Antibiotic Resistance in the Gut Microbiota

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

By

Fiona Mary Fouhy BSc

Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland

School of Microbiology, University College Cork, Cork, Ireland.

February 2014

Research Supervisors:

Dr. Paul D. Cotter, Prof. Catherine Stanton, Prof. Gerald F. Fitzgerald, Prof. R. Paul Ross

Table of Contents

Declaration

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 (*bla*TEM, *bla*OXA, *bla*SHV, *bla*ROB, and *bla*CTX-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 *Ruminoccoccaceae, 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:

Fouhy F, Ogilvie LA, Jones BV, Ross RP, Fitzgerald GF, Stanton C, Cotter PD. (2014). Identification of aminoglycoside and β-lactam resistance genes from within an infant gut functional metagenomic library. Under review.

Fouhy F, Ross RP, Fitzgerald GF, Stanton C, Cotter PD. (2014). A degenerate PCR-based strategy as a means of identifying homologues of aminoglycoside and β-lactam resistance genes in the gut microbiota.

BMC Microbiology Feb 5; 14 (25) doi:10.1186/1471-2180-14-25

Fouhy F, O'Connell Motherway M, Fitzgerald GF, Ross RP, Stanton C, van Sinderen D, Cotter PD. (2013). *In silico* assigned resistance genes confer *Bifidobacterium* with partial resistance to aminoglycosides but not to β-lactams.

PLoS One. Dec 6;8(12):e82653. doi: 10.1371/journal.pone.0082653.

Lapthorne S, Pereira-Fantini PM, **Fouhy F**, Wilson G, Thomas SL, Dellios NL, Scurr M, O'Sullivan O, Ross RP, Stanton C, Fitzgerald GF, Cotter PD, Bines JE. (2013). Gut microbial diversity is reduced and is associated with colonic inflammation in a piglet model of short bowel syndrome.

Gut Microbes. May-Jun;4(3):212-21. doi: 10.4161/gmic.24372.

Guinane CM, Tadrous A, **Fouhy F**, Ryan CA, Dempsey EM, Murphy B, Andrews E, Cotter PD, Stanton C, Ross RP. (2013). Microbial composition of human appendices from patients following appendectomy. MBio. Jan 15;4(1). pii: e00366-12. doi: 10.1128/mBio.00366-12.

Fouhy F, Guinane CM, Hussey S, Wall R, Ryan CA, Dempsey EM, Murphy B, Ross RP, Fitzgerald GF, Stanton C, Cotter PD. (2012). High-throughput sequencing reveals the incomplete, short-term recovery of infant gut microbiota following parenteral antibiotic treatment with ampicillin and gentamicin. Antimicrobial Agents and Chemotherapy. Nov;56(11):5811-20. doi: 10.1128/AAC.00789-12.

Fouhy F, Ross RP, Fitzgerald GF, Stanton C, Cotter PD. (2012). Composition of the early intestinal microbiota: knowledge, knowledge gaps and the use of high-throughput sequencing to address these gaps. Gut Microbes. May-Jun;3(3):203-20. doi: 10.4161/gmic.20169. Review.

Wall R, Marques TM, O'Sullivan O, Ross RP, Shanahan F, Quigley EM, Dinan TG, Kiely B, Fitzgerald GF, Cotter PD, **Fouhy F**, Stanton C. (2012). Contrasting effects of *Bifidobacterium breve* NCIMB 702258 and *Bifidobacterium breve* DPC 6330 on the composition of murine brain fatty acids and gut microbiota. American Journal of Clinical Nutrition. May;95(5):1278-87. doi: 10.3945/ajcn.111.026435

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 oxacilin

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

$Gent^R$: gentamicin resistant

GIT: gastrointestinal tract

GLS: gas liquid chromatography

GOS: galacto-oligosaccharides

h: hour

H_2O: 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

tet^{R}: 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

List of Figures

List of Tables

Chapter 7

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 focussing 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 (Grölund 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 Huërou-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 straight forward 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-*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 *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 *Lactobacilllus* 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% oligogalactosyl-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

Abrahamsson, T. R., Jakobsson, H. E., Andersson, A. F., Björkstén, B., Engstrand, L. & Jenmalm, M. C. 2011. Low diversity of the gut microbiota in infants with atopic eczema. *Journal of allergy and clinical immunology,* 129**,** 434-440.

Adlerberth, I., Strachan, D. P., Matricardi, P. M., Ahrné, S., Orfei, L., Åberg, N., Perkin, M. R., Tripodi, S., Hesselmar, B. & Saalman, R. 2007. Gut microbiota and development of atopic eczema in 3 European birth cohorts. *Journal of allergy and clinical immunology,* 120**,** 343-350.

Adlerberth, I. & Wold, A. E. 2009. Establishment of the gut microbiota in Western infants. *Acta Pædiatrica,* 98**,** 229-238.

Agostoni, C., Axelsson, I., Braegger, C., Goulet, O., Koletzko, B., Michaelsen, K. F., Rigo, J., Shamir, R., Szajewska, H. & Turck, D. 2004a. Probiotic bacteria in dietetic products for infants: a commentary by the ESPGHAN Committee on Nutrition. *Journal of pediatric gastroenterology and nutrition,* 38**,** 365-374.

Agostoni, C., Axelsson, I., Goulet, O., Koletzko, B., Michaelsen, K. F., Puntis, J. W. L., Rigo, J., Shamir, R., Szajewska, H. & Turck, D. 2004b. Prebiotic oligosaccharides in dietetic products for infants: a commentary by the ESPGHAN Committee on Nutrition. *Journal of pediatric gastroenterology and nutrition,* 39**,** 465-473.

Agostoni, C., Decsi, T., Fewtrell, M., Goulet, O., Kolacek, S., Koletzko, B., Michaelsen, K. F., Moreno, L., Puntis, J. & Rigo, J. 2008. Complementary feeding: a commentary by the ESPGHAN Committee on Nutrition. *Journal of pediatric gastroenterology and nutrition,* 46**,** 99-110.

Ajslev, T., Andersen, C., Gamborg, M., Sørensen, T. & Jess, T. 2011. Childhood overweight after establishment of the gut microbiota: the role of delivery mode, pre-pregnancy weight and early administration of antibiotics. *International Journal of Obesity,* 35**,** 522-529.

Alfaleh, K. M. & Bassler, D. 2010. Cochrane review: Probiotics for prevention of necrotizing enterocolitis in preterm infants. *Evidence Based Child Health: A Cochrane Review Journal,* 5**,** 339-368.

Allen, J. & Hector, D. 2005. Benefits of breastfeeding. *New South Wales Public Health Bulletin,* 16**,** 42-46.

Arslanoglu, S., Moro, G. E. & Boehm, G. 2007. Early supplementation of prebiotic oligosaccharides protects formula-fed infants against infections during the first 6 months of life. *Journal of Nutrition,* 137**,** 2420-2424.

Aureli, P., Capurso, L., Castellazzi, A. M., Clerici, M., Giovannini, M., Morelli, L., Poli, A., Pregliasco, F., Salvini, F. & Zuccotti, G. V. 2011. Probiotics and health: An evidence-based review. *Pharmacological Research,* 63**,** 366-376.

Bach, J. F. 2002. The effect of infections on susceptibility to autoimmune and allergic diseases. *New England journal of medicine,* 347**,** 911-920.

Barclay, A. R., Stenson, B., Simpson, J. H., Weaver, L. T. & Wilson, D. C. 2007. Probiotics for necrotizing enterocolitis: a systematic review. *Journal of pediatric gastroenterology and nutrition,* 45**,** 569-576.

Ben, X. M., Zhou, X. Y., Zhao, W. H., Yu, W. L., Pan, W., Zhang, W. L., Wu, S. M., Christien, M. & Anne, S. 2004. Supplementation of milk formula with galacto-oligosaccharides improves intestinal micro-flora and fermentation in term infants. *Chinese medical journal,* 117**,** 927-931.

Bennet, R., Eriksson, M. & Nord, C. E. 2002. The fecal microflora of 1–3-month-old infants during treatment with eight oral antibiotics. *Infection,* 30**,** 158-160.

Bezirtzoglou, E. & Stavropoulou, E. 2011. Immunology and probiotic impact of the newborn and young children intestinal microflora. *Anaerobe,* 17**,** 369-374.

Bezirtzoglou, E., Tsiotsias, A. & Welling, G. W. 2011. Microbiota Profile in Feces of Breast-and Formula-Fed Newborns by Using Fluorescence in situ Hybridization (FISH). *Anaerobe,* 17**,** 478-482.

Biasucci, G., Rubini, M., Riboni, S., Morelli, L., Bessi, E. & Retetangos, C. 2010. Mode of delivery affects the bacterial community in the newborn gut. *Early Human Development,* 186**,** 13-15.

Bin-Nun, A., Bromiker, R., Wilschanski, M., Kaplan, M., Rudensky, B., Caplan, M. & Hammerman, C. 2005. Oral probiotics prevent necrotizing enterocolitis in very low birth weight neonates. *The Journal of Pediatrics,* 147**,** 192-196.

Boehm, G. & Moro, G. 2008. Structural and functional aspects of prebiotics used in infant nutrition. *Journal of Nutrition,* 138**,** 1818S-1828S.

Boehm, G., Stahl, B., Jelinek, J., Knol, J., Miniello, V. & Moro, G. E. 2005. Prebiotic carbohydrates in human milk and formulas. *Acta Paediatrica,* 94**,** 18-21.

Böttcher, M., Nordin, E., Sandin, A., Midtvedt, T. & Björkstén, B. 2000. Microflora-associated characteristics in faeces from allergic and nonallergic infants. *Clinical & Experimental Allergy,* 30**,** 1591-1596.

Bourlioux, P., Koletzko, B., Guarner, F. & Braesco, V. 2003. The intestine and its microflora are partners for the protection of the host: report on the Danone Symposium" The Intelligent Intestine," held in Paris, June 14, 2002. *American Journal of Clinical Nutrition,* 78**,** 675-683.

Boyle, R., Bath Hextall, F., Leonardi Bee, J., Murrell, D. & Tang, M. 2009. Probiotics for the treatment of eczema: a systematic review. *Clinical & Experimental Allergy,* 39**,** 1117-1127.

Braga, T. D., Da Silva, G. a. P., De Lira, P. I. C. & De Carvalho Lima, M. 2011. Efficacy of Bifidobacterium breve and Lactobacillus casei oral supplementation on necrotizing enterocolitis in very-low-birth-weight preterm infants: a double-blind, randomized, controlled trial. *The American journal of clinical nutrition,* 93**,** 81-86.

Branton, D., Kasianowicz, J. J., Deamer, D. W. & Brandin, E. 1996. Characterization of Individual Polynucleotide Molecules Using a Membrane Channel. *Proceedings of the National Academy of Sciences,* 93**,** 13770-13773.

Brown, P. O. & Botstein, D. 1999. Exploring the new world of the genome with DNA microarrays. *Nature genetics,* 21**,** 33-37.

Cani, P. D., Bibiloni, R., Knauf, C., Waget, A., Neyrinck, A. M., Delzenne, N. M. & Burcelin, R. 2008. Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet–induced obesity and diabetes in mice. *Diabetes,* 57**,** 1470-1495.

Caplan, M. S., Lickerman, M., Adler, L., Dietsch, G. N. & Yu, A. 1997. The role of recombinant platelet-activating factor acetylhydrolase in a neonatal rat model of necrotizing enterocolitis. *Pediatric research,* 42**,** 779-783.

Caplan, M. S., Miller-Catchpole, R., Kaup, S., Russell, T., Lickerman, M. & Amer, M. 1999. Bifidobacterial supplementation reduces the incidence of necrotizing enterocolitis in a neonatal rat model. *Gastroenterology,* 117**,** 577-583.

Cardona, M. E. 2002. Effect of probiotics on five biochemical microflora-associated characteristics, in vitro and in vivo. *Food & Nutrition Research,* 46**,** 73-79.

Celedon, J. C., Fuhlbrigge, A., Rifas Shiman, S., Weiss, S. T. & Finkelstein, J. A. 2004. Antibiotic use in the first year of life and asthma in early childhood. *Clinical & Experimental Allergy,* 34**,** 1011-1016.

Claesson, M. J., Cusack, S., O'sullivan, O., Greene-Diniz, R., De Weerd, H., Flannery, E., Marchesi, J. R., Falush, D., Dinan, T. & Fitzgerald, G. 2011. Composition, variability, and temporal stability of the intestinal microbiota of the elderly. *Proceedings of the National Academy of Sciences,* 108**,** 4586-4591.

Clarke, J., Wu, H. C., Jayasinghe, L., Patel, A., Reid, S. & Bayley, H. 2009. Continuous base identification for single-molecule nanopore DNA sequencing. *Nature nanotechnology,* 4**,** 265-270.

Claud, E. C. & Walker, W. A. 2001. Hypothesis: inappropriate colonization of the premature intestine can cause neonatal necrotizing enterocolitis. *The FASEB Journal,* 15**,** 1398-1403.

Collado, M. C., Isolauri, E., Laitinen, K. & Salminen, S. 2010. Effect of mother's weight on infant's microbiota acquisition, composition, and activity during early infancy: a prospective follow-up study initiated in early pregnancy. *The American journal of clinical nutrition,* 92**,** 1023-1030.

Conroy, M. E., Shi, H. N. & Walker, W. A. 2009. The long-term health effects of neonatal microbial flora. *Current opinion in allergy and clinical immunology,* 9**,** 197-201.

Cremonini, F., Di Caro, S., Nista, E. C., Bartolozzi, F., Capelli, G., Gasbarrini, G. & Gasbarrini, A. 2002. Meta analysis: the effect of probiotic administration on antibiotic associated diarrhoea. *Alimentary pharmacology & therapeutics,* 16**,** 1461-1467.

D'souza, A. L., Rajkumar, C., Cooke, J. & Bulpitt, C. J. 2002. Probiotics in prevention of antibiotic associated diarrhoea: meta-analysis. *Bmj,* 324**,** 1361-1367.

De Filippo, C., Cavalieri, D., Di Paola, M., Ramazzotti, M., Poullet, J. B., Massart, S., Collini, S., Pieraccini, G. & Lionetti, P. 2010. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proceedings of the National Academy of Sciences,* 107**,** 14691-14697.

Deshpande, G., Rao, S. & Patole, S. 2007. Probiotics for prevention of necrotising enterocolitis in preterm neonates with very low birthweight: a systematic review of randomised controlled trials. *The Lancet,* 369**,** 1614-1620.

Deshpande, G., Rao, S., Patole, S. & Bulsara, M. 2010. Updated meta-analysis of probiotics for preventing necrotizing enterocolitis in preterm neonates. *Pediatrics,* 125**,** 921-930.

Dethlefsen, L., Huse, S., Sogin, M. L. & Relman, D. A. 2008. The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. *PLoS Biol,* 6**,** e280. doi: 10.137/journal.pbio.0060280.

Dominguez-Bello, M. G., Costello, E. K., Contreras, M., Magris, M., Hidalgo, G., Fierer, N. & Knight, R. 2010. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. *Proceedings of the National Academy of Sciences,* 107**,** 11971-11976.

Duncan, S., Lobley, G., Holtrop, G., Ince, J., Johnstone, A., Louis, P. & Flint, H. 2008. Human colonic microbiota associated with diet, obesity and weight loss. *International Journal of Obesity,* 32**,** 1720-1724.

Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S. R., Nelson, K. E. & Relman, D. A. 2005. Diversity of the human intestinal microbial flora. *Science,* 308**,** 1635-1638.

Edwards, C. A. & Parrett, A. M. 2002. Intestinal flora during the first months of life: new perspectives. *British Journal of Nutrition,* 88**,** 11-18.

Efsa 2007. Opinion of the Scientific Committee on a request from EFSA on the introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA. The EFSA Journal (2007) 587, 1-16.

Efsa 2010. EFSA on Dietetic Products, Nutrition and Allergies (NDA); Scientific Opinion on on the scientific

requirements for health claims related to gut and immune function.

Available online: www.efsa.europa.eu/efsajournal.htm.

Fagerås, M., Tomicic, S., Voor, T., Björkstén, B. & Jenmalm, M. C. 2011. Slow salivary secretory IgA maturation may relate to low microbial pressure and allergic symptoms in sensitized children. *Pediatric research,* 70**,** 572-577.

Fallani, M., Amarri, S., Uusijarvi, A., Adam, R., Khanna, S., Aguilera, M., Gil, A., Vieites, J. M., Norin, E. & Young, D. 2011. Determinants of the human infant intestinal microbiota after the introduction of first complementary foods in infant samples from five European centres. *Microbiology,* 157**,** 1385-1392.

Fallani, M., Young, D., Scott, J., Norin, E., Amarri, S., Adam, R., Aguilera, M., Khanna, S., Gil, A. & Edwards, C. A. 2010. Intestinal microbiota of 6-week-old infants across Europe: geographic influence beyond delivery mode, breast-feeding, and antibiotics. *Journal of pediatric gastroenterology and nutrition,* 51**,** 77-84.

Fao/Who 2001. Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. Report of a Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Foods Including Powder Milk with Live Lactic Acid Bacteria. Basel, Switzerland: World Health Organization, 2001

Favier, C. F., De Vos, W. M. & Akkermans, A. D. L. 2003. Development of bacterial and bifidobacterial communities in feces of newborn babies. *Anaerobe,* 9**,** 219-229.

Finegold, S. M. 1970. Interaction of antimicrobial therapy and intestinal flora. *American Journal of Clinical Nutrition,* 23**,** 1466-1471.

Fitzpatrick, F., Oza, A., Gilleece, A., O'byrne, A. & Drudy, D. 2008. Laboratory diagnosis of Clostridium difficile-associated disease in the Republic of Ireland: a survey of Irish microbiology laboratories. *Journal of Hospital Infection,* 68**,** 315-321.

Foliaki, S., Pearce, N., Björkstén, B., Mallol, J., Montefort, S. & Von Mutius, E. 2009. Antibiotic use in infancy and symptoms of asthma, rhinoconjunctivitis, and eczema in children 6 and 7 years

old: International Study of Asthma and Allergies in Childhood Phase III. *Journal of allergy and clinical immunology,* 124**,** 982-989.

Fox, P. F. & Kelly, A. L. 2006. Indigenous enzymes in milk: Overview and historical aspects--Part 2. *International Dairy Journal,* 16**,** 517-532.

Frank, D. N. & Pace, N. R. 2008. Gastrointestinal microbiology enters the metagenomics era. *Current opinion in gastroenterology,* 24**,** 4-10.

Fuller, R. 1989. Probiotics in man and animals. *The Journal of applied bacteriology,* 66**,** 365-378.

Gerding, D. N., Johnson, S., Peterson, L. R., Mulligan, M. E. & Silva, J. 1995. Clostridium difficile-associated diarrhea and colitis. *Infection control and hospital epidemiology,* 16**,** 459-477.

Gerrard, J., Geddes, C., Reggin, P., Gerrard, C. & Horne, S. 1976. Serum IgE levels in white and metis communities in Saskatchewan. *Annals of allergy,* 37**,** 91-100.

Gibson, G. R., Probert, H. M., Loo, J. V., Rastall, R. A. & Roberfroid, M. B. 2004. Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. *Nutrition research reviews,* 17**,** 259-275.

Gibson, G. R. & Roberfroid, M. B. 1995. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *The Journal of nutrition,* 125**,** 1401-1412.

Goldani, H. a. S., Bettiol, H., Barbieri, M. A., Silva, A. a. M., Agranonik, M., Morais, M. B. & Goldani, M. Z. 2011. Cesarean delivery is associated with an increased risk of obesity in adulthood in a Brazilian birth cohort study. *The American journal of clinical nutrition,* 93**,** 1344-1348.

Gooch, W. 1996. Effectiveness of five days of therapy with cefuroxime axetil suspension for treatment of acute otitis media. *The Pediatric infectious disease journal,* 15**,** 157-164.

Goodman, A. L., Kallstrom, G., Faith, J. J., Reyes, A., Moore, A., Dantas, G. & Gordon, J. I. 2011. Extensive personal human gut microbiota

culture collections characterized and manipulated in gnotobiotic mice. *Proceedings of the National Academy of Sciences,* 108**,** 6252-6258.

Gophna, U., Sommerfeld, K., Gophna, S., Doolittle, W. F. & Veldhuyzen Van Zanten, S. J. O. 2006. Differences between tissue-associated intestinal microfloras of patients with Crohn's disease and ulcerative colitis. *Journal of clinical microbiology,* 44**,** 4136-4141.

Grölund, M. M., Lehtonen, O. P., Eerola, E. & Kero, P. 1999. Fecal microflora in healthy infants born by different methods of delivery: permanent changes in intestinal flora after cesarean delivery. *Journal of pediatric gastroenterology and nutrition,* 28**,** 19-25.

Grześkowiak, Ł., Grönlund, M. M., Beckmann, C., Salminen, S., Von Berg, A. & Isolauri, E. 2011. The impact of perinatal probiotic intervention on gut microbiota: Double-blind placebo-controlled trials in Finland and Germany. *Anaerobe***,** doi:10.1016/j.anaerobe.2011.09.006.

Guarner, F. & Malagelada, J. R. 2003. Gut flora in health and disease. *The Lancet,* 361**,** 512-519.

Gueimonde, M., Sakata, S., Kalliomäki, M., Isolauri, E., Benno, Y. & Salminen, S. 2006. Effect of maternal consumption of lactobacillus GG on transfer and establishment of fecal bifidobacterial microbiota in neonates. *Journal of pediatric gastroenterology and nutrition,* 42**,** 166-170.

Haarman, M. & Knol, J. 2005. Quantitative real-time PCR assays to identify and quantify fecal Bifidobacterium species in infants receiving a prebiotic infant formula. *Applied and environmental microbiology,* 71**,** 2318-2324.

Hascoët, J. M., Hubert, C., Rochat, F., Legagneur, H., Gaga, S., Emady-Azar, S. & Steenhout, P. G. 2011. Effect of formula composition on the development of infant gut microbiota. *Journal of pediatric gastroenterology and nutrition,* 52**,** 756-762.

Hattori, M. & Taylor, T. D. 2009. The human intestinal microbiome: a new frontier of human biology. *Dna Research,* 16**,** 1-12.

Hoberman, A., Paradise, J. L., Burch, D. J., Valinski, W. A., Hedrick, J. A., Aronovitz, G. H., Drehobl, M. A. & Rogers, J. M. 1997. Equivalent efficacy and reduced occurrence of diarrhea from a new formulation of amoxicillin/clavulanate potassium (Augmentin (R)) for treatment of acute otitis media in children. *The Pediatric infectious disease journal,* 16**,** 463-470.

Holzapfel, W. H. & Schillinger, U. 2002. Introduction to pre-and probiotics. *Food Research International,* 35**,** 109-116.

Howie, P. W., Forsyth, J. S., Ogston, S. A., Clark, A. & Florey, C. 1990. Protective effect of breast feeding against infection. *British Medical Journal,* 300**,** 11-16.

Hoyos, A. B. 1999. Reduced incidence of necrotizing enterocolitis associated with enteral administration of Lactobacillus acidophilus and Bifidobacterium infantis to neonates in an intensive care unit. *International Journal of Infectious Diseases,* 3**,** 197-202.

Hussey, S., Wall, R., Gruffman, E., O'sullivan, L., Ryan, C. A., Murphy, B., Fitzgerald, G., Stanton, C. & Ross, R. P. 2011. Parenteral Antibiotics Reduce Bifidobacteria Colonization and Diversity in Neonates. *International journal of microbiology,* 2011**,** doi:10.1155/2011/130574.

Huurre, A., Kalliomaki, M., Rautava, S., Rinne, M., Salminen, S. & Isolauri, E. 2008. Mode of delivery-effects on gut microbiota and humoral immunity. *Neonatology,* 93**,** 236-240.

J Drychowski, W., Ga A , A., Whyatt, R. & Perera, F. 2006. The prenatal use of antibiotics and the development of allergic disease in one year old infants. A preliminary study. *International Journal of Occupational Medicine and Environmental Health,* 19**,** 70-76.

Jernberg, C., Lofmark, S., Edlund, C. & Jansson, J. K. 2010. Long-term impacts of antibiotic exposure on the human intestinal microbiota. *Microbiology,* 156**,** 3216-3223.

Jernberg, C., Löfmark, S., Edlund, C. & Jansson, J. K. 2007. Long-term ecological impacts of antibiotic administration on the human intestinal microbiota. *The ISME journal,* 1**,** 56-66.

Johansson, M. A., Sjögren, Y. M., Persson, J. O., Nilsson, C. & Sverremark-Ekström, E. 2011. Early colonization with a group of Lactobacilli decreases the risk for allergy at five years of age despite allergic heredity. *PLoS ONE,* 6**,** e23031.

Kalliomäki, M., Antoine, J. M., Herz, U., Rijkers, G. T., Wells, J. M. & Mercenier, A. 2010. Guidance for substantiating the evidence for beneficial effects of probiotics: prevention and management of allergic diseases by probiotics. *The Journal of nutrition,* 140**,** 713S-721S.

Kalliomaki, M., Carmen Collado, M., Salminen, S. & Isolauri, E. 2008. Early differences in fecal microbiota composition in children may predict overweight. *American Journal of Clinical Nutrition,* 87**,** 534-538.

Kassinen, A., Krogius-Kurikka, L., Mäkivuokko, H., Rinttilä, T., Paulin, L., Corander, J., Malinen, E., Apajalahti, J. & Palva, A. 2007. The fecal microbiota of irritable bowel syndrome patients differs significantly from that of healthy subjects. *Gastroenterology,* 133**,** 24-33.

Kitajima, H., Sumida, Y., Tanaka, R., Yuki, N., Takayama, H. & Fujimura, M. 1997. Early administration of Bifidobacterium breve to preterm infants: randomised controlled trial. *British Medical Journal,* 76**,** 101-107.

Koenig, J. E., Spor, A., Scalfone, N., Fricker, A. D., Stombaugh, J., Knight, R., Angenent, L. T. & Ley, R. E. 2011. Succession of microbial consortia in the developing infant gut microbiome. *Proceedings of the National Academy of Sciences,* 108**,** 4578-4586.

Kozyrskyj, A. L., Ernst, P. & Becker, A. B. 2007. Increased Risk of Childhood Asthma From Antibiotic Use in Early Life. *Chest,* 131**,** 1753-1759.

Kristiansen, A. L., Lande, B., Øverby, N. C. & Andersen, L. F. 2010. Factors associated with exclusive breast-feeding and breast-feeding in Norway. *Public Health Nutrition,* 1**,** 1-10.

Kukkonen, K., Savilahti, E., Haahtela, T., Juntunen-Backman, K., Korpela, R., Poussa, T., Tuure, T. & Kuitunen, M. 2007. Probiotics and prebiotic galacto-oligosaccharides in the prevention of allergic diseases: A randomized, double-blind, placebo-controlled trial. *Journal of allergy and clinical immunology,* 119**,** 192-198.

Kummeling, I., Stelma, F. F., Dagnelie, P. C., Snijders, B. E. P., Penders, J., Huber, M., Van Ree, R., Van Den Brandt, P. A. & Thijs, C. 2007. Early life exposure to antibiotics and the subsequent development of eczema, wheeze, and allergic sensitization in the first 2 years of life: the KOALA Birth Cohort Study. *Pediatrics,* 119**,** e225-e231.

Kurokawa, K., Itoh, T., Kuwahara, T., Oshima, K., Toh, H., Toyoda, A., Takami, H., Morita, H., Sharma, V. K. & Srivastava, T. P. 2007. Comparative metagenomics revealed commonly enriched gene sets in human gut microbiomes. *Dna Research,* 14**,** 169-181.

Lawlor, D. A., Smith, G. D., O'callaghan, M., Alati, R., Mamun, A. A., Williams, G. M. & Najman, J. M. 2007. Epidemiologic evidence for the fetal overnutrition hypothesis: findings from the mater-university study of pregnancy and its outcomes. *American Journal of Epidemiology,* 165**,** 418-424.

Le Huërou-Luron, I., Blat, S. & Boudry, G. 2010. Breast-v. formula-feeding: impacts on the digestive tract and immediate and long-term health effects. *Nutr Res Rev,* 23**,** 23-36.

Leahy, S., Higgins, D., Fitzgerald, G. & Sinderen, D. 2005. Getting better with bifidobacteria. *Journal of applied microbiology,* 98**,** 1303-1315.

Levene, M. J., Korlach, J., Turner, S. W., Foquet, M., Craighead, H. G. & Webb, W. W. 2003. Zero-mode waveguides for single-molecule analysis at high concentrations. *Science,* 299**,** 682-686.

Ley, R. E. 2010. Obesity and the human microbiome. *Current opinion in gastroenterology,* 26**,** 5-11.

Ley, R. E., Bäckhed, F., Turnbaugh, P., Lozupone, C. A., Knight, R. D. & Gordon, J. I. 2005. Obesity alters gut microbial ecology. *Proceedings of the National Academy of Sciences of the United States of America,* 102**,** 11070-11075.

Liepke, C., Adermann, K., Raida, M., Mägert, H. J., Forssmann, W. G. & Zucht, H. D. 2002. Human milk provides peptides highly stimulating the growth of bifidobacteria. *European Journal of Biochemistry,* 269**,** 712-718.

Lilly, D. M. & Stillwell, R. H. 1965. Probiotics: growth-promoting factors produced by microorganisms. *Science,* 147**,** 747-748.

Lin, H. C., Su, B. H., Chen, A. C., Lin, T. W., Tsai, C. H., Yeh, T. F. & Oh, W. 2005. Oral probiotics reduce the incidence and severity of necrotizing enterocolitis in very low birth weight infants. *Pediatrics,* 115**,** 1-4.

Lin, P. W. & Stoll, B. J. 2006. Necrotising enterocolitis. *The Lancet,* 368**,** 1271-1283.

Macfarlane, S., Macfarlane, G. T. & Cummings, J. H. 2006. Review article: prebiotics in the gastrointestinal tract. *Alimentary pharmacology & therapeutics,* 24**,** 701-714.

Maitra, A., Cohen, Y., Gillespie, S. E. D., Mambo, E., Fukushima, N., Hoque, M. O., Shah, N., Goggins, M., Califano, J. & Sidransky, D. 2004. The Human MitoChip: a high-throughput sequencing microarray for mitochondrial mutation detection. *Genome research,* 14**,** 812-819.

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

quantification of selected faecal bacteria. *Microbiology,* 149**,** 269-277.

Mangin, I., Suau, A., Gotteland, M., Brunser, O. & Pochart, P. 2010. Amoxicillin treatment modifies the composition of Bifidobacterium species in infant intestinal microbiota. *Anaerobe,* 16**,** 433-438.

Manichanh, C., Rigottier-Gois, L., Bonnaud, E., Gloux, K., Pelletier, E., Frangeul, L., Nalin, R., Jarrin, C., Chardon, P. & Marteau, P. 2006. Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. *Gut,* 55**,** 205-211.

Manning, T. S. & Gibson, G. R. 2004. Prebiotics. *Best Practice & Research Clinical Gastroenterology,* 18**,** 287-298.

Mardis, E. R. 2008a. The impact of next-generation sequencing technology on genetics. *Trends in Genetics,* 24**,** 133-141.

Mardis, E. R. 2008b. Next-generation DNA sequencing methods. *Annu. Rev. Genomics Hum. Genet.,* 9**,** 387-402.

Marra, F., Marra, C. A., Richardson, K., Lynd, L. D., Kozyrskyj, A., Patrick, D. M., Bowie, W. R. & Fitzgerald, J. M. 2009. Antibiotic use in children is associated with increased risk of asthma. *Pediatrics,* 123**,** 1003-1010.

Martín, R., Heilig, G. H. J., Zoetendal, E. G., Smidt, H. & Rodríguez, J. M. 2007. Diversity of the Lactobacillus group in breast milk and vagina of healthy women and potential role in the colonization of the infant gut. *Journal of applied microbiology,* 103**,** 2638-2644.

Martín, R., Langa, S., Reviriego, C., Jimínez, E., Marín, M. L., Xaus, J., Fernández, L. & Rodríguez, J. M. 2003. Human milk is a source of lactic acid bacteria for the infant gut. *The Journal of Pediatrics,* 143**,** 754-758.

Mckeever, T. M., Lewis, S. A., Smith, C. & Hubbard, R. 2002. The importance of prenatal exposures on the development of allergic disease: a birth cohort study using the West Midlands General Practice Database. *American journal of respiratory and critical care medicine,* 166**,** 827-832.

Midtvedt, T., Bjørneklett, A., Carlstedt-Duke, B., Gustafsson, B., Høverstad, T., Lingaas, E., Norin, K., Saxerholt, H. & Steinbakk, M. 1985. The influence of antibiotics upon microflora-associated characteristics in man and mammals. *Progress in clinical and biological research,* 181**,** 241-244.

Mohan, P. & Abrams, S. A. 2009. Oral lactoferrin for the treatment of sepsis and necrotizing enterocolitis in neonates. *Cochrane Database of Systematic Reviews***,** CD007138.

Mshvildadze, M. & Neu, J. 2010. The infant intestinal microbiome: Friend or foe? *Early Human Development,* 86**,** 67-71.

Neu, J. & Rushing, J. 2011. Cesarean versus vaginal delivery: long-term infant outcomes and the hygiene hypothesis. *Clinics in Perinatology,* 38**,** 321-331.

O' Hara, A. M. & Shanahan, F. 2006. The gut flora as a forgotten organ. *EMBO Rep,* 7**,** 688-693.

O' Toole, P. W. & Claesson, M. J. 2010. Gut microbiota: Changes throughout the lifespan from infancy to elderly. *International Dairy Journal,* 20**,** 281-291.

Overturf, K. 2009. Quantitative PCR. *Molecular Research in Aquaculture***,** 39-61.

Palmer, C., Bik, E. M., Digiulio, D. B., Relman, D. A. & Brown, P. O. 2007. Development of the human infant intestinal microbiota. *PLoS Biology,* 5**,** e177. doi:10.1371/journal.pbio. 0050177.

Parker, R. B. 1974. Probiotics, the other half of the antibiotic story. *Animal Nutrition and Health,* 29**,** 4-8.

Parracho, H., Mccartney, A. L. & Gibson, G. R. 2007. Probiotics and prebiotics in infant nutrition. *Proceedings of the Nutrition Society,* 66**,** 405-411.

Penders, J., Kummeling, I. & Thijs, C. 2011. Infant antibiotic use and wheeze and asthma risk-a systematic review and meta-analysis. *European Respiratory Journal,* 38**,** 295-302.

Penders, J., Thijs, C., Vink, C., Stelma, F. F., Snijders, B., Kummeling, I., Van Den Brandt, P. A. & Stobberingh, E. E. 2006. Factors influencing the composition of the intestinal microbiota in early infancy. *Pediatrics,* 118**,** 511-521.

Picard, C., Fioramonti, J., Francois, A., Robinson, T., Neant, F. & Matuchansky, C. 2005. Review article: bifidobacteria as probiotic agents–physiological effects and clinical benefits. *Alimentary pharmacology & therapeutics,* 22**,** 495-512.

Playne, M. J. & Crittenden, R. 1996. Commercially available oligosaccharides. *Bulletin-FIL-IDF (Belgium); International Dairy Federation.*

Preidis, G. A. & Versalovic, J. 2009. Targeting the human microbiome with antibiotics, probiotics, and prebiotics: gastroenterology enters the metagenomics era. *Gastroenterology,* 136**,** 2015-2031.

Prescott, S. L. & Björkstén, B. 2007. Probiotics for the prevention or treatment of allergic diseases. *The Journal of allergy and clinical immunology,* 120**,** 255-262.

Rastall, R. A., Gibson, G. R., Gill, H. S., Guarner, F., Klaenhammer, T. R., Pot, B., Reid, G., Rowland, I. R. & Sanders, M. E. 2005. Modulation of the microbial ecology of the human colon by probiotics, prebiotics and synbiotics to enhance human health: An overview of enabling science and potential applications. *FEMS microbiology ecology,* 52**,** 145-152.

Rautava, S., Kalliomäki, M. & Isolauri, E. 2002. Probiotics during pregnancy and breast-feeding might confer immunomodulatory protection against atopic disease in the infant. *Journal of allergy and clinical immunology,* 109**,** 119-121.

Reddy, B. S. 1999. Possible mechanisms by which pro-and prebiotics influence colon carcinogenesis and tumor growth. *Journal of Nutrition,* 129**,** 1478-1483.

Rinne, M. M., Gueimonde, M., Kalliomäki, M., Hoppu, U., Salminen, S. J. & Isolauri, E. 2005. Similar bifidogenic effects of prebiotic

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

Roberfroid, M., Gibson, G. R., Hoyles, L., Mccartney, A. L., Rastall, R., Rowland, I., Wolvers, D., Watzl, B., Szajewska, H. & Stahl, B. 2010. Prebiotic effects: metabolic and health benefits. *British Journal of Nutrition,* 104**,** S1-S63.

Roesch, L. F. W., Lorca, G. L., Casella, G., Giongo, A., Naranjo, A., Pionzio, A. M., Li, N., Mai, V., Wasserfall, C. H. & Schatz, D. 2009. Culture-independent identification of gut bacteria correlated with the onset of diabetes in a rat model. *The ISME journal,* 3**,** 536-548.

Roger, L. C., Costabile, A., Holland, D. T., Hoyles, L. & Mccartney, A. L. 2010. Examination of faecal Bifidobacterium populations in breast- and formula-fed infants during the first 18 months of life. *Microbiology,* 156**,** 3329-3341.

Russell, A. R. B. & Murch, S. H. 2006. Could peripartum antibiotics have delayed health consequences for the infant? *BJOG: An International Journal of Obstetrics & Gynaecology,* 113**,** 758-765.

Saavedra, J. M. 2007. Use of probiotics in pediatrics: rationale, mechanisms of action, and practical aspects. *Nutrition in Clinical Practice,* 22**,** 351-365.

Sakata, H., Yoshioka, H. & Fujita, K. 1985. Development of the intestinal flora in very low birth weight infants compared to normal full-term newborns. *European journal of pediatrics,* 144**,** 186-190.

Salminen, S., Gibson, G. R., Mccartney, A. L. & Isolauri, E. 2004. Influence of mode of delivery on gut microbiota composition in seven year old children. *British Medical Journal,* 53**,** 1388-1389.

Salminen, S., Ouwehand, A., Benno, Y. & Lee, Y. K. 1999. Probiotics: how should they be defined? *Trends in Food Science & Technology,* 10**,** 107-110.

Salvini, F., Riva, E., Salvatici, E., Boehm, G., Jelinek, J., Banderali, G. & Giovannini, M. 2011. A Specific Prebiotic Mixture Added to Starting

Infant Formula Has Long-Lasting Bifidogenic Effects. *The Journal of nutrition,* 141**,** 1335-1339.

Schadt, E. E., Turner, S. & Kasarskis, A. 2010. A window into third-generation sequencing. *Human molecular genetics,* 19**,** R227-R240.

Segarra-Newnham, M. 2007. Probiotics for Clostridium difficile-associated diarrhea: focus on Lactobacillus rhamnosus GG and Saccharomyces boulardii. *The Annals of pharmacotherapy,* 41**,** 1212-1221.

Sepp, E., Julge, K., Vasar, M., Naaber, P., Björkstén, B. & Mikelsaar, M. 1997. Intestinal microflora of Estonian and Swedish infants. *Acta Paediatrica,* 86**,** 956-961.

Shendure, J., Porreca, G. J., Reppas, N. B., Lin, X., Mccutcheon, J. P., Rosenbaum, A. M., Wang, M. D., Zhang, K., Mitra, R. D. & Church, G. M. 2005. Accurate multiplex polony sequencing of an evolved bacterial genome. *Science,* 309**,** 1728-1732.

Sinkiewicz, G. & Ljunggren, L. 2008. Occurrence of Lactobacillus reuteri in human breast milk. *Microbial Ecology in Health and Disease,* 20**,** 122-126.

Solís, G., De Los Reyes-Gavilan, C., Fernández, N., Margolles, A. & Gueimonde, M. 2010. Establishment and development of lactic acid bacteria and bifidobacteria microbiota in breast-milk and the infant gut. *Anaerobe,* 16**,** 307-310.

Stark, P. L. & Lee, A. 1982. The microbial ecology of the large bowel of breastfed and formula-fed infants during the first year of life. *Journal of medical microbiology,* 15**,** 189-203.

Strachan, D. P. 1989. Hay fever, hygiene, and household size. *British Medical Journal,* 299**,** 1259-1260.

Strachan, D. P. 2000. Family size, infection and atopy: the first decade of the'hygiene hypothesis'. *British Medical Journal,* 55**,** S2-S10.

Strausberg, R. L., Levy, S. & Rogers, Y. H. 2008. Emerging DNA sequencing technologies for human genomic medicine. *Drug discovery today,* 13**,** 569-577.

Survey, N. I. 2007. Breastfeeding Among U.S. Children Born 1999—2007, CDC National Immunization Survey

Tanaka, S., Kobayashi, T., Songjinda, P., Tateyama, A., Tsubouchi, M., Kiyohara, C., Shirakawa, T., Sonomoto, K. & Nakayama, J. 2009. Influence of antibiotic exposure in the early postnatal period on the development of intestinal microbiota. *FEMS Immunology & Medical Microbiology,* 56**,** 80-87.

Tankanow, R., Ross, M., Ertel, I., Dickinson, D., Mccormick, L. & Garfinkel, J. 1990. A double-blind, placebo-controlled study of the efficacy of Lactinex in the prophylaxis of amoxicillin-induced diarrhea. *The Annals of pharmacotherapy,* 24**,** 382-384.

Tarrant, R. C., Younger, K. M., Sheridan-Pereira, M., White, M. J. & Kearney, J. M. 2010. The prevalence and determinants of breast-feeding initiation and duration in a sample of women in Ireland. *Public Health Nutr,* 13**,** 760-770.

Thomas, C., Stevenson, M. & Riley, T. V. 2003. Antibiotics and hospital-acquired Clostridium difficile-associated diarrhoea: a systematic review. *Journal of antimicrobial chemotherapy,* 51**,** 1339-1350.

Thomas, R. K., Baker, A. C., Debiasi, R. M., Winckler, W., Laframboise, T., Lin, W. M., Wang, M., Feng, W., Zander, T. & Macconaill, L. E. 2007. High-throughput oncogene mutation profiling in human cancer. *Nature genetics,* 39**,** 347-351.

Thompson-Chagoyan, O. C., Fallani, M., Maldonado, J., Vieites, J. M., Khanna, S., Edwards, C., Doré, J. & Gil, A. 2011. Faecal Microbiota and Short-Chain Fatty Acid Levels in Faeces from Infants with Cow‘s Milk Protein Allergy. *International archives of allergy and immunology,* 156**,** 325-332.

Tjellström, B., Stenhammar, L., Högberg, L., Fälth-Magnusson, K., Magnusson, K., Midtvedt, T., Sundqvist, T. & Norin, E. 2005. Gut

microflora associated characteristics in children with celiac disease. *The American journal of gastroenterology,* 100**,** 2784-2788.

Tjellström, B., Stenhammar, L., Högberg, L., Fälth-Magnusson, K., Magnusson, K. E., Midtvedt, T., Sundqvist, T., Houlston, R., Popat, S. & Norin, E. 2007. Gut microflora associated characteristics in first-degree relatives of children with celiac disease. *Scandinavian journal of gastroenterology,* 42**,** 1204-1208.

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. P. 2008. A core gut microbiome in obese and lean twins. *Nature,* 457**,** 480-484.

Turnbaugh, P. J., Ridaura, V. K., Faith, J. J., Rey, F. E., Knight, R. & Gordon, J. I. 2009. The effect of diet on the human gut microbiome: a metagenomic analysis in humanized gnotobiotic mice. *Science translational medicine,* 1**,** 6ra14.

Vaishampayan, P. A., Kuehl, J. V., Froula, J. L., Morgan, J. L., Ochman, H. & Francino, M. P. 2010. Comparative metagenomics and population dynamics of the gut microbiota in mother and infant. *Genome Biology and Evolution,* 2**,** 53-66.

Van Nimwegen, F. A., Penders, J., Stobberingh, E. E., Postma, D. S., Koppelman, G. H., Kerkhof, M., Reijmerink, N. E., Dompeling, E., Van Den Brandt, P. A. & Ferreira, I. 2011. Mode and place of delivery, gastrointestinal microbiota, and their influence on asthma and atopy. *Journal of allergy and clinical immunology,* 128**,** 948-955.

Veereman-Wauters, G. 2005. Application of prebiotics in infant foods. *British Journal of Nutrition,* 93**,** 57-60.

Venter, J. C., Adams, M. D., Myers, E. W., Li, P. W., Mural, R. J., Sutton, G. G., Smith, H. O., Yandell, M., Evans, C. A. & Holt, R. A. 2001. The sequence of the human genome. *Science,* 291**,** 1304-1351.

Viljanen, M., Savilahti, E., Haahtela, T., Juntunen Backman, K., Korpela, R., Poussa, T., Tuure, T. & Kuitunen, M. 2005. Probiotics in the treatment of atopic eczema/dermatitis syndrome in infants: a double blind placebo controlled trial. *Allergy,* 60**,** 494-500.

Waligora-Dupriet, A. J., Campeotto, F., Nicolis, I., Bonet, A., Soulaines, P., Dupont, C. & Butel, M. J. 2007. Effect of oligofructose supplementation on gut microflora and well-being in young children attending a day care centre. *International journal of food microbiology,* 113**,** 108-113.

Wallace, T. C., Guarner, F., Madsen, K., Cabana, M. D., Gibson, G., Hentges, E. & Sanders, M. E. 2011. Human gut microbiota and its relationship to health and disease. *Nutrition reviews,* 69**,** 392-403.

Wang, M., Karlsson, C., Olsson, C., Adlerberth, I., Wold, A. E., Strachan, D. P., Martricardi, P. M., Åberg, N., Perkin, M. R. & Tripodi, S. 2008. Reduced diversity in the early fecal microbiota of infants with atopic eczema. *Journal of allergy and clinical immunology,* 121**,** 129-134.

Wang, X. & Gibson, G. R. 1993. Effects of the in vitro fermentation of oligofructose and inulin by bacteria growing in the human large intestine. *Journal of applied microbiology,* 75**,** 373-380.

Weber, J. L. & Myers, E. W. 1997. Human whole-genome shotgun sequencing. *Genome research,* 7**,** 401-409.

Who 2003. Global strategy on infant and young child feeding. *Available online at* (http://www.who.int/nutrition/publications/infantfeeding).

Who 2011. WHO obesity factsheet. Available online at (http://www.who.int/mediacentre/factsheets/fs311/en/index.html).

Zwielehner, J., Liszt, K., Handschur, M., Lassl, C., Lapin, A. & Haslberger, A. G. 2009. Combined PCR-DGGE fingerprinting and quantitative-PCR indicates shifts in fecal population sizes and diversity of Bacteroides, bifidobacteria and Clostridium cluster IV in institutionalized elderly. *Experimental gerontology,* 44**,** 440-446.

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

	Microflora associated characteristics	Culture-dependent techniques	Culture-independent techniques	High-throughput sequencing
Technique description	*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*	*Use of selective media to culture specific microorganisms or species of microorganisms* e.g. Man Rogosa Sharpe (MRS) media for lactobacilli growth	*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	*Sequencing based approaches used to rapidly identify bacteria using bacterial DNA as template e.g. 454, Illumina, SoLID, Ion torrent*
History of use	In the past has been predominantly used as an initial population screen or in epidemiological studies	Historically, the most frequently used approach to identify bacteria present in various environments	Increasingly popular in past 2 decades with increasing availability of computer based technologies and software programs	Became commercially available at the beginning of the 21st century and becoming increasingly popular ever since
Advantages	• Simple • Inexpensive • Suitable as initial screen to test a novel hypothesis • Useful for large population screens e.g. in epidemiology studies	• Quick • Inexpensive • Limited skill required • Limited equipment needed • Useful as the initial screen before more detailed investigations	• Relatively inexpensive • Relatively simple • More detailed results achievable	• Less biased results • Very detailed information • Bacterial profile in complex environments e.g. gut microbiota can be identified • Huge phylogenetic information provided • Relatively quick
Disadvantages	• Provides limited information • No bacterial species identification possible	• Up to 90% of bacteria non-culturable • Provides limited information • Need prior knowledge of bacteria to screen for • Requires further tests for species identification	• 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	• Extremely expensive • Data handling requirements are significant • Requires training on sample preparation and machine use and experience of interpreting results

Examples of studies using this technique	References (Böttcher et al., 2000, Cardona, 2002, Tjellström et al., 2007)	References (Bennet et al., 2002, Hascoët et al., 2011)	Reference (Fallani et al., 2011)	References (De Filippo et al., 2010, Vaishampayan et al., 2010)
Future use in infant gut microbiota research	Most likely to be used to test novel hypotheses and to be followed up with more detailed techniques	Likely to become infrequently used and to be mainly used in combination with and verified by newer technologies	Likely to remain popular in the coming decade, but decrease thereafter as increased availability and use of sequencing approaches occurs	Increased use since 2000 as cost is decreasing and likely to become the main approach used in the future

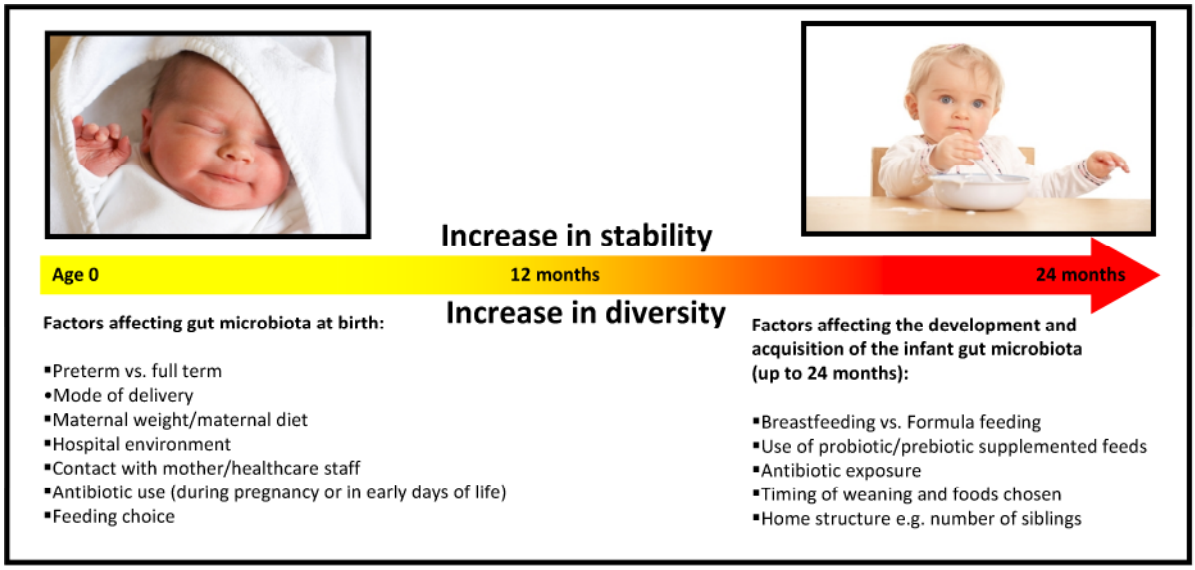

Figure 1 Factors contributing to changes in gut microbiota composition in the first 2 years of life

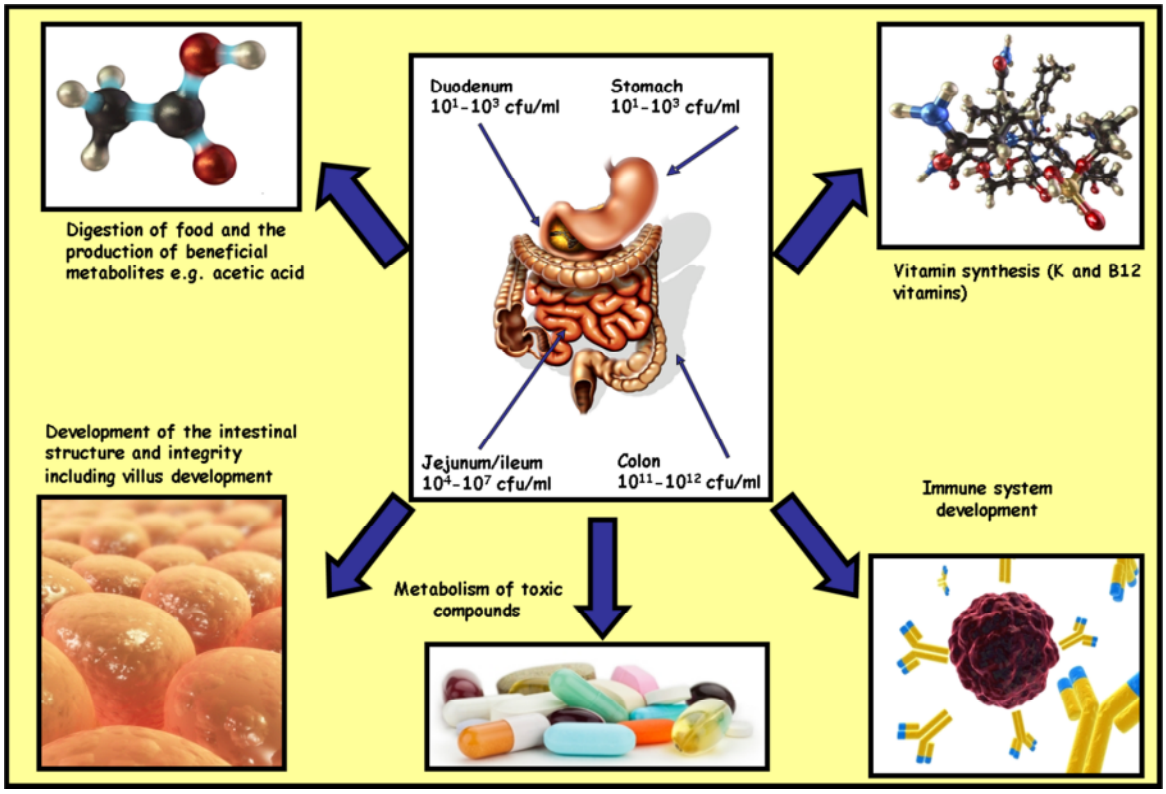

Figure 2 Location, concentrations and functions of human gut microbiota

Chapter 2

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

Published in Antimicrobial Agents & Chemotherapy, Volume 56, Issue 11, November 2012, Pages 5811-5820

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' TACCRGGGTHTCTAATCC), 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'-CGACCACTTCGGCAACCG), 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'-GGTGTTCTTCCCGATATCTACA) 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

Abrahamsson, T. R., Jakobsson, H. E., Andersson, A. F., Björkstén, B., Engstrand, L. & Jenmalm, M. C. 2011. Low diversity of the gut microbiota in infants with atopic eczema. *Journal of allergy and clinical immunology,* 129**,** 434-440.

Acred, P., Brown, D., Turner, D. & Wilson, M. 1962. Pharmacology and chemotherapy of ampicillin—a new broad-spectrum penicillin. *British journal of pharmacology and chemotherapy,* 18**,** 356-369.

Adlerberth, I. & Wold, A. E. 2009. Establishment of the gut microbiota in Western infants. *Acta Pædiatrica,* 98**,** 229-238.

Ajslev, T., Andersen, C., Gamborg, M., Sørensen, T. & Jess, T. 2011. Childhood overweight after establishment of the gut microbiota: the role of delivery mode, pre-pregnancy weight and early administration of antibiotics. *International Journal of Obesity,* 35**,** 522-529.

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research,* 25**,** 3389-3402.

Antonopoulos, D. A., Huse, S. M., Morrison, H. G., Schmidt, T. M., Sogin, M. L. & Young, V. B. 2009. Reproducible community dynamics of the gastrointestinal microbiota following antibiotic perturbation. *Infection and immunity,* 77**,** 2367-2375.

Bennet, R., Eriksson, M. & Nord, C. E. 2002. The fecal microflora of 1–3-month-old infants during treatment with eight oral antibiotics. *Infection,* 30**,** 158-160.

Bezirtzoglou, E., Tsiotsias, A. & Welling, G. W. 2011. Microbiota Profile in Feces of Breast-and Formula-Fed Newborns by Using Fluorescence in situ Hybridization (FISH). *Anaerobe,* 17**,** 478-482.

Blaser, M. 2011. Antibiotic overuse: Stop the killing of beneficial bacteria. *Nature,* 476**,** 393-394.

Boehm, G., Stahl, B., Jelinek, J., Knol, J., Miniello, V. & Moro, G. E. 2005. Prebiotic carbohydrates in human milk and formulas. *Acta Paediatrica,* 94**,** 18-21.

Bourlioux, P., Koletzko, B., Guarner, F. & Braesco, V. 2003. The intestine and its microflora are partners for the protection of the host: report on the Danone Symposium" The Intelligent Intestine," held in Paris, June 14, 2002. *American Journal of Clinical Nutrition,* 78**,** 675-683.

Bush, K. 2010. Alarming beta-lactamase-mediated resistance in multidrug-resistant Enterobacteriaceae. *Current opinion in microbiology,* 13**,** 558-564.

Celedon, J. C., Fuhlbrigge, A., Rifas Shiman, S., Weiss, S. T. & Finkelstein, J. A. 2004. Antibiotic use in the first year of life and asthma in early childhood. *Clinical & Experimental Allergy,* 34**,** 1011-1016.

Cramer, J. P., Burchard, G. D. & Lohse, A. W. 2008. Old dogmas and new perspectives in antibiotic-associated diarrhea. *Medizinische Klinik (Munich, Germany: 1983),* 103**,** 325-338.

Cremonini, F., Di Caro, S., Nista, E. C., Bartolozzi, F., Capelli, G., Gasbarrini, G. & Gasbarrini, A. 2002. Meta analysis: the effect of probiotic administration on antibiotic associated diarrhoea. *Alimentary pharmacology & therapeutics,* 16**,** 1461-1467.

De La Cochetiere, M., Durand, T., Lepage, P., Bourreille, A., Galmiche, J. & Dore, J. 2005. Resilience of the dominant human fecal microbiota upon short-course antibiotic challenge. *Journal of clinical microbiology,* 43**,** 5588-5592.

Dethlefsen, L., Huse, S., Sogin, M. L. & Relman, D. A. 2008. The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. *PLoS Biol,* 6**,** e280. doi: 10.137/journal.pbio.0060280.

Dethlefsen, L. & Relman, D. A. 2011. Incomplete recovery and individualized responses of the human distal gut microbiota to

repeated antibiotic perturbation. *Proceedings of the National Academy of Sciences,* 108**,** 4554-4561.

Dominguez-Bello, M. G., Costello, E. K., Contreras, M., Magris, M., Hidalgo, G., Fierer, N. & Knight, R. 2010. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. *Proceedings of the National Academy of Sciences,* 107**,** 11971-11976.

Donabedian, S., Thal, L., Hershberger, E., Perri, M., Chow, J., Bartlett, P., Jones, R., Joyce, K., Rossiter, S. & Gay, K. 2003. Molecular characterization of gentamicin-resistant enterococci in the United States: evidence of spread from animals to humans through food. *Journal of clinical microbiology,* 41**,** 1109-1113.

Echarri, P. P., Graciá, C. M., Berruezo, G. R., Vives, I., Ballesta, M., Solís, G., Morillas, I. V., Reyes-Gavilán, C. G. D. L., Margolles, A. & Gueimonde, M. 2011. Assessment of intestinal microbiota of full-term breast-fed infants from two different geographical locations. *Early Human Development,* 87**,** 511-513.

Fallani, M., Young, D., Scott, J., Norin, E., Amarri, S., Adam, R., Aguilera, M., Khanna, S., Gil, A. & Edwards, C. A. 2010. Intestinal microbiota of 6-week-old infants across Europe: geographic influence beyond delivery mode, breast-feeding, and antibiotics. *Journal of pediatric gastroenterology and nutrition,* 51**,** 77-84.

Favier, C. F., De Vos, W. M. & Akkermans, A. D. L. 2003. Development of bacterial and bifidobacterial communities in feces of newborn babies. *Anaerobe,* 9**,** 219-229.

Finegold, S. M. 1970. Interaction of antimicrobial therapy and intestinal flora. *American Journal of Clinical Nutrition,* 23**,** 1466-1471.

Foliaki, S., Pearce, N., Björkstén, B., Mallol, J., Montefort, S. & Von Mutius, E. 2009. Antibiotic use in infancy and symptoms of asthma, rhinoconjunctivitis, and eczema in children 6 and 7 years old: International Study of Asthma and Allergies in Childhood

Phase III. *Journal of allergy and clinical immunology,* 124**,** 982-989.

Fouhy, F., Ross, R. P., Fitzgerald, G., Stanton, C. & Cotter, P. D. 2012. Composition of the early intestinal microbiota: Knowledge, knowledge gaps and the use of high-throughput sequencing to address these gaps. *Gut microbes,* 3**,** 203-220.

Fujimura, K. E., Slusher, N. A., Cabana, M. D. & Lynch, S. V. 2010. Role of the gut microbiota in defining human health. *Expert review of anti-infective therapy,* 8**,** 435-454.

Gophna, U., Sommerfeld, K., Gophna, S., Doolittle, W. F. & Veldhuyzen Van Zanten, S. J. O. 2006. Differences between tissue-associated intestinal microfloras of patients with Crohn's disease and ulcerative colitis. *Journal of clinical microbiology,* 44**,** 4136-4141.

Grölund, M. M., Lehtonen, O. P., Eerola, E. & Kero, P. 1999. Fecal microflora in healthy infants born by different methods of delivery: permanent changes in intestinal flora after cesarean delivery. *Journal of pediatric gastroenterology and nutrition,* 28**,** 19-25.

Guarner, F. & Malagelada, J. R. 2003. Gut flora in health and disease. *The Lancet,* 361**,** 512-519.

Haarman, M. & Knol, J. 2005. Quantitative real-time PCR assays to identify and quantify fecal Bifidobacterium species in infants receiving a prebiotic infant formula. *Applied and environmental microbiology,* 71**,** 2318-2324.

Hill, D. A. & Artis, D. 2009. Intestinal bacteria and the regulation of immune cell homeostasis. *Annual review of immunology,* 28**,** 623-667.

Hill, D. A., Hoffmann, C., Abt, M. C., Du, Y., Kobuley, D., Kirn, T. J., Bushman, F. D. & Artis, D. 2010. Metagenomic analyses reveal antibiotic-induced temporal and spatial changes in intestinal microbiota with associated alterations in immune cell homeostasis. *Mucosal immunology,* 3**,** 148-158.

Huson, D. H., Auch, A. F., Qi, J. & Schuster, S. C. 2007. MEGAN analysis of metagenomic data. *Genome research,* 17**,** 377-386.

Hussey, S., Wall, R., Gruffman, E., O'sullivan, L., Ryan, C. A., Murphy, B., Fitzgerald, G., Stanton, C. & Ross, R. P. 2011. Parenteral Antibiotics Reduce Bifidobacteria Colonization and Diversity in Neonates. *International journal of microbiology* vol. 2011**,** doi:10.1155/2011/130574.

Huycke, M., Spiegel, C. & Gilmore, M. 1991. Bacteremia caused by hemolytic, high-level gentamicin-resistant Enterococcus faecalis. *Antimicrobial agents and chemotherapy,* 35**,** 1626-1634.

Ivanov, I. I., Frutos, R. L., Manel, N., Yoshinaga, K., Rifkin, D. B., Sartor, R. B., Finlay, B. B. & Littman, D. R. 2008. Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. *Cell host & microbe,* 4**,** 337-349.

Jakobsson, H. E., Jernberg, C., Andersson, A. F., Sjölund-Karlsson, M., Jansson, J. K. & Engstrand, L. 2010. Short-term antibiotic treatment has differing long-term impacts on the human throat and gut microbiome. *PLoS ONE,* 5**,** e9836. doi:10.1371/journal.pone.0009836

Jernberg, C., Lofmark, S., Edlund, C. & Jansson, J. K. 2010. Long-term impacts of antibiotic exposure on the human intestinal microbiota. *Microbiology,* 156**,** 3216-3223.

Johnson, C. C., Ownby, D. R., Alford, S. H., Havstad, S. L., Williams, L., Zoratti, E. M., Peterson, E. L. & Joseph, C. L. M. 2005. Antibiotic exposure in early infancy and risk for childhood atopy. *Journal of allergy and clinical immunology,* 115**,** 1218-1224.

Jureen, R., Top, J., Mohn, S. C., Harthug, S., Langeland, N. & Willems, R. J. L. 2003. Molecular characterization of ampicillin-resistant Enterococcus faecium isolates from hospitalized patients in Norway. *Journal of clinical microbiology,* 41**,** 2330-2336.

Kassinen, A., Krogius-Kurikka, L., Mäkivuokko, H., Rinttilä, T., Paulin, L., Corander, J., Malinen, E., Apajalahti, J. & Palva, A. 2007. The

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

Kim, B. J., Kim, H. Y., Yun, Y. J. & Kook, Y. H. 2010. Differentiation of Bifidobacterium species using partial RNA polymerase {beta}-subunit (rpoB) gene sequences. *International journal of systematic and evolutionary microbiology,* 60**,** 2697-2704.

Kozyrskyj, A. L., Ernst, P. & Becker, A. B. 2007. Increased Risk of Childhood Asthma From Antibiotic Use in Early Life. *Chest,* 131**,** 1753-1759.

Le Huërou-Luron, I., Blat, S. & Boudry, G. 2010. Breast-v. formula-feeding: impacts on the digestive tract and immediate and long-term health effects. *Nutr Res Rev,* 23**,** 23-36.

Ley, R. E. 2010. Obesity and the human microbiome. *Current opinion in gastroenterology,* 26**,** 5-11.

Mai, V. & Draganov, P. V. 2009. Recent advances and remaining gaps in our knowledge of associations between gut microbiota and human health. *World journal of gastroenterology: WJG,* 15**,** 81-85.

Mangin, I., Suau, A., Gotteland, M., Brunser, O. & Pochart, P. 2010. Amoxicillin treatment modifies the composition of Bifidobacterium species in infant intestinal microbiota. *Anaerobe,* 16**,** 433-438.

Matsuki, T., Watanabe, K., Fujimoto, J., Kado, Y., Takada, T., Matsumoto, K. & Tanaka, R. 2004. Quantitative PCR with 16S rRNA-gene-targeted species-specific primers for analysis of human intestinal bifidobacteria. *Applied and environmental microbiology,* 70**,** 167-173.

Mättö, J., Van Hoek, A. H. a. M., Domig, K. J., Saarela, M., Floréz, A. B., Brockmann, E., Amtmann, E., Mayo, B., Aarts, H. J. M. & Danielsen, M. 2007. Susceptibility of human and probiotic Bifidobacterium spp. to selected antibiotics as determined by the Etest method. *International Dairy Journal,* 17**,** 1123-1131.

Murphy, E., Cotter, P., Healy, S., Marques, T., O'sullivan, O., Fouhy, F., Clarke, S., O'toole, P., Quigley, E. & Stanton, C. 2010. Composition and energy harvesting capacity of the gut microbiota: relationship to diet, obesity and time in mouse models. *Gut,* 59**,** 1635-1642.

Nordmann, P. 1998. Trends in -lactam resistance among Enterobacteriaceae. *Clinical infectious diseases,* 27**,** S100-S106.

O' Sullivan, Ó., Coakley, M., Lakshmiwarayanaw, B., Claesson, M., Stanton, C., O'toole, P. & Ross, R. 2011. Correlation of rRNA gene amplicon pyrosequencing and bacterial culture for microbial compositional analysis of faecal samples from elderly Irish subjects. *Journal of applied microbiology,* 111**,** 467-473.

Olszak T, A. D., Zeissig S, Vera Pm, Richter J, Franke a, Glickman Jn, Siebert R, Baron Rm, Kasper Dl, Blumberg Rs. 2012. Microbial Exposure During Early Life Has Persistent Effects on Natural Killer T Cell Function. *Science***,** DOI: 10.1126/science.1219328

Parracho, H., Mccartney, A. L. & Gibson, G. R. 2007. Probiotics and prebiotics in infant nutrition. *Proceedings of the Nutrition Society,* 66**,** 405-411.

Penders, J., Thijs, C., Vink, C., Stelma, F. F., Snijders, B., Kummeling, I., Van Den Brandt, P. A. & Stobberingh, E. E. 2006. Factors influencing the composition of the intestinal microbiota in early infancy. *Pediatrics,* 118**,** 511-521.

Qin, X., Zerr, D. M., Weissman, S. J., Englund, J. A., Denno, D. M., Klein, E. J., Tarr, P. I., Kwong, J., Stapp, J. R. & Tulloch, L. G. 2009. Prevalence and Mechanisms of Broad-Spectrum {beta}-Lactam Resistance in Enterobacteriaceae: a Children's Hospital Experience. *Antimicrobial agents and chemotherapy,* 53**,** 3909-3914.

Rea, M. C., Dobson, A., O'sullivan, O., Crispie, F., Fouhy, F., Cotter, P. D., Shanahan, F., Kiely, B., Hill, C. & Ross, R. P. 2011. Effect of broad-and narrow-spectrum antimicrobials on Clostridium difficile

and microbial diversity in a model of the distal colon. *Proceedings of the National Academy of Sciences,* 108**,** 4639-4645.

Robinson, C. J. & Young, V. B. 2010. Antibiotic administration alters the community structure of the gastrointestinal microbiota. *Gut microbes,* 1**,** 279-284.

Sazawal, S., Dhingra, U., Hiremath, G., Sarkar, A., Dhingra, P., Dutta, A., Verma, P., Menon, V. P. & Black, R. E. 2010. Prebiotic and probiotic fortified milk in prevention of morbidities among children: community-based, randomized, double-blind, controlled trial. *PLoS ONE,* 5**,** e12164.

Schloss, P. & Handelsman, J. 2008. A statistical toolbox for metagenomics: assessing functional diversity in microbial communities. *Bmc Bioinformatics,* 9**,** 34-49.

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., Lesniewski, R. A., Oakley, B. B., Parks, D. H. & Robinson, C. J. 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and environmental microbiology,* 75**,** 7537-7541.

Schwiertz, A., Gruhl, B., Löbnitz, M. & Michel, P. 2003. Development of the intestinal bacterial composition in hospitalized preterm infants in comparison with breast-fed, full-term infants. *Pediatric research,* 54**,** 393-399.

Sekirov, I., Russell, S. L., Antunes, L. C. M. & Finlay, B. B. 2010. Gut microbiota in health and disease. *Physiological reviews,* 90**,** 859-904.

Sherman, P. M., Cabana, M., Gibson, G. R., Koletzko, B. V., Neu, J., Veereman-Wauters, G., Ziegler, E. E. & Walker, W. A. 2009. Potential roles and clinical utility of prebiotics in newborns, infants, and children: proceedings from a global prebiotic summit meeting, New York City, June 27-28, 2008. *The Journal of Pediatrics,* 155**,** S61-S70.

Suchodolski, J., Dowd, S., Westermarck, E., Steiner, J., Wolcott, R., Spillmann, T. & Harmoinen, J. 2009. The effect of the macrolide antibiotic tylosin on microbial diversity in the canine small intestine as demonstrated by massive parallel 16S rRNA gene sequencing. *BMC microbiology,* 9**,** 210-226.

Sullivan, Å., Edlund, C. & Nord, C. E. 2001. Effect of antimicrobial agents on the ecological balance of human microflora. *The Lancet infectious diseases,* 1**,** 101-114.

Tanaka, S., Kobayashi, T., Songjinda, P., Tateyama, A., Tsubouchi, M., Kiyohara, C., Shirakawa, T., Sonomoto, K. & Nakayama, J. 2009. Influence of antibiotic exposure in the early postnatal period on the development of intestinal microbiota. *FEMS Immunology & Medical Microbiology,* 56**,** 80-87.

Turnbaugh, P. J. & Gordon, J. I. 2009. The core gut microbiome, energy balance and obesity. *The Journal of physiology,* 587**,** 4153-4158.

Urich, T., Lanzén, A., Qi, J., Huson, D. H., Schleper, C. & Schuster, S. C. 2008. Simultaneous assessment of soil microbial community structure and function through analysis of the meta-transcriptome. *PLoS ONE,* 3**,** e2527. doi:10.1371/journal.pone.0002527

Weisser, M., Oostdijk, E. A., Willems, R. J. L., Bonten, M. J. M., Frei, R., Elzi, L., Halter, J., Widmer, A. F. & Top, J. 2012. Dynamics of ampicillin-resistant Enterococcus faecium clones colonizing hospitalized patients: Data from a prospective observational study. *BMC Infectious Diseases,* 12**,** 68-77.

Westerbeek, E. a. M., Van Den Berg, A., Lafeber, H. N., Knol, J., Fetter, W. P. F. & Van Elburg, R. M. 2006. The intestinal bacterial colonisation in preterm infants: a review of the literature. *Clinical Nutrition,* 25**,** 361-368.

Zhang, H., Dibaise, J. K., Zuccolo, A., Kudrna, D., Braidotti, M., Yu, Y., Parameswaran, P., Crowell, M. D., Wing, R. & Rittmann, B. E. 2009. Human gut microbiota in obesity and after gastric bypass.

Proceedings of the National Academy of Sciences, 106**,** 2365-2370.

Zoetendal, E., Rajilić-Stojanović, M. & De Vos, W. 2008. High-throughput diversity and functionality analysis of the gastrointestinal tract microbiota. *Gut,* 57**,** 1605-1615.

Table 1 Details of the infants in the trial

Infant	Sex*	Mode of delivery	Feeding method	Duration of antibiotic treatment (days)
A	M	Caesarean section	Breastfed	9
B	M	Caesarean section	Breasfed+Formula	5
C	M	Caesarean section	Breastfed	2
D	M	Vaginal delivery	Formula	2
E	F	Caesarean section	Formula	5
F	F	Vaginal delivery	Breastfed	2
G	F	Vaginal delivery	Breastfed	2
H	M	Caesarean section	Formula	2
I	M	Vaginal delivery	Formula	2
J	M	Vaginal delivery	Formula	-
K	M	Vaginal delivery	Formula	-
L	F	Vaginal delivery	Formula	-
M	F	Vaginal delivery	Formula	-
N	M	Vaginal delivery	Breastfed	-
O	F	Vaginal delivery	Breastfed	-
P	M	Vaginal delivery	Breastfed	-
Q	M	Vaginal delivery	Formula	-
R	F	Vaginal delivery	Formula	-

* 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

Data set	Treated week 4	Control week 4	Treated week 8	Control week 8
Similarity	97%	97%	97%	97%
Chao 1 richness estimation	243	364	334	490
Shannon's index for diversity	3.6	3.8	3.8	4.6
Good's coverage	96.1	94.3	93.2	88.6

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

Treated	Wk 4	Wk 8	P	Controls	Wk 4	Wk 8	P
B	$9.79x10^7$	$6.57x10^7$	0.7728	K	$6.23x10^7$	$7.35x10^7$	0.1489
F	$5.89x10^7$	$3.53x10^8$	0.0809	L	$2.19x10^7$	$3.61x10^8$	0.0809
G	$3.28x10^7$	$7.79x10^7$	0.0518	M	$2.37x10^7$	$2.18x10^8$	0.0518
H	$3.52x10^7$	$6.43x10^8$	0.1489	N	$9.05x10^7$	$5.75x10^6$	0.0765
Average	$4.78x10^7$	$2.48x10^8$	0.0005		$4.96x10^7$	$1.91x10^8$	0.0289

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

Treated	Wk 4	Wk 8	P	Controls	Wk 4	Wk 8	P
B	$1.49x10^{4}$	$1.76x10^{7}$	0.0814	K	$6.62x10^{7}$	$5.98x10^{5}$	0.0809
F	$7.32x10^{6}$	$1.10x10^{9}$	0.0809	L	$7.95x10^{6}$	$5.11x10^{4}$	0.0809
G	$2.51x10^{5}$	$1.93x10^{5}$	0.2472	M	$1.48x10^{6}$	$2.39x10^{8}$	0.0809
H	$5.55x10^{7}$	$4.57x10^{8}$	0.0304	N	$4.75x10^{6}$	$5.47x10^{6}$	0.7728
				O	$5.05x10^{7}$	$1.14x10^{8}$	0.0369
Average	$1.58x10^{7}$	$3.94x10^{8}$	0.4273		$2.62x10^{7}$	$7.18x10^{7}$	0.1548

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.

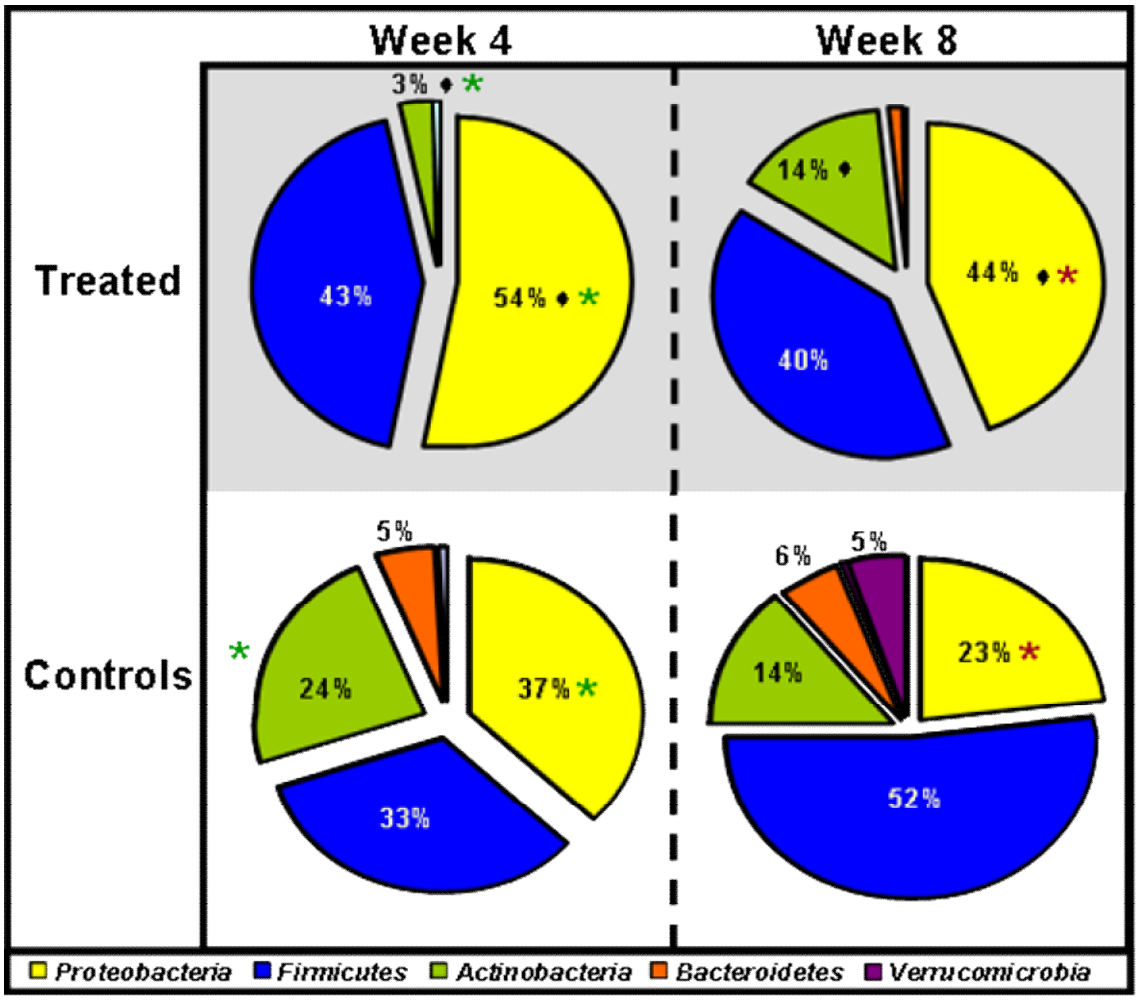

Figure 1 Microbial distributions at phylum level in the treated and control samples at week 4 and week 8. Statistically significant differences between treated samples and controls at week 4 are denoted by * (where $p < 0.05$). Statistically significant differences between treated samples and controls 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.

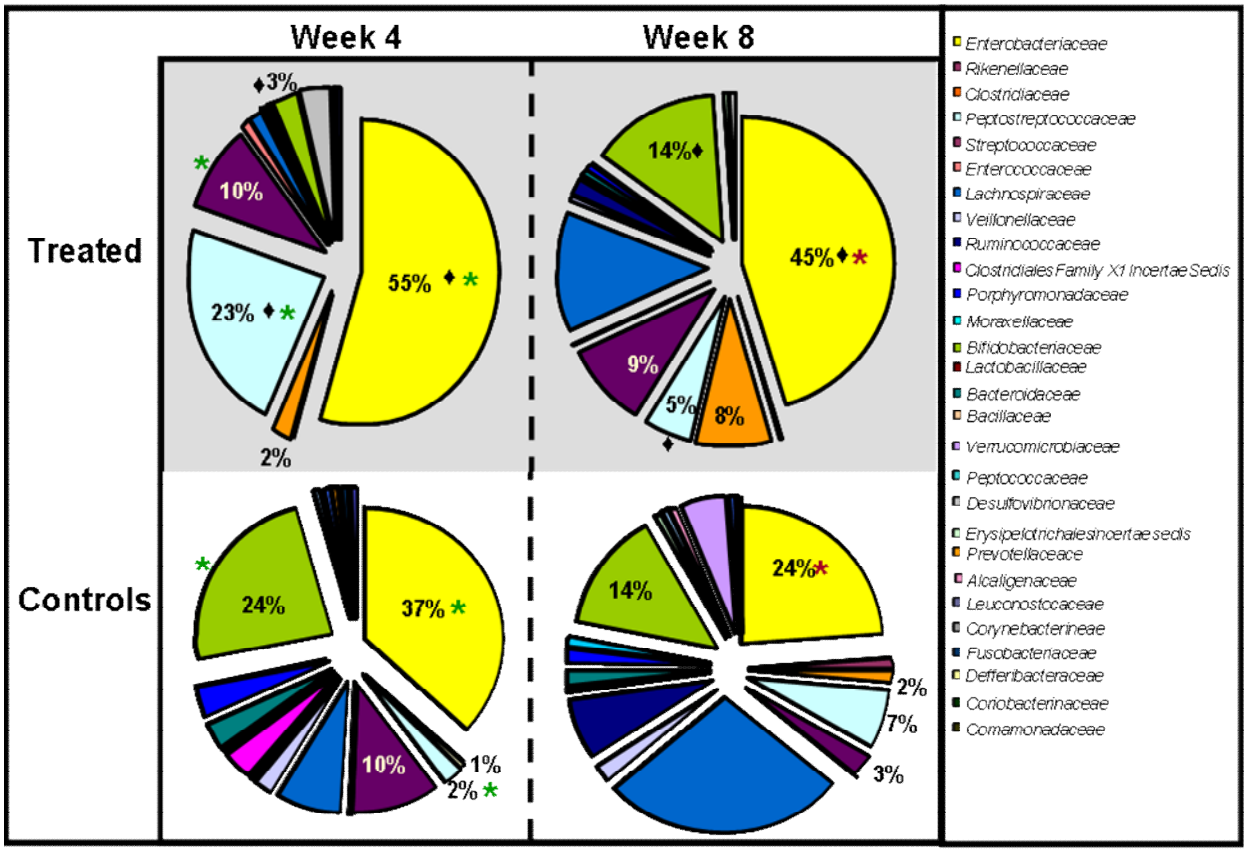

Figure 2 Microbial distributions at the family level in the treated and control samples at week 4 and week 8. Statistically significant differences between treated samples and controls at week 4 are denoted by * (where $p<0.05$). Statistically significant differences between treated samples and controls 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.

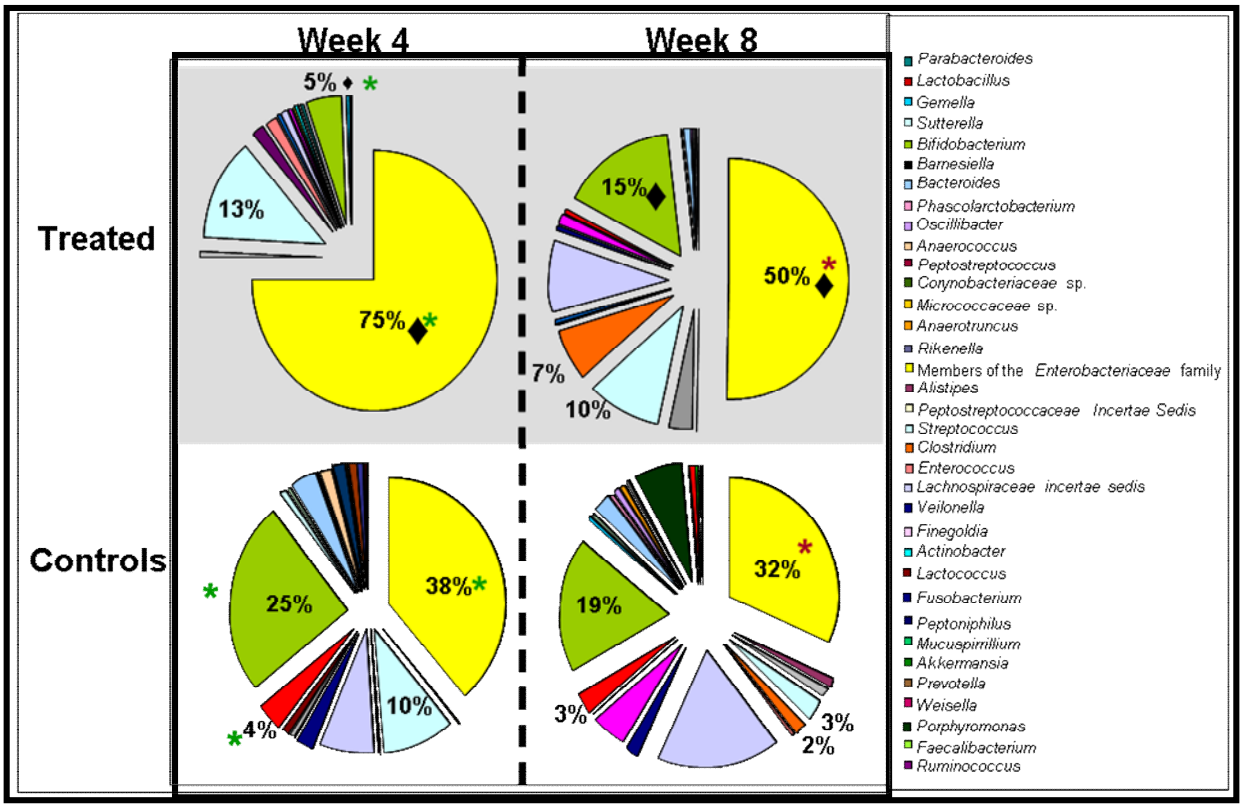

Figure 3 Microbial distributions at the genus level in the treated and control samples at week 4 and week 8. Statistically significant differences between treated samples and controls at week 4 are denoted by * (where $p<0.05$). Statistically significant differences between treated samples and controls 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.

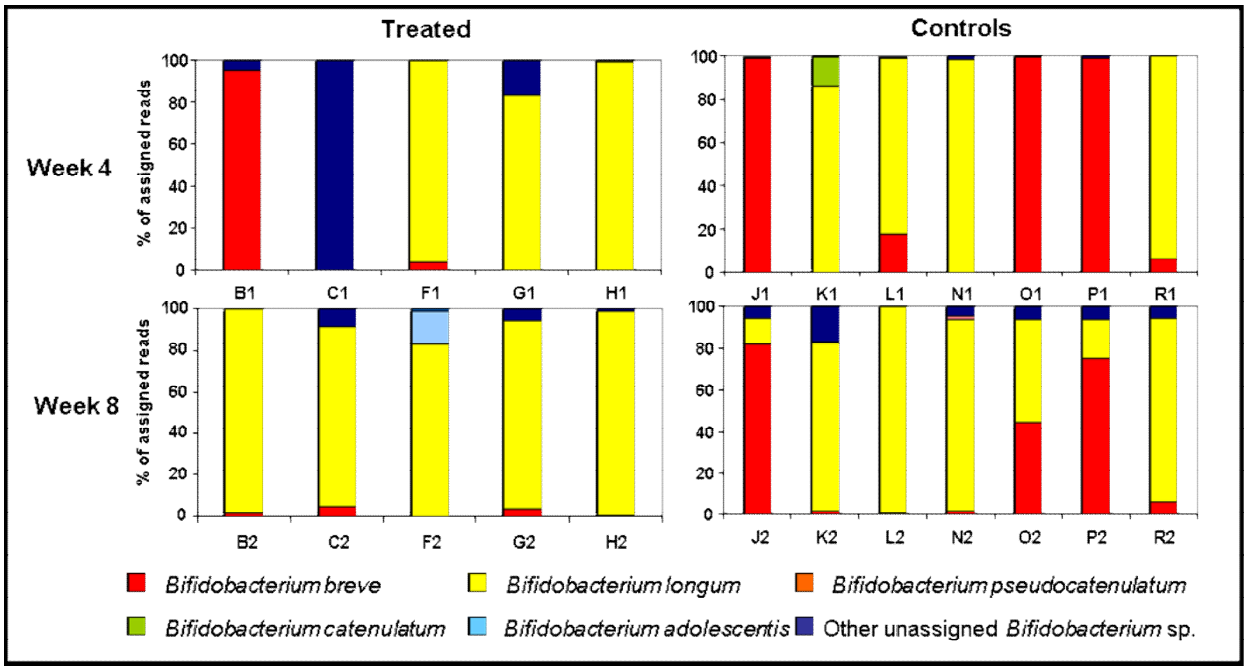

Figure 4 Individual distributions of bifidobacteria in the treated (B1-H1; B2-H2) and control samples (J1-R1; J2-R2) as detected using *rpoB* amplicons for 454-pyrosequencing. Values show the percentage of the different bifidobacteria species present in the individual samples. Treated samples show far 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 S1 *rpoB* primer sequences for 454-pyrosequencing of bifidobacteria populations

Name	Amplicon A adaptor	Barcode	Oligosequence
BC1	CGTATCGCCTCCCTCGCGCCATCAG	AGAGAGAG	TCGATCGGGCACATACGG
BC2	CGTATCGCCTCCCTCGCGCCATCAG	AGAGATGC	TCGATCGGGCACATACGG
BC3	CGTATCGCCTCCCTCGCGCCATCAG	AGAGCAGC	TCGATCGGGCACATACGG
BC4	CGTATCGCCTCCCTCGCGCCATCAG	AGAGCATG	TCGATCGGGCACATACGG
BC5	CGTATCGCCTCCCTCGCGCCATCAG	AGA TCATC	TCGATCGGGCACATACGG
BC6	CGTATCGCCTCCCTCGCGCCATCAG	AGATCTGC	TCGATCGGGCACATACGG
BC7	CGTATCGCCTCCCTCGCGCCATCAG	AGATGAGC	TCGATCGGGCACATACGG
BC8	CGTATCGCCTCCCTCGCGCCATCAG	AGATGATG	TCGATCGGGCACATACGG
BC9	CGTATCGCCTCCCTCGCGCCATCAG	AGATGCAG	TCGATCGGGCACATACGG
BC10	CGTATCGCCTCCCTCGCGCCATCAG	AGATGCTC	TCGATCGGGCACATACGG
BC11	CGTATCGCCTCCCTCGCGCCATCAG	AGCAGAGC	TCGATCGGGCACATACGG
BC12	CGTATCGCCTCCCTCGCGCCATCAG	AGCAGATG	TCGATCGGGCACATACGG
Rev 1	CTATGCGCCTTGCCAGCCCGCTCAG	None	CGACCACTTCGGCAACCG

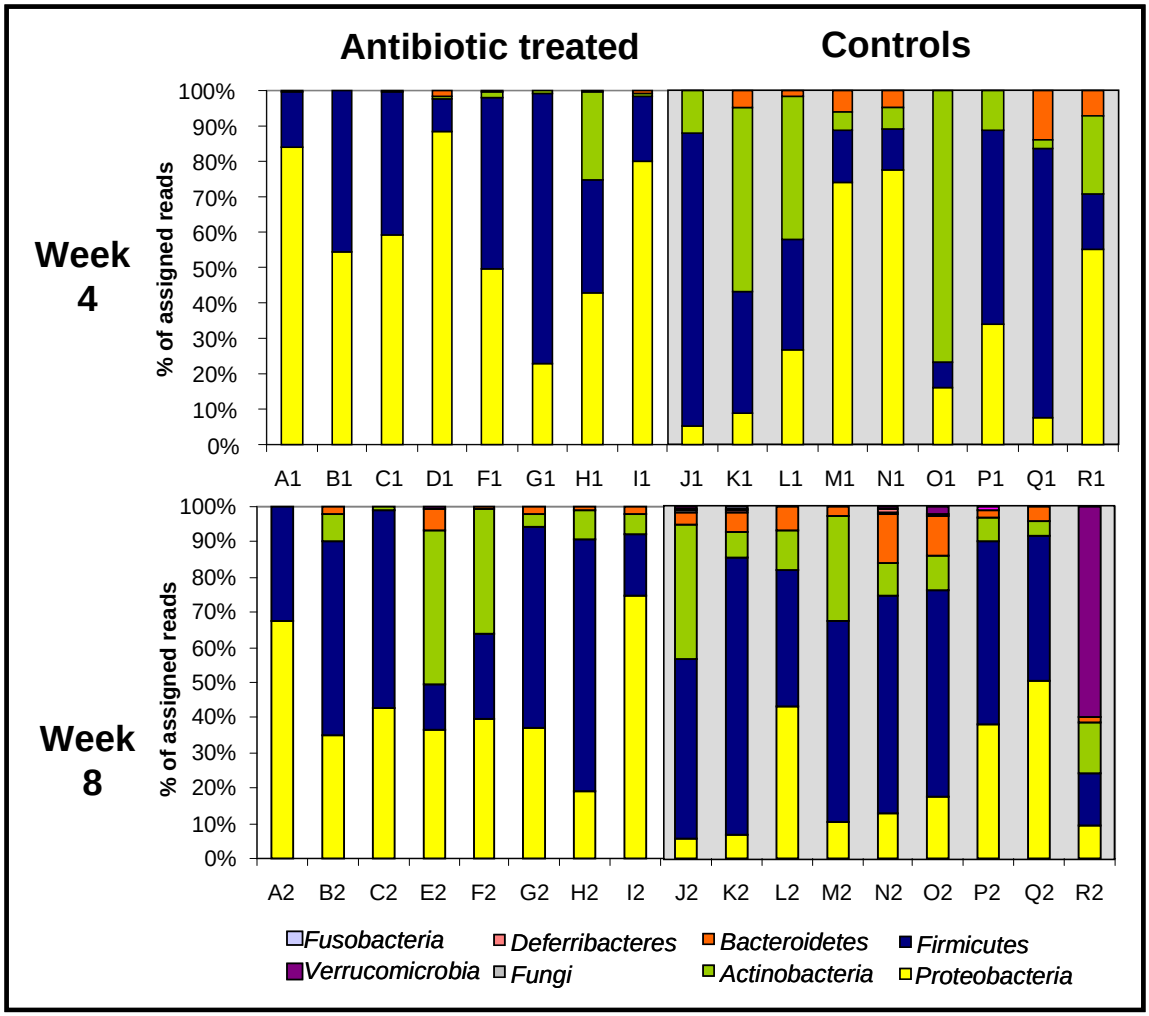

Figure S1 Distribution of microbiota at phylum level in the antibiotic-treated (A1-I1) and control infants (J1-R1) at week 4 and week 8, showing the limited number of phyla detected in the antibiotic-treated infants compared to the controls and also showing the individual variability that exists between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

Published in BMC Microbiology BMC Microbiology Feb 2014; 14 (25) doi:10.1186/1471-2180-14-25

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 (Kotra 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 *bla*$_{TEM}$ (Bailey et al., 2011, Tenover et al., 1994), *bla*$_{SHV}$ (Briñas et al., 2002) (both of which are classified as Bush group 2b β-lactamases), *bla*$_{CTX-M}$ (Monstein et al., 2009) (an extended spectrum β-lactamase (ESBL) which confers resistance to cefotaxime), *bla*$_{OXA}$ (Briñas et al., 2002) (ESBL, Bush group 2d ESBL) and *bla*$_{ROB}$ (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 *bla*$_{TEM}$ primer set 1 (RH605/606), *bla*$_{TEM}$ primer set 2 and *bla*$_{CTX-M}$, PCRs were completed as previously outlined. For the primers *bla*$_{OXA}$ and *bla*$_{ROB}$ 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 (*bla*$_{oxa}$) or 62°C (*bla*$_{ROB}$) and 72°C x 30s followed by 72°C x 10 mins and held at 4°C. For *bla*$_{SHV}$ 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)-VIa), *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')-*Ie*-*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*$_{TEM}$ 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*$_{TEM}$ 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*$_{TEM}$ 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*$_{TEM}$ primers also amplified genes that resembled *bla*$_{TEM}$ 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 *bla*$_{\text{TEM-116}}$, *bla*$_{\text{TEM-195}}$ and *bla*$_{\text{TEM-96}}$ amongst others, were also identified, with their closest homologues being members of the *Proteobacteria* (Table 2).

Using the *bla*$_{\text{SHV}}$ primers, multiple genes sharing homology with genes from members of the *Enterobacteriaceae*, and *Klebsiella* and *E. coli* in particular were detected. In addition, amplicons with low percentage identity to genes from *Alloprevotella rava* and *Parabacteroides johnsonii*, respectively, were also identified. This is again consistent with existing research which states that *Enterobacteriaceae* are the primary source of *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 *bla*$_{\text{SHV-132}}$, *bla*$_{\text{SHV-140}}$ and *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 β-lactamases (Table 2). A number of different CTX-M β-lactamases were detected, including CTX-M-1, CTX-M-15 and CTX-M-36. The fact that many of the β-lactamase genes detected using our approach share homology with resistance genes found in members of the phylum *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 $bla_{\text{CTX-M-15}}$ 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 *bla*$_{ROB}$ 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 *Staphylococcus haemolyticus bla*$_{ROB}$ gene. Finally, this study did not detect *bla*$_{OXA}$ 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.

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')-*Ie*-*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

Abraham, E. & Chain, E. 1940. An enzyme from bacteria able to destroy penicillin. *Nature,* 146**,** 837-837.

Babini, G. S. & Livermore, D. M. 2000. Antimicrobial resistance amongst Klebsiella spp. collected from intensive care units in Southern and Western Europe in 1997–1998. *Journal of antimicrobial chemotherapy,* 45**,** 183-189.

Bailey, J. K., Pinyon, J. L., Anantham, S. & Hall, R. M. 2011. Distribution of the blaTEM gene and blaTEM-containing transposons in commensal Escherichia coli. *Journal of antimicrobial chemotherapy,* 66**,** 745-751.

Bhullar, K., Waglechner, N., Pawlowski, A., Koteva, K., Banks, E. D., Johnston, M. D., Barton, H. A. & Wright, G. D. 2012. Antibiotic resistance is prevalent in an isolated cave microbiome. *PLoS ONE,* 7**,** e34953.

Bonnet, R. 2004. Growing group of extended-spectrum β-lactamases: the CTX-M enzymes. *Antimicrobial agents and chemotherapy,* 48**,** 1-14.

Boyd, D. A., Tyler, S., Christianson, S., Mcgeer, A., Muller, M. P., Willey, B. M., Bryce, E., Gardam, M., Nordmann, P. & Mulvey, M. R. 2004. Complete nucleotide sequence of a 92-kilobase plasmid harboring the CTX-M-15 extended-spectrum beta-lactamase involved in an outbreak in long-term-care facilities in Toronto, Canada. *Antimicrobial agents and chemotherapy,* 48**,** 3758-3764.

Briñas, L., Zarazaga, M., Sáenz, Y., Ruiz-Larrea, F. & Torres, C. 2002. β-Lactamases in ampicillin-resistant Escherichia coli isolates from foods, humans, and healthy animals. *Antimicrobial agents and chemotherapy,* 46**,** 3156-3163.

Broaders, E., Gahan, C. G. & Marchesi, J. R. 2013. Mobile genetic elements of the human gastrointestinal tract: Potential for spread of antibiotic resistance genes. *Gut microbes,* 4**,** 271-280.

Bush, K. 1989. Characterization of beta-lactamases. *Antimicrobial agents and chemotherapy,* 33**,** 259-263.

Bush, K. 2010. Alarming beta-lactamase-mediated resistance in multidrug-resistant Enterobacteriaceae. *Current opinion in microbiology,* 13**,** 558-564.

Cdc 2013. Antibiotic resistance threats in the United States

Cho, Y. J., Moon, D. C., Jin, J. S., Choi, C. H., Lee, Y. C. & Lee, J. C. 2009. Genetic basis of resistance to aminoglycosides in Acinetobacter spp. and spread of armA in Acinetobacter baumannii sequence group 1 in Korean hospitals. *Diagnostic microbiology and infectious disease,* 64**,** 185-190.

Coque, T., Baquero, F. & Canton, R. 2008. Increasing prevalence of ESBL-producing Enterobacteriaceae in Europe. *Euro surveillance: bulletin européen sur les maladies transmissibles= European communicable disease bulletin,* 13.

Coque, T. M., Oliver, A., Pérez-Díaz, J. C., Baquero, F. & Cantón, R. 2002. Genes Encoding TEM-4, SHV-2, and CTX-M-10 Extended-Spectrum β-Lactamases Are Carried by Multiple Klebsiella pneumoniae Clones in a Single Hospital (Madrid, 1989 to 2000). *Antimicrobial agents and chemotherapy,* 46**,** 500-510.

Cotter, P., Stanton, C., Ross, R. & Hill, C. 2012. The impact of antibiotics on the gut microbiota as revealed by high throughput DNA sequencing. *Discovery medicine,* 13**,** 193-199.

D'costa, V. M., King, C. E., Kalan, L., Morar, M., Sung, W. W. L., Schwarz, C., Froese, D., Zazula, G., Calmels, F. & Debruyne, R. 2011. Antibiotic resistance is ancient. *Nature***,** 457-461.

Dallenne, C., Da Costa, A., Decré, D., Favier, C. & Arlet, G. 2010. Development of a set of multiplex PCR assays for the detection of genes encoding important β-lactamases in Enterobacteriaceae. *Journal of antimicrobial chemotherapy,* 65**,** 490-495.

Davies, J. & Davies, D. 2010. Origins and evolution of antibiotic resistance. *Microbiology and Molecular Biology Reviews,* 74**,** 417-433.

De Fátima Silva Lopes, M., Ribeiro, T., Abrantes, M., Figueiredo Marques, J. J., Tenreiro, R. & Crespo, M. T. B. 2005. Antimicrobial resistance profiles of dairy and clinical isolates and type strains of enterococci. *International journal of food microbiology,* 103**,** 191-198.

De Vries, L. E., Vallès, Y., Agersø, Y., Vaishampayan, P. A., García-Montaner, A., Kuehl, J. V., Christensen, H., Barlow, M. & Francino, M. P. 2011. The gut as reservoir of antibiotic resistance: microbial diversity of tetracycline resistance in mother and infant. *PLoS ONE,* 6**,** e21644.

Dethlefsen, L., Huse, S., Sogin, M. L. & Relman, D. A. 2008. The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. *PLoS Biol,* 6**,** e280. doi: 10.137/journal.pbio.0060280.

Dijkshoorn, L., Nemec, A. & Seifert, H. 2007. An increasing threat in hospitals: multidrug-resistant Acinetobacter baumannii. *Nature Reviews Microbiology,* 5**,** 939-951.

Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S. R., Nelson, K. E. & Relman, D. A. 2005. Diversity of the human intestinal microbial flora. *Science,* 308**,** 1635-1638.

Ferretti, J. J., Gilmore, K. & Courvalin, P. 1986. Nucleotide sequence analysis of the gene specifying the bifunctional 6'-aminoglycoside acetyltransferase 2"-aminoglycoside phosphotransferase enzyme in Streptococcus faecalis and identification and cloning of gene regions specifying the two activities. *Journal of bacteriology,* 167**,** 631-638.

Fouhy, F., Guinane, C. M., Hussey, S., Wall, R., Ryan, C. A., Dempsey, E. M., Murphy, B., Ross, R. P., Fitzgerald, G. F. & Stanton, C.

2012. High-throughput sequencing reveals the incomplete, short-term, recovery of the infant gut microbiota following parenteral antibiotic treatment with ampicillin and gentamicin. *Antimicrobial agents and chemotherapy,* 56**,** 5811-5820.

Fouhy, F., Ross, Rp, Fitzgerald, Gf, Stanton C, Cotter Pd. 2013. PCR sequencing data of aminoglycoside and beta-lactam resistance genes. *BMC microbiology* [Online]. Available: http://dx.doi.org/10.6070/H42V2D1V.

Han, D., Unno, T., Jang, J., Lim, K., Lee, S.-N., Ko, G., Sadowsky, M. J. & Hur, H.-G. 2011. The occurrence of virulence traits among high-level aminoglycosides resistant Enterococcus isolates obtained from feces of humans, animals, and birds in South Korea. *International journal of food microbiology,* 144**,** 387-392.

Hegstad, K., Mikalsen, T., Coque, T., Werner, G. & Sundsfjord, A. 2010. Mobile genetic elements and their contribution to the emergence of antimicrobial resistant Enterococcus faecalis and Enterococcus faecium. *Clinical Microbiology and Infection,* 16**,** 541-554.

Heritage, J., M'zali, F. H., Gascoyne-Binzi, D. & Hawkey, P. M. 1999. Evolution and spread of SHV extended-spectrum β-lactamases in Gram-negative bacteria. *Journal of antimicrobial chemotherapy,* 44**,** 309-318.

Heuer, H., Krögerrecklenfort, E., Wellington, E., Egan, S., Elsas, J. V., Overbeek, L., Collard, J. M., Guillaume, G., Karagouni, A. & Nikolakopoulou, T. 2002. Gentamicin resistance genes in environmental bacteria: prevalence and transfer. *FEMS microbiology ecology,* 42**,** 289-302.

Hughes, V. M. & Datta, N. 1983. Conjugative plasmids in bacteria of the `pre-antibiotic' era. *Nature,* 302**,** 725-726.

Kotra, L. P. & Mobashery, S. 1998. β-Lactam antibiotics, β-lactamases and bacterial resistance. *Bulletin de l'Institut Pasteur,* 96**,** 139-150.

Lambert, T., Gerbaud, G. & Courvalin, P. 1994. Characterization of the chromosomal aac (6')-Ij gene of Acinetobacter sp. 13 and the aac

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

Lavollay, M., Mamlouk, K., Frank, T., Akpabie, A., Burghoffer, B., Redjeb, S. B., Bercion, R., Gautier, V. & Arlet, G. 2006. Clonal dissemination of a CTX-M-15 β-lactamase-producing Escherichia coli strain in the Paris area, Tunis, and Bangui. *Antimicrobial agents and chemotherapy,* 50**,** 2433-2438.

Leclercq, R. 1997. Enterococci acquire new kinds of resistance. *Clinical infectious diseases,* 24**,** S80-S84.

Matsumura, M., Katakura, Y., Imanaka, T. & Aiba, S. 1984. Enzymatic and nucleotide sequence studies of a kanamycin-inactivating enzyme encoded by a plasmid from thermophilic bacilli in comparison with that encoded by plasmid pUB110. *Journal of bacteriology,* 160**,** 413-420.

Mckay, G., Thompson, P. & Wright, G. 1994. Broad spectrum aminoglycoside phosphotransferase type III from Enterococcus: overexpression, purification, and substrate specificity. *Biochemistry,* 33**,** 6936-6944.

Mingeot-Leclercq, M. P., Glupczynski, Y. & Tulkens, P. M. 1999. Aminoglycosides: activity and resistance. *Antimicrobial agents and chemotherapy,* 43**,** 727-737.

Mokracka, J., Koczura, R. & Kaznowski, A. 2012. Multiresistant Enterobacteriaceae with class 1 and class 2 integrons in a municipal wastewater treatment plant. *Water research,* 46**,** 3353-3363.

Monstein, H.-J., Tärnberg, M. & Nilsson, L. E. 2009. Molecular identification of CTX-M and blaOXY/K1 β-lactamase genes in Enterobacteriaceae by sequencing of universal M13-sequence tagged PCR-amplicons. *BMC Infectious Diseases,* 9**,** 7-15.

Montecalvo, M. A., Horowitz, H., Gedris, C., Carbonaro, C., Tenover, F. C., Issah, A., Cook, P. & Wormser, G. P. 1994. Outbreak of vancomycin-, ampicillin-, and aminoglycoside-resistant

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

Morosini, M. I., Canton, R., Martinez-Beltran, J., Negri, M. C., Perez-Diaz, J. C., Baquero, F. & Blazquez, J. 1995. New extended-spectrum TEM-type beta-lactamase from Salmonella enterica subsp. enterica isolated in a nosocomial outbreak. *Antimicrobial agents and chemotherapy,* 39**,** 458-461.

Morris, D., Whelan, M., Corbett-Feeney, G., Cormican, M., Hawkey, P., Li, X. & Doran, G. 2006. First Report of Extended-Spectrum-ß-Lactamase-Producing Salmonella enterica Isolates in Ireland. *Antimicrobial agents and chemotherapy,* 50**,** 1608-1609.

Page, M. G. P. 2012. Beta-Lactam Antibiotics. *Antibiotic Discovery and Development***,** 79-117.

Park, C. H., Robicsek, A., Jacoby, G. A., Sahm, D. & Hooper, D. C. 2006. Prevalence in the United States of aac (6')-Ib-cr encoding a ciprofloxacin-modifying enzyme. *Antimicrobial agents and chemotherapy,* 50**,** 3953-3955.

Paterson, D. L., Hujer, K. M., Hujer, A. M., Yeiser, B., Bonomo, M. D., Rice, L. B. & Bonomo, R. A. 2003. Extended-spectrum β-lactamases in Klebsiella pneumoniae bloodstream isolates from seven countries: dominance and widespread prevalence of SHV- and CTX-M-type β-lactamases. *Antimicrobial agents and chemotherapy,* 47**,** 3554-3560.

Perez, F., Hujer, A. M., Hujer, K. M., Decker, B. K., Rather, P. N. & Bonomo, R. A. 2007. Global challenge of multidrug-resistant Acinetobacter baumannii. *Antimicrobial agents and chemotherapy,* 51**,** 3471-3484.

Perilli, M., Felici, A., Franceschini, N., De Santis, A., Pagani, L., Luzzaro, F., Oratore, A., Rossolini, G. M., Knox, J. R. & Amicosante, G. 1997. Characterization of a new TEM-derived beta-lactamase produced in a Serratia marcescens strain. *Antimicrobial agents and chemotherapy,* 41**,** 2374-2382.

Pitout, J., Sanders, C. & Sanders Jr, W. 1997. Antimicrobial resistance with focus on beta-lactam resistance in gram-negative bacilli. *The American journal of medicine,* 103**,** 51-59.

Pitout, J. D. D. & Laupland, K. B. 2008. Extended-spectrum [beta]-lactamase-producing Enterobacteriaceae: an emerging public-health concern. *The Lancet infectious diseases,* 8**,** 159-166.

Salyers, A. A., Gupta, A. & Wang, Y. 2004. Human intestinal bacteria as reservoirs for antibiotic resistance genes. *Trends in microbiology,* 12**,** 412-416.

Schmitz, F.-J., Fluit, A. C., Gondolf, M., Beyrau, R., Lindenlauf, E., Verhoef, J., Heinz, H.-P. & Jones, M. E. 1999. The prevalence of aminoglycoside resistance and corresponding resistance genes in clinical isolates of staphylococci from 19 European hospitals. *Journal of antimicrobial chemotherapy,* 43**,** 253-259.

Shaw, K., Cramer, C., Rizzo, M., Mierzwa, R., Gewain, K., Miller, G. & Hare, R. 1989. Isolation, characterization, and DNA sequence analysis of an AAC (6')-II gene from Pseudomonas aeruginosa. *Antimicrobial agents and chemotherapy,* 33**,** 2052-2062.

Shaw, K., Rather, P., Hare, R. & Miller, G. 1993. Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. *Microbiological Reviews,* 57**,** 138-163.

Sommer, M. O. A., Dantas, G. & Church, G. M. 2009. Functional characterization of the antibiotic resistance reservoir in the human microflora. *Science,* 325**,** 1128-1131.

Tenover, F. C., Huang, M. B., Rasheed, J. K. & Persing, D. H. 1994. Development of PCR assays to detect ampicillin resistance genes in cerebrospinal fluid samples containing Haemophilus influenzae. *Journal of clinical microbiology,* 32**,** 2729-2737.

Tipper, D. 1979. Mode of action of β-lactam antibiotics. *Review of Infectious Diseases,* 1**,** 39-53.

Tipper, D. J. & Strominger, J. L. 1965. Mechanism of action of penicillins: a proposal based on their structural similarity to acyl-D-alanyl-D-alanine. *Proceedings of the National Academy of Sciences of the United States of America,* 54**,** 1133-1141.

Ubukata, K., Yamashita, N., Gotoh, A. & Konno, M. 1984. Purification and characterization of aminoglycoside-modifying enzymes from Staphylococcus aureus and Staphylococcus epidermidis. *Antimicrobial agents and chemotherapy,* 25**,** 754-759.

Vakulenko, S. B., Donabedian, S. M., Voskresenskiy, A. M., Zervos, M. J., Lerner, S. A. & Chow, J. W. 2003. Multiplex PCR for detection of aminoglycoside resistance genes in enterococci. *Antimicrobial agents and chemotherapy,* 47**,** 1423-1426.

Vanhoof, R., Godard, C., Content, J., Nyssen, H. & Hannecart-Pokorni, E. 1994. Detection by polymerase chain reaction of genes encoding aminoglycoside-modifying enzymes in methicillin-resistant Staphylococcus aureus isolates of epidemic phage types. *Journal of medical microbiology,* 41**,** 282-290.

Vannuffel, P., Gigi, J., Ezzedine, H., Vandercam, B., Delmee, M., Wauters, G. & Gala, J.-L. 1995. Specific detection of methicillin-resistant Staphylococcus species by multiplex PCR. *Journal of clinical microbiology,* 33**,** 2864-2867.

Wong, M. H. Y., Liu, M., Wan, H. Y. & Chen, S. 2012. Characterization of Extended-Spectrum-β-Lactamase-Producing Vibrio parahaemolyticus. *Antimicrobial agents and chemotherapy,* 56**,** 4026-4028.

Zhao, W.-H., Hu, Z.-Q., Chen, G., Matsushita, K., Fukuchi, K. & Shimamura, T. 2007. Characterization of imipenem-resistant Serratia marcescens producing IMP-type and TEM-type beta-lactamases encoded on a single plasmid. *Microbiological research,* 162**,** 46-52.

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

Location	Primer	Sequence 5'-3'	Amplicon Size (bp)	Tm °C	Source
β-lactamase genes					
Bla$_{TEM}$	RH605	TTTCGTGTCGCCCTTATTCC	692	60	Bailey *et al.*
	RH606	CCGGCTCCAGATTTATCAGC			(2011)
	Bla_TEMF	TGGGTGCACGAGTGGGTTAC	526	57	Tenover *et al.*
	Bla_TEMR	TTATCCGCCTCCATCCAGTC			(1994)
Bla$_{ROB}$	Bla_ROBF	ATCAGCCACACAAGCCACCT	692	62	Tenover *et al.*
	Bla_ROBR	GTTTGCGATTTGGTATGCGA			(1994)
Bla$_{SHV}$	Bla_SHVF	CACTCAAGGATGTATTGTG	885	58	Briñas *et al.*
	Bla_SHVR	TTAGCGTTGCCAGTGCTCG			(2002)
Bla$_{OXA}$	Bla_OXAF	TTCAAGCCAAAGGCACGATAG	702	64	Briñas *et al.*
	Bla_OXAR	TCCGAGTTGACTGCCGGGTTG			(2002)
Bla$_{CTX-M}$	Bla_CTX-MF	CGTTGTAAAACGACGGCCAGTGAATGTG CAGYACCAGTAARGTKATGGC	600	55	Monstein *et al.* (2009)
	Bla_CTX-MR	TGGGTRAARTARGTSACCAGAAYCAGCG G			
AG resistant genes					
aac (3)-I	Faac3-1	TTCATCGCGCTTGCTGCYTTYGA	239	58	Heuer *et al.*
	Raac3-1	GCCACTGCGGGATCGTCRCCRTA			(2002)
aac (3)-II/VI	Faac3-2	GCGCACCCCGATGCMTCSATGG	189	58	
	Raac3-2	GGCAACGGCCTCGGCGTARTGSA			

	Facc3-6	GCCCATCCCGACGCATCSATGG			
	Raac3-6	CGCCACCGCTTCGGCATARTGSA			
aac (6')-II/Ib	Faac6	CACAGTCGTACGTTGCKCTBGG	235	58	
	Raac6	CCTGCCTTCTCGTAGCAKCGDAT			
ant (2")-I	Fant	TGGGCGATCGATGCACGGCTRG	428	58	
	Rant	AAAGCGGCACGCAAGACCTCMAC			
aph(2")-I	Faphc	CCCAAGAGTCAACAAGGTGCAGA	527	55	
	Faphd	GGCAATGACTGTATTGCATATGA	572	55	
	Raph	GAATCTCCAAAATCRATWATKCC			
aac(6')-*Ie-aph*(2")-*Ia*	aac-aphF	GAGCAATAAGGGCATACCAAAAATC	505	47	*De Fatíma Silva Lopes et al.* (2003)
	aac-aphR	CCGTGCATTTGTCTTAAAAAACTGG			
	aac6-aph2F	CCAAGAGCAATAAGGGCATACC	222	55	Schmitz *et al.* (1999)
	aac6-aph2R	CACACTATCATAACCATCACCG			

AG: aminoglycoside

Table 2 Homologues of β-lactamase genes detected in the human gut microbiota via PCR techniques

Accession #	Gene description	Closest homologue	E value	% identity
***Bla*$_{TEM}$**				
ADE18890.1	β-lactamase TEM-1	*S. enterica* subsp. *enterica*	$5e^{-154}$	99
AAS46844.1	β-lactamase TEM-1	*S. marcescens*	$2e^{-156}$	100
AEN02824.1	β-lactamase TEM-1	*K. pneumoniae*	$3e^{-111}$	99
AEN02817.1	β-lactamase TEM-1	*K. pneumoniae*	$1e^{-113}$	99
ACV88636.1	β-lactamase TEM-1	*E. coli*	$2e^{-151}$	99
AEL87577.1	ES β-lactamase TEM-116	*Vibrio parahaemolyticus*	$5e^{-154}$	99
AEQ55231.1	β-lactamase TEM-1	*E. coli*	$1e^{-35}$	45
ABQ14376.1	β-lactamase	*Uncultured soil bacterium*	$6e^{-05}$	83
ADN79104.1	β-lactamase TEM	*Escherichia vulneris*	$1e^{-15}$	86
WP_010157942.1	β-lactamase TEM	*Sar 86 cluster bacterium*	$9e^{-122}$	83
ACI29961.1	β-lactamase TEM-1	*E. coli*	$2e^{-153}$	99
AEQ39590.1	β-lactamase TEM-195	*E. coli*	$5e^{-93}$	96
AAM22276.1	β-lactamase TEM-96	*E. coli*	$7e^{-139}$	94
WP_019405145.1	β-lactamase TEM	*K. pneumoniae*	$4e^{-155}$	99
AEW28787.1	β-lactamase TEM-1	*Uncultured bacterium*	$1e^{-133}$	100
ABY81267.1	β-lactamase	*E. coli*	$4e^{-156}$	100
AAF74292.1	ES β-lactamase	*E. coli*	$5e^{-155}$	99
AFU53026.1	KPC-2 β lactamase	*S. marcescens*	$2e^{-112}$	98
ADE18896.1	β-lactamase TEM-1	*Salmonella enterica*	$2e^{-113}$	99
AEN02826.1	β-lactamase TEM-1	*K. pneumoniae*	$4e^{-113}$	99
***Bla*$_{ROB}$**				
YP_252228.1	Hypothetical protein SH0313	*S. haemolyticus*	$2e^{-33}$	44
***Bla*$_{SHV}$**				
WP_009348253.1	Hypothetical protein HMPREF 9332	*Alloprevotella rava*	$3e^{-07}$	56
WP_017896153.1	β-lactamase	*K. pneumoniae* subsp. *pneumoniae*	0.0	99
WP_008157744.1	Hypothetical protein	*Parabacteroides*	1.5	29

	HMPREF 1077	*johnsonii*		
CAJ47138.2	β-lactamase	*K. pneumoniae*	0.0	99
ADU15837.1	BlaSHV132	*K. pneumoniae*	0.0	99
AEK80394.1	β-lactamase SHV140	*K. pneumoniae*	0.0	99
ABS72351.1	β-lactamase SHV103	*K. pneumoniae*	0.0	99
AAP03063.1	β-lactamase SHV48	*K. pneumoniae*	0.0	99
AEG79634.1	ES β-lactamase SHV120	*E. coli*	0.0	99
***Bla*$_{\text{CTX-M}}$**				
ABG46354.1	ES β-lactamase	*E. coli*	$3e^{-139}$	99
AEZ49563.1	β-lactamase CTX-M-1	*E. coli*	$2e^{-138}$	99
AEZ49551.1	β-lactamase CTX-M-1	*K. pneumoniae*	$1e^{-139}$	100
ABG46356.1	ES β-lactamase	*K. pneumoniae*	$9e^{-139}$	97
ABW06480.1	ES β lactamase CTX-M-15	*K. pneumoniae*	$6e^{-51}$	94
AAB22638.1	β-lactamase penicillin hydrolase	*E. coli*	$9e^{-140}$	100
BAD16611.1	β-lactamase CTX-M-36	*E. coli*	$8e^{-139}$	99
YP_003717483.1	β-lactamase	*E. coli*	$2e^{-139}$	100
ABN09669.1	β-lactamase CTX-M-61	*S. enterica*	$2e^{-138}$	100

ESBL: extended spectrum β-lactamase

Table 3 Homologues of aminoglycoside resistance genes detected in the human gut microbiota via PCR techniques

Accession #	Gene description	Closest homologue	E value	% identity
***aac* (6)**				
AAA25680.1	AG 6'-N-acetyltransferase	*Pseudomonas fluorescens*	4 e^{-48}	98
WP_006234103.1	Hypothetical protein Colaer00186	*Collinsella aerofaciens*	0.0	95
AAS45464.1	6'-N-acetyltransferase	*A. baumannii*	$3e^{-33}$	75
***aac* (6')-*Ie-aph* (2")**				
WP_002304968.1	Phosphotransferase	*E. faecium*	$9e^{-108}$	100
WP_001028140.1	Acetyltransferase GNAT	*S. aureus*	$1e^{-107}$	99
WP_001028143.1	Acetyltransferase GNAT	*S. aureus*	$1e^{-107}$	99
WP_010729367.1	Bifunctional AAC/APH partial sequence	*E. faecium*	$5e-^{106}$	99
AAX82584.1	Bifunctional AG modifying enzyme	*Enterococcus faecalis*	$2e^{-112}$	100
WP_002417297.1	6' AG acetyltransferase	*E. faecalis*	$3e^{-111}$	97
AFR11868.1	Bifunctional AG 6'-N acetytransferase/2'-AG phosphotransferases	*S. epidermidis*	$1e^{-43}$	99
AFM29914.1	Gentamicin resistance protein	*Enterococcus* sp.	$7e^{-45}$	97
***aph* (2") Id**				
3SG8_A	Chain A crystal structure AG 2' phosphotransferases	*E. casseliflavus*	$1e^{-110}$	98
3N4T_A	Aph2'' chain a	*E. casseliflavus*	$2e^{-110}$	99
AAT77696.1	AG modifying enzyme	*E. faecium*	$1e^{-68}$	94
***Aph* (2")-Ic**				
3TDVA	AG phosphotransferase	*Enterococcus gallinarum*	$2e^{-83}$	97
***ant* (2") Ia**				
YP_005176240.1	AG 2'-O-adenyltransferase	*Pasturella mutocida*	$2e^{-97}$	100
WP_000314377.1	2' AG	*A. baumannii*	$3e^{-94}$	99

	nucleotidlytransferase			
WP_000946493.1	2' AG	*A. baumannii*	$1e^{-94}$	99
ACJ47203.1	AG adenyltransferase	*E. coli*	$6e^{-94}$	99
ACA48663.14	AG adenyltransferase	*Morganella morganii*	$2e^{-96}$	99
***aac*(3)-VI**				
AAA16194.1	aac 3-6	*Enterobacter cloacae*	$2e^{-05}$	77
WP_001642188.1	AG acetyltransferase	*S. enterica* subsp. *enterica*	$2e^{-20}$	98

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 (PBPs; (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 (≈ 1 x 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 ≈ 5 x 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 β-lactamase activity, nitrocefin tests were performed as previously described (Moubareck et al., 2005, Lee and Rosenblatt, 1983), i.e. β-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 *Hin*dIII and *Xba*I (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 *Bbr_0651* and *Bbr_1586*, rather than as an indirect consequence of the mutagenesis strategy, MICs were conducted on another insertion mutant created in *B. breve* UCC2003, namely *B. breve* UCC2003-*gos*G (O' Connell Motherway et al., 2013). This mutant was created previously using the same protocol that was used to create the mutants *Bbr_0651* and *Bbr_1586,* but in this instance the *Bbr_0529* (*gosG*) gene is disrupted. The antibiotic resistance phenotype of this mutant was similar to that of the wild-type *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 *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 *Bbr_0651*, *Bbr_0651+0650* or *Bbr_1586* into wild-type *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.,

2006, Xiao et al., 2010). 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 β-lactamase and aminoglycoside resistance proteins in the *Bifidobacterium* genus and to subsequently investigate if representative genes confer a resistant phenotype.

With respect to the putative β-lactamases, it was noted that several proteins of potential relevance have been assigned across the *Bifidobacterium* genus. However, none of these were clear representatives of any of the Ambler classes of β-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 β-lactamases. To detect such a high prevalence of putative β-lactamases amongst bifidobacteria was surprising given that previous laboratory based investigations have shown bifidobacteria to be sensitive to commonly prescribed β-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 *et al*. demonstrated that 23 investigated bifidobacterial strains were sensitive to all β-lactams tested (Xiao et al., 2010). In order to examine whether these annotated β-lactamase sequences resulted in a resistance phenotype, we selected a representative number of bifidobacteria strains, which had been identified in the *in silico* screen as containing putative β-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 *Bbr_0651+0650* or *Bbr_0651* was added to the wild-type *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 *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 *Bbr_1586,* which contribute to streptomycin resistance and are impacted upon in a polar manner following mutagenesis by plasmid insertion. The role of *Bbr_0651* and *Bbr_1586* as aminoglycoside resistance determinants was further confirmed through the provision of enhanced protection against at least one aminoglycoside upon their expression in *E. coli* XL1-blue.

Ultimately, it is evident that both *Bbr_0651* and *Bbr_1586* contribute to aminoglycoside resistance in *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

Abraham, E. & Chain, E. 1940. An enzyme from bacteria able to destroy penicillin. *Nature,* 146**,** 837-837.

Álvarez-Martín, P., O'connell-Motherway, M., Van Sinderen, D. & Mayo, B. 2007. Functional analysis of the pBC1 replicon from Bifidobacterium catenulatum L48. *Applied microbiology and biotechnology,* 76**,** 1395-1402.

Ambler, R. 1980. The Structure of beta-lactamases. *Philosophical Transactions of the Royal Society of London. B, Biological Sciences,* 289**,** 321-331.

Andorf, C., Dobbs, D. & Honavar, V. 2007. Exploring inconsistencies in genome-wide protein function annotations: a machine learning approach. *Bmc Bioinformatics,* 8**,** 284-296.

Andrews, J. 2009. BSAC standardized disc susceptibility testing method (version 8). *Journal of antimicrobial chemotherapy,* 64**,** 454-489.

Andrews, J. M. 2001a. BSAC standardized disc susceptibility testing method. *Journal of antimicrobial chemotherapy,* 48**,** 43-57.

Andrews, J. M. 2001b. Determination of minimum inhibitory concentrations. *Journal of antimicrobial chemotherapy,* 48**,** 5-16.

Andrews, J. M. 2001c. The development of the BSAC standardized method of disc diffusion testing. *Journal of antimicrobial chemotherapy,* 48**,** 29-42.

Brenner, S. E. 1999. Errors in genome annotation. *Trends in Genetics,* 15**,** 132-133.

Bryan, L., Kowand, S. & Van Den Elzen, H. 1979. Mechanism of aminoglycoside antibiotic resistance in anaerobic bacteria: Clostridium perfringens and Bacteroides fragilis. *Antimicrobial agents and chemotherapy,* 15**,** 7-13.

Bush, K. 1989. Classification of beta-lactamases: groups 1, 2a, 2b, and 2b'. *Antimicrobial agents and chemotherapy,* 33**,** 264-270.

Bush, K. 2012. Improving known classes of antibiotics: an optimistic approach for the future. *Current Opinion in Pharmacology,* 12**,** 527-534.

Bush, K. & Jacoby, G. A. 2010. Updated functional classification of β-lactamases. *Antimicrobial agents and chemotherapy,* 54**,** 969-976.

Bush, K., Jacoby, G. A. & Medeiros, A. A. 1995. A functional classification scheme for beta-lactamases and its correlation with molecular structure. *Antimicrobial agents and chemotherapy,* 39**,** 1211-1233.

Cani, P. D., Neyrinck, A., Fava, F., Knauf, C., Burcelin, R., Tuohy, K., Gibson, G. & Delzenne, N. 2007. Selective increases of bifidobacteria in gut microflora improve high-fat-diet-induced diabetes in mice through a mechanism associated with endotoxaemia. *Diabetologia,* 50**,** 2374-2383.

Chouraqui, J. P., Van Egroo, L. D. & Fichot, M. C. 2004. Acidified milk formula supplemented with Bifidobacterium lactis: impact on infant diarrhea in residential care settings. *Journal of pediatric gastroenterology and nutrition,* 38**,** 288-292.

Commission, E. 2008. Technical guidance prepared by the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) on the update of the criteria used in the assessment of bacterial resistance to antibiotics of human or veterinary importance. *The EFSA J***,** 1-15.

D'aimmo, M. R., Modesto, M. & Biavati, B. 2007. Antibiotic resistance of lactic acid bacteria and Bifidobacterium spp. isolated from dairy and pharmaceutical products. *International journal of food microbiology,* 115**,** 35-42.

Davies, J. & Davies, D. 2010. Origins and evolution of antibiotic resistance. *Microbiology and Molecular Biology Reviews,* 74**,** 417-433.

Davies, J. & Wright, G. D. 1997. Bacterial resistance to aminoglycoside antibiotics. *Trends in microbiology,* 5**,** 234-240.

Devos, D. & Valencia, A. 2001. Intrinsic errors in genome annotation. *Trends in Genetics,* 17**,** 429-431.

Fallani, M., Young, D., Scott, J., Norin, E., Amarri, S., Adam, R., Aguilera, M., Khanna, S., Gil, A. & Edwards, C. A. 2010. Intestinal microbiota of 6-week-old infants across Europe: geographic influence beyond delivery mode, breast-feeding, and antibiotics. *Journal of pediatric gastroenterology and nutrition,* 51**,** 77-84.

Fleming, A. 1979. On the antibacterial action of cultures of a penicillium, with special reference to their use in the isolation of B. influenzae. *British journal of experimental pathology,* 60**,** 3-16.

Fouhy, F., Guinane, C. M., Hussey, S., Wall, R., Ryan, C. A., Dempsey, E. M., Murphy, B., Ross, R. P., Fitzgerald, G. F. & Stanton, C. 2012. High-throughput sequencing reveals the incomplete, short-term, recovery of the infant gut microbiota following parenteral antibiotic treatment with ampicillin and gentamicin. *Antimicrobial agents and chemotherapy,* 56**,** 5811-5820.

Georgopapadakou, N. 1993. Penicillin-binding proteins and bacterial resistance to beta-lactams. *Antimicrobial agents and chemotherapy,* 37**,** 2045-2053.

He, T., Priebe, M., Zhong, Y., Huang, C., Harmsen, H., Raangs, G., Antoine, J. M., Welling, G. & Vonk, R. 2007. Effects of yogurt and bifidobacteria supplementation on the colonic microbiota in lactose-intolerant subjects. *Journal of applied microbiology,* 104**,** 595-604.

Huys, G., Botteldoorn, N., Delvigne, F., De Vuyst, L., Heyndrickx, M., Pot, B., Dubois, J. J. & Daube, G. 2013. Microbial characterization of probiotics–Advisory report of the Working Group “8651 Probiotics” of the Belgian Superior Health Council (SHC). *Molecular nutrition & food research,* 57**,** 1479-1504.

Jacoby, G. & Gorini, L. 1967. The effect of streptomycin and other aminoglycoside antibiotics on protein synthesis. *Mechanism of Action.* Springer.

Jones, B. V., Begley, M., Hill, C., Gahan, C. G. & Marchesi, J. R. 2008. Functional and comparative metagenomic analysis of bile salt hydrolase activity in the human gut microbiome. *Proceedings of the National Academy of Sciences,* 105**,** 13580-13585.

Jones, C. E., Brown, A. L. & Baumann, U. 2007. Estimating the annotation error rate of curated GO database sequence annotations. *Bmc Bioinformatics,* 8**,** 170-179.

Kailasapathy, K. & Chin, J. 2000. Survival and therapeutic potential of probiotic organisms with reference to Lactobacillus acidophilus and Bifidobacterium spp. *Immunology and Cell Biology,* 78**,** 80-88.

Kheadr, E., Bernoussi, N., Lacroix, C. & Fliss, I. 2004. Comparison of the sensitivity of commercial strains and infant isolates of bifidobacteria to antibiotics and bacteriocins. *International Dairy Journal,* 14**,** 1041-1053.

Kiwaki, M. & Sato, T. 2009. Antimicrobial susceptibility of *Bifidobacterium breve* strains and genetic analysis of streptomycin resistance of probiotic *B. breve* strain Yakult. *International journal of food microbiology,* 134**,** 211-215.

Kotra, L. P. & Mobashery, S. 1998. β-Lactam antibiotics, β-lactamases and bacterial resistance. *Bulletin de l'Institut Pasteur,* 96**,** 139-150.

Lambert, J. M., Bongers, R. S., De Vos, W. M. & Kleerebezem, M. 2008. Functional analysis of four bile salt hydrolase and penicillin acylase family members in Lactobacillus plantarum WCFS1. *Applied and environmental microbiology,* 74**,** 4719-4726.

Law, J., Buist, G., Haandrikman, A., Kok, J., Venema, G. & Leenhouts, K. 1995. A system to generate chromosomal mutations in Lactococcus lactis which allows fast analysis of targeted genes. *Journal of bacteriology,* 177**,** 7011-7018.

Lee, D. T. & Rosenblatt, J. E. 1983. A comparison of four methods for detecting β-lactamase in anaerobic bacteria. *Diagnostic microbiology and infectious disease,* 1**,** 173-175.

Lim, K., Huh, C. & Baek, Y. 1993. Antimicrobial susceptibility of bifidobacteria. *Journal of dairy science,* 76**,** 2168-2174.

Masco, L., Van Hoorde, K., De Brandt, E., Swings, J. & Huys, G. 2006. Antimicrobial susceptibility of Bifidobacterium strains from humans, animals and probiotic products. *Journal of antimicrobial chemotherapy,* 58**,** 85-94.

Mayrhofer, S., Mair, C., Kneifel, W. & Domig, K. J. 2011. Susceptibility of Bifidobacteria of Animal Origin to Selected Antimicrobial Agents. *Chemotherapy research and practice,* 2011**,** doi 10.1155/2011/989520.

Mazé, A., O'connell-Motherway, M., Fitzgerald, G. F., Deutscher, J. & Van Sinderen, D. 2007. Identification and characterization of a fructose phosphotransferase system in Bifidobacterium breve UCC2003. *Applied and environmental microbiology,* 73**,** 545-553.

Mingeot-Leclercq, M. P., Glupczynski, Y. & Tulkens, P. M. 1999. Aminoglycosides: activity and resistance. *Antimicrobial agents and chemotherapy,* 43**,** 727-737.

Mitsuoka, T. 1990. Bifidobacteria and their role in human health. *Journal of Industrial Microbiology,* 6**,** 263-267.

Moubareck, C., Gavini, F., Vaugien, L., Butel, M. & Doucet-Populaire, F. 2005. Antimicrobial susceptibility of bifidobacteria. *Journal of antimicrobial chemotherapy,* 55**,** 38-44.

Murphy, E., Cotter, P., Healy, S., Marques, T., O'sullivan, O., Fouhy, F., Clarke, S., O'toole, P., Quigley, E. & Stanton, C. 2010. Composition and energy harvesting capacity of the gut microbiota: relationship to diet, obesity and time in mouse models. *Gut,* 59**,** 1635-1642.

Nishimoto, M. & Kitaoka, M. 2007. Identification of N-acetylhexosamine 1-kinase in the complete lacto-N-biose I/galacto-N-biose metabolic pathway in Bifidobacterium longum. *Applied and environmental microbiology,* 73**,** 6444-6449.

O' Connell Motherway, M., Kinsella, M., Fitzgerald, G. F. & Sinderen, D. 2013. Transcriptional and functional characterization of genetic elements involved in galacto-oligosaccharide utilization by Bifidobacterium breve UCC2003. *Microbial Biotechnology,* 6**,** 67-79.

O' Connell Motherway, M., O'driscoll, J., Fitzgerald, G. F. & Van Sinderen, D. 2009. Overcoming the restriction barrier to plasmid transformation and targeted mutagenesis in Bifidobacterium breve UCC2003. *Microbial Biotechnology,* 2**,** 321-332.

O' Connell Motherway, M., Zomer, A., Leahy, S. C., Reunanen, J., Bottacini, F., Claesson, M. J., O'brien, F., Flynn, K., Casey, P. G. & Munoz, J. a. M. 2011. Functional genome analysis of Bifidobacterium breve UCC2003 reveals type IVb tight adherence (Tad) pili as an essential and conserved host-colonization factor. *Proceedings of the National Academy of Sciences,* 108**,** 11217-11222.

O' Hara, A. M. & Shanahan, F. 2006. The gut flora as a forgotten organ. *EMBO Rep,* 7**,** 688-693.

Page, M. G. P. 2012. Beta-Lactam Antibiotics. *Antibiotic Discovery and Development***,** 79-117.

Pitout, J., Sanders, C. & Sanders Jr, W. 1997. Antimicrobial resistance with focus on beta-lactam resistance in gram-negative bacilli. *The American journal of medicine,* 103**,** 51-59.

Richmond, M. & Sykes, R. 1973. The, B-lactamases of gram-negative bacteria and their possible physiological role. *Adv. Microb. Physiol,* 9**,** 31-88.

Salyers, A. A., Gupta, A. & Wang, Y. 2004. Human intestinal bacteria as reservoirs for antibiotic resistance genes. *Trends in microbiology,* 12**,** 412-416.

Sato, T. & Iino, T. 2010. Genetic analyses of the antibiotic resistance of Bifidobacterium bifidumstrain Yakult YIT 4007. *International journal of food microbiology,* 137**,** 254-258.

Schnoes, A. M., Brown, S. D., Dodevski, I. & Babbitt, P. C. 2009. Annotation error in public databases: misannotation of molecular function in enzyme superfamilies. *PLoS computational biology,* 5**,** e1000605.

Shaw, K., Rather, P., Hare, R. & Miller, G. 1993. Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. *Microbiological Reviews,* 57**,** 138-163.

Sommer, M. O. A., Dantas, G. & Church, G. M. 2009. Functional characterization of the antibiotic resistance reservoir in the human microflora. *Science,* 325**,** 1128-1131.

Talwalkar, A. & Kailasapathy, K. 2004. The role of oxygen in the viability of probiotic bacteria with reference to L. acidophilus and Bifidobacterium spp. *Current issues in intestinal microbiology,* 5**,** 1-8.

Tipper, D. 1979. Mode of action of β-lactam antibiotics. *Review of Infectious Diseases,* 1**,** 39-53.

Tipper, D. J. & Strominger, J. L. 1965. Mechanism of action of penicillins: a proposal based on their structural similarity to acyl-D-alanyl-D-alanine. *Proceedings of the National Academy of Sciences of the United States of America,* 54**,** 1133-1141.

Vankerckhoven, V., Huys, G., Vancanneyt, M., Vael, C., Klare, I., Romond, M.-B., Entenza, J. M., Moreillon, P., Wind, R. D., Knol, J., Wiertz, E., Pot, B., Vaughan, E. E., Kahlmeter, G. & Goossens, H. 2008. Biosafety assessment of probiotics used for human consumption: recommendations from the EU-PROSAFE project. *Trends in Food Science & Technology,* 19**,** 102-114.

Ventura, M., Turroni, F., Zomer, A., Foroni, E., Giubellini, V., Bottacini, F., Canchaya, C., Claesson, M. J., He, F. & Mantzourani, M. 2009. The Bifidobacterium dentium Bd1 genome sequence reflects its genetic adaptation to the human oral cavity. *PLoS genetics,* 5**,** e1000785.

Vlková, E., Rada, V., Popelářová, P., Trojanová, I. & Killer, J. 2006. Antimicrobial susceptibility of bifidobacteria isolated from gastrointestinal tract of calves. *Livestock Science,* 105**,** 253-259.

Walsh, C. 2000. Molecular mechanisms that confer antibacterial drug resistance. *Nature,* 406**,** 775-781.

Wang, K. Y., Li, S. N., Liu, C. S., Perng, D. S., Su, Y. C., Wu, D. C., Jan, C. M., Lai, C. H., Wang, T. N. & Wang, W. M. 2004. Effects of ingesting Lactobacillus-and Bifidobacterium-containing yogurt in subjects with colonized Helicobacter pylori. *The American journal of clinical nutrition,* 80**,** 737-741.

Xiao, J., Kondo, S., Takahashi, N., Miyaji, K., Oshida, K., Hiramatsu, A., Iwatsuki, K., Kokubo, S. & Hosono, A. 2003. Effects of milk products fermented by Bifidobacterium longum on blood lipids in rats and healthy adult male volunteers. *Journal of dairy science,* 86**,** 2452-2461.

Xiao, J., Takahashi, S., Odamaki, T., Yaeshima, T. & Iwatsuki, K. 2010. Antibiotic susceptibility of bifidobacterial strains distributed in the Japanese market. *Bioscience, biotechnology, and biochemistry,* 74**,** 336-342.

Yazid, A., Ali, A., Shuhaimi, M., Kalaivaani, V., Rokiah, M. & Reezal, A. 2001. Antimicrobial susceptibility of bifidobacteria. *Letters in applied microbiology,* 31**,** 57-62.

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

Aminoglycoside resistance gene classification groups	Representative sequences	β-lactamase gene classes	Representative gene name	Representative gene accession number
APH	M20305	**Class A**	TEM1	YP_209323.1
	V00618		TEM1	AFN82055.1
	M29953		SHV-2	YP_001966240.1
	X07753		PSE	YP_005086938.1
APH (6')	X05648		CepA	YP_210868.1
	X01702		Sme_1	CAA82281.1
AAC 3	X01385		Bla KPC	YP_003754012.1
	M55426	**Class B**	IMP-1	YP_005980003.1
	M22999		VIM-1	YP_003813035.1
AAC-Ia & Ib	L06157		CcrA	YP_004735262.1
AAC 6' Ic	M94066		L1	YP_006185056.1
ANT	X02340		CphA	YP_004391384.1
	X04555		Sph1	YP_005188946.1
		Class C	AMP C	AAG59351.1
		Class D	OXA-1	AFB82783.1
			OXA-10	YP_001715358.1
			OXA-23	YP_002317955.1

Table 2 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Ref or Source
***E.coli* strains**		
EC101	Cloning host, $repA^+$, kan^r	Law *et al.* (1995)
XL1-blue	Tet^r	Stratagene
XL1-blue-pBC1.2-Bbr_0651	Heterologous expression of *Bbr_0651*	This study
XL1-blue-pBC1.2-Bbr_0651+0650	Heterologous expression of *Bbr_0651+0650*	This study
XL1-blue-pBC1.2-Bbr_1586	Heterologous expression of *Bbr_1586*	This study
***B. breve* strains**		
UCC2003	Isolated from nursing stool	Mazé *et al.* (2007)
UCC2003-0651-tet	pORI19-0651-tet insertion mutant of *B. breve* UCC2003	This study
UCC2003-1586-tet	pORI19-1586-tet insertion mutant of *B. breve* UCC2003	This study
B. breve UCC2003-gosG	pORI19-tet-Bbr_0529 insertion mutant of UCC2003	O' Connell Motherway *et al.* (2013)
UCC2003-1586-tet-pBC1.2-Bbr_1586	pORI19-1586-tet insertion mutant complemented strain of *B. breve* UCC2003	This study
UCC2003-pBC1.2-Bbr_0651	pBC1.2-Bbr_0651 construct in *B. breve* UCC2003	This study
UCC2003-pBC1.2-Bbr_0651+0650	pBC1.2-Bbr_0651+0650 construct in *B. breve* UCC2003	This study
UCC2003-pBC1.2-Bbr_1586	pBC1.2-Bbr_1586 construct in *B. breve* UCC2003	This study
UCC2003-pBC1.2	*B. breve* UCC2003 harbouring pBC1.2	This study
Bifidobacteria strains		
B. gallicum DSM 20093	Contains putative β-lactamase protein	Teagasc Culture Collection
B. animalis subsp. *lactis*	Contains putative β-	Teagasc Culture

Bb12	lactamase and AG resistance proteins	Collection
B. angulatum DSM 20098	Contains putative β-lactamase and AG resistance proteins	Teagasc Culture Collection
B. pseudocatenulatum DSM 20438	Contains putative β-lactamase and AG resistance proteins	Teagasc Culture Collection
B. breve DSM 20213	Contains putative β-lactamase and AG resistance proteins	Teagasc Culture Collection
B. breve UCC2003	Contains putative β-lactamase and AG resistance proteins	Teagasc Culture Collection
Plasmids		
pAM5	pBC1-puC19-Tc^r	Alvarez-Martín *et al*. (2007)
pORI19	Em^r, $repA^-$, ori^+, cloning vector	Law *et al.* (1995)
pORI19-tet-0651	Internal 500bp fragments of *Bbr_0651* and tetW cloned in pORI19	This study
pORI19-tet-1586	Internal 400bp fragments of *Bbr_1586* and tetW cloned in pORI19	This study
pBC1.2	pBC1-pSC101-Cm^r	Alvarez-Martín *et al*. (2007)
pBC1.2-0651	*Bbr_0651* cloned in pBC1.2	This study
pBC1.2-0651+0650	*Bbr_0651+Bbr_0650* cloned in pBC1.2	This study
pBC1.2-1586	*Bbr_1586* cloned in pBC1.2	This study

AG: aminoglycoside

Table 3 *Bifidobacterium* derived β-lactamase protein sequences

***Bifidobacterium* strain**	**Accession number***	**Gene name**	**Assigned as**	**Pfam**
B. dentium ATCC 27679	ZP_07457312.1[a]	HMPREF0168_1872	Conserved hypothetical protein	PF13354
	ZP_07456818.1[b]	HMPREF0168_1378	β-lactamase	PF00144
	ZP_07455619.1[d]	HMPREF0168_0178	Hypothetical protein	Metal dependent hydrolase with PF07521
B. dentium Bd1	YP_003359579.1[a]	BDP_0063	Hypothetical protein	PF13354
	YP_003360049.1[b]	BDP_0556	Hypothetical protein	PF00144
	YP_003361167.1[d]	BDP_1754	Hypothetical protein	Metal dependent hydrolase with PF07521
B. dentium ATCC 27678	ZP_02917480.1[a]	BIFDEN_00760	Hypothetical protein	PF11354
	ZP_02916953.1[b]	BIFDEN_00213	Hypothetical protein	PF00144
	ZP_02918099.1[d]	BIFDEN_01398	Hypothetical protein	Metal dependent hydrolase with PF07521
B. gallicum DSM 20093	ZP_05965566.1[d]	BIFGAL_03078	Metallo-beta-lactamase family protein	Metal dependent hydrolase with PF07521
B. adolescentis L2-32	ZP_02027818.1	BIFADO_0224	Hypothetical protein	PF13354
	ZP_02029327.1[d]	BIFADO_01784	Hypothetical protein	PF07521
B. animalis subsp. *lactis* Bb12	YP_005575727.1[d]	BIF_01983	Hydrolase	Metal dependent hydrolase with PF07521
B. animalis subsp. *animalis* ATCC 25527	YP_006280466.1[d]	BANAN_06475	Hypothetical protein	Metal dependent hydrolase with PF07521

B. animalis subsp. *lactis* AD011	YP_002469408.1[d]	BLA_0533	β-lactamase-like protein	Metal dependent hydrolase with PF07521
B. bifidum BGN4	YP_006394858.1[f]	BBB_1387	Penicillin binding protein	PF13354
	YP_006395029.1[g]	BBB_1559	β-lactamase	PF13354
	YP_006393888.1[d]	BBB_0414	Ribo-nuclease J	Metal dependent hydrolase with PF07521
B. bifidum NCIMB 41171	ZP_07803038.1[g]	BBNG_01520	Conserved hypothetical protein	PF13354
	ZP_07803204.1[f]	BBNG_01686	β-lactamase	PF13354
	ZP_07801866.1[d]	BBNG_00347	Conserved hypothetical protein	Metal dependent hydrolase with PF07521
B. bifidum PRL 2010	YP_003971645.1[g]	BBPR_1582	β-lactamase	PF13354
	YP_003971485.1[f]	BBPR_1404	β-lactamase	PF13354
	YP_003970583.1[d]	BBPR_0437	Metal-dependent hydrolase	Metal dependent hydrolase with PF07521
B. longum subsp. *longum* JDM 301	YP_003660997.1	BLJ_0695	β-lactamase	PF13354
B. adolescentis ATCC 15703	YP_910171.1	BAD_1308	β-lactamase	PF13354
	YP_910159.1[d]	BAD_1296	Hypothetical protein	PF07521
B. breve UCC2003	ABE94945.1[e]	Bbr_0236	Conserved hypothetical protein with β-lactamase motif	PF13354
	ABE95207.1[d]	Bbr_0510	Metal-dependent hydrolase	Metal dependent hydrolase with PF07521

B. breve ACS 071 VSch8b	YP_005582166.1[e]	HMPREF9228_0250	Hypothetical protein	PF13354
	YP_005583195.1[d]	HMPREF9228_1387	Hypothetical protein	Metal dependent hydrolase with PF07521
B. breve DSM 20213	ZP_06595304.1[e]	BIFBRE_03112	Putative β-lactamase	PF13354
	ZP_06595596.1[d]	BIFBRE_03411	Metallo-beta-lactamase family protein	Metal dependent hydrolase with PF07521
B. breve CECT 7263	EHS86772.1[e]	CECT7263_10968	Putative β-lactamase	PF13354
	EHS85412.1[d]	CECT7263_11981	Metallo-beta-lactamase family protein	Metal dependent hydrolase with PF07521
B. catenulatum DSM 16992	ZP_03324536.1[c]	BIFCAT_01331	Hypothetical protein	PF00144
	ZP_03324350.1[d]	BIFCAT_01138	Hypothetical protein	Metal dependent hydrolase with PF07521
B. bifidum S17	YP_003939138.1[f]	BBIF_1359	β-lactamase	PF13354
	YP_003938240.1[d]	BBIF_0461	Metallo-beta-lactamase domain-containing protein	Metal dependent hydrolase with PF07521
	YP_003939303.1[g]	BBFI_1524	β-lactamase	PF13354
B. pseudocatenulatum DSM 20438	ZP_03741949.1[c]	BIFPSEUDO_02501	Hypothetical protein	PF00144
	ZP_03742801.1[d]	BIFPSEUDO_03375	Hypothetical protein	Metal dependent hydrolase with PF07521
B. longum NCC2705	NP696361.1[d]	BL_1192	Hypothetical protein	Metal dependent hydrolase with PF07521
B. longum subsp. *infantis* ATCC 55813	ZP_03976420.1[d]	HMPREF0175_0795	Metal dependent hydrolase	Metal dependent hydrolase

				with PF07521
B. longum BBMN68	YP_004000557.1[d]	BBMN68_955	Hydrolase	Metal dependent hydrolase with PF07521
B. longum DJ010A	YP_001954894.1[d]	BLD_0950	Metallo-beta-lactamase superfamily hydrolase	Metal dependent hydrolase with PF07521
B. longum subsp. *longum* JCM 1217	YP_004220181.1[d]	BLLJ_0420	Hypothetical protein	Metal dependent hydrolase with PF07521
B. longum subsp. *longum* JDM301	YP_00366798.1[d]	BLJ_0491	β-lactamase domain-containing protein	Metal dependent hydrolase with PF07521
B. longum subsp. *infantis* ATCC 15697	YP_005585858.1[d]	BLIJ_2111	Hypothetical protein	Metal dependent hydrolase with PF07521
	YP_002323794.1	BLon_2358	β-lactamase	PF12706 and 07521
B. animalis subsp. *lactis* HN019	ZP_02963481.1[d]	BIFLAC_07662	Hypothetical protein	Metal dependent hydrolase with PF07521
B. gallicum DSM 20093	ZP_05965566.1[d]	BIFGAL_03078	Metallo-beta-lactamase family protein	Metal dependent hydrolase with PF07521
B. angulatum DSM 20098	ZP_04447555.1[d]	BIFANG_02533	Hypothetical protein	Metal dependent hydrolase with PF07521

* Same superscript indicates proteins share >90% sequence percentage identity

Table 4 *Bifidobacterium* derived aminoglycoside resistance proteins

***Bifidobacterium* strain**	**Accession number***	**Gene name**	**Assigned as**	**Pfam**
B. longum DJ010A	YP_00195405.3[a]	BLD_0109	AG phospho-transferase	Proteins containing a protein kinase family domain, c109925
	ZP_00121257.2[a]	Blon_03001154	Hypothetical protein	Proteins containing a protein kinase family domain, c109925
	ZP_00121797.2[b]	BLD_1766	Hypothetical protein	Phosphotransferase family with PF 01636 and proteins containing a protein kinase family domain, c109925
B. longum BBMN68	YP_003999751.1[a]	BBMN68_137	AG phospho-transferases	Phosphotransferase family with PF 01636
	YP_004001272.1[b]	BBMN_1674	Homoserine kinase	Proteins containing a protein kinase family domain, c109925 and phosphotransferase family with PF 01636
B. longum subsp. *infantis* CCUG 52486	ZP_04663835.1[a]	BLIG_01916	Hypothetical protein	Proteins containing a protein kinase family domain, c109925
	ZP_04664566.1[b]	BLIG_01601	Hypothetical protein	Proteins containing a protein kinase family domain, c109925 and phosphotransferase family with PF 01636
B. longum NCC 2705	NP695320.1[a]	BL_0091	Hypothetical protein	Proteins containing a protein kinase family domain, c109925
	NP696793.1[b]	BL_1642	Desulfatase	Proteins containing a protein kinase family domain, c109925 and phosphotransferase family with PF 01636
B. longum KACC 91563	YP_005586893.1[a]	BLNIAS_00852	Hypothetical protein	Proteins containing a protein kinase family domain, c109925
B. adolescentis L2-32	ZP_02029839.1[i]	BIFADO_02300	Hypothetical protein	Proteins containing a protein kinase family domain, c109925
B. longum subsp. *infantis* ATCC 55813	ZP_03976875.1[a]	HMPREF0175_1250	AG phospho-transferase	Proteins containing a protein kinase family domain, c109925
B. longum subsp. *infantis* ATCC 15697	YP_002322254.1[a]	Blon_0773	AG phospho-transferase	Proteins containing a protein kinase family domain, c109925

	YP_002323612.1[a]	Blon_2173	AG phospho-transferase	Proteins containing a protein kinase family domain, c109925 and phosphotransferase family with PF 01636
B. longum subsp. *longum* JDM301	YP_003661654.1[a]	BLJ_1379	AG phospho-transferases	Proteins containing a protein kinase family domain, c109925
B. breve UCC2003	ABE95342.1[c]	Bbr_0651	Conserved Hypothetical secreted protein	Merozoite surface protein 1 (MSP1) C-terminus of the PF 07462 and proteins containing a protein kinase family domain, c109925
	ABE96255.1[d]	Bbr_1586	AG phospho-transferases	Proteins containing a protein kinase family domain, c109925 and phosphotransferase family with PF 01636
B. breve DSM 20213	ZP_06595772.1[c]	BIFBRE_03589	Conserved hypothetical protein	Merozoite surface protein 1 (MSP1) C-terminus of the PF 07462 and proteins containing a protein kinase family domain, c109925
	ZP_06596651.1[d]	BIFBRE_04498	Mucin desulfating sulfatase	Proteins containing a protein kinase family domain, c109925 and phosphotransferase family with PF 01636
B. breve CECT 7263	EHS85254.1[d]	CECT7263_14691	Mucin desulfating sulfatase	Proteins containing a protein kinase family domain, c109925 and phosphotransferase family with PF 01636
	EHS85519.1[c]	CECT7263_10981	Hypothetical protein	Merozoite surface protein 1 (MSP1) C-terminus of the PF 07462 and proteins containing a protein kinase family domain, c109925
B. breve ACS 071 VSch 8b	YP_005583039.1[c]	HMPREF9228_1217	Phospho-transferase enzyme domain protein	Merozoite surface protein 1 (MSP1) C-terminus of the PF 07462 and proteins containing a protein kinase family domain, c109925
	YP_005583418.1[d]	HMPREF9228_1637	Putative mucin-	Proteins containing a protein kinase family

			desulfating sulfatase	domain, c109925 and phosphotransferase family with PF 01636
B. animalis subsp. *lactis* Bb12	YP_005575653.1[e]	BIF_00526	Hypothetical protein	Proteins containing a protein kinase family domain, c109925 and phosphotransferase family with PF 01636
	YP_005576071.1[f]	BIF_01665	AG 3' phosphor-transferase	Proteins containing a protein kinase family domain, c109925 and phosphotransferase family with PF 01636
B. dentium ATCC 27678	ZP_02918244.1[g]	BIFDEN_01548	Hypothetical protein	Proteins containing a protein kinase family domain, c109925 and phosphotransferase family with PF 01636
B. dentium Bd1	YP_003361041.1[g]	BDP_1625	AG phosphor-transferase	Proteins containing a protein kinase family domain, c109925 and phosphotransferase family with PF 01636
B. dentium ATCC 27679	ZP_07455726.1[g]	HMPREF0168_0285	Conserved hypothetical protein	Proteins containing a protein kinase family domain, c109925 and phosphotransferase enzyme family of the PF 01636
B. dentium JCVHMP022	ZP_07696282.1[g]	HMPREF9003_0562	Conserved hypothetical protein	Proteins containing a protein kinase family domain, c109925 and phosphotransferase enzyme family of the PF 01636
B. catenulatum DSM 16992	ZP_03323625.1[h]	BIFCAT_00394	Hypothetical protein	Proteins containing a protein kinase family domain, c109925 and phosphotransferase family with PF 01636
B. pseudocatenulatum DSM 20435	ZP_03742521.1[h]	BIFPSEUDO_03094	Hypothetical protein	Proteins containing a protein kinase family domain, c109925 and phosphotransferase family with PF 01636

B. adolescentis ATCC 15703	YP_910027.1[l]	BAD_1164	Hypothetical protein	Proteins containing a protein kinase family domain, c109925 and phosphotransferase family with PF 01636
B. bifidum S17	YP_003938274.1[j]	BBIF_0495	Hypothetical protein	Proteins containing a protein kinase family domain, c109925 and phosphotransferase family with PF 01636
	YP_003938776.1[k]	BBIF_0997	Mucin de-sulfatase	Proteins containing a protein kinase family domain, c109925 and phosphotransferase family with PF 01636
	YP_003939526.1[l]	BBIF_1747	AG transferase	Phosphotransferase enzyme family of the PF 01636 and AG phosphotransferases of the aph family cd 05150
B. bifidum PRL 2010	YP_003970614.1[j]	BBPR_0470	AG phospho-transferase	Proteins containing a protein kinase family domain, c109925 and phosphotransferase family with PF 01636
B. bifidum BGN4	YP_006393921.1[j]	BBB_0447	AG phospho-transferase	Proteins containing a protein kinase family domain, c109925 and phosphotransferase family with PF 01636
	YP_006394449.1[k]	BBB_0978	N-acetyl hexosamine kinase	Proteins containing a protein kinase family domain, c109925 and phosphotransferase family with PF 01636
B. bifidum NCIMB 41171	ZP_07801902.1[j]	BBNG_00382	Conserved hypothetical protein	Proteins containing a protein kinase family domain, c109925 and phosphotransferase family with PF 01636
B. angulatum DSM 20098	ZP_04447474.1[m]	BIFANG_02451	Hypothetical protein	Proteins containing a protein kinase family domain, c109925
B. animalis subsp. *lactis* HN019	YP_002469703.1[e]	BLA_0835	AG phospho-transferase	Proteins containing a protein kinase family domain, c109925 and phosphotransferase

				family with PF 01636
	ZP_02963731.1[e]	BIFLAC_04950	Hypothetical protein	Proteins containing a protein kinase family domain, c109925 and phosphotransferase family with PF 01636
B. animalis subsp. *animalis* ATCC 25527	YP_006280402.1[e]	BANAN_06155	AG phospho-transferase	Proteins containing a protein kinase family domain, c109925 and phosphotransferase family with PF 01636
	YP_006279244.1[n]	BANAN_00270	AG phospho-transferase	Phosphotransferase family with PF 01636 and aminoglycoside phosphotransferases of the aph family cd 05150
B. longum subsp. *longum* JCM1217	YP_004221381.1[b]	BLLJ_1622	AG phospho-transferase	Proteins containing a protein kinase family domain, c109925 and phosphotransferase family with PF 01636
Bifidobacterium sp. 12_1_47BFAA	ZP_07941182.1[b]	HMPREF0177_00575	Phospho-transferase enzyme family protein	Proteins containing a protein kinase family domain, c109925 and phosphotransferase family with PF 01636
B. longum subsp. *infantis* 157F	YP_004209317.1[a]	BLIF_1400	Hypothetical protein	Proteins containing a protein kinase family domain, c109925

* Same superscript indicates proteins share >90% sequence percentage identity

AG: aminoglycoside

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

	Antibiotic (microgram/per disc)					
	β-lactams			Aminoglycosides		
Bifidobacteria species	**AMP 25**	**PEN 10 IU**	**KAN 30**	**GEN 200**	**STR 25**	**NEO 30**
B. breve DSM 20213	71mm	65mm	No zone	22mm	16mm	14mm
B. animalis subsp. *lactis* Bb12	65mm	55mm	No zone	28mm	21mm	20mm
B. pseudocatenulatum DSM 20438	61mm	56mm	8mm	10mm	13mm	20mm
B. gallicum DSM 20093	60mm	59mm	No zone	24mm	30mm	10mm
B. angulatum DSM 20098	64mm	65mm	4mm	23mm	16mm	10mm
B. breve UCC2003	67mm	56mm	No zone	26mm	21mm	10mm
B. breve UCC2003-0651-tet	52mm	57mm	10mm	40mm	33mm	14mm
B. breve UCC2003-1586-tet	62mm	57mm	9mm	41mm	31mm	15mm
B. breve UCC2003-1586-tet-pBC1.2-Bbr_1586	62mm	59mm	No zone	30mm	33mm	13mm

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)

	Antibiotic (mg/L)			
	GEN	**NEO**	**STR**	**KAN**
Sample	**1-1024**	**1-1024**	**2-4096**	**2-4096**
B. breve UCC2003 wild-type	>1024	>1024	1024	>4096
B. breve UCC2003-0651-tet	256	>1024	256	1024
B. breve UCC2003-1586-tet	256	>1024	256	1024
B. breve UCC2003-gosG	>1024	>1024	2048	>4096
B. breve UCC2003-1586-tet-pBC1.2-Bbr_1586	>1024	1024	256	4096
B. breve UCC2003 wild-type*	4096	4096	1024	4096
B. breve UCC2003-pBC1.2_Bbr_1586*	4096	4096	2048	8192
B. breve UCC2003-pBC1.2_Bbr_0651*	4096	4096	1024	4096
B. breve UCC2003-pBC1.2_Bbr_0651+0650*	4096	4096	1024	4096
E. coli XL1-blue-pBC1.2	<1	4	<2	<2
E. coli XL1-blue-pBC1.2_Bbr_0651+0650	2	8	<2	<2
E. coli XL1-blue-pBC1.2_Bbr_0651	2	8	<2	<2
E. coli XL1-blue-pBC1.2_Bbr_1586	<1	8	<2	<2

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

Primer name	Primer sequence	Source
Primers for insertions mutants		
Bbr_0651f (500bp insert product)	TGCGGAAAGCTTCGGTTCATCGCGAGGGACAG	This study
Bbr_0651r	CTATGCTCTAGACTCCATCTGCAACCATAG	This study
Bbr_1586f (400bp insert product)	TGCGGAAAGCTTGTATACAAATTCATCGAGCAC	This study
Bbr_1586r	CTATGCTCTAGAGCATGATGGTGTCGAGGTCG	This study
Pori19f	ATTGTGAGCGGATAACAATTTCAC	This study
Pori19r	GATTAAGTTGGGTAACGCCAG	Law *et al.* (1995)
Primers for complementation study and additional plasmid-encoded gene tests		
Bbr_0651fhind3	TCGCTTAAGCTTGCTGCGCTTGTTCCATGACC	This study
Bbr_0651rxbaI	GAACTGTCTAGACTCGTTGGTGCCCGTCGCCG	This study
Bbr_0651+ 0650r2xbaI	CTGCCATCTAGAGACCGATGAGGCCACCGTG	This study
Bbr_1586fhind3	TCGCTTAAGCTTGAGACCTTCGACCTTCAGCCCAG	This study
Bbr_1586xbaI(1586 complement strain)	GAACTGTCTAGAGCGCCGCCCGTAACCAGAACAGT	This study

Chapter 5

Identification of aminoglycoside and β-lactam resistance genes from within an infant gut functional metagenomic library

Under review

5.1 Abstract

The infant gut microbiota develops rapidly during the first 2 years of life, acquiring microorganisms from diverse sources. During this time, significant opportunities exist for the infant to acquire antibiotic resistant bacteria, which can become established and constitute the infant gut resistome. With increased antibiotic resistance limiting our ability to treat bacterial infections, investigations into resistance reservoirs are highly pertinent. The aim of this study was to explore the nascent resistome in antibiotically-naïve infant gut microbiomes, through a function-driven metagenomic survey, combining functional screening of a metagenomic fosmid bank with PCR analysis. Faecal samples from 22 six-month-old infants with no previous antibiotic exposure were used to construct a pooled metagenomic library, which was functionally screened for ampicillin and gentamicin resistance. Our library of ~220Mb was found to contain 0.45 ampicillin resistant hits/Mb and 0.059 gentamicin resistant hits/Mb. PCR-based analysis of recovered fosmid clones and uncloned infant gut metagenomic DNA, revealed a diverse and abundant aminoglycoside and β-lactam resistance reservoir within the infant gut, with resistance determinants exhibiting homology to those found in common gut inhabitants, including *Escherichia coli*, *Enterococcus* and *Clostridium difficile*, as well as to genes from cryptic environmental bacteria. Notably, the genes identified differed from those revealed when a purely sequence-driven PCR-based screen of metagenomic DNA was employed. Carriage of these antibiotic resistance determinants conferred

substantial but varied (2-512x) increases in antibiotic resistance (ampicillin and gentamicin) to their bacterial host. Overall, these data, provide insights into the infant gut resistome, revealing the presence of a varied aminoglycoside and β-lactam resistance reservoir even in the absence of selective pressure, indicating the infant gut resistome establishes early in life, perhaps even at birth.

5.2 Introduction

There is growing concern that we are rapidly approaching a post-antibiotic era. As a result every effort is being made to discover and investigate antibiotic resistance reservoirs with the aim of limiting the selection for, or dissemination of, antibiotic resistance genes. One such reservoir is the human gut microbiota. Colonized by trillions of bacteria representing hundreds of different species, this ecosystem has been identified as a source of antibiotic resistant bacteria (Sommer et al., 2009, Rolain, 2013, Penders et al., 2013). Though vastly less complex and considerably less stable than the adult gut microbiota, the gut microbiota of infants also has the potential to acquire and perhaps, disseminate antibiotic resistant genes. From the commencement of labour, the sterile infant gut rapidly becomes colonized (Koenig et al., 2011) and from birth through to 2 years of age the infant gut microbiota is dynamic, unstable and becomes increasingly complex, until it resembles that of an adult. The identity of the first colonizers of the infant gut depends on numerous factors (Fouhy et al., 2012b, Marques et al., 2010) including mode of delivery (Dominguez-Bello et al., 2010), and pre-term versus full-term gestation (Westerbeek et al., 2006) and, because of the aforementioned instability of this microbial population, many factors including feeding choice (Le Huërou-Luron et al., 2010), probiotic or prebiotic supplementation (Boehm and Moro, 2008) and antibiotic exposure (Tanaka et al., 2009) can significantly impact on its subsequent development. Indeed, in a previous study, we have demonstrated that the

infant microbiota becomes dominated by *Proteobacteria* following antibiotic administration in the first 48 hours of life (Fouhy et al., 2012a), which may be due to the known high prevalence of antibiotic resistant species within this phylum (Qin et al., 2009). Such findings suggest that the infant gut microbiota, though immature and in constant flux, can be a source of resistant bacteria which can become dominant following antibiotic exposure. Given the instability of the infant microbiota in early life, there is considerable opportunity for the infant gut to acquire resistant populations which, if they become established, could have significant effects on shaping the composition of the microbiota later in life.

While it is known that antibiotic resistance genes are present within the gut microbiota from early life (Gueimonde et al., 2006, Zhang et al., 2011), data with respect to the presence of antibiotic resistance genes in healthy infants with no antibiotic exposure is still limited. Furthermore, much of the existing data comes from studies investigating resistance genes in specific commensals, such as strains of *Escherichia coli* or *Lactobacillus* (Kirtzalidou et al., 2011, Roy et al., 2010), rather than the entire microbiota. Challenges in studying gut microbiota are well documented (Fouhy et al., 2012b) and include an inability to culture the majority of gut microbes in a laboratory environment (Eckburg et al., 2005). However, metagenomic libraries provide the opportunity to capture metagenomic DNA from complex environments and to functionally screen for phenotypes of interest. The limited existing data available from adult (Cheng et al., 2012, Sommer et al., 2009) and infant (De Vries et al.,

2011, Moore et al., 2013) metagenomic banks indicate that human gut microbiota is a source of diverse antibiotic resistance genes. However, there is a significant paucity in infant studies, particularly relating to infants free from antibiotic exposure. Thus, the aim of this study was to construct a fosmid bank using metagenomic DNA from 6-month-old infants who had never received antibiotic treatment and to screen for the presence of antibiotic resistance genes. Our goal was to provide an insight into the infant gut microbiota as a reservoir for resistance genes, when no antibiotic selective pressure exists. Due to the on-going focus of our research (Fouhy et al., 2012a), we specifically investigated resistance to aminoglycosides and β-lactam antibiotics. Using both a function-based and function-independent approach, we successfully identified a variety of aminoglycoside and β-lactam resistance genes in the gut microbiota of infants free from antibiotic exposure, providing insights into the infant gut resistome.

5.3 Materials and Methods

5.3.1 Recruitment of volunteers

Infants were recruited as part of the INFANTMET study. Parents of infant participants provided written informed consent. Approval for the INFANTMET study was received from the Clinical Research Ethics Committee of the Cork Teaching Hospitals, Cork, Ireland. Infants had no antibiotic exposure for the first 6 months of life. Fresh faecal samples were collected from 22 6-month old infants and immediately stored at -80°C until processed.

5.3.2 DNA extraction

Due to the small volume of each individual sample, it was necessary to pool faecal samples prior to DNA extraction. Faecal samples were homogenized and 500 mg from each were pooled to form one sample from which high molecular weight metagenomic DNA was extracted, using a previously described method (Jones and Marchesi, 2006, Ogilvie et al., 2013, Jones et al., 2008). Briefly, faecal samples were homogenised in PBS (Sigma Aldrich, Dublin, Ireland), centrifuged at 1000g x 5mins and the supernatant retained. Nycodenz (Axis Shield, UK) density gradient separation was performed to separate out the bacterial cells. Enzymatic digestion of the cells using lysozyme and mutanolysin (Sigma Aldrich) was performed, and subsequently the protein was removed using Proteinase K and ammonium acetate treatment (Sigma

Aldrich). DNA was then purified and precipitated using standard chloroform and ethanol precipitation procedures and was eluted in TE buffer.

5.3.3 Metagenomic bank creation

To allow functional screening of the metagenomic DNA from the infant gut, a fosmid metagenomic bank was created using the EpiCentre CopyControl Fosmid Library Production kit with the pCC1FOS vector, according to the manufacturer's instructions (Cambio, Cambridge, England). Briefly, size selection was performed on the metagenomic DNA using pulse field gel electrophoresis (PFGE) (0.5X TAE; 0.1initial/10 final switch times; 4V; 17 hours; 14°C) and fragments of ~40 kB were gel extracted from the low melting point agarose (Promega, Medical Supply Company, Dublin). Fragments were then ligated with the pCC1FOS vector according to the manufacturer's instructions and subsequently packaged into EPI300-T1^{R} *Escherichia coli* plating strain cells. These cells were then plated onto LB agar (Difco, Becton, Dickinson & Co, Oxford, England) + 12.5µg/ml chloramphenicol + IPTG + Xgal plates (Sigma Aldrich) and grown overnight aerobically at 37°C. To verify the diversity of the library, random white clones were selected and were digested with *Pst*1 and *Nde*1 restriction enzymes (New England Biolabs, UK), to determine if different DNA insert sequences were present in our metagenomic bank. The entire library was plated and then picked and stocked in 384-well-format using the QPix2-XT robotic system (Molecular

Devices, Berkshire, UK) and was stored at -80°C until screening. A library of ~ 220Mb of DNA was created.

5.3.4 Screening for antibiotic resistant clones

Due to the ongoing focus of our research, we chose to concentrate on the resistance of infant gut microbiota to 2 groups of antibiotics, namely the aminoglycosides and β-lactams. For screening of the metagenomic bank, the library was plated onto LB + 12.5 µg/ml chloramphenicol and inhibitory concentrations of ampicillin (50 µg/ml) or gentamicin (10 µg/ml) (Sigma Aldrich). These levels of antibiotics were chosen due to their inhibition of host EPI 300-T1^{R} *E. coli* cells. The library was plated in triplicate and plates were incubated aerobically at 37°C for 24-36 hours. Clones found to be consistently resistant over three replicate plates were selected, their resistance phenotype verified by re-streaking onto agar containing the relevant antibiotic and were stocked for further analysis (Figure 1).

5.3.5 PCR analysis

To investigate which resistance genes were present in the insert DNA that conferred resistance, PCRs were carried out on each of these clones using degenerate primers for aminoglycoside and β-lactam resistance genes (Table 1). For the aminoglycosides, we used degenerate primers designed to amplify the acetylation (AAC; *aac* (3)-I, *aac* (3)-II, *aac* (3)-III, *aac* (3)-VI and *aac* (6)), adenylation (ANT; *ant* (2")-Ia) and phosphorylation (APH; *aph* (2")-Ic and *aph* (2")-Id) genes (Heuer et al.,

2002), as well as the bifunctional gene *aac*(6')-*Ie*-*aph*(2") (De Fátima Silva Lopes et al., 2005, Schmitz et al., 1999). For β-lactam resistance the degenerate primer sets for the following genes were used bla_{TEM} (Bailey et al., 2011, Tenover et al., 1994), bla_{OXA} (Briñas et al., 2002), bla_{SHV} (Briñas et al., 2002), bla_{ROB} (Tenover et al., 1994) and bla_{CTX-M} (Monstein et al., 2009) (Table 1). Resistant clones were grown overnight in LB broth supplemented with chloramphenicol (12.5µg/ml) and either ampicillin (50 µg/ml) or gentamicin (10 µg/ml). Plasmids were extracted from the clones using the QIAprep Spin Mini Prep kit (Qiagen, Sussex, UK) and subsequently used as template DNA for PCR analysis. PCRs were performed using previously outlined protocols (Bailey et al., 2011, Briñas et al., 2002, De Fátima Silva Lopes et al., 2005, Heuer et al., 2002, Monstein et al., 2009, Schmitz et al., 1999, Tenover et al., 1994). Each reaction contained 25 µl of Biomix Red (MyBio, UK), 1 µl forward primer (10pmol), 1 µl reverse primer (10pmol), 64 ng plasmid DNA from the resistant clone, and PCR grade water (Bioline, Medical Supply Company, Dublin, Ireland) to a final reaction volume of 50 µl. All reactions were performed in duplicate. PCR products were visualized using gel electrophoresis (1.5% agarose, 1X TAE, 100V). Successful duplicate PCRs were pooled and cleaned using AMPure (Beckman Coulter UK) magnetic bead-based purification procedures.

The metagenomic DNA which was used for construction of the fosmid bank, was also used as template DNA for PCR analysis using the primers and protocols outlined above. Following PCR amplification, amplicons

were cloned using the TOPO TA cloning kit (Invitrogen, Dublin, Ireland) according to the manufacturer's instructions. TOPO cloning reactions were then transformed into *E. coli* TOP 10 cells and plated on LB agar containing antibiotics for the selection of the cloning vector (either Kanamycin 50 µg/ml or Ampicillin 50 µg/ml). Plasmids were then extracted from overnight cultures of the TOPO sub-clones using the QIAprep Spin Mini Prep kit (Qiagen, Sussex, UK) to facilitate subsequent DNA sequencing (Figure 1).

5.3.6 Sequencing and analysis

PCRs from each of the resistant fosmid clones were sent for Sanger sequencing to determine their closest homologue (Source Biosciences, Dublin, Ireland). Plasmid DNA from the TOPO sub-clones of the metagenomic DNA PCR products were also sent for Sanger sequencing. Sequencing reads were BLASTed against the NCBI non-redundant database using BLASTx (http://blast.ncbi.nlm.nih.gov/). In the event where multiple hits occurred, the BLAST hit which displayed greatest homology (based on E value) is reported.

5.3.7 Minimum inhibitory concentration assays

Minimum inhibitory concentration (MIC) tests were conducted on gentamicin and ampicillin resistant fosmid clones to determine the level of resistance conferred by the insert DNA. In order to determine the relative increase in resistance, MICs were compared to the control strain (EPI300-T1^{R} *E. coli* + empty pCC1FOS fosmid). MICs were performed

according to the British Society for Antimicrobial Chemotherapy guidelines (Andrews, 2001). Each of the resistant clones were grown to an OD_{600nm} of 0.5 and subsequently diluted to provide a final concentration of ≈$5x10^5$ cfu/well. 100 µl of Isosensitest broth (Oxoid) was added to each well of the 96-well-plate. 100 µl of antibiotic was added to the first well and serially diluted. Finally 100 µl of inoculum was added to each well and mixed. This resulted in an antibiotic range of 256-0.25 mg/L. Positive (broth + inoculum only) and negative controls (broth + antibiotic only) were included in each assay. Plates were incubated aerobically at 37 °C for 24 hours. The MIC was determined as the lowest concentration of antibiotic at which no visible growth occurred.

5.4 Results

5.4.1 Identification of gentamicin and ampicillin resistant clones within a functional, infant gut metagenomic bank

Metagenomic DNA was extracted from a pool of 22 infant faecal samples (pooled due to the small size of each individual sample) and was used to create a fosmid metagenomic library. Ten white clones were picked at random from LB + 12.5 µg/ml chloramphenicol + IPTG + X-gal plates, and underwent restriction digestion using *Pst*1 and *Nde*1 restriction enzymes. These randomly selected clones had unique restriction profiles (data not shown), establishing that the metagenomic library contained different insert DNA, demonstrating a diverse metagenomic fosmid bank had been successfully created. Our library contained ~ 220Mb of metagenomic DNA, of which ~1.8% of clones were positive for ampicillin resistance and ~0.2% of clones contained gentamicin resistance genes. To determine which genes were responsible for the resistance in the isolated clones, PCR analysis was conducted on each of the resistant clones using primers for β-lactam and aminoglycoside resistance genes. Furthermore, to determine if additional genes could be detected in the uncloned metagenomic DNA, PCR analysis was completed on this DNA with the same degenerate primers as used on the resistant clones.

5.4.2 PCR and Sanger sequencing of β-lactam resistant clones

Analysis of the one hundred Amp^R clones using bla_{TEM} primers, revealed that they all contained bla_{TEM} genes (Table 2). Based on sequencing

analysis, these genes shared closest homology with *bla*$_{TEM}$ genes from uncultured soil bacteria (clones 2, 3, 4, 5, 6, 10, 16, 17, 123, 128), *Shigella* sp. (clone 4), *Serratia maracescens* (clone 126) and *Clostridium freudii* (clone 17) (Table 2). Sequencing of amplicons generated using the same *bla*$_{TEM}$ primers and uncloned metagenomic DNA, revealed a number of different *bla*$_{TEM}$ genes that shared closest homology with genes from *Klebsiella pneumoniae*, *E. coli*, *Salmonella enterica* and *Staphylococcus aureus* (Table 3).

PCRs revealed that half of the Amp^{R} clones contained *bla*$_{CTX-M}$ genes, all of which shared homology with 1 of 3 *bla*$_{CTX-M}$ genes from *E. coli*. Sequencing of *bla*$_{CTX-M}$ PCR products generated directly from metagenomic DNA identified additional sources of these genes, detecting genes that shared homology with *bla*$_{CTX-M}$ genes present in *E. coli* (including one source that was not detected in the analysis of the Amp^{R} clones) and *K. pneumoniae* (Table 3).

Just 2 of the 100 Amp^{R} clones contained *bla*$_{OXA}$ genes, which resembled 2 different *bla*$_{OXA}$ genes from *E. coli*. No *bla*$_{OXA}$ genes were amplified when PCR was directly applied to the metagenomic DNA from the infant faecal samples. Six of the 100 Amp^{R} clones (clones 15, 50, 51, 61, 62 and 63) contained *bla*$_{ROB}$ genes and all shared closest homology (53% identity, 40% query cover) with a hypothetical protein from *Staphylococcus haemolyticus*. Direct PCR amplification using template metagenomic DNA failed to reveal any *bla*$_{ROB}$ genes. Finally, *bla*$_{SHV}$

primers did not generate amplicons from either the Amp^R clones or uncloned metagenomic DNA.

5.4.3 PCR and Sanger sequencing of aminoglycoside resistant clones

Degenerate PCR primers were employed to determine whether the fosmids conferring gentamicin resistance contained homologues of known aminoglycoside resistance genes. Acetylation, adenylation and phosphorylation genes were detected in the Gent^R clones (Table 2). With respect to aminoglycoside acetylation-associated genes, *aac* (3)-I, *aac* (3)-II, *aac* (3)-III and *aac* (3)-VI were not detected in either the 13 Gent^R clones or in the uncloned metagenomic DNA. However, all 13 Gent^R clones contained an *aac* (6) gene that most closely resembled a *Pseudomonas fluorescens* gene. Interestingly, the *aac* (6) primers did not generate amplicons when used directly with metagenomic DNA (Table 3), thereby again demonstrating the advantage of a multifaceted approach to detecting genes in complex environments. Aminoglycoside adenylation, *ant* (2")-Ia, genes were detected in 9 of the 13 Gent^R clones (Table 2). Eight of these 9 Gent^R clones shared closest homology with aminoglycoside 2' O adenyltransferase genes found in *Pasteurella mutocida*, while that from the remaining clone resembled an *E. coli* associated gene. Similar sources of these genes were also detected when PCR products amplified with the *ant* (2")-Ia primers and uncloned metagenomic template DNA, were sequenced (Table 3). Following

cloning of these amplicons, it was apparent that 4 of the 5 clones contained *ant* (2")-Ia genes sharing closest homology (99% identity, 97% query cover) with genes present in *P. mutocida* (identical to the source of these genes detected in the Gent^R clones). The fifth clone that was sequenced contained insert DNA sharing closest homology with a nucleotidyltransferase from *A. baumannii* (99% identity, 97% query cover).

When primers were applied to investigate the presence of aminoglycoside phosphorylation genes using *aph* (2")-*Id* primers, 10 of the 13 Gent^R clones contained these genes (Table 2). A number of these Gent^R clones (5, 6, 8, 11, 12, 13 and 34) shared closest homology with phosphorylation genes from *Clostridium difficile* (100% identity and 94% query cover). When these *aph* (2")-Id primers were used directly with metagenomic DNA, genes sharing homology with *C. difficile* (identical to those detected in the Gent^R clones), *Enterococcus faecium* and an unknown source of tobramycin resistant 2" aph genes were detected (Table 3).

Finally, using 2 different primer sets, it was found that all Gent^R clones contained the bifunctional gene *aac*(6')-*Ie*-*aph*(2''). These genes shared homology with bifunctional *aac*(6')-*Ie*-*aph*(2'') genes from *Enteroccus faecalis* (clones 3, 4, 5, 6 and 11), *Streptococcus epidermidis* (clones 4, 8 and 13), *E. faecium* (clone 10) and *Enterococcus* sp. (clone 5) (Table 2). Sequencing of the amplicons generated with uncloned metagenomic

DNA, identified genes sharing closest homology with bifunctional genes from *E. faecalis* (which were distinct from those detected in the Gent^R clones) and from *S. aureus* (WP_001028140.1), a source which was not detected using PCR analysis on the Gent^R clones (Table 3).

5.4.4 MIC analysis of ampicillin and gentamicin resistant clones

To determine the level of resistance conferred by the fosmid-cloned metagenomic DNA to ampicillin or gentamicin, we conducted MIC assays on a representative 22 of the 100 Amp^R clones (including representatives of those containing genes only detected in a limited number of clones e.g. *bla*$_{OXA}$ and those containing multiple β-lactam resistance genes clones) and on all 13 of the Gent^R clones, compared to the host EPI 300-T1^R *E. coli* cells containing the pCC1FOS fosmid.

The Amp^R clones exhibited a 2- (clone 6) to 512- (clone 2) fold increase in resistance to ampicillin compared to the empty fosmid control (Table 4). The large variation in MICs between the Amp^R clones highlighted the fact that different, but related, genes conferred different levels of resistance to the host *E. coli* cells, most likely due to differences in expression/translation in the surrogate *E. coli* host. For example, in the case of the only 2 clones that contained *bla*$_{OXA}$ genes, MICs of 4 mg/l and 32 mg/L, respectively, were observed. In the case of the 6 clones that contained *bla*$_{ROB}$ genes, 3 of these had an MIC of just 1 mg/ml, despite such genes being reported to confer high level resistance to ampicillin. Clones containing multiple β-lactam resistance genes (clones 2, 3, 4, 62,

128, 136 and 140), including *bla*$_{TEM}$, *bla*$_{CTX-M}$ and, in 1 case, *bla*$_{OXA}$ genes, were found to confer the highest levels of resistance to β-lactams. In contrast, when MICs were conducted on the 13 Gent^{R} clones, all clones showed similar levels of resistance to gentamicin with MICs of 0.5-1mg/L compared to 0.125mg/L for the control (Table 5), despite the apparent different origin of the Gent^{R} genes in these fosmid clones.

5.5 Discussion

The infant gut microbiota is in constant flux during the first two years of life (Palmer et al., 2007). During this time there is considerable opportunity for the infant to acquire antibiotic resistant populations which may persist. Given the opportunity, resistant populations could become dominant in the gut following antibiotic therapy or may contribute to antibiotic resistance gene dissemination. In this study we set out to use a metagenomic fosmid bank to functionally screen the gut microbiota of 22 healthy infants who had not been exposed to antibiotics, for antibiotic resistance genes. Using such an approach, we have demonstrated that the infant gut is a source of a variety of genes encoding resistance to aminoglycosides and β-lactams.

This metagenomic study has successfully identified acetylation, adenylation and phosphorylation genes conferring aminoglycoside resistance in the infant gut microbiota. The fact that these genes resemble genes from bacteria such as *E. coli*, *E. faecalis*, *S. epidermidis* and *C. difficile* is not surprising given past evidence of aminoglycoside resistance amongst these species (Schmitz et al., 1999, Vakulenko et al., 2003, Vanhoof et al., 1994). A recent study which employed a metagenomic approach to investigate antibiotic resistance in infants, also detected a high prevalence of aminoglycoside resistance genes (Moore et al., 2013). In that instance, they too identified aminoglycoside acetylation, adenylation and phosphorylation genes in the infant gut

which shared homology with *C. difficile* and *E. faecium*. As these bacteria may be among the first colonizers of the infant gut, these findings support previous research which suggests antibiotic resistance and the antibiotic resistome is established in early life, perhaps even from birth, irrespective of antibiotic exposure, and is closely associated with the microbes from the maternal and environmental sources it is exposed to during and immediately after birth (Zhang et al., 2011). This is consistent with the apparently ubiquitous nature of antibiotic resistance genes in the environment, with studies having demonstrated that antibiotic resistant isolates can be detected in individuals from remote areas of the world who lack, or who have minimal, antibiotic exposure (Pallecchi et al., 2008, Pallecchi et al., 2007, Bartoloni et al., 2009).

One hundred Amp^R clones were detected in the gut microbiota of infants through screening of the metagenomic fosmid bank. Applying a PCR-based approach we detected bla_{TEM}, bla_{OXA}, bla_{ROB} and $bla_{CTX\text{-}M}$ genes in these resistant isolates. Interestingly, the bla_{TEM} genes detected exhibited greatest homology with genes from uncultured bacteria, while the bla_{OXA} genes identified shared low percentage identity (38 and 71%) with bla_{OXA} genes found in *E. coli*. Results such as these highlighted the ability of our strategy to reveal genes from more cryptic sources, including β-lactam resistance genes from sources not accessible through classical culture-based approaches. It was also notable that we detected such a prevalence of ampicillin resistance genes, and that they appear to have originated from a diverse range of species. Nonetheless, the majority of

the β-lactam resistance genes identified shared homology with β-lactam resistance genes present in *E. coli*, *Shigella* and *Serratia*, which are common sources of such genes (Bonnet, 2004, Coque et al., 2002, Morosini et al., 1995).

In addition to screening the fosmid metagenomic bank, a PCR-based analysis of uncloned metagenomic DNA was also carried out. Previous studies demonstrated the success of such an approach with respect to detecting genes that were present at a low level and had not been captured in the metagenomic fosmid bank (De Vries et al., 2011). Additionally, this approach also addresses situations whereby certain genes may not be expressed or their products may not be functional in *E. coli*, e.g. as previously shown in the case of *strA* and *strB* (Donato et al., 2010). In this study, we have also highlighted the usefulness of employing a PCR-based approach to detect antibiotic resistance genes apparently originating from different species than those that were identified during the course of the functional screen. For example, the bla_{TEM} genes identified from the functional screen shared closest homology with genes from uncultured soil bacterium, whereas direct PCR-screening highlighted homologues of genes found in *K. pneumoniae*, *S. aureus*, *S. enterica* and *E. coli*. This demonstrates that combining different experimental approaches allows for the most comprehensive insight into the gut microbiota as a source of antibiotic resistance genes. It should also be noted that, if screened for, we expect that our metagenomic library would also uncover resistance genes for other major families of antibiotics.

This study is one of a limited number that has demonstrated the existence of antibiotic resistant genes in the healthy infant gut microbiota using a metagenomic library approach. Although used to investigate antibiotic resistance in environments as diverse as gull faeces (Martiny et al., 2011) or soil from apple orchards (Donato et al., 2010), to date the use of metagenomic banks to screen the human gut microbiota for resistance genes have been limited (Cheng et al., 2012, De Vries et al., 2011, Moore et al., 2013). In particular, our study adds considerably to the existing infant data by supporting and supplementing existing results (Moore et al., 2013), which have demonstrated that the infant gut is an antibiotic resistance reservoir. By studying 6-month-old infants with no antibiotic exposure, we were able to establish a comprehensive insight into the resistome of infants devoid of antibiotic selective pressure. Additionally, we have also demonstrated the benefits of supplementing functional screening with PCR and MIC assays, which enables the complexity of the infant resistome to be fully appreciated. The findings are important for several reasons. Firstly, as the infant gut is populated through complex bacterial acquisitions from maternal, dietary and environmental sources, there is the potential to control the populations colonizing infants, i.e. to maximize colonization with those bacteria that may be beneficial and pose less risk of contributing to the antibiotic resistome. Additionally, in cases where antibiotic administration is required, the route of antibiotic administration should be considered carefully when different options are available. Indeed, research using a

murine model has demonstrated that antibiotic administration through intravenous injection resulted in significantly less, or the delayed development of, antibiotic resistant genes in gut microbiota populations compared to oral administration (Zhang et al., 2013). It would also seem that further investigations with respect to links between maternal antibiotic exposure and subsequent antibiotic resistant populations in the infant would be beneficial. In one previous instance, tetracycline resistance (tet^R) in the gut microbiota of one mother-infant pair was studied (De Vries et al., 2011). More specifically, resistance genes from the gut microbiota of a vaginally delivered, exclusively breastfed male infant, one month after birth, were compared with those within the gut microbiota of his mother. Among the findings was an observation of some identical resistance genes in both populations, suggesting that the transfer of antibiotic resistant populations from the mother to the infant may occur, potentially through maternal microbiota transmission during delivery, physical contact or through breastfeeding (De Vries et al., 2011).

Despite not being exposed to antibiotics, the results of this study demonstrate that the gut microbiota of healthy 6-month-old infants harbors a diverse number of antibiotic resistance genes in their gut microbiota. Metagenomic fosmid bank creation allowed screening for resistance genes irrespective of their source and revealed genes from diverse sources including those previously linked with uncultured bacteria. Combining this sophisticated approach with PCR techniques, provided a particularly unique insight into the presence of resistance

genes in the infant gut microbiota. The results not only act as a cautionary insight into the prevalence of antibiotic resistance in infants, even in cases where no antibiotic selective pressure occurred, but also demonstrate the power of metagenomic approaches to provide important insights into the infant gut resistome.

References

Andrews, J. M. 2001. Determination of minimum inhibitory concentrations. *Journal of antimicrobial chemotherapy,* 48**,** 5-16.

Bailey, J. K., Pinyon, J. L., Anantham, S. & Hall, R. M. 2011. Distribution of the blaTEM gene and blaTEM-containing transposons in commensal Escherichia coli. *Journal of antimicrobial chemotherapy,* 66**,** 745-751.

Bartoloni, A., Pallecchi, L., Rodríguez, H., Fernandez, C., Mantella, A., Bartalesi, F., Strohmeyer, M., Kristiansson, C., Gotuzzo, E. & Paradisi, F. 2009. Antibiotic resistance in a very remote Amazonas community. *International journal of antimicrobial agents,* 33**,** 125-129.

Boehm, G. & Moro, G. 2008. Structural and functional aspects of prebiotics used in infant nutrition. *Journal of Nutrition,* 138**,** 1818S-1828S.

Bonnet, R. 2004. Growing group of extended-spectrum β-lactamases: the CTX-M enzymes. *Antimicrobial agents and chemotherapy,* 48**,** 1-14.

Briñas, L., Zarazaga, M., Sáenz, Y., Ruiz-Larrea, F. & Torres, C. 2002. β-Lactamases in ampicillin-resistant Escherichia coli isolates from foods, humans, and healthy animals. *Antimicrobial agents and chemotherapy,* 46**,** 3156-3163.

Cheng, G., Hu, Y., Yin, Y., Yang, X., Xiang, C., Wang, B., Chen, Y., Yang, F., Lei, F. & Wu, N. 2012. Functional screening of antibiotic resistance genes from human gut microbiota reveals a novel gene fusion. *FEMS microbiology letters,* 336**,** 11-16.

Coque, T. M., Oliver, A., Pérez-Díaz, J. C., Baquero, F. & Cantón, R. 2002. Genes Encoding TEM-4, SHV-2, and CTX-M-10 Extended-Spectrum β-Lactamases Are Carried by Multiple Klebsiella pneumoniae Clones in a Single Hospital (Madrid, 1989 to 2000). *Antimicrobial agents and chemotherapy,* 46**,** 500-510.

De Fátima Silva Lopes, M., Ribeiro, T., Abrantes, M., Figueiredo Marques, J. J., Tenreiro, R. & Crespo, M. T. B. 2005. Antimicrobial resistance profiles of dairy and clinical isolates and type strains of enterococci. *International journal of food microbiology,* 103**,** 191-198.

De Vries, L. E., Vallès, Y., Agersø, Y., Vaishampayan, P. A., García-Montaner, A., Kuehl, J. V., Christensen, H., Barlow, M. & Francino, M. P. 2011. The gut as reservoir of antibiotic resistance: microbial diversity of tetracycline resistance in mother and infant. *PLoS ONE,* 6**,** e21644.

Dominguez-Bello, M. G., Costello, E. K., Contreras, M., Magris, M., Hidalgo, G., Fierer, N. & Knight, R. 2010. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. *Proceedings of the National Academy of Sciences,* 107**,** 11971-11976.

Donato, J. J., Moe, L. A., Converse, B. J., Smart, K. D., Berklein, F. C., Mcmanus, P. S. & Handelsman, J. 2010. Metagenomic analysis of apple orchard soil reveals antibiotic resistance genes encoding predicted bifunctional proteins. *Applied and environmental microbiology,* 76**,** 4396-4401.

Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S. R., Nelson, K. E. & Relman, D. A. 2005. Diversity of the human intestinal microbial flora. *Science,* 308**,** 1635-1638.

Fouhy, F., Guinane, C. M., Hussey, S., Wall, R., Ryan, C. A., Dempsey, E. M., Murphy, B., Ross, R. P., Fitzgerald, G. F. & Stanton, C. 2012a. High-throughput sequencing reveals the incomplete, short-term, recovery of the infant gut microbiota following parenteral antibiotic treatment with ampicillin and gentamicin. *Antimicrobial agents and chemotherapy,* 56**,** 5811-5820.

Fouhy, F., Ross, R. P., Fitzgerald, G., Stanton, C. & Cotter, P. D. 2012b. Composition of the early intestinal microbiota: Knowledge,

knowledge gaps and the use of high-throughput sequencing to address these gaps. *Gut microbes,* 3**,** 203-220.

Gueimonde, M., Salminen, S. & Isolauri, E. 2006. Presence of specific antibiotic (tet) resistance genes in infant faecal microbiota. *FEMS Immunology & Medical Microbiology,* 48**,** 21-25.

Heuer, H., Krögerrecklenfort, E., Wellington, E., Egan, S., Elsas, J. V., Overbeek, L., Collard, J. M., Guillaume, G., Karagouni, A. & Nikolakopoulou, T. 2002. Gentamicin resistance genes in environmental bacteria: prevalence and transfer. *FEMS microbiology ecology,* 42**,** 289-302.

Jones, B. V., Begley, M., Hill, C., Gahan, C. G. & Marchesi, J. R. 2008. Functional and comparative metagenomic analysis of bile salt hydrolase activity in the human gut microbiome. *Proceedings of the National Academy of Sciences,* 105**,** 13580-13585.

Jones, B. V. & Marchesi, J. R. 2006. Transposon-aided capture (TRACA) of plasmids resident in the human gut mobile metagenome. *Nature Methods,* 4**,** 55-61.

Kirtzalidou, E., Pramateftaki, P., Kotsou, M. & Kyriacou, A. 2011. Screening for lactobacilli with probiotic properties in the infant gut microbiota. *Anaerobe,* 17**,** 440-443.

Koenig, J. E., Spor, A., Scalfone, N., Fricker, A. D., Stombaugh, J., Knight, R., Angenent, L. T. & Ley, R. E. 2011. Succession of microbial consortia in the developing infant gut microbiome. *Proceedings of the National Academy of Sciences,* 108**,** 4578-4585.

Le Huërou-Luron, I., Blat, S. & Boudry, G. 2010. Breast-v. formula-feeding: impacts on the digestive tract and immediate and long-term health effects. *Nutr Res Rev,* 23**,** 23-36.

Marques, T. M., Wall, R., Ross, R. P., Fitzgerald, G. F., Ryan, C. A. & Stanton, C. 2010. Programming infant gut microbiota: influence of dietary and environmental factors. *Current opinion in biotechnology,* 21**,** 149-156.

Martiny, A. C., Martiny, J. B., Weihe, C., Field, A. & Ellis, J. C. 2011. Functional metagenomics reveals previously unrecognized diversity of antibiotic resistance genes in gulls. *Frontiers in microbiology,* 2**,** 238. doi: 10.3389/fmicb.2011.00238.

Monstein, H.-J., Tärnberg, M. & Nilsson, L. E. 2009. Molecular identification of CTX-M and blaOXY/K1 β-lactamase genes in Enterobacteriaceae by sequencing of universal M13-sequence tagged PCR-amplicons. *BMC Infectious Diseases,* 9**,** 7-15.

Moore, A. M., Patel, S., Forsberg, K. J., Wang, B., Bentley, G., Razia, Y., Qin, X., Tarr, P. I. & Dantas, G. 2013. Pediatric Fecal Microbiota Harbor Diverse and Novel Antibiotic Resistance Genes. *PloS one,* 8**,** e78822.

Morosini, M. I., Canton, R., Martinez-Beltran, J., Negri, M. C., Perez-Diaz, J. C., Baquero, F. & Blazquez, J. 1995. New extended-spectrum TEM-type beta-lactamase from Salmonella enterica subsp. enterica isolated in a nosocomial outbreak. *Antimicrobial agents and chemotherapy,* 39**,** 458-461.

Ogilvie, L. A., Bowler, L. D., Caplin, J., Dedi, C., Diston, D., Cheek, E., Taylor, H., Ebdon, J. E. & Jones, B. V. 2013. Genome signature-based dissection of human gut metagenomes to extract subliminal viral sequences. *Nature communications,* 4.

Pallecchi, L., Bartoloni, A., Paradisi, F. & Rossolini, G. M. 2008. Antibiotic resistance in the absence of antimicrobial use: mechanisms and implications. *Expert review of anti-infective therapy,* 6**,** 725-732.

Pallecchi, L., Lucchetti, C., Bartoloni, A., Bartalesi, F., Mantella, A., Gamboa, H., Carattoli, A., Paradisi, F. & Rossolini, G. M. 2007. Population structure and resistance genes in antibiotic-resistant bacteria from a remote community with minimal antibiotic exposure. *Antimicrobial agents and chemotherapy,* 51**,** 1179-1184.

Palmer, C., Bik, E. M., Digiulio, D. B., Relman, D. A. & Brown, P. O. 2007. Development of the human infant intestinal microbiota. *PLoS Biology,* 5**,** e177. doi:10.1371/journal.pbio. 0050177.

Penders, J., Stobberingh, E. E., Savelkoul, P. H. & Wolffs, P. F. 2013. The human microbiome as a reservoir of antimicrobial resistance. *Frontiers in microbiology,* 4**,** doi: 10.3389/fmicb.2013.00087

Qin, X., Zerr, D. M., Weissman, S. J., Englund, J. A., Denno, D. M., Klein, E. J., Tarr, P. I., Kwong, J., Stapp, J. R. & Tulloch, L. G. 2009. Prevalence and Mechanisms of Broad-Spectrum {beta}-Lactam Resistance in Enterobacteriaceae: a Children's Hospital Experience. *Antimicrobial agents and chemotherapy,* 53**,** 3909-3914.

Rolain, J.-M. 2013. Food and human gut as reservoirs of transferable antibiotic resistance encoding genes. *Frontiers in Microbiology,* 4**,** 173 doi: 110.3389/fmicb.2013.00173.

Roy, S., Viswanathan, R., Singh, A., Das, P. & Basu, S. 2010. Gut colonization by multidrug-resistant and carbapenem-resistant acinetobacter baumannii in neonates. *European journal of clinical microbiology & infectious diseases,* 29**,** 1495-1500.

Schmitz, F.-J., Fluit, A. C., Gondolf, M., Beyrau, R., Lindenlauf, E., Verhoef, J., Heinz, H.-P. & Jones, M. E. 1999. The prevalence of aminoglycoside resistance and corresponding resistance genes in clinical isolates of staphylococci from 19 European hospitals. *Journal of antimicrobial chemotherapy,* 43**,** 253-259.

Sommer, M. O. A., Dantas, G. & Church, G. M. 2009. Functional characterization of the antibiotic resistance reservoir in the human microflora. *Science,* 325**,** 1128-1131.

Tanaka, S., Kobayashi, T., Songjinda, P., Tateyama, A., Tsubouchi, M., Kiyohara, C., Shirakawa, T., Sonomoto, K. & Nakayama, J. 2009. Influence of antibiotic exposure in the early postnatal period on the development of intestinal microbiota. *FEMS Immunology & Medical Microbiology,* 56**,** 80-87.

Tenover, F. C., Huang, M. B., Rasheed, J. K. & Persing, D. H. 1994. Development of PCR assays to detect ampicillin resistance genes

in cerebrospinal fluid samples containing Haemophilus influenzae. *Journal of clinical microbiology,* 32**,** 2729-2737.

Vakulenko, S. B., Donabedian, S. M., Voskresenskiy, A. M., Zervos, M. J., Lerner, S. A. & Chow, J. W. 2003. Multiplex PCR for detection of aminoglycoside resistance genes in enterococci. *Antimicrobial agents and chemotherapy,* 47**,** 1423-1426.

Vanhoof, R., Godard, C., Content, J., Nyssen, H. & Hannecart-Pokorni, E. 1994. Detection by polymerase chain reaction of genes encoding aminoglycoside-modifying enzymes in methicillin-resistant Staphylococcus aureus isolates of epidemic phage types. *Journal of medical microbiology,* 41**,** 282-290.

Westerbeek, E. a. M., Van Den Berg, A., Lafeber, H. N., Knol, J., Fetter, W. P. F. & Van Elburg, R. M. 2006. The intestinal bacterial colonisation in preterm infants: a review of the literature. *Clinical Nutrition,* 25**,** 361-368.

Zhang, L., Huang, Y., Zhou, Y., Buckley, T. & Wang, H. H. 2013. Antibiotic Administration Routes Significantly Influence the Levels of Antibiotic Resistance in Gut Microbiota. *Antimicrobial agents and chemotherapy,* 57**,** 3659-3666.

Zhang, L., Kinkelaar, D., Huang, Y., Li, Y., Li, X. & Wang, H. H. 2011. Acquired antibiotic resistance: are we born with it? *Applied and environmental microbiology,* 77**,** 7134-7141.

Table 1 Primers used in this study

Target gene	Primer name	Sequence 5'-3'	Annealing temp °C	Ref.
β-lactamase genes				
***Bla*$_{TEM}$**	RH605	TTTCGTGTCGCCCTTATTCC	60	Bailey *et al.* (2011)
	RH606	CCGGCTCCAGATTTATCAGC		
	Bla_TEM F	TGGGTGCACGAGTGGGTTAC	57	Tenover *et al. (*1994)
	Bla_TEM R	TTATCCGCCTCCATCCAGTC		
***Bla*$_{ROB}$**	Bla_ROB F	ATCAGCCACACAAGCCACCT	62	Tenover *et al.* (1994)
	Bla_ROB R	GTTTGCGATTTGGTATGCGA		
***Bla*$_{SHV}$**	Bla_SHV F	CACTCAAGGATGTATTGTG	58	Briñas *et al.* (2002)
	Bla_SHV R	TTAGCGTTGCCAGTGCTCG		
***Bla*$_{OXA}$**	Bla_OXA F	TTCAAGCCAAAGGCACGATAG	64	Briñas *et al.* (2002)
	Bla_OXA R	TCCGAGTTGACTGCCGGGTTG		
***Bla*$_{CTX-M}$**	Bla_CTX-MF	CGTTGTAAAACGACGGCCAGTGAATG TGCAGYACCAGTAARGTKATGGC	55	Monstein *et al.* (2009)
	Bla_CTX-MR	TGGGTRAARTARGTSACCAGAAYCAG CGG	60	
AG resistance genes				
***aac* (3)-I**	Faac3-1	TTCATCGCGCTTGCTGCYTTYGA	58	Heuer *et al.* (2002)
	Raac3-1	GCCACTGCGGGATCGTCRCCRTA		
***aac* (3)-II/VI**	Faac3-2	GCGCACCCCGATGCMTCSATGG	58	
	Raac3-2	GGCAACGGCCTCGGCGTARTGSA		
	Facc3-6	GCCCATCCCGACGCATCSATGG		
	Raac3-6	CGCCACCGCTTCGGCATARTGSA		
***aac* (6')-II/Ib**	Faac6	CACAGTCGTACGTTGCKCTBGG	58	
	Raac6	CCTGCCTTCTCGTAGCAKCGDAT		
***ant* (2")-I**	Fant	TGGGCGATCGATGCACGGCTRG	58	
	Rant	AAAGCGGCACGCAAGACCTCMAC		
***aph*(2")-I**	Faphc	CCCAAGAGTCAACAAGGTGCAGA	55	
	Faphd	GGCAATGACTGTATTGCATATGA	55	
	Raph	GAATCTCCAAAATCRATWATKCC	47	
aac*(6')-*Ie*-*aph*(2")-*Ia	aac-aphF	GAGCAATAAGGGCATACCAAAAATC		De Fatíma Silva Lopes

	aac-aphR aac6-aph2F aac6-aph2R	CCGTGCATTTGTCTTAAAAAACTGG CCAAGAGCAATAAGGGCATACC CACACTATCATAACCATCACCG	55	*et al.* (2003) Schmitz *et al*. (1999)

AG: aminoglycoside

Table 2 Identity of ampicillin and gentamicin resistant genes amplified from the infant gut metagenomic bank

Accession number	Clones in which genes detected	Closest homologue	E value	% ID
***bla*$_{ROB}$**				
WP_016930608.1	15,50,51,61,62, 63	Hypothetical protein *S. haemolyticus*	$1e^{-20}$	53
***bla*$_{TEM}$**				
AGH 19654.1	5,6,2	β-lactamase partial uncultured soil bacterium	$1e^{-134}$	100
AGH 19657.1	3,16,17	β-lactamase partial uncultured soil bacterium	$4e^{-139}$	99
AAP 93842.1	17,18	β-lactamase *C. freudii*	$5e^{-138}$	99
AEN 75339.1	4	β-lactamase TEM *Shigella* sp.	$4e^{-141}$	99
AGH 19655.1	2,123	β-lactamase uncultured soil bacterium	$2e^{-97}$	100
AGH 19650.1	3,4,10,128	β-lactamase uncultured soil bacterium	$7e^{-97}$	100
AAP 93841.1	126	β-lactamase *S. maracescens*	$2e^{-97}$	98
***Bla*$_{CTX-M}$**				
AEZ 49563.1	2,3	β-lactamase CTX-M 1 *E. coli*	$2e^{-116}$	99
BAD 16611.1	4,136	β-lactamase CTX-M 36 *E. coli*	$1.10e^{-127}$	99
AAB 22638.1	140,145	β-lactamase penicillin aminodo β-lactamase hydrolase *E. coli*	$2e^{-128}$	100
***bla*$_{OXA}$**				
AGN 75112.1	140	TEM 190 β-lactamase *E. coli*	$2e^{-04}$	38
ADZ 11076.1	132	β-lactamase TEM *E. coli*	$2e^{-40}$	71
***ant* (2”) Ia**				
YP_005176240.1	3,7,9,10,11,12, 33,34	AG 2 O’ adenyltransferase *P. mutocida*	$1e^{-79}$	96

WP_000292466.1	8	AG adenyltransferase *E. coli*	$5e^{-75}$	96
***aph* (2") Id**				
WP_0214010241.1	5, 6, 8,11,12,13, 34	aph *C. difficile*	$4e^{-95}$	100
***aac*(6)**				
AAA 25680.1	34,36	AG 6' N acetyltransferase *P. fluorescens*	$5e^{-33}$	97
***aac* (6')-*Ie-aph* (2")**				
WP_010714603.1	3,4,5,6,11	bifunctional *aac* (6')-*Ie-aph* (2") *E. faecalis*	$1e^{-96}$	100
AFR 11868.1	4,8,13	bifunctional 6' AG N acetyltransferase/2'' AG phosphotransferase *S. epidermidis*	$8e^{-32}$	98
AFM 29914.1	5	Gentamicin resistance protein *Enterococcus sp.*	$3e^{-30}$	96
WP_010782592.1	10	Bifunctional AAC/APH *E. faecium*	$1e^{-31}$	98

Table 3 Sequencing results from TOPO cloning of ampicillin and gentamicin resistant genes from metagenomic DNA

Accession #	Closest homologue	E value	% ID
***bla*$_{TEM}$**			
AAL 03985.1	ESBL TEM_71 *K. pneumoniae*	$7e^{-154}$	99
WP_004207849.1	β-lactamase TEM partial *K. pneumonia*	$3e^{-153}$	97
WP_017431996.1	β-lactamase partial *S. aureus*	$1e^{-112}$	84
WP_019405145.1	β-lactamase partial *K. pneumonia*	$6e^{-154}$	100
AEN 02824.1	β-lactamase TEM1 *K pneumonia*	$4e^{-111}$	99
ADE 18896.1	TEM-1 β-lactamase *S. enterica*	$7e^{-112}$	97
ABG 46354.1	ESBL *E. coli*	$1e^{-139}$	100
AEN 02826.1	β-lactamase TEM 1 *K. pneumonia*	$1e^{-108}$	99
***Bla*$_{CTX-M}$**			
AAB 22638.1	β-lactamase penicillin amido beta-lactam hydrolase *E. coli*	$1e^{-139}$	100
AEZ 49551.1	β-lactamase CTXM 1 *K pneumonia*	$5e^{-129}$	99
AEZ 49563.1	β-lactamase CTXM 1 *E. coli*	$8e^{-140}$	97
ABG 46356.1	ESBL *K. pneumonia*	$1e^{-138}$	99
***ant* (2”) Ia**			
YP_005176240.1	AG 2 O adenyltransferase *Pasturella mutocida*	$1e^{-95}$	99
WP_000946493.1	2 AG nucleotidltransferase *A. baumannii*	$3e^{-97}$	99
***aph* (2”) Id**			
AAW 59417.1	*E. faecium* aph2 Id	$5e^{-98}$	90
3Sg8_9	Crystal structure of AG 2'' phosphotransferase Tobramycin resistance gene	$5e^{-109}$	100
WP_021401024.1	aph 2 Id *C. difficile*	$6e^{-108}$	98
***aac* (6’)-*Ie-aph* (2”)**			
WP_ 001028140.1	GNAT family acetytransferase *S. aureus*	$2e^{-109}$	100
AAX 82584.1	Bifunctional AG modifying enzyme *E. faecalis*	$2e^{-106}$	99

WP_002417297.1	phosphotransferase enzyme family protein *E. faecalis*	$4e^{-113}$	99

ESBL: extended spectrum β-lactamase; AG: aminoglycoside

Table 4 MICs of ampicillin resistant clones

Amp^R clone	**MIC 256-0.25mg/L**
Control	**0.25**
2	128
3	32
4	64
5	1
6	0.5
10	4
15	1
16	8
17	16
18	8
50	1
51	4
61	16
62	64
63	1
123	16
126	4
128	32
132	4
136	64
140	32
145	2

Amp^R: ampicillin resistant

Table 5 MICs of gentamicin resistant clones

Gent^{R} clones	**MIC 256-0.125mg/L**
Control	**0.125**
3	1
5	1
7	1
10	1
4	1
6	1
8	1
9	1
11	1
12	1
13	0.5
33	0.5
34	1

Gent^{R}: Gentamicin resistant

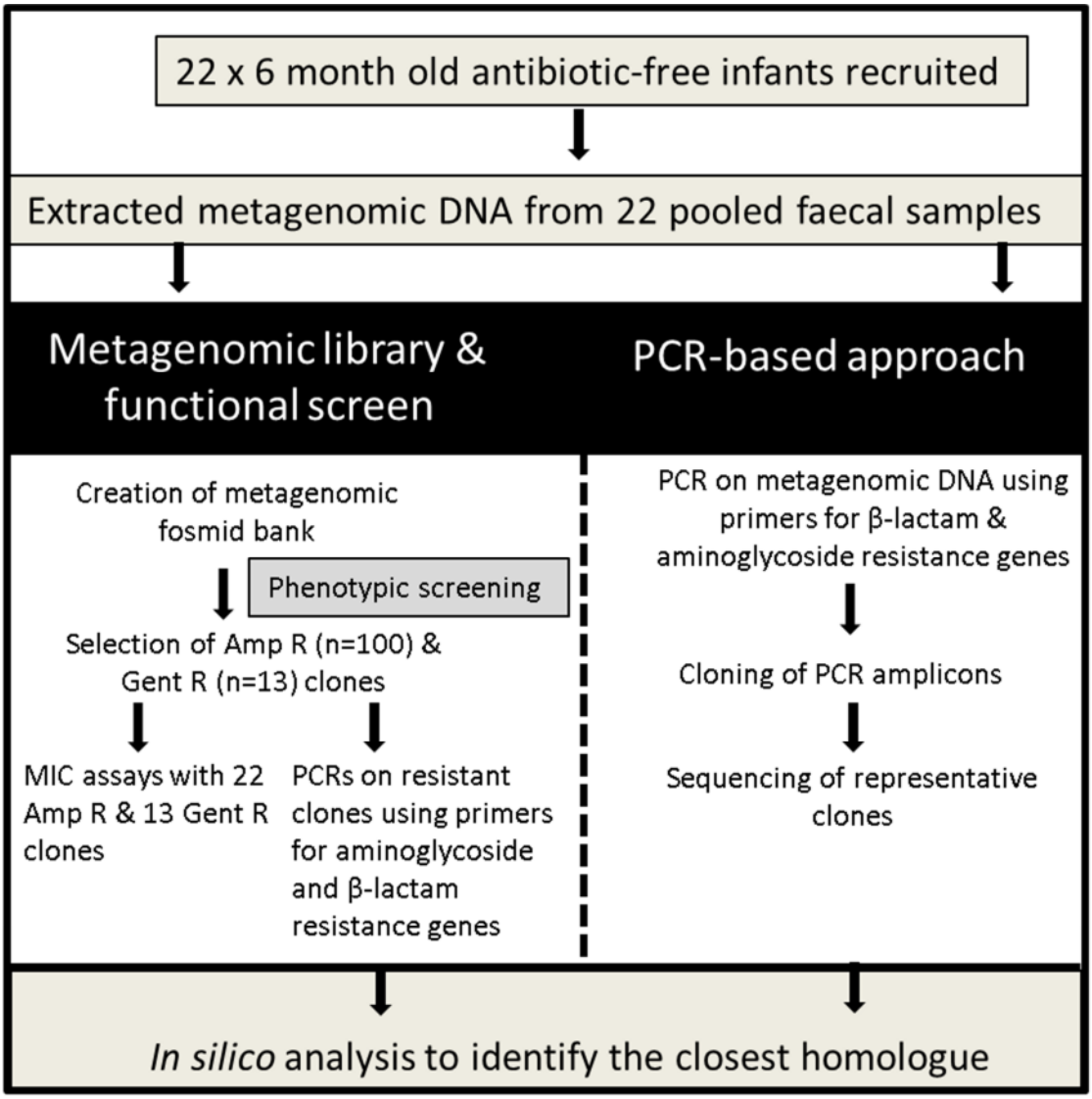

Figure 1 Schematic of the approach applied in this study

Chapter 6

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

Published in Gut Microbes, Volume 4, Issue 3, May-June 2013, Pages 212-221

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' AYTGGGYDTAAAGNG; 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* ($p = 0.0018$ and $p = 0.0115$) and a significant decrease in *Ruminococcaceae* ($p = 0.0012$ 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 ($p = 0.0015$ and $p = 0.0033$; 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* ($p = 0.0018$ and $p = 0.0013$) and significantly increased proportions of *Fusobacteria* ($p = 0.0072$ 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* ($p = 0.0477$ and $p = 0.0154$) and *Ruminococcaceae* ($p = 0.00001$ 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

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research,* 25**,** 3389-3402.

Begley, M., Gahan, C. G. M. & Hill, C. 2005. The interaction between bacteria and bile. *FEMS Microbiology Reviews,* 29**,** 625-651.

Bongaerts, G. P., Severijnen, R. S., Tangerman, A., Verrips, A. & Tolboom, J. J. 2000. Bile acid deconjugation by Lactobacilli and its effects in patients with a short small bowel. *J Gastroenterol,* 35**,** 801-4.

Buzoianu, S. G., Walsh, M. C., Rea, M. C., O'sullivan, O., Crispie, F., Cotter, P. D., Ross, R. P., Gardiner, G. E. & Lawlor, P. G. 2012. The Effect of Feeding Bt MON810 Maize to Pigs for 110 Days on Intestinal Microbiota. *PLoS ONE,* 7**,** e33668.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., Pena, A. G., Goodrich, J. K., Gordon, J. I., Huttley, G. A., Kelley, S. T., Knights, D., Koenig, J. E., Ley, R. E., Lozupone, C. A., Mcdonald, D., Muegge, B. D., Pirrung, M., Reeder, J., Sevinsky, J. R., Turnbaugh, P. J., Walters, W. A., Widmann, J., Yatsunenko, T., Zaneveld, J. & Knight, R. 2010. QIIME allows analysis of high-throughput community sequencing data. *Nat Methods,* 7**,** 335-6.

Chang, J. Y., Antonopoulos, D. A., Kalra, A., Tonelli, A., Khalife, W. T., Schmidt, T. M. & Young, V. B. 2008. Decreased Diversity of the Fecal Microbiome in Recurrent Clostridium difficile-Associated Diarrhea. *Journal of Infectious Diseases,* 197**,** 435-438.

Chen, Y., Yang, F., Lu, H., Wang, B., Lei, D., Wang, Y., Zhu, B. & Li, L. 2011. Characterization of fecal microbial communities in patients with liver cirrhosis. *Hepatology,* 54**,** 562-72.

Cole, C. R., Frem, J. C., Schmotzer, B., Gewirtz, A. T., Meddings, J. B., Gold, B. D. & Ziegler, T. R. 2010. The Rate of Bloodstream Infection Is High in Infants with Short Bowel Syndrome: Relationship with Small Bowel Bacterial Overgrowth, Enteral Feeding, and Inflammatory and Immune Responses. *The Journal of Pediatrics,* 156**,** 941-947.e1.

Cole, C. R., Hansen, N. I., Higgins, R. D., Ziegler, T. R., Stoll, B. J. & Network, F. T. E. K. S. N. N. R. 2008. Very Low Birth Weight Preterm Infants With Surgical Short Bowel Syndrome: Incidence, Morbidity and Mortality, and Growth Outcomes at 18 to 22 Months. *Pediatrics,* 122**,** e573-e582.

Compher, C., Rubesin, S., Kinosian, B., Madaras, J. & Metz, D. 2007. Noninvasive Measurement of Transit Time in Short Bowel Syndrome. *Journal of Parenteral and Enteral Nutrition,* 31**,** 240-245.

Craven, M., Egan, C. E., Dowd, S. E., Mcdonough, S. P., Dogan, B., Denkers, E. Y., Bowman, D., Scherl, E. J. & Simpson, K. W. 2012. Inflammation Drives Dysbiosis and Bacterial Invasion in Murine Models of Ileal Crohn's Disease. *PLoS ONE,* 7**,** e41594.

Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S. R., Nelson, K. E. & Relman, D. A. 2005. Diversity of the human intestinal microbial flora. *Science,* 308**,** 1635-8.

Flint, H. J., Duncan, S. H., Scott, K. P. & Louis, P. 2007. Interactions and competition within the microbial community of the human colon: links between diet and health. *Environ Microbiol,* 9**,** 1101-11.

Gerritsen, J., Smidt, H., Rijkers, G. & De Vos, W. 2011. Intestinal microbiota in human health and disease: the impact of probiotics. *Genes & Nutrition,* 6**,** 209-240.

Goulet, O., Colomb-Jung, V. & Joly, F. 2009. Role of the Colon in Short Bowel Syndrome and Intestinal Transplantation. *Journal of*

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

Goulet, O. & Joly, F. 2010. [Intestinal microbiota in short bowel syndrome]. *Gastroenterol Clin Biol,* 34 Suppl 1**,** S37-43.

Healey, K. L., Bines, J. E., Thomas, S. L., Wilson, G., Taylor, R. G., Sourial, M. & Pereira-Fantini, P. M. 2010. Morphological and functional changes in the colon after massive small bowel resection. *Journal of Pediatric Surgery,* 45**,** 1581-1590.

Heimesaat, M. M., Bereswill, S., Fischer, A., Fuchs, D., Struck, D., Niebergall, J., Jahn, H.-K., Dunay, I. R., Moter, A., Gescher, D. M., Schumann, R. R., Göbel, U. B. & Liesenfeld, O. 2006. Gram-Negative Bacteria Aggravate Murine Small Intestinal Th1-Type Immunopathology following Oral Infection with Toxoplasma gondii. *The Journal of Immunology,* 177**,** 8785-8795.

Höllwarth, M. E. 1999. Short bowel syndrome: pathophysiological and clinical aspects. *Pathophysiology,* 6**,** 1-19.

Huson, D. H., Auch, A. F., Qi, J. & Schuster, S. C. 2007. MEGAN analysis of metagenomic data. *Genome Research,* 17**,** 377-386.

Joly, F., Mayeur, C., Bruneau, A., Noordine, M.-L., Meylheuc, T., Langella, P., Messing, B., Duée, P.-H., Cherbuy, C. & Thomas, M. 2010. Drastic changes in fecal and mucosa-associated microbiota in adult patients with short bowel syndrome. *Biochimie,* 92**,** 753-761.

Joly, F., Mayeur, C., Messing, B., Lavergne-Slove, A., Cazals-Hatem, D., Noordine, M.-L., Cherbuy, C., Duée, P.-H. & Thomas, M. 2009. Morphological adaptation with preserved proliferation/transporter content in the colon of patients with short bowel syndrome. *American Journal of Physiology - Gastrointestinal and Liver Physiology,* 297**,** G116-G123.

Kalff, J. C., Turler, A., Schwarz, N. T., Schraut, W. H., Lee, K. K., Tweardy, D. J., Billiar, T. R., Simmons, R. L. & Bauer, A. J. 2003. Intra-abdominal activation of a local inflammatory response within

the human muscularis externa during laparotomy. *Ann Surg,* 237**,** 301-15.

Kararli, T. T. 1995. Comparison of the gastrointestinal anatomy, physiology, and biochemistry of humans and commonly used laboratory animals. *Biopharm Drug Dispos,* 16**,** 351-80.

Kaufman, S. S., Loseke, C. A., Lupo, J. V., Young, R. J., Murray, N. D., Pinch, L. W. & Vanderhoof, J. A. 1997. Influence of bacterial overgrowth and intestinal inflammation on duration of parenteral nutrition in children with short bowel syndrome. *The Journal of Pediatrics,* 131**,** 356-361.

Lapthorne, S., Macsharry, J., Scully, P., Nally, K. & Shanahan, F. 2012. Differential intestinal M-cell gene expression response to gut commensals. *Immunology,* 136**,** 312-324.

Livak, K. J. & Schmittgen, T. D. 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. *Methods,* 25**,** 402-408.

Ma, C. Y., Shi, G. Y., Shi, C. S., Kao, Y. C., Lin, S. W. & Wu, H. L. 2012. Monocytic Thrombomodulin Triggers LPS- and Gram-Negative Bacteria-Induced Inflammatory Response. *J Immunol*.

Manichanh, C., Rigottier-Gois, L., Bonnaud, E., Gloux, K., Pelletier, E., Frangeul, L., Nalin, R., Jarrin, C., Chardon, P., Marteau, P., Roca, J. & Dore, J. 2006. Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. *Gut,* 55**,** 205-211.

Mayeur, C., Gratadoux, J. J., Bridonneau, C., Chegdani, F., Larroque, B., Kapel, N., Corcos, O., Thomas, M. & Joly, F. 2013. Faecal d/l lactate ratio is a metabolic signature of microbiota imbalance in patients with short bowel syndrome. *PLoS ONE,* 8**,** e54335.

Mclaughlin, S. D., Walker, A. W., Churcher, C., Clark, S. K., Tekkis, P. P., Johnson, M. W., Parkhill, J., Ciclitira, P. J., Dougan, G., Nicholls, R. J. & Petrovska, L. 2010. The bacteriology of pouchitis: a

molecular phylogenetic analysis using 16S rRNA gene cloning and sequencing. *Ann Surg,* 252**,** 90-8.

Miller, E. R. & Ullrey, D. E. 1987. The pig as a model for human nutrition. *Annu Rev Nutr,* 7**,** 361-82.

Moughan, P. J., Birtles, M. J., Cranwell, P. D., Smith, W. C. & Pedraza, M. 1992. The piglet as a model animal for studying aspects of digestion and absorption in milk-fed human infants. *World Rev Nutr Diet,* 67**,** 40-113.

Murphy, E., Cotter, P., Healy, S., Marques, T., O'sullivan, O., Fouhy, F., Clarke, S., O'toole, P., Quigley, E. & Stanton, C. 2010a. Composition and energy harvesting capacity of the gut microbiota: relationship to diet, obesity and time in mouse models. *Gut,* 59**,** 1635-1642.

Murphy, E. F., Cotter, P. D., Healy, S., Marques, T. M., O'sullivan, O., Fouhy, F., Clarke, S. F., O'toole, P. W., Quigley, E. M., Stanton, C., Ross, P. R., O'doherty, R. M. & Shanahan, F. 2010b. Composition and energy harvesting capacity of the gut microbiota: relationship to diet, obesity and time in mouse models. *Gut,* 59**,** 1635-1642.

Ott, S. J., Musfeldt, M., Wenderoth, D. F., Hampe, J., Brant, O., Fölsch, U. R., Timmis, K. N. & Schreiber, S. 2004. Reduction in diversity of the colonic mucosa associated bacterial microflora in patients with active inflammatory bowel disease. *Gut,* 53**,** 685-693.

Pereira-Fantini, P., Thomas, S., Wilson, G., Taylor, R., Sourial, M. & Bines, J. 2011. Short- and long-term effects of small bowel resection: a unique histological study in a piglet model of short bowel syndrome. *Histochemistry and Cell Biology,* 135**,** 195-202.

Price, M. N., Dehal, P. S. & Arkin, A. P. 2010. FastTree 2 – Approximately Maximum-Likelihood Trees for Large Alignments. *PLoS ONE,* 5**,** e9490.

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

quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Research,* 35**,** 7188-7196.

Quirós-Tejeira, R. E., Ament, M. E., Reyen, L., Herzog, F., Merjanian, M., Olivares-Serrano, N. & Vargas, J. H. 2004. Long-term parenteral nutritional support and intestinal adaptation in children with short bowel syndrome: A 25-year experience. *The Journal of Pediatrics,* 145**,** 157-163.

Rasband, W. S. 1997-2012. *Image J* [Online]. U. S. National Institutes of Health, Bethesda, Maryland, USA. Available: http://imagej.nih.gov/ij/.

Ruifrok, A. C. & Johnston, D. A. 2001. Quantification of histochemical staining by color deconvolution. *Analytical and Quantitative Cytology and Histology,* 23**,** 291-9.

Schalamon, J., Mayr, J. M. & Höllwarth, M. E. 2003. Mortality and economics in short bowel syndrome. *Best Practice & Research Clinical Gastroenterology,* 17**,** 931-942.

Sokol, H., Pigneur, B., Watterlot, L., Lakhdari, O., Bermúdez-Humarán, L. G., Gratadoux, J.-J., Blugeon, S., Bridonneau, C., Furet, J.-P., Corthier, G., Grangette, C., Vasquez, N., Pochart, P., Trugnan, G., Thomas, G., Blottière, H. M., Doré, J., Marteau, P., Seksik, P. & Langella, P. 2008. Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. *Proceedings of the National Academy of Sciences*.

Stephens, A. N., Pereira-Fantini, P. M., Wilson, G., Taylor, R. G., Rainczuk, A., Meehan, K. L., Sourial, M., Fuller, P. J., Stanton, P. G., Robertson, D. M. & Bines, J. E. 2009. Proteomic Analysis of the Intestinal Adaptation Response Reveals Altered Expression of Fatty Acid Binding Proteins Following Massive Small Bowel Resection. *Journal of Proteome Research,* 9**,** 1437-1449.

Sukhotnik, I., Siplovich, L., Shiloni, E., Mor-Vaknin, N., Harmon, C. M. & Coran, A. G. 2002. Intestinal adaptation in short-bowel syndrome

in infants and children: a collective review. *Pediatric Surgery International,* 18**,** 258-263.

Wilson, K. H., Blitchington, R. B. & Greene, R. C. 1990. Amplification of bacterial 16S ribosomal DNA with polymerase chain reaction. *Journal of Clinical Microbiology,* 28**,** 1942-1946.

Zhang, H., Dibaise, J. K., Zuccolo, A., Kudrna, D., Braidotti, M., Yu, Y., Parameswaran, P., Crowell, M. D., Wing, R. & Rittmann, B. E. 2009. Human gut microbiota in obesity and after gastric bypass. *Proceedings of the National Academy of Sciences,* 106**,** 2365-2370.

Table 1 Surgical resection decreases the diversity of colonic bacteria

Data set	NOC Week 2	Sham Week 2	SBR Week 2	NOC Week 6	Sham Week 6	SBR Week 6
Chao 1 richness estimation	905±41	892±123	718±59	1224±82	1276±97	716±34
Shannon's index for diversity	6.9±0.2	6.5±0.6	5.7±0.3	7.1±0.1	7.4±0.2	5.5±0.2
Number of observed species	552±16	571±80	475±44	726±46	777±63	428±15
Simpson's diversity index	0.96±0.01	0.95±0.02	0.93±0.01	0.98±0.01	0.98±0.00	0.93±0.01

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

Weeks post-surgery	**Week 2**			**Week 6**		
Group	**NOC**	**Sham**	**SBR**	**NOC**	**Sham**	**SBR**
Phylum:						
Firmicutes	52.6±5.0	52.1±10.6	68.5±6.1	61.7±6.5	67.7±3.6	68.2±4.2
Proteobacteria	27.8±5.1	23.1±14.6	11.9±4.6	10.6±3.3	6.0±0.6	6.2±1.8
Bacteroidetes	17.9±2.5	19.1±5.8	10.1±1.7	22.3±2.6	23.2±1.9	10.0±1.6**††
Fusobacteria	0.9±0.4	3.8±3.1	8.5±2.7	4.7±2.3	2.8±1.4	15.4±2.4**††
Actinobacteria	0.7±0.2	2.0±1.5	1.0±0.4	0.7±0.2	0.3±0.1	0.3±0.1
Family:						
Enterobacteriaceae	31.8±6.9	5.8±2.5**	1.0±0.5***	6.7±2.7	1.9±0.7	4.0±2.1
Ruminococcaceae	28.1±3.1	27.0±4.2	5.2±2.5**†	41.5±5.2	36.1±4.1	3.9±1.6***†††
Veillonellaceae	19.5±4.9	36.7±8.4	70.5±7.1**†	9.9±2.9	28.0±6.5	64.2±6.6***††
Peptostreptococcaceae	4.6±1.5	2.1±1.5	0	2.8±0.6	3.7±1.4	0*†
Prevotellaceae	3.9±0.6	14.4±2.8*	7.1±0.5	2.0±0.9	9.7±1.8**	5.8±0.7**
Bacteroidaceae	3.5±0.8	1.1±0.6	2.4±1.1	9.0±2.1	6.0±0.9	2.9±1.2
Erysipelotrichaceae	2.5±0.9	1.4±0.7	0.4±0.2	10.0±6.0	3.8±1.3	0
Clostridiaceae	2.1±0.5	1.0±0.5	0	0.7±0.1	2.7±1.3	0.1±0.0
Lactobacillaceae	1.5±0.6	2.1±1.1	1.2±0.3	2.9±0.8	0.2±0.1*	0.8±0.4
Fusobacteriaceae	1.4±0.6	7.5±3.9	11.9±4.1	8.3±4.3	4.8±2.5	18.2±3.0**††
Peptococcaceae	1.1±0.5	0.9±0.3	0.4±0.4	6.3±1.9	3.2±0.7	0**
Genus:						
Enterobacteriaceae family members	48.8±8.7	9.4±3.7***	1.4±0.7***	11.6±2.8	3.6±1.1	5.7±2.9
Bacteroides	5.8±1.3	2.1±1.1	3.5±1.5	21.2±3.2	11.5±1.6	4.3±1.7
Parabacteroides	5.1±1.2	10.9±3.3	1.6±1.1	19.2±4.5	10.9±3.4	0.6±0.2*
Prevotella	6.7±1.6	23.2±4.5*	10.4±1.2	3.1±1.5	18.9±3.4*	9.6±0.9*
Clostridium	3.4±0.7	1.8±0.9	0	1.8±0.6	5.1±2.6	0.1±0.1†
Fusobacterium	2.7±1.4	12.4±6.6	17.7±6.2	11.9±5.1	9.0±4.8	29.9±4.3**††
Megasphaera	17.1±6.0	27.3±4.4	31.4±6.1	6.2±2.5	23.5±7.7	18.3±3.0*
Acidaminococcus	4.5±1.2	8.0±2.8	31.7±6.7**††	3.6±2.3	11.1±6.5	30.2±5.1***††
Peptococcus	1.9±0.9	1.5±0.6	0.7±0.6	14.9±5.7	6.1±1.7	0.1±0.1
Lactobacillus	4.0±1.8	3.4±1.8	1.7±0.4	6.4±1.9	0.4±0.2*	1.3±0.6

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

Bacterial alpha diversity	**IL-1β**	**IL-8**	**IL18**	**TNF-α**
Spearman r	-0.8286	-0.7714	-0.2571	-0.4857
p value	0.0583	0.1028	0.6583	0.3556

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

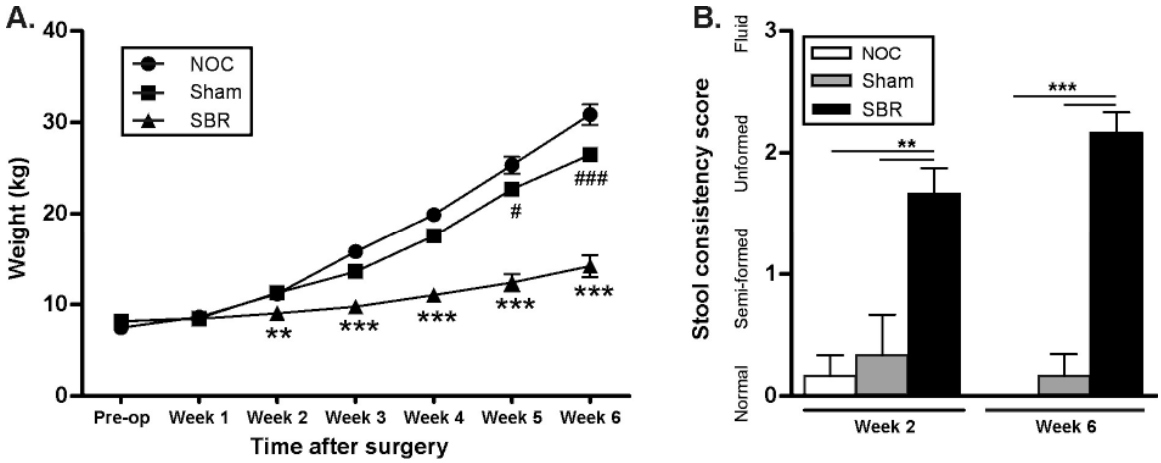

Figure 1 Clinical effects of small bowel resection. (**A**) The weight of piglets that received a small bowel resection (SBR) was significantly lower two weeks post-surgery than those that received a sham surgery (Sham) or no surgery (NOC; **p < 0.01). This suboptimal weight gain was sustained for the 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

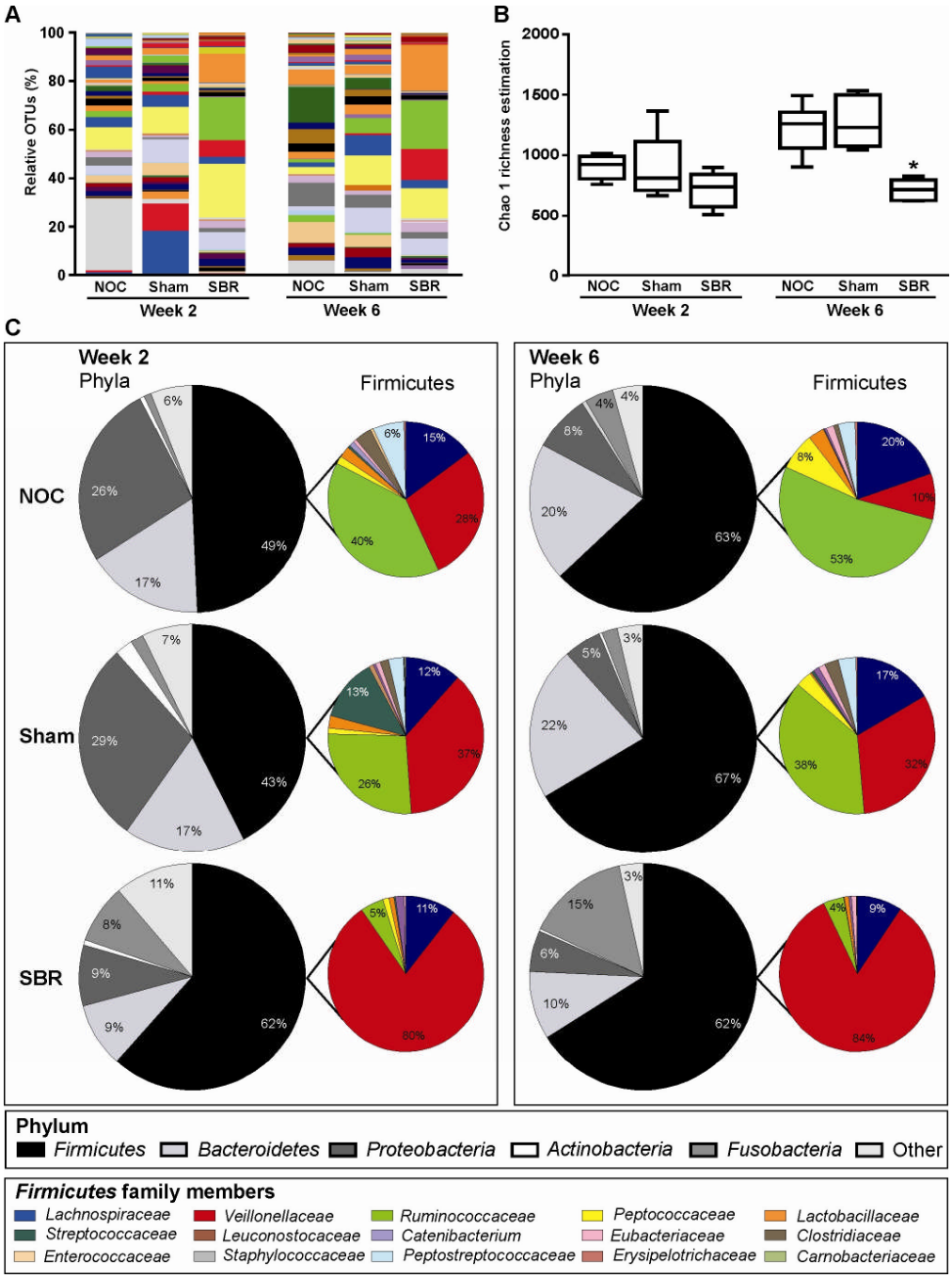

Figure 2 Surgical resection of the small intestine decreases the diversity of colonic bacteria and alters the relative proportion of families in the *Firmicutes* phylum. (**A**) There is a decrease in the number of distinct operational taxonomic units (OTUs) representing bacterial genera two and six 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

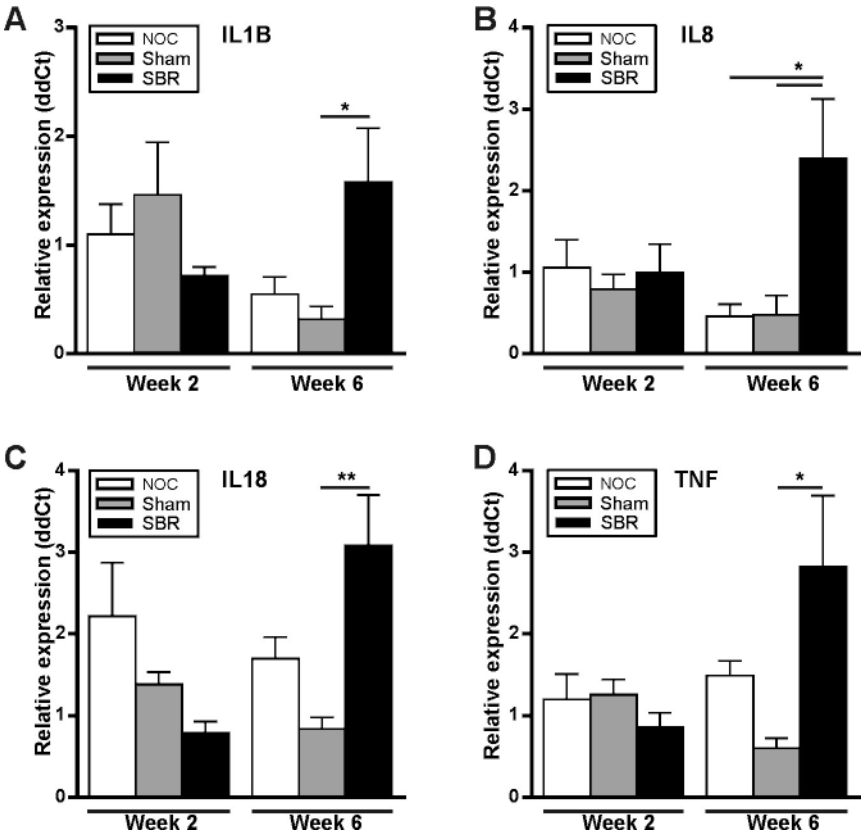

Figure 3 Surgical resection alters the expression of pro-inflammatory cytokines in the colon. In the colon, the gene expression of IL-1β (**A**), IL-18 (**C**) and TNF-α (**D)** was significantly higher in the SBR group compared to the sham group at six weeks (*p<0.05, **p<0.01). IL8 (**B**) was significantly 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

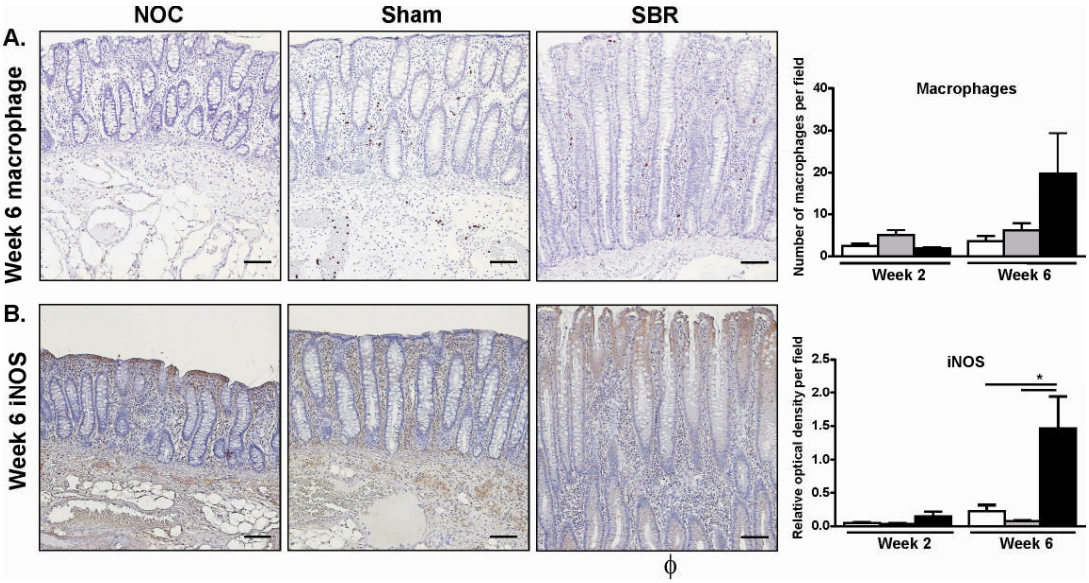

Figure 4 There is an increase in inflammatory cells and mediators in the colonic epithelium following small bowel resection. There was an increase in the number of macrophages (**A**) and the percentage of cytoplasmic inducible nitric oxide synthase (iNOS) staining (**B**) in the colonic epithelium six 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

Primer	**Sequence 5’ to 3’**	**UPL Probe**
RPL32 Forward RPL32 Reverse	aactggccatcagggtcac cacaactggaactcctgtctattc	#64
IL1B Forward IL1B Reverse	ccaattcagggaccctacc catggctgcttcagaaacct	#19
IL8 Forward IL8 Reverse	ttcttctttatccccaaactgg ccacatgtcctcaaggtagga	#41
IL18 Forward IL18 Reverse	actttactttgtagctgaaaacgatg tttaggttcaagcttgccaaa	#85
TNF Forward TNF Reverse	ttgtcgctacatcgctgaac ccagtagggcggttacagac	#32

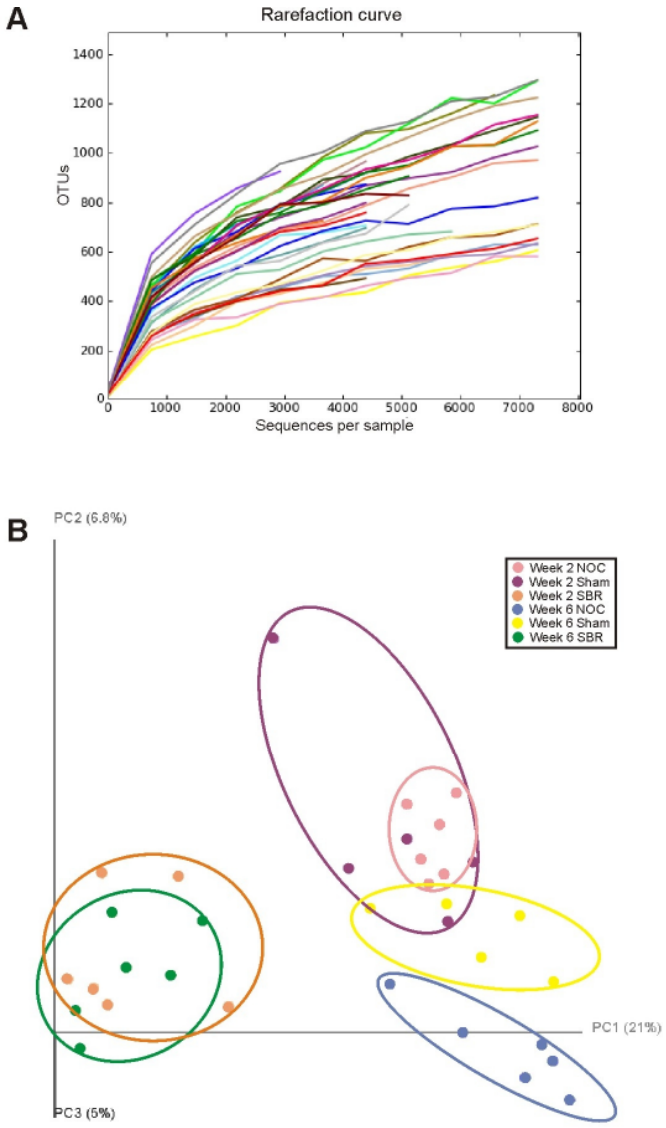

Figure S1 Rarefaction curve and principle coordinate analysis of samples sequenced by high-throughput DNA sequencing. (**A**) Rarefaction curves for each group at 97% similarity indicated that the total bacterial diversity present was well represented. Number of operational taxonomic units (OTUs) identified 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' CTACDSRGGTMTCTAATC) 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 10^9-10^2 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' AYTGGGYDTAAAGNG) (0.15 µM), 1 µl of the reverse primer R1 (5' TACCRGGGTHTCTAATCC) (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 *Deferribacteres*, *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). In

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 *Deferribacteres*, *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

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. 1990. Basic local alignment search tool. *J Mol Biol,* 215**,** 403-10.

Bik, E. M., Eckburg, P. B., Gill, S. R., Nelson, K. E., Purdom, E. A., Francois, F., Perez-Perez, G., Blaser, M. J. & Relman, D. A. 2006. Molecular analysis of the bacterial microbiota in the human stomach. *Proc Natl Acad Sci U S A,* 103**,** 732-7.

Bollinger, R. R., Barbas, A. S., Bush, E. L., Lin, S. S. & Parker, W. 2007. Biofilms in the normal human large bowel: fact rather than fiction. *Gut,* 56**,** 1481-2.

Bollinger, R. R., Everett, M. L., Palestrant, D., Love, S. D., Lin, S. S. & Parker, W. 2003. Human secretory immunoglobulin A may contribute to biofilm formation in the gut. *Immunology,* 109**,** 580-7.

Carr, N. J. 2000. The pathology of acute appendicitis. *Ann Diagn Pathol,* 4**,** 46-58.

Claesson, M. J., Cusack, S., O'sullivan, O., Greene-Diniz, R., De Weerd, H., Flannery, E., Marchesi, J. R., Falush, D., Dinan, T., Fitzgerald, G., Stanton, C., Van Sinderen, D., O'connor, M., Harnedy, N., O'connor, K., Henry, C., O'mahony, D., Fitzgerald, A. P., Shanahan, F., Twomey, C., Hill, C., Ross, R. P. & O'toole, P. W. 2011. Composition, variability, and temporal stability of the intestinal microbiota of the elderly. *Proc Natl Acad Sci U S A,* 108 Suppl 1**,** 4586-91.

Claesson, M. J., Jeffery, I. B., Conde, S., Power, S. E., O'connor, E. M., Cusack, S., Harris, H. M., Coakley, M., Lakshminarayanan, B., O'sullivan, O., Fitzgerald, G. F., Deane, J., O'connor, M., Harnedy, N., O'connor, K., O'mahony, D., Van Sinderen, D., Wallace, M., Brennan, L., Stanton, C., Marchesi, J. R., Fitzgerald, A. P., Shanahan, F., Hill, C., Ross, R. P. & O'toole, P. W. 2012. Gut microbiota composition correlates with diet and health in the elderly. *Nature*.

Cotter, P. D., Stanton, C., Ross, R. P. & Hill, C. The impact of antibiotics on the gut microbiota as revealed by high throughput DNA sequencing. *Discov Med,* 13**,** 193-9.

Diaz, P. I., Dupuy, A. K., Abusleme, L., Reese, B., Obergfell, C., Choquette, L., Dongari-Bagtzoglou, A., Peterson, D. E., Terzi, E. & Strausbaugh, L. D. 2012. Using high throughput sequencing to explore the biodiversity in oral bacterial communities. *Mol Oral Microbiol,* 27**,** 182-201.

Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S. R., Nelson, K. E. & Relman, D. A. 2005. Diversity of the human intestinal microbial flora. *Science,* 308**,** 1635-8.

Elhag, K. M., Alwan, M. H., Al-Adnani, M. S. & Sherif, R. A. 1986. Bacteroides fragilis is a silent pathogen in acute appendicitis. *J Med Microbiol,* 21**,** 245-9.

Fouhy, F., Guinane, C. M., Hussey, S., Wall, R., Ryan, A. C., Dempsey, E., Murphy, B., Ross, R. P., Fitzgerald, G. F., Stanton, C. & Cotter, P. D. 2012 High-throughput sequencing reveals the incomplete, short-term, recovery of the infant gut microbiota following parenteral antibiotic treatment with ampicillin and gentamycin. *Antimicrobial Agents and Chemotherapy,* In Press.

He, X., Tian, Y., Guo, L., Ano, T., Lux, R., Zusman, D. R. & Shi, W. In vitro communities derived from oral and gut microbial floras inhibit the growth of bacteria of foreign origins. *Microb Ecol,* 60**,** 665-76.

Huson, D. H., Auch, A. F., Qi, J. & Schuster, S. C. 2007. MEGAN analysis of metagenomic data. *Genome Res,* 17**,** 377-86.

Hussey, S., Wall, R., Gruffman, E., O'sullivan, L., Ryan, C. A., Murphy, B., Fitzgerald, G., Stanton, C. & Ross, R. P. 2011. Parenteral antibiotics reduce bifidobacteria colonization and diversity in neonates. *Int J Microbiol,* 2011.

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

Clostridium difficile recurrence. *Clin Gastroenterol Hepatol,* 9**,** 1072-7.

Laurin, M., Everett, M. L. & Parker, W. 2011. The cecal appendix: one more immune component with a function disturbed by post-industrial culture. *Anat Rec (Hoboken),* 294**,** 567-79.

Li, L., Hsiao, W. W., Nandakumar, R., Barbuto, S. M., Mongodin, E. F., Paster, B. J., Fraser-Liggett, C. M. & Fouad, A. F. Analyzing endodontic infections by deep coverage pyrosequencing. *J Dent Res,* 89**,** 980-4.

Marteau, P., Pochart, P., Dore, J., Bera-Maillet, C., Bernalier, A. & Corthier, G. 2001. Comparative study of bacterial groups within the human cecal and fecal microbiota. *Appl Environ Microbiol,* 67**,** 4939-42.

Merchant, R., Mower, W. R., Ourian, A., Abrahamian, F. M., Moran, G. J., Krishnadasan, A. & Talan, D. A. 2011. Association Between Appendectomy and Clostridium difficile Infection. *J Clin Med Res,* 4**,** 17-9.

Na, X. & Kelly, C. 2011. The vermiform appendix and recurrent Clostridium difficile infection: a curious connection. *Clin Gastroenterol Hepatol,* 9**,** 1017-9.

O' sullivan, O., Coakley, M., Lakshminarayanan, B., Claesson, M. J., Stanton, C., O'toole, P. W. & Ross, R. P. 2011. Correlation of rRNA gene amplicon pyrosequencing and bacterial culture for microbial compositional analysis of faecal samples from elderly Irish subjects. *J Appl Microbiol,* 111**,** 467-73.

Ota-Tsuzuki, C. & Alves Mayer, M. P. 2009. Collagenase production and hemolytic activity related to 16S rRNA variability among Parvimonas micra oral isolates. *Anaerobe,* 16**,** 38-42.

Pruesse, E., Quast, C., Knittel, K., Fuchs, B. M., Ludwig, W., Peplies, J. & Glockner, F. O. 2007. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res,* 35**,** 7188-96.

Rautio, M., Saxen, H., Siitonen, A., Nikku, R. & Jousimies-Somer, H. 2000. Bacteriology of histopathologically defined appendicitis in children. *Pediatr Infect Dis J,* 19**,** 1078-83.

Roberts, J. 1988. Quantitative bacterial flora of acute appendicitis. *Archives of Disease in Childhood,* 63**,** 536-540.

Schloss, P. D. & Handelsman, J. 2008. A statistical toolbox for metagenomics: assessing functional diversity in microbial communities. *BMC Bioinformatics,* 9**,** 34.

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., Lesniewski, R. A., Oakley, B. B., Parks, D. H., Robinson, C. J., Sahl, J. W., Stres, B., Thallinger, G. G., Van Horn, D. J. & Weber, C. F. 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol,* 75**,** 7537-41.

Simon, G. L. G., S.L. 1995. Normal alimentary tract microflora. *In M.J. Blaser, P.D. Smith, J.I. Rafdin, H.B. Greenberg abd R.L. Guerrant (ed.) Infections of the gastrointestinal tract,* Raven Press Ltd., New York, N.Y.

Smith, H. F., Fisher, R. E., Everett, M. L., Thomas, A. D., Bollinger, R. R. & Parker, W. 2009. Comparative anatomy and phylogenetic distribution of the mammalian cecal appendix. *J Evol Biol,* 22**,** 1984-99.

Swidsinski, A., Dorffel, Y., Loening-Baucke, V., Tertychnyy, A., Biche-Ool, S., Stonogin, S., Guo, Y. & Sun, N. D. Mucosal invasion by fusobacteria is a common feature of acute appendicitis in Germany, Russia, and China. *Saudi J Gastroenterol,* 18**,** 55-8.

Swidsinski, A., Dorffel, Y., Loening-Baucke, V., Theissig, F., Ruckert, J. C., Ismail, M., Rau, W. A., Gaschler, D., Weizenegger, M., Kuhn, S., Schilling, J. & Dorffel, W. V. 2011. Acute appendicitis is characterised by local invasion with Fusobacterium nucleatum/necrophorum. *Gut,* 60**,** 34-40.

Tap, J., Mondot, S., Levenez, F., Pelletier, E., Caron, C., Furet, J. P., Ugarte, E., Munoz-Tamayo, R., Paslier, D. L., Nalin, R., Dore, J. & Leclerc, M. 2009. Towards the human intestinal microbiota phylogenetic core. *Environ Microbiol,* 11**,** 2574-84.

Urich, T., Lanzen, A., Qi, J., Huson, D. H., Schleper, C. & Schuster, S. C. 2008. Simultaneous assessment of soil microbial community structure and function through analysis of the meta-transcriptome. *PLoS One,* 3**,** e2527.

Zhang, H., Dibaise, J. K., Zuccolo, A., Kudrna, D., Braidotti, M., Yu, Y., Parameswaran, P., Crowell, M. D., Wing, R., Rittmann, B. E. & Krajmalnik-Brown, R. 2009. Human gut microbiota in obesity and after gastric bypass. *Proc Natl Acad Sci U S A,* 106**,** 2365-70.

Table 1 Description of patients and appendix samples

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

* 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

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

*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)

Genus	A	B	C	D	E	F	G	S
Enterobacteriaceae members	9%	25%	36%	9%	49%	27%	25%	11%
Lachnospiracheae incertae sedis	*	10%	6%	*	11%	*	*	16%
Ruminococcaceae incertae sedis	8%	4%	3%	*	*	7%	4%	6%
Fusobacterium	3%	8%	9%	41%	*	*	7%	*
Lactobacillus	16%	4%	*	*	4%	23%	7%	*
Bacteroides	7%	9%	7%	7%	4%	4%	5%	18%
Bifidobacterium	4%	3%	3%	*	12%	*	*	15%
Eubacterium	*	*	*	10%	*	*	*	*
Streptococcus	*	*	*	*	10%	*	*	5%
Parvimonas	*	*	*	21%	*	*	18%	*
Parabacteroides	*	*	*	*	*	*	*	8%
Oscillibacter	*	*	*	*	*	3%	*	*
Gemella	5%	3%	*	*	*	*	*	*
Faecalibacterium	*	*	*	3%	*	4%	*	*
Alistipes	*	*	*	*	*	*	5%	*
Mucspirillium	5%	*	*	*	*	5%	*	*
Allobaculum	9%	*	*	*	*	*	*	*
Blautia	*	*	*	*	*	*	*	7%
Other genera	23%	34%	28%	9%	10%	27%	29%	14%
Not assigned	11%	*	8%	*	*	*	*	*

*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”

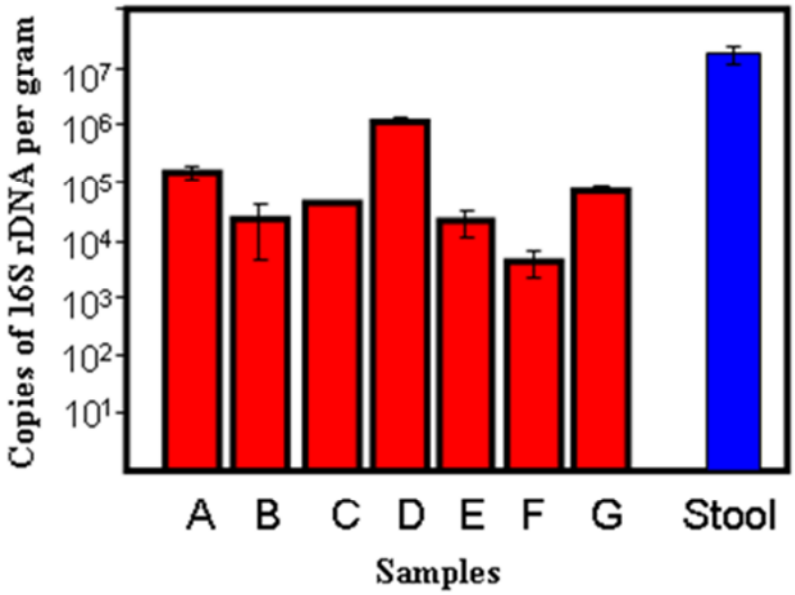

Figure 1 The numbers of bacteria quantified by real-time QPCR. Error bars represent the standard error of the mean (n=3). Red bars represent appendix samples A-G, Blue bar represents the stool sample

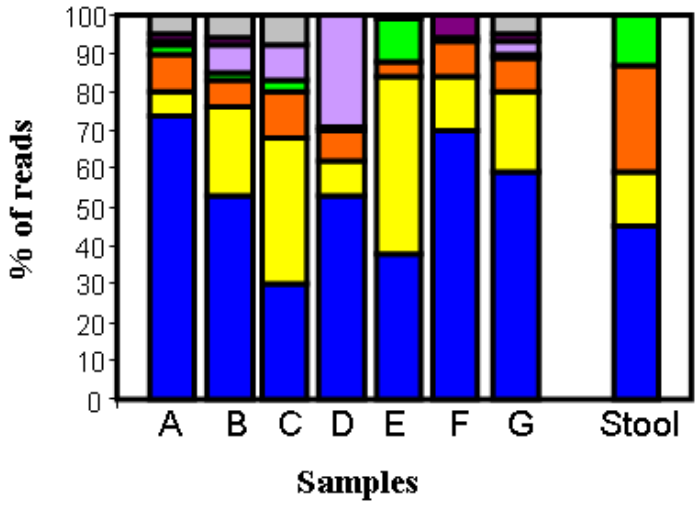

Figure 2 Phyla distribution among appendix samples (A-G) and the stool sample. *Firmicutes* ■ ; *Proteobacteria* ■ ; *Bacteroidetes* ■ ; *Actinobacteria* ■ ; *Fusobacteria* ■ ; Other phyla ■ ; Not assigned ■

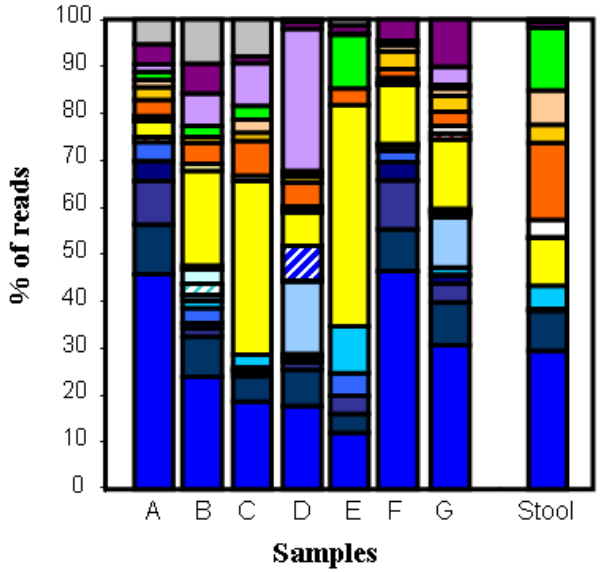

Figure 3 Family-level comparison of appendix samples A-G and the stool sample. Similar bar colours correspond to samples within a particular phylum. Families of lower abundance are grouped together. *Lachnospiraceae* ; *Ruminococcaceae* ; *Lactobacillaceae* ; *Erysipelotrichaceae* ; *Peptostreptococcaceae*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

Appendix

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

Published in The American Journal of Clinical Nutrition, Volume 95, Issue 5, May, 2012, Pages 1278-1287

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 (10^9 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 microorganims (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 dH_2O), a group fed with standard diet and *B. breve* DPC 6330 (approximate daily dose of 10^9 microorganisms) and a group fed standard diet and *B. breve* NCIMB 702258 (approximate daily dose of 10^9 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 *c*9, *t*11 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 ~10^9 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 ~2×10^{10} cells/ml in 15% (w/v) trehalose (Sigma) in dH_2O. 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 1×10^9 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 dH_2O). 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^{o}C followed by 10 ml 14% BF_3 in methanol (Sigma) for 10 min at 90^{o}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^{o}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 $\times$ 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^{o}C for 8 min then increased 8.5^{o}C/min to a final column temperature of 200^{o}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 dH_2O. 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'-AYTGGGYDTAAAGNG) and a combination of four reverse primers R1 (5'-TACCRGGGTHTCTAATCC), R2 (5'-TACCAGAGTATCTAATTC), R3 (5'-CTACDSRGGTMTCTAATC) and R4 (5'-TACNVGGGTATCTAATC) (RDP's Pyrosequencing Pipeline: http://pyro.cme.msu.edu/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

(Claesson et al., 2009). 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 1.1×10^6 CFU/g faeces and 8.2×10^6 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 1.2×10^5 CFU/g faeces and 3.4×10^5 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 7×10^5 CFU /g content and 1×10^6 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 *c*9, *t*11 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, Schwiertz 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

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research,* 25**,** 3389-402.

Bäckhed, F., Ding, H., Wang, T., Hooper, L. V., Koh, G. Y., Nagy, A., Semenkovich, C. F. & Gordon, J. I. 2004. The gut microbiota as an environmental factor that regulates fat storage. *Proceedings of the National Academy of Sciences of the United States of America,* 101**,** 15718-15723.

Barrett, E., Ross, R., Fitzgerald, G. & Stanton, C. 2007. Rapid screening method for analyzing the conjugated linoleic acid production capabilities of bacterial cultures. *Applied and environmental microbiology,* 73**,** 2333-2337.

Cani, P. D., Neyrinck, A., Fava, F., Knauf, C., Burcelin, R., Tuohy, K., Gibson, G. & Delzenne, N. 2007. Selective increases of bifidobacteria in gut microflora improve high-fat-diet-induced diabetes in mice through a mechanism associated with endotoxaemia. *Diabetologia,* 50**,** 2374-2383.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., Pena, A. G., Goodrich, J. K. & Gordon, J. I. 2010. QIIME allows analysis of high-throughput community sequencing data. *Nature methods,* 7**,** 335-336.

Claesson, M. J., O'sullivan, O., Wang, Q., Nikkilä, J., Marchesi, J. R., Smidt, H., De Vos, W. M., Ross, R. P. & O'toole, P. W. 2009. Comparative analysis of pyrosequencing and a phylogenetic microarray for exploring microbial community structures in the human distal intestine. *PloS one,* 4**,** e6669.

Coakley, M., Ross, R., Nordgren, M., Fitzgerald, G., Devery, R. & Stanton, C. 2003. Conjugated linoleic acid biosynthesis by

human-derived Bifidobacterium species. *Journal of Applied Microbiology,* 94**,** 138-145.

Collado, M. C., Isolauri, E., Laitinen, K. & Salminen, S. 2008. Distinct composition of gut microbiota during pregnancy in overweight and normal-weight women. *The American journal of clinical nutrition,* 88**,** 894-899.

D'argenio, G., Cosenza, V., Delle Cave, M., Iovino, P., Delle Valle, N., Lombardi, G. & Mazzacca, G. 1996. Butyrate enemas in experimental colitis and protection against large bowel cancer in a rat model. *Gastroenterology,* 110**,** 1727-1734.

Duncan, S., Lobley, G., Holtrop, G., Ince, J., Johnstone, A., Louis, P. & Flint, H. 2008. Human colonic microbiota associated with diet, obesity and weight loss. *International Journal of Obesity,* 32**,** 1720-1724.

Efsa 2009. Panel on Dietetic Products, Nutrition and Allergies. Scientific opinion of the panel on dietetic products, nutrition and allergies on a request from Mead Johnson Nutritionals on DHA and ARA in brain development. 1000**,** 1-13.

Emenaker, N. J., Calaf, G. M., Cox, D., Basson, M. D. & Qureshi, N. 2001. Short-chain fatty acids inhibit invasive human colon cancer by modulating uPA, TIMP-1, TIMP-2, mutant p53, Bcl-2, Bax, p21 and PCNA protein expression in an in vitro cell culture model. *The Journal of nutrition,* 131**,** 3041S-3046S.

Folch, J., Lees, M. & Sloane-Stanley, G. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J Biol chem,* 226**,** 497-509.

Furet, J.-P., Kong, L.-C., Tap, J., Poitou, C., Basdevant, A., Bouillot, J.-L., Mariat, D., Corthier, G., Doré, J. & Henegar, C. 2010. Differential Adaptation of Human Gut Microbiota to Bariatric Surgery–Induced Weight Loss Links With Metabolic and Low-Grade Inflammation Markers. *Diabetes,* 59**,** 3049-3057.

Galvez, J., Rodríguez-Cabezas, M. E. & Zarzuelo, A. 2005. Effects of dietary fiber on inflammatory bowel disease. *Molecular nutrition & food research,* 49**,** 601-608.

Henriksen, C., Haugholt, K., Lindgren, M., Aurvåg, A. K., Rønnestad, A., Grønn, M., Solberg, R., Moen, A., Nakstad, B. & Berge, R. K. 2008. Improved cognitive development among preterm infants attributable to early supplementation of human milk with docosahexaenoic acid and arachidonic acid. *Pediatrics,* 121**,** 1137-1145.

Hildebrandt, M. A., Hoffmann, C., Sherrill–Mix, S. A., Keilbaugh, S. A., Hamady, M., Chen, Y. Y., Knight, R., Ahima, R. S., Bushman, F. & Wu, G. D. 2009. High-fat diet determines the composition of the murine gut microbiome independently of obesity. *Gastroenterology,* 137**,** 1716-1724. e2.

Hoppu, U., Isolauri, E., Laakso, P., Matomäki, J. & Laitinen, K. 2012. Probiotics and dietary counselling targeting maternal dietary fat intake modifies breast milk fatty acids and cytokines. *European journal of nutrition,* 51**,** 211-219.

Hosseini, E., Grootaert, C., Verstraete, W. & Van De Wiele, T. 2011. Propionate as a health-promoting microbial metabolite in the human gut. *Nutrition reviews,* 69**,** 245-258.

Huson, D. H., Auch, A. F., Qi, J. & Schuster, S. C. 2007. MEGAN analysis of metagenomic data. *Genome research,* 17**,** 377-386.

Innis, S. M. 2007. Dietary (n-3) fatty acids and brain development. *The Journal of nutrition,* 137**,** 855-859.

Kalliomäki, M., Collado, M. C., Salminen, S. & Isolauri, E. 2008. Early differences in fecal microbiota composition in children may predict overweight. *The American journal of clinical nutrition,* 87**,** 534-538.

Kankaanpää, P. E., Yang, B., Kallio, H. P., Isolauri, E. & Salminen, S. J. 2002. Influence of probiotic supplemented infant formula on composition of plasma lipids in atopic infants. *The Journal of nutritional biochemistry,* 13**,** 364-369.

Kaplas, N., Isolauri, E., Lampi, A. M., Ojala, T. & Laitinen, K. 2007. Dietary counseling and probiotic supplementation during pregnancy modify placental phospholipid fatty acids. *Lipids,* 42**,** 865-870.

Kitajima, H., Sumida, Y., Tanaka, R., Yuki, N., Takayama, H. & Fujimura, M. 1997. Early administration of Bifidobacterium breve to preterm infants: randomised controlled trial. *British Medical Journal,* 76**,** 101-107.

Leahy, S., Higgins, D., Fitzgerald, G. & Sinderen, D. 2005. Getting better with bifidobacteria. *Journal of applied microbiology,* 98**,** 1303-1315.

Ley, R. E., Bäckhed, F., Turnbaugh, P., Lozupone, C. A., Knight, R. D. & Gordon, J. I. 2005. Obesity alters gut microbial ecology. *Proceedings of the National Academy of Sciences of the United States of America,* 102**,** 11070-11075.

Ley, R. E., Turnbaugh, P. J., Klein, S. & Gordon, J. I. 2006. Microbial ecology: human gut microbes associated with obesity. *Nature,* 444**,** 1022-1023.

Macfarlane, S. & Macfarlane, G. T. Regulation of short-chain fatty acid production. Proceedings-Nutrition Society of London, 2003. Cambridge Univ Press, 67-72.

Maekawa, M., Takashima, N., Matsumata, M., Ikegami, S., Kontani, M., Hara, Y., Kawashima, H., Owada, Y., Kiso, Y. & Yoshikawa, T. 2009. Arachidonic acid drives postnatal neurogenesis and elicits a beneficial effect on prepulse inhibition, a biological trait of psychiatric illnesses. *PLoS One,* 4**,** e5085.

Maragkoudakis, P. A., Mountzouris, K. C., Rosu, C., Zoumpopoulou, G., Papadimitriou, K., Dalaka, E., Hadjipetrou, A., Theofanous, G., Strozzi, G. P. & Carlini, N. 2010. Feed supplementation of *Lactobacillus plantarum* PCA 236 modulates gut microbiota and milk fatty acid composition in dairy goats—a preliminary study. *International journal of food microbiology,* 141**,** S109-S116.

Murphy, E., Cotter, P., Healy, S., Marques, T., O'sullivan, O., Fouhy, F., Clarke, S., O'toole, P., Quigley, E. & Stanton, C. 2010. Composition and energy harvesting capacity of the gut microbiota: relationship to diet, obesity and time in mouse models. *Gut,* 59**,** 1635-1642.

Park, P. & Goins, R. 1994. In situ preparation of fatty acid methyl esters for analysis of fatty acid composition in foods. *Journal of Food Science,* 59**,** 1262-1266.

Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K. S., Manichanh, C., Nielsen, T., Pons, N., Levenez, F. & Yamada, T. 2010. A human gut microbial gene catalogue established by metagenomic sequencing. *Nature,* 464**,** 59-65.

Santacruz, A., Marcos, A., Wärnberg, J., Martí, A., Martin-Matillas, M., Campoy, C., Moreno, L. A., Veiga, O., Redondo-Figuero, C. & Garagorri, J. M. 2009. Interplay between weight loss and gut microbiota composition in overweight adolescents. *Obesity,* 17**,** 1906-1915.

Schloss, P. D. & Handelsman, J. 2008. A statistical toolbox for metagenomics: assessing functional diversity in microbial communities. *BMC bioinformatics,* 9**,** 34.

Schwiertz, A., Taras, D., Schäfer, K., Beijer, S., Bos, N. A., Donus, C. & Hardt, P. D. 2010. Microbiota and SCFA in lean and overweight healthy subjects. *Obesity,* 18**,** 190-195.

Stams, A. J., Dijkema, C., Plugge, C. M. & Lens, P. 1998. Contribution of 13C-NMR spectroscopy to the elucidation of pathways of propionate formation and degradation in methanogenic environments. *Biodegradation,* 9**,** 463-473.

Topping, D. L. & Clifton, P. M. 2001. Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. *Physiological reviews,* 81**,** 1031-1064.

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.

P. 2008. A core gut microbiome in obese and lean twins. *Nature,* 457**,** 480-484.

Turnbaugh, P. J., Ley, R. E., Mahowald, M. A., Magrini, V., Mardis, E. R. & Gordon, J. I. 2006. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature,* 444**,** 1027-131.

Turroni, F., Foroni, E., Pizzetti, P., Giubellini, V., Ribbera, A., Merusi, P., Cagnasso, P., Bizzarri, B., De'angelis, G. L. & Shanahan, F. 2009a. Exploring the diversity of the bifidobacterial population in the human intestinal tract. *Applied and environmental microbiology,* 75**,** 1534-1545.

Turroni, F., Marchesi, J. R., Foroni, E., Gueimonde, M., Shanahan, F., Margolles, A., Van Sinderen, D. & Ventura, M. 2009b. Microbiomic analysis of the bifidobacterial population in the human distal gut. *The ISME journal,* 3**,** 745-751.

Urich, T., Lanzen, A., Qi, J., Huson, D. H., Schleper, C. & Schuster, S. C. 2008. Simultaneous assessment of soil microbial community structure and function through analysis of the meta-transcriptome. *PloS one,* 3**,** e2527.

Van Der Meulen, R., Adriany, T., Verbrugghe, K. & De Vuyst, L. 2006. Kinetic analysis of bifidobacterial metabolism reveals a minor role for succinic acid in the regeneration of NAD+ through its growth-associated production. *Applied and environmental microbiology,* 72**,** 5204-5210.

Velagapudi, V. R., Hezaveh, R., Reigstad, C. S., Gopalacharyulu, P., Yetukuri, L., Islam, S., Felin, J., Perkins, R., Borén, J. & Orešič, M. 2010. The gut microbiota modulates host energy and lipid metabolism in mice. *Journal of lipid research,* 51**,** 1101-1112.

Wall, R., Ross, R. P., Shanahan, F., O'mahony, L., O'mahony, C., Coakley, M., Hart, O., Lawlor, P., Quigley, E. M. & Kiely, B. 2009. Metabolic activity of the enteric microbiota influences the fatty acid composition of murine and porcine liver and adipose tissues. *The American journal of clinical nutrition,* 89**,** 1393.

Wall, R., Ross, R. P., Shanahan, F., O'mahony, L., Kiely, B., Quigley, E., Dinan, T. G., Fitzgerald, G. & Stanton, C. 2010. Impact of administered bifidobacterium on murine host fatty acid composition. *Lipids,* 45**,** 429-436.

Wolever, T., Brighenti, F., Royall, D., Jenkins, A. & Jenkins, D. 1989. Effect of rectal infusion of short chain fatty acids in human subjects. *The American journal of gastroenterology,* 84**,** 1027-1033.

Yurko-Mauro, K., Mccarthy, D., Rom, D., Nelson, E. B., Ryan, A. S., Blackwell, A., Salem Jr, N. & Stedman, M. 2010. Beneficial effects of docosahexaenoic acid on cognition in age-related cognitive decline. *Alzheimer's & Dementia,* 6**,** 456-464.

Zoetendal, E., Rajilić-Stojanović, M. & De Vos, W. 2008. High-throughput diversity and functionality analysis of the gastrointestinal tract microbiota. *Gut,* 57**,** 1605-1615.

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

	***B. breve* DPC 6330 fed mice**	***B. breve* NCIMB 702258 fed mice**	**Unsupplemented mice**
Initial weight (g)	26.5 ± 0.6	26.7 ± 0.6	25.4 ± 0.5
Final weight (g)	31.7 ± 0.8	33.4 ± 0.7	30.6 ± 0.8
Weight gain (%)	20.3 ± 4.5	25.1 ± 3.7	20.5 ± 3.1
Visceral fat mass (g)	$1.51 \pm 0.16^{A,B}$	1.71 ± 0.13^{A}	1.20 ± 0.10^{B}
Liver mass (g)	1.64 ± 0.05	1.55 ± 0.06	1.47 ± 0.05
Liver triglycerides (mg/g)	4.64 ± 0.73^{A}	$3.44 \pm 0.29^{A,B}$	2.93 ± 0.14^{B}

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

Serum parameter	*B. breve* DPC 6330 fed mice	*B. breve* NCIMB 702258 fed mice	Unsupplemented mice
Triglycerides (mg/dL)	78.1 ± 12.8	56.2 ± 11.8	61.3 ± 8.2
Glucose (mg/dL)	217.6 ± 17.6	186.0 ± 6.4	183.3 ± 2.8
Insulin (ng/mL)	1.4 ± 0.2	2.5 ± 0.8	1.7 ± 0.3
Leptin (ng/mL)	3.9 ± 0.8	5.2 ± 0.9[1]	2.4 ± 0.7[1]

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

SCFAs	*B. breve* DPC 6330 fed mice	*B. breve* NCIMB 702258 fed mice	Unsupplemented mice
Acetate	48.05 ± 3.58	45.05 ± 2.73	40.71 ± 2.43
Propionate	9.80 ± 0.51^{A}	9.28 ± 0.49^{A}	7.14 ± 0.40^{B}
Butyrate	18.24 ± 2.67	13.44 ± 1.45	13.71 ± 1.59
Iso-butyrate	$0.80 \pm 0.09^{A,B}$	0.88 ± 0.05^{A}	0.67 ± 0.02^{B}
Total acids	76.88 ± 6.39	68.65 ± 4.03	62.23 ± 4.14

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

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

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

FAME	Epididymal Adipose tissue		
g/100g FAME	***B. breve* DPC 6330**	***B. breve* NCIMB 702258**	**Unsupplemented mice**
C14:0	1.96 ± 0.24^{A}	1.44 ± 0.27^{A,B}	1.10 ± 0.15^{B}
C16:0	15.19 ± 0.94^{A}	12.78 ± 0.68^{A,B}	10.68 ± 0.52^{B}
C16:1c9	4.31 ± 0.39^{A}	3.75 ± 0.53^{A,B}	2.85 ± 0.12^{B}
C18:0	2.04 ± 0.13	2.26 ± 0.12	2.31 ± 0.03
C18:1c9	28.71 ± 0.92^{A}	32.22 ± 0.89^{B}	33.47 ± 0.48^{B}
C18:2*n*-6	33.57 ± 1.18	34.93 ± 1.13	36.66 ± 0.68
C18:3*n*-3	2.44 ± 0.16	2.42 ± 0.10	2.57 ± 0.10
C18:3*n*-6	0.16 ± 0.01	0.15 ± 0.01	0.17 ± 0.005
C20:3*n*-6	0.42 ± 0.02	0.42 ± 0.02	0.42 ± 0.01
C20:4*n*-6	0.77 ± 0.04	0.71 ± 0.05	0.72 ± 0.03
C20:5*n*-3	0.093 ± 0.01	0.072 ± 0.01	0.085 ± 0.01
C22:5*n*-3	0.29 ± 0.02	0.26 ± 0.03	0.25 ± 0.02
C22:6*n*-3	0.77 ± 0.04^{A}	0.66 ± 0.05^{A,B}	0.62 ± 0.05^{B}

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

FAME	**Liver**		
g/100g FAME	***B. breve* DPC 6330**	***B. breve* NCIMB 702258**	**Unsupplemented mice**
C14:0	0.65 ± 0.08	0.67 ± 0.03	0.67 ± 0.05
C16:0	31.87 ± 0.33	30.53 ± 0.66	30.37 ± 0.40
C16:1c9	2.81 ± 0.26	2.91 ± 0.24	2.60 ± 0.23
C18:0	9.32 ± 0.48	9.03 ± 0.30	9.61 ± 0.45
C18:1c9	13.48 ± 0.82	13.05 ± 0.92	10.85 ± 0.35
C18:2*n*-6	21.55 ± 0.56^{A}	$19.40 \pm 0.84^{A,B}$	19.07 ± 0.31^{B}
C18:3*n*-3	0.83 ± 0.05	0.71 ± 0.05	0.71 ± 0.03
C18:3*n*-6	0.30 ± 0.01	0.26 ± 0.02	0.28 ± 0.01
C20:3*n*-6	0.91 ± 0.05	0.96 ± 0.06	0.94 ± 0.02
C20:4*n*-6	7.07 ± 0.48	7.25 ± 0.24	7.81 ± 0.35
C20:5*n*-3	0.22 ± 0.01	0.22 ± 0.02	0.22 ± 0.01
C22:5*n*-3	0.38 ± 0.03	0.42 ± 0.03	0.41 ± 0.02
C22:6*n*-3	3.57 ± 0.27^{A}	$3.82 \pm 0.12^{A,B}$	4.48 ± 0.16^{B}

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.

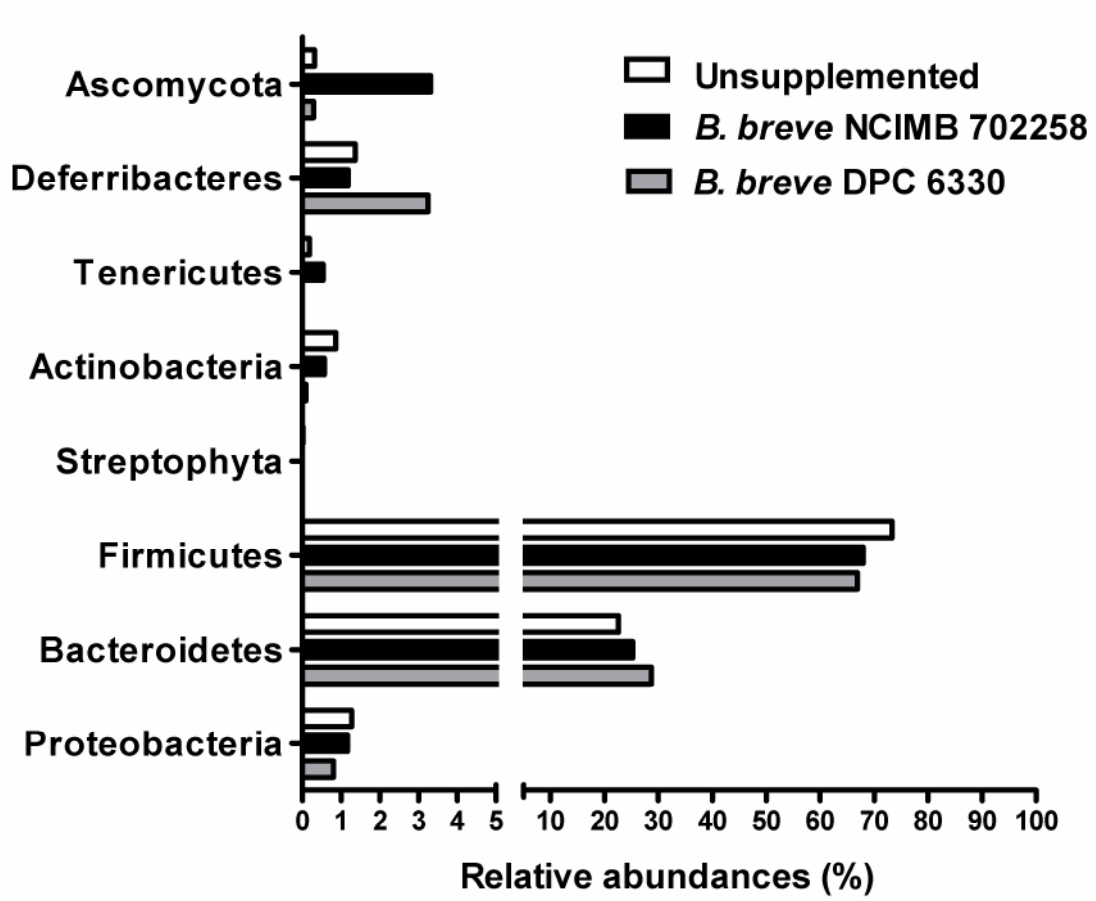

Figure 1 Phylum-level distributions of the microbial communities present in the caecum content, expressed as percentage of total population of assignable tags. *B. breve* DPC 6330 (n = 7), *B. breve* NCIMB 702258 (n = 7), unsupplemented mice (n = 8). No significant differences were observed

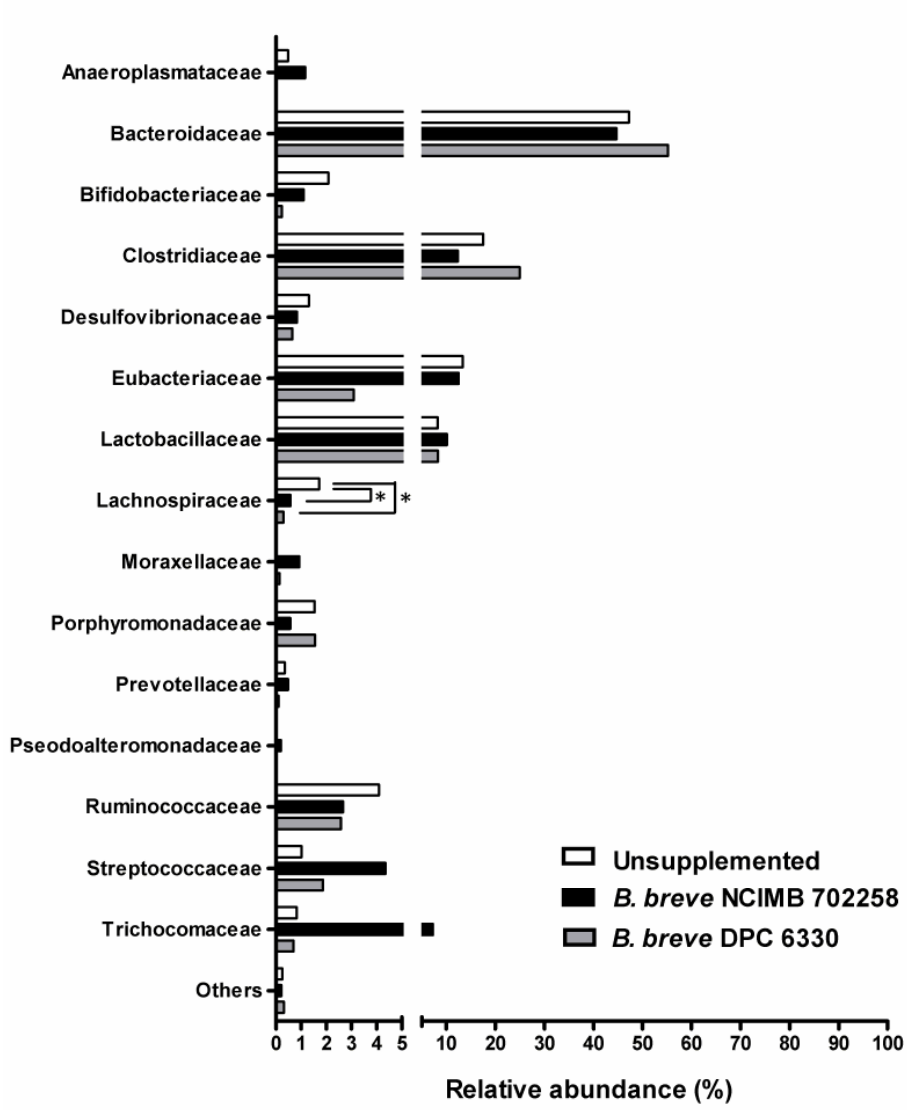

Figure 2 Family-level taxonomic distributions of the microbial communities in caecum content, expressed as percentage of total tags assignable at family level. *B. breve* DPC 6330 (n = 7), *B. breve* NCIMB 702258 (n = 7), unsupplemented mice (n = 8). *p<0.05, determined by Kruskal-Wallis test

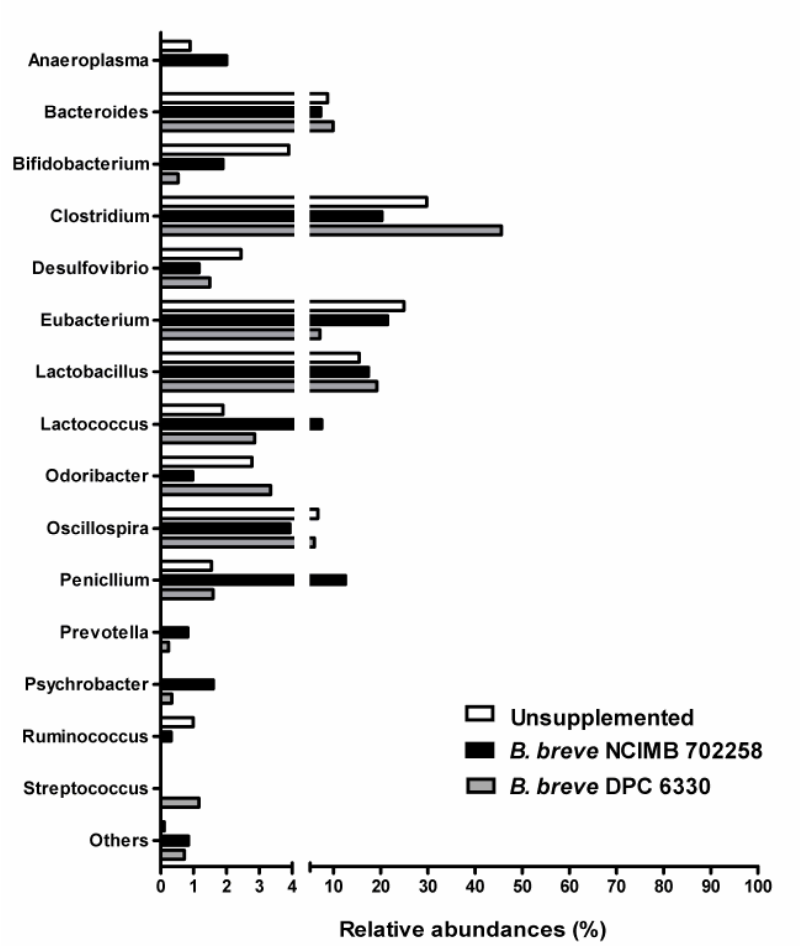

Figure 3 Genus-level taxonomic distributions of the microbial communities present in the caecum content, expressed as percentage of total tags assignable at genus level. *B. breve* DPC 6330 (n = 7), *B. breve* NCIMB 702258 (n = 7), unsupplemented mice (n = 8). No significant differences were observed

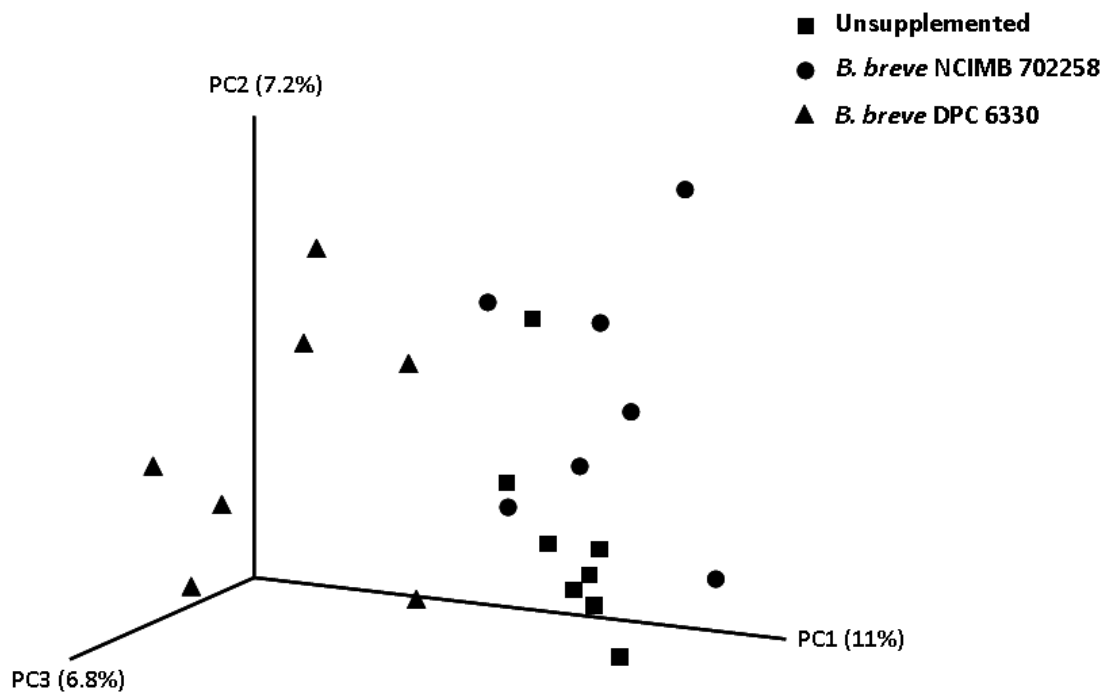

Figure 4 Principle coordinate analysis using unweighted UniFrac distances. *B. breve* DPC 6330 (n = 7), *B. breve* NCIMB 702258 (n = 7), unsupplemented mice (n = 8)

Appendix 1

Supplementary information

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

Data set	D1		D2		D3		D4		D5		D6		D7	
Similarity	97%	98%	97%	98%	97%	98%	97%	98%	97%	98%	97%	98%	97%	98%
Chao1 richness estimation	624	922	877	1366	402	543	731	1055	702	1033	556	741	748	1131
Shannon's index for diversity	5.2	5.6	5.6	6.1	5.1	5.5	5.2	5.7	5.5	5.9	5.2	5.7	5.4	5.9
Good's coverage	88%	82%	93%	89%	88%	82%	92%	89%	92%	87%	91%	87%	93%	89%

Data set	E1		E2		E3		E4		E5		E6		E7	
Similarity	97%	98%	97%	98%	97%	98%	97%	98%	97%	98%	97%	98%	97%	98%
Chao1 richness estimation	1099	1759	1013	1481	755	1048	922	1280	979	1595	861	1431	1050	1702
Shannon's index for diversity	5.8	6.2	5.8	6.2	5.3	5.8	5.6	6.1	5.7	6.2	5.6	6.1	5.8	6.3
Good's coverage	93%	88%	94%	90%	90%	86%	92%	88%	94%	90%	94%	90%	93%	89%

Data set	F1		F2		F3		F4		F5		F6		F7		F8	
Similarity	97%	98%	97%	98%	97%	98%	97%	98%	97%	98%	97%	98%	97%	98%	97%	98%
Chao1 richness estimation	1062	1759	974	1408	1563	2534	890	1382	1018	1633	935	1440	937	1559	987	1507
Shannon's index for diversity	5.8	6.3	5.6	6.0	6.0	6.6	5.6	6.1	5.7	6.1	5.6	6.1	5.8	6.3	5.7	6.2
Good's coverage	94%	90%	93%	88%	95%	91%	93%	89%	94%	90%	92%	88%	93%	88%	94%	90%

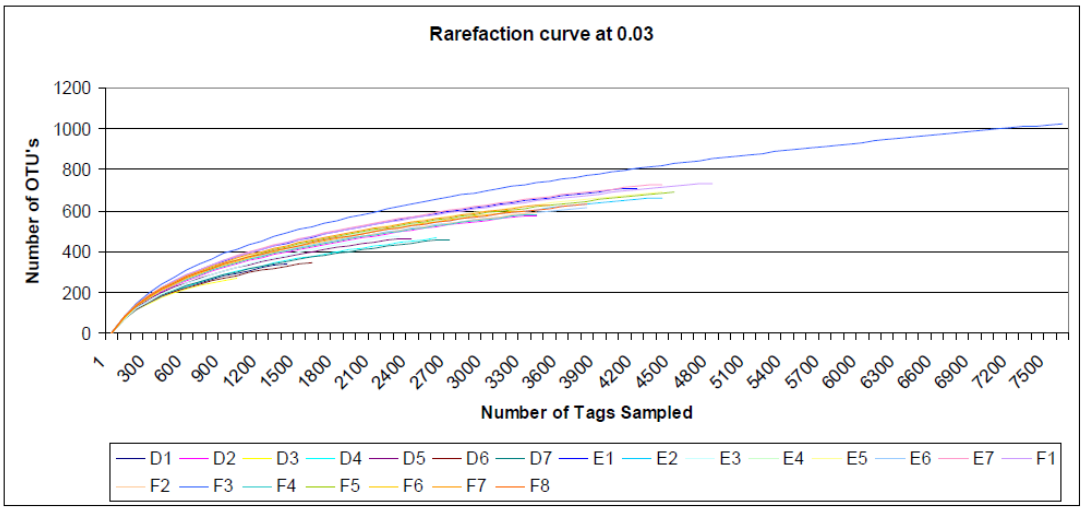

Figure S1 Rarefaction curves of samples sequenced by high-throughput DNA sequencing. Rarefaction curves at 97% similarity indicated that the total bacterial diversity present was well represented. Number of operational taxonomic units (OTUs) identified as a function of the number of sequence tags sampled

Appendix 2

The long- and short-term effects of small bowel resection on bile acid metabolism: experience with a piglet short bowel syndrome model

(In preparation for submission)

Abstract

Primary bile acids are synthesized from cholesterol in the liver and are then modified by bacteria in the gut. The bile acid composition in the gut of children with short bowel syndrome (SBS; a condition characterized by a significant reduction in the length of the bowel) is atypical in that it is composed predominantly of primary bile acids. The unusual gut microbiota of infants with SBS may be responsible for this change.

The aims of this study were to utilize a preclinical, porcine, model of SBS to investigate the temporal changes in bile acid composition as well as changes to key bacteria responsible for determining bile acid composition, following small bowel resection (SBR). DNA was extracted from the caecal contents of the short bowel resection, sham and non-operative control groups and high-throughput sequencing was performed to identify changes in bacteria with known bile salt hydrolase activity or 7 α-dehydroxylation ability. Bile acid composition was also determined for each of the groups.

Investigations of bile acid species within bile and portal samples revealed a compositional shift characterized by a dominance of primary bile acid species and a depletion of secondary bile acid species following SBR. The bile of SBS animals exhibited decreased levels of dihydroxy bile acids (including chenodeoxycholic acid and deoxycholic acid) and increased levels of trihydroxy bile acids (cholic acid and hyodeoxycholic acid). Additionally, SBS piglets exhibited a corresponding decrease in total levels of 7 α-dehydroxylating bacteria, and in *Clostridium* in particular, following SBR. Additionally, *Bacteroides* and *Lactobacillus* levels also decreased in SBS piglets, compared to the non-operative controls.

Ultimately, through this study we gain a greater insight into the SBR-induced changes in the composition of bile, and bile metabolising bacteria, which contribute to SBS.

Introduction

Short bowel syndrome (SBS) is the term used to describe a condition of malabsorption and malnutrition resulting from the loss of absorptive surface area following small bowel resection (SBR) (Sukhotnik et al., 2002). SBS is a devastating disease which, despite marked improvements in the care of infants and children, produces mortality rates in the 20-30% range (Spencer et al., 2008). After intestinal resection, a dynamic series of complex changes in morphology, mucosal function, gastrointestinal motility and hormones occur in the host in order to attempt to compensate for the loss of bowel length (Pereira and Bines, 2006). Paradoxically, despite displaying morphological and functional adaptation, adults and children with SBS may exhibit unresolved malnutrition due to malabsorption of fat and bile acids for years (Pakarinen et al., 2009, Ohkohchi et al., 1997). Factors related to intestinal resection, including loss of absorptive surface, increased intestinal transit and alterations in gut microbiota, are likely to significantly impact on bile acid composition.

Bile acid composition is determined by (1) the biosynthesis of primary bile acids from cholesterol in the liver and (2) bacterial modifications within the intestine, including deconjugation by bacteria with bile salt hydrolase (BSH) activity and the creation of secondary bile acids from primary bile acids by 7α-dehydroxylating bacteria. In humans the primary bile acids produced by the liver are cholic acid (CA) and chenodeoxycholic acid (CDCA), while the pig also produces hyocholic acid (HCA). Within the large intestine, bacteria convert CA to deoxycholic acid (DCA), CDCA to lithocholic acid (LCA) and ursodeoxycholic acid (UDCA) and HCA to hyodeoxycholic acid (HDCA). In contrast, children with SBS exhibit a switch to a bile acid composition dominated by primary bile acids (Ohkohchi et al., 1997) and more recent microbial studies, using qPCR and temperature gradient gel electrophoresis, describe a reduction in the number and diversity of *Clostridium* and *Bacteroides* species in patients

with SBS (Bongaerts et al., 1997, Joly et al., 2010). This is important and warrants further investigation as *Bacteroides* is a genus that contributes significantly to the deconjugation of bile acids and clostridia play an important role in the 7α-dehydroxylation of free-form primary bile acids in the intestine. The study of the interaction between bile acids and the gut microbiota in the context of SBS is essential as it may hold the key to understanding the aetiopathogenesis of the disease, thereby providing important new therapeutic options.

In our laboratory, we have established a preclinical model of SBS in infants and children using the juvenile piglet (Pereira-Fantini et al., 2011a, Pereira-Fantini et al., 2008a, Pereira-Fantini et al., 2008b, Lapthorne et al., 2013). The pig gastrointestinal tract has striking physiological similarities to humans and is considered to be one of the best models for the study of gastrointestinal disease in humans (Miller and Ullrey, 1987, Moughan et al., 1992). This study aimed to use our preclinical model of SBS to (1) detail temporal changes in bile acid composition in response to small bowel resection and (2) detail parallel changes in key bacteria responsible for determining bile acid composition. Quantitation of bile acid species within bile and portal samples revealed a compositional shift characterized by a dominance of primary bile acid species and depletion of secondary bile acid species following SBR. Furthermore, SBS piglets exhibited a corresponding decrease in levels of 7α-dehydroxylating bacteria following SBR. We conclude that significant changes in the microbiota and consequent bile acid dysmetabolism, contributes to the development of SBS.

Methods

Animals and experimental design

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 (Landrace/Large White cross; Aussie Pride Pork, Australia) and acclimatised prior to surgery. Piglets were housed at a temperature of 22°C with a 12 h light/dark cycle and fed a polymeric infant formula diet (Karicare De-Lact, Nutricia, Macquarie Park, Australia) supplemented to meet the daily requirements of piglets, as described previously (Pereira-Fantini et al., 2011b, Healey et al., 2010). The diets were isocaloric and isonitrogenous among the groups and were administered on a per kilogram basis. 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.

Clinical assessment and growth

Piglet weight was measured weekly before feeding. Faecal samples were collected fortnightly 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. Within the same stool sample, Laboratory Services assigned a semi-quantitative score indicating the presence of fat globules.

Experimental design

Four-week-old piglets underwent either a 75% proximal small bowel resection (SBR; n=6/group/time-point) or transection and re-anastomosis (sham; n=5/group/time-point) operation. One group of piglets did not receive any surgery (non-operative control; NOC; n=6/group/time-point). 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 ileocecal valve. Piglets received intramuscular amoxicillin (70 mg/kg; CSL Limited) 24 h pre-surgery. On the day of surgery, piglets were anesthetized 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 reintroduced from the third day post-operation. All piglets in the NOC group followed the same antibiotic and feeding regime.

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. Portal plasma, bile and colonic content samples were obtained on the day of sacrifice and frozen at -80ºC until required.

Determination of bile acid composition

Bile acid standards were purchased from Sigma Aldrich or Steraloids. Deuterated cholic acid (D-2452) and deuterated chenodeoxycholic acid (D-2772) were purchased from CDN Isotopes Inc. HPLC-grade chemicals were obtained from Fisher Scientific (Fair Lawn, NJ).

Bile acids were extracted from bile and portal plasma with 50% ice-cold methanol, followed by further extraction with ACN (5% NH_4OH). Ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS) was performed on the resultant supernatant using a modified method of Swann *et al*. (Swann et al., 2011) and samples analyzed using an Acquity UPLC system (Waters Ltd.) coupled online to an LCT Premier mass spectrometer (Waters MS Technologies, Ltd.) in negative electrospray mode with a scan range of 50–1,000 m/z. Analytes were quantified individually (three technical reads per sample) against known

bile acid standard curves. All quantitations were normalised relative to the internal standards.

High-throughput sequencing and bioinformatic analysis

High-throughput sequencing of colonic content was performed to identify changes in bacteria with known bile salt hydrolase activity (*Lactobacillus, Clostridium, Bacteroides)* or 7α-dehydroxylation ability (*Eubacterium, Bifidobacterium, Lactobacillus, Clostridium, Bacteroides*). Initially, 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 one forward primer and a combination of four reverse primers, as described previously (Murphy et al., 2010). 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 using a Genome Sequencer FLX platform (Roche Diagnostics Ltd.).

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). The Qiime suite of tools (Caporaso et al., 2010) was used to cluster data into operational taxonomical units (OTUs). Data is represented as the percentage of each bacterial species (OTUs)/total OTUs.

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 6.0). Sequencing analysis was completed using Minitab Release 15.1.1.0 (Minitab Inc. 2007). *P* values of < 0.05 were considered statistically significant. All statistical tests were performed using Graphpad Prism, version 6.0.

Results

Clinical assessment

All animals tolerated the procedure well, and there were no complications or mortality. Before surgery all piglets were of similar weight, however, although energy intake was comparable between groups, SBR piglets gained weight at a reduced rate after surgery (Figure 1A). From week 2 post-operation, SBR piglets exhibited significantly less weight gain when compared with both NOC or sham piglets and this continued throughout the time course studied. From week 4 post-operation sham piglets exhibited a small, but significant decrease in weight gain when compared with NOC piglets. As indicated by a consistently high stool consistency score, SBR piglets suffered from persistent diarrhoea throughout the time course (Figure 1B). The presence of stool fat globules was increased in SBR animals at both 2- and 6-weeks post-surgery when compared with NOC and sham-control piglets, suggestive of continuing fat malabsorption (Figure 1C).

Bile and portal bile acid composition

Given the degree of fat malabsorption and persistent diarrhoea observed throughout the time course, we next examined changes in the proportion of bile acids present in biliary and portal serum. The proportion of primary, secondary and conjugated bile acids in bile samples remained similar at both time points (Figure 2). In contrast, the proportion of bile acids present in portal samples was marginally altered at two weeks post-SBR with more pronounced changes evident at 6 weeks post-SBR (Figure 2).

Following this observation, we examined alterations in the concentration of specific bile acid species in portal samples obtained at either 2 (Table 1) or 6 weeks (Table 2) post-SBR. A sham control group was included in our experiments to allow us to differentiate between operation related

changes and those resultant from removal of 75% of the small bowel. Changes observed at two weeks following sham surgery included decreased CDCA and HCA concentration and increased LCA and conjugated UDCA concentration when compared with the NOC group (Table 1). Similarly at six weeks CDCA, UDCA and conjugated UDCA concentrations were decreased and HDCA and DCA concentration increased in sham-operated piglets, when compared with the NOC group (Table 2).

Comparison of SBR portal samples with both NOC and sham samples revealed an early increase in the concentration of primary bile acids, specifically unconjugated HCA and CA, and conjugated CDCA and HCA accompanied by decreased concentration of secondary bile acids including unconjugated LCA and HDCA and conjugated LCA, HDCA, DCA and UDCA (Table 1). A similar pattern persisted at 6 weeks post-SBR with the conjugated and unconjugated forms of the primary bile acids CDCA and HCA increased when compared to both NOC and SHAM control groups and secondary bile acid concentrations including unconjugated LCA and HDCA and conjugated HDCA and DCA decreased (Table 2 & Figure 3).

Analysis of bacterial composition

Portal bile acid composition is a reflection of bacterially mediated bile acid modification performed within the large intestine (Figure 4A). Therefore, following from our portal bile acid composition analysis, we next performed high-throughput sequencing of the colonic content to investigate changes in bacteria known to possess bile salt hydrolase activity and/or 7α-dehydroxylating ability. Two weeks following SBR, only the proportion of *Clostridium* was decreased when compared to NOC at genus level (Figure 4B). By six weeks post-SBR more extensive changes were evident, with a decrease in *Clostridium* species when compared to sham-controls, but not compared to the NOC. *Bacteroides* and

Lactobacillus species significantly decreased in the Sham group compared to the NOC, but not compared to the SBR group (Figure 4C). The proportion of *Eubacterium* was increased at 6 weeks post-SBR compared with either NOC or sham groups, where it was only detected in 2 of the piglets.

Discussion

Bile acids are necessary for a number of important physiological functions, including the solubilisation of cholesterol, fat soluble vitamins and other lipids in the intestine (Holt et al., 2003), and bile acid dysmetabolism is associated with fat malabsorption and diarrhoea (Ohkohchi et al., 1997) and liver injury (Bove et al., 2004, Monte et al., 2009). Few studies have examined the impact of small bowel resection (SBR) on bile acid metabolism. However, in a study of 13 children with SBS, Ohkohchi *et al*., detected a switch to primary bile acid dominance following small bowel resection (Ohkohchi et al., 1997) and first suggested resection-associated microbial dysbiosis as a contributor to bile acid dysmetabolism. Microbial studies to date have used culture- and microscopy-based techniques to examine changes post-SBR, however approximately 70-80% of gut bacteria are unculturable (Wilson et al., 1990). Recently we have employed high-throughput sequencing to provide a more comprehensive microbial fingerprint (Lapthorne et al., 2013). These studies described a decrease in microbial diversity which we hypothesised would influence the bile acid composition of SBS piglets (Lapthorne et al., 2013).

Bile acids are amphipathic sterols synthesized from cholesterol in the liver and secreted via bile into the intestine. Within the intestinal lumen conjugated bile acids play a crucial role in the absorption of fat by bringing into micellular solution, the fatty acids and monoglycerides that are the end products of the action of pancreatic lipase upon dietary triglyceride (Dietschy, 1968). Hence fat malabsorption is strongly associated with disturbance of bile acid metabolism (Hofmann and Poley, 1972). Fat malabsorption in patients following SBR is postulated to be due to the combined loss of absorptive surface area, compromised enterohepatic circulation and decreased bile acid pool (Yang et al., 2012). In the piglet SBS model, we observed persistent fat malabsorption and decreased weight gain despite increased intestinal surface area and

increased levels of conjugated bile acids. The bile of SBS animals exhibited decreased levels of dihydroxy bile acids (CDCA, DCA, HDCA, UDCA) and a concurrent increase in trihydroxy bile acids (CA, HCA), which may have impaired fat absorption as the higher critical micellar concentration of trihydroxy bile acids makes them less efficient at fat solubilisation when compared with dihydroxy bile acids (Hofmann, 1999).

In addition to the clinical outcomes of reduced weight and fat malabsorption, similar to patients with SBS, piglets who had undergone SBR exhibited persistent diarrhoea, which remained unresolved at six weeks post-SBR. We postulate that delivery to the intestine of increased concentrations of CDCA via bile may have contributed to the persistent diarrhoea observed in SBS piglets, as luminal CDCA administration shortens transit time in a canine model via stimulation of propulsive contractions (Kruis et al., 1986) and in rodents abolishes the ileal and colonic absorption of water and sodium (Caspary and Meyne, 1980) via activation of calcium sensitive chloride and potassium channels (Mauricio et al., 2000, Venkatasubramanian et al., 2001). Alternatively, the shift to a predominantly primary bile acid-based composition within the lumen may contribute to a protective, adaptive response, as conjugated CDCA has been shown to promote proliferation in an intestinal cell line (Ishizuka et al., 2012), whereas physiological concentrations of DCA in the intestine disturbs intestinal integrity resulting in leakage of ions and cellular components and eventually cell death (Islam et al., 2011). In addition DCA mediates colonic inflammation via inductions of COX-2 expression and prostaglandin synthesis in colonic fibroblasts, (Zhu et al., 2002) and in a human colon cancer cell line (Glinghammar et al., 2002).

Upon completion of their digestive tasks, most intestinal bile acids are recovered by active transport in the terminal ileum and returned to the liver via the portal circulation. Those that escape active transport in the small intestine enter the large intestine where they undergo bacterial modification. Bacterial bile acid transformation resulting in

dehydroxylation of bile acids at position C-7 is quantitatively the most important transformation, but is restricted to unconjugated primary bile acids (Monte et al., 2009). 7α-dehydroxylation results in the formation of DCA from CA, LCA from CDCA and HDCA from HCA. The deconjugation and 7α-dehydroxylation of bile acids increases their hydrophobicity, allowing them to be recovered by passive absorption across the colonic epithelium, (Monte et al., 2009) which may be important for maximising the retrieval potential of the colonic remnant in SBS.

In the piglet SBS model, we have observed a significant increase in portal unconjugated primary bile acid levels that occurs concurrent with a significant decrease in unconjugated secondary bile acids levels. This mirrors reports in children with SBS in whom the faecal bile acid profile shifts, such that primary bile acids account for more than 95% of the total bile acids, compared to 50-60% in the healthy state (Ohkohchi et al., 1997) and confirms the value of this model to explore the mechanisms underlying the disturbance in bile acid metabolism observed in SBS.

Studying the portal bile acid composition allowed us to elucidate the effect of alterations in bacteria with bile acid-modifying properties on bile acid composition. Quantification of the bacteria responsible for 7α-dehydroxylation of bile acids highlighted both a decrease in the total amounts of dehydroxylating bacteria following SBR and specifically demonstrated a persistent decrease in *Clostridium* species from two weeks post-SBR and long-term decreases in the genera *Bacteroides* and *Lactobacillus*. The decrease in the proportion of *Bacteroides* following SBR may be of particular relevance as it has been shown to enhance the 7α-dehydroxylase activity of other colonic bacteria (Hirano and Masuda, 1982). We have previously noted a significant decrease in overall colonic bacterial diversity in the piglet SBS model and similar decreases in *Clostridium* and *Bacteroides* have been observed in patients with SBS, (Bongaerts et al., 1997, Joly et al., 2010) possibly due to changes in the levels of bile acids, pH, nutrient levels and redox potential, as a result of

intestinal resection, all of which could impact the gut microbial community.

In conclusion, we propose a pathological scenario in which microbial dysbiosis following small bowel resection results in significant bile acid dysmetabolism and consequent outcomes including steatorrhea, persistent diarrhoea and liver damage (Figure 5). A potential novel therapy may be probiotic treatment with bacteria which have bile salt hydrolase activity, to restore the balance of primary to secondary bile acids and prevent SBS development through greater availability of unconjugated bile acids and increased 7α-dehydroxylation of unconjugated primary bile acids to produce secondary bile acids. However, preclinical testing in the piglet SBS model will be essential to ensure the safety of delivering live probiotic organisms to a potentially compromised gut, to confirm that increased levels of deconjugation do not worsen fat malabsorption and that enhanced levels of free bile acids do not exacerbate diarrhoea.

References

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research,* 25**,** 3389-3402.

Bongaerts, G. P., Tolboom, J. J., Naber, A. H., Sperl, W. J., Severijnen, R. S., Bakkeren, J. A. & Willems, J. L. 1997. Role of bacteria in the pathogenesis of short bowel syndrome-associated D-lactic acidemia. *Microb Pathog,* 22**,** 285-93.

Bove, K. E., Heubi, J. E., Balistreri, W. F. & Setchell, K. D. 2004. Bile acid synthetic defects and liver disease: a comprehensive review. *Pediatr Dev Pathol,* 7**,** 315-34.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., Peña, A. G., Goodrich, J. K. & Gordon, J. I. 2010. QIIME allows analysis of high-throughput community sequencing data. *Nature methods,* 7**,** 335-336.

Caspary, W. F. & Meyne, K. 1980. Effects of chenodeoxy- and ursodeoxycholic acid on absorption, secretion and permeability in rat colon and small intestine. *Digestion,* 20**,** 168-74.

Dietschy, J. M. 1968. Mechanisms for the intestinal absorption of bile acids. *J Lipid Res,* 9**,** 297-309.

Glinghammar, B., Inoue, H. & Rafter, J. J. 2002. Deoxycholic acid causes DNA damage in colonic cells with subsequent induction of caspases, COX-2 promoter activity and the transcription factors NF-kB and AP-1. *Carcinogenesis,* 23**,** 839-45.

Healey, K. L., Bines, J. E., Thomas, S. L., Wilson, G., Taylor, R. G., Sourial, M. & Pereira-Fantini, P. M. 2010. Morphological and functional changes in the colon after massive small bowel resection. *J Pediatr Surg,* 45**,** 1581-90.

Hirano, S. & Masuda, N. 1982. Enhancement of the 7 alpha-dehydroxylase activity of a gram-positive intestinal anaerobe by

Bacteroides and its significance in the 7-dehydroxylation of ursodeoxycholic acid. *J Lipid Res,* 23**,** 1152-8.

Hofmann, A. F. 1999. Bile Acids: The Good, the Bad, and the Ugly. *News Physiol Sci,* 14**,** 24-29.

Hofmann, A. F. & Poley, J. R. 1972. Role of bile acid malabsorption in pathogenesis of diarrhoea and steatorrhea in patients with ileal resection. I. Response to cholestyramine or replacement of dietary long chain triglyceride by medium chain triglyceride. *Gastroenterology,* 62**,** 918-34.

Holt, J. A., Luo, G., Billin, A. N., Bisi, J., Mcneill, Y. Y., Kozarsky, K. F., Donahee, M., Wang, D. Y., Mansfield, T. A., Kliewer, S. A., Goodwin, B. & Jones, S. A. 2003. Definition of a novel growth factor-dependent signal cascade for the suppression of bile acid biosynthesis. *Genes Dev,* 17**,** 1581-91.

Huson, D. H., Auch, A. F., Qi, J. & Schuster, S. C. 2007. MEGAN analysis of metagenomic data. *Genome Research,* 17**,** 377-386.

Ishizuka, S., Shiwaku, M., Hagio, M., Suzuki, T., Hira, T. & Hara, H. 2012. Glycochenodeoxycholic acid promotes proliferation of intestinal epithelia via reduction of cyclic AMP and increase in H2AX phosphorylation after exposure to gamma-rays. *Biomed Res,* 33**,** 159-65.

Islam, K. B., Fukiya, S., Hagio, M., Fujii, N., Ishizuka, S., Ooka, T., Ogura, Y., Hayashi, T. & Yokota, A. 2011. Bile acid is a host factor that regulates the composition of the cecal microbiota in rats. *Gastroenterology,* 141**,** 1773-81.

Joly, F., Mayeur, C., Bruneau, A., Noordine, M. L., Meylheuc, T., Langella, P., Messing, B., Duee, P. H., Cherbuy, C. & Thomas, M. 2010. Drastic changes in fecal and mucosa-associated microbiota in adult patients with short bowel syndrome. *Biochimie,* 92**,** 753-61.

Kruis, W., Haddad, A. & Phillips, S. F. 1986. Chenodeoxycholic and ursodeoxycholic acids alter motility and fluid transit in the canine ileum. *Digestion,* 34**,** 185-95.

Lapthorne, S., Pereira-Fantini, P. M., Fouhy, F., Wilson, G., Thomas, S. L., Dellios, N. L., Scurr, M., O'sullivan, O., Ross, R. P. & Stanton, C. 2013. Gut microbial diversity is reduced and is associated with colonic inflammation in a piglet model of short bowel syndrome. *Gut microbes,* 4**,** 1-10.

Mauricio, A. C., Slawik, M., Heitzmann, D., Von Hahn, T., Warth, R., Bleich, M. & Greger, R. 2000. Deoxycholic acid (DOC) affects the transport properties of distal colon. *Pflugers Arch,* 439**,** 532-40.

Miller, E. R. & Ullrey, D. E. 1987. The pig as a model for human nutrition. *Annual Review of Nutrition,* 7**,** 361-82.

Monte, M. J., Marin, J. J., Antelo, A. & Vazquez-Tato, J. 2009. Bile acids: chemistry, physiology, and pathophysiology. *World J Gastroenterol,* 15**,** 804-16.

Moughan, P. J., Birtles, M. J., Cranwell, P. D., Smith, W. C. & Pedraza, M. 1992. The piglet as a model animal for studying aspects of digestion and absorption in milk-fed human infants. *World review of nutrition and dietetics,* 67**,** 40-113.

Murphy, E., Cotter, P., Healy, S., Marques, T., O'sullivan, O., Fouhy, F., Clarke, S., O'toole, P., Quigley, E. & Stanton, C. 2010. Composition and energy harvesting capacity of the gut microbiota: relationship to diet, obesity and time in mouse models. *Gut,* 59**,** 1635-1642.

Ohkohchi, N., Andoh, T., Izumi, U., Igarashi, Y. & Ohi, R. 1997. Disorder of bile acid metabolism in children with short bowel syndrome. *Journal of Gastroenterology,* 32**,** 472-479.

Pakarinen, M. P., Koivusalo, A. I. & Rintala, R. J. 2009. Outcomes of intestinal failure--a comparison between children with short bowel and dysmotile intestine. *Journal of Pediatric Surgery,* 44**,** 2139-44.

Pereira-Fantini, P., Thomas, S., Wilson, G., Taylor, R., Sourial, M. & Bines, J. 2011a. Short- and long-term effects of small bowel resection: a unique histological study in a piglet model of short bowel syndrome. *Histochemistry and Cell Biology,* 135**,** 195-202.

Pereira-Fantini, P. M., Nagy, E. S., Thomas, S. L., Taylor, R. G., Sourial, M., Paris, M. C., Holst, J. J., Fuller, P. J. & Bines, J. E. 2008a. GLP-2 administration results in increased proliferation but paradoxically an adverse outcome in a juvenile piglet model of short bowel syndrome. *J Pediatr Gastroenterol Nutr,* 46**,** 20-8.

Pereira-Fantini, P. M., Thomas, S. L., Taylor, R. G., Nagy, E., Sourial, M., Fuller, P. J. & Bines, J. E. 2008b. Colostrum supplementation restores insulin-like growth factor -1 levels and alters muscle morphology following massive small bowel resection. *JPEN. Journal of Parenteral and Enteral Nutrition,* 32**,** 266-75.

Pereira-Fantini, P. M., Thomas, S. L., Wilson, G., Taylor, R. G., Sourial, M. & Bines, J. E. 2011b. Short- and long-term effects of small bowel resection: a unique histological study in a piglet model of short bowel syndrome. *Histochem Cell Biol,* 135**,** 195-202.

Pereira, P. M. & Bines, J. E. 2006. New growth factor therapies aimed at improving intestinal adaptation in short bowel syndrome. *Journal of Gastroenterology and Hepatology,* 21**,** 932-40.

Pruesse, E., Quast, C., Knittel, K., Fuchs, B. M., Ludwig, W., Peplies, J. & Glöckner, F. O. 2007. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Research,* 35**,** 7188-7196.

Spencer, A. U., Kovacevich, D., Mckinney-Barnett, M., Hair, D., Canham, J., Maksym, C. & Teitelbaum, D. H. 2008. Pediatric short-bowel syndrome: the cost of comprehensive care. *American Journal of Clinical Nutrition,* 88**,** 1552-9.

Sukhotnik, I., Siplovich, L., Shiloni, E., Mor-Vaknin, N., Harmon, C. M. & Coran, A. G. 2002. Intestinal adaptation in short-bowel syndrome in infants and children: a collective review. *Pediatric Surgery International,* 18**,** 258-263.

Swann, J. R., Want, E. J., Geier, F. M., Spagou, K., Wilson, I. D., Sidaway, J. E., Nicholson, J. K. & Holmes, E. 2011. Systemic gut

microbial modulation of bile acid metabolism in host tissue compartments. *Proc Natl Acad Sci U S A,* 108 Suppl 1**,** 4523-30.

Venkatasubramanian, J., Selvaraj, N., Carlos, M., Skaluba, S., Rasenick, M. M. & Rao, M. C. 2001. Differences in Ca(2+) signaling underlie age-specific effects of secretagogues on colonic Cl(-) transport. *Am J Physiol Cell Physiol,* 280**,** C646-58.

Wilson, K. H., Blitchington, R. & Greene, R. 1990. Amplification of bacterial 16S ribosomal DNA with polymerase chain reaction. *Journal of Clinical Microbiology,* 28**,** 1942-1946.

Yang, Q., Lan, T., Chen, Y. & Dawson, P. A. 2012. Dietary fish oil increases fat absorption and fecal bile acid content without altering bile acid synthesis in 20-d-old weanling rats following massive ileocecal resection. *Pediatr Res,* 72**,** 38-42.

Zhu, Y., Hua, P., Rafiq, S., Waffner, E. J., Duffey, M. E. & Lance, P. 2002. Ca2+- and PKC-dependent stimulation of PGE2 synthesis by deoxycholic acid in human colonic fibroblasts. *Am J Physiol Gastrointest Liver Physiol,* 283**,** G503-10.

Table 1 Alterations in the concentration of specific bile acid species in portal blood obtained at 2 weeks post sham-operation (SHAM), 75% small bowel resection (SBR) or from equivalent non-operation controls (NOC)

WEEK 2		Bile acid	NOC	SHAM	SBR
Primary Bile Acids	**Unconjugated**	CDCA	1.33±0.20	0.47±0.15**	0.81±0.09
		HCA	1.60±0.15	0.35±0.08***	2.82±0.18$^{***\#\#\#}$
		CA	0.05±0.01	0.02±0.01	0.00±0.00***
		Total Unconjugated Primary BA	**0.86±0.13**	**0.26±0.06****	**1.04±0.19*****
	Conjugated	CDCA	1.03±0.13	1.06±0.12	1.66±0.19$^{*\#}$
		HCA	0.78±0.24	0.35±0.09	1.83±0.30$^{**\#\#\#}$
		CA	0.03±0.01	0.02±0.01	0.02±0.01
		Total Conjugated Primary BA	**0.65±0.10**	**0.55±0.08**	**1.27±0.15$^{***\#\#\#}$**
Secondary/Tertiary Bile Acids	**Unconjugated**	LCA	0.70±0.06	0.99±0.11*	0.22±0.02$^{***\#\#\#}$
		HDCA	2.77±0.58	2.06±0.19	0.38±0.12$^{***\#\#}$
		DCA	ND	ND	ND
		UDCA	0.63±0.14	0.48±0.08	0.50±0.09
		Total Unconjugated Secondary/Tertiary BA	**1.22±0.21**	**1.08±0.12**	**0.37±0.05$^{***\#\#}$**
	Conjugated	LCA	0.02±0.01	0.04±0.01	0.00±0.00$^{\#\#}$
		HDCA	1.57±0.30	1.26±0.26	0.00±0.00$^{***\#\#\#}$
		DCA	1.03±0.18	0.79±0.11	0.08±0.04$^{***\#\#\#}$
		UDCA	0.12±0.02	0.20±0.02*	0.08±0.01$^{\#\#\#}$
		Total Conjugated Secondary/Tertiary BA	**0.75±0.13**	**0.71±0.11**	**0.03±0.01$^{***\#\#\#}$**

*p<0.05 versus NOC, **p<0.01 versus NOC, ***p<0.001 versus NOC. $^{\#}$p<0.05 versus SHAM, $^{\#\#}$p<0.01 versus SHAM, $^{\#\#\#}$p<0.001 versus SHAM.

Table 2 Alterations in the concentration of specific bile acid species in portal blood obtained at 6 weeks post sham-operation (SHAM), 75% small bowel resection (SBR) or from equivalent non-operation controls (NOC)

WEEK 6		Bile acid	NOC	SHAM	SBR
Primary Bile Acids	Unconjugated	CDCA	0.57±0.14	0.26±0.03	2.33±0.37$^{***###}$
		HCA	0.38±0.11	0.69±0.28	2.80±0.41$^{***###}$
		CA	ND	ND	ND
		Total Unconjugated Primary BA	**0.34±0.07**	**0.32±0.06**	**1.61±0.28$^{***###}$**
	Conjugated	CDCA	0.90±0.17	0.17±0.04**	1.74±0.17$^{***###}$
		HCA	0.21±0.05	0.34±0.04	0.73±0.12$^{***\#}$
		CA	0.01±0.01	0.02±0.01	0.02±0.01
		Total Conjugated Primary BA	**0.48±0.10**	**0.15±0.03**	**1.02±0.13$^{***###}$**
Secondary/Tertiary Bile Acids	Unconjugated	LCA	0.89±0.07	0.79±0.09	0.31±0.05$^{***###}$
		HDCA	1.76±0.44	2.12±0.33	0.33±0.05$^{**###}$
		DCA	ND	ND	ND
		UDCA	0.35±0.08	0.12±0.01*	0.42±0.05$^{\#\#}$
		Total Unconjugated Secondary/Tertiary BA	**0.93±0.13**	**0.93±0.16**	**0.35±0.03$^{**\#\#}$**
	Conjugated	LCA	0.03±0.02	0.00±0.00	0.02±0.02
		HDCA	1.28±0.24	0.67±0.17*	0.14±0.03***
		DCA	0.81±0.09	0.33±0.03***	0.11±0.03$^{***\#}$
		UDCA	0.30±0.07	0.05±0.01***	0.14±0.02*
		Total Conjugated Secondary/Tertiary BA	**0.67±0.10**	**0.26±0.06**	**0.12±0.01$^{***###}$**

*p<0.05 versus NOC, **p<0.01 versus NOC, ***p<0.001 versus NOC. $^{\#}$p<0.05 versus SHAM, $^{\#\#}$p<0.01 versus SHAM, $^{\#\#\#}$p<0.001 versus SHAM.

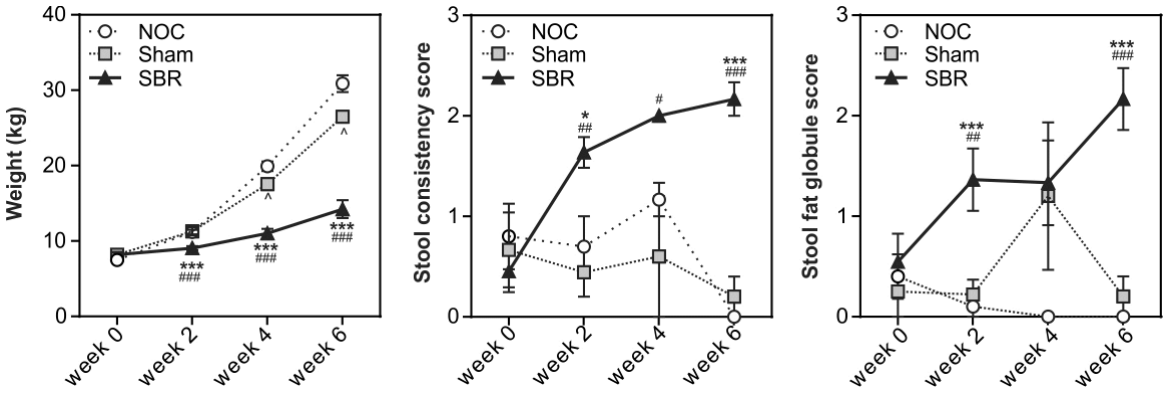

Figure 1 The clinical features of the SBS model include decreased weight (A), persistent diarrhoea (B) and fat malabsorption (C). $^{*}p<0.05$ versus NOC, $^{**}p<0.01$ versus NOC, $^{***}p<0.001$ versus NOC. $^{\#}p<0.05$ versus SHAM, $^{\#\#}p<0.01$ versus SHAM, $^{\#\#\#}p<0.001$ versus SHAM.

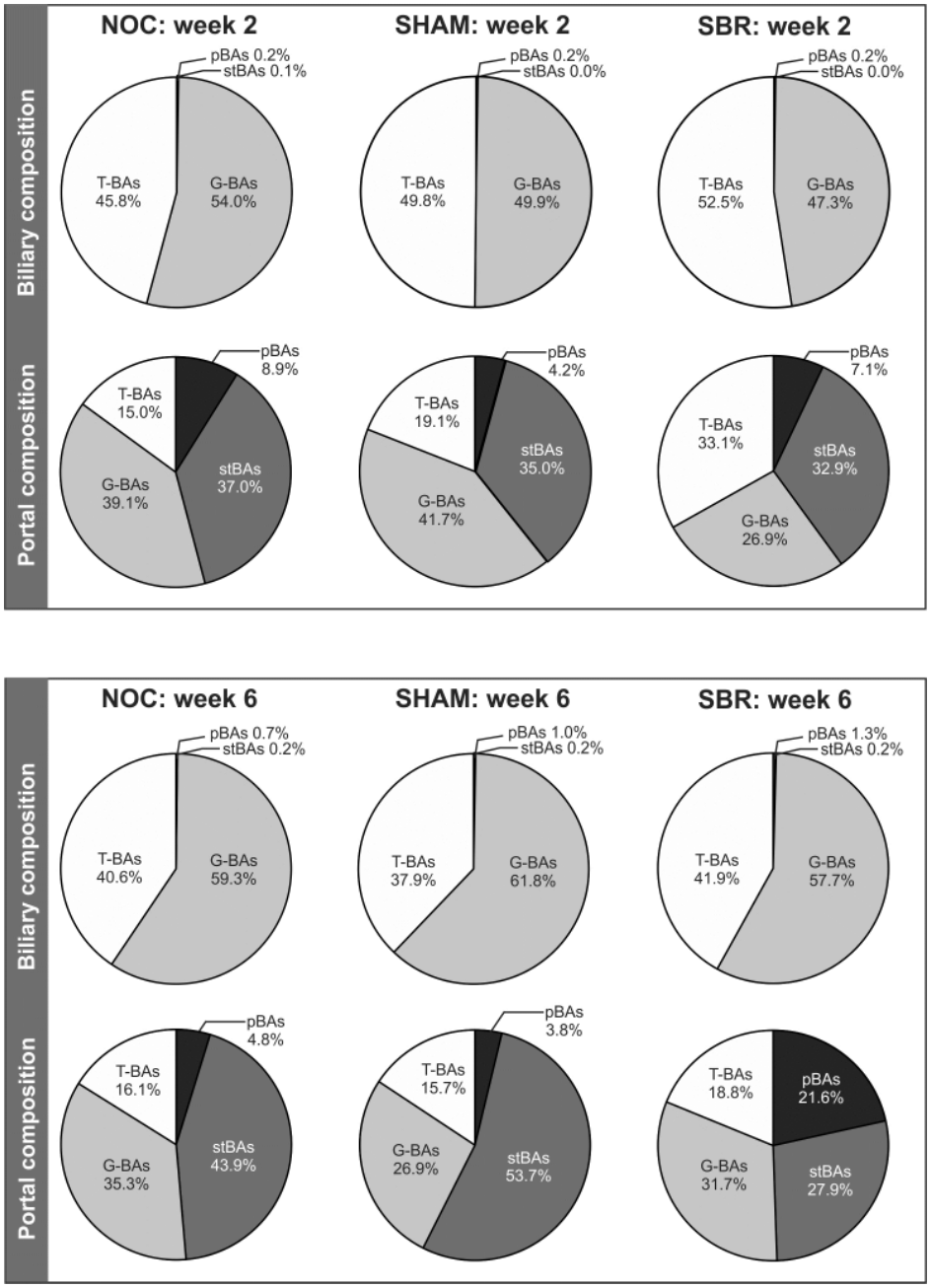

Figure 2 Changes in the proportion of biliary and portal primary bile acids (pBAs), secondary and tertiary bile acids (stBAs), glycine conjugated bile acids (G-BAs) and taurine-conjugated bile acid (T-BAs) in non-operation controls (NOC), sham-operated controls (SHAM) and animals which have undergone a 75% small bowel resection (SBR) at 2- or 6-weeks post-surgery

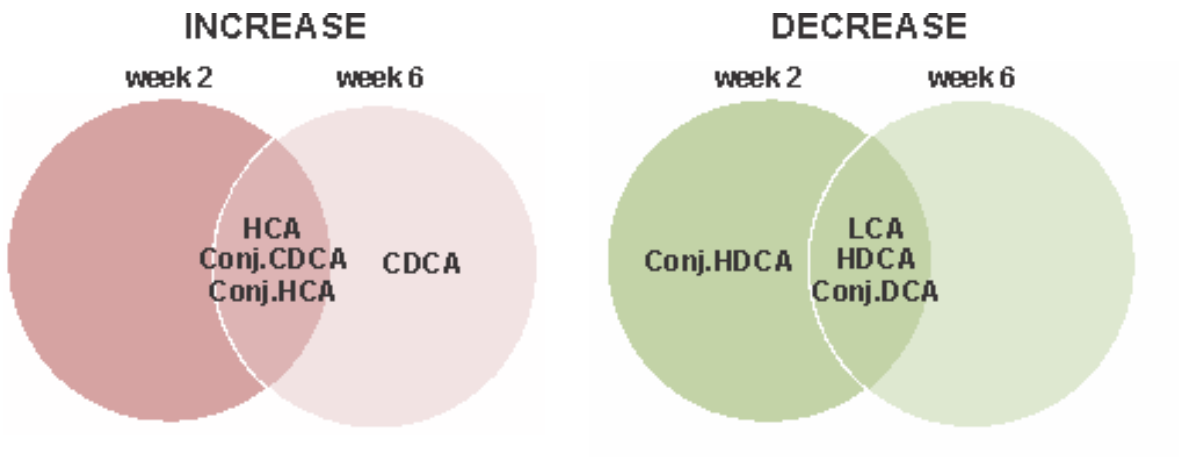

Figure 3 Venn diagram summarising changes in the concentration of specific bile acid species in SBR animals versus both NOC and SHAM control groups

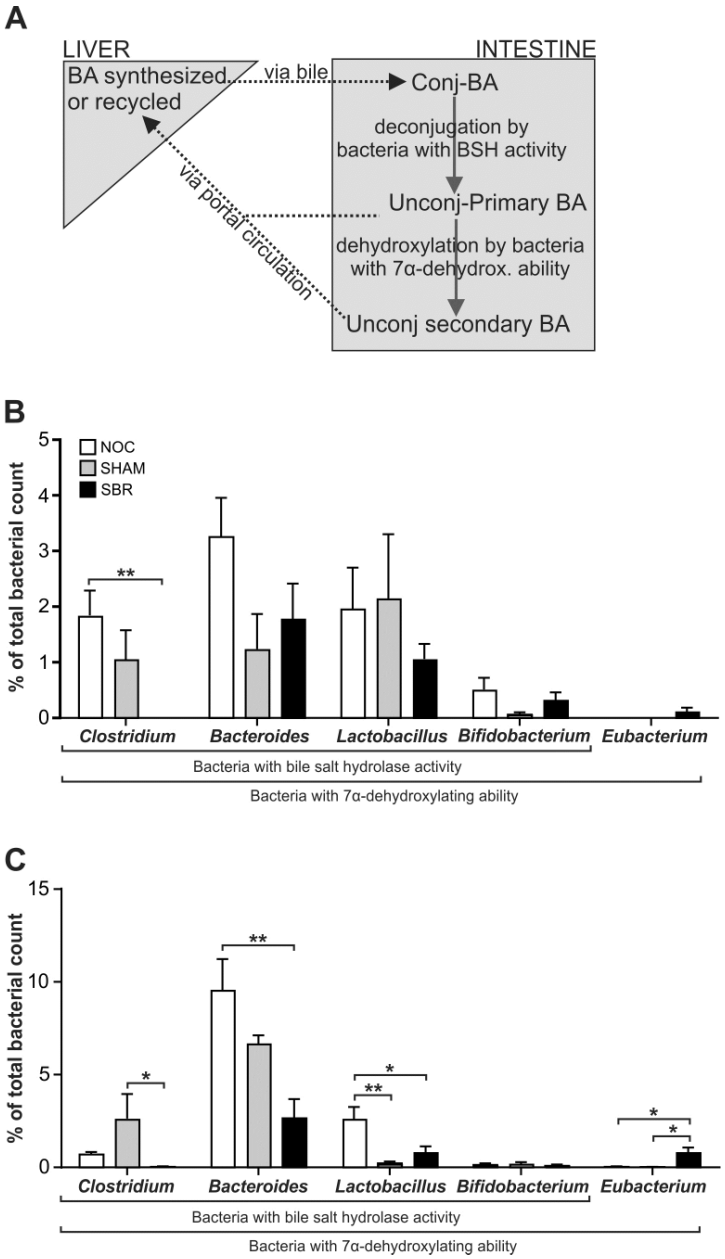

Figure 4 Simplified schematic depicting the microbial biotransformation of bile acids within the small intestine (A). Alterations in the proportion of bile acid bio-transforming bacteria at 2-weeks (B) and 6-weeks (C) post-surgery. $^{*}p<0.05$ and $^{**}p<0.01$

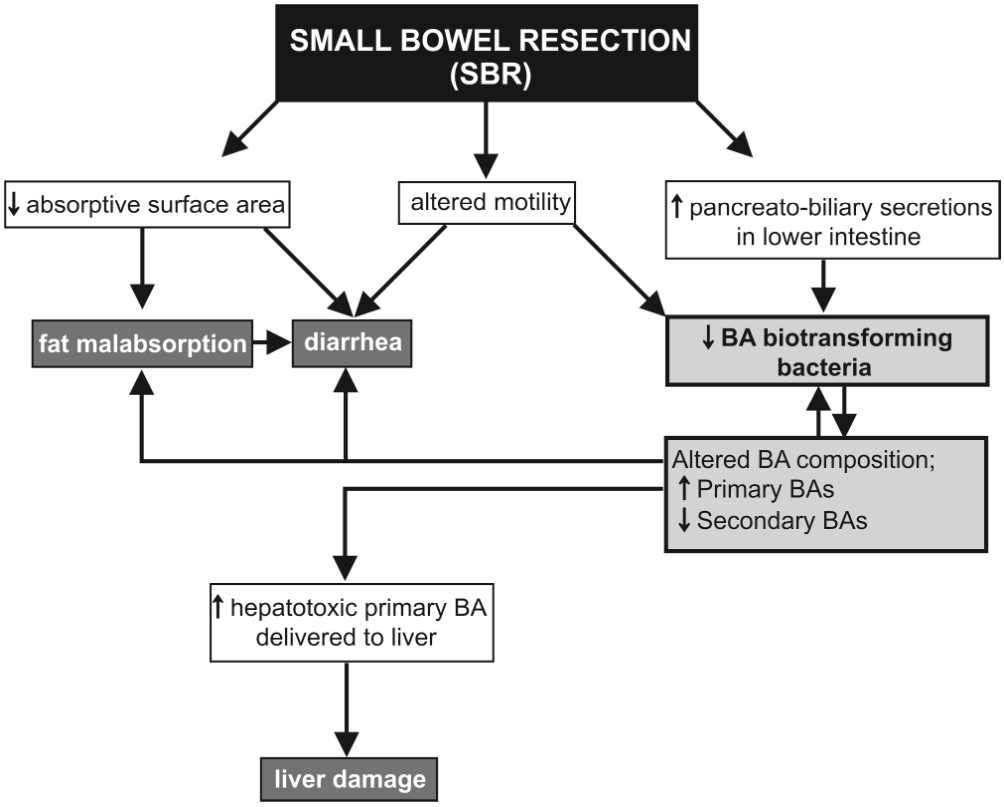

Figure 5 Schematic depicting the potential influence of bile acid dysmetabolism and microbial dysbiosis on important clinical aspects of SBS.

Appendix 3

The influence of minor surgery on the gut microbiota and inflammation: lessons learnt from a paediatric surgery model

(In preparation for submission)

Abstract

Minor abdominal surgery may be required during the infant period due to the development of acute intestinal conditions such as obstruction, perforation or intussusception, however the impact of neonatal abdominal surgery on the microbiota has not previously been studied. Given the previously demonstrated impact of early life bacterial dysbiosis on adult health, the aim of this study was to apply 454-pyrosequencing technology in the assessment of surgery-associated dysbiosis in a piglet model of minor abdominal surgery.

Four-week-old piglets were assigned to the no-surgery or minor abdominal surgery groups. Piglets received a polymeric infant formula diet for six weeks. 454-pyrosequencing was used to determine the microbial composition of colonic content and the gene expression of TNF, IL1B, IL18, IL12, IL8, IL6 and IL10 was determined in ileum, terminal ileum and colon tissues.

Minor surgery was associated with proportional reductions in family members *Enterobacteriaceae, Rhodospirillaceae* and *Bacteroidaceae* and at genus level members of the *Enterobacteriaceae* family, *Thalassospira, Alistipes* and *Bacteroides.* Conversely the proportion of *Prevotellacceae* family and *Prevotella* genus was increased following minor surgery. Furthermore, we observed a site-specific intestinal inflammatory response in the minor surgery group.

We have described microbial dysbiosis within the *Proteobacteria* and *Bacteroidetes* phylum and significant gastrointestinal inflammation following minor surgery performed in a paediatric model. As both the no-surgery control group and minor surgery group received identical antibiotic regimes, we postulate that additional oxygen, introduced during the transection procedure, may have resulted in an aerobic environment that is unable to support these anaerobic species.

Introduction

The gut microbiota is essential to human health, yet the acquisition of this microbial community during infancy remains poorly understood (Fouhy et al., 2012b). At birth, humans are essentially free of bacteria, with colonization of the gastrointestinal tract beginning during the birthing process as the newborn is exposed to maternal and environmental microbes (Tapiainen et al., 2006). The infant microbiota is marked by heterogeneity and instability until approximately 2-4 years of age (Koenig et al., 2011, Palmer et al., 2007), when it becomes more stable, resembling an adult microbiota (Mackie et al., 1999). Colonization of the newborn intestine plays a key role in the development and fine-tuning of the intestinal immune responses (Marques et al., 2010) and disruption of the gut microbiota has been linked to an increasing number of immune-related diseases, including inflammatory bowel disease, necrotizing enterocolitis, eczema, allergies and asthma (Young, 2012).

The development of the microbiota is known to be strongly influenced by early extrinsic factors including mode of infant delivery (Penders et al., 2006), type of feeding (Penders et al., 2005) and antibiotic therapy (Fouhy et al., 2012a). Acute intestinal conditions including intestinal obstruction, perforation and intussusception may necessitate surgical intervention during the neonatal or infant period, however the impact of neonatal abdominal surgery on the microbiota has not previously been studied. Given the previously demonstrated impact of early life bacterial dysbiosis on adult health, characterisation of the microbiota following surgery is vital.

To counteract the confounding variables inherent to human cohort studies, the majority of microbial-based research has been focussed on germ-free rodent models, in which the microbial community is repopulated using microorganisms originating from human faeces (Samuel and Gordon, 2006, Martin et al., 2008, Turnbaugh et al., 2009,

Faith et al., 2010, Goodman et al., 2011, Mcnulty et al., 2011). However, rodent models are limited by the many physiological differences between rodents and primates, and importantly do not typically display the clinical manifestations seen in human enteric diseases (Zhang et al., 2013). Over the last 15 years we have extensively studied gastrointestinal disease using a piglet model of disease (Lapthorne et al., 2013, Healey et al., 2010, Stephens et al., 2010, Pereira-Fantini et al., 2008). The advantage of pigs as an alternative model of human gastrointestinal disease lies in their physiological, (Moughan et al., 1992, Miller and Ullrey, 1987), bacteriological (Buzoianu et al., 2012) and immunological (Butler et al., 2002, Scharek et al., 2005) similarities to humans. In addition, the accelerated ageing rate of pigs when compared with humans, allows us to study the equivalent human timeframe of infancy to childhood within a two week study period.

The impact of minor surgical intervention in infancy on the development of the microbial community and consequent intestinal inflammation is unknown. The primary aim of the present study was to use 454-pyrosequencing technology to assess the impact of minor surgery on the colonic microbial population in a piglet model of minor abdominal surgery. Our secondary aim was to perform a multi-site assessment of molecular alterations in key gut inflammatory markers to assess if surgery-related microbial dysbiosis occurred concurrent with gastrointestinal inflammation.

Materials and Methods

Animals

This study was approved by the Animal Ethics Committee of the Murdoch Childrens Research Institute. Weaned female three-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 (Lapthorne et al., 2013, Healey et al., 2010, Stephens et al., 2010, Pereira-Fantini et al., 2008). The diets were isocaloric and isonitrogenous among the groups and were administered on a per kilogram basis. Water was given twice daily. Piglets were housed separately throughout the study.

Experimental Design

Four week old piglets were randomly allocated to a 'no-surgery control' group (n=6) or a 'surgery' group (n=5). During the surgical procedure, a conventional midline incision of the lower abdominal wall was made and the small intestine was transected at a site 225 cm proximal to the ileocaecal valve, a 2 cm segment was removed and the intestine was re-anastomosed. Both groups received intramuscular amoxicillin (70 mg/kg; CSL Limited) 24 hours pre-surgery, and for three days post-surgery in line with current clinical practise. In addition, both groups received oral rehydration salts (Sanofi-Aventis Australia) for three days post-surgery or equivalent date, with water and the polymeric infant formula diet re-introduced from the third day post-operation.

Sample collection

Animals in the surgical group were sacrificed two weeks post-surgery and at an age-matched time in the 'no-surgery control' group. Ileum tissue was collected 8 cm distal to the anastomosis in the surgical group and

217 cm proximal to the ileocaecal valve in the no-surgery control group. Terminal ileum tissue was collected 7 cm proximal to the ileocaecal valve and colon tissue was collected 3 cm distal to the caecum in both groups (Pereira-Fantini et al., 2011). Samples from each site were snap frozen in liquid nitrogen. Colonic content was collected from the proximal colon.

High-throughput sequencing

The 16S rRNA amplicons from colonic content were generated using a previously outlined approach (Murphy et al., 2010). Amplicons were generated using one forward primer and a combination of four reverse primers, as described previously (Murphy et al., 2010). 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 gene was sequenced at the Teagasc 454-Sequencing facility on a Genome Sequencer FLX platform (Roche Diagnostics Ltd.).

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 (Tsai et al.,

1995). A phylogenetic tree was generated using the FastTree package (Price et al., 2010).

Real-time reverse transcription PCR (qRT-PCR)

The muscle layer was stripped from the ileum, terminal ileum and colon tissue leaving the mucosa, which comprised the epithelium and the lamina propria. Total RNA was extracted from 100 mg of intestinal mucosa using TRIzol (Invitrogen). Complementary DNA (cDNA) was synthesised with the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science). PCR primers were designed against pig gene sequences using Roche Universal Probe Library Assay Design Centre (Roche Applied Science). Primer sequences and probe combinations are listed in Table 1. 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 in the surgical group relative to the non-surgical control group, using RPL32 as a housekeeping gene.

Statistical analysis

Sequencing analysis was completed using Minitab Release 15.1.1.0 (Minitab Inc 2007). Parametric unpaired t-test was employed to identify significant differences in the percentage of assignable reads at phylum, family and genus levels between non-surgery control and minor surgery groups (GraphPad Prism Software 6.0). Parametric unpaired t-tests were also employed to test for statistical significance in the relative gene expression of inflammation markers between non-surgery control and minor surgery groups. Statistical significance for all testing was accepted at $p < 0.05$.

Results

Microbial composition is significantly altered following minor surgery

High-throughput DNA sequencing was used to detect disturbances in microbial composition following minor surgery. Diversity and richness estimates were calculated for the non-surgery and minor surgery groups. The Chao 1 calculation is an estimator of phylotype richness in a dataset and the Simpson and Shannon index of diversity reflects both the richness and the community evenness (i.e. proportional phylotype abundance). As seen in Table 2, the overall alpha diversity of the colonic microbiota was unchanged following minor surgery as calculated by the Chao 1 richness estimate, Shannon's index for diversity, and Simpson's diversity index. Furthermore, there was no difference in the number of observed species.

16S rRNA sequence data obtained from colonic content samples was analysed to determine if minor abdominal surgery was associated with changes in the proportion of assignable reads at the phylum, family or genus level. There was no significant difference in composition at a phylum level between the no-surgery group and the minor surgery group (Figure 1). As is indicated by the colour distribution detailed in Figure 2, whilst the composition of families positioned within the *Firmicutes* (red shades), *Actinobacteria* (green shades) and *Fusobacteria* (yellow) remained unchanged following minor surgery, significant alterations in the proportion of *Proteobacteria* family members (blue shades) and *Bacteroidetes* family members (orange shades) were observed. These family level compositional shifts included a reduction in the proportion of *Enterobacteriaceae* (21.6±5.1 vs. 1.9±0.7, $p = 0.007$), and *Bacteroidaceae* (2.4±0.6 vs. 0.8±0.4, $p = 0.051$) and an increase in the proportion of *Prevotellaceae* (2.6±0.3 vs. 7.2±2.2, $p = 0.044$) in the minor surgery group when compared with the no-surgery control group (Figure

2). Of particular note, although *Rhodaspirillaceae* was detectable in 5/6 control samples, it was not detected amongst any of the minor-surgery samples. These changes were mirrored at the genus level with minor surgery associated with unchanged proportions of genera assigned to *Firmicutes, Actinobacteria* or *Fusobacteria* phyla and changes in genera assigned to the *Proteobacteria* and *Bacteroidetes* phyla (Figure 3). Specifically, minor surgery resulted in decreases in the proportion of assignable reads assigned to members of the *Enterobacteriaceae* family (21.5±5.1 vs. 1.9±0.7, p = 0.007), *Thalassospira* (0.4±0.1 vs. 0.0±0.0, p = 0.033), and *Bacteroides* (2.4±0.5 vs. 0.8±0.4, p = 0.051) and an increase in *Prevotella* (2.6±0.3 vs. 7.2±2.2, p = 0.045) compared to no surgery controls. Furthermore, whilst *Alistipes* was detected in all control samples, it was not detectable in the minor surgery samples.

A tissue-specific pattern of inflammation occurs following minor surgery

Disturbances of the gut microbial community are closely linked to inflammation, therefore given the detected bacterial dysbiosis in the surgery model we next examined changes in the mucosal gene expression of key inflammatory cytokines within the ileum, terminal ileum and colon. Within the ileum, the only detectable change was a decrease in interleukin 6 (IL6) gene expression following minor surgery (p = 0.038; Figure 4A). Amongst the pro-inflammatory cytokines expressed in the terminal ileum, gene expression of tumour necrosis factor (TNF) was decreased (p = 0.007), whilst interleukin 18 (IL18) and interleukin 12 (IL12) gene expression was increased following surgery (p = 0.012 and p = 0.043 respectively; Figure 4B). Gene expression of the anti-inflammatory cytokine interleukin 10 (IL10) was increased in terminal ileum samples from the minor surgery group (p = 0.005). Similar to the terminal ileum IL12 and IL10 gene expression was increased within the colon following surgery (p = 0.006 and p = 0.000 respectively; Figure 4C).

Discussion

Immediately after birth, the newborn gut environment is colonized by facultative anaerobic bacteria such as *Enterobacteriaceae* and *Streptococcaceae* (Marques et al., 2010). These bacteria gradually consume the oxygen in the intestine and produce new metabolites, preparing the intestinal environment for the establishment of a strict anaerobic population dominated by *Bifidobacterium, Clostridium* and *Bacteroides* sp., which may play a role in neonatal gut maturation (Marques et al., 2010). In the current study, we have utilized culture-independent high-throughput sequencing techniques to investigate the effect of abdominal surgery on the infant gut microbiota using a piglet transection model. The main finding of our studies was an altered establishment of the normal anaerobic gut microbiota, as indicated at a family level by surgery-related reductions in the proportion of *Enterobacteriaceae, Rhodaspirillaceae* and *Bacteroidaceae* and at a genus level by a proportional reduction in members of the *Enterobacteriaceae family, Thalassospira, Alistipes* and *Bacteroides.* Conversely, we also detected increases in the proportion of the *Prevotellacceae* family and *Prevotella* genus within the minor surgery group when compared to no-surgery controls.

Clinically, the reduction in the proportion of reads assigned to *Bacteroides* and *Alistipes* and the increase in the proportion of *Prevotella,* is highly significant. A similar microbial signature is also associated with HIV-1 infection (Lozupone et al., 2013). In early life, the intestinal microbiota of healthy infants displays a large abundance of *Bacteroides* (Adlerberth and Wold, 2009) and dominance of *Bacteroides* has been shown to be protective against allergy in a gnotobiotic mouse model of cow's milk allergy (Rodriguez et al., 2012). Our report of increased abundance of *Prevotellaceae* and *Prevotella* sp. is particularly disturbing in terms of future disease outcome. A number of studies have provided evidence that *Prevotella* is capable of acting as a pathobiont, a potentially

“pathogenic symbiont” within the microbiota (Lozupone et al., 2013). Whilst *Prevotella* is scarcely represented in healthy microbiomes, increased abundance of *Prevotella* is observed in the faecal microbiota of children with diarrhoea-dominant irritable bowel disease (Rigsbee et al., 2012), obesity and non-alcoholic steatohepatitis (Zhu et al., 2013) and in the salivary microbiota of inflammatory bowel disease patients (Said et al., 2013, Lucke et al., 2006) and those with periodontal disease (Kumar et al., 2003).

A second major observation of the current studies was a site-specific intestinal inflammatory response, initiated following minor surgery. Within the ileum, gene expression of the cytokine IL6 was undetectable in surgical samples, whilst within the terminal ileum gene expression of IL18, IL12 and IL10 was increased. In the colon, only IL12 and IL10 gene expression were raised. The site-specific nature of our results are in accordance with germ-free mice studies in which recolonization with an infant gut microbiota stimulated a T-cell response in the colon, but not in the ileum, at two weeks post-transplantation (Rodriguez et al., 2012). Colonization of the newborn intestine plays a key role in the development and fine-tuning of the intestinal immune responses (Marques et al., 2010). Disruption to this process, as occurs following surgery, may have long-term health consequences, giving rise to immune-related disorders such as eczema, allergic rhinitis and inflammatory bowel disease (Marques et al., 2010). *Bacteroides*, which were depleted following minor surgery, are believed to play a crucial role in the development of gastrointestinal-associated lymphoid tissues and the modulation of T-helper Th1/Th2/T-regulatory balance (Rodriguez et al., 2012). Whilst increased abundance of *Prevotella* or *Prevotellacceae,* as observed following minor surgery, is associated with increased numbers of colonic CD4 and CD8 T cells in HIV-1 disease and exacerbation of colitis in an inflammasone knockout mouse model (Dillon et al., 2014).

In conclusion, the enteric microbiota plays an important role in host health, being involved in nutritional, immunological and physiological functions. Early colonization of the infant gut is undoubtedly an important factor for the overall health of the infant and may also have effects on the health status in later life. To our knowledge this is the first study examining surgical-related microbial changes in infancy and provides valuable new information for consideration. We have described microbial dysbiosis within the *Proteobacteria* and *Bacteroidetes* phyla and significant gastrointestinal inflammation following minor surgery performed in a paediatric model. As both the no-surgery control group and minor surgery group received identical antibiotic regimes, we postulate that additional oxygen, introduced during the transection procedure may have resulted in an aerobic environment that is unable to support these anaerobic species. Further studies assessing the impact of post-surgical microbial changes on the development of childhood-associated immune diseases are imperative.

References

Adlerberth, I. & Wold, A. E. 2009. Establishment of the gut microbiota in Western infants. *Acta Paediatr,* 98**,** 229-38.

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research,* 25**,** 3389-3402.

Butler, J. E., Weber, P., Sinkora, M., Baker, D., Schoenherr, A., Mayer, B. & Francis, D. 2002. Antibody repertoire development in fetal and neonatal piglets. VIII. Colonization is required for newborn piglets to make serum antibodies to T-dependent and type 2 T-independent antigens. *J Immunol,* 169**,** 6822-30.

Buzoianu, S. G., Walsh, M. C., Rea, M. C., O'sullivan, O., Cotter, P. D., Ross, R. P., Gardiner, G. E. & Lawlor, P. G. 2012. High-throughput sequence-based analysis of the intestinal microbiota of weanling pigs fed genetically modified MON810 maize expressing Bacillus thuringiensis Cry1Ab (Bt maize) for 31 days. *Appl Environ Microbiol,* 78**,** 4217-24.

Dillon, S. M., Lee, E. J., Kotter, C. V., Austin, G. L., Dong, Z., Hecht, D. K., Gianella, S., Siewe, B., Smith, D. M., Landay, A. L., Robertson, C. E., Frank, D. N. & Wilson, C. C. 2014. An altered intestinal mucosal microbiome in HIV-1 infection is associated with mucosal and systemic immune activation and endotoxemia. *Mucosal Immunology*.

Faith, J. J., Rey, F. E., O'donnell, D., Karlsson, M., Mcnulty, N. P., Kallstrom, G., Goodman, A. L. & Gordon, J. I. 2010. Creating and characterizing communities of human gut microbes in gnotobiotic mice. *ISME J,* 4**,** 1094-8.

Fouhy, F., Guinane, C. M., Hussey, S., Wall, R., Ryan, C. A., Dempsey, E. M., Murphy, B., Ross, R. P., Fitzgerald, G. F. & Stanton, C. 2012a. High-throughput sequencing reveals the incomplete, short-

term, recovery of the infant gut microbiota following parenteral antibiotic treatment with ampicillin and gentamycin. *Antimicrobial agents and chemotherapy,* 56**,** 5811-5820.

Fouhy, F., Ross, R. P., Fitzgerald, G. F., Stanton, C. & Cotter, P. D. 2012b. Composition of the early intestinal microbiota: knowledge, knowledge gaps and the use of high-throughput sequencing to address these gaps. *Gut Microbes,* 3**,** 203-20.

Goodman, A. L., Kallstrom, G., Faith, J. J., Reyes, A., Moore, A., Dantas, G. & Gordon, J. I. 2011. Extensive personal human gut microbiota culture collections characterized and manipulated in gnotobiotic mice. *Proc Natl Acad Sci U S A,* 108**,** 6252-7.

Healey, K. L., Bines, J. E., Thomas, S. L., Wilson, G., Taylor, R. G., Sourial, M. & Pereira-Fantini, P. M. 2010. Morphological and functional changes in the colon after massive small bowel resection. *J Pediatr Surg,* 45**,** 1581-90.

Huson, D. H., Auch, A. F., Qi, J. & Schuster, S. C. 2007. MEGAN analysis of metagenomic data. *Genome Research,* 17**,** 377-386.

Koenig, J. E., Spor, A., Scalfone, N., Fricker, A. D., Stombaugh, J., Knight, R., Angenent, L. T. & Ley, R. E. 2011. Succession of microbial consortia in the developing infant gut microbiome. *Proc Natl Acad Sci U S A,* 108 Suppl 1**,** 4578-85.

Kumar, P. S., Griffen, A. L., Barton, J. A., Paster, B. J., Moeschberger, M. L. & Leys, E. J. 2003. New bacterial species associated with chronic periodontitis. *Journal of Dental Research,* 82**,** 338-44.

Lapthorne, S., Pereira-Fantini, P. M., Fouhy, F., Wilson, G., Thomas, S. L., Dellios, N. L., Scurr, M., O'sullivan, O., Ross, R. P., Stanton, C., Fitzgerald, G. F., Cotter, P. D. & Bines, J. E. 2013. Gut microbial diversity is reduced and is associated with colonic inflammation in a piglet model of short bowel syndrome. *Gut Microbes,* 4**,** 212-21.

Livak, K. J. & Schmittgen, T. D. 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. *Methods,* 25**,** 402-408.

Lozupone, C. A., Li, M., Campbell, T. B., Flores, S. C., Linderman, D., Gebert, M. J., Knight, R., Fontenot, A. P. & Palmer, B. E. 2013. Alterations in the gut microbiota associated with HIV-1 infection. *Cell Host and Microbe,* 14**,** 329-39.

Lucke, K., Miehlke, S., Jacobs, E. & Schuppler, M. 2006. Prevalence of Bacteroides and Prevotella spp. in ulcerative colitis. *Journal of Medical Microbiology,* 55**,** 617-24.

Mackie, R. I., Sghir, A. & Gaskins, H. R. 1999. Developmental microbial ecology of the neonatal gastrointestinal tract. *Am J Clin Nutr,* 69**,** 1035S-1045S.

Marques, T. M., Wall, R., Ross, R. P., Fitzgerald, G. F., Ryan, C. A. & Stanton, C. 2010. Programming infant gut microbiota: influence of dietary and environmental factors. *Curr Opin Biotechnol,* 21**,** 149-56.

Martin, F. P., Wang, Y., Sprenger, N., Yap, I. K., Lundstedt, T., Lek, P., Rezzi, S., Ramadan, Z., Van Bladeren, P., Fay, L. B., Kochhar, S., Lindon, J. C., Holmes, E. & Nicholson, J. K. 2008. Probiotic modulation of symbiotic gut microbial-host metabolic interactions in a humanized microbiome mouse model. *Mol Syst Biol,* 4**,** 157.

Mcnulty, N. P., Yatsunenko, T., Hsiao, A., Faith, J. J., Muegge, B. D., Goodman, A. L., Henrissat, B., Oozeer, R., Cools-Portier, S., Gobert, G., Chervaux, C., Knights, D., Lozupone, C. A., Knight, R., Duncan, A. E., Bain, J. R., Muehlbauer, M. J., Newgard, C. B., Heath, A. C. & Gordon, J. I. 2011. The impact of a consortium of fermented milk strains on the gut microbiome of gnotobiotic mice and monozygotic twins. *Sci Transl Med,* 3**,** 106ra106.

Miller, E. R. & Ullrey, D. E. 1987. The pig as a model for human nutrition. *Annu Rev Nutr,* 7**,** 361-82.

Moughan, P. J., Birtles, M. J., Cranwell, P. D., Smith, W. C. & Pedraza, M. 1992. The piglet as a model animal for studying aspects of digestion and absorption in milk-fed human infants. *World Rev Nutr Diet,* 67**,** 40-113.

Murphy, E. F., Cotter, P. D., Healy, S., Marques, T. M., O'sullivan, O., Fouhy, F., Clarke, S. F., O'toole, P. W., Quigley, E. M., Stanton, C., Ross, P. R., O'doherty, R. M. & Shanahan, F. 2010. Composition and energy harvesting capacity of the gut microbiota: relationship to diet, obesity and time in mouse models. *Gut,* 59**,** 1635-1642.

Palmer, C., Bik, E. M., Digiulio, D. B., Relman, D. A. & Brown, P. O. 2007. Development of the human infant intestinal microbiota. *PLoS Biol,* 5**,** e177.

Penders, J., Thijs, C., Vink, C., Stelma, F. F., Snijders, B., Kummeling, I., Van Den Brandt, P. A. & Stobberingh, E. E. 2006. Factors influencing the composition of the intestinal microbiota in early infancy. *Pediatrics,* 118**,** 511-21.

Penders, J., Vink, C., Driessen, C., London, N., Thijs, C. & Stobberingh, E. E. 2005. Quantification of Bifidobacterium spp., Escherichia coli and Clostridium difficile in faecal samples of breast-fed and formula-fed infants by real-time PCR. *FEMS Microbiol Lett,* 243**,** 141-7.

Pereira-Fantini, P., Thomas, S., Wilson, G., Taylor, R., Sourial, M. & Bines, J. 2011. Short- and long-term effects of small bowel resection: a unique histological study in a piglet model of short bowel syndrome. *Histochemistry and Cell Biology,* 135**,** 195-202.

Pereira-Fantini, P. M., Thomas, S. L., Taylor, R. G., Nagy, E., Sourial, M., Fuller, P. J. & Bines, J. E. 2008. Colostrum supplementation restores insulin-like growth factor -1 levels and alters muscle morphology following massive small bowel resection. *JPEN J Parenter Enteral Nutr,* 32**,** 266-75.

Price, M. N., Dehal, P. S. & Arkin, A. P. 2010. FastTree 2 – Approximately Maximum-Likelihood Trees for Large Alignments. *PLoS ONE,* 5**,** e9490.

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

quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Research,* 35**,** 7188-7196.

Rigsbee, L., Agans, R., Shankar, V., Kenche, H., Khamis, H. J., Michail, S. & Paliy, O. 2012. Quantitative profiling of gut microbiota of children with diarrhea-predominant irritable bowel syndrome. *American Journal of Gastroenterology,* 107**,** 1740-51.

Rodriguez, B., Prioult, G., Hacini-Rachinel, F., Moine, D., Bruttin, A., Ngom-Bru, C., Labellie, C., Nicolis, I., Berger, B., Mercenier, A., Butel, M. J. & Waligora-Dupriet, A. J. 2012. Infant gut microbiota is protective against cow's milk allergy in mice despite immature ileal T-cell response. *FEMS Microbiol Ecol,* 79**,** 192-202.

Said, H. S., Suda, W., Nakagome, S., Chinen, H., Oshima, K., Kim, S., Kimura, R., Iraha, A., Ishida, H., Fujita, J., Mano, S., Morita, H., Dohi, T., Oota, H. & Hattori, M. 2013. Dysbiosis of Salivary Microbiota in Inflammatory Bowel Disease and Its Association With Oral Immunological Biomarkers. *DNA Research*.

Samuel, B. S. & Gordon, J. I. 2006. A humanized gnotobiotic mouse model of host-archaeal-bacterial mutualism. *Proc Natl Acad Sci U S A,* 103**,** 10011-6.

Scharek, L., Guth, J., Reiter, K., Weyrauch, K. D., Taras, D., Schwerk, P., Schierack, P., Schmidt, M. F., Wieler, L. H. & Tedin, K. 2005. Influence of a probiotic Enterococcus faecium strain on development of the immune system of sows and piglets. *Vet Immunol Immunopathol,* 105**,** 151-61.

Stephens, A. N., Pereira-Fantini, P. M., Wilson, G., Taylor, R. G., Rainczuk, A., Meehan, K. L., Sourial, M., Fuller, P. J., Stanton, P. G., Robertson, D. M. & Bines, J. E. 2010. Proteomic analysis of the intestinal adaptation response reveals altered expression of fatty acid binding proteins following massive small bowel resection. *J Proteome Res,* 9**,** 1437-49.

Tapiainen, T., Ylitalo, S., Eerola, E. & Uhari, M. 2006. Dynamics of gut colonization and source of intestinal flora in healthy newborn infants. *APMIS,* 114**,** 812-7.

Tsai, H. H., Dwarakanath, A. D., Hart, C. A., Milton, J. D. & Rhodes, J. M. 1995. Increased faecal mucin sulphatase activity in ulcerative colitis: a potential target for treatment. *Gut,* 36**,** 570-576.

Turnbaugh, P. J., Ridaura, V. K., Faith, J. J., Rey, F. E., Knight, R. & Gordon, J. I. 2009. The effect of diet on the human gut microbiome: a metagenomic analysis in humanized gnotobiotic mice. *Sci Transl Med,* 1**,** 6ra14.

Young, V. B. 2012. The intestinal microbiota in health and disease. *Curr Opin Gastroenterol,* 28**,** 63-9.

Zhang, Q., Widmer, G. & Tzipori, S. 2013. A pig model of the human gastrointestinal tract. *Gut Microbes,* 4**,** 193-200.

Zhu, L., Baker, S. S., Gill, C., Liu, W., Alkhouri, R., Baker, R. D. & Gill, S. R. 2013. Characterization of gut microbiomes in nonalcoholic steatohepatitis (NASH) patients: a connection between endogenous alcohol and NASH. *Hepatology,* 57**,** 601-9.

Table 1 List of primer sequences and Universal Probe Library combinations used in this study

Primer	Sequence 5’ to 3’	UPL Probe
RPL32 Forward	aactggccatcagggtcac	#64
RPL32 Reverse	cacaactggaactcctgtctattc	
TNF Forward	ttgtcgctacatcgctgaac	#32
TNF Reverse	ccagtagggcggttacagac	
IL1β Forward	ccaattcagggaccctacc	#19
IL1β Reverse	catggctgcttcagaaacct	
IL18 Forward	actttactttgtagctgaaaacgatg	#85
IL18 Reverse	tttaggttcaagcttgccaaa	
IL12 Forward	gagggtgagtgagtgccttg	#62
IL12 Reverse	actccgcctaggttcgactt	
IL8 Forward	ttcttctttatccccaaactgg	#41
IL8 Reverse	ccacatgtcctcaaggtagga	
IL6 Forward	tgaactccctctccacaagc	#7
IL6 Reverse	ggcagtagccatcaccaga	
IL10 Forward	tccagttttacctggaagacg	#8
IL10 Reverse	ccttgatatcctccccatca	

Table 2 Estimation of diversity within non-surgery control and minor surgery groups

Data set	**No surgery control**	**Minor surgery**
Chao 1 richness estimation	905±41	892±123
Shannon's index for diversity	6.9±0.2	6.5±0.6
Simpson's diversity index	0.96±0.01	0.95±0.02
Number of observed species	552±16	571±80

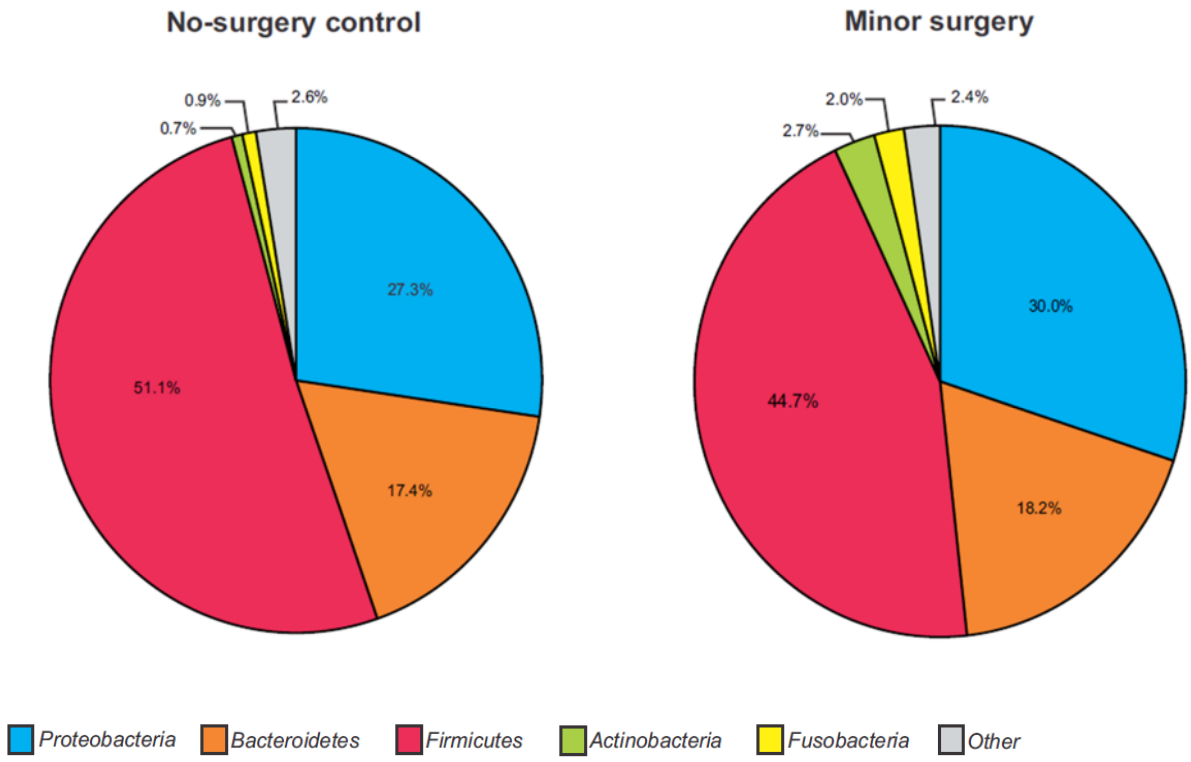

Figure 1 Microbial distributions at the phylum level in piglets which had undergone no-surgery or minor abdominal surgery. Percentages are based on the proportion of assignable reads

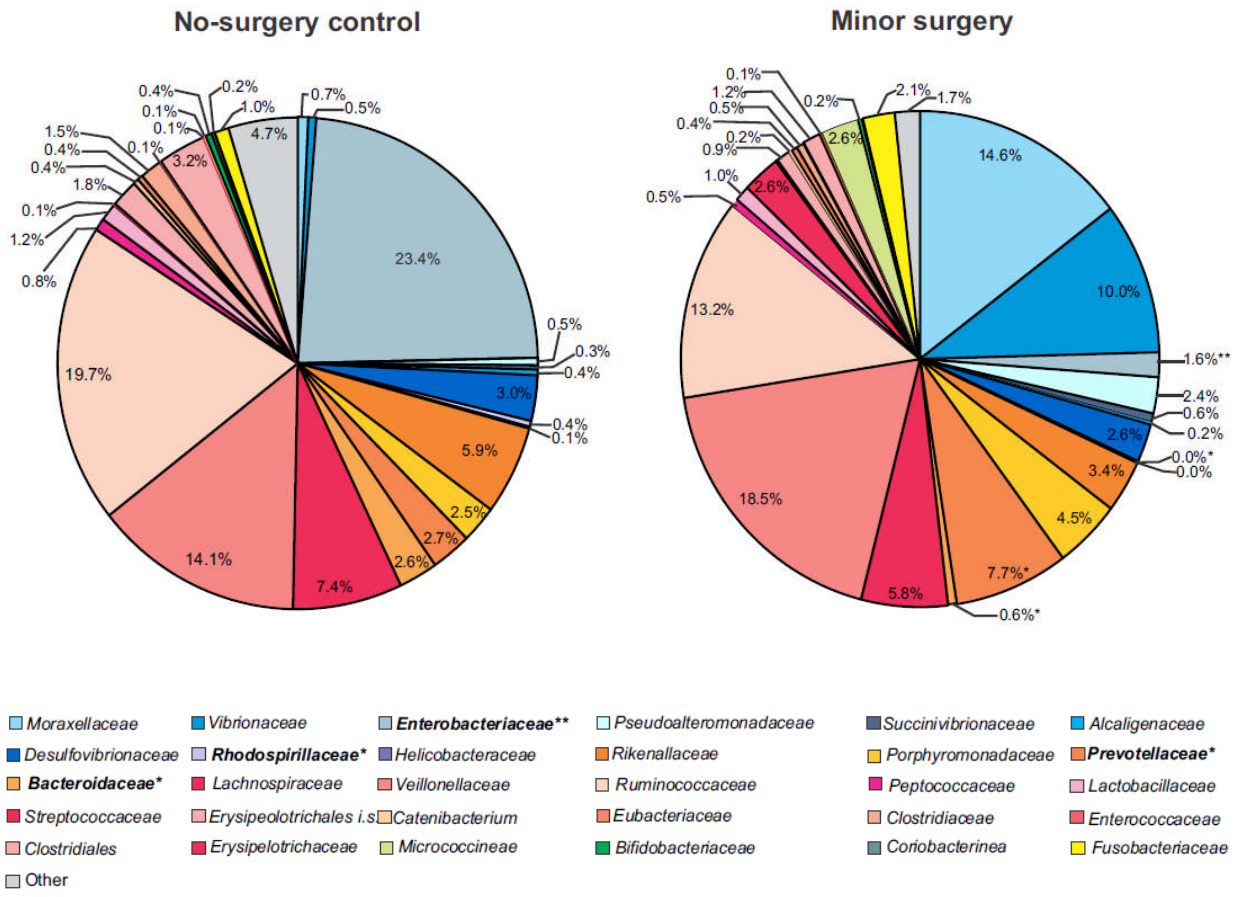

Figure 2 Microbial distributions at the family level in piglets which had undergone no-surgery or minor abdominal surgery. Statistically significant differences between the no-surgery control group and minor surgery group are indicated by asterisks. *P <0.05, **P < 0.01. Percentages are based on the proportion of assignable reads

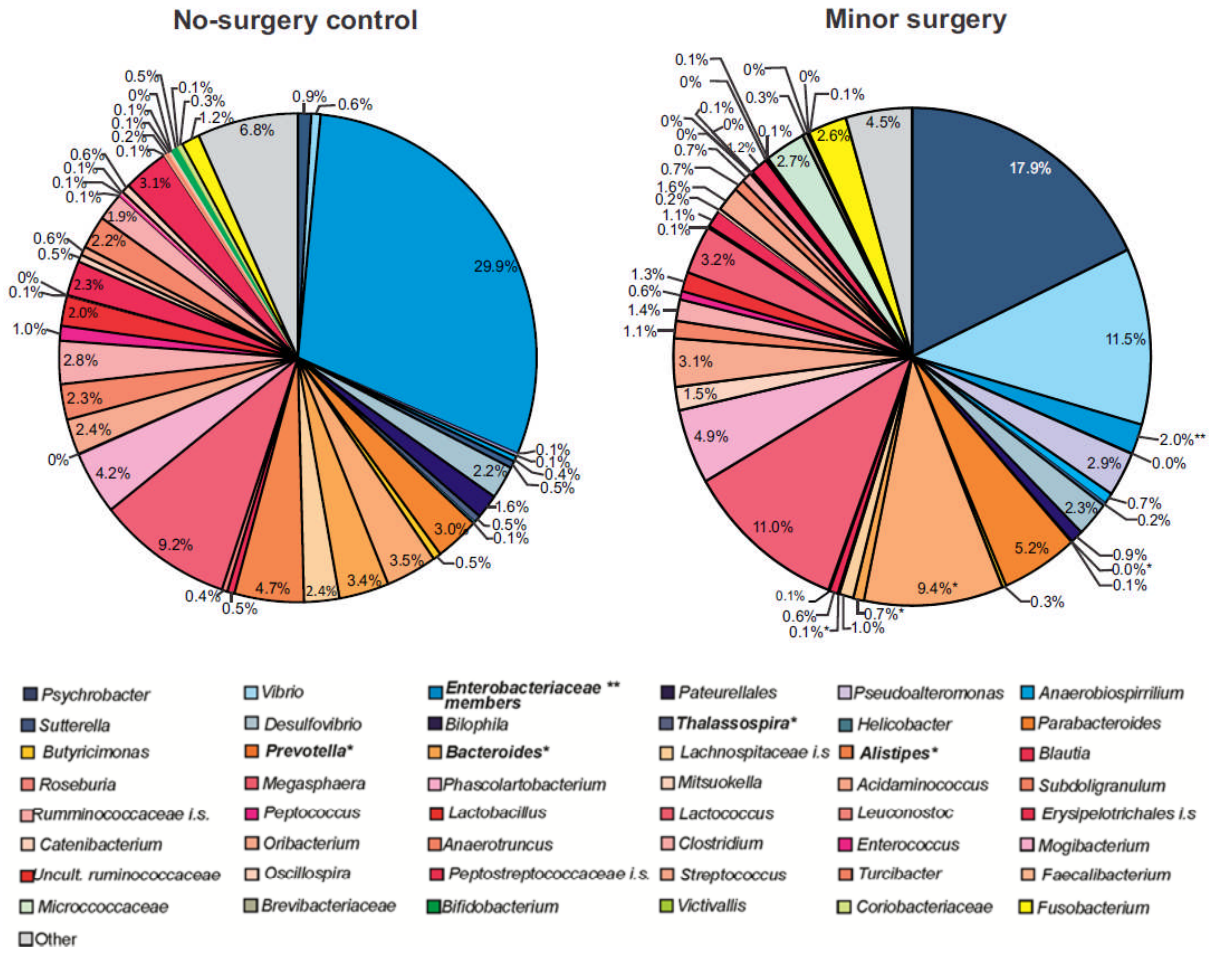

Figure 3 Microbial distributions at the genus level in piglets which had undergone no-surgery or minor abdominal surgery. Statistically significant differences between the non-surgery control group and minor surgery group are indicated by asterisks. *P <0.05, **P < 0.01. Percentages are based on the proportion of assignable reads

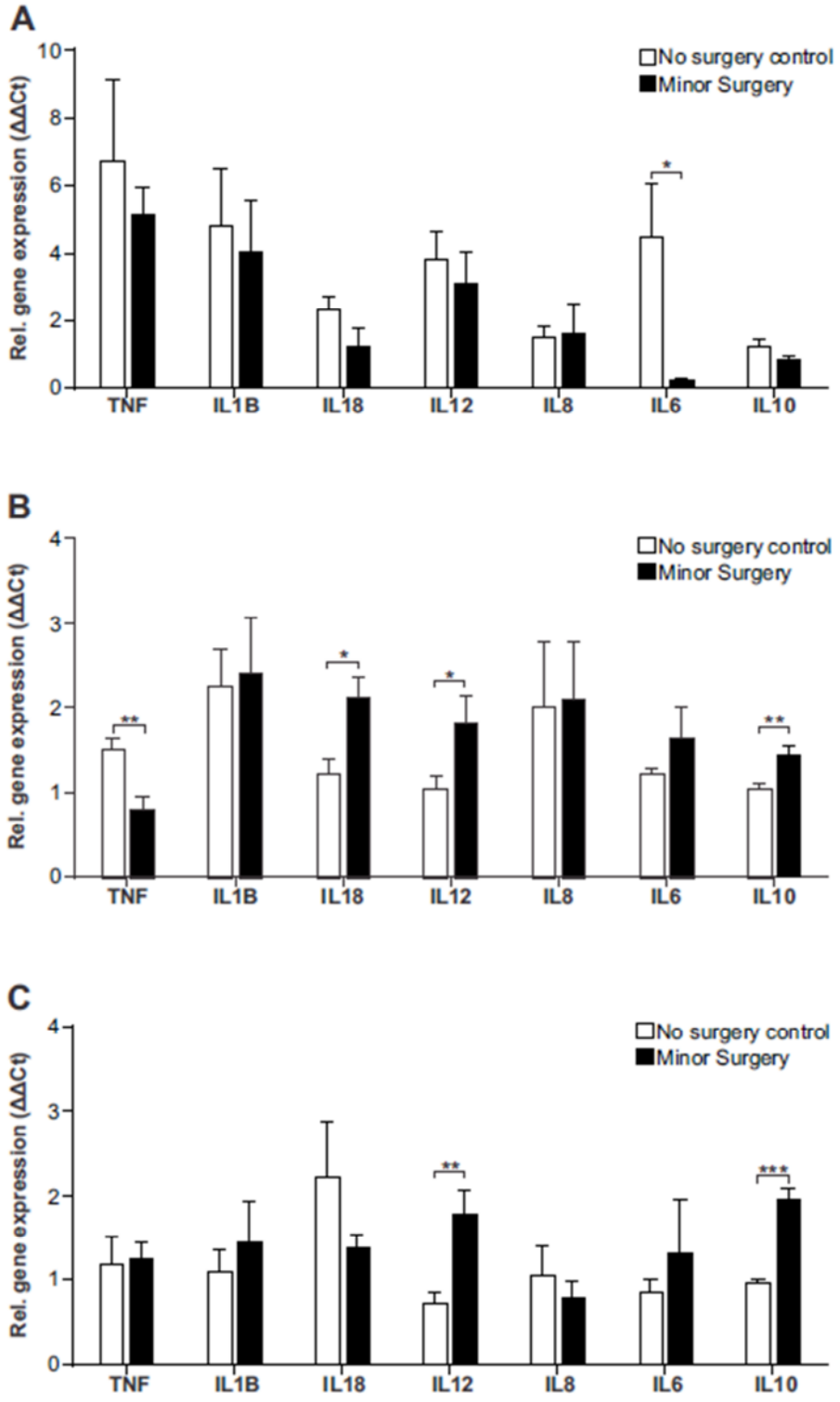

Figure 4 Relative gene expression of pro- and anti-inflammatory cytokines in (A) ileum, (B) terminal ileum and (C) colon from no-surgery control and minor surgery groups. Mean ± SEM. *P < 0.05, **P < 0.01

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 *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 *Proteobacteria*, at the expense of members of the *Actinobacteria* (most notably *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 *bla*$_{CTX-M}$, 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-*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.

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 β-lactams. Existing culture-based research suggested that bifidobacteria are sensitive to β-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 *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

Ajslev, T., Andersen, C., Gamborg, M., Sørensen, T. & Jess, T. 2011. Childhood overweight after establishment of the gut microbiota: the role of delivery mode, pre-pregnancy weight and early administration of antibiotics. *International Journal of Obesity,* 35**,** 522-529.

Antonopoulos, D. A., Huse, S. M., Morrison, H. G., Schmidt, T. M., Sogin, M. L. & Young, V. B. 2009. Reproducible community dynamics of the gastrointestinal microbiota following antibiotic perturbation. *Infection and immunity,* 77**,** 2367-2375.

Bennet, R., Eriksson, M. & Nord, C. E. 2002. The fecal microflora of 1–3-month-old infants during treatment with eight oral antibiotics. *Infection,* 30**,** 158-160.

Bolin, T. D., Wong, S., Crouch, R., Engelman, J. L. & Riordan, S. M. 2009. Appendicectomy as a therapy for ulcerative proctitis. *The American journal of gastroenterology,* 104**,** 2476-2482.

Bryan, L., Kowand, S. & Van Den Elzen, H. 1979. Mechanism of aminoglycoside antibiotic resistance in anaerobic bacteria: Clostridium perfringens and Bacteroides fragilis. *Antimicrobial agents and chemotherapy,* 15**,** 7-13.

Cheng, G., Hu, Y., Yin, Y., Yang, X., Xiang, C., Wang, B., Chen, Y., Yang, F., Lei, F. & Wu, N. 2012. Functional screening of antibiotic resistance genes from human gut microbiota reveals a novel gene fusion. *FEMS microbiology letters,* 336**,** 11-16.

D'aimmo, M. R., Modesto, M. & Biavati, B. 2007. Antibiotic resistance of lactic acid bacteria and Bifidobacterium spp. isolated from dairy and pharmaceutical products. *International journal of food microbiology,* 115**,** 35-42.

De La Cochetiere, M., Durand, T., Lepage, P., Bourreille, A., Galmiche, J. & Dore, J. 2005. Resilience of the dominant human fecal

microbiota upon short-course antibiotic challenge. *Journal of clinical microbiology,* 43**,** 5588-5592.

De Vries, L. E., Vallès, Y., Agersø, Y., Vaishampayan, P. A., García-Montaner, A., Kuehl, J. V., Christensen, H., Barlow, M. & Francino, M. P. 2011. The gut as reservoir of antibiotic resistance: microbial diversity of tetracycline resistance in mother and infant. *PLoS ONE,* 6**,** e21644.

Favier, C. F., De Vos, W. M. & Akkermans, A. D. L. 2003. Development of bacterial and bifidobacterial communities in feces of newborn babies. *Anaerobe,* 9**,** 219-229.

Foliaki, S., Pearce, N., Björkstén, B., Mallol, J., Montefort, S. & Von Mutius, E. 2009. Antibiotic use in infancy and symptoms of asthma, rhinoconjunctivitis, and eczema in children 6 and 7 years old: International Study of Asthma and Allergies in Childhood Phase III. *Journal of allergy and clinical immunology,* 124**,** 982-989.

Hussey, S., Wall, R., Gruffman, E., O'sullivan, L., Ryan, C. A., Murphy, B., Fitzgerald, G., Stanton, C. & Ross, R. P. 2011. Parenteral Antibiotics Reduce Bifidobacteria Colonization and Diversity in Neonates. *International journal of microbiology,* 2011**,** doi:10.1155/2011/130574.

Joly, F., Mayeur, C., Bruneau, A., Noordine, M.-L., Meylheuc, T., Langella, P., Messing, B., Duée, P.-H., Cherbuy, C. & Thomas, M. 2010. Drastic changes in fecal and mucosa-associated microbiota in adult patients with short bowel syndrome. *Biochimie,* 92**,** 753-761.

Kaneko, T., Bando, Y., Kurihara, H., Satomi, K., Nonoyama, K. & Matsuura, N. 1997. Fecal microflora in a patient with short-bowel syndrome and identification of dominant lactobacilli. *Journal of clinical microbiology,* 35**,** 3181-3185.

Kim, B. J., Kim, H. Y., Yun, Y. J. & Kook, Y. H. 2010. Differentiation of Bifidobacterium species using partial RNA polymerase {beta}-

subunit (rpoB) gene sequences. *International journal of systematic and evolutionary microbiology,* 60**,** 2697-2704.

Mangin, I., Suau, A., Gotteland, M., Brunser, O. & Pochart, P. 2010. Amoxicillin treatment modifies the composition of Bifidobacterium species in infant intestinal microbiota. *Anaerobe,* 16**,** 433-438.

Marra, F., Marra, C. A., Richardson, K., Lynd, L. D., Kozyrskyj, A., Patrick, D. M., Bowie, W. R. & Fitzgerald, J. M. 2009. Antibiotic use in children is associated with increased risk of asthma. *Pediatrics,* 123**,** 1003-1010.

Martiny, A. C., Martiny, J. B., Weihe, C., Field, A. & Ellis, J. C. 2011. Functional metagenomics reveals previously unrecognized diversity of antibiotic resistance genes in gulls. *Frontiers in microbiology,* 2**,** 238. doi: 10.3389/fmicb.2011.00238.

Masco, L., Van Hoorde, K., De Brandt, E., Swings, J. & Huys, G. 2006. Antimicrobial susceptibility of Bifidobacterium strains from humans, animals and probiotic products. *Journal of antimicrobial chemotherapy,* 58**,** 85-94.

Mazé, A., O'connell-Motherway, M., Fitzgerald, G. F., Deutscher, J. & Van Sinderen, D. 2007. Identification and characterization of a fructose phosphotransferase system in Bifidobacterium breve UCC2003. *Applied and environmental microbiology,* 73**,** 545-553.

Moore, A. M., Patel, S., Forsberg, K. J., Wang, B., Bentley, G., Razia, Y., Qin, X., Tarr, P. I. & Dantas, G. 2013. Pediatric Fecal Microbiota Harbor Diverse and Novel Antibiotic Resistance Genes. *PloS one,* 8**,** e78822.

Murphy, E. F., Cotter, P. D., Hogan, A., O'sullivan, O., Joyce, A., Fouhy, F., Clarke, S. F., Marques, T. M., O'toole, P. W. & Stanton, C. 2013. Divergent metabolic outcomes arising from targeted manipulation of the gut microbiota in diet-induced obesity. *Gut,* 62**,** 220-226.

O' Connell Motherway, M., Kinsella, M., Fitzgerald, G. F. & Sinderen, D. 2013. Transcriptional and functional characterization of genetic

elements involved in galacto-oligosaccharide utilization by Bifidobacterium breve UCC2003. *Microbial Biotechnology,* 6**,** 67-79.

O' Connell Motherway, M., O'driscoll, J., Fitzgerald, G. F. & Van Sinderen, D. 2009. Overcoming the restriction barrier to plasmid transformation and targeted mutagenesis in Bifidobacterium breve UCC2003. *Microbial Biotechnology,* 2**,** 321-332.

Penders, J., Stobberingh, E. E., Savelkoul, P. H. & Wolffs, P. F. 2013. The human microbiome as a reservoir of antimicrobial resistance. *Frontiers in microbiology,* 4**,** doi: 10.3389/fmicb.2013.00087

Pereira-Fantini, P. M., Thomas, S. L., Wilson, G., Taylor, R. G., Sourial, M. & Bines, J. E. 2011. Short-and long-term effects of small bowel resection: a unique histological study in a piglet model of short bowel syndrome. *Histochemistry and cell biology,* 135**,** 195-202.

Randal Bollinger, R., Barbas, A. S., Bush, E. L., Lin, S. S. & Parker, W. 2007. Biofilms in the large bowel suggest an apparent function of the human vermiform appendix. *Journal of theoretical biology,* 249**,** 826-831.

Schnoes, A. M., Brown, S. D., Dodevski, I. & Babbitt, P. C. 2009. Annotation error in public databases: misannotation of molecular function in enzyme superfamilies. *PLoS computational biology,* 5**,** e1000605.

Sommer, M. O. A., Dantas, G. & Church, G. M. 2009. Functional characterization of the antibiotic resistance reservoir in the human microflora. *Science,* 325**,** 1128-1131.

Sukhotnik, I., Siplovich, L., Shiloni, E., Mor-Vaknin, N., Harmon, C. & Coran, A. 2002. Intestinal adaptation in short-bowel syndrome in infants and children: a collective review. *Pediatric surgery international,* 18**,** 258-263.

Sun, Z., Baur, A., Zhurina, D., Yuan, J. & Riedel, C. U. 2012. Accessing the Inaccessible: Molecular Tools for Bifidobacteria. *Applied and environmental microbiology,* 78**,** 5035-5042.

Tian, B., Fadhil, N. H., Powell, J. E., Kwong, W. K. & Moran, N. A. 2012. Long-term exposure to antibiotics has caused accumulation of resistance determinants in the gut microbiota of honeybees. *mBio,* 3**,** e00377-12.

Torres-Cortés, G., Millán, V., Ramírez-Saad, H. C., Nisa-Martínez, R., Toro, N. & Martínez-Abarca, F. 2011. Characterization of novel antibiotic resistance genes identified by functional metagenomics on soil samples. *Environmental microbiology,* 13**,** 1101-1114.

Trasande, L., Blustein, J., Liu, M., Corwin, E., Cox, L. & Blaser, M. 2012. Infant antibiotic exposures and early-life body mass. *International Journal of Obesity,* 37**,** 26-23.

Xiao, J., Takahashi, S., Odamaki, T., Yaeshima, T. & Iwatsuki, K. 2010. Antibiotic susceptibility of bifidobacterial strains distributed in the Japanese market. *Bioscience, biotechnology, and biochemistry,* 74**,** 336-342.

Yazid, A., Ali, A., Shuhaimi, M., Kalaivaani, V., Rokiah, M. & Reezal, A. 2001. Antimicrobial susceptibility of bifidobacteria. *Letters in applied microbiology,* 31**,** 57-62.

Acknowledgements

First and foremost I wish to thank my main supervisor Dr. Paul Cotter for his supervision and guidance over the past 3.5 years. His invaluable advice, problem solving abilities and novel ideas, coupled with his generosity with his time and his patient nature made for an enjoyable and successful PhD. Thanks also for my first insight into the world of sequencing while on work placement, I haven't looked back since! To my additional supervisors Prof. Catherine Stanton, Prof. Paul Ross and Prof. Gerald Fitzgerald, thank you for all of your advice and support throughout the PhD.

To my funding bodies the Irish Research Council for my IRCSET Scholarship and to Teagasc for my Walsh Fellowship, both of which enabled this research and enabled me to present my research at conferences and symposiums both at home and abroad.

To those excellent scientists with whom I was lucky enough to collaborate with during my PhD: Dr. Brian Jones and Dr. Lesley Ogilvie for your invaluable help and training on metagenomic bank creation not only when I was hosted in your lab, but also with your follow up advice; Prof. Douwe van Sinderen and Dr. Mary O' Connell Motherway for hosting me in UCC and for your infinite knowledge regarding bifidobacteria; Dr. Susan Lapthorne and Dr. Prue Pereira-Fantini for a wonderful collaboration on SBS; and from Teagasc, Dr. Rebecca Wall (CLA trial) and Dr. Caitriona Guinane (appendix trial) both of whom were a pleasure to work with.

To those who helped, advised, encouraged and supported in any way throughout my PhD, thank you!

A special word of thanks to my original Vision 1 lab mates Alan, Siobhan, Lisa, Pete and Eileen. Thanks for all the help when I first started and for becoming wonderful friends over the years. From invaluable advice, to random chats, you made each day fun, unpredictable and memorable,

thanks!! To Dan for all the sports and pet related chats- you kept me sane! To everyone else in Vision 1, thanks for being such a great bunch to work with. To those in the student office, especially Elaine, who made me welcome from the start and who made the office a friendly and enjoyable workplace!

To my family, Mam, Dad, Caroline and Yvonne, for all your support not just throughout the PhD, but always. The chats, tea, love, encouragement and advice doesn't go unnoticed. Thank you.

Finally to Dave and Marmalade, my best friends and whose love (and in Marmalade's case the purring!!) made every day of this PhD better. Thank you!