Supplementary methods

The study population

This was a single centre cohort study of patients whose stool sample tested positive for Campylobacter spp. The main clinical study included all 155 eligible subjects who provided clinical details of their illness, psychological parameters, and bowel function. These factors were tested for their association with subsequent post-infective bowel dysfunction (PI-BD). We nested within the main study a detailed mechanistic study of the microbiota in those who provided adequate stool samples. Originally, we had planned to compare just 20 in each group but advances in technology and reduction in costs allowed us to expand our controls and we were able to compare 18 cases with 45 controls, chosen because they had provided the most complete set of stool samples.
The study was approved by the Nottingham Research Ethics Committee on 29th August S (13/EM/0310) and started 1st January 2014. The study was prospectively registered on clinicaltrials.gov (NCT02040922). Although the endpoints were not changed, we initially aimed to recruit 450 to provide adequate power to assess at the impact of antibiotic use during *Campylobacter* infection on prevalence of PI-IBS and to study in depth 20 cases. Despite the fact that we only recruited 129 by the time the funding ceased, and the study closed on Oct 2016, we were able to obtain 18 out of our planned 20 cases for detailed study, whose results are presented here along with the clinical features of 111 controls.

Participants

Inclusion / exclusion criteria
Eligible participants were aged >18, with a clinical syndrome suggestive of intestinal infection, *Campylobacter sp.* in the associated stool sample and living within the Nottingham postal code area. Patients were excluded if they: were pregnant (self-declared), had a pre-existing gastrointestinal disorder or previous resection of the gastrointestinal tract (excluding appendix and gallbladder), had a chronic condition likely to require antibiotics in the next 3 months, regularly used opioids, had used antibiotics or high dose laxatives in the 4 weeks preceding the infection, were unable to complete symptom questionnaires, or were, in the opinion of the investigator, unable to comply with the protocol.

Patient identification
Patients were identified from a stool culture positive for Campylobacter in the Public Health England Laboratory in Nottingham. Data protection laws prevented direct contact so every 2 weeks a batch of letters were sent to patients who had previously been informed of their positive stool culture, inviting them to contact the research team. We hoped to get a sample as early as possible in the illness and to compare this with a sample taken around 6 weeks and 12 weeks from the initial infection, a time span during which our previous study indicated that most people who were going to recover would do so (10).

Patient visits
Visit 1 was arranged as soon as possible after a positive culture, but in the event, there was a considerable delay. Visit 2 was aimed at being 6 weeks after diagnosis though in some cases, owing to delay in making the first appointment this was merged with visit 1 while

Visit 3 was the end of the study 12 weeks after the initial infection. Owing to the delay before patients received the invitation letter the first visit and faecal sample was mean 46 (range 17-93) days and the final sample as mean 97(range 57-160) days from the start of symptoms.

At Visit 1 eligibility was confirmed and written informed consent obtained. Demographics were documented and symptom questionnaires (see below) were completed.

Stool samples

Patients were asked to collect stool samples for each visit, either bringing them within 2 hours of passage to the laboratory for immediate freezing at -80°C or storing at home, double bagged in their domestic freezer at -180C, before bringing in an insulated bag to the laboratory for storage at -80°C prior to analysis.

If Visit 1 occurred within 5 weeks of diagnosis, patients were asked to return for Visit 2 at 6 weeks (typically one week later) to provide a further stool sample. At Visit 3, 12 weeks after diagnosis, patients were asked to complete a questionnaire on their bowel symptoms from the past week and provide a further stool sample

Recruitment

Eligible patients were recruited through the Nottingham University Hospitals Microbiology Laboratory by the Health Protection Team with an invitation letter, information sheet and questionnaires on gastrointestinal symptoms prior to and during the infection. Despite sending out 1286 invitations, recruitment was much slower than expected and after the allotted time of 2 years the study was closed with only 155 patients recruited, of which 99 completed the study (see manuscript Figure 1).

Symptom questionnaires

At Visit 1 patients completed questionnaires documenting demographics prior to infection including prior bowel habit using the Rome III questionnaire, anxiety and depression using the Hospital Anxiety and Depression scale (HADS) [1] and somatisation using the Patient Health Questionnaire -12 Somatic Symptom Scale (PHQ-12 SS) [2]. They were also asked about features of the acute illness with markers of severity including rectal bleeding, weight loss and duration of time off normal activities. We also documented any antibiotic treatment. At each visit they reported their bowel habit during the previous week. At Visit 3, cases were defined as those responding “No” to the question “have your bowels returned to normal” and controls were those who responded “Yes”.

Lack of significant difference between cases with PI-BD and those meeting Rome III criteria

Just under half of the cases (45%) met the Rome criteria III for IBS. These subjects (PI-IBS) were very similar to the remaining 12 not meeting the criteria (PI-BD) with no significant difference in age (mean 57, SD=±14 versus 53 ±15), anxiety (8 ± 6 versus 6 ± 3), depression (median (IQR), 4(2.8-8.5) versus 2(1-7.5)) nor PHQ-12 SS (5(2) versus 4(2)). Neither did the markers of severity differ significantly between PI-BD or PI-IBS, including fever (9/10 versus 9/12), blood in stool (1/10 versus 3/12), vomiting (3/10 versus 2/12) and antibiotic consumption (3/10 versus 6/12).

Microbiota sequencing

The sequencing reads were processed using R package mare and functions ProcessReads and TaxonomicTable. Here the quality filtering, chimera detection, and taxonomic annotation functions rely on USEARCH (version 8.1.1756_i86osx32), [3]. We used only the forward reads for the taxonomical annotation. After removing the primer sequence the reads were trimmed to 180 bases, which were then used for taxonomical assignment. [4] The reads were summed to ASV and reads with less than 68 replicates in all samples were excluded as potentially incorrect and removed from further analysis. The taxonomic annotation was performed using USEARCH by mapping the reads to the SILVA 16S rRNA reference database version 115, curated to contain only sequences matching to the forward and reverse primers that were used for sequencing. No other normalization method was applied after pre-processing. The sequencing controls were assessed and since all the sequencing controls contained less than 1000 reads, the potential contaminants were not removed from the original reads due to very low impact.

Statistical analysis:

In the statistical analysis we used the GroupTest and CovariateTest functions of mare package.[5] The CovariateTest function tests for associations between the desired taxa and a continuous variable. Whereas the group test uses each taxon to test the statistical difference between the studied groupings. Both functions consider the read depth of each sample and uses that as an offset for the model. All taxonomical levels were used as relative data in the analysis from genus level taxa up until phylum level data. The pre-processed data was not transformed in any other way.

There are several statistical models that can be applied in the GroupTest and CovariateTest functions. When having multiple samples from one subject, the subject ID information is used as and will use that variable as the random factor in the model. the function first attempts to fit a zero-inflated negative binomial model using the glmmADMB package. Violation against model assumptions e.g., homogeneity of residuals may lead to meaningless p-values and potentially false conclusions. Therefore, this is considered with in the GroupTest function. If the initial model fails to produce reliable results, glm.nb function, linear models (lm), generalised least squares models (gls), or linear mixed models (lme) are fitted, depending on the situation. If all tests fail, no p-values are given. All obtained p-values are corrected for multiple testing and q-values are produced.

We tested the association between alpha diversity and read counts. The sequenced samples had a median read count of 51893 (lower hinge of 46422 and upper hinge of 57773) indicating that there is not very much difference in the read depth of the sequenced samples. We tested the spearman correlation between read counts and both microbial richness (cor = 0.19, p = 0.03) and diversity (cor = 0.084, p = 0.33). Although the p-value of the correlation between read count and richness is bellow 0.05, the actual association is weak and is due to only few samples (data not shown). We therefore did not see it necessary to adjust the richness and diversity measures with read depth.

References:

1 Zigmond AS, Snaith RP. The hospital anxiety and depression scale. Acta Psychiatr Scand 1983;**67**:361-70.

2 Spiller RC, Humes DJ, Campbell E, Hastings M, Neal KR, Dukes GE*, et al.* The Patient Health Questionnaire 12 Somatic Symptom scale as a predictor of symptom severity and consulting behaviour in patients with irritable bowel syndrome and symptomatic diverticular disease. Aliment Pharmacol Ther 2010;**32**:811-20.

3 Edgar RC. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 2010;**26**:2460-1.

4 Korpela K, Blakstad EW, Moltu SJ, Strommen K, Nakstad B, Ronnestad AE*, et al.* Intestinal microbiota development and gestational age in preterm neonates. Sci Rep 2018;**8**:2453.

5 Korpela K. mare: Microbiota Analysis in R Easily. R package version 1.0. https://github.com/katrikorpela/mare DOI:10.5281/zendo.50310. 2016.