Composition of human faecal microbiota in resistance to Campylobacter infection

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Abstract

In mice, specific species composition of gut microbiota enhances susceptibility to Campylobacter jejuni but little is known about the specific composition of the human gut microbiota in providing protection from infections caused by enteropathogens. Healthy adult individuals, who travelled in groups from Sweden to destinations with an estimated high risk for acquisition of Campylobacter infection, were enrolled. Faecal samples, collected before travelling and after returning home, were cultured for bacterial enteropathogens, and analysed for Campylobacter by PCR and for the species composition of the microbiota by 16S amplicon massive parallel sequencing. The microbiota compositions were compared between persons who became infected during their travel and those who did not. A total of 63 participants completed the study; 14 became infected with Campylobacter, two with Salmonella and 47 remained negative for the enteropathogens tested. After exclusion of samples taken after antimicrobial treatment, 49 individuals were included in the final analyses. Intra-individual stability of the microbiota was demonstrated for samples taken before travelling. The original diversity of the faecal microbiota was significantly lower among individuals who later became infected compared with those who remained uninfected. The relative abundances of bacteria belonging to the family Lachnospiraceae, and more specifically its two genera Dorea and Coprococcus, were significantly higher among those who remained uninfected. The travel-related infection did not significantly modify the faecal microbiota composition. Species composition of human gut microbiota is important for colonization resistance to Campylobacter infection. Especially individuals with a lower diversity are more susceptible to Campylobacter infection.

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Introduction

It is becoming more and more certain that the composition of the gut microbiota not only plays an important role in many normal functions of the human body, such as the development of the intestinal immune system, but is also connected to a growing number of diseases [1–3]. Little is known, however, about the role of the species composition of the human intestinal microbiota in providing protection from enteropathogens. Although the disrupted indigenous gut microbiota...
in general, and that after antimicrobial use in particular, are known to be the central factors leading to the development of *Clostridium difficile* infection [4], only very recently some specific bacterial compositions of the gut community have been connected to this infection [5,6]. For *Salmonella*, murine studies indicate that the composition of the gut microbiota is important for colonization resistance [7]. Microbiota characteristics, such as a low complexity in general or high relative abundance of *Escherichia coli* [8], may lead to a higher susceptibility to *Salmonella* enterica. However, human studies are few.

In Europe, *Campylobacter* has been the most commonly reported bacterial enteropathogen in humans and the most commonly reported zoonotic agent since 2005; in 2013 the number of reported confirmed human *Campylobacter* cases in the EU was >200 000, *Campylobacter jejuni* and *Campylobacter coli* being the dominant pathogens [9]. The role of the human microbiota composition for the susceptibility to *Campylobacter* infection remains elusive. However, immunocompetent mice, naturally resistant to *Campylobacter*, could be successfully colonized with *C. jejuni*, resulting in inflammation, if their intestinal microbiota was first humanized [10]. Furthermore, in mice, elevated numbers of intestinal *E. coli* were shown to reduce colonization resistance to *C. jejuni* [11]. For the first time in humans, we recently showed that the faecal microbiota of highly exposed poultry abattoir workers who later became infected with *Campylobacter* had a significantly higher relative abundance of certain bacterial genera (e.g. *Bacteroides* and *Escherichia*) than the microbiota of those who remained *Campylobacter* negative [12]. In that particular study, we could also demonstrate that *Campylobacter* infection had long-term effects on the species composition of the microbiota.

In the present study, we assessed the species composition of the faecal microbiota in generally healthy adults before and after their international travel to destinations with high prevalence of enteropathogens. All the individuals were culture negative for enteropathogens at the beginning of the study but, during their travel, about a quarter developed bacterial enteritis. We detected clear differences in the initial composition of the faecal microbiota among those who later became infected compared with those who did not.

**Materials and methods**

**Participants and sample collection**

In this prospective, observational study, eight different groups of travellers with a total of 67 people were recruited (19 men and 48 women). Exclusion criteria included an age <18 or >65 years, a diagnosis of inflammatory bowel disease and failure to understand written Swedish. The participants were generally healthy people living in Sweden (aged 18–64 years; median age 24 years) who travelled from 1 week to 3 months (median 2 weeks) to a foreign destination with a reasonable risk of acquiring a bacterial enteropathogen (Egypt, Tunisia, Curacao, Turkey, Ecuador, Bangladesh or Tanzania) (Table 1). Only people travelling in groups (encompassing at least three individuals) were recruited, so that comparisons could possibly be made within the groups.

Faecal samples from each participant were collected twice before the trip (with a median of 10 days apart; the second sample collected 0–5 days, median 2 days, before travelling) and once after return to Sweden (range 0–25 days after arrival; median 3 days). On each sampling occasion, one sample was immediately sent for routine bacterial culture to the Clinical Microbiology Laboratory at Uppsala University Hospital, whereas the other sample taken in parallel was kept at −70°C for subsequent DNA extraction. On each sampling occasion the participants also filled in a short questionnaire and mailed it to the researchers. The questionnaires included information on previous medical conditions and intake of antibiotics. Those who became culture positive for enteropathogens during the study received an additional questionnaire including questions on intestinal symptoms and possible antimicrobial therapy.

All, except four participants from four different groups, delivered all three samples. Fourteen of these 63 individuals became infected with *C. jejuni* (11 culture positive and 3 only PCR positive) during their trip, two became positive for *S. enterica* and 47 remained uninfected. Given the alteration of the microbiota caused by antibiotics, faecal samples collected within 3 months of any antibiotic usage were excluded from further microbiota analyses. In the final analyses, 49 individuals were included.

Written informed consent was obtained from each participant. The regional board of the ethics committee at Uppsala University approved the study.

**Bacterial isolation**

All the faecal samples before and after travelling were cultured by routine diagnostic methods for *Campylobacter*, *Salmonella*, *Shigella* and *Yersinia*. All samples collected before travelling were culture negative for these bacterial agents.

**DNA extraction**

DNA was extracted from approximately 200 mg of faecal samples. Bead-beating was used to disrupt bacterial cell walls and was performed in tubes containing TE-buffer (pH 7.5) and 0.1-mm zirconia/silica beads (BioSpec products, Bartlesville, OK, USA) for 3–4 min at 20 Hz on a TissueLyser Adapter Set 2X24 (Qiagen, Düsseldorf, Germany). The DNA was then isolated and purified using an easyMAG NucliSENS extractor.
Participants and their samples included in the study

<table>
<thead>
<tr>
<th>Groups originally recruited according to travel destination (no of participants)</th>
<th>Finding in faecal culture/PCR after travelling for those who delivered all samples* (n = 67)</th>
<th>Included in analysis 1b (n = 57)</th>
<th>Included in analysis 2c (n = 51)</th>
<th>Included in analysis 3d (n = 49)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tourists</strong></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Egypt (n = 4)</td>
<td>Salmonella (n = 1)</td>
<td>Uninfected (n = 3)</td>
<td>Uninfected (n = 3)</td>
<td>Uninfected (n = 3)</td>
</tr>
<tr>
<td>Tunisia (n = 3)</td>
<td>Uninfected (n = 3)</td>
<td>Uninfected (n = 2)</td>
<td>Uninfected (n = 2)</td>
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<tr>
<td>Curacao (n = 6)</td>
<td>Uninfected (n = 5)</td>
<td>Uninfected (n = 5)</td>
<td>Uninfected (n = 5)</td>
<td>Uninfected (n = 5)</td>
</tr>
<tr>
<td>Turkey (n = 3)</td>
<td>Uninfected (n = 3)</td>
<td>Uninfected (n = 3)</td>
<td>Uninfected (n = 3)</td>
<td>Uninfected (n = 3)</td>
</tr>
<tr>
<td>All tourists (n = 16)</td>
<td>Salmonella (n = 1)</td>
<td>Uninfected (n = 13)</td>
<td>Uninfected (n = 13)</td>
<td>Uninfected (n = 13)</td>
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<tr>
<td><strong>Volunteers</strong></td>
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<tr>
<td>Ecuador (n = 7)</td>
<td>Campylobacter (n = 3)</td>
<td>Campylobacter (n = 3)</td>
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<td>Campylobacter (n = 3)</td>
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<tr>
<td>Bangladesh (n = 22)</td>
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<td>Uninfected (n = 4)</td>
<td>Uninfected (n = 4)</td>
<td>Uninfected (n = 4)</td>
</tr>
<tr>
<td>Ecuador (n = 17)</td>
<td>Salmonella (n = 1)</td>
<td>Salmonella (n = 1)</td>
<td>Salmonella (n = 1)</td>
<td>Salmonella (n = 1)</td>
</tr>
<tr>
<td>Tanzania (n = 5)</td>
<td>Uninfected (n = 9)</td>
<td>Uninfected (n = 7)</td>
<td>Uninfected (n = 5)</td>
<td>Uninfected (n = 5)</td>
</tr>
<tr>
<td>All volunteers (n = 51)</td>
<td>Campylobacter (n = 14)</td>
<td>Campylobacter (n = 14)</td>
<td>Campylobacter (n = 14)</td>
<td>Campylobacter (n = 12)</td>
</tr>
</tbody>
</table>

*All samples taken before travelling were culture-negative for Campylobacter, Salmonella, Shigella and Yersinia but after travelling some individuals showed positive faecal culture or positive Campylobacter PCR result.

**Subtyping of Campylobacter isolates and PCR analysis of faecal samples**

Campylobacter isolates originally cultured on modified charcoal cefoperazone deoxycholate agar plates (Oxoid, Basingstoke, UK) and identified by colony appearance, positive oxidase testing and microscopy, were further characterized by PCR analysis. Duplex-PCR using primers specific for C. jejuni (targeting mapA gene) and for C. coli (targeting cceE gene) were used [13,14]. Cycling conditions for PCR were 10 min at 95°C, 40 cycles of 30 s at 95°C, 90 s at 59°C and 1 min at 72°C, with a final extension step of 10 min at 72°C. PCR products were separated with gel electrophoresis on 1.5% agarose gel and bands were visualized using GelRed (Biotium, Hayward, CA, USA). Campylobacter jejuni (81-176) and C. coli (LMG6440) were used as positive controls and omission of template as a negative control. All isolates were shown to be C. jejuni.

DNA from all the samples collected after travelling was analysed by PCR for the presence of C. jejuni and C. coli as described above. Culture-negative samples of three participants after travelling were shown to be positive for C. jejuni by PCR; for these particular individuals both samples taken before travelling were also analysed by PCR and shown to be negative.

**16S rRNA analysis**

Sequencing libraries were prepared by amplifying the V3–V4 region of the 16S rRNA gene using the 341f-805r primers, described by Hugenholtz et al. [15]. After the initial amplification, a second PCR was performed to attach Illumina adapters as well as barcodes that allow for multiplexing. Samples were sequenced using the Illumina MiSeq platform producing 300 bp reads. Primer sequences were trimmed away and the paired-end reads produced by the sequencing instrument were merged using SeqPrep version 1.1 (https://github.com/jstjohn/SeqPrep) with default parameters and thereafter the merged sequences were processed with the QIIME 1.8.0 pipeline (Quantitative Insights Into Microbial Ecology) [16]. A closed reference operational taxonomic unit (OTU) strategy was used to assign sequences to OTUs. Using the UCLUST [17] algorithm built into the QIIME pipeline, sequences were clustered at 97% identity against the Greengenes reference database [18], which produced 5917 OTUS in the data set with an average of 20 222 reads per sample (range 11 422–24 869). Details on 16S rRNA gene primers, amplification conditions and sample barcodes were as published earlier [19].

**Statistical analysis**

Microbial diversity was calculated using Shannon’s index of diversity and phylogenetic diversity whole tree. QIIME was used to generate weighted UniFrac distances to monitor changes in β
diversity within an individual over time. The statistical software PAST was used for graphical illustrations of data and for significance testing between groups; Wilcoxon rank-sum test when two groups were compared and Kruskal–Wallis test when three groups were compared [20].

**Results**

**Stability of the composition of faecal microbiota before travelling**

The intra-individual stability of the microbiota composition in the two consecutive faecal samples collected before travelling at a median interval of 10 days was analysed. As samples taken within 3 months of any antimicrobial intake were excluded, paired samples of 57 individuals were included (Table 1). The samples were assessed by weighted UniFrac distance analysis (Fig. 1), and the analyses were also performed separately for the 13 tourists (attending a 1– to 2-week-long trip as tourists) and 44 volunteers (who studied/worked for 2–12 weeks abroad), respectively. There were no significant differences between the results of the tourists and the volunteers, and as the UniFrac distance for intra-individual samples did not significantly differ, the second samples taken immediately before travelling were regarded as representative and used in the further analyses.

**Enteropathogen findings after travelling**

During this study, 16 of the original 63 participants (25%) became infected with enteropathogens (Table 1) as shown by culture for *Salmonella* (*n* = 2), by culture for *Campylobacter* (*n* = 11) or by PCR only for *Campylobacter* (*n* = 3). A total of 47 participants remained culture and PCR negative. Of the *Campylobacter*-positive individuals (aged 18–29 years; median age 23 years), 11 were women and three were men. *Campylobacter* culture positive samples were collected 1–13 days (median 3 days) after returning home, and the three culture-negative but PCR-positive samples were collected 4, 5 and 12 days after returning to Sweden. Three of the 14 *Campylobacter*-positive individuals reported no intestinal symptoms during travelling; all three had faecal samples that were positive for *Campylobacter* both by culture and by PCR.

**Composition of the original faecal microbiota and susceptibility to enteropathogens**

To see whether the composition of faecal microbiota before travelling was different among those who later became infected compared with those who remained uninfected, the samples taken before travelling were compared between the infected and the uninfected groups. For this analysis, samples of 15 infected participants (14 positive for *C. jejuni* and one for *S. enterica*) and 36 uninfected individuals were analysed (Table 1).

The original taxon diversity of the faecal microbiota before travelling was significantly lower among the group of participants who later became infected as compared to those who remained uninfected (Wilcoxon’s test; Shannon diversity p 0.028 and phylogenetic diversity whole tree p 0.002). As analysed separately for the three groups of participants (infected, uninfected tourists and uninfected volunteers), the group of tourists who remained uninfected had the highest diversity; it was even significantly higher than that of the volunteers who remained uninfected (Fig. 2). These results remained significant even after exclusion of the sample of the only *Salmonella*-positive individual. The original composition of faecal microbiota among the three individuals, who became *Campylobacter* positive but remained asymptomatic during and after travelling, was not different from the microbiota of those who became *Campylobacter* positive and developed symptoms.

We also wanted to study specifically whether relative abundances of certain bacterial taxa differed between the participants who later became infected and those who remained uninfected. As infected individuals (after exclusion of

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**FIG. 1.** Intra-individual stability of microbiota composition in two consecutive faecal samples collected before travelling. The intra-individual stability was assessed by weighted UniFrac distance analysis for all participants and separately for volunteers and tourists. No significant differences between the groups of volunteers and tourists were detected. The horizontal line in the box plot represents the median value and the box is drawn from 25% to 75% quartiles. Whiskers show minimum and maximum values and circles represent outliers.
those treated with antimicrobials) could only be found among the volunteers (Table 1), these comparisons were performed between infected and uninfected volunteers only. Among the 23 participants who remained uninfected, higher relative abundances of bacteria belonging to the family Lachnospiraceae were detected (p 0.036, Fig. 3). Higher relative abundances were also demonstrated separately for two genera, Dorea (p 0.004) and Coprococcus (p 0.002), both belonging to Lachnospiraceae. These results remained significant even after exclusion of the sample of the only Salmonella-positive individual.

Influence of infection on the composition of faecal microbiota
To see if the faecal microbiota was changed in response to the imported infection, the samples collected after travelling were compared with those taken before the trip and the analyses were performed separately for the groups of infected and of uninfected individuals. As eight people had taken antibiotics during or after the trip and so were excluded from this analysis, 12 Campylobacter positive, 1 Salmonella positive and 36 uninfected participants were included (Table 1). These analyses were also performed separately for the volunteers only (12 Campylobacter positive, 1 Salmonella positive and 23 uninfected included). No significant changes in the composition of faecal microbiota were detected because of the imported infection (data not shown). Sequences classified as Campylobacter spp. were only found in very low relative abundances (0.004–0.4%) and in the samples of eight Campylobacter-positive (culture/PCR) individuals.

Discussion
Campylobacter jejuni is a leading cause of bacterial enteritis in the world but little is known about the pathogenic mechanisms involved [21]. We wanted to study the role of the human gut microbiota in the development of Campylobacter infection and prospectively followed Swedish travellers with analyses of their faecal microbiota compositions before and after travelling abroad. Significant differences were detected in the faecal microbiota compositions between individuals who became infected with Campylobacter and those who remained uninfected.

The general concept of colonization resistance, i.e. the microbiota’s capability to prevent and limit pathogen colonization and growth, has been well established for a long time, as the use of antibiotics was shown to immediately increase susceptibility to Salmonella infection in mice [22,23]. However, with the recent advances in sequencing technology, including high-throughput DNA sequencing and bioinformatics, it has now become possible to define the impact of specific bacterial genera [24]. Studies using mouse models for colonization resistance against C. jejuni indicate gut microbiota composition to have an important role and imply that similar aspects would be important for human C. jejuni infections as well [10,11] but results from human studies performed with next-generation sequencing techniques are very few [12,25].

The results of the present study are in line with those of Stecher et al. [8], who found that mice with microbiotas of high diversity were more resistant to S. enterica colonization than...
mice with microbiotas of low complexity or mice treated with antimicrobial agents. However, in our earlier study, which was also performed in humans, we could not find any significant difference in the diversity of the faecal microbiota between poultry abattoir workers who later became infected by *Campylobacter* and those who remained uninfected [12]. This discrepancy could be, at least to some extent, explained by the differences in the study populations in general and the presence of symptoms in particular. In our earlier study, all but one of the *Campylobacter*-positive abattoir workers were asymptomatic whereas in our present study 11 of the 14 *Campylobacter*-positive travellers reported intestinal symptoms of varying degree.

Higher relative abundance of bacteria belonging to the family *Lachnospiraceae* was demonstrated among the participants who remained uninfected. This is in line with our earlier study showing the same finding for unclassified *Lachnospiraceae* among poultry abattoir workers who remained *Campylobacter* negative [12]. In the present study, also higher relative abundances of two genera, *Dorea* and *Coprococcus*, belonging to the *Lachnospiraceae* family were shown for those who remained uninfected. However, significantly higher relative abundances of *Bacteroides* and *Escherichia*, earlier shown to be associated with a higher susceptibility for enteropathogens as demonstrated by us in the abattoir study [12] and by others in animal models [8,10,11], could not be verified in the present study. These findings, and actually the lack of them in the present study, highlight the complexity of faecal microbial communities.

Human gut microbiota in healthy adults is generally considered to be stable over time [26,27] but antimicrobial therapy rapidly causes significant changes in the taxonomic composition of gut microbiota [28,29]. In our study, all samples taken within 3 months of any antimicrobial use were excluded. Intra-individual stability was studied for the consecutive samples taken before travelling and no significant differences were detected.

Our aim was also to see how the original faecal microbiota was modified by the enteric infection obtained. In our study on poultry abattoir workers [12], *Campylobacter* infection was shown to cause long-term alterations to faecal microbiota compositions of the infected individuals whereas among participants who remained *Campylobacter* negative, microbiota compositions were stable even in samples taken more than 5 months apart. In a recent study [25], it was shown that the relative abundances of *Enterobacteriaceae* increased dramatically in the faecal microbiota of an individual with *Salmonella* infection and

**FIG. 3.** Relative abundances of taxa, which differed significantly between volunteers who became *Campylobacter/Salmonella* positive (red) and those who remained negative (brown) (Wilcoxon’s test). The horizontal line in the box plot represents the median value and the box is drawn from 25% to 75% quartiles. Whiskers show minimum and maximum values and circles represent outliers.
these microbiota alterations persisted for at least 3 months after Salmonella infection in the particular individual. However, in the present study, faecal microbiota compositions of individuals who developed Campylobacter infection did not show significant changes when the samples taken before travelling and those collected after return to Sweden were compared.

Human studies on colonization resistance against enteropathogens have been hampered by the lack of prospective samples collected before the infection. The strength of the present study is that enteropathogen-negative faecal samples taken before travelling were available for microbiota composition analyses.

There were only a few dropouts as 63 of the 67 individuals originally recruited to the study gave all three faecal samples. The infection rate was high among the participants but unevenly distributed between the two groups because all except one of the included infected individuals were actually detected among the volunteers. The clearly higher incidence of bacterial enteritis among the volunteers could have been due to a generally higher risk for Campylobacter infection in the particular destinations, usually longer travelling time (median 80 days), or possibly more primitive living conditions abroad compared with those of the tourist trips. It can only be speculated if the participants attending the tourist trips in this study were less susceptible for enteropathogens due to their intestinal microbiota composition (as showing significantly higher diversity of their original faecal microbiota compared with the volunteers who remained uninfected) or if they actually would have become infected when attending the volunteer journeys.

Although the exact mechanisms underlying human colonization resistance against C. jejuni presumably are multi-factorial and so far poorly understood, our current results give further insights into the interplay between C. jejuni and human gut microbiota. Our data might help to identify those individuals at a higher risk of developing Campylobacter infection.

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Contribution to Authorship

Study design: HR, LE, JD; recruitment and enrolment of participants: CK; acquisition of data: CK, JD; bioinformatic and biostatistical analyses: JD; study supervision: HR, LE; drafting of the manuscript: CK, HR; approval of final draft: all authors.

Transparency Declaration

The authors declare no conflicts of interest.

References


