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The response of the gut microbial community to therapeutic and nutritional interventions in preterm infants

BASHIR ABDULKADIR

PhD

2016

The response of the gut microbial community to therapeutic and nutritional interventions in preterm infants

BASHIR ABDULKADIR

A thesis submitted in partial fulfilment of the requirements of the University of Northumbria at Newcastle for the degree of Doctor of Philosophy

Research undertaken in the Faculty of Health and Life Sciences and in collaboration with the Royal Victoria Infirmary and Freeman Hospitals in Newcastle upon Tyne

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Publications relating to thesis

Publications

- ✓ Abdulkadir, B., Nelson, A., Skeath, T., Marrs, E. C. L., Perry, J. D., Cummings, S. P., Stewart, C. J. (2016). Routine Use of Probiotics in Preterm Infants: Longitudinal Impact on the Microbiome and Metabolome. Neonatology, 109(4), 239–247. http://doi.org/10.1159/000442936.

- ✓ Abdulkadir, B., Nelson, A., Skeath, T., Marrs, E. C. L., Perry, J. D., Cummings, S. P., Stewart, C. J. (2016). Stool bacterial load in preterm infants with necrotising enterocolitis. Early Human Development, 95, 1–2. http://doi.org/10.1016/j.earlhumdev.2016.01.018

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Abstract

The gut bacterial community plays a vital role in human health with a diverse and complex composition, sculpted by complex host – microbe interactions. Neonates born prematurely are vulnerable to various infections due to their weak immune system and the immaturity of the gut. The most significant diseases are necrotising enterocolitis (NEC) and sepsis. With the advent of molecular techniques the development of the microbial community were better characterised. Leading to better understanding of the microbial contribution to preterm diseases and how clinical and dietary interventions can be tailored to reduce their incidence. The aimed was to study the impacts of clinical and dietary interventions on the community structure and function of the preterm gut microbiota by using high throughput molecular techniques.

The microbial communities derived from clinical samples that are implicated in gastrointestinal disease were explored. By utilising ecological theory, high-throughput sequencing, metabolomic profiling, and statistical modelling to identify how the assembly, phylogenetic diversity, and overall function of these communities impact on disease state. The comparison of archeal and fungal diversity between preterm infants diagnosed with NEC and/or sepsis, compared to healthy controls, showed no significant differences in the community profiles between health and disease. To study if microbial load was associated with NEC, independent of community profiles, total bacterial load was quanitified temporally in NEC infants and matched. The results showed no significant differences in the bacterial load between NEC samples before or at diagnosis, compared to healthy controls, with the only difference occurring following diagnosis, with NEC samples showing significant reduced bacterial load

To better understand clinical intervention in shaping the developing microbial community and the resulting contribution to NEC and sepsis disease mechanisms, the commonly

administered antibiotics and probiotic supplementation were investigated. The result of the findings confirm existing publications, showing probiotic species administered to preterm infants significantly reduce the abundance of potentially pathogenic bacteria associated with NEC and LOS. In the first study of its kind, the study followed the same neonates longitudinally through probiotic administration, including post discharge several months after cessation. Crucially, this novel analysis revealed that supplemented *bifidobacteria* was able to colonise the gut long-term, but supplemented Lactobacilli did not. As well as changes in the bacterial profiles, further metabolomic profiling of functional small molecules confirmed that probiotic supplementation caused functional changes in the preterm gut microbiota. Antibiotic administration represents the most important element of current clinical practise that causes profound shifts in the gut microbiota. To further explore the routinely used antibiotics in neonatal intensive care units and to what extent this intervention alters the preterm gut microbiome, the infants were followed temporally through different antibiotic courses. This revealed that antibiotics differentially affected the preterm gut microbiome, with no single combination found to cause consistent changes between individuals. At a time when antibiotic use is under scrutiny, the outcomes show that much greater understanding of the short and long-term effects on the developing microbiome is necessary.

This thesis has combined a range of molecular methods to explore the microbial community in the developing preterm gut microbiota, including bacteria, fungi, and archaea, finding no association in pathogenesis of NEC. Metabolite profiling was also performed, determining how changes in the gut microbiota cause functional changes in the gut. Combining the data from these powerful analyses revealed probiotics help to modulate a healthy gut microbiome, but antibiotics may disturb the developing bacterial community. Ultimately, better understanding of the consequences of clinical intervention will lead to more refined and personalised care.

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Dedication

This thesis is dedicated to my elder brother (Bello Abdulkadir) and my step Mum (Fatima Abubakar- Gwaggo) who passed away during the period of my study, may Al-Mighty Allah forgive their shortcomings and make Jannatul- Firdaus to be their final abode.

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Declaration

I declare that the work contained in this thesis has not been submitted for any other award in other university or institution of learning and that it is all my own work. I also confirm that this work fully acknowledge contributions and ideas from the work of others. The work was done in collaboration with the Microbiology department at the Freeman hospital, Newcastle upon Tyne and the special care baby unit at the Royal Victoria Infirmary, Newcastle upon Tyne.

Name:

Signature:

Date:

List of abbreviations

ACN	Acetonitrile
ANOVA	One-way analysis of variance
AFG	Amoxicillin, Flucloxacillin, and Gentamicin
APS	Ammonium persulphate
bp	Base pair
BSA	Bovine serum albumin
cDNA	Complementarity DNA
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
DOL	Day of life
dH2O	distilled water
ELBW	Extremely low birth weight
EOS	Early onset sepsis
EFSA	European Food Safety Authority
ESI	Exploring soft ionization
EU	European Union
g	Gram(s)
GA	Gestational age
GC-MS	Gas column chromatography - mass spectrometry

GIT	Gastrointestinal tract
H'	Shannon diversity index
HCA	Histologic Chorioamnionitis
HMDB	Human metabolome data base
H_2O	Water
ISAPP	International Scientific Association for Probiotic and Prebiotic
IPA	International Probiotics Association
LB	Luria-Bertani medium
LC-MS	Liquid chromatography mass spectrometry
LOS	Late onset sepsis
M	Molar
mA	Milliamps
$MgCl_2$	Magnesium chloride
mRNA	Messenger RNA
MS	Mass spectrometry
NDA	Dietary products, Nutrition and Allergies
NEC	Necrotising enterocolitis
NGS	Next generation sequencing
NICU	Neonatal intensive care unit
NMR	Nuclear magnetic resonance

NTC	No template control
OTU	Operational taxonomical unit
PCA	Principal Component analysis
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
PD	Post discharge
PE	Paired-end
PLS-DA	Partial least squares discriminant analysis
PL	Preterm labour
PROM	Premature rupture of the membrane
PPROM	Preterm premature rupture of membrane
QIIME	Quantitative insights into microbial ecology
qPCR	Quantitative PCR
RDA	redundancy discriminate analysis
Rf	Retention factor
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RT-PCR	Reverse transcription polymerase chain reaction
RVI	Royal Victoria Infirmary
SOP	Standard operating procedure

spp.	Species
TAE	tris-acetate-ethylenediaminetetraacetic acid
TEMED	N,N,N',N'-Tetramethylethylenediamine
TIFF	Tagged image file format
UV	Ultraviolet
UPLC-MS/MS	Ultra-performance liquid chromatography mass spectrometry tandem mass-spectrometry
V	Volts
VC	Vancomycin and Ceftazidine
VCM	Vancomycin, Ceftazidine and Metronidazole
v/v	Volume per volume
VLBW	Very low birth weight
w/w	Weight per weight

1 CHAPTER ONE: Introduction

"Molecular biology has shown that even the simplest of all living systems on the earth today, bacterial cells, are exceedingly complex objects. Although the tiniest bacterial cells are incredibly small, weighing less than 10-12 gms, each is in effect a veritable micro-miniaturized factory containing thousands of exquisitely designed pieces of intricate molecular machinery, made up altogether of one hundred thousand million atoms, far more complicated than any machine built by man and absolutely without parallel in the non-living world."

— *Michael Denton, Evolution: A Theory in Crisis*

1.1 Preterm/prematurity

Neonates born at less than 37 weeks gestational age (GA) are said to be 'preterm' or exhibit the condition known as 'prematurity' (Goldenberg et al. 2009; Yeast & Lu 2007; Tucker & McGuire 2004). Preterm infants can be considered as early preterm (when born at less than 34 weeks GA) and late preterm (when born at 34 – 36 weeks GA) (Engle et al. 2007; Shapiro-Mendoza & Lackritz 2012). Preterm delivery is one of the leading causes of morbidity and mortality among the neonates in most developed countries (Goldenberg et al. 2009).

The possible causes of preterm delivery include: eclampsia and intrauterine growth restriction; Preterm premature rupture of the membrane (PPRM) (which accounts for 24% - 30% of such births) , delivery for maternal or fetal indications (30% -35% of the births) (Goldenberg et al. 2008; Ransom & Murtha 2012), and spontaneous preterm labour with intact membranes (40% - 45% of births) (Goldenberg & Mcclure 2010; Goldenberg et al. 2009). However, preterm labour can also be triggered by other factors including infections, haemorrhage, stress and immunological conditions (Sayres 2010). The number is higher in black women, those with previous preterm births, low maternal body-mass index and periodontal diseases can also be considered among the risk factors

responsible for spontaneous preterm delivery (Goldenberg et al. 2008; Goldenberg & Mcclure 2010). Additionally, poor nutritional status, low socio-economic status, multiple deliveries preceding infertility treatment can also increase risk factor (Van Den Broek et al. 2014; Behrman & Butler 2007; Goldenberg & Mcclure 2010), vaginal bleeding have also been reported as important risk factors resulting in prematurity (Goldenberg & Mcclure 2010; Sayres 2010; Behrman & Butler 2007). Severe illness from gut bacteria has also been associated to prematurity (Warner et al. 2016).

The cost implications to parents, social and health services for caring the neonates born prematurely also contribute toward the economics importance in prematurity (Blencowe et al. 2013; Tucker & McGuire 2004). Many interventions have been demonstrated to improve the health and reduce the incidence of preterm birth including ; smoking termination, progesterone therapy and cervical cerclage as well as other postnatal intervention (Simmons et al. 2010; Sayres 2010; Ransom & Murtha 2012; Wisanskoonwong et al. 2011).

1.2 Preterm infant infections

There are many infections related to prematurity including urogenital infections (Agger et al. 2014) and acute respiratory infections that account for most of the clinical disorders among the neonates born prematurely (Altman et al. 2013).

Neonates borne prematurely are vulnerable to infections due to their immature immune system (Groer et al. 2014) and other factors associated with their physiological environment (Sim et al. 2014). Necrotising enterocolitis (NEC) and sepsis are the most significant causes of morbidity and mortality among the preterm (Grishin et al. 2016; Ng et al. 2007). The sepsis has been classified in to two diseases states with different aetiological causes; these are: early onset infection and late on-set infection (QiLi et al.

2014). There are many factors that can influence the onset of neonatal infections among the preterm including: diet, age and other physiological functions (Lafeber et al. 2008). Moreover, a recent research shows that beneficial microbes were not fully established in the gut of preterm infants as such exposed them to infections (Singh, Brian Firek, et al. 2015).

1.3 Necrotising enterocolitis (NEC)

Necrotising enterocolitis is a life threatening infection that occurs when intestinal tissue becomes damaged and starts to die. NEC normally affects the premature babies within two weeks of delivery (Remon et al. 2014). In a serious case, a hole may appear in the intestinal wall enables bacteria to pass in to the abdomen to spread the infection (Jakaitis & Denning 2014; Fox & Godavitarne 2012). The timing of the onset of NEC is inversely proportional to the gestational age of the infants at birth (Russell & R. 2011). NEC has been associated with sequelae including severe neurodevelopmental delay, poor growth, intestinal obstruction due to scarring, short bowel syndrome, and potential liver failure due to prolonged hyperalimentation (Embleton & Yates 2008). The common symptoms of NEC include: swelling in the abdomen, bloody stool, diarrhoea, inflammation, presence of intestinal fluid and intestinal perforation (Hunter et al. 2008).

Necrotising enterocolitis is one of the catastrophic diseases affecting neonates and contributes to high morbidity and mortality rates among preterm infant globally (Berrington et al. 2014). NEC affects approximately 6–10% of VLBW babies, with a fatality rate as high as 20–40% (Meinzen-Derr et al. 2009). Infants of extremely LBW(less than 1000g) or very premature [<28 weeks gestational age (GA)] are at greater risk of NEC than those born closer to term (Wiedmeier et al. 2011). NEC only occurs following development of the gut microbiota, it has, therefore, been hypothesised that the disease is caused by inappropriate colonisation and dysbiosis of the premature intestine

(Fox & Godavitarne 2012; Claud & Walker 2001). NEC is uncommon in term infants (where it usually appears within 2-3 days after birth). In some studies, the pathophysiology of NEC was reported as multifactorial and since premature infants are at greatest risk, the immaturity of the intestine and abnormal bacterial colonisation are regarded as important contributing factor (Lin et al. 2008; Julia et al. 2010).

1.4 Pathogenesis of NEC

To date, no single causative pathogen has been identified accountable for NEC pathogenesis (McMurtry et al. 2015; Brower-Sinning et al. 2014; Leach et al. 2015; Carlisle et al. 2011). This is due to an inability to identify a single organism that is found in patients with NEC and not found among patients without the disease, thus fulfilling Koch's postulates of causality for microbial disease (Falkow 2004; Singh, Brian Firek, et al. 2015), but there are significant population of strict anaerobes found predominantly in the gut of preterm infants associated with NEC cases and a reduction in community diversity (McMurtry et al. 2015; Brower-Sinning et al. 2014). Another research demonstrated that certain infectious microbiota may be accountable for the NEC among the preterm infants (Singh, Brian Firek, et al. 2015). Viruses have also been associated with pathogenesis of NEC although their actual role in the aetiology of NEC have not been established (Torrazza & Neu 2013; Resta et al. 1985).

A recent study demonstrated that *Clostridium perfringens* and *Klebsiella* spp. of *Enterobacteriacaea* were experimentally examined using high through-put techniques to look at their relationship with NEC. After robust analysis comparing between NEC and control cases; *Clostridium* and *Klebsiella* spp. were found associated with NEC cases and absence in control cases, therefore, the researchers concluded that *Clostridium perfringens* has been regarded as a putative etiological pathogen associated with NEC (Sim et al. 2014) . Similarly, *Clostridium butyricum* is specifically associated with NEC

in preterm infant (Cassir et al. 2015). *Klebsiella pneumoniae* has been associated with the development of NEC (Torrazza et al. 2013). Moreover, a study demonstrated that *Clostridium perfringens and Bacteroides dorei* are associated with NEC. Whereas, Staphylococci are negatively associated with NEC in post-meconium sample (Heida et al. 2016). Another recent study showed that *Gammaproteobacteria* (GPB) has been positively associated with NEC. Whereas, a strict anaerobic bacteria specifically *Negativicutes* is negatively associated with NEC in very low birth weight preterm infants (Warner et al. 2016).

1.5 Sepsis

Neonatal sepsis is a major cause of morbidity and mortality during the early days of a preterm infant's life (Camacho-Gonzalez et al. 2013). It is usually occur as a result of direct bacterial translocation from the gut into the bloodstream (Mai et al. 2013). Sepsis can be associated with subsequent sequelae including prolonged ventilation and need for intravascular access, bronchopulmonary dysplasia, NEC, and an increased length of hospital stay (Satar & Özlü 2012). Different microorganisms are responsible for the neonatal sepsis depending on the age at onset (Satar & Özlü 2012; Paolucci et al. 2012). It can cause long-term complications to the new-born and premature infants during their stay at intensive care unit (Tappero & Johnson 2010).

The initial sign and symptoms are non-specific and can easily be confused with the other conditions from the infants (Tappero & Johnson 2010). The prevalence of the disease is attributed to various factors including geographical region, maternal and neonatal risk factors (Shane & Stoll 2014). The smaller preterm infants are at higher risk of sepsis (Russell & R. 2011). Similarly, preterms with very low birth weight are prone to sepsis due to their immature organs and compromised of immune system (Shane & Stoll 2014). Almost 20% - 30% of all very-low-birth-weight (VLBW; <1500g) that have been

hospitalised in NICU will suffer from sepsis at some stage; however, the risk will increases up to 35% in preterm of extremely-low-birth-weight (ELBW; <1000g) and to closely to 50% in infants of less than 750g (Stoll et al. 2004).

The gut flora of preterm infants diagnosed with sepsis is quite different from that of healthy infants, with an increased incidence of *Proteobacteria* and *Firmicutes* (Russell & R. 2011). Unlike in the case of predominant organism in NEC, *Staphylococcus* spp. are regarded as the most serious bacterial species in the pathogenesis of sepsis globally (Venkatesh & Abrams 2010).

1.6 Types of sepsis

The neonatal sepsis can be classified in to two types depending on the onset of symptoms (Stefanovic 2011), with different aetiological causes; these are: early onset infection and late on-set infection (Samuelsson et al. 2014; QiLi et al. 2014).

1.6.1 Early on-set sepsis (EOS)

Early on-set sepsis (EOS) normally occur during the first 1-3 days of life (Vergnano & Heath 2013). They are associated with very low birth weights of <1500g and are usually caused by pathogens which across the placenta and infect the baby (Zuhair 2012). The major pathogens responsible for EOS infections are the Group B *Streptococci* and *Escherichia coli* (Hornik et al. 2012; Simonsen et al. 2014). In addition, other organisms have been reported to cause the sepsis depending on the region and environment especially *Klebsiella pneumonia*, coagulase negative *Staphylococcus*, *Pseudomonas* spp., *Micrococcus* spp., and *Alcaligenes faecalis* , but however, Group *B Streptococci* and *E. coli* which are the common pathogens causing EOS in the western countries were not detected (Ananthakrishnan & Gunasekaran 2009; Samuelsson et al. 2014). Transmission

of the pathogens occurs mostly during labour (Juretić. 2010), and EOS infection is characterised by symptoms of respiratory disorder or fever during the early hours of life (Samuelsson et al. 2014; Chacko & Sohi 2001). Sometimes the symptoms are delayed more especially if the mother has been treated with antibiotics (Cortese et al. 2015; John et al. 2006).

1.6.2 **Late on-set sepsis (LOS)**

Late onset sepsis (LOS) occur between 48 hrs to 90 days after birth (Cortese et al. 2015). They are most common in very low birth weight (VLBW) preterm infants, or term infants that require prolonged neonatal intensive care (Inna et al. 2010). Late on-set infections are usually associated with nosocomial infections (Samuelsson et al. 2014), and studies shows that preterm infants are more susceptible than full term babies and the mortality rate is greatest in infections that occur soon after birth (Cortese et al. 2015). However, the outcomes depend on the duration of exposure and virulence of the causative organism (Ananthakrishnan & Gunasekaran 2009). Gram positive organisms are the principal pathogens responsible for LOS (Hornik et al. 2012; Zuhair 2012). The most frequently encountered pathogens include: coagulase negative Staphylococci which are responsible for majority of the infection (Ananthakrishnan & Gunasekaran 2009) and *Enterobacteriaceae* such as *Escherichia coli, Klebsiella pneumoniae* and *Acinetobacter baumannii* (Inna et al. 2010). Many factors may contribute to increased LOS incidence including complications during birth, prolonged labour, ventilation, exposure to antibiotics and parenteral nutrition among others (Hornik et al. 2012; Cortese et al. 2015).

1.7 Invasive fungal infection

Invasive fungal infections are usually caused by pathogenic fungi most commonly *Candida* Species (Koh 2013; Kaufman et al. 2014). Some studies demonstrated that fungal infections accounts for almost 30% of infections in preterm infants with very low birth weight that result in mortality (Iliev & Underhill 2013; Koh 2013).

The incidence of invasive fungal sepsis is rapidly increasing and becoming common among preterm babies receiving neonatal care (Cetinkaya et al. 2014). This is due to the ability of fungi to colonize the skin surface, mucosal membrane and vascular catheters associated with VLBW (Aydemir et al. 2011). Preterm infants that are immunosuppressed or who require invasive therapies, are exposed to parenteral feeding and broad spectrum antibiotics are vulnerable to fungal infection (Kaufman et al. 2014). In some healthcare systems, prophylactic antifungal and topical prophylaxis are routinely prescribed to reduce the risk of invasive fungal infections (Kaufman et al. 2014; Al Tawil et al. 2010).

1.8 Development of gut microbial communities

1.8.1 Gut Microbiome

Gut microbiomes are the collection of microorganisms including bacteria, archaea, virus and fungi found within the gastrointestinal tract (GIT) together with their complete genetic material (Actis 2014; Galland 2014). It is estimated that the GIT comprises of 70% of most of the microbes found in the human body, this makes it complex environment with a large population of microorganisms (Brown & Allen-Vercoe 2011). It plays a significant role in health as well as affecting the physiological functions and psychological changes in our lives (Actis 2014; Christian et al. 2015). However, the advent of modern molecular techniques has helped to expand our knowledge and understanding of the composition of gut microbiome and its impact on health and disease

(Mulle et al. 2013; Icaza-Chávez 2013). A recent report demonstrated that the human gut colonisation may be initiated in *utero* by a distinct microbial communities already present in placenta and amniotic fluid (Collado et al. 2016). It has been reported recently that, *Streptococcus* spp. found to be dominant in the gut microbiota of maternal antibiotic treatment mothers while *Enterococcus faecalis* dominated the GIT microbiota of maternal antibiotic infant (Gonzalez-Perez et al. 2016).

1.8.2 **Colonisation and composition of gut microbiomes**

The microbial colonization from our cohort study could be classified in to: **Beneficial Microbes**- specifically *Bifidobacteria* and Lactobacilli as the key bacteria of our research study, **potentially pathogenic microbes**- mainly *Enterobacteriaceae, Escherichia, Enterococcus, Bacteriodes, Streptococcus*, and **Pathogenic microbes** which include *Staphylococcus* and *Clostridia* (Westerbeek et al. 2006). It is worth mention that the bacterial colonization occurs during birth through inoculation from maternal microbiota, environment, and other factors (de Almada et al. 2015; Barrett & Guinane 2013; Nyangale et al. 2012).

Many environmental factors influence the composition and colonisation of the GIT by microorganisms including: geography, medication and general life style (Davenport et al. 2014). The gut microbial colonisation by vaginal and faecal bacteria starts during and immediately after delivery such that the early gut microbiome resembles that of the maternal microbiota (Rigon et al. 2012). It has been reported recently that the composition of infants gut microbiota begins to resemble that found in colostrum (Collado et al. 2016). In infants delivery by caesarean section, the microbiome is significantly influenced by maternal skin contact and from the environment (Nyangale et al. 2012; Mshvildadze et al. 2010). In particular, some work shows that the colonisation of the preterm gut microbiome differs over time and between hospital environments which could be relevant

to patients outcomes (Taft et al. 2014). Interestingly, after weaning, the composition of the gut microbiota becomes almost identical to that of adults and remains relatively stable throughout life depending on other environmental factors (Thompson-Chagoyán et al. 2007).

Factors associated with premature delivery can affect the composition of gut microbiota (Stewart et al. 2012). There is limited information related to the microbial communities and their subsequent evolution and dynamics from meconium during the early life of preterm infants (Moles et al. 2013).

However, work on term infants indicates microbial colonization is dominated by facultative anaerobes during the first day(s) of life, subsequently as oxygen is depleted in the GIT; obligate anaerobic bacterial isolates are observed (Ventura et al. 2012). Bacterial communities are found to colonize the human gut more than archaeal and fungal communities, however, other biological components such bacteriophage are present in very high titres and may exert significant ecological effects on the gut microbial communities in ways that are still not clear (Brown & Allen-Vercoe 2011).

1.8.3 **Microbiome and preterm gut**

The gut of an unborn child is regarded as sterile (Rigon et al. 2012) and the recent research shows that colonization begins as soon as the unborn child swallows amniotic fluid containing microbes from the gut of the mother (Rodrı et al. 2015), this is evidenced by meconium samples that are not sterile but harbour diverse microbial communities (Rodrı et al. 2015; Ardissone et al. 2014; Moles et al. 2013). However, antibiotic treatment in pregnant mothers affects the colonisation of their infants gut microbiota (Gonzalez-Perez et al. 2016).

1.8.4 **Implication of gut microbiome in health and diseases**

The Microbial community of the human gastrointestinal tract (GIT) plays a vital role in human health due to its significance in digestion, nutrition and maintenance of host physiology (Brown & Allen-Vercoe 2011). Preterm gut microbiome has been reported to have a short term health effects immediately after birth at NICU and long-term effects during post discharge (Stewart et al. 2015).

The gut microbiome has significant impacts on the health by stimulating the bacterial proteins to interact with human antigens to affect the responses of the adaptive immune system and production of neurotoxic metabolites (D-lactic acid & ammonia) by bacterial enzymes (Galland 2014; Cong et al. 2015). However, it has been recently reported that the GIT microbiota play a vital role in regulating adaptive immune functions, but its role against systemic viral infections is not clear (Gonzalez-Perez et al. 2016). Additionally, the gut microbiome produce hormones that influence microbial growth and virulence (Galland 2014), it also stimulate afferent neurones of the enteric nervous system to send a signals to the brain through different mechanisms which help to shape the psychological behaviour of the host (sleep, stress, mood and cognition) (Cong et al. 2015; Christian et al. 2015).

On the other hand, the GI flora has also been implicated in the pathogenesis of disease (Claud et al. 2013; Magne et al. 2005). Premature infants are particularly vulnerable to infections and other neonatal sepsis (Cortese et al. 2015) due to the fact that, they have low immune system and yet fully matured organs as well as having small number of beneficial microbes (Singh, Brian Firek, et al. 2015).

1.8.5 Factors affecting the preterm gut microbiome

Preterm infants are normally cared for in neonatal intensive care units (NICU), As a result, they are exposed to a different bacterial community compared to full term healthy infants in a general ward who are rapidly discharged in to a domestic environment

(Berrington et al. 2013). The factors that affect an infant's microbiota include: mode of delivery, delayed enteral feedings, exposure to pathogens in the hospital, exposure to their mother's oral and skin microbiota as well as breast milk (Rigon et al. 2012), antibiotics, type of feeding and the function of the host immune system (Berrington et al. 2013; Ventura et al. 2012). It has also been shown that long exposure to a NICU is another factor contributing to the vulnerability of preterm infants to pathogenic bacteria (Beken 2015).

1.8.5.1 Delivery mode

Delivery mode affects the gut microbiome and level of colonisation in preterm infants, it determines the initial gut microbiome (Actis 2014). Premature babies born via vaginal canal will always harbour a bacterial community resembling that of mother's vaginal environment (Dominguez-Bello et al. 2010). In contrast, those borne via Caesarean section have microbial community derived from the maternal skin with *Clostridium, Escherichia, Streptococcus* and *Staphylococcus* predominating (Thompson-Chagoyan et al. 2007). *Lactobacillus* and *Bifidobacterium* are the dominant bacteria found to colonize the gut of preterm infants who were delivered vaginally in the early days of life (Dominguez-Bello et al. 2010; Ventura et al. 2012). Nevertheless, a study reported by co-researchers who compared the mode of delivery among the infants and observed that there is no significant difference in microbial diversity between Caesarean and Vaginal delivery (Mshvildadze and Neu, 2010).

Moreover, a comparative study on delivery mode was conducted among the infants and observed that there is no significant difference in microbial diversity between Caesarean and Vaginal delivery *(P= 0.5)* (Mshvildadze & Neu 2010). Another previous work also shows no significant difference between the bacterial diversity and mode of delivery (P= 0.14) (Mshvildadze et al. 2010).

1.8.5.2 Nutrition

The nutrition given to preterm infants has significant impacts on the composition and level of colonisation of the gut microbiome (Galland 2014). It has been reported that, there is a high abundance of Lactobacilli and *Bifidobacteria* in the gut of preterm infants in tandem with low numbers of *Clostridium* spp. In infants fed with maternal breast milk compared to preterm infants who receive formula feeding (Penders et al. 2005). Formula fed infants usually demonstrate higher microbial diversity with an increase in facultative anaerobes, specifically *Bacteroides*, *Clostridium*, and *Enterococcus*. However, feeding the preterm with artificial formula rather than expressed maternal breast milk (EBM) exposed them to the risk of infections. Remarkably, the gut flora of breast fed babies changes to resemble that of formula fed infants after weaning period (Adlerberth & Wold 2009).

The duration of complete enteral feeding and using nutritional supplements (pro and prebiotics) may stimulate the gut of preterm infants to function, and influence the colonization of beneficial bacteria (Actis 2014; David et al. 2014).

1.8.6 Microbiology of breast milk

Breast milk is an essential nutrient to all new born neonates. The use of breast milk to preterm during their early days of life helped to: reduce the incidence of NEC, faster tolerance of enteral feeding and reduced the need of parenteral diet (Lindemann et al. 2004). Breast milk microbiota is composed of viable skin and non-skin bacteria and some of them plays a vital role in enhancing immunity and liberating the nutrient contents (LaTuga et al. 2014), they also influence the establishment and stability of the gut bacterial community (Ward et al. 2013). *Bifidobacterium* species has been shown to be

effective in preventing certain disease associated with breast milk (Breast milk Jaundice) (Tuzun et al. 2013).

It has been reported that breast milk contains a reasonable amount of Lactobacilli in the gut of an infant, and that new born babies acquire Lactobacilli through oral contamination from vaginal strains during delivery and later to transmit such bacteria to the maternal breast during breast feeding (Martin et al., 2007). Breast milk can sometimes transmit viral and bacterial pathogens leading to the cause of morbidity and mortality to preterm infants (Widger et al. 2010). However, there is a strong correlation between the concentration of *Bifidobacterium bifidum* in breast milk and faecal samples of infants (Tuzun et al. 2013). Moreover, a current study demonstrated that the microbial relationship between the mother and new born baby is continue immediately after delivery by microbes found in breast milk (Collado et al. 2016).

1.9 Management of neonatal infections/sepsis

1.9.1 NEC

1.9.2 Clinical diagnosis of NEC

NEC diagnosis is done by careful observations from clinicians to look for swelling, pain and tenderness as well as conducting abdominal X-ray, radiography and ultrasound for the symptoms of inflammation (Bohnhorst 2013; Santos & Tristram 2015). Laboratory stool tests can be conducted to see the presence of blood (Ng et al. 2015). Blood test for measuring the white blood cells and platelets level can also be helpful in the diagnosis of NEC (Ng 2013).

1.9.3 **Treatment**

As the exact pathogenesis of NEC is yet to be confirmed, its treatment and prevention remains challenging (Lin & Stoll 2006; Lin et al. 2008). NEC treatment depends on the following factors: the severity of the infection, the gestational age of infants and the general health condition of the baby (Harpavat et al. 2012). The treatment can include dietary interventions (antibiotics & probiotics), intravenous fluids or sometimes surgery in severe cases (Bozeman et al. 2013). Moreover, cessation or delaying in enteral feeds, gastric decomposition with intermittent suction and prompt antibiotic therapy can be effective in treating the NEC cases (Morgan et al. 2014). Administering oral feeds with human milk and probiotics supplementation has also been reported to be effective in the prevention of NEC (Torrazza et al. 2013).

Furthermore, the maternal breast milk is one of the important key factor considered as a natural prevention of NEC and facilitating the healthy gut microbiome (Martín et al. 2009; Roger et al. 2010), this is because it composed of many immuno-protective and growth factors, prebiotics oligosaccharides, bioactive immune-modulatory cells and other ‘immunonutrients’ including amino acids, fatty acids, lysozyme, lactoferrin, minerals and metals such as zinc (Hall 2013; Bhatia 2010). It has been previously reported that oral lactoferrin can be used in the prevention of NEC in preterm infants (Pammi & Sa 2011; Venkatesh & Abrams 2010). Arginine supplementation also been reported to be helpful in prevention of NEC (Shah & Shah 2007). NEC can also be treated by administering of broad-spectrum antibiotics after diagnosis for a minimum of 5 days. If the symptoms continue, surgery is an option to remove the dead tissue or necrotic bowel segments (Bhatia 2010; Pierro 2005).

1.10 Sepsis

1.10.1 Clinical diagnosis and treatment of neonatal sepsis

Diagnostic approach mostly focuses on the review of suspected symptoms and patient history during the onset (Stefanovic 2011). However, diagnosis is based on blood culture techniques (Satar & Özlü 2012), but the advent of molecular techniques, enable identification of the possible pathogens to become more accurate and rapid (Camacho-Gonzalez et al. 2013; E. 2010; Tappero & Johnson 2010). LOS is diagnosed by the manifestation of various clinical symptoms including hyperglycaemia, abnormal white blood count, feeding intolerance among others (Samuelsson et al. 2014). Sepsis is managed by antibiotic treatment for a minimum of 5 days (Tappero & Johnson 2010).

Empirical antibiotics therapy remain an effective treatment for suspected cases of LOS (Dong & Speer 2015). They are usually prescribed to a preterm who show signs of infections and may help in reducing the incidence and severity of the infections (Cortese et al. 2015). Intrapartum antibiotic prophylaxis is very effective against Group *B Streptococci* (Stefanovic 2011; E. 2010). Sepsis can also be treated using antifungal prophylaxis for at least 5 days for the management of fungal mediated sepsis. In cases of negative blood culture with the evidence of symptomatic infection, treatment is around 5 ±3 days (Cordero & Ayers 2003; Paolucci et al. 2012). Oral lactoferrin has also been used in the prevention of neonatal sepsis (Venkatesh & Abrams 2010).

1.11 Dietary intervention in preterm infections/sepsis

For effective management of neonatal infections/sepsis, intervention including the administration of probiotics and the use of antibiotic is reported to play a role in the management of neonatal sepsis (Angelakis et al. 2013). The following measures were reported in the management of neonatal sepsis: to attack and prevent the spread of pathogenic microbes, to enhance and improve the immune response to the host, and to

adapt the use of supplementary live microbes and dietary intervention (probiotics) (Robinson 2014).

1.12 **Probiotics**

Probiotics are the live microbial supplements that when given in an appropriate amount exert a beneficial health impact to the host (Costeloe et al. 2015). *Lactobacillus* spp*.,* *Bifidobacterium* spp*. and Staphylococcus* spp. are the most common types of microbes used as probiotics, though certain yeasts and bacilli can also be used (Julia et al. 2010). Probiotics compete with pathogenic bacteria for host binding sites and nutrients while also stimulating host defence mechanisms and enhancing intestinal maturation (Jakaitis & Denning 2014; Liu et al. 2015). They may also protect against systemic bacterial invasion by decreasing permeability of the GI wall (Klaenhammer et al. 2012; Julia et al. 2010).

Lactobacillus and *Bifidobacterium* spp. are the main probiotic groups (Abdulkadir et al. 2016). However, the probiotic potentialities of *Pediococcus, Lactococcus, Bacillus* and Yeasts has been reported (Soccol et al. 2010) and that some probiotics strains exhibit anti-inflammatory and anti-allergic properties (Soccol et al. 2013). The potentiality of probiotics as a drug agent in pharmaceutical industries has also been reported (Sarkar 2013).

The safety of routinely used probiotics has been extensively reviewed and four safety areas were highlighted: i- pathogenicity and infectivity, ii- deleterious metabolic activities, iii- excessive immune response and iv- potential gene transfer (Zhang et al. 2008).

1.13 Current scientific dilemma in "probiotic concept"

The 'Probiotics concept' is presently in a scientific dilemma, this is due to the initial definition of probiotics given by FAO/WHO in 2001 as "Live microorganisms which when administered in adequate amounts confer a health benefit on the host". Since then, this concept was widely used and accepted globally in the clinical and industrial field (Hill et al. 2014). However, there is a discussion on the misuse of the term 'probiotic' by many nutritional products without properly fulfilling the criteria of that concept. The debate is based on the position that the term 'probiotic' can be considered as a "Health claim" or "Nutritional claim". According to EFSA (European Food Safety Authority), claims of effects on immunity, pathogen, suppression and gastrointestinal functions, has led to scientific studies that fail to establish links to reducing the risk factors of disease (Sanz 2016). As a consequence of this debate by various scientific research groups and other organisations with an interest in advocating the benefits, to health and nutrition of probiotics, guidelines have been published in 2011 and recently updated by a EFSA and NDA (Dietary products, Nutrition and Allergies) panel that address: i- functional claims related to the role of food in maintenance/improvement of a physiological function and ii- disease risk reduction claims related to the role of a food in reducing a risk factor for disease. Specific claims addressed in the guidance include those on functions of the immune system (based on the essentiality of nutrients), functions of the gastro-intestinal tract (discomfort, gas accumulation, normal defecation and digestion and /or absorption of nutrients), defences against pathogens and reduction of risk factor for infections. Claims evaluated by the panel with a favourable opinion have been used to provide guidance to applicants on the scientific requirements for the substantiation, whereas those evaluated with an unfavourable opinion have been used to illustrate the shortcomings that prevented their substantiation (Sanz 2016).

Furthermore, a series of discussions were organised between interested parties related to the ‘probiotic concept’ such as ISAPP (International Scientific Association for Probiotic and Prebiotic), gastroenterologist, microbiologist, etc. with expertise from WHO/FAO. Currently, the dialogue is ongoing between the EU (European Union) and EFSA (European Food Safety Authority) to resolve the issue at stake. Nevertheless, some useful guidelines to clinicians and consumers on the appropriate use and scope of the term probiotics were drafted and reach a consensus agreement (Hill et al. 2014).

Within the European Union there is a *de facto* ban on the use of the word ‘probiotic’ when presenting, labelling or advertising probiotic foods. This has led the industry and some member states to reflect on a Europe-wide solution and to consider the future design of the probiotic food industry. However, efforts have been made in collaboration with IPA-Europe (International Probiotics Association- Europe) to resolve Europe’s probiotics issues and create a stable framework for its probiotic industry. The following solutions were outlined to end the ban and explain how the probiotic industry is working to restore trust in its products.

- The source of the problem – why the European Commission decided ‘contains probiotics’ constituted a health claim
- A step towards a solution – the legal options now under consideration and lessons from beyond the EU
- The state of play – signs that some member states and the commission want to find a solution and re-launch innovation
- Next steps – the industry’s commitment to prevent misuse of the word ‘probiotic’ and introduce conditions for its use (Lambert 2016).

1.14 **Prebiotics**

Prebiotics are selective ingredients that induce the growth and activity of beneficial microorganisms that exert health benefit to the host (Drakoularakou et al. 2011). Typically, they are carbohydrates, indigestible and non-fermented food substances found in the GIT and usually fermented by colonic bacteria but not in the upper part of GIT (Bronsky. 2011). They are selectively used to support the growth of some beneficial bacteria and influence the health status of the host (Quigley 2010).

Prebiotics can alter the composition of the gut microbiome and prevent intestinal infections (Licht et al. 2012). The most common type of prebiotic is from soluble dietary fibre inulin found in plant material and frequently consumed in vegetables (Bultosa 2016). Roots and tuber flours of some plant have also been shown to be source of prebiotic compounds (Sousa et al. 2015).

Prebiotics and probiotics play a significant role in brain functions (Saulnier et al. 2013), and have been shown to impact on mucosal immunity and serve as a biomarkers for measuring the human immune responses (Klaenhammer et al. 2012). The role of pre and probiotics is in modulating the activity of the gut microbiota and how it interacts with the enteric and central nervous system (Saulnier et al. 2013; Madsen 2011; Vieira et al. 2013; Scaldaferri et al. 2013; Chen & Sears 2014; Shukla et al. 2011; Quigley 2010; Vyas & Ranganathan 2012). Prebiotics have been applied in inflammatory skin conditions by reducing the incidence of atopic dermatitis in infants (Nole et al. 2014).

The prebiotics, have a great impact on gut microbiota and gene expressions (Paturi et al. 2015), and it influenced the gut microbiota to maintained the signal communication that exist between the GIT and brain (Mayer et al. 2015). However, the neurological behaviour (anxiety, stress, autism, learning, mood and memory) have also been modulated by prebiotics and nutritional diets (Burokas et al. 2015; Liu et al. 2015). The prebiotics have

also been reported to be effective in treating chronic inflammatory diseases and maintaining the gastrointestinal health such as celiac disease, vasculitis lupus, COPD etc (Patel & Dupont 2015).

1.15 Application of probiotics to preterm infants

Many reports show how routinely used probiotic supplements reduced NEC and other sepsis without serious adverse effect to the neonates (Janvier et al. 2014; Neu 2014; Gaul 2008). Some reports also demonstrated the effect of prebiotics alone or when combining it with probiotics intervention to reduce the incidence of NEC among preterm infants (Partty et al. 2013; Szajewska 2010; Beken 2015).

Probiotics help to reduce morbidity and mortality rate of infection in preterm infants, by improving nutrients utilization; in animal models, they increase short term growth. In addition, some strains of probiotics have a significant impact on gut microbial compositions that are related to obesity and overweight (de Almada et al. 2015; Picaud 2013b). Low diversity of gut microbiota may increase the risk of bacterial translocation across the gut wall, probiotic administration helps to increase the diversity of gut microbiota which in turn prevents translocation (Picaud 2013a). Furthermore, a recent study demonstrated how the probiotics can alter the gut microbiota in restoring the balance of intestinal flora and its effectiveness in prevention of NEC in VLBW infants and treatment of various chronic neurological disorders; this can be achieved by either direct antimicrobial effects, enhancement of mucosal barrier integrity or through immune modulation (Patel & Dupont 2015).

1.16 **Antibiotics**

Antibiotics are the compound molecules derived from natural, synthetic or semi synthetic origin that can kill or inhibit the growth of microorganisms without toxic effect to the host. They can either be bacteriostatic or bactericidal and those with bactericidal actions are usually preferred in the treatment of immunocompromised patients including preterm infants (Alexander Mankin 2011).

Prophylactic antibiotics are normally prescribed and given to the preterm infants during their stay in neonatal care units (Greenwood et al. 2014). Furthermore, the intake of enteral feeding and prophylactic antibiotics by some mothers during delivery influence the health of the baby (Torrazza et al. 2013). Antibiotics were reported to have short and long term impact to the gastrointestinal microbiota, the course of antibiotics administered may vary based on clinical experience and other physiological factors (Jernberg et al. 2010; Jernberg et al. 2007).

1.16.1 **Impacts of antibiotics on gut flora**

A recent report shows that the composition of the intestinal microbiota is relatively stable throughout life but it can be changed due to number of factors including antibiotics therapy, dietary intervention and microbial infections (Rodrı et al. 2015). The gut flora has been recognised as an important reservoir host of antibiotics resistance genes of the human infants (Gosalbes et al. 2016). The routine use of antibiotics in preterm infants is one of the factors influencing the colonisation of microbial flora in the early days of life and affects their composition (Ward etal., 2014). Exposure to antibiotics also affects the microbial diversity and affect the rate of colonization in the preterm gut (Torrazza & and Josef Neu 2012). It has been reported recently that maternal antibiotic treatment during pregnancy affects the composition of gut micro flora in both mothers and infants (Gonzalez-Perez et al. 2016).

Antibiotics are either intended to target selected pathogenic bacteria or to provide a broad spectrum of antibacterial activity. When administered, they can inhibit the growth of beneficial bacteria long after the treatment is stopped which may alter the host- microbial relationship (Willing et al. 2011). Important antibiotic factors that are reported to influence the composition of gut flora include the combination of drugs, time and number of doses (Jernberg et al. 2010). Antibiotic therapy can cause shift in gut microbial flora and may predispose preterm to infection later on (Beken 2015), they may also affect the chemical composition of faeces (Antunes et al. 2011).

1.17 The PCR-DGGE Analysis for Archaeal and Fungal Microbial communities

Archaea are prokaryotic microorganisms that are genetically distinct from bacteria and eukaryote (Breu et al. 2008). Some archaeal taxa are found in the GIT of humans and other animals (Matarazzo et al. 2012; Cavicchioli et al. 2003). The archaeal communities that colonise the human gastro intestinal tract are limited to few groups, particularly methane-producing archaea (Matarazzo et al. 2012; Eme & Doolittle 2015). Despite their unique characteristics, the potentiality of archaea as a pathogens is questionable, based on how well to addressed and achieved the following assumptions: the diversity nature of archaea and its pathogenicity, its relation to eukaryotes, immune response to archaea, possession of molecular fingerprints of a known pathogens by archaea and the evidences to prove the known disease caused by archaea (Cavicchioli et al. 2003). The presence and absence of methanogenic archaea in the human gut determines the level of health and dysbiosis of intestinal microbiota respectively (Berrington et al. 2014).

It has been demonstrated that archaea are widely distributed and there is the interaction relationship with eukaryotes (Reed & Hicks 2011; Cavicchioli et al. 2003). Archaea found to be present in human gastrointestinal tract (Hoffmann et al. 2013) and human vaginal environment (Finster 2008; Belay et al. 1990). It is well established that anaerobic

methanogens inhabits eukaryotic hosts, including humans and high numbers have been found in the gastrointestinal tract (Pikuta 2011; Cavicchioli et al. 2003), which might cause some dysbiosis in that environment

Fungi are a diverse group of eukaryotic single-celled or multi nucleated organisms that live by decomposing and absorbing the organic materials. They are numerous in the environment and appear in different structural forms. Fungi play a vital role as part of the normal human gut microbiota (Cui et al. 2013; Ott et al. 2008) and can be commensal or pathogenic (Cui et al. 2013; Koh 2013; Huffnagle & Noverr 2013; Iliev & Underhill 2013; Lukes et al. 2015; Luan et al. 2015).The diversity of fungal microbiota in the human gut is less than that of bacteria (Huffnagle & Noverr 2013; Parfrey et al. 2014). The fungal community has been associated with human gastrointestinal tract infections (Koh 2013) and other related infections in health and diseases (Cui et al. 2013). The knowledge of advanced molecular techniques helps to identified various species of fungi to cause some diseases (*Candida albicans*, *Aspergillus fumigatus* and dimorphic fungi). Specific organs are also known to harbour different fungi example lung, other species of fungi (*Penicillium, Aspergillus* Species) are found to be useful in pharmaceutical industries.

The diverse nature of fungal communities from the human gut has been analysed by using PCR-DGGE fingerprints (Gouba et al. 2013; Walker et al. 2011; Scanlan & Marchesi 2008; Luan et al. 2015) and other high throughput techniques that help to revolutionised the understanding, the phylogenetic and functional diversity of fungi (Cui et al. 2013; Koh 2013; Iliev & Underhill 2013). Though, there is wide range of literature on gut microbial communities, the number of studies investigating specifically fungal microbiota is quite limited (Ott et al. 2008; Koh 2013) and was not intensely studied (Koh 2013; Iliev & Underhill 2013; Caporaso et al. 2012), this drawback resulted to the limited fungal profiles from the intestinal samples (Luan et al. 2015).

Analyses of the microbial communities using PCR-DGGE techniques have been widely used to determine their diversity and molecular identification from different habitat (Zhongtang et al. 2008; Ranjard et al. 2000), including stool samples (Brown & Allen-Vercoe 2011). The analysis of stool samples by PCR-DGGE for assessing fungal and bacterial in the preterm gut associated with NEC or sepsis has been undertaken. This study showed the viable and total bacterial and fungal communities to differ in preterm infants and may contribute to better understanding of NEC and sepsis (Christopher James Stewart et al. 2013).

During our cohort study, the DGGE community profile analysis was used, PCR of 16S / 28S rRNA genes for the both archaeal and fungal community respectively as well as analysis of microbial DGGE products. A previous study illustrated different set of primers with many variable regions and found that the V3 region of Archaea using GC-ARC-344F/519R produced highest and clear DGGE bands (Zhongtang et al. 2008).

Moreover, low abundance of fungal communities from the gut of preterm infants during our cohort study was observed; this could be attributed to the fact that, there is limited data and little molecular research conducted on this group more especially with regards to the intestinal fungi associated with diseases. This assumption has been confirmed by some recent studies (Huffnagle & Noverr 2013; Iliev & Underhill 2013; Luan et al. 2015). Though, a different study conducted on fungal DGGE profiles showed a higher fungal diversity in patients with Crohn's disease in comparison with their control (Ott et al. 2008). Another work demonstrated a low microbial diversity of archaea and fungal communities compared to that of bacterial community using PCR-DGGE finger prints (Brown & Allen-Vercoe 2011).

1.18 The trend in the study of gut microbiota

The methods for the study of gut microbiomes among the preterm infants were classified as: culture based techniques or culture independent techniques. Historically, studies relied on culture based techniques to cultivate and identify the bacteria present in the gut of preterm (de Almada et al. 2015; Singh, Brian Firek, et al. 2015; Rougé et al. 2010). Recently, culture independent techniques have helped to reveal the interaction and ecology of the GIT bacterial community (Mshvildadze et al. 2010; Zdzislawa Libudzisz 2010). For the purpose of these studies, focussed was made only on the culture independent techniques with different molecular finger print to analyse the association of gut microbial communities and relation to preterm infant infections.

1.19 High throughput-techniques and its application to gut microbiome

The advent of modern technology using advanced molecular techniques have contributed immensely toward the understanding of gut microbial communities (Johnson & Versalovic 2012). Such culture independent techniques include: metabolomics profiling, next generation sequencing (NGS), denaturing gradient gel electrophoresis (DGGE), polymerase chain reaction (PCR) and real time PCR to mentioned but a few (Rougé et al. 2010; Brower-Sinning et al. 2014; Mshvildadze et al. 2010).

Molecular techniques as a modern research tool have been utilised clinically in the gut microbiome and have radically altered our understanding of these communities to that of culture based techniques (Petrosino et al. 2009). These techniques allow the microbial community to be identified and observed without prior knowledge of its structure or composition. The foundation of these techniques is the DNA amplification (DNA and/or RNA) processed from stool samples (Nechvatal 2008). DNA analysis is more common as a result of the added difficultly and potential for degradation when working with RNA. It is feasible that an microorganism is present in a dormant state and so not metabolically

active, but when environmental conditions shift they can become active and play a part in the community function (Prosser et al. 2007). Likewise, to achieve full coverage and understanding of the viral communities, the DNA and RNA need to be thoroughly investigated. However, in order to gain the most comprehensive insight into the pathogenesis of infectious diseases such as NEC, analysis of both DNA and RNA is required.

After DNA extraction from biological samples, subsequent diversity studies require amplification by polymerase chain reaction (PCR), typically based on the 16S ribosomal RNA (rRNA) gene. These amplified regions (amplicons) are then further differentiated into different regions that take part in to a pre-defined similarity to one another called as operational taxonomic units (OTUs). The 16S rRNA gene codes for the small subunit in the prokaryotic ribosome (Kang et al. 2010). This ribosomal gene is functionally conserved and it is ubiquitously expressed in bacteria. It represents a rational target for comparative studies between bacterial communities and is also the most extensively used gene for bacterial classification and identification (Isenbarger et al. 2008). The gene contains nine different regions known as 'hypervariable regions' with substantial sequence diversity, this is then fringed by 'conserved regions' of homologous sequence (Chakravorty et al. 2007). Furthermore, it allows universal PCR primers that become complimentary to conserved regions, to be designed, facilitating the amplification of the intervening hypervariable regions. Bacterial classification can then be determined by comparing the amplicons to homologous 16S rRNA sequences from characterised bacteria within open access sequence databases (Brookman-Amissah et al. 2012)

It is worth mentioning that the multiple copies of the same target gene in PCR analysis are not restricted to the bacteria. The linking of cell abundance and PCR amplicons abundance is also limited by the multiple copy nature and intragenomic variability of the

common molecular marker of fungi; the ITS region of the rRNA gene from the 28S subunit (Amend et al. 2010).

1.19.1 Denaturing gradient gel electrophoresis (DGGE)

Denaturing gradient gel electrophoresis (DGGE) was one of the first generation molecular fingerprinting techniques that have the ability to separate amplified DNA products not exceeding 500 bp (Strathdee & Free 2013; Temmerman & Masco 2003). DGGE is widely used as a fingerprint techniques in studying microbial communities from different environments (Sakai 2008; Ying et al. 2005). The polymerase chain reaction of environmental DNA can generate templates of differing DNA sequence that represent many of the dominant bacterial organisms (Enwall & Hallin 2009; Toledo 2004). DGGE is a rapid and sensitive technique for easy detection of nucleotide sequence variation using differential melting activities (Strathdee & Free 2013; Miller et al. 1999). Therefore, the variable 3 (V3) region within the 16S rRNA gene is ideally suited to this analysis with primers targeting the conserved regions at positions 341 to 518 (*E. coli* 16S rDNA position) (Sakai 2008; Muyzer G et al. 1993). Molecular fingerprinting approaches exploit the different number of intermolecular hydrogen bonds between G-C and A-T base pairs. G-C base pairs have three hydrogen bonds and A-T have two, thus G-C base pairs require greater denaturant concentration to dissociate the extra hydrogen bond. Following electrophoresis, fragments with a higher G-C content will travel further through a denaturing gel, separating amplicons on the basis of their base pair content (Strathdee & Free 2013; Muyzer G et al. 1993). The gels can be further stained with relevant staining solutions and viewed under UV to see the position of the amplicons, which look as bands. DGGE is a semi-quantitative technique so the intensity of the band positively correlates with the abundance of the OTU in the sample (Scanlan & Marchesi 2008).

However, the technique is capable of separating amplicons with a single base pair difference. Nevertheless, there are some limitations with regards to the use of DGGE

technique. The first is multiple sequences migrating to the same position on a gel and as a result only one band is detected. This leads to an underrepresentation of the actual number of unique OTUs in a mixed population sample, masking the true diversity (Enwall & Hallin 2009; Muyzer & Smalla 1998; Papen et al. 1998). A single band which incorporates mixed sequences will also lead to false classification of abundance, potentially leading to the assumption of a highly abundant OTU. A second limitation includes the involvement of a single organism with a multiple copies of the target gene. If the multiple copies are homogenous then this may lead to an overestimation of the true abundance of an organism, as described above. Also, if the multiple copies of the target gene are heterogeneous then multiple bands from the same organism will appear on the gel at different positions (Kang et al. 2010). This will lead to an over representation of the true diversity of samples. Additionally, if sequence information is required for taxonomic classification from the molecular fingerprint then individual bands need to be excised and sequenced (Sakai 2008). This process can be time consuming and the added expense can be relatively high. Furthermore, the hypervariable regions utilised typically generate short amplicons of around 200 bp which limits the resolution of taxonomic identification (Strathdee & Free 2013; Temmerman & Masco 2003). It is worth mentioning that, the protocols of DGGE finger print is the same as that for bacteria and fungi organisms with the exception of the electrophoresis conditions (Seiya Tsushima 2010).

1.19.2 **Polymerase chain reaction (PCR)**

The polymerase chain reaction (PCR) is a technique used to amplify a single copy of DNA over different orders of magnitude; leading to produce millions copies of a specific DNA fragment.

PCR amplifies a specific region of a DNA strand (the DNA target). Most PCR methods typically amplify DNA fragments of between 0.1 and 10 (kbp), although some techniques allow for amplification of fragments up to 40 kbp in size. The amount of amplified product is determined by the available substrates in the reaction, which become limiting as the reaction progresses (Agne et al. 2009). PCR technology is widely used to aid in quantifying DNA because the amplification of the target sequence allows for greater sensitivity of detection than could otherwise be achieved. In an optimized reaction, the target DNA quantity will approximately double during each amplification cycle (Brookman-Amissah et al. 2012). PCR has some shortcomings as DNA polymerase is prone to error, which in turn causes mutations in the PCR fragments that are made. Additionally, the specificity of the PCR fragments can mutate to the template DNA, due to nonspecific binding of primers. Furthermore prior information on the sequence is necessary in order to generate the primers, Bias is introduced by universal primers through differential annealing in the amplification of a heterogeneous template, affecting the amplification efficiency of some OTUs (Wang & Qian 2009).

1.19.3 **Real time PCR (qPCR)**

Quantitative real-time PCR (qPCR) has become the most precise and accurate method for analysing gene expression. Before the advent of qPCR, the most common methods for determining expression levels were northern blotting, RNase protection assays, or traditional, endpoint reverse transcription (RT) PCR. Endpoint RT-PCR was an improvement over the older methods due to its ease of use and the much smaller amounts of RNA needed for the reaction (Smith & Osborn 2009). However, with this method, expression levels can only be observed by performing agarose gel electrophoresis on a sample of the product at the end of the entire reaction. While traditional RT-PCR can be useful for determining the presence or absence of a particular gene product, qPCR has the advantage of measuring the starting copy number and detecting small differences in expression levels between samples (Palmer et al. 2007; Bucher et al. 2011). Additionally,

qPCR quantifies the PCR products while the amplification is in progress. Fluorescent reagents allow amplification to be measured while the reaction is occurring through use of a fluorescence detector in conjunction with the thermal cycler. This allows analysis of the entire amplification curve rather than only the end point (Smith & Osborn 2009).

In real-time PCR (QPCR), the amount of amplified product is linked to fluorescence intensity using a fluorescent reporter molecule. The point at which the fluorescent signal is measured in order to calculate the initial template quantity can either be at the end of the reaction (endpoint semi- quantitative PCR) or while the amplification is still progressing (real- time QPCR). The technique relies on the detection of fluorescence, where the signal intensity is relative to the number of amplicons generated on completion of each cycle in the PCR. The concentration of target DNA in the sample is then calculated based on the exponential phase of the PCR when reaction components are in abundance by calculating the threshold cycle (Smith & Osborn 2009).

Moreover, real-time PCR has the following benefits: easiness during the quantification of amplified DNA, greater sensitivity, rapid and quick analysis, accuracy, precision and reproducibility, maintenance of quality during the process and less risk of contamination of the products (Agne et al. 2009).

1.19.4 Next Generation Sequencing (NGS)

Different NGS platforms are available to amplify single fragments and perform sequencing reactions on the amplified fragments (Siqueira et al. 2012). In NGS, the DNA fragments are ligated to platform-specific oligonucleotide adapters needed to perform the sequencing biochemistry, requiring as little as 90 minutes to complete (Claesson et al. 2010; Illumina 2012).

One of the common amplicon applications in NGS is sequencing the bacterial 16S rRNA gene across a number of species, a widely used method for studying phylogeny and

taxonomy, particularly in diverse metagenomic samples. This method has been used to evaluate bacterial diversity in a number of environments, allowing researchers to characterize microbiomes from samples that are otherwise difficult or impossible to study. An overwhelming majority of the world's microorganisms have evaded cultivation, but sequencing-based metagenomic analyses are finally making it possible to investigate their ecological, medical, and industrial relevance (Illumina 2012).

The knowledge of 16S sequencing techniques plays a vital role in discovering new microbial communities within GIT and beyond. A Scientist called Carl Woese initially proposed the three Domain of life as: Archaea, Bacteria, and Eukarya – and he founded his work based on sequence information. The rRNA gene is the least variable DNA among all the other cells. There is similarity of rDNA sequence from microorganisms that are closely related to each other as such the sequence can be aligned for easy identification. This is why; genes that encode the rRNA have been used widely to determine nomenclature, phylogeny and help to estimate the differences among the bacterial species (Woese 2007).

One challenge associated with sequencing small genomes is the lack of reference genomes available for most species (Claesson et al. 2010). To overcome this challenge, some NGS platforms offer paired-end (PE) sequencing protocols, where both ends of a DNA fragment are sequenced, as opposed to single-read sequencing where only one end is sequenced (Wang et al. 2010).

Presently, one of the problems facing NGS is the bioinformatics processing of the raw sequencing reads. As large amounts of data can be generated in relatively short period of time, the computing power needed to analyses the data can be a bottleneck in the process. A number of bioinformatics software platforms for the processing and analysis of data are now available, for instance, Mothur and QIIME (quantitative insights into microbial

ecology) are the leading software packages in microbial ecology (Kozich et al. 2013; Schloss et al. 2011; Caporaso et al. 2010; Siqueira et al. 2012)

1.19.5 Metabolomics

The advent of metabolomics is another 'omic' discipline that has revolutionised the field of molecular analyses. Metabolomics is the analysis of functional metabolites found in biological samples (Ganna et al. 2014; Antonucci et al. 2009; V.V. Tolstikov 2002). Metabolome analysis is commonly used to detect the detail of chemical and molecular substances from biological system (Inna et al. 2010; Gomase et al. 2008; Villas-Bôas, Rasmussen & Lane 2005). The word 'metabolome' was first recommended by British scientist called Stephen Oliver in 1998, to designate the set of low-molecular-mass compound substances that have been synthesized by a microorganisms (Morrow et al. 2013; Villas-Bôas, Rasmussen, Lane, et al. 2005). Subsequently, detailed work on metabolomics profiling was published , offering new terminologies and protocols for the better understanding of metabolomics as well as proposing the process for quantification and identification of metabolites from different group of organism (Xie et al. 2013; Fiehn 2002).

After undergoing robust validation, it has become clear that developing a metabolomics finger print is not an easy task due to the complex chemical nature and diversity of even biological systems (Lagomarcino et al. 2013; Idle & Gonzalez 2007; Fiehn 2002). Some researches maintain the view that metabolomics should regarded in a broader sense as a new phase of scientific research rather than considering it an analytical approach only (Antonucci et al. 2009; Villas-Bôas, Rasmussen & Lane 2005). They therefore, give a comprehensive definition of metabolomics as " The characterization of metabolic phenotypes (the metabolome) under specific sets of conditions (i.e. developmental stages, environmental conditions, genetic modifications) and the linking of these phenotypes to

their correspondent genotypes (integrating or not with gene expression and protein patterns)" (Ganna et al. 2014; Gomase et al. 2008; Villas-Bôas, Rasmussen & Lane 2005).

However, currently the metabolome is further characterised analytically narrowing its scope for better understanding. A metabolite profile in that sense is a set of known or unknown derivatives that where identified by analysing a biological sample using a specific analytical procedure (Marincola et al. 2012; Xie et al. 2013; Roessner et al. 2000; Villas-Boas et al. 2005).

Metabolite profiling was applied in the field of pharmaceutical sciences when discovering new drugs and its efficacy, microbial physiology, cytology and its pathogenesis, clinical diagnosis and other taxonomic studies (Villas-Bôas, Rasmussen & Lane 2005; Becker et al. 2012). There are different analytical platforms used in the study of metabolomics profiling which include: nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry (MS), Gas column chromatography - mass spectrometry(GC-MS), Liquid chromatography- mass spectrometry (LC-MS) alongside chemometric software that provide a simultaneous identification of different compound substances from biological samples (Zhou et al. 2013; Idle & Gonzalez 2007; Xie et al. 2013; V.V. Tolstikov 2002). The most widely established platform is the GC-MS technique (Ogura & Sakamoto 2007) more especially in plant materials. However, LC-MS techniques have been developed exploring soft ionization methods like electro spray (ESI) or photo ionization (APPI) and, simultaneously, mass spectrometers have become more robust and sophisticated for daily routine use (Ganna et al. 2014; Zhou et al. 2013).

The interactive relationship between gut microbial communities and their systemic function with other metabolic pathways using metabolomics signalling has significant potential for increasing and deciphering useful information in clinical applications (Becker et al. 2012; Xie et al. 2013).

The aim and objectives of this research are:

1- To study the impacts of clinical and dietary interventions, on the community structure and function of the preterm gut microbiota by using high throughput molecular techniques.
2- To study the microbial communities derived from clinical samples that are implicated in gastrointestinal disease.
3- Using ecological theory, high-throughput sequencing and statistical modelling to identify the assembly, functional and phylogenetic diversity and contribution these communities make to the disease state.
4- Using molecular methodology including: PCR-DGGE, Real-Time PCR (qPCR), 16S /28S rRNA gene amplification and next generation sequencing. To compare the results obtained within phylogenetic communities and to observe the increase in bacterial load from the samples collated.
5- Determine if the onset of NEC is associated with alterations in the structure of the gut microbial community by analysing stool before and after the onset on NEC.
6- Examine the quantitative changes in the microbial community pre and post onset of infections compared to healthy controls.
7- Observe the impact of archaeal and fungal community and whether it has any significance effect to the gut microbiota of preterm infants.

2 CHAPTER TWO: General Methods

2.1 Sample collection and patient cohort study

Faecal samples were collected from a preterm cohort with <32 weeks gestation admitted to the neonatal intensive care unit (NICU) of the RVI. Stool samples were collected directly from the nappy and immersed in RNA later (Ambion) as soon as possible. RNA later is a bacteriostatic agent offering immediate RNA stabilisation and protection. After collecting the samples, were transferred to Department of Microbiology at the Freeman Hospital Newcastle upon-Tyne where they were kept at -20°C.

The ethical approval was duly obtained from NHS via National Research Ethics Service, County Durham and Tees Valley Research Ethics Committee (Research ethics project number: RE-HLS-13-140303-53143b935c9f8) and informed parental consent on behalf of the babies.

Furthermore, clinical characteristics of preterm demographic data were obtained concurrently with the new stool samples. The demographic data covers the clinical characteristics of the patient in the study cohort including: full names, gender, mode of delivery (caesarean or vaginal birth), gestational ages, birth weight, patient’s day of life (DOL), pathogenic organisms, incidence of NEC or Sepsis, antibiotics courses and administration, antifungal prophylaxis, mortality, and whether the clinicians (Janet Berrington and Nicholas Embleton) independently classified the NEC status and other clinical diagnosis. Sepsis was also confirmed by obtaining positive blood culture laboratory diagnosis.

2.2 Comparison of the experimental technique

2.2.1 Archaeal and Fungal DGGE preliminary study

The stool samples already collated stored at -80°C were sorted in to a total of 43 (25 NEC, 8 Sepsis and 10 Nec/Sepsis) samples and 24 Controls (NEC and Sepsis). The DNA samples were identified from the demographic data and subsequently analysed by PCR-DGGE for assessment of the total archaeal and fungal communities.

2.2.2 Probiotic and non- probiotic diversity: DGGE

A total of 10 patients contributing 85 stool samples contributed to probiotics and non-probiotic diversity analysis. 7 patients (57 stool samples) were all probiotics babies and 3 patients contributing 28 samples serve as matched control babies. All the stool samples were further analysed using DGGE analysis for bacterial diversity.

2.2.3 Stool bacterial load study

In 10 NEC babies (37 samples) and 10 healthy controls (35 samples) samples were studied to determine stool bacterial load. The total 72 stool samples were classified by t time interval as (i) prior, (ii) during and (iii) after NEC diagnosis and further analysed for qPCR technique.

2.2.4 Metabolomics profiling and molecular analysis of probiotic studies

A total of 10 preterm infants were enrolled in the study contributing a total of 88 samples for sequencing, 75 samples for qPCR and 40 stool samples for metabolomics analysis. Patients were split into two groups; 7 who received probiotics and 3 matched controls. All the babies involved in this cohort were breast-fed with maternal milk. The samples were further divided in to four groups: (i) before (ii) during and (iii) after probiotic supplementation and (iv) post-discharge samples.

2.2.5 **Antibiotics course study**

A total of 38 patients contributing 141 stool samples were involved in the antibiotics cohort study. All infants were less than 30 weeks gestational age and less than 1500g birth weight. The commonly prescribed antibiotic courses identified are: Vancomycin, Ceftazidine and Metronidazole (VCM) received by 12 patients contributing 41 samples,, Vancomycin and Ceftazidine (VC) given to 13 patients producing 51 samples and Amoxicillin, Flucloxacillin, and Gentamicin (AFG) having 13 patients with the total of 49 stool samples. All the samples were collected at different time intervals and analysed using 16S rRNA gene profiling.

There are two regimes treatment added to cover for what the clinicians know are the most likely infections after the first few days of delivery, but the babies get different things if they were infected before delivery versus after (usually). Furthermore, penicillin and gentamicin are been used for the first days after delivery, but after that AFG regime is been used if the babies are unwell without a lot of medical 'plastic' (such as umbilical or long lines for drugs or feed solutions) or VC regime if they are unwell and have a lot of plastic in. The Vancomycin (V) covers the CONS (coagulase negative Staphylococcus) which is most commonly seen with lots of plastic in the baby. Metronidazole is been added to either regime if there are specific concerns about the abdomen, in case there is an anaerobic element - so if there is worried about NEC then the patient get metronidazole.

2.3 Analysis of Faecal microbiota composition by NGS during probiotic study

In order to ensure and to accurately determine the probiotics diversity of preterm babies compare with the control group. The entire extracted DNA was processed and sequenced using the benchtop MiSeq (Illumina) platform. Extracted DNA from the samples was aliquot into 96 well plates and was vortexed briefly and span down before being placed in the thermocycler (BioRad CFX96 Touch). PCR was carried out using the following cycling conditions; initial step at 95 °C for 2 min, then 30 cycles of 95 °C for 20 seconds, 55°C for 15 seconds, extension at 72 °C for 5 min, and a final extension at 72 °C for 10 min. Successful PCR was confirmed using an agarose gel (1%) on a subset of 12 samples per plate. The details of the PCR reaction was briefly described below (2.5). However, the preparation of 1% agarose gel, its running buffer and dye as well as visualisation of band is given on next pages under 2.6 (confirmation of successful DNA and PCR).

Normalisation was performed by transferring 18 µL of PCR product to the corresponding well on a normalisation plate. Library quality control was carried out using a Bioanalyser (Agilent bioanalyser 2100) and qPCR using KAPA SYBR FAST qPCR Master Mix (Anachem Ltd.). qPCR was carried out using the following cycling conditions; initial activation step at 95 °C for 5 min, then 35 cycles of denaturation at 95 °C for 30 seconds and annealing at 60°C for 45 seconds.

Raw data generated using next generation sequencing technology was processed to reduce the error rate and removing reads with a poor quality score using different steps of Mothur sequence reads-fastq files as contained in the Illumina Miseq SOP (available at - http://www.mothur.org/wiki/MiSeq_SOP), (Kozich et al. 2013). The protocol involves; the stability files are processed through set of files and making contigs to read a forward & reverse fastq file, this will generate MG-RAST & extract the contiged by running to split in to stability trim contig fast groups. The quality of the sequences will

be summarised in an aligned/unaligned fast files following the screening of the sequence, this will remove any sequences with ambiguous bases and anything longer than 275 bp. From there it would lead to the unique sequences found in a fast formatted files and will indicate the identical sequences to the reference sequence. At this time, it will calculate the distance and cluster each of the sequence. The count sequence will count the number of sequence represented by reference sequence.

The columns that are not included in calculating distances will be filters and remove from the alignments. Pre-cluster will also remove sequences that are likely due to pyrosequencing errors and remove large number of sequences making the distance calculation much faster. This can be achieved through using a special version of algorithm. Afterward, chimeric sequences followed via uchime command to remove it from the count file. The above removed sequences will take the list of sequence names and fastq group in to a new file that does not contain the sequences in the list and further classify the sequences through different methods in order to assign their sequences to the taxonomy outline page. Later the remove lineage will generates a new file that contains only the sequences not containing that taxon and the Mothur will generate those files in to distance matrix and use cluster command to assign sequences in to OTUs at certain distance from all other sequences within the OTU. A shared file will be created for each group and classify the out to get consensus taxonomy for an out (Kozich et al. 2013). The protocol for miseq data analysis was presented in detail in the next pages under 2.17 (next generation sequencing) and 2.19 (Analysis of data).

2.4 DNA extraction of faecal specimen

Microbial DNA was extracted from 100 mg of faecal sample for analysis of the total bacterial load using the PowerLyzer™ PowerSoil® DNA Isolation Kit (MoBio). Faecal samples were added directly into glass bead tubes (0.1 ml), followed by the addition of 750 µL of bead solution and gently vortexed to disperse the sample in the bead solution. Solution C1 was heated at 60°C to dissolved the precipitation before 60 µL of it was added into the sample (Solution C1 helps in cell lysis as its contain SDS and other disruption agents that will break down fatty acids and lipids associated with the cell membrane of many organisms).The bead tubes were shaken horizontally using the MoBio vortex adapter at the maximum speed for 10 minutes for complete homogenization and mechanical breakdown of the microbial cells. Then, the glass beads were centrifuged for 2 minutes at 10,000 x g and 400-500 µL of supernatant was aseptically transferred into a sterile 2ml collection tube. 250 µL of solution C2 was added and shaken to mix briefly before being incubated at 4ºC for 5 minutes (This will precipitate organic and in-organic particles including humic substances, cell debris and proteins that may inhibit downstream DNA analyses and reduce its purity). The tubes were centrifuged for 1 minute at 10,000 x g at room temperature. To obtain high quality DNA, transferring any pellet was avoided as it still contains some non-DNA organic and inorganic material. 600 µL of clear supernatant was transferred into another sterile 2 ml collection tube, then 200 µL of solution C3 was added and briefly vortexed to mix, it was then incubated at 4ºC for 5 minutes to further remove contaminants including leftover humic acid, cell debris and proteins. It was then centrifuged at room temperature for 1 minute at 10,000 x g before 750 µL of the clear supernatant was transferred into another new sterile 2ml collection tube. Solution C4 was mixed before 100 µL (1.2ml) was added to the supernatant and vortexed briefly, this solution has a high concentration of salt that will allow DNA to bind to the silica membrane on to the spin filters. 675 µL of supernatant mixture was loaded

on to a spin filter and centrifuged at 10,000 x g for 1 minute at room temperature. The flow through was discarded carefully and the whole process was repeated. As there is the need to further clean the DNA and remove residual salt, humic acid and other contaminants from the silica membrane leaving only DNA, solution C5 an ethanol based wash solution (500 µL) was added and centrifuged at 10,000 x g for 30 seconds at room temperature, the flow through was discarded and centrifuged again for an additional 1 minute with same condition to remove all the traces of ethanol which can interfere with downstream DNA applications. The spin filter was carefully placed onto a new clean sterile 2ml collection tube before 100 µL of solution C6 was added to the centre of the filter membrane to release the bound DNA. Solution C6 is an elution buffer which contains no EDTA and salt and then it was centrifuged at 10,000 x g for 30 seconds at room temperature and the flow through containing eluted DNA was kept frozen at -20°C for further downstream application.

2.5 Polymerase chain reaction (PCR)

All PCR was carried out aseptically in a PCR Workstation™ (C.B.S. Scientific) to control the risk of contamination. The C1000 Touch™ thermal cycler (Bio-Rad) was used in all analyses to make sure there was no variation during the amplification when using different instruments.

2.5.1 Archaeal 16S rRNA amplification

PCR amplification of the V3 region of the archaeal 16S rRNA gene profiling was conducted to analyse the archeal community from the gut of preterm infants. Different forward and reverse primers with clamp and without clamp before were optimised which yielded clear bands. For the forward primers used: YUArch344FC(5'CGCCCGCCGCGCCGCGGCGGGCGGGGCGGGGGCACGGGGGGACGGGGYGCAGCAGGCGCGA), YU-ARCH 344 (ACGGGGYGCAGGCGCGA 3'), RC Arc 344 f (5'-TCGCGCCTGCTGCRCCCCGT 3') andCS-Arc344FGC (5'-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCACGGGGCGCAGCAGGCGCGA-3') and for the reverse primers: RC519RGC(5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCAGCMGCCGCGGTAATWC 3') and CS 519 R (5'-GWATTACCGCGGCKGCTG 3') (Zhongtang et al. 2008). In order to run the PCR product on DGGE gel the forward primer contained a 40 bp GC-clamp attached to the 5' end. The reaction was performed with 0.5 mM each primer 1x EX-Taq buffer, 0.3 mM each dNTP, 1 mM of $MgCl_2$, 500 mg BSA, 1.25 U Ex-Taq (Takara) and 1 µL of gDNA or cDNA template made up to 50 µL with sterile 18.2 MΩ H_20.

The thermo cycler conditions used for Archeal 16S rRNA DNA amplification included initial denaturation for 5 min at 95 ˚C followed by 10 cycles of denaturation at 95 °C for 30 s, annealing at 61 °C for 30 s, elongation at 72 °C for 1 min and another 25 cycles of 94 °C denaturation for 30 s, annealing at 56 °C for 30 s and elongation at 72 °C for 1 min with a final extension at 72 °C for 10 min. The final extension step was included in all PCR cycles that were to be analysed by DGGE to prevent visualisation of spurious double bands which would obstruct accurate analysis of the gel images and make excision of bands difficult (Janse et al. 2004).

2.5.2 Bacterial 16S rRNA amplification

PCR amplification of the V3 region of the bacterial 16S rRNA gene was performed with the following primers (V3FC (5'- CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG -3') with a 40 bp GC clamp attached to the 5' end and V3R (5'- ATT ACC GCG GCT GCT GG -3') (Muyzer et al. 1993). The reaction was performed with 0.5 mM each primer 1x EX-Taq buffer, 0.3 mM each dNTP, 1 mM of $MgCl_2$, 500 mg BSA, 1.25 U Ex-Taq (Takara) and 1 µL of gDNA or cDNA template made up to 50 µL with sterile 18.2 MΩ H_20. The cycling conditions include initial denaturation for 5 min at 95 ˚C followed by 20 cycles of 95 °C for 1 min, 65 °C for 1 min and 72 °C for 30 s followed by 15 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 3 min with a final extension at 72 °C for 30 min. The 30 minute final extension step was included in all PCR cycles that were to be analysed by DGGE to prevent visualisation of spurious double bands which would hinder accurate analysis of the gel images and make excision of bands difficult (Janse et al. 2004).

2.5.3 Fungal 28S rRNA amplification

The fungal community 28S rRNA amplification was performed with the following primers (5'- GTG AAA TTG TTG AAA GGG AA -3') with a 40bp GC clamp at the 5' end and 28SRC (5'- CGC CCG CCG CGC GCG CGG CGG GCG GGG CGG GGG CAC GGG GGG GAC TCC TTG GTC CGT GTT -3') (Sandhu et al. 1995). The reaction was performed with 0.5 µM each primer 1x Ex Taq buffer, 0.3 mM each dNTP, 1 mM of $MgCl_2$, 500 mg BSA, 1.25 U Ex Taq (Takara) and 1 µL of cDNA template, the reaction was made up to make the final volume of 50 µL with sterile 18.2 MΩ H_20. The cycling conditions used were an initial denaturation for 5 min at 95 ˚C followed by 10 cycles of 95 °C for 1 min, 60 °C (-1 °C per cycle) for 1 min and 72 °C for 30s followed by 25

cycles of 95 °C for 1 min, 50 °C for 1 min and 72 °C for 30 s with a final extension at 72 °C for 30 min (Janse et al. 2004).

2.6 Confirmation of successful DNA extraction and PCR

A 1% (w/v) agarose gel was prepared to confirm that nucleic acid extraction had been successful. Briefly, The agarose gel was submerged in 1× TAE and a 5 µL aliquot of nucleic acid was added to 1 µL of 6× bromophenol blue (Appendix 1), mixed by pipetting, and then loaded into the wells of the agarose gel alongside 5 µL of Hyperladder 1 (Bioline). The electrophoresis tank was run at a constant current of 120 mA for 24 minutes. For DNA staining, 5 µL of SYBR Safe (Invitrogen; 10,000×) was added to the molten agarose prior to it setting. The gel was stained in the dark for 30 minutes at room temperate with gentle agitation. Gels were viewed under U.V. light using the Gel Doc 2000 gel documentation system (Bio-Rad) and quantity one™ software (v4.1.1.) to confirm the presence of nucleic acid product of correct size by comparison with fragments from the standard ladder.

2.7 Denaturing gradient gel electrophoresis (DGGE)

The DGGE technique involves the following stages:

2.7.1 Culture of common stool isolates used for DGGE ladder

In order to create a "ladder", some common bacterial isolates from the gut of preterm infants were cultured that could be used for loading on to a DGGE gel alongside with samples to allow alignment across multiple gels (Tourlomousis & Kemsley 2010). The isolates used are shown in Table 2.1. Cultured isolates were obtained from the Freeman hospital.

Table 2-1: Organisms used to generate DGGE ladder

Bacterial species[a]	Fungal species[b]
Enterobacter cloacae	*Candida parapsilosis*
Serratia marcescens	*Candida dubliniensis*
Citrobacter freundii	*Candida albicans*
Staphylococcus epidermidis	*Candida glabrata*
Clostridium perfringens	*Aspergillus fumigatus*
Bacteroides fragilis	*Exophiala dermatitidis*
Sercina ventriculi	*Scedosporium apiospermum*

[a]Ladder used only for alignment of multiple gels. Sequencing of bands was carried out for identification.

[b]Ladder used for alignment and identification.

2.7.2 Preparation of the stock solution for DGGE

The details of the stock solution and other electrophoresis conditions for DGGE were given in Table 2.1 and 2.2. The other protocols for the preparation of DGGE reagents are presented in the appendix 1.

Table 2-2: Electrophoresis conditions for DGGE

The following are the protocols for making up a 12% acrylamide denaturing solution (200ml)

Condition	For bacteria	For fungi
Denaturing concentration gradient	35% - 55%	40% - 60%
Temperature	60°C	60°C
Voltage	200 V	70 V
Time	4.5 hrs	17 hrs

Table: 2-3 Composition of the gel stock solution for DGGE

Reagent	For bacteria		For fungi	
	35% denaturing solution	55% denaturing solution	40% denaturing solution	60% denaturing solution
40% acrylamide (37.5:1)	60 ml	60 ml	60 ml	60 ml
50× TAE	4 ml	4 ml	4 ml	4 ml
Formamide (deionized)	28 ml	44 ml	32 ml	48 ml
Urea(electrophoresis grade)	29.4 g	463.2 g	33.6 g	50.4 g
Distilled water	Fill up to 200 ml	Fill up to 200 ml	Fill up to 200 ml	Fill up to 200 ml

2.7.3 Assembly of Gel cast

The (Bio-Rad) protocol was used to cast and run the DGGE gels were placed in the centre of the flat plane. Glass plates were first cleaned using ethanol before use to remove any residue and static that may interfere with the gel casting. A thin layer of silicon grease was applied to the 1mm spacers and the sponge in the casting stand to prevent leakage of the denaturant during casting and running (Brinkhoff & Hannen 2001). The alignment card was inserted between the glass plates and the sandwich clamps were tightened to each side of the glass plates. The alignment card was then removed and the gel cast was securely fixed in the casting stand ensuring the base of the glass plates was sealed. The sandwich clamps on the long and short plates were fitted into the notches in the clamps. The screws were tightened.

2.7.4 Casting Gels

To cast DGGE gels, a 19 gauge needle fitted with a tube and Y-fitting was attached to the centre of the plates. Two plastic 50 mL tubes, labelled high and low, were placed upright in a rack and the denaturant solutions described below were added to the tubes accordingly, the lids were secured, and inverted to mix. Bacterial DGGE analysis utilised a denaturant gradient of 34 – 55% denaturant, whereas fungal DGGE employed a denaturant gradient of 40 – 60% denaturant (with 100% denaturant corresponding to 7 mol l^{-1} urea plus 40% v/v formamide). In all cases 12% polyacrylamide gels were used (See Table 1.2 for the composition of DGGE gel stock solutions).

Table 2-4: DGGE reagents

Reagent	**High**
DCODE dye (Appendix 1)	100 µL
Denaturing solution (Appendix 3)	25 mL
APS (10% w/v)	216 µL
TEMED	21.6 µL

The high and low denaturing solutions were each drawn up into a separate 25 mL syringe with rubber tubing. Each syringe was then attached to the Y-fitting before being secured in a Model 475 gradient former (Bio-Rad) and the cam wheel turned to dispense the solutions into the gel cast. The solution was injected until it reached the upper edge of the plate. Once the solutions had been dispensed into the cast, the needle was removed and a well comb was placed between the two glass plates, ensuring no bubbles existed in the gel. The gel was left for a minimum of 1.5 hours to polymerise.

2.7.5 **Running the DGGE Gel**

The control module was placed on the electrophoresis tank, the heater was turned on to set up the temperature to 60 ºC at least 5 ºC higher than the electrophoresing temperature and the stirrer was switched on. The comb was carefully pulled out from the polymerized gel. The gel was attached to the core of the DCode. A 15 µL aliquot of sample was mixed with an equal volume of 2x DGGE loading buffer (Appendix 1) and loaded onto gel. The control module was replaced and once the temperature had returned to 60 ºC the power pack was set to 200 V for 4.5 hours for bacterial community analysis or 70 V for 17 hours for fungal community analysis.

2.7.6 **Staining**

A 25 µL aliquot of SYBR gold (Invitrogen, 10,000x) was diluted in 250 mL of 1x TAE in a staining container. The gel was removed from the glass plates and submerged in the staining solution in the dark for 30 minutes at room temperate with gentle agitation. The gel was de-stained in distilled water to remove any excess stain and viewed under U.V. light using the Gel Doc 2000 gel documentation system (Bio-Rad) and quantity one™ software (v4.1.1.).

2.7.7 **Preparation of the DGGE Ladder**

The denaturing gradient used for loading the fungal DGGE ladder is 40% - 60% and that of archaeal ladder was 35% - 55% which gives desire result as contained in Table 1.1 & 1.2. The organisms used to generate the ladder were known common isolates for both bacterial and fungal species (Table 2.1). The organisms were pulled together, vortexed properly and loaded approximately 30 µl each on the three different wells, two wells from the both ends and one at the middle, the prepared gels was run accordingly. Clear bands were observed and saved.

2.7.8 **Statistical analysis of DGGE gels (TotalLab Phoretix 1D)**

Images captured using quantity one™ software (v4.1.1.) were exported in tagged image file format (.TIFF) at 276 dots per inch (DPI) into a new experiment folder. The lanes of the gels were created using the automatic lane creation function and the frames manually adjusted so that all bands were central in the lanes. The background from the lanes was subtracted using a rolling ball method with a radius of 100 pixels. The band detection setting was applied to the gels using a minimum slope of 100, noise reduction of 4, and a % max peak of 2, then bands were added / removed and the band width adjusted manually. As explained previously, a ladder of known organisms was loaded to the outside and middle lanes on each DGGE gel in the experiment so that successful gel alignment could be achieved (Tourlomousis & Kemsley 2010). Bands from the DGGE ladder were assigned standard retention factor (Rf) values based upon the distance they had migrated through the gel, each corresponding band from all gels in the experiment was given the same value. A minimum of five bands per standard has been suggested for accurate interpolation of multiple gels which was exceeded for both the bacterial and fungal bands analysis (Tourlomousis & Kemsley 2010).

A new database was created in Phoretix 1D Pro based on the analysed gels. The successful alignment of the gels was confirmed by matching all lanes in the database then creating a dendrogram based on the Dice coefficient. Alignment of multiple gels was deemed successful if the ladders clustered together. An OTU matrix was then created by the software using the Rf values of all bands and the relative intensities, which was exported to Microsoft Excel 2010 to be used for subsequent analysis.

2.8 PCR-DGGE Analysis for Archeal microbial communities

2.8.1 Primers trial test

For a robust analysis of archeal community different sets of primers were compared in our cohort (all the primers used were ordered). Initially, two new archeal primers were ordered for both forward andbackward:CS-Arc344FGC(5'CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCACGGGGCGCAGCAGGCGCGA-3')and:RC519RGC(5'CGCCCGCCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCAGCMGCCGCGGTAATWC 3'). After conducting normal PCR (as described previously) alongside with eight different DNA samples selected from demographic data by using the above set of primers; there was no any band observed in all the samples including the negative control. Same samples, thermo cycler conditions and other protocol maintained to carry out another PCR using new forward primer: YUArch344FC (5'CGCCCGCCGCGCCGCGGCGGGCGGGGCGGGGGCACGGGGGGACGGGGY GCAGCAGGCGCGA). After running the agarose gel for this new primer, same result was observed.

To optimise the above primers, the DGGE was set up and used the two set of PCR products from the above set of primers, they were arranged serially and loaded in the wells by alternating the samples bearing same numbers i.e. 1 & 1 loaded in the first and

second well from the first and second primers respectively, 2 & 2….8 & 8. The DGGE was run accordingly. After gel was viewed, clear bands were observed from the second wells bearing the second primer (YUArch344FC) indicating that it seems to be better than the first primer (CS-Arc344FGC) as such it was maintained and used for subsequent archeal studies.

2.8.2 Preparation of Archeal Ladder

Because common archeal isolates were unavailable to be used in generating ladder, an improvised ladder was used for subsequent analysis. Therefore, the desired bands obtained from known bacterial isolates were cut off and processed accordingly, a normal PCR was performed using the prepared products above, after agarose gel was ran, some clear bands appeared. Another gel was prepared, a ladder was made by aliquoting 2µl of each PCR products mixed with 15µl of 2X loading dye, vortexed properly and loaded 30µl on to the first lane as a ladder, then 10 selected PCR products were prepared as DNA templates (3 µl DNA+ 2 µl sterile 18.2 MΩ H_2O+ 5 sterile 18.2 MΩ H_2l of 2X loading dye) then 10 µl of the PCR product was loaded on each well and ran the electrophoresis for 4.5hrs at 200 volts, after run finished , it was viewed, focussed and saved. Clear desired bands were observed. Furthermore, the DNA samples of the three corresponding PCR products above that produced good bands were selected and prepared another PCR in quadrant to increase the volume of the ladder (all the other conditions and working solutions were maintained). Agarose gel was performed; one amplified DNA was selected to represents each set up products, clear bands were seen. Therefore, all the PCR products were pooled together and vortexed properly as our desired archeal ladder for subsequent used in DGGE analysis.

2.9 Sequencing excised Bands from DGGE Gels

2.9.1 Gel storage

Excess moisture was removed carefully before the gel was placed between two A4 sheets of acetate, which was then wrapped in cling film. The gel was then placed in a labelled A4 plastic sleeve and stored in a folder at -80 °C.

2.9.2 Band excision

The desired bands were excised from previously stored or freshly run DGGE gels. Whilst exposed to U.V. light, bands of interest were excised from the gels using a sterile blue (1 mL) pipette tip and placed in a sterile 1.5 mL microfuge tube. The bands were immersed in 10 µL of 18.2 MΩ H_20 and vortexed for 1 minute. The tubes were stored at 4 ºC overnight to allow DNA to elute from the gel.

2.9.3 Amplification and clean-up of excised band

After overnight incubation, the tubes containing the excised band were thoroughly mixed and centrifuged prior to the full 10 µL aliquot of the eluate being used as the template for PCR. The PCR was conducted following the normal protocols as described previously with the exception of the primer containing the 40 bp GC-clamp which was replaced by the unclamped primer. Once confirmation of successful PCR was carried out, the PCR product was subject to ExoSAP-IT PCR clean-up (Affymetrix). Briefly, 5 µL of PCR product was mixed with 2 µL of ExoSAP-IT and incubated in a c1000 Touch™ thermal cycler (Bio-Rad) at 37 °C for 15 minutes to degrade unused primers and nucleotides. The reaction was then heated to 80 °C for 15 minutes to inactivate the ExoSAP-IT.

2.10 Cloning

2.10.1 Preparation of Top 10 competent cells

The TOP10 chemically competent *E. coli* cells are prepared in series of steps over number of days. A sterile 30 mL glass universal containing 10 mL LB media was inoculated with a single fresh colony of TOP10 *E. coli* cells (Invitogen). The culture was incubated at 200 rpm for 2 – 3 hours at 37°C. When the OD600 reached 0.35 – 0.4 nm, the cells were then transferred aseptically into two sterile ice cold universals and incubated on ice for 10 minutes. The cells were then centrifuged for 10 min at 2,700 x g, 4 °C. The supernatant was discarded and the each of the pelleted cells were re-suspended in 7.5 ml sterile ice cold $MgCl_2$ by gentle mixing. The cells were pelleted again by centrifugation (10 min at 2,700 x g, 4°C). The supernatant was discarded and each of the cell pellets were re-suspended in 0.5 mL of sterilised ice cold $CaCl_2$ and incubated on ice for at least 1.5 h to become competent. For long term storage the appropriate volume of sterile 50% (v/v) glycerol was added to give a final concentration of 15% (v/v). Since transformation required the cells at 50 µL per reaction, 65 µL of cells (in 50% (v/v) glycerol at a final concentration of 15% (v/v)) was aliquoted into individual sterilised 1.5 mL micro centrifuge tubes and stored at -80°C for downstream application.

2.10.2 Ligation

The ligation was carried out following the reaction set up as in the Table (2.2) below. ExoSAP-IT treated PCR product was cloned using the pGEM-T® Easy Vector and the 2X Rapid Ligation Buffer (Promega). Tubes were centrifuged to collect the contents at the bottom. The ligation reaction was mixed by pipetting and incubated overnight at 4 °C to increase number of transformants after the process.

Table 2-5: Ligation reaction set up

Reaction Component	Standard Reaction	Positive Control
2X Rapid Ligation Buffer,	5 µL	5 µL
pGEM®-T Easy Vector (50 ng)	1 µL	1 µL
PCR product	3 µL	-
Control Insert DNA	-	2 µL
T4 DNA Ligase (3 Weiss units/µL)	1 µL	1 µL
18.2 MΩ H_20	-	1 µL

2.10.3 **Transformation**

Aliquots of previously prepared TOP10 competent cells were removed from storage (-80 °C) and thawed on ice for approximately 5 minutes. Meanwhile, 2 µL of ligation reaction were added to a sterile 1.5 mL microfuge tube on ice. The 65 µL aliquot of TOP10 competent cells were carefully added to the ligation reaction and mixed by gentle flicking. The tubes were incubated on ice for 20 minutes and then heat-shocked at 42 °C for 50 seconds and immediately returned to ice for a further 2 minutes. 950 µL of room temperature SOC medium (Appendix 4) was then added and the tubes were incubated for 1.5 hours at 37 °C in an orbital incubator with shaking at 150 rpm. Duplicate LB/ampicillin/IPTG/X-Gal plates (Appendix 5) were warmed in an incubator at 37 °C for 30 minutes prior to spreading a lawn from 100 µL of the ligation reaction onto each of the two plates which were incubated at 37 °C overnight (16-24 hours). The presence of white colonies (Blue-white screening technique) was an indicator of successful incorporation of the PCR product in to the plasmid but this must be confirmed by carrying restriction enzyme digest.

2.10.4 **Inoculation and confirmation of successful incorporation of insert**

When the white colony was successfully obtained, most of them were inoculated into an LB/ampicillin broth (Appendix 5) which was incubated at 37 °C overnight. PCR amplification was performed to confirm successful incorporation of the insert. The primers used were M13 Forward (5'- CGC CAG GGT TTT CCC AGT CAC GAC -3') and M13 Reverse (5'- TCA CAC AGG AAA CAG CTA TGA C -3'). An initial PCR was set up containing 0.5 mM each primer 1x EX-Taq buffer, 0.3 mM each dNTP, 1 mM of $MgCl_2$, 500 mg BSA, made up to 49.75 µL with sterile 18.2 MΩ H_20 in the absence of template DNA and 1.25 U Ex-Taq (Takara). Using a sterile pipette tip, the remainder of the white colony was transferred to the PCR reaction before the Ex-Taq was added and

the PCR was initiated. The cycling conditions used were an initial denaturation for 5 min at 95 ˚C followed by 35 cycles of 95 °C for 30 s, 65 °C for 30 s and 72 °C for 3 min and 72 °C for 3 min with a final extension at 72 °C for 10 min. An agarose gel was carried out following the previous protocols; successful visualisation of a band corresponding to the correct fragment length was used to confirm successful incorporation of the insert.

2.11 Plasmid DNA purification (PureYield™ Plasmid Miniprep kit)

Plasmid DNA was purified using the PureYield™ Plasmid Miniprep kit (Promega). Antibiotic selection broth (5 ml) was prepared and individual broths were inoculated with a single white colony from the transformation stage and incubated overnight at 37 ˚C. Prior to beginning of the experiment, the cell lysis buffer was warmed to 37 ˚C and inverted to dissolve any precipitate. Briefly, A 1.5 mL of the LB/ampicillin overnight bacterial culture was added to a 2 mL microfuge tube and centrifuged at 13,000 × g for 30 seconds and the supernatant discarded, this step was repeated once to process a total volume of 3 mL. The cell pellet was re-suspended in 600 µL of sterile 18.2 MΩ H_20 and 100 µL of cell lysis buffer was added before the tube was inverted 6 times until the solution changed from opaque to clear blue. The cell lysis buffer contains SDS to lyse cell membrane and sodium hydroxide which breaks down the cell wall but also causes DNA to depolymerise by breaking hydrogen bonds. Within 2 minutes, 350 µL of cold neutralization solution was added and the solution was mixed by inversion causing a precipitate to form. The neutralisation solution contains potassium acetate which reduces the alkalinity of the solution causing renaturation of the plasmid DNA but leaving gDNA linearised allowing it to be separated from the plasmid DNA by centrifugation. The solution was centrifuged at 13,000 × g for 3 minutes to pellet the precipitate and the supernatant (~900 µL) was transferred to a PureYield™ Mini-column. The column was centrifuged at 13,000 × g for 15 seconds and the flow through discarded. 200 µL of

Endotoxin removal wash was added to the column and centrifuged for 15 seconds, followed by addition of 400 µL of column wash solution to the column and a 30 second centrifugation at 13,000 × g. The column was transferred to a 1.5 mL micro centrifuge tube and 30 µL of elution buffer was added to the column membrane which was incubated at room temperature for 1 minute. The column was centrifuged at 13,000 × g for 15 seconds and the eluted DNA was stored at -20 ˚C for further downstream applications.

2.12 DNA quantification (NanoDrop 1000)

The plasmid DNA is required in a concentration of 50 - 100 ng/µL in a total volume of 15 µL, to calculate the amount needed for an appropriate standard curve for the qPCR reaction. To quantify the plasmid DNA a NanoDrop 1000 Spectrophotometer (Thermo Scientific) was used. The purified plasmid DNA diluted by 50% and 1 µL was applied to the pedestal and readings were measured in triplicate and recorded. An average of the triplicates was calculated and the concentration adjusted as appropriate for sequencing. Samples were either diluted in sterile 18.2 MΩ H_20 or concentrated using a RVC 2-18 rotational vacuum concentrator at 60 °C.

2.13 Restriction enzyme (RE) digest

An analytical scale restriction enzyme digest is usually performed in a volume of 20 µl between 0.2 – 1.5 µg of substrate DNA. The reaction was set up in the following order: Sterile deionized water 12.3 µl, 2.0 µl of RE 10X Buffer, 0.2 µl Acetylated BSA (10 µg/ µl), DNA 1 µl/ µl, the above reaction mixture was gently mixed by pipetting and 0.5 µl of restriction enzyme was added up to make the final volume of 20 µl. The reaction mixture was gently mixed by pipetting and centrifuged; it was then incubated at 37 °C for 90 minutes followed by a heat inactivation step at 65 °C for 10 minutes. After the experiment digestion products were visualised on an agarose gel (1% w/v).

2.14 Quantitative Polymerase Chain Reaction (QPCR)

2.14.1 Preparation of qPCR standard curve

To generate the plasmid DNA standard of the total microbial load from the samples, conventional end point PCR was performed on the extracted DNA using 5 µL of X10 EX-Taq Buffer, 2.5 µL each primer, 4 µL each dNTPs, 0.25 µL each Ex-Taq, 2.0 µL each mgcl2, 2.5 µL BSA, 0.5 µL DNA template and 30.75 µL sterile 18.2 MΩ H_2O to made up to 50 µL. The PCR conditions were as follows: 1 cycle at 95 °C for 3 minutes, followed by 34 cycles of 95 °C, denaturation for 30 secs, primer annealing at 65 °C for 30 secs, and DNA extension at 72 °C for another 30 secs, then a final extension cycle step at 72 °C for 6 minutes. The PCR products were cleaned to remove unincorporated dNTPs and primers using Exo-SAP-IT and cloned using the p-GEMT easy vector cloning kit (Promega). Plasmid DNA was purified using the PureYield™ Plasmid Miniprep kit (Promega). The purified plasmids were then pooled into a single micro centrifuge tube. Concentration and quality of extracted plasmid DNA was determined using a NanoDrop 1000 Spectrophotometer (Thermo Scientific). An average of the triplicates was calculated and the concentration adjusted as appropriate for Real –Time PCR and sequencing.

To achieve absolute quantification of the total microbial load of faecal samples, quantitative real-time polymerase chain-reactions (qPCR) was conducted on the extracted DNA from the samples from NEC and control specimens. The quantification was done using SYBR green methodology as described in qPCR protocol Guide (Illumina), with universal 16S primers (forward 5'- ACTCCTACGGGAGGCAGCAG-3' and reverse 5'-ATTACCGCGGCTGCTGG-3'). A standard curve with a 3 fold dilution series was generated. This requires the calculation of plasmid DNA which is needed to generate the standards. The relative fluorescence units (RFU) and then relative Ct values (ΔCt) as well as cycle threshold (Ct) were obtained, relative to NEC and control samples. Values were normalized by the mass of faecal material used for DNA extraction. Data was then

multiplied by a constant 0.1 gram of stool (1000) (Appendix 7 Table for qPCR raw data for total bacterial load).

A standard curve with 10 fold dilutions was generated. This requires the calculation of plasmid DNA which is needed to generate the standards. Firstly, the size of the plasmid DNA in base pairs (bp) was calculated based on the combined length of the PCR amplicon and the plasmid (p-GEMT easy vector is 3015 bp).

The mass of the plasmid was then calculated by multiplying the size of the plasmid (bp) by the average weight of one bp which is 1.096×10^{-21} (g/bp). The average weight of one bp was calculated by dividing the average molecular weight of a double stranded DNA molecule (660 g) by Avogadro's number (6.023×10^{23}). The mass of plasmid containing the copy number needed to achieve a suitable standard curve was then calculated based on a standard curve quantifying between 3×10^{6} and 30 copies/g in 10-fold dilutions. Therefore, to calculate what mass of plasmid will contain the copy number of interest, the previously calculated mass of the plasmid was multiplied by the desired copy number for the standard curve.

The figure produced by this calculation could then be divided by the required volume in the final PCR reaction mix to give the final concentration of the plasmid DNA (g/µL). It was then possible to prepare a standard curve by rearranging the formula $V_1C_1 = V_2C_2$ to $V_1 = C_2 \times V_2 / C_1$. Where V1 is the initial volume required to achieve the concentration (unknown), C1 is the initial concentration of the plasmid which was calculated in the previous step, C2 is the final concentration of the stock plasmid, and V2 is the final volume in which the plasmid will be diluted. This protocol was maintained throughout when calculating the plasmid DNA needed in preparing qPCR standard during the cohort studies.

Table 2-6 Primers used in qPCR assay for probiotics study

Species	Name of Primer	Sequence (5'-3')	Target sequence	Annealing Temp (°C)	Product Size (bp)	Reference
B. bifidum	BiBIf- 1	CCACATGATCGCATGTGATTG	16S rDNA	62	278	(Maria et al. 2003)
	BiBIF- 2	CCGAAGGCTTGCTCCCAAA				
L. acidophilus	Acidfor	AGCGAGCTGAACCAACAGAT	16S rDNA	60	227	(Tabasco et al. 2007)
	Acidrev	AGGCCGTTACCCTACCAACT				

Table 2-7 Primers used in qPCR assay for the quantification of total bacterial loads

Name of Primer	**Target species**	**Sequence (5'-3')**	Target sequence	Annealing Temp (°C)	Product Size (bp)	Reference
EUB517R	All bacteria	ATTACCGCGGCTGCTGG	16S rDNA	65	180	Muyzer *et al.*, 1993
EUB338	All bacteria	ACTCCTACGGGAGGCAGCAG	16S rDNA	65	180	J Lane, 1991)

2.14.3 **Setup qPCR reaction mix**

When the standard curve had been properly prepared, the reaction mix for the standard curve and reaction could be setup. All preparation was carried out aseptically in a PCR Workstation. Pipette filter tips (Fisher) and pipettes designated solely for qPCR setup were used to reduce the possible contamination and errors during the reaction. No template controls (NTCs) were included in every reaction to check for contamination of all the reagents, plastics and pipettes used in preparing the reaction. Each reaction was performed in triplicate to ensure accuracy. The qPCR protocol was based on the one previously described (Baxter & Cummings 2008). The unknown DNA samples were diluted 1:20, dilutions were performed in micro centrifuge tubes using sterile 18.2 MΩ PCR grade water. These diluted samples and an aliquot of the plasmid DNA stock was heated at 95 °C for 10 minutes to ensure any tertiary structures which may have formed had been eliminated so that efficient amplification was possible. The plasmid DNA was then used to prepare the serial dilutions as calculated previously. The unknown samples (diluted 1:20) were further diluted 1:5 and the equivalent volume of sterile 18.2 MΩ water was used as the NTC. Once the dilutions of the standard curve and unknown samples was complete and the NTC was prepared, the reaction mix was setup ensuring that ABsolute™ QPCR SYBR® mix (Thermo Scientific) had limited exposure to light to prevent degradation. The final reaction mix contained 1x ABsolute™ QPCR SYBR® Green Mix (Thermo-Start™ DNA Polymerase, 3 mM $MgCl_2$) , 0.35 mM each primer, 12.5 µg BSA and 5 µl of DNA in a final volume of 25 µl.

For total bacterial count, the cycling conditions used as 1 cycle at 95 °C for 3 minutes, 34 cycles of 95 °C denaturation for 3secs, primer annealing at 65 °C for 3secs, and DNA

extension at 72 °C for another 3secs, then a final extension cycle step at 72 °C for 6 minutes.

Furthermore, to generate the standard for the *Lactobacillus acidophilus* and *Bifidobacterium bifidum* specific analysis during the probiotic studies, conventional end point PCR was carried out on the extracted DNA using 5 µL of X10 EX-Taq Buffer, 2.5 µL each primer, 4 µL each dNTPs, 0.25 µL each Ex-Taq, 1 µL DNA template and 34.75 µL sterile 18.2 MΩ H_2O to made up to 50 µL. PCR amplification was carried out by applying the following PCR temperature profiles: After an initial denaturation step of 1 cycle at 94 °C for 5 minutes, followed by 35 cycles of 95 °C for 30s, primer annealing at 60 °C for 30s, and DNA extension at 72 °C for 30s, then a final extension cycle step at 72 °C for 10 min (Tabasco et al., 2007). The protocol was the same for both *Lactobacillus acidophilus* and *Bifidobacterium bifidum* with minor modifications, for *Bifidobacterium bifidum;* annealing temperature was increased to 62 °C (Bielecka et al. 2003) and the number of cycles were reduced from 35 to 30. The target copy numbers for each reaction were calculated from the standard curve and were used to ascertain the number of copies per g of stool then log transformed. Standard deviation was determined on the replicate threshold cycle (*CT*) value and reactions repeated if the deviation was above 0.4. Samples were considered to be below reasonable limits of detection if the *CT* value was above 30 cycles (Karlen et al. 2007).

2.14.4 **Normalisation of qPCR data**

The mean copy numbers from the triplicate reactions were calculated using the qPCR standard and the value for the refrigerated samples was paired with the same room temperature sample and the two values were added up. The concentrations for the two temperatures were then divided by the two added together to normalise the values and obtain a value between 0 and 1 to reduce the noise from biological variance.

2.15 Analysis of Faecal *Lactobacillus acidophilus* and *Bifidobacterium bifidum* by Quantitative PCR

Bacterial DNA was extracted from 100 mg of faecal sample for analysis of the total community using the PowerLyzer™ PowerSoil® DNA Isolation Kit (MoBio) and performed according to manufacturer's instructions. To generate the standard for the *Lactobacillus acidophilus* and *Bifidobacterium bifidum* specific analysis, conventional end point PCR was carried out on the extracted DNA using 5 µL of X10 EX-Taq Buffer , 2.5 µL each primer, 4 µL each dNTPs, 0.25 µL each Ex-Taq, 1 µL DNA template and 34.75 µL sterile 18.2 MΩ H_2O to made up to 50 µL. PCR amplification was carried out by applying the following PCR temperature profiles: After an initial denaturation step of 1 cycle at 94 °C for 5 minutes, followed by 35 cycles of 95 °C for 30s, primer annealing at 60 °C for 30s, and DNA extension at 72 °C for 30s, then a final extension cycle step at 72 °C for 10 min (Tabasco et al., 2007). The protocol same for both *Lactobacillus acidophilus* and *Bifidobacterium bifidum* with minor modifications, for *Bifidobacterium bifidum;* annealing temperature was increased to 62 °C (this is due to its higher PCR product size of 278 bp compared to *Lactobacillus* that has 227 bp) (Maria et al. 2003) and the number of cycles were reduced to 30 instead of 35. Successful PCR was confirmed by 1% agarose gel electrophoresis. The PCR products were cleaned to remove unincorporated dNTPs and primers using Exo-SAP-IT and cloned using the p-GEMT easy vector cloning kit (Promega). Plasmid DNA was purified using the PureYield™ Plasmid Miniprep kit (Promega). The purified plasmids were then pooled into a single microcentrifuge tube. Concentration and quality of extracted plasmid DNA was determined using a NanoDrop 1000 Spectrophotometer (Thermo Scientific). An average of the triplicates was calculated and the concentration adjusted as appropriate for Real – Time PCR and sequencing.

To achieve absolute quantification of *L. acidophilus* and *B. bifidum;* quantitative real-time polymerase chain-reactions (qPCR) was conducted. For quantification of *L. acidophilus* and *B. bifidum,* Taqman® methodology using species specific primers for each was done at an annealing temperature of 60°C (Tabasco et al. 2007) and 62 °C (Maria et al. 2003) respectively. The whole quantification was done using SYBR green methodology as described in qPCR protocol Guide (Illumina). A standard curve with 3 fold dilutions series was generated. This requires the calculation of plasmid DNA which is needed to generate the standards.

2.16 **Quantitative PCR analysis of Eubacteria**

To generate the plasmid DNA standard, conventional end point PCR was performed on the extracted DNA using 5 μL of X10 EX-Taq Buffer, 2.5 μL each primer, 4 μL each dNTPs, 0.25 μL each Ex-Taq, 2.0 μL each mgcl2, 2.5 μL BSA, 0.5 μL DNA template and 30.75 μL sterile 18.2 MΩ H_2O to made up to 50 μL. DNA amplification was performed as follows: 1 cycle at 95 °C for 3 minutes, 34 cycles of 95 °C denaturation for 3secs, primer annealing at 65 °C for 3secs, and DNA extension at 72 °C for another 3secs, then a final extension cycle step at 72 °C for 6 minutes. PCR products were visualised on a 1% agarose gel. The PCR products were cleaned using Exo-SAP-IT and cloned using the p-GEMT easy vector cloning kit (Promega). Plasmid DNA was purified using the PureYield™ Plasmid Miniprep kit (Promega). The purified plasmids were then pooled into a single microcentrifuge tube. Concentration and quality of extracted plasmid DNA was determined in triplicate using a NanoDrop 1000 Spectrophotometer (Thermo Scientific). An average of the triplicates was calculated and the concentration adjusted as appropriate for Real – Time PCR and sequencing.

To achieve absolute quantification of total microbial load of faecal samples, quantitative real-time polymerase chain-reactions (qRT-PCR) was conducted on the extracted DNA from the faecal samples from NEC and control patients. The quantification was performed using SYBR green methodology with universal 16S primers; EUB 517R and EUB 338 (forward 5' ACTCCTACGGGAGGCAGCAG-3' and reverse 5'-ATTACCGCGGCTGCTGG-3'). Theses primers were all ordered (see Table 2.17 for the primers detail). A standard curve with a 3 fold dilutions series was generated. With plasmid DNA, The relative fluorescence units (RFU) and then relative Ct values (ΔCt) as well as cycle threshold (Ct) were obtained, relative to NEC and control samples. Values were normalized to 0.1 gram of stool. (Appendix 7 Table for qPCR raw data for total bacterial load).

2.17 Next Generation Sequencing (NGS) Technique

In order to accurately determine the diversity and relative abundance of microbial community in the gut of preterm infants, the DNA sample was processed and sequenced using the benchtop MiSeq (Illumina) platform. The whole NGS process was carried out by the "NUOMICS" facility at Northumbria University.

2.17.1 MiSeq

The 16S sequencing on the MiSeq platform was carried out in house, based on the 'Schloss wet-lab MiSeq SOP' (available at - http://www.mothur.org/wiki/MiSeq_SOP), (Kozich et al. 2013; Kozich et al. 2014). Paired end indexed reads were used to multiplex 192 samples per run (188 samples, 2 positive, and 2 negative controls). Briefly, extracted DNA from the samples was aliquoted into 96 well plates with the last two wells left empty for controls. In a new 96 well plate, 17 μL of Accuprime Pfx Supermix (Life Technologies Ltd.) was dispensed into each well, before 1 μL of the DNA template and 2 μL of each paired set of index primers was transferred to the corresponding well. 1 μL of PCR grade

dH_2O was added to the negative control well and 1 µL of a mock community was added to the positive control well on each plate. Plates were vortexed briefly and spun down before being placed in the thermo cycler (BioRad CFX96 Touch). PCR was carried out using the following cycling conditions; initial step at 95 °C for 2 min, then 30 cycles of 95 °C for 20 seconds, 55°C for 15 seconds, extension at 72 °C for 5 min, and a final extension at 72 °C for 10 min. Successful PCR was confirmed by visualising products on an agarose gel (1%) on a subset of 12 samples per plate.

Normalisation was performed by transferring 18 µL of PCR product to the corresponding well on a normalisation plate. 18 µL of binding buffer was then transferred and the contents mixed by pipetting and vortexing before being spun down. Plates were incubated at room temperature for 1 hour. Being careful not to touch the sides of the wells, liquid was removed and 50 µL wash buffer was added and briefly mixed by pipetting and removed immediately removed leaving no residue. 20 µL of elution buffer was then added and mixed by pipetting and vortexed before being spun down. Following incubation at room temperate for 5 minutes 5 µl from each well was pooled and the plates frozen for later use.

Library quality control was carried out using a Bioanalyser (Agilent bioanalyser 2100) and qPCR using KAPA SYBR FAST qPCR Master Mix (Anachem Ltd.). The pooled library underwent serial dilutions to generate the following dilutions; 1:1, 1:10, 1:1000, 1:2000, and 1:4000. For the Bio analyser, the gel dye mix, ladder, and 1 µL of the 1:1 and 1:10 dilutions were loaded into the necessary wells of a high sensitivity chip. For Kapa qPCR library quantification reactions were carried out in a 10 µL reaction volume with 6 µL of master mix and 4 µL of standards and library dilutions, in triplicate. qPCR was carried out using the following cycling conditions; initial activation step at 95 °C for 5 min, then 35 cycles of denaturation at 95 °C for 30 seconds and annealing at 60°C for 45

seconds. From these results a further dilution was carried out on the median pool dilution amongst the standards, as all pools were normalised to the lowest dilution pool selected.

However, for the sequencing a 500 cycle reagent cartridge and all reagents and samples were thawed prior to set up. 3.4 µL of read 1 sequencing primers was placed in well 12, 3.4 µl of the index primer was placed in to well 13, and 3.4 µl of read 2 sequencing primers was placed in well 14. Sampled were prepared by mixing 10 µl of library and 10 µl of 0.2 NaOH and the PhiX spike was prepared by mixing 2 µl of PhiX, 3 µl PCR grade H_2O, and 5 µl of NaOH. Following 5 min incubation, samples and PhiX were made up to 1 ml with HT1 and then HT1 was used to dilute the library and PhiX to 10 pM. A 5% PhiX run was used so 950 µl of 3.5 pM library and 50 µl PhiX were mixed in a tube and 600 µl of this mixture was loaded into well 17. The flow cell was rinsed with Milli-Q water, wiped with 80% ethanol, and carefully dried prior to placement within the MiSeq instrument. The cartridge, flow cell, and PR2 bottle were then loaded and the instruments followed accordingly (Kozich et al. 2014; Kozich et al. 2013).

2.17.2 **Processing the raw sequencing reads**

Raw data generated using next generation sequencing technology was bioinformatically processed to reduce the error rate by trimming the primer and barcode sequences and removing reads with a poor quality score. The Mothur platform was used in the processing of raw sequence reads.

2.17.3 **Mothur**

For both the Probiotics and Antibiotics studies, the fastq files generated were processed using Mothur version 1.31.2 (Kozich et al. 2014; Schloss 2009; Schloss et al. 2011). The Schloss MiSeq SOP was followed according to the following criteria: 1) no ambiguous bases; 2) maximum length of 275 bp; 3) maximum of 8 homopolymers; 4) within 2 mismatches of the sequence being considered. Detection of potentially chimeric

sequences was performed using Chimera uchime and chimeric sequences were removed from downstream analysis. Alignment was generated via the Silva database (Kozich et al. 2013) (accessed Jan 2015).

2.18 Metabolomics profiling

Metabolomics analyses were conducted using two main steps:

- Extraction & preparation of faecal samples and
- Running the samples (this was conducted by the "NUOMICS" facility at Northumbria University.

Briefly, metabolites were extracted from 100mg stool and homogenised in cold 80% methanol by vortexing for 15 min at 4ºC. The suspension was then centrifuged at 10,000×g for 10 min at 4ºC, passed through a 0.2 µm cellulose acetate filter (Minisart, Sigma-Alrich), and lyophilised in a freeze dryer before storage at -80ºC. Samples were re-suspended in 1 ml and diluted a further 1:1 in initial start phase buffer (5% ACN). L-methionyl-arginyl-phenylalanyl-alanine Acetate (MRFA; Sigma-Alrich) was added to each sample in a final concentration of 0.5ng/µl to act as an internal standard.

Stool metabolite profiling was performed using reverse-phase ultra-performance LC-MS tandem mass-spectrometry (UPLC-MS/MS). An Accucore C18 column (2.6 µm, 150 × 2.1 mm) was used at 40 ºC with a 3.0 µl injection and 300 µl/min flow rate throughout. A multi-step LC gradient was used with 5% CAN (v/v) increasing to 95% ACN (v/v) over 22 minutes, with a further 95% ACN (v/v) for 4 minutes followed by a final 4 minutes re-equilibration at 5% CAN (v/v). Samples were run in triplicate and the order of samples in each triplicate sequence was randomised. A blank consisting of LC-MS grade water underwent the same procedure and an aliquot of every sample was used as a pool. Prior to each run a blank and 5 pools were processed to equilibrate the system, then blanks and pools were processed periodically every 20 samples for background

subtraction and quality control, respectively. A Q-Exactive (Thermo) was used for the MS and subsequent data dependant MS/MS. Metabolomics profiling was performed using HESI with high resolution (70,000) positive and negative switching. The mass range was set from 100 – 1000 m/z. MS/MS was subsequently employed with data dependency based on the metabolites of interest.

SIEVE (Version 2.2 *beta*) was used to process the Thermo RAW files by component extraction. The first, middle, and last blank were used in background subtraction. Positive and negative data were processed individually and combined prior to downstream analysis. An intensity threshold of 1,000,000 was applied detecting a total of 11612 components (8343 positive and 3269 negative). Putative identification of metabolites was based on component mass primarily using the Human Metabolome Database (HMDB). Following MS/MS acquisition of significant components identification was performed using mz cloud.

2.19 Analysis of data

2.19.1 Statistical analysis of Probiotic study

The bacterial DGGE and 16S rRNA gene profiles were analysed by multivariate partial least squares discriminant analysis (PLS-DA) using SIMCA 13.0 (Umetrics, Stockholm, Sweden) (Eriksson et al. 2006) and the results are expressed as copy of OTU-matrix and Graphs. All variables, either OTU or component, were automatically transformed within SIEVE. Analysis of variance (ANOVA) was used to determine whether numbers of *L. acidophilus* and *B. bifidum* (qPCR) increased during probiotic treatment with post-hoc Tukey's applied for multiple pairwise comparisons (Minitab 17), and the results are expressed with means plot and Graphs.

2.19.2 Diversity indices

Diversity indices were calculated for data generated using all techniques to examine the structure of the microbial communities present in the preterm gut using PAST (Hammer & Harper 2001). Species richness (R) was calculated based on the total number of different species present per sample. For culture this was based on the number of different isolates, DGGE was based on the total number of distinct bands, and NGS was based on the number of unique OTUs. For DGGE and NGS, where abundance information was also obtained, Shannon diversity was also calculated. The Shannon diversity index (*H'*) was calculated using the formula;

$$H' = -\Sigma (p_i \log[p_i])$$

The symbol *pi* is the relative intensity of each species. The log of the relative intensity was multiplied by the relative intensity for every species in all of the samples (*pi*log[*pi*]). The sum of these values for each lane was taken and multiplied by -1 (-Σ) which gives the Shannon diversity of the sample.

2.19.3 **Multivariate analysis**

OTU matrix files were generated in Microsoft Excel for data generated using all techniques. The OTU matrix for culture data contained the identity of all cultured isolates and either a 1 or 0 to represent presence or absence, respectively. For DGGE and NGS data, where abundance data was obtained, the OTU matrix contained the normalised abundance of each band (for DGGE) or OTU (for NGS). Unless otherwise stated, for the DGGE analysis, all bands were included based on the Rf value and where possible the actual identity of the band replaced the Rf value.

2.19.4 **SIMCA**

OTU matrices generated from both DGGE and NGS data also underwent multivariate partial least squares discriminant analysis (PLS-DA) using SIMCA 13.0 (Eriksson et al. 2006). OTU matrices were uploaded into SIMCA and each sample in the analysis was assigned to a specific group, with the scores of the model visualised in a score plot. The loadings plot was used to determine which OTUs were associated with each of the variables based on the assigned grouping. To check that data was adhering to multivariate normalities, Hotelling's T^2 tolerance limits were calculated and set at 0.95. Plots were edited within Windows picture viewer to aid clarity according to important variables.

2.19.5 **Tukey's test**

Tukey's test was used to compare the mean bacterial load of both *Bifidobacteria* spp. and Lactobacilli spp. during probiotics study. The samples were analysed based on the different treatment (before, during, and after probiotic supplementation and post discharge) and compared with controls. This test is a multiple comparisons procedure, used in conjunction with one-way analysis of variance (ANOVA), to find means that are significantly different from each other. This analysis was computed in Minitab 17 (v. 17) with a 95.0 confidence interval and a family error rate of 5.

2.19.6 **One- Way Analysis of variance (ANOVA)**

The normalised concentration from the qPCR data were then put into Minitab (version.17) alongside with mean bacterial load and time relative to diagnosis, while for probiotics qPCR the data was put in Minitab with different variables as treatment type, time and mean load. Minitab was also used during analysis of Antibiotics data. A One-Way ANOVA was used. This generated a report that contained the mean, standard deviation for each treatment as well as a p-value depicting the difference between the two means. A p-value of 0.001 – 0.009 shows highly significant relationships and p-value

between 0.05 – 0.09 indicated significance while those (that were 0.05 or greater were) not deemed to be significant.

2.20 Ethical Approval

The research studies were carried out after obtaining an ethical approval from NHS via National Research Ethics Service, County Durham and Tees Valley Research Ethics Committee (Research ethics project number: RE-HLS-13-140303-53143b935c9f8) and informed parental consent on behalf of the babies. All the stool samples were collected from preterm infants hospitalised in the Neonatal Intensive Care Unit at RVI Newcastle.

3 CHAPTER THREE

3.1 Quantitative analysis of gut microbial flora in preterm infants associated with Necrotising enterocolitis

Abstract

Aims: Necrotising enterocolitis (NEC) is one of the major causes of morbidity and mortality in preterm infants. There is no data that shows a particular trend or pattern in terms of specific taxa or community structures that are associated with NEC. The aim was to identify whether the total bacterial load in the gut of preterm infants is significantly correlated with the incidence of NEC. A secondary aim was to quantify the bacterial communities' structure and dynamics over time. It was hypothesized that, if the bacterial load in the gut of preterm infants increase over time, it might have an impact on the development of NEC and the subsequent clinical management of the disease.

Methods: Stool samples were collected from 37 preterm infants with confirmed NEC plus 35 controls, matched by gestational age, all of which were being treated in the NICU at the RVI, Newcastle Upon-Tyne. Bacterial DNA was extracted from 100mg of faecal sample for analysis of total bacterial load, using PowerLyzer™ PowerSoil® DNA Isolation Kit. Quantitative polymerase chain reaction (qPCR) was used to quantify faecal bacterial load using SYBR green with universal 16S rRNA primers.

Results: The quantification analysis of the total bacterial load over time revealed inconsistent patterns. No significant differences were observed between bacterial load at 1 & 2 week, before diagnosis, on the day of NEC diagnosis and 2 weeks after NEC versus controls. However, our study showed a difference in total microbial loads in NEC versus control at 1 week after NEC diagnosis ($p < 0.05$ at 5%). Total bacterial loads between control and disease cohorts were also compared which showed no significant difference between NEC and control at 5% ($p > 0.05$).

Conclusion: There is no distinct trend of total bacterial load associated with NEC over time; there are higher bacterial counts at 1 week after NEC diagnosis in control samples. Therefore, further work needs to be done in quantifying specific microbial signatures associated with NEC based on time intervals as our study applied universal approach.

3.2 Background

Necrotising enterocolitis (NEC) affects the gastrointestinal tract of preterm infants and is one of the leading causes of morbidity and mortality in preterm babies (Leach et al. 2015; Neu 2007; Lagomarcino et al. 2013). It has been reported that there is no consistent trend pattern of total microbial load found among the preterm infants with NEC (Singh, Firek, Brooks, Castelle1, et al. 2015). But there is variability in the diversity and abundance of opportunistic pathogens (Brower-Sinning et al. 2014). However, the evidence suggest that bacteria contribute to the pathogenesis of NEC (Leach et al. 2015).

Studies have been carried out to quantify the bacterial load from different samples utilising the real-time polymerase chain reaction (qPCR). Recently, a study was conducted to assess the correlation between bacterial load of *Clostridium difficile* and Bristol stool; No relationship was observed between Bristol stool and the bacterial load of *C. difficile* across the time points (Thabit & Nicolau 2015). Another study explored, using real-time PCR, the bacterial load of *Ureaplasma parvum* in amniotic fluid and the level of inflammatory responses among the preterm infants, that showed a positive correlation between the amount of *U. parvum* and the magnitude of inflammation (Kasper et al. 2010a). The increase in bacterial load was correlated to various factors including; premature rupture of the membrane (PROM), preterm labour (PL), early-onset sepsis and histologic Chorioamnionitis (Kasper et al. 2010b). Furthermore, the histologic Chorioamnionitis (HCA) in women with preterm premature rupture of membrane (PPROM), is associated with a higher bacterial load of genital mycoplasmas (Kacerovsky et al. 2011).

Several animal models have been studied to understand physiological and histological changes associated with NEC in preterm infants. These animal models have been developed to find effective measures and potentialities for the management of NEC (Tian

et al. 2010; Sodhi et al. 2008). Work conducted on pups model showed that the presence of some Gram negative bacteria (*Citrobacter, Klebsiella* and *Tatumella*) was associated with NEC (Carlisle et al. 2011). However, a study using preterm pigs with induced NEC versus control revealed the colonisation of *Clostridium perfringens* in high abundance among the pigs with NEC which suggests that *C. perfringens* is an opportunistic pathogens associated with NEC (Cilieborg et al. 2012). In addition, another study has demonstrated a higher abundance of total microbial load and greater *Clostridium perfringens* densities in piglets with NEC versus healthy controls which suggests their positive correlation to be associated with NEC severity (Cathrine et al. 2015).

Therefore, it was hypothesized that, applying advanced molecular techniques in faecal analysis of microbial flora from the gut of preterm infants associated with NEC may show the abnormal quantitative colonization and variation based on time and might have some implications in the development of NEC (Rougé et al. 2010; Torrazza et al. 2013). Therefore, the aim was to quantify the total bacterial load by evaluating the faecal samples before, during and after NEC diagnosis.

3.3 Methods

3.3.1 Study design

This study was carried out after obtaining an ethical approval from the NHS via the Research Ethics Committee (County Durham and Tees Valley Research Ethics Committee) and informed parental consent on behalf of the babies. All the stool samples were collected from preterm infants hospitalised in the Neonatal Intensive Care Unit (NICU) at the Royal Victoria Infirmary (RVI) Newcastle. The protocols involved in this study ensured good sampling based on time intervals from NEC diagnosis (+/- 2 days) as well as +/- 2 weeks before and after NEC depending on the availability and suitability of the samples obtained. In total, 72 samples were obtained which constituted 37 from

confirmed NEC cases and 35 as their corresponding controls from matched infants. Controls were selected and matched with NEC cases by gestational age, birth weight, day of life and delivery mode. Where the desired sample was not available, then a sample from a baby which had the optimal matching characteristics was selected (Table 3.1 – 3.2).

Table 3-1: Clinical Characteristics of the Infants in the cohort study (Median)

Characteristic	NEC (n=37)	Control (n=35)
Birth weight (g)	725(500-1470)	825(570-1455)
Gestational age (wks)	25(23-30)	25(23-30)
Delivery mode- no/total (%)		
Vaginal	24/37(64.9)	24/35(68.6)
C-section	13/37(35.1)	11/35(31.4)
Sex –no/total no. (%)		
Male	11/37(29.7)	15/35(42.9)
Female	26/37(70.3)	20/35(57.1)
Day of life of development of NEC	19(5-31)	NA
Day of life for sample collected(median value-lowest to highest)		
2 weeks before	9(4-16)	14(5-20)
1 week before	19(9-27)	18(9-27)
1 week after	28(21-36)	23(15-36)
2 weeks after	37(17-47)	35(16-48)

Table 3-2 Summary of the Cohort demographic data

Patient	Number of samples obtained	Gestational Age(wks)	Birth Weight(g)	Sex	Delivery Mode	NEC day onset	Case	Total no. of antibiotics prior to last sample (days)
139	5	30	1470	M	CS	28	NEC	6
161	5	25	700	F	V	31	NEC	26
171	5	26	790	F	V	19	NEC	26
180	4	23	500	F	V	16	NEC	32
188	5	24	750	F	V	15	P NEC	13
199	4	25	725	F	V	25	NEC	24
178	3	26	525	F	CS		P COMPLX	33
281	4	25	620	M	CS	22	NEC	5
303	1	25	960	M	V	5	NEC	17
315	1	24		M		10	NEC	21

140	3	30	1455	F	CS	CON	5
223	5	25	885	F	V	CON	7
176	5	26	880	M	CS	CON	2
181	4	23	570	F	V	CON	14
222	5	24	620	F	V	CON	11
152	4	25	800	M	V	CON	4
186	3	26	840	F	CS	CON	2
307	4	25	810	M	V	CON	10
229	1	25	910	M	V	CON	2
292	1	24	680	M	V	CON	10

PNEC= potential NEC and P COMPLEX = potential complex cases (see details inappendix 7 A & B- demographic data of Eubacteria qPCR).

Table 3-3 qPCR based quantification of total bacterial load

The Table below shows the mean copies/g of individual patient between NEC and control babies.

PATIENT	COPIES/G AT 0(NEC)	CASE
140	1966376365	CONTROL
139	149685193.6	NEC
223	271117654.2	CONTROL
161	3247230518	NEC
176	694790839.3	CONTROL
171	65318060.14	NEC
181	748408802.9	CONTROL
180	617695143.9	NEC
222	3634911474	CONTROL
188	2082634076	NEC
152	1096865860	CONTROL
199	7361043187	NEC
186	3423746058	CONTROL
178	768520288.7	NEC
307	319513441.9	CONTROL
281	552673102	NEC
229	142906181.6	CONTROL
303	14165864.54	NEC
292	1972369568	CONTROL
315	187954912.3	NEC

Table 3-4 Primers used in the study

The primers used in qPCR assay during this study for the quantification of total bacterial loads are tabulated below:

Name of Primer	Target species	Sequence (5'-3')	Target sequence	Temperature of Annealing(°C)	Product Size (bp)	Reference
517R	All bacteria	ATTACCGCGGCTGCTGG	16S rDNA	65	180	(Muyzer G et al. 1993)
EUB338	All bacteria	ACTCCTACGGGAGGCAGCAG	16S rDNA	65	180	(Weisburg et al,. 1991)

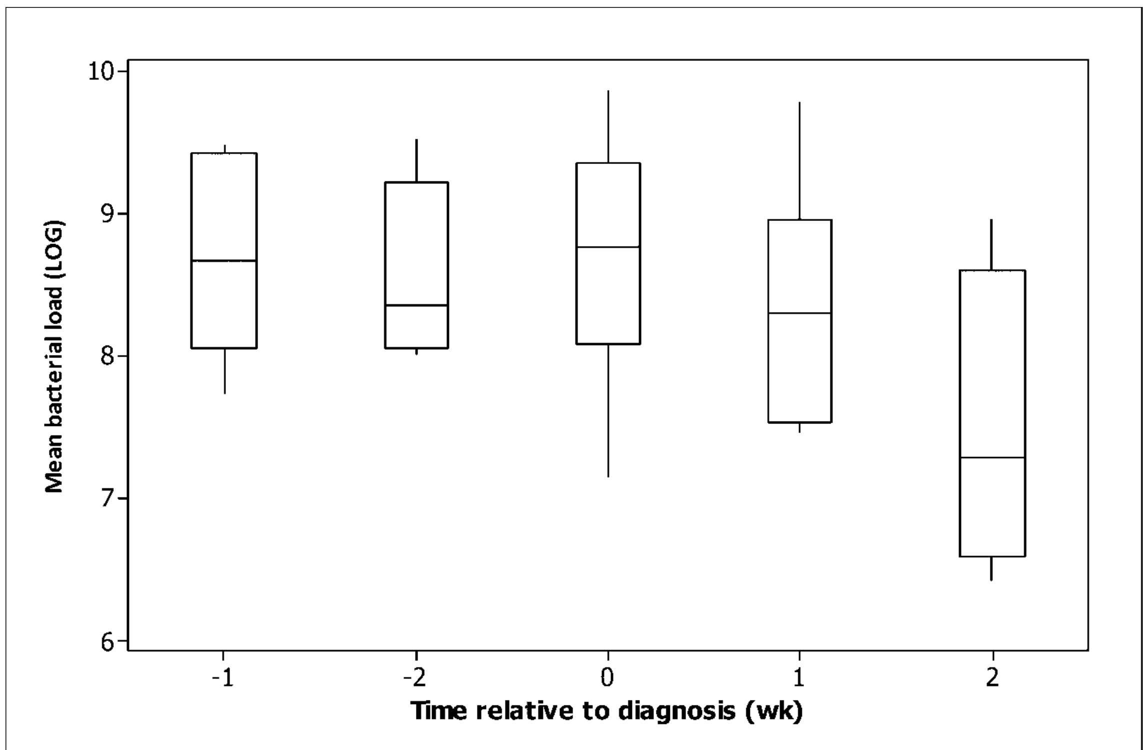
3.4 Statistical analysis

Paired student's *t*- test was used for qPCR mean copies data between NEC and control, excel and SIMCA-13(Umetrics) were used to analysed the exported qPCR raw data as appropriate. Minitab-17 was used to analyse and compared the data between NEC and control babies and the results were expressed as one-way analysis of variance (ANOVA) with Boxplot of copies/g. Excel was used to evaluate clinical characteristics and demographic of the preterm infants in the cohort study. The two-tailed P-values were calculated using Mann-Whitney- U Test software (equivalent to the Wilcoxon rank sum test) as ($p > 0.05$) which was considered to be not statistically significant between NEC and control.'

3.5 Results

A total of 72 faecal specimens from 20 babies were analysed, 37 with NEC and 35 controls. The total bacterial load present in the samples was estimated by measuring the normalised qPCR data (Table 3).

Boxplot A - bacterial load of disease in NEC babies



Boxplot B - bacterial load of Control babies

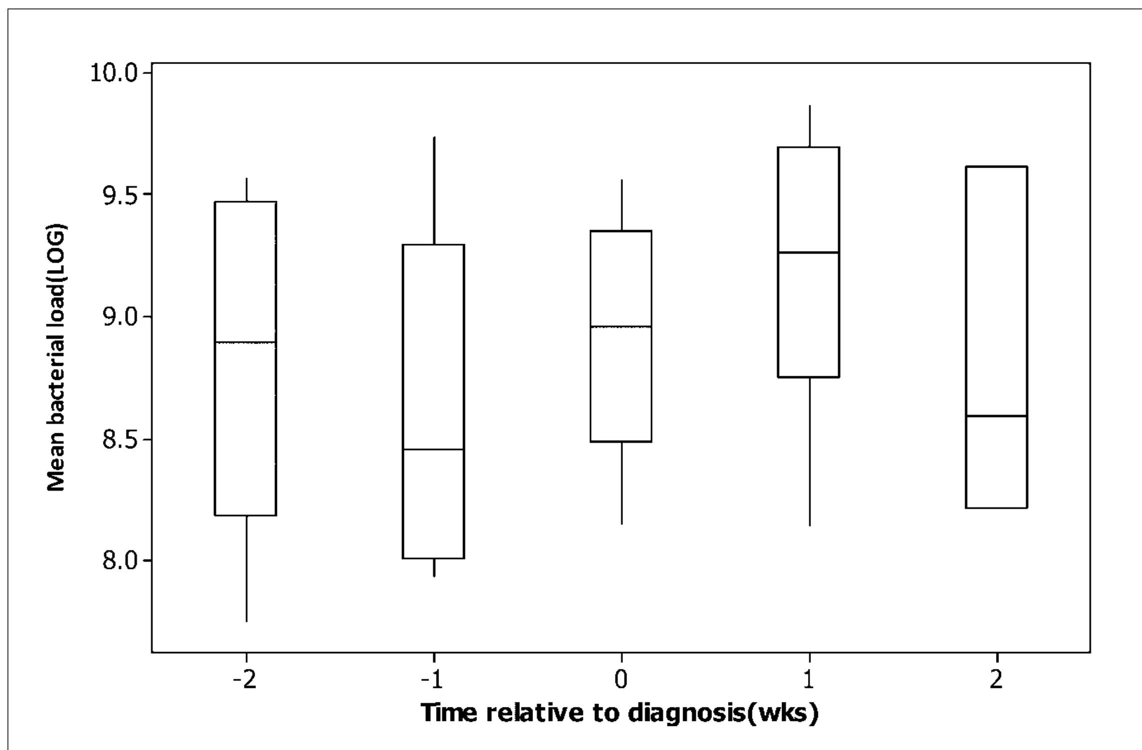


Figure 3-1(A & B): Time relative to diagnosis (wks) of disease in NEC and control infants.

Boxplot A is the quantification data for the NEC babies and B for controls. The horizontal lines inside the boxes represent the median value of bacterial load. The total bacterial loads were determined in NEC patients and matched controls 2 and 1 weeks prior to diagnosis in the cohort, then at diagnosis (0) and 1 and 2 weeks following diagnosis of NEC.

However, the above Figures shows the pattern of bacterial load across the time interval between NEC cases (A) and control cases (B). both trend shows indefinite distinct pattern and variation between within the group, but there is increased in bacterial load at diagnosis compared to the time prior to diagnosis in both NEC and control with marginal decrease after two weeks of diagnosis in both cases. To make the clear comparison see the table below:

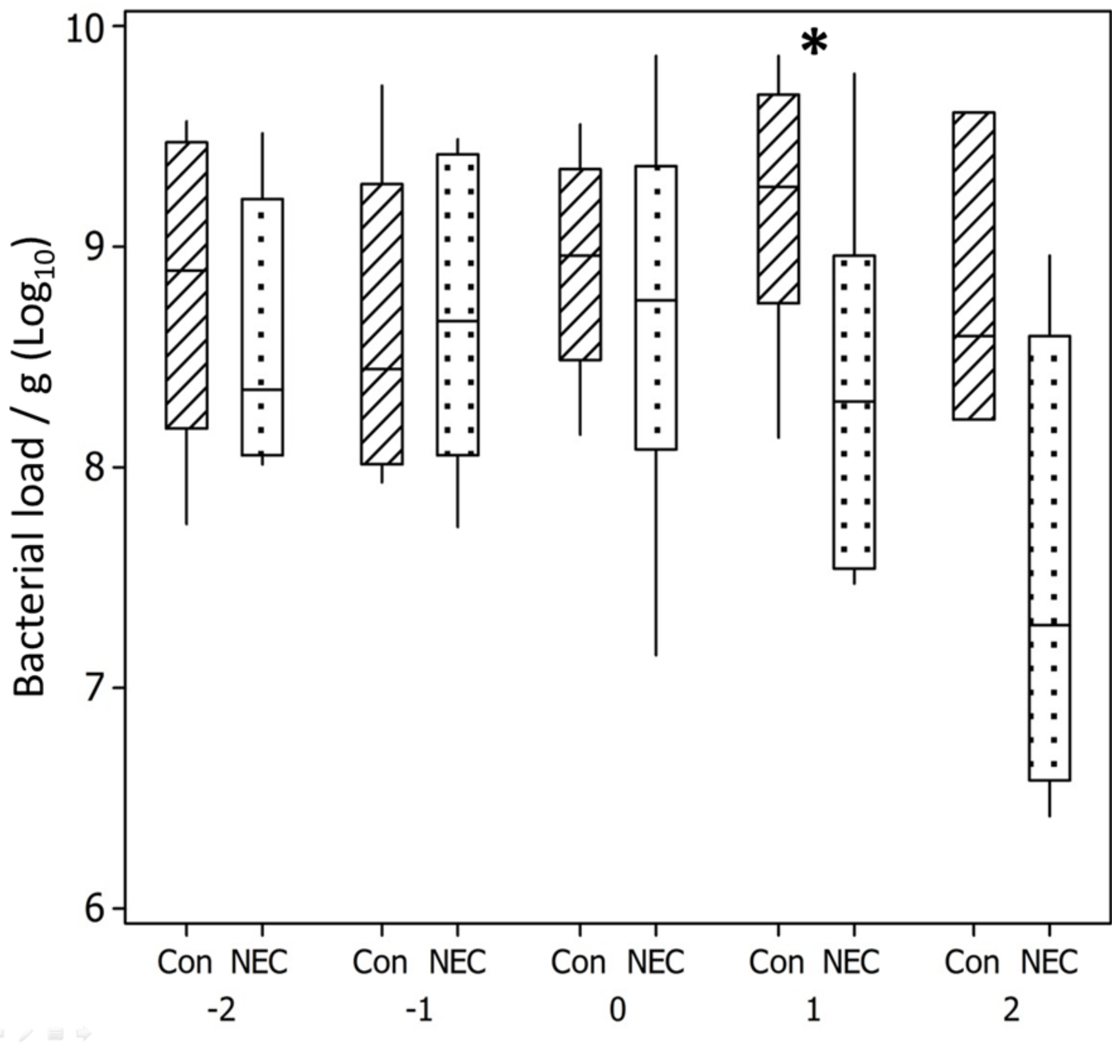


Figure 3-2 Combined total bacterial load between and within NEC and control samples
Horizontal lines inside the boxes represent the median; bars indicate upper and lower quartiles. Asterisks represent an outlier. * denotes significance. The NEC and Con indicates necrotising enterocolitis and control respectively. The number -2 to 2 refer to time intervals in week relevant to NEC diagnosis.

Comparatively, the total bacterial loads between control and disease cohorts were not statistically significant ($p > 0.05$) over time, except for values obtained from the samples between disease and control at 1 week after NEC diagnosis ($p < 0.05$) that shows that the load is marginally greater in controls (Figure 3.2).

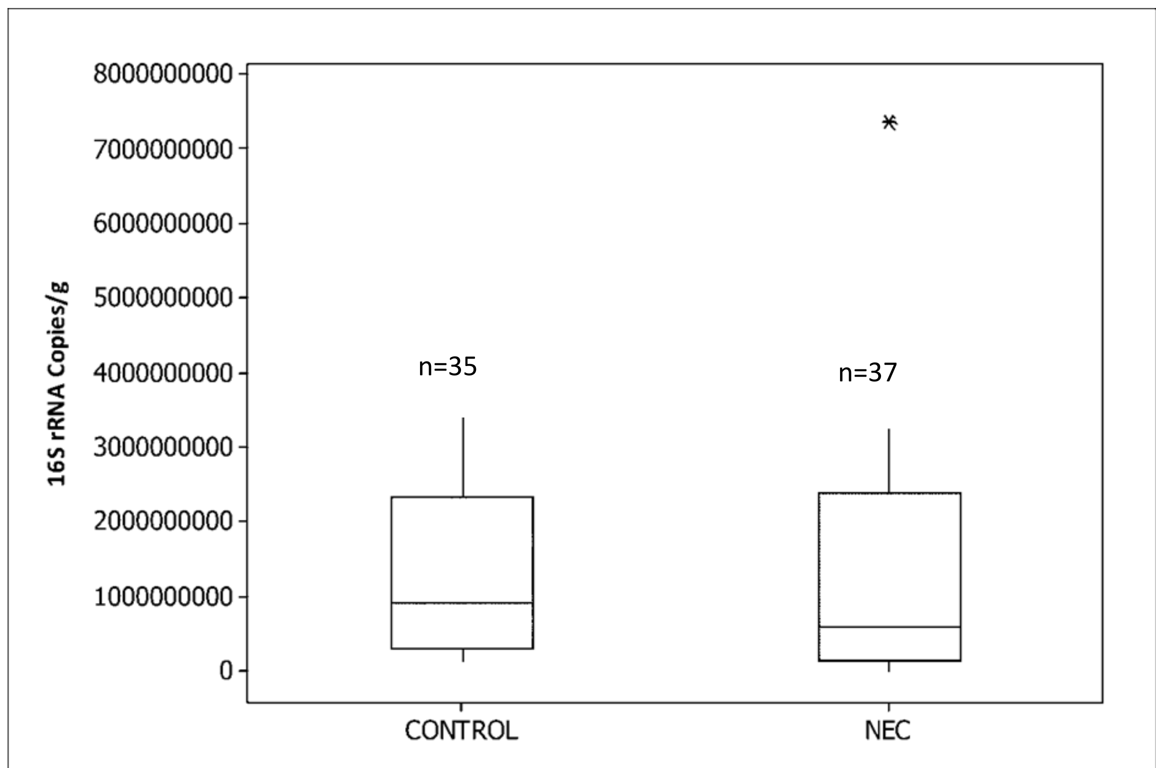


Figure 3-3 qPCR for total microbial load in copies/g of 16S gene sequences in NEC and control samples

*Bars indicate upper and lower quartiles. * Asterisks represent the outlier. The horizontal lines inside the boxes represent the median value of bacterial load.* The 'n' inside the box represent the patient number against each case.

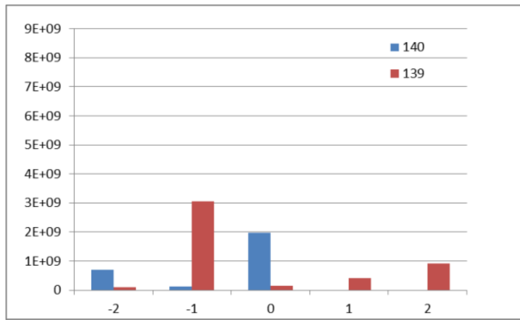
However, from the above Figure, the total bacterial load of the entire NEC cases irrespective of their group across the time points and the entire controls during the cohort study was compared, and the results shows no significant difference between NEC and diseases cohorts *(P= 0.92).*

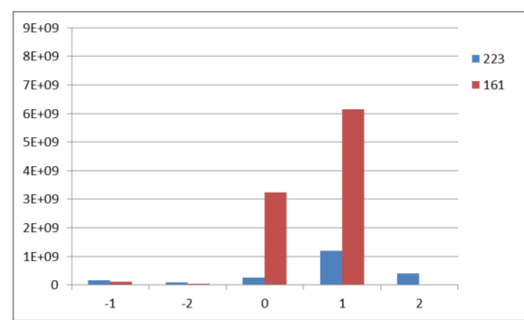
3.6 Comparison of bacterial load between NEC and matched control based on time intervals

The following Figure (Figure 3.4) indicates the comparison of bacterial load and analysis between individual NEC patients with a corresponding matched control at different time intervals. It shows the indefinite pattern of bacterial load across the time interval in the cohort, as variability was observed between NEC samples compared to their matched control with the exception of patient NO 180 & 181 that depicted a comparable pattern of bacterial load at before and after diagnosis (Fig. 3- D). However, in some patients; the stool samples not available in the cohort at prior and after diagnosis for both NEC and their matched controls as such the graph devoid of bars (Fig. 3- I & J).

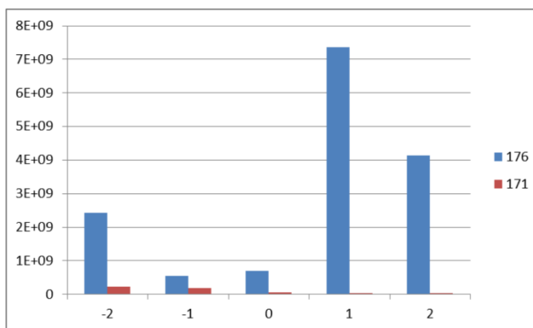
A B

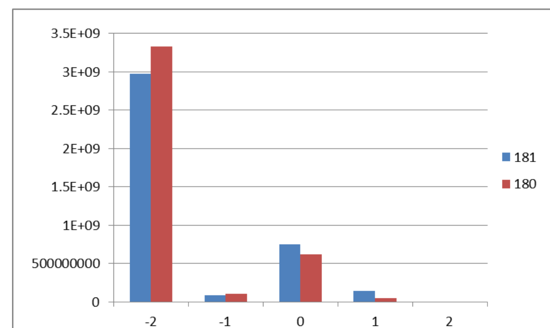
Bacterial number with units



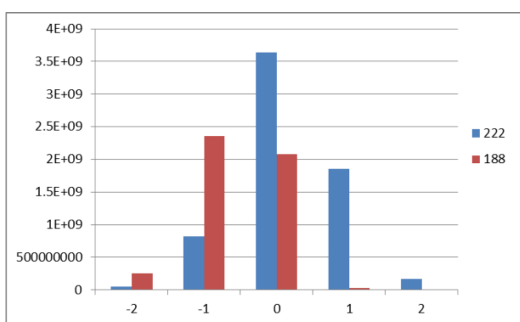


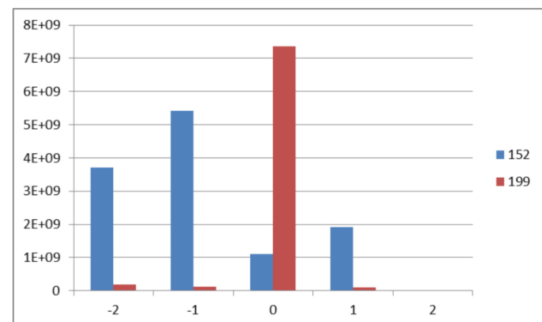
C D



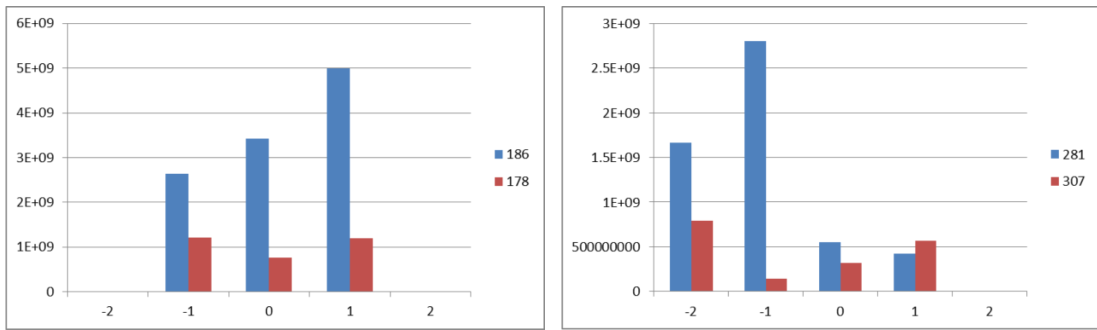


E F

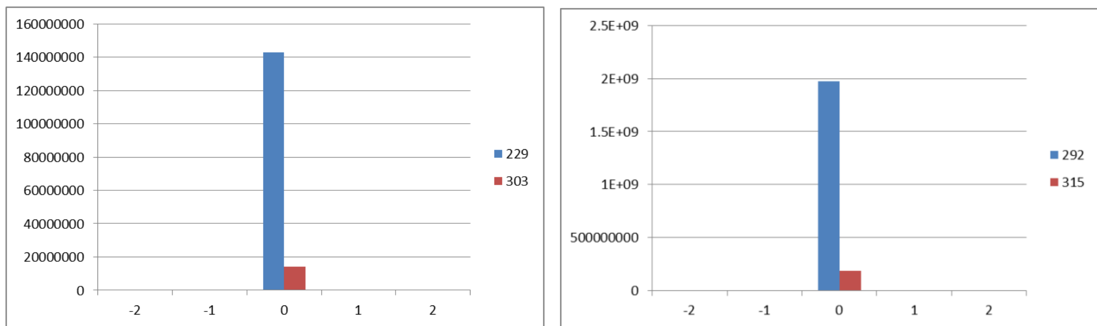




G H



I J



Time intervals

Figure 3-4 (A-J): Comparison of total bacterial load based on time intervals between individual NEC babies with their matched control.

All the bars marked in red and blue colours represent NEC & control respectively. The ages attest when samples collected are shown at the bottom of each bar chart in weeks, started as -2 , -1 refers to weeks prior to diagnosis, while 0 indicates time at diagnosis and 1 & 2 refers to week after diagnosis of NEC.

3.7 Discussion

To date, the actual aetiological pathogens if any responsible for NEC are not known. It is important therefore, to explore if the total bacterial load irrespective of a particular bacterial taxa present might be important etiologically as suggested in studies conducted on the resected gut tissue of NEC samples (Brower-Sinning et al. 2014). Therefore, the need to accurately quantify the bacterial load in the stool samples of preterm infants with confirmed cases of NEC compared with those of matched controls.

It was hypothesized that an accurate real time-PCR assay for quantification of total bacterial load based on time intervals could facilitate the monitoring and management of NEC and allow further studies in understanding preterm gut microbiota. This was informed by previous work in a pig model that showed a relationship between the total bacterial load and the incidence of NEC among preterm pigs (Cilieborg et al. 2012).

Our data based on stool showed no significant difference ($P = 0.92$) between NEC and matched controls at diagnosis, where the total bacterial load per gram of stool was 1.5×10^9 (range 1.4 × 107–7.4×10^9) in NEC and 1.4×10^9 (range 1.4×10^8–3.6×10^9) in control.

Furthermore, the analysis showed no significance *($P > 0.05$)* differences between the bacterial load in the stool samples of infants who went on to develop NEC versus control based on the time interval studied in our cohort and that the resected gut tissue in NEC has a decrease in bacterial load compared to healthy controls. This finding disagrees with the previous work that found an increased in total bacterial load of NEC samples than the controls (Brower-Sinning et al. 2014). However, another study conducted using an animal model indicated that the pathological changes of NEC were noticed from the gut of preterm rabbit and there are significant differences between NEC and control *($P < 0.0001$)* which showed that the incidence and severity of NEC-like damage increased with time

and completeness of gastrointestinal dysfunction (Bozeman et al. 2013). Nevertheless, our study is limited as a universal approach was used in quantifying the bacterial loads over time intervals; this could detect all the pathogenic and beneficial bacteria associated with NEC and other sepsis, as universal primers were used in detecting total bacterial count without specification. (Though, in another cohort- probiotic chapter, the qPCR technique utilising a specific primers targeting only the beneficial bacteria was explored).

Furthermore, there is no consistent trend observed in the bacterial load among the individual patients as the bacterial load indicated variably intermittent among the NEC and control, only that in the Figure 3D (patient No 180 & 181) observed similar pattern which goes concurrently, the microbial load increases 2 weeks before NEC and decreases significantly at a week before NEC with the loads in NEC infants slightly higher than those of the matched control. The pattern continues during and after NEC diagnosis with the counts in NEC slightly lower than the control, but generally it shows microbial load decreases over time; the reasons behind this could be due to the fact that the patients are twin babies which are expected to have similar genetic backgrounds, maternal and other clinical exposures associated with their intestinal microbial ecology (See appendix 7 for details of their demographic data). Furthermore, no significant difference observed between two samples ($p > 0.05$) from the twin babies (patient No 180 & 181). These therefore, suggests that, no specific pattern of bacterial load associated with NEC during our cohort, this data supports the previous finding that demonstrated no specific trends of microbial community associated with NEC (Brower-Sinning et al. 2014).

Generally, there was no significant difference between bacterial load of NEC and matched control during our study. Moreover, our data supports recent reports that found the gut microbial communities of preterm infants differ significantly over time and between different environments. It was found that individuals exhibited very dynamic gut communities while others maintained the same community composition through time. It

also showed these kind of microbial shift and pattern varied widely from one hospital to another during within the cohort (Taft et al. 2014).

Additionally, qPCR technique was explored in a recent study comparing the bacterial load between NEC and non-NEC individuals and the outcomes suggested that; there is no single pattern of microbial diversity associated with the NEC (McMurtry et al. 2015; Brower-Sinning et al. 2014). This finding supports the findings within our cohort.

However, as mentioned earlier that there was no significance difference of total bacterial load between NEC and control within our cohort and an increased in bacterial load from control with decrease in NEC cases following one week of NEC diagnosis, this finding is contrary to a recent study that indicated an increase of bacterial load in both NEC and control using intestinal mucosal sample of preterm pigs but irrespective of time interval (Cathrine et al. 2015). In contrast, research conducted to determine the level of bacterial colonisation on the age of preterm infants showed a distinct bacterial pattern in NEC cases during the first two weeks of life prior to NEC diagnosis, with low bacterial population (de la Cochetiere et al. 2004). This is contrary to our findings as our data shows no distinct pattern and no differences in bacterial load between NEC and control prior to NEC diagnosis and after the disease.

Nevertheless, It has been shown previously in a study conducted on term infants that the total bacterial load is stable after the first week of life (Palmer et al. 2007) and in our cohort, the preterm developed NEC at a median postnatal age of 20 days. Fascinatingly, the previous study found the copy number per gram in term neonates generally persisted in the range of 10^9 to 10^{10}, an order of magnitude higher than the 10^8–10^9 per gram of stool in our infants. The time at which the preterm infants increase to a load comparable to term is unknown, but appears to be after our study ended at a median postnatal age of 34 days.

3.8 Conclusions

No unique or characteristic trend in microbial signatures that might be responsible for causing NEC in preterm infants. Our findings revealed that before and at diagnosis total bacterial loads in babies with NEC fluctuating widely over time and were not significantly different to bacterial loads in control samples ($p > 0.05$). Although, the overall bacterial loads analysed at one week after NEC diagnosis showed that, the load is significant different ($p < 0.05$) between NEC and control. Further work should be conducted utilising specific primer set for targeting particular bacteria based on time intervals that were suggested as putative pathogens responsible for NEC.

4 CHAPTER FOUR

4.1 Molecular characterisation of Probiotics supplementation from the gut of preterm infants and its impacts in the development of NEC and sepsis

Abstract

Probiotics are live microbial supplements that colonize the gut and potentially exert health benefit to the host. It was hypothesized that probiotics strains (Infloran: containing two species of *Lactobacillus acidophilus*-NCIMB 701748 and *Bifidobacterium bifidum*-ATCC 1569 6) would successfully colonize the gut and protect the infants from developing Gastrointestinal tract (GIT) disease. Only a few studies have reported on the efficacy of probiotic supplements to reduce late on-set sepsis in Very Low Birth Weight (VLBW) infants.

Aims: To use high throughput techniques to analyse probiotic diversity and the impact it makes in the gut of preterm infants, with its possible effects on Necrotizing Enterocolitis (NEC) and late onset sepsis (LOS). Also, quantification of the probiotic strains and its relevance in the health and disease was performed.

Methods: Microbial DNA was extracted from stool samples and further analysed with specific primers. Samples were classified into four groups: (i) before, (ii) during and (iii) after probiotic intake with matched controls, and (iv) Post discharge samples. The 88 samples underwent analyses of their bacterial community composition, utilizing 16S rRNA gene profiling. A subset of 75 samples underwent quantitative Polymerase Chain Reaction (qPCR). For probiotic diversity, another subset of 85 samples were collected and analysed by PCR-DGGE (denaturing gradients gel electrophoresis).

Results: QPCR analysis showed significant higher numbers of *B. bifidum* in infants who received probiotic treatment compared to controls *($p<0.001$)*, but no significant increase was observed for *L. acidophilus* within the groups *($p=0.575$)* and also between probiotic

and control groups *(p=0.153)* in the cohort study. The result from 16S rRNA gene library indicated a greater number of *Bifidobacteria* in the bacterial community during supplementation (15.1%) compared to the control group (4.0%). A small number of *Lactobacillus* (4.2%) was detected in probiotic babies but none (0.0%) or little (1.7%) in controls and in groups prior to probiotics administration was detected. There was also reduction of *Lactobacillus* after probiotic was stopped; however, the *Bifidobacterium* contribution to the bacterial community remained in post discharge. The Shannon diversity (H′) from the DGGE profiles indicated a significantly reduced diversity *(p=<0.001)* between the probiotic and non-probiotic babies. Probiotic babies have therefore, statistically lower diversity compared to infants not receiving probiotics.

Conclusions: Probiotic strains (*Bifidobacterium* and *Lactobacillus*) were found to colonize the gut of preterm infants with different levels of abundance (15.1% and 4.2%) and they all increase with probiotic supplements. *B. bifidum* was found to colonize the gut before administering probiotics and were more prevalent in the gut of preterm infants compared to Lactobacilli. Our findings also demonstrated the long term effects of *Bifidobacterium* strain in the preterm gut during post discharge samples. It was therefore conclude that, the findings in this study suggest that probiotics have the potentiality to alter the gut bacterial community in preterm infants.

4.2 **Background**

Probiotics are live microorganisms which when administered in a controlled amount may give health benefits to the recipient (FAO/WHO 2001; de Almada et al. 2015; Wang et al. 2015). Probiotics have been shown to affect the composition of gut microbiota which may confer health benefits (Villarreal et al. 2013; Herbel et al. 2013; Binns 2013; Picaud 2013a; Wang et al. 2015). *Lactobacillus* spp., *Bifidobacterium* spp. *and Staphylococcus* spp. are the most common types of microbes used as probiotics (Herbel et al. 2013), though certain yeasts and bacilli can also be used (Soccol et al. 2013; Julia et al. 2010; Binns 2013).

However, Species of *Bifidobacteria* and Lactobacilli are the two principal probiotic bacteria (Herbel et al. 2013; Chen 2011; de Almada et al. 2015; Alona Bin-Nun et al. 2005). Probiotics can also increase the population of Lactobacilli and *Bifidobacteria* in the gut of preterm infant (Christopher J. Stewart et al. 2013). Probiotics can be taken as a food or supplements and are normally administered after antibiotics treatment in certain infections (Soccol et al. 2013). The susceptibility of probiotic bacteria to antibiotics has been reported; thus suggesting the association of maternal antibiotics to NEC (Beken 2015). Generally, the gut microbial communities of breast-fed infants are primarily composed of Lactic acid bacteria (LAB) including *Bifidobacteria and* Lactobacilli (Turroni et al. 2014), unlike formula-fed infants which is widely diverse of *Bacteriodes*, *Clostridium* and *Enterobacteriaceae* (Haarman & Knol 2005; Groer et al. 2014).

It has been reported that probiotics therapy increases the commensal bacteria and promotes human gut health (Saxon 2015). Probiotics compete with pathogenic bacteria for host binding sites and nutrients while also stimulating host defence mechanisms and enhancing intestinal maturation as well as strengthening the host immune system (Soccol et al. 2013). They may also protect against systemic bacterial invasion by decreasing the

permeability of the GI wall (Julia et al. 2010; Binns 2013). Some probiotic strains have been reported to have an impact in reshaping the gut microbiome on a mice (Wang et al. 2015). However, for probiotics to work effectively and confer a health benefits to the host, it has to be taken regularly (Binns 2013) and with caution more especially in VLBW infants (Zbinden et al. 2015), as high doses may be unfavourable to the host (Neu 2007).

Preterm infants have been shown to have delayed colonisation with potentially important 'beneficial bacteria' such as Bifidobacteria and Lactobacilli (Butel et al. 2007). Some evidences shows that probiotics reduce the risk of NEC/Sepsis in preterm infants with less than 33 weeks GA and in very low birth weights between 1000-1500g (Deshpande & Patole 2013; Picaud 2013b; Alona Bin-Nun et al. 2005; Millar et al. 2003; Yang et al. 2014; Shlomai et al. 2014; Picaud 2013a; Neu 2007). Other studies have shown that the mortality rate of the preterm infants receiving probiotics was significantly lower than controls *(P=0.003)* (Yang et al. 2014; Neu 2007).

Despite the benefits of probiotic administration, there are concerns as some strains of probiotics can cause bacteraemia in infants, children, adults and older patients (Singh, Firek, Brooks, Castelle, et al. 2015; Picaud 2013a; Herbel et al. 2013). Specifically, *Bifidobacterium longum* was detected in the blood culture of the preterm infants alongside probiotics intake (Zbinden et al. 2015). Though, a meta-analysis study of probiotic supplementation, showed that probiotics may have no (Costeloe et al. 2015) or minimal adverse effect on normal feeding and growth among the preterm infants (Shlomai et al. 2014; Yang et al. 2014).

Currently, the routine use of probiotic prophylaxis in clinical practice is still insufficiently understood despite it being reported as beneficial in the therapy of NEC (Costeloe et al. 2015; Zbinden et al. 2015). As such probiotic supplementation should be adopted cautiously (Shlomai et al. 2014), as there is no definitive evidence whether different

strains of the routinely used probiotics produce variable outcomes in health and diseases (Costeloe et al. 2015; Shlomai et al. 2014; Beken 2015).

Lactobacilli *acidophilus* has been examined intensively as a probiotic supplement, it was found to increase the number of Lactobacilli in an infant gut (Breitbart et al. 2008). When combined with Lactitol (beta-galactosido-sorbitol) a non-absorbable disaccharide , it increased both the Lactobacilli *and Bifidobacteria* numbers (van Zanten et al. 2014; Ouwehand et al. 2009; Bjorklund et al. 2012). It was first isolated in 1990 and name *Bacillus acidophilus.* It is part of the normal flora of human GIT and as important probiotic strains detected in human breast milk (Cárdenas et al. 2015).

Bifidobacteria are residents of the human and animal gastrointestinal tract, dental caries, vagina and oral cavity (Barrett & Guinane 2013; Amin et al. 2013). Several *Bifidobacterium* strains are now being used as probiotics including the species of *Bifidobacterium animalis*, *B. bifidum, B. breve*, *B. longum* fermentum and some other species (Amin et al. 2013). *B. bifidum* is found to colonize the infant gut more especially those receiving breast-milk (Millar et al. 2003; Turroni et al. 2014).

High through-put NGS techniques have extensively improved our understanding of intestinal microbiota and the impact of probiotics in health and diseases (Villarreal et al. 2013; de Almada et al. 2015; Herbel et al. 2013; Ju & Zhang 2015). The use of qPCR assays and other molecular techniques has identified and quantified the faecal *Bifidobacterium* and Lactobacilli species (Haarman & Knol 2005; van Zanten et al. 2014; Wall et al. 2007; Herbel et al. 2013) as well as other probiotic strains (Tobin et al. 2013).

Here the use of high throughput techniques to study probiotic diversity and babies receiving Infloran capsule compared to a control group was explored. Infloran is used as a food supplement in some NICUs and contains *Lactobacillus acidophilus* and *Bifidobacterium bifidum*. The aimed was to investigate the bacterial load in the preterm

gut in babies whose diet is supplemented with Infloran and the long term effects of this supplementation. There is earlier research that has used Infloran supplementation (Repa et al. 2015), containing different species of Bifidobacterium as *Bifidobacterium longum* instead of *B. bifidum*; but with the same species of Lactobacillus as used in our cohort (Zbinden et al. 2015).

4.3 Methods

4.3.1 Study design

Samples Identification for probiotic DGGE study: - Demographic data was provided from the infant cohort collected from neonatal intensive care unit of the RVI (Table 4.1). A total of 85 samples (57 Probiotic and 28 Non-Probiotic) stool samples were identified. (See the demographic data in the appendix 8)

4.3.2 Samples identification for NGS and qPCR in probiotics study

The study protocol compared a cohort of preterm infants, who had been admitted in the NICU and received probiotics with a control group who did not receive the probiotic. Informed consent was obtained from parent as well as ethical approval. Eighty eight samples collected from the patients who received Infloran as a prophylactic probiotic containing the two species of bacteria: *Lactobacillus acidophilus* and *Bifidobacterium bifidum*. Bacterial DNA from each samples were subjected to NGS (60 probiotics and 28 controls). Subsets of 75 samples were subjected to qPCR analysis, (50 from probiotics groups and 25 samples from control group). All the stool samples collected were from preterm infants born between 27 to 31 weeks gestational age. The two techniques were explored in order to know the relative abundance of the desired organisms (in case of NGS data) and the actual bacterial load of those organisms (in case of qPCR data) in the gut of preterm infants in our cohort.

4.4 Probiotic qPCR and NGS study

Table 4-1: Demographic data for the clinical cohort

	Control			Probiotic						
Patient No.	263	271	272	270	273	274	275	276	277	278
Gestational Age	27	31	31	25	27	24	28	28	24	24
Birth weight	550	2030	1535	750	945	700	1100	1150	620	620
Delivery	CS	V	V	V	CS	V	CS	CS	CS	CS
Sex	F	F	M	M	F	M	F	M	F	M
Total No. of Samples	10	7	8	8	7	12	5	4	3	7
Post-discharge (age in months)	N	N	N	Y (25)	Y	Y	N	N	N	Y

4.4.1 **Characteristics of preterm infants participants**

The probiotic supplement was given to preterm infants of less than 32 weeks gestation soon after initial introduction of feeds until it reached 34 weeks corrected. The dose contain a half of an Infloran capsule which was administered twice a day, equating to 125 mg b.d at 10^9 organisms per dose. All the infants received maternal breast milk. All babies in this study had a birth weight of less than 1500g. A total of 75 stool samples were collected from the cohort, the samples were divided in to the following groups during the cohort study: before, during, and after exposure and a control groups as well as post-discharge (Post discharge is after they leave the NICU as follow up samples) Table 4.2 and Table 4.3 shows demographic and clinical data for the different groups by which the samples were classified in relation to probiotic intake and the number of samples collated within each group during the cohort.

Table 4-2: Frequency of probiotics groups in the cohort study

Probiotic schedule	Number of sample
Before probiotic	**5**
During probiotic	**30**
After probiotic	**11**
Post-discharged	**4**
Control	**25**
Total	**75**

Table 4-3: Clinical characteristics of preterm infant's cohort

Characteristics	Frequency of Occurrence (Average range)	
	Probiotics	**Control**
Total stool samples	50	25
Total samples by Gender: Male/Female	22/28	8/17
Birth weight (g)	835.6(620-1150)	1371.1(550-2030)
Gestational age (weeks)	26(24-28)	29(27-31)
Delivery: Vaginal/ Caesarean	22(44%)/28(56%)	15(60%)/10(40%)
Feeding: Breast milk/ Formula	50/0	25/0

4.5 **Results**

4.5.1 **Probiotic and Non-Probiotic diversity study**

4.5.1.1 DGGE Analysis of microbial community in Probiotic and non-probiotic diversity

The Molecular fingerprinting by DGGE analyses of the 10 patients with the total of 85 samples (57 probiotics and 28 non-probiotics samples) yielded 22 distinct bands for the probiotic samples (4-12 bands per lane) (Fig. 4.1) and 36 distinct bands for non-probiotic samples (5-18 bands per lane) (Fig. 4.2) respectively. Each DGGE band was assumed to be a single taxon or operational taxonomic unit (OTU) from which measurements of species richness and evenness could be derived.

Qualitative observation from the DGGE profile of probiotic diversity (Fig. 4.1) showed that the bands were distributed towards the middle and lower region of the gel in non – probiotic samples (Fig. 4.2). The band matrix was analysed using PCA (unconstrained) which showed that the non-probiotic babies have large variation in the DGGE profiles whereas the probiotic babies all develop comparable DGGE profiles (Fig. 4.3). All the probiotic babies cluster together while the non-probiotic babies were widely distributed with a large variation within and outside the circle. However, the two groups cluster independently, indicating distinct DGGE profiles between the treatment groups. To determine if there was a statistically significant diversity between treatments, Shannon diversity (H′) indices were calculated and showed that probiotics babies have reduced diversity when compared to non-probiotics $P = <0.001)$ (Fig. 4.4).

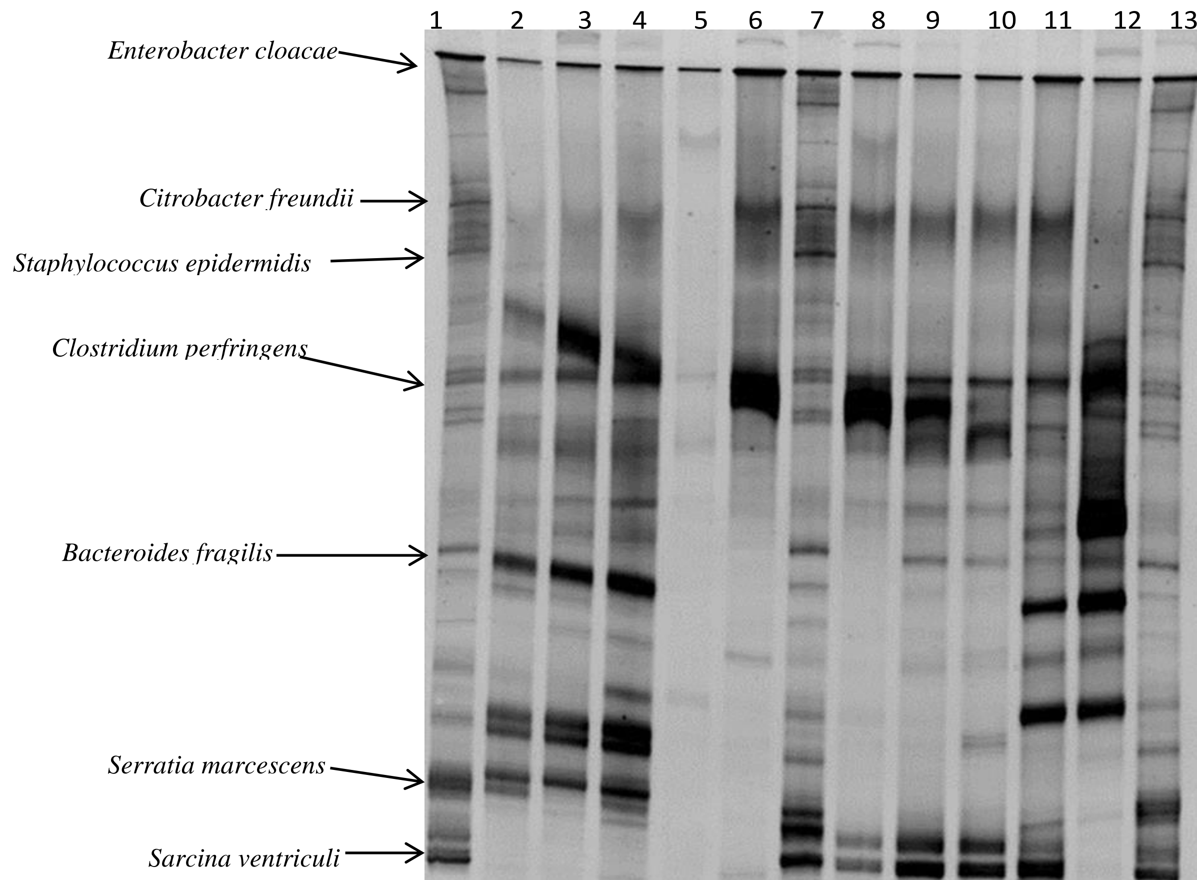


Figure 4-1 representative DGGE profile on 35% - 55% denaturing gradient showing different bands of Probiotic samples

The gel contains 13 Lanes. L 1, L 7 and L 13 representing the Bacterial ladder of the isolates, while the remaining 10 lanes on the image represent the probiotic samples loaded. Lane numbers 2-Probiotic sample (PS-327), 3-PS 3253, 4 – PS3248, 5 –PS 3242, 6 – PS 3343, 8 – PS 3322, 9 – PS 3310, 10 – PS 3300, 11– PS 3331, - 12 - PS 3263.

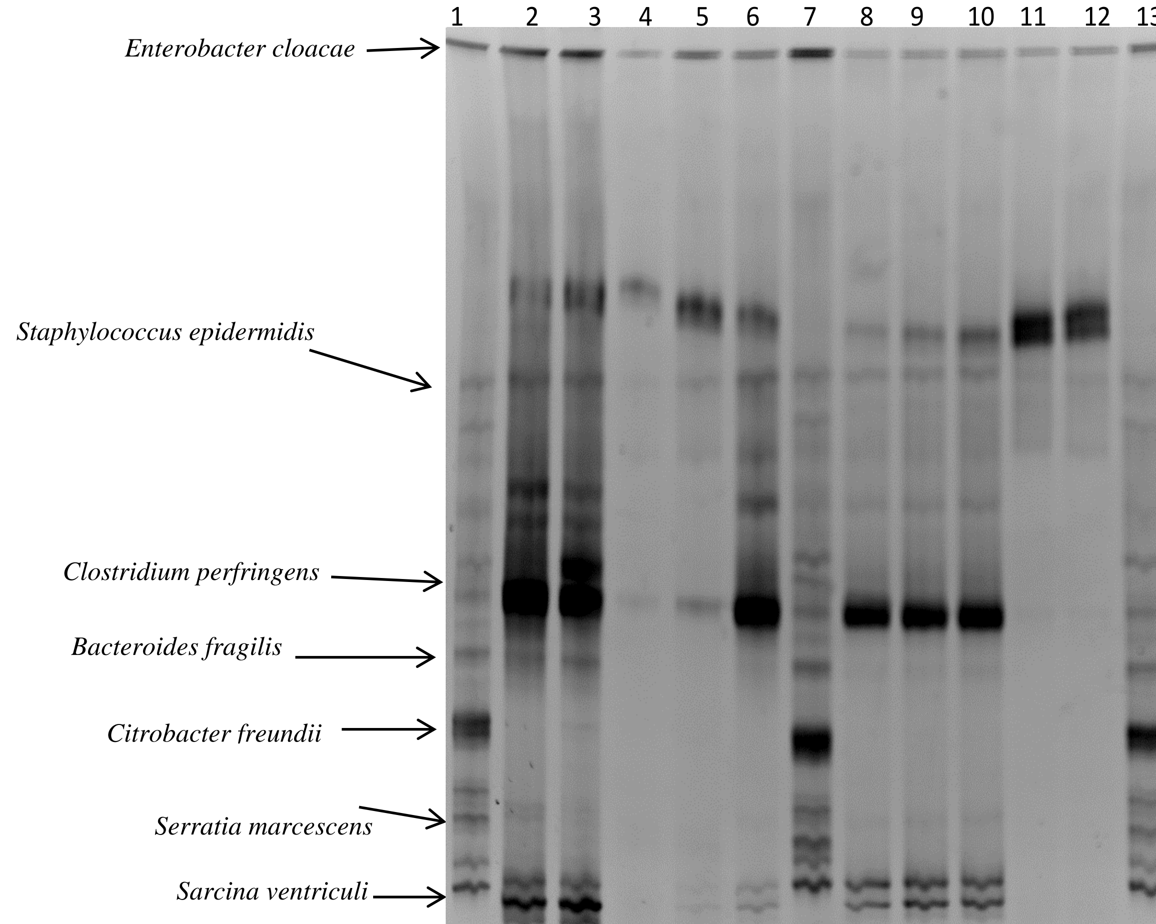


Figure 4-2: A representative DGGE profile on 35% - 55% denaturing gradient showing different bands of non- probiotic samples

The gel contains 13 Lanes. L 1, L 7 and L 13 representing the Bacterial ladder of the isolates, while the remaining 10 lanes on the image represent the non- probiotic samples loaded. Lane number: 2-Probiotic sample (PS-3258), 3-PS 3234, 4 – PS 3226, 5 –PS 3215, 6 – PS 3197, 8 – PS 3174, 9 – PS 3149, 10 – PS 3090, 11– PS 3320, - 12 - PS 3337.

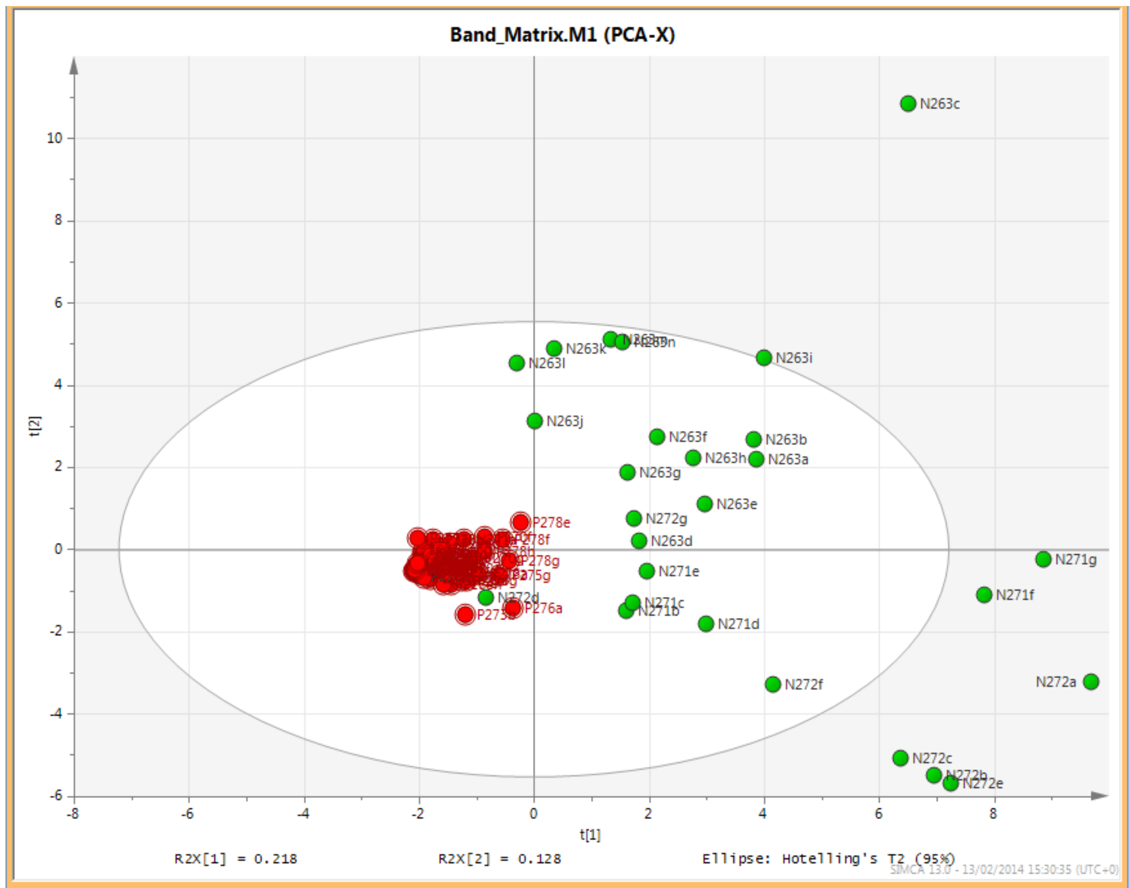


Figure 4-3: Band Matrix (PCA) of Probiotic and Non-Probiotic DGGE Profiles

Green circles represent Non-probiotic babies and red circles represent probiotic babies. The numbers associated with the circles i.e. 263- 278 represents the patient's unique identification number in the cohort study. Thus; 263(a-n), 271(a- g) and 272(a-g) stands for non-probiotic babies while, the number 270(a-h), 273(a-i), 274(a-p), 275(a-e), 276(a-g), 277(a-c) and 278(a-i) stands for probiotic babies.

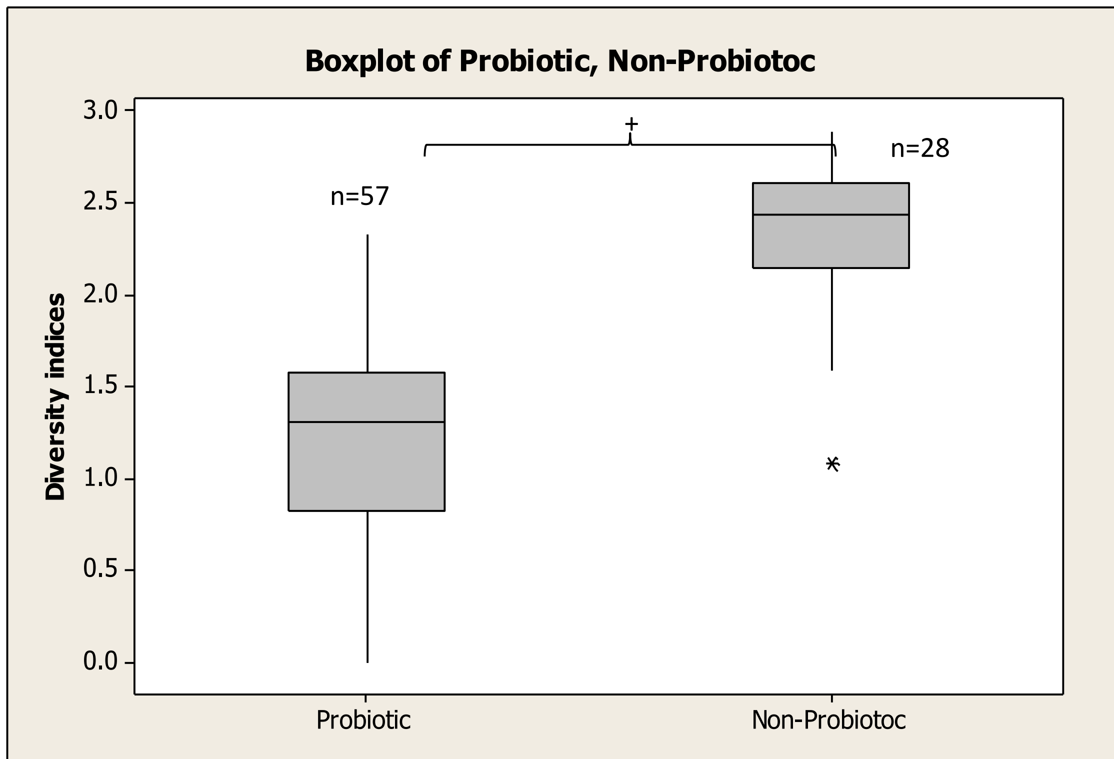


Figure 4-4: Boxplot of Probiotic and Non-Probiotic data

Asterisks represent the outlier; the horizontal lines inside the boxes represent the median value of diversity indices. + denotes significance (P = <0.001). The 'n' inside the box represent the patient number.

During the research, it was then sought to examine how the bacterial community differed between babies receiving probiotic and those that do not during their stay in NICU and the impacts on the preterm gut. To achieve this, the data was modelled using PLS analyses (Fig. 4.5).

In PLS analysis, an R value of 0.8 or above is suggestive of securing an adequate (comprehensive) set of formative measures assuming (adequate loading), and an R value of 0.9 or above indicates an extremely strong result. Q2 >0.5 implies the model has predictive relevance, whereas Q2<0.5 represents a lack of predictive relevance (Falfari, Alessandro 2010). Forming a model based on the DGGE data shows high predictive value, i.e. an unknown sample would be identified as receiving probiotic or not. Well modelled variables have high green bars, R2, and high blue bars, Q2, at levels of 0.5 or above. Here R2 indicates how well the variation of a variable is explained, while Q2 indicates how well a variable can be predicted.

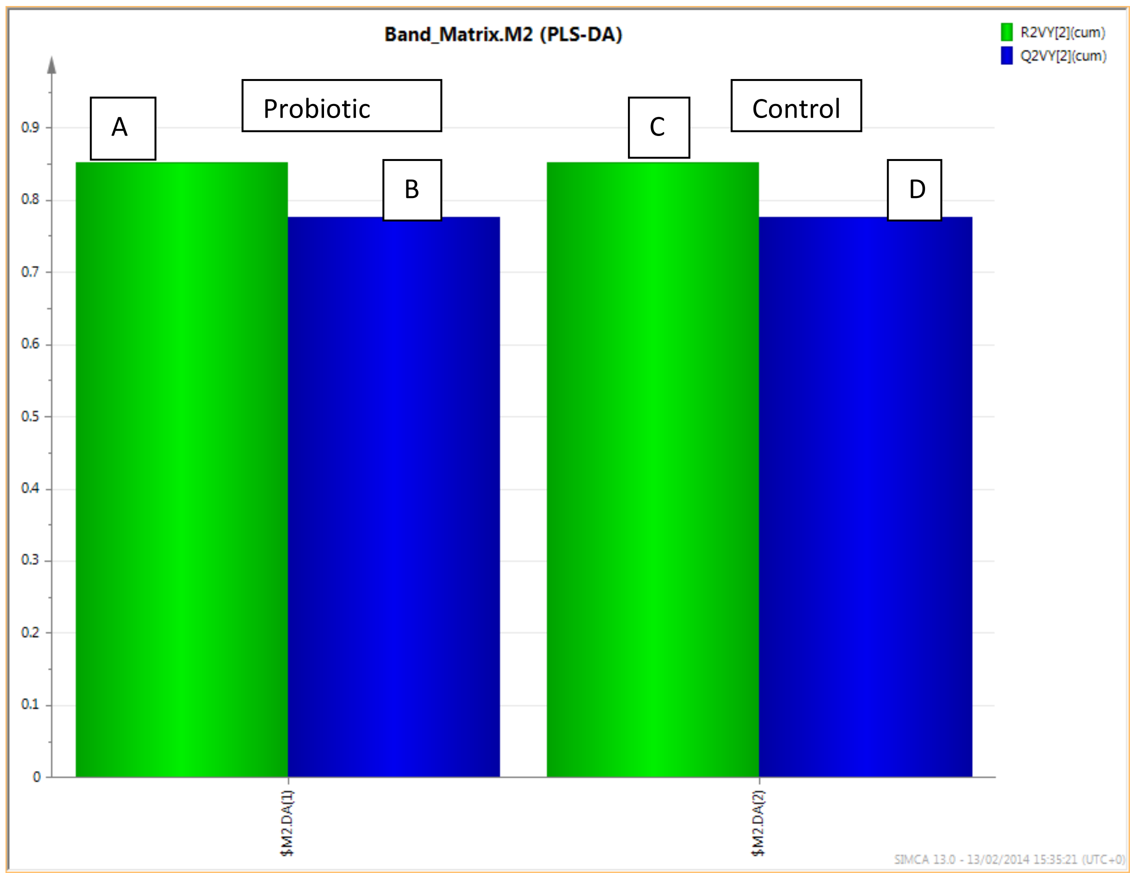


Figure 4-5: PLS Bar chart model of Probiotic and Non-Probiotic DGGE

The data showing valid prediction of babies receiving probiotic, the green bars of (A & C) in R2 imply that receiving probiotic treatment has a significant impact on preterm babies. While the blue bars (B & D) in Q2, implies that hypothetical assumption model is valid significantly in predictive sense base on the data generated during the analysis. (As all the variables are above 0.5)

4.5.2 Quantification of *L. acidophilus*-(NCIMB 701748) by qPCR in stool samples

The qPCR analysis of the stool samples detected quantifiable numbers of *L. acidophilus* in 2 samples during probiotic administration and 3 in the controls. ANOVA analysis of the data shows no significant difference within the groups in the cohort study (Fig. 4.6). While, between probiotics and controls (Fig.4.7) displays few outliers in both cases, the grouping by Tukey's family error rate has *(p =0.153)* at 95% confidence level. This shows no significant difference between the probiotic and control groups in the cohort study (mean Log=0.00±0.00).

However, *Lactobacillus species* Not detected prior to probiotic intake and after administration as well as at post discharge samples, they were only detected in control babies and small number during administration (Fig. 4.6). But *Lactobacillus species* were detected higher in control group compared to probiotic samples (Fig. 4.7).

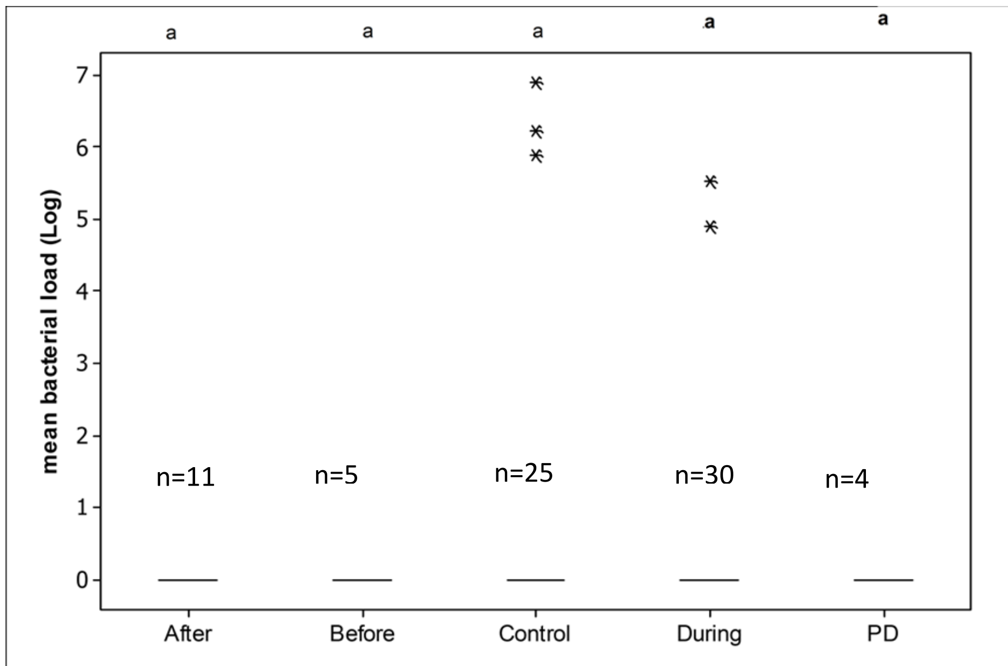


Figure 4-6: Boxplot of Lactobacterial load based on time interval to Probiotics intake.

* Asterisks represent the outliers. “a” represents the grouping by Tukey’s family.

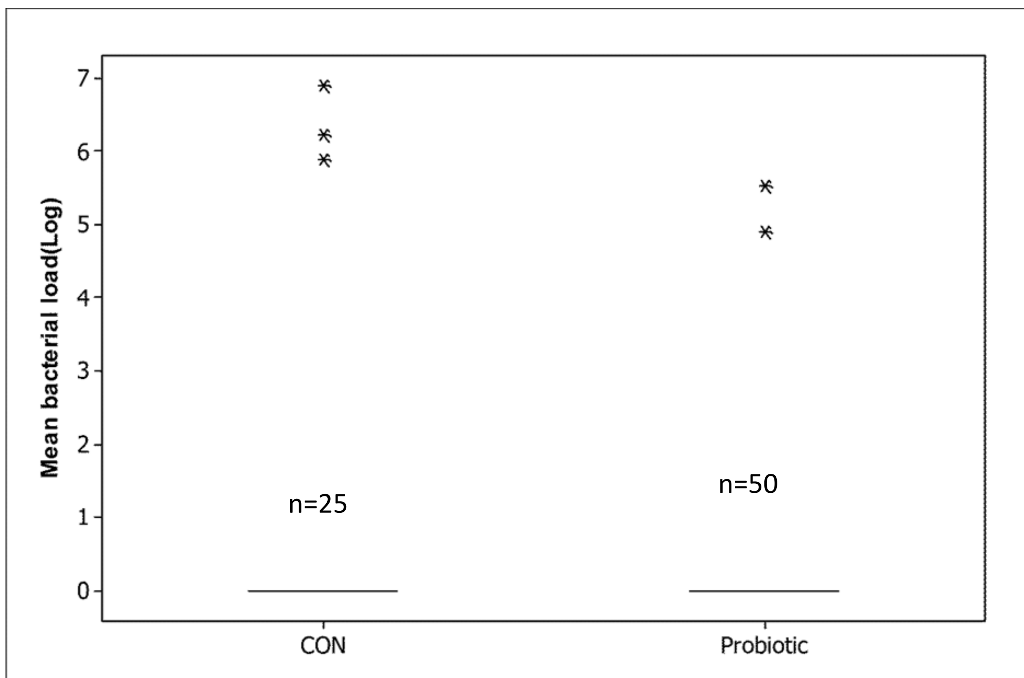


Figure 4-7: Boxplot of Lactobacterial load between Probiotics and Control group.

* The horizontal lines inside the boxes represent the median value of bacterial load. “a” represents the grouping by Tukey’s family. *L. acidophilus* was detected in only two samples during probiotic administration and in 3 control samples, while absence in the remaining time points during the cohort. The numbers ‘n’ inside the boxes represent the patients number with respect to each goup.

4.5.3 Quantification of *B. bifidum*-(ATCC 1569 6) by qPCR in stool samples

Quantification results of *B. bifidum* (ATCC 1569 6) as revealed by Minitab analysis showed the mean bacterial load among the groups in the cohort study. The *B. bifidum* was detected in one probiotic baby before administration but was absent in the control cohort. *B. bifidum* load was also significantly (p = <0.001) higher at all-time points following administration of probiotic.

Figure 4.8 displays no outliers probiotic supplementation; before, control or in post discharge samples. The *Bifidobacterial* load after probiotic intake has highest median with 1 outlier, then decrease a bit during post discharge samples. Also 6 outliers have been observed from the load during probiotic intake, it also recorded lower or less at before and control respectively, the grouping by Tukey's family error rate has *(P <0.01)* at 95% confidence level. This shows that it is highly significant.

Figure 4.9 displays no box at control group, while observing a reasonable load counts of *Bifidobacteria* been detected from the gut of infants who received probiotic supplements with only few numbers not detected. The grouping by turkey's family error rate *(P <0.01)* at 95% confidence level which indicates highly significant difference between probiotic and control group in the cohort study.

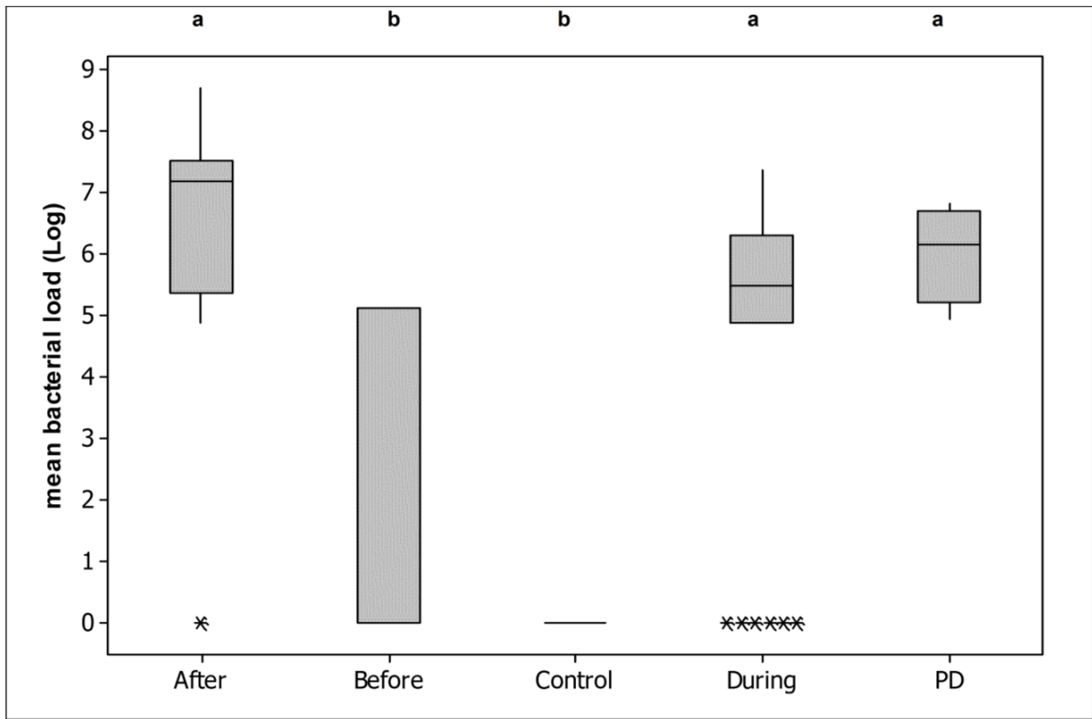


Figure 4-8: Boxplot of Bifidobacterial load based on time to Probiotics intake

* Asterisks represent the outliers, "**a & b**" = represents the grouping by turkey's family. The horizontal lines inside the boxes represent the median value of bacterial load.

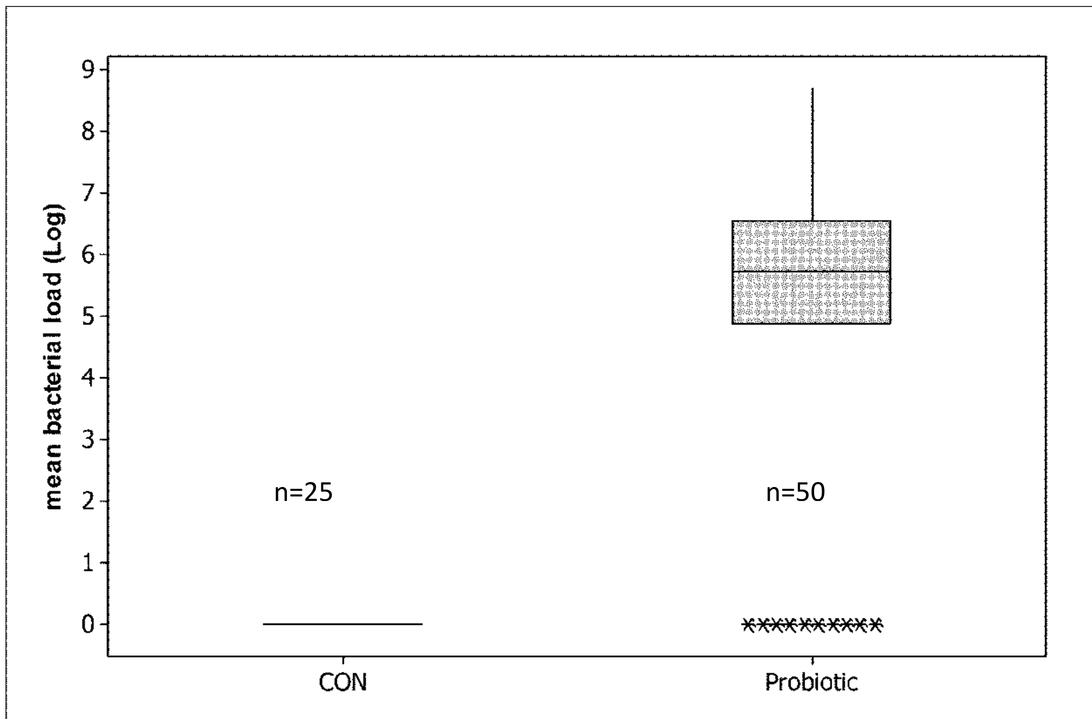


Figure 4-9: Boxplot of Bifidobacterial load between Probiotics and Control group.

* Asterisks represent the outliers. The horizontal lines inside the boxes represent the median value of bacterial load. The ‘n’ inside the bo0x represent the patient number.

4.6 Probiotic Next generation sequencing study

NGS analyses showed significant change in bacterial communities (Fig. 4.10) in the different group studied. The most common taxa in the sample are: *Enterococcus, Escherichia, Enterobacteriaceae, Staphylococcus* and *Bifidobacterium.* Some taxa only appear post discharge: *Bacteriodes, Proteus* and *Lachnospiracea*, while, the probiotic taxa are: *Bifidobacterium* and Lactobacilli and the most common taxa appear in the control group are: *Bifidobacterium, Enterobacteriaceae, Enterococcus, Escherichia, Staphylococcus, Clostridium* and *Veillonella*. The differences between experimental samples and controls are: Lactobacilli*, Bacteriodes, Lachnospiracea* and *Proteus* (Table 4.4).

Bifidobacterium spp. increase sequentially with probiotic supplementation with an 11.1% contribution before supplementations are given, which grow to 15.0% and 19.3% during and after supplementation regimen. But fall down in Post-discharge sample 14.2% and remain low in control (4.0%) groups. *Lactobacillus* taxa contribute 1.7%, 4.8% and 6.1 % to total bacterial community before, during and after probiotics respectively. Very little or no *Lactobacillus* spp. was found among post-discharged and control groups (Table 4.4). Therefore, *Bifidobacterium* species recorded high population numbers from the gut of preterm infants. While *Lactobacillus* taxa detected from the gut of preterm infants (Fig. 4.10) who received probiotic during the cohort study. The samples in the post discharge (PD) group were more diverse (with different bacterial taxa classified as ‘others’ in (Fig. 4.10) as the babies associate with domestic environment and begins to eat solid food.

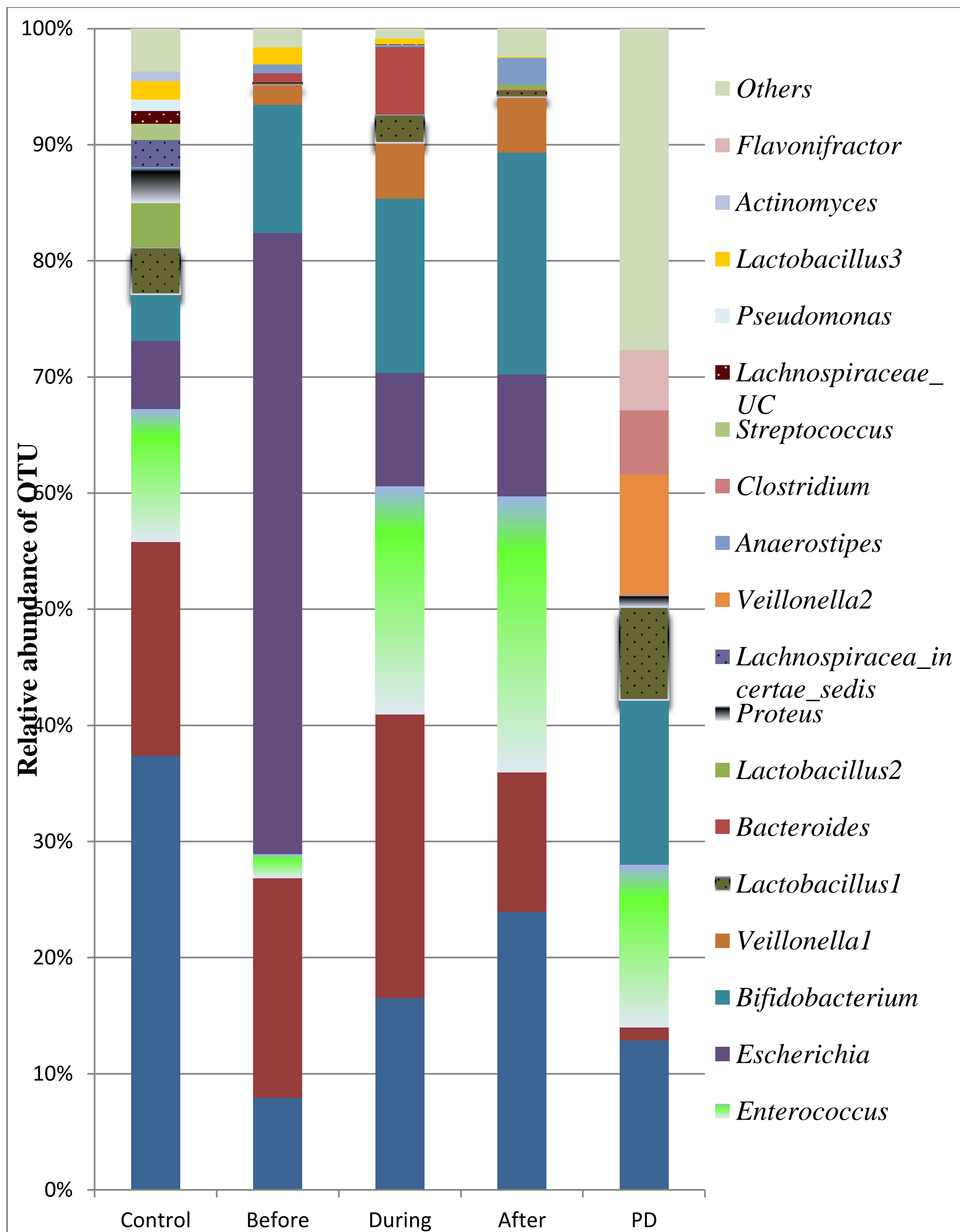


Figure 4-10: Relative abundance of Bacterial Taxonomy at genera level from MiSeq analysis.

Proportion of OTUs matching the 20 most frequently observed bacteria across the samples. Colours on the charts matching with the bacterial taxa on the legend represent the diversity in percentage. Sequences matching other bacterial taxa with less abundance OTUs are classified as "others" shown on the legend with their matching colour on the chart. The 'UC' on the legend indicates unclassified OTUs. The final bar labelled as PD represents post discharge samples. (The data were sent in to MG-RAST with accession number 4615668.3-4615749.3)

Table 4.4: Abundance of Most common taxa in samples

Bacterial Taxa	Probiotic (%) (n=60)	Control(%) (n=28)	Before (%) (n=10)	During (%) (n=32)	After (%) (n=14)	Post-disch. (%) (n=4)
Bifidobacterium	15.1	4.0	11.1	15.0	19.3	14.2
Lactobacillus	4.2	0.0	1.7	4.8	6.1	0.0
Enterobacteriacaea	18.8	37.4	7.9	17.4	31.1	12.8
Bacteriodes	0.0	0.0	-	0.0	-	10.3
Enterococcus	17.4	18.4	18.9	24.9	8.2	1.2
Escherichia	11.9	11.4	2.1	18.4	15.3	14.0
Lachnospiracea	0.0	0.0	0.0	0.0	0.0	5.1
Proteobacteria	0.7	1.6	1.4	0.5	0.0	0.0
Proteus	0.0	0.0	0.0	0.0	0.0	5.5
Staphylococcus	25.2	5.9	53.4	9.6	12.6	0.0
Clostridium	0.2	3.8	0.0	0.0	0.6	0.0
Veillonella	1.08	4.0	0.2	2.4	0.7	7.10
Total	**94.58**	**86.5**	**96.7**	**93**	**93.9**	**70.2**

The probiotic column stand for the entire babies receiving probiotic supplements during the cohort (before, during and after) to compare them with their healthy matched controls in the subsequent column (control), then between the control and the individual groups for easy comparison (see the descriptive narration : 4.6, on the previous pages concerning this table: 4.4 and previous Figure 4.10 under).

4.6.1 Faecal microbial diversity on probiotics babies by MiSeq

The gut microbiome was distinct between preterm infants who received probiotics with their healthy control babies (R2Y = 0.85, Q2 = 0.60), the gut bacteria within preterm infants receiving probiotic supplement remained relatively comparable (R2Y = 0.36, Q2 = 0.27), when comparing to sampling point before probiotic supplementation (R2Y = 0.18, Q2 = 0.02) and after supplementation stopped (R2Y = 0.25, Q2 = 0.09). Therefore, R2Y here denotes how well the variable is explained, and Q2 signifies how well the variable is predicted as a result of probiotic administration. The PLS-DA matrix illustrated the bacterial OTUs associated with each group with different colours that cluster separately according to individual groups in the cohort (Fig. 4.12) with the exception of post-discharge samples. When the PD samples were included, all NICU cluster away from the PD samples (Fig. 4.13); this indicates that bacterial OTUs associated with post-discharged are different compared to other groups in the cohort study. Nevertheless, PLS-DA analysis of control group compared to other probiotics groups revealed that 4x *Lactobacillus* spp.(otu0007, otu0011, otu0013 & otu0044) were detected in probiotic babies and none was detected from the matched control while only 1x *Bifidobacterium species* detected from probiotic group (otu0003). Interestingly, *Streptococcus* and *Clostridium* spp. were increased in controls (Fig. 4.15). The green bar in R2 implies that administering probiotic supplement has a significant impact on preterm babies (Fig. 4.16). While the blue bars in Q2, implies that hypothetical assumption model is valid significantly in predictive sense base on the data generated during the analysis (Fig 4.16.).

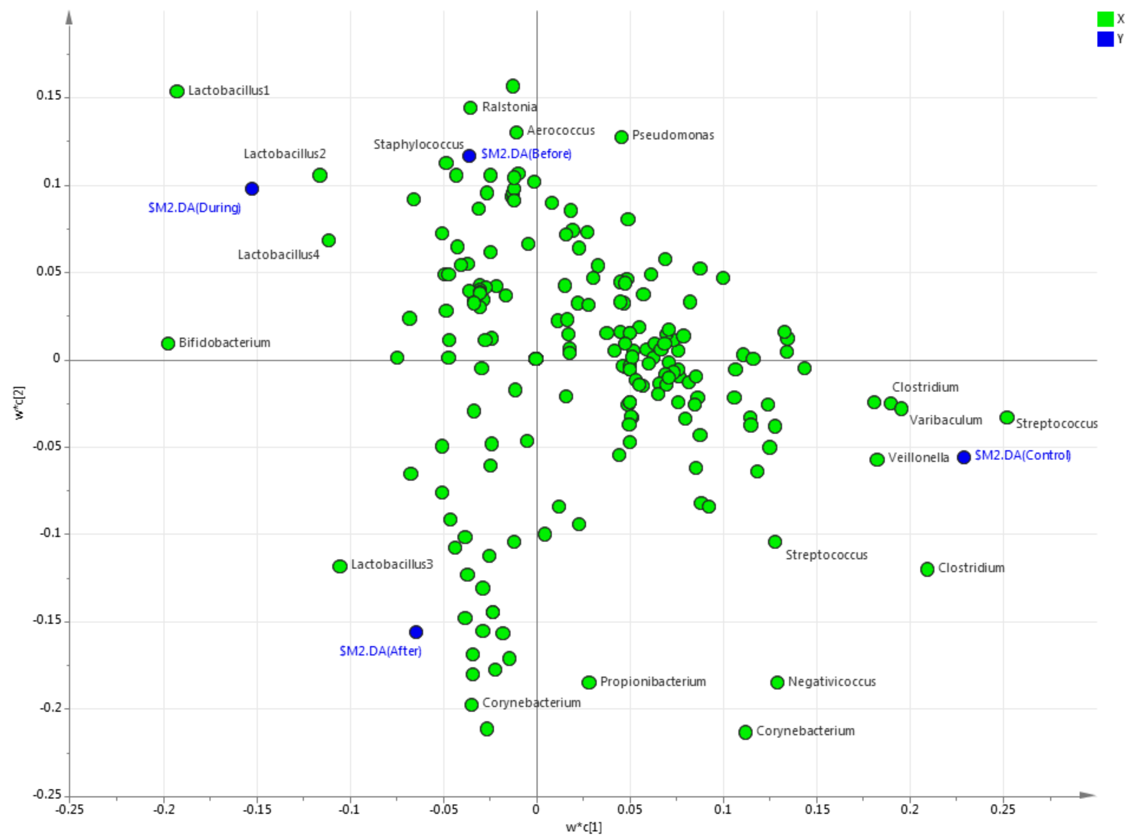


Figure 4-11: Loading plot showing the relationship of bacterial OTUs within the groups.

(PLS-DA Coloured according to model terms), the blue circles representing the groups as Control, before, during and after. The green circles representing the individual samples during the cohort. OTUs associated with during are 3x *Lactobacillus* spp. (otu 007, otu 0013 & otu 0044) and 1x *Bifidobacterium* spp. (otu 003). The details shown in the Figure 4.12 below:

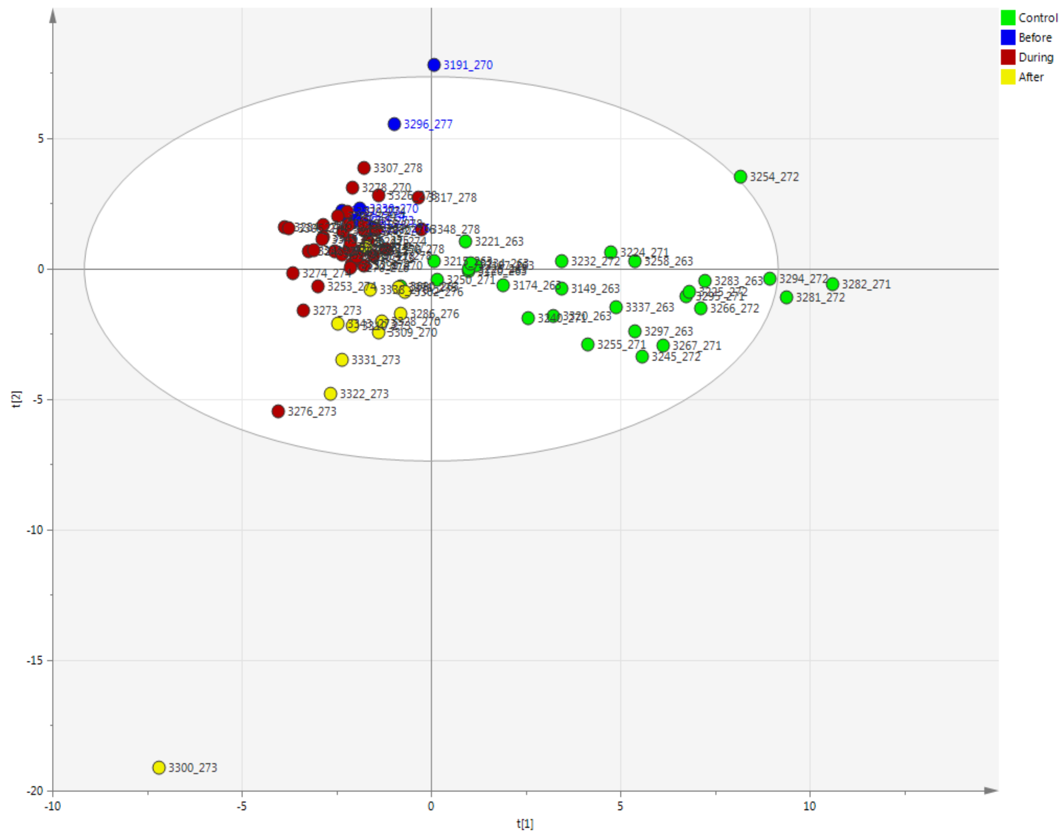


Figure. 4-12: The OTU Matrix showing the relationship of bacterial OTUs associated with each group.

(PLS-DA Coloured according to classes in M3), the colours indicating the groups but post-discharge samples removed. The green = control, blue = before, red = during and yellow = after. The numbers attached to each coloured circle representing the actual sample & patient numbers from the cohort study.

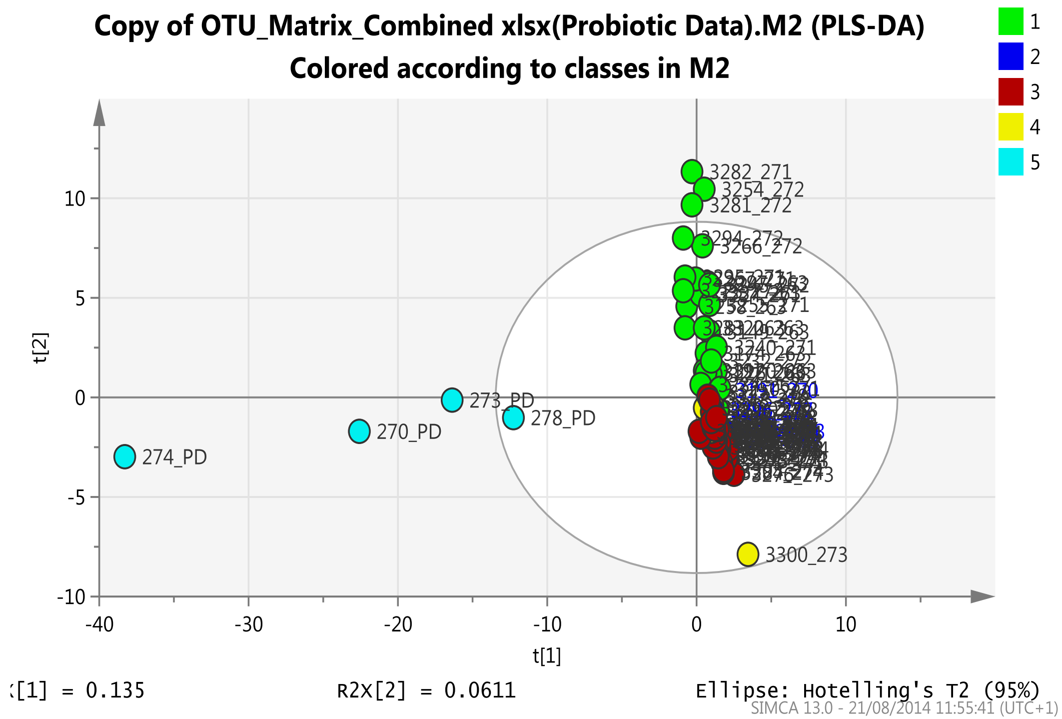


Figure 4-13: The bacterial samples showing the relationship of each group including post-discharge samples.

The coloured circles and numbers associated with them represents: 1=Control, 2=before, 3= during, 4= after and 5= Post-discharged. Most of the samples condensed closely related within the circle, while most of the post-discharge samples displaced apart outside the circle.

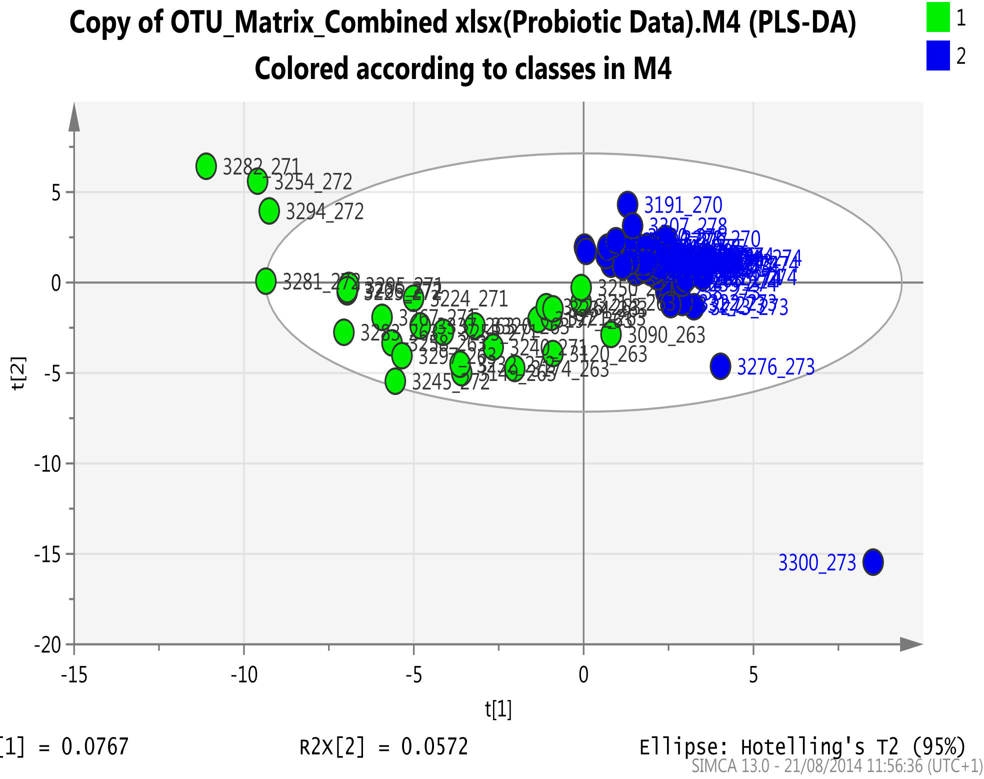


Figure 4-14: The OTUs copy shows some few control groups outside the circle. The numbers attached to green colour (1) represents control, while those with blue(2) represents Probiotics. See the details of their loading plots in the figure below:

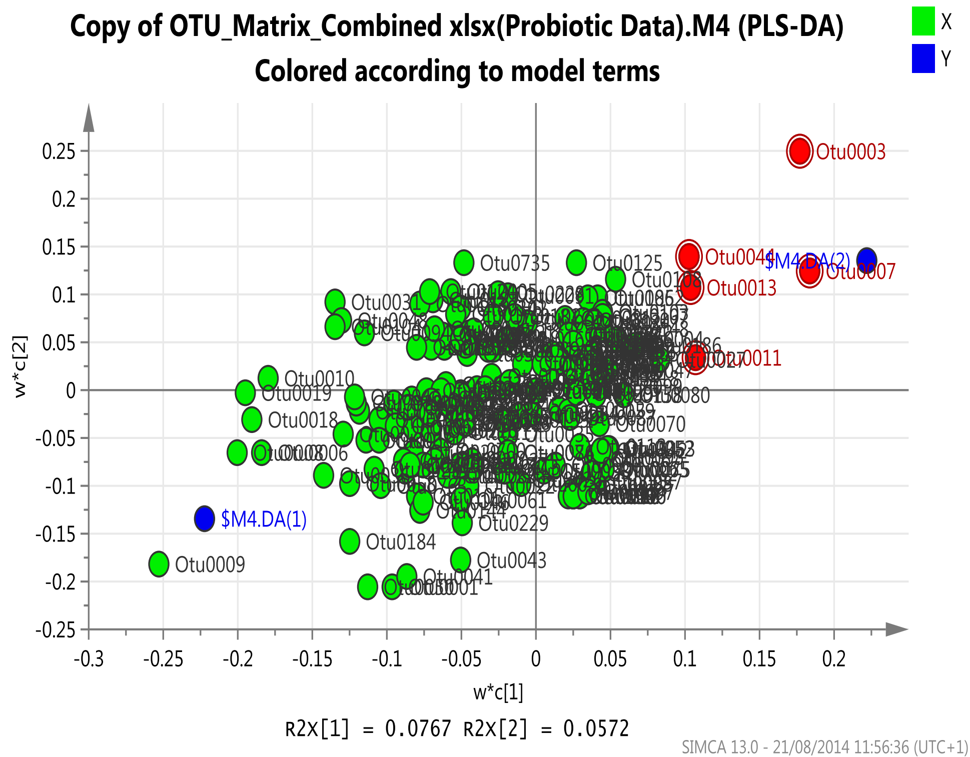


Figure 4-15: Loading plot showing the relationship of bacterial OTUs between probiotics and controls.

All the OTUs on the blue region (1) associated with control while those on blue region (2) with probiotics. Red circles = 4x *Lactobacillus* spp. (otu 0007, out 0011, otu 0013 and otu 0044) and 1x *Bifidobacterium* spp. (otu 0003).

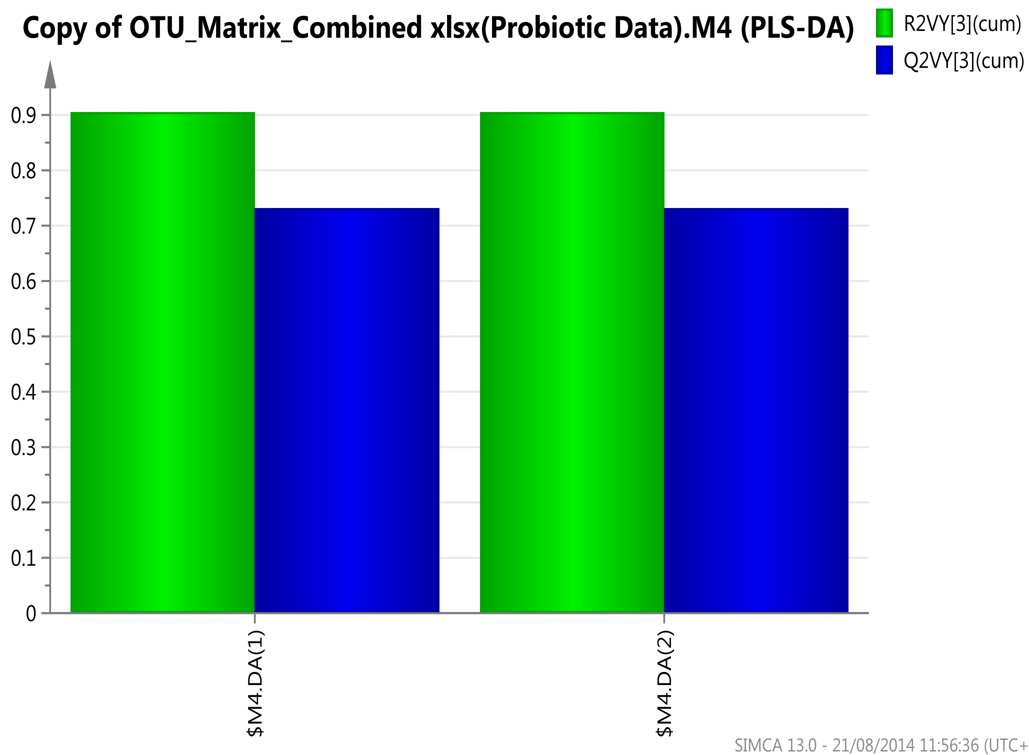


Figure 4-16: PLS Bar chart model of Probiotic and control MiSeq.

The data showing valid prediction of babies receiving probiotics (R2= 0.9 & Q2 = 0.73). Well modelled variables have high green bars, R2, and high blue bars, Q2, at levels of 0.5 or above. Here R2 indicates how well the variation of a variable is explained, while Q2 indicates how well a variable can be predicted.

4.7 Discussion

This study explored the impact of routinely used probiotics on the gut bacterial communities of preterm infants in the NICU at the RVI, Newcastle upon-Tyne during their administration and post- discharge. The application of non-culture base techniques was utilised to determine the bacterial diversity in stool sample and to quantify the long-term effects of probiotic supplementation as well as to determine whether they are implicated in microbial dysbiosis of preterm infants gut communities which may impact on clinical outcomes.

The results obtained from DGGE analysis shows that there is a significant reduction in diversity between the babies receiving probiotics (P = <0.001) compared to those babies that do not (Fig. 5). This suggests that probiotics have the potential to alter the gut microbial community. This may correlate with earlier work that shows some groups of probiotic significantly reduced NEC in very preterm infants (Jacobs et al. 2013). Also a significant decrease in the incidence of sepsis after administering of probiotics to preterm infants was noted in several studies (Wang et al. 2009; Lin et al. 2008; Alona Bin-Nun et al. 2005; Mai et al. 2011; Mihatsch et al. 2012; Braga et al. 2011; Stenger et al. 2011).

Probiotic strains analysis of *B. bifidum* and *L. acidophilus* showed both taxa were detected in the stool of preterm infants during the cohort study. There is significant difference seen with respect to *B. bifidum* in the gut of preterm infants when comparing profiles before and after probiotics administration *(P<0.005).* However, no significant differences was observed for *L. acidophilus* from the stool of probiotics babies in the same time interval *(P>0.005).* Nonetheless, the detection of probiotic strains from the preterm gut does not necessarily indicate the colonisation of the gut but rather may indicate that the organisms were shed after probiotic intake. It has been demonstrated that some probiotics strains

were found to be shed a month after probiotics supplementation was stopped with different level of colonisation (Tobin et al. 2013).

The results from 16S gene profiling and qPCR assays both agree with one another in respect to *B. bifidum* (Fig. 4.6, 4.8 and 4.10) and for *L.acidophilus* (Fig. 4.6, 4.7, 4.10 and Table 4.4). NGS analyses indicated fewer OTUs reads were detected prior to probiotic intake and during the post discharge samples with none detected in control groups (Fig. 10 & Table 4.4). However, qPCR showed *Bifidobacteria* were found to increase during and after probiotics were stopped (Fig. 4.8).

However, quantitative analysis from our study demonstrated the ability of *Bifidobacterium* to successfully colonise the gut of preterm infants in high numbers even during post discharge (Fig. 4.10 and Table 4.4) which is not the case in *Lactobacillus*. This agrees with previous findings that showed an increased in the number of *Bifidobacterium* among breast- fed preterm compare to *Lactobacillus* (Barrett & Guinane 2013). However, there are conflicting reports that demonstrate increased colonisation of *Lactobacillus* over *Bifidobacterium* in the preterm gut in breast-fed infants (Chen 2011). Additionally, our research also showed a reduction in the number of *Clostridium* among the preterm babies receiving probiotics (Table 4.4). The role of *Clostridium* in NEC has been identified in recent papers; this finding may suggest by reducing *Clostridium*, probiotics can reduce NEC. Some studies have indicated an association of between *Clostridia* and the establishment of NEC (Sim et al. 2014; Cassir et al. 2015; Mai et al. 2013). Also *Clostridium* and *Streptococcus* were increased in control group compared to probiotic fed babies (Fig. 4.10). This shows the potential of probiotics to impact on the bacterial community in the gut (Table 4.4 shows 3.3% & 0.2% of *Clostridium* at control and probiotic groups respectively). Also *Staphylococcus* has 53.4% before probiotic start and falls down to 12.6% after probiotic stopped.

Diversity of bacterial taxa

Notwithstanding, our study focused on the abundance of *Bifidobacterium bifidum* and *Lactobacillus acidophilus* from the gut of preterm infant and their effects on the gut bacterial community in our cohort; however, other bacterial taxa were detected in high abundance which may shed more light on dysbiosis studies associated with preterm infants. Our data (Fig. 4.10 and Table 4.4) indicated that the *Enterobacteria, Enterococcus, Staphylococcus* and *Escherichia* were frequently detected in the gut of preterm babies. This may explain the higher incidence of NEC and sepsis in preterm babies as the findings of (Mshvildadze & Neu 2010) showed high numbers of *Enterococcus* were frequently detected in NEC compared to control groups. Conversely, *Klebsiella* was frequently detected in control group compared with NEC cases *(P= 0.06).* While, detection of high abundance of *Proteobacteria* (61%) and *Actinobacteria* (3%) before NEC dignosis compared to the control having 19% and 0.4% respectively and less abundance of *Bifidobacteria* and *Bacteriodes* before NEC (Mai et al. 2013). More recently the abundance of *Enterobacteriaceae* among the NEC babies only has been reported (Brower-Sinning et al. 2014).

However, many factors are found to be associated with the diversity of microbes in the preterm gut such as mode of delivery, birth weight, feeding habit, gestational age and sex (Rigon et al. 2012). These factors may work with and against any impact of probiotic supplementation and may be confounders of understanding the impact of such supplements on health and disease.

Delivery mode

Table 4.3 showed 44% and 56% of babies who received probiotics were delivered through vaginal and caesarean respectively. While 60% and 40% of control babies were born via vaginal and caesarean section respectively (Table 4.3). A previous study showed that

infants delivered through the vaginal canal always harboured a gut microbial community similar to that of mother's vaginal tract. Whereas, caesarean delivered babies had communities that resemble the maternal skin microbiota (Dominguez-Bello et al. 2010) and gut colonization is delayed (Thompson-Chagoyan et al. 2007). *Clostridium, Escherichia, Streptococcus* and *Staphylococcus* were found to be prevalent in the gut of preterm infants delivered through Caesarean section (Fig. 10) which agrees with the previous work (Thompson-Chagoyan et al. 2007). Likewise different studies were in agreement with our finding with some variations in the taxa and level of colonisation depending on the delivery mode. *Klebsiella, Enterobacter* and *Clostridium* were detected after caesarean delivery and high numbers of *Bifidobacteria* after vaginal delivery (Ventura et al. 2012). Other report showed low number of *Bacteriodes* and *Clostridia* in preterm infants delivered by caesarean section (Westerbeek et al. 2006), and low number of *Clostridium* with high number of *Bifidobacteria* and *Bacteriodes* from the infant delivered vaginally (John et al. 2006). *Lactobacillus* was also found to be dominant among the preterm infants who were delivered vaginally (Rougé et al. 2010). A study conducted on microbial diversity based on delivery mode from an animal model showed reduction of *E-coli, Clostridia* and Lactobacilli among others from Caesarean delivery pigs (Cilieborg et al. 2012).

In terms of bacterial load, qPCR quantified the *Bifidobacteria* and Lactobacilli from the stool of breast-fed infants and observed that there is an increased in *Bifidobacteria* among both vaginally and caesarean delivered groups; this finding comply to the previous study (Chen et al. 2007).

Impact of birth weight

All the probiotic babies in our cohort weighed less than 1500g, while more than half of control group weighed above 1500g (Table 4.1 and 4.3), this may impact on the

colonisation of the infant gut, as a more immature GIT may facilitate or confound attachment and colonisation by different bacterial taxa. However, a previous work shows no significant difference between the bacterial diversity, sex, birth weight & gestational age *(P= 0.42)* (Mshvildadze et al. 2010).

Breast milk

In our study, all the babies were breast - fed, this could be why great numbers of *Bifidobacteria* (11.1%) compare to low Lactobacilli (1.7%) were detected even before starting probiotic supplementation (Fig. 10). It was previously reported that the gut of breast-fed preterm infants was predominantly colonized by *Bifidobacterium* and *Lactobacillus* which potentially suppress pathogenic bacteria (Westerbeek et al. 2006). However, in case of NGS data, no *Lactobacillus* and only a small number of *Bifidobacterium* (4.0%) in breast-fed controls and no *Lactobacillus* in Post-discharge babies (Table 5) were detected. This implies that, the feeding alone does not significantly increase *Lactobacillu*s and *Bifidobacterium* but when coupled with probiotic supplements it can influence the bacterial colonization in the gut of preterm babies. This finding agreed with previous research which indicated that, the feeding is not significantly related to bacterial colonization in the gut of preterm infants (Westerbeek et al. 2006).

However, the speed at which complete enteral feeding is achieved, or the addition of other supplementary dietary inputs may stimulate the gut of preterm infants to function and mature more rapidly (Cilieborg et al. 2012; Westerbeek et al. 2006). This could influence the colonization of the gut. *Bifidobacterial* colonization has no significant differences associated with birth weight, mode of delivery and feeding habit, but gestational age was significantly associated with *Bifidobacterial* colonization at birth (Butel et al. 2007). In contrast, another study showed the high abundance number of Lactobacilli and

Bifidobacteria in the gut of preterm infants with decreased number of *Clostridium* which has been associated with maternal breast milk (John et al. 2006).

However, regardless of probiotics intake, *Bifidobacterium* was found in our research to colonize the gut of preterm infants (4.0% & 11.1% for control and before administering probiotic respectively) thus, it concurs with other work that revealed that *Bifidobacterium* is dominant in colonising the infants' gut more especially those receiving breast milk (Turroni et al. 2014). It also support a study that shows that *Lactobacillus* as common beneficial bacteria that is found to colonise the gut of breast-fed infants (Cárdenas et al. 2015).

Moreover, the adoption of probiotics prophylaxis routinely in neonatal care units must be undertaken with great care in order to reduce the NEC incidence when its properly administered (Shlomai et al. 2014). Recently, some research suggested that preterm infants with NEC who were fed with breast milk while receiving Infloran as probiotic supplements had significantly reduced NEC incidence *(P= 0.027)* but it did not have a similar significant effect on the incidence of NEC for infants fed entirely with formula *(P=0.345)* (Repa et al. 2015). It was also reported recently that exposure to maternal antibiotics increased the risk of NEC but when followed by post natal probiotics supplement, NEC was decreased among the preterm infants (Beken 2015).

The quantitative analysis of both *Lactobacillus* and *Bifidobacterium* comparing between probiotics and control groups (Fig. 4.7, 4.9) showed lower detectable levels of *Lactobacillus* in probiotic infants compared to control group (Fig. 4.6). While, for *Bifidobacterium* quantification, we detected reasonable loads among the preterm receiving probiotics and none at the control group (Fig. 4.9). A study that compared quantitatively, the *Bifidobacteria* and Lactobacilli load counts between preterm patients and their healthy control groups; detected significantly lower *Bifidobacteria* and

Lactobacilli counts than their healthy control groups and also observed significant differences between two groups among intestinal *Bifidobacteria* and Lactobacilli (Chen 2011).

Despite the impact of probiotics intake in reducing the NEC and suppressing some potential pathogens (Amin et al. 2013), another viable bacteria (*Bifidobacterium longum*) that was used in the combination of prophylactic probiotics was detected in the blood culture of preterm infants who developed NEC (Zbinden et al. 2015). Therefore, care has to be taken and other clinical characteristics need to be considered while administering the probiotics more especially in VLBW infants to avoid other complications.

4.8 **Conclusions**

Our findings demonstrated that Probiotic babies have statistically lower diversity compared to non-probiotic. It also revealed *Bifidobacterium* and *Lactobacillus* colonize the gut of preterm infants at different levels during and after the treatment and they all increase with probiotic supplements, which contribute to the decrease of the relative abundance of the microbial communities associated with these diseases and hence may reduce NEC or LOS in preterm infants. *B.bifidum* was found to colonize the gut before probiotics where administered and *Bifidobacteria* are more prevalent in the gut of preterm infants and found to proliferate long-term compared to Lactobacilli. Multiple OTUs associated with *Lactobacillus* were detected and qPCR was unable to robustly quantify the *L. acidophilus* used in Infloran raising potential questions about the reported quality control of available probiotics. Future studies should explore this concept in advance to better understand the systematic role of probiotic supplementation.

5 CHAPTER FIVE

5.1 Systematic functional analysis of the gut of preterm infants by probiotic metabolomics profiles

Abstract

Metabolomics is one of the modern techniques used to study the function of gut microbial communities by analysing different metabolites associated with a particular sample. Metabolites may have the potential to serve as biomarkers in clinical diagnosis as well as determining the nutritional status of an individual in response to clinical interventions.

Aims

The aimed was to determine metabolite profile from the gut of preterm infants receiving probiotics and any functional changes associated with NEC or sepsis. It was hypothesized that probiotics administration may result in functional shifts in the gut microbiome and affect the preterm gut metabolites signature.

Methods

A robust study of stool metabolomic profiles using ultra-performance liquid chromatography mass spectrometry tandem mass-spectrometry (UPLC-MS/MS) technique was performed.

Results

Exploring the samples showed each group of metabolites to cluster discretely. Groups of metabolites were found specifically in controls. While the samples taken during probiotics administration, showed distinct metabolites associated with probiotic supplements. Therefore, the metabolomic profiles showed variable composition associated with probiotics administration compared to that of control samples.

Conclusions

Metabolite profiles clustered separately, with distinct metabolites associated with probiotic administration, but their identification was not available. Our findings also suggest that probiotics have some systemic functions and may play a significant role in the gut microbial communities.

5.2 Background

Metabolomics involves a comprehensive analysis and systematic identification of different metabolites and their physiological changes in a particular sample (Inna et al. 2010). The metabolome refers to the complete set of small-molecule metabolites in a biological system (Xie et al. 2013). The study of low-weight molecules (< 1,500 Da) which are the intermediates or end products of metabolism may serve as biomarkers for common infections and disease states affecting human population including preterm infants (Ganna et al., 2014). A previous study showed that metabolomic fingerprints of some early microbial dysbiosis may serve as a biomarker for predicting NEC among preterm infants (Lagomarcino et al. 2013). A recent study also demonstrated the usefulness of metabolomics analysis as a biomarker in determining the nutritional value and predicting the health status as well as in the management of diseases affecting neonate in clinical practices (Dessì et al. 2014; Marincola et al. 2012; Ganna et al. 2014).

Metabolomics study serve as a mirror image of the genome and its interaction with its ecological environment (Xie et al. 2013). Evaluation at the metabolomics level would allow us to determine functional changes as a result of antibiotics therapy, probiotics supplementation and other physiological conditions (Dessì et al. 2014). Recent advances in metabolomics helps us to study the small complex molecules present in metabolome and have shown that intestinal microbiome can influence the metabolomics profiles of the individual host (Lagomarcino et al. 2013; Del Chierico et al. 2015; Antunes et al.

2011; Jansson et al. 2009). Moreover, the application of high-throughput metabolomics technique was used by a previous study to demonstrate the interaction between the intestinal microbiome and the individual host (Antunes et al. 2011).

Metabolomics data are mostly analysed conveniently using principal component analysis (PCA) or partial least square (PLS) as part of a multivariate analysis (Trivedi 2012; Worley & Powers 2012). Although a recent study demonstrated that univariate analysis such as *t* –test or ANOVA are starting to become prominent in metabolomics data analysis. This is due to the need to test the significant differences among different metabolites within a particular biological sample (Saccenti et al. 2013). In order to have a comprehensive biological mechanism of a given metabolomics sample, the data obtained should be integrated in a pathway analysis for proper comparison with other 'omics' studies (Zhou et al. 2013).

Most of the metabolites identification techniques involve: matching spectral features of the unknown compounds to curated spectral database of reference compounds (known as 'identification of known unknowns'), identification of truly novel compounds (identification of unknown unknowns) is more difficult and requires advanced computational approaches (Wishart 2009; Waterman et al. 2009). Metabolites identification is one of the major challenges facing metabolomics studies due to the diverse structure of different metabolites alongside with diversity in their physical and chemical properties (Zhou et al. 2013; Becker et al. 2012).

The choice of method (LC/MS) in the analysis of metabolites from faecal samples of probiotic infants is one of the most promising techniques used for the detection of large number of compounds in biological samples (Ganna et al. 2014). LC/MS is a robust techniques that has been proven in the analysis of the complex compound in biological samples (Becker et al. 2012; Ogura & Sakamoto 2007). LC-MS approaches provide an

accurate quantitation and identification of metabolites by evaluating the metabolic changes in a given sample (Zhou et al. 2013). However, there are difficulties faced during compound detection by MS technique at very low concentration is due to ion suppression. Though, it is easy to measure the mass of metabolites detected in LC-MS base technique, the ability to identify compounds is hindering the application of the approach (Zhou et al. 2013).

5.3 Methods

5.3.1 Study design

A Probiotic supplement was given to preterm infants of less than 32 weeks gestation. All had received some maternal breast milk during their stay at neonatal intensive care unit (NICU) of the RVI, Newcastle upon-Tyne. All the infants were born within a 3 month period. Overall, 60 stool samples were analysed from 9 patients (6 probiotics and 3 control babies). 40 are the study samples while 20 are the trial samples. The 20 trial samples involved only probiotic babies and were only used to optimise the extraction protocols as such were not involved in the analysis.

The 40 study samples were further divided in to probiotics (28 samples) and their matched controls (12 samples). All the 40 study samples underwent metabolomics profiling and further analysis (the data presented in this chapter is based on these 40 samples). The patient's clinical demographics data are summarised in Table 5.1- 5.2.

Table 5-1: Demographic data for the clinical cohort

	Control			Probiotic					
Patient No	263	271	272	270	273	274	275	276	278
Gestational Age	27	31	31	25	27	24	28	28	24
Birth weight	550	2030	1535	750	945	700	1100	1150	620
Delivery	CS	V	V	V	CS	V	CS	CS	CS
Sex	F	F	M	M	F	M	F	M	M
Total NO. of Samples	4	4	4	5	4	5	4	4	6
Post dis (age in months)	N	N	N	Y (25)	Y	Y	N	N	Y
Feed	All	BM							

Table 5-2: Frequency of probiotic metabolomics groups

The Table below shows different groups on which the entire samples were classified in relation to probiotics intake and the number of samples collated within each group during the cohort study:

Group	Number of stool samples
Before probiotic	1
During probiotic	17
After probiotic	6
Post-discharge	4
Control (pre-discharge)	12
Overall total	**40**

5.4 Trial test of metabolomics analysis

Twenty metabolomics stool samples were sorted out from seven babies receiving probiotic supplementation - the probiotic supplementation was given to preterm infants of less than 32 weeks gestational age immediately after initial introduction of feeds until it reached 34 weeks corrected. The dose contain a half of an Infloran capsule which was administered twice a day, equating to 125 mg b.d at 10^9 organisms per dose - (see the appendix for demographic data of metabolomics trial test) to test the protocols as a trial for optimisation of the metabolite extraction/preparation stage. The samples were extracted in triplicate and analyses using the LCMS concurrently (see the methods chapter for the detail metabolomics protocols). The samples were marked A, B and C to identify the modified preparation protocols. The results are in Table 5.3.

Table 5-3: Trial test for stool metabolomics technique

Sample groups					Remarks
Procedure	Test A	Test B	Test C	Experimental samples	
Homogenisation	50% MeoH	80% MeoH	80% MeoH	80% MeoH	80% gives better result
Vortexing	15 mins at $4^{o}c$	15 mins at $4^{o}c$	15 mins at $4^{o}c$	15 mins at $4^{o}c$	Adapter was taken to cold room
Centrifugation	500g/15 mins at $4^{o}c$	500g/15 mins at $4^{o}c$	3,000g/10 mins at $4^{o}c$	3,000g/10 mins at $4^{o}c$	In the cold room or using thermos centrifuge
Filtration	No Filtration	No Filtration	Filtered with 0.2 whatsize	No Filtration	No filtration gives better result
Lyophilisation	Speed vac at 1,1/2hr for $30^{o}c$	Speed vac at 1,1/2hr for $30^{o}c$	Freeze drying over night	Freeze drying over night	Freeze drying work better
Re-suspension	1000 µl of 5% ACN	1000 µl of 5% ACN	1000 µl of 5% ACN	1000 µl of 5% ACN	95% A (water): 5% B (ACN)

5.5 Statistical analysis

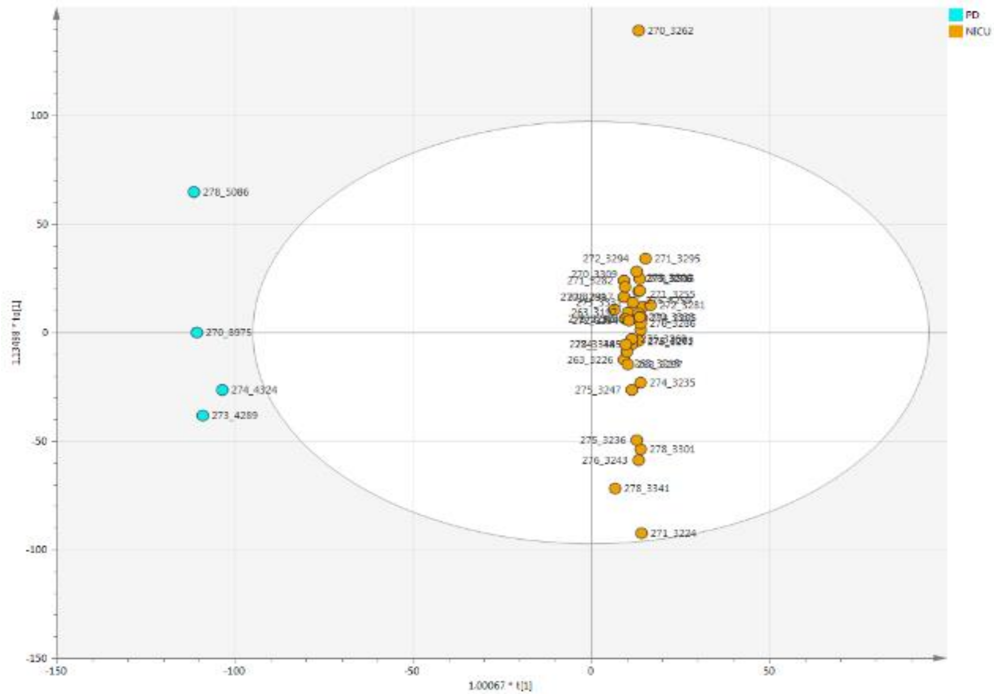
The bacterial profiles were analysed by multivariate partial least squares discriminant analysis (PLS-DA) and the metabolite profiles underwent orthogonal PLS-DA (OPLS-DA) using SIMCA 13.0 (Umetrics, Stockholm, Sweden) - (Eriksson et al. 2006). All variables, either operational taxonomic unit (OTU) or component, were automatically transformed within SIEVE. To check that data was adhering to multivariate normalities, Hotelling's T^2 tolerance limits were calculated and set at 0.95. To remove the high amounts of noise from the metabolomics dataset, only variables >1 in the variable importance plot (VIP) were included from the important variables plot (Trivedi 2012).

5.6 Results

5.6.1 Metabolomics profiling of the gut microbiome

Metabolite profiles demonstrated uniformity among the study group in the cohort. The loadings plot showed a cluster of metabolites associated with samples during probiotic intake (Fig. 5.1). The post-discharge (PD) samples were found to have a distinct metabolite profile when compared with NICU samples by OPLS-DA (R2Y = 0.99, Q2 = 0.8), with a strong association of unique metabolites and metabolites present in much greater abundance compared with NICU samples (Fig. 5.2). Due to low amount of sample in the 'before' samples (and the skew caused by the PD samples), these 2 groups were omitted from OPLS-DA of the probiotic and control groups. Due to the inherently similar metabolomics profiles, OPLS-DA was unable to robustly separate samples taken during or after probiotic administration, and control groups (Figure 5.2: R2Y = 0.62, Q2 = 0.31). However, following removal of metabolites with a VIP of <1 (leaving only significant metabolites with large differences in the relative intensities), the groups could be separated more robustly (Figure 3B: R2Y = 0.80, Q2 = 0.56).

A



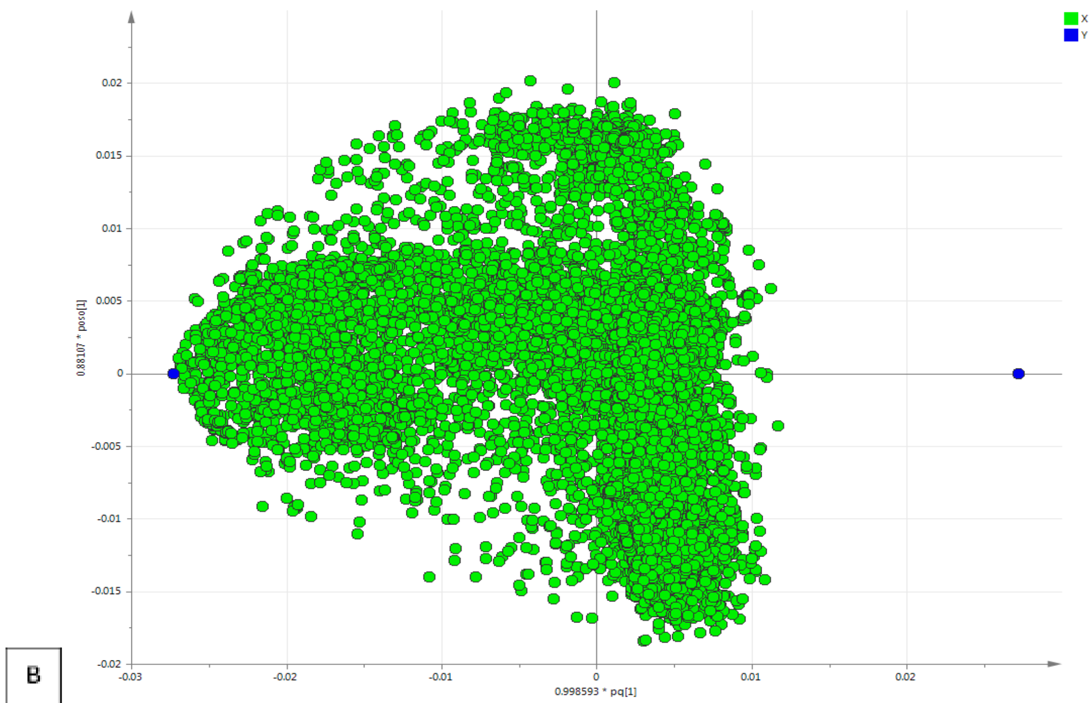


Figure 5-1 PLS-DA of metabolite profiles

The data comparing samples collected on the NICU and post discharge. R2Y = 0.99, Q2 = 0.8. **A**) Score scatter plot**. B)** Loadings plot. The green circles in ‘A’ represent the samples collected during post discharge period, while the orange circles in ‘A’ represent

also the samples collected at NICU. All the samples associated with each group cluster separately.

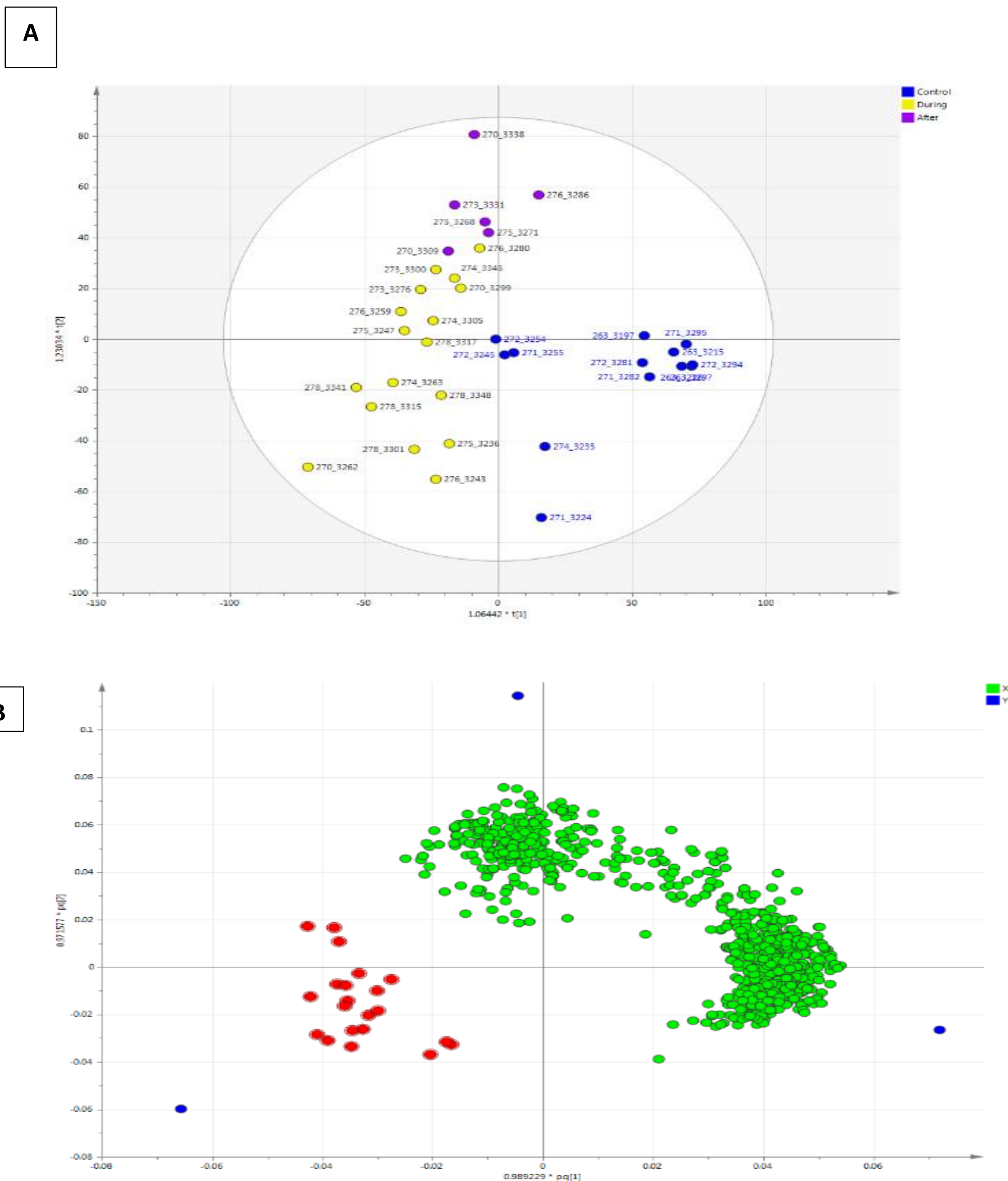


Figure 5-2: PLS-DA of the metabolite profiles

Before and post discharge not included**. A)** Raw core scatter plot of the showing the relationship of samples associated with each group (R2Y = 0.62, Q2 = 0.31).The colours assigned in the Figure represent; Blue= control, Yellow= during & Purple= after. The numbers associated with each circle represents the patient and sample number during the cohort study. **B)** Loading plot of detected metabolites associated with each group (during, after and control) to cluster separately with distinct metabolites following VIP removal

of noise (R2Y = 0.80, Q2 = 0.56). Red metabolites associated with probiotic administration.

With regards to relative abundance of the OTUs metabolites (Fig. 3), because of the low amounts of samples involved in the group before probiotic supplementation, the 'before' not included during the analysis. However, the metabolites are more diverse in control group followed by during probiotic intakes and then after supplementation. While post discharge metabolites are less in abundance compared to other groups. Moreover, despite the detection and distinct separation of the metabolites during the analysis, their identification is challenging in the human metabolome data base (HMDB) library, hence; the matched colour numbers in the legend key, represent the samples number in the cohort (Fig. 3).

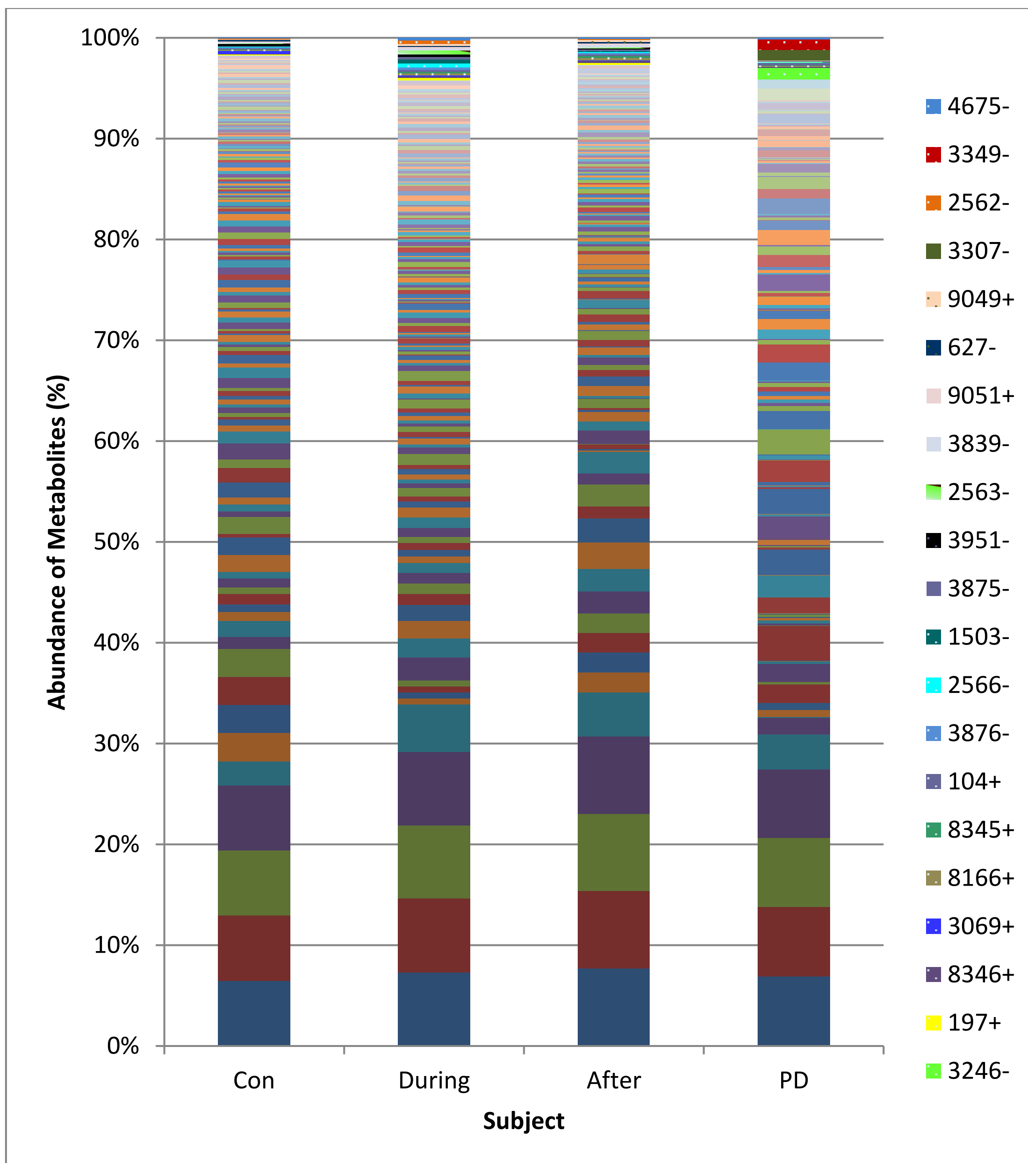


Figure 5-3: Relative abundance of metabolomics profiles from MiSeq analysis.

Proportion of OTUs matching the 200 most frequently observed metabolites across the samples. Colours on the chart matched with the metabolites data abundance in percentage. Sequences matching other metabolites with less frequently observed in the samples are not shown on the chart and represents minor and unclassified OTUs.

5.7 Discussion

In order to expand our understanding of the impact of probiotics on the preterm gut microbiome function, the metabolomic profiles was analysed to obtain a snapshot of the effect of probiotics before, during and after supplementation. A metabolomic profiles utilising UPLC-MS from preterm stool sample to determine how functional metabolites shift are associated with the intervention and to begin to elaborate the mechanistic role of probiotics in neonates was explored. This study is unique as most of the previous studies utilising same technique have exploited human biological samples such as serum and urine rather than stool (Ganna et al. 2014; Ogura & Sakamoto 2007). Other work has utilised urine sample to performed metabolomic profiling in preterm infants and how it could be used clinically in neonatal disorders (Antonucci et al. 2009).

As our study utilised the stool samples by exploring LC-MS approach to analyse the metabolite shifts and its relation with OTUs bacterial profiles. Other study utilised H-NMR approach to determine the preterm stool and its correlation with OTUs microbial profiles and metabolomic changes (Del Chierico et al. 2015). unlike one of the previous study that focussed on the blood metabolites and showed the great effects of gut microbiota on the human blood metabolites (Wikoff et al. 2009).

In accordance with a small number of published studies related to preterm cohorts, the metabolomics profiles were more comparable and stable than bacterial profiles (Turnbaugh et al. 2009; Morrow et al. 2013), and were comparable to those of controls, as certain metabolites were detected with an increased abundance during probiotic administration.

However, a previous study showed that metabolic pathways were affected by antimicrobial agents and altered systematic functions of the gut (Antunes et al. 2011). It was also reported that antibiotics treatment affects a high percentage of detectable

metabolites from the human gut demonstrating the impact of antibiotic therapy on the function of the intestinal metabolome (Antunes et al. 2011).

Here, the relationship between gut microbial communities and stool metabolites in preterm infants was explored and the result showed that distinct metabolites cluster independently between the samples collected in the NICU and during post-discharge (Fig. 5.2). This could be attributed to many factors including nutrition, environment and maturity of the microbiome associated with probiotics metabolites. During PD (post discharge), the preterm gut matures and consumption of solid food begins. Environment may also contribute as the NICU is more hygienically managed than a domestic milieu. Moreover, the gut microbiome becomes more diverse during PD as antibiotics therapy and pre and probiotics supplements are removed.

None of the metabolites detected within our cohort could be matched with the existing ones and hence could not be identified based on the reference sample library available. This is one of the limitations of our study as further work will be needed to identify these compounds, and identifying whether they may be connected with potential regulatory pathways. Thus, our detectable metabolites fall under the metabolomics analysis term 'unknown unknowns compounds' (Wishart 2009) and highlights the limitations of this technique.

Previous studies have shown that > 70 % of detected ions in LC-MS analyses were either unidentified or had multiple putative identification making it difficult to identify a particular metabolite (Zhou et al. 2013). Therefore, identification of metabolites is one of the challenges in the field of metabolomics analysis (Zhou et al. 2013). However, due to recent advances in technologies, more techniques are being introduced and applied that is making metabolite identification simpler more robust (Becker et al. 2012; Wishart

2011; Marincola et al. 2012). These techniques include NMR and MS approaches (Wishart 2009; Xie et al. 2013; He et al. 2014).

5.8 Conclusions

It was demonstrated that metabolites increased during probiotics supplementation, but the identity of these metabolites was not achieved. Further study will be required to robustly identify these and other metabolites of interest using additional techniques or known standards. And also exploring functional metabolite changes may provide important information on mechanisms of action of interventions such as probiotics, and should be considered for inclusion into future interventional trials.

6 CHAPTER SIX

6.1 Impact of antibiotics course combination on the gut microbial community in preterm infants

Abstract

Antibiotics are usually prescribed to preterm infants during their early days of life in neonatal intensive care units (NICU). The effects of this intervention on the developing gut microbiome are poorly understood, but might have important consequences for health.

Aim: The aimed was to explore how routinely used antibiotics in a neonatal intensive care units impacts on the preterm gut microbiome.

Methods: The three most commonly prescribed antibiotic combinations were analysed VCM (Vancomycin, Ceftazidine and Metronidazole), VC (Vancomycin and Ceftazidine) and AFG (Amoxicillin, Flucloxacillin, and Gentamicin). Sampling was performed at four time points: 2-3 days before the course started (Pre), the last day of administration (During), 1-2 days after antibiotic was given (After), and one week later than or as late as possible before next antibiotic course. In total, 141 stool samples were collected from 38 patients and bacterial profiling was performed by 16S rRNA gene sequencing (Miseq, Illumina)

Results: Bacterial diversity increased after the VC and VCM course were stopped .Diversity was reduced for all antibiotic treatment during their administration *($P > 0.05$).* Generally, VCM and VC were comparable with lower bacterial taxa when compared to AFG which recorded higher bacterial taxa.

Conclusion: The three antibiotics courses differentially affected the preterm gut microbiome, causing reductions in the diversity. Further work is necessary to determine

the contribution of these changes to health and how medical intervention can be tailored to achieve optimal outcomes for preterm infants.

6.2 Background

Preterm infants are typically treated with antibiotics during the early days of life. Antibiotics produce short- and long-term impacts on the gut microbial communities (Jernberg et al. 2010; Jernberg et al. 2007), though the long-term health implications are still not clear (Fouhy et al. 2012). The impact of antibiotics on the gut microbiome are manifested during the early days of an infant's life through decreases in the number of *Bifidobacterium and Bacteriodes* as well as general decrease in bacterial diversity (John et al. 2006; Johnson & Versalovic 2012).

Some studies showed that preterm gut microbiota contain; more pathogens than healthy term infants; few numbers of beneficial bacteria and low bacterial diversity (Mai et al. 2011; Morowitz et al. 2011) but the extent to which this observation can be attributed to antibiotics exposure is not clear (Greenwood et al. 2014). It was therefore, sought to explore the impact of commonly prescribed antibiotic combinations on preterm infant's gut microbiome. Antibiotics affect the intestinal microbial composition depending on a number of factors including: drug combination, mode of delivery, treatment time and dosage (Jernberg et al. 2010). Intestinal antibiotics may cause dysbiosis (Johnson & Versalovic 2012; Dethlefsen & Relman 2010; Jernberg et al. 2007; Jernberg et al. 2010), as well as affecting the host-microbe's interaction and intestinal homeostasis (Antunes et al. 2011). It has been recently reported that antibiotic therapy during pregnancy influence the gut microbial colonisation of maternal mothers and infants (Gonzalez-Perez et al. 2016).

The routine antibiotic exposure of preterm infants is one of the factors influencing the colonisation of gut bacteria in the early days of life that affects the community

composition and structure (Ward et al., 2014). Early exposure to antibiotics by preterm infants may lead to increases in the incidence of morbidity and mortality when compare to those not receiving antibiotics (Greenwood et al. 2014). Furthermore, antibiotics are routinely prescribed to very extremely preterm (less than 34 weeks GA) infants within the first two days of life without symptoms of infection and then may subsequently be administered later if symptoms of infection were manifested (Gosalbes et al. 2016; Greenwood et al. 2014). Antibiotics in the gut of preterm infants has been linked to the pathogenesis of NEC (Torrazza et al. 2013). Furthermore, care has to be taken when prescribing antibiotics to both preterm infants and pregnant women in order to avoid their detrimental effects on the bacterial gut community (Johnson & Versalovic 2012). A recent study demonstrated how certain antibiotics resistance genes present among the pregnant women can be transmitted and established in the gastrointestinal tract of infants (Gosalbes et al. 2016). Moreover, antibiotics resistance genes were reported to persist in the human intestinal microbiome after antibiotic administration for up to two years after treatment stopped (Jernberg et al. 2007; Jernberg et al. 2010). It has been reported that the alteration of gut microbiota due to antibiotic treatment may cause immune dysregulation leading to autoimmune disorders (Willing et al. 2011; Wu & Wu 2012).

The application of molecular techniques may help in understanding the gut microbiome better and the impact of clinical interventions (Willing et al. 2011). Studying the effects of antibiotics using molecular techniques illustrates how antibiotics affect the intestinal physiology and its interactions with gut microbiota resulting in significant impacts on the intestinal metabolites (Antunes et al. 2011a).

Some routinely used antibiotic courses with and without metronidazole were selected and looked at their impact on preterm gut microbiology over time. Metronidazole was the key antibiotic that our study focussed on which when combined with other antibiotics is instrumental in the management of NEC/Sepsis during infants stay at NICU. Identifying

the best antibiotic course could lead to better outcome in the treatment of mixed infections. Metronidazole is the antibiotic of choice globally in clinical practice against anaerobic infections and, is commonly used in the treatment of severe complications of prematurity, such as NEC, (Löfmark et al, 2010). Metronidazole has previously been shown to alter the rat intestinal microbial community by significantly increasing the number of *Bifidobacterium and Enterobacteria* (Pélissier et al. 2010). Different clinical trials have been carried out using probiotics strains to test the clinical responses of infections to metronidazole either alone or combined with other antimicrobial agents (Löfmark et al., 2010; Zar et al., 2007).

To further explore the effects of antibiotics in preterm infants, three different antibiotic combinations were analysed, which were the most frequently prescribed in the NICU: VCM (Vancomycin, Ceftazidine and Metronidazole), VC (Vancomycin and Ceftazidine) and AFG (Amoxicillin, Flucloxacillin, and Gentamicin). It was hypothesise that distinct changes in the gut microbiota will occur as a direct result of antibiotic administration, which may have important consequences for the short and long term outcomes of premature infants.

6.3 Methods

6.3.1 Ethical approval

Ethical approval was obtained from the County Durham and Tees Valley Research Ethics Committee. The consent form was duly processed and endorsed by parents facilitating sample collection.

6.3.2 Study design

All the preterm infants in the cohort study were cared for in the neonatal intensive care unit (NICU) of the Royal Victoria Infirmary Newcastle upon Tyne. There is a standard

feeding practice in the unit; antibiotics, antifungal as well as probiotics were used depending on the clinical diagnosis. All the infants in the cohort were <30 weeks gestational age and <1500g birth weight with the exception of patients 207 who was 1580g and patient 294 who was 1650g (Appendix 13). Babies were grouped into before, during and after antibiotic treatment and 1 week post antibiotics treatment (Table 6.1). Overall 141 stool samples and clinical data were collected from 38 infants, out of which 12 preterm infants received VCM (41 samples), 13 VC (51 samples) and 13 (49 samples) AFG course. Three different antibiotic combinations were identified during the studies as: VCM (Vancomycin, Ceftazidine and Metronidazole), VC (Vancomycin and Ceftazidine) and AFG (Amoxicillin, Flucloxacillin & Gentamicin).

Table 6-1: Antibiotics sampling at different time points

Sampling was undertaken at four time points.

Time points	Description
I	2-3 days before course started
II	last day of administration
III	1-2 days after antibiotic was given
IV	1 week after last antibiotic treatment, or as late as possible before next antibiotic course

I = Pre

II = During

III = After

IV = Post antibiotics

The study involved 38 patients from which 141 samples were collected (See the summary in Table 6.1 and the details in the appendix 13 for clinical characteristics and demographic data during the antibiotics cohort study).

6.4 Sample analysis

6.4.1 Next Generation sequencing (NGS)

16S rRNA gene bacterial profiling was performed on all samples in the study. The entire stool samples underwent nucleic acid extraction on 100 mg of stool sample using the PowerLyzer™ PowerSoil® DNA Isolation Kit (MoBio) following the manufacturer's guidelines. After DNA extraction, the NGS analysis was carried out by NU-OMICS (Northumbria University) based on the Schloss wet-lab MiSeq standing operating protocol (available at - http://www.mothur.org/wiki/MiSeq_SOP). Raw fastq data were processed using Mothur (version 1.31.2) as described in the MiSeq SOP. Chimeric sequences were detected by Chimera.uchime and removed from downstream analysis. Alignment was generated via the Silva database. The data obtained was subjected further to statistical analysis using SIMCA, Minitab, and past.exe and excel.

Table 6-2: Demographic data of cohort infants (Average Mean)

Antibiotics course	No of Patient	No of Sample	GA (weeks)	BW (weeks)	Antibiotics (No of Pt.)	Time	points	
					I	II	III	IV
VCM	12	41	24.6(24-27)	777.1(620-1180)	12	7	12	10
VC	13	51	25.9(24-28)	922.5(790-1310)	10	11	13	17
AFG	13	49	26.0(23-29)	1013.8(695-1650)	13	12	13	11
TOTAL	**38**	**141**			**35**	**30**	**38**	**38**

*The alphabets represent the name of antibiotics course given as: VCM (Vancomycin, Ceftazidine and Metronidazole), VC (Vancomycin and Ceftazidine) and AFG (Amoxicillin, Flucloxacillin, and Gentamicin).

Table 6-3: Antibiotics and their Target Microorganisms

The Table below shows the individual antibiotic used to treat infants during the cohort study alongside their usual bacterial targeted.

	Antibiotics	Target Microorganisms
1	**Vancomycin**	*Listeria monocytogenes*, *Streptococcus pyogenes*, *Streptococcus pneumoniae* , *Streptococcus agalactiae*, *Actinomyces* spp., and *Lactobacillus* spp., *Enterococci.*
2	**Ceftazidine**	*Pseudomonas, Lactobacillus* and *Enterobacteriaceae,* with intermediate activity versus *Veillonella*
3	**Metronidazole**	*Anaerobic bacteria, Helicobacter pylori,* and *Veillonella.*
4	**Amoxicillin**	*Streptococcus pyogenes*, *S. pneumoniae*, *Staphylococcus* spp., *H. influenzae*, *E. coli*, *P. mirabilis*, or *E. faecalis.* , *Veillonella*.
5	**Flucloxacillin**	*Anaerobic bacteria* and *aerobic bacteria, Staphylococcus* spp., and *Streptococci, Clostridia* spp.
6	**Gentamicin**	*Proteus* spp., *E. coli*, *Klebsiella-Enterobactor-Serratia* spp., *P. aeruginosa*, *Citrobacter* spp. and *Staphylococcus* spp.

6.5 Results

6.5.1 Comparison of most abundant taxa over time

It was observed that *Staphylococcus* had a significantly higher *(P = 0.004)* number of OTU in the VCM treatment across the early time points but this was reduced 1 week after treatment stopped and showed no significant difference *(P = 0.157)* compared to other taxa except with *E. coli (P = 0.006)* (Fig. 6.1). However, the other taxa (*Pseudomonas, Enterococcus and Enterobacteriaceae)* increased at 1 week after VCM regimen stopped but not reached significantly so (Fig. 6.1).

*Staphylococcus a*nd *Enterococcus* OTUs were high in the VC regimen across all the time points when compared to other taxa (Fig. 6.2). All taxa increased at 1 week after VC regimen was stopped except *Enterococcus*, (Fig. 6.2). *Pseudomona*s OTUs were lower in AFG treatment across the time points when compared to other taxa (Fig. 6.3). All taxa have stable OTUs in AFG treatment at 1 week after treatment stopped except *Enterococcus* OTUs that increased significantly *(P = 0.02)* (Fig. 6.3).The other most abundant taxa showed inconsistent patterns across the time points between the different the entire antibiotic courses (Fig. 6.1- 6.3 and Table 6.4).

Generally, *Staphylococcus* was the most abundant taxa with high OTUs in almost all the antibiotic mixtures in the cohort. Also there is overall increase in OTUs number with antibiotic treatment at 1 week after regimens were stopped with the exception of *Staphylococcus* (in VCM treatment) and *Enterococcus* (in VC treatment) Figure 6.1- 6.3.

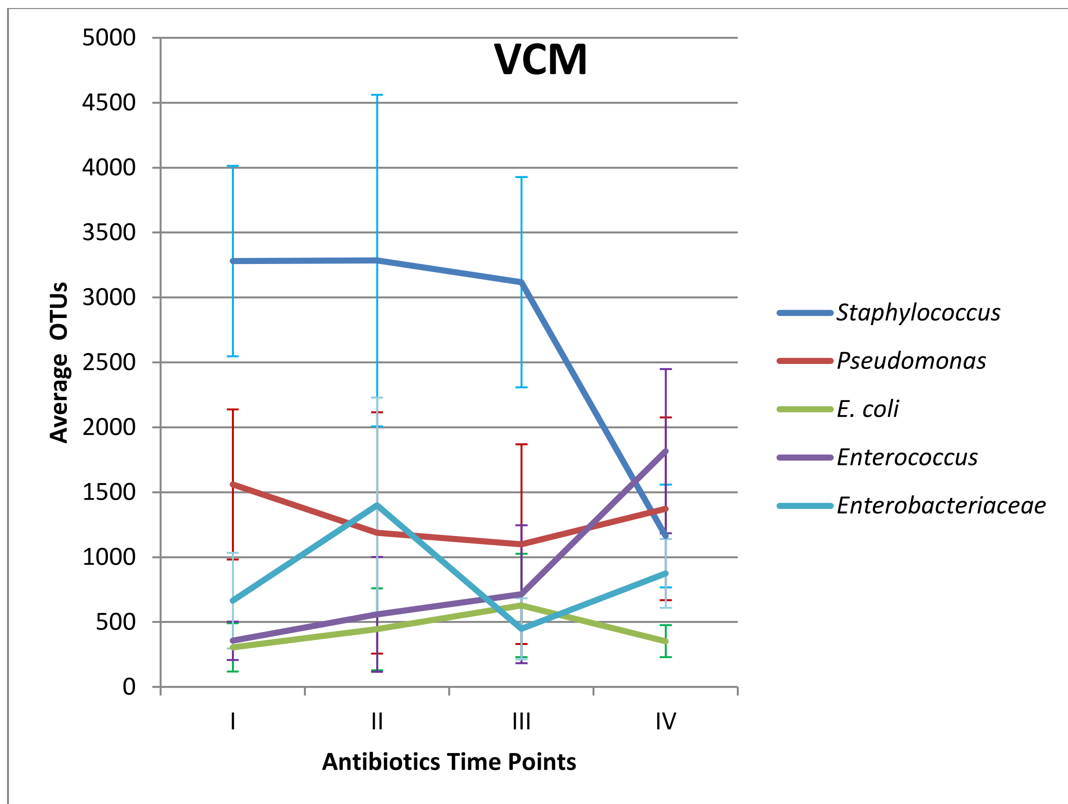


Figure 6-1: The most abundant organisms over time for VCM regimen.

Figure 6.1 above indicates the average OTUs of the highest relative abundance OTUs and the effect of antibiotic treatment to microbial community in the cohort study. Proportion of OTUs matching the 5 most frequently observed taxa over time among the antibiotic course. Colours on the charts matching with the bacterial taxa on the legend represent the diversity in percentages. While the letters I, II, III and IV on the bottom of each chart representing different sampling points of the antibiotics I, II, III and IV represents pre, during, after and week after respectively**.**

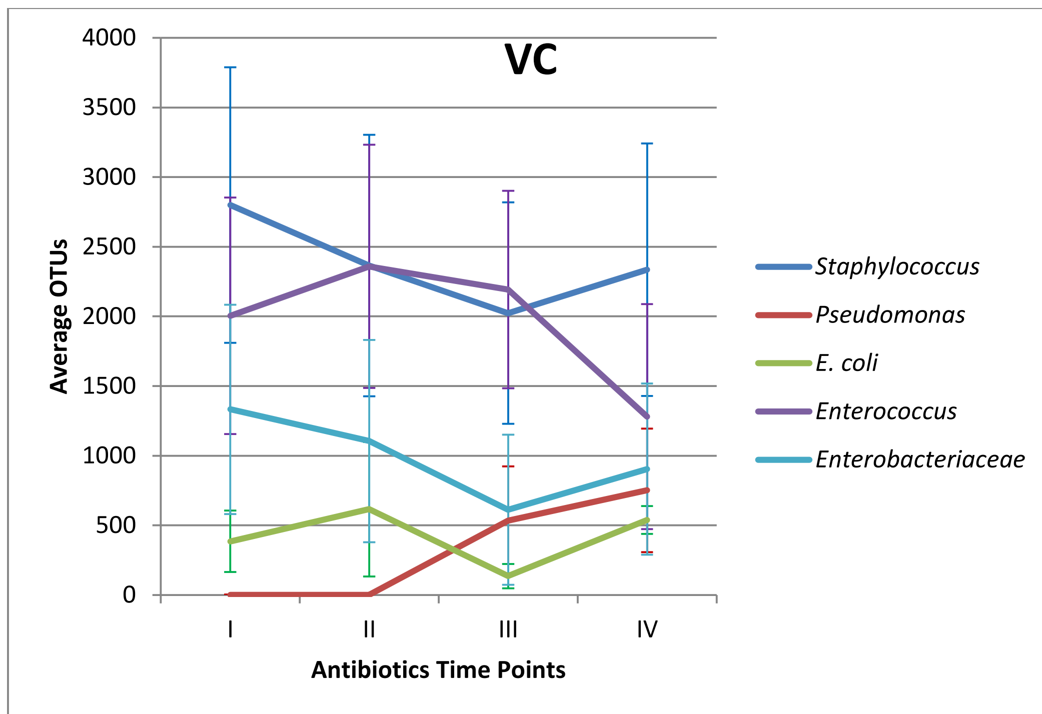


Figure 6-2: The most abundant organisms over time for VC regimen.

Figure 6.2 above indicates the average OTUs of the highest relative abundance OTUs and the effect of antibiotic course to microbial community in the cohort study. Proportion of OTUs matching the 5 most frequently observed taxa over time among the antibiotic course. Colours on the charts matching with the bacterial taxa on the legend represent the diversity in percentages. While the letters I, II, III and IV on the bottom of each chart representing different sampling points of the antibiotics I, II, III and IV represents pre, during, after and 1 week after respectively.

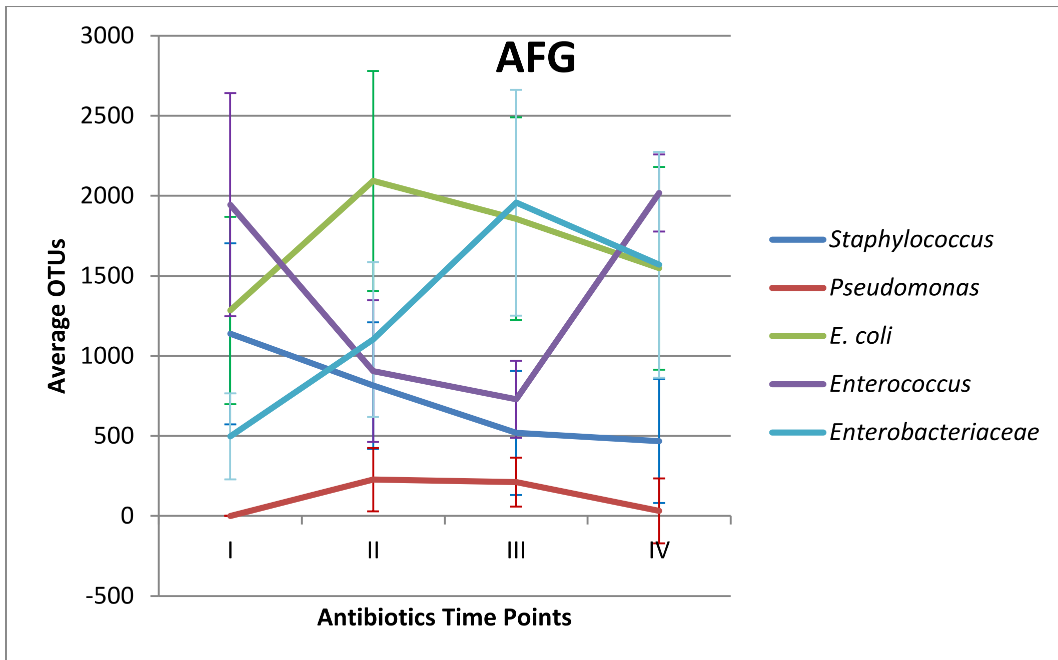


Figure 6-3: The most abundant organisms over time for AFG regimen.

Figure 6.3 above indicates the average OTUs of the highest relative abundance OTUs and the effect of antibiotic course to microbial community in the cohort study. Proportion of OTUs matching the 5 most frequently observed taxa over time among the antibiotic course. Colours on the charts matching with the bacterial taxa on the legend represent the diversity in percentages. While the letters I, II, III and IV on the bottom of each chart representing different sampling points of the antibiotics I, II, III and IV represents pre, during, after and 1 week after respectively.

Table 6-4: Average OTUs of the bacterial community in patients treated with VCM, VC & AFG at different time points

	VCM				VC				AFG			
Organisms	**I**	**II**	**III**	**IV**	**I**	**II**	**III**	**IV**	**I**	**II**	**III**	**IV**
Staphylococcus	3280±	3285±	3118±	1162	2798±	2365±	2023±	2335	1138±	814±	518±	467
Pseudomonas	1560±	1186±	1100±	1371	16±	14±	6943±	751	0.307±	226±	211±	31
E. coli	305±	444±	628±	352	384±	615±	136±	538	1283±	2092±	1856±	1547
Enterococcus	356±	559 ±	714±	1816	2004±	2359±	2192	1280	1945±	904±	728±	2018
Enterobacteriaceae	665±	1400±	448±	874	1332±	1105±	612±	902	497±	1102±	1957±	1569

6.5.2 **Shannon diversity for antibiotic courses over time**

Shannon diversity did not significantly change across different sampling points in stools from infants treated with VCM, VC or AFG (Fig. 6.4 – 6.6). The median value was decreased during antibiotic administration while there is an increase after VC treatment was stopped (Fig. 6.5). A comparable pattern was observed between VCM (Fig.6.4) and AFG (Fig. 6.6) with general decreased in diversity across the time points.

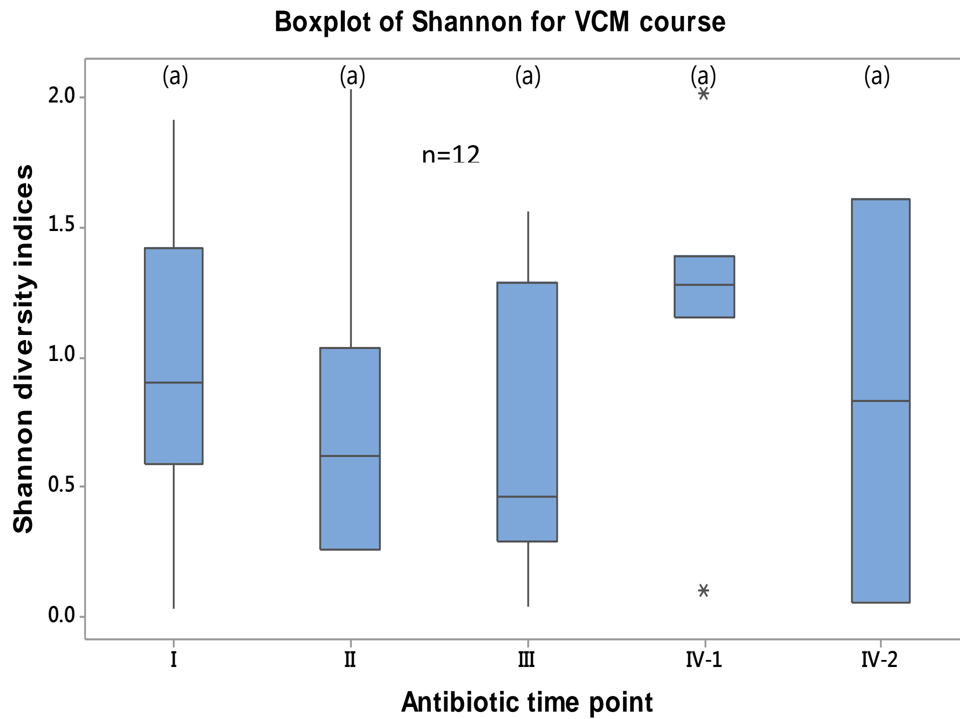


Figure 6-4: Shannon diversity for VCM course combination across the antibiotic time points

The letters below on the X axis represent time points; where I = pre, II = during, III = after, IV-1 = 1 week after and IV-2 = more than 1 week before next antibiotic course. * Asterisks represent the outliers, horizontal lines in the boxes represent the median value, shade boxes indicates upper and lower quartiles and the vertical lines extending from the boxes represent highest and lower whiskers. Diversities not significantly different between time points ***(P = 0.3).*** The 'n' inside the bos represent the patient number that received AFG regime in the cohort.

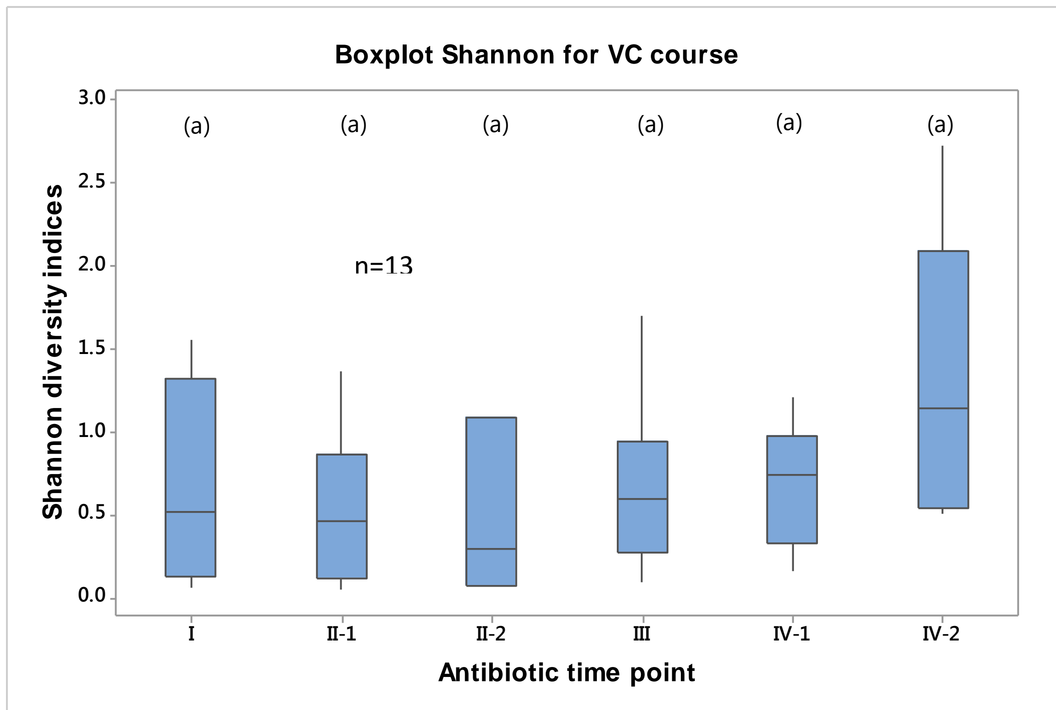


Figure 6-5: Shannon diversity for VC course combination across the antibiotic time points.

The letters below on the X axis represent time points; where I = pre, II-1 = 1-2 day during antibiotics administration, III = after, IV-1 = 1 week after and IV-2 = more than 1 week before next antibiotic course. The horizontal lines in the boxes represent the median value, shade boxes indicates upper and lower quartiles and the vertical lines extending from the boxes represent highest and lower whiskers. Diversities were not significantly different between time points ***(P-value 0.1).*** The 'n' inside the bos represent the patient number that received AFG regime in the cohort.

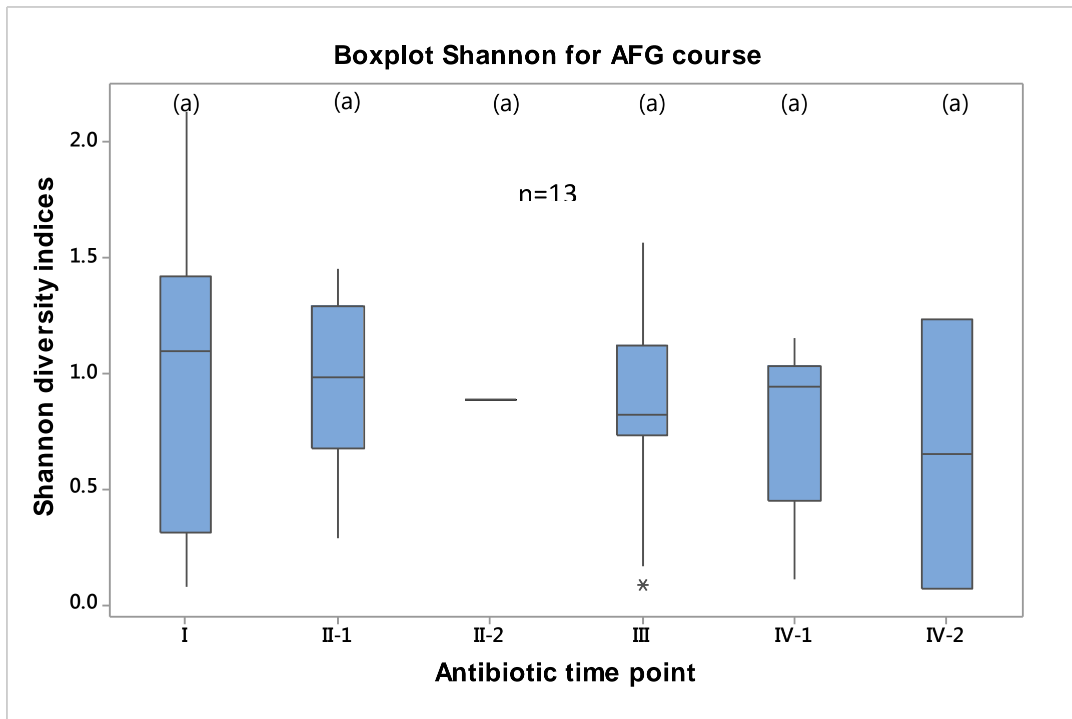


Figure 6-6: Shannon diversity for AFG course combination across the antibiotic time points.

The letters below on the X axis represent time points; where I = pre, II-1 = 1-2 day during antibiotics administration, III = after, IV-1 = 1 week after and IV-2 = more than 1 week before next antibiotic course. * Asterisks represent the outliers, horizontal lines in the boxes represent the median value, shade boxes indicates upper and lower quartiles and the vertical lines extending from the boxes represent highest and lower whiskers. Diversities are not significantly different between time points ***(P- value 0.9).*** The 'n' inside the bos represent the patient number that received AFG regime in the cohort.

6.5.3 **Average Shannon diversity of microbial taxa**

Although, there was a general trend of decreased diversity, this was not significant between treatment time points for any antibiotic mixture (Fig. 6.4 – 6.6). Overall average diversity between antibiotic mixtures was calculated, the mean, standard deviation and mean error was also calculated (Fig. 6.7). A comparable pattern was observed between VCM and VC treatment across the time points with VC lower in diversity than VCM and AFG (Fig. 6.7). Generally, there is decrease in microbial diversity with all treatment during antibiotics administration and increase after the treatment was stopped except in AFG regimen (Fig. 6.7).

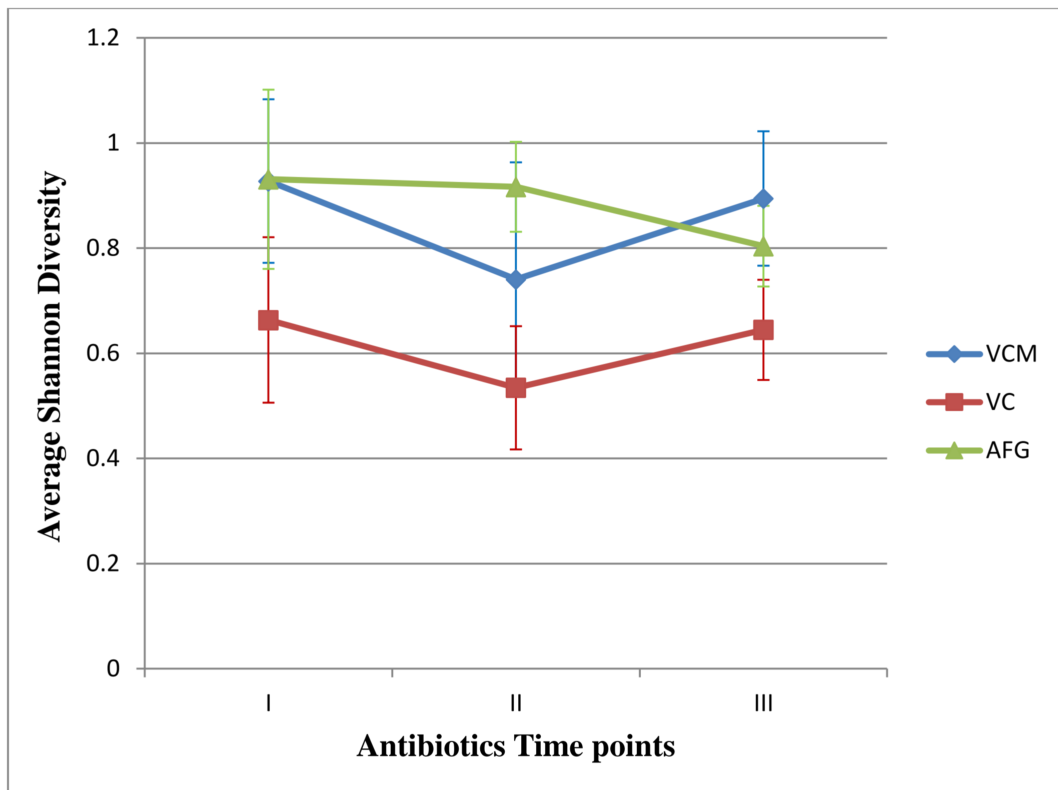


Figure 6-7: Comparison of the overall Antibiotics combination with respects to their microbial diversity over time.

A comparison of the gut community diversity measured in stool in response to the three antibiotic treatments (Fig. 6.7). Although there was a general trend to decreased diversity, this was not significant between treatment time points for each antibiotic mixture as illustrated in Figs 6.4 – 6.6.

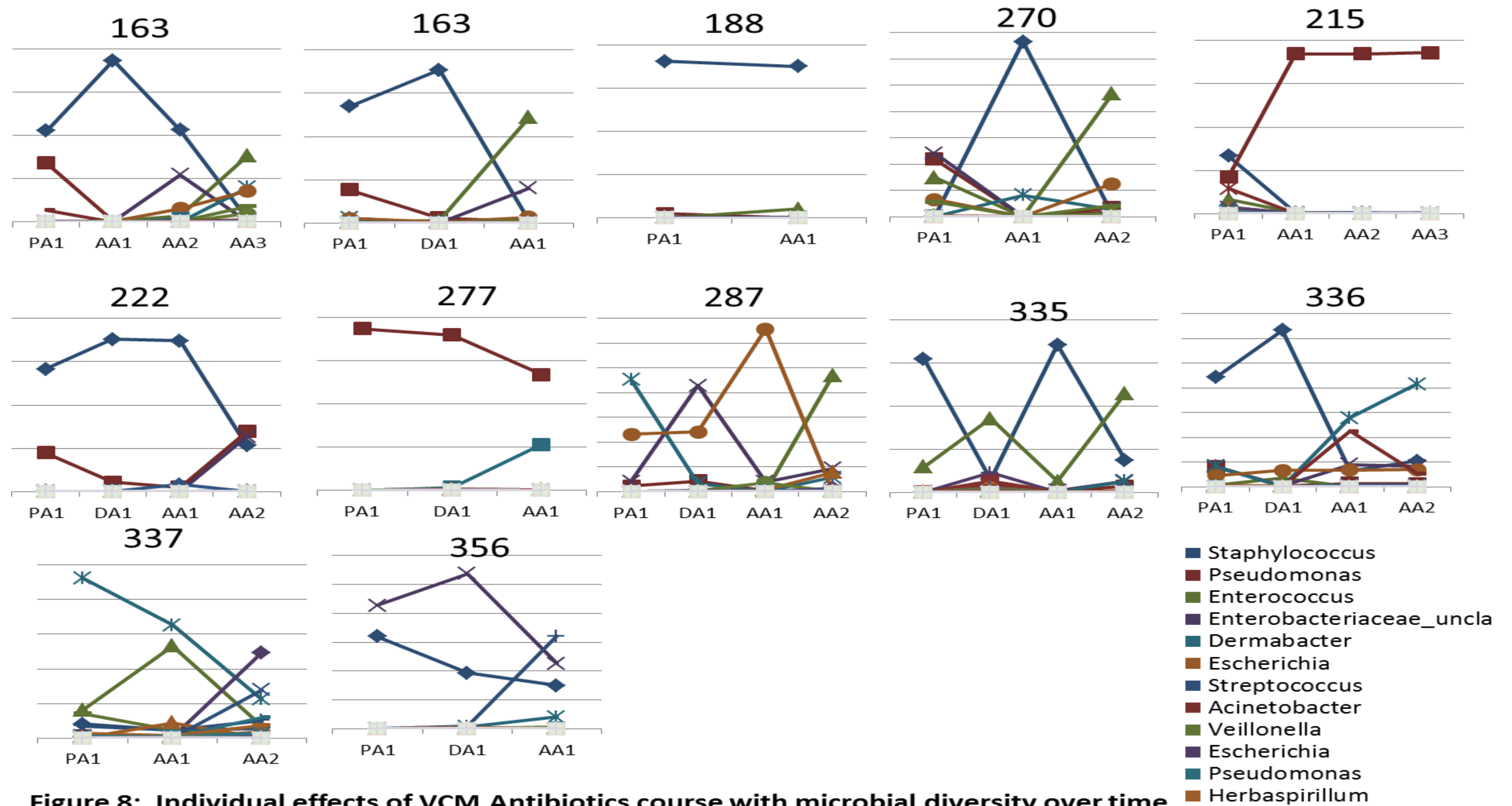


Figure 6-8: Individual effects of VCM antibiotics with microbial diversity over time

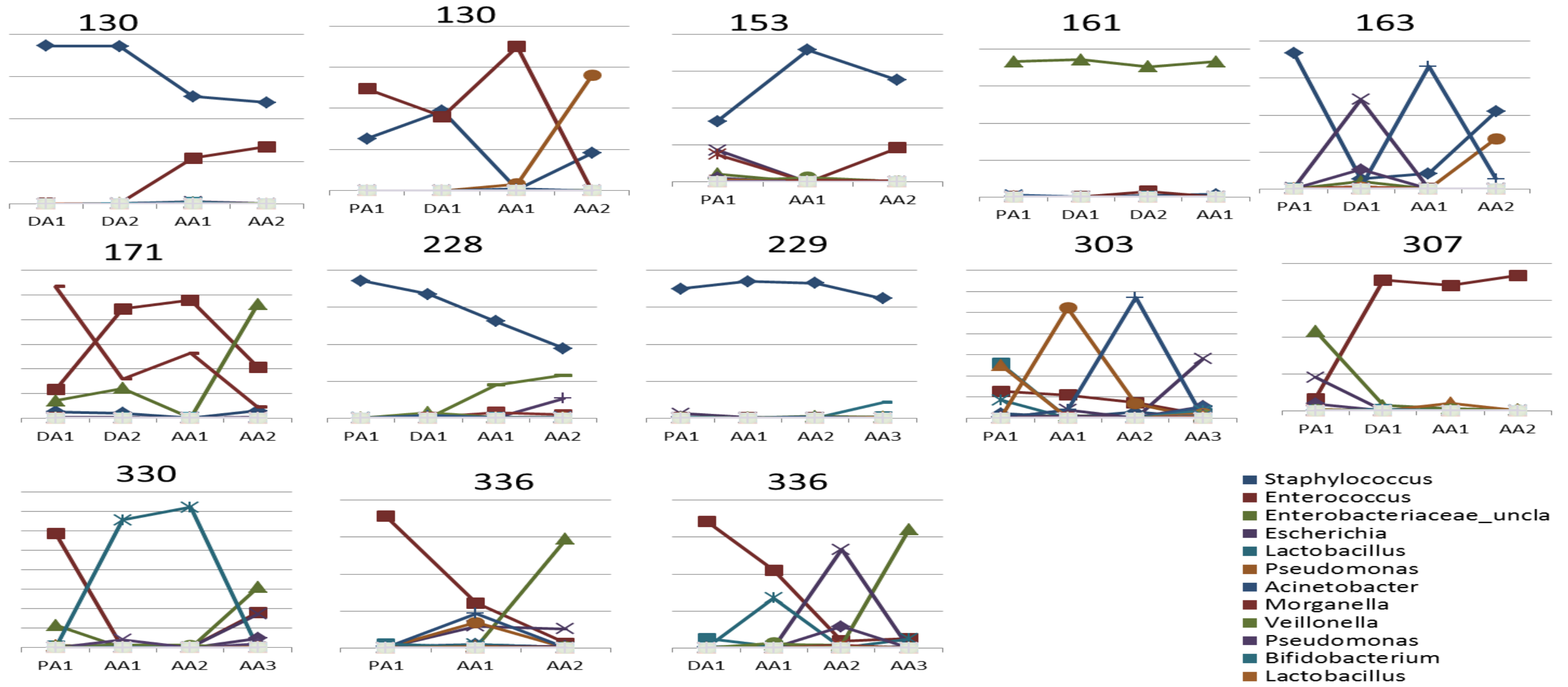


Figure 6-9: Individual effects of VC antibiotics with microbial diversity over time

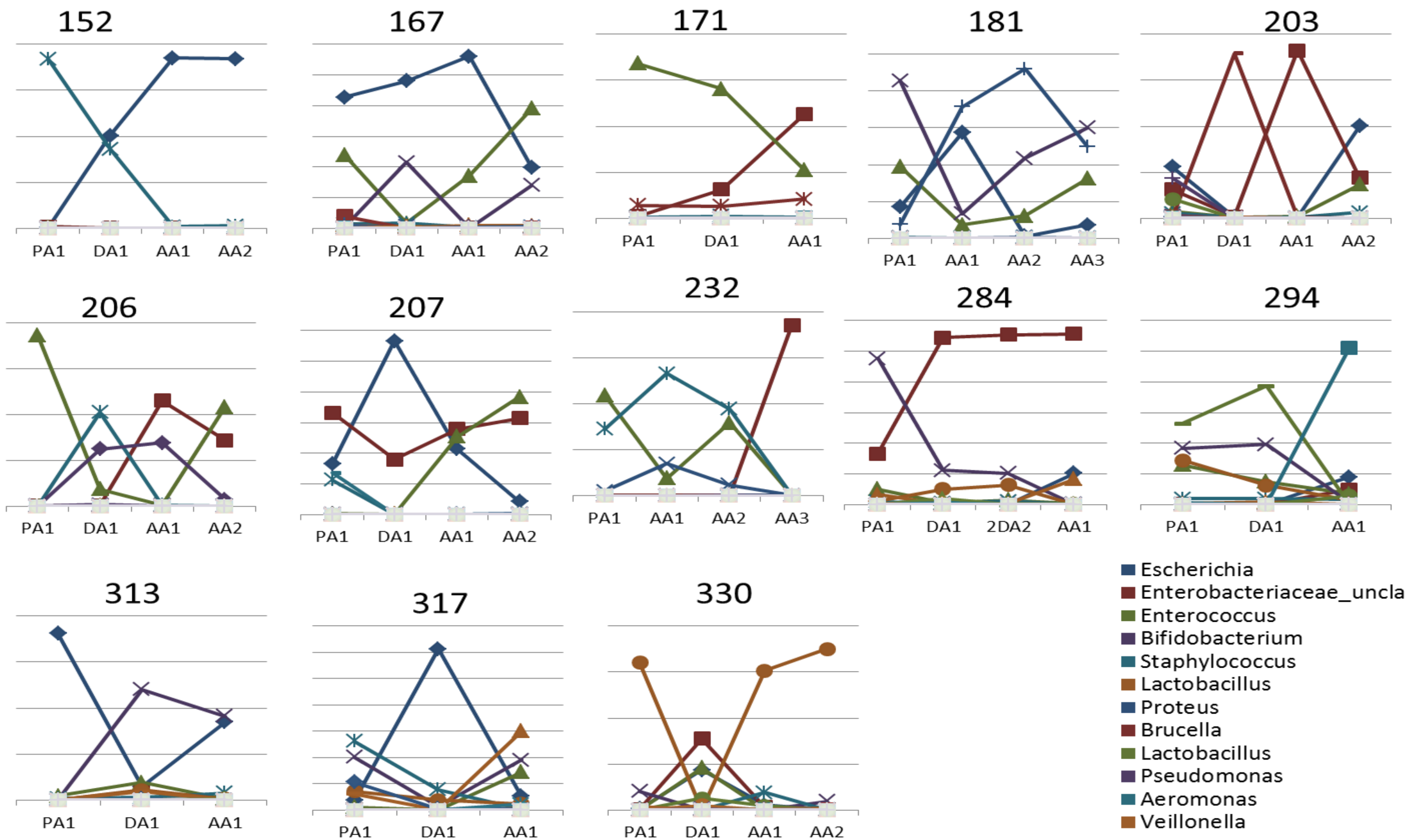


Figure 6-10: Individual effects of AFG antibiotics with microbial diversity over time

Figures 6.8 - 6.10 above indicates the individual data for each patient treated with the antibiotic course VCM, VC and AFG respectively showing the microbial diversity alongside with time points during the analysis

However, the Figures 6.8 – 6.10 illustrated the proportion of OTUs matching the 12 most frequently observed taxa across individual patients treated with VCM, VC and AFG in our cohort. Sequences matching other bacterial taxa represent less frequently (< 1 %) in the samples are not shown on the legend. The alpha-numeric numbers on the bottom of each figure represent the time point on which antibiotic course was given. PA indicates pre antibiotic, DA indicates during antibiotic and AA indicating after antibiotic. Numerical numbers on the top of each line chart represents the patient number in the cohort.

The data shows that specific taxa varied widely across the time course for each antibiotic regimen. The individual variation suggests no coherent trends and highlights the fact that bacterial gut communities are very variable from one baby to another and significantly affected by host factors.

6.6 Discussion

The faecal samples derived from 38 premature infants were robustly explored by subjecting them to 16S rRNA sequencing and further statistical analysis. A comparison was made between three antibiotic regimens which including one incorporating metronidazole to look at their impact on bacterial community in the gut of these infants. Our hypothesis was that certain antibiotic regimen may alter the preterm gut microbiota.

Overall comparison between antibiotic courses: The result from average Shannon diversity showed the following distinct points:

i- There is general decrease in microbial diversity across the entire course during the antibiotics administration and increase after the treatment stopped except in AFG, but these differences are not significant *(P = 0.9).*

ii- The overall response of the gut bacterial communities showed comparable patterns between VCM and VC regimen. However, the VC regimen had a lower diversity, but not significantly so *(P = 0.1).*

iii- The, AFG regimen had a higher diversity at sampling points when compared to other treatments, but this decreased after treatment was stopped though no statistical difference between the means was found *(P = 0.5)* (Fig. 6.7).

Moreover, with regards to the most abundant taxa, *Staphylococcus* had a high OTU number in VCM and VC regimens across the sampling points with a decrease one week after VCM treatment was terminated. *Enterococcus* also decreased in VC one week after treatment but with no significant difference *(P = 0.181)* when compared with other abundant taxa. *Pseudomonas* was lower in AFG regimen with a significant difference between its mean value and that of *Enterococcus, E. coli* and *Enterobacteriaceae* across the time points *(P = 0.02)*. *Staphylococcus* was significantly different when compared with *Pseudomonas (P*

= *0.05)* at point 1, but statistically not different at remaining sampling points *(P = 0.27)* (Fig. 6.3).

Our findings are closely related to previous study which observed a significant reduction in the diversity of bacterial populations within the gut of preterm infants (Greenwood et al. 2014). That study showed a reduction in bacterial diversity during antibiotic intake with an increased contribution of *Enterobacter* in to the community (Greenwood et al. 2014). Other studies have reported low microbial diversity with an increase in *Proteobacteria* in the preterm infants associated with NEC (Wang et al. 2009), and that the abundance of *Bifidobacterium* in the gut is reduced as a result of antibiotic usage which may result in dysbiosis (Kheadr et al., 2007). Similarly, the effect of antibiotics treatment in reducing the bacterial diversity as well as that of *Bifidobacterium* and *Bacteriodes* among infants of less than 12 month of age (Johnson & Versalovic 2012) has been reported. It has also been shown that the pattern of bacterial colonisation after one week of antibiotics treatment resemble that of at the initial point of administration (Dethlefsen & Relman 2010).

Furthermore, our research demonstrated how certain antibiotics combinations altered the composition and structure of the bacterial communities from the gut of preterm infants during the early days of life (Fig. 6.2 – 6.4 and Table 6.4). This supports with work reporting the use of antibiotics course combination within the 48 hrs of birth among preterm infants significantly altered the gut microbial community (Fouhy et al. 2012).

Previous work has demonstrated that the intestinal microbiome of infants who received antibiotics treatment had high percentage of *Proteobacteria* and lower percentages of *Actinobacteria*, *Bifidobacterium* and *Lactobacillus* than infants that did not, even after the antibiotic dose was stopped. However, two month later, *Actinobacteria, Bifidobacterium and Lactobacillus* recovered to levels observed before the antibiotic treatment (Fouhy et al. 2012). Similarly, a commensal microbiome in the human gut were shown to stabilised a few weeks after antibiotics treatment ceased (Jernberg et al. 2010).

During this study, some bacterial taxa (Fig. 6.1 & Table 6.3) were detected differentially in high abundance prior to antibiotics administration; these might be passed across from the maternal microbiota during birth or acquired immediately after delivery as it is recognised previously that the preterm GIT often harbour a microbiota resembling that of mother's skin or vaginal community dependent on the mode of delivery (Nyangale et al. 2012; Mshvildadze et al. 2010). However, it has been reported that preterm gut microbiome consists of more pathogens when compared to healthy term infants (Greenwood et al. 2014; Mai et al. 2011; Morowitz et al. 2011).

Metronidazole was described as one of the most commonly used antibiotics for the treatment of pathogenic anaerobic bacteria. Its clinical importance relates to it being cheap, its mechanisms of action being against anaerobic infections, that it had less adverse effects than many alternating and ease of application (Löfmark et al. 2010). However, combining it with other antibiotics is clinically important during the therapy of both aerobic and anaerobic infections (Zar et al. 2007; Löfmark et al. 2010). In our cohort, it was observed that when metronidazole was added to the VC course, a distinct bacterial community pattern was found enriched in *Staphylococcus* with fewer *Enterococcus*, compared to VC treatment alone. Also, there is significant $(P = 0.02)$ increase in *Pseudomonas* in VC versus VCM regimen after treatment stops. This signifies that metronidazole when combine with ceftazidine has a great effect against *Pseudomonas.* Moreover, *Staphylococcus* and *E. coli* were reduced in VCM versus VC regimens after treatment stops which imply the effects of Metronidazole against anaerobic bacteria (Table 6.3). However, in AFG regimen, *Staphylococcus* decrease across the time points indicating the effect of antibiotics mixture against *Staphylococcus* as one of the target organism (Table 6.3). With regards to *Lactobacilussus, Veillonella, Bifidobacterium, Acinetobacter* and *Enterobacteriacaea*; there is an inconsistent trend across all the sample points during the cohort study which indicates a large amount of variation within individuals in term of their gut bacterial

community and how it responds to antibiotic treatment (Berrington et al. 2014) (Fig. 9.8, 6.9 and 6.10).

Microbial diversity: Generally, AFG recorded higher diversity, followed by VCM and then VC with least diversity in the cohort study (Fig. 6.7). While with respect to sampling points, microbial diversity was higher prior to antibiotics intake and reduced during the antibiotics intake in the entire courses with variation after the dose was finished. For VCM and AFG, it was decreased across the time points but increased after VC course was stopped (Fig. 6.1 to 6.5).

Though, a short-term recovery of some bacteria was noticed from the preterm gut after antibiotics treatment, it varied and were inconsistent depending on the antibiotic combination. There is limited published data reported on the short-term recovery of microbiome from the infant's gut after antibiotics therapy (Fouhy et al. 2012) although some studies demonstrated both short and long term impacts of antibiotics treatment on gut microbiome (Jernberg et al., 2010 & Jansson et al., 2007) with also few studies on long-term impacts (Jernberg et al. 2007). However, a lack of a definite pattern of bacterial recovery after antibiotics treatment has also been reported (Johnson & Versalovic 2012).

Our study involved cross sectional samples from preterm infants receiving different antibiotics combinations. However, the limitations of our study include the low number of the preterm infants who received different antibiotics regimen and the limited antibiotics combination that could be studied. In future, it will be necessary to explore more antibiotic regimen in larger cohorts and compare that data with the present study.

6.7 Conclusions

Generally, there is decrease in microbial diversity during antibiotics intake for the entire course and an increased at one week after the treatment terminated in all the antibiotic mixture with the exception of AFG regimen. VCM and VC showed similar pattern of microbial diversity across the time points. It was therefore conclude that antibiotics administration may alter the microbial diversity from the gut of preterm infants. Further study is necessary on other antibiotic regimens that are routinely given to preterm infants and their clinical impacts in health and disease as our study is limited to particular course due to unavailability of the desired samples from other combinations.

7 CHAPTER SEVEN: Concluding remarks

7.1 Summary

The study focused on the development of the preterm gut microbiome and its response to therapeutic and nutritional interventions. These multi-disciplinary studies explored the use of culture and non-culture fingerprinting and molecular profiling to determine microbial colonisation and metabolomics to determine overall functional changes resulting from microbial-host interaction in the preterm gut environment. This study utilised the ecological theory and statistical modelling to identify the assembly, functional and phylogenetic diversity and contribution these communities make to the disease state. It was also aimed to study the impacts of clinical and dietary interventions on the community structure, function and how it alter the preterm gut microbiota by using a robust approach to analyse stool before diagnosis, at diagnosis, and after onset of NEC. However, sampling in different time points including post discharge pave way toward better understanding, comparisons and observing the long term effects of these interventions and how it can be tailored accordingly.

The bacterial mediated pathogenesis of NEC remains elusive, but several studies including our own demonstrated some potential bacteria to be associated as the aetiological pathogens of NEC (*Clostridium, Klebsiella, Bacteroides and Gammaproteobacteria*). However, our qPCR study to determine the stool bacterial load from preterm infants with NEC matched to healthy controls demonstrated the bacterial load is not associated with NEC in preterm infants. Probiotic supplementations and antibiotics therapy are found to be promising interventions in the management of NEC and alter dysbiosis as well as the functional shifts in the preterm gut microbiome. Administration of probiotics in this breast fed population was not sufficient for

eradicating NEC or LOS, but a reduction in the relative abundance of organisms previously associated with these diseases was observed. Thus therapeutics should be tailored to optimise healthy microbial communities, rather than suppressing and potentially limiting microbial diversity through the use of broad-spectrum antibiotics. Exploring the function metabolites in conjunction with microbial profiling may offer important information on how the probiotic strains are interacting with the gut ecosystem, both with other microbes and with the host.

Table 7-1– Summary of the bacterial species select from OTUs

	Control	NICU Probiotic			PD
		Before	During	After	
Acinetobacter	**1.38**	0.02	0.00	0.00	0.00
Actinomyces	**2.36**	0.00	0.00	0.00	0.00
Anaerostipes	0.00	0.00	0.00	0.00	**4.37**
Bacteroides	0.00	0.00	0.03	0.00	**10.36**
Bifidobacterium	4.02	**11.06**	**15.03**	**19.10**	**14.16**
Clostridium1	**3.84**	0.03	0.02	0.47	0.00
Clostridium2	**0.81**	0.00	0.00	0.00	0.08
Enterococcus	**18.41**	**18.93**	**24.38**	12.04	1.16
Escherichia	11.44	2.06	19.66	**23.77**	14.02
Klebsiella	**37.41**	7.93	16.54	23.92	12.84
Lachnospiracea	0.00	0.00	0.00	0.00	**5.10**
Lactobacillus1	0.00	1.73	**4.81**	**4.77**	0.00
Lactobacillus2	0.00	0.77	**5.80**	0.08	0.00
Lactobacillus3	0.00	0.73	0.20	**2.32**	0.01
Lactobacillus4	0.00	0.06	**0.19**	**0.24**	0.00
Proteus	0.00	0.00	0.00	0.00	**5.53**
Pseudomonas	**1.58**	**1.44**	0.49	0.03	0.01
Staphylococcus	5.86	**53.49**	9.73	10.52	0.02
Streptococcus1	**3.09**	0.01	0.01	0.00	1.04
Streptococcus2	**1.00**	0.00	0.00	0.00	0.00
Veillonella	0.00	0.00	0.00	0.00	**4.47**

The above table suggest a summarized bacterial species associated with NEC and how these change with time or intervention during the cohort study. The bold numbers in the table indicate the OTUSs associated with sampling time according to PLS-DA loading plot. From the previous findings and as confirmed by our research study, the following bacteria were reported to be associated with the development of NEC in preterm infants: ***Clostridium* spp.*,*** ***Klebsiella* spp.,** and ***Bacteriodes* spp.** which were all detected in our cohort study.

7.1.1 Archeal/Fungal PCR-DGGE study

Following the stool analysis from the gut of preterm infants by exploring PCR-DGGE fingerprinting of the archeal16S rRNA and fungal 28S rRNA fragments revealed low diversity of archaeal and fungal communities in preterm stool samples. Both archaeal and fungal microbial communities were found to colonize the gut of preterm infants in low abundance compare to that of bacterial microbiota with archaea in less abundance than fungi.

7.1.2 Stool bacterial load study

Resected gut tissue is not valuable as a diagnostic tool and is only taken from the most severe NEC cases, but stool bacterial load has not been explored before. If the results from stool matched to those from resected gut tissue then there is the potential for this finding to be exploited in clinical diagnostics. The aimed was to determine changes in bacterial load in preterm infant stool **t**o determine if the onset of NEC is associated with alterations in the structure and bacterial load of the preterm gut community**.** Quantitative PCR (qPCR) was carried out on longitudinal stool samples from preterm infants with established NEC cases and matched to healthy control infants to accurately quantify the total bacterial copy number (bacterial load). The stool was analysed before and after the

onset on NEC. The outcome of this study showed that no unique or characteristic trend in microbial signatures that might be responsible for causing NEC in preterm infants. Our findings also revealed that before and at diagnosis total bacterial loads in babies with NEC fluctuated widely over time and were not significantly different to bacterial loads in control samples ($p > 0.05$). Although, the overall bacterial loads analysed at one week after NEC diagnosis showed that, the load is significant lower ($p < 0.05$) between NEC and control, this is most likely due to clinical intervention with antibiotics. It was therefore conclude that more work needs to be done in quantifying specific microbial signatures associated with NEC based on time intervals as our study applied universal approach.

7.1.3 **Probiotics study**

Our findings demonstrated that Probiotic babies have statistically lower diversity compared to non-probiotic. It also revealed *Bifidobacterium* and *Lactobacillus* colonize the gut of preterm infants at different levels during and after the treatment and they all increase with probiotic supplements, which contribute to the decrease of the relative abundance of the microbial communities associated with these diseases and hence may reduce NEC or LOS in preterm infants. *B.bifidum* was found to colonize the gut before probiotics where administered and *Bifidobacteria* are more prevalent in the gut of preterm infants and found to proliferate long-term compared to Lactobacilli. Multiple OTUs associated with *Lactobacillus* were detected and qPCR was unable to robustly quantify the *L. acidophilus* used in Infloran raising potential questions about the reported quality control of available probiotics. It was conclude that, the findings in this study suggest that probiotics have the potentiality to alter the gut bacterial community in preterm infants and further work should explore this concept in advance to better understand the systematic role of probiotic supplementation.

7.1.4 **Metabolomics study**

To achieve the above recommendation, the LC-MS metabolomics approach was explored. The results showed that metabolite profiles clustered separately, with distinct metabolites associated with probiotic administration, but their identification was not available. It was suggest that probiotics have some systemic functions and play significant role in the gut microbial communities. It was demonstrated that metabolites increased during probiotics supplementation, but the identity of these metabolites was not achieved.

7.1.5 **Antibiotics study**

In order to explore the routinely used antibiotics that are usually prescribed to preterm infants in a neonatal intensive care unit and to what extent this intervention alters the preterm gut microbiome. The 16S rRNA gene profiling was utilised to analyse three commonly antibiotics course given to preterm infants in our cohort study (VCM, VC and AFG).These three antibiotics courses differentially affected the preterm gut microbiome, causing reductions in the diversity. Generally, there is decrease in microbial diversity during antibiotics intake for the entire course and an increased at one week after the treatment terminated in all the antibiotic mixture with the exception of AFG regimen. VCM and VC showed similar pattern of microbial diversity across the time points. It was therefore conclude that antibiotics administration may alter the microbial diversity from the gut of preterm infants.

7.1.6 **Future work**

Based on the findings in our study, the following recommendations and projections can be considered into future study:

- ✓ Sampling from other NICUs for comparisons of different clinical and nutritional intervention can be tailored accordingly.
- ✓ Further molecular studies exploring metabolomics approach and next generation sequencing to investigate systematic functions and genetic profiles of the archeal and fungal microbial communities from the gut of preterm infants should be employed.
- ✓ Further work should be conducted utilising specific primer set for targeting particular bacteria based on time intervals that were suggested as putative pathogens responsible for NEC. Future studies should aim to compare resected tissue and stool to determine if bacterial load is involved in NEC pathogenesis and if stool provides an accurate means of quantification.
- ✓ Exploring functional metabolite changes may provide important information on mechanisms of action of interventions such as probiotics, and should be considered for inclusion into future interventional trials. Future studies should explore this concept further to better understand the mechanistic role of probiotics supplementation
- ✓ Further study is necessary on other antibiotic regimens that are routinely given to preterm infants and their clinical impacts in health and disease as our study is limited to particular course due to unavailability of the desired samples from other combinations. Also future work is necessary to determine the contribution of these changes to health and how medical intervention can be tailored to achieve optimal outcomes for preterm infants.

References

Abdulkadir, B. et al., 2016. Routine Use of Probiotics in Preterm Infants: Longitudinal Impact on the Microbiome and Metabolome. *Neonatology*, 109(4), pp.239–247. Available at: http://www.ncbi.nlm.nih.gov/pubmed/26859305 [Accessed February 16, 2016].

Actis, G.C., 2014. The gut microbiome. *Inflamm Allergy Drug Targets*, 13(4), pp.217–223. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24953716.

Adlerberth, I. & Wold, a E., 2009. Establishment of the gut microbiota in Western infants. *Acta Paediatr*, 98(2), pp.229–38.

Agger, W. a et al., 2014. Epidemiologic factors and urogenital infections associated with preterm birth in a midwestern U.S. population. *Obstetrics and gynecology*, 124, pp.969–977.

Agne, M. et al., 2009. Principles and applications of polymerase chain reaction in medical diagnostic fields: A review. , pp.1–11.

Alexander Mankin, 2011. Definition of antibiotics. *Centre for pharmaceutical biotechnology, University of Illinoise at Chicago*.

de Almada, C.N. et al., 2015. Characterization of the intestinal microbiota and its interaction with probiotics and health impacts. *Applied microbiology and biotechnology*, pp.4175–4199. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25895093 [Accessed April 29, 2015].

Alona Bin-Nun, R.B. et al., 2005. Oral probiotics prevent necrotizing enterocolitis in very low birth weight neonates. , pp.192–196.

Altman, M. et al., 2013. Risk factors for acute respiratory morbidity in moderately preterm infants. *Paediatric and Perinatal Epidemiology*, 27, pp.172–181.

Amend, A.S., Seifert, K. a & Bruns, T.D., 2010. Quantifying microbial communities with 454 pyrosequencing: does read abundance count? *Mol Ecol*, 19(24), pp.5555–65.

Amin, M.S. et al., 2013. Identification of Bifidobacterium animalis; Bifidobacterium adolescentis and Bifidobacterium bifidum from Stool of Children and Detection of Their Antibacterial Properties. *Advances in Infectious Diseases*, 03(03), pp.200–204.

Ananthakrishnan, S. & Gunasekaran, D., 2009. Etiology and risk factors for early onset neonatal sepsis. *Indian Journal of Medical Microbiology*, 27(3), p.279. Available at: http://www.ijmm.org/article.asp?issn=0255-0857\nyear=2009\nvolume=27\nissue=3\nspage=279\nepage=279\naulast=Ananthakrishnan.

Angelakis, E., Merhej, V. & Raoult, D., 2013. Related actions of probiotics and antibiotics on gut microbiota and weight modification. *The Lancet. Infectious diseases*, 13(10), pp.889–99. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24070562 [Accessed February 22, 2015].

Antonucci, R. et al., 2009. Metabolomics: a new tool for the neonatologist. *The Journal of Maternal-Fetal & Neonatal Medicine*, 22, pp.50–53.

Antunes, L.C.M. et al., 2011. Effect of antibiotic treatment on the intestinal metabolome. *Antimicrobial Agents and Chemotherapy*, 55(4), pp.1494–1503. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3067180&tool=pmcent

rez&rendertype=abstract [Accessed May 18, 2015].

Ardissone, A.N. et al., 2014. Meconium microbiome analysis identifies bacteria correlated with premature birth. *PloS one*, 9(3), p.e90784. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3948723&tool=pmcentrez&rendertype=abstract [Accessed January 28, 2016].

Aydemir, C. et al., 2011. Randomised controlled trial of prophylactic fluconazole versus nystatin for the prevention of fungal colonisation and invasive fungal infection in very low birth weight infants. *Archives of disease in childhood. Fetal and neonatal edition*, 96(3), pp.F164–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20659937.

Barrett, E. & Guinane, C., 2013. Microbiota diversity and stability of the preterm neonatal ileum and colon of two infants. *Microbiologyopen*.

Baxter, J. & Cummings, S.P., 2008. The degradation of the herbicide bromoxynil and its impact on bacterial diversity in a top soil. *J Appl Microbiol*, 104(6), pp.1605–16.

Becker, S. et al., 2012. LC-MS-based metabolomics in the clinical laboratory. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, 883-884, pp.68–75. Available at: http://dx.doi.org/10.1016/j.jchromb.2011.10.018.

Behrman, R.E. & Butler, A.S., 2007. *Preterm Birth: Causes, Consequences, and Prevention*,

Beken, S., 2015. The ProPre-Save Study: Effects of Probiotics and Prebiotics Alone or Combined on Necrotizing Enterocolitis in Very Low Birth Weight Infants €. , pp.1–8.

Belay, N. et al., 1990. Methanogenic Bacteria in Human Vaginal Samples de f e e). , 28(7), pp.1666–1668.

Berrington, E.J. et al., 2014. The neonatal bowel microbiome in health and infection. *Current opinion in infectious diseases*, 27(3), pp.236–43. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24751892 [Accessed June 5, 2014].

Berrington, J.E. et al., 2013. Gut microbiota in preterm infants: assessment and relevance to health and disease. *Arch Dis Child Fetal Neonatal Ed*, 98(4), pp.F286–90. Available at: http://fn.bmj.com/content/98/4/F286.short [Accessed September 8, 2013].

Bhatia, J., 2010. Strategies to prevent necrotising enterocolitis. *Chin Med J (Engl)*, 123(20), pp.2759–2765.

Bielecka, M., Markiewicz, L. & Wasilewska, E., 2003. Evaluation of primers applied for PCR identification of Bifidobacterium spp. *Polish Journal of Food and Nutrition Sciences*, 12(48 89), pp.10–16.

Binns, N., 2013. *Probiotics, Prebiotics and the gut microbiota* G. R. Gibson, M. E. Sanders, & N. Delzenne., eds., Belgium: International Life Sciences Institute Europe. Available at: www.ilsi.eu.

Bjorklund, M. et al., 2012. Gut microbiota of healthy elderly NSAID users is selectively modified with the administration of Lactobacillus acidophilus NCFM and lactitol. *Age (Dordr)*, 34(4), pp.987–999. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21853265.

Blencowe, H. et al., 2013. Born too soon: the global epidemiology of 15 million preterm births. *Reproductive health*, 10 Suppl 1, p.S2. Available at:

/pmc/articles/PMC3828585/?report=abstract.

Bohnhorst, B., 2013. Usefulness of abdominal ultrasound in diagnosing necrotising enterocolitis. *Archives of disease in childhood. Fetal and neonatal edition*, 98, pp.F445–50. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23572342.

Bozeman, A.P. et al., 2013. An animal model of necrotizing enterocolitis (NEC) in preterm rabbits. *Fetal and pediatric pathology*, 32(2), pp.113–22. Available at: http://informahealthcare.com/doi/abs/10.3109/15513815.2012.681426#.UxB8O_DNZLo.mendeley [Accessed February 28, 2014].

Braga, T.D. et al., 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(1), pp.81–86.

Breitbart, M. et al., 2008. Viral diversity and dynamics in an infant gut. *Res Microbiol*, 159(5), pp.367–73. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18541415 [Accessed June 24, 2013].

Breu, F., Guggenbichler, S. & Wollmann, J., 2008. ARCHAEA: Archaea (overview). *Vasa*, pp.1–23. Available at: http://medcontent.metapress.com/index/A65RM03P4874243N.pdf.

Brinkhoff, T. & Hannen, E., 2001. Use of silicone grease to avoid “smiling effect” in denaturing gradient gel electrophoresis. *J Rapid Meth Auto Microbio*, 9(4), pp.259–261.

Van Den Broek, N.R., Jean-Baptiste, R. & Neilson, J.P., 2014. Factors associated with preterm, early preterm and late preterm birth in Malawi. *PLoS ONE*, 9.

Brookman-Amissah, N. et al., 2012. *PrimeTime qPCR Application Guide*,

Brower-Sinning, R. et al., 2014. Mucosa-associated bacterial diversity in necrotizing enterocolitis. *PloS one*, 9(9), p.e105046. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4159227&tool=pmcentrez&rendertype=abstract [Accessed September 26, 2014].

Brown, E. & Allen-Vercoe, E., 2011. Analysis of the fungal, archaeal and bacteriophage diversity in the human distal gut. *Studies by Undergraduate Researchers at Guelph*, 4(2), pp.75–82.

Bucher, B.T. et al., 2011. Bacterial DNA content in the intestinal wall from infants with necrotizing enterocolitis. *J Pediatr Surg*, 46(6), pp.1029–33.

Bultosa, G., 2016. Functional Foods: Dietary Fibers, Prebiotics, Probiotics, and Synbiotics. In *Reference Module in Food Science*. Available at: http://www.sciencedirect.com/science/article/pii/B9780081005965002456.

Burokas, A. et al., 2015. Microbiota regulation of the mammalian gut-brain axis. *Advances in Applied Microbiology*, 91, pp.1–62.

Butel, M., Suau, A. & Campeotto, F., 2007. Conditions of bifidobacterial colonization in preterm infants: a prospective analysis. *J Pediatr Gastroenterol Nutr*, 44(5), pp.577–82.

Camacho-Gonzalez, A., Spearman, P.W. & Stoll, B.J., 2013. Neonatal Infectious Diseases. Evaluation of Neonatal Sepsis. *Pediatric Clinics of North America*, 60, pp.367–389.

Caporaso, J., Kuczynski, J. & Stombaugh, J., 2010. QIIME allows analysis of high-throughput community sequencing data. *Nature*, 7(5), pp.335–336.

Caporaso, J.G. et al., 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *The ISME journal*, 6(8), pp.1621–4. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3400413&tool=pmcentrez&rendertype=abstract [Accessed July 9, 2014].

Cárdenas, N. et al., 2015. Relationships between the genome and some phenotypical properties of Lactobacillus fermentum CECT 5716, a probiotic strain isolated from human milk. *Applied microbiology and biotechnology*, 99(10), pp.4343–53. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25661998 [Accessed May 18, 2015].

Carlisle, E.M. et al., 2011. Gram Negative Bacteria Are Associated with the Early Stages of Necrotizing Enterocolitis. *PLoS One*, 6(3), p.e18084.

Cassir, N. et al., 2015. Clostridium butyricum strains and dysbiosis linked to necrotizing enterocolitis in preterm neonates. *Clinical Infectious Diseases*, 10(1093).

Cathrine, F.A. et al., 2015. Necrotizing Enterocolitis in Preterm Pigs Is Associated with Increased Density of Intestinal Mucosa-Associated Bacteria Including Clostridium perfringens. *Neonatology*, 108(3), pp.188–195.

Cavicchioli, R. et al., 2003. Pathogenic archaea: do they exist? *Bioessays*, 25(11), pp.1119–1128. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14579252.

Cetinkaya, M. et al., 2014. Efficacy of Prophylactic Fluconazole Therapy in Decreasing the Incidence of Candida Infections in Extremely Low Birth Weight Preterm Infants. *American Journal of Perinatology*.

Chacko, B. & Sohi, I., 2001. Early Onset Neonatal Sepsis. , pp.23–26.

Chakravorty, S. et al., 2007. A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. *J Microbiol Methods*, 69(2), pp.330–9.

Chen, J., Cai, W. & Feng, Y., 2007. Development of intestinal bifidobacteria and lactobacilli in breast-fed neonates. *Clin Nutr*, 26(5), pp.559–566. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17507117.

Chen, L.A. & Sears, C.L., 2014. Prebiotics, Probiotics, and Synbiotics. In *Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases*. pp. 19–25.

Chen, R.W. and J., 2011. Early colonization of intestinal bifidobacteria and lactobacilli in the postoperative neonates with congenital intestinal atresia. *Saudi Medical Journal*, 32.

Del Chierico, F. et al., 2015. Phylogenetic and Metabolic Tracking of Gut Microbiota during Perinatal Development. *Plos One*, 10(9), p.e0137347. Available at: http://dx.plos.org/10.1371/journal.pone.0137347.

Christian, L.M. et al., 2015. Gut microbiome composition is associated with temperament during early childhood. *Brain, Behavior, and Immunity*, 45, pp.118–127.

Cilieborg, M.S., Boye, M. & Sangild, P.T., 2012. Bacterial colonization and gut development in preterm neonates. *Early human development*, 88 Suppl 1, pp.S41–9. Available at: http://www.sciencedirect.com/science/article/pii/S0378378211003975 [Accessed November 11, 2015].

Claesson, M.J. et al., 2010. Comparison of two next-generation sequencing technologies for resolving highly complex microbiota composition using tandem variable 16S rRNA gene regions. *Nucleic acids research*, 38(22), p.e200. Available at:

http://nar.oxfordjournals.org/content/early/2010/09/29/nar.gkq873.abstract [Accessed June 8, 2015].

Claud, E.C. et al., 2013. Bacterial community structure and functional contributions to emergence of health or necrotizing enterocolitis in preterm infants. *Microbiome*, 1(1), p.20. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3971604&tool=pmcentrez&rendertype=abstract.

Claud, E.C. & Walker, W.A., 2001. Hypothesis: inappropriate colonization of the premature intestine can cause neonatal necrotizing enterocolitis. *The FASEB Journal*, 15(8), pp.1398–1403.

Collado, M.C. et al., 2016. Human gut colonisation may be initiated in utero by distinct microbial communities in the placenta and amniotic fluid. *Scientific Reports*, 6(October 2015), p.23129. Available at: http://www.nature.com/articles/srep23129 [Accessed March 22, 2016].

Cong, X. et al., 2015. Early Life Experience and Gut Microbiome: The Brain-Gut-Microbiota Signaling System. *Advances in neonatal care : official journal of the National Association of Neonatal Nurses*, 15(5), pp.1–10. Available at: http://www.ncbi.nlm.nih.gov/pubmed/26240939.

Cordero, L. & Ayers, L., 2003. Duration of empiric antibiotics for suspected early-onset sepsis in extremely low birth weight infants. *Infect Control Hosp Epidemiol*, 24(9), pp.662–6.

Cortese, F. et al., 2015. Early and Late Infections in Newborns: Where Do We Stand? A Review. *Pediatrics and neonatology*, pp.1–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/26750406 [Accessed January 16, 2016].

Costeloe, K. et al., 2015. Bifidobacterium breve BBG-001 in very preterm infants: a randomised controlled phase 3 trial. *The Lancet*.

Cui, L., Morris, A. & Ghedin, E., 2013. The human mycobiome in health and disease. *Genome medicine*, 5(7), p.63. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3978422&tool=pmcentrez&rendertype=abstract.

Davenport, E.R. et al., 2014. Seasonal variation in human gut microbiome composition. *PLoS ONE*, 9.

David, L.A. et al., 2014. Diet rapidly and reproducibly alters the human gut microbiome. *Nature*, 505, pp.559–63.

Deshpande, G. & Patole, S., 2013. Probiotic for preventing necrotising enterocolitis in preterm neonates-The past, present, and the future. *Eastern Journal of Medicine*, 15(4), pp.168–174.

Dessì, A. et al., 2014. Clinical metabolomics and nutrition: the new frontier in neonatology and pediatrics. *BioMed research international*, 2014, p.981219. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4163466&tool=pmcentrez&rendertype=abstract.

Dethlefsen, L. & Relman, D.A., 2010. Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation.

Dominguez-Bello, M.G. et al., 2010. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. *Proc Natl Acad Sci U S A*, 107(26), pp.11971–5. Available at:

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2900693&tool=pmcentrez&rendertype=abstract [Accessed February 28, 2013].

Dong, Y. & Speer, C.P., 2015. Late-onset neonatal sepsis: recent developments. *Archives of disease in childhood. Fetal and neonatal edition*, 100, pp.F257–63. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4413803&tool=pmcentrez&rendertype=abstract.

Drakoularakou, A., Rastall, R. & Gibson, G., 2011. 19 - Functional foods for the gut: probiotics, prebiotics and synbiotics. In M. Saarela, ed. *Functional Foods (Second edition)*. Woodhead Publishing, pp. 449–470. Available at: http://www.sciencedirect.com/science/article/pii/B9781845696900500199.

E., J., 2010. Early-onset neonatal sepsis. *Gynaecologia et Perinatologia, Supplement*, 19(SUPPL. 1), pp.S61–S67. Available at: http://www.embase.com/search/results?subaction=viewrecord&from=export&id=L360103682\nhttp://sfx.library.uu.nl/utrecht?sid=EMBASE&issn=13310151&id=doi:&atitle=Early-onset+neonatal+sepsis&stitle=Gynaecol.+Perinatol.+Suppl.&title=Gynaecologia+et+Perinatologia.

Embleton, N.D. & Yates, R., 2008. Probiotics and other preventative strategies for necrotising enterocolitis. *Semin Fetal Neonatal Med*, 13(1), pp.35–43.

Eme, L. & Doolittle, W.F., 2015. Archaea. *Current Biology*, 25(19), pp.R851–R855.

Engle, W.A., Tomashek, K.M. & Wallman, C., 2007. "Late-preterm" infants: a population at risk. *Pediatrics*, 120, pp.1390–1401.

Enwall, K. & Hallin, S., 2009. Comparison of T-RFLP and DGGE techniques to assess denitrifier community composition in soil. *Letters in Applied Microbiology*, 48(1), pp.145–148.

Eriksson, L., Johansson, E. & Kettaneh-Wold, N., 2006. *Multi-and Megavariate Data Analysis, Part 2, Advanced Applications and Method Extensions*, MKS Umetrics AB.

Falfari, Alessandro, J.E.G.K.H.M., 2010. Handbook of Partial Least Squares Concepts Methods and Applicationsmore. *Springer Handbooks of Computational Statistic*.

Falkow, S., 2004. Molecular Koch's postulates applied to bacterial pathogenicity--a personal recollection 15 years later. *Nature reviews. Microbiology*, 2(1), pp.67–72.

FAO/WHO, 2001. *Probiotics in food - Health and nutritional properties and guidelines for evaluation* Cordoba, A., Argentina.

Fiehn, O., 2002. Metabolomics – the link between genotypes and phenotypes. , pp.155–171.

Finster, K., 2008. Anaerobic bacteria and archaea in cold ecosystems. In *Psychrophiles: From Biodiversity to Biotechnology*. Springer Berlin Heidelberg, pp. 103–119.

Fouhy, F. et al., 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*, 56(11), pp.5811–20. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3486619&tool=pmcentrez&rendertype=abstract [Accessed April 9, 2015].

Fox, T.P. & Godavitarne, C., 2012. What really causes necrotising enterocolitis? *ISRN gastroenterology*, 2012, p.628317. Available at:

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3534306&tool=pmcentrez&rendertype=abstract.

Galland, L., 2014. The Gut Microbiome and the Brain. *Journal of medicinal food*, 17(12), pp.1261–1272. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25402818.

Ganna, a. et al., 2014. *A workflow for UPLC-MS non-targeted metabolomic profiling in large human population-based studies*, Available at: http://biorxiv.org/lookup/doi/10.1101/002782 [Accessed March 25, 2014].

Gaul, J., 2008. Probiotics for the prevention of necrotizing enterocolitis. *Neonatal Network: The Journal of Neonatal Nursing*, 27(2), pp.75–80.

Goldenberg, R.L. et al., 2008. Epidemiology and causes of preterm birth. *The Lancet*, 371(9606), pp.75–84.

Goldenberg, R.L. et al., 2009. Preterm Birth 1: Epidemiology and Causes of Preterm Birth. *Obstetric Anesthesia Digest*, 29(1), pp.6–7.

Goldenberg, R.L. & Mcclure, E.M., 2010. The Epidemiology of Preterm Birth. In *Preterm Birth: Prevention and Management*. Wiley-Blackwell, pp. 22–38.

Gomase, V.S. et al., 2008. Metabolomics. *Curr. Drug Metab.*, 9, pp.89–98.

Gonzalez-Perez, G. et al., 2016. Maternal Antibiotic Treatment Impacts Development of the Neonatal Intestinal Microbiome and Antiviral Immunity. *The Journal of Immunology*. Available at: http://www.jimmunol.org/cgi/doi/10.4049/jimmunol.1502322 [Accessed April 2, 2016].

Gosalbes, M.J. et al., 2016. High frequencies of antibiotic resistance genes in infants' meconium and early fecal samples. *Journal of developmental origins of health and disease*, 7(1), pp.35–44.

Gouba, N., Raoult, D. & Drancourt, M., 2013. Plant and Fungal Diversity in Gut Microbiota as Revealed by Molecular and Culture Investigations Y. Sanz, ed. *PLoS ONE*, 8(3), p.e59474. Available at: http://dx.plos.org/10.1371/journal.pone.0059474 [Accessed March 25, 2013].

Greenwood, C. et al., 2014. Early Empiric Antibiotic Use in Preterm Infants Is Associated with Lower Bacterial Diversity and Higher Relative Abundance of Enterobacter. *The Journal of pediatrics*. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24529620 [Accessed March 20, 2014].

Grishin, A. et al., 2016. Roles of nitric oxide and intestinal microbiota in the pathogenesis of necrotizing enterocolitis. In *Journal of Pediatric Surgery*. W.B. Saunders, pp. 13–17.

Groer, M.W. et al., 2014. Development of the preterm infant gut microbiome: a research priority. *Microbiome*, 2(1), p.38. Available at: http://www.microbiomejournal.com/content/2/1/38 [Accessed October 14, 2014].

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(5), pp.2318–24. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1087546&tool=pmcentrez&rendertype=abstract.

Hall, D., 2013. Satellite Symposium Nutrition of the Preterm Infant Revisited.

Hammer, Ø. & Harper, D., 2001. PAST: Paleontological statistics software package for education and data analysis. *Palaeo Electronica*, 4(1), pp.1–9.

Harpavat, S., Pammi, M. & Gilger, M., 2012. Novel treatments for NEC: Keeping IBD in mind. *Current Gastroenterology Reports*, 14, pp.373–379.

He, Q. et al., 2014. NMR-based metabolomic analysis of spatial variation in soft corals. *Marine drugs*, 12(4), pp.1876–90. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4012446&tool=pmcentrez&rendertype=abstract [Accessed May 18, 2015].

Heida, F.H. et al., 2016. A necrotizing enterocolitis-associated gut microbiota is already present in the meconium: results of a prospective study. *Clinical Infectious Diseases Advance Acc*, pp.1–29.

Herbel, S.R. et al., 2013. Timely approaches to identify probiotic species of the genus Lactobacillus. *Gut Pathog*, 5(1), p.27. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24063519.

Hill, C. et al., 2014. Expert consensus document: The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nature reviews. Gastroenterology & hepatology*, 11, p.9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24912386.

Hoffmann, C. et al., 2013. Archaea and fungi of the human gut microbiome: correlations with diet and bacterial residents. C. Pan, ed. *PloS one*, 8(6), p.e66019. Available at: http://dx.plos.org/10.1371/journal.pone.0066019 [Accessed September 19, 2013].

Hornik, C.P. et al., 2012. Early and late onset sepsis in very-low-birth-weight infants from a large group of neonatal intensive care units. *Early human development*, 88 Suppl 2(SUPPL.2), pp.S69–74. Available at: http://www.scopus.com/inward/record.url?eid=2-s2.0-84861474088&partnerID=tZOtx3y1 [Accessed February 10, 2016].

Huffnagle, G.B. & Noverr, M.C., 2013. The emerging world of the fungal microbiome. *Trends in microbiology*, 21(7), pp.334–41. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3708484&tool=pmcentrez&rendertype=abstract [Accessed May 28, 2015].

Hunter, C.J. et al., 2008. Understanding the susceptibility of the premature infant to necrotizing enterocolitis (NEC). *Pediatric Research*, 63(2), pp.117–123.

Icaza-Chávez, M.E., 2013. [Gut microbiota in health and disease]. *Revista de gastroenterología de México*, 78(4), pp.240–8. Available at: http://www.revistagastroenterologiamexico.org//es/microbiota-intestinal-salud-enfermedad/articulo/S0375090613001468/.

Idle, J.R. & Gonzalez, F.J., 2007. Metabolomics. *Cell Metabolism*, 6(5), pp.348–351. Available at: http://www.sciencedirect.com/science/article/pii/S1550413107002987.

Iliev, I.D. & Underhill, D.M., 2013. Striking a balance: fungal commensalism versus pathogenesis. *Current opinion in microbiology*, 16(3), pp.366–73. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3742553&tool=pmcentrez&rendertype=abstract [Accessed November 3, 2015].

Illumina, 2012. *An Introduction to Next-Generation Sequencing Technology Welcome to Next-Generation Sequencing*,

Inna, S. et al., 2010. Gut Microbiota in Health and Disease. *Physiology review*, 90:,

pp.859–904.

Isenbarger, T. a et al., 2008. The most conserved genome segments for life detection on Earth and other planets. *Orig Life Evol Biosph*, 38(6), pp.517–33.

J., B., 2011. The role of prebiotics in prevention of allergy in children. *Pediatrie pro Praxi*, 12(5), pp.359–360. Available at: http://www.pediatriepropraxi.cz/pdfs/ped/2011/05/18.pdf\nhttp://ovidsp.ovid.com/ovidweb.cgi?T=JS&PAGE=reference&D=emed10&NEWS=N&AN=2012270196.

Jacobs, S.E. et al., 2013. Probiotic effects on late-onset sepsis in very preterm infants: a randomized controlled trial. *Pediatrics*, 132, pp.1055–62. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24249817.

Jakaitis, B.M. & Denning, P.W., 2014. Commensal and probiotic bacteria may prevent NEC by maturing intestinal host defenses. *Pathophysiology*, 21, pp.47–54.

Janse, I., Bok, J. & Zwart, G., 2004. A simple remedy against artifactual double bands in denaturing gradient gel electrophoresis. *J Microbiol Methods*, 57(2), pp.279–81.

Jansson, J. et al., 2009. Metabolomics reveals metabolic biomarkers of Crohn's disease. *PloS one*, 4(7), p.e6386. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19636438.

Janvier, A., Malo, J. & Barrington, K.J., 2014. Cohort study of probiotics in a North American neonatal intensive care unit. *The Journal of pediatrics*, 164(5), pp.980–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24411521 [Accessed May 27, 2014].

Jernberg, C. et al., 2007. Long-term ecological impacts of antibiotic administration on the human intestinal microbiota. *The ISME journal*, 1(1), pp.56–66. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18043614 [Accessed August 7, 2015].

Jernberg, C. et al., 2010. Long-term impacts of antibiotic exposure on the human intestinal microbiota. *Microbiology (Reading, England)*, 156(Pt 11), pp.3216–23. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20705661 [Accessed June 2, 2015].

John, P. et al., 2006. Factors influencing the composition of the intestinal microbiota in early infancy. *Pediatrics*, 118(2), pp.511–21. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16882802 [Accessed October 1, 2014].

Johnson, C.L. & Versalovic, J., 2012. The human microbiome and its potential importance to pediatrics. *Pediatrics*, 129(5), pp.950–60. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22473366 [Accessed September 3, 2015].

Ju, F. & Zhang, T., 2015. 16S rRNA gene high-throughput sequencing data mining of microbial diversity and interactions. *Applied microbiology and biotechnology*, 99(10), pp.4119–29. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25808518 [Accessed April 30, 2015].

Julia, C.A. et al., 2010. Using a Mathematical Model to Analyze the Role of Probiotics and Inflammation in Necrotizing Enterocolitis: e10066. *PloS one*, 5(4).

Kacerovsky, M. et al., 2011. The microbial load with genital mycoplasmas correlates with the degree of histologic chorioamnionitis in preterm PROM. *American Journal of Obstetrics and Gynecology*, 205.

Kang, Y. et al., 2010. Multiple copies of 16S rRNA gene affect the restriction patterns and DGGE profile revealed by analysis of genome database. *Microbiol*, 79(5), pp.655–662.

Karlen, Y. et al., 2007. Statistical significance of quantitative PCR. *BMC bioinformatics*, 8.

Kasper, D.C. et al., 2010a. The bacterial load of Ureaplasma parvum in amniotic fluid is correlated with an increased intrauterine inflammatory response. *Diagnostic Microbiology and Infectious Disease*, 67(2), pp.117–121.

Kasper, D.C. et al., 2010b. The bacterial load of Ureaplasma parvum in amniotic fluid is correlated with an increased intrauterine inflammatory response. *Diagnostic microbiology and infectious disease*, 67(2), pp.117–21. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20207094 [Accessed May 17, 2015].

Kaufman, D. et al., 2014. Fungal Infections in Preterm Infants. *Medscape*.

Kheadr, E. et al., 2007. Antibiotic susceptibility profile of bifidobacteria as affected by oxgall, acid, and hydrogen peroxide stress. *Antimicrobial agents and chemotherapy*, 51(1), pp.169–74. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1797697&tool=pmcentrez&rendertype=abstract [Accessed September 2, 2015].

Klaenhammer, T.R. et al., 2012. The impact of probiotics and prebiotics on the immune system. *Nature Reviews Immunology*, 12, pp.728–734. Available at: http://dx.doi.org/10.1038/nri3312.

Koh, A.Y., 2013. Gastrointestinal Colonization of Fungi. *Current Fungal Infection Reports*, 7(2), pp.144–151. Available at: http://link.springer.com/10.1007/s12281-013-0133-2 [Accessed November 3, 2015].

Kozich, J., Arbor, A. & Patrick, S., 2014. *16S Sequencing with the Illumina MiSeq Personal Sequencer*,

Kozich, J.J. et al., 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Applied and environmental microbiology*, 79(17), pp.5112–20.

de la Cochetiere, M.-F. et al., 2004. Early intestinal bacterial colonization and necrotizing enterocolitis in premature infants: the putative role of Clostridium. *Pediatric research*, 56(3), pp.366–70. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15201403 [Accessed October 6, 2014].

Lafeber, H.N. et al., 2008. Nutritional factors influencing infections in preterm infants. *The Journal of nutrition*, 138(9), p.1813S–1817S.

Lagomarcino et al., 2013. Early microbial and metabolomic signatures predict later onset of necrotizing enterocolitis in preterm infants. *Microbiome,*, 1(13), p.13. Available at: http://www.microbiomejournal.com/content/1/1/13 [Accessed April 25, 2013].

Lambert, C., 2016. Towards a new design for probiotic foods in Europe. In *Probiota Conference*.

Lane, D., 1995. 16S/23S rRNA sequencing. In M. Stackebrandt, E. and Goodfellow, ed. *Nucleic Acid Techniques in Bacterial Systematics*. New York: NY, John Wiley and Sons, pp. 115–175.

LaTuga, M.S., Stuebe, A. & Seed, P.C., 2014. A review of the source and function of microbiota in breast milk. *Seminars in Reproductive Medicine*, 32(1), pp.68–73.

Leach, S.T. et al., 2015. Multiple Opportunistic Pathogens, but Not Pre-existing Inflammation, May Be Associated with Necrotizing Enterocolitis. *Digestive diseases and sciences*, 60(12), pp.3728–34. Available at: http://www.ncbi.nlm.nih.gov/pubmed/26250832 [Accessed November 9, 2015].

Licht, T.R., Ebersbach, T. & Frøkiær, H., 2012. Prebiotics for prevention of gut infections. *Trends in Food Science and Technology*, 23, pp.70–82.

Lin, P.W., Nasr, T.R. & Stoll, B.J., 2008. Necrotizing enterocolitis: recent scientific advances in pathophysiology and prevention. *Semin Perinatol*, 32(2), pp.70–82.

Lin, P.W. & Stoll, B.J., 2006. Necrotising enterocolitis. *The Lancet*, 368, pp.1271–1283. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17027734.

Lindemann, P.C., Foshaugen, I. & Lindemann, R., 2004. Characteristics of breast milk and serology of women donating breast milk to a milk bank. *Archives of disease in childhood. Fetal and neonatal edition*, 89, pp.F440–F441.

Liu, X., Cao, S. & Zhang, X., 2015. Modulation of Gut Microbiota-Brain Axis by Probiotics, Prebiotics, and Diet. *Journal of Agricultural and Food Chemistry*, 63(36), pp.7885–7895.

Löfmark, S., Edlund, C. & Nord, C.E., 2010. Metronidazole is still the drug of choice for treatment of anaerobic infections. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*, 50 Suppl 1(Suppl 1), pp.S16–23. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20067388 [Accessed August 8, 2015].

Luan, C. et al., 2015. Dysbiosis of fungal microbiota in the intestinal mucosa of patients with colorectal adenomas. *Scientific reports*, 5, p.7980. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25613490 [Accessed November 3, 2015].

Lukes, J. et al., 2015. Are Human Intestinal Eukaryotes Beneficial or Commensals? *PLoS Pathog*, 11(8).

Madsen, K., 2011. The International Scientific Conference on Probiotics and Prebiotics. *Expert review of gastroenterology & hepatology*, 5, pp.571–3. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21910573.

Magne, F. et al., 2005. Fecal microbial community in preterm infants. *J Pediatr Gastroenterol Nutr*, 41(4), pp.386–92.

Mai, V. et al., 2013. Distortions in Development of Intestinal Microbiota Associated with Late Onset Sepsis in Preterm Infants. *PLoS ONE*, 8(1).

Mai, V. et al., 2011. Fecal microbiota in premature infants prior to necrotizing enterocolitis. *PLoS One*, 6(6), p.e20647.

Maria, B., Wasilewska Ewa & Lidia, M., 2003. Evaluation of primers applied for PCR identification of Bifidobacterium Spp. *Polish Journal of Food and Nutrition Sciences*, 12(53), pp.10–16.

Marincola, F.C. et al., 2012. A metabolomic study of preterm human and formula milk by high resolution NMR and GC/MS analysis: preliminary results. *J Matern Fetal Neonatal Med*, 25(Suppl 5), pp.62–67. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23025771.

Martin, R. et al., 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(6), pp.2638–2644.

Martín, R. et al., 2009. Isolation of bifidobacteria from breast milk and assessment of the bifidobacterial population by PCR-denaturing gradient gel electrophoresis and quantitative real-time PCR. *Appl Environ Microbiol*, 75(4), pp.965–9.

Matarazzo, F. et al., 2012. The domain Archaea in human mucosal surfaces. *Clinical Microbiology and Infection*, 18(9), pp.834–840.

Mayer, E.A., Tillisch, K. & Gupta, A., 2015. Gut/brain axis and the microbiota. *Journal of Clinical Investigation*, 125(3), pp.926–938.

McMurtry, V.E. et al., 2015. Bacterial diversity and Clostridia abundance decrease with increasing severity of necrotizing enterocolitis. *Microbiome*, 3, pp.1–8. Available at: http://www.microbiomejournal.com/content/3/1/11.

Meinzen-Derr, J. et al., 2009. Epidemiology of necrotizing enterocolitis temporal clustering in two neonatology practices. *The Journal of pediatrics*, 154(5), pp.656–61.

Mihatsch, W.A. et al., 2012. Critical systematic review of the level of evidence for routine use of probiotics for reduction of mortality and prevention of necrotizing enterocolitis and sepsis in preterm infants. *Clin Nutr*, 31(1), pp.6–15. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21996513.

Millar, M., Costeloe, K. & Wilks, M., 2003. Probiotics for preterm infants? *Arch Dis Child Fetal Neonatal Ed.*, 88. Available at: group.bmj.com.

Miller, K.M. et al., 1999. Denaturing Gradient Gel Electrophoresis (DGGE): A Rapid and Sensitive Technique to Screen Nucleotide Sequence Variation in Populations Research Report. , 27(5).

Moles, L. et al., 2013. Bacterial diversity in meconium of preterm neonates and evolution of their fecal microbiota during the first month of life. *PloS one*, 8(6), p.e66986. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3695978&tool=pmcentrez&rendertype=abstract [Accessed August 28, 2015].

Morgan, J., Young, L. & McGuire, W., 2014. Delayed introduction of progressive enteral feeds to prevent necrotising enterocolitis in very low birth weight infants. *The Cochrane database of systematic reviews*, 12.

Morowitz, M.J. et al., 2011. Strain-resolved community genomic analysis of gut microbial colonization in a premature infant. *Proceedings of the National Academy of Sciences of the United States of America*, 108(3), pp.1128–33. Available at: http://www.pnas.org/cgi/content/long/108/3/1128 [Accessed January 15, 2016].

Morrow, A.L. et al., 2013. Early microbial and metabolomic signatures predict later onset of necrotizing enterocolitis in preterm infants. *Microbiome*, 1(1), p.13. Available at: http://www.microbiomejournal.com/content/1/1/13 [Accessed April 25, 2013].

Mshvildadze, M. et al., 2010. Intestinal microbial ecology in premature infants assessed with non-culture-based techniques. *The Journal of pediatrics*, 156(1), pp.20–5. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3628625&tool=pmcentrez&rendertype=abstract [Accessed August 20, 2014].

Mshvildadze, M. & Neu, J., 2010. The infant intestinal microbiome: friend or foe? *Early human development*, 86 Suppl 1(1), pp.67–71. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3586601&tool=pmcentrez&rendertype=abstract [Accessed August 30, 2014].

Mulle, J.G., Sharp, W.G. & Cubells, J.F., 2013. The gut microbiome: A new frontier in autism research. *Current Psychiatry Reports*, 15.

Muyzer G, De Waal E. C & Uitterlinden A. G., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol*, 59(3), pp.695–700.

Muyzer, G. & Smalla, K., 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie van Leeuwenhoek*, 73(1), pp.127–41. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9602286.

Neu, J., 2007. Gastrointestinal development and meeting the nutritional needs of premature infants. *The American journal of clinical nutrition*, 85(2), p.629S–634S. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17284768.

Neu, J., 2014. Probiotics and necrotizing enterocolitis. *Clin Perinatol*, 41(4), pp.967–978.

Ng, P.C., 2013. Biomarkers of Neonatal Infection and Necrotising Enterocolitis-The State of the Art Approach. *Annals of the Academy of Medicine, Singapore*, 42, pp.S47–S47. Available at: http://ovidsp.ovid.com/ovidweb.cgi?T=JS&PAGE=reference&D=emed11&NEWS=N&AN=75000532.

Ng, P.C. et al., 2007. IP-10 is an early diagnostic marker for identification of late-onset bacterial infection in preterm infants. *Pediatric research*, 61(1), pp.93–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17211148 [Accessed May 18, 2015].

Ng, P.C., Ma, T.P.Y. & Lam, H.S., 2015. The use of laboratory biomarkers for surveillance, diagnosis and prediction of clinical outcomes in neonatal sepsis and necrotising enterocolitis. *Archives of Disease in Childhood - Fetal and Neonatal Edition*, pp.1–6. Available at: http://fn.bmj.com/cgi/doi/10.1136/archdischild-2014-307656.

Nole, K.L.B., Yim, E. & Keri, J.E., 2014. Probiotics and prebiotics in dermatology. *Journal of the American Academy of Dermatology*, 71, pp.814–821.

Nyangale, E.P., Mottram, D.S. & Gibson, G.R., 2012. Gut microbial activity, implications for health and disease: the potential role of metabolite analysis. *Journal of proteome research*, 11(12), pp.5573–5585.

Ogura, T. & Sakamoto, Y., 2007. *Application of Metabolomics Techniques using LC / MS and GC / MS Profi ling Analysis of Green Tea Leaves*,

Ott, S.J. et al., 2008. Fungi and inflammatory bowel diseases: Alterations of composition and diversity. *Scandinavian journal of gastroenterology*, 43(7), pp.831–41. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18584522 [Accessed November 4, 2015].

Ouwehand, A. et al., 2009. Influence of a combination of Lactobacillus acidophilus NCFM and lactitol on healthy elderly: intestinal and immune parameters. *Br J Nutr*, 101(3), pp.367–375. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18634707.

Palmer, C. et al., 2007. Development of the human infant intestinal microbiota. *PLoS Biol*, 5(7), pp.1556–73.

Pammi, M. & Sa, A., 2011. Oral lactoferrin for the prevention of sepsis and necrotizing enterocolitis in preterm infants (Review). , (10).

Paolucci, M., Landini, M.P. & Sambri, V., 2012. How Can the Microbiologist Help in Diagnosing Neonatal Sepsis? *International Journal of Pediatrics*, 2012, pp.1–14.

Papen, H. et al., 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie van Leeuwenhoek*, 73(1), pp.123–130. Available at: http://dx.doi.org/10.1023/A:1000669317571\nhttp://dx.doi.org/10.1023/A:1004243

810473.

Parfrey, L.W. et al., 2014. Communities of microbial eukaryotes in the mammalian gut within the context of environmental eukaryotic diversity. *Frontiers in microbiology*, 5(June), p.298. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4063188&tool=pmcentrez&rendertype=abstract [Accessed September 29, 2015].

Partty, A. et al., 2013. Effects of early prebiotic and probiotic supplementation on development of gut microbiota and fussing and crying in preterm infants: a randomized, double-blind, placebo-controlled trial. *J Pediatr*, 163(5), p.1272. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23915796.

Patel, R. & Dupont, H.L., 2015. New approaches for bacteriotherapy: Prebiotics, new-generation probiotics, and synbiotics. *Clinical Infectious Diseases*, 60, pp.S108–S121.

Paturi, G. et al., 2015. Differential effects of probiotics, prebiotics, and synbiotics on gut microbiota and gene expression in rats. *Journal of Functional Foods*, 13, pp.204–213.

Pélissier, M.-A. et al., 2010. Metronidazole effects on microbiota and mucus layer thickness in the rat gut. *FEMS microbiology ecology*, 73(3), pp.601–10. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20579100 [Accessed August 6, 2015].

Penders, J. et al., 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(1), pp.141–7.

Petrosino, J.F. et al., 2009. Metagenomic pyrosequencing and microbial identification. *Clin Chem*, 55(5), pp.856–66.

Picaud, J.-C., 2013a. Nutrition of preterm infants revisited. In *Satellite symposium presented during the 54th Annual meeting of the European society for paediatric research*. Portugal: Science for better nutrition. Available at: www.Nestlenutrition-institute.org.

Picaud, J.-C., 2013b. Why, How and When Should we use Probiotics in Preterm Infants? *Nestle Nutrition Institute- Science for better Nutrition*.

Pierro, A., 2005. The surgical management of necrotising enterocolitis. In *Early Human Development*. pp. 79–85.

Pikuta, E. V, 2011. Overview of Archaea. *SPIE Proceedings - Microbial Extremophiles I*, 8152.

Prosser, J.I. et al., 2007. The role of ecological theory in microbial ecology. *Nat Rev Microbiol*, 5(5), pp.384–92.

QiLi, Z. et al., 2014. Comparative study on the relevant factors for early-onset and late-onset neonatal sepsis. *Modern Preventive Medicine*, 41(20), pp.3700–3702.

Quigley, E.M.M., 2010. Prebiotics and probiotics; modifying and mining the microbiota. *Pharmacological Research*, 61, pp.213–218.

Ranjard, L., Poly, F. & Nazaret, S., 2000. Monitoring complex bacterial communities using culture-independent molecular techniques: application to soil environment. *Research in Microbiology*, 151(3), pp.167–177. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0923250800001364.

Ransom, C.E. & Murtha, A.P., 2012. Progesterone for Preterm Birth Prevention. *Obstetrics and Gynecology Clinics of North America*, 39, pp.1–16.

Reed, A.J. & Hicks, R.E., 2011. Microbial ecology of Lake Superior Bacteria and Archaea: An overview. *{AQUATIC} {ECOSYSTEM} {HEALTH} & {MANAGEMENT}*, 14(4), pp.386–395.

Remon, J. et al., 2014. Acute drop in blood monocyte count differentiates NEC from other causes of feeding intolerance. *Journal of perinatology : official journal of the California Perinatal Association*, 34(7), pp.549–54. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24674979.

Repa, A. et al., 2015. Probiotics (Lactobacillus acidophilus and Bifidobacterium bifidum) prevent NEC in VLBW infants fed breast milk but not formula. *Pediatr Res*, 77(2), pp.381–388.

Resta, S. et al., 1985. Isolation and propagation of a human enteric coronavirus. *Science Magazine- Journal*, 229(4717), pp.978–981.

Rigon, G. et al., 2012. Maternal factors pre- and during delivery contribute to gut microbiota shaping in newborns. *Frontiers in cellular and infection microbiology*, 2(July), p.93. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3417649&tool=pmcentrez&rendertype=abstract [Accessed May 18, 2015].

Robinson, J., 2014. Cochrane in context: probiotics for prevention of necrotizing enterocolitis in preterm infants. *Evid Based Child Health*, 9(3), pp.672–674. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25236308.

Rodrı, J.M. et al., 2015. The composition of the gut microbiota throughout life, with an emphasis on early life. , 1, pp.1–17.

Roessner, U. et al., 2000. Simultaneous analysis of metabolites in potato tuber by gas chromatography-mass spectrometry. *The Plant Journal*, 23(1), pp.131–142. Available at: http://doi.wiley.com/10.1046/j.1365-313x.2000.00774.x.

Roger, L.C. et al., 2010. Examination of faecal Bifidobacterium populations in breast- and formula-fed infants during the first 18 months of life. *Microbiology*, 156(Pt 11), pp.3329–41.

Rougé, C. et al., 2010. Investigation of the intestinal microbiota in preterm infants using different methods. *Anaerobe*, 16(4), pp.362–70. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20541022 [Accessed November 7, 2010].

Russell, B. & R., A., 2011. Neonatal sepsis. *Paediatrics and Child Health*, 21, pp.265–269.

Saccenti, E. et al., 2013. Reflections on univariate and multivariate analysis of metabolomics data. *Metabolomics*, 10(3), pp.361–374.

Sakai, K., 2008. Denaturing Gradient Gel Electrophoresis (DGGE) Molecular microbiological methods on community profiling.

Samuelsson, a et al., 2014. Late-onset neonatal sepsis, risk factors and interventions: an analysis of recurrent outbreaks of Serratia marcescens, 2006-2011. *The Journal of hospital infection*, 86(1), pp.57–63. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24332914 [Accessed February 9, 2016].

Sandhu, G.S. et al., 1995. Molecular probes for diagnosis of fungal infections. *J Clin Microbiol*, 33(11), pp.2913–9.

Santos, R.P. & Tristram, D., 2015. A Practical Guide to the Diagnosis, Treatment, and Prevention of Neonatal Infections. *Pediatric Clinics of North America*, 62, pp.491–508.

Sanz, Y., 2016. EFSA update on the scientific requirements for the substantiation of health claims related to immunity, pathogens and gastrointestinal function. In *Probiota Conference*.

Sarkar, S., 2013. *Potential of probiotics as pharmaceutical agent: A review*, Available at: http://www.scopus.com/inward/record.url?eid=2-s2.0-84886799507&partnerID=40&md5=a7ef271cd2c192123e81fbc690bc7bf5.

Satar, M. & Özlü, F., 2012. Neonatal sepsis: A continuing disease burden. *Turkish Journal of Pediatrics*, 54, pp.449–457.

Saulnier, D.M. et al., 2013. The intestinal microbiome, probiotics and prebiotics in neurogastroenterology. *Gut microbes*, 4, pp.17–27.

Saxon, E., 2015. Beyond bar charts. *BMC biology*, 13(1), p.60. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4526301&tool=pmcentrez&rendertype=abstract [Accessed August 10, 2015].

Sayres, W.G., 2010. Preterm labor. *American Family Physician*, 81, pp.477–484.

Scaldaferri, F. et al., 2013. Gut microbial flora, prebiotics, and probiotics in IBD: Their current usage and utility. *BioMed Research International*, 2013.

Scanlan, P.D. & Marchesi, J.R., 2008. Micro-eukaryotic diversity of the human distal gut microbiota: qualitative assessment using culture-dependent and -independent analysis of faeces. *The ISME journal*, 2(12), pp.1183–93. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18670396 [Accessed November 3, 2015].

Schloss, P., 2009. A high-throughput DNA sequence aligner for microbial ecology studies. *PLoS One*, 4(12), p.e8230.

Schloss, P.D., Gevers, D. & Westcott, S.L., 2011. Reducing the Effects of PCR Amplification and Sequencing Artifacts on 16S rRNA-Based Studies. *PLoS One*, 6(12), p.e27310.

Seiya Tsushima, Y.M., 2010. Technical Report on the PCR-DGGE Analysis of Bacterial and Fungal Soil Communities Table of Contents.

Shah, P. & Shah, V., 2007. Arginine supplementation for prevention of necrotising enterocolitis in preterm infants. *Cochrane Database of Systematic Reviews*.

Shane, A.L. & Stoll, B.J., 2014. Neonatal sepsis: Progress towards improved outcomes. *Journal of Infection*, 68.

Shapiro-Mendoza, C.K. & Lackritz, E.M., 2012. Epidemiology of late and moderate preterm birth. *Seminars in Fetal and Neonatal Medicine*, 17, pp.120–125.

Shlomai, O. et al., 2014. Probiotics for preterm neonates: what will it take to change clinical practice? *Neonatology*, 105(1), pp.64–70. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24281648.

Shukla, S. et al., 2011. Meta-analysis: The effects of gut flora modulation using prebiotics, probiotics and synbiotics on minimal hepatic encephalopathy. *Alimentary Pharmacology and Therapeutics*, 33, pp.662–671.

Sim, K. et al., 2014. Dysbiosis anticipating necrotizing enterocolitis in very premature infants. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*, p.ciu822–. Available at: http://cid.oxfordjournals.org/content/early/2014/10/23/cid.ciu822.abstract [Accessed October 29, 2014].

Simmons, L.E. et al., 2010. Preventing Preterm Birth and Neonatal Mortality: Exploring the Epidemiology, Causes, and Interventions. *Seminars in Perinatology*, 34,

pp.408–415.

Simonsen, K.A. et al., 2014. Early-onset neonatal sepsis. *Clinical Microbiology Reviews*, 27, pp.21–47.

Singh, T.R.-S.A., Firek, B., Brooks, B., Castelle, C.J., et al., 2015. <Gut bacteria are rarely shared by co-hospitalized premature infants, regardless of necrotizing enterocolitis development (1).pdf>. *Genomics and evolutionary biology | Microbiology and infectious disease*, 4(e05477).

Singh, T.R.-S.A., Brian Firek, B.B., et al., 2015. Gut bacteria are rarely shared by co-hospitalized premature infants, regardless of necrotizing enterocolitis development. *Genomics and evolutionary biology | Microbiology and infectious disease*, 4(e05477).

Singh, T.R.-S.A., Firek, B., Brooks, B., Castelle1, C.J., et al., 2015. Gut bacteria are rarely shared by co-hospitalized premature infants, regardless of necrotizing enterocolitis development. *Genomics and evolutionary biology | Microbiology and infectious disease*, 4(e05477).

Siqueira, J.F., Fouad, A.F. & Rôças, I.N., 2012. Pyrosequencing as a tool for better understanding of human microbiomes. *J Oral Microbiol*, 4, pp.1–15.

Smith, C. & Osborn, A., 2009. Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbial ecology. *FEMS Microbiol Ecol*, 67(1), pp.6–20. Available at: http://onlinelibrary.wiley.com/doi/10.1111/j.1574-6941.2008.00629.x/full [Accessed July 26, 2013].

Soccol, C.R. et al., 2013. The Potential of Probiotics : A Review. , 48(4), pp.413–434.

Soccol, C.R. et al., 2010. The potential of probiotics: A review. *Food Technology and Biotechnology*, 48(4), pp.413–434.

Sodhi, C. et al., 2008. The development of animal models for the study of necrotizing enterocolitis. *Dis Model Mech*, 1(2-3), pp.94–98. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19048070.

Sousa, S. et al., 2015. In vitro evaluation of yacon (Smallanthus sonchifolius) tuber flour prebiotic potential. *Food and Bioproducts Processing*, 95, pp.96–105.

Stefanovic, I.M., 2011. Neonatal sepsis. *Biochemia medica*, 21, pp.276–81. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22420241.

Stenger, M.R. et al., 2011. Probiotics and prebiotics for the prevention of necrotizing enterocolitis. *Curr Infect Dis Rep*, 13(1), pp.13–20. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21308450 [Accessed March 9, 2011].

Stewart, C.J. et al., 2013. Bacterial and fungal viability in the preterm gut: NEC and sepsis. *Arch Dis Child Fetal Neonatal Ed*. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23426613 [Accessed June 14, 2013].

Stewart, C.J. et al., 2013. Development of the Preterm Gut Microbiome in Twins at Risk of Necrotising Enterocolitis and Sepsis. *PLoS ONE*, 8.

Stewart, C.J. et al., 2015. Preterm gut microbiota and metabolome following discharge from intensive care. *Scientific reports*, 5(February 2016), p.17141. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4657104&tool=pmcentrez&rendertype=abstract [Accessed February 11, 2016].

Stewart, C.J. et al., 2012. The preterm gut microbiota: changes associated with necrotizing enterocolitis and infection. *Acta paediatrica*, 101(11), pp.1121–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22845166 [Accessed May 23,

2013].

Stoll, B.J. et al., 2004. Neurodevelopmental and growth impairment among extremely low-birth-weight infants with neonatal infection. *JAMA*, 292(19), pp.2357–65.

Strathdee, F. & Free, A., 2013. Denaturing gradient gel electrophoresis (DGGE). *Methods Mol.Biol.*, 1054, pp.145–157.

Szajewska, H., 2010. Probiotics and prebiotics in preterm infants: Where are we? Where are we going? *Early Human Development*, 86(SUPPL. 1).

Tabasco, R. et al., 2007. Selective enumeration and identification of mixed cultures of Streptococcus thermophilus, Lactobacillus delbrueckii subsp. bulgaricus, L. acidophilus, L. paracasei subsp. paracasei and Bifidobacterium lactis in fermented milk. *International Dairy Journal*, 17(9), pp.1107–1114.

Taft, D.H. et al., 2014. Intestinal microbiota of preterm infants differ over time and between hospitals. *Microbiome*, 2(1), p.36. Available at: http://www.microbiomejournal.com/content/2/1/36 [Accessed October 15, 2014].

Tappero, E. & Johnson, P., 2010. Laboratory evaluation of neonatal sepsis. *Newborn and Infant Nursing Reviews*, 10(4), pp.209–217.

Al Tawil, K. et al., 2010. Neonatal necrotizing fasciitis and fungal infections in preterm infants. *Journal of Neonatal-Perinatal Medicine*, 3(2), pp.147–151.

Temmerman, R. & Masco, L., 2003. Development and validation of a nested-PCR-denaturing gradient gel electrophoresis method for taxonomic characterization of bifidobacterial communities. *Appl Environ Microbiol*, 69(11), pp.6380–6385.

Thabit, A.K. & Nicolau, D.P., 2015. Lack of Correlation between Bristol Stool Scale and Quantitative Bacterial Load in Clostridium difficile Infection. *Infectious diseases*, 8, pp.1–4. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4346303&tool=pmcentrez&rendertype=abstract.

Thompson-Chagoyan, O.C., Maldonado, J. & Gil, A., 2007. Colonization and impact of disease and other factors on intestinal microbiota. *Dig Dis Sci*, 52(9), pp.2069–2077. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17420934.

Thompson-Chagoyán, O.C., Maldonado, J. & Gil, A., 2007. Colonization and impact of disease and other factors on intestinal microbiota. *Dig Dis Sci*, 52(9), pp.2069–77.

Tian, R. et al., 2010. Characterization of a necrotizing enterocolitis model in newborn mice. , 3(4), pp.293–302.

Tobin, J.M. et al., 2013. Rapid assay to assess colonization patterns following in-vivo probiotic ingestion. *BMC Res Notes*, 6, p.252. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23830222.

Toledo, U. of, 2004. Denaturing Gradient Gel Electrophoresis (DGGE). *Department of Earth, Ecological and Environmental Sciences*, pp.1–7.

Torrazza, R.M. et al., 2013. Intestinal microbial ecology and environmental factors affecting necrotizing enterocolitis. *PloS one*, 8(12), p.e83304. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3875440&tool=pmcentrez&rendertype=abstract [Accessed January 23, 2014].

Torrazza, R.M. & and Josef Neu, 2012. NIH Public Access. *Clinical perinatol*, 40(1), pp.93–108.

Torrazza, R.M. & Neu, J., 2013. The Altered Gut Microbiome and Necrotizing Enterocolitis. *Clin Perinatol.*, 14(1), pp.93–108.

Tourlomousis, P. & Kemsley, E., 2010. electrophoresis of complex microbial communities: A two-step approach to address the effect of gel-to-gel variation and allow valid comparisons across a large dataset. *Microb Ecol*, 59(4), pp.776–86.

Trivedi, D.K., 2012. The Application of SIMCA P+ in Shotgun Metabolomics Analysis of ZIC®HILIC-MS Spectra of Human Urine - Experience with the Shimadzu IT-T of and Profiling Solutions Data Extraction Software. *Journal of Chromatography & Separation Techniques*, 03(06).

Tucker, J. & McGuire, W., 2004. ABC of preterm birth Epidemiology of preterm birth. *BMJ*, 329(7467), pp.675–678.

Turnbaugh, P.J. et al., 2009. A core gut microbiome in obese and lean twins. , 457(32089), pp.480–484.

Turroni, F. et al., 2014. Bifidobacterium bifidum as an example of a specialized human gut commensal. *Front Microbiol*, 5(August), p.437. Available at: http://journal.frontiersin.org/article/10.3389/fmicb.2014.00437/abstract [Accessed April 15, 2015].

Tuzun, F. et al., 2013. Breast milk jaundice: effect of bacteria present in breast milk and infant feces. *Journal of pediatric gastroenterology and nutrition*, 56(3), pp.328–32. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23132163.

V.V. Tolstikov, N.T. and O.F., 2002. Metabolomics LC-MS Development analysis. *Anal.Biochem.*, 301, pp.298–307.

Venkatesh, M.P. & Abrams, S. a, 2010. Oral lactoferrin for the prevention of sepsis and necrotizing enterocolitis in preterm infants. *Cochrane Database Syst Rev*, 5(6), p.CD007137.

Ventura, M. et al., 2012. Host-microbe interactions that facilitate gut colonization by commensal bifidobacteria. *Trends in microbiology*, 20(10), pp.467–76. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22902802 [Accessed October 4, 2012].

Vergnano, S. & Heath, P.T., 2013. Fetal and neonatal infections. *Medicine*, 41, pp.723–729. Available at: http://www.sciencedirect.com/science/article/pii/S1357303913002648.

Vieira, A.T., Teixeira, M.M. & Martins, F.S., 2013. The role of probiotics and prebiotics in inducing gut immunity. *Frontiers in Immunology*, 4.

Villarreal, M.L.M. et al., 2013. Advantageous direct quantification of viable closely related probiotics in petit-suisse cheeses under in vitro gastrointestinal conditions by Propidium Monoazide--qPCR. *PloS one*, 8(12), p.e82102.

Villas-Bôas, S.G., Rasmussen, S., Lane, G.A., et al., 2005. Metabolomics or metabolite profiles? *Trends Biotechnol*, 23(8), pp.385–386. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15939497.

Villas-Boas, S.G., Rasmussen, S. & Lane, G.A., 2005. Metabolomics or metabolite profiles? *Trends Biotechnol*, 23(8), pp.385–386. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15939497.

Villas-Bôas, S.G., Rasmussen, S. & Lane, G.A., 2005. Metabolomics or metabolite profiles? *Trends in Biotechnology*, 23(8), pp.385–386.

Vyas, U. & Ranganathan, N., 2012. Probiotics, prebiotics, and synbiotics: Gut and beyond. *Gastroenterology Research and Practice*.

Walker, A.W. et al., 2011. High-throughput clone library analysis of the mucosa-associated microbiota reveals dysbiosis and differences between inflamed and non-

inflamed regions of the intestine in inflammatory bowel disease. *BMC microbiology*, 11(1), p.7. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3032643&tool=pmcentrez&rendertype=abstract [Accessed September 9, 2015].

Wall, R. et al., 2007. Genomic diversity of cultivable Lactobacillus populations residing in the neonatal and adult gastrointestinal tract. *FEMS microbiology ecology*, 59(1), pp.127–37. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16978242 [Accessed May 18, 2015].

Wang, J. et al., 2015. Modulation of gut microbiota during probiotic-mediated attenuation of metabolic syndrome in high fat diet-fed mice. *ISME J*, 9(1), pp.1–15. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24936764.

Wang, Y. et al., 2009. 16S rRNA gene-based analysis of fecal microbiota from preterm infants with and without necrotizing enterocolitis. *ISME J*, 3(8), pp.944–54.

Wang, Y. & Qian, P.-Y., 2009. Conservative fragments in bacterial 16S rRNA genes and primer design for 16S ribosomal DNA amplicons in metagenomic studies. *PLoS One*, 4(10), p.e7401.

Wang, Z., Gerstein, M. & Snyder, M., 2010. RNA-Seq : a revolutionary tool for transcriptomics. , 10(1), pp.57–63.

Ward, Doyle V, Dirk Gevers, David S. Newburg, Ardyth L. Morrow, & K.S., 2014. Impact of Antibiotic administration on the establishment and development of infant Gut flora. *Infants Gut Metagenomics initiative, Broad institute.org*.

Ward, T.L. et al., 2013. Human milk metagenome: a functional capacity analysis. *BMC Microbiology*, 13(1), p.116. Available at: http://www.biomedcentral.com/1471-2180/13/116.

Warner, B.B. et al., 2016. Gut bacteria dysbiosis and necrotising enterocolitis in very low birthweight infants: a prospective case-control study. *The Lancet*, 6736(16), pp.1–8. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0140673616000817 [Accessed March 10, 2016].

Waterman, D.S., Bonner, F.W. & Lindon, J.C., 2009. Spectroscopic and statistical methods in metabonomics. *Bioanalysis*, 1(9), pp.1559–1578.

Weisburg, W.G. et al., 1991. <16S Ribosomal DNA Amplification for Phylogenetic Study.pdf>. *JOURNAL OF BACTERIOLOGY,*, Vol. 173(No. 2), pp.697–703.

Westerbeek, E. a M. et al., 2006. The intestinal bacterial colonisation in preterm infants: a review of the literature. *Clinical nutrition (Edinburgh, Scotland)*, 25(3), pp.361–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16677741 [Accessed May 18, 2015].

Widger, J., O’Connell, N.H. & Stack, T., 2010. Breast milk causing neonatal sepsis and death. *Clinical Microbiology and Infection*, 16(12), pp.1796–1798.

Wiedmeier, J.E. et al., 2011. Early postnatal nutrition and programming of the preterm neonate. *Nutrition Reviews*, 69(2), pp.76–82. Available at: http://dx.doi.org/10.1111/j.1753-4887.2010.00370.x.

Wikoff, W.R. et al., 2009. Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. *Proceedings of the National Academy of Sciences of the United States of America*, 106(10), pp.3698–3703. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2656143&tool=pmcentrez&rendertype=abstract.

Willing, B.P., Russell, S.L. & Finlay, B.B., 2011. Shifting the balance: antibiotic effects on host-microbiota mutualism. *Nature reviews. Microbiology*, 9(4), pp.233–43. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21358670 [Accessed September 8, 2015].

Wisanskoonwong, P., Fahy, K. & Hastie, C., 2011. The effectiveness of medical interventions aimed at preventing preterm birth: A literature review. *Women and Birth*, 24, pp.141–147.

Wishart, D.S., 2011. Advance in metabolite identification. *Bioanalysis*, 3(15), pp.1769–1782.

Wishart, D.S., 2009. Computational strategies for metabolite identification in metabolomics. *Bioanalysis*, 1(9), pp.1579–1596.

Woese, C., 2007. 16 rRNA Sequencing to identify unknown microorganism. *online seq anal*, pp.1–5.

Worley, B. & Powers, R., 2012. Multivariate Analysis in Metabolomics. *Current Metabolomics*, 1, pp.92–107.

Wu, H. & Wu, E., 2012. The role of gut microbiota in immune homeostasis and autoimmunity © 2012 Landes Bioscience . Do not distribute . © 2012 Landes Bioscience . Do not distribute . , 3(1), pp.4–14.

Xie, G. et al., 2013. Metabolomics approaches for characterizing metabolic interactions between host and its commensal microbes. *Electrophoresis*, 34(19), pp.2787–2798.

Yang, Y. et al., 2014. A meta-analysis of probiotics for preventing necrotizing enterocolitis in preterm neonates. *Brazilian Journal of Medical and Biological Research*, 47(9), pp.804–810.

Yeast, J.D. & Lu, G., 2007. Biochemical Markers for the Prediction of Preterm Delivery. *Clinics in Perinatology*, 34(4), pp.573–586.

Ying, Z. et al., 2005. Study of microbial community structures in UASB sludge treating municipal wastewater by denaturing gradient gel electrophoresis of 16S rDNA. , 48(ii), pp.128–135.

van Zanten, G.C. et al., 2014. Synbiotic Lactobacillus acidophilus NCFM and cellobiose does not affect human gut bacterial diversity but increases abundance of lactobacilli, bifidobacteria and branched-chain fatty acids: a randomized, double-blinded cross-over trial. *FEMS microbiology ecology*, 90(1), pp.225–36. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25098489 [Accessed September 2, 2014].

Zar, F. a et al., 2007. A comparison of vancomycin and metronidazole for the treatment of Clostridium difficile-associated diarrhea, stratified by disease severity. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*, 45(3), pp.302–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17599306 [Accessed September 2, 2015].

Zbinden, A. et al., 2015. Case Series of Bifidobacterium longum Bacteremia in Three Preterm Infants on Probiotic Therapy. *Neonatology*, 107(1), pp.56–59.

Zdzislawa Libudzisz, 2010. *Microbiota of Human Gastrointestinal Tract*,

Zhang, Z., Liu, C. & Guo, X., 2008. Safety of probiotics--a review. *Wei sheng wu xue bao = Acta microbiologica Sinica*, 48(2), pp.257–261.

Zhongtang, Y. et al., 2008. Evaluations of different hypervariable regions of archaeal 16S rRNA genes in profiling of methanogens by Archaea-specific PCR and denaturing gradient gel electrophoresis. *Applied and environmental microbiology*,

74(3), pp.889–93. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2227698&tool=pmcentrez&rendertype=abstract [Accessed September 2, 2014].

Zhou, B. et al., 2013. LC-MS-based metabolomics. *Mol Biosyst.*, 8(2), pp.470–481.

Zuhair, O.E., 2012. Early- and Late-Onset Neonatal Sepsis: Risk Factors and Outcome Study. *Kerbala Journal of Medicine*, 5(1), pp.1341–1356.

Appendices

Appendix 1- Dyes

Bromophenol blue (6x concentrate)

Preparation: Bromophenol blue was prepared at 6× concentrate and diluted appropriately with the sample as required as follows:

0.025g Bromophenol blue

4.0g Sucrose

dH_2O to 1 L

DCode Dye

DCode dye was added to the „high" denaturing solution so that the efficacy of mixing between the low and high solutions when pouring the gradient for the DGGE gel could be established. The solution was prepared as follows;

0.05g Bromophenol blue

0.05g Xylene cyanol

1x TAE up to 10 ml

DGGE loading dye (2× concentrate)

DGGE loading dye was prepared at 2× concentrate and diluted appropriately with the sample as required. The solution was prepared as below:

Step 1 - 2% (w/v) solution of bromophenol blue and xylene cyanol

A 2% (w/v) solution of bromophenol blue and a 2% (w/v) solution of xylene cyanol were prepared by dissolving 0.002g of each solid in 1 mL dH_2O.

Step 2 – Make up to 10 mL

0.25ml 2% (w/v) bromophenol blue

0.25ml 2% (w/v) xylene cyanol

7.0ml 100% glycerol

2.5ml dH2O

Appendix 2- TAE Buffer

TAE buffer was prepared at 50x concentrate then diluted as required.

100ml EDTA pH 8.0;

Step 1

18.61g EDTA

100ml dH2O

The beaker containing the EDTA and 50ml dH2O was placed on to a magnetic stirrer and the pH was measured throughout. Sodium hydroxide pellets were added to the solution until the solution was at pH 8.0. Addition dH2O was added as required to achieve a final volume of 100ml.

Step 2

242g Tris base ultrapure

57.1ml Glacial acetic acid

100ml EDTA pH 8.0 dH2O to 1L

The Tris base was weighed and placed into a 1L Duran bottle along with the glacial acetic acid and the EDTA which was prepared fresh as described above. The buffer was then made up to 1L with dH2O.

To make 1x TAE dilute 1 part 50x TAE in 49 parts dH2O

200 mL EDTA pH 8.0

Step 1

37.22 g EDTA

200 mL dH_2O

A beaker containing the EDTA and ~150 mL dH_2O was placed on to a magnetic stirrer and the pH was measured throughout. Sodium hydroxide pellets were added to the solution until the solution was at pH 8.0. dH_2O was added to achieve a final volume of 200 mL.

Step 2 – Make up 2 L

484g Tris base ultrapure

114.2 mL Glacial acetic acid

200 mL EDTA pH 8.0 dH_2O to 2L

The Tris base was weighed and placed into a 1L Duran bottle along with the glacial acetic acid and the EDTA which was prepared fresh as described above. dH_2O was added to achieve a final volume of 2 L.

To make 1 L of 1× TAE: dilute 20 mL of 50x TAE in 980 mL dH2O.

Appendix 3 – DGGE denaturing solutions

Reagent	Bacterial		Fungal	
	34%	55%	40%	60%
40% (v/v) acrylamide (37.5:1 acrylamide:bisacrylamide)	30 mL	30 mL	30 mL	30 mL
50x TAE	2 mL	2 mL	2 mL	2 mL
Deionised formamide	13.6 mL	22 mL	16 mL	24 mL
Urea (electrophoresis grade)	14.28 g	23.1 g	16.8 g	25.2 g
dH2O	To 100 mL	To 100 mL	To 100 mL	To 100m L

Appendix 4 – SOC media

Step 1 – Prepare solutions

Prepare the following solutions:

1M NaCl

0.5844 g NaCl

dH_2O to 10 mL

1M KCl

0.7455 g KCl

DH_2O to 10 mL

2M Mg2+ stock

2.330 g $MgCl_2 \cdot 6H_2O$

2.465 g $MgSO_4 \cdot 7H_2O$

dH_2O to 10 mL filter sterilise with a 0.22 µM filter

2M glucose

3.603 g Glucose

dH_2O to 10 mL filter sterilise with a 0.22 µM filter

Step 2 – Make the media

To make the media add;

2.0 g Tryptone

0.5 g Yeast extract

1 mL 1M NaCl

1 mL 1M KCl

dH_2O to 100 mL

Autoclave and allow to cool to room temperature. Then add;

1 mL 2M Mg^{2+}

1 mL 2M glucose

Check the pH is 7.0, adjust accordingly if it is not.

Appendix 5 – Luria-Bertani media

Basic recipe (per Litre)

Tryptone 10 g

Yeast Extract 5 g

Sodium Chloride 5 g

Agar 15 g (For broth omit agar from the recipe)

Autoclave at 121°C for 45 minutes and allows cooling to 50 °C and pouring ~ 20 mL in to each Petri dish.

JM109 LB plates (LB/ampicillin/IPTG/X-Gal)

Once the media from the basic recipe has cooled to 50 °C add Ampicillin (100 µg/mL), IPTG (0.5 mM), and X-Gal (80 µg/mL) to the media and pour as described above.

Antibiotic selection broth

Omit agar from the basic recipe and then proceed as described above. When the media has cooled to 50 °C add Ampicillin (100 µg/mL). For plates pour as described above and for broths dispense 5mL aliquots into sterile glass universals.

Appendix 6 - The PCR-DGGE Analysis for Archaeal and Fungal Microbial diversity from the gut of preterm infants with Necrotising enterocolitis and Sepsis

Summary

The aim was to compare the archaeal and fungal diversity from patients that have had NEC or sepsis compared to healthy controls. Due to the fastidious nature of Archaea and their low abundance in most microbial community and the lack of data on archaeal communities in relation to preterm infants, it was aimed to map the diversity of Archaea in the gut of preterm infants to identify any significant correlation between their presence and disease outcomes.

A total of 43 (NEC, Sepsis and Nec/Sepsis) test DNA samples and 24 Controls (NEC and Sepsis) were identified from the demographic data. The parameters used to identify infants for the study and controls include: Mode of delivery, Sex, Birth weight, Gestational Age. Emphasis was given to samples analysed on two days, one and two weeks before and after the occurrence of NEC/Sepsis respectively. DNA extracts were analysed by PCR-DGGE for assessment of the total archaeal and fungal communities by analysis of 16S bacterial profiling and 28S fungal rRNA genes respectively. Relevant Primers were used for the analysis.

After the analysis, the DGGE data were subjected to statistical software using Phoretix ID and Conoco to analyse the association and impact between archeal and fungal communities in the cohort study. No significant community profiles for either archeal or fungal communities were obtained from the analysis. Sequencing of excised DGGE bands from archeal analysis did not yield any recognisable Archaea-like sequences. In the analysis of fungal communities, low diversity in the fungal DGGE was found.

It was conclude that, DGGE analysis of the archaeal community in the preterm gut showed low diversity with no characteristic community profiles distinguishable. The fungal DGGE from the preterm cohort study likewise had low diversity. Further work has to be done to investigate systematic function molecular analysis of the archaeal and fungal microbiota from the gut of preterm infants using other established molecular techniques.

Archaeal PCR- DGGE analysis

After producing the archeal ladder, series of DNA samples from our cohort study were selected and undergo PCR-DGGE analysis Most of the gels produce no any desired bands. A clean amplified DNA sample produced was selected and prepared for sequencing. The results showed no archaeal-like distinguishable profiles.

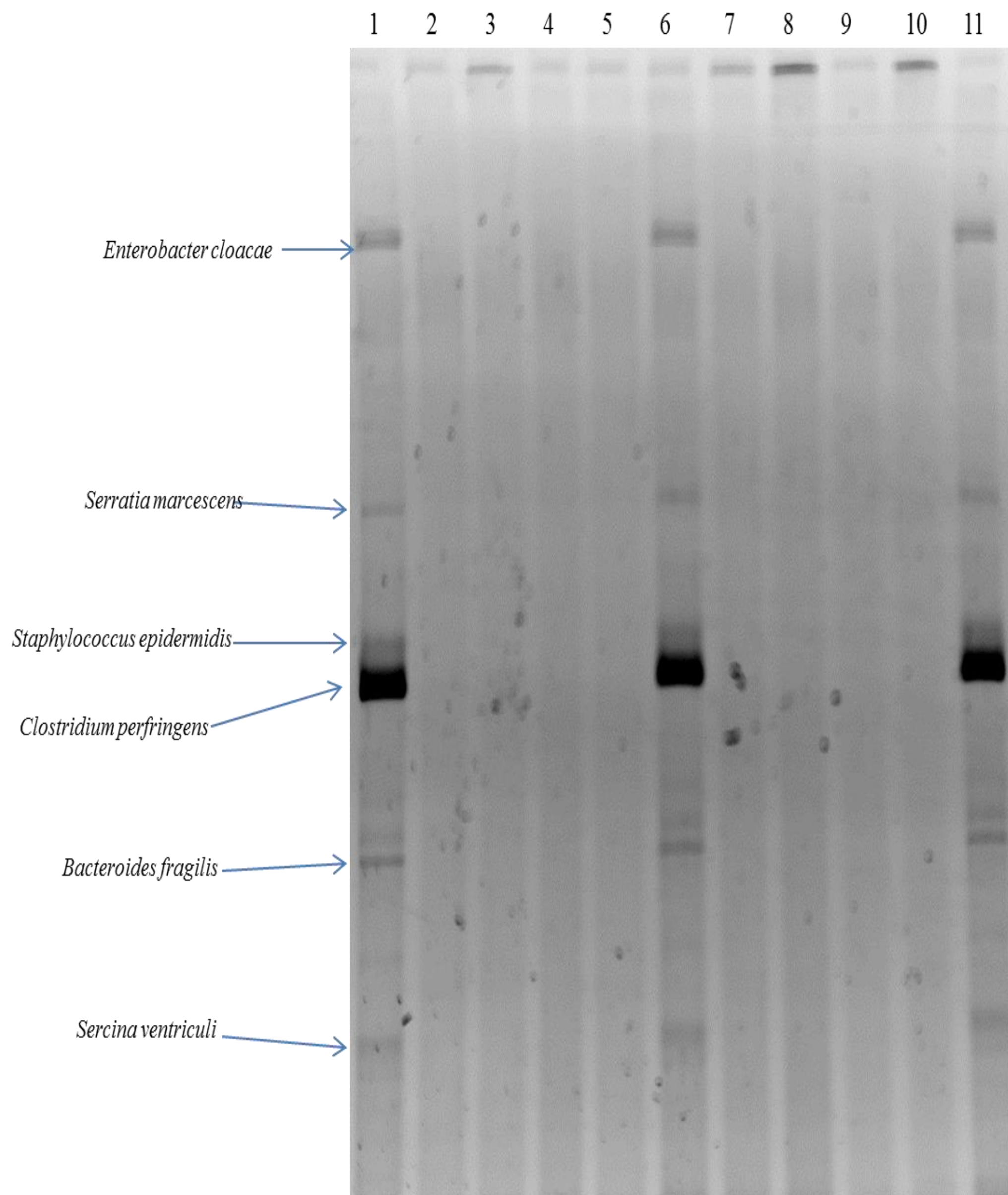


Figure 1 : DGGE profile on 35% - 55% denaturing gradient showing different bands of archaeal ladder . The gel contains 11 Lanes. L 1, L 6 and L 11 representing the Bacterial ladder of the isolates, while the remaining lanes on the image represent the samples loaded.

Fungal PCR- DGGE Analysis

After confirming the fungal ladder, the desired amplified DNA samples were loaded alongside with the DGGE Ladder. The DGGE analyses of the fungal community produced few distinct band positions on some lanes corresponding to the band position from the ladder while others produces the bands at the top or below the ladder bands (Figure 10). Low diversity of fungal community from the gut of preterm infants after DGGE analysis using phoretix ID was observed.

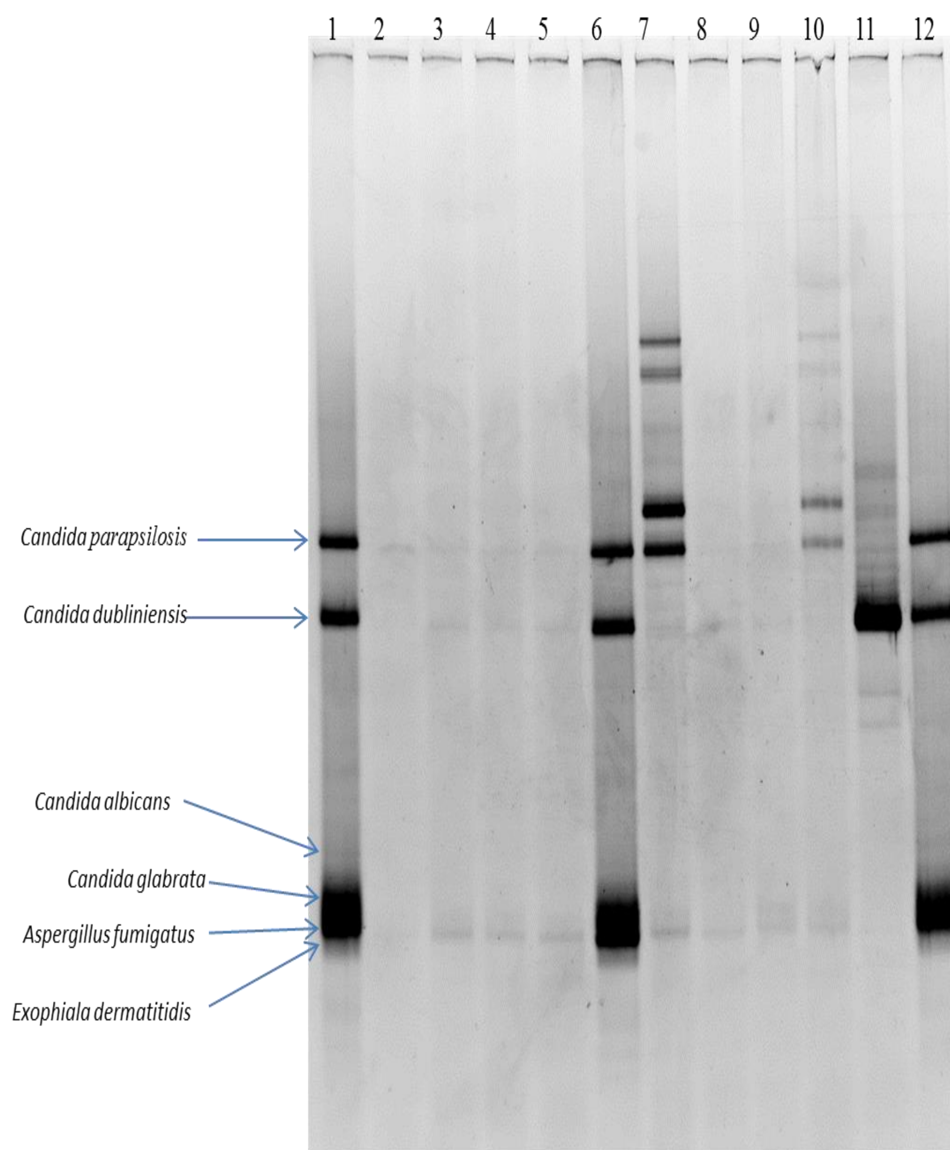


Figure 2 : DGGE profile on 40% - 60% denaturing gradient showing different bands of fungal ladder . The gel contains 12 Lanes. L 1, L 6 and L 12 representing the fungal ladder of the isolates, while the remaining lanes on the image represent the samples loaded.

Appendix 7 - Quantitative analysis of gut microbial flora in preterm infants associated with Necrotising enterocolitis

qPCR raw data for Eubacteria

PATIENT NO	SAMPLE	MEAN CQ	MEAN SD	MEAN COPIES	DILUTION 1	DILUTION 2	COPIES 0.1G STOOL	COPIES 1G STOOL
139	554	16.78263	0.353494	103187.6841	20	5	10318768.41	103187684.1
	590	11.94395	0.882166	3055011.276	20	5	305501127.6	3055011276
	619	16.22536	0.282551	149685.1936	20	5	14968519.36	149685193.6
	678	14.75834	0.400781	411954.1584	20	5	41195415.84	411954158.4
	716	13.54159	0.126836	924692.5656	20	5	92469256.56	924692565.6
140	555	13.93422	0.213551	710592.169	20	5	71059216.9	710592169
	592	16.68139	0.968853	122648.3069	20	5	12264830.69	122648306.9

	621	13.07236	2.025927	1966376.365	20	5	196637636.5	1966376365
161	838	16.64041	0.299257	112971.8903	20	5	11297189.03	112971890.3
	868	17.68956	0.134863	54641.97324	20	5	5464197.324	54641973.24
	961	11.70303	0.172915	3247230.518	20	5	324723051.8	3247230518
223	2283	16.18425	0.111225	152401.5905	20	5	15240159.05	152401590.5
	2331	16.85005	0.119727	96812.28639	20	5	9681228.639	96812286.39
	2442	15.33753	0.053719	271117.6542	20	5	27111765.42	271117654.2
	2499	13.1481	0.060106	1207015.326	20	5	120701532.6	1207015326
	2615	14.79711	0.219668	394693.8472	20	5	39469384.72	394693847.2
171	1085	15.68618	0.651839	226842.7872	20	5	22684278.72	226842787.2
	1113	16.06468	0.813804	180619.6895	20	5	18061968.95	180619689.5

	1168	17.42679	0.116099	65318.06014	20	5	6531806.014	65318060.14
	1272	18.49748	0.127985	31485.14995	20	5	3148514.995	31485149.95
176	1239	12.12836	0.184127	2431299.587	20	5	243129958.7	2431299587
	1349	12.7186	0.27159	7360645.222	20	5	736064522.2	7360645222
	1435	13.50389	0.125228	4132200.925	20	5	413220092.5	4132200925
180	1257	13.84325	0.436919	3328573.342	20	5	332857334.2	3328573342
	1293	18.55365	0.222149	108489.3812	20	5	10848938.12	108489381.2
	1325	16.16197	0.343799	617695.1439	20	5	61769514.39	617695143.9
	1388	19.74462	0.372085	46579.90192	20	5	4657990.192	46579901.92
181	1258	13.95778	0.019626	2969773.325	20	5	296977332.5	2969773325
	1299	18.89762	0.371241	85828.54411	20	5	8582854.411	85828544.11

	1327	15.91561	0.442012	748408.8029	20	5	74840880.29	748408802.9
	1377	18.28296	0.582099	138062.509	20	5	13806250.9	138062509
188	1394	17.38801	0.150232	250505.4647	20	5	25050546.47	250505464.7
	1446	14.27733	0.097237	2361642.697	20	5	236164269.7	2361642697
	1476	14.45283	0.124016	2082634.076	20	5	208263407.6	2082634076
	1509	20.36723	0.290105	29450.62401	20	5	2945062.401	29450624.01
222	2173	19.45075	0.026734	56288.80227	20	5	5628880.227	56288802.27
	2235	15.73972	0.09322	821534.9019	20	5	82153490.19	821534901.9
	2398	13.69541	0.274813	3634911.474	20	5	363491147.4	3634911474
	2263	14.64686	0.395441	1856358.558	20	5	185635855.8	1856358558
	2301	17.98807	0.329352	165013.2285	20	5	16501322.85	165013228.5

191	1579	17.77467	0.336532	191554.0296	20	5	19155402.96	191554029.6
	1720	18.26061	0.126361	133183.1688	20	5	13318316.88	133183168.8
	1776	12.71592	0.255265	7361043.187	20	5	736104318.7	7361043187
	1959	18.75103	0.60517	99754.37561	20	5	9975437.561	99754375.61
152	780	17.43428	0.311192	3704796.491	20	5	370479649.1	3704796491
	850	17.04649	0.910074	5425353.021	20	5	542535302.1	5425353021
	957	19.29823	0.964772	1096865.86	20	5	109686586	1096865860
178	1268	19.18543	0.937519	1208518.138	20	5	120851813.8	1208518138
	1343	19.71087	0.684755	768520.2887	20	5	76852028.87	768520288.7
	1376	18.98545	0.113309	1201231.321	20	5	120123132.1	1201231321
186	1421	18.07124	0.854738	2639998.775	20	5	263999877.5	2639998775

	1491	17.69373	0.92379	3423746.058	20	5	342374605.8	3423746058
	1526	17.04032	0.502501	4986576.828	20	5	498657682.8	4986576828
281	3362	18.82548	1.111896	1664343.312	20	5	166434331.2	1664343312
	3369	17.89848	0.67491	2800138.537	20	5	280013853.7	2800138537
	3382	20.09416	0.337063	552673.102	20	5	55267310.2	552673102
	3407	20.52689	0.618198	422576.7866	20	5	42257678.66	422576786.6
307	3645	22.13913	0.886655	144023.2861	20	5	14402328.61	144023286.1
	3667	21.12232	1.236884	319513.4419	20	5	31951344.19	319513441.9
	3685	20.05603	0.318486	565968.2699	20	5	56596826.99	565968269.9
229	2472	21.96441	0.213252	142906.1816	20	5	14290618.16	142906181.6
315	3699	21.83649	1.163065	187954.9123	20	5	18795491.23	187954912.3

292	3450	18.31156	0.299822	1972369.568	20	5	197236956.8	1972369568
176	1250	19.31453	0.456952	279225.4321	40	5	55845086.43	558450864.3
161	984	16.02937	0.403188	3072523.619	40	5	614504723.8	6145047238
176	1287	19.11622	0.753285	347395.4197	40	5	69479083.93	694790839.3
307	3589	18.79154	0.147156	396654.8337	40	5	79330966.75	793309667.5
152	822	18.08165	1.687845	956512.7024	40	5	191302540.5	1913025405
171	1183	21.363	0.518433	63359.90589	10	5	3167995.295	31679952.95
161	1081	23.0327	1.264422	24114.74707	10	5	1205737.354	12057373.54
188	1615	25.5922	2.226755	5274.085298	10	5	263704.2649	2637042.649
303	3590	22.65555	1.143021	28331.72908	10	5	1416586.454	14165864.54

Demographic data of Eubacteria qPCR

A - NEC Babies

Samples	Storenum	Johnptnur	Ageattest	Deliverym	Birthweig	GA	Reviewed	NEC	NECdayon
90015518	554	139	14	CS Breeh p	1470	30	JB	Y (medica	28
90015518	590	139	21	CS Breeh p	1470	30	JB	Y (medica	28
90015518	619	139	27	CS Breeh p	1470	30	JB	Y (medica	28
90015518	678	139	36	CS Breeh p	1470	30	JB	Y (medica	28
90015518	716	139	42	CS	1470	30	JB	Y (medica	28
90016504	838	161	16	V	700	25	JB	Y (Sx)	31
90016504	868	161	19	V	700	25	JB	Y (Sx)	31
90016504	961	161	32	V	700	25	JB	Y (Sx)	31
90016504	984	161	35	V	700	25	JB	Y (Sx)	31
90016504	1081	161	47	V	700	25	JB	Y (Sx)	31
90017219	1085	171	9	V	790	26	JB	Y	19
90017219	1113	171	12	V	790	26	JB	Y	19
90017219	1168	171	19	V	790	26	JB	Y	19
90017219	1183	171	21	V	790	26	JB	Y	19
90017219	1272	171	36	V	790	26	JB	Y	19
91169570	1257	180	7	V	500	22	JB	Y(sx_)	16
91169570	1293	180	11	V	500	23	JB	Y(sx_)	16
91169570	1325	180	15	V	500	23	JB	Y(sx_)	16
91169570	1388	180	23	V	500	23	JB	Y(sx_)	16
90018108	1394	188	4	V	750	24	JB	Possible	15
90018108	1446	188	10	V	750	24	JB	Possible	15
90018108	1476	188	15	V	750	24	JB	Possible	15
90018108	1509	188	19	V	750	24	JB	Possible	15
90018108	1615	188	29	V	750	24	JB	Possible	15
90018699	1579	199	11	V	725	25	JB	Y (Sx)	25
90018699	1720	199	19	V	725	25	JB	Y (Sx)	25
90018699	1776	199	24	V	725	25	JB	Y (Sx)	25
90018699	1959	199	38	V	725	25	JB	Y (Sx)	25
90017650	1268	178	14	CS	525	26	JB	P (complex!))	
90017650	1343	178	24	CS	525	26	JB	P (complex!))	
90017650	1376	178	28	CS	525	26	JB	P (complex!))	
90029077	3362	281	8	CS	620	25		N	22
90029077	3369	281	21	CS	620	25		N	22
90029077	3382	281	28	CS	620	25		N	22
90029077	3407	281	37	CS	620	25		N	22
90031153	3590	303	17	V	960	25		N	5
90032123	3699	315	9			24		N	10

B Control Babies

Samples	Storenum	Johnptnur	Ageattest	Deliverym	Birthweig	GA	Reviewed	NEC
90015519	555	140	14	CS poor GI	1455	30	JB	N
90015519	592	140	21	CS poor GI	1455	30	JB	N
90015519	621	140	27	CS	1455	30	JB	N
90019862	2283	223	15	V	885	25		N
90019862	2331	223	20	V	885	25		N
90019862	2442	223	31	V	885	25		N
90019862	2499	223	36	V	885	25		N
90019862	2615	223	48	V	885	25		N
90017582	1239	176	10	CS	880		JB	N
90017582	1250	176	12	CS	880	26	JB	N
90017582	1287	176	16	CS	880	26	JB	N
90017582	1349	176	25	CS	880	26	JB	N
90017582	1435	176	35	CS	880	26	JB	N
91169448	1258	181	8	V	570	23	JB	N
91169448	1299	181	12	V	570	23	JB	N
91169448	1327	181	17	V	570	23	JB	N
91169448	1377	181	23	V	570	23	JB	N
90019802	2173	222	5	V	620	24	JB	N
90019802	2235	222	11	V	620	24	JB	N
90019802	2398	222	28	V	620	24	JB	N
90019802	2263	222	15	V	620	24	JB	N
90019802	2301	222	19	V	620	24	JB	N
	780	152	14	V	800	25	JB	N
	822	152	20	V	800	25	JB	N
	850	152	24	V	800	25	JB	N
	957	152	39	V	800	25	JB	N
90017984	1421	186	14	CS	840	26	JB	N
90017984	1491	186	23	CS	840	26	JB	N
90017984	1526	186	28	CS	840	26	JB	N
90031465	3589	307	#VALUE!	V	810	25	JB	N
90031465	3645	307	#VALUE!	V	810	25	JB	N
90031465	3667	307	#VALUE!	V	810	25	JB	N
90031465	3685	307	#VALUE!	V	810	25	JB	N
90020240	2472	229	16	V	910	25	JB	N
90029891	3450	292	9	V	680	24	JB	N

Appendix 8 - Molecular characterisation of Probiotics supplementation from the gut of preterm infants and its impacts in the development of NEC and sepsis

A – Probiotics NGS Demographic data

Probiotic Start	Probiotic Stop	Sample	Patient number	Age attest	Remark	Delivery mode	Sex	Birth weight	GA
Control	**Control**	3090	**263**	**12**	**Control**	**CS**	**F**	**550**	**27**
Control	**Control**	3094	**263**	**13**	**Control**	**CS**	**F**	**550**	**27**
Control	**Control**	3099	**263**	**15**	**Control**	**CS**	**F**	**550**	**27**
Control	**Control**	3106	**263**	**16**	**Control**	**CS**	**F**	**550**	**27**
Control	**Control**	3108	**263**	**17**	**Control**	**CS**	**F**	**550**	**27**
Control	**Control**	3120	**263**	**19**	**Control**	**CS**	**F**	**550**	**27**
Control	**Control**	3127	**263**	**20**	**Control**	**CS**	**F**	**550**	**27**
Control	**Control**	3134	**263**	**22**	**Control**	**CS**	**F**	**550**	**27**
Control	**Control**	3140	**263**	**23**	**Control**	**CS**	**F**	**550**	**27**
Control	**Control**	3149	**263**	**26**	**Control**	**CS**	**F**	**550**	**27**

Control	Control	3153	263	27	Control	CS	F	550	27
Control	Control	3159	263	28	Control	CS	F	550	27
Control	Control	3162	263	29	Control	CS	F	550	27
Control	Control	3168	263	32	Control	CS	F	550	27
31	61	3191	270	9	Before	V	M	750	25
31	61	3230	270	28	Before	V	M	750	25
31	61	3262	270	39	During	V	M	750	25
31	61	3278	270	46	During	V	M	750	25
31	61	3299	270	59	During	V	M	750	25
31	61	3309	270	65	After	V	M	750	25
31	61	3328	270	71	After	V	M	750	25

31	**61**	3338	**270**	**77**	**After**	**V**	**M**	**750**	**25**
31	**61**	PD	**270**	**PD**	**After**	**V**	**M**	**750**	**25**
Control	**Control**	3224	**271**	**3**	**Control**	**V**	**F**	**2030**	**31**
Control	**Control**	3240	**271**	**9**	**Control**	**V**	**F**	**2030**	**31**
Control	**Control**	3250	**271**	**11**	**Control**	**V**	**F**	**2030**	**31**
Control	**Control**	3255	**271**	**14**	**Control**	**V**	**F**	**2030**	**31**
Control	**Control**	3267	**271**	**18**	**Control**	**V**	**F**	**2030**	**31**
Control	**Control**	3282	**271**	**26**	**Control**	**V**	**F**	**2030**	**31**
Control	**Control**	3295	**271**	**35**	**Control**	**V**	**F**	**2030**	**31**
Control	**Control**	3225	**272**	**3**	**Control**	**V**		**1535**	**31**
Control	**Control**	3232	**272**	**6**	**Control**	**V**		**1535**	**31**

Control	**Control**	3245	**272**	**10**	**Control**	**V**		**1535**	**31**
Control	**Control**	3254	**272**	**14**	**Control**	**V**		**1535**	**31**
Control	**Control**	3266	**272**	**18**	**Control**	**V**		**1535**	**31**
Control	**Control**	3281	**272**	**25**	**Control**	**V**		**1535**	**31**
Control	**Control**	3290	**272**	**31**	**Control**	**V**		**1535**	**31**
14	**28**	3231	**273**	**9**	**Before**	**CS**	**F**	**945**	**27**
14	**28**	3238	**273**	**12**	**Before**	**CS**	**F**	**945**	**27**
14	**28**	3273	**273**	**25**	**During**	**CS**	**F**	**945**	**27**
14	**28**	3276	**273**	**27**	**After**	**CS**	**F**	**945**	**27**
14	**28**	3300	**273**	**40**	**After**	**CS**	**F**	**945**	**27**
14	**28**	3310	**273**	**47**	**After**	**CS**	**F**	**945**	**27**

14	28	3322	273	50	After	CS	F	945	27
14	28	3331	273	54	After	CS	F	945	27
14	28	3343	273	63	After	CS	F	945	27
14	28	3347	273	66	After	CS	F	945	27
12	70	3235	274	9	Before	V	M	700	24
12	70	3242	274	12	During	V	M	700	24
12	70	3248	274	13	During	V	M	700	24
12	70	3253	274	16	During	V	M	700	24
12	70	3263	274	20	During	V	M	700	24
12	70	3269	274	23	During	V	M	700	24
12	70	3274	274	25	During	V	M	700	24

12	70	3279	274	27	During	V	M	700	24
12	70	3284	274	31	During	V	M	700	24
12	70	3288	274	33	During	V	M	700	24
12	70	3298	274	38	During	V	M	700	24
12	70	3305	274	43	During	V	M	700	24
12	70	3316	274	48	During	V	M	700	24
12	70	3333	274	53	During	V	M	700	24
12	70	3339	274	59	During	V	M	700	24
12	70	3345	274	64	During	V	M	700	24
12	70	PD	274	PD	During	V	M	700	24
3	12	3236	275	6	During	CS		1100	28

3	12	3244	275	9	During	CS	1100	28
3	12	3247	275	10	During	CS	1100	28
3	12	3268	275	19	After	CS	1100	28
3	12	3271	275	21	After	CS	1100	28
8	26	3302	276	37	After	CS	1150	28
8	26	3237	276	7	Before	CS	1150	28
8	26	3243	276	9	During	CS	1150	28
8	26	3259	276	15	During	CS	1150	28
8	26	3270	276	21	During	CS	1150	28
8	26	3280	276	24	During	CS	1150	28
8	26	3286	276	29	After	CS	1150	28

14	**26**	3296	**277**	**6**	**Before**	**CS**	**620**	**24**
14	**26**	3318	**277**	**16**	**During**	**CS**	**620**	**24**
14	**26**	3340	**277**	**27**	**After**	**CS**	**620**	**24**
5	**73**	3301	**278**	**7**	**During**	**CS**	**620**	**24**
5	**73**	3307	**278**	**13**	**During**	**CS**	**620**	**24**
5	**73**	3315	**278**	**15**	**During**	**CS**	**620**	**24**
5	**73**	3317	**278**	**16**	**During**	**CS**	**620**	**24**
5	**73**	3326	**278**	**19**	**During**	**CS**	**620**	**24**
5	**73**	3330	**278**	**20**	**During**	**CS**	**620**	**24**
5	**73**	3341	**278**	**29**	**During**	**CS**	**620**	**24**
5	**73**	3348	**278**	**35**	**During**	**CS**	**620**	**24**

5	**73**	3350	**278**	**38**	**During**	**CS**	**620**	**24**
5	**73**	PD	**278**	**PD**	**During**	**CS**	**620**	**24**

B – Probiotics Diversity Demographic data

Sample	Patient Number	Age attest	Probiotic Start	Probiotic Stop	Remark
3090	**263**	**12**	**Control**	**Control**	**Control**
3094	**263**	**13**	**Control**	**Control**	**Control**
3099	**263**	**15**	**Control**	**Control**	**Control**
3106	**263**	**16**	**Control**	**Control**	**Control**
3108	**263**	**17**	**Control**	**Control**	**Control**
3120	**263**	**19**	**Control**	**Control**	**Control**
3127	**263**	**20**	**Control**	**Control**	**Control**
3134	**263**	**22**	**Control**	**Control**	**Control**
3140	**263**	**23**	**Control**	**Control**	**Control**
3149	**263**	**26**	**Control**	**Control**	**Control**
3153	**263**	**27**	**Control**	**Control**	**Control**
3159	**263**	**28**	**Control**	**Control**	**Control**
3162	**263**	**29**	**Control**	**Control**	**Control**
3168	**263**	**32**	**Control**	**Control**	**Control**
3191	**Patient 270**	**9**	**31**	**61**	**Before**
3230	Patient 270	**28**	**31**	**61**	**Before**

3262	Patient 270	39	31	61	During
3278	Patient 270	46	31	61	During
3299	Patient 270	59	31	61	During
3309	Patient 270	65	31	61	After
3328	Patient 270	71	31	61	After
3338	Patient 270	77	31	61	After
3224	Patient 271	3	Control		Control
3240	Patient 271	9	Control		Control
3250	Patient 271	11	Control		Control
3255	Patient 271	14	Control		Control
3267	Patient 271	18	Control		Control
3282	Patient 271	26	Control		Control
3295	Patient 271	35	Control		Control
3225	Patient 272	3	Control		Control
3232	Patient 272	6	Control		Control
3245	Patient 272	10	Control		Control
3254	Patient 272	14	Control		Control
3266	Patient 272	18	Control		Control
3281	Patient 272	25	Control		Control
3290	Patient 272	31	Control		Control

3231	Patient 273	9	14	28	Before
3238	Patient 273	12	14	28	Before
3273	Patient 273	25	14	28	During
3276	Patient 273	27	14	28	During
3300	Patient 273	40	14	28	After
3310	Patient 273	47	14	28	After
3322	Patient 273	50	14	28	After
3331	Patient 273	54	14	28	After
3343	Patient 273	63	14	28	After
3235	Patient 274	9	12	70	Before
3242	Patient 274	12	12	70	During
3248	Patient 274	13	12	70	During
3253	Patient 274	16	12	70	During
3263	Patient 274	20	12	70	During
3269	Patient 274	23	12	70	During
3274	Patient 274	25	12	70	During
3279	Patient 274	27	12	70	During
3284	Patient 274	31	12	70	During
3288	Patient 274	33	12	70	During
3298	Patient 274	38	12	70	During

3305	**Patient 274**	**43**	**12**	**70**	**During**
3316	**Patient 274**	**48**	**12**	**70**	**During**
3333	**Patient 274**	**53**	**12**	**70**	**During**
3339	**Patient** 274	**59**	**12**	**70**	**During**
3345	**Patient 274**	**64**	**12**	**70**	**During**
3236	**Patient** 275	**6**	**3**	**12**	**During**
3244	**Patient 275**	**9**	**3**	**12**	**During**
3247	**Patient** 275	**10**	**3**	**12**	**During**
3268	**Patient 275**	**19**	**3**	**12**	**After**
3271	**Patient** 275	**21**	**3**	**12**	**After**
3302	**Patient 276**	**37**	**8**	**26**	**After**
3237	**Patient 276**	**7**	**8**	**26**	**Before**
3243	**Patient 276**	**9**	**8**	**26**	**During**
3259	**Patient 276**	**15**	**8**	**26**	**During**
3270	**Patient 276**	**21**	**8**	**26**	**During**
3280	**Patient** 276	**24**	**8**	**26**	**During**
3286	**Patient 276**	**29**	**8**	**26**	**After**
3296	**Patient 277**	**6**	**14**	**26**	**Before**
3318	**Patient 277**	**16**	**14**	**26**	**During**
3340	**Patient** 277	**27**	**14**	**26**	**After**

3301	**Patient 278**	**7**	**5**	**73**	**During**
3307	**Patient 278**	**13**	**5**	**73**	**During**
3315	**Patient 278**	**15**	**5**	**73**	**During**
3317	**Patient 278**	**16**	**5**	**73**	**During**
3326	**Patient 278**	**19**	**5**	**73**	**During**
3330	**Patient** 278	**20**	**5**	**73**	**During**
3341	**Patient 278**	**29**	**5**	**73**	**During**
3348	**Patient** 278	**35**	**5**	**73**	**During**
3350	**Patient 278**	**38**	**5**	**73**	**During**

C - Probiotics QPCR Demographic data

Sample	Patient Number	Age attest	GA	Probiotic Start	Probiotic Stop	Remark
3090	263	12	27	Control	Control	Control
3149	263	26	27	Control	Control	Control
3197	263	41	27	Control	Control	Control
3215	263	48	27	Control	Control	Control
3221	263	53	27	Control	Control	Control
3226	263	57	27	Control	Control	Control
3258	263	69	27	Control	Control	Control
3297	263	89	27	Control	Control	Control
3320	263	100	27	Control	Control	During
3337	263	108	27	Control	Control	Control
PD	270	PD	25	PD	PD	PD
3230	270	28	25	31	61	Before
3262	270	39	25	31	61	During
3278	270	46	25	31	61	During
3299	270	59	25	31	61	During
3309	270	65	25	31	61	After

3328	270	71	25	31	61	After
3338	270	77	25	31	61	After
3224	271	3	31	Control	Control	Control
3240	271	9	31	Control	Control	Control
3250	271	11	31	Control	Control	Control
3255	271	14	31	Control	Control	Control
3267	271	18	31	Control	Control	Control
3282	271	26	31	Control	Control	Control
3295	271	35	31	Control	Control	Control
3225	272	3	31	Control	Control	Control
3232	272	6	31	Control	Control	Control
3245	272	10	31	Control	Control	Control
3254	272	14	31	Control	Control	Control
3266	272	18	31	Control	Control	Control
3281	272	25	31	Control	Control	Control
3294	272	35	31	Control	Control	Control
PD	273	PD	27	PD	PD	PD
3231	273	9	27	14	28	Before
3238	273	12	27	14	28	Before
3276	273	27	27	14	28	During

3300	273	40	27	14	28	After
3310	273	47	27	14	28	After
3330	273	40	27	14	28	After
3331	273	54	27	14	28	After
PD	274	PD	24	PD	PD	PD
3235	274	9	24	12	70	Before
3242	274	12	24	12	70	During
3248	274	13	24	12	70	During
3253	274	16	24	12	70	During
3263	274	20	24	12	70	During
3279	274	27	24	12	70	During
3284	274	31	24	12	70	During
3298	274	38	24	12	70	During
3305	274	43	24	12	70	During
3316	274	48	24	12	70	During
3333	274	53	24	12	70	During
3345	274	64	24	12	70	During
3236	275	6	28	3	12	During
3244	275	9	28	3	12	During
3247	275	10	28	3	12	During

3268	275	19	28	3	12	After
3271	275	21	28	3	12	After
3243	276	9	28	8	26	During
3259	276	15	28	8	26	During
3280	276	24	28	8	26	During
3286	276	29	28	8	26	After
3296	277	6	24	14	26	Before
3318	277	16	24	14	26	During
3340	277	27	24	14	26	After
PD	278	PD	24	PD	PD	PD
3301	278	7	24	5	73	During
3307	278	13	24	5	73	During
3315	278	15	24	5	73	During
3317	278	16	24	5	73	During
3326	278	19	24	5	73	During
3341	278	29	24	5	73	During
3348	278	35	24	5	73	During
3350	278	38	24	5	73	During

Appendix 9 : *Lactobacillus spp*. qPCR raw data in the cohort

Sample	Patient number	SQ Mean	SQ Std. Dev	mean (Log)	Standard deviation (log)	Age attest	Probiotic Start	Probiotic Stop	Remark
270-PD		0.000	0.000	0.00	0.00	PD	PD	PD	PD
273-PD		0.000	0.000	0.00	0.00	PD	PD	PD	PD
274-PD		0.000	0.000	0.00	0.00	PD	PD	PD	PD
278-PD		0.000	0.000	0.00	0.00	PD	PD	PD	PD
3090	263	0.000	0.000	0.00	0.00	12	Control	Control	Control
3120	263	0.000	0.000	0.00	0.00	19	Control	Control	Control
3149	263	0.000	0.000	0.00	0.00	26	Control	Control	Control

3197	263	0.000	0.000	0.00	0.00	41	Control	Control	Control
3215	263	0.000	0.000	0.00	0.00	48	Control	Control	Control
3221	263	0.000	0.000	0.00	0.00	53	Control	Control	Control
3224	271	0.000	0.000	0.00	0.00	3	Control	Control	Control
3225	272	0.000	0.000	0.00	0.00	3	Control	Control	Control
3226	263	0.000	0.000	0.00	0.00	57	Control	Control	Control
3230	270	0.000	0.000	0.00	0.00	28	31	61	Before
3231	273	0.000	0.000	0.00	0.00	9	14	28	Before
3232	272	0.000	0.000	0.00	0.00	6	Control	Control	Control
3235	274	0.000	0.000	0.00	0.00	9	12	70	Before
3236	275	0.000	0.000	0.00	0.00	6	3	12	During

3238	273	0.00000	0.00000	0.00	0.00	12	14	28	Before
3240	271	7921483.09259	832340.32746	6.90	5.92	9	Control	Control	Control
3242	274	80452.99627	8736.04045	4.91	3.94	12	12	70	During
3243	276	0.000	0.000	0.00	0.00	9	8	26	During
3244	275	0.000	0.000	0.00	0.00	9	3	12	During
3245	272	0.000	0.000	0.00	0.00	10	Control	Control	Control
3247	275	0.000	0.000	0.00	0.00	10	3	12	During
3248	274	0.000	0.000	0.00	0.00	13	12	70	During
3250	271	0.000	0.000	0.00	0.00	11	Control	Control	Control
3253	274	0.00000	0.00000	0.00	0.00	16	12	70	During
3254	272	0.00000	0.00000	0.00	0.00	14	Control	Control	Control

3255	271	0.00000	0.00000	0.00	0.00	14	Control	Control	Control
3258	263	0.00000	0.00000	0.00	0.00	69	Control	Control	Control
3259	276	347925.46389	40255.21687	5.54	4.60	15	8	26	During
3262	270	0.00000	0.00000	0.00	0.00	39	31	61	During
3263	274	0.00000	0.00000	0.00	0.00	20	12	70	During
3266	272	1707784.44464	106561.73176	6.23	5.03	18	Control	Control	Control
3267	271	787843.16177	118520.59161	5.90	5.07	18	Control	Control	Control
3268	275	0.00000	0.00000	0.00	0.00	19	3	12	After
3271	275	0.00000	0.00000	0.00	0.00	21	3	12	After
3276	273	0.00000	0.00000	0.00	0.00	27	14	28	During
3278	270	0.00000	0.00000	0.00	0.00	46	31	61	During

3279	274	0.00000	0.00000	0.00	0.00	27	12	70	During
3280	276	0.00000	0.00000	0.00	0.00	24	8	26	During
3281	272	0.00000	0.00000	0.00	0.00	25	Control	Control	Control
3282	271	0.00000	0.00000	0.00	0.00	26	Control	Control	Control
3284	274	0.00000	0.00000	0.00	0.00	31	12	70	During
3286	276	0.00000	0.00000	0.00	0.00	29	8	26	After
3294	272	0.00000	0.00000	0.00	0.00	35	Control	Control	Control
3295	271	0.00000	0.00000	0.00	0.00	35	Control	Control	Control
3296	277	0.00000	0.00000	0.00	0.00	6	14	26	Before
3297	263	0.00000	0.00000	0.00	0.00	89	Control	Control	Control
3298	274	0.000	0.000	0.00	0.00	38	12	70	During

3299	270	0.000	0.000	0.00	0.00	59	31	61	During
3300	273	0.000	0.000	0.00	0.00	40	14	28	After
3301	278	0.000	0.000	0.00	0.00	7	5	73	During
3305	274	0.00000	0.00000	0.00	0.00	43	12	70	During
3307	278	0.000	0.000	0.00	0.00	13	5	73	During
3309	270	0.000	0.000	0.00	0.00	65	31	61	After
3310	273	0.000	0.000	0.00	0.00	47	14	28	After
3315	278	0.000	0.000	0.00	0.00	15	5	73	During
3316	274	0.000	0.000	0.00	0.00	48	12	70	During
3317	278	0.000	0.000	0.00	0.00	16	5	73	During
3318	277	0.000	0.000	0.00	0.00	16	14	26	During

3320	263	0.000	0.000	0.00	0.00	100	Control	Control	Control
3326	278	0.000	0.000	0.00	0.00	19	5	73	During
3328	270	0.000	0.000	0.00	0.00	71	31	61	After
3330	273	0.000	0.000	0.00	0.00	40	14	28	After
3331	273	0.000	0.000	0.00	0.00	54	14	28	After
3333	274	0.000	0.000	0.00	0.00	53	12	70	During
3337	263	0.000	0.000	0.00	0.00	108	Control	Control	Control
3338	270	0.000	0.000	0.00	0.00	77	31	61	After
3340	277	0.000	0.000	0.00	0.00	27	14	26	After
3341	278	0.000	0.000	0.00	0.00	29	5	73	During
3345	274	0.000	0.000	0.00	0.00	64	12	70	During

3348	278	0.000	0.000	0.00	0.00	35	5	73	During
3350	278	0.00000	0.00000	0.00	0.00	38	5	73	During

Appendix 10: *Bifidobacterium spp*. qPCR raw data in the cohort

Sample	Patient Number	SQ Mean	Mean (Log)	SQ Std. Dev	St deviation (Log)	Age attest	Probiotic Start	Probiotic Stop	Remark
3268	275	225,888	5.35	166168.86969	5.22	19	3	12	After
3271	275	15,511,928	7.19	40323.43811	4.61	21	3	12	After
3286	276	487,850,010	8.69	3033391.64732	6.48	29	8	26	After
3300	273	25,168,965	7.40	123291.04233	5.09	40	14	28	After
3309	270	658,850	5.82	112250.62990	5.05	65	31	61	After
3310	273	17,346,830	7.24	82069.08700	4.91	47	14	28	After
3328	270	6,292,852	6.80	3206394.42357	6.51	71	31	61	After

3330	273	52,193,995	7.72	10559838.49398	7.02	40	14	28	After
3331	273	32,034,908	7.51	484526.82979	5.69	54	14	28	After
3338	270	76,263	4.88	7062.52023	3.85	77	31	61	After
3340	277	0	0.00	0.000	0.00	27	14	26	After
3230	270	0	0.00	0.000	0.00	28	31	61	Before
3231	273	0	0.00	0.000	0.00	9	14	28	Before
3235	274	133,105	5.12	3694.96386	3.57	9	12	70	Before
3238	273	0	0.00	0.000	0.00	12	14	28	Before
3296	277	130,002	5.11	9758.40879	3.99	6	14	26	Before
3090	263	0	0.00	0.000	0.00	12	Control	Control	Control
3120	263	0	0.00	0.000	0.00	19	Control	Control	Control

3149	263	0	0.00	0.000	0.00	26	Control	Control	Control
3197	263	0	0.00	0.000	0.00	41	Control	Control	Control
3215	263	0	0.00	0.00000	0.00	48	Control	Control	Control
3221	263	0	0.00	0.000	0.00	53	Control	Control	Control
3224	271	0	0.00	0.000	0.00	3	Control	Control	Control
3225	272	0	0.00	0.000	0.00	3	Control	Control	Control
3226	263	0	0.00	0.000	0.00	57	Control	Control	Control
3232	272	0	0.00	0.000	0.00	6	Control	Control	Control
3240	271	0	0.00	0.000	0.00	9	Control	Control	Control
3245	272	0	0.00	0.000	0.00	10	Control	Control	Control
3250	271	0	0.00	0.000	0.00	11	Control	Control	Control

3254	272	0	0.00	0.000	0.00	14	Control	Control	Control
3255	271	0	0.00	0.000	0.00	14	Control	Control	Control
3258	263	0	0.00	0.000	0.00	69	Control	Control	Control
3266	272	0	0.00	0.000	0.00	18	Control	Control	Control
3267	271	0	0.00	0.000	0.00	18	Control	Control	Control
3281	272	0	0.00	0.000	0.00	25	Control	Control	Control
3282	271	0	0.00	0.000	0.00	26	Control	Control	Control
3294	272	0	0.00	0.000	0.00	35	Control	Control	Control
3295	271	0	0.00	0.000	0.00	35	Control	Control	Control
3297	263	0	0.00	0.000	0.00	89	Control	Control	Control
3320	263	0	0.00	0.000	0.00	100	Control	Control	Control

3337	263	0	0.00	0.000	0.00	108	Control	Control	Control
3236	275	74,894	4.87	9075.33954	3.96	6	3	12	During
3242	274	79,564	4.90	2895.98919	3.46	12	12	70	During
3243	276	90,437	4.96	18702.04048	4.27	9	8	26	During
3244	275	1,568,726	6.20	5198.74564	3.72	9	3	12	During
3247	275	1,950,779	6.29	8983.19734	3.95	10	3	12	During
3248	274	0	0.00	0.00000	0.00	13	12	70	During
3253	274	2,419,817	6.38	3352.21227	3.53	16	12	70	During
3259	276	4,964,745	6.70	495588.53475	5.70	15	8	26	During
3262	270	2,021,199	6.31	2369.52959	3.37	39	31	61	During
3263	274	0	0.00	0.000	0.00	20	12	70	During

3276	273	23,436,334	7.37	75084.95157	4.88	27	14	28	During
3278	270	1,162,462	6.07	123695.73014	5.09	46	31	61	During
3279	274	207,123	5.32	19579.01791	4.29	27	12	70	During
3280	276	22,512,857	7.35	137823.45821	5.14	24	8	26	During
3284	274	17,761,359	7.25	23096.25195	4.36	31	12	70	During
3298	274	163,703	5.21	21220.29048	4.33	38	12	70	During
3299	270	1,537,540	6.19	522465.01326	5.72	59	31	61	During
3301	278	91,554	4.96	11387.16884	4.06	7	5	73	During
3305	274	682,730	5.83	26010.93129	4.42	43	12	70	During
3307	278	73,497	4.87	1487.58565	3.17	13	5	73	During
3315	278	82,420	4.92	1475.16989	3.17	15	5	73	During

3316	274	0	0.00	0.000	0.00	48	12	70	During
3317	278	431,310	5.63	31263.95858	4.50	16	5	73	During
3318	277	0	0.00	0.000	0.00	16	14	26	During
3326	278	0	0.00	0.00000	0.00	19	5	73	During
3333	274	222,935	5.35	30407.54183	4.48	53	12	70	During
3341	278	1,095,837	6.04	2284.43704	3.36	29	5	73	During
3345	274	1,946,796	6.29	7938.05726	3.90	64	12	70	During
3348	278	0	0.00	0.00000	0.00	35	5	73	During
3350	278	3,000,712	6.48	83442.00484	4.92	38	5	73	During

Appendix 11: MG-RAST Accession number for Probiotic data

Your MG-RAST IDs: 4615661.3, 4615662.3, 4615663.3, 4615664.3, 4615665.3, 4615666.3, 4615667.3, 4615668.3, 4615669.3, 4615670.3, 4615671.3, 4615672.3, 4615673.3, 4615674.3, 4615675.3, 4615676.3, 4615677.3, 4615678.3, 4615679.3, 4615680.3, 4615681.3, 4615682.3, 4615683.3, 4615684.3, 4615685.3, 4615686.3, 4615687.3, 4615688.3, 4615689.3, 4615690.3, 4615691.3, 4615692.3, 4615693.3, 4615694.3, 4615695.3, 4615696.3, 4615697.3, 4615698.3, 4615699.3, 4615700.3, 4615701.3, 4615702.3, 4615703.3, 4615704.3, 4615705.3, 4615706.3, 4615707.3, 4615708.3, 4615709.3, 4615710.3, 4615711.3, 4615712.3, 4615713.3, 4615714.3, 4615715.3, 4615716.3, 4615717.3, 4615718.3, 4615719.3, 4615720.3, 4615721.3, 4615722.3, 4615723.3, 4615724.3, 4615725.3, 4615726.3, 4615727.3, 4615728.3, 4615729.3, 4615730.3, 4615731.3, 4615732.3, 4615733.3, 4615734.3, 4615735.3, 4615736.3, 4615737.3, 4615738.3, 4615739.3, 4615740.3, 4615741.3, 4615742.3, 4615743.3, 4615744.3, 4615745.3, 4615746.3, 4615747.3, 4615748.3, 4615749.3

Appendix 12 - Systematic functional analysis of the gut of preterm infants by probiotic metabolomics profiles

7.3 Demographic data of probiotics metabolomics study

7.4

Sample	Patient Number	Age attest	Probiotic Start	Probiotic Stop	Remark
4289	273	PD	PD	PD	PD
4324	274	PD	PD	PD	PD
5086	278	PD	PD	PD	PD
8975	270	PD	PD	PD	PD
3197	263	41	Control	Control	Control

3215	263	48	Control	Control	Control
3226	263	57	Control	Control	Control
3297	263	89	Control	Control	Control
3262	270	39	31	61	During
3299	270	59	31	61	During
3309	270	65	31	61	After
3338	270	77	31	61	After
3224	271	3	Control	Control	Control
3255	271	14	Control	Control	Control

3282	271	26	Control	Control	Control
3295	271	35	Control	Control	Control
3245	272	10	Control	Control	Control
3254	272	14	Control	Control	Control
3281	272	25	Control	Control	Control
3294	272	35	Control	Control	Control
3276	273	27	14	28	During
3300	273	40	14	28	During
3331	273	54	14	28	After

3235	274	9	12	70	Before
3263	274	20	12	70	During
3305	274	43	12	70	During
3345	274	64	12	70	During
3236	275	6	3	12	During
3247	275	10	3	12	During
3268	275	19	3	12	After
3271	275	21	3	12	After
3243	276	9	8	26	During

3259	276	15	8	26	During
3280	276	24	8	26	During
3286	276	29	8	26	After
3301	278	7	5	73	During
3315	278	15	5	73	During
3317	278	16	5	73	During
3341	278	29	5	73	During
3348	278	35	5	73	During

Demographic data of metabolomics trial samples

Sample No	Pt NO.	Age attest	Probiotic Start	Probiotic Stop	Remark
3328-A	270	**71**	31	61	After
3328-B	270	**71**	31	61	After
3328-C	270	**71**	31	61	After
3322-A	273	**54**	14	28	After
3322-B	273	**54**	14	28	After
3322-C	273	**54**	14	28	After
3321-A	274	**49**	12	70	During
3321-B	274	**49**	12	70	During
3321-C	274	**49**	12	70	During

3244-A	275	9	3	12	During
3244-B	275	9	3	12	During
3244-C	275	9	3	12	During
3237-A	276	7	8	26	Before
3237-B	276	7	8	26	Before
3237-C	276	7	8	26	Before
3340-A	277	**27**	14	26	After
3340-B	277	**27**	14	26	After
3350-A	278	**38**	5	73	During
3350-B	278	**38**	5	73	During
3350-C	278	**38**	5	73	During

Appendix 13: Impact of antibiotics course combination on the gut microbial community in preterm infants

Table: Demographic data of Antibiotics course combination

Pts No	GA (weeks)	BW (g)	Antibiotics course	Antibiotics course combination*
222	24	620	VCM	0(G6M6A6) 15(V5C5) 26(V4M4C4)
188	24	750	VCM	0(P2G2) 12 (A2F2G2) 14 (M9V9C9)
270	25	750	VCM	0(P,G2) 3 (C5,V7,M8) 14 (C5,V5,M5) 28 (C2,V2) 33 (C2,V2)
215	27	1180	VCM	0(P2G2) 3(v5C5M5)
163	24	760	VCM	0(P2,G2)8(M7,V7,C7)20(A2,F2,G2)22(V3,C5)42(V7,M7,C7)69(V2,C2)77(V2,C2,M2)86(F2,G2)

163	24	760	VCM	0(P2,G2)8(M7,V7,C7)20(A2,F2,G2)22(V3,C5)42(V7,M7,C7)69(V2,C2)77(V2,C2,M2)86(F2,G2)
277	24	620	VCM	0(P2G2) 12(V7,C7,M7)
287	24	670	VCM	0 (P6G5Cx6) 4(F7) 17 (A1G1F1) 18(C2M6V6) 19(mero4)
335			VCM	0(P5G5) 21(A5F5) 26(C6V6M6) 48(C13F13)61(L33)
336			VCM	0(P2G2) 7(C3V3) 12(C2V2) 14(A8) 28(V15C15M4) 31(A6)
337			VCM	0(P2G2) 4(V5C5M5) 44 (V3C3M3) 63 (V7C7M7) Y
356			VCM	0(P2G2) 4(F4G4A4) 27(V10C10M10) 60(A3F3G3) 87 (F2)
130	27	1000	VC	0(P2G2)1(A7,M7)8(V3,C3)21(V2,C2)23(F14)31(G6)63(A2,F2,G2)
130	27	1000	VC	0(P2G2)1(A7,M7)8(V3,C3)21(V2,C2)23(F14)31(G6)63(A2,F2,G2)
153	28	1310	VC	8(C2,V2)

161	25	700	VC	0(P2,G2)6(A3,F3,G3)19(V6,C6)20(M5)27(A10,G10,M10)61(A5)
163	24	760	VC	0(P2,G2)8(M7,V7,C7)20(A2,F2,G2)22(V3,C5)42(V7,M7,C7)69(V2,C2)77(V2,C2,M2)86(F2,G2)
171	26	790	VC	0(P3,G3)9(V2C2)13(A3,G3,F3)17(A4,G5,F4)19(M8)21(V6C6)
228	25	910	VC	0(P2,G2,M2)7(V2,C5)98(A2,F2,G2)118(A5,F6,G5)
229	25	910	VC	0(P2,G2,M2)3(V6,C8)11(Mer6)20(F3,A3,G3)25(T6)27(M5)38(F1,A1,G1)46(Trimethoprim prophylaxis)
303	26	990	VC	0 (P2G2) 5 (M8A8G7) 14 (A1) 15 (V6C6)
307	25	810	VC	0 (P2G2) 2(A3F3) 32 (C5V5)
330	26	990	VC	0(P5G5) 2(F5) 7(V5C5) 16 (A3F3G3) 24 (V2C2) 37 (taz5)
336			VC	0(P2G2) 7(C3V3) 12(C2V2) 14(A8) 28(V15C15M4) 31(A6)
336			VC	0(P2G2) 7(C3V3) 12(C2V2) 14(A8) 28(V15C15M4) 31(A6)

152	25	800	AFG	0(P2,G2)5(F2,G2,A2)
167	27	1290	AFG	0(P2,G2)43(A2,F2,G2)
171	26	790	AFG	0(P3,G3)9(V2C2)13(A3,G3,F3)17(A4,G5,F4)19(M8)21(V6C6)
181	23	570	AFG	0(P2,G2)13(V3)14(F9)27(T1,C1,V2,G4,Mer17)33(G3)37(G3)41(G3)53(A2,F2,G2)61(A2,F2,G2)
203	26	1130	AFG	0(P2,G2)4(F3,A3,G2)
206	28	1255	AFG	0(P1,G2)5(A2,F2,G2)13(A3,F3,G3)
207	29	1580	AFG	0(P2,G2)10(A3,F3,G3)
232	24	695	AFG	0(P2,G2)7(G2,F6,A2)14(G3,F3,A3)24(A2,F2,G2)31(A5,F5,G5)
284	25	710	AFG	0(P2G2) 2(C10V10) 27(A3F12G5)
294	30	1650	AFG	0(P2G2) 28(A2F7G2)

313	28	1120	AFG	0 (P2G2) 10 (A2 F2G2) 21 (A4G2M9) 24(V7 C7) 34 (V3C2M3) 43 (mero2V2) 54 (Mero7 V7) 56 (M5)
317	28	780	AFG	0(P2G2) 21 (A2F2G2)
330	26	990	AFG	0(P5G5) 2(F5) 7(V5C5) 16 (A3F3G3) 24 (V2C2) 37 (taz5)

*Antibiotic course combination with the number inside the bracket indicating how many times (days) each specific antibiotic was given, while the number outside the bracket indicates the day of life during which antibiotic was given. The alphabets represent the name of antibiotics as: A – Amoxicillin, C – Ceftazidine, F – Flucloxacillin, G – Gentamicin, L – Linezolid, M – Metronidazole, Me – Meropenem, P – Penicillin, T – Tazocin, and V – Vancomycin.

Table: Clinical characteristics of the infants in Antibiotics cohort study

Patient Number	Frequency of occurrence	Sample Number	Day Of Life	Gestational Age (weeks)	Birth Weight (g)	Sex	Delivery Mode	Antibiotics Age (days)	Antibiotics Course	Duration of Antibiotics Treatment (days)
222	4	2362	25	24	620	F	V	26	VCM	4
		2411	29							
		2429	32							
		2475	36							
188	2	1459	12	24	750	F	V	14	VCM	9
		1578	39							
270	3	3191	9	25	750	M	V	14	VCM	5
		3230	28							
		3262	39							
215	4	1984	10	27	1180	M	FD	3	VCM	5
		2013	13							
		2047	16							
		2054	17							

163	4	1109	40	24	760	M	CS	42	VCM	7
		1188	50							
		1227	58							
		1238	60							
163	3	1362	76	24	760	M	CS	77	VCM	2
		1383	78							
		1409	81							
277	3	3296	6	24	620	M	CS	12	VCM	7
		3318	16							
		3340	27							
287	4	3390	16	24	670	M	V	18	VCM	6
		3421	23							
		3430	25							
		3445	30							
335	4	3899	22					26	VCM	6
		3919	30							

		3936	33							
		3945	39							
336	4	3922	26					28	VCM	15
		3975	42							
		4010	46							
		4020	50							
337	3	3974	40					44	VCM	3
		4029	49							
		4037	51							
356	3	4228	26					27	VCM	10
		4244	35							
		4261	44							
130	4	429	8	27	1000	F	CS	8	VC	3
		435	11							
		439	13							
		453	18							

130	4	454	19	27	1000	F	CS	21	VC	2
		461	22							
		467	26							
		474	28							
153	4	736	5	28	1310	M	CS	8	VC	2
		763	11							
		775	13							
		803	17							
161	4	849	17	25	700	F	CS	19	VC	6
		868	19							
		869	20							
		961	32							
163	4	867	9	24	760	F	CS	22	VC	5
		1010	27							
		1089	35							
		1109	40							

171	4	1085	9	26	790	M	V	9	VC	2
		1106	11							
		1125	13							
		1149	16							
228	4	2378	6	25	910	M	V	7	VC	5
		2435	12							
		2467	15							
		2521	19							
229	4	2348	3	25	910	M	V	3	VC	6
		2413	10							
		2449	14							
		2488	17							
303	4	3568	4	25	960	M	V	15	VC	6
		3597	22							
		3599	23							
		3608	27							

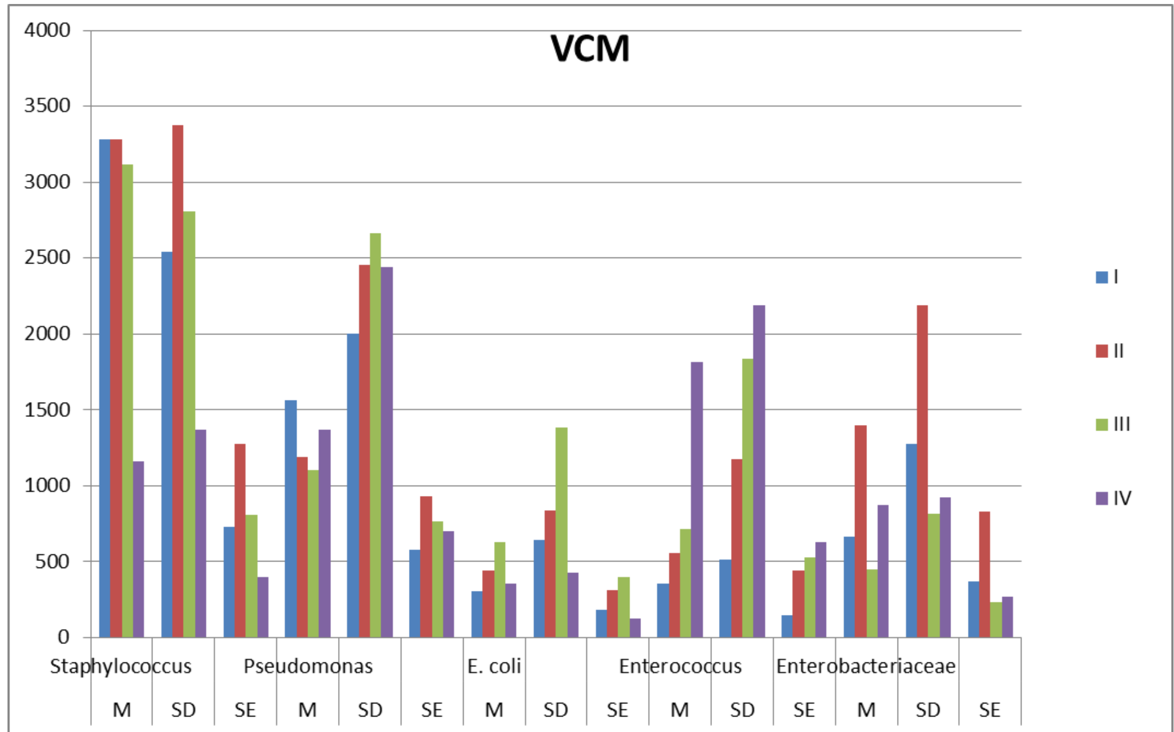
307	4	3677	30	25	810	F	V	32	VC	5
		3685	37							
		3689	39							
		3697	41							
330	4	3794	5	26	990	M	V	7	VC	3
		3805	11							
		3813	13							
		3829	18							
336	3	3842	6			M		7	VC	3
		3869	12							
		3887	16							
336	4	3881	7			M		7	VC	3
		3878	14							
		3880	15							
		3906	21							
152	4	731	4	25	800	F	V	5	AFG	2

		739	6							
		750	9							
		778	13							
167	4	1228	40	27	1290	F	CS	43	AFG	2
		1265	45							
		1286	48							
		1320	52							
171	4	1113	12	26	790	M	V	13	AFG	3
		1142	15							
		1168	19							
		1183	21							
181	4	1643	54	23	570	M	V	61	AFG	2
		1693	66							
		1719	68							
		1746	71							
203	4	1690	2	26	1130	M	V	4	AFG	3

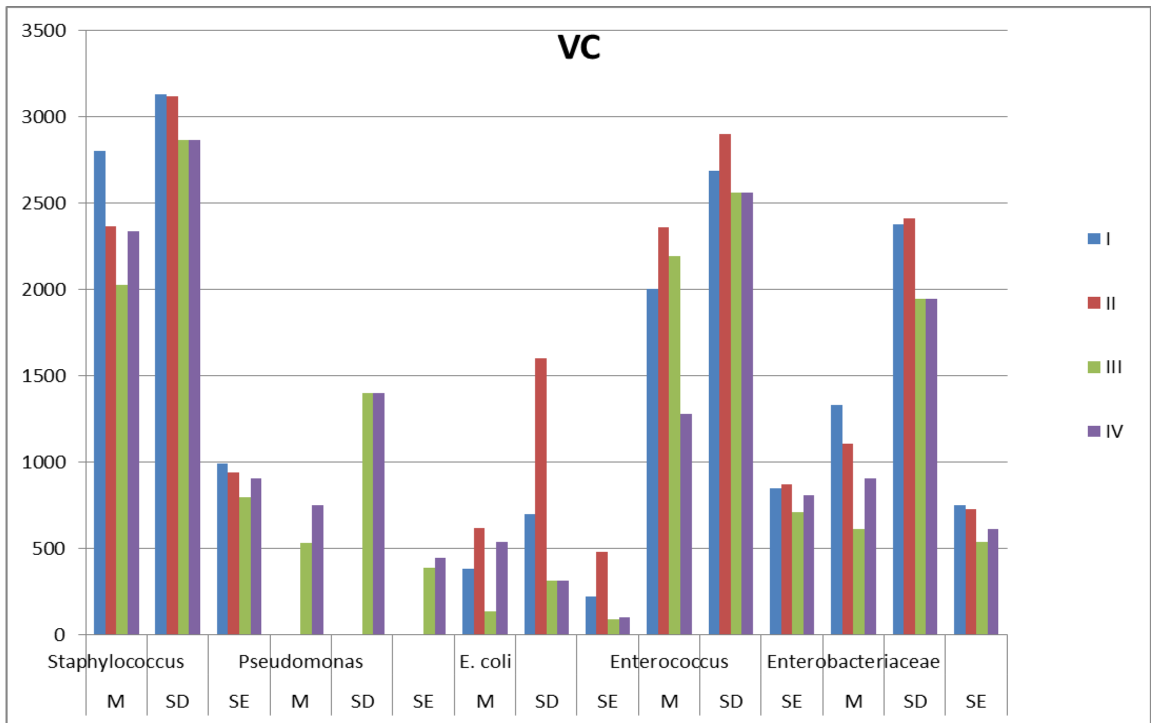
		1761	7							
		1767	9							
		1824	14							
206	4	1736	4	28	1255	M	V	5	AFG	2
		1766	7							
		1790	9							
		1834	13							
207	4	1806	9	29	1580	M	CS	10	AFG	3
		1831	12							
		1885	16							
		1930	19							
232	4	2469	6	24	695	M	V	7	AFG	2
		2517	10							
		2539	12							
		2561	14							
284	4	3398	25	25	710	M	CS	27	AFG	3

		3422	29							
		3426	30							
		3435	34							
294	3	3479	26	30	1650	M	CS	28	AFG	2
		3483	29							
		3488	36							
313	3	3668	9	28	1120	F	CS	10	AFG	2
		3678	12							
		3683	18							
317	3	3704	20	28	780	F	CS	21	AFG	2
		3707	22							
		3712	31							
330	4	3815	14	26	990	M	V	16	AFG	3
		3829	18							
		3846	21							
		3867	26							

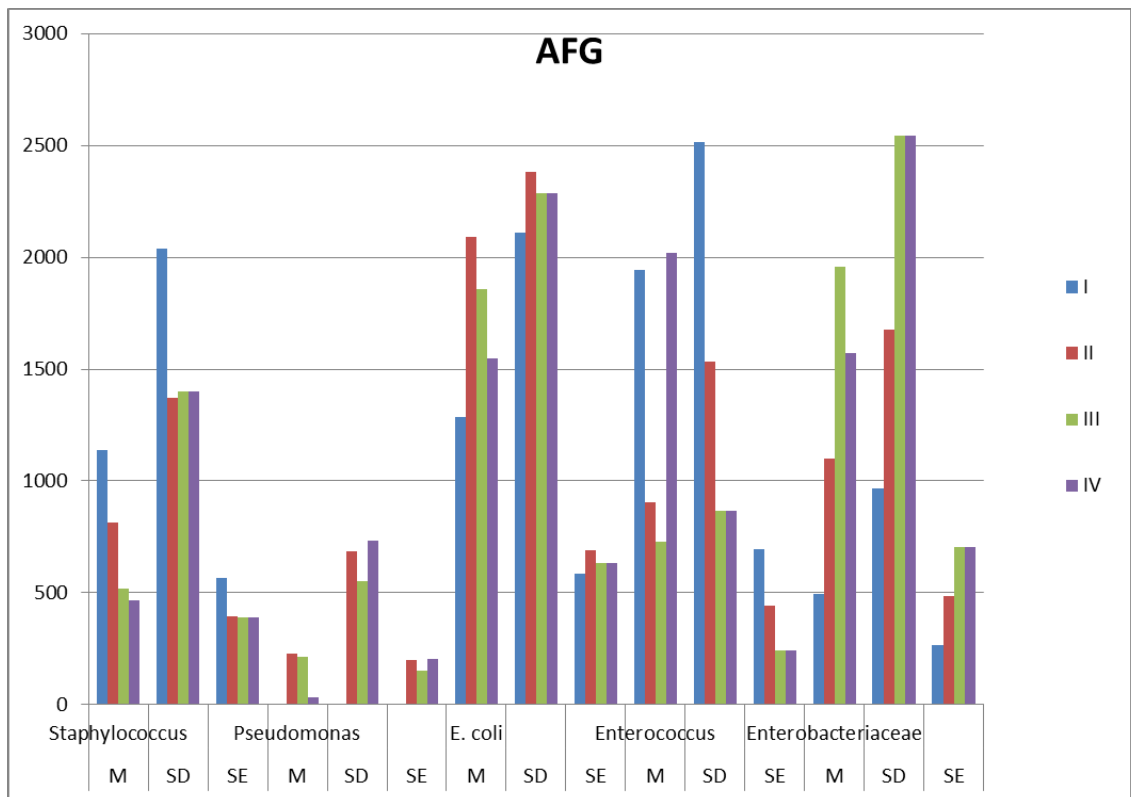
Comparison between Mean, Standard Deviation and Standard Error of the Most Abundance Taxa



Comparison between Mean, Standard deviation and Standard error of the VCM course among the most abundance taxa, the colour at the chart matched with the Roman numerals colour of the time points, where I =prior to antibiotic intake, II = during, III = immediately after and IV = one week after administration. M = OTUs mean, SD = standard deviation and SE = standard error.



Comparison between Mean, Standard deviation and Standard error of the VC course in the most abundance taxa, the colour at the chart matched with the Roman numerals colour of the time points, where I =prior to antibiotic intake, II = during, III = immediately after and IV = one week after administration. M = OTUs mean, SD = standard deviation and SE = standard error.



Comparison between Mean standard deviation and standard error of the AFG course in the most abundance taxa, the colour at the chart matched with the Roman numerals colour of the time points, where I =prior to antibiotic intake, II = during, III = immediately after and IV = one week after administration. M = OTUs mean, SD = standard deviation and SE = standard error.