

1 **Supplementary methods**

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3 The study population

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5 This was a single centre cohort study of patients whose stool sample tested positive for
6 *Campylobacter* spp. The main clinical study included all 155 eligible subjects who provided
7 clinical details of their illness, psychological parameters, and bowel function. These factors
8 were tested for their association with subsequent post-infective bowel dysfunction (PI-BD).
9 We nested within the main study a detailed mechanistic study of the microbiota in those
10 who provided adequate stool samples. Originally, we had planned to compare just 20 in
11 each group but advances in technology and reduction in costs allowed us to expand our
12 controls and we were able to compare 18 cases with 45 controls, chosen because they had
13 provided the most complete set of stool samples.

14 The study was approved by the Nottingham Research Ethics Committee on 29th August S
15 (13/EM/0310) and started 1st January 2014. The study was prospectively registered on
16 clinicaltrials.gov (NCT02040922). Although the endpoints were not changed, we initially
17 aimed to recruit 450 to provide adequate power to assess at the impact of antibiotic use
18 during *Campylobacter* infection on prevalence of PI-IBS and to study in depth 20 cases.
19 Despite the fact that we only recruited 129 by the time the funding ceased, and the study
20 closed on Oct 2016, we were able to obtain 18 out of our planned 20 cases for detailed
21 study, whose results are presented here along with the clinical features of 111 controls.

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23 Participants

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25 Inclusion / exclusion criteria

26 Eligible participants were aged >18, with a clinical syndrome suggestive of intestinal
27 infection, *Campylobacter* sp. in the associated stool sample and living within the
28 Nottingham postal code area. Patients were excluded if they: were pregnant (self-declared),
29 had a pre-existing gastrointestinal disorder or previous resection of the gastrointestinal tract
30 (excluding appendix and gallbladder), had a chronic condition likely to require antibiotics in
31 the next 3 months, regularly used opioids, had used antibiotics or high dose laxatives in the
32 4 weeks preceding the infection, were unable to complete symptom questionnaires, or
33 were, in the opinion of the investigator, unable to comply with the protocol.

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35 Patient identification

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

43

44 Patient visits

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

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

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

54 Stool samples

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

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

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65 Recruitment

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

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74 Symptom questionnaires

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

85

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

87 Just under half of the cases (45%) met the Rome criteria III for IBS. These subjects (PI-
88 IBS) were very similar to the remaining 12 not meeting the criteria (PI-BD) with no
89 significant difference in age (mean 57, SD=±14 versus 53 ±15), anxiety (8 ± 6 versus 6 ± 3),
90 depression (median (IQR), 4(2.8-8.5) versus 2(1-7.5)) nor PHQ-12 SS (5(2) versus 4(2)).

91 Neither did the markers of severity differ significantly between PI-BD or PI-IBS, including
92 fever (9/10 versus 9/12), blood in stool (1/10 versus 3/12), vomiting (3/10 versus 2/12) and
93 antibiotic consumption (3/10 versus 6/12).

94

95 Microbiota sequencing

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

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110 Statistical analysis:

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

119

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

130

131 We tested the association between alpha diversity and read counts. The sequenced
132 samples had a median read count of 51893 (lower hinge of 46422 and upper hinge of
133 57773) indicating that there is not very much difference in the read depth of the sequenced
134 samples. We tested the spearman correlation between read counts and both microbial
135 richness ($cor = 0.19$, $p = 0.03$) and diversity ($cor = 0.084$, $p = 0.33$). Although the p-value of
136 the correlation between read count and richness is bellow 0.05, the actual association is
137 weak and is due to only few samples (data not shown). We therefore did not see it
138 necessary to adjust the richness and diversity measures with read depth.

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