from each participant from the first and second cohorts (n=93) was fixed in water-free methanol-Carnoy solution, paraffin-embedded and sectioned. Following de-waxing, slides with sections were stained with either hematoxylin-eosin (H&E), Alcian blue/periodic acid-Schiff (AB-PAS), toluidine blue, Gram stain, or with one or more of the following antibodies for fluorescent microscopy: *Brachyspira* antiserum (dilution 1:10,000; gift from Lars Engstrand, Karolinska Institute, Stockholm, Sweden),[13] anti-CLCA1 (1:2,000; ab129283, clone 1C4, , Abcam, Cambridge, UK), anti-MUC2 (1:500; developed in-house),[14] anti-lipoteichoic acid (1:50; MA1-7401, clone G35C, Thermo Fisher, Eugene, OR) or anti-*Pseudomonas aeruginosa* (1:800; ab68538, Abcam). Anti-rabbit/antimouse IgG conjugated with fluorophores Alexa Fluor 488 or 555 (Thermo Fisher) were used as secondary antibodies (1:1,000 dilution). DNA was counter-stained with Hoechst 34580 (Thermo Fisher; 1 µg/ml). Sections were examined using an Eclipse E-1000 epifluorescent microscope (Nikon, Tokyo, Japan) and images captured using NIS elements (v. 4.20, Nikon).

The local immune response was quantified by differential counting of the number of immune cells per 5 high-power fields (magnification x 600) in the lamina propria, or in/adjacent to the surface epithelium, using sections stained with H&E and – for mast cells – toluidine blue. The analysis was restricted to participants where *Brachyspira* colonization could be confirmed/rejected by two independent methods (with the exception of three cases which were negative on real-time PCR analysis with no positive results). For H&E-stained sections, a subset of *Brachyspira*-negative IBS patients and healthy volunteers were randomly selected for analysis from among sections deemed to be of sufficient quality for assessment. Quantification of lamina propria immune cells was performed in 15 controls, 21 IBS patients without *Brachyspira*, 5 patients with mucus-associated spirochetosis,

and 9 patients with membrane-associated spirochetosis. Sub-/epithelial immune cell populations were counted in 14 controls, 19 IBS patients without *Brachyspira*, 5 patients with mucus-associated spirochetosis, and 9 patients with membrane-associated spirochetosis. There were no significant differences in the distribution of IBS subtypes between participants selected vs. not selected for lamina propria immune cell quantification on H&E stained sections (IBS-C: 24% vs. 33%, p=0.46; IBS-D: 29% vs. 33%, p=1.0; IBS-M: 43% vs. 22%, p=0.31; IBS-U= 5% vs. 11 %, p=0.59). Median overall IBS-SSS scores were also comparable (325 vs. 318; p=0.86).

Quantification of mucosal mast cells on toluidine blue stained sections was attempted for all IBS patients where *Brachyspira* colonization could be confirmed/rejected with high confidence; and was possible in 85% of these cases. Mast cells were also quantified in a subset of healthy volunteers. Altogether, numbers of total/activated mast cells were assessed in 15 controls, 33 IBS patients without spirochetosis, 3 patients with mucus-associated spirochetosis, and 9 patients with membrane-associated spirochetosis. Among IBS patients without *Brachyspira*, IBS severity did not significantly differ between cases where section quality precluded (n=6) vs. allowed mast cell quantification (median IBS-SSS 249 for cases with missing data vs. 325 for cases with conclusive results, p=0.52). There were also no significant differences in the distribution of IBS subtypes among cases with vs. without conclusive results from mast cell quantification (IBS-C: 27% vs. 33 %, p=1.0; IBS-D: 30% vs. 33%, p=1.0; IBS-M: 36% vs. 17%, p=0.64; IBS-U= 6% vs. 17%, p=0.40). Mast cells were counted in two locations: the subepithelial and deep portions of the lamina propria. For the latter case, mast cells in the muscularis mucosae were also included.

Transmission electron microscopy

Biopsies were fixed for 24 hours in modified Karnovsky's fixative (2% paraformaldehyde, 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.2), followed by sequential staining using 1% OsO_4 for 4 h, 1% tannic acid for 3 h, and 1% uranyl acetate overnight at 4°C. Samples were dehydrated and embedded in epoxy resin (Agar 100, Agar Scientific, Stansted, UK). Sections (50 nm) were microtome-cut (Ultracut E, Reichert, New York, NY) and collected on copper mesh support grids. The sections were contrasted using lead citrate and uranyl acetate, and electron microscopy was

conducted on the Leo 912 Omega with a lanthanum hexaboride gun (Carl Zeiss) at 120 kV. Images were acquired using a MegaView III CCD camera (SiS, Münster, Germany).

DNA isolation from Carnoy-fixed paraffin-embedded tissue, fresh frozen biopsies and faecal material

DNA was isolated from methanol-Carnoy fixed paraffin-embedded tissue in a total of 74 participants (first cohort: 28, second cohort: 46). Briefly, for each tissue sample five 10 µm sections were cut and collected in a 1.5 ml DNA free microcentrifuge tube. Samples were briefly stored at -20°C before DNA isolation. The microtome blade was replaced after each sample, to prevent contamination of the subsequent sample. Sections were de-paraffinised using xylene and washed in 99.5% ethanol. Following evaporation of excess ethanol, DNA was isolated using QIAamp DNA micro kit (Qiagen, Hilden, Germany), following manufacturer's instructions, including incubation at 90°C for one hour following the proteinase K digestion step. Eluted DNA was stored at -20°C until use.

In addition, PCR analysis was performed with DNA extracted from fresh frozen biopsies (10 participants from the first cohort and 7 from the second cohort) and faecal material (4 patients; explorative cohort: 3, second cohort: 1). DNA was isolated from frozen biopsies and 250 mg of faecal material using QIAamp DNA micro kit and QIAamp PowerFecal kit (Qiagen), respectively, following manufacturer's guidelines, and stored at -20°C until use.

The quality of the DNA extracted from fresh frozen and fixed colonic tissue was assessed by real-time PCR analysis of the human *GAPDH* gene (forward primer: 5´-GCTCTTAAAAAGTGCAGGGTCTG-3´; reverse primer: 5´-TGCTGTAGCCAAATTCGTTGTC-3´), resulting in the exclusion of three samples from healthy volunteers (Data file S1).

Primer design and PCR analysis

The presence of *Brachyspira* spp. was detected via melting curve-based species identification as described by Westerman et al.[15] The primers 5´-TGGATAAGTTAGCGGCGAACTG-3´(forward) and 5´-TAGGCCGCAGGCTCAT-3´ (reverse) amplify an 82 base pair fragment of the *Brachyspira* 16S genes. Differences in the melting temperatures of the generated products allow for discrimination

between the human pathogenic *B. aalborgi, B. pilosicoli*, and (the unconfirmed) *B. hominis* species. Twenty microliter reactions were performed using SsoFast™ EvaGreen® Supermix (Bio-Rad, Hercules, CA) and contained 2.5 μM of each primer. The amplification program consisted of 98°C for 5 minutes followed by 45 cycles (98°C for 10 seconds, 55°C for 10 seconds and 72°C for 20 seconds). However, only results obtained within the first 40 cycles were considered for the analyses. Following amplification, a melting curve was generated from 65°C to 95°C with a 0.5°C ramp rate and continuous fluorescence acquisition.

In order to increase the specificity of PCR detection of *Brachyspira* in tissue and faecal samples, a multiplex hydrolysis probe assay specific for *B. aalborgi* and *B. pilosicoli* was designed. Available 16S sequences for *B. aalborgi* and *B. pilosicoli* were obtained from GenBank and were aligned using Clustal Ω.[16] Primers targeting the 16S gene from both *B. aalborgi* and *B. pilosicoli* were designed (forward: 5´-ACCCATGGAAACATGGACTA-3´, reverse: 5´-TAGGCCGCAGGCTCAT-3´) along with hydrolysis probes specific for the two species (*B. aalborgi* 5´-TxRed-ACCGCATATACTCTTGACGCTAAAGCGT-BHQ2, *B. pilosicoli* 5´-FAM-ACCGCATATACTCTTGCTACATAAGTAGA-BHQ1). Primers and probes were designed using Primer3 and specificity was checked using the BLAST algorithm.[17] Twenty microliter multiplex hydrolysis probe reactions were performed using SsoAdvanced™ Universal Probes Supermix (Bio-Rad) and contained 2 μM of each primer along with 0.25 μM of each probe. The following amplification protocol was utilized: 95°C for 3 minutes followed by 45 cycles (95°C for 10 seconds, 55°C for 10 seconds and 72°C for 20 seconds). Fluorescence acquisition in the FAM and Texas Red channels was performed following each amplification cycle. Again, only results obtained within the first 40 cycles were considered.

All PCR reactions were carried out in triplicate, using a Bio-Rad CFX96 Real-Time System (Bio-Rad). Analysis was performed with Bio-Rad CFX Manager version 3.1 (Bio-Rad).

16S rDNA sequencing of faecal samples

Microbial DNA extracted from faecal material was amplified using 515F and 806R barcoded primers targeting the variable V4 region of the 16S rRNA gene. Sequencing analysis was performed on an Illumina MiSeq (Illumina RTA v1.17.28; MCSv2.5, Illumina Inc., San Diego, CA, USA). Illumina paired-end reads were merged using PEAR, and quality filtered to remove reads that had at least one base with a q-score lower than 20 and that were shorter than 220 nucleotides or longer than 350 nucleotides. Sequences were clustered into operational taxonomic units (OTUs) at a 97% identity threshold using an open-reference OTU picking approach in QIIME 1 (version 1.9.1) with UCLUST against the Greengenes reference database (13_8 release).[18] All sequences that failed to cluster when tested against the Greengenes database were used as input for picking OTUs de novo. Representative sequences for the OTUs were Greengenes reference sequences or cluster seeds and were taxonomically assigned using the Greengenes taxonomy and the Ribosomal Database Project Classifier. Representative OTUs were aligned using PyNAST and used to build a phylogenetic tree with FastTree. Chimeric sequences were identified with ChimeraSlayer and excluded from all downstream analyses. Very low abundant sequence rRNA genes (relative abundance <0.005%) were also excluded. To correct for differences in sequencing depth, the same amount of sequences was randomly sub-sampled for each group of samples (rarefaction; maximum depth: 35000).

Mucus penetrability analysis by confocal microscopy

The method used to assess colonic mucus penetrability has been previously described.[5] One biopsy from each participant in the discovery cohort was analysed; however conclusive results were obtained for 14 IBS patients where Brachyspira colonization could be confirmed (n=6) or rejected (n=8) with high confidence. Briefly, sigmoid biopsies were transported in oxygenated Krebs buffer and directly incubated in a perfusion chamber for 20 min. A suspension of 2 µm green beads (Fluospheres, Thermo Fisher) was then added to the apical surface, and allowed to sediment through the mucus for 40 min. The tissue was stained by Calcein Violet Blue (1 µl/ml in the serosal perfusate; Thermo Fisher). Confocal z-stack images were acquired using an upright Axio Examiner Z.1 LSM 700 confocal imaging system equipped with a Plan-Apochromat ×20/1.0DIC water objective (Zeiss, Oberkochen,

Germany). The impenetrable mucus thickness was quantified as the average distance to the 20 most penetrating beads. The distance from the tissue surface to individual beads was calculated using the Volocity software (v. 6.1.1; Perkin Elmer, Waltham, MA).[19]

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Figures

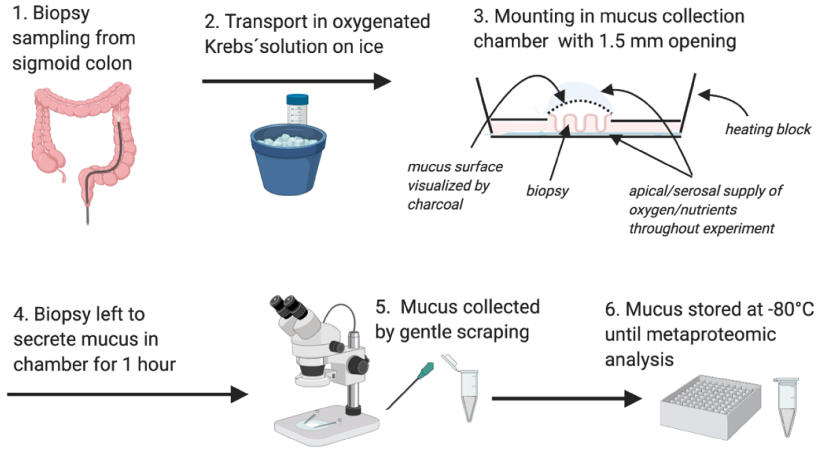

Figure S1 *Graphical depiction of mucus collection from ex vivo sigmoid colon biopsies.*

The figure was created with BioRender.com.

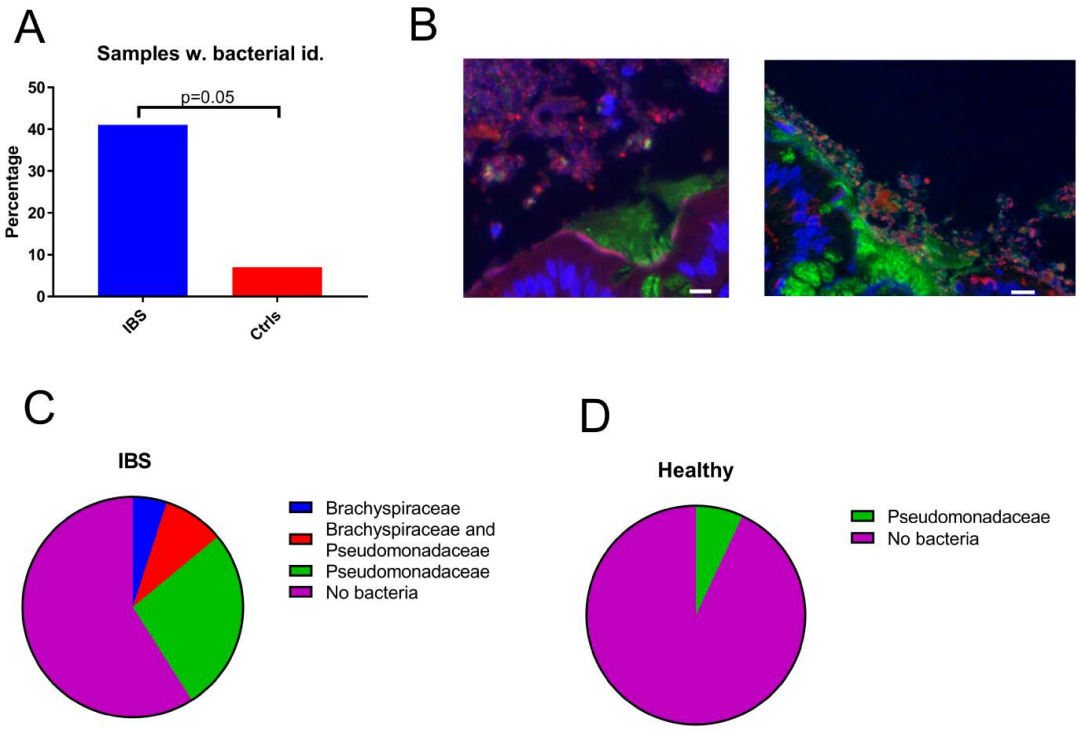

Figure S2 *Proteomic analysis identified potentially pathogenic bacteria in a substantial proportion of IBS mucus samples.*

(A) Bacteria were identified in 41% (9/22) of mucus samples from IBS patients, vs. 7% (1/14) of controls, using mass spectrometry (odds ratio: 9.0; 95% confidence interval: 1.0-81.6). Groups were compared by Fisher's exact test. *(B)* Immunofluorescent staining of *Pseudomonas* (red) in mucus from patients with positive identifications of Pseudomonadaceae peptides in the mass spectrometric analysis. Sections were co-stained with antiserum against mucus component CLCA1 (green) and DNA counterstained by Hoechst (blue). Scale bars: 10 µm. *(C)* Species from the Pseudomonadaceae family were detected in 8/22 (36%) IBS mucus samples; Brachyspiraceae species in 3/22 (14%) samples. *(D)* Pseudomonadaceae was identified in 1/14 (7%) samples from healthy individuals, whereas Brachyspiraceae was not detected in any control sample.

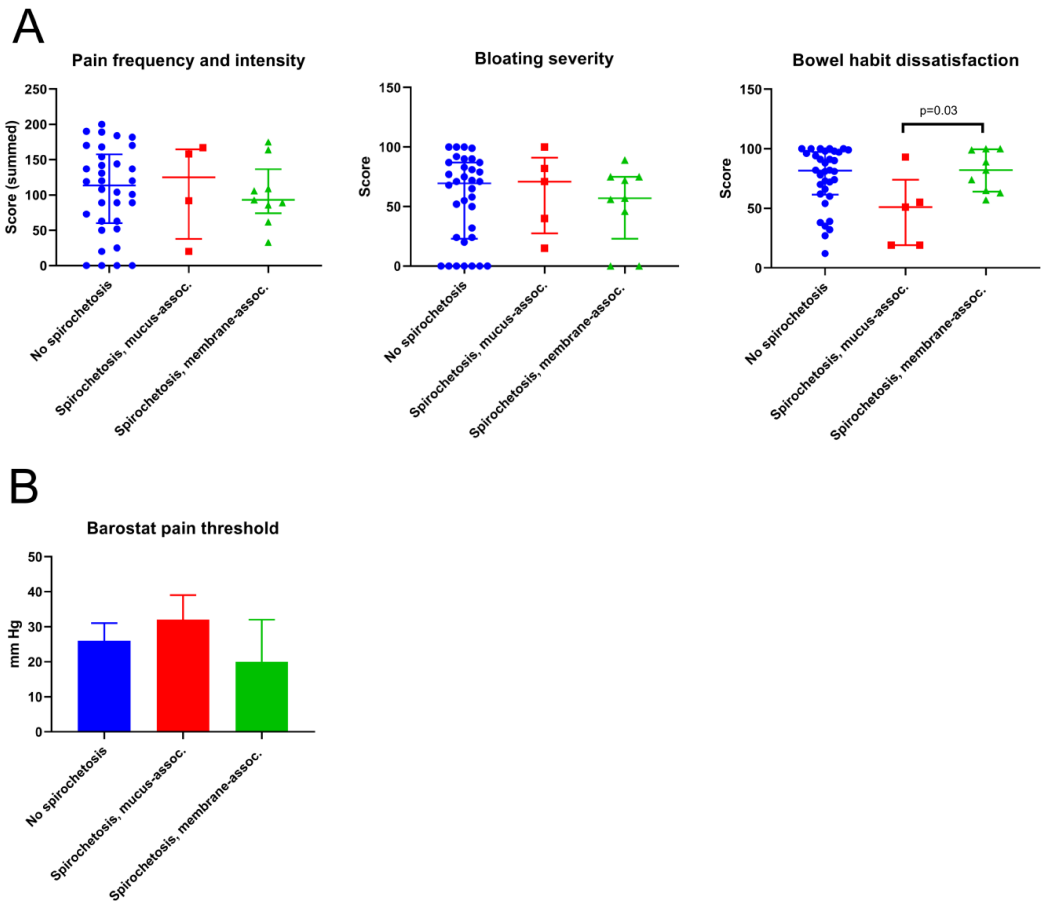

Figure S3 *Individuals with spirochetosis did not differ from other IBS patients with regard to self-reported intestinal symptoms or rectal sensitivity.*

(A) There were no significant differences between individuals with mucus- or membrane-associated spirochetosis and other IBS patients regarding the individual items of IBS-SSS. Dots refer to individual observations; lines represent the median and error bars the inter-quartile range (IQR). P-values were obtained by the Mann-Whitney U-test, with an adjusted significance threshold of 0.017. *(B)* Rectal pain thresholds, as measured by barostat testing, did not differ between IBS patients with and without spirochetosis. Results were available for 28 IBS patients without *Brachyspira* colonization, 4 patients with mucus-associated spirochetosis and 5 patients with membrane-associated spirochetosis. Bars represent the median rectal pain threshold in mmHg; error bars the IQR. Figures *(A)* and *(B)* show data from both cohorts; but are restricted to participants where Brachyspira colonization could be confirmed/rejected by two independent tests, or where real-time PCR was negative with no conclusive results from other methods.

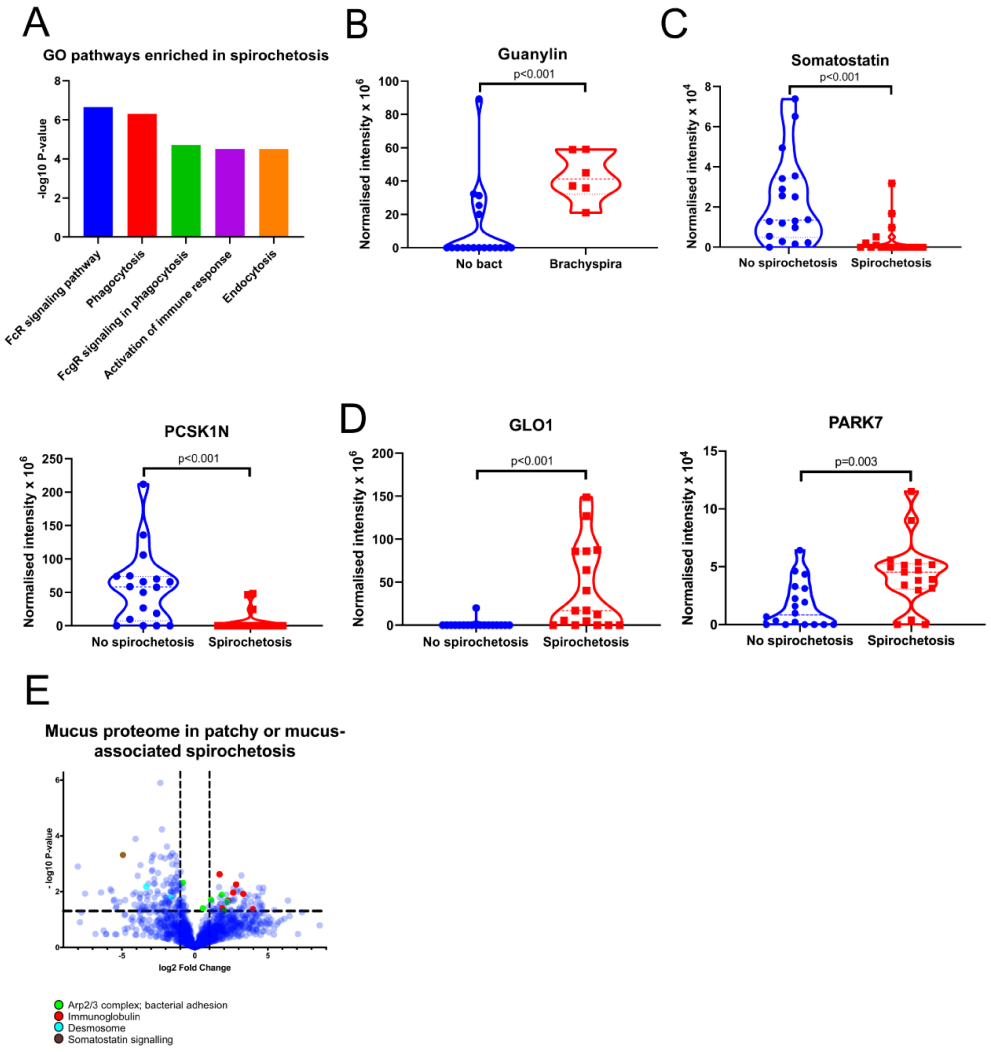

Figure S4 *Mucus proteome alterations in IBS patients with Brachyspira*

(A) Prediction of upregulated host gene ontology biological pathways based on protein enrichment in mucus samples where *Brachyspira* was identified by metaproteomics. P-values were obtained by a modified version of Fisher's exact test (EASE score), using DAVID bioinformatics tool. Minimum two-fold upregulated proteins at a significance level <0.10 were included in the analysis. *(B)* Guanylin (GUCA2A) was significantly more abundant in mucus samples positive for *Brachyspira*. *(C)* Proteins associated with somatostatin (SST) signaling were reduced in mucus samples from IBS patients with spirochetosis. *(D)* Proteins associated with methylglyoxal detoxification were more abundant in spirochetosis. For all violin plots, the midline represents the median and the upper and lower lines the interquartile range (25th and 75th percentile). Groups were compared by the Mann-Whitney U-test. *(E)* Spirochetosis patients without continuous brush border involvement exhibited enrichment of mucus immunoglobulins, and reduced somatostatin levels. However, in contrast to patients with dense brush border *Brachyspira* colonization, no significant apical membrane remodelling, reduction of glycocalyx components or augmented guanylin levels were detected. Transformed p-values were obtained by Welch's unequal variances t-test.

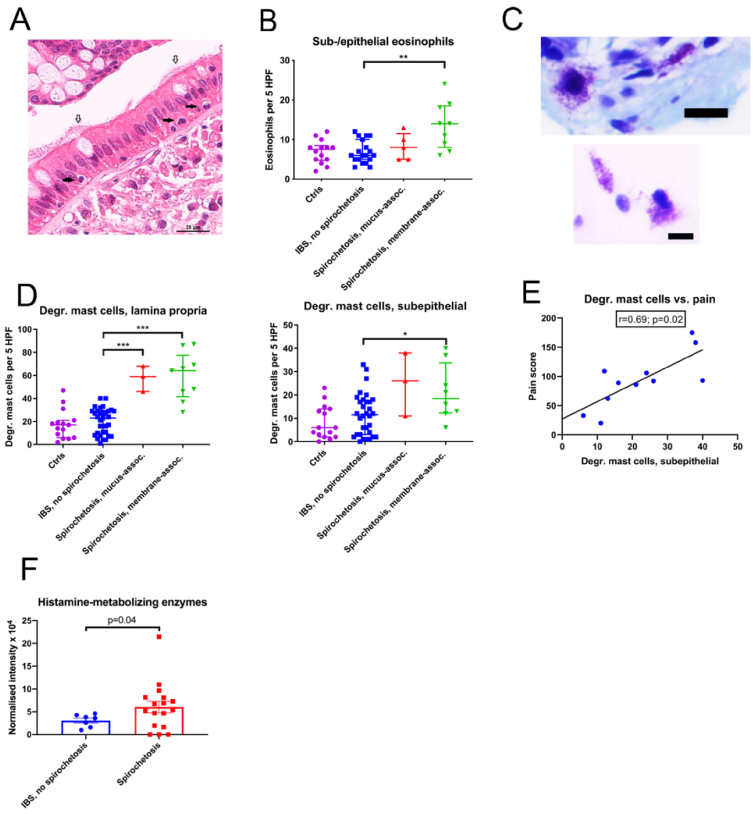

Figure S5 *Mucosal eosinophil and mast cell activation in spirochetosis*

(A) Intraepithelial eosinophils (black arrows) in an H&E-stained section from a patient with membrane-associated spirochetosis (white arrows). Scale bar: 25 µm. *(B)* Increased numbers of (sub-)epithelial eosinophils in membrane-associated spirochetosis as compared to IBS patients without *Brachyspira*. *(C)* Mast cells undergoing degranulation, stained by toluidine blue. Scale bars: 10 µm. *(D)* Activated mast cell counts were increased in the deep and sub-epithelial lamina propria of patients with spirochetosis as compared to IBS patients without *Brachyspira*. Individual observations are overlaid by the median and interquartile range (IQR). P-values were obtained by the Mann-Whitney U-test, *p≤0.05; **p≤0.01; ***p≤0.001. The analysis was restricted to participants with consistent results from ≥2 independent methods for the identification of *Brachyspira* colonization; or participants where real-time PCR was negative with no conclusive results from other methods. Inferential statistical analyses were limited to the comparison of IBS patients without spirochetosis with IBS patients with mucus- and membrane-associated spirochetosis, respectively. *(E)* Correlation between activated mast cells and pain (sum of pain frequency and intensity components of the IBS-SSS) in spirochetosis patients (n=11). The r value refers to Spearman's rank correlation coefficient. *(F)* Enzymes involved in histamine metabolism (HNMT, AOC1 and ALDH2) were more abundant in mucus samples from IBS patients with spirochetosis than in IBS patients without *Brachyspira,* based on nano-liquid chromatography-tandem mass spectrometry. The bar and error bar represent the mean and SEM of the summed normalized intensity levels; groups were compared by Welch's unequal variances t-test.

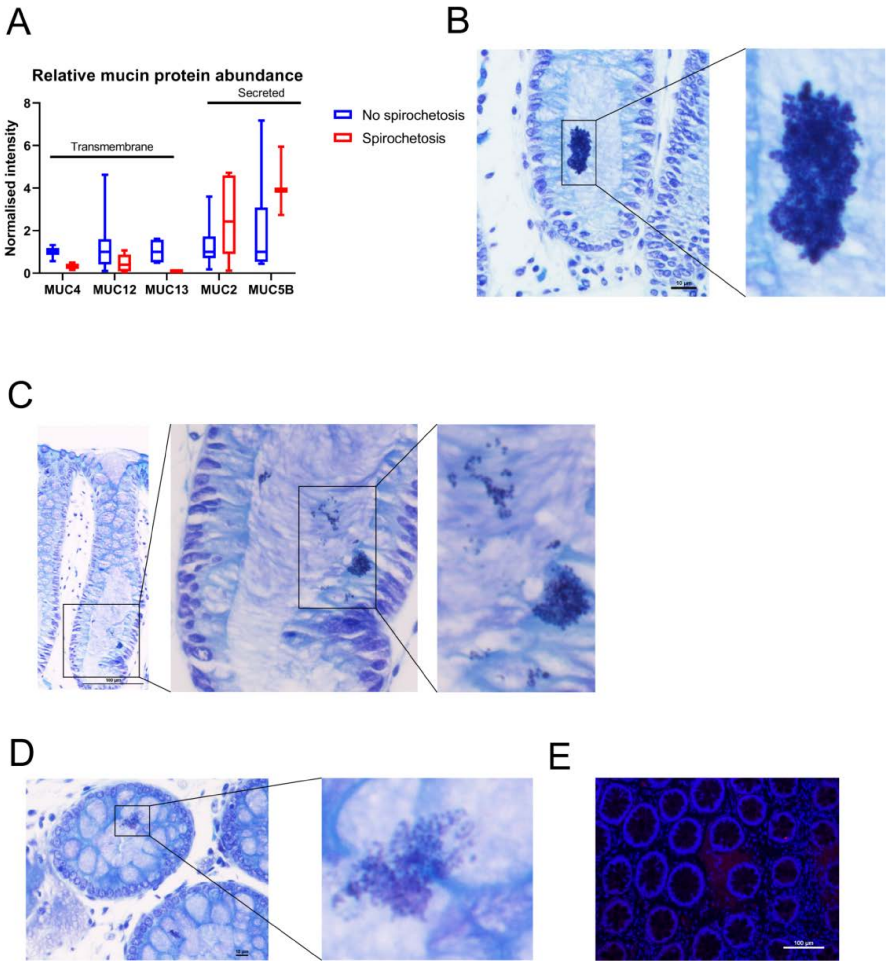

Figure S6 *Individuals with spirochetosis had altered abundance of mucin proteins, and signs of crypt and goblet cell invasion by other bacterial genera.*

(A) Relative abundance of transmembrane and secreted mucins in mucus samples where *Brachyspira* could vs. could not be identified (n=6 and n=18 samples, respectively), based on analysis by nano-liquid chromatography-tandem mass spectrometry. To facilitate visualization, the median intensity of each mucin in individuals without spirochetosis was set to 1.0. *(B)* Toluidine blue staining of sigmoid colon section from an IBS patient with spirochetosis, demonstrating bacterial invasion into the crypt lumen. Scale bar: 10 µm. *(C)* Toluidine blue staining showing bacteria in the base of a crypt, and inside a goblet cell, in an IBS patient with spirochetosis. Scale bar: 100 µm. *(D)* Bacteria inside crypt goblet cells; toluidine blue stained sigmoid colon section from another IBS patient with spirochetosis. Scale bar: 10 µm. *(E) Brachyspira* immunostaining (red) of an untreated spirochetosis patient with bacterial findings in crypts and goblet cells according to Gram stain. There was no sign of invasion of spirochetes into crypts or goblet cells. DNA was counterstained with Hoechst. Scale bar: 100 µm.

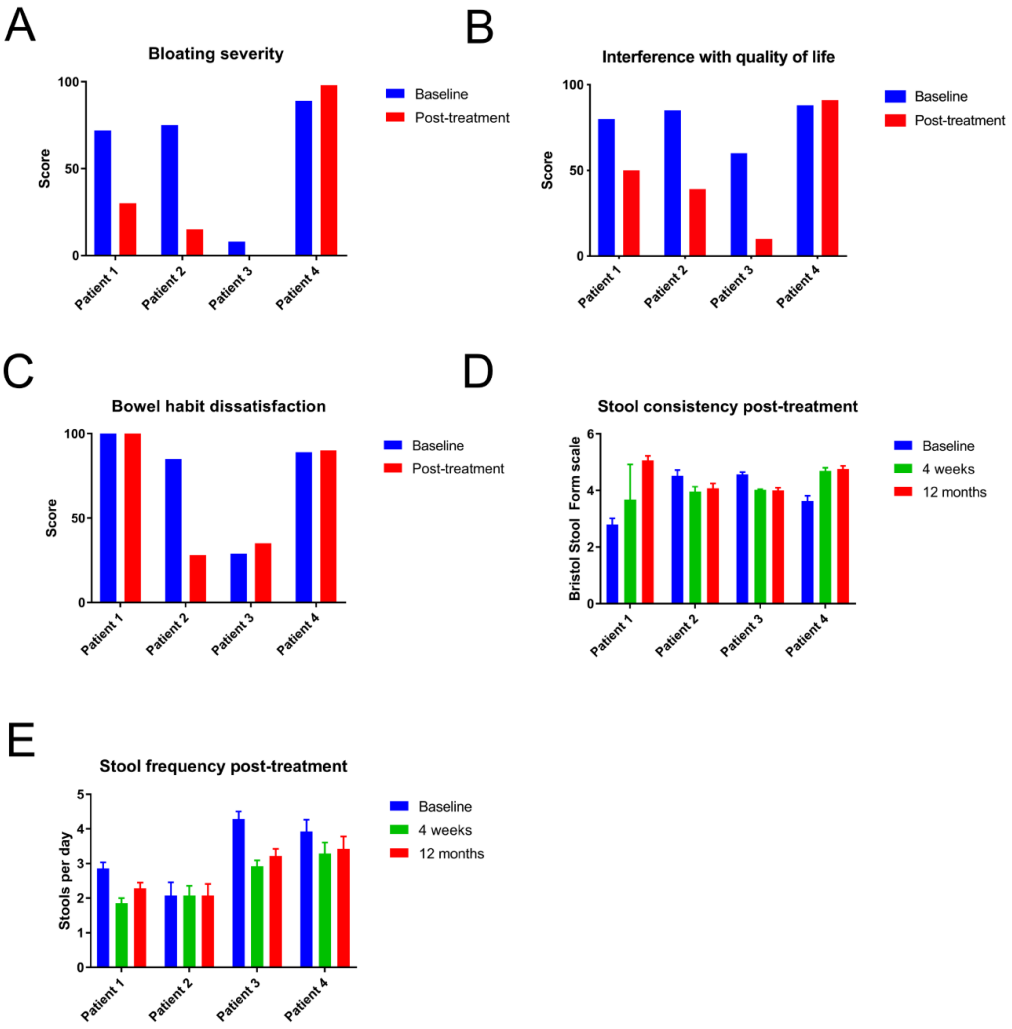

Figure S7 *Alterations of symptom scores and bowel habits following antibiotic treatment*

(A) Bloating tended to improve after antibiotic treatment. Bars show bloating severity for each IBS patient treated for spirochetosis with metronidazole, at baseline and 15 months post-treatment *(B)* The three long-term responders reported a reduction in IBS interference with quality of life, after antibiotic treatment. *(C)* Bowel habit dissatisfaction was unchanged after treatment in all but one of the patients (Patient 2). In *(A-C)* results at baseline and 15 months post-antibiotics are shown. *(D)* Stool consistency had improved (i.e., become more formed) in patients 2 and 3 one year after treatment ($p=0.11$ and $p<0.001$) but had become looser in patients 1 and 4 ($p<0.001$ for both). *(E)* Two patients (Patient 1 and 3) had a reduction in the average number of stools per day at four weeks ($p<0.001$ for both) and twelve months ($p=0.03$ and 0.002) after the completion of metronidazole treatment. For (D) and E) bars represent averages, error bars the standard error of the mean (SEM). Time-points were compared separately for each patient using Welch's t-tests.

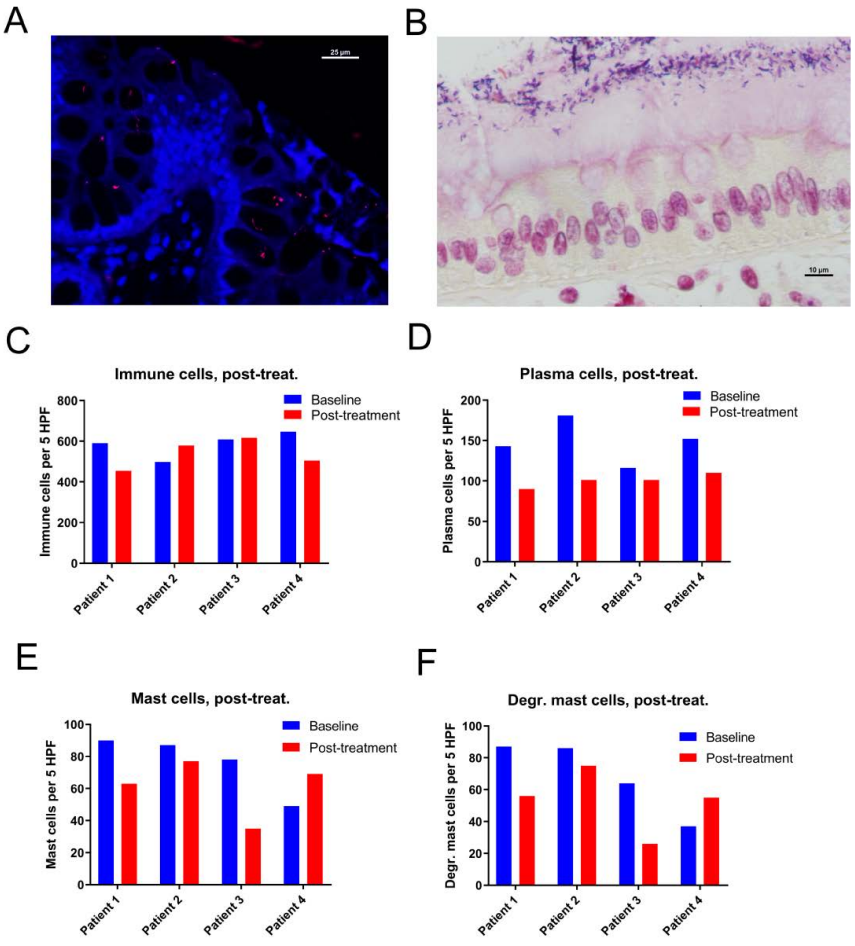

Figure S8 *Observations from follow-up of IBS patients with spirochetosis six weeks after completion of metronidazole treatment.*

(A) Brachyspira immunostaining (red) showing spirochetes inside sigmoid colon crypt goblet cells six weeks after metronidazole treatment; section from Patient 4. DNA was counterstained with Hoechst (blue). Scale bar: 25 µm. *(B)* Gram staining after treatment did not show signs of invasion of Gram-positive bacteria into goblet cells, indicating that this is not a general phenomenon, but rather involves specific bacterial genera. The mucus barrier appeared to be restored after treatment. Scale bar: 10 µm. Pre-treatment images from the same patient, showing invasion of bacteria, some of which Gram-positive, into crypts and goblet cells are provided in Figure S6B and C, and in Figure 6D. *(C)* Overall numbers of lamina propria immune cells were not consistently altered six weeks after the conclusion of metronidazole therapy, as compared to baseline values. *(D)* The number of lamina propria plasma cells was reduced in all patients following treatment. In the three responders (patients 1-3) there was a reduction in the number of total *(E)* and activated *(F)* mast cells in the lamina propria post-treatment. Counting was performed in 5 high-power fields from hematoxylin-eosin (immune cells, plasma cells) or toluidine blue (mast cells) stained sigmoid colon sections (magnification x 600).

Tables

Table S1 *Tabulation of missing data stratified by participant group (IBS vs. healthy) and Brachyspira colonization status*

The upper row for each method shows missing data for entire study population; the lower row missing data among cases where *Brachyspira* colonization was confirmed/rejected based on consistent results from at least two methods. Missing results due to a method being purposely applied only to a subset of the population (meta-proteomics, histology, TEM, 16S rDNA sequencing of faecal samples) are not included in the table. Fisher's exact test was used to compare proportions of missing data between different participant groups (IBS vs. healthy) and between participants with vs. without *Brachyspira*. The mucus penetrability analysis was only performed for participants in the explorative cohort (n=36). Note that the OATT and rectal sensitivity results reported in Figure 4D and Figure S3B are restricted to IBS patients with consistent results from at least two methods for *Brachyspira* identification (n=50), or patients with negative results from real-time PCR, where no other analyses were performed (n=3).

	Conclusive results			**Missing/inconclusive**			**IBS vs. healthy**	***Brachy*[1] vs. no *Brachy*.**
	IBS with spirochetosis	*IBS without spirochetosis*	*Healthy volunteers*	*IBS with spirochetosis*	*IBS without spirochetosis*	*Healthy volunteers*		
real-time PCR	17/19 (89%)	37/43 (86%)	25/31 (81%)	2/19 (11%)	6/43 (14%)	6/31 (19%)	p=0.54	p=0.73
	14/14 (100%)	33/36 (92%)	25/30 (83%)	0/14 (0%)	3/36 (8%)	5/30 (17%)	p=0.14	p=0.34
IF[1]	18/19 (95%)	38/43 (88%)	28/31 (90%)	1/19 (5%)	5/43 (12%)	3/31 (10%)	p=1.0	p=0.68
	14/14 (100%)	34/36 (94%)	27/30 (90%)	0/14 (0%)	2/36 (6%)	3/30 (10%)	p=0.36	p=0.58
OATT[1]	19/19 (100%)	41/43 (95%)	NA	0/19 (0%)	2/43 (5%)	NA	NA	p=1.0
	14/14 (100%)	34/36 (94%)	NA	0/14 (0%)	2/36 (6%)	NA	NA	p=1.0
Rectal sensitivity	13/19 (68%)	32/43 (74%)	NA	6/19 (32%)	11/43 (26%)	NA	NA	p=0.76
	9/14 (64%)	25/36 (69%)	NA	5/14 (36%)	11/36 (31%)	NA	NA	p=0.75
Mucus penetrability	8/9 (89%)	9/13 (69%)	8/14 (57%)	1/9 (11%)	4/13 (31%)	6/14 (43%)	p=0.27	p=0.23
	6/7 (86%)	8/12 (67%)	8/14 (57%)	1/7 (14%)	4/12 (33%)	6/14 (43%)	p=0.46	p=0.38

[1]IF, immunofluorescence; OATT, oro-anal transit time; *Brachy.*, *Brachyspira.*

Table S2 *Identifications of bacterial proteins and peptides in the inner mucus layer of IBS patients and controls*

Median numbers of Brachyspiraceae and Pseudomonadaceae proteins/peptides identified per positive sample are shown. Quality criteria for peptides: unique, non-modified, family-specific, with an ion score >20. At least one such peptide identification was required for a protein to fulfill quality criteria. For the identification of a bacterial family in a sample, the following criteria were set: 1) at least three proteins, each identified by a minimum of one peptide fulfilling the above criteria or 2) at least one protein identified by a minimum of one unique, family-specific, non-modified peptide with an ion score >45.

	Brachyspiraceae	**Pseudomonadaceae**
Median number of proteins [p25-p75][1]	6 [4-9]	9 [6-18]
Median number of proteins fulfilling quality criteria [p25-p75][1]	4 [3-7]	7 [4-13]
Median number of peptides [p25-p75][1]	10 [7-18]	12 [8-39]
Median number of peptides fulfilling quality criteria [p25-p75][1]	5 [4-11]	8 [5-18]

[1]p25, 25th percentile; p75, 75th percentile.

Table S3 *Identifications of Brachyspira proteins and peptides by metaproteomic analysis of mucus samples.*

Peptides with an ion score >20 are highlighted in red color. Protein identifications unique to the *Brachyspira* genus are marked in *italics.*

Protein name	Gene name	Peptides	Number of patients
Flagellar filament core protein flaB2/flaB3	*flaB2/flaB3*	INRAGDDASGLAVSEK	3
		AGDDASGLAVSEK	3
		AGDDASGLAVSEKMR	1
		QRADLGAYQNR	1
Flagellar filament core protein flaB1	*flaB1*	MVINNNISAINAQR	3
50S ribosomal protein L7/L12	*rplL*	IALIKEVR	2
		AVSGLGLKEAK	2
		DAVEKGGETIK	1
		QLEAAGGKVEVK	2
Flagellar filament outer layer protein flaA1	*flaA1*	LDSLGFYR	2
		MIPSVKLDSLGFYR	1
Elongation factor Tu	*tuf*	VAYDSVAK	2
		EHVLLSR	2
50S ribosomal protein L11	*rplK*	QLEEIAQEK	1
		GTSTSTLIK	1
		QIQIAAFVK	1
Probable transcriptional regulatory protein BHWA1_01533	*BHWA1_01533*	GIEPESAEIVR	1
50S ribosomal protein L5	*rplE*	LIFIALPR	1
		MYDFLER	1
30S ribosomal protein S10	*rpsJ*	LALPAGVDVQLK	1
		VSGPIPLPTSIR	1
30S ribosomal protein S20	*rpsT*	YASALDKAAR	1
30S ribosomal protein S18	*rpsR*	NIALLPYETRY	1
50S ribosomal protein L19	*rplS*	LGVKIPK	1
		AKLYYLR	1

Table S4A *Prevalence rates of Brachyspira colonization in IBS patients and healthy volunteers*

	IBS	Controls	IBS-C	IBS-D	IBS-M	IBS-U
First cohort	9/22 (41%)	0/14 (0%)	1/5 (20%)	4/10 (40%)	3/5 (60%)	1/2 (50%)
Second cohort	10/40 (25%)	0/17 (0%)	1/8 (13%)	6/15 (40%)	2/14 (14%)	1/3 (33%)
All	19/62 (31%)	0/31 (0%)	2/13 (15%)	10/25 (40%)	5/19 (26%)	2/5 (40%)

Table S4B *Prevalence rates of Brachyspira colonization based on two independent methods*

Only cases where the presence of *Brachyspira* in colonic mucus/epithelium could be confirmed or rejected by at least two independent diagnostic methods were considered for this analysis.

	IBS	Controls	IBS-C	IBS-D	IBS-M	IBS-U
First cohort	7/19 (37%)	0/14 (0%)	1/5 (20%)	4/9 (44%)	1/3 (33%)	1/2 (50%)
Second cohort	7/31 (23%)	0/16 (0%)	1/8 (13%)	4/10 (40%)	1/10 (10%)	1/3 (33%)
All	14/50 (28%)	0/30 (0%)	2/13 (15%)	8/19 (42%)	2/13 (15%)	2/5 (40%)

Table S4C *Prevalence of membrane-associated spirochetosis in IBS patients and healthy volunteers*

Based on positive staining for *Brachyspira* at the apical epithelial cell surface, using immunofluorescence.

	IBS	Controls	IBS-C	IBS-D	IBS-M	IBS-U
First cohort	6/19 (32%)	0/12 (0%)	0/4 (0%)	4/9 (44%)	1/4 (25%)	1/2 (50%)
Second cohort	5/37 (14%)	0/16 (0%)	0/8 (0%)	3/14 (21%)	2/12 (17%)	0/3 (0%)
All	11/56 (20%)	0/28 (0%)	0/12 (0%)	7/23 (30%)	3/16 (19%)	1/5 (20%)

Table S4D *Prevalence of membrane-associated spirochetosis based on two independent methods*

Based on epithelial surface staining for *Brachyspira*, using immunofluorescence; with the presence/absence of Brachyspira colonization verified by at least one additional diagnostic method. Thus, patients with mucus-associated spirochetosis were not included in the analysis.

	IBS	Controls	IBS-C	IBS-D	IBS-M	IBS-U
First cohort	6/16 (38%)	0/12 (0%)	0/3 (0%)	4/8 (50%)	1/3 (33%)	1/2 (50%)
Second cohort	3/27 (11%)	0/15 (0%)	0/7 (0%)	2/8 (25%)	1/10 (10%)	0/2 (0%)
All	9/43 (21%)	0/27 (0%)	0/10 (0%)	6/16 (38%)	2/13 (15%)	1/4 (25%)

Table S5 *Sensitivity of methods for the diagnosis of intestinal spirochetosis, based on cases where Brachyspira colonization was detected by at least two methods*

For PCR, borderline cases were defined as samples with amplification after cycle 40; for immunofluorescence, borderline cases were considered as positive by one out of two independent analysts.

Participant	IBS subtype	Cohort	# methods with pos. results	# methods with neg. results	PCR MC[1]	PCR HP[2]	IF[3]	Proteomics	TEM[4]	*PCR MC + PCR HP*	*PCR MC + IF*	*PCR HP + IF*
1	*D*	*2nd*	*5*	*0*	***pos.***	***pos.***	***membrane***	***pos.***	***pos.***	*pos.*	*pos.*	*pos.*
2	*U*	*1st*	*5*	*0*	***pos.***	***pos.***	***membrane***	***pos.***	***pos.***	*pos.*	*pos.*	*pos.*
3	*D*	*1st*	*5*	*0*	***pos.***	***pos.***	***membrane***	***pos.***	***pos.***	*pos.*	*pos.*	*pos.*
4	*M*	*1st*	*5*	*0*	***pos.***	***pos.***	***membrane***	***pos.***	***pos.***	*pos.*	*pos.*	*pos.*
5	*D*	*2nd*	*3*	*0*	***pos.***	***pos.***	***membrane***			*pos.*	*pos.*	*pos.*
6	*M*	*2nd*	*3*	*0*	***pos.***	***pos.***	***membrane***			*pos.*	*pos.*	*pos.*
7	*D*	*1st*	*3*	*2*	***pos.***	***pos.***	***membrane***	*neg.*	*neg.*	*pos.*	*pos.*	*pos.*
8	*D*	*1st*	*3*	*2*	***pos.***	***pos.***	***membrane***	*neg.*	*neg.*	*pos.*	*pos.*	*pos.*
9	*C*	*2nd*	*2*	*1*	*borderline*	***pos.***	***mucus***	*neg.*		*pos.*	*pos.*	*pos.*
10	*U*	*2nd*	*2*	*1*	*borderline*	***pos.***	***mucus***	*neg.*		*pos.*	*pos.*	*pos.*
11	*D*	*1st*	*2*	*2*	***pos.***	*neg.*	***membrane***	*neg.*	*scant Brachyspira*	*pos.*	*pos.*	*pos.*
12	*D*	*2nd*	*2*	*0*	***pos.***	***pos.***	*borderline mucus*			*pos.*	*pos.*	*pos.*
13	*D*	*2nd*	*2*	*1*	***pos.***	***pos.***	*neg.*			*pos.*	*pos.*	*pos.*
14	*C*	*1st*	*2*	*2*	***pos.***	***pos.***	*neg.*	*neg.*	*scant Brachyspira*	*pos.*	*pos.*	*pos.*
Sensitivity					*86%*	*93%*	*79%*	*40%*	*50%*	*100%*	*100%*	*100%*
Sensitivity,borderline results as pos.					*100%*	*93%*	*86%*	*40%*	*75%*	*100%*	*100%*	*100%*

[1]MC, melting curve [2] HP, hydrolysis probe [3] IF, immunofluorescence [4] TEM, transmission electron microscopy

Table S6A *Differential count of lamina propria immune cells.*

Immune cells were counted in five high-power fields (magnification x 600) in hematoxylin and eosin stained sections from the sigmoid colon in healthy controls (n=12), IBS patients without *Brachyspira* colonization (n=9) and patients with membrane-associated spirochetosis (n=8).The median number of cells and the interquartile range (25th percentile and 75th percentile) are provided. Note that the full differential counting of lamina propria immune cells was done in a smaller area than the confirmatory counting by a different observer shown in Figure 5. This variation is explained by different modes of compensation for areas covered by crypts.

	Healthy individuals	IBS without spirochetosis	Membrane-associated spirochetosis
# Immune cells	176 (142-197)[1]	185 (153-229)[1]	277 (201-330)[1]
# Eosinophils	8 (6-13)	7 (4-8)	9 (5-20)
# Lymphocytes	48 (39-68)	57 (48-81)	78 (58-134)
# Macrophages	55 (41-62)	52 (41-57)	57 (46-96)
# Plasma cells	56 (47-66)[1]	50 (41-61)[1]	85 (65-111)[1]

[1] Significant difference between participant categories according to the Kruskal Wallis test.

Table S6B *Differential count of (sub-)epithelial immune cells.*

Immune cells in or adjacent to the surface epithelium were counted in five high-power fields in hematoxylin-eosin stained sections from the sigmoid colon, at a magnification x 600. Full differential counting was performed in 12 healthy controls, 7 IBS patients without *Brachyspira* colonization and 9 patients with membrane-associated spirochetosis. The median number of cells and the interquartile range (25th percentile and 75th percentile) are provided.

	Healthy individuals	IBS without spirochetosis	Membrane-associated spirochetosis
# Immune cells	90 (76-104)	93 (84-96)	69 (58-108)
# Eosinophils	3 (1-5)[1]	4 (4-6)[1]	8 (8-15)[1]
# Lymphocytes	27 (23-44)	41 (28-42)	26 (19-36)
# Macrophages	56 (27-63)	30 (20-43)	30 (20-38)
# Plasma cells	9 (5-16)	8 (7-16)	7 (3-14)

[1] Significant difference between participant categories according to the Kruskal Wallis test.

Table S7 *Demographic and clinical baseline characteristics of patients treated with metronidazole for intestinal spirochetosis*

Stool score represents summed average stool frequency (stools/24 hours) and consistency (according to the Bristol Stool Form Scale), as recorded by the patient in a structured two-week bowel habit diary.

	Gender	Age	IBS subtype	IBS-SSS	IBS severity	Stool score	Species
Patient 1	M	27	IBS-M	416	Severe	5	*B. aalborgi*
Patient 2	M	22	IBS-U	353	Severe	7	*B. aalborgi*
Patient 3	F	23	IBS-D	206	Moderate	12	*B. aalborgi*
Patient 4	F	26	IBS-D	355	Severe	10	*B. hominis*

Table S8A *Quantification of Brachyspira in endoscopic biopsies at baseline and six weeks after completion of metronidazole treatment.*

Quantities refer to the number of *Brachyspira* 16S copies per 20 ng DNA.

	Baseline	Post-treatment
Patient 1	1.35×10^5	6.80×10^1
Patient 2	4.96×10^4	9.70×10^1
Patient 3	1.70×10^4	4.49×10^1
Patient 4	3.07×10^5	5.40×10^1

Table S8B *Quantification of Brachyspira in stool at baseline and at six weeks, six months and one year after completion of metronidazole treatment.*

Quantities refer to the number of *Brachyspira* 16S copies per 10 ng DNA.

	Baseline	6 weeks	6 months	12 months
Patient 1	3.36×10^9	2.13×10^1	1.33×10^4	Not detected
Patient 2	3.09×10^6	5.62×10^1	Not detected	Not detected
Patient 3	2.68×10^{10}	7.06×10^{-1}	Not detected	Not detected
Patient 4	1.76×10^8	3.79×10^2	Not detected	Not detected

Data file S1 *Compilation of Brachyspira colonization status for each study participant, stratified by the different diagnostic methods used in the study.*

For the real-time PCR analysis, positive results obtained during cycles 41 and 42 are marked as borderline. Provided as separate excel file.

Data file S2 *Results of 16S rDNA sequencing analysis of faecal samples from four patients with confirmed membrane-associated spirochetosis, before and after antibiotic treatment.*

Results are provided as percentages, on the family level. Brachyspiraceae were not detected in stool either before or after antibiotic (metronidazole) treatment. Provided as separate excel file.

Data file S3 *Mucus proteome in healthy individuals and IBS patients with and without Brachyspira colonization.*

Exclusion criteria for the analysis were: 1) patients with proteomic identifications of bacteria other than *Brachyspira,* 2) borderline or contradictory results with regard to *Brachyspira* identification by other methods, 3) individuals where spirochetosis could not be confirmed/ rejected by two independent methods and 4) technical failures, defined as <200 protein identifications. In total, 18 samples from an equal number of individuals without spirochetosis (11 healthy individuals and 7 IBS patients) and 17 samples from 10 IBS patients with spirochetosis were analyzed. *Brachyspira* could be detected by the proteomic analysis in 6 samples from 4 patients; these are marked in the excel file. In another 6 patients spirochetosis was diagnosed by other methods. Only human proteins are included. Intensity values for each protein were normalized against the summed intensity of all proteins for each sample. Proteins belonging to the functional groups that were found to be altered in spirochetosis, as shown in Figure 5, are highlighted in the excel file.