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The ELDERMET Biobank: Isolation and Characterization of Intestinal Microbiota from Elderly Irish Subjects

A Thesis Presented to the National University of Ireland for the Degree of Doctor of Philosophy

By

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ABSTRACT

The human gastrointestinal (GI) tract is colonized by a dense and diverse bacterial community, the commensal microbiota, which plays an important role in the overall health of individuals. This study was an investigation of selected intestinal microbiota, mainly the *Bifidobacterium* and *Lactobacilli* communities of elderly Irish subjects, using both traditional culture-dependent and next generation sequencing technologies. The degree of correlation between measurements from both methods (**Chapter 2a**) suggested that a single method is capable of profiling intestinal *Bifidobacterium* spp., *Lactobacillus* spp. and Enterobacteriaceae populations. However, both methods have advantages that justify their use in tandem.

Antibiotic administration has been associated with profound disturbances of the GI microbiota. **Chapter 2b** evaluates the impact of antibiotic therapy on the composition of the intestinal microbiota of a cross-section of elderly Irish subjects, using both culture-dependent (plating) and culture-independent (compositional) approaches. Culturable bifidobacterial population in antibiotic-treated group decreased 7-fold when compared with the antibiotic-untreated group and the culturable lactobacilli numbers increased 2.6-fold in antibiotic-treated subjects. Exploring the impact of antibiotic types revealed that the nucleic acid synthesis inhibitors had the most dramatic effect on the levels of *Bifidobacterium* spp., showing a 23-fold decrease. In contrast the cell envelope antibiotics increased the levels of *Lactobacillus* spp. by 10-fold. Therefore, while antibiotics remain an essential medical tool, more targeted antimicrobial therapies should be explored to prevent intestinal dysbiosis.

The temporal stability of the gut microbiota is of importance to host health and consequently maintenance of the balance of this bacterial community is desirable. For this reason, the stability of culturable *Bifidobacterium* spp., *Lactobacillus* spp. and Enterobacteriaceae in faecal samples from 65 elderly subjects was monitored over a 6 month period (**Chapter 3**). Unexpectedly antibiotic administration did not result in major perturbations in the counts of the 3 bacterial groups. Regular probiotic consumption resulted in a significant increase in *Bifidobacterium* spp. counts by month 6, while there were no alterations in the other 2 bacterial groups studied. Overall, the studied bacterial groups remained relatively stable over a 6 month period; reinforcing a previous study by the ELDERMET team.

Chapter 4 investigated the isolation of bacteriocin-producing LAB of human intestinal origin that may be evaluated for probiotic use. A number of previously characterized bacteriocins, including ABP-118 and salivaricin B (from *Lactobacillus salivarius*), enterocin B

(*Enterococcus faecium*), lactacin B (*L. acidophilus*), gassericin T and a variant of gassericin A (*L. gasseri*) were identified. Interestingly, two antimicrobial-producing species, not generally associated with intestinally derived microorganisms were also isolated: *Lactococcus lactis* producing nisin Z and *Streptococcus mutans* producing mutacin II. The results presented are important due to growing evidence indicating bacteriocin production as a potential probiotic trait by virtue of strain dominance and/or pathogen inhibition in the mammalian intestine.

While ELDERMET aimed to enumerate bifidobacteria, lactobacilli and Enterobacteriaceae from faecal samples of elderly subjects, the fortuitous isolation of the clinically significant *C. perfringens* at high levels, on medium selective for bifidobacteria, from a sub-set of these elderly subjects motivated this spin-off study (**Chapter 5**). Of 368 faecal samples analysed during this study, 28 samples (7.6% of samples from 19 subjects), yielded colonies that were atypical of *Bifidobacterium* spp. on the *Bifidobacterium* selective medium, which were confirmed as *C. perfringens* using 16S rRNA sequencing. Microbiota profiling revealed some significant compositional changes across both the family and genus taxonomic levels between the *C. perfringens* positive and negative data-sets. The isolation frequency of *C. perfringens* from subjects in long-stay residential care with a shedding level in excess of 10^6 cfu/gram faeces suggest that such a high viable count may be indicative of a less healthy microbiota in the intestine of elderly people residing in longstay residential care.

The selection pressure applied on the bacterial population during antibiotic usage is the driving force for the emergence of antibiotic resistant bacteria. Investigation of lactic acid bacteria (LAB) isolated from over one hundred faecal samples for their resistance against the clinically important antibiotics, ciprofloxacin and amoxicillin (**Chapter 6**) identified 21 LAB isolates with increased ciprofloxacin resistance. The *parC* and *gyrA* genes of the quinolone resistance determining region (QRDR) for the isolates with increased ciprofloxacin resistance, showed no typical mutations in the amino acid codons encoding *gyrA*. However, a single amino acid change in the *parC* gene was determined. Identification of antibiotic resistant isolates opens up the possibility of using such probiotics to offset the problems caused by antibiotics to the gut microbiota.

LIST OF PUBLICATIONS

Isolation and characterization of bacteriocin-producing bacteria from the intestinal microbiota of elderly Irish subjects (2013) - **B. Lakshminarayanan**, C.M. Guinane, P.M. O'Connor, M. Coakley, C. Hill, C. Stanton, P.W. O'Toole and R.P. Ross. *Journal of Applied Microbiology* 114 (3): 886-898.

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Correlation of rRNA gene amplicon pyrosequencing and bacterial culture for microbial compositional analysis of faecal samples from elderly Irish subjects (2011) – Ó. O'Sullivan, M. Coakley, **B. Lakshminarayanan**, M.J. Claesson, C. Stanton, P.W. O'Toole and R.P. Ross. *Journal of Applied Microbiology* 111 (2): 467-473.

Alterations in intestinal microbiota of elderly Irish subjects post-antibiotic therapy (2013) - Ó. O'Sullivan, M. Coakley, **B. Lakshminarayanan**, S. Conde, M.J. Claesson, S. Cusack, A.P. Fitzgerald, P.W. O'Toole, C. Stanton, and R.P. Ross. *Journal of Antimicrobial Chemotherapy* 68 (1): 214-221.

Compositional dynamics of the human microbiota with ageing: Implications for human health (2013) - **B. Lakshminarayanan**, C. Stanton, P.W. O'Toole and R.P. Ross. *Journal of Nutrition, Health and Ageing* (Accepted).

PUBLICATION NOT INCLUDED IN THIS THESIS

Gut microbiota composition correlates with diet and health in the elderly (2012) - Claesson MJ, Jeffery IB, Conde S, Power SE, O'Connor EM, Cusack S, Harris HM, Coakley M, **Lakshminarayanan B,** O'Sullivan O, Fitzgerald GF, Deane J, O'Connor M, Harnedy N, O'Connor K, O'Mahony D, van Sinderen D, Wallace M, Brennan L, Stanton C, Marchesi JR, Fitzgerald AP, Shanahan F, Hill C, Ross RP, O'Toole PW *Nature* 488(7410):178-184.

GLOSSARY OF TERMS

AAD	Antibiotic-associated diarrhoea
ATCC	American Type Culture Collection
BHI	Brain Heart Infusion
BLAST	Basic Local Alignment Search Tool
BMI	Body mass index
bp	base pair
CD	Crohn's disease
CDAD	*Clostridium difficile*-associated disease
CFU	Colony forming unit
CPA	*Clostridium perfringens* alpha toxin
CPB	*Clostridium perfringens* beta toxin
CPB2	*Clostridium perfringens* beta2 toxin
CPE	*Clostridium perfringens* enterotoxin
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
DPC	Dairy Products Research Centre
dsDNA	double-stranded DNA
ETX	*Clostridium perfringens* epsilon toxin
FISH	Fluorescence *in situ* hybridization
GI	Gastrointestinal
GIT	Gastrointestinal tract
Glu	Glutamic acid
Gly	Glycine
GRAS	Generally recognized as safe
hr	Hours
IBD	Inflammatory bowel disease
IBS	Inflammatory bowel syndrome
IL	Interleukin
Ile	Isoleucine
ITX	*Clostridium perfringens* iota toxin
L	Litre
LAB	Lactic acid bacteria
LBS	Lactobacillus Selective Agar
Leu	Leucine
Lys	Lysine
min	Minutes
MALDI	Matrix laser desorption ionization
MEGAN	MetaGenome Analyzer
µg ml^{-1}	Micrograms per milliliter
µl	Microlitre
mM	Micromolar
mg	Milligrams
mg ml^{-1}	Milligrams per milliliter
ml	Millilitre
MRS	de Man-Rogosa-Sharpe agar
mMRS	modified de Man-Rogosa-Sharpe agar
MRSA	Methicillin resistant *Staphylococcus aureus*

MS	Mass spectrometry
NaOH	Sodium hydroxide
NEB	New England Biolabs
OD	Optical density
ORF	Open reading frame
OTUs	Operational taxonomic units
P	P-value
PCR	Polymerase chain reaction
PFGE	Pulsed field-gel electrophoresis
qPCR	Quantitative polymerase chain reaction
RDP	Ribosomal database project
RNA	ribonucleic acid
RPM	Revolutions per minute
RSM	Reconstituted skim milk
rDNA	Ribosomal DNA gene
rRNA	Ribosomal RNA gene
SCFA	Short-chain fatty acids
SD	Standard Deviation
Ser	Serine
TGGE	Temperature gradient gel electrophoresis
TOF	Time of flight
T-RFLP	Terminal restriction fragment length polymorphism
TTGE	Temporal temperature gradient gel electrophoresis
UC	Ulcerative colitis
VRBD	Violet red bile dextrose
v/v	Volume per volume
WHO	World Health Organization
w/v	Weight per volume

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CHAPTER 1

COMPOSITIONAL DYNAMICS OF THE HUMAN MICROBIOTA WITH AGEING: IMPLICATIONS FOR HUMAN HEALTH

A draft of this chapter has been accepted in
Journal of Nutrition, Health and Ageing
B. Lakshminarayanan, C. Stanton, P.W. O'Toole and R.P. Ross

1.1 ***Abstract***

The human gut contains trillions of microbes which form an essential part of the complex ecosystem of the host. This microbiota is relatively stable throughout adult life, but may fluctuate over time with aging and disease. The gut microbiota serves a number of functions including roles in energy provision, nutrition and also in the maintenance of host health such as protection against pathogens. This review summarizes the age-related changes in the microbiota of the gastrointestinal tract (GIT) and the link between the gut microbiota in health and disease. Understanding the composition and function of the gut microbiota along with the changes it undergoes overtime should aid the design of novel therapeutic strategies to counteract such alterations. These strategies include probiotic and prebiotic preparations as well as targeted nutrients, designed to enrich the gut microbiota of the aging population.

1.2 *Introduction*

The human gut is home to a complex ecological system harboring trillions of bacteria which vary according to their location in the gastrointestinal tract (GIT). This ecological niche provides an excellent site for complex interactions between the host and its microbial inhabitants. Molecular methods indicate that there are approximately 1,100 prevalent bacterial species in the intestine with as many as 10^{12}-10^{14} microorganisms present, of which 70-80% remain uncultured (1, 2, 3, 4, 5, 6). In its entirety, the human intestinal microbiota is estimated to contain 150-fold more genes than its own host's genome (7). The mutualistic relationship between the gut microbiota and the human host contributes to the maintenance of health and well-being in a number of ways. These include protecting the host against colonization of pathogenic bacteria, metabolizing complex carbohydrates, producing bioactive peptides and lipids, synthesizing vitamins and hormones and stimulating the immune system (8, 9, 10, 11). Reduced proportions of protective bacteria such as lactobacilli and bifidobacteria in the gut have been linked to the severity of disorders such as inflammatory bowel diseases or colonic inflammation (12, 13). This review summarizes the age-related changes that occur in the human GIT and makes the link between the bacterial communities and human health in an effort to understand the relationship between the two.

1.3 *Changes and succession of human gut microbiota from infancy to elderly*

The establishment of a stable microbial population involves complex processes such as bacterial succession and host-microbe interactions (14, 15). The colonization of the microbial population in the human gut begins immediately after birth, and results in an unstable composition in infants, which then undergoes marked changes until it develops into a relatively stable community in the adult (15, 16). The initial bacterial population of an infant depends on a number of factors including mode of delivery, feeding type, antibiotic usage and the surrounding environment (17, 18). The microbiota of infants delivered by Caesarean (C-) section have been reported to harbour relatively

lower numbers of bifidobacteria and higher numbers of *Clostridium difficile* and *Escherichia coli* than infants delivered vaginally (17). Colonization is also delayed in infants delivered by C-section when compared to vaginally delivered infants. Not surprisingly, bacteria involved in the initial colonization of the gut of vaginally delivered infants were predominated by lactobacilli and *Prevotella* species which have been shown to reflect their mother's faecal and vaginal bacteria (19). Antibiotic administration has also been shown to influence the intestinal colonization in infants, whereby antibiotic treated infants have lower numbers of enterococci and lactic acid bacteria (17).

The diet of a new born is one of the major factors affecting the intestinal colonization and studies using molecular techniques showed that the gut microbiota of the breast-fed infants is predominantly comprised of *E. coli*, streptococci and bifidobacteria and the gut microbiota of formula-fed babies is predominantly comprised of *Bacteroides*, clostridia and enterobacteria (17, 20). Changes to the intestinal microbiota then occur after the introduction of solid food leading to a remarkably complex and stable gut microbial population (20). The impact of diet on the gut microbiota of European children (1-6 years of age) consuming a Western diet was compared to the gut microbiota of children from a rural African village consuming a diet rich in fibre (21). The Western diet was associated with reduced microbiota diversity, higher proportions of *Firmicutes* and *Proteobacteria* and lower proportions of *Bacteroidetes* and *Actinobacteria* compared with the African diet.

After several years of development into the typical adult microbiota, the species composition of the bacterial community remains relatively stable (14) but can be transiently altered by some extrinsic factors (4, 22, 23). While both anaerobic and aerobic bacteria inhabit the GIT (3), the majority of genera are composed of anaerobic bacteria such as bifidobacteria, lactobacilli, clostridia, and *Bacteroides* (2). Recent 16S rRNA gene compositional studies had shown that the diversity of the healthy adult intestinal microbiota was mostly distributed among the phyla *Firmicutes* and *Bacteroidetes* followed by *Actinobacteria*, *Proteobacteria* and others (24, 25, 26).

The composition of the intestinal microbiota in elderly subjects changes from that of healthy adults due to factors related to aging, including nutritional behaviour, lifestyle modification, mobility, dentition, stress and reduced intestinal functionality (27, 28). Alterations in the gut morphology and physiology also occur during the aging process. These include difficulty in swallowing, decreased gastrointestinal motility, prolongation of gastric emptying and decline in splanchnic blood flow (28, 29). The microbial composition in elderly subjects was typically characterized by a decreased diversity of bacterial species, including the levels of beneficial bacteria (30, 31). Assessment of the gut microbiota of the elderly revealed lower levels of bifidobacteria and lactobacilli and higher levels of Enterobacteriaceae and clostridia when compared to younger adults (32, 33). This age related breakdown in the balance between the beneficial and detrimental bacteria has been associated with an increase in many intestinal inflammatory disorders. Indeed, a healthy intestinal microbial population supports human longevity. A recent Italian study supports the hypothesis that complex remodeling of metabolism and gut microbiota functionality are the key regulatory processes that marks longevity in humans (34).

Changes in the intestinal microbiota of the elderly may derive from dietary changes, changes in immune response, hospitalization, prolonged intestinal transit times, lack of physical activity, recurrent infections, and frequent use of antibiotics and other medications (31, 35, 36, 37, 38). The most dramatic changes in the composition of the intestinal microbiota in the elderly include reduction in numbers and species diversity of *Bacteroides* and bifidobacteria, and increased abundance of *Ruminococcus*, enterobacteria and lactobacilli (30, 32, 39, 40). Interestingly, a high level of total presumptive aerobes were recorded in the gut microbiota of elderly when compared to the younger adult (41). Isolation of potential pathogens such as *Clostridium perfringens* and *C. difficile* (causative agent of *C. difficile* associated diarrhoea (CDAD)) from elderly subjects has also been reported (33, 42).

The gut microbiota of the elderly has been reported to show different microbial composition and greater inter-individual variations compared to younger adults (31, 43). A Finnish study showed that members of *Firmicutes, Bacteroidetes* and *Actinobacteria* were reported to be more abundant in the intestinal microbiota of younger adults compared to the elderly (44). Similarly, Claesson *et al* have also reported that the *Firmicutes*/*Bacteroidetes* ratio was lower in elderly people when compared to young adults (31). However, Biagi *et al* did not find significant differences among the *Firmicutes*/*Bacteroidetes* ratios of Italian centenarians, elderly and young adults (36).

Age related changes in the intestinal microbial composition have also been shown to be location/geography-dependent (45). The intestinal abundance of *Clostridium* cluster XIVa has been reported to decrease in elderly Japanese, Italian and Finnish people (44, 45, 46), whereas an inverse trend was noted in elderly German people (45). An increase in the relative abundance of *Bacteroides* group was also observed in the Austrian elderly (47), whereas decreased proportions of *Bacteroides* group were reported in elderly Italian people (45). Benno *et al* reported that elderly Asian subjects had lower levels of bifidobacteria and higher levels of lactobacilli and Enterobacteriaceae (48).

One of the well documented aging effects is the decrease in the prevalence of *Faecalibacterium prausnitzii*, a member of *Clostridium* cluster IV, as found in the elderly Austrian and Italian subjects (36, 45, 47). Analysis of the faecal microbiota of IBD patients previously showed reduced numbers of *Faecalibacterium prausnitzii* (49, 50). A decrease in the *Faecalibacterium* population is also associated with hospitalization and antibiotic therapy (35). The gut ecosystem is dynamic. In order to fully understand the human biology in respect to the human microbiota, it is crucial to understand the impact of the gut microbiota on the host health. Summarization of the earlier studies on elderly has reported a large inter individual variations among older subjects (2, 31, 36). Any deviation in the diversity of the intestinal microbiota profile seems to be one of the indicators of

aging process and have been reported to be associated with inflammatory disorders. Overall, the maintenance of microbial homeostasis in the GI tract is essential for healthy ageing.

1.4 *Pathogenesis and alterations in the gut microbiome during intestinal dysbiosis*

The gut microbiota plays an important role in both human health and disease (51) and a vital role in the maturation of host immunity and defence against enteropathogens (52, 53, 54, 55). The stability that coexist between the host and the gut microbiota has a profound impact on the human health status, in ageing and longevity; as alteration of the intestinal microbiota composition (sometimes referred to as "dysbiosis") (56) is associated with pathogenesis of many diseases, including liver disease (57), Inflammatory Bowel Disease (IBD) (58), cancer (59, 60), malnutrition (61, 62), diabetes (25) imbalance in the regulation of body weight (63, 64) and several chronic conditions including obesity, frailty, sarcopenia and cognitive impairment among others. In addition, changes in the diet and lifestyles are also involved in disease manifestation. The changes of gut microbial communities during these diseased states are discussed in detail.

Bowel Diseases

The absence or low levels of bifidobacteria in the intestine of the elderly may have health consequences for the host, such as affecting the immune system or colonization resistance in the bowel (39). Irritable Bowel Syndrome (IBS) is an intestinal disorder that is characterized by recurrent intestinal pain, diarrhoea and / or constipation with other non-colonic symptoms, which includes features such as constant backache, urinary symptoms, non-cardiac chest pain and fibromyalgia (65, 66). Abdominal bloating, passage of mucus and faecal urgency are the most common features by IBS and are often rated as the worst symptoms by IBS patients (66). Several studies have implicated the role of the gut microbiota in immune changes in IBS (67, 68, 69, 70, 71).

Crohn's disease (CD) and Ulcerative Colitis (UC), are collectively known as chronic inflammatory bowel diseases (IBD) of the GIT. Diagnostic methods are the same as for other age groups. It would be of benefit to elderly IBD patients to receive proper diagnosis as misdiagnosis in the initial stages of IBD are more common, which can explained by the higher prevalence of number of clinical conditions that may mimic IBD, particularly ischemic colitis or colitis associated with diverticular disease (66, 72). The IBD pathogenesis includes impairment of mucosal barrier function, superficial or deep ulcers, rectal bleeding, higher rates of anaemia, electrolyte disturbance and malnutrition, which are more common in older patients compared to patients younger than 65 years (73) .

Crohn's disease can involve any part of the gastrointestinal tract from the mouth to the anus; however, the colon seems to be most affected in the elderly (74). Severity of recurrence after surgery is usually common in CD. Depending on the localization and disease extent, the clinical picture can be variable and the patients may present with abdominal pain, tiredness, fever, anemia, weight loss and symptoms of bowel obstruction (74). Although the initial mortality rate in elderly CD patients is similar to that in younger patients, the elderly do show a higher mortality rate few years after the onset of CD (72). Ulcerative colitis generally affects the rectum and colon to a varying extent, and can be cured by surgery. The pathogenesis involves impairment of mucosal barrier function and superficial or deep ulcers. Common features include bloody diarrhea, passage of mucous, abdominal pain and sometimes fever (74). Pouchitis is a common complication occurring in mostly in the first 10 years of disease (74).

In general, a large reduction in the microbial diversity was reported in patients with CD, including a decline in proportions of *Bacteroidetes* and *Firmicutes* (49, 58, 75). Similarly, studies observed the disruption of the commensal microbiota in UC, including a decreased proportion of bifidobacteria (76), reduction in the proportions of members belonging to the phyla *Bacteroidetes* and *Firmicutes* (58), decreased levels of *F. prausnitzii* (50), along with increased *Peptostreptococcus* species

numbers compared to healthy individuals (76) and the occurrence of *C. difficile* during relapse of UC (77).

Antibiotic-associated diarrhoea (AAD) occurs in 5-30% of patients receiving antibiotic therapy (78). The common risk factors include hospitalization, health of the host and exposure to pathogens, such as toxigenic *C. difficile*, a spore-forming gram-positive toxigenic anaerobic bacterium that is commonly associated with patients in hospitals and long-term care facilities (42). The overgrowth of this bacterial species in the intestinal tract disrupts the normal microbiota leading to gastrointestinal illness, decreased digestion, infections including sepsis, perforation of the colon, pseudo membranous colitis and sometimes death (79, 80, 81, 82).

Colon Cancer

The most common bowel cancer is colon or colo-rectal cancer (CRC),which is the second largest cause of cancer deaths in Western countries (83). Colorectal cancer (CRC) is a major source of morbidity and mortality in the elderly population with advancing age (84). More likely both colon and rectal cancer in the older adults are diagnosed at an advanced stage, which may contribute to a decreased survival among this population (85). The standard treatment for advanced colorectal cancer includes chemotherapy or combination of therapies, during which the patients experience diarrhoea, vomiting, especially neurotoxicity, which can cause functional decline and poorer quality of life (86). It has been suggested that both dietary supplements and intestinal bacteria may play a role in the initiation of colon cancer through production of carcinogens (87). Results from a human study indicated that *Bacteroides* species (*B. vulgatus* and *B. stercoris*) are associated with a higher risk of colon cancer (87) and especially with a high-fat diet; mainly because fat stimulates bile flow, which in turn is thought to stimulate the growth of *Bacteroides* species. Other studies have supported a similar finding with members of *Bacteroides* species being shown to convert bile to metabolites and fecapentaenes, which are considered co-carcinogenic or mutagenic (88). Studies

have observed changes in the bacterial species of individuals that are associated with CRC. Scanlan *et al.* observed that colon cancer patients exhibit a significantly increased diversity and reduced stability of clostridia compared to healthy individuals (89). Interestingly, Sobhani *et al* demonstrated (using qPCR) that all the bacterial species belonging to the *Bacteroides*/*Prevotella* group were more abundant in colon rectal cancer patients than in normal individuals (90).

Obesity

The prevalence of obesity is increasing in all age groups. In general, obese individuals with a BMI $\geq$ 30 are considered to have a higher mortality risk than do those who are considered overweight (BMI: 25.0–29.9) (91). In older adults, obesity can cause serious medical complications, which can lead to considerable cardiovascular morbidity, osteoarthritis, hypertension and impaired quality of life (91, 92). Increased BMI is associated with an increased risk of knee osteoarthritis and higher rates of certain types of cancers including breast, colon, renal and cervical in older population (91). Obesity increases the risk of frailty, sarcopenia in older adults (92). Obesity is also a recognized contributing factor to urinary incontinence in older population, especially in older women (93). Increased abdominal obesity in older people can cause metabolic changes which lead to insulin resistance, a key risk factor in the development of type II diabetes (92). Aging causes a progressive decrease in renal function and obesity is a significant risk factor for end-stage renal disease (92). Increased chest wall stiffness in obese adults may contribute to difficulty in breathing (93).

On the whole, obesity is a complex and increasingly alarming health issue in humans so it is not surprising that obesity-related gut microbiota studies have received a lot of attention in recent years. Ley and co-workers characterized the faecal microbiota of 12 obese individuals consuming low calorie diets and observed significantly higher proportions of *Firmicutes* than *Bacteroidetes* when compared to the faecal microbiota of lean individuals (63). This was in agreement with the findings by Turnbaugh *et al*, where they observed individuals with high BMI have decreased proportion of

Bacteroidetes compared to lean individuals (64). In contrast, a study by Schwiertz *et al* showed that the obese individuals showed higher proportions of *Bacteroidetes* compared to lean counterparts. They also observed that the concentrations of SCFAs were higher in the obese individuals than the lean individuals. The study concluded that rather than the *Firmicutes*/*Bacteroidetes* ratio, the metabolism of SCFAs might play a considerable role in the development of obesity (94). However, these specific changes remain controversial as other studies found no evidence that the proportions of *Bacteroidetes* and *Firmicutes* are associated with obesity (95, 96).

Diabetes

Type-2 diabetes is a metabolic disease associated with insulin resistance and compositional changes in the intestinal microbiota. Aging is associated with a reduced ability to metabolize glucose from food, which makes type-2 diabetes one of the most prevalent conditions in elderly (97). It is considered as the sixth-leading cause of death among the aged population, as it associated with increased risk of multiple medical conditions in older adults including cardiovascular events, fatal hypoglycemia, dementia and Alzheimer's disease (98, 99). Hypoglycemia is linked to cognitive impairment and incidence of dementia among older population (100). Age related insulin resistance appears to be associated with oral and dental issues, sarcopenia, under nutrition due to altered taste and smell, swallowing difficulties, hearing impairment (100).

Larsen and co-workers observed that the proportion of *Firmicutes* and *Clostridium* species were significantly reduced in diabetic patients compared to non-diabetic subjects (25). In addition, the *Bacteroidetes*/*Firmicutes* ratio and the ratio of the *Bacteroides-Prevotella* group to *C. coccoides-Escherichia rectale* group demonstrated a positive correlation with plasma glucose concentration (25). Recently, a study highlighted that diabetic individuals were characterized by a decrease in the abundance of butyrate-producing bacteria and an increase in several opportunistic pathogens, as well as an increase in functions relating to oxidative stress response and sulphate reduction (101).

Other diseases

Intestinal permeability and increased bacterial translocation (migration of bacteria from the intestinal lumen to mesenteric lymph nodes or other extra-intestinal sites) especially by *E. coli*, *Klebsiella*, enterococci and other streptococci species are a common complication in patients with cirrhosis(102).Other frequent complications observed in these patients include spontaneous bacterial peritonitis (an infection of ascitic fluid), urinary tract infections, respiratory tract sepsis (pneumonia and spontaneous bacterial empyema) and bacteraemia (102, 103).

Celiac disease is a chronic inflammatory disorder of the small intestine, where patients show permanent intolerance to cereal gluten proteins. The only therapy is adherence to a strict lifelong gluten-free diet. Recent studies on healthy adults consuming a gluten-free diet (GFD) have shown reductions in beneficial gut bacteria populations (including bifidobacteria and lactobacilli) and corresponding alterations in host immunity (104). Similar results were seen in a preliminary study, where subjects on a GFD showed decreased populations of beneficial bacteria, while populations of *E. coli* and total Enterobacteriaceae increased. In addition, production of pro-inflammatory cytokines and chemokines (TNFα, IFNγ and IL-8) and anti-inflammatory cytokines (IL-10) were remarkably reduced as a consequence of the GFD (105).

Using a combination of flow cytometry, 16S rRNA hybridization and DNA-staining, Vaahtovuo and colleagues compared the composition of the intestinal microbiota of patients with early rheumatoid arthritis (RA) and fibromyalgia (FM). The RA patients had significantly lower levels of bifidobacteria, members of the *Bacteroides-Porphyromonas-Prevotella* group, *B. fragilis* subgroup and *Eubacterium rectale–C. coccoides* group when compared to patients with FM (106).

Although it is clear from the studies described above that the intestinal microbiota are likely to be involved in various diseases but is difficult to draw definite conclusions on the role of any particular

bacterial groups. Further well-designed studies are required to examine the disease progression and outline the link between the gut microbiota and various health measures.

Geriatric syndromes

It has been suggested that decreased levels of SCFA production by the gut microbiota of older people may contribute to the onset of some distinctive conditions such as frailty, malnutrition, diabetes and sarcopenia (107). Frailty can be generally defined as state of decreasing reserves to functions in the elderly such as mobility, physical fitness, comorbidity, hearing, weight loss and vision and stress factors (108). Frailty in older men is associated with poor health conditions, less cognitive function and greater mortality (109). It is typically a multisystem impairment and its prevalence advances with age. Serum levels of IL-6 and CRP have shown to be elevated in the community-dwelling frail older adults (110).

Cognitive decline occurs at varying degrees in older adults (111). The basic cognitive functions most affected by age are attention and memory. The other factors associated with cognitive aging include hypertension, diabetes mellitus, and dietary factors such as vitamin D deficiency (112, 113). The study conducted by Barnes *et al* included women aged 65 and above being assessed for cognitive function of a 15 year period. About 9 % maintained optimal cognitive function, 58 % showed minor decline and 33 % experienced major decline. The group with optimal cognitive function was shown to have access to a positive social network, lack of diabetes, lack of hypertension and have moderate alcohol consumption (113). Maintaining a balanced diet, regular exercising have been considered as protective factors for cognitive decline related to aging (112).

Sarcopenia is an important syndrome characterized by progressive loss of skeletal muscle mass and strength, which occurs as a consequence of aging and is usually associated with decreased motility and poor physical inactivity (114). IL-6 is shown to be strongly associated with adverse physiologic effects such as sarcopenia, loss of weight and increased susceptibility to infections (110).

Nutritional supplementation in particular dietary protein intake is relevant for the maintenance of this condition (114).

Immune disorders

The survival of the host against the effects of pathogenic microbes depends on the protective immune system. Aging is a complex process that negatively impacts the functionality of the immune system resulting in a low-grade inflammatory status often referred to as inflamm-aging (115). This causes a persistent inflammation of the intestinal mucosa, increased susceptibility to diseases such as type-2 diabetes, sarcopenia, arthritis, osteoporosis and Alzheimer's diseases (116). The high prevalence of cardiovascular risk factors and morbidity increases with age and significantly contributes to the higher circulating levels of proinflammatory cytokines in older people (117). Increased levels of proinflammatory cytokines including interleukin-6 (IL-6) and C-reactive protein (CRP) can influence Alzheimer's disease and mortality in the elderly (118). It has been shown that higher production of IL-10 has a significant influence in the attainment of longevity (119). Immunosenescence (deterioration of the immune system) may lead to significant biological changes in the elderly population (115, 120, 121), which are detailed in Table 1.

The aging process also affects innate immunity, with a reduction in the levels of natural killer (NK) cells and phagocytes making the elderly more prone to infections (122, 123). In this respect, the composition of the commensal microbiota may be disrupted which may favour the growth of opportunistic pathogens (pathobionts) (124). Aging has been shown to be associated with reduced antigen-specific IgA antibody responses (125), involuntary weight loss (126), diminished ability to generate high affinity antibodies after immunization (120), reduced secretion of IL-7, an essential cytokine for development of lymphocyte responses and altered composition/type of lymphocytes in the spleen and lymph nodes (121).

In healthy human intestine, a fine homeostatic balance exists between the immune cells and the gut microbes (127). The interaction of an altered microbiota could contribute to maintaining a low-grade, systemic inflammation (128). The aged gut microbiota enriched in facultative anaerobes including streptococci, staphylococci, enterococci, and enterobacteria (often classified as pathobionts) and depleted in immune modulatory species belonging to the *Clostridium* clusters IV and XIVa are hypothesized to contribute to the development of an overall pro-inflammatory profile (107).

In this context, in a recent study a comparison of aged microbiota between community-dwelling and long-stay individuals showed that the microbiota of people in a long-stay care environment had a high proportion of *Bacteroidetes,* whereas individuals living in the community had a high level of *Firmicutes* (37). Levels of clinical markers such as IL-6 and IL-8 and C-reactive protein (CRP) were significantly high in long-stay subjects than in community dwellers expressing the status of systemic inflammation In addition, metabolome data suggested that changes in gut microbial populations are responsible for an altered production of short-chain fatty acids, which was more pronounced in community group than in long-stay subjects. Taken together, the major trends in the microbiota that separated healthy community subjects from less healthy long-term care correlated with increased frailty, inflammation and other clinical markers (37).

Fermentation of non-digestible prebiotic substances by certain anaerobic bacteria accounts for the production of short chain fatty acids (SCFA), in particular butyrate, acetate and propionate. In aged people, reduction of butyrate levels was correlated with decreased amounts of *F. prausnitzii*, and bacteria belonging to the *E. rectale*/Roseburia group, which are butyrate producers. Therefore, the decrease of anti-inflammatory SCFA-producing bacteria may lead to an easier entry of pathogens into the intestinal mucosa, especially Enterobacteriaceae, which has been positively correlated with the serum levels of two proinflammatory cytokines in old Italian people (36). It has also been demonstrated that the level of *Bifidobacterium* species is negatively correlated with the serum levels

of the pro-inflammatory cytokine TNF-α and the regulatory cytokine IL-10, indicating that the modulation of the faecal *Bifidobacterium* microbial population may represent a mean of influencing the inflammatory responses (129). Although, it seems plausible that the intestinal microbiota play an important role in regulating the inflammatory and immune responses, more studies are required for a better understanding of the intricate relationship between the human gut microbiota and the gut immune cells in the elderly.

1.5 *Influence of diet on the gut microbiota*

Dietary habits are considered as one of the major factors that influence the intestinal microbiota composition in human studies (21, 37) and animal models (130) and this review provides a link on the changes in the gut microbiome of elderly and other subjects based on diet. The study by De Filippo *et al* indicated that both the composition and the fermentation pattern of the gut microbes were influenced by diets rich in dietary fibre. The African children had significantly higher levels of *Actinobacteria* and *Bacteroidetes*, which possess enzymes that encode for hydrolysis of complex polysaccharides. Furthermore, higher abundance of Enterobacteriaceae, *Prevotella* and *Xylanibacter* species that possess enzymes required for cellulose hydrolysis were found in the African children which were absent from the European population (21). The authors postulated that the microbiota of the rural African population allow them to maximize energy extraction from the consumed fibre rich diet.

A more recent molecular study by our ELDERMET consortium compared the dietary pattern and faecal microbiota of elderly community-dwelling subjects (consuming diet rich in fibre but low in fat) and elderly subjects in long-term residential care (consuming diet low in fibre but rich in fat). Those in long-term care had a less diverse microbiota with a higher proportion of the phylum *Bacteroidetes*, while the microbiota of community subjects exhibited a far greater level of diversity with a higher proportion of phylum *Firmicutes*. The microbiota of the community-dwelling subjects

had an abundance of bacteria from the genus *Prevotella*, similar to the rural Burkina Faso children (21), confirming the association between carbohydrate rich diet and the genus *Prevotella* (37). Notably, the microbiota of elderly in long-term care was significantly less diverse and a loss of the community-associated microbiota correlated with increased frailty and progression of disease in older people and hence indicates the relationship between diet, gut microbiota and the health status among the elderly (37).

In the same context, van Tongeren and colleagues assessed the faecal microbiota composition of 23 elderly volunteers (median age of 86 years) living in the same environment and receiving similar diet. Based on the Groningen Frailty Indicator, the subjects were stratified into two groups, 13 subjects with low frailty score and 10 subjects with high frailty score. More differences in faecal microbiota composition were observed between elderly subjects with low and high frailty scores. Statistically significant reduction was seen in the number of lactobacilli (26-fold), *F. prausnitzii* (4-fold) and *Bacteroides*/*Prevotella* group (3-fold) were seen in the high frailty volunteers. In contrast, members of Enterobacteriaceae *showed* a 10-fold increase in high frailty group (131).

Diet and dietary patterns fluctuate over time, especially in older life, and can be modulated by mobility, appetite, taste and smell. Malnutrition is considered as one of the major factors leading to reduced immune responses in all ages of people (132). Both amino acid and protein deficiencies has been associated with impaired cellular immunity and decreased antibody response respectively (132). Gastric atrophy or *Helicobacter pylori* infection in the elderly has been associated with the malabsorption of vitamin B12 (133). A nutritionally-imbalanced diet may result in reduced mastication and taste sensation (134), coupled with dysphagia (135). Constipation, a common problem with advancing age, may be associated with inappropriate diet, depression, decreased physical activity resulting from chronic diseases and multiple medications (136, 137). Constipation maybe readily improved by laxative intake or by non-pharmological measures including increased fluid and fibre intake, consumption of legumes, fruits and vegetables (137).

Diet appears to be the main environmental modifier for microbiota composition. It has been shown by Wu *et al* that dietary protein and animal fat favour the growth of *Bacteroides* while carbohydrate is associated with increase in *Prevotella* (138). Diets rich in fat result in a phylogenetic shift in the intestinal microbiome associated with obesity (139). Diets low in vegetables but rich in fat and processed meat have been shown to be associated with the increase the faecal excretion of N-nitroso compounds, a promoter of colon cancer (140). A low fibre diet and low levels of calcium and selenium may also relate to the incidence of colonic diseases (141).

Diet influences metabolism of the bacterial species and specific items in the diet may have selective effects in the microbiota, which may be important for host health, irrespective of their age. It is been suggested that reducing the intake of food items rich in sulphur containing amino acids (such as cheese and eggs) from the diet of UC patients resulted in substantial therapeutic benefits (142). The ingestion of sulphur-rich food encourages the production of sulphide, which can damage the colonic mucosa by inhibiting butyrate oxidation- this is a characteristic defect in UC patients (142). Dietary protein may also reach the colon undigested and is then fermented by the gut microbiota to produce end products including ammonia, indoles, cresol and phenols, which favour the growth of malignant cells and may play a role in the etiology of bladder and bowel cancer (143). In contrast, a high-fibre diet was shown to lower urinary phenol and cresol concentrations in humans (144).

Kruis *et al* observed that a high-sugar diet (165 grams/day) prolonged the gut transit time and significantly increased the fermentative colonic bacterial activity and concentration of intestinal bile acids in the human colon (145). De Palma and co-workers observed that GFD could contribute to a reduction in pro-inflammatory signals (TNF-α, interferon-γ, IL-10 and IL-8) which was associated with modifications in the microbiota composition. These include decreased levels of bifidobacteria and lactobacilli and increased levels of *E. coli* (104).

1.6 *Analysis of microbiota diversity in the gut*

The complexity of the microbial communities in the human gut makes their study challenging. Traditionally, the composition of the gut microbiota was analysed using culture based techniques. Molecular approaches have opened a new window to explore and understand the composition of the intestinal microbiota (146).

Metagenomic approaches for analyzing the gut microbiota

Until a decade ago, research on the intestinal microbiota relied on the use of culture dependent methodologies. Triggered by a growing awareness that only certain microbial species can be cultured under standard laboratory conditions, various molecular methods have been evaluated depending on the rationale of the scientific study. These include Polymerase Chain Reaction (PCR) (147), real-time PCR or quantitative PCR (qPCR) (148, 149), Fluorescent *in situ* hybridization (FISH) (45, 150), DNA microarrays (151), dot-blot hybridization (152) and metagenomics . Various functional microbiomic approaches, such as metabolomics, metaproteomics and metatranscriptomics has allowed an in-depth analysis of the microbial communities and their metabolic outputs/consequences (153, 154, 155, 156).

The most employed and accepted molecular marker gene for bacterial classification and identification is the 16S ribosomal RNA gene, which is conserved in all eubacteria, and is of reasonable size (approximately 1.5 kb) for comparative sequence analysis. The diversity of the gastrointestinal microbiota can be thoroughly characterized using a full-length 16s rRNA sequencing (Sanger sequencing) method. The PCR technique is a widely used technique, which involves the amplification of the target DNA sequence using oligonucleotide primers. The amplified DNA fragments can then be visualized using agarose gel electrophoresis (157). The technique where the DNA amplicon resulting from the PCR reaction can be digested using restriction enzymes resulting in fragments of different sizes is referred as Restriction Fragment

Length Polymorphism (RFLP). This approach has been used to measure variations within the microbial communities among individuals (158).

Real-time or qPCR is another molecular biology tool, where the targeted DNA molecule of specific bacteria present in a bacterial community is simultaneously amplified and quantified. It is also the method of choice for rapid determination of bacterial number for microbiome sampling. Quantification is done using fluorescence-labelled group or species specific probes or DNA binding dye (ethidium bromide) (159). The fluorescence or dye intensity is proportional to the concentration of the target DNA, which depends on the prevalence of the target bacterial species. Molecular fingerprinting techniques that combine PCR-amplification of 16S rRNA gene and separation of amplicons using Denaturing Gradient Gel Electrophoresis (PCR-DGGE) have been successfully proven to analyse variations in the microbial community (160), while dot blot hybridization employs labelled oligonucleotide probes targeting the 16S rRNA gene, to measure the abundance of particular taxa (159). Using specific fluorescence probes (*in situ* hybridization) or primers (qPCR), quantitative analysis of the bacterial population can be achieved. FISH has been widely used to target different bacterial groups. A study by Mueller *et al* analysed the faecal microbiota using a set of group and species specific 16s rRNA targeted probes by FISH coupled to flow cytometry (45). Fluorescein-labelled oligonucleotide probes that target the 16S rRNA genes are frequently used for FISH analysis. Each probe is specific to a particular group of bacteria. The advantage of flow cytometry includes its high-speed process and accuracy and that there is no requirement for DNA extraction or amplification. The bacterial cells are fixed in a liquid and hybridized with fluorescein labelled antibodies or probes or stained with DNA-binding dye. Another promising quantitative technique that is used to study the bacterial composition is real-time PCR, which exhibits an increased numeric accuracy in comparison with the FISH technique (161).

Temporal temperature gradient gel electrophoresis (TTGE), temperature gradient gel electrophoresis (TGGE) and DGGE are related methods that have been widely used to determine

the identity of bacterial species present in the complex intestinal microbial communities without the need for sequence information (14, 162). The amplicons become denatured according to their GC content during gel migration. In TGGE and DGGE, a temperature gradient and denaturing gradient of urea and formamide are used, respectively for separation of DNA fragments (163). More recently, microarray technology is being used to study the diversity of microbial ecosystems. DNA microarray technology is a multiplex technology consisting of an arrayed series of thousands of probes which determine relative abundance of nucleic acid sequences in the target. This quantitative technique has been used to investigate the profile of the infant gut microbiota (164) and the genes involved in carbohydrate metabolism (165).

In general, DNA sequencing is the most powerful method for the identification of gut microbial composition (2) and has transcended all other techniques. The technology has developed further using next generation sequencing to study complex microbial communities (7). High throughput sequencing technologies (also known as next generation sequencing) includes three platforms that are presently widespread: the Roche/454, Illumina and SOLid (Applied Biosystems) (166, 167). The Roche-454 platform is based on pyrosequencing chemistry, which provides 100 fold higher throughput than Sanger sequencing targeting amplicons of the highly variable regions of the 16s rRNA gene (e.g. V2, V3, V4 or V6 regions) using bar-coded primers (168). Pyrosequencing has revealed in-depth information on the ecological impact of antimicrobial agents on the human intestinal microbiota (169). In a relatively new 454 pyrosequencing technique, parallel sequencing of a large number of templates can be performed without need for cloning. The illumina sequencing platform has been applied to metagenomic analysis, which produces read length of 100 bases (HiSeq system) to 150 bases (MiSeq system) (168, 170). The new technologies are evolving all the time and are helping to open new areas of research, including the investigation and characterization of microbial diversity and identification of variation in the complex genomes.

Conventional methods and molecular methodologies

Historically, culture-based approaches have provided significant contributions towards the understanding of gut microbiology. The advantages of culture dependent methods include reduced cost, enumeration of the bacterial populations and the possibility of performing various biochemical and physiological tests on the isolated strains (e.g. assessing antimicrobial production, inhibitory spectrum, antibiotic susceptibility testing and ability to utilize different substrates). However, these culture-dependent methods have several drawbacks in that they are laborious, time-consuming and technically challenging (171). Reasons for such cultivation problems include inherent limitations related to issues with the growth of the bacterial species, the selectivity of the media and stress factors (159). In addition, the nutritional interdependence of different species present in the human intestine makes the quantification by culture technique an inaccurate reflection of the available population. Nevertheless, traditional methods may not decline in usage in the future, as there is a need for the in-depth study of the physiology of particular bacterial isolates, which cannot be achieved without pure bacterial cultures.

To circumvent the limitations associated with culturing, molecular methodologies have accelerated progress by enabling the rapid simultaneous analysis of the complex intestinal microbiome (171, 172). Undoubtedly, one of the major advantages of these methods is its independence of variations in the growth conditions of the microbes. These techniques allow a high throughput approach to samples processing and exhibit various levels of discriminatory power from phylum to species level. As mentioned, the 16S rRNA gene is the predominant basis for phylogenetic determination; however, the ribosomal sequences may not be divergent enough to allow robust discrimination of species of the same genus (173). Most PCR-based methods, however, fail to reveal the ratio between the live and dead bacteria, as the amplification is dependent on intact nucleic acid rather than viable cells (174). The method may induce bias in relation to the primers used. It should also be emphasized that the results achieved with these molecular approaches are significantly

influenced by the efficacy of the protocol for the extraction of the genetic material from samples, as even a DNA contaminant may serve as a PCR template (175).

The major issue when using DGGE or TGGE is that the species diversity may be underestimated; in that a single band can represent more than one strain due to incomplete separation of amplicons (176). Disadvantages of FISH are its low sensitivity and that only few probes can be used per analysis (177). In addition to low sensitivity, flow cytometry analysis is limited mostly to liquid samples. qPCR has provided insight to the microbial community by amplifying and simultaneously targeting DNA molecule of specific bacterial group (25). One of the major limitations of qPCR using bacterial 16S rRNA is the presence of multiple copies of 16s rRNA in their genome resulting in either under or over estimation of the targeted taxa concentration. Also, the numbers of microbes quantified do not represent only viable microbiota as the primers and probes amplify DNA of all the cells present which can lead to an inaccurate viable count. In addition, there can be a question of specificity in relation to the primers and probes used. Although the technological improvement in the sequencing field has made a huge impact on microbial genetics, it still has its limitations. One of the major limitations of the available metagenomic approaches is that the composition of the predominant bacterial community is host specific (14). Also, it is an expensive approach and quiet challenging as the analysis of the complex data generated is far from easy and so requires extensive bioinformatics skills for the relevant representation of the complete microbiome (178). Comparative analysis of both culture dependent and culture independent techniques is presented in Table 2. Technologies continue to improve and the culture independent methods are particularly useful to establish the composition of the gut microbiota by identifying bacterial species that are present in the GI tract at relatively low abundance and difficult to culture. The shift from culture dependent methods to these molecular approaches has resulted in rapid growth in the field of GIT microbiota research.

1.7 *Effect of antibiotic therapy on the intestinal microbiota of elderly subjects*

While the development of antibiotics has lengthened the lifespan of humans, their use has led to a pervasive impact on the gut microbiota for a long period of time, sometimes up to one or two years, depending on the antibiotics used (179). Antibiotic therapy for the main part drastically alters the composition of the microbiota and can provide a pathway for the proliferation of pathogens. The intensity of the impact on the commensal bacteria depends on the antibiotic type, dosage, route and duration of therapy (180).

Assessing the temporal changes in the gut microbiota due to administration of broad spectrum antibiotics is one of the emerging fields of research and is a target for highly sophisticated molecular techniques. Several studies have demonstrated the long-term ecological impact on the gut microbiota of antibiotic therapy (181, 182). Dethlefsen *et al* demonstrated that a short course of the antibiotic ciprofloxacin (member of the Fluoroquinolones) reduced the diversity and abundance of the intestinal microbiota with significant effect on about one-third of the bacterial taxa (26). Although the majority of the bacterial community returned to the pretreatment state within a period of four weeks, failure of several other species to recover demonstrated the permanent damage and changes caused by antibiotic therapy (26). In another study, the impact of the same antibiotic on 3 individuals was assessed over a 10 month period. The study highlighted that the microbiota response varied across the subjects with reduced diversity among the microbial population in all subjects. This diversity was restored following the completion of antibiotic treatment to the point that they had stabilized by end of the study (183). Using molecular approaches, Donskey *et al* have also demonstrated the disturbances in the gut population due to administration of different antibiotics (184). The study indicated that the anaerobic microbiota was minimally affected by ciprofloxacin but markedly reduced by clindamycin therapy.

It is important to understand the need for antibiotics in the elderly, in particular those living in long-term care facilities, as these vulnerable people are more prone to bacterial infections due to immunosenescence and compromised health. In the process of eliminating the pathogenic microbes, the antibiotic therapies extend their effect to the gut microbiota as a whole, leading to unintentional dysbiosis. Several studies have looked at the impact of antibiotics on the intestinal microbiota in the elderly. Antibiotic therapy decreased the proportion of *Desulfovibrio* and *Faecalibacterium* species (35) along with increasing levels of *Lactobacillus* species (185). Recently, Claesson *et al* investigated the overall impact of antibiotic administration on the gut microbial composition of the elderly and revealed that antibiotic therapy reduced proportions of *Firmicutes* and *Proteobacteria* and increased the proportion of *Bacteroidetes* (31). Bartosch *et al* also reported a 2.5-fold decrease in the *Bifidobacterium* species in elderly hospitalized subjects receiving antibiotics compared to those not on antibiotics (35).

As a consequence of the potential damaging effect of the antibiotics, a number of investigations have focused on the introduction of antimicrobials over classical antibiotics that can cause less collateral damage. One such potent narrow spectrum antimicrobial peptide is Thuricin CD, a recently identified bacteriocin produced by *Bacillus thuringiensis*, which is specifically active against *C. difficile* (186). The efficacy of broad spectrum antimicrobials, such as vancomycin, metronidazole, lacticin 3147 and the Thuricin CD bacteriocin, on the gut microbiota composition, in particular *C. difficile* was demonstrated in a human distal colon model (187). The introduction of broad spectrum antibiotics resulted in a major disturbance in the overall population with a dramatic proportional increase in *Proteobacteria* and Enterobacteriaceae and a decrease in the numbers of *Bacteroidetes* and *Firmicutes*. In contrast, the introduction of the Thuricin CD bacteriocin caused no significant alterations in the relative proportions of the dominant microbial population but had potent anti-*C.difficile* activity.

Another major concern with the use of antibiotics is the emergence of antibiotic resistant bacteria, and the potential transfer of antibiotic resistance genes to pathogenic bacteria (182, 188). Bacterial resistance to antimicrobial agents can be either intrinsic (inherent or natural) or acquired. The microbes develop intrinsic resistance by altering the target sites to avoid binding of the antibiotic or by decreasing the permeability of their cell walls and/or developing efflux pumps to pump out the antibiotic. In acquired resistance, the microbes acquire genes to produce different enzymes, such as beta-lactamases which deactivate the beta-lactam ring of penicillin. Treating the rapidly emerging antibiotic resistant bacteria, especially the hospital acquired microbes, is becoming more challenging (189). Sjölund *et al* demonstrated that all *Enterococcus* species isolated immediately after treatment with clarithromycin had high level resistance to the antibiotic due to the presence of the *ermB* gene. It was also observed that resistant enterococci persisted for one to three years even after treatment in 3 patients (190).

The frequency and severity of infectious diseases are higher in older people compared to younger individuals (189). In this respect, antibiotic administration is the cause of profound disturbances in the indigenous bacterial population which undoubtedly lead to compromised colonization resistance, causing adverse effects including AAD (191), unintentional state of dysbiosis (56), risk of CDAD especially in elderly hospitalized patients (42, 192, 193), bowel dysfunction (194), intestinal malabsorption (195), nephrotoxicity, ototoxicity, seizures, skin rashes, nauseas, abdominal cramps, renal dysfunction and acute liver injury (196, 197). The mechanism of action and the adverse effects of different antibiotics are presented in Tables 3 and 4, respectively. The benefits of antibiotic usage is straight-forward, nevertheless negative impacts like the promotion of antibiotic resistance and disruption of the ecology within the gut are quiet common. Antibiotic alternatives are an active area of research in terms of developing new approaches. The use of prebiotics and/or probiotics could be a promising effect in restoring impaired functions or enhancing desirable functions of the microbiota.

1.8 *Modulation of the elderly human gut microbiota using probiotics, prebiotics and synbiotics*

The increased prevalence of diseases and disorders associated with the gut microbial imbalance due to factors such as dietary habits, lifestyle and drug usage can be modulated through supplementation with probiotics, prebiotics or synbiotics.

Probiotics

Probiotics, a term derived from the Greek meaning 'for life', are defined as "live microorganisms, which, when consumed in adequate amounts, confer a health benefit on the host" (198). The most promising probiotics include organisms of the genera *Lactobacillus*, *Bifidobacterium*, *Lactococcus*, *Streptococcus* and non-pathogenic yeasts. Probiotic strains should be natural inhabitants of the host species and have some of the following properties, technologically suitable for industrial processes, acid-fast and bile-fast, viable, adhere to the gut epithelial tissue, modulate immune responses (199, 200), regulate cytokine secretion and produce antimicrobial substances such as bacteriocins (201, 202). A number of mechanisms have contributed to the health benefits of probiotic bacteria, including production of SCFAs, vitamins, bioactive peptides, bacterial-host signalling molecules, antimicrobial substances and triggering the immune response (200, 203).

Prebiotics

The term prebiotic was first introduced by Gibson and Roberfroid in 1995 and are defined as "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or limited number of bacteria in the colon, and thus improves the health of the host" (204). Prebiotic usage avoids the drawbacks of using probiotic bacteria, such as maintaining viability during storage or en route to the intestine (204). Prebiotics of proven efficacy and commercially available are lactulose, galacto-oligosaccharides (GOS), fructo-oligosaccharides (FOS) and inulin, of which the last two are the most studied and well recognized (204). These

carbohydrates, when fermented by gut bacteria can serve as energy sources for the intestinal epithelial cells. Through stimulation of bacterial growth and fermentation, prebiotics can influence many aspects of bowel functionality (205).

Health benefits of probiotics and prebiotics in elderly

Probiotic intervention is a promising dietary approach for exerting health benefits such as prevention of ADD (206, 207), restoring the intestinal population in IBS patients (208) and enhancement of intestinal immunity (209, 210, 211). Probiotics have been considered as a promising approach to modify the gut microbiota and its overall functions for decades (212). Sood and colleagues reported that the probiotic preparation VSL#3 reduced UC symptom severity in some patients and induced remission in patients with mild-to-moderately active UC (213).

Lactobacilli and bifidobacteria are well known for their beneficial effects as probiotics in human health and can cause significant improvements in providing longevity and alleviation of age related disorders. As a key member of the intestinal microbiota, bifidobacteria were shown to inhibit the growth of enteric pathogens and may prevent gastroenteritis in humans (214). Supplementation with *Bifidobacterium* strains have been shown to increase the levels of health-promoting bacteria in the elderly (215, 216). Some strains of the genus *Bifidobacterium* exhibit powerful anti-inflammatory properties by restoring appropriate cytokine production (217). Certain members of the genus *Bifidobacterium* have been involved in functional foods, conferring health-promoting effects, in particular reduction of chronic inflammatory diseases including UC and Pouchitis, reducing symptoms of allergy and lactose intolerance (218). A Finnish study has shown an increase in the frequency of bowel movements in elderly nursing home residents with natural food supplies containing the two probiotic strains, *B. longum* and *B. lactis* (219). Intervention studies with *B. lactis* HN019 have also shown positive effects on the immune system of the elderly, such as increased phagocytic activity and number of NK cells (209, 220).

A more recent study demonstrated that one-month consumption of a probiotic biscuit, containing mixture of two probiotic strains, *B. longum* Bar33 and *L. helveticus* Bar13 was effective in not only restoring some of the age-related dysbiosis of the intestinal microbiota but also reverted the age-related increase in the opportunistic pathogens *Clostridium* cluster XI, *C. difficile*, *C. perfringens*, *E. faecium* and *Campylobacter* (221). Probiotic supplements containing lactic acid bacteria such as *L. rhamnosus* and *L. acidophil*us has been shown to alleviate constipation in aging people (222, 223). A randomized control study also demonstrated that dietary supplementation with *L. casei* DN-114001 in elderly subjects for three weeks showed potential for a 20% reduction in the duration of ‘‘winter infections’’ (gastrointestinal or respiratory) compared to the controls (224).

Elderly people may be regarded as immune compromised due to their decreased ability to fight infections- this is one of the main targets for researchers to develop strategies that can boost their immunity level (225). Probiotic intervention is a potential nutritional approach to modulate immune functions in the elderly population. The probiotic strains *Lactobacillus rhamnosus* HN001 and *B. lactis* HN019 have improved innate immune functions in elderly subjects (226, 227). Recent studies have demonstrated that the consumption of probiotic cheese containing *L. rhamnosus* HN001 and *L. acidophilus* NCFM by elderly volunteers resulted in significantly enhanced innate immune function (228) and modified subpopulations of faecal lactobacilli and *C. difficile* (229). Some probiotics are able to increase the activity of NK cells (230) especially in those habitual smokers affected by decreased NK cell activity (231). Other studies addressing the use of specific probiotic strains and their involvement in improving the host health are shown in Table 5.

Prebiotics have been shown to exert beneficial effects on host health by minimizing the disruption to the baseline gut bacterial community. Guigoz *et al* reported an increase in levels of faecal bifidobacteria accompanied by a significant rise in total lymphocyte counts in a group of frail elderly subjects treated with the prebiotic FOS for three weeks (232). Kleessen *et al* found that the intake of unabsorbed carbohydrates such as inulin improved constipation in elderly and

significantly increased the bifidobacterial population (233). Consumption of the prebiotic mixture trans-galacto-oligosaccharide (B-GOS) showed a beneficial effect, by significantly increasing the level of bifidobacteria and the immune response in healthy elderly volunteers (234). Similarly, a four week ingestion of short chain fructo-oligosaccharides (scFOS) significantly increased the levels of faecal bifidobacteria in healthy elderly volunteers (235). A progressive increase in inulin ingestion from 20 grams (g)/day (d) to 40 g/d for 19 days in an *in vivo* study significantly increased the population of bifidobacteria without altering the total bacterial counts in elderly female subjects suffering from constipation (233). Inulin has also been used as an aid to treat UC and to inhibit *C. difficile* infections (236).

Synbiotic therapy is proving promising in the management of some health conditions. The use of synbiotic supplementation (combination of *B. longum* and fructo-oligosaccharides/inulin) in a placebo-controlled study was shown to reduce inflammation in patients with active UC (237). Similarly, their use in the treatment of CD has been documented, where the patients receiving synbiotics exhibited reduced CD activity (238).The consumption of the synbiotic combination with *L. acidophilus* NCFM and lactitol had an effect on the microbiota composition (239) further reinforcing the effects on microbiota reported previously for this combination(240). Bartosch *et al* have demonstrated that consumption of synbiotics modified the composition of intestinal bifidobacterial populations in healthy elderly volunteers(241). A similar synbiotic preparation has been used to increase the levels of bifidobacteria and lactobacilli (242). Supplementation with pre/pro/synbiotics has been proven to promote species diversity and increase resilience of microbial communities to challenges including various pathological conditions and antibiotic therapy.

1.9 *Conclusions*

The human gut is made up of a complex consortium of microorganisms that perform multiple important functions, including governing the health of the host by acting as a barrier against pathogens and improving the host immune system. With rapidly developing metagenomic methods, an in depth understanding of the physiological and metabolic activities of the intestinal microbes is being uncovered. Several factors promote shifts in the microbial diversity during aging, such as the use of antibiotics, dietary supplements and life style, resulting in susceptibility to infections and diseases. Studies hold promise that administration of functional foods containing probiotics and/or prebiotics on its own or in combination (synbiotics) as possible approaches to restore homeostasis between the gut microbiota and immune system of the elderly. However, there is very little evidence for long term colonization with probiotics suggesting that their effects are transient and occur at times following consumption. Future research and applications should help serve not only to investigate the complex diversity of the microbial community, but also to focus on the functional properties ensuring health benefits for the host.

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Table 1 Age-related immune changes

Affected cell type	Change	Reference
Phagocytes	Reduced in number of cells	(122, 243)
B-lymphocytes	Reduced antibody production	(120, 123)
Lymphokines	Declined production of IL-2	(120)
Antigens*	Declined proliferation of T-cells	(121, 123)
Memory cells	Decreased functionality	(120)
Cytokines profile	Increased production of proinflammatory cytokines	(243)

*response to stimulation of antigens

Table 2 Comparative analysis of culture dependent and independent techniques

Techniques	Advantages	Disadvantages
Culture dependent methods	• Inexpensive, limited equipment is required • Organisms are available for further analysis • Possible to obtain knowledge about physiological or functional properties of strains • Can identify novel species	• Bias of selective enrichment media as no appropriate defined media is available for culturing most GI organisms • Restricted to detection of only culturable organisms • Time consuming and labour intensive • Samples require immediate processing
Culture independent methods	• More sensitive – can detect less abundant and uncultivable organisms • Many samples can be analysed simultaneously • Can target specific bacterial groups (FISH, qPCR, microarray) • Samples can be frozen for later analysis	• Bias in the selection of primers and oligonucleotide probes and differences in extraction efficiency • Expensive • Extensive bioinformatics analysis is required for example-454 pyrosequencing requires training and experience for interpretation of results • Cannot identify novel groups of bacteria (qPCR, FISH) • Both living and dead cells are quantified

Table 3 Mode of action of antibiotics commonly prescribed to the elderly

Antibiotic	Interferes with	Target site	References
Pencillins, cephalosporins, carbapenems and glycopeptides	Cell wall synthesis	Peptidoglycan layer	(244)
Fluoroquinolone	Nucleic acid	DNA gyrase enzyme	(245)
Rifampicin	synthesis	RNA polymerase	(246)
Macrolides	Protein synthesis	Ribosomal subunits	(245)
Sulfonamides	Folic acid synthesis	Folic acid	(247)
Aminoglycosides	Elongation process	Peptidoglycan layer	(248)

Table 4 Side effects of antibiotic therapy in the elderly

Antibiotic	Side effects
Beta-lactams: penicillins, cephalosporins and carbapenems	Bronchitis, diarrhoea and rashes
Fluoroquinolones: ciprofloxacin, ofloxacin andgatifloxacin	Nausea, vomiting and seizures
Rifampicin	Drug interactions
Macrolides: erythromycin, clarithromycin and azithromycin	Gastrointestinal intolerance
Trimethoprim-Sulfamethoxazole	Rashes and drug induced fever
Aminoglycosides: amikacin, streptomycin and kanamycin	Nephrotoxicity and ototoxicity

Table 5 Effect of probiotic therapy on human health

Probiotic strain or probiotic mix	Therapeutic effect	Reference
L. casei DG	Prevents recurrence of diverticular disease of the colon	(249)
L. rhamnosus GG, Saccharomyces boulardii and probiotic mixtures	Reduces the development of AAD	(250)
L. johnsonii La1 (NCC533)	Improves immune system and reduces duration of infection	(251)
L. casei DN 114001, *L. bulgaricus* and *Streptococcus thermophilus*	Reduces the incidence of AAD and CDAD	(252)
L. acidophilus SDC 2012 and *L. acidophilus* SDC 2013	Reduces abdominal discomfort in patients with IBS	(253)
L. casei DN 114001	Improves antibody responses to influenza vaccination	(254)
L. bulgaricus	Increases NK cell activity and reduces risk of common cold	(255)
L. acidophilus 145 and *Bifidobacterium* sp.420	Prevents recurrence of diverticular disease of colon	(256)
L. casei DN 114001	Reduces duration of respiratory infection	(257)
Bifidobacterium longum BB536	Increases NK cell activity and neutrophilic function	(258)

CHAPTER 2A

Correlation of rRNA gene amplicon pyrosequencing and bacterial culture for microbial compositional analysis of faecal samples from elderly Irish subjects

Bhuvaneswari Lakshminarayanan Chapter 4A contribution:

- Participated in the microbiological analysis including media preparation, isolation and microbial enumeration

- Analysed all data in relation to 16S compositional sequencing analysis with the aid of the staff bioinformatician

- Collaborated with the authors to write the manuscript relating to the analysis of the microbial composition through culture dependent method

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2.1 Abstract

Aims

The aim of this investigation was to establish the degree of correlation between measurements from culture-dependent microbiological techniques and from next generation sequencing technologies.

Methods and Results

Data generated by both techniques were collected from faecal samples from 185 elderly Irish people involved in the on-going ELDERMET study (http://eldermet.ucc.ie). The results for three groups of intestinal bacteria were compared. *Bifidobacterium* spp., *Lactobacillus* spp. and *Enterobacteriaceae* were enumerated on selective media through culture dependent techniques, whereas proportions of these bacteria were determined through sequencing technology against the background of other bacteria. The Spearman's rank correlation coefficient determined a good correlation between results from culture-dependent microbiology and culture independent techniques for all three bacterial groups assessed (correlation coefficients for *Bifidobacterium* spp., *Lactobacillus* spp. and *Enterobacteriaceae* were 0.380, 0.366 and 0.437, respectively).

Conclusion

Correlation between the two methods implies that a single method is capable of profiling intestinal *Bifidobacterium, Lactobacillus* and *Enterobacteriaceae* populations. However, both methods have advantages that justify their use in tandem.

Significance and Impact of Study

This is the first extensive study to compare bacterial counts from culture-dependent microbiological techniques and from next generation sequencing technologies.

2.2 Introduction

The human gastrointestinal tract harbours a complex microbial ecosystem consisting of a large number of bacteria (Eckburg et al. 2005; Qin et al. 2010; Tiihonen et al. 2010) that have been shown to contribute to various aspects of human health, including immunity, nutrition, digestion and even well-being (Curtis 2007; Sekirov et al. 2010; Cryan and O'Mahony 2011). From its establishment in infancy, the human intestinal microbiota is thought to undergo a period of stability in adulthood until it again changes in old age (Mitsuoka 1992; Hopkins and Macfarlane 2002; Hopkins et al. 2002; Silvi et al. 2003; Makivuokko et al. 2010; O'Toole and Claesson 2010). The ELDERMET consortium, founded in 2008 (http://eldermet.ucc.ie) was established to investigate the composition of the intestinal microbiota specific to the elderly Irish population, with a view to developing functional foods for this societal group. Initial results have revealed a core gut microbiome rich in *Bacteroidetes* and *Firmicutes* with large inter-individual variability but intra-individual temporal stability (Claesson et al. 2011). Additionally the core elderly microbiota has been demonstrated to have a distinct pattern from its younger counterparts (Claesson et al. 2009; Claesson et al. 2010).

Culture based techniques have been routinely used to monitor bacterial populations and to determine the bacterial composition of faeces as an indicator of the composition of the intestinal microbiota. The term “plate count anomaly” was introduced in 1985 (Staley and Konopka 1985) to describe the difference (sometimes in orders of magnitude) between the numbers of cells from natural environments that form colonies on agar media and those from direct cell counts under the microscope. Molecular techniques based on the analysis of 16S rRNA genes directly amplified from faecal DNA have estimated that less than 25 % of the faecal bacterial populations have been cultured to date (Suau et al. 1999). Estimations of the total culturable gut bacteria range 10-50 % therefore, despite constant evolution and improvement of culturing techniques, the full spectrum of the bacteria in any given environment cannot be accurately established (O'Toole and Claesson

2010). There have been improvements in culture independent techniques such as fluorescence *in situ* hybridisation (FISH), quantitative real-time PCR (qPCR), phylogenetic microarrays and direct sequencing of 16S rRNA which aim to solve this "plate count anomaly" through the identification of so-called uncultivable bacteria (Tyson and Banfield 2005; Mardis 2008; Petrosino et al. 2009). However, culture independent methods are not without their limitations (e.g. choice of target gene, detection of live/dead cells, choice of primers and availability of accurate databases) (Juste et al. 2008).

For the ELDERMET study, it was proposed that culture independent techniques would be used in concert with culture dependent techniques (Mardis 2008) and in this study the results from 185 elderly subjects were compared to examine the correlation between culture dependent and culture independent approaches. Culture dependent techniques were employed whereby a selective medium was used to determine the total numbers of *Bifidobacterium* spp., *Lactobacillus* spp. and Enterobacteriaceae in fresh faecal samples from elderly Irish subjects. The counts determined in this way were then compared to relative abundance measurements of *Bifidobacterium* spp., *Lactobacillus* spp. and Enterobacteriaceae determined by 16S rRNA amplicon pyrosequencing. We decided to focus on these microorganisms because bifidobacteria and lactobacilli are natural inhabitants of the healthy human intestinal microbiota and there is mounting evidence demonstrating the role of these bacteria as potential probiotics (Gill et al. 2001; Klaenhammer et al. 2008; Turroni et al. 2008; O'Flaherty and Klaenhammer 2010; Aureli et al. 2011). Additionally, fluctuations in the levels of bifidobacteria and lactobacilli have been reported in the elderly intestine or faeces (Mitsuoka 1992; Hopkins and Macfarlane 2002; Woodmansey 2007), particularly during antibiotic usage where their numbers rapidly decline. Members of the family Enterobacteriaceae constitute an important part of the human intestinal microbiota (Qin et al. 2010) and while many have been demonstrated to be important factors in gut health (Lupp et al. 2007; Garrett et al. 2010) some also represent important pathogenic species (e.g. *Salmonella* spp. and *Escherichia coli*).

Determination of a correlation between the culture dependent and culture independent techniques would render both methods equally efficient at detecting levels of *Bifidobacterium* spp., *Lactobacillus* spp. and Enterobacteriaceae in the intestinal microbiota. However, the ability of culture dependent techniques to target and isolate bacteria with probiotics potential from the intestinal tract and the ability of culture independent methods to profile the entire intestinal microbiota (both culturable and uncultivable) justifies using both in parallel.

2.3 Materials and Methods

Subject Recruitment and Sample Collection

One hundred and eighty-five subjects aged 65 yr and older were recruited and 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, from next of kin, in accordance with the local Clinical Research Ethics Committee guidelines. This study was approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals.

Sequencing and Bioinformatics

DNA was extracted from faecal samples according to a standard protocol (Qiagen, West Sussex, UK) using Qiagen QIAamp DNA Stool Mini Kit. Universal 16S rRNA primers, designed to amplify from highly conserved regions corresponding to those flanking the V4 region (region and primers selection based on previous study (Claesson et al. 2009)), i.e. the forward primer F1 (5'-AYTGGGYDTAAAGNG) and a combination of four reverse primers R1 (5'-TACCRGGGTHTCTAATCC), R2 (5'-TACCAGAGTATCTAATTC), R3 (5'-CTACDSRGGTMTCTAATC) and R4 (5'-TACNVGGGTATCTAATC) (RDP's Pyrosequencing Pipeline: http://pyro.cme.msu.edu/) were used for Taq-based PCR amplification. Standard PCR reaction conditions were employed for reactions with Taq polymerase – 2 mM MgCl2, 200 nM each primer, 200 µM dNTPs. The PCR conditions were 94°C for 50 seconds (initialization and denaturing) followed by 40°C for 30 seconds (annealing), 72°C for 60 seconds in 35 cycles (extension), and a final elongation step at 72°C for 5 minutes. Two negative control reactions containing all components, but water instead of template, were performed alongside all test reactions, and were routinely free of PCR product, demonstrating lack of contamination with post-

PCR product. The optimal annealing temperature for the primers, which included 454 adapters and barcode sequences, was empirically determined by gradient PCR using control reactions with initially purified bacterial genomic DNA, and validated on faecal microbial community DNA (data not shown). 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 (Claesson et al. 2009). Twenty five subjects were sequenced per sequencing run generating, on average, 41000 raw sequence reads per person. Raw sequencing reads were quality trimmed using a locally installed version of the Ribosomal Database Project (RDP) Pyrosequencing Pipeline applying the following criteria (Cole et al. 2009): (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 analyzed by BLAST (Altschul et al. 1990) against a previously published 16S rRNA gene sequence-specific database (Urich et al. 2008) using default parameters. The resulting BLAST output was parsed using MEGAN (Huson et al. 2007). 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 rRNA gene sequence data (Urich et al. 2008). Total number of pyrosequencing reads assigned to taxa in the 185 individuals ranged from 10368 to 26071. Counts for total bifidobacteria (genus level), lactobacilli (genus level) and Enterobacteriaceae (family level) were extracted from the MEGAN output of each subject.

Isolation and enumeration of Bifidobacterium spp. through bacteriological culture

The faecal sample for microbiological analysis was collected by the ELDERMET subject into a sterile container and stored at 4^{o}C until delivery to the laboratory; storage times varied from 3 to 193 hr and were on average 28 hr. One gram of the faecal sample was placed in a small Seward stomacher bag (VWR, Dublin, Ireland) and mixed well with 9 gram maximum recovery diluents

(MRD, Oxoid Ltd, Basingstoke, Hampshire, UK), prior to diluting further through ten-fold dilutions. To enumerate total culturable bifidobacteria, dilutions of the faeces were spread-plated (100 µl) onto de Man, Rogosa, Sharpe (MRS, Difco Laboratories, Detroit, MI, USA) agar supplemented with 0.05% (wt/vol) L-cysteine hydrochloride (Oxoid Ltd., Basingstoke, Hampshire, UK) (denoted mMRS agar) supplemented with 100 µg/ml mupirocin (Oxoid), 50 Units nystatin (Sigma) and 1.5 % (wt/vol) agar (Oxoid). The agar plates were incubated anaerobically (anaerobic jars with Anaerocult A gas packs, Merck, Darmstadt, Germany) at 37°C for 72 hr. Bacterial counts were recorded as colony forming units (CFU) per gram of faeces and data were expressed as log CFU *gram*$^{-1}$ *faeces.*

Isolation and enumeration of Lactobacillus spp. through bacteriological culture

To enumerate total culturable lactobacilli, the required dilutions of the faeces were spread-plated onto *Lactobacillus* selective agar (LBS, Becton Dickinson Co, Cockeysville, USA) with 50 Units nystatin. Agar plates were incubated anaerobically at 37°C for 5 days. Bacterial counts were recorded as described previously.

Isolation and enumeration of Enterobacteriaceae through bacteriological culture

To enumerate total culturable Enterobacteriaceae, the required dilutions of the faeces were pour-plated onto Violet Red Bile Glucose Agar (VRBG agar, Merck, Darmstadt, Germany). Agar plates were incubated semi-aerobically at 37°C overnight. Bacterial counts were recorded as described earlier.

Confirmation of bacterial strain identity

Bacterial isolates from the *Bifidobacterium*-selective and *Lactobacillus*-selective media were identified through sequencing. Putative lactobacilli and putative bifidobacteria were selected from a number of ELDERMET subjects. Lactobacilli were confirmed using the CO1 and CO2 universal

primers under conditions previously outlined (Simpson et al. 2003). The BIF-specific and 23S_bif primers were used to amplify the *Bifidobacterium* 16S and ITS spacer sequences, as previously outlined (Turroni et al. 2009). Sequence analysis was then carried out by Beckman Coulter (Essex, UK) with the CO1 and CO2 primers (for *Lactobacillus* (Simpson et al. 2003)) and the bif-sec and 23S-bif primers (for *Bifidobacterium* (Turroni et al. 2009)). Sequences obtained were used to identify the isolates using the NCBI BLAST database (http://blast.ncbi.nlm.nih.gov).

Statistical Methods

Statistical analyses were performed using Minitab Release 15.1.1.0 (Minitab Inc. 2007). The Spearman's rank correlation coefficient was used to estimate the relationship between the variables. Statistical significance was accepted at $P<0.05$.

2.4 Results

Bifidobacterium numbers enumerated by bacteriological culture ranged from <3.0 log CFU gram^{-1} faeces (limit of detection) to 10.7 log CFU gram^{-1} faeces, .The selective medium used to enumerate bifidobacteria proved effective, because when a selection of isolates (n=39) were identified by full length 16S rRNA gene sequencing, all were identified as *Bifidobacterium* spp. (data not shown). Other bacteria can grow on this medium but they can be distinguished, based on colony size on the selective medium (Simpson et al. 2004) or by microscopic examination (unpublished data). *Bifidobacterium* numbers established by pyrosequencing were expressed as a relative number of the total reads assigned to taxa (number of actual bifidobacteria reads counted ranged from 0 to 2153). These relative numbers ranged from 0 to 0.08 (mean = 0.005) for the 185 ELDERMET subjects whose faecal microbiota amplicons were sequenced. Based on Spearman's rank correlation coefficient (0.38) there was a significant correlation between data from pyrosequencing and the bacteriological culture (Figure 1a, P<0.001, Table 1).

Lactobacillus populations enumerated using bacteriological culture ranged from <3.0 log CFU gram^{-1} faeces (limit of detection) to 9.74 log CFU gram^{-1} faeces. The selective medium used to recover and enumerate lactobacilli proved effective as, when a selection of isolates (n=37) were identified through 16S rRNA gene sequencing all were identified as *Lactobacillus* spp. (data not shown). Using pyrosequencing, the levels of *Lactobacillus* spp. detected ranged from 0 to 0.01 (mean=0.0006; total reads of lactobacilli ranged from 0 to 1699). Using the Spearman's rank correlation coefficient (0.366) there was a significant correlation between the counts obtained from the pyrosequencing method and the counts from bacteriological culture (Figure 1b, P<0.001, Table 1).

Enterobacteriaceae numbers established by culture techniques ranged from <2.0 log CFU gram^{-1} faeces (limit of detection) to 10.0 log CFU gram^{-1} faeces. The relative quantities of

Enterobacteriaceae detected using pyrosequencing ranged from 0.0 to 0.11 (mean = 0.003; total reads of Enterobacteriaceae ranged from 0 to 4449). Based on the Spearman's rank correlation coefficient (0.437) there was a significant correlation between the counts obtained from the pyrosequencing methods and those from the culture technique (Figure 1c, $P<0.001$, Table 1).

2.5 Discussion

This study aimed to investigate the degree of correlation between culture dependent and culture independent methods for quantifying selected bacterial genera or families as part of the on-going ELDERMET study. Numbers of *Bifidobacterium* spp., *Lactobacillus* spp. and Enterobacteriaceae established by both methods were compared. Data from the amplicon pyrosequencing were assigned to genus level to determine read proportions for *Bifidobacterium* spp. and *Lactobacillus* spp. and to family level for the Enterobacteriaceae.

For the culture dependent results the average *Bifidobacterium* count (7.8 log CFU gram^{-1} faeces) are comparable to that observed in other studies of elderly subjects where faecal samples were assessed (Hopkins et al. 2001; Guigoz et al. 2002; Bouhnik et al. 2007; Schiffrin et al. 2007). The average *Lactobacillus* spp. count recorded (6.27 log CFU gram^{-1}) are comparable to counts from other studies of elderly subjects (Hopkins and Macfarlane 2002; Schiffrin et al. 2007; Stsepetova et al. 2011). Additionally taxonomic proportions elucidated through pyrosequencing in this study are consistent with those previously reported for the intestinal microbiota (Gill et al. 2006; Ley et al. 2006).

In this study populations of three bacterial groups were investigated, and these were chosen due to their probiotic potential (*Bifidobacterium* spp. and *Lactobacillus* spp.) and possible use as indicators of gut health (Enterobacteriaceae). While these groups are important; they represent a small proportion of the intestinal microbiota. The correlation demonstrated within these groups may not be transferable to other bacterial groups. Additionally we investigated one culture independent method, pyrosequencing, and a single selective medium for each bacterial group. If other methods such as qPCR, FISH, and phylogenetic arrays or different selective media were chosen this correlation may differ.

There are a number of factors which may affect the ability of pyrosequencing techniques to efficiently detect taxa. Firstly, universal primers used to detect all bacteria have been demonstrated to have a bias against high GC bacteria (Farris and Olson 2007). 16S rRNA genes are chosen due to their ubiquitous nature; however they may not have sufficient variability to assign taxonomy to genus level (Juste et al. 2008). Additionally 16S rRNA genes can exist in multiple copies in bacteria which may affect detection levels of these bacteria (Riesenfeld et al. 2004). In the case of *Bifidobacterium* spp. there is a low mean figure of 0.005 using pyrosequencing, relative to the culture dependent counts; this may be due to the fact that universal primers were used, as opposed to *Bifidobacterium* spp. specific primers. As previously discussed, universal primers may be biased against high GC content bacteria, in particular members of the phylum *Actinobacteria* (Farris and Olson 2007).

This investigation demonstrated that there is a correlation between culture dependent techniques and contemporary culture independent techniques, when enumerating *Bifidobacterium* spp., *Lactobacillus* spp. and Enterobacteriaceae (Spearman's rank correlation coefficients were 0.380, 0.366 and 0.437, respectively). It is important to emphasize that for bacterial species resident in low numbers in the gut, pyrosequencing may not always detect these species, while selective culturing methods may prove useful (Riesenfeld et al. 2004). This is clearly demonstrated in our study; where no counts were observed from pyrosequencing, but the culture method consistently returned a count (illustrated in Figure 1). Correlation will also depend on factors such as the selectivity of the culture medium, the ability of species to grow on the culture medium (unculturable or non-viable cells), and the efficiency of universal primers in identifying all members of a taxon. Whilst culture dependent techniques remain valuable for isolating bacterial species for further analysis (e.g. novel probiotic strains), the correlation between the culture dependent and independent techniques highlights that a single method is sufficient to enumerate selected major elements of the intestinal microbiota.

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Table 1: Spearman's rank correlation coefficient comparing culture techniques (log CFU gram^{-1} faeces) and results from pyrosequencing (relative number).

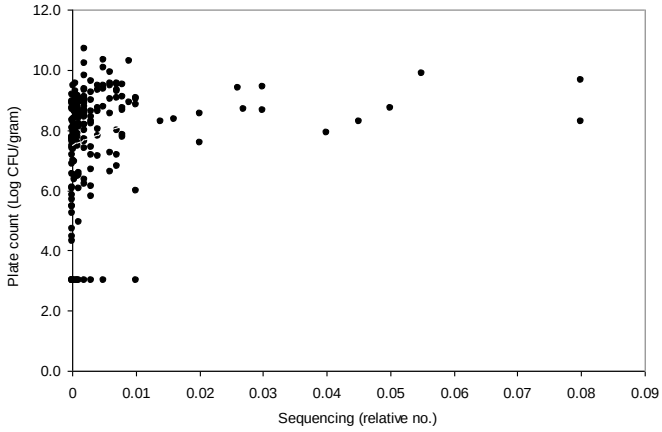
Group	***Bifidobacterium spp.***	***Lactobacillus spp.***	***Enterobacteriaceae***	***No. of subjects***
Total	0.38 (<0.001)*	0.366 (<0.001)	0.437 (<0.001)	185

*Spearman's rank correlation coefficient with p-value in brackets.

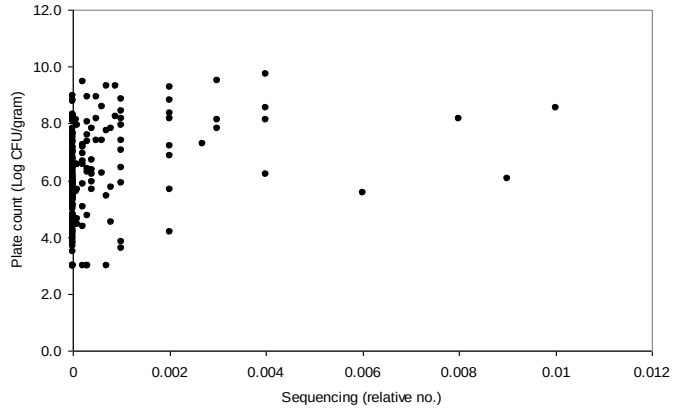
Figure Legends

Figure 1: Scatterplot of (a) *Bifidobacterium* spp., (b) *Lactobacillus* spp. and (c) Enterobacteriaceae counts from 185 datasets representing counts from culture methods (log CFU gram^{-1}) and from pyrosequencing methods (relative number) (P<0.001).

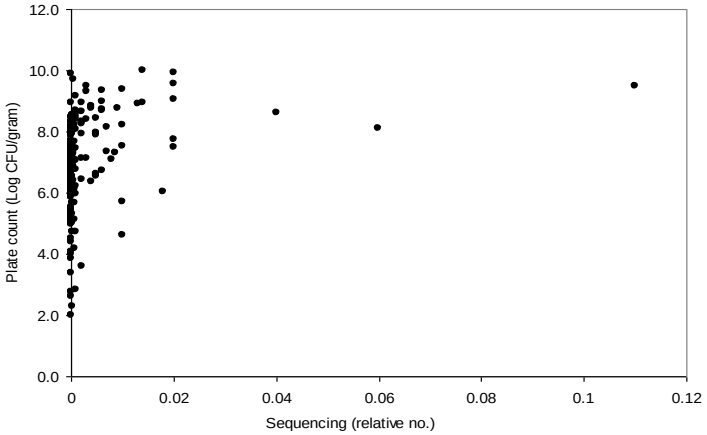
(a)



(b)



(c)



CHAPTER 2B

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

Bhuvaneswari Lakshminarayanan Chapter 4B contribution:

- Participated in the microbiological analysis including media preparation, isolation and microbial enumeration

- Collaborated with the authors to write the manuscript relating to the analysis of the microbial composition through culture dependent method

- Helped in compiling all graphical interpretations relating to the composition of bacteria and antibiotic therapy

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O. O'Sullivan, M. Coakley, **B. Lakshminarayanan,** S. Conde, M.J. Claesson, S. Cusack, A.P. Fitzgerald, P.W. O'Toole, C. Stanton, and R.P. Ross (2013)

2.8 Synopsis

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 yr) was investigated, taking into consideration their residence location.

Methods: Forty-two of the 185 elderly subjects were treated with at least one antibiotic within one 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 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 to untreated subjects (n=79). More alterations in the intestinal microbiota were observed post nucleic acid 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.

2.9 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 aging, even taking extensive inter-individual variation into account.[4,9,10] In general, Bacteroides[11-14] 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 aging 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.[22-25] Indeed, recent studies confirm the short and long-term impacts of antibiotic therapy on the human intestinal microbiota.[23,26] It has been demonstrated that antibiotic therapy reduces overall bacterial diversity, affecting up to 33% of the microbial population[5] and that it can also have an individualised effect on the intestinal microbiota.[27] More specifically, antibiotic therapy reduced *Bacteroides* numbers as well as altering the composition of the *Bacteroides* group[22] and reducing *Bifidobacterium* spp., *Desulfovibrio* spp., *Clostridium* spp. and *Faecalibacterium* spp. populations.[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[15] to 74.[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 *Bifidobacterium* spp., *Lactobacillus* spp. and Enterobacteriaceae in faecal samples, whilst culture independent 16S rRNA gene amplicon sequencing was employed to investigate the global intestinal microbiota. *Bifidobacterium* spp. and *Lactobacillus* spp. were investigated due to their presence in the gastro-intestinal tract being associated with positive health effects[29-32], and Enterobacteriaceae because they form a major component of the gut microbiota.[33-35] This study demonstrated that antibiotic therapy resulted in significantly reduced levels of culturable *Bifidobacterium* spp., as well as perturbing the relative abundance of 9 genera, including *Lactobacillus* spp., in the intestine of older subjects.

2.10 Materials and Methods

Subject Recruitment and Sample Collection

Subjects aged 65 years and older 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 institutionalised care (long-stay; > 6 weeks; n=48); (ii) short-term rehabilitation hospital care (rehabilitation; stay of < 6 weeks; n=24); (iii) occasionally attending an out-patient 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 one 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 as Supplementary data in Table S1). 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 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 inhibitors and cell envelope antibiotics and one subject received a combination of protein synthesis inhibitors and cell envelope antibiotics (Table S1, available as Supplementary data at JAC Online). A breakdown of the subjects by residence location and antibiotic treatment is represented in Table S2 (provided as Supplementary data).

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^{o}C until delivery to the laboratory. Storage times varied and were on average 28 h. Total culturable *Bifidobacterium* spp., *Lactobacillus* spp. and Enterobacteriaceae were enumerated as outlined previously.[36] Bacterial counts were recorded as colony forming units (cfu) per gram of faeces and results expressed as log cfu/gram faeces. The lower limit of detection for the culture technique was 3.0 log cfu/gram for *Bifidobacterium* spp. and *Lactobacillus* spp. and 2.0 log cfu/gram for Enterobacteriaceae.

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.[37] 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, (ii) no ambiguous bases (Ns), and (iii) read-lengths no shorter than 150 bp. Trimmed FASTA sequences were then BLASTed[38] against a previously published 16S-specific database[39] using default parameters. The resulting BLAST output was parsed using MEGAN.[40] MEGAN assigns reads to National Centre for Biotechnology Information (NCBI) taxonomies by employing the lowest common ancestor algorithm. Bit scores were used from within MEGAN to filter the results prior to tree construction and summarisation; a cut-off bit-score of 86 was implemented.[39] Phylum, family and genus counts for each subject were extracted from MEGAN. Operational taxonomic unit assignment (OTU), chimera-checking, clustering and alpha and beta diversities of reads were implemented with Qiime.[41]

Statistical Methods

Non-parametric statistical analyses (Mann Whitney, Chi-Square 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 FDR functions (phylum and family level) in the R statistical package (version 2.13.1) using the Benjamini & Hochberg method.[42]

2.11 Results

Relationship between antibiotic therapy and residence location

The elderly subjects were divided into four groups based on their residence location. Table S2 (provided as Supplementary data) 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-Square test statistic: χ^2_3 =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* spp. counts ranged from <3.00 to 10.70 log cfu/gram faeces for individual subjects. *Lactobacillus* spp. counts ranged from <3.00 to 9.70 log cfu/gram and Enterobacteriaceae levels similarly ranged from <2.00 to 10.00 log cfu/gram (Figure S1, provided as Supplementary data).

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/gram reducing to 7.15 log cfu/gram; 7-fold reduction post antibiotic therapy). *Lactobacillus* spp. and Enterobacteriaceae numbers recovered from the elderly faecal samples were not significantly different between antibiotic treated and untreated groups

(Figure S2). No correlation was established between the time since cessation of antibiotic therapy within the one month prior to attending the ELDERMET clinic and the counts recovered for culturable *Bifidobacterium* spp., *Lactobacillus* spp. or Enterobacteriaceae (data not shown). The effect of nucleic acid inhibitors (n=12), cell envelope antibiotics (n=10) and combinations of these 2 antibiotic types (n=11) on a selection of culturable bacteria was assessed. Subjects receiving only nucleic acid inhibitors had significantly lower levels of *Bifidobacterium* spp. (p=0.004, 23-fold decrease), as did subjects receiving a combination of the 2 antibiotic types (p=0.012, 13-fold reduction), whereas 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 to 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 1a. For subjects not receiving antibiotics (first 4 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/gram) and lowest in the long-stay group (6.27 log cfu/gram) (p=0.008). *Bifidobacterium* numbers were significantly lower for the long-stay (p=0.047) and day hospital (p=0.046) subjects receiving antibiotics, compared to 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 1b. The levels of *Lactobacillus* spp. detected were not significantly related 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 both long-stay ($p=0.015$) and community-dwelling subjects ($p=0.027$) 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 1c. 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 operational taxonomical unit (OTU) based on 97% identity and further assigned taxonomy (at the phylum, family and genus level) 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 to untreated subjects, both as a whole and when broken down by residence location, no significant changes were observed (Table S3, provided as Supplementary data). To investigate similarities or differences in the microbial composition between antibiotic treated and untreated subjects, principal coordinates analysis (PCoA) based on weighted-Unifrac beta 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 S3, provided as Supplementary data), but did confirm separation of microbiota based on residence location[43]. Also there was no clustering of subjects based on antibiotic type (Figure S4, provided as Supplementary data). Additionally, PCoA revealed no difference in the microbiota composition based on time since cessation of antibiotic therapy (Figure S5, provided as Supplementary data).

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 intestinal microbiota of the untreated group, while 58 genera were identified in the antibiotic treated group. Nine genera showed significant differences in abundance in the antibiotic treated subjects relative to the untreated subjects (Figure S6 and Table S4, both provided as Supplementary data). Read numbers corresponding to *Lactobacillus* spp. were significantly different ($p=0.031$), showing a relative increase in the antibiotic treated group (Figure 2c).

When considering subjects on a single antibiotic only, those subjects receiving a nucleic acid inhibitor had significant differences in the levels of *Anaerococcus* ($p=0.008$), *Denitrobacterium* ($p=0.008$), *Faecalibacterium* ($p<0.001$), *Lactinofactor* ($p<0.001$) and *Proteus* ($p=0.008$), when compared with the antibiotic untreated subjects. Those subjects receiving a cell envelope antibiotic had only 2 significant differences at the phylum level, Ascomycota ($p<0.001$) and Elusmicrobia ($p<0.001$). However, members of these phyla were found in only one antibiotic treated subject and in no antibiotic untreated subject. Subjects receiving a combination of nucleic acid inhibitors and cell envelope antibiotics had significantly different levels of Staphylococcaceae ($p<0.001$) at the

family level and *Staphylococcus* ($p<0.001$) and *Proteus* ($p<0.001$) at the genus level. However again, members of these taxa were only found in one antibiotic treated subject and in no antibiotic untreated subject.

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* ($p <0.001$), *Butyrvibrio* ($p=0.017$) 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, provided as Supplementary data).

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 to 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 to the untreated subjects. There were no significant differences, across all taxonomical levels, in the rehabilitation group.

2.12 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 less than one 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 to 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 hospitalised subjects receiving antibiotics (n=21) compared with those not in receipt of antibiotics (n=38).[13] 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*[11], who recorded a 100-fold increase in *Lactobacillus* spp. levels in antibiotic treated subjects (n=10) compared to those not receiving antibiotics (n=6).

The impact of the antibiotic types on the intestinal *Bifidobacterium* spp. revealed that the nucleic acid inhibitors had the most dramatic effect, with levels reduced following the administration of nucleic acid inhibitors (p=0.004, 23-fold decrease), and a combination of nucleic acid synthetic inhibitors and cell envelope antibiotics (p=0.012, 13-fold decrease). This suggests the effect of the antibiotic combinations was due to the nucleic acid synthetic inhibitors, as cell envelope antibiotics

did not impact culturable *Bifidobacterium* spp. numbers. Conversely, *Lactobacillus* spp. levels were only affected by cell envelope antibiotics ($p=0.047$, 10-fold increase).

Looking at the intestinal microbiota profiles, notably there was a decrease in *Faecalibacterium* populations in subjects treated with nucleic acid synthetic inhibitors only, compared to the antibiotic untreated group. *Faecalibacterium* spp. have been associated with a protective effect against ulcerative colitis[44] and colorectal cancer.[45] A decrease in the *Faecalibacterium* population as demonstrated here precludes such positive associations occurring in antibiotic treated older subjects. In general, cell envelope antibiotics appeared to have less impact on specific taxa of the intestinal microbiota than nucleic acid inhibitors.

The consequences of an altered intestinal microbiota post antibiotic therapy are not fully understood, but include increased carriage of antibiotic resistance genes[46-48], increased susceptibility to antibiotic associated diarrhoea[49], increased enteric infection[7] and an altered inflammatory response.[50,51] Therefore, while antibiotics remain an essential medical tool, more targeted anti-microbial therapies should be explored to prevent the intestinal dysbiosis, most notably the 7-fold reduction in *Bifidobacterium* spp. numbers observed in the current study, the long-term implication of which remains unknown. Furthermore, these data suggest that probiotic and/or prebiotic use, in particular targeting the *Bifidobacterium*-population, may be one approach to correct the altered intestinal microbial composition resulting from antibiotic therapy in the elderly.

2.13 Acknowledgements

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Madsen KL, Doyle JS, Tavernini MM et al. Antibiotic therapy attenuates colitis in interleukin 10 gene–deficient mice. *Gastroenterol* 2000; **118**: 1094-105.

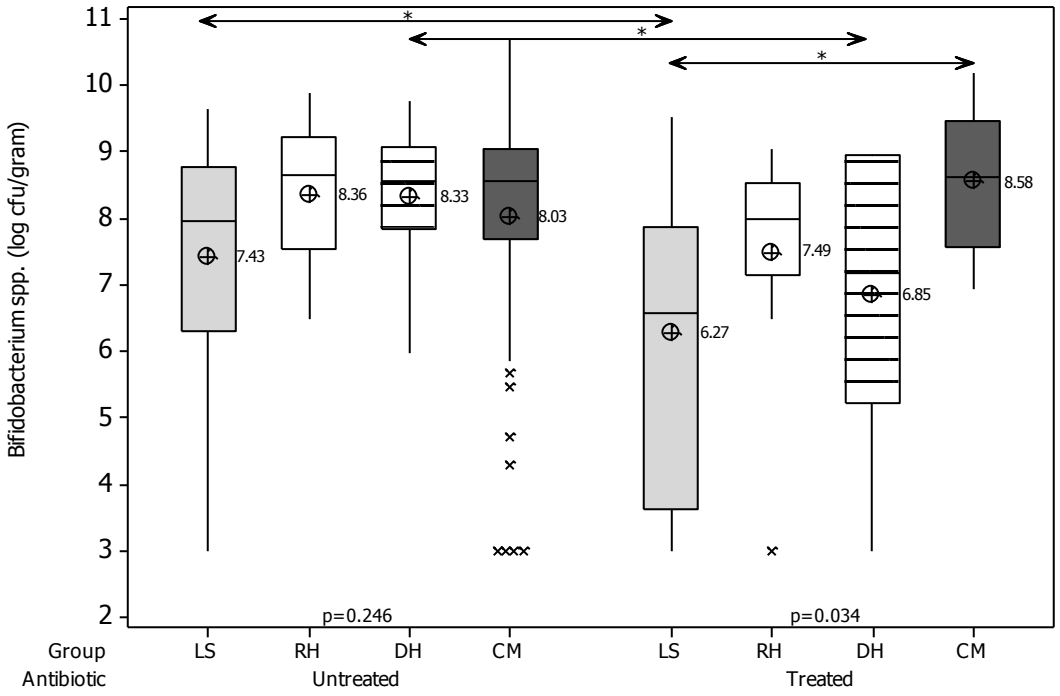
Table 1. Comparison of microbiota for antibiotic treated subjects versus antibiotic untreated subjects, grouped by residence location. Only significant differences are shown.

Residence Location	Organism	Significance (p-value)	Proportional change in antibiotic treated group
Long Stay	*Lachnospiraceae (f)*	<0.001	Decrease
Day hospital	*Blautia (g)*	<0.001	Decrease
Day hospital	*Faecalibacterium (g)*	<0.001	Decrease
Day hospital	*Lactobacillus (g)*	<0.001	Increase
Day hospital	*Mogibacterium (g)*	<0.001	Increase
Day hospital	*Moryella (g)*	<0.001	Increase
Day hospital	*Peptoniphilus (g)*	<0.001	Increase
Day hospital	*Victicallis (g)*	<0.001	Increase
Day hospital	*Weisella (g)*	<0.001	Increase
Community	*Bacteroidetes (p)*	0.020	Increase
Community	*Firmicutes (p)*	0.020	Decrease
Community	*Proteobacteria (p)*	0.009	Decrease
Community	*Euryarchaeota (p)*	0.009	Increase
Community	*Rhodocyclaceae (f)*	0.012	Increase

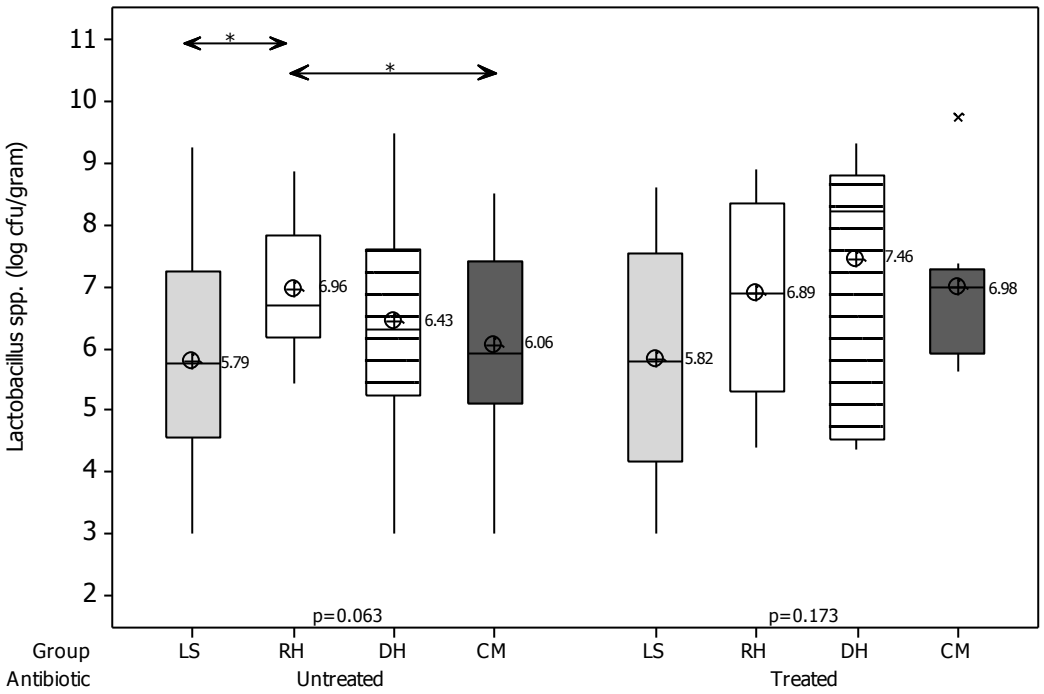
p=phylum level, f=family level, g=genus level

Figure 1: Levels of (a) *Bifidobacterium* spp., (b) *Lactobacillus* spp. and (c) Enterobacteriaceae enumerated (log cfu/gram faeces) from faecal samples from 185 elderly subjects, separated by antibiotic therapy (data for untreated subjects on left of the 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 enclosed cross = mean; letter cross = outliers; asterik = significant difference between residence locations.

(a)



(b)


Lactobacillus spp. (log cfu/gram)
11
10
9
8
7
6
5
4
3
2
*
*
5.79
6.96
6.43
6.06
5.82
6.89
7.46
6.98
p=0.063
p=0.173
Group
LS
RH
DH
CM
LS
RH
DH
CM
Antibiotic
Untreated
Treated

(c)

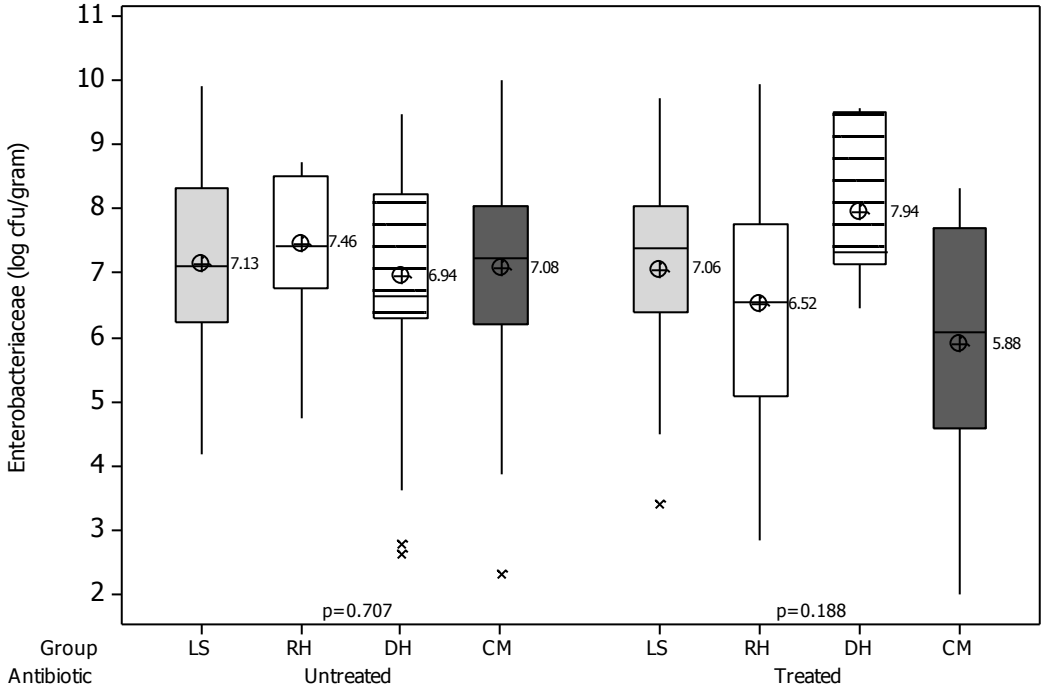
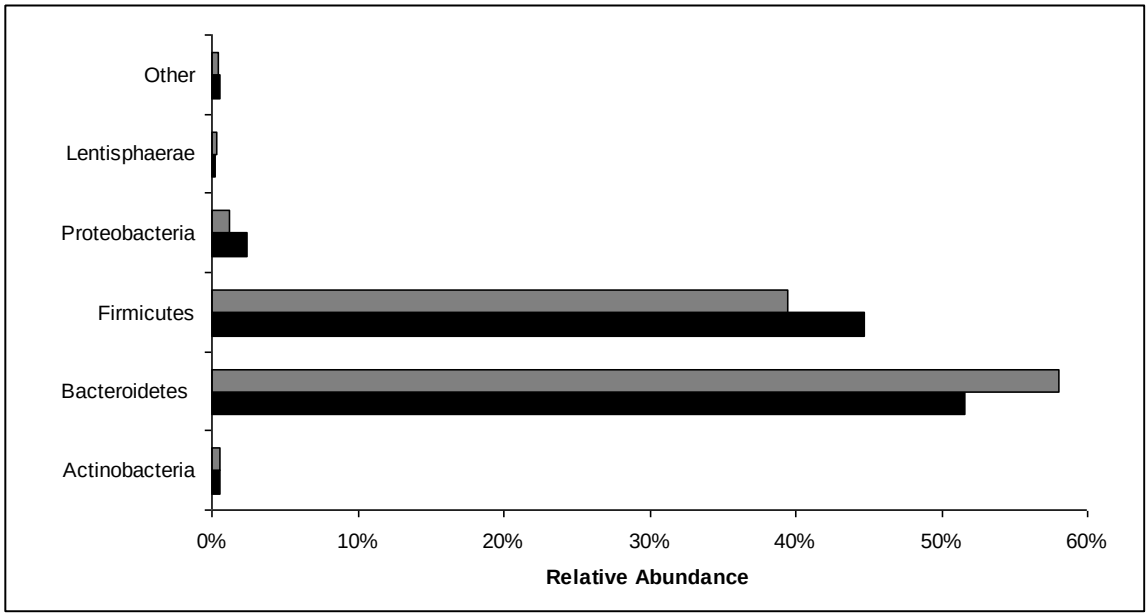
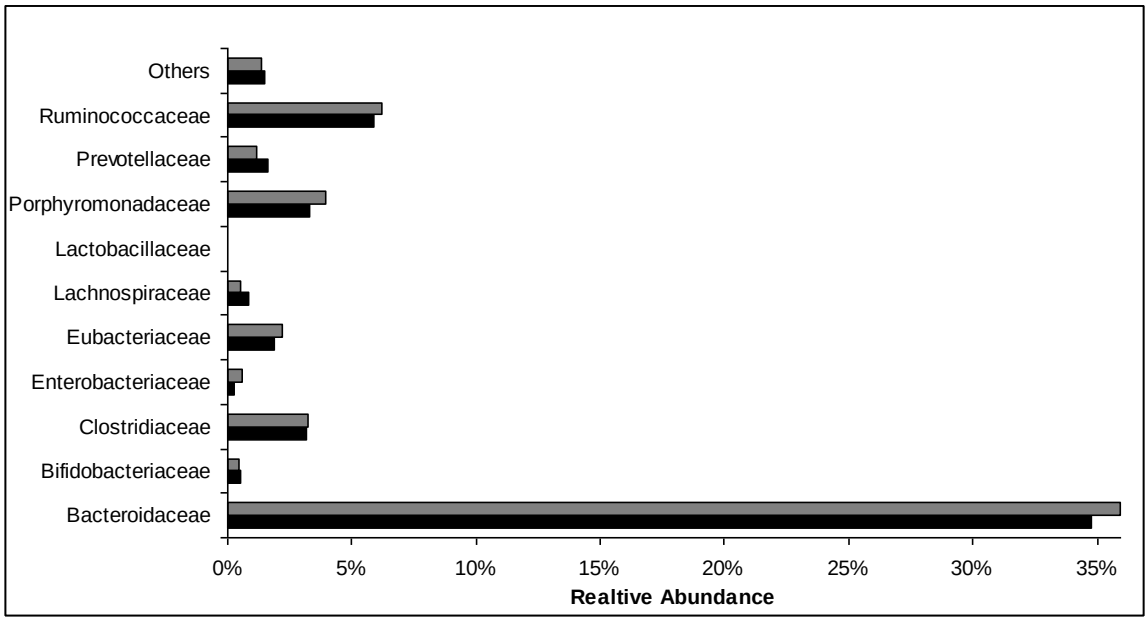

Enterobacteriaceae (log cfu/gram)
11
10
9
8
7
6
5
4
3
2
7.13
7.46
6.94
7.08
7.06
6.52
7.94
5.88
p=0.707
p=0.188
Group
LS
RH
DH
CM
LS
RH
DH
CM
Antibiotic
Untreated
Treated

Figure 2: Relative abundance in 42 antibiotic treated subjects (grey bars) and 143 antibiotic untreated subjects (black bars) at (a) phylum, (b) family and (c) genus level. Only major taxonomic groups are shown.

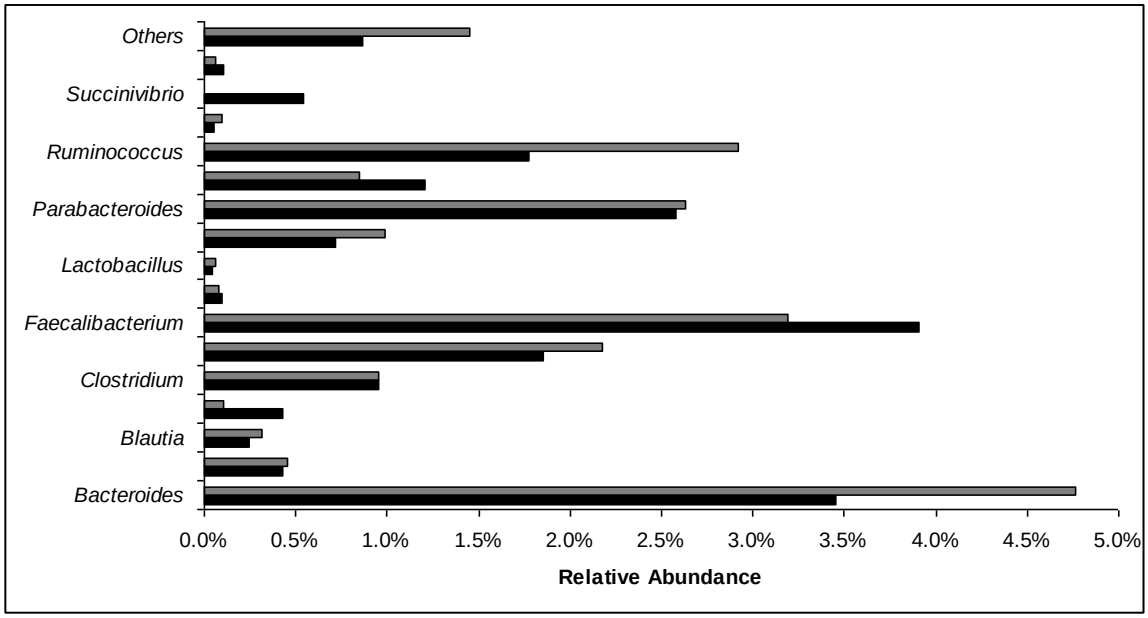
(a)



(b)



(c)


Others
Succinivibrio
Ruminococcus
Parabacteroides
Lactobacillus
Faecalibacterium
Clostridium
Blautia
Bacteroides
0.0%
0.5%
1.0%
1.5%
2.0%
2.5%
3.0%
3.5%
4.0%
4.5%
5.0%
Relative Abundance

SUPPLEMENTARY DATA

Supplementary Tables for Chapter 2B

Table S1. Antibiotics administered to 37 of the subjects in this study (administration details are unknown for 5 of the 42 subjects). Antibiotics are listed with the most recently administered antibiotic first, when the subject was treated with more than one antibiotic during this period.

Subject	Antibiotic name	Antibiotic type[a]
EM005[b]	Trimethoprim and Truoxin[c]	combination (nasi and cea)
EM031[b]	Macrodantin and Augmentin	combination (nasi and cea)
EM067	Amoxicillin	cell envelope antibiotic
EM068[b]	Ciprofloxacin	nucleic acid synthesis inhibitor
EM076	Augmentin	cell envelope antibiotic
EM079	Trimethoprim	nucleic acid synthesis inhibitor
EM080	Moxifloxacin	nucleic acid synthesis inhibitor
EM085	Augmentin duo	cell envelope antibiotic
EM101[b]	Trimethoprim and Cephradine[c]	combination (nasi and cea)
EM103	Clarithromycin	protein synthesis inhibitor
EM108	Clonamox	cell envelope antibiotic
EM110	Fluconazole	others
EM111[b]	Trimethoprim	nucleic acid synthesis inhibitor
EM112[b]	Amoxicillin, Tazocin, Flagyl and Ciprofloxacin	combination (nasi and cea)
EM114	Augmentin	cell envelope antibiotic
EM115	Augmentin and Klacid	combination (psi and cea)
EM117	Ciprofloxacin	nucleic acid synthesis inhibitor
EM124[b]	Tazocin and Flagyl	combination (nasi and cea)
EM125[b]	Augmentin	cell envelope antibiotic
EM126[b]	Nitrofurantoin	nucleic acid synthesis inhibitor
EM131[b]	Calvapen	cell envelope antibiotic
EM132	Augmentin and Ciprofloxcin	combination (nasi and cea)
EM133	Augmentin, Co-amoxicillin and Ciproxin	combination (nasi and cea)
EM135	Moxifloxacin	nucleic acid synthesis inhibitor
EM139	Ciproxin and Augmentin	combination (nasi and cea)
EM147	Tazocin	cell envelope antibiotic
EM149	Flucloxacillin	cell envelope antibiotic
EM160	Co-amoxicillin	cell envelope antibiotic
EM163[b]	Ciproxin	nucleic acid synthesis inhibitor
EM164[b]	Cephradine and Trimethoprim[c]	combination (nasi and cea)
EM165	Moxifloxacin	nucleic acid synthesis inhibitor
EM170[a]	Moxifloxacin	nucleic acid synthesis inhibitor
EM186	Trimethoprim and Augmentin	combination (nasi and cea)
EM200	Trimethoprim	nucleic acid synthesis inhibitor
EM222	Klacid	protein synthesis inhibitor
EM281	Nitrofurantoin	nucleic acid synthesis inhibitor
EM290[b]	Trimethoprim and Cephradine[c]	combination (nasi and cea)

[a]Antibiotics were classified as nucleic acid synthesis inhibitors, cell envelope antibiotics, protein synthesis inhibitors or others. Those on multiple antibiotics are listed as combination (nasi and cea) for those on a combination of .nucleic acid synthesis inhibitors and cell envelope antibiotics and combination (psi and cea) for those on a combination of protein synthesis inhibitors cell envelope antibiotics.

[b]These subjects were treated with antibiotics at the time of attendance at the ELDERMET Clinic (n=14).

[c]These two antibiotics were alternated on an on-going basis.

Table S2. χ^2 tabulation of residence location and antibiotic treatment data for elderly subjects studied (n=185).

Group	Antibiotic treatment[a]		Total no. of subjects
	No	***Yes***	
Long-stay	32 (66.7%)	16 (33.3%)	48
Rehabilitation	14 (58.3%)	10 (41.7%)	24
Day Hospital	33 (82.5%)	7 (17.5%)	40
Community	64 (87.7%)	9 (12.3%)	73
Total	***143 (77.3%)***	***42 (22.7%)***	***185***

[a]subjects administering antibiotics within one month prior to visiting the ELDERMET Clinic.

Table S3. Comparison of α diversity metrics for antibiotic untreated versus treated subjects as a whole and broken down by residence location. The top line of each section is the p-value and the bottom line is the mean value for antibiotic untreated / antibiotic treated subjects, respectively.

Diversity metric	All locations	Long-Stay	Rehabilitation	Day-Hospital	Community
Shannon	0.720 5.051/4.861	0.829 5.054/4.893	0.720 5.056/4.79	0.720 4.884/4.641	1.000 5.146/5.052
Simpson	0.720 0.923/0.912	1.000 0.920/0.905	0.720 0.923/0.918	0.750 0.918/0.913	1.000 0.925/0.918
Chao1	0.720 384.437/363.323	0.720 414.965/394.812	0.720 380.536/337.039	0.720 317.335/279.883	1.000 404.626/380.871
Phylogenetic diversity	0.720 22.352/20.619	0.720 23.053/21.949	0.720 22.067/19.167	0.720 20.203/17.660	1.000 23.172/22.171
Observed species	0.720 326.902/304.286	0.792 351.281/334.438	0.720 332.643/282.900	0.720 274.848/231.286	1.000 340.297/331.222

Table S4. Changes in the relative genus abundances in antibiotic treated versus untreated subjects. Only genera with significant changes are presented.

Genus	**Significance (p-value)**	**Proportional change in antibiotic treated group**
Acidaminococcus	0.045	decrease
Anaerococcus[a]	0.031	increase
Butyrvibrio	0.031	decrease
Lachnospira	0.045	decrease
Lactonifactor[a]	0.031	increase
Lactobacillus	0.031	increase
Megasphaera[a]	0.023	increase
Penicillium[a]	0.031	increase
Proteus[a]	0.031	increase

[a]present in low numbers of subjects.

Table S5. p-values from pair-wise comparison of significantly different genera broken down by residence location in the antibiotic untreated subjects.

Microbe	Long-stay vs. Day hospital	Long-stay vs. Rehabilitation	Long-stay vs. Community	Day hospital vs. Rehabilitation	Day hospital vs. Community	Rehabilitation vs. Community
Anaerofilum (g)	0.185	0.042[a]	0.021[a]	0.192	0.149	0.306
Anaerotruncus (g)	0.201	0.007[a]	<0.001[a]	0.016[a]	<0.001[a]	0.017[a]
Blautia (g)	0.221	0.042[a]	0.039[a]	0.192	0.194	0.333
Butyrvibrio (g)	0.127	0.205	0.059	0.077	0.031[a]	0.180
Coprobacillus (g)	0.149	0.042[a]	0.001[a]	0.333	0.062	0.042[a]
Coprococcus(g)	0.115	0.049[a]	0.039[a]	0.017[a]	0.017 [a]	0.356
Desulfovibrio (g)	0.117	0.234	0.017 [a]	0.192	0.326	0.043[a]
Eggerthella (g)	0.228	0.031[a]	0.017[a]	0.089	0.017[a]	0.218
Enterococcus (g)	0.356	0.142	0.017[a]	0.185	0.036[a]	0.142
Faecalibacterium (g)	0.195	0.001[a]	0.001[a]	0.042[a]	0.078	0.142
Oscillospira (g)	0.222	0.263	0.014[a]	0.142	0.017[a]	0.045[a]
Peptococcus (g)	0.042[a]	0.205	0.285	0.017[a]	0.042[a]	0.142
Porphyromonas (g)	0.234	0.132	0.089	0.080	0.050[a]	0.356
Prevotella (g)	0.283	0.042[a]	0.356	0.062	0.285	0.059
Spirochaeta (g)	0.248	0.039[a]	0.015[a]	0.042[a]	0.020[a]	0.201
Synergistes (g)	0.183	0.192	0.178	0.068	0.031[a]	0.317

[a]denotes significance; g=genus level

Supplementary Figures for Chapter 2B

Figure S1. Range of *Bifidobacterium* spp., *Lactobacillus* spp. and Enterobacteriaceae (log cfu/g) recovered from faecal samples of elderly human subjects (n=185; circle with enclosed cross = mean; cross = outlier).

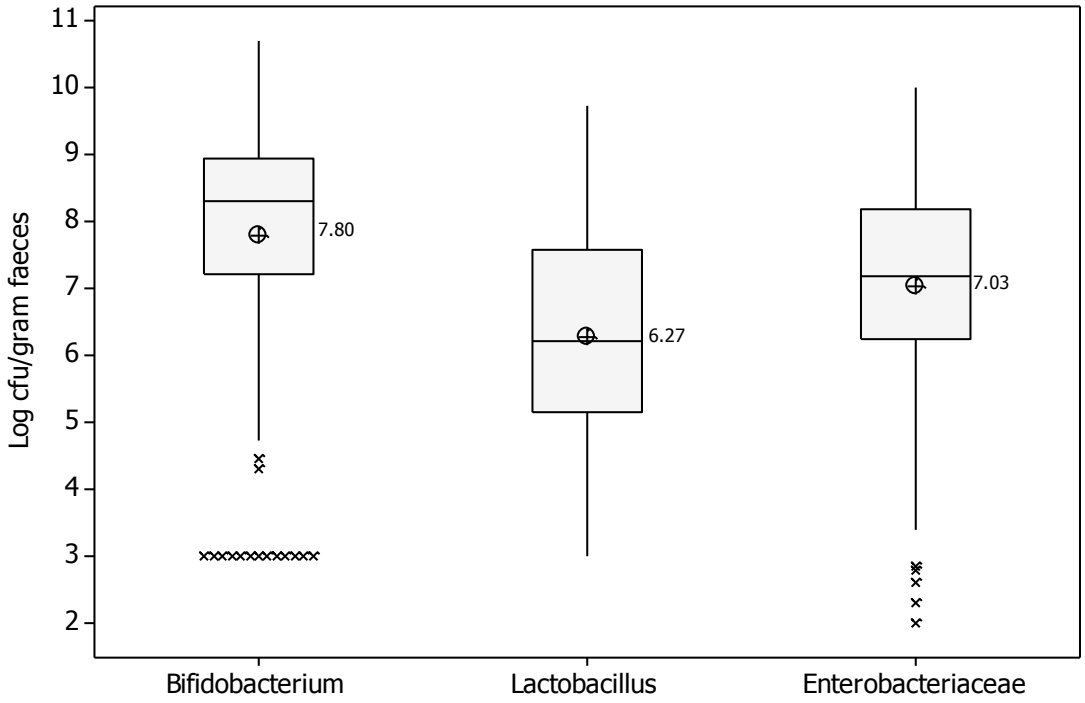


Figure S2. Box plot representation of the numbers of *Bifidobacterium* spp., *Lactobacillus* spp. and Enterobacteriaceae (log cfu/g) recovered from 185 faecal samples from subjects treated (n=42) and untreated (n=143) with antibiotics, independent of residence location (circle with enclosed cross = mean; cross = outlier).

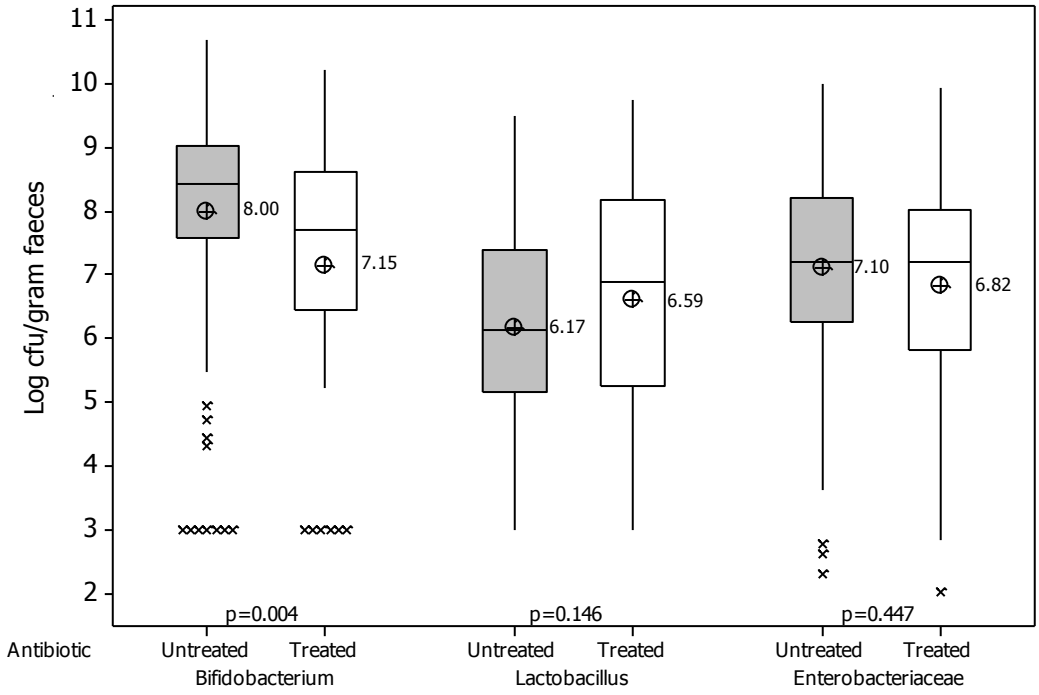


Figure S3. PCoA plots based on weighted–UniFrac distances for a) antibiotic treated (blue circle) and antibiotic untreated subjects (red circle), b) broken down by residence location: long-stay (light red circle: untreated, dark red circle: treated); rehabilitation (orange circle: untreated, brown circle: treated), day hospital (yellow circle: untreated, light yellow circle: treated) and community (dark green circle: untreated, light green circle: treated) and c) individual PCoA plots for each residence location (colour coding as before).

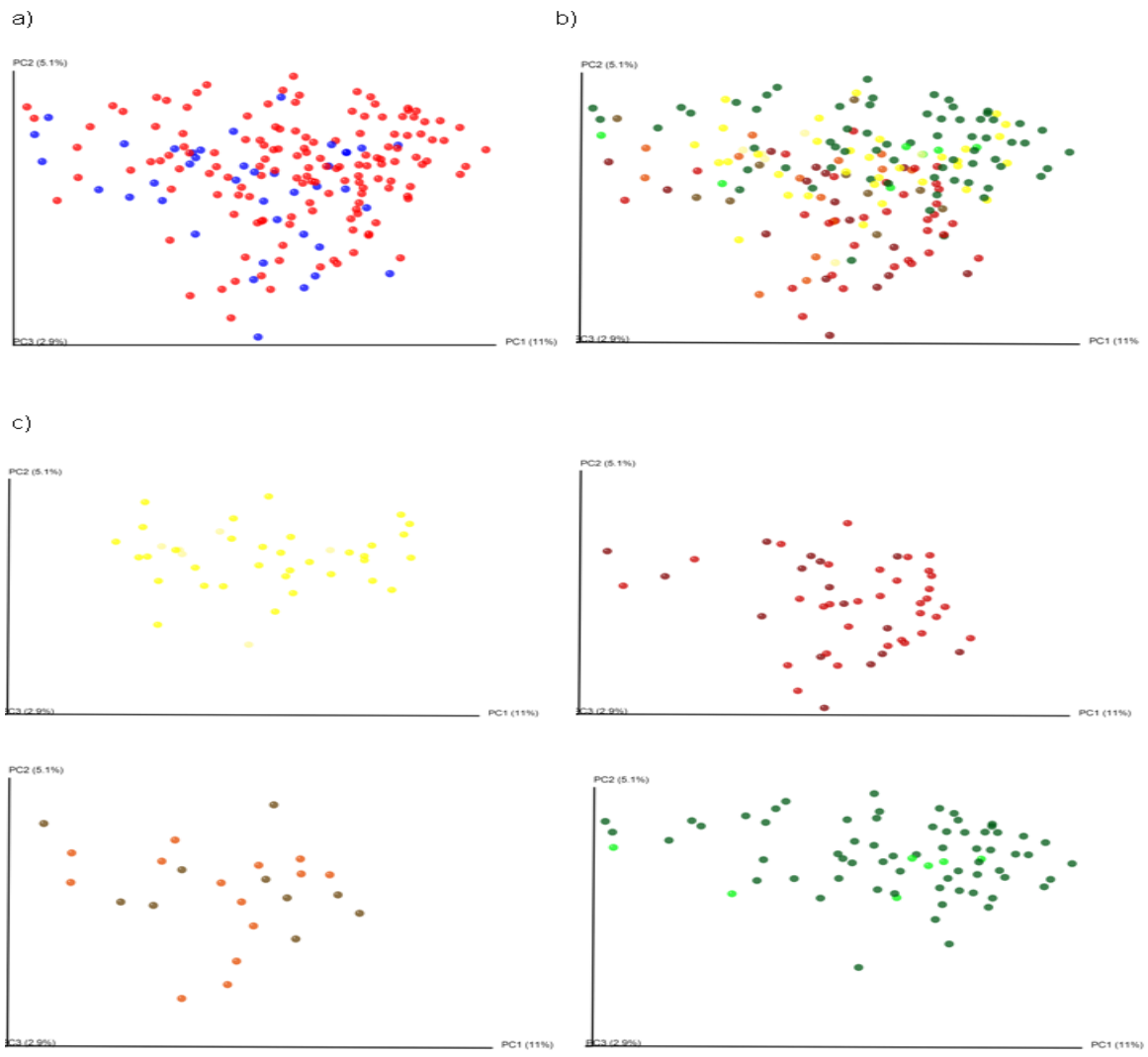


Figure S4. PCoA plots based on a) bray-curtis, b) unweighted–UniFrac and c) weighted UniFrac distances. Antibiotics are grouped into nucleic acid synthesis inhibitors (red), cell envelope antibiotics (blue), protein synthesis inhibitors (orange), others (purple) and combination of antibiotics (green).

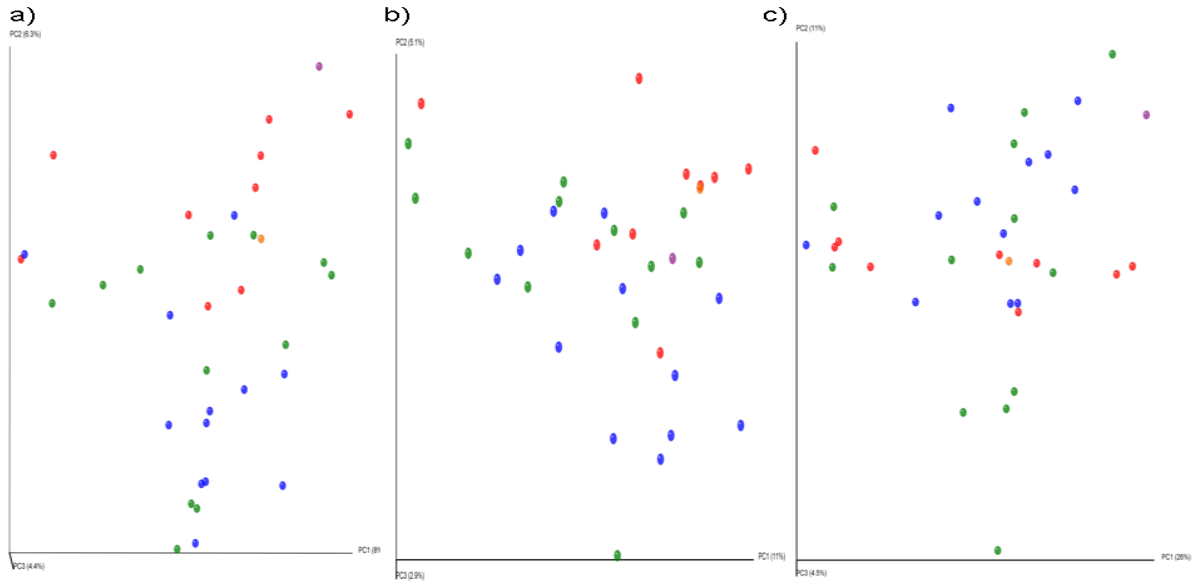


Figure S5. PCoA plot based on weighted-Unifrac β-diversity distance. Subjects are colour coded based on time since cessation of antibiotic therapy; currently on antibiotics (blue circle), ceased antibiotic therapy within: 1 week (orange circle), 2 weeks (green circle), 3 weeks (purple circle) and 4 weeks (yellow circle).

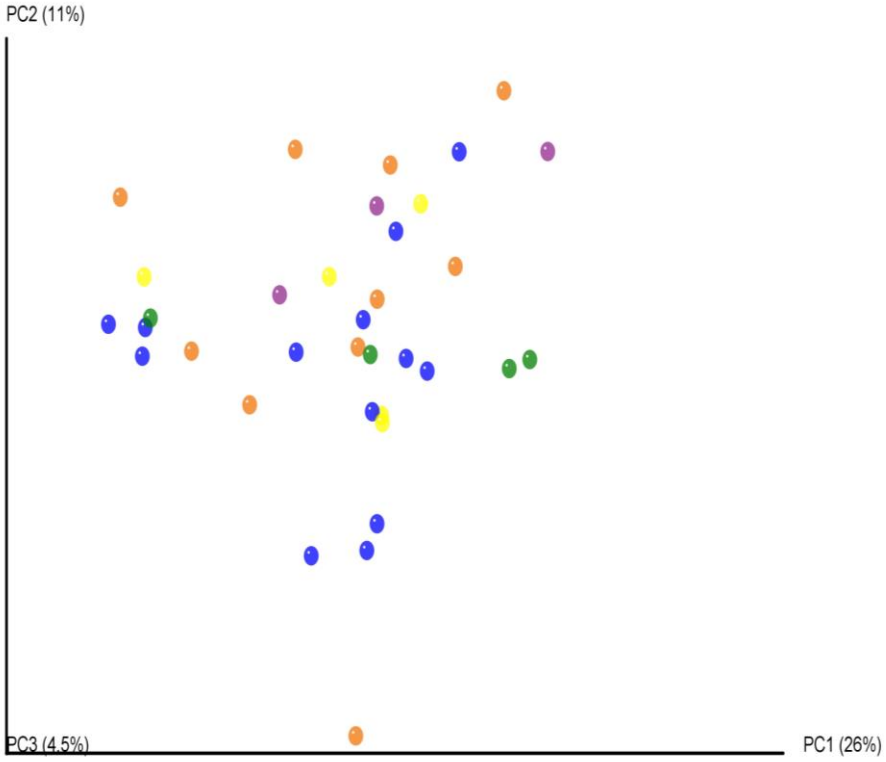
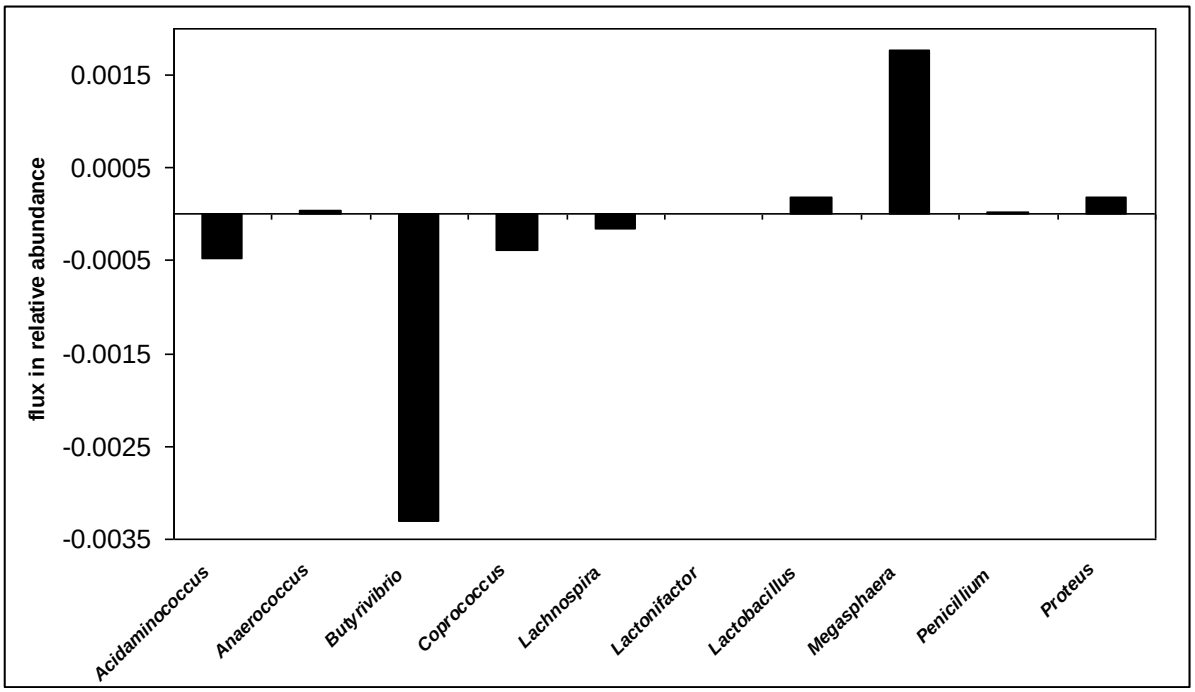


Figure S6. Relative change in the abundance of selected genera in antibiotic treated subjects relative to untreated subjects. Only genera with significant differences in population numbers are illustrated.



CHAPTER 3

TEMPORAL STABILITY OF CULTURABLE *BIFIDOBACTERIUM* SPP., *LACTOBACILLUS* SPP. AND ENTEROBACTERIACEAE IN ELDERLY IRISH SUBJECTS

This chapter will be combined with other ELDERMET research and submitted to

Letters in Applied Microbiology

3.1 Abstract

The intestinal microbiota plays a pivotal role in human health and well-being, with many health-promoting properties being associated with this community. Factors such as antibiotic therapy, aging and disease can negatively impact on this bacterial population. The temporal stability of this microbiota is thought to be of importance to host health and consequently maintenance of the balance of this bacterial community is desirable. Probiotic supplementation is one strategy commonly employed to contribute to a healthy intestinal environment. It is well known that ingestion of probiotic preparations consisting of bifidobacteria and/or lactobacilli have been associated with a number of positive health effects. For this reason, the temporal stability of culturable *Bifidobacterium* spp. and *Lactobacillus* spp. in faecal samples from 65 elderly subjects was monitored over a 6 month period. The levels of another important intestinal group, Enterobacteriaceae were also monitored over this period. The impact of probiotic intake, antibiotic therapy and residence location on these inhabitants of the GIT were also determined over time. Contrary to data that indicates that the microbiota of the elderly can be easily perturbed by extrinsic factors, this study demonstrates that the levels of the 3 bacterial groups analysed remained relatively stable over the 6 month period. Surprisingly, antibiotic administration did not result in perturbations in the counts of the 3 bacterial groups. Regular probiotic consumption resulted in a significant increase in *Bifidobacterium* spp. counts by month 6, while there were no alterations in the other two bacterial groups studied.

3.2 Introduction

The gastrointestinal tract (GIT) is inhabited by a complex microbial community known as the gut microbiota that is thought to have a profound impact on host health, immunity and nutrition (Guarner & Malagelada 2003; Sekirov *et al.* 2010; Tiihonen *et al.* 2010). At birth the GIT is initially sterile and is then rapidly colonised by bacteria of maternal origin and from environmental exposure (Palmer *et al.* 2007). Then, an ecological succession takes place within the human intestinal microbiota over the first few years of life, which after a period of relative flux converges into a complex adult pattern (Favier *et al.* 2002; Palmer *et al.* 2007; Koenig *et al.* 2011). Studies on the human gut microbiota have suggested that in the absence of perturbations, the microbial communities are relatively stable during adulthood (Zoetendal *et al.* 1998; Vanhoutte *et al.* 2004; Costello *et al.* 2009), yet the bacterial profiles are unique in that there is a lot of inter-individual variation (McCartney *et al.* 1996; Eckburg *et al.* 2005). Variations in the composition of the gut microbiota have been linked to the aging process (Hopkins *et al.* 2001; Woodmansey *et al.* 2004; Biagi *et al.* 2010). Indeed a number of extrinsic factors such as diet (De Filippo *et al.* 2010; Wu *et al.* 2011), geographic origin (Mueller *et al.* 2006; De Filippo *et al.* 2010), living environment (Bartosch *et al.* 2004; Claesson *et al.* 2012), antibiotic therapy and health conditions such as inflammatory bowel disease, obesity and colon cancer (Ley *et al.* 2006; Jernberg *et al.* 2007; Scanlan *et al.* 2008; Jeffery *et al.* 2012) can contribute to significant changes in the faecal bacterial communities in the elderly. Age-related changes in the gut microbiota composition include decreased species diversity and a decline in the abundance of intestinal *Bifidobacterium* spp. (Hopkins *et al.* 2001; Woodmansey 2007; Biagi *et al.* 2010; Claesson *et al.* 2012). In contrast, the population of *Lactobacillus* spp. remain stable or increase (Mitsuoka 1992; Hayashi *et al.* 2003; Mariat *et al.* 2009; Claesson *et al.* 2012).

Antibiotic use is often associated with adverse GI symptoms and can induce disturbances in the intestinal microbiota especially in hospitalized subjects (Bartosch *et al.* 2004; Young & Schmidt 2004; O'Sullivan *et al.* 2013). The impact of antibiotic treatment is also a factor in the prevalence of pathogenic bacteria such as *Clostridium difficile* (Rea *et al.* 2012). Recent studies have revealed that short and long term antibiotic administration can lead to ecological disturbances of the gut microbiota that can persist for up to two years (Jakobsson *et al.* 2010; Jernberg *et al.* 2010).

The balance and composition of the intestinal microbiota is important for the well-being of the host. Attempts to increase the natural resistance of the host to infection and minimize alterations in the bacterial community include the consumption of probiotics. Both *Lactobacillus* spp. and *Bifidobacterium* spp. are the key components of the gut microbiota and have received attention due to their associated health-promoting properties (Salminen *et al.* 1998; Rastall 2004; O'Flaherty & Klaenhammer 2010). They are commonly used as probiotic supplements and in that capacity they may restore the microbial balance that is negatively affected by aging. As such they may be considered as potential modulators of the gut microbiota and may be particularly effective in elderly populations (Ouwehand *et al.* 2002; Lahtinen *et al.* 2009; Guillemard *et al.* 2010; Namba *et al.* 2010).

The stability of the gut microbiota is known to change at certain key times in life such as early and late life, infection and inflammation. The temporal stability of this gut microbiota has been evaluated by repeated sampling of faecal communities over time, which suggests that the predominant bacterial community remains relatively stable and unique in healthy individuals over a prolonged period of time (Zoetendal *et al.* 1998; Vanhoutte *et al.* 2004). Culture independent methods during initial ELDERMET studies demonstrated temporal stability of the faecal microbiota at 3 months for the majority of subjects (85 %), while there was substantial inter-individual variation (Claesson *et al.* 2011). Using culture dependent

methods, this study aimed to investigate the temporal dynamics (0, 3 and 6 months) of a selection of bacteria (*Bifidobacterium* spp., *Lactobacillus* spp. and Enterobacteriaceae) in faecal samples from elderly Irish subjects (n=65) as impacted by antibiotic therapy, residence location and probiotic intake. Extremes of residence location (long-stay versus community) and probiotic intake (regular versus none) were compared. Some subjects ceased antibiotic therapy during the 6 month period and the impact of this on the 3 bacterial groups was also determined.

3.3 Materials and Methods

Subject recruitment and sample collection

Subjects aged 65 yr and older were recruited and examined at ELDERMET Clinics at two local hospitals (Cork University Hospital and St. Finbarr's Hospital, Cork) as previously outlined (Claesson *et al.* 2011). This study determined the levels of culturable *Bifidobacterium* spp., *Lactobacillus* spp. and Enterobacteriaceae at month 0, 3 and 6 (T0, T3 and T6) for 65 of these elderly subjects. Subjects were recruited from long-term institutionalised care (long-stay; more than 6 wk), short-term rehabilitation centers (rehab; under 6 wk stay), out-patient day hospitals and from the general elderly community. Residence locations and details of antibiotic therapy by the subjects are outlined in Table 1. Subjects receiving antibiotics within one month prior to visiting an ELDERMET Clinic were classified as antibiotic treated. In total 20 subjects received antibiotics at 1, 2 or all 3 time points. These subject were divided into 4 groups based on the type of antibiotic administered: Group 1 (cell envelope inhibitors (CE) at 1 time point), Group 2 (protein synthesis inhibitors (PSI) at 1 time point), Group 3 (nucleic acid synthesis inhibitors (NAI) at 1 or 2 time points) and Group 4 (combinations of antibiotics at 1 to 3 time points) (Table 2). Probiotic intake varied among the 65 subjects and details were unavailable for 20 subjects. Frequency of probiotic consumption was graded from never, to infrequent (max. 1 portion per month), to irregular (1 to 2 portions per week) and regular probiotic intake (from 0.7 to 2 portions per day) (Table 3).

Isolation and enumeration of bacteria by culture dependent methods

Stool samples were collected into sterile containers and stored at 5^{o}C until transported to the laboratory. *Bifidobacterium* spp., *Lactobacillus* spp. and Enterobacteriaceae were enumerated as outlined previously (O'Sullivan *et al.* 2011). Bacterial counts were recorded as colony forming units (CFU) per gram of faeces and results expressed as log CFU gram^{-1} faeces. The lower limit of detection for the culture technique was 3.0 log CFU gram^{-1} for *Bifidobacterium* spp. and *Lactobacillus* spp. and 2.0 log CFU gram^{-1} for Enterobacteriaceae.

Statistical Methods

Non-parametric statistical analyses (Mann Whitney, Chi-Square and Kruskal-Wallis tests) were applied using Minitab Release 15.1.1.0 (Minitab Inc. 2007), to determine the impact of time, antibiotic therapy or residence location of the individuals on the levels of bacteria recovered. Statistical significance was accepted at $P<0.05$.

3.4 Results and Discussion

During this study the effect of external factors such as antibiotic therapy, residence location and probiotic intake on the levels of culturable *Bifidobacterium* spp., *Lactobacillus* spp. and Enterobacteriaceae recovered from faecal samples of 65 elderly human subjects were assessed. We focused on these subjects as the bacterial counts were available at 3 times points over a 6 month period.

Temporal stability of Bifidobacterium spp., Lactobacillus spp. and Enterobacteriaceae

Compositional alterations that disturb the normal balance of the intestinal microbiota (dysbiosis) are partly considered responsible for adverse health conditions (Sekirov *et al.* 2010). Hence, ensuring appropriate balance and stability of the intestinal microbiota is considered an effective means of maintaining host health. In this study, we concentrated on *Bifidobacterium* spp. and *Lactobacillus* spp. as they are autochthonous members of the human gut microbiota and have been positively associated with a range of health-promoting effects (Kleerebezem & Vaughan 2009). Enterobacteriaceae were also investigated as they form a major component of gut microbiota and are used as indicators of hygiene and health (Lupp *et al.* 2007; Al-Mutairi 2011).

Culturable *Bifidobacterium* spp., *Lactobacillus* spp. and Enterobacteriaceae recovered from the faecal samples of elderly human subjects over time are presented in Figure 1. These bacterial counts are presented irrespective of residence location, antibiotic therapy or probiotic intake. There was a large inter-individual variation in the numbers of bacteria recovered from the 65 subjects. *Bifidobacterium* spp. counts ranged from <3.0 to 10.7 log CFU gram^{-1} faeces for individual subjects; *Lactobacillus* spp. counts ranged from <3.0 to 9.7 log CFU gram^{-1} and Enterobacteriaceae levels ranged from 2.3 to 10.0 log CFU gram^{-1} (Figure 1).

The numbers of *Bifidobacterium* spp., *Lactobacillus* spp. and Enterobacteriaceae remained stable over the 6 month period, with no significant difference observed between the counts at the 3 time points (*P* values for comparison of T0, T3 and T6 in Figure 1). Furthermore no significant differences were observed when 2 individual time points were compared (e.g. T0 compared to T3, T0 compared to T6, etc). This result reinforces a previous ELDERMET study, where Claesson *et al* observed using compositional sequencing that the faecal microbiota of elderly individuals was relatively stable over a 3 month period in the majority of the subjects. In this case it was noted that the microbiota composition of the elderly was characterised by higher inter-individual variation when compared to younger control subjects (Claesson *et al.* 2011).

Effect of antibiotic therapy on the temporal stability of Bifidobacterium spp., Lactobacillus spp. and Enterobacteriaceae

Antibiotic therapy is of fundamental importance to modern medicine and is pivotal in treating many infectious diseases. Both molecular and culture based techniques have revealed the perturbations and resilience of gut microbiota following antibiotic administration (De La Cochetiere *et al.* 2005; Jernberg *et al.* 2007; Dethlefsen & Relman 2011). In this study, no significant difference was observed between the levels of *Bifidobacterium spp., Lactobacillus* spp. or Enterobacteriaceae over the 6 month period when the impact of antibiotic therapy was considered i.e. in the antibiotic treated group or in the untreated group (sees Figure 2). In addition, there was no significant difference between the levels of *Bifidobacterium* spp., *Lactobacillus* spp. or Enterobacteriaceae between the antibiotic treated and untreated subjects at the corresponding time points. However, in a previous ELDERMET study, we observed that the *Bifidobacterium* spp. numbers were significantly reduced following antibiotic therapy (P=0.004) (O'Sullivan *et al.* 2013) . This difference may be due to a larger subject number in the initial study and thus more subjects on antibiotic therapy (42 of 185 subjects on

antibiotics (23 %) versus 7 to 9 of 65 subjects (average 12 %) on antibiotics). Even though there was no significant difference observed in the three bacterial groups studied, the levels of *Bifidobacterium* spp. and *Lactobacillus* spp. tended to be lower (albeit not significantly) for the subjects on antibiotic therapy compared to antibiotic untreated subjects (Figure 2).

Alterations to and recovery of the bacterial groups post antibiotic therapy

Even though antibiotic therapy as a whole did not affect the temporal stability of the 3 bacterial groups, the elderly subjects receiving antibiotics (n=20) were individually assessed based on antibiotic type (group 1 to 4) and antibiotic combination. In this study, 19 subjects were treated with various antibiotics and antibiotic combinations at 1 or 2 time points. Only one subject received antibiotics at all 3 time-points (EM101, Table 2). Antibiotic types and combinations included cell envelope inhibitors (Group 1; n=5), protein synthesis inhibitors (Group 2; n=4), nucleic acid synthesis inhibitors (Group 3; n=2) and combination of antibiotics (Group 4; n=7).

Figure 3(a) represents the subjects receiving only cell envelope inhibitors (Group 1). Subjects EM085 and EM185 were on antibiotic therapy prior to the study and at T0, but had ceased by month 3. In these cases, *Bifidobacterium* spp. levels increased slightly following completion of antibiotic therapy (0.5 log by month 3 for EM185 and 1 log by month 6 for EM085). Enterobacteriaceae levels increased by 0.5 log in both subjects when antibiotic treatment ceased (T3) but returned to the original level by T6. In subject EM085, the levels of *Lactobacillus* spp. decreased when antibiotic therapy ceased (2 log decrease by T3 and another 2 log decrease by T6) (Figure 3(a)). This reflects the observation that *Lactobacillus* spp. can increase with antibiotic therapy (O'Sullivan *et al.* 2013). In contrast, for subject EM137, when antibiotics were introduced at T3, the *Lactobacillus* spp. levels declined by 1 log and then increased again when antibiotic therapy ceased (T6). Levels of *Bifidobacterium*

spp. and Enterobacteriaceae in faecal microbiota of subject EM137 were not impacted immediately by antibiotic therapy, but levels had dropped by over 1 log by T6. Introducing antibiotics at T6 for subject EM096 did not impact levels of *Bifidobacterium* spp. *and Lactobacillus* spp.; however, Enterobacteriaceae declined by 2 logs. All bacterial groups assessed in subject EM181 decreased by the introduction of antibiotics at T6, with levels dropping over 100 fold in all cases.

In subjects on protein synthesis inhibitor therapy (PSI, Group 2) there was no major impact on the three bacterial groups, with the exception of subject EM059, where the counts of *Bifidobacterium* spp. decreased by up to 6 log following commencement of antibiotic therapy just prior to T3. *Bifidobacterium* spp. levels had increased slightly by T6 once antibiotic therapy ceased (Figure 3(b); Group 2). Of the two elderly subjects receiving nucleic acid inhibitors (Group 3), subject EM200 received antibiotics at only T0, whereas subject EM090 received antibiotics at T3 and T6. There was no noticeable impact of antibiotics on the bacterial classes in subject EM090 over time. However, for subject EM200, the levels of *Bifidobacterium* spp. *and Lactobacillus* spp. initially increased at T3 but then decreased by 1 to 2 log by T6 (Figure 3(c); Group 3). The impact of the combination of antibiotics (Group 4) was limited; of the 7 subjects, only 2 showed a 1 to 2 log decrease in bacterial counts, which eventually recovered ((Figure 3(d); Group 4).

Overall, in some subjects there was still an effect on bacterial counts up to 3 and 6 months following therapy, re-enforcing the long-term impact of antibiotics on the gut microbiota observed in other studies (Jernberg *et al.* 2007; Hussey *et al.* 2010). In some subjects, the recovery of the microbiota was slow and in some subjects it stabilized relatively quickly, suggesting that the resilience of the gut microbiota to antibiotic treatments varies considerably between individuals. Studies have reported bacterial resilience following antibiotic treatment (Dethlefsen *et al.* 2008; Antonopoulos *et al.* 2009; Jakobsson *et al.*

2010). A recent study by Dethlefsen *et al* outlined the response of distal gut communities to antibiotic administration across three individuals, where the gut community returned to a stable state similar, yet distinct to the pre-treatment state (Dethlefsen & Relman 2011). Similarly, our results suggest that the resilience of the microbiota can vary considerably across individuals and between the antibiotic treatment periods in the same individual.

Impact of residence location on the temporal stability of Bifidobacterium spp., Lactobacillus spp. and Enterobacteriaceae

The 65 elderly subjects resided in 4 different living environments and we compared the 2 more diverse residence locations (long-stay versus community). The long-stay subjects were resident in institutionalised care for greater than 6 weeks (n=21 at all-time points) and the community subjects were members of the general public living in the community (n=35, 36 and 36 at T0, T3 and T6, respectively). To analyse the effect of residence location on *Bifidobacterium* spp., *Lactobacillus* spp. and Enterobacteriaceae, the bacterial counts were also divided based on antibiotic therapy, as it was demonstrated previously that the levels of culturable *Bifidobacterium* spp. are affected by antibiotic therapy (O'Sullivan *et al.* 2013).

Statistical analysis was not carried out on the antibiotic treated group as the sample numbers were limited (n=4, 5 and 2 for the for long-stay subjects at T0, T3 and T6 respectively and n=3 for community subjects at all time-points). However, the culture results for these antibiotic treated subjects are graphically represented in Figure 4(b), (d) and (f). Enterobacteriaceae and *Bifidobacterium* spp. levels were maintained over 6 months in the long-stay and community groups on antibiotic therapy, but levels of *Lactobacillus* spp. were found to decline as time progressed (in both stratifications).

The levels of *Bifidobacterium* spp., *Lactobacillus* spp. and Enterobacteriaceae in the antibiotic untreated groups in both residence locations were maintained over the 6 month

period (*P* values detailed in Figure 4(a), (c) and (e)). When the residence locations were compared at the individual time-points, *Bifidobacterium* spp. were significantly higher in the community compared to the long-stay residence location at T0 (P=0.047). This significant difference was maintained at T3 (P=0.020) and T6 (P=0.002). This result was in agreement with Bartosch *et al.,* 2004 who reported that the elderly living in the community had higher levels of faecal *Bifidobacterium* spp. than hospitalised subjects (Bartosch *et al.* 2004). *Lactobacillus* spp. and Enterobacteriaceae levels were not significantly different at any time point, when the two residence locations were compared.

Effect of probiotic intake on the temporal stability of Bifidobacterium spp., Lactobacillus spp. and Enterobacteriaceae

The evidence for the use of probiotics to maintain health and well-being in the elderly is growing and has been shown to cover a wide range of diseases and ailments (Fujimura *et al.* 2010; Aureli *et al.* 2011; Gerritsen *et al.* 2011). The health benefits derived by the consumption of foods containing probiotic bacteria are well documented (Gill *et al.* 2001; Ahmed *et al.* 2007), and most probiotics belong to the two genera *Bifidobacterium* spp. and *Lactobacillus* spp., which are generally regarded as safe (Ventura *et al.* 2008; Kleerebezem & Vaughan 2009; Kim *et al.* 2013). In the last 10 years, there has been an increasing interest in the consumption of probiotics and functional foods. Probiotic bacteria are known to suppress potentially pathogenic microorganisms in the gastrointestinal tract and enhance the population of beneficial microorganisms (Culligan *et al.* 2009; Gerritsen *et al.* 2011).

Levels of culturable *Bifidobacterium* spp., *Lactobacillus* spp. and Enterobacteriaceae were enumerated to establish whether regular probiotic intake had an effect on the numbers of bacteria detected in faecal samples from elderly subjects. The food frequency questionnaire (FFQ) enabled the elderly subjects to document whether they consumed a probiotic-based

drink or food. Details were unavailable for some of the 65 elderly subjects in this study (Table 3). Levels of culturable *Bifidobacterium* spp., *Lactobacillus* spp. and Enterobacteriaceae in faecal samples from subjects that regularly consumed a probiotics-based product at all 3 time points (T0, T3 and T6; n=5) were compared to subjects that did not consume probiotics at any time point (n=26). Statistical analysis (P>0.05 for T0, T3 and T6 on Figure 5) indicated no significant difference between the subject groups. When the levels of the three bacterial groups were compared with respect to probiotic intake, only *Bifidobacterium* spp. numbers at month 6 were significantly higher (P=0.012) in the group with regular probiotic consumption (8.85 compared to 7.58 log CFU gram^{-1}). Although statistically there was no difference in the count of *Bifidobacterium* spp. observed at T0, (P=0.248) and T3 (P=0.126), an increase in counts was observed (Figure 5(a)). Similarly, the average numbers of culturable *Lactobacillus* spp. in the subjects on probiotics were higher than the subjects not on probiotics, but there was no statistical difference (P>0.05).

The study showed that the bacterial counts remained stable over the 6 month period for the groups assessed in all subjects, irrespective of probiotic intake. This stability of the gut microbiota was in contrast to the observed response of the gut microbiota of both elderly and infants fed with probiotics, in which the gut microbiota composition was considerably affected by probiotics administration (Lahtinen *et al.* 2009; Cox *et al.* 2010). Indeed, in a short-term dietary intervention study, controlled feeding of the probiotic diet over 10 days also showed a rapid and marked change in the microbiome composition within a day of the intervention (Wu *et al.* 2011).

Overall, monitoring the stability of specific key members of the human gut bacterial community from 65 elderly subjects over a 6 month time period demonstrated the temporal stability of 3 bacterial groups (*Bifidobacterium* spp., *Lactobacillus* spp. and Enterobacteriaceae). The population of these bacterial communities fluctuated unpredictably

with antibiotic usage, wherein the microbiota of some subjects recovered slowly and others reached a stable state relatively quick, suggesting that the resilience of the gut microbiota varied among the subjects. Studies have examined variations in the faecal microbiota using high-throughput sequencing technologies (Caporaso *et al.* 2010; Dethlefsen & Relman 2011) and have shown that despite fluctuations in the microbiota composition over time and shifts due to disturbances, the overall bacterial community is maintained in the longer term (Turnbaugh *et al.* 2008; Dethlefsen & Relman 2011).

3.5 Conclusion

The ability of the human gut microbiota to remain stable is thought to be an important factor to maintain the host health. In this respect, it was found that microbial diversity was inversely related to health, based on a number of physiological and biological parameters. In this study, we monitored the stability of specific key members of the human gut bacterial community (*Bifidobacterium* spp., *Lactobacillus* spp. and Enterobacteriaceae) from 65 elderly subjects over a 6 month time period and demonstrated the temporal stability of these 3 bacterial groups. The administration of antibiotics did not significantly alter the temporal stability of the bacterial communities. However, the population of these bacterial communities fluctuated unpredictably with the use of different antibiotic types and the resilience of the gut microbiota varied between subjects. Regular probiotic intake by the elderly subjects demonstrated a significant increase in the counts of *Bifidobacterium* spp. at only one time point (month 6). In contrast, there was no significant effect on culturable *Lactobacillus* spp. and Enterobacteriaceae due to regular probiotic consumption. One of the major limitations of this study was the lack of molecular characterisation of the isolates to strain level, where undoubtedly even larger changes in the microbial population could have occurred. Further longitudinal studies assessing the bacterial species composition and diversity, and function of the gut microbiota in these elderly subjects may help to better understand the activity and the contribution of the microbiota to human health and well-being.

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Table 1: Residence location and details of antibiotic therapy for 65 elderly subjects at month 0, 3 and 6

Residence location	**Time (month)**		
	0	**3**	**6**
Long-stay	21 (4)*	21 (5)	21 (2)
Rehab	4 (2)	5 (1)	4 (2)
Day Hospital	5 (0)	3 (0)	4 (0)
Community	35 (3)	36 (3)	36 (3)
Total	65 (9)	65 (9)	65 (7)

*number of subjects on antibiotic therapy in each residence location is outlined in brackets.

Table 2: Antibiotics administered to 20 elderly subjects in this study. Antibiotics were classified as nucleic acid inhibitors (NAI), cell envelope antibiotics (CE), protein synthesis inhibitors (PSI) or others. Groups were defined by the type of antibiotic administered - Group 1: CE at 1 time point; Group 2: PSI at 1 time point; Group 3: NAI at 1 or 2 time points and Group 4: combination of antibiotics at 1, 2 or 3 time points.

Subject	Group	T0	T3	T6
EM085	1	CE	none	none
EM096	1	none	none	CE
EM137	1	none	CE	none
EM181	1	none	none	CE
EM185	1	CE	none	none
EM047	2	none	PSI	none
EM053	2	none	PSI	none
EM059	2	none	PSI	none
EM118	2	none	none	PSI
EM090	3	none	NAI	NAI
EM200	3	NAI	none	none
EM101	4	CE, NAI & CE	CE & NAI	CE & NAI
EM110	4	CE, NAI, PSI & others	none	none
EM126	4	NAI	none	CE
EM133	4	CE, CE & NAI	none	none
EM135	4	NAI, NAI & CE	none	none
EM166	4	CE, CE, NAI, CE & NAI	none	none
EM169	4	none	NAI & CE	NAI
EM159	NA*	none	others	none
EM168	NA	none	others	none

*NA – not applicable.

Table 3: Probiotic intake by 65 elderly subjects over time.

Frequency of probiotic consumption	**Time (month)**		
	0	**3**	**6**
Never	31	40	38
Infrequent (max. 1 portion/month)	9	13	10
Irregular (1- 2 portions/week)	2	3	4
Regular (0.7-2 portions/day)	6	8	9
Data not available	17	1	4

Figure 1: Culturable *Bifidobacterium* spp., *Lactobacillus* spp. and Enterobacteriaceae (log CFU gram^{-1}) recovered from faecal samples from elderly subjects over time (0 (□), 3 (▒) and 6 (■) months) (n=65; ⊕ = mean; × = outliers).

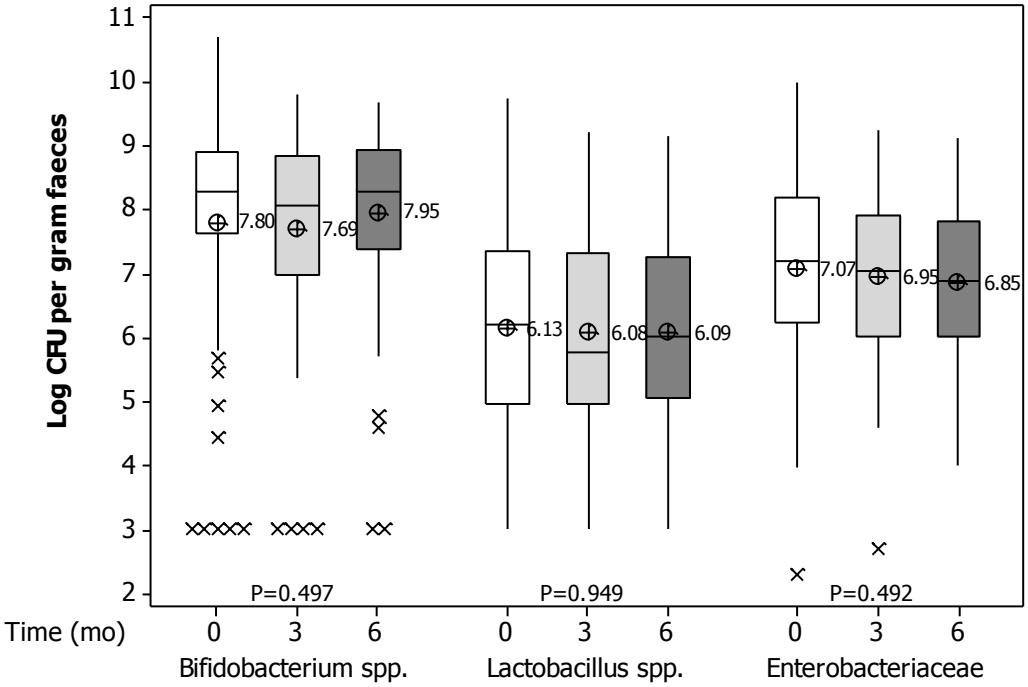
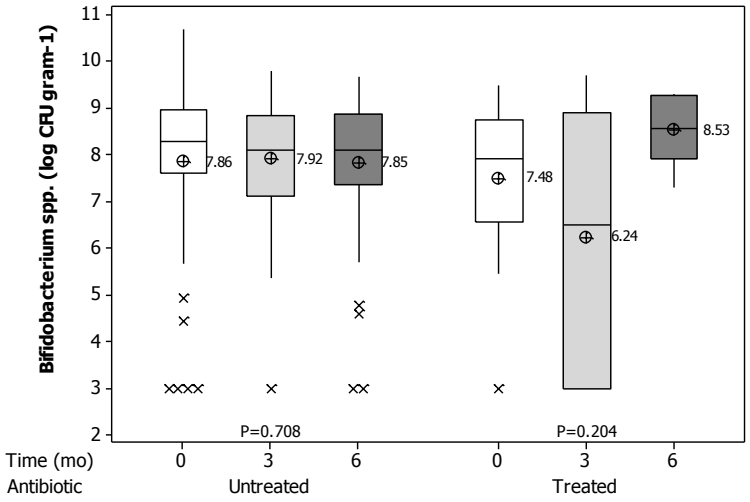
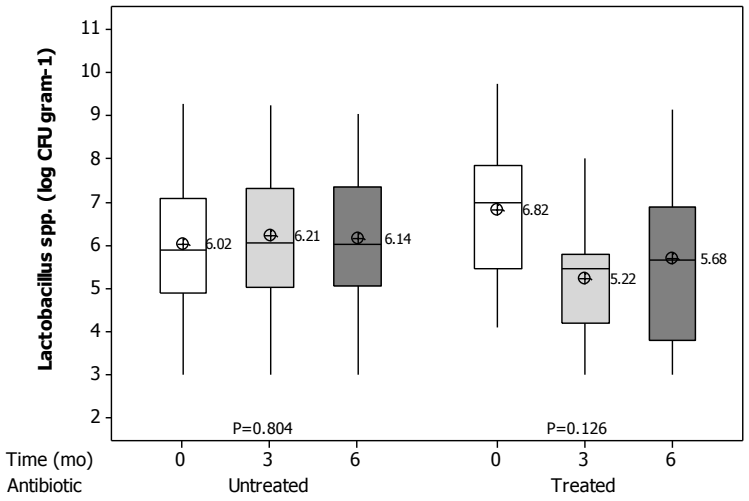


Figure 2: Box plot representation of the numbers of (a) *Bifidobacterium* spp., (b) *Lactobacillus* spp. and (c) Enterobacteriaceae (log CFU $gram^{-1}$) recovered from faecal samples from 65 elderly subjects treated and untreated with antibiotics at T0 (□; n=9 and n=56, respectively), T3 (■; n=9 and n=56, respectively) and T6 (■; n=7 and n=58, respectively), independent of residence location (⊕ = mean; × = outliers).

(a)


Bifidobacterium spp. (log CFU gram-1)
11
10
9
8
7
6
5
4
3
2
7.86
7.92
7.85
7.48
6.24
8.53
P=0.708
P=0.204
Time (mo)
0
3
6
0
3
6
Antibiotic
Untreated
Treated

(b)


Lactobacillus spp. (log CFU gram-1)
11
10
9
8
7
6
5
4
3
2
6.02
6.21
6.14
6.82
5.22
5.68
P=0.804
P=0.126
Time (mo)
0
3
6
0
3
6
Antibiotic
Untreated
Treated

(c)

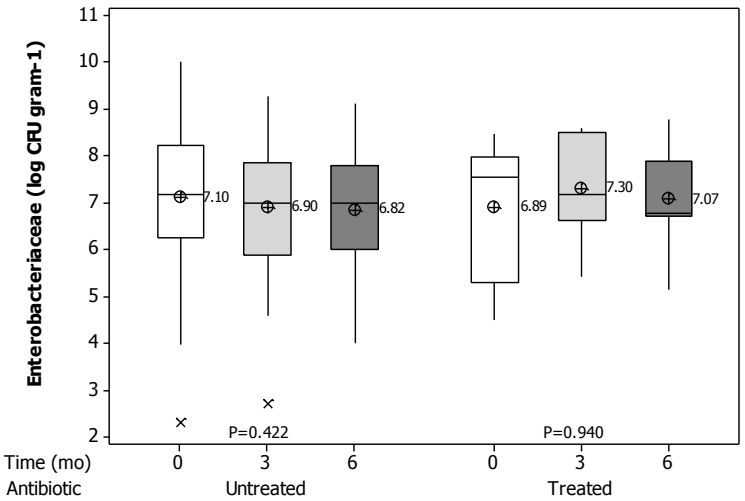
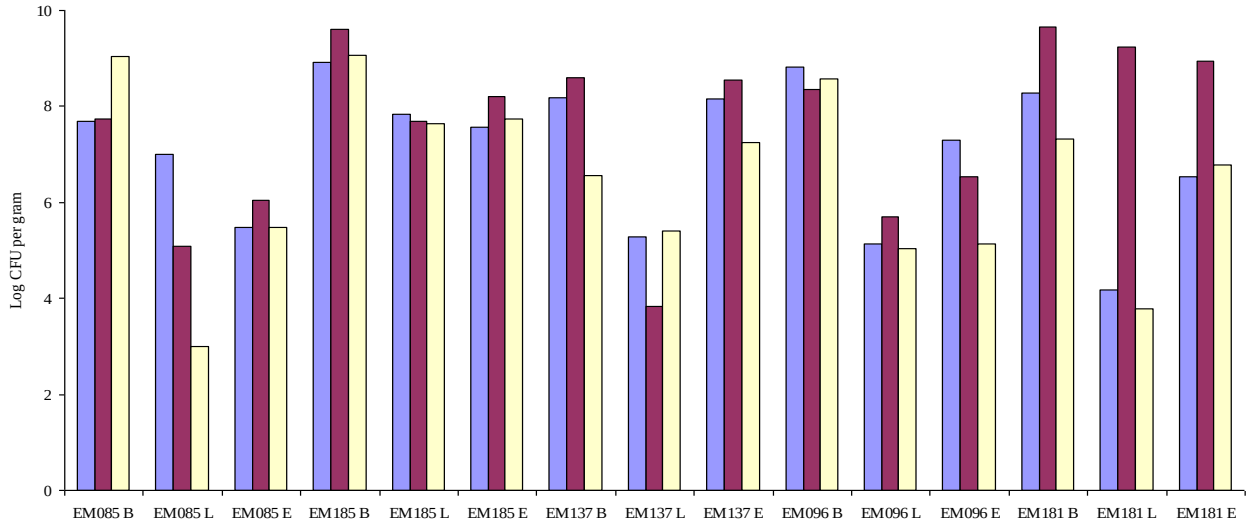
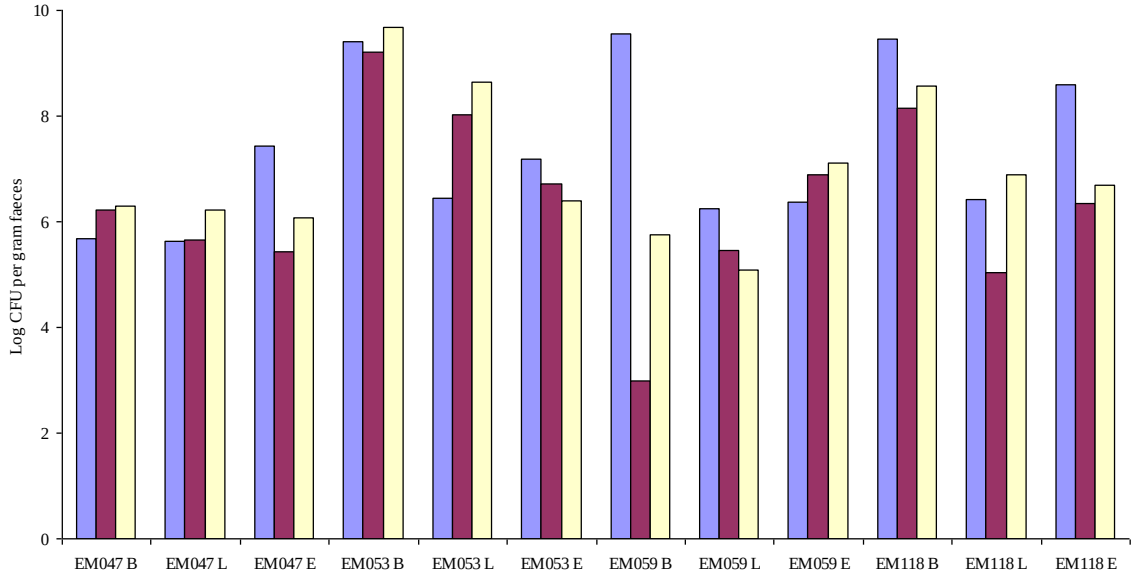

Enterobacteriaceae (log CFU gram-1)
11
10
9
8
7
6
5
4
3
2
7.10
6.90
6.82
6.89
7.30
7.07
P=0.422
P=0.940
Time (mo)
0
3
6
0
3
6
Antibiotic
Untreated
Treated

Figure 3: Levels of *Bifidobacterium* spp. (B), *Lactobacillus* spp. (L) and Enterobacteriaceae (E) in faecal samples from elderly subjects on antibiotic therapy at one or more time point. Subjects are divided into (a) Group 1: treatment with cell envelope (CE) antibiotics at one time point (EM085 at T0, EM185 at T0, EM137 at T3, EM096 at T6 and EM181 at T6); (b) Group 2: treatment with protein synthesis inhibitors (PSI) at one time point (EM047 at T3, EM053 at T3, EM059 at T3 and EM118 at T6); (c) Group 3: treatment with nucleic acid synthesis inhibitors (NAI) at one or two time points (EM200 at T0 and EM090 at T3 and T6) and (d) Group 4: treatment with a combination of antibiotics at one or more time points (EM110 at T0, EM133 at T0, EM135 at T0, EM166 at T0, EM126 at T0 and T6, EM169 at T3 and T6 and EM101 at T0, T3 and T6). Counts were recorded at month 0 (■), month 3 (■) and month 6 (■).

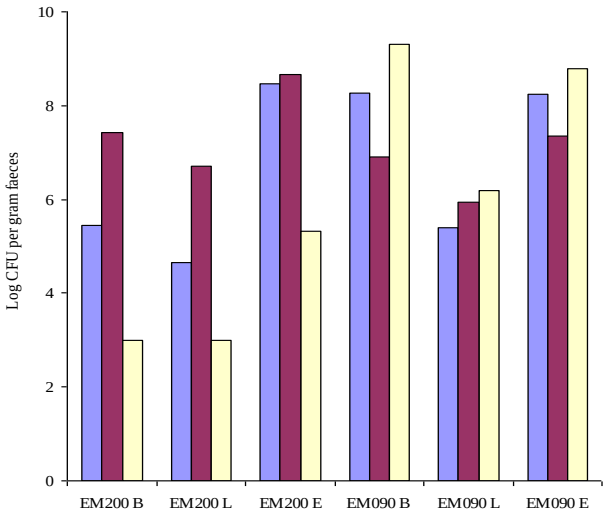
(a) Group 1



(b) Group 2



(c) Group 3



(d) Group 4

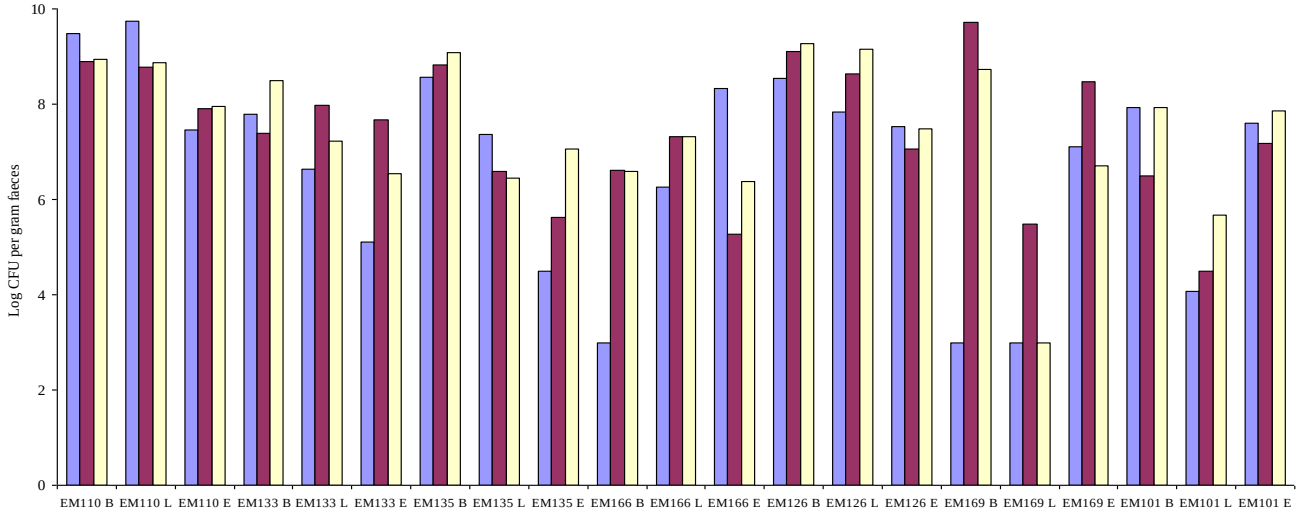
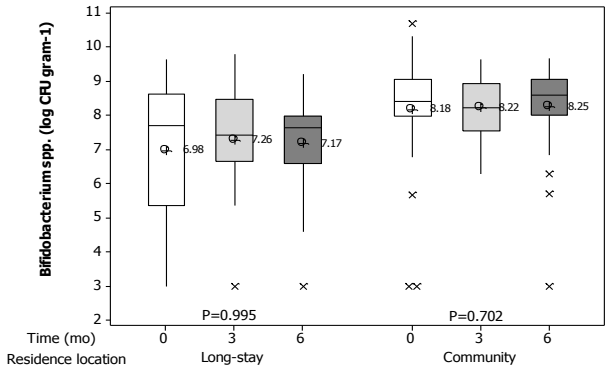


Figure 4: Levels of culturable *Bifidobacterium* spp., *Lactobacillus* spp. and Enterobacteriaceae (log CFU $gram^{-1}$ faeces) in faecal samples from 65 elderly subjects separated by antibiotic therapy and residence location. Figures 4(a), 4(c) and 4(e) represent levels of *Bifidobacterium* spp., *Lactobacillus* spp. and Enterobacteriaceae, respectively, in the antibiotic untreated subjects from the long-stay (n=17 (T0), 16 (T3) and 19 (T6)) and community (n=32 (T0), 33 (T3) and 33 (T6)) residence locations. Figures 4(b), 4(d) and 4(f) represent levels of *Bifidobacterium* spp., *Lactobacillus* spp. and Enterobacteriaceae, respectively, in the antibiotic treated subjects from the long-stay (n=4 (T0), 5 (T3) and 2 (T6)) and community (n=3 (T0), 3 (T3) and 3 (T6)) residence locations. (⊕ = mean; × = outliers).

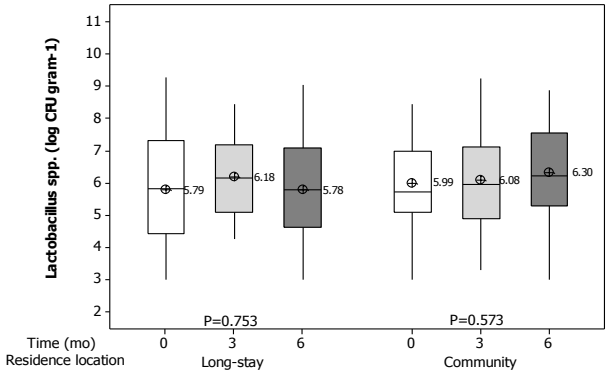
Antibiotic Untreated

Antibiotic Treated

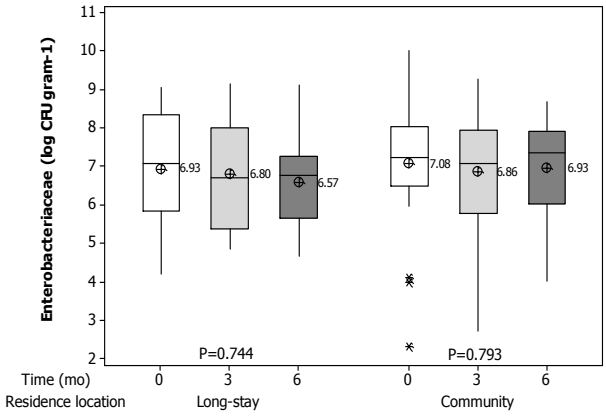
(a)



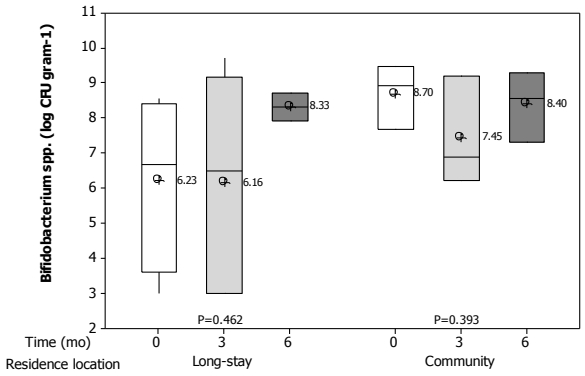
(b)



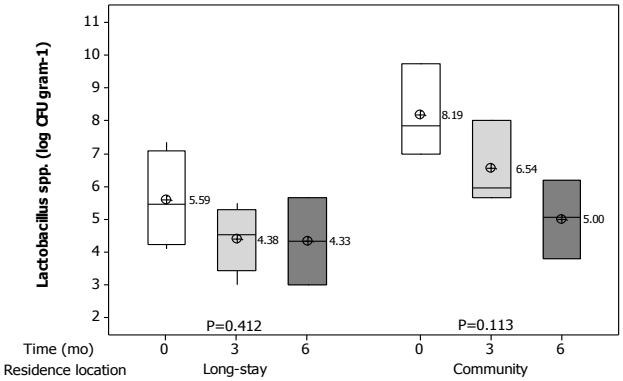
(c)



(d)



(e)



(f)

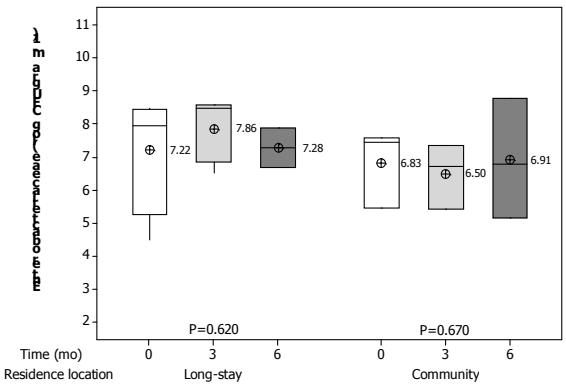
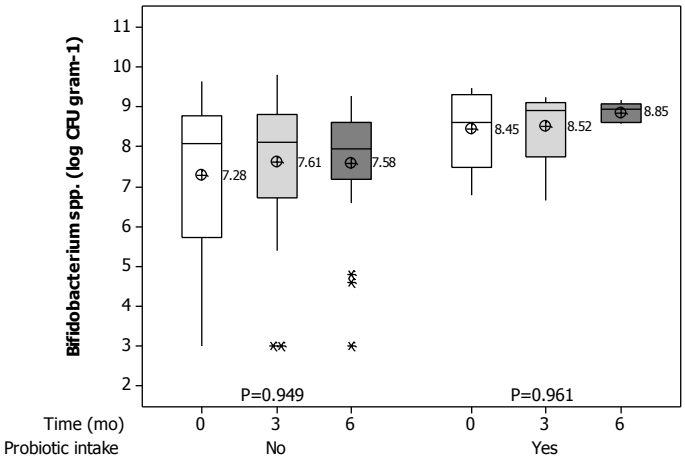
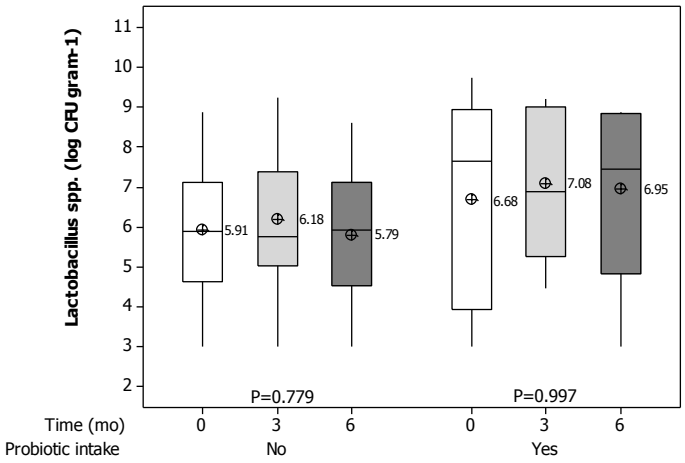


Figure 5: Levels of culturable (a) *Bifidobacterium* spp., (b) *Lactobacillus* spp. and (c) Enterobacteriaceae in faecal samples from elderly subjects. Subjects were assessed based on probiotic intake, where no probiotic intake at any time point (n=26) was compared to regular probiotic intake at all 3 time points (n=5). Counts were recorded at 0 (□), 3 (▒) and 6 (■) months.

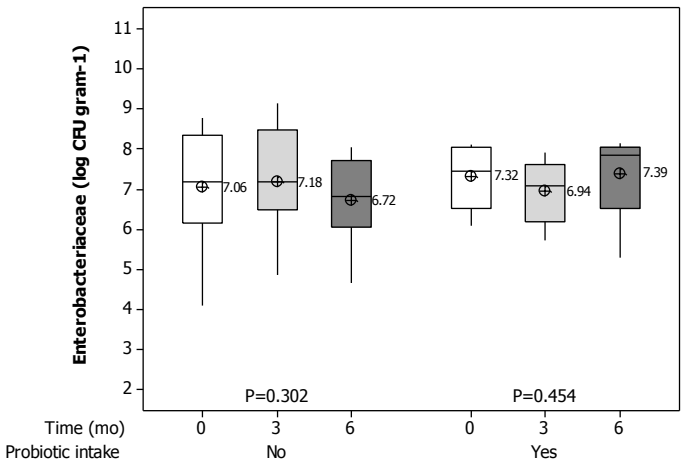
(a)


Bifidobacterium spp. (log CFU gram-1)
11
10
9
8
7
6
5
4
3
2
7.28
7.61
7.58
8.45
8.52
8.85
P=0.949
P=0.961
Time (mo)
0
3
6
0
3
6
Probiotic intake
No
Yes

(b)


Lactobacillus spp. (log CFU gram-1)
11
10
9
8
7
6
5
4
3
2
5.91
6.18
5.79
6.68
7.08
6.95
P=0.779
P=0.997
Time (mo)
0
3
6
0
3
6
Probiotic intake
No
Yes

(c)


Enterobacteriaceae (log CFU gram-1)
11
10
9
8
7
6
5
4
3
2
7.06
7.18
6.72
7.32
6.94
7.39
P=0.302
P=0.454
Time (mo)
0
3
6
0
3
6
Probiotic intake
No
Yes

CHAPTER 4

ISOLATION AND CHARACTERIZATION OF BACTERIOCIN-PRODUCING BACTERIA FROM THE INTESTINAL MICROBIOTA OF ELDERLY IRISH SUBJECTS

This chapter has been published in

Journal of Applied Microbiology

B. Lakshminarayanan, C.M. Guinane, P.M. O'Connor, M. Coakley, C. Hill, C. Stanton, P.W. O'Toole and R.P. Ross (2013)

4.1 Abstract

Aims: To isolate and characterize bacteriocins produced by predominant species of lactic acid bacteria (LAB) from faeces of elderly subjects.

Methods and Results: Screening over 70,000 colonies, from faecal samples collected from 266 subjects, using the indicator organisms *Lactobacillus bulgaricus* LMG 6901 and *Listeria innocua* DPC 3572, identified 55 antimicrobial-producing bacteria. Genomic fingerprinting following *Apa*I digestion revealed 15 distinct strains. The antimicrobial activities associated with 13 of the 15 strains were sensitive to protease treatment. The predominant antimicrobial-producing species were identified as *Lactobacillus salivarius*, *Lactobacillus gasseri*, *Lactobacillus acidophilus*, *Lactobacillus crispatus* and *Enterococcus* spp. A number of previously characterized bacteriocins, including ABP-118 and salivaricin B (from *Lb. salivarius*), Enterocin B (*Ent. faecium*), Lactacin B (*Lb. acidophilus*), Gassericin T and a variant of Gassericin A (*Lb. gasseri*), were identified. Interestingly, two antimicrobial-producing species, not generally associated with intestinally-derived microorganisms were also isolated: *Lactococcus lactis* producing Nisin Z and *Streptococcus mutans* producing Mutacin II.

Conclusion: These data suggest that bacteriocin production by intestinal isolates against our chosen targets under the screening conditions used was not frequent (0.08%).

Significance and Impact of the Study: The results presented are important due to growing evidence indicating bacteriocin production as a potential probiotic trait by virtue of strain dominance and/or pathogen inhibition in the mammalian intestine.

4.2 Introduction

Bacteriocins produced by lactic acid bacteria (LAB) are ribosomally synthesised peptides, which generally have inhibitory action against closely related organisms and to which the producing strain is immune (Cotter *et al.* 2005; De Vuyst and Vandamme 1994). A number of bacteriocins produced by LAB have potential as novel antimicrobial agents in various practical applications from food to medicine (De Martinis *et al.* 2002; Joerger 2003; Klaenhammer *et al.* 2005; O'Sullivan *et al.* 2002; Todorov and Dicks 2007). Many LAB have been proven to function as probiotics, which are of benefit to human health when ingested in sufficient quantities. The isolation of potential bacteriocin-producing strains from the intestinal microbiota (comprising both Gram-negative and Gram-positive bacteria) has been previously reported (Gordon and O'Brien 2006; O'Shea *et al.* 2009). A diverse range of Gram-positive bacteriocin producers including lactobacilli, enterococci and streptococci were isolated in the latter study, with a high incidence (up to 70%) of repeated isolation for some bacteriocin producers. With respect to Gram-negative bacteria, the frequency of production of bacteriocins (such as colicins and microcins) by the *E. coli* population component of the microbiota can vary from 10-80% (Gordon and O'Brien 2006).

Bacteriocin production may confer a significant advantage to the producing strain by allowing it to dominate complex microbial populations (O'Shea *et al.* 2009). The added advantage of using bacteriocin-producing probiotics over bacteriocins themselves is that *in situ* production potentially overcomes the adverse effect of proteolysis of the released peptide during gastric transit. A number of studies have demonstrated the potential use of bacteriocins in the control of important gastric pathogens including *Salmonella* spp. (Casey *et al.* 2004), *Campylobacter jejuni* (Stern *et al.* 2006; Svetoch *et al.* 2011), *Listeria monocytogenes* (Allende *et al.* 2007; Corr *et al.* 2007), *Escherichia coli* O157:H7 (Brashears *et al.* 2003) and *Clostridium difficile* (Rea *et al.* 2007). With regard to the latter, Rea *et al.*

demonstrated the potential of a narrow spectrum bacteriocin to control *C. difficile* in a human colon model, without causing collateral damage to the surrounding microbiota (Rea *et al.* 2011).

The intestinal microbiota is considered a rich source of potential probiotic bacteria and there is an increasing interest in the isolation of new bacteriocin-producing strains of human origin. Indeed, intestinal source has been reported as one of the primary criteria in selecting probiotic strains, as these strains may function better in an environment similar to its origin (Saarela *et al.* 2000). While, there has been considerable research on LAB bacteriocins to date, there are few studies on the screening for bacteriocin-producing LAB from the human intestinal tract (Birri *et al.* 2010; Flynn *et al.* 2002; Verdenelli *et al.* 2009). The increasing interest in the isolation of bacteriocin-producing LAB of human intestinal origin that could be evaluated for probiotic effects was one of the motivating factors for this research.

The ELDERMET consortium was established in 2008 (http://eldermet.ucc.ie), and aims to determine the intestinal microbiota of 500 elderly (>65 yr) Irish people. To date, this project has elucidated links between microbiota composition, diet and health (Claesson *et al.* 2011; Claesson *et al.* 2012), and has generated a large collection of intestinal isolates (including bifidobacteria and lactobacilli), suitable for screening for use as probiotics for the elderly population. The aim of this present study was to isolate and characterize bacteriocin-producing isolates from the ELDERMET biobank that may have potential applications for improving gut health in the elderly. Bacteriocin-like substances were characterized based on their spectrum of inhibition and identified by DNA sequencing and Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (MS) with a view to determining their potential for probiotic applications.

4.3 Materials and Methods

Subject recruitment and sample collection

Subjects aged 65 yr and older were recruited and examined at ELDERMET Clinics at two local hospitals (Cork University Hospital and St. Finbarr's Hospital, Cork) as previously outlined (Claesson *et al.* 2011). The subjects included patients in long-term institutional care, rehabilitation centres, out-patient day hospitals, people being treated with antibiotics and a group of comparatively healthy elderly individuals. A total of 410 faecal samples from 266 elderly subjects were assessed. In addition to the initial sample (T0), faecal samples were also collected from some subjects at 3 (T3) and 6 months (T6). Stool samples were collected into a sterile container, stored at 5^{o}C until transported on ice to the laboratory.

Bacterial strains, media and growth conditions

LAB strains were grown anaerobically at 37^{o}C for 24 to 48 h on de Man, Rogosa, Sharpe (MRS, Difco Laboratories, Detroit, MI, USA) agar supplemented with 0.05% (w/v) L-cysteine hydrochloride (Sigma Chemical Co., St. Louis, MO, USA) (denoted mMRS agar). All other bacterial strains were cultivated in Brain Heart Infusion (BHI) medium and were grown aerobically at 37^{o}C. Where solid media was required, 1.5% agar (w/v) (Oxoid Ltd., Basingstoke, Hampshire, UK) was added and for softer overlay medium, 0.8% (w/v) agar was added.

Enumeration of culturable bacteriocin-producing strains from faecal samples

One gram of the faecal sample was mixed with 9 ml maximum recovery diluent (MRD, Oxoid) in a Seward stomacher bag and subsequently diluted 10-fold as outlined previously (O'Sullivan *et al.* 2011). To enumerate total culturable LAB strains, the dilutions were spread

plated (100 µl) onto mMRS agar. Agar plates were incubated anaerobically at 37°C for 48 h. Bacterial counts were recorded as colony forming units (CFU) per gram of faeces and counts were expressed as log CFU g^{-1} faeces. These plates were overlaid with the indicator strains and were incubated at 37°C for 24 h. The indicator strains used were *Lactobacillus delbrueckii* subsp. *bulgaricus* LMG 6901 (for all samples) (BCCM/LMG Culture Collection, Gent, Belgium), *Bifidobacterium breve* NCIMB 8807 (for 228 samples) (National Collection of Industrial, Marine and Food Bacteria, Aberdeen, Scotland), *Bifidobacterium lactis* Bb12 (for 30 samples) (Chr. Hansen, Little Island, Cork, Ireland), *Escherichia coli* O157:H7 NCTC 12900 (for 11 samples) (National Collection of Type Cultures, Central Public Health Laboratory, London, UK) and *Listeria innocua* DPC 3572 (for 123 samples) (Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland). Bacterial colonies generating a zone of inhibition against the indicator strain on the agar plates were selected and cultured in mMRS broth for 24 h at 37°C. These isolates were stocked in the ELDERMET Culture Collection for further characterization.

Antimicrobial activity assay

The antimicrobial activity of the cell-free supernatant (CFS) of the bacteriocin-producing strains was determined by the agar well diffusion method (Ryan *et al.* 1996). To eliminate the possibility of a zone of inhibition due to acid production, all culture supernatants were adjusted to pH 6.5 with 1 N NaOH and filter-sterilised (0.45 µm). Antimicrobial activity was assessed against a selection of Gram-positive and Gram-negative strains (*Lb. bulgaricus* LMG 6901, *L. innocua* DPC 3572, *L. monocytogenes* DPC 3785, *Staphylococcus aureus* DPC 5245, *Salmonella typhi* DPC 6452, *S. typhi* DPC 6046, *E. coli* DPC 6239 and *Cronobacter sakazakii* DPC 6440). All agar plates were examined for a zone of inhibition following overnight incubation.

Influence of heat treatment and enzymes on antimicrobial activity

To evaluate the heat stability of the bacteriocins, the neutralised supernatants from the bacteriocin-producing strains were heated to 100^{o}C for 30 min. The untreated bacteriocin was used as a positive control. All culture supernatants were adjusted to pH 6.5 with 1 N NaOH and were mixed with an equal volume of each of the enzymes proteinase K, pepsin and catalase (25 mg ml^{-1}; Sigma) and incubated at 37^{o}C for 3 h. Following enzyme and heat treatment, the residual antimicrobial activity in the treated supernatant was assessed using the agar-well diffusion assay with the indicator strain *Lb. bulgaricus* LMG 6901, in comparison with the untreated sample.

Genetic characterization of bacterial isolates

Genomic DNA was extracted as outlined previously (Coakley *et al.* 1996), and RNAse (0.75 µl, Sigma) was added to the extracted DNA, incubated for 1 h at 37^{o}C and then denatured at 65^{o}C for 15 min. Bacterial isolates were identified by amplification of the 16S rRNA gene using the 16S Eubacterial primers CO1 and CO2 (Table 1; Simpson *et al.* 2003) and the complete sequence of the 16S rRNA gene was determined by Sanger sequencing (Beckman Coulter Genomics). The species was determined by nucleotide alignments (>98%) with deposited species in the NCBI database (http://blast.ncbi.nlm.nih.gov) and using the Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu/). For *Enterococcus* isolates further PCR analysis was used to distinguish the species present using previously published species-specific primers (Jackson *et al.* 2004). Primers specific to structural genes of known bacteriocins were also used (Table 1). All PCR reactions were performed in a G-Storm PCR machine (Gene Technologies, Essex, UK) and PCR products sequenced by Beckman Coulter Genomics. Sequences were aligned to the structural bacteriocin genes using the Lasergene 6

software programme and subsequently analysed by BlastP analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Pulsed-field gel electrophoresis (PFGE) of bacterial isolates

PFGE of isolates demonstrating antimicrobial activity in the CFS was performed as previously described (Simpson *et al.* 2002) using the *Apa*I restriction endonuclease. A low range molecular weight DNA marker (9.42–194.0 Kb; New England Biolabs, Beverly, MA, USA) was used to determine the band size. Electrophoresis was performed using the Biorad Chef-DR II instrument (Biorad, USA). For each PFGE pattern obtained, one strain was selected for further characterization.

Purification and identification of bacteriocins

Purification of antimicrobial peptides was performed by reverse-phase high-performance liquid chromatography (RP-HPLC). The bacteriocins were purified from an overnight culture of the producing strain in mMRS broth. Bacterial cells were harvested by centrifugation and resuspended in 70% (v/v) propan-2-ol and 0.1% (v/v) trifluoroacetic acid (TFA) and mixed at room temperature for 3-4 h. Following removal of the propan-2-ol by rotary evaporation, 20 ml of the sample was applied to a 5 g STRATA C18-E SPE column (Phenomenex, Cheshire, UK) which was pre-equilibrated with methanol and water. The column was washed with 20 ml 40% (v/v) ethanol and the bacteriocin activity was eluted with 70% (w/v) propan-2-ol. Following removal of the propan-2-ol from the preparation, aliquots were applied to a semi-preparative Proteo Jupiter (4 u Proteo, 90 Å, 250 x 10 mm) RP HPLC column, previously equilibrated with 30% propan-2-ol containing 0.1% TFA. The column was subsequently developed in a gradient of 30% (v/v) propan-2-ol, containing 0.1% (v/v) TFA to 60% (v/v) propan-2-ol, containing 0.1% (v/v) TFA over 40 min. Bioactive fractions were collected at 1 min intervals and bacteriocin activity was monitored by the well diffusion assay. For some

strains, the supernatant of the overnight culture was used to determine the bacteriocin activity. MALDI-TOF mass spectrometry was performed on HPLC fractions as described previously (Cotter *et al.* 2006).

4.4 *Results*

Screening and isolation of bacteriocin-producing bacteria

Screening of over 70,000 colonies from 410 faecal samples (from 266 elderly subjects) resulted in the detection of 276 colonies exhibiting antimicrobial activity against *Lb. bulgaricus* LMG 6901 (273 colonies) and *L. innocua* DPC 3572 (3 colonies), representing an isolation frequency of 0.4% in the initial screening. Despite exhaustive parallel screening, colonies showing inhibitory activity against *Bif. breve* NCIMB 8807, *Bif. lactis* Bb12 and *E. coli* strains were not detected in this study. The lack of activity against the indicator strain *E. coli* was not surprising due to the protective nature of the cell membrane in Gram-negative bacteria against the action antimicrobial compounds (Gao *et al.* 1999). In addition, the culture conditions in this study focused mainly on the isolation of LAB bacteria which generally kill or inhibit closely related Gram-positive strains (Klaenhammer 1988; Riley and Wertz 2002).

The 276 inhibitory isolates were subsequently reduced in number to 55 isolates (from 32 subjects) following neutralization of the CFS, representing a frequency of successful probable bacteriocin isolation of 0.08%. Further analysis of the 55 isolates by PFGE resulted in 15 individual strains (from 14 subjects) with distinct PFGE profiles (Figure 1), corresponding to a probable bacteriocin detection frequency of 0.02%. Strains with similar PFGE patterns were isolated from multiple subjects, as shown in Table 2. A high incidence of repeated isolation was observed for *Lb. gasseri* (12), *Lb. crispatus* (15) and *Ent. durans* (6). Strains with identical PFGE patterns were confirmed to produce the same/similar bacteriocin by cross-sensitivity assays, since each isolate was cross-immune to the antimicrobial compound produced by the other (data not shown).

Species identification of bacterial strains

The 16S rDNA gene sequences of the isolates were determined and compared with the available 16S rDNA sequences in the NCBI database. One of the most predominant species isolated in this study was *Lb. gasseri*. Other organisms identified included *Lb. salivarius, Lb. acidophilus, Strep. mutans, L. lactis* and *Enterococcus* spp. (Table 3). In addition to 16S rDNA gene sequencing, the identified enterococcal isolates were further typed using species-specific PCR primer sets for sequence variations in manganese dependent superoxide dismutase genes of *Enterococcus*, as previously described (Jackson *et al.* 2004). Based on this method, two of the enterococcal strains (EM342-BC-1 and EM297-BC-T6-1) were determined to be *Ent. faecium* and EM133-BC-1 to be *Ent. durans*.

Inhibitory spectrum

The 15 antimicrobial-producing strains with distinct PFGE patterns were selected for further characterization. The inhibitory spectra of the strains using a range of indicator organisms were investigated and revealed a strain-dependent variation (Table 4). All the strains exhibited zones of inhibition against *Lb. bulgaricus* and other strains including *Lb. salivarius, L. lactis* and *Enterococcus* spp. also exhibited relatively wide spectra of activity, inhibiting *L. innocua* and *Staph. aureus*. As expected, none of the strains tested exhibited antimicrobial activity against the Gram-negative indicator organisms, including strains of *S. typhi, C. sakazakii* or *E. coli.*

Enzyme and heat sensitivity

The isolated crude bacteriocins exhibited stability at 100^{o}C for 30 min, with the exception of bacteriocins from the *Lb. crispatus* strains. None of the inhibitory zones were affected by catalase, indicating that hydrogen peroxide was not involved in the inhibitory action. In

contrast, the antimicrobial activity associated with 13 of the 15 strains was found to be sensitive to proteinase K, indicating the presence of antimicrobial compound of a protein nature in these cases (Table 3). The two *Lb. crispatus* strains were again exceptions, as their culture supernatants demonstrated antimicrobial activity even after proteinase K treatment.

Identification and purification of bacteriocins

Following species identification of the bacteriocin-producing isolates, primers specific to known bacteriocin structural genes from the isolated bacterial species were employed in test amplifications (Table 1). PCR analysis and subsequent DNA sequencing were used as an initial method to identify the putative bacteriocin structural genes present in each strain. This resulted in identification of ten different putative bacteriocins from the selected 15 isolates (Table 3). In parallel, the bacteriocins produced by these strains were purified by RP HPLC, and the molecular mass of the peptides was determined using MALDI-TOF MS. Precise matching of these peptide masses to the theoretical masses was taken as evidence that the identified bacteriocin structural genes encoded the active bacteriocin. Certain bacteriocin producers were selected for further characterization based either on their unusual presence in the human intestinal microbiota (*L. lactis* EM089-BC-T6-1 and *Strep. mutans* EM315-BC-T3-2), on their broad inhibitory spectra (*Lb. salivarius* EM100-BC-T3-1 and EM253-BC-T6-2), or due to the production of multiple bacteriocins (*Ent. faecium* EM297-BC-T6-1).

PCR and amplicon sequence analysis of *Lb. salivarius* EM100-BC-T3-1 and EM253-BC-T6-2 confirmed the presence of the three bacteriocin structural genes; these encoded salivaricin B and both peptides of the ABP-118 bacteriocin (α and β) in all strains. MALDI-TOF MS of the pure peptides, following RP-HPLC purification, confirmed molecular masses of 4,096 and 4,332 Da (Figures 2a and 2b), which correspond to the previously characterized ABP-118α and ABP-118β peptides of the ABP-118 bacteriocin, respectively (Flynn *et al.*

2002). This was in accordance with the PCR and sequence analysis, which indicated the presence of the structural genes (100% identity) for this two-peptide bacteriocin within the strain. In addition, MS analysis also confirmed the presence of another previously characterized peptide, salivaricin B (Cataloluk 2001) showing a corresponding mass of 4,433 Da (Figure 2c). However, this peptide neither inhibited the indicator strain *Lb. bulgaricus* nor displayed anti-listerial activity, which confirmed recent findings in our laboratory.

PCR analysis and DNA sequencing data for *Ent. faecium* EM342-BC-1 confirmed the presence of the Enterocin B gene, a previously characterized class IIc bacteriocin (Casaus *et al.* 1997). Interestingly, another *Ent. faecium* strain EM297-BC-T6-1, was confirmed to harbour the structural genes for a recently identified bacteriocin, Enterocin M (Marekova *et al.* 2007) and also the genes for the two-component bacteriocin Enterocin 62-6, which is closely related to Enterocins L50A and L50B (Dezwaan *et al.* 2007). MS analysis of the bacteriocins from *Ent. faecium* EM297-BC-T6-1 identified proteins of two different masses corresponding to two putative bacteriocins. A peptide of 4,628 Da was identified corresponding to Enterocin M, which is an anti-listerial class IIa pediocin-like bacteriocin (Figure 2f) and a second bacteriocin consisted of two peptides (5,206 and 5,219 Da), corresponding to the two-peptide class IIc bacteriocins produced by a vaginal isolate of *Ent. faecium* 62-6 (Figure 2g).

The sequence of the amplified PCR product from the *L. lactis* isolates revealed 100% amino acid identity to the translated structural gene of the well characterized lantibiotic, Nisin Z (Mulders *et al.* 1991) and was further investigated. MS analysis of bioactive RP-HPLC fractions purified from *L. lactis* EM089-BC-T6-1 revealed a peptide with a molecular mass of 3,332 Da, which corresponds exactly to the peptide mass value expected for the nisin variant, Nisin Z (Figure 2d). 16S rDNA sequencing identified the ELDERMET isolate EM315-BC-T3-2 as *Strep. mutans*. PCR and sequence analysis indicated the presence of the structural

gene for the bacteriocin, Mutacin II, whose expression was verified by MS analysis of the bacteriocin. The purified peptide (3,244 Da) from this isolate corresponded to the mass of the lanthionine-containing mutacin peptide, Mutacin II (Novak *et al.* 1994) (Figure 2e).

In this study, three *Lb. gasseri* strains were shown to produce bacteriocins; *Lb. gasseri* EM081-BC-T3-1, EM315-BC-T6-1 and EM301-BC-T3-1 (Table 3). Of the three strains, *Lb. gasseri* EM081-BC-T3-1 generated the expected-sized PCR product when amplified with Gassericin T primers, which was confirmed by DNA sequencing. The other two strains were initially screened for the presence of the Acidocin B and Gassericin A structural genes using specific primers (Table 1). Due to the high similarity (>95% amino acid identity) between these two bacteriocins (Kawai *et al.* 1998b), both PCRs successfully generated a product in all strains. However, subsequent sequence analysis confirmed the presence of a structural gene with 98% amino acid identity to both Acidocin B and Gassericin A, indicating a new variant of this family of bacteriocins (Figure 3). MS analysis of the purified antimicrobial peptides revealed molecular mass of 5,683 Da, inferring the protein to be a variant of Gassericin A, a previously characterized class IIc bacteriocin (Kawai *et al.* 1998b) (Figure 2h). PCR amplification of DNA from *Lb. acidophilus* EM066-BC-T3-3 confirmed the presence of the structural gene for Lactacin B and the protein alignments indicated 100% amino acid identity with the Lactacin B structural gene product, identified previously in *Lb. acidophilus* La-5 (Tabasco *et al.* 2009). The strain isolated from this study, demonstrated a narrow inhibitory spectrum, inhibiting only *Lb. bulgaricus*, which is similar to the Lactacin B bacteriocin (Barefoot and Klaenhammer 1983).

4.5 *Discussion*

As intestinal origin is generally considered a desirable attribute for probiotic strains for human consumption, this screening study was undertaken to isolate human intestinally-derived bacteria with antimicrobial activity. The availability of a large number of faecal samples from older subjects recruited in the ELDERMET study enabled a comprehensive screening for such strains specifically associated with the intestinal niche of older subjects of different health states, and resulted in the successful isolation of a broad range of bacteriocin producers of human intestinal origin. The initial activity screening of 70,000 colonies against *Lb. bulgaricus* LMG6901 resulted in the isolation of 273 isolates (representing an isolation frequency of 0.4%), which were subsequently reduced to 15 distinct strains through further characterization.

The bacteriocinogenic isolates were identified from the total bacterial count (on mMRS agar incubated anaerobically) ranging between log 10^6 to log 10^9 CFU per gram faeces. The isolation frequency of the bacteriocinogenic isolates was found to be low than expected (0.4% from total culturable bacteria) and did not represent a major part of bacteria culturable by the above methods. Due to the high number of colonies screened, the number of indicator organisms was restricted in this study. In addition, it does have to be noted that conventional culture methods can limit a screening study to the 'easy to culture' organisms from the intestine. However, it has also been widely documented that the elderly intestinal microbiota differs from younger adults (Biagi *et al.* 2011; Claesson *et al.* 2011) and that the compromised stability of the intestinal microbiota in the elderly (Biagi *et al.* 2012; Mueller *et al.* 2006) may have resulted in the lower isolation of bacteriocin-producing strains.

The majority of bacteriocin producers isolated in this study were *Lactobacillus* spp., with *Lb. gasseri* being the predominant species isolated. This is in agreement with previous

studies which consider *Lb. gasseri* to be one of the most commonly detected LAB in the human intestinal tract (Reuter 2001; Wall *et al.* 2007). Other bacteriocin producers identified included *Lb. crispatus*, a common vaginal isolate (Pavlova *et al.* 2002), *Lb. salivarius*, *Enterococcus* spp., *Strep. mutans* and *L. lactis*. All the isolated bacteriocins exhibited stability at 100^{o}C for 30 min and were sensitive to proteases with the exception of bacteriocins from the *Lb. crispatus* strains. Further analysis is required to determine the nature of the antimicrobial compound produced by these strains.

Certain bacteriocin producers were selected for further characterization, including *L. lactis* EM089-BC-T6-1, *Lb. salivarius* EM100-BC-T3-1, *Lb. salivarius* EM253-BC-T6-2, *Ent. faecium* EM297-BC-T6-1 and *Strep. mutans* EM315-BC-T3-2. All four of the *Lb. salivarius* isolates (Table 3) from different ELDERMET subjects demonstrated inhibitory activity against *Lb. bulgaricus* LMG 6901, *L. innocua* DPC 3572 and the pathogenic *L. monocytogenes* DPC 3785 (Table 4). The presence of the three peptides ABP-118α, ABP-118β and salivaricin B were confirmed in these *Lb. salivarius* isolates. ABP-118 is a well characterized two-component Class II heat-stable bacteriocin, produced by *Lb. salivarius* subsp. *salivarius* UCC118 (Flynn *et al.* 2002) that inhibits the food-borne pathogen *L. monocytogenes* (Corr *et al.* 2007). In contrast to the ABP-118α and ABP-118β peptides, the salivaricin B did not exhibit anti-listerial activity, which is in agreement with a recent study concerning another *Lb. salivarius* strain (O'Shea *et al.* 2011). These results demonstrate that the production of a two-component class II bacteriocin may be a common feature among intestinal *Lb. salivarius* strains.

Bacteriocin-producing species not commonly isolated from the human intestine, including *L. lactis* and *Strep. mutans*, were identified in this screening study. The bacteriocin produced by *L. lactis* EM089-BC-T6-1 (Nisin Z) exhibited broad spectrum inhibitory activity against the indicator strains *Lb. bulgaricus* LMG 6901, *L. innocua* DPC 3572, *L.*

monocytogenes DPC 3785 and *Staph. aureus* DPC 5245. Although it is well documented that nisin is anti-listerial (Cai *et al.* 1997), the first human-derived nisin reported previously (Millette *et al.* 2007) did not show activity against *Listeria* spp. and was sensitive to temperatures greater than 70^{o}C. This outcome was explained by the fact that the nisin concentration produced by the strain was insufficient to inhibit the food pathogen (Millette *et al.* 2008). Further studies on this human-derived Nisin Z from *L. lactis* EM089-BC-T6-1 isolate are necessary to determine its efficacy. Additionally, further characterization of bacteriocin production by the *Lb. crispatus* and *Ent. durans* isolated in this study is also required, as limited information is available on bacteriocin production by these strains (Hu *et al.* 2008; Tahara and Kanatani 1997; Yanagida *et al.* 2005).

Strep. mutans is one of the principal aetiological agents of dental caries (Kamiya *et al.* 2005) and although the presence of *Strep. mutans* in the human intestine has already been reported (Hamada and Slade 1980), to our knowledge this is the first time a bacteriocin-producing *Strep. mutans* (producing Mutacin II) has been identified from a human intestinal source. In this respect, it is noteworthy that mutacin production has been shown to be a colonization factor in the human oral cavity (Hillman *et al.* 1987).

Both the *Ent. faecium* bacterial isolates, EM342-BC-1 and EM297-BC-T6-1, exhibited inhibitory activity against *Lb. bulgaricus* LMG 6901, *L. innocua* DPC 3572 and *L. monocytogenes* DPC 3785. MS analysis of the purified antimicrobial peptides produced by *Ent. faecium* EM297-BC-T6-1 confirmed molecular masses for two putative bacteriocins. While it is characteristic of enterococci to produce different types of bacteriocins (Franz *et al.* 2007), little has been reported about the production of bacteriocins by enterococci of human intestinal origin (Moon *et al.* 2000). Enterocin producers are being characterized as novel probiotics and have been studied for their antagonistic nature against major food pathogens such as *L. monocytogenes* (Achemchem *et al.* 2005; Franz *et al.* 1999; Kang and Lee 2005;

Renye *et al.* 2009). The present study reports the characterization of two active bacteriocins exhibiting anti-listerial activity and suggests the need to assess such strains for their beneficial effect on the gastrointestinal microbiota.

The bacteriocins produced by the *Lb. gasseri* isolated in this study exhibited a narrow spectrum of activity, inhibiting just the *Lb. bulgaricus* assessed (Table 4). Sequence analysis identified Gassericin bacteriocins, Gassericin T and a new variant of Gassericin A, in agreement with the MS data. In general, *Lb. gasseri* is considered one of the most predominant and commonly detected species of *Lactobacillus* in the human gastrointestinal tract (Reuter 2001) and is commonly associated with bacteriocin production (Kawai *et al.* 1998a). It was recently demonstrated that the combined effect of both Gassericin A and Gassericin T along with glycine could be used as a preservative for the food industry (Arakawa *et al.* 2009). Similarly, the Gassericin producing strains isolated in this study may be of benefit for the food industry.

Bacteriocin production can confer a competitive advantage to the producing strain, enhancing its dominance over other species in an ecological niche. To date, only a limited number of studies relating to bacteriocin production by strains of human intestinal origin have been performed. This study has highlighted the diverse range of bacteriocinogenic strains present in the human intestinal environment, which may help these strains to predominate in the bacterial population. Overall, following preliminary characterization, genotyping of all of the 55 bacteriocin-producing strains isolated revealed 15 different individual strains indicating a higher incidence of repeated isolation of similar strains. In addition to the known predominant species of the human intestinal environment, including *Lb. gasseri* and *Enterococcus* spp., this study has highlighted the isolation of unexpected species such as *L. lactis* and *Strep. mutans*. This screening has resulted in the isolation of a

panel of bacteriocin-producing human strains with the potential to alter the gut microbiota when introduced as probiotic cultures.

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Table 1 Primers used in this study

Primers	Sequence (5'-3')	Annealing Temp. (^{o}C)	Amplicon size (bp)	Reference
CO1-Forward CO2-Reverse	AGTTTGATCCTGGCTCAG TACCTTGTTAC GACT	50	1500	(Simpson *et al.* 2003)
118α-For 118im-Rev	ATGATGAAGGAATTTACA G CCACGCTCTCACATAAC	50	700	(Barrett *et al.* 2007)
salivaricin B-For salivaricin B-Rev	AGCGGACAGTTTGGTTATGA TTCAGCACATAGAACACATCCTC	55	900	(O'Shea *et al.* 2011)
Lactacin B-For Lactacin B-Rev	TTGAGTTATGTAATTGGTGGAG CTGCTGAGCCTTTGATAACG	60	181	(Tabasco *et al.* 2009)
Acidocin-B-For Acidocin B-Rev	GTTGGAATAGTATGGTTTTCG ATTCATATCCGGCACCAACG	58	700	This study
Gassericin A-For Gassericin A-Rev	CACTCGTAATGGATTTCCTA GCCTTT TTTGAATACTCTATTC	55	350	(Kawai *et al.* 2001)
Gassericin T-For Gassericin T-Rev	TGGATTTAAATTGCCTGAAAC CATTCCCCCACTTGTTTCC	58	645	This study
Nisin Z-For Nisin Z-Rev	AACGGCTCTGATTAAATTC CTATTTAGCAACCCTAAATAAC	50	215	This study
Enterocin B-For Enterocin B-Rev	GAAAATGATCACAGAATGCCTA GTTGCATTTAGAGTATACATTTG	58	159	(Toit *et al.* 2000)
Enterocin 62-6-For Enterocin 62-6-Rev	GTGGAAAGCTAGTATTTGCAAC AGCGTTAAGCCGAATGTTTACAC	49	511	(Dezwaan *et al.* 2007)
Mutacin II-For Mutacin II-Rev	AACGCAGTAGTTTCTTTGAA TTCCGGTAAGTACATAGTGC	52	444	(Kamiya *et al.* 2005)
Enterolysin A-For Enterolysin A-Rev	GGACAACAATTCGGGAACACT GCCAAGTAAAGGTAGAATAAA	55	1007	(Nigutova *et al.* 2007)

Table 2 Diversity of bacteriocin-producing strains among individual elderly subjects

Strains	Number of isolates	Number of subjects	Number of pulsotypes	PFGE profile
Lb. gasseri	12	6	3	A, B & C
Lb. salivarius	5	3	4	D, E, F & G
Lb. acidophilus	9	7	1	H
Ent. faecium	4	2	2	I & J
L. lactis	3	2	1	K
Strep. mutans	1	1	1	L
Lb. crispatus	15	7	2	M & N
Ent. durans	6	4	1	O

Table 3 Predicted bacteriocins and their enzyme sensitivity

Strains identified through 16S rDNA sequencing	Strain ID	PFGE profile	Predicted bacteriocin	†Amino acid identity (%)	Proteinase K	Pepsin
Lb. gasseri	EM081-BC-T3-1	A	Gassericin T	99	+	+
	EM301-BC-T3-1	B	Acidocin B	100	+	+
	EM315-BC-T6-1	C	Acidocin B	100	+	+
Lb. salivarius	EM100-BC-T3-1	D	ABP-118 & Salivaricin B	100	+	+
	EM253-BC-T6-2	E	ABP-118 & Salivaricin B	100	+	+
	EM347-BC-T3-1	F	ABP-118 & Salivaricin B	100	+	+
	EM351-BC-5	G	ABP-118 & Salivaricin B	100	+	+
Lb. acidophilus	EM066-BC-T3-3	H	Lactacin B	98	+	+
Ent. faecium	EM342-BC-1	I	Enterocin B	100	+	+
	EM297-BC-T6-1	J	Enterocin 62-6 & Enterocin M	100	+	+
L. lactis	EM089-BC-T6-1	K	Nisin Z	100	+	+
Strep. mutans	EM315-BC-T3-2	L	Mutacin II	100	+	+
Lb. crispatus	EM047-BC-T6-4	M	ND*	ND	-	-
	EM200-BC-T3-1	N	ND	ND	-	-
Ent. durans	EM133-BC-1	O	ND	ND	+	+

* ND Not determined
+ Sensitive to enzyme
- Resistant to enzyme
† Amino acid alignments of the sequenced structural genes of strains identified in this study with structural genes from characterised bacteriocins (NCBI).

Table 4 Inhibitory spectrum of ELDERMET isolates

ELDERMET isolates	Indicator organism			
	Lb. bulgaricus **LMG 6901**	***L. monocytogene*** **DPC 3785**	***L. innocua*** **DPC 3572**	***S. aureus*** **DPC 5245**
Lb. gasseri	+	-	-	-
Lb. crispatus	+	-	-	-
Lb. acidophilus	+	-	-	-
Lb. salivarius	+	+	+	+
Ent. durans	+	-	-	-
Ent. faecium	+	+	+	-
L. lactis	+	+	+	+
Strep. mutans	+	-	-	-

+ Strains exhibiting antimicrobial activity against indicator organism

- Strains not exhibiting antimicrobial activity against indicator organisms. No antimicrobial activity was observed against *S. typhi*, *C. sakazakii* and *E. coli*

Figure 1 Grouping of the macro-restriction patterns of the bacteriocin-producing strains generated 15 PFGE pulsotypes; termed A to O, on digestion of chromosomal DNA with *Apa*I restriction enzyme (Lane 1 is the marker (Ma)).

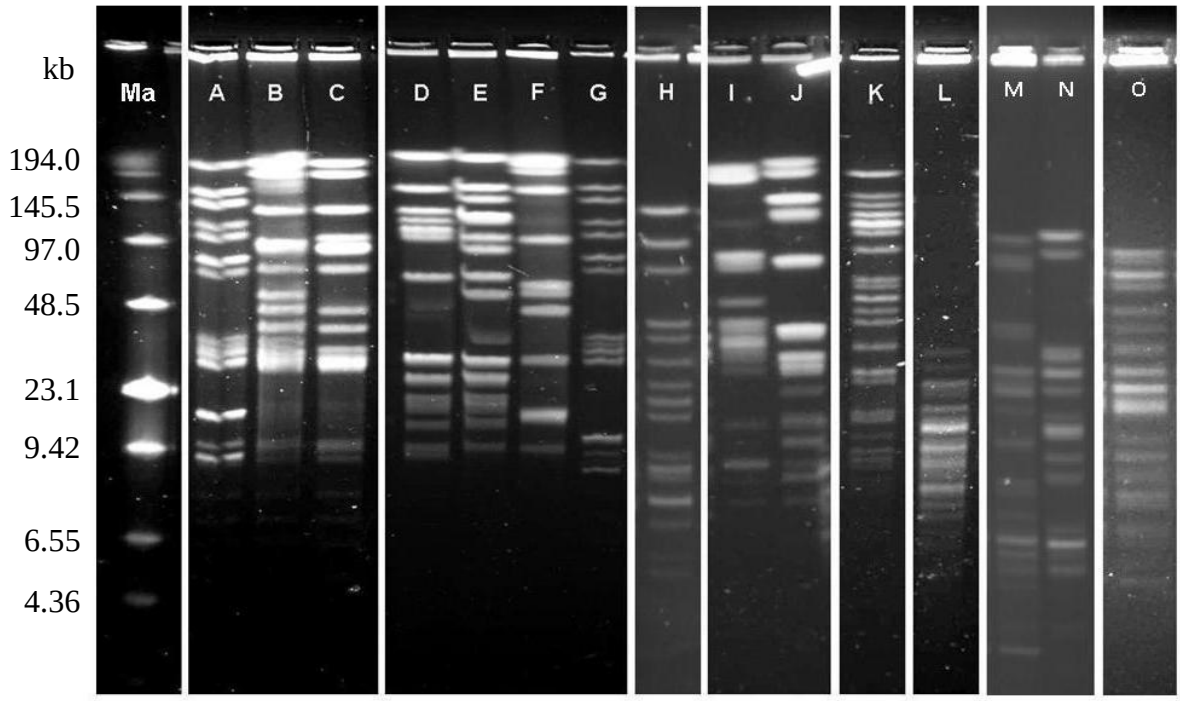
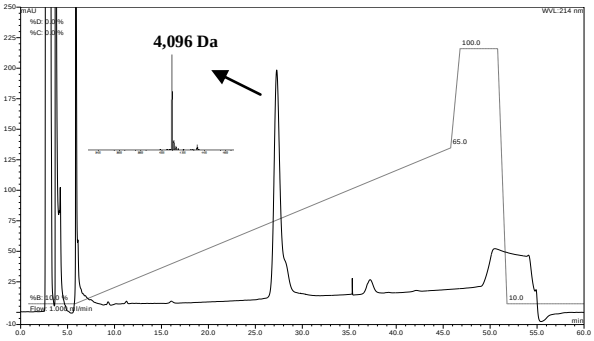
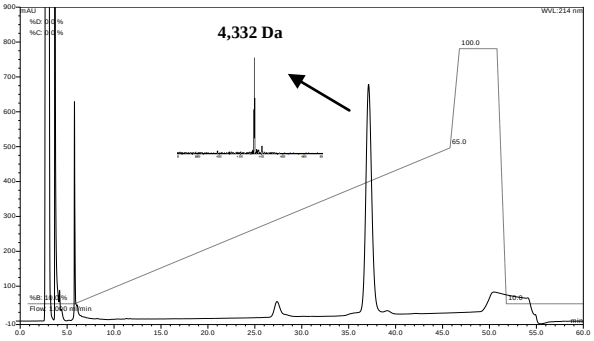


Figure 2 RP-HPLC chromatograms and MALDI-TOF MS data (inserted panel) of (a) S1n1 produced by *Lb. salivarius* EM100-BC-T3-1, (b) S1n2 produced by *Lb. salivarius* EM100-BC-T3-1, (c) Salivaricin B produced by *Lb. salivarius* EM100-BC-T3-1, (d) Nisin Z produced by *L. lactis* EM089-BC-T6-1, (e) Mutacin II produced by *Strep. mutans* EM315-BC-T3-2, (f) Enterocin M produced by *Ent. faecium* EM297-BC-T6-1, (g) Enterocin 62-6 produced by *Ent. faecium* EM297-BC-T6-1 and (h) a variant of Gassericin A produced by EM301-BC-T3-1. Arrows indicate location of the antimicrobial peptides.

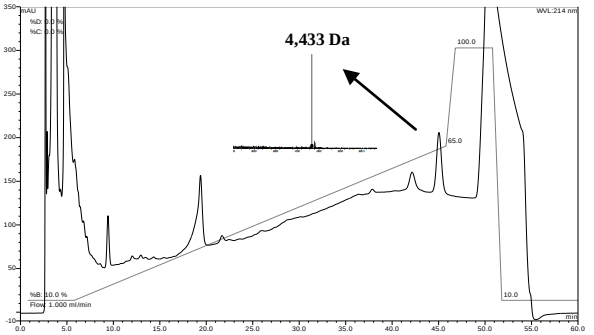
(a)


4,096 Da

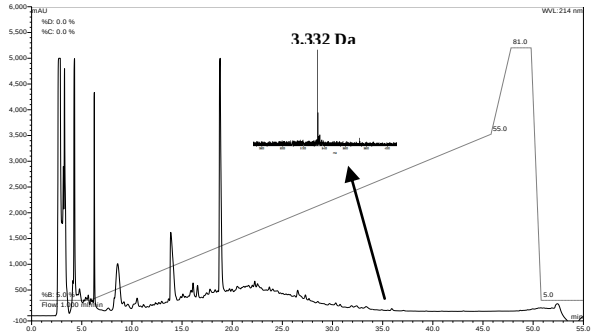
(b)


4,332 Da

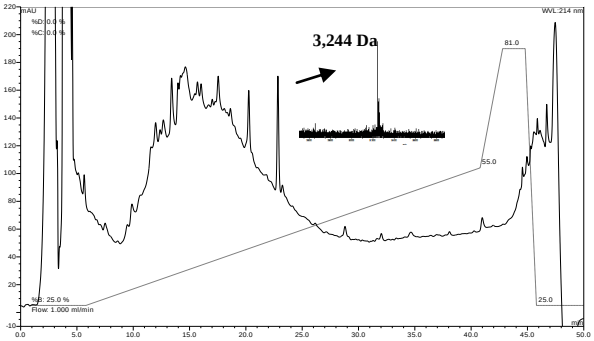
(c)


4,433 Da

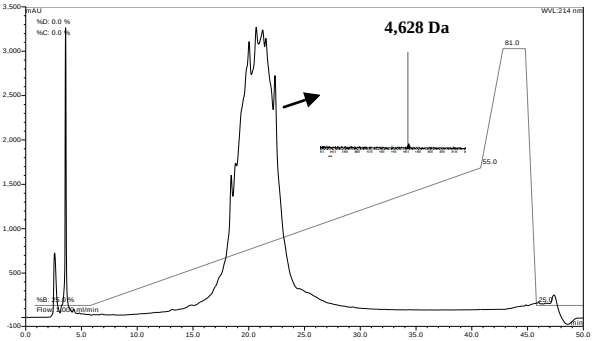
(d)


3,332 Da

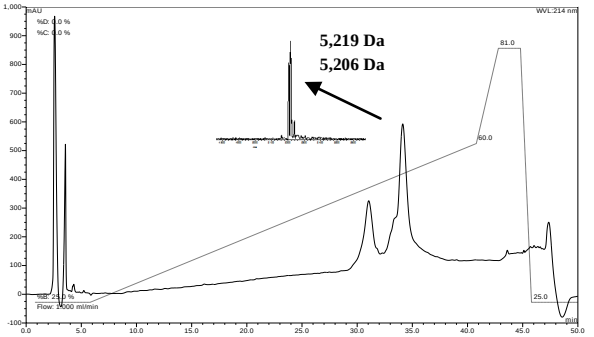
(e)


3,244 Da

(f)


4,628 Da

(g)


5,219 Da
5,206 Da

(h)

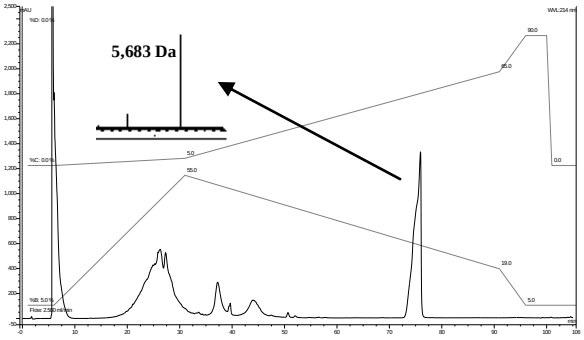

5,683 Da

Figure 3 Comparison of amino acid sequences of Gassericin A (mature peptide) and variant produced by *Lb. gasseri* EM301-BC-T3-1 and *Lb. gasseri* EM315-BC-T6-1. Amino acid differences are bold and underlined.

Gassericin A : MASGASLGTAFA**A**ILGVTLPAWALAAAGALGATAA Mass: 5,652 Da

EM301-BC-T3-1: MASGASLGTAFA**T**ILGVTLPAWALAAAGALGATAA Mass: 5,683 Da

EM315-BC-T6-1: MASGASLGTAFA**T**ILGVTLPAWALAAAGALGATAA Mass: 5,683 Da

CHAPTER 5

PREVALENCE AND CHARACTERIZATION OF *CLOSTRIDIUM PERFRINGENS* FROM THE FAECAL MICROBIOTA OF ELDERLY IRISH SUBJECTS

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5.1 Abstract

The aim of this study was to investigate the diversity and composition of the intestinal microbiota of elderly subjects using a combination of culture dependent techniques and 16S rRNA gene amplicon sequencing. The study was performed as part of the ELDERMET project, in which 368 faecal samples were assessed for viable numbers of *Bifidobacterium* spp., *Lactobacillus* spp. and *Enterobacteriaceae* on selective agar. However, the *Bifidobacterium* selective medium (BSM) also supported the growth of *Clostridium perfringens*, which appeared as distinct colonies and were subsequently characterised phenotypically and genotypically. All the isolates were confirmed as toxin biotype A producers. In addition, three isolates tested also had the genetic determinants for the beta-2 (β2) toxin. Of the 368 faecal samples assessed, *C. perfringens* was detected in 28 samples (7.6%). Moreover, *C. perfringens* was observed in samples from subjects in all the residence locations assessed; but was most prevalent in subjects from longstay residential care, with 71.4% of the samples (63.2% of the subjects) being from this residence location, and with a shedding level in excess of 10^6 c.f.u. g^{-1} faeces. Microbiota profiling revealed some significant compositional changes across both the family and genus taxonomic levels between the *C. perfringens* positive and negative data-sets. Levels of culturable *Bifidobacterium* spp. and *Lactobacillus* spp. were significantly ($P<0.05$) lower in the *C. perfringens* positive samples. Sequence based methods also confirmed a significant difference in the *Bifidobacterium* spp. level ($P<0.05$) between both the datasets. Taken together, these data suggest that a high viable count (in excess of 10^6 c.f.u. g^{-1} faeces) of *C. perfringens* in stool samples may be indicative of a less healthy microbiota in the intestine of elderly people in longstay residential care.

5.2 Introduction

Although the composition of the intestinal microbiota can vary greatly between individuals, the dominant population in the adult intestinal microbiota has been shown to be relatively stable over time (Zoetendal *et al.,* 1998). However, it is known that ageing impacts the composition of the gut microbiota (Biagi *et al.,* 2010; Claesson *et al.,* 2011). Recently, it has been shown that variations in the dietary pattern and health parameters also impact the gut microbiota of elderly individuals (Claesson *et al.,* 2012).

Previous studies have demonstrated modulations in the elderly gut microbiota, with a decline in the population of bifidobacteria and lactobacilli (Mitsuoka 1992; Hopkins & Macfarlane 2002; Woodmansey 2007) and an increase in the populations of *Enterobacteriaceae* (Hopkins *et al.,* 2002) and obligate anaerobes such as clostridia (Guigoz *et al.,* 2008). Despite being a normal constituent of the intestinal flora, when overpopulated the *Clostridium* spp. may be responsible for serious infections including mild to severe diarrhoea and pseudomembranous colitis caused by *Clostridium difficile* (Rupnik *et al.,* 2009) or food borne illnesses caused by *C. perfringens* enterotoxin (Sparks *et al.,* 2001).

The ELDERMET project (http://eldermet.ucc.ie) aims to elucidate the intestinal microbiota of 500 elderly (≥65 yr) Irish subjects. Work to date has revealed a core gut microbiota rich in *Bacteroidetes* and *Firmicutes* with large inter-individual variability (n=161) (Claesson *et al.,* 2011) and with a distinctive pattern when compared to the younger Irish population (Claesson *et al.,* 2009; Claesson *et al.,* 2010). In parallel to the sequence based methods, culture dependent techniques enumerated levels of *Bifidobacterium* spp., *Lactobacillus* spp. and *Enterobacteriaceae* in the faecal samples (O'Sullivan *et al.,* 2011; O'Sullivan *et al.,* 2012). This study mainly focuses on *Bifidobacterium* spp. and *Lactobacillus* spp. among other human intestinal microbiota because of their potential probiotic role (Aureli *et a*l., 2011; Turroni *et al.,*

2008).In this respect, several selective media have been previously recommended for the isolation of *Bifidobacterium* spp. (Beerens 1990; Nebra & Blanch 1999; Rada & Petr 2000; Mikkelsen *et al.,* 2003; Simpson *et al.,* 2004; Thitaram *et al.,* 2005; Ferraris *et al.,* 2010) from various sample types (dairy products, faeces, intestinal samples, animal feed). In this study, de Man, Rogosa, Sharpe (MRS) medium supplemented with L-cysteine hydrochloride and mupirocin, termed *Bifidobacterium* selective medium (BSM) was used. This medium was previously used for the growth and enumeration of *Bifidobacterium* spp. from human faecal samples (Rosberg-Cody *et al.,* 2004; Wall *et al.,* 2008).

While one aim of the ELDERMET project was to enumerate bifidobacteria from faecal samples of elderly subjects, the fortuitous isolation of the clinically significant *C. perfringens* at high levels from a sub-set of these elderly subjects motivated the current study. Culture independent techniques were used to compare the microbiota of the *C. perfringens* positive subjects and negative subjects, to determine whether the presence of *C. perfringens* had an impact on the intestinal microbiota as a whole.

5.3 Materials and Methods

Subject recruitment and sample collection

Subjects aged 65 yr and older, were recruited and examined at ELDERMET Clinics at two local hospitals (Cork University Hospital and St. Finbarr's Hospital, Cork) as previously outlined (Claesson *et al.*, 2011). Residence locations of the subjects were defined as long-term residential care (longstay, >6 weeks), short-term rehabilitation centres (rehab, <6 weeks), out-patient in day hospitals and community dwelling. A sub-set of these subjects were undergoing antibiotic therapy within the month prior to faecal sampling. All subjects were screened for the presence of *C. difficile*. Informed consent was obtained from all subjects, or in cases of cognitive impairment, from next of kin in accordance with the local Clinical Research Ethics Committee guidelines. This study was approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals. Faecal samples were collected from some subjects at two further time points (at month 3 (T3) and month 6 (T6)). Stool samples were collected in a sterile container, stored at 5 ^{o}C until transported on ice packs to the laboratory. Storage time varied with a mean time of 28 h.

Bacterial strains, media and growth conditions

Bifidobacterium breve NCIMB 8807 from the (National Collection of Industrial, Marine and Food Bacteria, Aberdeen, Scotland), *B. bifidum* NCIMB 700795, *Lactobacillus delbrueckii* subsp. *bulgaricus* LMG 6901 from the (BCCM/LMG Culture Collection, Gent, Belgium), *C. perfringens* NCTC 8239 from the (National Collection of Type Cultures (NCTC), PHLS Central Public Health Laboratory, UK), *C. perfringens* LMG 10468, *C. perfringens* LMG 11264, a selection of *C. perfringens* strains (n=10) from (Alimentary Health Limited, based at

University College Cork, Ireland; isolated as described previously (Pruteanu *et al.,* 2011)), *Listeria innocua* DPC 3572 from (Teagasc Food Research Centre), *C. perfringens* WIT 495 (Waterford Institute of Technology, Waterford, Ireland) and *Streptococcus agalactiae* LMG 14694 were used in this study. *Bifidobacterium* spp. and *Lactobacillus* spp. were grown anaerobically at 37 ^{o}C for 24 to 48 h on de Man, Rogosa, Sharpe (MRS, Difco Laboratories, Detroit, MI, USA) agar supplemented with 0.05% (w/v) L-cysteine hydrochloride (Sigma Chemical Co., St. Louis, MO, USA) (denoted mMRS agar). Where solid medium was required, 1.5% agar (w/v) (Oxoid Ltd., Basingstoke, Hampshire, UK) was added. To encourage selectivity for *Bifidobacterium* spp. from the faecal samples, the medium was supplemented with 100 µg mupirocin ml^{-1} (Oxoid) as outlined previously (Rosberg-Cody *et al.,* 2004; Wall *et al.,* 2008). The antifungal agent nystatin (50 Units ml^{-1}, Sigma) was also included. All the *Clostridium* strains were grown anaerobically at 37 ^{o}C for 24 to 48 h in Trypticase Soy agar (Merck, Germany) containing 7% sheep blood (TCS Biosciences Ltd, Buckingham, UK). All other bacterial strains were cultivated in Brain Heart Infusion (BHI) medium and were grown aerobically at 37 ^{o}C.

Isolation and enumeration of Bifidobacterium spp. and Lactobacillus spp.

Total culturable *Bifidobacterium* spp. and *Lactobacillus* spp. were enumerated as outlined previously (O'Sullivan *et al.,* 2011). The agar plates were incubated anaerobically for 3 or 5 days at 37 °C for *Bifidobacterium* spp. or *Lactobacillus* spp., respectively. Bacterial counts were recorded as c.f.u. g^{-1} of faeces and results expressed as log c.f.u. g^{-1} of faeces.

Phenotypic characterization of putative C. perfringens isolates

Fructose 6-phosphoketolase assay

A fructose 6-phosphoketolase (F-6-PPK) assay was performed as previously described (Bibiloni *et al.*, 2000). *B. breve* NCIMB 8807 and *Lb. bulgaricus* DPC 6901 were used as positive and negative controls, respectively. A positive reaction, which is typical of *Bifidobacterium* spp., resulted in the development of a red-violet colour.

Stormy Clot assay

C. perfringens was inoculated at 1% (v/v) into 10 ml reconstituted skim-milk in a test tube, which was then plugged with 2% agar and incubated at 37 °C overnight. Coagulation of the milk resulting in a stormy clot was indicative of *C. perfringens* (Mac Faddin 1976).

Reverse CAMP test

C. perfringens cultures were streaked at right angles to within 1-2 mm of a β-haemolytic group B *Streptococcus* (*S. agalactiae* LMG 14694) on sheep blood agar plates. The agar plates were incubated anaerobically for 18-24 h. A positive reverse CAMP test was indicated by the formation of a bow-tie or reverse arrow pattern of haemolysis at the junction of the two cultures (Buchanan 1982).

Mupirocin resistance of C. perfringens

To assess the sensitivity of a selection of *C. perfringens* strains to mupirocin, the putative *C. perfringens* ELDERMET isolates and the *C. perfringens* strains from Alimentary Health Ltd (n=10), together with *C. perfringens* NCTC 8239, *C. perfringens* WIT 495, *C. perfringens* LMG 10468 and *C. perfringens* LMG 11264 were streaked onto agar plates with increasing concentrations of mupirocin (from 100 μg ml^{-1} to 500 μg ml^{-1}). *B. breve* NCIMB 8807 and *B. bifidum* NCIMB 795 were used as positive controls. *Lb. bulgaricus* LMG 6901 and *L. innocua* DPC 3572 were used as negative controls.

Genotypic characterization

Isolation of genomic DNA

Genomic DNA was extracted as outlined previously (Coakley *et al.,* 1996). RNAse (0.75 µl, Sigma) was added to the extracted DNA, incubated for 1 h at 37 ^{o}C, and denatured at 65 ^{o}C for 15 min. The DNA was stored at -20 ^{o}C.

16S rRNA gene sequence analysis to identify bacterial isolates

The full length 16S rRNA gene was amplified from genomic DNA of each strain using 16S Eubacterial primers (Simpson *et al.,* 2003). The complete sequence of the 16S region was determined by Sanger sequencing (Beckman Coulter Genomics). The species was identified by nucleotide alignments (>98% identity cut-off) with deposited species in the NCBI database and using RDP classifier (Wang *et al.,* 2007).

PFGE

Following overnight incubation, 200 µl of each culture was inoculated into 10 ml of BHI broth (Merck, Darmstadt, Germany), which had previously been boiled and cooled under anaerobic conditions, and grown for 5 h at 37 ºC. Genomic DNA was prepared in agarose plugs, lyzed and subsequently digested with *Sma*1 as described previously (Lukinmaa *et al.,* 2002). Electrophoresis was performed using the Biorad Chef-DR II instrument for 20 h at 5.5 Vcm^{-1} with initial and final pulse times of 0.5 s to 40 s respectively. A lambda ladder PFG marker (New England BioLabs Inc., UK) ranging from 48.5 to 727.5 kb was used as the molecular weight marker and DNA macro-restriction profiles visualised using the Alpha Imaging system. The DNA banding patterns were analysed with Bionumerics software version 6.5 (Applied Maths). The Dice coefficient of similarity was calculated and the unweighted pair group method with arithmetic averages (UPGMA) was used for cluster analysis with optimisation and

position tolerance set at 1.5%. A cut-off at 90% similarity of the Dice co-efficient was used to indicate identical PFGE patterns.

Assessment of toxin production by the C. perfringens isolates

Detection of the cpe enterotoxin gene

PCR was used to detect the presence of the *cpe* enterotoxin gene in the *C. perfringens* isolates as outlined by Lukinmaa *et al*. (Lukinmaa *et al.,* 2002). *C. perfringens* NCTC 8239 was used as a positive control. Presence of the *cpe* enterotoxin gene was indicated by the amplification of a 933 bp fragment.

Enterotoxin immunoassay

Prior to the entertoxin test, all isolates were grown in modified Duncan-Strong sporulation medium (Duncan & Strong 1968) to promote enterotoxin production. Enterotoxin production was assessed with the reverse passive latex agglutination kit (PET_RPLA kit, Oxoid).

Detection of other toxin genes

Primers corresponding to the alpha toxin (*cpa*), beta toxin (*cpb*) or epsilon toxin (*etx*) genes were used as previously described (Wu *et al.,* 2009) to assess the *C. perfringens* isolates for toxin production capability. In addition, production of the β2 toxin, encoded by *cpb2* gene was assessed as previously outlined (Greco *et al.,* 2005).

Amplicon sequencing and bioinformatics

DNA was extracted from faecal samples according to a standard protocol (Qiagen, West Sussex, UK). Pyrosequencing yielded an average of 32,677 reads per sample. Amplicon sequencing analysis was performed as previously outlined (Claesson *et al.,* 2011). Trimmed

FASTA sequences were then RDP classified from phylum down to genus level (Wang *et al.*, 2007) using Bergey's taxonomy (Garrity *et al.,* 2004). Species level data was generated using a combination of the Qiime package (http://qiime.org/) and BLAST. First, individual reads were grouped into operational taxonomic units (OTUs) at 97% sequence similarity using UCLUST, and then a representative sequence was chosen from each OTU using the default method. These representative sequences were analysed using BLASTed against a custom-made species database to identify species from the genus *Clostridium* and any other species of interest. OTU assignment, clustering, correspondence analysis were implemented with Qiime. The data generated from BLAST and the RDP Classifier were stored in a MYSQL database.

Statistical Methods

Statistical analysis for the bacteriological culture data was performed using Minitab Release 15.1.1.0 (Minitab Inc. 2007). Significance was calculated with the Mann-Whitney test and statistical significance was accepted at $P<0.05$. The R statistical software version 2.13.1 was used for the amplicon sequencing statistical analyses. For each subject, all reads at phylum, family and genus level were converted to percentage values of the total number of reads. The Mann-Whitney *U* test was used to test for statistical differences between the *C. perfringens* positive data-set (n=27) and the *C. perfringens* negative data-set (n=254) for each taxon in the phylum, family and genus level data-sets. The P-values were adjusted to account for multiple testing. Two related methods were used – estimation of q-values using the FDR (false discovery rate) function in R for genus level and Benjamini and Hochberg correction for phylum and family levels (Benjamini & Hochberg 1995).

5.4 Results and Discussion

Prevalence of C. perfringens in the faecal microbiota of older subjects

Of 368 faecal samples analysed (from 184 elderly subjects at T0, T3 and/or T6) during this study, 28 samples (7.6% of samples from 19 subjects; Table 1) upon culture, yielded colonies that were atypical of *Bifidobacterium* spp. on the *Bifidobacterium* selective medium (BSM). These atypical colonies were translucent, irregular shaped (4-5 mm diameter), yellow colonies with a distinct odour; as opposed to typical bifidobacterial colonies, which were white/cream, convex and circular in shape (2-3 mm diameter). From these 19 subjects, atypical colonies were observed at two time points for 5 subjects and at all three time points for 2 subjects, indicating the stability of the presence of this bacterium over time in the intestinal environment of some subjects. In some cases (n=15), these isolates grew on the selective medium in conjunction with the *Bifidobacterium* spp., but in other cases (n=13) only these unusual colonies were observed. However, they were reliably distinguishable from bifidobacteria both macroscopically and microscopically (larger box-car shaped rods). Moreover, these isolates tested negative in a fructose-6-phosphate phosphoketolase assay (a positive result is indicative of *Bifidobacterium* spp.) demonstrating that they were not bifidobacteria.

Phenotypic and genotypic characterisation of C. perfringens

Approximately 3 to 4 colonies per sample were taken for further characterisation. All of the atypical isolates demonstrated phenotypic traits characteristic of *C. perfringens* such as the stormy clot formation when grown in 10% skim-milk and an arrow-head shaped haemolytic pattern in the reverse CAMP test. All isolates were subsequently confirmed as *C. perfringens* using 16S rRNA sequencing. *C. perfringens* isolates that have the potential to cause *C.*

perfringens associated diarrhoea, by the production of an enterotoxin, are normally identified by confirming the presence of the *cpe* gene. However none of the isolates from this study were positive for the *cpe* gene, indicating that all were non-enterotoxigenic. This was further confirmed by testing all the isolates for enterotoxin production using a RPLA kit, which demonstrated that none produced enterotoxin. It is reported that CPE-producing *C. perfringens* are only occasionally isolated, representing < 5% of all *C. perfringens* isolates (Smedley III & McClane 2004).

Genotyping of toxin produced by C. perfringens

In general, all *C. perfringens* types produce α toxin; additionally, type B *C. perfringens* produce β and ε toxins, type C produce β toxin, type D produce ε toxin and type E produce ι toxin (Petit *et al.,* 1999). Type A *C. perfringens* has been shown to predominate in the environment and can cause gas gangrene in humans, while the other types (B-E) are usually restricted to animals. As type E strains rarely cause disease in humans (Petit *et al.,* 1999; Smedley III & McClane 2004), none of the *C. perfringens* isolates from this study were tested for this toxin. The *cpa* gene, encoding α toxin, was detected by PCR in all of the isolates, but none of the other genes, including the *cpb* gene (encoding β toxin in types B and C), or the *etx* gene (encoding ε toxin in types B and D) were detected. This confirms that all the isolates belonged to *C. perfringens* type A. In addition to the α toxin, three of the isolates (EM156-T6, EM177-T0, and EM200-T6) produced β2 toxin and were then classified as *C. perfringens* type A and β2 toxin positive. PCR and sequence analysis revealed 100% amino acid identity to the *cpb2* structural gene of the previously characterised β2 toxin (Shimizu *et al.*, 2002) indicating the presence of this toxin in all three isolates. Although the β2 toxin positive *C. perfringens* have been linked with intestinal disorders in animals (Waters *et al.,* 2003) and humans (Fisher *et al.,* 2005), their presence does not always result in an adverse effect on the intestine (Lebrun

et al., 2007; Carman *et al.*, 2008). In this respect, the impact of the β2 toxin appears to be subject dependent and circumstance related.

Relatedness and stability of C. perfringens isolates

Genomic fingerprinting by PFGE is a valuable tool when establishing strain similarities and has been used for strain identification in *C. perfringens* related diarrhoeal outbreak situations (Lukinmaa *et al.*, 2002). Diversity among the *C. perfringens* isolates from this study was observed, as they were resolved into 17 distinct strains (A-Q) using the Dice coefficient (≥90 % similarity). This diversity is noteworthy because these strains were isolated from 19 *C. perfringens* positive subjects residing in different residence locations.

The *C. perfringens* PFGE profiles from four subjects - EM156-T0, EM165-T0, EM168-T3 and EM169-T0 were confirmed as being 100% identical (Fig. 1). Even though these subjects were sampled over a 9 month period, all were from the same residence location (long-term residential care) and even the same ward. Similarly, the profiles from subjects EM186-T0 and EM200-T0 were 100% identical. Interestingly at the time of sampling these two subjects were residing on the same ward and were sampled within 15 days of each other.

In some cases, the persistence of the *C. perfringens* strains was confirmed by isolating similar pulsotypes from the same subject at multiple time points as recorded for EM082 (T3 and T6), EM151 (T0, T3 and T6), EM156 (T0 and T6), EM168 (T3 and T6) and in EM182 (T3 and T6), indicating their stability of colonization over time. However, diversity among the strains was observed over time in a few subjects. For instance, the faecal samples collected at all three time points for EM159, showed strains with two distinct PFGE patterns (one at T0 and another at each of T3 and T6). In EM200, where two distinct strains of *C. perfringens* were observed at T0 and T6, *C. perfringens* was not detected at the intermediate time (T3). This subject was on the same ward at all time-points but was receiving antibiotics at T0 and T6 (Fig 1); the time-

points where *C. perfringens* was detected. Overall, the PFGE analysis revealed a diverse range of pulsotypes from the different residence locations.

Mupirocin resistance of C. perfringens isolates and co-association with bifidobacteria

In general, supplementation of selective media with mupirocin enables the recovery of *Bifidobacterium* spp. from faecal samples. However, in this study *C. perfringens* was isolated on the BSM despite using a high concentration of mupirocin (100 μg ml^{-1}). The growth of these isolates on this selective medium was reasonably distinguishable, both macroscopically and microscopically. To assess the resistance of *C. perfringens* in general to mupirocin, a number of strains including *C. perfringens* NCTC 8239, *C. perfringens* LMG 10468, *C. perfringens* LMG 11264, a selection of *C. perfringens* strains (n=10) and *C. perfringens* WIT 495 were grown on agar plates with varying concentrations of mupirocin. All the *C. perfringens* isolates assessed grew at all mupirocin concentrations up to 500 μg ml^{-1}, indicating a high level of resistance (Table 2). This high level of mupirocin resistance may provide an opportunity to exploit the antibiotic as a selective agent for *C. perfringens*. However, this study does point to the limitations of using mupirocin as a selective agent for *Bifidobacterium* spp.

During the broader ELDERMET study, BSM has been used to enumerate *Bifidobacterium* spp. from 366 faecal samples (O'Sullivan *et al.*, 2012). The levels of *Bifidobacterium* spp. were below the limit of detection (<3.0 log c.f.u. g^{-1}) in 5.2% of these faecal samples. When analysing the samples, where *C. perfringens* was detected on BSM (n=28), *Bifidobacterium* spp. were below the limit of detection for 46.0% of these samples. This indicates that the presence of the *C. perfringens* in the elderly is inversely associated with the numbers of *Bifidobacterium* spp. recovered. In addition, when the levels of culturable *Bifidobacterium* spp. and *Lactobacillus* spp. were compared in the *C. perfringens* positive and *C. perfringens*

negative groups, significantly lower levels ($P<0.05$) of both species were observed in the *C. perfringens* positive group.

Prevalence of C. perfringens as a function of subject health status

C. perfringens was detected in subjects from all of the residence locations assessed (longstay, rehabilitation, day hospital and community) and at varying levels (4.0 to 9.5 log c.f.u.g^{-1} faeces; Table 1). However, the longstay subjects accounted for 71.4% of the samples where *C. perfringens* was detected. This is in agreement with the study by Stringer *et al*. (1985), reporting high numbers of *C. perfringens* in elderly hospitalized patients (Yamagishi *et al.*, 1976; Stringer *et al.*, 1985). It has also been reported that the carriage rate of *C. perfringens* was significantly higher in the elderly (Benno *et al.*, 1989). *C. perfringens* type A food poisoning outbreaks are usually reported in institutionalised settings involving larger numbers of elderly patients (Smith 1998; Lund *et al.*, 2000).

Microbiota alterations and C. perfringens culture status

Microbiota composition analysis by amplicon sequencing was used to determine whether the presence of *C. perfringens* was associated with an altered intestinal microbiota. Pyrosequencing data were available for 27 *C. perfringens* positive samples and 254 *C. perfringens* negative samples. The taxonomical profile of each sample was elucidated using a combination of BLAST and RDP classifier and resulting taxa were compared at the phylum, family and genus levels. While there were some observed differences at the phylum level, none of these were statistically significant. The major observed differences were; *Bacteroidetes and Proteobacteria* proportions of 52.2% and 2.7%, respectively in the *C. perfringens* negative data-set compared to 48.4% and 1.5% in the *C. perfringens* positive data-set, whilst *Firmicutes* were higher at 44.0% in the negative population compared to 48.6% in the positive population. At the family level, some significant changes in compositional data were observed; Members

of the families *Clostridiaceae* (P=0.0003), *Eubacteriaceae* (P=0.0033), *Coriobacteriaceae* (P=0.0103), *Enterobacteriaceae* (P=0.0337), *Desulfovibrionaceae* (P=0.0374) and *Porphyromonadaceae* (P=0.0383) were higher in the *C. perfringens* positive data-set compared to the negative data-set. Members of the families *Lachnospiraceae* (P=0.0011) and *Veillonellaceae* (P=0.0383) were lower in the *C. perfringens* positive subject microbiota when compared to the negative subjects (Fig. 2 and Table 3).

There were many significant differences in the proportions of individual genera between the *C. perfringens* positive and negative data-sets; 10 genera appeared to be higher in the *C. perfringens* positive population including *Clostridium* spp. (P<0.0001), *Eubacterium* (P=0.0022), *Parabacteroides* (P=0.0084). In contrast, *Coprococcus* (P=0.0233), *Roseburia* (P=0.0006), *Catenibacterium* (P=0.0363) and *Bifidobacterium* (P=0.0363) were lower in the positive group (Fig. 3 and Table 3).

Levels of *Bifidobacterium* spp. were significantly lower in the *C. perfringens* positive population (P=0.0363; Table 3), which is in agreement with the culture-based techniques as discussed above. In contrast to the culture results, no significant difference was observed in levels of *Lactobacillus* spp. (P=0.9375). To establish the similarities in the total microbial composition between the *C. perfringens* positive and negative samples, correspondence analysis on the proportions at phylum, family and genus level was employed. This failed to show any major differences in the microbiota composition between these two groups (Fig. S1- provided as Supplementary data).

In conclusion, this study focuses on the isolation of high levels of *C. perfringens* from faecal samples of elderly Irish subjects, on a selective medium aimed at isolating *Bifidobacterium* spp. The subjects were from different residence locations with the isolation of *C. perfringens* being more frequent in subjects in long-term residential care and having a higher shedding

level (up to 10^9 c.f.u. g^{-1}). The isolation of similar *C. perfringens* strains from individual subjects located in the same residence location suggests horizontal transmission within the hospital. The presence of *C. perfringens* negatively correlated with the numbers of *Bifidobacterium* spp. recovered, suggesting that *Bifidobacterium* spp. replacement warrants investigation as a possible means of reducing *C. perfringens* carriage. However, overall, there was no significant diversity among the microbial community between the *C. perfringens* positive and negative subjects.

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Table 1. Data on the 28 ELDERMET samples from which *C. perfringens* was cultured on BSM

No.	Subject	Time (month*)	Residence location	Antibiotic therapy†	*Bifidobacterium* spp. (log c.f.u. gram^{-1})	*Lactobacillus* spp. (log c.f.u. gram^{-1})	*C. perfringens* (log c.f.u. gram^{-1})	*C. perfringens* Pulsotypes
1	EM059-T3	T3	day hospital	yes	<3.00‡	5.45	6.15	A
2	EM063-T6	T6	community	no	<3.00	6.00	6.30	B
3	EM082-T3	T3	community	no	6.30	7.42	6.18	C
4	EM082-T6	T6	community	no	5.70	7.87	6.85	C
5	EM095-T0	T0	community	no	<3.00	4.77	5.11	D
6	EM124-T3	T3	longstay (CD+)§	no	<3.00	<3.00	5.79	E
7	EM133-T0	T0	rehab	yes	7.78	6.64	8.15	F
8	EM144-T6	T6	longstay	no	4.60	4.23	7.10	G
9	EM151-T0	T0	longstay	no	8.41	7.07	9.46	H
10	EM151-T3	T3	longstay	no	<3.00	4.86	8.32	H
11	EM151-T6	T6	longstay	no	6.60	5.54	7.59	H
12	EM156-T0	T0	longstay (CD+)	no	5.81	5.83	6.13	I
13	EM156-T6	T6	longstay (CD+)	no	8.11	7.09	8.69	I
14	EM159-T0	T0	longstay	no	<3.00	<3.00	7.81	J
15	EM159-T3	T3	longstay	no	<3.00	<3.00	4.53	K
16	EM159-T6	T6	longstay	no	4.77	<3.00	5.73	K
17	EM165-T0	T0	longstay	yes	<3.00	5.43	6.62	I
18	EM168-T3	T3	longstay	no	<3.00	5.08	8.26	I
19	EM168-T6	T6	longstay	no	7.89	4.99	7.57	I
20	EM169-T0	T0	longstay	no	<3.00	<3.00	8.15	I
21	EM177-T0	T0	community	no	5.48	5.83	6.61	L
22	EM182-T3	T3	longstay	no	7.60	<3.00	7.60	M
23	EM182-T6	T6	longstay	no	7.03	3.00	5.90	M
24	EM186-T0	T0	longstay (CD+)	no	<3.00	7.61	6.73	N
25	EM200-T0	T0	longstay	yes	5.45	4.64	5.36	N
26	EM200-T6	T6	longstay	no	3.00	3.00	4.00	O
27	EM297-T0	T0	longstay	yes	<3.00	-	6.92	P
28	EM322-T0	T0	rehab	No	<3.00	-	7.18	Q

*T0=Month 0, T3=Month 3 and T6=Month 6; †on antibiotics within 1 month of attending the ELDERMET Clinic; ‡<3.00 indicates the lower limit of detection; §CD+ *C. difficile* detected in the faecal sample.

Table 2. Details of *C. perfringens* strains with resistance to 500 µg mupirocin ml^{-1}.

No.	*C. perfringens* strain ID	Source
1	UC 3.1	subject with ulcerative colitis, UC3
2	UC 8.1	subject with ulcerative colitis, UC8
3	UC 18	subject with ulcerative colitis, UC18
4	CD 19.1	subject with Crohn's disease, CD19
5	AH 36.1	healthy volunteer, AH36
6	AH 36.2	healthy volunteer, AH36
7	AH 55.1	healthy volunteer, AH55
8	AH 55.2	healthy volunteer, AH55
9	AH 95.2	healthy volunteer, AH95
10	AH 95.3	healthy volunteer, AH95
11	NCTC 8239	unknown
12	LMG 10468	lamb, intestinal contents
13	LMG 11264	bovine
14	WIT 495	pig manure composite

Table 3. Significant differences in faecal microbiota abundance between the *C. perfringens* positive and *C. perfringens* negative subjects. Only taxa with significant differences are shown.

	P-value*	**Observation in *C. perfringens* positive data-set†**
Family		
Clostridiaceae	0.0003	increase
Lachnospiraceae	0.0011	decrease
Eubacteriaceae	0.0033	increase
Coriobacteriaceae	0.0103	increase
Enterobacteriaceae	0.0337	increase
Desulfovibrionaceae	0.0374	increase
Porphyromonadaceae	0.0383	increase
Veillonellaceae	0.0383	decrease
Genus		
Clostridium	0.0000	increase
Acetanaerobacterium	0.0005	increase
Butyricicoccus	0.0005	decrease
Roseburia	0.0006	decrease
Anaerostipes	0.0018	increase
Eubacterium	0.0022	increase
Lactonifactor	0.0033	increase
Anaerotruncus	0.0070	increase
Parabacteroides	0.0084	increase
Coprobacillus	0.0116	increase
Ethanoligenens	0.0233	increase
Escherichia	0.0233	increase
Coprococcus	0.0233	decrease
Parasporobacterium	0.0233	decrease
Bifidobacterium	0.0363	decrease
Anaerosporobacter	0.0363	decrease
Catenibacterium	0.0363	decrease

*<0.05 is considered a significant result; †compared to the *C. perfringens* negative data-set.

Figure 1. Dendographic analysis of PFGE profiles of *C. perfringens* from elderly Irish subjects and their pulsotypes.

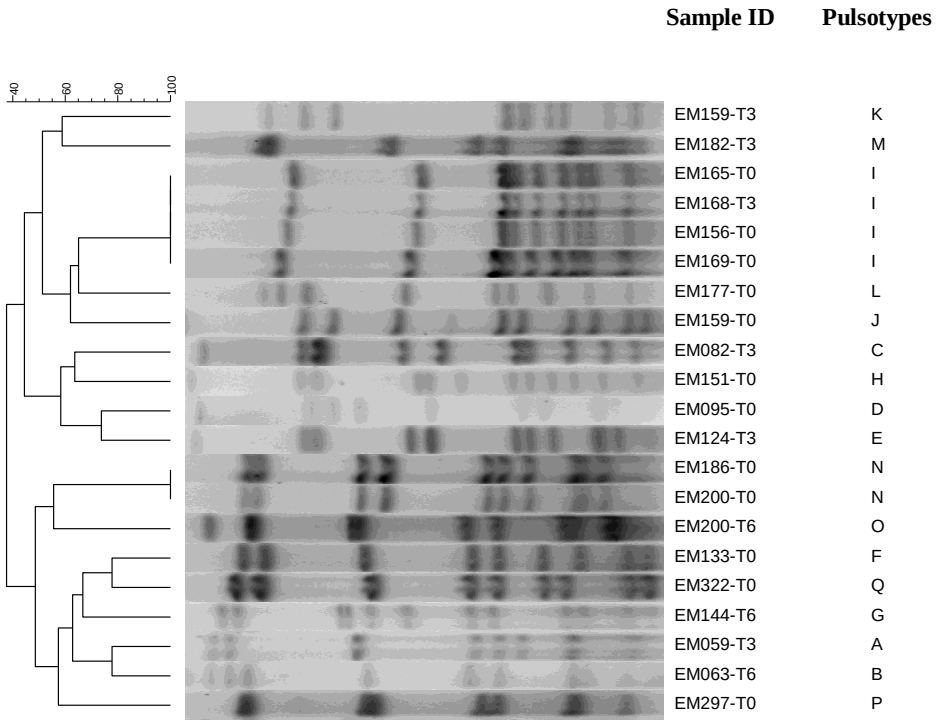
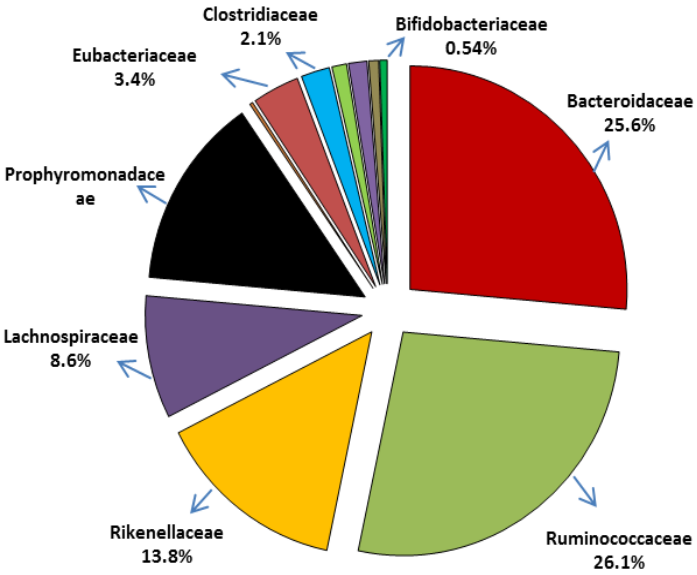


Figure 2. Family level composition of the faecal microbiota from (a) *C. perfringens* positive subjects (aggregated) and (b) the *C. perfringens* negative subjects (aggregated).

(a)



(b)

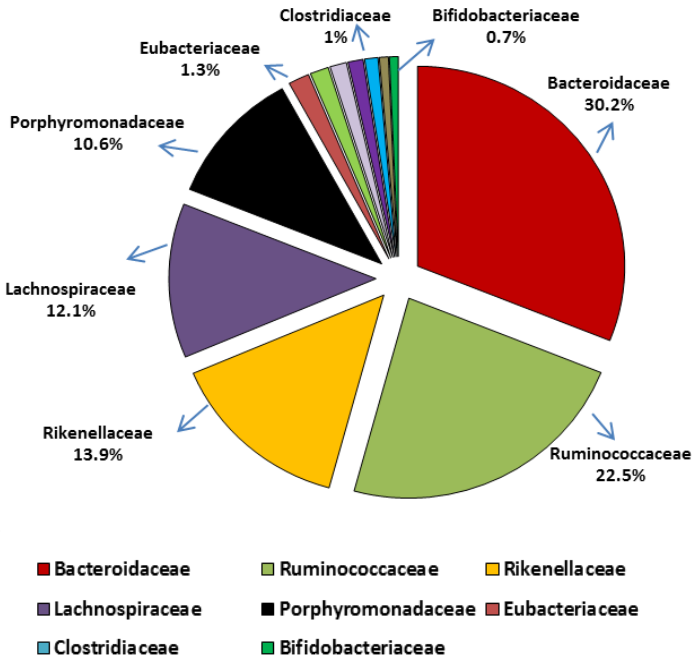
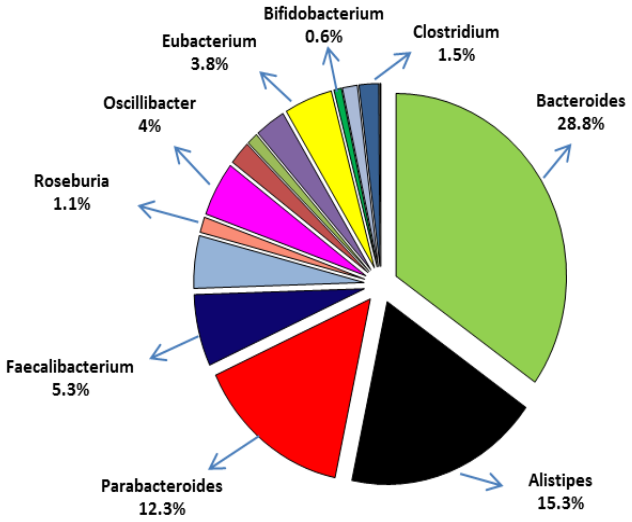
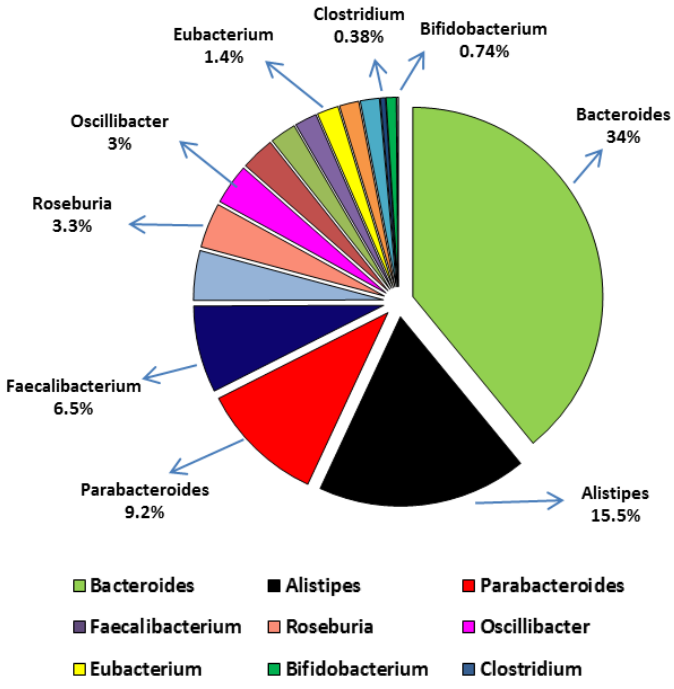


Figure 3. Genus level composition of the faecal microbiota of the (a) *C. perfringens* positive subjects compared to the (b) *C. perfringens* negative subjects, expressed as percentage reads.

(a)



(b)

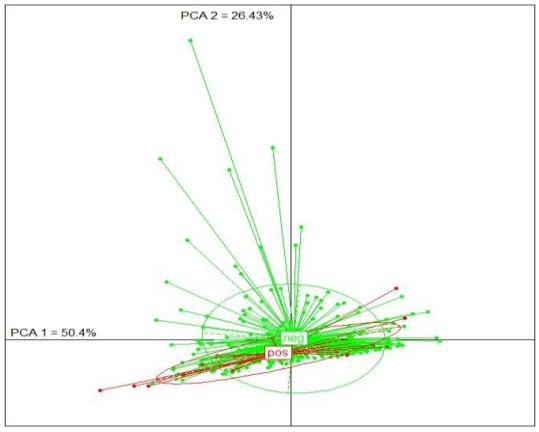


SUPPLEMENTARY DATA

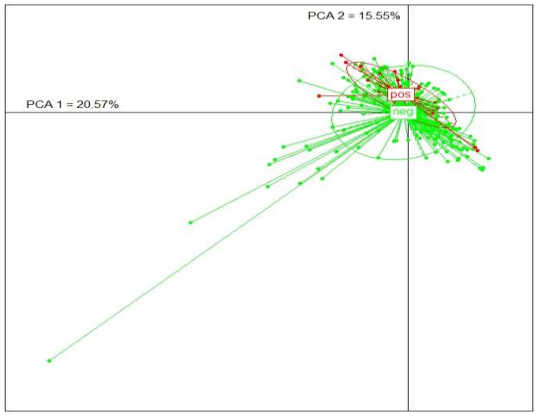
Supplementary Figures for Chapter 5

Supplementary Figure S1. Correspondence analysis of the proportions of (a) phylum, (b) family and (c) genus level of *C. perfringens* positive (■) and *C. perfringens* negative groups (■).

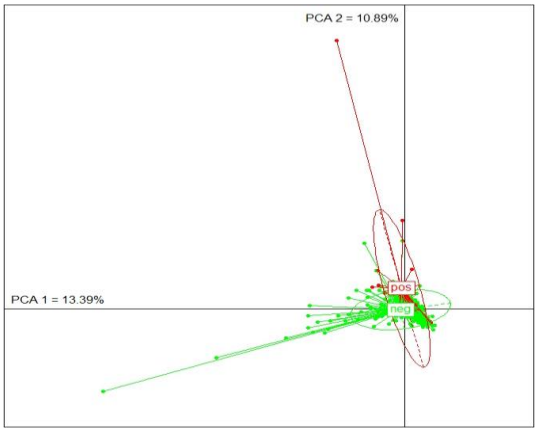
(a) Phylum



(b) Family



(c) Genus



CHAPTER 6

ISOLATION AND MOLECULAR CHATACTERIZATION OF CIPROFLOXACIN RESISTANT LACTIC ACID BACTERIA FROM EDERLY IRISH SUBJECTS

6.1 Abstract:

Lactic acid bacteria (LAB) isolated from over one hundred faecal samples from elderly Irish subjects as a part of ELDERMET project (http://eldermet.ucc.ie) were investigated for their resistance against the clinically important antibiotics, ciprofloxacin and amoxicillin. Ciprofloxacin represents the widely used members of the fluoroquinolone family. Screening over 25,000 colonies identified 21 LAB isolates with increased ciprofloxacin resistance; however, none of the isolates screened exhibited resistance against amoxicillin. The predominant isolates with increased ciprofloxacin resistance were identified by 16S rRNA sequencing as *Lactobacillus gasseri*, *Enterococcus* and *Pediococcus* spp. The minimum inhibitory concentrations (MIC) values for all of the isolates exhibiting ciprofloxacin resistance ranged between 90-300 µg/ml. The *parC* and *gyrA* genes of the quinolone resistance determining region (QRDR) for the isolates with increased ciprofloxacin resistance were analyzed using PCR primers specific for the respective genes. We found no typical mutations in the amino acid codons of quinolone-resistance determining regions of the genes encoding *gyrA*. However, in *parC*, a single amino acid change related with the substitution of Ser at position 80 to isoleucine was determined. These resistant strains could potentially be co-administered with ciprofloxacin to maintain a positive microbiota which reduces the gastrointestinal side effects in the elderly subjects, being treated with antibiotics.

6.2 Introduction:

Lactic Acid Bacteria (LAB) are a heterogeneous group of bacteria found widely in nature. Indeed, they are commonly found among the resident microbiota of the gastrointestinal tract (GIT) of humans and animals. Members of LAB have been exploited for millennia in the production of fermented foods and beverages (Leroy & De Vuyst 2004). In addition, LAB have been used as probiotic bacterial cultures (Collins *et al.* 1998), which are intentionally added to the diet to positively influence human health and have been shown to have a number of health benefits associated with ingestion of particular strains. However, concerns have been raised in relation to LAB being a potential source for transferring antibiotic resistance to other bacterial strains including pathogenic microorganisms (Danielsen & Wind 2003; Mathur & Singh 2005; Gevers *et al.* 2006). Henceforth, evaluating potentially probiotic strains for the presence of transmissible antibiotic resistance genes is an important safety measure.

In recent years, researchers have focused on characterizing the presence of antibiotic resistance in LAB of different origin (Axelsson *et al.* 1988; Scott *et al.* 2000; Delgado *et al.* 2005; Belletti *et al.* 2009). Among the LAB, the subject of intense study is the antibiotic resistance among enterococci, as they play a major role in imparting serious infections in humans such as urinary tract infections (Petersen & Jensen 2006; Hummel *et al.* 2007a; Sood *et al.* 2008; Ribeiro *et al.* 2011; Chang *et al.* 2012). The molecular determinants underlying antibiotic resistance among lactobacilli is of growing interest (Hummel *et al.* 2007b; Fukao *et al.* 2009).

Bacterial resistance to antibiotics can either be natural (intrinsic or innate), acquired (through the acquisition of exogenous DNA) or mutational (mutation of indigenous genes) (Ashraf & Shah 2011). Intrinsic resistance is an inherent trait of bacteria and can be due to a number of

mechanisms including reduced permeability of certain antibiotics across the bacterial cell wall, absence of the antibiotic affinity targets (Džidić *et al.* 2008). Intrinsic resistance to antibiotics is generally not horizontally transferable as it is chromosomally encoded. In contrast, a bacterial strain can acquire resistance either by mutation in genes or by the uptake of resistance genes by horizontal transfer from other bacteria (Egervärn *et al.* 2009).

Although LAB have a long history of safe use and have acquired the 'generally recognized as safe (GRAS) status, some LAB have demonstrated intrinsic or acquired antibiotic resistance (Levy 1997; Delgado, Flórez *et al.* 2005; Kastner *et al.* 2006; Ouoba *et al.* 2008). For example, many lactobacilli seem to be intrinsically resistant to quinolones, for example, ciprofloxacin and nalidixic acid, by an unidentified resistance mechanism (Hummel, Hertel *et al.* 2007b).

Fluoroquinolones have been used for antibacterial therapy for almost two decades. Of these, ciprofloxacin, gatifloxacin, ofloxacin and moxifloxacin represent the widely used members of the family (Stahlmann & Lode 2010). Ciprofloxacin is a second generation fluoroquinolone, that inhibits the activity of the bacterial enzymes DNA gyrase (encoded by *gyrA* and *gyrB)* and DNA topoisomerase (encoded by *parC* and *parE* - referred to as *grlA* and *grlB* respectively in *Staphylococcus aureus*), which are essential for DNA replication (Cambaul & Gurmannz 1993; Blanche *et al.* 1996). DNA gyrase and topoisomerase IV share extensive amino acid sequence homology, including highly conserved regions in both subunits A and B (Bagel *et al.* 1999). Interestingly, the quinolone target varies in gram positive and gram negative microorganisms, being primarily DNA gyrase for gram negative bacteria, and topoisomerase IV in gram positive bacteria (Ruiz 2003).

Ciprofloxacin, being one of the most important member of the quinolone family have been tremendously used in the treatment of urinary, respiratory, gastrointestinal and skin infections (Dalhoff 2012). They are particularly effective, given their broad spectrum activity against different microbes, and for their use as both oral and parenteral preparations and for their relative safety (Rodríguez-Julbe *et al.* 2010). However, resistance to fluoroquinolone has emerged relatively rapid among almost all gram positive and gram negative species. The most common mode of resistance to quinolones is through mutations in the genes, *gyrA* and *parC*, which encode for subunit A of the DNA gyrase and topoisomerase IV, respectively. These regions are referred to collectively as the Quinolone Resistance Determining Region (QRDR).

The ELDERMET consortium (http://eldermet.ucc.ie) was established in 2008 and aims to explicate the intestinal microbiota of 500 elderly Irish subjects. Work to date has revealed links between the microbial composition, health and diet of the elderly Irish population (Claesson *et al.* 2011; Claesson *et al.* 2012). In addition, investigating the impact of antibiotic therapy on the composition of the intestinal microbiota of elderly Irish subjects (n=185) revealed a notable reduction in the numbers *of Bifidobacterium* spp., emphasizing the potential need of probiotics or prebiotics to rectify this aspect of the intestinal microbial composition (O'Sullivan *et al.* 2013). The objective of this study was not only to screen and isolate lactic acid bacterial strains from elderly Irish subjects with increased resistance to clinically important antibiotics, including ciprofloxacin and amoxicillin, but to also elucidate the underlying genetic mechanism. These antibiotics were chosen for this study mainly because of their intensive use in the elderly in treating a wide range of infections.

6.3 Materials and Methods

Subject recruitment and sample collection

Subjects aged ≥ 65 years were recruited and examined at ELDERMET clinics at two local hospitals (Cork University Hospital, Cork, Ireland and St. Finbarr's Hospital, Cork, Ireland) as outlined previously (Claesson, Cusack *et al.* 2011). The subjects included patients in long-term institutional care (long-stay, >6 weeks), short-term rehabilitation centers (rehab, <6 weeks), outpatient day hospitals and community dwelling subjects. A subset of these subjects was undergoing antibiotic therapy within the month prior to faecal sampling. A total of 107 samples from 91 elderly subjects were assessed. In addition to the initial sample (T0), faecal samples were collected from some subjects at two further time points - at month 3 (T3) and month 6 (T6). Stool samples were collected in a sterile container and stored at 5^{o}C until transported on ice to the laboratory.

Isolation and enumeration of bacterial isolates from faecal samples

All of the faecal samples were processed as outlined previously (O'Sullivan *et al.* 2011). To enumerate total culturable LAB strains, the dilutions were spread plated (100µl) onto de Man, Rogosa, Sharpe agar supplemented with 0.05% L-cysteine hydrochloride (Sigma Chemical Co., St Louis, MO, USA) (denoted mMRS agar). For the enumeration of bifidobacteria, the dilutions were spread plated (100µl) onto mMRS agar supplemented with mupirocin (Oxoid) at a concentration used in our earlier study (O'Sullivan, Coakley *et al.* 2011). mMRS medium incorporating the antibiotics of interest were used to isolate antibiotic resistant strains. The antibiotics, Ciprofloxacin and Amoxicillin (Sigma) were added to the selective medium at a concentration of 75µg ml^{-1}, following an initial screening, where several samples were assessed

on mMRS agar with a range of antibiotic concentrations (25µg ml^{-1} to 150µg ml^{-1}). The concentration of 75µg ml^{-1} was then chosen as the optimum concentration for both antibiotics, as this concentration yielded a considerable number of amoxicillin and ciprofloxacin resistant isolates. The agar plates were incubated anaerobically at 37°C for 3 or 5 days for *Bifidobacterium* spp. and *Lactobacillus* spp. respectively. Bacterial counts were recorded as colony forming units (CFU) per gram of faeces and the results expressed as log CFU g^{-1} faeces. Bacterial colonies that grew on the mMRS agar supplemented with the antibiotics were selected and cultured in mMRS broth for 24 hr at 37°C. These isolates were then stocked in the ELDERMET Culture Collection for further characterization.

Genetic characterization of bacterial isolates

Genomic DNA was extracted as outlined previously (Coakley *et al.* 1996). RNase (0.75µl; Sigma) was added to the extracted DNA, incubated for 1 hr at 37°C and denatured at 65°C for 15 min. Bacterial isolates were identified by amplifying the full-length 16S rRNA gene using the 16S eubacterial primers (Simpson *et al.* 2003). The complete sequence of the 16S rRNA gene region was determined by Sanger sequencing (Beckman Coulter, Essex, UK). The resulting sequences were compared with the deposited GenBank database using the BLAST program and the identity of the isolates was determined on the basis of the highest scores (>98%). The presence of antibiotic resistance genes for ciprofloxacin (*gyrA* and *parC*) were amplified by PCR using previously designed primers (Hummel, Hertel *et al.* 2007b). PCR products and the deduced amino acid sequences were aligned with those retrieved from the GenBank database using the Lasergene 6 software program.

Minimum inhibitory concentration (MIC) assays

MIC determinations were carried out in triplicate in 96 well micro-titer plates. For preparation of antibiotic stock solutions, ciprofloxacin was dissolved in 0.01M HCl and amoxicillin in water to a concentration of 30mg ml^{-1}. Fresh overnight bacterial strains were diluted to a final concentration of 10^6 CFU ml^{-1}. A further (1:1000) dilution was made into double strength mMRS broth, and a 0.1 ml inoculum was added into each well containing the serially diluted antibiotics. Positive (mMRS medium with bacterial culture) and negative (mMRS medium without bacterial culture) controls were included in the micro-titer plates. The 96 well plates were incubated in an anaerobic chamber at 37°C, with the exception of *Enterococcus* spp., which were incubated aerobically at 37°C. MIC was interpreted as the minimum concentration of antibiotic required for total inhibition of visible bacterial growth after incubation for 48 hr.

Pulsed-field gel electrophoresis (PFGE) of bacterial isolates

Pulsed-field gel electrophoresis of the resistant isolates was performed as previously described (Simpson *et al.* 2002) using the *Apa*1 restriction endonuclease. A low-range molecular weight DNA marker (New England Biolabs; 4.36-194.0 Kb) was used to determine band size, and the DNA macro restriction profiles were visualized using the Alpha Imaging System. For each PFGE pattern observed, one strain with a high MIC for ciprofloxacin was selected for further characterization.

6.4 Results and Discussion

The emergence of bacterial resistance to antibiotics presents a serious public health threat. Resistance can be an inherent property of the bacteria themselves or it can be acquired. Acquired bacterial antibiotic resistance can be caused by gene mutation or acquisition of foreign resistance genes or by both mechanisms. A variety of genes can be involved in antibiotic resistance as there are several targets or pathways for the antibiotic entry into the bacterial cell and each access point requires expression of several genes (Džidić, Šušković *et al.* 2008). Ciprofloxacin and other quinolones are considered the most important antibiotics mainly because of their low level resistance (Cirz *et al.* 2005). However, resistance to these antibiotics is evolving at an alarming rate due to alterations in the targets of quinolones through mutations in gyrase or topoisomerase IV and/or an over expression of the efflux system (Džidić, Šušković *et al.* 2008). Both of these mechanisms are chromosomally mediated. It has been documented that intrinsic resistance and resistance by mutations in the chromosomal genes presents a low risk of horizontal dissemination (Ammor *et al.* 2007).

Isolation and species identification of antibiotic resistant bacteria

Initial screening over 25,000 isolates from 107 faecal samples (from 91 elderly subjects) resulted in the detection of 21 isolates (from 15 subjects) with increased antibiotic resistance to ciprofloxacin, representing an isolation frequency of (0.08% from total culturable bacteria). Further analysis of the 21 isolates by PFGE resulted in five individual strains (from five different subjects) with distinct PFGE profiles (Figure 1), corresponding to ciprofloxacin resistance frequency of 0.02%. None of these five subjects were on ciprofloxacin or amoxicillin. Strains showing similar PFGE profile were isolated from multiple subjects, as shown in Table 1. Of the

bacterial strains detected with increased ciprofloxacin resistance, *Lactobacillus gasseri* was the most predominant species in this study (Table 1), suggesting that this trait is more common among isolates of these species (Wall *et al.* 2007; Lakshminarayanan *et al.* 2013). Other bacterial strains identified included *Enterococcus faecalis*, *Pediococcus* spp. and *Lb. casei* (Table 1). Despite intense parallel screening, no bacterial isolates exhibiting resistance against amoxicillin were detected in this study. Other studies have also demonstrated the susceptibility of lactic acid bacteria to the β-lactam group of antibiotic, which includes penicillin, amoxicillin and ampicillin (Liasi *et al.* 2009; Sieladie *et al.* 2011).

Determination of MIC and identification of antibiotic resistant determinants

The MIC value for ciprofloxacin for the strains was assessed and is summarized in Table 2, which ranged from 90-300µg ml^{-1} in our strains. The MIC values obtained in this study were found to be comparatively higher than the results shown in recent studies, which assessed ciprofloxacin resistance in different *Lactobacillus* spp., *Pediococcus* spp., and *Enterococcus* spp. (Rojo-Bezares *et al.* 2006; Hammad & Shimamoto 2010; Chang, Wang *et al.* 2012). The five individual strains exhibiting high levels of resistance to ciprofloxacin were then further selected for molecular analysis of the *gyrA* and *parC* genes in the QRDR.

Fluoroquinolone resistance in Gram-positive bacteria has been associated with mutations in the QRDR of the *gyrA* or *parC* genes, resulting in alterations in the amino acid sequences. (Kanematsu *et al.* 1998; Schmitz *et al.* 1998; Hooper 2002; Kawamura *et al.* 2003; Petersen & Jensen 2006; Hummel, Hertel *et al.* 2007b). The presence of point mutations in the QRDR of both gyrA and *parC* regions were investigated and the deduced amino acid sequences of both genes are presented (Table 3).

It is postulated that high level resistance to quinolones in Gram-positive bacteria is linked to a substitution in the *gyrA* QRDR sequence of Ser83 to Arg and/or Glu87 to Gly or Lys (Petersen & Jensen 2006). The mutation in this region of the *gyrA* gene alters the structure of the gyrase protein near its active site. Although this typical substitution could not be observed for any of the ciprofloxacin resistant isolates obtained in this study, an amino acid variation of Glu87 to Leu was detected in *Lb. gasseri* and *Ent. faecalis* isolates (Table3). This kind of substitution was previously identified in *Lb. acidophilus* BFE 7429, however this substitution has not yet been reported to be associated with increase in quinolone resistance (Hummel, Hertel *et al.* 2007b).

It is postulated that other variations in the QRDR of the ParC subunit of topoisomerase IV, such as substitution of Ser80 with Leucine or Isoleucine can also be responsible for high level resistance to quinolone (Petersen & Jensen 2006) (Table 3). Interestingly, the substitution of Ser80 to Isoleucine was observed in all the resistant strains isolated in this study (Table 3). Similar alterations (Ser to Arg/Ile) in *Ent. faecalis* have been reported earlier (Kanematsu, Deguchi *et al.* 1998). Although mutations in the *parC* gene are shown to confer resistance to quinolones, it should be noted that previous studies have suggested that mutations in topoisomerase IV may simply be a contributor to high level resistance along with other mechanisms such as reduced permeability of quinolones across the cell membrane (Kumagai *et al.* 1996; Lee *et al.* 2005). Further work would be required to confirm the role of parC in quinolone resistance observed in our isolates.

In the recent decade, increased usage of antibiotics has been implicated as a major cause of the emergence of antibiotic resistant strains. Knowledge on the antibiotic resistance of LAB is still limited. Since LAB are naturally present in fermented food and are also used as probiotic bacteria in the food industries, concerns have been raised about the antibiotic resistance in these

beneficial bacterial strains. In contrast where LAB is present in the gastrointestinal tract in large amounts, their resistant to certain antibiotics could potentially benefit the host organism, in cases, where other beneficial microbes are killed. This study opens up the possibility for using antibiotic resistant probiotics to offset the problems which antibiotics cause to the gut flora. Such strains could be particularly effective to treat people on long term antibiotic treatment such as cystic fibrosis sufferers. Of course, this hypothesis would need to be tested in rigorous human clinical studies where readouts would include both health and microbial measurements.

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Table 1: Diversity of antibiotic resistant strains among individual elderly subjects

Strains	**Number of isolates**	**Number of subjects**	**Number of isolates**	**Number of subjects**
L. gasseri	8	5	1	A
L. casei	3	2	1	B
Ent. faecalis	4	2	1	C
Pediococcus pentosaceus	3	3	1	D
P. acidilactici	3	3	1	E

Table 2: Minimum inhibitory concentrations of ciprofloxacin (µg ml^{-1}) of all bacterial strains isolated from this study

Bacterial strains	**MIC (µg/ml)**
Ent. faecalis EM358-1	150
L. gasseri EM296-T6-1	200
L. casei EM324-1	200
P. pentosaceus EM350-1	250
P. acidilactici EM341-1	250

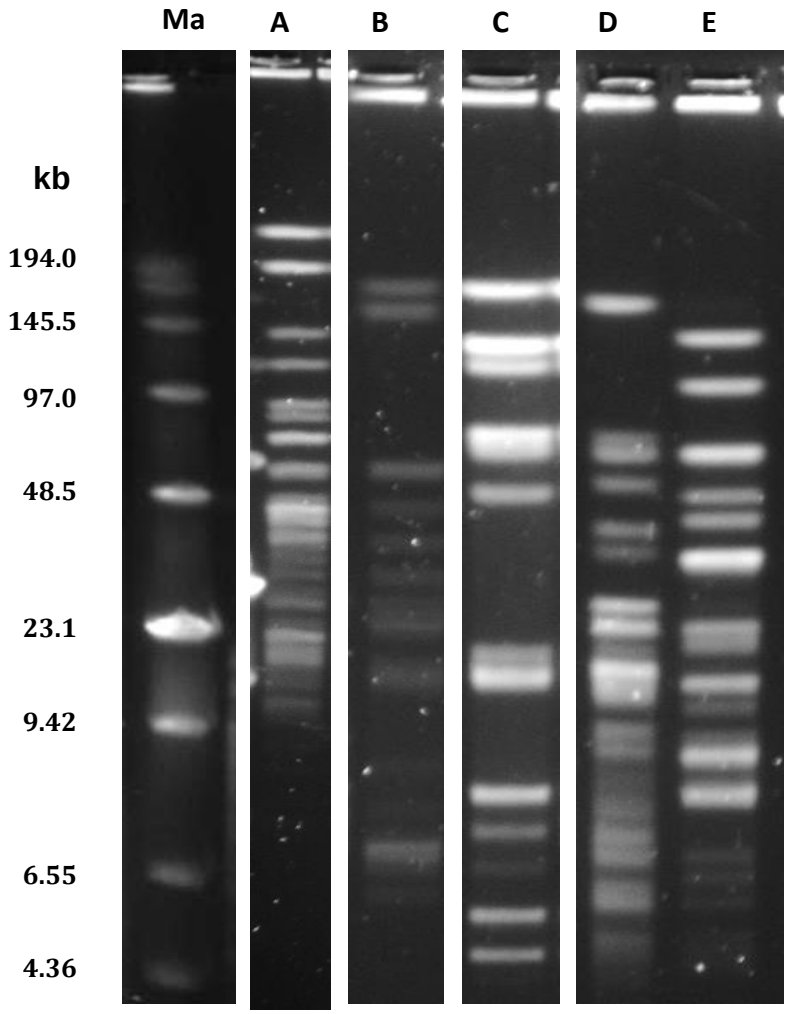
Table 3: Amino acid alignments of partially sequenced *parC (*codons 74-92) and *gyrA* (codons 73-87) genes in the quinolone resistance determining regions (QRDRs) are presented

Bacterial strains	Resistance phenotype[a]	Partial amino acid sequence[b]	Source or reference
GyrA			
Ent. faecalis ATCC 19433	S	VMGKYHPHGDSAIYE	(Petersen & Jensen 2006)
Ent. faecalis E52	R	VMGKYHPHGDSAIY**G**	(Petersen & Jensen 2006)
Ent. faecalis E82	R	VMGKYHPHGDSAIY**G**	(Petersen & Jensen 2006)
L. acidophilus BFE 7429	R	VMGKFHPHGDSSIY**L**	(Hummel, Hertel *et al.* 2007b)
Ent. faecalis EM358-1	R	VMGKFHPHGDSSIY**L**	
L. gasseri EM296-T6-1	R	VMGKFHPHGDSSIY**L**	This study
L. casei EM324-1	R	VMGKFHPHGDSSIY**L**	This study
P. pentosaceus EM350-1	R	VMGKYHPHGDSSIYE	This study
P. acidilactici EM341-1	R	VMGKYHPHGDSSIYE	This study
ParC			
Ent. faecalis ATCC 19433	S	YHPHGDSSIYEAMVRLSQD	(Petersen & Jensen 2006)
Ent. faecalis E52	R	YHPHGD**I**SIYEAMVRLSQD	(Petersen & Jensen 2006)
Ent. faecalis E82	R	YHPHGD**I**SIYEAMVRLSQD	(Petersen & Jensen 2006)
Ent. faecalis EM358-1	R	YHPHGD**I**SIYEAMVRLSQD	This study
L. gasseri EM296-T6-1	R	YHPHGD**I**SIYEAMVRLSQD	This study
L. casei EM324-1	R	YHPHGD**I**SIYEAMVRLSQD	This study
P. pentosaceus EM350-1	R	YHPHGD**I**SIYEAMVRLSQD	This study
P. acidilactici EM341-1	R	YHPHGD**I**SIYEAMVRLSQD	This study

[a] R, resistant; S, sensitive

[b] Amino acid substitutions resulting in resistant phenotype are bold and underlined.

Figure 1 Grouping of the macro-restriction patterns of the antibiotic resistant strains generated 5 PFGE pulsotypes; termed A to E, on digestion of chromosomal DNA with *Apa*I restriction enzyme (Lane 1 is the marker (Ma)).



DISCUSSION AND CONCLUSIONS

Discussion

The ageing population is dramatically changing with a constant increase in the proportion of elderly people globally. At the global level, the number of those over the age of 60 is projected by the UN Population Division to increase from just fewer than 800 million (representing 11% of world population) to just over 2 billion in 2050 (representing 22% of world population). The world population is projected to increase 3.7 times from 1950 to 2050, but the number of those aged 60 and over will increase by a factor of nearly 10 (Bloom *et al.* 2011). Therefore it is not surprising that understanding the gastrointestinal microbiota of the elderly has received increased attention in recent years (Tiihonen *et al.* 2010; Forssten *et al.* 2011; Biagi *et al.* 2012b). ELDERMET (http://eldermet.ucc.ie) is an innovative project based at University College Cork (UCC), Cork area hospitals and Teagasc Food Research Centre, Fermoy, Co. Cork focuses on the elderly. This project aimed to determine the baseline composition of the faecal microbiota of the elderly Irish population and how it is influenced by both intrinsic and extrinsic factors. The project demonstrated that diverse dietary patterns may be responsible for the intestinal microbiota variation between community-dwellers and individuals in long-term residential care. This indicates a strong role for diet-driven microbiota towards health and in the progression of disease and frailty in older people (Claesson *et al.* 2012). The research in this thesis focused on selected culturable bacteria (*Bifidobacterium* spp., *Lactobacillus* spp. and Enterobacteriaceae) in faecal samples from elderly Irish subjects. We decided to focus on these three bacterial groups because of their probiotic potential (*Bifidobacterium* spp. and *Lactobacillus* spp.) and possible use as indicators of intestinal health (Enterobacteriaceae) (Lupp *et al.* 2007; Turroni *et al.* 2008; O'Flaherty & Klaenhammer 2010; Aureli *et al.* 2011).

The human gastrointestinal tract (GIT) is a complex ecosystem housing a dynamic and diverse microbial consortium estimated at up to 100 trillion (10^{14}) bacterial cells (Clemente *et al.* 2012). The consequences of disturbances in the microbiota may sometimes result in the pathogenesis of chronic gastrointestinal disease including IBD, colon cancer, allergy and obesity (Aspinall & Meddings 2003; Ley *et al.* 2006; Mongan *et al.* 2010). **Chapter 1** reviewed the current literature regarding the baseline composition and function of the gut microbiota along with the changes it undergoes and the therapeutic strategies employed to counteract these alterations. Novel sequencing technologies such as pyrosequencing have provided more in-depth analysis of the biodiversity of the intestinal microbiota (Claesson *et al.* 2010). The exploration of the human gut microbiota will possibly lead in the long-term to the identification of novel probiotics that possess more health benefits but more importantly to dietary strategies to modulate the composition of the microbiota in a positive direction.

Culture dependent techniques had been the traditional method of exploration of the human gut microbiota up until relatively recently. Indeed, they have been extensively used to quantify lactic acid bacteria (LAB) from faecal material (Wall *et al.* 2007; O'Shea *et al.* 2008; Birri *et al.* 2010). The great advantage with cultivation is that isolates can be recovered and further characterised in detail including analysis of their antibiotic susceptibility pattern and bacteriocin production. However, molecular methods are now increasingly being applied since they give a truer representation of the entire microbiota including the uncultivable bacteria (Amor *et al.* 2007). In **Chapter 2a** we investigated the degree of correlation between the culture dependent and culture independent methods for quantifying selected bacterial genera or families including *Bifidobacterium* spp., *Lactobacillus* spp. and Enterobacteriaceae. The result showed a large variation in the levels of *Bifidobacterium* spp., *Lactobacillus* spp. and Enterobacteriaceae among

the subjects studied (n=185). The range of counts varied from <3.0 log (limit of detection) to 10.7 log c.f.u. g^{-1} faeces for total bifidobacteria; from <3.0 log to 9.74 log c.f.u. g^{-1} faeces for total lactobacilli and from <2.0 log (limit of detection) to 10.0 log c.f.u. g^{-1} faeces for total Enterobacteriaceae. This investigation demonstrated that there is a correlation between culture dependent techniques and contemporary culture independent techniques, when enumerating *Bifidobacterium* spp., *Lactobacillus* spp. and Enterobacteriaceae (Spearman's rank correlation coefficients were 0.380, 0.366 and 0.437, respectively).

While these bacterial groups are important, they represent only a small proportion of the overall intestinal microbiota. The correlation demonstrated within these groups may not be transferable to other bacterial groups as these groups are generally cultivable unlike many of the other intestinal inhabitants. It is also important to emphasize that for bacterial species resident in low numbers in the gut, pyrosequencing with general bacterial primers is not useful (Riesenfeld *et al.* 2004). This is clearly demonstrated in our studies, where on some occasions no counts were observed from pyrosequencing (due to lack of sequencing depth); however, the culture method returned a count (albeit low). Correlation also depend on factors such as the selectivity of the culture medium, the ability of species to grow on the culture medium (uncultivable or non-viable cells), and the efficiency of universal primers in identifying all members of a taxon. Whilst culture dependent techniques remain valuable for isolating bacterial species for further analysis (e.g. novel probiotic strains), the correlation between the culture dependent and independent techniques highlights that a single method is sufficient to enumerate some selected major elements of the intestinal microbiota. However, both methods have advantages that justify their use in tandem. This was the first extensive study that compared the bacterial counts using both culture dependent methodologies and next generation sequencing technologies.

The balance and composition of the intestinal microbiota is important for the well-being of the host. In this respect, the ability of the human gut microbiota to remain stable in the face of continuous and potentially disruptive perturbations (such as antibiotic exposure, infection and inflammation) is thought to be highly important for health (Bäckhed *et al.* 2012). Hence, the temporal stability of the three groups of intestinal bacteria (*Bifidobacterium* spp., *Lactobacillus* spp. and Enterobacteriaceae) in faecal samples from 65 elderly subjects was monitored over a 6 month period (**Chapter 3**). Contrary to data that indicate that the microbiota of the elderly can be easily perturbed by extrinsic factors (Jakobsson *et al.* 2010; Jernberg *et al.* 2010; Rea *et al.* 2012; O'Sullivan *et al.* 2013), this study demonstrated that the levels of the 3 bacterial groups analysed remained stable over the 6 month period reinforcing previous observations from pyrosequencing results (Claesson *et al.* 2011). Antibiotic administration did not significantly alter the temporal stability of the bacterial communities, however, the levels of *Bifidobacterium* spp. and *Lactobacillus* spp. tended to be lower (albeit not significantly) for the subjects on antibiotic therapy compared to antibiotic-untreated subjects. A recent study by Dethlefsen *et al* outlined the response of distal gut communities to antibiotic administration across three individuals, and found that the gut community returned to a stable state similar, yet distinct to the pre-treatment state (Dethlefsen & Relman 2011). Similarly, the population of the bacterial groups in our study fluctuated unpredictably with the use of different antibiotic types and treatment periods suggesting that the resilience of the gut microbiota varied between subjects. Regular probiotic consumption resulted in a significant increase in *Bifidobacterium* spp. by month 6, while there were no alterations in the other two bacterial groups studied. One of the major limitations of this study was the lack of molecular characterisation of the isolates to strain level, where undoubtedly even larger changes in the microbial population could have occurred.

The production of antimicrobials including organic acids, fatty acids and bacteriocins are often considered an important probiotic trait. In the last few decades much research has been undertaken to characterize the antimicrobial nature of many bacteriocins produced by LAB (Millette *et al.* 2007; O'Shea *et al.* 2008). Bacteriocins are a diverse group of ribosomally-synthesized antimicrobial peptides against which the producing strains possess specific immunity (Cotter *et al.* 2005). The ability of bacteriocin producers to inhibit pathogens and protect the host against infections has been well documented (Corr *et al.* 2007; Millette *et al.* 2008). **Chapter 4** involved the isolation and characterization of bacteriocin-producing LAB of human intestinal origin. Faecal samples from subjects from diverse residence locations, including community and long-stay nursing homes were analysed. The predominant antimicrobial-producing species were identified as *Lactobacillus salivarius, Lb. gasseri, Lb. acidophilus, Lb. crispatus* and *Enterococcus* spp. A number of previously characterized bacteriocins, including ABP-118 and salivaricin B (from *Lb. salivarius*), Enterocin B (*Ent. faecium*), Lactacin B (*Lb. acidophilus*), Gassericin T and a variant of Gassericin A (*Lb. gasseri*), were identified. Interestingly, two antimicrobial-producing species, not generally associated with intestinally-derived microorganisms were also isolated: *Lactococcus lactis* producing Nisin Z and *Streptococcus mutans* producing Mutacin II. The isolation frequency of the bacteriocinogenic isolates was found to be lower than expected (0.4% from total culturable bacteria) and did not represent a major part of bacteria culturable by the method employed in the study. It has also been widely documented that the elderly intestinal microbiota differs from younger adults (Biagi *et al.* 2011; Claesson *et al.* 2011) and that the compromised stability of the intestinal microbiota in the elderly (Mueller *et al.* 2006; Biagi *et al.* 2012a) may have resulted in the lower isolation of bacteriocin-producing strains. However, this study has resulted in the isolation of a panel of

bacteriocin producers with the potential to alter the gut microbiota when introduced as probiotic cultures.

An unusual aspect of this thesis work was the isolation of the clinically significant *Clostridium perfringens* at high levels from a subset of these subjects (**Chapter 5**). Of the 368 faecal samples analysed, 28 samples (7.6% of samples) from 19 subjects yielded colonies that were atypical of *Bifidobacterium* spp. on *Bifidobacterium* selective medium, which were subsequently confirmed as *C. perfringens*. Interestingly, the isolation of similar *C. perfringens* strains from subjects residing in the same residence location suggested horizontal transmission within the hospital environment. The shedding level of *C. perfringens* in elderly hospitalized patients was in agreement with the previous studies (Yamagishi *et al.* 1976; Stringer *et al.* 1985; Benno *et al.* 1989), where a high count was reported (in excess of 10^6 c.f.u. g^{-1} faeces). The results suggest that such a high viable count (in excess of 10^6 c.f.u. g^{-1} faeces) may be indicative of a less healthy microbiota in the intestine since elderly people residing in long-stay residential care were more frequent shedders. Microbiota composition analysis by amplicon sequencing was used to determine if the presence of *C. perfringens* was associated with an altered intestinal microbiota. There were many significant differences in the proportions of individual genera between the *C. perfringens* positive and negative subjects. A significant higher level of *Clostridium* spp. ($P<0.0001$) and significant lower level of *Bifidobacterium* ($P=0.0363$) were observed in the positive group. Furthermore isolation of all the *C. perfringens* strains from medium with higher concentration of mupirocin (up to 500 μg ml^{-1}) suggests the limitation of using this antibiotic as a selective agent for *Bifidobacterium* spp.

The discovery of antibiotics has revolutionized the treatment of infections and diseases and is one of the primary factors associated with extended life expectancy of humans. Knowledge of

the effect of antibiotic therapy on the composition of the intestinal microbiota is fundamental to the development of targeted treatments for improved health. **Chapter 2b** of this thesis assessed the impact of antibiotic therapy on the intestinal microbiota of 185 elderly Irish subjects from various residence locations using a combination of culture dependent and culture independent approaches. Echoing previous studies (Bartosch *et al.* 2004; Woodmansey *et al.* 2004) antibiotic therapy had a deleterious effect on *Bifidobacterium* spp. populations (a 7 fold decrease). In contrast, increased the levels of culturable lactobacilli populations (a 2.6 fold increase) were reported following antibiotic therapy. Assessment of the different antibiotic types revealed that the nucleic acid synthesis inhibitors had the most dramatic effect on *Bifidobacterium* spp. resulting in a 23 fold decrease. In contrast, the cell envelope antibiotics demonstrated a 10 fold increase in the *Lactobacillus* spp. levels. The consequences of an altered intestinal microbiota due to antibiotic therapy are not fully understood, but could include increased carriage of antibiotic resistance genes (Salyers *et al.* 2004; Jernberg *et al.* 2010). The selection pressure applied on the bacterial population during antibiotic usage is undoubtedly the driving force for the emergence of resistant bacteria (Levy & Marshall 2004).

As mentioned by Felix Marti-Ibanez (1955)

"*Antibiotic therapy, if indiscriminately used, may turn out to be a medicinal flood that temporarily cleans and heals, but ultimately destroys life itself"*.

Since LAB are present in the GI tract in large amounts, there is growing interest in the possible role of LAB acting as reservoirs of antibiotic resistance genes (Mathur & Singh 2005). In addition to the properties that define a bacterial strain as being "probiotic", the antibiotic resistance and the ability to act as a donor of antibiotic resistance genes must also be assessed.

Thus, the combination of antibiotic and probiotic intake can provide a potential advantage in the treatment of bacterial disorders by aiding the recovery of intestinal microbial imbalance. Thus the objective of **Chapter 6** was to isolate and characterize LAB with increased resistance to clinically important antibiotics including ciprofloxacin and amoxicillin and also elucidate the molecular determinants underlying the resistance. The *gyrA* genes of the quinolone resistance determining region (QRDR) for the isolates with increased ciprofloxacin resistance showed no typical mutations in the amino acid codons. However, in *parC*, a single amino acid change was discovered, suggesting that mutation in topoisomerase IV may simply be a contributor to high level resistance along with other mechanisms such as reduced permeability of quinolones across the cell membrane (Kumagai *et al.* 1996; Lee *et al.* 2005). Further work would be required to confirm the role of parC in quinolone resistance observed in our isolates. This study opens up the possibility of using antibiotic resistant probiotics to offset the problems which antibiotics cause to the gut microbiota. Such strains could be particularly effective to treat people on long term antibiotic treatment such as cystic fibrosis sufferers. Of course, this hypothesis would need to be assessed in rigorous human clinical studies.

Culture collection and gene banks are at the very heart of efforts to conserve bacterial diversity. One of the major parts of thesis work was the generation of a large bank of selected beneficial gut bacteria (lactobacilli and bifidobacteria) from hundreds of elderly subjects. This ELDERMET Culture Collection (EMCC) will be screened for potential probiotics and can be used to develop the next generation of functional foods for the elderly. A total of 6,500 bacterial isolates are currently stored in the ELERMET culture collection, which constitutes an invaluable bank of bacterial cultures for future characterization and exploitation.

Overall Conclusions

The adaptation of the gut microbiota to our changing life-style is probably the reason for the large inter-individual variation observed among different people. Since the gut microbiota plays an essential role in interactions with host metabolism, it is of utmost importance to explore this relationship. The elderly intestinal microbiota has been the subject of a number of studies in recent years. The results presented in this thesis have further contributed to the expansion of knowledge related to gut microbiota research highlighting the combined effect of culture based and molecular methods as powerful tools for understanding the true impact of microbes. Bacteriocins have shown great promise as alternatives to traditional antibiotics. In this respect, the isolation and characterisation of bacteriocinogenic and antibiotic resistant strains can be exploited for their potential as possible agents in the control of infections and to improve the intestinal microbial environment. Future work is required to explore the culture collection housing thousands of bacterial isolates as a valuable source of potential probiotics for use for the elderly Irish community.

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