Alterations in intestinal microbiota of elderly Irish subjects post-antibiotic therapy

Órla O’Sullivan†, Mairead Coakley†, Bhuvaneswari Lakshminarayanan1,2, Susana Conde3, Marcus J. Claesson2,4, Siobhán Cusack2, Anthony P. Fitzgerald3,5, Paul W. O’Toole2,4, Catherine Stanton1,4 and R. Paul Ross1,4* on behalf of the ELDERMET Consortium‡

1Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland; 2Department of Microbiology, University College, Cork, Ireland; 3Department of Statistics, School of Mathematical Sciences, University College, Cork, Ireland; 4Alimentary Pharmabiotic Centre, University College, Cork, Ireland; 5Department of Epidemiology and Public Health, University College, Cork, Ireland

*Corresponding author. Tel: +00353-(0)2542229; Fax: +00353-(0)2542340; E-mail: paul.ross@teagasc.ie
†Ó. O’Sullivan and M. Coakley contributed equally to the manuscript.
‡Additional Principal Investigators of the ELDERMET Consortium are listed in the Acknowledgements section.

Received 30 July 2012; accepted 31 July 2012

Objectives: The human intestinal microbiota composition alters naturally with age, but is unusually perturbed by antibiotic therapy. The impact of antibiotic therapy on the composition of the intestinal microbiota of a cross-section of elderly Irish subjects (n = 185, ≥65 years) was investigated, taking into consideration their residence location.

Methods: Forty-two of the 185 elderly subjects were treated with at least one antibiotic within 1 month prior to faecal microbiota profiling. The residence locations of the subjects varied from long-term nursing care and rehabilitation wards to day hospitals and the community.

Results: Culture-dependent methods indicated that faecal Bifidobacterium spp. numbers were significantly reduced following antibiotic treatment (P = 0.004, 7-fold reduction), while levels of Lactobacillus spp. and Enterobacteriaceae were unaffected. The largest decrease in Bifidobacterium spp. numbers was linked to the administration of nucleic acid synthesis inhibitors (P = 0.004, 23-fold reduction). Microbiota profiling revealed a significant compositional change across nine genera following antibiotic therapy, including a relative increase in Lactobacillus spp. (P = 0.031), as well as a decrease in the number of genera identified in the antibiotic-treated subjects (n = 58), when compared with untreated subjects (n = 79). More alterations in the intestinal microbiota were observed post-nucleic acid synthesis inhibitor therapy, most notably a decrease in relative Faecalibacterium spp. numbers (P < 0.001).

Conclusions: The impact of antibiotic therapy on the intestinal microbiota in the elderly should be considered for long-term health effects, and differential susceptibility may require the development of products (e.g. prebiotics and probiotics) for at-risk subjects.

Keywords: antibiotics, culturable, unculturable, 16S rRNA gene amplicon sequencing

Introduction

The human gastrointestinal tract accommodates a vast number (>1000 species) and diverse range of bacteria,1–3 which can be profiled by high-throughput sequencing technologies.4,5 Microorganisms constituting the intestinal microbiota of healthy individuals are associated with numerous health benefits, including roles in nutrition and metabolism, conditioning of the immune system and protection against pathogens.6,7 Perturbations in the microbiota (dysbiosis) have been linked to adverse health conditions, such as obesity and inflammatory bowel disease.7,8 Changes have been identified in the intestinal microbiota composition with ageing, even taking extensive inter-individual variation into account.5,9,10 In general, Bacteroides11–16 and Bifidobacterium spp.11,15,16 decline with advancing age, whilst levels of clostridia, lactobacilli, streptococci and Enterobacteriaceae were observed to increase.10,17,18

In addition to the natural changes that occur during the ageing process, due to alterations in diet, lifestyle, digestive physiologies and immune function,10,16,19–21 the composition of the
intestinal microbiota in the elderly is impacted by antibiotic therapy. Broad-spectrum antibiotic therapy affects not only the target pathogenic bacterium but also the entire intestinal microbiota.\textsuperscript{22–25} Indeed, recent studies confirm the short- and long-term impacts of antibiotic therapy on the human intestinal microbiota.\textsuperscript{23,26} It has been demonstrated that antibiotic therapy reduces the overall bacterial diversity, affecting up to 33\% of the microbial population,\textsuperscript{5} and that it can also have an individualized effect on the intestinal microbiota.\textsuperscript{27} More specifically, antibiotic therapy reduced \textit{Bacteroides} numbers as well as altering the composition of the \textit{Bacteroides} group\textsuperscript{22} and reducing \textit{Bifidobacterium} spp., \textit{Desulfovibrio} spp., \textit{Clostridium} spp. and \textit{Faecalibacterium} spp. populations.\textsuperscript{13}

Knowledge of the effect of antibiotic therapy on the composition of the intestinal microbiota of the elderly is fundamental to the development of targeted treatments for improved health, e.g. prebiotics and probiotics. Previous reports on the impact of antibiotics on the gut microbiota of the elderly have varied in their approach and extent; studies used a combination of culture-dependent and culture-independent techniques, with subject numbers varying from 5\textsuperscript{15} to 74.\textsuperscript{28} In this study, the impact of antibiotics on the intestinal microbiota of 185 elderly Irish subjects from various residence locations was assessed using a combination of culture-dependent and culture-independent techniques. Culture-dependent methods were used to specifically enumerate viable populations of \textit{Bifidobacterium} spp., \textit{Lactobacillus} spp. and \textit{Enterobacteriaeae} in faecal samples, whilst culture-independent 16S rRNA gene amplicon sequencing was employed to investigate the global intestinal microbiota. \textit{Bifidobacterium} spp. and \textit{Lactobacillus} spp. were investigated due to their presence in the gastrointestinal tract being associated with positive health effects,\textsuperscript{29–32} and \textit{Enterobacteriaeae} because they form a major component of the gut microbiota.\textsuperscript{33–35} This study demonstrated that antibiotic therapy resulted in significantly reduced levels of culturable \textit{Bifidobacterium} spp., as well as perturbing the relative abundance of nine genera, including \textit{Lactobacillus} spp., in the intestine of older subjects.

### Methods

#### Subject recruitment and sample collection

Subjects aged \( \geq 65 \) years were recruited and clinically examined at ELDERMET Clinics at two local hospitals (Cork University Hospital and St Finbarr’s Hospital, Cork). Informed consent was obtained from all subjects or, in cases of cognitive impairment, next of kin, in accordance with the local research Ethics Committee guidelines. This study was approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals. Subjects completed a validated food-frequency questionnaire, provided a medical history, underwent a full physical examination and provided faecal, blood, saliva and urine samples.

The residence locations of subjects (\( n = 185 \)) were defined as: (i) long-term institutionalized care (long-stay; \( \geq 6 \) weeks; \( n = 48 \)); (ii) short-term rehabilitation hospital care (rehabilitation; stay of <6 weeks; \( n = 24 \)); (iii) occasionally attending an outpatient day hospital (day hospital; \( n = 40 \)); or (iv) community-dwelling (\( n = 73 \)). The mean age of the subjects (\( n = 185 \)) was 78 ± 7 years, with a range of 65–95 years. Subjects receiving antibiotics within 1 month prior to visiting an ELDERMET Clinic (\( n = 42 \)) were further classified as antibiotic treated. These subjects received various antibiotics (details for 37 of the 42 subjects are provided in Table S1, available as Supplementary data at JAC Online). The subjects were treated with 1 (\( n = 25 \)), 2 (\( n = 10 \)), 3 (\( n = 1 \)) or 4 (\( n = 1 \)) antibiotics. The antibiotics administered were grouped into nucleic acid synthesis inhibitors (\( n = 12 \) subjects), cell envelope antibiotics (\( n = 10 \)), protein synthesis inhibitors (\( n = 2 \)) and others (\( n = 1 \)); a further 11 subjects received a combination of nucleic acid synthesis inhibitors and cell envelope antibiotics and 1 subject received a combination of protein synthesis inhibitors and cell envelope antibiotics (Table S1). A breakdown of the subjects by residence location and antibiotic treatment is represented in Table S2 (available as Supplementary data at JAC Online).

#### Isolation and enumeration of bacteria by culture

Faecal samples for microbiological analysis were collected by the ELDERMET subjects into sterile containers and stored at 4°C until delivery to the laboratory. Storage times varied and were on average 28 h. Total culturable \textit{Bifidobacterium} spp., \textit{Lactobacillus} spp. and \textit{Enterobacteriaeae} were enumerated as outlined previously.\textsuperscript{36} Bacterial counts were recorded as cfu/g faeces and results expressed as log cfu/g faeces. The lower limit of detection for the culture technique was 3.0 log cfu/g for \textit{Bifidobacterium} spp. and \textit{Lactobacillus} spp. and 2.0 log cfu/g for \textit{Enterobacteriaeae}.

#### Amplicon sequencing and bioinformatics

DNA was extracted from faecal samples according to a standard protocol (Qiagen, West Sussex, UK) and, subsequently, the V4 region of the 16S rRNA gene was amplified and sequenced by Roche Diagnostics (West Sussex, UK) according to 454 protocols, as previously described.\textsuperscript{37} Raw sequencing reads were quality trimmed using a locally installed version of the Ribosomal Database Project Pyrosequencing Pipeline, applying the following criteria: (i) exact matches to primer sequences and barcode tags; (ii) no ambiguous bases (Ns); and (iii) read lengths no shorter than 150 bp. Trimmed FASTA sequences were then BLAST\textsuperscript{ed} against a previously published 16S-specific database\textsuperscript{39} using default parameters. The resulting BLAST output was parsed using MEGAN.\textsuperscript{40} MEGAN assigns reads to National Center for Biotechnology Information (‘NCBI’) taxonomies by employing the lowest common ancestor algorithm. Bit scores were scored from within MEGAN to filter the results prior to tree construction and summarization; a cut-off bit score of 86 was implemented.\textsuperscript{39} Phylum, family and genus counts for each subject were extracted from MEGAN. Operational taxonomic unit (OTU) assignment, chimera checking, clustering and \( \alpha \) and \( \beta \) diversities of reads were implemented with QIIME.\textsuperscript{41}

#### Statistical methods

Non-parametric statistical analyses (Mann–Whitney, \( \chi^2 \) and Kruskal–Wallis tests) were applied using Minitab Release 15.1.1.0 (Minitab Inc., 2007) and SPSS PASW Statistics version 18, to determine the impact of antibiotic therapy and residence location of the individuals on the levels of bacteria recovered. Statistical significance was accepted at \( P < 0.05 \). Adjustment for multiple testing was estimated using the \( q \) value (genus level) and the false discovery rate (‘FDR’) functions (phylum and family level) in the \( R \) statistical package (version 2.13.1) using the Benjamini and Hochberg method.\textsuperscript{42}

### Results

#### Relationship between antibiotic therapy and residence location

The elderly subjects were divided into four groups based on their residence location. Table S2, available as Supplementary data at JAC Online, presents a cross tabulation of residence location by...
antibiotic therapy for these subjects and indicates (as expected) that there was a clear association between these two factors for the subjects ($\chi^2$-test statistic = 13.103, $P = 0.004$), with many more subjects in the long-stay (33.3%) and rehabilitation (41.7%) groups receiving antibiotic therapy than those in the community (12.3%) or attending a day hospital (17.5%).

Variation in levels of culturable *Bifidobacterium* spp., *Lactobacillus* spp. and *Enterobacteriaceae*

There was a large inter-individual variation in the numbers of bacteria recovered from faecal samples of the 185 ELDERMET subjects assessed. With respect to culturing, *Bifidobacterium*

![Box plots](image-url)

**Figure 1.** Levels of (a) *Bifidobacterium* spp., (b) *Lactobacillus* spp. and (c) *Enterobacteriaceae* enumerated (log cfu/g faeces) from faecal samples from 185 elderly subjects, separated by antibiotic therapy (data for untreated subjects on left of figure and treated subjects on right) and residence location [long-stay (LS), n = 32 and 16, light grey boxes; rehabilitation (RH), n = 14 and 10, white boxes; day hospital (DH), n = 33 and 7, white boxes with horizontal lines; and community (CM), n = 64 and 9, dark grey boxes]. Circle with an enclosed cross = mean; cross = outlier; asterisk = significant difference between residence locations.
Impact of antibiotic therapy on the gut microbiota of the elderly

Figure 1. Continued.

spp. counts ranged from <3.00 to 10.70 log cfu/g faeces for individual subjects. Lactobacillus spp. counts ranged from <3.00 to 9.70 log cfu/g and Enterobacteriaceae levels similarly ranged from <2.00 to 10.00 log cfu/g (Figure S1, available as Supplementary data at JAC Online).

Levels of culturable Bifidobacterium spp., Lactobacillus spp. and Enterobacteriaceae were enumerated and assessed to establish whether antibiotic treatment had an effect on the numbers of bacteria detected (Figure S2, available as Supplementary data at JAC Online). Bifidobacterium spp. numbers were significantly reduced following antibiotic administration (P=0.004), with reduced bacterial numbers recovered from faecal samples, compared with those from non-antibiotic-treated subjects (average of 8.00 log cfu/g, reducing to 7.15 log cfu/g; 7-fold reduction post-antibiotic therapy). Lactobacillus spp. and Enterobacteriaceae numbers recovered from the elderly faecal samples were not significantly different between the antibiotic-treated and untreated groups (Figure S2). No correlation was established between the time since cessation of antibiotic therapy within 1 month prior to attending the ELDER-MET clinic and the counts recovered for culturable Bifidobacterium spp., Lactobacillus spp. and Enterobacteriaceae (data not shown). The effect of nucleic acid synthesis inhibitors (n=12), cell envelope antibiotics (n=10) and combinations of these two antibiotic types (n=11) on a selection of culturable bacteria was assessed. Subjects receiving only nucleic acid synthesis inhibitors had significantly lower levels of Bifidobacterium spp. (P=0.004, 23-fold decrease), as did subjects receiving a combination of the two antibiotic types (P=0.012, 13-fold reduction). Those subjects receiving only cell envelope antibiotics had no significant alterations in levels of culturable Bifidobacterium spp. Subjects receiving only cell envelope antibiotics had significantly altered levels of Lactobacillus spp. (P=0.047, 10-fold increase).

None of the antibiotic types or combinations affected the levels of Enterobacteriaceae compared with the control group. To further analyse the effect of antibiotic therapy on Bifidobacterium spp., Lactobacillus spp. and Enterobacteriaceae, the bacterial count data were considered separately by residence location and antibiotic use (Figure 1). Bifidobacterium spp. numbers separated by residence location and antibiotic treatment are represented in Figure 1(a). For subjects not receiving antibiotics (first four box-plots, Figure 1a), there was no significant difference between the numbers of Bifidobacterium spp. based on residence location. In contrast, for subjects receiving antibiotics, numbers of Bifidobacterium spp. varied with residence location (P=0.034). Counts of Bifidobacterium spp. were highest in the community-dwelling group (8.58 log cfu/g) and lowest in the long-stay group (6.27 log cfu/g) (P=0.008). Bifidobacterium spp. numbers were significantly lower for the long-stay (P=0.047) and day hospital (P=0.046) subjects receiving antibiotics, compared with the equivalent groups not receiving antibiotics.

The levels of Lactobacillus spp. cultured as a function of residence location and antibiotic therapy are represented in Figure 1(b). The levels of Lactobacillus spp. detected were not significantly different when all four residence locations were compared simultaneously, either in antibiotic-treated or untreated subjects. Pair-wise comparisons revealed that the levels of Lactobacillus spp. recovered from the rehabilitation subjects were significantly higher than those from both the long-stay (P=0.015) and community-dwelling (P=0.027) subjects in the antibiotic-untreated group. Importantly, in antibiotic-treated subjects, the numbers of Lactobacillus spp. were not significantly different in the individual residence locations (P>0.05 for all). Recovery of Enterobacteriaceae as a function of residence location and antibiotic use is represented in Figure 1(c).
Enterobacteriaceae numbers were not significantly related to residence location, either in the subjects receiving antibiotics ($P=0.188$) or the untreated subjects ($P=0.707$). The numbers of Enterobacteriaceae did not vary significantly between the different residence locations following antibiotic therapy.

**Microbiota differences determined by amplicon sequencing**

Each sequence read was assigned to an OTU based on 97% identity and further assigned taxonomy (at the phylum, family and genus levels) based on the BLAST homology searches. To determine whether antibiotic treatment impacted on intra-subject diversity, we calculated five diversity measures (Shannon diversity, Simpson index, Chao1, phylogenetic diversity and observed species). While there was a decrease in the mean microbial diversity in antibiotic-treated subjects compared with untreated subjects, both as a whole and when broken down by residence location, no significant changes were observed (Table S3, available as Supplementary data at JAC Online). To investigate similarities or differences in the microbial composition between antibiotic-treated and untreated subjects, principal coordinates analysis (PCoA) based on weighted-UniFrac β diversity distance was employed. This did not reveal any difference between the antibiotic-treated and untreated subjects as a whole or when broken down by residence location (Figure S4, available as Supplementary data at JAC Online). To investigate similarities or differences in the microbial composition between antibiotic-treated and untreated subjects, principal coordinates analysis (PCoA) based on weighted-UniFrac β diversity distance was employed. This did not reveal any difference between the antibiotic-treated and untreated subjects as a whole or when broken down by residence location (Figure S4, available as Supplementary data at JAC Online). To investigate similarities or differences in the microbial composition between antibiotic-treated and untreated subjects, principal coordinates analysis (PCoA) based on weighted-UniFrac β diversity distance was employed. This did not reveal any difference between the antibiotic-treated and untreated subjects as a whole or when broken down by residence location (Figure S4, available as Supplementary data at JAC Online). 

Also, there was no clustering of subjects based on antibiotic type (Figure S4, available as Supplementary data at JAC Online). Additionally, PCoA revealed no difference in the microbiota composition based on time since cessation of antibiotic therapy (Figure S5, available as Supplementary data at JAC Online). Microbial composition assignment revealed a large inter-individual variability in the phylogenetic profile of both antibiotic-treated and untreated groups. Bacteroidetes ranged from 4% to 92% of the total microbiota in the untreated group and from 0.1% to 91% in the antibiotic-treated group. The Firmicutes ranged from 8% to 96% in the untreated group and from 9% to 99% in the antibiotic-treated group. All phyla remained at approximately equal proportions across the datasets (Figure 2a). At the family phylogenetic level, no significant change in the microbiota of the antibiotic-treated subjects was observed (Figure 2b). At the genus level, reads were assigned to 79 genera in the intestine of antibiotic-treated subjects, both as a whole and when broken down by residence location, no significant changes were observed (Figure S6 and Table S3, available as Supplementary data at JAC Online). 

In the antibiotic-treated group, no significant differences were observed across residence locations. In antibiotic-untreated subjects, there were some differences across residence locations; at the genus level, 16 genera were significantly altered across the residence locations, including *Desulfovibrio* ($P=0.036$), *Faecalibacterium* ($P<0.001$), *Butyrivibrio* ($P=0.017$) and *Prevotella* ($P=0.012$). The largest differences appeared to be between the long-stay and community groups, with 11 out of the 16 genera being significantly different between these groups (Table S5, available as Supplementary data at JAC Online).

Considering the residence locations individually, most significant differences between the antibiotic-treated and untreated populations were observed in the day hospital population (Table 1). *Lactobacillus* spp. proportionately increased ($P<0.001$) in the day hospital antibiotic-treated subjects compared with the antibiotic-untreated group; in addition to alterations in seven other genera (Table 1). In the community residence location, *Bacteroidetes* ($P=0.020$), *Firmicutes* ($P=0.020$), *Proteobacteria* ($P=0.009$) and *Euryarchaeota* ($P=0.009$) populations were significantly different at the phylum level, while *Rhodocyclaceae* populations ($P=0.012$) were proportionately increased at the family level in the antibiotic-treated group. In the long-stay group, at the family level, *Lachnospiraceae* ($P<0.001$) populations were significantly reduced in the antibiotic-treated subjects compared with the untreated subjects. There were no significant differences, across all taxonomic levels, in the rehabilitation group.

**Discussion**

In this study, the impact of antibiotic therapy on the intestinal microbiota of a large cohort of elderly subjects was investigated, based on faecal microbiota analysis, using both culture-dependent (plating) and -independent (compositional) approaches. The antibiotics used varied considerably across individuals and the inclusion criteria simply stipulated that the treatment was administered $<1$ month before sampling. Time since cessation of antibiotic therapy had no impact on the composition of the microbiota. The impact of residence location on the microbiota of antibiotic-treated subjects was limited; this may be a consequence of antibiotic therapy having a blanket effect on the entire intestinal microbiota and not just the target pathogen.

Overall, it was found that the culturable *Bifidobacterium* spp. population in faecal samples from the antibiotic-treated group decreased 7-fold when compared with the untreated group. In this respect, previous studies have reported a deleterious effect on bifidobacterial populations, e.g. Bartosch et al. reported a 2.5-fold decrease in *Bifidobacterium* spp. in elderly hospitalized subjects receiving antibiotics ($n=21$) compared with those not in receipt of antibiotics ($n=38$). Although no significant difference
was observed in culturable *Lactobacillus* spp., the numbers recovered increased 2.6-fold in antibiotic-treated subjects as a whole. Indeed, such a finding was previously reported by Woodmansey et al., who recorded a 100-fold increase in *Lactobacillus* spp. levels in antibiotic-treated subjects (*n*=10) compared with those not receiving antibiotics (*n*=6).

![Figure 2](image-url)
targeting the *Bifidobacterium* spp. population, may be one approach to correct the altered intestinal microbial composition resulting from antibiotic therapy in the elderly.

### Acknowledgements
This work has been presented as a poster at the Eighth INRA-ROWET Symposium, Clermont-Ferrand, France, 2012 (Poster P2).

We are grateful to all those who participated in this study. We are also grateful to Elibhis O’Connor, Ian Jeffery, Edel Flannery, Mary Rea, Caitriona Guinan, Rachel Greene, Jennifer Deane, Nessa Gallwey, Karen O’Donovan and Patricia Egan for technical and clinical help.

### Additional Principal Investigators of the ELDERMET Consortium (http://eldermet.ucc.ie)
Colin Hill, Ted Dinan, Gerald Fitzgerald, Denis O’Mahony, Cillian Twomey, Douwe van Sinderen and Julian Marchesi.

### Funding
This study was performed as part of the ELDERMET project (http://eldermet.ucc.ie) and was funded by the Department of Agriculture, Fisheries and Food and the Health Research Board, through the Food and Health Initiative 2007–2011. M. J. C. is funded by a fellowship from the Health Research Board of Ireland.

### Transparency declarations
None to declare.

### Supplementary data
Tables S1 to S5 and Figures S1 to S6 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

### References
Impact of antibiotic therapy on the gut microbiota of the elderly


