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

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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 gentamycin (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 gentamycin in early life can have significant

64 effects on the evolution of the infant gut microbiota, the long-term health
65 implications of which remain unknown.

Introduction

It is becoming increasingly evident that the composition of the human gut microbiota can have a significant impact on health and disease (47, 64, 69, 74). 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 (27, 30). Furthermore, there have been a number of studies which have suggested associations between an altered gut microbial composition and Crohn's disease (28), irritable bowel syndrome (42), obesity (46, 51) 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 (54). 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 (3). 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 (56). A myriad of factors that affect this composition have been investigated and include mode of delivery (56), feeding choice (i.e. breast versus formula feeding) (8, 45), prematurity (63, 72), and the administration of probiotics (11, 15, 55, 60) and prebiotics (10, 31, 65). 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 (7, 23, 35). 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 (9), 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) (23) 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 (6, 17, 18, 51, 58, 66). 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 (67). While antibiotic administration in adults can have a number of gut microbiota-mediated consequences, such as an increased susceptibility to *Clostridium difficile* associated diarrhoea (14) 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 (1, 44, 59). 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 gentamycin within the first 48 hours of birth.

Materials and Methods

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 (35). Briefly, 18 infants were recruited, 9 of whom had received parenteral antibiotic treatment with a combination of ampicillin and gentamycin 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).

Generation of 16S rRNA amplicons for high-throughput sequencing

The generation of 16S rRNA amplicons was performed as described previously (51). Total bacterial DNA was extracted from the faecal samples using the QIAamp DNA Stool Mini Kit (Qiagen, West Sussex, UK) (35). 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.

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 (43), 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.

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 (53). Trimmed FASTA sequences were then BLASTed (5) against a previously published 16S-specific database (70) using default parameters. The BLAST output was then parsed using MEGAN software (version 4.6)(34), which assigns reads to NCBI taxonomies by

193 employing the Lowest Common Ancestor algorithm. Bit scores from within
194 MEGAN were used to filter the results prior to tree construction and
195 summarization. A bit-score of 86 was selected, as previously used for 16S
196 ribosomal sequence data (70). Phylum, family and genus counts for each subject
197 were extracted from MEGAN. Clustering and diversity analysis of the sequence
198 data was performed using the MOTHUR software package (61, 62). For
199 *Bifidobacterium* analysis, raw *rpoB* sequencing reads were quality trimmed as
200 above, with read-lengths for the *rpoB* amplicon above 300 bp being used.
201 Trimmed FASTA sequences were then BLASTed (5) against the NCBI non-
202 redundant database using default parameters. The resulting BLAST output was
203 parsed through MEGAN using default parameters (34).
204
205 **qPCR-based determination of total bacteria and total bifidobacteria**
206 **numbers**
207 Absolute quantification of total bacterial numbers (from 8 representative infants,
208 infants B, F, G, H, K-N) and total bifidobacteria numbers (from 9 representative
209 infants, infants B, F, G, H, K-O) was carried out by qPCR using the Roche 480
210 Lightcycler platform. To determine total bifidobacteria counts, the primers g-Bifid-
211 F (5'-CTCCTGGAAACGGGTGG) and g-Bifid-R (5'-
212 GGTGTTCTTCCCGATATCTACA) were used (49). *Bifidobacterium longum*
213 ATCC 8809 was used as a reference strain to generate a standard curve for total
214 bifidobacteria quantification (21). *B. longum* was grown overnight anaerobically at
215 37°C in modified MRS broth (Difco) (0.05% cysteine) (Sigma Aldrich). Total
216 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 (73). 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.

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 $p<0.05$.

Results

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 gentamycin within 48 hours of birth, and 9 controls who had received no antibiotic treatment, were recruited (35). 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).

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) (Fig. 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 (Fig. 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 (Fig. 1 & SI Fig. 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 (Fig. 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 (Fig. 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.

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) (Fig. 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 (Fig. 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) (Fig. 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) (Fig. 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).

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).

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 (43) 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 (Fig. 4).

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 (9). Short-term health effects include antibiotic associated diarrhoea, gastrointestinal discomfort, gastritis and glossitis (24) as well as the possible development of antibiotic resistant bacteria

406 populations in the gut (39). Furthermore, it has been suggested that a number of
407 long-term health effects are influenced by the development of the gut microbiota
408 (38) and, in turn, the immune system in early life (32, 33, 37, 64), with data
409 suggesting that antibiotic administration contributes to the risk of developing
410 asthma and allergy (13, 25, 40) in addition to heightened risk of obesity (4) later
411 in life. The risks associated with disrupting the gut microbiota may be especially
412 great in young infants, as antibiotic administration can impact on the commensal
413 microbiota at a time when this population is in rapid flux and can easily be
414 unbalanced. Despite this concern, there have been no studies to date which have
415 used powerful next generation sequencing technologies to assess the microbiota
416 of infants who have been administered antibiotics. This study was performed with
417 a view to addressing this issue by employing 454-pyrosequencing, together with
418 qPCR analysis. The results of this relatively small study are important and
419 highlight the apparently major impact that treatment with a combination of
420 ampicillin and gentamycin can have on the gut microbiota of infants. It is evident
421 that the treated infants suffered significant reductions in potentially beneficial
422 bacteria belonging to the phylum *Actinobacteria*, including *Bifidobacterium,* as
423 well as some members of the *Firmicutes* phylum, including *Lactobacillus*. These
424 appeared to be replaced by members of the *Proteobacteria*, including members
425 of the *Enterobacteriaceae* family, thereby resembling trends previously noted in a
426 terminal restriction fragment length polymorphism-based study of antibiotic-
427 treated infants (68). The dominance of the *Proteobacteria*, and an overall
428 reduced microbial diversity, continued to be evident even 8 weeks after antibiotic
429 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 (51, 58). 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 (22, 51, 58). Notably, the frequency of beta-lactam antibiotic resistance among *Enterobacteriaceae*, as a consequence of the production of beta-lactamases, has been well established (12, 52, 56, 57). 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 (41, 71) and gentamycin (20, 36) 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 (2) 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 (16, 23). However, the fact that some recovery was evident in this and previous trials (16, 35) 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 (22, 23, 48). More specifically, in agreement with previous DGGE-based analysis (35), *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 gentamycin, strains of *B. longum* have been found to be more ampicillin resistant than other bifidobacteria (50). 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 (3, 19, 26). 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* (19)). 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 (29). 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 (8, 56), 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 (13, 25). 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.

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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 Ref 29.

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 controls 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		$4.96x10^7$	$1.91x10^7$	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	$1.58x10^7$	$3.94x10^8$			$2.62x10^7$	$7.18x10^7$	

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 legends.

FIG. 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.

FIG. 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.

FIG. 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.

FIG. 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.