Antibiotic Resistance in the Gut Microbiota

A thesis presented to the National University of Ireland for the Degree of Doctor of Philosophy

By

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I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of PhD is entirely my own work, and has not been submitted for another degree, either at University College Cork or elsewhere.

Signed:________________________________________________

Fiona Mary Fouhy

Date:__________________________________________________
Abstract
The human gut microbiota is altered by numerous factors, not least antibiotic exposure. This thesis sets out to investigate the effects of antibiotics on gut microbiota composition and its role as a reservoir for antibiotic resistance genes and, in doing so, highlight how new sequencing and molecular approaches can greatly enhance our knowledge in these areas.

Currently, there is a paucity of studies applying high-throughput sequencing technologies to assess the effects of antibiotics on the infant gut microbiota. Thus, our aim was to investigate the effects of ampicillin and gentamicin treatment on these populations, compared to those in healthy controls, 4 and 8 weeks after antibiotic therapy ceased. DNA extracted from faecal samples was amplified using 16S rRNA and rpoB primers and was sequenced using 454-pyrosequencing. Four weeks post antibiotic therapy there was a significant increase in Proteobacteria and a significant decrease in Bifidobacterium in the gut microbiota of antibiotic treated infants, compared to the no antibiotic controls. By week 8 the levels of Actinobacteria had recovered, though Proteobacteria remained dominant in the treated infants. This is the first high-throughput sequencing study to demonstrate the significant short-term effects of antibiotics on infant gut microbiota.

It is notable that it has recently been proposed that the human gut microbiota acts as a reservoir for antibiotic resistance genes. Our second goal was to investigate the presence of aminoglycoside and β-lactam
resistance genes in the gut microbiota of healthy adult males. To facilitate this, metagenomic DNA was extracted from faecal samples of adult males without antibiotic exposure and degenerate primers were used to screen for the presence of β-lactam (blaTEM, blaOXA, blaSHV, blaROB, and blaCTX-M) and aminoglycoside resistance genes (acetylation, adenylation and phosphorylation genes). PCR successfully detected resistance genes, without the need for prior screening for resistant isolates. Moreover, the results indicate that the adult microbiota is a significant reservoir for β-lactam and aminoglycoside resistance genes, with members of the *Proteobacteria* being significant sources of these genes. This was the first study to use direct PCR analysis on metagenomic DNA thereby eliminating the need for initial screening for resistant isolates from complex environments.

Following on from this, and to determine if *Bifidobacterium* sp. contain aminoglycoside or β-lactam resistance genes, we conducted an *in silico* based screen of *Bifidobacterium* using the NCBI Protein database. A high prevalence of putative aminoglycoside and β-lactam resistance proteins in *Bifidobacterium* was observed. Laboratory investigations found no β-lactam resistance phenotype in any of the bifidobacteria containing these putative β-lactam resistance proteins. We concluded that such sequences have been mis-annotated. In contrast, when we disrupted, through insertional inactivation, the 2 genes responsible for aminoglycoside resistance proteins in *B. breve* UCC2003, a reduction in aminoglycoside
resistance resulted. This was the first study to identify genes contributing to the innate aminoglycoside resistance of *Bifidobacterium* species.

In our 4th study, the expertise gained with respect to working with metagenomic DNA, infant samples and screening for antibiotic resistance and associated genes was combined. More, specifically we constructed a fosmid metagenomic bank using infant faecal microbiota from healthy 6 month old infants who had no antibiotic exposure, and screened for ampicillin and gentamicin resistant clones. Resistant isolates underwent PCR and sequencing analysis. Additional PCRs were also completed directly on metagenomic DNA from the infant faecal samples to determine if additional genes would be detected, which had not been captured or were not expressed in the *E. coli* host. The results demonstrate that the gut microbiota of healthy 6 month old infants is a sizable reservoir of β-lactam and aminoglycoside resistance genes and that a metagenomic fosmid bank enables the detection of such genes from diverse sources.

Although this thesis focuses primarily on investigating antibiotics, antibiotic resistance and the gut microbiota, other factors that impact on the gut microbiota, i.e. surgery/short bowel syndrome, the microbes present in the appendix and conjugated linoleic acid, were also investigated. Short bowel syndrome is a condition characterized by a significant reduction in the length of the bowel, which culture-based studies have suggested results in a significant increase in *Lactobacillus* in
the gut microbiota. The aim of our study was to use a porcine model of
the infant gut to investigate the effects of surgical resection on gut
microbiota and inflammation. A significant increase in *Firmicutes*
occurred, while *Veillonellaceae* were dominant at family level. Bacterial
diversity was also reduced following resection, which was associated with
increased inflammation. No increased *Lactobacillus* levels occurred
following short bowel resection.

The human appendix has generally been regarded as an evolutionary
vestige. Recently however, it was proposed that the appendix could act
as a microbial safe house and could potentially replenish the gut with
commensals following perturbations to the normal gut microbiota, e.g.
following antibiotic therapy. Our aim was to investigate this microbiota
using high-throughput DNA sequencing techniques. Seven appendices
were sequenced, with significant inter-individual differences observed
between their diverse and complex microbiota. Some trends did emerge,
with *Firmicutes* being the dominant phylum and *Ruminococcaceae*,
*Fusobacterium* and *Bacteroides* comprising the majority of family and
genus sequences. Given the location at the distal colon and the
complexity and individual nature of the appendix microbiota demonstrated
in our study, it is highly probable that the human appendix has an
important role to play in the human body, one which has yet to be
elucidated.
Finally, given the importance of gut microbes in health and daily functioning, extensive research has been conducted to investigate ways to manipulate this environment in a positive manner. *Bifidobacterium* have received significant attention as potential probiotics. In this study, 2 conjugated linoleic acid producing probiotic *B. breve* strains (DPC 6330 and NCIMB 702258) were administered to mice for 8 weeks and the effects on gut microbiota, fat distribution and composition were investigated. The results indicated that the 2 strains exerted different effects on gut microbiota, with DPC 6330 supplementation resulting in increased proportions of *Clostridiaceae* and decreased *Eubacterium*, compared to mice receiving the NCIMB 702258 strain. The results also demonstrated that fatty acid metabolism is dependent on the probiotic strain administered.

Thus, the data presented in this thesis highlights the negative consequences of antibiotic therapy and surgery on gut microbiota composition, while also identifying the infant and adult gut microbiota as reservoirs of antibiotic resistance genes. Furthermore, we have demonstrated ways in which the gut may be repopulated, via the appendix, or beneficially manipulated.
List of publications

This thesis generated the following publications:


Glossary of terms

AAC: acetylation enzyme
aac: acetylation gene
AAD: antibiotic associated diarrhoea
AG: aminoglycoside
Amp^R^: ampicillin resistant
ANOVA: Analysis of variance
ANT: adenylation enzyme
ant: adenylation gene
APH: phosphorylation enzyme
aph: phosphorylation gene
ARA: arachidonic acid
ATCC: American Type Culture Collection
β-lactam: beta-lactam
bla: beta-lactamase resistance gene
bla_{CTXM}^: extended spectrum β-lactamase conferring resistance to cefotaxime, ceftazidime, ceftriaxone or cefepime
bla_{OXA}^: β-lactamases which hydrolyse oxacillin
bla_{ROB}^: β-lactamase conferring high level ampicillin resistance
bla_{SHV}^: β-lactamases which hydrolyse penicillin
BLAST: Basic local alignment search tool
bla_{TEM}^: β-lactamases which hydrolyse penicillin
bp: base pair
BSAC: British Society for Antimicrobial Chemotherapy
BSH: bile salt hydrolase
CA: chenodeoxycholic acid
CDAC: *Clostridium difficile* associated diarrhoea
cDNA: Complementary deoxyribonucleic acid
cfu/ml: colony forming unit per ml
CFU: colony forming unit
CLA: conjugated linoleic acid
DAB: 3,3-Diaminobenzidine
DGGE: denaturing gradient gel electrophoresis
DHA: docosahexanoic acid
DNA: deoxyribonucleic acid
DPC: Dairy Production Collection
e.g.: example
EFSA: European Food Safety Authority
ESBL: extended spectrum β-lactamase
ESPGHAN: European Society for Paediatric Gastroenterology Hepatology and Nutrition
FAME: fatty acid methyl ester
FAO: Food and Agricultural Organization
FCM: flow cytometry
FISH: fluorescent *in situ* hybridization
FOS: fructooligosaccharides
g/dL: gram per deciliter
g: gravitational force or gram
GALT: gut associated lymphoid tissue
Gent\textsuperscript{R}: gentamicin resistant
GIT: gastrointestinal tract
GLS: gas liquid chromatography
GOS: galacto-oligosaccharides
h: hour
H\textsubscript{2}O: water
HCA: hyocholic acid
HDCA: hyodeoxycholic acid
HMOS: human milk oligosaccharides
Ig: immunoglobulin
IL: Interleukin
iNOS: Inducible nitric oxide synthase
LAB: lactic acid bacteria
LCA: lithocholic acid
LPS: Lipopolysaccharide
MAC: microflora associated characteristics
MEGAN: metagenome analyzer
mg/L: milligram per litre
MIC: Minimum inhibitory concentration
µg/ml: microgram per millilitre
µg: microgram
µl: microliter
µM: micro molar
MID: distinct multiple identifier

min(s): minute(s)

mm: millimeter

mMRS: modified de Man-Rogosa-Sharpe agar

MRS: de Man-Rogosa-Sharpe agar

NCBI: National Centre for Biotechnology Information

NCIMB: National Collection of Industrial Food and Marine Bacteria

NEC: necrotizing enterocolitis

NOC: Non operation control

OTU: operational taxonomic unit

PBPs: Penicillin binding proteins

PCoA: principle coordinate analysis

PCR: polymerase chain reaction

Pfam: protein family

PFGE: pulse field gel electrophoresis

pH: Power of Hydrogen

qPCR: quantitative PCR

qRTPCR: Quantitative reverse transcription polymerase chain reaction

RCT: Randomized control trial

RDP: Ribosomal database project

rpoB: RNA (Ribonucleic Acid) Polymerase Beta Subunit

rRNA/g: ribosomal RNA per gram

rRNA: ribosomal RNA

SBR: Small bowel resection
SBS: Short bowel syndrome

SCFA: Short chain fatty acid

SEM: mean plus standard error

SI: supplementary information

tetR: tetracycline resistance gene

TGGE: temperature gradient gel electrophoresis

Th: T helper cell

TNF: Tumour necrosis factor

UDCA: ursodeoxycholic acid

UPLC-MS: ultra-performance liquid chromatography tandem mass spectrometry

v/v: volume per volume

V4 region: variable region of the 16S rRNA gene

VLBW: very low birth weight

vs.: versus

w/v: weight per volume

WHO: World Health Organization

y: years
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Chapter 1

Literature review:
Composition of the early intestinal microbiota: knowledge, knowledge gaps and the use of high-throughput sequencing to address these gaps

Published in Gut Microbes, Volume 3, Issue 3, May-June 2012, Pages 203-220
1.1 Abstract
The colonization, development and maturation of the newborn gastrointestinal tract that begins immediately at birth and continues for 2 years, is modulated by numerous factors including mode of delivery, feeding regime, maternal diet/weight, probiotic and prebiotic use and antibiotic exposure pre-, peri- and post-natally. While in the past, culture-based approaches were used to assess the impact of these factors on the gut microbiota, these have now largely been replaced by culture-independent DNA-based approaches and most recently, high-throughput sequencing-based forms thereof. The aim of this review is to summarise recent research into the modulatory factors that impact on the acquisition and development of the infant gut microbiota, to outline the knowledge recently gained through the use of culture-independent techniques and, in particular, highlight advances in high-throughput sequencing and how these technologies have, and will continue to, fill gaps in our knowledge with respect to the human intestinal microbiota.
1.2 Introduction

Following birth, the gut microbial composition undergoes remarkable alterations during the first two years of life. More specifically, the human gastrointestinal tract (GIT) changes from being initially sterile, to possessing an adult-like stable microbiome by the time the infant reaches 2 years of age (Adlerberth and Wold, 2009). Despite being home to more than $10^{14}$ bacterial cells (outnumbering the total amount of human cells in the body ($10^{13}$)) (Vaishampayan et al., 2010), which contribute up to 60% of faecal mass, the human gut contains a surprisingly limited number of dominant phyla (i.e. *Firmicutes* and *Bacteroidetes*). A diverse number of factors contribute to the development of the gut microbiota and impact on the unique composition that each individual develops (Figure 1).

The infant gut is initially an aerobic environment. However, through colonization the environment is altered, resulting in a reduction in oxygen levels thereby creating an environment suitable for the growth of anaerobes. The initial gut composition is simple, dynamic and very unstable and undergoes marked fluctuations (Palmer et al., 2007). Nonetheless, evidence exists that the initial colonization influences subsequent immune system development by influencing intestinal morphology and gut associated lymphoid tissue (GALT) function. Furthermore, more recently it has also been suggested that the gut microbiota play a significant role in the regulation of the immune system (something which will be returned to later) (Fagerås et al., 2011). Thus, an altered gut microbiota composition can potentially predispose the
infant to more frequent infections and allergic disease risk (Conroy et al., 2009).

Given the enormity of the bacterial population present in the gut, it has been proposed that the gut bacterial microbiome be considered a “super organism” (O’ Hara and Shanahan, 2006). While the composition of the species present within this microbiome is of great significance, the overall diversity of this population can also be of critical importance. In the majority of cases, the interaction between the bacteria and the human host can be regarded as mutualistic, in that both the bacteria and the host benefit from a mutual relationship. The vast array of functions which these bacteria are capable of are still being elucidated but many benefits have been well documented (Guarner and Malagelada, 2003), and these functions can be divided into those which are metabolic, trophic or protective (Guarner and Malagelada, 2003). The mechanisms by which these functions occur are outside the scope of this review, but examples of some of these diverse functions have been summarised in Figure 2.

1.3 Advances in techniques to study gut microbiota

In the past, investigations into the infant gut microbiome were culture-based and therefore the insight provided was limited by a lack of knowledge with respect to the growth requirements of the majority of microbes present in the gut. Selection of the correct media, temperature, oxygen content and time for growth all impacted on the ability to generate accurate culture-based results. It has since been estimated that as little
as 10-50% of the entire gut bacteria are easily cultured (Eckburg et al., 2005). With our increasing knowledge of the growth requirements of a vast number of microbes, as well as the availability of specialised culturing media, we can now successfully culture increasing numbers of different microbes, as recently demonstrated by Goodman et al. (Goodman et al., 2011). Culture-based approaches are still being employed in some studies (despite the availability of much more sophisticated and complex technologies, some of which will be outlined below; Table 1), though most often in combination with culture-independent techniques such as fluorescent in situ hybridization (FISH) or flow cytometry. Despite these advances in culturing capabilities, this approach is still unsuitable for characterising the microbiota as a whole, especially in complex environments such as the human gut.

Another approach that has been taken has involved the study of differences in the composition or presence/absence of microbiota associated characteristics (MACs) between different subject groups. The concept of MACs was first proposed in 1978 (Midtvedt et al., 1985) and some examples include mucin degradation, conversion of cholesterol to coprostanol and inactivation of tryptic activity. One of the most commonly studied MACs are short chain fatty acids (SCFAs). These are a sub-group of fatty acids that contain 6 or less carbons on their aliphatic side chain. They include acetic, butyric and propionic acids and are produced as a result of fermentation of dietary fibre by bacteria in the large intestine. Differences in bacteria populations could result in alterations in
the type and amount of faecal SCFAs present. The comparison of MACs has been a major component of studies investigating the contribution of the microbiome in, for example, coeliacs relative to controls (Tjellström et al., 2005, Tjellström et al., 2007), probiotic or antibiotic treated infants/children compared to controls (Bezirtzoglou and Stavropoulou, 2011, Cardona, 2002) and even to identify changes in gut microbiota-related functionality due to allergic disease (Böttcher et al., 2000, Thompson-Chagoyan et al., 2011). While MACs are useful as a tool for screening large populations, such as in epidemiological studies, they are most useful when supplemented with detailed insights into gut microbial composition.

Due to the limitations associated with culture-based approaches, researchers began to develop and utilize culture-independent, DNA-based approaches to gain such detailed insights. There are a variety of such DNA-based approaches available. Among the most popular of those employed initially were temperature gradient gel electrophoresis (TGGE) and denaturing gradient gel electrophoresis (DGGE) (Cani et al., 2008). These systems work by the separation of amplicons (often of the 16S ribosomal RNA gene (rRNA)) based on their GC content, to reveal distinctive patterns. The 16S gene allows phylogenetic identification of the bacteria present, as this gene is present in all prokaryotes and contains conserved and variable regions which facilitates amplicon generation and differentiation (O’ Toole and Claesson, 2010). These techniques are rapid and provide an overview of the composition of
microbial populations. Downstream analysis to identify specific components of the population can be facilitated by band excision and sequencing, however, despite this, these approaches usually only provide limited phylogenetic information and, as with all PCR based strategies, can be subject to PCR bias. Dot-blot hybridization technologies have also been utilized to investigate the infant gut microbiota (Malinen et al., 2003). In this case, RNA is isolated, immobilised and assessed qualitatively and quantitatively using oligonucleotide-labelled probes. This approach is not subject to PCR bias, but the resolution of results can be limited, and it is focussed on specific populations rather than the microbiota as a whole. Furthermore, results depend on the ability to firstly generate reference sequences to facilitate the design of probes. Similarly, FISH approaches have been used and have also provided valuable information, but, as with dot-blot hybridization, the results are again focussed on specific populations and reference sequence generation is again required (Duncan et al., 2008, Manichanh et al., 2006). Quantitative PCR (qPCR) is now also frequently used, which measures the accumulation of products through measurement of fluorescently labelled primers or probes (Overturf, 2009). Studies have also employed several techniques in combination, such as dot-blot hybridization together with qPCR to allow for the quantification of bacterial numbers as well as identification of the different species present (Zwielehner et al., 2009). Other studies have used qPCR and FISH in combination, yielding significantly more detailed and enlightening results (Collado et al., 2010).
The next step in the evolution of culture-independent technologies involved the use of phylogenetic microarrays (Palmer et al., 2007). Microarrays are similar to the previously described approaches, but are more advantageous in that they allow hybridisation of greater numbers of sequences to the one slide, thus allowing extensive data generation from the one read. Briefly, the sequences are attached to the glass slide, using a robotic arrayer. These sequences are fluorescently labelled and their expression can be measured using a fluorescence assay (Brown and Botstein, 1999). Thus it is clear that a shift in gut microbiota research has occurred in recent decades, to focusing more specifically on the bacterial 16S ribosomal RNA (rRNA) gene (Frank and Pace, 2008, Mardis, 2008b).

As highlighted by an extensive review in 2008 (Frank and Pace, 2008), the investigation into the gastrointestinal microbiota has moved into the “metagenomic era”, with increasing numbers of studies employing DNA sequencing-based techniques. Sequencing of the 16S rRNA gene has the advantage of providing the gene sequence itself (and, thus, valuable information regarding the identity of microbes present) rather than the indirect, and less accurate, information provided by DGGE and TGGE. When carried out on a larger scale, DNA sequencing can reveal detailed information relating to the overall microbial population in a particular environment e.g. the human gut which contrasts with targeted approaches such as dot-blotting, FISH, qPCR and, to a lesser extent, phylogenetic microarrays. The earliest sequencing-based approaches
were based on cloning of full length 16S rRNA genes into a plasmid, its introduction into a host (most often *Escherichia coli, E. coli*), followed by conventional, capillary-based, Sanger sequencing thereof. While this technique allows the identification of bacterial species, it is slow and expensive. This process can take up to 3 weeks from the generation of purified DNA to the generation of results (Strausberg et al., 2008). Today the focus has shifted to high-throughput sequencing which, because of the scale at which sequence data is generated, provides a greater insight into the precise composition of the microbiota present (Mardis, 2008b). High-throughput sequencing technologies (also known as next generation sequencing), such as those supplied by Roche/454 and Illumina, have been used extensively for gut microbiota-related studies. The Roche/454 pyrosequencing approach is based on sequencing by synthesis. For 16S sequencing, purified DNA is used to generate an amplicon library which then undergoes an emulsion based clonal PCR. This PCR uses beads coated in oligonucleotides, which are specific to adaptor sequences attached to the amplicons. Following bead recovery and enrichment, the amplicon coated beads are added to a picotitre plate and sequencing ensues. Sequencing involves an enzymatic reaction and, as each nucleotide is sequentially added, pyrophosphate is released and ATP is subsequently generated. This then enables the conversion of luciferin and the emission and detection of photons of light (Strausberg et al., 2008). For Illumina sequencing, single stranded DNA fragments are generated with oligo-ligated adaptors attached. These are then attached
to a glass flow cell, onto which oligonucleotides complementary to the adaptor region of the amplicons are attached. Heating and cooling cycles follow, after which incubation with reagents and a polymerase to hybridise the DNA fragments to the oligonucleotides occurs. The flow cell, when placed into a cassette, is then sequenced and the incorporation of the nucleotides (each of which is fluorescently labelled) is measured using imaging technologies (Mardis, 2008a). However, these techniques also have their own inherent limitations. In the case of amplicons, they are prone to PCR bias. One also requires extensive bioinformatic capabilities to handle the vast amount of bioinformatic data generated and the associated platforms are expensive to purchase and run. In addition to this, while these technologies provide valuable information with respect to the proportions of different populations present, qPCR is often required to generate absolute quantification data.

While amplicon-based 16S compositional sequencing has been most frequently used for human studies, another option is shotgun sequencing whereby the metagenomic DNA (i.e. all of the DNA from the microbial population) is first fragmented into short lengths and sequenced randomly (Weber and Myers, 1997, Hattori and Taylor, 2009). This approach involves the sequencing of random fragments of DNA rather than specifically targeted regions and provides valuable information regarding the functional potential and, to some degree, the identity of the microbes present in a particular niche and, if carried out on a sufficiently large enough scale, entire genomic sequences can be generated (Kurokawa et
al., 2007, Venter et al., 2001). As with target-specific approaches, shotgun sequencing has also benefited enormously from the availability of high-throughput sequencing technologies (Turnbaugh et al., 2008, Turnbaugh et al., 2009, Claesson et al., 2011, Vaishampayan et al., 2010).

Other high-throughput sequencing technologies have, or will shortly, emerge. Examples include the Ion torrent (Schadt et al., 2010), SOLid (Applied Biosystems) (Shendure et al., 2005), SMRT (Pacific Biosystems) (Levene et al., 2003) and nano pore sequencers (Branton et al., 1996, Clarke et al., 2009). These techniques aim to provide longer, or greater numbers of reads, more rapidly and/or at a lower cost. While the exact mechanisms, advantages, disadvantages and differences between these new culture-independent techniques are outside the scope of this review (and are covered extensively in other reviews cited herein), it is worth noting that these technologies will undoubtedly revolutionise the way in which we study the human gut microbiota in the future. Indeed, in the past few years alone, these high-throughput sequencing technologies have already been employed to study the gut microbiota associated with different diseases including, but not limited to, diabetes (Roesch et al., 2009), Crohn’s disease (Gophna et al., 2006), irritable bowel syndrome (Kassinen et al., 2007), cancer (Maitra et al., 2004, Thomas et al., 2007) and obesity (Ley, 2010, Ley et al., 2005, Turnbaugh et al., 2008) and to investigate the effects of diet (De Filippo et al., 2010, Turnbaugh et al., 2009) and antibiotics (Dethlefsen et al., 2008) on the gut microbiota.
Thus, it is clear that in the past 15 years researchers have progressed from relying heavily on culture-based approaches to utilizing sophisticated high-throughput sequencing technologies to investigate the microbial world within us. Before proceeding, it should also be noted that while there has been enormous progress made, one limitation that still remains with respect to studying the gut microbiota, is accessing a representative bacterial sample to study. Most frequently, the composition of the gut microbiota of infants is assessed following the collection of stool samples and the extraction of DNA. However, there are limitations to this approach, as faecal samples are most representative of the bacteria present in the lower colon but less representative of the bacteria of the stomach and upper intestine. However, despite this limitation, faecal samples are very useful with respect to identifying the majority of bacteria present in the colon, which is where the preponderance of intestinal bacteria reside (due to transit time, pH, nutrient availability etc.) and, in the absence of other alternatives, faecal-based assessments remain the approach of choice. This review will focus on the infant gut microbiota development, based on results generated using culture-independent approaches and will highlight how the results generated using these different technologies compare to those generated using older approaches.

1.4 Shaping the early intestinal microbiota: Effect of mode of delivery
Infants undergo rapid colonization during delivery and in the first few hours following birth. Initially the infant is colonized by aerobes, followed by facultative anaerobes and, as the oxygen level is diminished, strict anaerobes predominate (Grölund et al., 1999, Edwards and Parrett, 2002, Salminen et al., 2004). Some of the earliest colonizers include *E. coli* and enterococci and, once the oxygen has been consumed, they are followed by strict anaerobes including bifidobacteria, *Bacteroides* and *Clostridium spp.* (Stark and Lee, 1982, Adlerberth and Wold, 2009). However, while these general patterns of colonization occur, colonization of the infant’s gut is altered by birth mode. Infants born vaginally are colonized with vaginal and faecal microbes from their mother and this has been shown to result in a strong maternal signature, which contrasts with the microbiota of Caesarean born infants (Adlerberth and Wold, 2009). It is generally accepted that infants born by Caesarean section have no access to the mother’s microbiota, although there have been suggestions that the swallowing of amniotic fluid allows some colonization of the infant’s gut *in utero* (Mshvildadze and Neu, 2010). Caesarean delivered infants are instead colonized by microbes from the environment, such as those from healthcare staff, wards and other infants. A recent study of 9 women and their 10 infants (i.e. including one set of twins) was completed using high-throughput sequencing (Roche/454) of the variable 2 (V2) region of the bacterial 16S rRNA gene (Dominguez-Bello et al., 2010). The authors sequenced 34 samples from the mother and 46 from their infants, resulting in 157,915 partial 16S sequences. The study found
that there was a strong vertical transmission of vaginal microbes from the mother to the infant when birth was by vaginal delivery, resulting in a dominant number of lactobacilli within hours of birth. In contrast, in the gut of Caesarean delivered infants there was a strong presence of maternal skin microbes, with staphylococci being dominant in these infants (Dominguez-Bello et al., 2010). This study advances our understanding of the relationship between the mother’s microbiota and that of her infant and highlights the benefits of employing high-throughput sequencing for such purposes.

Other DNA-based studies have also been completed that support the aforementioned results. A study of over 1000 infants in the Netherlands examined, using qPCR, the potential of over 16 factors to alter the composition of the infant gut microbiota at age 1 month (Penders et al., 2006). When the gut microbiota of infants that were vaginally born was compared with those born by Caesarean section, it was apparent that the latter group had 100 fold lower bifidobacteria and Bacteroides fragilis numbers. In addition, birth by Caesarean delivery was also associated with a 100 fold increased colonization with Clostridium difficile. C. difficile is a Gram positive spore-forming anaerobic pathogen, which has been shown to be capable of producing toxins and is frequently a cause of diarrhoea and colitis (Fitzpatrick et al., 2008, Gerding et al., 1995, Thomas et al., 2003). Notably, a follow-on study by the same group found a positive association between mode of delivery, the gut microbiota and atopy risk (Van Nimwegen et al., 2011). Another such study focussed on
the microbial composition of even younger infants (i.e. 3 days old) (n=46) (Biasucci et al., 2010). The TGGE- and DGGE-based approaches employed again highlighted the strong impact of delivery mode on the microbial composition, with vaginally born, exclusively breastfed infants, having the highest bifidobacteria levels and lowest *C. difficile* counts of all infants (Biasucci et al., 2010). Surprisingly, although subject to bias and the inherent limitations outlined previously, culture-based approaches have revealed similar trends in that, when Adlerberth *et al.* examined over 300 infants across 3 European cohorts, they found that Caesarean delivered infants are colonized with greater numbers of clostridia and *Klebsiella* and decreased *E. coli*, bifidobacteria and *Bacteroides* compared to vaginally delivered infants (Adlerberth *et al.*, 2007). A FISH-based study of 168 one month old Finnish infants provided a somewhat different set of results in that the *Clostridium, Bacteroides* and *Lactobacillus* populations in the gut were found to be similar in both vaginally and Caesarean born infants (Huurre *et al.*, 2008). Notably, however, it was again apparent that bifidobacteria were greatly impacted upon by delivery mode, with a 1300 fold higher level observed in infants born vaginally. This is significant as bifidobacteria, along with lactobacilli, are the microorganisms most frequently employed as probiotics. Bifidobacteria were first characterised in the period 1899-1900 and, since then, have been shown to predominate in vaginally delivered, breastfed infants. The health promoting properties of specific lactobacilli and bifidobacteria have been reported and include the combat of diarrhoea,
increasing resistance to pathogenic microorganisms, decreased occurrence of urinary, gastrointestinal and respiratory infections, alleviating lactose intolerance symptoms, reducing constipation and boosting immune functioning (Leahy et al., 2005, Picard et al., 2005, Liepke et al., 2002). However, as has been highlighted recently by EFSA (European Food Safety Authority), it is critical that the health claims pertaining to each specific strain are rigorously tested (Efsa, 2007). Nonetheless, research to date does suggest that a delivery-mode mediated variation in the numbers and diversity of both lactobacilli and bifidobacteria occurs and considerable research has been carried out with a view to determining the significance of these differences. Moving forward this area of research will benefit from more detailed investigations to determine precisely which populations are influenced by delivery modes and to establish which of these populations can be specifically associated with subsequent health-related impacts.

Culture-based studies have shown that the influence of delivery mode on the gut microbiota can persist for some time and thus may impact on the subsequent health of the infant (Grönlund et al., 1999). Recent culture-independent approaches have also shown this to be the case, once more showing the ability to verify the results of culture-based approaches using new culture-independent and high-throughput sequencing based technologies. More specifically, a FISH-based study of 60 children at age 7 years, in which 31 had been born by Caesarean section and 29 vaginally (Salminen et al., 2004), revealed that vaginally born infants had
increased levels of clostridia compared to those delivered by Caesarean section. The authors reported that lower clostridia levels appear in those infants being treated for asthma at age 7, while healthy children had higher numbers of clostridia, thereby highlighting a potential long-term consequence of the impact of delivery mode on the gut microbiota. A recent birth cohort supports these gut microbiota findings of a long-term consequence on health due to delivery mode (Goldani et al., 2011). The study examined the association between Caesarean delivery and the subsequent risk of being obese at age 23-25 years. After controlling for sex, birth weight, activity, income, smoking and maternal factors (schooling and smoking during the pregnancy), it was revealed that those born by Caesarean section had a 58% increased risk of obesity compared to vaginally born infants, thus highlighting the long-term effects of a factor that impacts on the infant’s gut microbiota (Goldani et al., 2011). This topic has also been the focus of a recent review (Neu and Rushing, 2011).

1.5 Effect of early feeding regime

The impact of feeding choice, i.e. breastfeeding versus formula feeding, and weaning on the infant gut microbiota has also been investigated. The World Health Organisation (WHO) recommends exclusive breastfeeding of all infants up to 6 months of age and continued supplemented breastfeeding up to 12 months of age (Who, 2003). Despite this, there are large variations in the rates of breastfeeding from one country to
another. Presently, Scandinavian countries have some of the highest levels of breastfeeding with, for example, recent data for Norway suggesting that 96% of infants are breastfed at birth, of which 84% are exclusively breastfed (Kristiansen et al., 2010). In comparison to these high levels, many developed countries have much lower rates of breastfeeding, with recent Irish data suggesting that rates of exclusive breastfeeding currently stand at just 47% at hospital discharge and drop to between 6.5 and 9.4% of women partially breastfeeding during the first 6 months of the infant’s life (Tarrant et al., 2010). In the USA, the 2007 National Immunisation Survey found that at 3 months of age, just 33% of infants were exclusively breastfed, and that this level falls further at 6 months to just 13% being exclusively breastfed (Survey, 2007).

Breastfeeding is accepted as being highly beneficial to both mothers and infants (Allen and Hector, 2005). Breastmilk is a nutritious food for the newborn, the composition of which varies in response to the infant’s changing nutritional requirements and age. In addition to containing the appropriate nutrients for the growing infant, breastmilk can have a significant impact on the gut microbial composition by virtue of being a source of prebiotics (non digestible food ingredients that beneficially effect the host by selectively stimulating the growth of one or a limited number of bacteria in the colon), lactoferrin (an antimicrobial protein) and lysozyme (an enzyme found naturally in milk, tears and sweat that is capable of digesting the cell walls of bacteria) (Fox and Kelly, 2006). Thus, the constituents of milk may play a determining role in the gut
microbial composition and development. Significantly, there are considerable differences in the oligosaccharide composition of human breastmilk and cows’ milk although it has been revealed that the addition of prebiotics to cows’ milk based infant formulas can reduce these differences somewhat (Manning and Gibson, 2004). The ability of prebiotics to modulate the infant gut microbiota in a manner similar to that associated with breastmilk will be discussed in greater depth later in this review.

Thirty years ago, Stark and Lee pioneered the research into the influence of different approaches to feeding on the infant gut microbiota (Stark and Lee, 1982). This culture-based study paved the way for the more recent investigations and was notable in that it revealed that bifidobacteria levels varied greatly depending on feeding method, with breastfed infants having higher bifidobacteria levels compared to formula fed controls. Although it is now apparent that the feeding regime is not the sole determinant of the levels of bifidobacteria and lactobacilli in the infant gut (Adlerberth and Wold, 2009), it is clear that feeding does have a crucial impact. The findings of this initial culture-based study by Stark and Lee (Stark and Lee, 1982), have been corroborated by several more recent culture-independent studies and reviews, which confirm that bifidobacteria are more dominant (and, in at least some cases, more diverse) in the gut of breastfed infants (Bezirtzoglou et al., 2011, Fallani et al., 2010, Le Huêrou-Luron et al., 2010, Roger et al., 2010, Penders et al., 2006). These studies have also revealed that *E. coli* and clostridia
counts, including *C. difficile*, are lower in breastfed infants than those fed infant formula. Notably, when formula was supplemented with oligosaccharides it resulted in greater bifidobacteria counts in the faecal samples of the associated infants than was present in samples provided by the unsupplemented control group (Penders et al., 2006). Thus, in this case and as is often the case, the use of culture-independent techniques has resulted in the validation of earlier culture-dependent studies but has also significantly advanced our understanding of the broader consequences of feeding method choice with respect to the infant gut microbiota. Thus, while culture-based approaches are rapid and relatively straightforward and therefore are useful as preliminary investigations, the more advanced techniques provide us with the greatest insight into the complex interaction between feeding method and gut microbiota.

Other patterns have also been noted in that a review by Adlerberth and Wold (2009) noted trends towards higher levels of *Lactobacillus rhamnosus* in partially breastfed infants compared to weaned infants, observed that staphylococci are also more common in breastfed infants, while also establishing that higher levels of *Klebsiella* and *Nitrobacteria* are seen in formula fed infants (Adlerberth and Wold, 2009). Fallani *et al.* have reported that *Bacteroides* were dominant in the gut microbial population of 6 week old formula fed infants (Fallani *et al.*, 2010) and it has also been noted that the microbiota of formula fed infants is, in general, more diverse than that of their breastfed counterparts (Bezirtzoglou *et al.*, 2011). Perhaps most notably of all, it has also been
established that the consumption of breastmilk can significantly reduce the risk of necrotizing enterocolitis (NEC) (by 3-10 fold) in infants relative to those who are formula fed (Lin and Stoll, 2006).

In addition to the fact that the composition of milk consumed influences infant gut microbial composition, it has also been claimed that breastmilk contains microbes such as staphylococci, streptococci, lactobacilli, micrococci and bifidobacteria (Martín et al., 2003) and thus may be a direct source of the lactobacilli and bifidobacteria that become established in the infant gut (Martín et al., 2007, Sinkiewicz and Ljunggren, 2008). These earlier studies have been supported by a 2010 study which provided further evidence of the role of breastmilk-associated microbes in the establishment of lactic acid bacteria (LAB; which includes the lactobacilli) and bifidobacteria in the immature infant gut (Solís et al., 2010). In addition to the benefits of nutrients, oligosaccharides and, perhaps, microbes present in breastmilk on the infant gut microbiota, the antimicrobial impact of lactoferrin, as alluded to earlier, may be beneficial.

In 2009, a review found that oral treatment with lactoferrin reduced the incidence of sepsis and NEC in very low birth weight (VLBW) infants (usually including infants 1-1.5 kg in weight). Significant reductions in sepsis and NEC were apparent when lactoferrin was supplemented along with the probiotic L. rhamnosus GG (Mohan and Abrams, 2009).

The introduction of solid foods (recommended by the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) to occur not before 17 weeks of age and no later than 26 weeks) (Agostoni
et al., 2008) is also known to induce alterations in the gut composition of infants (Edwards and Parrett, 2002, Koenig et al., 2011). Koenig and colleagues performed high-throughput sequencing of faecal DNA from one infant over a 2.5 year period (Koenig et al., 2011). The study employed 454-pyrosequencing to generate 318,620 16S rRNA gene sequences from sixty samples and over 500,000 metagenomic sequences from 12 samples. The study identified so called “steps” at which dramatic alterations occurred in the infant’s GIT microbiota which they found could be attributed to a significant life event (Koenig et al., 2011). An example of this was “Step 3”, which took place around days 170-290, when the introduction of formula and peas to the diet of the previously exclusively breastfed infant resulted in a significant increase in *Bacteroidetes*. Overall, the study found that the introduction of solid foods was associated with an increase in *Bacteroidetes* and *Firmicutes*. It was also again suggested that by 2.5 years the gut microbiota closely resembled that of an adult. The impact of weaning has also been investigated by others. In one case, this involved a study of 605 children from 5 European countries (Fallani et al., 2011). The infants were examined 4 weeks after weaning commenced and were compared to the same infants prior to weaning, with results being generated using FISH and flow cytometry. The study found *Bifidobacterium*, the *Clostridium coccoides* group and *Bacteroides* to predominate after weaning but it was noted that the relative proportions of these were affected by the approach to pre-weaning feeding i.e. infants who had been breastfed had higher
levels of bifidobacteria and decreased *Bacteroides* compared to infants who had been formula fed prior to weaning. The authors noted that despite weaning having a noticeable modifying effect on the gut microbiota of infants, other modulating factors such as mode of delivery, continued to exert measurable effects during the weaning period (Fallani et al., 2011). Roger and colleagues have also reported an increase in the diversity of bifidobacteria corresponding to the introduction of solid foods (Roger et al., 2010).

Finally, an alternative approach to the investigation of the impact of diet was demonstrated by De Filippo et al. in a study which compared diet, and its effects on the gut microbiota, of children (aged 1-6 years) from Europe (n=15) compared to those from rural Africa (n=14) (De Filippo et al., 2010). The African diet was low in fat and protein from animal sources and was high in fibre and starch and these children also differed in that they were breastfed up to 2 years of age. The authors performed pyrosequencing of the V5 and V6 hyper-variable regions of the bacterial 16S rRNA gene and generated 438,219 gene sequences, corresponding to 15,111 sequences per sample. The study found that the lack of diversity in the Western diet, and its over-reliance on nutrient dense, processed and refined foods, appears to have an effect on the gut microbial composition. More specifically, the Western diet was associated with a reduced microbial diversity, with the European gut microbiota containing higher proportions of *Firmicutes* and *Proteobacteria* and lower proportions of *Bacteroidetes* and *Actinobacteria* (De Filippo et al., 2010).
Despite the increased knowledge gained in recent years, it is clear that there is a need to more closely investigate the gut microbial composition of breast and formula fed infants as well as the effects of weaning and other diet-related issues. Investigations are also required to determine the duration of such effects and the short- and long-term impact that they have on infant health. It is anticipated that high-throughput sequencing technologies will provide significant clarity in this regard.

1.6 Impact of family structure

Though studied to a lesser extent, a factor that is emerging as a possible contributor to the composition of an infant’s gut microbiota is family structure. One relevant concept is known as the ‘sibling effect’ which is an adjunct to the hygiene hypothesis and postulates that allergic disease is lower in children from larger families (Strachan, 1989). However, this theory remains controversial, particularly as the studies which have been performed to test this theory have been carried out in a myriad of different ways. Thus, to date, definitive evidence of the effects of family size, structure and birth order has yet to be established. Nonetheless, as part of the ALLERGYFLORA study (Adlerberth et al., 2007), it was found (using culture-based approaches) that infants without older siblings had increased proportions of non-\textit{E. coli} enterobacteria as well as clostridia in the gut, but also had a lower anaerobe to facultative anaerobe ratio, resembling that of Caesarean delivered infants (Adlerberth et al., 2007). In 2006, Penders \textit{et al.} had reported the presence of greater
bifidobacteria concentrations in infants with older siblings than those without. While family order and the environment have been implicated in allergic disease development (Mckeever et al., 2002, Strachan, 2000), the link between these effects and the composition of the intestinal microbiota requires further investigation. Further culture-independent studies are needed, for example, to identify if the gut microbiota of infants without older siblings is significantly different from that of other infants and if this predisposes them to later health risks. By providing answers to such questions, it will then become possible to address problems, that might previously have been overlooked, by positively influencing the gut microbiota (e.g. through probiotics use). As has been shown in the previous sections, the results of culture-based approaches are often verified by newer approaches, though these new technologies are advantageous as they provide a more detailed and less biased insight into such complex interactions between environmental factors and the gut microbiota of infants. Thus, there is an obvious opportunity to employ sequencing approaches to identify the gut microbiota of these infants and its relationship to health outcomes.

1.7 Effect of maternal weight/diet

The WHO released startling figures in 2010, which were updated in 2011, relating to the state of the world’s obesity crisis (Who, 2011). The statistics showed that obesity levels have doubled since 1980 and that, as of 2008, 200 million men and 300 million women were obese.
Childhood obesity was also highlighted, with 43 million children under 5 years being obese in 2010. Surprisingly, 65% of the world’s populations now live in countries where more deaths occur due to obesity rather than being underweight (Who, 2011). Obesity appears to be a vicious cycle, as an obese mother is more likely to have an obese infant, who in turn has an increased risk of becoming an obese adult (Lawlor et al., 2007). Work by Gordon, Cani and others have shown that obesity is influenced by the microbial composition of the gut (Cani et al., 2008, Ley et al., 2005). The effects of childhood obesity on the composition of the child’s gut microbiota have recently been studied (Kalliomaki et al., 2008). This culture-independent study (comparing FISH and flow cytometry in combination, to results from microscopic detection and qRT-PCR) examined participants (n=25) and controls (n=24) at birth, at 3, 6, 12, 18 and 24 months and again when aged 4 and 7 years (at which time BMI was calculated). The controls (i.e. normal weight children) were matched for birth mode, gestational age, probiotic treatment, breastfeeding duration, antibiotic treatment, atopic disease prevalence and cohort at age 7 years. Faecal samples were analysed and it was shown that children who were classified as being of normal weight had, and continued to have, higher levels of bifidobacteria than those who were, or who became, obese. They also noted lower Staphylococcus aureus levels in normal weight infants. This therefore provides a further indicator for the role of gut microbiota in obesity development and highlights the
possibility of modulating disease risk through the alteration of the gut microbiota.

A recent study has taken the alternative approach of investigating if a mother’s weight before or during pregnancy could impact on her infant’s gut microbiota (Collado et al., 2010). The results from this 2010 study showed that infants of overweight mothers tended to be overweight or heavier at birth than those of normal weight mothers, while also revealing that overweight mothers had infants with decreased numbers of gut bacteria from the Bacteroides-Prevotella group at age 1 month, but had higher levels of Clostridium histolyticum in their gut at age 6 months. Similar results were observed among infants whose mothers underwent significant weight gain during pregnancy. The gut microbiota of the offspring of overweight mothers contained higher Clostridium leptum, lower Clostridium perfringens and higher S. aureus levels than that of the infants of normal weight mothers. In contrast, at 6 months bifidobacteria counts were higher in the infants of normal weight mothers than in those of overweight mothers. While this culture-independent study provides intriguing evidence of the effect that obese mothers have on their infant’s gut microbiota, research in this area still remains limited and additional culture-independent studies are required. There is an opportunity to apply high-throughput sequencing approaches to this area of research to compare the microbial profile of the mother at birth, and at later time points, with that of her infant and to track the changes in gut microbiota and the weight profile of both. There is a clear opportunity to exploit these
technologies to considerably advance our knowledge of this complex interaction between gut microbiota and weight. Finally, it has also been shown that a mother can modulate her infant’s gut microbiota through the consumption of probiotics (Gueimonde et al., 2006) or the use of antibiotics during pregnancy (Mangin et al., 2010). The impact of probiotics and antibiotics on the infant’s gut microbial population will be addressed later in this review.

1.8 Probiotics

The word probiotic is derived from the Latin “pro” meaning for and from the Greek “biotic” meaning living. Having undergone numerous alterations (Parker, 1974, Fuller, 1989, Salminen et al., 1999) since the first proposed definition in 1965 (Lilly and Stillwell, 1965), today the most generally accepted, and most widely used definition, is that provided by the Food and Agricultural Organization (FAO) who define probiotics as “live microorganisms which, when consumed in adequate amounts as part of food, confer a health benefit on the host” (Fao/Who, 2001). Additionally, criteria have been proposed to allow for a more systematic identification of probiotics and these have been outlined by a review in 2007 (Parracho et al., 2007). To date, representatives of the lactobacilli have been most extensively studied with a view to their use as probiotics (Holzapfel and Schillinger, 2002).
The issue of the health benefits associated with the consumption of a probiotic has been the focus of great attention in recent years. Since 2006, EFSA has implemented regulations pertaining to nutrition and health claims, including claims relating to probiotics. They have outlined that with respect to health claims relating to the ability of a probiotic to modulate the gut microbiota positively, they expect that the changes induced should have a specific health benefit, such as a reduction in specific (potentially) pathogenic microorganisms within the gut, which is clearly related to the consumption of the product under investigation (Efsa, 2010). They do not, however, support the claim that increased levels of bifidobacteria or lactobacilli are beneficial to overall health _per se_, due to a lack of specific scientific evidence to support such a claim. Thus, in many cases, further evidence is needed to prove the role of specific probiotic strains in the gut and thus allow health claims relating to them. EFSA also require all scientific documents presented in the dossier supporting the health claim to specifically relate to the species and strain of probiotic microorganisms being examined. Thus, while there is considerable evidence supporting the role of some probiotics in gut microbiota modulation (as discussed below), care needs to be taken when making associated health claims.

Given the recent EFSA rulings, it is not surprising that the specific mechanisms by which probiotics exert beneficial health effects on the host continues to be the focus of much attention (Aureli et al., 2011, Preidis and Versalovic, 2009, Rastall et al., 2005). There are several
proposed modes of action including the production of bacteriocins and other antimicrobials which inhibit other bacteria or the alteration of immune function, possibly through altered GALT function or through a physical enhancement of the mucosal barrier function (O’ Hara and Shanahan, 2006). Indeed, the specific mechanism(s) involved will vary depending on the specific strain administered, further highlighting the importance of assessing each probiotic strain individually. Regardless of the precise mechanism(s) involved, a vast array of data exists relating to the beneficial impact of probiotics on host health (Fuller, 1989, Holzapfel and Schillinger, 2002, Wallace et al., 2011). However, for the purpose of this review, the focus will be confined to the benefits to infant gut microbiota and subsequent health. With respect to healthy, full term infants, this review has previously outlined the transition that the infant gut undergoes from being initially sterile to having a composition that is relatively stable and resembles that of an adult by 2 years of age. It is during this initial transition phase that probiotics may be most beneficial. It is also notable, however, that in many cases the proposed benefit has related to increasing levels of lactobacilli and bifidobacteria in the gut, which, as highlighted above, is not accepted as a health claim by EFSA. Given the ongoing debate regarding the significance of the ability of a probiotic to alter the composition of the gut microbiota (other than alterations in levels of specific pathogens), we have presented just a few examples to highlight the considerable degree to which some probiotics can bring about change. In one instance, a RCT examining the effects of
supplementing the diet of infants with *Bifidobacterium breve* Bb12 for the first 28 days of life showed, using culturing techniques, that gut colonization patterns were altered compared to those of infants in the placebo group (Kitajima et al., 1997). As one might expect, *B. breve* colonization commenced more quickly in these infants but, in addition, after 6 weeks *Lactobacillus* colonization rapidly increased. In contrast, *Enterobacteriaceae* decreased over the supplementation period in treated infants compared to controls (Kitajima et al., 1997). Investigations have also taken place to determine if probiotic administration to pregnant mothers affects the gut microbiota of their infants. In 2006 a study investigated (using qPCR) the impact of probiotic administration of *L. rhamnosus* GG to pregnant mothers on the gut microbiota of their infants (Gueimonde et al., 2006). The probiotic was fed to 29 mothers 2-4 weeks prior to delivery and up to 3 weeks after delivery, while the control group consisted of the infants of 24 mothers not in receipt of probiotics. Results showed that supplementation of the mother’s diet with the probiotic had a significant impact on the infants’ gut microbial composition, i.e. significantly increased bifidobacteria numbers and diversity in these infants at day 5 and a trend towards increased *B. breve* levels at age 3 weeks, relative to the controls. Thus, probiotics have the potential to have a significant impact regardless of whether they are administered to the mother during pregnancy or directly to the infant, via supplemented formula after birth. A recent study was conducted using qPCR and flow cytometry coupled with FISH (FCM-FISH) to analyze the faecal
microbiota of infants in Finland and Germany who received perinatal probiotic treatment (Grześkowiak et al., 2011). This study of over 150 infants found that the perinatal administration of probiotics did impact on the gut microbiota of the infants, but also found that the consequences depended on the feeding method employed (either breastfed or formula fed) as well as the microbiota present in the infant’s gut prior to probiotic administration. This area of research lends itself perfectly to further investigation through high-throughput sequencing, which will provide information with respect to the impact of these probiotics on gut microbes other than bifidobacteria and lactobacilli.

In recent years there has been an increase in allergies and atopic diseases, which have paralleled a corresponding decrease in infectious diseases. In 1976 John Gerrard first proposed the hygiene hypothesis (Gerrard et al., 1976), although it was not until 1989, when David P Strachen published his paper in the BMJ which focussed on hay fever, hygiene and household size that the hygiene hypothesis really began to gain scientific interest (Strachan, 1989). Strachen’s paper suggested that decreased exposure to environmental challenges in early life, due to improved sanitation and hygiene practices, resulted in the reduced exposure of the immature immune system to the challenges necessary to develop tolerance and resistance to everyday environmental challenges e.g. dust, pollen etc. Additionally, in 1997, the hygiene hypothesis was extended to incorporate the relationship between gut microbiota and immune regulation (Sepp et al., 1997). This hypothesis is still being
debated and studied today. However, given the knowledge we have of the influential role that gut microorganisms play in the establishment, maturation and regulation of the infant immune system, studies have once more returned to the hygiene hypothesis to determine if alterations in the gut microbial composition could result in alterations in the development of the immune system, which result in an altered allergy risk. In 2003, Bourlioux et al. reviewed the evidence up to that point which related to the role of the intestinal microbiota in immune function (Bourlioux et al., 2003). The authors reminded us that alterations in the ratio of T helper 1/T helper 2 cells can have adverse consequences for the host i.e. increased Th2 levels result in an increased risk of allergy, while increased Th1 levels increases autoimmune disease risk e.g. diabetes mellitus. Studies have shown that having lower counts of bifidobacteria and atopy risk are associated and it has been proposed that bifidobacteria alter the level of Th2 development and inhibit the Th2 type response (Saavedra, 2007). Similarly, it has also been revealed that, in children with allergic parents, higher levels of lactobacilli in early life did reduce the risk of allergy development at age 5 years (Johansson et al., 2011). It has also been suggested that the beneficial roles of specific commensal bacteria in allergic disease prevention may be due to alterations in the immune regulation process. The regulatory role that gut microbes play in the immune system has been convincingly demonstrated through studies involving gnotobiotic mice as well as human trials and, most recently, it was also shown to influence secretory
IgA levels and subsequent allergic symptom development (Fagerås et al., 2011). Such a role of gut microbes in the regulation of the immune system would help explain why it is not only Th2 mediated allergic diseases, but also Th1 associated illnesses such as Type 1 diabetes, which are increasing globally (Bach, 2002).

Following on from findings such as these, scientists have investigated the potential to favourably alter the infant gut microbiota in early life to decrease allergic disease risk. Notably, several studies have shown benefits in treating atopic children with probiotics and thus, modulation of the infant gut could potentially reduce the risk of them becoming allergic to environmental stimuli. The proposed regulatory role of gut microbes would occur predominantly during infancy and this may also explain why the effects of probiotics are more clearly observed in infants than in adults. This review will now summarise a number of relevant studies that address this topic.

In a study published in 2002, L. rhamnosus GG was provided to pregnant women who had a family history of atopic diseases (Rautava et al., 2002). The supplement was consumed for the last 4 weeks of pregnancy and throughout the breastfeeding period, until 3 months after the birth of the infant. The study found that the risk of the infant developing eczema was significantly reduced, i.e. 15% compared to 47% incidence in the control group up to 2 years of age. A subsequent study again involved supplementation with L. rhamnosus GG but, on this occasion, L. rhamnosus LC705 (DSM 7061), B. breve Bb99 (DSM 13692)
and *Propionibacterium freudenreichii* ssp. *shermanii* JS (DSM 7076) were also provided to expectant mothers who had a family history of atopy (Kukkonen et al., 2007). Once born, these infants also received this combination of probiotics, plus galactooligosaccharides (prebiotics). The authors found that compared to controls, probiotic treatment reduced the frequency of IgE associated (atopic) diseases, with an odds ratio (OR) of 0.71; 95% CI 0.5-1.00, though not significantly. Probiotic treatment did significantly reduced the risk of eczema with an OR of 0.74 CI 0.55-0.98, \( p < 0.035 \). The authors also noted the frequent colonization of bifidobacteria and lactobacilli in the gut of supplemented infants. In 2005, a study of 230 infants (aged 1.4-11.9 months) investigated the use of probiotics in the reduction in the symptoms of atopic eczema/dermatitis (Viljanen et al., 2005). Unlike the previous studies in which mothers received probiotics, this study specifically investigated the effect of directly treating the infants with probiotics. Treatment was either with *Lactobacillus* GG (LGG), LGG in combination with 3 other probiotics or a placebo. Participants were randomised into the 3 groups and treated for 4 weeks. Although the authors noted that symptoms improved, they did so in all three groups and only a non-significant improvement was observed when the probiotic treated group was compared with the control groups. The study found there to be potential for probiotics with respect to decreasing symptoms in IgE sensitised individuals but showed little benefit in non-IgE sensitised infants.
Despite the fact that, as noted above, some positive outcomes have been reported, a 2007 review of this topic concluded that the studies to date are conflicting and inconclusive (Prescott and Björkstén, 2007) and a Cochrane meta-analysis of the effects of probiotics in the treatment of eczema found no significant benefit of probiotic treatment (Boyle et al., 2009). In a recent paper on this topic evidence of the benefits of providing probiotics in order to prevent atopic eczema was quite convincing. However, the authors did agree that weaker associations have been shown between probiotics and other atopic diseases (Kalliomäki et al., 2010). The inconsistent findings to date most likely reflect differences with respect to the probiotic strains employed in the studies reviewed. It is apparent that large RCTs involving infants are needed to investigate fully and to specifically determine the benefits of treatment with specific probiotics in the context of allergy and atopic diseases. Use of high-throughput sequencing of faecal samples from affected versus unaffected individuals could be employed to determine if differences in symptoms are due to altered gut microbial compositions. Given that the hypothesis is that probiotic treatment alters gut microbiota, thus reducing allergy risk, one would presume that it is only a matter of time before culture-independent strategies are employed to investigate the link between probiotic use, alterations to the gut microbiota and subsequent impacts on allergy. It is also notable that the studies to date have often been limited to the use of lactobacilli as probiotics and thus the inclusion of other genera or the use of strains in combination may also be beneficial.
While there has been a focus on the impact of specific strains, a consistent observation across many studies is the reduced microbial diversity in the gut of allergic infants (Wang et al., 2008). This reduction in diversity and allergic status relates well to the research on the association between early antibiotic exposure, the accompanying reduction in gut microbiota diversity and subsequent allergic disease risk (Jernberg et al., 2010, Jernberg et al., 2007).

While debate continues as to the specific beneficial health effects of many probiotics, one area where more convincing evidence exists is with respect to NEC. NEC, though first characterised over 100 years ago, still remains a poorly understood disease. The condition is characterised by abdominal distension, bleeding of the intestines and ulcer formation (Claud and Walker, 2001, Lin and Stoll, 2006). There has been considerable interest in the use of probiotics to prevent NEC by normalising the intestinal microbiota of preterm infants, i.e. trying to change its composition to resemble that of healthy, full term infants (Deshpande et al., 2007, Braga et al., 2011). Notably, trials using animal models of NEC have shown the benefits of introducing probiotic supplemented diets (Caplan et al., 1997, Caplan et al., 1999). In one instance the animals, which were fed $10^9$ organisms/animal/day, had significantly reduced cases of NEC with just 7/24 in the treatment group suffering from NEC, compared to 19/27 in the control group. Corresponding human studies have also been completed (Lin et al., 2005). In one case, the benefits of feeding *Lactobacillus acidophilus* in
combination with *B. infantis* (no strain details provided) to infants was tested (Hoyos, 1999). This large, year long, study of over 1000 infants revealed that the cases of NEC, as well as the mortality rates in the treated group, were reduced compared to the controls. In 2005, a trial was conducted to examine the effects of some probiotics and NEC prevention but in low birth weight infants (Bin-Nun et al., 2005). The study found that a reduction in NEC cases in treated infants occurred with a reduction in the incidence of NEC from 17% in controls to 4% in the treated group. They noted that levels of clinically significant NEC (classified as Bell Stage 2 or 3) in the treated group (1/72 i.e. 1%) were statistically significantly reduced compared to the control group (10/73 i.e. 14%). Several meta-analysis and systematic reviews have been conducted on this topic to date (Alfaleh and Bassler, 2010, Barclay et al., 2007, Deshpande et al., 2010) and they have provided support for the use of probiotics in preterm infants to prevent NEC. Despite this, questions relating to what changes occur in the gut microbiota composition of NEC affected infants as well as the changes that occur following probiotic treatment remain unanswered and this is one knowledge gap that lends itself to the utilization of modern DNA based approaches. It is anticipated that in the future, in addition to assessing the ability of different strains to prevent NEC, attention will also begin to focus on unravelling the specific mechanism(s) via which probiotics can prevent this disease.
Some studies have also been carried out to investigate the potential benefits of using probiotics to prevent or treat antibiotic associated diarrhoea (AAD). The concept is that probiotics could temporarily colonize the gut, to compensate for the collateral damage to the gut microbiota resulting from antibiotic use, thus reducing the risk of diarrhoea due to altered digestion and absorption. It is estimated that between 8 and 30% of children suffer from AAD (Gooch, 1996, Hoberman et al., 1997). Two systematic reviews on this topic concluded that when probiotics and antibiotics were co-administered, AAD risk was reduced (Cremonini et al., 2002, D'souza et al., 2002). However, these reviews were based mainly on studies in adults. Cremonini and colleagues also highlighted the lack of RCTs, especially with respect to infants and noted that generalisations could not be made about probiotic effects, as different strains exerted different effects (Cremonini et al., 2002). The studies which have taken place which relate to children have provided conflicting outcomes. In 1990, a small study on children treated with *L. acidophilus* and *Lactobacillus helveticus* (administered prophylactically as Lactinex) found that they did not have a significant effect with respect to the prevention of AAD (Tankanow et al., 1990). In contrast in 2004, ESPGHAN concluded that there is promising evidence to suggest that some probiotics can contribute to the prevention of AAD (Agostoni et al., 2004a). In a 2007 review, it was concluded that (based on 6 RCTs at the time), co-treatment with probiotics did result in a reduced risk of AAD compared to those who received antibiotics alone (28.5% to 11.9% reduction in risk) (Saavedra,
2007). This meta-analysis found that the most significant beneficial effects occurred when *Lactobacillus GG*, *Saccharomyces boulardii* and *Bifidobacterium lactis* and *Streptococcus thermophilus* were administered. They did not however, see any significant beneficial effect from administering *Lactobacillus acidophilus/Bifidobacterium infantis* or *L. acidophilus/Lactobacillus bulgaricus*. This again further emphasises the species and strain specific effects of probiotics and the need for rigorous testing of each proposed probiotic rather than making generalisations that all probiotics are beneficial to health. A recent Cochrane review on this topic, which also looked at the above mentioned species, again found evidence of a protective effect from concomitant treatment with probiotics during antibiotic therapy. However, once again the authors emphasised the species and strain specific effects that occurred and the need for further high quality studies on this topic before routine administration of probiotics to infants/children could be recommended. Thus, there is still a considerable gap in our knowledge in this area with respect to the specific impact of probiotic administration on the composition of the gut microbiota of infants in receipt of antibiotics. Further research is required to establish if temporary colonization by probiotics occurs, to identify which microbial populations are impacted on by antibiotic administration and probiotic supplementation, to assess the duration of microbiota-related changes and to definitively establish the merits of probiotic administration in such circumstances. Finally, there is a strong need to carry out further studies to assess the success with which probiotics can prevent *C. difficile*
associated diarrhoea (CDAD) in infants and children. This was also the conclusion of a meta-analysis on this topic (Segarra-Newnham, 2007).

1.9 Prebiotics

In 1995, Gibson and Roberfroid defined a prebiotic as “a non digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improves health” (Gibson and Roberfroid, 1995). Based on this definition a substance must escape digestion or degradation in the stomach and small intestine and reach the colon intact, where it must only act as a stimulant for the growth of beneficial bacteria, which must then result in a measurable benefit to the host. The substances that have received the greatest attention to date have been oligosaccharides. Oligosaccharides are composed of repeating sugar units (2-20 units generally) and they exist naturally in breastmilk at a level of 10-12g/l (Veereman-Wauters, 2005). It is notable however that the human milk oligosaccharides present in human breastmilk have yet to be produced commercially, and instead it has been fructooligosaccharides (FOS) and galactooligosaccharides (GOS) which have been the most studied as potential prebiotics. In the past, lactulose was investigated with a view to its use as a potential prebiotic, however such investigations have become more limited due to associated laxative effects at high doses. Although FOS and GOS are naturally present in foods such as bananas, celery, chicory and artichoke (Manning and Gibson, 2004), the amount present is
too small to be beneficial and thus there is considerable interest in incorporating prebiotics into functional foods following their extraction from plant sources or synthesis thereof (Playne and Crittenden, 1996). This review will focus on the studies relating to the effect of prebiotics on infant health, through modulation of their gut microbiota.

Oligosaccharides are unusual in that they consist of a β-glycosidic bond which is resistant to degradation in the human GIT, due to a lack of appropriate enzymes to digest this bond. They remain intact until they reach the colon where they are fermented by a subset of bacteria which are capable of degrading this bond. This fermentation results in short chain fatty acids, primarily acetate, butyrate and propionate. On the basis of culture-based studies, it has been known for quite some time now that the gut microbes which benefit from supplementation with prebiotics, such as bifidobacteria, proliferate at the expense of other gut microbes including Bacteroides, clostridia or coliforms, thus resulting in what is being suggested as being a more favourable gut microbial composition (Wang and Gibson, 1993). Indeed, most research to date has focused on the ability of prebiotics to increase bifidobacteria and, to a lesser extent, lactobacilli numbers. However, it has been suggested that moving forward the increase in the numbers of other bacteria such as Roseburia and Eubacterium needs consideration also (Roberfroid et al., 2010). Furthermore, as highlighted previously in this review, details on the actual benefit to health from increased levels of specific populations of
microorganisms are needed i.e. simply targeting an increase in the numbers of specific microbes cannot be employed as a health claim. Interest in prebiotics has increased as a consequence of evidence of several potential benefits, including the possibility of decreased colon cancer risk (Reddy, 1999), improved host resistance to pathogens, improved calcium absorption, altered blood lipids and altered immunological responses (Arslanoglu et al., 2007, Gibson et al., 2004), the majority of which still require further testing before EFSA will fully approve these claims. However extensive investigations have occurred and have been of considerable value (Macfarlane et al., 2006). To date GOS and FOS have been the most extensively studied as prebiotics for supplementation to infant formula. ESPGHAN have concluded that the inclusion of 0.8g/100ml of oligosaccharide (combination of 90% oligagalactosyl-lactose and 10% high molecular weight oligofructosyl-saccharose) in infant formulas poses no major risk to the infant (Agostoni et al., 2004b). The review also showed evidence that 0.4g/dL, 0.8g/dL or 1g/dL mix of 90:10 GOS:FOS ratio brought about a significant increase in faecal bifidobacteria levels. It appears that a combination of long chain and short chain FOS/GOS and the ratio they appear in plays an important role in the efficiency of the prebiotic and its ability to exert beneficial effects. Extensive studies have repeatedly shown that prebiotics increase bifidobacteria and lactobacilli levels (Ben et al., 2004, Waligora-Dupriet et al., 2007) and examples of these studies will be described below. Before proceeding, it should again be noted that lactobacilli and bifidobacteria
constitute only a small proportion of the overall gut microbiota and future studies will need to investigate the global impact of prebiotics on the infant gut microbiota. Furthermore, as noted before, an increase in bifidobacteria and lactobacilli levels is not regarded as a valid health claim by EFSA.

In 2008, a review of studies investigating the impact of prebiotics on infant health was completed (Boehm and Moro, 2008). The authors highlighted the benefits of consuming human milk oligosaccharides (HMOS) with respect to infant health. These included decreased incidence of gastroenteritis and respiratory infections (Howie et al., 1990). It was also noted that specific combinations of prebiotics, including short chain (sc) GOS/ long chain (lc) FOS, increased bifidobacteria and lactobacilli levels to the extent that, in some cases, levels of these genera were comparable to those observed in the gut of breastfed infants (Boehm et al., 2005, Haarman and Knol, 2005, Parracho et al., 2007, Rinne et al., 2005, Salvini et al., 2011). This impact was apparent despite the fact that these prebiotics had structures which differed from those of HMOS. In one such study qPCR and FISH were used in combination to identify and quantify the bifidobacteria in faecal samples from infants fed GOS and FOS supplemented feeds (Haarman and Knol, 2005). This study revealed significant increases in faecal bifidobacteria in treated infants compared to controls and once again highlighted the ability of prebiotic supplemented formula to mimic breastfeeding effects on the gut microbiota, thus corroborating the results from earlier culture-based
studies. As this review has shown, newer studies are now employing culture-independent methods (e.g. FISH and qPCR in combination or separately) to investigate more specifically the effects of prebiotics on the gut microbiota of infants, which may lead to greater insights compared to those provided by earlier culture-based approaches. There is also however, an opportunity to use sequencing technologies to more accurately assess the effects of prebiotics on the gut microbiota (and not just the effects on bifidobacteria and lactobacilli levels).

1.10 Antibiotics

While probiotics and prebiotics can potentially modulate the infant’s gut microbiota in a positive manner, antibiotics can exert a detrimental effect on the infant’s commensal microbiota. The use of antibiotics has increased dramatically and consequently the effects of specific antibiotics on the gut microbiota of infants are a significant concern. A culture-based study in 1970 was one of the first to examine the effects of various antimicrobials on the gut microbial composition (Finegold, 1970). The study suggested that the intestinal microbiota was altered to different degrees depending on the spectrum of specificity of the antimicrobial administered, the duration of treatment and the route of administration. Interestingly in this early study, and also in some infant related studies since, it has been shown that penicillin exerts a less significant effect (and in some cases no significant effect) on the gut microbiota (Bennet et al., 2002) relative to other antibiotics, once more stressing the need to
investigate the effect of the different antimicrobials commonly prescribed during childhood in turn in order to determine the specific impact that they have on the gut microbiota. Since the initial Finegold et al. study highlighted the effects of antibiotics on the gut microbiota, several other culture-based studies have also supported these findings (Bennet et al., 2002, Sakata et al., 1985). Following on from these culture-based approaches, culture-independent approaches were completed and, in the majority of cases, they corroborated the results of the earlier studies while also providing an even greater insight (Mangin et al., 2010). In 2009, a culture-independent study examining the effects of antibiotics on the infant gut microbiota in the early postnatal period was published (Tanaka et al., 2009). This study involved 26 infants, 5 of whom had been treated with antibiotics. Faecal samples were analysed for the first 5 days of life and then monthly for 2 months. The impact on the gut microbiota was assessed using qPCR targeting the V1-V3 regions of the 16S rRNA gene. The study found that antibiotic treated infants (treated with cefalexin 50mg/kg four times daily, for the first four days of life) had significantly lower bifidobacteria until 1 month of age and had increased Enterococcus levels compared to antibiotic free controls. The impact of antibiotic administration on colonization patterns has also been the subject of attention. In one instance this involved a study which focussed on an infant in receipt of clavulanic acid and amoxicillin (Augmentin®) for 13 days, followed by trimethoprim and sulfamethoxazol (Bactrimel®) for 12 days (Favier et al., 2003). This study employed both culture-based and
culture-independent techniques to examine the effects of antibiotics on gut microbial composition. The antibiotic treated infant had an extremely unstable microbiota up to the age of 1 month, with *E. coli* being dominant in the early colonization period. However, the most significant difference between the antibiotic treated infant and the controls was the apparent absence of gut bifidobacteria. Indeed, up to 5 months of age no bifidobacteria were detected, highlighting the prolonged effects of antibiotic treatment on some commensal bacteria (Favier et al., 2003). Following on from the previously outlined negative effect of antibiotic treatment on bifidobacteria populations, another study in 2010 showed that treatment of infants with parenteral antibiotics (a combination of ampicillin and gentamicin), administered within 48 hours of birth, reduced, but did not completely eliminate bifidobacteria, i.e. some bifidobacteria such as *B. bifidum* survived antibiotic treatment (Hussey et al., 2011). At 8 weeks of age those who had been treated with antibiotics continued to have a less diverse population of bifidobacteria relative to controls. Recent reviews have indicated that the recovery of microorganisms after antibiotic administration can be delayed (Jernberg et al., 2010) and that in some cases some bacteria (namely *Bacteroides*) may not re-establish (Penders et al., 2006). In a recent longitudinal study of 28,354 mother-child pairs in the Danish national birth cohort it was found that antibiotics administered in the first 6 months of life were positively associated with an increased obesity risk in children of normal weight mothers by the time the children reached age 7 (Ajslev et al., 2011). Thus, the changes to the
infant’s gut microbiota in the initial months of life could predispose the infant to chronic illness in later life.

The impact of administering antibiotics to expectant mothers with respect to the gut microbiota and/or health of their infants has also been investigated (J Drychowski et al., 2006). Furthermore, in 2002, McKeever and colleagues examined the effect of maternal antibiotic use on the risk of allergic disease in their infants (Mckeever et al., 2002). The study was part of the general practice research database in the UK and included 24,690 children. The authors noted that 3 or more exposures to antibiotics during pregnancy was associated with an increased hazard ratio for asthma (1.36; 95% CI 1.16-1.60), eczema (1.19; 95% CI 1.02-1.39) and hay fever (1.33; 95% CI 1.0-1.77) in the infant. The positive association between antibiotic use in the first year of the child’s life and subsequent asthma and allergy risk was also demonstrated in a 2009 study, when it was established among 193,412 children that such antibiotic use was associated with an increased risk of asthma and allergies at age 6 or 7 (Foliaki et al., 2009). Previous studies have also supported this association between early life exposure to antibiotics and increased asthma and allergy risk (Kozyrskyj et al., 2007, Marra et al., 2009). Russell and Murch have also reviewed the impact of peripartum antibiotics on the gut microbiota of infants and in turn, their health effects and have suggested that administration of peripartum antibiotics could alter the initial colonization of the infant gut, resulting in an alteration to the GALT and a shift towards Th2 differentiation. Such a shift is known to
result in an increased risk of atopy (Russell and Murch, 2006). Furthermore, as highlighted previously in this review, changes to the gut microbiota in early life could affect the regulation of the immune system, which in turn could cause health effects. A complication in studying the effects of antibiotics and atopic disease is called “reverse causation”. The concept is that antibiotics may have preceded the atopic disease or may have been prescribed in response to symptoms. Thus, it is difficult to separate these to identify cause and effect.

While these studies demonstrate the negative effects of antibiotics on the gut microbiota, there is considerable merit in carrying out further investigations using the newer technologies available to us. Thus, by not having to select for specific microorganisms, as is the case when culture- or hybridisation-based approaches are employed, one could generate an overall profile of the impact of antibiotics on infant gut microbiota. While the studies to date are significant, and it is notable that a recent meta-analysis of 18 studies also found a weak positive association between antibiotic use in infancy and asthma and wheeze risk (OR 1.27 95% CI 1.12-1.43) (Penders et al., 2011), there are a number of other studies, such as that by Celedon et al. (Celedon et al., 2004), that failed to reveal the existence of an association. Similarly in 2007, the Koala Birth Cohort Study of over 2,700 families, once more failed to demonstrate any association between antibiotic use and eczema or asthma risk (Kummeling et al., 2007). In contrast, the most recent publication on this topic found that the limited diversity in the gut microbiota of infants arising
through antibiotic exposure before 1 month of age, was positively associated with atopic eczema risk by the age of 2 years (Abrahamsson et al., 2011). This again provides support for the theory that it may be reductions in the diversity of the gut microbiota (rather than in any particular species of bacteria) which results in an altered development and regulation of the immune system and subsequently results in long-term health consequences. Antibiotics by their very nature cause alterations to the microbiota present in the individual. However, the extent of the effects on beneficial microbiota, during and after the treatment period, needs to be examined further. As a consequence of the research on the concomitant use of probiotics and antibiotics and the observed benefits (such as decreased antibiotic associated diarrhoea risk), the use of specific probiotics in conjunction with antibiotics to benefit the host by minimising the negative effects on gut microbiota composition, may become even more prevalent in the future. New technologies such as high-throughput sequencing will also improve our ability to more accurately study the gut microbiota of antibiotic treated subjects in a less biased manner, than culture-dependent techniques. In particular, the impact of antibiotics on the GIT of preterm infants requires greater attention. Knowing the impact of different antibiotics on fullterm and preterm infant’s gut microbiota acquisition and development could lead to the selection of particular antibiotics on the basis of their efficiency in dealing with the illness, while having a minimal impact on the infant’s microbiota.
1.11 Conclusions

The infant’s gut microbiota undergoes rapid and radical changes during the first 2 years of life. During this period, changes at both the phylum and species levels occur. The changes which occur are determined by the factors that have been outlined in this review. Some of the most influential factors appear to be mode of delivery, feeding practices and the use of probiotics and prebiotics to potentially modulate the infant gut in what appears to be a positive manner (though there is a need for further research to determine what constitutes a ‘normal healthy’ gut microbiota). However, in contrast, antibiotics have clearly been shown to have detrimental and often prolonged effects. Thus, the first 2 years of the infant’s life may pose a unique window of opportunity, during which time the gut microbial composition can be positively modulated through diet and lifestyle factors. However, a number of questions remain. As scientists we must question what is the ideal composition of the infant gut microbiota? Do we know what core gut microbes will lead to the most favourable health outcomes? Does the core gut microbiome depend on the age of the infant (i.e. should we have milestones of colonization to aim for), and should this change depending on other factors such as ethnicity? Notably, breastmilk has been shown to be the optimum nutrition source for infants, thus investigating the composition of the breastfed infant’s gut microbiota at different time points may provide targets to aim for. If we know the benefits of breastfeeding should we try to prolong these effects and mimic them in the weaning period? While
this review has outlined the significant advances in our understanding of
the acquisition and development of the infant gut microbiota, it is evident
that large knowledge gaps still exist. Most notably we still struggle to
define what constitutes a beneficial or normal gut microbiota. In the
future, additional research of the long-term impacts on health arising from
an altered infant gut microbiota and, of the duration of the effects of
different factors (such as breastfeeding or antibiotic use) on these
populations is needed. To date, high-throughput sequencing technologies
have been relatively under-utilized with respect to investigations of the
infant gut microbiota, despite their widespread use in other fields of
microbial ecology, or even the gut microbiota of adults. Where these
technologies have been utilized, in many cases the results correlate
closely with those from culture-based approaches, thus showing the
ability to corroborate and supplement the culture-based results with those
generated using newer technologies. Additionally, it is evident the
significantly more comprehensive insights that can be achieved by using
the new technologies available. Accordingly, it is anticipated that our
movement into the metagenomic era will provide us with detailed insights
into the gut microbiota of infants, and the factors which influence this
microbiota, in the very near future. The value of such research has the
potential to be immense.
References


Malinen, E., Kassinen, A., Rinttila, T. & Palva, A. 2003. Comparison of real-time PCR with SYBR Green I or 5'-nuclease assays and dot-blot hybridization with rDNA-targeted oligonucleotide probes in


supplemented partially hydrolyzed infant formula and breastfeeding on infant gut microbiota. *FEMS Immunology & Medical Microbiology*, 43, 59-65.


Table 1 Techniques used to investigate the human gut microbiota; advantages, disadvantages and examples of use

<table>
<thead>
<tr>
<th>Technique description</th>
<th>Microflora associated characteristics</th>
<th>Culture-dependent techniques</th>
<th>Culture-independent techniques</th>
<th>High-throughput sequencing</th>
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</thead>
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<tr>
<td><strong>History of use</strong></td>
<td>The use of characteristics associated with microbes e.g. SCFA production to identify if differences exist in the gut microbial populations between different subject groups</td>
<td>Use of selective media to culture specific microorganisms or species of microorganisms e.g. Man Rogosa Sharpe (MRS) media for lactobacilli growth</td>
<td>Identify bacteria through isolation and amplification of bacterial DNA e.g. 16S rRNA gene. Includes: PCR, DGGE, TGGE, qPCR, dot blot hybridization, FISH, flow cytometry</td>
<td>Sequencing based approaches used to rapidly identify bacteria using bacterial DNA as template e.g. 454, Illumina, SoLID, Ion torrent</td>
</tr>
<tr>
<td><strong>Advantages</strong></td>
<td>In the past has been predominantly used as an initial population screen or in epidemiological studies</td>
<td>Historically, the most frequently used approach to identify bacteria present in various environments</td>
<td>Increasingly popular in past 2 decades with increasing availability of computer based technologies and software programs</td>
<td>Became commercially available at the beginning of the 21st century and becoming increasingly popular ever since</td>
</tr>
<tr>
<td><strong>Disadvantages</strong></td>
<td>• Simple • Inexpensive • Suitable as initial screen to test a novel hypothesis • Useful for large population screens e.g. in epidemiology studies</td>
<td>• Quick • Inexpensive • Limited skill required • Limited equipment needed • Useful as the initial screen before more detailed investigations</td>
<td>• Relatively inexpensive • Relatively simple • More detailed results achievable</td>
<td>• Less biased results • Very detailed information • Bacterial profile in complex environments e.g. gut microbiota can be identified • Huge phylogenetic information provided • Relatively quick</td>
</tr>
<tr>
<td></td>
<td>• Provides limited information • No bacterial species identification possible</td>
<td>• Up to 90% of bacteria non-culturable • Provides limited information • Need prior knowledge of bacteria to screen for • Requires further tests for species identification</td>
<td>• Prone to PCR bias • Requires more sophisticated equipment and training on their use • May need several methods in combination to get appropriate level of details in results</td>
<td>• Extremely expensive • Data handling requirements are significant • Requires training on sample preparation and machine use and experience of interpreting results</td>
</tr>
<tr>
<td>Examples of studies using this technique</td>
<td>References (Böttcher et al., 2000, Cardona, 2002, Tjellström et al., 2007)</td>
<td>References (Bennet et al., 2002, Hascoët et al., 2011)</td>
<td>Reference (Fallani et al., 2011)</td>
<td>References (De Filippo et al., 2010, Vaishampayan et al., 2010)</td>
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<tr>
<td>Future use in infant gut microbiota research</td>
<td>Most likely to be used to test novel hypotheses and to be followed up with more detailed techniques</td>
<td>Likely to become infrequently used and to be mainly used in combination with and verified by newer technologies</td>
<td>Likely to remain popular in the coming decade, but decrease thereafter as increased availability and use of sequencing approaches occurs</td>
<td>Increased use since 2000 as cost is decreasing and likely to become the main approach used in the future</td>
</tr>
</tbody>
</table>
Figure 1 Factors contributing to changes in gut microbiota composition in the first 2 years of life

Factors affecting gut microbiota at birth:
- Preterm vs. full term
- Mode of delivery
- Maternal weight/maternal diet
- Hospital environment
- Contact with mother/healthcare staff
- Antibiotic use (during pregnancy or in early days of life)
- Feeding choice

Factors affecting the development and acquisition of the infant gut microbiota (up to 24 months):
- Breastfeeding vs. formula feeding
- Use of probiotic/prebiotic supplemented feeds
- Antibiotic exposure
- Timing of weaning and foods chosen
- Home structure e.g. number of siblings
Figure 2 Location, concentrations and functions of human gut microbiota
Chapter 2

High-throughput sequencing reveals the incomplete, short-term, recovery of the infant gut microbiota following parenteral antibiotic treatment with ampicillin and gentamicin

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2.1 Abstract

The infant gut microbiota undergoes dramatic changes during the first two years of life. The acquisition and development of this population can be influenced by numerous factors, with antibiotic treatment being suggested as being among the most significant. Despite this, however, there have been relatively few studies which have investigated the short-term recovery of the infant gut microbiota following antibiotic treatment. The aim of this study was to use high-throughput sequencing (employing both 16S rRNA and *rpoB* specific primers) and quantitative PCR to compare the gut microbiota of 9 infants who underwent parenteral antibiotic treatment with ampicillin and gentamicin (within 48 hours of birth), 4 and 8 weeks after the conclusion of treatment, relative to that of 9 matched healthy controls. The investigation revealed that the gut microbiota of the antibiotic-treated infants had significantly higher proportions of *Proteobacteria* (*p*=0.0049) and significantly lower *Actinobacteria* (*p*=0.00001), (and the associated genus *Bifidobacterium* (*p*=0.0132)) as well as the genus *Lactobacillus* (*p*=0.0182) compared with the untreated controls 4 weeks after the cessation of treatment. By week 8, the *Proteobacteria* remained significantly higher in the treated infants (*p*=0.0049) but the *Actinobacteria, Bifidobacterium* and *Lactobacillus* levels had recovered and were similar to the control samples. Despite this recovery in total *Bifidobacterium* numbers, *rpoB*-targeted pyrosequencing revealed that the number of different *Bifidobacterium* species present in the antibiotic-treated infants was reduced. It is thus apparent that the
combined use of ampicillin and gentamicin in early life can have significant effects on the evolution of the infant gut microbiota, the long-term health implications of which remain unknown.
2.2 Introduction

It is becoming increasingly evident that the composition of the human gut microbiota can have a significant impact on health and disease (Sekirov et al., 2010, Mai and Draganov, 2009, Turnbaugh and Gordon, 2009, Zoetendal et al., 2008). Indeed, several studies have highlighted the role gut microbes play in diverse and important functions in the body including, for example, vitamin synthesis, immune system development and toxin metabolism (Guarner and Malagelada, 2003, Fujimura et al., 2010). Furthermore, there have been a number of studies which have suggested associations between an altered gut microbial composition and Crohn’s disease (Gophna et al., 2006), irritable bowel syndrome (Kassinen et al., 2007), obesity (Ley, 2010, Murphy et al., 2010) and other diseases/syndromes. These studies have highlighted the importance of developing and maintaining a ‘healthy’ gut microbiota. Indeed, it has recently been established that the fact that the immune system of germ free mice is not exposed to commensal microbes in early life can lead to increased numbers of invariant natural killer T cells, which in turn caused inflammation on exposure to particular microbes, resulting in an increased risk of both colitis and asthma (Olszak T, 2012). The infant gut microbiota is established early in life such that, although the infant gut is sterile in utero, by the time the infant reaches the age of two years, this microbiota resembles that of an adult (Adlerberth and Wold, 2009). Consequently, this period of the infant’s life represents a unique window of opportunity during which time the gut microbiota may be
modified with implications for health outcomes (Penders et al., 2006). A myriad of factors that affect this composition have been investigated and include mode of delivery (Penders et al., 2006), feeding choice (i.e. breast versus formula feeding) (Bezirtzoglou et al., 2011, Le Huërou-Luron et al., 2010), prematurity (Schwiertz et al., 2003, Westerbeek et al., 2006), and the administration of probiotics (Bourlioux et al., 2003, Cremonini et al., 2002, Parracho et al., 2007, Sazawal et al., 2010) and prebiotics (Boehm et al., 2005, Haarman and Knol, 2005, Sherman et al., 2009). It is also thought that exposure to antibiotics can have a significant negative influence on the composition and development of the gut microbiota in early life (Bennet et al., 2002, Favier et al., 2003, Hussey et al., 2011). Antibiotics by their very nature are designed to target and inhibit microorganisms in a variety of ways. The majority of those used clinically have a broad spectrum of activity and, as a consequence, in addition to controlling pathogenic bacteria, have the potential to inflict collateral damage on commensal gut bacteria (Blaser, 2011), including genera that can often have health-promoting roles, such as the bifidobacteria and lactobacilli. Thus far, the most in-depth investigations into the nature and extent of this collateral damage have relied on the use of denaturing gradient gel electrophoresis (DGGE) (Favier et al., 2003) and have revealed that antibiotic exposure in infancy results in significant decreases in bifidobacteria, lactobacilli and Bacteroides levels compared to control infants.
More recently, the impact of antibiotic administration on the gut microbiota has been revealed in even greater depth as a consequence of the use of high-throughput sequencing technologies in both animal and human trials (Antonopoulos et al., 2009, Dethlefsen et al., 2008, Dethlefsen and Relman, 2011, Murphy et al., 2010, Rea et al., 2011, Suchodolski et al., 2009). These studies have shown that antibiotics can dramatically alter the gut microbiota, with the effects depending on factors such as the specific antibiotic administered, the spectrum of inhibition and the duration of treatment (Sullivan et al., 2001). While antibiotic administration in adults can have a number of gut microbiota-mediated consequences, such as an increased susceptibility to Clostridium difficile associated diarrhoea (Cramer et al., 2008) there is also evidence to suggest that perturbation of the infant gut microbiota during its rapid developmental phase can have even more significant consequences. Indeed, for example, an association between antibiotic administration in early life and an increased risk of asthma and allergies, such as atopic eczema, in later life has been noted previously (Kozyrskyj et al., 2007, Robinson and Young, 2010, Abrahamsson et al., 2011). Thus, developing a detailed understanding of the impact of specific antibiotics on the infant gut microbiota is vital in order to begin to understand the mechanism(s) by which these changes could increase the risk of disease. It is thus notable, that the impact of antibiotics on the composition of the infant gut microbiota has yet to be assessed through high-throughput sequencing technologies. Here we address this issue, by using 454-pyrosequencing
technology together with quantitative PCR (qPCR), to reveal the short-term (4-8 weeks) consequences of the treatment of infants with a combination of ampicillin and gentamicin within the first 48 hours of birth.
2.3 Materials and Methods

2.3.1 Participants

Approval for this trial was received from the Clinical Research Ethics Committee of the Cork Teaching Hospitals, Cork, Ireland. Details on inclusion criteria, sample collection and storage have been outlined previously (Hussey et al., 2011). Briefly, 18 infants were recruited, 9 of whom had received parenteral antibiotic treatment with a combination of ampicillin and gentamicin within 48 hours of birth, and 9 untreated controls. Exclusion criteria included premature birth, requiring oral antibiotics, being on nil by mouth, infants who required surgery or those with congenital abnormalities. Faecal samples were collected 4 and 8 weeks after the cessation of antibiotic treatment. Of the 18 infants, 8 had been breastfed and 10 formula fed, while 13 were born vaginally and 5 by Caesarean section (Table 1).

2.3.2 Generation of 16S rRNA amplicons for high-throughput sequencing

The generation of 16S rRNA amplicons was performed as described previously (Murphy et al., 2010). Total bacterial DNA was extracted from the faecal samples using the QIAamp DNA Stool Mini Kit (Qiagen, West Sussex, UK) (Hussey et al., 2011). DNA was frozen at -80°C prior to PCR amplification. 16S rRNA bacterial gene amplicons (V4) were generated with a view to high-throughput sequencing using the Roche Genome Sequencer FLX platform. These amplicons, 239 nucleotides in length,
were generated using one forward, i.e. F1 (5’ AYTGGGYDTAAAGNG), and a combination of 4 reverse primers, R1 (5’ TACCRGGGHTHTCTAATCC), R2 (5’ TACCAGAGTATCTAATTC), R3 (5’ CTACDSRGGTMTCTAATC) and R4 (5’ TACNVGGGTATCTAATC). These primers also contained an A (F primer) or B (R primers) adapter and different versions of the F primer (each containing a distinct multiple identifier (MID)) were employed for each sample. PCRs were completed on a G-storm PCR machine under the following conditions: heated lid 110°C, 94°C for 2 min followed by 35 cycles of 94°C for 1 min, 52°C for 1 min and 72°C for 1 min followed by a temperature step of 72°C for 2 min and held at 4°C. PCRs had a final volume of 50µl made up of 25 µl of Biomix Red (MyBio, Ireland), 1 µl forward primer (0.15µM), 1 µl reverse primer (0.15µM) (mix of 4), template DNA and sterile PCR grade water. All samples were completed in duplicate. PCR products were analysed by agarose gel electrophoresis (1.5% in 1x TAE buffer). Following this, PCR products were cleaned using Agentcourt AMPure kit (Beckman Coulter Genomics, UK) as per manufacturer’s protocol. Samples were then quantified using the Quant-iT Picogreen quantification kit (Biosciences, Ireland) and the Nanodrop 3300 (Thermo Scientific, Ireland). Equimolar solutions of samples were then pooled for sequencing. These pooled samples were then cleaned and re-quantified (as before). Emulsion based clonal amplification was completed as part of the 454-pyrosequencing process. Sequencing took place at the Teagasc 454 Sequencing facility on a Genome Sequencer FLX platform (Roche
Diagnostics Ltd, West Sussex, UK) according to the manufacturer’s protocols.

2.3.3 Generation of Bifidobacterium-derived rpoB amplicons for high-throughput sequencing

A set of PCR primers, which have been used previously to facilitate the identification of bifidobacteria (Kim et al., 2010), and which amplify a 351 bp region from the Bifidobacterium RNA polymerase β-subunit gene, rpoB, were also utilized in this study. Twelve week 4 and twelve week 8 samples were selected and amplified using these primers which had MID tags and 454 adapters attached, allowing pooling of the samples for sequencing while also enabling downstream separation of individual results for analysis (Table S1). PCRs were completed on a G-storm PCR machine under the following conditions: heated lid 110°C, 94°C for 2 min followed by 35 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min followed by a temperature step of 72°C for 2 min and held at 4°C. PCRs had a final volume of 50µl containing 25 µl of Biomix Red (MyBio, Ireland), 1 µl forward primer (0.15µM) (BC1 5’-TCGATCGGGCACATACGG), 1 µl reverse primer (0.15µM) (Rev 1 5’-CGACCACCTTCGGCAACCG), template DNA and sterile PCR grade water. All samples were completed in duplicate. All other steps for sequencing (cleaning, quantifying, pooling etc.) were completed as outlined above.

2.3.4 Bioinformatic analysis
Raw 16S rRNA sequencing reads were quality-trimmed using a locally installed version of the Ribosomal Database Project (RDP) Pyrosequencing Pipeline applying the criteria as previously described (O’ Sullivan et al., 2011). Trimmed FASTA sequences were then BLASTed (Altschul et al., 1997) against a previously published 16S-specific database (Urich et al., 2008) using default parameters. The BLAST output was then parsed using MEGAN software (version 4.6) (Huson et al., 2007), which assigns reads to NCBI taxonomies by employing the Lowest Common Ancestor algorithm. Bit scores from within MEGAN were used to filter the results prior to tree construction and summarization. A bit-score of 86 was selected, as previously used for 16S ribosomal sequence data (Urich et al., 2008). Phylum, family and genus counts for each subject were extracted from MEGAN. Clustering and diversity analysis of the sequence data was performed using the MOTHUR software package (Schloss and Handelsman, 2008, Schloss et al., 2009). For *Bifidobacterium* analysis, raw *rpoB* sequencing reads were quality trimmed as above, with read-lengths for the *rpoB* amplicon above 300 bp being used. Trimmed FASTA sequences were then BLASTed (Altschul et al., 1997) against the NCBI non-redundant database using default parameters. The resulting BLAST output was parsed through MEGAN using default parameters (Huson et al., 2007).

### 2.3.5 qPCR-based determination of total bacteria and total bifidobacteria numbers
Absolute quantification of total bacterial numbers (from 8 representative infants, infants B, F, G, H, K-N) and total bifidobacteria numbers (from 9 representative infants, infants B, F, G, H, K-O) was carried out by qPCR using the Roche 480 Lightcycler platform. To determine total bifidobacteria counts, the primers g-Bifid-F (5’-CTCCTGGAAACGGGTGG) and g-Bifid-R (5’-GGTGTTCCTCCGATATCTACA) were used (Matsuki et al., 2004). *Bifidobacterium longum* ATCC 8809 was used as a reference strain to generate a standard curve for total bifidobacteria quantification (Echarri et al., 2011). *B. longum* was grown overnight anaerobically at 37°C in modified MRS broth (Difco) (0.05% cysteine) (Sigma Aldrich). Total bacterial DNA was then isolated using High Pure PCR template preparation kit (Roche Diagnostics, West Sussex, United Kingdom) as per manufacturer’s instructions, and used to establish a standard curve on the Lightcycler 480 platform (Roche Diagnostics, West Sussex, United Kingdom). Total bifidobacteria numbers were quantified using the following programme: 95°C for 5 min followed by 50 cycles of 95°C for 10s, 60°C for 20s and 72°C for 20s followed by melting curve analysis of 95°C for 5s, 65°C for 1 min and 97°C continuously, followed by cooling at 40°C for 10s. Reactions took place in a 20 µl volume made up of 3 µl PCR grade water, 1 µl g-Bifid-F (0.15 µM), 1 µl g-Bifid-R (0.15 µM), 5 µl DNA template and 10 µl SYBR green (Roche Diagnostics, West Sussex, United Kingdom). To quantify total 16S rRNA bacterial counts, a standard curve was established using copy numbers of 16S rRNA/µl from $10^9$-$10^2$
copies 16S rRNA/µl. Values were then converted to copies 16S rRNA/g wet stool using a previously outlined calculation (Zhang et al., 2009). The following programme was used to quantify total bacterial numbers: 95°C for 5 minutes followed by 35 cycles of 95°C for 20s, 51°C for 20s and 72°C for 20s followed by melting curve analysis of 95°C for 5s, 46°C for 1 min and 97°C continuously and a final cooling at 40°C for 10s. Samples contained 2 µl of PCR grade water, 1 µl of forward primer F1 (5’-AYTGGGYDTAAAGNG) (0.15 µM), 1 µl of the reverse primer R1 (5’-TACCRGGGTHTCTAATCC) (0.15 µM), 1 µl template DNA and 5 µl of SYBR green (Roche Diagnostics, West Sussex United Kingdom), giving a final reaction volume of 10 µl. Samples were run in quadruplicate, while negative controls (where template DNA was replaced with PCR grade water) and standards were run in triplicate.

2.3.6 Statistical Analysis

Minitab Release 15.1.1.0 (Minitab Inc. 2007) was used to perform non-parametric statistical analysis (Mann Whitney test) when comparing 2 specific subject groups to determine the impact of antibiotic treatment on the microbiota. Statistical significance was accepted at p<0.05.
2.4 Results

2.4.1 High-throughput sequencing of 16S rRNA amplicons from the faecal samples of antibiotic-treated and control infants

Eighteen infants, 9 of whom had been treated with a combination of parenteral ampicillin and gentamicin within 48 hours of birth, and 9 controls who had received no antibiotic treatment, were recruited (Hussey et al., 2011). Faecal samples were collected 4 and 8 weeks after the cessation of antibiotic treatment and faecal DNA was extracted and used as a template to generate 16S rRNA amplicons, with a view to determining the composition of the gut microbiota through next generation sequencing. Diversity, richness, coverage and evenness estimations were calculated for all data sets (Table 2). The Chao 1 calculation is an estimator of phylotype richness in a dataset and the Shannon index of diversity reflects both the richness and the community evenness (i.e. proportional phylotype abundance). The diversity index was above 3.6 in all samples, indicating an overall high level of biodiversity (Table 2). The Good’s coverage, a measure of sampling completeness, at the 97% similarity level ranged between 88.6-96.1% for the datasets. The lowest value was obtained for the control samples at week 8 and is a reflection of the more diverse nature of the microbiota present (Table 2).

2.4.2 Composition of the gut microbiota of antibiotic-treated and control infants 4 weeks after the conclusion of treatment
Bioinformatic analysis of 16S rRNA sequence data revealed that there were significant differences in the gut microbiota of antibiotic-treated infants compared with untreated controls 4 weeks following the cessation of antibiotic treatment. Statistically higher proportions of reads corresponding to the phylum *Proteobacteria* were detected in the antibiotic-treated samples compared with the control samples (*p*=0.0049) (Figure 1). Indeed, the gut microbiota of the antibiotic-treated infants was dominated by *Proteobacteria*, accounting for 54% of all bacteria present, compared to just 37% in the untreated controls (Figure 1). While *Proteobacteria*, *Firmicutes* and *Actinobacteria* were found in all antibiotic-treated infants at week 4, *Bacteroidetes* were detected in less than half of these infants and, in those where they were detected, levels were notably low (Figure 1 & SI Figure 1). *Actinobacteria* were also significantly lower in the antibiotic-treated samples than in the controls (3% vs. 24%; *p*=0.00001).

At the family level, the antibiotic-treated samples had significantly higher numbers of *Enterobacteriaceae* (55% vs. 37%; *p*=0.0073) and *Peptostreptococcaceae* (23% vs. 2%; *p*=0.0381) compared to the control infants at week 4 (Figure 2). Significantly lower numbers of *Bifidobacteriaceae* (3% vs. 24%; *p*=0.0132) were also evident in the antibiotic-treated samples at week 4. In addition antibiotic treatment also resulted in significant differences at genus level relative to the controls at this time (Figure 3). Significantly higher levels of *Bifidobacterium* (25% vs. 5%; *p*=0.0132) and *Lactobacillus* (4% vs. 1% *p*=0.0088) were present in
the untreated controls compared to the antibiotic-treated infants. Additionally, the gut microbiota of the antibiotic-treated infants displayed limited diversity, as they were dominated by genera within the Enterobacteriaceae family, with levels of these bacteria being statistically significantly higher in the antibiotic-treated infants compared to the controls (p=0.0073). This pattern was also apparent with respect to proportions of the Firmicutes-associated genus Clostridium (p=0.0033). Additionally, there was a significantly higher level of enterococci in the treated infants compared to the controls at week 4 (p=0.0172). Despite the fact that the diversity of antibiotic-treated and control samples did not differ significantly (p=0.5752) (Table 2), the overall numbers of genera detected in the antibiotic-treated samples was notably lower than in the controls, reflecting the restriction in diversity and the dominance of the members of the Proteobacteria and the persistent effects of antibiotic treatment 4 weeks after administration ceased.

2.4.3 Composition of the gut microbiota of antibiotic-treated and control infants 8 weeks after the conclusion of treatment

Bioinformatic analysis of the 16S rRNA sequence data revealed that, the week 8 samples from the antibiotic-treated infants contained significantly higher proportions of Proteobacteria (44%) compared to controls (23%) (p=0.0049). Eight weeks after the cessation of antibiotic treatment Proteobacteria continued to be the dominant phylum present in antibiotic-treated infant samples despite the fact that the proportions of
Proteobacteria reads decreased significantly between week 4 and week 8 (p=0.0136). During the same period the proportion of Actinobacteria reads increased significantly (p=0.0055) in the antibiotic-treated infant samples, to the extent that they no longer differed significantly from those in the control samples (p=0.1164). Nonetheless, a more diverse gut microbe population was observed in the controls relative to the antibiotic-treated samples 8 weeks after antibiotic treatment (Shannon’s index for diversity was 3.8 and 4.6 in the treated and control infants, respectively) (Figure 1 & Table 2). Analysis of data from individual infants also revealed that the recovery of the infant gut microbiota to one more comparable to that of the controls was also dependent on the duration of treatment (data not shown). For example, the gut microbiota of the infant who underwent the longest antibiotic treatment period (infant A, treated for 9 days) displayed the most limited recovery of all treated infants. This infant’s gut microbiota was populated predominantly by Proteobacteria, and this phylum remained dominant at week 8, at which time it accounted for 67% of all of the bacteria detected (Figure S1).

At the family level at week 8, the Enterobacteriaceae remained dominant in the antibiotic-treated infants (45%), despite having significantly decreased in proportion relative to week 4 (p=0.0136) (Figure 2). During the same interval, proportions of Enterobacteriaceae decreased in the control infants (37% at week 4 vs. 24% at week 8). In the antibiotic-treated group, there was also a significant decrease in levels of Peptostreptococcaceae between week 4 and week 8 (p=0.0014) whereas
a significant increase (p=0.0182) in the *Bifidobacteriaceae* levels occurred during this 4 week interval, to the extent that the proportions of this family in the antibiotic-treated and control samples no longer differed significantly by week 8 (p=0.3927).

At genus level, the gut microbiota of the antibiotic-treated infants remained predominantly populated with members of the *Enterobacteriaceae* family, which accounted for half of all of the genera detected at week 8. The numbers of these bacteria were significantly higher in the antibiotic-treated infants than in the control samples at week 8 (p=0.0061). In contrast, *Bifidobacterium* numbers were similar in the controls and antibiotic-treated samples at this time (19% vs. 15%, p=0.3927). This was as a consequence of the fact that the proportions of *Bifidobacterium* had increased significantly in the antibiotic-treated samples during this 4 week interval (p=0.0182). Significant differences in the levels of *Lactobacillus* no longer existed at week 8 between the 2 groups (p=0.3253) (Figure 3), due to a trend towards a significant recovery in *Lactobacillus* proportions in the antibiotic-treated samples (p=0.059) during this interval. In addition, *Clostridium* proportions remained higher in the treated infants compared to the controls at week 8 (7% vs. 2%; p=0.0345), as a consequence of the fact that there was no significant change in the levels of *Clostridium* in the antibiotic-treated infants between weeks 4 and 8 (p=0.6132). By week 8 there was no longer a significant difference in the proportions of enterococci seen in the treated infants compared to the controls (p=0.1105).
2.4.4 qPCR-based determination of total bacteria and total bifidobacteria numbers

To determine the impact of antibiotic treatment on the total number of bacteria and of bifidobacteria, absolute quantification was completed using qPCR, with a representative subset of samples. The qPCR results revealed that all infants, i.e. both treated and controls, had $10^7$-$10^8$ copies of the 16S rRNA gene/g wet stool (Table 3) and established that no significant differences existed between total 16S rRNA gene copies (which is representative of total bacteria numbers) when values for antibiotic-treated infant samples were compared to those for controls at week 4 ($p=0.7667$) or week 8 ($p=0.7918$). However, a statistically significant increase in total 16S rRNA values did occur in the antibiotic associated samples ($p=0.0005$) between weeks 4 and 8. With respect to total bifidobacteria numbers, it was established that counts in both the treated and control samples ranged from $10^6$-$10^7$ CFU/g wet stool (Table 4). There was no significant difference in the average bifidobacteria numbers of the antibiotic-treated infants relative to the controls at week 4 ($p=0.4273$) or at week 8 ($p=0.1548$). Furthermore, in the majority of individual infants, the total bifidobacteria numbers did not differ significantly between the two time points (Table 4).

2.4.5 Specific assessment of the composition of the gut Bifidobacterium population in antibiotic-treated and control infants
Given the health benefits that have been attributed to many strains of *Bifidobacterium*, a strategy was implemented to specifically assess the impact of antibiotic treatment on gut bifidobacteria. This again relied on the use of high-throughput sequencing but in this instance focused on the sequencing of amplicons corresponding to a region of the *Bifidobacterium* sp. RNA polymerase β-subunit gene, *rpoB*, using a set of primers which have been used previously for bifidobacterial species identification (Kim et al., 2010) but, in this instance, contain adapters and MIDs to facilitate the sequencing process. These primers demonstrated excellent specificity with 99% of the reads at phylum level being assigned to the *Actinobacteria*. The total number of reads for the antibiotic-treated infants at week 4 was 80,034, averaging at 6670 reads per subject and for week 8 was 36,557, averaging at 3046 reads per subject. While the 16S rRNA data presented above showed that antibiotic treatment decreased the proportion of bifidobacteria present in the gut microbiota of infants, the *rpoB* data provides further, more detailed insights. More specifically, this analysis revealed that only 2 species were detected in the majority of cases in the antibiotic-treated infants, namely *B. longum* and *Bifidobacterium breve*. In contrast, the controls showed a more considerable variation in the composition of individual samples, and even between samples from the same individuals at different time points (Figure 4).
2.5 Discussion

Antibiotics are of fundamental importance to modern medicine and their use has been pivotal to the prolongation of human life. Despite this, there are ever increasing concerns with respect to the negative consequences of antibiotic utilization, including issues revolving around the collateral damage inflicted on the commensal microbiota and the implications thereof (Blaser, 2011). Short-term health effects include antibiotic associated diarrhoea, gastrointestinal discomfort, gastritis and glossitis (Finegold, 1970) as well as the possible development of antibiotic resistant bacteria populations in the gut (Jernberg et al., 2010). Furthermore, it has been suggested that a number of long-term health effects are influenced by the development of the gut microbiota (Jakobsson et al., 2010) and, in turn, the immune system in early life (Hill and Artis, 2009, Hill et al., 2010, Ivanov et al., 2008, Sekirov et al., 2010), with data suggesting that antibiotic administration contributes to the risk of developing asthma and allergy (Celedon et al., 2004, Foliaki et al., 2009, Johnson et al., 2005) in addition to heightened risk of obesity (Ajslev et al., 2011) later in life. The risks associated with disrupting the gut microbiota may be especially great in young infants, as antibiotic administration can impact on the commensal microbiota at a time when this population is in rapid flux and can easily be unbalanced. Despite this concern, there have been no studies to date which have used powerful next generation sequencing technologies to assess the microbiota of infants who have been administered antibiotics. This study was
performed with a view to addressing this issue by employing 454-pyrosequencing, together with qPCR analysis. The results of this relatively small study are important and highlight the apparently major impact that treatment with a combination of ampicillin and gentamicin can have on the gut microbiota of infants. It is evident that the treated infants suffered significant reductions in potentially beneficial bacteria belonging to the phylum Actinobacteria, including *Bifidobacterium*, as well as some members of the Firmicutes phylum, including *Lactobacillus*. These appeared to be replaced by members of the Proteobacteria, including members of the *Enterobacteriaceae* family, thereby resembling trends previously noted in a terminal restriction fragment length polymorphism-based study of antibiotic-treated infants (Tanaka et al., 2009). The dominance of the Proteobacteria, and an overall reduced microbial diversity, continued to be evident even 8 weeks after antibiotic treatment, despite the fact that populations of potentially beneficial bacteria (including *Bifidobacterium*) recovered somewhat. Given the fact that sequencing data provides results revealing the proportions of different populations present, rather than their absolute number, the question as to whether the dominance of Proteobacteria is reflective of an outgrowth of this population, or its numbers remaining stable among a total bacterial population which is diminished in number, exists and some of the more recent sequencing based studies have begun to address this issue (Murphy et al., 2010, Rea et al., 2011). It is thus important that in this instance qPCR data establishes that there is no significant difference
between the total 16S rRNA counts in the treated infants compared to the controls, thereby implying *Proteobacteria* outgrowth, presumably as a consequence of reduced competition from other more antibiotic-sensitive gut microbes. Others have also documented a corresponding phenomenon of *Proteobacteria* outgrowth as a consequence of antibiotic administration (Rea et al., 2011, Fallani et al., 2010, Murphy et al., 2010). Notably, the frequency of β-lactam antibiotic resistance among *Enterobacteriaceae*, as a consequence of the production of β-lactamases, has been well established (Bush, 2010, Nordmann, 1998, Penders et al., 2006, Qin et al., 2009). The presence of significantly higher levels of enterococci in the antibiotic-treated samples 4 weeks after treatment ended is also consistent with the fact that ampicillin (Jureen et al., 2003, Weisser et al., 2012) and gentamicin (Donabedian et al., 2003, Huycke et al., 1991) resistant *Enterococcus* have been identified on numerous occasions. The ability of the administered antibiotics, and especially ampicillin, to significantly alter the gut microbiota is also reflective of their activity profile. Following parenteral administration, ampicillin is rapidly and widely distributed throughout the body resulting in high levels in bile (Acred et al., 1962) and, once excreted, in the gut.

It was notable that while the 16S rRNA sequencing data and the total bacteria qPCR data correlated well, the assessment of the impact of antibiotic administration on relative or total bifidobacteria numbers, as determined by sequencing and qPCR respectively, was not consistent.
More specifically, qPCR analysis at week 4 revealed no significant difference between total bifidobacteria values in the antibiotic-treated infants compared to the controls, while the 16S sequencing data detected significantly lower proportions in infants that had undergone antibiotic treatment. These differences may be accounted for by the fact that only a subset of the 18 infants were included in the rpoB-based qPCR analysis and, as outlined earlier, individual variations occur in response to antibiotic treatment. Furthermore, differences regarding primer specificity between those used for qPCR and for total bacterial 16S rRNA sequencing may also have contributed to this result.

The altered gut microbial composition of antibiotic-treated infants is a concern given that several members of the Bifidobacterium and Lactobacillus genera have been found to possess health-promoting properties, to the extent that they are frequently employed as probiotic cultures, whereas many Proteobacteria have the potential to become pathogenic given a suitable environment. This study also demonstrated that the collateral damage inflicted on the gut microbiota through the use of broad spectrum antibiotics is not rapidly repaired, as significant differences between the composition of antibiotic-treated and control populations were apparent at 4 and 8 weeks post treatment. Previous studies, employing temperature gradient gel electrophoresis or DGGE, have also shown that antibiotic treatment causes short- to medium-term effects, in some cases with no bifidobacteria being detected 28 days after treatment ceased (Favier et al., 2003, De La Cochetiere et al., 2005).
However, the fact that some recovery was evident in this and previous trials (Hussey et al., 2011, De La Cochetiere et al., 2005) indicates that the infant microbiota, despite being much less stable than that of an adult, is somewhat resilient. Indeed, on average, bifidobacteria populations recovered to the extent that both sequencing- and qPCR-based analysis revealed that their levels were no longer significantly reduced in the antibiotic-treated samples, relative to the controls, at week 8. Critically, however, it was apparent that the composition of these *Bifidobacterium* populations differed from one another. This is consistent with previous studies highlighting the differing susceptibilities of species of bifidobacteria to antibiotics (Fallani et al., 2010, Favier et al., 2003, Mangin et al., 2010). More specifically, in agreement with previous DGGE-based analysis (Hussey et al., 2011), *B. longum* was found to be more dominant in samples from antibiotic-treated infants. This may be due to the fact that while all bifidobacteria have previously been found to display comparably high levels of sensitivity to gentamicin, strains of *B. longum* have been found to be more ampicillin resistant than other bifidobacteria (Mättö et al., 2007). The significant impact of antibiotic administration on the *Bifidobacterium* population at the species level suggests that many other species are similarly impacted upon by antibiotic administration, something which warrants further investigation.

It is important to note that factors other than antibiotic administration may also contribute to the differences in the gut microbial composition of the cohort of infants that were the focus of this investigation. From this
perspective, it is notable that the majority of antibiotic-treated infants were delivered by Caesarean section, while the controls were all delivered vaginally. This is particularly relevant as numerous studies have noted the presence of an altered gut microbiota profile in Caesarean delivered infants (Adlerberth and Wold, 2009, Dominguez-Bello et al., 2010, Fouhy et al., 2012). Caesarean delivered infants have significantly altered profiles compared to vaginally delivered infants, due to a lack of colonization with their mother’s vaginal microbiota during delivery and are instead colonized by skin microbiota (predominantly *Staphylococcus* and *Corynebacterium* (Dominguez-Bello et al., 2010)). However, it has also been previously demonstrated that while levels of *Bifidobacterium* were significantly lower in Caesarean delivered infants compared to vaginally delivered infants, *Bifidobacterium* levels were comparable by 1 month of age (Grölund et al., 1999). This was not the case in our study, in that all antibiotic-treated infants (regardless of delivery mode) had significantly lower levels of *Bifidobacterium* at 1 month of age and, even at 2 months of age, possessed a gut microbiota which was altered relative to the control group. It is also worth noting that although our microbiota-related data is presented as an average, we also possessed the microbiota-related data (both high-throughput sequencing and qPCR derived) from each infant. Analysis of this data failed to reveal significant differences between the microbial populations of the vaginally delivered and Caesarean delivered infants subgroups of the antibiotic treated infants (data not shown). Thus, while we acknowledge that delivery mode may
influence the microbial composition of the infants studied, it would seem not to be as significant a factor as antibiotic administration. Another factor that merits consideration relates to breastfeeding. While there are a considerable numbers of publications regarding the benefits of breastfeeding with respect to the development of the infant gut microbiota (Bezirtzoglou et al., 2011, Penders et al., 2006), in this study breastfeeding did not provide any additional protection to the infant gut microbiota against the impact of antibiotic treatment. A failure to observe protection could well be due to the relatively small subgroup of our infants who were breastfed. Regardless, this is a topic which warrants further investigation. Regardless of the extent to which factors other than antibiotic administration influence these results, there is a concern that these short-term changes to the microbiota may in turn have long-term health consequences in the form of allergies, asthma etc. later in life (Celedon et al., 2004, Foliaki et al., 2009). While follow up analysis of these infants was outside the scope of this short-term study, we hope to return to this topic in future studies. In conclusion, this study has shown the altered microbiota, over 8 weeks, of a group of infants who were in receipt of parenteral antibiotic treatment within the first 48 hours of life. To our knowledge, this is the first study to use high-throughput sequencing of 16S rRNA and/or rpoB amplicons to accurately assess these impacts. While the results may reflect a combination of several environmental effects in early life, it would appear
that antibiotic administration is the most influential factor. It would thus seem that, where available, the use of narrow spectrum antibiotics coupled with the use of pre and probiotics should be considered with a view to minimizing the risk of long-term health effects. While it is evident that the study of the composition of the infant gut microbiota and the consequence of antibiotic treatment on this population requires further investigation, it is anticipated that the further application of high-throughput sequencing technologies (including those used in long-term follow up trials) will shed additional light as to the optimal strategies to employ to control infection, while minimising the risks to commensal microbes.
References


fecal microbiota of irritable bowel syndrome patients differs significantly from that of healthy subjects. *Gastroenterology*, 133, 24-33.


### Table 1 Details of the infants in the trial

<table>
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<th>Infant</th>
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<th>Feeding method</th>
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<td>M</td>
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<tr>
<td>D</td>
<td>M</td>
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<td>Formula</td>
<td>2</td>
</tr>
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<td>Formula</td>
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</tr>
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<td>F</td>
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<td>2</td>
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<td>Formula</td>
<td>2</td>
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<td>Formula</td>
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* M, male; F, female. Table adapted with permission from Hussey et al. 2011
### Table 2  Estimation of diversity within the treated and control groups at week 4 and week 8

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<th>Data set</th>
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<th>Control week 4</th>
<th>Treated week 8</th>
<th>Control week 8</th>
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<td>97%</td>
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<td>334</td>
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**Table 3** Total bacteria numbers given as copies of 16S rRNA/g wet stool in treated and control samples at week 4 and week 8

<table>
<thead>
<tr>
<th>Treated</th>
<th>Wk 4</th>
<th>Wk 8</th>
<th>P</th>
<th>Controls</th>
<th>Wk 4</th>
<th>Wk 8</th>
<th>P</th>
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<td>K</td>
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<td><strong>Average</strong></td>
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P values based on Mann Whitney analysis, with statistical significance determined as p< 0.05. P values are indicating if statistically significant differences exist between total bacterial numbers in each infant between week 4 and week 8.
Table 4 Total bifidobacteria numbers as CFU/g infant stool in the treated and control samples at week 4 and week 8

<table>
<thead>
<tr>
<th>Treated</th>
<th>Wk 4</th>
<th>Wk 8</th>
<th>P</th>
<th>Controls</th>
<th>Wk 4</th>
<th>Wk 8</th>
<th>P</th>
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P values based on Mann Whitney analysis, with statistical significance determined as p< 0.05. P values are calculated with respect to differences in total bifidobacteria numbers within individual infants between week 4 and week 8.
Figure 1 Microbial distributions at phylum level in the treated and control samples at week 4 and week 8. Statistically significant differences between treated samples and controls at week 4 are denoted by * (where p< 0.05). Statistically significant differences between treated samples and controls at week 8 are denoted by *. A statistically significant difference between treated samples at week 4 and at week 8 (i.e. the recovery of the treated samples) is denoted by ♦. Percentages are based on proportion of assignable tags.
Figure 2 Microbial distributions at the family level in the treated and control samples at week 4 and week 8. Statistically significant differences between treated samples and controls at week 4 are denoted by * (where p<0.05). Statistically significant differences between treated samples and controls at week 8 are denoted by *. A statistically significant difference between treated samples at week 4 and at week 8 (i.e. the recovery of the treated samples) is denoted by ♦. Percentages are based on proportion of assignable reads.
Figure 3 Microbial distributions at the genus level in the treated and control samples at week 4 and week 8. Statistically significant differences between treated samples and controls at week 4 are denoted by * (where p<0.05). Statistically significant differences between treated samples and controls at week 8 are denoted by *+. A statistically significant difference between treated samples at week 4 and at week 8 (i.e. the recovery of the treated samples) is denoted by ♦. Percentages are based on proportion of assignable reads.
Figure 4 Individual distributions of bifidobacteria in the treated (B1-H1; B2-H2) and control samples (J1-R1; J2-R2) as detected using rpoB amplicons for 454-pyrosequencing. Values show the percentage of the different bifidobacteria species present in the individual samples. Treated samples show far less variability both between treated samples and also between week 4 and week 8 compared to the controls. X-axis indicates the individual infants; y-axis percentage of total bifidobacteria assigned to each species.
Chapter 2

Supplementary information
<table>
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Figure S1 Distribution of microbiota at phylum level in the antibiotic-treated (A1-I1) and control infants (J1-R1) at week 4 and week 8, showing the limited number of phyla detected in the antibiotic-treated infants compared to the controls and also showing the individual variability that exists between samples, particularly in the controls.
Chapter 3

A degenerate PCR-based strategy as a means of identifying homologues of aminoglycoside and β-lactam resistance genes in the gut microbiota

3.1 Abstract
The potential for the human gut microbiota to serve as a reservoir for antibiotic resistance genes has been the subject of recent discussion. However, this has yet to be investigated using a rapid PCR-based approach. In light of this, here we aim to determine if degenerate PCR primers can detect aminoglycoside and β-lactam resistance genes in the gut microbiota of healthy adults, without the need for an initial culture-based screen for resistant isolates. In doing so, we would determine if the gut microbiota of healthy adults, lacking recent antibiotic exposure, is a reservoir for resistance genes.

Degenerate PCR primers were used to detect and amplify aminoglycoside and β-lactam resistance genes from human gut microbiota samples. Cloning and Sanger sequencing of the PCR amplicons was also conducted to assess levels of homology to known resistance genes.

The strategy employed resulted in the identification of numerous aminoglycoside (acetylation, adenylation and phosphorylation) and β-lactam (including bla_{OXA}, bla_{TEM}, bla_{SHV} and bla_{CTX-M}) resistance gene homologues. On the basis of homology, it would appear that these genes originated from different bacterial taxa, with members of the Enterobacteriaceae being a particularly rich source. The results demonstrate that, even in the absence of recent antibiotic exposure, the human gut microbiota is a considerable reservoir for antibiotic resistance genes.
This study has demonstrated that the gut can be a significant source of aminoglycoside and β-lactam resistance genes, even in the absence of recent antibiotic exposure. The results also demonstrate that PCR-based approaches can be successfully applied to detect antibiotic resistance genes in the human gut microbiota, without the need to isolate resistant strains. This approach could also be used to rapidly screen other complex environments for target genes.
3.2 Introduction

Almost as soon as the widespread therapeutic use of antibiotics occurred, bacteria displaying diverse and complex mechanisms of resistance became problematic (Davies and Davies, 2010, Abraham and Chain, 1940). As the human gut is one of the most densely populated microbial environments, it has been postulated that it can act as a considerable reservoir for antibiotic resistance genes (Salyers et al., 2004). Thus, gut microbes may disseminate antibiotic resistance genes to other commensals or to bacteria transiently colonizing the gut (Broaders et al., 2013). Given that antibiotics are known to exert significant and sustained negative effects on the gut microbiota (Dethlefsen et al., 2008, Cotter et al., 2012), possessing resistance genes can provide a significant selective advantage to a subpopulation of microorganisms in individuals undergoing antibiotic treatment (Sommer et al., 2009). The aminoglycosides and β-lactams are two large families of antibiotics which are frequently employed in clinical settings. The aminoglycosides, which were first characterised in 1944, (Mingeot-Leclercq et al., 1999) function by binding to the 30S subunit of the prokaryotic ribosome resulting in disruption to protein synthesis. Resistance to aminoglycosides can be through reduced aminoglycoside uptake or enzymatic modification of the aminoglycoside through acetylation (AAC), adenylation (ANT) or phosphorylation (APH). β-lactam antibiotics include the penicillins and cephalosporins and inhibit bacteria through disruption of cell wall biosynthesis (Page, 2012, Tipper and Strominger, 1965). Resistance to β-
lactams can be due to alterations to penicillin binding proteins or to the porins in the outer membrane (in Gram negative targets) or alternatively through the production of β-lactamases, which hydrolyse the eponymous β-lactam ring rendering the antibiotic inactive (Bush, 1989, Bush, 2010). The question of the evolutionary origin of antibiotic resistance genes has been the subject of much attention (Kotron and Mobashery, 1998, Page, 2012, Tipper, 1979). For quite some time it was thought that resistance evolved following exposure of bacteria to new antibiotics (Hughes and Datta, 1983). However, it is now apparent that repositories of antibiotic resistance genes exist such that, following the development and application of new antibiotics, bacteria possessing or acquiring such genes will gain a selective advantage and thus resistance will increase over time (Bhullar et al., 2012, D’costa et al., 2011). Previous studies have employed PCR to detect resistance genes in specific pathogens (Dallenne et al., 2010, Vannuffel et al., 1995), though studies employing PCR to detect resistance genes in complex microbial environments have been limited. In one instance, a PCR-based approach was used to investigate the prevalence of gentamicin resistance genes in resistant isolates from sewage, faeces (from cattle and chickens), municipal and hospital sewage water and coastal water (Heuer et al., 2002). The utilization of a PCR approach in that instance resulted in the identification of diverse genes encoding gentamicin modifying enzymes from across a broad host range, thus demonstrating the suitability of a PCR-based approach to investigate resistance genes present in complex
environments. However, the study did not investigate antibiotic resistance genes in human gut microbiota and, to our knowledge, to date no such PCR-based studies exist. Given these findings and other indications that there exist large natural pools of antibiotic resistance genes within complex microbial populations, it is likely that the human gut also contains many such genes. However, until now, PCR-based strategies to detect antibiotic resistance genes in the gut microbiota have involved an initial culture-based screen for resistant isolates, followed by subsequent PCR-based approaches to identify the associated resistance genes. This does not take into consideration the fact that the vast majority of gut microbes are not easily cultured (Eckburg et al., 2005), and thus antibiotic resistance genes from such microorganisms would typically be overlooked.

Here we utilize degenerate PCR primers to investigate the presence of β-lactam resistance genes and each of the three categories of aminoglycoside modifying enzymes within human metagenomic DNA and in doing so demonstrate that the human gut microbiota is a reservoir for antibiotic resistance genes. Additionally, we establish that a PCR-based approach allows the rapid detection of such genes in the complex gut microbiota environment, without the need for an initial isolation of strains.
3.3 Materials and Methods

3.3.1 Recruitment of volunteers

Forty adults were recruited and each provided written, informed consent for participation in this study. Approval for this trial was received from the Clinical Research Ethics Committee of the Cork Teaching Hospitals, Cork, Ireland. Volunteers were aged 28.8 ± 3.8 years, were free from gastrointestinal disorders and had not been treated with antibiotics in the 6 months prior to sample collection. Fresh faecal samples were collected and stored at -80°C until processed.

3.3.2 DNA Extraction

Stool samples were weighed, homogenised and due to the total volume provided by each individual, samples had to be pooled to achieve the required volume for our metagenomic DNA extraction protocol. To facilitate this, an equal volume (250mg) from each individual was taken and pooled to form one sample, from which metagenomic DNA was extracted. The DNA extraction procedure used was optimised for total bacterial genomic DNA extraction from stool samples. The stool sample was homogenized in PBS and centrifuged at 1000g x 5 mins and the supernatant was removed and retained. This was repeated 3 times. The supernatant then underwent Nycodenz (Axis Shield, UK) density gradient centrifugation separation, to separate out the bacterial cells from faecal matter. Following enzymatic lysis of bacterial cells using lysozyme and mutanolysin (Sigma Aldrich, Dublin, Ireland) protein precipitation using
Proteinase K and ammonium acetate (Sigma Aldrich) was completed. Bacterial DNA was then precipitated and washed using standard chloroform and ethanol procedures. DNA was eluted in TE buffer.

3.3.3 PCR-based detection of β-lactam resistance gene homologues

PCR-based detection of β-lactam resistance genes was completed using primers (MWG Eurofins, Germany) for the genes \( \text{bla}_{\text{TEM}} \) (Bailey et al., 2011, Tenover et al., 1994), \( \text{bla}_{\text{SHV}} \) (Briñás et al., 2002) (both of which are classified as Bush group 2b β-lactamases), \( \text{bla}_{\text{CTX-M}} \) (Monstein et al., 2009) (an extended spectrum β-lactamase (ESBL) which confers resistance to cefotaxime), \( \text{bla}_{\text{OXA}} \) (Briñás et al., 2002) (ESBL, Bush group 2d ESBL) and \( \text{bla}_{\text{ROB}} \) (Tenover et al., 1994) (confers high level ampicillin resistance) (Table 1). PCRs were completed using bacterial metagenomic DNA and all PCRs were performed in triplicate. PCRs were completed on a G-storm PCR machine and for the primer sets \( \text{bla}_{\text{TEM}} \) primer set 1 (RH605/606), \( \text{bla}_{\text{TEM}} \) primer set 2 and \( \text{bla}_{\text{CTX-M}} \), PCRs were completed as previously outlined. For the primers \( \text{bla}_{\text{OXA}} \) and \( \text{bla}_{\text{ROB}} \) the PCR conditions were as follows: heated lid 110°C, 94°C x 5 mins followed by 30 cycles of 94°C x 30s, 64°C x 30s (\( \text{bla}_{\text{oxa}} \)) or 62°C (\( \text{bla}_{\text{ROB}} \)) and 72°C x 30s followed by 72°C x 10 mins and held at 4°C. For \( \text{bla}_{\text{SHV}} \) PCRs were performed as follows: heated lid 110°C, 94°C x 5 mins followed by 35 cycles of 94°C x 30s, 58°C x 30s and 72°C x 30s followed by a final extension step of 72°C x 10 mins and held at 4°C. All PCRs contained 25µl Biomix Red (Bioline, UK), 1 µl forward primer (10pmol
concentration), 1 µl reverse primer (10pmol concentration), metagenomic DNA (64ng) and PCR grade water (Bioline, UK), to a final volume of 50µl. Negative controls were completed for all primer sets. Gel electrophoresis was performed on all samples using 1.5% agarose gel in 1X TAE buffer.

3.3.4 PCR-based detection of aminoglycoside resistance gene homologues

For the detection of aminoglycoside resistant genes, degenerate primer sets were used which had previously been designed and shown to amplify all known genes encoding gentamicin-modifying enzymes and similar, but as yet undiscovered, sequences (Heuer et al., 2002). PCRs were completed using primer sets (MWG Eurofins, Germany) for genes belonging to each group of aminoglycoside modifying enzymes namely, acetylation, adenylation and phosphorylation enzymes. DNA from positive controls (kindly gifted to us from the Smalla laboratory, JKI, Braunschweig) namely *Escherichia coli* S17-1 pAB2002 (*aac*(3)-Ia), *Pseudomonas aeruginosa* 88.341F (*aac*(3)-Ib), *Enterobacter aerogenes* 17798 VDK (*aac*(3)-IIa), *E. coli* DH5α pSCH4203 (*aac*(3)-IIb), *E. coli* DH5α pSCH4101 (*aac*(3)-Vla), *P. aeruginosa* PST-1 (*aac*(3)-IIIa), *Acinetobacter baumannii* LBL.3 (*aac*(6')-Ib), *P. aeruginosa* F-03 (*aac*(6')-IIa), *E. coli* DH5α pSCH5102 (*aac*(6')-IIb), *E. coli* CV600 pIE723 (*ant*(2'')-I), *E. coli* DH5α pAM6306 (*aph*(2'')-Ic) and *E. coli* NC95 (*aph*(2'')-Id) were used as positive controls for the PCR reactions. This ensured the specificity of the respective primer pairs. PCRs for the detection of
acetylation genes **aac (3)-I, aac (3)-II, aac (3)-III, aac (3)-VI** and **aac (6)**, adenylation genes **ant (2”)-Ia** and phosphorylation genes **aph (2”)-Ic** and **aph (2”)-Id** were completed as previously outlined (Heuer et al., 2002) (Table 1). Additionally, PCRs using primers for the bifunctional gene **aac(6’)-le-aph(2”)** (De Fátima Silva Lopes et al., 2005, Schmitz et al., 1999) (which encodes enzymes responsible for high level gentamicin resistance, as well as concomitant resistance to tobramycin and kanamycin) (Matsumura et al., 1984, Ubukata et al., 1984, Schmitz et al., 1999, Hegstad et al., 2010, Ferretti et al., 1986) were completed as follows: heated lid 110°C, 94°C x 5 mins followed by 30 cycles of 94°C x 30s, 47°C x 30s, 72°C x 30s, with a final extension step of 72°C x 10 mins and held at 4°C. All PCRs contained 25µl Biomix Red (Bioline, UK), 1 µl forward primer (10pmol concentration), 1 µl reverse primer (10pmol concentration), metagenomic DNA (64ng) and PCR grade water (Bioline, UK), to a final volume of 50µl. Negative controls were run for all primer sets. All PCRs were performed in triplicate and analysed using gel electrophoresis, as described above.

### 3.3.5 Cloning of PCR amplicons

Triplicate samples from successful PCR reactions were pooled and cleaned using AMPure magnetic bead-based PCR clean up kit (Beckman Coulter, UK). TOPO cloning reactions were performed on purified PCR products using the TOPO TA cloning kit (Invitrogen, Dublin, Ireland) to facilitate the sequencing of individual gene fragments. TOPO cloning
reactions were then cloned into TOP10 *E. coli* (Invitrogen) as per the manufacturer’s instructions and plated onto LB (Difco) containing the appropriate antibiotic (either ampicillin 50µg/ml or kanamycin 50µg/ml; Sigma Aldrich, Dublin, Ireland) to select for the presence of the cloning vector. Transformants were selected from each TOPO cloning reaction and grown overnight in LB broth containing the suitable selective antibiotic (either ampicillin 50µg/ml or kanamycin 50µg/ml). Plasmids were extracted from overnight samples using QIAprep Spin Mini Prep kit (Qiagen, Sussex, UK) according to the manufacturer’s instructions and sent for Sanger sequencing (Source BioSciences, Dublin, Ireland).

### 3.3.6 Bioinformatic analysis

Following Sanger sequencing, sequence reads were analysed using the NCBI protein database (BlastX; [http://blast.ncbi.nlm.nih.gov/]). In the event where multiple hits occurred, the BLAST hit which displayed greatest homology is reported.
3.4 Results and Discussion

3.4.1 A PCR-based approach highlights the presence of β-lactamase gene homologues in the gut microbiota

The results of the β-lactamase-specific PCRs demonstrated the presence and diversity of class 2 β-lactamase genes in the gut microbiota of healthy adults (Table 2 (Fouhy, 2013)). Of the β-lactam primers used, the primers designed to amplify bla \textsubscript{TEM} genes yielded the greatest number of unique sequence hits (42% of selected TOPO sub-clones gave a unique hit). The majority of these genes exhibited a high percentage identity with genes from various members of the Proteobacteria including E. coli, Klebsiella, Salmonella, Serratia, Vibrio parahaemolyticus and Escherichia vulneris. The resistance of strains of Salmonella and Serratia to β-lactams via bla \textsubscript{TEM} genes has been noted (Morris et al., 2006, Perilli et al., 1997, Zhao et al., 2007) and such strains have been associated with nosocomial infections (Morosini et al., 1995). In contrast, there have been relatively few studies of bla \textsubscript{TEM} genes in Vibrio parahaemolyticus and Escherichia vulneris (Wong et al., 2012, Mokracka et al., 2012). The identification of genes homologous to those from Enterobacteriaceae is not surprising given the prevalence of resistance genes among members of this family (Bush, 2010). It was notable that the bla \textsubscript{TEM} primers also amplified genes that resembled bla \textsubscript{TEM} genes from some more unusual sources, including two genes from uncultured bacteria and from a Sar 86 cluster (a divergent lineage of γ-Proteobacteria) bacteria. This approach can thus provide an insight into possible novel/unusual sources of
resistance genes, including those that culture-based approaches would fail to detect. Such results also highlight that had initial screening for resistant isolates been completed prior to PCR amplification of the resistance genes, such unusual sources of resistance genes may have been overlooked. Additionally, genes encoding ESBLs, including \( \text{bla}_{\text{TEM-116}} \), \( \text{bla}_{\text{TEM-195}} \) and \( \text{bla}_{\text{TEM-96}} \) amongst others, were also identified, with their closest homologues being members of the \textit{Proteobacteria} (Table 2).

Using the \( \text{bla}_{\text{SHV}} \) primers, multiple genes sharing homology with genes from members of the \textit{Enterobacteriaceae}, and \textit{Klebsiella} and \textit{E. coli} in particular were detected. In addition, amplicons with low percentage identity to genes from \textit{Alloprevotella rava} and \textit{Parabacteroides johnsonii}, respectively, were also identified. This is again consistent with existing research which states that \textit{Enterobacteriaceae} are the primary source of \( \text{bla}_{\text{SHV}} \) genes (Coque et al., 2002, Paterson et al., 2003, Heritage et al., 1999, Babini and Livermore, 2000, Pitout et al., 1997). Furthermore, the amplicons sequenced resembled various different types of ESBL-encoding SHV genes, including \( \text{bla}_{\text{SHV-132}} \), \( \text{bla}_{\text{SHV-140}} \) and \( \text{bla}_{\text{SHV-48}} \), thus again highlighting the genuine degeneracy of the primers used.

Additional PCRs were completed to identify other ESBLs, specifically CTX-M- and OXA-type \( \beta \)-lactamases (Table 2). A number of different CTX-M \( \beta \)-lactamases were detected, including CTX-M-1, CTX-M-15 and CTX-M-36. The fact that many of the \( \beta \)-lactamase genes detected using our approach share homology with resistance genes found in members of the phylum \textit{Proteobacteria} is not surprising as, despite being typically
less common than the *Bacteroidetes* or *Firmicutes* in the gut microbiota of healthy adults (Eckburg et al., 2005), members of this genus have been identified as sources of antibiotic resistance genes and have been frequently associated with nosocomial infections and outbreaks (Coque et al., 2002, Morosini et al., 1995, Bonnet, 2004, Pitout and Laupland, 2008). In the 1990s, TEM- and SHV-type ESBLs were the β-lactamases most frequently observed among *Enterobacteriaceae* (Dallenne et al., 2010). However, more recently, CTX-M-type ESBLs have spread rapidly and are now the most prevalent ESBL in *Enterobacteriaceae* in several parts of the world (Coque et al., 2008). In a recent report on antibiotic resistance threats in the USA, the Centre for Disease Control stated that ESBL-producing *Enterobacteriaceae* were a serious public health threat (Cdc, 2013). The report estimates that 26,000 infections and 1,700 deaths that occur each year in the United States are attributable to ESBLs and that upwards of 140,000 health-care related *Enterobacteriaceae* infections occur annually. Therefore the detection of homologues of ESBL-encoding genes in the gut microbiota of healthy individuals is significant and provides evidence of the ubiquitous nature of these resistance genes, even in the absence of recent antibiotic exposure. With respect to the CTX-M-type ESBLs, it is particularly notable that homologues of the *bla*$_{CTX-M-15}$ gene were detected, as these have received significant attention due to their recent rapid spread and their association with multi-drug resistant *E. coli* responsible for outbreaks of antibiotic resistant infections (Boyd et al., 2004, Lavollay et al., 2006).
In such cases, these genes have been found on multi-drug resistance-encoding regions of plasmids, thus facilitating the rapid transfer of these genes. The presence of such genes within the gut microbiota raises concerns that horizontal gene transfer may occur between commensals or to bacteria passing through the gut. If the resistance genes detected in our study are, or were to become, mobile, it would enable the gut to act not only as a source of resistance genes, but also as a site of resistance gene transfer. Although outside the scope of this study, studies investigating whether these genes are located on or near mobile genetic elements would be pertinent to ascertain the risk of the gut acting as a site for horizontal gene transfer.

When the \textit{bla}\textsubscript{ROB} primer set was employed to detect the presence of homologues of these ampicillin resistance-encoding genes, all amplicons sequenced were identical and shared 44% identity to \textit{Staphylococcus haemolyticus bla}\textsubscript{ROB} gene. Finally, this study did not detect \textit{bla}\textsubscript{OXA} gene homologues in our metagenomic sample. These findings are unexpected and may have occurred as a result of the particular affinity of the primer sets used.

\textbf{3.4.2 A PCR-based approach highlights the presence of aminoglycoside resistance encoding gene homologues in the gut microbiota}

Degenerate primers were selected that amplify genes encoding aminoglycoside modifying enzymes from each of the enzyme modification
groups, namely acetylation, adenylation and phosphorylation (Fouhy, 2013). When primers were applied to detect acetylation-associated genes, it was established that the primers designed to target $aac\,(3)$-I, $aac\,(3)$-II, and $aac\,(3)$-III homologues did not generate amplicons. In each of these PCR reactions the positive controls successfully amplified, thus we are satisfied that the lack of amplification products for our metagenomic sample is a true result. However, a number of distinct $aac\,(6)$ and $aac\,(3)$-VI homologues were detected and were found to resemble genes from a variety of genera, including *Acinetobacter*, *Pseudomonas* and *Enterobacter* (Table 3). The presence of aminoglycoside acetylation genes within these genera has been noted previously (Cho et al., 2009, Lambert et al., 1994, Shaw et al., 1989, Park et al., 2006). The detection of resistance genes resembling those seen in *A. baumannii* is a concern, as many strains of this species have been shown to exhibit multi-drug resistance (Dijkshoorn et al., 2007, Perez et al., 2007). In addition, homologues of genes from *Collinsella* and *Salmonella* were also detected. Primers designed to amplify bifunctional $aac(6')$-$le-aph(2'')$ genes were also employed. Our investigations revealed the presence of homologues of such genes, resembling those from *S. aureus*, *E. faecium* and *S. epidermidis*, all of which are known sources of these genes (Vakulenko et al., 2003, Vanhoof et al., 1994, Schmitz et al., 1999).

Homologues of aminoglycoside phosphorylation-encoding genes were also detected using a PCR-based approach, with both $aph\,(2'')$-Ic and
aph (2\")-Id like genes being detected. These genes shared homology with genes from *Enterococcus* species, including *E. faecium* and *E. casseliflavus*. Aminoglycoside resistant *E. faecium* have received significant attention due to their role in nosocomial infections (Han et al., 2011, Montecalvo et al., 1994). Notably, the role of mobile genetic elements in the maintenance and dissemination of multi-drug resistance in *Enterococcus faecalis* and *E. faecium* has previously been highlighted (Hegstad et al., 2010, Leclercq, 1997, Mckay et al., 1994). While it is not certain that the genes identified in this study are also associated with mobile elements, the possibility that resistance genes could be transferred to commensals is a concern. Homologues of aminoglycoside adenylation genes, *ant* (2\")-Ia, were also successfully detected. These resembled genes from *Pasteurella*, *Acinetobacter* and *E. coli* (Table 3), and the findings are thus consistent with previous research showing that these genes are most frequently detected in Gram negative bacteria (Shaw et al., 1993). Overall, the results demonstrate that the gut microbiota is a source of diverse aminoglycoside and β-lactam resistance genes, despite having had no recent antibiotic exposure. If these genes are expressed there is the potential that if antibiotic exposure occurred, bacteria containing these resistance genes would become the dominant component of the gut microbiota, as has been shown in previous studies (Dethlefsen et al., 2008, Fouhy et al., 2012).
3.5 Conclusions

This study has highlighted the merits of applying a PCR-based approach to detect antibiotic resistance genes within the human gut microbiome. The results clearly demonstrate that the human gut microbiota is a considerable reservoir for resistance genes. Further studies are required to determine the exact sources of these genes and to determine if they have the potential to become mobile. Additionally, we have highlighted the successful application of a PCR-based screen of a complex environment without prior isolation of resistant isolates. The possibility exists to couple this approach with lower throughput next-generation sequencing strategies, such as that provided by the Ion PGM 314 chip, in instances where great diversity is likely. Our approach could also be used in conjunction with functional screening of metagenomic libraries to enable the detection of genes present in a complex environment at a low threshold and that may have avoided capture in the metagenomic library, as shown in a recent study (De Vries et al., 2011). Such a PCR-based approach is not being proposed as a substitute for ultra-deep high-throughput shotgun sequencing of metagenomic DNA, rather it is a lower cost, more targeted, alternative which facilitates the detection and in silico analysis of specific gene sets of interest. Finally, while this study demonstrates that the gut microbiota is a source of diverse resistance genes, further studies are required to investigate the exact sources of these genes, their expression and whether they have the potential to become mobile. As the scientific community continues to gain knowledge
with respect to the genetic mechanisms involved in providing resistance to various antibiotics, the design of additional sets of degenerate primers will be possible and will provide further opportunities for the use of PCR to rapidly and efficiently detect antibiotic resistance genes in complex microbial environments, including the human gut microbiota.
References


Cdc 2013. Antibiotic resistance threats in the United States


(6')-Ih plasmid gene of Acinetobacter baumannii. *Antimicrobial agents and chemotherapy*, 38, 1883-1889.


Enterococcus faecium bacteremia in an adult oncology unit. *Antimicrobial agents and chemotherapy*, 38, 1363-1367.


Table 1 Primers used for the detection of β-lactamase and aminoglycoside resistant genes

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AG: aminoglycoside

*De Fatima Silva Lopes et al. (2003)*

*Schmitz et al. (1999)*
Table 2: Homologues of β-lactamase genes detected in the human gut microbiota via PCR techniques

<table>
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<th>Accession #</th>
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ESBL: extended spectrum β-lactamase
Table 3 Homologues of aminoglycoside resistance genes detected in the human gut microbiota via PCR techniques

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AG: aminoglycoside
Chapter 4

*In silico* assigned resistance genes confer *Bifidobacterium* with partial resistance to aminoglycosides but not to β-lactams

Published in PLoS ONE, Volume 8, Issue 12, doi: 10.1371/journal.pone.0082653, December, 2013
4.1 Abstract

Bifidobacteria have received significant attention due to their contribution to human gut health and the use of specific strains as probiotics. It is thus not surprising that there has also been significant interest with respect to their antibiotic resistance profile. Numerous culture-based studies have demonstrated that bifidobacteria are resistant to the majority of aminoglycosides, but are sensitive to β-lactams. However, limited research exists with respect to the genetic basis for the resistance of bifidobacteria to aminoglycosides. Here we performed an in-depth *in silico* analysis of putative *Bifidobacterium*-encoded aminoglycoside resistance proteins and β-lactamases and assess the contribution of these proteins to antibiotic resistance.

The *in silico*-based screen detected putative aminoglycoside and β-lactam resistance proteins across the *Bifidobacterium* genus. Laboratory-based investigations of a number of representative bifidobacteria strains confirmed that despite containing putative β-lactamases, these strains were sensitive to β-lactams. In contrast, all strains were resistant to the aminoglycosides tested. To assess the contribution of genes encoding putative aminoglycoside resistance proteins in *Bifidobacterium* sp. two genes, namely *Bbr*_0651 and *Bbr*_1586, were targeted for insertional inactivation in *B. breve* UCC2003. As compared to the wild-type, the UCC2003 insertion mutant strains exhibited decreased resistance to gentamicin, kanamycin and streptomycin.
This study highlights the associated risks of relying on the *in silico* assignment of gene function. Although several putative β-lactam resistance proteins are located in bifidobacteria, their presence does not coincide with resistance to these antibiotics. In contrast however, this approach has resulted in the identification of two loci that contribute to the aminoglycoside resistance of *B. breve* UCC2003 and, potentially, many other bifidobacteria.
4.2 Introduction

Following the discovery of penicillin by Alexander Fleming (Fleming, 1979), exponential antibiotic discovery and development occurred which revolutionized medicine. However, during this same period, target bacteria developed sophisticated mechanisms of resistance against many of the most commonly prescribed antibiotics (Davies and Davies, 2010). It is thus not surprising that considerable efforts have been and are still being made to investigate the genetic mechanisms involved in the transfer, acquisition and expression of antibiotic resistance genes, in order to curtail or prevent the further development of resistance (Bush, 2012, Walsh, 2000).

The mechanisms underlying resistance to aminoglycosides and to β-lactams are among those that have been the focus of particular attention. Briefly, aminoglycosides are a family of broad spectrum antibiotics that were first reported in 1944 (Mingeot-Leclercq et al., 1999), whose bactericidal activity results from their binding to the 30S subunit of the prokaryotic ribosome and the subsequent impairment of protein synthesis (Jacoby and Gorini, 1967, Mingeot-Leclercq et al., 1999). Aminoglycoside resistance can be mediated through reduced aminoglycoside uptake (Davies and Wright, 1997), or through enzymatic modification of the aminoglycoside through the activity of the N-acetyltransferases (AAC), O-nucleotidyltransferases (ANT) or O-phosphotransferases (APH). Aminoglycoside resistance genes have been classified based on the enzymatic modification mechanism used by the resultant protein and the
chemical position at which the aminoglycoside is modified (Shaw et al., 1993).

β-lactam antibiotics are a class of broad spectrum antibiotics which include the penicillins and cephalosporins (Kotra and Mobashery, 1998). β-lactams inhibit bacteria by their interference with normal cell wall synthesis, via disruption of the final cross-linking stage of cell wall peptidoglycan formation, resulting in a significantly weakened cell wall polymer, ultimately leading to bacterial cell death (Page, 2012, Tipper, 1979, Tipper and Strominger, 1965). β-lactam resistance can arise through mutation of target penicillin binding proteins (PBP; (Georgopapadakou, 1993, Pitout et al., 1997)), as well as through the production of β-lactamases (Abraham and Chain, 1940), which catalyze the hydrolysis of the eponymous β-lactam rings present in β-lactam antibiotics, rendering the antibiotic inactive. β-lactamase classification has undergone significant rounds of change from the initial Ambler classification proposed in 1973 (Richmond and Sykes, 1973) and the classification schemes of Bush and colleagues (Ambler, 1980, Bush, 1989, Bush and Jacoby, 2010, Bush et al., 1995).

The antibiotic resistance genes of pathogenic bacteria have been the focus of greatest attention. Similarly, antibiotic sensitivity is regarded as a desirable trait among candidate probiotic strains for the feed (Commission, 2008) and human (Huys et al., 2013, Vankerckhoven et al., 2008) markets. Such a phenotype ensures that their consumption does not further increase the risk of antibiotic resistance gene dissemination,
especially in situations where such genes are located on mobile genetic elements. Gut-associated bifidobacteria are generally viewed as beneficial microbes and many strains have been attributed with health-promoting characteristics (Chouraqui et al., 2004, He et al., 2007, Wang et al., 2004, Xiao et al., 2003). Thus, it is not surprising that many bifidobacteria are used, or have been studied with a view to their potential use, as probiotics in functional foods (Kailasapathy and Chin, 2000). As a consequence, there has been considerable interest in determining if certain bifidobacteria possess antibiotic resistance genes (Kheadr et al., 2004, Mayrhofer et al., 2011, Xiao et al., 2010, Sato and Iino, 2010). These studies established that the tested bifidobacteria strains are generally resistant to aminoglycoside antibiotics (Yazid et al., 2001), but are sensitive to β-lactams (D’aimmo et al., 2007, Kheadr et al., 2004, Vlková et al., 2006, Xiao et al., 2010). In a previous study, we found that combined ampicillin and gentamicin treatment in infants, caused a significant decrease in the proportion of bifidobacteria present 4 weeks after antibiotic administration ceased, while also significantly altering the bifidobacteria species present (Fouhy et al., 2012). We were therefore interested in investigating differences in the distribution of genes encoding β-lactam or aminoglycoside resistance proteins among members of the Bifidobacterium genus.

To date little is known about the genetic mechanisms that underlie aminoglycoside resistance in bifidobacteria. Despite the existence of some specific studies (Kiwaki and Sato, 2009, Sato and Iino, 2010,
Masco et al., 2006), the presence of antibiotic resistance genes has been more frequently inferred through the annotation of DNA sequences and the identification of genes bearing some homology to genes previously assigned as being potential resistance determinants. Given the risks associated with relying exclusively on rapid in silico assignments, here we present an in-depth bioinformatic analysis of putative β-lactam and aminoglycoside resistance proteins that are Bifidobacterium-encoded. We have investigated if a correlation exists between these proteins and antibiotic resistance and, in the case of aminoglycoside resistance, have demonstrated the contribution of the assigned resistance genes to this phenotype.
4.3 Materials and Methods

4.3.1 NCBI database search for *Bifidobacterium*-associated β-lactam and aminoglycoside resistance proteins

Using the NCBI protein database, a search for putative β-lactamases and aminoglycoside resistance proteins associated with bifidobacteria was completed using the terms ‘beta-lactamase’ and ‘*Bifidobacterium*’ (searched on 28/8/12) and ‘aminoglycoside’ and ‘*Bifidobacterium*’ (search completed on 29/8/12). This approach was taken so that all such proteins, regardless of the basis upon which they were assigned, would be revealed. Following the removal of duplicates and sequences that did not originate from *Bifidobacterium*, all remaining sequences were used as drivers for subsequent rounds of BLAST investigations. All subsequent distinct sequences detected were employed for additional BLAST-based investigations until a finalized list was achieved. Additionally, further BLAST-based investigations using known β-lactamase and aminoglycoside resistance proteins as drivers were completed to ensure no additional sequences were overlooked.

4.3.2 Classification of β-lactamases and aminoglycoside resistance protein sequences from bifidobacteria

Putative *Bifidobacterium*-associated β-lactamase and aminoglycoside resistance proteins were subjected to *in silico* analysis with a view to classifying them using the Ambler method for β-lactamases (Ambler, 1980), or assigning them into one of the 3 main enzyme modification
groups associated with aminoglycoside resistance (Shaw et al., 1993). To this end, the putative *Bifidobacterium*-associated resistance determinants were aligned (MegAlign Clustal W, LaserGene) against representative sequences from each class (A-D for the β-lactamases) and from each of the 3 enzyme groups (AAC, APH and ANT for the aminoglycosides) (Bush and Jacoby, 2010, Bush et al., 1995) (Table 1).

**4.3.3 Laboratory based assessments of antibiotic resistance**

The antibiotic susceptibility of bifidobacteria strains was investigated in a number of different ways. Disc diffusion assays were carried out according to the British Society for Antimicrobial Chemotherapy (BSAC) guidelines (Andrews, 2009, Andrews, 2001c, Andrews, 2001a). Briefly the bifidobacteria strains were cultured overnight anaerobically and delivered onto Iso-Sensitest agar plates (Oxoid, Fisher Scientific, Dublin, Ireland) using a swab in three directions. Antimicrobial discs containing ampicillin (25 µg), penicillin (10 µg) (VWR International, Dublin, Ireland), neomycin (30 µg), gentamicin (200 µg), kanamycin (30 µg) and streptomycin (25 µg) (Fisher Scientific, Dublin, Ireland) were dispensed manually onto the agar plates. Following anaerobic incubation at 37°C for 48 hours, the diameters of the zones of inhibition (mm) were measured. All tests were carried out in triplicate.

Minimum inhibitory concentration tests (MICs) using 4 aminoglycosides i.e. neomycin, gentamicin, streptomycin and kanamycin (Sigma Aldrich, Dublin, Ireland) were performed as per the micro-dilution method, as
described in detail by others (Andrews, 2001b). Briefly, bifidobacteria were grown overnight anaerobically at 37°C in MRS broth supplemented with 0.05% cysteine (Sigma Aldrich, Wexford, Ireland). Cultures were adjusted to an OD$_{600}$ of 0.1 ($\approx 1 \times 10^5$ cfu/ml) in fresh MRS broth (media pH 6.8). Stock solutions of each of the aminoglycoside antibiotics were prepared in sterile distilled water and a 2-fold dilution series was performed. An inoculum of 100 µl of culture was added to each well of the 96 well plate (resulting in a final concentration of $\approx 5 \times 10^4$ cfu/ml) (Sarstedt, Wexford, Ireland). Additionally, each 96 well plate contained positive (MRS + culture) and negative controls (MRS only), and tests were carried out in triplicate. Plates were incubated anaerobically (using anaerobic gas jars and Anaerocult P anaerobic gas pack inserts (Merck Millipore Ltd, Cork, Ireland)) at 37°C for 24 hours and the MIC was determined as the lowest concentration of antimicrobial agent at which no visible growth was recorded. MICs were also carried out on E. coli XL1-blue which had been transformed with plasmid-encoded copies of the putative aminoglycoside resistance genes Bbr_0651, Bbr_1586 and Bbr_0651+0650. Protocols were as described above except that LB broth (pH 7.1) (Difco, Fisher Scientific, Ireland) was used for culturing and growth conditions were 24 hours aerobically at 37°C.

To test for $\beta$-lactamase activity, nitrocefin tests were performed as previously described (Moubareck et al., 2005, Lee and Rosenblatt, 1983), i.e. $\beta$-lactamase nitrocefin sticks (Fisher Scientific, Ireland), were dipped into a single colony for each species being tested and assessed for 1-2
minutes and again after 15 minutes for the appearance of a pink colour, indicative of β-lactamase activity. *Staphylococcus aureus* DPC 5286 was used as the positive control.

4.3.4 Disruption of the *Bbr_0651* and *Bbr_1586* genes from *B. breve* UCC2003

Site specific homologous recombination was used to disrupt 2 genes present in *B. breve* UCC2003, namely *Bbr_0651* and *Bbr_1586*, using protocols similar to those previously described (O’Connell Motherway et al., 2009, Mazé et al., 2007). Briefly, internal fragments of *Bbr_0651* and *Bbr_1586*, were amplified by PCR using specifically designed primers (MWG Eurofins, Germany) (Table S1), resulting in 500bp and 400bp products respectively. These fragments were cloned into the pORI19 vector and a tetracycline resistance marker (*tetW* gene) from the pAM5 vector (Álvarez-Martín et al., 2007) was subcloned to generate the plasmids pORI19-tet-0651 and pORI19-tet-1586 (Table 2). The correct sequence of each cloned insert was verified by sequencing (Source BioScience, Dublin, Ireland).

Being derivatives of pORI19 these plasmids cannot replicate in *B. breve* UCC2003, due to a lack of a functional replication protein (Law et al., 1995), and instead are utilized with a view to integrating into and disrupting target genes. To facilitate methylation, the pORI19 plasmids were introduced via electroporation into EC101 *E. coli* cells containing pNZ-M.BbrII-M.BbrIII. The resulting methylated pORI19-tet-0651 and
pORI19-tet-1586 constructs were electroporated into *B. breve* UCC2003. Transformants were selected based on presence of tetracycline resistance. Transformants were expected to carry *Bbr_0651* or *Bbr_1586* gene disruptions, respectively. To verify the suspected chromosomal integration of these pORI19 constructs, colony PCRs were performed on a selection of tetracycline resistant transformants, using a forward primer upstream of the integration region and a reverse primer based on pORI19 (Table S1).

### 4.3.5 Complementation studies

DNA fragments containing the gene *Bbr_1586* and its native promoter region were generated by PCR amplification from *B. breve* UCC2003 chromosomal DNA, using Pfu Ultra II Hotstart Mastermix (Agilent Technologies, Cork, Ireland) and sequence specific primers (Table S1). The amplicons and the pBC1.2 plasmid were digested with *HindIII* and *XbaI* (Roche Diagnostics, Sussex, UK) and subsequently ligated using T4 DNA ligase (Roche Diagnostics, Sussex, UK). This resulted in the complementation plasmid pBC1.2-Bbr_1586 (Table 2). The dialysed ligations were electroporated into *E. coli* XL1-blue and the resulting plasmids verified by PCR and restriction digest analysis. Finally, the plasmid pBC1.2-Bbr_1586 was electroporated into competent *B. breve* UCC2003-1586-tet cells. Transformants from the complemented strain were selected and the presence of the construct confirmed.
4.3.6 Studies of wild-type *B. breve* UCC2003 with additional copies of aminoglycoside resistance genes

Studies were also completed to investigate if the addition of extra plasmid-encoded copies of the putative aminoglycoside resistance genes *Bbr_0651*, *Bbr_0651+0650* or *Bbr_1586* would result in enhanced resistance of the wild-type *B. breve* UCC2003. Competent *B. breve* UCC2003 cells were prepared and transformed with the constructs pBC1.2-0651, pBC1.2-0651+0650 or pBC1.2-1586. Transformants were selected and the presence of the plasmid inserts was confirmed.

4.3.7 Heterologous expression of putative aminoglycoside resistance genes in *E. coli*

Plasmid-encoded copies of the entire putative aminoglycoside resistance genes *Bbr_0651*, *Bbr_0651+0650* and *Bbr_1586*, along with their native promoters were transformed via electroporation into competent *E. coli* XL1-blue. Following confirmation of the presence of the correct plasmid insert in the transformants, MIC assays were completed, using the protocol outlined above.
4.4 Results

4.4.1 Putative β-lactamases associated with *Bifidobacterium* species

In order to identify *Bifidobacterium*-associated proteins which have been annotated, or possibly mis-annotated, as β-lactamases, the NCBI protein database was screened for *Bifidobacterium*-associated proteins which had been annotated as β-lactamases or which had been noted to contain β-lactamase associated motifs (searched on 28/8/12). The proteins identified were in turn employed as drivers for BLAST analysis (of non-redundant proteins), to identify and assess the distribution of related *Bifidobacterium*-associated proteins. Subsequent rounds of BLAST analysis, employing the related, yet distinct, protein sequences as drivers, ultimately resulted in saturation. To ensure that other potential β-lactamases were not overlooked, further BLAST-based investigations, using known β-lactamase proteins as drivers, were also carried out to screen all publically available *Bifidobacterium* genomes.

The resultant proteins fell into a number of different categories (Table 3). The most common protein was that annotated variably as a metallo-beta-lactamase family protein, a metal-dependent hydrolase or ribonuclease J such as HMPREF0168_0178 from *B. dentium* ATCC 27679. This protein is conserved, at high (>90%) percentage identity, across almost all publically available *Bifidobacterium* genomes and is a member of the protein family 07521 (Pfam07521; RNA-metabolising metallo-beta-lactamases). A considerable number of other proteins are linked by virtue of containing domains typical of Pfam13354 (a β-lactamase enzyme
family of proteins). These proteins are not highly conserved, with distinct subgroups such as those represented by HMPREF0168_1872 from B. dentium ATCC 27679, BBB_1387 from B. bifidum BGN4, BBB_1559 from B. bifidum BGN4 and Bbr_0236 from B. breve UCC2003, respectively, being apparent. Other unique members of Pfam13354 are BIFADO_0224 (B. adolescentis L2-32), BLJ0695 (B. longum subsp. longum JDM 301) and BAD_1308 (B. adolescentis ATCC 15703). B. dentium genomes also share a conserved protein, representative of Pfam00144 (a β-lactamase family), such as HMPREF0168_1378 from B. dentium ATCC 27679. B. catenulatum DSM 16992 (BIFCAT_01331) and B. pseudocatenulatum DSM 20438 (BIFPSEUDO_02501) also contained proteins from this family (PF00144) which were highly conserved (>90% identity). However, these were distinct from other PF00144 family proteins associated with B. dentium ATCC 27679. The remaining protein of potential relevance is Blon_2358 from B. longum subsp. infantis ATCC 15697. This protein has been assigned as a β-lactamase but, unlike the other proteins referred to above, its closest homologues are not other Bifidobacterium-associated proteins but, rather, are proteins that have been found in the genomes of various clostridia, enterococci and lactobacilli. In addition to containing domains corresponding to Pfam07251, this protein is also representative of Pfam12706, i.e. the lactamase_B_2 family of proteins.
4.4.2 Putative aminoglycoside resistance proteins associated with *Bifidobacterium* species

An identical approach to that taken for the β-lactamases, was taken to identify *Bifidobacterium*-associated proteins which had been annotated, or potentially mis-annotated, as aminoglycoside resistance proteins. A search of the NCBI protein database using the terms ‘aminoglycoside’ and ‘*Bifidobacterium*’ was completed (search completed on 29/8/12). The analysis revealed that putative aminoglycoside resistance proteins are widely distributed across the *Bifidobacterium* genus, and are particularly common among strains of *B. longum* (Table 4). Furthermore, it appears that all putative *Bifidobacterium*-associated aminoglycoside resistance proteins can be broadly classified into 3 groups i.e. those containing proteins of the family Pfam01636 (phosphotransferase enzyme family), proteins containing a protein kinase family domain, c109925, or those which appear to contain both. While some of these proteins appeared to be highly conserved within or across bifidobacteria strains and species, some proteins appear to be much more distantly related. The results indicated that only one putative protein was solely associated with the protein family Pfam01636, namely BBMN_137 from *B. longum* BBMN68. In a number of other instances proteins which were members of Pfam01636 and which also contained the c109925 domain, were noted. In some cases these proteins were annotated as aminoglycoside phosphotransferases, e.g. BIF_01665 (*B. animalis* subsp. *lactis* Bb12), while in other cases they were annotated as desulfatases, e.g. BL_1642
(B. longum NCC 2705), or homoserine kinases, e.g. BBMN_1674 (B. longum BBMN8). In addition, B. bifidum BGN4 BBB_0978 and B. bifidum S17 BBIF_0997 also exhibit characteristics of Pfam01636 and possess a protein kinase domain, but have been annotated as an N-acetyl hexosamine kinase and a mucin desulfatase, respectively. In this instance, laboratory-based investigations have previously established that this gene does indeed encode N-acetyl hexosamine kinase (Nishimoto and Kitaoka, 2007). Some sequences which were annotated as being from Pfam01636 and also contained a protein kinase family domain were highly conserved (with >90% percentage identity) e.g. BLD_1766 (B. longum DJ010A) and BLIG_01601 from B. longum subsp. infantis CCUG 52486). However, in other instances, these proteins were more distantly related e.g. BBIF_0997 (B. bifidum S17) and Bbr_1586 (B. breve UCC2003).

Proteins containing a protein kinase family domain, c109925, only and also annotated as aminoglycoside phosphotransferase or hypothetical proteins are also widely distributed across Bifidobacterium species. Some of these, such as BLD_0109 (B. longum DJ010A), Blon_0773 (B. longum subsp. infantis ATCC 15697) and BLJ_1379 (B. longum subsp. longum JDM301), are highly conserved while others, such as BLJ_1379 (B. longum subsp. longum JDM301) and BIFANG_02451 (B. angulatum DSM 20098), are more distantly related. Finally, 4 proteins (Bbr_0651, BIFBRE_03589, CECT7263_10981 and HMPREF9228_1217) were annotated as containing both a protein kinase family domain from
c109925, while also containing a protein from the Pfam07462 (merozoite surface proteins). These 4 proteins were very highly conserved within the B. breve species sharing >99% percentage identity, while being more distantly related to proteins from other Bifidobacterium species, e.g. BIFANG_02451 from B. angulatum DSM 20098, which did not contain any protein of the Pfam07462.

We also investigated if the β-lactamases and aminoglycoside resistant protein sequences detected in bifidobacteria, could be classified according to the Ambler classes A-D for β-lactamases and acetylation, adenylation and phosphorylation enzymes for aminoglycosides. However, due to insufficient similarity with the sequences of known β-lactamases and aminoglycoside resistance proteins from other genera, such classifications were not possible.

4.4.3 Laboratory-based assessment of the antibiotic resistance of representative bifidobacterial strains

Laboratory tests were conducted with a number of representative Bifidobacterium species to determine if the presence of putative antibiotic resistance proteins corresponded to antibiotic resistance. The specific strains used had been determined, on the basis of the in silico screen, to contain putative β-lactam and/or aminoglycoside resistance genes. The use of different species and strains enabled us to determine if the results were genus, species or strain specific. The strains tested were B. breve UCC2003, B. breve DSM 20213, B. gallicum DSM 20093, B. animalis
subsp. *lactis* Bb12, *B. angulatum* DSM 20098 and *B. pseudocatenulatum* DSM 20438 (Table 2). Disc diffusion assays were performed using both aminoglycoside [kanamycin (30µg), gentamicin (200 µg), streptomycin (25 µg) and neomycin (30 µg)] and β-lactam antibiotic discs [ampicillin (25 µg) and penicillin (10 µg)]. Following anaerobic incubation at 37°C for 48 hours, zones of inhibition were measured (Table 5). All tests were performed in triplicate. The results indicated that all strains tested were highly sensitive to the β-lactam antibiotics tested (all zones ≥ 52mm in diameter), thus establishing that the annotated β-lactamase genes did not confer resistance to the β-lactam antibiotics in the strains tested. Additionally, the β-lactamase nitrocefin tests also demonstrated a lack of β-lactamase activity among the bifidobacteria strains tested. In contrast, when these strains were tested using aminoglycoside antibiotic discs, each of the strains were shown to be highly resistant to each of the antibiotics, i.e. zone of inhibition was small or absent (Table 5).

### 4.4.4 Disruption of the *Bbr_0651* and *Bbr_1586* genes of *B. breve* UCC2003

An insertional inactivation approach was implemented to determine to what extent putative aminoglycoside resistance genes contribute to the observed aminoglycoside resistance in bifidobacteria. *B. breve* UCC2003 was selected as a target, due to the success with which gene disruptions have been previously created in this strain (O’Connell Motherway et al., 2011, O’Connell Motherway et al., 2013). The genes *Bbr_0651* and
Bbr_1586 were targeted for disruption. The gene Bbr_0651 encodes a putative conserved hypothetical secreted protein which shares 99% identity with other putative phosphotransferase enzymes (e.g. BIFBRE_03589 from B. breve DSM 20213) and also shares 71% identity with an aminoglycoside phosphotransferase from B. longum subsp. longum ATCC 55813 (HMPREF0175_1250). The gene Bbr_1586 encodes a putative phosphotranferase family enzyme, which also shares 91% identity with a putative aminoglycoside phosphotransferase from B. longum subsp. longum ATCC 55813 (HMPREF0175_1250).

To determine if disruptions to the genes Bbr_0651 and Bbr_1586 which encode putative aminoglycoside resistance proteins impact on the aminoglycoside resistant phenotype of B. breve UCC2003, disc diffusion assays were carried out. Zones of inhibition were measured and compared to the wild-type, B. breve UCC2003. Differences in the inhibition zones were noted between the mutants and the wild-type, suggesting reduced aminoglycoside resistance in the mutants as compared to the wild-type B. breve UCC2003 (Table 5). Additionally, MICs were performed to compare aminoglycoside resistance of the wild-type to that of the two insertion mutants. As shown in Table 6, after 24 hours incubation, the insertion mutants were more sensitive to gentamicin, streptomycin and kanamycin, but not neomycin, as compared to the wild-type strain. These results thereby demonstrate that both Bbr_0651 and Bbr_1586 contribute to aminoglycoside resistance and can be assigned as aminoglycoside resistance determinants. To verify that
the observed changes to phenotype were as a direct result of disruption to the genes \textit{Bbr\_0651} and \textit{Bbr\_1586}, rather than as an indirect consequence of the mutagenesis strategy, MICs were conducted on another insertion mutant created in \textit{B. breve} UCC2003, namely \textit{B. breve} UCC2003-gosG (O’Connell Motherway et al., 2013). This mutant was created previously using the same protocol that was used to create the mutants \textit{Bbr\_0651} and \textit{Bbr\_1586}, but in this instance the \textit{Bbr\_0529} (gosG) gene is disrupted. The antibiotic resistance phenotype of this mutant was similar to that of the wild-type \textit{B. breve} UCC2003 (Table 6).

To further confirm that the observed reduction in aminoglycoside resistance of the insertion mutant was as a direct result of disruption to the putative AG resistance proteins, complementation studies were performed with one of the mutants. The MIC results demonstrate that following complementation, the resistance of the insertion mutant \textit{Bbr\_1586} was restored to levels almost identical to those of the wild-type (Table 6). Additionally, MICs were determined upon addition of extra plasmid-encoded copies of the putative aminoglycoside resistance genes \textit{Bbr\_0651}, \textit{Bbr\_0651+0650} or \textit{Bbr\_1586} into wild-type \textit{B. breve} UCC2003 to determine if enhanced resistance to aminoglycosides would occur (Table 6). The results established that the addition of the construct pBC1.2-Bbr\_1586 resulted in a 2-fold increased resistance to both streptomycin and kanamycin, relative to that of the parental strain. No increase in resistance to either gentamicin or neomycin was observed. Furthermore, the addition of either pBC1.2-Bbr\_0651+0650 or pBC1.2-
Bbr_0651 did not increase the resistance of UCC2003 to any of the tested aminoglycosides. Finally, the introduction of Bbr_0651 or Bbr_0651+0650 into E. coli XL1-blue resulted in a 2-fold increased resistance to gentamicin and neomycin, while the introduction of Bbr_1586 also increased resistance to neomycin by 2-fold, relative to the control E. coli XL1-blue-pBC1.2 strain (Table 6).
4.5 Discussion

The human microbiota contributes to numerous vital gut functions including nutrient metabolism, vitamin biosynthesis and immune system development (O’Hara and Shanahan, 2006). However, it has more recently been postulated that this complex microbial population is also a sizeable reservoir for antibiotic resistance genes (Salyers et al., 2004, Sommer et al., 2009), and that microbes containing such genes can become dominant in the human gastrointestinal tract following antibiotic exposure (Fallani et al., 2010, Murphy et al., 2010, Fouhy et al., 2012). There is also a risk that such genes could be transferred to other microbes, including those passing through the gastrointestinal tract, and thus could contribute to the dissemination of antibiotic resistance genes (Salyers et al., 2004). Commensal bifidobacteria have received significant attention as a consequence of frequent reports of the beneficial impact of particular species or strains on health (Cani et al., 2007, He et al., 2007, Mitsuoka, 1990), with only one species, B. dentium, being a known human (cariogenic) pathogen (Ventura et al., 2009). Furthermore, given the frequent use of Bifidobacterium strains as probiotics, any association between these microbes and potentially transferrable antibiotic resistance would be a cause for concern.

Several studies have utilized culture-based approaches to determine the resistance or sensitivity of bifidobacteria to various families of antibiotics, though the genetics underlying this resistance has not been examined extensively (Kheadr et al., 2004, Moubareck et al., 2005, Vlková et al.,...
The exceptional studies that exist have focused on mutations to genes encoding specific targets and the resulting increased antibiotic resistance. In one instance the genetic basis for the enhanced resistance of mutants of *B. bifidum* Yakult strain YIT4007 was investigated (Sato and Iino, 2010). Briefly, YIT 4007 was isolated from the progenitor strain YIT 4001 by screening mutants of YIT 4001 for enhanced resistance to neomycin, erythromycin and streptomycin. To investigate the potential transfer of resistance, genetic tests on the mutants were also performed. The study identified several chromosomal mutations, namely mutations on 3 copies of the 23S ribosomal RNA genes, an 8bp deletion of the *rluD* gene and a mutation on the *rspL* gene, which they considered to be responsible for the observed increased resistance to aminoglycoside antibiotics, at levels at which the progenitor strain was sensitive. As these mutations were not located on mobile genetic elements, it was concluded that this strain posed no risk of antibiotic resistance transfer. Another study investigated antibiotic resistance levels in 26 *B. breve* strains and found that a Yakult probiotic strain demonstrated atypically high resistance to streptomycin (Kiwaki and Sato, 2009). Genetic analysis determined that a mutation to the *rpsL* gene, which encodes the ribosomal protein S12, was responsible. In light of the general rarity of studies investigating the genetic basis for innate aminoglycoside resistance in bifidobacteria, this study examined the contribution of *in silico* assigned aminoglycoside resistance proteins to the resistance phenotype of bifidobacteria. Indeed, to our knowledge,
ours is the first study that utilizes a targeted in silico based approach to assess the existence and prevalence of putative \( \beta \)-lactamase and aminoglycoside resistance proteins in the \textit{Bifidobacterium} genus and to subsequently investigate if representative genes confer a resistant phenotype.

With respect to the putative \( \beta \)-lactamases, it was noted that several proteins of potential relevance have been assigned across the \textit{Bifidobacterium} genus. However, none of these were clear representatives of any of the Ambler classes of \( \beta \)-lactamases. When all of the sequences were considered it appeared they could be grouped broadly into one of three groups, i.e. those which were members of Pfam 00144, those of Pfam 07521 or Pfam 12706. Most frequently these sequences were annotated as hypothetical proteins, while others were annotated as \( \beta \)-lactamases. To detect such a high prevalence of putative \( \beta \)-lactamases amongst bifidobacteria was surprising given that previous laboratory based investigations have shown bifidobacteria to be sensitive to commonly prescribed \( \beta \)-lactams (Kheadr et al., 2004, Vlková et al., 2006, Xiao et al., 2010, Moubareck et al., 2005, Lim et al., 1993). Indeed, for example, in 2010 Xiao \textit{et al.} demonstrated that 23 investigated bifidobacterial strains were sensitive to all \( \beta \)-lactams tested (Xiao et al., 2010). In order to examine whether these annotated \( \beta \)-lactamase sequences resulted in a resistance phenotype, we selected a representative number of bifidobacteria strains, which had been identified in the \textit{in silico} screen as containing putative \( \beta \)-lactamases, and studied
these further. Using a culture-based approach, the results indicated that none of the representative bifidobacterial strains which were tested were resistant to the β-lactam antibiotics. These results draw into question the significance of the high frequency of putative β-lactamases or hypothetical proteins closely related to β-lactamases in bifidobacteria genomes. The fact that the tested bifidobacteria were sensitive to β-lactam antibiotics and showed no β-lactamase activity (as assessed using the nitrocefin test), despite the presence of annotated β-lactams in their genome, as well as the lack of sequence homology when compared to known β-lactamase sequences, led us to conclude that this is most likely due to significant mis-annotation of protein sequences across publically available Bifidobacterium genomes. Alternatively, it could be proposed that these β-lactamase genes are repressed in bifidobacteria. While this possibility could be assessed by expression-based studies, which may be investigated in future studies, we think it more likely that the mis-annotation of these putative resistance genes is the basis for the absence of resistance. Indeed, there are previous examples of the mis-assignment of genes as penicillin resistance genes, such as the mis-annotation of the bile salt hydrolase genes as penicillin acylases (Jones et al., 2008, Lambert et al., 2008). With the development of high-throughput genome sequencing methods, automated approaches to annotation became increasingly popular (Schnoes et al., 2009). However, this study provides an example of how mis-annotation of the first bifidobacteria genomes has led to further mis-annotation of subsequent genome sequences. Notably,
several studies have investigated the extent of mis-annotation of genomes and noted the frequency of this issue (Andorf et al., 2007, Brenner, 1999, Devos and Valencia, 2001, Jones et al., 2007), with one study finding an 8% error rate across just 340 genes (Brenner, 1999). Such an approach, which is likely to continue as sequencing becomes even more efficient and cost effective, and is coupled to automated annotation, could cause undue concern about the safety of a species, for example, in the case where antibiotic resistance protein sequences are detected in a potential probiotic bacterium. Thus, our results highlight the necessity for laboratory-based investigations into the function of annotated proteins.

Various culture-based studies have demonstrated that bifidobacteria are resistant to the aminoglycoside family of antibiotics (Kheadr et al., 2004, Vlková et al., 2006, Xiao et al., 2010). This phenomenon was also apparent in the representative strains employed for this study. This resistance has been suggested to be due to the absence of appropriate cytochrome-mediated transport systems in bifidobacteria for aminoglycoside uptake (Bryan et al., 1979). This theory was first proposed in 1979, when it was demonstrated that Bacteroides fragilis and Clostridium perfringens were resistant to aminoglycoside antibiotics due to an inability to synthesize cytochrome structures and thus cannot utilize electron transport mediated transfer that is proposed to facilitate the entry of aminoglycosides into the cells (Bryan et al., 1979). It has since been accepted that bifidobacteria are intrinsically resistant to aminoglycoside
antibiotics by the same mechanism (Talwalkar and Kailasapathy, 2004). However, we hypothesized that the resistance proteins detected in our *in silico* screen could be providing additional resistance beyond this intrinsic resistance and thus could contribute to the survival of bifidobacteria at higher concentrations of aminoglycosides.

The *in silico* screen highlighted the prevalence of putative aminoglycoside resistance proteins across members of the *Bifidobacterium* genus. Though a high frequency of aminoglycoside resistance proteins and related hypothetical proteins were detected, the sequences could be broadly categorised as those which were members of the Pfam 01636, those containing a protein kinase family domain c109925 and those which belonged to the Pfam 01636 and contained the domain c109925.

To investigate the hypothesis that these putative resistance proteins contribute to aminoglycoside resistance in bifidobacteria, putative aminoglycoside resistance genes from one strain were mutated. More specifically, using *B. breve* UCC2003 as a representative strain, we disrupted the 2 genes present in this strain, which were detected in the *in silico* screen as being the genes potentially encoding aminoglycoside resistance proteins. Following confirmation that successful homologous recombination had occurred (at the targeted gene specific sites) within *B. breve* UCC2003, aminoglycoside resistance of the respective mutants was tested. These experiments demonstrated that disruption of either of these 2 aminoglycoside resistance genes impacted on the resistance phenotype of *B. breve* UCC2003 (Table 5). Thus, we propose that while
the lack of cytochrome-mediated transport of the aminoglycosides into the cells may be an important contributor to the observed resistance phenotype among bifidobacteria and alone are sufficient to result in the strains being considered to be clinically resistant, these annotated aminoglycoside resistance proteins are true aminoglycoside resistance proteins, which further enhance this intrinsic resistance. To investigate this hypothesis further, MICs were conducted to compare the resistance of the mutants compared to the wild-type at higher levels of aminoglycoside antibiotics. The results established that the mutants exhibited greater sensitivities to gentamicin, streptomycin and kanamycin compared to the wild-type strain (Table 6). Unfortunately, the strategy employed precluded the creation of a double mutant that lacks both Bbr_1586 and Bbr_0651. Should methods be developed to create deletion mutants in Bifidobacterium in the future, such a mutant can be created in order to determine if the inactivation of both aminoglycoside resistance genes results in a more pronounced aminoglycoside sensitive phenotype. Through complementation studies, it was demonstrated that reintroduction of the Bbr_1586 gene restored resistance to gentamicin and kanamycin to levels which were essentially identical to those of the wild-type (Table 6). Additionally, when an extra, plasmid-borne copy of the gene Bbr_1586 was added to wild-type B. breve UCC2003, a 2-fold increased resistance was seen for streptomycin and kanamycin. However, additional copies of Bbr_1586 did not enhance resistance of the wild-type B. breve UCC2003 to neomycin and gentamicin. This may
be due to the fact that the resistance of the wild-type to these antibiotics was already high (Table 6), and thus the aminoglycoside resistance proteins may have been saturated or unable to provide additional resistance to such high levels of antibiotics. Moreover, when an additional copy of either \textit{Bbr\_0651}+\textit{0650} or \textit{Bbr\_0651} was added to the wild-type \textit{B. breve} UCC2003, no additional enhanced resistance occurred for any of the aminoglycosides tested. This suggests that the genome-encoded copy of this gene is already performing its function optimally. The results in relation to \textit{Bbr\_1586} and streptomycin resistance are puzzling in that, while disruption to the putative aminoglycoside resistance genes resulted in a reduction in streptomycin resistance and additional plasmid-encoded copies of these genes increased the resistance to streptomycin compared to wild-type levels, complementation failed to restore streptomycin levels to those seen in the wild-type. One possible explanation is that there are additional genes downstream of \textit{Bbr\_1586}, which contribute to streptomycin resistance and are impacted upon in a polar manner following mutagenesis by plasmid insertion. The role of \textit{Bbr\_0651} and \textit{Bbr\_1586} as aminoglycoside resistance determinants was further confirmed through the provision of enhanced protection against at least one aminoglycoside upon their expression in \textit{E. coli} XL1-blue.

Ultimately, it is evident that both \textit{Bbr\_0651} and \textit{Bbr\_1586} contribute to aminoglycoside resistance in \textit{B. breve} UCC2003. Importantly however, given that these resistance genes are not located on or near mobile genetic elements, they are unlikely to pose a risk of transferring antibiotic
resistance to other bacteria populations. In fact it may be beneficial for species of *Bifidobacterium* to possess such non-transferable aminoglycoside resistance genes. Such species would survive higher levels of aminoglycosides than species without this additional genetic resistance, and so they may be more suitable as potential probiotics for use during aminoglycoside therapy. The results of this study re-emphasise the fact that annotation of genomes is a predictive process and that the results generated must be interpreted cautiously. Nonetheless, this approach did accurately predict the presence of aminoglycoside resistance proteins in bifidobacterial genomes. Crucially, laboratory based experiments were carried out to validate these annotations and similar such laboratory experiments are required to assess other putative antibiotic resistance genes in bifidobacteria and other genera.
References


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Table 1: Representative sequences used as drivers for Blast based investigations into *Bifidobacterium*-associated aminoglycoside resistant proteins and β-lactamases

<table>
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<th>Aminoglycoside resistance gene classification groups</th>
<th>Representative sequences</th>
<th>β-lactamase gene classes</th>
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<th>Representative gene accession number</th>
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AG: aminoglycoside
Table 3 *Bifidobacterium* derived β-lactamase protein sequences

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<td>NP696361.1</td>
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<td>Accession</td>
<td>Description</td>
<td>Notes</td>
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<tr>
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<td>BLD_0950 Metallo-beta-lactamase</td>
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<td>BLJ_0491 β-lactamase domain-</td>
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<tr>
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<tr>
<td><em>B. gallicum</em> DSM 20093</td>
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<tr>
<td><em>B. angulatum</em> DSM 20098</td>
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<td>BIFANG_02533 Hypothetical protein</td>
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* Same superscript indicates proteins share >90% sequence percentage identity
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<th>Bifidobacterium strain</th>
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<th>Gene name</th>
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<td>ZP_00121797.2a</td>
<td>BLD_1766</td>
<td>Hypothetical protein</td>
<td>Phosphotransferase family with PF 01636 and proteins containing a protein kinase family domain, c109925</td>
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<tr>
<td><strong>B. longum BBMN68</strong></td>
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<td>BBMN68_137</td>
<td>AG phosphotransferases</td>
<td>Phosphotransferase family with PF 01636</td>
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<td></td>
<td>YP_00400127.2.1b</td>
<td>BBMN_1674</td>
<td>Homoserine kinase</td>
<td>Proteins containing a protein kinase family domain, c109925 and phosphotransferase family with PF 01636</td>
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<tr>
<td><strong>B. longum subsp. infantis CCUG 52486</strong></td>
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<td>BLIG_01601</td>
<td>Hypothetical protein</td>
<td>Proteins containing a protein kinase family domain, c109925 and phosphotransferase family with PF 01636</td>
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<tr>
<td><strong>B. longum NCC 2705</strong></td>
<td>NP695320.1a</td>
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<td>Proteins containing a protein kinase family domain, c109925</td>
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<tr>
<td></td>
<td>NP696793.1b</td>
<td>BL_1642</td>
<td>Desulfatase</td>
<td>Proteins containing a protein kinase family domain, c109925 and phosphotransferase family with PF 01636</td>
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<tr>
<td><strong>B. longum KACC 91563</strong></td>
<td>YP_00558689.3.1a</td>
<td>BLNIAS_100852</td>
<td>Hypothetical protein</td>
<td>Proteins containing a protein kinase family domain, c109925</td>
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<td><strong>B. adolescentis L2-32</strong></td>
<td>ZP_02029839.1a</td>
<td>BIFADO_0230</td>
<td>Hypothetical protein</td>
<td>Proteins containing a protein kinase family domain, c109925</td>
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<tr>
<td><strong>B. longum subsp. infantis ATCC 55813</strong></td>
<td>ZP_03976875.1a</td>
<td>HMPREF0175 _1250</td>
<td>AG phosphotransferase</td>
<td>Proteins containing a protein kinase family domain, c109925</td>
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<td><strong>B. longum subsp. infantis ATCC 15697</strong></td>
<td>YP_00232225.4.1a</td>
<td>Blon_0773</td>
<td>AG phosphotransferase</td>
<td>Proteins containing a protein kinase family domain, c109925</td>
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<td>Gene ID</td>
<td>Description</td>
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<td>YP_002323612.1a</td>
<td>Blon_2173</td>
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<td></td>
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<td><em>B. breve</em> UCC2003</td>
<td>YP_003661654.1a</td>
<td>BLJ_1379</td>
<td>AG phosphotransferases</td>
<td></td>
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<td>ABE95342.1c</td>
<td>Bbr_0651</td>
<td>Conserved Hypothetical secreted protein</td>
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<td>ABE96255.1a</td>
<td>Bbr_1586</td>
<td>AG phosphotransferases</td>
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<tr>
<td><em>B. breve</em> DSM 20213</td>
<td>ZP_06595772.1c</td>
<td>BIFBRE_03589</td>
<td>Conserved hypothetical protein</td>
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<td>ZP_06596651.1d</td>
<td>BIFBRE_04498</td>
<td>Mucin desulfating sulfatase</td>
<td></td>
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<tr>
<td><em>B. breve</em> CECT 7263</td>
<td>EHS85254.1a</td>
<td>CECT7263_14691</td>
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<td>EHS85519.1c</td>
<td>CECT7263_10981</td>
<td>Hypothetical protein</td>
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<tr>
<td><em>B. breve</em> ACS 071 VSch 8b</td>
<td>YP_005583039.1c</td>
<td>HMPREF9228_1217</td>
<td>Phosphotransferase enzyme domain protein</td>
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<td></td>
<td>YP_005583418.1a</td>
<td>HMPREF9228_1637</td>
<td>Putative mucin-</td>
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Proteins containing a protein kinase family domain, c109925 and phosphotransferase family with PF 01636
Merozoite surface protein 1 (MSP1) C-terminus of the PF 07462 and proteins containing a protein kinase family domain, c109925
Mucin desulfating sulfatase
Phosphotransferase enzyme domain protein
Putative mucin-protein kinase family
<table>
<thead>
<tr>
<th></th>
<th>Accession</th>
<th>Identifier</th>
<th>Domain/Function</th>
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<tr>
<td><em>B. animalis</em> subsp. <em>lactis</em> Bb12</td>
<td>YP_00557565</td>
<td>BIF_00526</td>
<td>desulfating sulfatase Hypothetical protein</td>
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<td></td>
<td>YP_00557607</td>
<td>BIF_01665</td>
<td>AG 3' phosphotransferase Hypothetical protein</td>
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<tr>
<td><em>B. dentium</em> ATCC 27678</td>
<td>ZP_02918244</td>
<td>BIFDEN_0154</td>
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<td><em>B. dentium</em> Bd1</td>
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<td>BDP_1625</td>
<td>AG phosphotransferase</td>
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<td><em>B. dentium</em> ATCC 27679</td>
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<td>HMPREF0168</td>
<td>Conserved hypothetical protein</td>
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<td><em>B. dentium</em> JCVHM P022</td>
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<td><em>B. catenulatum</em> DSM 16992</td>
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<td>Hypothetical protein</td>
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<tr>
<td><em>B. pseudocatenulatum</em> DSM 20435</td>
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<td>BIFPSEUDO_03094</td>
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<td>BBB_0447</td>
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<td>YP_00639444 9.1</td>
<td>BBB_0978</td>
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<td>B. animalis subsp. animalis ATCC 25527</td>
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<td>BIFLAC_04950</td>
<td>Hypothetical protein</td>
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<td>YP_006280402.1&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>AG phosphotransferase</td>
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<td></td>
<td>YP_006279244.1&lt;sup&gt;n&lt;/sup&gt;</td>
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<td>YP_006279244.1&lt;sup&gt;n&lt;/sup&gt;</td>
<td>BANAN_00270</td>
<td>AG phosphotransferase</td>
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<td>Bifidobacterium sp. 12_1_47BFAA</td>
<td>ZP_079411821&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Phosphotransferase enzyme family protein</td>
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<tr>
<td>B. longum subsp. infantis 157F</td>
<td>YP_004209317.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>BLIF_1400</td>
<td>Hypothetical protein</td>
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<sup>*</sup> Same superscript indicates proteins share >90% sequence percentage identity

AG: aminoglycoside
Table 5 Antibiotic resistance of bifidobacteria strains as assessed through antibiotic disc assays

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<tr>
<th>Bifidobacteria species</th>
<th>Antibiotic (microgram/per disc)</th>
<th>β-lactams</th>
<th>Aminoglycosides</th>
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<tr>
<td></td>
<td>AMP 25</td>
<td>PEN 10 IU</td>
<td>KAN 30</td>
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<tr>
<td><strong>B. breve DSM 20213</strong></td>
<td>71mm</td>
<td>65mm</td>
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<tr>
<td><strong>B. animalis subsp. lactis Bb12</strong></td>
<td>65mm</td>
<td>55mm</td>
<td>No zone</td>
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<tr>
<td><strong>B. pseudocatenulatum DSM 20438</strong></td>
<td>61mm</td>
<td>56mm</td>
<td>8mm</td>
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<td><strong>B. gallicum DSM 20093</strong></td>
<td>60mm</td>
<td>59mm</td>
<td>No zone</td>
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<td><strong>B. angulatum DSM 20098</strong></td>
<td>64mm</td>
<td>65mm</td>
<td>4mm</td>
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<tr>
<td><strong>B. breve UCC2003</strong></td>
<td>67mm</td>
<td>56mm</td>
<td>No zone</td>
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<tr>
<td><strong>B. breve UCC2003-0651-tet</strong></td>
<td>52mm</td>
<td>57mm</td>
<td>10mm</td>
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<tr>
<td><strong>B. breve UCC2003-1586-tet</strong></td>
<td>62mm</td>
<td>57mm</td>
<td>9mm</td>
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<td><strong>B. breve UCC2003-1586-tet-pBC1.2-Bbr_1586</strong></td>
<td>62mm</td>
<td>59mm</td>
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AMP, ampicillin; PEN, penicillin; KAN, kanamycin; GEN, gentamicin; STR, streptomycin; NEO, neomycin

Values are average of triplicate plate results (SD±1mm for all samples, on all antibiotics)
**Table 6** MIC values (mg/L) of wild-type *B. breve* UCC2003 compared to mutants as determined by broth micro-dilution assay (MRS+cysteine for *Bifidobacterium* and LB broth for *E. coli* cultures)

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<th>Sample</th>
<th>GEN 1-1024</th>
<th>NEO 1-1024</th>
<th>STR 2-4096</th>
<th>KAN 2-4096</th>
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<td>&gt;1024</td>
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<td>&gt;4096</td>
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<td><em>B. breve</em> UCC2003-0651-tet</td>
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<td>&gt;1024</td>
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<td><em>B. breve</em> UCC2003-1586-tet</td>
<td>256</td>
<td>&gt;1024</td>
<td>256</td>
<td>1024</td>
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<td><em>B. breve</em> UCC2003-gosG</td>
<td>&gt;1024</td>
<td>&gt;1024</td>
<td>2048</td>
<td>&gt;4096</td>
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<td><em>B. breve</em> UCC2003-1586-tet-pBC1.2-Bbr_1586</td>
<td>&gt;1024</td>
<td>1024</td>
<td>256</td>
<td>4096</td>
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<td><em>B. breve</em> UCC2003 wild-type*</td>
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<td>4096</td>
<td>1024</td>
<td>4096</td>
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<td><em>B. breve</em> UCC2003-pBC1.2_Bbr_1586*</td>
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<td>4096</td>
<td>2048</td>
<td>8192</td>
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<td>4096</td>
<td>1024</td>
<td>4096</td>
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<td><em>B. breve</em> UCC2003-pBC1.2_Bbr_0651+0650*</td>
<td>4096</td>
<td>4096</td>
<td>1024</td>
<td>4096</td>
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<tr>
<td><em>E. coli</em> XL1-blue-pBC1.2</td>
<td>&lt;1</td>
<td>4</td>
<td>&lt;2</td>
<td>&lt;2</td>
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<td>2</td>
<td>8</td>
<td>&lt;2</td>
<td>&lt;2</td>
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<td>2</td>
<td>8</td>
<td>&lt;2</td>
<td>&lt;2</td>
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<tr>
<td><em>E. coli</em> XL1-blue-pBC1.2_Bbr_1586</td>
<td>&lt;1</td>
<td>8</td>
<td>&lt;2</td>
<td>&lt;2</td>
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</table>

GEN, gentamicin; NEO, neomycin; STR, streptomycin; KAN, kanamycin

Values based on triplicate readings, which were identical in all cases

* Higher ranges of antibiotics used to test effect of additional gene copies on MICs compared to wild-type (High range used: 256-16384mg/L for Gent/Neo; 1024-65536mg/L for Strep/Kan)
Chapter 4

Supplementary information
Table S1 Primers used in this study

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<th>Primer name</th>
<th>Primer sequence</th>
<th>Source</th>
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<tr>
<td>Bbr_0651f (500bp insert product)</td>
<td>TGCGGAAGCTTTCGTTCATCGCGAGGACAG</td>
<td>This study</td>
</tr>
<tr>
<td>Bbr_0651r</td>
<td>CTTGCTCTAGACTCCATCGAACCATG</td>
<td>This study</td>
</tr>
<tr>
<td>Bbr_1586f (400bp insert product)</td>
<td>TGCAGAGCTTTGTATAACAAATTCATCGAGCAC</td>
<td>This study</td>
</tr>
<tr>
<td>Bbr_1586r</td>
<td>CTATGCTCTAGACTGATGTCGAGGTGC</td>
<td>This study</td>
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<tr>
<td>Pori19f</td>
<td>ATTTGAGCGAGATAACAAATTTCA</td>
<td>This study</td>
</tr>
<tr>
<td>Pori19r</td>
<td>GATTAAGTTGGGTACGCCAG</td>
<td>This study</td>
</tr>
<tr>
<td>Primers for complementation study and additional plasmid-encoded gene tests</td>
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<td></td>
</tr>
<tr>
<td>Bbr_0651fhind3</td>
<td>TCGCTTAAGCTTGCTCGTTGTTCCATGACC</td>
<td>This study</td>
</tr>
<tr>
<td>Bbr_0651rxbal</td>
<td>GAACGTCTAGACTCGTTGTCGCCGTCGCCG</td>
<td>This study</td>
</tr>
<tr>
<td>Bbr_0651 + 0650r2xbal</td>
<td>CTGCCATCTAGACGGAGAGGACCCCTGCCG</td>
<td>This study</td>
</tr>
<tr>
<td>Bbr_1586fhind3</td>
<td>TCGCTTAAGCTTGAGACCTCGTTGCCAGACCTGAGCAG</td>
<td>This study</td>
</tr>
<tr>
<td>Bbr_1586xbal (1586 complement strain)</td>
<td>GAACTGTCTAGCGCGCCGTAACCAGAAGCAG</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>Source (1995)</td>
<td></td>
</tr>
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Please note that Chapter 5 (pp.215-253) is unavailable due to a restriction requested by the author.
Chapter 6

Gut microbial diversity is reduced and is associated with colonic inflammation in a piglet model of Short Bowel Syndrome

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6.1 Abstract

Following small bowel resection (SBR) the luminal environment is altered, which contributes to clinical manifestations of short bowel syndrome (SBS) including malabsorption, mucosal inflammation and bacterial overgrowth. However, the impact of SBR on the colon has not been well-defined. The aims of this study were to characterise the colonic microbiota following SBR and to assess the impact of SBR on mucosal inflammation in the colon.

Female (4-week old) piglets (5-6/group) received a 75% SBR, a transection (sham) or no surgery. Compositional analysis of the colonic microbiota was performed by high-throughput sequencing, two- and six-weeks post-surgery. The gene expression of the pro-inflammatory cytokines interleukin (IL)-1β, IL-6, IL-8, IL-18 and tumour necrosis factor (TNF)-α in the colonic mucosa was assessed by qRT-PCR and the number of macrophages and percentage inducible nitric oxide synthase (iNOS) staining in the colonic epithelium were quantified by immunohistochemistry.

Analysis of the colonic microbiota demonstrated that there was a significant level of dysbiosis both two- and six-weeks post-SBR, particularly in the phylum *Firmicutes*, coupled with a decrease in overall bacterial diversity in the colon. This decrease in diversity was associated with an increase in colonic inflammation six weeks post-surgery. SBR significantly decreased the diversity of the colonic microbiota and this was associated with an increase in colonic mucosal inflammation.
This study supports the hypothesis that SBR has a significant impact on the colon and that this may play an important role in defining clinical outcome.
6.2 Introduction

Short bowel syndrome (SBS) is the clinical state of malabsorption and malnutrition that occurs following small bowel resection (SBR) (Sukhotnik et al., 2002). Surgical resection of the small bowel may be required for the treatment of a range of conditions including congenital bowel abnormalities and necrotising enterocolitis in children, and Crohn’s disease, trauma and malignancy in adults (Quirós-Tejeira et al., 2004). Although the underlying reason for SBR between adults and children may differ, the clinical manifestations and consequences of SBS are similar. The symptoms of SBS reflect the loss of absorptive surface area and functional disturbance of the remaining intestine and include diarrhoea, vomiting, malabsorption, dehydration and electrolyte imbalance, malnutrition and bacterial overgrowth.

Most cases of SBS occur in the neonatal age group and the mortality associated with the condition ranges from 15-25% (Höllwarth, 1999, Schalamon et al., 2003). The gut microbiota is known to be a major factor in determining the clinical outcome in children (Goulet and Joly, 2010). The effects of an alteration in the microbiota on the small intestine are well studied in this cohort and include increased risk of bacterial overgrowth, bacteraemia, villous atrophy and small intestinal mucosal inflammation, which can result in a loss of intestinal epithelial barrier function and can lead to sepsis (Cole et al., 2008, Cole et al., 2010, Kaufman et al., 1997). In both adult patients with SBS and a piglet SBS
model, the colon is known to play a critical role in determining clinical outcome, and significant morphological adaptation of the colonic mucosa has been observed following resection (Goulet et al., 2009, Joly et al., 2009, Healey et al., 2010). However, the actual impact of changes in the microbiota on the colon itself is poorly understood. Anecdotal evidence suggests that changes in the composition of the colonic microbiota contribute to the generation of symptoms including malnutrition due to decreased bile acid deconjugation, insufficient breakdown of nutrients, and diarrhoea and play a key role in development of serious complications such as septicemia. However, very little is known about specific changes to the colonic microbiota following SBR or the impact these changes may have on colonic mucosal inflammation, the adaptive response and severity of symptoms in SBS.

Despite an obvious clinical need, there are a number of barriers to the study of gut function and immune regulation in SBS. Patients with SBS are a complex, heterogeneous group and access to tissue is limited. Thus, many studies are based on animal models. Rodent SBS models, although useful for molecular and cellular analysis, may not accurately reflect the physiology and pathophysiology of the human intestine. The pig is accepted as the best model for the study of human intestinal biology and diet (Kararli, 1995, Miller and Ullrey, 1987, Moughan et al., 1992) and, with a similar intestinal microbiota to that of humans (Buzoianu et al., 2012, Eckburg et al., 2005, Flint et al., 2007) it is an ideal model for the study of complex gastrointestinal diseases.
The aims of this study were (1) to utilize high-throughput sequencing to characterise changes in the colonic diversity and microbial composition following SBR, (2) to examine the influence of surgical resection on colonic mucosal inflammation and (3) to examine whether there is a correlation between microbial differences and inflammation in a pre-clinical piglet model of SBS in children.
6.3 Materials and Methods

6.3.1 Animals

This study was approved by the Animal Ethics Committee of the Murdoch Childrens Research Institute. Weaned female 3-week-old piglets (Landrace/Large White cross; Aussie Pride Pork) were transported to the University of Melbourne Centre for Animal Biotechnology and acclimatised prior to surgery. Piglets were fed a polymeric infant formula diet (Karicare De-Lact, Nutricia) supplemented to meet the daily requirements for piglets as described previously (Pereira-Fantini et al., 2011, Healey et al., 2010, Stephens et al., 2009). Water was given twice daily. Piglets were housed separately throughout the study to allow accurate daily monitoring of food and water intake and stool output.

6.3.2 Clinical Assessment and Growth

Piglet weight was measured weekly before feeding. Faecal samples were collected weekly and stool consistency was scored by the Royal Children's Hospital Laboratory Services, Melbourne, Australia using the following scale: 0=formed, 1=semi-formed, 2=unformed, and 3=fluid.

6.3.3 Experimental Design

At 4 weeks of age, piglets underwent either a 75% proximal small bowel resection (SBR, n=12) or a transection and re-anastomosis (sham, n=10) operation. One group of piglets did not receive any surgery (non-
operation control; NOC, n=12). The 75% SBR included the removal of the small bowel from 90 cm distal to the ligament of Treitz to 225 cm proximal to the ileocaecal valve. During the sham procedure the intestine was transected and re-anastomosed at a site 225 cm proximal to the ileocaecal valve. Piglets received intramuscular amoxicillin (70 mg/kg; CSL Limited) 24 hours pre-surgery. On the day of surgery, piglets were anaesthetised and given amoxicillin. Piglets received amoxicillin and oral rehydration salts (Sanofi-Aventis Australia) for three days post-surgery in line with current clinical practice. Water and the polymeric infant formula diet were re-introduced from the third day post-operation. All piglets in the NOC group followed the same antibiotic and feeding regime.

6.3.4 Sample collection
Animals in the SBR and sham groups were sacrificed either two- or six-weeks post-surgery and at age-matched times in the NOC group. Colonic tissue was collected 3 cm and 10 cm distal to the caecum in the two-week and six-week groups, respectively, at locations optimised for age, as based on a previous study (Pereira-Fantini et al., 2011). A 3 cm section from each site was divided in half longitudinally and fixed in 10% neutral buffered formalin (Australian Biostain Pty Ltd) or snap frozen in liquid nitrogen. Colonic content was collected from the excised colonic tissue sample.

6.3.5 DNA extraction and amplicon sequencing
DNA was extracted from colonic content using the standard QIAamp DNA Stool Mini Kit protocol (Qiagen, 51504), with the addition of an initial bead beating step. The 16S rRNA amplicons were generated using a previously outlined approach (Murphy et al., 2010a). Amplicons were generated using one forward primer and a combination of four reverse primers as described previously (Murphy et al., 2010b). Each primer contained a distinct multiple identifier (MID) allowing pooling of the amplicons and subsequent separation of the results for analysis. Duplicate PCR products were pooled and cleaned using Agencourt AMPure kit (Beckman Coulter, A63880). Quantification was completed using Quant-iT Picogreen quantification kit (Invitrogen, P7589) and the Nanodrop 3300 (Thermo Scientific). The V4 region of the 16S rRNA was sequenced at the Teagasc 454-Sequencing facility on a Genome Sequencer FLX platform (Roche Diagnostics Ltd.).

6.3.6 Bioinformatic analysis

Raw sequencing reads were quality trimmed using the 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 base pairs. Trimmed FASTA sequences were then BLASTED (Altschul et al., 1997) against the SILVA (v100) database for 16S reads (Pruesse et al., 2007). Phylum, family and genus counts were extracted from MEGAN (Huson et al., 2007) using a bit score cut-off of 86 (Pruesse et al., 2007). Clustering into operational
taxonomical units (OTUs), alignments, chimera-checking and alpha diversities were implemented using the Qiime suite of tools (Caporaso et al., 2010). A phylogenetic tree was generated using the FastTree package (Price et al., 2010) and principal coordinate analysis (PCoA), measuring dissimilarities at phylogenetic distances based on unweighted Unifrac analysis, was performed with Qiime suite of tools (Caporaso et al., 2010). PCoA plots were visualised with KiNG software package (http://kinemage.biochem.duke.edu/software/king.php).

6.3.7 Quantitative PCR

Absolute quantification was completed using the Roche LightCycler 480 platform (Roche Diagnostics). Samples consisted of 2 µl PCR grade water, 1 µl forward primer F1 (5’ AYTGGGYDAAAGNG; 0.15 µM), 1 µl reverse primer R1 (5’TACCRGGGTHTCTAATCC; 0.15 µM), 1 µl template DNA and 5 µl SYBR green (Roche Diagnostics, 04887352001). Bacteria were quantified using 16S rRNA counts based on a standard curve, using a previously outlined calculation (Zhang et al., 2009). All reactions were run in triplicate.

6.3.8 Real-time reverse transcription PCR (qRT-PCR)

The muscle layer was stripped from 100 mg of colonic tissue leaving the colonic mucosa, which comprised the epithelium and the lamina propria. Total RNA was extracted from the mucosa using TRIzol (Invitrogen, 15596-026). Complementary DNA (cDNA) was synthesised with the
Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, 04897030001). PCR primers were designed against pig gene sequences using Roche Universal ProbeLibrary Assay Design Centre (Roche Applied Science, 04683633001). Primer sequences and probe combinations are listed in Table S1. PCR reactions were performed in triplicate on the LightCycler 480. The $2^{\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) was used to calculate relative changes in gene expression using RPL32 as a housekeeping gene and relative to a pre-operation group (n=6).

6.3.9 Microscopic assessment of inflammation

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded colon tissues sectioned at 5 µm. To identify macrophages, antigen retrieval was performed with proteinase K followed by addition of primary antibody (MAC387; 1:1000; Abcam, ab22506) for two hours at room temperature. Staining of inducible nitric oxide synthase (iNOS) was carried out by performing antigen retrieval in citrate buffer (pH 6.0) and adding primary antibody (iNOS; 1:100; Abcam, ab15323) overnight at 4°C. Secondary staining and 3,3-Diaminobenzidine (DAB) detection was performed using Histostain-Plus 3rd Gen IHC Detection Kit (Invitrogen, 85-9073). Negative controls were included in which the primary antibody was substituted with antibody diluent. Slides were viewed under an apochromat 10x objective lens using a Nikon Eclipse 80i microscope (Nikon Instruments Inc), equipped with a DS-Ri1 CCD camera (Nikon)
and controlled by NIS-Elements acquisition software version 4.00 (Nikon).

For each set of analyses, ten fields of view of the epithelium were collected. Macrophages were quantified by counting the number of positively-stained cells in the colonic epithelium using ImageJ (Rasband, 1997-2012). The amount of iNOS epithelial cytoplasmic staining was quantified by colour deconvolution using ImageJ (Ruifrok and Johnston, 2001).

6.3.10 Statistical analysis

Data are presented as mean values with their standard error (SEM). Statistical analysis was performed using one-way ANOVA at each time-point, followed by Tukey’s post-hoc test (GraphPad Prism Software 5.0). Sequencing analysis was completed using Minitab Release 15.1.1.0 (Minitab Inc 2007). To identify if significant differences occurred at phylum, family and genus levels between the three groups, non-parametric Kruskal-Wallis or Mann-Whitney tests were performed followed by Tukey’s post-hoc test. This test was also used for comparing the changes in total bacterial counts in the three groups. The non-parametric Spearman rank correlation was used to determine relationships between diversity and inflammation. Statistical significance was accepted at p < 0.05.
6.4 Results

6.4.1 Piglets that received a small bowel resection had a poorer clinical outcome

Food intake was monitored throughout the study and all diets were isocaloric and isonitrogenous. However, piglets that received a small bowel resection (SBR) had suboptimal weight gain compared to the non-operation control (NOC) and sham-operated piglets (Figure 1A). This was significant from the 2-week time-point \( (p = 0.004) \), up until and including the 6-week time-point \( (p < 0.0001) \). The sham group also had a significantly lower mean weight than the NOC group at the 5-week and 6-week time-points \( (p = 0.041 \) and \( p < 0.0001) \). Piglets in the SBR group failed to resolve diarrhoea at either the 2-week \( (p = 0.002 \) and \( p = 0.005) \) or 6-week \( (p < 0.0001) \) time-points (Figure 1B).

6.4.2 Total colonic bacterial number is unaltered following small bowel resection

Absolute quantification, using qPCR, was used to determine the impact of surgery on total numbers of bacteria. The results indicate that there were no significant differences in the total 16S rRNA gene copies (representative of total bacteria numbers) between any of the groups at either week two \( (p = 0.7845) \) or week six \( (p = 0.8784; \) data not shown).
6.4.3 Surgical resection of the small intestine decreases bacterial diversity in the colon

The colonic content from NOC, sham and SBR piglets was sequenced two- and six-weeks post-surgery. A total of 318,784 V4 16S sequence reads were generated, averaging at 9,376 per sample. Species richness, coverage and diversity estimations were calculated for each data set (Table 1). Rarefaction curves for each sample were parallel or approaching parallel, with the x axis indicating total bacteria diversity was well represented (Figure S1A). There was a trend towards a decrease in the level of diversity in the SBR group compared to the NOC and sham groups at the two-week time-point as assessed by the Chao 1 calculation (Figure 2A and B and Table 1), and the Simpson and Shannon indices (Table 1). By six weeks, however, there was a significant decrease in diversity in the SBR group compared to the NOC and sham groups as demonstrated by the Chao 1 calculation (p = 0.004; Figure 2A and B), the Simpson index (p = 0.008) and the Shannon index (p = 0.003; Table 1). Principle component analysis revealed that the SBR groups at week two and week six clustered together, and were distinct from the NOC and sham groups, which clustered together at both times (Figure S1B).

6.4.4 The composition of the pig microbiota is altered two weeks after small bowel resection

Two weeks post-SBR, the majority of changes were detected at the family and genus levels. There were significant differences at the family
level between the SBR group and the NOC and sham groups (Figure 2C; Table 2). These included a significant increase in *Veillonellaceae* \( (p = 0.0018 \text{ and } p = 0.0115) \) and a significant decrease in *Ruminococcaceae* \( (p = 0.0012 \text{ and } p = 0.0244) \) in the SBR group compared to the NOC and sham groups (Figure 2C; Table 2). *Peptostreptococcaceae* was undetectable in any of the piglets in the SBR group at week two (Table 2). At the genus level, there was a significant increase in *Acidaminococcus* in the SBR group compared to the NOC and sham groups \( (p = 0.0015 \text{ and } p = 0.0033; \text{ Table 2}) \).

**6.4.5 Small bowel resection leads to dysbiosis of the gut microbiota six weeks post-surgery**

Six weeks post-surgery, there were significant decreases in the proportions of *Bacteroidetes* \( (p = 0.0018 \text{ and } p = 0.0013) \) and significantly increased proportions of *Fusobacteria* \( (p = 0.0072 \text{ and } p = 0.0032) \) in the SBR group compared to the NOC and sham groups at this time-point (Figure 2C; Table 2). The majority of differences were observed at the level of the family, particularly in members of the *Firmicutes* phylum (Figure 2C; Table 2). At six weeks, there was a significant decrease in members of the *Peptostreptococcaceae* \( (p = 0.0477 \text{ and } p = 0.0154) \) and *Ruminococcaceae* \( (p = 0.00001 \text{ and } p = 0.0002) \) families in the six-week SBR group compared to the six-week NOC and sham groups. *Peptococcaceae* were undetectable in the majority of SBR piglets at week six. Conversely, there was a significant increase in the proportions
of Veillonellaceae (p = 0.00001 and p = 0.0007) and Fusobacteriaceae (p = 0.0072 and p = 0.0032) in the SBR group compared to the NOC and sham groups at the six-week time-point. Increases at the family level were reflected at the genus level, with increases in the proportion of Megasphaera (p = 0.0156) in the SBR group compared to the NOC group, and Acidaminococcus (p = 0.0006 and p = 0.0063), and Fusobacterium (p = 0.0073 and p = 0.0032) compared to both the NOC and sham groups. Clostridium proportions were significantly decreased in the SBR group compared to the sham group (p = 0.0433).

6.4.6 There is an increase in colonic inflammation following small bowel resection, which is associated with decreased diversity

The gene expression of a panel of pro-inflammatory cytokines was assessed in the colon. Two weeks after surgery, there was no difference in inflammatory gene expression amongst groups (Figure 3A-D). There was, however, a significant increase in the expression of IL-1β (p = 0.045), IL-18 (p = 0.006) and TNF-α (p = 0.036) in the SBR group compared to the sham group six-weeks post-surgery (Figures 3A, C, D), and IL-8 was significantly increased in the SBR group compared to both the NOC and sham groups (p=0.025 and p=0.034; Figure 3B). In the case of IL-1β and IL-8, the increase in these pro-inflammatory cytokines correlated with the observed decrease in colonic bacterial diversity (r = -0.829 and r = -0.771, respectively; Table 3). The increase in colonic pro-inflammatory cytokines was also associated with a
concomitant increase in the total number of macrophages present in the colonic epithelium of the SBR group compared to the NOC and sham groups at six weeks (Figure 4A). At this time-point, there was also a significant increase in the amount of the inflammatory marker iNOS in the colonic epithelium of the SBR group compared to both the NOC and the sham (p = 0.03; Figure 4B).
6.5 Discussion

This study provides the first comprehensive description of the changes in the colonic microbiota following small bowel resection. Not only does this study take advantage of high-throughput sequencing to identify and characterise the dysbiosis that occurs following SBR using an established preclinical piglet model of SBS in children, it also links these changes with local mucosal inflammatory responses.

Following SBR, the luminal environment becomes altered due to rapid shunting of luminal content from the upper intestine into the lower intestine, due to shortened bowel length and increased intestinal transit (Compher et al., 2007). This exposes the colon and its resident bacteria to both digesta and intestinal and pancreatico-biliary secretions that would normally be broken down or absorbed in the upper intestinal tract, which could potentially impact on the composition of bacteria in the lumen (Begley et al., 2005). Evidence on the impact of SBR on bacterial composition is largely anecdotal; however any changes in bacterial composition are likely to have important implications for patients with SBS, in terms of management of their condition.

This study has established that there is a significant decrease in overall bacterial diversity in the colon in a piglet model of SBS that is particularly evident six weeks post-resection. This is consistent with other inflammatory conditions including Crohn’s disease (Ott et al., 2004) ulcerative colitis, (McLaughlin et al., 2010) and antibiotic-associated
diarrhoea (Chang et al., 2008) and has previously been described in adult patients with SBS (Joly et al., 2010). A decrease in microbial diversity also corresponds to decreased metabolic diversity, which has potential implications for SBS patients including insufficient breakdown of dietary components such as complex polysaccharides leading to malabsorption, reduced energy availability and decreased production of short-chain fatty acids (Gerritsen et al., 2011).

This decrease in diversity is due to certain species becoming dominant in the colon, to the detriment of others. At two weeks post-surgery, members of the *Firmicutes* phylum underwent the most significant alterations, with an increase in the proportion of *Veillonellaceae* family and a decrease in the proportion of *Ruminococcaceae* family in the SBR group relative to the NOC and sham group. Major differences in bacterial composition were observed six weeks post-surgery. The phylum *Fusobacteria* was significantly increased in the SBR group relative to the NOC and sham groups. Gram negative bacteria in this phylum express pro-inflammatory molecules such as lipopolysaccharide, which is known to trigger inflammation in the gut (Heimesaat et al., 2006, Ma et al., 2012, Lapthorne et al., 2012). At the family level, the majority of changes were again within the *Firmicutes* phylum, with a decrease in the proportion of *Clostridiaceae, Peptostreptococcaceae* and *Peptococcaceae* in the SBR group compared to the sham group at six weeks. These Gram positive anaerobic bacteria may provide CpG DNA with immunomodulatory activities and their decrease or absence is postulated to disrupt the
crosstalk between the host mucosal immune system and the microbiota (Manichanh et al., 2006). The increase in the Veillonellaceae family and the decrease in Ruminococcaceae family observed in the SBR group at two weeks was sustained at the six week time-point. Several studies have linked alterations in each of these families with systemic and local inflammation (Chen et al., 2011, Sokol et al., 2008). As the delicate balance between host epithelial cells and resident bacteria is already disturbed in patients with SBS, due to the unfavourably altered luminal environment, these specific changes in key bacterial families may further exacerbate the clinical manifestations of SBS.

Interestingly, there were also alterations in some bacterial families in the sham surgery group. The proportion of Enterobacteriaceae was significantly decreased and the proportion of Prevotellaceae was significantly increased in the sham group compared to the NOC group at the two week time-point. This increase in Prevotellaceae was sustained at the six week time-point and there was also a decrease in the proportion of Lactobacillaceae at this time. It is known that surgical trauma often results postoperative ileus and in the activation of resident macrophages and the upregulation of functional activity of pro-inflammatory cytokines (Kalff et al., 2003). Thus, the sham surgery, although relatively minor compared to the massive small bowel resection surgery, would also have an effect on the gut microbiota.
A limited number of studies have examined the bacterial community in SBS patients using culture- and microscopy-based techniques. These studies suggest that there is a shift in the microbiota, and that the microbiota of SBS patients is composed primarily of Gram positive organisms such as lactobacilli (Bongaerts et al., 2000). More recent studies that used quantitative PCR to identify changes in the microbiota in adult patients with SBS, confirmed that there is enrichment in lactobacilli in these patients (Joly et al., 2010, Mayeur et al., 2013). Using high-throughput DNA sequencing, we observed no significant difference in the proportion of *Lactobacillus* spp. at either time-point. Given that a purported 70-80% of gut bacteria are unculturable, our results may reflect the more comprehensive identification of previously unidentified bacterial content (Wilson et al., 1990) and may also indicate that there are important differences between adult and paediatric SBS patients in terms of their microbiota.

Despite significant differences in pro-inflammatory bacteria in the SBR group two weeks post-surgery, there was no increase in pro-inflammatory colonic cytokine expression at this time-point. However, six weeks post-SBR, there were significant increases in the pro-inflammatory cytokines IL-1β, IL-8, IL-18 and TNF-α in the SBR group. This was coupled with an increase in the number of macrophages in the colon, as well as an increase in colonic iNOS in the SBR group. This increase in mucosal inflammation coincided with a marked decrease in diversity and dysbiosis at the later time-point. Consistent with this observation, there was a
correlation between a decrease in bacterial diversity and an increase in colonic IL-8 and IL-1β. Several studies have shown a link between diversity and inflammation, although there is debate as to which is the causative agent (Craven et al., 2012, Ott et al., 2004, Manichanh et al., 2006). In our study, the decrease in diversity was evident two weeks post-surgery, but significant markers of inflammation did not occur until six weeks post-SBR. Thus, it is our hypothesis that the colonic microbiota is negatively impacted by the altered luminal environment following the surgical resection, resulting in a decrease in diversity, which initiates a pro-inflammatory response in the colon.

This study has identified and characterised dysbiosis in the colon that occurs following SBR and challenges current accepted theories regarding specific alterations in the microbiota of patients with SBS. We have shown a reduction in diversity following SBR due to certain bacterial species establishing dominance and described inflammation in the colon that persists for six weeks after SBR. We suggest that this inflammation is a consequence, rather than a trigger of the dysbiosis and that the overall composition of the microbiota may be more relevant than the presence or absence of a single species. This study has highlighted that the colon is significantly impacted by proximal SBR and the role that the colon may play in determining the clinical outcome following a resection has been underappreciated.
References


Pediatric Gastroenterology and Nutrition, 48, S66-S71
10.1097/MPG.0b013e3181a118ef.


Pruesse, E., Quast, C., Knittel, K., Fuchs, B. M., Ludwig, W., Peplies, J. & Glöckner, F. O. 2007. SILVA: a comprehensive online resource for


Table 1 Surgical resection decreases the diversity of colonic bacteria

<table>
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<tr>
<th>Data set</th>
<th>NOC Week 2</th>
<th>Sham Week 2</th>
<th>SBR Week 2</th>
<th>NOC Week 6</th>
<th>Sham Week 6</th>
<th>SBR Week 6</th>
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<tr>
<td>Chao 1 richness estimation</td>
<td>905±41</td>
<td>892±123</td>
<td>718±59</td>
<td>1224±82</td>
<td>1276±97</td>
<td>716±34</td>
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<td>Shannon’s index for diversity</td>
<td>6.9±0.2</td>
<td>6.5±0.6</td>
<td>5.7±0.3</td>
<td>7.1±0.1</td>
<td>7.4±0.2</td>
<td>5.5±0.2</td>
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<td>Number of observed species</td>
<td>552±16</td>
<td>571±80</td>
<td>475±44</td>
<td>726±46</td>
<td>777±63</td>
<td>428±15</td>
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<td>Simpson’s diversity index</td>
<td>0.96±0.01</td>
<td>0.95±0.02</td>
<td>0.93±0.01</td>
<td>0.98±0.01</td>
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Estimation of diversity within the non-operation control (NOC), sham and small bowel resection (SBR) groups at two- and six-weeks post surgery as assessed by Chao 1 richness estimation, Shannon’s index for diversity, number of observed species and Simpson’s diversity index.
Table 2 Small bowel resection alters the composition of the colonic microbiota over time

<table>
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<tr>
<th>Weeks post-surgery</th>
<th>Week 2</th>
<th>Week 6</th>
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<td>Group</td>
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<tr>
<td>Phylum:</td>
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<tr>
<td>Firmicutes</td>
<td>52.6±5.0</td>
<td>52.1±10.6</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>27.8±5.1</td>
<td>23.1±14.6</td>
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<tr>
<td>Bacteroidetes</td>
<td>17.9±2.5</td>
<td>19.1±5.8</td>
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<tr>
<td>Fusobacteria</td>
<td>0.9±0.4</td>
<td>3.8±3.1</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>0.7±0.2</td>
<td>2.0±1.5</td>
</tr>
<tr>
<td>Family:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>31.8±6.9</td>
<td>5.8±2.5**</td>
</tr>
<tr>
<td>Ruminococcaceae</td>
<td>28.1±3.1</td>
<td>27.0±4.2</td>
</tr>
<tr>
<td>Veillonellaceae</td>
<td>19.5±4.9</td>
<td>36.7±8.4</td>
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<tr>
<td>Peptostreptococcaceae</td>
<td>4.6±1.5</td>
<td>2.1±1.5</td>
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<tr>
<td>Prevotellaceae</td>
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<td>3.5±0.8</td>
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</tr>
<tr>
<td>Genus:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>48.8±8.7</td>
<td>9.4±3.7***</td>
</tr>
<tr>
<td>family members</td>
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<td></td>
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<tr>
<td>Bacteroides</td>
<td>5.8±1.3</td>
<td>2.1±1.1</td>
</tr>
<tr>
<td>Parabacteroides</td>
<td>5.1±1.2</td>
<td>10.9±3.3</td>
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<tr>
<td>Prevotella</td>
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<td>23.2±4.5</td>
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<tr>
<td>Clostridium</td>
<td>3.4±0.7</td>
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<tr>
<td>Fusobacterium</td>
<td>2.7±1.4</td>
<td>12.4±6.6</td>
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<tr>
<td>Peptococcus</td>
<td>1.9±0.9</td>
<td>1.5±0.6</td>
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<tr>
<td>Lactobacillus</td>
<td>4.0±1.8</td>
<td>3.4±1.8</td>
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The composition of the colonic microbiota is altered two and six weeks post-surgery in the small bowel resection (SBR) group. Values are relative proportions of operational taxonomic units (OTUs) ± SEM, n=5-6/group/time-point. Statistical significance was determined using a general linear model ANOVA; *p < 0.05; **p < 0.01; ***p < 0.001 compared to non-operation control (NOC) group; †p < 0.05, ††p < 0.01, †††p < 0.001 compared to sham group.
Table 3 A decrease in colonic bacterial diversity is associated with an increase in inflammatory cytokines

The expression of colonic IL-1β and IL-8 negatively correlate with colonic bacterial diversity as assessed by Spearman’s rank correlation coefficient.

<table>
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<tr>
<th>Bacterial alpha diversity</th>
<th>IL-1β</th>
<th>IL-8</th>
<th>IL-18</th>
<th>TNF-α</th>
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<tr>
<td>Spearman r</td>
<td>-0.8286</td>
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<tr>
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<td>0.6583</td>
<td>0.3556</td>
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Figure 1 Clinical effects of small bowel resection. (A) The weight of piglets that received a small bowel resection (SBR) was significantly lower two weeks post-surgery than those that received a sham surgery (Sham) or no surgery (NOC; **p < 0.01). This suboptimal weight gain was sustained for the duration of the experiment (***p < 0.001 at week 3, P < .0001 at weeks 4, 5 and 6). The sham piglets also had a significantly lower weight gain at weeks 5 (#p < 0.05) and 6 (###p < 0.0001) compared to the NOC group. Values are expressed as mean ± SEM; n=12 NOC, n=10 sham, n=12 SBR at pre-op, week 1 and week 2; n=6 NOC, n=5 sham, n=6 SBR at weeks 3, 4, 5 and 6. (B) Piglets in the SBR group had a significantly higher stool consistency score than the sham and NOC groups two weeks (**p < 0.01) and six weeks (***p < 0.001) post-surgery. Values are expressed as mean ± SEM; n=5-6/group/time-point.
Figure 2 Surgical resection of the small intestine decreases the diversity of colonic bacteria and alters the relative proportion of families in the Firmicutes phylum. (A) There is a decrease in the number of distinct operational taxonomic units (OTUs) representing bacterial genera two and six weeks post-surgery in the small bowel resection (SBR) group.
compared to the non-operation control (NOC) and sham group. This is translated into a decrease in overall bacterial diversity as calculated by the Chao 1 richness estimation (B). (C) Pie charts representing the major bacterial phyla and the relative proportion of families in the *Firmicutes* family at the two- and six-week time-point in the NOC, sham and SBR groups.
Figure 3 Surgical resection alters the expression of pro-inflammatory cytokines in the colon. In the colon, the gene expression of IL-1β (A), IL-18 (C) and TNF-α (D) was significantly higher in the SBR group compared to the sham group at six weeks (*p<0.05, **p<0.01). IL8 (B) was significantly higher in the colon in the small bowel resection (SBR) group compared to the non-operation control (NOC) and sham group at six weeks (*p<0.05). Values are expressed as mean ± SEM; n=5-6/group/time-point
Figure 4 There is an increase in inflammatory cells and mediators in the colonic epithelium following small bowel resection. There was an increase in the number of macrophages (A) and the percentage of cytoplasmic inducible nitric oxide synthase (iNOS) staining (B) in the colonic epithelium six weeks post-surgery. Immunohistochemistry images illustrate representative staining six weeks post-surgery. Scale bar represents 100 µm. Values are expressed as mean ± SEM; n=5-6/group
Chapter 6

Supplementary information
**Table S1** List of primer sequences and Universal ProbeLibrary probe combinations used in this study

<table>
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<th>Primer</th>
<th>Sequence 5’ to 3’</th>
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<tr>
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<td>cacaactggaactctgtctatc</td>
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<tr>
<td>IL1B Forward</td>
<td>ccaattcagggacacctacc</td>
<td>#19</td>
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</tr>
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<td>IL8 Forward</td>
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<td>TNF Reverse</td>
<td>ccagtagggcgggtaacag</td>
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</tbody>
</table>
Figure S1 Rarefaction curve and principle coordinate analysis of samples sequenced by high-throughput DNA sequencing. (A) Rarefaction curves for each group at 97% similarity indicated that the total bacterial diversity present was well represented. Number of operational taxonomic units (OTUs) identified as a function of the number of sequence tags sampled. (B) Principle component analysis revealed that the small bowel resection
(SBR) groups at week 2 and week 6 clustered together, and were distinct from the non-operation control (NOC) and sham groups.
Chapter 7

The microbial composition of the human appendix from patients following appendectomy

Published in mBio, Volume 4, Issue 1, January 2013, e00366-12
7.1 Abstract

The human appendix is historically considered a vestige of evolutionary development with an unknown function. While limited data are available on the microbial composition of the appendix, it has been postulated that this organ could serve as a microbial reservoir for repopulating the gastrointestinal tract in times of necessity. We aimed to explore the microbial composition of the human appendix, using high-throughput sequencing of the 16S rRNA gene V4 region. Seven patients aged 5y-25y presenting with symptoms of acute appendicitis were included in this study. Results showed considerable diversity and inter-individual variability among the microbial composition of the appendix samples. In general however, *Firmicutes* were the dominant phylum with the majority of additional sequences being assigned at varying levels to *Proteobacteria, Bacteroidetes, Actinobacteria* and *Fusobacteria*. Despite the large diversity of microbiota found within the appendix, however, a few major families and genera were found to comprise the majority of the sequences present. Interestingly also, certain taxa not generally associated with the human intestine including the oral-associated pathogens *Gemella, Parvimonas*, and *Fusobacterium* were identified among the appendix samples. The prevalence of genera such as *Fusobacterium* could also be linked to the severity of inflammation of the organ. We conclude that the human appendix contains a wealth of microbiota distinct from other niches within the human microbiome. The microbial composition of the human appendix is subject to extreme
variability and holds a diversity of biota that may play an important, as-yet-unknown role in human health.
7.2 Introduction

It has recently been hypothesised that the human appendix functions as a reservoir of beneficial microbes for recovery, following events of pathogen colonization, diarrhoeal disease or antibiotic treatment (Laurin et al., 2011, Smith et al., 2009). Parker and co-workers theorize that the vermiform appendix serves as microbial reservoir or ‘safe-house’ for beneficial bacteria capable of repopulating the gut. The associated lymphoid tissue of the appendix has been recognised to provide an ideal environment for bacterial growth in biofilms acting as an enteric reservoir (Bollinger et al., 2003, Bollinger et al., 2007). Furthermore, the presence of the appendix may reduce the risk of Clostridium difficile recurrence (Im et al., 2011), the protective effect being attributed to the presence of beneficial microbial biofilms and/or due to an immune defence (Na and Kelly, 2011). Conversely, however, another recent study suggested that the appendix may actually promote C. difficile acquisition, carriage and disease (Merchant et al., 2011).

Acute appendicitis is one of the most common causes of abdominal pain with surgical appendectomy being the standard choice of treatment and still considered a clinical emergency. It is now indicated that obstructions in the organ are unlikely to be the primary cause of appendicitis (Carr, 2000) and the role of bacterial infection is believed central to appendix inflammation (Swidsinski et al., 2011). Despite this however, there are limited data on the causal agents of acute appendicitis and of the microbial composition of the human appendix. Culturing-dependent
studies have documented the dominance of *Bacteroides* species in both healthy and inflamed appendices (Elhag et al., 1986, Roberts, 1988), in addition to *Escherichia coli* and *Streptococcus* sp. being recovered from the tissue (Roberts, 1988). Recent studies using fluorescence *in situ* hybridisation (FISH) reported that local invasion with species of *Fusobacterium* is the cause in the majority of cases of suppurative appendicitis (Swidsinski et al., 2011, Swidsinski et al.). The presence of *Fusobacterium* sp. in the mucosal lesions correlated positively with the severity of acute appendicitis and other faecal-associated organisms including members of *Bacteroides, Eubacterium rectale, Faecalibacterium prausnitzii* and *Akkermansia muciniphila* inversely correlated with inflammation associated with the organ (Swidsinski et al., 2011).

In this study, we determined the microbial composition of the human appendix, using next-generation sequencing technologies. The data presented provides, to our knowledge, the first account of the entire microbiota of this organ and provides insight into the diversity of its associated taxa. Important information regarding the bacterial diversity of inflamed appendices is presented and although full conclusions cannot yet be made, it is a first step towards assigning a role for the microbiota of the appendix in human health.
7.3 Materials and Methods

7.3.1 Participants

Approval for the study protocol was obtained from the Clinical Research Ethics Committee of the Cork Teaching Hospitals, Cork, Ireland. Informed consent was obtained from all subjects or from parents of children, in accordance with the local Clinical Research Ethics Committee guidelines. The subject details, pre-operative antibiotics administered and the macro- and micro-scopic details of the appendices are outlined in Table 1. Material from 7 appendices removed during laparoscopic emergent appendectomy was used in this study. One stool sample (patient B) was also taken for comparative analysis. All patients were fasting >15 hr prior to surgery. Samples were transported under anaerobic conditions and kept at 4°C until processing.

7.3.2 Generation of 16S rRNA amplicons for 454 sequencing

Total bacterial metagenomic DNA was extracted from appendix and stool samples (triplicate samples for each subject) using the QIAamp DNA Stool Mini Kit (Qiagen) (Hussey et al., 2011). 16S rRNA bacterial gene amplicons (V4) were generated with a view sequencing using the Roche Genome Sequencer FLX platform. Amplicons of 239 bp were generated using a combination of one forward, i.e. F1 (5’ AYTGGGYDTAAAGNG), and a combination of 4 reverse, R1 (5’ TACCRGGGTHTCTAATCC), R2 (5’ TACCAGAGTATCTAATTC), R3 (5’ CTACDSRGGTTMTCTAATC) and R4 (5’ TACNVGGGTATCTAATC), primers. These primers also contained
an A (F primer) or B (R primers) adapter and different versions of the F primer, each containing a distinct multiple identifier (MID), were employed for each sample. PCRs were performed using Biomix Red (MyBio) and conditions were as follows: 94°C for 2 min followed by 35 cycles of 94°C for 1 min, 52°C for 1 min and 72°C for 1 min followed by a temperature step of 72°C for 2 min. All samples were completed in duplicate. PCR products were cleaned using Agentcourt AMPure kit (Beckman Coulter Genomics) as per manufacturer’s instructions. Samples were quantified using Quant-iT Picogreen quantification kit (Biosciences, Ireland) and the Nanodrop 3300 (Thermo Scientific). Equimolar solutions of samples were pooled for sequencing, cleaned and re-quantified (as above). Emulsion based clonal amplification was completed as part of the 454-pyrosequencing process. Sequencing was performed at the Teagasc 454 Sequencing facility on a Genome Sequencer FLX platform (Roche Diagnostics Ltd) according to the manufacturer’s protocols.

7.3.3 Bioinformatic analysis

16S rRNA sequencing reads were quality trimmed using a locally installed version of the Ribosomal Database Project (RDP) Pyrosequencing Pipeline applying the criteria as previously described (O'sullivan et al., 2011). Trimmed FASTA sequences were BLASTed (Altschul et al., 1990) against a locally installed version of SILVA 16S rRNA database (Pruesse et al., 2007). The resulting BLAST output was parsed using MEGAN (version 4.6) (Huson et al., 2007) using modified
accession look-up tables for mapping the SILVA assignments to NCBI taxonomy. MEGAN assigns reads to NCBI taxonomies by employing the Lowest Common Ancestor algorithm. Bit scores (bit-score of 86) as previously described for 16S rRNA data (Urich et al., 2008) were used from within MEGAN to filter the results prior to tree construction and summarization of taxa. Phylum, family and genera counts for each subject were extracted from MEGAN. Clustering and diversity analysis of the sequence data was performed using the MOTHUR software package (Schloss and Handelsman, 2008, Schloss et al., 2009).

7.3.4 qPCR-based analysis

Absolute quantification of total bacterial numbers was performed by qPCR using the Roche 480 Lightcycler platform. To quantify total 16S rRNA bacterial counts, a standard curve was established using copy numbers of 16S rRNA/µl from 10⁰-10² copies 16S rRNA/µl. Values were then converted to copies 16S rRNA/g appendix or wet stool using the previous calculation (Zhang et al., 2009). Samples were made up of 2 µl of PCR grade water, 1 µl of forward primer F1 (5’ AYTGGGYDAAAAGNG) (0.15 µM), 1 µl of the reverse primer R1 (5’ TACCRRGGGHTCTAATCC) (0.15 µM), 1 µl DNA and 5 µl of SYBR green (Roche Diagnostics Ltd), giving a final reaction volume of 10 µl. All samples, negative controls (where template DNA was replaced with PCR grade water) and standards were run in triplicate.
7.3.5 Statistical Analysis

Minitab Release 15.1.1.0 (Minitab Inc. 2007) was used to run non-parametric statistics (Kruskal-Wallis tests) to determine differences between the microbial numbers from the appendices as determined by PCR. Statistical significance was accepted at p<0.05.
7.4 Results

The 7 patients from whom appendices were surgically removed had been fasting prior to surgery and received pre-operative antibiotics (Table 1). Both the macroscopic appearance of the appendices and the microscopic histopathology was used to determine the severity of the disease of the organ. Samples A, B and D were deemed macroscopically to be the most inflamed with the pathohistology indicating a diagnosis of acute appendicitis whereas samples C, E, F and G appeared not as red or inflamed and therefore are considered as healthier samples.

7.4.1 QPCR analysis of appendix samples

Shifts in phyla may be due to a depletion or increase of bacteria which may alter the final bacterial numbers. QPCR, therefore, was performed to determine total bacterial numbers of the 7 appendices. Absolute quantification revealed that all appendix samples harboured between $10^4$- $10^6$ copies of 16S rRNA/g appendix (Figure 1). Statistical analysis, using the Kruskal-Wallis test revealed that there was a significant difference in total 16S rRNA gene copies across the 7 appendix samples ($p<0.01$) with sample D having the highest total numbers (Figure 1). The microbial load of the appendices was compared to the faecal sample from patient B. The stool sample from patient B had $10^7$ copies of 16S rRNA/g wet stool whereas the comparative appendix sample just harboured $10^4$ copies. This value for the stool sample is comparable to results obtained by
similar methods for faecal samples (Fouhy et al., 2012), despite the use of antibiotics in the current study.

7.4.2 Compositional high-throughput sequencing

The microbial content of the appendices and stool sample was investigated by high-throughput sequencing (Roche-454 GS-FLX Titanium) of 16S rRNA (V4) amplicons generated from extracted DNA. The sequence reads averaged at 9,934 reads per appendix sample and 11,010 reads represented the stool sample. Diversity, richness and coverage estimations were calculated for each data set at 97% similarity levels (Table 2). The Chao1 estimator of species richness indicated a sufficient level of overall phylotype diversity (Table 2). The Shannon index is reflective of both species numbers and evenness of their abundance and therefore the index is increased by having additional unique species or having greater species evenness. The Shannon indices indicated a high level of overall diversity within samples with all values exceeding 4.2. The Good's coverage, a measure of sampling completeness, ranged from 85.7-93.8% for all samples, indicating sufficient overall sampling.

7.4.3 Individual variation of the microbiota of the human appendix

The overall phyla distribution of the appendix samples are shown in Figure 2 and indicate that the individual composition data sets show much variation with respect to each other. Across the 7 appendix samples, there was considerable diversity observed with 15 phyla in total
represented. In general, however, the appendices were dominated by 5 major phyla; *Firmicutes, Proteobacteria, Bacteroidetes, Fusobacteria* and *Actinobacteria* with others detected at low levels (<1-2%) in certain samples including *Deferrribacteres, Verrucomicrobia, Deinococcus-thermus, Chloroflexi, Lentisphaerae, Viridiplantae, Spirochaetes*, Candidate division TM7, OP10 and OP11.

*Firmicutes* were generally the dominant phylum comprising 30%-75% of the total assignable sequences among the 7 appendix samples. *Proteobacteria*, were also well represented, however, and in sample C (37%) and E (46%) were the predominant phylum. Although the average *Actinobacteria* represented was just 3%, sample E harboured relatively high levels (11%) of this phylum. *Bacteroidetes* varied between samples (4%-12%) but generally were not largely represented (Figure 2). The levels of the phylum *Fusobacteria* varied considerably between samples, with levels ranging from <1%-29% of assignable reads. *Fusobacteria* was most represented in the inflamed sample D.

### 7.4.4 Sub-populations of the microbiota of the appendix

At the family level, it appeared that the sub-populations of the assigned phyla also differed somewhat between samples (Figure 3). In general, *Lachnospiraceae* were the dominant sub-group of *Firmicutes* and corresponded to 46% of total assignable sequences in sample A, however, >12 sub-populations of *Firmicutes* were identified within the 7 appendices with the remaining majority of reads being assigned to the
families Ruminococcaceae, Lactobacillaceae and Streptococcaceae (Figure 3). Of the sub-populations of Proteobacteria identified, there was a considerable large proportionate amount of reads assigned in each sample to the family Enterobacteriaceae. The families Bacteroidaceae, Fusobacteriaceae and Bifidobacteriaceae comprised the dominant or only family of the phyla Bacteroidetes, Fusobacteria and Actinobacteria respectively in all samples (Figure 3).

At genus level, despite the large diversity of bacteria represented, a low number of taxa comprised the majority of the bacteria found within the samples (Table 3). Although a large number of phyla (>15), families (>40) and their subpopulations are represented within the 7 samples tested indicating a high level of microbial diversity, it is evident that in certain samples a few major families and genera comprise the majority of the sequences (Table 3). It should be noted, however, that percentages are based on the sequences that can be assigned at this level and assignments to genus level are difficult owing to limited taxonomic assignments within certain families and phyla. Members of the families Enterobacteriaceae, Lachnospiraceae and the genera Fusobacterium, Lactobacillus, Bacteroides and Bifidobacterium are represented in the majority of samples at varying levels (Table 3). Fusobacterium sp., in particular Fusobacterium nucleatum, are generally regarded as oral pathogens but are also commonly found in the lining of the gut, have been identified in the stomach (Bik et al., 2006) and most notably as the likely etiological agents of acute appendicitis (Swidsinski et al., 2011).
addition, the genus *Gemella* usually associated with mucous membranes of oral cavities (Diaz et al., 2012) is represented in samples A and B. *Parvimonas* was identified in high numbers in samples D and G (Figure 4) which is also generally considered with the oral microbiome and as a peridontal pathogen (Ota-Tsuzuki and Alves Mayer, 2009). *Parvimonas micros*, however, has been previously identified from appendix tissue of patients with acute appendicitis (Rautio et al., 2000).

**7.4.5 Comparative analysis of the microbial composition of the appendix with the microbiota of the gut**

The microbiota of the healthy adult gut is generally regarded to be dominated by a small number of phyla with considerable inter-individual variation (Eckburg et al., 2005, Tap et al., 2009, Claesson et al., 2011, Claesson et al., 2012). Studies on young adults have indicated the majority of bacteria belong to the phylum *Firmicutes* or *Bacteroidetes* followed by *Proteobacteria* and *Actinobacteria* (Eckburg et al., 2005, Tap et al., 2009). The results from the appendix samples in this study indicate in addition to the above phyla, the substantial presence of *Fusobacteria* at phylum level and a more diverse biota at family and genus levels when compared to data previously presented (Claesson et al., 2011) (Figures 2-4).

For further comparison, we used one faecal sample (patient B) and the microbial composition was compared to its corresponding appendix sample at phyla (Figure 2), family (Figure 3) and genus levels (Figure 4).
At phylum level, the stool sample harboured *Firmicutes, Bacteroidetes, Proteobacteria* and *Actinobacteria* (Figure 2). The appendix sample from the same patient however had, in addition to the above 4 phyla, a significant proportion of sequences designated to the phylum *Fusobacteria* (7%) and 2% of reads were also assigned to the other phyla *Deferrribacteres, Verrucomicrobia, Viridiplantae* and Candidate division OP11. Examining the subpopulations (Figure 3, Table 3), it is also clear that there is more diversity within the appendix sample than the faecal sample. Of the subpopulations of the phylum *Firmicutes*, just *Lachnospiraceae, Ruminococcaceae* and *Streptococcaceae* were represented in the stool whereas the subpopulation of *Firmicutes* in the appendix sample comprised of 11 families with the most reads being assigned to *Lachnospiraceae* (Figure 3). Taken together, these data suggest that there is a more diverse microbial composition in the human appendix than in the human intestine. It is also evident however that despite some apparent differences, the microbiota represented in both samples share some of the dominant biota represented at phylum, family and genus levels (Figures 2, 3 and Table 3).
7.5 Discussion

This study represents a ‘first look’ at the bacterial composition of the human appendix by next-generation sequencing technologies. Overall, this work reveals that the appendix harbours a wealth of microbiota distinct from other niches within the human microbiome and shows considerable inter-individual variation. However, comparison of the differences between samples may be limited, given that the patients in this study varied in age, gender and clinical presentation (Table 1). Also, antibiotic use has been widely documented to disturb the gut microbiota (for review see (Cotter et al.)) and due to the nature of the clinical admittance of the patients participating in this study, it was unavoidable that antibiotics were taken prior to surgery. However, it should be emphasized that the appendix may “protect” the microbiota from full antibiotic exposure given that it is outside of the main flow of intestinal contents. It cannot be ruled out that the composition of the stool sample used in this study was not affected by antibiotic use however the microbiota of the stool sample in this study is not dramatically different to those presented in previous studies (Eckburg et al., 2005, Claesson et al., 2011, Tap et al., 2009).

qPCR analysis of the total bacterial numbers suggests that the appendix contains fewer bacteria within the tissue than found in the comparative faecal sample (Figure 1). Culturing techniques also indicated a lower overall bacterial count within the appendix than found with the comparative stool sample (data not shown). This is expected as the
bacterial density increases from the upper (ileum, jejunum) to lower (cecum) colonic sites (Marteau et al., 2001, Simon, 1995). Compositional sequencing revealed a diverse microbiota with at least 15 phyla identified within the human appendix. In addition to the 4 major phyla associated with the gut, *Fusobacteria* were largely represented among samples (Figure 2). The presence of this phylum and the proportionate reads assigned to the genus *Fusobacterium* is interesting as species of *Fusobacterium* have been suggested to be a causative agent of acute appendicitis (Swidsinski et al., 2011, Swidsinski et al.). Appendicitis is a clinical diagnosis and therefore its uniformity cannot be assumed. The severity of the disease of the appendix samples in this study was based on a macroscopic and pathohistological analysis (Table 1). The levels of *Firmicutes*, *Proteobacteria* and *Bacteroidetes* varied among samples, regardless of disease state (Figure 2), whereas the levels of *Actinobacteria* were most represented in the non-perforated sample E (11%). The phylum *Fusobacteria*, and at genus level *Fusobacterium* sp., were most represented in sample D (Table 3). Appendix sample D presented as a red and inflamed appendix and a clinical diagnosis of acute appendicitis was given. *Fusobacterium* sp. were also found at varying levels among other appendix samples except in samples E and F, which were deemed to be healthier samples in this study (Tables 1 and 3).

Is there a role for the microbiota of the human appendix in gut health? The function of the appendix as a microbial reservoir for the gut has been
proposed and has recently received much attention (Laurin et al., 2011, Smith et al., 2009). Based on this study, the appendix appears to harbour a large diversity of gut microbes including significant amounts of ‘beneficial bacteria’ or indicators of gut health including the genera Bacteroides, Lactobacillus and Bifidobacterium (Figure 4). It is plausible that these bacteria present in biofilms on the epithelial layer of the appendix may serve as a reservoir when eradicated from the gut. The genus Clostridium was not largely represented in any appendix sample (Figure 4) and therefore these results would not indicate that the appendix serves as an organ to carry this organism as previously suggested (Merchant et al., 2011).

The composition of the appendix differed somewhat from the stool sample in this study (Figures 2-4). This is not surprising, however, based on their differing ecological conditions. The appendix is an extension of the cecum which has a lower pH and higher fatty acid content and in consequence has been found to harbour varying bacteria from those found in the faecal samples (Marteau et al., 2001). However, despite some variation, the profiles were not radically different and the appendix does contain a high proportion of intestinal-associated microbes. In addition to these however, there were also a number of phyla found that are not generally regarded as gut microbes including Deinococcus-thermus, Spirochaetes and Chloroflexi. Deinococcus-related organisms are largely associated with extreme environments, however have recently been identified in the human stomach (Bik et al., 2006) and in endodontic
infections (Li et al.). The presence of these phyla indicates environmentally resistant organisms reside within the appendix. At genus level, *Parvimonas* and *Gemella* were represented which are generally considered pathogenic organisms of the oral cavity (Diaz et al., 2012, Ota-Tsuzuki and Alves Mayer, 2009). The high incidence of oral pathogens in the appendix samples is interesting as microbial community analysis has previously implied that the oral cavity and GI tract share few bacterial species (He et al.).

This study presents a first comprehensive view of the microbiota of the human appendix. It would appear that this organ harbours a diverse microbiota and, although it shares a substantial amount of microbes with the intestinal tract, it has its own defined microbiome. The study presented here provides some insights into the composition of the human appendix and information on pathogens present that may possibly contribute to appendicitis. The microbial diversity may be shaped through the co-evolution of the microbial communities and specific ecological factors of the appendix tissue. It may also be the case that the microbial dynamics of surrounding intestinal niches influence the microbiota of this organ. Further studies of this diverse biota may be fruitful in ultimately assigning it a role in gut health.
References


Im, G. Y., Modayil, R. J., Lin, C. T., Geier, S. J., Katz, D. S., Feuerman, M. & Grendell, J. H. 2011. The appendix may protect against


Table 1 Description of patients and appendix samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Gender</th>
<th>Age (y)</th>
<th>Macroscopic Appearance</th>
<th>†Microscopic Analysis</th>
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<tr>
<td>A</td>
<td>M</td>
<td>14</td>
<td>congested appendix</td>
<td>acute appendicitis</td>
<td>Amoxicillin/clavulanate</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>mucosal haemorrhagic, red inflamed appearance no perforation</td>
<td>acute suppurative appendicitis</td>
<td>Amoxicillin/clavulanate + Gentamicin</td>
</tr>
<tr>
<td>B*</td>
<td>M</td>
<td>17</td>
<td>mucosal haemorrhagic, red inflamed appearance no perforation</td>
<td>no evidence of active inflammation acute appendicitis</td>
<td>Amoxicillin/clavulanate</td>
</tr>
<tr>
<td>C</td>
<td>F</td>
<td>20</td>
<td>congested appendix, red appearance</td>
<td>no evidence of active inflammation acute appendicitis</td>
<td>Amoxicillin/clavulanate</td>
</tr>
<tr>
<td>D</td>
<td>M</td>
<td>13</td>
<td>congested appendix, red appearance</td>
<td>serositis</td>
<td>Amoxicillin/clavulanate + Gentamicin + Metronidazole</td>
</tr>
<tr>
<td>E</td>
<td>M</td>
<td>25</td>
<td>no perforation, congested surface</td>
<td>no evidence of inflammation no pathological changes</td>
<td>Amoxicillin/clavulanate</td>
</tr>
<tr>
<td>F</td>
<td>M</td>
<td>5</td>
<td>no perforation</td>
<td></td>
<td>Amoxicillin/clavulanate</td>
</tr>
<tr>
<td>G</td>
<td>F</td>
<td>14</td>
<td>no perforation</td>
<td></td>
<td>Amoxicillin/clavulanate</td>
</tr>
</tbody>
</table>

* Stool sample received for patient
† Result of the pathohistological analysis of the appendices
Table 2 Estimation of diversity at 97% similarity level within each data set

<table>
<thead>
<tr>
<th>Sample*</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>Stool</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chao1</td>
<td>790</td>
<td>697</td>
<td>466</td>
<td>705</td>
<td>358</td>
<td>834</td>
<td>727</td>
<td>694</td>
</tr>
<tr>
<td>Richness</td>
<td>5.5</td>
<td>5.3</td>
<td>4.7</td>
<td>4.8</td>
<td>4.2</td>
<td>5.3</td>
<td>5.3</td>
<td>5</td>
</tr>
<tr>
<td>Shannon</td>
<td>86.8</td>
<td>86.7</td>
<td>90.8</td>
<td>93.8</td>
<td>85.7</td>
<td>92.4</td>
<td>89.4</td>
<td>93.9</td>
</tr>
<tr>
<td>Index</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Good's</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>Coverage</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Appendix samples A-G and comparative Stool sample B
Table 3 Comparison of the dominant subpopulations of the appendix samples A-G and the stool sample (S)

<table>
<thead>
<tr>
<th>Genus</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enterobacteriaceae members</td>
<td>9%</td>
<td>25%</td>
<td>36%</td>
<td>9%</td>
<td>49%</td>
<td>27%</td>
<td>25%</td>
<td>11%</td>
</tr>
<tr>
<td>Lachnospiraceae incertae sedis</td>
<td>*</td>
<td>10%</td>
<td>6%</td>
<td>*</td>
<td>11%</td>
<td>*</td>
<td>*</td>
<td>16%</td>
</tr>
<tr>
<td>Ruminococcaceae incertae sedis</td>
<td>8%</td>
<td>4%</td>
<td>3%</td>
<td>*</td>
<td>*</td>
<td>7%</td>
<td>4%</td>
<td>6%</td>
</tr>
<tr>
<td>Fusobacterium</td>
<td>3%</td>
<td>8%</td>
<td>9%</td>
<td>41%</td>
<td>*</td>
<td>*</td>
<td>7%</td>
<td>*</td>
</tr>
<tr>
<td>Lactobacillus</td>
<td>16%</td>
<td>4%</td>
<td>*</td>
<td>4%</td>
<td>23%</td>
<td>7%</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Bacteroides</td>
<td>7%</td>
<td>9%</td>
<td>7%</td>
<td>7%</td>
<td>4%</td>
<td>4%</td>
<td>5%</td>
<td>18%</td>
</tr>
<tr>
<td>Blidobacterium</td>
<td>4%</td>
<td>3%</td>
<td>3%</td>
<td>*</td>
<td>12%</td>
<td>*</td>
<td>*</td>
<td>15%</td>
</tr>
<tr>
<td>Eubacterium</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>10%</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Streptococcus</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>10%</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Parvimonas</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>21%</td>
<td>*</td>
<td>*</td>
<td>18%</td>
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</tr>
<tr>
<td>Parabacteroides</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Oscillibacter</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>3%</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Gemella</td>
<td>5%</td>
<td>3%</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Faecalibacterium</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>3%</td>
<td>*</td>
<td>4%</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Alistipes</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>5%</td>
<td>*</td>
</tr>
<tr>
<td>Mucspirillium</td>
<td>5%</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>5%</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Aliobaculum</td>
<td>9%</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Blautia</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>7%</td>
</tr>
<tr>
<td>Other genera</td>
<td>23%</td>
<td>34%</td>
<td>28%</td>
<td>9%</td>
<td>10%</td>
<td>27%</td>
<td>29%</td>
<td>14%</td>
</tr>
<tr>
<td>Not assigned</td>
<td>11%</td>
<td>*</td>
<td>8%</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

*Only genera representing >3% of assignable sequences. Percentages are representative of the relative abundances of total sequences assignable at genus level. All genera present in <3% are grouped together as “Other genera”
**Figure 1** The numbers of bacteria quantified by real-time QPCR. Error bars represent the standard error of the mean (n=3). Red bars represent appendix samples A-G, Blue bar represents the stool sample.
Figure 2 Phyla distribution among appendix samples (A-G) and the stool sample.

Firmicutes ■; Proteobacteria □; Bacteroidetes ■; Actinobacteria ■; Fusobacteria ■; Other phyla ■; Not assigned ■
Figure 3 Family-level comparison of appendix samples A-G and the stool sample. Similar bar colours correspond to samples within a particular phylum. Families of lower abundance are grouped together. *Lachnospiraceae* □; *Ruminococcaceae* ■; *Lactobacillaceae* □; *Erysipelotrichaceae* ■; *Peptostreptococcaceae* □; *Streptococcaceae* □; *Clostridiales family XI incertae sedis* □; *Veillonellaceae* □; *Staphylococcaceae* □; *Clostridiales* □; *Eubacteriaceae* □; *Enterobacteriaceae* □; *Desulfovibrionaceae* □; *Succinivibrionaceae* □; *Alcaligenaceae* □; *Bacteroidaceae* □; *Rikenellaceae* □; *Porphyromonadaceae* □; *Bifidobacteriaceae* □; *Fusobacteriaceae* □; Other families □; Not assigned □
Appendix 1

Contrasting effects of *Bifidobacterium breve* NCIMB 702258 and *Bifidobacterium breve* DPC 6330 on the composition of murine brain fatty acids and gut microbiota

Abstract

We previously demonstrated that microbial metabolism in the gut influenced the composition of bioactive fatty acids in host adipose tissue. This study compared the impact of dietary supplementation for 8 weeks with human-derived *Bifidobacterium breve* strains on fat distribution and composition and the composition of the gut microbiota in mice. C57BL/6 mice (*n*=8/group) received either *B. breve* DPC 6330 or *B. breve* NCIMB 702258 (10⁹ microorganisms) daily for 8 weeks compared with unsupplemented controls. Tissue fatty acid composition was assessed by gas-liquid chromatography and 16S rRNA pyrosequencing was employed to investigate microbiota composition.

Visceral fat mass, and brain stearic-, arachidonic- and docosahexaenoic acids were higher in *B. breve* NCIMB 702258 supplemented mice compared with the other two groups (p<0.05). In addition, both *B. breve* DPC 6330 and *B. breve* NCIMB 702258 supplementation resulted in higher propionate in caecum, compared with unsupplemented controls (p<0.05). Compositional sequencing of the gut microbiota revealed a tendency for greater proportions of *Clostridiaceae* (25% vs. 12% vs. 18%; p=0.08) and lesser proportions of *Eubacteriaceae* (3% vs. 12% vs. 13%; p=0.06) in *B. breve* DPC 6330 supplemented mice compared with *B. breve* NCIMB 702258 supplemented mice and unsupplemented controls.

The response of fatty acid metabolism to administration of bifidobacteria is strain-dependent, and strain-strain differences are important factors with respect to modulation of the gut microbial community by ingested microorganisms.
**Introduction**

The human gut microbiota comprises trillions of microorganisms, reaching cell numbers that outnumber that of host cells, and contains at least 100-fold more genes than the human genome (Qin et al., 2010). The collective genome of these microorganisms (the microbiome) contributes to a broad range of metabolic and biochemical functions that the host could not otherwise perform (Ley et al., 2006). Recent research has revealed an interaction between the gut microbiota and host metabolism, energy utilization and fat storage, suggesting that intestinal microbes play a direct role in the development of obesity (Bäckhed et al., 2004, Ley et al., 2005, Ley et al., 2006, Turnbaugh et al., 2006). Metagenomic analyses have also revealed that the caecal microbiota of ob/ob mice and obese individuals are more efficient at energy extraction from the diet and at producing short chain fatty acids (SCFAs) than those of normal phenotype (Schwiertz et al., 2010, Turnbaugh et al., 2006). These SCFAs can then be utilized for *de novo* synthesis of lipids and glucose (Wolever et al., 1989), thus providing an additional source of energy for the host. While some studies of bacterial communities in the gut microbiota in both mouse models and in humans, using compositional sequencing, have revealed an increase in the ratio of *Firmicutes* to *Bacteroidetes* in obese subjects (Ley et al., 2006, Turnbaugh et al., 2008), the identity of the microbial populations that are associated with obesity continues to be the subject of much debate, with others reporting a decreased *Firmicutes* to *Bacteroidetes* ratio in overweight and obese individuals (Murphy et al., 2010, Schwiertz et al., 2010). Another study reported no link between the proportion of *Firmicutes* and *Bacteroidetes* and human obesity (Duncan et al., 2008). Whether alterations in the microbiota are a cause, or consequence of obesity, is controversial, with the role of the gut microbiota in fat metabolism and obesity being more complex than first considered (Duncan et al., 2008, Hildebrandt et al., 2009, Murphy et al., 2010). Thus, the interrelation between the gut microbial composition, diet, and host adiposity has to be further
investigated. Knowledge of the interactions between energy intake and specific microbial populations, and their influence on body weight is limited to small-scale clinical trials (Ley et al., 2006). Among the gastrointestinal bacteria, *Bifidobacterium* is an important commensal group, accounting for an estimated 3% of the intestinal microbiota from an average adult (Turroni et al., 2009a, Turroni et al., 2009b). Due to their well-documented beneficial health effects (Leahy et al., 2005), bifidobacteria have attracted significant interest for probiotic applications in pharmaceutical and dairy products. In relation to host energy metabolism, higher numbers of bifidobacteria have been documented in normal-weight adults and adolescents than in their overweight counterparts (Collado et al., 2008, Kalliomäki et al., 2008). However, some recent studies suggest that the role of bifidobacteria in weight management may be species specific. Thus, Santacruz *et al.* reported that weight loss was associated with a reduction in the numbers of *Bifidobacterium breve* and *Bifidobacterium bifidum* and an increase in the numbers of *Bifidobacterium catenulatum* (Santacruz et al., 2009).

A promising mechanism by which the manipulation of the gut microbiota can affect host metabolism and fat storage is the modulation of fatty acid composition of host cellular membranes. We have previously shown that dietary supplementation with a conjugated linoleic acid (CLA)-producing *Bifidobacterium* strain of human origin (*B. breve* NCIMB 702258) influenced the composition of bioactive fatty acids in host liver and adipose tissue, in different animal species (Wall et al., 2010, Wall et al., 2009). Thus, in this study, we compared the impact of administering two different CLA-producing strains of the same species, i.e. *B. breve* DPC 6330 and *B. breve* NCIMB 702258, on host fat distribution and composition. Furthermore, we performed a high-throughput pyrosequence-based assessment of the impact of oral administration of these *B. breve* strains on the diversity of the resident gut microbiota.
Materials and Methods

Animals and treatment

Wild-type C57BL/6 male mice, aged 7-8 weeks, were obtained from Charles River (L’arbresle, France) and housed under barrier-maintained conditions within the Biological Services Unit, University College Cork (UCC). All animal experiments were approved by the UCC Animal Ethics Committee and experimental procedures were conducted under the appropriate license from the Irish Government. Mice were allowed to acclimatise for 1 week before the start of the study and were fed ad libitum with Teklad Global rodent standard diet (Harlan Laboratories, Madison, WI, USA, #2018S) with free access to water at all times. Mice were housed in groups of four per cage and kept in a controlled environment at 25°C under a 12-hr-light/12-hr-dark cycle. After 1 week of acclimatisation, the mice were divided into three groups (n=8/group): a control group fed with standard diet and placebo freeze-dried powder (15% w/v trehalose in dH₂O), a group fed with standard diet and B. breve DPC 6330 (approximate daily dose of 10⁹ microorganisms) and a group fed standard diet and B. breve NCIMB 702258 (approximate daily dose of 10⁹ microorganisms). The diet contained the following nutrient composition: crude protein (18.6%), carbohydrate (44.2%), fat (6.2%), crude fibre (3.5%), neutral detergent fibre (14.7%), and ash (5.3%). The fatty acids present in the diet included palmitic acid (C16:0, 0.7%), stearic acid (C18:0, 0.2%), oleic acid (C18:1n-9, 1.2%), linoleic acid (C18:2n-6, 3.1%), and linolenic acid (C18:3n-3, 0.3%). Body weight and food intake was assessed weekly. Following 8 weeks on experimental diets, the animals were sacrificed by decapitation. Liver, brain, fat pads (epididymal, perirenal and mesenteric), gastrointestinal tract (GIT) from stomach to anus, and caecal contents were removed, blotted dry on filter paper, weighed and flash-frozen immediately in liquid nitrogen. All samples were stored at -80°C until processed. Blood samples were...
collected from fasted animals, and allowed to clot for 2 hours at 4°C before centrifuging for 20 min at 2000 x g.

**Preparation and administration of B. breve NCIMB 702258 and B. breve DPC 6330**

We have previously shown that *B. breve* NCIMB 702258 and *B. breve* DPC 6330 are efficient CLA producers, converting up to 65% and 76%, respectively, of linoleic acid to c9, t11 CLA when grown in 0.5 mg/ml linoleic acid *in vitro* (Barrett et al., 2007, Coakley et al., 2003). Rifampicin resistant variants of *B. breve* NCIMB 702258 and *B. breve* DPC 6330 were isolated by spread-plating ~10⁹ colony forming units (CFU) from an overnight culture onto MRS agar (de Man, Rogosa & Sharpe; Difco Laboratories, Detroit, MI, USA) supplemented with 0.05% (w/v) L-cysteine hydrochloride (98% pure; Sigma Chemical Co., Poole, Dorset, UK) (mMRS) containing 500 µg/ml rifampicin (Sigma). Following anaerobic incubation (anaerobic jars with Anaerocult A gas packs; Merck, Darmstadt, Germany) at 37°C for 3 days, colonies were stocked in mMRS broth containing 40% (v/v) glycerol and stored at -80°C. To confirm that the rifampicin resistant variant was identical to the parent strain, molecular fingerprinting using pulse-field gel electrophoresis was employed.

Prior to freeze drying, *B. breve* NCIMB 702258 and *B. breve* DPC 6330 were grown in mMRS by incubating overnight at 37°C under anaerobic conditions. The culture was washed twice in phosphate buffered saline (PBS) and resuspended at a concentration of ~2×10¹⁰ cells/ml in 15% (w/v) trehalose (Sigma) in dH₂O. One millilitre aliquots were freeze-dried using a 24 hr programme (freeze temp. -40°C, condenser set point -60, vacuum set point 600 m Torr). Each mouse that received the bacterial strains consumed approximately 1×10⁹ live microorganisms per day. This was achieved by resuspending appropriate quantities of freeze-dried powder in water which mice consumed *ad libitum*. Mice that did not
receive the bacterial strains received placebo freeze-dried powder (15% (w/v) trehalose in dH₂O). Water containing either the bacterial strains or placebo freeze-dried powder was the only water supply provided to the animals throughout the trial. Freeze dried powders with the bacterial strains were undergoing continuous quality control of cell counts for the duration of the trial by plating serial dilutions on mMRS agar supplemented with 100 µg of mupirocin (Oxoid)/ml and 100 µg rifampicin (Sigma)/ml and incubating plates anaerobically for 72 hr at 37°C.

**Culture-dependent microbial analysis**

Fresh faecal samples were taken from C57BL/6 mice every week for microbial analysis. Microbial analysis of the faecal samples involved enumeration of the *B. breve* strains by plating serial dilutions on mMRS agar supplemented with 100 µg of mupirocin (Oxoid)/ml, 100 µg rifampicin (Sigma)/ml and 50 U/ml nystatin (Sigma). Agar plates were incubated anaerobically for 72 hr at 37°C. In addition, proximal colonic contents were sampled at sacrifice for enumeration of the administered *B. breve* strains.

**Lipid extraction and fatty acid analysis**

Lipids were extracted with chloroform:methanol (2:1 v/v; Fisher Scientific, Dublin, Ireland) according to the method by Folch *et al.* (Folch *et al.*, 1957). Fatty acid methyl esters (FAME) were prepared using first 10 ml 0.5N NaOH (Sigma) in methanol for 10 min at 90°C followed by 10 ml 14% BF₃ in methanol (Sigma) for 10 min at 90°C (Park and Goins, 1994)(23). FAME were recovered with hexane (Fisher Scientific). Prior to gas-liquid chromatography (GLC) analysis, samples were dried over 0.5 g of anhydrous sodium sulphate (Sigma) for 1 hr and stored at -20°C. FAME were separated by GLC (Varian 3800, Varian, Walnut Creek, CA, USA) fitted with a flame ionization detector, using a Chrompack CP Sil 88 column (Chrompack, Middleton, The Netherlands, 100 m × 0.25 mm i.d., 0.20 µm film thickness) and Helium as carrier gas. The column oven was
programmed to be held initially at 80°C for 8 min then increased 8.5°C/min to a final column temperature of 200°C. The injection volume used was 0.6 μl, with automatic sample injection on a SPI 1093 splitless on-column temperature programmable injector. Peaks were integrated using the Varian Star Chromatography Workstation version 6.0 software and peaks were identified by comparison of retention times with pure FAME standards (Nu-Chek Prep, Elysian, MN, USA). The percentage of individual fatty acids was calculated according to the peak areas relative to the total area (total fatty acids were set at 100%). All fatty acid results are shown as mean ± standard error of the mean (SEM) g/100 g FAME.

SCFA analysis
Approximately 100 mg caecal content was vortex mixed with 1.0 mL Milli-Q water and, after standing for 10 min at room temperature, centrifuged at 10 000g for 5 min to pellet bacteria and other solids. The supernatant was collected, 3.0 mM 2-ethylbutyric acid (Sigma) added as internal standard, and samples filtered before being transferred to clean vials. Calibration was done using standard solutions containing 10.0 mM, 8.0 mM, 6.0 mM, 4.0 mM, 2.0 mM, 1.0 mM and 0.5 mM of acetic acid, propionic acid, iso-butyric acid and butyric acid (Sigma). The concentration of SCFA was determined using a Varian 3500 GC flame ionization system, fitted with a Nukol-FFAP column (30 m x 0.32 mm x 0.25 μm; Sigma). Helium was used as the carrier gas at a flow rate of 1.3 mL/min. The initial oven temperature was 100°C for 0.5 min, raised to 180°C at 8°C/min and held for 1 min, then increased to 200°C at 20°C/min, and finally held at 200°C for 5 min. The temperature of the detector and the injector were set at 250°C and 240°C, respectively. Peaks were integrated using the Varian Star Chromatography Workstation version 6.0 software. Standards were included in each run to maintain the calibration.
Measurement of triglycerides in liver
Liver lipids were extracted and purified according to the method of Folch et al. (Folch et al., 1957). After mixing thoroughly, the samples were dried under nitrogen and resolubilised in 5% (v/v) Triton X-100 in dH2O. The concentration of triglycerides was determined using a commercial kit (EnzyChrom Triglyceride Assay kit, BioAssay Systems, CA, USA). Results were normalised to the weight of the samples.

Measurements of serum parameters
Serum variables were determined with commercial kits. Glucose in serum was determined using QuantiChrom glucose assay kit (BioAssay Systems), serum insulin concentration was measured using Ultra Sensitive Mouse ELISA kit (Crystal Chem Inc., IL, USA), leptin in serum was measured using Mouse Leptin ELISA kit (Crystal Chem Inc.), and serum triglyceride concentrations using EnzyChrom Triglyceride Assay kit (BioAssay Systems).

Culture-independent microbial analysis
For analysis of the microbial community composition of caecal contents, total DNA was extracted from the caecal contents of all mice using the QIAamp DNA stool mini kit according to the manufacturer’s instructions (Qiagen, West Sussex, UK) coupled with an initial bead-beating step. Universal 16s rRNA primers, designed to amplify from highly conserved regions corresponding to those flanking the V4 region, i.e. the forward primer F1 (5’-AYTGGGYDAAAAGNG) and a combination of four reverse primers R1 (5’-TACCRGGGTHTCTAATCC), R2 (5’-TACCAGGTATCTAATTC), R3 (5’-CTACGSGGTTCTAATC) and R4 (5’-TACNVGGGTATCTAATC) (RDP’s Pyrosequencing Pipeline: http://pyro.cme.msu.edu/pyro/help.jsp) were used for Taq-based PCR amplification. Sequencing was performed on a Roche 454 GS-FLX using Titanium chemistry by the Teagasc 454 Sequencing Platform. Resulting raw sequences reads were quality trimmed as previously described.
Trimmed FASTA sequences were then BLASTed (Altschul et al., 1997) against a previously published 16S-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 ribosomal sequence data (Urich et al., 2008). Phylum and family counts for each subject were extracted from MEGAN. Clustering and alpha diversities were generated with the MOTHUR software package (Schloss and Handelsman, 2008). Beta diversities and principle coordinate analysis (PCoA) of sequence reads were calculated using the Qiime suite of tools (Caporaso et al., 2010).

**Statistical analysis**

Results in the text, tables and figures are presented as Mean per group ± SEM. To assess if differences between treatment groups were significant, data were analysed using one-way analysis of variance (ANOVA) followed by post hoc Tukey’s multiple comparison tests using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA, USA). Statistical analyses of compositional data were performed using Minitab release 15.1.1.0 (www.minitab.com). The non-parametric Kruskal-Wallis test was used to estimate the relationships between different groups. Statistical significances were accepted at p<0.05, and trends for statistically significant differences were recognized at p<0.10.
Results

Survival and transit of *B. breve* DPC 6330 and *B. breve* NCIMB 702258 in C57BL/6 mice

Quantification of the numbers of the administered *B. breve* strains in the faeces of mice confirmed their gastrointestinal transit and survival. Stool recovery of *B. breve* DPC 6330 and *B. breve* NCIMB 702258 were approximately $1.1 \times 10^6$ CFU/g faeces and $8.2 \times 10^6$ CFU/g faeces after two weeks of feeding the respective strain and remained at similar numbers for week four and six. At week eight, there was a decline in the numbers of excreted *B. breve* strains, with stool recovery of *B. breve* DPC 6330 and *B. breve* NCIMB 702258 being $1.2 \times 10^5$ CFU/g faeces and $3.4 \times 10^5$ CFU/g faeces, in their respective groups.

At sacrifice, *B. breve* DPC 6330 and *B. breve* NCIMB 702258 were detected in the large intestine at $7 \times 10^5$ CFU/g content and $1 \times 10^6$ CFU/g content, respectively, in the mice administered the strains.

Dietary supplementation with *B. breve* NCIMB 702258, but not *B. breve* DPC 6330, increases visceral host fat storage

The weight of visceral body fat; the sum of epididymal, mesenteric and perirenal fat pads, was significantly higher in mice that were fed *B. breve* NCIMB 702258 compared to unsupplemented mice (1.71 ± 0.13 g vs. 1.20 ± 0.10 g; p<0.05, Table 1). This increase in visceral fat mass correlated with a tendency for greater circulating leptin for *B. breve* NCIMB 702258 supplemented mice compared with unsupplemented controls (5.2 ± 0.9 ng/mL vs. 2.4 ± 0.7 ng/mL; p=0.06, Table 2). There was no significant difference in body mass between the groups over the 8-week feeding period (Table 1). No effect on liver mass or liver triglycerides was observed following administration of *B. breve* NCIMB 702258. In contrast, administration of *B. breve* DPC 6330 was associated with a higher concentration of liver triglycerides compared to unsupplemented mice (p<0.05; Table 1). Neither of the administered *B.
breve strains affected the levels of circulating insulin, glucose or triglycerides, compared with unsupplemented mice (Table 2). In addition, no difference in feed intake was observed between the groups (data not shown).

Higher gut levels of SCFAs following administration of B. breve DPC 6330 and B. breve NCIMB 702258
Analysis of caecal SCFAs, the major fermentation end-products and source of energy for the host, showed that propionate was significantly higher in mice that received B. breve DPC 6330 and B. breve NCIMB 702258 compared with unsupplemented mice (p<0.05; Table 3). Higher concentrations of iso-butyrate were also observed in mice supplemented with B. breve NCIMB 702258 compared with unsupplemented controls (p<0.05; Table 3). In addition, the mean total SCFA concentration (acetate, propionate, butyrate, iso-butyrate) detected in the caecal contents was 24% higher in mice fed B. breve DPC 6330 compared with unsupplemented controls (p=0.07; Table 3).

Contrasting effects on host tissue fatty acid composition of dietary supplementation with B. breve DPC 6330 and B. breve NCIMB 702258
To investigate the effects of dietary supplementation with B. breve DPC 6330 and B. breve NCIMB 702258 on fatty acid composition of host tissues, fatty acid profiling was performed on brain, epididymal adipose tissue and liver. Significant differences in tissue fatty acid composition were observed following supplementation with the B. breve strains (Table 4, Table 5 and Table 6). Mice that were administered B. breve NCIMB 702258 had significantly higher stearic acid (C18:0), arachidonic acid (ARA, C20:4n-6) and docosahexaenoic acid (DHA, C22:6n-3) in brain compared with both unsupplemented mice (p<0.05) and mice supplemented with B. breve DPC 6330 (p<0.05; Table 4), whereas dietary supplementation with B. breve DPC 6330 resulted in higher
myristic acid (C14:0) in brain compared with both unsupplemented mice and mice supplemented with *B. breve* NCIMB 702258 (p<0.05; Table 4). However, both groups of *B. breve* supplemented mice exhibited significantly lower palmitic acid (C16:0), palmitoleic acid (C16:1c9) and dihomo-γ-linolenic acid (C20:3n-6) in brain compared with unsupplemented controls (p<0.05; Table 4).

Mice that received *B. breve* DPC 6330 exhibited significantly higher myristic acid, palmitic acid, palmitoleic acid and DHA in epididymal adipose tissue compared to unsupplemented mice (p<0.05; Table 5). In contrast, these fatty acids were not higher in epididymal adipose tissue of the mice supplemented with *B. breve* NCIMB 702258. Mice that received *B. breve* DPC 6330 also had significantly lower oleic acid (C18:1c9) in epididymal adipose tissue compared with both unsupplemented mice and mice supplemented with *B. breve* NCIMB 702258 (p<0.05; Table 5). In contrast to a higher concentration of DHA in epididymal adipose tissue of mice fed *B. breve* DPC 6330, these mice had a lower concentration of DHA in liver compared with unsupplemented mice (p<0.05; Table 6). There was a tendency for higher concentrations of c9, t11 CLA in the livers of mice receiving *B. breve* NCIMB 702258 compared to mice supplemented with *B. breve* DPC 6330 and unsupplemented controls (0.031 ± 0.006 g/100g FAME vs. 0.015 ± 0.007 g/100g FAME, and 0.013 ± 0.006 g/100g FAME, respectively; p=0.06).

**Contrasting effects on gut microbiota composition of dietary supplementation with *B. breve* NCIMB 702258 and *B. breve* DPC 6330**

At the end of the 8 week study, the microbial composition of the gut microbiota of individual mice was elucidated through high-throughput pyrosequencing (Roche-454 Titanium) of 16S rRNA (V4) amplicons generated from DNA extracted from the caecal content. A total of 103,711 reads were sequenced, averaging at 4509 reads per animal. Species-richness, coverage, and diversity estimations were calculated for each
data set (Table S1). At the 97% similarity level, the Shannon index, a metric for community diversity, revealed a high level of overall biodiversity within all samples with values exceeding 5.1. The Good's coverage at the 97% similarity level ranged between 88-95% for all the datasets. The Chao1 richness also indicated a sufficient level of overall diversity (Table S1). Rarefaction curves for each dataset were parallel or approaching parallel with the x axis, indicating that the total bacterial diversity present within these is well represented and that additional sampling would yield a limited increase in species richness (Figure S1).

In agreement with previous work (Ley et al., 2005, Ley et al., 2006, Turnbaugh et al., 2006), taxonomy-based analysis showed that at the phylum level, the mouse gut microbiota was dominated by *Firmicutes* and *Bacteroidetes* (together harbouring on average 94% of sequences; Figure 1). At the family level, the most dominant groups were *Bacteroidaceae, Clostridiaceae, Eubacteriaceae* and *Lactobacillaceae* (Figure 2).

A comparison of the composition of the microbiota of *B. breve* NCIMB 702258 supplemented mice, *B. breve* DPC 6330 supplemented mice and unsupplemented mice showed that administration of these strains altered the composition of the gut microbiota differently at both phylum, family and genus levels. There was a tendency for a reduction in the *Firmicutes* population from 74% in the unsupplemented mice to 68% in *B. breve* NCIMB 702258 supplemented mice and 67% in *B. breve* DPC 6330 fed mice (p=0.08, Figure 1). All other phyla remained at relatively similar proportions across the groups. At the family level, administration of both *B. breve* DPC 6330 and *B. breve* NCIMB 702258 resulted in a significantly lower proportion of *Lachnospiraceae* compared with unsupplemented mice (p<0.05, Figure 2). Furthermore, *Eubacteriaceae* tended to be less abundant in the caecum of mice supplemented with *B. breve* DPC 6330 compared with both *B. breve* NCIMB 702258 supplemented mice and unsupplemented controls (3% vs. 12% vs. 13%; p=0.06, Figure 2). Other observed differences at the family level included
a tendency for an apparent enrichment in the *Clostridiaceae* population in *B. breve* DPC 6330 supplemented mice (25%) compared with *B. breve* NCIMB 702258 supplemented mice (12%) and unsupplemented mice (18%, p=0.08, Figure 2). At the genus level, *Eubacterium* tended to be lower following dietary supplementation with *B. breve* DPC 6330 compared with no supplementation and supplementation with *B. breve* NCIMB 702258 (p=0.06, Figure 3).

PCoA generated using unweighted UNIFRAC distances demonstrated that mice clustered into relatively distinct groups based on whether they received *B. breve* DPC 6330, *B. breve* NCIMB 702258 or no supplementation. Mice that received *B. breve* DPC 6330 (illustrated as triangles, Figure 4) clustered closely together and showed a clear separation from the unsupplemented mice (illustrated as squares, Figure 4) and *B. breve* NCIMB 702258 supplemented mice (illustrated as circles, Figure 4). This suggests that supplementation with *B. breve* DPC 6330 had a greater effect on the gut microbiota than supplementation with *B. breve* NCIMB 702258.
Discussion
This study demonstrates that the response of fatty acid metabolism to administration of bifidobacteria is strain-dependent and furthermore highlights the importance of strain-strain differences in modifying the gut microbiota composition of the host. Administration of *B. breve* NCIMB 702258 increased visceral fat mass and weight gain in C57BL/6 mice, whereas administration of *B. breve* DPC 6330 did not. In general, beneficial effects have been attributed to *Bifidobacterium* in connection with obesity and weight management and reports support the preventative role of bifidobacteria in promoting fat mass development and body weight gain (Cani et al., 2007, Collado et al., 2008, Kalliomäki et al., 2008). However, some studies contradict the preventative role of *Bifidobacterium* in body weight gain (Furet et al., 2010, Santacruz et al., 2009) and question the role of specific *Bifidobacterium* species in obesity and weight management (Santacruz et al., 2009). Since the *Bifidobacterium* genus is complex, it is possible that certain bifidobacterial species, or even strains, as observed in the present study, promote fat mass storage and body weight more efficiently than others through diverse regulatory roles in fat absorption and distribution. Notably, strains of *B. breve* have previously been shown to improve weight gain in very low birth weight infants (Kitajima et al., 1997). However, it must be acknowledged that this study is limited to two strains of *B. breve*, therefore further studies are required to draw conclusions about the role of specific *Bifidobacterium* species and strains in fat/energy absorption and their impact on weight gain in animal models of obesity and in humans.

Previous studies have demonstrated that the gut microbiota affects the composition and quality of fat in the host, as well as the quantity (Velagapudi et al., 2010). We have demonstrated that manipulation of the gut microbiota by administering a single metabolically active strain, *B. breve* NCIMB 702258, to different animal species influenced the composition of bioactive fatty acids in host liver and adipose tissue (Wall
et al., 2009). In the present study, we compared the impact of *B. breve* NCIMB 702258 and *B. breve* DPC 6330 on fatty acid composition of different host tissues in mice. We observed that administration of these *B. breve* strains altered the fatty acid composition in distinct ways. While administration of *B. breve* DPC 6330 had a greater influence on the fatty acid composition of epididymal adipose tissue, with higher palmitic acid, palmitoleic acid and DHA, administration of *B. breve* NCIMB 702258 had a greater impact on the fatty acid composition of brain. Intriguingly, mice that were supplemented with *B. breve* NCIMB 702258 demonstrated significantly higher concentrations of ARA and DHA in brain compared with both *B. breve* DPC 6330 fed mice and unsupplemented mice. This observation of increased ARA and DHA in the brain of mice administered with *B. breve* NCIMB 702258 is consistent with our previous findings using a different model (Wall et al., 2010). ARA and DHA play important roles in neurogenesis, neurotransmission, and protection against oxidative stress (Innis, 2007, Maekawa et al., 2009) and their concentrations in the brain influences cognitive processes such as learning and memory (Henriksen et al., 2008, Yurko-Mauro et al., 2010). The importance of these fatty acids in neurodevelopment has led to their supplementation in infant formula (Efsa, 2009). The present study supports our previous observations (Wall et al., 2010, Wall et al., 2009), and findings by others (Kankaanpää et al., 2002, Kaplas et al., 2007), in which manipulation of the gut microbiota alter the composition of fat in the host. Interactions between fatty acids and members of the gut microbiota might affect the biological roles of both and such interactions may therefore result in physiological consequences for the host.

The mechanism by which these ingested strains mediate the changes in fatty acid composition observed in the present study is uncertain and remains to be elucidated. Possible explanations include modulations of fat absorption processes in the small intestine and/or desaturase activities involved in the metabolism of fatty acids to the longer chain unsaturated derivatives caused either directly by the strains administered
or by alterations in the gut microbiota. Interestingly, a previous study in lactating goats demonstrated that administration of a Lactobacillus plantarum strain resulted in changes in the faecal microbiota and modulated the milk fatty acid composition with a higher content of polyunsaturated fatty acids (Maragkoudakis et al., 2010). Furthermore, a recent study reported that dietary supplementation of lactating women with Lactobacillus rhamnosus GG and Bifidobacterium lactis Bb12 in combination with rapeseed oil resulted in higher gamma-linolenic acid of breast milk, compared with fatty acid-supplementation alone (Hoppu et al., 2012).

The present study demonstrated not only significant differences in the composition of the gut microbiota between B. breve-fed mice and non-B. breve-fed mice but also differences between mice fed different B. breve strains. At the phylum level, the Firmicutes population tended to be less abundant in the caecum of mice supplemented with B. breve DPC 6330 and B. breve NCIMB 702258 compared with unsupplemented mice. At the family level, the proportions of Eubacteriaceae were reduced in the animals receiving B. breve DPC 6330, but not in those receiving B. breve NCIMB 702258, compared with unsupplemented controls. In addition, administration of B. breve DPC 6330 lead to an apparent enrichment in Clostridiaceae compared to the unsupplemented mice, whereas this enrichment was absent in B. breve NCIMB 702258 supplemented mice. Penicillium were found to be present in the B. breve NCIMB 702258 supplemented mice, however this genus is not regarded as typical members of the gut microbiota, and factors contributing to their presence in the gut as found in this instance require further investigation. PCoA analysis demonstrated that mice administered with B. breve DPC 6330 had a more divergent clustering pattern and were separated from the unsupplemented mice and from mice fed B. breve NCIMB 702258. This suggests that supplementation of B. breve DPC 6330 has a greater impact on the composition of the murine gut microbiota than supplementation with B. breve NCIMB 702258. Our observations suggest
that administration of a single strain can play a role in determining the composition of gut bacterial populations in vivo and furthermore that strain-strain differences are important factors with respect to modulation of the gut microbial community. Although changing the intestinal microbiota may be more difficult in free-living individuals compared to laboratory models, it is important to perform extensive microbiota-related studies on bifidobacteria-associated probiotics in human trials, especially in groups where shifts in the composition have been observed due to the state of physiology (i.e. obesity, diabetes etc), particularly since an alteration of the gut microbiota at lower taxonomic levels is still likely to have important functional consequences for the host. While it is acknowledged that pyrosequencing of the 16S rRNA genes, as undertaken in this study, did not provide quantitative population data, it did yield an overview of the effects of administration of these strains on the entire microbial population.

SCFAs (acetate, propionate, butyrate, iso-butyrate) are major products from fermentation processes from gut microbiota of non-digestible carbohydrates in the intestine. These fatty acids have recently attracted significant interest because of their positive effect on human gastrointestinal health and diseases including colon cancer, gastrointestinal infections and inflammatory bowel disease (D'argenio et al., 1996, Emenaker et al., 2001, Galvez et al., 2005, Topping and Clifton, 2001). SCFAs are also believed to be a driver of energy sparing and are portrayed as a potential mechanism involved in the increase of fat mass storage in microbiota-bearing mice (Turnbaugh et al., 2006). The differences in stool SCFA concentrations between lean and obese people have been considerable. The mean total SCFA concentration in faecal samples of obese volunteers was shown to be more than 20% higher compared with lean volunteers, with the highest increase observed for propionate being 41% higher in obese volunteers (Schwiertz et al., 2010). Administration of B. breve DPC 6330 and B. breve NCIMB 702258 led to an increase in propionate in the caecum, being 37% higher in mice
supplemented with *B. breve* DPC 6330 and 30% higher in mice supplemented with *B. breve* NCIMB 702258 compared with unsupplemented mice. Noteworthy, neither of these *B. breve* strains produce propionate *in vitro*, suggesting that administration of these strains may result in an increase of propionate-producing bacteria in the GIT. Well-known propionate producers belong to the genera *Bacteroides* spp., *Prevotella* spp., and *Propionibacterium* spp. (Hosseini et al., 2011, Schwertz et al., 2010). However, since many metabolic properties are shared between lower microbial taxa, it is difficult to link the capacity of producing specific SCFAs, such as propionate, to phylogenetic information obtained in the present study (Zoetendal et al., 2008). Increases in propionate in the *B. breve*-fed mice could also be due to cross-feeding, in which one bacterial species metabolises the fermentation products of another, thus producing a different end product, a common cooperation in a complex microbial community. Indeed, *Bifidobacterium* are known to produce lactate, ethanol and succinate (Macfarlane and Macfarlane, 2003, Van Der Meulen et al., 2006), all of which could be used as substrates for the production of propionate by other bacteria such as *Bacteroides* spp., *Propionibacterium* spp. and *Clostridium propionicum* in a sequential fermentation, thus increasing the propionate concentration in the gut (Hosseini et al., 2011, Stams et al., 1998).

In conclusion, our results demonstrate that the impact of bifidobacteria on host fatty acid metabolism is dependent on the strain administered and that strain-strain differences are important factors with respect to modulation of the gut microbial community by ingested microorganisms. Further studies are needed to draw conclusions about the role of specific *Bifidobacterium* species and strains in obesity and weight management.
References


Turnbaugh, P. J., Hamady, M., Yatsunenko, T., Cantarel, B. L., Duncan, A., Ley, R. E., Sogin, M. L., Jones, W. J., Roe, B. A. & Affourtit, J.


Table 1 Body mass, fat mass, liver mass and liver triglyceride levels of mice fed *B. breve* DPC 6330, *B. breve* NCIMB 702258 and unsupplemented diet for 8 weeks

<table>
<thead>
<tr>
<th></th>
<th><em>B. breve</em> DPC 6330 fed mice</th>
<th><em>B. breve</em> NCIMB 702258 fed mice</th>
<th>Unsupplemented mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial weight (g)</td>
<td>26.5 ± 0.6</td>
<td>26.7 ± 0.6</td>
<td>25.4 ± 0.5</td>
</tr>
<tr>
<td>Final weight (g)</td>
<td>31.7 ± 0.8</td>
<td>33.4 ± 0.7</td>
<td>30.6 ± 0.8</td>
</tr>
<tr>
<td>Weight gain (%)</td>
<td>20.3 ± 4.5</td>
<td>25.1 ± 3.7</td>
<td>20.5 ± 3.1</td>
</tr>
<tr>
<td>Visceral fat mass (g)</td>
<td>1.51 ± 0.16&lt;sup&gt;A,B&lt;/sup&gt;</td>
<td>1.71 ± 0.13&lt;sup&gt;A&lt;/sup&gt;</td>
<td>1.20 ± 0.10&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>Liver mass (g)</td>
<td>1.64 ± 0.05</td>
<td>1.55 ± 0.06</td>
<td>1.47 ± 0.05</td>
</tr>
<tr>
<td>Liver triglycerides (mg/g)</td>
<td>4.64 ± 0.73&lt;sup&gt;A&lt;/sup&gt;</td>
<td>3.44 ± 0.29&lt;sup&gt;A,B&lt;/sup&gt;</td>
<td>2.93 ± 0.14&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Visceral fat mass include epididymal, perirenal, and mesenteric fat pads.

Results are expressed as Means ± SEM, n = 8 mice/group. Means not sharing a common superscript letter are significantly different at p<0.05 based on ANOVA followed by post hoc Tukey’s multiple comparison tests.
Table 2 Serum parameters in mice fed *B. breve* DPC 6330, *B. breve* NCIMB 702258 and unsupplemented diet for 8 weeks

<table>
<thead>
<tr>
<th>Serum parameter</th>
<th><em>B. breve</em> DPC 6330 fed mice</th>
<th><em>B. breve</em> NCIMB 702258 fed mice</th>
<th>Unsupplemented mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>78.1 ± 12.8</td>
<td>56.2 ± 11.8</td>
<td>61.3 ± 8.2</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>217.6 ± 17.6</td>
<td>186.0 ± 6.4</td>
<td>183.3 ± 2.8</td>
</tr>
<tr>
<td>Insulin (ng/mL)</td>
<td>1.4 ± 0.2</td>
<td>2.5 ± 0.8</td>
<td>1.7 ± 0.3</td>
</tr>
<tr>
<td>Leptin (ng/mL)</td>
<td>3.9 ± 0.8</td>
<td>5.2 ± 0.9</td>
<td>2.4 ± 0.7</td>
</tr>
</tbody>
</table>

Blood samples were collected after fasting. Results are expressed as means ± SEM, n = 8 mice/group. No significant differences were observed. \(^{1}p=0.06\), based on ANOVA followed by post hoc Tukey’s multiple comparison tests.
Table 3 SCFA concentrations (μmol/g) of caecum content

<table>
<thead>
<tr>
<th>SCFAs</th>
<th>B. breve DPC 6330 fed mice</th>
<th>B. breve NCIMB 702258 fed mice</th>
<th>Unsupplemented mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
<td>48.05 ± 3.58</td>
<td>45.05 ± 2.73</td>
<td>40.71 ± 2.43</td>
</tr>
<tr>
<td>Propionate</td>
<td>9.80 ± 0.51&lt;sup&gt;A&lt;/sup&gt;</td>
<td>9.28 ± 0.49&lt;sup&gt;A&lt;/sup&gt;</td>
<td>7.14 ± 0.40&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>Butyrate</td>
<td>18.24 ± 2.67</td>
<td>13.44 ± 1.45</td>
<td>13.71 ± 1.59</td>
</tr>
<tr>
<td>Iso-butyrate</td>
<td>0.80 ± 0.09&lt;sup&gt;A, B&lt;/sup&gt;</td>
<td>0.88 ± 0.05&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.67 ± 0.02&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total acids</td>
<td>76.88 ± 6.39</td>
<td>68.65 ± 4.03</td>
<td>62.23 ± 4.14</td>
</tr>
</tbody>
</table>

Results are expressed as Means ± SEM; B. breve DPC 6330 (n=8), B. breve NCIMB 702258 (n=7), unsupplemented mice (n=8). Means not sharing a common superscript letter are significantly different at p<0.05 based on ANOVA followed by post hoc Tukey’s multiple comparison tests.
Table 4 Fatty acid profile in brain of mice fed *B. breve* DPC 6330, *B. breve* NCIMB 702258 or unsupplemented diet for 8 weeks

<table>
<thead>
<tr>
<th>FAME</th>
<th>Brain</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>g/100g</td>
<td><em>B. breve</em> DPC 6330</td>
<td><em>B. breve</em> NCIMB 702258</td>
<td>Unsupplemented mice</td>
<td></td>
</tr>
<tr>
<td>FAME</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C14:0</td>
<td>3.66 ± 0.21&lt;sup&gt;A&lt;/sup&gt;</td>
<td>1.36 ± 0.05&lt;sup&gt;B&lt;/sup&gt;</td>
<td>1.90 ± 0.08&lt;sup&gt;C&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>C16:0</td>
<td>22.34 ± 0.80&lt;sup&gt;A&lt;/sup&gt;</td>
<td>21.46 ± 0.18&lt;sup&gt;A&lt;/sup&gt;</td>
<td>26.75 ± 0.45&lt;sup&gt;B&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>C16:1c9</td>
<td>0.69 ± 0.03&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.72 ± 0.03&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.90 ± 0.04&lt;sup&gt;A&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>C18:0</td>
<td>10.80 ± 0.18&lt;sup&gt;A&lt;/sup&gt;</td>
<td>11.99 ± 0.15&lt;sup&gt;B&lt;/sup&gt;</td>
<td>11.08 ± 0.16&lt;sup&gt;A&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>C18:1c9</td>
<td>9.83 ± 0.17&lt;sup&gt;A&lt;/sup&gt;</td>
<td>10.48 ± 0.07&lt;sup&gt;B&lt;/sup&gt;</td>
<td>10.22 ± 0.13&lt;sup&gt;A,B&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>C18:2&lt;sup&gt;n-6&lt;/sup&gt;</td>
<td>0.49 ± 0.02&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.58 ± 0.02&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.49 ± 0.01&lt;sup&gt;A&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>C18:3&lt;sup&gt;n-9&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
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<tr>
<td>C18:3&lt;sup&gt;n-6&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>C20:3&lt;sup&gt;n-6&lt;/sup&gt;</td>
<td>0.33 ± 0.01&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.37 ± 0.01&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.27 ± 0.01&lt;sup&gt;B&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>C20:4&lt;sup&gt;n-6&lt;/sup&gt;</td>
<td>5.70 ± 0.31&lt;sup&gt;A&lt;/sup&gt;</td>
<td>6.55 ± 0.07&lt;sup&gt;B&lt;/sup&gt;</td>
<td>5.27 ± 0.07&lt;sup&gt;A&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>C20:5&lt;sup&gt;n-3&lt;/sup&gt;</td>
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<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>C22:5&lt;sup&gt;n-3&lt;/sup&gt;</td>
<td>0.24 ± 0.04</td>
<td>0.21 ± 0.04</td>
<td>0.20 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>C22:6&lt;sup&gt;n-3&lt;/sup&gt;</td>
<td>17.22 ± 0.35&lt;sup&gt;A&lt;/sup&gt;</td>
<td>18.58 ± 0.32&lt;sup&gt;B&lt;/sup&gt;</td>
<td>17.19 ± 0.10&lt;sup&gt;A&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

Results are expressed as percentage of total identified fatty acids. Data are Means ± SEM g/100g FAME, n = 8 mice/group. Means not sharing a common superscript letter are significantly different at p<0.05 based on ANOVA followed by post hoc Tukey’s multiple comparison tests. FAME = fatty acid methyl esters. C14:0 myristic acid; C16:0 palmitic acid; C16:1c9 palmitoleic acid; C18:0 stearic acid; C18:1c9 oleic acid; C18:2n-6 linoleic acid; C18:3n-3 linolenic acid; C18:3n-6 γ-linolenic acid; C20:3n-6 dihomo-γ-linolenic acid; C20:4n-6 arachidonic acid; C20:5n-3 eicosapentaenoic acid; C22:5n-3 docosapentaenoic acid; C22:6n-3 docosahexaenoic acid. ND; not detected.
Table 5 Fatty acid profile in epididymal adipose tissue of mice fed with *B. breve* DPC 6330, *B. breve* NCIMB 702258 or unsupplemented diet for 8 weeks

<table>
<thead>
<tr>
<th>FAME</th>
<th>Epididymal Adipose tissue</th>
<th>B. breve DPC 6330</th>
<th>B. breve NCIMB 702258</th>
<th>Unsupplemented mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Average ± SEM g/100g</td>
<td>Average ± SEM g/100g</td>
<td>Average ± SEM g/100g</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C14:0</td>
<td>1.96 ± 0.24^A</td>
<td>1.44 ± 0.27^A,B</td>
<td>1.10 ± 0.15^B</td>
<td></td>
</tr>
<tr>
<td>C16:0</td>
<td>15.19 ± 0.94^A</td>
<td>12.78 ± 0.68^A,B</td>
<td>10.68 ± 0.52^B</td>
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<tr>
<td>C16:1c9</td>
<td>4.31 ± 0.39^A</td>
<td>3.75 ± 0.53^A,B</td>
<td>2.85 ± 0.12^B</td>
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</tr>
<tr>
<td>C18:0</td>
<td>2.04 ± 0.13</td>
<td>2.26 ± 0.12</td>
<td>2.31 ± 0.03</td>
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<tr>
<td>C18:1c9</td>
<td>28.71 ± 0.92^A</td>
<td>32.22 ± 0.89^B</td>
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<tr>
<td>C18:2n-6</td>
<td>33.57 ± 1.18</td>
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</tr>
<tr>
<td>C18:3n-3</td>
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</tr>
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<td>0.42 ± 0.02</td>
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</tr>
<tr>
<td>C20:4n-6</td>
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<td>C20:5n-3</td>
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<td>C22:6n-3</td>
<td>0.77 ± 0.04^A</td>
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Results are expressed as percentage of total identified fatty acids. Data are Means ± SEM g/100g FAME, n = 8 mice/group. Means not sharing a common superscript letter are significantly different at p<0.05 based on ANOVA followed by post hoc Tukey’s multiple comparison tests.
Table 6 Fatty acid profile in liver of mice fed *B. breve* DPC 6330, *B. breve* NCIMB 702258 or unsupplemented diet for 8 weeks

<table>
<thead>
<tr>
<th>FAME</th>
<th><strong>Liver</strong></th>
<th><strong>B. breve DPC 6330</strong></th>
<th><strong>B. breve NCIMB 702258</strong></th>
<th><strong>Unsupplemented mice</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>g/100g</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FAME</td>
<td></td>
<td><strong>C14:0</strong></td>
<td><strong>C16:0</strong></td>
<td><strong>C16:1c9</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.65 ± 0.08</td>
<td>0.67 ± 0.03</td>
<td>0.67 ± 0.05</td>
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<tr>
<td></td>
<td></td>
<td>21.55 ± 0.56</td>
<td>19.40 ± 0.84</td>
<td><strong>19.07 ± 0.31</strong></td>
</tr>
<tr>
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<td>0.38 ± 0.03</td>
<td>0.42 ± 0.03</td>
<td>0.41 ± 0.02</td>
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Results are expressed as percentage of total identified fatty acids. Data are Means ± SEM g/100g FAME, n = 8 mice/group. Means not sharing a common superscript letter are significantly different at p<0.05 based on ANOVA followed by post hoc Tukey’s multiple comparison tests. FAME = fatty acid methyl esters.
Figure 1  Phylum-level distributions of the microbial communities present in the caecum content, expressed as percentage of total population of assignable tags. *B. breve* DPC 6330 (n = 7), *B. breve* NCIMB 702258 (n = 7), unsupplemented mice (n = 8). No significant differences were observed.
Figure 2  Family-level taxonomic distributions of the microbial communities in caecum content, expressed as percentage of total tags assignable at family level. *p<0.05, determined by Kruskal-Wallis test.
Figure 3 Genus-level taxonomic distributions of the microbial communities present in the caecum content, expressed as percentage of total tags assignable at genus level. *B. breve* DPC 6330 (n = 7), *B. breve* NCIMB 702258 (n = 7), unsupplemented mice (n = 8). No significant differences were observed.
Figure 4 Principle coordinate analysis using unweighted UniFrac distances. *B. breve* DPC 6330 (n = 7), *B. breve* NCIMB 702258 (n = 7), unsupplemented mice (n = 8)
Appendix 1

Supplementary information
Table S1  Species-richness, coverage, and diversity estimations for each data set

<table>
<thead>
<tr>
<th>Data set</th>
<th>D1</th>
<th>D2</th>
<th>D3</th>
<th>D4</th>
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<td>88%</td>
<td>93%</td>
<td>89%</td>
<td>88%</td>
<td>82%</td>
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<table>
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<td>97%</td>
<td>98%</td>
<td>97%</td>
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<td>97%</td>
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<tr>
<td>Chao2 richness estimation</td>
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<td>1759</td>
<td>1013</td>
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<td>932</td>
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<td>6.2</td>
<td>5.8</td>
<td>6.2</td>
<td>5.3</td>
<td>5.8</td>
<td>5.6</td>
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<tr>
<td>Good’s coverage</td>
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<td>88%</td>
<td>94%</td>
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<td>90%</td>
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<td>92%</td>
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<tr>
<td>Similarity</td>
<td>97%</td>
<td>98%</td>
<td>97%</td>
<td>98%</td>
<td>97%</td>
<td>98%</td>
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<tr>
<td>Chao2 richness estimation</td>
<td>1062</td>
<td>1759</td>
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<td>Shannon’s index for diversity</td>
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<td>6.3</td>
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<td>Good’s coverage</td>
<td>94%</td>
<td>90%</td>
<td>93%</td>
<td>88%</td>
<td>95%</td>
<td>91%</td>
<td>93%</td>
<td>89%</td>
</tr>
</tbody>
</table>
Figure S1 Rarefaction curves of samples sequenced by high-throughput DNA sequencing. Rarefaction curves at 97% similarity indicated that the total bacterial diversity present was well represented. Number of operational taxonomic units (OTUs) identified as a function of the number of sequence tags sampled.

Please note that Appendix 2 & Appendix 3 (pp.366-416) are unavailable due to a restriction requested by the author.
Discussion
The threat posed to human health by antibiotic resistance cannot be overstated. Antibiotics are fundamental to our ability to treat common bacterial infections and are also central to the success of surgical procedures, chemotherapy and organ transplantation (Penders et al., 2013). Intensive investigations into antibiotic resistance reservoirs are underway, with the aim of minimising the further dissemination of resistance genes, thereby enabling the continued application of the remaining effective antibiotics. Given the complexity of the human gut microbiota, as well as its exposure to antibiotics, there is every opportunity for the gut microbiota to develop or acquire antibiotic resistance. To fully appreciate the likelihood that gut microbes will develop antibiotic resistance, one must first understand the effects antibiotics have on gut bacteria. This topic was reviewed with respect to infants in chapter 1 and was the focus of the first study described in this thesis.

Our initial study was designed as a follow up to an existing study (Hussey et al., 2011), in which infants treated with ampicillin and gentamicin were shown to have significantly reduced bifidobacteria colonization and species diversity. Unlike this previous DGGE-based study, our aim was to take advantage of high-throughput 454-pyrosequencing, to identify the effects of ampicillin and gentamicin treatment on the entire infant gut microbiota. Research at the time suggested that antibiotics cause significant alterations to infant gut microbiota (Bennet et al., 2002, Favier et al., 2003, Mangin et al., 2010), though no sequencing-based studies
had been carried out to investigate this phenomenon. Currently, due to the length of the 16S rRNA amplicons generated, accurate assignment at species level cannot be completed. Due to our interest in comparing our results to those detected in the earlier study, regarding the effects of these antibiotics on bifidobacteria species, we not only amplified and sequenced the V4 region of the 16S rRNA gene, but also the \textit{rpoB} gene, which enabled us to determine which bifidobacteria species were present (Kim et al., 2010).

The results of this study demonstrate the significant dominance of the \textit{Proteobacteria}, at the expense of members of the \textit{Actinobacteria} (most notably \textit{Bifidobacterium}) following short-term antibiotic exposure. Though only treated with antibiotics for between 2 and 9 days, the infant gut was dramatically altered, with the effects still being observed 8 weeks after treatment ceased. Thus, even short-term antibiotic treatment can cause significant perturbations to the gut microbiota, which are slow to resolve. While previous studies have demonstrated the resilience of the adult human gut microbiota (De La Cochetiere et al., 2005), here we have shown that for a period of at least 8 weeks after antibiotic therapy, the infant gut microbiota remains altered. A concern when interpreting high-throughput sequencing data is whether the results relate to changes in absolute numbers or relative proportions of bacteria. As the sequences represent proportions of sequencing reads assigned to a particular phylum, family or genus, it is important to consider if the total bacteria numbers have decreased. To address this, we conducted qPCR analysis
on copies of the 16S rRNA gene/g faeces and noted no difference in total bacteria numbers following antibiotic therapy and thus our results represent actual increases/decreases in bacteria following antibiotic therapy. Using the rpoB sequences, we noted that overall bifidobacteria diversity was decreased 4 and 8 weeks after treatment, though some species, namely B. longum, appeared more resistant to antibiotic therapy compared to others.

While this study represents an important insight into the short-term effects of antibiotic treatment as identified using high-throughput sequencing, there is a need for longer follow up trials to determine the duration of the dysbiosis in gut microbiota. It has been suggested that early life exposure to antibiotics results in an increased risk of atopic disease, allergies and obesity (Ajslev et al., 2011, Foliaki et al., 2009, Marra et al., 2009, Trasande et al., 2012), and hence understanding the duration for which the perturbations appear in the infant gut, compared to healthy controls, is vital. Importantly, sequencing-based approaches to identify the bacteria that are most affected following antibiotic treatment, such as those presented here, afford the opportunity to develop approaches (such as probiotic therapy) with the aim of minimising the negative effects on gut microbiota during and after antibiotic exposure.

The findings from our initial infant study highlighted the ability of certain bacteria populations to become dominant following antibiotic exposure. Previous studies have also demonstrated such trends (Antonopoulos et
al., 2009, Murphy et al., 2013). To investigate if the gut naturally harbours antibiotic resistant populations, enabling such bacteria to become dominant upon exposure to antibiotics, we conducted a study investigating the presence of antibiotic resistance genes in the gut microbiota of healthy adult males. Following on from the previous study, aminoglycoside and β-lactam resistance genes were specifically targeted. Using a PCR-based approach, we detected genes encoding enzymes for the modification of aminoglycosides (including acetylation, adenylation and phosphorylation enzymes) and β-lactamases. Thus, it was evident that the human gut microbiota is a source of antibiotic resistance, which supports previous findings generated using alternative approaches (Cheng et al., 2012, Sommer et al., 2009). Our approach of using direct PCR analysis on metagenomic DNA, without screening for resistant isolates, is a rapid and cost-effective method that is suitable for initial investigations of any environment for genes of interest. Though not applied in our study, this initial PCR analysis can be combined with lab-scale high-throughput sequencing platforms such as the Ion Personal Genome Machine or Illumina MiSeq, to provide additional insights into genes and their sources.

In the majority of cases the detected genes shared closest homology with genes found in members of the Enterobacteriaceae family, which is consistent with the findings of our infant study in which these bacteria became dominant following exposure to aminoglycosides and β-lactams. Given that many of the detected β-lactamase genes, including ESBLs
such as \textit{bla}_{\text{CTX-M}}, are plasmid-encoded, the potential exists for these to disseminate to commensals or to microbes transiently colonizing the gut. Thus, there is a concern that the gut microbiota, most often studied for its beneficial roles in health, could be contributing to antibiotic resistance. A recent CDC report on antibiotic resistance in the USA noted that an estimated 2 million people are infected with antibiotic resistant bacteria annually, resulting in an estimated 23,000 deaths. Of these, they estimate that 26,000 infections and 1,700 deaths are due to ESBL producing-\textit{Enterobacteriaceae}. Thus, understanding the sources and the spread of such resistance genes is highly pertinent. Moreover, recognizing that such resistant populations exist in the human gut can help us to predict the effects of certain antibiotics on the gut microbiota and enable strategies to be developed to minimise such effects.

\textit{In silico} analysis is now routinely employed to identify potential sources of genes of interest. Following our initial study, we were interested in determining if a genetic mechanism of resistance could account for the variations in the response of different bifidobacteria species to aminoglycosides and \(\beta\)-lactams. Existing culture-based research suggested that bifidobacteria are sensitive to \(\beta\)-lactams and resistant to aminoglycosides (D'aimmo et al., 2007, Masco et al., 2006, Xiao et al., 2010, Yazid et al., 2001). Resistance to the aminoglycosides amongst \textit{Bifidobacterium} has been accepted as being intrinsic and due to a lack of a cytochrome-mediated mechanism (Bryan et al., 1979). However, such resistance would not explain the differences observed in the gut of the
aminoglycoside treated infants, whereby bifidobacteria species responded differently to aminoglycoside treatment. Through the use of in silico analysis, the widespread prevalence of putative aminoglycoside and β-lactam resistance proteins in the Bifidobacterium genus was evident. Notably, however, antimicrobial resistance assays failed to detect β-lactam resistance amongst the representative bifidobacteria that were tested, which contained in silico detected putative β-lactam resistance proteins. Hence we concluded that these proteins have been mis-annotated. As sequencing speed and efficiency increased, genome annotation moved from the time-intensive manual approach, to automated annotation. Several studies have highlighted the high frequency of mis-annotation which is now occurring as a result (Schnoes et al., 2009). The detection of such a prevalence of mis-annotated sequences amongst bifidobacteria highlights the need for these sequences to be correctly annotated to enable their true function to be determined.

With respect to the aminoglycosides, all of the tested bifidobacteria which contained putative aminoglycoside resistance proteins were found to be resistant. Therefore, our aim was to determine if the resistance proteins encode additional resistance, above intrinsic levels. Difficulties in the genetic manipulation of bifidobacteria have greatly hampered research into this genus (Sun et al., 2012). Recently, however, there have been several publications demonstrating the successful manipulation of genes in B. breve UCC2003 (Mazé et al., 2007, O’Connell Motherway et al.,
2013, O’Connell Motherway et al., 2009) via an insertional inactivation approach. Our aim was to use this novel approach to disrupt the 2 genes present in *B. breve* UCC2003, which encode putative aminoglycoside resistance proteins and to determine the effects on the resistance phenotype. A decreased resistance to aminoglycosides was observed, thereby demonstrating for the first time an enzymatic role in aminoglycoside resistance in bifidobacteria. Based on this, differences in aminoglycoside resistance genes present in various bifidobacteria could account for their individual response to aminoglycoside exposure. Moreover, bifidobacteria containing such resistance genes could be investigated as potential probiotics for use during aminoglycoside therapy, with the aim of minimising the effects of these antibiotics on gut bifidobacteria populations. The use of molecular approaches, such as those applied in this study, facilitate insights into the genetics of families of bacteria such as *Bifidobacterium*, which until now have remained somewhat elusive.

While high-throughput sequencing has significantly enhanced our ability to gain comprehensive insights into gut microbiota, metagenomic libraries/banks have also become an increasingly popular and valuable approach with respect to capturing and expressing genes from bacteria in complex environments. Initially this approach was used to investigate the soil as a source of novel genes (Torres-Cortés et al., 2011), providing considerable insights which culture-based approaches could not. Since these initial soil-based studies, metagenomic banks have been used to
investigate antibiotic resistance genes present in diverse environments from gull faeces to the gut microbiota of honey bees and humans (Martiny et al., 2011, Sommer et al., 2009, Tian et al., 2012). However, there have been very few studies in which the infant gut microbiota has been examined using metagenomic library-based approaches, with just 2 published when our study was conducted (De Vries et al., 2011, Moore et al., 2013). The first study demonstrated the transfer of antibiotic resistance genes from mother to infant (De Vries et al., 2011), while the second demonstrated that the infant gut contained bacteria expressing resistance genes to all major families of antibiotics (Moore et al., 2013). Our aim was to determine if aminoglycoside and β-lactam resistance genes are present in 6 month old infants with no antibiotic exposure. By 6 months of age, infants have been exposed to bacteria potentially containing resistance genes from maternal, environmental and dietary sources. This study thus provides insight into the development of the infant resistome devoid of antibiotic exposure.

The results clearly demonstrate that by 6 months of age, an infant resistome has already established. Use of a high-throughput robotic approach allowed us to screen our entire library to rapidly detect ampicillin and gentamicin resistant isolates. The resistance genes detected shared closest homology with genes present in bacteria from the Firmicutes and Proteobacteria phyla. However, we also detected genes sharing homology with those found in more obscure sources, including uncultured soil bacterium. These results highlight the
considerable insight that metagenomic libraries can provide with respect to resistance genes and the human gut. It should also be noted that while our focus was on aminoglycoside and β-lactam resistance, the metagenomic bank could be screened for resistance to all known families of antibiotics and indeed for any other phenotype of interest for which a culture-based, high-throughput screen exists, or can be developed. Thus, once created, metagenomic banks become an invaluable resource for future studies. Furthermore, it is evident that through investigations using metagenomic libraries, insights into previously unexplored resistance reservoirs can be gained.

While the primary focus of this thesis was antibiotics, antibiotic resistance and gut microbiota, we also performed studies on related topics regarding gut microbiota repopulation and manipulation. Short bowel syndrome (SBS) is a condition characterized by a significant reduction in the length of the bowel. While it can be congenital, in the majority of cases it occurs in infants as a result of the surgical removal of a large portion of the bowel, most often due to necrotizing enterocolitis. Culture-based research has consistently stated that short bowel syndrome results in increased faecal levels of *Lactobacillus* (Joly et al., 2010, Kaneko et al., 1997). Bowel resection results in reduced colon length, increased oxygen content, altered pH and altered nutrient availability. All of these factors make it highly probable that the gut microbiota will also be significantly altered. A comprehensive understanding of the gut microbiota present in infants with SBS could help explain the associated health effects.
including diarrhoea, weight loss and increased immunological inflammation (Sukhotnik et al., 2002). Our study took advantage of a recently developed piglet model of infants with SBS (Pereira-Fantini et al., 2011). The advantages of a piglet model lie not only in their physiological and immunological similarities to humans, but also in the fact that due to their lifecycle, you can study the human equivalent of infancy in a 2 week period.

Our study demonstrated increased *Acidaminococcus*, *Fusobacterium* and *Veillonellaceae* and decreased *Ruminococcaceae* at 2 and 6 weeks after surgery relative to controls. The detection of high levels of *Veillonellaceae* 6 weeks after resection is interesting and could relate to their particularly successful utilization of carbohydrates, the levels of which are increased in infants with SBS. Interestingly, contrary to existing research, significant changes in *Lactobacillus* levels in the SBS piglets were not detected in our study. Our results may differ from published research, as our approach enables the detection of changes to the entire gut microbiota, which culture-based approaches cannot achieve. Reductions in bacterial diversity were associated with increased inflammation 6 weeks post-surgery, with increased levels of pro-inflammatory cytokines IL-1β, IL-8, IL-18 and TNF-α. This study also investigated the effects of minor surgery on gut microbiota. While minor surgery was associated with some changes in gut microbiota, these were minor compared to the dramatic dysbiosis observed in the SBS piglets. We were conscious of the implications of the use of antibiotics in this trial, given their effects on the
gut microbiota. However, all piglets, including the controls, received the same treatment and the results clearly indicate that the effects of antibiotics could not account for the changes that were only observed in the SBS piglets. Furthermore, as antibiotic treatment occurs in infants with SBS, our results provide a true representation of the combined effects of surgery and antibiotic exposure, on the gut microbiota of infants with SBS. It has been suggested that the remaining portion of the colon adapts rapidly following resection. However, while this may be true of the physiological adaptations, significant changes to the gut microbiota 6 weeks post-operative were observed in this study, indicating prolonged dysbiosis in this condition.

While this thesis has demonstrated the significant dysbiosis that results in the gut as a result of antibiotics and surgery, we were also interested in investigating ways via which the gut might be repopulated. The human appendix has recently been suggested as a source of commensals capable of recolonizing the gut following e.g. antibiotic or diarrhoeal perturbations (Randal Bollinger et al., 2007). Such a theory is supported by the observations that individuals with ulcerative colitis or proctitis who underwent appendectomy exhibited a significant improvement in symptoms, potentially due to the lack of bacterial stimulation of the immune system in the absence of the appendix (Bolin et al., 2009). Despite the renewed interest in the human appendix, a paucity of research identifying the microbiota it contains existed. Our aim was to conduct the first study applying high-throughput sequencing to investigate
the microbiota of the human appendix. Seven appendices were studied and the results highlighted not only the inter-individual differences between the samples but also the diverse and complex microbiota present in the human appendix. It was found that Bacteroidetes, Proteobacteria and Fusobacteria were commonly detected though the Firmicutes were the dominant phylum. At family level, Lachnospiraceae, Enterobacteriaceae and Streptococcaceae were noted frequently. It was also interesting that we detected certain bacteria including Gemella and Deinococcus-thermus that are not usually associated with the gut microbiota. In one individual where the faecal microbiota was sequenced and compared to the microbiota of their appendix, it was interesting to note the higher microbial diversity present in the appendix compared to faeces. Overall our study provides the first comprehensive insight into the microbiota of an organ often regarded as an evolutionary vestige. Considering our results and the location of the appendix, it is highly probable that the appendix plays a role in host health, one which has yet to be fully elucidated.

Finally, with ever increasing interest in, and consumer demand for, probiotics, knowledge regarding the effects of different potential probiotic strains on the gut microbiota is vital. The effects of probiotics on the infant gut microbiota have been described in the literature review in chapter 1 of this thesis. One of the fundamental requirements for a probiotic bacterial strain is its ability to enable an increase in beneficial microbes, without increasing potential pathogens. Thus studies investigating the effects of
different strains of probiotics on gut microbiota are of increased importance. In this final study, 2 CLA-producing probiotic strains of *B. breve* (NCIMB 702258 and DPC 6330) were administered to mice and the effects on fatty acid metabolism, fat deposition and gut microbiota were studied. The high-throughput sequencing results demonstrate the strain-specific effects of these 2 probiotic strains on gut microbiota composition, which may relate to fatty acid metabolism. The insights provided by this study into the effects of different strains on gut microbiota demonstrates the need to carefully consider not just which probiotic species, but which strain, to use. As regulations surrounding probiotic use heightens, data regarding specific strains and their effects on host metabolism and gut microbiota will become increasingly important.

The area of molecular microbiology has been revolutionized over the past decade with high-throughput sequencing and molecular techniques that facilitate the creation of metagenomic banks, enabling unprecedented insights into the true microbial complexity of environments, not least the human gut. This thesis presents an insight into the harmful effects of antibiotics on the gut microbiota and the resistant bacterial populations inhabiting the human gut. These results act as a stark reminder of the ubiquitous nature of resistance genes that may be present in the infant gut from birth and can remain with us for life.
References


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