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# **Gut bacterial activity in a cohort of preterm infants in health and disease**

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## Summary

### Introduction

Randomised controlled trials administering probiotic supplements to preterm infants to prevent sepsis and necrotising enterocolitis are already underway, despite the lack of a robust evidence base of normative values for gut microbiota, bacterial metabolites, and markers of inflammation and immunity. There are increasing calls for observational studies to establish baseline data in these infants. Most of these studies to date have involved the measurement of these analytes individually. In the studies presented in this thesis, we measured a range of stool markers collectively in a cohort of preterm infants in health and disease.

### Design

56 infants at <32 week gestation and less than 1500g birth weight were sequentially recruited from all three Glasgow Neonatal Units within week one of life after commencement of enteral feeds. Anthropometric, dietary and treatment data were collected. Stool samples were taken once weekly for the first four weeks, testing: short chain fatty acids; calprotectin, secretory immunoglobulin A; and microbial diversity by temporal temperature gel electrophoresis.

### Results

Out of 61 live births meeting the study criteria, 56 infants were enrolled in the study, 62.5% of whom were female. 19.6% were between 24-26 weeks gestation, 28% were 26-28 weeks, 30% were 28-30 weeks, and 21% were 30-32 weeks. 5.3% were between 490-600g in birth weight, 17.8% were 600-800g, 21.4% were 801-1000g, 39.2% 1001-1250g, and 16% were between 1251-1500g. Feed regimen was heterogeneous, comprising 5 combinations of maternal, donor and formula milks. The highest social deprivation level as measured by the Carlisle 'Depcat' scoring system of level 7 was significantly higher in the study group than Glasgow or Scotland-wide averages. Sepsis rates were low, with a group median of only 1 per infant. Overall mortality: 7%. 32 with any NEC (56%), 20 with Bells'  $\geq 2a$  NEC. 8 (14%) with surgically treated NEC, 5 (8%) underwent ileostomy. SCFAs: (n=56) there were no correlations between gestation, weekly totals, feed type, or NEC and SCFA concentration. Acetate and lactate dominated each sample. Few significant changes were noted with respect to NEC, and these were in the less dominant SCFAs: stage 2a NEC showed higher concentrations of propionate in week 4 than week 3, and lower valerate in week 4 than 2. Stage 3b levels of isobutyrate and heptanoate were significantly

lower in week 4 than 3. FC: (n=56) there were no significant differences in FC levels between each week in infants with or without NEC, although the former illustrated a trend to lower levels by week 4. There were no significant differences in NEC before and after clinical signs were apparent, or in those before NEC and after stoma formation for stage 3b NEC. However, significantly lower FC levels were noted in stage 3b NEC requiring ileostomy compared to the immediate pre-operative sample. SIgA: (n=34) Levels rose significantly week on week, and were considerably higher in weeks three and four than week one. There were no significant differences in stool SIgA concentration between infants with and without NEC. A significant increase in mean stool SIgA concentration appeared from week 2 to week 3 in NEC infants, and from week 1 to week 2 for those without. For all breastfed preterm neonates (n=6), the level of milk SIgA was significant higher on week 1 (colostrum) than week 2 and week 3. TTGE: (n=22) There was large variability between number (1-17) and species diversity (25-36 different species). Bacterial composition varied largely between the 2 sample points. No difference in species richness or similarity within the 2 feeding groups was observed. 4 bands were identified in >50% of infants. Intra-individual similarity varied greatly and ranged from a similarity index (Cs) of 0% to 66.8%. There was no statistical difference between the similarity indices of the feeding groups or between those with and without NEC. There were no significant correlations between any of the analytes.

## Conclusions

Only extreme prematurity and extremely low birth weight were associated with NEC, which was at a strikingly high incidence. A limitation was therefore the unexpected onset of severe NEC resulting in prolonged paralytic ileus with low stool production. No correlations were found between analytes, indicating that each set of stool investigations may signify independent physiological, biochemical and immunological gut processes. Despite the severity of NEC, the levels of each analyte were remarkably consistent. High levels of deprivation within the study population may provide the constellation for an as of yet undefined genetic and epigenetic predisposition to NEC in this cohort, similar to that of other illnesses endemic to different geographical areas – notably Multiple Sclerosis in the North East of Scotland – and both follow up of these infants into childhood as well as further analysis of future inborn infants with NEC is planned.

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Dedicated to the memories of

Morag Beattie Strachan

March 29<sup>th</sup> 1941 – February 26<sup>th</sup> 2009

and

Rebecca Margaret McKeown

October 14<sup>th</sup> 2007 – December 2<sup>nd</sup> 2009

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Andy and my daughters Kate and Zed: for everything; for without whom, this is all meaningless.



Alicia, Study Baby 59, at age 2 – taken and included at parental suggestion

‘Keep calm and carry on’

- British World War II propaganda poster, 1939

Declaration:

I declare that, except where explicit reference is made to the contribution of others, that this dissertation is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Lynne Mary Beattie

## **Study Concept, Design and Completion**

The original concept for this project was identified by Dr Andrew Barclay, after the 2007 publication of his systematic review of probiotic trials in preterm infants (Barclay, Stenson et al. 2007). This premise was further extrapolated by myself, and refined in consultation with Dr Douglas Morrison (DJM), Professor Christine Edwards (CAE), Dr Judith Simpson (JHS), Dr Kostas Gerasimidis (KG), and Dr Helen Mactier. I then wrote the ethics proposal, attended the subsequent REC panel hearing, and secured funding for a two-year Clinical Research Fellowship with the University of Glasgow. Furthermore, I secured funding for consumables from The NICU Research Fund at Yorkhill, and another small grant from the University of Glasgow.

I performed all recruitment, and collection of clinical and demographical data. Nursing staff very kindly took all stool samples from the nappies, which I then collected from each NICU on a daily basis, returning each day to the Department of Child Health at Yorkhill, where they were stored. SCFA protocols were performed and developed by myself and DJM, under the tutelage of KG. FC ELISA was performed by me after instruction by KG. SIgA ELISA was performed by myself and Miss Wen Wen (MW, MSc student), under the supervision of Dr Aspray-Combet. TGGE was performed chiefly by Dr Gerasimidis, Miss Bruner (KB, MSc student), and myself. Please note that although offshoots of the SIgA and TTGE analyses lead to MSc projects for KB and MW, the actual contribution of these to this thesis is considered by all to be minimal. Data was reviewed by me, with verification by DJM and CAE. I performed all statistics for the SCFA and calprotectin data, and for the SIgA and TTGE data did so with MW and KB. These were periodically cross-checked with Dr David Young, DM and CE. Dr Young attempted multivariate analysis on these complex results, and it was agreed between all that given the heterogeneity of histograms and variation in non-normal data, multivariate analysis would be inappropriate.

This thesis has been written in its entirety by me, with comments from DJM, CE, JS, MW, KB and KG. Dr Richard Russell kindly edited the calprotectin background and data. Within the body of the background text, I performed all systematic reviews of the evidence as presented in table form and discussed thereafter, as well as creating all figures and tables. Graphs and tables for the SCFA and FC results were created by me. All others were created by me and collaborators KG, KB and MW.

## Common Abbreviations

AEDF	Absent End Diastolic Flow
ANOVA	Analysis of Variance
APGAR	Apgar Score
BCFA	Branched Chain Fatty Acids
BIFS	British Intestinal Failure Study
BMI	Body mass index
CH <sub>3</sub> CN	Acetonitrile
CMV	Cytomegalovirus
CPAP	Continuous Positive Airway Pressure
CRIB	Clinical Risk Index in Babies score
CRP	C-reactive protein
CS	Caesarean Section
C <sub>s</sub>	Similarity index
D	Donor milk
DEBM	Donor Expressed Breast Milk
DEF	Donor, Expressed maternal and Formula feeding
DepCat	Deprivation category
DNA	Deoxyribonucleic acid
DPO	Diphosphoric acid
ECA	Enzyme conjugate antibody
EF	Exclusive Formula
EFM	Expressed breast milk and Formula Mixed
EL	Evidence level
ELBW	Extreme low birth weight
ELLUSCS	Elective Lower Uterine Segment Caesarean Section
ELISA	Enzyme Linked Immunosorbant Assay
EMLUSCS	Emergency Lower Uterine Segment Caesarean Section
FA	Fatty acid
FC	Faecal Calprotectin
FID	Flame ionisation detector

FISH	Fluorescent in situ hybridisation
FOB	Faecal Occult Blood test
FOS	Fructo-oligosaccharide
GC	Gas Chromatography
GCMS	Gas Chromatography - Mass Spectrometry
GGC	Greater Glasgow and Clyde
GGNHSB	Greater Glasgow National Health Service Board
GI	Gastro-Intestinal
GIT	Gastro-Intestinal Tract
GOS	Galacto-oligosaccharide
GP	Glucose polymer formula
HCl	Hydrochloric acid
HIV	Human Immuno-deficiency Virus
HMO	Human Milk Oligosaccharides
HPGC	High Performance Gas Chromatography
H <sub>2</sub> O	Water
IBD	Inflammatory Bowel Disease
IFN- $\gamma$	Interferon Gamma
ISD	Information Services Division
IUGR	In utero Growth Restriction
IQR	Interquartile range
IVF	In-vitro fertilisation
IVH	Intraventricular haemorrhage
LAC	Lactose formula
lcFOS	Long chain fructo-oligosaccharides
LBW	Low Birth Weight
LDL	Low density lipoprotein
LPL	Lipoprotein lipase
MBL-2	Mannose binding lectin-2
mg/L	Milligrams per litre
mls/day	Millilitres per day
mmoles/L	Millimoles per litre
MEBM	Maternal Expressed Breast Milk
Med	Median
MeSH	Medical subject headings

MCT-1	Monocarboxylate transporter 1
MCDA/DCDA/MCMA	Monochorionic diamniotic/dichorionic diamniotic monochorionic monoamniotic
MF	Mixed fed
MOD	Method of delivery
MS	Multiple sclerosis
MTBSTFA	N-methyl-N-tert-butyltrimethylsilyltrifluoroacetamide
mV	Millivolts
NA	Not Applicable
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate oxidase
NaOH	Sodium hydroxide
NEC	Necrotising enterocolitis
NICE	National Institute for Health and Clinical Excellence
NON	Non-NEC
NPEU	National Perinatal Epidemiology Unit
NS	Not specified
PCR	Polymerase Chain Reaction
PET	Pre-eclampsia
PGHN	Paediatric Gastroenterology Hepatology and Nutrition
PIH	Pregnancy Induced Hypertension
pNPP	p-Nitrophenylphosphate
PN/TPN	Parenteral nutrition/total parenteral nutrition
PPROM	Preterm Prolonged Rupture of Membranes
PR	Per rectum
PRMH	Princess Royal Maternity Hospital
qPCR	Quantitative Polymerase Chain Reaction
RCTs	Randomised Controlled Trials
R+D	Research and Development
RHSC	Royal Hospital for Sick Children
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SCFA	Short Chain Fatty Acids
SD	Standard deviation
SEM	Standard error of the mean

SGH	Southern General Hospital
SIgA	Secretory Immunoglobulin A
SIGN	Scottish Intercollegiate Guideline Network
Sp.	species
SVD	Spontaneous vaginal delivery
TAE	Tris-acetate-EDTA (ethylenediaminetetraacetic acid)
tBDMS	tert-Butyldimethylsilyl
TCD	Thermal conduction detector
TCR	T-cell receptor
TLR-4	Toll-like receptor 4
TTGE	Temperature Temporal Gradient Electrophoresis
UIFABP	Urinary intestinal fatty acid binding protein
UK	United Kingdom
UNICEF	United Nations Children's Fund
UVC/UAC	Umbilical venous catheter/umbilical arterial catheter
USA	United States of America
VLBW	Very Low Birth Weight
VLDL	Very low density lipoprotein
VRE	Vancomycin Resistant Enterococcus
Vs	Versus
2EB/3MV	2-ethyl butyric acid/3methyl-valeric acid
μmoles/Kg	Micromoles per kilogram
μg/g	Micrograms per gram
μL	Microlitre
>	More than
<	Less than
=	Equals
≥	Equal to or more than
≤	Equal to or less than

# Chapter 1

## Background

### 1.1) Introduction

In preterm infants, the gut microbiota (also known as the dominant gut bacterial consortia) in the first few months of life number far fewer bacterial species than infants born at term. Observational studies also suggest that the type and concentration of metabolites produced by these bacteria are significantly different in preterm than term infants, which could in turn indicate differences in gut immunology and inflammation, and may act as diagnostic and/or prognostic markers of gut dysfunction. However, whether these differences are physiological or pathological is yet to be defined, and there are no normative data for these values in 'healthy' preterm infants, without infection, gut necrosis, or poor weight gain.

With the establishment of trials of enterally administered 'probiotic' supplements (bacteria with benefits to the host) to term infants aiming to treat and/or prevent allergy, eczema and colitis, trial supplementation is now focussed upon preterm infants in order to prevent NEC, the most devastating disease of the gut of early life, affecting 6-10% of preterm, VLBW infants, but with mortality rates of up to and beyond 50%. In the last 5 years, repeated meta-analyses of these RCTs suggest that the supplementation of milk with probiotics significantly reduces their risk of NEC. However, with no defined normative microbiological, metabolic, immunological and inflammatory data, it is difficult to ascribe this benefit solely to probiotic supplementation, given the well-established effect of exclusive maternal breast milk feeding in preventing NEC and sepsis in preterm infants. Notably, none of the meta-analyses to date can extrapolate data according to feed type. As such, this effect requires ascertainment with comparative analyses in 'healthy' preterm infants without probiotic supplementation. The stool analyses of: metabolites (short and branched chain fatty acids), bacteria (transient temperature gradient electrophoresis), an immunological marker (secretory immunoglobulin A), and an inflammatory marker (calprotectin) are seen individually in observational studies to vary in preterm infants with and without NEC and sepsis. As a panel however, they had not, at the inception of this project, been tested concurrently in a cohort of preterm infants over the first month of life, assessing correlations with nutrition and environment. This study aims to do just that.

## **1.2) Definition and Evolution of Gut Microbiota**

### 1.2.1) Definition

The term gut ‘microbiota’ is a collective noun describing the all-inclusive commensal gut bacterial consortia. The gut microbiota is a powerful and complex collection of microorganisms. Numbering ten times that of the cells in the entire adult human body, the gut microbiota could be considered an organ in its own right, given a metabolic capacity equivalent to the liver (Edwards and Parrett 2002). Within each individual adult there are more than 1000 known species, with around 2 million genes (the so-called ‘microbiome’ – the human microbial genome) (Xu and Gordon 2003). Once established in infancy, more than 99 % of the gut microbiota comprises anaerobic bacteria. Once stabilised and established in healthy humans, usually by the age of 2 years, the components of the gut microbiota remain relatively consistent throughout life, although high interindividual variation exists (Rambaud and Buts 2006). Fungi, protozoa and viruses are also gut commensals, but little is known about their function. The most heavily colonised area of the human body by surface area is the digestive tract (Hill 1985). An estimated 60% of dry faecal mass is composed purely of bacteria. The gut microbiota has been implicated in protection against cardiovascular, inflammatory, allergic and malignant conditions in later life (Isolauri 2012). Conversely, adverse alterations in the microbiota may be linked to a range of chronic, non-infectious conditions including malignancy, obesity, cardiovascular events and autoimmune disease (Ley, Backhed et al. 2005, Bezirtzoglou and Stavropoulou 2011, Shanahan 2012, Wong, Esfahani et al. 2012). Homeostasis of the gut microflora is generally adversely affected by GI pathologies (such as inflammatory bowel disease, colonic malignancies, gastroenteritis and dysentery), yet, conversely, evidence exists linking abnormal gut microbiota to the development of these very illnesses. Additionally, changes in nutrition (for example according to cultural or religious need, or in other physiological states such as pregnancy) and enterally administered medications, particularly antibiotics are also noted to have profound effects upon the gut microflora. The symbiotic relationship between microbiota and host is currently undergoing extensive further scrutiny owing to developments in molecular and metabolic techniques allowing higher resolution analyses and new information on species type and abilities (Satokari, Vaughan et al. 2003, Vanhoutte, De Preter et al. 2006). The dominant microbiota in adult humans is illustrated in the following so-called ‘phylogenetic tree’ – linking taxa from bacteria with similar phenotypical and genotypical features as illustrated in Figure 1:

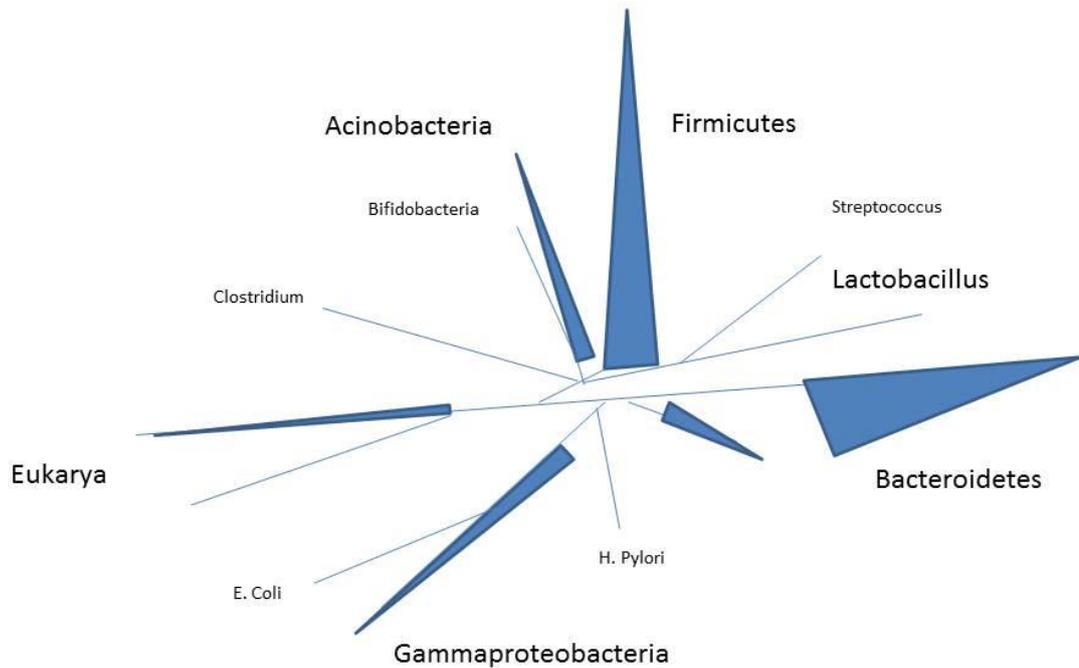


Figure 1, Phylogram: Major unrooted phylogenetic tree illustrating gut microbiota components in healthy adults; size of triangle indicates relative abundance, and orientation of limbs denotes similar morphology.

### 1.2.2) Functions

The human microbiota has a wide variety of potential influences including immunological, metabolic, trophic, anticarcinogenic, as well as, paradoxically pro-carcinogenic and pro-inflammatory. Most of these require interaction between the microbiota and immune system – so-called ‘cross-talk’. Identification of species and function are now considered as ‘metatranscriptomics’ – the study of the relationship between the gut microbiome and its bacterial metabolites. A glossary of definitions of bacterial ‘cross-talk’ is seen in table 1. There is potential for manipulation of the microbiota to establish permanent effects on the host – particularly in early life (Ouwehand, Isolauri et al. 2002, Gueimonde, Kalliomaki et al. 2006). Mode of delivery at birth has been shown in observational studies to be associated with significant differences in microbiota even in adulthood (Huurre, Kalliomaki et al. 2008, Biasucci, Rubini et al. 2010, Dominguez-Bello, Costello et al. 2010, Fallani, Young et al. 2010). Observational studies indicate that the microbiota composition can be influenced by consistent, long term administration of microbes (probiotics), antibiotics, or diet (for example fibre, or prebiotics) (Rambaud and Buts 2006). This raises the intriguing possibility that manipulation of microbiota in the neonatal period can influence adult illnesses – even more so than lifestyle changes implemented

later on in life (Barker 2001). However, many of the benefits of probiotic administration are seen to regress once stopped (Walker and Lawley 2013)

<b>Parameter</b>	<b>Definition</b>
Microbiota	Dominant bacterial consortia
Genome	Entire gene sequence
Transcriptome	Set of all RNA molecules
Microbiome	Microbial genetic elements
Metabolome	Metabolites within an organism
Metabolomics	Study of metabolic processes
Metabonomics	Qualitative analysis of all measurable metabolites
Proteome	Set of proteins expressed by a genome

Table 1: Table adapted from *The Core Microbiome*, by Turnbaugh et al, Nature, 2009 (Turnbaugh, Hamady et al. 2009)

Gut bacterial metabolism serves not simply as a consequence of bacterial energy consumption, but describes the processes employed by bacteria in order to produce energy and nutrients from which to survive. This can involve a host of strategies according to both species and strain type, and production, accordingly, enables bacterial identification. Such metabolites, as illustrated in figure 2, may be as diverse as ethanol, lactate and hydrogen, depending on the sources of energy and pathways utilised, according to environmental conditions (Resta 2009).

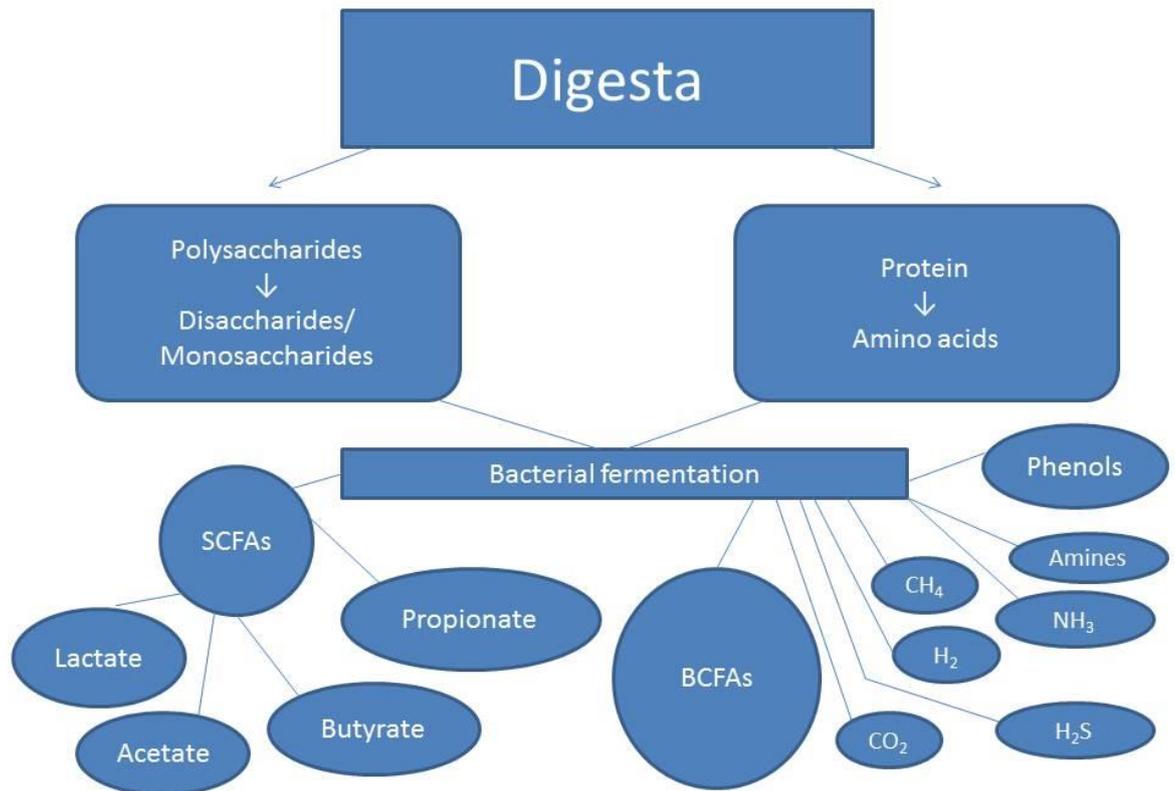


Figure 2: Gut bacterial metabolism, depicting fermentation of carbohydrate and protein. (Abbreviations: SCFAs = short chain fatty acids; BCFAs = branched chain fatty acids; CH<sub>4</sub> = methane; H<sub>2</sub> = hydrogen; CO<sub>2</sub> = carbon dioxide; NH<sub>3</sub> = ammonia; H<sub>2</sub>S = hydrogen sulphide)

## i) Fermentation, energy absorption and micronutrient production

### a) Carbohydrates

- *Animal Models and Adults*

The fermentation of unabsorbed carbohydrate is achieved by enzymatic pathways absent from the human genome, and specific to the gut microbiota. Higher non-digestible carbohydrate and fibre intake results in a lower colonic pH, with resultant alteration in bacterial metabolism and growth, promoting species including *Lactobacillus* and *Bifidobacteria*. The gut microbiota ferments non-digestible carbohydrates into short chain fatty acids (SCFAs), as a means of electron disposal in the absence of oxygen and as an electron acceptor. Indeed, germ-free rat models (i.e. those lacking microbiota) have shown a 30% higher calorific requirement than conventional animals in order to maintain body weight which suggests the importance of the bacteria in energy assimilation (Sears 2005). Trials of intestinal microbiota transfer in humans from lean to obese donors reveal significant changes in body mass index, glucose tolerance, and associated gut butyrate

levels, and obesity-specific SCFA trends have been observed, notably lower levels of propionate, acetate, and butyrate (Achour, Flourie et al. 1994, Arora, Sharma et al. 2011, Vrieze, Van Nood et al. 2012). Similarly, dietary differences in SCFA profiles have been recognised in those using carbohydrate restriction in order to lose weight, notably lower total SCFAs and butyrate concentrations. In other studies, high levels of SCFAs and butyrate are associated with adverse gastrointestinal disorders, such as necrotising enterocolitis (Lin 2004) (Brinkworth, Noakes et al. 2009).

‘Prebiotics’ are a collection of non-digestible substances, mainly dietary carbohydrates, that stimulate the growth of selective bacteria, often the same types as those used in ‘probiotics’ – bacteria that display benefits to the host (Araya 2001). *In vitro* studies of selective fermentation of ‘prebiotic’ oligosaccharides by gut microbiota reveal higher concentrations of lactate, presumed secondary to their bifidogenic and lactobacillogenic effects (Grimoud, Durand et al. 2010, Russo, de la Luz Mohedano et al. 2012, Garrido, Ruiz-Moyano et al. 2013). Many studies, however, are still in animal models, although increasingly, paired data matching qualitative and quantitative molecular analyses with metabolites confirms the ability of prebiotics to promote growth of selective strains, and, in adults, producing beneficial butyrate and reducing parameters linked with protein fermentation (Vitali, Ndagijimana et al. 2012, Walton, Lu et al. 2012). Other studies of the fermentation of other food substrates (for example soy-based products, complex carbohydrates including type 3 resistant starch (Topping and Clifton 2001, Scheiwiller, Arrigoni et al. 2006) illustrate the production of a host of other trophic products for uptake by the colonic mucosa. Such is the ubiquity of prebiotic supplementation that their addition is becoming commonplace in the commercial setting, and oligosaccharides are now added to sweeteners, baking products, yoghurts, and milkshakes (Sangwan, Tomar et al. 2011).

- *Infants: Term and Preterm*

Infants delivered at term have higher concentrations of short chain fatty acids earlier in infancy than those born prematurely, owing to a faster rate of colonisation. Marked differences are noted according to feed type – particularly between infants exclusively breast or formula fed. (Heavey, Savage et al. 2003, Donovan, Wang et al. 2012). Spectrum of stool SCFAs in infants exclusively breast milk fed illustrate higher levels of propionic and n-butyric acids, and lower levels of lactic acid than infants who are exclusively formula fed. These differences continue for the first month of life. From the establishment of weaning, however, these differences are lost, and a new, consistent microbiota is established (Edwards, Parrett et al. 1994).

Preterm infants are known to have few species at low abundance in the first months of life, and, unsurprisingly, lower levels of energy-yielding products of bacterial fermentation, which may in turn contribute to their lower weight gain until term equivalent. Infants who develop NEC and/or who require antibiotics in the neonatal period are seen in observational studies to be colonised with even fewer gut commensals, although certain products of bacterial fermentation such as butyrate may be raised, indicative of enteropathogenic activity such as *Clostridium butyricum*, while others may be lower secondary to a paucity of commensal and indeed beneficial strains of *Bifidobacteria*, which predominate in breast fed infants (Wang, Shoji et al. 2007, Underwood, Salzman et al. 2009).

*b) Protein*

Fermentation of protein by gut microflora yields a host of potentially toxic metabolites, the effects of which have been analysed mainly in animal models and adult studies (Phua, Rogers et al. 1984, Hughes, Magee et al. 2000, Huang, Shu et al. 2012). Such metabolites include phenol, cresol, para-cresol, ammonia, hydrogen sulphide, and branched and short chain fatty acids (Meyer and Hostetter 2012, Windey, De Preter et al. 2012). Animal models have noted abnormal neurology in rats administered intrathecal propionate, and other studies of protein-derived SCFAs have revealed hepatotoxicity at physiological levels (Jolly, Ciurlionis et al. 2004). Fermentation of protein by the gut microbiota yields approximately 15g nitrogenous faecal material daily in a healthy adult. Adults also both ferment and recycle the products of protein metabolism, including hydrolysis of urea, deamination of amino acids, and recycling of ammonia. Nitrosation reactions of secondary amines from amino acid fermentation are associated with an increased risk of colo-rectal cancer (Hughes, Magee et al. 2000, Kuhnle and Bingham 2007, Kuhnle, Story et al. 2007, Lunn, Kuhnle et al. 2007, Joosen, Kuhnle et al. 2009). Given the multiple mechanisms of absorption and excretion of these compounds, it is possible to measure a variety of colonic protein metabolites in blood, stool and urine. Toxic products of protein fermentation are now recognised in observational studies of adults with chronic kidney disease, and are associated with heightened cardiovascular morbidity and mortality (Huang, Shu et al. 2012, Meyer and Hostetter 2012). Hydrogen sulphide in the gut is implicated in the development of ulcerative colitis and colonic carcinomas, yet, paradoxically, recent research in adults and animals notes multiple beneficial effects of hydrogen sulphide including neuroprotective, cardioprotective, and anti-inflammatory (Windey, De Preter et al. 2012). So far most observational studies of protein fermentation products have been performed in animal models and adults, with few focussing upon infancy. As such, it is

theoretically possible that neonates may accumulate potentially toxic metabolites including phenols, cresols, indoles, branched chain amino acids, SCFAs (especially propionate) and hydrogen sulphide, which can be absorbed into plasma with resultant systemic effects. However, this has not yet been explored in neonatal studies. Localised effects upon the gut mucosa are uncertain, although animal studies forming models of NEC suggest that protein-derived SCFAs may cause or at least contribute to localised inflammation (Hughes, Magee et al. 2000).

c) *Lipids*

The gut microflora may affect body fat composition via a variety of endocrine, metabolic, and fermentation mechanisms, including suppression of LPL inhibitors by certain commensal species; metabolism of oligosaccharides by microbiota producing SCFA profiles inhibiting liver triglyceride and VLDL synthesis, thus lowering circulating triglyceride and cholesterol levels; and the hydroxylation and hydrogenation of lipids (Kaddurah-Daouk, Baillie et al. 2011, Fava, Gitau et al. 2012, Wong, Esfahani et al. 2012). Most dietary cholesterol is esterified and therefore not absorbed from the gut (Trapani, Segatto et al. 2012, Tanaka, Yasuda et al. 2013). Of the cholesterol that is absorbed by the gut, 50% of that oxidised by the liver into bile acids is reabsorbed by the small intestine into the blood stream. A diet rich in fibre is recognised to enlarge the bile acid pool, binding and excreting more cholesterol at a higher rate (Kumar, Nagpal et al. 2012). Gut microbiota are also pivotal in recycling of bile acids thus metabolising cholesterol (Ley, Backhed et al. 2005, Turnbaugh, Backhed et al. 2008). Conversely, reduced microbial metabolism of cholesterol is associated with severe colonic disorders: colitis, bacterial overgrowth, and malabsorption (Schippa, Iebba et al. 2010, Scaldaferrri, Pizzoferrato et al. 2012, Shanahan 2012). Observational studies have shown an association with increased fat accumulation in adults and an ‘abnormal’ gut microbiota comprising a reduction in *Bacteroidetes*, and increase in *Firmicutes* (Ley, Backhed et al. 2005, Turnbaugh, Backhed et al. 2008). Lean individuals are observed to have higher levels of *Bacteroidetes*, with clinically obese patients exhibiting higher abundance of clostridia (Tilg 2010). One theory is that the by-product of this loss of major *Bacteroidetes* strains appears to be an increased fermentation of polysaccharides to SCFAs, thus providing additional energy and so weight gain in obese subjects. In addition, metabolism of phosphatidylcholine to lecithin has been shown to promote the deposition of atherosclerotic plaques – with a resultant increase in cardiovascular morbidity and mortality (Wang, Klipfell et al. 2011).

d) *Micronutrients*

- **Vitamins**

By separate pathways, the gut microbiota also produces vitamins (particularly biotin and Vitamin K) and facilitate absorption by the host through the absorption and storage of lipids, necessary for the solubility of certain vitamins (A, D, E, and K) (Strozzi and Mogna 2008, Resta 2009). Bacteria usually produce vitamins through the 2-methyl-D-erythritol-4-phosphate pathway. Human stores of vitamins K and B12 are also produced by the gut microbiota, particularly lactobacillus species (Vaughan, Heilig et al. 2005, Leblanc, Milani et al. 2012). Various gut commensal bacteria produce vitamins by acting through the coenzymes NAD and NADPH to facilitate the production of niacin, pantothenic acid, and folic acid. Certain probiotic bacteria can promote vitamin D production by stimulating vitamin D receptors in the gut both with and without SCFAs. SCFAs can induce expression of the vitamin D receptor, which acts as a key regulator of calcium absorption and intracellular storage. A positive feedback cycle can thus be proffered: bacteria thrive in a SCFA-rich environment of low pH, and as such commensal bacteria produce more SCFAs, with a resultant increase in cellular energy and more intracellular calcium binding proteins. This theoretically results in extra calcium storage in the body, particularly teeth and bones. Thus treatment with probiotic bacteria in adult trials is associated with reduction in chronic joint inflammation and higher bone density as measured by bone density index (Scholz-Ahrens, Ade et al. 2007, Mandel, Eichas et al. 2010).

ii) **Trophic factors**

‘Trophic factor’ is a generic term used to describe an array of endogenous substances that can stimulate intestinal growth and function. Although mainly peptides, this blanket term includes an array of phytochemicals utilising unique pathways. *Bifidobacteria* sp. facilitate the production of specific trophic factors, and so are associated with improved growth and reduced time to intestinal adaptation when administered to infants recovering from intestinal failure and short bowel syndrome (Barclay, Beattie et al. 2011). Lectins and equol, a non-steroidal oestrogen produced from the bacterial metabolism of isoflavones found commonly in soyabean products, act as hormonal intestinal trophic factors. Similarly, phytoestrogen production as a consequence of microbiota metabolism of isoflavones, are seen to regulate cell differentiation and growth of the gut lumen. This is of particular consequence given the presence of isoflavones in soy-based infant formula milks – the greatest dietary source at any stage of life (Setchell, Zimmer-Nechemias et al. 1997).

Much of the ability of gut microflora to promote gastrointestinal growth may be via SCFAs. Through various mechanisms, the gut microbiota are seen to effect development of the villus microvasculature, promoting gut perfusion (Sakata 1987). This may in part be due to the transition from use of glucose and glutamine to butyrate as an energy substrate. In 1987, Sakata et al produced experimental translocated colon and small intestine samples, and measured the resultant SCFA production. A significantly thicker mucosa and muscularis layer, with a three to four-fold increased crypt cell production rate, was closely associated with higher levels of SCFAs. Their subsequent research in this field further delineated butyric acid as a main stimulant of epithelial cell proliferation (Inagaki and Sakata 2005), which has, in the intervening years, been consolidated by other research groups (Scheppach, Bartram et al. 1992, Ichikawa, Shineha et al. 2002). In observational studies, germ-free animals are also seen to have thinner villi with deeper crypts (Stappenbeck, Hooper et al. 2002). Other studies have investigated differences in adults post-disease (for example, those in recovery from IBD and gastro-intestinal malignancy, versus controls) histological colonic specimens with and without probiotic supplementation. It appears that certain strains have the ability to effect villus growth and even inhibit colonic tumour growth (Bindels, Porporato et al. 2012, Ou, DeLany et al. 2012, Thirabunyanon and Hongwittayakorn 2013). For infants' post-SBS or with NEC with prolonged recovery, or intestinal failure, the potential for probiotics to elongate villus length is an exciting prospect.

### **iii) Immunological, antibiotic and anti-inflammatory**

The gut microbiota have important anti-enteropathogenic effects, achieved mainly by a competitive 'barrier effect' whereby harmful microorganisms are unable to thrive due to the competitive binding actions of beneficial bacteria binding to the gut mucosa (Chow, Lee et al. 2010, Fukuda, Toh et al. 2012). Dominant microbiota species' in infancy, such as *Bifidobacteria* and *Lactobacillus* sp. stimulate key immunological effects, both local and systemic, possibly preventing clinical eczema, but to a lesser extent for other allergy and inflammatory disorders, later in life (Osborn and Sinn 2007). The gut microbiota is also responsible for cell signalling in immunity, promoting maturation of immune cells, which affect macrophage function on the intestinal mucosa, and even traverse the blood brain barrier (Diamond, Huerta et al. 2011). Germ-free mice exhibit immature lymphatic systems, less Peyer's patches and fewer isolated lymphoid follicles (Cebra, Periwai et al. 1998, Ouwehand, Isolauri et al. 2002, Bouskra, Brezillon et al. 2008). Several communities of commensal bacteria are also seen to strengthen the colonic defence barrier by reinforcing the tight junctions at a cellular level, by clustering between the lamina propria

and the lumen (Prakash, Rodes et al. 2011). Infective viral gastroenteritis is less commonly observed in infants who are exclusively breast fed rather than formula, thought to be mainly from the properties of *Bifidobacteria* and *Lactobacillus* species to lower colonic pH and so produce an acidic environment hostile to enteric viruses (Plenge-Bonig, Soto-Ramirez et al. 2010). Other properties also include the bacterial production of bacteriocins.

Bacteriocins are anti-enteropathogenic proteins produced by commensal bacteria in the gut – chiefly lactic acid producing bacteria (Hammami, Fernandez et al. 2012). However, bacteriocins produced by bacteria can also inhibit members of the same strain. Most bacteriocins appear to be directed against gram positive enteropathogens (although gram positive bacteria can also produce bacteriocins), and activity profiles suggest many are more effective than conventional antibiotics (Borrero, Brede et al. 2011). Class I lantibiotics comprise post-translationally modified amino acids; Class II non-lantibiotics refer to nonmodified amino acids; and Class III are large, heat-labile proteins. Commercial efforts are now focussed upon large-scale production of bacteriocins for medical purposes (Velazquez 2012).

Additionally, the enteropathogenic role of pH, mediated chiefly by acetate production from an abundance of *Bifidobacteria* species in the gut microbiota of infants exclusively breast fed, is seen to play a pivotal role in the inhibition of major known enteropathogens such as *E.Coli* 0157 (Fukuda, Toh et al. 2011), *Clostridia jejuni* (Baffoni, Gaggia et al. 2012), and rotavirus (Balamurugan, Magne et al. 2010). Paradoxically, prophylactic probiotic administration to infants has not yet been seen to reduce their incidence of gastrointestinal infection, and probiotics administered to infants with short gut syndrome were at increased risk of translocating those strains to the bloodstream – accounting for several case series’ of clinically septic infants with the sole identification of probiotic strains in blood samples; so-called probiotic-related ‘sepsis’ (Thompson, McCarter et al. 2001, Sherman 2010, Lee and Siao-Ping Ong 2011).

#### **iv) Anti-carcinogenic effects**

Strains of *Lactobacilli* are known to produce a host of factors that inhibit the proliferation of tumour cells, degrade carcinogens, and successfully compete for mucosal binding sites with microorganisms that produce pro-carcinogens. Various strains of *Lactobacillus* and *Bifidobacteria* sp. which predominate in the gut microbiota of infants are also known to release antioxidants, such as glutathione and superoxide dismutase, which also exert anti-carcinogenic effects (Kullisaar, Zilmer et al. 2002, Achuthan, Duary et al. 2012). SCFAs

such as butyrate have also been observed in *in vivo* studies to be associated with suppression of cancer cells (Tang, Chen et al. 2011, Leonel and Alvarez-Leite 2012, Matthews, Howarth et al. 2012). Studies investigating the *in vitro* administration of probiotics note a reduction in the proliferation of tumour cells, particularly colonic, although the remainder of the evidence base is mainly evidence level 2: controlled data in case series or reports. Observational studies of adults with colorectal cancers reveal diminished populations of gut commensals such as *F. prausnitzii* (a butyrate producer) and *E. rectale*, and higher abundance of known carcinogen associated enteropathogens, such as *Bacteroides-Prevotella* populations – although whether this is cause or effect has yet to be ascertained (Balamurugan, Rajendiran et al. 2008, Sobhani, Tap et al. 2011). Many animal studies are investigating these associations (Topping and Clifton 2001). Studies of propionate show similar anticarcinogenic properties in adult subjects and *in vitro* work, but to a lesser extent (Cousin, Jouan-Lanhouet et al. 2012, Matthews, Howarth et al. 2012). SCFAs generically lower colonic pH, and this acidity is noted to have an important anticarcinogenic effect. Recently, colonic pH has subsequently been shown to alter efficacy of oral chemotherapeutic agents (Ashwanikumar, Kumar et al. 2012, Madhusudana Rao, Mallikarjuna et al. 2013), particularly for drugs such as 5 fluorouracil. This also enables controlled release of these drugs, targeted at certain areas of small and large bowel according to the rate of activation dependent on luminal acidity (Deepa, Thulasidasan et al. 2012). Preterm infants are at higher risk of malignancies in adult life than infants born at term, although this may be secondary to their higher incidence of Syndrome X, also known as Metabolic Syndrome, and higher waist-to-hip ratio, both of which are in turn associated with higher levels of colonic and other malignancies in general (Griffin and Cooke 2012).

**v) Reduction of serum cholesterol and morbid obesity**

Elements of the gut microbiota, especially an abundance of *Firmicutes* and diminished growth of *Bacteroides* species', have been associated with lower serum cholesterol and leanness in adulthood, theoretically in turn leading to less cardiac risk and lowering malignancy (Cani and Delzenne 2009, Parnell, Raman et al. 2012). Most studies analysing the effects of altered gut microbiota on cholesterol synthesis have been animal studies (De Smet, Van Hoorde et al. 1995, Kumar, Nagpal et al. 2012, Pavlovic, Stankov et al. 2012). Identifying the species implicated in these cholesterol-reducing effects allows commercial production as dietary probiotic supplements. Subsequently, observational and *in vitro* studies have identified *Lactobacillus gasseri*, acidophilus, and *Bifidobacterium bifidum* as exhibiting the most pronounced effect on lowering serum cholesterol and promoting a

healthy BMI in adults (Klaver and van der Meer 1993, Usman and Hosono 1999). In addition, the ability of *Bifidobacteria* and *Lactobacillus* species to create an acidic colonic environment aids the excretion and impairs the absorption of dietary cholesterol. Mechanisms utilised by *Lactobacillus acidophilus* for this purpose include the inhibition of a rate-limiting enzyme of endogenous cholesterol biosynthesis, and promotion of excretion of dietary cholesterol via precipitation in bile acid, hepatic lipolysis, or (theoretically) even absorption by the organism itself (Gilliland, Nelson et al. 1985, De Rodas, Gilliland et al. 1996). Other mechanisms include increased faecal bile acid loss, with resultant greater utilisation of liver cholesterol to replace lost bile acids, in doing so reducing cholesterol deposition; promotion of bile salt hydrolase, resulting in deconjugation of bile and resultant co-precipitation with cholesterol; and reduced reabsorption of cholesterol in the small intestine, both associated with increased colonic propionate production (Jones, Martoni et al. 2012, Kumar, Nagpal et al. 2012). In observational studies, the dominance of *Bifidobacteria* in the adult gut microbiota is associated with a higher incidence of leanness. Two mechanisms for this are proposed: gut colonisation suppresses expression of a fasting-induced adipose factor released from the epithelium, in turn increasing the activity of lipoprotein lipase with a resultant increase in triglyceride storage. Secondly, gut bacteria may increase hepatic lipogenesis as a byproduct of dietary polysaccharide degradation, thus promoting obesity. Disordered gut microflora are implicated in the development of metabolic disease, particularly in the western world, where obesity is epidemic (Tremaroli and Backhed 2012). Dominance of known enteropathogens in the gut microflora is conversely associated with increased plasma cholesterol levels and obesity (Santacruz, Collado et al. 2010), and may even be aetiologically, when observed in obese and lean twins (Turnbaugh, Hamady et al. 2009). Several animal studies have also noted an increase in acetate production yet concurrent decrease in propionate production in obese subjects (mainly mice), although whether this is as a result of or implicating in causing obesity, is unclear. Certain studies postulate a future therapeutic role for propionate as a hypophagic agent (Hong, Nishimura et al. 2005, Arora, Sharma et al. 2011, Darzi, Frost et al. 2011, Lin, Frassetto et al. 2012).

#### **vi) Hormonal interactions**

The gut is intimately associated with the endocrine system, and produces hormones by specialist enteroendocrine cells from stomach to distal colon. Despite these numbering less than 1% of the cells of the entire GIT, their production is prolific, accounting for around 70% of postprandial insulin secretion alone (Vilsboll and Holst 2004). GI hormones include gastric inhibitory peptide, glucagon-like peptide-1, peptide YY and oxytomodulin

(CCK). Multiple neuroenteroendocrine loops have been postulated and investigated in human and animal studies, and impairment of these has been implicated in the pathogenesis of type 1 insulin-dependent diabetes mellitus (Boerner and Sarvetnick 2011, Holzer, Reichmann et al. 2012, Vaarala 2012). Many are intimately related to glycaemic control, lipid metabolism, appetite control, and so are also associated with disease states such as Metabolic Syndrome, NIDDM and morbid obesity (Manco, Putignani et al. 2010, Tilg 2010, Vijay-Kumar, Aitken et al. 2010, Tremaroli and Backhed 2012). Bacteria can influence the release of hormones and hormone-like molecules, including gut microbiota-derived signalling molecules, and biologically active peptides, particularly in the regulation of appetite, and gastric perfusion (Holzer, Reichmann et al. 2012). Transfer of intestinal microflora has also been observed to correlate with a transfer of insulin resistance between individuals with metabolic syndrome (Vrieze, Van Nood et al. 2012, Aroniadis and Brandt 2013) Faecal transplantation of microbiota from obese mice has been shown to result in a greater increase in body fat than microflora from lean mice (Vrieze, Holleman et al. 2010).

Various species within the microbiota are also thought to maintain homeostasis between thyroid and oestrogen-based hormones and the host, through promotion of enzymes metabolising these – although these are very new observations, mostly in animal studies (Van de Wiele, Vanhaecke et al. 2005, Mori, Nakagawa et al. 2012, Awaisheh, Khalifeh et al. 2013). Multiple observational studies and randomised controlled trials have attempted to identify differences in composition and function of gut microbiota communities in populations of pre and post-menopausal women, without significant results (Bonorden, Greany et al. 2004, Vrieze, Holleman et al. 2010). Such studies aim to identify associations with breast and gynaecological cancers that could potentially be ameliorated or prevented by alteration of the gut microbiota by probiotic supplementation. Studies in pregnant women have so far examined mother-infant stool pairs, placental samples, and cord blood immunological markers. Few have examined maternal or fetal endocrine effects on gut microflora profiling or supplementation with probiotics during pregnancy (Lindsay, Gibney et al. 2012, Stojanovic, Plecas et al. 2012). Any differences may also be gestation dependent, and this important confounder is yet to be investigated in observational or randomised controlled trials. Notably, the incremental effects of antenatally produced progesterone frequently result in relaxation of maternal smooth muscle, resulting in physiological ileus and constipation, the effects of which upon the gut microflora have not been investigated.

### **vii) Modulation of neurological development**

The gut microbiota is interlinked with immunology and thus neurology (Diamond, Huerta et al. 2011, Diaz Heijtz, Wang et al. 2011). Germ-free and gnotobiotic mice have been reported to be immunosuppressed yet resistant to autoimmune diseases in several studies (Walton, Galanko et al. 2006, Tlaskalova-Hogenova, Stepankova et al. 2011). Gut microbiota have also been theoretically implicated in alteration of immune signalling from the gut, including cytokine release, production of immune cells such as helper T cells and macrophages which in turn stimulate the brain to produce cholinergic anti-inflammatory agents via the vagus nerve – which also innervates the gut (Tracey 2010). *In utero* there may be a fixed ‘window’ during which the gut microbiota can influence the growing fetal brain (Diaz Heijtz, Wang et al. 2011). Observational studies reveal that the gut microbiota can induce systemic immune responses that may in turn influence development of glial and neuronal pathways, as well as the cerebral vasculature (Greenwood, Heasman et al. 2011). Fledgling investigations using mouse models of the relapsing-remitting inflammatory disease multiple sclerosis implicate changes in the commensal gut flora in this regard (Berer, Mues et al. 2011, Berer and Krishnamoorthy 2012, Rook 2012).

By definition a commensal benefits one partner, yet simultaneously neither benefits nor negatively impacts upon the other. In humans only eight bacterial divisions exist within the microbiota, (Backhed, Ley et al. 2005), which can be affected by both horizontal genetic changes (in microbes) and vertical changes (in environment: for example mutations and deletions). In mammals, *Firmicutes* and *Bacteroidetes* predominate, as identified by the 16S rRNA sequence work in major studies (Leser, Amenuvor et al. 2002, Eckburg, Bik et al. 2005). Bacterial phenotypes are furthermore affected by the host and transmitted to new bacterial cells – progeny. ‘Functional redundancy’ occurs when bacteria have overlapping roles such that if one strain is absent then others can assume similar functions. This is particularly important for humans with fewer species, in whom colonisation is delayed – in particular, preterm infants.

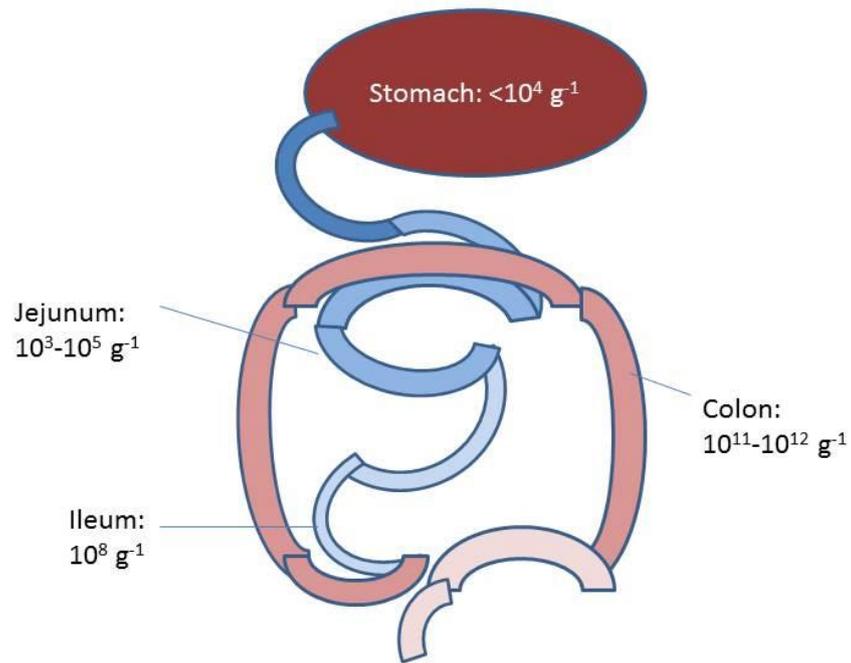


Figure 3: Anatomical quantification of the gut microbiota in healthy adults (Abbreviations:  $\text{g}^{-1}$  = grams to the power of -1)

Differences are emerging between numbers of faecal commensal bacteria in the elderly, young adulthood (see figure 3 above), and infancy with reduced quantities at the extremes of lifespan (Tiihonen, Ouwehand et al. 2010, Biagi, Candela et al. 2012, Brussow 2013). The long-term relevance of this prognostically to preterm infants is as of yet unknown.

### 1.3) Microbiota, Metabolites and Markers of Gut Inflammation

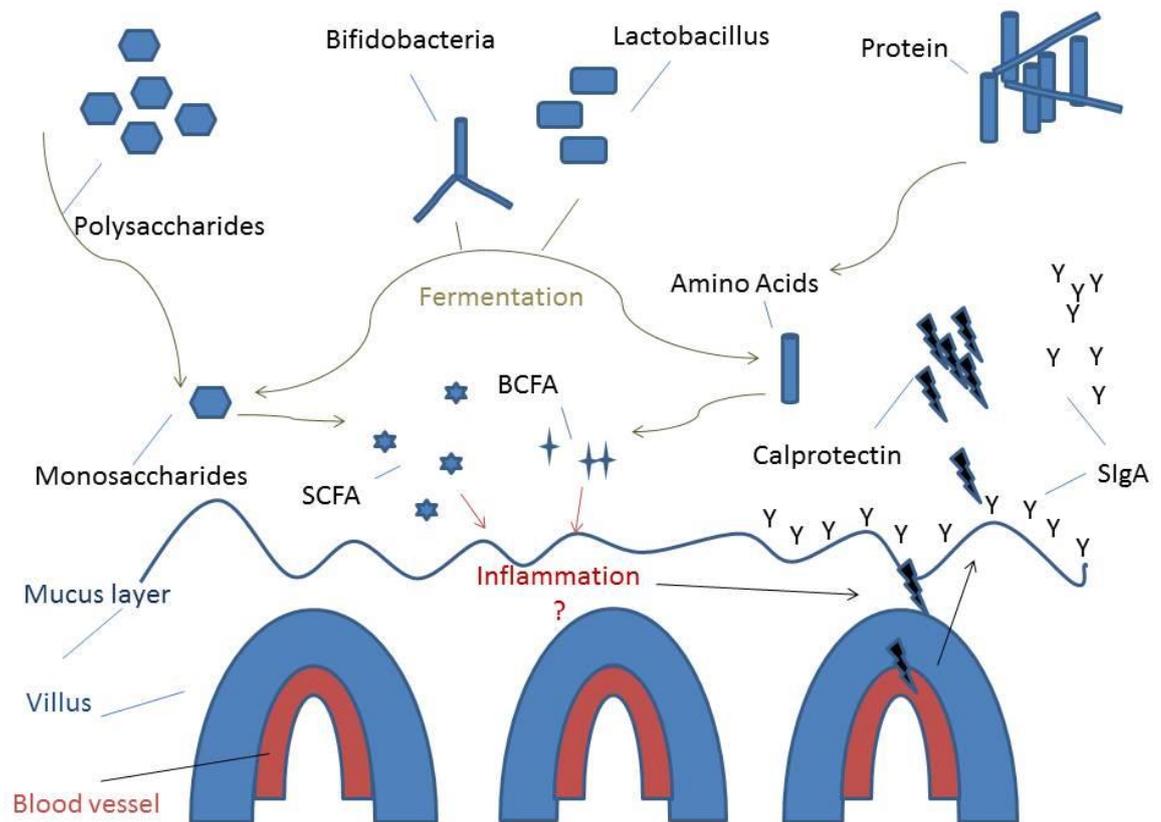


Figure 3: Interaction between gut microbiota, metabolites, inflammation and immunity at the gut mucosa. (Abbreviations: SCFA = short chain fatty acids; BCFA = branched chain fatty acids; SIgA = secretory immunoglobulin A)

#### 1.3.1) Short Chain Fatty Acids: Definition and Relevance

Although first identified more than 100 years ago (Brieger 1878), the relevance of SCFAs to health and disease has been discussed since the 1980's. As far back as the beginning of last century, high SCFA values have been identified in infant stool (Bahrtdt 1914, Goiffon 1921). Fatty acids (FAs) are organic compounds comprising a hydrocarbon chain and a terminal carboxyl group, varying from a single hydrogen molecule to nearly 30 carbons. They are the constituents of a range of dietary lipids. Their properties are determined by chain length, degree of saturation (the presence of one or more double bonds), and the branching of chains.

Short chain fatty acids (SCFAs) are fatty acids with less than six carbon atoms, and include formic, acetic, propionic, butyric, valeric and caproic acids. Isomeric forms (isovaleric, isobutyric and isocaproic) are also known as Branched Chain Fatty Acids (BCFAs)

produced from the fermentation of amino acids. SCFAs are produced mainly in the colon by the fermentation of dietary carbohydrate, particularly dietary fibre, starch and oligosaccharides, allowing the recovery of carbon and thus provision of energy for colonocytes and body systems (Rambaud and Buts 2006). Conversely, high levels of certain SCFAs have been associated with colonic injury in animal models, giving rise to the potential for disease markers in inflammatory conditions such as IBD and NEC (see figure 3) (Waligora-Dupriet, Dugay et al. 2005, Peng, He et al. 2007), although research in adults with IBD strongly points to the therapeutic commodity of butyrate (Wong, de Souza et al. 2006, Hamer, Jonkers et al. 2008, Vieira, Leonel et al. 2012). Colonic anaerobes also ferment amino acids to BCFAs by a series of reduction/oxidation and transamination reactions. In the intestine, SCFAs are present mainly as negatively charged anions and not free acids, given the pH of the colonic environment (between 5.5 – 7.0) (Duncan, Louis et al. 2009). They are also miscible with water and so readily transported directly to the portal vein via MCT-1 and sodium dependent transporters during fermentation as an energy source for liver and muscle tissue. As such, a cascade can be postulated: different carbohydrates are fermented by selective bacteria to specific SCFAs – thus acting as markers of nutrition and infection. Acetate is oxidised by muscle, and propionate is sequestered almost entirely by the liver. Butyrate, however, is absorbed and oxidised by colonocytes to the liver, and BCFAs are partially excreted in faeces. Although concerns have been raised regarding the possibility of gut lactate being absorbed into the blood stream, these are in patients with short bowel syndrome, who are already at increased risk of D-lactic acidosis (Uribarri, Oh et al. 1998, Munakata, Arakawa et al. 2010). Lactate is chiral, with two optical isomeric forms: L (+)-lactate, physiologically produced from pyruvate in response to anaerobic metabolism, and D (-)-lactate. D-lactate is also produced by colonic fermentation of carbohydrates, and can be absorbed into the systemic circulation. In situations of small bowel bacterial overgrowth, which are commonly due to short gut syndrome and a resultant overgrowth of lactic acid producing bacteria (such as *Lactobacilli*), D-lactic acidosis is at significant risk of developing, leading primarily to encephalopathy (Petersen 2005). Other more recent probiotic trials in infants have so far noted no raised levels of urinary D- or L-lactate in those fed lactic acid producing strains, such as *Lactobacillus Johnsonii*, but this is obviously an important potential risk requiring further investigation (Mack 2004, Haschke-Becher, Brunser et al. 2008).

### 1.3.2 Branched Chain Fatty Acids and Products of Protein Degradation

Branched chain fatty acids (BCFAs) are a subset of saturated SCFAs with at least one methyl branch on the carbon chain, but can also be produced from the degradation of

protein, and are found in substances directly relevant to the newborn such as vernix and human milk (Ran-Ressler, Devapatla et al. 2008). These are isomeric forms of SCFAs, and those of note in preterm infants appear to be isobutyric, isovaleric and isocaproic acids. BCFAs account for approximately 20% of total SCFA production from protein. This represents a small component of SCFA in the large bowel (see figure 3 for schematic). However no other studies have addressed the fate of branched chain fatty acids in VLBW preterm infants, nor any correlation to types of milk fed, or possible links to NEC. Recently, animal models have noted a reduction in NEC associated with the enteral administration of 20% BCFA feed. Given the presence of BCFAs in the gut lumen at birth in term infants secondary to the development and deglutition of vernix, which occurs exclusively in the last trimester, the hypothesis that BCFAs may protect the gut from NEC is under development by this research group (Ran-Ressler, Khailova et al. 2011). Phenol, cresol and ammonia are toxic bacterial metabolites of protein fermentation, and their role in the premature gut is unknown. Preterm infants display high levels of proteolysis, contributing to lower growth rates (Denne 2007, Hay and Thureen 2010). Only one paper was identified which assessed such stool products in term infants, revealing significantly higher levels of ammonia and beta-glucuronidase (a faecal bacterial enzyme) activity in those breast fed in comparison with formula fed (Heavey, Savage et al. 2003). Para-cresol is a product of proteolysis and aromatic amino acid fermentation by *Clostridium* sp. that has been identified in the stool of preterm infants with NEC (Phua, Rogers et al. 1984).

The SCFA type and concentration also vary within the host according to anatomical location. The oral cavity, oesophagus, stomach and small intestine host mainly facultative anaerobic or aerobic eubacteria (Hobson and Stewart 1997), and as such SCFA production is low. However the highest microbial production of SCFAs takes place in the proximal colon (Hintz, Schryver et al. 1978). In the large intestine most anaerobes ferment carbohydrates to SCFAs by the Embden Meyerhof pathway. Animal and human studies models have illustrated that SCFAs can expedite gut transit time, most likely by stimulating release of polypeptide YY (Richardson, Delbridge et al. 1991, Cuche, Cuber et al. 2000, Cherbut 2003).

### **1.3.3 General functions of short chain fatty acids:**

SCFA function in two general respects: 1) they form energy substrates for the gut mucosa; 2) they act as signalling molecules. They therefore have the potential to be diagnostic and prognostic markers of disease. With these three elements in mind, a systematic review of the evidence base for the role of and associations with SCFAs in term and preterm infants was undertaken. The following table highlights the paucity of this evidence.

*Main Identities and Properties of FAs C1-C11, With Isomeric Forms, in Infancy*

Individual Stool SCFAcid	Bacteria implicated in fermentation of individual SCFA	Effects in Term Infant Studies	Effects in Preterm Infant Studies
Acetic (C2)	<i>Acetobacteraceae</i> e.g. <i>acetobacter</i>	<p>↑ in prebiotic sup studies (Knol, Scholtens et al. 2005, Holscher, Czerkies et al. 2012);</p> <p>↔ probiotics (Underwood, Salzman et al. 2009)</p> <p>Age-dependent ↑ (Midtvedt and Midtvedt 1992)</p>	<p>↑ in probiotic trials (Mohan, Koebnick et al. 2006, Wang, Shoji et al. 2007);</p> <p>↔ with feed type (Favre, Szylit et al. 2002)</p>
Propionic (C3)	<i>Propionibacterium</i> e.g. <i>Propionibacterium propionicus</i>	<p>↑ in prebiotic sup studies (Holscher, Faust et al. 2012)</p> <p>↑ in well formula-fed infants (Edwards, Parrett et al. 1994)</p> <p>↑ in mixed-fed term infants (Ogawa, Ben et al. 1992)</p> <p>↓ in prebiotic RCT (Knol, Scholtens et al. 2005)</p> <p>Age-dependent ↑ (Midtvedt and Midtvedt 1992)</p>	<p>↔ with probiotic administration (Wang, Shoji et al. 2007);</p> <p>↔ with feed type (Favre, Szylit et al. 2002)</p>
Butyric (C4)	Obligate anaerobic bacteria e.g. <i>Clostridium butyricum</i> , <i>Fusobacterium nucleatum</i>	<p>↑ in prebiotic sup studies (Holscher, Faust et al. 2012)</p> <p>↑ in well formula fed infants (Edwards, Parrett et al. 1994).</p> <p>Age-dependent ↑ (Midtvedt and Midtvedt 1992)</p>	<p>↓ in probiotic trial (Wang, Shoji et al. 2007)</p> <p>↓ in EBM fed (Favre, Szylit et al. 2002)</p> <p>↑ in PR bleeds; ↑ in healthy prems 2<sup>nd</sup> – 3<sup>rd</sup> week of life (Szylit, Maurage et al. 1998)</p>
Caproic (C5)	<i>Clostridium</i> e.g. <i>klyveri</i> ; <i>Fibrobacter</i> e.g. <i>succinogenes</i>	Marker for clostridia levels (Madan and Slifkin 1988)	Nil significant
Valeric (C6)	<i>Clostridia</i> , <i>Eubacterium pyruvativerrans</i>	No studies found	No studies found
Heptanoic (C7)	<i>Clostridium</i> e.g. <i>sporosphaeroides</i>	No studies found	No studies found
Octanoic (C8)	Nil significant	No studies found	No studies found

Lactic (C12)	<i>Lactobacillus, Lactococcus</i>	↓ in well formula-fed infants (Ogawa, Ben et al. 1992, Edwards, Parrett et al. 1994).	↔ probiotic trial (Wang, Shoji et al. 2007). ↑ in probiotic trial (Mohan, Koebnick et al. 2006)
Isobutyric (iC4)	<i>Lactobacillus</i>	No studies found	No studies found
Isocaproic (iC6)	<i>Clostridium difficile</i>	↑ in allergic infants (Bottcher, Nordin et al. 2000)	Nil significant
Isovaleric (iC5)	<i>Bacteroides, Prevotella</i>	No studies found	No studies found

Table 2: The evidence base for the bacteria-specific production of stool Short Chain Fatty Acids and their associations in term and preterm infant studies (↑=higher SCFA; ↓=lower SCFA; ↔=no difference in SCFA)

## **1.4) Evolution and Identification of the Gut Microflora**

### **1.4.1) Introduction**

Given the high level of obligate and strict anaerobes in the gut microbiota, it has until relatively recently been difficult to identify many bacterial species and strains owing to limitations of culture techniques. In the 1960's the majority of strains resident in the gut were thought to have been identified, yet it is now evident that methods of anaerobic culture used at this time were of low yield (Dubos and Schaedle 1964, Dubos, Savage et al. 1967, Rambaud and Buts 2006). However, as molecular techniques have flourished, the proportion of unidentified species has, paradoxically, expanded, with new species and strains being identified on a daily basis (see figure 4 for schematic) (Rodrigues da Cunha, Fortes Ferreira et al. 2012, Turrone, Peano et al. 2012). This heightened level of identification allows demographical links to be explored amongst cohorts with specific illnesses, as well as family studies – for example, trends in abnormal gut microbiota have been noted between individuals with Crohn's Disease and their unaffected first degree relatives (Hedin, Stagg et al. 2012). Since 2008, large studies such as the European Metagenomics of the Human Intestinal Tract (MetaHIT) has been investigating links between the gut microbiota and both IBD and obesity using culture-independent techniques (2010). Given that individual bacterial cells are at least ten-fold smaller than human skin cells, the estimated weight of the entire human gut microbiome is only around 200 grams (Hooper, Midtvedt et al. 2002). Given the lack of interindividual similarity, and the increasingly remote likelihood of there being a 'core' human microbiome (given a mere 0.9% shared species abundance within the gut of any two individuals), charting the microbiome may be a lengthy process dependent upon stratifying for ethnicity, diseases, and cohabitation states (Hamady and Knight 2009). Nonetheless, a core metagenome may still hold, with core functions at gene level, yet diversity at 16s rRNA stage.

### **The Human Microbiome Project**

These techniques are also leading to large observational studies in healthy individuals. The Human Microbiome Project is a 5 year feasibility study funded by the USA National Institutes of Health. It aims to characterise the microbial community from five different anatomical sites (oral, skin, vaginal, gut, nasal/lung), exploring broad as well as deep sequencing of the microbiome. Similarly, this is also the focus of several research groups with interests in familial links. Tandem research on the gut microbiome of identical twins is flourishing, and expertly placed to attempt to identify environmental versus hereditary microbiome associations. Turnbaugh et al have produced much work on this topic, identifying shared core microbiomes in monozygotic twins through deep sequencing, and

further mining the gut microbiota in lean and obese twins (Turnbaugh and Gordon 2009, Turnbaugh, Hamady et al. 2009). Other projects examining monozygotic versus dizygotic twins have also indicated significant similarity in the former, albeit within the constraints of TTGE analysis, comparing only bands yielded and not strains identified (Stewart, Chadwick et al. 2005). Turnbaugh et al also in 2010 published their findings of marked differences in the microbiome of adult identical twins, strongly suggesting that some external environmental factor alters the microbiota very early on in life – and alters it permanently (Turnbaugh, Quince et al. 2010). When combined with the spectrum of bacterial metabolic and immunological functions, correlations between microbiota composition, species, genome, and intensity seem limitless.

#### 1.4.2) Methods of Identification:

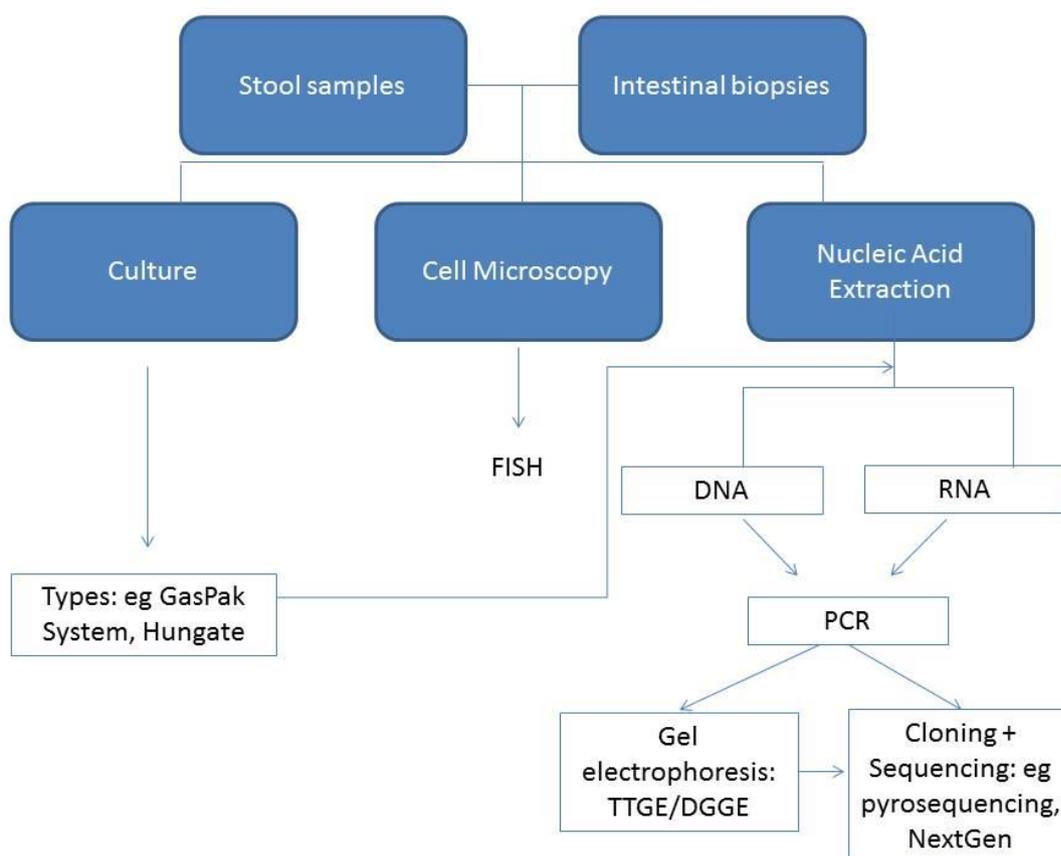


Figure 4: Culture and culture-independent methods of gut microbiota identification and quantification, illustrating the continued relevance of culture as both independent identification, as well as substrate for further molecular data. (Abbreviations: DNA = deoxynucleic acid; RNA = ribonucleic acid; PCR = polymerase chain reaction; TTGE = transient temperature gel electrophoresis; DGGE = denaturing gradient gel electrophoresis)

### **i) Culture**

A major limitation in the study of gut microbiota has been difficulty in culturing these organisms, requiring the development of molecular techniques. In 1974 an estimated 93% of all faecal microbiota were considered 'culturable' according to published data. By 1999, however, it was evident through the use of molecular methods of identification that an estimated 60-80% of gut microbiota are 'unculturable' in conventional anaerobic laboratory conditions (Rambaud and Buts 2006). Despite the prolific number and variation in molecular methods, culture methods are not obsolete, and still form a significant proportion of published work on gut microbiota (Bjorkstrom, Hall et al. 2009). Continuing culture-based methods becomes of particular importance given its ubiquity in clinical practice, since hospital microbiology departments within the NHS are still hugely reliant on culture as a first line investigation.

### **ii) Culture-independent identification**

#### *Molecular Methods of Analysis: Identification of Species Diversity and Prevalence*

The evolution of molecular biology is becoming the key to evolution of our understanding of the gut microbiota, its acquisition, and development in the early stages of life. While great interindividual variation makes the identification of a full microbiota 'genome' a slow process (Turnbaugh, Hamady et al. 2009), new bacterial strains are being identified on a regular basis in premature infants (Jacquot, Neveu et al. 2011, Arboleya, Ang et al. 2012). Molecular methods based on the 16S ribosomal RNA gene (rDNA) are particularly sensitive, given its greater hybridisation potential for primers on conserved regions. Such conserved regions act to anchor gene targets closely to hypervariable regions, as such making them good targets for phylogenetic profiling. Once extracted, DNA can be amplified by PCR to make multiple copies in order to be defined by fingerprinting techniques. These include Denaturing or Temperature Gradient Gel Electrophoresis (DGGE/TGGE), which separates the dominant bacterial consortia into 'bands' within a strip of gel, known together as a 'community profile'. These bands are then cross-referenced to control bands of known species catalogued within clone libraries. However, gel electrophoresis does not directly identify microbes, and while intensity of each band correlates with abundance of the strain or species, hybridisation techniques (including Fluorescent In-Situ Hybridisation [FISH] and qPCR) allow precise quantification of each known species. A newer comprehensive method has managed to combine both qualitative and quantitative analysis of the community profile. Whole genome pyrosequencing can run vast numbers of genetic sequences, becoming quantitative once enough sequences have been revealed in order to limit sampling error (Sundquist, Ronaghi et al. 2007). High

throughput sequencing techniques are expanding rapidly, from multiple sources, to whole genome reads. However, the vast amount of data generated requiring specialist bioinformatics support is a limitation of this approach. A major benefit of molecular work, conversely, is the wealth of statistical analyses available concerning species type, acquisition and growth, which is becoming increasingly accessible with ever-diminishing costs.

## **1.5) Acquisition of Gut Microbiota:**

### **1.5.1) Influencing the Infant Microbiota Perinatally**

Management of the neonatal gut and nutrition begins in utero, with even small changes at this time potentially following the individual into adulthood (Barker 2001). The progression of establishment of the gut microbiota will be discussed in fetal, infantile (preterm and term) and adult life. By the age of 2 years, both qualitatively and quantitatively the gut microflora has reached maturity, changing very little during the remainder of childhood and adulthood in healthy individuals (Agans, Rigsbee et al. 2011, Guarino, Wudy et al. 2012, Isolauri 2012). However, as molecular methods of identification expand, it is likely that differences will eventually become apparent.

#### ***i) In utero effects: maternal dietary pre- and probiotic supplementation***

The diet of mothers during pregnancy should take into account not just her nutritional needs but also those of her fetus (Ota, Tobe-Gai et al. 2012). For instance, periconceptual folate has been shown to prevent neural tube defects (Lane 2011). Energy supplementation can reduce the prevalence of low birth weight (Ota, Tobe-Gai et al. 2012). Iron supplementation will reduce the risks of anaemia (Pena-Rosas, De-Regil et al. 2012). The effect of antenatal pre- and probiotics is only beginning to be investigated. New evidence is challenging the widely held belief that the gut of the fetus remains sterile until colonisation once the maternal membranes have ruptured. In 2008, Isolauri et al identified strains of *Bifidobacteria* and *Lactobacillus* species in frozen sections of placentae, from both 'cold' caesarean sections (without rupture of membranes), and vaginal deliveries (Satokari, Gronroos et al. 2009). Although this has not been replicated in other studies, Keski-Nisula et al in 1997 identified strains of *Lactobacillus* sp. in amniotic fluid specimens taken in theatre under aseptic technique (Keski-Nisula, Kirkinen et al. 1997).

One observational study qualitatively and quantitatively analysing the gut microbiota in a cohort of 50 pregnant women identified differences in gut microflora composition, plasma cholesterol levels, ferritin and folic acid levels in obese versus normal BMI women at 24 weeks gestation (Santacruz, Collado et al. 2010). So far, U.K. national Royal College of Obstetrics and Gynaecology guidelines neither recommend nor discourage pre- or probiotics during pregnancy. One study has investigated the effect of antenatal prebiotic supplementation. Shadid et al performed a randomised controlled trial administering the prebiotics galactooligosaccharide (GOS) and long chain fructooligosaccharide (lcFOS) to pregnant women (n = 48) from 25 weeks of gestation until delivery. None of the infants were of low birth weight, although one was preterm (Shadid, Haarman et al. 2007). With the use of qualitative FISH analysis and quantitative PCR, they concluded that although supplementation increased the maternal faecal bifidobacterial counts, it had no effect on neonatal carriage of *Bifidobacteria*, although individual strains were not assessed. A few studies have assessed the effect of maternal probiotic supplementation during pregnancy on subsequent neonatal colonisation in term infants (Thum, Cookson et al. 2012). One study illustrated temporary infant colonisation (n=6) with *Lactobacillus GG* in samples taken at one, six, twelve and twenty-four months of age, despite stopping supplementation at delivery, and despite the disappearance of maternal faecal *Lactobacillus GG* by one month postpartum (Schultz, Gottl et al. 2004).

Other studies have, however, demonstrated potential immunological benefits of antenatal probiotic supplementation to the term neonate (Kopp, Goldstein et al. 2008, Prescott, Wickens et al. 2008). One illustrated a rise in immune factors such as IFN-gamma at term, although the clinical relevance of this is unknown (Prescott, Wickens et al. 2008). Similar work relative to immune factors implicated in childhood atopic eczema have not illustrated *in utero* benefits of antenatal probiotic supplementation (Boyle, Ismail et al. 2011). The hypothesis that antenatal probiotic administration lowers infant blood pressure at six months of life was not supported by the evidence (Aaltonen, Ojala et al. 2008). A recent Cochrane review concluded that no reduction in the incidence of preterm labour with antenatal probiotic supplementation could be shown, despite the theoretical reduction in bacterial vaginosis, a relatively common cause of premature labour (Othman, Neilson et al. 2007). Several studies have investigated the possibility of desensitisation of term infants of atopic mothers given probiotic therapy during pregnancy and lactation, but because of the postpartum continuation of therapy, this effect cannot be assumed to originate *in utero* (Abrahamsson, Jakobsson et al. 2007, Huurre, Laitinen et al. 2008, Kopp, Goldstein et al. 2008). To date, no studies have explored antenatal diet and pre- or probiotic consumption

with subsequent gut colonisation in preterm infants, and data concerning only one preterm infant of normal birth weight (36.4 weeks and >2.5Kg) could be extrapolated from existing work (Gronlund, Grzeskowiak et al. 2011).

## ii) **Establishment of the Microbiota at Birth: Beneficial Bacteria Versus Enteropathogenic Micro-organisms**

The main wave of perinatal gut colonisation is thought to occur once the maternal membranes have ruptured (Magne, Suau et al. 2005). Primarily, bacteria from the maternal birth canal, skin, and colon transfer to the infant after delivery, and are acquired by the gut in a cranio-caudal fashion owing in part to the wave patterns of peristalsis (Blakey, Lubitz et al. 1982). Initially aerobic species thrive, particularly *E. coli* and *Streptococci*, and create an environment conducive to subsequent colonisation by anaerobic species by consuming oxygen, mainly *Bifidobacterium*, *Clostridium* and *Bacteroides* (Edwards and Parrett 2002). Unsurprisingly, the proportion of gut anaerobes increases with distal anatomical location, given that the oxygen partial pressure in the colonic mucosa is less than a quarter of room air oxygen concentration. The resultant anaerobic microbial activity reduces redox potential in the distal gut of between -200 mV to -300 mV (Schroeder, Wu et al. 2011). Species and quantities of bacteria vary both according to anatomical gut location, and situation either within the lumen or on the mucosa.

## iii) **Ex Utero Influences: Nutrition and Environment**

As discussed later, various perinatal factors are known to alter establishment of the microbiota, including gestation, method of delivery, intra- and postpartum antibiotics, and, theoretically, incubator care (Edwards and Parrett 2002, Schumann, Nutten et al. 2005, Penders, Thijs et al. 2006, Fallani, Young et al. 2010). However, the greatest and most studied effect is that of diet.

### a) **Nutrition**

It is well documented that exclusively breast-fed term infants have a predominance of lactic acid-producing bacteria (mainly *Bifidobacterium* and *Lactobacilli* sp.), becoming established by day seven in healthy term infants delivered by spontaneous vaginal delivery (Rambaud and Buts 2006, Bezirtzoglou, Tsiotsias et al. 2011, Isolauri 2012, Turrone, Peano et al. 2012). *Lactobacilli* are gram positive rods, and have several functions including bacterial adherence, and the regulation of intraluminal acidosis and mucus

binding proteins (Rambaud and Buts 2006). *Bifidobacteria*, also a gram positive rod but with a characteristic V or Y-shape, has been seen to account for up to 91% of the bacterial consortia in breast fed (and up to 75% in formula fed) term infants (Harmsen, Wildeboer-Veloo et al. 2000). Members of this genus are thought to activate dendritic cells to produce interleukin 10, and also augment the function of immunoglobulin E (Ewaschuk, Diaz et al. 2008). Maternal milk confers many benefits to the microbiota that have proven difficult to reproduce in commercially available formulae. The bifidogenic effects of human milk are well recognised, including lowering colonic pH (which also functions as a hostile environment for enteropathogens), and providing natural ‘prebiotics’ in the form of human milk oligosaccharides, which provide an alternate energy source. Although the iron content of human milk is lower than in formulae, its bioavailability is much higher, thus maintaining infant stores but depriving *Bacteroides* and *Enterobacteria* species of utilising it for their growth. This effect is augmented by the presence in maternal milk of lactoferrin, which binds any unabsorbed iron thus rendering it unavailable to such bacteria in the colon (Butler 1979). Additionally, secretory immunoglobulin A (SIgA) and lysozyme found exclusively in breast milk also inhibit the growth of various enteropathogens (Brandtzaeg 2003, Groer, Davis et al. 2004).

Exclusive breast feeding rates by 3 months of age vary hugely within Europe, from over 90% in Hungary, to barely 10% in the UK (OECD 2009). The UK continues to have one of the lowest breast feeding rates in Europe, as reported by the 2010 UK Infant Feeding Survey from the Office of National Statistics, and by the age of 6 months only 1% of infants were exclusively fed, despite the UK Department of Health recommendation to exclusively breast feed until 6 months of age (McAndrew 2012). The complex constellation of reasons for the elective discontinuation of exclusive breast feeding by mothers include the need to return to work before 6 months, reliance on cultural and religious observances, and social acceptability (Li, Fein et al. 2008). Paradoxically, it remains the case that mothers of lower social class in developed countries are less likely to breast feed – despite the obvious financial savings associated with breast feeding (McDonald, Pullenayegum et al. 2012). As such, multiple interventional trials aimed at encouraging the establishment and continuation of breast feeding have now merited meta-analysis (Jolly, Ingram et al. 2012). According to the United Nations International Children’s Emergency Fund (UNICEF), early breast feeding is associated with lower neonatal mortality in developed and developing countries (Oddy, Kendall et al. 2003, Oddy, Sly et al. 2003).

However, within the last 15 years an important discovery of a particular additional commodity within breast milk has come to light with the advent of molecular methods of anaerobic bacterial identification. The identification of naturally occurring known *Bifidobacteria* and *Lactobacillus* strains within breast milk raise the distinct possibility that it can also function as a probiotic supplement (Fernandez, Langa et al. 2012). This is a curious finding considering that these strains are strict anaerobes, and thus unable to survive passage to the aerobic environment of the areola. However, the biofilm theory serves as an explanation for the ability of these anaerobes to thrive. Previous studies have illustrated the bacterial production of a surrounding ‘biofilm’ an aggregate of bacteria upon a surface (e.g. the mammary ductules, or gut mucosa) from which a matrix of extracellular polymeric substances (EPS) are produced. Such EPS are designed to promote the properties of the bacteria within as they are shielded from external threats and thus able to cooperate in different manners in order to thrive (Kleessen and Blaut 2005, Macfarlane, Bahrami et al. 2011). For enteropathogens, biofilm formation is therefore of great advantage since they are resistant to many antibacterial agents (Smith, Perez et al. 2009, Ramage, Culshaw et al. 2010). Biofilm formation is ubiquitous throughout the eukaryotic world, and recent research indicates that biofilms produced by *Lactobacillus* strains go on to express anti-inflammatory and anti-enteropathogenic factors (Spinler, Taweechoitipatr et al. 2008, Jones and Versalovic 2009). However, biofilm formation within the mammary ductules may also be associated with pathogens implicated in mastitis (Delgado, Arroyo et al. 2009, Gutierrez, Martinez et al. 2012). The source of these bacteria are the subject of paired fecal/breast milk studies in lactating women, indicating that the enteromammary circulation may facilitate the transport of these bacteria from gut to gland (Albesharat, Ehrmann et al. 2011, Urbaniak, Burton et al. 2012). This is already the subject of several studies noting that *Bifidobacteria* and *Lactobacillus* species from the gut microbiota are seen to translocate to other mucosal surfaces, as well as into the bloodstream (Kochan, Chmielarczyk et al. 2011, Strus, Chmielarczyk et al. 2012). Similarly, animal models illustrate the ability of *Lactobacillus* species to effect extra-intestinal immunological processes (Marranzino, Villena et al. 2012). In 2012, these studies and implications therein were discussed in our leading article ‘Mothers, Babies and Friendly Bacteria’, published in Archives of Disease in Childhood from which the ‘Beattie and Weaver Postulates’ were defined, encapsulating the consequences of this discovery. As further strains have been identified, satellite studies are testing the properties of these strains for their commercial probiotic potential (Olivares, Diaz-Ropero et al. 2006, Diaz-Ropero, Martin et al. 2007, Beattie and Weaver 2011).

### *The perfect 'synbiotic': breast milk*

Human milk is 'synbiotic', containing both prebiotics (oligosaccharides) that promote the growth of probiotics as well as protect against infection, and probiotics (live microorganisms of benefit to the host) (Araya 2001). Human milk oligosaccharides (HMOs) in human milk have been reported at levels of up to 15g/L (Rambaud and Buts 2006). There are a growing number of studies identifying natural 'probiotic' bacteria resident in human milk (Martin, Jimenez et al. 2006, Gueimonde, Laitinen et al. 2007, Martin, Heilig et al. 2007), assessing mother-infant pairs at term, as well as the placenta, reinforcing a 'horizontal' method of transmission (Gueimonde, Sakata et al. 2006, Martin, Jimenez et al. 2006, Satokari, Gronroos et al. 2009). It is postulated that these strict anaerobes are allowed to thrive in the breast owing to mammary changes that occur with increasing gestation, such as increased blood flow and hypertrophy that are conducive to the creation and maintenance of a 'biofilm' enabling bacterial adherence. The importance of maternal milk is further highlighted by the ingenuity of the enteromammary circulation, a cycle that integrates the maternal immune response to enteropathogens with resultant protection of her nursing infant against those bacteria specifically. When the mother ingests an enteropathogenic bacteria, Peyer's patches in the small intestine secrete activated B lymphocytes that are transported in the blood to the mammary glands, where, as plasma cells, they secrete pathogen-specific neutralising antibodies into milk (Butler 1979, Van de Perre 2003). The method of transmission of non-enteropathogenic endogenous 'probiotic' bacteria between mother and nursing infant is debatable, but recent work suggests that there is a cycle of transmission, from mother's milk to baby and back again, and that skin lactic-acid bacteria do not contribute to the neonatal flora (Martin, Heilig et al. 2007).

Conversely, those fed exclusively formula milks have greater numbers of potential enteropathogens, including *Bacteroides*, *Clostridia* sp., and *Enterobacteriaceae*. On the contrary, very little is known about the gut flora of those who are fed both maternal and formula milks (i.e. 'mixed-fed'), and these comprise most infants in the UK (Fallani, Young et al. 2010). Weaning triggers the beginning of further change, and, once this is complete, microbiota patterns between formerly breast-fed and formula-fed term infants are less distinct. However, breast feeding is associated with less respiratory disease, gastrointestinal infections, allergy, obesity and cardiovascular disease in later life, all potentially related to microbial facilitation (Armstrong, Reilly et al. 2002, Rudnicka, Owen et al. 2007, Parikh, Hwang et al. 2009, Plenge-Bonig, Soto-Ramirez et al. 2010, Risch 2012, Sonnenschein-van der Voort and Duijts 2012). The minimal exclusive breast feeding

rates in parts of the developed world make mixed feeding the pre-weaning reality for the majority of infants in the UK.

#### *Maternal post-partum and lactational probiotic supplementation*

Other randomised controlled trials have assessed the use of antenatal and postnatal probiotics on term neonates. Several promising trials have reported an increase in secretory IgA titres in early lactation, and an increase in neonatal protective immune factors at term such as IFN-gamma, in response to maternal probiotic supplementation (Prescott, Wickens et al. 2008). Some of these studies are confounded by breastfeeding, and the concurrent administration of neonatal probiotics, but nonetheless they show benefits to neonatal immunity and reduction in childhood atopy (Huurre, Laitinen et al. 2008). Several recent studies have correlated identical strains of lactic-acid producing bacteria in mother-infant pairs, analysing both milk and stool.

#### **b) Antibiotics**

Antibiotic administration, both oral and intravenous, is known to affect the gut microbiota adversely, albeit temporarily (Dethlefsen, Huse et al. 2008, Looft and Allen 2012). Evidence suggests that even a month long course of oral ciprofloxacin can make certain taxa extinct for up to 6 months (Dethlefsen, Huse et al. 2008). There is no evidence to support a greater effect on the microbiota from either oral or intravenous administration. *In vivo* work suggests that *Bifidobacteria* remain sensitive to broad spectrum antibiotics, including penicillins, macrolides, and vancomycin, but that around 70% of isolates are resistant to fusidic acid, and metronidazole (Moubareck, Gavini et al. 2005, Zhou, Pillidge et al. 2005). *Lactobacillus* sp. is known to be resistant to vancomycin, an antibiotic commonly used in the treatment of coagulase negative *Staphylococcus* sepsis. So far, this natural resistance would appear to occur via a route unrelated to that of Vancomycin Resistant *Enterococci* (VRE) (Patel 2000). Controversy exists as to whether probiotics require antibiotic resistance in order to survive in the gut, and, notably, future issues may concern transferable resistance to other enteropathogenic bacterial strains (Salminen, von Wright et al. 1998, Borriello, Hammes et al. 2003). Many conditions in which disturbance of gut microbiota are implicated in aetiology and pathogenesis, and thus for which pre and/or probiotic supplementation would be potentially advantageous also require regular courses of antibiotics, both orally and intravenously administered. These in turn further disturb the natural gut ecosystem, and are of particular importance in conditions for which antibiotic administration paradoxically can make some symptoms worse (for example

aggravation of diarrhoea in Crohn's Disease, urticarial rashes in atopy) (Choi, Han et al. 2010, De Vroey, De Cassan et al. 2010, Khan, Ullman et al. 2011).

Although antibiotic transmission in breast milk is well established, there is no evidence for the effects of antibiotic administration on the infant gut microbiota. For the newborn infant, antibiotics may be administered orally or intravenously for the prophylaxis of infection in high-risk infants, or the treatment of perinatal infection in infants for whom there are clinical signs. Evidence for the subsequent alteration in infant gut microbiota shows reduced numbers of *Bifidobacteria* species and generally lower microbiota species variety and intensity (Favier, de Vos et al. 2003, Fallani, Young et al. 2010, Hussey, Wall et al. 2011, Fouhy, Guinane et al. 2012). Notably, there is no evidence to suggest a link between mastitis and altered infant microbiota.

**c) Environment:**

The constant exposure of neonates to bacteria through environmental contact is an important building block for future immunity. Skin commensal transfer from mother to infant is well-recognised, as are the marked differences in the gut microbiota of neonates delivered by caesarean section, as opposed to those delivered vaginally. Those delivered surgically have a well-recognised delay in the establishment of gut colonisation for several months thereafter (Huurre, Kalliomaki et al. 2008, Biasucci, Rubini et al. 2010, Dominguez-Bello, Costello et al. 2010, Lif Holgerson, Harnevik et al. 2011, Pandey, Verma et al. 2012). This factor may have a more dominant effect on repression of the gut microbiota than other interventions around the time of delivery, including antibiotic administration to the neonate and even feed type within the first crucial week of life (Biasucci, Rubini et al. 2010).

According to the UK national statistics for birth, preterm infants were commonly delivered by caesarean section, which, as described, significantly delays the development of gut colonisation in term infants. However the effect of method of delivery is not seen to be as dominant a cause of the delay and reduction in species variety and intensity seen in the gut microbiota of infants born prematurely in comparison with those at term (Cilieborg, Boye et al. 2012). Additionally, the majority of preterm infants weighing less than 1.5Kg at birth also require incubation to maintain core body temperature. Sick term infants are also generally nursed in incubators to facilitate monitoring, nursing and medical care. This naturally reduces the 'skin-to-skin' contact and thus cross-colonisation of commensal bacteria from mother to infant. The effects of incubation upon the gut microbiota are only

beginning to be investigated, although several animal models show significant differences in young nursed with and without their parents (Benson, Kelly et al. 2010). In 2007, Butel et al emphasised the importance of environmental cross-colonisation in their observational study of *Bifidobacteria* strains found in a cohort of preterm infants. They found no significant differences in type or growth of strains according to method of delivery or gestation, but did identify *B. animalis* subspecies *lactis* in four infants from the same neonatal unit. This strain had never before been identified in human gut microbiota, but is a common commercially available probiotic strain. They postulated that environmental cross-contamination from staff, parents, and/or visitors who had consumed these products were the primary source (Butel, Suau et al. 2007). Pilot work for the UK's first probiotic RCT in preterm infants showed that at the end of the 6 week administration period, 79% of intervention infants were colonised, as well as 35% of the control group. Similarly, this level of control group colonisation was attributed to cross-contamination in the milk kitchen and through other environmental sources (Costeloe).

This finding is surprising given the strictly anaerobic nature of most such bacteria, but can be explained by two methods: firstly, that anaerobes can survive in normal air for several minutes before dying; and secondly, the fact that mere fragments of probiotic bacterial DNA can affect host gut mucosal responses (Zhang, Li et al. 2005, Adams 2010, Ou, Lin et al. 2011, Orlando, Refolo et al. 2012, Tareb, Bernardeau et al. 2013). This has further implications for other environmental aspects of neonatal unit care, including kangaroo care (skin-to-skin contact with parents), and duration of ventilation (see figure 5). Critics would however note a major confounder of this is that the meticulous ability to detect such small remains of bacterial DNA may not indicate those that affect a mucosal response – rather simply identifying a previous microbial transferred within the environment.

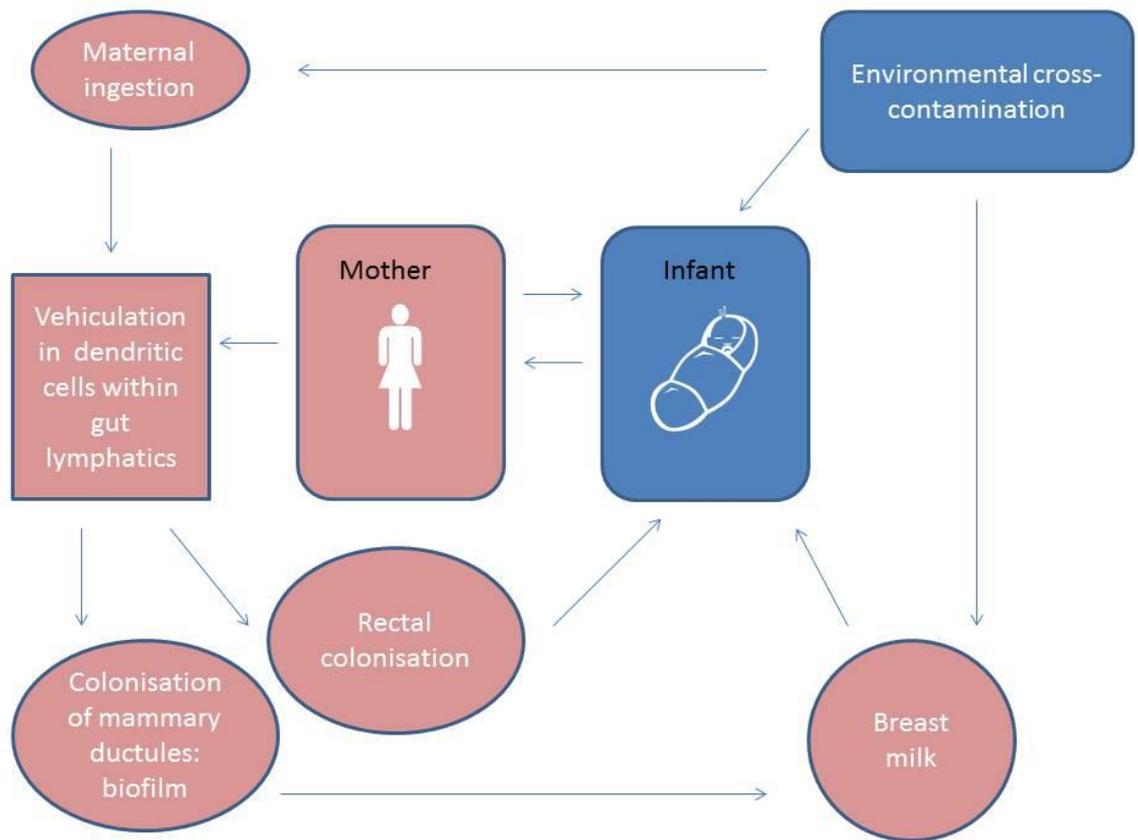


Figure 5: Colonisation patterns between mother, infant and environment

## 1.6) GUT MICROBIOTA AND PRETERM INFANTS

### 1.6.1) Demographics and Definitions

Approximately 12.27 infants are born per 1000 head of population annually in the United Kingdom, of which 92% are term (delivered at more than 37 weeks gestation), and 8% are preterm. Of those extremely preterm, 89% are less than 1.5kg in birth weight.

Parameter	Term infants	Preterm infants
Gestational age	>37 weeks	<37 weeks
Birth weight	Normal: >2500g Low: 1500 – 2499g Very Low: 1000 – 1499g Extremely low: <1000g	
Relative to gestation	Small for Gestational age: <10 <sup>th</sup> centile Large for Gestational Age: >90 <sup>th</sup> centile	
Relative to in utero growth	In Utero Growth Restriction: <10 <sup>th</sup> centile for Estimated Fetal Weight	

A breakdown of these figures for Scotland between 1998 and 2009 is shown in the table below (Information Services Division 2011).

	Absolute # delivered		Percentage			Absolute #	Percentage		
	Total	All Prem	< 1500g	1500- 2499g	2500g +		All Full Term	< 1500g	1500 – 2499g
<b>2009</b>	<b>58243</b>	4586	16.0	46.7	36.7	53657	0.0	2.5	97.4
<b>2008</b>	<b>57844</b>	4437	17.5	45.6	36.1	53407	0.0	2.6	97.3
<b>2007</b>	<b>55016</b>	4199	16.0	45.9	37.7	50817	0.0	2.6	97.3
<b>2006</b>	<b>53056</b>	4332	16.7	47.8	35.2	48724	0.0	2.9	97.0
<b>2005</b>	<b>53395</b>	4183	16.4	46.0	37.4	49212	0.0	2.7	97.3
<b>2004</b>	<b>52716</b>	4344	16.7	48.2	34.8	48372	0.0	2.9	97.0
<b>2003</b>	<b>51004</b>	3976	17.0	48.2	34.3	47028	0.0	2.8	97.2
<b>2002</b>	<b>50846</b>	3867	16.3	47.5	36.1	46979	0.0	2.9	97.1
<b>2001</b>	<b>52571</b>	4061	17.9	45.8	36.2	48510	0.0	2.8	97.2
<b>2000</b>	<b>54112</b>	4082	16.3	47.7	35.7	50030	0.0	2.8	97.2
<b>1999</b>	<b>56588</b>	4232	16.8	46.5	36.4	52356	0.0	2.8	97.2
<b>1998</b>	<b>58388</b>	4263	16.3	48.0	35.6	54125	0.0	2.7	97.3

Table 3: a) (top) Table adapted from ISD Scotland, Birth Statistics 2009, showing the proportions of preterm and very low birth weight infants delivered in Scotland through a ten year period.

International statistics show that although mortality in VLBW preterm infants has reduced significantly over the last 10 years, morbidity has maintained a plateau, mainly due to increasing numbers of surviving ex-preterm infants who are malnourished, mostly secondary to necrotising enterocolitis – one of the most devastating diseases of the gut in early life. This cohort of infants often requires tertiary paediatric gastroenterology and hepatology care (Costeloe, Hennessy et al. 2000, Fanaroff, Hack et al. 2003, Berrington, Hearn et al. 2012).

## 1.6.2) Effects of Prematurity on the Development and Composition of the Gut Microbiota

### i) *Gestation*

Prematurity and being of very low birth weight are factors associated with delayed establishment of gut bacteria, as well as the presence of fewer species present at lower abundance (Edwards and Parrett 2002, Jacquot, Neveu et al. 2011, Arboleya, Binetti et al. 2012). The reason for this is unclear, but several hypotheses exist. Firstly, until 34 weeks gestation, premature infants lack co-ordinated, effective peristalsis, due to incomplete migration of vital gut neuromotor innervation (Sanderson 1999, Pena, Parks et al. 2010). Hence this limits the 'mixing' abilities within the gut, with less opportunity for milk to be digested and presented to the epithelium, where mucus layers require agitation in order to permit the diffusion of vital nutrients. Secondly, the protective barrier functions of these mucus layers are lacking in premature infants, resulting in higher absorption of hydrogen ions (leading to systemic acidosis), and increasing the likelihood of bacterial translocation (resulting in sepsis), the effects of which are compounded by the immaturity of the premature host's systemic immune function (Vieten, Corfield et al. 2006, Sherman 2010, McElroy and Weitkamp 2011). Immature hepatic, biliary and pancreatic functions further limit both digestion and absorption, particularly of lipids (Nishiura, Kimura et al. 2010). Preterm infants, particularly those delivered before 30 weeks gestation, require small volumes of 'trophic' feeds initially: volumes that do not suffice as a sole source of hydration and nutrition, but are given to stimulate GI motility, increase feed tolerance, as well as to protect the premature gut, both by enhancing gut maturation and reducing the incidence of NEC (Bombell and McGuire 2009). This, consequentially, elongates the period of parental nutrition, and the resultant lack of breast milk delays the instillation of breast milk-related *Bifidobacteria* and *Lactobacillus*, as well as the prebiotic effect of breast milk oligosaccharides. Given the intricacies and specialised nature of bacterial analyses, published work has mainly focussed upon observational case series' with small patient numbers. Despite the advances in methods of molecular identification and quantification, relatively little is known about how these bacteria develop and function in preterm infants (see figure 6 for postulated factors affecting colonisation of the preterm newborn gut).

### ii) *Prematurity Versus Small for Gestational Age*

Term infants of low birth weight (LBW: <2.5Kg), very low birth weight (VLBW: <1.5Kg) or extreme low birth weight (ELBW: <1Kg) are at increased risk of morbidity and mortality when compared with their normal birth weight counterparts (Fanaroff, Stoll et al.

2007). VLBW infants comprise 65% of all neonatal unit admissions (Lucas 1997). Such term infants are at greater risk of gastrointestinal diseases, such as the inflammatory and infective condition necrotising enterocolitis (NEC) and doubt has recently been cast on the benefits of ‘catch-up’ growth (Simmer 2007). The role and development of gut microbiota in this cohort is poorly defined. One study has attempted to make comparisons between VLBW yet mainly premature infants with term infants of normal birth weight, noting similar initial colonisation patterns with *Streptococci* and *Enterobacteria* sp., but delayed appearance of *Bifidobacteria* in VLBW infants (Schwartz, Gruhl et al. 2003). It cannot simply be assumed that these infants have a microflora comparable to that of ‘healthy’, non-growth restricted premature infants. The terms LBW, VLBW and ELBW are often synonymous with prematurity, but it is important to note that differences in species type and timing in term growth-restricted infants may subsequently become apparent in the future.

iii) *Effect of Method of Delivery and Incubation on the Gut Microbiota of Preterm Infants*

Although in healthy term infants gut colonisation is strongly affected by method of delivery (Huurre, Kalliomaki et al. 2008), this effect is muted in premature infants, according to observational studies. Premature infants are immunologically immature, and environmental colonisation relative to the need for incubation, particularly of species such as staphylococci and yeasts, often acts as the foci of systemic infection. However, to date no studies have considered the effect of duration of incubation on the gut microflora although one has noted an association between the higher incidence of gram-negative bacterial colonisation and longer inpatient stay (Hoy, Wood et al. 2000). The importance of the environmental impact on the microflora of these infants cannot be underestimated. Little data exists correlating other clinical factors, such as duration of ventilation, with patterns of colonisation, although associations between type and duration of antibiotic courses are the subject of several observational studies (Sakata, Yoshioka et al. 1985, Sakata, Fujita et al. 1986, Penders, Thijs et al. 2006, Arboleya, Binetti et al. 2012).

iv) *Maternal environment*

Various perinatal factors are known to influence the establishment of the microbiota in preterm infants, including method of delivery, intra- and post-partum antibiotics, and the relatively sterile incubator environment in which they are cared (Edwards and Parrett 2002, Magne, Suau et al. 2005). Method of delivery seems to be less significant in premature infants, perhaps because of their higher rate of LUSCS and theoretically quicker

spontaneous vaginal delivery (Sakata, Yoshioka et al. 1985, Hall, Cole et al. 1990, Gewolb, Schwalbe et al. 1999, Edwards and Parrett 2002, RCOG 2004). However, few data exist correlating other environmental factors such as length of cot incubation and skin-to-skin maternal contact with patterns of colonisation. A recent study noted the presence of *Bifidobacteria longum* subspecies *lactis* in the faecal flora of four premature infants from the same neonatal unit. This strain has not previously been identified in premature neonates or probiotic therapy in randomised controlled trials, but is routinely used in commercially available probiotic foodstuffs, raising the intriguing possibility that consumption of these products by neonatal unit staff may actually affect the microbiota of these infants (Butel, Suau et al. 2007). This is relevant considering the importance and popularity of ‘kangaroo care’, whereby stable preterm infants have regular skin-to-skin contact with their mothers (Conde-Agudelo, Belizan et al. 2011, Karlsson, Heinemann et al. 2012). It is also a curious finding, given the necessity of the strictly anaerobic environment required for this strain to thrive (Rambaud and Buts 2006). However, given the theoretical possibility that probiotics may not need to be ‘live’ microorganisms in order to exert a beneficial effect on the host, and that ‘killed’ bacteria or even bacterial DNA may suffice and indeed, be safer – this finding may have particular consequences to neonatal care (Adams 2010).

v) *Antibiotics*

The majority of infants born at very or extreme low birth weight, and/or who are less than 35 weeks gestation are administered intravenous antibiotics, at least for the first forty-eight hours of life, although this is mainly a prophylactic process (Craft, Finer et al. 2000, Clark, Bloom et al. 2006, Tagare, Kadam et al. 2010). Inhibition of protective flora naturally leads to the proliferation of adverse bacteria, including *Clostridium* and *Staphylococci*, with associated morbidity. Unsurprisingly, several studies illustrate that the duration of antibiotic therapy after delivery is closely correlated with reduced bacterial diversity and intensity by the end of the first month of life in term infants (Sakata, Yoshioka et al. 1985, Vlkova, Nevoral et al. 2005, Mullie, Romond et al. 2006). One theory for the marked delay in *Bifidobacteria* colonisation is antibiotic sensitivity, with several studies correlating type and length of antibiotic courses with this delay (Penders, Thijs et al. 2006). Another study has illustrated the delay in detection of *Lactobacillus* in infants receiving simple penicillin during the first four days of life (Sakata, Yoshioka et al. 1985). Disturbingly, antibiotic therapy may promote the development of antibiotic resistant opportunistic enteropathogens (Morelli, Cesena et al. 1998, Salminen, von Wright et al. 1998, Saarela, Mogensen et al. 2000, Bonnemaïson, Lanotte et al. 2003).

vi) *Nutrition*

As with those at term, it is postulated that colonisation of premature infants is heavily influenced by the type of milk fed: maternal, donor, or formula. Exclusively breast-fed term infants have a predominance of beneficial lactic acid-producing bacteria (mainly the facultative anaerobes *Lactobacilli* and *Bifidobacterium* sp.). Conversely, term infants fed exclusively formula milks have greater numbers of potential enteropathogens, including *Bacteroides*, *Clostridia* and *Enterobacteriaceae* (Edwards and Parrett 2002, Magne, Suau et al. 2005). However, this difference is less marked in preterm infants, and no studies have sought to document the microbiota of those mixed-fed, despite this being a reality for most preterm infants in the first weeks of life (Schanler, Lau et al. 2005, Maayan-Metzger, Avivi et al. 2012).

Of the seven studies identified that examined the microflora of premature infants, only two considered comparisons between those exclusively breast or formula milk fed. In both instances patient numbers were too small to identify significant differences. No studies have analysed the microbiota in solely mixed fed premature infants. Although Schwartz et al compared the gut flora of premature neonates with exclusively breast fed term infants, no mention was made of the premature infants' feed regimen (Schwartz, Gruhl et al. 2003). Given the reality of considerable difficulties in maintaining breast milk supplies for premature infants, most are therefore fed a mix of breast and formula milks. Breast milk from mothers of preterm infants is known to be of more dilute, less calorific and with a lower fat content. However, whether there are differences in the nature and quantity of probiotic bacteria in preterm breast milk are yet to be ascertained. However, it is likely that the acquisition of probiotic bacteria in breast milk is gestation-dependent.

a) *Donor Expressed Breast Milk*

The development of an alternate microbiota is an increasing possibility with the advent of breast milk donation. Donor Expressed Breast Milk (DEBM) banks are increasing, both in number and supply. Seventeen such milk banks exist throughout the United Kingdom, providing premature infants from the country's 250 Neonatal Units with around 5000 litres of Donor Expressed Breast Milk annually (UKIAMB 2013). The effect of this type of milk on gut colonisation has not yet been studied, but is likely to be highly variable, since donors tend to be a mix of term and preterm mothers, at different time points after delivery. All DEBM in the UK undergoes pasteurisation and frequent microbial testing to ensure sterility from bacterial and viral enteropathogens. Studies examining the safety aspects of treatment and handling of DEBM have focussed mainly upon viruses, such as HIV,

hepatitis B, and cytomegalovirus. However, no studies that focus upon the effect of pasteurisation of milk, including ultrasound and flash heating, on its 'probiotic' bacterial content can be identified in extensive literature searching. Holder Pasteurisation (heating at a minimum of 62.5<sup>0</sup>C for 30 minutes) affects the bioavailability of nutritional and immunological components, and is a controversial practice that is by no means universal (Stein, Cohen et al. 1986, Modi 2006, Andersson, Savman et al. 2007). The omission of bacteriological analysis of human milk 'probiotics' before and after pasteurisation is important, given the propensity for DEBM to lower the incidence of necrotising enterocolitis and neonatal sepsis in several systematic reviews and meta-analyses (Quigley, Henderson et al. 2007, Schanler 2007, Sullivan, Schanler et al. 2010). The correlation between the pasteurisation of DEBM and lower incidence of NEC in preterm infants consuming donor milk requires further analysis of the gut microflora to explain. However, probiotic bacteria need not be 'live' in order to exert an effect on the host (Adams 2010). Toll-like receptor 4 (TLR4) is a protein cell receptor found in leucocytes and the placenta that plays a key role in pathogen sensing and subsequent immune activation. Evidence suggests that TLR4 receptors can be stimulated by fragments of bacterial DNA – one of the key effects of vaccination (Villena, Suzuki et al. 2012). TLR4 failure is also associated with NEC (Hackam, Good et al. 2013). *In vivo* studies of heat-killed lactic acid producing bacteria note their immunomodulatory properties, particularly in the production of interferon alpha and a host of interleukins. Recent research also suggests an antiproliferative and proapoptosis effect, of significance in cancer research (Ou, Lin et al. 2011, Orlando, Refolo et al. 2012), but so far these are only evident from animal models and *in vivo* work. In addition, routine pasteurisation of DEBM for preterm infants may not be required for virological purposes, given that all donors are screened for HIV and hepatitis viruses. However long-term follow-up studies of ex-preterm infants who have suffered neonatal CMV infection show poorer long term adverse motor effects into childhood than ex preterm infants without CMV infection, although still in the normal range (Bevot, Hamprecht et al. 2012, Resch 2012). Indeed, in term infants CMV exposure is often referred to as a 'natural immunisation' (personal communication, A Williams). Alternatively, with the combination of donor milk screening and questionnaires, it may be prudent to test donor milk for bacterial enteropathogens and CMV only, and pasteurise if significant growth. It is ironic that the United Kingdom Association of Milk Banks guidelines for handling human milk apply only to donated and not maternal human milk. In addition, there is no consensus on sterilisation of equipment used to express milk for women lactating at any gestation.

b) *Maternal Postnatal Probiotic Supplementation*

Although, as described earlier, a number of RCTs have explored the effect of administration of probiotics to postpartum lactating mothers, none of these trials include preterm infants, although one study, currently recruiting in Israel, is assessing the effect of lactational probiotics on preterm infants (Dollberg 2010).

**1.6.3) Evidence for gut microbiota species diversity and abundance in preterm infants**

In extensive systematic literature searching, utilising the SIGN methodology (Network 2013), only seven studies exclusively considering gut microbiota in VLBW preterm infants were identified (see Table 4). These studies also suggested that pathogenic bacteria (such as *Clostridia*, *Staphylococcus*, *Pseudomonas* and *Klebsiella*) appear sooner than beneficial ‘probiotic’ strains in premature infants. Given difficulties in sampling, few studies have compared microflora at different levels of the gastrointestinal tract in premature infants without NEC, although one study found low levels of bacteria in the stomach similar to that of the faecal microbiota, and one further study examined microflora solely from the duodenum of premature infants via nasoduodenal tube placement (Kraeft, Roos et al. 1985, Hoy, Wood et al. 2000).

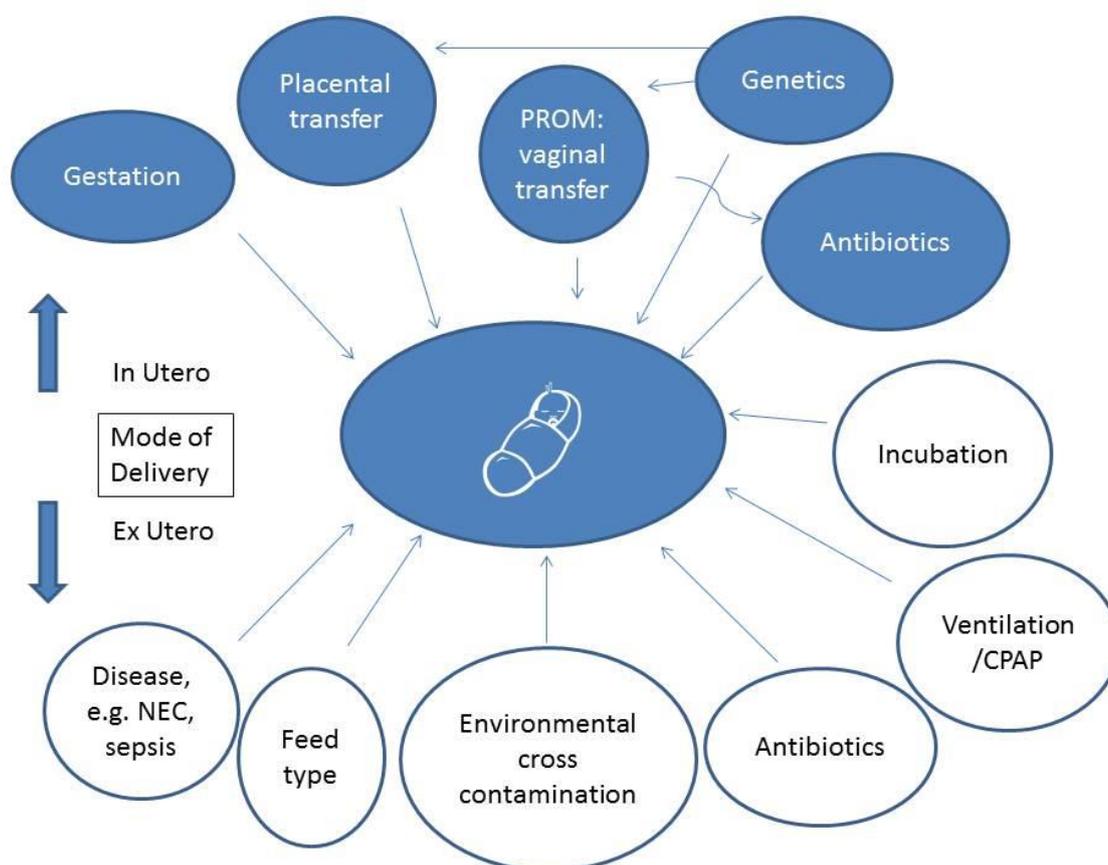


Figure 6: In utero and ex utero factors affecting gut colonisation in preterm infants (Abbreviations: NEC = necrotising enterocolitis; CPAP = continuous positive airway pressure; PROM = prolonged rupture of membranes).

In order to explore the evidence base for these *in utero* and *ex utero* influences on the gut microbiota of preterm infants, I undertook a systematic literature review at the beginning of my research period (2009). This was updated before thesis submission (2013). Pubmed and Medline were searched for all titles concerning the identification of stool bacteria in preterm infants (< 37 weeks gestation), regardless of culture or culture independent method. MeSH keywords and combinations therein were: preterm; gut bacteria; microflora; microbiota; stool. All abstracts were reviewed, and those without associated full publication, those of evidence level four (expert opinion) and those that were not translated into English were excluded. This left ten articles which were assessed in conjunction with the Scottish Inter-Collegiate Guideline Network methodology (Network 2013).

Table 4: Evidence Base for Components of and Factors Influencing the Gut Microbiota of Preterm Infants Without NEC (Blakey, Lubitz et al. 1982, Stark and Lee 1982, Rotimi, Olowe et al. 1985, Sakata, Yoshioka et al. 1985, Hall, Cole et al. 1990, Gewolb, Schwalbe et al. 1999, Schwiertz, Gruhl et al. 2003, Magne, Abely et al. 2006, Chang, Shin et al. 2011, Barrett, Guinane et al. 2013)

Study, Journal + Evidence Level	Year	n =	Gestation in weeks (median, range)	Birth weight (g)	Feed Type	SVD	Antibiotics given	Incubation period	Postnatal age of sample(s)	Methods + samples	Stool bacteria identified	Main results (p<0.05)
Stark PL J Hygiene (Lond) EL 3	1982	11	33 (30-35)	1920 (1440-2300)	All DEBM	NS	None	NS	3-6 specimens during week 1; thereafter weekly for inpatient stay	Culture Stool samples	<i>Bifido</i> ; <i>bacteroides</i> ; <i>Clostridia</i> ; G+ <i>anaerobes</i>	High counts anaerobes; delayed <i>Bifids</i> in prems
Blackey JL J Med Microbiol EL 3	1982	28	30 (25-36)	1125 (560-1500)	17 EBM/D 2 F 1 TPN	NS	23	NS	Twice weekly for 3 weeks	Culture Throat, gastric aspirate, stool, blood	<i>Bacteroides</i> , <i>E.Coli</i> , <i>clostridia</i> , <i>lactobacillus</i> , <i>staph aureus</i> , <i>klebsiella</i>	↓ colonisation if antibiotics or PN. No <i>Lactobacillus</i> if antibiotics
Rotimi VO J Hygiene (Lond) EL 3	1985	23	29.01 (24-36)	1728 (750-2400)	9 E/F 2 F 12 NS	12	15	NS	Days 1, 2, 3, + 6	Culture Swabs of mouth, umbilicus + rectum	<i>E.Coli</i> , <i>strep faecalis</i> , <i>staph epi</i> , <i>candida</i> , <i>klebsiella</i> , <i>bifido</i> , <i>bacteroides</i> , <i>clostridia</i>	↓ colonisation with LUSCS; High G-bacteria, esp. <i>Clostridia</i>
Sakata H Eur J Ped EL 2	1985	7	29.5 (25.4-34.7)	810-1350	All E/D	3	2	NS	Days 1-7	Culture Stool samples	<i>Enterococci</i> , <i>strep</i> , <i>staph</i> , <i>bifido</i> , <i>lactobacillus</i> , <i>bacteroides</i> , <i>clostridium</i>	Longer period <i>enterococci</i> + <i>strep</i> than term; Delayed <i>bifids</i> + higher <i>staph</i> in prems
Hall MA ADC EL 2	1990	46	32 (25-33)	1440 (620-2510)	23 EBM/D 5 E/F 14 F	18	30 (71)	36 days (86)	10 days + 30 days	Culture + gas liquid chromatography Stool only	<i>Coliforms</i> > <i>Lactobacilli</i> > <i>Bifidobacteria</i>	↓ <i>Lactobacilli</i> if incubated +/-or antibiotics
Gewolb IH ADC EL 2	1999	29	E 26.4 (2.2) F 26.0 (1.8)	E 814 (117) F 747 (136)	E 15 F 14	E 9 F 9	E 18.5 (5.5) d F 21.4 (6.6) d	NS	Days 10, 20 + 30	Culture Stool samples	<i>Enterococcus faecalis</i> , <i>E.coli</i> , <i>Staph epidermidis</i> , <i>Enterbacter cloacae</i> , <i>Klebsiella</i> + <i>Staph haemolyticus</i>	E: ↑ colonisation with time <i>Lacto</i> and <i>Bifido</i> in only 1 infant Colonisation delayed in all with ↑ antibiotic duration
Schwartz A Pediatri Res EI 2	2003	29	24-37	830-2635	All E/D	11	29	'At least 4/52'	Days 1-14, 17, 21, 24, 28	PCR-DGGE (16s rRNA)	<i>E.Coli</i> , <i>enterococcus</i> , <i>klebsiella</i>	High interindividual similarity prems

												over time, due to environmental cross contamination
Magne F FEMS Microbiol Ecol EL 3	2006	16	28.5 (27-36)	1265 (640- 2300)	E 3 F 6 E/F 7	6	10	'At least 4/52'	NS – 1 sample per infant	PCR-TTGE + 16s rRNA	<i>Enterobacter</i> ; <i>enterococcus</i> , <i>strep</i> , <i>staph</i> . <i>Bifids</i>	High interindividual variation; 3.25 species (mean) per infant; low bifids.
Chang JY JPGN EL 3	2011	10	29+3 – 34+3	970-1495	E: 5 M: 5	0	All Range: 2-7 days	NS	72 hours, 2 weeks, and 1 month	16s rRNA pyrosequencing	<i>Gammaproteob</i> <i>acteria</i> , <i>bacilli</i> , <i>clostridia</i> , <i>bacteroides</i> , <i>Escherichia</i> , <i>enterobacter</i> , <i>enterococcus</i> , <i>veillonella</i> , <i>serratia</i> , <i>staph</i> , <i>roseburia</i>	21.9% species unclassified; <i>Clostridia</i> and <i>Bacteroides</i> ubiquitous 72 hours after birth; Pathogenic bacilli present at all stages
Barrett E ADC EL 3	2013	10	27-31	NS	E 7 E/F 2 E/D 1	2	10	NS	Days 10-14, then day 24- 28	16s rRNA pyrosequencing stool samples	Phylum/family/ genus of: <i>proteobacteria</i> ' <i>Firmicutes</i> , <i>Bacteroides</i> , <i>acinobacter</i> ; <i>bacteroides</i> , <i>clostridia</i> , <i>staph</i> , <i>enterococcus</i> , <i>bifids</i> , <i>lacto</i> .	Lack of <i>Bifids</i> and <i>Lacto</i> ; large interindividual variation in prems – more so than previously reported. Huge variation in proportion phyla/families/ genera.

Abbreviations: E = Expressed Breast Milk; D = Donor Expressed Breast Milk; F = Formula;

Table 4: Evidence Base for Components of and Factors Influencing the Gut Microbiota of Preterm Infants without NEC; 5 were of EL 2, and 5 of EL 3. Six comprised culture and 4 culture independent methods. All considered samples within the first month of life. As publications progressed chronologically, so the interindividual variation widened.

This heterogeneous group of studies include four case-control series, six case series', but no randomised controlled trials, published over a period of 21 years. Notably, as molecular methods have expanded, so have the number and type of phyla, families, and genera identified in preterm infants. Although the range of gestations and birth weights is wide, all sampling has occurred within the first month of life. There is congruence in the consistent finding of a delay in the appearance of *Bifidobacteria*, and dominance of anaerobes and gram negative bacteria. As the molecular methods used advance, however, the interindividual variation appears to increase in each cohort.

#### **1.6.4) Evidence for Normative Data in Stool Metabolites, Inflammatory and Immunological Markers of Gut Health in Preterm Infants**

##### *i) Variation in Stool Bacterial Metabolites in Healthy Preterm Infants*

The role of Short Chain Fatty Acids (SCFAs) as the products of bacterial fermentation of undigested carbohydrate in the colon in preterm infants in health and disease is under much dispute, given the ‘butyrate paradox’ (Kien 1996), whereby butyrate has been observed to have both detrimental and therapeutic effects. SCFAs are present at birth from the primary metabolism of lipids, and are generally seen to rise after feeding is commenced, but the effect of other environmental and specific nutritional differences has not yet been investigated. Preterm infants illustrate ineffective and uncoordinated peristalsis, leading to a surfeit of undigested milk, which theoretically acts as a substrate for bacterial fermentation of complex carbohydrates and protein to short and branched chain fatty acids. Trends may therefore emerge in future studies to signify the effect of probiotics and differences in feed regimen in preterm infants on their SCFA profiles.

In order to investigate the relevance of stool SCFA analysis to neonatology, I undertook a literature search (using combinations of the MeSh key words: short chain fatty acid; branched chain fatty acids; neonate/infant; preterm; stool; faecal; necrotising enterocolitis; butyrate) into Pubmed and Medline. Major reviews in the area were also cross-referenced. Levels of evidence were appraised using the SIGN guideline methodology ([www.sign.co.uk](http://www.sign.co.uk)), excluding EL four (expert opinion), abstracts without publication, and papers not translated into English. This was performed at both the beginning of my research period (October 2009) and then updated before thesis submission (Anyon and Clarkson 1971, Kien, Liechty et al. 1990, Stansbridge, Walker et al. 1993, Favre, Szylit et al. 2002, Wang, Shoji et al. 2007, Mohan, Koebnick et al. 2008, Underwood, Salzman et al. 2009).

Study, Journal + Evidence Level	Year	n =	Gestation (weeks)	Birth weight (g)	SVD	Incubation	Antibiotics	Feed type	Postnatal age of samples	Samples	Methods of analysis	Results
Anyon CP Aust Paediatric J EL 3	1971	10	<37	2.5Kg	NS	NS	NS	Cow's milk/water, then ↑ratios sucrose	Days 5-7, 10-15, and 20-22	Stool	GC, clinitest + lactate method	Age related ↓ acetate and butyrate
Kien CL Gastroenterology EL 1	1990	15	28-32	'Weight and length >10 <sup>th</sup> centile'	NS	NS	NS	7: LAC 8: GP	2-4 weeks – '4 day excreta period'	Stool samples	GLC	LAC = ↑acetate + total SCFA 90% fecal carbohydrate energy from bacterial fermentation
Stansbridge EM ADC EL 1	1993	20	Gp A: 30.5 (26-33) Gp B: 30 (24-33)	Gp A: 1445 (800-2560) Gp B: 1500 (830-2150)	12	NS	15	17: EF 3: F	Days 7, 14, 21, 28, 35 'or adjacent days'	Stool and urine	GC	'No detrimental effects' upon SCFAs in probiotic group: no significant differences
Favre A JPEN EL 2	2002	28	Gp I: 34 (1.12) Gp II: 34 (0.89) Gp III: 31 (0.7)	I: 1953 (381) II: 2038 (257) III: 1453 (229)	NS	NS	NS	33-37: E 33-37: Nutramigen <33: E	Days 8-21	Stool	GC	↑butyric with nutramigen beyond day 17. ↓ total SCFA concentration if <33 weeks
Wang C JPGN EL 1	2007	66	23-36	414-2124	6	NS	Some	All mixed E/F	0, 2 + 4 weeks	Stool	HPLC	↓butyric in probiotic group
Mohan R Ped Res EL 1	2008	69	Gp I: 31.05(2.31) Gp II: 31.27(2.56)	I: 1449(343) II: 1398(331)	8	NS	18	E: 58 F: 11	Daily for 21 days	Stool	HPGC + ELISA for lactate	Probiotic group: ↑total SCFA wks 2+3, ↑propionic, lactic and butyric;
Underwood MA JPGN EL 1	2009	90	Gp I: 29.3(2.6) Gp II: 29.5 (2.6) Gp III: 30.2 (2.4)	I: 1393 (363) II: 1394 (356) III: 1461 (372)	27	NS	Total days: I: 5 (2,10) II: 4 (2,8) III: 3 (2,7)	I: E 11 F II: E 9 F 4 III: E 10 F 5	Week 4	Stool	HPLC	No differences in probiotics vs placebo

Abbreviations: LAC = lactose formula; GP = Glucose polymer formula

Table 5: Evidence base for the relevance of stool SCFA analysis in preterm infants without NEC; five studies were of EL 1, and one each of ELs 2 and 3. There was a wide variation in reported SCFA trends with probiotic administration, ranging from no differences to higher butyric, lactic and propionic acids. The two feed trials showed conflicting results, with alternately increased or decreased total SCFA concentrations with feed type.

Given that SCFA analysis is well established by gas chromatography, there are few methodological differences within the systematic review of these studies. Most involved GC analysis, and used a variety of internal standards (including 2-ethylbutyric, and 3-methyl valeric acids). Of the seven studies, five were randomised controlled trials (four concerning probiotics, one using formula feeds), one was a controlled case series', and one was a case series. Dominant SCFAs throughout the studies were acetate and lactate, and higher levels were associated with lactose formula feeding, and probiotic administration in one study. Four studies found age- and gestation-dependent increase in total SCFA concentrations. Butyrate levels were generally low in samples from the infants in this cohort generating the theory that butyrate is not employed as an anti-inflammatory agent in preterm infants as it is for adults. None of the infants involved in these studies incurred gut pathologies therefore the 288 infants included in these studies represent normative data. Four of the studies used molecular methods to concurrently correlate bacteria with metabolites.

The evidence for the relevance and measurement of stool BCFA in preterm infants, is, however lacking. BCFAs are found in abundance in vernix caseosa, the waxy, white substance found coating the skin of newborn infants at term. Vernix is an exclusively human finding, and commensurate with the shedding of skin epithelium and lanugo during the third trimester. Preterm infants who have missed out on much of the last trimester characteristically retain lanugo, lack a robust dermis, and have less dermal fat deposition, associated with the lower production of vernix, which generally starts from 20 weeks gestation onwards. Approximately 25-30% of the wet weight of vernix is composed of BCFA (Pickens, Warner et al. 2000), and the composition of vernix in preterm versus term infants varies, with higher levels of squalene (a naturally occurring hydrocarbon/triterpene combination) and a higher wax to sterol ester ratio in term infants (Schachner 1999). One additional theory of relevance of vernix, rather than an incidental finding or by-product, is that it is entero-protective. Ran-Ressler et al in 2008 propagated their theory that the absence of vernix in preterm infants predisposes them to NEC. Their published study suggested that skin sloughing in late trimester, leaving epithelial cells suspended in amniotic fluid, begins the deposition of vernix. Amniotic fluid also contains lipids, of which 17/154 mg/L are BCFA according to observational studies (Biezenski, Pomerance et al. 1968, Ran-Ressler, Devapatla et al. 2008) Since swallowing mainly starts at >34 weeks (around 200-500 mls/day) (Pritchard 1966), the combination of skin sloughing and amniotic fluid ingestion then results in the deposition of BCFA-laden vernix, and the subsequent presence of BCFAs in the fetal colon. However GCMS studies comparing

BCFAs in amniotic fluid and in meconium show less metabolism within meconium, strongly suggesting that the BCFAs are utilised by the fetus, possibly for energy (as are other SCFAs for enterocytes). BCFAs have also been identified in human milk, but are not added to most formulae; their presence in maternal milk has been postulated to be protective, although the evidence for this is lacking (Ran-Ressler, Devapatla et al. 2008). Ran-Ressler et al (2011) examined the BCFA-enriched colons of preterm rats with NEC induced by hypothermic stress, and reported that ileal biopsies showed less NEC than those without BCFAs. In association with this finding, BCFA-producing bacteria were fivefold greater in those supplemented than those without (Ran-Ressler, Khailova et al. 2011).

## ii) **Inflammation and Immunoprotection: Calprotectin and Secretory Immunoglobulin A**

### a) *Calprotectin*

Calprotectin is a calcium and zinc-binding protein released during neutrophil activation or death. It is found in higher levels in the gut lumen at times of intestinal inflammation, given the preference for dying neutrophils to migrate across the gut wall (see figure 3) (Fliedner, Cronkite et al. 1964, Fagerhol 2000, Josefsson, Bunn et al. 2007), and has bacteriostatic and fungistatic properties (Steinbakk, Naess-Andresen et al. 1990). It is a well-established marker of disease activity in children with Inflammatory Bowel Disease (IBD), but its role in the health and disease in infants is yet to be defined. Calprotectin can be detected in an array of body fluids, including urine, cerebrospinal fluid, synovial fluid, faeces and blood. In one study, calprotectin was detected in breast milk, albeit in low levels (Olafsdottir, Aksnes et al. 2002). Faecal calprotectin can also be raised during malignant conditions in adulthood, such as colon cancer, thus assuming the potential to be a diagnostic tool and marker of disease activity in all age groups. Since its identification in 1980, (Desai, Faubion et al. 2007) a host of the protective functions of calprotectin have also been ascertained, including regulation of immunity, antimicrobial actions, and even anti-tumour activities (Bando, Hiroshima et al. 2010, Brophy, Hayden et al. 2012, Srikrishna 2012). This ubiquitous collection of properties implies that calprotectin also confers benefits to the host, rather than simply acting as a by-product of inflammation.

Paradoxically, high faecal calprotectin levels similar to adults with acute exacerbations of IBD have been recorded in healthy term neonates. Instead of reflecting an underlying neonatal gut inflammation, this is thought to occur due to the lag period after birth before gut bacterial colonisation and formation of a robust gut barrier becomes established in the neonatal period (Nissen, van Gils et al. 2004). Beneficial probiotic gut bacteria play an important role in reinforcing the barriers of the gut wall, warding off potentially pathological organisms. This process can take several weeks even in healthy breast fed term infants, especially if delivered by caesarean section. It is possible that during this time calprotectin also exerts a protective role, rejecting harmful bacteria and other toxins, although proof of active calprotectin expression without neutrophil death has yet to be observed.

### *Calprotectin in Prematurity*

In order to fully investigate the evidence base for the measurement of FC in preterm infants, I performed a systematic review, in line with the SIGN methodology assessment (Network 2013). Pubmed and Medline were searched for articles concerning preterm infants (<37 weeks) with FC measured beginning within the first month of life. Abstracts that did not lead to a publication, were not in English, or were of EL 4 (expert opinion) were excluded (Mohan, Koebnick et al. 2008, Bjorkstrom, Hall et al. 2009, Rouge, Piloquet et al. 2009, Rouge, Butel et al. 2010, Campeotto, Suau et al. 2011, Shulman, Ou et al. 2011, Terrin, Passariello et al. 2011, Bukulmez, Dogru et al. 2012).

Study, Journal + Evidence Level	Year	n =	Gestation (weeks)	Birth weight (g)	SVD	Incubation	Antibiotics	Feed type	Postnatal age of samples	Methods of analysis	Results (Levels in µg/g)
Mohan Ped Res EL 1	2008	69	Gp I: 31.05(2.31) Gp II: 31.27(2.56)	I: 1449(343) II: 1398(331)	8	NS	18	E: 58 F: 11	Daily for 21 days	ELISA, PhiCal	Levels ↓ in probiotic group, especially in combination with antibiotics (specific numbers NS)
Rouge Am J Clin Nutr EL 1	2009	93	Probiotics: 28.1 1.9 Control: 28.1 1.8	1115 251 1057 260	17 14	NS	Antibiotic days: P:11.7 14.4 C:10.2 9.7	NS	2 weekly until discharge	ELISA, Calprest	In >1000g infants: P: 154 (84) C: 103 (90) No diff at any point
Bjorkstrom Acta Paediatrica EL 3	2009	48	27 (2.3)	910 (250)	8	NS	NS	NS	Days 0, 7, 14, 21	ELISA, Calprest	Levels not associated with any bacterial species
Rouge PloS One EL 3	2010	47	29 (27-29)	110 (880-1320)	31	NS	39 due to maternofetal infection; 25 for nosocomial infection	17 E 1 F 29 Mixed	2 week intervals until discharge	ELISA, Calprest	138 (15-811) ↓ with ↑ feed tolerance ↓ with antibiotics ↑ with clostridia and staph stool colonisation
Shulman RJ Neonatology EL 3	2011	50	28.6 (2.2)	1200 (330)	NS	NS	NS	8: E + 27 E + F + 10 F 5 E + F	Weekly until discharge	ELISA, Genova	No correlation in levels and feeding types, regimens, gastric residuals, gut permeability, gastric emptying or abdo distension
Terrin Clin Immunol EL 2	2011	201: Septic:62 Not:29 Controls: 110	S:29.2 (28.7-29.9) N:30.1 (29.1-31.1) C:29.2 (28.7-29.6)	S:1082 (1029-1135) N: 1178 (1080-1275) C:1088 (1045-1131)	S: 10 N: 3 C: 13	NS	NS	NS	1 serum sample after diagnosis of sepsis or possible sepsis	ELISA, Calprest	S: 3.1 (1) mcg/ml N:1.1 (0.3) C:0.91 (0.58) Septic>non + controls Sens: 89% Spec: 96% for sepsis
Campeotto Br J Nutr EL 1	2011	58: 24 Probiotic formula 34 preterm formula	30-35: 33.5 (1.3) 33.4 (1.1)	1912 (354) 1926 (386)	13 15	NS	4.5 days (3.6) 5.6 days (2.5)	E: 6 E: 10	Weekly	ELISA, Calprest	257 (16-1240) ↓ levels in Probiotic formula + without antibiotics
Bukulmez* Am J Perinatol EL 2	2012	44: Phototherapy: 29 Non: 19	42: term 44: preterm	NS*	NS*	NS*	NS*	NS*	1 sample 24 hours after commencement of phototherapy	ELISA	Phototherapy had no effect on levels

Table 6: Evidence base for the measurement of stool calprotectin in preterm infants; \*only available in abstract form; NS = not specified

Preterm infants who are of Very Low Birth Weight are born with several deficits in gut function in comparison with those born at term, and this may account for their higher levels of calprotectin during the neonatal period. Firstly, a lack of peristalsis, the normal wave patterns of muscular contraction within the gut wall designed to propel content throughout the digestive tract, impairs their ability to deliver nutrients to the inner bowel wall for absorption of nutrients (Patole 2007). Secondly, preterm infants have a deficiency of protective layers of mucin on the inner lining of the bowel, normally present as a filter to bacterial and viral enteropathogens while permitting the passage of vital nutrients and other essential factors (Siggers, Siggers et al. 2011). The defective nature of this barrier is referred to as ‘increased permeability’ of the gut wall, and may facilitate neutrophil passage, thus accounting for higher faecal calprotectin levels found in ‘healthy’ preterm infants. Thirdly, preterm neonates experience a delay in the establishment of normal gut bacteria. This postpones the favourable effects of ‘probiotic’ bacteria which are known to reinforce this barrier by adhering to gut wall cells and excluding pathogens and toxins (Corridoni, Pastorelli et al. 2012).

Given this increased gut permeability, it is theoretically possible that neutrophil migration from the bloodstream into the gut at times of blood infection may also result in higher levels of faecal calprotectin, as postulated in a population of ‘sick’ neonates without NEC (Terrin, Passariello et al. 2011). Conversely, increased neutrophil migration across the gut wall has been seen to heighten gut permeability in patients with IBD (Chin, Lee et al. 2008), although this ‘reverse’ effect has not been investigated in preterm infants. Paradoxically, prematurity is also associated with defective neutrophil migration, possibly contributing to their lower immunity in comparison with those at term (Turkmen, Satar et al. 2000). This dampened immune system is associated with a higher incidence of blood infections.

Other seemingly innocuous factors may increase calprotectin levels in the delicate preterm gut, and these require further analysis in observational studies. Birth asphyxia is associated with an increase in faecal calprotectin in term infants, due to a temporary loss of blood flow to the gut followed by reperfusion – restitution of blood flow often associated with injury. Theoretically, other factors associated with loss of blood flow to the preterm gut (thereby increasing the risk of NEC) may increase calprotectin, including congenital gut abnormalities, low blood pressure (as can occur during blood infections), and a cardiac condition commonly associated with prematurity known as Patent Ductus Arteriosus.

Premature neonates exclusively fed breast milk have lower rates of NEC and other gastrointestinal infections, in the neonatal period, but there is as of yet no evidence for the continuation of this into childhood. This may be due to the resultant dominance of lactic acid producing bacteria secondary to breast milk. Preterm infants exclusively fed formula milk have lower levels of 'probiotic' gut bacteria, and theoretically higher levels of calprotectin. However, another paradox may exist. Two studies investigated changes in faecal calprotectin with milk type in term neonates (Dorosko, Mackenzie et al. 2008, Rosti, Braga et al. 2011). The first found no significant difference between infants who were breast fed with those given standard or prebiotic infant formulae, although the highest values (860  $\mu\text{g/g}$ ) were found in breast fed infants (Rosti, Braga et al. 2011). The second article, however, found significantly higher faecal calprotectin levels in term infants exclusively fed breast milk ( $p=0.01$ ) (Dorosko, Mackenzie et al. 2008). They proposed that in a cohort of 41 samples from 32 healthy neonates, calprotectin had a protective role aiding gut adaptation. However, a major confounding feature of these results was the use of oral medications within the breast fed group, including the gut motility agent simethicone and the H<sub>2</sub> antagonist ranitidine. In 2002, Olafsdottir *et al* detected calprotectin in breast milk, albeit at low levels ( $1.0\pm 1.6\text{mg l}^{-1}$ ) (Olafsdottir, Aksnes et al. 2002).

The evidence for normal reference ranges of fecal calprotectin in preterm infants consists mainly of small case series. Twenty studies have considered faecal calprotectin levels in preterm neonates, both with and without gastroenterological illnesses (Carroll, Corfield et al. 2003, Mohan, Koebnick et al. 2006, Campeotto, Kalach et al. 2007, Josefsson, Bunn et al. 2007, Yang, Smith et al. 2008, Bjorkstrom, Hall et al. 2009, Campeotto, Baldassarre et al. 2009, Rouge, Piloquet et al. 2009, Thuijls, Derikx et al. 2010, Campeotto, Suau et al. 2011, Shulman, Ou et al. 2011, Terrin, Passariello et al. 2011, Westerbeek, Morch et al. 2011, Aydemir, Cekmez et al. 2012, Bukulmez, Dogru et al. 2012, Dabritz, Jenke et al. 2012, Reisinger, Van der Zee et al. 2012, Selimoglu, Temel et al. 2012, Zoppelli, Guttel et al. 2012). Of these, 10 discuss levels in preterm infants without gastrointestinal disease; 1 defines levels in preterm infants with and without gastrointestinal illnesses; 8 compare levels in infants with necrotising enterocolitis; and 5 compare levels in preterm infants enterally administered probiotics or prebiotics with controls. In comparison with the normal adult range ( $<50 \mu\text{g/g}$ ) the studies examining well preterm infants conclude the following: 1) high levels in meconium (first infant stool), tailoring to adult levels by the end of infancy; 2) higher levels in preterm infants and in infants with birth asphyxia, regardless of gestation; 3) lower levels in infants administered probiotics in randomised

controlled trials. Undeniably, more observational trials are required for meta-analyses in order to establish 'normal' ranges in the healthy neonatal population. In term infants, a temporal relationship has been observed, with higher levels within the first month of life, and no differences with regard to type of milk fed (Campeotto, Kalach et al. 2007). During heightened intestinal permeability within the first month of life, the rise in faecal calprotectin may be secondary to granulocyte migration, or from cross-reactivity by other potent metabolites produced by the establishment of the microbiota, such as formyl-methionyl-leucyl-phenylalanine (FMLP), causing false positive tests (Olafsdottir, Aksnes et al. 2002). One article illustrated higher levels in the meconium of term infants with perinatal asphyxia, presumably secondary to reperfusion-induced inflammation (Cui and Li 2012). Espinoza et al in 2003 observed higher calprotectin levels in amniotic fluid of mothers of preterm infants delivered after chorioamnionitis, suggesting an antenatal influence (Espinoza, Chaiworapongsa et al. 2003).

## ***b) Secretory Immunoglobulin A***

- *Structure and Purpose of SIgA*

Immunoglobulin A (Ig A) is the most prolific immunoglobulin found throughout the human gastrointestinal tract, where it plays a critical role in mucosal immunity (see figure 3). It holds key viro- and bacteriostatic properties (Fagarasan and Honjo 2003, Fagarasan and Honjo 2004, Fagarasan 2006). Accounting for 75% of the total body immunoglobulin, its highest concentrations are found in colostrum, saliva, and tears (Forchielli and Walker 2005). The term ‘secretory’ IgA (SIgA) is given to its dimeric form that protects the molecule from degradation by the proteolytic environment of the gut. It incorporates more than four binding sites, allowing determination of SIgA adherence. Studies have indicated that the faecal SIgA level is representative of the colonic SIgA content (Grewal, Karlsen et al. 2000). Notably, it is not absorbed, but adheres to the gut mucosa, promoting the effects of beneficial commensals, and impairing the abilities of enteropathogens to exert adverse effects (Macpherson and Uhr 2004, Macpherson, Geuking et al. 2011). Latterly a number of strategies have been employed within randomised controlled trials aiming to increase stool SIgA in healthy term infants through the administration of enteral supplements (particularly prebiotics and probiotics), but there remains a paucity of information regarding normative data in healthy preterm infants according to feed type (Bakker-Zierikzee, Tol et al. 2006).

- *SIgA in Human Milk*

The enteromammary circulation refers to the circular process by which maternal immunoglobulins are produced from B cells within Peyer’s patches of the gut lymphatics in response to ingested maternal gut enteropathogens. The pathogen-specific type A immunoglobulins are then passed on to the infant through breast milk. After ingestion, this IgA is neither absorbed nor destroyed but remains in the neonatal gut lumen to deter an identical infection (Nathavitharana, Catty et al. 1994). As such, the effect of maternal breast milk in preterm infants, who by definition are immunocompromised, cannot be underestimated. The effects of donor expressed breast milk in this regard are unknown, but likely to be diminished considering that most donors are producing mature non-colostrum milk, many at term, and these factors are known to reduce the SIgA content. However, although numerous studies have recognised higher SIgA titres in milk of mothers of preterm infants (Araujo, Goncalves et al. 2005, Thibeau and D’Apolito 2012), recent studies have suggested that the SIgA content of the milk of mothers of preterm infants

delivered less than 30 weeks gestation may be even lower than mothers of infants at term (Castellote, Casillas et al. 2011). No trials could be found assessing the effects of maternal administration of pre- or probiotics upon subsequent titres of breast milk, although there are several observational studies noting similarities in the maternal and neonatal gut microbiota in breast-feeding mother-infant pairs (Sanz 2011, Thum, Cookson et al. 2012) and several others noting the efficacy of strategies to increase breast milk supply in mothers of preterm infants do not include methods of increasing nutrient or immunological quotients (Donovan and Buchanan 2012). No evidence for corresponding alteration in antenatal variations in faecal SIgA, or correlation with antenatal gut microbiota and enteropathogens could be identified.

- ***Variation in Stool Secretary Ig A levels in Preterm Infants***

The level of maternal serum SIgA increases at the beginning of pregnancy, but falls at the end of the second trimester, prior to rising again until delivery. As a result, preterm infants who have missed this final trimester wave of SIgA have lower serum and faecal SIgA levels than infants delivered at term (Goldman, Garza et al. 1982). So far, no studies have examined paired analyses of maternal milk SIgA with neonatal stool SIgA in term or preterm infants. Given that preterm infants are often fed trophically, maternal milk supply often far exceeds demand in the first month of life. No guidelines exist as to whether colostrum should be used preferentially, given the higher concentrations of SIgA in colostrum. In addition, storage and sterilisation procedures are known to alter the SIgA level by as much as 30% (Lawrence 1999, Akinbi, Meinzen-Derr et al. 2010). No studies have yet shown the spectrum of SIgA in DEBM, which is likely to be broad given the heterogeneous selection of donors at different gestations, and mandatory pasteurisation in all UK centres.

Only two studies could be identified examining the SIgA levels in stool samples from preterm infants. In 2008, Mohan et al published their analyses of the SIgA levels of 69 preterm infants as part of a randomised control trial of the effectiveness of probiotic supplementation with *Bifidobacterium lactis* Bb12 upon body weight, fecal pH, acetate, lactate, calprotectin and IgA in preterm infants. Samples were taken directly from the nappy weekly for the first 3 weeks of life. Thirty-seven of these infants were enterally administered probiotics, and most were exclusively breast milk fed. There was no significant difference in proportions of breast and formula feeding between the two groups. Standard ELISA kits from Immunodiagnostik AG, Bensheim, Germany, were used. SIgA levels in those fed probiotics were 44% higher than controls, and this group also showed a

significant increase in stool SIgA levels between weeks 1 and 2, but only in the subgroup of infants who did not require antibiotic administration (Mohan, Koebnick et al. 2008).

Secondly, Campeotto *et al* in 2011, published their randomised control trial comparing SIgA, calprotectin and colonisation changes in preterm infants fed a fermented formula (containing *Bifidobacterium breve* C50 and *Streptococcus*) versus an unnamed preterm formula (manufactured by the company 'Bledina', France). The groups did not differ in clinical or demographical aspects, and necrotising enterocolitis was not mentioned in any patient. SIgA titres showed high interindividual variation, and there were no significant differences between the two groups (fermented formula fed infants: median 27 (range: 1-474 µg/g); preterm formula fed infants: median 12 (range: 1-350 µg/g)). They did, however, note that infants who were partially breast fed showed higher SIgA levels from week 2 in those who were also fed the fermented formula instead of the standard preterm milk (3038 (range 1225-6040) Versus 1473 (range 30-2655)) (Campeotto, Suau et al. 2011).

### **1.6.5) Necrotising Enterocolitis**

#### *i) NEC: Definition and incidence*

NEC occurs in approximately 10% of all very and extremely low birth weight infants (Lin and Stoll 2006, Kovacs 2007), and has a high mortality, quoted in some studies to be as high as 80% (Hintz, Kendrick et al. 2005, Holman, Stoll et al. 2006). In survivors, this has several well-established long-term effects to the gut and growth in general. Breast milk fed infants have a significantly reduced incidence of NEC, likely owing to a combination of natural beneficial bacteria, immunological factors, and so the cultivation of an anti-enteropathogenic colonic environment. There is evidence to suggest that ‘trophic’ enteral nutrition (slow feeding with diminutive volumes increased at small increments) prevents NEC (Bombell and McGuire 2009). However, the rate at which trophic feeding should start and proceed is an unknown quantity subject to a variety of trials listed currently. Prolonged parenteral nutrition also adversely affects the premature gut by: 1) prolonging the need for intravenous access, with associated increased risk of line-related sepsis and resultant septic ileus, and 2) TPN cholestasis and intestinal failure-related liver disease.

#### *ii) Associations with Morbidity and Mortality*

Over the last ten years mortality rates for VLBW premature infants have fallen substantially, although this has not been mirrored by a similar decline in morbidity. This is despite a drop in the incidence of severe chronic lung disease and intra-ventricular haemorrhage (Fanaroff, Stoll et al. 2007). This morbidity plateau has instead been attributed to the increasing numbers of surviving ex-premature infants who are simply malnourished, many secondary to NEC and its sequelae, including Short Bowel Syndrome, and the need for sustained parental nutrition leading to repeated central venous line infections, liver disease, and consequent failure to thrive. As such, with greater numbers of those surviving extreme prematurity, it is anticipated that nutrition and care of the neonatal gut will become increasingly important in the future. Tertiary Paediatric Gastroenterology, Hepatology and Nutrition Units are expanding, increasing in number from 3 general and specialist consultants in Scotland in the mid 80’s, to 11 tertiary subspecialists at the current time, plus general paediatricians with an interest in PGHN who regularly see patients in this regard for shared care. Concurrently, the British Intestinal Failure Study, established in 2003, aims to identify all children in the UK with intestinal failure, which will start to give an impression of a conservative estimate of the long term effects of survival after NEC in ex-preterm infants (AR Barclay, personal communication).

iii) *Aetiology*

Various theories abound as to the most likely combination of pathophysiologies instigating NEC. The Santulli hypothesis marries three major theories of NEC pathogenesis: intestinal ischaemia, gut microbiota, and luminal substrate (Santulli, Schullinger et al. 1975). The Lawrence theory highlights the contribution of bacterial toxins to NEC (Lawrence, Bates et al. 1982), a theory extrapolated by Lin *et al*, in 2004 (Lin 2004). A variation on both, by Claud and Walker, offers a more generalised triad of prematurity, enteral feeding, and bacterial colonisation (Claud and Walker 2001). It is likely that these are the lynch pins from which all other associations, such as reduced gut perfusion (e.g. from patent ducts arteriosus, or systemic hypotension during the first 24-48 hours of life, or during episodes of sepsis) contribute to a vicious cycle of intestinal ischaemia, both contributing to and coinciding with gut dysmotility, compounded by abnormal colonisation (i.e. reduced species variety and intensity), leading to inflammation, potentiating a pathological ileus, and therefore further gut dysmotility, limited gut colonisation, and so on. A representation of these interactions is illustrated in figure 7 below.

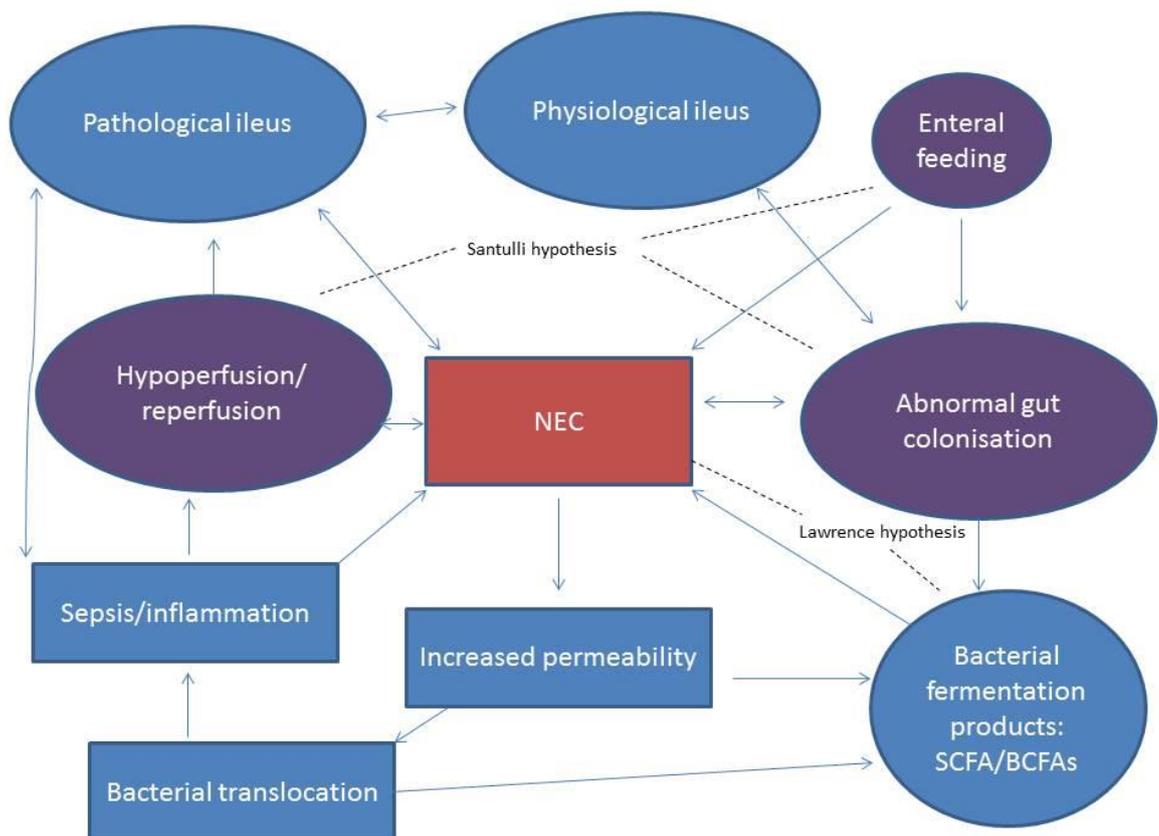


Figure 7: Summary of the possible pathogenesis of necrotizing enterocolitis (Abbreviations: NEC = necrotizing enterocolitis; SCFA = short chain fatty acids; BCFA = branched chain fatty acids).

Whatever the aetiological spectrum, the clinical outcome is both an inflammatory and infective bowel condition that can lead to ischaemia and necrosis. Notably, the

histopathology of NEC has not changed since first observations at the turn of the 20<sup>th</sup> century, although immunohistochemical methods of identifying immunological factors associated with NEC have led to an increase in the understanding type and sequence of inflammatory processes – with the potential to influence them (Vieten, Corfield et al. 2005, Vieten, Corfield et al. 2006).

*iv) Diagnosis and Management of NEC*

In the absence of biomarkers of NEC, diagnosis and thus management rests upon a constellation of clinical, biochemical, haematological and radiological features. The Modified Bell's Criteria (see Table 7) categorises NEC according to established clinical and radiological features, and is used internationally as the foremost NEC classification system, but has more recently fallen out of favour due to its use of Faecal Occult Blood testing, which is now virtually obsolete in neonatal units due to high numbers of positive results found in well infants, and also its low specificity for NEC (Pinheiro 2003). It is important to note that NEC can be an elusive diagnosis, with treatment of mild cases often comprising drip-suck, antibiotic administration, and then a 'wait and see' approach. Seemingly minor episodes of NEC can lead to significant and long term impairment in gut function, including ischaemic stricture formation and malabsorption. Other potential blood markers of NEC include the general inflammatory marker C-reactive protein (CRP), a protein created by the liver and released into the blood stream during inflammation or infection, and a raised white cell count. However the rise in both is often seen to lag after the clinical onset of NEC, and is not NEC-specific. Similarly, radiological evidence of NEC is part of the Modified Bell's Criteria, but recent research suggests that x-ray features commonly held as pathognomonic of NEC are highly specific but of low sensitivity (Coursey, Hollingsworth et al. 2008). Some observational studies of NEC are beginning to re-define the illness with terms such as 'enteropathy', although this then makes comparison of studies designed to assess NEC prevention or management strategies difficult, particularly when trying to perform meta-analyses to glean sensitivity/specificity values on potential diagnostic and prognostic markers (Campeotto, Baldassarre et al. 2009).

Stage	Classification	Systemic Signs	Intestinal Signs	Radiological Signs
1a	Suspected	Pyrexia/apnoea/ bradycardia/lethargy	Large aspirates/vomiting/ mild abdominal distension/FOB+ stool	Normal or mild dilatation Mild ileus
1b	Suspected	1a	Gross PR blood	1a
2a	Proven	1a	1b + Absent bowel sounds +/- Abdominal tenderness	Intestinal dilatation Ileus Pneumatosis
2b	Proven	2a + Mild metabolic acidosis Mild thrombocytopenia	2a + Definite abdominal tenderness	2a + Portal gas +/- ascites
3a	Advanced	2b+ Hypotension, bradycardia, apnoea, mixed acidosis, DIC, neutropenia	2b + Peritonitis Abdominal distension Abdominal tenderness	2b + Ascites
3b	Advanced	3a	3a	3a + pneumoperitoneum

Table 7: Modified Bell's Criteria, as reported by Bell et al in 1978 (Bell, Ternberg et al. 1978, Walsh and Kliegman 1986)

It may well be that there is no one unifying diagnostic marker, and that instead a panel of investigations together hold the highest sensitivity and specificity in anticipating and diagnosing this unpredictable illness. Several studies have started to explore such panels – including immunological markers in both stool and serum samples (Cetinkaya, Ozkan et al. 2011, Eras, Oguz et al. 2011). Naturally, the benefit of using serum samples is the immediacy with which they can be taken and analysed, in comparison with stool samples.

#### 1.6.6 Trends in Microbiological Stool Studies of Preterm Infants with NEC

Several studies have highlighted trends in pathogenic bacteria in NEC – chiefly *Clostridia* sp. (de la Cochetiere, Piloquet et al. 2004). Similarly, animal models designed to replicate the premature intestine have noted NEC-like lesions with the addition of both enteropathogens (*Clostridium*, *E. coli* and *Klebsiella* sp.) and short chain fatty acids (Lin, Peng et al. 2005, Waligora-Dupriet, Dugay et al. 2005). However, as well as identifying specific enteropathogens associated with NEC, the sequence of gut colonisation and acquisition of beneficial species and strains of *Bifidobacteria* and *Lactobacillus* may be as

important to understand in order to establish preventative practices and identify best evidence-based treatments.

In total, ten studies have examined the gut microbiota in preterm infants with NEC, as identified by systematic review as described earlier. These are summarised and critiqued in the table 8 (Westra-Meijer, Degener et al. 1983, Millar, Linton et al. 1996, Hoy, Wood et al. 2000, de la Cochetiere, Piloquet et al. 2004, Wang, Hoenig et al. 2009, Mshvildadze, Neu et al. 2010, Mai, Young et al. 2011, Smith, Bode et al. 2011, Stewart, Marrs et al. 2012, Normann, Fahlen et al. 2013).

Study, Journal + Year	Evidence Level	n =	Gestation (weeks)	Birth weight (g)	SVD	Incubation	Antibiotics	Feed type	Postnatal age of samples	Samples	Methods of analysis	Results
Westra-Meijer ADC EL 2		1983 77	NEC: 29.44±2.8	1262±382	NS	NS	20 (83)	NS	12.3±69 10.8±6.8 11.2±2-6	Stool	Culture	↑ <i>Klebsiella</i> in NEC;
			NEC: 24	13864±345			7 (58)					
			?NEC: Non:	?NEC: 31.4±2.7			36 (88)					
			Non: 30.3±2.0									
Hoy C ADC EL 2		1990 90	NEC: 28 (27-30)	NEC: 995 (660-1160)	NS	NS	3 infants	NS	752 stool samples	Stool	Culture	↓species up to 72 hrs before onset of NEC; thereafter <i>enterobacteriaceae</i> found in samples 48 hrs before definite NEC, 2 episodes with new isolates
			NEC: 7				before 3 of 8 episodes possible					
			?NEC: 7 (26-30)	Non: 725 (620-1440)			NEC; 1 of actual NEC					
Millar MR J Clin Microbiol EL 2		1996 32	24-34	NS	NS	NS	NS	NS	Weekly samples for mean 5.3 weeks	Stool	Culture, PCR-DGGE + 16s rRNA	>90% sequences were <i>Strep. Salivarius</i> ; PCR identified no extra species than culture
de la Cochetiere Pediatric Res EL 2		2004 12	NEC: 28.5 (2.1)	NEC: 880 (170)	NS	NS	9: initial antibiotics	NS	Weekly from first stool	Stool	PCR-TTGE	Close match to <i>Clostridia perfringens</i> in NEC cases (95% similarity)
			Non: 27.7 (2.0)	Non: 940 (320)								
Wang Y ISME J EL 2		2009 20	25-32	NS	3	NS	NS	E: 10 F: 10	4 – 49 days	Stool	PCR-RFLP	↓diversity in NEC; ↑gammaproteobacteria; ↑mean antibiotic days
Mshvildadze M J Pediatr EL 2		2010 23	29.9 (2.5)	1394 (420)	10	NS	19	EBM: 15 F: 8	5.2 (3.3) per infant	Stool	DGGE -16s rRNA pyrosequencing	Microbial DNA detected in meconium; <i>citrobacter</i> + enterococcus in NEC only; ↑ <i>klebsiella</i> in

													controls
Smith B BMC Microbiol EL 3	2011	24	29 (25-40)	1030 (600-3660)	10	NS	NS	NS	NS	NS	Intestinal specimens	Laser capture microdissection + FISH	Large variability, but no differences in NEC; ↑ <i>Clostridium butyricum</i> + <i>paraputrificum</i> with pneumatosis intestinalis
Mai V PloS 1 EL 2	2011	19	26.7 (23-30)	960 (570-1269)	9	NS	15	E: 13	From first stool and weekly; All post-NEC within 72 hrs	Stool	High throughput rRNA sequencing	34% ↑ <i>Proteobacteria</i> + 32% ↓ <i>Firmicutes</i> in NEC; unique bacterial signature found: γ-proteobacteria	
Stewart CJ Acta Paediatrica EL 2	2012	38	27 (23-31+6)	895 (520-1850)	18	NS	All 48 hrs antibiotics 29 antifungals	35 'received breast milk'	Varied – some before and some after NEC; no 'protocol'. First stool and weekly.	Stool	Culture + 16s rRNA	Meconium not sterile. NEC: ↑enterococcus, staph and CoNS	
Normann E Acta Paediatrica EL 2	2013	20	NEC: 23+5 (22+0-25+5) Non: 23+5 (22+5-25+6)	NEC: 582 (487-965) Non: 570 (440-892)	NEC: 6 NEC: 6	NS	NEC: 6 (m) Non: 7 (m)	All mixed E/D/F	Weekly for 1 <sup>st</sup> 7 weeks Plus 1 maternal sample	Stool	Bar-coded pyrosequencing	No significant differences between NEC and controls; <i>Enterococcus</i> , <i>Bacillales</i> and <i>Enterobacter</i> predominated	

Table 8: Evidence base for the identification of and associations with gut microbiota in preterm infants with NEC; all were controlled data bar one case series. Results were extremely heterogeneous and varied with specificity of molecular analysis. Higher levels of known enteropathogens were found in those with NEC in 9 studies. *Clostridia* species were however only identified in one publication. (Abbreviations: NEC = necrotising enterocolitis; E = expressed breast milk; D = donor breast milk; M = mixed breast and formula milk; CoNS = coagulase negative *staphylococcus*; EL = evidence level; FISH = fluorescent in situ hybridisation; PCR = polymerase chain reaction; TGGE = temperature gradient gel electrophoresis; DGGE = denaturing gradient gel electrophoresis; RFLP = restriction fragment length polymorphism; NS = not specified; rRNA = ribosomal ribonucleic acid).

Given the 25 year range of these studies, and the rapid advancement of molecular methods of identification, it is not surprising that more bacterial phyla, families and genera were identified in later studies. In total, none of the studies included were evidence level one RCTs, nine were evidence level two controlled studies, and one was an evidence level three case series', representing 355 preterm infants, including 88 with NEC. Demographically, there was a wide range of gestational ages, birth weights, and ages at production of samples, and all studies encountered issues in low stool production relative to physiological and pathological ileus. None of the articles specifically discussed changes in ileostomy fluid, although one noted that several infants had undergone stoma formation. One study considered the bacteria in peritoneal fluid cultured intraoperatively in infants with surgically managed NEC (Coates, Karlowicz et al. 2005). An ideal forum through which to examine the gut microbiota in preterm infants with NEC are the growing number of clinical trials assessing preventative and management strategies – particularly in the administration of probiotics, which, by definition, should be seen to colonise the stool flora in order to ascertain whether any of these effects are because of such supplements.

#### **1.6.7) Potential Biomarkers of NEC**

##### *i) Bacterial Metabolites: Toxic Products or Innocent Bystanders of Inflammation?*

The seemingly endless search for serum diagnostic and prognostic biomarkers of NEC (including amyloid A, procalcitonin, interleukins six and 10, and CRP) has been heavily confounded by their high levels seen with sepsis and/or pneumonia (Romagnoli, Frezza et al. 2001, Pourcyrus, Korones et al. 2005, Eras, Oguz et al. 2011) with some studies showing highest levels only at later stages once NEC has been well-established clinically (Cetinkaya, Ozkan et al. 2011). Tests of breath hydrogen, a by-product of bacterial fermentation, were trialled in the 1980's as possible markers for NEC, but without success (Garstin and Boston 1987, Cheu, Brown et al. 1989). Simple analyses such as stool pH may in the future reveal significant trends for premature and low birth weight neonates once significant numbers have been recruited to observational studies in order to identify a correlation with NEC. A wide variety of alternate prospective markers within stool samples are currently under investigation in preterm infants: metabolites of commensal and pathogenic bacteria. Recent work by Andrew Ewer et al investigating trends in volatile organic compounds identified a significant reduction in their number in the days before and after the diagnosis of NEC in 6 infants. In addition they found that 4 specific esters were absent in their stool samples in the 4 days prior to the onset of NEC (Garner 2009). Generally, such bacterial metabolites are important in three regards. Firstly, they may exert local effects, both beneficial and detrimental; secondly, they may be absorbed

systemically, with generalised effects; and lastly, they may act as markers of illness – in particular, of NEC. Only those products most commonly represented in extensive literature searching will be discussed.

ii) *Short Chain Fatty Acids: Friend or Foe?*

It is widely recognised that in different populations, these metabolites have a dual identity, being recognised as both beneficial (lactate indicates the presence of lactate-producing bacteria such as *Lactobacilli* and *Bifidobacteria*) and detrimental (butyrate has been seen to rise in animal models of NEC (Waligora-Dupriet, Dugay et al. 2009) to host health. It is unclear, however, whether some SCFAs themselves are causative agents in neonatal gastrointestinal disease, or merely reflective of the metabolically active bacteria which cause disease. The only trend to be noted in regard to dietary differences is the predominance of acetic acid in breast fed term infants (Edwards, Parrett et al. 1994). This has yet to be explored in premature neonates. However, butyrate is known to increase intercellular junctional integrity in adults (thus preserving intestinal permeability), yet has been seen to rise in animals with NEC (Mariadason, Kiliass et al. 1999). It may therefore be an ‘innocent bystander’, detected in tandem with another pathological inflammatory process, or could be an indicator of butyrate-producing bacteria which increases the risk of NEC. Similarly, neonatal studies have detected an increase in stool interleukin 8 at times of intestinal inflammation (Butel, Roland et al. 1998, Fusunyan, Quinn et al. 1999, Pender, Quinn et al. 2000). Animal models have, however, illustrated damage locally to the intestinal mucosa, and even systemically, with the intrathecal administration of butyrate causing autistic behaviour in rats (Butel 2001, MacFabe, Cain et al. 2007, Hamer, Jonkers et al. 2008). Only one published study could be identified assessing stool SCFA levels as markers of NEC in preterm infants (Szyliet, Maurage et al. 1998). In 1998, Szyliet et al published their observational study of 46 preterm infants, of whom 31 had ‘digestive disorders’. Although NEC was not defined using the Modified Bell’s Criteria, the digestive disorders reported included ‘abdominal distension’, or ‘rectal bleeding’. Nonetheless, the term ‘NEC’ was referred to within the text. In total 46 preterm infants who had commenced enteral feeds were studied. 31 of these incurred ‘digestive disorders’, and their SCFA stool profiles were compared, making this an evidence level two study. Mean gestational age of all infants included was 32.8 weeks (range 30-36 weeks), and a mean birth weight of 1920g (range 1170-2570g). Their feed regimen comprised donor breast milk as soon as possible after delivery, until 35 weeks corrected gestational age, in VLBW infants (who were stipulated as <1800g, rather than the now conventional <1500g). Some infants were supplemented with a protein hydrolyzate fortifier after a week of life. Those who incurred digestive disorders persisting for more than 24 hours were recommenced on feeds using a lactose-free formula containing a glucose polymer, alongside the protein hydrolyzate fortifier. Antibiotic courses were noted. As such, for the purposes of analysis, there were two distinct groups: group I comprised 2 subgroups of infants, one as healthy

controls, the other as healthy controls who required phototherapy. None of these infants were administered antibiotics. Conversely, group II was divided by clinical abdominal signs: one group with rectal bleeding, and the other with abdominal distension. All had immediate postnatal period antibiotics for treatment of materno-fetal infection, but none were treated for confirmed or suspected NEC. Samples were taken daily over the first 20 days of life, inclusive of meconium, and analysed using gas chromatography, using 2-ethyl butyric acid as the internal standard.

The mean total SCFA concentrations ranged between 0 to 80  $\mu\text{moles/kg}$  wet weight of sample, and wide interindividual variation was noted. Higher levels were associated with lower birth weight (mean 3  $\mu\text{moles/kg}$  vs 0.9  $\mu\text{moles/kg}$ ). Significant differences in profiles occurred in those receiving phototherapy, with higher total concentrations during 11-20 days of life, alongside lower concentrations of butyrate and minor SCFAs. Antibiotic therapy was further seen to reduce SCFA concentrations within 1 – 2 days of commencement. 31 infants in total had digestive disorders. Of those who developed intestinal pathology beyond 10 days of life, an altered SCFA profile was observed to precede that, comprising a dominance of acetate and butyrate. Furthermore, in infants with colonic bleeding, butyric and caproic acids increased significantly more than controls. Once again, preceding differences were noted in SCFA profiles prior to gastrointestinal bleeding, mainly a butyric acid concentration 38% higher than that observed in controls, peaking 24 to 48 hours prior to the onset of abdominal distension or GI bleeding: 25.3 ( $\pm$  16.2)  $\mu\text{moles per kg}$ , versus 42.6 ( $\pm$  12.3)  $\mu\text{moles per kg}$  respectively. Confounding factors not accounted for in this study were the dominance of more mature and larger infants than other studies; indeed some were of normal birth weight despite slight prematurity, and certain infants would not be considered at risk of NEC at all in some UK Neonatal Units. All infants with potentially early onset NEC were excluded given the exclusion of any infants undergoing abdominal surgery with the first week of life. Nonetheless the high frequency of stool samples and thus analytes, with close proximity to the clinical onset of digestive disorders makes this observation of spike in butyrate extremely interesting. Unfortunately, no other studies have considered this in preterm infants with and without NEC in the subsequent 14 years.

iii) *Calprotectin in NEC*

Calprotectin is remarkably stable at room temperature over periods of up to a week, such that patients with IBD can send stool ‘spots’ in the post on Guthrie-style cards directly to laboratories from home, making surveillance of this extremely sensitive and specific marker of IBD in children relatively easy and accessible. In recent years, the development of rapid-acting test kits makes the possibility of cotside testing a reality in the neonatal unit, but currently cost and lack of evidence to pre-clinical diagnosis of NEC precludes routine use. No studies could be found using rapid acting kits in the neonatal unit setting. Alternately, with newer ELISA kits requiring increasingly small stool samples (some as small as 30 mg wet weight), the possibility of using rectal swabs in neonates for whom stool production is low or absent, may in future make the calprotectin analysis more accessible. It is important to note that rectal swabs are a routine standard of neonatal care, and, as such would not be an additional invasive test. Ultimately, the current vogue for randomised controlled trials gives a huge opportunity to assess the properties of calprotectin to diagnose NEC pre-clinically, to prognosticate definite NEC, and to assist feed regimen and antibiotic duration in recovery. A large randomised controlled trial is currently underway in the United Kingdom piloting oral probiotics to preterm VLBW infants to reduce the incidence of NEC. The study aims to recruit 1294 patients, at 90% power; such a number would likely be necessary to investigate the positive and negative predictive values as well as the sensitivity and specificity of faecal calprotectin in NEC (Costeloe). Eleven studies considering this as a diagnostic marker of NEC are presented in the following table, and thereafter critiqued in the text. They were identified using the systematic review technique outlined previously (Carroll, Corfield et al. 2003, Campeotto, Kalach et al. 2007, Josefsson, Bunn et al. 2007, Yang, Smith et al. 2008, Campeotto, Baldassarre et al. 2009, Thuijls, Derikx et al. 2010, Westerbeek, Morch et al. 2011, Aydemir, Aydemir et al. 2012, Reisinger, Van der Zee et al. 2012, Selimoglu, Temel et al. 2012, Zoppelli, Guttel et al. 2012).

Study, Journal + Evidence Level	Year	n =	Gestation (weeks)	Birth weight (g)	SVD	Incubation	Antibiotics	Feed type	Postnatal age of samples	Methods of analysis	Results (Levels in µg/g)
Carroll D Lancet EL 2	2003	14 NEC 7	30 +4	NS	NS	NS	7; 7 NS	NS	Day 12	ELISA Calprest	NEC: 288.4 (49.1) Non: 98 (60.6)
Josefsson JPGN EL 2	2007	59 NEC 7	27.2(2.6)	939 (273)	10	NS	Daily	96% EBM 88% DEBM 12% Preterm formula 2% formula	Every stool for first 4 weeks; thereafter 24 hrly	ELISA, Calprest	Meconium: 332(12-9386) NEC: >2000 in 3 cases
Campeotto Acta Paediatrica EL 2	2007	34	30 (27-34)	1480 (780-2900)	NS	NS	NS	2 E 18 F 14 Mixed	1 sample weekly for 4 weeks	ELISA, Calprest	Group: 196 (15-928) Healthy: 160 (<15-650) GI bleed: 417 (177-604) Diarrhoea: 645 (334-928) Abdo dis: 342 (116-729)
Yang Neonatology EL 2	2008	14	26.6 (2.1)	982.1 (289.4)	5	NS	NS	NS	At least twice weekly for 4 weeks	ELISA, PhiCal	>350 with GI injury Not sick: 122.8 ± 98.9 Sick: 380.4 ± 246.3
Campeotto JPGN EL 2	2009	126	33 (25.7-35)	1760 (730-2750)	NS	NS	NS	NS	Weekly for 1 <sup>st</sup> 4 weeks	ELISA, Calprest	226 (16-4775) if birth wt <1800g ROC Cutoff for 'intestinal distress': 363 (Sens: 0.65; Spec: 0.82)
Thuijls Ann Surg EL 2	2010	35: 14 NEC 21 Other	N: 30+5 (27+2-38+2) O:30+5 (25-41+2)	N:1465 (860-1960) O: 1198 (585-3570)	NS	NS	NS	NS	Daily	ELISA, Hycult	NEC cut off point: 286.2 (Sens: 0.86/Spec: 0.93). Only 21 infants produced pre-NEC samples
Westerbeek Ped Res EL 1	2011	113	<32	<1500	NS	NS	NS	NS	Days 7, 14, 30	ELISA, Buhlmann	Levels correlated with stool IL 8. No difference with prebiotics. ↑ levels in NEC.
Aydemir J Mat Fetal Neo Medicine EL 2	2012	50 NEC: 25 Non: 25	NEC: 28.3 +2.5 Non: 28.9 (2.0)	NEC: 1048 (323) Non: 1044 (232)	NEC: 12 Non: 5	NS	NEC: all Non: NS	E, or preterm formula Numbers NS	At diagnosis of NEC and 3-5 days later	ELISA, Buhlmann	NEC: 1282 (241-3337) Non: 365 (58-1006) A level of 792 was 76% sensitive, 92% specific for NEC
Selimoglu Paediatric critical care med EL 2	2012	37: NEC: 14 Non: 23	NS	NS	NS	NS	NS, but any septic infants excluded	24: E 15: F 1: TPN	At first clinical signs NEC + same age matched controls	ELISA, PhiCal	Not predictive or diagnostic of NEC: 167.56 (143.43) Vs 172.2 (171.25) No differences in feed type
Zoppelli	2012	206	28.5	1057	NS	NS		NS	Alt days for first	ELISA,	Levels depend upon gestation +

Neonatology EL 2		NEC: 14 F-NEC: 5 GID: 47 Non: 140	26.6 (23.7-33.1) 25.4 (24.3-27.0) 27.1 (23.3-32.4) 29.2 (24.4-35.7)	859 (295) 672 (187) 888 (296) 1145 (253)					5.8 days (1.6) 7 days (0) 4.8 days (2.8) 4.5 days (2.7)	28 days of life	Bensheim, Germany	postnatal age; Cut offs: 180 for intestinal distress, 210 for NEC. Fulminant NEC however: <24
Reisinger J Pediatr Surg EL 2	2012	62 NEC: 29 Non: 33	215 days (175- 289) NEC: 215 (184- 268) Non: 213 (175- 289)	1328 (585- 3570) 1400 (860- 1960) 1270 (585- 3570)	NS	NS	NS	NS		At time of NEC diagnosis – but only in 35/62 infants	ELISA, Hycult	Promising as diagnostic of NEC in combination with urinary IFABP; NEC: 402.2 (107.6-847.6) versus 79.6 (1- 625.1); Cut off for NEC: 286.3 (sens 81%, spec 79%). With UIFABP: sens 63%, spec 100%)

Table 9: The evidence base for calprotectin as a marker of NEC; given the heterogeneity in NEC definitions, sensitivity and specificity analyses were deemed inappropriate. (Abbreviations: NEC = necrotising enterocolitis; ELISA = enzyme linked immunosorbant assay; NS = not specified; UIFABP = urinary intestinal fatty acid binding protein; EBM = expressed breast milk; DEBM = donor expressed breast milk; GI = gastrointestinal; EL = evidence level).

In total, this systematic review included one article describing a randomised controlled trial (evidence level 1), and ten with controlled data (evidence level 2). Although cumulatively these studies compare calprotectin levels in 1485 infants, the heterogeneity of NEC definitions makes direct comparison in metaanalysis inappropriate. In two studies by the same research group, different definitions of NEC are used in each publication, including terms such as ‘intestinal distress’ and ‘enteropathy’ (Campeotto, Kalach et al. 2007, Campeotto, Baldassarre et al. 2009). Similarly, the significance of potential confounders of high calprotectin levels is not accounted for in some studies (for example postnatal age, antibiotic usage, and episodes of concurrent sepsis). Each published study uses ELISA testing, and most use the same commercially available kit by Calprest (Eurospital, Trieste, Italy). One article was identified that considered serum calprotectin levels rather than stool concentrations, and identified a significant peak during episodes of sepsis (Terrin, Passariello et al. 2011). Eight of the 20 studies found that stool levels rose in tandem with definite NEC – but only after that diagnosis had been made clinically. The median calprotectin concentrations for infants with confirmed NEC varied widely between 210 µg/g, to over 2000 µg/g. Some studies quoted a cut-off level for definite NEC, which was far lower than other studies. Although some of the studies stratified levels by severity of NEC, comparing stage 2 and stage 3, only one considered the effect of perforation – perforation accounted for in only stage 3b NEC and not 3a. Curiously, the paper by Zoppelli et al in 2012, noted high levels with definite NEC, but a sharp decrease in calprotectin concentration with fulminant perforated NEC. The reasons for this are unclear from the subsequent discussion within the article, but could be: 1) fluid stool samples – e.g. diluted with frank blood; 2) total ileus resulting in decreased production of any stool or effluent; 3) perforation leading to peritoneal contamination with stool and thus calprotectin; 4) loss of permeability of necrotic bowel, thus neutrophilic apoptosis redirected back into the circulation (Zoppelli, Guttel et al. 2012). Further studies, in particular those powered to NEC, are ideally placed to investigate the predictive and prognostic potential for calprotectin.

*iv) SIgA and necrotizing enterocolitis*

The high mortality and morbidity associated with NEC have given rise to a host of potential immunotherapies, but no studies could be identified assessing changes in SIgA according to infants with and without NEC. However, within the last ten years, a vogue for the oral administration of pooled immunoglobulin and combinations therein of Ig G and Ig A has dwindled, with repeated updated Cochrane Reviews showing no benefits in the

treatment or prevention of necrotising enterocolitis, despite eligible trials involving a total of 2095 preterm infants (Foster and Cole 2004). In the mammalian intestine, colonisation with commensal microbes is considered to enable the development of both humoral and cellular mucosal immune systems during neonatal life (Cebra 1999, Stagg, Hart et al. 2003) – hence the potential for therapeutic probiotic administration to increase colonic SIgA levels, as seen by Mohan et al (Mohan, Koebnick et al. 2008), and Retnaningtyas (Retnaningtyas 2008).

#### **1.6.8) Management of NEC**

Treatment of NEC is therefore multifactorial, involving manipulations of diet, antibiotics, and surgery. Given the unpredictability of NEC, wherein relatively ‘mild’ cases of NEC can evolve into fulminant necrosis and death within hours, the importance of prevention cannot be overemphasised. However, despite advances in management, the incidence of NEC has remained relatively constant throughout the last two decades (Fanaroff, Hack et al. 2003). The economic impact of NEC-related morbidity cannot be underestimated, nor the dietary impact therein. Projected cost analysis in one study reported there to be an estimated 3440 more infants per year with NEC in the United States if fed exclusively formula instead of breast milk, to the tune of \$3.5 million dollars extra in the cost of care (Arias 2002, Meizen-Derr, Poindexter et al. 2009). Furthermore, the estimated cost of treating an infant with short bowel syndrome secondary to NEC is 1.5 million dollars over five years. As such, preventative strategies are in development, focussing on immunoglobulin administration, oral aminoglycosides, glutamine, feed regimes, and manipulation of the gut microbiota.

### 1.6.9) Animal models: relevance to the research of necrotising enterocolitis

In the absence of ethically approved observational and interventional human studies regarding the aetiology and evolution of NEC, there are increasing numbers of published animal models, inducing NEC lesions biochemically and microbiologically, in order to assess the diagnostic and therapeutic potentials. The gnotobiotic and monobiotic quail model of NEC has been used repeatedly in studies by Waligoria-Dupriet et al, with the advantage of being able to precisely control feed type and frequency, in a consistently temperate environment, and to closely correlate stool and serum biomarkers with the emergence of NEC lesions. In one study, Waligoria-Dupriet et al were able to chart the histological sequence of events in the evolution of NEC after inoculation with *Clostridium butyricum*, observing mononuclear cell infiltrates, followed by the emergence of heterophilic cells, promptly followed by gaseous necrosis and cystic change (Waligoria-Dupriet, Dugay et al. 2005). This study followed from their work published in 2005, identifying the gross histopathological intestinal effects of lesions produced by the actions of 6 different bacterial strains implicated in NEC in human observational studies (*Klebsiella pneumonia*, *Clostridium perfringens*, *difficile*, *paraputrificum* and *butyricum*). Once again, *Clostridium butyricum* was linked to direct visual evidence of pneumatosis, as seen in other trials in both animal models and preterm humans (Waligoria-Dupriet, Dugay et al. 2005, Azcarate-Peril, Foster et al. 2011, Smith, Bode et al. 2011) Similar induction of NEC intestinal lesions in preterm pigs have begat a cohort of trials in this regard. Several studies have validated the use of piglet models according to their similar oesophageal motility and feed performance to human neonates (Di Lorenzo, Bass et al. 1995, Vicente, Da Rocha et al. 2001, Sangild, Siggers et al. 2006, Sangild, Tappenden et al. 2006, Rasch, Sangild et al. 2010). Moreover, the close histological similarity of NEC-lesions in preterm piglets closely mirrors those of preterm infants. Methods of inducing these lesions are now being tested, and include formula milks, hypoxic-ischaemic insults, hypothermic stress, and administration of bacterial toxins – for example *clostridium perfringens* beta-toxin (Miclard, Jaggi et al. 2009).

Animal models of NEC are therefore also developing therapeutic options of management. So far, therapies trialled in animal models of NEC include: L-arginine (Akisu, Ozmen et al. 2002, Cekmez, Purtuloglu et al. 2012); nitric oxide (Cekmez, Purtuloglu et al. 2012); n-acetyl cysteine (Koivusalo, Kauppinen et al. 2002, Ozdemir, Yurttutan et al. 2012, Tayman, Tonbul et al. 2012); and cyclosporine (Gill, Lee et al. 2012, Gill, Manouchehri et al. 2012). In turn, these have led to fledgling human trials in preterm infants, mainly in L-

arginine administration in preterm infants, which so far shows no clear benefit in preventing or ameliorating NEC (Shah and Shah 2007). However, the anticipated translation of animal trials to human trials does not appear to progress when assessing the international clinical trials databases. Registered trials at the clinicaltrials.gov website citing interventions with the primary outcome of prevention and/or treatment of NEC, include: the administration of docosahexanoic acid in the prevention of NEC (Garcia et al, National Council of Science and Technology, Mexico); vitamin A in the treatment of NEC (Johns Hopkins University, Maryland); and pentoxifylline as a treatment of NEC (Shaare Zedek Medical Center, Israel) (Health 2013).

However, one contentious potential therapeutic option preventing NEC in preterm infants is currently undergoing extensive trials in both animal models and humans: the administration of pre- and probiotics. Prebiotic animal trials have utilised gnotobiotic quail, rats, and piglets, with increased growth of beneficial species of Bifidobacteria, and reduced numbers of clostridia associated with lower butyric acid concentrations (Butel 2001). Catala et al in 1999 inoculated gnotobiotic quails with microflora from preterm twins, and assessed the subsequent effect of oligofructose on the resultant microbiota, which showed a significant increase in the growth of Bifidobacteria species and, conversely, a reduction in *E.coli* and *Clostridia perfringens* (Catala, Butel et al. 1999). Probiotic animal models to date have used strains including *Bifidobacterium bifidum* and *Lactobacillus reuteri* with positive results. Rat models have shown similarities in the production of antimicrobial colonic peptides produced in humans with NEC, and administration of Bifidobacteria strains have observed the induction of higher levels of lysozyme and secretory phospholipase A2 (Underwood, Kananurak et al. 2012). The advantage of such trials is the ability to directly assess the histopathological and immunological effects of these bacteria, including downregulation of inflammatory markers IL-6, and TNF  $\alpha$ , and enhanced expression of the anti-inflammatory cytokine IL-10 (Khailova, Dvorak et al. 2009, Khailova, Mount Patrick et al. 2010, Liu, Fatheree et al. 2012). However, the method of inducing NEC in animal models may be a significant confounder of these studies. Most are induced using asphyxia or hypothermia, but this may not replicate typical NEC as found histologically in preterm infants. This was acknowledged by Zhou et al in 2011, when they compared three different methods of inducing NEC in rats, including hypothermic stress, varying proportions of feeds and nitrogen gas, intraperitoneal saline, and intraperitoneal lipopolysaccharide. They found that the combination of artificial feeding with hypoxia and hypothermia most closely replicated

intestinal injury correlating with NEC in humans (Zhou, Zheng et al. 2011). These studies have naturally led to trials of pre- and probiotics to term and then preterm infants.

### **1.6.10) Therapeutics:**

#### *i) Prebiotics*

Prebiotics, as defined by Roberfroid et al, are a ‘selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health’ (Roberfroid 2007). These are typically, but not exclusively, carbohydrates found naturally in a wide variety of food stuffs, especially vegetables. Various carbohydrate-based substances are now incorporated into commercial prebiotic preparations. Inulins, a group of polysaccharides found commonly in vegetables such as chicory, onion and garlic, are a popular commercial choice. Lactulose, an osmotic laxative, has been seen in adults to exert a prebiotic effect, stimulating the growth of *Bifidobacteria* and *Lactobacillus* (Saarela, Mogensen et al. 2000, Vanhoutte, De Preter et al. 2006). This also raises the possibility of changing the SCFA profile, as seen in adults (Hamer, Jonkers et al. 2009). In both the UK and the USA, prebiotic preparations are neither classed as foodstuffs or drugs. A variety of trials in animals claim to reduce the incidence of malignancies and IBD, and to boost immunity and cardiovascular health, although the specific mechanisms by which these occur remain unknown in most cases (Liong 2008, Ellis, Rutledge et al. 2010, Lam, Moulder et al. 2012, Hemarajata and Versalovic 2013, Whelan and Quigley 2013). It is generally theorised that these benefits occur secondary to increased SCFA production by the proliferation of bacteria such as clostridia and bacteroides.

Breast milk contains natural prebiotics in the form of human milk oligosaccharides (HMOs). Although many are excreted in the urine intact, some remain undigested in the small intestine, and enter the colon to undergo bacterial fermentation to short chain fatty acids. The resultant environment promotes the growth of beneficial bacteria which preferentially ferment these carbohydrates for energy, but this effect is not seen to be strain-specific. The resultant bacterial strains from breast milk are then repeatedly isolated for further testing of their properties in order to achieve probiotic status (Beattie and Weaver 2011, Fernandez, Langa et al. 2012). However, the ability of breast milk probiotic bacteria to be transferred to the gut microbiota of the nursing infant is difficult to replicate in exogenous administration, given the propensity for commercially produced, enterally administered probiotic bacteria to be destroyed by stomach acid and bile salts. This has led to the development of ‘microcapsulation’ techniques to preserve the integrity of the probiotic until it reaches the small bowel, and various studies have used different coatings in this regard (Cook, Tzortzis et al. 2012, Piano, Carmagnola et al. 2012).

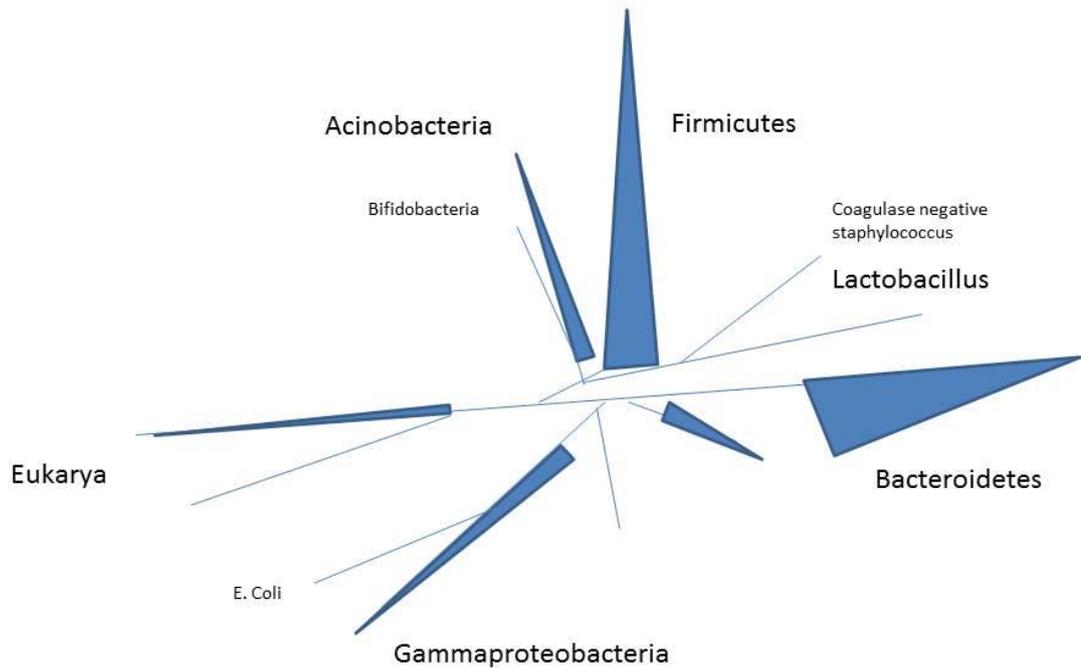


Figure 8: Phylogenetic tree of common gut commensals found in preterm infants. The size of each leaf corresponds to abundance in the stool of preterm infants according to literature; bacteria are linked by boughs according to similarity in morphology.

ii) *Probiotics*

Probiotics are defined by the World Health Organisation as being ‘live microorganisms which, when administered in adequate amounts confer a health benefit on the host’ (Araya 2001). It is possible that this definition will be refined as further properties and functions of such bacteria are established. Currently, probiotic preparations, as for prebiotics, are classified neither as foodstuff or drugs, although recently the portmanteau ‘nutraceuticals’ has been coined to appreciate its value as both. *In vitro* studies have illustrated that the effects of probiotic bacteria are similar regardless as to whether they are live, heat-killed, or even in DNA form (Orlando, Refolo et al. 2012, Tareb, Bernardeau et al. 2013). Certain forms of heat-treated *Lactobacillus* strains have even been observed to have enhanced stability and immunological effects after heat-treatment (Fujiki, Hirose et al. 2012). In particular certain *Bifidobacterium* strains stimulate the production of interleukin 10 and immunoglobulin E (Young, Simon et al. 2004). *Lactobacillus* is seen to reduce pathogen adherence, as well as effecting a protective immunoglobulin A layer effect over other surfaces such as respiratory, nasal, oral and vaginal mucosae (Perdigon, Alvarez et al. 1999, Kotani, Shinkai et al. 2010, Strus, Chmielarczyk et al. 2012). Figure 8 illustrates the

relationship between ‘probiotic’ species and other commonly identified gut microbiota in preterm infants. In order to qualify as probiotic, a bacterial strain must have several properties as defined by Teitelbaum et al in table 10:

A probiotic should:
Be of human origin
Be in nature
Be resistant to destruction by technical processing
Be resistant to destruction by gastric acid and bile
Adhere to intestinal epithelial tissue
Be able to colonize the gastrointestinal tract, if even a short time
Produce antimicrobial substances
Modulate immune responses
Influence human metabolic activities (i.e., cholesterol, assimilation, vitamin production, etc.)

Table 10: Defining Criteria of Microorganisms That Can Be Considered Probiotics (Teitelbaum and Walker 2002)

Given newer culture techniques, different functions of *Lactobacillus* and *Bifidobacteria* strains have been identified, including their ability to inhibit growth of several urogenital pathogens, and their adherence to Caco-2 cells, reflecting their innate ability to adhere to the intestinal mucosa (Martin, Jimenez et al. 2006, Cadieux, Burton et al. 2009). Probiotic bacteria may have different properties *in vivo* and *in vitro*, as evidenced by laboratory and animal studies (Ibnou-Zekri, Blum et al. 2003). Identical probiotic bacterial strains have also been seen to exhibit different functions depending on the host – such that the same strain in a preterm infant can display different properties in an elderly adult (Romeo, Nova et al. 2010). Of further interest still is the finding that enterally administered probiotics can colonise remote mucosal surfaces after absorption through Peyer’s patches, and vehiculation in the lymphatic circulation to the respiratory and urogenital surfaces (Rescigno, Rotta et al. 2001, Macpherson and Uhr 2004, Perez, Dore et al. 2007, Pennisi 2008).

Recent studies have suggested that not only could these strains not have to be alive in order to exert beneficial effects on the host, but that fragments of bacterial DNA have the capacity to bind to and activate mucosal receptors – that is, in live and ‘killed’ forms. This emphasises the importance of the surrounding environment, and cross-contamination (Lammers, Brigidi et al. 2003, Zhang, Li et al. 2005, Broekaert, Nanthakumar et al. 2007). The T-Cell receptor (TCR) response may be important in this regard, given the increasingly convincing evidence of host-specific and strain-specific probiotics and their ability to stimulate an immune cascade, which includes cytokines (such as interleukin-4) acting as directors of a host of other innate and systemic immune factors (Kalliomaki, Kirjavainen et al. 2001).

It is important to note that certain strains of so-called enteropathogens are also considered ‘probiotic’, including *E. coli* M17, *E. coli* Nissle 1917, and *Clostridium butyricum* (Araki, Andoh et al. 2004, Fitzpatrick, Small et al. 2008). Similarly, strains of exogenously administered probiotic bacteria have been implicated in case reports of sepsis, and also one randomised controlled trial of probiotics in adults (Gooszen, Simmermacher et al. 2004, Lee and Siao-Ping Ong 2011, Mehta, Rangarajan et al. 2012).

### *iii) Synbiotics*

Synbiotics are simply a combination of pre and probiotics, both endogenous and exogenous, breast milk being the most obvious naturally occurring in nature. As the roles of newfound metabolites are defined, ‘synbiotic’ may be used as a collective term for all metabolites of microbial fermentation processes of benefit to the gut. It is postulated that the benefit of combining prebiotics with probiotics is not so that prebiotics can stimulate the growth of probiotics within these preparations, but that they work on different areas of the gut – prebiotics mainly on the colon, and probiotics on the small intestine (Roberfroid 2007).

## **1.6.11) Therapeutic alteration in the gut microbiota of the preterm infant**

### *i) Prebiotics*

In premature infants the establishment of enteral nutrition is often slow, and as such they are known to be deficient in micronutrients such as glutamine, vitamins E and A despite supplementation within parenteral nutrition (Powers 1993, Kositamongkol, Suthutvoravut et al. 2011). However, evidence for benefits of supplements designed to alter the gut microflora in preterm VLBW infants is unclear (Tubman, Thompson et al. 2005, van den Berg, van Elburg et al. 2007, Li, Bauer et al. 2012). It is possible that these effects are

simply overwhelmed by other stronger factors, such as the influence of antibiotics, and type of milk fed. One study concerning term formula fed neonates revealed higher levels secretory immunoglobulin A in those supplemented with prebiotics (Bakker-Zierikzee, Tol et al. 2006). Most preterm infant formulae now include prebiotics in the form of galacto-oligosaccharides and fructo-oligosaccharides (GOS and FOS). Outcomes are awaited from an Israeli RCT assessing the effect of prophylactic lactulose administration on microbiota and NEC in premature infants (Dollberg 2010).

ii) *Probiotics*

It is postulated that manipulation of dietary factors with the addition of probiotics (live bacterial colonies of favourable species) may reduce the incidence of short and long-term illness in these infants, particularly NEC (Deshpande, Rao et al. 2007). However, the types of favourable species and strains have yet to be defined in healthy preterm infants (figure 8). Several studies have illustrated inhibition of NEC-like lesions in animal models by *Bifidobacterium* supplementation (Butel, Roland et al. 1998, Caplan, Miller-Catchpole et al. 1999), as well as a reduction in faecal butyrate (Wang, Shoji et al. 2007). The probiotics used in preterm neonatal RCTs to date have employed combinations of different strains of *Lactobacillus* and *Bifidobacterium* sp., owing to their immunogenic, adhesive, and antienteropathogenic properties. Undeniably, they are also used because of their wide commercial availability. The results of these trials indicate that probiotics could reduce NEC in VLBW infants. However, there are two very different interpretations of these trials. On one hand, the latest metaanalysis (including 11 trials) concludes that the evidence for their efficacy is so great that no further RCTs are required before routine implementation. However, a previous systematic review of probiotic therapy to prevent NEC in premature infants found only six inclusive randomised control trials, but noted that although a trend towards reduction of NEC in the treatment groups was observed, the heterogeneity of probiotic types, doses, and frequency and duration of administration made comparisons impossible (Barclay, Stenson et al. 2007). Fundamentally, the diversity of these trial designs, using different species, strains, doses, frequencies and durations, makes comparison inappropriate. As Guarner *et al* noted in 2007: ‘the effect of a bacterium is strain-specific and cannot be extrapolated even to other strains of the same species’ (Guarner 2007). These trials are also confounded by feeding practices and the widespread use of prebiotic formula milks, and it is impossible to separate data on extremely preterm infants (Beattie, Hansen et al. 2010). Moreover, several of these studies are confounded by environmental and maternal nutritional factors that also affect the composition and rate of

acquisition of the gut microflora. Most of these have been developed from the methodology of previous RCTs, and are limited by commercial availability of strains.

The United Kingdom's first randomised control trial of probiotics in preterm infants is currently underway, aiming to recruit 650 infants to each wing (Costeloe). Previous reviews of probiotic therapy for NEC have postulated that between 750 and 2000 infants would require to be exposed in order for a significant reduction in NEC to be evaluated (Barclay, Stenson et al. 2007, Neu and Shuster 2010).

One recent study has attempted to assess metabolic and inflammatory effects of probiotic supplementation in preterm infants, although was not powered to NEC, nor does it mention cases of this illness within the cohorts. In 2008, Mohan et al enrolled 69 low birth weight infants into a randomised control trial using *Bifidobacterium lactis* B12 and a placebo. They found significantly higher faecal lactate and acetate, and faecal IgA, as well as lower faecal pH and calprotectin in the treatment group. Interestingly, higher body weight was only seen in infants who had received antibiotics as well as probiotic therapy (Mohan, Koebnick et al. 2008). Following from this, it is possible that the manipulation of other nutritional, clinical and environmental factors, and establishment of associations between bacteria, their products, and inflammatory markers may further research in this important area. As highlighted by Rouge et al in 2010 (Rouge, Goldenberg et al. 2010), given the high interindividual variability in gut microbiota, the combination of these techniques gives rise to the exciting possibility of personalised therapy.

#### a) *Probiotic Safety*

None of the preterm RCTs have documented probiotic-related sepsis. Other milder adverse reactions, such as diarrhoea and flatulence, are difficult to interpret. Several neonatal case reports documenting sepsis with 'probiotic' bacteria have not been related to probiotic administration (Brook, Frazier et al. 1991, Thompson, McCarter et al. 2001), but were considered gut translocation through natural acquisition. However, two further case reports have declared *Bifidobacterium* sepsis secondary to probiotic administration (Ohishi 2010, Jenke 2012) - the latter concerning a preterm infant. Given the difficulty in culturing these organisms, cases of probiotic bacteraemia merit further analyses to establish the 'pathogenicity' of these strains.

Most importantly, the advent of probiotic therapy must be tempered with the recognition of potential short and long-term adverse effects. Future implications of supplementing the diet of the preterm infant with probiotics have lifelong potential. Organisms first colonising the gut after delivery may achieve permanence, as has been shown in studies of children years after probiotic supplementation in term neonates (Kalliomaki, Kirjavainen et al. 2001, Kalliomaki and Isolauri 2003). This has yet to be proven in preterm infants. Conversely, studies in adults and infants have shown certain probiotic strains to have a short half-life – sometimes days (Alander, Satokari et al. 1999, Schultz, Gottl et al. 2004, Saxelin, Lassig et al. 2010).

The possibility of probiotic-related sepsis is a theoretical yet very realistic possibility in immunologically immature preterm infants. Only one case report could be found describing *Lactobacillus acidophilus* sepsis in a premature infant (Thompson, McCarter et al. 2001). Other cases have been limited to adults in whom *Bifidobacterial* strains have caused wound abscesses after obstetric and gynaecological procedures (Brook, Frazier et al. 1991), and a large randomised control trial of prophylactic probiotics for acute pancreatitis in adults, in which the treatment group had a significantly higher mortality.

Previous rashly implemented therapeutic interventions for premature infants include high-dose dexamethasone therapy, subsequently shown to cause cerebral palsy (Halliday, Ehrenkranz et al. 2009). In short, no probiotic studies for preterm infants have justified: 1) the best type or combinations of bacteria and strains to be used; 2) the most appropriate doses; or 3) when to use them, and how long to use them for. The safest and most appropriate method prior to testing probiotics on premature infants is through the use of *in vivo* and animal models. These are essential points of information required for any pharmaceutical intervention, and we believe that manipulation of gut microbiota in any administered form should undergo rigorous examination in this regard.

- b) Current Randomised Controlled Trials Registered on International Clinical Trials Database ([www.clinicaltrials.gov](http://www.clinicaltrials.gov)):

Type of Nutraceutical	Country	Status	Number for recruitment	Gestation + Birth weight	Age at enrolment	Protocol	Primary Outcomes	Stool analytes
<i>Bifidobacterium breve</i> C50 + <i>streptococcus</i>	Paris, France	Completed	58	30-35 weeks	>3 days	Not specified	Stool colonisation	Microbial, immune, inflammatory
<i>ProBioPlus: Lactobacillus acidophilus, Bifidobacterium longum, Bifidobacterium infantis, and Bifidobacterium bifidum</i> plus inulin <i>Lactobacillus rhamnosus GG</i> plus fructo-oligosaccharide, ConAgra	California, USA	Completed	90	<35 weeks 750-2000g	<8 days	Twice daily for 28 days	Weight gain	Microbial + butyric acid
<i>ProLactPlus: human milk-derived oligosaccharides Galacto-oligosaccharide Bifidobacterium infantis Bifidobacterium animalis</i>	USA	Recruiting	36	<33 weeks + <1500g	<15 days	Each increases weekly	Stool colonisation	PCR for <i>Bifidobacteria</i> + total bacteria
Galacto-oligosaccharide	Israel	Recruiting	260	'Preterm' + <1750g	<8 days	1.3g/kg/d from start of enteral feeds until 35 weeks CGA	NEC	Bifidobacteria; calprotectin; urinary IFABP
<i>Bifidobacterium Bb12</i>	London	Recruiting	1300	<31 weeks	<48 hours	Daily from recruitment until 36 weeks CGA	NEC, death, infection	Stool flora

Table 11: Current Registered Randomised Controlled Trials of probiotic and prebiotic preparations for preterm infants (Health 2013). (Abbreviations: USA = United States of America; g = grams; NEC = necrotising enterocolitis; < = less than).

This collection of international registered clinical trials not only illustrates the heterogeneity of ‘nutraceuticals’ targeted to preterm infants, but also the variety of primary outcomes. This indicates the wide ranging effects of prebiotics, probiotics, and therefore synbiotics in this important patient group. However, given that the effects of single probiotic strains on NEC, allergy, feed tolerance, weight gain and sepsis in preterm infants is as of yet unascertained, the increasing popularity of RCTs of multiple probiotic and symbiotic combinations makes comparison in meta-analyses inappropriate. What is encouraging is that all of these trials are concurrently measuring stool markers, not only to ensure colonisation of these infants, but also to assess the immunological and inflammatory effects. Whether there are enough preterm infants of extreme prematurity and with extreme low birth weight to assess their response to these supplements, and their resultant stool microbiota and analytes in healthy controls to glean ranges of normative gestation and/or birth weight dependent data in order to establish these as diagnostic and prognostic markers of disease.

### **1.7) Introduction to Study Purpose**

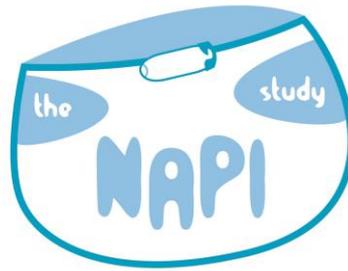
With the escalation in number and type of randomised controlled trials designed to assess the clinical effects of prebiotics, probiotics, and synbiotics on the health of preterm infants, there are still many confounders of establishment and maintenance of the gut microbiota that may easily skew the results of these trials. The emphasis of feed type, administration of antibiotics, duration of ventilation and incubation are all important and necessary factors of neonatal care. Feed type is most commonly influenced by maternal choice, despite the establishment of donor milk banks, with increasing accessibility and supply. The synbiotic effect of breast milk, and the recognised risks of cross-contamination of probiotic bacteria between Neonatal Intensive Care Unit patients make the investigation of these effects upon the gut microflora and metabolites of infants without symbiotic administration imperative if we are to separate what can already be controlled and augmented with nutritional and environmental trials, and what is added by the use of nutraceuticals. As stipulated earlier, details such as precise feed ratios and types, duration of incubation, types and durations of antibiotic courses are often absent from comparison between case and control groups, despite being important associations with NEC and sepsis.

Additionally, the significance of changes in the gut microbiota, metabolites and inflammatory markers in preterm infants – in particular NEC, sepsis, poor weight gain, and mortality therein – highlight their potential as biomarkers of these diseases. Observational studies, such as the NAPI Study described in this thesis, are therefore required to illustrate the relevance of including stool SCFA, calprotectin, SIgA and TTGE analyses for inclusion in the many evolving randomised controlled trials aiming to alter the gut microbiota by enteral supplementation.

## Chapter 2

### Methodology

#### THE NAPI STUDY: Neonatal microflorA in Preterm Infants



#### 2.1) Introduction

Despite the increasing popularity of RCTs aiming to alter the gut microbiota in preterm infants, there is a paucity of normative data concerning gut bacteria, bacterial metabolites, immunological and inflammatory markers in preterm infants in health and disease. As such, the NAPI Study was designed as an observational cohort study of inborn infants throughout Glasgow's three neonatal units. The following chapter describes the study hypotheses, design, methodology, and statistics.

##### 2.1.1) Hypotheses:

###### a) Primary:

Levels of stool metabolites, markers of intestinal inflammation, and diversity of gut flora of infants born before 32 weeks of gestation vary over the first month of life according to the type of nutrition and environment.

###### b) Secondary:

Stool parameters:

1. All analytes will vary with gestation, age, feed type, NEC and sepsis, and will co-correlate
2. BCFAs will be detected in stool samples, indicating protein fermentation
3. SIgA levels in milk will correlate with those in infant stool
4. Each of the analytes will vary with severity of NEC. It should be noted that this hypothesis was added only after recruitment ended and the high incidence of NEC was noted.

## **2.2) Study Design and Methodology:**

### 2.2.1) Study Design

The Neonatal MicroflorA in Preterm Infants (NAPI) Study was designed as an observational cohort study of sequentially recruited VLBW, preterm infants delivered at less than 32 weeks gestation. It was approved by the Glasgow Royal Infirmary Research and Ethics Committee on April 14<sup>th</sup> 2009 (REC reference number: 09/S0904/15), and was also approved by the Greater Glasgow and Clyde NHS Research and Development Board on April 20<sup>th</sup>, 2009 (R+D reference number: GN09NN090). Recruitment commenced on April 24<sup>th</sup>, 2009, and finished on February 20<sup>th</sup> 2010. Furthermore, after one study patient transferred to Crosshouse Hospital in Kilmarnock, local R+D approval was sought and gained from the NHS Ayrshire and Arran Research and Development department on June 11<sup>th</sup> 2009 (Reference Number: 2009AA028).

Glasgow city's population is currently around 600,000, and the birth rate between all 3 maternity hospitals within the city was over 7000 in 2011. Infants were sequentially recruited from the three main Glasgow Neonatal Units: Queen Mother's Hospital, Princess Royal Maternity Hospital, and the Southern General Hospital. Given that the observational nature of the project, along with a lack of similar studies, did not lend itself to reliable power calculations, we decided to recruit at least 60 infants annually based upon a) the relative numbers of inborn infants within the inclusion criteria per year according to local audit data; b) timing and cost of the sample analysis; c) comparable studies in preterm infants, the highest number in which included 69 neonates (Mohan, Koebnick et al. 2006).

#### **i) Recruitment**

Inclusion criteria comprised:

- <32 weeks gestation
- <1500g birth weight
- CRIB score < 15
- Fed within the first 7 days of life

Exclusion criteria comprised:

- 32 weeks gestation
- >1500g at birth
- Nil by mouth within the first week of life
- CRIB score > 15
- Any major congenital abnormality

Major congenital abnormalities were considered on a case-by-case basis. The Clinical Risk Index in Babies Score was considered as a marker of illness severity and was applied within the 12 hours preceding consideration for recruitment. A CRIB Score of more than 15, equivalent to projected mortality risk of more than 50%, excluded infants from recruitment at that point (Patrick, Schumacher et al. 2013).

### **Recruitment Process**

Infants were recruited within the first week of life. Infants fitting the inclusion criteria were identified by me, and parents were initially approached by a leaflet describing the project. Once inclusion criteria were confirmed, parents were asked in person for their consent for their baby to take part. The mothers also completed a short questionnaire with me, assessing their consumption of probiotic foodstuffs. The parents of any infants who scored >15 on the Clinical Risk Index for Babies illness severity scoring system (CRIB), were not approached for consent. All infants were delivered as clinically appropriate, and no additional monitoring occurred for the sake of this trial either intra- or post-partum. Implicit in obtaining consent, I discussed each baby's current severity of illness with Nursing Staff caring directly for the infant, as well as the emotional status of the parents. In the event that it would clearly be insensitive or an imposition to discuss the project with the parents of a critically unwell infant (or, indeed, a critically unwell mother), or one who was not expected to survive, I did not approach for consent, and these infants were excluded until stabilisation. The greatest care was taken not to approach fragile parents, and it should be noted that all members of staff involved had extensive daily experience in counselling and discussing issues of care with parents of ill preterm infants.

#### **ii) Sample collection:**

Samples of normally voided stool, and expressed breast milk were taken non-invasively: stool was retrieved from the nappy during routine nursing care, and immediately stored in freezers at -20<sup>0</sup>C; milk was retrieved from the milk freezers by me. Samples were then transported in thermal bags.

**Stool:** samples for four weeks, aiming to analyse one per week, from recruitment.

**Milk:** Maternal breast milk was sampled at least once weekly for four weeks.

Sample analysis and interpretation were split into four epochs as follows:

Days:

- 1 – 7;
- 8 – 14;
- 15 – 21;
- 22 – 28.

### 2.2.2) Analyses

The following analyses were performed:

Stool:

- Stool short chain fatty acids and branched chain fatty acids by gas chromatography/mass spectrometry;
- Calprotectin and secretory IgA analysis by Enzyme Linked Immunosorbant Assay (ELISA);
- Bacterial diversity by Temporal Temperature Gel Electrophoresis.

Milk:

- SIgA and calprotectin by ELISA kits.
- Milk samples were also stored for future molecular analysis.

Data: demographical, clinical, nutritional and environmental factors.

### 2.2.3) Necrotising Enterocolitis:

Recruited infants who incurred the gut inflammation and infective disorder known as necrotising enterocolitis (estimated 10% - a projected maximum of 6) had this diagnosis made by two independent physicians using the internationally renowned Modified Bell Criteria (table 7). In the event of discrepancies in diagnosis, a consultant acted as arbiter. Part of the Modified Bell Criteria involved the interpretation of an abdominal x-ray, and the opinion of a consultant paediatric radiologist was taken for this purpose. One aspect of the Bell's Criteria involves testing the stool for blood using the Faecal Occult Blood test (FOB), which can identify microscopic amounts of blood not visible to the naked eye. In Glasgow this test is now obsolete given the over-sensitivity in preterm infants, in whom blood associated mild gastritis or even nasogastric tube passage resulted in positive tests in the absence of NEC (Pinheiro 2003). As such, the presence of any visible, fresh gastrointestinal tract bleeding was taken in lieu of this sign.

#### **2.2.4) Demographical and Clinical Data**

Demographical and clinical data was retrieved from medical and nursing records. Demographics were sub-classified into: basic data (such as gender, birth weight, gestation); social data (including the Deprat score, a Scottish deprivation scale (McLoone 2004). It is based upon demographical information including four census variables depicting employment status, overcrowding, social class, and material hardship, and is stratified according to postcode. A higher score equates to higher deprivation); and antenatal data (including PPRM, intrapartum antibiotics, multiparous pregnancy). Clinical data was further stratified into: management data (for example Apgar score - interpretation of scores is more difficult in prematurity, but still performed. Ten minute Apgar scores of less than 3 are associated with a poor outcome - duration of ventilation and incubation, PDA ligation); nutritional data (including feed type and volume, time to full feeds, addition of fortifier); sepsis data (including number of episodes; type of bacteria cultured; highest CRP during study period; number of antibiotic days); and NEC data (stage by Modified Bell's criteria; day of first onset; type of surgery).

Of note, antibiotic regimen varied according to Neonatal Unit preference, age at use, and clinical features. Since preterm delivery is a risk factor for sepsis in itself, all infants in the study were given antibiotics for at least 48 hours after birth. In the PRMH and QMH units, these were benzylpenicillin and gentamicin, whereas SGH preferred to use cefotaxime. For infants requiring antibiotics after this initial 48 hour period, vancomycin and gentamicin were used. For infants requiring antibiotics for suspected NEC, the combination of vancomycin, gentamicin and metronidazole were used in all cases in all units. Infants were also occasionally given antibiotics different to these in cases of treatment-resistant coagulase negative staphylococcus sepsis (including for example rifampicin), or according to colonisation patterns and specific antibiotic sensitivities or resistance. Fluconazole was used as prophylaxis against yeast infection in SGH and RHSC units but not PRMH. Given the difficulties in comparing multiple antibiotics in a small cohort, I elected to examine simply number of antibiotic days as a parameter for comparison.

## 2.3) Methodology

### 2.3.1) Stool Samples

Each stool sample was collected by nursing staff from the nappy in universal containers and immediately frozen in a  $-20^{\circ}\text{C}$  freezer. An estimated weight of one gram was needed for each sample in order for all analyses to be performed. No sample lay for more than 48 hrs at  $-20^{\circ}\text{C}$ . All samples were anonymised and labelled by study number and date of sample alone. If a date was not specified (e.g. not recorded by nursing staff) then date range was specified, and assigned to one of the four weeks of recruitment. This happened on approximately 20% of occasions.

Prior to testing, all samples were thawed at room temperature, vortexed by Fisher Whirl Mixer, Heidolph REAX top (Fisher Scientific, Loughborough, UK), weighed and divided into four samples within a flow cabinet which was cleaned and sterilised beforehand with detergent and UV light, and decanted into separate eppendorfer and/or five ml bijoux tubes with autoclaved wooden or bamboo picks.

Not every sample was large enough to merit all analyses, so they were prioritised as follows in figure 9.

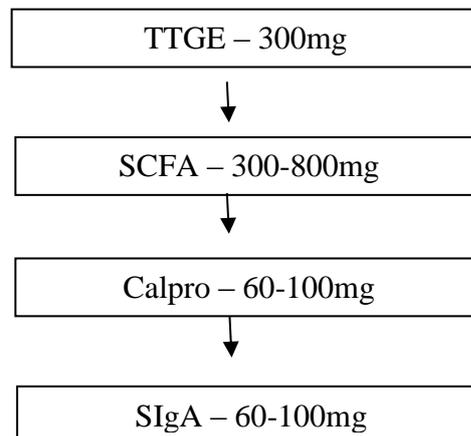


Figure 9, Quorum Chart: Standard sample operating procedure. (Abbreviations: TTGE = transient temperature gel electrophoresis; SCFA = short chain fatty acids; Calpro = calprotectin; SIgA = Secretory immunoglobulin A).

### **2.3.2) Breast Milk Samples**

These samples were taken with specific maternal consent; no more than 10% of the existing supply was taken at any point. Each sample was expressed by the mothers either within the NICU or at home. It was subject to the normal handling regimen as per each unit. It was immediately frozen at  $-20^{\circ}\text{C}$  before transfer in sterile universal containers and contained within freezer bags to RHSC, whereupon it was stored in a  $-80^{\circ}\text{C}$  freezer. After thawing at room temperature in a sterile flow cabinet with UV light, each sample was divided into 3 and one millilitre each was decanted with sterile pipette tips into sterile eppendorfer containers for the purposes of molecular, SIgA and calprotectin analyses. All were refrozen at  $-80^{\circ}\text{C}$  until testing was complete.

### 2.3.3) *Short Chain Fatty Acid Analysis: Gas Chromatography/Mass Spectrometry*

#### i) **Measurement of SCFAs**

At the end of the 19<sup>th</sup> century, volatile FAs were initially eluted by steam distillation of intestinal contents. However, over the last 60 years the mainstay of SCFA analysis has been gas chromatography: the separation of different components of a mixture with the use of a heated carrier gas flowing through a metal column coated with a liquid or polymer known to react with the mixture and ‘elute’ different components at different times (the ‘retention’ times). Components can then be identified according to the sequence in which they appear. A detector, such as a flame ionisation (FID) or thermal conductivity detector (TCD), uses combustion or heats a filament in order to detect ions and so identify components. Importantly, the FID burns and so destroys the sample; a TCD does not, thus allowing sample to continue to another detector if needs be. Alongside Mass Spectrometers (GC-MS) as detectors, the immediate identification of components can occur by determining their mass-to-charge ratio of charged particles (Kotani, Miyaguchi et al. 2009, Garcia-Villalba, Gimenez-Bastida et al. 2012). This has greater specificity than GC alone, although both methods provide quantitative analysis. Newer methods are in the process of development involving high throughput, rapid analysis chromatography methods (Tan, Ju et al. 2006, Olivero and Trujillo 2011).

The short and medium chain fatty acids C1 to C12 (see Appendix 2) are generally found in neonatal stool samples in both term and preterm infants. However, lactate is also an important acid in neonatal stool. High levels of lactose are present in both breast and formula milks, and, in addition, preterm infants are generally deficient in intestinal lactase before the age of 32 weeks gestation. However, whereas C1 to C12 can be measured readily in their native form by GC, lactate requires derivatisation to volatilise it for GC analysis. Thus we aimed to alter our established SCFA GC protocol to analyse lactate in addition to C1 – C12 by GC alone, thus increasing through-put, cutting duration and cost of analyses. Lactate also requires a higher temperature to elute thus requires a different column within the GC, as it is usually last to appear among SCFAs C1 – C12 (Garcia-Villalba, Gimenez-Bastida et al. 2012).

*Initial pre-extraction sample handling protocol for all samples:*

Samples were thawed at room temperature and vortexed. Between 300 and 800mg wet weight faeces was weighed into 5ml bijoux containers and vortexed again for 30 seconds. The wet weight was recorded and 1M sodium hydroxide (NaOH) was added at a 1:1 ratio to bring the pH above nine to prevent loss of SCFA by evaporation. The container was vortexed again for one minute and then stored in -80C freezer. Within a week the samples were freeze dried, and homogenised with bamboo sticks before being stored in vacuum packed containers at room temperature.

Some general issues were noted during handling of the samples.

- After freeze drying, most samples were noted to be markedly ‘stickier’ than those of adults or indeed older children. Samples of meconium in particular required prolonged vortexing (4 – 5 minutes) in order for mixing to occur after the addition of 1M NaOH. In addition, after addition of ether during the SCFA assay, most samples expanded, illustrating a large floating volume of fatty material consuming much ether in its density. This meant that, on occasion, only small volumes of ether could be extracted, requiring several repeats for the same sample.
- The use of crimp top caps rather than eppendorfer caps proved important as evaporation of such small volumes of ether was rife. Notably, at 200 ul of ether the samples evaporated over a matter of minutes, leaving inadequate volumes for injection.

**ii) Lactate analysis by GC: Trial Protocols**

In order to incorporate lactate analysis by gas chromatography, the column on the GC was changed to a Dimethylpolysiloxane column (ZB-5, Phenomenex, Cheshire, England): 30 m, 0.25mm ID, 0.25 um Film Thickness. A number of different elution protocols were trialled to attain separation of reagent and SCFA peaks over an appropriate run-time. In order to produce distinct peaks, we adapted the original GC protocol as follows:

- 1) 2M sodium hydroxide (NaOH) rather than 1M NaOH was used to counteract the acidity of the external standards in order to match the pH of the sample
- 2) We substituted hydrochloric (HCl) acid for orthophosphoric acid (PO) as we discovered that PO contaminates ether and produces an augmented peak with tBDMS.

- 3) Acetonitrile (CH<sub>3</sub>CN) was added to improve the mixing of reagents and polarity of the solvent mix in order to optimise the tert-Butyldimethylsilyl derivative group.

As such, the successful reagent was methyltertiarybutylsilyl trifluoroacetamide (MTBSTFA). The protocol was as follows (see Appendix 2 for preceding trial protocols and external standards):

### iii) tBDMS: Final Protocol

25mg dry weight stool sample was diluted with 100 ul distilled H<sub>2</sub>O. A further 100 ul HCl and 20 ul internal standard were added, and vortexed. 1000 ul ether was then added, and 800 ul immediately extracted. 100 ul of this was seconded into a separate glass tube with 200 mcl of 20% acetonitrile/tBDMS (160 ul acetonitrile, 40 ul tBDMS), resulting in 300 ul in total. The bottles were then placed on a hotplate for 60 mins at 60<sup>0</sup>C, prior to being loaded onto the GCMS. Chromatography was performed on a Trace GC 2000 Flame ionisation detector Gas Chromatograph with an Autosampler AS2000 by ThermoQuest CE Instruments, 1998. Chrom-Card for TRACE software was employed (Thermoquest CE instruments, 1998, Milan for Windows 1995, Version 1.00). Two different columns were used in the development of these protocols. These were: Zebron Capillary Column Nitroterephthalic acid modified polyethylene glycol by Phenomenex, Cheshire, England. 15m x 0.53mm Internal Diameter x 1.00 um Film Thickness; and a Dimethylpolysiloxane column (ZB-1, Phenomenex, Cheshire, England): 30 m, 0.25mm ID, 0.25 um Film Thickness. Each employed fitted needles with 10ul syringes, with methanol used as needle cleaner. The following carrier gases and their flow rates were used (from the right carrier only): Nitrogen (30ml/min), Air (350ml/min), and Hydrogen (35ml/min).

Peak identification was the most difficult aspect of the method development, requiring meticulous and numerous repeated analyses (see Appendix 2). During several GC trial protocols, the internal standard peak was too small to integrate, and then once an appropriate peak was established through manipulation of reagent/sample ratios, the peaks of caproic acid and the internal standard could not be baseline separated to ensure accurate quantification of both. Despite several attempts at many different run and ramp time/temperature combinations (see Appendix 2), the peaks corresponding with C5 (valeric acid, commonly found in formula milks) and our internal standard (2EB) could not be adequately separated, leaving quantification of these SCFAs impossible. At a GC Ramp 2

run of 5.0, 3.5, 3.0, 2.5 and 10.<sup>0</sup>C/min, the best separation that could be achieved was 2 peaks, around 10 seconds apart, but with no clear baseline separation between. Since it was considered that C5, as a SCFA found in formula milks, was likely to be seen in significant quantities in the stool samples from our population, delineation of this peak without interference from other SCFAs or reagents was imperative to ensure accurate measurements.

As such, the same dry weight samples were extracted and re-run using the GCMS. 3-methylvaleric acid (3MV) was added as the internal standard as per the tBDMS protocol after several trials ensuring that its peak could be separated from 2-ethylbutyric acid (given they are isomeric) according to manipulation of run and ramp times.

**iv) Method development: derivatisation by tetra-Butyldimethylsilyl (tBDMS) to identify lactate by GCMS**

The GCMS extraction protocol for the GCMS was identical to that of the GC. As such, the following run and ramp times were used for both protocols: Run time: 10.67 mins; initial hold time: 1 min at 80<sup>0</sup>C; Ramp 1: increasing by 15C/min to 210<sup>0</sup>C; Hold time: 1 min; Equilibration time: 0.25 mins; Max Temp: 260<sup>0</sup>C. Given the use of both GC and GCMS methods, we further assessed the intra-assay variability of these methods, once standardisation of protocols and concentrations was applied to all samples, thus evaluating the potential for all future samples to be performed using GCMS, and render the GC-FID redundant. The GCMS settings are detailed in Appendix 2.

#### **2.3.4) Calprotectin Analysis by Enzyme Linked Immunosorbant Assay**

After initial decanting of thawed samples, their wet weights were recorded and were frozen at  $-80^{\circ}\text{C}$  until analysis. Within a washed extractor hood, they were thawed again at room temperature and analysed per patient recruited. The PhiCal Calprotectin ELISA kits were used for precision assay (PhiCal, from Calpro, Norway, distributed via Firefly Scientific, Manchester, UK), and their protocols were used with minor in-house adaptations.

##### **Protocol**

Samples were thawed at room temperature within the extractor hood, weighed and transferred by loop into conical tubes. A faecal extraction buffer was prepared as such: 90 mls FEB added to 135 mls distilled  $\text{H}_2\text{O}$  into a sterile bottle and immediately refrigerated. A washing solution was then prepared adding 50 mls of pack solution to 950mls distilled water, and refrigerated. A sample diluent was made up using 20mls of pack diluent added to 180 mls of distilled water, covered with aluminium foil to prevent light degradation. Other solutions pre-made in the pack and ready to use comprised: an enzyme antibody; and p-nitrophenyl phosphate.

The centrifuge (Thermo Heraeus Fresco 21) was switched on to allow its temperature to fall to  $-5^{\circ}\text{C}$  for combined refrigeration of samples during centrifuging. The appropriate adjusted FEB volume per wet weight of faecal sample was pipetted into each blue conical tube (between 2- 6 mls – see Appendix 3) All stool dilutions were adjusted for a factor of 0.049 in order to allow precision to the milligram per wet weight sample. Each tube was hand shaken and then vortexed for 30 seconds. All tubes were then mounted on a shaker @ 1200 rpm for 30 mins. Three sets of clip cap eppendorfers were placed into 3 racks, and 1 to 1.5 mls of homogenate was extracted per tube. These tubes were placed in the centrifuge after balancing @ 10,000 xg for 20 mins @  $2 - 8^{\circ}\text{C}$ . One ml of supernatant was then seconded into a second eppendorfer tube. These were again placed in a refrigerated centrifuge at 5 mins @ 10,000 xg @  $2-8^{\circ}\text{C}$ . A further 500  $\mu\text{l}$  was decanted into a third eppendorfer. These were then centrifuged once more in a mini-centrifuge at room temperature for 5 mins.

Samples were stored as required at this point in a  $-20^{\circ}\text{C}$  freezer. Thereafter, 980  $\mu\text{l}$  of sample diluent was pipetted into new tubes. 20  $\mu\text{l}$  of supernatant was added to each 980  $\mu\text{l}$  sample diluent tube, rinsing the tip in the 980  $\mu\text{l}$  tube. All the other tubes at this point from

sets 1 + 2 were discarded in incinerator bins. Taking the plate and sealing foils from the PhiCal kit, the plate was labelled horizontally from 1 to 12 as per the guidance sheet. Precision assay yellow 100 µl Finntips were used for pipetting from this point onwards. Each small bottle of standards were removed from the kit and shaken by hand. 100 µl of was pipetted into the appropriate wells according to the labels, including 2 blanks. 100 µl of Sample Diluent only was added to the blank wells. Each sample of stool supernatant was again vortexed and 100 µl was pipetted into each well. A seal was placed on top of the plate, which was then placed on an incubator/agitator for 45 minutes. A large filter needle was used to filter 1M NaOH into a 10 ml universal container, and the wells were aspirated with a purpose built multichannel pipette, and then washed with 250 µl washing solution on five occasions. The plate was inverted after each wash-out onto green towels and tapped on all well openings to remove any washing solution. The Enzyme Conjugate Antibody (ECA) bottle was inverted and 100 µl ECA was added to each well with Eppendorfer multichannel pipette and finntips. The reverse pipetting technique was employed in order to ensure precision measurement of a high viscosity liquid – the pipette was fully discharged and sample taken up, before being pressed to the half point mark to accurately decant into the well, before taking up the next sample in releasing the pipette plunger fully to its original position. The plate was then re-sealed again and placed on the horizontal plate shaker for 45 mins. All washing steps were repeated as before. 100 µl pNPP was then added to each well. The plate was covered with foil and left at room temperature in darkness for 20 – 30 minutes, with 5 minute checks for reaction. At point of reaction, the plate was retrieved and 100 µl of 1M NaOH was added to each well as a stopping solution. The plate was then read by a microtiter plate reader (Multiskan, Thermo) plate reader, under Skanit software. Each plate was read at a cubic spline calibration curve and those with an intra-assay variability of >10 % were rechecked on a second plate. The mean of the two duplicates was taken as the final reading.

Calprotectin has been detected in breast milk in previous studies (albeit at extremely low levels (Bjorkstrom, Hall et al. 2009)) and, as such, this assay was also performed upon human breast milk from mothers of infants at varying gestations up to 32 weeks, as per the inclusion criteria. For this, the 100 µl sample was decanted after thawing at room temperature in a sterile extractor hood, and treated as per the faecal samples in terms of additions and supernatant extractions.

### **2.3.5) Secretary IgA Analysis by ELISA**

This part of the study was performed by Miss Mandy Wenwen, Dr Emilie Combet-Aspray, and myself, and was also presented as Miss Wenwen's Master's project, 2010. Given that samples for SIgA were taken last on the study protocol, 34 infants were included in this part of the project.

Total SIgA titres were determined by a quantitative enzyme linked immune assay (ELISA) by Immunodiagnostik AG, Bensheim, Germany. Stool samples were collected over the first 4 weeks of life from 34 of the 56 recruited infants, and stored at -20°C. Approximately 100 mg of stool sample were weighed with loops and transferred into plastic tubes, before resuspension in 5ml wash buffer from the ELISA kits, mixing and vortexing (Fisher Whirli-mix) for at least 30 seconds. Accurate weights were recorded for dilution purposes. After centrifugation of 1 ml stool suspension for five minutes at 13000 rpm, the supernatant was diluted 1:250 in wash buffer for analysis. The final dilution factor was approximately 1:12500 (for a 100 mg sample), and the samples analysed in this study also included one milk sample per week for four weeks from six mothers who expressed breast milk for their infants, of whom five who were exclusively breast milk fed until the end of the recruitment period. 100 µl of breast milk from each sample was diluted to 1:20000 for analysis.

The assay procedure was performed according the manufacturer's instructions. Controls, standards (100 µL) and diluted samples (stool or milk) were added to the ELISA plates in duplicate. After incubation on a horizontal shaking mixer for 1 hour at room temperature, samples were washed with 250 µL wash buffer, prior addition of 100 µL conjugate (peroxidase-labelled mouse anti-SIgA). The plate was incubated for one hour, and washed as described previously. The substrate (100 µL of tetramethylbenzidine) was added and incubated at room temperature for 10 minutes before addition of 50 µL of 'stop' solution: sulphuric acid. The absorbance was determined with a microtiter plate reader (Multiskan, Thermo) at 450 nm against 620 nm as reference.

In order to obtain the concentration of SIgA in different samples, a calibration curve was conducted according to the concentrations of standardised samples and their corresponding absorbencies. The sample concentration was calculated using the standard curve and

multiplying the results by the dilution factor. Samples with high coefficient variation (5%) were reanalyzed if sample volume was sufficient.

### 2.3.6) *Molecular techniques: Temporal Temperature Gel Electrophoresis*

The following protocol was developed and performed by internal collaborators Dr Kostas Gerasimidis, Clinical Lecturer in Clinical Nutrition, and postgraduate student Miss Katja Brunner, in conjunction with myself. This collaboration led to Miss Brunner's Master's project in 2010. Due to time and cost constraints, 4 gels were run, containing samples from 22 infants. Meconium samples were excluded. Bands were compared before and after NEC in two groups of infants: those mixed versus exclusively maternal milk fed.

#### i) **DNA extraction from faeces**

DNA was extracted from 200 mg of faeces per sample. A probiotic powder (VSL3 containing 8 strains: *Streptococcus thermophiles*, *Bifidobacterium breve*, *longum*, and *infantis*, *Lactobacillus acidophilus*, *plantarum*, *paracasei*, and *delbrueckii* (subspecies *bulgaris*; Ferring Pharmaceuticals, West Drayton, UK) served as a standard for TTGE analysis. Faeces were suspended in 250µL of 4 M Guanidine Thiocyanate 4 M/Tris-Cl 0.1 M (pH 7.5) and 40 µL 10% N-Lauroylsarcosine, vortexed (Fisher Whirl Mixer, Heidolph REAX top), shortly centrifuged, and incubated for 10 mins at room temperature. 500 µL of 5% N-Lauroylsarcosine/Phosphate buffer 0.1 M (pH 8.0) was then added, and the resultant slurry homogenised and incubated on a dry bath (Techne Dri-Block) at 70°C for one hour. 750 mg 0.1mm sterile zirconia/silica beads were added and bacterial cells were ruptured twice with the Bead-Beater (MP FastPrep-24) for 3 mins at 4.5 m/s with intermittent incubation on ice. 15 mg polyvinylpyrrolidone (PVPP) was added, shaken (IKA Vibrax VXR Basic; ~1300 motions/min) and centrifuged for 3 minutes at 15 000 x g and 4°C. The supernatant was recovered in a new sterile 2 mL tube and the pellet was washed three times with 450µL TEN-P buffer. The recovered liquid was then centrifuged for 10min at 20 000 x g and 4°C and the supernatant split in two 2mL and one 1.5mL tubes (2 x 750µL/1 500 µL). DNA was precipitated by incubating for 10 minutes in isopropanol (2 x 1000µL/ 1 x 750µL). The solution was then centrifuged for 5 minutes at 15.000 xg, the supernatant was discarded, and the pellet was resuspended in 225 µL phosphate buffer 0.1 M (pH 8) and 25 µL potassium acetate 5 M, and left in the fridge overnight. The next day, the three tubes were reunited into one new 2 mL tube. Five µL RNase (10 mg/mL) was added and incubated at 35°C in a dry bath for 45 minutes. DNA was precipitated with 50 µL 3 M sodium acetate and 1 mL ice cold 100% ethanol and incubated in the freezer for 1 hour. The sample was centrifuged for 10 minutes at 15000 x g, the supernatant was discarded and the pellet washed 3 times with 800 µL 70% ethanol. DNA yield was left to

dry under the sterile bench for 1 hr and was then resuspended in 100  $\mu$ L sterile water and stored at -20°C.

The DNA extract was measured by spectrometry at 260nm to estimate DNA quantity, and the ratio of 260 nm/280 nm and 260 nm/230 nm was taken to estimate impurity, with protein and phenol respectively. Results were compared with those received by agarose gel electrophoresis (1 % agarose gel, stained with ethidium bromide). For seven subjects the agarose gel could not detect DNA.

## ii) PCR amplification and optimisation of the protocol

A hot star PCR was performed to amplify the V6-V8 regions of bacterial 16S rDNA gene. Primer sequence and thermocycler conditions are shown in Table 1. For the initial protocol each PCR reaction tube contained 15  $\mu$ L Hot star Taq Mastermix (Qiagen, France), 0.6  $\mu$ L primer L, 0.6  $\mu$ L primer U, 12.8  $\mu$ L water and 1  $\mu$ L DNA template (1:100 dilution) and run in the thermocycler (MJ research, USA) with 30 cycles. PCR yield was tested on a 1.5% agarose gel stained with ethidium bromide.

Primer	Forward primer (5'-3')	U968-5' Gcclamp GAA CGC GAA GAAa CCT TAC
	Reverse primer (3'-5')	L1401-GCG TGT GTA CAA GAC CC
Thermocycles	Activation	• 95°C for 15min
	Denaturation	• 97°C for 1 min
	Annealing	• 58°C for 1min
	Elongation	• 72°C for 1 min 30sec
		• 72°C for 15 min
	• 4°C until end	

} 30/35 cycle

**Table 12: Primer sequence and conditions of the PCR thermocycler**

The initial conditions for PCR reaction failed to obtain PCR products for most of the samples, due possibly to the presence of PCR inhibitors found in the stool samples, such as phenolic compounds, EDTA, fats, and/or bile acids (Kreader 1996, Al-Soud 2005, Oikarinen 2009). In the SCFA analysis, it was noted that prior to ether extraction, diluted stool samples were markedly ‘fatty’, and it is considered that this is the cause of the lack of DNA yield in seven members of our cohort. In order to optimise this protocol a series of alterations were performed and tried on a subset of samples.

The optimisation process included the following steps (Appendix 4):

1. Changes in the dilution of the DNA template to either increase the amount of DNA or reduce the amount of potential PCR inhibitors.
2. Changes of reagents and increased volume of Mastermix and primer.
3. Addition of bovine serum albumin (BSA) in a concentration of 0.04 % w/v to bind PCR inhibitors (Al-Soud 2005).
4. A second extraction of the DNA with Phenol/Chloroform method:  
DNA was emulsified three times with equal volume phenol:chloroform:isoamylalcohol (25:24:1) and separated by centrifuging at 20,000 xg for 5 mins. The aqueous phase was recovered and washed twice with equal volume of chloroform. DNA was precipitated on ice with 0.1 volumes of 3M sodium acetate and 2 volumes of 100 % ethanol.
5. Incubation in SDS and Proteinase K:  
20 µL DNA extract was diluted in 280 µL water. 36 µL 10% w/v SDS and 16 µL 10 mg/mL Proteinase K solution were added and incubated at 45°C for 2 hours. DNA was precipitated on ice with 0.1 volumes of 3M sodium acetate and 2 volumes of 100 % ethanol.
6. Increase in number of PCR cycles from 30 to 35 cycles.

### **iii) Optimised PCR protocol**

DNA extracts were incubated in SDS and Proteinase K. PCR reaction tubes contained 15 µL Mastermix, 0.6 µL of each primer, 11.6 µL milliQ water, 1.2 µL BSA 50 % w/v solution and 1 µL DNA template (Dilution 1:50). PCR reaction was run for 35 cycles. For those samples that did not show PCR products on the agarose gel, the PCR reaction was repeated with a DNA template dilution of 1:20 and the best result was then used for TTGE analysis (Table 13).

	Infants	DNA Dilution	
		Sample 1	Sample 2
<b><u>EBM</u></b>	A	1:50	1:20
	B	1:50 <sup>\$</sup>	1:20
	C	1:20	1:50
	D	1:50	1:20
	E	1:50	1:50
	F	1:20	1:50
	G	1:50	1:50
	H	1:50	1:50
<b><u>MF</u></b>	I	1:50 <sup>\$</sup>	1:20
	J	1:50 <sup>\$</sup>	1:20
	K	1:50	1:50
	L	1:50 <sup>\$</sup>	1:50
	M	1:50 <sup>\$</sup>	1:50
	N	1:50 <sup>~</sup>	1:20
	O	1:50 <sup>\$</sup>	1:50
	P	1:50	1:50
	Q	1:50	1:50
	R	1:50 <sup>\$</sup>	1:50
	S	1:20	1:50
	T	1:50	1:20
	U	1:50	1:50
	V	1:50	1:50

\$ no DNA yield on agarose plate, ~ no PCR band

**Table 13: DNA dilutions for PCR, noting those with and without DNA yield** (Abbreviations: DNA = deoxyribonucleic acid; PCR – polymerase chain reaction; EBM = expressed breast milk; MF = mixed fed)

**iv) Temporal temperature gradient gel electrophoresis (TTGE)**

For the TTGE polyacrylamide gel 19.6 g of urea was diluted in 22.6 mL of MilliQ water and 1.17 mL 50x TAE buffer was added once the urea was dissolved. 9.7 mL acrylamide:bisacrylamide was added and the mixture was filtered under vacuum. On ice, 402  $\mu$ L 10% ammonium persulfate (APS) and 40.2  $\mu$ L tetramethylethylenediamine (TEMED) were quickly added to the solution above and filled in a glass plate sandwich assembly (1mm) with the help of a syringe and then left to polymerise. After polymerisation the gel was repeatedly washed with 1.25 % TAE buffer. The TTGE tank with 1.25 % TAE buffer was preheated to 66°C and the washed gel was loaded with a mixture of PCR product (20  $\mu$ L for samples, 10  $\mu$ L for VSL3 standard) and the same amount of loading dye (0.05 % bromophenol blue/ 0.05 % xylene cyanol).

For alignment of the samples, a pre-run was performed at 66°C on constant 20V for 20 mins. After that the TTGE was run at constant 64V and 0.3°C ramp rate for 16 hours until the temperature reached 70.5°C. The gel was stained with 200 mL 1.25 % TAE and 20  $\mu$ L SYBR Green (Roche Diagnostics, Germany) for 20 mins under constant shaking and then destained in 1.25 % TAE buffer for 5 mins. Images were captured under UV light (Syngene InGenius LHR Gel Documentation System) with GeneSnap (see figure 95 e) for a simplified schematic of the process).

**v) Analysis**

Gels were analysed by using Quantity One software (Version 4.5.0, BioRad). Bands were detected by comparing several pictures taken at different exposure values and manual changes of contrast, light and gamma values. Results were interpreted as followed:

- *Species richness* was defined as the number of species present in a community (Begon M 2006) and was therefore calculated by summing up the number of bands detected per lane.
- *Species turnover* was calculated by the band profile of one individual at two different time points. Bands were divided in resistant species (present in Sample 1 and 2), extinct species (present in Sample 1 but not 2) and immigrant species (present in Sample 2 but not 1) to estimate fluctuations of the bacterial community over time (Begon M 2006).

- *Similarity index* was calculated by Quantity one using the  $C_s$ -similarity equation:

$$C_s = \frac{2j}{(a+b)} \times 100$$

- where 'a' and 'b' represent the number of bands for the first sample and second sample respectively and 'j' the number of common bands between the two samples (Schwiertz, Gruhl et al. 2003). The similarity index was calculated for intra-individual comparison to estimate changes during the two time points within the same infant and for inter-individual comparison of the two groups for the second sample only.  $C_s$  can only be calculated for samples run on the same gel. TTGE gel 1 and gels 2+3 were run with a sample set of each individual of the EBM and MF group, respectively and TTGE gel 4 contained the second sample of all MF infants.

- *Relative abundance* of species within the population was defined as the number of samples presenting a species divided by the total number of samples on the gel.

### **2.3.7) General Statistical Analyses and Data Interpretation**

Microsoft Excel 2010, SPSS 16.0.2, and Minitab 16 were employed to interpret and display results. All were both encrypted and anonymised. The following group sets were considered: data from the entire cohort by week; gestational groupings; feed regimen (exclusively maternal expressed breast milk versus mixed breast milk and formula); presence or absence of necrotising enterocolitis, by all-stages of NEC, and stages  $\geq 2a$ . Each group was also considered by weekly analysis. All data sets were subject to normality tests using the Anderson-Darling method. For normally distributed data, paired or unpaired T-tests were performed, and means with standard deviations reported. Kruskal Wallis tests were used for non-normally distributed multiple group analyses, followed by Mann-Whitney U testing if significant p values were identified. Medians and interquartile ranges were therefore reported. For the SIgA analyses, non-normal data were log transformed prior to using T-tests, yielding mostly normalised data. This was secondary to the personal preference of the collaborators involved in these analyses. Proportionate analyses were performed using either chi-squared or Fisher's exact T-test. Where applicable, the Yates' correction was applied to the chi-squared test, and the resultant p value recorded. Correlations were performed using Pearson's test. For all boxplot graphs, the midline represents the median, the upper and lower borders of each box denote the interquartile range, and the whiskers signify the reaches of the fourth quartile. Outliers are signified by asterisks (\*). For bar charts, the upper limit of each bar represents the mean or median, and the whiskers denote either the standard deviation, or interquartile range, respectively. These are clarified on each legend. It should be recognised that within each graph, significant p values of note are represented by the symbol '↓'. Within each table of results, the asterisk symbol '\*' is used to denote significant p values. In all cases, p values of  $<0.05$  were considered significant. In conjunction with Dr David Young, statistician at NHS Greater Glasgow and Clyde, and the University of Strathclyde, further multivariate analysis was attempted, but considered inappropriate given the wide variation in non-normal histograms.

### **Group and National Weight Z Scores**

Two different types of weight Z scores were calculated for the cohort over 5 separate time points (birth, and the ends of week 1, 2, 3 and 4). Where the phrase 'z score' is used, this in all cases refers to the 'weight z score'. The first of these were labelled as either 'group' or 'cohort' z scores and was calculated by subtracting the mean from the raw score and then

dividing by the standard deviation (using the ‘standardize’ function in Excel). The second Z score was derived from national data, used with kind permission by H Pan and TJ Cole (Cole 1998, Pan 2012). For this, the ‘LMS Growth’ add-in programme was downloaded (Pan 2012), and each infant’s gestational age at birth, and weight at each of the five time points was inserted into the calculator in order to generate the corresponding z score. For the purposes of this thesis, these z scores were then referred to as ‘National’ z scores.

### **Post-hoc analyses**

Since no prior audits could be ascertained, the ‘resting’ incidence of NEC and severity was unknown in these neonatal units. As such, once the spike in NEC was observed in this cohort, it was clear that we needed to explore correlations between clinical and demographical data, as well as all analytes; the former was performed to attempt to elicit a common cause of such a high incidence of NEC, and the latter to investigate the potential for these to act as diagnostic and/or prognostic ‘biomarkers’, whilst accepting that this study was not powered to NEC.

Specifically, the following statistical tests were performed post-hoc:

- 1) Bell’s Stage NEC: clinical and demographical comparisons by severity (P163-165)
- 2) All SCFA ratiometric analyses. After multi- and univariate analyses revealed significant differences in (albeit low) BCFA concentrations, we sought to determine ratiometric changes according to demographics, feed type, and NEC. In so far as can be ascertained, ratiometric analyses have not been published in this patient group to date, but are often used in studies on adults (Walker 2005) (P189-204).
- 3) Calprotectin analyses by Bell’s Stage severity of NEC, in particular levels before and after stoma formation (P239-242).
- 4) SIgA analyses: correlations between stool and milk titres illustrating a significant difference between later stool and earlier milk levels (P255).

## Chapter 3

### Clinical and Demographical Results

#### Introduction

The results are presented in three separate chapters: clinical and demographical results; bacteria and bacterial metabolites; and inflammation and immunological markers. Each includes a detailed discussion thereafter, including comparison with the evidence base (as discussed in chapter 1), and in reference to the methodologies (as described in chapter 2).

#### 3.1) Study population

Numbers recruited, excluded and included are illustrated in figure 10 below. Data gathered included clinical attributes (ventilation, antibiotics, feeds, for example), demographical information (for example: gestation, gender, birth weight, and maternal deprivation level), and markers of illness (including sepsis, IVH and NEC). Correlations were then sought between clinical and demographical factors of interest, after which stool analytes were correlated with particular clinical and demographical features of note. All data was compared between the individual recruiting NICUs.

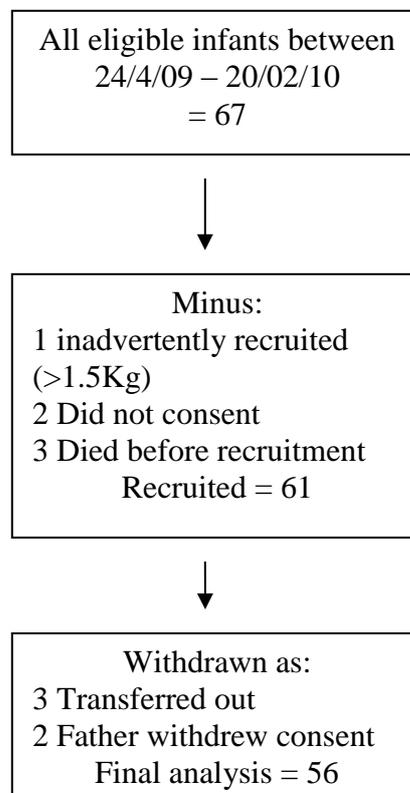


Figure 10: Quorum chart of recruitment sequence

**Table 14: Included Infants – Whole Study Population Demographics (n=56)**

<i>Parameter</i>	<b>Raw data</b>	<b>CI/p value</b>
Male/female: n (%)	20/36 (37.5/ 62.5)	♀>♂ 0.031
CRIB 12 hours before recruitment (M,sd)	2.5, 2.4	1.856, 3.144
DepCat Score (M,sd)	4.7, 1.98	4.182, 5.247
AEDF (n,%)	8, 14	NS
IUGR (n,%)	7, 12	NS
MOD: SVD Vs LUSCS (n,%)	EMLUSCS: 37, 66 ELLUSCS: 5, 8.9 SVD: 14, 25	<0.00001
PPROM (n,%)	11, 19.6	NS
Intrapartum antibiotics given (n,%)	13, 23.2	NS
Gestation, weeks (M,sd)	28.0, 2.2	27+3.36 days, 28+4.6 days
Birth weight, g (M,sd)	1029.3, 258.2	960.2, 1098.5
Apgar at 10 (M,sd)	8.7,1.79	8.46, 9.059
Umbilical lines? (n=UVC only/None/Both)	9,18,29	Both>None+UVC: <0.05
Days Ventilated (M,sd)	9,12.7	5.51, 11.38
Days CPAP (M,sd)	9.4, 10.5	5.88, 10.62
PDA surgical ligation? (n,%)	8, 14	NS
ROP laser surgery? (n,%)	5, 9	NS
IVH? (n,%)	Total: 12, 21.4 Grade I: 5; Grade II: 5; Grade III: 0; Grade IV: 2	NS
<b>Mortality (n,%)</b>	4, 7.1	0.0018, 0.14

There were significantly more girls than boys within the cohort (p = 0.032), and most infants were delivered by LUSCS (p<0.00001).

Note all infants had antenatal Doppler studies as per GG+C policy pre-2010 RCPCH Guidelines. (Abbreviations: CRIB = Clinical Risk Index in Babies score; Depcat = deprivation category; AEDF = absent end diastolic flow; IUGR = intrauterine growth restriction; PDA = patent ductus arteriosus; ROP = retinopathy of prematurity; MOD = method of delivery; SVD = spontaneous vaginal delivery; LUSCS = low uterine segment caesarean section; IVH = intraventricular haemorrhage; CPAP = continuous positive airway pressure; UVC = umbilical venous catheter; PPROM = premature prolonged rupture of membranes).

## Significant Study Population Demographical and Clinical Parameters:

### 3.1.1) Gender by gestation and birth weight:

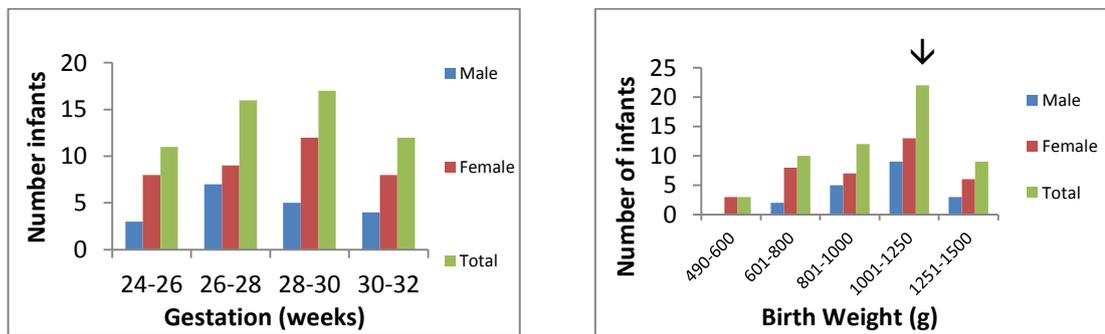


Figure 11: a) (left) Gender by gestation; there were significantly more girls than boys in the cohort ( $p=0.032$ ); b) (right) Gender by birth weight; the 1001-1250g category had the most infants ( $\downarrow$ :  $p=0.004$ ).  $\downarrow$  denotes a significant p value.

There were significantly more girls than boys in the study group and throughout all gestations (see figure 11; study group: 36 Vs 20,  $p = 0.032$ ; all gestation categories  $p<0.05$ ). No gestational group differed significantly in male/female ratio. There were significantly more infants within the 1001-1250g weight category than any other group ( $p = 0.004$ ). The sub-600g birth weight group were notably exclusively female.

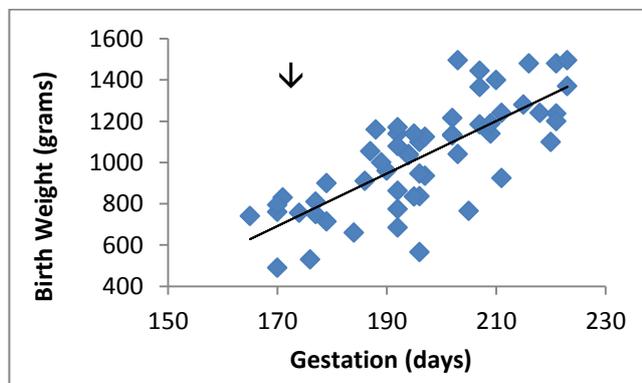


Figure 12: Gestation versus birth weight, showing a significant correlation with  $p<0.0001$ .

The study group was normally distributed with respect to birth weight and gestation (see figure 12). There was a positive correlation between increasing gestation and higher birth weight. IUGR and SGA levels were low.

### 3.1.2) CRIB in the preceding 12 hours prior to recruitment:

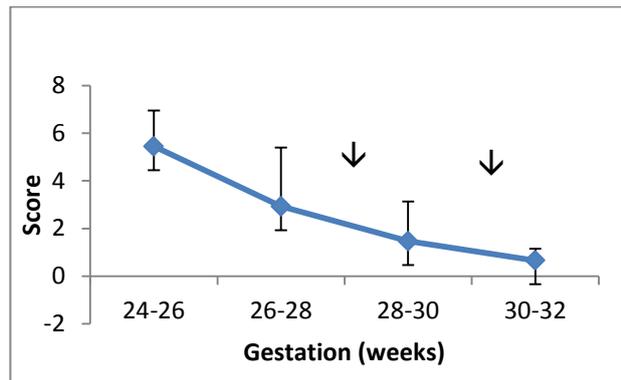


Figure 13: CRIB scores by gestation; those at lower gestations showed significantly higher scores: 26-28 week group were significantly higher than those of the 28-30 week group ( $p=0.0001$ ), and of the 30-32 week group ( $p<0.00001$ ). ↓ denotes a significant p value.

The Clinical Risk Index in Babies Score was developed by The International Neonatal Network as a prognostic tool for illness severity within the first 12 hours of life (Parry 2003). It was adapted for the purposes of this study using parameters in the 12 hours preceding recruitment as a marker of illness requested by the Ethics Committee to ensure that the parents of the sickest infants were not approached. Infants with a CRIB of more than 15 (therefore >50% predicted mortality) were not recruited. This occurred on one occasion. This infant also had a Congenital Diaphragmatic Hernia and as such would have been excluded anyway on the basis of major congenital anomaly. Sadly, this infant died. As expected CRIB score was significantly lower for those born at higher gestations (see figure 13: 24-26 Vs 26-28: NS; 24-26 Vs 28-30:  $p = 0.0001$ ; 24-26 Vs 30-32:  $p < 0.00001$ ).

### 3.1.3) Method of Delivery:

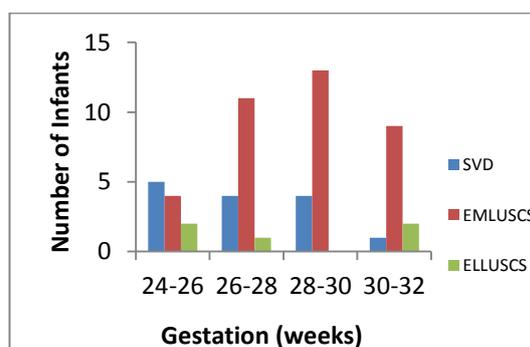


Figure 14: Method of delivery, by gestation; significantly more infants were delivered by LUSCS ( $p < 0.00001$ ) than SVD. Most LUSCS were performed as emergencies ( $p < 0.00001$ ).

Most infants were delivered by caesarean section (Figure 14: 75% Vs 25%,  $p < 0.00001$ ). Of these, 88% were performed as emergencies ( $n=37$ , Vs  $n=5$ ;  $p < 0.00001$ ). This is significantly higher than Scottish statistics for live singleton births at all gestations, whereby 61% of all deliveries in 2010 were SVD ( $p < 0.00001$ ). The incidence of elective LUSCS is higher in multiple births at all gestations (35% elective and 30% emergency LUSCS) in the UK (RCOG 2004). Note one SVD occurred precipitously at home.

### 3.1.4) Multiparity and Chorionicity:

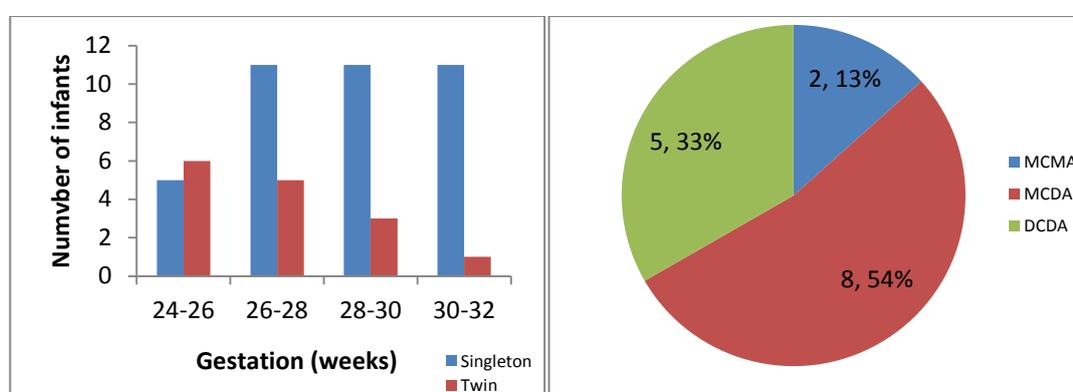


Figure 15: a) (left) Singletons by Gestation; there were significantly more singletons than multiples ( $p=0.0013$ ), and twins were more likely to be of lower gestational age (significantly more twins at 24-26 weeks than 30-32 weeks:  $p=0.031$ ); b) (right) Chorionicity of twins within the cohort; most were dichorionic and diamniotic. MCMA = monochorionic monoamniotic; MCDA = monochorionic diamniotic; DCDA = dichorionic diamniotic.

Multiparous pregnancies are at high risk of complications. Those sharing a placenta have a 15% risk of twin to twin transfusion, and in pregnancies in which one twin has died in utero, the resultant morbidity and mortality risk for the surviving twin escalates. As figure 15 shows, in this cohort there were significantly more singleton infants ( $p = 0.0013$ ), and twins were most likely to be of lower gestation (24-26 weeks versus 30-32 weeks:  $p = 0.031$ ; 24-26 versus 28-30 weeks,  $p = 0.041$ ). Notably two infants were delivered as the surviving twin after the in utero demise of their sibling.

### 3.1.5) Depcat Scores:

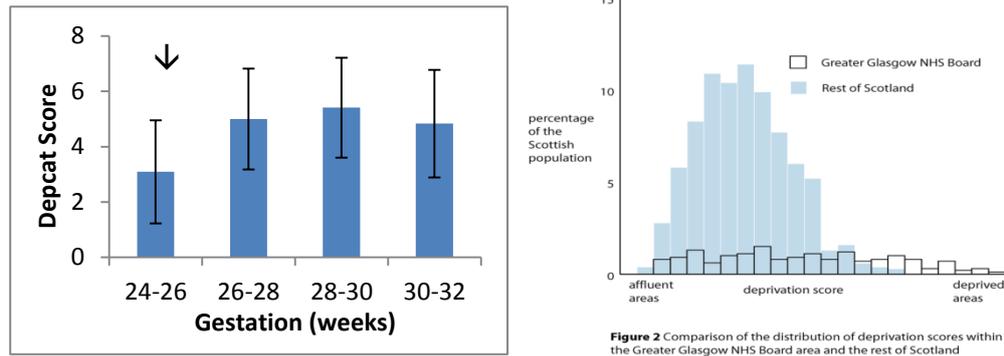


Figure 16: a) (left) Group Depcat scores by Gestation; scores were significantly lower at lower gestations when comparing those at 24-26 weeks with all other gestations ( $p < 0.0172$ ). ↓ denotes a significant p value. b) (right) Glasgow versus Scotland Depcat Scores from the Carlisle Report, illustrating high levels of deprivation in Glasgow (McLoone 2004)

The Depcat scoring system was designed as Scotland-specific measure of social deprivation. It is based upon demographical information including four census variables depicting employment status, overcrowding, social class, and material hardship, and is stratified according to postcode (see figure 16 b)). A higher score equates to higher deprivation. The mean score was 4.2 (sd 1.98). Those between 24-26 weeks had significantly lower Depcat scores than at other gestations (Figure 16 a): 24-26 Vs 26-28:  $p = 0.0172$ ; 24-26 Vs 28-30:  $p = 0.0034$ ; 24-26 Vs 30-32:  $p = 0.0391$ ). This was contrary to our hypothesis that poor maternal health and social standing would result in higher rate of birth at lower gestations. This is believed to be due to the number of affluent couples conceiving by IVF, which carries with it a higher risk of complications and of multiparous pregnancy. Given this is a Scotland-specific score, there are no methods of comparison nationally, but it is clear from the updated 2004 report that Glasgow and Greater Glasgow, as defined by postcode sectors from the Greater Glasgow NHS Board areas, dominate the most deprived areas, with 30 % of the GGNHSB population contained within the highest deprivation score of 7 for Scotland. The study cohort very closely fit the Glasgow statistics, as illustrated below, but have a higher proportion of infants within the highest category of deprivation, as illustrated in figure 17:

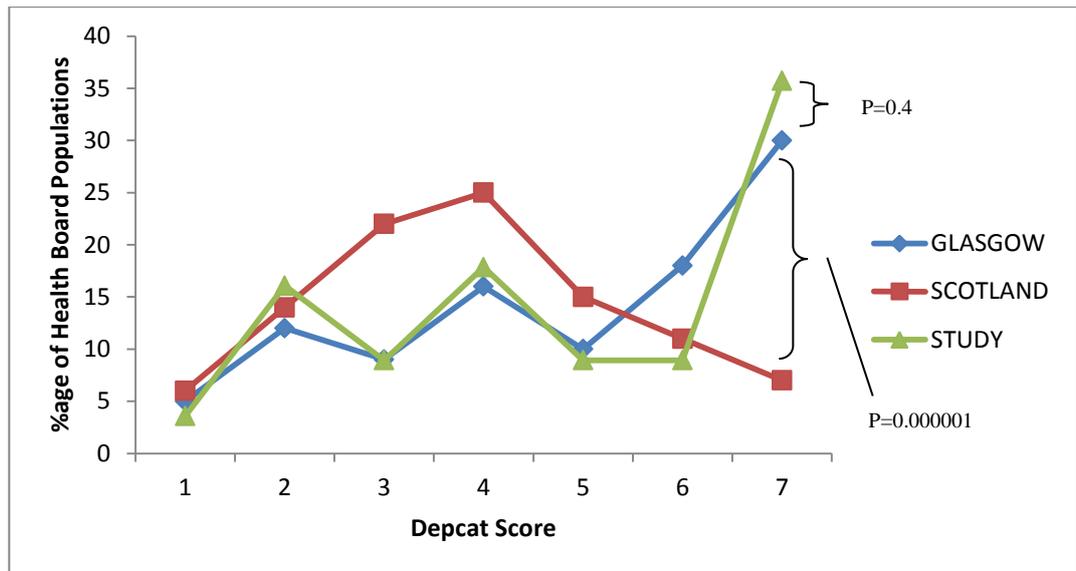
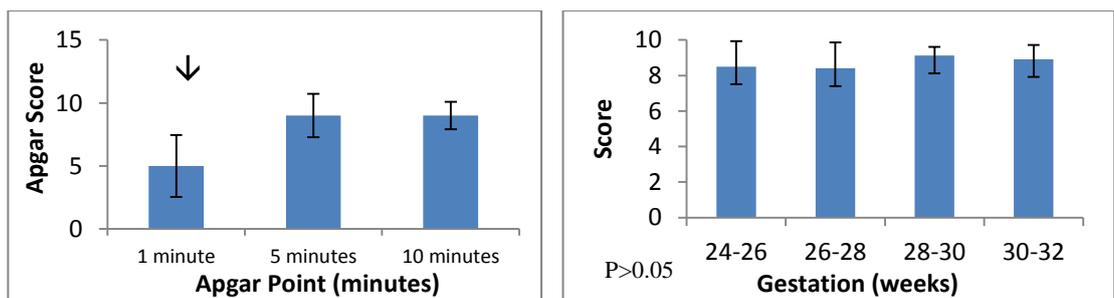


Figure 17: Assimilated from study data, and the Carstairs Report, 2001, with raw data taken from Table 8, p 13: Depcat Scores by percentage of health board populations within each category, comparing the study cohort, Glasgow and Scotland; the study cohort follows the Glasgow proportions closely, yet the study cohort at depcat 7 are significantly higher than the scottish figures (p=0.000001).

Figure 17 shows that in category 7, that of the highest deprivation, there was no significant difference between proportion of study group and proportion of Glasgow health board population (p = 0.45). However the proportion of study group infants in category 7 is significantly higher than that nationally (p < 0.0000011).

### 3.1.6) Apgars:



Figures 18: a) (left) Mean Apgar scores at minutes 1, 5 and 10 of life; the score at minute 1 was significantly lower than minutes 5 or 10 throughout the cohort (p=0.00001); ↓ denotes a significant p value. b) (right) Apgar score at 10 minutes by gestation, showing no significant differences.

The Apgar score originated in 1953 as a method of newborn assessment, and is still recorded locally (Apgar 1953). Interpretation of scores is more difficult in prematurity, but still performed. Ten minute Apgar scores of less than 3 are associated with a poor outcome. As figure 18 shows, there were no differences between Apgars in gestational

groupings, and all gestations showed a significant rise in Apgar between 1 and 5 minute scores ( $p = 0.00001$ ).

### 3.1.7) PPRM:

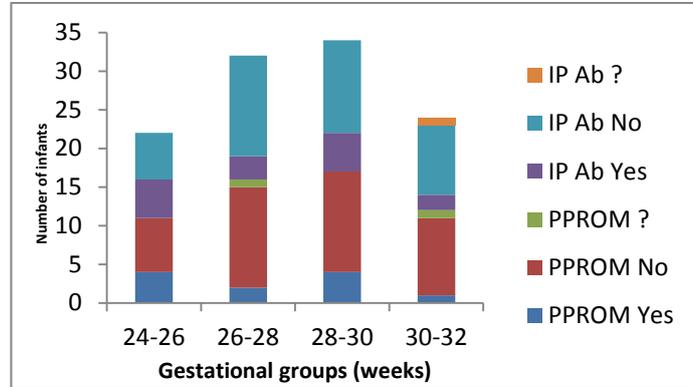


Figure 19: PPRM and intrapartum antibiotics by gestation. No significant gestational differences were noted.

Prolonged preterm rupture of membranes (PPROM) was defined as the prolonged, premature rupture of membranes before 37 weeks gestation for more than 72 hours, as per the regional guideline. As shown in figure 19, the incidence of PPRM was not significant in our cohort ( $p > 0.05$ ), and there were no significant differences in frequency of PPRM according to gestational comparisons using chi squared analyses. There were no significant differences in administration of intrapartum antibiotics when stratifying according to gestation. All 11 mothers with PPRM were given intrapartum antibiotics, and an additional 4 mothers with urinary tract infections were also administered antibiotics starting in the immediate antepartum period, continuing before and after delivery.

### 3.1.8) Pregnancy Induced Hypertension Contributing to Preterm Delivery:

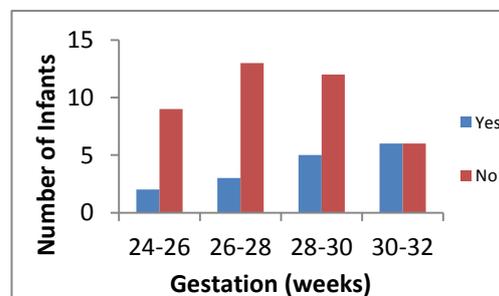


Figure 20: Mothers with PIH contributing to preterm delivery. There were no significant gestational differences.

Sixteen infants were delivered prematurely secondary to maternal pregnancy induced hypertension (28 %). This was not a significant proportion of the group. There were no significant differences between gestations (Figure 20:  $p > 0.05$ ).

### 3.1.9) Presence of Umbilical Lines, by gestation:

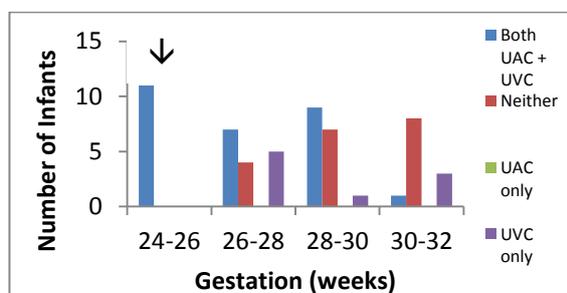
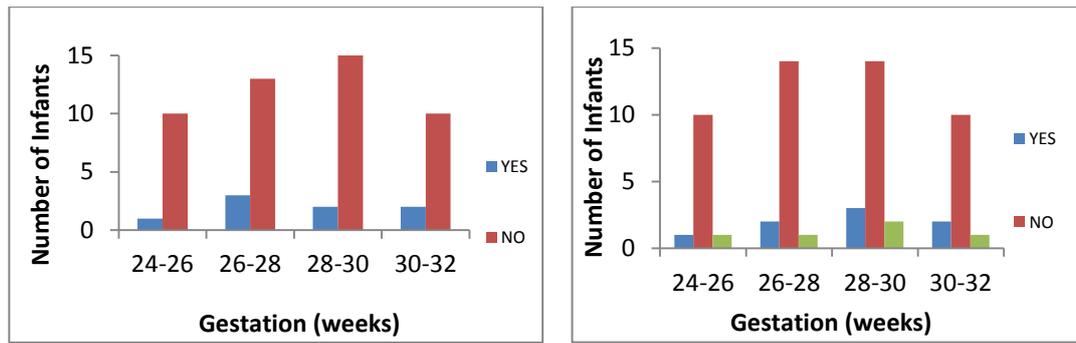


Figure 21: UAC and UVC insertion by gestation; those of lower gestations were significantly more likely to have umbilical lines inserted (24-26 weeks versus all other groups:  $p < 0.003$ ). ↓ denotes a significant p value.

Although there are no national guidelines for UAC and/or UVC placement in preterm infants, there is a regional guideline recommending attempted insertion for all preterm infants requiring respiratory support, and line insertion was significantly more common at lower gestations. In this study 100 % of extremely preterm infants had both UAC and UVC sited (Figure 21: 24-26 weeks vs 28-30 weeks,  $p = 0.0001$ ; 24-26 weeks vs 28-30 weeks,  $p = 0.004$ , 24-26 vs 30-32 weeks,  $p = 0.003$ ). Fewer more mature infants (30-32 week group) had both umbilical lines sited (1 of 12, 8 %). Only 5 infants had cord gases immediately after delivery, although routine cord sampling for this purpose is not part of a recommendation by the RCOG.

### 3.1.10) IUGR and AEDF:

IUGR was defined as being  $<10^{\text{th}}$  centile for weight, as stated by the NICE Guidelines for Routine Care for the Healthy Pregnant Woman (Health 2008). Additional definitions of asymmetrical or symmetrical growth restriction were not considered.



Figures 22: a) (left) IUGR by gestation was not a significant phenomenon within the cohort; b) (right) AEDF by gestation also showed no significant differences

The term intrauterine growth restriction rather than ‘small for gestational age’ was used to denote infants in whom a pathological cause of IUGR, rather than a constitutional SGA was evident owing to their prematurity. None of the infants with IUGR were delivered because of their poor IU growth. The absence of end diastolic flow was defined by consultant obstetricians and senior sonographers in each unit. As figure 22 illustrates, very few infants were growth restricted or had AEDF in utero, and there were no significant gestational differences. IUGR was not significant within the study cohort ( $p > 0.05$ ), at 12.5 %. AEDF did not correspond closely with IUGR. Eight infants were noted antenatally to have AEDF (14 %,  $p > 0.05$ ). It is possible that more would have been identified had these pregnancies progressed with more scans able to identify these factors.

### 3.1.11) Duration of Incubation:

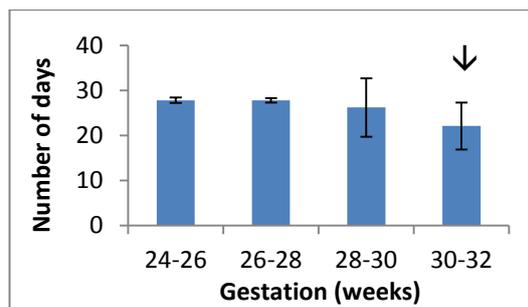


Figure 23: Duration of incubation, by gestation; infants at 30-32 weeks gestation showed an earlier transition to cot care than those at 24-26 weeks ( $p=0.0014$ ). ↓ denotes a significant p value. No other gestational differences were noted.

Most infants remained incubated for the duration of the study period, but as expected there was a trend to earlier transfer to cot care in more mature infants (Figure 23: 24-26 versus 30-32 weeks medians 27.8 vs 22.1 days,  $p = 0.0014$ ).

### 3.1.12) Duration of Invasive and Non-invasive Ventilation:

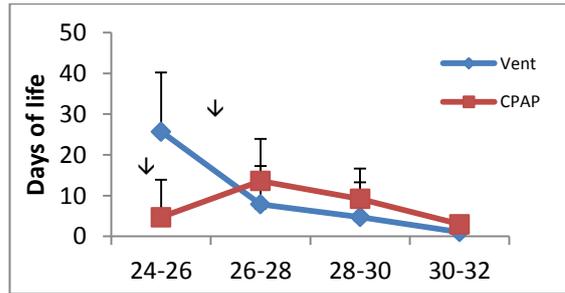
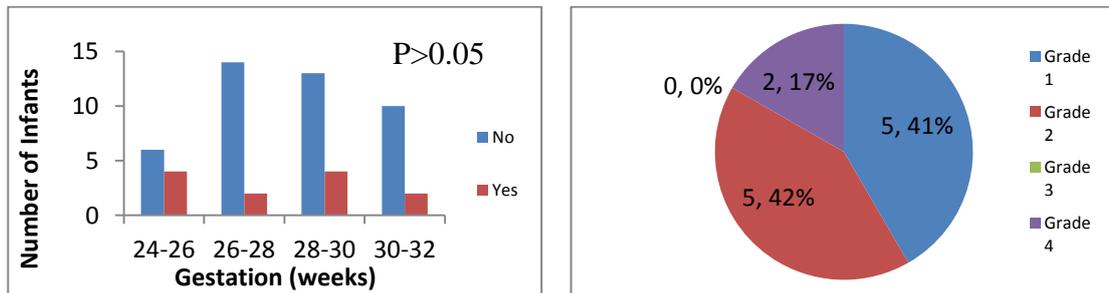


Figure 24: Duration of invasive and non-invasive ventilation, by gestation; those between 24-26 weeks were ventilated for longer than all other groups ( $p < 0.0015$ ). Conversely, they had a significantly lower duration of non-invasive (CPAP) ventilation over the study period ( $p < 0.01$ ), bar those at 30-32 weeks ( $p = 0.10$ ).

As expected, the 24-26 week gestation infants were ventilated for significantly longer than those in any other gestational grouping (Figure 24: 24-26 versus all other gestational groupings:  $p < 0.0015$ ). There were no significant differences between those at 26-28 weeks and those at 28-30 weeks, or 28-30 versus 30-32 weeks ( $p = 0.06$  and  $0.36$ ). The duration of CPAP non-invasive ventilation was significantly shorter in the 24-26 week gestation group as more infants remained invasively ventilated for longer (24-26 < 26-28  $p = 0.014$ ; 24-26 < 28-30  $p = 0.016$ ; 26-28 < 30-32  $p = 0.01$ ; 28-30 < 30-32  $p = 0.02$ ) and similar to the 30-32 week gestation group ( $p = 0.10$ ). The highest median in the 26-28 week group reflects their shorter period of invasive ventilation but considerable levels of RDS requiring respiratory support. The incidence of RDS and thus any types of ventilation lessened for the later gestations.

### 3.1.13) Intraventricular Haemorrhage:



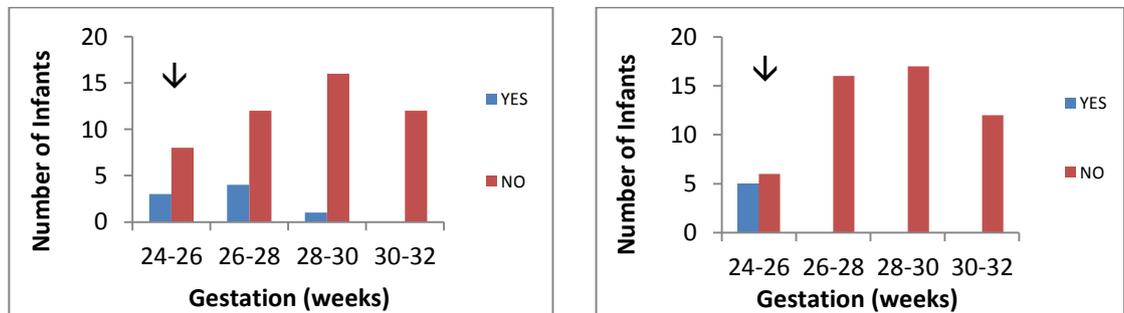
Figures 25: a) (left) IVH, by gestation; there was a low incidence of IVH within the cohort, and no significant gestational differences were noted. b) (right) Grades of IVH; note no infant incurred Grade 3 IVH.

IVH diagnoses were taken from radiologist scans. Where these reports were not available, the opinion of the most senior doctor performing the scans was taken. The 24-26 week group had fewer but more severe IVHs, although this did not reach statistical significance

(Figure 25:  $p > 0.05$  in all gestation comparisons). Both infants with Grade IV IVH also incurred periventricular leukomalacia.

### 3.1.14) PDA ligation and Laser Surgery for Retinopathy of Prematurity

The end points of PDA ligation and ROP surgery were taken as absolutes, as it was accepted that the decision to treat the duct pharmacologically or to perform surgical duct ligation is not consistent throughout units in the UK. Since the majority of preterm infants have a persistent ductus arteriosus in the neonatal period, many inconsequential, simply the presence of the duct was not recorded. ROP laser surgery however, has specific indications and as such is considered consistent throughout the UK.



Figures 26: a) Surgical PDA ligation, by gestation; significantly more infants at 24-26 weeks received ligation than those at 30-32 ( $p=0.027$ ); ↓ denotes a significant p value. b) Laser surgery for ROP was significantly more common at 24-26 weeks than at any other gestation ( $p<0.03$ ). ↓ denotes a significant p value.

There were significantly more infants at 24-26 weeks than 30-32 weeks gestation who underwent PDA ligation (Figure 26 a):  $p = 0.027$ ), but other gestational comparisons were not significant. This may have been skewed by the higher morbidity and mortality rates in those of 24-26 weeks, affecting their ability to undergo surgery. All infants requiring laser surgery for ROP were less than 25 weeks gestation, as such far less than the surveillance parameters ( $<30$  weeks and  $<1.5\text{Kg}$ ), indicating excellent vigilance to national guidelines (Health 2008). This was significantly greater than at any gestation (Figure 26 b):  $p < 0.03$ ), although there were no other significant gestational differences.

### Demographical and Clinical Data Omissions

Few data was missing from demographical collection given the meticulousness of the nursing and medical records. Two missing drug prescription charts for two mothers meant that administration of intrapartum antibiotics could not be confirmed.

### **3.1.15) Mortality**

Four infants in the study died. The first two were twin girls (MCDA) delivered at 25 weeks gestation. One died from *E.coli* sepsis on day 26, and her sister with respiratory failure on day 62. Both incurred but did not succumb to NEC. The third infant was delivered at 23 weeks and required surgical resection of NEC with ileostomy formation, and died suddenly on day 105 of life after a likely embolic event causing gut necrosis. Her parents declined the option of post-mortem examination. The fourth baby died on day 69 of life after reorientation of care secondary to respiratory failure. She had previously recovered from stage 2b NEC. All four infants weighed 1000 g or less at birth. Given that one of these infants died from late-onset, post-ileostomy NEC, thus the mortality from all-stage NEC throughout this observational study was 1.7 % (1 of n = 56 included infants). Mortality wherein the deceased infant had incurred NEC at any point was 5.3 %. All-cause infant mortality was 7 %, far lower than the national averages as reported by the EpiCure Study albeit these figures apply only to infants up to 26 weeks gestation (Costeloe, Hennessy et al. 2000). The rates quoted by the Office for National Statistics (England and Wales) are lower than the study mortality, which is attributed to their inclusion of infants between 32 and 36 weeks gestation (Modi 2008). Records for Scotland, as published in the Scottish Perinatal and Infant Mortality and Morbidity Report of 2010, showed that 10.8 % of all infants delivered between 24 and 32 weeks gestation died in the neonatal period, and these deaths accounted for 41 % of all neonatal deaths regardless of gestation for the year 2010.

### 3.1.16) Study Population: Feed Types

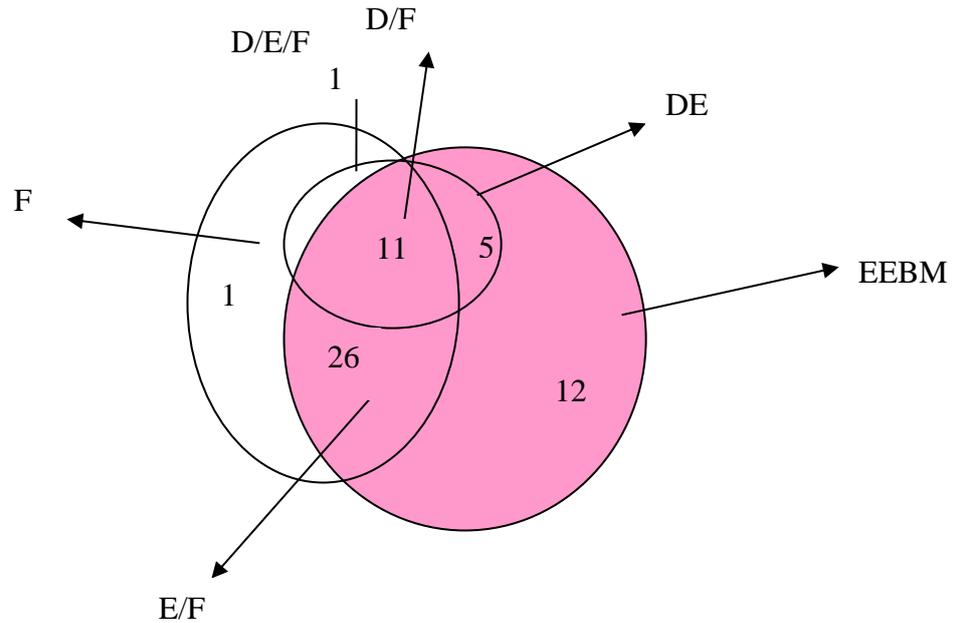


Figure 27: Venn diagram illustrating types of feed administered to study patients, showing the heterogeneity of milks used. The numbers indicate total number of infants fed using each type of milk over the study period. Most infants were fed a mixture of breast and formula milks. (Abbreviations: D/E/F = donor EBM/maternal EBM/formula; D/F = donor EBM/formula; DE = donor EBM/maternal EBM; EEBM = exclusive maternal EBM; E/F = expressed maternal EBM/formula; F = formula. Note none of the cohort were exclusively fed donor EBM for the duration of the study).

The Venn diagram in figure 27 illustrates the heterogeneity of feeds for the study group, contrary to prior local audit data. 21 % were fed solely maternal EBM. 49 mothers expressed milk for their infants (87.5 %), of which 12 were exclusively fed maternal EBM during the study period (21 % of infants, vs 33 % in audit data). Note no infants were exclusively fed DEBM for the duration of the study period (vs 33 % in local audit). The total volume of DEBM used by all patients during the study period was just over 18 litres. Formula milk used was *Nutriprem 1 (Cow and Gate)* bar one infant who received less than 100 ml of *Aptamil (Milupa)* – versus 33 % in local audit data.

In a questionnaire, only four mothers reported being aware of ‘probiotic’ products and only two consumed these regularly with the purpose of replenishing their gut microbiota – one drank Actimel yoghurt drinks and the other took over-the-counter probiotic tablets from the high street retailers Holland and Barrett. The latter did so as she had previously delivered a daughter at 26 weeks who incurred NEC and although made a full recovery was seriously unwell for some months. However, given that all natural yoghurt contains

some probiotic bacteria, it is likely that they consumed probiotics without knowing or intending to do so.

### i) Feed Regimen: By Volume

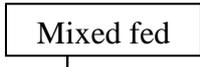
Further data analysis revealed the following breakdown of feeds during the study period:

Type of feed(s) given	Infants (n,%)	Total feed over study period (L)	Total Feed Volume (L)			
			Week 1	Week 2	Week 3	Week 4
EEBM	12	21.813	2.352 Med: 0.078	4.925 Med: 0.341	6.075 Med: 0.506	8.460 Med: 1.718
EFM	1	6.685	.798	1.893	1.817	2.177
Mixed E/F	26	135.064 % EBM: 53%	14.402 Med: 517.1	34.035 Med: 1.303	41.247 Med: 1.618	45.378 Med: 1.823
DEBM	0	0	0	0	0	0
Mixed D/E	5	6.965 % EBM: 50%	0.360 Med: 0.038	0.474 Med: 0.098	2.761 Med: 0.615	3.369 Med: 1.004
Mixed D/E/F	11	33.491 % EBM: 10% D: 41%	2.787 Med: 0.163	6.995 Med: 0.786	10.669 Med: 1.101	13.038 Med: 1.272
D/F	1	5.895 %D: 11%	0.466 NA	1.561 NA	1.723 NA	2.145 NA
TOTAL	56	209.914 E: 101.36 D: 18.308	21.167	49.884	64.293	74.568

Table 15: Feed regimen by volume; except for the single infant fed a mix of donor EBM and formula, the other infants who were mixed fed received at least 50% of their feed as breast milk during the study period. (Abbreviations: EEBM=exclusive expressed breast milk; EFM=exclusive formula milk; E/F expressed EBM/formula; DEBM=donor expressed breast milk; D/E=donor/expressed breast milk; D/E/F=donor/expressed/formula; D/F=donor/formula).

During this time period, the West of Scotland Donor EBM bank reported issuing 132 litres of donor breast milk to all 3 recruiting neonatal units, of which the DEBM usage by study patients accounts for only 13 % of this total. The remaining 77 % was devoted to use by the infants with Short Gut Syndrome and/or Intestinal Failure in RHSC NICU (personal communication, Debbie Barnett, 2012). It is therefore worth noting that in total, the 90 litre shortfall in EBM that was replaced by formula milk could have been covered by existing supplies from the Donor Bank. During this study there were no unit or regional feed guidelines, and as such feed increments were made on a case-by-case basis by individual staff members. A regional trust-wide feed policy is currently under review.

ii) Demographics According to Feed Regimen



Feed regimen n =	EEBM 12	EF 27	F 1	DE 5	DEF 10	DF 1	P values EEBM Vs Mixed
Gestation (m,sd)	27.2, 2.1	28.7, 2	31.1	26.7, 0.7	26.7, 1.8	31.5	NS
Birth weight (m,sd)	895, 241	1151.7, 211.1	1240	821.6, 145.3	896.1, 254.4	1200	NS
Female (n,%)	9, 75	14, 51	0	4, 80	7, 70	1, 100	NS
AEDF (n,%)	3, 25	2, 7.4	0	1, 20	2, 20	0	NS
IUGR (n,%)	3, 25	1, 3.7	0	2, 40	2, 20	0	NS
DEPCAT Score (m,sd)	4.67, 2.38	5.04, 1.67	4, 0	4.6, 2.5	3.8, 2.09	7	NS
Mat PROM (n,%)	2, 16.6	4, 14.8	0	2, 40	2, 20	0	NS
Intrapartum antibiotics (n,%)	3, 25	7, 25.9	0	2, 40	2, 20	0	NS
CRIB Score (m,sd)	3.33, 2.46	1.4, 1.5	1, 0	5.4, 2.07	3.4, 2.83	1, 0	NS
Apgars at 10 mins (m,sd)	8.72, 0.6	8.5, 2.9	7, 0	8.4, 1.94	8.9, 1.44	9, 0	NS
Singletons (n,%)	8, 66.6	22, 81.4	1, 100	4, 80	5, 50	1, 100	NS
Days incubated (m,sd)	25.5, 7.7	24.7, 6.3	25, 0	28, 0	27.8, 0.6	18, 0	NS
Days ventilated (m,sd)	15.6, 12	4.3, 7.6	2, 0	12.2, 14.4	10.8, 12.19	1, 0	NS
Days CPAP (m,sd)	13.08, 12.9	6.23, 7.9	4, 0	15.6, 14.2	10.9, 9.9	2, 0	NS
IVH (% ,n)	3, 25	4, 14.8	0	2, 40	2, 20	1, 100	NS
PDA requiring ligation (m,sd)	4, 33.3	3, 11.1	0	1, 20	0	0	0.05*
ROP requiring laser surgery (m,sd)	1, 8.33	2, 7.41	0	0	2, 20	0	NS
No. episodes sepsis (m,sd)	1.42, 1.62	0.42, 1.02	0	1.6, 0.54	0.7, 1.05	0	NS
Types of sepsis	CNS, E.Coli	CNS, E.Coli, Strep faecalis	NA	CNS	CNS, E.Coli, Staph. Aureus	NA	NA
Days Abx (m,sd)	18.17, 9.76	7.52, 7.41	5, 0	16, 6.74	12.7, 10.29	5, 0	0.01*
Highest CRP (m,sd)	76.5, 69.76	30.6, 54.3	15, 0	24.3, 16.76	48.07, 56.4	19, 0	NS
% MEBM Given (% ,mls)	100, 21813.45	56.5, 77451.4	0	50.5, 3520	10.4, 3541.6	0	NA
Day 1 <sup>st</sup> feed (m,sd)	7, 9.8	2, 1.03	6, 0	2.8, 0.83	3.5, 1.26	3, 0	NS
Day to full (m,sd)	19.6, 8.9	8.37, 6.22	3, 0	21, 4.69	13.5, 5.4	6, 0	0.002*
Fortifier supplementation (n,%)	2, 16.6	10, 37	0	0	3, 30	0	NS
Meds 1 (m,sd)	0.08, 0.28	0.44, 0.57	2, 0	0	1.0, 1.05	0	0.035*
Meds 2 (m,sd)	1.08, 1.37	2.37, 1.04	3, 0	0.6, 1.3	2.2, 1.75	2, 0	0.0246*
Meds 3 (m,sd)	1.5, 1.73	2.74, 1.12	2, 0	2.6, 2.6	3.6, 1.89	3, 0	0.012*
Meds 4 (m,sd)	1.75, 2.05	3.15, 1.58	2, 0	3.4, 3.13	3.9, 1.52	3, 0	0.02*
NEC (n,%)	8, 66.6	11, 40	0	5, 100	7, 70	0	NS
Mortality (n,%)	2, 16.6	1, 3.7	0	1, 20	0	0	NS

Table 16: Demographics by feed regimen; those mixed fed had shorter durations of antibiotics despite no significant reduction in sepsis. They also reached full feeds quicker, and tolerated more oral medications than those EEBM fed. These findings may be skewed by smaller numbers within the EEBM group.

(Abbreviations: EEBM = exclusive maternal expressed breast milk; EF = expressed breast milk and formula; F = formula; DE = donor expressed breast milk and maternal expressed breast milk; DEF = donor, maternal and formula milks; DF = donor and formula milks; CRP = c-reactive protein; PROM = prolonged rupture of membranes; NEC = necrotising enterocolitis; AEDF = absent end diastolic flow; IUGR = intrauterine growth restriction; PDA = patent ductus arteriosus; ROP = retinopathy of prematurity; DepCat = deprivation category; CRIB = clinical risk index in babies score; IVH = intraventricular haemorrhage; MEBM = maternal expressed breast milk; Abx = antibiotics; NS = not specified.)

### 3.1.17) Birth Weight and Weight Gain During the Study Period

Birth weights, weekly weights and all z scores were normally distributed. As a cohort, weight fell significantly between birth and the end of week 1, rising significantly week-on-week thereafter. By week 2, the mean weight was then significantly higher than at birth ( $p < 0.01$ ). Group Z score means varied between 0.18 to -0.06 throughout the study period, with no significant differences between any time points. However, in comparison with national data (WHO 1990 reference data, reanalysed 2009), z scores were consistently negative, and varied between -0.622 at birth, falling significantly to -1.34 at the end of week 1 ( $p < 0.0001$ ). However, all other weeks showed no significant differences.

<b>Parameter</b>	<b>Weight (g) (m, sd)</b>	<b>Z score Study Group (m, sd)</b>	<b>Z score for UK (m, sd)</b>
<b>Birth</b>	1029.357, 258.1978	0.07, 0.993	-0.622, 0.97
<b>End of wk 1</b>	1008.25 235.4517	0.18, 1.055	-1.34, 0.93
<b>End of wk 2</b>	1155.824 239.5414	-0.02, 1.0	-1.33, 1.05
<b>End of wk 3</b>	1287.291 339.3607	-0.06, 1.0	-1.32, 1.03
<b>End of wk 4</b>	1452.415 404.1188	-0.03, 1.0	-1.27, 1.10
<b>P Values</b>	Birth<1, 2<3, 3<4, birth< 2/3/4: <0.01	NS	Birth>Wk 1: <0.0001* All others: >0.05

Table 17: Weights and weight Z scores throughout the study period; as expected, mean weight of the study group fell between birth and week 1, rising again consistently throughout the remainder of the study period. However, this was not reflected in group z scores, but did occur with national z scores between birth and week 1. (Annotations: \* significant p value; Abbreviations: g = grams; m = mean; sd = standard deviation; wk = week; NS = not specified.)

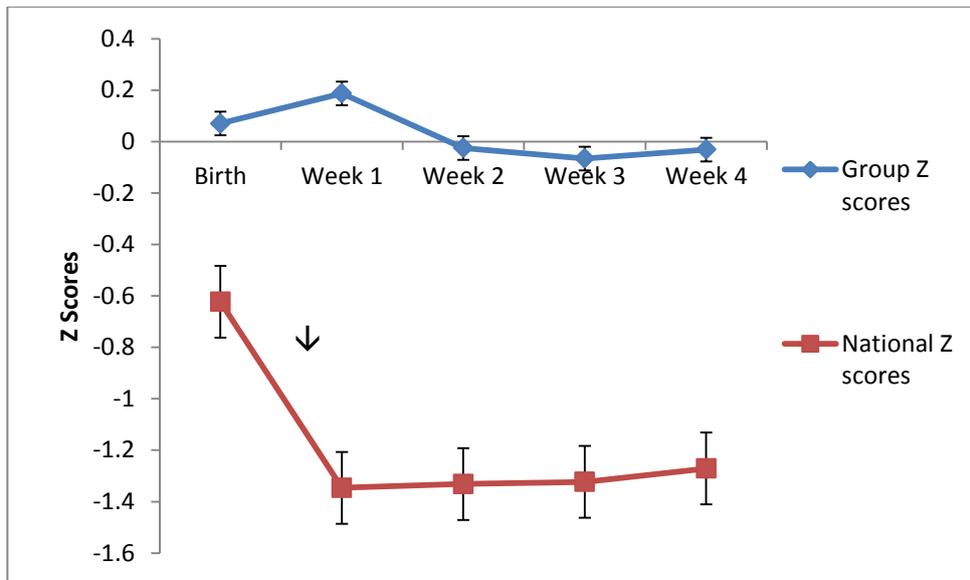
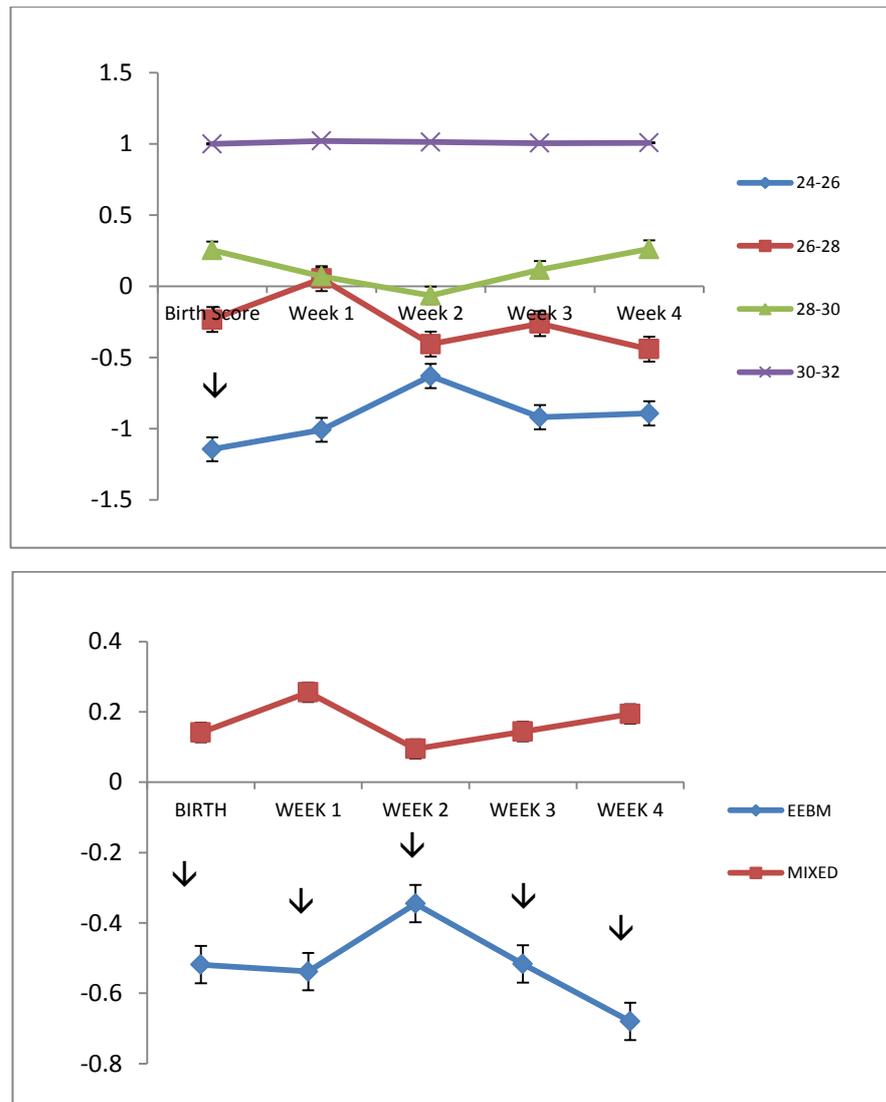


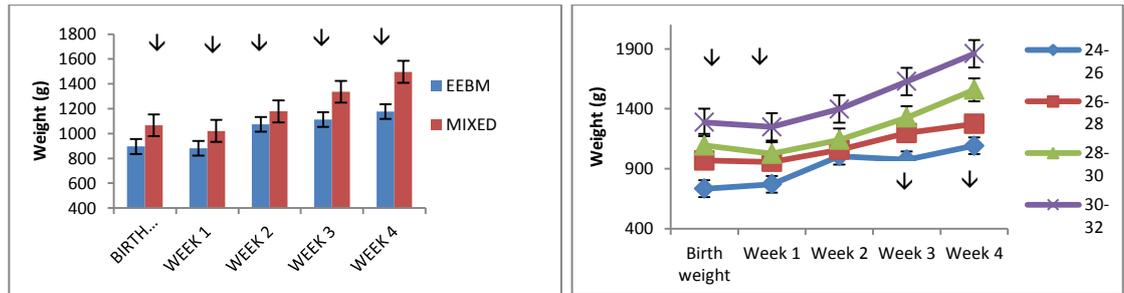
Figure 28: Graph of group weight z scores and national weight z scores; no significant differences were noted between the group's own z scores at each time point. Significant differences were revealed upon comparison with national data. By national z score, the cohort fell significantly between birth and week 1 of life ( $p < 0.0001$ ). The error bars represent the SEM. ↓ denotes a significant p value.

*i) By gestation and feed type*

In the 24-26 week gestation there were no significant differences in Z scores at each time point. For the other gestational groupings over each time point, significant differences were not seen between birth and all 4 other weeks. When comparing Z scores across the gestations by week, the 24-26 week group unsurprisingly were significantly lower than any other group in week 1 ( $p < 0.0002$ ). The differences over the study period were most marked between the gestations on weeks 1, 3 and 4 scores and weights (table 17 and figures 28 and 29). Data by gestational groupings was normally distributed, and, unsurprisingly, significantly lower at lower gestations, in all time points except week 2 in the 26-28 versus 28-30 week groups (Figure 29 a):  $p = 0.08$ ). When stratified for feed regimen, there were no differences over the study period for those either EEBM or mixed fed, but when comparing the 2 groups by week, those fed EEBM were significantly lighter at the end of weeks 1 and 4 (Figure 29 b):  $p < 0.03$ ).



Figures 29: a) (top) Group's own Z scores by gestation, weeks 1-4; those of 24-26 weeks had a significantly lower score than any other group at birth and week 1 ( $p < 0.0002$ ). All other time points were closely correlated with gestation, except week 2 when those at 26-28 and 28-30 weeks showed no difference. The error bars represent the SEM. ↓ denotes a significant p value. b) (bottom) Z scores by feed type, weeks 1-4; those EEBM fed were significantly lower than mixed fed at all time points ( $p < 0.03$ ). The error bars represent the SEM. ↓ denotes a significant p value. (Abbreviations: EEBM = exclusive expressed maternal breast milk; Mixed = mixed maternal breast milk and formula).

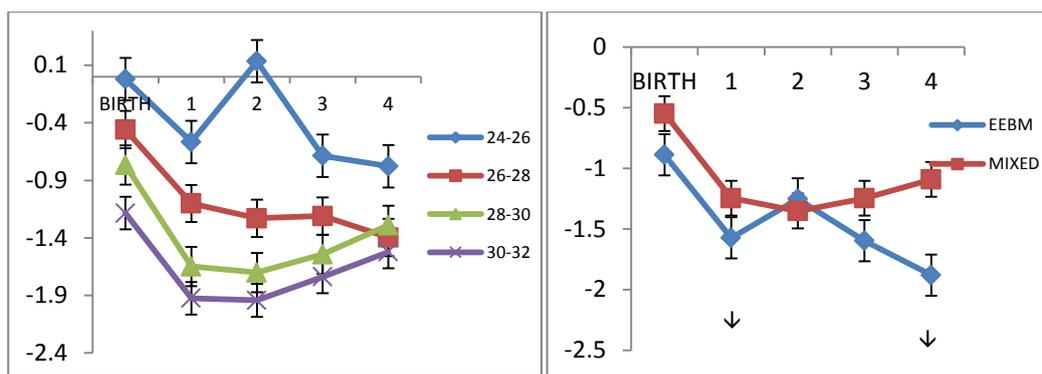


Figures 30: a) (left) Weights by feed type; those EEBM fed were significantly lighter at all time points. The error bars represent the SEM. ↓ denotes a significant p value. b) (right) Weights by gestation; those at 24-26 weeks gestation were significantly lighter than any other group except during week 2 ( $p < 0.015$ ). The error bars represent the SEM. ↓ denotes a significant p value. (Abbreviations: SEM = standard error of the mean; EEBM = exclusive expressed maternal breast milk; Mixed = mixed maternal breast milk and formula).

## ii) Comparison with National Z Scores

### *By gestation and feed type*

A significant trend was seen (figure 31) with higher scores at lower gestations. This is considered to represent the well-grown 24-26 week gestation infants, who were of lower deprivation score. The reason for the peak at 2 weeks in those of 24-26 weeks gestation is unclear, and may be explained by soft tissue oedema secondary to increased illness (for example NEC and sepsis) in this group. When considering each group over the study period, no significant differences between each data point were noted for the 24-26 week and 28-30 week gestation groups. In the 26 week gestation group, z scores were significantly higher at birth than any other week ( $p < 0.02$ ). Similarly those in the 30-32 week group scores at birth were higher than in weeks 1, 2 and 3. With respect to feed type, those exclusively fed MEBM had significantly lower scores than those mixed fed at weeks 1 and 4 ( $p < 0.03$ ).



Figures 31: a) (left) Cohort national z scores by gestation; these were clearly stratified, with the 24-26 weeks group illustrating higher scores than any other gestation. By the fourth week, no differences were seen between those at 26 weeks onwards. The error bars represent the SEM. b) (right) Study national z scores by feed type; significantly lower scores were seen in those EEBM fed at weeks 1 and 4 ( $p < 0.03$ ). The error bars represent the SEM. ↓ denotes a significant p value. (Abbreviations: EEBM = exclusive expressed maternal breast milk; Mixed = mixed maternal breast milk and formula).

### 3.1.18) Sepsis

#### i) By gestation and feed type

Early and late onset sepsis rates were generally low throughout the study period. Sepsis was defined as positive pure growth of bacteria from blood culture (at more than  $10 \times 10^6$  per ml). Most episodes cultured coagulase negative staphylococci (76.9 %). Those born at 24-26 weeks gestation had significantly more episodes than infants of greater than 28 weeks gestation (Figure 32:  $p < 0.0007$ ). Sepsis was less likely as the gestation lengthened, and there was a direct correlation between gestation and highest CRP reached during the study period. Correspondingly, infants at lowest gestations experienced longer total antibiotic courses than those at higher gestations.

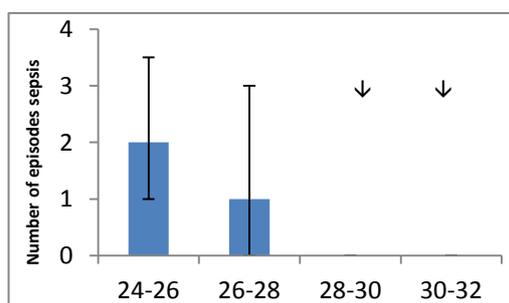
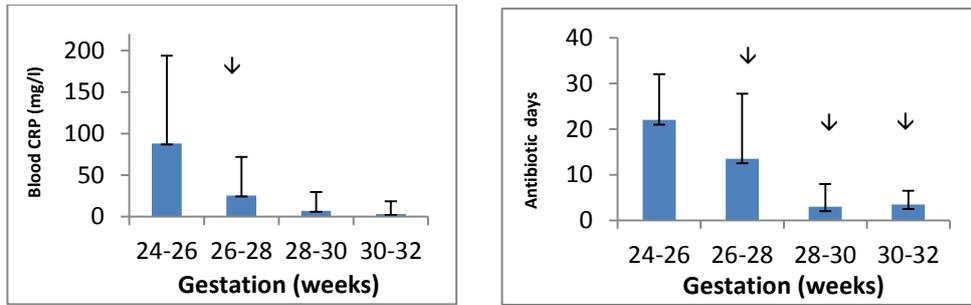


Figure 32: Number of episodes of sepsis by gestation; the 24-26 week group had significantly more episodes than those of 28 weeks and above ( $p < 0.0007$ ). ↓ denotes a significant p value.



Figures 33: a) (left) Highest CRP by gestation; this was higher in those of 24-26 weeks than any other group ( $p < 0.002$ ). The error bars represent the SEM. ↓ denotes a significant p value. b) (right) Number of antibiotic days by gestation; this was also higher in the 24-26 week group than any other ( $p < 0.018$ ). The error bars represent the SEM. ↓ denotes a significant p value. (Abbreviations: CRP = c-reactive protein; mg/L = milligrams per litre; SEM = standard error of the mean).

With regard to feed regimen, there were no significant differences in rates of sepsis or highest CRP during the study between those exclusively MEBM fed, and those mixed fed. Infants exclusively EBM fed incurred longer durations of antibiotic courses ( $p < 0.01$ ), presumed secondary to the high rate of NEC in this group (66 % had any stage of NEC), as per figure 33.

### 3.1.19) Demographics per Unit: SGH, QMH/RHSC, PRMH

Parameter	QMH/RHSC (n=7)	SGH (n=16)	PRMH (n=33)	P values
<b>Gestation (days)[m,sd,CI]</b>	204.2, 16.2,189.28/219.29	193.6, 16.4,185.13/202.62	196.2, 14.7,191.03/201.52	NS
<b>Birth weight (g)[m,sd,CI]</b>	1099, 309,812/1385	1015.8, 222.6,897.2/1134.4	1021.2, 268.7,925.9/1116.5	NS
<b>IUGR (n, %)</b>	2, 28.5	2, 12.5	4, 12	NS
<b>AEDF (n, %)</b>	2, 28.5	0, 0	6, 18	P>S, 0.023
<b>PPROM (n, %)</b>	1, 14.2	2, 12.5	8, 24	NS
<b>IP Abx (n, %)</b>	2, 28.5	3, 18.7	10, 30.3	NS
<b>PET (n, %)</b>	4, 57	4, 25	7, 21	NS
<b>LUSCS (n, %)</b>	4, 57.1	14, 87.5	24, 72	NS
<b>Apgars @10m (m, sd,CI)</b>	8.28, 1.49,6.902/9.669	8.86, 0.74,8.455/9.278	8.81, 1.14,8.399/9.266	NS
<b>CRIB (m, sd,CI)</b>	1.85, 1.86,0.133/3.581	2.68, 2.21,1.508/3.867	2.54, 2.62,1.615/3.476	NS
<b>Depcat (m, sd,CI)</b>	5.28, 1.97,3.458/7.113	4.5, 2.33,3.254/5.746	4.69, 1.84,4.043/5.351	NS
<b>Umbilical line insertion (n, %)</b>	6, 85.7	13, 81.2	18, 54	NS
<b>Days incubated (m, sd,CI)</b>	26.2, 3.73,22.84/29.73	26.56, 3.79,24.541/28.584	25.9, 5.4,23.96/27.85	NS
<b>Days ventilated (med, IQR,CI)</b>	2, 23,-2.97/19.55	5.5, 24,5.13/17.62	2, 7 ,3.38/10.74	NS
<b>Days CPAP (med,IQR,CI)</b>	3, 7,-2.49/15.63	4, 14,2.86/12.02	5, 15,5.6.36/14.55	NS
<b>Number episodes sepsis (med,IQR,CI)</b>	0, 1,-0.689/2.689	0.5, 2,0.436/2.064	0, 1,0.264/0.827	NS
<b>Number antibiotic days (med,IQR,CI)</b>	15, 19,6.25/24.04	20.5, 23,10.5/21.75	5, 10,5.54/10.89	S>P, 0.01; Q>P, 0.05
<b>Highest CRP (mg/L)[med,IQR,CI]</b>	19, 95,2.1/103.0	49.5, 87.5,24.6/99.9	15, 37.86,13.57/48.32	NS
<b>EEBM (n, %)</b>	3, 42.8	6, 37.5	3, 9	S>P, 0.01; Q>P, 0.04
<b>Fortifier (n, %)</b>	0, 0	0, 0	17, 51	P>Q/S, 0.001
<b>PDA ligation (n, %)</b>	2, 28.5	4, 25	2, 6	NS
<b>ROP laser surgery (n, %)</b>	1, 14.2	2, 12.5	2, 6	NS
<b>NEC (n, %)</b>	4, 57	10, 62.5	16, 48	NS
<b>Week 1 weight (g) [med, IQR,CI]</b>	1057.3,243.5 801.8, 1312.9	1007.6 (216.4) 892.3,1123	998.7 (249.8) 905.5,1092.1	NS
<b>Week 2 weight (g) [med, IQR,CI]</b>	1237.5 (234.6) 991.3, 1483.7	1167.5 (198.7) 447,3138	1132.4 (263.4) 1032.3,1232.7	NS

<b>Week 3 weight (g)</b>	1424.7 (330.8)	1321.8 (295.4)	1239.9 (360.5)	NS
<b>[med, IQR,CI]</b>	1119, 1731	1164.4, 1479.3	1109.9,1369.9	
<b>Week 4 weight (g)</b>	1335.7 (675.9)	1464.5 (371.4)	1406.9 (420.1)	NS
<b>[med, IQR,CI]</b>	1273, 1999	1250.1, 1679.0	1255.4,1558.4	
<b>Week 1 Z score</b>	-1.65, 1.65,	-1.1, 0.76,	-1.41, 0.83,	NS
<b>(med, IQR,CI)</b>	-3.389,0.079	-1.51,-0.695	-1.725,-1.103	
<b>Week 2 Z score</b>	-1.43, 1.82	-0.87, 1.06	-1.56, 0.76	S>P, 0.03
<b>(med, IQR,CI)</b>	-3.346,0.486	-1.438,-0.306	-1.857,-1.272	
<b>Week 3 Z score</b>	-1.27, 1.92	-0.93, 0.85	-1.52, 0.81	S>P, 0.03
<b>(med, IQR,CI)</b>	-3.049,0.503	-1.391,-0.477	-1.823,-1.236	
<b>Week 4 Z score</b>	-0.92, 2.06	-1.14, 0.78	-1.35, 0.99	NS
<b>(med, IQR,CI)</b>	-3.407,1.24	-1.597,-0.696	-1.718,-1.001	
<b>Mortality (n, %)</b>	0, 0	3, 18.7	1, 3	NS

Table 18: Unit Demographics. All CIs are 95%. Significantly more infants at PRMH had AEDF (p=0.023); antibiotic administration was shorter in PRMH than the other units (p<0.05); z scores were significantly higher in SGH than PRMH during weeks 2 and 3 (p=0.03); EEBM usage was lower in PRMH (p<0.04), and PRMH also had a fortifier policy, which was not shared by the other units. (Abbreviations: EEBM = exclusive expressed maternal breast milk; Mixed = mixed maternal breast milk and formula; IP Abx: intrapartum antibiotics; in the ‘p values’ column: p = Princess Royal Maternity Hospital; S = Southern General Hospital; Q = Queen Mother’s Hospital).

### 3.1.20) Necrotising Enterocolitis: Demographical and Clinical Associations

*Significant clinical and demographical findings with respect to all stage NEC*

32 infants displayed any signs of necrotising enterocolitis by the Modified Bell's criteria (56%). Of these 6 had stage 1a, 6 stage 1b, 5 stage 2a, 5 stage 2b, 4 stage 3a, and 6 stage 3b. The features of infants with all stage NEC versus those without are presented below.

Parameter	All stage NEC (32)	Non-NEC (24)	P value
Gestation (weeks+days)[m, sd, CI]	27+2.9, 14 d,26.4/28	29+1.9, 12 d,28.2/29.9	0.001
Birth weight (g)[m, sd, CI]	912.5, 205,861.7/1010.2	1150, 271.8,1039.1/1268.7	0.001
Female (n, %)	19, 59	17, 70	NS
Apgar at 10 mins (med, IQR, CI)	9, 0,8.9/9.1	9, 0,8.6/9.2	NS
CRIB Score (med, IQR, CI)	3, 4,2.3/4.0	1, 0,0.72/2.44	0.01
PPROM (n, %)	5, 15.6	6, 25	NS
IP Antibiotics (n, %)	6, 18.75	9, 37.5	NS
SVD (n, %)	9, 28	5, 20	NS
Singleton (n, %)	21, 65	20, 62.5	NS
Deprivation Score (med, IQR, CI)	5, 4,2.5,3.8/5.4	4, 3,4/5.5	NS
In-Utero Growth Restriction (n, %)	6, 18.75	2, 8.3	NS
AEDF (n, %)	4, 12.5	4, 16.6	NS
Umbilical lines (n, %)	6, 18.75	11, 45.8	0.02
Exclusively EBM fed (n, %)	8, 25	4, 16.6	NS
No. infants with any DEBM (n, %)	12, 37.5	3, 12.5	NS
Day feeds started (med, IQR, CI)	2, 1,2.5,2.4/3.4	2, 2,1.7/2.5	0.02
Day full feeds achieved (med, IQR, CI)	14, 14,2.5,13.5/19.5	6, 3,5,5.8/8.5	0.00001
Days ventilated (med, IQR, CI)	6, 25,5,7.7/16.2	1, 1,0.52/6.9	0.02
Feed Vol Wk 1 (med, IQR, CI)	122.3, 305,114.7/266.1	626.6, 577.25,469.6/786.7	0.00001
Feed Vol WK 2 (med, IQR, CI)	519.5, 944,405.4/757.3	1386, 641.3,1135.5/1512.7	0.00001
Feed Vol Wk 3 (med, IQR, CI)	868, 1174,598/1034	1673, 479.3,1399.7/1735.6	0.00001
Feed Vol Wk 4 (med, IQR, CI)	1097.3, 942,762/1225	1875.3, 571,1548/1962	0.00001
Days incubated (med, IQR, CI)	28, 0,26.9/28	28, 3,5,21.4/27.1	0.01
PDA ligated (n, %)	7, 21	1, 4	0.048
ROP surgery (n, %)	8, 25	0, 0	0.03
Fortifier given (n, %)	4, 12.5	10, 41.6	0.02
IVH (n, %)	5, 15.6	5, 20.8	NS
Length of first antibiotic course in days (m, sd, CI)	3.3, 2.9 2.2, 4.4	2.6, 1.2 2.1, 3.1	NS
Episodes sepsis (med, IQR, CI)	1, 2,0.78/1.78	0, 0,0.005/0.32	0.0002

Highest CRP during study [mg/L] (med, IQR, CI)	25.5, 66.5,29.5/76.4	3.5, 2.5,4.82/23.9	0.0014
Antibiotic days (med, IQR, CI)	19.5, 16,13.25/19.8	3.4, 16,3.35/5.64	0.00001
Weight Wk 1 (med, IQR, CI)	880, 340,874.6/1036.2	1090, 204,966/1182	0.04
Weight Wk 2 (med, IQR, CI)	1040, 366,719/2160	1247.5, 248.75,1129/1355	0.03
Weight Wk 3 (med, IQR, CI)	1160, 545,1083.5/1300.7	1415, 326.25,1260/1563	0.01
Weight Wk 4 (med, IQR, CI)	1290, 463,1194.7/1451.2	1623, 400,1450/1809	0.002
Z score: Birth (med, IQR, CI)	-0.46, 1.02,-0.93/-0.19	-0.65, 0.95,-1.08/-0.31	NS
Z score: Week 1 (med, IQR, CI)	-1.08, 1.01,-1.48/-0.71	-1.54, 0.98,-1.96/-1.34	NS
Z score: Week 2 (med, IQR, CI)	-1.23, 1.09,-1.53/-0.62	-1.45, 0.90,-1.98/-1.33	NS
Z score: Week 3 (med, IQR, CI)	-1.25, 1.15,-1.62/-0.77	-1.43, 0.93,-1.84/-1.12	NS
Z score: Week 4 (med, IQR, CI)	-1.08, 1.20,-1.64/-0.72	-1.28, 1.11,-1.79/-0.95	NS
Mortality (n, %)	4, 12.5	0, 0	NS

Table 19: Demographic and clinical features of those with all-stage NEC versus those without; significant p values are quoted and discussed within the text.

### i) All Stage NEC Associations

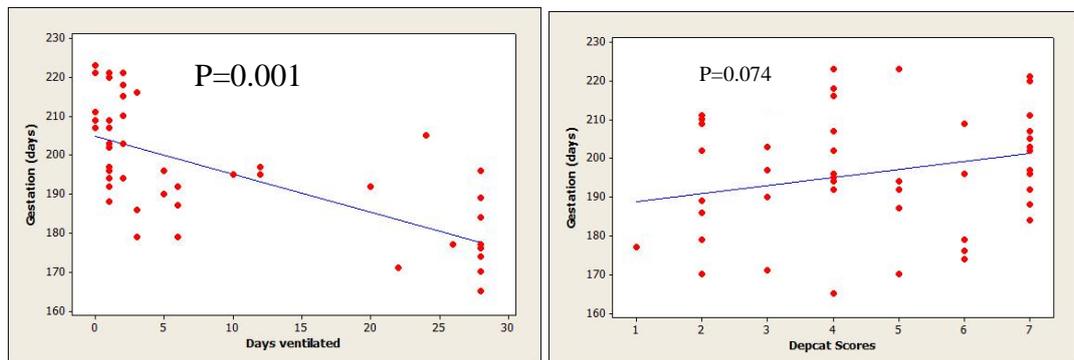


Figure 34: a) (left) All stage NEC, gestation versus days ventilated; there was a clear correlation ( $p=0.001$ ); b) (right) All stage NEC, gestation versus Depcat scores approached significance at  $p=0.074$ .

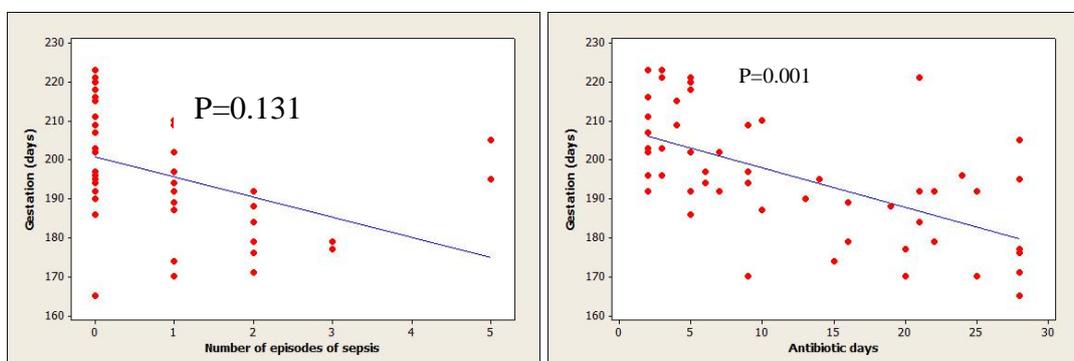


Figure 35: a) (left) All stage NEC, gestation versus episodes of sepsis did not show a significant correlation ( $p=0.131$ ); b) (right) All stage NEC, gestation versus antibiotic days showed a clear correlation, with longer courses at lower gestations ( $p=0.001$ ).

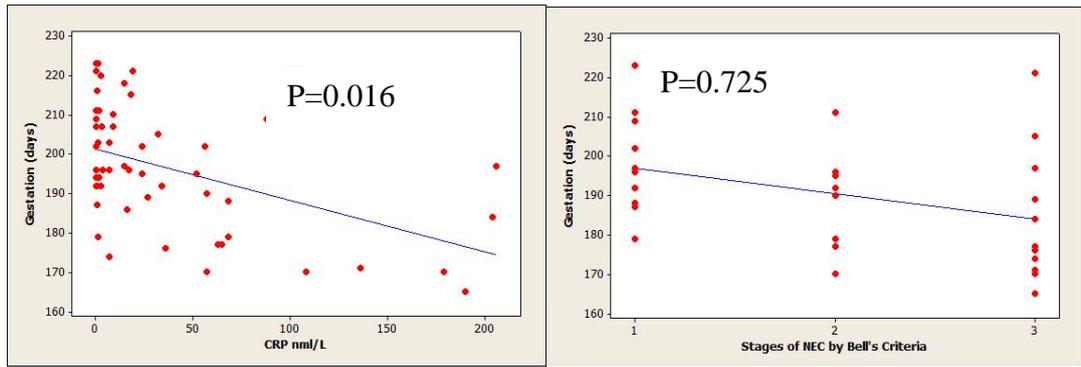


Figure 36: a) (left) All stage NEC, gestation versus CRP level; there were closely correlated ( $p=0.016$ ). b) (right) All stage NEC, gestation by Bell's Criteria; this did not show a significant correlation ( $p=0.725$ ).

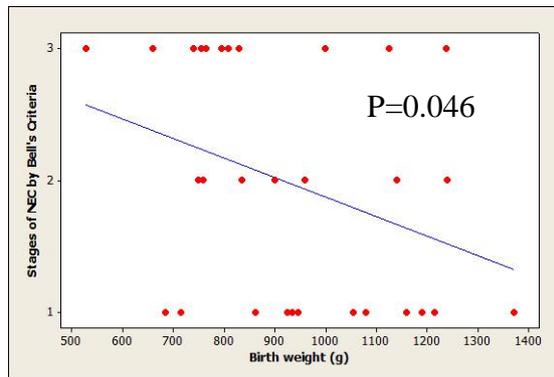


Figure 37: Stages of NEC by birth weight; more severe NEC occurred at lower birth weights ( $p=0.046$ )

All-stage NEC demographics and clinical correlations are illustrated in figures 34 – 37.

As such, 20 were confirmed radiologically as  $\geq$  Stage 2a (i.e. 'definite' NEC). 8 out of 20 underwent laparotomy: 2 with bowel resection and primary anastomosis, 5 with resection and ileostomy formation; 1 with biopsies and ileostomy formation. The clinical features of infants with  $\geq$  stage 2a NEC are displayed in table 20.

Patient	Bell's Stage	Gestation (weeks + days)	Birth weight (grams)	Day of 1 <sup>st</sup> NEC	Type of feed	Day of 1 <sup>st</sup> feed	Day to full feeds	Management
1	2a	28+0	836	8	EEBM	2	13	Medical
2	2a	25+2	750	8	EEBM	2	never	Medical
3	2a	30+1	1240	3	MIXED	2	10	Medical
4	2a	27+1	960	14	MIXED	3	10	Medical
5	2a	25+4	900	10	MIXED/D	3	18	Medical
6	2b	27+3	1140	3	EEBM	2	18	Medical
7	2b	27+6	1140	7	MIXED	3	36	Medical
8	2b	27+6	835	3	MIXED/D	2	11	Medical
9	2b	24+2	760	35	MIXED/D	4	11	Medical
10	3a	25+1	530	16	EEBM/D	4	28	STOMA
11	3a	31+4	1238	9	EEBM	3	28	STOMA
12	3a	23+4	740	18	MIXED	6	15	STOMA
13	3a	27+0	1000	5	EEBM/D	4	23	Medical*
14	3a	24+2	795	12	EEBM/D	3	10	1 ANAST
15	3a	26+2	660	44	EEBM/D	2	16	1 ANAST
16	3b	24+3	830	22	EEBM	7	28	Medical*
17	3b	25+2	810	34	EEBM	2	never	Medical*
18	3b	29+2	766	5	EEBM	4	28	STOMA
19	3b	24+6	756	4	MIXED	1	10	STOMA
20	3b	28+1	1125	9	MIXED	4	28	STOMA
Med/IQR		26.6±2.82	832.5±272.25	9±11.5	35% EEBM	3±2	17±18	60% Medical

Annotation: \*too sick to transfer to surgical unit

Table 20: Comparison of demographical and clinical features in infants with stage 2a, 2b, 3a and 3b NEC. (Abbreviations: EEBM = exclusive expressed maternal breast milk; Mixed = mixed maternal breast milk and formula; D = donor expressed breast milk).

## ii) Surgical management

All histological diagnoses of NEC were confirmed on biopsy. None of these infants had an alternate or contributing surgical diagnosis as an adjuvant or alternate diagnosis to NEC. All stomas formed were ileostomies, and all ileocaecal valves remained intact. Primary resections involved jejunum and part of the ileum in one infant, and ileum in the other. One

infant had an ileostomy formed but no gut resected. Accordingly seven infants subsequently developed Short Gut Syndrome.

The following figures 38 a and b are anonymised x-rays of study patients with confirmed stage 3a NEC or more.

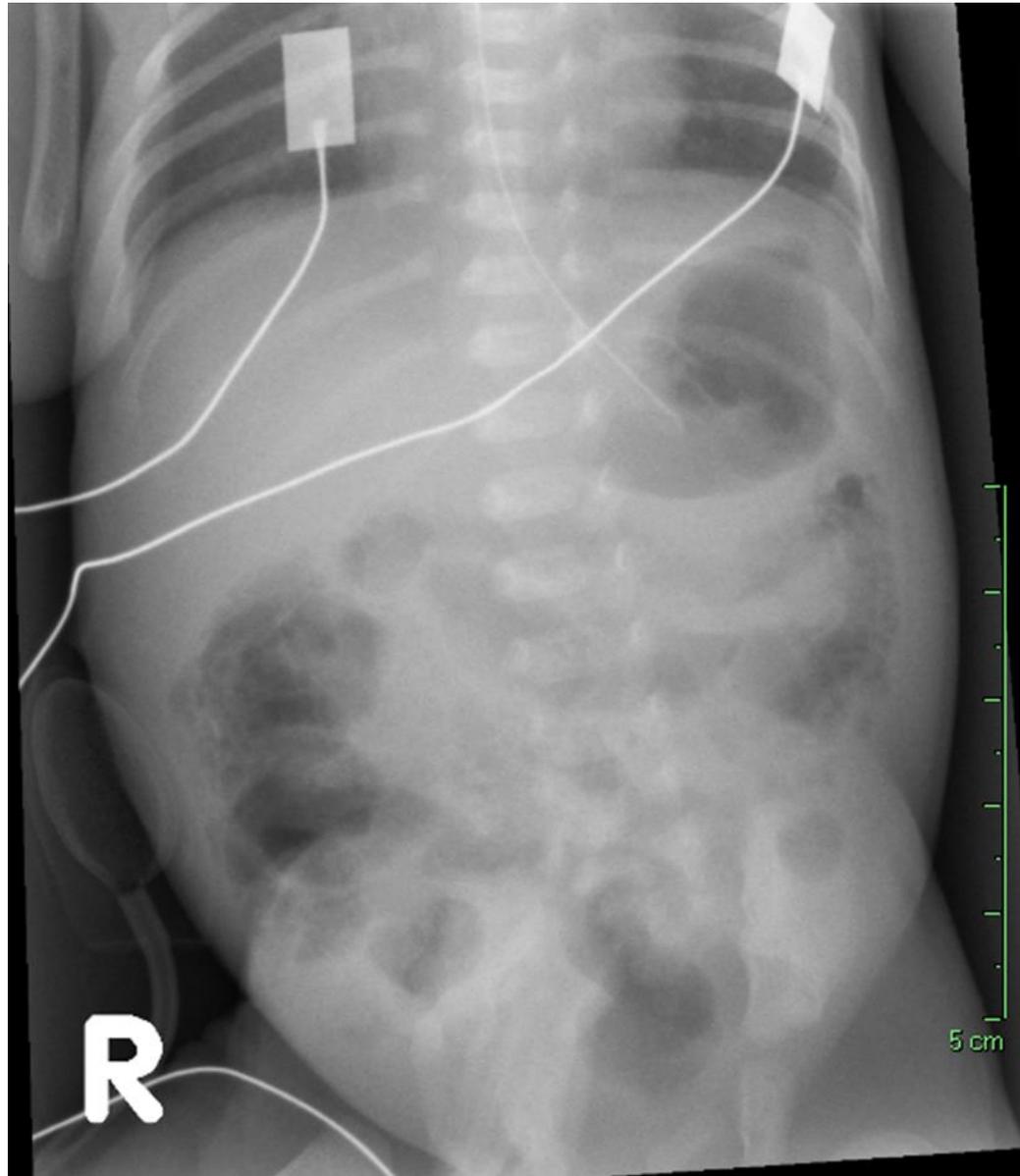


Figure 38: a) X-ray of NEC stage 3a (patient number 48)



Figure 38: b) X-ray of NEC stage 3b (patient number 39).

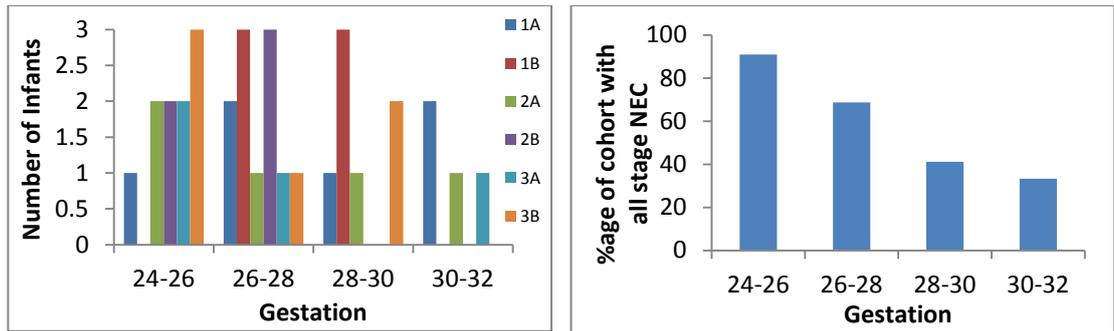
*iii) Significant clinical and demographical features in those with  $\geq$ Stage 2a NEC versus those without NEC*

Parameter	$\geq$ Stage 2a NEC (20)	Non-NEC (24)	P value
Gestation (weeks+days)[m, sd,CI]	26.6, 19.75 d,25.5/27.5	29+1.9, 12 d,28.2/29.8	0.0005
Birth weight (g)[m, sd,CI]	832.5, 272.2,798.1/983	1150, 271.8,1039/1268	0.0001
Female (n, %)	13, 65,	17, 70	NS
Apgar at 10 mins (med, IQR,CI)	9, 1.25,6.68/8,91	9, 0,8.6/9.2	0.05
CRIB Score (med, IQR,CI)	4, 4,2.55.4,74	1, 0,0.72/2.44	0.0023
PPROM (n, %)	4, 20	6, 25	NS
IP Antibiotics (n, %)	5, 25	9, 37.5	NS
SVD (n, %)	6, 30	5, 20	NS
Singleton (n, %)	13, 65	20, 62.5	NS
Deprivation Score (med, IQR,CI)	4, 4,2.5,3/5.2	4, 3,4.03/5.54	NS
In-Utero Growth Restriction (n, %)	5, 25	2, 8.3	NS
AEDF (n, %)	3, 15	4, 16.6	NS
Umbilical lines (n, %)	18, 90	11, 45.8	0.005
Exclusively EBM fed (n, %)	11, 55	4, 16.6	0.01
No. infants with any DEBM (n, %)	7, 35	3, 12.5	NS
Day feeds started (med, IQR,CI)	3, 2,2.3/3.7	2, 2,1.7/2.5	0.02
Day full feeds achieved (med, IQR,CI)	17, 18,13.1/21.2	6, 3.5,5.8/8.5	0.0001
Days ventilated (med, IQR,CI)	23, 22.5,11.8/22.6	1, 1,0.52/6.9	0.0003
Feed Vol Wk 1 (med, IQR,CI)	58, 146.7,53.9/264.9	626.6, 577.25,469.9/786.7	0.00001
Feed Vol Wk 2 (med, IQR,CI)	209.8, 799.6,237/671	1386, 641.3,1135.5/1512.7	0.00001
Feed Vol Wk 3 (med, IQR,CI)	611.4, 940.3,384/911	1673, 479.3,1399.7/1735.6	0.00001
Feed Vol Wk 4 (med, IQR,CI)	796.5, 938.8,502/1093	1875.3, 571,1548/1962	0.00001
Days incubated (med, IQR,CI)	28, 0,26.6/28.2	28, 3.5,21.4/27.1	NS
PDA ligated (n, %)	6, 30	1, 4	0.05
ROP surgery (n, %)	7, 35	0, 0	0.006
Fortifier given (n, %)	1, 5	10, 41.6	0.01
IVH (n, %)	5, 25	5, 20.8	NS
Episodes sepsis (med, IQR,CI)	1, 2,0.71/2.1	0, 0,0.005/0.32	0.0004
Highest CRP during study [mg/L] (med, IQR,CI)	54.5, 95.25,41.7/108.8	3.5, 2.5,4.8/23.9	0.001
Duration of first antibiotic course in days (m, sd, CI)	2.85, 2.0, 1.89, 3.8	2.6, 1.2 2.1, 4.4	NS
Antibiotic days (med, IQR,CI)	21, 12.5,16.2/23.5	3.4, 16,3.35/5.64	0.00001
Weight Wk 1 (med, IQR,CI)	840, 329.5,812.9/1019.1	1090, 204, 966.8/1182.5	0.03
Weight Wk 2 (med, IQR,CI)	1000, 450,406/2947	1247.5, 248.75, 1129.1/1355.1	NS

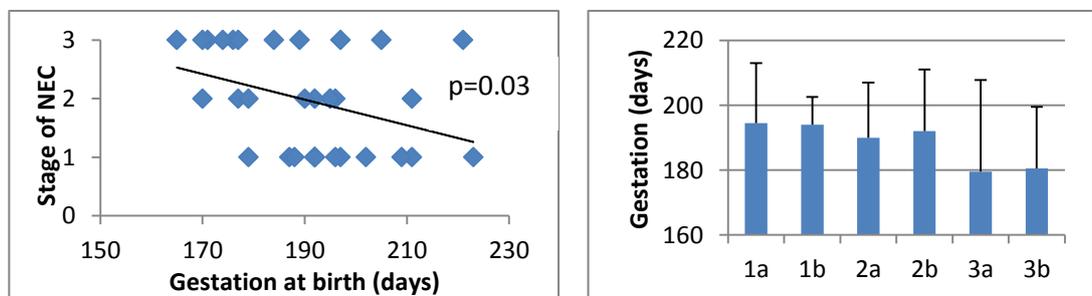
Weight Wk 3 (med, IQR,CI)	1000, 589,1008/1316.7	1415, 326.25,1260.5/1563.3	0.02
Weight Wk 4 (med, IQR,CI)	1132, 469.51129.9/1477.8	1623, 400,1450.7/1809.9	0.008
Z score: Birth (med, IQR,CI)	0.23, 1.61,-0.919/0.07	-0.65, 0.95,-1.08/-0.31	NS
Z score: Week 1 (med, IQR,CI)	-0.80, 1.54,-1.52/-0.46	-1.54, 0.98,-1.96/-1.34	0.04
Z score: Week 2 (med, IQR,CI)	-0.65, 1.5,-1.43/-0.06	-1.45, 0.90,-1.98/-1.33	0.04
Z score: Week 3 (med, IQR,CI)	-1.21, 1.77,-1.59/-0.33	-1.43, 0.93,-1.84/-1.12	NS
Z score: Week 4 (med, IQR,CI)	-1.02, 1.46,-1.68/-0.35	-1.28, 1.11,-1.79/-0.95	NS
Mortality (n, %)	4, 20	0, 0	NS

Table 21: Clinical and demographical features of those with  $\geq$ stage 2a NEC versus those without NEC. CIs are at 95%. (Abbreviations: EBM = expressed breast milk; CRIB = Clinical Risk Index in Babies; PPROM = prolonged premature rupture of membranes; IP = intra-partum; SVD = spontaneous vaginal delivery; AEDF = absent end diastolic flow; DEBM = donor EBM; PDA = patent ductus arteriosus; ROP = retinopathy of prematurity; IVH = intraventricular haemorrhage; NS = not specified)

#### iv) NEC Infants: Significant Demographical and Clinical Correlations



Figures 39: a) (left) All-stage NEC, by gestation; b) (right) Percentage of cohort with all stage NEC, by gestation



Figures 40: a) (left) Gestation versus NEC stages 1, 2 and 3 showed significant correlation with lower gestation ( $p=0.03$ ); b) (right) Number of infants with each stage of NEC, according to gestation.

NEC was closely correlated with gestational age (Figure 40); infants of lower gestational age incurred a higher incidence of NEC, and was more likely to be severe (figure 40 b). A less significant correlation was seen between birth weight and stage of NEC (figures 37 and 41).

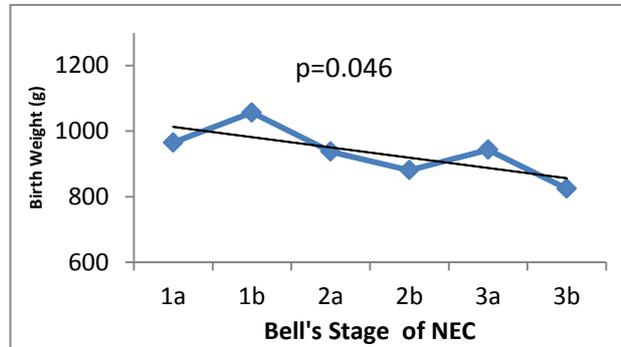


Figure 41: NEC stages by birth weight showed a positive correlation with birth weight ( $p=0.046$ )

A significant correlation was seen between NEC stage and the day of life on which the infants first became unwell with any stage of NEC (Figure 42:  $p=0.034$ ).

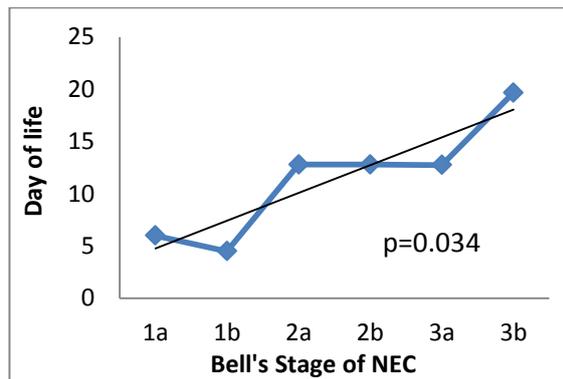


Figure 42: Day of first NEC, by highest NEC stage; early onset clinical suspicion was associated with milder NEC ( $p=0.034$ ).

NEC that developed later on in the study period was more severe, whereas early-onset NEC tended to be milder. There was no significant difference in day of first onset of NEC between those who were medically or surgically managed. Given the significant heterogeneity of feed types, no correlations were seen with regard to severity of NEC. Several other gestation-related correlations were observed in infants with all-stage NEC, illustrating the bias to this group from extreme prematurity. Extreme prematurity showed strong correlations with duration of ventilation ( $p=0.001$ ), deocat score ( $p=0.0074$ ), sepsis ( $p=0.0131$ ), duration of antibiotics ( $p=0.001$ ), and CRP ( $p=0.016$ ) (data not illustrated).

Relevant associations were further stratified according to stage of NEC. Given the ambiguous nature of stages 1a and 1b, and the further clinical difficulties in separating 2a from 2b, and 3a from 3b, 1a and 1b were excluded from further analysis, and stages 2a and 2b were paired together, as were stages 3a and 3b.

*Stage 2a and b*

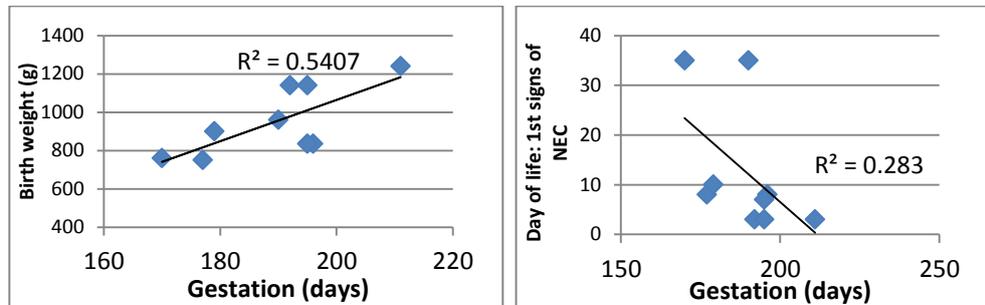


Figure 43: a) (left) Stage 2a+b infants' gestation versus birth weight strongly correlated ( $p=0.024$ ); b) (right) Stage 2a+b infants' gestation versus day of life of first emergence of NEC also showed a significant correlation ( $p=0.035$ ).

*Stage 3a and b*

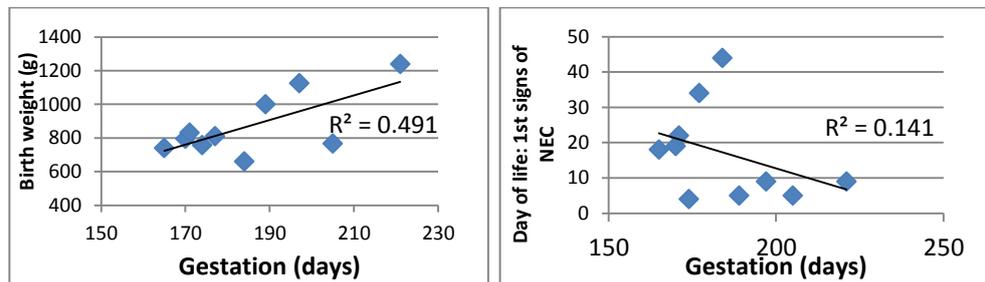


Figure 44: a) (left) Stage 3a+b infants' gestation did not correlate with birth weight ( $p=0.081$ ); b) (right) Stage 3a+b infants' gestation versus day of life of first emergence of NEC also did not correlate ( $p=0.172$ )

66 % of infants with stage 3a NEC were fed exclusively maternal EBM for the study duration. Figure 47 illustrates correlations between gestation, birth weight, and day of first signs of NEC. 50 % of these infants with stage 3b NEC were fed solely maternal EBM. Figure 48 shows attempts to correlate gestation, birth weight and day of first signs of NEC in those with stage 3b. There was no significant difference in date of onset of NEC between medically and surgically managed patients (Day 2 vs Day 3 (med),  $p=0.754$ ). Most NEC occurred within the first 2 weeks of life (median 14 days).

### v) Demographical Associations in Infants with $\geq$ Stage 2a NEC

In combination, all infants with  $\geq$ stage 2a NEC showed a strong correlation between gestation and birth weight, as well as gestation versus day of first clinical emergence of NEC, as illustrated in figure 45 below:

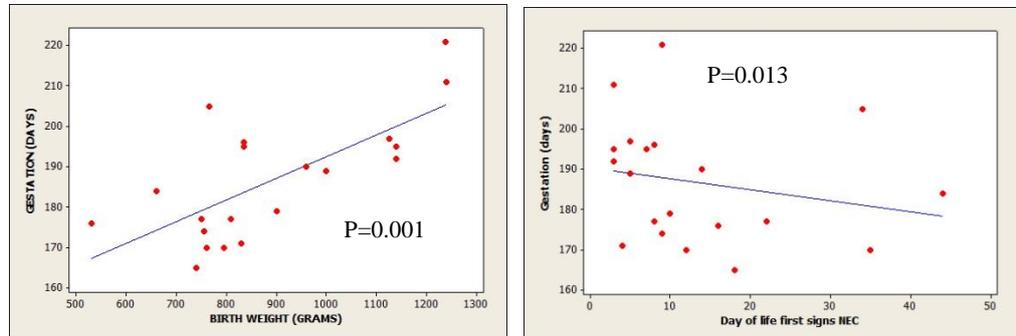


Figure 45: a) (left) Gestation versus birth weight in  $\geq$ stage 2a NEC correlated closely ( $p=0.001$ ); b) (right) Gestation versus day of first signs of NEC,  $\geq$ stage 2a NEC also correlated strongly ( $p=0.013$ ).

No other notable demographical associations were found in infants with  $\geq$ stage 2a NEC, as shown in figures 50 – 52, including: gestation versus Depcat score; ( $p = 0.068$ ); gestation versus number of days ventilated ( $p = 0.257$ ); gestation versus number of episodes of sepsis ( $p = 0.763$ ); gestation versus antibiotic days ( $p = 0.18$ ), gestation versus highest CRP ( $p = 0.189$ ).

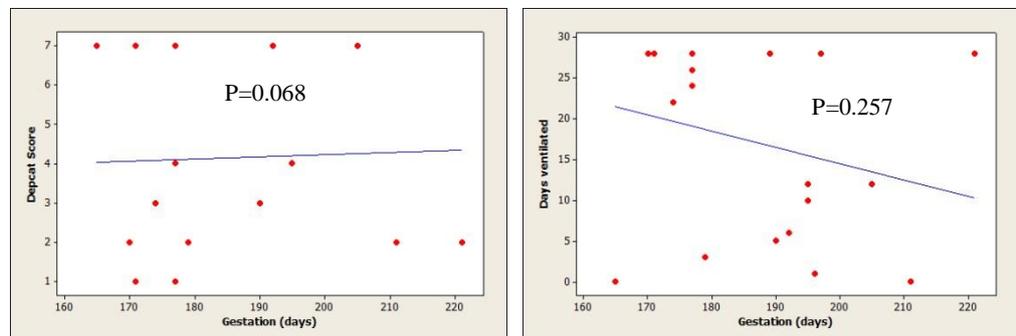


Figure 46: a) (left) Gestation versus Depcat score, infants with  $\geq$ stage 2a NEC was close to significance ( $p=0.068$ ); b) (left) Gestation versus days ventilated, infants with  $\geq$ stage 2a NEC did not correlate ( $p=0.257$ ).

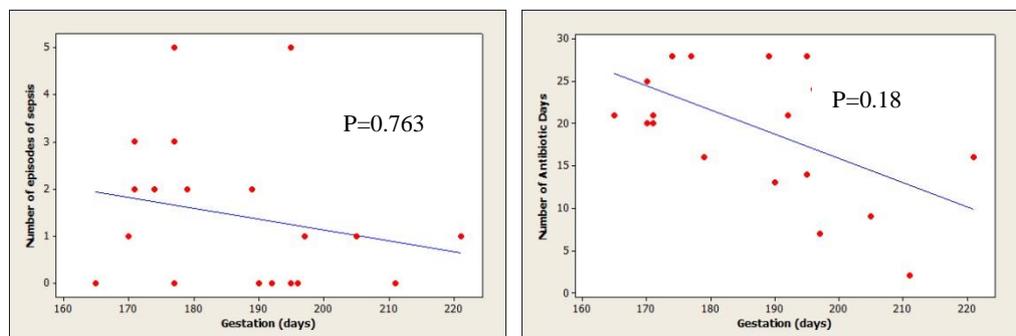


Figure 47: a) (left) Gestation versus episodes sepsis, infants with  $\geq$ stage 2a NEC; b) (right) Gestation versus number of antibiotic days, infants with  $\geq$ stage 2a NEC. Neither showed significant correlations.

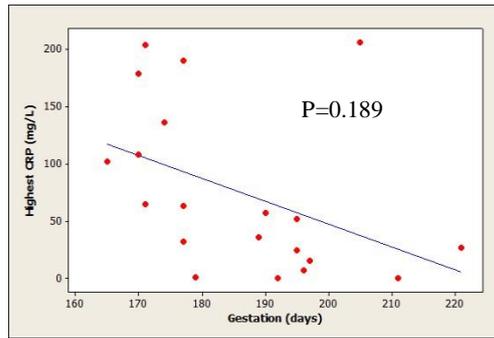


Figure 48: Gestation versus highest CRP, infants with  $\geq$ stage 2a NEC did not show a significant correlation ( $p=0.189$ ).

### Further multivariate analyses:

Upon discussion with a statistician, it was agreed that given the heterogeneity of feed types, and wide range of non-normally distributed data, further multivariate analysis would be inappropriate.

### **3.1.21) Discussion**

#### *i) Demographical Factors*

The high rate of recruitment (83%) reflected the observational nature of this study. We excluded out-born infants, and, as such, this realistically represents the local population, bar one infant, who was transferred in utero from Inverness at the onset of preterm labour given the lack of a local tertiary neonatal unit. The low study rates of IUGR and AEDF were surprising given Glasgow's status of extreme deprivation – the so-called 'Glasgow Effect' – with deprivation levels amongst the highest in Europe (Gray L 2009). Depcat scores were significantly lower at lower gestational ages, which likely reflected the higher rate of these infants conceived through assisted conception, which in turn is associated with higher social class and lower deprivation scores. This was unexpected considering the strong regional and national association between social class and prematurity. Birth weight was closely correlated with gestation, and, as such, the cohort was not significantly small for their gestational age. Not all infants with IUGR had AEDF, and vice versa, which, again was surprising for this cohort anecdotally.

Most of the cohort was delivered by caesarean section, mainly for reasons of maternal health, and as such were not perinatally compromised. PPROM complicates 2% of pregnancies, but accounts for 40% of preterm births. As such, the incidence of PPROM was higher in comparison (19%), but the use of antenatal and intrapartum antibiotics was also high (100% of mothers with PPROM, plus 4 mothers with antenatal urinary tract infections before, during and after delivery), in accordance with regional and national guidelines to prevent the establishment of chorioamnionitis (Gynaecologists 2006). Curiously, pregnancy induced hypertension was more common as a cause of preterm delivery at later gestations, although this did not reach statistical significance.

In total 15 twins were recruited. One infant was the second and smaller twin, of whom the elder brother was too heavy to be recruited. Two other infants were surviving twins who had respective siblings who sadly died in utero. It is a recognised phenomenon that studies of preterm infants commonly include multiples, which may, if unaccounted for, skew resultant demographical and clinical data. However, recent evidence suggests that if twins number less than 10-20% of the study population, that no further statistical alterations are required in addition to standard univariate and multivariate methodologies (Marston 2009,

Shaffer M 2009). Notably, 54% of the twins were monochorionic, diamniotic: naturally conceived identical twin pregnancies.

### *ii) Clinical features*

Theoretically, preterm infants commonly require stabilisation at delivery, in contrast with resuscitation (Vento 2010). Our cohort, however, showed a significantly lower Apgar score at 1 minute of life than 5 or 10, indicating a need for intervention at the resuscitaire. This did not appear to be gestation-dependent. The Apgar score at 5 minutes has recently been adopted as a diagnostic marker in the definition of hypoxic ischaemic encephalopathy, and are now considered to be prognostic of neurological outcome in term infants (Apgar 1953). Although there is often intraobserver disparity in scoring preterm infants, the Apgar score at 10 minutes is now utilised by the National Neonatal Database system 'Badger' as part of its Clinical Risk Index Score in Babies.

When the duration of invasive and non-invasive ventilation was considered, the lengths of each dovetailed according to gestation: infants of extreme prematurity had significantly higher durations of invasive ventilation, while those at 30-32 weeks gestation had longer duration of non-invasive ventilation. As expected, the duration of incubation was longer at lower gestations. 28 % of the study cohort was noted to have intraventricular haemorrhage, slightly above the current considered incidence of 20 % (Fanaroff, Stoll et al. 2007). Intraventricular haemorrhage was not gestation-dependent, but the more severe haemorrhages were incurred by infants of extreme prematurity. The incidence of duct ligation was similarly gestation dependent, with significantly more extremely preterm infants undergoing surgery than those between 30-32 weeks gestation. At 12.5 % of the whole cohort, this is a lower incidence than reported nationally or internationally (Fanaroff, Hack et al. 2003). Surgery for retinopathy of prematurity occurred in only five infants (8 %), lower than the national average (Health 2008). All infants were less than 26 weeks gestational age.

### *iii) Unit differences in demographics*

There were reassuringly few clinical and demographical differences in study populations between each NICU involved in the study (table 18). The PRMH NICU catchment area for deliveries includes the most deprived areas in Glasgow, and thus was expected to have a significantly higher deprivation score than the other NICUs. However, there was no

significant difference between the scores of any unit, although this was partly confounded by the QMH/RHSC and SGH units merging during the recruitment period. The unexpected similarity in Depcat score is considered secondary to the number of affluent parents conceiving after assistance from the GRI fertility unit, thereafter booking and receiving antenatal care in PRMH. The study infants born in PRMH did, however, have significantly higher levels of AEDF than those at SGH, which, although associated with NEC, did not correlate with a higher rate of NEC in the PRMH population. Both SGH and QMH units had significantly higher rates of exclusive maternal breast milk feeding than PRMH, although there is no specific unit policy that would appear to be the cause of this. Fortifier, on the other hand, is only used in PRMH, and was not associated with a higher incidence of NEC.

There were some unexpected unit differences, including a considerably shorter duration of antibiotic usage for PRMH infants, and higher weight Z scores in SGH infants during weeks 2 and 3, despite the higher formula usage in PRMH, which may be considered likely to increase weight gain at a faster rate.

#### *iv) Feeds*

Although local audit suggested that there would be three distinct feed groups – exclusively MEBM fed; DEBM fed; and formula fed – the actual groups were far more heterogeneous. Given there are no regional or national feed guidelines, this was not surprising, although the volume of DEBM used accounted for less than 14 % of the total generated for the DEBM bank over the same time period, leaving ample supply to cover the equivalent volume of formula fed to the cohort. The remaining supply was distributed to ELBW, extremely preterm infants throughout other units throughout Scotland, and local surgical infants at all gestations. Some of these infants would still have received formula feeds as calorific supplements, although this can also be achieved through use of fortifiers. Supplementation for ‘catch-up growth’ is a controversial topic, and divisive amongst neonatologists. Recent studies have supported the use of an exclusive breast milk diet to maintain adequate growth in surviving VLBW infants, either donor or maternal (Schanler, Shulman et al. 1999, Sullivan, Schanler et al. 2010, Underwood 2013). Catch up growth may not be advantageous to preterm infants in the long term, with studies revealing an increased incidence of metabolic syndrome and its sequelae, particularly adverse cardiovascular events (Simmer 2007, Griffin and Cooke 2012).

However, other issues regarding the use of DEBM are pertinent to discuss. Although there are no requirements for parents within the UK to consent to blood or blood product transfusions, consent is taken in our neonatal units for the use of DEBM, despite strict screening of all donors for the same virological infections as per blood donors. However, during the study further advertising was undertaken by the DEBM bank to increase visibility through each of the neonatal units – with one of the study babies who was almost exclusively fed DEBM featured on promotional leaflets and fact sheets. Ironically, despite strict handling and processing of all DEBM, there are no aseptic guidelines for MEBM, which is not pasteurised in the same way that all national supplies of DEBM are. There are still no clear guidelines on appropriateness for use, but there is increasing interest in the use of DEBM and human-derived milk fortifiers, creating an ever-complex array of feeding blends and options. Only 1 human milk derived fortifier exists, but is only licensed for use within the USA. During the duration of this study, further changes in milk expression guidelines occurred within NHS GG+C, including a change of breast pump washing policy, which no longer needed to be sterilised and autoclaved, but simply washed. In the absence of a definitive RCT or metaanalysis, clinician preference on the use of donor milk may have added to the feed type heterogeneity.

The sampling process for this study raised other unexpected issues regarding feeds. It was noted that many of the mothers of preterm infants produced far more milk than required by their infants, many of whom required prolonged trophic feeds. As a result, most of their colostrum was sequestered at the back of each milk freezer. As such, the most mature milk was often used first. The differences between mature and first milk are well-established, as are differences between milk produced by mothers of preterm infants (Castellote, Casillas et al. 2011). This observation in the discrepancy of use was highlighted to nurses in each NICU with the aim of preferentially using colostrum first in this regard.

As expected, there was a higher rate of MEBM feeding from the SGH and RHSC units, which was not surprising considering their locations in parts of the city with lower Depcat scores. However, there was statistically no difference in Depcat scores between these families, which may be because of these units merging half way through the recruitment period. The newer unit at the SGH then observed a surge in their delivery rate, as mothers chose antenatally to transfer their care from other units outside the city. The mixture of feeds in the majority of infants is important to emphasise as often trials utilise strict feed

groups, when the need for parental consent dictates feed type in the majority of infants, for at least part of their inpatient admission.

*v) Growth*

Birth weight was closely aligned to gestational age, indicating the appropriate growth of these infants. Group z scores were calculated to assess changes in the group over time. At all gestations, z scores fell between birth and first week of life, but those between 24-26 weeks remained the closest to 0, and were the only gestational grouping to show a rise in z score during week 2. This likely reflects fluid gain as these were the sickest infants within the cohort. As anticipated, when stratified by feed type, those mixed fed had higher z scores than EBM, although again during week 2, their z scores dovetailed, likely reflecting increased fluid gain by those at 24-26 weeks, most of whom were EEBM fed. The nearest comparator that could be found on reviewing the evidence base for UK z scores in preterm infants is described by Wood et al in 2003 (Wood, Costeloe et al. 2003), as part of the EPICure Study. They describe weight in terms of number of standard deviations above the mean for their population of 283 infants between 23 and 25 weeks gestation, in comparison with national data as published by The Child Growth Foundation, using 1990 growth reference ranges from NPEU (Foundation 1996). Each gestational group (when stratified for gender and plurality) showed standard deviations above, rather than below, the mean for the UK. Analysis of variance revealed this to be significantly higher than the national average. The figures in our study, however, were compared with more recent data as described by Pan et al in 2009 (Pan 2012), using the LMS Growth Programme (Pan 2012), and included infants up to 32 weeks gestation. Nonetheless, it is significant that in comparison to national scores, our cohort were consistently negative at birth, reflecting poor in utero gain, and throughout the study period - the sharpest drop being between birth and the end of week 1.

*vi) Sepsis*

Proven bacterial sepsis rates were extremely low within the study cohort, regardless of neonatal unit, and correlated closely with gestational age. Over three quarters of episodes were from coagulase negative staphylococci. The precautionary use of antibiotics was noted to be higher in the SGH and RHSC units than in PRM, but with no difference in sepsis rates. Since preterm delivery is in itself a risk factor for early onset sepsis, very few infants escaped antibiotics in the immediate post-partum period. When sepsis was

considered in regard to feed type, surprisingly there was no correlation with EEBM, despite the fact that EEBM feeding was strongly associated with extreme prematurity. This may simply be due to extremely low rates of sepsis. However, those between 24-26 weeks gestation received longer courses of antibiotics, which is expected given their significantly higher CRP levels. Again CRP levels were not significantly different between feed types, likely reflecting the higher rates of NEC in those mixed fed, in combination with low numbers of infants exclusively fed EBM. Sepsis, higher CRP levels, and length of antibiotic treatment were also significantly more likely to occur in infants with confirmed NEC.

### **vii) NEC: Demographical and Clinical Factors**

The strikingly high rate of NEC was a surprise to all involved in this project. Although naturally observed bias could have accounted for some of the considerations of stage 1 NEC, it is unanimous that the ten infants who incurred stage 3 NEC were all appropriately diagnosed – of whom 8 were confirmed histologically. Even excluding all infants with NEC who did not have this histologically confirmed, the incidence would still be over 14 %, far more than the 6-10 % range quoted in large trials from developed countries (Kawase, Ishii et al. 2006, Lin and Stoll 2006, Kovacs 2007, Rennie 2012). Two immediate theories for this high incidence were dispelled after statistical analysis: 1) that early onset, pre-feeding NEC within the first week of life reflected high rates of intrapartum and antenatal distress; 2) that the extreme deprivation of the study population was more likely within those who incurred NEC. Firstly, the median age at first onset of NEC was older with increasing NEC severity – such that milder episodes of NEC early on in life were far less likely to lead to definite NEC. There was also no association between IUGR or AEDF and NEC, although the incidence of both was so low as to make this analysis unreliable. There was no difference in Depcat Scores, and this may be confounded by the opening of the new SGH unit, with associated migration of deliveries according to maternal request. Notably, the Depcat scoring system is Scotland-specific, so there is no way to compare scores for similarly deprived cities in the rest of England, Wales and Northern Ireland.

One possibility is that NEC is underreported in other centres (Lin and Stoll 2006, Kovacs 2007). It is well recognised that other neonatal and infantile illnesses have a similarly variable range of incidence and prevalence data – particularly intestinal failure, which has

been reported at between 70-77 and 1300 new cases per year (Barclay, Paxton et al. 2009). In future, diagnoses of both illnesses will become clearer with the UK-wide NICU data system 'Badger', which encompasses a daily record of meticulous feed, PN and NEC data, although it should be noted that the options for recording NEC are at the moment limited to a somewhat binary 'yes' or 'no'. According to UK Badger data from 2011, there were 809 infants dependent upon PN for more than 28 days – which by definition means 809 infants with intestinal failure. Concurrent BIFS data however do not support this from a tertiary referral perspective – which either means these infants are not being referred, or are not being identified at NICU level (Modi, Barclay, personal communication).

Part of the discrepancy in NEC diagnosis is the laxity in the Modified Bell's Criteria, which although extensive, has with progression of neonatology since its inception in 1976, been subject to no further alterations. The presence of apnoea and bradycardia is high in preterm infants without NEC, thus many infants who do not progress to stage 2 or 3 NEC can be categorised with stage 1a with mild abdominal distension or bilious vomits, both of which can be common physiological features in preterm infants. A positive Faecal Occult Blood test (FOB) delineates stage 1b NEC from stage 1a, although many NICUs have stopped using the FOB tests as they have extremely high sensitivity but low specificity, with many false positives secondary to mild gastric trauma from NG tube placement, or rectal bleeding from fissures. In the infants in our study, it was anecdotally observed that the appearance of gross PR blood was actually a late sign – whereas the Modified Bell's Criteria places the FOB positive test in stage 1. As a result, our compromise was to replace the FOB test with any visible PR or NG blood in conjunction with the other criterion. Similarly, perforation is considered the end-stage of the Bell's Criteria, and differentiates between stages 3a and 3b. However, this confuses the diagnosis in a small portion of infants who develop focal intestinal perforation, who remain clinically stable but have confirmed pneumoperitoneum on x-ray, most secondary to gastric perforation in association with CPAP (Novack, Waffarn et al. 1994, Kawase, Ishii et al. 2006). Within our cohort, those for whom FIP was suspected were then re-classified after histopathology confirmed NEC. Equally, the potential for laparotomy depends upon proximity to a neonatal surgical unit, as well as their preference for surgical management. A recent Cochrane Review of Laparotomy and resection/enterostomy versus peritoneal drain for treatment of surgical NEC showed no advantage of laparotomy (Rao, Basani et al. 2011). In our study two infants with perforated NEC were too sick to move to a neonatal surgical

unit, and although peritoneal drain insertion was considered in both, combined surgical and medical opinion decided on conservative management.

### *New NEC definitions?*

Although the Bell's Criteria is the most commonly known and used NEC scoring system in research and radiology, its use in clinical practice is waning. Newer studies highlighting discrepancies between radiologists reporting NEC may eventually make the Modified Bell's Criteria obsolete (Coursey, Hollingsworth et al. 2008). As such, other scoring systems have been postulated, but none so far have taken precedent. Some studies simply split infants with NEC according to whether they have been medically or surgically managed. Some observational studies searching for diagnostic and prognostic markers of NEC have begun to categorise NEC using the terms 'intestinal distress', and 'mild/moderate or severe enteropathy'. However, these terms are at best vague, and potentially confuse the metaanalyses of studies. Upon literature searching, other NEC scoring systems appear to be related to mortality (Bell, Ternberg et al. 1978, Kessler 2006). The article by Kessler et al, from 2006 emphasises the likelihood that there is no one specific parameter, or biological marker that is prognostic of the outcome of surgical NEC, but several, encompassing Bell's Criteria, stool markers, CRP, arterial lactate and platelet count.

If we view the study population assuming observer bias on NEC diagnosis and staging therein, then we would expect clinical data to show few differences between the way that the groups were managed by clinical staff, particularly: feed volume and attrition; growth; day to full feeds; episodes of sepsis, CRP levels and duration of antibiotics. When clinical and demographical details for infants with all-stage NEC were compared to those without NEC, it was clear that there were still differences in management and clinical response consistent with their recorded diagnosis of NEC. These infants were significantly older when feeds were started, and took longer to establish full enteral nutrition. They were more likely to have septic episodes, alongside higher CRP levels and, accordingly, longer duration of antibiotics. When all infants with stage 1a and 1b NEC were excluded from these analyses, the associations were even stronger, and new differences became apparent.

In comparison with all-stage NEC, infants with 'definite' NEC (i.e.  $\geq$ stage 2a Bell's) had significantly lower 10 minute Apgar scores, were more likely to be EBM fed, and to have a

significantly lower z score at the end of weeks 1 and 2. Whereas those with all stage NEC were significantly lighter at the end of week two than infants without NEC, those with stage  $\geq 2a$  NEC were not, which, again, is attributed to their higher fluid gain – partly pathological and associated with NEC, and partly iatrogenic.

However, hypervigilance to signs of NEC in ELBW and extremely preterm infants could explain the differences in these populations (whether all-stage or  $\geq$ stage 2a NEC), given that in each case, those with NEC were significantly smaller and more preterm than those without. If the clinical and demographical results are looked at from this perspective, most of the observations may be tributaries from extreme prematurity, bar the Apgar score at 10 minutes (just significantly lower in  $\geq 2a$  NEC, at  $p=0.05$ ), and CRIB score within 12 hours before recruitment (significantly higher in  $\geq$ stage 2a), both of which indicate that these infants were sicker at delivery. Although numbers were small, when demographical data was compared between those with Stage 3a and b NEC and infants without NEC, many of these associations were lost, given this was a heterogeneous group comprising a wide range of gestations and birth weights.

### *Feeds*

NEC in preterm infants commonly occurs after feeding, yet feeding is vital in order for the gut to adapt to postnatal life. Multiple studies on restriction of enteral feeds, prolongation of PN and clear fluids have shown no benefit in delaying the commencement of enteral feeds beyond the classical first 28 days of the neonatal period, when most preterm infants are likely to incur NEC (Lin and Stoll 2006). Repeated Cochrane Reviews of feeding regimens illustrate that there is no consensus on how quickly to escalate feeds – but that trophic feeding with maternal EBM is associated with a lower incidence of NEC (Bombell and McGuire 2009). In reality, as our study cohort shows, mixed feeding is the norm for preterm infants. Similarly, there is no consensus on whether fortifier is protective or associated with NEC, but the infants in our study who received fortifier were all from the same unit, and did so because they were tolerating enough enteral feed to merit starting – therefore, by definition were already less likely to get NEC before starting fortifier. Nonetheless, its addition was not noted to be associated with an increased risk of NEC thereafter. Within the regional NICU guidelines there is no unified feed volume policy, although it is currently under review. Similarly, the proceeds from the National Neonatal

Network and ESPGHAN Committees were examined, and they too reflect a spectrum of practices nationally and internationally.

#### *Antibiotic administration*

As expected, there were longer and more numerous antibiotics courses in those with NEC. There is, as of yet, no unified antibiotic policy within the Glasgow neonatal units. The main differences are in administration during the immediate postnatal period. Until its union with RHSC, the SGH NICU commonly used cefotaxime. However, the numbers of infants administered cefotaxime were too small to merit analysis. Such was the wide variety of antibiotics used according to blood culture and skin colonisation pathogens that it became impossible to stratify. The phenomenon of antibiotic resistance is more likely in preterm infants, in whom there are fewer microbiota species growing at lower intensity than in healthy term breast fed infants. One study by Kuppala et al in 2011 confirmed their hypothesis that preterm infants administered antibiotics without culture positive sepsis were at higher risk of subsequent NEC. They postulated this was due to the suppression of protective commensals, and this was most likely due to continuation of existing antibiotic administration rather than new, shorter courses (Kuppala 2011). One of the recruiting NICUs for our study showed statistically shorter durations of antibiotic usage than the others, but with no significant difference in NEC rate, or CRP.

#### *Mortality*

Four infants in our whole study population (n=56) died (7.1 %). All four had incurred NEC (12.5 % of all-stage NEC, 20 % of  $\geq$ stage 2a), and this was the cause of death in one infant, at more than 100 days of life post-ileostomy. In all, 1 infant died as a direct result of NEC (1.7 % of study cohort; 5 % of  $\geq$ stage 2a NEC). When this is considered in light of recent preterm neonatal mortality figures as quoted by Embleton et al in 2012 (21 %), the all-cause mortality rate in our study is considerably lower, and mortality secondary to NEC was much lower (Berrington, Hearn et al. 2012).

## Chapter 4

### Bacteria and Bacterial Metabolites

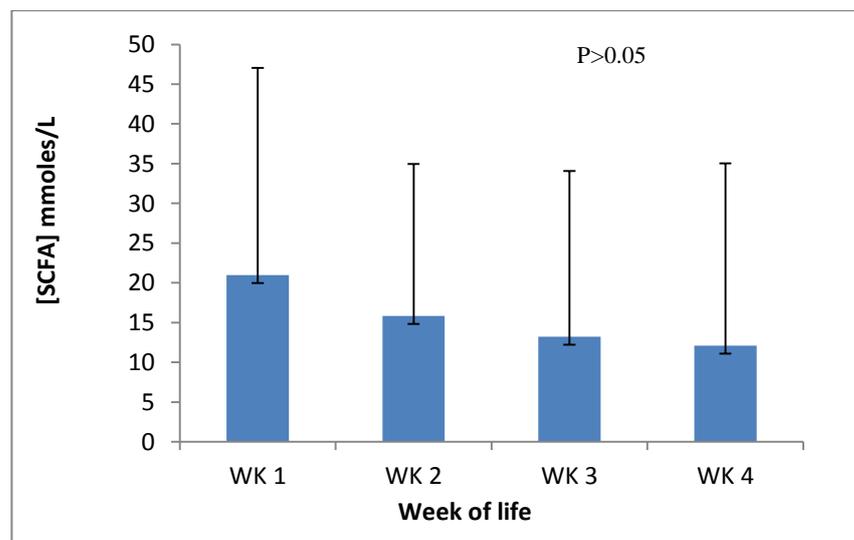
#### 4.1) Metabolites: SCFAs and BCFAs

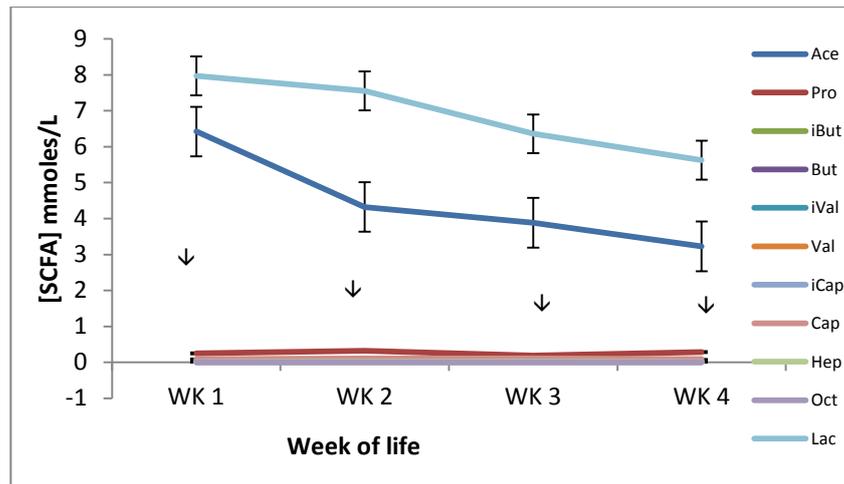
##### Introduction

The first half of this chapter describes the results from SCFA analysis by GCMS (using methodology as discussed in Chapter 2) for all 56 infants included within the cohort. Results are described first according to total SCFA concentrations, followed by individual acids, each considered temporally. They are then stratified according to temporal differences in gestation, feed type, and finally NEC. Ratiometric analyses are mentioned at the end of each part. Finally, a discussion section considers these results both in isolation and in light of the evidence base, as gleaned during the systematic reviews included in chapter 1.

##### 4.1.1) SCFA Concentrations: Totals

Total SCFA concentrations were measured in mmol/L wet weight sample. Formic acid (C1) was excluded from the totals given its ubiquity and thus potential for contamination. As such, the following SCFAs/BCFAs were included in these totals: acetic, propionic, butyric, isobutyric, valeric, isovaleric, caproic, isocaproic, heptanoic, octanoic, and lactic acids. Furthermore, ratiometric analysis was also undertaken, namely: ‘lactic:all branched chain fatty acids’ (isobutyric, isocaproic, and isovaleric acids); ‘acetic:all BCFAs’; and both lactic and acetic acids versus each BCFA individually.



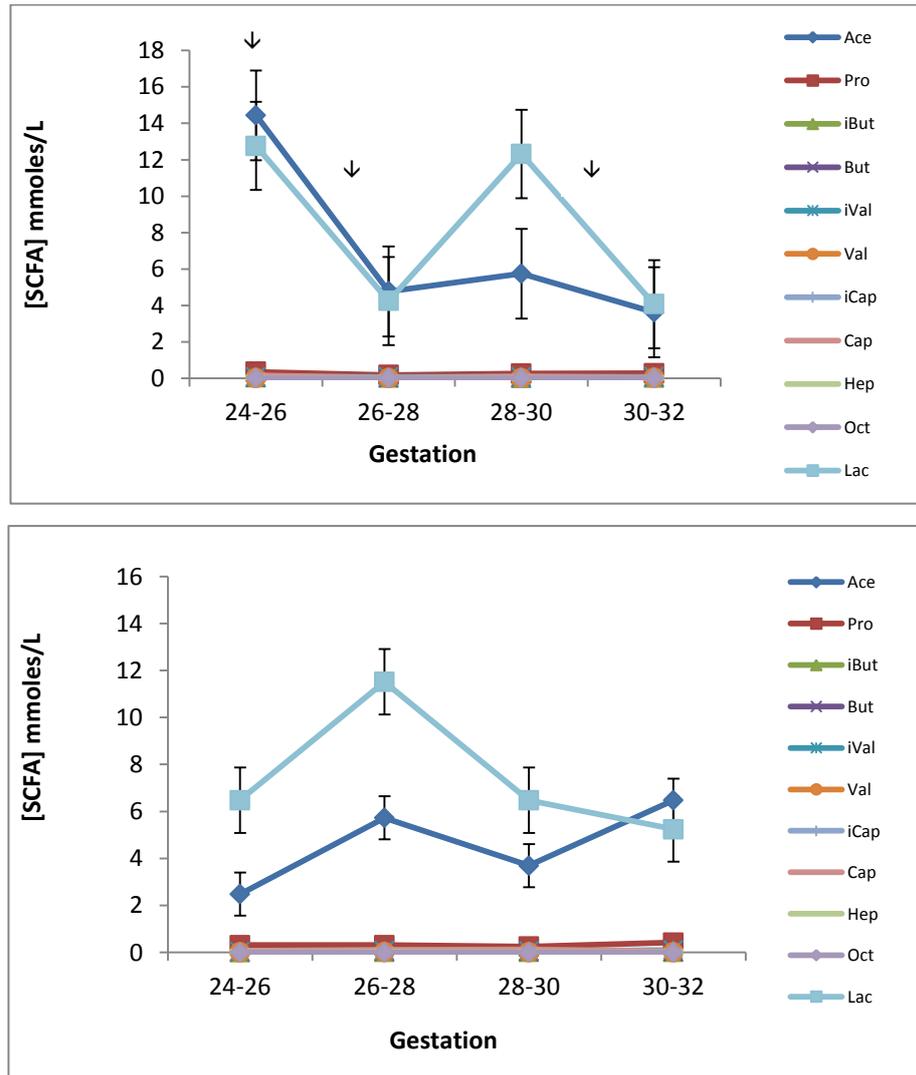


Figures 49: a) (top) Median group total weekly SCFA concentrations (with IQRs) showed no significant differences; b) (bottom) Median group individual SCFA concentrations (with IQRs) showed acetate and lactate to be significantly higher than all other SCFAs ( $p < 0.05$ ), although were not significantly different from one another throughout the study period. ↓ denotes a significant p value.

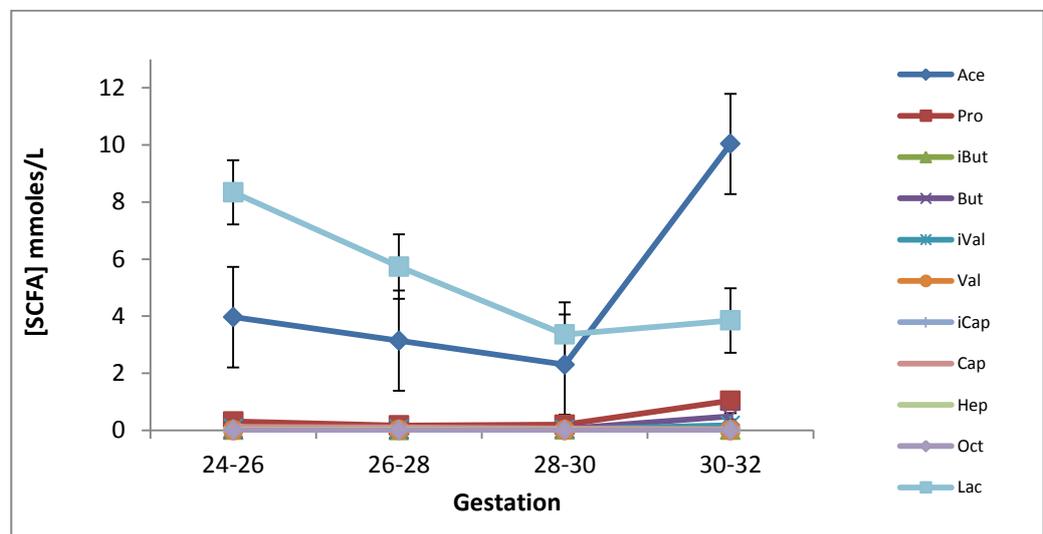
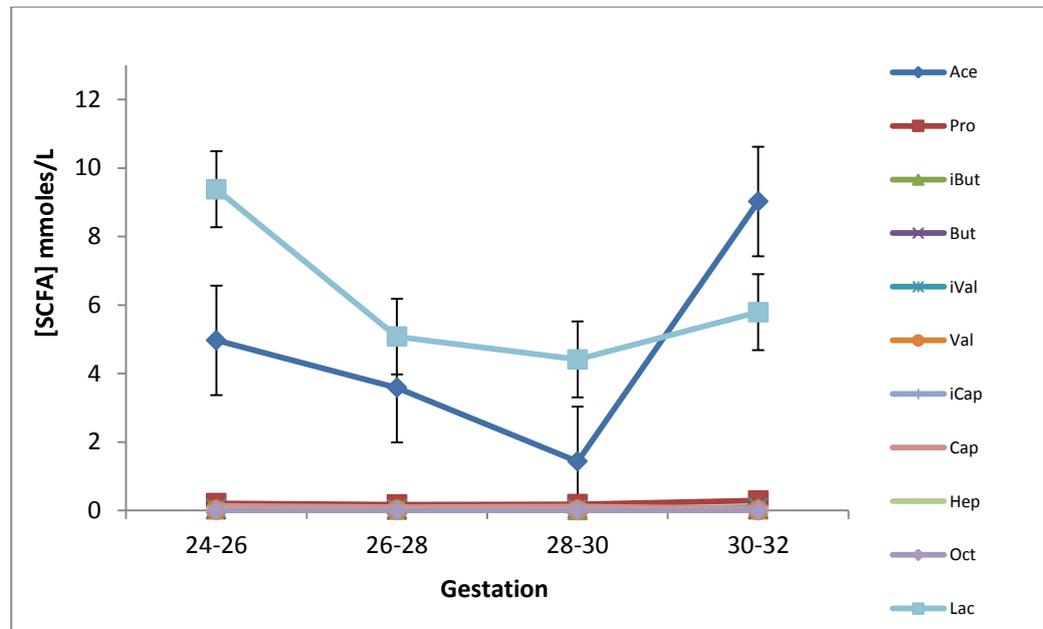
Figure 49 above shows that the total SCFA concentrations fell from week to week throughout the study period, but not significantly so ( $p > 0.05$ ). Lactic and acetic acids were higher than any other SCFAs throughout the study period ( $p < 0.05$ ), although were not significantly different from each other throughout. Acetic acid fell between weeks 1 and 2, but not significantly so ( $p = 0.5$ ). Comparison of individual acid totals per week showed this also to remain consistent, without significant changes. There were very low concentrations of each BCFA, which did not vary significantly in the cohort from week to week ( $p > 0.05$ ). Therefore ratiometric analyses of lactic and acetic acids versus BCFA individually and in combination, were assessed. These analyses for the study cohort each week showed no significant differences for either lactic or acetic acids, either by total or individual BCFA over time.

#### 4.1.2) By Gestation

SCFA production was then stratified according to gestation, week by week.



Figures 50: a) (top) Median SCFA concentrations by gestation week 1 (with IQR); lactic acid was significantly higher in 24-26 week group than 26-28 weeks ( $p=0.042$ ), or those at 30-32 weeks ( $0.017$ ).  $\downarrow$  denotes a significant p value. b) (bottom) Median SCFA concentrations week 2 (with IQR); no gestational differences were seen in acetic or lactic acids, but isocaproic acid was higher in the 26-28 week group than those at 30-32 weeks ( $p=0.006$ ).

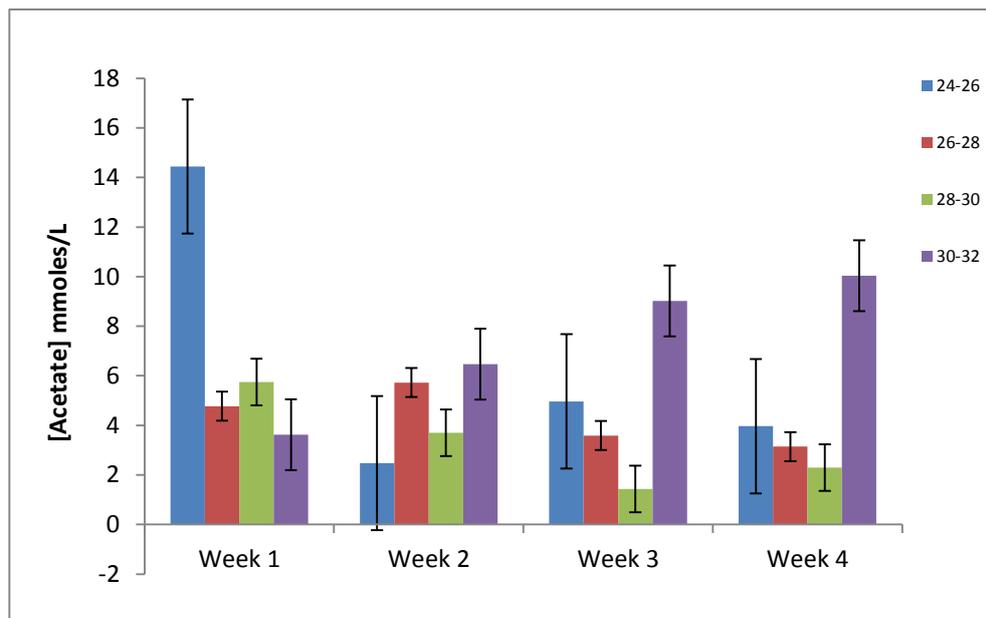
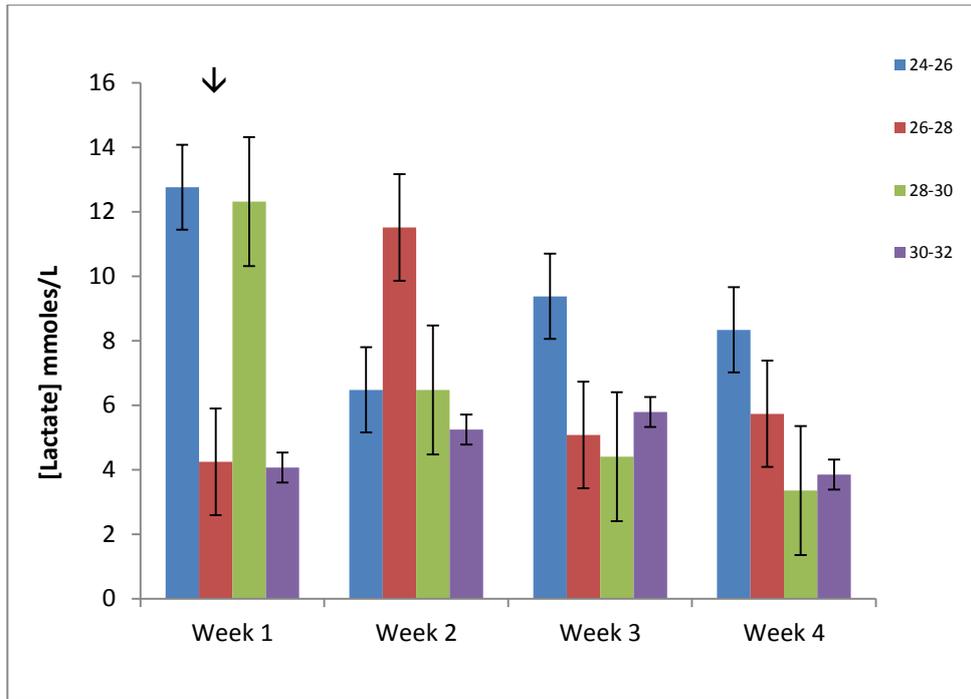


Figures 51: a) (top) Median SCFA concentrations by gestation week 3 (with IQR); b) (bottom) Median SCFA concentrations week 4 (with IQR). In both weeks lactic and acetic acids were significantly higher than all other SCFAs, but no gestational differences were detected. Significant differences were detected in minor SCFAs (see later).

### i) Week by week analysis

#### Week 1

In week 1 (figure 52 a), significant differences were observed in lactic acid concentrations between different gestational ages. Lactic acid was higher in the 24-26 week infants than those between 26-28 or 30-32 weeks ( $p = 0.042$  and  $0.017$  respectively). This accounted for the significantly higher total SCFA concentration in those at lower gestations (24-26 > 26-28 weeks,  $p = 0.03$ ; see figure 54).



Figures 52: a) (top) Median lactic acid concentrations by gestation, weeks 1-4 (with IQR); infants at 24-26 and 28-30 weeks showed higher concentrations than other groups during week 1 ( $p < 0.04$ ). ↓ denotes the significant p value. b) (bottom) Median acetic acid concentrations by gestation, weeks 1-4 (with IQR). No significant differences were noted

However, further gestational differences in lactate disappeared through the remaining weeks (figure 52 a). No significant differences were seen in acetic acid concentrations between gestations at any time point (figure 52 b).

Significant gestation-dependent differences were nonetheless seen in one of the ratiometric analyses. Ratiometric analysis revealed higher lactic to total BCFA ratio in the 28-30 week group than those at 26-28 weeks (figure 53 a:  $p = 0.0067$ ). This was due to their higher lactate:isobutyric acid ratio (figure 53 b:  $p = 0.01$ ).

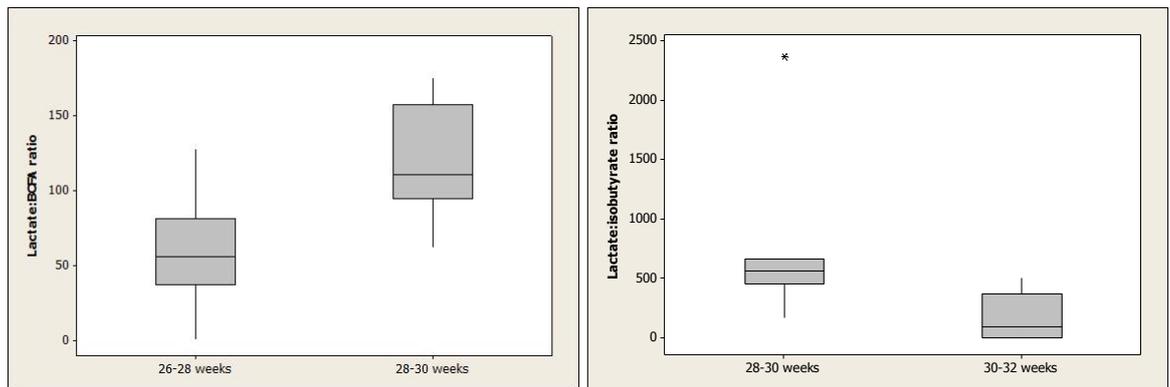


Figure 53: a) (left) Week 1 ratiometric analysis of lactate:BCFA in 26-28 versus 28-30 week gestation groupings, showing a significantly higher ratio in the 28-30 week group ( $p=0.0067$ ); b) (right) Week 1 lactate:isobutyrate was higher at 28-30 weeks than those at 30-32 weeks ( $p=0.01$ ).

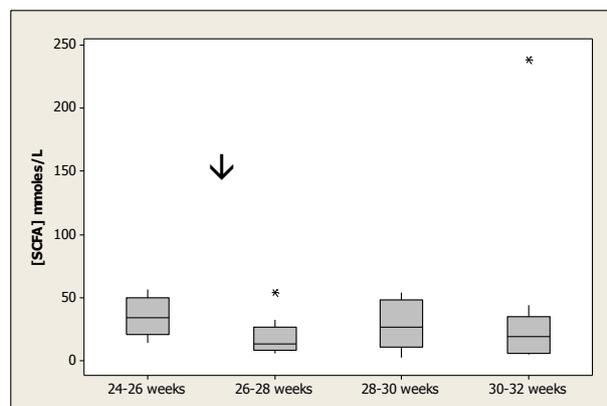


Figure 54: Week 1 total SCFA concentrations, by gestation; those between 24-26 weeks gestation had a significantly higher total than those at 26-28 weeks ( $p=0.03$ , as denoted by '↓').

## Week 2

No differences were seen in the major acids acetic, lactic or propionic between any gestational group during week 2, nor in their total concentrations. However, a significant difference was observed in the branched chain fatty acid isocaproic, which was higher in those at 26-28 weeks than the 30-32 week group ( $p = 0.006$ ). No ratiometric differences

were noted when considering total BCFAs, but individual changes were seen with regard to isocaproic acid. As shown in figure 59 a), the lactate:isocaproic acid ratio was higher in those at 24-26 weeks than 26-28 weeks ( $p = 0.0035$ ), and higher in those at 28-30 than 26-28 weeks ( $p = 0.0015$ ). Similar gestation-dependent trend was seen in figure 59 b) for the acetate:isocaproic acid ratios in this week, with higher levels in those at 24-26 weeks gestation than those at 26-28 weeks ( $p = 0.0093$ ).

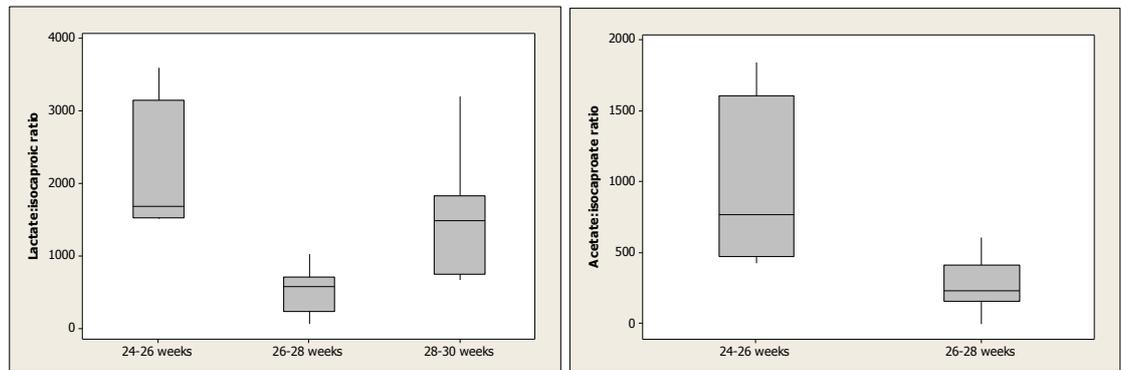


Figure 55: a) (left) Week 2 lactate:isocaproic ratio illustrating higher levels in those at 24-26 weeks than 26-28 weeks ( $p=0.0035$ ), and the 28-30 group than those at 26-28 weeks ( $p=0.0015$ ). b) (right) Week 2 acetate:isocaproic acid ratios were higher in those at 24-26 weeks than 26-28 weeks gestation ( $p=0.0093$ ).

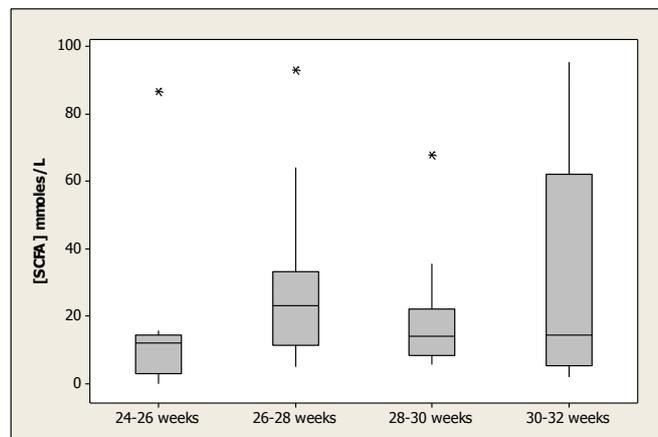


Figure 55: c) Week 2 total SCFA concentrations, by gestation; there were no significant gestational differences

### Week 3

No significant differences were seen in acetic, propionic, butyric, heptanoic, octanoic or lactic acids between gestations, or in their total concentrations. Similarly no significant ratiometric changes were noted (see figure 56).

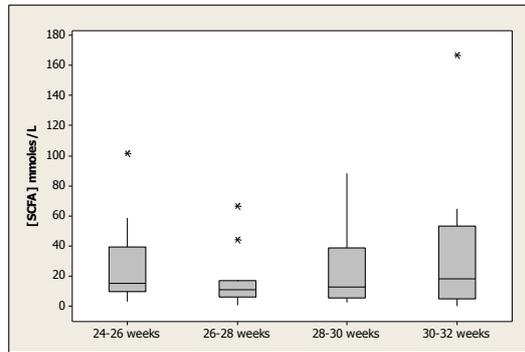


Figure 56: Week 3 total SCFA concentrations, by gestation; no gestational differences were noted

## Week 4

Again, no significant gestational differences were seen in acetic, propionic, butyric, isobutyric, heptanoic, octanoic, or lactic acids (see figure 58). Total concentrations also showed no gestational differences. During week four (see figure 57 a)), ratiometric analysis revealed higher lactate versus total BCFA in those of 24–26 weeks gestation than those of 28-30 weeks ( $p = 0.015$ ), those at 24 versus 26 weeks ( $p = 0.012$ ), and those at 26 versus 28 week groups ( $p = 0.037$ ). On further analysis (figure 57 b)) the ratios of lactate to isobutyrate showed gestational trends (higher in 24-26 weeks than 28-30 weeks:  $p = 0.012$ ; 24-26 > 30-32 weeks,  $p = 0.011$ ; 26-28 weeks > 30-32 weeks,  $p = 0.03$ ; 26-28 weeks > 28-30 weeks,  $p = 0.037$ ), as did lactate:isovalerate ratios (Figure 57 c): 24-26 > 30-32 weeks,  $p = 0.016$ ; 26-28 weeks > 30-32 weeks,  $p = 0.02$ ).

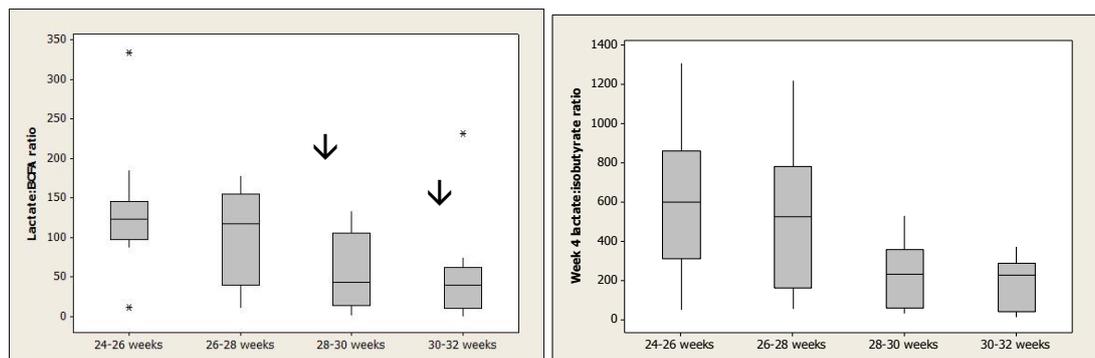


Figure 57: a) (left) Week 4 ratiometric analysis of lactate:BCFA by gestation revealed higher ratios at 24-26 weeks than 28-30 weeks ( $p=0.012$ ), and 26-28 weeks ( $p=0.012$ ). The ratio was also higher in 26-28 week group versus 28-30 group ( $p=0.037$ ). b) (right) Lactate:isobutyrate ratios were higher at lower gestations (24-26 weeks than 28-30 weeks:  $p = 0.012$ ; 24-26 > 30-32 weeks,  $p = 0.011$ ; 26-28 weeks > 30-32 weeks,  $p = 0.03$ ; 26-28 weeks > 28-30 weeks,  $p = 0.037$ ).

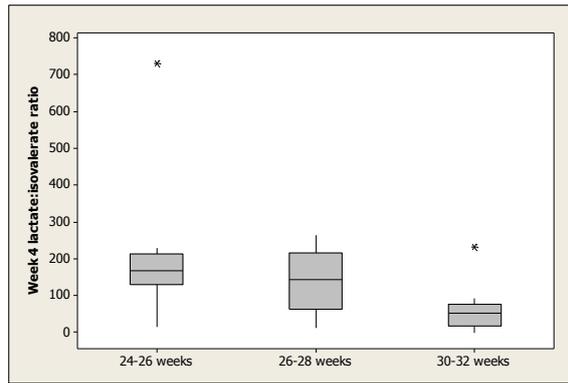


Figure 57 c) (top) During week 4, lactate:isovalerate ratios were higher at lower gestations (24-26 > 30-32 weeks,  $p = 0.016$ ; 26-28 weeks > 30-32 weeks,  $p = 0.02$ ).

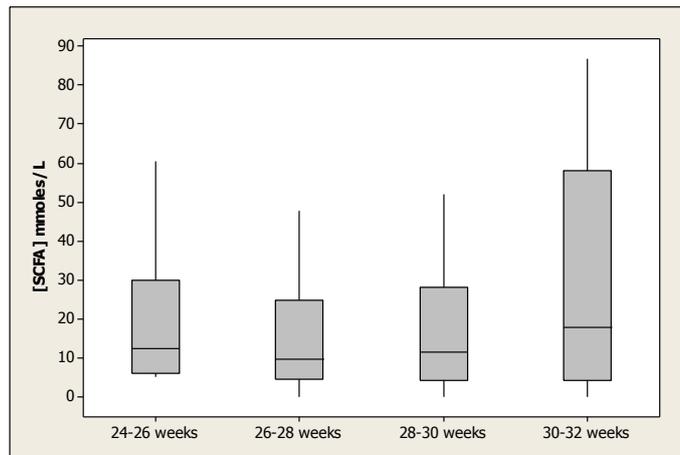
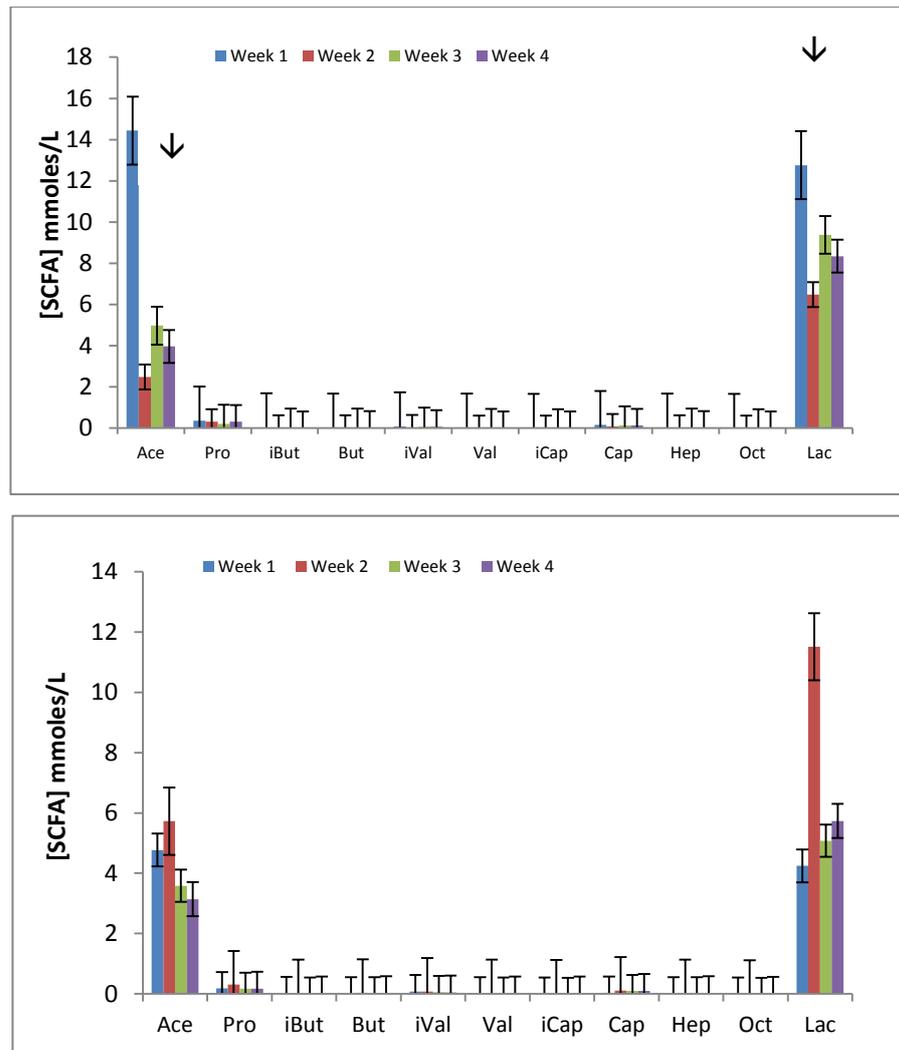


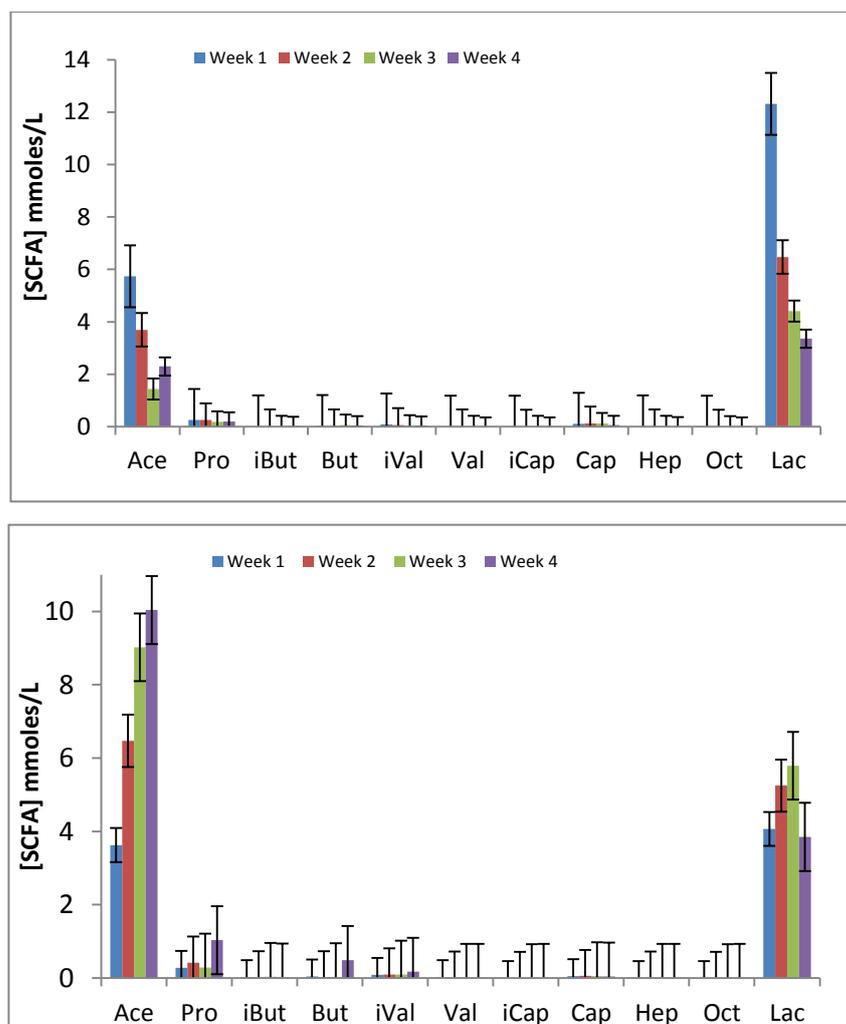
Figure 58: No significant differences were noted between total SCFA concentrations at each gestation during week 4.

## ii) Week-on-week comparisons by gestation

SCFA concentrations for each gestational grouping were also compared week by week, and followed similar patterns, as seen in figures 59 and 60:



Figures 59: a) (top) Median SCFA levels in infants 24-26 weeks (with IQR); there was a significantly higher total SCFA concentration in week 1 than either weeks 2 or 4 ( $p=0.019$  and  $p=0.04$  respectively); ↓ denotes a significant p value. b) (bottom) Median SCFA levels in infants 26-28 weeks (with IQR). Acetic and lactic acids were significantly higher throughout all time points ( $p<0.05$ ).



Figures 60: a) (top) Median SCFA levels in infants 28-30 weeks (with IQR); b) (bottom) Median SCFA levels in infants 30-32 weeks (with IQR). Both gestational groups showed higher levels of acetic and lactic acids, but there were no week-to-week differences.

For each gestational grouping, acetic and lactic acids were consistently higher than other acids throughout the study period. Total SCFA concentrations remained consistent throughout the study period at each gestation. Only the 24-26 week gestation grouping showed higher total concentrations in week 1 than weeks 2 or 4 (figure 60 a):  $p = 0.019$  and  $p = 0.04$ ). Their week 1 total SCFA concentration was significantly higher than infants in the 26-28 week group at the same time point ( $p = 0.03$ ; see figure 54 a)).

### iii) Ratiometric data

Ratiometric analysis of each gestation week-by-week showed significant differences in infants at 24-26 weeks and 28-30 weeks gestation. As shown in figure 61, those at 24 weeks gestation had significantly higher acetic:isocaproic levels in week 1 than week 3 ( $p = 0.02$ ), and also acetic:isovaleric levels in week 1 than week 2 ( $p = 0.05$ ). In the 28-30

week group, lactic:total BCFA ratios were higher in weeks 1 and 2 than week 4 (Figure 62:  $p = 0.01$  and  $0.02$  respectively). When the ratio of lactic:isobutyric acid was made at those of 28 weeks, this was considerably higher in week 1 than week 4 (Figure 63 a):  $p = 0.0075$ ), as was the same ratiometric analysis when substituting acetic acid for lactic (Figure 63 b):  $p = 0.01$ ).

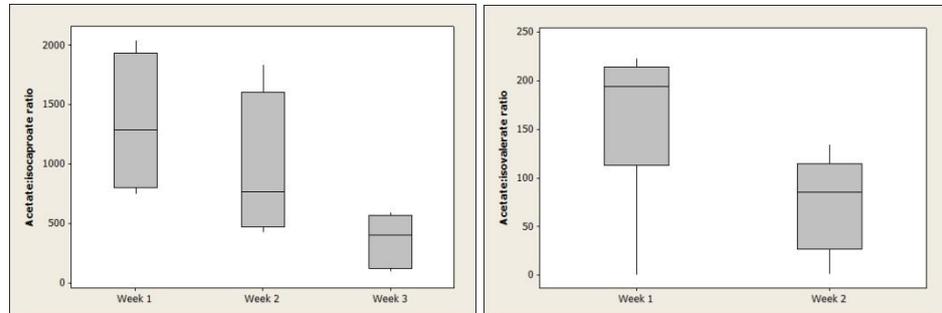


Figure 61: a) (left) Acetate:isocaproate ratio 24-26 weeks gestation; levels at weeks 1 and 2 were higher than 4 ( $p < 0.02$ ). b) (right) Acetate:isovalerate ratio 24-26 weeks gestation; week 1 levels were significantly higher than week 2 ( $p = 0.05$ ).

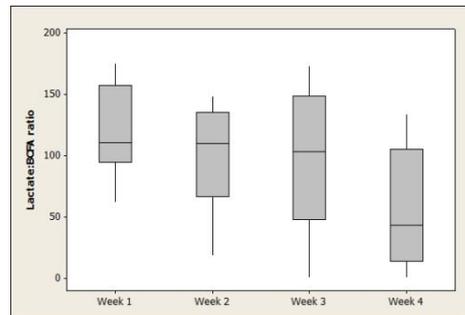


Figure 62: 28-30 weeks: lactate:BCFA ratio weeks 1-4; levels were significantly lower in both weeks 1 and 2 than week 4 ( $p < 0.02$ ).

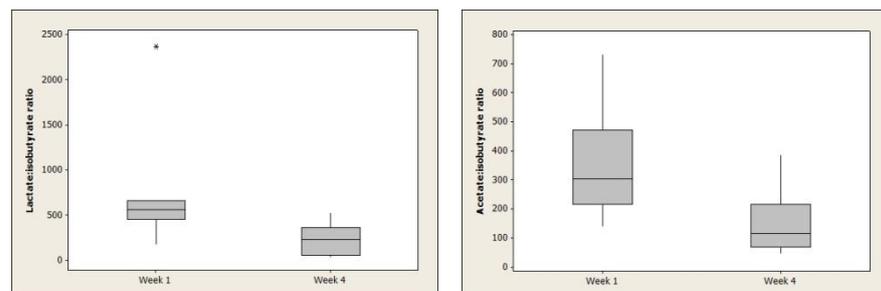


Figure 63: a) (left) 28-30 weeks: lactate:isobutyrate; week 1 levels were higher than week 4 ( $p = 0.0075$ ). b) (right) 28-30 weeks gestation acetate:isobutyrate; levels were significantly higher in week 1 than week 4 ( $p = 0.01$ )

#### 4.1.3) By Feed Type

##### i) EEBM by week

Total SCFA concentration for infants exclusively EBM fed in week 1 was significantly higher than during week 4 (Figures 64 and 65 a):  $p = 0.02$ ). No ratiometric differences were noted.

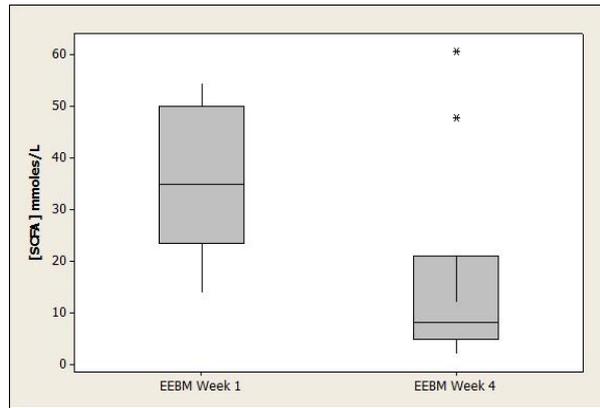
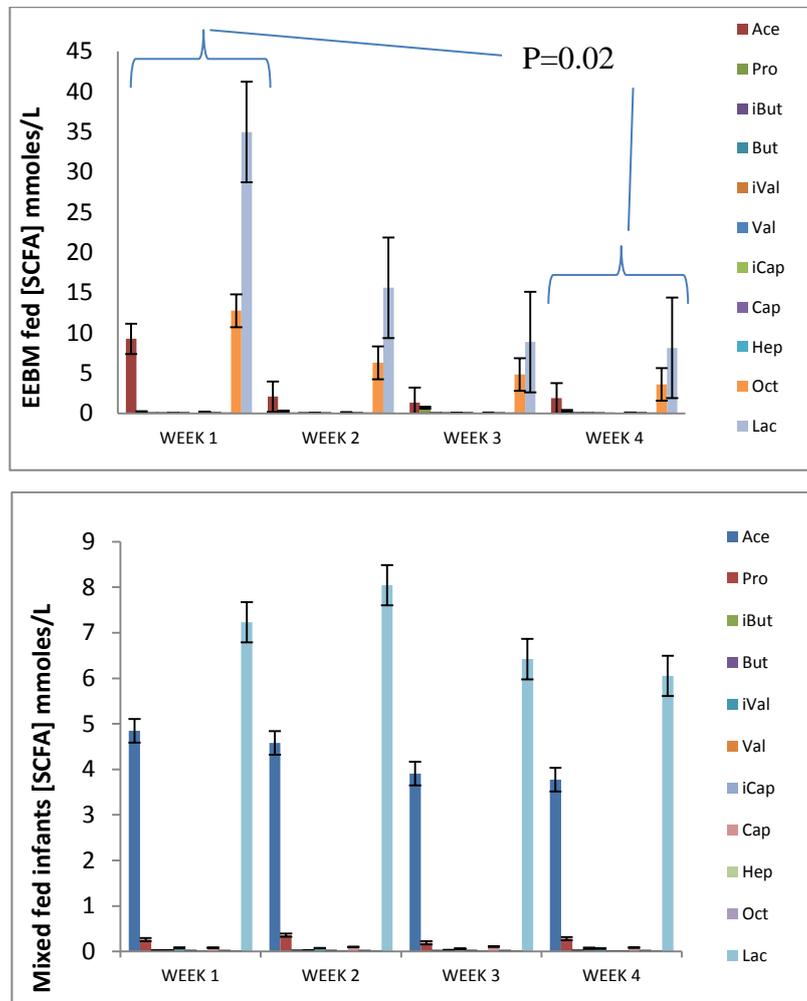


Figure 64: Comparison between week 1 and week 4 total SCFA concentrations in infants exclusively fed EEBM showed significantly higher concentrations in week 1 ( $p=0.02$ );

ii) EEBM and Mixed SCFAs, by Week



Figures 65: a) (top) Median weekly SCFA concentrations in those fed EEBM was significant only between weeks 1 and 4 ( $p=0.02$ ); b) (bottom) Median weekly SCFA concentrations in those mixed fed. No significant differences were observed.

### iii) Ratiometric Data

No significant differences were seen in total SCFA concentrations for infants mixed fed, but when ratiometric analysis was considered, acetic:total BCFA level was significantly higher in week 2 than week 4 (Figure 66 a):  $p = 0.05$ ). Week by week and ratiometric comparisons between those EEBM and mixed fed showed no significant differences, although comparison of levels at week 1 approached significance, at  $p = 0.065$ . Analysis of individual SCFAs revealed acetic acid concentrations to be significantly higher in mixed fed infants in week 4 than those fed EEBM (Figure 66 b):  $p = 0.03$ ).

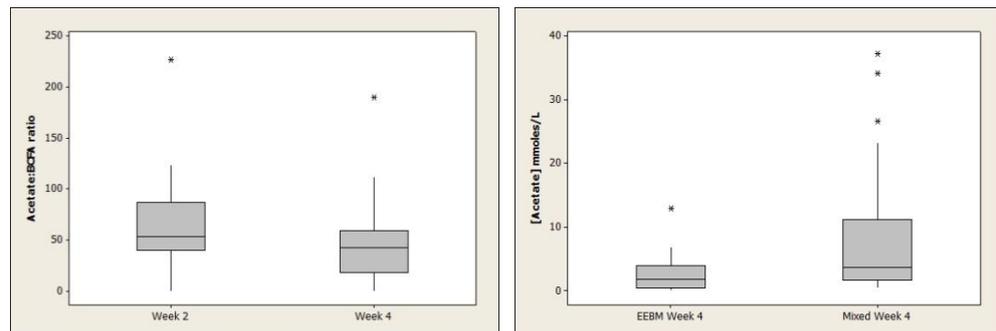


Figure 66: a) (left) Mixed fed infants acetate:BCFA ratio; week 2 levels were significantly higher than week 4 ( $p=0.05$ ) b) (right) EEBM levels of acetic acid were higher than mixed fed infant acetic acid levels in week 4 ( $p=0.03$ )

Although lactate levels during week 4 were higher in those EEBM than mixed fed, this did not reach statistical significance ( $p = 0.07$ ).

#### 4.1.4) By NEC: Stage $\geq 2a$

##### i) Total SCFA: NEC $\geq 2a$ versus those without NEC

When comparing total SCFA concentrations in those infants with  $\geq$ stage 2a NEC and those without NEC, there were no significant differences. However, as noted by the interquartile ranges, there were hugely variable levels, making the likelihood of significant differences remote (see figure 67).

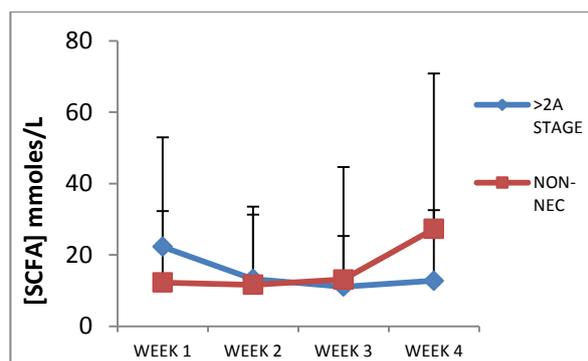
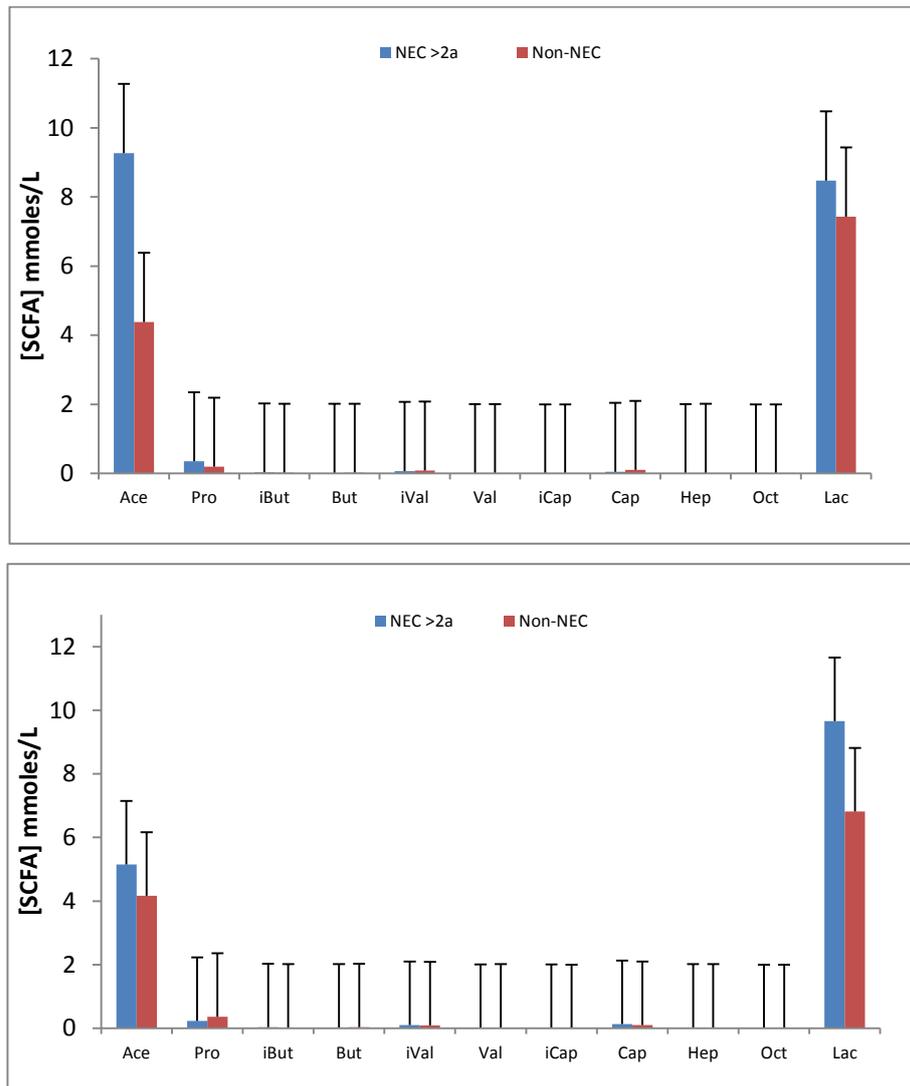


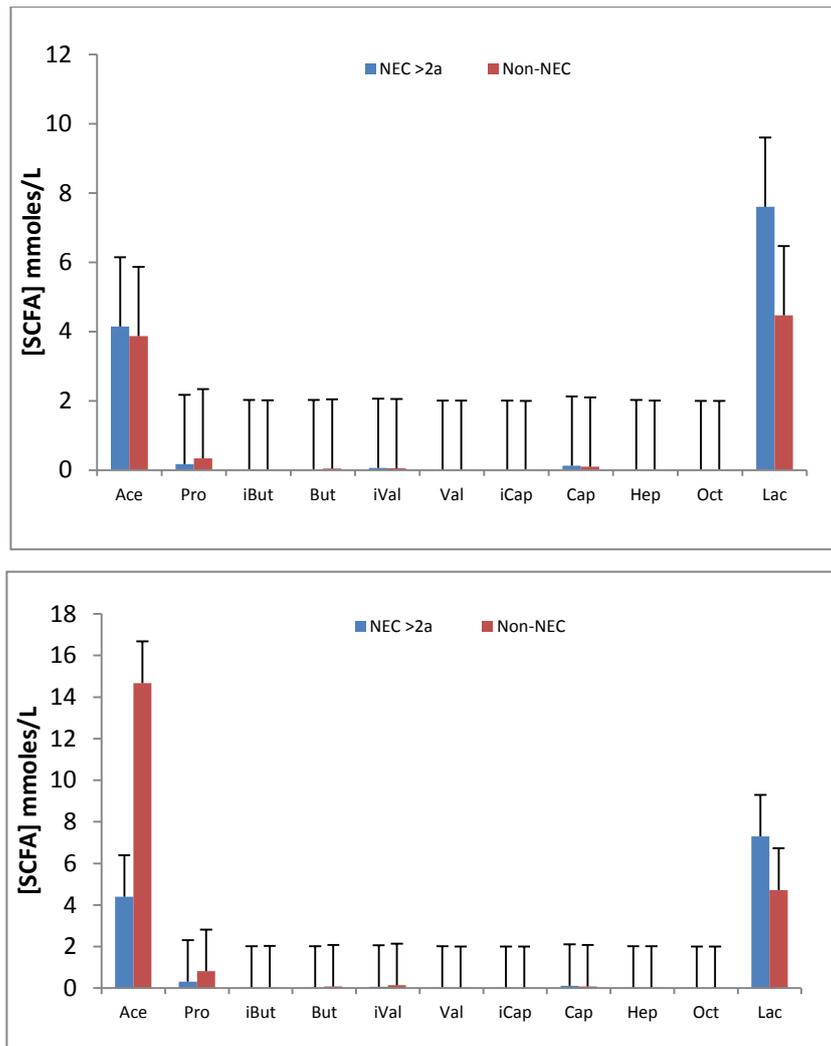
Figure 67: Median SCFA totals Stage 2a vs Non-NEC, weeks 1-4 (with IQR). No significant differences were noted.

There were also no significant differences observed between those with  $\geq$ stage 2a NEC and non-NEC with regard to individual SCFAs throughout the study period. Wide interquartile ranges were noted throughout both groups (figures 68 and 69).

*ii) Weekly comparisons of those with  $\geq$ Stage 2A NEC and those without NEC*



Figures 68: a) (top) Median Individual SCFAs  $\geq$ Stage 2a NEC Vs Non-NEC, week 1 (with IQRs); b) (bottom) Median Individual SCFAs  $\geq$ Stage 2a NEC Vs Non-NEC, week 2 (with IQRs). In each case, no significant differences were noted.



Figures 69: a) (top) Median individual SCFAs  $\geq$ Stage 2a NEC Vs Non-NEC, week 3 (with IQRs); b) (bottom) Median individual SCFAs  $\geq$ Stage 2a NEC Vs Non-NEC, week 4 (with IQRs). In each case, no significant differences were noted.

### iii) Ratiometric Data

However, when ratiometric concentrations were compared between those with  $\geq$  stage 2a NEC and those without NEC several differences were observed – mainly in acetic acid ratios. During week 1, the acetic:total BCFA ratio was significantly higher in those with  $\geq$  2a NEC versus those without (Figure 70 a):  $p = 0.0059$ ), as was the acetic:isovaleric acid ratio (Figure 70 b):  $p = 0.0079$ ). A significant difference was also seen in the lactic:isocaproic acid ratio during week 2, with a higher level in  $\geq$ 2a NEC (Figure 71 a):  $p = 0.05$ ). No other differences were noted until week 4, when the lactic:isobutyric acid ratio was significantly higher than those without NEC (Figure 71 b):  $p = 0.05$ ).

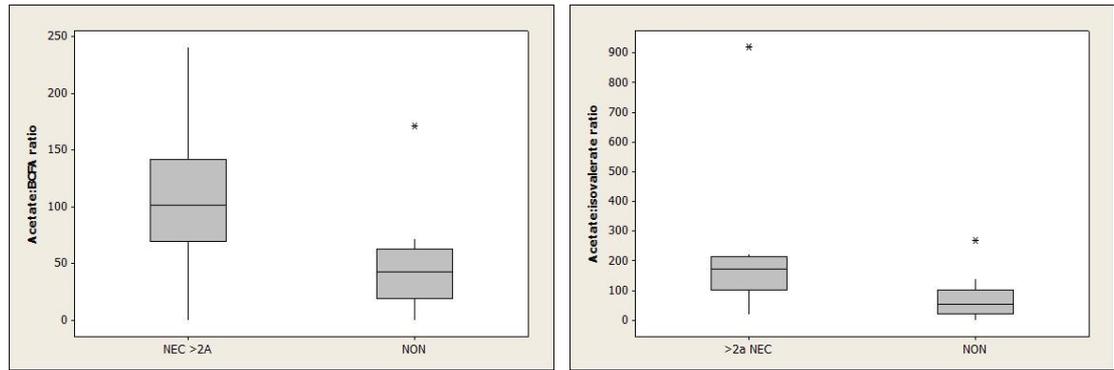


Figure 70 a) (left) NEC  $\geq 2a$  versus Non, acetate:BCFA ratio, week 1; NEC was significantly higher than non ( $p=0.0059$ ) b) (right) NEC  $\geq 2a$  versus Non, acetate:isovalerate ratio, week 1; NEC was significantly higher than non ( $p=0.0079$ )

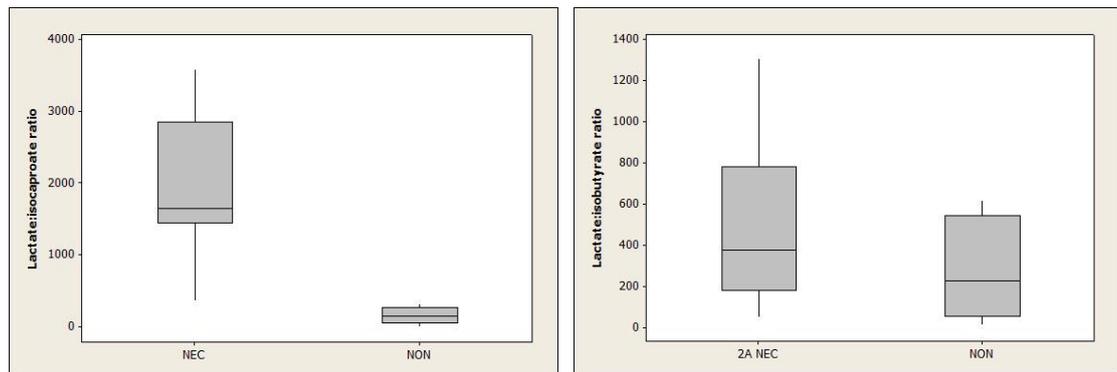


Figure 71: a) (left) NEC  $\geq 2a$  versus Non, lactate:isocaproate week 2; NEC levels were significantly higher than non ( $p=0.05$ ) b) (right)  $\geq 2a$  NEC versus Non, lactate:isobutyrate ratio, week 4; NEC levels were significantly higher than non ( $p=0.05$ )

When weekly ratiometric levels of acetic:total BCFA were considered in those with  $\geq$  stage 2a NEC (Figure 72 a)), levels at week 1 were significantly higher than week 2 ( $p = 0.04$ ), and week 3 ( $p = 0.03$ ). Acetic ratios with isovaleric acid were higher in week 1 than weeks 2 and 4 (Figure 72 b):  $p = 0.04$ , and  $p = 0.01$ ). Ratios were also considered in infants without NEC. Again, most of the significant differences were observed in acetic ratios. Acetic:total BCFA level was significantly lower during week 1 than 2 (Figure 73 a):  $p = 0.0003$ ). Acetic:isocaproic ratio at week 1 was significantly lower than in weeks 3 or 4 (Figure 73 b):  $p = 0.0091$ , and  $p = 0.0083$  respectively). Differences in lactic ratios were temporally analogous, showing significantly lower lactic:isocaproic ratios in week 1 than weeks 2 and 4 (Figure 74:  $p = 0.0001$ , and  $p = 0.0008$ ).

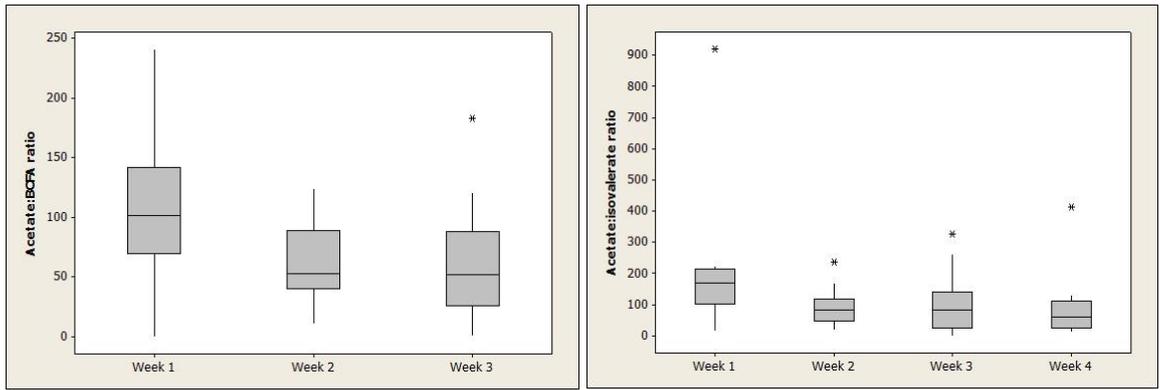


Figure 72: a) (left)  $\geq 2a$  NEC acetate:BCFA ratios weeks 1-3; levels in week 1 were higher than weeks 2 and 3 ( $p=0.04$  and  $0.03$  respectively) b) (right)  $\geq 2a$  NEC acetate:isovalerate ratio weeks 1-4; levels at week 1 were higher than weeks 2 and 4 respectively ( $p=0.04$  and  $0.01$  respectively)

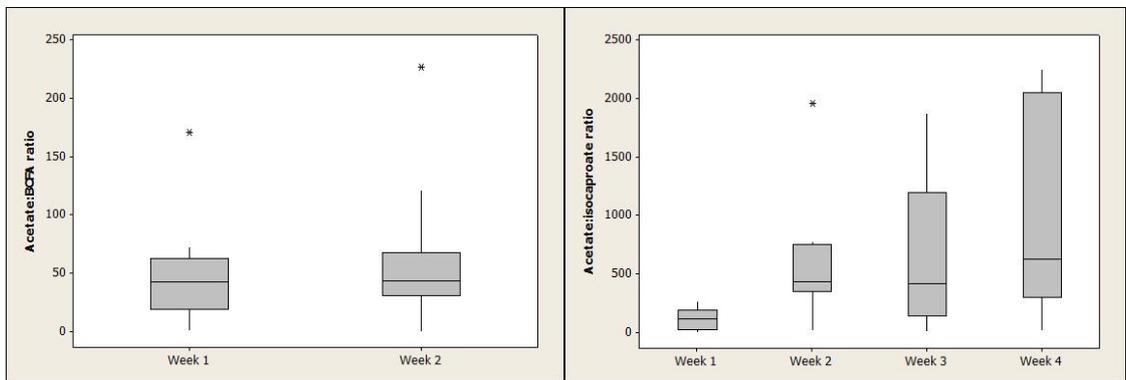


Figure 73: a) (left) Acetate:BCFA ratios, week 1 were significantly higher than 2 in those without NEC ( $p=0.003$ ); b) (right) Acetate:isocaproate ratios, week 1 was significantly lower than week 3 ( $p=0.0091$ ) and week 4 in those without NEC ( $p=0.0083$ )

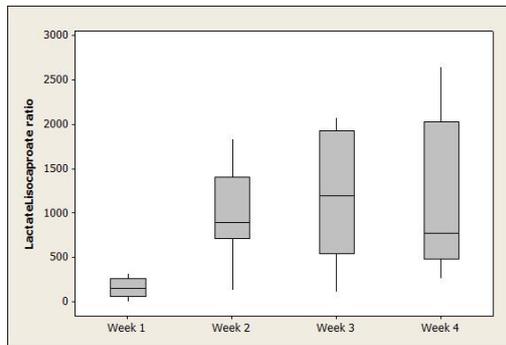
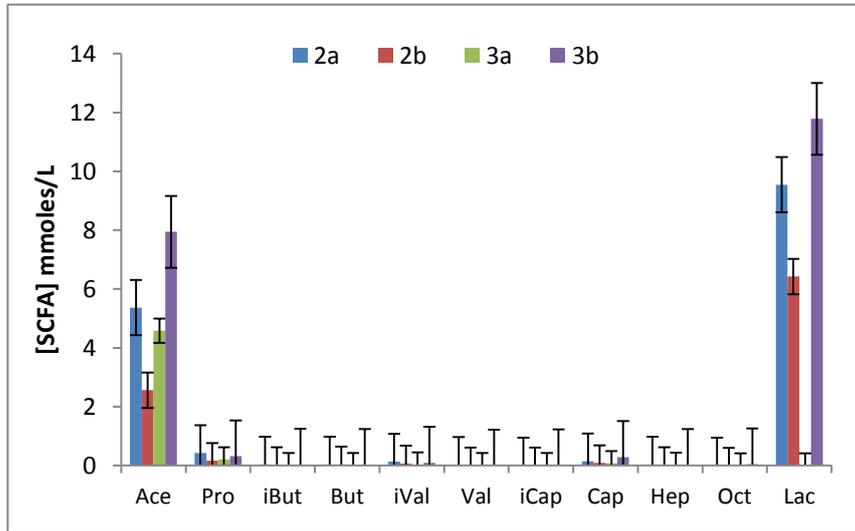
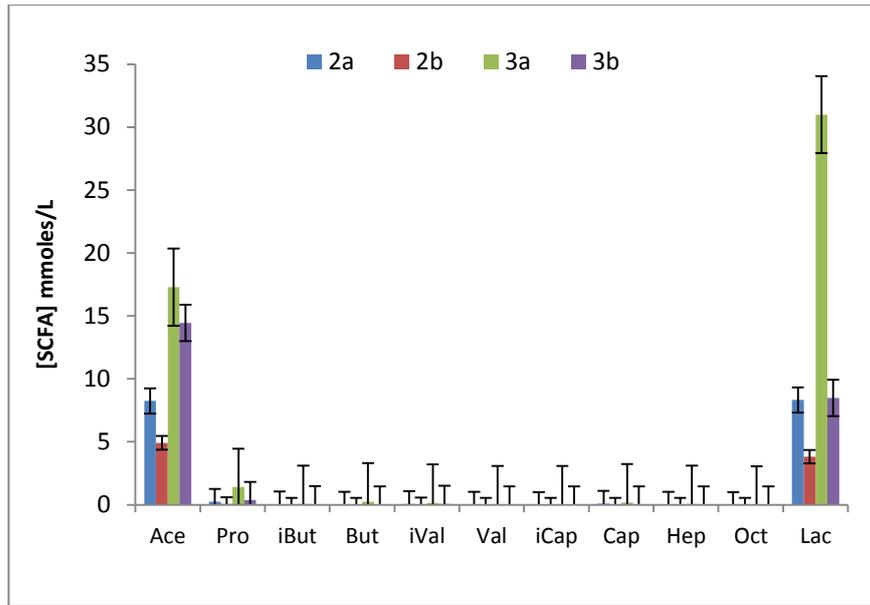


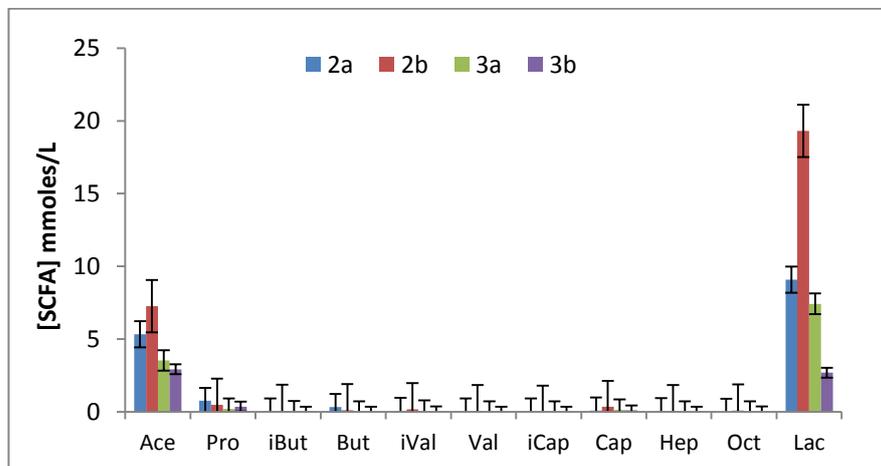
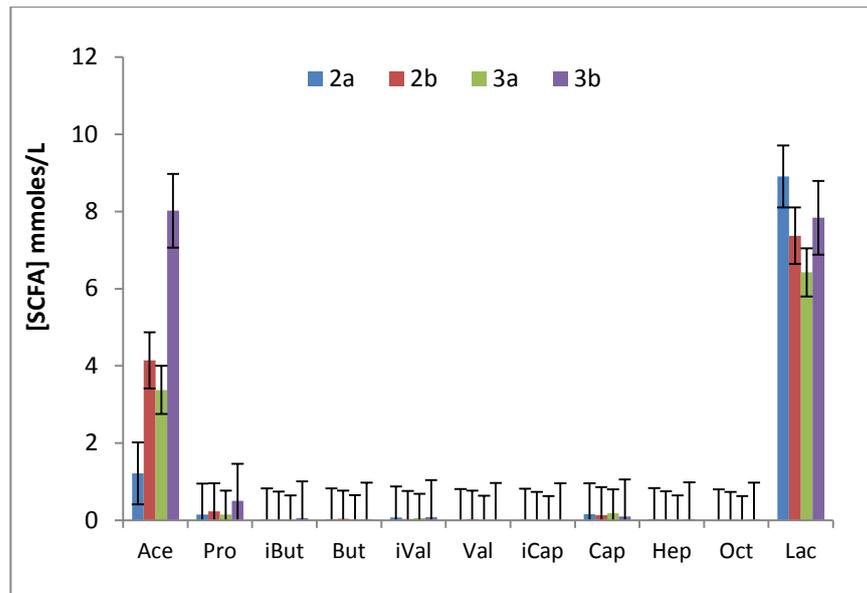
Figure 74: Non lactate:isocaproate ratios, week 1 was significantly lower than weeks 2, 3 and 4 ( $p<0.0008$ )

*iv) Stage-by-stage comparisons: 2a, 2b, 3a and 3b NEC*

SCFA concentrations were further compared between the four ‘definite’ stages of NEC: 2a, 2b, 3a and 3b (Figure 75 and 76). Once again, the same trends were noted as for other comparisons: acetic and lactic acids predominate through all stages of NEC.



Figures 75: a) (top) Median individual SCFAs by NEC Stage, week 1 (with IQRs); no significant differences were noted b) (bottom) Median individual SCFAs by NEC stage, week 2 (with IQRs); valeric acid levels were significantly higher in those with 2A than 3B NEC ( $p=0.02$ ).



Figures 76: a) (top) Median individual SCFAs by NEC Stage, week 3 (with IQRs); b) (bottom) Median individual SCFAs by NEC stage, week 4 (with IQRs); all acids apart from lactate and acetate were higher in those with 2A than other stages of NEC.

Univariate analyses were then performed to assess whether there were any significant week by week changes in individual SCFAs by NEC stage, considering only those with definite NEC. Again, stages 2a and b, and 3a and b were combined.

## Stage 2a and b

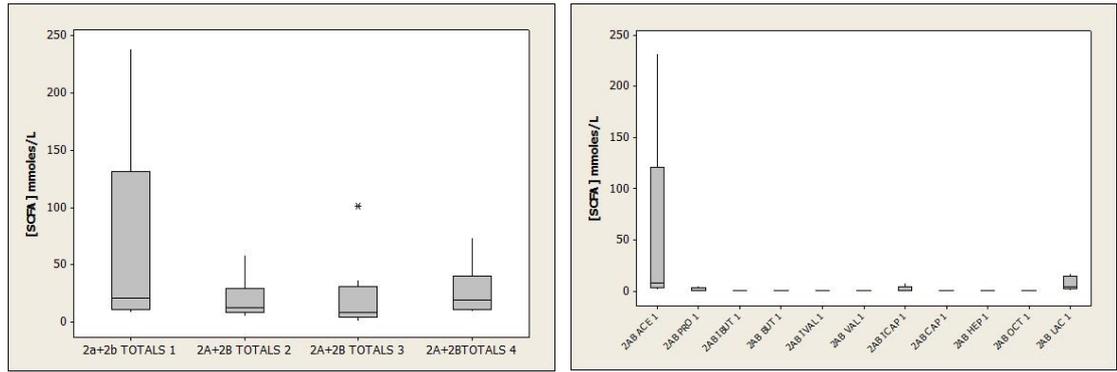


Figure 77: a) (left) Stage 2a+b NEC: Total SCFA concentrations over the study period; b) (right) Individual SCFAs, week 1, stage 2a+b NEC. In both cases, other than acetic and lactic being higher, no significant differences were noted.

There were no significant differences in SCFA totals over the study period (see figure 77 a).

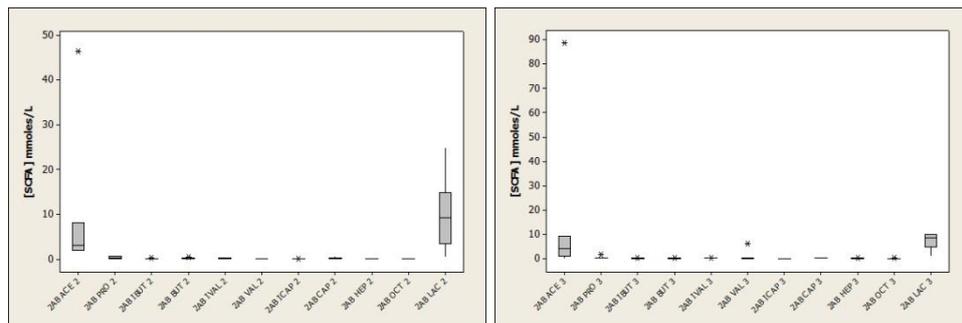


Figure 78: a) Stage 2a+b NEC: individual SCFA concentrations week 2; b) Stage 2a+b NEC: individual SCFA concentrations week 3. None were found to be significant, other than acetic and lactic higher than all other SCFAs.

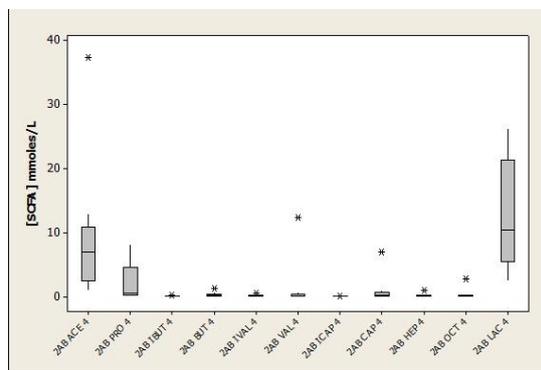


Figure 79: Stage 2a+b Individual SCFA Concentrations, week 4; acetic and lactic were significantly higher than others, but no other changes were noted.

Although acetic and lactic acid were higher than the other SCFAs each week, no other significant differences were noted.

## Stages 3a + b

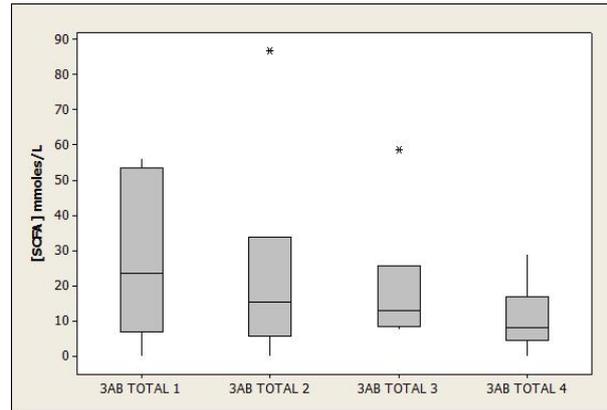
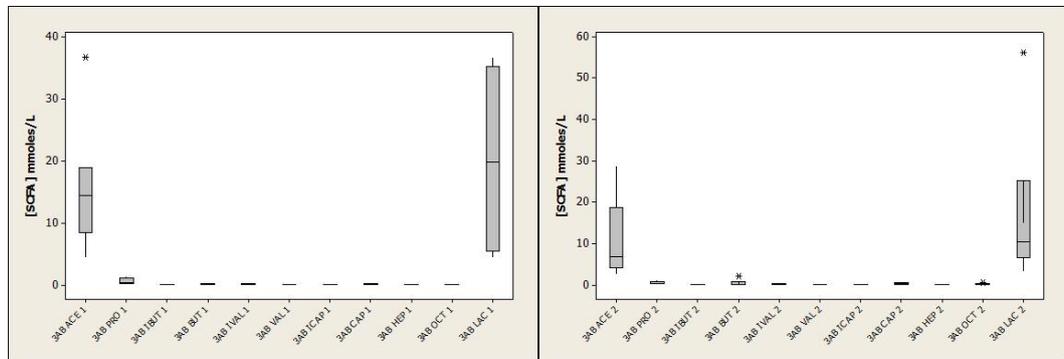


Figure 80: Total SCFA levels in weeks 1 – 4 in infants with stage 3a+b NEC; no significant differences could be identified.



Figures 81: a) Individual SCFA concentrations in infants with 3a+b NEC, week 1; b) Individual SCFA concentrations in infants with 3a+b NEC, week 2; in both cases acetic and lactic dominated.

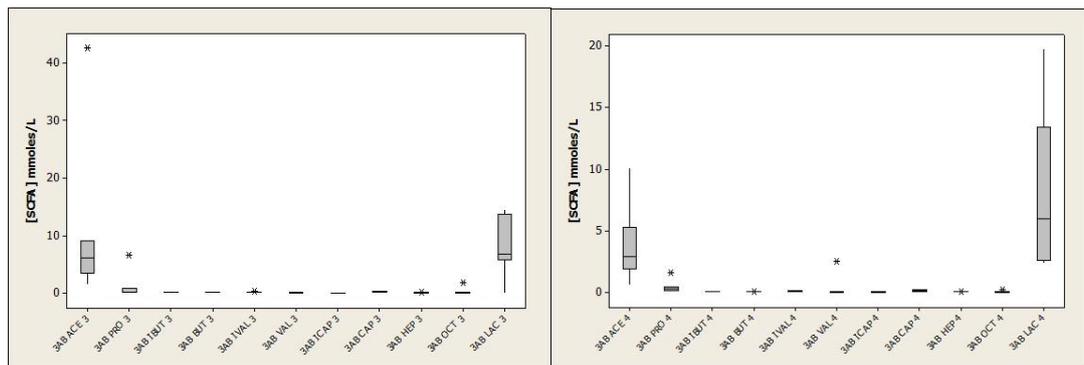


Figure 82: a) Individual SCFA concentrations in infants with 3a+b NEC, week 3; b) Individual SCFA concentrations in infants with 3a+b NEC, week 4; in both cases acetic and lactic dominated.

For those with stage 3a+b NEC, acetic and lactic acids were not significantly different from one another throughout the study period. However, a number of significant differences were noted concerning acetic and lactic acids in these infants:

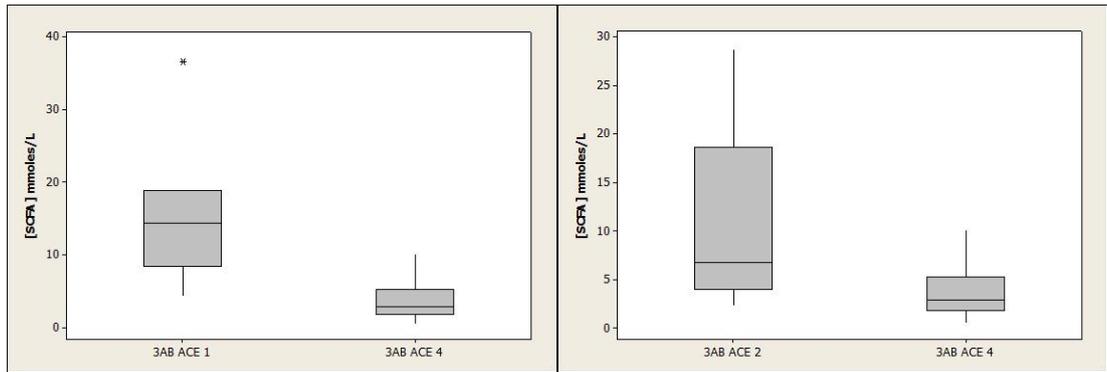


Figure 83 a): Concentrations of acetic acid were significantly higher in week 1 than week 4 in those with 3a + b NEC ( $p=0.005$ ); Figure 83 b): Concentrations of acetic acid were also significantly higher in week 2 than week 4 in those with 3a + b NEC ( $p=0.05$ ).

*Comparisons of SCFA concentrations in stage 2a+2b NEC with stages 3a+3b NEC*

Trends were observed when considering individual SCFAs within each weekly epoch. In each case, those with 2a+2b NEC showed significantly higher levels than those with 3a+3b NEC, and all during week 4 – by which point those with 3a+b were post-laparotomy (figures 84 and 85).

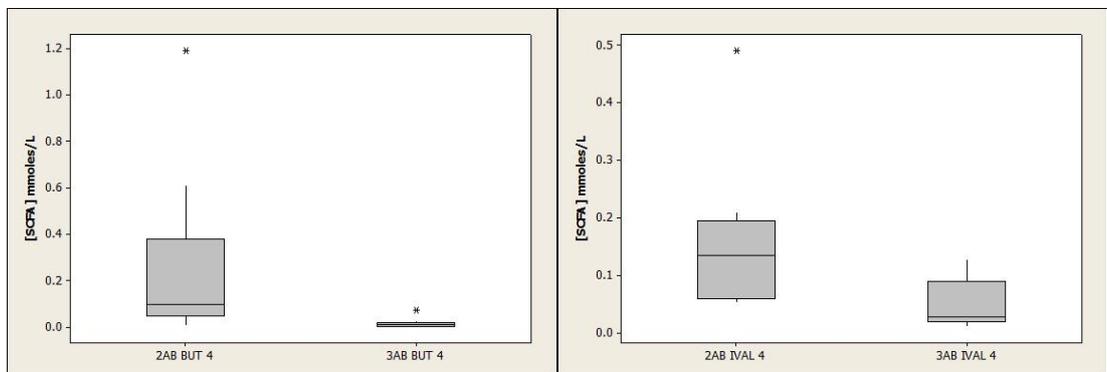


Figure 84 a): Butyric acid levels in those with 2a+b NEC were significantly higher than in those with stage 3a+b NEC ( $p=0.0047$ ); b): Similarly, levels of isovaleric acid were also significantly higher in those with stage 2a+b than 3a+b during week 4 ( $p=0.0273$ ).

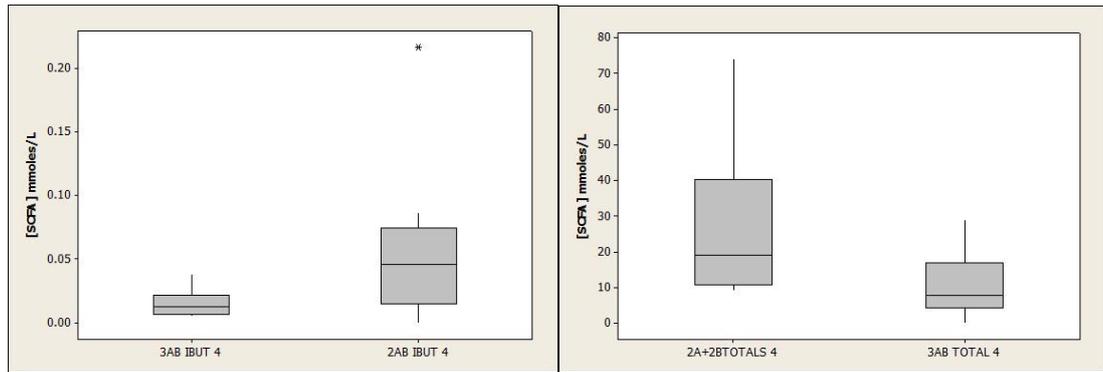


Figure 85 a): Concentrations of isobutyric acid were significantly higher in those with stages 2a+b NEC than those with stages 3a+b during week 4 ( $p=0.05$ ); b): Similarly, total SCFA concentrations were significantly higher in those with stage 2a+b NEC versus 3a+b NEC ( $p=0.0247$ ).

### v) Before and After the Clinical Diagnosis of NEC

Samples from those with  $\geq$ stage 2a NEC were also analysed before and after their diagnosis was made. No differences were seen in either total weekly or individual SCFAs. In samples taken post-diagnosis, only valeric acid was significantly higher in those with stage 1a NEC versus those with stage 3b (Figure 86:  $p = 0.02$ ).

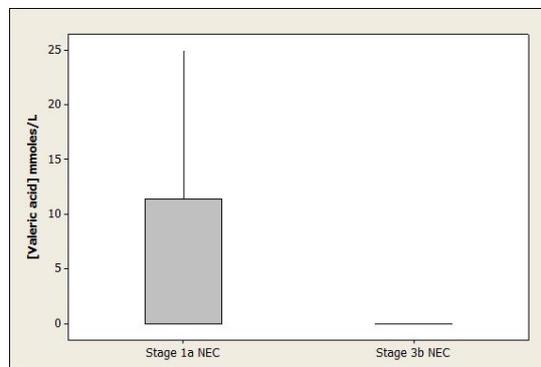


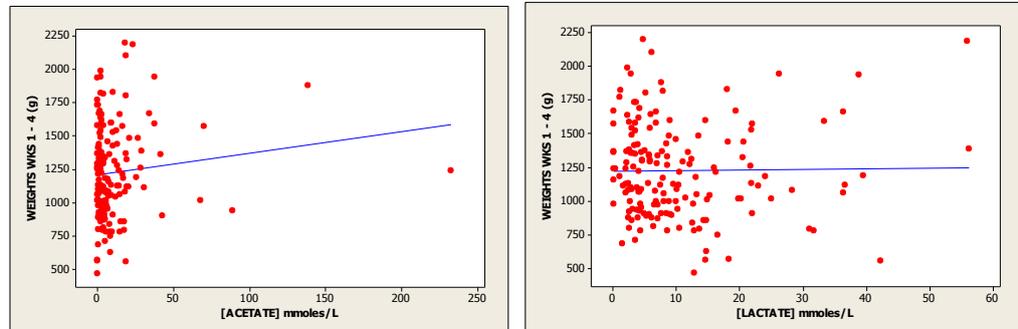
Figure 86: Valeric acid levels in infants with stage 1a NEC were higher than those with stage 3b NEC, post-diagnosis ( $p=0.02$ )

### *Before and After Ileostomy Formation*

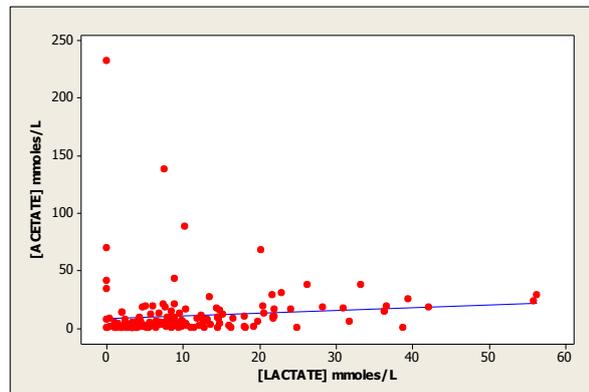
SCFA levels were measured in separate samples taken before and after ileostomy formation. No significant changes were noted in either total or individual SCFA concentrations.

#### 4.1.5) Correlations between SCFAs and demographical data

A number of associations between total SCFA data, individual SCFAs and demographical data were investigated, although none showed a significant correlation. A number of examples are shown below (Figures 87 and 88):



Figures 87: a) (left) Infant weights versus acetate, weeks 1-4; no significant correlation was noted ( $p=0.16$ ); b) (right) Infant weights versus lactate, weeks 1-4; no significant differences were noted ( $p=0.47$ )



Figures 88: Acetate levels versus lactate levels; no significant correlation was noted ( $p=0.28$ ).

#### 4.1.6) Discussion

Despite the severity of NEC, and wide range of gestations, birth weights, and antibiotics, total SCFA concentrations were remarkably consistent through each week, especially considering the heterogeneity of feed types. These concentrations were similar to those published elsewhere in regard to preterm infants, although the trends and correlations noted were very different. Given there is no evidence in literature for the effect of mixed feeding on these SCFA profiles, this strongly suggests that this factor may explain the consistency in results. It is possible that any ‘contamination’ with formula affects establishment of the microbiota, and therefore SCFA production. Unfortunately since no infants were exclusively DEBM, and only a single infant was formula fed, thus these comparisons could not be made. The levels and associations found in our study will be discussed first, followed by an analysis of this data with regard to the evidence base.

##### *i) Individual and Total SCFA Analyses: Gestational and Feeding Differences*

Acetate and lactate dominated most stool samples. In week one, a higher lactate level resulted in a higher total SCFA concentration in infants aged between 24-26 weeks gestation, but this association was lost for the remaining weeks. This could be explained by the higher rate of exclusive breast milk feeding in these infants, in combination with a lower rate of peristalsis. Gestation-specific trends were noted within the other minor, isomeric and branched chain fatty acids throughout the weeks. Although the higher levels of caproic acid in those at 24-26 weeks gestation can be explained by their higher content in breast milk, the reason for the consistently higher levels of isovaleric acid in these infants is unclear, but may be due to higher levels of protein fermentation. Isovaleric acidaemia is a known inherited metabolic disorder, but none of these infants were diagnosed as such. Upon examining the evidence base for identification of isovaleric acid in preterm stool, no articles could be found. However, given that breast feeding is permitted for infants with isovaleric acidaemia, levels generated by the leucine content of breast milk are assumed to be low or absent (Huner, Baykal et al. 2005). This leaves us with the possibility that raised levels are due to microbiological differences in gut microflora, particularly strains of *Clostridia*. However, other significant SCFAs produced by such enteropathogens (namely valeric, isocaproic and butyric acids) were lower at earlier gestations throughout the study period, possibly suggesting an association with a specific strain, such as that of

*Clostridia* sp. The consistency of the SCFA profiles in light of high intraindividual variability in feeds, antibiotics, and natural changes with age strongly suggests that their primary aetiology is from the gut microflora. Particularly given our initial hypothesis that the SCFA profile would change according to feed type, we would have expected changes in the second half of the study period, when the transition between EBM and formula feeding occurred in most mixed-fed infants. This was partly observed when considering the total SCFA concentrations in those EEBM fed, which were significantly higher in week 1 than week 4. Similarly, the acetic acid levels were higher in mixed-fed infants than those EEBM fed in week 4, commensurate with the higher acetate content of formula milks. Whether this is also compounded by a lower acetate level in mature milk from mothers of preterm infants, or simply high variability within our cohort, is yet to be assessed on extensive literature searching.

*ii) Comparisons between infants with and without NEC*

When considering the SCFA levels in regard to NEC, again, these were remarkably consistent, with the dominance of acetate and lactate. No significant differences were noted in totals, which may well simply be from missing relevant ‘spikes’ given the weekly sampling period. When individual acids were considered, those with stage 2a NEC were noted to have an rise in propionic acid between weeks 3 and 4, and a fall in valeric acid between weeks 2 and 4. These correspond with the onset of NEC, and require further studies to assess their potential as biomarkers. However, this effect may have been underestimated due to a number of missing data given the high incidence of paralytic ileus. For those with the most severe stage of NEC, stage 3b, there were more complete data sets given their consistent production of stoma fluid. Isobutyric and heptanoic acids were noted to fall between weeks 3 and 4, corresponding to stoma formation in most of these infants. That these acids were detected at all suggests that they are not just produced by the large bowel. The only difference seen in any individual acid between stages of NEC was in valeric acid, which was higher in those with stage 1a than 3b – perhaps simply reflecting the absence of the colon through ileostomy formation; or impending small bowel bacterial overgrowth, for which these infants are at risk. On post-hoc analysis this had no other obvious clinical or demographical correlation.

### *iii) Ratiometric Analyses*

Ratiometric analyses revealed some interesting trends, and may be of more relevance than individual SCFAs. Unfortunately there are no published data in preterm infants with which to compare. All ratios were generally higher during week one analyses, and when considering gestational groups, both lactate and acetate:BCFA ratios were higher at lower gestations, and considerably higher during week one than at any other time points. The 24-26 week group showed higher acetate:isocaproate and acetate:isovalerate ratios during week one than other weeks, whereas the 28-30 week group showed higher lactate:BCFA, acetate:BCFA and lactate:isobutyrate levels. A simple explanation could be the difference in feed type, since the 24-26 week group were mainly EEBM fed, in contrast with the 28 week gestation group who were mainly mixed fed. This is supported by the higher acetate concentration during week 4 in those mixed fed – although there were no ratiometric differences between either the EEBM or mixed fed groups. The mixed fed group appeared to have higher acetate:BCFA levels in week 2 than week 4, which reflects higher BCFA levels in week 4 – possibly due to the establishment of potential enteropathogens. This is supported by similar ratiometric trends when comparing all infants with  $\geq$  stage 2a NEC than those without NEC, particularly their higher acetate:BCFA ratio in week 1.

Changes in a variety of lactate and acetate ratios were notably higher in those with  $\geq$  stage 2a NEC than those without, throughout the study period. Commensurate with this observation, acetate ratios in those with  $\geq$ 2a NEC were consistently highest at week one; conversely, all acetate and some lactate ratios were higher in week 4 in those without NEC. During week 2 the acetate:isovalerate ratio was also higher in those with  $\geq$ stage 2a NEC than those without. Also during week 2, the lactate ratios began to change, with higher lactate:isocaproate ratios, followed by higher lactate:isobutyrate ratios in week 4. This shift is unlikely to be due to the differences in feed type, since those with NEC were fed more EEBM, and considerably lower volumes during these time points, and similar gestational trends were not noted. This leaves an aberrant gut microbiota either instigating or secondary to NEC, as the most likely cause. During the earlier weeks, this effect could be explained by their longer antibiotic courses. In addition, when those with and without  $\geq$ stage 2a NEC and were compared week-on-week, they showed opposite trends. Infants with  $\geq$ stage 2a NEC had higher acetate:BCFA in week 1, falling through the other time points. This was the direct

opposite for infants without NEC, in whom acetate:isocaproate and lactate:isocaproate ratios were also noted to rise through the later weeks.

Ratiometric analyses showed stronger relationships than considering the individual or total analytes in univariate analyses – and may well point to ratiometric analysis as the principle consideration in future studies of preterm infants. This disagrees with one of our original hypotheses – that those with NEC would have greater levels of protein fermenting bacteria, with higher BCFA and lower SCFA concentrations as a result. Instead, proportionately lower BCFA levels were observed in those with NEC, insinuating that in these patients, less proteolytic and more sacrolytic bacteria are present. Given the absolute values are so small, ratiometric changes are better placed to identify these.

*iv) Comparison with the evidence base*

This work has identified similar levels to those found in other studies concerning SCFAs in preterm infants that have also found samples to be dominated by acetate and lactate, also been found in infants randomised to enteral probiotic administration. Our figures were similar, medians measuring: total SCFA concentrations of 20  $\mu\text{moles/g}$ ; lactate levels of 8  $\mu\text{moles/g}$ ; acetate levels of 6  $\mu\text{moles/g}$ ; and all other SCFAs were  $<1.0 \mu\text{moles/g}$ . In particular, butyrate levels were significantly lower than in other studies, and mostly less than 1  $\mu\text{moles/g}$ . Total SCFA concentrations in those EEBM fed showed a median of 35  $\mu\text{moles/g}$ , decreasing to a median of 10  $\mu\text{moles/g}$  in week 4. No significant differences were seen between levels in infants with and without  $\geq$  stage 2A NEC (median: 20  $\mu\text{moles/g}$  in  $\geq$  stage 2A NEC vs 18  $\mu\text{moles/g}$  in those without). Changes were observed in minor SCFAs when comparing different stages of NEC, but these concentrations were extremely low – with valeric, butyric, isovaleric, isobutyric and heptanoic acids all measuring less than 1  $\mu\text{moles/g}$ . No normative BCFA data in preterm infants could be ascertain on extensive systematic literature searching. As such, our data would appear to be the first.

Comparison of other studies with ours is immediately confounded by the use of multiple other units of measurement, including: mEq/100g; Kcal/Kg/day; mumoles/g dry weight;  $\mu\text{moles/g}$  wet weight; and  $\mu\text{moles/mg}$ . Only three studies shared the same unit of measurement ( $\mu\text{moles/g}$ ): Favre in 2002, Wang in 2007, and Mohan in 2008

(Favre, Szylit et al. 2002, Mohan, Koebnick et al. 2006, Wang, Shoji et al. 2007). These will be discussed specifically, and all other trends from the literature will be noted generally.

Favre et al in 2002 randomised 28 preterm infants to either breast milk or nutramigen. Three groups were considered: preterm infants with breast milk (group 1); preterm infants with nutramigen (group 2); and extremely preterm infants breast milk fed (group 3). Levels were quoted in  $\mu\text{moles/g}$ . Total concentrations were similar to ours, with all those breast milk fed: 24 (1.3-118.8), vs all nutramigen fed: 23 (3-73.3) showing no significant difference. The total SCFA concentration was significantly lower in group 1 than any other: 7.4 (0.3-37.4). Increased levels of butyric acid were observed in those fed nutramigen, by 30%. None of our population were fed nutramigen formula milk.

Wang et al in 2007 randomised 66 preterm infants to the probiotic *Bifidobacterium BB12* or a placebo, and used HPLC to analyse samples at 0, 2 and 4 weeks of life, using  $\mu\text{moles/g}$  units. They found similar total SCFA levels to ours, with significantly higher totals in controls at 4 weeks. These were further stratified by birth weight (ELBW; VLBW; and LBW). ELBW infants had: 20.4 (0-88.5); VLBW infants: 33.4 (11.4-54.7); L: 43.2 (25.8-79.4) – all  $p < 0.05$ . Higher total SCFA levels in controls were also seen at 2 weeks in VLBW and LBW infants: 2.1 (0-11.4) and 6.9 (0-55.7). Decreased levels of butyrate were seen in those administered the probiotic in BB12 in both ELBW and VLBW infants: ELBW: 2.4 (0-17.9) vs control 0.1 (0-0.3),  $p < 0.05$ ; VLBW: 2 (0-6.6) vs control 0.1 (0-0.4),  $p < 0.05$ . Our butyrate levels were very similar to their control groups, at  $< 1.0$ . Wang et al also performed some ratiometric analyses, although they did not consider BCFA but the total SCFAs. They showed that the ratios of acetic:SCFAs at week 4 were higher in those given the probiotic at all weight ranges: LBW: 96.8  $\pm$  3.4% vs control 81.7  $\pm$  7.6; VLBW: 97.8  $\pm$  3.5% vs control 5.8  $\pm$  6.9; ELBW: 99.3  $\pm$  0.7% versus 88.6  $\pm$  6.3% (all  $p < 0.05$ ). They concluded that *Bifidobacterium BB12* lowered butyrate thus protecting against NEC. However, this trial was not powered to NEC, and no NEC data were displayed.

Mohan et al in 2008 randomised 69 preterm infants to either placebo or *Bifidobacterium BB12*. They performed GC analyses twice weekly, declaring weeks 1,

2 and 3 data. Lactate was measured by ELISA. All SCFAs were reported as  $\mu\text{moles/g}$ , but whether wet or dry weight was not specified. As with our analyses, acetic acid was the dominant SCFA comprising 90% of the total. Data for propionic and butyric acids were not mentioned specifically, but was far less than acetic acid ( $p < 0.05$ ). Ratiometric analysis was used, but compared acetic, propionic, butyric, and valeric acids, and was quoted at 90:4:3.2:2 for the total cohort. Values were displayed in graph form only, therefore the following are estimates. The levels of lactate were 38% higher in those administered probiotics than those given the placebo. The median level of total SCFAs was 11 for the probiotic group and 7 for the controls – considerably lower than our own figures. Differences were seen in infants with and without antibiotic administration. Infants without antibiotic administration had considerably higher lactate levels. Given that most of our study cohort was administered antibiotics during the study period, we were unable to perform a similar comparison. Those given probiotics in Mohan's study showed higher total SCFA levels during wk 2, with  $\sim 32 \mu\text{moles/g}$  vs  $\sim 25 \mu\text{moles/g}$  of placebo infants. In week 3, this difference was more pronounced, and similar to our own figures: probiotics  $\sim 47 \mu\text{moles/g}$  vs placebo  $\sim 27 \mu\text{moles/g}$ . Higher acetic acid levels were noted in week 2 in those given probiotics:  $\sim 30 \mu\text{moles/g}$  vs  $20 \mu\text{moles/g}$  in placebo. Again, by week 3 this difference was more pronounced, with probiotic group  $\sim 41 \mu\text{moles/g}$  vs the placebo group median of  $\sim 22 \mu\text{moles/g}$ . These concentrations of acetic acid were, on the contrary, 5 to 6 fold higher than those found in our study. All infants within the Mohan study were mixed fed. Although Mohan et al found a significant correlation between levels of lactate and acetate ( $p = 0.0001$ ), as well as *Bifidobacterial* numbers and both lactate and acetate ( $p = 0.05$  and  $p = 0.01$ ). We were not able to replicate these correlations.

Trends found by other studies were highly variable. These studies comprised one observational study, and two RCTs – one of feed type, and one of probiotic supplementation. There were few gestational and age differences in SCFA individual and total concentrations within our cohort. Most other studies did not find or express such differences either. Anyon et al in 1971 (Anyon and Clarkson 1971) observed 10 preterm infants of less than 37 weeks and 2.5kg birth weight, over three epochs within the first month of life. They measured GC output using  $\text{mEq}/100\text{g}$ , and recorded little variation in totals: 1<sup>st</sup> epoch:  $84.7 \pm 124.7$ ; 2<sup>nd</sup> epoch:  $69.2 \pm 11.7$ , 3<sup>rd</sup> epoch:  $80.7 \pm 16.4$ . There was a significant decline in acetic acid levels: 1<sup>st</sup> epoch:  $62.2 \pm 0.8$  2<sup>nd</sup> epoch:

48.2±7.6; 3<sup>rd</sup> epoch: 48.7±2.7. Levels of propionic acid unexpectedly rose through the epochs: 1<sup>st</sup> epoch: 6.3±4.8; 2: 8.9±3.1; 3: 14.6±2.7. but: 13.2±3.1 2: 12.1±2.8; 3: 11.2±0.8. Lactic acid was in far lower levels than our study, and sharply declined to the 3<sup>rd</sup> epoch: 1<sup>st</sup> epoch: 1.3±1.2; 2<sup>nd</sup> epoch: 0.45±0.1; 3<sup>rd</sup> epoch: 0.23±0.8. They concluded that fermentation of carbohydrate was normal in preterm infants but disappears during weeks 2 – 3, presumably secondary to better absorption of sugars by the gut. NEC was not noted in the cohort.

Kien et al in 1996 (Kien 1996) measured SCFAs by GC, quoting kcal/kg/day - values which are not comparable with our study. They performed a trial administering a lactose formula to 15 preterm infants between 28-32 weeks gestation, measuring stool samples between 2 – 4 weeks of age. In those given the lactose formula, they saw an increased acetate level by 77% (p = 0.03), and higher total SCFA concentrations by 54% (p = 0.04). They concluded that 90% of fecal carbohydrate energy is large molecular weight compounds from bacterial fermentation.

Stansbridge et al in 1993 (Stansbridge, Walker et al. 1993) underwent a RCT of a *Lactobacillus* probiotic, recruiting 20 preterm infants, and observing stool samples over the 1<sup>st</sup> 28 days of life. They quoted their results in µmoles/g dry wet sample. Their results showed no differences in (probiotic group vs control group): acetic acid 173 (trace – 799) vs 166 (trace – 700); propionic acid: 44 (trace – 169) vs 37 (11-229); butyric acid: 31 (5-107) vs 37 (2-118).

More recently, Underwood et al in 2009 (Underwood, Salzman et al. 2009) performed a RCT of prebiotic/probiotic combinations in preterm infants, and recruited 90 in all. These were stratified into three groups: controls (group 1); those given *Lactobacillus* sp. and FOS (group 2); and those given *Lactobacillus*, *Bifidobacterium*, and FOS (group 3). Samples were measured using HPLC, and values were reported as nmoles/mg of sample. Comparisons showed no significant differences in acetic, propionic or butyric acids. Medians were quoted as follows: acetic: Group 1 - 2.5; Group 2 – 2; Group 3 - 3; propionic: Groups 1, 2 and 3 all had a median of 1; butyric: Group 1 – 6; Group 2 – 6; Group 3 - 4. Although seven infants were noted to develop any stage of NEC, these were not considered within the analysis.

v) SCFAs in NEC: the evidence base

As discussed within the background chapter, only one published study has considered SCFA analysis with respect to NEC in preterm infants (Szyliet, Mauraage et al. 1998). In their observational study, Szyliet et al considered differences in 46 enterally fed preterm infants with and without ‘digestive disorders’ using GC and quoting mmoles/Kg wet weight of sample. However, they did not consider the Modified Bell’s Criteria as their staging system for NEC, and instead used more numerous and different descriptions of ‘digestive disorders’. Their cohort included infants who were also more mature and heavier at delivery than in our own study (mean 32.8 weeks gestation and mean birth weight 1920 g). All infants who could not be fully fed maternal EBM were supplemented only with DEBM – unlike in our study, where formula was also used. In addition, few infants within the study by Szyliet et al were administered antibiotics, in stark contrast with our own cohort. Their mean total SCFA levels were however lower than ours, with a range of 0 – 80 mmoles/kg, and were normally distributed. They used 2-ethylbutyric acid as their internal standard. They too noticed a wide inter-individual variation in total and individual levels. The total SCFA levels from the infants in their cohort fell significantly with increasing birth weight (LBW 3 mmoles/Kg vs NBW 0.9 mmoles/kg). It also increased with phototherapy, and declined with antibiotic usage, neither of which we considered in regard to our SCFA data since both were ubiquitous throughout our cohort. Infants without digestive disorders showed an increasing butyric acid ratio from 7 % to 24 % throughout their study period.

Szyliet et al noted a significant ‘spike’ in butyric acid prior to GI bleeding ( $42.6 \pm 12.3$  mmoles/Kg vs  $25.3 \pm 16.2$  mmoles/Kg). These levels, when converted to our unit of  $\mu\text{moles/L}$  are still likely to be significantly higher than the butyric acid levels seen within our cohort at any time point. The infants studied by Szyliet et al showed a trend to higher acetic acid before the onset of GI haemorrhage, and higher butyric and caproic acid levels in these infants than those without digestive signs. However a major confounder of their study was the exclusion of infants with laparotomy first week. If we had excluded these infants then two infants with stage 3B NEC would have been omitted from our analyses.

#### **4.1.7) Conclusions**

The consistency and lack of significant differences in data with respect to demographical information supports the notion that much of the stool SCFA measured in our cohort is generated by bacterial fermentation, likely influenced heavily by the predominance of mixed EBM and formula milk feeds, and the ubiquity of antibiotic administration. Our figures also corroborate similar studies quoting the same units of measurement ( $\mu\text{moles/g}$ ). It is extremely encouraging that despite the high incidence of NEC, the total and individual SCFA levels in our study are comparable to those from published studies of infants administered probiotics – studies which conclude these levels to be in deference to this supplementation. This may point to the greater value of EBM HMOs, endogenous EBM probiotic bacteria, and possibly cross contamination from staff consuming probiotic products, given that the majority of mothers of the infants recruited to our study did not use probiotic foodstuff or supplements. This is the first study to report levels of BCFAs in preterm infants with and without NEC, and also the first to combine these in ratiometric form. The significant differences seen with regard to NEC give these measurements promise for further development as diagnostic and prognostic markers of NEC in the future.

## **4.2) MOLECULAR ANALYSIS OF STOOL SAMPLES VIA TEMPORAL TEMPERATURE GEL ELECTROPHORESIS**

### **4.2.1) Introduction**

Given the time-consuming and expensive nature of this method of molecular analysis, all 56 infants did not have samples analysed for this satellite study. Instead, patients were selected from this cohort so that ‘case’ and ‘control’ groups could be compared. The external collaborators were therefore not blinded to the NEC status of any infant. In addition, it was decided to exclude meconium samples, given the lack of gut colonisation in these samples from other studies – although it is appreciated that certain observational studies have yielded bacteria from meconium, despite the absence of PPRM in the mother (Jimenez 2008). As such, samples were compared between non-meconium stool from week 1 and week 4.

### **4.2.2) Clinical and Demographical Results**

In total 44 stool samples from 22 infants were analysed for the purpose of this study. There was no significant difference in demographics, weight and clinical characteristics between EBM and MF infants but there was a large inter-individual variability (Table 22). As expected, there was a strongly positive correlation between gestational age and birth weight ( $p = 0.0001$ ). In the EBM group 62% of the infants had been diagnosed with NEC and 50% in the MF group but the difference was not significant ( $p = 0.6749$ ) (Table 22). Infants with NEC had a significantly lower birth weight ( $p = 0.0027$ ) and gestational age ( $p = 0.0092$ ) compared to those without. These findings were significant in the MF group ( $p = 0.0022$  and  $p = 0.0049$ , respectively) but not in the EBM group ( $p = 0.7656$  versus  $p = 1.0$ ). Most infants were born by caesarean section, and in each group two infants were delivered by SVD (Table 22). Five of the mothers in the MF group had received antibiotics during their pregnancy and delivery, while none of the mothers of the EBM infants had intrapartum antibiotic treatment (Table 22). All infants received TPN for the first few days of life. Enteral feeding was introduced within the first three days of life in most infants but was delayed in three infants with NEC to day six and seven (two EBM, one MF) (Table 22). There was no significant difference in the age at the commencement of enteral feeding between EBM and MF infants ( $p = 0.459$ ). The infants’ age at sample collection varied within individuals but the median difference between the first and second samples was significant for all individuals (22

days;  $p = 0.003$ ). Notably, this only includes samples with an available collection date (Table 22), but it should be noted that due to daily collections, the longest any sample rested in a  $-20^{\circ}\text{C}$  freezer in situ at the birth hospital was 48 hours (i.e. over a weekend). All second samples from infants with NEC were collected after diagnosis.

**Table 22: Demographical and clinical characteristics of infants**

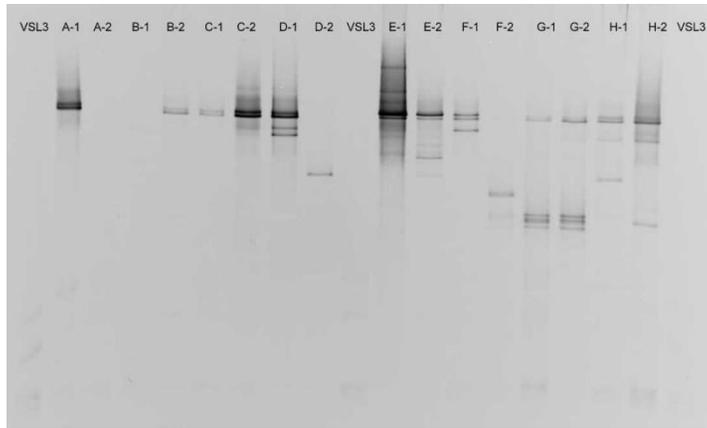
	Infant	Hospital	Gender	Mode of delivery	Gestational age (weeks)	Birth weight (g)	Maternal antibiotics	NEC/ NON	Feeding start (DOL)	Sample 1 (DOL)	Sample 2 (DOL)
<b>EBM</b>	A	QMH	M	CS	28.0	836	no	NEC	2	8	29
	B	SGH	F	SVD	24.4	830	no	NEC	7	13(MEC)	22-28
	C	SGH	M	CS	27.4	1140	no	NEC	2	15	22-28
	D	SGH	M	CS	27.4	1080	no	NEC	6	16(MEC)	22-28
	E	SGH	F	CS	30.1	1240	no	NEC	2	12	27
	F	PRMH	F	SVD	26.7	1055	no	NON	2	2-7	22-28
	G	PRMH	M	CS	27.7	1034	no	NON	2	2-7	30
	H	PRMH	F	CS	29.0	1040	no	NON	3	3-7	22-28
	Median (range)		4M/ 4F	2 SVD/ 6 CS	27.6 (24.4-30.1)*	1078 (830-1240)*	All no	5 yes/3 no	2 (2-7)*		
<b>MF</b>	I	PRMH	F	CS	27.9	835	no	NEC	2	9(MEC)	30
	J	PRMH	F	CS	30.1	925	no	NEC	2	3	28
	K	PRMH	F	SVD	28.1	935	no	NEC	2	2-7	22-28
	L	PRMH	F	CS	27.4	685	no	NEC	2	8(MEC)	31
	M	PRMH	F	CS	25.6	715	yes	NEC	6	14	31
	N	PRMH	M	CS	25.6	900	yes	NEC	3	8(MEC)	32
	O	SGH	M	CS	27.1	960	no	NEC	3	8(MEC)	22-28
	P	PRMH	M	CS	31.9	1370	no	NON	2	7	28
	Q	PRMH	F	CS	31.6	1480	yes	NON	2	9	31
	R	SGH	F	CS	30.9	1480	-	NON	2	7(MEC)	30
	S	PRMH	F	CS	29.6	1185	yes	NON	1	5	36
	T	SGH	F	CS	31.4	1100	no	NON	2	9(MEC)	22-28
	U	PRMH	M	CS	30.7	1280	no	NON	2	9(MEC)	22-28
	V	PRMH	M	SVD	28.9	1135	yes	NON	1	8	22-28
	Median (range)		5 M/ 9 F	2 SVD/ 12 CS	29.3 (25.6-31.9)*	1030 (685-1480)*	5 yes/ 8 no	7 yes/7 no	2 (1-6)*		

Annotations: - missing value, \* values given in median (range); Abbreviations: EBM = expressed maternal breast milk; MF = mixed breast milk and formula fed; CS = caesarean section; SVD = spontaneous vaginal delivery; DOL = day of life; MEC = meconium; NEC = necrotising enterocolitis; NON = no necrotising enterocolitis).

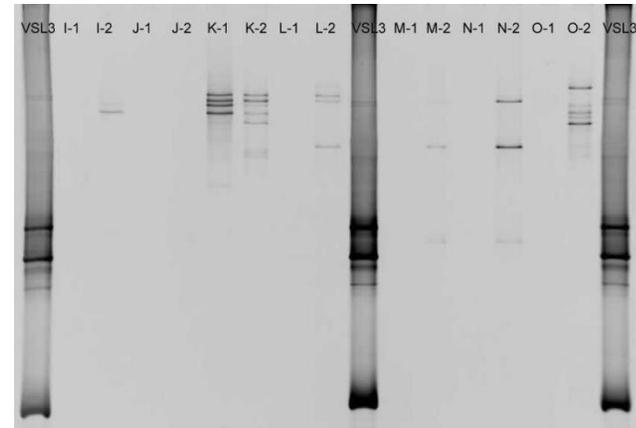
### **4.2.3) Outcomes of TTGE analysis**

Band analysis could be conducted for a total of 39 samples. For seven of the samples, genomic DNA was not detected with ethidium bromide stain, but four still presented results by TTGE. No bands could be detected in the three remaining DNA-negative samples, even with the use of TTGE-Sybr Green, a stain 25 times more sensitive. As such, these negative samples were excluded from the analysis. One sample had illustrated products on the DNA agarose gel but PCR and TTGE gel could not obtain a result so that was also excluded from analysis (Figure 89 a-d).

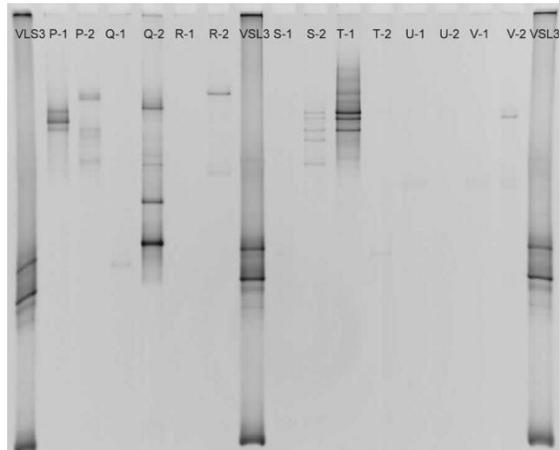
**Gel 1: Figure 89 a)**



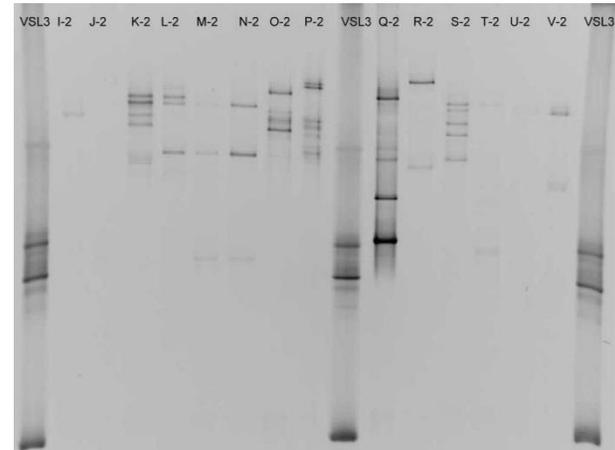
**Gel 2: Figure 89 b)**



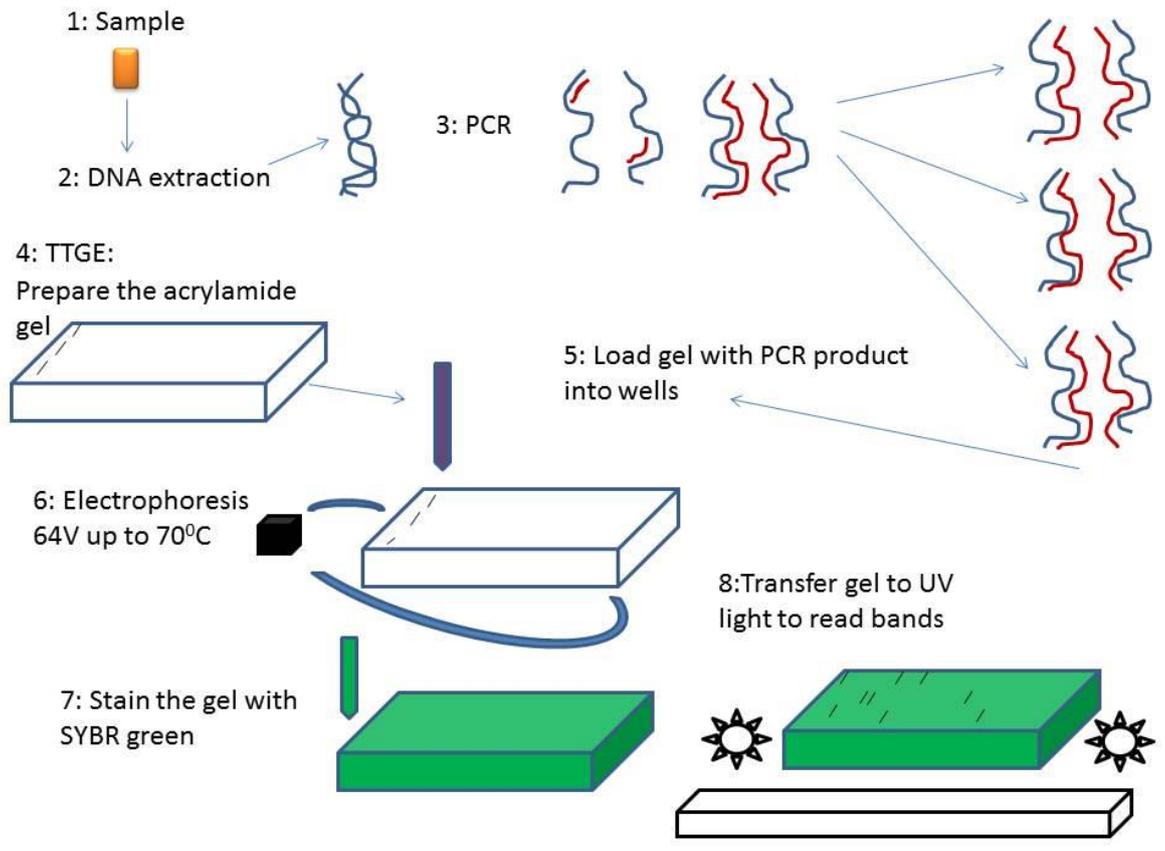
**Gel 3: Figure 89 c)**



**Gel 4: Figure 89 d)**



**Figure 89 a-d): TTGE Gels 1-4. 'VSL3' was used as standard. Letters stand for each participant and numbers for the time of sample collection. Gel 4 illustrates the second samples from all mixed fed infants. VSL-3 refers to the probiotic reference sample.**



**Figure 90:** Annotated schematic example of TTGE steps. Note one fecal sample was introduced per well. Photographs were then taken of each gel, and bands analysed as described within the text.

**i) Number of species present at both sample points**

Species richness was defined as the number of bands per sample. This varied greatly within individuals with a minimum 1 to a maximum value of 17 species (Table 23). No significant differences in microbial richness could be detected between Sample 1 (collected during week 1) and Sample 2 (collected during week 4;  $p = 0.453$ ) in the cohort, or in those with and without NEC ( $p = 0.6111$ ). Similarly, no significant differences were noted by feeding mode ( $p = 1.0$ ).

**Table 23: Number of species present at the two sample points**

		Species richness		
		Sample 1	Sample 2	
<b><u>EBM</u></b>	A	9	1	
	B	2	3	
	C	2	7	
	D	7	1	
	E	7	8	
	F	3	7	
	G	10	6	
	H	7	13	
		7 (1-10)*	6.5 (1-13)*	$p=1.000$
<b><u>MF</u></b>	I	-	4	
	J	-	3	
	K	11	9	
	L	-	6	
	M	-	5	
	N	-	5	
	O	2	10	
	P	8	8	
	Q	1	17	
	R	2	4	
	S	3	10	
	T	10	4	
	U	4	2	
	V	2	4	
		3 (1-11)*	5 (2-17)*	$p=0.294$

Annotations: - missing value, \* values given in mean (range). (Abbreviations: EBM = expressed maternal breast milk; MF = mixed expressed maternal breast and formula milks).

There was no significant increase in numbers of species found at the two sample points in either feed groups (Table 23). Within both feeding groups, there was considerable interindividual variation, with some individuals developing an increasing microbial richness, whereas others became more spartan. This occurred regardless of the presence or absence of NEC (Figure 91).

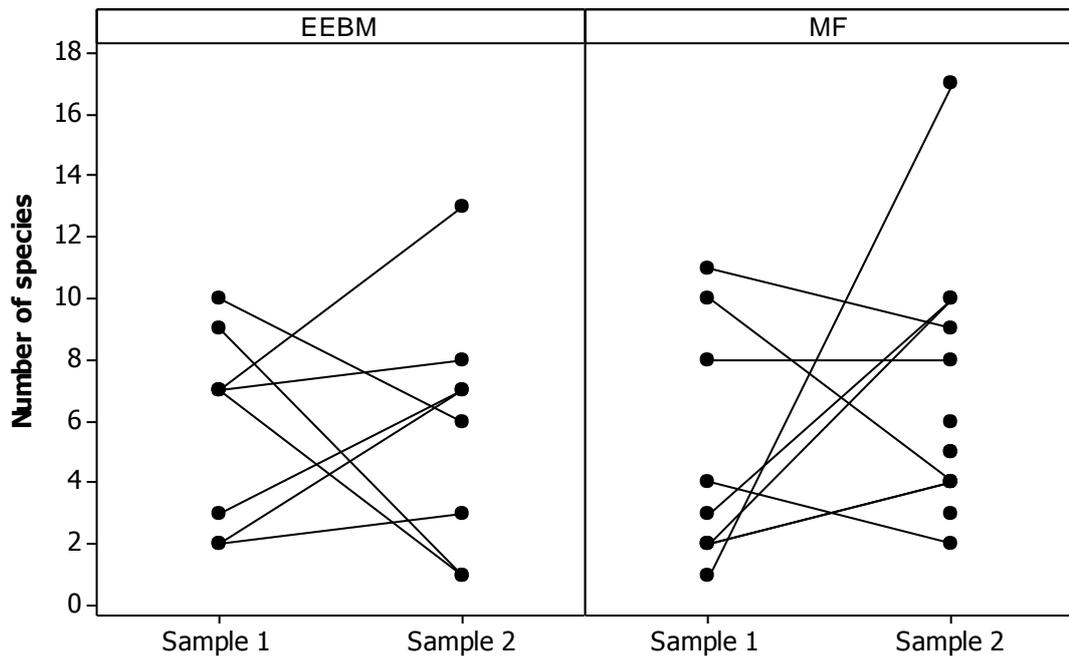


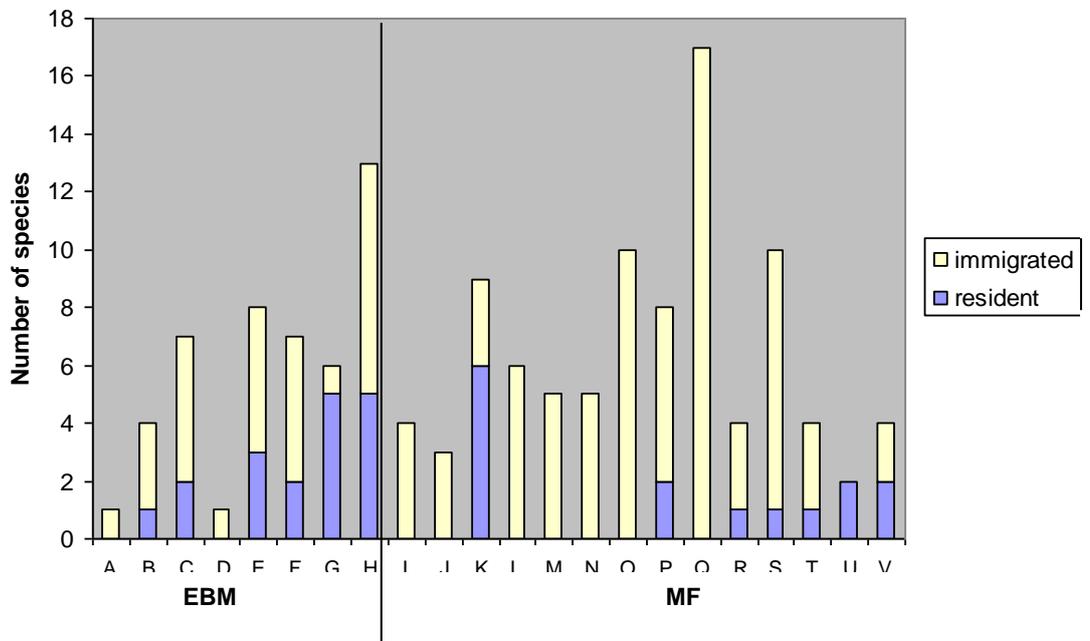
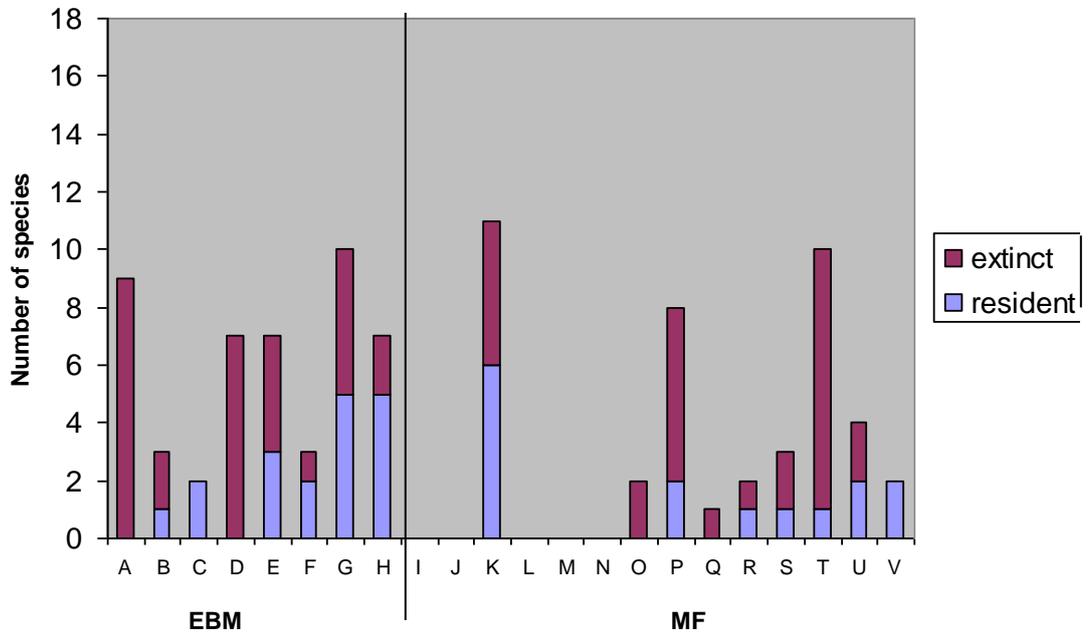
Figure 91: Changes in number of species present between each sample; no differences were noted. (Abbreviations: EEBM = exclusive expressed maternal breast milk fed; MF = mixed breast and formula milk fed).

## ii) Changes within the microbial community over time

In order to get an idea of species turnover, the detected bands were categorised into: 1) those that remained the same between sample 1 and sample 2 (persistent species); 2) those that were present in sample 1 but not in sample 2 (extinct species); and 3) those that were not present in sample 1 but then appeared in sample 2 (immigrated species) (Figure 92). The corresponding values of species turnover (i.e. the numbers of resident, extinct and immigrated species) did not correlate with each other and there was no difference between the feeding groups regarding the number of resident ( $p = 0.5637$ ), extinct ( $p = 0.7003$ ), or immigrated species ( $p = 0.441$ ).

**Figure 92: Species turnover. Values show number of species present in:**

- Sample 1 and 2 (resident)
- Sample 1 but not 2 (extinct)
- Sample 2 but not 1 (immigrated)



Letters A-V represent the subjects. (Abbreviations: EBM = expressed maternal breast milk; MF = mixed breast and formula milk).

### iii) Inter-individual similarity

Intra-individual similarity varied greatly and ranged from a similarity index ( $C_s$ ) of 0 % to 66.8 % (see Appendix 4). There was no statistical difference between the similarity indices of the feeding groups ( $p = 0.885$ ) or between those with and without NEC ( $p = 0.171$ ) (Figure 93).

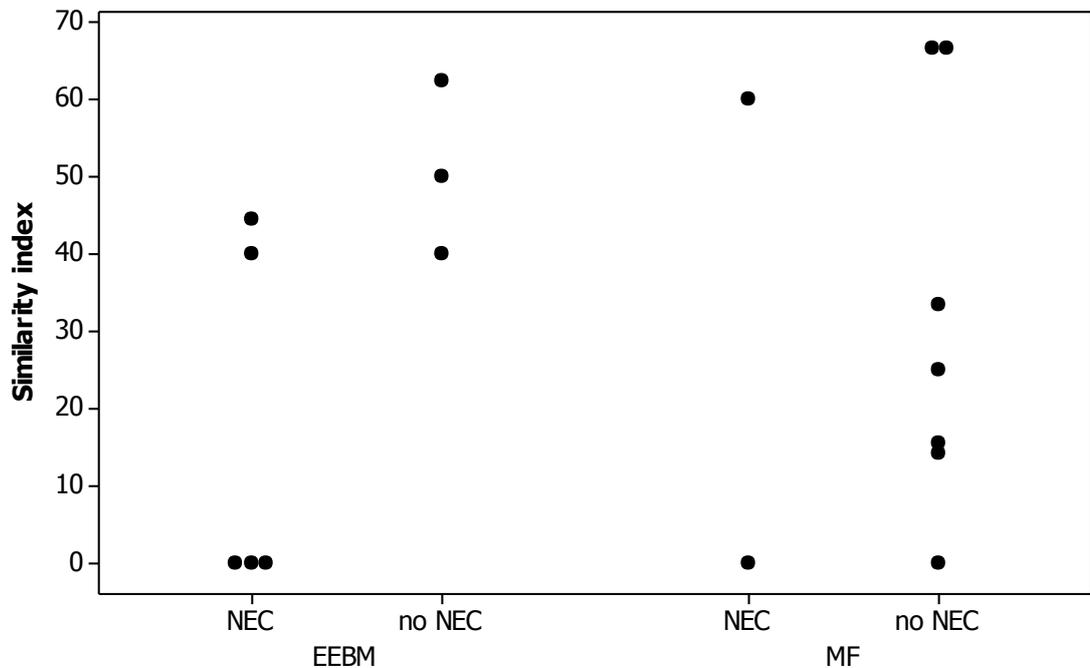


Figure 93: Interindividual similarity indices of EEBM and MF fed infants showed no difference with respect to NEC. (Abbreviations: EEBM = exclusive expressed maternal breast milk; MF = mixed breast and formula milk fed).

Inter-individual similarity was calculated only for Sample 2 due to the number of missing values in the first sample set. Single  $C_s$  had a great variance and ranged from 0 % to 66.8 %, but the mean inter-individual similarity did not differ between the feeding groups (Figure 94).

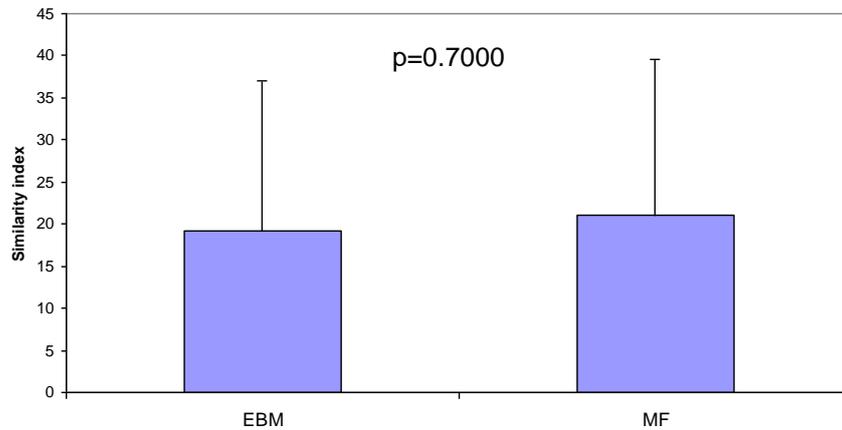


Figure 94: Mean percentage of interindividual similarities of EBM and MF infants (with error bars representing SD); no significant differences were noted. (Abbreviations: EEBM = exclusive expressed maternal breast milk; MF = mixed breast and formula milk fed).

#### iv) Relative abundance of species in the study population

The TTGE profile of the second samples identified a total number of 26 different species within the EBM group and 31 species within the MF group. Most species detected in the second sample of EBM and MF infants had a low abundance across the sample population but three bands in the EBM group and one band in the MF group were detected in more than 50 % of the subjects. The relative abundance of species did not differ between the groups (Figure 95), and their numbers were too small to note any significant changes in abundance or similarity according to stage of NEC.

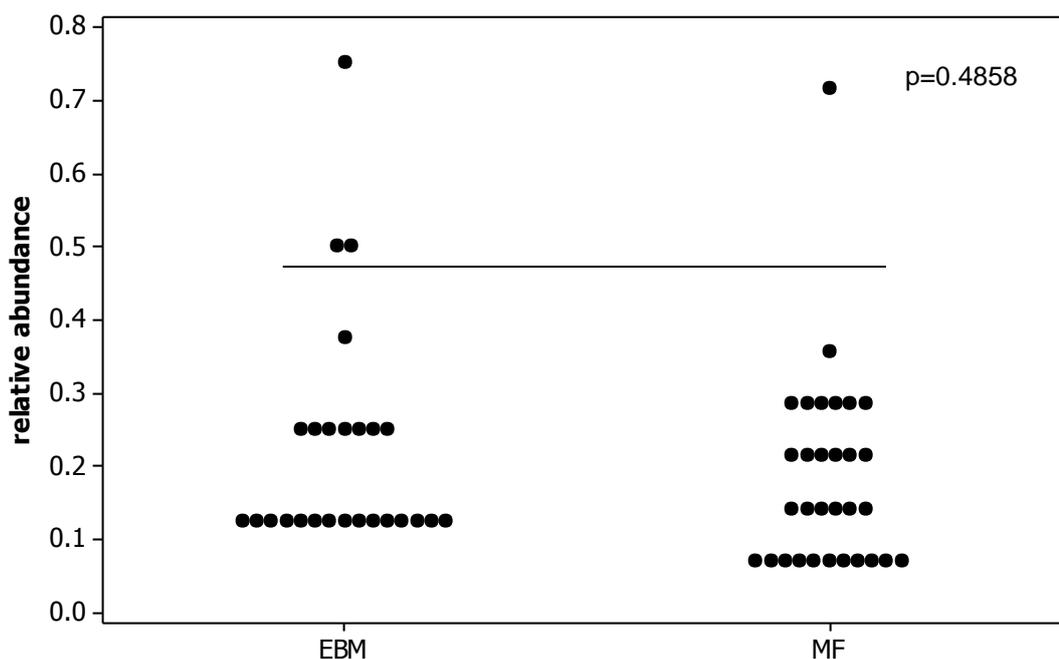
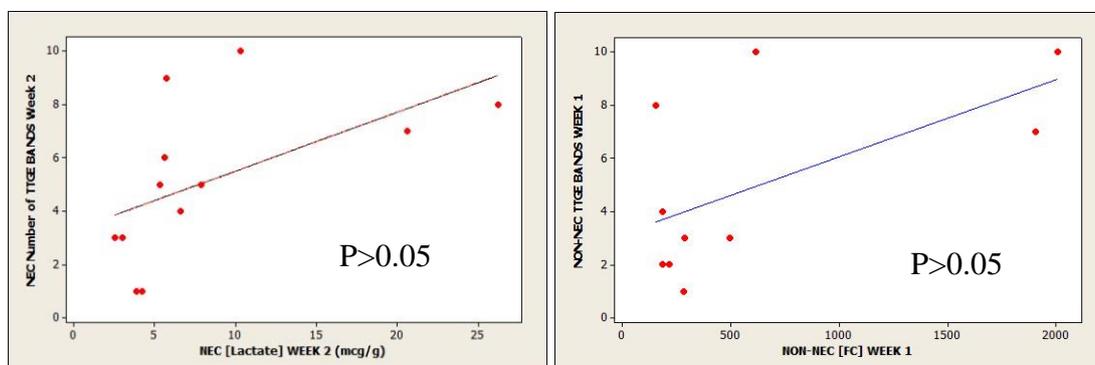


Figure 95: Individual value plot: relative abundance of species from EBM and MF infants. (Abbreviations: EEBM = exclusive expressed maternal breast milk; MF = mixed breast and formula milk fed).

#### v) Correlations between TTGE parameters and other analytes

No correlations at all were found between the number of bands and any other metabolic or inflammatory analytes on univariate analysis (Figure 96 a)). A non-significant correlation was observed between number of bands in those without NEC and their corresponding calprotectin levels during their week 1 sample (Figure 96 b):  $p = 0.065$ , Pearson correlation 0.604).



Figures 96: a) (left) Bands versus lactate in infants with all-stage NEC; b) (right) Bands versus FC in infants without NEC; in each case, no significant differences were observed.

When these analyses were compared by Neonatal Unit, there were no significant differences, but this may be due to small numbers. None of the NICUs were noted to have any unusual bacteria colonising these or other patients.

#### 4.2.4) Discussion

##### *i) Introduction*

This subgroup analysis corroborates earlier similar studies using TTGE, revealing fewer species, at lower intensities, and with wide intra and interindividual variety. The aim of this study was to gain an insight into the early intestinal bacterial colonisation of VLBW preterm neonates and to investigate a potential impact of the feeding mode on gut microbiota development and its relationship with the risk of NEC. Although the bands obtained by TTGE changed rapidly, this could indicate the tremendous amount of microbial flux that occurs during the first month of life – more so than at any other age. In general, the evidence base suggests that the gut microbiota of preterm infants shows a dominance of anaerobes and gram negative bacteria, the delayed appearance of *Bifidobacteria* sp., and increased interindividual variation in healthy infants. Observational studies also note reduced colonisation in infants delivered by LUSCS, those administered antibiotics, and those incubated after delivery (all of which occurred commonly for our cohort) – particularly diminished populations of *Lactobacillus*. Our results will be considered by data set analysis in tandem with studies of preterm infants both with and without NEC, as already documented in pages 65 – 67 and 86 – 88.

##### *ii) DNA yield*

Stool from our cohort in the first week of life from non-meconium samples was analysed and a TTGE band pattern could be obtained in 17 of 22 subjects. The number of species found in those samples varied greatly within individuals (range 1-11), suggesting that the colonisation of the gut starts rapidly after birth but there is also a large inter-individual variability. This was in keeping with Magne et al in 2006, who found a mean of 3.25 species per infant from 16 preterm neonates using two different techniques: TTGE and PCR. The samples were collected at the fourth week of life and showed a bacterial richness of 1 to 9 species with TTGE analysis and 1 to 8 species with 16S rRNA sequencing technique (Magne, Abely et al. 2006). Species identified included *Enterococcus*, *Streptococcus*, *Staphylococcus*, and *Bifidobacteria* sp.

There was no significant difference or increased trend between the two times of sample collection in our study. Species richness found in our study did not vary largely from findings of other studies. This is supported by Mshvildadze et al from 2010, who obtained DGGE results in 21 of 23 first meconium stool samples of very premature neonates of less than 30 weeks gestational age (Mshvildadze, Neu et al. 2010). This is to date the only

study to identify bacterial DNA in meconium. We chose not to use meconium as there was likely to be a higher yield in later, changing stool of enterally fed infants. Mshvildadze et al also found that DNA could be extracted in all second samples, and the number of species identified by DGGE varied between 1 and 17 species. The main species identified in those with NEC were *Citrobacter*, *Enterobacter*, whereas those without NEC showed higher levels of *Klebsiella*. Similarly, Schwiertz et al in 2003 identified a range of between 5 to 20 species using denaturing gradient gel electrophoresis (DGGE) analysis in stool collected during the first four weeks of preterm neonates. In contrast to our study, the numbers of species were lower at the third day of life and there was an increased trend of bacterial richness over time (Schwiertz, Gruhl et al. 2003). Another recent prospective study also reported an increased diversity score of 0.45 units/week ( $p < 0.0001$ ) in extremely low birth weight infants (Jacquot, Neveu et al. 2011). Our results did not show an increased trend with time - perhaps due to variations in the infants' chronological age at the time of sample collection. In agreement with other findings, there was no association in species richness and gestational age or birth weight (Schwiertz, Gruhl et al. 2003, Magne, Abely et al. 2006).

### *iii) Intra-individual and Inter-individual Similarities*

Most infants within our sub-group analysis showed a low similarity index between first and second sample, and illustrated a large degree of species turnover with low numbers of resistant and large numbers of extinct and immigrated species. These findings did not differ between those exclusively breast milk fed, and mixed-fed infants, indicating that the early microbiota community of these preterm neonates was unstable and underwent constant changes regardless of the type of milk fed. This is in agreement with early findings, albeit in term infants by Harmsen et al, as published in 2000 (Harmsen, Wildeboer-Veloo et al. 2000). The bacteria composition during the first days of life of term infants was found to be equally heterogeneous between breast and mixed-fed infants and a stable gut microbiota community, with a significant pattern between the feeding groups not developed before 12-20 days of life. Although it was reported that the profile of intestinal microbiota between breast-fed term infants has a low similarity index (mean 11.2 %) at day 6 of life (Schwiertz, Gruhl et al. 2003), it is seen to develop a homogeneous colonisation pattern that can be distinct to those of formula-fed infants, as reported by Harmsen et al in 2000. This increased stability of the microbiota community however was not detected in our study on this particularly interesting group of preterm neonates. The

mean similarity index at four weeks of age remained low and did not show a distinct pattern between the feeding groups.

This low similarity between infants differs greatly from what had been reported by Schwiertz et al, in 2003. For that study, serial stool samples were collected for PCR-DGGE analysis over the first four weeks of life from 29 preterm neonates. They found a significant increase in the subjects' inter-individual similarities over time ( $p < 0.05$ ), with the mean similarity index increasing between 18.1% at day three to 57.4 % at day 28. The values at week four are therefore far higher than those conducted in our study regardless of feed type (EBM: mean 19.2 % SD 17.9 %, versus MF: mean 21.0 % SD 18.5 %). Schwiertz et al also suggested that the increased similarity in the band profiles observed in their preterm neonates was as a result of hospitalisation and due to bacterial cross-transmission in the neonatal unit – particularly since the main species identified were *E. coli* and *Klebsiella*. In contrast to Schwiertz et al, our study was of multi-centre design encompassing three NICUs, two with very different antibiotic regimens. This might present a possible explanation for the much lower similarity index that have been found in the study population, as there are significant differences in management protocols of the three centres. Of the 14 subjects in the MF group, 11 were recruited from the same neonatal unit. However the mean similarity index of only those subjects was not significantly higher than of the total MF group (mean 24.5 % SD 19.0 %;  $p = 0.3083$ ) and did not indicate a large impact from care within different neonatal units.

Further differences in the evidence base were reported by Chang et al in 2011. Using 16s rRNA pyrosequencing of 10 preterm infant, 5 fed EEBM and 5 formula fed, they quoted that 21.9 % of species identified were 'unclassifiable', and that *Clostridium* and *Bacteroides* sp. were ubiquitous throughout the cohort by the age of 72 hours. Given their enteropathogenicity, these are concerning results (Chang, Shin et al. 2011). However, as methods develop, identification of the unclassifiable species may change these results once again. Indeed, work recently published by Smith et al in 2011 noted wide interindividual variability in those with NEC, and only the dominance of *Clostridia perfringens* in those with pneumatosis coli (Smith, Bode et al. 2011). This was supported by Barrett et al, in 2013 (Barrett, Guinane et al. 2013). The most recent publication, by Normann et al, published in 2013, used pyrosequencing to analyse bacterial composition in a cohort of ten extremely preterm infants with NEC, in comparison with matched controls. A low

diversity of microflora was seen throughout all patients, with no significant differences in those with NEC (Normann, Fahlen et al. 2013).

*iv) Differences in Feed Type*

In accordance with Mshvildadze et al's published work of 2010, our study does not support a different stool microbial development between opposing feeding regimens in preterm neonates. In our study, the number of species in sample 1: EEBM fed 7 (1-10) vs mixed 3 (1-11); in sample 2: EEBM 6.5 (1-13) vs mixed 5 (2-17). In their study, they analysed the microbiotal composition of 23 preterm neonates through combined DGGE and 16S ribosomal RNA pyrosequencing. Samples taken at 7 days postnatal age showed no difference in microbial diversity when using the Simpson diversity index as compared between breast milk and formula-fed infants (mean 9.09, SD 2.03 and mean 9.04, SD 2.13, respectively;  $p=0.96$ ) (Mshvildadze, Neu et al. 2010). In contrast to that, in 1999 Gewolb et al (Gewolb, Schwalbe et al. 1999) reported significantly lower numbers of gut microflora species in 14 formula-fed extremely low birth weight infants, compared with 15 babies fed exclusively human breast milk ( $p<0.05$ ). Cultures of stool bacteria on day 10, 20 and 30 of life showed that breast-fed infants had a significantly increased total number of bacteria species at day 30 compared to day 10 or day 20 (mean 4.2, SD 0.45, mean 2.5, SD 0.34, mean 3.13, SD 0.38, respectively;  $p<0.001$ ). Formula-fed infants, in comparison, had no increase in species richness. However the number of species detected by culture seemed to be more consistent than the findings of our study, perhaps due to uncultivable species. The increased sensitivity and qualitative nature of their techniques may be responsible for these opposing results.

*v) Band Number*

The number of bands detected on our TTGE gels ranged from 22 to 38, but the relative abundance of each species in the gel population was generally low, and most bands were only present in one or two of the samples. In comparison with other studies, Magne et al in 2006 distinguished 25 operational taxonomic units (OTUs) with a 16S rRNA technique but, in line with our results, the relative abundance in their study population was significantly low. Twenty-two OTUs were present in only one to three of the samples (out of a possible 16) which suggests that their preterm neonates harboured a distinct bacteria community early in life (Magne, Abely et al. 2006). Four bands (three from infants EBM fed, and one from infants MF) were identified to have a relative abundance of over 50 % in

our sample population. The identification of those species with cloning and sequence techniques should be explored in a future study, alongside the corresponding breast milk flora in the lactating mothers. It would be of interest to see if there is a difference in those species between the two feeding groups, and a correlation to breast milk commensals. A quantitative analysis of those bands with real time PCR could give further information of their distribution in the microbial community. Those bands with higher abundance in this study are located in the first half of the TTGE gel. *Bifidobacterium sp.* bacterial 16 rDNA genes tend to denature at higher temperature and are therefore expected to migrate further and are generally found at the bottom of the gel (Roudiere, Jacquot et al. 2009). It can be tentatively speculated that these bands might not be *Bifidobacterium sp.*, but it is inappropriate to draw firm conclusions without identification with gene sequencing method.

*vi) Correlation with metabolic analytes*

Although Mohan et al in 2008 showed a significant correlation between *Bifidobacteria* counts and both lactate and acetate concentrations, we could not replicate this same in our study.

*vii) Study Limitations*

This sub-group study has several limitations: chiefly that the study population was extremely diverse in important clinical and demographical factors including gestational age, mode of delivery and incidence and severity of NEC. It is possible that these factors influence the microbial development and have therefore influenced the outcome of this study. Mixed-fed infants received differing proportions of formula, pasteurised donor and/or maternal non-pasteurised expressed breast milk, hence present an extremely variable group, for whom two groups were assimilated (EBM versus Mixed) in order to make significant comparisons. It has been speculated that the pasteurisation process of donor milk alters its components, and thus it is accepted that it may change its effect on the gut colonisation pattern compared to non-pasteurised EBM (Andersson, Savman et al. 2007). However this has not yet been established in published work to date, and numbers were too small to merit analyses within this study. Notably, within the study group as a whole, none of the infants were exclusively fed DEBM. The infants' chronological age at sample collection was not available for some individuals, but was always within a 48 hour period. As this generally varied within individuals, it could explain why bacterial richness

did not increase within the two sample points in contrast to what had been reported in other studies (Gewolb, Schwalbe et al. 1999, Jacquot, Neveu et al. 2011). The methodology used in this study provides qualitative information about the microbial community in binary form: hence the presence and absence of a species is obtained. However, more specific quantitative analysis could add a wealth of additional information and further describe the ecological community and evaluate the distribution of bacterial counts of each species present in this ecological system. With this additional information it would be possible to calculate microbial biodiversity of the samples, as defined by Begon et al in 2006 (Begon M 2006). It is possible that some species are more prominent than others, and that this could lead to an underestimated similarity within the total bacteria community between the groups in this study. In our study almost half of the infants were diagnosed with NEC and whether their altered microbiotal community was part of their disease process or due to their treatments (antibiotics, nil by mouth, prolonged parenteral nutrition) was unable to be assessed in this study due to the wide range of treatments administered. Gewolb et al in 1999 found a significantly inverse correlation between the number of days with antibiotic treatment and the bacterial richness ( $R=0.491$ ,  $p<0.007$ ) (Gewolb, Schwalbe et al. 1999). It is as such very likely that this may also have affected the study outcome. Maternal antibiotic treatment was only present in MF infants and could also impair the resultant data.

Part of this study was to evaluate the impact of breast milk on intestinal microbiota, due to a possible link with the prevention of NEC. Results of this study do not support a link between feed type, microbial development and subsequent NEC. Review of the evidence base revealed only two articles using similar techniques to our study. De la Cochetiere et al in 2004 found high rates of *Clostridia* sp. with 95% similarity in those with NEC, using PCR after TTGE (de la Cochetiere, Piloquet et al. 2004). Mshvildadze's study using DGGE and pyrosequencing, published in 2011, has already extensively been discussed. In 1996, Miller et al published their comparison of species yield by TTGE and culture, finding no extra species by TTGE (Millar, Linton et al. 1996). It is therefore readily evident that type of method of bacterial identification and quantification is crucial, no matter the clinical or demographical features of the cohorts. As the incidence of SBS increases, studies considering the microbiota of those with ileostomy formation may develop. As of yet, none of the studies of gut microbiota in NEC have considered this important sub-population.

#### **4.2.5) Conclusions**

Concurrently, there are increasing numbers of randomised studies trialling arbitrarily chosen probiotic bacteria, given in varying doses by different regimen to prevent NEC and reduce NEC-related morbidity and mortality by altering the gut microbiota in this unique patient group. These probiotic products are chosen mainly on pharmaceutical availability, and not evidence base, given that little is known about the normal microbial ecology in preterm infants without NEC, and these trials (the majority of which are based on clinical outcomes) do not consider either quantitative or qualitative analysis of the developing gut microbiota and metabolites. Since more than 90 % of infants who develop NEC have been enterally fed, it could be speculated that the limited time frame taken in most cases to establish feeds might not be sufficient to form a protective microbiota community responsible for the prevention of NEC (Lin and Stoll 2006). It is probable that other beneficial factors within breast milk – in particular secretory IgA, lactoferrin, and alpha-lactalbumin - have a larger implication in the onset of NEC than colonisation of the gut. This thesis investigates molecular associations between the microbiota in infants fed EBM or mixed feeds, with and without NEC, which has contributed to the growing evidence base that will enable these trials to be targeted toward supplementation of the acquisition, composition and function of the microbiota in the early stages of life. In only the last two years, four studies have already been published reporting the use of high through-put pyrosequencing techniques in preterm infants, yielding many thousands of sequences. The increasing accessibility of these methods, as their popularity drops the cost, makes the evidence-based identification of probiotic bacteria appropriate for use in trials to prevent NEC a closer reality.

## Chapter 5

### Gut Inflammatory and Immunological Markers

#### 5.1) Calprotectin Analyses

##### *Introduction*

This section describes the FC levels in all 56 study group infants, as performed by ELISA (see methodology chapter). Levels are presented temporally, and then stratified according to gestation, feed type, and NEC, including: levels before and after NEC; by NEC stage; and before and after ileostomy formation. Finally, several regression analyses are presented to identify correlations between FC levels and relevant demographical and clinical data.

##### 5.1.1) Totals over study period

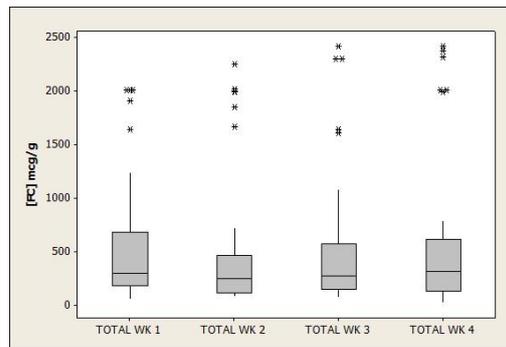
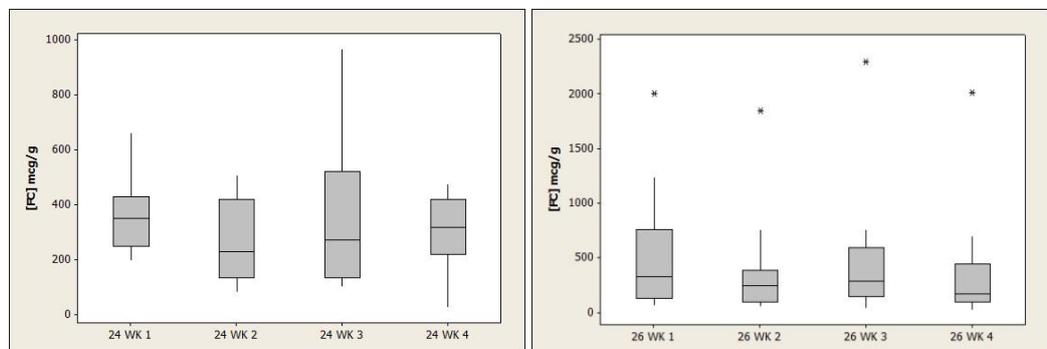


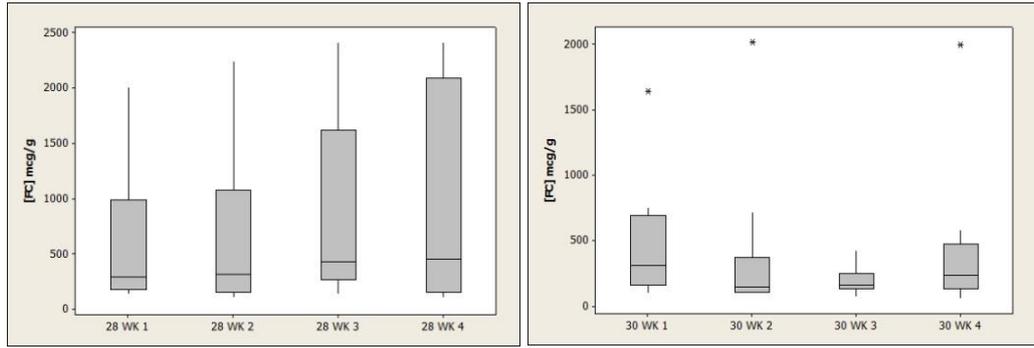
Figure 97: Total FC levels weeks 1 – 4; no significant differences were noted.

No significant differences were noted in total FC levels over the study period (Figure 97). Levels were further stratified according to gestation, feed type, and presence of NEC.

##### 5.1.2) Totals by Gestation



Figures 98: a) (left) FC levels weeks 1-4 in infants between 24-26 weeks gestation; no differences were noted. b) (right) FC levels weeks 1-4 in infants between 26-28 weeks gestation. Again, no differences were noted.

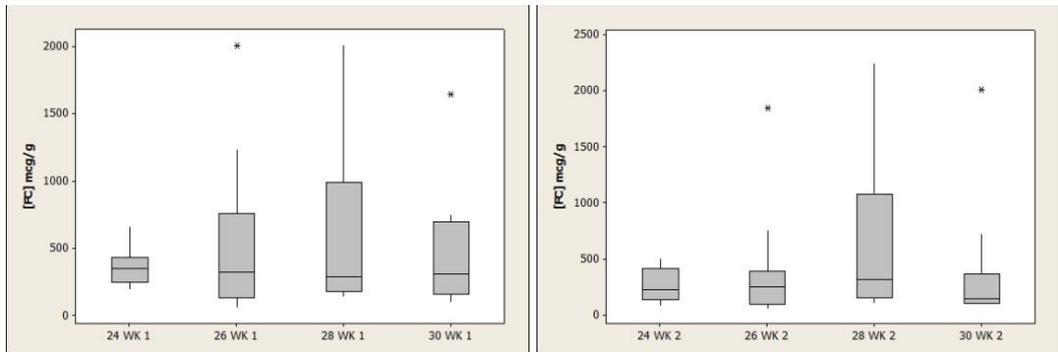


Figures 99: a) (left) FC levels weeks 1-4 in infants between 28-30 weeks gestation; no differences were noted. b) (right) FC levels weeks 1-4 in infants between 30-32 weeks gestation; no differences were noted.

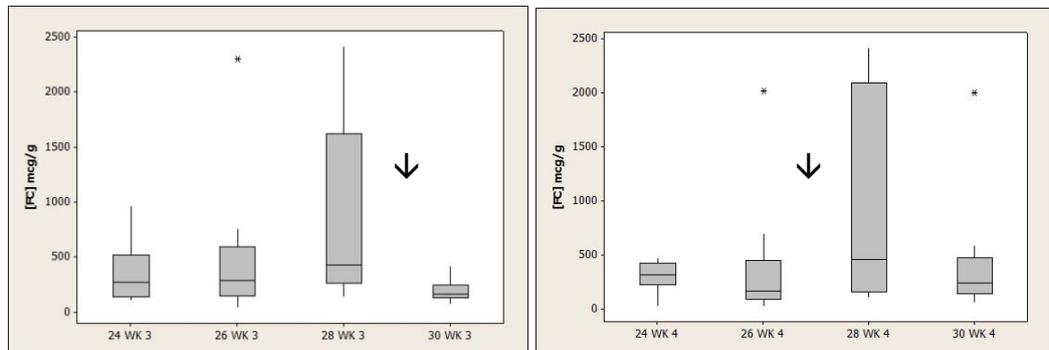
No significant differences were observed between weeks 1 – 4 at any gestation (Figures 98 and 99).

### 5.1.3) Week on week totals, by gestation

Comparisons were also made between each gestational group by week (Figures 100 and 101).



Figures 100: a) (left) FC levels by gestation, week 1; no significant differences were observed. b) (right) FC levels by gestation, week 2. Again, no significant differences were noted.



Figures 101: a) (left) FC levels by gestation, week 3; those at 28-30 weeks gestation had significantly higher levels than their 30-32 week counterparts ( $p=0.003$ ). b) (right) FC levels by gestation, week 4; levels were higher in the 28-30 week group than those at 26-28 weeks ( $p=0.04$ ).

Over each week's analyses, there were no significant differences between the gestational groupings during weeks 1 or 2 (Figure 100). Week 3 showed significant differences between those at 28-30 and 30-32 weeks gestation, with higher FC levels in 28-30 weeks (Figure 100 a):  $p = 0.003$ ). Similarly higher FC levels were seen in week 4 at infants of 28-30 weeks in comparison with those at 26-28 weeks gestation (Figure 101 b):  $p = 0.04$ ).

No correlations were seen between FC levels and birth weight.

5.1.4) Totals by feed type:

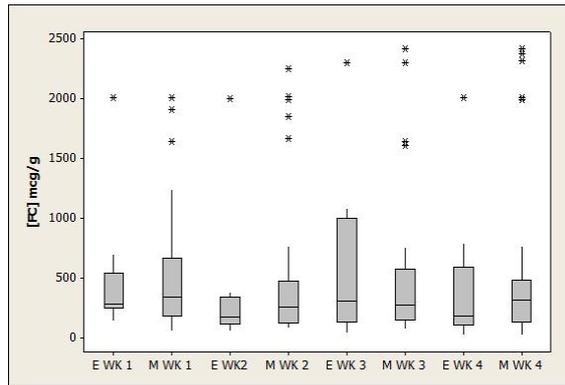
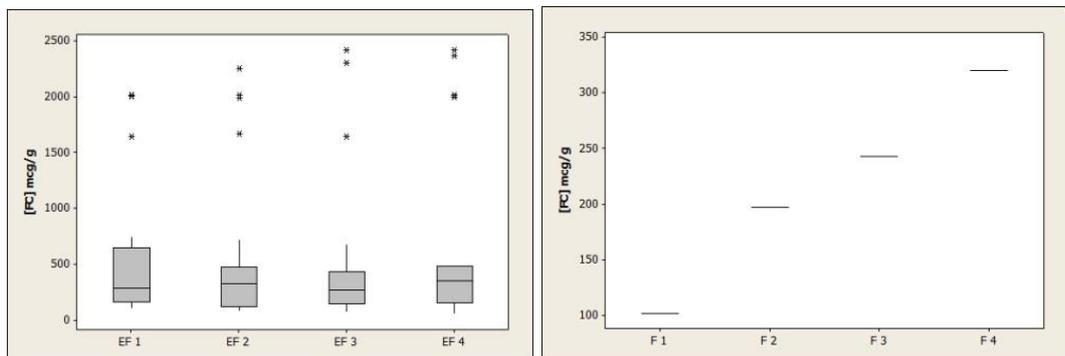
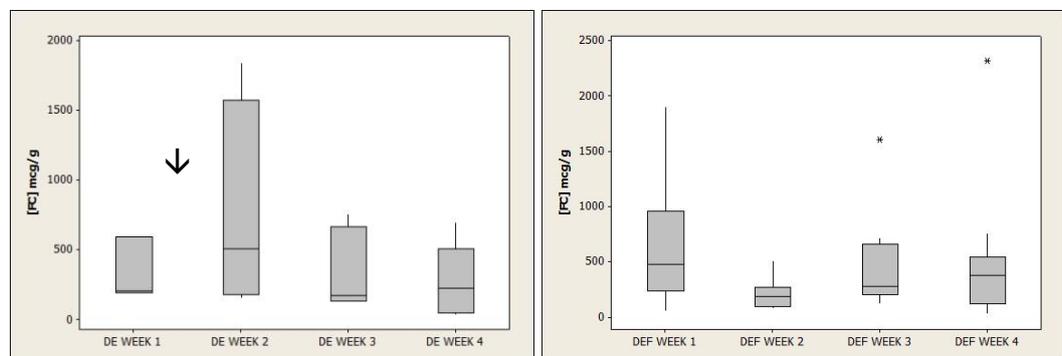


Figure 102: FC levels by feed type, weeks 1-4; no significant differences were seen at any time point.

There were no significant differences between those exclusively fed EEBM and those fed a mix of milks (Figure 102). When changes in each group were compared week by week, no differences were observed. Further analyses of mixed feeding subtypes showed the following trends, although numbers were too small in the formula and DEBM/formula groups to merit significance (Figures 103 and 104):



Figures 103: a) (left) FC levels in EF infants, weeks 1-4; no significant differences were seen. b) (right) levels in F fed infants, weeks 1-4; numbers were too small for analysis.



Figures 104: a) (left) FC levels in DE fed infants, weeks 1-4; levels rose significantly between weeks 1 and 2 ( $p=0.048$ ). ↓ denotes a significant p value. b) (right) FC levels in DEF fed infants, weeks 1-4; no differences were detected.

In the DE group week 1 FC levels were lower than week 2 (Figure 104 a):  $p = 0.048$ ). However, no significant differences were seen in other weeks. No other significant difference were seen week-on-week in the other mixed feed groups (Figure 105).

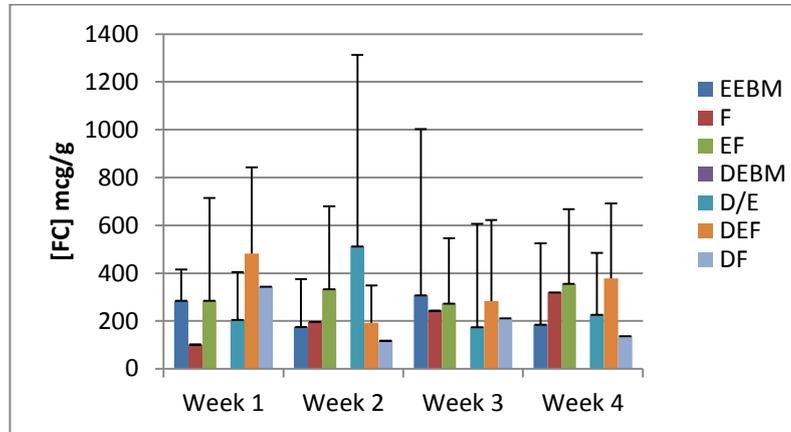
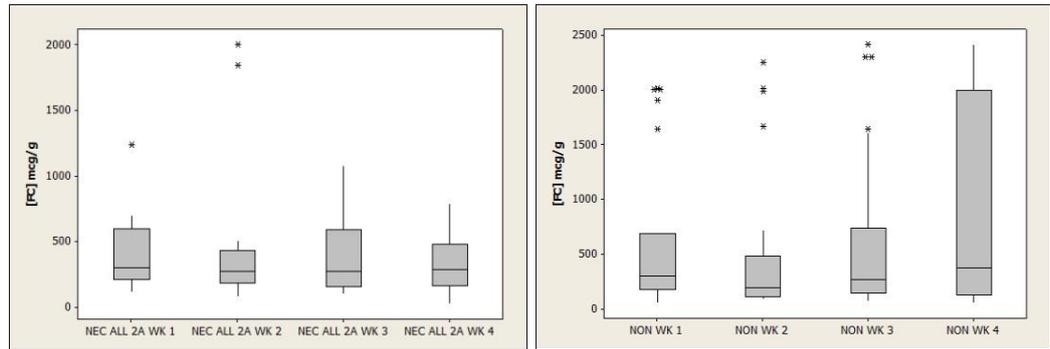


Figure 105: Median FC levels by feed type, weeks 1-4 (with IQRs). (Abbreviations: EEBM = exclusive expressed maternal breast milk; F = exclusive formula milk; EF = expressed maternal breast and formula milks; DEBM = exclusive donor expressed breast milk; D/E = donor and maternal expressed breast milks; DEF = donor, maternal breast and formula milks; DF = donor expressed breast and formula milks.)

5.1.5) Totals by  $\geq$  Stage 2a Necrotising Enterocolitis



Figures 106: a) (left) FC levels in infants with  $\geq$ stage 2a NEC, weeks 1-4; b) (right) FC levels in infants without NEC over weeks 1 – 4; in each case, no significant differences were noted.

No significant differences were noted using non-parametric analyses in both groups, or when comparing them week by week, although those with stage 2a NEC or more illustrated a trend to lower levels by week 4 (Figure 107:  $p = 0.096$ ).

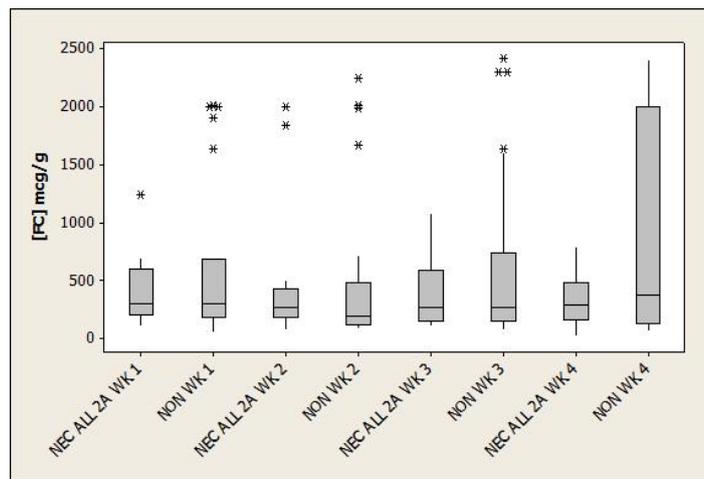
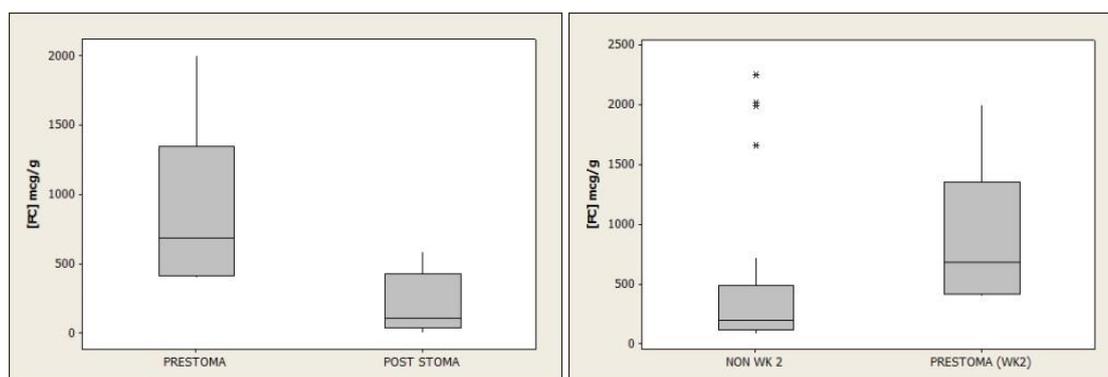


Figure 107: FC levels in infants'  $\geq$ stage 2a NEC versus those without NEC, weeks 1-4; no significant differences were observed.

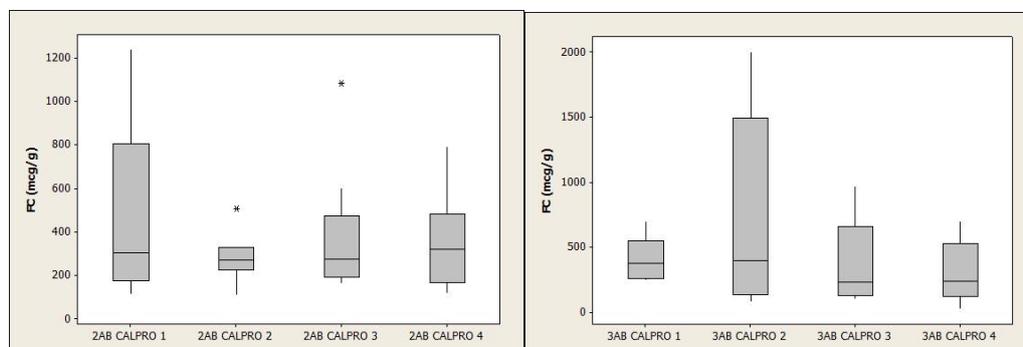


Figures 108: a) (left) FC levels in those with NEC fell significantly after stoma formation ( $p=0.0327$ ); b) (right) FC levels in infants without NEC ('NON'), week 2, and those before stoma formation ('PRESTOMA (WK2)'); levels were significantly lower in those without NEC ( $p=0.05$ ).

Many NEC infants developed concurrent ileus with lower stool production. There were no significant differences in calprotectin levels in infants with NEC before and after clinical signs were apparent ( $p = 0.1179$ ), or in those before NEC and after stoma formation for stage 3b NEC ( $p = 0.3026$ ). However, significantly lower calprotectin levels were noted for infants who had stage 3b NEC requiring ileostomy formation after surgery compared to the immediate sample before (Figure 112 a):  $p = 0.0327$ ). Also, those with stage 3b NEC prior to stoma formation illustrated significantly higher levels than those without NEC at week 2 (Figure 108 b):  $p = 0.05$ ).

#### i) FC levels by stages of NEC

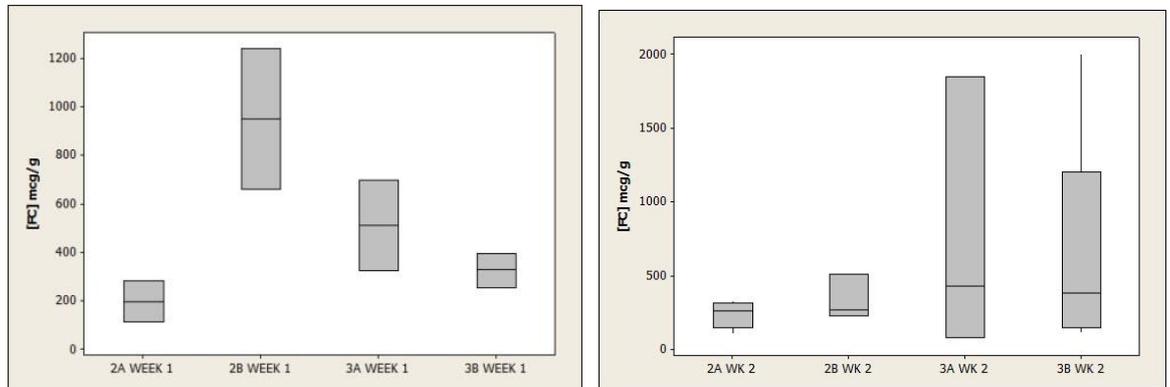
When considering infants at different stages of NEC, those with 2a were combined with 2b, as were 3a with 3b (Figures 109):



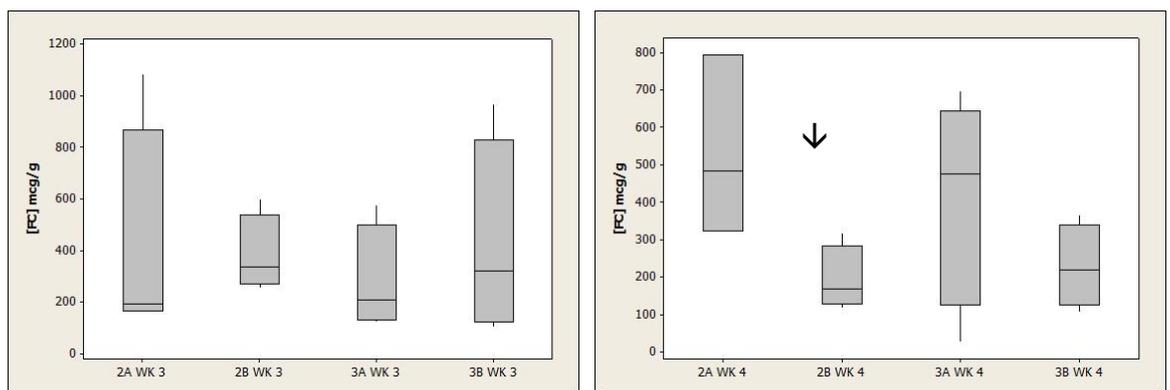
Figures 109: a) (left) FC levels weeks 1-4 in infants with stage 2a+b NEC. No significant differences were observed; b) (right) FC levels weeks 1-4 in infants with stage 3a+b NEC; in each case, no significant differences were observed.

No differences were noted comparing 2a/b with 3a/b, or comparing them to infants without NEC over weeks 1 to 4.

*Weekly comparison between FC levels at different stages of NEC*



Figures 110: a) (left) FC Levels during week 1 by NEC stage; b) (right) FC levels during week 2, by NEC stage in each case, no significant differences were noted.

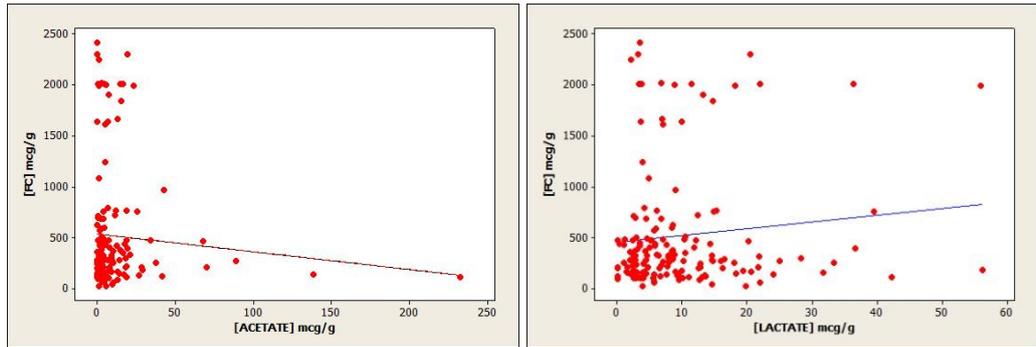


Figures 111: a) (left) FC Levels during week 3 by NEC stage; no significant differences were noted. b) (right) FC levels during week 4, by NEC stage; levels were significantly lower in 2B than 2A patients (p=0.05) ↓ denotes a significant p value.

The only significant difference in FC levels by NEC stage was noted during week 4, when 2a FC levels were higher than 2b patients (Figure 111 b): p = 0.05).

**5.1.6 Regression Analyses:**

Correlations between FC and other analytes and demographics were sought, namely: lactate; acetate; and weight increment. These were illustrated as follows in figures 112 and 113:



Figures 112: a) (left) Correlation between FC and acetate levels; no correlation was found. b) (right) Correlation between FC and lactate levels; again, no significant correlation was found.

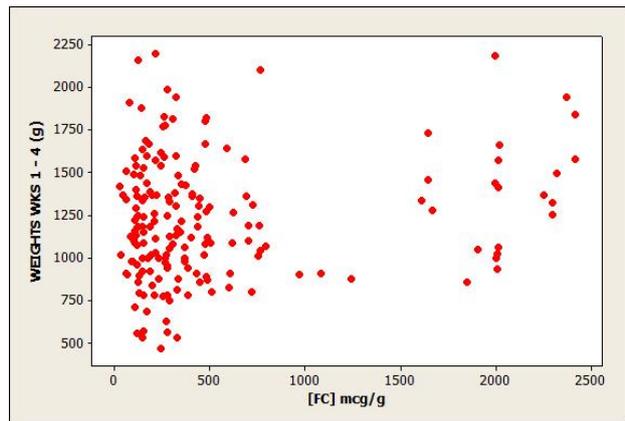


Figure 113: Correlation between calprotectin and infant weights at each time point; no correlation was observed.

No correlation was observed between FC and acetate levels (Figure 113 a):  $p = 0.374$ ), or lactate levels (Figure 113 b):  $p=0.173$ ) in the cohort as a whole.

### **5.1.7) Discussion**

#### *i) Introduction*

The recent articles as described in depth within the aforementioned systematic review of FC measurement in preterm infants with NEC showed some promise in this as a diagnostic and prognostic marker. However, there are two major confounders of this: 1) the lack of normative data, and potential for significant temporal changes within the first months of life; 2) the heterogeneity of NEC definitions. In this discussion, the important findings of our FC analyses will be considered, along with a comparison of these values in light of the evidence base.

#### *ii) Study levels and significant findings*

Despite the dominance and severity of NEC, calprotectin levels remained remarkably consistent, with total weekly medians measuring around 250 µg/g. This level is at least five-fold higher than the internationally agreed maximum upper limit of normal for adults (50 µg/g). Wide interquartile ranges were noted throughout, as found in the SCFA data. We noted a small gestational ‘spike’ for the infants at 28 - 30 weeks gestation during weeks three and four, but no obvious clinical reason for this could be identified. No correlation with birth weight was noted. A minor yet statistically significant increase in FC was seen between weeks one and two in the infants who were fed a mix of EEBM and DEBM. This may be secondary to the occurrence of NEC at this time period in this cohort, but unfortunately numbers were small to merit analysis. Just as for the SCFA analyses, since this study was not powered to NEC, a pre-NEC ‘spike’ could easily have been missed in performing weekly analyses. We identified no other specific gestation, birth weight or feed dependent trends, but this may have been confounded by NEC, in which the only significant FC differences could be detected. These were subtle. By week 4, the calprotectin levels were higher in those with 2a NEC than 2b, which could be accounted for by the later onset of NEC in more severe cases, since it was clear in each group that more severe NEC occurred at a later point in the study period. A ‘spike’ was observed in infants with severe NEC before stoma formation, also confirming that calprotectin is not simply a large bowel protein, and raises the possibility that calprotectin may be detectable in other gastrointestinal fluids – for example gastric acid or saliva, as already found in studies of children with dental caries (Striz and Trebichavsky 2004, Toomarian, Sattari et al. 2011). We could not detect calprotectin in preterm breast milk within this study, but other studies have measured calprotectin in milk of mothers of infants at term only, indicating that this may be gestation-dependent (Olafsdottir, Aksnes et al. 2002).

### *iii) Comparison with the evidence base*

There is a lack of plentiful normative data of FC levels in healthy children, and as such the ‘adult’ maximum limit of 50 µg/g is still recognised as a cut-off for further investigation for Inflammatory Bowel Disease. However, recently this has been reconsidered in the literature, with some units now considering up to 200 µg/g as normal (Henderson, Casey et al. 2012). The studies showed consistency in method (ELISA), and unit employed (µg/g). In the published evidence to date, (see pages 66, 67, 86 and 87) seven articles have considered FC in health and 11 in NEC. Only one showed differences with gestation or age (Zoppelli, Guttel et al. 2012), although one found a lower FC level in those less than 1800 g at birth (Campeotto, Baldassarre et al. 2009). One article showed no differences with duration of phototherapy (Bukulmez, Dogru et al. 2012). Two articles found lower FC levels in infants enterally administered probiotics (Mohan, Koebnick et al. 2006, Campeotto, Suau et al. 2011), but one found no significant difference (Rouge, Piloquet et al. 2009). Only Mohan showed significant correlations between FC, total SCFA and SIgA levels – but none of these infants incurred NEC. Our FC figures could not replicate these correlations.

When considering the articles concerned FC as a marker of NEC, we previously noted that our systematic review identified multiple definitions of NEC, so making comparison of data inappropriate. The range of medians or means within these articles was highly variable. Those with NEC quoted ‘cut-off’ values of: 288.4 mcg/g, > 350 mcg/g, 380.4 mcg/g in ‘sick’ infants, 363 mcg/g, 286.2 mcg/g, 792 mcg/g, 210 mcg/g, and 286.3 mcg/g. One article quoted a significantly lower FC level for infants with fulminant, perforated NEC (< 24 mcg/g). The highest quoted mean was 9386 mcg/g in meconium. The medians or means for infants without NEC was also diverse: 98 mcg/g, 160 mcg/g, 122.8 mcg/g, 365 mcg/g, and 172.2 mcg/g. The studies powered to NEC showed similar stool collection periods, aiming to collect most or all stool samples within the first 28 days of life, which we did not do. Thuijls et al incurred the same issue as we did with low stool production in infants with NEC: only 21 out of 35 infants produced pre-NEC samples (Thuijls, Derikx et al. 2010).

### **5.1.8) Conclusions**

Our study did not support the use of FC as a diagnostic or prognostic marker of NEC in our population, but this may have been confounded by the weekly sample frequency. It's possible that if most or all of the stool samples were collected, 'spikes' in FC before NEC could have been identified, and so a future study appropriately designed would be well placed to investigate this. The fall observed in FC after ileostomy could be considered to confirm the necessity of resection. This fall was also significantly lower than that of other infants with medically treated NEC at the same median time point.

Given the popularity and increasing use of FC in the diagnosis of IBD, the commercial production of ELISA kits is increasing, with more sensitive assays and the ability to perform these on smaller samples. Newer rapid acting kits are progressively coming into vogue, and further production will hopefully reduce costs and make these more accessible to the NHS – possibly replacing the FOB test within Bell's Criteria.

## **5.2) Secretary IgA**

### *5.2.1) Introduction*

Given its status as the last of the four analytes to be taken from each sample within our standard operating procedure, not all samples were large enough to merit SIgA analysis. As such, samples were selected in order to produce a case-control analysis. ELISAs were performed by Miss Ma WenWen, MSc student, Dr Emilie Combet, Lecturer in Human Nutrition, and I. Statistical analyses were performed using SPSS. All non-normally distributed data was log transformed prior to analysis.

### *5.2.2) Clinical and Demographical Features*

34 preterm neonates were involved in this study, including 20 with NEC and 14 without. The numbers of those with NEC were too small to analyse according to their stage of NEC, and, as such are considered collectively. Table 24 shows the clinical and demographical characteristics of the infants studied. Controls were matched to cases in so far as could be ascertained – mainly by gestation and birth weight. Unsurprisingly, low gestation and extreme low birth weight were closely correlated, and associated strongly with NEC. When stratifying by method of delivery, as per the rest of the study cohort, most infants were delivered by caesarean section (SVD 26.5 % versus LUSCS 73.5 %) There was no significant relationship between method of delivery and incidence of NEC ( $p = 0.307$ ). As with the cohort as a whole, exclusive breast feeding was the minority, with only six infants exclusively breast fed, of whom four incurred NEC. However, when compared in regard to presence or absence of NEC, feed type did vary from the cohort as a whole, with no significant difference in the type of feeding in infants with or without NEC for the purposes of this sub-group study of SIgA. As a self-fulfilling prophesy, the smallest and most preterm infants were, appropriately, more likely to be exclusively breast milk fed, yet also carry the highest risk of NEC – rather than a new association between breast milk and NEC.

Table 24: Clinical and Demographical Features; those with NEC were significantly lighter and more premature than those without (p=0.001 and p=0.012 respectively)

Demographic	Total (N=34)	NEC (N=20)	NON – NEC (N=14)	Pearson Chi-square
Gender				
Male	14	9	5	0.588
Female	20	11	9	
Birth Weight (Mean±SD)	1.046±0.258	0.941±0.207	1.195±0.258	
< 1kg				0.001
1-1.5kg	14	13 (92.9%)	1 (7.1%)	
	20	7 (35.0%)	13 (65.0%)	
Gestation (days) (Mean±SD)	197.65±16.98	191.50±16.11	206.43±14.53	
< 196 (28wks)				0.012
196-224 (28-32wks)	16	13 (81.3%)	3 (18.8%)	
	18	7 (38.9%)	11(61.1%)	
MOD				
Vaginal (%MOD)	9 (26.5%)	4	5	0.307
Caesarean section (%MOD)	25(73.5%)	16	9	
Mode of Feeding				
EBF	6	4	2	0.667
MF	28	16	12	

Abbreviations: NEC = necrotising enterocolitis; NON = no necrotising enterocolitis; MOD = method of delivery; EBF = exclusively maternal breast milk fed; MF = mixed breast and formula fed; SD = standard deviation.

By ranking the birth weight as ‘less than 1kg’ and ‘between 1kg to 1.5kg’, 92.9 % (13) of the infants whose birth weight were less than 1kg suffered from NEC. On the other hand, 65.0 % (13) of the infants whose birth weight were between 1 - 1.5kg did not have NEC. With an increase of birth weight, the preterm infants had a significantly lower risk of NEC (P=0.001). A similar phenomenon also happened when considering ranked gestational age groups, with a higher gestational age leading to a lower incidence of NEC (p=0.012). As expected, there was statistically significant positive correlation (p = 0.001) between gestational age, birth weight and NEC (Figure 114).

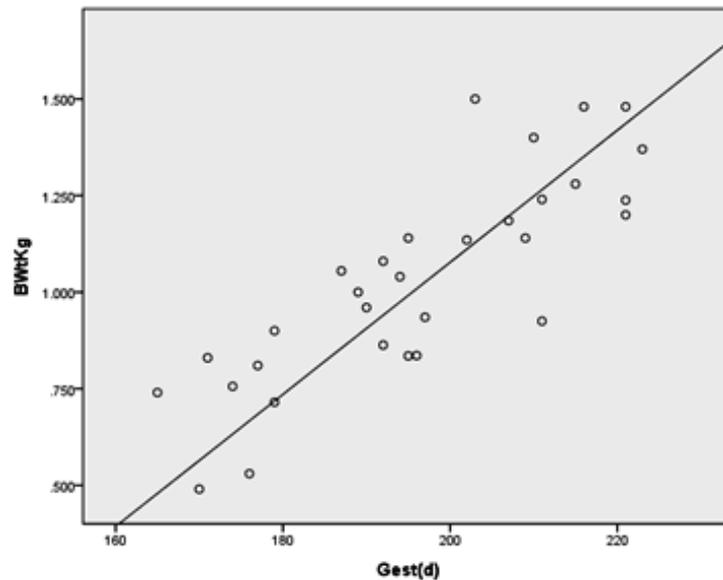


Figure 114: The relationship between gestation (in days) and birth weight (in kg) in the cohort of infants from the SIgA study

### 5.2.3) Results

#### *i) Stool SIgA titres*

Among all preterm infants (both with and without NEC), stool SIgA concentration were significantly higher in week 3 ( $p = 0.020$ ) and week 4 ( $p = 0.027$ ) than week 1. No significant correlations were found between stool SIgA level and gestational age, or birth weight during all four weeks of sampling ( $p > 0.05$ ). In addition, no significant difference was found between stool SIgA level and gender.

Figure 115 shows the mean stool concentration of SIgA in those infants with NEC ( $n = 20$ ) and without NEC ( $n = 14$ ). In comparison with the difference between the means, data were log transferred to normally distributed data sets. As shown in Figure 120, it seems that infants who had experienced NEC have a lower SIgA concentration in week 2, but higher in week 1 and week 3 than those infants who did not incur NEC. However, in comparison of all four weeks, there were no significant differences in stool SIgA concentration between infants who developed NEC and those who did not (Table 25:  $p > 0.05$ ). In week four in particular, despite all infants having complete data sets, no difference was shown between them with regard to NEC ( $p = 0.902$ ).

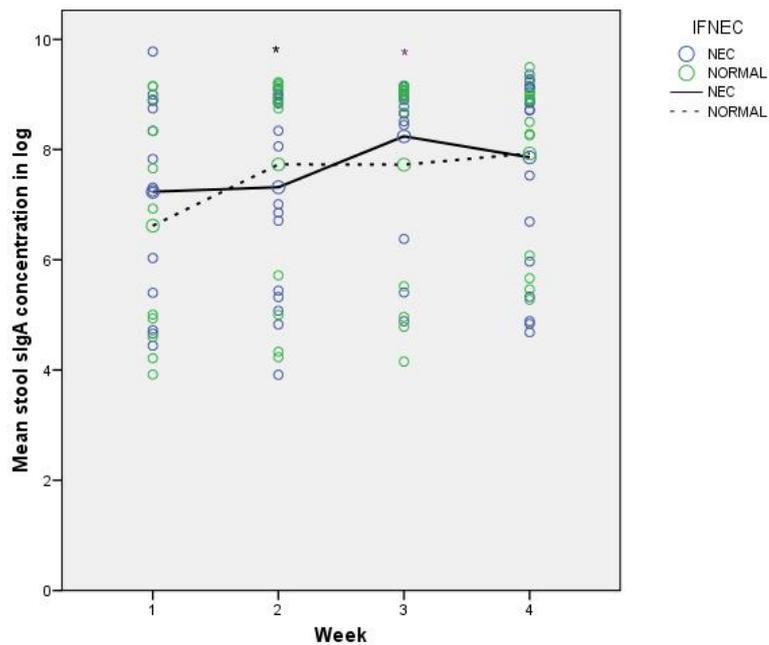


Figure 115: Repeated stool SIgA means (in log) in infants with and without NEC neonates over a period of four weeks after birth. (Data were transferred to normally distributed figures in log for analysis. Week 1: NEC: n=14, Normal: n=11; week 2: NEC: n=17, Normal: n=13; week 3: NEC: n=16, Normal: n=13; week 4: NEC: n=20, Normal: n=14; NEC is defined as Bell criteria  $\geq 2a$ ; \* significant at  $p < 0.05$ )

When comparing each time point, there was a significant rise in the mean stool SIgA concentration from week 2 to week 3 ( $p = 0.048$ ) in NEC infants, and from week 1 to week 2 ( $p = 0.005$ ) for infants without NEC. After this change, no significant differences were detected (Figure 115).

Sample	NEC status	N	Mean	Median	Standard Deviation	P value
Week 1	NEC	14	7.23	7.57	1.85	0.45
	NON	11	6.62	6.93	2.12	
Week 2	NEC	17	7.32	8.06	1.80	0.56
	NON	13	7.73	8.90	2.05	
Week 3	NEC	16	8.24	8.84	1.38	0.44
	NON	13	7.73	8.99	2.02	
Week 4	NEC	20	7.86	8.85	1.74	0.90
	NON	14	7.93	8.68	1.56	

Table 25: T-test for equality of means of four weeks stool SIgA concentration (in log) between infants with and without NEC.

ii) Mode of feeding and stool SIgA

Since the concentration of stool SIgA in week 4 (n=34) of this subgroup study cohort was normally distributed, a two-factor ANOVA was performed to analyse significant factors therein (Figure 119). There was no significant main effect of the presence or absence of NEC ( $p > 0.05$ ) or whether the infants were EBM or mixed fed ( $p > 0.05$ ), as well as no significant interaction between the two factors ( $p > 0.05$ ). It was also evident that only 4% of the variability between the scores can be explained by the independent variables (R Squared = 0.040).

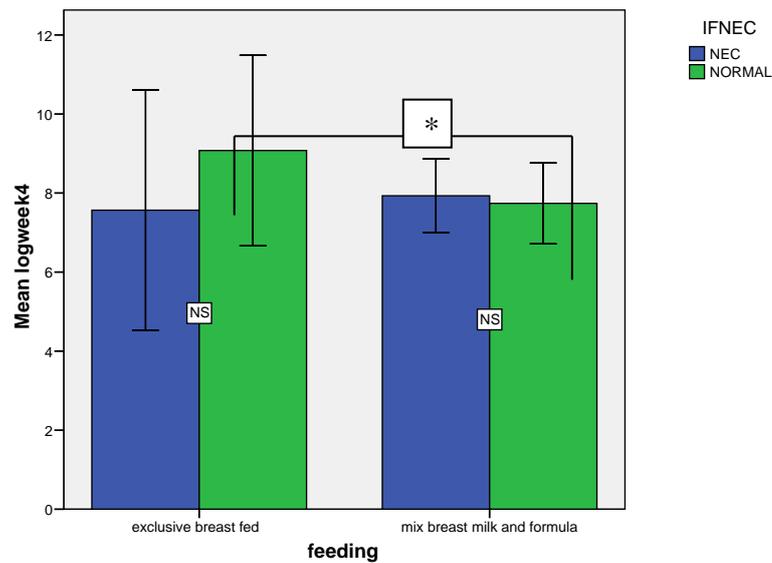


Figure 116: The effects of feeding methods and NEC status on the stool concentration of SIgA in week 4. (Week 4: NEC=20, NON=14; NEC defined by Bell's criteria  $>2a$ ; stool SIgA concentration was transformed to log values).

Table 26 compared stool SIgA concentration (log transformed) in exclusively breast fed and mixed breast milk and formula fed preterm neonates. Significant differences between the feeding methods were detected in week 2 ( $p = 0.036$ ) and week 3 ( $p = 0.006$ ). According to the mean values from the cohort as a whole, infants exclusively breastfed tended to have a higher stool SIgA level in week 2 and week 3.

Sample	Feeding	N	Mean	Standard Deviation	P value
Week 1	EBM	3	6.83	2.12	0.902
	MIX	22	6.98	1.98	
Week 2	EBM	6	8.48	0.90	0.036*
	MIX	24	7.25	2.00	
Week 3	EBM	5	8.96	0.26	0.006*
	MIX	24	7.81	1.79	
Week 4	EBM	6	8.07	1.68	0.778
	MIX	28	7.85	1.67	

Table 26: Stool SIgA concentration (log transformed) in exclusively breast fed and mix breast milk and formula fed preterm neonates (total n=34). (Abbreviations: EBM = exclusive expressed maternal breast milk fed; MIX = mix maternal breast and formula milks).

When testing differences between stool SIgA levels in the infants without NEC, those exclusively breastfed illustrated a significantly higher concentration in week 2 ( $p = 0.046$ ), week 3 ( $p = 0.030$ ) and week 4 ( $p = 0.021$ ) compared with those fed a mix of breast milk and formula (table 27). Conversely, these differences were obsolete in the infants with NEC during the same time periods when considering their feed types ( $p > 0.05$ ).

	Feeding	N	Mean	Standard Deviation	P value
Week 1	EBM	1	6.93	No value produced	No value produced
	MIX	10	6.59	2.23	
Week 2	EBM	2	9.02	0.27	0.046*
	MIX	11	7.50	2.16	
Week 3	EBM	2	9.08	0.51	0.030*
	MIX	11	7.48	2.11	
Week 4	EBM	2	9.08	0.27	0.021*
	MIX	12	7.74	1.61	

Table 27: Differences of stool SIgA concentration (log transformed) in healthy infants without NEC, and their related feeding methods. (Total n=14). (Abbreviations: EBM = exclusive expressed maternal breast milk fed; MIX = mix maternal breast and formula milks).

*iii) Breast Milk SIgA and Correlation with Neonatal Stool Titres*

For all breastfed preterm neonates (n=6) in the first four weeks of life, the level of milk SIgA was significant higher on week 1 (colostrum) than week 2 (p = 0.021) and week 3 (p = 0.034) (Figures 117). However, there were no significant differences of stool SIgA between the weeks (p > 0.05). Conversely, the SIgA level found in colostrum was significantly higher than the concentration measured in infants' stool in week 1 (p = 0.035, table 28).

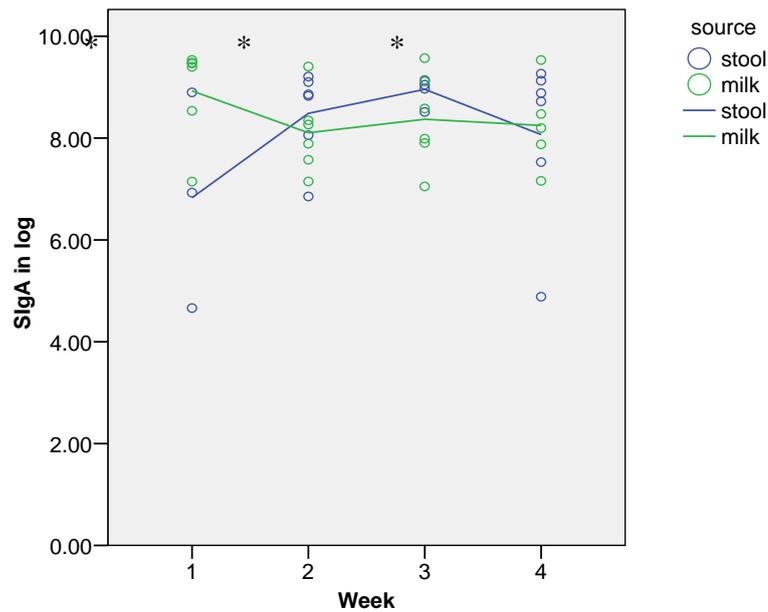


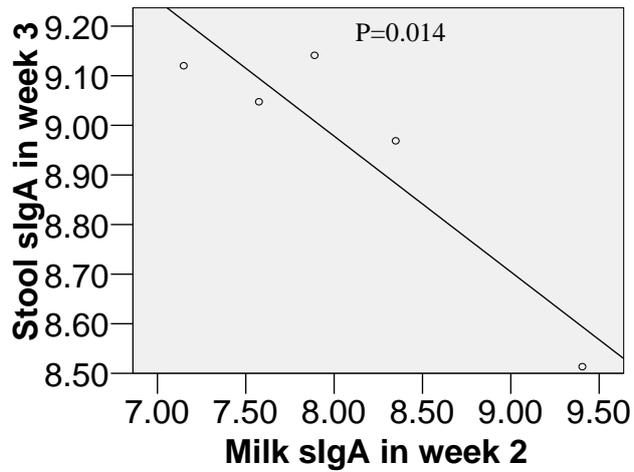
Figure 117: Comparison of the mean SIgA levels (log transformed) between stool and milk for all breast fed preterm infants (n=6) during first four weeks after birth. (Week 1: stool: n=3, milk: n=6; Week 2: stool: n=6, milk: n=6; Week 3: stool: n=5, milk: n=6; Week 4: stool: n=6, milk: n=5) No significant differences can be drawn for the stool SIgA level among the four weeks, but milk SIgA was significantly higher at week 1 than weeks 2 or 3 (p=0.021 and p=0.034 respectively). In addition, milk SIgA in week 1 was significantly higher than that found in infant stool samples at the same time point (p=0.035);\*: significant p values.

	Sources	N	Mean	Standard Deviation	P value
week1	Stool	3	6.82	2.11	
	Milk	6	8.92	0.95	0.035
week2	Stool	6	8.48	0.89	
	Milk	6	8.10	0.77	0.226
week3	Stool	5	8.95	0.25	
	Milk	6	8.37	0.91	0.092
week4	Stool	6	8.06	1.67	
	Milk	5	8.24	0.86	0.418

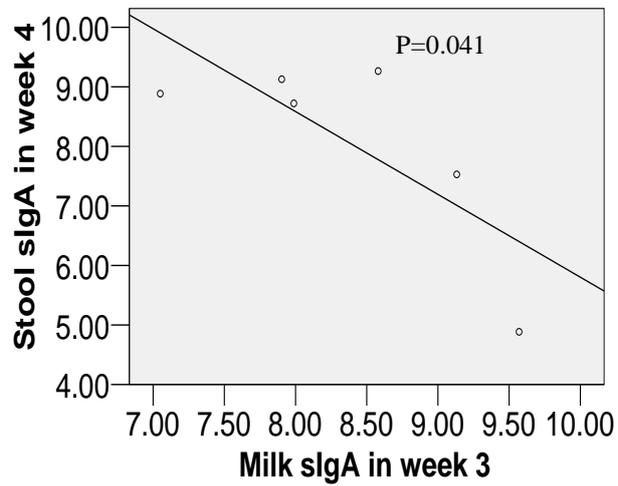
Table 28: SIgA titres (log transformed) measured by quantitative ELISA in stool and milk (week 1 = colostrum) samples from six exclusively breastfed preterm neonates.

During each week, no significant differences were observed in milk SIgA levels between infants with and without NEC (p > 0.05). However, when comparing stool and milk SIgA levels some correlations were noted. Although no correlation was found between samples in parallel weeks (i.e. stool week 1 versus milk week 1), on closer observation, there was a trend between earlier milk SIgA and later stool samples, with correlations noted between milk SIgA in week 2 and stool SIgA in week 3 (p = 0.014), milk SIgA in week 3 and stool

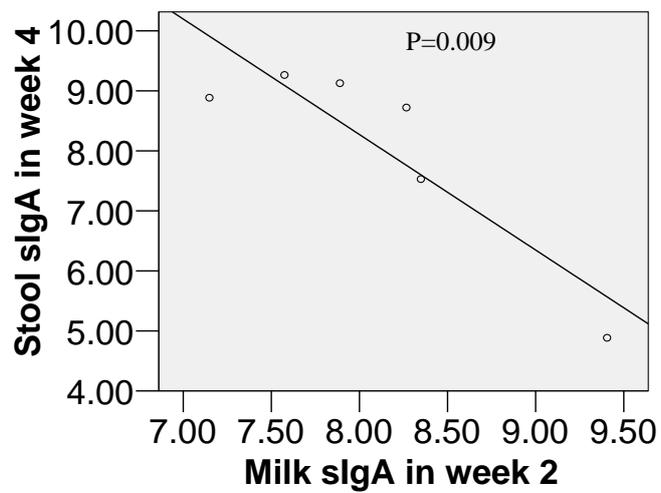
SIgA in week 4 ( $p = 0.041$ ), as well as milk SIgA in week 2 and stool SIgA in week 4 ( $p = 0.009$ , Figures 122 a, b, and c), which replicates expected observations physiologically.



a)



b)

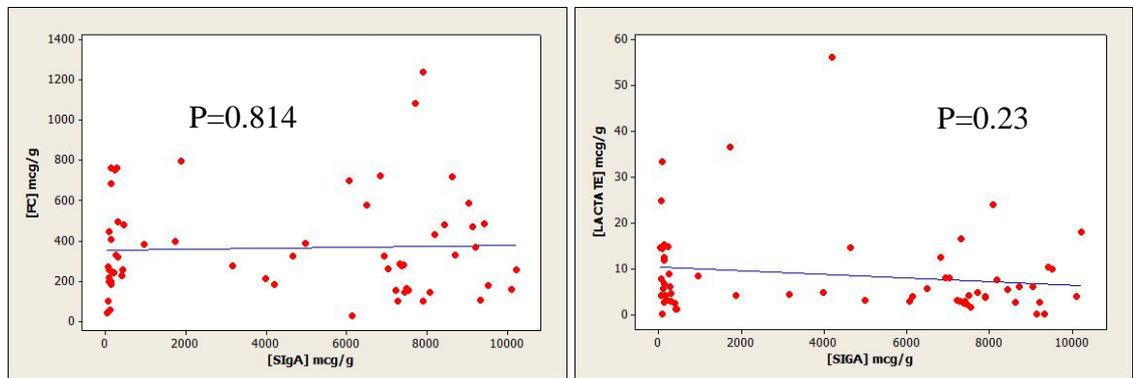


c)

Figure 118: (A, B, C) The correlation relationship between stool and milk SIgA level at individual time points in six preterm infants fed with breast milk exclusively. Positive correlations were seen in each case: milk SIgA in week 2 with stool SIgA in week 3 ( $p=0.014$ ); milk SIgA in week 3 with stool SIgA in week 4 ( $p=0.041$ ); and milk SIgA in week 2 with stool SIgA in week 4 ( $p=0.009$ ).

#### 5.2.4) Correlations between SIgA and other analytes:

As seen below, no correlations were seen between SIgA and any of the analytes, despite extensive comparisons. This was performed using multiple univariate analyses, since multivariate test were deemed inappropriate given the wide variation in histograms from Anderson-Darling normality tests for the vast majority of the data. Examples included: FC vs SIgA (Figure 119 a):  $p = 0.814$ ); lactate versus SIgA (Figure 119 b):  $p = 0.237$ ); and acetate vs SIgA (Figure 120:  $p = 0.124$ ). No correlations were noted between number of TTGE bands and calprotectin or SIgA.



Figures 119: a) FC versus SIgA; b) Lactate versus SIgA; no significant correlations were identified

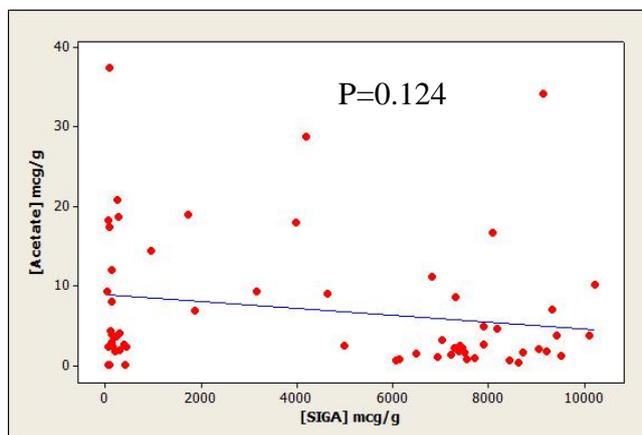


Figure 120: Acetate versus SIgA; no significant correlations were identified

### **5.2.5) Discussion**

#### *i) Introduction*

Despite being the most prolific immunoglobulin within the human body, the gut's first defence against pathogens, and a rich component of breast milk, little is published about the measurement of SIgA in preterm infants. This subgroup study has illustrated the temporal changes in stool and breast milk SIgA within the first month of life. This discussion will consider both the values and significant trends revealed in this analysis, and its relationship to the current evidence base, as already displayed on pages 79 - 81, 95 – 96.

#### *ii) Stool SIgA and feeding mode*

In this subgroup analysis, nearly half of those studied were of less than 28 weeks gestational age, with 81.3% of these infants developing NEC. In this study, we did not find any significant differences between stool SIgA level in infants with and without NEC, nor correlations with gestation or birth weight. However, this may be because this study was not powered to NEC, and that as such NEC-specific alterations in titres were missed, which also acted as a significant confounder for the comparison of the stool SIgA level before, during and after NEC in those infants affected, as well as stage-specific changes.

To explore the reasons for this similarity between those with and without NEC, these groups were stratified according to feed type. We conducted a two-way analysis of variance for week 4 according to the feeding methods between those with and without NEC, but no significant difference was noted. However, when we consider the missing data in week 1, week 2 and week 3, there appears a significant ( $p < 0.05$ ) result from Leven's test, which illustrates that the variances in the three weeks are significantly different. This makes the two-way ANOVA unsuitable for the first three weeks of our data. Moreover, the missing data resulted in a small sample size, which was not appropriate to conduct a multivariate model utilising feeding methods, NEC status, gestational age, and birth weight. Similarly, the numbers of those with NEC were too small to merit sub-group analysis. As a consequence, we tested the difference between feeding methods individually with independent sample T test. The significant results indicated that exclusive breast milk feeding could be more conducive to higher stool SIgA levels in healthy preterm infants without NEC. However, since there were only six infants exclusively breast milk fed in this sub-group analysis, larger numbers were required to further assess this.

### *iii) SIgA in those without NEC*

Preterm infants not exclusively fed with breast milk also appeared to have a high level of stool SIgA, although significantly lower than that of breast milk fed infants in week 2 (0.036) and week 3 (0.006). As such, it would be important for further analysis of the *Nutriprem 1* formula (*Cow and Gate*) that they were fed, which may be due to its blend of prebiotic oligosaccharides, which have been suggested the administration to preterm infants may result in a higher level of faecal SIgA (Bakker-Zierikzee, Tol et al. 2006). Moreover, temporal observations in our study showed that preterm infants diagnosed with NEC during the first month after birth may reach their peak stool SIgA concentration later than infants without NEC (week 3 versus week 2 respectively) during the first four weeks after birth. This may indicate the synergy of the disease process consuming SIgA – or that more SIgA is retained on the mucosa itself, and less excreted in stool. Either way, conversion of all infants to full exclusive breast feeding would appear to have a favourable influence on both processes.

### *iv) Milk SIgA*

For term neonates, the breast milk SIgA of mothers is seen to peak during the first day after birth, and contains around 2–5 mg SIgA/ml in colostrum. Observational studies show that the concentration later decreases gradually at around 0.5–1 mg/ml (Goldman, Garza et al. 1982). For our analysis of nine mother's milk samples, since the data set was not normally distributed, the median SIgA of colostrum (n=9) was 12.1 mg/ml and 3.6 mg/ml in week 4 (n=5) in our study. The difference between term and preterm neonates predicts a gestation-dependent immunological adaptation for preterm infants, whom are known to be immunodeficient, lacking in the placental transfer of IgG and IgM during the third trimester. This supports the results from Araujo et al in 2005 (Araujo, Goncalves et al. 2005). However, given there were milk samples from only nine mothers of preterm infants, more participants would be required to assess this fully. Nonetheless, this is the first study to correlate preterm stool IgA and maternal milk IgA with regard to prematurity.

As shown before, the significant differences of milk SIgA level between each week in exclusively breastfed infants could reflect the decrease in concentration of milk SIgA from colostrum to week 2 and week 3 in preterm infants, in turn exacerbating their immunodeficiency and making them more susceptible to both infection and NEC. However, as described earlier, within each NICU there was no method of using the breast

milk according to chronology of expression, and as such, although likely that ‘freshest’ milk was used preferentially, thus mirroring the time periods of expression with stool production, it is possible that on some occasions infants were given older colostrum – particularly if breast milk supplies were running low.

When comparing the stool and milk SIgA level in the six exclusively breast fed preterm infants, the significant higher SIgA level colostrum than that found in the corresponding infants stools in week 1 suggests that milk SIgA at this time does not contribute to the stool SIgA at the same week. In addition with the negative correlation observed, it should be considered that the breast milk SIgA may have no positive effect on the stool SIgA level. However, the concentration of milk SIgA could be affected by maternal health status and habits - even stress and mood have the potential to influence milk SIgA level (Groer, Davis et al. 2004). Furthermore, Brandtzaeg et al in 2003 stated that SIgA in breast milk directly corresponded with mother’s previous and current immunity and infection status (Brandtzaeg 2003). Given the high rates of mastitis among all breast feeding women within the first month of life, it is possible that this could be a confounder. Mastitis is known to be higher in women manually expressing breast milk, which would account for all of the women in our study, given that their infants were too premature to latch on within the first month of life (Amir, Forster et al. 2007). In addition, as discussed in the introduction and methods chapters earlier, the storage of the samples (which were mainly at -20C within the NICUs, but -80C within the university laboratories) may further reduce the SIgA concentration measured.

#### *v) Comparison with evidence base*

Despite systematically reviewing the literature at both the beginning and end of this research period (2009-2013), scant evidence could be identified investigating SIgA in preterm infants within the literature base. Only two articles could be identified, as previously discussed. Both concerned healthy preterm infants. As part of their multi-analyte RCT study, Mohan et al in 2008 measured SIgA in 69 infants with and without *Bifidobacterium BB12* administration, finding higher levels in week 2 than week 1 in infants who weren’t administered antibiotics. As with our study, values were reported in mg/kg, but raw data were not reported and instead were illustrated in graph form. As such, estimated mean levels were around 5 mg/kg for those randomised to the probiotic group, and around 3 mg/kg in those given the placebo. In total, the SIgA titres were around 44%

higher in those given probiotics than placebo. Most of these infants were exclusively breast milk fed and there was no significant difference in proportions of breast and formula feeding between the two groups (Mohan, Koebnick et al. 2008).

Secondly, Campeotto *et al* in 2011 published their randomised control trial comparing SIgA, calprotectin and colonisation changes in preterm infants fed a fermented formula (containing *Bifidobacterium breve* C50 and *Streptococcus*) versus an unnamed preterm formula (manufactured by the company 'Bledina'). The groups did not differ in clinical or demographical aspects, and necrotising enterocolitis was not mentioned in any patient. SIgA titres showed high interindividual variation, and there were no significant differences between the two groups (fermented formula fed infants: median 27 (range: 1-474 µg/g); preterm formula fed infants: median 12 (range: 1-350 µg/g)). They did, however, note that infants who were partially breast fed showed higher SIgA levels from week 2 in those who were also fed the fermented formula instead of the standard preterm milk (3038 (range 1225-6040) versus 1473 (range 30-2655)) (Campeotto, Suau et al. 2011).

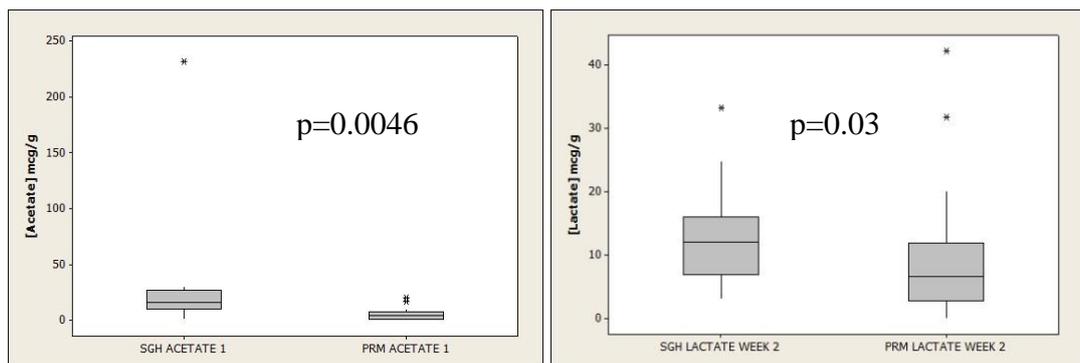
The high mortality and morbidity associated with NEC have given rise to a host of potential immunotherapies, but no studies could be identified assessing changes in SIgA according to infants with and without NEC. However, within the last ten years, a vogue for the oral administration of pooled immunoglobulin and combinations therein of Ig G and Ig A has dwindled, with repeated updated Cochrane Reviews showing no benefits in the treatment or prevention of necrotising enterocolitis, despite eligible trials involving a total of 2095 preterm infants (Foster and Cole 2004).

#### **5.2.6) Conclusions**

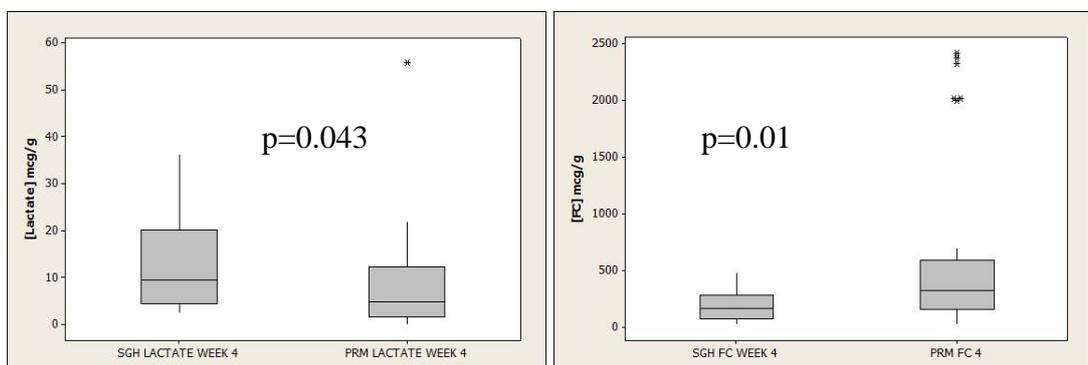
There is still much to be learned about the acquisition of SIgA from mother to preterm infant within the crucial first month of life, and this study illustrates the importance of feeding colostrum to these fragile infants. Greater numbers in future studies may lead to further detail about factors likely to increase the SIgA yield in maternal milk, as well as its enhancement in the gut of preterm infant, and its potential abilities to safeguard or indeed promote the growth of *Lactobacillus* and *Bifidobacteria* species as well as reduce the likelihood of NEC.

### 5.3) Unit Comparison of Analytes

There were few statistically significant differences in analytes between the three neonatal units. No differences of note were found in the number of TTGE bands, although this was heavily confounded by small numbers. In regard to SCFA concentrations, three significant differences were noted. During week 1, acetate levels were significantly higher in infants born at SGH than those born in PRMH (Figure 121 a): SGH med: 16.2 mmoles/L vs PRM med: 4.3 mmoles/L,  $p = 0.0046$ ). In week 2 samples, SGH lactate concentrations were significantly higher than PRM levels (Figure 121 b): SGH med: 12.08 mmoles/L vs PRM med: 6.68 mmoles/L,  $p = 0.03$ ), as was also the case in week 4 (Figure 122 a): SGH med: 9.57 mmoles/L vs PRM med: 4.88 mmoles/L,  $p = 0.043$ ). Calprotectin levels were significantly lower in samples from infants born at SGH than those from PRM (Figure 122 b): SGH med: 169.9 mmoles/L vs PRM med: 323 moles/L,  $p = 0.01$ ).



Figures 121: a) (left) SGH and PRM acetate levels, week 1; levels were significantly higher in SGH patients ( $p=0.0046$ ) b) (right) SGH and PRM lactate levels, week 2; levels were significantly higher in SGH infants ( $p=0.03$ )



Figures 122: a) (left) SGH and PRM lactate levels, week 4; levels were significantly higher in SGH infants ( $p=0.043$ ) b) (right) SGH and PRM calprotectin levels, week 4; levels were significantly lower in SGH infants ( $p=0.01$ ).

Demographically, notable significant differences between the Units were the lower duration of antibiotic usage, exclusive breast feeding, and Unit preference of fortifier at PRMH. There were no differences in NEC rates, and as such it is likely that the lower concentrations of acetate, lactate and SIgA reflect the lower incidence of exclusive breast milk feeding in PRMH. Given the lack of differences in NEC rates, reasons for the significantly higher calprotectin levels in PRMH during week 4 are more difficult to ascertain, and possibly due in part to their use of fortifier. Similarly bacterial diversity analysis using TTGE bands showed no specific bacterial differences between the Units to account for the higher calprotectin levels, and no correlation between number of bands and FC level. This, however, may be due to small numbers within this subgroup analysis.

## Chapter 6

### General Discussion

#### 6.1) Introduction

My initial hypothesis was that the stool microbiota, metabolites and inflammatory analytes would vary with nutritional and environmental aspects of neonatal care in our cohort of preterm, very low birth weight infants. According to local audit analysis, and in respect of national and international data reporting an incidence of up to 10%, we expected a maximum of 6 infants with any stage of NEC in our study. Instead, the emergence of 20 infants with NEC posed many more questions than can be answered by this study alone. The high incidence of NEC in this cohort was unexpected, and as such additional comparisons were made in these results comparing cases of NEC and those without. However, given that this study was not powered to NEC, the weekly sample analysis may have missed significant changes in analytes, and that future observational studies in our regional neonatal units would be better placed to anticipate this NEC incidence and take daily samples – or even, as per other studies, attempt to take all stool samples produced by these infants. The following issues will be discussed in turn: NEC, in light of major demographical and clinical findings; study analytes; inter-unit differences; study limitations and strengths; and future work.

##### *6.1.1) Clinical and demographic associations with the incidence of NEC*

The high incidence of NEC in this study was unexpected, given recent publications reporting the incidence to be between 6 and 10 % last 10 years. Although observer bias could be considered, these would only account for the infants without histopathological confirmation from laparotomy. As such, excluding these infants left eight with confirmed NEC – still more than the maximum of five infants expected. When the demographical and clinical data for these infants was investigated, several factors lessened the likelihood of observer bias, and reinforced confidence in these cases as ‘true positives’. None, however, pointed towards a cause for this rise. Rates of IUGR and AEDF were low and there appeared to be no significant difference in Depcat scores.

As expected, extreme prematurity was closely associated with NEC, which was typically more severe and first onset of clinical signs occurred later in the study period than those with milder NEC. Of note, these infants had significantly higher CRIB scores, and lower 10-minute Apgars, indicating that they had a more traumatic perinatal period. This is all

the more important a feature considering that in all other respects, those with NEC were at no higher risk than those without, being no quicker to establish full enteral feeds, no earlier commenced on feeds than those without NEC, and actually less received formula milk. There are currently no national guidelines on the rate of acceleration of trophic feeds. The ‘SIFT’ trial is currently aiming to recruit infants to just such a study. In addition, the use of fortifier was not more likely in those with NEC. Much of the remainder of their clinical details, however, can simply be attributed to extreme prematurity, especially longer duration of ventilation, more episodes of sepsis, and a longer duration of antibiotic administration, and incubation. Along these lines, more infants with NEC underwent PDA ligation and laser therapy for ROP.

However their far higher CRP levels would suggest that they suffered a genuine inflammatory response, although the clarification of septic ileus versus NEC could not entirely be made in those infants without histopathological diagnosis. Pneumatosis coli is not pathognomonic of NEC, neither is the absence of pneumatosis is 100% specific.

Given the inappropriateness of multivariate analysis, the influence of gestation on some of the important demographical and clinical features was examined only in univariate form. We expected the correlations with gestation, deplat score, duration of ventilation, and incidence of sepsis to get stronger through the more severe stages of NEC. This, however did not appear to be the case. Instead, with regard to the infants incurring stages 3a and 3b NEC in particular, these appeared to be a heterogeneous group, with a wide range of gestation and birth weight. The smallest infant in the cohort did not develop any signs of NEC at all. Of interest, the Z score comparisons between those with  $\geq$ stage 2a NEC and those without were contrary to anticipated, with lower scores in weeks 1 and 2, but no significant differences in weeks 3 and 4. It is considered that this is due to infants with severe NEC who gained weight by oedema.

#### *i) Genetic and Epigenetic Factors? Analogy with Multiple Sclerosis*

The comparison between clinical and demographical information suggests that the most important risk factors for the development of NEC are simply ELBW and extreme prematurity. However, these associations are similar throughout the UK, yet the high incidence of NEC would appear to be specific to Glasgow. Subsequent data collection from the neonatal surgical unit at RHSC shows similar levels of NEC in VLBW infants at

less than 32 weeks gestation referred from SGH and PRMH units: 10 infants between January 1<sup>st</sup> 2012 and January 1<sup>st</sup> 2013 (5 in each unit), and then 4 between January 1<sup>st</sup> 2013 and January 1<sup>st</sup> 2014. 5 of those in 2012 required surgical intervention; all survived to discharge. All 4 of those referred in 2013 required surgery; again, all survived to discharge. These numbers exclude referrals from other neonatal units in Scotland, and do not include infants with medically treated NEC who remained in the SGH and PRMH units.

Analogies can be drawn between other illnesses and their geographical predominance. The north-east of Scotland records the highest international incidence and point-prevalence of Multiple Sclerosis (MS). Although MS is known to be higher in patients living further away from the equator, the high levels of MS in the north east have merited epidemiological studies over the last 40 years (Shepherd 1979, Dean, Goodall et al. 1981, Handel, Jarvis et al. 2011). These suggest that not only do those born in this area have a higher risk of MS, but that people emigrating out with the north-east carry their higher risk with them, and, conversely, people immigrating into this area increase their MS risk. This strongly suggests that there is not simply a genetic, but also an ‘epigenetic’ environmental factor that synergistically combines to cause this location-specific phenomenon. Genetic studies of NEC are yet to identify associations, although it is noted that there are now genetic associations in other gut pathologies similar to NEC, such as Inflammatory Bowel Disease, which have only been established in the last 10 years (Imielinski, Baldassano et al. 2009, Henderson, van Limbergen et al. 2011). Genetic studies of infants with NEC are far less well established, but have so far postulated polymorphisms of a variety of genes encoding gut enzymes and anti-inflammatory agents, including Mannose-Binding Lectin 2 (MBL-2), carbamoyl phosphate synthetase 1, and toll-like receptor pathways (Moonen, Paulussen et al. 2007, Sampath, Le et al. 2011, Prencipe, Azzari et al. 2012). In Scotland, the ‘PINE’ study (Preterm Infants with Necrotising Enterocolitis) attempted to identify associations between genes encoding TNF alpha and other cytokines, without positive results. It is possible, in conclusion, that the high Glasgow-wide incidence of NEC has a similar epidemiology to that of MS in the North-East of Scotland.

### **6.1.2) Study Analytes**

#### *i) Stool production*

Stool production in preterm infants is often delayed (Arnoldi, Leva et al. 2011). In our cohort, a delay of up to one week was not unusual or associated with congenital gut

pathology (for example hirschsprungs or atresias). Given that many samples were less than 1 gram (as required to perform all analytes), although splitting days of the week (as with Mohan et al in 2008 (Mohan, Koebnick et al. 2008)) for different samples would have yielded more complete data sets, correlation of the analytes would not have been as reliable. We were prevented from using rectal swabs given the specific instructions from the Research and Ethics Committee before the study began, despite the fact that rectal swabs are standard practice in neonatal care for infection control management. Most infants who incurred paralytic ileus were of extreme prematurity and ELBW. Infants who then developed NEC, regardless of gestation or birth weight, developed ileus due to iatrogenic gut rest.

#### *ii) SCFA analyses*

SCFAs were detectable in week 1, when the majority of infants were only trophically fed, and had not yet established full enteral nutrition. Although this level was not significantly higher than in other weeks, there was a noticeable trend to lower levels by week 4. This strongly suggests that bacterial fermentation has produced these SCFAs, which in most samples were dominated by lactate and acetate. In addition, consistent gestational trends in total and individual SCFAs were noted, with consistent week-on-week trends, suggesting differences in the bacterial composition between these groups, changing over time, such as the higher caproic and isovaleric acids and lower valeric and isocaproic acids in those 24-26 weeks gestation declining and rising respectively over the study period. Given the heterogeneity of feed types, few differences were seen when considering EEBM fed versus mixed fed, although there was a higher acetic acid level in those EEBM fed than mixed in week 4. That these levels were similar to those of infants in probiotic trials is extremely encouraging. However, ratiometric analyses in regard to NEC showed the most significant trends, particularly higher acetic:BCFA ratio during week 1, higher acetic:isovaleric and lactate:isocaproic levels during week 2, and higher lactate:isobutyrate levels in week 4. These strongly suggest that the microbiota is afflicted with less proteolytic and higher levels of sacrolytic bacteria, in stark contrast with the healthy infants who did not incur NEC, who with lower ratios move further towards a less hostile and pathogenic gut environment. The ubiquity of antibiotic usage amongst the cohort made comparisons obsolete.

### *iii) Calprotectin levels*

FC levels showed little significant changes, despite an abundance of severe NEC and extreme prematurity. Although at least five-fold higher than the adult maximum cut-off value for colitis, these median levels within the cohort were similar to values seen in other similar studies. Although small gestational spikes were observed, no temporal changes were noted, and as for the SCFA data, it is possible that very diverse milk types administered may be a confounding factor in the lack of significant differences with feeds. To date, this is the first study to measure FC in ileostomy fluid in preterm infants, suggesting it is not limited to colonic excretion. Although FC was not a diagnostic marker in this cohort, given the lack of power to NEC, an appropriately designed future study may well change this.

### *iv) TTGE*

Although limited, TTGE analysis illustrated that the first month of life in our cohort of preterm infants was a time of great microbial flux within the gut. This was demonstrated by a high variability in immigrated and emigrated species, as well as a low similarity index between time points. Influence of antibiotics was however difficult to discern due to ubiquitous use throughout the cohort, and as such there was 'control' group who had not been administered antibiotics. Unlike in Mohan et al's work from 2008, no correlation was noted with metabolites, likely indicating that far greater numbers require to be recruited in order to make these associations. The lack of correlation with feeds was surprising considering the evidence base, but by volume the 'mixed' fed group were given more than 50 % maternal EBM; so this lack of significance is perhaps not that surprising. It is more than likely that deeper mining of the microbiota is the key, and extremely promising that even in the short time since our research was conducted, huge leaps in type and affordability of pyrosequencing have occurred.

### *v) SIgA*

Despite a scant evidence base, our study illustrates the importance of acquisition of SIgA and its temporal nature. Although subgroup analysis, it is the first of its kind to examine paired neonatal stool and maternal SIgA titres in a preterm cohort. The increase in those with NEC between weeks 2 and 3 may simply reflect ileus formation secondary to the disease, but may also indicate an exudative, protective 'last-ditch' attempt by the gut as it staves off the initial subclinical throes of the disease process. The expected lag between

stool and milk SIgA emphasises the importance of using colostrum – much of which remains stored as the infants take far longer to establish trophic and then full enteral feeds than their mothers take to express mature milk beyond the first 3 days post-partum. The higher titres in milk of mothers of preterm infants reflects the body's 'natural selection' to protect its preterm young, and in itself stresses the importance of SIgA as a robust first line of gut defence.

*vi) Unit differences in Analytes*

Although few, there were some unit differences in analytes that have mainly been explained by the variation in feed and antibiotic regimen. However, the insignificant differences in NEC, sepsis and mortality indicate that our cohort was subject to much the same clinical management decisions, and that these did not hugely influence the results.

**6.1.3) Confounders of the study**

As noted through each part of this discussion, given the unexpectedly high rate of NEC, the associated onset of ileus resulted in a paucity of samples around and after the onset of disease. In comparison, sample collection in infants without NEC was extremely high, with few missed samples. After the establishment of full enteral feeding, samples from infants at any stage were abundant. If the incidence of NEC had been expected, then merit would have been held in categorising samples by analyte according to days of the week, or a system of alternate samples. Alternatively, the project could have been focussed to marry the molecular with SCFA or calprotectin analyses, since these require only small weights of stool. However, in order to appropriately investigate these as biomarkers for NEC, undoubtedly the most robust standard operating procedure would have been to attempt to take every stool sample produced, alongside rectal swabs during times of ileus. This would perhaps also have simplified matters for the nursing staff, for whom there would have been no confusion over when a sample was due to be taken. Notably, newer molecular techniques are advancing ever-quickly, utilising high throughput sequences, and becoming cheaper. Unfortunately, this study was restricted both by time and finances, as well as to the techniques available on hand within the department. It is also important to note that stool microbiota may not reflect mucosal colonisation. Given the status of RHSC's NICU as a tertiary and quaternary neonatal surgical unit, it would be valid and accessible to obtain intraoperative biopsy samples in future to compare.

#### **6.1.4) Study strengths**

This study is the only work to date (as published in abstract or article form), to recognise and correlate each of these analytes in a cohort with and without NEC. This study also reports the highest number of infants in a cohort series to analyse SCFA profiles with regard to NEC, as well as being the first study to compare secretory Ig A titres in maternal milk and infant stool pairs, as well as BCFA analyses.

## **6.2) Conclusions and future research**

The consistent lack of correlation between these analytes in this high NEC-laden cohort indicates their independence from one another, and points to increasingly florid gut activity within the crucial first four weeks of life in preterm, VLBW infants. Their individual consistency, particularly of the SCFA and calprotectin profiles, indicates that no one clinical or demographical factor exerts a preferential influence on these analytes. In contrast, the high interindividual and intra-individual variation in microbial species, with flight of immigration and emigration, suggests that from a molecular perspective, huge changes occur within this first month. The next obvious step in this study would be to extrapolate this, increasing the power and diversity with a modified standard operating procedure to other centres throughout the UK. Although such a task would be costly, if combined with an observational study on NEC, or other NEC-prevention strategy studies (for example, prospective UK feeding trials) not only could clinical and demographical data be shared, but the information gained from these stool tests would greatly enhance our understanding of data in well preterm infants, as well as exploring the potential for these as diagnostic and prognostic markers for those with NEC. Given that this study identified only minor changes with NEC, no corresponding sample size calculations can be made. It would be important to anticipate the high incidence of NEC and to plan any further studies within Glasgow meticulously to include frequent sample collection in order to capture disease onset. Molecular techniques involved in qualitative and quantitative bacterial analysis are developing timeously, although the diversity of these methods may influence comparison of studies in the future.

Further smaller intra-departmental projects from this work will include species identification from TTGE, as well as breast milk bacteria, possibly using NextGen sequencing in collaboration with external colleagues; the analysis of the remaining milk supplies for other immune factors, particularly IGF-1. Similarly, the stability of the existing stool samples give rise to testing for other potential inflammatory markers, for example i-Claudin. Analyses of metabolites have the potential for a wealth of applications, particularly in the identification of possible biomarkers of NEC. Use of all of these tools at other sites, for example on gastric aspirates may yield further important information. Given the increasing drive from NICUs to acquire and use various probiotic preparations, it may be that these studies have to occur in tandem with their advent – in doing so creating perhaps the biggest confounder of studies on the natural evolution of the human gut

microflora at the beginning of life. Animal models have proven valuable, and would appear to provide a wealth of opportunity to study new techniques and therapies prior to use in premature infants.

The REC approval for a database to track the clinical and growth parameters of the infants who survived NEC will give us a wealth of information on the long term effects of this disease. Furthermore, the CalRap Study, in collaboration with Dr Richard Russell, has ethically approval to perform rapid acting cotside calprotectin testing of the existing samples. Validation of this test would facilitate its use within each NICU, and make testing considerably quicker and more accessible.

There is much still to be learned about the effects of physiology and management of prematurity upon the gut microbiota, particularly in those with NEC. Although the routine use of probiotics is an exciting and tempting prospect, an important underlying fact in the quest to develop new molecular and biochemical tools is the discovery of new bacterial strains and metabolites, carrying with them the potential to define new functions and interactions. In doing so, we may be able to unlock the doors to a host of disease processes, not to mention therapeutic interventions.

## Appendix 1

### NAPI Consent and Information



#### Consent Form

##### Patient Identification Number:

I confirm that I have read and understand the Parent Information Leaflet dated 18/3/9, for The NAPI Study, have had the opportunity to ask questions and have had them answered satisfactorily.

I consent to (baby's name): \_\_\_\_\_  
taking part in the NAPI Study, giving (delete as appropriate):

samples of his/her stool

samples of his/her stomach fluid

I understand that relevant sections of my and my baby's medical records may   
looked at by individuals including the Investigator and/or supervisors for this study,  
regulatory authorities, or the NHS Trust, where it is relevant to my taking part in this  
research. I give permission for these individuals to have access to our medical records.

I understand that you may in the future wish to use these samples for other   
studies. I agree that you can contact me in the future for my consent for this use.

I consent to the testing of my own breast milk

I consent to taking part in your questionnaire

I agree to my GP being informed of our participation in this study.

I understand that my participation is voluntary and that I am free to withdraw at   
any time without giving any reason, without my or my baby's medical care or  
legal rights being affected.

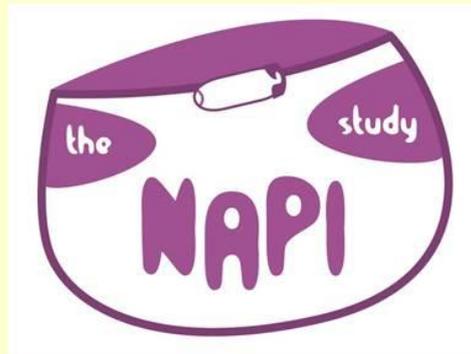
Signed (parent/guardian): \_\_\_\_\_

Date: \_\_\_\_\_

Signed (recruiter): \_\_\_\_\_

Date: \_\_\_\_\_

# Neonatal MicroflorA in Premature Infants



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University  
of Glasgow



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## What is this study about?

The NAPI Study is a 2 year project aiming to identify normal 'good' gut bacteria (so-called 'microflora') present in stool, stomach fluid, and breast and formula milks. These bacteria also produce other substances which will also be measured in these samples. These will be measured in infants who weigh less than 1.5Kg at birth, over a four week period from the commencement of milk feeds.

## Why are you doing this study?

- The presence of 'good bacteria' in the gut plays a crucial role in the health of premature babies. These bacteria appear in the gut in the first few days and months of life, and are known to stop the growth of harmful bacteria. They also produce substances that protect babies, particularly against infection. We have little knowledge of how or why this happens.
- Other studies have identified different factors that affect the type of 'good' bacteria and when they grow in the gut of premature babies. We are assessing the effect of differences in their environment (e.g. length of time spent in an incubator) and milks (e.g. formula, mum's milk, donor breast milk, or combinations of these) on the growth of these good bacteria, and the substances they produce.
- Eventually it is thought that 'good' bacteria, known to be lacking in premature babies, could be given to them as an oral medicine, similar to the adult probiotic drinks that can be purchased from supermarkets. However, before that can occur, we need to know a lot more about which good bacteria grow, when they appear, which substances they produce, and how they repel harmful bacteria.



University  
of Glasgow





## What will happen to my baby?

Your baby will be fed according to **your** preference of milk: breast, donated breast milk, or formula. Your baby's routine day-to-day care will not be disrupted or changed in any way.

## Which samples will be taken?

Three different types of samples will be collected: stool (poo), stomach fluid, and milk, twice weekly for four weeks once feeding has started.

1. Stool

We aim to collect at least 2 stool samples per week for 4 weeks. Another sample may be taken at 8 weeks of age. These will be collected directly from the nappy as they are produced, and will cause no change to your baby's routine. If you are changing your baby's nappy, we would ask that you notify your son or daughter's nurse, who will place the sample in one of our special containers that are stored in the incubator or cot.

2. Stomach fluid

We will take 2 samples of stomach fluid per week for 4 weeks. These will be taken from the nasogastric tube (the tube already inserted from nose to stomach for your baby's care) and will likely be just a few millilitres in volume. A nasogastric tube will not be inserted just to take the sample, and the act of removing the fluid is not painful or uncomfortable for your baby.

3. Milk

We will take small samples of your baby's milk twice weekly for 4 weeks. For those babies receiving mum's milk, these will only be taken if there is obviously enough milk for your baby first.



## What if I don't want my breast milk to be analysed?

- If you prefer for your breast milk not to be analysed, then we will not do so. The samples of milk taken are tiny, and will not deprive your baby of vital nutrients.

## Will my baby have a nasogastric (nose-to-stomach) tube passed just in order to obtain the sample of stomach fluid?

- No. Nasogastric tubes will only be passed if your baby requires one for feeds. They will not be passed in order simply to take a sample.

## When will the results be available?

- The tests measured will be performed in batches, and as such the results will take up to one year to process.

## Will the results affect the clinical care of my baby?

- No. The tests performed are not done routinely in the Neonatal Unit. Part of this project is assessing the relevance of these tests to premature babies. Some results will be available within a few weeks, and others will take up to one year. The results will not affect the care of your baby.





## Will my baby be identifiable to anyone working on the study?

- Your baby will have a unique identifying number, linked to their name, unit number, date of birth and other similar details stored on NHS documents. All other samples and tests will use this number. Your baby's confidential details will be held on a password-protected computer within a locked room within the Department of Child Health, at the Royal Hospital for Sick Children at Yorkhill. Only the Project Co-ordinator and Supervisor will be able to link the unique reference number with your baby. Once the study and work arising from it is complete, these details will be destroyed.

## How long will you store these details for?

- We will store these details for a maximum of 4 years, in line with national guidelines. We will not share any of these details with any other institutions.

## How will the results be used?

- We aim to collate the results and present them at medical conferences, and to publish them in medical journals. Your baby will not be identifiable through these results. We will as a matter of course let your GP know that your baby is in this study.

## Will my baby be identifiable from these documents and presentations?

- No. All results presented and published will be fully anonymised.



## Does my baby have to take part?

No. Your baby will only take part with your consent.

## Can I change my mind later?

Yes. You can decide to remove your baby from this study at any point. We will keep the samples and tests that we have done, but will not take any further.

## How long will you store these samples for?

These samples can be stored for up to 3 years.

## Will these samples be used for any other studies?

If in the future it appears that these samples could be of interest to another study, we will only analyse them with your consent.

## How will these samples be disposed of?

These samples will be destroyed at Yorkhill Hospital in line with national guidelines.





## Mum's questionnaire

- A small part of our study involves a short face-to-face questionnaire for mums. We can do this at the same time that you sign the consent form, and it should take less than 5 minutes to complete.
- We will ask:
- If you are aware of food and drink products that contain 'good' bacteria, such as yoghurts and yoghurt drinks;
- If you consume these products;
- If so, which ones, and how often per week.



## Contacts

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## Appendix 2

### SCFA:

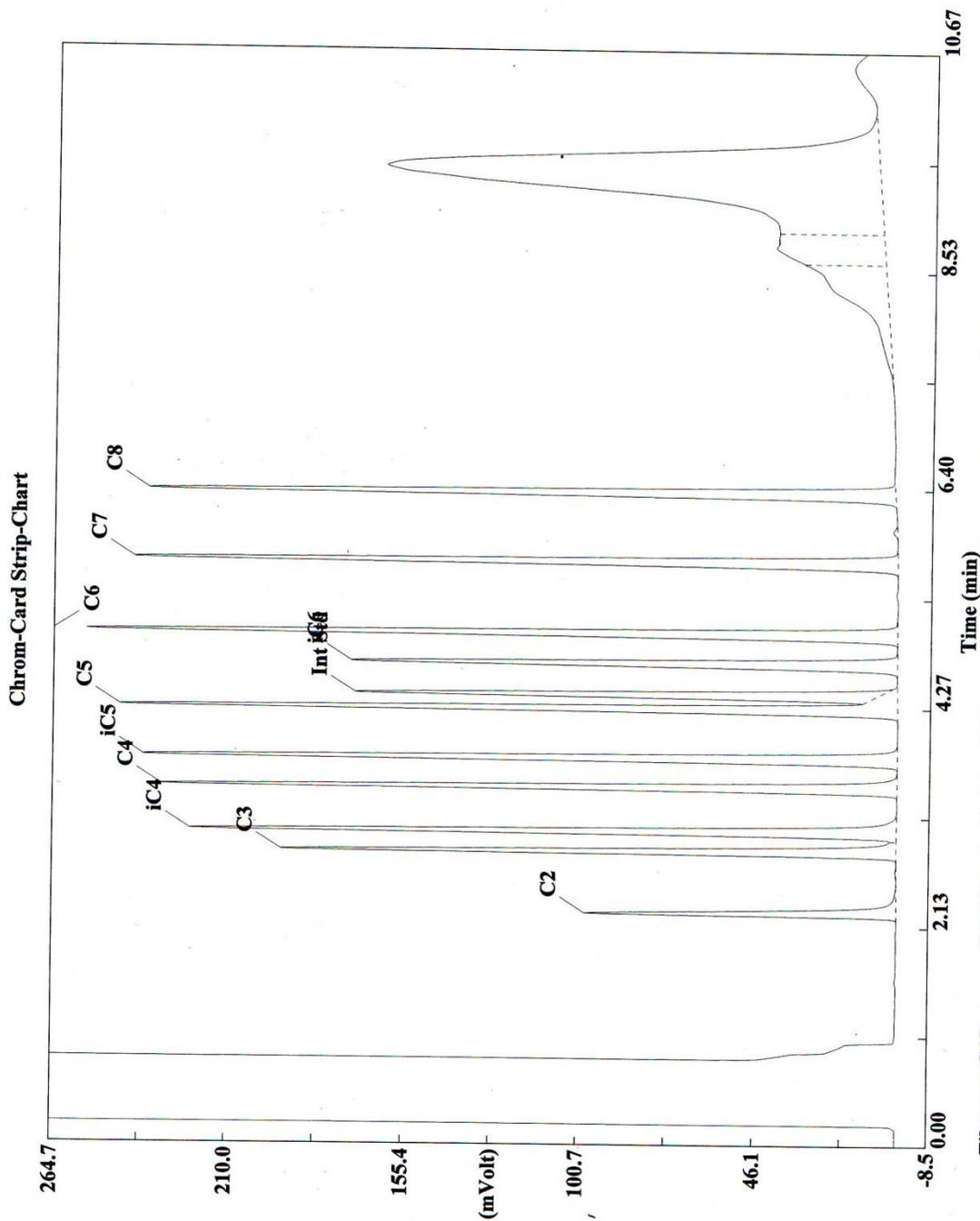
#### tBDMS External Standards: successful ES protocol

- 1) the following was added to 10 mls 2M NaOH:

Systematic name	Trivial name	umoles	ml 2M NaOH	mM	umoles	mg	ul
Methanoic acid	Formic	1000	10	100	1000	46.03	38
Ethanoic acid	Acetic	1000	10	183.3	1833	110.0717	105
Propanoic acid	Propionic	1000	10	133.2	1332	98.67456	100
Butanoic acid	Butyric	1000	10	104.2	1042	91.81062	96
Pentanoic acid	Valeric	1000	10	86.3	863	88.13819	95
Hexanoic acid	Caproic	1000	10	74	740	85.9584	93
Heptanoic acid	Enanthic	1000	10	64.7	647	84.23293	93
Octanoic acid	Caprylic	1000	10	57.5	575	82.92075	91
2-Methylpropanoic acid	Isobutyrate	1000	10	102	1020	89.8722	93
3-Methylbutyric acid	Isovalerate	1000	10	86	860	87.8318	95
4-Methylvaleric acid	Isocaproic	1000	10	50	500	58.08	63
2-Hydroxypropanoic acid	Lactate	1000	10	50	500	45.04	38

- 2) Then add: 100mcl ES
- 3) Add 100mcl HCl
- 4) Add 100 mcl 3Methylvaleric
- 5) VORTEX
- 6) Add 1000 mcl ether
- 7) VORTEX
- 8) Leave to settle 30 mins
- 9) Extract 800 mcl into store glass tube
- 10) Extract 100 mcl into glass tube with 160 mcl ACN, 40 mcl tBDMS
- 11) Hotplate for 60 mins
- 12) GCMS

# Example Chromatogram from the Initial GC Run



Filename C:\Program Files\Thermo Finnigan\Chrom-Card Trace-Focus GC\data\SCFA's\Lynne Beattie\040810\Cal2.dat  
Sample name :EXTSTD Analyzed :08/04/2010 12:09

## GCMS Settings

Sample inlet: GC  
Injection source: GC ALS  
Injection volume: 1ul  
Syringe: 10uls  
Washes: Preinjection solvent A  
          Post injection solvent B  
          Pumps 2  
Inlet B 250C (Injector)  
Oven Equilibration time: 0.5 mins  
Oven Max 300C Ambient 25C

### Run temperatures:

Level	Rate C/min	Final temp	Final Time
1	15	100	0
2	15	200	0
3	15	210	0

Initial temp 80C Initial time 1 min Next run time 9.67 mins

Inlet B: constant flow on. Pressure 20.9psi@temp 140C

Column 30m Phenomenex ZB-5MX diameter 0.220mm He gas

Oven temp 140C Pressure 20.9psi Flow 1ml/min Velocity 37.7cm/sec Split ration 20:1

### Sim Parameters

Group	Time	Resolution	m/Z	Dwell	Plot
1	3.00	Low	103	100	window 1
2	3.60		117		
3	4.20		131		
4	4.90		145		
5	5.70		159		
6	6.55		173		
7	7.80		187		
8	8.71		201 and 261		

## Appendix 3

### Calprotectin ELISA dilutions

Patient	No	Sample	Dilution	20PRMH13	2	103.1	5.05	43PRMH27	6	82.9	4.06
02QMH01	1	80	3.92	20PRMH13	3	102.8	5.04	44SGH11	7	115.6	5.66
02QMH01	2	60.6	2.97	20PRMH13	4	95.5	4.68	44SGH11	8	79.9	3.92
02QMH01	3	95	4.66	20PRMH13	5	115.1	5.64	44SGH11	9	93	4.56
02QMH01	4	82	4.02	21PRMH14	6	103	5.05	45PRMH28	10	77.3	3.79
02QMH01	5	80	3.92	21PRMH14	7	119	5.83	45PRMH28	11	85.1	4.17
02QMH01	6	66	3.23	21PRMH14	8	83.5	4.09	45PRMH28	12	71.5	3.50
02QMH01	7	59.6	2.92	21PRMH14	9	119	5.83	45PRMH28	13	117.1	5.74
03PRMH02	9	79.4	3.89	22SGH04	10	89.9	4.41	46PRMH29	14	97.6	4.78
03PRMH02	10	78.7	3.86	22SGH04	11	101.1	4.95	46PRMH29	15	76.1	3.73
03PRMH02	11	110	5.39	22SGH04	12	62.8	3.08	46PRMH29	16	95	4.66
03PRMH02	12	99	4.85	22SGH04	13	90.8	4.45	46PRMH29	17	79.5	3.90
03PRMH02	13	65	3.19	22MILK	14	120	5.88	47SGH12	18	77.6	3.80
04QMH02	14	93	4.56	24SGH06	15	104.8	5.14	47SGH12	19	61.9	3.03
04QMH02	15	54.2	2.66	24SGH06	16	95.8	4.69	47SGH12	20	84.3	4.13
04QMH02	16	93	4.56	24SGH06	17	102.4	5.02	47SGH12	21	95.7	4.69
04QMH02	17	59.7	2.93	24SGH06	18	85.5	4.19	48QMH07	22	71.4	3.50
05PRMH03	18	87.9	4.31	24SGH06	19	3.4	0.1666	48QMH07	23	101.7	4.98
05PRMH03	19	64	3.14	25QMH05	20	106.7	5.23	49PRMH30	24	117.4	5.75
05PRMH03	20	98	4.80	25QMH05	21	48.6	2.38	49PRMH30	25	87.7	4.30
05PRMH03	21	89.9	4.41	25QMH05	22	93	4.56	49PRMH30	26	90	4.41
05PRMH03	22	70	3.43	25QMH05	23	95.2	4.66	50PRMH31	27	107.2	5.25
06PRMH04	23	70	3.43	25QMH05	24	63.6	3.12	50PRMH31	28	77.7	3.81
06PRMH04	24	98	4.80	25QMH05	25	108.6	5.32	50PRMH31	29	77.1	3.78
06PRMH04	25	66	3.23	26PRMH15	26	80.2	3.93	50PRMH31	30	83.8	4.11
06PRMH04	26	75	3.68	26PRMH15	27	98.3	4.82	51PRMH32	31	112.3	5.50
07PRMH05	28	40	1.96	26PRMH15	28	79.3	3.89	51PRMH32	32	88.7	4.35
07PRMH05	29	120	5.88	26PRMH15	29	103.6	5.08	51PRMH32	33	73	3.58
07PRMH05	30	100	4.90	27PRMH16	30	71.1	3.48	52PRMH33	34	110.5	5.41
07PRMH05	31	102	5.00	27PRMH16	31	93.5	4.58	52PRMH33	35	105.2	5.15
07PRMH05	32	65	3.19	27PRMH16	32	77.5	3.80	53SGH13	36	50.6	2.48
08SGH01	33	82	4.02	27PRMH16	33	105.9	5.19	53SGH13	37	52.2	2.56
08SGH01	34	70	3.43	28SGH07	34	109.6	5.37	53SGH13	38	52.3	2.56
08SGH01	35	51.4	2.52	28SGH07	35	84.2	4.13	54SGH14	1	81.6	4.00
08SGH01	36	66	3.23	28SGH07	36	112.3	5.50	54SGH14	2	94.3	4.62
09SGH02	37	99	4.85	28SGH07	37	73	3.58	54SGH14	3	86.5	4.24
09SGH02	38	50	2.45	45PRMH MI	38	115.2	5.64	55PRMH34	4	55.4	2.71
09SGH02	39	76.8	3.76	31SGH08	1	80.3	3.93	55PRMH34	5	96.4	4.72
09SGH02	40	83.7	4.10	31SGH08	2	116.4	5.70	55PRMH34	6	70.2	3.44
10PRMH06	1	89.4	4.38	31SGH08	3	103.9	5.09	55PRMH34	7	50.7	2.48
10PRMH06	2	88	4.31	32PRMH19	4	81.9	4.01	56PRMH35	8	60.5	2.96
10PRMH06	3	79.4	3.89	32PRMH19	5	96.2	4.71	56PRMH35	9	68.3	3.35
10PRMH06	4	119	5.83	32PRMH19	6	108.9	5.34	56PRMH35	10	62	3.04
11PRMH07	5	42.2	2.07	32PRMH19	7	81	3.97	56PRMH35	11	77.9	3.82
11PRMH07	6	69.2	3.39	33PRMH20	8	87.1	4.27	56PRMH35	12	112.8	5.53
11PRMH07	7	87.7	4.30	33PRMH20	9	75.5	3.70	13PRMH08	13	119.8	5.87
11PRMH07	8	62.7	3.07	33PRMH20	10	70.4	3.45	57SGH15	14	93.7	4.59
11PRMH07	9	74.5	3.65	33PRMH20	11	71.2	3.49	57SGH15	15	55	2.70
12SGH03	10	94.1	4.61	36PRMH22	12	90.6	4.44	57SGH15	16	24.3	1.19
12SGH03	11	75	3.68	36PRMH22	13	80.8	3.96	57SGH15	17	88.8	4.35
12SGH03	12	62.7	3.07	36PRMH22	14	95.1	4.66	58SGH16	18	54.7	2.68
12SGH03	13	58	2.84	36PRMH22	15	91.7	4.49	58SGH16	19	84.2	4.13
13PRMH08	14	74.4	3.65	37PRMH23	16	40.7	1.99	28SGH07	20	90	4.41
13PRMH08	15	51.1	2.50	37PRMH23	17	103.6	5.08	58SGH16	21	85.6	4.19
13PRMH08	16	108	5.29	37PRMH23	18	108.5	5.32	59PRMH36	22	42.6	2.09
13PRMH08	17	114.1	5.59	37PRMH23	19	102.4	5.02	59PRMH36	23	50.6	2.48
14PRMH09	18	77.7	3.81	38PRMH24	20	102.3	5.01	59PRMH36	24	98.3	4.82
14PRMH09	19	64.6	3.17	38PRMH24	21	84.7	4.15	59PRMH36	25	93.5	4.58
14PRMH09	20	90.8	4.45	38PRMH24	22	95.6	4.68	60PRMH37	26	75	3.68
14PRMH09	21	54	2.65	38PRMH24	23	86.3	4.23	60PRMH37	27	67.3	3.30
16QMH03	22	95.8	4.69	38PRMH24	24	88.6	4.34	60PRMH37	28	69	3.38
16QMH03	23	91.1	4.46	39QMH06	25	63.7	3.12	60PRMH37	29	81.1	3.97
16QMH03	24	110	5.39	39QMH06	26	77.1	3.78	61PRMH38	30	101.1	4.95
16QMH03	25	79.7	3.91	39QMH06	27	86.9	4.26	61PRMH38	31	79	3.87
17PRMH11	26	90	4.41	39QMH06	28	65.5	3.21	61PRMH38	32	89.6	4.39
17PRMH11	27	93.6	4.59	40PRMH25	29	104.3	5.11	61PRMH38	33	85.5	4.19
17PRMH11	28	80	3.92	40PRMH25	30	109	5.34	62SGH17	34	45.5	2.23
17PRMH11	29	90.4	4.43	40PRMH25	31	86.2	4.22	62SGH17	35	50	2.45
17PRMH11	30	102	5.00	41PRMH26	32	86.4	4.23	62SGH17	36	81	3.97
18QMH04	31	68	3.33	41PRMH26	33	95.8	4.69	62SGH17	37	60.1	2.94
18QMH04	32	79.5	3.90	41PRMH26	34	84.7	4.15	2QMH01	38	94.2	4.62
18QMH04	33	74.3	3.64	41PRMH26	35	75.2	3.68				
18QMH04	34	56.3	2.76	41PRMH26	36	91.1	4.46				
19PRMH12	35	96.3	4.72	42SGH10	37	94.8	4.65				
19PRMH12	36	73.4	3.60	42SGH10	38	64.5	3.16				
19PRMH12	37	73	3.58	42SGH10	1	92.9	4.55				
08SGH01	38	100.9	4.94	43PRMH27	2	114.6	5.62				
19PRMH12	1	100.9	4.94	43PRMH27	3	86.9	4.26				
				43PRMH27	4	99.7	4.89				
				43PRMH27	5	86.4	4.23				

## Appendix 4

### TTGE: DNA Extraction Spectrometry Measurements

Spectrometry measurements							
Infant	Sample	DNA ( $\mu\text{g/mL}$ )		260/280nm		260/230	
		mean	SD	mean	SD	mean	SD
A	1	580	204	2,2	0,2	0,7	0,0
A	2	545	71	1,6	0,1	0,3	0,0
B	1	326	14	1,4	0,0	0,2	0,0
B	2	928	90	2,4	0,1	0,6	0,0
C	1	208	23	1,5	0,1	0,2	0,0
C	2	776	414	2,1	0,3	0,5	0,0
D	1	202	60	1,3	0,0	0,2	0,0
D	2	397	153	1,8	0,1	0,5	0,0
E	1	324	65	1,5	0,0	0,4	0,0
E	2	533	40	1,6	0,0	0,4	0,0
F	1	1279	212	1,2	0,3	0,3	0,0
F	2	760	184	1,5	0,2	0,5	0,0
G	1	1791	134	2,6	0,4	0,6	0,0
G	2	1984	54	3,2	0,3	0,5	0,0
H	1	860	120	1,7	0,0	0,5	0,0
H	2	1757	19	2,2	0,0	0,6	0,0
I	1	579	113	1,2	0,0	0,2	0,0
I	2	1800	280	3,3	0,7	0,5	0,0
J	1	455	71	1,3	0,0	0,2	0,0
J	2	922	120	1,8	0,3	0,7	0,0
K	1	900	123	1,8	0,3	0,6	0,0
K	2	955	73	1,7	0,3	0,6	0,1
L	1	761	67	1,4	0,3	0,3	0,0
L	2	546	76	2,0	0,0	0,8	0,1
M	1	200	56	1,1	0,0	0,2	0,0
M	2	373	48	1,5	0,0	0,4	0,0
N	1	1121	44	1,8	0,0	0,3	0,0
N	2	997	141	2,1	0,0	0,7	0,0
O	1	742	76	1,1	0,2	0,2	0,0
O	2	885	150	1,7	0,3	0,6	0,0
P	1	595	124	1,1	0,0	0,2	0,0
P	2	665	78	1,4	0,5	0,4	0,0
Q	1	658	111	1,7	0,1	0,5	0,2
Q	2	574	20	1,6	0,0	0,4	0,0
R	1	1096	29	1,8	0,1	0,3	0,1
R	2	495	6	1,6	0,0	0,3	0,0
S	1	500	100	1,6	0,3	0,6	0,0
S	2	920	41	1,7	0,4	0,5	0,1
T	1	397	62	1,6	0,1	0,4	0,0
T	2	1003	171	2,2	0,0	0,6	0,1
U	1	1020	126	1,8	0,3	0,7	0,0
U	2	608	62	1,4	0,2	0,4	0,0
V	1	843	164	2,0	0,1	0,8	0,1
V	2	907	99	2,0	0,1	0,7	0,0

Interindividual Similarity Indices: Infants By Feed Regimen

Inter-individual similarity indices (EBM)							
	A	B	C	D	E	F	G
B	50						
C	0	20					
D	0	0	0				
E	0	18	40	22			
F	0	20	28	0	26		
G	0	22	30	0	28	61.5	
H	14	25	50	0	38	20	21.1
	.3				.1		

Inter-individual similarity indices (MF)													
	I	J	K	L	M	N	O	P	Q	R	S	T	U
J	0												
K	16	0											
	.7												
L	25	0	37										
			.5										
M	0	57	26	54									
		.1	.7	.5									
N	0	66	28	20	66								
		.7	.6		.7								
O	0	0	22	0	0	16							
			.2			.7							
P	0	0	40	37	26	28	33.3						
			.5	.7	.6								
Q	15	0	47	23	25	13	21.1	28.6					
	.4		.6	.5	.3								
R	0	0	28	20	0	0	0	28.6	26				
			.6						.7				
S	0	0	52	13	14	30	35.3	52.6	40	15			
			.6	.3	.3	.8				.4			
T	0	40	30	22	50	57	0	15.4	14	0	16		
			.8	.2	.1				.3		.7		
U	50	0	16	25	28	33	0	16.7	30	0	18	40	
			.7		.6	.3			.8		.2		
V	33	0	14	20	22	25	0	14.3	26	0	15	28	66.7
	.3		.3		.2				.7		.4	.6	

## **Glossary**

Microbiota:	(plural noun) the dominant bacterial consortia
Transcriptome:	the set of all RNA molecules in a population of cells
Metabolome:	the complete set of metabolites within a single organism
Metagenomics:	the study of metagenomes: genetic material recovered from environments
Metabolomics:	the study of metabolites and their genetic precursors within an environment
Probiotic:	live microorganisms with benefits to the host
Prebiotic:	non-digestible foods stimulating the growth of probiotics

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## Publications and dissemination arising as a result of this thesis

### Original Articles

- 1) Beattie LM, Hansen R, Barclay AR. Probiotics for Preterm Infants: Confounding Features Warrant Caution. *Pediatrics*. 2010 Sept; 126(3): E742-E743.
- 2) Beattie LM, Weaver LT. Mothers, Babies and Friendly Bacteria. *Archives of Disease in Childhood. Fetal Neonatal Ed*. 2011 May;96(3):F160-3.

### Abstracts

- 1) Brunner K, Beattie LM, Morrison DJ, Barclay AR, Simpson JH, Edwards CA, Gerasimidis K. Detection of Gut Microbiota in Preterm Infants in the First Month of Life Using Transient Temperature Gel Electrophoresis. *Archives of Disease in Childhood* 2013;98:Suppl 1 A 91. (BSPGHAN at RCPCH, Glasgow, 2013)
- 2) Beattie LM, Gerasimidis K, Barclay AR, Morrison DJ, Edwards CA, Russell RK, Simpson JH. Stool SCFA Concentrations in Preterm Infants With and Without Necrotising Enterocolitis. *Archives of Disease in Childhood* 2013;98:Suppl 1 A 92. (BSPGHAN at RCPCH, Glasgow, 2013)
- 3) Beattie LM, Gerasimidis K, Barclay AR, Morrison DJ, Edwards CA, Russell RK, Simpson JH. Stool Calprotectin Levels in Preterm Infants With and Without Necrotising Enterocolitis. *Gastroenterology*. 2012 May;142(5):S732. (DDW, San Diego 2012)
- 4) Brunner K, Beattie LM, Morrison DJ, Barclay AR, Simpson JH, Edwards CA, Gerasimidis K. Detection of Gut Microbiota in Preterm Infants in the First Month of Life Using Transient Temperature Gel Electrophoresis. *Gastroenterology*. 2012 May;142(5):S713. (DDW, San Diego 2012)
- 5) Wenwen M, Beattie LM, Edwards CA, Barclay AR, Morrison DJ, Simpson JH, Combet E. Stool Secretory IgA Levels in Preterm Infants With and Without Necrotising Enterocolitis. *Gastroenterology*. 2012 May; 142(5):S714. (DDW, San Diego 2012)
- 6) Beattie LM, Barclay AR, Russell RK. Faecal calprotectin as a marker of intestinal disease in preterm infants: a systematic review. *Journal of Pediatric Gastroenterology and Nutrition*. 2010 June; 50(2): E88-E89. (ESPGHAN, Istanbul 2010)