Supplement to

Association between *Brachyspira* and irritable bowel syndrome with diarrhoea

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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

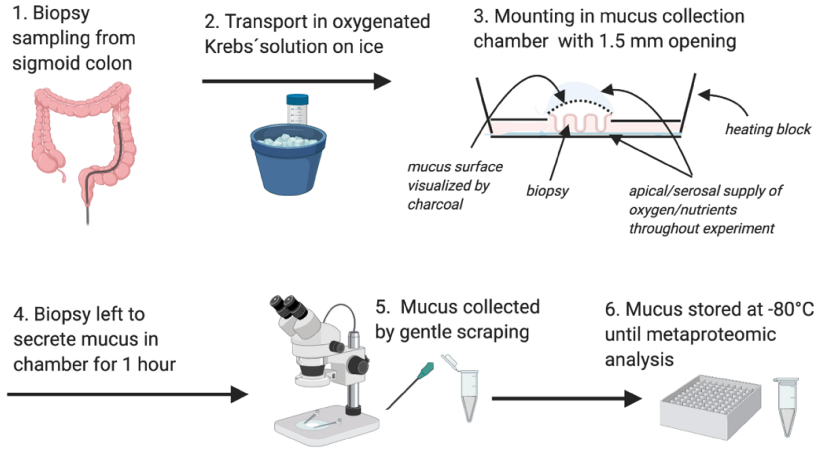

Figure S1 *Graphical depiction of mucus collection from ex vivo sigmoid colon biopsies.*

The figure was created with BioRender.com.

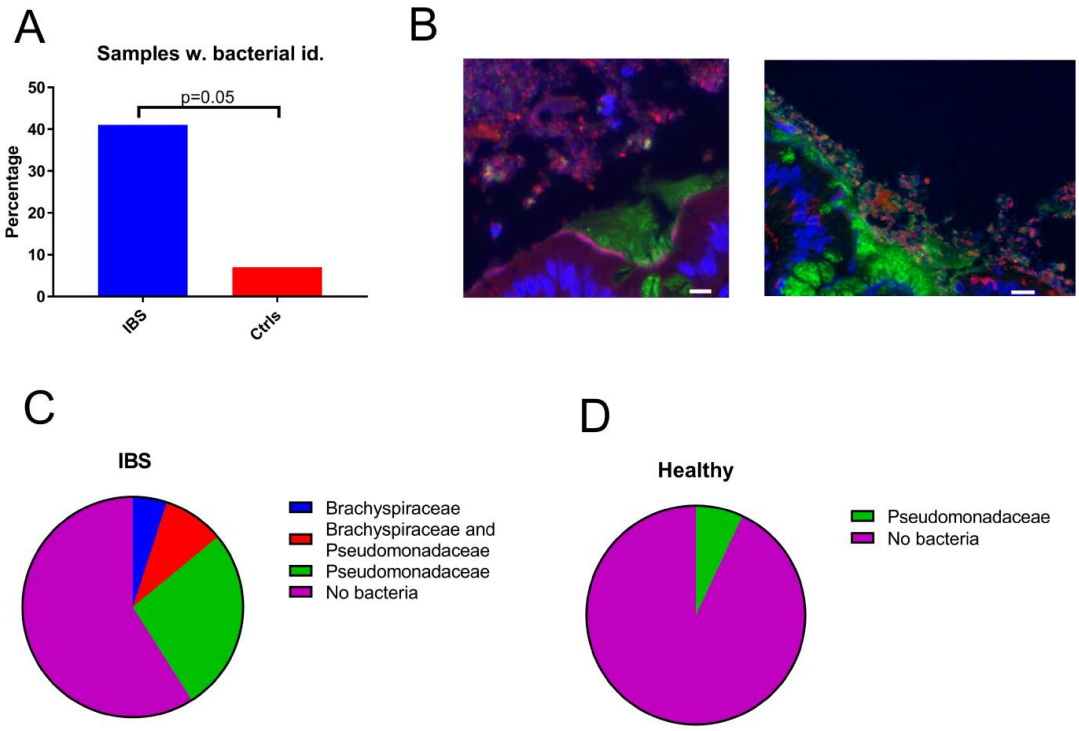

Figure S2 *Proteomic analysis identified potentially pathogenic bacteria in a substantial proportion of IBS mucus samples.*

(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.

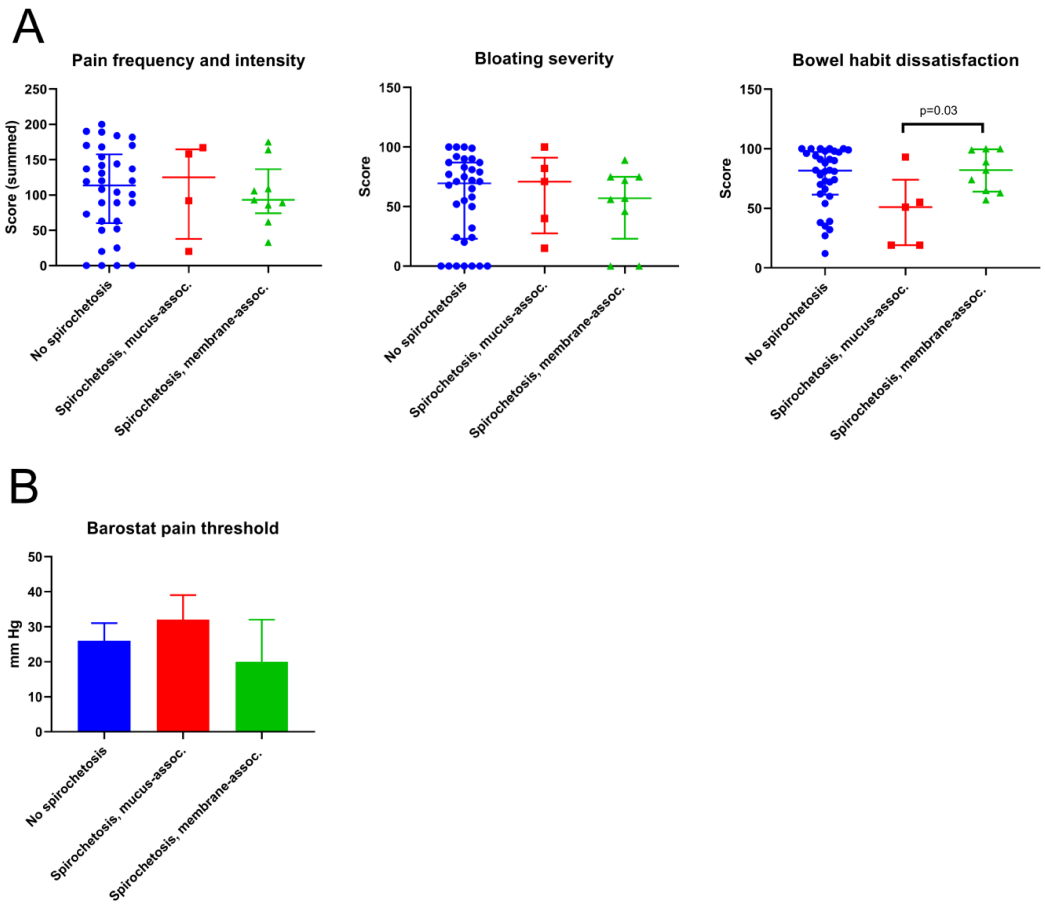

Figure S3 *Individuals with spirochetosis did not differ from other IBS patients with regard to self-reported intestinal symptoms or rectal sensitivity.*

(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.

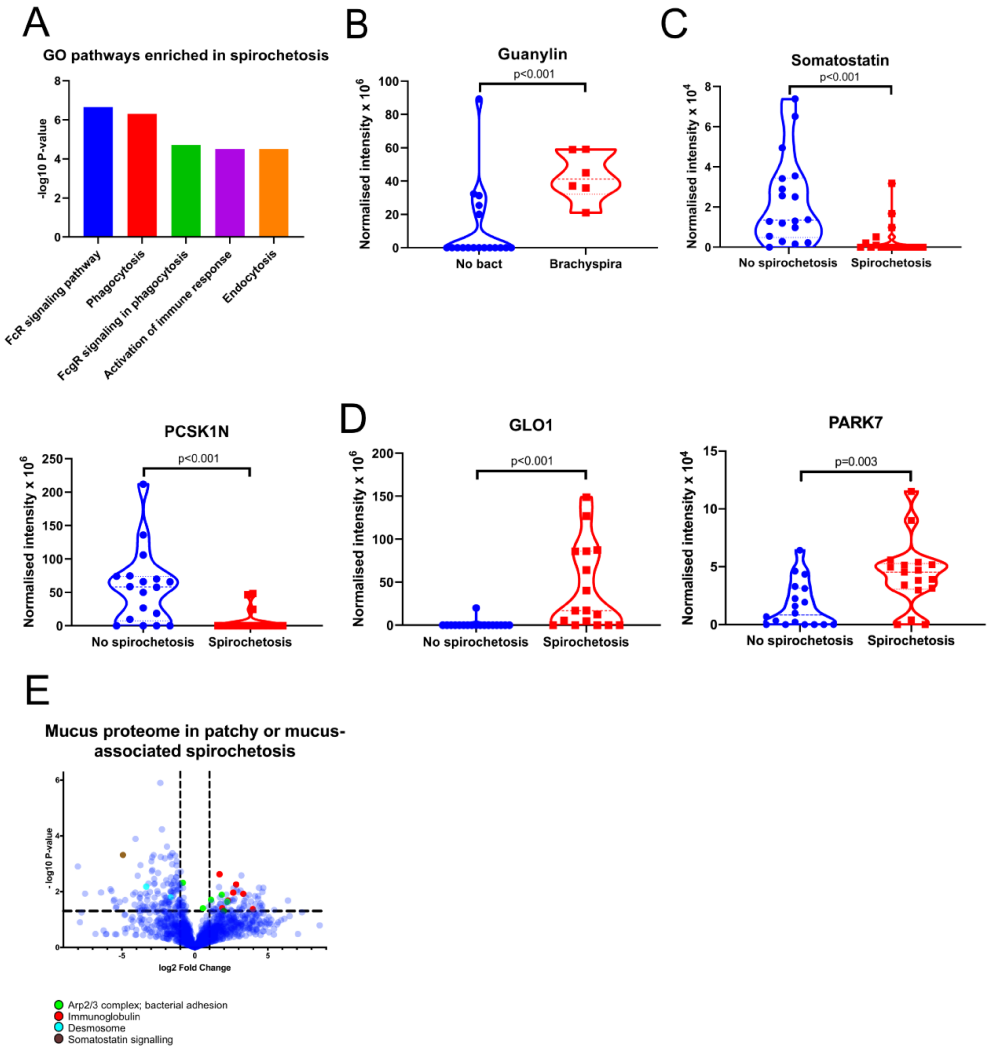

Figure S4 *Mucus proteome alterations in IBS patients with Brachyspira*

(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.

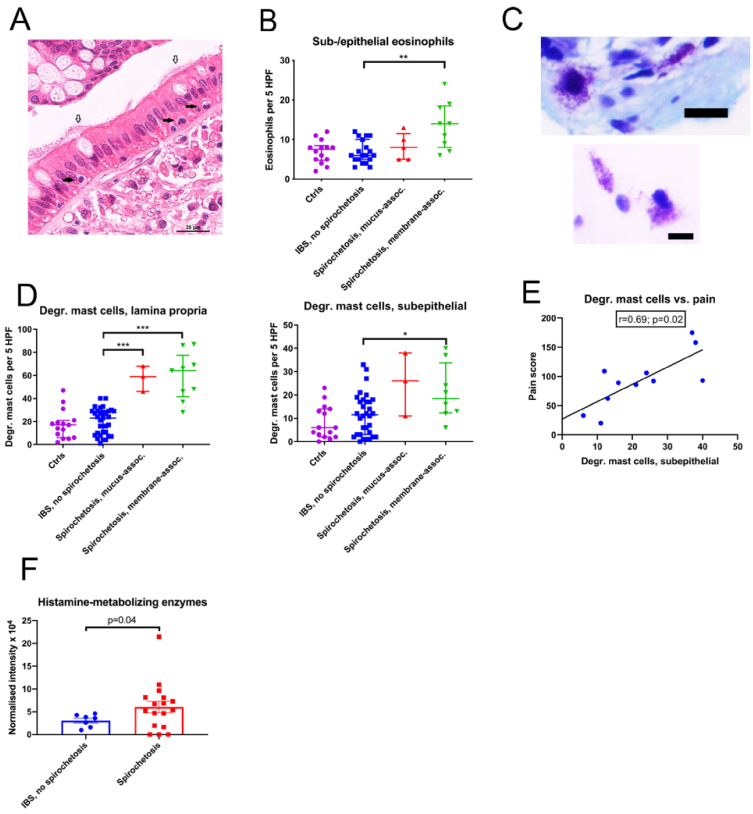

Figure S5 *Mucosal eosinophil and mast cell activation in spirochetosis*

(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.

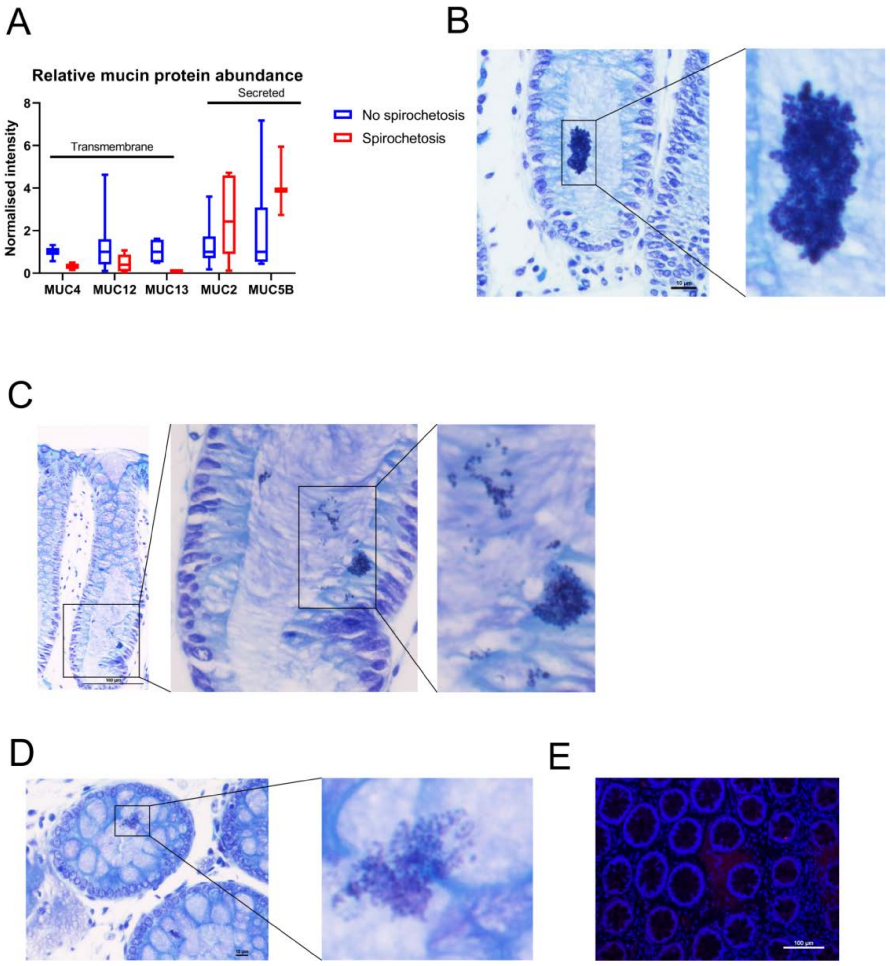

Figure S6 *Individuals with spirochetosis had altered abundance of mucin proteins, and signs of crypt and goblet cell invasion by other bacterial genera.*

(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.

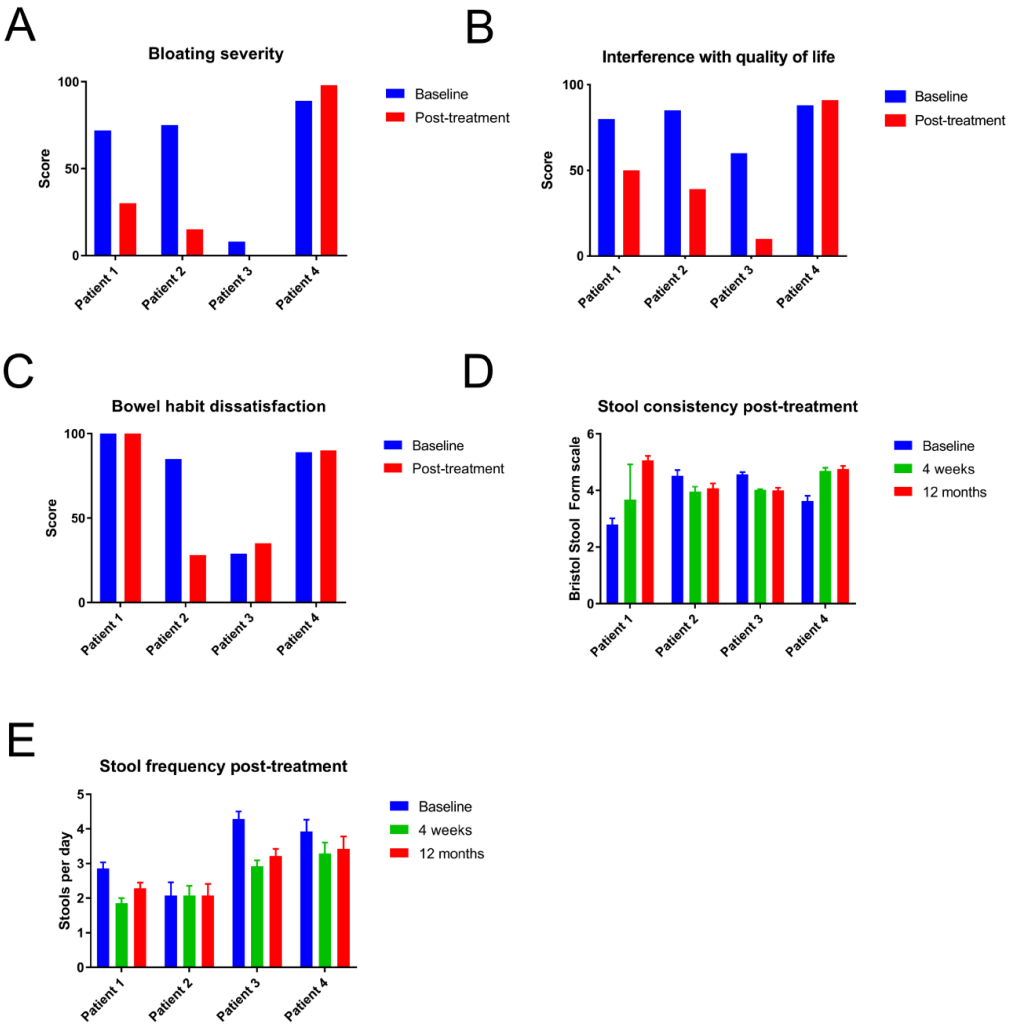

Figure S7 *Alterations of symptom scores and bowel habits following antibiotic treatment*

(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.

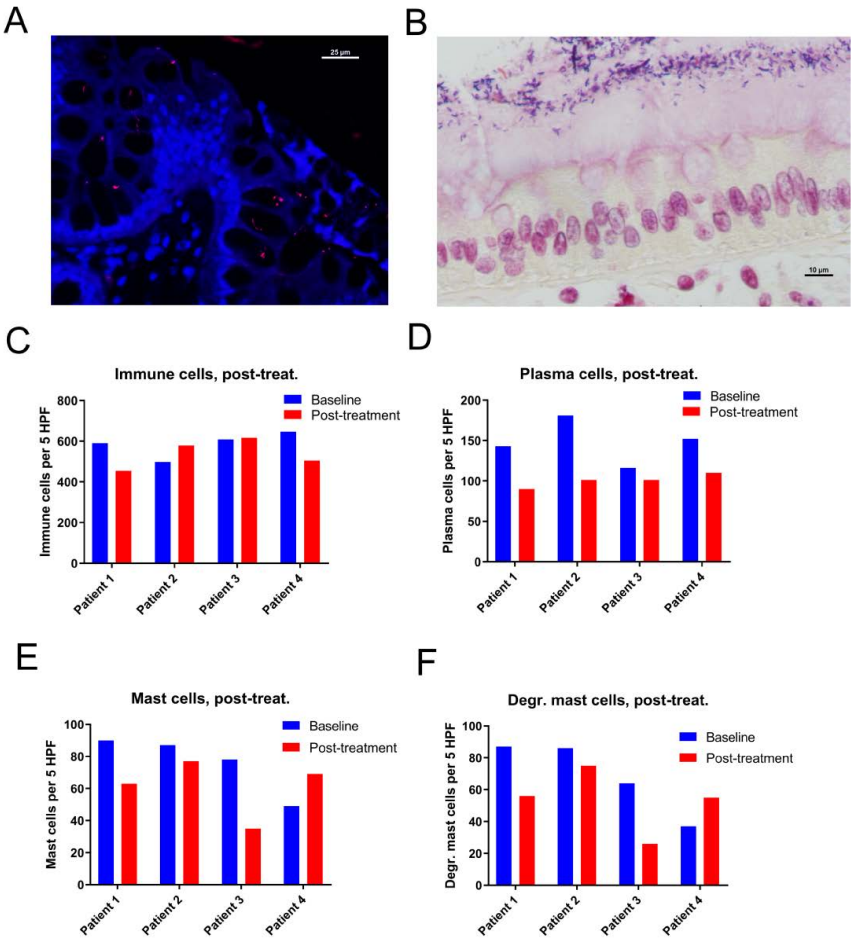

Figure S8 *Observations from follow-up of IBS patients with spirochetosis six weeks after completion of metronidazole treatment.*

(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.