CLOSTRIDIUM DIFFICILE CARRIAGE IN ELDERLY SUBJECTS AND ASSOCIATED CHANGES IN THE INTESTINAL MICROBIOTA

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Abstract

Clostridium difficile is an important nosocomial pathogen associated particularly with diarrhoeal disease in elderly individuals in hospitals and long-term care facilities. We examined the carriage rate of Clostridium difficile by culture as a function of faecal microbiota composition in elderly subjects recruited from the community, outpatient and short term respite and long term hospital stay. The carriage rate ranged from 1.6% (n=123) for subjects in the community, 9.5% (n=43) in outpatient settings increasing to 21% (n=151) for patients in short- or long-term care in hospital. The dominant ribotype was carried by 43% (12/28) of subjects while the hyper-virulent strain R027 (B1/NAP1/027) was isolated from 3 subjects (11%), two of whom displayed CDAD symptoms at the time of sampling. Emerging ribotypes with enhanced virulence (078 and 018) were also isolated from 2 asymptomatic subjects. Pyrosequencing of rRNA gene amplicons was used to determine the composition of the faecal microbiota as a surrogate for the microbial population structure of the distal intestine. Asymptomatic subjects (n=20) from whom C. difficile was isolated showed no dramatic difference at phylum or family taxonomical level when compared with those that were culture-negative (n=252). However, in contrast, a marked reduction in microbial diversity at genus level was observed in patients who had been diagnosed with CDAD at the time of sampling and from whom C. difficile R027 was isolated.
Introduction

Changes in gut physiology and function occur during aging, and the decline in the immune system (so called ‘inflammaging’) may be the result of dysbiosis of the gut microbiota (12). A number of studies have reported changes in gut microbiota composition of the elderly at both phylum and species level (16, 39, 40). Initial studies by the ELDERMET consortium (http://eldermet.ucc.ie) compared the faecal microbiota composition of 161 elderly subjects and 9 healthy young controls through pyrosequencing, which demonstrated that while each individual showed a distinct profile, at phylum level the microbiota of the elderly subjects is dominated by Bacteroidetes while in the younger subjects analysed, the Firmicutes was dominant (7).

Antibiotic treatment has been shown to alter the gut microbiota causing severe dysbiosis, which can have a long term health impact (20). The classic example of the protective role played by a normal gut microbiota is demonstrated by the dysbiosis of the gut microbiota that occurs after antibiotic treatment leading to C. difficile infection. Following antibiotic therapy, virulent strains of C. difficile can colonise the gut, producing toxins and shedding spores, leading to a wide spectrum of illness from mild diarrhoea to fulminant relapsing diarrhoea and pseudomembranous colitis (PMC)(29). Current antibiotic therapy for C. difficile associated diarrhoea (CDAD) almost exclusively involves the use of the broad spectrum antibiotics vancomycin and metronidazole. While the development of resistance to these antibiotics is infrequent, there is a trend towards decreased susceptibility which is often manifested as increased recurrence of symptoms (5, 13, 18). In a study by Baines et al (2008) 24% of 001 ribotypes of C. difficile demonstrated reduced susceptibility to metronidazole which may question the use of metronidazole to treat very refractory cases. (3).
increased rate of disease recurrence has driven the search for alternative treatments, and antibiotics such as rifaximin, nitazoxanide, tigecycline and fidaxomicin (OPT-80) are currently being proposed as different therapies with varying degrees of success (31). In addition, bacteriocins have been suggested as substitute or adjunct therapies to conventional antibiotics (4, 25-27).

The risk of developing CDAD varies between individuals and is thought to be dependant on host factors and the type, dose and duration of the antibiotic treatment (37). *C. difficile* infection is primarily regarded as a nosocomial or healthcare related disease that is most prominent among the elderly population (29). This is most likely due to a reduced immune status, an increased likelihood of antibiotic use, and more frequent hospitalisation. *C. difficile* is the major cause of nosocomial diarrhoea in adults in the developed world, being responsible for virtually all cases of PMC and up to 25% of all antibiotic associated diarrhoea (11, 22). The worldwide emergence of the hyper virulent strain R027 (B1/NAP1/027) has resulted in an increased number and severity of outbreaks of CDAD (15). Numerous studies have reported *C. difficile* carriage from both healthy and diseased adults, ranging from 2% for asymptomatic healthy adults in the community to 10-51% of patients in hospitals or long term care facilities (10, 28, 30, 38).

The ELDERMET consortium (http://eldermet.ucc.ie) is using culture-independent methods to determine the composition of the faecal microbiota from 500 subjects aged 65 years and older, and a baseline microbiota composition survey for several hundred subjects has been published recently (7-9). In this phase of the project, we analysed faecal samples for culturable *C. difficile* to determine the carriage rate among healthy elderly subjects and those attending out-patient clinics in hospitals, short term in-patient respite care or long stay patients. While the *C. difficile* isolates
were characterised in terms of ribotype and potential to produce toxin the main focus of the study was to investigate whether carriage of *C. difficile* was related to the composition of the faecal microbiota of elderly subjects. In addition, the faecal microbiota of two subjects who had active CDAD at the time of sampling was also investigated.

**Materials and Methods**

*Subject recruitment and sample collection*

This study was approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals. Subjects aged 65 years and older were recruited and clinically investigated in two local hospitals. Exclusion criteria included a history of alcohol abuse, participation in an investigational drug evaluation within the previous 30 days, or advanced organic disease (i.e. patients who have end stage renal failure, end stage pulmonary disease, life threatening tumours or anything deemed terminal). Informed written consent was obtained from all subjects and, in cases of cognitive impairment, next of kin in accordance with the local research ethics committee guidelines. Data collected included clinical history and status and medication history including antibiotic usage. Some *C. difficile* culture positive subjects were on a variety of antibiotics in the 4 weeks prior to sampling; these antibiotics are listed in Table 1.

*Isolation of C. difficile*

Samples were collected from subjects and stored at refrigeration temperatures prior to delivery on ice to the laboratory within 24 h of sampling; at the laboratory they were frozen immediately at -20°C before ethanol shocking and plating on Cycloserine Cefoxitin Egg Yolk Agar (Lab M Bury UK) as described previously (10). Presumptive *C. difficile* isolates i.e. Gram positive anaerobic spore forming rod
shaped bacteria with typical ‘horse stable’ odour, non haemolytic, lecithinase negative and L-proline aminopeptidase positive which fluoresced under long wave UV light were subsequently confirmed as *C. difficile* by 16s ribosomal DNA sequencing using the primer pairs and PCR cycling conditions as described by Simpson *et al.*, (2003).

Strains were maintained on Microbank Beads at -80°C. For routine use strains were sub-cultured onto Fastidious Anaerobic Agar (Lab M) containing 7.5% defibrinated horse blood and grown anaerobically in a Don Whitley Anaerobic Chamber at 37°C.

**Bacterial strains used**

*C. difficile* VPI 10463 (ATCC 43255; A⁺/B⁻), *C. difficile* CUG 20309 (A⁻/B⁺) and *C. difficile* ATCC43593 (A⁻/B⁻) were used as positive and negative controls for toxin production *in vitro* and for the presence of tdcA and tcdB genes.

**PCR Ribotyping**

PCR-ribotyping for this study was carried out by the Microbiology Department, Leeds Teaching Hospital NHS Trust, Old Medical School, Leeds General Infirmary, Leeds LS1 3EX, West Yorks., UK.

**Toxin testing**

Toxin production, *in vitro*, was assessed from culture supernatants of strains grown in Reinforced Clostridium Medium (Merck) for up to 48h using the following commercial kits Toxin A⁺/B⁻: ProSpecT II (Remel Lexana, KS, USA) and Toxin A⁺: ImmunoCard Stat! *Clostridium difficile* Toxin A (Meridian Bioscience, Inc. 3471 River Hills Drive, Cincinnati, OH 45244, USA). Assays were carried out according to the manufacturers’ instructions.

Detection of tcdA and tcdB by PCR
C. difficile toxin genes tcdA and tcdB were amplified from the isolates as described by Terhes et al 2004 (35) with minor modifications. The DNA was extracted using 5% Chelex 100 as follows: 5-6 colonies from an over night culture of C. difficile grown on fastidious anaerobic agar containing 7% defibrinated horse blood were resuspended in 200µl 5% Chelex 100 (Sigma), heated for 30 min at 56ºC and subsequently boiled for 8 min before centrifugation at 13,000rcf for 3 minutes.

**Sequencing and Bioinformatics**

Two hundred and seventy four faecal samples were collected, 22 from C. difficile culture-positive subjects and 252 from C. difficile culture-negative subjects. Pyrosequencing yielded on average 28,000 reads per sample. DNA was extracted from faecal samples according to standard protocol (Qiagen, West Sussex, UK).

Universal 16s primers, designed to amplify from highly conserved regions corresponding to those flanking the V4 region, i.e. the forward primer F1 (5'-'AYTGGGYDTAAAGNG) and a combination of four reverse primers R1 (5'-'TACCAGAGTATCTAATCC), R2 (5'-'TACCAGAGTATCTAATTC), R3 (5'-'CTACDSRGGGTATTACATCTAAT), and R4 (5'-'TACNVGGGTATCTAATC) (RDP's Pyrosequencing Pipeline: [http://pyro.cme.msu.edu/pyro/help.jsp](http://pyro.cme.msu.edu/pyro/help.jsp)) were used for Taq-based PCR amplification. Subsequently, the V4 region of the 16S rRNA gene was amplified and sequenced on a 454 Genome Sequencer FLX platform (Roche Diagnostics) according to 454 protocols as previously described (8). Raw sequencing reads were quality trimmed using a locally installed version of the Ribosomal Database Project (RDP) Pyrosequencing Pipeline applying the following criteria: (i) exact matches to primer sequences and barcode tags, no ambiguous bases (Ns), and (iii) read-lengths no shorter than 150 bp. Trimmed FASTA sequences were then BLASTed (1) against a previously published 16s-specific database (36) using default
parameters. The resulting BLAST output was parsed using MEGAN (19). MEGAN is a tool for taxonomic classification based on BLAST output. Whilst not a true phylogenetic representation (branch lengths are not illustrative of evolutionary time) the MEGAN graphic exploits a tree-like representation to illustrate the taxonomical spread of samples in a hierarchical manner (i.e. Phylum-Class-Order-Family-Genus-Species). MEGAN assigns reads to NCBI taxonomies by employing the Lowest Common Ancestor algorithm. Bit scores were used from within MEGAN for filtering the results prior to tree construction and summarization. A bit-score of 86 was selected as previously used for 16s ribosomal sequence data (36). Phylum and family counts for each subject were extracted from MEGAN. Each subject’s DNA was individually sequenced and analysed; the resulting data was subsequently pooled for further examination.

Statistical Methods

Statistical analyses were carried out using Minitab Release 15.1.1.0 (Minitab Inc. 2007). P-values were calculated using the non parametric Kruskal-Wallis test and statistical significance was accepted at p<0.05.

Results

Characterization of C. difficile isolates

In this study faecal samples of patients, who were recruited primarily as part of the Eldermet Consortium, were analyzed to determine whether carriage of C. difficile impacted on the gut microbiota. Carriage of C. difficile was determined through standard culturing techniques and subsequently the gut microbiota of culture positive

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(n=22) and culture negative subjects was sequenced using 454 sequencing to
determine the impact, if any, of *C. difficile* carriage on the gut microbiota.
Subjects recruited from the community were classified as healthy (n=123), subjects
attending outpatient clinics/day wards in hospitals as outpatients (n=43), those with 6
weeks hospitalisation or less were classified as in rehabilitation (n=48) and those who
had spent greater than 6 weeks hospitalised in geriatric units were termed long stay
subjects (n=103). *C. difficile* was isolated from 28 subjects (9%). Table 1 summarises
the data obtained in this study on the subjects which were culture positive for *C.
difficile* carriage with regards to 1) location of the subject, sex, age and previous
antibiotic usage and 2) the isolates i.e. ribotype, *in vitro* toxin production and the
presence of pathogenicity locus. Two subjects (EM304 and EM306) were clinically
diagnosed with CDAD at the time of sampling and 2 subjects. (EM124 and EM186)
had been clinically diagnosed as suffering from CDAD approximately 9 months
previously all other culture positive subjects had no previous history of CDAD. The
carriage rate for community, outpatients, and rehabilitation and long stay patients was
1.6%, 9.5% 8% and 13%, respectively. While the most dominant ribotype isolated
from asymptomatic patients was 072, R027 was isolated from 3 subjects with 2 of
whom were diagnosed with CDAD at the time of sampling. Both symptomatic
subjects had a history of metronidazole and vancomycin usage within the 4 weeks
prior to sampling (Table 1).

Sequence-based faecal microbiota composition from *C. difficile* culture-positive and
culture-negative subjects

The V4 region of the 16S rRNA gene was amplified and sequenced from DNA
isolated from the stool samples of 22 *C. difficile* culture-positive subjects and the
microbiota composition established bioinformatically was compared to that of 252
subjects from whom *C. difficile* was not detected by culture. The phylogenetic profile of each subject was elucidated using a combination of BLAST and MEGAN algorithms, and resulting taxa were compared at the phylum, family and genus levels.

At the phylum level, the only statistically significant difference was a reduction in the proportion of *Spirochaetes* in the *C. difficile* positive population (p=0.017). All other phyla remained at approximately the same abundance across the populations, with the exception of the *Proteobacteria*, which was elevated from 2.3% in the *C. difficile* negative population to 4.3% in the *C. difficile* positive population.

Comparing subjects, there was large variability in the individual phylogenetic profile, both in the *C. difficile* negative and positive groups (Figure 1). At the phylum level, *Bacteroidetes* ranged from 4-92% of the total microbiota in the culture negative group and from 0.1-79% in the positive group. The *Firmicutes* ranged from 8-96% in the negative group and from 19-99% in the positive group. The *Actinobacteria* ranged from 0-8% in the negative group and from 0-1.2% in the positive group.

At the family level, some significant differences in the compositional data were observed (Fig 2). The families *Erysipelotrichaceae* (p=0.011), *Aerococaccae* (p=0.000) and *Flavobacteriaceae* (p=0.032) were elevated in the *C. difficile* positive population compared with the culture-negative group. However, *Flavobacteriaceae* and *Aerococaccae* were only present in one subject each in the culture-positive group and in none of the culture-negative group. Conversely, the levels of *Enterococceae* (p=0.009), *Leuconostocaceae* (p=0.026), *Prevotellaceae* (p=0.018) and *Spirochaetaceae* (p=0.045) were reduced in the culture-positive population when compared to the *C. difficile* culture-negative population. At the genus level, significant differences were observed only in genera that were present in low numbers of subjects; these differences are listed in Table 2. In total, the faecal microbiota of 22
subjects from whom *C. difficile* was isolated was determined by amplicon sequencing. Eighteen of these were asymptomatic carriers, 2 subjects had a previous episode of CDAD recorded but were asymptomatic at the time of sampling, and 2 subjects had active CDAD at the time of sampling. We analysed the microbiota compositional differences at the genus level between the *C. difficile* culture-negative subjects (A), asymptomatic carriers of *C. difficile* (B), those 2 subjects with a previous episode of CDAD recorded (C and D) and the two subjects with active CDAD at time of sampling (E and F) (Fig 3). The most obvious difference between the microbiota of these subjects was the dramatic alterations identified at the genus level in those subjects with active CDAD. The dominant genus in the subjects with active CDAD is *Parabacteroides* (97.4% and 61.5% compared to the 14.97% and 8.5% for the *C. difficile* culture-negative group and the asymptomatic carriers, respectively). However in those subjects with a previous history of CDAD, the dominant species were either *Catenibacterium* (33.0 %) or *Ruminococcus* (65.6%). These genera were present at <2% (*Catenibacterium*) and <10% (*Ruminococcus*) in the *C. difficile* culture-negative or asymptomatic carrier groups. In addition no *Bifidobacteria* were detected in subjects with current or a previous history of CDAD but they were present in the asymptomatic carriers (1.8%) and the *C. difficile* culture-negative group (2.6%). Where *C. difficile* was isolated from the same subject at 3 and 6 months, there appeared to be little alteration in the microbiota composition over time (Suppl. Fig. 1), which indicates the stability of the gut microbiota of these individuals. This observation was apparent at the phylum (Suppl. Fig.1), family and genus levels (data not shown).

*C. difficile* R027 was detected by culture in just three subjects two of whom had active CDAD at the time of sampling and one of whom was an asymptomatic carrier.
of *C. difficile*. A comparative MEGAN illustration of the overall gut microbiota of these three subjects was performed in which the asymptomatic carrier of ribotype 027 is depicted in blue and those subjects with active *C. difficile* subjects depicted in red and orange (Fig. 4). There are obvious differences, in that the subjects with active CDAD have a reduced overall number of assigned taxa compared with the asymptomatic carrier of R027. In the *Bacteroides* phyla, the active CDAD subjects harbour *Parabacteroides* as the dominant genus, while *Lactobacilli* are only present in the active *C. difficile* subjects and these subjects also have increased *Enterobacteriaceae* populations. Members of the *Lachnospiraceae*, *Succinivibrionaceae* *Ruminococcaceae*, *Synergistaceae*, *Bifidobacteriaceae* and *Victivallaceae* families are only present in the asymptomatic carrier group.

**Discussion**

The study outlined here examined *C. difficile* carriage in the elderly population in the Cork region of Ireland, based upon mixed urban-suburban-country town catchment areas. While the main focus of the study was to determine the effect of carriage of *C. difficile* among the elderly Irish population, preliminary characterisation of the isolates was carried out to determine ribotype and toxin production capability. While 5 ribotypes (027, 078, 018, 014, 026) which have been associated with CDAD outbreaks (17) were isolated from the largely asymptomatic population of elderly people, of particular interest here was the isolation of the hyper-virulent ribotype 027 from 3 subjects, one of whom was a day hospital patient and asymptomatic at the time of sampling. Ribotype 027 has come to prominence since the beginning of this millennium and has been isolated in at least 16 European countries (21) their increased virulence being associated with increased toxin and spore production. (13), (6, 23)
The human gut microbiota is a complex ecosystem whereby an estimated 15,000-36,000 different species live in a mutualistic relationship with each other and with the human host. While traditional culturing techniques identify only a small proportion of the total intestinal microbiota (<25%) (33) recent advances in sophisticated culture independent techniques allow us to make a more global assessment of the composition of the intestinal microbiota. The emerging method of choice to study the complexity of the human microbiome is high throughput amplicon sequencing. This high-throughput method is not, however, without its limitations; a previous study by this group (24) discussed both the strengths and limitations of both culture independent methods such as the one implemented here and culture dependent methods in-depth. These limitations include the inability of amplicon sequencing to identify population present in an environment below levels of $10^6$ and inability to assign to data to species level.

A study by Andersson et al (2008) of healthy adults showed that in six faecal samples the phylum Firmicutes dominated (81%) followed by Actinobacteria (14.6%), Bacteroidetes (2.5%) and Proteobacteria 1.7%. The dominant class among the Firmicutes was Clostridium (92%) and the genera Ruminococcus, Clostridium and Eubacterium were well represented (2). Similarly a study by Tap et al (2009) of seventeen healthy adults showed a similar phylum distribution(34) More recently a study by Claesson et al 2011, showed that the dominant phylum of 9 healthy subjects between 28 and 46 years old was also Firmicutes (51%) while in contrast Bacteroidetes (57%) dominated in 161 elderly subjects and the Firmicutes phylum was reduced to 40% of the total number of reads assigned (7).

While there were no dramatic differences between the C. difficile culture positive and culture negative groups at phylum level however, there was dramatic inter-individual
variation in the gut microbiota of these elderly subjects which has been reported previously for Eldermet subjects (7). While some significant differences were observed at family level between the culture positive and negative subjects these groups comprised a very small percentage of the total families identified (range from 0.03 to 0.05 % of total population). Therefore the isolation of ribotypes from asymptomatic subjects, which have capacity to produce TcdA and TcdB, suggest that the commensal flora in these subjects have the capacity to protect the host by preventing those potentially pathogenic C. difficile from overcoming colonisation resistance, proliferating within the colon and producing TcdA and TcdB. Antibiotic therapy is generally considered a risk factor for the development of CDAD, however 50% of the culture positive but asymptomatic subjects had a history of broad spectrum antibiotic usage within the previous 4 weeks (Table 1) however no usage of clindamycin was recorded by these subjects which is frequently associated with development of CDAD. Broad spectrum antibiotic therapy targets not only the pathogenic bacteria but also impacts on the wider microbiota of the gut leading to perturbations of the commensal flora and therefore decreasing their colonisation resistance potentially leading to outgrowth of pathogens such as C. difficile. In this study ribotype 027 was isolated from 3 subjects two of whom had CDAD at the time of sampling. A Megan comparison of the bacterial populations in these three subjects shows a dramatically decreased in taxa assigned in the patients with active CDAD when compared with the asymptomatic carrier of 027, their microbiota being dominated by members of the Bacteroidetes phylum and to lesser extent members of the Lactobacillaceae family (Fig 4). However it must be stated here that these 2 subjects were on antibiotics prior to sampling (Table 1) which does pose the question is the reduced number of taxa due to prior antibiotic therapy leading to the
proliferation of *C. difficile* or is the reduction due to antibiotic treatment for CDAD

However the more diverse microflora in the asymptomatic subject carrying ribotype 027 who was not receiving antibiotics supports the theory that the gut microbiota may have protected this subject from developing CDAD.

A number of studies have looked at the microbiota composition of patients with CDAD using a variety of culture dependant/ independent methods in an effort to elucidate the impact of gut flora on CDAD. A study by Hopkins and MacFarlane (2002) used cellular fatty acid profiles to identify bacteria from faecal samples to species level. CDAD patients were characterised by decreased numbers of *Bacteroides*, *Prevotella* and *Bifidobacteria* together with increase numbers of facultative species, *Clostridia* and *Lactobacilli*. These results are largely in agreement with our study, we also see decreased numbers of *Bacteroides*, *Prevotella* and *Bifidobacteria* in *C. difficile* positive subjects, and whilst our study cannot identify species, at the genus level, in agreement with the Hopkins and McFarlane study, we observe a decrease in *Lactobacillus* sp. However, in contrast to the Hopkins and McFarlane study there is an increase in *Clostridium* sp in *C. difficile* positive subjects. Chang *et al* (2008) using culture independent phylogenetic analysis of the 16SrRNA-encoding gene sequences compared the faecal communities of control subjects with those with initial or recurrent CDAD and demonstrated that the faecal microbiota of patients with recurrent CDAD was shown consistently to have a decreased phylotype richness compared to the controls and interestingly this reduction in species richness was not observed in patients with antibiotic associated diarrhoea not linked to *C. difficile*. However, unlike our study, 16s phylotypes related to *C. difficile* dominated the faecal microflora. The absence of reads assigned to *C. difficile* in our study may be explained by the antibiotic usage (metronidazole and vancomycin) by these subjects.
prior to sampling, and also the inability of pyrosequencing to assign reads to species level (24). In contrast a study by Manges et al (2010) using ribosomal RNA microarrays compared the differences in the microbiota of control subjects with the microbiota of subjects prior to and after development of CDAD taking a number of risk factors into account. Probe intensities for the phyla Firmicutes, Proteobacteria and Actinobacteria were increased in CDAD patients while the phylum Bacteroides decreased however when adjustment was made for the use of non-steroidal anti-inflammatory drugs and fluoroquinolones only a small subset of organisms within the Bacteroidetes and Firmicutes phyla remained significantly and independently associated with CDAD. These authors did however make an association between increased probe intensities in Lactobacillaceae and Enterococcaceae but not with Enterobacteriaceae. Our study demonstrates a decrease in Enterococcaceae but an increase in Lactobacillaceae and Enterobacteriaceae in C. difficile positive subjects. The differences observed in our study compared to Manges et al could be accounted for by the fact that the dominant phylum changes from Firmicutes in a younger population to Bacteroidetes in the elderly population. The authors also accept that in the study reported here patients with active or a history of CDAD were sampled after the disease was established and in the case of the two patients with active CDAD after the administration of the antibiotics vancomycin and metronidazole which may contribute to the differences observed at phylum and genus level and species richness for the symptomatic versus the asymptomatic group.

The four subjects who had active or a history of C. difficile infection had no reads assigned to the genus Bifidobacterium. These data are in broad agreement with a culture based study for patients with CDAD, where Bifidobacterium were not detected in significant numbers. In addition, in this study there was a trend in those subjects
with active or a history of CDAD to harbour lower numbers of *Faecalibacteria* (17.53% and 0.25% in those with a history of CDAD, 0% in those with a current CDAD versus 18.48% for the asymptomatic group). Levels of *Faecalibacterium praunzitzi* and *Bifidobacterium* are reduced in patients with chronic gut inflammation and *F. praunzitzi*, in particular, has been shown to exert anti-inflammatory effects on inflammatory bowel disease models of colitis (14, 32).

Studies such as Eldermet are investigating, in depth, the gut microbiota of healthy and diseased elderly subjects, including the changes in the gut microbiota of patients with bowel disease including CDAD. By understanding the changes that occur in the microbiota of patients suffering from intestinal disorders these studies may facilitate the development of prophylactic therapies for elderly patients prior to antibiotic therapy, through supplementation of their diet with pre/probiotics to encourage the growth of beneficial or protective bacteria such as *Bifidobacterium* and *F. praunzitzi*. As a result, this may reduce the major dysbiosis of the gut microbiota caused by antibiotic treatment and therefore reduce the chances of susceptible patients developing CDAD.

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References


Figure 1: Bar chart of inter-individual variability of *C. difficile* negative (a) and *C. difficile* positive (b) subjects. Legend is below.

Figure 2: Family level assignments of gut communities of *C. difficile* culture-positive subjects [A (n = 22)] compared with *C. difficile* culture-negative controls [B (n = 252)] expressed as a percentage of assignable tags.

Figure 3: Genus level assignments of gut communities in: A- 252 *C. difficile* negative subjects; B- 18 asymptomatic carriers of *C. difficile*; C and D- individual subjects with previous documented incidence of *C. difficile* infection; E and F- individual subjects with active *C. difficile* infection at the time of sampling.

Figure 4: MEGAN comparison of 3 (EM011, EM304 and EM306) subjects harbouring *C. difficile* strain R027. This MEGAN illustration provides a comparison view in which each node shows the number of reads assigned to it for each of the datasets. Pie charts at each node display the relative abundance for each taxonomical level. Reads are assigned according to BLAST assignments. EM304 and EM306 were diagnosed with CDAD at the time of sampling. EM3011 was an asymptomatic carrier with no prior history of CDAD.

Suppl. Figure 1. Relative phylum abundance of 2 *C. difficile* positive subjects (255 & 243) at 3 time points (T0- time zero, T3- 3 months, T6- 6 months).
<table>
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<tr>
<th>Subject</th>
<th>Sex</th>
<th>Age</th>
<th>Ribotype</th>
<th>Stratification</th>
<th>Antibiotic usage within the 4 weeks prior to sampling</th>
<th>tcdA/tcdB</th>
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<td>EM011</td>
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<td>027</td>
<td>D&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>++/+</td>
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</tr>
<tr>
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</tr>
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</tr>
<tr>
<td>EM140</td>
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</tr>
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<tr>
<td>EM148</td>
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<tr>
<td>EM149</td>
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<td>82</td>
<td>010</td>
<td>C&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Fluoxacillin</td>
<td>-/-</td>
</tr>
<tr>
<td>EM152</td>
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<td>050</td>
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</tr>
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</tr>
<tr>
<td>EM186</td>
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<td>Trimetoprin, Augmentin</td>
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<td>Augmentin DUO, Augmentin</td>
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<tr>
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<td>Klacid, Clindamycin, Fluocxacillin, PenicillinV, Metronidazole, Trimetoprin, Ciprofloxacin, Vancomycin, Co-Amoxiclav, Tazocin</td>
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<td>EM324</td>
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<tr>
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<td>70</td>
<td>216</td>
<td>C</td>
<td>Clarithromycin, Co-Amoxicin</td>
<td>++/+</td>
</tr>
</tbody>
</table>

<sup>a</sup>subjects in hospital as day patient; <sup>b</sup>subjects in rehabilitation i.e. less than six weeks as in-patient; <sup>c</sup>long stay i.e. subjects more than six week as in-patient; <sup>d</sup>community dwelling subjects.

Table 1 Summary data of subjects from whom *C. difficile* was isolated and classification of isolates regarding ribotype and presence of pathogenicity locus
Table 2: Significant differences in abundance of genera between *C. difficile* culture positive and culture negative populations.

<table>
<thead>
<tr>
<th>Genus</th>
<th>Significance (p-value)</th>
<th>Change in Abundance in <em>C. difficile</em> positive population</th>
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</thead>
<tbody>
<tr>
<td><em>Aerococcus</em></td>
<td>0.001</td>
<td>Increase</td>
</tr>
<tr>
<td><em>Anaerococcus</em></td>
<td>0.028</td>
<td>Increase</td>
</tr>
<tr>
<td><em>Enterococcaceae</em></td>
<td>0.002</td>
<td>Decrease</td>
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<tr>
<td><em>Helcococcus</em></td>
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<td>Increase</td>
</tr>
<tr>
<td><em>Leuconostoc</em></td>
<td>0.009</td>
<td>Decrease</td>
</tr>
<tr>
<td><em>Weeksella</em></td>
<td>0.001</td>
<td>Increase</td>
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