The Respiratory Microbiota of Preterm Infants at Risk of Chronic Lung Disease of Prematurity

PhD

2019

David Gallacher

Thesis Overview

Extreme preterm infants are at risk of developing chronic lung disease of prematurity (CLD). This thesis examines the bacterial colonisation of the respiratory tract in extreme preterm infants.

Longitudinal sampling was performed from three anatomical sites within the respiratory tract of preterm infants over the first 28 days of life (Nasopharyngeal aspirates (NPA), tracheal aspirates (TA) and bronchoalveolar lavages (BAL)). Sequencing the bacterial 16S rRNA gene identified bacteria within the samples. IL-6 and IL-8 concentrations were measured using ELISA on TA and BAL samples.

Widespread antibiotic use appeared to suppress bacterial load at all sites with most samples appearing sterile. Most samples successfully sequenced showed a dominant bacterial genus, consistent with previous studies of neonatal bacterial colonisation. In all samples, organisms from the Proteobacteria and Firmicutes phyla were most abundant with smaller number of Tenericutes and Actinobacteria.

NPA samples demonstrated a clear effect of delivery mode over early colonisation patterns and a progression from Firmicutes to Proteobacteria dominated profiles over time. Tracheal aspirate samples showed evidence of an infectious process or dysbiosis associated with bacterial colonisation, with higher concentrations of IL-6 and IL-8 in samples successfully sequenced. BALs sampling more distally in the lungs verified these results suggesting pulmonary infection in preterm infants may be more common than clinically suspected.

Comparing the three anatomical sites sampled revealed significant differences in bacterial community structure suggesting that these are separate bacterial niches.

Also presented is work analysing the effect of extracellular apoptosis-associated speck-like protein containing a caspase activation domain (ASC), a protein involved in the initiation and propagation of the innate immune response. ASC was demonstrated to be present and bioactive in BAL supernatant from preterm infants. ASC induced IL-6 and IL-8 secretion in cell culture models and is a potential therapeutic target for preventing CLD in preterm infants.

Acknowledgements

I would first like to thank the parents of the infants who gave permission for their new-born baby to be enrolled in this study. Without this selfless decision, taken at a very stressful time, this work would not have been possible.

I would also like to thank my supervisors Prof Sailesh Kotecha and Prof Julian Marchesi. Prof Kotecha strongly encouraged me to pursue research, and without this I would never have started this project. Without his support, direction and encouragement I would never have finished it. Prof Marchesi has given his time and expertise to bring an important non-clinical perspective to the project.

Thanks go to Prof Kathy Triantafilou and Dr Martha Triantafilou for providing direction and advice with my work on innate immune system activation.

Dr Eamon McGreal and Dr Brad Spiller provided help in turning a clinician into a scientist along with Dr Matthew Stott who helped me with laboratory techniques and demonstrated a lot of patience.

Without the help of collaborators this work would have been impossible. Dr Richard Wach worked relentlessly as the principle investigator at the Bristol recruiting site. I will be forever grateful for his efforts in recruiting infants and collecting samples. I would like to thank Prof Nigel Klein, Dr Dagmar Alber and Dr Grace Logan from the Institute of Child Health laboratories, University College London for their work on 16S rRNA gene sequencing. For supplying and extracting DNA from the tracheal aspirate samples in Newcastle I would like to thank Dr Janet Berrington, Dr Clare Lanyon and Dr Greg Young. Prof Kate Fitzgerald kindly supplied the plasmid to enable rASC production.

Particular thanks goes to my colleagues in the Department of Child Health namely Dr Sarah Kotecha, Dr John Lowe, Dr John Watkins, Kylie Hart, Dr Michael Cousins, Louise Yendle and Gillian Willets for the support and encouragement in the good times and the bad. Dr Emma Mitchell deserves a special mention for putting up with me and assisting me as we worked through our PhD's together.

The team in the MMI group were instrumental in helping me understand microbiology, master laboratory techniques and provide suggestions for the project as it evolved. Dr Laura Osbourne, Dr Rebecca Weiser, Dr Jane Mikhail, Dr Ann Smith, Alexandra Campbell, Dr Guilia Masetti, Dr Gordon Webster, Dr Matthew Bull, Dr Richard Brown and Duaa Al-Dulaimy were all instrumental.

I cannot forget my wonderful family. My wonderful wife Dr Jennifer Miller-Gallacher has made many sacrifices to allow me to complete this thesis. She has been a pillar of support throughout this project and I could not have done it without her. She is an amazing mother to our two wonderful children, Hannah and Lucy, who have been born during my research. My parents, Professor John and Anne Gallacher, continue to love me unconditionally and are always there to support us as a family. My brother Dan used his expertise to provide helpful emergency statistics advice when I could not make R work.

Finally, I want to thank the faithful and loving God in whom I choose to trust and without whom nothing in this life holds any meaning.

Abbreviations

ASC	Apoptosis-associated speck-like protein containing a caspase activation domain
BAL	Bronchoalveolar lavage
BSA	Bovine serum albumin
BW	Birth weight
CAPS	Cyropyrin associated periodic syndromes
CBA	Cytometric bead array
CD	Cluster of differentiation
CF	Cystic fibrosis
CHAPS	3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate
CI	Confidence interval
CLD	Chronic lung disease
COPD	Chronic obstructive pulmonary disease
CRIB-II	Clinical risk index for babies (scoring system 2)
CRP	C-reactive protein
CRISPR	Clustered regularly interspaced short palindromic repeats
CS	Caesarean section
CXCL-8	Chemokine (C-X-C motif) ligand 8
DAMP	Danger associated molecular patterns
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EBM	Expressed breast milk
ECL	Electrochemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ELBW	Extremely low birth weight
ELISA	Enzyme-linked immunosorbent assay
ET	Endotracheal
FasR	Fas receptor
FCS	Fetal calf serum
HRP	Horseradish peroxidase
IL	Interleukin
IPTG	Isopropyl β-D-1-thiogalactopyranoside

IQR	Interquartile range
IRF3	Interferon regulatory factor 3
Iκβ	Inhibitor of NF-κB
LPS	Lipopolysaccharide
MyD88	Myeloid differentiation primary response gene 88
NBT	North Bristol NHS Trust
NEC	Necrotising enterocolitis
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NG	Nasogastric
NGS	Next generation sequencing
NLR	Nod like receptor
NLRP	Nod like receptor contain a pyrin domain
NMDS	Non-metric multidimensional scaling
NP-40	Nonyl phenoxypolyethoxylethanol 40
NPA	Nasopharyngeal aspirate
OR	Odds ratio
OTU	Operational taxonomic unit
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen associated molecular patterns
PAS	Protein A sepharose
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDA	Patent ductus arteriosus
PERMANOVA	Permutational multivariate analysis of variance
PRR	Pattern recognition receptors
PYCARD	PYD and CARD Domain containing protein
qPCR	Quantitative polymerase chain reaction
RDP	Ribosomal database project
RDS	Respiratory distress syndrome
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute media

rRNA	Ribosomal ribonucleic acid
RVI	Royal Victoria Infirmary (Newcastle)
S	Svedberg units
SCFA	Short chain fatty acids
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SE	Standard error
siRNA	Specific interfering ribonucleic acid
TA	Tracheal aspirate
TE	Tris EDTA
TGF	Transforming growth factor
TIR	Toll/IL-1 receptor
TLR	Toll like receptor
TNF	Tumour necrosis factor
UHW	University Hospital of Wales
UNG	Uracil-DNA glycosylase
VEGF	Vascular endothelial growth factor
VLBW	Very low birth weight

Table of Contents

THESIS OVERVIEW ..... **I**

ACKNOWLEDGEMENTS ..... **II**

ABBREVIATIONS ..... **III**

TABLE OF CONTENTS ..... **VI**

TABLE OF FIGURES ..... **XII**

1 INTRODUCTION **1**

1.1 INTRODUCTION 1
1.2 PREMATURITY 1
1.3 CHRONIC LUNG DISEASE OF PREMATURITY 1
1.3.1 Introduction *1*
1.3.2 Normal lung development *3*
1.3.2.1 Embryonic. 3
1.3.2.2 Pseudoglandular 3
1.3.2.3 Cannalicular 3
1.3.2.4 Saccular 3
1.3.2.5 Alveolar 3
1.3.3 Old and new chronic lung disease of prematurity *4*
1.3.4 Risk factors for chronic lung disease of prematurity *4*
1.3.4.1 Gestation 4
1.3.4.2 Intrauterine growth gestriction 5
1.3.4.3 Chorioamnionitis 5
1.3.4.4 Postnatal infection 5
1.3.4.5 Ventilation 6
1.3.4.6 Inspired oxygen concentration 7
1.3.4.7 Inflammation 7
1.3.4.8 Patent ductus arteriosus (PDA) and fluid balance 8
1.3.4.9 Genetics 8
1.3.5 Lung inflammation in chronic lung disease of prematurity *8*
1.3.5.1 Initiation 8
1.3.5.2 Cellular response 10
1.3.5.3 Resolution of inflammation 11
1.3.6 Summary *12*
1.4 THE MICROBIOME 13
1.4.1 Introduction *13*
1.4.2 Taxonomic classification *13*
1.4.3 The 16S ribosome *13*
1.4.4 DNA sequencing *15*
1.4.5 Sequencing the 16S rRNA gene *17*
1.4.6 Microbiota identification workflow *17*
1.4.7 Microbiome statistical analysis *19*
1.4.8 Microbiome and disease *20*
1.4.9 The human gut microbiota in preterm infants *21*
1.4.9.1 Acquisition of the preterm gut microbiota 22
1.4.9.2 Necrotising enterocolitis 23
1.4.10 Summary *23*
1.5 THE RESPIRATORY MICROBIOME 23
1.5.1 Introduction *23*
1.5.2 Healthy adult respiratory microbiota *24*
1.5.3 Source of the respiratory microbiota *25*
1.5.4 Comparison of upper and lower airways microbiota *26*

1.5.5 Respiratory microbiome influence over pulmonary health ... 27
1.5.5.1 Cystic fibrosis ... 27
1.5.5.2 Chronic obstructive pulmonary disease ... 27
1.5.5.3 Asthma ... 28
1.5.6 Respiratory microbiota in new-born infants ... 28
1.5.6.1 Antenatal colonisation ... 28
1.5.6.2 Respiratory colonisation in the neonatal period ... 29
1.5.6.3 The constituents of the airway microbiota in the neonatal period ... 30
1.5.6.4 Factors affecting the respiratory colonisation in the neonatal period ... 31
1.5.6.5 Lung microbiota and chronic lung disease of prematurity ... 32
1.5.7 Host-microbiome interactions ... 34
1.5.7.1 Host influence over microbiome communities ... 34
1.5.7.2 Microbiome influences on host immunity ... 35
1.5.8 Summary ... 36
1.6 THE INNATE IMMUNE SYSTEM ... 37
1.6.1 Introduction ... 37
1.6.2 Pattern recognition receptors of the innate immune system ... 37
1.6.2.1 Toll-like receptors ... 37
1.6.2.2 TLR expression ... 40
1.6.2.3 TLR signalling ... 40
1.6.3 Nod-like receptors ... 42
1.6.4 The inflammasome ... 42
1.6.4.1 Introduction ... 42
1.6.4.2 The NLRP3 inflammasome ... 42
1.6.4.3 Activation of the NLRP3 inflammasome ... 43
1.6.5 Apoptosis-associated speck-like protein containing a caspase-recruitment domain ... 45
1.6.5.1 Extracellular ASC ... 45
1.6.5.2 Extracellular ASC specks and disease ... 46
1.6.6 Inflammasomes in lung inflammation ... 46
1.6.7 Innate immunity in new-born infants ... 47
1.6.7.1 Toll-like receptors in new-born infants ... 48
1.6.7.2 NLRs/Inflammasomes ... 48
1.6.7.3 Cellular Responses ... 49
1.6.8 Summary ... 49
1.7 BRONCHOALVEOLAR LAVAGE ... 49
1.7.1 Introduction ... 49
1.7.2 Safety ... 49
1.7.3 Reliability ... 50
1.7.4 Comparing bronchoalveolar lavage and tracheal aspirate samples ... 51
1.7.5 Summary ... 52
1.8 SUMMARY ... 52
1.9 AIMS AND HYPOTHESES ... 53
1.9.1 Hypotheses ... 53
1.9.3 Specific aims: ... 54

2 MATERIALS AND METHODS ... 57

2.1 PATIENT RECRUITMENT ... 57
2.2 SAMPLE COLLECTION ... 58
2.2.1 Bronchoalveolar lavage ... 58
2.2.2 Tracheal aspirate ... 58
2.2.3 Nasopharyngeal aspirate ... 59
2.3 PROCESSING OF SAMPLES ... 59
2.3.1 Cardiff ... 59
2.3.1.1 Bronchoalveolar lavage ... 59
2.3.1.2 Nasopharyngeal aspirate ... 60
2.3.1.3 Tracheal aspirate ... 60
2.3.2 Bristol ... 60

2.3.2.1 Nasopharyngeal aspirate and tracheal aspirate samples 60
2.4 DIFFERENTIAL CELL COUNTS 61
2.5 SOLUTIONS 62
2.5.1 Phosphate buffered saline 62
2.5.2 Sodium dodecyl sulphate solution 62
2.5.3 Sample reducing buffer for SDS-polyacrylamide gel electrophoresis (PAGE) 62
2.5.4 Phosphate buffered saline-Tween 62
2.5.5 SDS-PAGE running buffer 62
2.5.6 Western blotting transfer buffer 62
2.5.7 Stripping buffer 62
2.5.8 Sodium phosphate buffer 63
2.5.9 DNA extraction buffer 63
2.5.10 Hi-Trap column deactivation buffers 63
2.5.11 Hi-Trap column elution buffer 63
2.5.12 Hi-Trap column coupling buffer 63
2.5.13 STET buffer 63
2.5.14 ELFO buffer 64
2.5.15 NP-40 lysis buffer 64
2.5.15.1 NET buffer 64
2.5.15.2 600 mM CHAPS solution 64
2.5.15.3 10 mM iodoacetamide 64
2.5.15.4 10% (w/v) NP-40 64
2.6 DNA EXTRACTION FROM RESPIRATORY SAMPLES 64
2.6.1 Cardiff and Bristol samples 64
2.6.2 Newcastle samples 66
2.7 DNA QUANTIFICATION 66
2.8 PREPARATION OF *E. COLI* DNA FOR USE IN QPCR 67
2.9 QUANTIFICATION OF BACTERIAL LOAD BY QPCR 68
2.10 SEQUENCING OF BACTERIAL 16S RRNA GENE 70
2.10.1 qPCR inhibition check 70
2.10.2 Amplification of the 16S rRNA gene using barcoded primers and adaptors 71
2.10.3 Purification of PCR product 73
2.10.4 Quantification of PCR product and library pooling 74
2.10.5 Quantification and quality checking of library 74
2.10.6 Denaturing, diluting and loading of library into MiSeq 75
2.10.7 Post sequencing data processing 75
2.11 ENZYME-LINKED IMMUNOSORBENT ASSAYS 78
2.11.1 IL-6 ELISA 78
2.11.2 IL-8 ELISA 80
2.12 WESTERN BLOTTING 81
2.12.1 Stripping and re-probing membrane 82
2.12.2 Antibodies and reagents used in Western blotting 82
2.12.3 Densitometry of Western blot images 83
2.13 CELL CULTURE AND STIMULATIONS 84
2.13.1 Maintenance of cells in culture 84
2.13.2 Cell stimulations 85
2.13.3 Measurement of cytokines in supernatant of cell stimulations 86
2.14 IMMUNOPRECIPITATION OF ASC FROM POOLED BRONCHOALVEOLAR LAVAGE SUPERNATANT 86
2.15 PURIFICATION OF RASC 87
2.15.1 Growing E. coli containing ASC plasmid 87
2.15.2 Purification by immunoaffinity chromatography 88
2.15.3 Determining protein concentration 89
2.15.4 Determining lipopolysaccharide contamination of rASC Solution 89
2.16 ATTEMPTS TO KNOCK DOWN OF TLRS IN MAMMALIAN CELLS 90
2.16.1 Transformation of competent E. coli cells 90

2.16.2 Purification of plasmid DNA using a phenol-chloroform DNA extraction 90
2.16.3 Transfection of mammalian cells 91
2.17 STATISTICAL ANALYSIS 91
2.1 CONTRIBUTIONS OF COLLABORATORS 92

3 BACTERIAL COLONISATION OF THE LOWER AIRWAYS AND LUNGS OF INTUBATED PRETERM INFANTS 95

3.1 INTRODUCTION 95
3.2 AIMS 96
3.3 PATIENT CHARACTERISTICS 97
3.4 TRACHEAL ASPIRATE SAMPLES FROM NEWCASTLE 98
3.5 SAMPLES FROM CARDIFF AND BRISTOL 100
3.6 SEQUENCING RESULTS FROM TRACHEAL ASPIRATE SAMPLES 101
3.7 BACTERIAL LOAD OVER TIME 102
3.8 TAXONOMY OF BACTERIAL ORGANISMS IDENTIFIED IN TRACHEAL ASPIRATE SAMPLES 103
3.9 EFFECTS OF ANTIBIOTICS ON TRACHEAL ASPIRATE SAMPLES 107
3.10 INFLAMMATION ASSOCIATED WITH BACTERIAL COLONISATION 109
3.11 B-DIVERSITY TO COMPARE INDIVIDUAL INFANTS 112
3.12 COMPARING COLONISATION IN DIFFERENT CENTRES 112
3.12.1 Bacterial load differences. 113
3.12.2 Comparing bacterial community structure 113
3.12.3 Comparison of individual genera between sites 114
3.12.4 Comparing alpha diversity indices 115
3.12.5 Sensitivity analysis removing infants culture positive for Acinetobacter baumannii 115
3.13 EFFECT OF DELIVERY MODE ON AIRWAY COLONISATION 117
3.14 SUMMARY OF TRACHEAL ASPIRATE SAMPLE RESULTS 118
3.15 BRONCHOALVEOLAR LAVAGE SAMPLES 118
3.16 PATIENT RECRUITMENT 118
3.17 BRONCHOALVEOLAR LAVAGE SAMPLE COLLECTION AND PROCESSING 119
3.19 FACTORS ASSOCIATED WITH FAILURE TO AMPLIFY BACTERIAL DNA 121
3.20 PRO-INFLAMMATORY RESPONSE ASSOCIATED WITH BACTERIAL COLONISATION OF THE LUNGS 124
3.21 BACTERIAL LOAD WITHIN BRONCHOALVEOLAR LAVAGE SAMPLES 126
3.22 SEQUENCING RESULTS FROM BRONCHOALVEOLAR LAVAGE SAMPLES 127
3.23 COMPARING QPCR AND SEQUENCING RESULTS 133
3.24 EFFECT OF MODE OF DELIVERY ON BRONCHOALVEOLAR LAVAGE RESULTS 133
3.25 CHANGES IN DIVERSITY WITHIN THE LUNGS OF INTUBATED PRETERM INFANTS OVER TIME 134
3.26 B-DIVERSITY OF BRONCHOALVEOLAR LAVAGE SAMPLES 136
3.27 BACTERIAL LOAD AND CYTOKINE CONCENTRATIONS IN INDIVIDUAL INFANTS 138
3.28 AVERAGE IL-6 AND IL-8 CONCENTRATION IN BRONCHOALVEOLAR LAVAGE SUPERNATANT OVER TIME 141
3.29 HIGHEST IL-6 AND IL-8 CONCENTRATIONS ASSOCIATED WITH DOMINANCE WITH SPECIFIC GENERA 142
3.30 LUNG COLONISATION PREDICTIVE OF LONG-TERM RESPIRATORY SUPPORT 143
3.31 ATTEMPTING TO IDENTIFY ORGANISMS TO SPECIES LEVEL 144
3.32 DISCUSSION 145
3.32.1 Recruitment 145
3.32.2 Validity of cytokine data from tracheal aspirate samples 146
3.32.3 Sequencing data 148
3.32.4 Sequencing results 150
3.32.5 Bacterial load 151
3.32.6 Effect of delivery mode on respiratory colonisation 152
3.32.7 Differences between recruitment centres 152
3.32.8 Average cytokine concentration 153
3.32.9 Strengths and weaknesses 153
3.33 CONCLUSION 154

4 BACTERIAL COLONISATION OF THE UPPER AIRWAYS IN PRETERM INFANTS 157

4.1 Introduction 157
4.2 Aims 158
4.3 Recruitment and demographics 158
4.4 Bacterial load within nasopharyngeal aspirate samples 160
4.5 Sequencing data 160
4.6 Samples unable to undergo 16S rRNA gene sequence analysis 161
4.7 Relative abundance of organisms within nasopharyngeal aspirate samples 162
4.8 Changes over time in relative abundance in nasopharyngeal aspirate samples 165
4.9 Effect of delivery mode on nasopharyngeal aspirate samples 168
4.10 Effect of antibiotics on nasopharyngeal colonisation 170
4.11 Effect of gender on nasopharyngeal colonisation 173
4.12 Effect of probiotics on nasopharyngeal colonisation 173
4.13 Effect of location on nasopharyngeal colonisation 175
4.14 Discussion 177
4.14.1 Strengths and weaknesses 179
4.15 Conclusion 179

5 COMPARING THE UPPER AND LOWER AIRWAY MICROBIOTA IN PRETERM INFANTS 182

5.1 Introduction 182
5.2 Aims 182
5.3 Methodology of combining data from different samples 182
5.4 Differences in community structure 183
5.5 Differences in bacterial load 184
5.6 Comparing dominance of individual genera between sample types 185
5.7 Differences in alpha diversity indices 185
5.8 Comparing individual samples 186
5.9 Discussion 188
5.9.1 Validity of sequencing data 188
5.9.2 Colonisation at different anatomical sites 188
5.9.3 Bacterial load comparisons between nasopharyngeal, bronchoalveolar lavage and tracheal aspirate samples 189
5.9.4 Using nasopharyngeal aspirate samples to identify lung pathogens 190
5.9.5 Strengths and weaknesses 191
5.10 Conclusions 191

6 INVESTIGATING EXTRACELLULAR ASC AS A POTENTIAL MECHANISM OF THE EFFECT OF THE MICROBIOTA OVER INNATE IMMUNITY 194

6.1 Introduction 194
6.2 Aims 196
6.3 Identifying inflammasome components within BAL supernatant 196
6.4 ASC and inflammasome components in BAL fluid over time 197
6.5 ASC in BAL fluid has pro-inflammatory stimulatory properties 204
6.6 The pro-inflammatory potential of neonatal bronchoalveolar lavage supernatant on respiratory epithelial cells in culture 204
6.7 The effect of immunoprecipitation of ASC from BAL in cell stimulation experiments 206
6.8 Producing recombinant ASC 208
6.8.1 Thiobarbiturate assay to exclude LPS contamination of rASC 211
6.9 Cell stimulation using ASC depleted BAL with rASC 212
6.10 Attempts to characterise the mechanism of ASC induced cytokine stimulation 214
6.11 Discussion 216
6.11.1 Inflammasome components as a biomarker for CLD within the lungs of preterm infants 216
6.11.2 Producing rASC 218
6.11.3 Cell Stimulations 218
6.11.4 Role of TLRs 220

6.11.5 Direction of further work *221*
6.12 CONCLUSION 222

7 GENERAL DISCUSSION 225

7.1 INTRODUCTION 225
7.2 EARLY BACTERIAL COLONISATION OF THE RESPIRATORY TRACT OF PRETERM INFANTS 225
7.3 MICROBIOTA AND INNATE IMMUNITY 229
7.4 STRENGTHS AND WEAKNESSES 230
7.5 SUGGESTIONS FOR FURTHER RESEARCH 231
7.6 SUMMARY OF MAIN FINDINGS 234

8 REFERENCES 236

9 APPENDIX 271

9.1 APPENDIX 1: ETHICAL APPROVAL FOR SAMPLE COLLECTION 271
9.2 APPENDIX 2: PARENT INFORMATION SHEET AND CONSENT FORM - CARDIFF 275
9.3 APPENDIX 3: PARENT INFORMATION SHEET AND CONSENT FORM - BRISTOL 280
9.4 APPENDIX 4: SEQUENCES OF PRIMERS USED IN 16 BACTERIAL RNA GENE SEQUENCING 285
9.5 APPENDIX 5: SYNTAX FOR MOTHUR V1.39.5 286
9.6 APPENDIX 6: RSCRIPT FOR USE IN R-STUDIO WITH R V3.4.2 289
9.7 APPENDIX 7: PAPERS PUBLISHED DURING RESEARCH 292

Table of Figures

FIGURE 1-1: STAGES OF NORMAL LUNG DEVELOPMENT (ILIODROMITI ET AL. 2013) 4
FIGURE 1-2: SECONDARY STRUCTURE OF THE 16S RRNA OF E. COLI DEMONSTRATING THE 9 HYPERVARIABLE REGIONS (BOLD TYPEFACE) (YARZA ET AL. 2014) 14
FIGURE 1-3: SEQUENCING USING SANGER AND NGS METHODS. 16
FIGURE 1-4: GENERAL WORKFLOW OF A MICROBIOME STUDY, FROM DESIGN TO DATA ANALYSIS. THIS SCHEMATIC IS SPECIFIC FOR TARGET GENE SEQUENCING-BASED STUDIES (E.G. 16S RRNA GENE) (AHO ET AL. 2015). 18
FIGURE 1-5: TLRS FOUND IN HUMANS AND THEIR LIGANDS. 38
FIGURE 1-6 TLR SIGNALLING PATHWAYS DEMONSTRATING THE MYD88-DEPENDENT AND MYD88-INDEPENDENT PATHWAYS (CARMODY AND CHEN 2007). 41
FIGURE 1-7: THE STRUCTURE OF THE NLRP3 INFLAMMASOME (B. K. DAVIS, WEN, AND TING 2011) 43
FIGURE 1-8: PRIMING THE NLRP3 INFLAMMASOME FOR ACTIVATION (ELLIOTT AND SUTTERWALA 2015). 44
FIGURE 2-1: IMAGES OF CELL TYPES OBSERVED FROM CYTOSPINS OF BAL SAMPLES. 61
FIGURE 2-2: IMAGE OF GEL ELECTROPHORESIS OF DNA EXTRACTED USING BEAD BEATING + MAXWELL 16 INSTRUMENT 66
FIGURE 2-3: EXAMPLE OF GENERATION OF STANDARD CURVE FROM QPCR EXPERIMENT. 70
FIGURE 2-4: STRUCTURE OF PRIMERS USED TO AMPLIFY THE V3-V4 REGION OF THE BACTERIAL 16S RRNA GENE IN PREPARATION FOR PAIRED END SEQUENCING 71
FIGURE 2-5: PRIMER LAYOUT FOR SEQUENCING OF SAMPLES WITHIN A 96 WELL PLATE RESULTING IN A UNIQUE PRIMER COMBINATION FOR EACH WELL 73
FIGURE 2-6: GRAPHICAL RESULTS OUTPUT FROM TAPESTATION. 74
FIGURE 2-7: SUMMARY OF WORKFLOW IN MOTHUR V1.39.5 FOR PROCESSING OF SEQUENCING DATA 76
FIGURE 2-8: SCHEMATIC DIAGRAM DEMONSTRATING THE PROCESS OF ELISA ASSAY (R&D-SYSTEMS 2005) 78
FIGURE 2-9: STANDARD CURVE GENERATED FROM ELISA EXPERIMENT MEASURING IL6 CONCENTRATION IN BAL AND TA SUPERNATANTS. R^2 VALUES FOR THE CURVE-FIT OF THE STANDARD CURVE WERE ALWAYS >0.99. 80
FIGURE 2-10: STANDARD CURVE GENERATED FROM ELISA EXPERIMENT MEASURING IL8 CONCENTRATION WITHIN BAL AND TA SAMPLES. R^2 VALUES FOR THE CURVE FIT OF THE STANDARD CURVE WERE ALWAYS >0.99. 81
FIGURE 2-11: DENSITOMETRY OF WESTERN BLOT IMAGE USING IMAGE J SOFTWARE. 84
FIGURE 2-12: PURIFICATION OF RASC BY IMMUNOAFFINITY CHROMATOGRAPHY 88
FIGURE 2-13: STRUCTURE OF SIRNA PLASMID INCORPORATING ZEOCIN RESISTANCE GENE 90
FIGURE 3-1: PHYLUM LEVEL ABUNDANCE OF ORGANISMS PRESENT IN TA SAMPLES FROM THE COHORT OF PATIENTS FROM NEWCASTLE SHOWING CONTAMINATION OF THE 2ND AND 3RD BATCHES OF DNA EXTRACTION. ARROWS INDICATE THE DNA EXTRACTION CONTROL SAMPLES. NO AMPLIFICATION WAS SEEN IN THE EXTRACTION CONTROL SAMPLE FROM BATCH 1. 99
FIGURE 3-2: NMDS PLOT OF TA SAMPLES FROM NEWCASTLE COLOURED BY BATCH OF DNA EXTRACTION. SAMPLES ORDINATED BY BRAY-CURTIS DISSIMILARITY INDEX. STRESS VALUE = 0.144. 100
FIGURE 3-3: FLOW-CHART OF TA SAMPLE ANALYSIS 101
FIGURE 3-4: HEATMAP DEMONSTRATING JUSTIFICATION FOR EXCLUSION OF A SINGLE SAMPLE DUE TO CO-CLUSTERING WITH NEGATIVE CONTROL SAMPLES 102
FIGURE 3-5: COMPARING THE PRESENCE OF DETECTABLE BACTERIAL DNA BY TWO METHODS IN TRACHEAL ASPIRATE SAMPLES FROM INTUBATED PRETERM INFANTS. A – AVERAGE BACTERIAL LOAD AGAINST TIME. B – PERCENTAGE OF SAMPLES THAT WERE SUCCESSFULLY AMPLIFIED FOR DNA SEQUENCING AGAINST TIME. 103
FIGURE 3-6: RELATIVE ABUNDANCE OF BACTERIAL PHYLA WITHIN TA SAMPLES FROM INTUBATED PRETERM INFANTS BORN AT <32 WEEKS GESTATIONAL AGE. 105
FIGURE 3-7: RELATIVE ABUNDANCE OF BACTERIAL GENERA WITHIN TA SAMPLES FROM INTUBATED PRETERM INFANTS <32 WEEKS GESTATIONAL AGE. BACTERIAL PHYLA IDENTIFIED BY SEQUENCING OF THE 16S RRNA GENE. SAMPLES ARE DISPLAYED COMBINED IN THREE GROUPS - THE FIRST WEEK OF LIFE, SECOND WEEK OF LIFE AND THIRD/FOURTH WEEK OF LIFE. 106
FIGURE 3-8: EFFECT OF ANTIBIOTICS ON ABILITY TO AMPLIFY BACTERIAL DNA FROM TRACHEAL ASPIRATE SAMPLES FROM PRETERM INFANTS. 107
FIGURE 3-9: EFFECT OF ANTIBIOTICS ON PHYLUM AND GENUS ABUNDANCE WITHIN TA SAMPLES FROM PRETERM INFANTS. SAMPLES TAKEN ON ANTIBIOTICS N = 220, SAMPLES TAKEN OFF ANTIBIOTICS N = 58. 108
FIGURE 3-10: EFFECT OF ANTIBIOTICS ON ALPHA DIVERSITY MEASURES IN TA SAMPLES FROM PRETERM INFANTS. 109

FIGURE 3-11: AMPLIFIABLE BACTERIAL DNA IS ASSOCIATED WITH HIGHER IL-6 AND IL-8 CONCENTRATION IN TA SAMPLES 110
FIGURE 3-12: PARALLEL INCREASES IN IL-6, IL-8 CONCENTRATIONS AND BACTERIAL LOAD IN TA SAMPLES FROM FOUR INDIVIDUAL INFANTS 111
FIGURE 3-13: NMDS PLOT OF TA SAMPLES ORDINATED USING BRAY-CURTIS DISSIMILARITY INDEX. 112
FIGURE 3-14: COMPARING BACTERIAL LOAD ON TA SAMPLES THAT WERE SUCCESSFULLY SEQUENCED FOR 16S RRNA GENE BETWEEN TWO RECRUITMENT CENTRES. 113
FIGURE 3-15: NMDS PLOT COMPARING THE COLONISATION PATTERNS BETWEEN THE 2 RECRUITING CENTRES, STRESS VALUE = 0.18. PERMAOVA R^2 = 0.044, P=0.026. 114
FIGURE 3-16: COMPARING THE MEAN PROPORTION OF THE MOST ABUNDANT 10 GENERA IN TA SAMPLES TAKEN BETWEEN 2 RECRUITING CENTRES. NUMBER OF SAMPLES: UHW = 21, NBT = 26. 114
FIGURE 3-17: EFFECT OF RECRUITMENT CENTRE ON ALPHA DIVERSITY MEASURES, NBT N=26 UHW N=21. 115
FIGURE 3-18: NMDS PLOT OF TA SAMPLES FROM INFANTS NOT COLONISED WITH ACINETOBACTER BAUMANNII. ORDINATED BY BRAY-CURTIS DISSIMILARITY INDEX. STRESS = 0.195. PERMANOVA R^2 = 0.058, P=0.027. UHW N=11, NBT N=26. 116
FIGURE 3-19: DIFFERENCE IN COLONISATION OF INDIVIDUAL GENERA BETWEEN RECRUITMENT SITE WITH INFANTS CULTURE POSITIVE FOR ACINETOBACTER BAUMANNII EXCLUDED. UHW N=11, NBT N=26. 117
FIGURE 3-20: EFFECT OF DELIVERY MODE ON THE MEAN PROPORTION OF THE MOST ABUNDANT 10 GENERA IN TA SAMPLES. VAGINAL DELIVERY N=36, CAESAREAN DELIVERY N=11. 117
FIGURE 3-21: FLOW CHART DEMONSTRATING SAMPLES COLLECTED AND ANALYSED DURING THE STUDY 120
FIGURE 3-22: COMPARING PERCENTAGE OF SAMPLES CONTAINING AMPLIFIABLE BACTERIAL DNA BETWEEN SAMPLES TAKEN ON AND OFF ANTIBIOTICS IN ALL BAL SAMPLES COLLECTED. 121
FIGURE 3-23: PERCENTAGES OF BAL SAMPLES WITH AMPLIFIABLE BACTERIAL DNA BY DAY OF LIFE 122
FIGURE 3-24: IL-6 AND IL-8 CONCENTRATION BETWEEN SAMPLES POSITIVE AND NEGATIVE FOR DETECTABLE BACTERIAL DNA 124
FIGURE 3-25: ASSOCIATION OF SEPSIS MARKERS WITH BAL SAMPLES POSITIVE FOR BACTERIAL DNA 125
FIGURE 3-26: BACTERIAL LOAD MEASUREMENTS BY QPCR IN BAL SAMPLES OF PRETERM INFANTS. 126
FIGURE 3-27: AVERAGE BACTERIAL LOAD OVER TIME IN BAL AND TA SAMPLES 127
FIGURE 3-28: RELATIVE ABUNDANCE OF BACTERIAL PHYLA WITHIN BAL SAMPLES BY DAY OF LIFE. 131
FIGURE 3-29: RELATIVE ABUNDANCE OF INDIVIDUAL GENERA WITHIN BAL SAMPLES FROM PRETERM INFANTS, USING ONLY THE TOP 20 GENERA TO GENERATE FIGURE. 132
FIGURE 3-30: COMPARISON OF SAMPLES POSITIVE FOR 16S RRNA GENE BY DIFFERENT METHODOLOGIES 133
FIGURE 3-31: PROPORTION OF INDIVIDUAL PHYLUM AND GENERA WITHIN BAL SAMPLES FROM PRETERM INFANTS COMPARING DIFFERENCE BY MODE OF DELIVERY 134
FIGURE 3-32: ALPHA DIVERSITY CHANGES OVER TIME IN BAL SAMPLES 135
FIGURE 3-33: NMDS PLOTS OF INDIVIDUAL BAL SAMPLES ORDINATED BY BRAY-CUTIS DISSIMILARITY INDEX. STRESS=0.16. 137
FIGURE 3-34: PAIRED GRAPHS SHOWING CYTOKINE CONCENTRATION AND BACTERIAL LOAD FOR INDIVIDUAL INFANTS OVER TIME 139
FIGURE 3-35: AVERAGE IL-6 AND IL-8 CONCENTRATION IN BAL SUPERNATANT FROM INTUBATED PRETERM INFANTS. 141
FIGURE 3-36: IL-6 CONCENTRATION IN BAL FLUID SUPERNATANT BY DOMINANT BACTERIAL GENUS IDENTIFIED IN THE SAMPLE 142
FIGURE 3-37: IL-8 CONCENTRATION IN BAL FLUID SUPERNATANT BY DOMINANT BACTERIAL GENUS IDENTIFIED IN THE SAMPLE 143
FIGURE 3-38: MEAN PROPORTIONS OF INDIVIDUAL GENERA IN BAL SAMPLES PREDICTIVE OF RESPIRATORY SUPPORT REQUIREMENT AT 36 WEEKS CORRECTED GESTATIONAL AGE. 144
FIGURE 3-39: COMPARING IL-6 AND IL-8 CONCENTRATIONS IN BAL AND TA SAMPLES TAKENWITHIN 24HOURS OF EACH OTHER FROM VENTILATED PRETERM INFANTS 147
FIGURE 4-1: AVERAGE BACTERIAL LOAD WITHIN NPA SAMPLES FROM PRETERM INFANTS BY DAY OF LIFE. 160
FIGURE 4-2: BOXPLOT OF COPY NUMBER OF 16S RRNA GENE CONCENTRATION AGAINST NPA SAMPLES THAT AMPLIFIED ADEQUATELY FOR SEQUENCING. 161
FIGURE 4-3: PERCENTAGE OF ALL NPA SAMPLES TAKEN THAT HAD SUCCESSFUL BACTERIAL 16S RRNA GENE AMPLIFICATION AND SEQUENCING. CHI-SQUARED P<0.001. 162
FIGURE 4-4: RELATIVE ABUNDANCE OF ORGANISMS WITHIN NPA SAMPLES AT PHYLUM LEVEL. 163

Figure 4-5: Relative abundance of genera within NPA samples..... 164
Figure 4-6: Average abundance of individual phyla within NPA samples over time 165
Figure 4-7: Changes in proportion of Staphylococcus, Enterobacteriaceae (unclassified) and Corynebacterium over time in NPA samples from preterm infants. (Staphylococcus p=0.024, Enterobacteriaceae (unclassified) p= 0.029, Corynebacterium p=$1.62x10^{-4}$)..... 166
Figure 4-8: Changes in alpha diversity over time in bacteria detected within NPA samples from preterm infants. 168
Figure 4-9: Differences in microbiota in NPA samples between infants delivered by caesarean section and vaginally delivered infants. 169
Figure 4-10: Effect of delivery mode over early colonisation patterns in NPA samples from preterm infants. 170
Figure 4-11: Effect of antibiotics on microbiota profiles in NPA samples from preterm infants at phylum and genus level. 171
Figure 4-12: Effect of antibiotics on alpha diversity indices in NPA samples. 172
Figure 4-13: Differences in individual phyla and genera comparing samples from male and female preterm infants 173
Figure 4-14: Bacterial Load in NPA samples taken from UHW site showing the difference between samples taken when infant receiving oral probiotic preparation compared to when not receiving oral probiotic. 174
Figure 4-15: Comparing the relative abundance of bacteria at phylum and genus level between NPA samples from preterm infants collect on and off oral probiotic..... 175
Figure 4-16: Effect of recruitment centre on microbiota of NPA samples from preterm infants..... 176
Figure 5-1: NMDS plot comparing bacterial community structure from 3 different sites within the respiratory tract..... 183
Figure 5-2: Comparing average bacterial load over time in NPA, BAL and TA samples that were successfully amplified for sequencing of the 16S rRNA gene collected from preterm infants. Error bars indicate standard error. 184
Figure 5-3: Comparing alpha diversity indices between samples from different sites within the respiratory tract of preterm infants..... 186
Figure 5-4: Bar plot comparing abundance of most abundant genus present within TA and NPA samples taken from the same infant within the 24 hours of each other. 187
Figure 5-5: Paired dyads of BAL and TA samples taken within 24 hours from the same infant showing only top 10 most abundant OTUs. (BAL – Bronchoalveolar lavage, TAF – Tracheal Aspirate Fluid) 188
Figure 6-1: Western blot image demonstrating the detection of extracellular ASC in the supernatant of BAL fluid from preterm infants. 197
Figure 6-2: Example Western blot images using antibodies targeted to inflammasome components in BAL fluid from preterm infants. 198
Figure 6-3: Total protein concentration of BAL supernatant samples from day 1 used in Western blotting199
Figure 6-4: Box-plots of densitometry data obtained from Western blotting of BAL supernatants for inflammasome components by outcome group. All samples taken on day 1 of life. 200
Figure 6-5: Densitometry data from Western blots of inflammasome components comparing preterm and term infants. BAL samples taken on day 1 of life. 202
Figure 6-6: Protein adjusted densitometry scores obtained from Western blotting for inflammasome components on supernatant of BAL samples obtained from day 2 and 7 of life..... 203
Figure 6-7: Cytokine concentration in supernatant of cultured A549 cells and BEAS-2B cells following stimulation overnight with pooled BAL fluid compared with unstimulated controls. 205
Figure 6-8: Representative image of Western-blot to confirm success of immunoprecipitation from pooled BAL fluid from preterm infants. 206
Figure 6-9: Cytokine concentrations in supernatant of cells stimulated with neat BAL and ASC depleted BAL. 207
Figure 6-10: SDS-PAGE gel and Western blot image of purified rASC. 208
Figure 6-11: Cytokine concentrations in supernatant of BEAS-2B cells stimulated with varying concentrations of recombinant ASC 209

FIGURE 6-12: CYTOKINE CONCENTRATIONS IN SUPERNATANT OF A549 CELLS STIMULATED WITH VARYING CONCENTRATIONS OF RECOMBINANT ASC. 210
FIGURE 6-13: STANDARD CURVE GENERATED FROM KNOWN CONCENTRATIONS OF E. COLI LPS IN THE THIOBARBITUATE ASSAY 211
FIGURE 6-14: CYTOKINE CONCENTRATIONS IN SUPERNATANTS FROM BEAS-2B AND A549 CELLS STIMULATED WITH BAL FLUID. 213
FIGURE 6-15: GEL ELECTROPHORESIS OF PURIFIED DNA PLASMIDS FOR THE PRODUCTION OF SIRNA OF TLR2 AND TLR4. 215
FIGURE 6-16: WESTERN BLOT IMAGES OF LYSATES OF A549 CELLS FOLLOWING ATTEMPTED KNOCKDOWN OF TLR 2 AND 4. 216

Chapter 1: Introduction

1 Introduction

1.1 Introduction

In this chapter, the background to Chronic Lung Disease of Prematurity (CLD) will be presented, beginning with the definition of CLD and fetal lung development. The risk factors for CLD and the evidence of inflammatory processes occurring in the lungs which may influence CLD will be summarised. The concept of the microbiome and the effect microorganisms have over human health, particularly in relation to pulmonary disease and in new-born infants will be presented. The published work regarding innate immune system activation and signalling, particularly with reference to Toll-like receptors and inflammasomes will be discussed. The work in this thesis investigates the microorganisms living in the airways and lungs of preterm infants and studies a potential mechanism of innate immune system signalling.

1.2 Prematurity

Preterm birth is classified as delivery before 37 weeks' of gestation. Prematurity is the leading cause of perinatal morbidity and mortality in developed countries (Goldenberg et al. 2008). The rate of preterm birth in England and Wales is 7.1% (EURO-PERISTAT 2010). The rate of preterm birth showed a rising trend in the 1980s and 90s in many countries (Goldenberg et al. 2008; Gray et al. 2007); possibly in part due to a higher rate of multiple pregnancy as a result of increased rates of in-vitro fertilisation and earlier delivery for maternal reasons.

Infants born at less than 28 weeks' of gestational age are classed as extremely preterm. These Infants have the highest rates of complications (Costeloe et al. 2012). Preterm birth is associated with a high mortality rate and long term neurodevelopmental problems among survivors (Blencowe et al. 2013).

1.3 Chronic lung disease of prematurity

1.3.1 Introduction

CLD, also known as bronchopulmonary dysplasia, affects up to 68% of infants born at ≤28 weeks' gestation (Stoll et al. 2010; Laughon et al. 2009). Infants with CLD face lifelong morbidity. Such infants are more likely to be re-admitted to hospital with respiratory symptoms (Greenough 2012); have raised pulmonary pressures throughout infancy (Poon, Edwards, and Kotecha 2013); and display impaired lung function into adulthood (Northway et al. 1990; S. J. Kotecha et al. 2013). CLD affected infants experience a poorer neurodevelopmental outcome compared to infants without CLD (Jeng et al. 2008).

CLD was first described in 1967 as advances in neonatal medicine resulted in preterm born children surviving (Northway, Rosan, and Porter 1967). CLD is defined as a requirement for oxygen supplementation in a preterm infant at 28 days of life or 36 weeks' corrected gestational age. The traditional CLD definition of the need for oxygen supplementation at 28 days of age was challenged by Shennan et al. Oxygen requirement at 36 weeks' corrected gestational age was proposed as an alternative definition for CLD (Shennan et al. 1988). This allows for an oxygen requirement due to lung immaturity rather than CLD for infants born extremely preterm. However, infants born at a more advanced gestational age need oxygen for a shorter period to be diagnosed with CLD.

In 2001 a collaboration between the National Institute for Child Health and Human Development; the National Heart, Lung and Blood Institute and the Office of Rare Diseases agreed on a consensus definition of CLD that included an oxygen requirement for at least 28 days with the concentration of inspired oxygen at 36 weeks' corrected gestational age used to define CLD severity (Alan Jobe and Bancalari 2001). The disease severity classification defining criteria are shown below in Table 1-1.

Table 1-1: The 2001 consensus definition of CLD providing a classification for disease severity (Alan Jobe and Bancalari 2001)**.**

Gestational Age	<32 Weeks'	>32 Weeks'
Time point of assessment	36 weeks' gestational age or discharge home, whichever comes first	> 28 days but <56 days postnatal age or discharge home, whichever comes
	Treatment with oxygen >21% for at least 28 days plus:	
Mild CLD	Breathing room air at 36 weeks' gestational age or discharge, whichever comes first	Breathing room air by 56 days of age or discharge, whichever comes first
Moderate CLD	Need for >30% oxygen at 36 weeks' gestational age or discharge, whichever comes first	Need for >30% oxygen at 56 days of age postnatal age or discharge, whichever comes
Severe CLD	Need for >30% oxygen and/or positive pressure, at 36 weeks' gestational age or discharge, whichever comes first	Need for >30% oxygen and/or positive pressure, at 56 days of age or discharge, whichever comes first

The consensus definition has become the most widely used definition of CLD due to the fact this definition allows stratification of disease severity and prevents lung immaturity from being wrongly misclassified as CLD. The weakness of this definition is the potential for variation in clinical practice between units is defining need for supplemental oxygen. This has led to the development of a physiological definition of CLD with an objective test for oxygen

dependency, an oxygen reduction test, performed on each infant to define need for supplemental oxygen (Walsh et al. 2004).

1.3.2 Normal lung development

Normal lung development can be split into 5 phases (Carlson 2009) as shown in Figure 1-1.

1.3.2.1 Embryonic.

Weeks' 4-7 gestational age. Lung development begins with the formation of the respiratory diverticulum, as a bud from the foregut. This then branches further to form all the major bronchopulmonary segments.

1.3.2.2 Pseudoglandular

Weeks' 8-16 gestational age. The bronchopulmonary branches further subdivide during a period of major growth of the duct system. The pulmonary vasculature begins to develop alongside the developing ducts.

1.3.2.3 Cannalicular

Weeks' 17-26 gestational age. Respiratory bronchioles form from budding of the branches already developed. Capillaries become closely associated with the walls of developing vessels.

1.3.2.4 Saccular

Weeks' 26-36 gestational age. Alveoli begin to branch off respiratory bronchioles. The epithelial lining cells of the alveoli begin to differentiate into specialised adapted cells (type 1 and 2 pneumocytes). The type 2 cells begin to secrete surfactant within the alveoli.

1.3.2.5 Alveolar

Weeks' 36-40 gestational age. Exponential increase in the respiratory surface area of the lung due to rapidly increasing numbers of alveoli present. Alveolar development continues after birth until around 2 years of age.

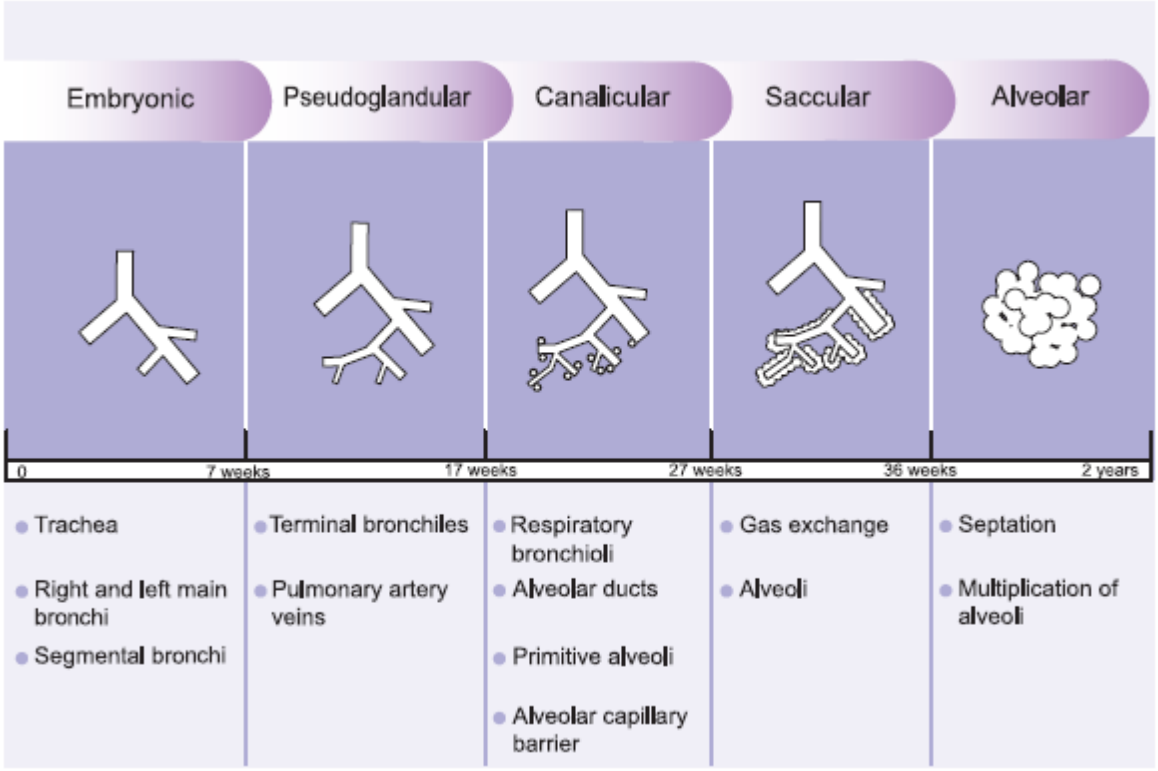


Figure 1-1: Stages of normal lung development **(Iliodromiti et al. 2013)**

1.3.3 Old and new chronic lung disease of prematurity

The original histological description of CLD featured areas of marked fibrosis; varying hyperinflation and atelectasis; severe epithelial lesions and decreased alveolarisation, leading to reduced surface area for gas exchange (Northway, Rosan, and Porter 1967). Since the introduction of antenatal steroids, surfactant, new ventilation strategies and improved nutrition for preterm infants, there is a widely held belief that the pathophysiology of the disease has changed and the term 'new CLD' has become accepted (Kinsella, Greenough, and Abman 2006; A Jobe 2006). Histologically specimens of new CLD infants show less fibrosis, less heterogeneity of lung disease and larger, fewer alveoli than old CLD infants (Alan Jobe 1999). During this evolution, the overall incidence of CLD has remained stable. Infants born at earlier gestations are now surviving routinely. CLD is almost exclusively now seen in infants born at less than 30 weeks' gestation. Previously such preterm infants were not surviving.

1.3.4 Risk factors for chronic lung disease of prematurity

CLD is the resulting pulmonary insufficiency from damage to developing lungs. A diverse range of factors have been shown to contribute to the multifactorial disease process.

1.3.4.1 Gestation

There is no doubt that the risk of CLD increases with increasing prematurity. Infants born at 23 weeks' gestational age have an incidence of CLD of 73%, with 56% of infants having severe

disease (Trembath and Laughon 2012). In contrast, those born at 28 weeks' gestation the incidence of CLD was 23% with only 8% of infants with severe disease (Trembath and Laughon 2012). Alveolar development does not begin until the 26th week of gestation. Damage to the lungs prior to and during this crucial phase may explain why these infants are at greater risk. Male infants have a higher risk of developing CLD as compared to females of the same gestation (C. Bose et al. 2009).

1.3.4.2 Intrauterine growth restriction

A heterogeneous number of maternal and fetal conditions such as pre-eclampsia, maternal smoking and congenital infection and can cause infants to be born small for gestational age. In preterm infants <28 weeks' gestation, small for gestation age infants are at higher risk of CLD than appropriately grown infants (C. Bose et al. 2009).

1.3.4.3 Chorioamnionitis

Chorioamnionitis is the leading cause of preterm birth (Goldenberg, Hauth, and Andrews 2000). Foetuses *in-utero* are able to mount an inflammatory response to chorioamnionitis (Hecht et al. 2011). Injection of endotoxin to the amniotic fluid of sheep, to induce chorioamnionitis in the womb, results in lung inflammation, injury and alveolar remodelling in new-born lambs (Kramer et al. 2002). Antenatal inflammation enhances lung maturation, leading to reduced rates of respiratory distress syndrome (RDS) (Kramer et al. 2009), the name given to respiratory distress seen in many preterm infants primarily caused by surfactant deficiency. However, rates of CLD are increased in infants exposed to histologically confirmed chorioamnionitis (Van Marter et al. 2002) and mean IL-8 levels in tracheobronchial fluid in the first months of life are higher in preterm infants exposed to antenatal infection than those not exposed (Groneck et al. 2001). Chorioamnionitis results in reduced alveolarisation and may increase the risk of CLD. Antenatal infection may help mature the premature lung, but prime the lung for postnatal injury (Chakraborty, McGreal, and Kotecha 2010).

Infection with specific organisms is shown to be associated with higher risk of CLD. *Ureaplasma* is the most commonly cultured organism from placental samples in chorioamnionitis (Sanchez 1993). Injecting *Ureaplasma* into the amniotic fluid of mice induced an inflammatory response and tissue damage within the lungs of the pups (Normann et al. 2009). Human studies have shown conflicting results attempting to demonstrate a causal link between *Ureaplasma* species and CLD (Aaltonen et al. 2006; Beeton et al. 2011; Pandey et al. 2007; Honma et al. 2007). A meta-analysis of 39 studies assessing the association between *Ureaplasma* infection and CLD showed a significant association (Lowe et al. 2014).

1.3.4.4 Postnatal infection

Systemic bacterial infection and localised infection within the lungs are likely to cause damage to lungs of preterm infants contributing to the risk of CLD. The immune response to bacterial

infection is also likely to contribute to the risk of CLD. Systemic blood culture positive sepsis has been identified in several studies as a risk factor for CLD (Klinger et al. 2010; Lahra, Beeby, and Jeffery 2009; Schlapbach et al. 2011). The mechanism for sepsis causing CLD could be due to the systemic immune response affecting lung development or prolonged ventilatory requirements for infants suffering with sepsis.

In neonatal mouse models injection of lipopolysaccharide (LPS), a component of bacterial cell walls, into the lungs to mimic bacterial infection leads to influx of neutrophils to the lungs and LPS-injured lungs show evidence of decreased alveolarisation and reduced total lung volumes (Franco et al. 2002).

Bacterial DNA is detected more frequently in bronchoalveolar lavage (BAL) samples from infants who go on to develop CLD compared to those without CLD. This study also shows an association between detection of bacterial DNA and peak concentrations of pro-inflammatory cytokines suggesting infection rather than colonisation is being detected (Beeton et al. 2011).

Culture independent studies using tracheal aspirates from intubated preterm infants consistently identify the presence of dominant bacterial organisms suggesting possible respiratory infection. These studies are discussed in more detail in Section 1.5.6.5.

Viral pathogens have also been investigated as a potential cause of CLD. Cytomegalovirus is known to cause a pneumonitis when contracted *in-utero* and has been linked to CLD in preterm infants (Sawyer, Edwards, and Spector 1987). The presence of adenovirus within the tracheal aspirates during the first week of life has also been shown to be associated with developing CLD in preterm infants (Couroucli et al. 2000). Subsequent work has, however, conflicted with these findings. Prösch et al shows that although preterm infants are frequently infected with cytomegalovirus and adenovirus, these pathogens are not associated with CLD (Prosch et al. 2002).

One study suggests that postnatal respiratory bacterial infections may not contribute to CLD. Mean IL-8 and IL-1 concentrations in tracheal aspirate samples from infants who developed culture positive respiratory infections are no higher than those obtained from infants with sterile tracheal aspirate samples (Groneck et al. 2001).

1.3.4.5 Ventilation

Any mechanical ventilation has the risk of causing lung damage. Physiologically air is drawn into the lungs by negative pressure. Ventilators use positive pressure to maintain lung expansion and create a tidal volume. The abnormal forces exerted on the lungs result in tissue disruption, inflammation and oedema (Attar and Donn 2002; Brew et al. 2013). Modern ventilation strategies have sought to reduce this damage to lung tissue. Adequate positive end expiratory

pressures aim to prevent atelectotrauma, lower peak pressures (Donn and Sinha 2006) to avoid barotrauma and volume limited ventilation to prevent volutrauma. High frequency oscillated ventilation has been tried as a strategy to reduce CLD with disappointing results (Cools et al. 2009).

1.3.4.6 Inspired oxygen concentration

The effect of exposure to high concentrations of oxygen in preterm infants has long been suspected as a contributor to the pathogenesis of CLD (Northway, Rosan, and Porter 1967). Animal models have demonstrated exposure to excessive inspired oxygen concentrations in the neonatal period can alone result in histological changes similar to those of CLD (Bonikos et al. 1975; D'Angio et al. 1997). Production of reactive oxygen species which trigger an inflammatory response in the premature lung is thought to be the mechanism by which inspired oxygen concentration affects risk of CLD (Iliodromiti et al. 2013). The persistent significant incidence of CLD in preterm infants, despite efforts to limit oxygen exposure, demonstrates that hyperoxia is not a prerequisite for CLD (Chess et al. 2006). A randomised control trial of the antioxidant N-acetylcysteine administered in the first week of life failed to protect against CLD (Ahola et al. 2003). Other antioxidants including intratracheal administration of recombinant super-oxide dismutase demonstrate no effect on the rate of CLD (J. Davis et al. 1997).

Hypoxia can also restrict alveolar development and may contribute to CLD (Burri and Weibel 1971).

1.3.4.7 Inflammation

The result of lung damage from all of the above factors is an inflammatory reaction within the lung. Many pro-inflammatory mediators such as IL-8 and IL-6 have been shown to be raised in bronchoalveolar lavage fluid and tracheal aspirate fluid from infants who went on to develop CLD (S. Kotecha et al. 1995; Chakraborty et al. 2013; Koksal et al. 2012; Schneibel et al. 2013). These cytokines act as chemoattractant to neutrophils and other inflammatory cells.

The inflammatory response in CLD is characterised by infiltration of neutrophils within the alveoli. This response is prolonged in those infants who develop CLD. The effect of inflammation is covered in more detail subsequently.

Corticosteroid therapy, used to suppress pulmonary inflammation, is known to be beneficial for respiratory outcomes in preterm infants. Studies optimising the choice of drug, route of administration, dose and timing of administration of corticosteroids continue to be undertaken due to the adverse neurodevelopmental effects of these drugs (Doyle, Ehrenkranz, and Halliday 2014; Onland et al. 2011; Baud et al. 2016).

1.3.4.8 Patent ductus arteriosus (PDA) and fluid balance

Pulmonary oedema is associated with developing CLD (Brown et al. 1978). PDA results in increased pulmonary blood flow and pulmonary oedema. Serum levels of pro inflammatory cytokine TNF-α are higher in preterm infants with PDA than those without (Gonzalez et al. 1996). Strategies to close a PDA, both surgical and medical, show conflicting results with regard to CLD outcomes (Knight 2001; J. Harris et al. 1982). Fluid overload can have a similar effect as a PDA on pulmonary blood flow. Randomised trials of restricted fluid intake also show conflicting results for improved rates of CLD (Kavvadia et al. 2000; Tammela and Koivisto 1992).

1.3.4.9 Genetics

Understanding any genetic contribution to CLD is complicated by the fact that CLD is associated with prematurity and RDS. Both have genetic components to their aetiology (Parton et al. 2006). Two twin studies have indicated that genetic predisposition contributes significantly to CLD (Bhandari et al. 2006; Lavoie, Pham, and Jang 2008). Candidate genes coding for surfactant proteins and inflammatory pathway constituents have been suggested. However, a genome wide association study failed to identify any single-nucleotide polymorphisms associated with CLD (H. Wang et al. 2013). A polymorphism of VEG-F has been shown to be associated with an increased risk of CLD (Fujioka et al. 2014).

1.3.5 Lung inflammation in chronic lung disease of prematurity

The inflammatory reaction within the neonatal lung is known to be a mechanism for lung injury and a predictor of CLD. A large body of work exists towards understanding the inflammatory mechanisms and cells that are present in the lungs of preterm infants, and the impact these factors have in the pathogenesis of CLD. Modifying the inflammatory response has also been suggested as a possible target for therapeutic agents to prevent CLD.

Roman encyclopaedist Celsus documented the four classic features of inflammation – redness, swelling, heat and pain (Spencer 1936). These are now understood to be due to increased blood flow, raised cellular metabolism, vasodilation, release of soluble mediators, extravasation of fluid and cellular influx (Ferrero-Miliani et al. 2007). Inflammation is part of the non-specific response to any injury to the body or to any pathogenic threat to the body.

All inflammatory processes show an initiation in response to tissue damage or invading pathogen; a sustained response and a resolution phase, or failure of resolution resulting in chronic inflammation.

1.3.5.1 Initiation

Neutrophils, the main effector cell of the innate immune system are seen to enter the lungs of premature infants within the first few hours after birth. The influx of immune cells to the lungs

is important as these cells produce cytokines, proteases and toxic reactive oxygen species (Ryan, Ahmed, and Lakshminrusimha 2008). A decrease in circulating neutrophils at one hour of age in preterm infants is associated, not only with severity of RDS, but also with the development of CLD (Ferreira et al. 2000).

The chemotactic activity for neutrophils of tracheal aspirate fluid from infants with prolonged respiratory distress is higher than those with resolved RDS. The study found higher concentrations of known chemo-attractants in the CLD infants (Groneck et al. 1994). One of the most important neutrophil chemo-attractants is interleukin 8 (IL-8) (also known as CXCL-8). IL-8 concentration is significantly higher within the BAL fluid (S. Kotecha et al. 1995; Beeton et al. 2011) and tracheal aspirates (Munshi et al. 1997) of preterm infants who develop CLD compared to those infants with RDS that resolves. Within the tracheal aspirate the rise in IL-8 concentration precedes the influx of neutrophils (Munshi et al. 1997). IL-8 levels in serum are also predictive of death or CLD in the first few days of life with higher levels associated with adverse outcomes (Ambalavanan et al. 2009).

IL-8 is produced from a 99 amino-acid precursor. Several active forms of IL-8 are produced within humans, each differing by the number of amino acids at the amino terminal. Cells of the immune system secrete IL-8_{72}, while the longer and less potent IL-8_{77} is secreted by non-immune cells. Within the preterm lung the shorter isoforms predominate, despite evidence that the longer form is found within the circulation of term infants (Maheshwari et al. 2009). Development of CLD is associated with a larger proportion of the short isoform (Chakraborty et al. 2014).

IL-8 levels show peaks and troughs within the BAL fluid of preterm infants. Peaks of IL-8 are associated with the presence of the bacterial ribosomal ribonucleic acid (rRNA) gene, suggesting a bacterial infective cause. The presence of these peaks in IL-8 concentration is associated with later development of CLD (Beeton et al. 2011).

IL-6 is a further pro-inflammatory cytokines expressed within the lungs of preterm infants who develop CLD. Concentrations of IL-6 in BAL fluid from intubated preterm infants are highest at 10 days of age (S. Kotecha, Wilson, et al. 1996), but also an early peak has been observed at day 3 of life in tracheal aspirate fluid (Munshi et al. 1997). The pro-inflammatory cytokines IL-1β and TNF-α are also raised within the lungs of infants who develop CLD. Pro-inflammatory cytokines have the effect of up regulating cell adhesion molecules, to facilitate chemotaxis of immune cells, primarily neutrophils, and act along cell signalling pathways to amplify the response of the immune system.

Other powerful neutrophil chemo-attractants that are raised within the lungs of preterm infants who develop CLD include the complement anaphylatoxin C5a and the fatty acid leukotriene B4 (Groneck, Oppermann, and Speer 1993).

1.3.5.2 Cellular response

The influx of neutrophils to the neonatal lung during the initiation phase of inflammation is seen both in those infants who develop CLD and those who do not progress to CLD. By one week of age the neutrophil count of BAL fluid typically has fallen in infants who will recover; however, it is persistently raised in those who will develop CLD (Ogden et al. 1984).

Neonatal neutrophils survive longer than those of adults due to reduced apoptosis. (Ryan, Ahmed, and Lakshminrusimha 2008; S. Kotecha et al. 2003). Neutrophil cell death is mediated via the Fas receptor (FasR). Neonatal neutrophils have reduced expression of FasR compared to adult neutrophils (Hanna et al. 2005). This may prolong the contribution of the neutrophil to the inflammatory process. The BAL fluid of infants who develop CLD has a reduced potential to induce apoptosis in healthy neutrophils compared to BAL fluid from infants who do not develop CLD (S. Kotecha et al. 2003).

Macrophages are important in regulating inflammatory responses. More macrophages are found in the lungs of infants with RDS who do not go on to develop CLD compared to those who do progress to CLD (S. Kotecha et al. 2003). Early preterm delivery is associated with higher number of macrophages, but a lower proportion of macrophages displaying an anti-inflammatory phenotype. The more mature and differentiated macrophage expressing CD14/CD36 or CD17/HLA-DR are considered to be involved in the resolution of inflammation and exert an anti-inflammatory effect. These cells are present in higher proportions in infants who do not have CLD compared to those that develop CLD (Prince et al. 2014). Macrophages activity in regulating and resolving inflammation in the preterm lung is impaired in those with CLD.

Lymphocytes are the primary cells of the adaptive immune system. Activation of lymphocytes is typically associated with chronic inflammation. In CLD ongoing inflammation is witnessed within the lung; however, an influx of lymphocytes is not typically seen. Evidence suggest that lymphocytes may have a role to play in the pathogenesis of CLD. Two studies have performed serial analysis of lymphocyte subpopulations in serum from preterm infants. Ballabh et al identified a significantly lower level of total lymphocytes throughout the first two weeks of life in infants who went on to have CLD compared to those who did not (Ballabh et al. 2003). Both studies identified a role for activated T-cells. CD62L is expressed by immature inactive T cells (Smalley and Ley 2005). CD54 is as adhesion molecule expressed on activation (Picker and Butcher 1992). Ballabh et al showed decreased expression of CD62L on CD4 cells in infants

who went on to have CLD (Ballabh et al. 2003). Turunen et al showed increased CD54 expression on CD4 and CD8 cells from infants who went on to have CLD (Turunen et al. 2009). Both studies suggesting greater activation of lymphocytes is associated with an increased risk of CLD.

1.3.5.3 Resolution of inflammation

Inflammatory responses usually undergo a phase of resolution when repair to tissue damaged during the period of inflammation occurs. Tight control is needed during the repair phase as an excessive remodelling can lead to fibrosis (Magnan et al. 1996). Many studies report an association between growth factors and developing CLD. The transforming growth factor (TGF) family is most studied. TGF-β is involved in regulating the branching seen in the embryonic, pseudo-glandular and canalicular phases of lung development (Bartram and Speer 2004).

TGF-β is present at significantly higher concentrations in the bronchoalveolar lavage and tracheal aspirate fluid of infants who develop CLD compared to those with RDS (S. Kotecha, Wangoo, et al. 1996; Jónsson et al. 2000). Levels peak in the third week of life (Lecart et al. 2000). TGF-β may influence the pathogenesis of CLD during the inflammatory repair phase in several ways. In mice, increased TFG-β1 expression within the lung leads to macrophage and monocyte infiltration (Sureshbabu et al. 2015). Macrophages are known to have an important role in wound healing. Lymphocyte inflammatory responses are reduced by TGF-β (Rook et al. 1986; Kehrl et al. 1986) and an excess of TGF-β known to result in severe pulmonary fibrosis (Sime et al. 1998). In a mouse model of CLD inducing TGF-β expression led to apoptotic cell death in type 2 pneumocytes, impaired alveolarisation and was associated with increased mortality (Sureshbabu et al. 2015).

Vascular endothelial growth factor (VEGF), involved in promoting angiogenesis within the developing lung, has also been implicated in developing CLD. The vascularisation of the lung is now recognised as an important part of CLD development, when previously CLD was considered a disease of the airways and lung parenchyma. Inhibiting angiogenesis is associated with impaired alveolarisation in an animal model (Jakkula et al. 2000). The role of VEGF in CLD was first suspected following the finding that VEGF secretion is decreased by neonatal hypoxia (Maniscalco et al. 1997). VEGF treatment has been shown to improve lung structure in animal models of CLD (Kunig et al. 2005). Evidence in humans suggests that infants who die from CLD have significantly lower levels of VEGF expression in lung tissue compared to infants who die from non-respiratory causes (Bhatt et al. 2001). More recently cord blood levels of a further angiogenic growth factor, placental growth factor, were shown to be associated with CLD, but VEGF levels in cord blood not related to developing CLD (Yang et al. 2015). Many

other growth factors affecting angiogenesis within the lungs have been implicated in developing CLD (De Paepe et al. 2008; Bhatt et al. 2001).

Pulmonary growth factors have also been suggested to play a role in the pathogenesis of CLD including connective tissue growth factor, keratinocyte growth factors and hepatocyte growth factors (Speer 2003).

1.3.6 Summary

Understanding CLD remains an important area for neonatal research. The multifactorial nature of the condition requires a holistic approach to management and prevention. CLD has short- and long-term implications for preterm infants affecting quality of life and life expectancy. The pro-inflammatory reaction occurring in the lungs of intubated preterm infants is crucial in the development of CLD.

1.4 The microbiome

1.4.1 Introduction

The microbiome refers to all the microorganisms, specifically their genomes, and the organisms interactions with the particular context or environment inhabited (Marchesi and Ravel 2015). The term was first used to encompass all pathogenic and commensal organisms existing on a human host including all bacteria, fungi and viruses (Lederberg and McCray 2001). The term microbiota is now used to specifically refer to the organisms themselves (Marchesi and Ravel 2015). The extent of the variety in the composition of the microbiota between individuals and between anatomical sites has been realised since the introduction of culture independent techniques (The Human Microbiome Project Consortium 2012; Costello et al. 2009). The reason for this variety is not understood; however genetic, environmental and geographic factors are all likely to contribute.

1.4.2 Taxonomic classification

All life, including bacteria, can be classified taxonomically from kingdom to species level. An example of taxonomic classification of the bacterial species Escherichia Coli is shown below in Table 1-2.

Table 1-2: Taxonomic classification of Escherichia Coli

Taxonomic Rank	Classification
Kingdom	Bacteria
Phylum	Proteobacteria
Class	Gammaproteobacteria
Order	Enterobacteriales
Family	Enterobacteriaceae
Genus	*Escherichia*
Species	*Coli*

1.4.3 The 16S ribosome

The ribosome is the site of protein synthesis found in all cells. Ribosomal components are measured and identified in Svedberg units (S), based on rate of sedimentation during

centrifugation. The ribosome of the prokaryotic cell is 70S and made up of a large 50S and small 30S subunit. The small unit is further divided containing the 16S subunit. The typical bacterial 16S rRNA gene contains around 1500 nucleotides (Amann, Ludwig, and Schleifer 1995). The gene is divided into 9 hypervariable regions (V1-V9) which demonstrate differences between bacterial species and genera, flanked by conserved regions which are nearly identical in all bacterial species (Chakravorty et al. 2007). Figure 1-2 below demonstrates the secondary structure of the 16S ribosomal RNA following transcription.

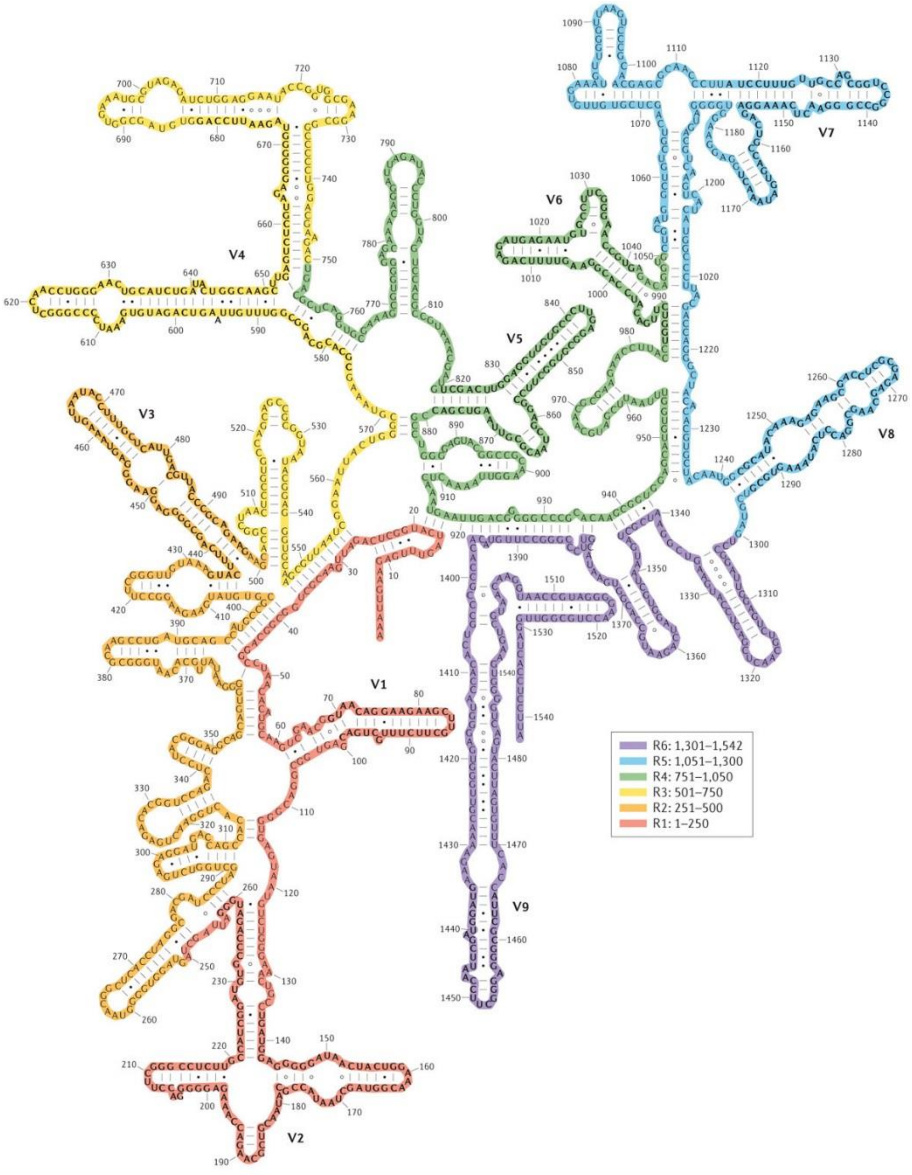


Figure 1-2: Secondary Structure of the 16S rRNA of E. coli demonstrating the 9 hypervariable regions (bold typeface) **(Yarza et al. 2014)**

The availability of polymerase chain reaction (PCR) technology has provided the ability to study bacterial communities not previously possible. Earlier work relied on culture-based methodology. These techniques were designed to identify individual pathogens rather than

bacterial communities. Uncultivatable and slow growing species are difficult to culture in vitro. Estimates of bacterial populations using culture-based techniques inevitably fail to detect uncultivatable organisms despite using multiple types of growth media to maximise the number of species identifiable. An estimated 60-80% of bacteria many not be detected by culture based techniques (Suau et al. 1999). PCR technology amplifying the 16S rRNA gene from all the bacteria present allow identification of all bacteria present in a sample.

Early attempts to establish a system for bacterial identification and classification based on gene sequencing used the 5S RNA molecule (Stahl et al. 1984). The ribosomal gene was initially targeted as the ribosome is an integral component of the prokaryotic cell, making it a good candidate to be well preserved throughout the phylogenic tree (Olsen and Woese 1993). The slow rate of change over time allows comparison of distantly related species (Woese and Fox 1977). Evolutionary theory states that all bacteria evolved from a common ancestor. 16S rRNA gene analysis shows evidence for this with the difference in sequences used to infer branching to form new species. The branches resulting from this interpretation do not correspond with the understanding of related organisms from phenotypic appearances that was previously used. The 16S rRNA gene has been shown to be representative of bacterial phylogeny using many bacterial genes (Snel, Bork, and Huynen 1999).

1.4.4 DNA sequencing

Sanger et al published a method of sequencing DNA in 1977 using chain terminating dideoxy nucleotides (Sanger, Nicklen, and Coulson 1977). Sanger sequencing, as it came to be known, was the gold standard for DNA sequencing for many years (McGinn and Gut 2013). Incorporating a dideoxy nucleotide during a PCR reaction prevents further elongation of the DNA strand. Using gel electrophoresis or capillary separation DNA is separated by fragment length. Sequences are revealed by labelling the terminating nucleotide radioactively or with fluorescence.

Next generation sequencing (NGS) refers to a number of technologies building on Sanger sequencing (Rothberg et al. 2011; Shendure et al. 2005; Ronaghi, Uhlén, and Nyrén 1998). NGS allows the up scaling of sequencing with reduced time scales of sample processing and high throughput. The sequencing-by-synthesis approach is most widely used gathering sequence data in real time during a PCR amplification. Chain terminating fluorescent bases are incorporated to the strand of DNA and the fluorescent signal generated. A restoration step removed the fluorescent tag and allows further extension to the DNA strand (Muzzey, Evans, and Lieber 2015). Figure 1-3 below demonstrates the steps involved in both Sanger and sequencing-by amplification sequencing methods.

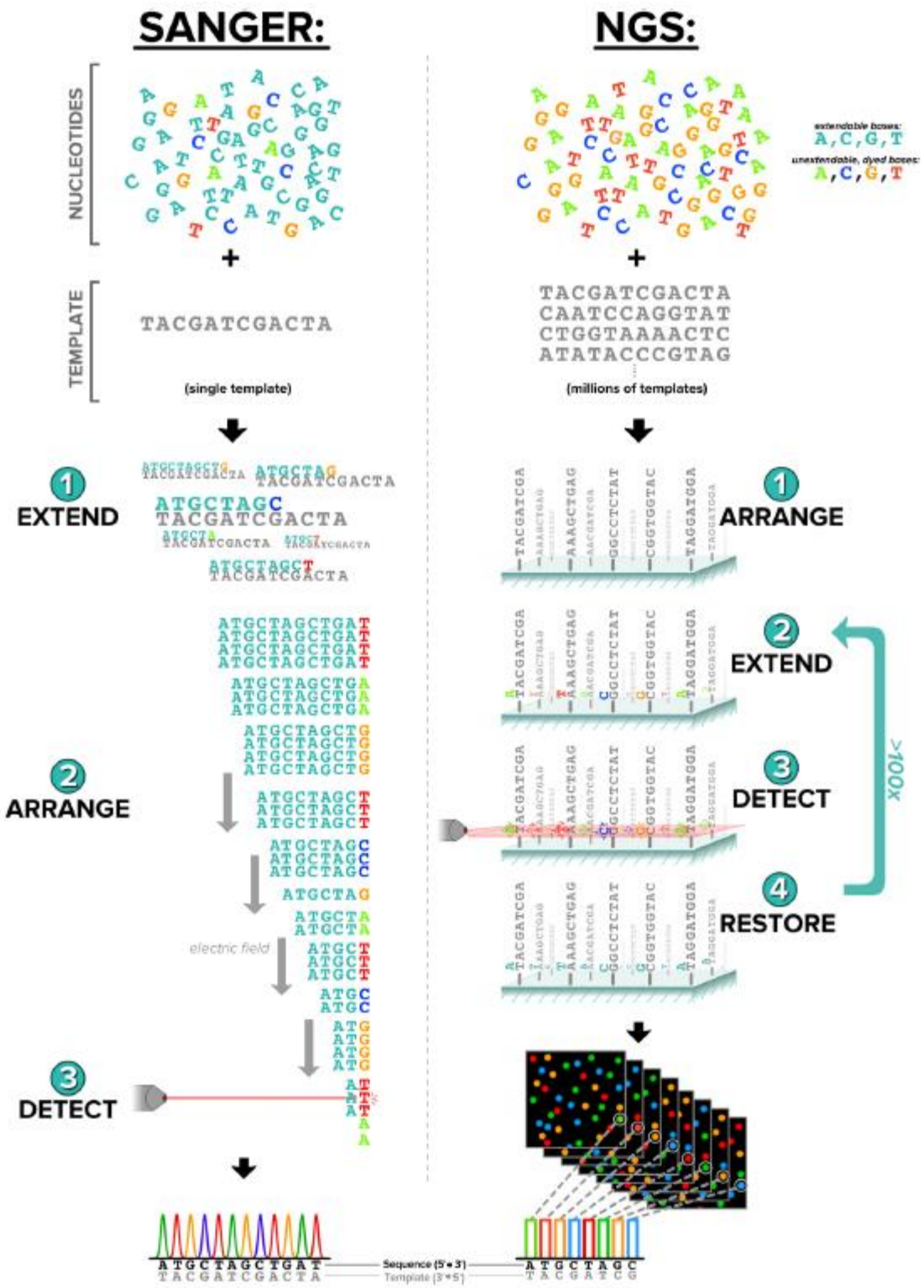


Figure 1-3: Sequencing using Sanger and NGS methods.
In both methodologies, a polymerase copies template molecules by incorporating nucleotides from a pool, that is, either partially (Sanger) or entirely (NGS) composed of dyed and unextendible bases. Extension, arrangement, and detection are shared steps in both protocols but occur in different order, with NGS alone having a restoration step that converts bases to the undyed and extendable form **(Muzzey, Evans, and Lieber 2015).**

1.4.5 Sequencing the 16S rRNA gene

16S rRNA gene sequence data is often expressed in terms of operational taxonomic units (OTU) when assessing bacterial communities. A single OTU is a species or group of species defined by DNA sequence data. The 16S rRNA gene is usually sequenced using a single pass resulting in the possibility of at least one sequencing error. Defining organisms as different at a threshold of 100% identical sequences will result in virtually clonal genomes being classified as different organisms (Morgan and Huttenhower 2012). Given this, the definition of difference between sequences to classify differing species is usually arbitrarily set between 1-3% (Pei et al. 2010; Stackebrandt and Ebers 2006).

Using the 16S rRNA gene for species analysis and identification of bacterial communities has a number of weaknesses. It is relatively common for organisms within a single species to have 16S rRNA gene sequences that differ by greater than 1% (Pei et al. 2010) possibly leading to an overestimation of diversity within a community if the threshold of difference is set at 1%. Chimeric sequences form as a hybrid of 16S rRNA genes from different species within a sample. This can lead to overestimation of species diversity if chimeric sequences are recognised as a unique organism despite attempt to identify and remove chimeric sequences during bioinformatic data processing (Haas et al. 2011). Copy number of the 16S rRNA gene is known to show wide variation between 1-15 copies per organism (Klappenbach et al. 2001). At genera and family level, the copy number variability is very wide; however, within species the copy number is largely conserved (Větrovský and Baldrian 2013).

Other target genes have been used to overcome these weaknesses. Housekeeping genes with a single copy number such as RNA polymerase and amino-acylsynthetase, in theory overcome the issue of multiple copy number (Santos and Ochman 2004). The rpoB gene has been demonstrated to give equal or improved phylogenic resolution than the 16S gene (Case et al. 2007). The 16S rRNA gene remains the most commonly used molecular marker for OTU identification due to the large body of work and in-depth sequence data existing for this gene.

1.4.6 Microbiota identification workflow

Studies to identify the microbiota from any environment using sequencing of the 16S rRNA gene follow a similar workflow summarised in Figure 1-4 below. Once the study has been designed to answer the research question samples are collected from the appropriate site. DNA extraction from the sample is then performed. Amplification of the bacterial 16S rRNA gene is then performed using PCR and the amplicon purified. Sequencing of the amplicon is then undertaken. Data processing is required to exclude poor quality and erroneous reads from the data set before OTUs can be formed. Statistical tests and graphical representation of the results can then be performed.

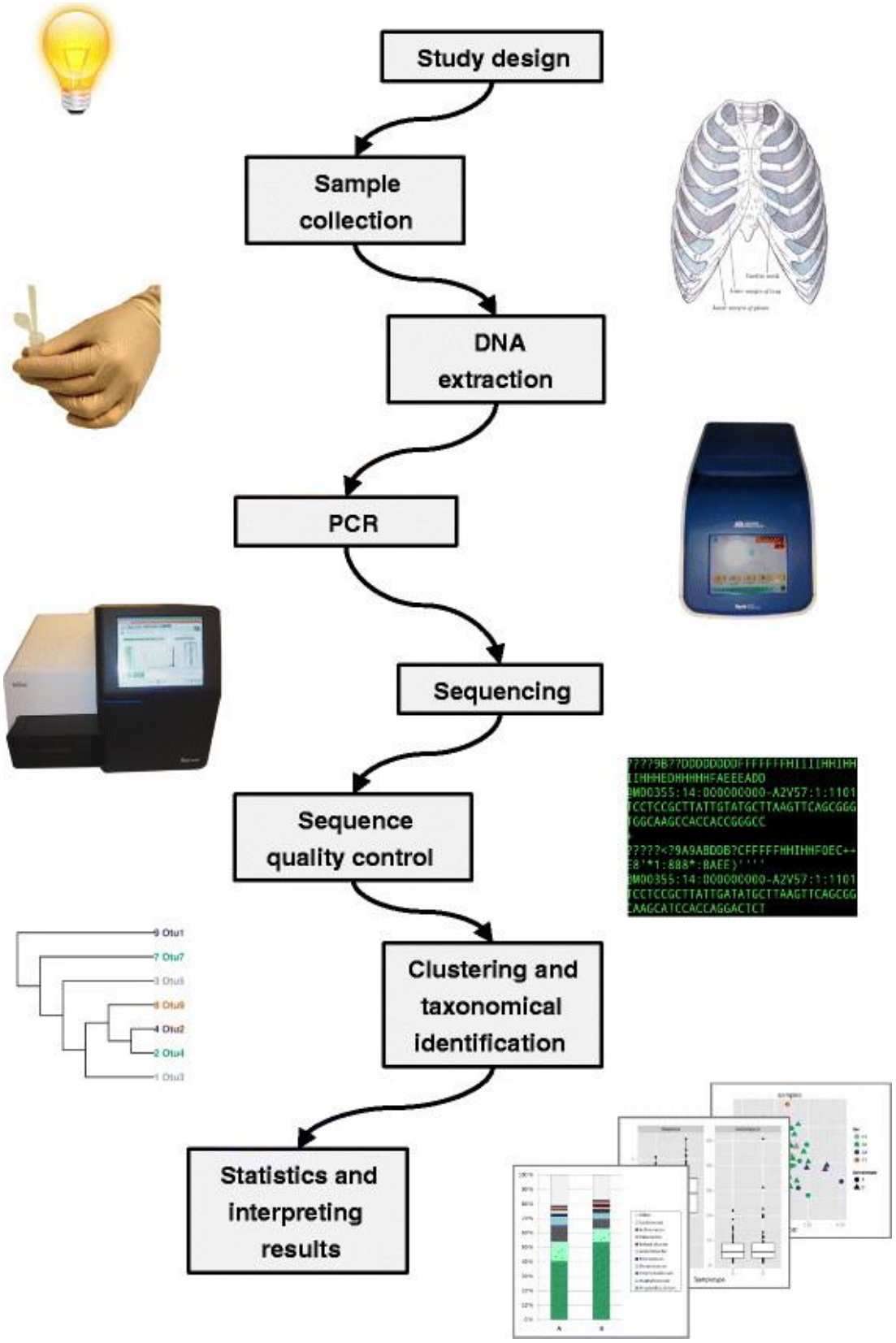


Figure 1-4: General workflow of a microbiome study, from design to data analysis. This schematic is specific for target gene sequencing-based studies (e.g. 16S rRNA gene) **(Aho et al. 2015).**

1.4.7 Microbiome statistical analysis

Comparing bacterial communities between samples or between environments requires statistical measures to define groups. The methodology utilised by ecologists studying diversity of macro-organisms within environments has been widely applied to microbiological data (Hughes et al. 2001). Diversity refers to the number of species of bacteria within a sample. This is normally expressed in terms of richness and evenness. The richness of a samples measures or estimates the number of different species present. Evenness considers the relative abundance of species identified.

Diversity can also be compared within a single population, referred to as alpha diversity, or as a similarity score between populations, referred to as beta-diversity. The most commonly used mathematical measures of α-diversity are the Shannon Diversity Index and the Simpson Diversity Index.

The Shannon diversity index was first proposed in 1948 to quantify the uncertainty in predicting the next letter in a string of text, with more letters available there is greater uncertainty (Shannon 1948). Applying this index to microbiology results in quantifying the uncertainty of identification of an individual sequence taken at random from the dataset. It is expressed mathematically with p_i as the proportion of organisms belonging to the $_i$th species in the dataset as:

$$H' = -\sum_{i=1}^{R} p_i \ln p_i$$

The Simpson diversity index was devised for use in ecology also attempting to mathematically quantify diversity of species within a sample by quantifying the chance that 2 organisms picked at random will be of the same species (Simpson 1949). Mathematically it is calculated by the following formula, where p_i is the proportion of organisms belonging to the $_i$th species in the dataset.

$$\lambda = \sum_{i=1}^{R} p_i^2$$

This equation gives a larger result for less diverse samples and a smaller value in the more diverse samples. Due to this counterintuitive result the inverse of the Simpson index is often used ($1/\lambda$). Simpson's Index is sensitive to the abundance of more plentiful species in a sample, and has been regarded as a measure of "dominance concentration" (Hill 1973).

The Chao1 index is an estimate of richness based on the assumption that some species have not been identified in each sample (Chao 1984). The index uses the number of organisms counted only once or twice within the sample to estimate the total number of different organisms found in the sampled niche. This index is useful as a measure of richness as it can be used on non-parametric data and on samples dominated by a single organism where important biodiversity exists with the rarely occurring species. The formula for calculation of the Chao1 index is below, where S_{obs} = number of species observed in the samples, n=number of samples being considered, F_1= number of singletons and F_2= number of species with two observed individuals in the sample:

$$\hat{S}_{Chao1} = S_{obs} + \left(\frac{n-1}{n}\right)\frac{F_1^2}{2F_2}$$

The Bray-Curtis dissimilarity index is most commonly used to define β-diversity (Bray and Curtis 1957).

$$BC_{ij} = \frac{S_i + S_j - 2C_{ij}}{S_i + S_j}$$

Where S_i and S_j are the number of species in populations i and j, and C_{ij} is the total number of species at the location with the fewest species. Beta diversity indices are often displayed as ordination plots with samples with more similar bacterial communities plotting close together and those more dissimilar further apart.

Alternative methods to the use of OTUs in microbiome analysis are to use a phylogenetic approach. Phylogenetic analysis quantifies diversity in terms of the total breadth or depth of the phylogenetic tree contained within a microbiome, allowing a series of highly related 16S rRNA gene sequences, which may be treated as a single OTU, to be considered as spanning a short evolutionary distance (Morgan and Huttenhower 2012).

1.4.8 Microbiome and disease

Dysbiosis is defined as an imbalance in the microbes present in a particular niche due to a change in conditions predisposing to disease (Rogers et al. 2015). The most studied and understood human microbiome is that of the gut due to ease of sampling from stool samples. The composition of the gut microflora is also susceptible to change in response to a change in diet (Walker et al. 2011) and shows considerable differences across geographical regions (Yatsunenko et al. 2012). The gut microbiome has been shown to be disordered in terms of species diversity and relative abundance in many disease states including inflammatory bowel disease (Cenit et al. 2014), obesity (Moran and Shanahan 2014) and conditions such as rheumatoid arthritis and mental health conditions (J. Kelly et al. 2016) which affect sites anatomically distant from the gut.

Some authors now argue that the concept of dysbiosis is flawed. Dysbiosis implies a causality between the observed difference in the microbiota and the disease state, without evidence that the difference is not due to reverse causality or confounding. Reduced diversity of the microbiome is the most commonly sited example of dysbioisis, but this is not necessarily bad. For the term dysbiosis to be useful, differences in the microbiota need to be shown to predict or improve disease. (Olesen and Alm 2016; Shanahan and Hill 2019).

The microbiota-gut-brain axis theory suggests organisms within the gut can influence the host by host absorption of metabolites, a phenomenon first documented by Heijtz et al (Heijtz et al. 2011). Evidence exists for other similar axes affecting other body systems including the microbiota-gut-lungs axis and microbiota-gut-liver axis (Marsland, Trompette, and Gollwitzer 2015; Young, Hopkins, and Marsland 2016).

Short chain fatty acids (SCFA) are have been studied as metabolites potentially acting as mediators of a microbiota effect on distant body systems. SCFA are produced during microbial fermentation of polysaccharides indigestible by the host. SCFA have local effects to improve intestinal epithelial barrier integrity (Peng et al. 2007) and can affect the immune response by influencing T-cell cytokine production (Bird et al. 1998) and neutrophil chemotaxis and function (Vinolo, Hatanaka, et al. 2009; Vinolo, Rodrigues, et al. 2009) but may have a systemic role in influencing host metabolism to affect disease states (T. Chen et al. 2019).

Correcting a dysbiosis or manipulating the microbiota for a beneficial clinical effect is a therapeutic strategy currently being investigated for many conditions. Faecal microbiota transplantation has become a common rescue therapy for *Clostridium Difficile* infection (Staley et al. 2019). Microbiota differences associated with disease states may reflect the underlying different physiological and pathological processes at work, rather than suggesting a causal role for the bacteria. The clinical changes witnessed as a result faecal microbiota transplantation suggests that the microorganisms may be directly affecting the disease rather than responding to a different environment.

1.4.9 The human gut microbiota in preterm infants

The healthy adult gut microbiome is known to contain a diverse and variable number of bacterial species. Within neonatal literature all studies published use stool samples or rectal swabs to study the gut microbiome, therefore only the distal microbiota has been analysed. Culture independent studies of healthy term infants have shown that species of the Actinobacteria phylum, mainly *Bifidobacterium*, dominate the stool microbiome from 1 week of age (Barrett et al. 2013).

Many factors have been shown to influence the pattern of neonatal gut microorganism colonisation. Infants born by elective caesarean section have stool colonisation with organisms

found on maternal skin compared to vaginally delivered infants (Dominguez-Bello et al. 2010; Adlerberth et al. 2006). Further studies show a delay in colonisation with *E. coli* and *Bacteroides* (Adlerberth et al. 2006) and lower levels of *Bifidobacterium* species in infants born by caesarean section (Biasucci et al. 2010). Differences in the stool microbiota between infants born by caesarean section and those born vaginally can be seen even at 12 months of age, with lower levels of *Bacteroides* in the caesarean group (Adlerberth et al. 2007). However a more recent study showed that by 6 weeks of age the bacterial communities in stool samples have expanded and increased in complexity since birth, but there is no significant effect of mode of delivery (D. M. Chu et al. 2017). A systematic review conducted due to such conflicting results identified a significant role for mode of delivery over shaping gut colonisation of infants up to six months of age (Rutayisire et al. 2016).

C. difficile, *Bacteroides*, *Enterococci*, *Enterobacteriaceae*, and *Enterobacter* are more common in bottle-fed infants, while *Staphylococci* and *Bifidobacterium* are more numerous in breastfed infants, however results are not consistent between studies (Adlerberth and Wold 2009; Westerbeek et al. 2006; John Penders et al. 2006). Antibiotic exposure in the perinatal period in term infants results in slower *Bifidobacterium* colonisation and an overgrowth of *Enterococci*. Difference in stool colonisation between antibiotic free and antibiotic exposed infants were still detectable at one month of age (Tanaka et al. 2009). Marked difference have been observed between the colonisation patterns in different geographical areas. Sanitary conditions may explain why infants born in the developing world have a higher number of species and a faster rate of turnover of species than those from developed nations (Adlerberth et al. 1998).

Accurate determination of the source of organisms colonising the neonatal gut requires identification of an individual strain both in the neonatal stool and in the source. *E. coli* strains in infant stools have been traced to originate from the maternal gut microbiota (up to 50% of strains), other infants on the same ward, staff caring for infants and environmental sources (Fryklund et al. 1992; Bettelheim and Lennox-King 1976; Adlerberth et al. 1998). Lactobacilli and Group B streptococci strains within neonatal stools have both been matched to those within the maternal vaginal microbiota, suggesting this site as the likely source (Tsolia et al. 2003; Matsumiya et al. 2002). Parental skin is the likely source of *Staphylococcus aureus* in over 90% of infants (Lindberg et al. 2004).

1.4.9.1 Acquisition of the preterm gut microbiota

Across many studies, preterm infants show lower levels of colonisation with *Bifidobacterium* species than term infants (Jacquot et al. 2011; John Penders et al. 2006; Barrett et al. 2013). *Lactobacilli* colonisation is also delayed, with lower levels persisting at 30 days of age (M. Hall et al. 1990). By one week of age Proteobacteria and Firmicutes predominate (Barrett et al. 2013). Potential pathogens such as *E. coli* and C*lostridia* are more commonly detected in

preterm than term infants (Adlerberth and Wold 2009). Diversity of species within the bowel of preterm infants is reduced compared to that of term infants with delivery by caesarean section also associated with reduced diversity (Jacquot et al. 2011).

1.4.9.2 Necrotising enterocolitis

Necrotising enterocolitis (NEC) is a devastating bowel disease affecting preterm infants associated with immune dysregulation, enteral feeding and a dysbiosis of gut communities (C. J. Stewart et al. 2013). Many studies have attempted to identify a causative organism or microbial signature to guide treatment prior to the onset of clinical symptoms.

One study noted a sudden increase in Proteobacteria and a decrease in Firmicutes one week prior to NEC onset, not seen in healthy controls (Mai et al. 2011). The Proteobacteria family have also been implicated in NEC being detected in high numbers in excised inflamed bowel samples (B. Smith et al. 2011) and over represented in the stools of NEC patients compared to controls (Yunwei Wang et al. 2009). *E. coli*, within the Proteobacteria phylum, has specifically been implicated in one study, with a significant increase in numbers prior to the diagnosis being made (Jenke et al. 2013). Two distinct patterns of dysbiosis were suggested by one further study. All infants who went on to suffer with NEC displayed a lack of Proprionibacterium and had either an early (day 4-9 of life) overgrowth of Firmicutes, or late (day 10-16) overgrowth of Proteobacteria, with 25% of controls displaying this trend (A. Morrow et al. 2013).

The evidence across many studies suggests a dominant organism and reduced species diversity as predictive for later NEC, with organisms of the Proteobacteria phylum frequently implicated. It is not clear that the change in microbiota is causative for NEC as it may reflect a change in the milieu of the bowel secondary to a causative influence.

1.4.10 Summary

The human body is host to many communities of microorganisms. The gut microbiome is the most studied and links between colonisation patterns and disease have been demonstrated. Preterm infants differ in the gut colonisation compared to healthy term infants, with a wide range of environmental exposure differences between the two groups probably explaining this. The example of NEC demonstrates that the microbiome can influence the health of preterm infants.

1.5 The respiratory microbiome

1.5.1 Introduction

Traditional culture-based methods led to the belief that the lungs were a sterile environment, with no colonisation by microorganisms; the presence of detectable bacteria indicating a disease state (Thorpe et al. 1987). Culture-based techniques failed to identify the low level carriage of

bacteria that is now known to inhabit the airways and lungs of healthy individuals. The introduction of molecular PCR based techniques has revolutionised this thinking (Beck, Young, and Huffnagle 2012). It is now understood that a wide variety of microorganisms live within the lungs of healthy individuals and those with pulmonary disease. This community is referred to as the microbiota of the lung.

1.5.2 Healthy adult respiratory microbiota

Several studies have attempted to characterise the microbiome of healthy adult lungs. The most commonly occurring phyla detected are Bacteroides and Firmicutes making up around 80% of organisms and a smaller proportion of Proteobacteria (Erb-Downward et al. 2011; Morris et al. 2013). These phyla dominate most sites in the human host.

The lung microbiome is characterised by a relatively lower level of Actinobacteria compared to the upper respiratory tract. Genera predominating in the lungs of healthy adults are *Prevotella*, *Veillonella*, *Streptococcus* and *Pseudomonas* (Dickson, Erb-Downward, and Huffnagle 2013). Comparing bacterial load between the upper and lower respiratory tract has demonstrated a 2 to 4 $\log_{10}$ lower biomass in the lungs (Charlson et al. 2011).

The composition of the gut microbiome has shown wide geographic variation (Yatsunenko et al. 2012), however this has not yet been sufficiently studied with regard to the lung. There is evidence from sputum samples of Cystic Fibrosis (CF) patients of a difference between organisms observed in patients from the USA and UK (Stressmann et al. 2011).

The lung microbiome in adults and older children has been studied mainly using the technique of bronchoscopic bronchoalveolar lavage (J. K. Harris et al. 2007; Hogan et al. 2016; Twigg et al. 2013; Marsh et al. 2016). This involves inserting a fibre-optic endoscope through the nose or mouth to access the lower airways and suctioning out instilled saline to wash out the alveoli (de Blic et al. 2000). Sputum sample analysis and bronchial brushing are alternatives (Schmidlin et al. 2015; Hogan et al. 2016; J. Zhao et al. 2012). All methods of collecting samples from the lower airways for microbiome analysis are potentially compromised by contamination by upper airway organisms. To overcome this, studies have been performed on surgically excised lung tissue (Erb-Downward et al. 2011; Goddard et al. 2012). The presence of the same organisms from explanted lung tissue as from BAL samples suggests that the BAL samples accurately reflect the organisms present in the lung (Erb-Downward et al. 2011). Such studies also indicate that the lung microbiome is similar in composition but distinct in relative abundance of organisms compared to that of the upper airways (Dickson, Erb-Downward, and Huffnagle 2013).

Probably the most comprehensive study of the healthy human respiratory microbiota was performed by Dickson et al. Careful use of controls, close attention to preventing contamination

and sequential sampling from different level of the respiratory tract of 8 healthy individuals provided in depth data on the colonisation at different sites. This study demonstrated that bronchoscopic BAL samples do reflect respiratory colonisation in healthy individuals, rather than contamination. It also provided evidence of micro-aspiration being the primary source of the respiratory microbiota, due to the similarity between the mouth and the carina being greater than the mouth and proximal trachea. Gravity resulting in aspirated saliva more likely to be contributing to the carina colonisation in an upright individual (Dickson et al. 2017).

Microbiome analysis from different niches of skin and different areas of the gut shows marked variability between anatomical sites of both organs (Grice and Segre 2011; Zhigang Zhang et al. 2014). BAL in infants usually is aimed at sampling the right lower lobe (de Blic et al. 2000; Grigg, Arnon, and Silverman 1992); and in adults often the right middle lobe in research studies with the lobe sampled in clinically indicated BAL guided by radiological evidence and clinical features (Du Rand et al. 2013). Samples taken from multiple sites within explanted lungs of chronic obstructive pulmonary disease (COPD) patients showed significant difference between bacterial communities identified within the same lung (Erb-Downward et al. 2011). In contrast, Charlson et al demonstrated very similar bacterial communities within different lobes of the lung, all with marked similarity to the oral microbiome (Charlson et al. 2011). Pulmonary tuberculosis is well known to have a predilection for upper lobe involvement (Goodwin 1983). Bacterial communities within different lobes of the lungs may be isolated from each other. Using 10 pairs of explanted lungs from cystic fibrosis patients undergoing lung transplant, the dominant organism, *Pseudomonas aeruginosa*, displayed different antibiotic resistance patterns between lobes of the same lung (Jorth et al. 2015). Analysis of the organism's genomes showed a difference in single nucleotide polymorphims between colonies in different lobes. This suggests that over time, within a cystic fibrosis affected lung, the *Pseudomonas aeruginosa* colonising the lungs evolves independently in different lobes of lungs, evidence of little mixing of bacteria between lobes of the lung. BAL samples from infants may therefore not be representative of communities beyond the right lower lobe if bacterial communities in the lung are specific to each lobe.

The lung microbiome of healthy term infants has not been studied due to the inaccessibility of the lungs and the ethical issues involved in taking invasive samples from well infants (Warner and Hamvas 2015).

1.5.3 Source of the respiratory microbiota

In healthy adult subjects who underwent microbiome analysis from the oral cavity, nasal cavity, lungs and stomach, the lung microbiome was found to be more closely associated with the microbiome of the oral cavity than other sites (Bassis et al. 2015). Micro-aspiration of saliva into the airway is known to occur during sleep in healthy individuals of volumes likely to

contain physiologically significant numbers of bacteria (Gleeson, Eggli, and Maxwell 1997). This would suggest that the lower airways could be colonised from bacteria inhabiting the oropharynx. A study of 78 children (mean age 2.2 years) including children with healthy lungs and those with chronic suppurative lung disease concluded that the microbiota detected by bronchoalveolar lavage contained components of both the nasopharyngeal microbiota and the oral microbiota (Marsh et al. 2016).

Evidence from cystic fibrosis patients followed up until 21 months of age, suggested that species colonising the airway (oropharyngeal samples) are first present in the stool (Madan et al. 2012). This suggests that the airway microbiota may be established from that of the gut, the so-called gut-lung axis.

Three factors determine the organisms present within the lower airways. Microbial immigration into the airways, elimination from the airways and the reproduction rates of the organisms in the airways (Huffnagle, Dickson, and Lukacs 2017). A dysbiotic change in the lungs is a result of a change in one of these factors.

1.5.4 Comparison of upper and lower airways microbiota

The easy accessibility of the upper respiratory tract has led to a greater understanding of the organisms commonly found in the upper respiratory tract than in the lungs. The nasal cavity, nasopharynx, oropharynx and oral cavity may all the classed as the upper respiratory tract, so care is needed when comparing studies especially as several studies have shown differences between these sites (Stearns et al. 2015; Marsh et al. 2016; Dominguez-Bello et al. 2010).

Some studies have used results from throat samples or nasopharyngeal samples as a proxy for lung bacterial communities (Cox et al. 2010). However, the upper airways may not have similar bacterial colonisation patterns to the lungs. A study of 10 CF patients undergoing lung transplantation analysed throat swabs, sputum samples and lung explant tissue for the bacterial communities using molecular methods. The throat swabs poorly reflect the microbiota of the lungs. Throat specimens demonstrate higher levels of microbial diversity than lung samples obtained just a few hours later and the predominant species in the throat swabs does not match that identified in the explanted lungs (Goddard et al. 2012).

The nasal cavity, specifically the middle meatus, of healthy adults was positive for bacterial colonisation in all 28 patients swabbed in one study. The most prevalent and abundant organisms present were *Staphylococcus aureus, Staphylococcus epidermidis* and *Propionibacterium acnes* (Ramakrishnan et al. 2013). In Chinese undergraduate students the nasopharyngeal microbiota was dominated by *Corynebacterium, Dolosigranulum,* and *Staphylococcus* (Ling et al. 2013). The nasopharyngeal microbiota is known to change over time. Parents and children within the same family showed marked difference in bacterial load

and species diversity, with children having less diversity but three times larger bacterial load than the adults (Stearns et al. 2015).

1.5.5 Respiratory microbiome influence over pulmonary health

The lung microbiome in several pulmonary disease states has been analysed. Research to date has focused on adult and paediatric chronic lung diseases. Mouse models have also been used to demonstrate the role of the microbiota in the lungs. Mice raised to be bacteria free have smaller lungs and a reduced number of mature alveoli than bacteria exposed mice, with alveolarisation returning to normal following exposure to *Lactobacillus* species (Wostmann 1981; Yun et al. 2014). Exposure to microorganisms may therefore play a role in morphological lung development.

1.5.5.1 Cystic fibrosis

CF is the most common lethal genetically inherited disease in the Western world (O'Sullivan and Freedman 2009). Studies in CF have revealed a much greater diversity in the array of species identified than had previously been appreciated and identified possible pathogenic organisms that could be responsible for exacerbations previously undetected by culture-based methods.

Longitudinal studies have demonstrated reduced microbial diversity with increasing age and worsening lung function (Cox et al. 2010; Coburn et al. 2015; Paganin et al. 2015; Zemanick et al. 2017). In a further decade long study, diversity was maintained in those with less severe disease progression, compared to those who deteriorated (J. Zhao et al. 2012). An Italian study comparing the microbiome between individuals with a recent decline in lung function compared to stable individuals showed significant difference between the two groups. The stable patients showed a more interconnected community than those with a decline in lung function possibly suggesting a higher resilience of communities within the lung of stable patients (Bacci et al. 2016). It is not clear from these studies if the microbiome is influencing the disease course or a sign of the changing habitat within the lung as the disease progresses.

1.5.5.2 Chronic obstructive pulmonary disease

COPD is characterised by progressive fixed airway obstruction with frequent exacerbations. Cigarette smoking is strongly associate with COPD. There is evidence that smoking affects the composition of the microbiome in anatomical sites distant from the airways (Biedermann et al. 2014; Brotman et al. 2014). The lungs of healthy smokers contain less species diversity than non-smokers (Erb-Downward et al. 2011). It has been suggested that community dysbiosis could provoke the inflammatory response that is characteristic of COPD (Erb-Downward et al. 2011).

Viral infection is well established as a cause for exacerbations of COPD (Dickson, Martinez, and Huffnagle 2014). Culture independent studies have shown changes within the composition of the microbiome during an exacerbation (Dickson, Martinez, and Huffnagle 2014; Jubinville et al. 2018). Introduction of new strains of bacteria, rather than new species, have been implicated in causing exacerbations (Sze, Hogg, and Sin 2014).

1.5.5.3 Asthma

Members of the Proteobacterium phylum (including *Haemophilus* and *Moraxella* species) are over represented in the airways of asthmatics in both children and adults, compared to controls (Hilty et al. 2010). In this study, all asthmatics were on inhaled corticosteroids which could have influenced the result.

Evidence also suggests that the microbiome has increased diversity in the airways of poorly controlled asthmatics compared to controls. The diversity positively correlates with degree of bronchial hyper-responsiveness (Huang et al. 2011). This could explain why asthmatic control appears to improve following macrolide antibiotics. This evidence indicates a role of host-microbe interaction in the pathogenesis and disease process of asthma.

1.5.6 Respiratory microbiota in new-born infants

1.5.6.1 Antenatal colonisation

Tissier concluded infant stool colonisation was via breast milk (Tissier 1900). Following this the amniotic sac was thought to be a sterile environment. The introduction of molecular based technologies has led some to question the sterility of the *in-utero* environment (Wassenaar and Panigrahi 2014). Amniotic fluid is continuous with fluid in the lungs of the foetus *in-utero.* Detection of bacteria in amniotic fluid may indicate their presence in the lung. In a study of women undergoing amniocentesis for small for gestational age pregnancies, 6% of samples had detectable bacterial DNA, with all the samples negative to routine culture (DiGiulio et al. 2010). Analysis of amniotic fluid, obtained by amniocentesis, from women in preterm labour showed 11.4% had detectable bacterial DNA (DiGiulio et al. 2008). The presence of bacteria was associated with chorioamnionitis which is a risk factor for preterm birth. These studies selected high risk groups; however, one study of 15 healthy pregnancies undergoing elective caesarean section (CS), amniotic fluid and placental tissue both collected at the time of CS demonstrated the presence of bacterial communities within both samples in all cases (Collado et al. 2016). A larger study looking for bacterial colonisation of placental tissue detected the presence of bacteria in all 48 placental samples analysed (Aagaard et al. 2014). This study used placentas from health term born, vaginally delivered infants as a control group, comparing this to a cohort with preterm birth and a history of antenatal infection. Interestingly, the placentas from healthy pregnancies had detectable bacterial colonisation. Multiple anatomical sites were studied with

the placental bacterial communities most similar to the oral bacterial communities of the mothers (Aagaard et al. 2014).

Placental samples taken after delivery may be subject to contamination during labour or caesarean section. However, the study by Aagard et al identified distinct patterns of bacterial colonisation within the placenta compared to swabs taken from within the birth canal. A further study, compared small numbers of placental and fetal membrane samples from vaginal verses CS deliveries. 50% (5/10) of term vaginal deliveries were positive for bacterial DNA, but none of the term infants born by CS had detectable bacterial DNA (Jones et al. 2009). This finding suggests that contamination may be responsible for bacteria detected in vaginally delivered placentas.

Bacterial DNA has also been detected in the first pass of meconium (Hansen et al. 2015) in 66% of cases using fluorescent *in-situ* hybridisation. First pass meconium may be expected to be sterile if the *in-utero* environment is sterile. However, first pass meconium is also subject to contamination, with samples from this study taken directly from the nappy. Commensal bacteria have also been detected within umbilical cord blood samples taken following caesarean section delivery (Jiménez et al. 2005). This finding is more difficult to explain by contamination. An in-depth review of this literature concluded that the evidence for in utero-colonisation was weak due to inadequate use of negative control samples and the high risk of contamination of low biomass samples (Perez-Muñoz et al. 2017). More recent evidence from placental samples indicates that there is no microbiota associated with the placenta, with the most detected organisms explained by contamination. Some placentas do, however, contain potential pathogens such as Group B streptococcus (de Goffau et al. 2019).

In-utero colonisation of the fetus could conceivably establish an airway and lung microbiota before birth. The concept of *in-utero* bacterial colonisation remains controversial but any bacterial colonisation occurring *in-utero* will be of a very low level.

Chorioamnionitis is a major cause of preterm delivery (Goldenberg, Hauth, and Andrews 2000). The airways of infants born following intrauterine infection are more likely to be colonised than infants unaffected by infection.

1.5.6.2 Respiratory colonisation in the neonatal period

If the *in-utero* environment is sterile, the microbiota of the airways and lung is acquired at the point of delivery from the exposure to maternal organisms during passage through the birth canal or through postnatal environmental exposures in infants delivered by caesarean section.

Relatively little is known regarding the acquisition of the lung microbiota in term or preterm infants. Immediately after birth (less than 5 mins) bacteria are detectable within the upper

airways and mouth of new-born infants (Dominguez-Bello et al. 2010), suggesting that colonisation of the upper airway has already begun at this early stage.

Preterm infants are frequently intubated. This process is likely to affect early colonisation. An early attempt to look for the presence of bacterial DNA in the lower airways of intubated preterm infants showed the presence of a diverse array of bacteria in the lower respiratory tract within the first week of life (Payne et al. 2010). Subsequently, bacterial DNA was detected in all tracheal aspirate samples taken immediately after intubation at birth from preterm infants (Lohmann et al. 2014). One study comparing ventilated term and preterm infants showed the presence of colonising bacteria with the first day of life in all 33 infants tested (Lal et al. 2016). In contrast, in a further study of intubated preterm infants, 2 of 10 tracheal aspirate samples taken at <72 hours of age contained detectable bacterial DNA. At 7 days of age all 10 tracheal aspirates from the same infants contained detectable bacterial DNA (Mourani et al. 2011). This evidence suggests that colonisation of the airways is detectable soon after delivery but that bacterial load may be low at birth.

Ureaplasma species, a possible cause of neonatal respiratory infection in preterm infants, have been detected by molecular methods in tracheal aspirates samples at 24 hours of age confirming bacterial presence in the airways at this age (Patterson et al. 1998). *Ureaplasma* species are also implicated in chorioamnionitis and preterm delivery, so this may reflect *in-utero* infection of the foetus rather than being evidence of bacterial colonisation at 24 hours of age.

No studies have analysed bacterial communities present within bronchoalveolar lavage fluid in intubated preterm infants.

1.5.6.3 The constituents of the airway microbiota in the neonatal period

The concept of a 'normal' microbiota of a preterm airway is a fallacy. In common with every aspect of prematurity, developing a normal reference standard is challenging. There is very little which is normal regarding preterm infants' inadequately developed lungs, being intubated, receiving positive pressure ventilation, antibiotic exposure and surviving outside the womb from early gestational ages. These factors and many more will influence the microbiota of an infant's airways; however, a physiological, as opposed to pathological state, may exist. Limited data are available regarding the organisms colonising the airways and lungs in the early days of life.

Within the respiratory tract the nasopharyngeal microbiota has been studied in the most depth. The nasopharynx of healthy term infants showed very similar colonisation to other body sites within five minutes of birth, with the organisms detected influenced by delivery mode. *Lactobacillus, Prevotella, Atopobium,* or *Sneathia* species were identified in samples from infants delivered vaginally and organisms typically found on skin including *Staphylococcus*

species were found in samples from CS born infants (Dominguez-Bello et al. 2010). A longitudinal study over 6 months demonstrated a similar influence of delivery mode over nasopharyngeal colonisation at birth, but at 1 week of age *Staphylococcus aureus* is dominant in most infants. By 4 months more stable profiles with *M. catarrhalis*, *Corynebacterium* species and *Dolosigranulum* species emerged (Bosch et al. 2016).

A study of ten preterm infants, found a dominant organism was present (>50% of total sequences) in 31 of 32 tracheal aspirate samples. The most common dominant genus was *Staphylococcus* species in 19 samples. Of these 17 were identified as coagulase negative *Staphylococcus*. *Ureaplasma* species dominated in 9 samples from 6 subjects. Other species that predominated in a single sample, all between 14-21 days of life were *Pseudomonas aeruginosa*, *Enterococcus faecalis*, and *Escherichia coli*. The Shannon diversity index ranged from 0.08-2.9 in this study (Mourani et al. 2011). A dominant organism was also found in a separate study using tracheal aspirates collected during the first week of life. The most common organisms identified using the 16S rRNA gene were *Staphylococcus haemolyticus* and *Staphylococcus epidermidis*, both coagulase negative Staphylococci (Payne et al. 2010).

A further study comparing tracheal aspirate samples between infants with and without CLD showed a predominance of a dominant organisms in many samples. Most commonly species within the Proteobacteria phylum were dominant, *Acinetobacter* the most abundant genus. Firmicutes were also dominant in some samples (Lohmann et al. 2014).

Lal et al took tracheal aspirates from ventilated full-term infants and extremely low birth weight (ELBW) infants within the first day of life from a single centre and showed very similar patterns of colonisation. Firmicutes and Proteobacteria organisms predominated with smaller proportions of Actinobacteria, Bacteroidetes, Tenericutes, Fusobacterium, Cyanobacteria, and Verrucomicrobia. No difference was identified between the Shannon diversity index between the ELBW group and the term infants ($p = 0.46$, t-test) (Lal et al. 2016).

The presence of a dominant organism is repeated across several studies suggesting a pioneering coloniser is established within the airways; however, the identity of the organism can vary. More work is needed to investigate early bacterial colonisation of the upper respiratory tract in preterm infants to understand if this follows similar patterns to that in term infants.

1.5.6.4 Factors affecting the respiratory colonisation in the neonatal period

The nasopharynx of term-born infants share the same initial colonising organisms as skin sites and the mouth, all demonstrating a significant influence of the delivery method (Dominguez-Bello et al. 2010). A difference with delivery mode was also seen on day one of life in a longitudinal study using NPA samples in healthy term infants (Bosch et al. 2016). Exposure to chorioamnionitis was associated with a trend towards decreasing species diversity in tracheal

aspirates of mechanically ventilated preterm infants, although this trend did not reach statistical significance (Lohmann et al. 2014). An overgrowth of pathogenic species in infants exposed to chorioamnionitis may reduce diversity within the sample.

The use of antibiotics is very common in preterm infants and antibiotic exposure is known to reduce bacterial diversity in the neonatal bowel (Tanaka et al. 2009). Antibiotics induce significant changes in sputum microbiota of cystic fibrosis patients (D. J. Smith et al. 2014). The impact of antibiotics on the lung microbiota of preterm infants is unknown, however, in the upper airways of infants under 12 months of age those who had received antibiotics in the preceding 4 weeks displayed a reduced proportion of *Alloiococcus* and *Corynebacterium*, with an increased proportion of potential pathogens including *Haemophilus*, *Streptococcus* and *Moraxella* (Teo et al. 2015).

Breast feeding is currently promoted due to multiple health benefits for both the mother and the infant (Shamir 2016). Breast milk has the potential not only to pass from mother to child desirable colonising bacteria but also to provide oligosaccharides to promote development of a healthy microbiota (Jost et al. 2015). The evidence for an effect of breast feeding on the nasopharyngeal microbiota is mixed. One study comparing exclusively breast fed and exclusively formula fed infants showed detectable changes in the nasopharyngeal microbiota at 6 weeks of age (Biesbroek, Bosch, et al. 2014).

Studies analysing the nasopharyngeal microbiome of healthy infants have demonstrated an association between bacterial colonisation patterns with the season of the year (Mika et al. 2015), exposure to cigarette smoke (Greenberg et al. 2006), and vaccination status (Biesbroek, Wang, et al. 2014).

Further work is needed to understand factors that influence bacterial colonisation of the airways and lungs of preterm infants. The effect of an intensive care environment and antibiotic exposure is likely to impact on colonisation patterns.

1.5.6.5 Lung microbiota and chronic lung disease of prematurity

A small number of studies have sought to define a relationship between the microbiota of the neonatal lung and the development of CLD. Two early small studies testing the methodology of sequencing the 16S rRNA gene in tracheal aspirate samples from intubated preterm infants recruited infants at risk of CLD. All the infants in both studies had an oxygen requirement at 36 weeks' corrected gestational age precluding any comparison between infants with and without CLD as is often the case in longitudinal studies of preterm infants (Mourani et al. 2011; Stressmann et al. 2011).

A further study looked at tracheal aspirate samples in 25 infants born with a gestational age 24-32 weeks'. Ten infants developed CLD. At the point of intubation infants who went on to develop CLD had a lower diversity of species in terms of both species count (23±4.5 vs 37±12; p=0.02) and Shannon diversity index (1.73±0.65 vs 2.34±0.87; p=0.094). At a phylum level Firmicutes increased and Proteobacterium decreased over time in those infants who developed CLD (Lohmann et al. 2014). No correlation with cytokines or inflammatory profiles was found questioning the expected mechanism by which the microorganisms may influence CLD. However, details of the cytokines were limited in the publication.

Another study identified that organisms of the *Lactobacillus* genus are less abundant in the early airway microbiome of infants who later develop CLD. Tracheal aspirate samples were taken within the first six hours of life from preterm infants at risk of CLD. Five extremely preterm infants who went on to develop CLD from this study had serial samples taken to follow changes in the microbiota over time. A consistent dysbiotic change was identified with a decrease in Firmicutes and increase in abundance of Proteobacteria over time in these infants (Lal et al. 2016). This is in direct contrast to the work of Lohmann et al who showed the opposite patterns of colonisation associated with CLD.

Wagner et al used routinely collected tracheal aspirate samples from 155 ventilated preterm infants to identify the presence of bacteria using the 16S rRNA gene. Infants were stratified into mild, moderate or severe CLD. Cross sectional analysis of samples obtained from day 7 of life failed to show any association between severity of CLD and Shannon diversity index, bacterial load or relative abundance of individual taxa. However longitudinal analysis demonstrated that infants with severe CLD had a higher turnover, were colonised with less *Staphylococcus* species in the first days of life and had higher initial relative abundance of *Ureaplasma* (Wagner et al. 2017).

A recent systematic review assessing the influence of the respiratory microbiota over CLD included the 6 studies listed above. The review concluded that microbial dysbiosis may be associated with the progression and severity of CLD but more work was needed to identify if and when an optimum respiratory microbiota is established (Pammi et al. 2018).

Other studies have used culture-based techniques to assess bacterial colonisation of the airway to identify colonisation patterns associated with CLD. A Japanese study used routinely collected tracheal aspirate samples from intubated preterm infants to compare the bacterial species cultured from infants with mild/moderate CLD to those with severe CLD. Severe CLD was defined as >30% inspired oxygen concentration at 36 weeks' corrected gestational age. Of the 9 organisms identified only *Corynebacterium* species showed significant difference between the 2

groups with *Corynebacterium* species associated with severe CLD (Imamura et al. 2016). This study did not attempt to culture *Ureaplasma* species from the samples.

An American study also using retrospective data from tracheal aspirate cultures collected for clinical reasons compared the presence of Gram-negative rods and Gram-positive cocci with respiratory outcomes (Tramper et al. 2016). Those infants who had evidence of Gram-negative rods prior to 36 weeks' gestational age were at higher risk of needing long term oxygen than those without Gram-negative rod positive cultures. Gram-positive cocci had no significant effects. This study provides evidence for an effect of respiratory infection on respiratory outcomes rather than colonisation, due to the use of culture-based techniques and the focus on pathogenic organisms. Using groups such as "Gram-negative rods" may mask the effect of specific species on the developing lung.

Indirect evidence for a potential relationship between the respiratory microbiome and CLD exists from a retrospective cohort analysing antibiotic use during the first 2 weeks of life in very low birth weight (VLBW) infants. Every additional day of empiric antibiotic therapy was associated with an increased risk of CLD and was also related to CLD severity (Cantey et al. 2016). The authors attempted to control for disease severity at birth using CRIB-II scores, however this may not have adequately reflected respiratory disease severity and postnatally acquired conditions such as pneumonia. Despite the limitations, a possible explanation for the association between increased antibiotic use and increased risk of CLD could be due to antibiotic manipulation of the respiratory microbiota affecting early colonisation patterns.

The dysbiosis witnessed in the respiratory tract of preterm infants in the neonatal period may be longer lasting than previously appreciated. Recent evidence found differences in the microbiota of induced sputum from young adults between those born preterm with a CLD diagnosis and term born controls (Rofael et al. 2019). The clinical relevance of these differences remains unclear.

The effect of early bacterial colonisation on the risk of developing CLD is still unclear with the published evidence showing conflicting and inconsistent results. More work is needed to understand if early colonisation is associated with pulmonary inflammation.

1.5.7 Host-microbiome interactions

1.5.7.1 Host influence over microbiome communities

The immune system is designed to attack and destroy potential threats including bacteria but recognise host molecular signatures. Colonising organisms present a significant challenge to the host immune system. The symbiotic relationship existing between host and microbe is important for the host to maintain. It is however important that the host recognises the bacteria as foreign and maintains mechanisms to control potential infection by microorganisms. The interaction

between the host immune response and microbiome has been studied in most depth in the gut. Mechanisms within the gut to control bacterial proliferation and protect the host include secretion of mucus from Goblet cells (Hooper, Littman, and Macpherson 2012) and secretion of specific IgAs by dendritic cells and secretion of α-defensins by small intestinal Paneth cells (Salzman et al. 2003).

The lungs and airways are far less studied than the gut regarding host-microbiome interaction. It is, however, likely that mechanisms exist for host control over commensal organisms at these sites. BAL fluid of healthy adults is known to contain white blood cells indicating their presence within the airways and alveoli of healthy individuals (Balbi et al. 2007). Respiratory epithelial cells are capable of secreting molecules with antimicrobial effects to limit bacterial survival in the lungs and airways. These molecules include the complement system, lactoferrin, antimicrobial peptides, lysozyme, secretory leukoprotease inhibitor and surfactant proteins -A and -D (Bals and Hiemstra 2004; Mackay et al. 2019). It is unclear, if such proteins are produced only during periods of infection or are involved in regulating the microbiota.

1.5.7.2 Microbiome influences on host immunity

Small intestinal isolated lymphoid follicles do not develop in germ-free mice (Hooper, Littman, and Macpherson 2012). Mouse models for various diseases are known to be dependent on colonisation by microorganisms. Germ free mice have diminished disease in arthritis and autoimmune encephalitis models (H.-J. Wu et al. 2010; Y. K. Lee et al. 2011). In contrast, the risk of ovalbumin-induced asthma is increased in germ free mice due to altered natural killer cell function. This effect was reversible with neonatal colonisation of mice, but not if colonisation was delayed until adulthood (Olszak et al. 2012). Exposure to microorganisms in mice is important in developing appropriate immune responses especially during the neonatal period. The artificial germ-free status and the use of mice makes it difficult to assume any clinically significant effect in humans.

Some human studies have demonstrated notable results in the area of microbiome-host interaction. The “early programming” theory states that early life adverse exposures can determine disease susceptibility in later life (Williams and Drake 2015). The most well-known example of this is the Barker hypothesis that low birth weight, reflecting poor intrauterine nutrition, results in a higher risk of cardiovascular and endocrine disease in later life (Barker 1986). This approach has been adapted to look at the influence of the early neonatal colonization over later disease. Infants with hypopharyngeal colonisation with *S. pneumoniae, H. influenzae, or M. catarrhalis* (detected by culture) at 1 month of age are more likely to develop bronchiolitis and pneumonia by the age of 3 years (Vissing, Chawes, and Bisgaard 2013) and develop asthma by 5 years than those not colonised (Bisgaard et al. 2007). Interestingly the colonisation status at 12 months of age was not related to later asthma

(Bisgaard et al. 2007). This highlights the importance of early airway colonisation and demonstrates that there may be a window of opportunity for intervention in the creation of a 'healthy' microbiome to promote long term health. The mechanisms for this programming effect are not fully understood but the study demonstrated the T helper cell type induced by colonisation varied. *M. catarrhalis* and *H. influenzae* induced a mixed T helper cell response. No effect of *S. pneumoniae* was observed (Følsgaard et al. 2013). By inducing a mixed T helper cell response, the Th1 responses needed for intracellular bacterial destruction may be impaired, potentially leading to chronic inflammation (Følsgaard et al. 2013). Further work showed differences in systemic inflammatory profiles in infants colonised with respiratory pathogens in the first 6 months of life (Rahman-Fink et al. 2018).

Several studies indicate that childhood atopy, a frequent precursor to asthma, is as affected by the early colonisation patterns of the gut. Finding high levels of *E. coli* or *C. difficile* is associated with increased risk of atopy (J Penders et al. 2006; Kalliomäki et al. 2001). The lack of *Lactobacillus* or *Bifidobacterium* in neonatal faecal samples also increases the risk of atopy (Sjögren et al. 2009). It seems that anomalous airway and gut microbiome colonisation early in life prime the individual for atopy and asthma later in childhood.

One study has extrapolated data from 16S rRNA gene sequencing to create a probable metagenomic profile within TA from preterm infants and studied metabolomic signatures within the same samples (Lal et al. 2018). This identified metabolome differences affecting the risk of CLD. Metagenomic profile suggested differences in lipid and hormonal metabolism may be targets to explore in modulating risk of CLD. The methodology of creating the metagenomic profile used in this paper requires many assumptions and further work with directly sequenced profiles are likely to be more accurate.

1.5.8 Summary

Culture-independent techniques have repeatedly demonstrated that the lungs are not a sterile environment. The environmental conditions of the nasal passages are distinctly different to that at the alveoli and the respiratory tract is likely to harbour many individual niches along its course.

In the neonatal population several studies have attempted to characterise typical respiratory colonisation patterns of preterm infants, particularly looking for associations between bacterial colonisation and CLD. Further longitudinal work is needed to identify whether an optimum lung microbiota is established and the timing of when this occurs.

The interactions between microbiota and host continue to be the source of ongoing research. The mechanisms discussed may explain some of the effects of dysbiosis over many host body systems. The gut microbiome has been more thoroughly investigated with regard to these

effects, but it is possible that organisms colonising the lung may exhibit similar effects. The role of the early microbiome identified by Vissing *et al* and Bisgaard *et al* demonstrates the importance of microbiota research using new-born infants.

1.6 The innate immune system

1.6.1 Introduction

The human body uses a complex series of interacting mechanisms to recognise and overcome potential microbiological threats. These mechanisms have traditionally been divided into the innate and adaptive immune system. The adaptive immune system utilises B and T lymphocytes to mount a response to an invading pathogen (Koenderman, Buurman, and Daha 2014). This immune response takes time to react to a potential threat but is remembered by the host to prevent against future infection by that specific organism. The adaptive immune system is only seen in higher organisms (Buchmann 2014).

The innate immune system is found in almost all multicellular organisms. It reacts more quickly than the adaptive immune system to pathogens overcoming the physical barrier of skin and mucosal surfaces.

1.6.2 Pattern recognition receptors of the innate immune system

The innate immune system utilises a series of pattern recognition receptors (PRR) to identify the presence of threats to the host, thus activating the innate immune response. The motifs recognised by PRR are known as pathogen-associated molecular patterns (PAMP). Non-pathogen related signals activating the innate immune response are known as danger associated molecular patterns (DAMP). These allow initiation of immune response to non-infectious tissue damage such as trauma. PRRs can be classified into five main groups, namely Toll-like receptors (TLRs), nucleotide binding and oligomerization domain (NOD) like receptors (NLRs), retinoic acid-inducible gene-I like receptors, absent-in-melanoma like receptors and C-type lectins (Barbé, Douglas, and Saleh 2014).

1.6.2.1 Toll-like receptors

In 1996 Toll was demonstrated to be critical in resisting fungal infection in *Drosophila* (Lemaitre et al. 1996). The TLRs in mammalian cells are named because of their similarity to Toll. A year later, the molecule now known as TLR4 was shown to be capable of inducing an innate immune response (Medzhitov, Preston-Hurlburt, and Janeway 1997). Animal models using mice were used to identify the function of TLRs. By ablating the TLR4 gene they were able to show that mice without functioning TLR4 were unable to respond to the Gram-negative bacterial cell wall constituent LPS, a known endotoxin. This demonstrated TLR4 as a PRR for LPS. Since then it has been shown that each TLR detects specific PAMP.

Toll-like receptors are trans-membrane proteins with a common structure. The extracellular domain contains a horseshoe-shaped leucine-rich repeat domain responsible for ligand recognition. The intracellular domain is similar to that of the IL-1 receptor (known as the Toll/IL-1 receptor (TIR)) and is involved in signal transfer. The intra- and extra-cellular domains are linked a single transmembrane domain (McClure and Massari 2014).

Figure 1-5 below shows the different TLRs and their activating ligands. The variety of activating ligands enables the innate immune response to react to a variety of invading pathogens.

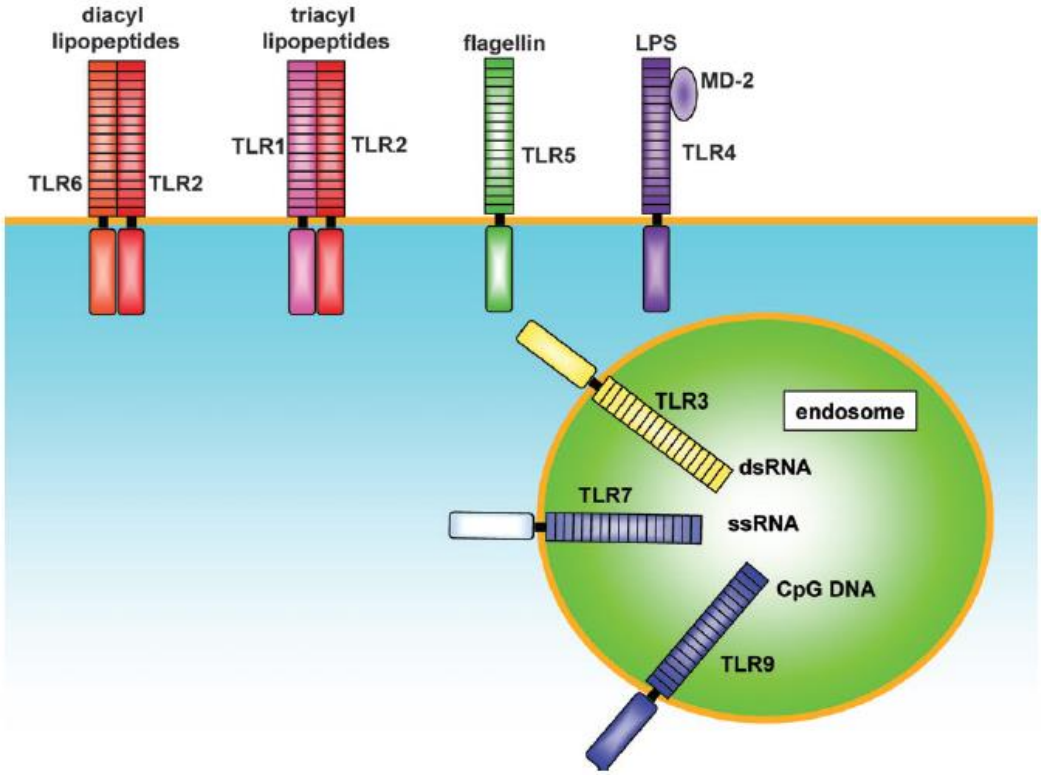


Figure 1-5: TLRs found in humans and their ligands.
***TLR2 is essential in the recognition of microbial lipopeptides. TLR1 and TLR6 cooperate with TLR2 to discriminate subtle differences between triacyl and diacyl lipopeptides, respectively. TLR4 is the receptor for LPS. TLR9 is essential in CpG DNA recognition. TLR3 is implicated in the recognition of viral dsRNA, whereas TLR7 and TLR8 are implicated in viral-derived ssRNA recognition. TLR5 recognizes flagellin. TLRs 1,2,4,5 and 6 are located on the cell surface, TLRs 3,7 and 9 are located intracellularly within the membrane of the endosome* (Takeda and Akira 2005).**

1.6.2.1.1 TLRs 1, 2 and 6

TLR2 has been demonstrated to recognise a range of microbial derived molecules. These include lipoproteins from Gram-negative bacteria (Aliprantis et al. 1999), peptidoglycan and lipoteichoic acid from Gram-positive bacteria (Schwandner et al. 1999), lipoarabinomannan

from mycobacteria (Means et al. 1999) and zymosan from fungi (Underhill et al. 1999). TLR2 operates in combination with other member of the TLR family to discriminate between microbial components. TLR2 and TLR6 immunoprecipitate together suggesting a physical interaction between the molecules (Ozinsky et al. 2000). The co-operation between TLR2 and TLR6 allows differentiation between triacyl and diacyl lipopeptidases (O Takeuchi et al. 2001). TLR1 has also been shown to functionally associate with TLR2 in identifying triacyl lipopeptidases (Osamu Takeuchi et al. 2002).

1.6.2.1.2 TLR3

TLR3 binds double stranded RNA (Alexopoulou et al. 2001) allowing the host to respond to viruses that incorporate double stranded RNA into their replication cycle.

1.6.2.1.3 TLR4

TLR4 was initially described as recognising LPS from Gram-negative bacterial cell walls (Qureshi et al. 1999). It has subsequently been revealed that LPS alone does not activate TLR4, but must first form a complex with LPS binding protein, present in serum (Takeda, Kaisho, and Akira 2003). TLR4 has also been shown to recognise fusion proteins of Respiratory Syncytial Virus and a number of host derived molecules including heat shock proteins (Ohashi et al. 2000).

1.6.2.1.4 TLR5

Flagella are found on bacteria acting as propellers to help move bacteria and aid in the attachment of bacteria to host cells (Takeda, Kaisho, and Akira 2003). TLR5 recognises flagellin, a component of bacterial flagella (Hayashi et al. 2001). TLR5 has been shown to be important at detecting these structures at the epithelium within the gut and lung.

1.6.2.1.5 TLRs 7, 8 and 9

TLR7, TLR8 and TLR9 are all located within the endosome of the cell. They are highly conserved in terms of structure and function, all being activated by nucleic acids. TLR7 and TLR8 are activated by single stranded RNA from viral pathogens, identified due the high guanosine or uridine content, with different viral species activating TLR7 and 8 (Heil et al. 2004). TLR9 binds to unmethylated CpG-dinucleotide-containing DNA sequences found commonly in the bacterial genome (Bauer et al. 2001).

1.6.2.1.6 Other TLRs

TLR10 is the only known human expressed TLR for which the ligand is still to be identified. It is expressed on the cell surface. Current evidence suggests that it is involved in the recognition of bacterial and viral pathogens but may also have an anti-inflammatory effect. (S. M. Y. Lee et al. 2014; Oosting et al. 2014) TLR11, TLR12 and TLR13 have been shown to be expressed in mice, but are not thought to be expressed in humans (Brubaker et al. 2015).

1.6.2.2 TLR expression

TLRs are expressed throughout all cells of the innate immune system. Monocytes and macrophages express all TLRs except TLR3 (Muzio et al. 2000). Phagocytes express an abundance of TLRs. Within human dendritic cells myeloid dendritic cells express TLR1, TLR2, TLR4, TLR5, and TLR8, and Plasmacytoid dendritic cells exclusively express TLR7 and TLR9 (Kadowaki et al. 2001). Selective expression of TLRs at different epithelial sites within the body is documented through detection of the TLRs themselves and transcriptional evidence for their production using messenger RNA. Table 1-3 below demonstrates the TLRs shown to be expressed from human epithelium at different sites of the respiratory tract and bowel.

Table 1-3: TLR expression on Respiratory and Bowel epithelial cells demonstrating variable expression of TLRs between different epithelial tissues (McClure and Massari 2014).

		Protein	mRNA
Respiratory			
	Nasal	**TLR 1-10**	**TLR 2,3 and 4**
	Tracheal/Bronchial	**TLR 1-10**	**TLR 1,2,3,4,5,6,7,9 and 10**
	Lung	**TLR 1,2,4,5 and 6**	**TLR 2,4 and 5**
Bowel			
	Oesophageal	**TLR 1-5**	**TLR 1-5**
	Gastric	**TLR 2,4 and 5**	**TLR 2,4 and 5**
	Intestinal	**TLR 1-10**	**TLR 2,3,4,5 and 9**

1.6.2.3 TLR signalling

TLR activation leads to a change in the intracellular TIR domain beginning a cascade of signalling molecules, see Figure 1-6. TLRs act primarily via the myeloid differentiation primary response gene 88 (MyD88)-dependent pathway and the MyD88-independent pathway. In the MyD88-dependent pathway, TIR domain containing adaptor proteins (TIRAP) and MyD88 are initially recruited. MyD88 further recruits members of the interleukin-1 receptor-associated kinase (IRAK) family of molecules, specifically IRAK4 is essential for TLR signalling (Lin, Lo, and Wu 2010). The resulting cascade causes phosphorylation of inhibitor of NF-κB protein (Iκβ) protein causing degradation of Iκβ. Functional Iκβ acts to prevent nuclear factor kappa-

light-chain-enhancer of activated β cells (NF-κβ) from entering the nucleus. The degradation of Iκβ allows NF-κβ to enter the nucleus and act as a transcription factor for pro-inflammatory cytokines (Carmody and Chen 2007).

Each TLR recruits a specific combination of adaptor molecules to enable activation of different transcription factors leading to the suitable response to pathogens. All TLRs except TLR3 are able to signal via the MyD88-dependent pathway. The MyD88-independent pathway signals using TRAF molecules to result in activation of nuclear transcription factors including NF-κB and Interferon Regulatory Factor 3 (IRF3) (Kawai and Akira 2007).

Figure 1-6 below demonstrates the MyD88-dependent and MyD88-independent pathways visually.

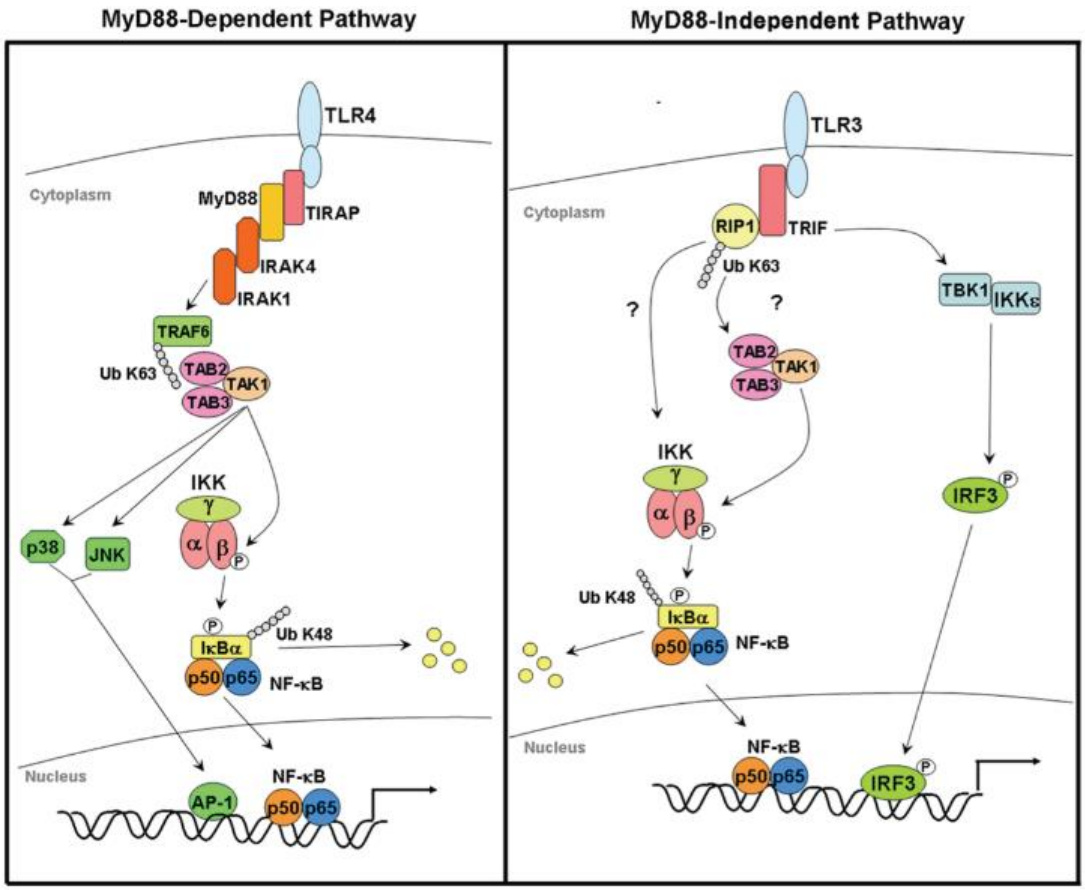


***Figure 1-6 TLR signalling pathways demonstrating the MyD88-dependent and MyD88-independent pathways* (Carmody and Chen 2007).**

The result of NF-κβ entering the nucleus and altering transcription is the production of many cytokines and effects on both the cell being stimulated and on neighbouring cells. The number of genes known to be upregulated by NF-κβ is currently several hundred (Gilmore 2007) including IL-6 and IL-8. The overall result is to upregulate the inflammatory processes within the cell and to signal to neighbouring cells regarding the danger sensed by the TLR.

1.6.3 Nod-like receptors

A further class of PRRs are the Nod-Like receptors (NLRs). These intracellular molecules sense PAMPs and DAMPs within the cytoplasm. The structure of NLRs is split in to 3 functional domains. The C-terminal domain is rich in leucine repeats and binds the activating ligand to senses the presence of intracellular threat. The central nucleotide binding and oligomerization domain is necessary for oligomerization. The N-terminal protein–protein interaction domain is involved in signal transduction. In the absence of ligand, the C-terminal domain masks the N-terminal domain, preventing signal transduction (Martinon, Burns, and Tschopp 2002).

1.6.4 The inflammasome

1.6.4.1 Introduction

In 2002 Martinon et al described complex macromolecules within the cytoplasm responsible for caspase recruitment and IL-1β production. These complexes were named inflammasomes (Martinon, Burns, and Tschopp 2002). Inflammasomes consist of multiple activated NLR molecules which bind the adaptor molecule apoptosis-associated speck-like protein containing a caspase activation domain (ASC). This recruits and activates caspase-1. This activation leads to the processing of IL-1β and IL-18 from their inactive forms to activated cytokines (Kayagaki et al. 2011; Proell et al. 2013). Once activated cytokines act as cell signalling molecules to amplify the immune response in local tissue. The structure of the inflammasome is demonstrated in Figure 1-7 below, with the NLRP3 inflammasome used as an example. The result of inflammasome activation for the activated cell is cell death and release of the cytoplasmic contents (B. K. Davis, Wen, and Ting 2011). This mechanism of cell death is termed pyroptosis (Galluzzi et al. 2012). The consequences of inflammasome activation are therefore significant for the cell and on a larger scale for the host organism as a whole.

1.6.4.2 The NLRP3 inflammasome

The most studied and best understood inflammasome is the NLRP3 inflammasome. It is expressed in myeloid cells. Unlike other inflammasomes which are understood to have specific PAMPs/DAMPs leading to activation, the NLRP3 inflammasome is activated by a diverse range of pathogenic stimuli and also a series of self-derived molecules. The known pathogen derived stimuli include molecules from bacteria, viruses, fungi and protozoa (Kanneganti et al. 2006; Muruve et al. 2008; Dostert et al. 2009; Gross et al. 2009). In addition, inorganic molecules and ultraviolet radiation can also stimulate inflammasome assembly (Barbé, Douglas, and Saleh 2014). Figure 1-7 includes a list of activating molecules and non-molecular stimulants for the NLRP3 inflammasome. The broad range of molecular structure of the known initiators of NLRP3 have led some to question if NLRP3 is a true receptor (Lamkanfi and Dixit 2014). No direct evidence of ligand binding exists to refute this possibility. NLRP3 may sense a general change within the cytoplasmic milieu, rather than having specific binding sites, as a classical

receptor is understood to function (Latz 2010).

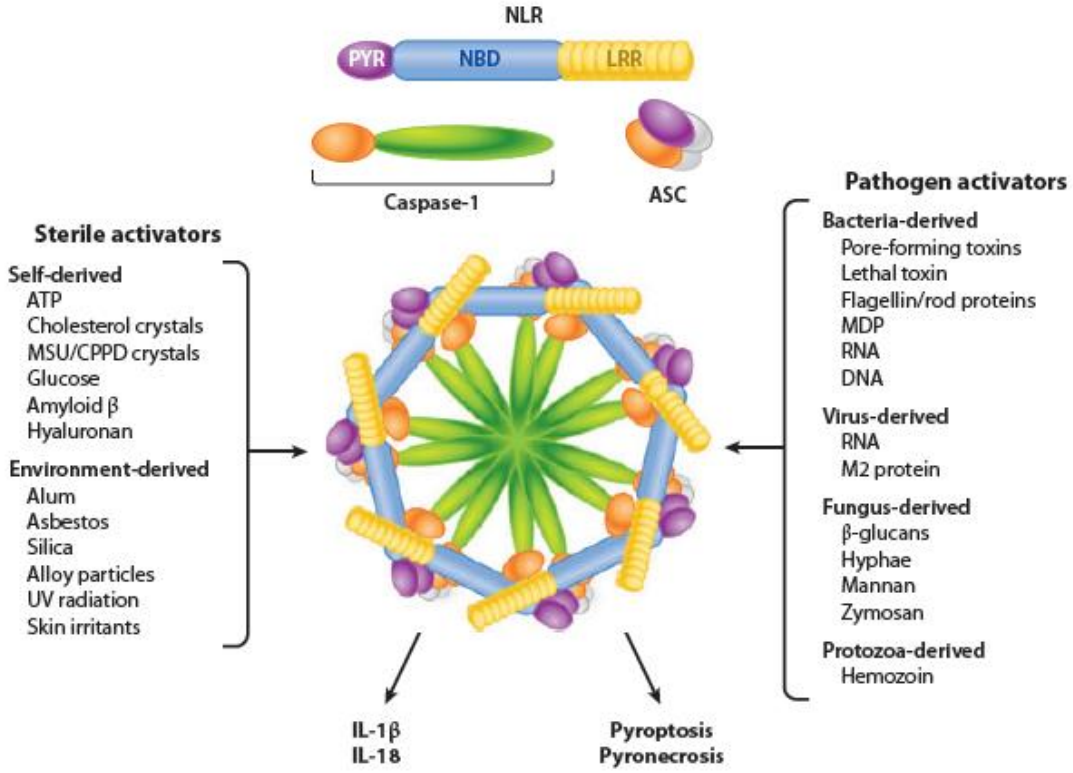


Figure 1-7: The structure of the NLRP3 Inflammasome (B. K. Davis, Wen, and Ting 2011)

1.6.4.3 Activation of the NLRP3 inflammasome

Complete understanding of the activation mechanism of NLRP3 is yet to be elucidated. The catastrophic consequence of pyroptosis for the cell hosting inflammasome activation results in tight regulation of inflammasome formation. Two signals are required for NLRP3 inflammasome formation.

1.6.4.3.1 Priming of the NLRP3 inflammasome

The first signal, also known as priming of the inflammasome, is achieved via activation of the MyD88-dependent pathway or MyD88-independent pathway (Bauernfeind et al. 2009; Franchi, Eigenbrod, and Núñez 2009), leading to NF-κβ driven changes in nuclear transcription. Activation of these pathways is most commonly through TLR activation, but also through the IL-1 receptor, tumour necrosis factor receptor and NOD2. The original model of priming resulted in upregulation of the components of the inflammasome. A more rapid mechanism of priming, not dependent on translation has also been demonstrated, utilising de-ubiquitination of NLRP3 (Juliana et al. 2012; Schroder et al. 2012). A conceptual model of inflammasome priming can be seen in Figure 1-8 below.

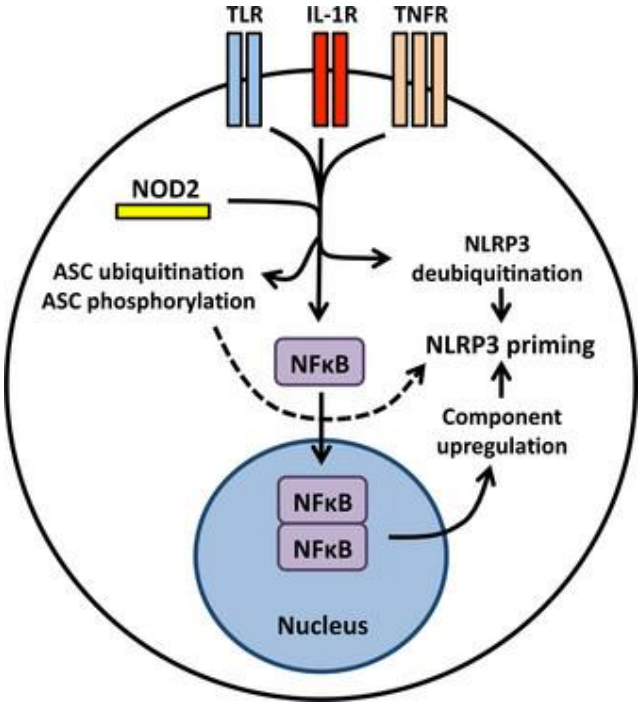


Figure 1-8: Priming the NLRP3 inflammasome for activation **(Elliott and Sutterwala 2015).**

1.6.4.3.2 Signal-two for inflammasome activation

Studies of inflammasome activation have revealed several independent ways of providing the second signal to cause assembly of the NLRP3 inflammasome *in-vivo*. This process is not yet fully understood (Próchnicki, Mangan, and Latz 2016), and more than one possible activating mechanisms may explain how many different ligands have been shown to activate inflammasomes.

Lysosomal rupture is a suggested mechanism. Hornung et al demonstrated lysosomal disruption from hypotonic incubation, chemical treatment and particulate phagocytosis led to NLRP3 activation (Hornung et al. 2008). Secondly movement of potassium across the cell membrane has also been shown to activate the NLRP3 inflammasome. Potassium efflux from the cell, induced by pore forming toxins or extracellular ATP activate the inflammasome (Mariathasan et al. 2006). Blocking potassium channels reduces NLRP3 activity (Lamkanfi et al. 2009). Calcium signalling is also theorised as an independent mechanism for inflammasome activation. Calcium influx to the cell is required for ultraviolet radiation and cholesterol-dependent cytolysin induced NLRP3 activation (J. Chu et al. 2009; Feldmeyer et al. 2007). NLRP3 inflammasome activation in response to high extracellular calcium concentrations has been reported in two studies (Rossol et al. 2012; G.-S. Lee et al. 2012).

Mitochondrial dysfunction has also been suggested as a mechanism for activation of inflammasomes. Evidence suggests that the activated inflammasome is localised to the mitochondria within the cell (Zhou et al. 2011). Mitochondria could be a source of reactive

oxygen species (Zhou et al. 2011) or of adenosine triphosphate, also shown to be important in inflammasome assembly (Duncan et al. 2007). However, activated NLRP3 and caspase-1 cause mitochondrial damage making it difficult to study which features of mitochondrial dysfunction are a cause rather than the result of inflammasome formation (Yu et al. 2014).

1.6.5 Apoptosis-associated speck-like protein containing a caspase-recruitment domain

Apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC) also known as PYD and CARD Domain protein (PYCARD) is a 22 kDa adaptor protein essential to many pathways of innate immune system (Masumoto et al. 1999). It is an essential adaptor molecule within NLRP3 inflammasome activity. In response to the detection of PAMP/DAMP within the cell, activation of NLRP3 causes rapid polymerisation of ASC into large helical filaments by facilitating interactions of the pyrin domains (Franklin et al. 2014). This facilitates significant caspase-1 activation, via the CARD domain, causing the polymerization and filament formation of pro-caspase-1 resulting in its self-activation (Fernandes-Alnemri et al. 2007; Lu et al. 2014). ASC is also essential in activating caspase in the NLRP1 and NLRC4 inflammasomes, despite the presence of a caspase activation domain in the respective NLRs (Compan et al. 2014). In these cases, ASC appears to form a ring like structure around the macromolecular complex (Man et al. 2014).

Following activation of the inflammasome, ASC dimers may polymerise to form 1-2 μm macromolecular specks within the cytoplasm. These have been termed pyroptosomes, as macrophages quickly succumb to pyroptosis following ASC speck formation (Fernandes-Alnemri et al. 2007). Following pyroptosis, the ASC specks are released into the extracellular space.

1.6.5.1 Extracellular ASC

ASC specks released from a cell retain their functionality and can activate extracellular caspase-1 (Franklin et al. 2014). Baroja-Mazo et al suggested that extracellular ASC is released in oligomeric form, but also concluded that extracellular ASC is able to activate caspase-1 (Baroja-Mazo et al. 2014). Neighbouring cells can phagocytose ASC specks and the large protein structures of the specks act in a similar way to crystals, causing lysosomal damage, leading to the inflammasome of NLRP3 activation (Franklin et al. 2014). Cytosolic ASC specks were also witnessed to aggregate ASC from the host cell after phagocytosis. This is suggesting that ASC may act in a prion like way to recruit soluble ASC from within the cell (Franklin et al. 2014). ASC thus acts as a cell signalling molecule propagating the inflammatory response. Both ASC and NLRP3 fragments have been shown to be released by macrophage cells prior to pyroptosis, suggesting that cell death may not be necessary for these molecules to act on adjacent cells.

1.6.5.2 Extracellular ASC specks and disease

In mice, ASC speck have been detected within the extracellular space of lymph nodes following injection of *Pseudomonas auriginosa*, suggesting they may play a role in propagating inflammation in response to infection (Franklin et al. 2014).

Within the lungs, ASC specks have been detected in the supernatant of BAL fluid in patients with COPD, but not seen in BAL samples from healthy donors (Franklin et al. 2014). Cigarette smoke has been shown to provoke the release of ASC and soluble NLRP3 into the BAL supernatant in mice (Eltom et al. 2014).

Cryopyrin-associated periodic syndromes (CAPS) are known to be caused by a genetic mutation within the gene encoding NLRP3 (Hoffman, Wanderer, and Broide 2001). Both NLRP3 particles and ASC specks have been detected in the serum of patients with active CAPS. There were however significantly lower levels of both proteins in healthy volunteers and patients with other similar inherited autoimmune diseases such as familial Mediterranean periodic fever syndrome (Baroja-Mazo et al. 2014).

1.6.6 Inflammasomes in lung inflammation

Inflammasome activation was first identified within macrophages and initially studied in sepsis models. Further work demonstrates that inflammasome activation in the lung is involved in lung inflammation both in response to infection but also in non-infective inflammatory lung diseases.

Inflammasome activation has been demonstrated in models of mechanical ventilation *in-vitro.* A mouse model of ventilation demonstrates that alveolar macrophages may sense cyclical stretch and respond with activation of the innate immune system via the NLRP3 inflammasome (J. Wu et al. 2013). Murine macrophage autophagy, mediated via NLRP3, has been demonstrated to propagate lung inflammation in response to hyperventilation (Y. Zhang et al. 2014). A probable role for NLRP3 activation has also been demonstrated using BAL samples in adults with ventilator-associated lung injury demonstrating increased transcription of NLRP3 and ASC. The same study demonstrated partial protection from ventilator-associated lung injury in mice deficient of NLRP3 (Kuipers et al. 2012).

NLRP3 has also been implicated in the inflammatory response to hyperoxia. Mice with NLRP3 deficiency have a reduced inflammatory response to hyperoxia compared to wild type mice (Fukumoto et al. 2013). NLRP3 inflammation in response to hyperoxia is responsible for the increased cellular permeability in hyperoxia induced lung injury (Kolliputi, Shaik, and Waxman 2010).

In adult patients with acute respiratory distress syndrome, caspase-1-dependent IL-18 production has been demonstrated to play an important role in pathogenesis (Dolinay et al.

2012). A mouse model of ARDS also found evidence of inflammasome mediated inflammation, with NLRP3 activation contributing to acute lung injury (Grailer et al. 2014).

Inflammasome activation is implicated in response to many inhaled irritants/carcinogens including cigarette smoke, asbestos, diesel exhaust particles and engineered nanoparticles (Ather et al. 2013). Studies evaluating a role for inflammasome activity in asthma have conflicting results (Allen et al. 2012; Tran et al. 2012), with studies focused on infective exacerbations and atopic asthma. One theory suggests that asthmatic individuals may have reduced innate immune system activity in the airways, making them more susceptible to bacterial infections (Im and Ammit 2014).

NLRs also have an important role within the lung in sensing and responding to infection. The NLRP3 inflammasome has been shown to be activated by lung pathogens including, *K. pneumoniae, S. pneumoniae, S. aureus, C. pneumoniae, M. tuberculosis, L. pneumophila*, influenza virus, rhinovirus, respiratory syncytial virus, and *Aspergillus fumigatus* (Chaput et al. 2013).

Preterm infants who develop CLD are often ventilated, possibly for prolonged periods and are exposed to hyperoxia and infection. Each of these factors is associated with inflammasome activation. In a thorough assessment of the role of NLRP3 in the development of CLD, Liao et al demonstrated an important role for inflammasome activation in CLD development. In a mouse model, preterm-mice deficient for NLRP3 are protected from the effects of hyperoxia demonstrating no caspase-1 activity, no IL-1β production and no inflammatory response to hyperoxia. NLRP3 deficient mice also have normally developed alveolarisation, in contrast to wild type mice (Liao et al. 2015). IL-1β:IL-1βra (IL-1β receptor antagonist) ratio can be used as a measure of inflammation following NLRP3 activation. In a baboon model, Liao et al demonstrated a significant increase in this ratio in the preterm baboons ventilated for 14 days and given supplementary oxygen when compared to matched controls. In humans IL-1β, IL-1ra and IL-1b:IL-1ra ratio in the first 1–3 days of life are all significantly higher in infants who go on to develop CLD compared to infants who do not develop CLD (Liao et al. 2015). Caffeine, a therapy given to most preterm infants as a respiratory stimulant has also been shown to prevent CLD (Schmidt et al. 2006). Recent evidence has shown that caffeine supresses NLRP3 activity providing a possible mechanism for the therapeutic effect (W. Zhao et al. 2019).

1.6.7 Innate immunity in new-born infants

The innate immune system is tightly controlled *in-utero* and after delivery. The preterm neonate heavily is dependent on the innate immune response for protection against pathogens in early life as adaptive immunity is immature at this age (Siegrist and Aspinall 2009). Defective regulation of neonatal innate immunity can result in excessive IL-1β, which can result in

neonatal onset multisystem inflammatory disorder, characterised by low birth weight, urticaria, aseptic meningitis and hearing loss (Goldbach-Mansky et al. 2006). Work to understand inflammasome activation and innate immune function has come from cell culture models, murine studies and *in-vivo* work on adult diseases. Some work has attempted to understand the innate immune activation in the new-born period.

1.6.7.1 Toll-like receptors in new-born infants

One study compared TLR responses from cord blood monocytes between term born infants and infants born <33 weeks' gestation. Stimulation of various TLRs demonstrated that preterm monocytes produced less pro-inflammatory cytokines than term monocytes. No effect of priming from exposure to chorioamnionitis or a gender difference was witnessed (Sharma et al. 2014). This corroborates the finding of reduced cytokine secretion from preterm cord monocytes in response to *S. epidermidis* (Strunk et al. 2012). *S. epidermidis* colonises all infants within the first days of life and causes a sepsis in a number of preterm infants <32 weeks' gestation (Vergnano et al. 2011) but is a non-pathogenic commensal in more mature infants and immunocompetent adults. The expression of TLR4 on preterm monocytes and their phagocytic activity was no different to that of adults but the cytokine response to stimulation varied with gestation. Extreme preterm infants (<30 weeks' gestation) secreted less pro-inflammatory cytokines (IL-1β, IL-6, IL-8, and TNF-α) than moderate preterm infants (31-33 weeks' gestation) with term infants producing even higher concentrations. The diminished TLR function is possibly to be due to reduced expression of MyD88 witnessed in preterm monocytes (Yan et al. 2004).

Within the lungs a murine study demonstrated that TLR2 and TLR4 expression was virtually undetectable before the equivalent of 30 weeks' human gestation (Harju, Glumoff, and Hallman 2001).

1.6.7.2 NLRs/Inflammasomes

A study of cord blood analysing the ability of monocytes to form inflammasomes demonstrated a change in monocyte subset expression with gestation. Before 33 weeks' gestation immature low CD14 expressing/CD16 positive monocytes predominated which failed to produce pro-IL-1β in response to LPS. Cord monocytes from infants of greater than 33 weeks' gestation were more mature with high CD14 expression. These monocytes were capable of expressing pro-IL-1β, but unable to convert this to mature IL-1β. NLRP3 induction following TLR stimulation was also reduced (Sharma et al. 2015).

The same study showed that after birth the ability of monocytes to secrete IL-1β increases to adult levels soon after birth. Using samples from peripheral blood taken at day 7-35 from preterm infants born at 24-29 weeks' gestation demonstrated no difference compared to adult monocytes following stimulation with LPS (Sharma et al. 2015). Further work has shown that

defective inflammasome responses in VLBW infants may increase the risk of late onset sepsis (Zasada et al. 2018).

1.6.7.3 Cellular Responses

The reduced phagocytic activity of neonatal neutrophils is probably due to reduced opsonisation by soluble factors than a deficiency of neutrophil phagocytic capacity (Strunk et al. 2011). The lower intracellular bactericidal capacity of preterm neutrophils compared to adults appears to be a consequence of neutrophil immaturity (Källman et al. 1998; Peden et al. 1987). In contrast, neonatal monocytes appear to have phagocytic and intracellular bacterial killing ability equal to adult monocytes (Gille et al. 2006).

1.6.8 Summary

The human innate immune system has evolved several mechanisms for recognising threats including the TLRs and NLRs. Inflammasomes are known to be important in pulmonary inflammation including contributing to CLD. Of interest is the adaptor molecule ASC and the ability of ASC to maintain activity following release from the cell.

1.7 Bronchoalveolar lavage

1.7.1 Introduction

BAL is a technique for sampling the epithelial lung fluid from distal airways and alveoli (Connett 2000). It has been used in many pulmonary disease states to obtain samples for evaluation of inflammation and infection. In adults and older children, samples are usually collected by direct vision via a flexible bronchoscope. In the neonatal period non-bronchoscopic bronchoalveolar lavage is used in ventilated infants (de Blic et al. 2000; Grigg, Arnon, and Silverman 1992; S. Kotecha et al. 1995).

Non-bronchoscopic BAL is performed by inserting a suction catheter through the endotracheal tube of a ventilated infant, advancing until resistance is felt. Sterile saline is instilled into the lung before being aspirated and collected. Lying the infant supine with the infant's head to the left side increases the chance of the catheter entering the right lung (Placzek and Silverman 1983; Soong and Hwang 1991), with the aim of sampling from the right lower lobe.

1.7.2 Safety

The safety of this procedure in preterm infants has been evaluated. At the point of lavage, bradycardia and oxygen desaturation are known risks (B. Morrow and Argent 2001; S. Kotecha 1999), however, infants recover from this rapidly similarly to routine tracheal suctioning. Some may require transiently increased inspired oxygen concentration. At one-minute post lavage, one study identified that heart rate had returned to baseline, with mean arterial blood pressure elevated and oxygenation significantly decreased. Both mean arterial blood pressure and

oxygenation showed partial recovery by three minutes of age (Grigg, Arnon, and Silverman 1992). Long term complications from repeated sampling washing out surfactant were evaluated by comparing chest x-rays of infants who underwent this procedure with controls. No difference was found between chest x-ray scores of the right lower lobe (Vyas et al. 2002). Many studies report using this technique in the neonatal period with no adverse events (Grigg, Arnon, and Silverman 1992; Mildner et al. 2001; D'Angio et al. 2002; Davies et al. 2010; S. Kotecha et al. 1995).

1.7.3 Reliability

Non-bronchoscopic BAL aims to wedge the catheter within the right lower lobe bronchus, enabling aspiration of lung lining fluid from the distal airways after flushing with saline. Flushing is usually performed twice in neonatal patients. The first sample has been demonstrated to contain different cellular components to subsequent samples in both non-bronchoscopic and bronchoscopic BAL (Ratjen et al. 1994; Pohunek, Pokorna, and Striz 1996; Grigg, Arnon, and Silverman 1992). Typically, the first sample shows a higher proportion of neutrophils and a lower proportion of lymphocytes. This is hypothesised to indicate that the first sample preferentially collects epithelial lining fluid from the bronchi, with subsequent flushes sampling from more distal airways (de Blic et al. 2000). Radiological evidence from adults also supports this theory. Serial were x-rays taken during and after 3 serial instillations of contrast media via a bronchoscope in adult males. The initial sample distributed more proximally than subsequent samples (C. A. Kelly et al. 1987). Marsh et al detected no significant differences in the microbiota sampled between 2 lavages collected sequentially during a single bronchoscopy on young children (Marsh et al. 2016), but it is uncertain if new-born infants would have similar results.

In the neonatal population volumes of saline recovered are smaller than in older children or adults so samples are usually pooled for analysis (S. Kotecha 1999). Fractional analysis in BAL samples from neonatal patients showed no difference in volume recovered or estimate epithelial lining fluid recovered between 2 serial aliquots (Grigg, Arnon, and Silverman 1992).

BAL analysis is complicated by the variable dilution of epithelial lining fluid. Levels of urea and albumin have been used historically to calculate a dilution factor. This is, however, unreliable as factors such as dwell time of instilled saline and airway disease have been shown to affect these concentrations (Ward et al. 1992). The European Respiratory Society taskforce recommends reporting constituents of compounds within the supernatant of BAL in concentration per millilitre of BAL recovered (de Blic et al. 2000).

Little analysis has been done on the repeatability of BAL. One study looked at infants less than 15 months old receiving extracorporeal membranous oxygenation (Mildner et al. 2001). BAL

samples were obtained simultaneously from both lungs. This showed a trend towards higher cytokine concentrations on the right side. It also demonstrated that recovered BAL volume was the most important source of variability between variations in cytokine concentrations. This was not a significant factor in variation in cell counts, for which age was important.

1.7.4 Comparing bronchoalveolar lavage and tracheal aspirate samples

The lungs are an inaccessible anatomical site for collecting samples. BAL is one technique which has been used to attempt to collect samples which accurately reflect the milieu of the lower airways and alveoli. Other techniques that have been tried are open lung biopsy, tracheal aspirate (TA), sputum sample analysis, bronchial brushing and upper respiratory tract sampling.

One study showed a difference in total protein concentration and IgA secretory component; known to be secreted from proximal airways; between TA and BAL samples in infants (Dargaville, South, and McDougall 1999). This suggests that the two techniques sample different environments. In the same study, the concentration of surfactant proteins, secreted distally was not different between TA and BAL. A further study showed comparable cell counts and cytokine levels between TA and BAL taken from preterm infants (D'Angio et al. 2002). For some indices TA may be equivalent to BAL.

No comparison has been done on the difference in microbiome sampled using the two techniques in infants. A small adult study using healthy volunteers found no difference in the composition of the microbiome within the whole the respiratory tract. More distal sources showed a lower number of total organisms, but the diversity and composition were similar. Intensive sampling analysed specimens from nasopharyngeal swabs, oral swabs, oral wash, glottis bronchoscopic BAL of the right middle lobe and protected bronchial brushing (Charlson et al. 2011). The authors conclude that the lung microbiota is a continuation of the upper respiratory tract microbiota. Another study, however, did to find differences between the microbiota of anatomically sites in close proximity (oral and nasal cavity) within the upper respiratory tract, challenging the assertion that the whole respiratory tract has the same microbiota (Bassis et al. 2014).

Using culture independent techniques on samples obtained via BAL is well established (Morris et al. 2013; Erb-Downward et al. 2011; Dickson, Erb-Downward, Freeman, et al. 2014; Bassis et al. 2015). Even compared with modern culturing protocols, culture independent techniques increase the detection rate of bacteria within BAL fluid from 80.4% to 95.7% (Dickson, Erb-Downward, Prescott, et al. 2014).

1.7.5 Summary

BAL is an established procedure used to sample the distal airways and lungs for research and clinical indications. Non-bronchoscopic BAL the preferred technique in new-born infants. In preterm infants non-bronchoscopic BAL has been shown to reliably sample the right lower lobe and the evidence suggests that it is a safe procedure. TA is more routinely performed method of obtaining samples of lung lining fluid in ventilated preterm infants. Evidence is inconsistent as to whether TA samples provide the comparable results as BAL samples.

1.8 Summary

CLD is a major cause of long-term morbidity in surviving preterm infants, affecting mainly those born extremely preterm. Due to the improved survival of the most preterm infants CLD rates have not improved despite recent advances in neonatal care. Understanding the influence the microbiota can have over human pathology has improved the understanding of many disease processes. The microbiota may have a contributing role in the development of CLD with current work showing conflicting results. Further work is needed to explore if an optimum respiratory microbiota can be established in the airways and lungs and the if so the timing of the acquisition of the microbiota. Few studies have tested the microbiota of the upper respiratory tract in extreme preterm infants in the early weeks of life and no data exists on the relationship between the bacterial colonisation of the upper and lower respiratory microbiota in preterm infants.

The inflammatory response within the lung is central to the pathophysiology of CLD. The microbiota has been shown to modulate the host immune response in other disease processes including other lung diseases. More work is needed to understand the effect of early bacterial colonisation within the lung over the innate immune system activation. Better understanding of the microbiome the host immune response to colonisation and the interaction between microorganisms and host may lead to identification of targets for therapeutic intervention.

1.9 Aims and hypotheses

1.9.1 Hypotheses

1. Infants who develop CLD have a different respiratory tract microbiota from infants who do not develop CLD.

2. The net effect of bacterial colonisation of the respiratory tract in preterm infants is to cause progressive inflammation which may contribute to CLD.

3. The respiratory microbiota displays variation between the upper and lower respiratory tract.

4. The innate immune system is activated within the neonatal lung via the NLRP3 inflammasome.

5. Extracellular ASC acts as propagator of inflammation in CLD.

1.9.3 Specific aims:

1. To take serial samples from the lower airways of preterm infants at risk of CLD and identify the pattern of bacterial colonisation in the lower airways and lungs.

2. To measure bacterial load within samples from the lower airways and lungs of preterm infants and relate this to inflammatory profiles.

3. To compare the lung microbiota in infants who do and do not go on to develop CLD as well as a control group of ventilated term infants.

4. Analyse the effect of antibiotic treatment on respiratory and lung colonisation in ventilated preterm infants.

5. To compare airway colonisation in ventilated preterm infants between different recruitment centres.

6. To test the depth of sequencing of the rRNA 16S gene to attempt to identify bacteria to species level.

7. To analyse serial samples from the upper airways of preterm infants and a term control group for the presence and relative abundance of bacteria and identify the pattern of bacterial colonisation.

8. To measure bacterial load within samples from the upper airways of preterm infants and analyse changes over time.

9. Analyse the effect of delivery mode, antibiotics and probiotics on upper respiratory tract colonisation in preterm infants.

10. To compare upper airway colonisation in preterm infants between two different recruitment centres.

11. To compare bacterial community structure between NPA, TA and BAL samples from preterm infants.

12. To compare total bacterial load between NPA, TA and BAL samples from preterm infants.

13. To compare measures of alpha diversity between NPA, TA and BAL samples from preterm infants.

14. To compare concordance of the dominant organism identified from individual samples from the upper and lower respiratory tract taken within 24 hours from the same infant.

15. To attempt to identify the presence of inflammasome components within BAL fluid from preterm infants.

16. To examine the pro-inflammatory potential of ASC in neonatal BAL fluid from preterm infants.

17. To produce ASC recombinantly for use in cell stimulation experiments.

18. To determine the mechanism of action of the pro-inflammatory effect of extracellular ASC on respiratory epithelial cells in culture.

Chapter 2: Materials and Methods

2 Materials and Methods

2.1 Patient recruitment

Patients were recruited from the University Hospital of Wales Cardiff, between November 2014 and March 2017. Patients were recruited into one of 3 groups:

- Preterm infants, ventilated. Infants born at ≤32 weeks' gestation and required ventilation within the first 24 hours of life for respiratory distress syndrome.
- Term infants, ventilated. Infants born ≥37 weeks' gestational age admitted to the neonatal unit and ventilated within 24 hours of age for a non-respiratory condition. E.g abdominal surgical problem.
- Term infants, not ventilated. Infants born at ≥37 weeks' gestational age, with no known pathology, who are considered healthy and on the post-natal ward.

Any infants with respiratory pathology such as congenital adenomatous lesions or diaphragmatic hernias were not recruited to the study. Cases of meconium aspiration and hypoxic ischaemic encephalopathy were also excluded. Those infants with suspected underlying chromosomal anomalies or who were not expected to survive, as determined by the treating clinicians, were also not recruited.

Parents were approached to enter their infant in the study either during threatened preterm delivery or after delivery. An information sheet was provided (Appendix 2 and 3) and written informed consent was obtained prior to the infant being entered into the study.

An outbreak of *Acinetobacter baumannii* in the University Hospital of Wales resulted in limited admissions to the neonatal unit. An amendment to the study protocol to include Southmead Hospital, Bristol as a recruitment centre received ethical approval and infants were recruited between May 2015 and September 2016. A second amendment to the protocol received ethical approval in August 2016 to allow collection of stool samples from infants on the postnatal ward.

Ethical approval for the study was given by the Wales Ethics Committee 2, reference number 14WA0190, and the study was approved by the research and development committees of the Cardiff and Vale University Health Board and North Bristol NHS Trust. A copy of confirmation of ethical approval is contained in Appendix 1.

2.2 Sample collection

2.2.1 Bronchoalveolar lavage

BAL samples were collected following the European Respiratory Society taskforce guidelines (de Blic et al. 2000) and as per previous studies in the Kotecha research group (Davies et al. 2010; Beeton et al. 2011).

Intubated preterm infants on the neonatal unit in Cardiff routinely receive surfactant therapy at birth and if necessary, again at 12 hours of age. To avoid interfering with this treatment the initial BAL was aimed to be performed at either 12 hours of age before additional surfactant therapy or in cases where informed consent had not been obtained by 12 hours of age at 24 hours of age.

The procedure has been widely used in other studies studying neonatal lung inflammation (S. Kotecha, Wilson, et al. 1996; Davies et al. 2010; Vyas et al. 2002). The infant was placed supine with the head to the left side and the ventilator briefly disconnected from the endotracheal tube. A size 6Fr nasogastric (NG) feeding tube (Intervene, Chesterfield, UK) was advanced through the endotracheal tube until it met resistance. 1 ml/kg of sterile 0.9 % sodium chloride for injection (B.Braun Medical Ltd, Sheffield, UK) was instilled through the catheter. After a brief pause, a suction pressure of 5-7 kPa is applied to the NG tube. The tube was slowly withdrawn with the aspirated material collected in a mucous specimen trap (Pennine Healthcare, Derby, UK) and the infant reconnected to the ventilator. After a pause to allow the infant to recover, the procedure was repeated and the samples pooled. I performed the procedure on all recruited infants to reduce variability.

The samples were immediately placed on ice and transported to the laboratory for processing.

2.2.2 Tracheal aspirate

Tracheal Aspirate (TA) samples from Cardiff were collected by nursing staff as a part of routine clinical care, according to local policy. The endotracheal tube (ET) was disconnected from the ventilator and 1 ml/kg of sterile 0.9 % sodium chloride (B.Braun Medical Ltd, Sheffield, UK) was instilled via the ET. A sterile suction catheter (TenderTip, GBUK, Yorkshire, UK) was then inserted to a depth equal to the length of the ET tube and a suction pressure of 5-7 kPa applied as the catheter was slowly withdrawn. The infant was then reconnected to the ventilator and after a pause to allow the infant to recover, the procedure was repeated and the sample pooled.

TA samples from Bristol were also collected as part of routine care. The local policy utilised a sterile in-line suction device (Halyard UK, Surrey, UK) changed every 24 hours. The infant remained connected to the ventilator during suctioning. Samples for the study were only collected immediately after the device had been changed to prevent contamination of samples

from previous aspirations. As in Cardiff, 1 ml/kg of saline was instilled to the ET tube via a port on the in-line suction device. The catheter was advanced to a depth equal to the length of the ET tube and a suction pressure of 5-7 kPa applied as the catheter was slowly withdrawn. After a pause to allow the infant to recover the procedure was repeated and the sample pooled. Samples were then immediately frozen at -20 °C prior to transfer to the laboratory.

TA samples were collected from ventilated preterm infants on the neonatal unit at the Royal Victoria Infirmary (RVI) as part of the ELFIN trial. Ethical approval and informed consent had been obtained for further testing of TA samples. 107 TA samples from 29 infants were selected to be analysed for this study. Infants were chosen based on the same inclusion criteria for infants recruited from the Cardiff and Bristol sites. Where multiple samples were collected from an individual infant, samples were chosen to mirror the sampling frequency of infants recruited from Cardiff and Bristol. TA samples from the RVI were collected as part of routine clinical care. The whole suction catheter was placed into a universal container and stored at -80 °C prior to DNA extraction. TA samples from the RVI were kindly provided by Dr J Berrington (Newcastle Upon Tyne NHS Trust) and Dr C Lanyon (University of Northumbria).

2.2.3 Nasopharyngeal aspirate

Nasopharyngeal aspirate (NPA) samples from Cardiff were collected contemporaneously with BAL samples. A size 6Fr nasogastric feeding tube (Intervene, Chesterfield, UK) was gently inserted via the nostril to the nasopharynx. A suction pressure of 5-7 kPa was applied and the tube slowly withdrawn. Each nostril was suctioned. 2 ml of sterile sodium chloride for injection (B.Braun Medical Ltd, Sheffield, UK) was aspirated through the NG tube to flush any mucous within the catheter into a mucous specimen trap (Pennine Healthcare, Derby, UK). The sample was placed on ice and transported to the laboratory for processing.

NPA samples collected in Bristol were collected according to the local departmental guidelines. 2 ml/kg of sterile saline for injection (B.Braun Medical Ltd, Sheffield, UK) was instilled directly through the nostrils. A sterile suction catheter was used to aspirate the fluid from the nasopharynx under a suction pressure of 5-7 kPa. Samples were immediately frozen at -18 °C prior to transportation to the laboratory.

2.3 Processing of samples

2.3.1 Cardiff

2.3.1.1 Bronchoalveolar lavage

Samples were transported on ice immediately following collection to the laboratory. Processing was completed within 1 hour of collection. Samples were weighed on arrival and the empty sample traps re-weighed after transferring samples to a microcentrifuge tube (Eppendorf UK, Stevenage, UK) to calculate the weight of the sample. The volume was calculated using an approximated density of 1 g/mL.

A cell count was performed using a haemocytometer by mixing 20 µL of the sample with an equal volume of Trypan Blue solution (Life Technologies (Gibco), Paisley, UK). A haemocytometer is device used to estimate cell concentrations. It consists of a grid of perpendicular lines etched in the middle of a thick glass microscope slide. The slide has specific dimensions, so the area covered by the grid is known making it possible to count the number of cells in a specific volume of solution. An average of 3 squares of the haemocytometer count was taken and the number of cells per ml calculated.

Cytospin slides were prepared loading 20 µL of BAL fluid, followed by 100 µL of phosphate buffered saline (PBS) with 10 mM Ethylenediaminetetraacetic acid (EDTA) (Fisher Scientific. Leicestershire, UK) into the well of the cassette. Polysine adhesion slides (VWR, Leicestershire, UK) were used to enhance the adherence of the cells to the slide. If the cell count was greater than $1.2x10^6$ cells/ml then a smaller volume of BAL fluid was used to give a cell concentration of $0.3\text{-}1.2x10^6$ cells/ml. The slides were air dried overnight before being stored at -20 °C.

The remaining BAL fluid was centrifuged at 10,000 x*g* for 10 minutes at 4 °C to produce a cell pellet and cell free supernatant. The supernatant was removed and stored in 25 µL aliquots. Both the cell pellet and supernatant were labelled appropriately and stored at -80 °C until further processing.

2.3.1.2 Nasopharyngeal aspirate

Samples were transported on ice to the laboratory. On arrival the BAL fluid was centrifuged at 10,000 x *g* for 10 minutes at 4 °C to leave a cell pellet and cell free supernatant. The supernatant was removed and stored in 25 µL and 100 µL aliquots. Both the cell pellet and supernatant were stored at -80 °C until further processing.

2.3.1.3 Tracheal aspirate

Tracheal aspirate samples were taken by nursing staff and immediately placed at 4 °C awaiting collection. All samples were collected from the unit within 2 hours of sampling. Samples were transferred to the laboratory on ice and immediately stored at -80 °C until further processing.

2.3.2 Bristol

All samples taken in Bristol (TA, NPA and stool samples) were immediately frozen at -18 °C. Periodically, samples were transferred frozen to the laboratory in Cardiff using a heat resistant box and freezer packs pre-frozen at -80 °C to prevent thawing of samples. TA, NPA and stool samples were stored at -80 °C on arrival in Cardiff.

2.3.2.1 Nasopharyngeal aspirate and tracheal aspirate samples

At the point of DNA extraction, NPA and TA samples were defrosted, and the samples weighed. The samples were transferred to sterile microcentrifuge tubes (Eppendorf UK,

Stevenage, UK) then centrifuged at 10,000 x *g* for 10 mins at 4 °C to leave a cell pellet and cell-free supernatant. The empty universal container was re-weighed to calculate sample weight and volume using an approximated density of 1 mg/mL. The supernatant was removed and stored at -80 °C. The cell pellet was immediately used for DNA extraction.

2.4 Differential cell counts

The prepared cytospin slides were defrosted prior to fixation and staining with Haemacolor cell staining kits. (Merck KGaA, Darmstadt, Germany) This utilises a methanol-based fixative followed by a red nuclear stain and a blue cytoplasmic stain. The slides were immersed in the three solutions for 1 min each, prior to washing with tap water. Images of the stained cells were obtain using a Nikon Eclipse microscope (Nikon Instruments UK, Surrey, UK) at 40x magnification. Differential cell counts were obtained by counting a minimum of 300 cells from the digital images. Examples of cells from cytospin slides are shown in Figure 2-1.

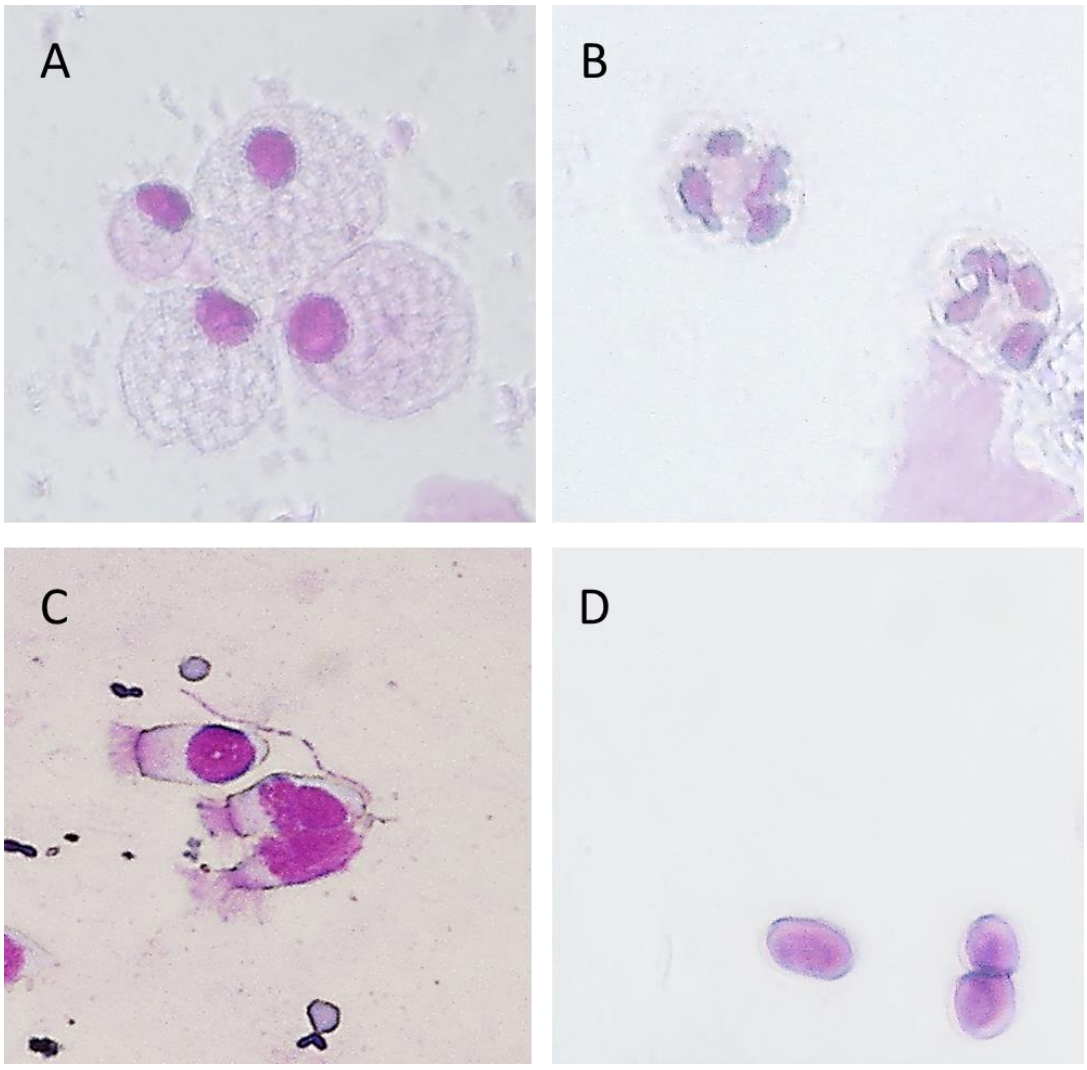


Figure 2-1: Images of cell types observed from cytospins of BAL samples.
A= Mononuclear cells, B= Polymorphonuclear cells, C= Epithelial cells, D= Red blood cells.

2.5 Solutions

2.5.1 Phosphate buffered saline

1 L of 10x PBS solution was prepared by dissolving 100 g of NaCl (Fisher Scientific. Leicestershire, UK), 2.5 g KCl (Fisher Scientific, Leicestershire, UK), 14.4 g anhydrous Na_2HPO_4 (Fisher Scientific. Leicestershire, UK) and 2.5 g KH_2PO_4 (Fisher Scientific, Leicestershire, UK) in distilled water.

2.5.2 Sodium dodecyl sulphate solution

A 10% Sodium dodecyl sulphate (SDS) solution (w/v) was prepared by dissolving 10 g of SDS (Sigma-Aldrich, Dorset, UK) in 100 mL of deionised H_2O.

2.5.3 Sample reducing buffer for SDS-polyacrylamide gel electrophoresis (PAGE)

A working solution of sample reducing buffer was prepared combining 10 ml 0.5 M Tris solution (pH 6.8), 8 ml of 10% SDS solution, 5 g of glycerol (Sigma-Aldrich, Dorset, UK), 2.0 ml of 14.3 M β-mercaptothanol (Sigma-Aldrich, Dorset, UK) and 5 mg of bromophenol blue (Sigma-Aldrich, Dorset, UK).

2.5.4 Phosphate buffered saline-Tween

10 ml of Tween 20 (Fisher Scientific, Leicestershire, UK) was added to 1 L of 10x PBS. This was diluted 1 in 10 with distilled water to give a 1x working solution prior to use.

2.5.5 SDS-PAGE running buffer

A 10x concentrated pre-prepared stock solution of running buffer containing 0.25 M Tris, 1.92 M Glycine and 1% SDS (National Diagnostics UK, Yorkshire, UK) was diluted with deionised water to produce a 1x concentration by diluting the stock 1 in 10. Freshly prepared prior to use.

2.5.6 Western blotting transfer buffer

A 2x concentrated stock solution was prepared by dissolving 4.88 g of Tris (Fisher Scientific, Leicestershire, UK), 20 ml of the 10% SDS solution and 400 ml of isopropanol (Fisher Scientific, Leicestershire, UK) to a total volume of 1000 ml in deionised H_2O. The solution was adjusted to pH 8.3 with acetic acid.

2.5.7 Stripping buffer

Antibody stripping buffer prepared containing 100 mM mercaptoethanol (Sigma-Aldrich, Dorset, UK), 2% SDS (Sigma-Aldrich, Dorset, UK) and 62.5 mM Tris-HCl (Fisher Scientific, Leicestershire, UK) made up with deionised water.

2.5.8 Sodium phosphate buffer

150 mM Sodium Phosphate buffer was made by dissolving 4.45 g of $NaH_2PO_4*2H_2O$ (Sigma-Aldrich, Dorset, UK) and 17.25 g of Na_2HPO_4 (Sigma-Aldrich, Dorset, UK) in deionised water. The solution was adjusted to pH 8.0.

2.5.9 DNA extraction buffer

A DNA extraction buffer was prepared according to the protocol for DNA extraction from soil supplied by Promega. A buffer containing 5 M guanidine thiocyanate (Fisher Scientific. Leicestershire, UK), 1% Na-Lauroylsarcosine (Sigma-Aldrich, Dorset, UK), 100 mM ethylenediaminetetraacetic acid (EDTA) (Fisher Scientific, Leicestershire, UK) and 1% polyvinylpyrrolidone K30 (Sigma-Aldrich, Dorset, UK) was made using 150 mM sodium phosphate buffer and stored at 4 °C.

2.5.10 Hi-Trap column deactivation buffers

Buffers prepared using protocol obtained from column manufacturers.

Buffer A – 0.5 M ethanolamine, 0.5 M Sodium Chloride. pH 8.3. Prepared by dissolving 15.09 mL of Ethanolamine (density 1.012 g/mL) (Sigma-Aldrich, Dorset, UK) and 14.61 g of sodium chloride (Fisher Scientific, Leicestershire, UK) in 500 mL of deionised H_20.

Buffer B – 0.1 M sodium acetate, 0.5 M Sodium Chloride. pH 4. Prepared by dissolving 4.1 g of sodium acetate (Fisher Scientific, Leicestershire, UK) and 14.61 g of sodium chloride (Fisher Scientific, Leicestershire, UK) in 500 mL of deionised H_2O.

2.5.11 Hi-Trap column elution buffer

Buffer containing 15 mM triethanolamine and 140 mM sodium chloride was prepared by dissolving 0.59 g of triethanolamine (Sigma-Aldrich, Dorset, UK) and 1.64 g of sodium chloride (Fisher Scientific, Leicestershire, UK) in 200 ml of deionised H_2O. pH adjusted to 11.5 with NaOH.

2.5.12 Hi-Trap column coupling buffer

Buffer containing 0.2 M sodium bicarbonate and 0.5 M sodium chloride was prepared by dissolving 0.17 g of sodium bicarbonate and 2.92 g of sodium chloride (Fisher Scientific, Leicestershire, UK) in 100 mL of deionised H_2O.

2.5.13 STET buffer

Buffer prepared by dissolving 8 g of sucrose (Fisher Scientific, Leicestershire, UK), in H_2O and mixing with 500 µL of Triton X (Sigma-Aldrich, Dorset, UK), 10 ml of 0.5 M EDTA solution (pH 8) and 1 ml of 1 M Tris solution (pH 8). Made up to a total volume of 100 mL with H_20.

2.5.14 ELFO buffer

Buffer prepared at 50x concentration by dissolving 242 g of Tris (Sigma-Aldrich, Dorset, UK) in deionised H_2O, adding 100 ml of 0.5 M EDTA solution, made up to a total volume of 1 L at pH 7.7. A 1x working solution was produced by diluting the stock solution using deionised H_2O.

2.5.15 NP-40 lysis buffer

100 mL of buffer was prepared by combining 10 mL of NET buffer, 1 mL of 600 mM CHAPS solution, 1 mL of 10 mM iodoacetamide, 10 mL of 10% (w/v) NP-40, 50 µL of 50 mM Phenylmethylsulfonyl fluoride and 77.95 mL of deionised H_2O. The preparation of constituent solutions is described below. Final concentration within lysis buffer is 50 nM Tris, 1% NP-40, 6 mM CHAPS, 150 mM NaCl, 5 mM EDTA, 50 µM phenylmethylsulfonyl fluoride and 0.1 mM iodoacetamide.

2.5.15.1 NET buffer

Buffer prepared by dissolving 15.14 g of Tris (Sigma-Aldrich, Dorset, UK), 21.92 g of NaCl (Fisher Scientific, Leicestershire, UK) and 4.56 g of EDTA (Fisher Scientific. Leicestershire, UK) to a final volume of 250 mL of deionised H_2O and corrected to pH 8.0. The final concentration of solutes is 500 mM Tris, 1.5 M NaCl and 50 mM EDTA.

2.5.15.2 600 mM CHAPS solution

1.8 g of 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate (CHAPS) (Sigma-Aldrich, Dorset, UK) dissolved in 5 ml of deionised H_2O.

2.5.15.3 10 mM iodoacetamide

92.5 mg of Iodoacetamide (Sigma-Aldrich, Dorset, UK) dissolved in 50 mL of deionised H_2O.

2.5.15.4 10% (w/v) NP-40

10 g of NP-40 (Sigma-Aldrich, Dorset, UK) dissolved in 100 ml of deionised H_2O.

2.6 DNA extraction from respiratory samples

2.6.1 Cardiff and Bristol samples

Cell pellets from BAL, TA and NPA samples were resuspended in 0.5 ml DNA extraction buffer. The samples were transferred to 0.5 ml sterile skirted tubes (Starlabs UK, Milton Keynes, UK) containing 0.1 g of 0.1 mm zirconia beads (Biospec, OK, USA). Samples were bead beaten to break open bacterial cells for 3x30 seconds at 6.5 m/s using a FastPrep 24 device (MP Biomedicals (Fisher Scientific, Leicestershire, UK)). Centrifugation of samples at 10,000 x*g* for 60 seconds to remove the beads was performed. The supernatants were loaded into the Maxwell 16 automated DNA extraction device (Promega UK, Southampton, UK). 300 µL of the supplied elution buffer was added to the elution chamber. Samples were stored at -80 °C prior to further analysis.

No respiratory samples from infants or adults were available for optimising DNA extraction methods. Saliva and laboratory cultures were used to compare different DNA extraction techniques. The table below compares DNA extraction from adult saliva samples comparing with Maxwell 16 instrument (no bead beating) to the manual Fast Spin kit for Soil (MP Biomedicals (Fisher Scientific, Leicestershire, UK)).

Table 2-1: Comparison of automated verses manual DNA extraction techniques

Method of DNA extraction	Elution Volume (µL)	Mean DNA Concentration (ng/µL)	Mean DNA Yield (µg)
Maxwell 16	180	3.02	0.54
Fast Spin Kit	90	1.14	0.10

DNA extracted from saliva samples in duplicate as per manufacturer's instructions. DNA quantified using Qubit fluorometer.

Using saliva is useful to demonstrate the DNA extraction works using biological samples however recovered DNA could be purely host DNA. To ensure DNA from both Gram-Positive and Gram-Negative bacteria was extracted pure growths of *E. coli* and *S. aureus* were used to test effectiveness of bacterial DNA extraction. Bacterial cultures were grown as per the protocol in Section 2.8. Cultures were serially diluted prior to DNA extraction. Drop counts from these cultures estimated the *E. coli* culture contained $1.42x10^9$ colony forming units and the *S. aureus* culture contained $7.02x10^8$ colony forming units. The effect of adding a bead beating step using zirconia beads (6.5 m/s for 30 seconds repeated for a total of 3 times) to the protocol was also assessed.

Table 2-2: Comparing yield of DNA from E. coli and S. aureus cultures, demonstrating the effect of bead beating. DNA extracted in duplicate and quantified using Qubit fluorometer.

Dilution	Maxwell 16 Only (Mean DNA Concentration ng/µL)		Maxwell 16 + Bead Beating (Mean DNA Concentration ng/µL)	
	E. coli	*S. aureus*	*E. coli*	*S. aureus*
10^{-2}	15.7	0.26	18.9	3.78
10^{-3}	1.51	<0.05	0.968	0.303
10^{-4}	0.119	<0.05	0.152	0.051

This demonstrated that the bead beating increased the yield of DNA when used in combination with the Maxwell 16 instrument. Visualisation of the extracted DNA from the experiment in

Table 2-1 on an agarose gel showed that adding bead beating to the protocol resulted in a yield of genomic DNA, without damaging the DNA as a clear band was seen at the appropriate size for both organisms (Figure 2-2).

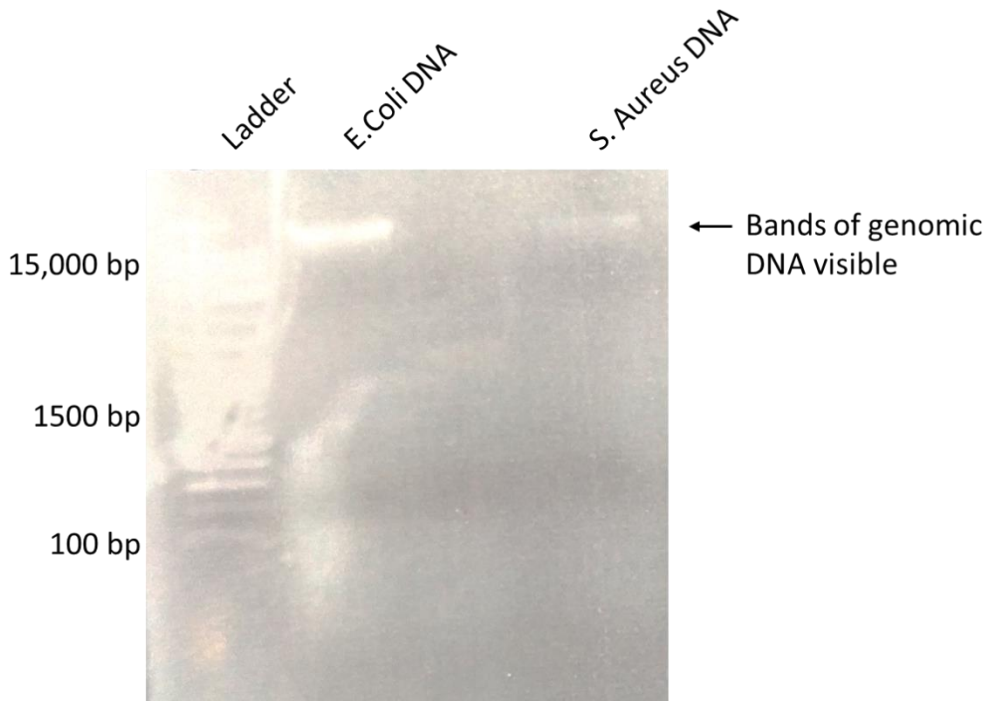


Figure 2-2: Image of gel electrophoresis of DNA extracted using bead beating + Maxwell 16 instrument

2.6.2 Newcastle samples

DNA extraction of samples from Newcastle was kindly performed by Greg Young, PhD Student at the University of Northumbria. DNA was extracted using the Powerfoods Microbial DNA extraction kit (MoBio (Qiagen), Hilden, Germany) as per the manufacturers instructions. This kit utilises a bead beating step and a spin column to isolate bacterial genomic DNA. DNA was stored at -80 °C and transferred to the laboratories of Prof N Klein in University College London for 16S rRNA gene sequencing.

2.7 DNA quantification

DNA concentration was measured using a Qubit fluorometer (original model) (Life Technologies, UK). A master-mix was prepared using 0.5 µL of Quantifluor dye (Promega UK, Southampton, UK) per reaction with 99.5 µL of TE buffer per reaction. In a thin walled 0.5 mL volume microcentrifuge tube 100 µL of TE was mixed with 98 µL of the prepared solution and 2 µL of sample DNA solution to give a final reaction volume of 200 µL. Standards were prepared using 2 µL of 1xTE solution and 1 µL of λ DNA solution supplied with the kit. After calibration with the standards the Qubit fluorometer was used to quantify DNA concentration within the samples.

2.8 Preparation of *E. coli* DNA for use in qPCR

A pure culture of *E. coli* was obtained from Prof J Marchesi. *E. coli* was grown on plates of nutrient agar (Oxoid, ThermoFisher, Leicestershire, UK) overnight incubated at 37 °C in 5% CO_2. A freezer stock was prepared using tryptic soy broth (Oxoid, ThermoFisher, Leicestershire, UK) and DMSO (Sigma-Aldrich, Dorset, UK). A single colony was selected and grown in nutrient broth (Oxoid, ThermoFisher, Leicestershire, UK) overnight at 37 °C in 5% CO_2. *E. coli* DNA was extracted using the Maxwell 16 instrument by the method described in paragraph 2.6. Concentration of the extracted DNA was obtained using a Qubit fluorometer as described in paragraph 2.7. The *E. coli* genome contains 7 copies of the 16S rRNA gene (Klappenbach et al. 2001) and the total genome contains $4.6x10^6$ base pairs (Blattner et al. 1997). The average molar mass of a single base pair is 650 g/mol. The number of copies of the 16S rRNA gene in the extracted DNA solution was calculated as below. In this example the concentration of DNA extracted from a pure growth of *E. coli* was measured to be 6.125 µg/mL.

Calculate weight of 1 mole of *E. coli* genome = Size of *E. coli* genome x Molar mass per base pair

$= 4.6x10^6$ base pairs x 650 g/mol/bp

$= 2.99x10^9$ g/mol

Calculate Molarity of *E. coli* genome within solution of extracted DNA

$$= \frac{\text{Conc. of DNA in solution}}{\text{Molar mass of } E.\ coli \text{ genome}} = \frac{6.125x10^{-3}\ g/L}{2.99x10^9\ g/Mol} = 2.04849x10^{-12}\ M$$

Multiply by Avogadro's constant to calculate number of copies of *E. coli* genome in solution

$= 2.14849x10^{-12} \times 6.022x10^{23} = 1.2336x10^{12}$ copies/L

$= 1.2336x10^9$ copies/mL

Multiply by 7 (as there are 7 copies of the 16S rRNA gene within the *E. coli* genome)

$= 1.2336x10^9 \times 7 = 8.635x10^9$ copies of 16S rRNA gene/ml

The equations $C_1V_1=C_2V_2$ was used to calculate the volume of solution and volume of sterile H_2O required to prepare a solution containing $4x10^5$ copies of the 16S rRNA gene per mL. The qPCR reaction used 2.5 µL of this solution giving $1x10^6$ copies of the 16S rRNA gene in the reaction.

A serial dilution of *E. coli* genomic DNA was prepared from $1x10^6$ copies 16S rRNA gene in 2.5 µL to $0.5x10^2$ copies 16S rRNA gene in 2.5 µL.

2.9 Quantification of bacterial load by qPCR

The assay to quantify bacterial load within samples was based on the BactQuant protocol (Liu et al. 2012) amplifying the V3-V4 region of the 16S rRNA bacterial gene. Experiments were performed in qPCR specific reaction tubes (ThermoFisher, Leicestershire, UK) using the Chromo4 thermal cycler (Bio Rad, Hertfordshire, UK). The Opticon Monitor 3 software package (Bio Rad, Hertfordshire, UK) was used to manage the experiment and analyse the results. A master mix was prepared using the volumes listed in Table 2-3.

Table 2-3: Constituents of master mix for qPCR reaction

Volume	Reagent
5 µL	Platinum qPCR Supermix – UDG (Thermo Scientific, Leicestershire, UK)
2.12 µL	Nuclease Free H_2O (Severn Biotech Ltd, Worcestershire, UK)
0.18 µL	Forward Primer (100 µM) (Eurofins, Ebersberg, Germany)
0.18 µL	Reverse Primer (100 µM) (Eurofins, Ebersberg, Germany)
0.0225 µL	TaqMan Probe (ThermoFisher, Leicestershire, UK)

The use of a master mix containing uracil-DNA glycosylase (UNG) and dUTP nucleotides allows prevention of cross contaminations by DNA from previous PCR reactions containing dUTP nucleotides. During an initial incubation phase UNG causes the degradation of dUTP in PCR products from previous amplifications, causing fragmentation of the DNA. Template DNA which does not contain dUTP bases remains unaffected. The initial denaturation step of the PCR causes inactivation of UNG allowing PCR amplification to continue (Tetzner 2009). The sequences of the primers used in this assay:

Forward Primer: 5′- CCTACGGGDGGCWGCA-3′

Reverse Primer: 5′- GGACTACHVGGGTMTCTAATC -3′

Primers were obtained in lyophilised form (Eurofins, Ebersberg, Germany) and reconstituted to 100 µM concentration using nuclease free H_2O. The use of degenerate bases within the primers (D,W,H and V) potentially allows the 16S rRNA gene region of more organisms to be identified. The International Union of Biochemistry defined the use of letters to represent different possible bases (D=G, A or T, W=A or T, H=A, T or C, V=G, A or C). Using primers

with slightly different sequences should allow 16S rRNA genes from organisms with subtle variations in the target sequences to be amplified.

A TaqMan Probe incorporating a 6-FAM (6-Carboxyfluorescein) reporter and a Molecular-Groove Binding Non-fluorescence Quencher (MGBNFQ) was used (ThermoFisher, Leicestershire, UK).

Sequence: (6FAM) 5′-CAGCAGCCGCGGTA-3′ (MGBNFQ)

7.5 µL of the master mix was added to each reaction well. 2.5 µL of sample DNA solution was added to give a total reaction volume of 10 µL.

The thermal cycling condition used are detailed below:

- 3 min at 50 °C for UNG treatment
- 10 min at 95 °C for Taq activation
- 40 cycles of
    - 15 s at 95°C for denaturation
    - 1 min at 60°C for annealing and extension

A standard curve was generated by running in triplicate a serial dilution of *E. Coli* genomic DNA. See example in Figure 2-3. The graph shows good reproducibility of replicates and good PCR efficiency.

Samples were run in triplicate. Threshold of fluorescence to define the reaction reaching above background fluorescence (cT threshold) was set to 0.01 units of fluorescence for each run of the experiment.

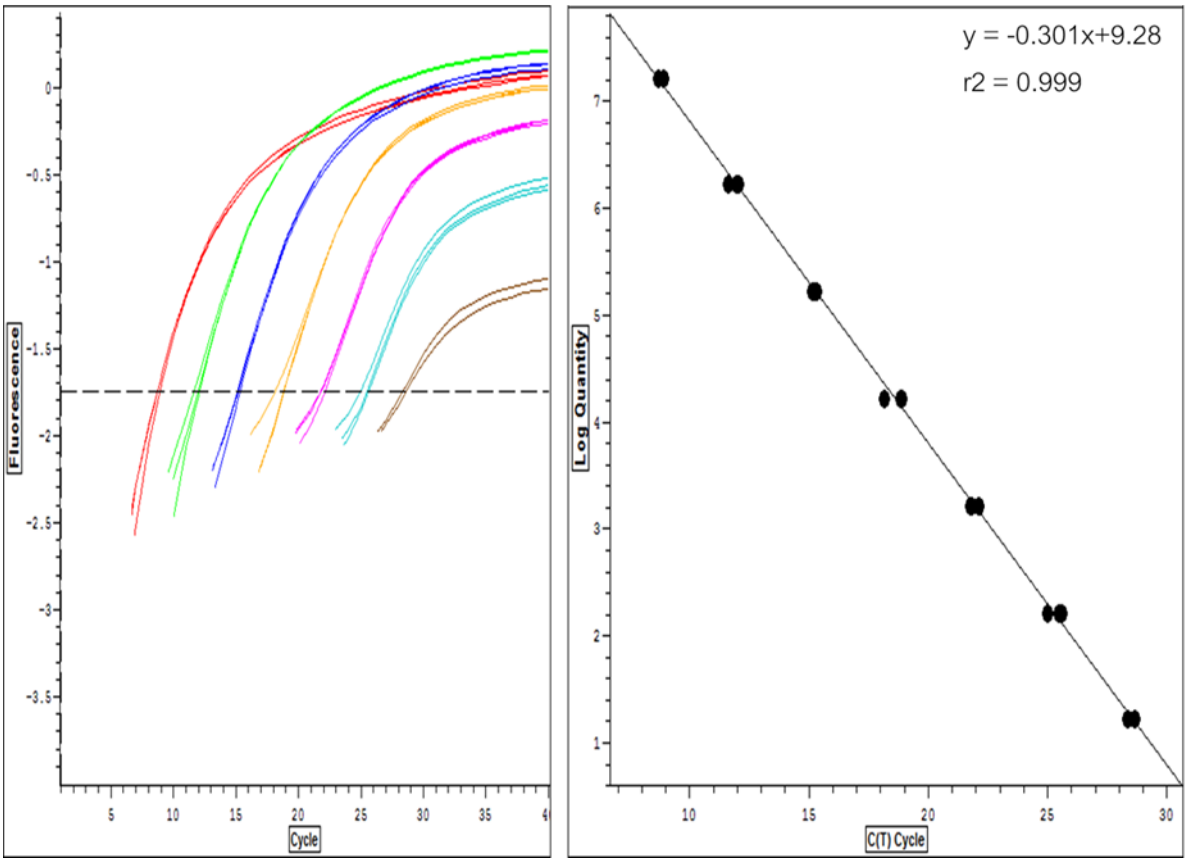


Figure 2-3: Example of generation of standard curve from qPCR experiment. Each colour represents a 10 fold dilution of E. Coli genomic DNA. Efficiency of reaction = 99.9%. Background fluorescence suppressed in this figure.

2.10 Sequencing of bacterial 16S rRNA gene

Preparation of the extracted DNA and the sequencing of the bacterial 16S rRNA gene of the BAL and TA samples was performed on my behalf by Prof Nigel Klein's team in the Institute of Child Health laboratories of University College London. Particular thanks to Dr Dagmar Alber and Dr Grace Logan who performed the amplification and sequencing. The preparation for sequencing of the NPA samples was undertaken by myself at the institute of Child Health following the same protocol.

2.10.1 qPCR inhibition check

A qPCR assay using a selection of samples was performed to ensure DNA within the samples would amplify and to check for inhibition of the PCR reaction. A PowerSYBR Green PCR master mix (ThermoFisher, Leicestershire, UK) was used with serial dilutions of extracted DNA (neat, 1:10, 1:100 and 1:1000) with samples run in duplicate.

Each reaction consisted of 10 µL of Master mix, 6 µL nuclease free H_2O (Bioline, London, UK), 1 µL of forward and reverse primers at 10 pmol concentration and 2 µL sample DNA to give a 20 µL reaction volume. The thermal cycling conditions used were 95 °C for 10 mins

followed by 45 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. The qPCR device used for the assay was a CF96 Touch Real Time PCR Detection System (BioRad, Hertfordshire, UK). The cT values for each dilution were plotted against dilution factor. A straight line demonstrated no inhibition to the PCR reaction.

2.10.2 Amplification of the 16S rRNA gene using barcoded primers and adaptors

The 16S rRNA gene is amplified using a PCR reaction prior to sequencing. To allow multiplexing of samples and to facilitate the binding of amplicons to the flow cell of the Miseq device, specific primers were designed incorporating barcodes and adaptors. Barcodes are a sequence of bases read during the sequencing process to allow identification of which sample a specific sequence originated from, adaptors introduce a 5' overhang to the sequence to allow binding of the DNA to the complementary strand of DNA on the flow cell of the Miseq. The primer pad allows adjustment of the melting temperature of the primer.

The structure of an example primer is shown below in Figure 2-4. A full list of all primer sequences used are shown in Appendix 4. The sequences of the 16S rRNA sequence specific components of the primers designed to amplify the V3-V4 region of the gene are 341F primer: CCTACGGGNGGCWGCAG and 805R primer: GACTACHVGGGTATCTAATCC. These sequences were originally designed to study marine bacterial communities (Herlemann et al. 2011) but have been extensively used in next generation sequencing and have been verified capture the majority of bacterial phyla (Klindworth et al. 2013). The primers were manufactured by Sigma-Aldrich (Sigma-Aldrich, Dorset, UK). The amplification reaction utilised a Taq PCR Core Kit (Qiagen, Hilden, Germany) with a master mix prepared using the components listed in Table 2-4.

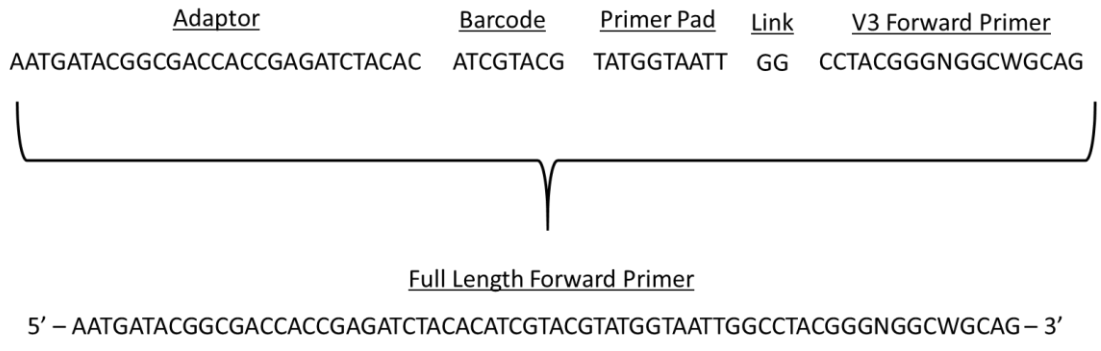


Figure 2-4: Structure of primers used to amplify the V3-V4 region of the bacterial 16S rRNA gene in preparation for paired end sequencing

Table 2-4: Components of the master mix for the PCR reaction used to amplify the 16S rRNA gene (V3-V4 region) in preparation for sequencing

Reagent	Volume per Reaction (µL)
Taq Polymerase	0.25
$MgCl_2$ Solution (25 mM)	1.0
dNTP Mix (10 mM each)	1.0
Q Solution	10.0
10x Buffer	5.0
Nuclease Free H_20 (Bioline, London, UK)	25.25

42.5 µL of master mix was aliquoted into the relevant wells of a 96 well PCR plate (Elkay, Hampshire, UK). 1.25 µL of the relevant primers at a concentration of 20 pM was then added to each well as outlined below (

	Forward Primer 1	Forward Primer 2	Forward Primer 3	Forward Primer 4	Forward Primer 5	Forward Primer 6	Forward Primer 7	Forward Primer 8	Forward Primer 9	Forward Primer 10	Forward Primer 11	Forward Primer 12
Reverse Primer 1												
Reverse Primer 2												
Reverse Primer 3												
Reverse Primer 4												
Reverse Primer 5												
Reverse Primer 6												
Reverse Primer 7												
Reverse Primer 8												

Figure 2-5) to give a unique combination of primers in each well. Two sets of reverse primers were available allowing two full 96 well plates to receive unique barcodes. Samples for amplification were performed in batches of 24-36 samples per run with a negative control and a DNA extraction control run with each batch. Any runs with a positive negative control sample were discarded and the PCR repeated.

	Forward Primer 1	Forward Primer 2	Forward Primer 3	Forward Primer 4	Forward Primer 5	Forward Primer 6	Forward Primer 7	Forward Primer 8	Forward Primer 9	Forward Primer 10	Forward Primer 11	Forward Primer 12
Reverse Primer 1												
Reverse Primer 2												
Reverse Primer 3												
Reverse Primer 4												
Reverse Primer 5												
Reverse Primer 6												
Reverse Primer 7												
Reverse Primer 8												

Figure 2-5: Primer layout for sequencing of samples within a 96 well plate resulting in a unique primer combination for each well

The thermal cycling conditions used during the amplification are shown below in Table 2-5. The thermocycler used for amplification was a CFX96 Touch (Biorad UK, Hertfordshire, UK).

Table 2-5: Thermal cycling conditions used during amplification of the bacterial 16S rRNA gene (V3-V4 region) in preparation for sequencing

Step	Temperature (°C)	Time	Cycles
Initial denaturation	95	3 mins	
Denaturation	95	30 secs	x30
Annealing	54	30 secs	
Extension	72	1 min	
Final extension	72	10 mins	
Hold	12	∞	

2.10.3 Purification of PCR product

Purification of the PCR product was achieved using AMPure beads (Beckman Coulter (UK), High Wycombe, UK). 35 µL of resuspended AMPure beads were added to each well and incubated for 5 minutes at room temperature to allow the DNA to bind to the beads. The PCR plate was then placed on a magnetic stand causing the beads and associated DNA to form a pellet. The supernatant was removed and the bead pellet washed twice with 200 µL 80% ethanol for 30 seconds each time. Care was taken to remove all ethanol and the beads were air dried at room temperature for 15 mins. The plate was removed from the stand and the beads resuspended in 50 µL of AE buffer (Qiagen, Hilden, Germany). The resuspended beads were incubated at room temperature for 2 minutes before being placed on the magnetic stand to pellet the beads. The eluted DNA solution was then transferred to a new plate.

2.10.4 Quantification of PCR product and library pooling

The concentration of DNA obtained following the PCR reaction was measured using the Qubit analyser (ThermoFisher, Leicestershire, UK) in a similar method that previously discussed. The Qubit high sensitivity assay kit (ThermoFisher, Leicestershire, UK) was used with 2 µL of DNA solution per sample.

Samples with DNA concentrations >0.3 ng/µL post PCR were considered to have amplified adequately for pooling. For the BAL samples this threshold was lowered to 0.1 ng/µL due to difficulty in amplifying these samples. Each sample was diluted to the threshold concentration and 10 µL of each diluted DNA solution was combined into a single tube to produce an amplicon library.

2.10.5 Quantification and quality checking of library

The pooled library was quality control checked prior to denaturation using the TapeStation dsDNA assay (Agilent Technologies, CA, USA). In wells of a 96 well plate, 2 µL of reagent buffer was combined with 2 µL of ladder or library. The sample was mixed on a plate mixer and briefly centrifuged prior to running the assay. The TapeStation conducted an electrophoresis of the DNA and ladder to allow visualisation of the DNA. This revealed a clean band at the expected size of the DNA amplicon. An estimate of the concentration of DNA within the sample was also made from the size of the band. An example of the results output from the TapeStation is shown below in Figure 2-6. Primer dimers would be represented by a small peak with a much lower fragment size. If primer-dimers were identified the library was cleaned using the AMPure beads as previously described and the TapeStation procedure repeated to confirm removal of primer dimers.

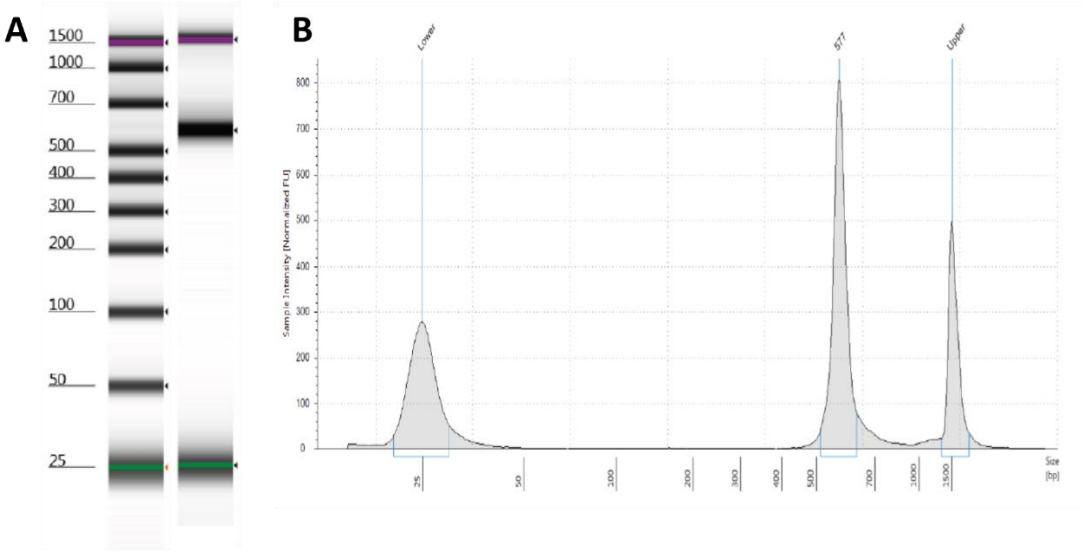


Figure 2-6: Graphical results output from TapeStation.
A. Electrophoresis demonstrating ladder and single band of DNA amplicon in library. B. Line graph of library demonstrating a single peak associated at appropriate amplicon size, with other peaks suggesting no primer dimers within library.

A further quantification of the library and to confirm the presence of primers that will bind to the Illumina sequencer on the amplicons, the library was quantified using the Next Library QuantKit for Illumina (New England Biolabs, MA, USA). This assay ran a qPCR reaction using four known standards to accurately quantify the concentration of DNA containing Illumina primers within the library. Dilutions of 1:1,000, 1:10,000 and 1:100,000 of the library were run in triplicate. The assay was run with an initial denaturation step of 95 °C for 1 minute followed by 35 cycles of 95 °C for 15 seconds and 63 °C for 45 seconds using a CF96 Touch Real Time PCR Detection System (BioRad, Hertfordshire, UK). The amplification of the library confirmed the presence of Illumina primers on the amplicons and the concentration of the library was interpolated from the standard curve generated. The library concentration was corrected for the amplicon size, as the standards contain a 399 bp amplicon, using the equation below:

Adjusted Concentration = Calculated Concentration x 399 / amplicon size (bp)

2.10.6 Denaturing, diluting and loading of library into MiSeq

Sequencing was performed on a MiSeq instrument (Illumina UK, Cambridge, UK). Prior to loading on the cartridge for sequencing, the library was denatured by diluting with an equal volume of 0.2N Sodium Hydroxide and Tris-HCL. The library was further diluted to 4 pM by diluting with the hybridization HT1 buffer (Illumina UK, Cambridge, UK). The diluted and denatured library was combined with denatured PhiX Control v3 (Illumina UK, Cambridge, UK) DNA to act as an internal control alongside the low-diversity amplicon library. The final library was loaded into the MiSeq cartridge (Illumina UK, Cambridge, UK) along with the custom primers. A clean flowcell (Illumina UK, Cambridge, UK) was also required.

2.10.7 Post sequencing data processing

Processing of the 16S rRNA gene sequence data was performed using Mothur v1.39.5 (Schloss et al. 2009) . Mothur is an open-source software used for bioinformatic processing sequencing data designed to be used in microbial ecology. Mothur was run on the HIVE multicore processor computer owned by Cardiff University School of Biosciences. File transfer was achieved using the WinSCP programme and PuTTy was used as a terminal emulator to run the analysis.

A full copy of the Mothur commands used in the analysis is shown in Appendix 5. A summary of the data processing pathway is shown below in Figure 2-7.

Data output from DNA sequencing contains multiple sequences of letters encoding the bases from a strand of DNA, each sequence representing a copy of the target gene. Each individual sequence is known as a "read" of the target gene, in this case the 16S rRNA gene.

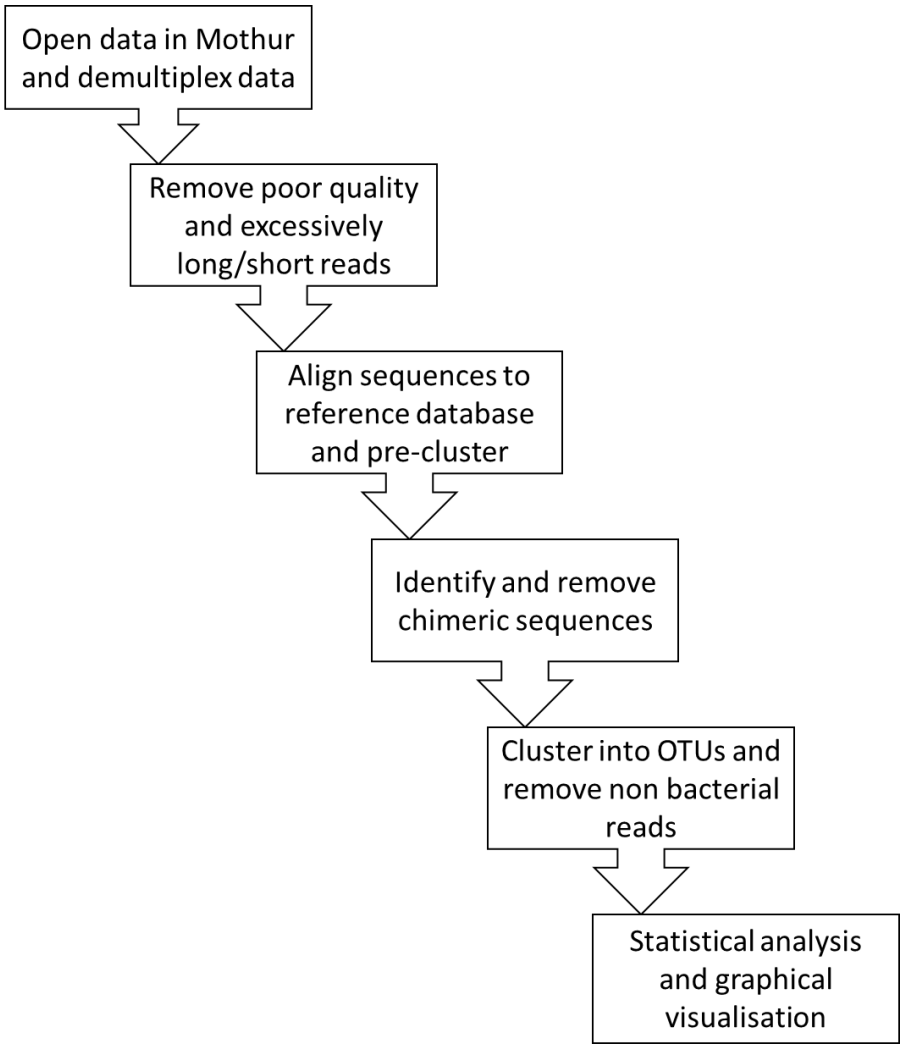


Figure 2-7: Summary of workflow in Mothur v1.39.5 for processing of sequencing data

The workflow used in Mothur is designed to remove sequences amplified in error, before grouping into OTUs prior to data analysis. Initially de-multiplexing of the data was performed to identify from which samples each read originated. Excessively long sequences and those sequences with excessive numbers of ambiguous bases were removed to exclude poor quality reads. Unique sequences within the dataset were identified to prevent duplication during the analysis. Sequences were then aligned to the SILVA reference database of 16S rRNA gene sequences (Quast et al. 2013). Sequences not covering the target V3-V4 region were removed. Also removed were sequence data from beyond the V3-V4 region. Sequences differing in up to 2 bp were assumed to be identical, with the small difference due to errors in sequencing and so such sequences were grouped together with the most abundant sequence. Chimeric sequences were screened for and removed. OTUs, with a cut off of 3% differences in base pairs, were

then formed and the phylogenic identification of each OTU made from kingdom to genus level using the 16S Training Set reference database of 16S rRNA gene sequences downloaded from the Ribosomal Database Project (Cole et al. 2014). A phylogenetic tree using representative sequences of each OTU was produced using FastTree software (Price, Dehal, and Arkin 2009).

Attempts to identify organisms to a species level was undertaken using the USEARCH program v10 (Edgar 2010). Comparing the representative sequences from each OTU to sequences within the Ribosomal Database Project (Cole et al. 2014), matches of greater than 97% were considered to be the same species. Representative sequences of OTUs were manually compared to sequences of several species from the identified genus using Bioedit v7.0.5 (T. A. Hall 1999) to confirm if there was more than one species with >97% match. Identification to a species level was considered not possible if >1 species had a match >97% with the representative species.

Further analysis was performed using Phyloseq v1.20.0 (P. J. McMurdie and Holmes 2013) within R v 3.4.1 (R Core Team 2017) accessed via RStudio v1.0.143. R is a free software programme and programming language used for statistical analysis and graphical presentation of data. Phyloseq is an open access software package that is used within R to enable the analysis and graphical display of microbiome data.

As each sample will have different numbers of successful and good quality reads normalisation of the data is required. This was achieved using rarefication. Rarefication is the process of equalising the number of reads in all samples by choosing a minimum number of reads required for each sample. Samples with less than this threshold are discarded. The remaining reads from each sample are subsampled without replacement such that they all have this minimum number of reads.

The taxonomy and shared files created in Mothur, the tree file created using representative sequences and a metadata file were imported into Phyloseq. Within Phyloseq rarefication of sample reads, calculations of alpha and beta diversity and production of graphical figures was performed. Alpha diversity indices calculated from the sequencing data were the Shannon diversity index, inverse Simpson diversity index and the Chao 1 diversity index. Total number of OTUs was also used as a measure of richness. The Bray-Curtis dissimilarity index was used as a measure of beta-diversity. Data was exported from Phyloseq for use in other analysis or graphical visualisation of results.

Microsoft Excel v1908 was used for creation of stacked bar charts. Permutational Multivariate Analysis of Variance Analysis (PERMANOVA) was conducted within R using the vegan package v2.4-4 (Oksanen et al. 2016). Ordination plots were drawn using the R package ggplot2 (Wickham 2016).

STAMP v2.1.3 (statistical analysis of metagenomic profiles) computer package (Parks and Beiko 2010) was used to compare microbiome profiles from samples between different groups visualising results using extended error bar plots.

Heatmaps were drawn using ClustViz online software (Metsalu and Vilo 2015).

2.11 Enzyme-linked immunosorbent assays

The concentration of IL6 and IL8 in the supernatant of respiratory samples was tested using an enzyme-linked immunosorbent assay (ELISA). ELISA assays utilise a capture antibody to the desired analyte fixed to a plate. During incubation with the sample, analyte present is bound to the immobilised primary antibody. A biotinylated secondary detection antibody is then used to bind to the fixed analyte and streptavidine-horseradish peroxidase (HRP) binds to the detection antibody. Between each step plates are washed with a weak detergent solution to remove unbound or non-specifically bound protein. Tetramethylbenzidine substrate solution added to the wells results in a blue colour developing in proportion to the amount of analyte present in the sample. Colour development is stopped turning the colour in the wells to yellow using stop solution. Optical density at a specific wavelength can then be measured. The diagram in Figure 2-8 demonstrates this process schematically.

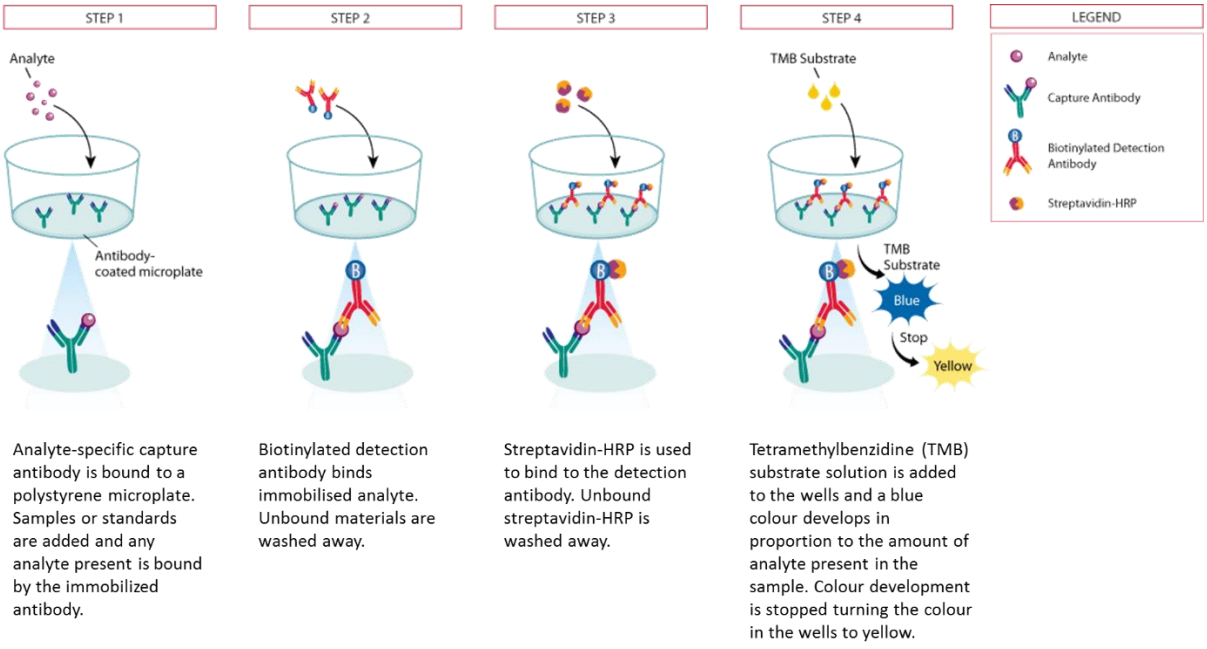


Figure 2-8: Schematic diagram demonstrating the process of ELISA assay **(R&D-Systems 2005)**

2.11.1 IL-6 ELISA

A DuoSet IL-6 ELISA kit (R&D Systems, MN, USA) was used to measure the IL-6 concentration in BAL and TA supernatants as per the manufacturer's instructions. All

antibodies and reagents were provided in the ELISA kit. The plate was prepared by diluting the capture antibody to a concentration of 2 µg/ml in plate coating buffer (1x PBS) and adding 100 µL per well to the plate. The sealed plate was incubated overnight at room temperature. A wash step was performed using 200 µL of wash buffer (x3). The plate was blocked using 200 µL of reagent diluent (1% Bovine Serum Albumin (BSA)) solution for 1 hour at room temperature. A further wash step was repeated before adding 100 µL of standards, samples and blanks (reagent diluent) in duplicate. IL-6 Standards were prepared using a stock of recombinant human IL-6 supplied with the kit. A 2x serial dilution of IL-6 was prepared from 600 pg/ml – 9.375 pg/ml, diluting in reagent diluent. Samples were diluted 1 in 10 with further dilutions performed if values beyond the range of the standard curve were obtained. Following a 2-hour incubation at room temperature, a further wash step was performed and 100 µL of biotinylated detection antibody diluted in reagent diluent to a concentration of 50 ng/ml was added to the wells. Following a 2-hour incubation at room temperature, the wash step was repeated and 100 µL of streptavidine-HRP (diluted 1 in 40 from stock in reagent diluent) was then incubated in each well for 20 minutes at room temperature in the dark. A further wash step was performed. Substrate solution was prepared using equal volumes of reagent A and reagent B and 100 µL of this solution was added to each well and the plate was incubated in the dark for 20 minutes. Wells containing IL-6 turned blue. A volume of 50 µL of stop solution (2N-sulfuric acid) was added to each well, turning the blue to yellow. The optical density of the wells was measured using a microplate reader (Dynex Corporation, Worthing, UK) at 450 nm with a wavelength correction at 570 nm. The value of blanks was subtracted from the standards and samples and a standard curve drawn from the values generated from the known standards using a 4-parameter logistic curve fit. The concentration of the samples was interpolated from this curve. Standard curve and interpolation of unknown values was performed using Graphpad Prism software. An example of the standard curve generated from an ELISA experiment for IL-6 concentration is shown below (Figure 2-9). The range of detection of the assay was 600 pg/ml – 9.375 pg/ml. Samples and standards were run in duplicate.

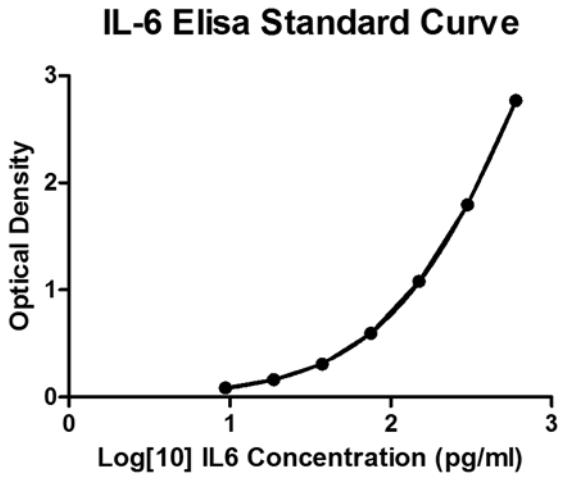


Figure 2-9: Standard curve generated from ELISA experiment measuring IL6 concentration in BAL and TA supernatants. R^2 values for the curve-fit of the standard curve were always >0.99.

2.11.2 IL-8 ELISA

A DuoSet IL-8 ELISA kit (R&D Systems, MN, USA) was used to measure the IL-8 concentration in BAL and TA supernatants as per the manufacturers instructions. All antibodies and reagents were provided in the ELISA kit. The plate was prepared by diluting the capture antibody to a concentration of 4 µg/ml in plate coating buffer (1x PBS) and adding 100 µL per well to the plate. The sealed plate was incubated overnight at room temperature. A wash step was performed using 200 µL of wash buffer (x3). The plate was blocked using 200 µL of block buffer (1% BSA) solution for 1 hour at room temperature. A further wash step was repeated before adding 100 µL of standards, samples and blanks (reagent diluent, 0.1% BSA in Tris-buffered saline, 0.05% Tween 20) in duplicate. IL-8 standards were prepared using a stock of recombinant human IL-6 supplied with the kit. A 2x serial dilution of IL-6 was prepared from 2000 pg/ml – 31.25 pg/ml, diluting in reagent diluent. Samples were diluted 1 in 10 with reagent diluent and further dilutions performed if values beyond the range of the standard curve obtained. Following a 2-hour incubation at room temperature a further wash step was performed and 100 µL of biotinylated detection antibody diluted in reagent diluent to a concentration of 20 ng/ml was added to the wells. Following a 2-hour incubation at room temperature the wash step was repeated and 100 µL of streptavidine-HRP (diluted 1 in 40 from stock in reagent diluent) was then incubated in each well for 20 minutes at room temperature in the dark. A further wash step was performed. Substrate solution was prepared using equal volumes of reagent A and reagent B and 100 µL added to each well and incubated in the dark for 20 minutes. Wells containing IL-8 turned blue. A volume of 50 µL per well of stop solution (2N-sulfuric acid) was added turning the blue colour yellow. The optical density of the wells was measured using a microplate reader (Dynex Corporation, Worthing, UK) at 450 nm with a wavelength correction at 570 nm. The value of blanks was subtracted from the

standards and samples and a standard curve drawn from the values generated from the known standards using a 4-parameter logistic curve fit. The concentration of the samples was interpolated from this curve. Generation of the standard curve and interpolation of unknown values was performed using Graphpad Prism software. An example of the standard curve generated from an ELISA experiment for IL-8 concentration is shown below (Figure 2-10). The range of detection of the assay was 2000 pg/ml – 31.25 pg/ml. Samples and standards were run in duplicate.

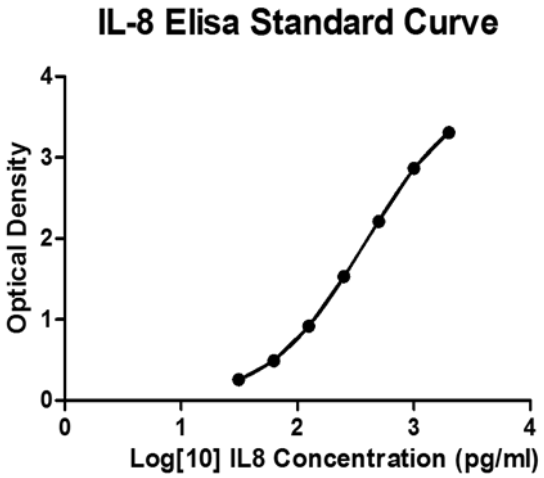


Figure 2-10: Standard curve generated from ELISA experiment measuring IL8 concentration within BAL and TA samples. R^2 values for the curve fit of the standard curve were always >0.99.

2.12 Western blotting

Western blotting is a technique to identify specific proteins within a sample allowing visualisation of free proteins, protein in oligomeric form or in complex with other proteins. Proteins are initially separated by molecular weight on an SDS-polyacrylamide gel using a voltage gradient. Proteins are blotted onto a nitrocellulose membrane before being visualised using fluorescent or radiolabelled antibodies. Components of the inflammasome within neonatal BAL fluid were studied by using Western blotting of BAL supernatant within a mini-PROTEAN Western blotting system (Bio-Rad, Hertfordshire, UK). Compatible precast gradient gels of 4-15% SDS were used to run the Western blot (Sigma-Aldrich, Dorset, UK). 20 µL of BAL supernatant was incubated with 10 µL of reducing buffer at 100 °C for 10 mins immediately prior to loading into gel to ensure protein was linearised. A voltage gradient of 120 V was applied across the gel for 45 minutes or until the dye front reached the end of the gel.

The proteins within the gel were transferred to nitrocellulose membrane using a fixed current of 210 mV for 60 minutes.

The membrane was placed in 5% skimmed milk (Marvel, Lincolnshire, UK) blocking solution made up with 1x PBS-Tween for 60 minutes at room temperature. Following two 15 minute washes in PBS-Tween at room temperature, the membrane was incubated in primary antibody over night at 4 °C.

Two 15-minute washes in 1xPBS-T were performed. The membrane was incubated in secondary antibody (1:4000) and streptavidin-HRP (1:10,000) for 60 mins at room temperature. Twelve 10 min washes in PBS-T were performed. The membrane was incubated with electrochemiluminescence (ECL) Prime detection reagents (GE, Buckinghamshire, UK) for 2 minutes. Photographic film (Amersham Hyperfilm) (GE, Buckinghamshire, UK) was used to detect the light emitted from the ECL reaction in a dark room.

2.12.1 Stripping and re-probing membrane

Due to the limited availability of neonatal BAL samples, the nitrocellulose membrane was stripped of antibody and probed for alternative proteins. The membrane was incubated in pre-heated stripping buffer for 10 minutes at 50 °C. Two washes in large volumes of PBS-T were performed. The membrane was incubated in a 5% milk blocking solution following by primary antibody as described above. The procedure for Western blotting was followed unchanged.

To check the efficiency of the stripping process, the stripped membrane was incubated with ECL reagents and photographic film was used to detect light emitted. No signal was detected. This indicates the secondary antibody had been removed adequately. To test the removal of the primary antibody, a membrane that had been stripped of antibody was incubated with the secondary antibody that had been previously used and only very faint bands of protein were visible. To prevent this interaction a different species of primary antibody was used for each component of the inflammasome probed. The new secondary antibody used would not detect any remaining primary antibody from the previous experiment.

2.12.2 Antibodies and reagents used in Western blotting

The antibodies used in Western blotting experiments of neonatal BAL fluid in probing for inflammasome components are shown below in Table 2-6.

Table 2-6: Antibodies and reagents used for Western blotting of inflammasome components with BAL fluid

Description	Manufacturer	Ref Number	Lot Number
Streptavidin HRP	GE Healthcare	RPN1231	
Polyclonal IgG Rabbit anti-Caspase 1 p10	Santa Cruz	SC515	A1514
Monoclonal IgG Mouse anti-ASC	Santa Cruz	SC271054	C1213
Polyclonal IgG Goat anti-NLRP3	Santa Cruz	SC34498	J2011
Polyclonal Rabbit anti-Mouse IgG	Dako Cytomation	P0260	00020228
Polyclonal Goat anti-Rabbit IgG	Jackson	111-035-045	95678
Polyclonal Rabbit anti-Goat IgG	Dako Cytomation	P0449	20011796

2.12.3 Densitometry of Western blot images

Western blotting can be semi-quantified using densitometry analysis. This involves using software to measure the density of the bands on an image of the Western blot membrane. This relies on the assumption that the density of band is directly proportional to the quantity of the target protein in the sample as the primary and secondary antibodies bind in proportion to the amount of the target on the membrane.

Western blot images on developed X-ray film were digitally scanned and Image J (Schneider, Rasband, and Eliceiri 2012) software was used to perform the densitometry analysis. For each sample the lane of the gel is selected and the background signal excluded by drawing a horizontal line at the relevant peak. Samples from membranes run, transferred, co-incubated with blocking reagents and antibodies, and co-washed together can be compared in this way. Figure 2-11 taken from Image J demonstrates this procedure.

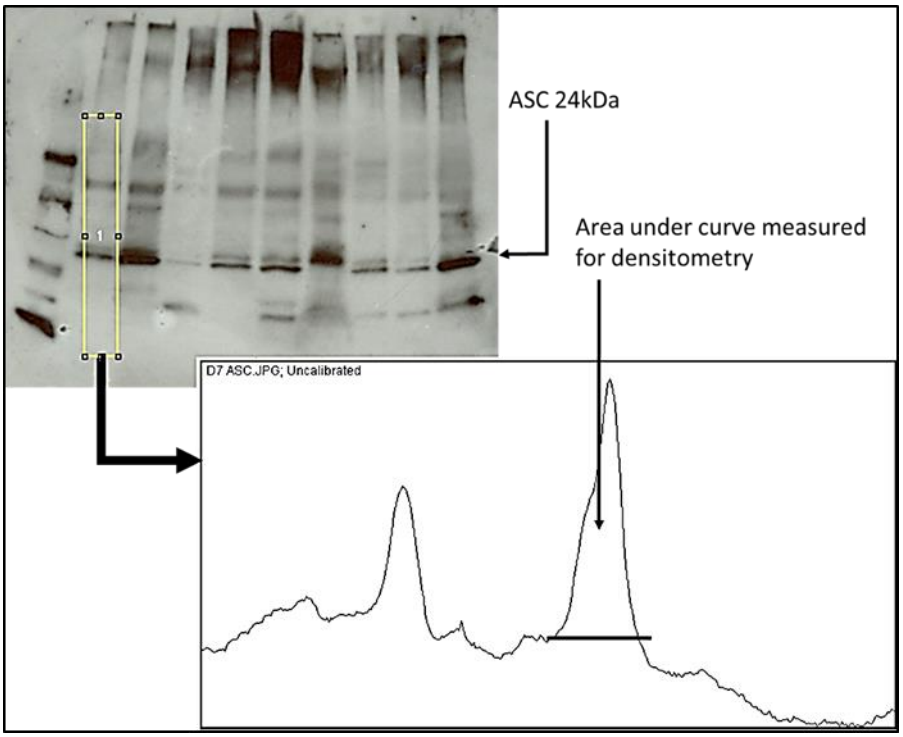


Figure 2-11: Densitometry of Western blot image using Image J software. Each lane of the gel is selected, and the image J software transforms the density of the bands into a linear graph. A horizontal line is drawn to remove the effect of background signal and the area under the curve of the representing the density of the selected band from the gel is measured by the software.

2.13 Cell culture and stimulations

2.13.1 Maintenance of cells in culture

Frozen stocks of A549 cell line and BEAS-2B cell line were kindly supplied by Dr M Triantifalou and Prof K Triantifalou. Both cell lines had previously been obtained from the American Type Culture Collection (ATCC, Virginia, USA). Both cell lines are immortalised cells originating from the respiratory tract. The A549 cell line originate from culturing human alveolar basal epithelial cells from an explanted adenocarcinoma from a 58-year-old caucasian male (Giard et al. 1973). A549 are adherent cells that form monolayers when cultured in flasks. A549 cells have been widely used as an *in vitro* model of alveolar epithelial cells (Zhihong Zhang et al. 2005; Somborac-Bačura et al. 2018).

BEAS-2B cells are immortalised cells originating from human bronchial epithelium (Reddel et al. 1987). The BEAS-2B cell line is commonly used as an in *vitro model* of human bronchial epithelial cells (Peeters et al. 2013; C. E. Stewart et al. 2012). These cell lines were chosen due to extensive use of these cells in the literature particularly in the study of pulmonary inflammation and to allow comparison of an alveolar epithelial cell line and a bronchial epithelial cell line.

A549 and BEAS-2B cell lines were maintained in 25 cm^2 flasks (Life Technologies (Nunc), Paisley, UK), using Roswell Park Memorial Institute 1640 (RPMI) media with glutaMAX (Life Technologies (Gibco), Paisley, UK) supplemented with 10% (v/v) fetal calf serum (FCS) (Life Technologies (Gibco), Paisley, UK) and 3% (v/v) amino acids (Life Technologies (Gibco), Paisley, UK). Preparation of cells was achieved by defrosting of cells and immediately diluting the freezing medium with supplemented RPMI media in a 15 ml Falcon tube (Life Technologies (Nunc), Paisley, UK). After briefly mixing the cell suspension by hand, the cells were centrifuged for 5 minutes at 5000 x *g*. The supernatant was removed and cells were seeded into flasks and incubated at 37 °C in 5% CO_2 with 5 ml of supplemented RPMI media.

Confluent flasks were split using 0.05% trypsin-EDTA (Life Technologies (Gibco), Paisley, UK). The previous media was removed and the cells were washed with sterile PBS (Life Technologies (Gibco), Paisley, UK) prior to trypsinisation. Cells were split 1:4 and incubated with 5 ml RPMI media per flask.

Freezer stocks of each cell line were prepared by trypsinisation of a flask of cells at 90% confluency during exponential growth phase. The cell suspension was centrifuged at 5000 x *g* for 5 minutes and the supernatant discarded. The cell pellet was resuspended in 1 ml of freezing medium composed of FCS supplemented with 10% DMSO (Sigma-Aldrich, Dorset, UK) to prevent ice-crystal formation. Cells were frozen at -80 °C immediately after resuspension in freezing medium.

2.13.2 Cell stimulations

Cell stimulation experiments were performed using both A549 and BEAS-2B cells maintained in culture as previously described in section 2.13.1. In preparation for stimulation, cells were seeded to a 24-well plate (LifeTechnologies (Nunc), Paisley, UK). After trypsinisation of cultured cells a haemocytometer was used to estimate the concentration of cells. The cell suspension was centrifuged at 1500 rpm for 5 minutes to create a cell pellet. The cell pellet was resuspended in RPMI to give a cell count of $1x10^5$ cells/mL. 200 µL of this cell suspension was seeded to each well and 1 mL of media was added to each well. Plates were incubated overnight in RPMI media at 37 °C in 5% CO_2 to achieve 80-90% confluency.

All cell stimulations were performed in un-supplemented human endothelial serum free media (LifeTechnologies (Gibco), Paisley, UK). Cell stimulations using BAL fluid and rASC were incubated overnight at 37 °C in 5% CO_2.

Cell stimulations were terminated by aspiration of the supernatant and the addition of 300 µL ice-cold lysis buffer to each well. Lysis buffer was incubated for 60 minutes before aspiration. Cell supernatants and lysates were stored at -20 °C until further analysis.

2.13.3 Measurement of cytokines in supernatant of cell stimulations

Cytokine concentrations in supernatant from cell stimulation experiments was measured using the Human Inflammatory Cytokine Kit (BD Biosciences, Oxford, UK) as per the manufacture's instructions. This cytometric bead array (CBA) kit uses beads coated in antibodies for 6 specific pro-inflammatory cytokines (IL-8, IL-1β, IL-6, IL-10, TNF, IL-12p70). When incubated with a sample and secondary antibody, the fluorescence intensity of the beads was proportional to the concentration of the cytokine in the sample.

20 µL of suspended mixed cytokine specific beads were mixed with 20 µL of sample and 20 µL of secondary antibody. The tubes were incubated protected from light for 3 hours at room temperature with regular vortexing to resuspend beads. Samples were centrifuged at 5000 x *g* for 5 min to pellet beads, before being washed in the supplied wash buffer.

A FACSCalibur flow cytometer (BD Biosciences, Oxford, UK) was used to measure the fluorescence intensity of beads. A minimum of 3000 beads per sample was used to calculate the cytokine concentration. Standard curves for each cytokine were generated from serial dilutions of standards provided with the kit and run with beads from the same kit lot number. The BD CBA Analysis software (BD Biosciences, Oxford, UK) was used to interpolate the median fluorescence intensity of the beads for each cytokine from the standard curves.

2.14 Immunoprecipitation of ASC from pooled bronchoalveolar lavage supernatant

Immunoprecipitation uses antibodies to deplete a sample of the target protein. The antibody binds specifically to the target protein before adding protein A sepharose (PAS) beads which bind antibodies non-specifically.

ASC was depleted from BAL samples prior to cell stimulation experiments to implicate ASC on cytokine production by the cultured cells. PAS beads (ThermoFisher, Leicestershire, UK) were prepared by suspending 0.1 g of beads in 1 mL of sterile PBS. Preclearing of the samples to assess for non-specific binding to the beads was achieved by incubating 20 µL of suspended beads with 50 µL of pooled BAL supernatant for 45 mins at 4 °C with regular flicking to resuspend beads. Samples were centrifuged at 10000 x*g* for 2 minutes and the supernatant removed. The preclear step was repeated twice with each sample.

Polyclonal Rabbit anti-ASC IgG antibody (Santa Cruz Biotechnology, Heidelberg, Germany) was used to bind ASC in the sample. The sample was incubated with 5 µL of antibody overnight at 4 °C.

PAS beads readily bind immunoglobulins. 30 µL of PAS beads were mixed with the sample for 60 minutes at 4 °C with regular flicking to resuspend beads. The sample was centrifuged at

5000 x *g* for 2 minutes. The supernatant was aspirated and stored at -70 °C for future use in cell stimulation experiments.

The pellet of beads and the preclear pellets were washed five times in lysis buffer. The beads were resuspended in 1 mL of lysis buffer and vortexed for 60 seconds. Centrifugation of the sample at 10000 x *g* for 2 minutes was performed and the supernatant discarded. After the final wash the beads were dried using tissue paper without disturbing the pellet.

The pellets were then prepared for running on an SDS-PAGE gel for Western Blotting under reducing conditions as described in section 2.12. The PAS beads suspended in the reducing buffer were placed into the wells of the gel. The antibodies used to identify ASC in the Western blot membrane were the same as those used to identify ASC within clinical samples (see section 2.12.2). Successful immunoprecipitation was confirmed by identification of bands at the correct molecular weight for ASC in the immunoprecipitation sample, but not in the preclear sample.

2.15 Purification of rASC

2.15.1 Growing *E. coli* containing ASC plasmid

A stock of *E. coli* transformed to contain a plasmid containing the gene for ASC production was kindly donated by Prof K Fitzgerald, University of Massachusetts Medical School. The ASC gene within the plasmid was under lac operator control and the plasmid contained an ampicillin resistance gene to enable selection of organisms containing the plasmid.

Luria broth and agar was prepared by dissolving 10 g of Bacterial Peptone (Oxoid/Thermoscientific, Leicestershire, UK), 5 g of yeast (Oxoid/Thermoscientific, Leicestershire, UK) and 5 g of NaCl (Fisher Scientific. Leicestershire, UK) in deionised H_2O to total volume of 1 L. To prepare Luria agar, 15 g of agar (Oxoid/Thermoscientific, Leicestershire, UK) was added to the prepared broth. The preparations were autoclaved to sterilise prior to use.

E. coli was cultured on Luria agar plates containing 100 µg/mL ampicillin overnight at 37 °C. A single colony was picked and grown in 15mL Luria Broth containing 15 µL of 1000 mg/mL ampicillin and 7.5 µL of isopropyl β-D-1-thiogalactopyranoside (IPTG), to induce ASC synthesis. Two 15ml growths were prepared and incubated at 37 °C in a shaking incubator overnight, or until the solution was cloudy. Two 500 ml bottles of Luria broth were prepared with 500 µL of ampicillin and 250 µL of IPTG added to each broth. The 15 mL cultures were added to the 500 mL bottles and incubated at 37 °C in a shaking incubator for a minimum of 5 hours, until cloudy. The cultures were centrifuged at 10,000 x *g* for 45 minutes to create a cell pellet and the supernatant discarded. The cell pellet was resuspended in 1xPBS and stored at -20 °C.

The *E. coli* organisms were lysed using a freeze/thaw technique allowing the suspension to completely freeze and thaw 6 times.

2.15.2 Purification by immunoaffinity chromatography

ASC was purified by immunoaffinity chromatography. Lysate from *E. coli* cells described above was passed over immunoaffinity columns consisting of polyclonal ASC antibody covalently bound to N-hydroxysuccinimide activated 1 mL Hi-Trap columns (GE, Buckinghamshire, UK). Columns were prepared using 1 mM hydrochloric acid to wash out the isopropanol within the column. 1 mL of rabbit polyclonal IgG anti-ASC antibody 200 mg/mL (Santa Cruz Biotechnology, Heidelberg, Germany) was injected onto the column and incubated for 30 minutes at room temperature. A further 1 mL of antibody was injected into the column and incubated for 2 hours at room temperature. The column was deactivated using alternate injections of alkaline and acidic buffers, buffers A and B, as per the manufacturer's instructions. A final injection of 1xPBS with 0.1% NaN_3 pH 7 was added to neutralise the column pH and preserve the column prior to use. The column was stored at 4 °C.

Lysate was passed through the column in 1 mL aliquots and incubated for 2 mins at room temperature to allow the rASC protein to bind to the antibody within the column. The column was then washed with 1xPBS and ASC was eluted using the alkaline elution buffer as the change in pH causes release of the rASC from the antibody. The eluted protein solution was neutralised with concentrated hydrochloric acid to prevent protein denaturation. The column was then restored to a neutral pH with PBS and the procedure was repeated for all the lysate. Figure 2-12 demonstrates the purification process.

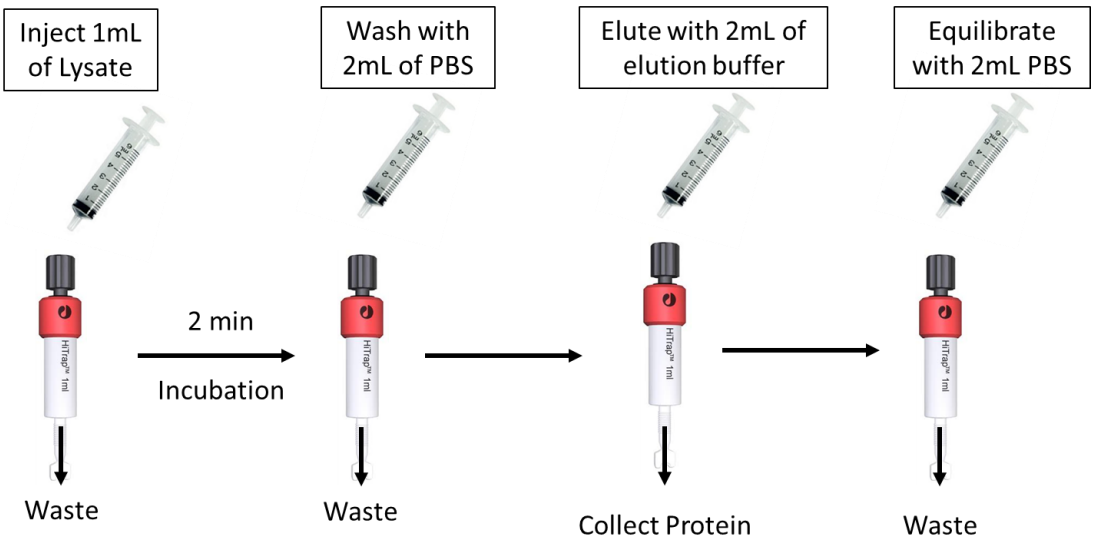


Figure 2-12: Purification of rASC by immunoaffinity chromatography

rASC was concentrated using Centricon Plus 20 centrifugal filter (Millipore, MA, USA). Protein solution was centrifuged at 1125 x*g* at 4 °C. Buffer exchange to 1 x PBS was

performed by adding 1x PBS to the concentrated protein solution. The ASC was concentrated in a final volume of 2.5 mL of 1x PBS.

2.15.3 Determining protein concentration

After buffer exchange, an ultraviolet spectrophotometer was used to determine the concentration of protein within the solution using the formula below. The theory behind this formula is the amino acid residues tyrosine and tryptophan within proteins demonstrate maximum absorption at 280nm wavelength. While proteins vary in the proportion of these amino acids most proteins have an extinction coefficient within the range of 0.4-1.5. One of Beer-Lambert's law equations states:

Absorbance = extinction coefficient x concentration of solution x length of solution

Nucleic acids have an absorbance 10 times that of protein at this wavelength so a small amount of nucleic acid can influence absorbance. The formula corrects for this by detracting the absorbance at 260nm (the peak absorbance of nucleic acids) from that at 280nm using ratios determined by early researchers (Stoscheck 1990; Layne 1957).

Protein conc. (mg/ml) = $1.55 \times A_{280} - 0.76 \times A_{260}$

2.15.4 Determining lipopolysaccharide contamination of rASC Solution

To ensure the rASC solution was not contaminated with significant concentrations of lipopolysaccharide (LPS), a thiobarbiturate assay was used to detect the presence of keto-3-deoxycytulosonic acid, a component of the core domain in the LPS of many bacteria including *E. coli*, the organism from which the rASC was generated.

Mild acid hydrolysis of the glycosidic bond of LPS was achieved by incubating 10 µL of sample with 250 µL of 10 mM H_2SO_4 (Fisher Scientific, Leicestershire, UK) at 100 °C for 30 mins. Samples were then cooled to room temperature and incubated with 125 µL of 40 mM periodic acid (H_5IO_6) (Sigma-Aldrich, Dorset, UK) in 60 mM H_2SO_4 (Fisher Scientific, Leicestershire, UK). 125 µL of 0.2 M $NaAsO_2$ (Sigma-Aldrich, Dorset, UK) in 0.5 M HCl (Fisher Scientific, Leicestershire, UK) was added resulting in the transient precipitation of iodine. Once the yellow/brown colour had disappeared, 250 µL of 0.6% aqueous thiobarbituric acid (Sigma-Aldrich, Dorset, UK) was added and samples were incubated at 100 °C for 15 mins. Finally 500 µL of DMSO (Sigma-Aldrich, Dorset, UK) was added to each sample. After mixing and cooling to room temperature the absorbance was measured at 549 nm using a spectrophotometer, subtracting the values of a blank. A standard curve from known concentrations of commercially available *E. coli* LPS (Sigma-Aldrich, Dorset, UK) was drawn and used to interpolate the concentration of the unknown samples.

2.16 Attempts to knock down of TLRs in mammalian cells

Short interfering RNA (siRNA) can be used to inhibit gene expression of specific genes. First demonstrated using double stranded RNA in muscles of nematodes (Fire et al. 1991), many products are now commercially available to knockdown the expression of specific genes. SiRNA work by interfering with mRNA preventing translation and so inhibiting protein production.

Plasmids encoding siRNA for TLR2 (psiRNA-hTLR2) and TLR4 (psiRNA-hTLR4) were obtained (Invivogen, Toulouse, France). The plasmids contained a zeocin resistance gene. The structure of the plasmid is seen below in Figure 2-13.

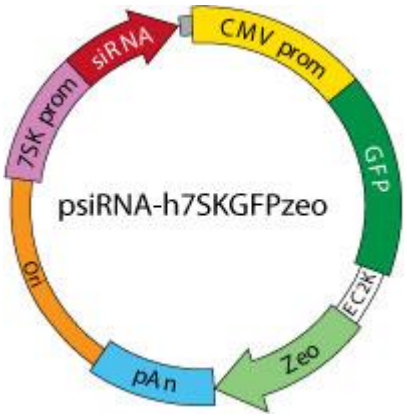


Figure 2-13: Structure of siRNA plasmid incorporating zeocin resistance gene.

2.16.1 Transformation of competent *E. coli* cells

Luria Broth and agar were prepared, as in section 2.15.1, with 25 µg/ml of zeocin (Invivogen, Toulouse, France) as the selection antibiotic. Competent *E. coli* cells (Chemicorp GT116, Lot # G6C-37-01A) (Invivogen, Toulouse, France) were incubated with the plasmid solution on ice for 30 mins. The cells were heated to 42 °C for 1 minute, returned to the ice for 2 minutes to induce phagocytosis of the plasmid by the cells. The cells were incubated for 1 hour at 37 °C after 400 µL of Luria broth was added to the cells. The bacteria were spread over a plate of Luria broth containing zeocin and incubated overnight at 37 °C. Single colonies from these plates were picked and grown overnight in Luria broth containing zeocin 25 µg/ml in universal containers.

2.16.2 Purification of plasmid DNA using a phenol-chloroform DNA extraction

Cultures of *E. coli* were centrifuged at 15000 x *g* for 30 minutes to form a pellet of bacterial cells. The pellets were resuspended in 400 µL of STET buffer. The organisms were lysed using 10 µL of Lysozyme (50 mg/ml) and heating to 100 °C in a water bath for 1 minute. Samples were immediately cooled on ice for 5 minutes. The samples were centrifuged for 30 minutes at 15000 x*g* and the resulting cell pellet of debris from the lysed cells removed. RNA

was removed from the samples using 5 µL of RNAse A (20 mg/ml) incubated at 37 °C for 30 mins.

An equal volume of phenol was well mixed with the sample before centrifugation at 15000 x*g* for 15 mins. The aqueous upper layer was aspirated and kept. To this an equal volume of chloroform was well mixed before centrifugation at 15000 x *g* for 15 mins. Sodium acetate solution (20 µL of 2 Molar concentration) was added to the samples and 1 mL of ethanol. Samples were placed at -80 °C for 1 hour to precipitate the DNA. After thawing the samples were centrifuged at 15,000 x *g* for 20 minutes and all the supernatant removed. Sterile H_2O (60 µL) was used to dissolve the purified DNA.

To assess the success of isolation of the recombinant plasmid a sample of the DNA was run on a 1% agarose gel prepared with ELFO buffer. Ethidium bromide (ThermoFisher, Leicestershire, UK) was added to the gel prior to setting the gel using 10 µL per 100 mL of buffer. Ten microliters of DNA from each extraction was mixed with 2 µL of gel loading dye (NE Biolabs, MA, USA). A 1kb DNA ladder (NE Biolabs, MA, USA) was run alongside the extracted DNA to allow estimation of length of extracted DNA. The gel was electrophoresed at 80 V on a Subgel GT mini device (Bio Rad, Hertfordshire, UK) for roughly 60 mins and viewed using a Bio Doc It ultraviolet transilluminator (UVP, Cambridge, UK).

2.16.3 Transfection of mammalian cells

A549 and Beas-2B cells were grown in small flasks to 70-80% confluency as previously described. A transfection cocktail was prepared in 2 microcentrifuge tubes. In tube "A" 10 µL of plasmid DNA solution was mixed with 90 µL of Opti-MEM 1 media (Life Technologies (Gibco), Paisley, UK). In tube "B" 10 µL of Lipofectamine 2000 (Life Technologies (Invitrogen), Paisley, UK) was mixed with 90 µL of Opti-MEM 1 media. Both tubes were incubated at room temperature for 5-10 minutes. Tubes "A" and "B" were combined and incubated for 20 minutes. During this incubation cells were washed twice with Opti-MEM-1 media and left in 1 mL Opti-MEM 1 media. The transfusion cocktail containing Lipofectamine and plasmid DNA was then added to the flask and incubated overnight at 37 °C. The cells were washed in RPMI media (Life Technologies (Gibco), Paisley, UK) and allowed to grow in RPMI media for 2 days. RPMI media with zeocin antibiotic (100 µg/ml) (Life Technologies (Gibco), Paisley, UK) was used to select cells containing the plasmid.

Confirmation of successful knockdown of TLR was assessed using Western blotting of cell lysates generated as in 2.13.2 and using the protocol in section 2.12.

2.17 Statistical analysis

Statistical analysis of data was performed using the statistical package R version 3.4.2 (R Core Team 2017) unless otherwise stated.

Significance was taken as p value < 0.05 throughout.

Normality testing was performed using the Shapiro-Wilk test. Normally distributed variables were analysed and presented using means, standard deviations and a t-test comparing means. Variables not normally distributed were presented using medians and inter-quartile ranges to reduce the impact of outlying results. Variables not normally distributed were compared using non-parametric test such as independent Mann-Whitney U test for two groups or the Kruskal-Wallis test for more than two groups. Categorical variables were compared using the chi-squared test. Dunn's test was used as a post-hoc test following a significant result in a Kruskal-Wallis test due to the non-parametric nature of Dunn's test.

Odds ratios were calculated using an online calculator and the results expressed with 95% confidence intervals and a p value (MedCalc 2010).

Comparison of bacterial communities between different anatomical sites and different recruitment centres was performed using PERMANOVA in the Vegan package within R (Oksanen et al. 2016). PERMANOVA is a non-parametric multivariate statistical test that uses dissimilarity measurements to compare the position of centroids and dispersion around the centroids of groups. The dissimilarity measurement used in this work was the Bray-Curtis dissimilarity index. With PERMANOVA a p value <0.05 means that either the centroid and/or the spread of the objects is different between the groups (Anderson 2017).

Visualisation of differences between groups using the Bray-Curtis dissimilarity index were made using non-metric multidimensional scaling (NMDS) plots in Phyloseq within R (P. J. McMurdie and Holmes 2013). NMDS plots are ordination plots displaying objects, in this case samples, characterized by multiple variables so that similar objects are close to each other and dissimilar objects, or samples, are farther from each other. NMDS plots utilise many variables and define the distances between the objects in multi-dimensional space. A plot is then constructed in two dimensions and the distances on this plot compared to those in multi-dimensional space. The difference between the two distances is the stress on the data points. The position of each object is then adjusted to optimise the stress for all data points. The lower the final stress value the more representative the 2-dimensional plot is of the distances in the original multi-dimensional space. Stress values less than 0.25 are usually considered acceptable.

2.1 Contributions of collaborators

Preparing the study protocol and obtaining research permission was undertaken by myself. Collection of BAL samples and processing of all samples from Cardiff and Bristol including DNA extraction was performed by myself. DNA extraction from TA samples from Newcastle was kindly completed by Dr G Young. Bacterial load quantification was undertaken by myself.

I am grateful that amplification for 16S rRNA sequencing of BAL and TA samples was performed by Dr D Alber and Dr G Logan. Amplification and quality control checking for sequencing of the NPA samples was undertaken by myself kingly supervised by Dr D Alber. I competed the post-sequencing data processing and all statistical analysis. Combining the sequencing data from the different sample types was completed jointly by myself and Dr E Mitchell as data from stool samples was also combined. Cytokine concentration measurements using ELISA were performed by myself. All work related to the innate immune activation was completed by myself.

Chapter 3: Bacterial Colonisation of the Lower Airways and Lungs of Intubated Preterm Infants

3 Bacterial Colonisation of the Lower Airways and Lungs of Intubated Preterm Infants

3.1 Introduction

Colonisation by bacteria of the lower airways and lungs of adults is now generally accepted (Pragman et al. 2012; Erb-Downward et al. 2011). The microbiota in healthy adult lungs is characterised by the presence of Proteobacteria, Firmicutes, and Bacteroidetes at the phylum level. *Pseudomonas*, *Streptococcus*, *Prevotella*, *Fusobacteria* and *Veillonella* predominate at genus level (Beck, Young, and Huffnagle 2012).

Access to sampling the lung microbiota is usually via bronchoscopic bronchoalveolar lavage in children and adults (Connett 2000). However, sputum analysis has been used as an alternative methodology (Hogan et al. 2016; Balbi et al. 2007) avoiding an invasive procedure. In new-born infants, there is limited experience of bronchoscopic bronchoalveolar lavage (Hysinger et al. 2019) with the size of airways in preterm infants making it a technically difficult procedure. A non-bronchoscopic approach is generally undertaken as a research technique to obtain samples for microbiological and inflammatory research (S. Kotecha 1999). Community-wide microbiota analysis from the lungs of preterm infants has not been previously undertaken with previous studies utilising TA samples (Lal et al. 2016; Lohmann et al. 2014; Wagner et al. 2017) and NPA samples taken from studies involving term infants (Biesbroek, Bosch, et al. 2014; Teo et al. 2015). Studies analysing the lower respiratory tract using TA samples have shown the presence of dominant phyla and dominant OTUs within most samples. The most commonly occurring phyla are Proteobacteria and Firmicutes with Actinobacteria the next most abundant found consistently across several studies (Lohmann et al. 2014; Lal et al. 2016; Wagner et al. 2017). 16S rRNA gene sequencing of BAL samples from preterm infants has previously attempted to identify only a dominant organism (Beeton et al. 2011; Davies et al. 2010).

Pulmonary infection is a common complication of ventilation in preterm infants occurring in 2.7 to 10.9 episodes per 1,000 ventilator days (Cernada et al. 2014). Wide variation is explained by the risk of gestation with earlier gestations at higher risk. The most commonly isolated pathogens in culture based studies are *Pseudomonas aeruginosa* and *Staphylococcus aureus* with *Klebsiella pneumoniae* and *Escherichia coli* also reported (Apisarnthanarak et al. 2003; Cernada et al. 2013).

3.2 Aims

1. To take serial samples from the lower airways of preterm infants at risk of CLD and identify the pattern of bacterial colonisation in the lower airways and lungs.

2. To measure bacterial load within samples from the lower airways and lungs of preterm infants and relate this to inflammatory profiles.

3. To compare the lung microbiota in infants who do and do not go on to develop CLD as well as a control group of ventilated term infants.

4. Analyse the effect of antibiotic treatment on respiratory and lung colonisation in ventilated preterm infants

5. To compare airway colonisation in ventilated preterm infants between different recruitment centres

6. To test the depth of sequencing to attempt to identify bacteria to species level

3.3 Patient characteristics

Table 3-1: Demographics of all preterm infants recruited from three neonatal units in the UK.

	NBT	RVI	UHW	p
Preterm Infants recruited	35	29	20	
Male, n (%)	24 (68.6)	10 (34.5)	12 (60)	0.03
Gestation, Med (IQR)	25.9 (24.7 - 26.7)	24.6 (23.5-26.6)	26.8 (25.0-29.5)	0.008
BW, Med (IQR)	746 (674 - 890)	630 (530-715)	835 (685 - 1187.5)	<0.001
Maternal Age, Mean (SD)	31 (6)	32 (6)	27 (5)	0.05
Infant from a multiple pregnancy, n (%)	11 (31.4)	15 (51.7)	6 (30)	0.17
Apgar at 1 min, Med (IQR)	6 (5-6)	NA	5 (3-5)	-
Apgar at 5 min, Med (IQR)	8 (7-9)	NA	7 (6-8)	-
Ventilation Days, Mean (SD)	21 (19)	31 (17)	34 (50)	0.08
Non-Invasive Respiratory Support Days, Mean (SD)	51 (27)	48 (35)	44 (37)	0.38
Low flow O_2 days, Mean (SD)	18 (15)	14 (16)	9 (12)	0.04
Length of Hospital stay (days), Mean (SD)	98 (35)	95 (41)	104 (61)	0.99
CS delivery, n (%)	16 (45.7)	10 (34.5)	10 (50)	0.51
O_2 at 28 days, n (%)	34 (97.1)	29 (100)	16 (80)	0.009
O_2 at 36 weeks, n (%)	33 (94.3)	27 (93.1)	13 (65)	0.004
Complete course antenatal steroids, n (%)	26 (74.3)	20 (69)	11 (55)	0.27
Survival to hospital discharge, n (%)	30 (85.7)	25 (86.2)	17 (85)	0.99
Postnatal steroids for ventilator dependence, n (%)	2 (5.7)	6 (20.7)	9 (45.0)	0.002
Home O_2, n (%)	17 (48.6)	11 (37.9)	3 (15)	0.045
NEC ≥ Grade 2, n (%)	6 (17.1)	8 (27.6)	1 (5)	0.040
EBM Ever, n (%)	35 (100)	27 (93.1)	20 (100)	0.14
Home exclusive breast milk, n (%)	11 (31.4)	10 (34.5)	6 (30)	0.97

NBT = North Bristol Trust, RVI = Royal Victoria Infirmary, UHW = University Hospital of Wales, CS = Caesarean section, NEC = Necrotising enterocolitis, EBM = Expressed breast milk. P values calculated using Kruskal-Wallis test for continuous variables and chi-squared test for binary variables.

Table 3-1 above shows the demographics of infants recruited from the three neonatal units. This shows that the infants recruited from the neonatal unit at RVI were had a lower both weight and were of an earlier gestation. Despite having on average more mature infants recruited, infants were ventilated for longer and had the longest median stay in hospital in UHW suggesting likely

differences in clinical practice between the units. Rates of CLD were high in recruited infants (65.0-94.3% based on a definition of oxygen requirement at 36 weeks corrected gestational age), probably due to the number of babies <26 weeks recruited.

3.4 Tracheal aspirate samples from Newcastle

DNA extraction of samples from Newcastle was kindly performed by Greg Young, PhD Student at the University of Northumbria, and sequencing of the V3-V4 region of the bacterial 16S rRNA gene was performed by Dr D Alber of University College London in the laboratories of Prof N Klein. I was responsible for the data analysis. Sequencing data was processed using Mothur to exclude sequencing errors, sequences containing ambiguous bases and chimeric sequences as per the methodology in Section 2.10.7. The assigned OTUs were used in further analysis.

107 TA samples from 29 infants were selected to be analysed for this study. Samples from Newcastle underwent DNA extraction in three batches, each with an appropriate DNA extraction negative control. 70 samples (65.4%) were successfully amplified. qPCR data showed that the DNA extraction control associated with batches two and three was positive for bacterial DNA, suggesting contamination of the control by bacterial DNA and therefore possible contamination of the samples. The proportion of samples positive for bacterial DNA using qPCR was higher for the samples associated with extraction controls 2 and 3 than control 1, also suggesting contamination. The decision was made to proceed with sequencing and exclude contamination of clinical samples bioinformatically. The relative abundance of the OTUs at a phylum level of samples associated with each DNA extraction control are shown in Figure 3-1. The first batch of samples demonstrates dominant phyla (>50% of reads from a single phylum) present in most samples with the identity of the dominant phylum either Firmicutes, Proteobacteria, or Actinobacteria. This is consistent with previous studies which have identified these organisms within TA samples from intubated preterm infants (Lohmann et al. 2014).

The samples associated with extraction controls 2 and 3, however, contain a greater number of organisms per sample but display less diversity between samples. There is no correlation between age of the infant or day of life sample taken between the different batches. The pattern of organisms identified in the majority of samples matches that of the extraction control. The control sample does not contain contamination by one OTU, but by a variety of OTUs from several phyla. This is suggesting that many of the samples have been contaminated with the same organisms found in the extraction control.

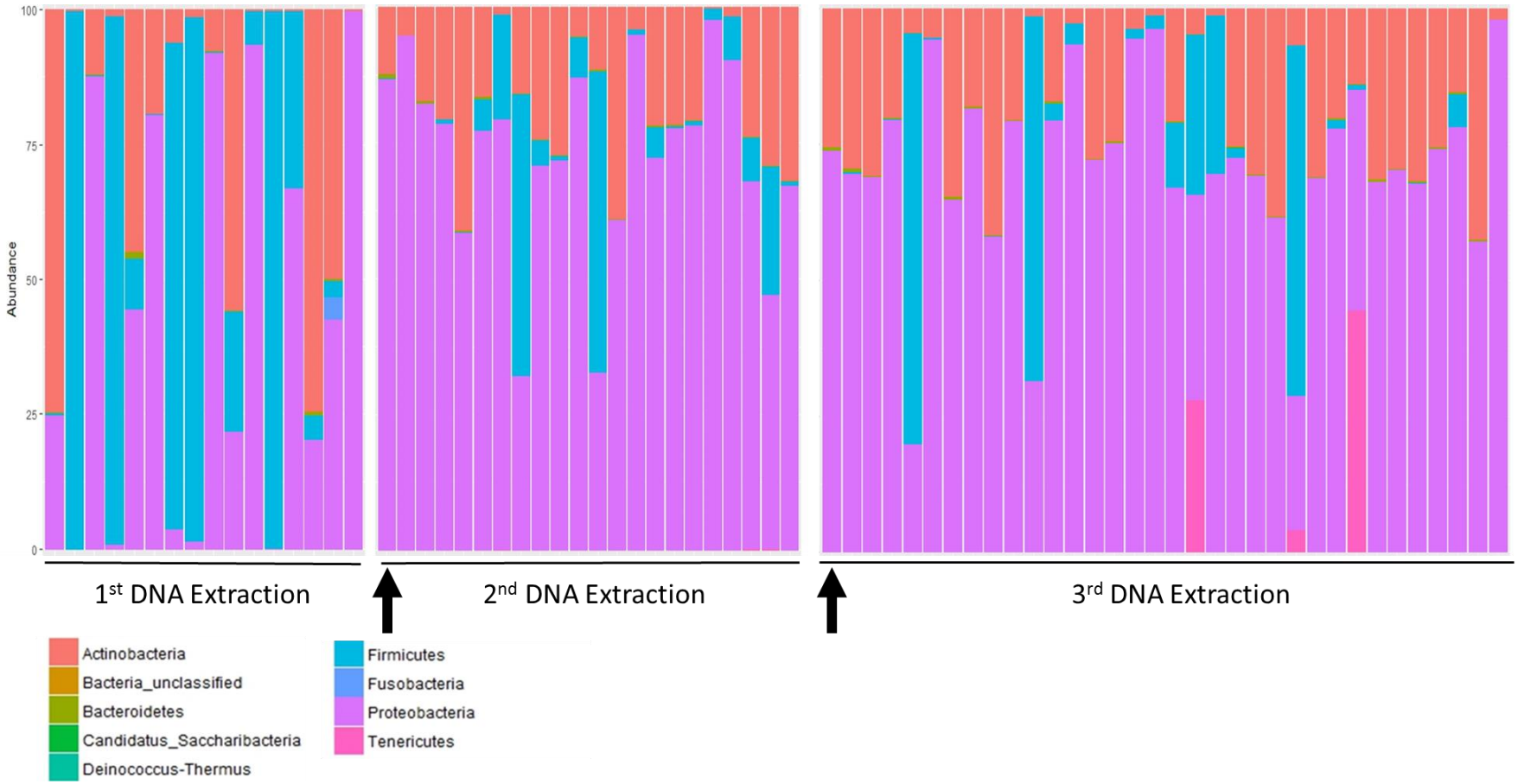


Figure 3-1: Phylum level abundance of organisms present in TA samples from the cohort of patients from Newcastle showing contamination of the 2nd and 3rd batches of DNA extraction. Arrows indicate the DNA extraction control samples. No amplification was seen in the extraction control sample from batch 1.

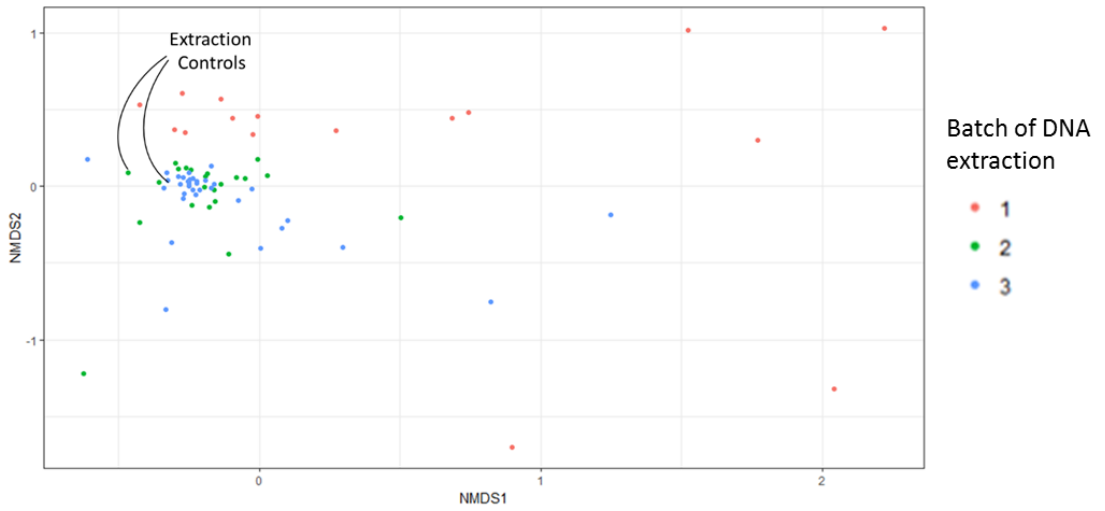


Figure 3-2: NMDS plot of TA samples from Newcastle coloured by batch of DNA extraction. Samples ordinated by Bray-Curtis dissimilarity index. Stress Value = 0.144.

Figure 3-2 shows a non-metric multidimensional scaling (NMDS) plot of all the samples ordinating by Bray-Curtis dissimilarity index identifies a clear grouping of the samples from batch 2 and 3 of DNA extraction, compared to batch 1 which have a greater spread. This suggests there is a systematic difference between the samples extracted in batch 2 and 3 from those in batch 1. Given that the batches were not related to any clinical parameter and the concerns over contamination of batches 2 and 3, it was concluded that samples from batches 2 and 3 contain DNA likely to arise from contamination at the point of DNA extraction. The samples from Newcastle were therefore excluded from further analysis.

3.5 Samples from Cardiff and Bristol

Tracheal aspirate samples were obtained from 35 preterm infants in Bristol and 20 preterm infants in Cardiff. A total of 278 TA samples were collected with 276 suitable for DNA extraction (1 sample empty, 1 cracked). 47 samples (17%) had adequate amplification of the bacterial 16S rRNA gene to be sequenced. This indicates that a large proportion of samples had undetectable levels of bacterial colonisation. Figure 3-3 shows the number of samples excluded at each stage of the analysis.

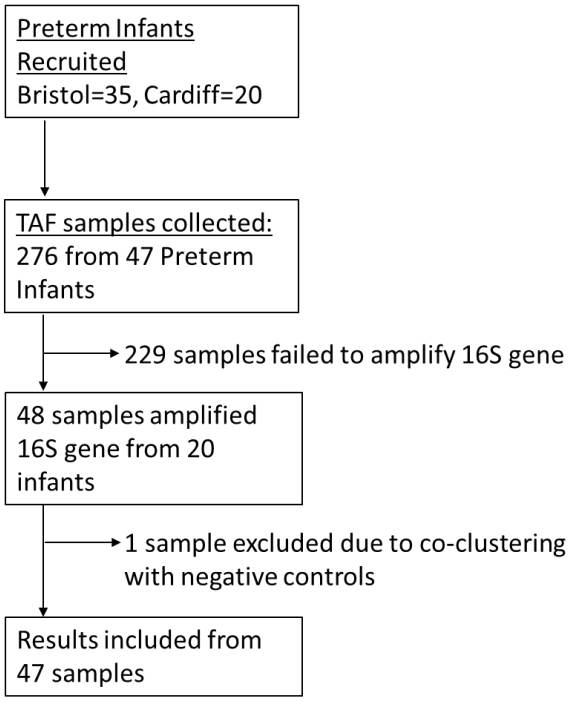


Figure 3-3: Flow-chart of TA sample analysis

3.6 Sequencing results from tracheal aspirate samples

Following removal of low-quality reads and chimeric sequences, the number of reads ranged from 8,665 to 426,696 reads per sample with an average of 109,457 reads per sample. The number of ambiguous bases per sequence accepted was set to zero. One sample was discarded due to co-clustering with negative control samples (see Figure 3-4 below). Results from the three DNA extraction controls contained 2 to 7558 reads per sample. Rarefication to 5000 reads per sample was performed prior to further analysis.

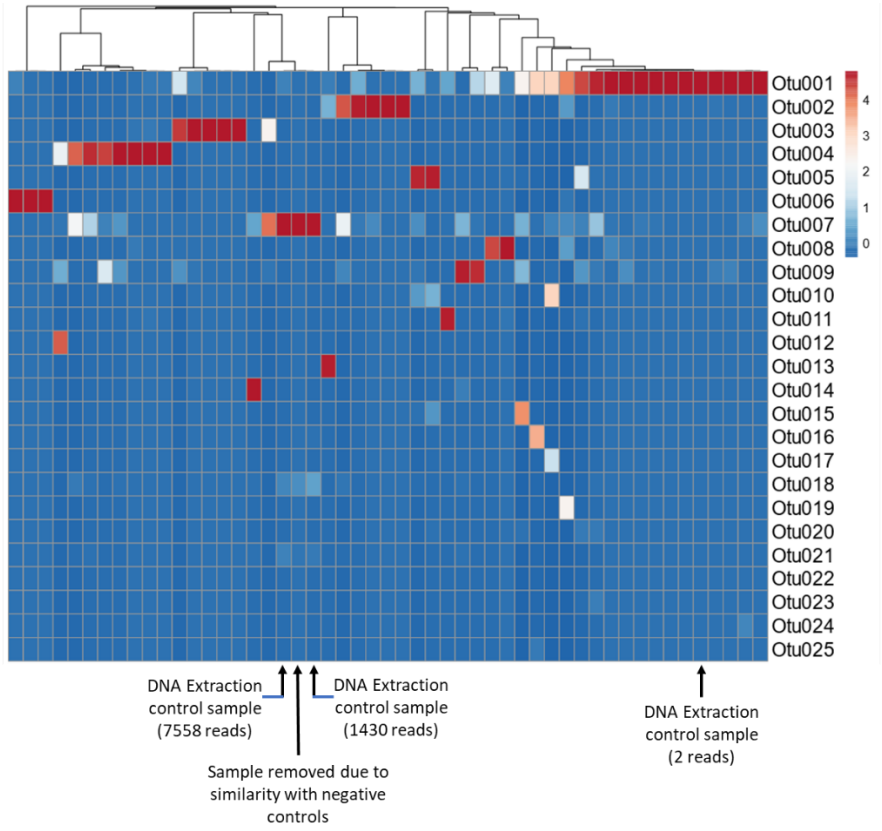


Figure 3-4: Heatmap demonstrating justification for exclusion of a single sample due to co-clustering with negative control samples
Each column represents a single clinical TA sample or DNA extraction control sample. Only the most abundant 25 OTUs shown. Heatmap drawn using ClustViz software (Metsalu and Vilo 2015), Clustering distance using correlation.

3.7 Bacterial load over time

Bacterial load within TA samples was calculated using qPCR analysis quantifying the number of copies of the 16S rRNA gene within the samples. Figure 3-5A demonstrates the changes in bacterial load over time within the TA samples. This shows that the average bacterial load in samples on day 1-3 was very low. This indicates that bacterial colonisation only commences during this period. Controversy exists regarding the possibility of antenatal colonisation of infants within the womb (Perez-Muñoz et al. 2017). This is indirect evidence against the theory of antenatal colonisation. If antenatal colonisation of the airways was occurring, then the early samples would be expected to have a detectable bacterial load. It is important to note, however, that all recruited preterm infants were on antibiotics during the first 48 hours of life. This may have influenced the results in samples taken early in life. The average bacterial load shows an increase over time peaking at days 8-14 despite antibiotic treatment being continued in the vast majority of infants. Average bacterial load then falls. This pattern is mirrored in Figure 3-5B which shows the percentages of samples that amplified the 16S rRNA gene adequately to be sequenced. The fact that the same pattern is seen using two different methodologies suggests this is a genuine finding.

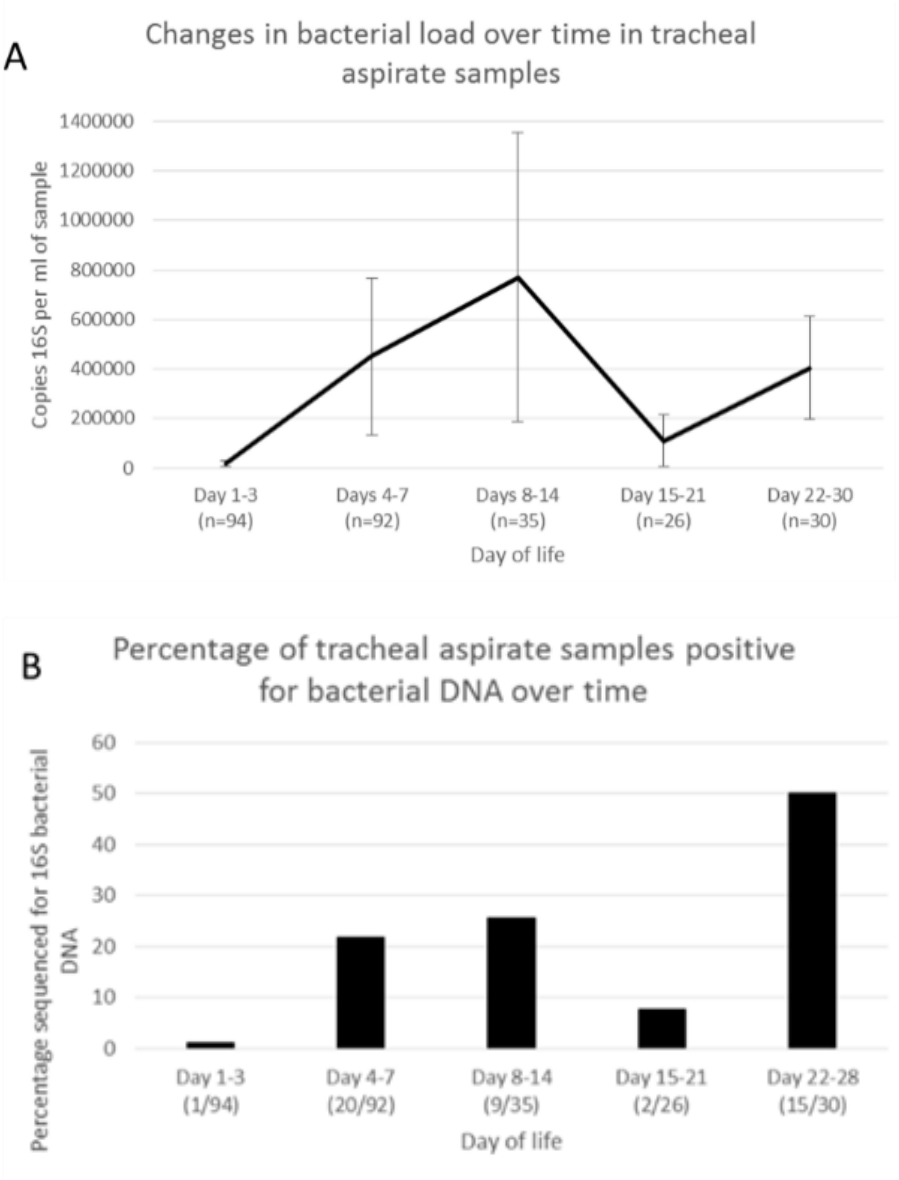


Figure 3-5: Comparing the presence of detectable bacterial DNA by two methods in tracheal aspirate samples from intubated preterm infants. A – Average bacterial load against time. B – Percentage of samples that were successfully amplified for DNA sequencing against time.

3.8 Taxonomy of bacterial organisms identified in tracheal aspirate samples

Representative sequences from the OTUs identified within TA samples were used to identify the taxonomy of the bacteria present in the samples. Figure 3-6 and Figure 3-7 demonstrate the identity of the bacterial phyla and genera identified.

The overwhelming majority of samples 46/47 (97.9%) contained a dominant phylum (>50% of reads from a single phylum) with 31/47 (66.0%) samples where the dominant phylum represented >90% of reads within the sample. In 44/47 (93.6%) samples a dominant OTU was present. The most commonly occurring phyla were Proteobacteria and Firmicutes. No

significant changes were seen comparing the relative abundance of Proteobacteria and Firmicutes over time.

The most commonly identified genus within Proteobacteria were unclassified *Enterobacteriacae* and *Acinetobacter*, while *Staphylococcus* dominated the identified Firmicutes.

Tenericutes organisms, *Ureaplasma* species and *Mycoplasma* species, were detected in 30/47 (63.8%) samples. These organisms were the dominant genera in 8 samples from 4 infants with samples with dominant Tenericutes found throughout the first month of life.

Identifying Proteobacteria and Firmicutes with a lower abundance of Actinobacteria present as the dominant genera is expected as all human colonised niches are dominated with these three phyla. These are also the phyla identified in previous studies looking at airway colonisation in intubated preterm infants (Lohmann et al. 2014; Wagner et al. 2017).

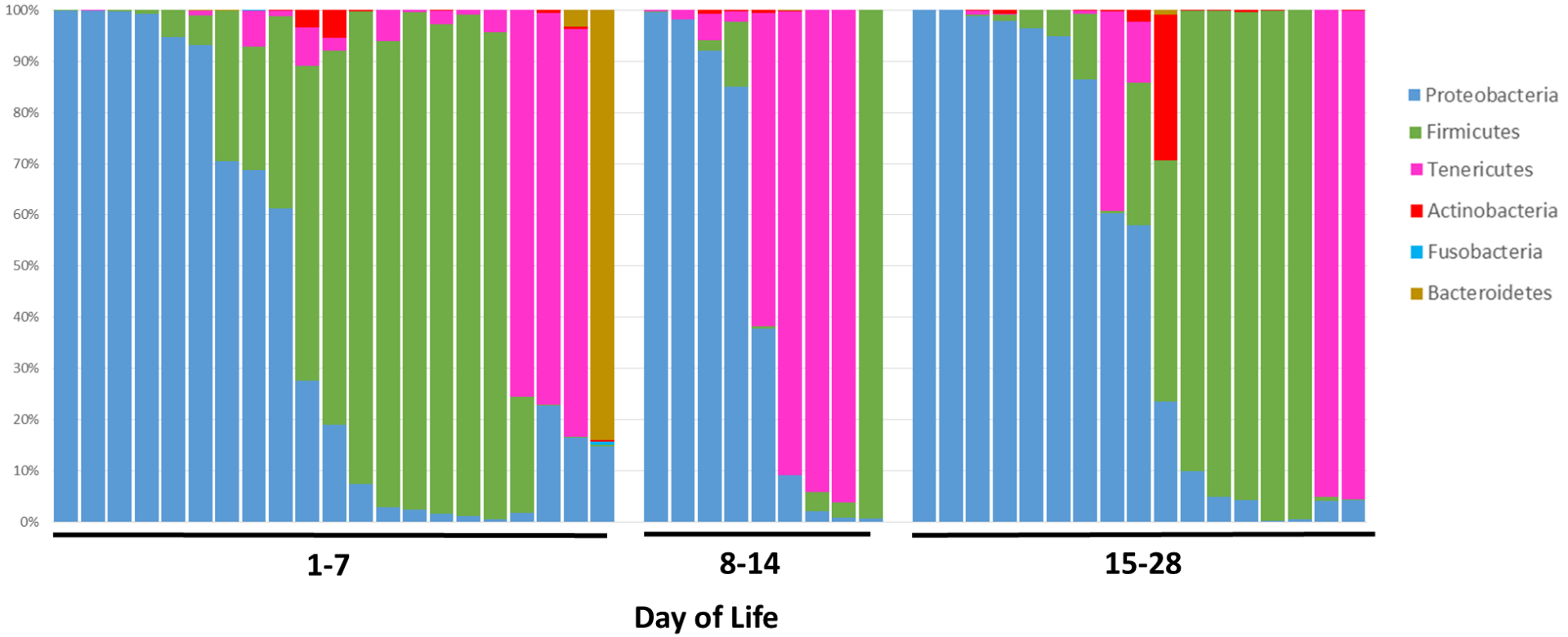


Figure 3-6: Relative abundance of bacterial phyla within TA samples from intubated preterm infants born at <32 weeks gestational age. Bacterial phyla identified by sequencing of the 16S rRNA gene. Samples are displayed combined in three groups - the first week of life, second week of life and third/fourth week of life.

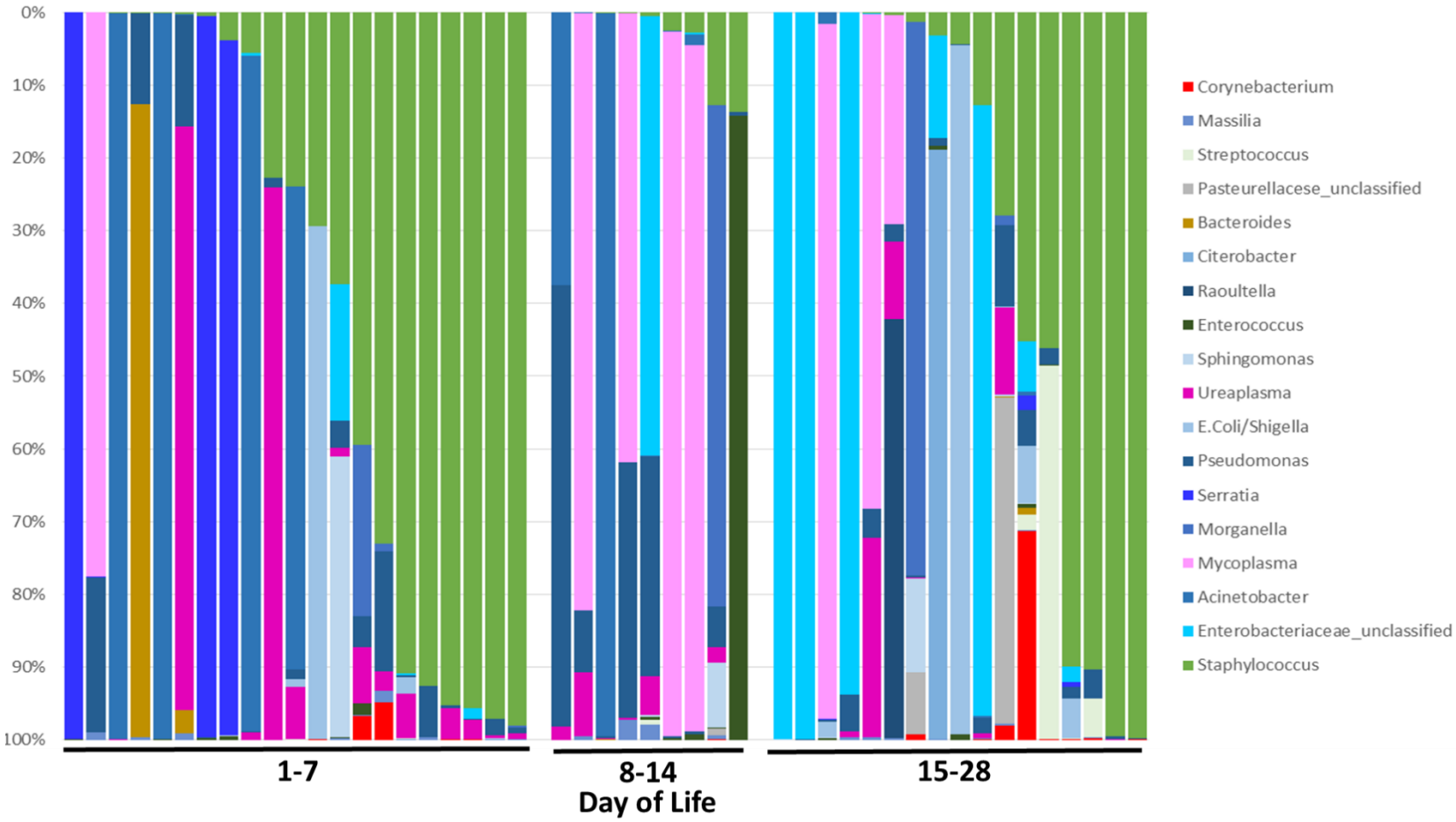


Figure 3-7: Relative abundance of bacterial genera within TA samples from intubated preterm infants <32 weeks gestational age. Bacterial phyla identified by sequencing of the 16S rRNA gene. Samples are displayed combined in three groups - the first week of life, second week of life and third/fourth week of life.

3.9 Effects of antibiotics on tracheal aspirate samples

This study had a high rate of samples where the 16S rRNA gene could not be amplified (82.9%). The high rate of sterile samples may be partly explained by the widespread use of antibiotics in preterm infants. 219 (79.3%) TA samples were taken while the infant was receiving antibiotic therapy. Samples taken off antibiotics were significantly more likely to be positive for bacterial DNA than samples taken on antibiotics (OR=2.91, 95%CI=1.48-5.73, p=0.002), (See Figure 3-8). Antibiotics may supress bacterial growth and colonisation within the airways of preterm intubated infants and affect diversity of bacterial communities. The high rate of antibiotic use in the early days of life in preterm infants (100% of infants studied had IV antibiotics on day 1 and 2) may also contribute to the very low bacterial loads detected on days 1-3 of life (Figure 3-5).

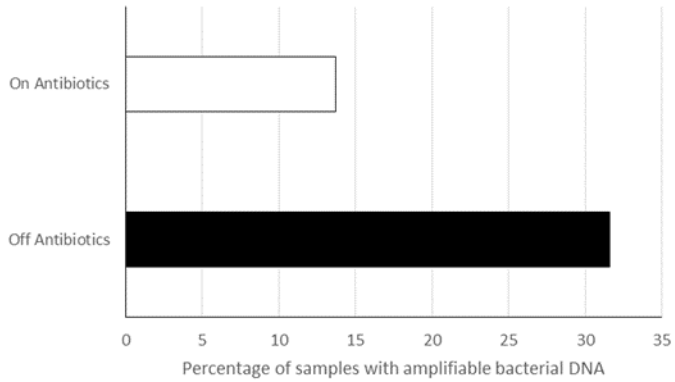


Figure 3-8: Effect of antibiotics on ability to amplify bacterial DNA from tracheal aspirate samples from preterm infants.
Samples taken on antibiotics n = 220, Samples taken off antibiotics n = 58. OR = 2.91, 95%CI = 1.48-5.73, p=0.002.

Figure 3-9 shows that at a phylum level Tenericutes were significantly more abundant in TA samples taken from infants on antibiotics than those taken from infants off antibiotics. At a genus level, significantly higher proportion of *Mycoplasma* and *Pseudomonas* were identified in TA samples taken while on antibiotics than those taken when not on antibiotics. *Mycoplasma* is not targeted by usual empirical antibiotic regimens used in most neonatal units (National Institute for Health and Clinical Excellence 2012) so it is likely therefore, that *Mycoplasma* species are able to proliferate when other genera are suppressed by antibiotic therapy. *Pseudomonas* organisms are known to cause respiratory infections in preterm infants. The significantly higher proportion of *Pseudomonas* organisms detected in TA samples taken while infants are on antibiotics may represent the bloom of this organism during a respiratory infection that is being treated or the organism may be resistant to the antibiotics being administered. Bacterial DNA may remain detectable even after empiric antibiotic treatment. Due to the small number of positive samples and the range of different antibiotic regimens that the infants received during the study, it has not been possible to compare the effects of specific

antibiotics or antibiotic regimens but in general a combination of broad spectrum antibiotics are used in preterm infants.

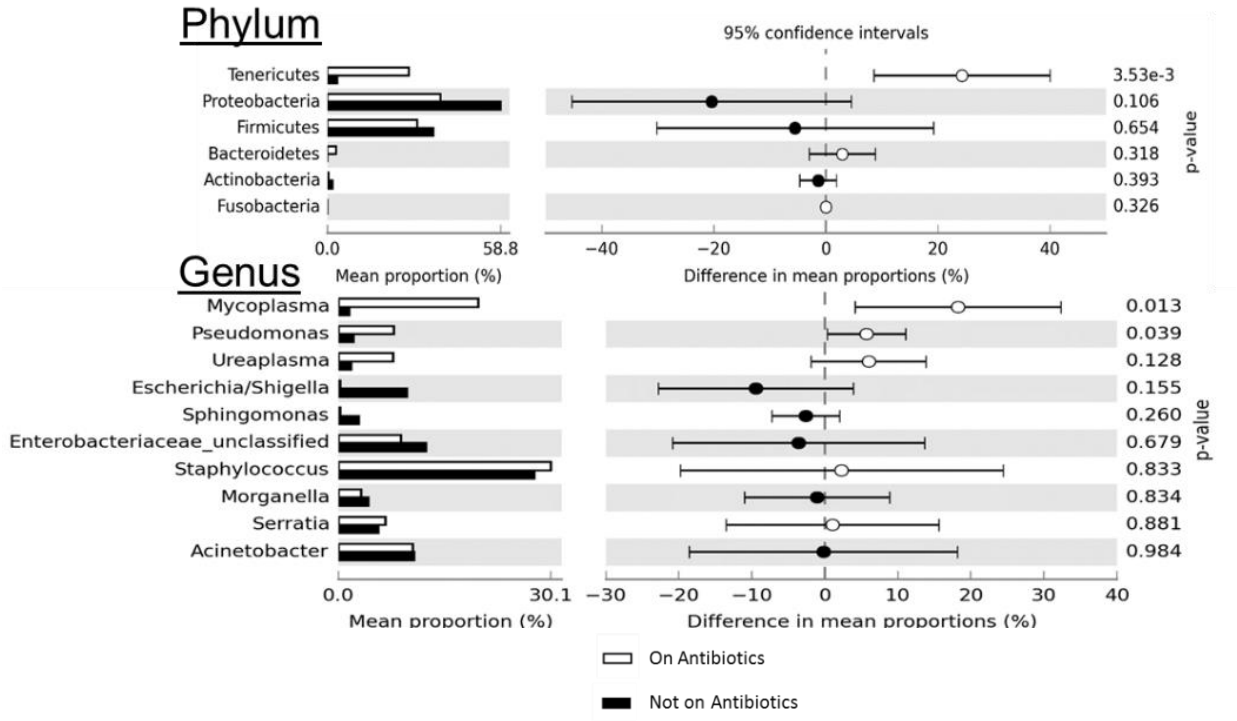


Figure 3-9: Effect of antibiotics on Phylum and Genus abundance within TA samples from preterm infants. Samples taken on antibiotics n = 220, Samples taken off antibiotics n = 58.

The alpha diversity indices from TA samples were low due to the presence of very dominant OTUs within most samples. The median number of species observed in all the samples was 9 (IQR 7-13). The median number of species observed comparing samples taken with infants on antibiotics compared to samples taken when infants not receiving antibiotics were 10 (IQR 6-16) and 9 (IQR 7-12). Antibiotics had no significant effect on any of the alpha diversity indices studied within the TA samples successfully sequenced (Figure 3-10) thus suggesting that antibiotics had suppressed overall bacterial load rather than reducing diversity by eliminating only certain organisms.

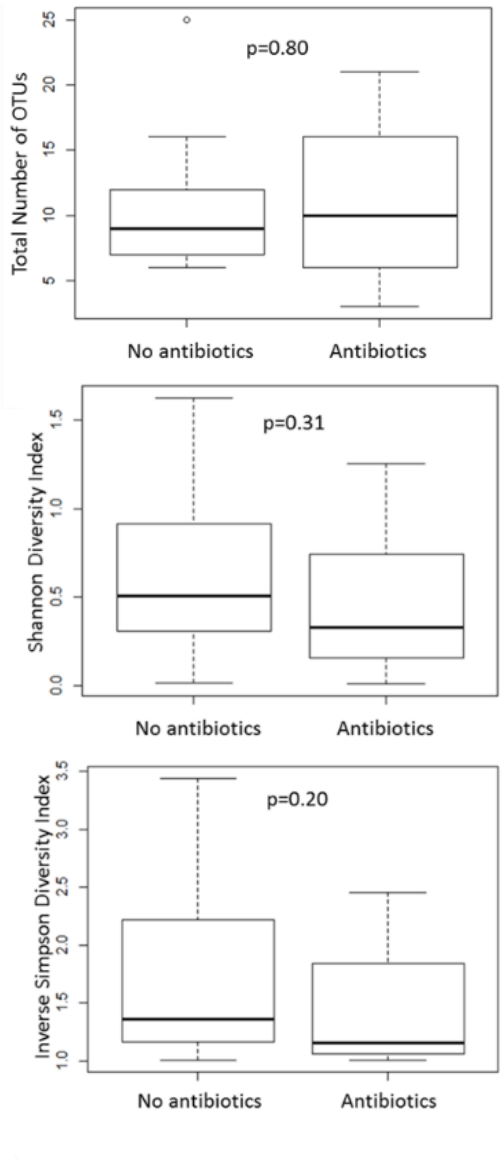


Figure 3-10: Effect of antibiotics on alpha diversity measures in TA samples from preterm infants.
(Samples taken on antibiotics = 29, no antibiotics = 18)

3.10 Inflammation associated with bacterial colonisation

IL-6 and IL-8 concentrations were measured in the supernatant of TA samples. Concentration of both cytokines were significantly higher in samples successfully sequenced compared to samples with no detectable bacterial DNA (Figure 3-11). This suggests that the bacterial organisms detected are causing an inflammatory reaction within the lungs of preterm infants, thus an infectious process. Individual infants are known to have episodic peaks of IL-6 and IL-8 during the first two weeks of life when measured serially within bronchoalveolar lavage samples (S. Kotecha, Wilson, et al. 1996; S. Kotecha et al. 1995). Figure 3-12 shows that similar patterns of cytokine concentrations are seen in four individual infant's TA samples from

this study. This figure also shows that cytokine concentrations show parallel increases with bacterial load in TA samples. 20 episodic peaks of IL-8 concentration within TA supernatant >50,000 pg/mL were witnessed in 19 infants. 13 episodes (65%) were associated with bacterial DNA being successfully sequenced within 24 hours of the peak cytokine concentration.

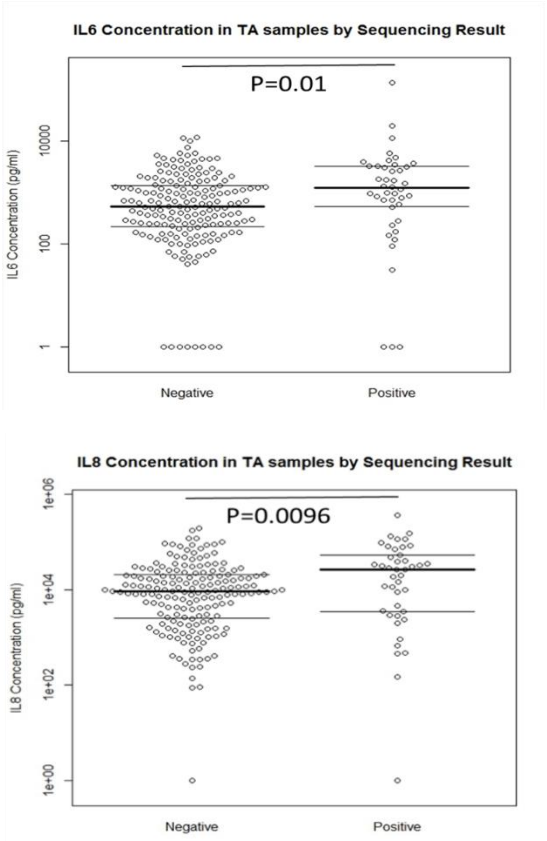


Figure 3-11: Amplifiable bacterial DNA is associated with higher IL-6 and IL-8 concentration in TA samples. Negative = 16S rRNA not detected, Positive = 16S rRNA detected.

The cytokine data suggest that an infective process rather than symbiotic colonisation was being detected in the preterm airways. The detection of bacterial DNA was associated with peaks of IL-6 and IL-8 concentration suggesting a host inflammatory response was initiated to the presence of bacteria. An inflammatory response to a bloom in bacterial presence is what would be expected in an infective process. The presence of a dominant OTU in the majority of samples may identify an infecting organism.

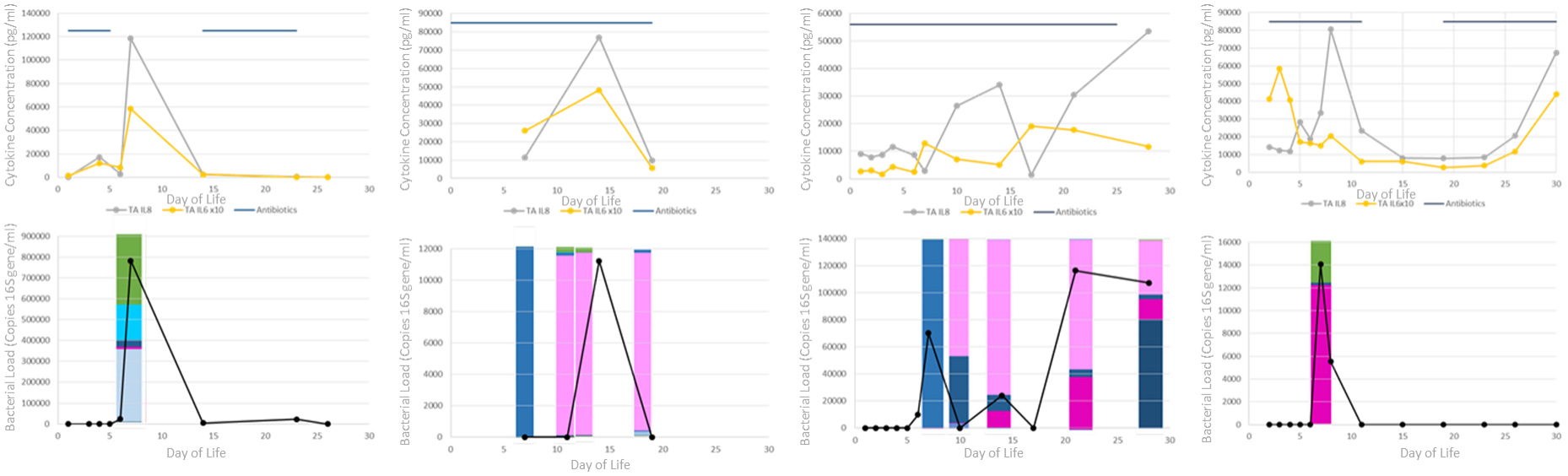


Figure 3-12: Parallel increases in IL-6, IL-8 concentrations and bacterial load in TA samples from four individual infants. IL-6 concentration multiplied by 10 to allow plotting on same scale as IL-8 concentration. The colours of the bacterial genus shown in the stacked bar charts are as in Figure 3-7. The blue line on cytokine figure indicates the duration of time the infant was receiving antibiotic treatment.

3.11 β-Diversity to compare individual infants

Figure 3-13 is an NMDS plot using the Bray-Curtis Dissimilarity index to ordinate each TA sample. Colouring samples from the same infant and linking them with polygons demonstrates that most infants' samples group together with relatively little overlap of the polygons. Therefore, each infant appears to have a separate colonisation pattern which is seen repeatedly in serial samples causing the grouping together on the NMDS plot.

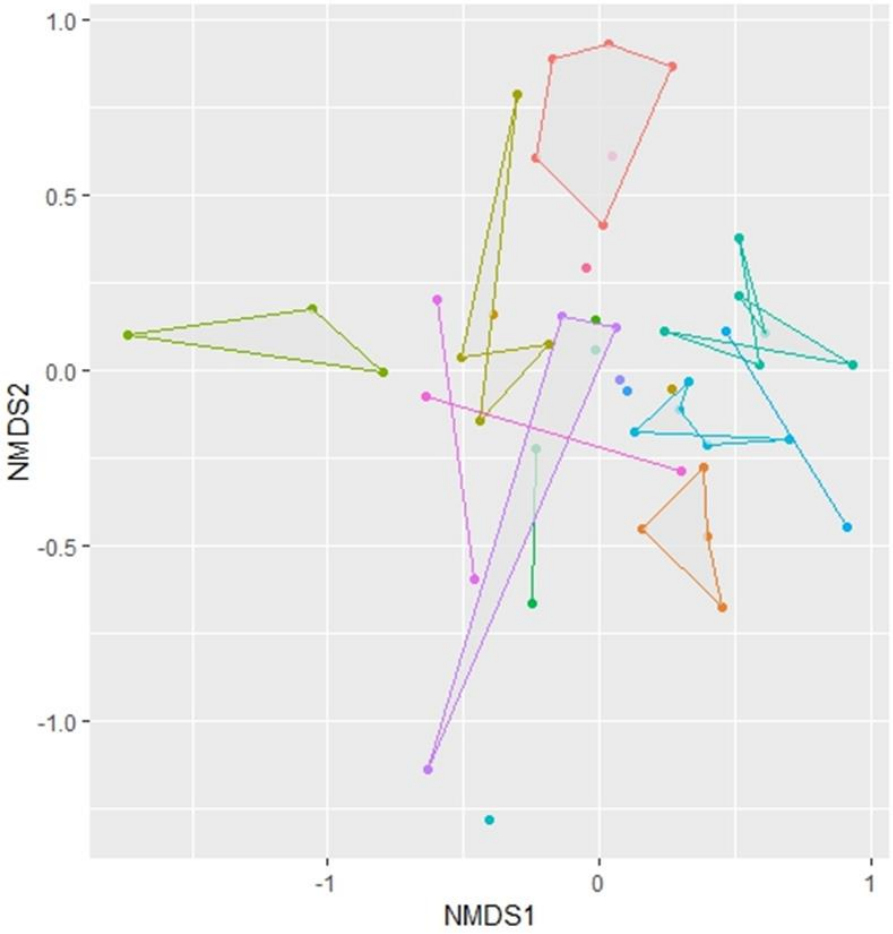


Figure 3-13: NMDS plot of TA samples ordinated using Bray-Curtis dissimilarity index. Each colour and polygon represents samples from an individual infant. Stress = 0.18.

3.12 Comparing colonisation in different centres

Sample collection from 2 centres allows comparison of colonisation patterns between the different centres. The number of samples from each centre where sequencing of the bacterial 16S rRNA gene was successful was 26/215 (12.1%) from Bristol and 21/61 (34.4%) from Cardiff ($p<0001$, chi squared test). The Cardiff recruitment centre had an outbreak of *Acinetobacter Baumannii*, which is known to cause respiratory infections, possibly contributing to this difference.

3.12.1 Bacterial load differences.

Figure 3-14 demonstrates that there was no difference between the two recruiting sites for the bacterial loads of samples that were successfully sequenced for 16S rRNA gene.

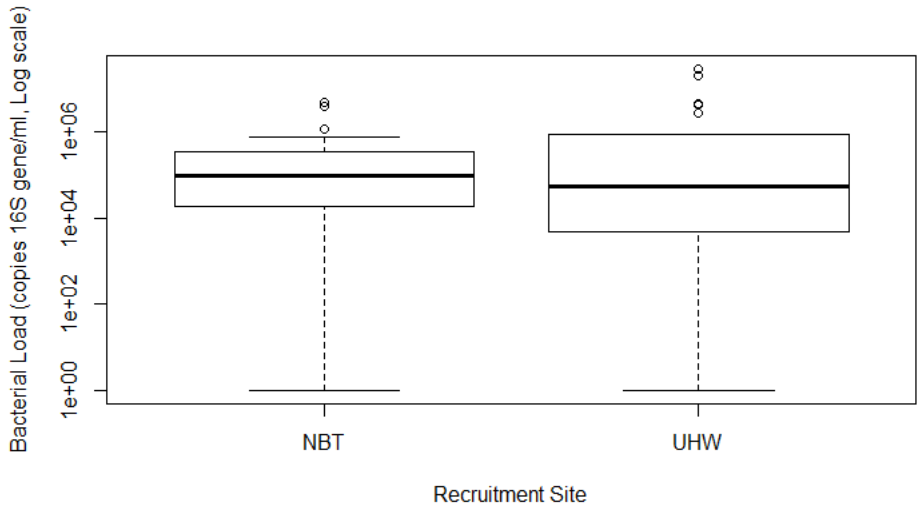


Figure 3-14: Comparing bacterial load on TA samples that were successfully sequenced for 16S rRNA gene between two recruitment centres.
NBT= North Bristol Trust (n= 26), UHW = University Hospital of Wales (n=21). Samples with undetectable bacterial load were given a value of 1 to allow plotting on a logarithmic scale. p=0.9 (Calculated with Wilcoxon test)

3.12.2 Comparing bacterial community structure

Figure 3-15 shows an NMDS ordinating each sample by the Bray-Curtis dissimilarity index. The samples are coloured by recruitment centre with the centroid of each group plotted with spikes to each sample. Univariate PERMANOVA analysis of this difference shows an R^2 = 0.044, p=0.026. A permutation test for homogeneity of multivariate dispersions, p= 0.73, indicates this statistically significant effect is not explained by different dispersion around the centroid of the 2 groups. Therefore, this figure shows that there is a significant difference between the bacterial communities detected between the two recruitment sites.

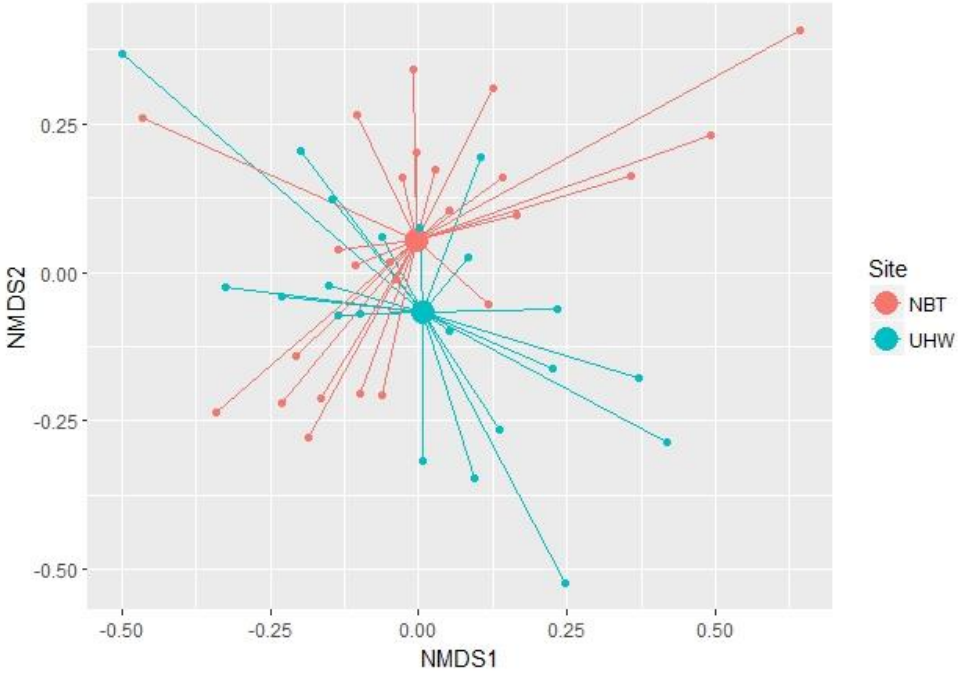


Figure 3-15: NMDS plot comparing the colonisation patterns between the 2 recruiting centres, stress value = 0.18. PERMANOVA R^2 = 0.044, p=0.026.

3.12.3 Comparison of individual genera between sites

Figure 3-16 shows only the most abundant 10 genera in the dataset comparing average abundance between the two collection sites. Using the 10 most abundant genera included 91.8% of reads within the dataset. The only significantly different genus is *Acinetobacter*. This can be explained by the presence of an *Acinetobacter baumannii* outbreak at the Cardiff recruiting centre during the samples collection period. This result provides evidence for the reliability of the data as the infection outbreak is identified by the results. The absence of other differences indicates there was no differences in the organisms identified between the two sites.

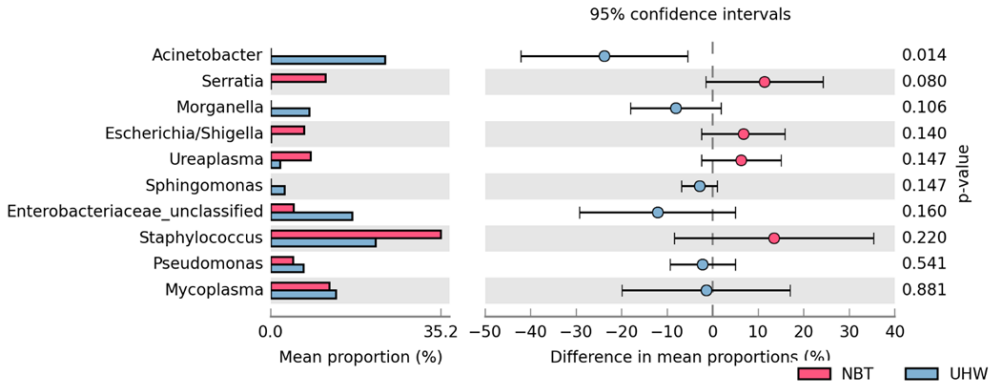


Figure 3-16: Comparing the mean proportion of the most abundant 10 genera in TA samples taken between 2 recruiting centres. Number of samples: UHW = 21, NBT = 26.

3.12.4 Comparing alpha diversity indices

Further evidence for the lack of a difference between recruiting centres was that no statistically significant differences were seen in alpha diversity measures between the two sites (Figure 3-17).

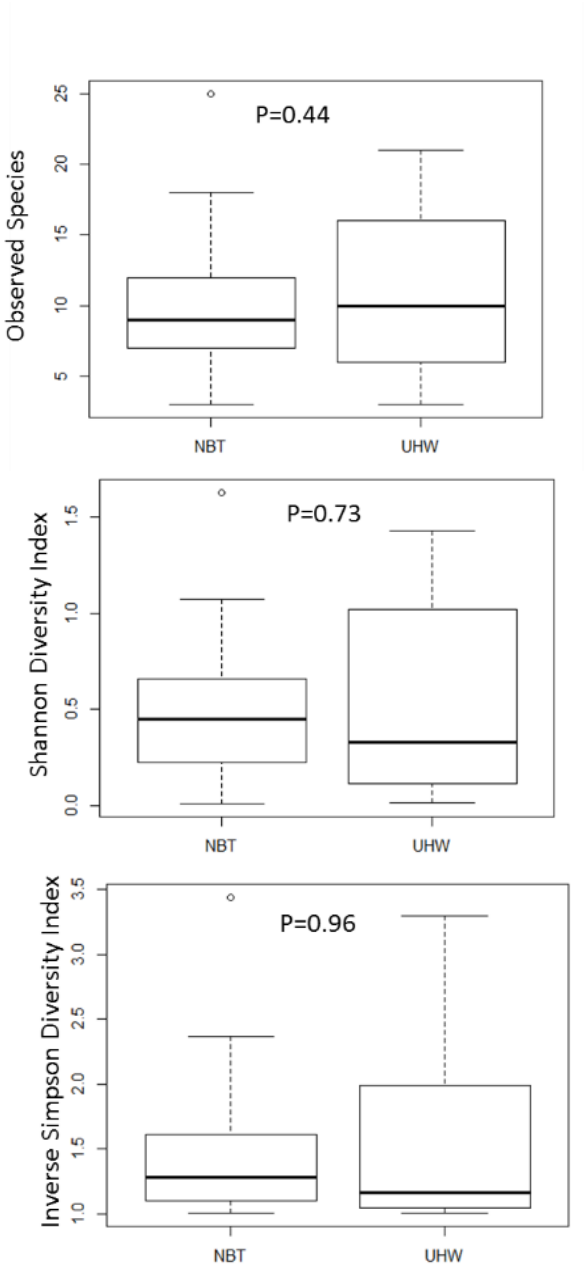


Figure 3-17: Effect of recruitment centre on alpha diversity measures, NBT n=26 UHW n=21.

3.12.5 Sensitivity analysis removing infants culture positive for *Acinetobacter baumannii*

The differences witnessed in the PERMANOVA analysis and analysis of the most abundant genera between sites may be explained by the *Acinetobacter baumannii* outbreak that occurred at one of the recruiting sites (UHW) during the study period. To remove the effect of this, the four infants who were known to have *Acinetobacter* colonisation/infection from culture results

from screening performed on all infants at the affected site were removed and the sensitivity analysis performed. Of the four infants colonised with *Acinetobacter baumannii*, three had tracheal aspirate samples with successful 16S rRNA sequencing.

Figure 3-18 shows an NMDS plot of samples from both recruitment centres with infants culture positive for *Acinetobacter baumannii* excluded. This shows the difference between centres remains. PERMANOVA analysis shows that this difference remains statistically significant. Figure 3-19 shows differences in the top 10 individual genera between recruitment centres following the removal of infants culture positive for *Acinetobacter baumannii*. no longer being a difference in proportions of *Acinetobacter* between the two recruitment sites. A statistically significant difference is now present in the proportion of Mycoplasma species which may explain the differences in the NMDS plot and the PERMANOVA analysis.

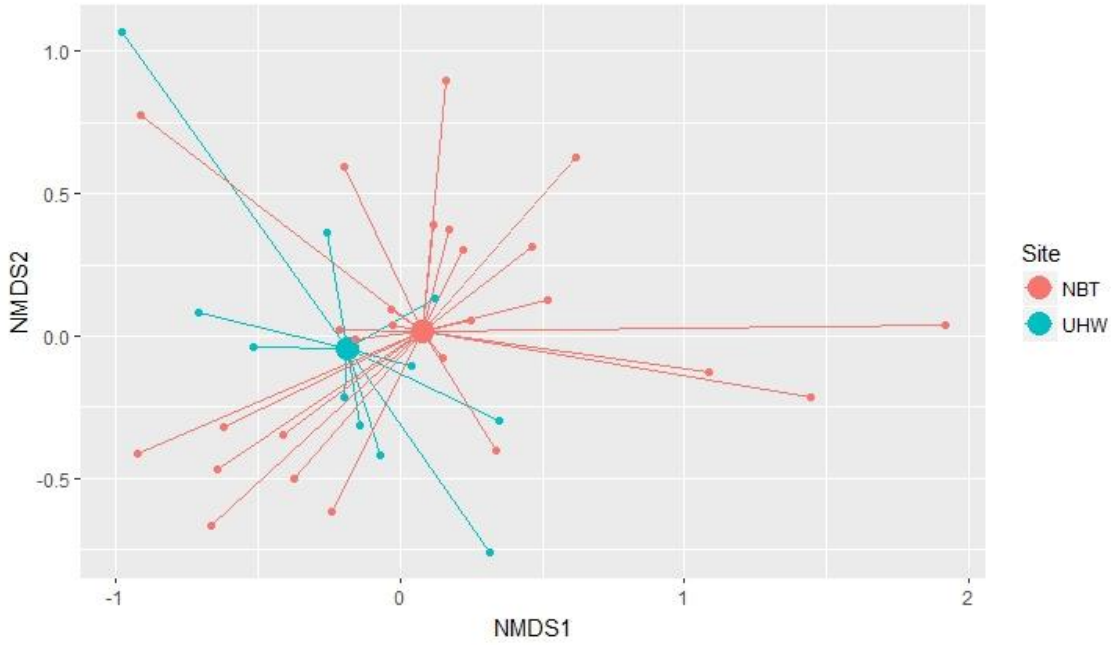


Figure 3-18: NMDS plot of TA samples from infants not colonised with Acinetobacter baumannii. Ordinated by Bray-Curtis dissimilarity index. Stress = 0.195. PERMANOVA R^2 = 0.058, p=0.027. UHW n=11, NBT n=26.

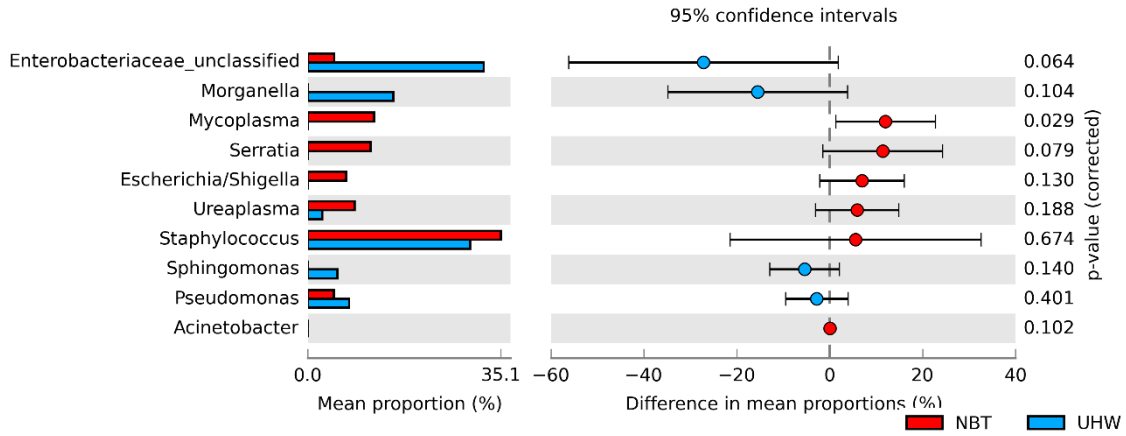


Figure 3-19: Difference in colonisation of individual genera between recruitment site with infants culture positive for Acinetobacter baumannii excluded. UHW n=11, NBT n=26.

The *Acinetobacter baumannii* outbreak did not fully explain the difference between bacterial community structure between the two recruitment sites and this data is suggesting that there is an effect of recruitment centre over bacterial colonisation of the airways.

3.13 Effect of delivery mode on airway colonisation

Delivery mode is known to influence early life colonisation across many anatomical sites in new-born infants (Dominguez-Bello et al. 2010; Biasucci et al. 2010; Rutayisire et al. 2016). I examined the role of delivery mode over airway colonisation and found an increase in Gram negative genera (*Acinetobacter* and *Pseudomonas*) and *Mycoplasma* in those infants born by vaginal delivery (See Figure 3-20). Colonisation by these organisms would potentially occur during vaginal delivery. Infants born by caesarean section had a larger proportion of *Staphylococcus*. Skin organisms such as those within the *Staphylococcus* genera would be more likely to be the primary colonisers for infants who are not exposed to vaginal bacteria.

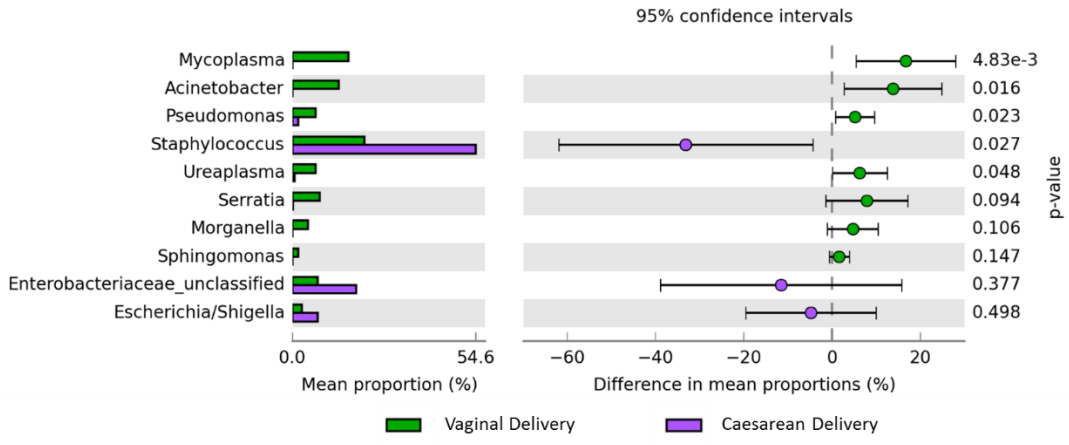


Figure 3-20: Effect of delivery mode on the mean proportion of the most abundant 10 genera in TA samples. Vaginal delivery n=36, Caesarean delivery n=11.

3.14 Summary of tracheal aspirate sample results

The data from the TA samples suggests an infective process has been detected rather than a colonisation by commensal organisms. This is further evidenced by the presence of dominant OTUs within the majority of samples, an association between positive samples and raised IL-6 and IL-8 and the clustering of samples from individual infants on the NMDS plot. Patterns from individual infants support this hypothesis of an infective process. The high rate of samples with no detectable bacterial DNA suggests that any colonisation occurring between the detected infective episodes is below the level of detection of the methodology. Comparing recruiting centres showed differences in bacterial communities between the two sites that was not fully explained by an outbreak of *Acinetobacter baumannii* in Cardiff, suggesting environmental factors may affect bacterial colonisation of the airways. Delivery mode was shown to have a significant effect on organisms detected in TA samples.

3.15 Bronchoalveolar lavage Samples

Due to the suggestion of an infective process occurring during early colonisation of the airway, non-bronchoscopic bronchoalveolar lavage samples were used to analyse deeper within the respiratory tract to look at lung colonisation and reduce the impact of ET tube colonisation confounding results.

3.16 Patient recruitment

Recruitment of infants from the neonatal unit of the University Hospital of Wales occurred between November 2014 and August 2016. A total of 20 infants were recruited into the preterm group and 3 infants into the ventilated term control group. Recruitment was compromised by the unit being shut for prolonged periods due to a series of infection control issues including the outbreak of *Acinetobacter baumannii* previously described.

A total of 96 BAL samples were collected from 22 infants (one infant extubated before the collection of BAL fluid possible). A table demonstrating the characteristics of recruited infants is shown in Table 3-2.

Table 3-2: Demographics of infants recruited from Cardiff.

	Preterm	Term	P
n	20	3	-
Birth Weight (g), mean (SD)	967 (390)	2783 (750)	<0.001
Gestation (weeks), mean (SD)	26.8 (2.8)	38.9 (2.1)	<0.001
Male, n (%)	12 (60)	2 (66.6)	0.85
Maternal Age (years), mean (SD)	27 (5)	22 (5)	<0.001
Maternal IV antibiotics in labour, n (%)	4 (20)	0 (0)	0.39
Maternal GBS Colonisation, n (%)	4 (20)	0 (0)	0.39
Caesarean Delivery, n (%)	10 (50)	2 (66.6)	0.59
Apgar 1, mean (SD)	5 (2)	7 (3)	<0.001
Apgar 5, mean (SD)	7 (2)	8 (1)	<0.001
Doses of surfactant, mean (SD)	1.7 (0.6)	0 (0)	0.01
CLD at 28 days, n (%)	16 (80)	0 (0)	<0.001
CLD at 36 weeks, n (%)	13 (65)	-	-
CLD: n (%)			
Mild	3 (15)	-	-
Moderate	3 (15)	-	-
Severe	10 (50)	-	-
Ventilated Days, median (IQR)	25 (3-37)	3 (3-3)	<0.001
Non-Invasive Respiratory support days, median (IQR)	38 (20-54)	0 (0-1)	<0.001
Home oxygen, n (%)	3 (15)	0 (0)	0.47
Died Before Discharge, n (%)	3 (15)	0 (0)	0.47
Length of Hospital Stay (days), mean (SD)	104 (61)	23 (12)	<0.001

P-values calculated using Student's t-test for normally distributed variables, chi-squared test for binary variables and Wilcoxon test for non-normally distributed variables. (CLD = Chronic Lung Disease of Prematurity)

Characteristics associated with prematurity such as birth weight, gestation and administration of surfactant are, as expected, statistically significantly different between the groups. Apgar scores are also unsurprisingly lower in the preterm group. It is also expected to see a difference between the two groups regarding the length of admission to hospital and the respiratory parameters of length of time receiving ventilation and non-invasive respiratory support. Maternal age was significantly higher in the preterm infant group. This is unlikely to have affected the results.

Only three term infants were recruited due to the closure of the neonatal unit for a prolonged time.

3.17 Bronchoalveolar lavage sample collection and processing

BAL samples were collected and processed as described in Sections 2.2.1 and 2.3.1.1. DNA extraction and qPCR for bacterial load was performed by myself. The extracted DNA was sent for 16S rRNA gene sequencing by Dr D Alber in the laboratory of Prof N Klein in University College London. Sequencing and data processing were performed as described in Section 2.10.

Of the 96 BAL samples collected, 36 (37.5%) samples from 11 infants were found to contain amplifiable bacterial DNA using universal primers for the bacterial 16S rRNA gene. The flow chart in Figure 3-21 demonstrates the samples excluded from the study.

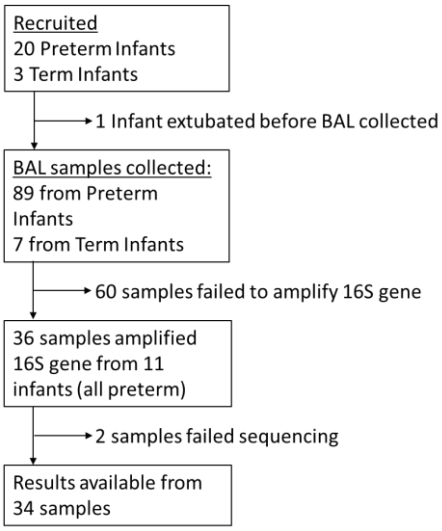


Figure 3-21: Flow chart demonstrating samples collected and analysed during the study

Amplification of the 16S rRNA gene from BAL samples was challenging due to small biomass of original samples and low DNA concentrations obtained following DNA extraction. Tests for PCR inhibitors performed by Dr Alber demonstrated that PCR inhibition was not responsible for poor amplification. The range of DNA concentrations obtained from BAL samples was <0.05 to 3.04 µg/ml with a median of 0.164 µg/ml (IQR = 0.095 – 0.332 µg/ml). This was expected due to the small volume of samples and the low biomass within the samples. The DNA extracted is also likely to be mainly host DNA.

The concentration of DNA obtained from DNA extraction was very low and the lack of template DNA explains the difficulty in amplification. The threshold of samples considered to have amplified adequately for sequencing was lowered to a DNA concentration of 0.1 ng/µL following the PCR amplification reaction using barcoded primers. This is lower than the MiSeq guidelines but enabled data collection from the samples with some amplification.

3.19 Factors associated with failure to amplify bacterial DNA

Figure 3-21 shows that 37.5% of samples amplified adequately for sequencing with final sequencing results available for 35.4% of samples. Factors associated with samples being successfully amplified for sequencing were analysed to identify the reasons why so many samples failed to be sequenced.

As was seen with the TA samples, the strongest identified factor associated with samples containing detectable bacterial DNA was whether the sample was taken while the infant was receiving antibiotics (Figure 3-22). This includes any antibiotics regardless of route of delivery, but most courses of antibiotics are administered intravenously to preterm infants. Some infants included in the study did receive inhaled colomycin for culture positive *Acinetobacter baumannii* infection. The influence of antibiotics may explain why bacterial DNA was unable to be amplified from a large proportion of BAL samples. Samples taken when infants were not on antibiotics had an odds ratio indicating they were 5.6 times more likely to be successfully amplified than BAL samples taken while on antibiotics (95%CI = 1.9-16.4, p = 0.0017).

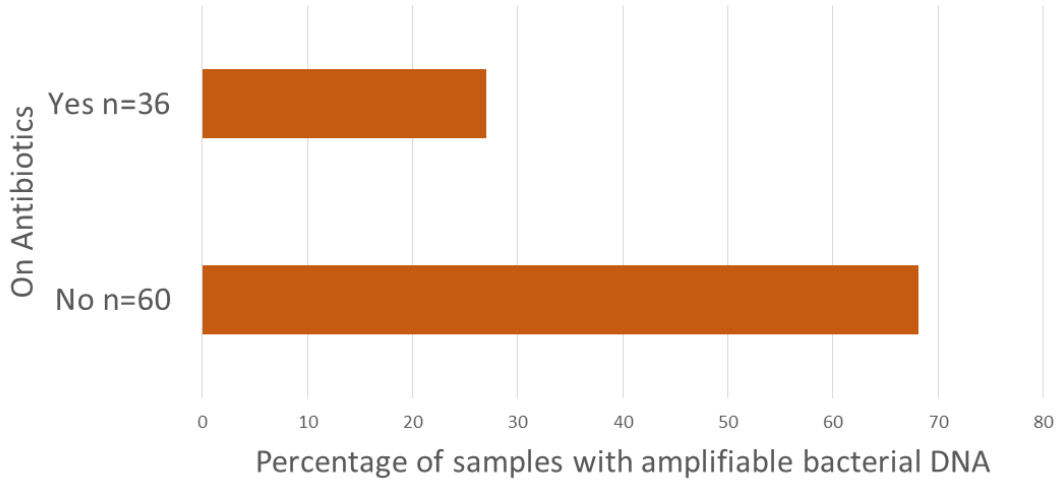


Figure 3-22: Comparing percentage of samples containing amplifiable bacterial DNA between samples taken on and off antibiotics in all BAL samples collected.
P-value using chi-squared test with continuity correction = 0.001.

Figure 3-23 below demonstrates an increasing likelihood of samples having detectable bacterial DNA with increasing day of life. As all preterm infants recruited received antibiotics routinely at birth, as per the local protocol, this trend may well reflect antibiotics usage decreasing with age. The peak of samples positive for bacterial DNA on day 4-7 may well represent the age at which antibiotics are often stopped allowing bacteria to reach detectable levels, before suspected late onset sepsis results in antibiotics being restarted.

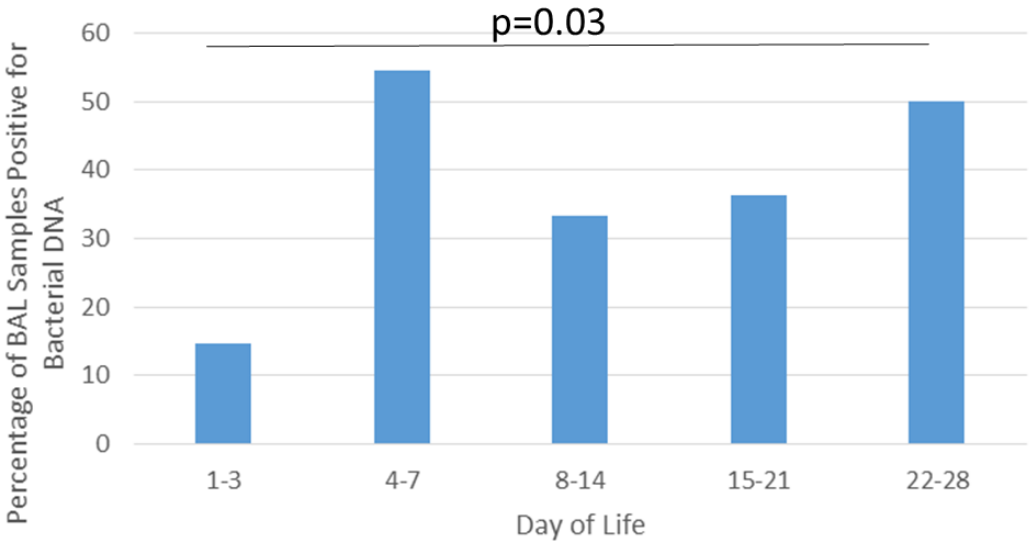


Figure 3-23: Percentages of BAL samples with amplifiable bacterial DNA by day of life Generalised linear model demonstrates statistically significant difference between days 1-3 and days 22-28. Comparing each other day group to the day 1-3 group was not statistically significant.

Other factors that were analysed to identify associations with amplifiable bacterial DNA within BAL samples from preterm infants are shown in Table 3-3 below. The data included only the samples from preterm infants as many factors such as working weight, gestation and feeding are not comparable between preterm and term infants. Factors associated with sample collection were not significantly associated with detectable bacterial DNA in samples.

Table 3-3: Table comparing samples with amplifiable bacterial DNA and samples with no amplifiable bacterial DNA, demonstrating association with clinical and non-clinical variables over samples having amplifiable bacterial DNA in BAL samples from preterm infants only.

Variable	Test	P
Percentage of saline volume returned	t-test	0.57
Probiotics	Chi-Sq	0.04
Enteral Feeding	Chi-Sq	0.015
Inspired O_2 concentration	Wilcoxon	0.77
Serum Albumin concentration	Wilcoxon	0.58
Days current ET tube in situ	Wilcoxon	0.004
Corrected Gestational Age	Wilcoxon	0.097
DNA Extraction kit lot	Chi-Sq	0.37
Completed course of antenatal steroids	Chi-Sq	1.0

The standard care in the recruiting centres was to withhold enteral feed in the first couple of days of life and not feed sick or deteriorating infants. A probiotic containing a mixture of *Lactobacillus acidophilus* and *Bifidobacterium infantis* was prescribed in the Cardiff recruiting centre when the infant tolerated trophic feeds. Probiotics were not used at the Bristol recruiting centre. Whether the infant was on feed and the use of probiotics were significantly associated with detecting amplifiable bacterial DNA in preterm infants. Being on feed and receiving probiotics made it more likely that an infant would have amplifiable bacterial DNA in the sample. This is likely to be a confounding effect of antibiotics and age. Infants would usually have feeds and probiotics withheld during the first 48hrs life whilst on antibiotics. Feed and probiotics would be stopped if the infant was clinically unwell or nor tolerating feeds and would likely start antibiotics at this point. However, probiotics and feeding may be an important source of bacteria colonising preterm infants. The difference witnessed between infants receiving probiotics and not receiving probiotics may reflect a difference between recruiting centres.

DNA extraction kit lot was not associated with samples being successfully amplified for the 16S rRNA gene, demonstrating that contamination of a specific kit was unlikely to be affecting the results.

An analysis was performed using samples taken on day 1-3 of life to see if antenatal steroids had any effect on early rates of detectable bacterial DNA in BAL samples. 34 samples were taken on day 1-3 of life, of which 30 were taken from preterm infants. A complete course of

antenatal steroids had no effect on the chances of samples having amplifiable bacterial DNA (p= 0.85).

3.20 Pro-inflammatory response associated with bacterial colonisation of the lungs

The pro-inflammatory cytokines IL-6 and IL-8 were measured in the supernatant of all BAL samples by ELISA as per the methodology described in Section 2.11. Figure 3-24 below shows that samples positive for bacterial DNA were found to have higher IL-6 and IL-8 concentrations in BAL supernatant than samples negative for bacterial DNA, suggestive of an infective process.

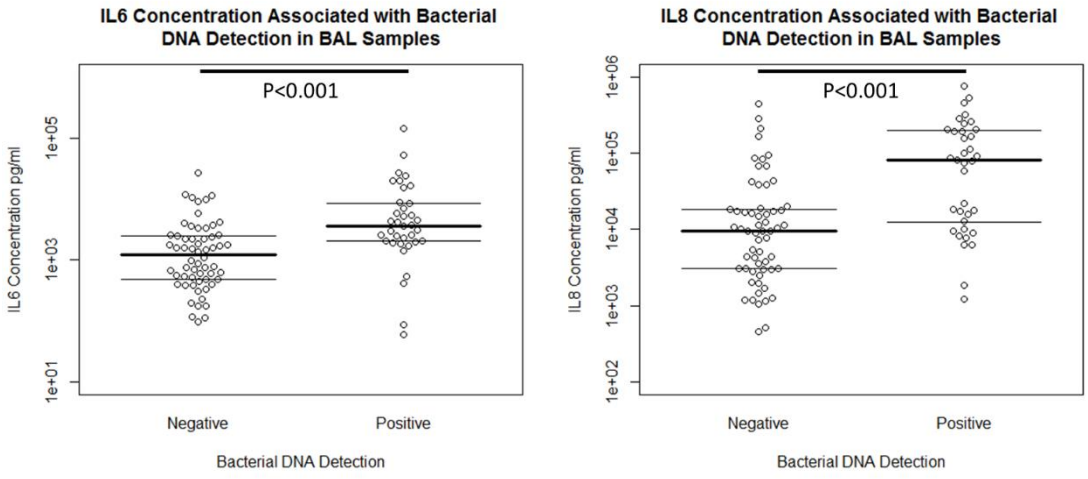


Figure 3-24: IL-6 and IL-8 Concentration between samples positive and negative for detectable bacterial DNA
P-values calculated using Wilcoxon rank sum test with continuity correction. Negative n=60, Positive n=36.

The association between samples positive for bacterial DNA and other markers of infection, both respiratory and systemic, are displayed below in Figure 3-25. The total cell count and polymorphonuclear cell count within BAL samples showed a trend towards higher cell counts in samples with detectable bacterial DNA but failed to reach statistical significance. This is likely to be due to the relatively small number of samples in the study. Total white blood cell count in blood, a systemic marker of infection, had no association with detectable bacterial DNA, however, counterintuitively, serum C-reactive protein (CRP) measurement was significantly higher in infants without bacterial DNA detectable within samples. This is most likely as result of the clinical practice to start IV antibiotics in response to a rise in CRP. The effect of antibiotics on detection of bacterial DNA has been shown in Figure 3-22, resulting in sterile BAL samples.

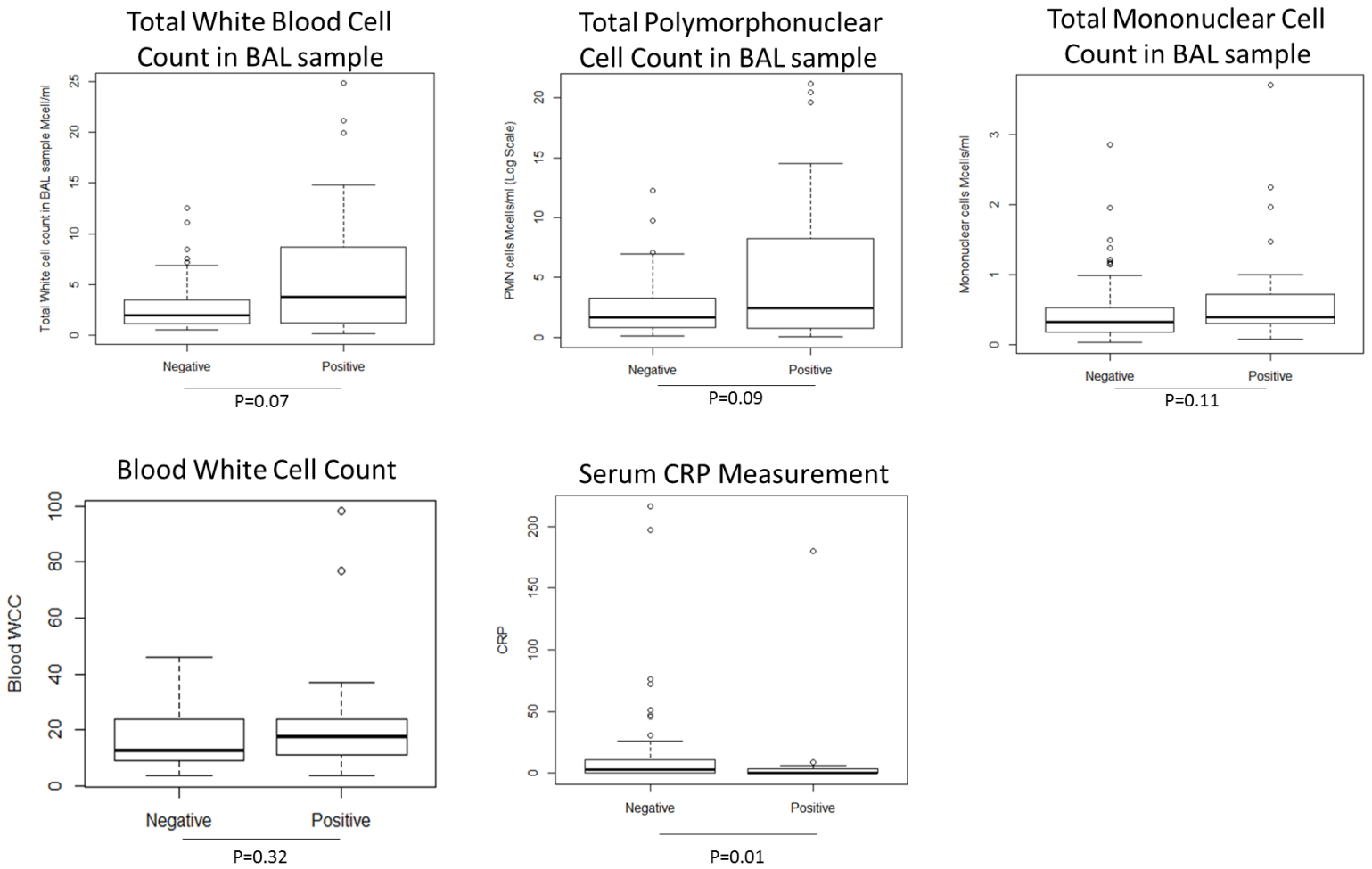


Figure 3-25: Association of sepsis markers with BAL samples positive for bacterial DNA

P-values calculated using Wilcoxon rank sum test with continuity correction. Negative n=60, Positive n=36.

3.21 Bacterial load within bronchoalveolar lavage samples

The total bacterial load within each BAL sample was measured using qPCR with primers targeted to the V3-V4 region of the bacterial 16S rRNA gene. Details of the methodology are given in Section 2.9. Of the 96 samples available, 34 samples were positive for bacterial DNA, defined as greater than the limit of detection of the assay (50 copies of 16S rRNA gene in 2.5 µL reaction volume).

As in the TA samples, episodic peaks of high bacterial load were detected within the BAL samples from individual infants as visualised in Figure 3-26. This is suggestive of episodes of bacterial overgrowth likely to be seen in infection. The colonisation of the lungs by bacteria was not detected between the episodic peaks, suggesting that any bacterial colonisation of the airways in preterm infants is occurring at levels below the limit of detection of the assay.

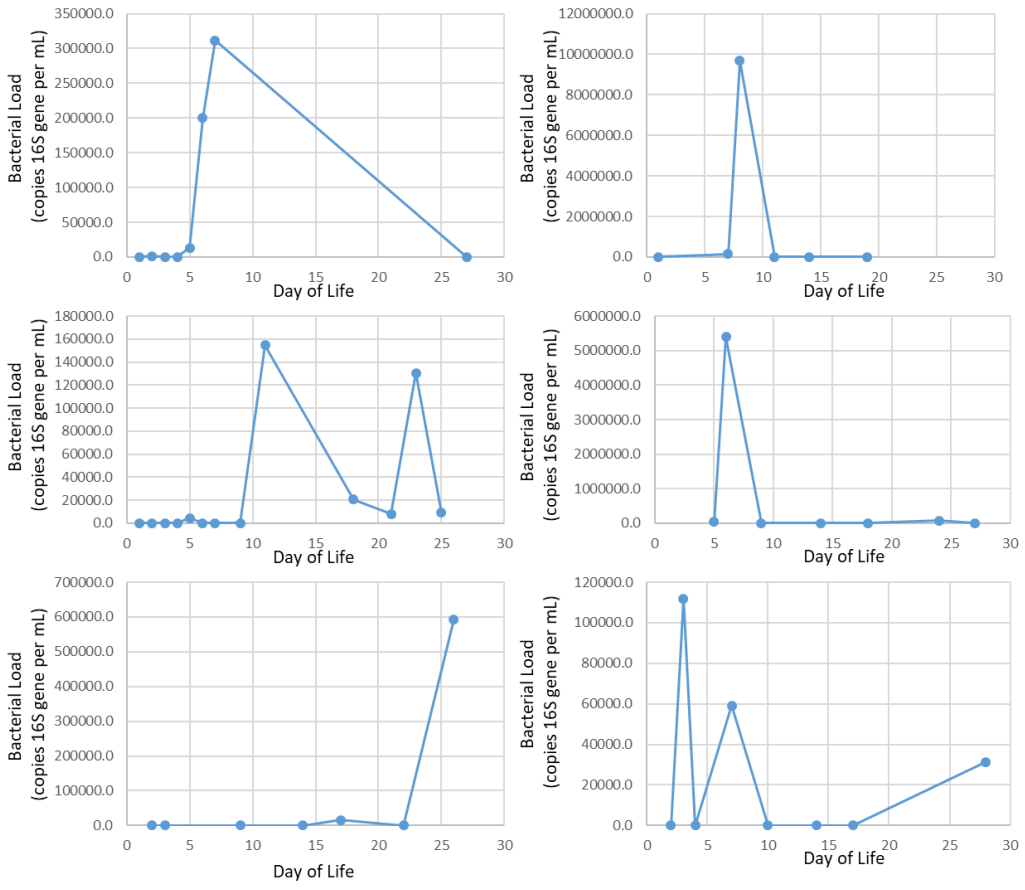


Figure 3-26: Bacterial load measurements by qPCR in BAL samples of preterm infants. Each figure presents the bacterial load detected in serial BAL samples from an individual infant over time.

The average bacterial load over time shows a very similar pattern between TA and BAL samples (Figure 3-27). Early bacterial colonisation is very low followed by an increase over the first two weeks of life followed by a fall in average bacterial load. As the results suggest an

infective process is being detected rather than colonisation, this is likely to show the timing of most respiratory infections. Results may be affected by the correlation between severity of lung disease and antibiotic usage in infants intubated for a prolonged period.

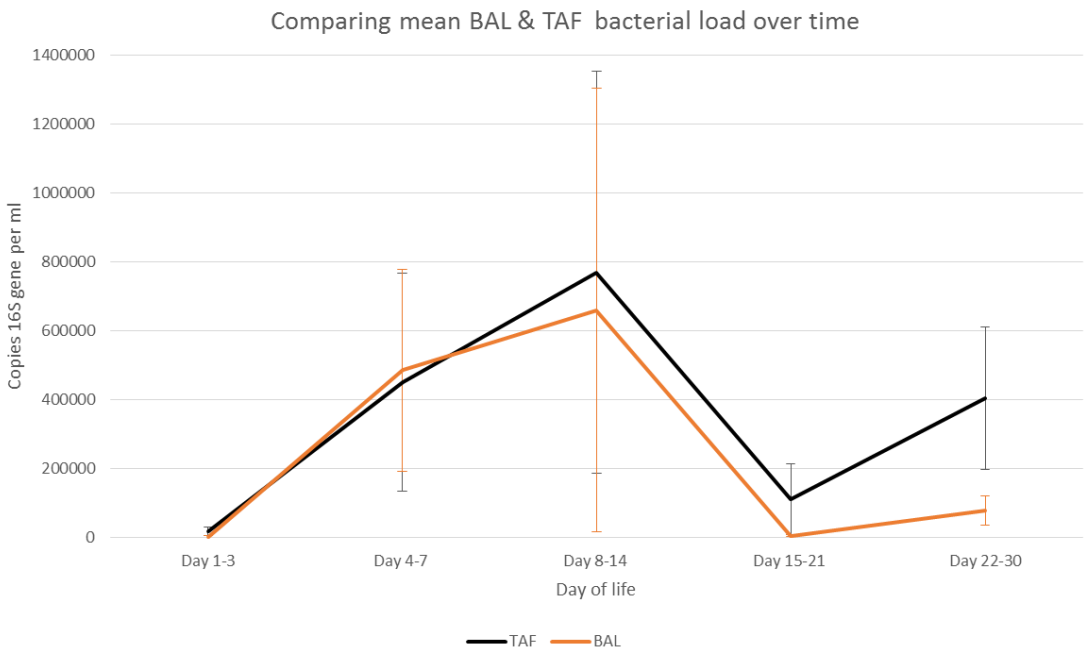


Figure 3-27: Average bacterial load over time in BAL and TA samples
Day 1-3: TAF n= 94, BAL n=31; Day 4-7: TAF n= 92, BAL n=18; Day 8-14: TAF n= 35, BAL n=15; Day 15-21: TAF n=26, BAL n=11; Day 22-30: TAF n=30, BAL n=14.

3.22 Sequencing results from bronchoalveolar lavage samples

DNA from samples containing amplifiable 16S rRNA genes were sequenced. Sequencing was performed as described in Section 2.10 for the V3-V4 region of the bacterial 16S rRNA gene. Table 3-4 below demonstrates the number of reads obtained from sequencing for each sample and the numbers of sequences remaining in the dataset following processing of the data using Mothur to exclude ambiguous bases, chimeric sequences and other random noise or sequencing errors. The threshold for the number of ambiguous bases that was considered acceptable was set at 12, which excluded 5% of the sequences.

Alongside the DNA from clinical samples, two DNA extraction controls and one template free control underwent amplification of the V3-V4 region of the 16S rRNA gene and the PCR products were sequenced. The two DNA extraction controls were samples of the same saline used to collect the clinical samples, aspirated through the sample collection equipment used to collect the BAL samples. These samples were stored and processed alongside the clinical samples. This enables contamination of regents and the DNA extraction kit to be assessed. The no template control reflects only contamination of the PCR and sequencing reagents and was

made up of the PCR reagents used during amplification of the V3-V4 region of the 16S rRNA gene using sterile water in place of extracted DNA.

After processing with Mothur, the read number for all three control samples was low (maximum=588). The most commonly occurring OTUs found within the negative controls were of the genera *Pseudomonas* (maximum = 446) and *Acinetobacter* (maximum = 77 reads). Other genera detected in negative controls were *Cellumonas*, *Variovorax*, *Pasturellaceae* and *Massilia*, most of which are known contaminants of PCR reagents and DNA sequencing kits (Salter et al. 2014). Ideally, the OTUs of contaminating genera detected in the negative controls would be removed from further processing. However, *Pseudomonas* and *Acinetobacter* are known pathogens within the neonatal population and infants within the study were culture positive during the study period for organisms within these genera from TA samples taken for clinical indication. Therefore, these OTUs remain of clinical relevance. When these OTUs appear in clinical samples, the read numbers are many orders of magnitude greater than the numbers detected within the negative controls. To remove the effect of contamination on clinical samples, double the maximum number of reads of each OTU found in a control sample was subtracted from the corresponding number of reads within a clinical sample. For example, if the maximum number of reads for a given OTU in a control sample was 15, then 30 was taken off the total of reads for that OTU in each clinical sample.

Following this, OTUs with less than 10 reads across the whole data set were removed as these were presumed to be sequencing errors. Samples with <1000 reads were discarded. Rarefication of reads to a random selection of 1225 reads per sample was performed. This figure was chosen as it included all samples with reads >1000 with resulting coverage values >0.99. This excluded two samples from further analysis. The coverage values obtained by rarefying to 1225 reads per sample is shown below in Table 3-4. Coverage values >0.99 indicate that an adequate number of reads have been used to capture >99% of the OTUs within each sample.

Table 3-4: Read numbers and coverage values for sequenced BAL samples.

Sample	Initial Reads	Reads Post Mothur Processing	Reads after Negative Control Sequences accounted for	Coverage after rarefying to 1225 sequences
02B11C	751	27	27	-
02B25G	3815	1238	1225	0.9992
02B2B	7035	1643	1573	1.0000
03B28C	110098	72309	72271	0.9984
04B5D	117475	58962	58961	0.9992
05B5E	29599	7159	7144	1.0000
05B6F	151397	89992	89974	0.9992
05B7G	59895	35979	35963	0.9984
06B11I	233139	66387	66383	0.9992
06B18J	33891	16148	16125	0.9984
06B1A	40650	10241	10122	0.9984
06B21K	6903	2667	2598	0.9992
06B23L	183593	97507	97477	0.9984
06B3C	34088	11241	11126	1.0000
06B4D	13903	3981	3919	0.9992
06B5E	29295	7559	7559	1.0000
06B7G	9018	2879	2687	0.9984
10B13F	207903	84893	83845	0.9992
10B28L	197849	112585	111476	1.0000
10B6D	218110	142501	142331	1.0000
10B7E	74395	46330	46176	1.0000
12B26G	171698	95594	95538	1.0000
12B2F	49596	26233	26049	0.9967
19B19F	10359	3546	3496	0.9992
19B7B	54821	38127	37968	1.0000
19B8C	135993	92215	92061	0.9992
27B24F	2907	156	95	-
27B27G	5770	1730	1715	0.9984
27B5A	54223	25860	25607	1.0000
27B6B	82514	57445	57291	1.0000
34B2A	41672	16793	16583	1.0000
34B9B	258417	112330	112083	0.9992
35B14F	297020	177875	177831	0.9992
35B17G	37876	19541	19513	1.0000
35B28H	72602	25784	25747	1.0000
35B3B	73120	38378	38378	0.9976
NTC	2121	48	48	-
Neg1	135	7	7	-
Neg2	1702	588	588	-

Negative 1 and Negative 2 are extraction controls that were handled identically to clinical samples. NTC = No Template control, nuclease free H_20.

The most commonly occurring phyla within the BAL samples were Proteobacteria and Firmicutes (Figure 3-28), the same as the TA samples. These organisms predominate in most human microbiome studies and have been shown to predominate in culture-independent studies of the neonatal respiratory tract (Lal et al. 2016; Lohmann et al. 2014). The other phylum detected in my samples which predominated in at least one sample were Tenericutes, known pathogens in neonatal lungs, and Bacteroidetes. A small number of Actinobacteria and Fusobacterium were detected in some samples.

The pattern of phylum detected did not change over the first 28 days of life with 3/6 (50%) positive samples in day 1-3 showing a predominance of Proteobacteria and 4/7 (57%) in days 22-28 positive for Proteobacteria. There was no association between the proportion of individual phyla and antenatal steroid, maternal antibiotic use, infant feeding or infant probiotic use.

At genus level, dominant genera, defined as >50% of reads from a single genus, were identified within the majority of samples. Figure 3-29 demonstrates that within a dominant genus, a single OTU is also present within the samples (defined as >50% of reads within a sample from a single OTU). The most abundant genus within the dataset was *Acinetobacter*, followed by *Enterobacteriaceae* (unclassified) and *Staphylococcus*. Within these genera are known pathogens and commensals of the respiratory tract.

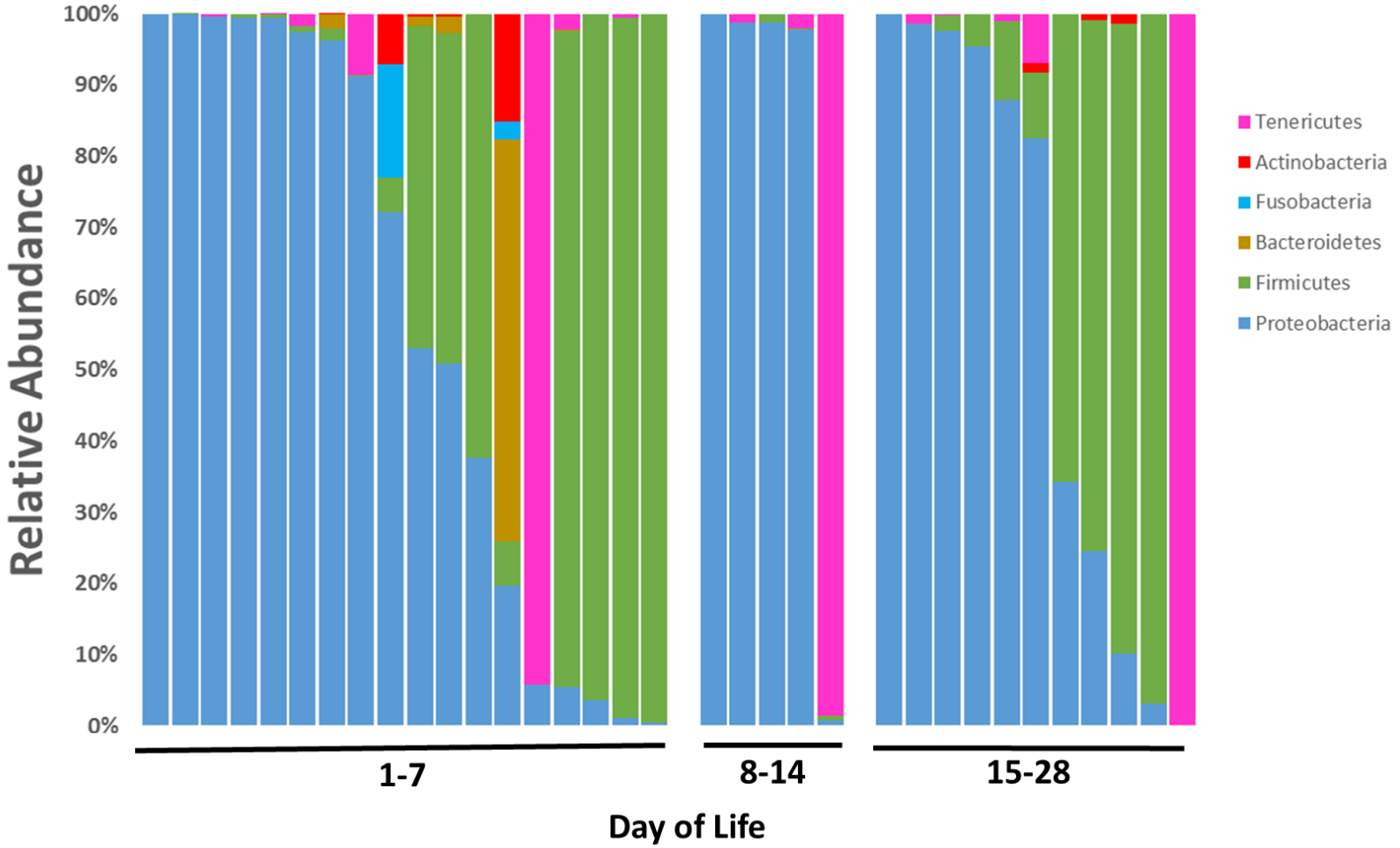


Figure 3-28: Relative abundance of Bacterial Phyla within BAL Samples by Day of Life. Within each day of life group, samples are ordered by relative abundance of proteobacteria.

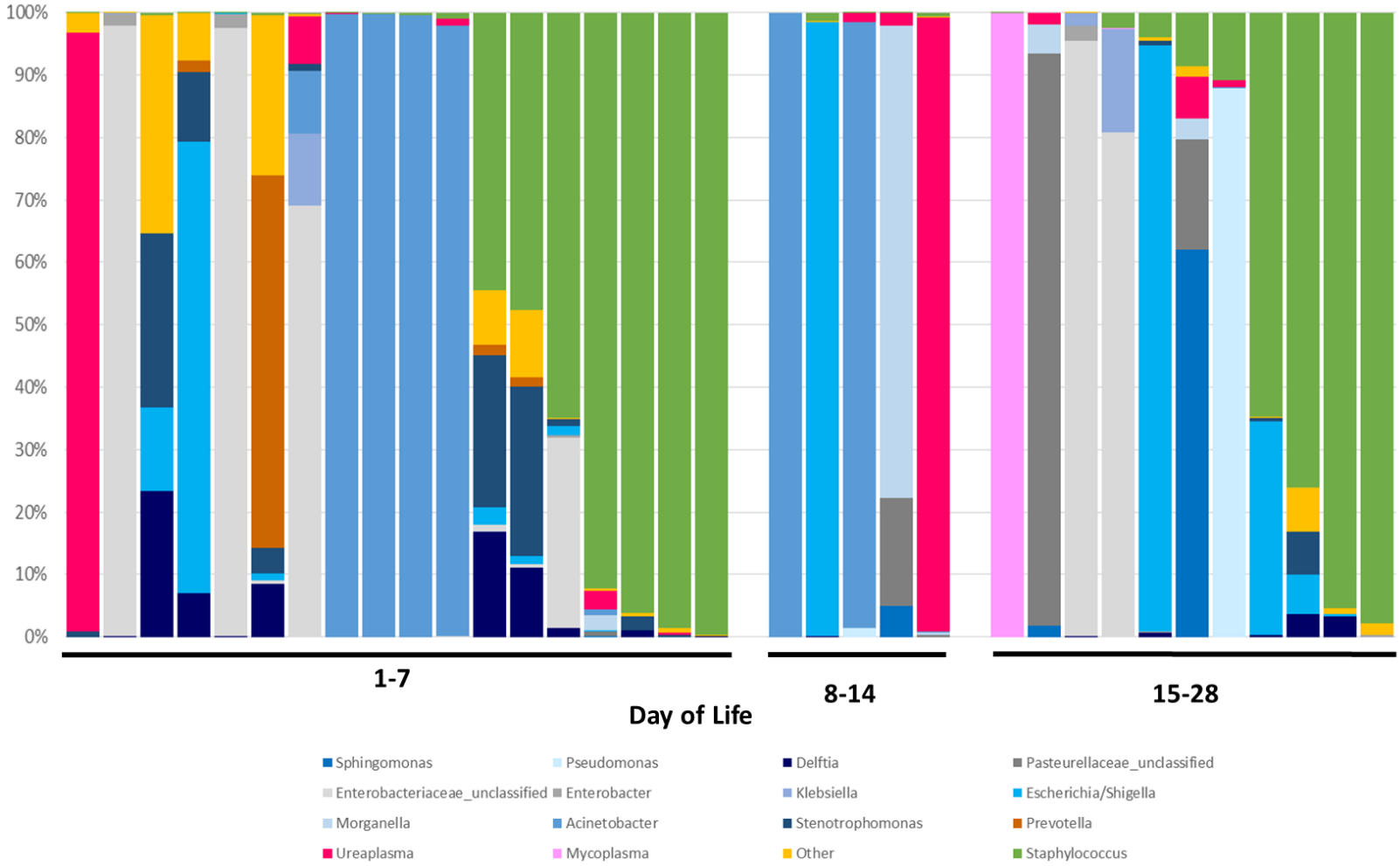


Figure 3-29: Relative abundance of individual genera within BAL samples from preterm infants, using only the top 20 genera to generate figure. Samples are ordered by dominant genus. The top 15 OTUs are shown individually with the remaining OTUs pooled as "other".

3.23 Comparing qPCR and sequencing results

Of the 36 samples which were amplified for sequencing 23 were found to have detectable bacterial DNA using the qPCR methodology described in Section 2.9 (Figure 3-30A). The boxplot in Figure 3-30B shows that the difference samples positive by qPCR that failed to amplify had a lower bacterial load than those that were successfully amplified for sequencing. This suggests that the samples were near the threshold of detection of the amplification assay and may explain why they were successfully amplified by qPCR but not for sequencing.

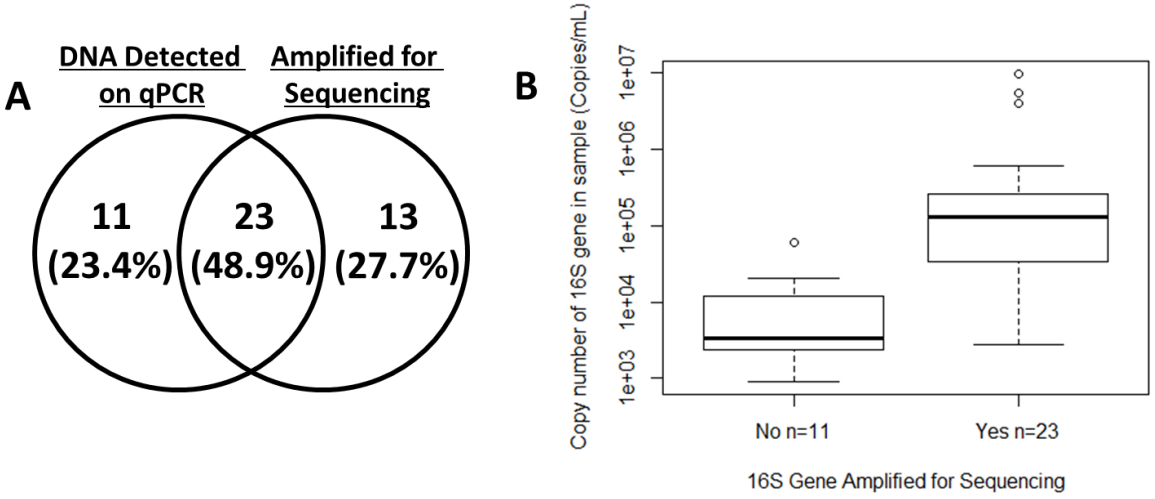


Figure 3-30: Comparison of samples positive for 16S rRNA gene by different methodologies
A- Venn Diagram showing numbers of samples positive for the 16S rRNA gene by qPCR and samples that amplified for sequencing. Percentages of all 96 samples. B – Comparison of copy number per mL of the 16S rRNA gene between samples successfully and unsuccessfully amplified for sequencing. Only including samples positive by qPCR. P<0.001 between the 2 groups.

3.24 Effect of mode of delivery on bronchoalveolar lavage results

No differences were seen in the proportions of individual phyla when comparing different modes of delivery (See Figure 3-31). However, the proportion of *Acinetobacter* was significantly different between modes of delivery with more of this genus witnessed in infants born vaginally. The outbreak of *Acinetobacter* at the unit was thought to have been spread by direct contact and poor infection control practices by staff on the unit. It may be however that babies born vaginally were more susceptible to *Acinetobacter* infection.

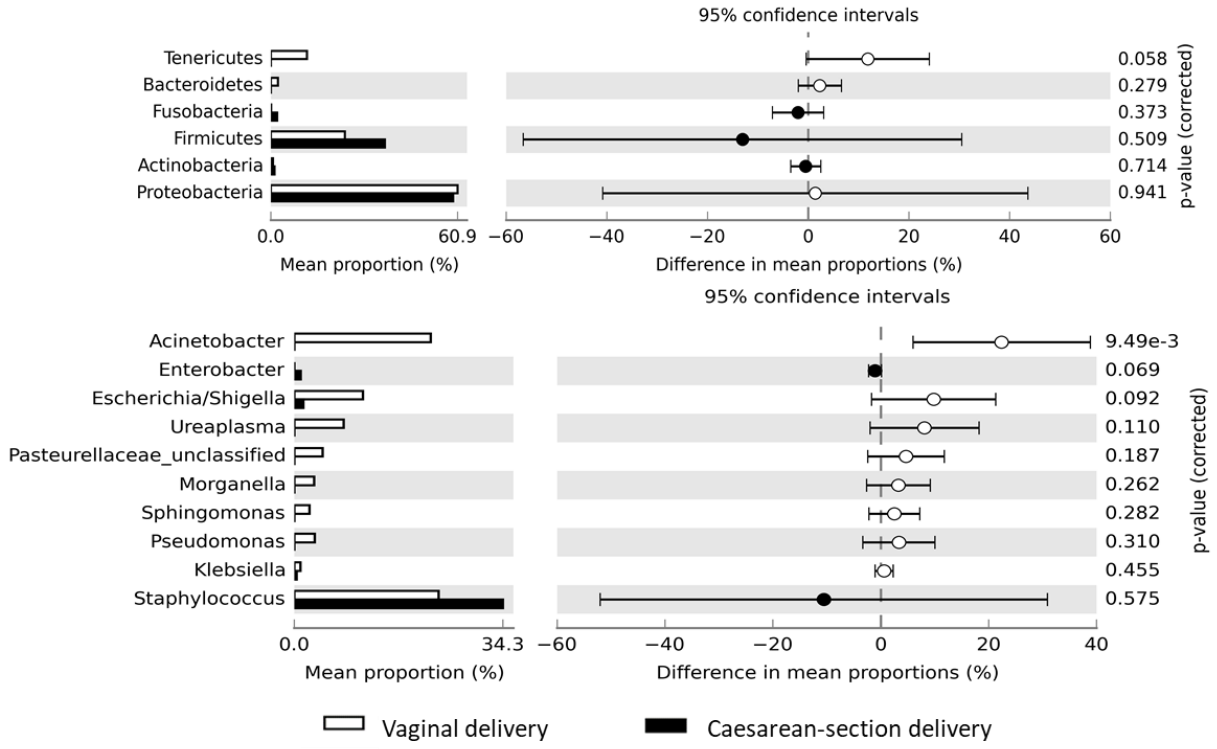


Figure 3-31: Proportion of individual phylum and genera within BAL samples from preterm infants comparing difference by mode of delivery
Vaginal delivery n=27, Caesarean section delivery n = 7. Only top 10 genera shown in lower figure (Top 10 genera represent 97.9% of all reads)

3.25 Changes in diversity within the lungs of intubated preterm infants over time

Measures of alpha diversity applied to the samples with successful sequencing show lower diversity than that shown in previous studies using TA of preterm infants. Low diversity scores are unsurprising given the large numbers of samples with a strongly dominant OTU. Species richness (the number of species observed in a sample) ranged from 1 to 17, with a median of 5. Low richness indices are seen in periods of infection. Samples were grouped by day of life and the mean diversity score +/- standard deviation was plotted (Figure 3-32). For infants with more than one sample in a group the mean of the diversity score for samples was taken.

Using all measures of diversity, the greatest diversity was witnessed in days 1-3 of life, however, the differences over time were not statistically significant. This is likely to be due to the small numbers of samples at each time point. Most samples from days 1-3 were negative, and in the samples that were positive the diversity was greatest. It is possible the lower airways of preterm ventilated infants are colonised with a wide variety of organisms before one dominant organism outcompetes the others resulting in a loss of diversity. This process is associated with a rise in the proinflammatory cytokines IL-6 and IL-8 indicating the host is mounting an inflammatory response most likely to the presence of the dominant bacterium.

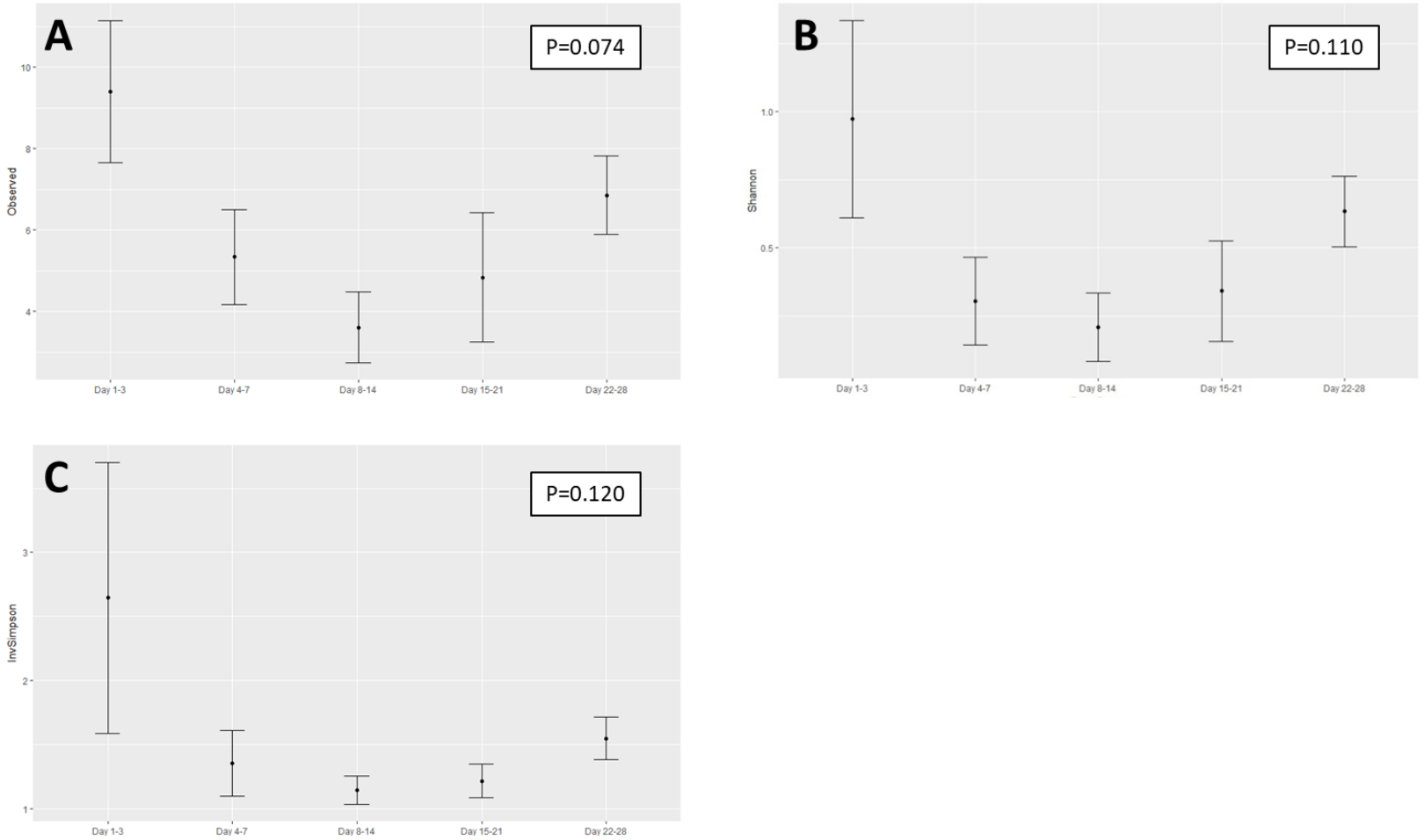


Figure 3-32: Alpha diversity changes over time in BAL samples

A=Species observed. B = Shannon-Diversity Index. C= Inverse Simpson Index. Plotted as mean value +/- SD. Day 1-3 n = 5, Day 4-7 n= 6, Day 8-14 n= 5, Day 15-21 n= 3, Day 22-28 n= 7. If more than one sample from the same infant in each group, then the average diversity index score of the samples was used. P-values calculated using Kruskal Wallis Test.

3.26 β-diversity of bronchoalveolar lavage samples

An NMDS plot ordinating using the Bray-Curtis dissimilarity index demonstrates several clusters (Figure 3-33). These demonstrate clustering by dominant bacterial genus and by infant. The fact that an infant's samples cluster together independently of dominant bacterial genus shows that the other organisms in the sample remain more similar between samples of the same infant than between infants.

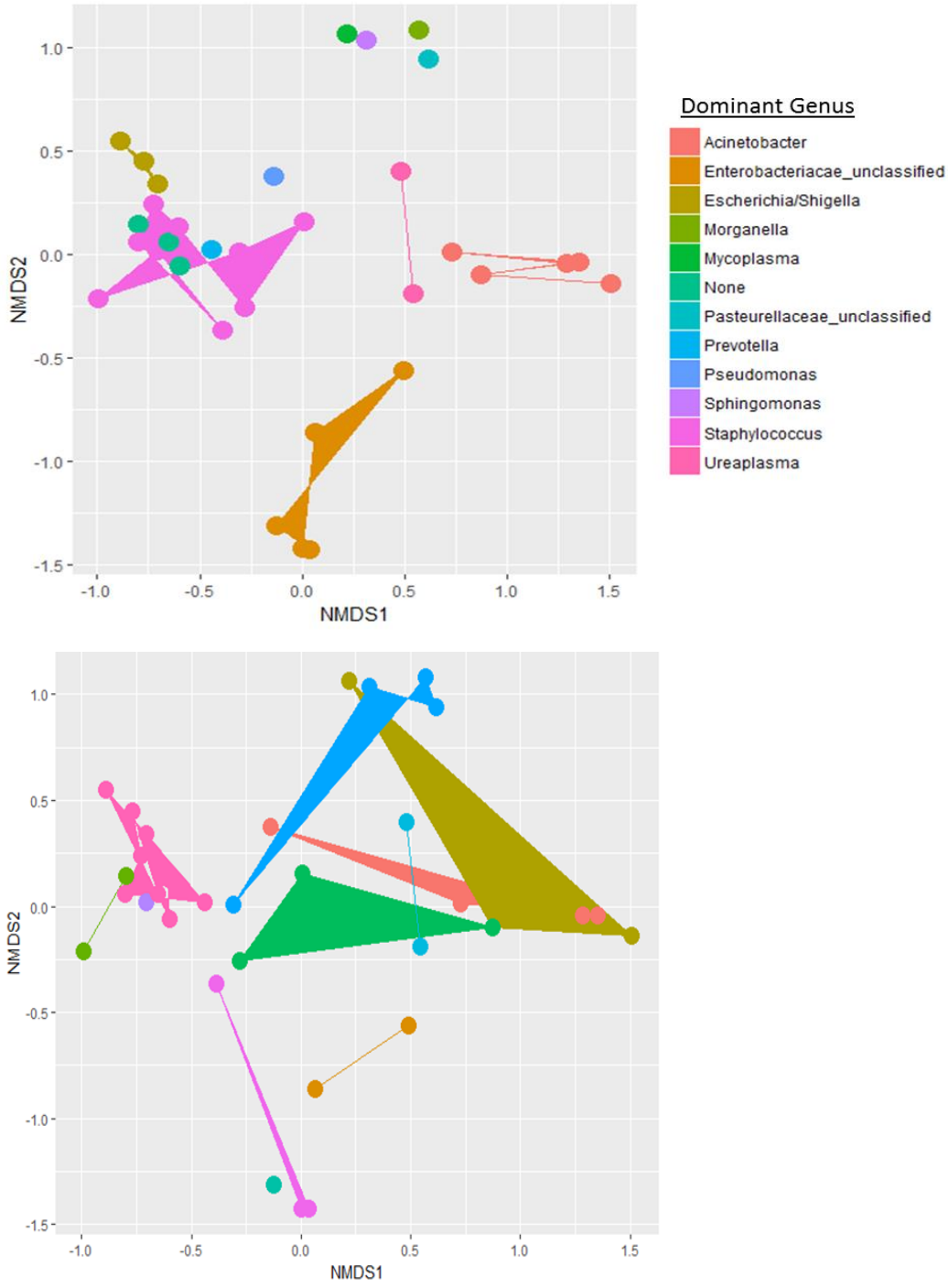


Figure 3-33: NMDS plots of individual BAL samples ordinated by Bray-Cutis dissimilarity index. Stress=0.16.
A= Dominant genus within sample linked by polygons and colour. B = Same plot with serial samples from the same infant coloured and joined by polygon.

3.27 Bacterial load and cytokine concentrations in individual infants

Evidence from the whole dataset suggest that the BAL samples have detectable bacterial DNA associated with raised IL-6 and IL-8 concentrations within BAL fluid suggestive of inflammation associated with the presence of bacteria. To confirm that this was witnessed on an individual patient level plots were made combining the relative abundance of phyla in each sample successfully sequenced with the bacterial load estimated using qPCR and the IL-6 and IL-8 concentration for each infant over time. Examples of such figures for individual infants is shown below in Figure 3-34. These figures show the peaks and troughs of bacterial load coincided with peaks and troughs of cytokine concentration. It was noted that even in consecutive days the dominant genus can be different in the same infant, with some infants showing a dynamically changing population of bacteria within the lower airways and lungs.

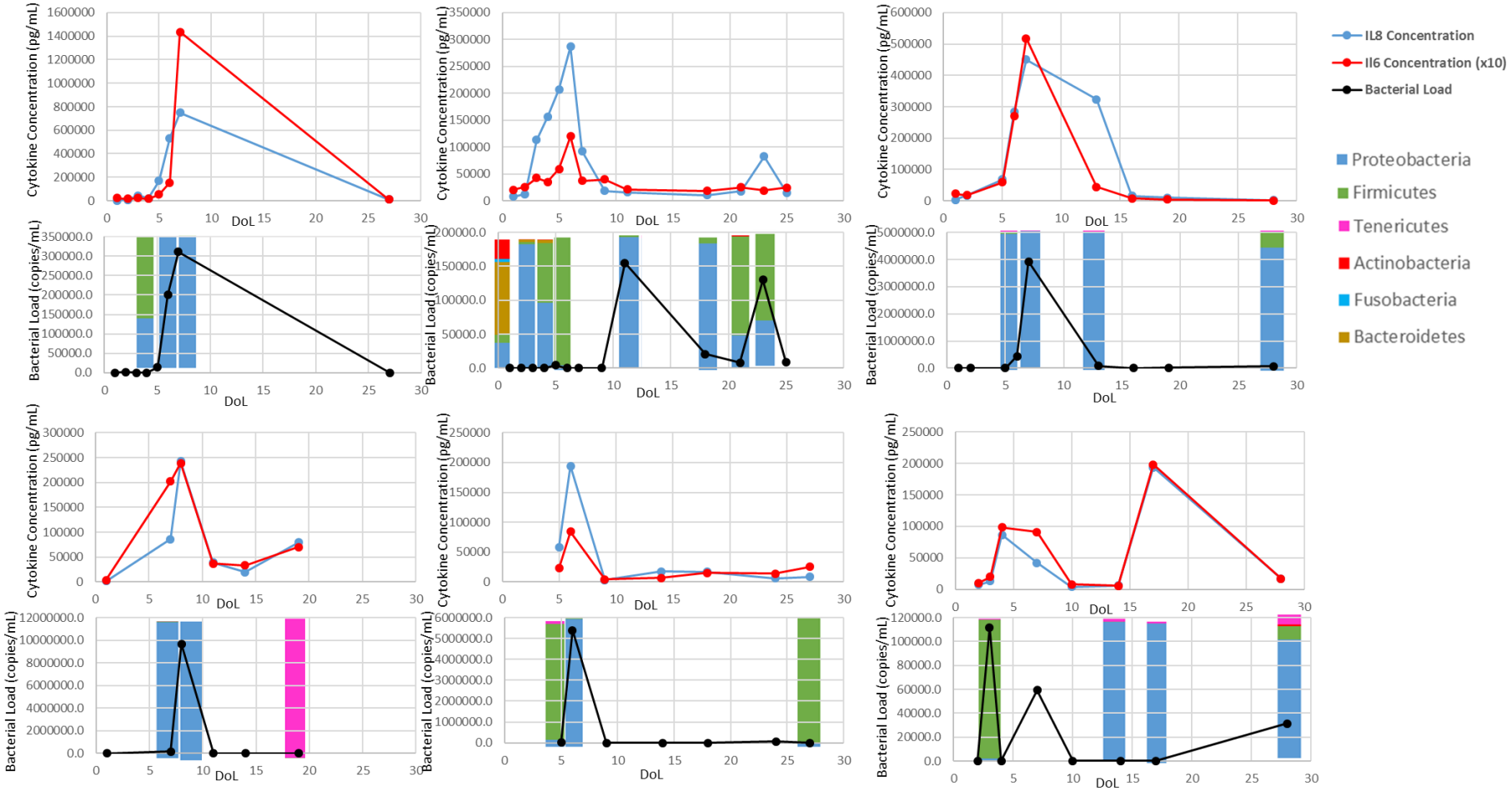


Figure 3-34: Paired graphs showing cytokine concentration and bacterial load for individual infants over time
Cytokine concentrations are given as pg/ml. IL-6 concentrations (in red) have been plotted as 10 times the measured value. Bacterial load graphs have y axis units of copies 16S rRNA gene per ml of sample. The bar plots behind the bacterial load graphs demonstrate the relative abundance of bacterial organisms detected within the sample when sequencing was successful.

I have shown that detectable bacterial DNA is associated with higher IL-6 and IL-8 concentrations in BAL fluid (Section 3.20) and that the episodic peaks in bacterial load are associated with peaks in IL-6 and IL-8 concentrations. I investigated whether all the peaks in IL-6 and IL-8 were associated with bacterial presence. A threshold of >50,000 pg/ml of IL-8 and >3,000 pg/ml of IL-6 were taken as cut offs for a peak of cytokine concentration. These thresholds were set arbitrarily set using the graphs of cytokine concentration to determine when a peak had occurred. In the 19 preterm infants for whom cytokine concentrations were available from BAL samples 14 peaks of IL-8 were witnessed in 11 infants. In the same cohort 14 peaks of IL-6 were witnessed in 12 infants. For both IL-6 and IL-8, 12 of the 14 peaks were associated with amplifiable bacterial DNA. This suggest that bacterial colonisation resulting in an infection is responsible for the rise in episodic rise in pro-inflammatory cytokine concentrations seen within BAL.

3.28 Average IL-6 and IL-8 concentration in bronchoalveolar lavage supernatant over time

Previous studies have shown the average concentration of IL-6 and IL-8 in BAL samples from intubated preterm infants peak around 10 days of age (S. Kotecha, Wilson, et al. 1996; S. Kotecha et al. 1995). Figure 3-35 below shows that this study replicates this data with a clear peak in average cytokine concentration on day 7 of life.

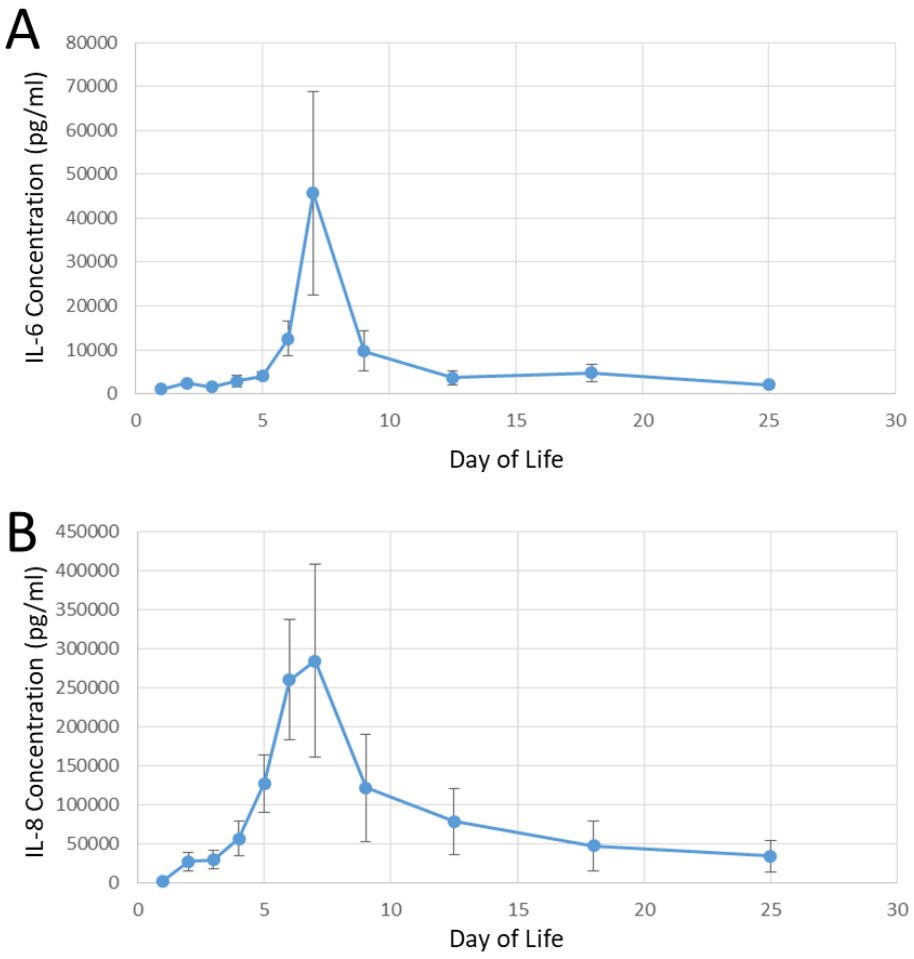


Figure 3-35: Average IL-6 and IL-8 concentration in BAL supernatant from intubated preterm infants.
Mean cytokine concentration plotted with SE displayed with error bars. Days 1-7 plotted individually (Day 1 n=10, Day 2 n=14, Day 3, n=10, Day 4 n=6, Day 5 n=6, Day 7 n= 5), Days 8-10 (n=6), 11-14 (n=7), 15-21 (n=8) and 22-28 (n=9) combined. A = IL-6 Concentrations, B = IL-8 Concentrations.

3.29 Highest IL-6 and IL-8 concentrations associated with dominance with specific genera

The IL-6 and IL-8 concentrations with analysed by dominant genus found in the sample (Figure 3-36 and Figure 3-37). *Acinetobacter* was associated with the highest median concentration of IL-6 and IL-8 followed by unclassified *Enterobacteriaceae*. If levels of inflammation are higher during infections with these organisms then this may result in worse long term respiratory outcomes for infants infected with these organisms.

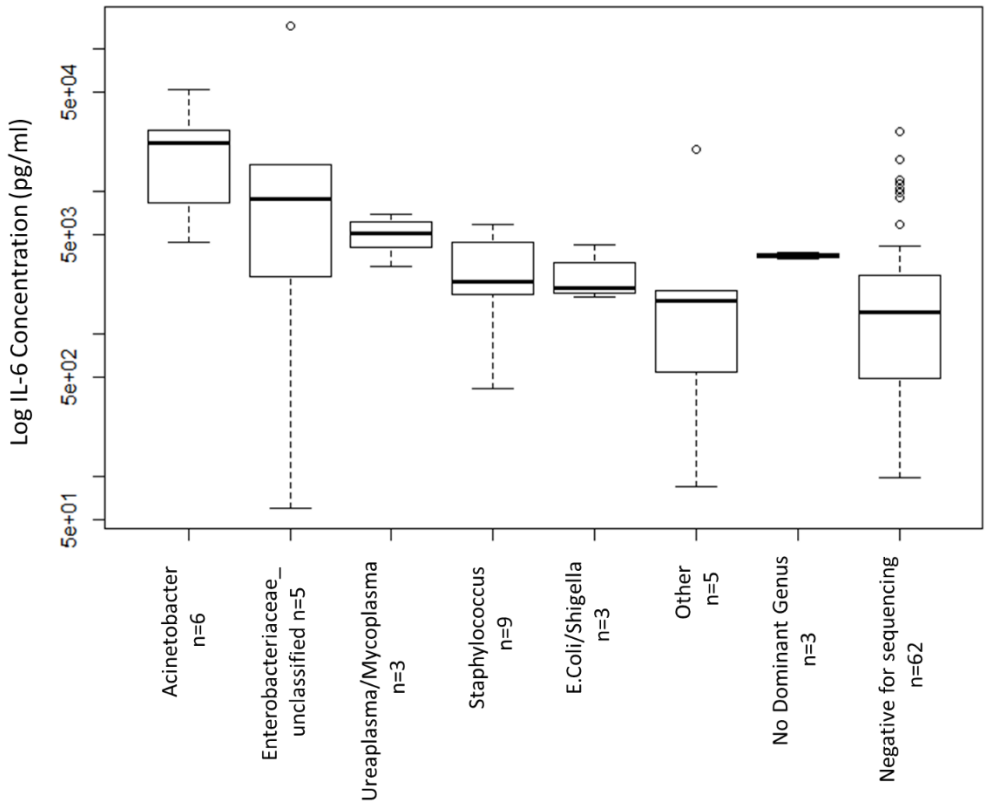


Figure 3-36: IL-6 concentration in BAL fluid supernatant by dominant bacterial genus identified in the sample

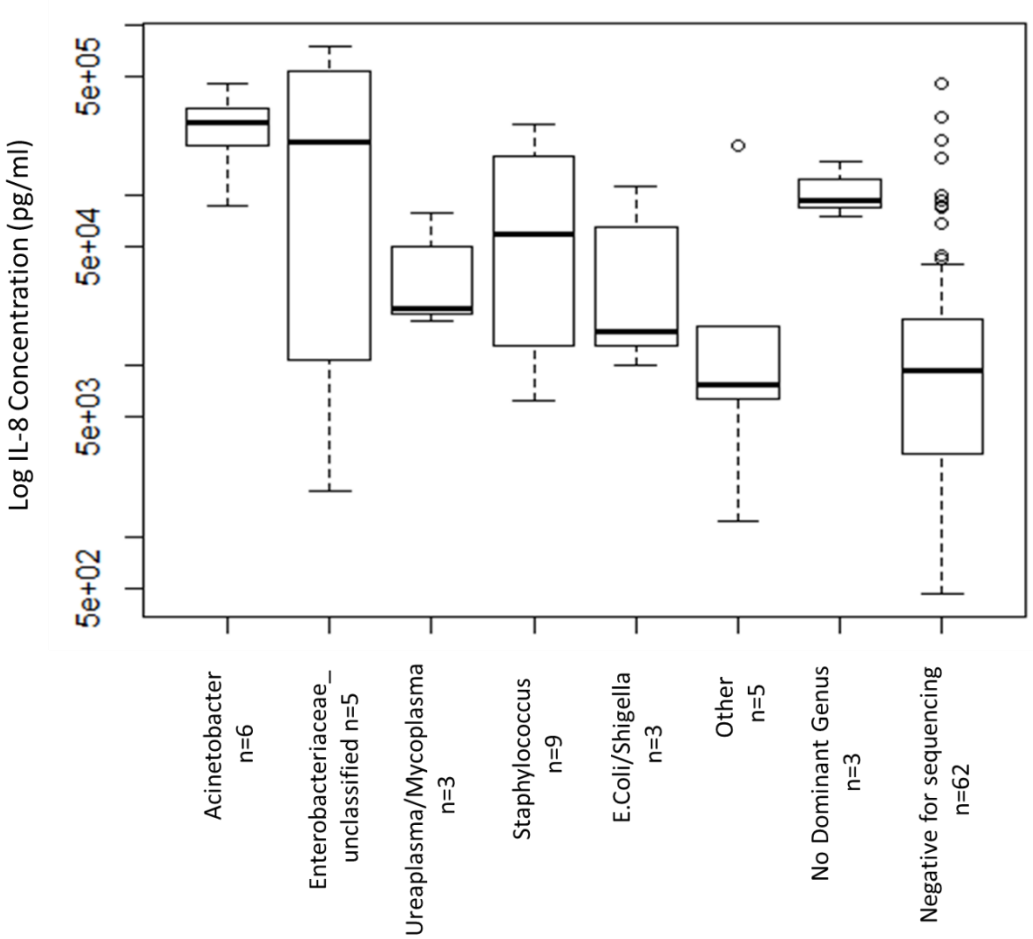


Figure 3-37: IL-8 concentration in BAL fluid supernatant by dominant bacterial genus identified in the sample

3.30 Lung colonisation predictive of long-term respiratory support

I was unable to compare the colonisation results between infants with and without CLD due to over recruitment of extremely immature infants at high risk of developing CLD. Only one infant of the 11 infants for whom sequencing data is available from at least one BAL sample did not develop CLD with 8 of the 11 infants classified as having severe CLD. The 8 infants with severe CLD required prolonged respiratory support defined as the need for non-invasive respiratory support or ventilation at 36 weeks' corrected gestational age. Figure 3-38 shows the mean proportion of individual genera in samples comparing those requiring respiratory support at 36 weeks' gestational age compared to those who did not require respiratory support at 36 weeks' gestational age. None of the top 15 genera show a statistically significant difference between the two groups.

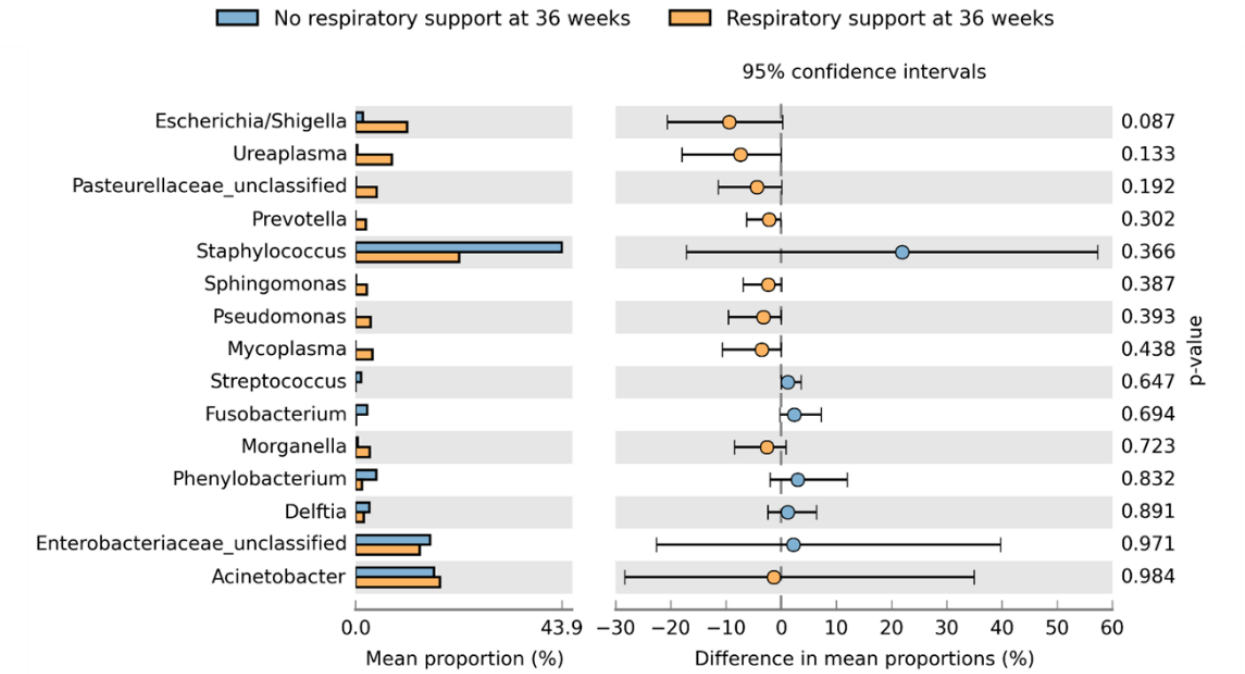


Figure 3-38: Mean proportions of individual genera in BAL samples predictive of respiratory support requirement at 36 weeks corrected gestational age.
Most abundant 15 genera shown ordered by effect size. P values calculated using a 2-sided White's non-parametric t-test with 95% confidence intervals calculated using bootstrapping. The no respiratory support at 36 weeks group contained 6 samples from 3 infants. The respiratory support at 36 weeks contained 28 samples from 8 infants.

3.31 Attempting to identify organisms to species level

An attempt was made to identify whether the sequencing depth from the V3-V4 region was adequate to identify beyond a genus level to the species level using the methodology described in Section 2.10.7. Identification to species level would allow diagnosis of infecting organisms and guide clinical treatment of infection. The 7 most abundant OTUs within the dataset were compared to sequences within the RDP database. Two of the top 7 OTUs compared to the RDP database was shown in Table 3-5 below. For each of the top 7 OTUs, more than one species showed >97% similarity of base pairs compared to the representative sequence in the dataset. Therefore, the sequencing depth was insufficient to identify organisms at species level. The taxonomic level at which organisms are reliably identified is, therefore, at genus level.

Table 3-5: Comparison of representative sequences from OTUs in dataset to aligned sequences of species within the same genus in the RDP database

RDP Database Identified Species	Similarity to representative sequence of OTU1	RDP Database Identified Species	Similarity to representative sequence from OTU3
Acinetobacter calcoaceticus	0.962	*Staphylococcus epidermidis*	0.99
Acinetobacter baumannii	1	*Staphylococcus aureus*	0.99
Acinetobacter haemolyticus	0.981	*Staphylococcus haemolyticus*	0.995
Acinetobacter junii	0.969	*Staphylococcus hominis*	0.993
Acinetobacter guillouiae	0.969	*Staphylococcus epidermidis*	0.993

.

Values expressed as ratios with 1= representative sequence and database sequence identical, to 0 = No matching bases in the aligned sequences.

3.32 Discussion

3.32.1 Recruitment

Recruitment for this study was more difficult than anticipated. Similar studies using the same methodology of sample collection have been performed historically at the same centre without such marked recruitment difficulties (Beeton et al. 2011; Davies et al. 2010). There is no doubt that the repeated and prolonged closure of the neonatal unit in Cardiff limited the number of infants available to be recruited. I circumvented this by adding a second recruitment centre in Bristol. Changing neonatal practice is also likely to have influenced recruitment. The adverse effects of ventilation are more widely recognised in recent years, with reduced number of infants being intubated, clinicians choosing to manage more preterm infants with non-invasive methods of respiratory support (Stoll et al. 2015). The introduction of techniques to deliver surfactant with either only a brief period of intubation or without intubation has led to less infants needing intubation for the purposes of surfactant delivery (Kribs et al. 2015; Ali et al. 2016). Modern neonatal practice also shows a trend towards extubating preterm infants earlier

(Robbins et al. 2015). Reduced numbers of infants remaining intubated and, therefore, less TA and BAL samples were collected from infants recruited to the study.

For the same reasons given above both centres over recruited the most extreme preterm infants and under recruited infants in the 28-32 weeks' gestational age range. The median gestation of recruitment was 25.9 weeks in Bristol and 26.8 weeks in Cardiff. The only infants intubated for long enough to be recruited to the study were in the extreme preterm range. These infants are also more likely to develop CLD, and this, therefore, also explains the high rates of CLD witnessed by infants in the study. The lack of infants who did not develop CLD prevented comparison between a CLD and non-CLD group however, I was however able to serially study infants at risk of CLD. Participation in the study is unlikely to have caused a high rate of CLD in the recruited infants as TA sampling was no different to the routine care the infants received and the BAL procedure has been investigated for safety and is an established research tool (Vyas et al. 2002). The rate of CLD was higher in the recruitment centre that did not perform BAL sampling on infants than the centre where BAL samples were collected, meaning BAL sampling was unlikely to have contributed to the high rates of CLD.

3.32.2 Validity of cytokine data from tracheal aspirate samples

BAL samples were centrifuged prior to freezing. This was not possible with the TA samples which were frozen prior to centrifugation and processing. Cell rupture during the freeze-thaw process may have resulted in inaccurate cytokine concentrations in the TA samples. All published studies using cytokine concentrations from respiratory samples were centrifuged to remove cellular material prior to freezing. To test the validity of the cytokine results measured in TA samples a comparison was made of the concentrations of IL-6 and IL-8 in samples taken from the same infant within the same 24-hour period. This is shown below in Figure 3-39. Good correlation was seen between the IL-8 and IL-6 cytokines. The Bland-Altman plot suggests that IL-6 measurements were less concordant at higher concentrations of cytokines. Based on these results it was felt the cytokine concentrations from TA samples were reliable enough to use.

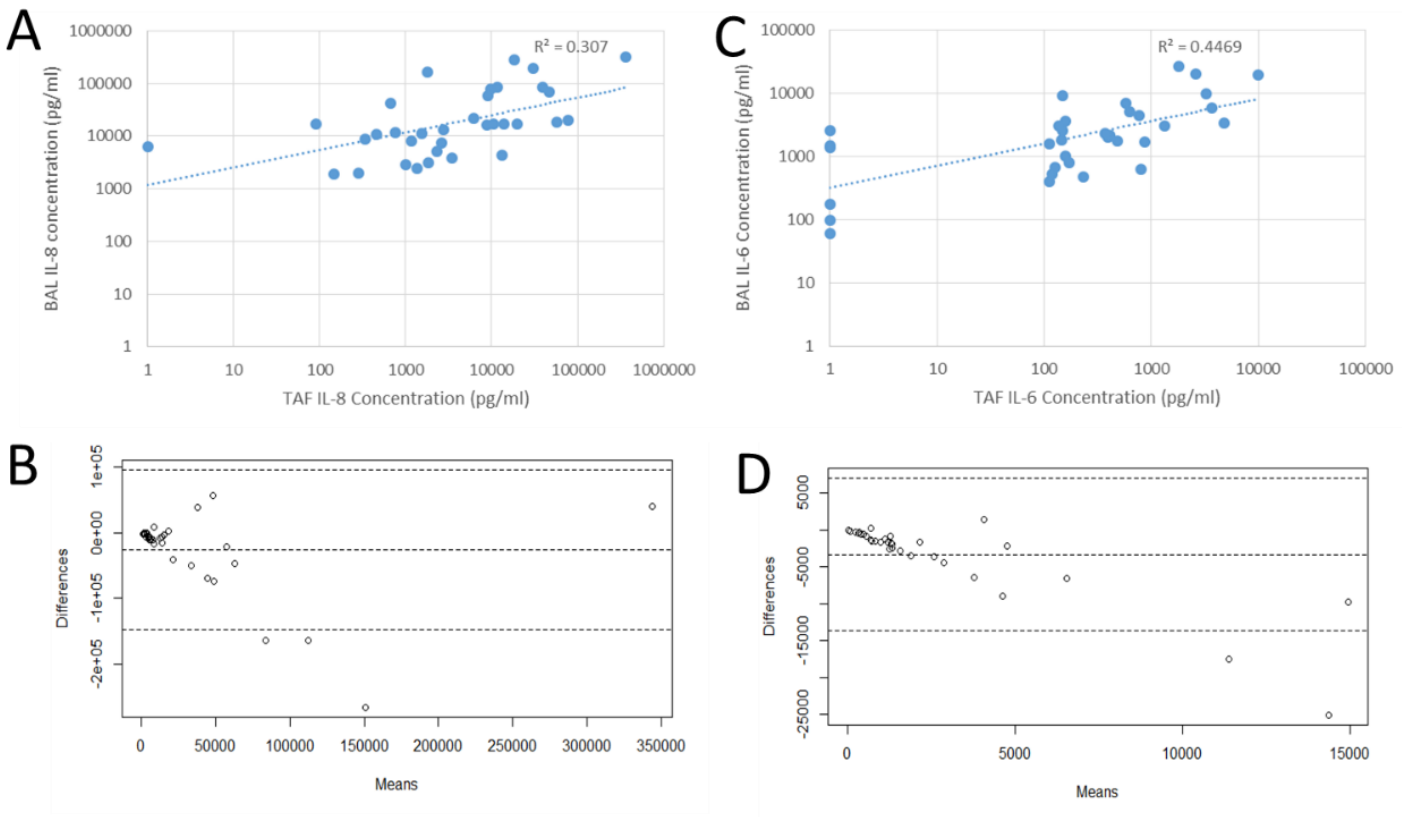


Figure 3-39: Comparing IL-6 and IL-8 concentrations in BAL and TA samples takenwithin 24hours of each other from ventilated preterm infants A= Correlation of IL-8 measurements. B= Bland-Altman plot of IL-8 measurements. C= Correlation of IL-6 measurements, D= Bland-Altman plot of IL-8 measurements.

3.32.3 Sequencing data

The is the first time 16S rRNA gene sequencing has been used to attempt to study bacterial communities in the lungs using BAL samples from preterm infants. The majority of both the BAL and TA samples did not contain amplifiable 16S rRNA gene in the samples. PCR inhibition was not responsible for this issue, thought to be due to the low biomass and low bacterial load within the samples. The TA samples contained higher concentration of amplified bacterial DNA than the BAL samples, however the bacterial load estimates suggested a similar number of organisms were present in each sample (Figure 3-27).

The low bacterial load and difficulty amplifying bacterial DNA resulted in very low concentrations of amplified DNA being pooled for sequencing with BAL samples. The quality control scores and cluster density on the sequencing run were suboptimal. However, despite this adequate numbers of reads were achieved to provide a coverage value of >0.99 in 34 samples when rarefying to 1225 reads per sample. The number of ambiguous bases permitted in the Mothur pipeline for the TA samples was 12 (to exclude 5% of reads). Therefore, the quality of many reads was suboptimal. This was probably because of the low-quality control scores achieved on the sequencing run. However, despite allowing extra ambiguous bases through the processing pipeline the samples have formed OTUs satisfactorily.

The TA samples amplified the bacterial 16S rRNA gene to a greater concentration and had better quality control scores and cluster density scores during the sequencing run. All reads containing ambiguous bases during data processing were excluded. One negative control sample had a significant number of reads but due to this analysis of the samples was performed and one sample excluded due to similarity with the negative control sample.

Despite these issues, the sequencing results do not appear to be the result of contamination or mis-amplification. The same genera dominated the TA and BAL sample datasets when performed on different runs. The same phylum and genera were found in previous studies of the airway microbiota in preterm infants, with a similar pattern of most sample dominated by a single genus (Lal et al. 2016; Lohmann et al. 2014). The same dominant organism was found in serial samples from the same infant at times and infants cluster individually on the NMDS plots for both samples (Figure 3-13 and Figure 3-33). An increased proportion of Acinetobacter was detected at the site with an outbreak of Acinetobacter during the study. The association between peaks of IL-6 and IL-8 concentration and the detection of bacterial DNA showed very similar patterns measured by two different methodologies. Also, the effect of mode of delivery showed a pattern of increased *Staphylococcus* in caesarean section deliveries and increased Proteobacteria in vaginal deliveries previously documented across many body sites in term infants (Dominguez-Bello et al. 2010). Each of the points above suggest that the

results of both TA and BAL sequencing is genuine amplification of bacterial DNA from within the collected samples.

One important observation of the study was the high rate of samples with no amplification of the 16S rRNA gene. The results suggest that antibiotics suppressed bacterial growth within the lungs to a level below the threshold of detection of the methodology. Other studies have also demonstrated a high proportion of samples negative for bacterial DNA, including one study looking just for predominant bacteria within neonatal BAL samples (78/164 contained 16S rRNA gene (47.6%)) (Beeton et al. 2011).

The finding of high rates of sterile samples may represent the fact that the airways of preterm infants are sterile in the absence of infection. If colonisation of the airways occurs postnatally then a low bacterial load may be expected in the first days and weeks as colonisation occurs over time. Other reasons for an undetectably low bacterial load would include the small biomass obtained from neonatal BAL and TA samples.

Alternatively, the methodology may not have the sensitivity to detect low levels of bacterial colonisation. The choice of automated DNA extraction using the Maxwell 16 instrument combined with a bead beating step was based on experiments using pure cultures of bacteria and tests on saliva samples from adults. Both have much larger bacterial loads than the neonatal respiratory samples and laboratory strains of bacteria may behave differently to bacteria found in clinical samples. It maybe that while the Maxwell instrument performed equally well as manual techniques with the higher bacterial load samples, it was less efficient with lower bacterial loads and an alternative methodology would have yielded higher concentrations of DNA. The widespread use of antibiotics in the first month of life in preterm infants has also been shown to have suppressed bacterial detection within the lungs.

Data from BAL and TA samples was rarefied prior to statistical analysis and graphical representation. Rarefication is a controversial area with some arguing that the discarding of sequence data is inadmissible (P. McMurdie and Holmes 2014). The alternatives to rarefication are to use proportions of samples or to model uncertainty using Poisson-Gamma or Binomial-Beta models and normalization performed using variance- stabilizing transformations (P. McMurdie and Holmes 2014). Comparing samples with vastly different read numbers without some normalisation procedure is problematic. Rarefication remains the most widely used method of normalising sequence counts within 16S rRNA gene sequencing literature despite the previously stated concerns. Rarefying is recommended for datasets with a large difference between the largest and smallest libraries which was the case for the BAL dataset (Weiss et al. 2017).

The majority of BAL and TA samples contained a dominant OTU. 16S rRNA gene sequencing may overestimates the predominance of a dominant organism due to the exponential amplification of the target DNA. In a perfectly efficient PCR reaction, each copy of the gene will be amplified in each cycle, but in reality not each copy is amplified in each cycle. When a single organism dominates, the DNA from this organism is more likely to be amplified at each cycle resulting in a potential overestimation of the dominance of the OTU. My samples would be prone to this effect and the results need to be interpreted being aware of this potential source of bias, although the dominance was very high with different organisms amplified so thus unlikely.

3.32.4 Sequencing results

Both the TA and BAL datasets showed many similarities. The majority of both types of samples showed a dominant OTU with *Staphylococcus*, *Acinetobacter* and *Enterobacteriacae_ unclassified* the most commonly occurring genera in each sample dataset. Both sample types had episodic peaks of the proinflammatory cytokines IL-6 and IL-8 associated with peaks of bacterial load. Serial samples from individual infants clustered separately on NMDS plots for both types of samples. My data suggest that 16S rRNA sequencing of BAL and TA sampling has identified infective organisms thriving in the lungs and airways of intubated premature infants resulting in an inflammatory response within the lungs/airways of preterm infants.

On days 1-3 of life, samples were rarely positive for bacterial DNA. The alpha diversity data for BAL samples collected in the first 3 days of life were the most diverse. This suggests that the preterm infant is exposed to many bacterial species in the first few days following birth, but within a few days a single organism has become established as a dominant or infecting species, resulting in reduced diversity. Lohmann et al, utilising TA samples, witnessed the same pattern of colonisation with higher diversity in the first three days of life than in later days (Lohmann et al. 2014).

This study has the weakness of no control group of “normal healthy” infants. Attempts were made to recruit ventilated term control infants for the BAL study, however only three were ever recruited and all six BAL samples failed to amplify the bacterial 16S rRNA gene. It is possible that in the lungs of healthy term infants who are not ventilated that an inflammatory response is generated in response to the colonisation by microorganisms. Given the difficulties of sampling the lungs of healthy infants not requiring a ventilator it is currently impossible to determine if the features of colonisation/infection seen in this study are witnessed in healthy term infants. Early colonisation of the skin is associated with causing the rash of erythema toxicum neonatorum when *Staphylococci* species enter skin tissue via hair follicles and cause a pro-inflammatory reaction (Marchini et al. 2005). It is feasible that a similar process may occur following birth in the lungs.

One aim of this work was understand the development of the "normal" microbiome in ventilated preterm infants. The results indicate that dominant organisms associated with inflammation prevent the development of a "normal" microbiome in these infants. Targetting these organisms with antibiotics may reduce lung inflammation and reduce the development of CLD.

In this study the V3-V4 region of the bacterial 16S rRNA gene was sequenced to identify bacterial organisms. This led to adequate sequencing depth to analyse at genus level but sequencing depth was inadequate to identify bacterial species. One clinical implication of this work would be to replace culture based microbiological testing with molecular based techniques that sequence DNA to identify organisms. My work has shown that this technique is able to identify the presence of a dominant genus, but increased depth of sequencing would be required for a clinically useful result. A clinician would need to know if the organism detected is a strongly virulent organism such as *Staphylococcus aureus* or a less virulent commensal organism such as *Staphylococcus epidermidis*. The depth of sequencing I achieved is unable to distinguish between these species.

3.32.5 Bacterial load

The bacterial load within samples showed a consistent pattern of peaking at day 8-14 and then falling in both TA and BAL samples. The influences of several factors may explain this phenomenon. As already discussed, an infective process has been detected in the lung of preterm infants. Bacterial load peaking during the second week of life may reflect the time at which respiratory infection is most likely to occur in preterm infants. All infants in the study were started on empirical antibiotics at birth to treat or prevent early onset sepsis. Antibiotics are more likely to be stopped by the second week of life allowing bacterial proliferation. Colonisation of the respiratory tract may be delayed in preterm infants explaining the lower bacterial load initially. Overall the influence of bacterial load within the lungs and airways of preterm infants is unknown. High bacterial loads might be assumed to be evidence of infection, but bacterial load changes are not seen during infective exacerbations in cystic fibrosis (Twomey et al. 2013). The immune responses of preterm infants to bacterial colonisation of the respiratory tract is also poorly understood but neonatal white blood cells are thought to have reduced phagocytosis ability compared to adults (Ballinger, Peters-Golden, and Moore 2011; Lambert and Culley 2017). The fluctuations in bacterial load may reflect poorly regulated immunes responses within the lungs and airways responding to early colonisation. Individual infants had individual patterns of bacterial load. Therefore, when combining the data from all the infants the individual patterns were no seen.

3.32.6 Effect of delivery mode on respiratory colonisation

Respiratory colonisation was significantly different at both phylum and genera level in TA samples, but not in the BAL samples. The results of the TA samples are consistent with the findings of previous literature in term infants suggesting that vaginal delivery is associated with a higher proportion of Proteobacteria, with caesarean section delivered infants having great colonisation with Firmicutes, specifically of the *Staphylococci* genus (Costello et al. 2009). This finding has also been witnessed across other body sites including in stool samples of new-born infants (Rutayisire et al. 2016; Costello et al. 2009). This effect is likely to be due to colonisation by the bacteria that the infant is exposed to during or soon after delivery, as Proteobacteria colonise the birth canal and Firmicutes predominate on adult skin. This effect has not been shown before in the airways of preterm infants. The fact that the BAL samples did not show this effect may be because the BAL samples are taken from deeper in the respiratory tract and the effect of early life exposure may not penetrate so deeply into the lung, however it is more likely due to an insufficient number of samples to draw conclusions. Only 7 samples were from infants who had been born by caesarean section making comparisons difficult.

3.32.7 Differences between recruitment centres

Contamination issues in the samples from Newcastle made it impossible to compare the colonisation of the lower airways between three recruitment centres. Interpretation of the samples from Newcastle compared to those from Cardiff and Bristol would have been complicated by the differences in sample collection, sample storage and DNA extraction. A clear difference was found between the colonisation patterns of the respiratory tract between the two recruitment sites for which data was obtained. The geographical distance between the two units is around 35 kilometres so climate and population demographic differences are unlikely to explain the dissimilarity in airway microbiota identified. Both sites recruited throughout all seasons of the year, excluding seasonal variation as explaining this difference. The recruiting centres are part of different neonatal networks and different NHS trusts/health boards resulting in policy and procedural difference that may affect clinical care. Notably the choice of first line antibiotics for early and late onset sepsis was the same between the two sites, so while clinical practice may differ between the units first line antibiotic prescribing is standardised. The sensitivity analysis showed that differences remained in the bacterial communities colonising the airways between the two recruitment centres when infants colonised with *Acinetobacter baumannii* were excluded (Figure 3-18 and Figure 3-19). The differences between the units suggest that the environment and the staff in a neonatal unit can influence respiratory microbiota colonisation. This is an important clinical implication of this study. This study is unable to identify what the critical factors are in promoting optimum respiratory microbiota colonisation, but it should cause clinicians to think about the influence

of their actions on respiratory microbiota colonisation when managing preterm infants. Exposure to different staff and environmental exposures on the neonatal unit are likely to be significant factors which may influence respiratory colonisation. Previous studies analysing differences in microbiota between difference recruitment centres have focused on centres in different continents and climates, specifically to looks for effects of these variables (Stressmann et al. 2011). By studying two centres in close geographical proximity the effect of different clinical practice has become apparent.

3.32.8 Average cytokine concentration

The average IL-6 and IL-8 concentration observed over time within the supernatant of BAL samples shows very similar results to those demonstrated 20 years ago in similar samples from preterm infants (S. Kotecha, Wilson, et al. 1996; S. Kotecha et al. 1995). This would indicate that the changes in ventilation strategies aiming for lung protection introduced during this time have made little difference in the levels of inflammation in the lungs of intubated preterm infants at risk of CLD.

3.32.9 Strengths and weaknesses

This study is the first to use 16S rRNA gene sequencing to analyse lung colonisation using BAL samples from preterm infants. Serial sampling from infants at risk of CLD combined with measuring the concentration of proinflammatory cytokines has shown important findings relating bacterial colonisation of the airways with a pro-inflammatory response which may contribute to developing CLD.

One potential weakness of this study is the large number of samples from which the 16S rRNA gene could not be amplified, however this may be accurately reflecting low bacterial loads early in life. Using bacterial DNA to identify organisms' risks identifying dead or non-viable bacteria. Bacterial DNA from organisms not colonising the respiratory tract, for example aspirated organisms, would be detected by the methodology of this is study. Studies using bacterial RNA to identify organisms would overcome this issue as RNA does not have the potential to remain detectable after the organism is non-viable.

The lack of a control group of healthy infants has already been discussed as a potential weakness. The contamination during DNA extraction of the samples from Newcastle made comparing colonisation across three recruitment sites impossible. This contamination issue highlights the difficulties of analysing low biomass samples and the importance negative controls. One DNA extraction control from the TA samples showed a high number of reads. After further evaluating these samples for potential contamination it was clear that only 1 sample was at risk of similar contamination, so this sample was eliminated from further analysis.

The high rate of infants who went on to develop CLD precluded comparing early respiratory colonisation in infants who developed CLD and those that did not, however it did allow serial sampling from infants at risk of CLD.

3.33 Conclusion

The aims of this chapter were to present evidence of patterns bacterial colonisation from serial sampling of the airways and lungs from preterm infants, to measure bacterial load and relate this to the inflammatory profiles, to compare the lung microbiota between infants to did and did not develop CLD, to analyse the effect of antibiotics on respiratory colonisation and to compare airways colonisation between difference recruitment centres. I have successfully demonstrated the serial sampling and analysis of the bacterial colonisation of the lower respiratory tract in ventilate preterm infants at risk of CLD. The colonisation of the lower airways of preterm infants was not detectable for most BAL and TA samples in the absence of a dominant organisms with evidence of an infective process occurring in the lungs. These episodes of possible infection prevented the detection of a "normal" colonisation of the respiratory tract.

I have measured the bacterial load and shown that the concentration of proinflammatory cytokines IL-6 and IL-8 were significantly higher in samples with detectable bacterial DNA. Peaks and troughs of bacterial load were associated with parallel increases and decreases in IL-6 and IL-8 concentration. Inflammatory changes within the lungs of preterm infants increase the risk of CLD. Targeting the dominant organisms which may be causing the increase pro-inflammatory cytokines may reduce CLD.

Antibiotics reduce the bacterial carriage within the lungs. With most preterm infants receiving multiple courses of antibiotics during the first month of life this impacts on early respiratory tract colonisation within the lung and contributed to the sterility of many of the TA and BAL samples collected.

Bacterial colonisation of the lower airways and lungs in ventilated preterm infants is characterised by the presence of dominant OTUs in most samples. Even in infants not on antibiotics who are not suspected to have an infection evidence of an inflammatory response to the presence of bacteria has been detected. Therefore, preterm lung infection is probably more common than clinically detected. Dominant OTUs are consistently identified within preterm respiratory samples and this may be a normal feature of colonisation of the respiratory tract. The difficulty in obtaining samples from the lower respiratory tract of healthy, unventilated, infant born at term limits the interpretation of this. The rise in cytokines associated with lung colonisation may also be normal however this is controversial. The absence of data from healthy term infants make it difficult to prove that the evidence of colonisation detected in this

study is an example of dysbiosis. The data is suggestive of a dysbiotic colonisation pattern within the airways and lungs of intubated preterm infants, but more evidence is needed of the bacterial colonisation causing harm.

Contamination issues prevented samples from Newcastle from being included in the analysis. However, evidence for the role of the environmental exposures on the neonatal unit has been shown due to the differences identified between the Cardiff and Bristol sites. Evidence also exists for the important role delivery mode, antibiotic treatment and general neonatal management in neonatal respiratory colonisation. With the long term impacts of early microbiological exposures known to have over longer term respiratory health (Teo et al. 2015; Biesbroek, Tsivtsivadze, et al. 2014) neonatologists need to be mindful of the impact of interventions which may affect respiratory health beyond the infant's stay on the neonatal unit.

Eventually molecular based techniques are likely to replace traditional bacterial cultures for pathogen identification. This study has shown the importance of identification of lung pathogens in new-born infants highlighting the infective process occurring in the lungs of preterm infants. In this study identification of bacterial organisms to genus level was possible. Identification of organisms at species level, with advanced sequencing techniques able to detect resistance patterns from the organism's genome would be required before culture-based work can be halted. Molecular tests would also need to be confident of the viability of detected organisms before such tests replace culture-based pathogen identification.

Chapter 4: Bacterial Colonisation of the Upper Airways in Preterm Infants

4 Bacterial Colonisation of the Upper Airways in Preterm Infants

4.1 Introduction

The nasopharynx is anatomically much closer to the skin and external environment than the lower airways and lungs. Deposition of microparticles in the nose prevent such material from entering the lungs. The upper airway colonisation is more likely to be subject to environmental influences than the lungs and lower airways. The ease of sampling from the nasal passages, nasopharynx and oral cavity of infants has resulted in these anatomical niches being studied in far greater depth than the lower airways with many studies focusing on the effect of the upper respiratory microbiota on respiratory tract infections in infancy (Perez et al. 2017; Teo et al. 2015; Hilty et al. 2012).

Studies of term infants have found evidence of nasopharyngeal colonisation within 5 minutes of delivery (Dominguez-Bello et al. 2010). At this early stage delivery mode was predictive of bacterial community structure, as with other anatomical sites studied immediately after birth (Dominguez-Bello et al. 2010). A subtle effect of delivery mode was seen persisting until 6 months after delivery by caesarean section with lower abundance of *Corynebacterium* and *Dolosigranulum*, both potentially beneficial commensals (Bosch et al. 2016). A longitudinal study using nasal swabs from term infants during the first year of life demonstrated increasing bacterial density but decreasing diversity over time. No effect of delivery mode was evaluated but an effect of seasonal variation was noted (Mika et al. 2015). A further longitudinal study using nasopharyngeal swabs from term infants over two years showed that adult-like colonisation patterns were not established within this timeframe. Most samples were dominated by a single genus with clear succession patterns identified over time (Biesbroek, Tsivtsivadze, et al. 2014).

Within the first week of life, studies have found wide variation between individuals regarding the bacteria identified from the nasopharynx (Bosch et al. 2016; Biesbroek, Tsivtsivadze, et al. 2014). A dominant OTU/genus is frequently identified from nasopharyngeal samples in infants. *Streptococcus* and *Staphylococcus* species predominate in caesarean section-born infants and Gram negative gut organisms predominate in vaginally delivered infants (Dominguez-Bello et al. 2010; Bosch et al. 2016). There are no published studies of early nasal or nasopharyngeal colonisation of preterm infants during the stay on the neonatal unit. One study showed difference between ex-preterm infants and term born infants when NPA sampling was undertaken at 6 months of age. Preterm infants had greater heterogenicity between samples and increased Proteobacteria and decreased Firmicutes compared to term infants (Perez et al. 2017).

4.2 Aims

1. To analyse serial samples from the upper airways of preterm infants and a term control group for the presence and relative abundance of bacteria and identify the pattern of bacterial colonisation

2. To measure bacterial load within samples from the upper airways of preterm infants and analyse changes over time

3. Analyse the effect of delivery mode, antibiotics and probiotics on upper respiratory tract colonisation in preterm infants

4. To compare upper airway colonisation in preterm infants between two different recruitment centres

4.3 Recruitment and demographics

NPA samples were taken from preterm and term infants from two recruitment centres (UHW and NBT). In addition, a cohort of well infants from the postnatal ward from UHW were recruited as a term control group. NPA samples were taken at the time of routine "cares" from infants admitted to the neonatal unit, at the same time as BAL/TA samples were collected for intubated infants. Serial sampling was performed with NPA samples taken daily during the first week of life and then twice weekly until 28 days or discharge from a recruiting centre, whichever was first. No serial sampling was possible on infants from the postnatal ward due to early discharge from hospital.

Table 4-1 summarises the demographics of all recruited infants. Statistical analysis comparing the preterm infants recruited between the two sites showed no significant differences between the two units in terms of baseline characteristics of the infants. There was a non-significant difference in the birth-weight between the centres with lower birth-weight infants recruited from NBT. Mothers of infants recruited from NBT were on average older than from UHW. Outcome measures showed that NBT had a greater median days of supplementary oxygen use and, therefore, a higher rate of infants with CLD at 36 weeks' gestation. This is probably explained by the differences in birth weight in the two groups. The differences in postnatal corticosteroid use and home oxygen use probably reflect the difference in patient admissions between the two units.

Table 4-1: Demographics table of infants included in the NPA study

	NBT (Preterm Infants)	UHW (Preterm Infants)	Term Controls	P value (comparing NBT v UHW preterm infants)
Preterm Infants recruited	35	20	8	-
Number of NPA Samples	390	149	8	-
Male, n(%)	24 (68.6)	12 (60)	7 (88)	0.73
Gestation, Med (IQR)	25.86 (24.7 - 26.7)	26.78 (25.0-29.5)	39.7 (39.1-40.2)	0.15
BW, Med (IQR)	746 (674 - 890)	835 (685 - 1187.5)	3560 (3338-3752)	0.07
Maternal Age, Mean (SD)	30.71 (5.7)	27.35 (5.3)	32.6 (4.4)	0.04
Infant from a multiple pregnancy, n(%)	11 (31.4)	6 (30)	0 (0)	1
CS delivery, n(%)	16 (45.7)	10 (50)	1 (13)	0.82
Apgar at 1 min, Med (IQR)	6 (5-6)	5 (3-5)	9 (9-9)	0.06
Apgar at 5 min, Med (IQR)	8 (7-9)	7 (6-8)	10 (10-10)	0.08
Vent Days, Mean (SD)	20.83(18.98)	34.15 (49.86)	0 (0)	0.26
Non Invasive Respiratory Support Days, Mean (SD)	50.66(26.54)	44.15 (36.92)	0 (0)	0.49
Low flow O2 days, Mean (SD)	18.2(15.08)	9.0 (11.63)	0 (0)	0.01
Length of Hospital stay (days), Mean(SD)	97.83 (35.41)	103.5 (60.93)	2.3 (1.0)	0.71
O2 at 28 days, n(%)	34 (97.1)	16 (80)	0 (0)	0.10
O2 at 36 weeks, n(%)	33 (94.3)	13 (65)	NA	0.01
Complete course antenatal steroids, n(%)	26 (74.3)	11 (55)	0 (0)	0.18
Survival to hospital discharge, n(%)	30 (85.7)	3 (85%)	8 (100)	1
PN steroids for vent dependence, n(%)	2 (5.7)	9 (45)	0 (0)	0.002
Home O2, n(%)	17 (48.6)	3 (15)	0 (0)	0.03
EBM Ever, n(%)	35 (100)	20 (100)	8 (100)	1
Home exclusive breast milk, n(%)	11 (31.4)	6 (30)	8 (100)	1

Shapiro-Wilk test used to determine normal distribution of continuous variables. Normally distributed variables given as mean with standard deviation, p values calculated using t-test. Variables with significant Shapiro-Wilk test displayed as median with interquartile range, p values calculate with Wilcoxon signed rank test. Binary variables p values calculated with chi-squared test.

4.4 Bacterial load within nasopharyngeal aspirate samples

The bacteria load within NPA samples was calculated using the qPCR procedure detailed in Section 2.9 on DNA extracted from the cell pellet. Figure 4-1 shows the average bacterial load in all the NPA samples from preterm (both UHW and NBT recruitment sites).

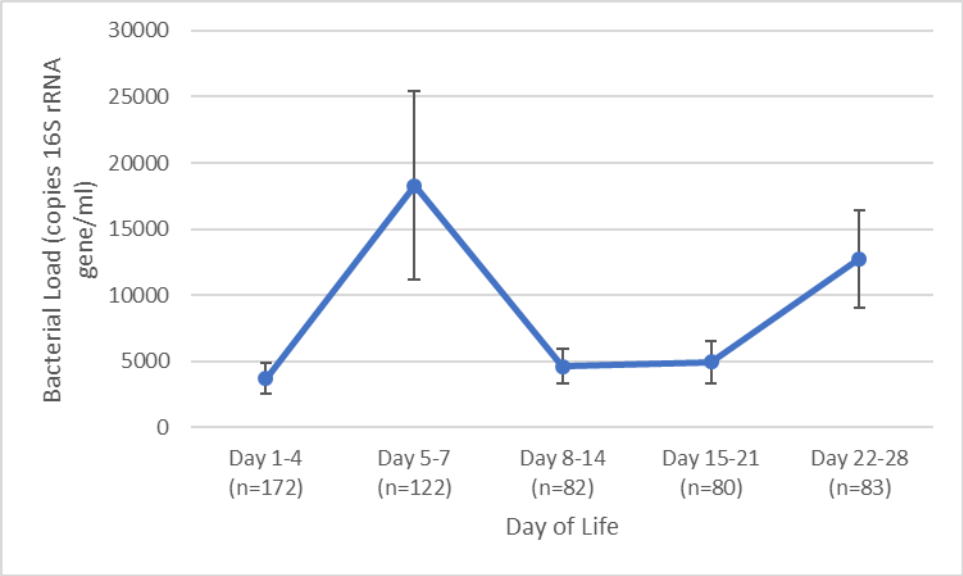


Figure 4-1: Average bacterial load within NPA samples from preterm infants by day of life. Error bars display standard error.

The results from Figure 4-1 show that bacterial load within NPA samples does not increase over time in a linear way. The highest average bacterial load detectable in NPA samples was on days 5-7 of life, with bacterial load subsequently falling to levels similar to those witnessed on days 1-4 of life, before rising again after day 22 of life.

4.5 Sequencing data

Sample were amplified for sequencing in batches of 24. Samples had already undergone qPCR analysis targeting the 16S rRNA gene. The samples with the highest bacterial load were amplified for sequencing first. Amplification of bacterial DNA for sequencing was performed on NPA samples with sequentially lower bacterial loads until no samples successfully amplified for sequencing on two consecutive runs. Of the 564 samples 265 samples were attempted to be amplified for sequencing. The remaining samples with a lower or undetectable bacterial load on qPCR were assumed to have insufficient bacterial DNA for sequencing.

Sequencing of the bacterial 16S rRNA gene was performed on DNA from samples that amplified successfully as per the methodology in Section 2.10. I performed the sample preparation and sequencing supervised by Dr D Alber in the laboratory of Prof N Klein at the Institute of Child Health, UCL. Of the 264 NPA samples tested, 126 samples amplified successfully. Figure 4-2 demonstrates a difference in bacterial load between those samples that amplified successfully and those that did not amplify successfully showing that those samples

with a significantly higher bacterial load were more likely to be successfully amplified for sequencing. The threshold for successful amplification was set at 0.3ng/µL of DNA post amplification and purification.

During bioinformatic processing of the sequencing data, reads with any ambiguous bases were excluded. Of the 126 samples which were pooled, the read numbers of from samples following removal of the ambiguous bases, chimeric sequences and mis-reads ranged from 5,952 – 222,539 with an average of 60,295 per sample. Five extraction controls and 1 no template control were amplified alongside the samples. The number of reads within these controls ranged from 8 – 580 with an average of 171 reads.

Rarefication of samples to 1000 reads per sample was performed prior to further analysis. Rarefication to 1000 reads per sample maintained coverage >0.99 for every sample.

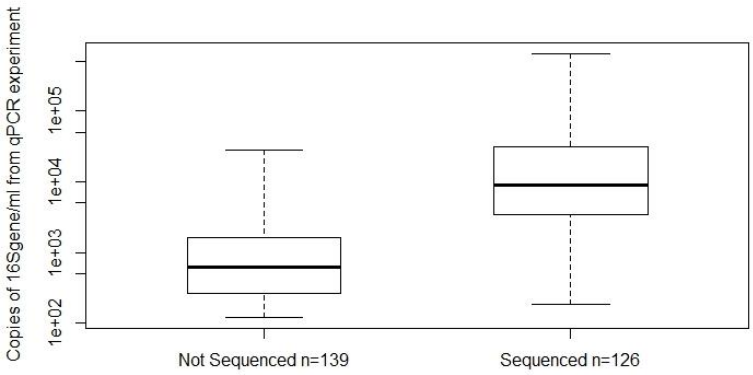


Figure 4-2: Boxplot of copy number of 16S rRNA gene concentration against NPA samples that amplified adequately for sequencing.
Only samples when amplification for sequencing was attempted have been included. P<0.001.

4.6 Samples unable to undergo 16S rRNA gene sequence analysis

A large proportion (77.6%) of the NPA samples were unable to be sequenced due to low bacterial load, similar to BAL and TA samples. No samples from the term control group were able to be sequenced due to low bacterial load so further analysis was only possible with regard to the preterm infants recruited. As with BAL and TA samples, antibiotics use was significantly associated with NPA samples having an inadequate bacterial load to successfully obtain sequence data. NPA samples taken when the infant was not on antibiotics were significantly more likely to be sequenced successfully for the bacterial 16S rRNA, odds ratio = 3.23, 95% CI 2.14-4.87, p<0.0001. Samples taken at an older day of life were more likely to be successfully sequenced. See Figure 4-3 below.

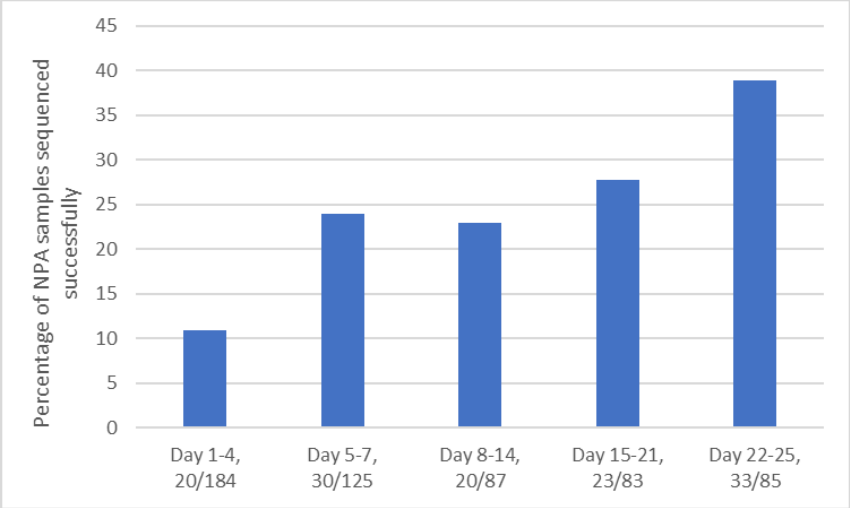


Figure 4-3: Percentage of all NPA samples taken that had successful bacterial 16S rRNA gene amplification and sequencing. Chi-Squared p<0.001.

4.7 Relative abundance of organisms within nasopharyngeal aspirate samples

Showing similarity to the abundance data previously presented for TA and BAL samples, the majority of NPA samples contained a single dominant OTU. 122/126 (96.8%) samples contained a dominant OTU (>50% of reads from a single OTU). Extreme dominance of samples (>90% of reads from a single OTU) was witnessed in 66/126 (52.4%) NPA samples.

At a phylum level, Proteobacteria and Firmicutes dominated most NPA samples with Bacteroidetes, Actinobacteria and Tenericutes present in fewer samples (see Figure 4-4).

The most abundant genus detected was *Staphylococcus* (50.8% of reads within the dataset before rarefication). *Staphylococcus* was by far the most abundant genus within the Firmicutes phylum (88.3% of Firmicutes reads). In contrast, the Proteobacteria phylum was much more evenly distributed between many different genera. The most abundant genera within Proteobacteria was *Escherichia/Shigella* making up 32.7% of Proteobacteria reads.

Identification of bacteria beyond genus level to allow species identification was not possible.

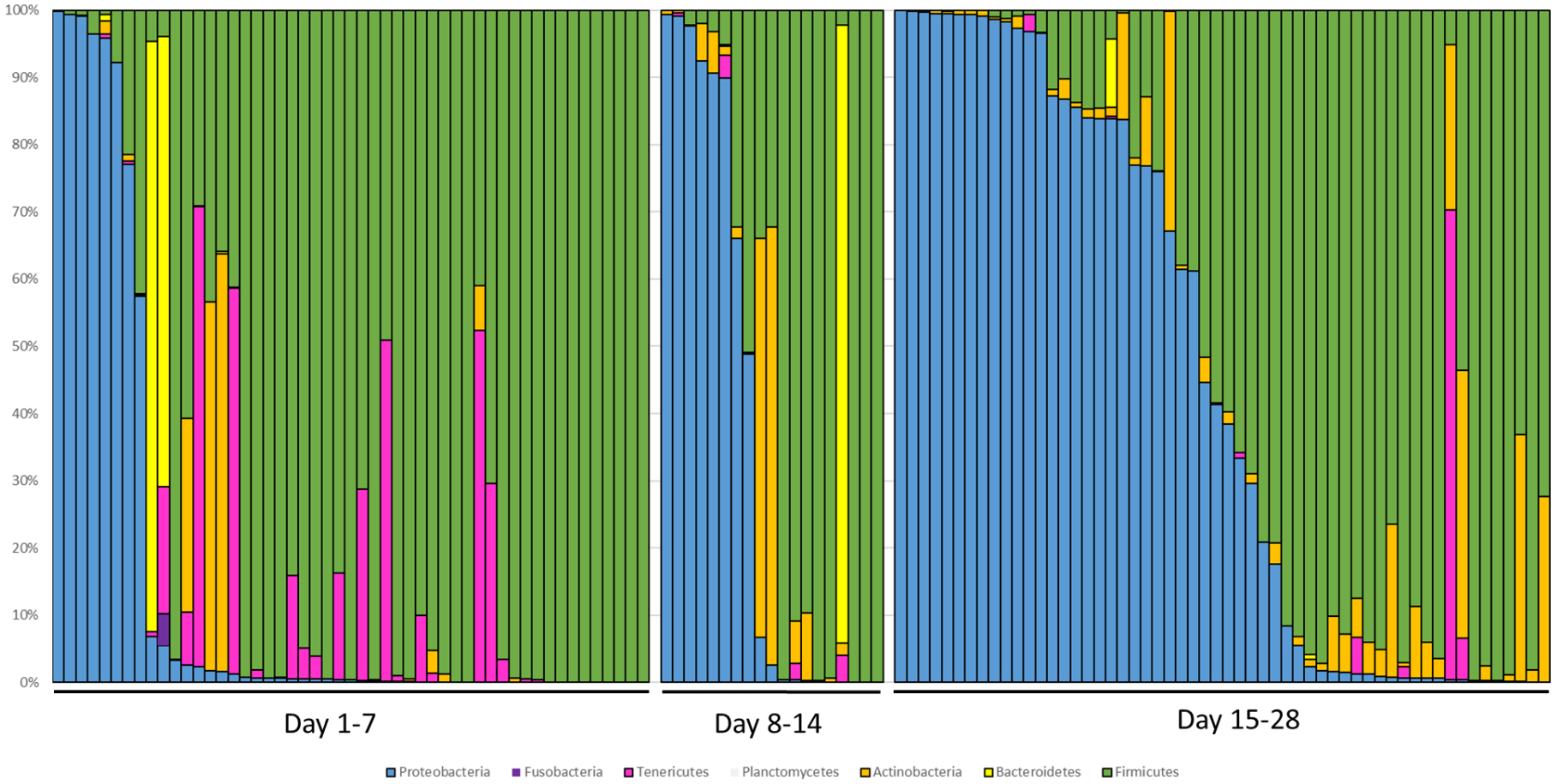


Figure 4-4: Relative abundance of organisms within NPA samples at phylum level.
Each bar represents 1 NPA sample. Samples ordered by proportion of Proteobacteria at each timepoint.

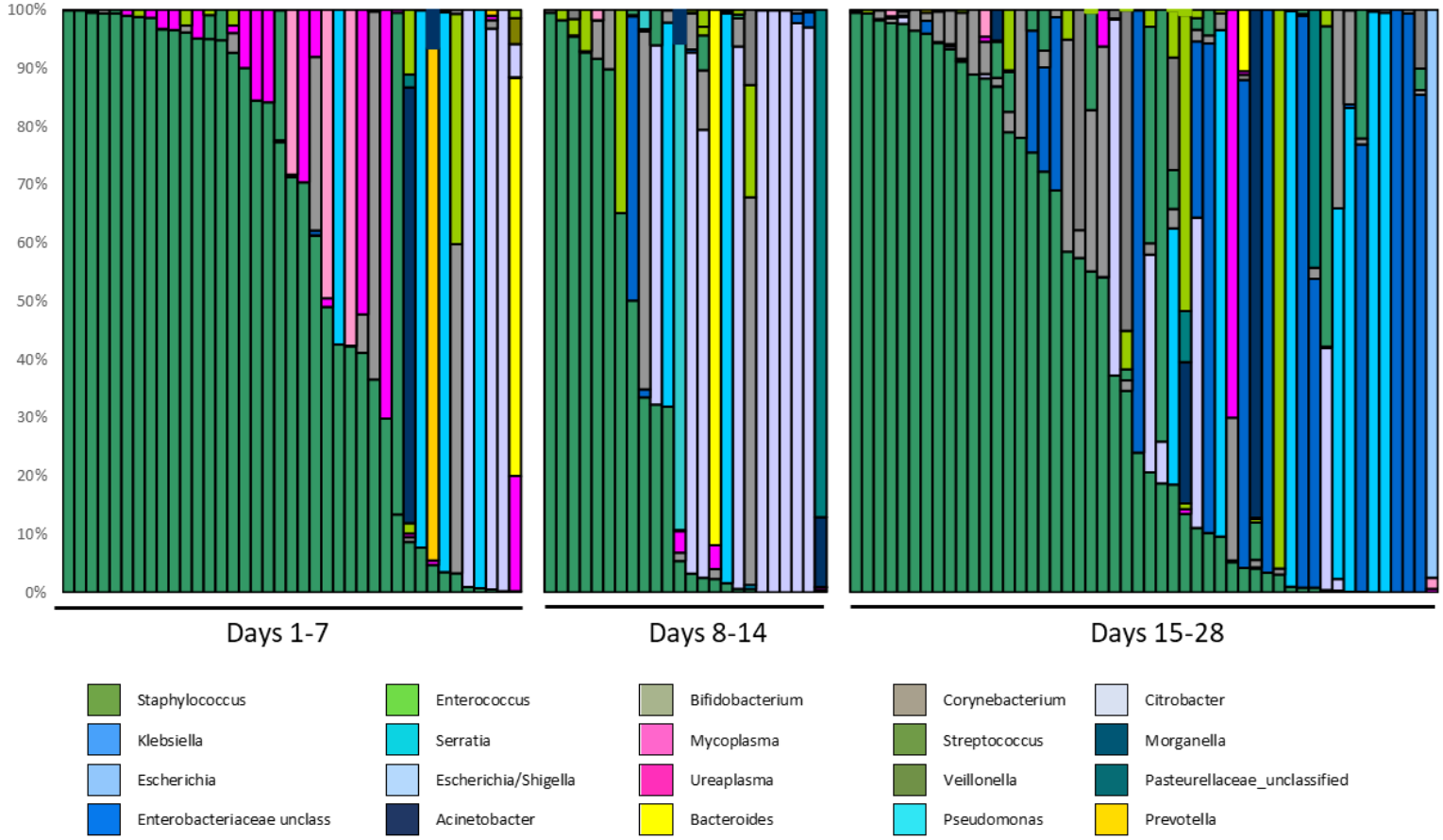


Figure 4-5: Relative abundance of genera within NPA samples.
Top 20 most abundant genera within dataset shown. Each bar represents a single NPA sample

4.8 Changes over time in relative abundance in nasopharyngeal aspirate samples

Figure 4-4 above suggests an increase in average proportion of Proteobacteria and a decrease in Firmicutes over time. Figure 4-5 suggests the decreasing trend in Firmicutes is due to a falling proportion of the *Staphylococcus* genus over time. By increasing the number of time points analysed, it was possible to identify this trend more precisely and decrease the impact of multiple samples obtained from the same infant in the same timepoint. Figure 4-6 below shows the average abundance of each phyla in samples grouped across five timepoints. This demonstrates a clear increase in the average proportion of Proteobacteria over time and a decrease in Firmicutes over time.

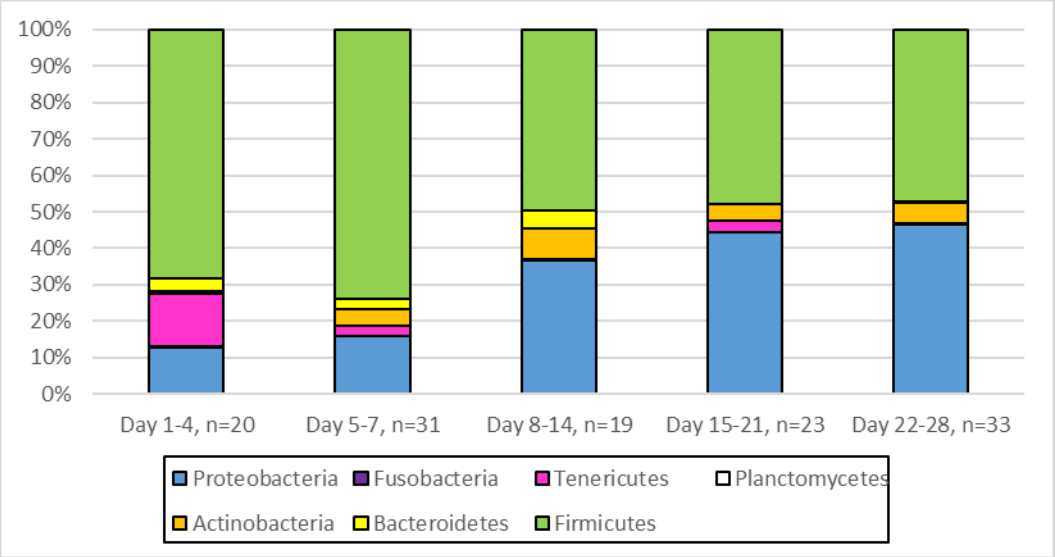


Figure 4-6: Average abundance of individual phyla within NPA samples over time

Using Kruskal-Wallis test the p-value for significance of increasing Proteobacteria between the 5 groups (with Bonferroni correction for multiple testing) was $p=8.75 \times 10^{-4}$. Post hoc testing shows that the samples in groups day 1-4 and day 5-7 are both significantly different to those in days 22-28.

The same tests applied to Firmicutes show corrected p=0.024. Post hoc testing failed to show significant differences between individual groups.

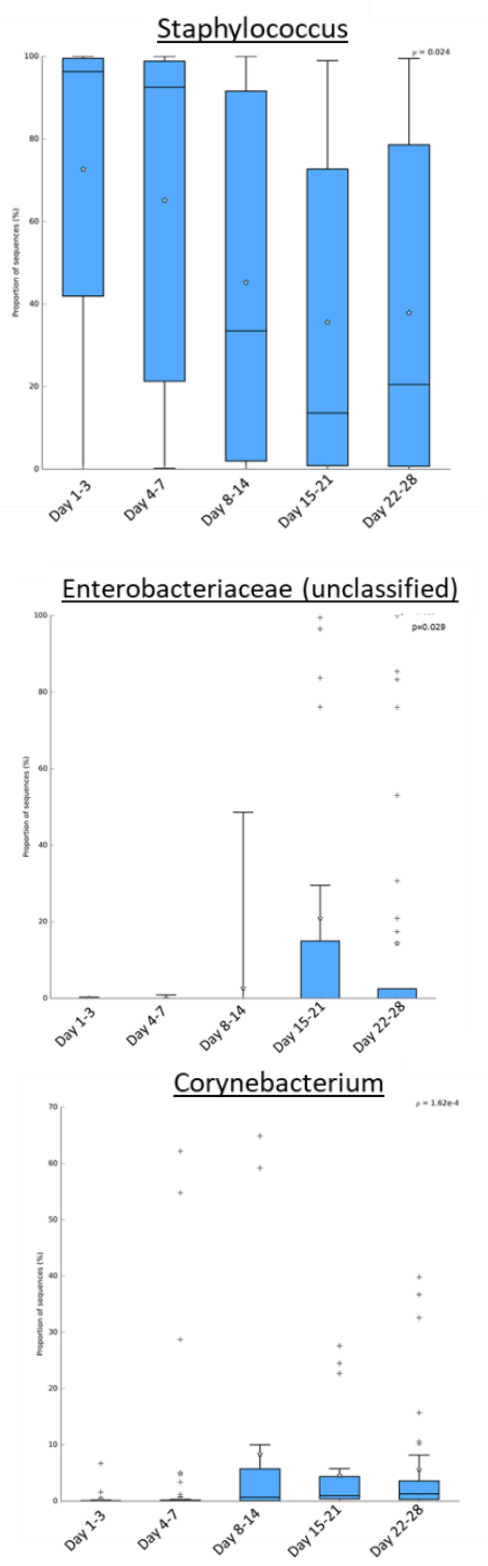


Figure 4-7: Changes in proportion of Staphylococcus, Enterobacteriaceae (unclassified) and Corynebacterium over time in NPA samples from preterm infants. (Staphylococcus p=0.024, Enterobacteriaceae (unclassified) p= 0.029, Corynebacterium p=1.62x10^{-4})

At a genus level, only three genera showed statistically significant changes over time. Box plots showing the changes in relative abundance over time of these genera are shown in Figure 4-7. This figure shows a decrease in the proportion of *Staphylococcus* in over time. Post hoc analysis

shows significant differences exist between the earliest time point and both group day 15-21 and day 22-28. Within Proteobacteria the only statistically significant increase over time was in unclassified *Enterobacteriaceae*. Post hoc analysis showed this subtle effect was significantly different between both groups within the first week of life and the day 15-21 group. *Corynebacterium*, within the Actinobacteria phylum, also showed a statistically significant increase over time.

Changes in measures of alpha diversity over time are shown in Figure 4-8. Low diversity was seen in all samples. Figure 4-5 shows the dominance of a single genus within the majority of samples. The median number of species observed was 5 per sample (range=1-15).

Both measures of richness (observed species count, Chao1 index and Fisher index) and evenness (Shannon diversity index and Inverse Simpson diversity index) showed a statistically significant increase over time. Post hoc testing using Dunn's test showed significant differences ($p<0.05$) between day 1-4 and day 22-28 groups, and day 5-7 and day 22-28 groups for observed species, Chao1 index, Fisher Index and Shannon diversity index. No significant differences were identified between groups for the inverse Simpson diversity index. The median number of observed species in samples taken day 1-4 = 3.5 (IQR 2-4.5). On days 22-28 the median number of observed species = 7 (IQR 5-9) ($p<0.001$).

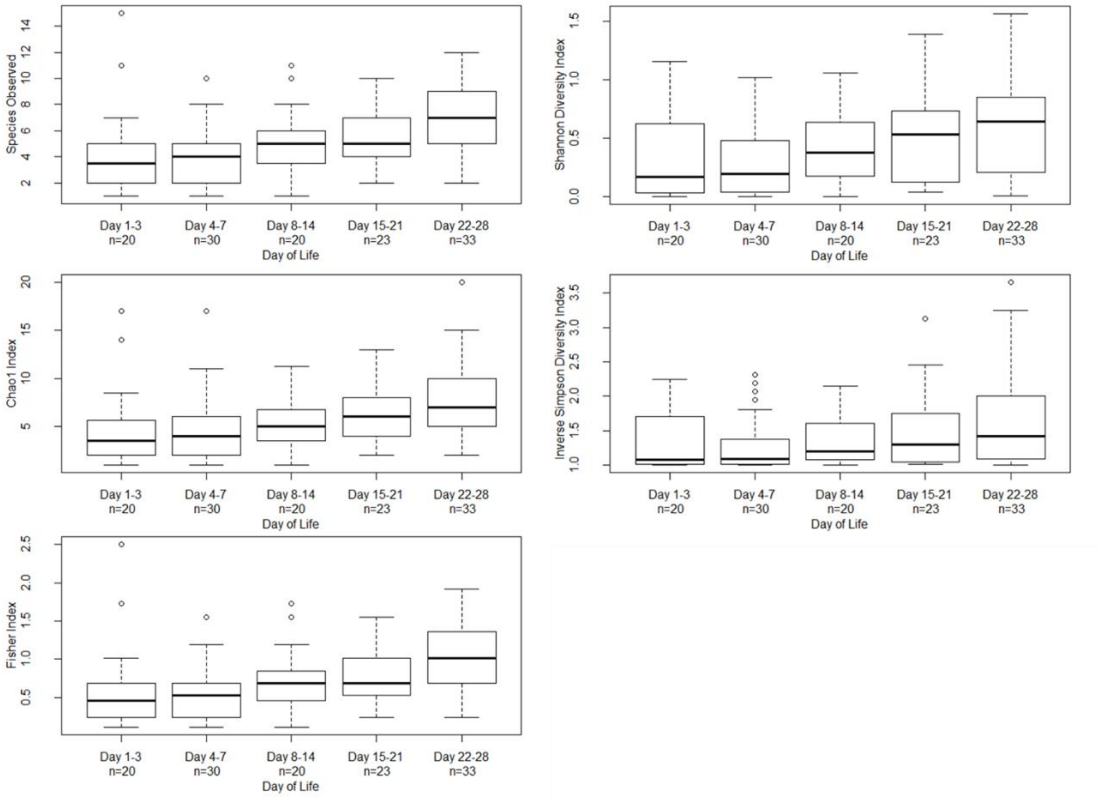


Figure 4-8: Changes in alpha diversity over time in bacteria detected within NPA samples from preterm infants.
Kruskal-Wallis tests: Species observed p<0.001, Chao1 p=0.003, Fisher Index p<0.001, Shannon Diversity Index p=0.02, Simpson p=0.04.

4.9 Effect of delivery mode on nasopharyngeal aspirate samples

Previous studies have identified differences in the microbiota composition within the nasopharyngeal cavity between term infants born by caesarean section and those born by vaginal delivery (Bosch et al. 2016; Dominguez-Bello et al. 2010). Therefore, I analysed my data for changes due to delivery mode. Figure 4-9 shows the difference between mean proportions of each phyla and the top 10 genera including all the NPA samples for which sequence data is available. This shows a higher proportion of Firmicutes, of the genera *Staphylococcus*, in infants born by caesarean section than those vaginally delivered and a lower proportion of Tenericutes and Actinobacteria. At a genus level, *Serratia*, *Corynebacterium* and *Ureaplasma* were all present in greater proportions in samples from vaginally born infants compared to caesarean section delivered infants.

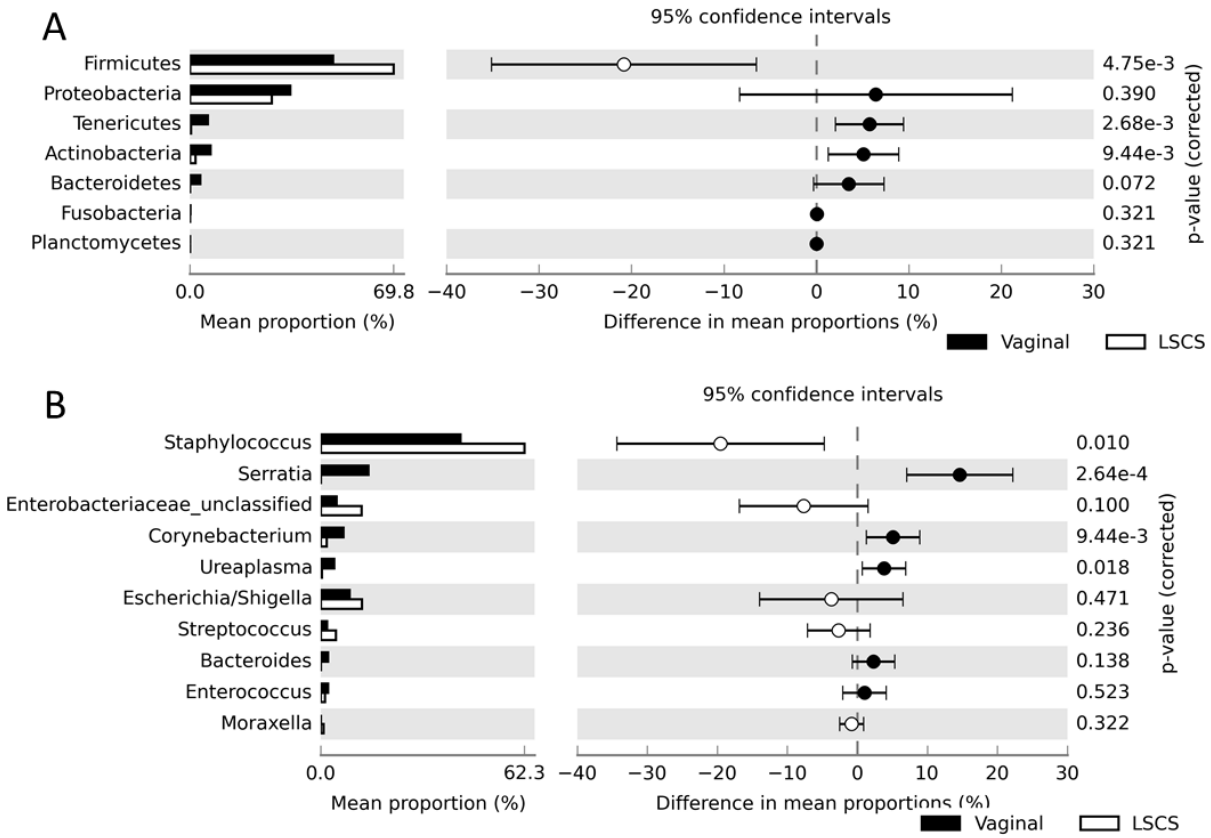


Figure 4-9: Differences in microbiota in NPA samples between infants delivered by caesarean section and vaginally delivered infants.
A= Differences at phylum level. B= Differences at genus level, top 10 most abundant genera displayed. Vaginal delivery n= 74, LSCS delivery n=52.

A sensitivity analysis was performed to exclude the effect of multiple sampling and the potential confounding from later post-natal exposures the analysis. Only the first sample that was positive from each infant within the first 7 days of life was included leaving 28 infants (10 born by CS and 18 delivered vaginally).

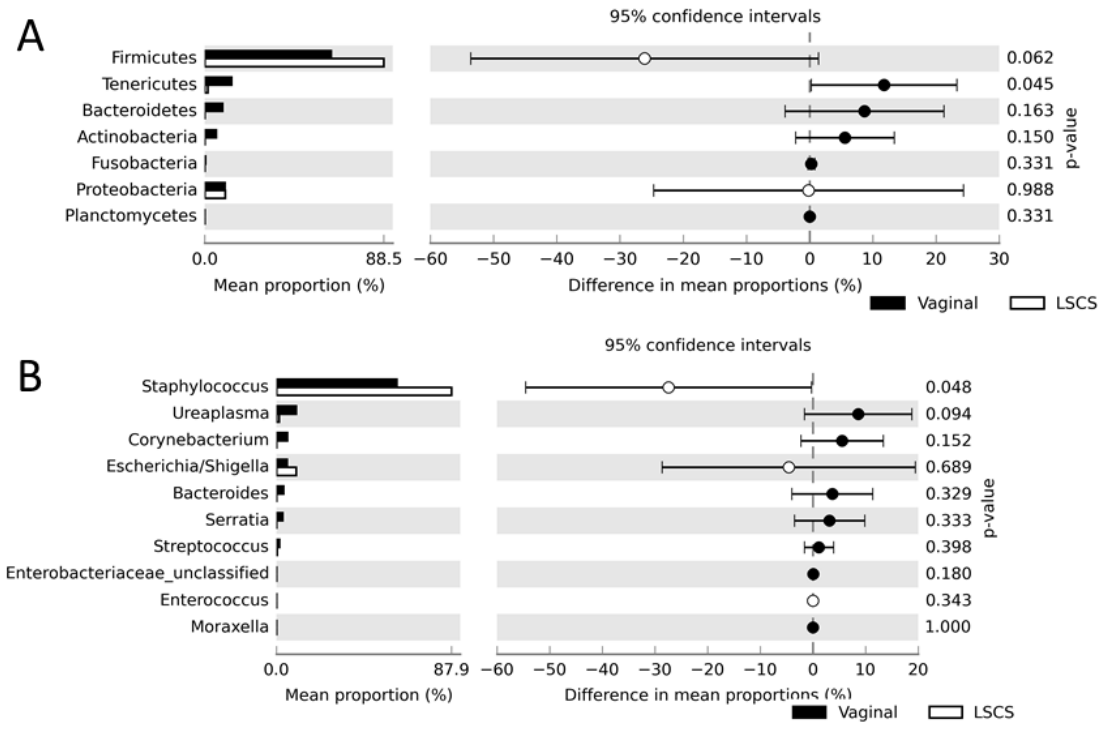


Figure 4-10: Effect of delivery mode over early colonisation patterns in NPA samples from preterm infants.
Only samples taken in the first 7 days of life included with only the earliest samples from each infant included if multiple samples available within the first 7 days. Vaginal deliveries n= 18, CS deliveries n=10.

Figure 4-10 above shows that with only the first week of life samples, the power to detect differences was reduced. At phylum level, there was a trend towards increased proportion of Firmicutes in samples from infants delivered by caesarean section. The significant difference in Tenericutes remained. At genus level the proportion of *Staphylococcus* remains significantly higher in infants born by caesarean section compared to vaginally born infants.

4.10 Effect of antibiotics on nasopharyngeal colonisation

All the recruited preterm infants received antibiotics after birth. It was, therefore, not possible to compare microbiota profiles from antibiotic-naïve and antibiotic-exposed preterm infants. It was possible to compare samples from infants who were receiving antibiotics and those not on antibiotics at the time of sample collection. Figure 4-11 demonstrates an increase in the proportion of Protobacteria in samples taken while infants were not receiving antibiotics. At a genus level the only significant difference identified was an increase in *Escherichia*/*Shigella* in infants on antibiotics.

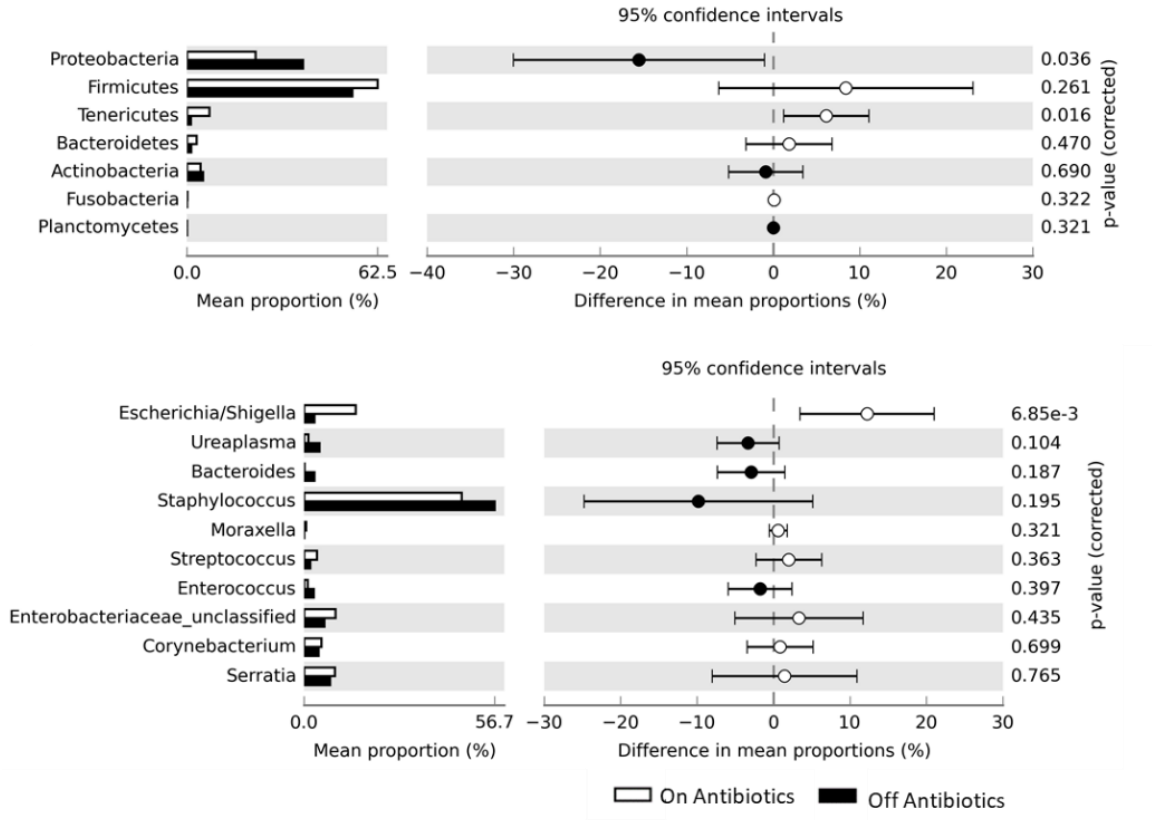


Figure 4-11: Effect of antibiotics on microbiota profiles in NPA samples from preterm infants at phylum and genus level.
A- Phylum level, B=Genus level, top 10 most abundant genera shown only. On antibiotics n=51, Off antibiotics n=75.

No differences were identified comparing alpha diversity indices between samples taken on antibiotics and samples taken off antibiotics. See Figure 4-12 below.

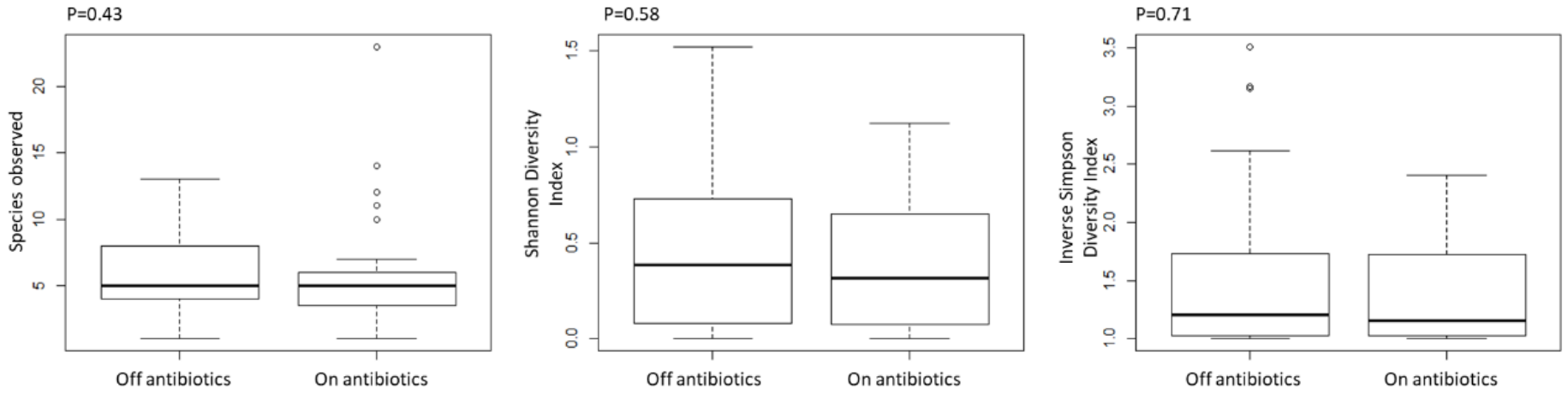


Figure 4-12: Effect of antibiotics on alpha diversity indices in NPA samples.
On antibiotics n=51, Off antibiotics n=75.

4.11 Effect of gender on nasopharyngeal colonisation

Male infants made up 65% of recruited participants. More male infants are born preterm than female infants with males typically making up 55% of preterm births (Zeitlin et al. 2002). Males are also known to have a worse prognosis than females in terms of survival and neurodevelopmental outcomes (Kent, Wright, and Abdel-Latif 2011). I analysed the colonisation patterns comparing NPA samples from male and female infants. Figure 4-13 shows that male infants had a higher proportion of Proteobacteria than female infants. At genus level, the *Escherichia*/*Shigella* genus was statistically significantly more abundant in males than females. Male infants could be at higher risk of nasal carriage of this potential pathogen than females.

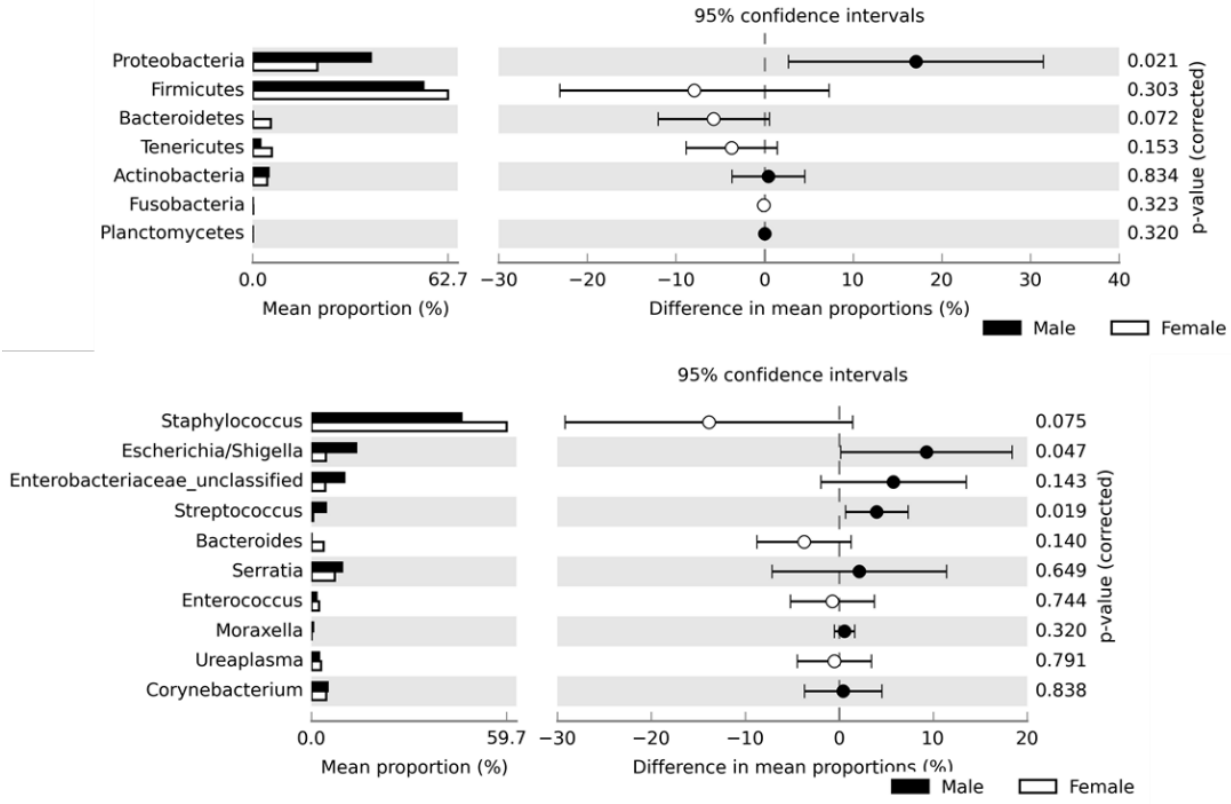


Figure 4-13: Differences in individual phyla and genera comparing samples from male and female preterm infants

4.12 Effect of probiotics on nasopharyngeal colonisation

During the recruitment phase, one of the recruitment sites routinely prescribed an oral probiotic product (Infloran) to all preterm infants <32 weeks' gestational age or birth-weight <1500g for prevention of NEC. The probiotic contained a mixture of *Lactobacillus acidophilus* and *Bifidobacterium infantis* and was started when the infant tolerated trophic feeds. I analysed the results for influence of probiotic usage over colonisation patterns.

Probiotic use was not associated with the chances of successfully sequencing the 16S bacterial rRNA gene from NPA samples. Analysing just samples from the recruitment centre where probiotics were used, odds ratio for successful sequencing of bacterial DNA from NPA samples comparing samples taken on and off probiotics was 0.78 (95% CI = 0.27-2.29, p=0.66). Probiotics were also not associated with a difference in bacterial load in NPA samples (Figure 4-14).

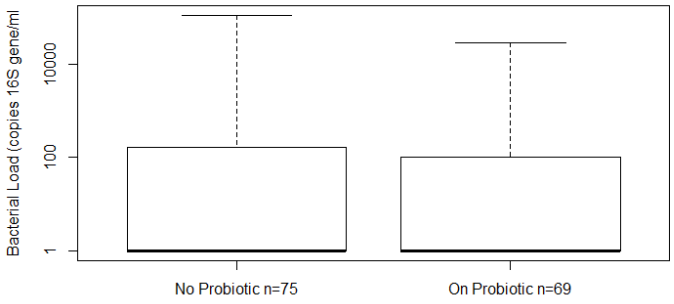


Figure 4-14: Bacterial Load in NPA samples taken from UHW site showing the difference between samples taken when infant receiving oral probiotic preparation compared to when not receiving oral probiotic.
Samples with undetectable bacterial load given a bacterial load of 1 to enable plotting on logarithmic scale. Wilcoxon test p=0.44.

Only 7 of the 126 (5.6%) samples successfully sequenced were taken from an infant receiving oral probiotic. Comparing the relative abundance of the identified bacteria within the samples show that when not on probiotics bacteria from genera known to have pathogenic organisms are present in greater proportions (Figure 4-15). *Esherichia/Shigella*, *Serratia* and *Enterobacteriacae_unclassifed* are statistically significantly more abundant when not on probiotics.

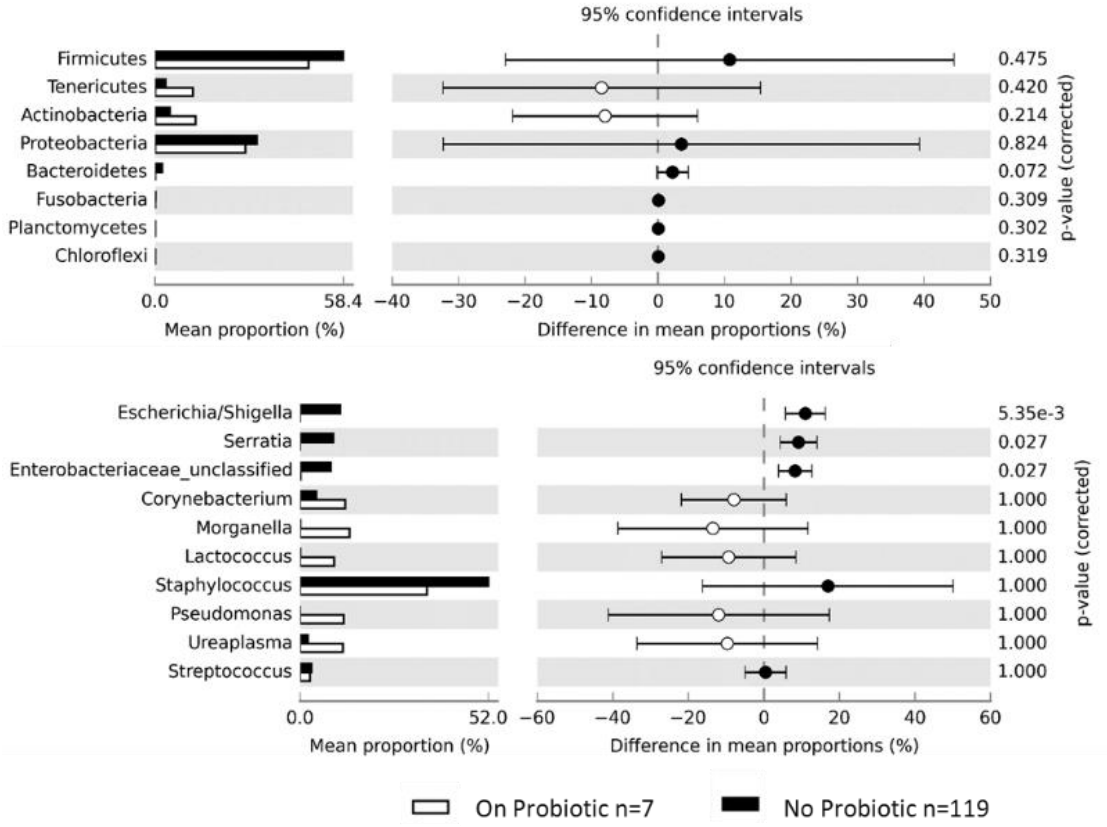


Figure 4-15: Comparing the relative abundance of bacteria at phylum and genus level between NPA samples from preterm infants collect on and off oral probiotic.

4.13 Effect of location on nasopharyngeal colonisation

A significant effect comparing locations between the two recruiting was previously shown for TA samples (Section 3.12). Therefore, I compared the microbiota profiles obtained from NPA samples between the two recruiting centres. There was no significant difference between the two sites in the number of samples from infants born by caesarean section or vaginal deliveries ($p=0.46$), day of life that samples were collected on ($p=0.97$), or the number of samples taken on antibiotics ($p=0.63$) for samples where sequencing data was available. Samples were, however, more likely to sequence successfully from NBT than UHW ($p<0.001$).

Figure 4-16A demonstrates the difference between microbiota profiles as a whole between the two centres. A PERMANOVA analysis comparing the centroids of from the two sites shows $R^2=0.03$, $p=0.005$. A Multivariate homogeneity of groups dispersions test shows this difference is not explained by difference dispersion patterns around the centroid ($p=0.103$).

Figure 4-16B and Figure 4-16C show the difference between the two sites was mainly in the proportion of Firmicutes, of which *Staphylococcus* was significantly different. *Serratia* was also present in significantly larger proportions in NPA samples taken at NBT.

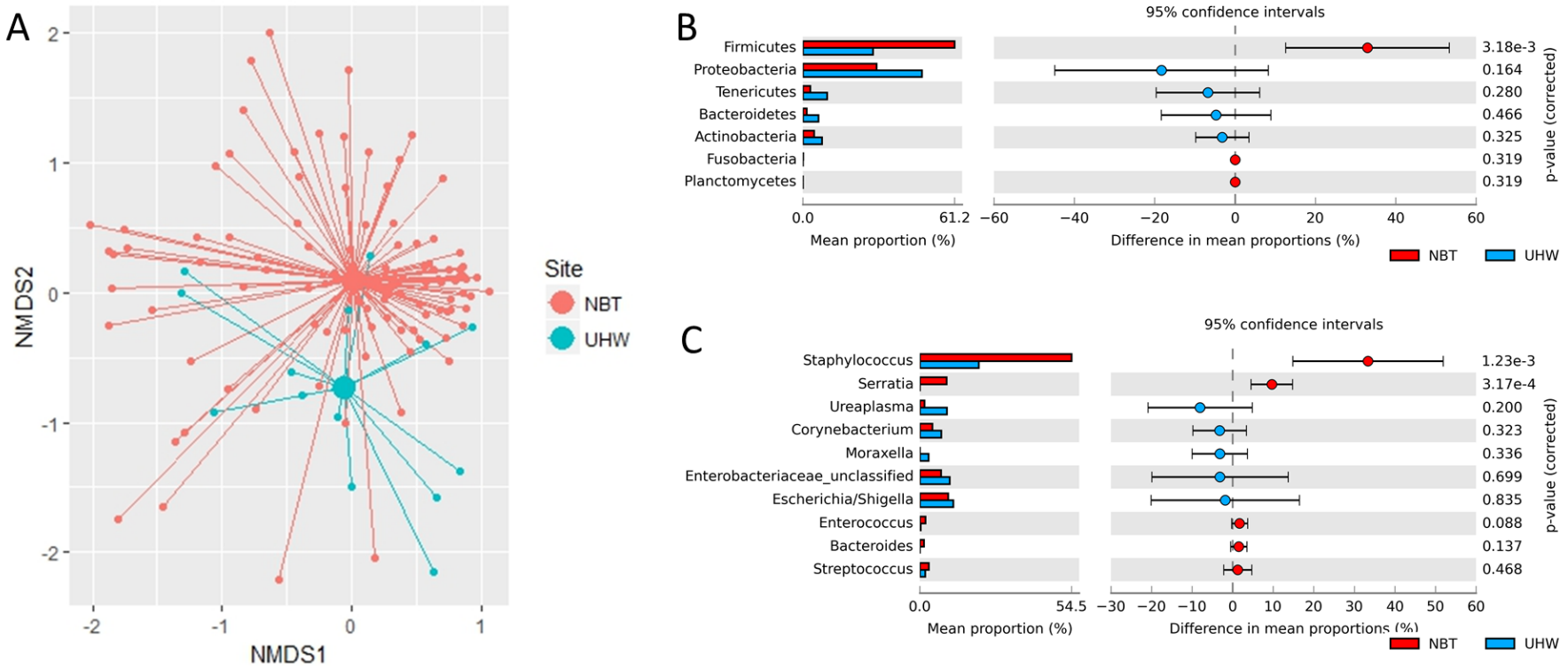


Figure 4-16: Effect of recruitment centre on microbiota of NPA samples from preterm infants
A= NMDS plot comparing two recruitment sites ordinated using Bray-Curtis Dissimilarity index, Stress = 0.22. B= Difference in mean proportions of individual Phyla between recruitment centres. C= Difference in mean proportions of most abundant 10 genera between recruitment centres. NBT=North Bristol Trust (n=112), UHW = University Hospital of Wales, (n=14).

4.14 Discussion

This study sought to identify early colonisation of the nasopharynx in preterm infants. Preterm infants from two centres were successfully recruited and serial sampling undertaken. One weakness of the study is the lack of data from a control group. Eight term infants who were on the postnatal ward were recruited to provide a control group. The lack of successful sequencing any samples from these infants has resulted in an inability to compare the results from preterm infants directly with healthy term infants.

The large proportion of samples where sequencing was unsuccessful limits the power of the study to identify differences between groups, however the number of samples negative for bacterial DNA may be important evidence of delayed colonisation in preterm infants. The exposure of the nasopharynx to the external environment and the ability of other studies to sequence successfully from the nasopharynx of term infants from samples taken within 5 minutes of life (Dominguez-Bello et al. 2010) suggested that colonisation may have been easier to detect from NPA rather than TA and BAL samples. This study mainly looked at preterm infants nursed in incubators which may have limited exposure to environmental organisms. The effect of antibiotics, the potential for DNA extraction yielding sub-optimal amounts of DNA and the challenges of working with low biomass samples have already been discussed with regard to TA and BAL samples. The fact that many samples were negative for bacterial DNA, particularly early in life supports the notion of colonisation of infants occurring postnatally.

Due to the fact that 22 preterm infants had no NPA samples that amplified successfully for 16 bacterial rRNA sequencing and serial sampling had been performed on all preterm infants, it was necessary to include more than one sample per infant when available in the analysis. The sensitivity analysis performed on samples from day 1-7 where only one sample from each infant was included did not fundamentally change the results.

Low biomass microbiome studies are susceptible to contamination from bacterial DNA within PCR reagents and from the DNA extraction process. These results are unlikely to represent contamination due to the difference between read numbers between samples and negative controls. The increased proportion of Firmicutes, namely *Staphylococci*, in infants born by caesarean section corroborates other studies and indicates these sequencing results are a genuine finding.

Over time there was a clear change in the microbiota profiles identified. The proportion of Firmicutes (of the *Staphylococcus* genus) decreased over time and the proportion of Proteobacteria increased over time. This shows a progression of the nasopharyngeal microbiome during the first month of life in preterm infants. A steady increase in richness during the first month of life was also evidence of maturation of the microbiome and the acquisition of new organisms. Evenness did not change over time. Therefore, on average NPA

samples acquired more OTU's over time but remained dominated by a single OTU throughout the first month of life. It may be the case, as with TA and BAL samples, that organisms from respiratory infections have been detected. No cytokine data is available to support or refute this. The changes in diversity over time would however suggest that colonisation is being detected.

Antibiotic exposure is a significant issue for preterm infants with concerns over changes to early bacterial colonisation (Jacquot et al. 2011), increasing the risk of NEC (C. Cotten et al. 2009), increased risk of invasive candidiasis (C. Cotten et al. 2006) and concerns regarding antibiotic resistance (Tzialla et al. 2012). Antibiotic use had a significant effect in reducing bacterial colonisation within the nasopharynx to below the threshold for detection in this study. No changes in evenness or richness were seen comparing samples taken with infants receiving antibiotics and those not receiving antibiotics suggesting that there is a general suppressive effect of antibiotics rather than antibiotics suppressing certain organisms and not others. The genus and phylum data comparing the effect of antibiotics are interesting. Antibiotics appeared to suppress Proteobacteria but were associated with an increase in *Escherichia*/*Shigella* (of the Proteobacteria genus). This could be explained by over proliferation by *Escherichia*/*Shigella* organisms due to antibiotic resistance. Alternatively, antibiotics may have been prescribed to treat infection with *Escherichia*/*Shigella* organisms, thus were detected. Due to a large number of different antibiotics and combinations of antibiotics used for recruited infants, it is not possible to analyse the effect of individual antibiotic treatments.

The difference in microbiota profiles between recruitment centres showed similarities to that identified between recruitment centres from TA samples (Section 3.12). The PERMANOVA showed a significant difference between the two centroids on the NMDS plot but the R^2 value of 0.03 showed only a small proportion of the variance is explained by the model. The fact that significantly more samples were sequence-able from NBT compared to UHW may be as a result of sampling techniques at the two centres or may represent differences in colonisation between the two recruitment sites. The number of samples from UHW (n=14) was far too small to draw firm conclusions.

It is difficult to explain the differences between male and female infants based on environmental exposures on the neonatal unit. Both are nursed in identical incubators and cared for using the same nursing staff and unit policies. A higher proportion of the boys recruited were born by caesarean section (37% of males, 26% of females) with *Escherichia*/*Shigella* more likely to be colonised during a vaginal delivery than a caesarean section delivery. Alternatively, boys may be susceptible to colonisation by the potentially pathogens in the *Escherichia/Shigella* genus.

It is difficult to interpret the effect of probiotics on NPA colonisation due to the small number of samples successfully sequenced from infants receiving probiotics. There was however a statistically significant difference between samples taken from infants on probiotics and those

taken when not receiving probiotics. Location is a clear confounding factor as only one of the sites was using probiotics, and clear evidence that location influenced colonisation has been shown in Section 4.12. It may be that the effect of probiotics is to suppress the growth of *Escherichia/Shigella*, *Serratia* and *Enterobacteriaceae*. It maybe however that the small number of samples successfully sequenced while on probiotics is a consequence of the probiotics suppressing bacterial load to below the level of detection.

4.14.1 Strengths and weaknesses

The study is the first to study the nasopharyngeal microbiota in extreme preterm infants during the first month of life. A total of 539 NPA samples were taken from 55 preterm infants making this the largest study of the preterm respiratory microbiota. Serial sampling has shown changes in bacterial load and a maturation of the microbiota during the first 4 weeks of life. Using NPA samples to study respiratory bacterial colonisation does not require the presence of an ET tube to collect the sample. This has allowed samples from infants following extubation to be included, something which was not possible from the BAL and TA samples.

A potential weakness is the number of samples with no detectable bacterial DNA, but this may also be an important finding showing delayed acquisition of the nasopharyngeal microbiota in preterm infants. The failure to amplify bacterial DNA from a term control group has precluded comparing the results from preterm infants with healthy term infants.

4.15 Conclusion

The aims of this chapter were to analyse serial samples from the upper airways of preterm infants and a term control group and identify the pattern of bacterial colonisation specifically looking for the influence of delivery mode, antibiotics, probiotics and recruitment centre. A further aim was to analyse bacterial load changes in upper airways of preterm infants over time.

The nasopharyngeal microbiome in preterm infants demonstrated a clear change over the first 28 days of life with an increasing proportion of Proteobacteria and a decreasing proportion of Firmicutes. A trend of decreasing Firmicutes and increasing Proteobacteria over time has previously been described in healthy term infants (Bosch et al. 2016), however this the first time the finding has been replicated in extreme preterm infants.

The exposures of antibiotics and the complex influences of preterm birth, invasive ventilation and other deviations from “normality” associated with extreme prematurity have not influenced the microbiota to prevent this colonisation pattern. Delivery mode has also been shown to influence nasopharyngeal colonisation for the first time in preterm infants with greater Firmicutes in infants delivered by caesarean section.

Studies of healthy term infants have suggested an association between early upper respiratory tract colonisation patterns and risk of future respiratory pathology (Teo et al. 2015; Vissing, Chawes, and Bisgaard 2013; Bisgaard et al. 2007). This study is not designed to identify such effects but the potential implications for neonatologists to encourage healthy respiratory microbiome development through judicious use of antibiotics and promoting breast feeding cannot be ignored. The role of probiotics is not proven, with this study showing that respiratory pathogens may be suppressed by oral probiotics. Further study is warranted on the role of probiotics in respiratory colonisation, long term respiratory health and whether delivering probiotics directly to the lungs would have any benefit.

Chapter 5: Comparing the Upper and Lower Airway Microbiota in Preterm Infants

5 Comparing the Upper and Lower Airway Microbiota in Preterm Infants

5.1 Introduction

Studies comparing the microbiota within the upper and lower respiratory tract have shown conflicting results. Some have identified similarities between colonisation of the different anatomical sites, but most have concluded that there are important differences and that using samples from the upper respiratory tract to identify pathogens in the lungs is not reliable (Ahmed et al. 2018; Boutin et al. 2015). No study has compared the upper and lower respiratory tract colonisation in preterm infants. I, therefore, analysed my data for differences between the TA, BAL and NPA samples.

5.2 Aims

1. To compare bacterial community structure between NPA, TA and BAL samples from preterm infants

2. To compare total bacterial load between NPA, TA and BAL samples from preterm infants

3. To compare measures of alpha diversity between NPA, TA and BAL samples from preterm infants

4. To compare concordance of the dominant organism identified from individual samples from the upper and lower respiratory tract taken within 24 hours from the same infant

5.3 Methodology of combining data from different samples

Combining the datasets of the different respiratory samples was undertaken jointly between myself and Dr Emma Mitchell as this was done also incorporating 16S rRNA sequencing data from stool samples from the same infants which Dr Emma Mitchell was analysing for a PhD project. She was interested the gut-lung axis while I was interested in comparing the different sites within the respiratory tract.

NPA, BAL and TA samples were analysed on different sequencing runs with separate data processing using the same Mothur pipeline. OTUs from each sample type were, therefore, not directly comparable due to the method of deriving OTUs based the differences between sequences within the dataset. Combining the sequence data at OTU level was, therefore, not possible. To allow accurate comparisons between the sequencing data of each sample type rarefication to 1000 reads per sample was performed prior to pooling the sample data. The sample read data were then combined at genus level for the top 100 OTUs in each dataset by

combining any OTUs that were from the same genus within the same sample. This included >95% of the sequence reads from each dataset.

5.4 Differences in community structure

The majority of samples of all three samples types contained a dominant genus. Community structure was, therefore, heavily influenced by the identification of the dominant genus. Figure 5-1 below demonstrates the differences in community structure comparing the three sample types. The overall PERMANOVA analysis comparing the three samples types was statistically significant (p=0.001). Table 5-1 demonstrates the post hoc analysis of this analysis showing the p values to be significant for each comparison. The R^2 value is smallest comparing the TA and BAL samples, which is unsurprising as they plot closer together on the NMDS chart and both are samples from the lower airways.

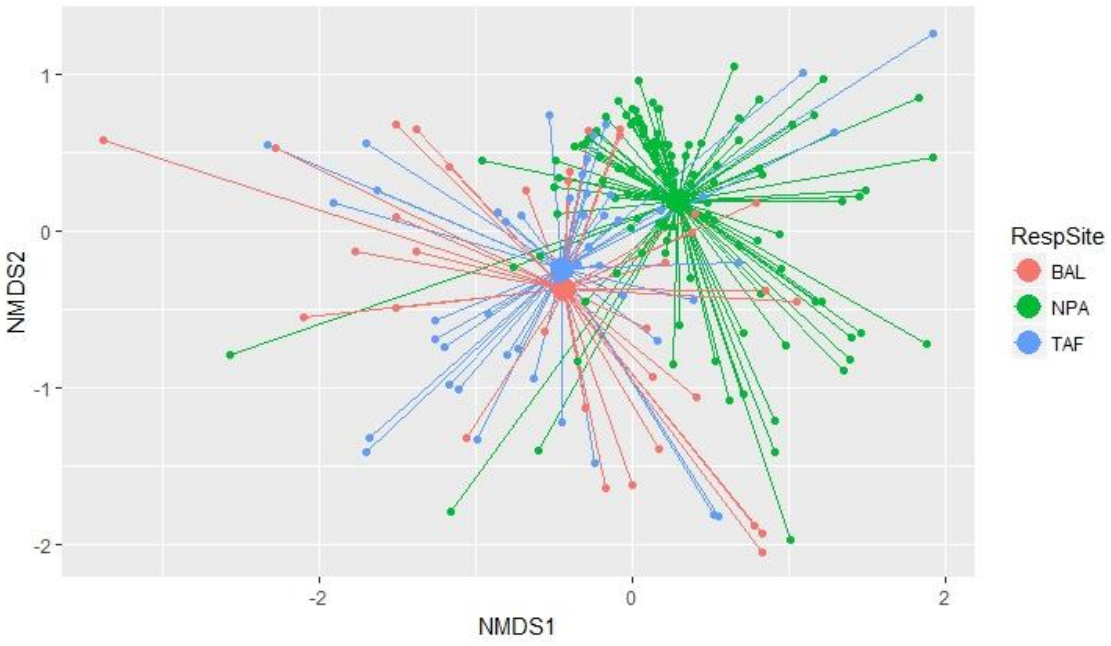


Figure 5-1: NMDS plot comparing bacterial community structure from 3 different sites within the respiratory tract.
Samples ordinated using the Bray-Curtis dissimilarity index. Stress = 0.21. PERMANOVA p=0.001, R2=0.052.

Table 5-1: Post hoc analysis results from PERMANOVA comparing community structure between samples from different sites within the respiratory tract of preterm infants.

Comparison	R2 value	P value	Adjusted p value
BAL vs TA	0.041	0.012	0.012
BAL vs NPA	0.071	0.001	0.0015
TA vs NPA	0.055	0.001	0.0015

5.5 Differences in bacterial load

Bacterial load estimation was performed on BAL, TA and NPA samples. Figure 5-2 below demonstrates the changes over time in average bacterial load of the samples that were successfully sequenced for the 16S rRNA gene. In days 1-3 of life, each sample type had a very low average bacteria load. Each sample type had an increase in bacterial load during the first one to two weeks of life, with the average bacterial dropping by days 21-23 to levels similar to those on days 1-3. Peak bacterial load was observed at days 4-7 for NPA samples but later at days 8-14 for TA and BAL samples. BAL samples had consistently the lowest average bacterial load. NPA samples had a slightly higher average bacterial load on days 1-3, but the highest average bacterial load on days 8-14 was in the TA samples.

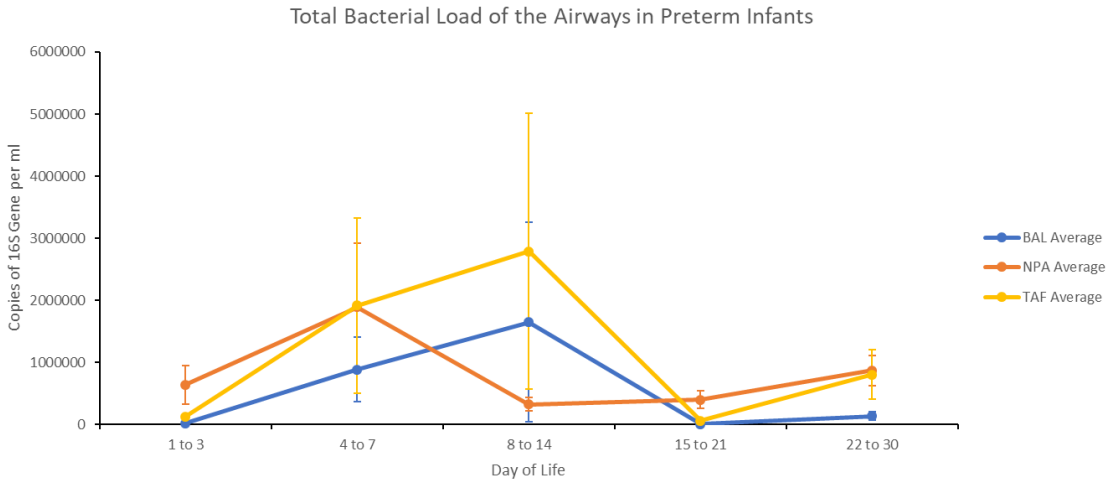


Figure 5-2: Comparing average bacterial load over time in NPA, BAL and TA samples that were successfully amplified for sequencing of the 16S rRNA gene collected from preterm infants. Error bars indicate standard error.
Day 1-2: BAL n=6, TAF n=1, NPA n=9; Day 4-7: BAL n=12, TAF n=20, NPA n=41; Day 8-14: BAL n=5, TAF n=9, NPA n=20; Day 15-21 BAL n=4, TAF n=2, NPA n=23; Day 22-30: BAL n=7, TAF n=15, NPA n=33.

5.6 Comparing dominance of individual genera between sample types

The BAL, TA and NPA samples have been shown to contain dominant OTUs. Table 5-2 below shows the percentage of each sample type that had a single OTU dominating showing the different levels of dominance of a single OTU. This highlights the extreme dominance of single OTUs in all the respiratory samples. Over 40% of the BAL and NPA samples had a single OTU making up ≥95% of the reads. Slightly less TA samples were dominated by a single OTU at each threshold.

Table 5-2: Dominance of a single OTU within respiratory samples at different thresholds of dominance

Dominance of single OTU	BAL (% of samples)	TA (% of samples)	NPA (% of samples)
≥95%	44.1	30.6	43.7
≥90%	58.8	46.9	52.4
≥80%	64.7	59.2	66.7
≥70%	73.5	69.4	76.2
≥50%	91.2	91.8	96.8

5.7 Differences in alpha diversity indices

Figure 5-3 shows four different measures of alpha diversity between the different sample types. Alpha diversity indices were calculated for each sample type individually using the OTU level data presented in previous chapters not the combined dataset at genus level used Section 5.4. Each index was calculated three times to show overall differences when all the samples with available sequencing data were included and comparing diversity within the first week of life and in the fourth week of life. BAL samples consistently showed higher alpha diversity scores for richness and NPA samples showed the lowest alpha diversity scores for richness. The statistically significant differences for richness were all seen within the first 7 days of life. No differences were identified between the samples for the Shannon or inverse Simpson indices, both measures of evenness. This is unsurprising as a similar level of dominance of individual OTUs in each type of sample has been demonstrated.

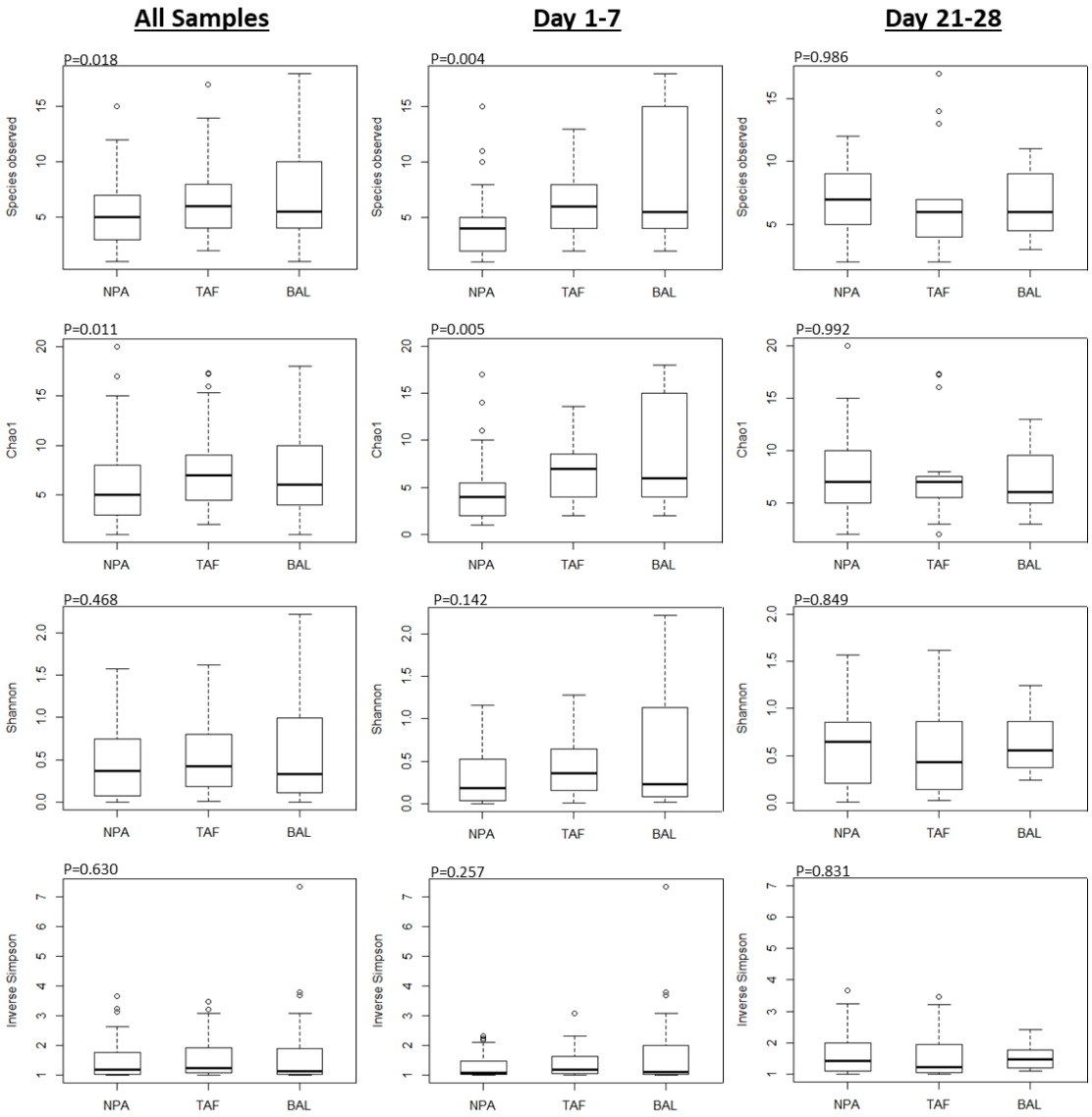


Figure 5-3: Comparing alpha diversity indices between samples from different sites within the respiratory tract of preterm infants.
1st column includes all samples (NPA n=126, TAF n=47, BAL n=34), 2nd column only samples taken within the first 7 days of life (NPA n=51, TAF n=21, BAL n=18), 3rd column only samples from the 4th week of life (NPA n=39, TAF n=16, BAL n=9). NPA – Nasopharyngeal Aspirate, TAF - Tracheal Aspirate Fluid, BAL – Bronchoalveolar lavage. Kruskal-Wallis test used to calculate p values.

5.8 Comparing individual samples

The results presented above suggest that the three anatomical sites sampled in this study were separate compartments with differences in bacterial colonisation. However, I was also interested in whether samples from individual infants taken contemporaneously showed concordance between the upper and lower airways. Successful 16S rRNA gene sequencing from TA and BAL was samples was associated with an increase in IL-6 and IL-8 suggesting possible infection, with the most dominant OTU likely the infective organism. I compared TA and NPA samples from the same infant collected within 24 hours of each other to see if the dominant

genus identified within each sample was concordant. 22 pairs of samples were identified when sequencing had been successful from a TA and NPA sample from the same infant taken within 24 hours. Figure 5-4 shows the sample dyads with the bars representing the relative abundance of the most abundant genus within each sample. In 14 pairs (64%) the most abundant genus is concordant between the TA and NPA samples. In 8 pairs (36%) the most abundant genus was discordant. This suggests that there is a degree of similarity between the two anatomical sites, despite the differences identified in Section 5.4. Similar analysis was attempted comparing BAL vs NPA samples but only six pairs of samples when sequencing had been successful on both a BAL and an NPA sample within 24 hours were available which was considered too few to draw conclusions.

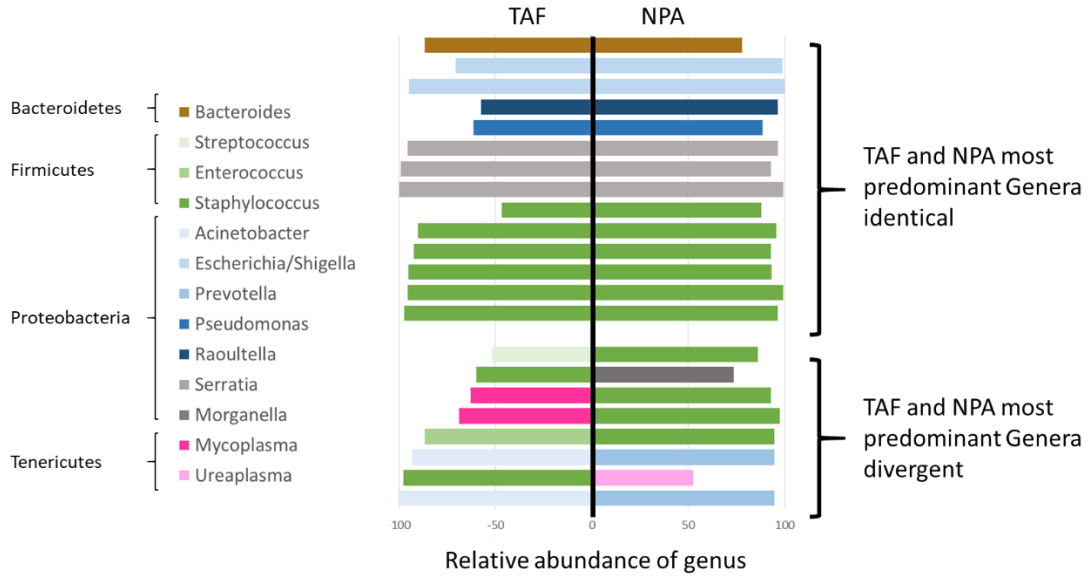


Figure 5-4: Bar plot comparing abundance of most abundant genus present within TA and NPA samples taken from the same infant within the 24 hours of each other.
TAF = Tracheal aspirate fluid, NPA = Nasopharyngeal aspirate.

Nine dyads of samples were available for comparison between BAL and TA samples taken from the same infant within 24 hours. Figure 5-5 below shows that seven dyads had the same most abundant genus in both TA and BAL sample. Identification of the same genus in samples from the same infant in two separate samples processed independently, sequenced in different runs on the MiSeq device, with independent identification of OTUs was evidence for the reliability of the sequence data in both datasets.

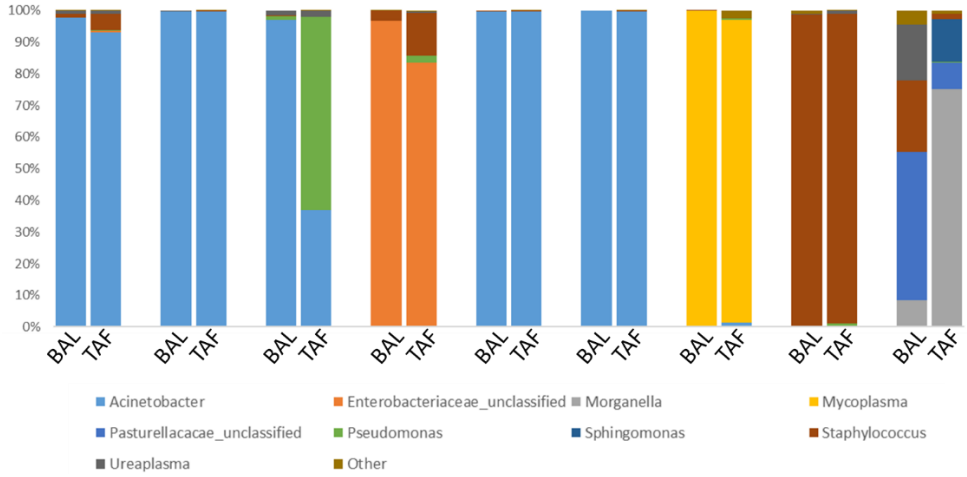


Figure 5-5: Paired dyads of BAL and TA samples taken within 24 hours from the same infant showing only top 10 most abundant OTUs. (BAL – Bronchoalveolar lavage, TAF – Tracheal Aspirate Fluid)

5.9 Discussion

5.9.1 Validity of sequencing data

The data presented comparing individual samples in terms of the dominant genus detected between NPA and TA samples and between BAL and TA samples provides some evidence for the validity of the sequencing data. The samples shown were taken within 24 hours of each other from the same infant but had DNA extracted at different times, sequencing for the 16S rRNA gene performed on separate sequencing runs on the MiSeq device and had OTU allocation and identification performed independently. However, on 7/9 (78%) pairs of BAL – TA samples and 14/22 (64%) pairs of TA – NPA samples the most predominant genus was identical. This is a validation of the methodology in identifying bacterial genera within the airways of preterm infants.

The datasets were combined at genus level rather than OTU level. This limits the sensitivity of the analysis. The decision to combine the data from each sample type at genus level was based on the pragmatic approach. Each dataset had already demonstrated that identification to species level was not possible with the length of the 16S rRNA gene that had been sequenced. Combining the datasets at genus level would lead to an underestimate in differences between the different sample types as several OTUs may be combined into a single genus, reducing the diversity and the sensitivity of the NMDS plot to identify differences between samples. The NMDS plot (Figure 5-1) and associated PERMANOVA analysis did identify differences between the three sample types despite the weaknesses of using genus abundance data.

5.9.2 Colonisation at different anatomical sites

Each anatomical site demonstrated similar levels of dominance with an individual OTU (see Section 5.3). NPA samples had consistently the highest percentage of samples with a dominant

OTU regardless of the threshold of dominance used. This finding was also corroborated in analysis of alpha diversity analysis between the different samples. Indies of richness were significantly lower in NPA samples than BAL and TA samples during the first month of life with the main difference being identified in the first week of life. This unexpected finding suggests that fewer species of bacteria colonise the NPA tract than the lower airways during the first week of life in preterm infants. When measures of evenness were compared between the sample types no significant differences were identified for any of the time periods analysed.

The nasopharynx is located closer to the outside environment than the lower airways and lungs, with species colonising the lower airways and lungs likely to have gained access to the lungs via the upper airways, greater richness was expected in the NPA samples compared to the lower airways samples. All recruited infants were intubated during the first days of life, therefore, air was not passing through the nasal passages and nasopharynx which may have affected bacterial colonisation of the nasopharynx. The effect of the ET tube may also have influenced TA and BAL samples. It has been previously discussed that the positive BAL and TA samples may reflect an increase in bacterial load associated with infection. The successfully sequenced NPA samples may also reflect episodes of infection. It may be that the dominance of a single OTU in the NPA samples is due to greater suppression of other bacterial species in the nasopharynx in episodes of infection compared to the lower airways and lungs. A recent study of children with cystic fibrosis showed no difference in any alpha diversity indices between throat swabs and bronchial brushings (Ahmed et al. 2018), in contrast another study of children with cystic fibrosis showed much greater richness in nasal swabs compared to sputum samples, used as a proxy for lower airway colonisation (Boutin et al. 2015).

Figure 5-1 shows the differences in average community structure between NPA, TA and BAL samples. This demonstrates that TA and BAL samples were more closely related in terms of community structure with the centroids close together. Statistical analysis using PERMANOVA showed the differences between each sample type to be significant. It is unsurprising that bacterial community structure within BAL and TA samples are more closely related to each other than NPA samples given that both BAL and TA samples are aiming to sample from the lower airways. The statistical difference between the TA and BAL samples does, however, indicate that a separate niche has been identified and that BAL samples are not just identifying organisms colonising the ET tube.

5.9.3 Bacterial load comparisons between nasopharyngeal, bronchoalveolar lavage and tracheal aspirate samples

Figure 5-2 demonstrates that each sample type shows an increase in average bacterial load over the first 1-2 weeks of life with average bacterial load then falling to levels seen in days 1-4 of life. Bacterial load would be affected by the difference in sampling practices between each

sample. Anecdotally the BAL and TA samples generated greater biomass in terms of volume of mucous and visible material aspirated than the NPA samples. Despite this during days 1-3 the NPA samples showed higher bacterial load than the BAL and TA samples. The nose and nasopharynx are known to be host to an established microbiome in adults and children and are close to the external environment. Therefore, NPA samples are more likely to be colonised by micro-organisms entering via the nares than TA and BAL samples. TA and BAL samples follow a very similar pattern, validating the methodology. TA samples had consistently greater average bacterial load than BAL samples. This again may reflect the distance from the external environment of the anatomical sampling area, and is a finding previously described (Charlson et al. 2011).

The fall in bacterial load seen at day 8-14 in NPA samples and at day 15-21 in BAL and TA samples is more difficult to explain. As already discussed in previous chapters, the use of antibiotics does affect bacterial load, but this was not identified as the cause for the bacterial load of BAL and TA samples to drop after the first week of life. The developing neonatal immune system may take time to establish the control needed for healthy levels of mucosal colonisation therefore allowing a peak in bacterial load. There may be an advantage in allowing greater bacterial colonisation over the early days of life as the infant generates sensitising exposures to colonising bacteria programming the immune system with T cell and dendritic cell sensitisation (Torow et al. 2017). Comparing the bacterial load in healthy term infants would be useful to identify whether or not this phenomenon is pathological or physiological.

5.9.4 Using nasopharyngeal aspirate samples to identify lung pathogens

Many studies have hypothesised that using samples from the upper airway can identify lung pathogens (Goddard et al. 2012; Prevaes et al. 2017; Stralin et al. 2014). This would be a clinically useful application of culture independent techniques on upper respiratory tract samples, preventing the need for invasive lower airway sampling using BAL and ability to obtain diagnostic samples from non-intubated patients. Published studies show conflicting results regarding the ability to detect pathogens causing lower respiratory tract infections from samples from the upper respiratory tract, possibly due to the differences in underlying disease states and the specific types of samples analysed. The work presented in this chapter suggests that upper airway samples from preterm infants are unlikely to be a useful proxy for more invasive sampling. In 14/22 (64%) cases the most abundant genus was identical in NPA and TA samples taken within 24 hours. Therefore 36% of cases a misleading result may be obtained. The biggest weakness of this comparison is that it is only at genus level. With many bacterial species within each genus it is not possible to identify if the same species has been detected. Species level identification is needed for clinically useful pathogen identification. Samples included in this analysis were not necessarily taken at the same time, with samples collected within 24 hours included. This may have affected the concordance between samples as data

from lower airway samples showed that the colonisation was a dynamic process with the dominant organism changing regularly in some infants.

There are insufficient numbers of pairs of BAL:NPA or BAL:TA samples collected within the same 24 hour period to conclude if these samples reliably identify the same organisms. If BAL and TA samples are reliably identifying the same organisms, then it would need to be established whether both samples are identifying lung colonisation/infection or whether both are identifying the organisms colonising the ET tube. The only way to identify lung colonisation without risking detecting ET tube colonisation would be to use sampling from open lungs – something that would not be justified ethically. More acceptable would be a study targeting preterm infants with suspected ventilator associated infection to compare culture independent sequencing results from NPA and BAL or TA samples collected contemporaneously.

5.9.5 Strengths and weaknesses

This chapter has combined the results from the previous two chapters, with sequencing results available from a total of 207 samples. No previous study has compared bacterial colonisation from different sites in the respiratory tract of preterm infants. The work in this chapter has demonstrated that the methodology of 16S rRNA gene sequencing is reliable, with very similar result obtained from the different sites within the respiratory tract from the same baby taken on the same day.

The main weakness of this work is the combining of the data from each sample type at genus level rather than at OTU level. The effect of the methodology used would be reduce any differences between the sample types as several OTUs may have been combined into a single genus.

A further weakness is the fact that BAL samples were only collected from one recruitment centre and TA and NPA samples were collected from two recruitment centres. Analysis of both the TA and NPA samples showed a significant effect of location of bacterial community structure. Comparing these samples to BAL samples from only one location may induce a confounding effect of location into the analysis.

Despite the identified weaknesses this analysis shows important differences between the different sample types which are important for gaining an understanding of bacterial colonisation of the respiratory tract.

5.10 Conclusions

In this chapter I aimed to compare the data from TA, BAL and NPA samples with regard to bacterial load, bacterial community structure and alpha diversity measurements.

Comparing the nasopharyngeal bacterial community structure to that lower in the respiratory tract showed evidence of the NPA, TA and BAL samples identifying different bacterial communities, with the TA and BAL bacterial communities being more closely linked than the NPA bacterial communities. This is the first study in preterm infants to be able to compare BAL samples with samples taken more proximally within the respiratory tract.

The bacterial load with NPA, BAL and TA samples showed clear patterns of peaking within the first two weeks then returning to lower levels. Bacterial load within NPA samples peaked earlier than the samples from the lower respiratory tract. Further work is needed to understand why this happens and if this is a physiological process seen in all infants, or if it is a phenomenon limited to preterm infants.

Alpha diversity indices showed NPA samples contained the lowest measures of richness in the first week of life, however there was no difference in evenness comparisons at any time point.

The work in this chapter also aimed to identify if sequencing the 16S rRNA gene from NPA samples would be a useful diagnostic test in cases of lower respiratory tract infection. Using paired samples this study suggests that NPA samples detect the same dominant genus as TA samples in 64% of cases, too low a value to be clinically useful. Studies capable of pathogen identification at a species level with contemporaneous sampling are required before clinically useful information can be obtained from next generation sequencing data of this type. Timely workflows could however shorten the time taken to get results using culture-independent techniques compared to culture-based pathogen identification.

Chapter 6: Investigating Extracellular ASC as a Potential Mechanism of the Effect of the Microbiota Over Innate Immunity

6 Investigating Extracellular ASC as a Potential Mechanism of the Effect of the Microbiota Over Innate Immunity

6.1 Introduction

The bacterial communities colonising the human body are known to have a complex interaction with the host (Geuking et al. 2014; Belkaid and Hand 2014). Sections 3.10 and 3.20 demonstrated that early bacterial colonisation of the lower respiratory tract was associated with a pro-inflammatory response with higher levels of IL-8 and IL-6 present in BAL and TA samples when bacterial DNA was detected. The innate immune system is involved in sensing the presence of bacterial antigens through a series of PRRs including TLRs and NOD like receptors (Wiersinga et al. 2014). The innate immune system responding to pulmonary microbes may therefore contribute to pulmonary inflammation leading to CLD. Strict controls of the resulting immune response may allow communities of bacteria to survive in symbiosis with the host without activating the host immune response inappropriately. I therefore undertook further work to study one aspect of the innate immune system to understand how the inflammation generated by bacterial colonisation may be propagated through the lung.

Inflammasomes are intracellular macromolecular complexes assembled within cells in response to a perceived threat (Latz 2010). Inflammasome activation of the immune system was first identified via the activation of cytokines IL-1β and IL-18 (Martinon, Burns, and Tschopp 2002). These cytokines are released from the cell and act as cell signalling molecules to activate other immune cells and generate a response to the threat. The work of Franklin et al and Baroja-Mazo et al suggest that components of the inflammasome propagate an immune response extracellularly when released from the cell following cell death by pyroptosis (Baroja-Mazo et al. 2014; Franklin et al. 2014).

Franklin and colleagues demonstrated that ASC specks retain extracellular activity, being able to activate extracellular caspase-1. Also demonstrated was the ability of neighbouring cells to phagocytose extracellular ASC specks resulting in NLRP3 activation within the cell. In addition phagocytosed ASC specks were shown to aggregate cytosolic ASC, acting in a prion like way to propagate the immune response within the host cell (Franklin et al. 2014). Baroja-Mazo's paper looked at inflammasome release from a cell and demonstrated oligomeric structures containing ASC were released from mouse bone-marrow-derived macrophages within five minutes of stimulating the inflammasome. The supernatants from activated macrophages demonstrated increased ability to activate caspase suggesting that ASC remained active after being released from the cells (Baroja-Mazo et al. 2014).

Franklin et al attempted to identify the presence of ASC specks and oligomers in the BAL fluid from adults with COPD, pneumonia and pulmonary hypertension as well as healthy donors. BAL fluid from patients with COPD and pneumonia contained extracellular preassembled ASC specks, while BAL from patients with pulmonary hypertension and from healthy donors did not contain ASC specks (Franklin et al. 2014).

Activation of inflammasomes within the lungs of a preterm mouse model of CLD was demonstrated by Liao et al. NLRP3 deficient mice were shown to be protected from the harmful effects of hyperoxia with improved alveolarization and less caspase activation than wild type mice. NLRP3 deficient mice also showed less neutrophilic cellular infiltration to the lungs and reduced IL-1β and IL-18 production than wild type mice. Similar results were obtained by pharmacological blocking of NLRP3 activation with glyburide. Using a baboon model of prematurity the IL-1β:IL-1ra ratio in tracheal fluid was significantly increased in the preterm birth group compared to the term birth group. In human subjects the IL-1β:IL-1ra ratio in tracheal aspirates was higher in preterm infants who developed CLD than those who did not develop CLD. Inflammasome activation was then inferred to be greater in the those infants developing CLD (Liao et al. 2015). A further study in mice identified an up regulation in gene expression of NLRP3 and ASC detecting higher mRNA levels in animals ventilated with high tidal volumes compared to standard lower tidal volumes. NLRP3 inflammasome deficiency partially protected mice from ventilator associated lung injury (Kuipers et al. 2012).

IL-1β, produced by the activated NLRP3 inflammasome from the inactive pro-IL-1β form, has been shown to be present on day 1 of life within tracheal aspirate samples of preterm infants exposed to chorioamionitis (Cayabyab et al. 2003). IL-1β was found in higher concentration in bronchoalveolar lavage samples from infants who developed CLD than infants who did not (S. Kotecha, Wilson, et al. 1996). These studies indirectly identify inflammasome activation within the lungs of preterm infants and add to the evidence of a role for inflammasome activation in the pathogenesis of CLD.

6.2 Aims

1. To attempt to identify the presence of inflammasome components within BAL fluid from preterm infants

2. To examine the pro-inflammatory potential of ASC in neonatal BAL fluid from preterm infants

3. To produce ASC recombinantly for use in cell stimulation experiments

4. To determine the mechanism of action of the pro-inflammatory effect of extracellular ASC on respiratory epithelial cells in culture

6.3 Identifying inflammasome components within BAL supernatant

Previous work has demonstrated the presence of IL-8, IL-6, IL-1β and other pro-inflammatory cytokines within BAL fluid from preterm infants. BAL fluid from preterm infants has been used to study the inflammatory activity within the lungs extensively (Davies et al. 2010; S. Kotecha, Wilson, et al. 1996; Bagchi et al. 1994). I investigated BAL from preterm infants for the presence of ASC and inflammasome components. BAL samples from a historical cohort of patients collected by Dr P Davies and Dr N Maxwell were used with permission of Prof S Kotecha. Ethical approval for the storage and further testing was in place for these samples. Four samples were selected for testing using Western blot analysis. Samples were chosen based on results from previous work of IL-8 concentration, measured using the same ELISA assay described in Section 2.11.2, within the sample. This was used as a measure of inflammatory activity within the sample. Two samples were chosen due to the high IL-8 concentration and two samples with low IL-8 concentration.

Table 6-1: BAL samples selected for Western blotting to determine the presence of extracellular ASC within the supernatant of BAL fluid from preterm infants.

Sample	IL-8 Concentration (pg/ml)	Inflammatory Activity
35.7	1520	Low
32.1	1138	Low
19.7	230599	High
35.9	56812	High

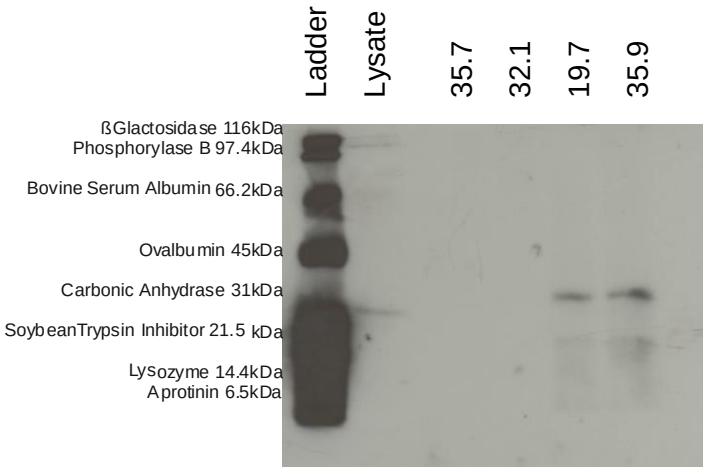


Figure 6-1: Western blot image demonstrating the detection of extracellular ASC in the supernatant of BAL fluid from preterm infants.
Note that the samples considered to have a strong pro-inflammatory profile (high IL-8) were the samples where ASC was detected with a prominent between 20-30 kDa.

Equal volumes (20 µL) of BAL supernatant was used in the Western blot. Also run as positive control was lysate from a cell stimulation experiment which had undergone inflammasome activation using nigericin, a potent activator of NLRP3 (Mariathasan et al. 2006). Primary antibody used – mouse monoclonal IgG, secondary antibody – monoclonal rabbit anti mouse HRP. The result of the western blot is shown in Figure 6-1.

This image demonstrates a clear band at the appropriate molecular weight for ASC in the positive control and in the two samples chosen for their high inflammatory activity. This experiment demonstrated that extracellular ASC was detectable in BAL supernatants obtained from preterm infants. The detection of ASC only in the two samples with high IL-8 concentrations, therefore the samples with presumed greater inflammatory activity, suggested that inflammasome components could be used as biomarkers for inflammatory activity and possibly to predict the chance of infants developing CLD.

6.4 ASC and inflammasome components in BAL fluid over time

To further investigate the role of extracellular ASC and the inflammasome in CLD a series of Western blot experiments were performed probing for ASC, NLRP3 and caspase-1p10 using BAL samples from infants collected by Dr P Davies and Dr N Maxwell from previous cohort studies. I compared preterm infants who developed CLD, preterm infants who did not develop CLD and term infants ventilated for abdominal surgery, with normal lungs. I selected days 1, 2, and 7 of age for testing due to the known influence of inflammation in early life over CLD

outcomes (S. Kotecha, Wilson, et al. 1996; S. Kotecha et al. 1995; C. L. Bose, Dammann, and Laughon 2008).

For each day of life tested, 20 µL of BAL supernatant was used in each Western blot. The protein concentrations of these samples had previously been measured using a microBCA kit by Dr P Davies. The IL-8 concentration was also previously measured by Dr P Davies using a Duoset ELIZA methodology.

The antibodies used for each target molecule in the Western blot are shown in Section 2.12.2. Examples of the images obtained from Western blots from these experiments are shown below in Figure 6-2.

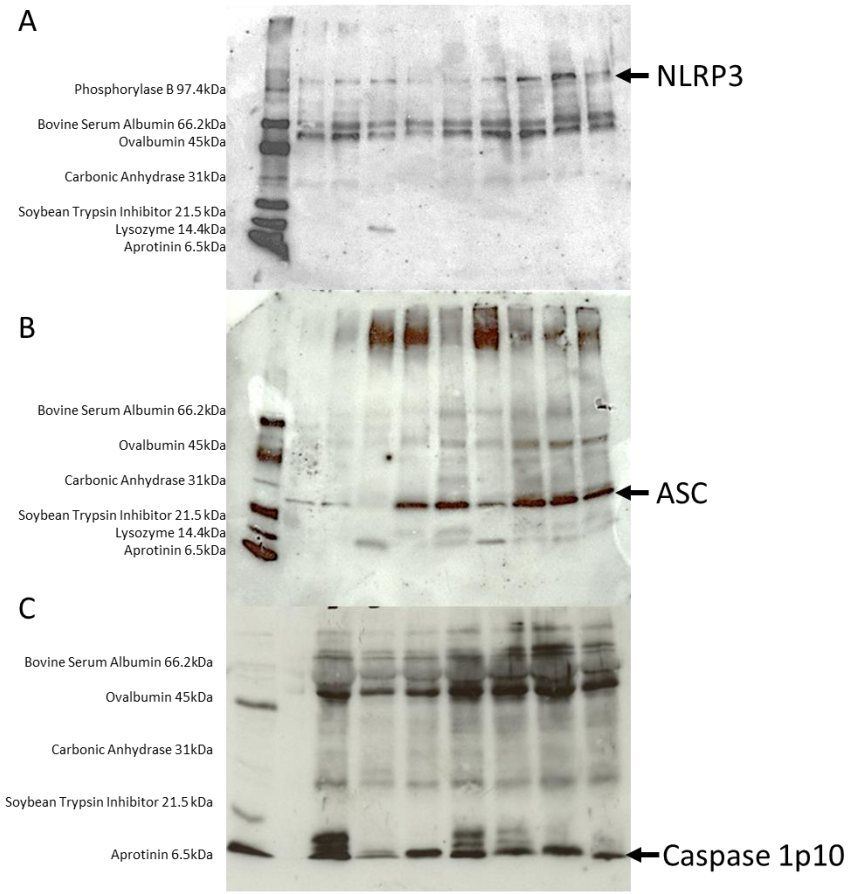


Figure 6-2: Example Western blot images using antibodies targeted to inflammasome components in BAL fluid from preterm infants.
The first lane in each image is the ladder, samples run in random order in each gel. A- Probing for NLRP3 using BAL samples from day 1 of life. B - Probing for ASC using BAL samples from day 7 of life. C - Probing for caspase-1p10 using BAL samples from day 2 of life.

Western blot images demonstrate the presence and absence of the target molecule within the BAL fluid. The relative density of the bands detected on the same exposure can be used to semi-quantify the concentration of the target molecules relative to the other samples tested in arbitrary units. To do this densitometry analysis the Image J programme was used. The film obtained by Western blotting was scanned in using a digital computer scanner.

Densitometry data was used to compare the density of bands between infants who developed CLD and infants who did not develop CLD. Comparisons were made between preterm and term infants. Samples from day 1, 2 and 7 were compared between infants.

The Western blots were run with equal volume loading of each BAL sample, uncorrected for protein concentration. A difference was witnessed between the protein concentrations in samples from day 1 of life of the preterm and term groups that approached statistical significance (see Figure 6-3). For this reason, both the raw densitometry values and the values corrected for protein concentration (obtained by dividing the raw value by the protein concentration in µg/mL) are given.

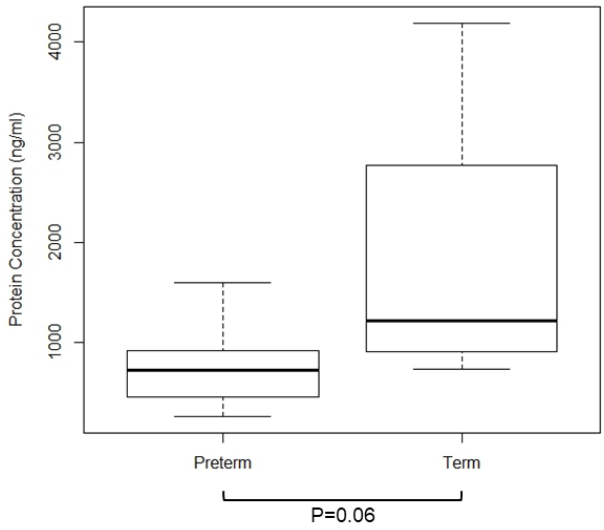


Figure 6-3: Total protein concentration of BAL supernatant samples from day 1 used in Western blotting

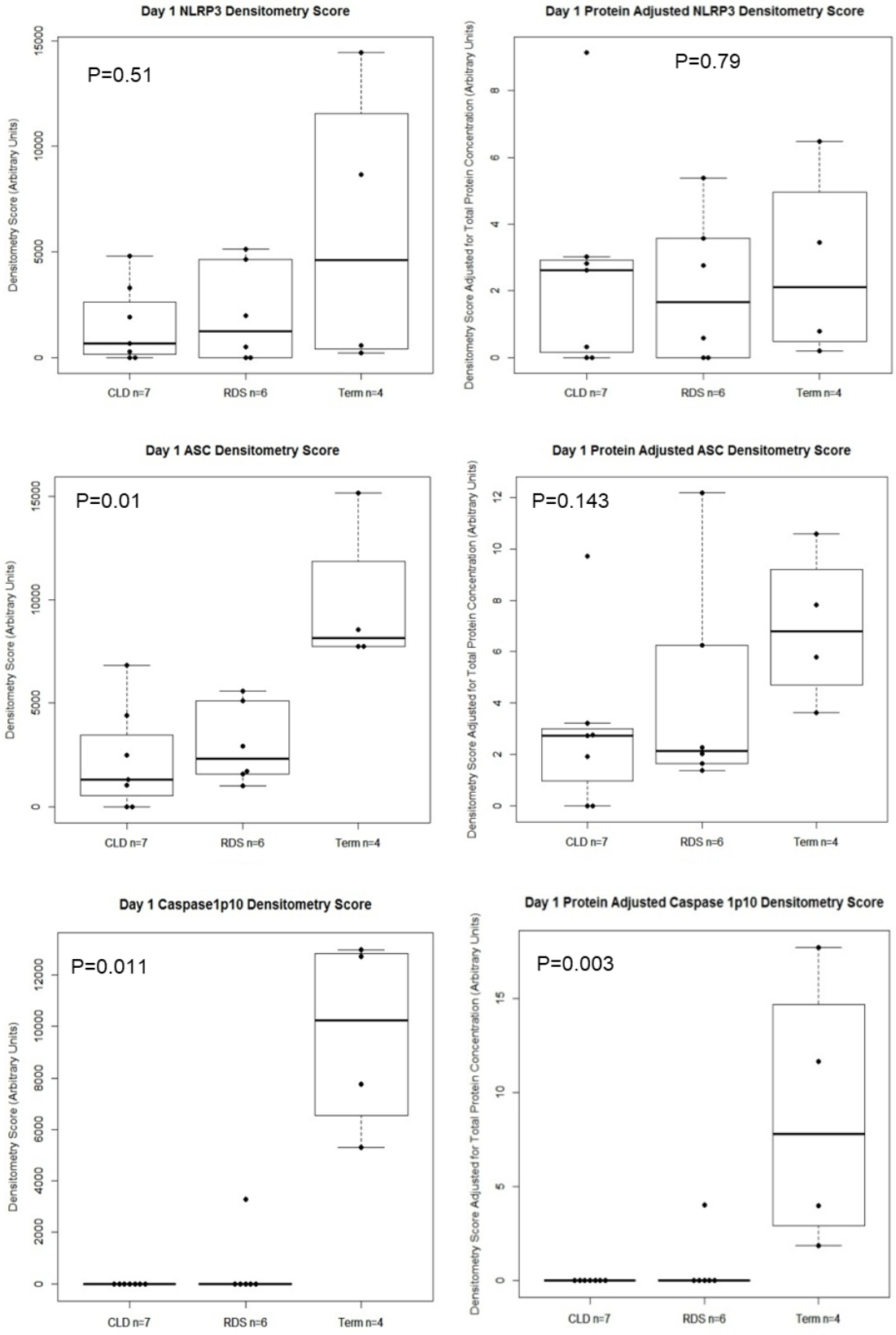


Figure 6-4: Box-plots of densitometry data obtained from Western blotting of BAL supernatants for inflammasome components by outcome group. All samples taken on day 1 of life.
P values calculated using the Kruskal-Wallis rank sum test.

Figure 6-4 above demonstrates that of the three inflammasome components tested caspase-1p10 and ASC showed a statistically significant difference between the outcome groups. The effect for ASC was not maintained when densitometry scores were corrected for protein concentration.

A consistent pattern was noted that for each inflammasome component that the results the median values of the CLD and RDS were very similar with the term group appearing to differ. No significant differences were detected between the RDS and CLD groups with any of the probed for inflammasome components on any day. This would suggest that in the first week of life extracellularly detectable NLRP3 inflammasome components are not useful as a biomarker for predicting CLD. The data for subsequent days is displayed only comparing preterm and term infants. Protein corrected values have been used due to the difference between the protein concentrations identified on the samples from day one of life between term and preterm infants.

Due to the similarity of the results obtained between preterm infants with RDS and those who went on to develop CLD in all the inflammasome components probed for across each day tested, the results were re-analysed comparing just two groups preterm and term infants (Figure 6-5). This demonstrated significantly greater differences between the amount of ASC and caspase-1p10 in the lungs of term infants compared to preterm infants using raw densitometry values. When comparing the two groups using protein corrected values only caspase-1p10 maintains significance, with ASC just above the threshold of significance ($p=0.06$). Given that caspase-1p10 was largely undetectable in the preterm infants correcting for protein concentration would not have a meaningful effect if the caspase-1p10 was below the limits of detection due to low total protein load. The small numbers involved in this experiment make interpretation difficult.

Caspase-1p10 is released from cells during pyroptosis. Higher levels of caspase-1p10 in BAL fluid collected on day 1 may indicate increased inflammatory activity within the lungs of term infants compared to preterm infants on the first day of life. Other studies have shown higher levels of pro-inflammatory markers in BAL samples from preterm infants than term infants (S. Kotecha, Wilson, et al. 1996; Davies et al. 2010) during the first week of life.

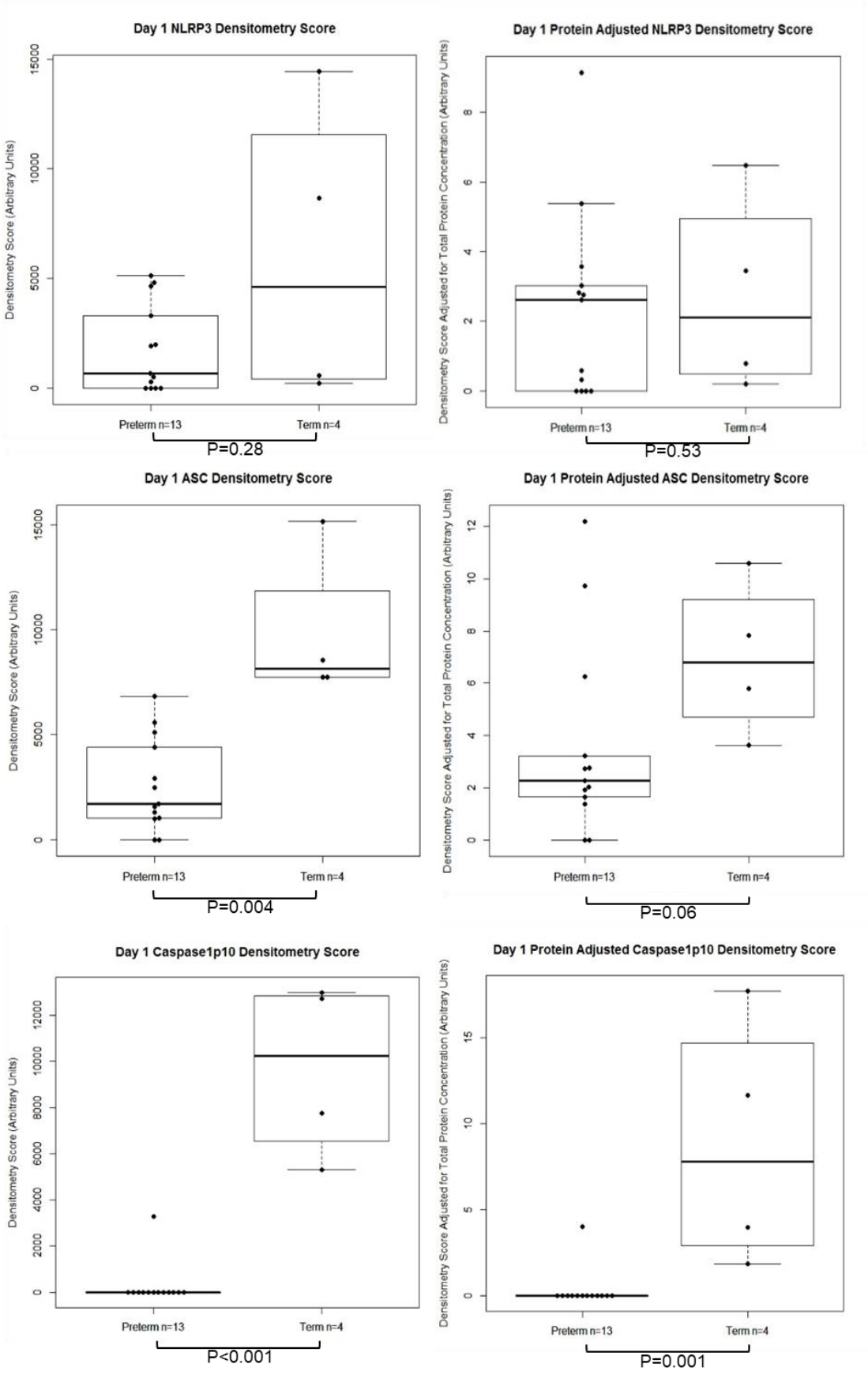


Figure 6-5: Densitometry data from Western blots of inflammasome components comparing preterm and term infants. BAL samples taken on day 1 of life.
p values calculated using the Wilcoxon signed rank test.

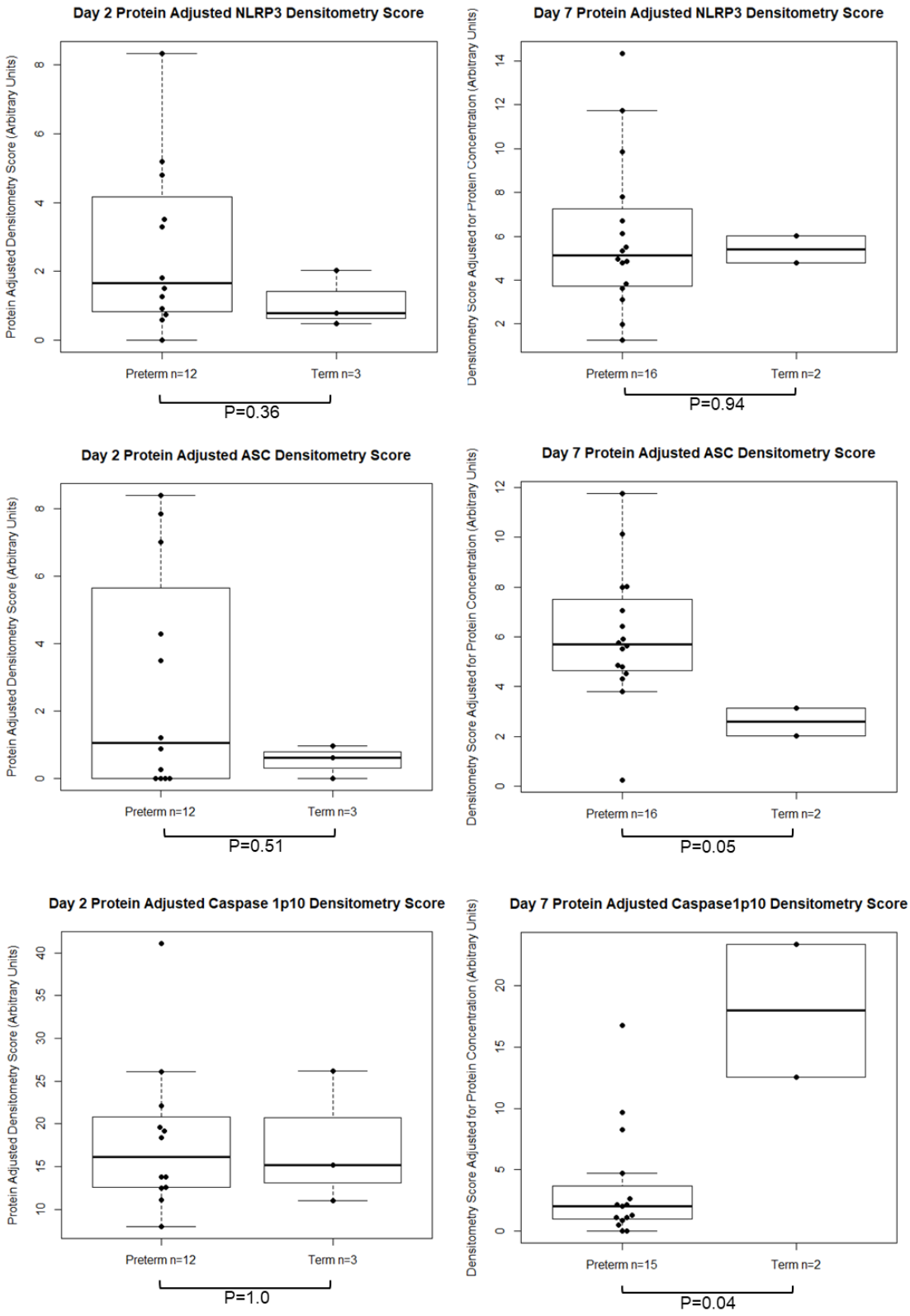


Figure 6-6: Protein adjusted densitometry scores obtained from Western blotting for inflammasome components on supernatant of BAL samples obtained from day 2 and 7 of life. P values calculated using the Wilcoxon signed rank test.

The results obtained from the day two samples (Figure 6-6) indicate no differences between the concentration of any of the inflammasome components between preterm and term infants. The day 7 results are difficult to interpret due to the presence of only two infants in the term group. There is a difference between the groups with higher levels of ASC in the preterm infants but higher levels of caspase-1p10 in the term infants. It would be expected that all the inflammasome components probed for would show the same trend if NLRP3 activation was occurring which is suggested by the detection of NLRP3. Inflammation within the lungs of preterm infants peaks between days 7-10 as seen in my data from IL-6 and IL-8 concentrations from the BAL and TA samples (Section 3.28) and in previous published work (S. Kotecha, Wilson, et al. 1996; Beeton et al. 2011). Greater densitometry scores may then be expected in the preterm group at day 7 if inflammasome mediated inflammation is contributing to pulmonary inflammation in preterm infants. However, term infants continuing to receive ventilation at day 7 may have developed respiratory pathology by this stage due to the complications of ongoing ventilation.

This work has demonstrated that inflammasome components, specifically ASC, are detected in the cell-free supernatant of BAL fluid from preterm infants.

6.5 ASC in BAL fluid has pro-inflammatory stimulatory properties

To further investigate the role of ASC as an extracellular signalling molecule in CLD, experiments were designed to use BAL supernatant to stimulate cells in culture. Comparing the effect of BAL fluid depleted of ASC by immunoprecipitation with complete BAL fluid the actions of ASC can be identified.

6.6 The pro-inflammatory potential of neonatal bronchoalveolar lavage supernatant on respiratory epithelial cells in culture

The A549 cell line has been widely used to as an *in-vitro* model of alveolar epithelial cells (Zhihong Zhang et al. 2005; Somborac-Bačura et al. 2018). The BEAS-2B cell line is commonly used as an in vitro model of bronchial epithelial cells (Peeters et al. 2013; C. E. Stewart et al. 2012). These cell lines were cultured and proliferated as described in Section 2.13.1. A preliminary experiment was performed using pooled BAL fluid from preterm infants to stimulate cells in culture to determine the cytokine response generated by the cells. Pooled BAL was obtained by selecting four samples from preterm infants. Day one and two samples were excluded due to the failure to detect ASC in the earliest samples as documented above. Samples with many aliquots were chosen to allow the experiment to be repeated using the identical samples.

This experiment was performed in duplicate and repeated to on separate occasions three times. The cytokine concentration of the supernatant following overnight incubation comparing stimulated and unstimulated cells is shown in Figure 6-7 below.

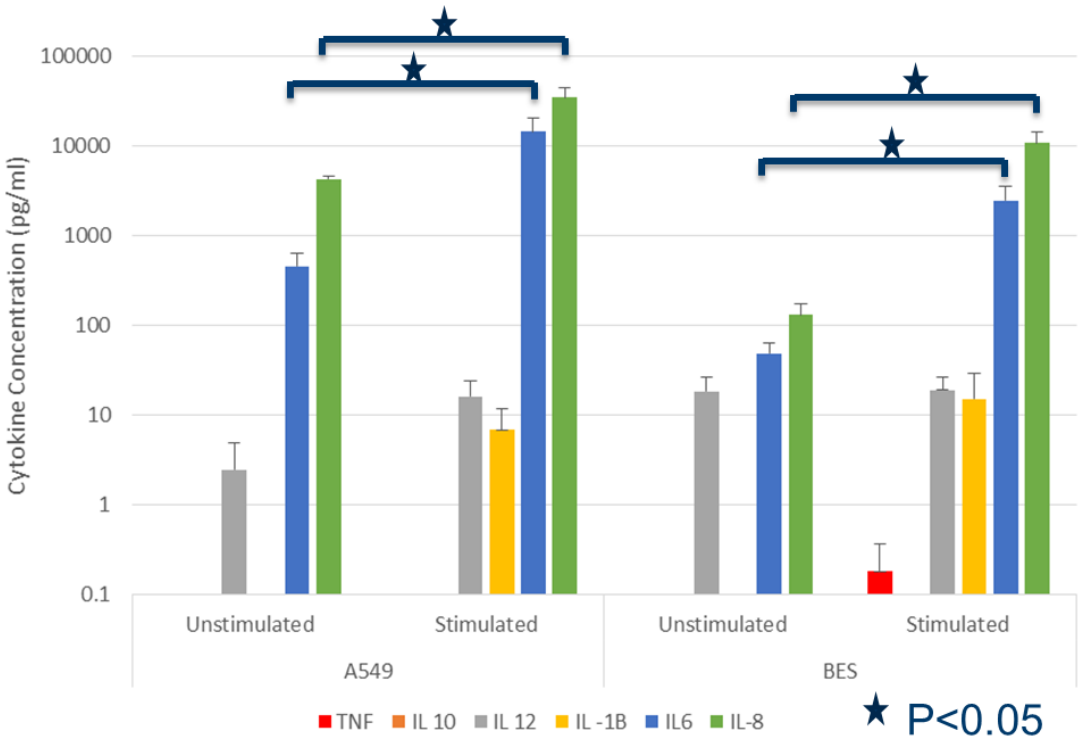


Figure 6-7: Cytokine concentration in supernatant of cultured A549 cells and BEAS-2B cells following stimulation overnight with pooled BAL fluid compared with unstimulated controls. Cell stimulations performed in serum free media. Cytokine concentrations measured using a cytometric bead array. P value calculated using Wilcoxon Signed rank test.

This demonstrates a basal level of IL-6, IL-8 and IL12 production by unstimulated cells in both cell lines. Following stimulation with BAL fluid the secretion of IL-6 and IL-8 markedly increased with IL-1β also produced by both cell lines. A low level of IL-10 was detected following stimulation of the A549 cell line only. Samples from preterm infants lungs are known to contain many factors including LPS and cytokines (Lohmann et al. 2014; Beeton et al. 2011) capable of inducing a pro-inflammatory response in this cell model. The difference between the concentrations of cytokines between the stimulated and unstimulated is unlikely to represent just the addition of this cytokine as each well received the equivalent of 20 µL of BAL, which was diluted in serum free media to give a total volume of 400 µL. The hundred times increase in cytokine concentration cannot be explained by the addition of the cytokine already in the BAL and represents the induction of cytokine production by the cells.

6.7 The effect of immunoprecipitation of ASC from BAL in cell stimulation experiments

ASC was immunoprecipitated from BAL using the procedure detailed in Section 2.14, and the success of the immunoprecipitation was confirmed using Western blot analysis detecting ASC in the immunoprecipitant. Representative images of these results are shown below in Figure 6-8. Cell stimulations were performed in serum-free media incubating the cells with the BAL fluid overnight. Unstimulated cells incubated in serum-free media only were used as a negative control.

The results are shown below in Figure 6-9. Depleting the samples of ASC resulted in lower IL-6 and IL-8 concentrations in the supernatant of the A549 cells. The BEAS-2B cells showed significantly lower IL-6 concentration following stimulation with ASC depleted BAL compared to neat BAL. This is suggesting that ASC is contributing to the IL-6 and IL-8 secretion from the cultured respiratory epithelial cells.

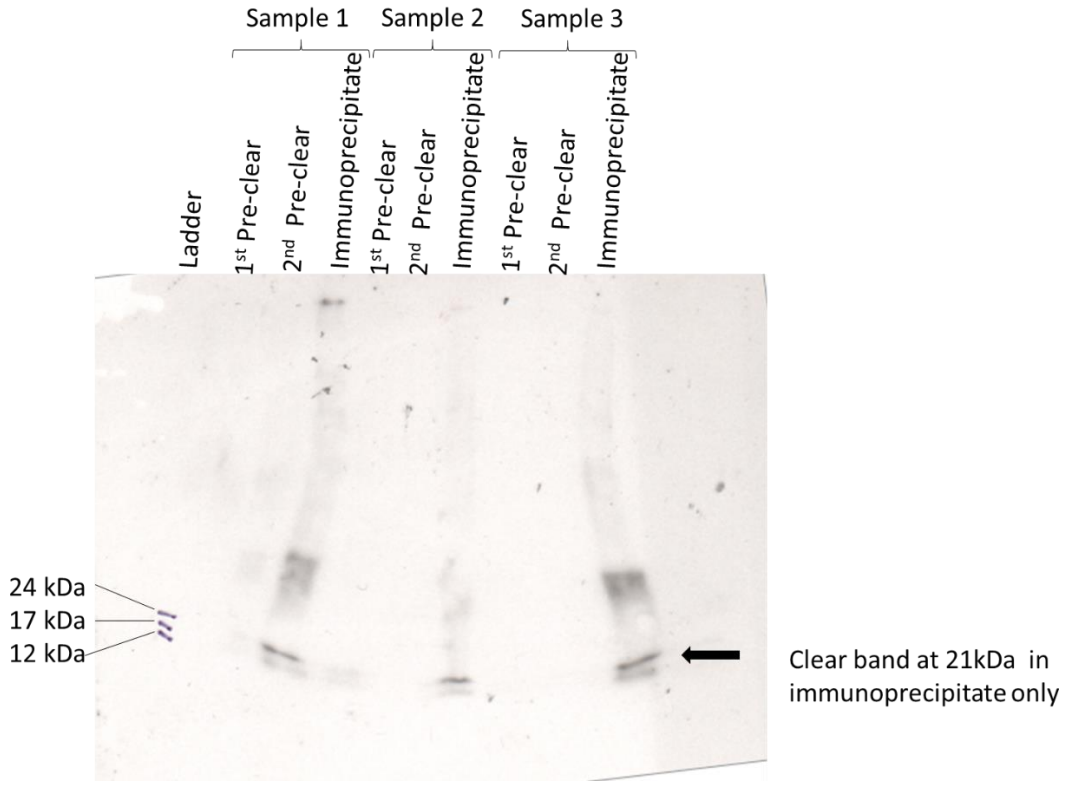


Figure 6-8: Representative image of Western-blot to confirm success of immunoprecipitation from pooled BAL fluid from preterm infants.
The presence of a band at 21kDA in the immunoprecipitated but not in the pre-clear samples suggests the ASC has been successfully depleted from the pooled BAL samples.

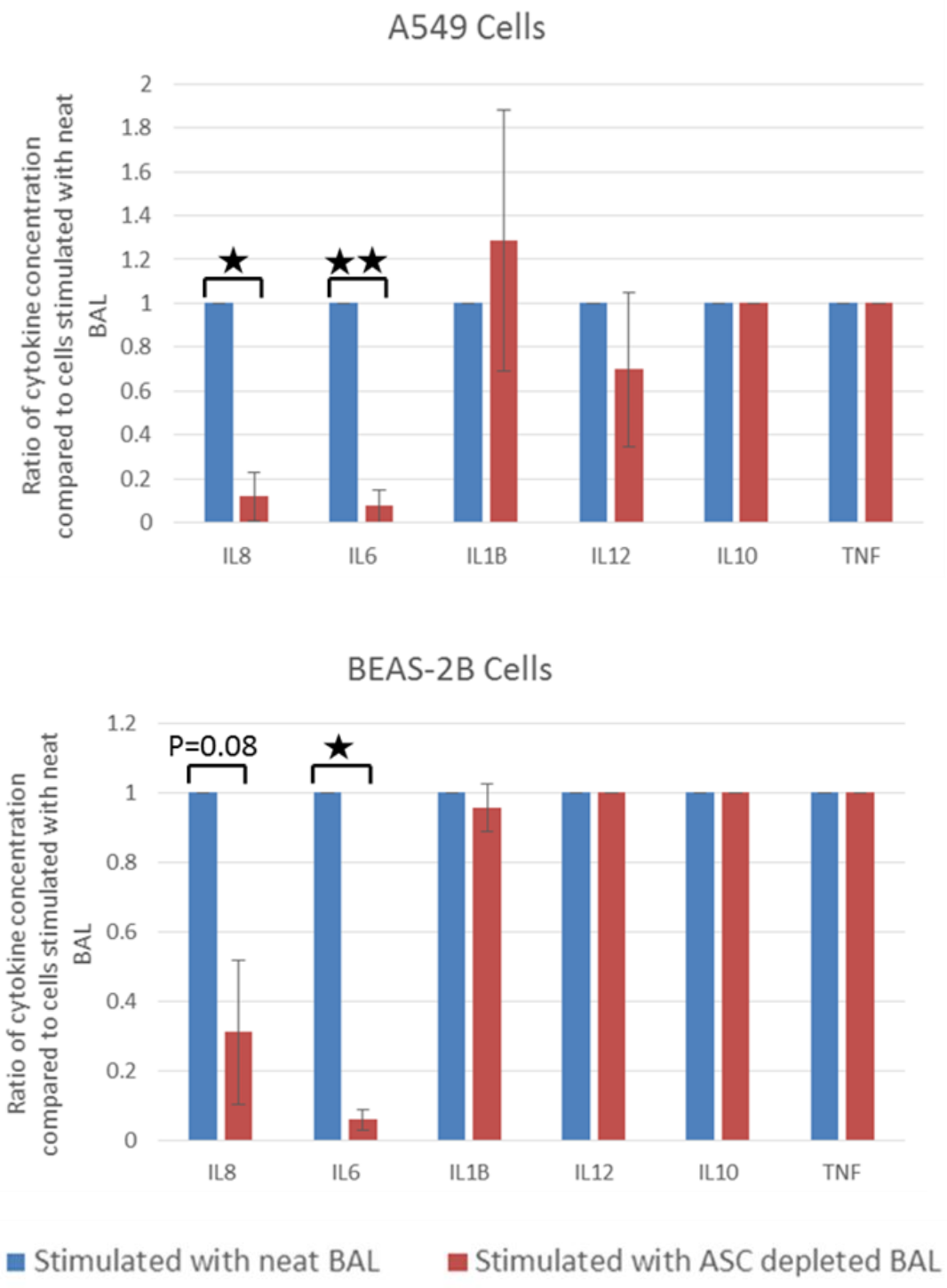


Figure 6-9: Cytokine concentrations in supernatant of cells stimulated with neat BAL and ASC depleted BAL.
Cytokine concentrations expressed as a ratio of cytokine concentration in cells stimulated with neat BAL after subtracting the concentration of cytokine in unstimulated cells. Error bars represent SE of the ratios. P values calculated using Wilcoxon signed rank test. * p<0.05, **p<0.01.

6.8 Producing recombinant ASC

To confirm the role of ASC in the production of IL-6 and IL-8 recombinant ASC (rASC) was produced and added to the BAL fluid in cell stimulation experiments. A plasmid containing the gene for ASC was kindly donated by Prof K Fitzgerald, University of Massachusetts Medical School. The plasmid was supplied within a frozen culture of *E. coli* bacteria. Details of the process by which the organism was grown and the ASC protein purified by immune-affinity chromatography is described in Section 2.16. Success of rASC purification was confirmed using SDS-PAGE to visualise a band of protein at the correct molecular weight and Western blot analysis to demonstrate specific and ASC antibody binding at the same molecular weight. Images taken from these analyses are shown below in Figure 6-10.

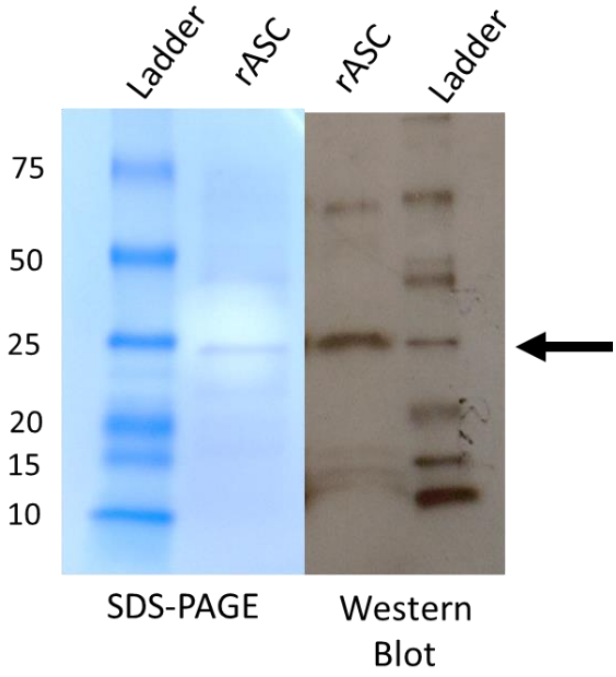


Figure 6-10: SDS-PAGE gel and Western blot image of purified rASC.

To test the bioactivity of the purified recombinant ASC, an experiment was designed to use different concentrations of the recombinant ASC to stimulate A549 and BEAS-2B cells. Each concentration was tested in duplicate in each cell line and the experiment repeated 3 times independently. The concentration of pro-inflammatory cytokines in the supernatant of the media following the cell stimulation was analysed using a cytometric bead array kit measuring IL-6, IL-8, IL-10, IL-12, IL-1β and TNF.

Figure 6-11 and Figure 6-12 below demonstrate the concentration of each cytokine in the supernatant following overnight stimulation in each cell line.

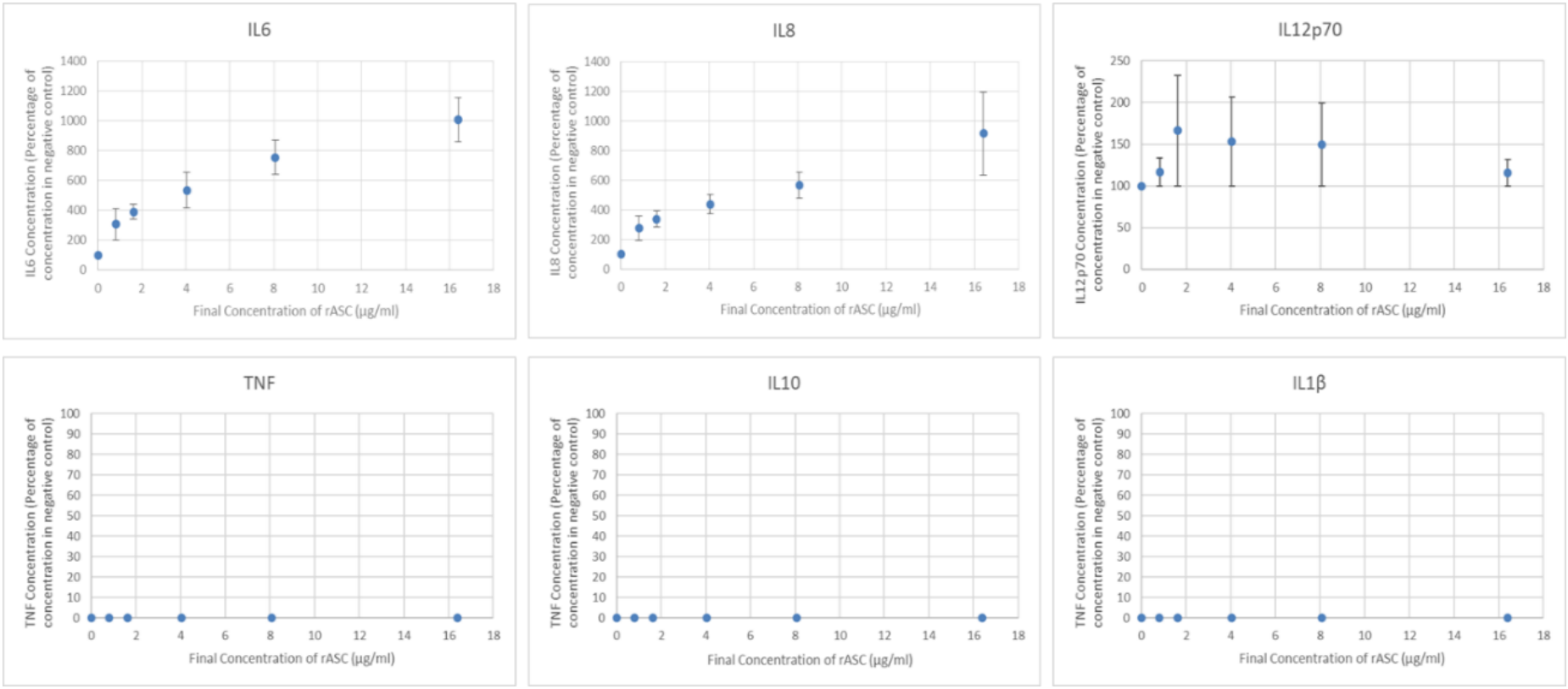


Figure 6-11: Cytokine concentrations in supernatant of BEAS-2B cells stimulated with varying concentrations of recombinant ASC

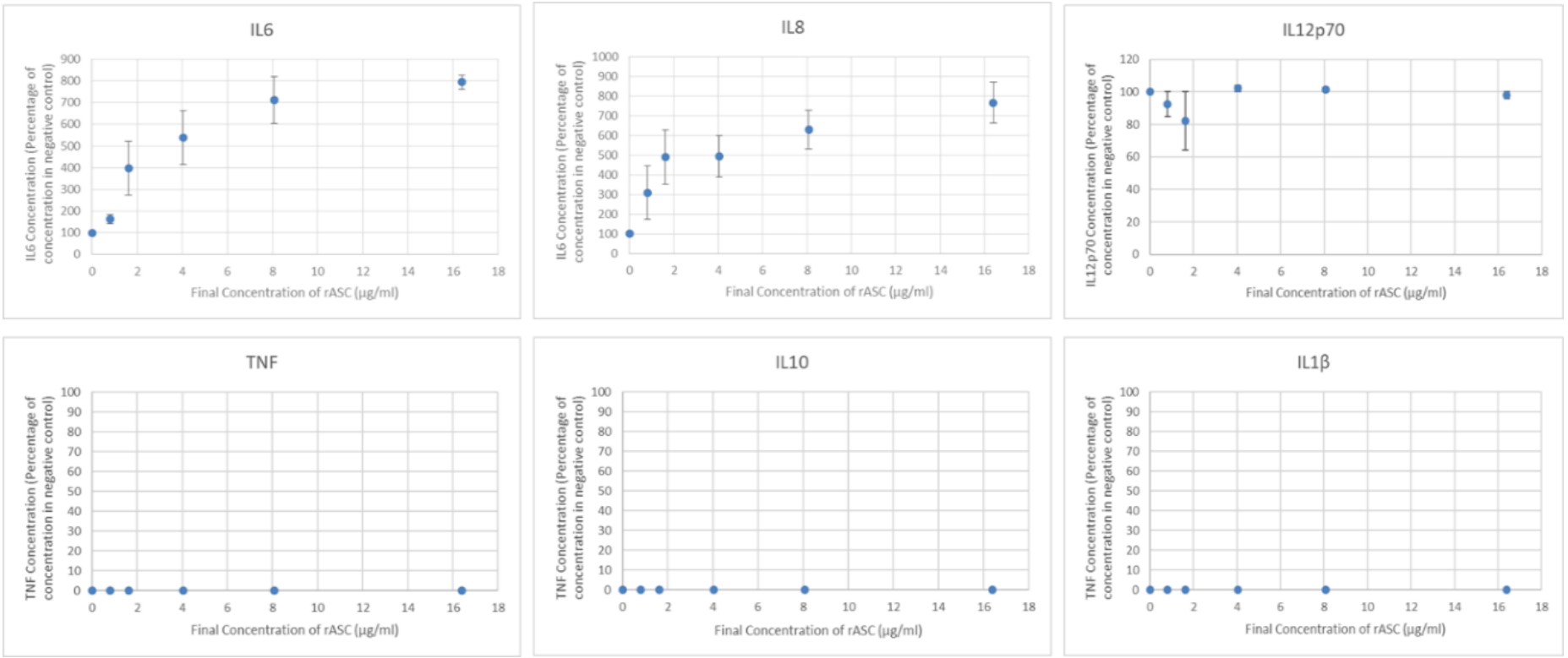


Figure 6-12: Cytokine concentrations in supernatant of A549 cells stimulated with varying concentrations of recombinant ASC.

Increasing the final concentration of ASC added to the cells, resulted in a dose-dependant increase in production of IL-6 and IL-8. There was relationship between ASC stimulation and IL-12 production. No IL-10, TNF or IL-1B were produced by either cell line in response to ASC stimulation even at the highest dose. The bioactivity pattern of the recombinant ASC is consistent with the cell stimulation experiments suggesting that ASC is capable of inducing cells to secrete IL-6 and IL-8 but not IL-1β, TNF, or IL-12. This suggests that the most likely mechanism of action of extracellular ASC is activating the NF-kB pathway resulting in IL-6 and IL-8 production.

6.8.1 Thiobarbiturate assay to exclude LPS contamination of rASC

To exclude contamination by LPS as being responsible for the induction of IL-6 and IL-8 in the previous experiments, the concentration of LPS within the rASC solution was calculated using a thiobarbiturate assay as described in Section 2.15.4. A standard curve from 0.25µg/ml to 1.5µg/ml of LPS from *E. coli* was produced. This can be seen below in Figure 6-13.

A further dilution of 25ng/ml of LPS was prepared but this was below the limit of detection of the assay. The rASC was tested in duplicate. Both samples demonstrated lower absorbance than the blank indicating that LPS was not detectable in the solution.

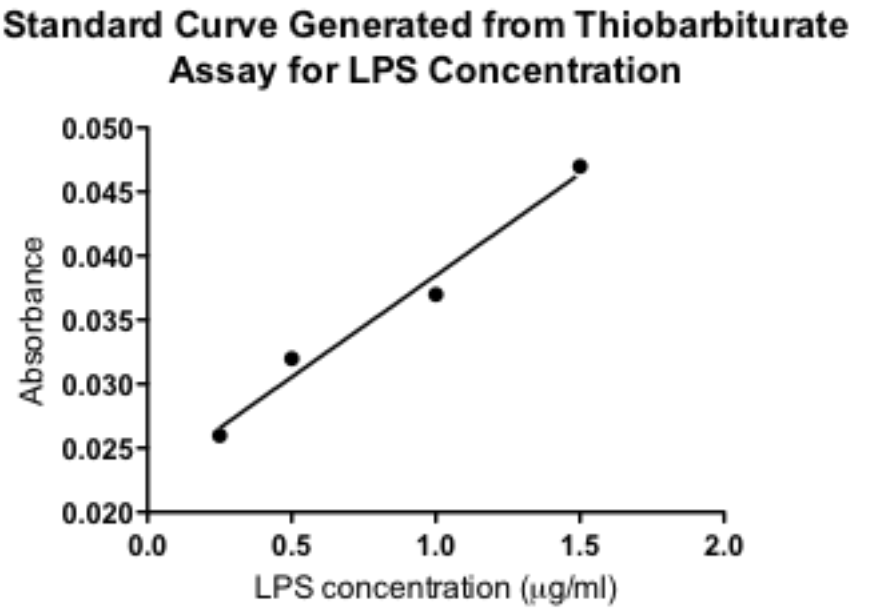


Figure 6-13: Standard curve generated from known concentrations of E. coli LPS in the thiobarbituate assay.
R^2 value = 0.98.

6.9 Cell stimulation using ASC depleted BAL with rASC

To confirm the role of ASC in the production of IL-6 and IL-8 rASC was re-added to ASC depleted BAL fluid. A549 and BEAS-2B cells were stimulated overnight under 4 conditions:

1. Negative control – serum free media only
2. BAL fluid (neat)
3. BAL fluid depleted for ASC
4. ASC depleted BAL fluid, replete with rASC at a concentration of 15 µg/mL

A549 cells were tested in triplicate in three independent experiments, and the BEAS-2B cells were tested in four independent experiments. In analysing the results the average concentration value of each cytokine obtained from the negative control wells was subtracted from the average of the cytokine concentration from each of the stimulation wells. To permit comparisons between the experiments, the values of the cytokines from the neat BAL wells were set at 1 and the other wells were expressed as decimal fractions of this value. The results of these experiments are shown in Figure 6-14 below.

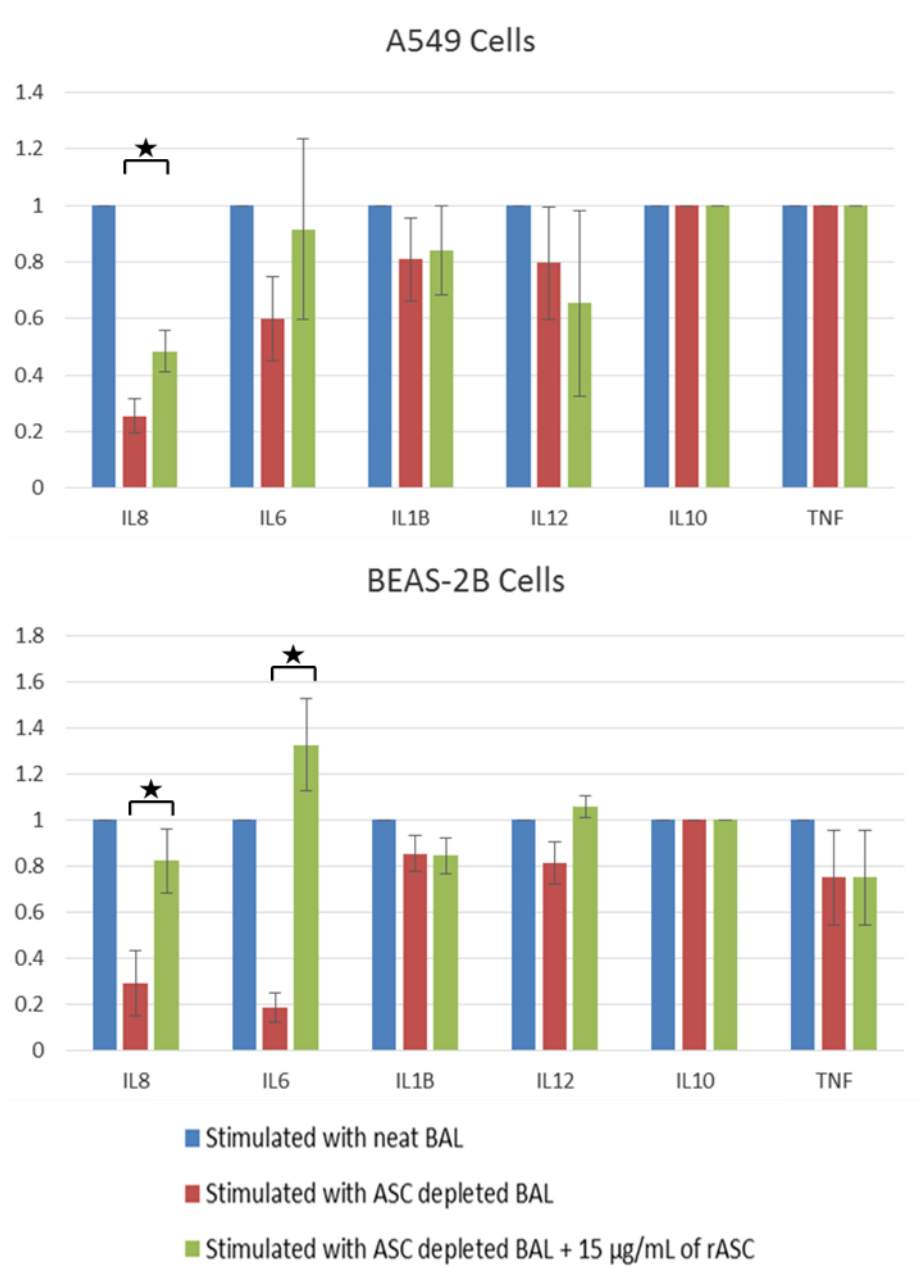


Figure 6-14: Cytokine concentrations in supernatants from BEAS-2B and A549 cells stimulated with BAL fluid.
The cytokine concentration of unstimulated cells was subtracted from the cytokine concentrations obtained from stimulated cells and expressed as a ratio of the cytokine concentration of the cells stimulated with neat BAL fluid. Error bars represent the SE of the ratios.*p<0.05.

This figure demonstrates reduced IL-6 and IL-8 secretion in cells stimulated by ASC depleted BAL fluid compared cells stimulated by undepleted BAL fluid. It also demonstrates that adding rASC to the BAL fluid results in an increase of IL-6 and IL-8 secretion compared to stimulation by ASC depleted BAL to levels similar to stimulation by un-depleted BAL especially in the BEAS-2B cells. IL-1β, IL-12, IL-10 and TNF were unaffected by the different stimulation conditions.

6.10 Attempts to characterise the mechanism of ASC induced cytokine stimulation

The cell stimulation experiments consistently demonstrated that ASC stimulated IL-6 and IL-8 secretion from A549 cells, as demonstrated by the reduction in cytokine secretion following ASC depletion of BAL fluid and the effect of rASC. IL-8 and IL-6 are most likely produced following induction of the NF-κB pathway leading to increased transcription of mRNA coding for IL-6 and IL-8. I hypothesised that ASC was binding a cell surface receptor, and triggering activation of the NF-κB pathway. I sought to further elucidate the mechanism of this action by blocking potential receptors which ASC could be binding to and activating the NF-κB pathway. The receptors known to activate the NF-κB pathway include, TLRs, IL-1β receptor, T-cell receptor, B-cell receptor, TNF receptor 1 and CD40. I decided to target TLR2 and TLR4 as cell surface TLRs most likely to bind ASC. I set out to knock down the TLR2 and TLR4 expression and stimulate knockdown and wild type cells with ASC and analyse differences in cytokine secretion.

Specific inhibitory RNA (siRNA) molecules are produced from plasmids in transfected cells preventing the translation of RNA from the nucleus into protein. I used commercially available plasmids for siRNA directed at TLR2 and TLR4 to attempt to knock down expression of these receptors in A549 and BEAS-2B cells. The procedure for producing the plasmid in *E. coli*, purification and transfection of the plasmid into A549 and BEAS-2B cells is given in Section 2.16. Transformation and propagation of the TLR2 and TLR4 knockdown plasmids within E. coli was successful and plasmids were purified using a phenol-chlorophorm DNA extraction technique. Successful purification of the plasmids was shown by agarose gel electrophoresis (see Figure 6-15 below).

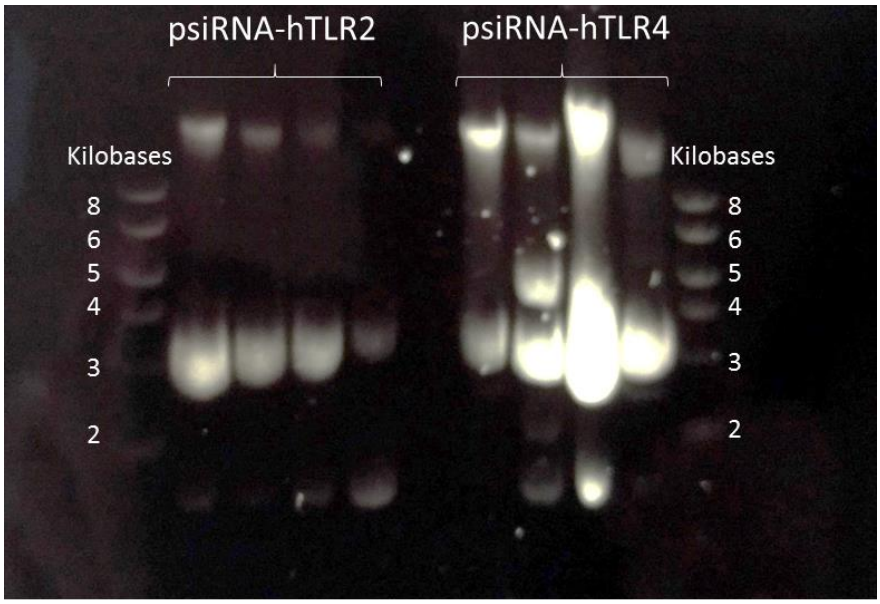


Figure 6-15: Gel electrophoresis of purified DNA plasmids for the production of siRNA of TLR2 and TLR4.
Ethidium Bromide staining visualised under UV light. Molecular weight of each plasmid = 3591bp.

Western blot of equal protein concentrations from cell lysates of transfected and wild type cells were used to determine success of transfection and knocking down of the relevant TLR. Despite attempting multiple transfection conditions using lipofectamine 2000 I was unable to successfully knockdown TLR2 or TLR4 expression. Western blotting images demonstrated no difference in bands between transfected and wild type cells in the A549 cells when probing for TLR2 and TLR4 (Figure 6-16). All attempts to transfect BEAS-2B cells resulted in cell death for all cells during the transfection process despite altering the time cells were incubated in Optimem reduced serum media.

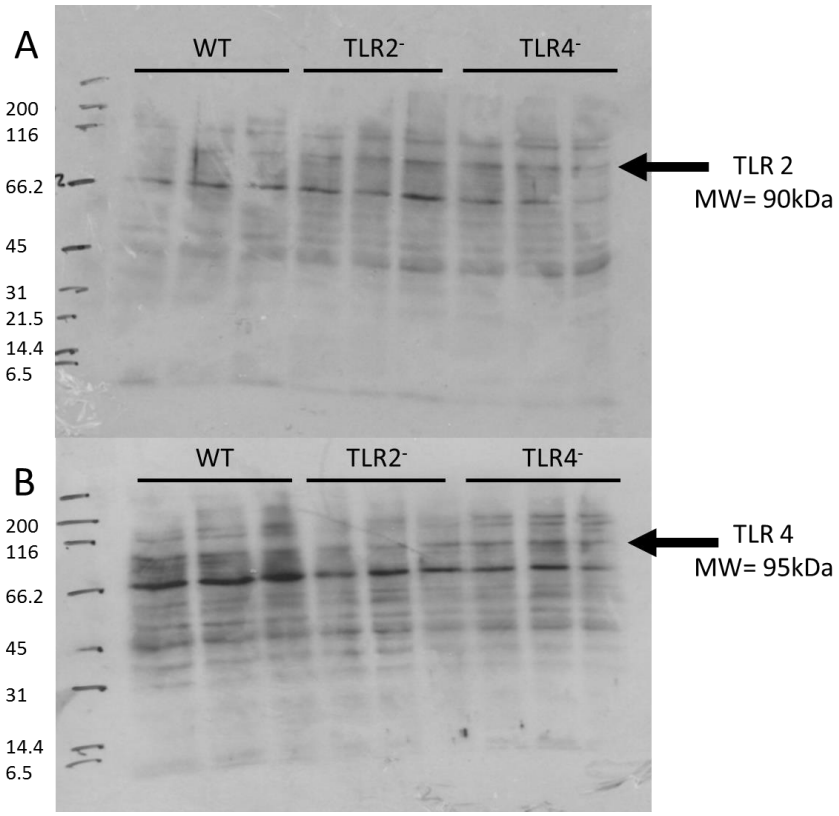


Figure 6-16: Western blot images of lysates of A549 cells following attempted knockdown of TLR 2 and 4.
A- Probing for TLR2. B- Probing for TLR4. WT = Wild type cells. TLR2$^-$ = cells attempted to knockdown TLR2. TLR4$^-$ = cells attempted to knockdown TLR4. Lysates loaded equalised for protein concentration. Images show bands of similar density to WT cells at correct molecular weight for TLR 2 and 4 in lanes containing lysates of cells attempting to knock down production of the targeted proteins. This demonstrates the failure of the knockdown process.

6.11 Discussion

6.11.1 Inflammasome components as a biomarker for CLD within the lungs of preterm infants

The data from Western blotting for inflammasome components within BAL supernatant suggests that components of the NLRP3 inflammasome are poor biomarkers for CLD as no differences were detected between the CLD and RDS groups. The Western blots used to generate the presented data were run using equal sample volume loading of wells. Using equal sample volumes and equal sample protein have been previously published using neonatal BAL samples (Davies et al. 2010). Total protein concentration of samples may vary with volume of lung lining fluid returned, a factor difficult to control for. Most Western blots are loaded with equal amounts of protein. However, protein concentration has been shown to be a poor indicator of lung lining fluid concentration in BAL samples due to protein leak in inflammatory disorders (Watts and Bruce 1995). The ERS guidelines for BAL in children (de Blic et al. 2000) suggest not correcting concentration values for protein (or other measures of lung lining fluid such as urea) partly because protein concentration within the lungs of preterm infants is affected by

CLD disease severity and episodes of sepsis (Watts and Bruce 1995). The presented data for day 1 demonstrated the difference between correcting for protein concentration and not doing so. The protein corrected results were used in this study as the pattern of results (with higher amounts of ASC and caspase-1p10 detected in the term infants) is likely to be explained by the increased protein concentration and not by a biological difference.

Previous work has found an important role for NLRP3 inflammasome activation in the development of CLD in preterm infants (Liao et al. 2015). The results of the Western blots for components of the NLRP3 inflammasome show no difference between the infants with and without CLD. Inflammasome activation is an intracellular process with release of inflammasome components only occurring after cell death. My study utilised cell free supernatant from BAL samples centrifuged prior to freezing. Any inflammasome components detected within these samples, therefore, originated from the infant's extracellular lung lining fluid and must then come from cells already dead and lysed prior to sample centrifugation. As the samples were transported and processed immediately it can be inferred that the detected inflammasome components are from cells already dead when the sample was collected.

Many studies have demonstrated an exaggerated and disorganised inflammatory response in the lungs of preterm infants who develop CLD. It is also known that greater numbers of neutrophils are present during the first week of life in the lungs of infants who go on to have CLD compared to those who do not get CLD (S. Kotecha et al. 2003). Therefore, it might be expected that more inflammasome components would be released from the greater numbers of white blood cells in the lungs of infants in the CLD group. It has been shown, however, that neutrophils of infants with CLD survive longer than infants who develop RDS (S. Kotecha et al. 2003), which may explain why no difference was witnessed between the two groups. Preterm infants are also known to have increased protease activity within the lungs compared to term infants. This may result in increased degradation of extracellular inflammasome components contributing to the results. Extracellular ASC has been shown to be phagocytosed by white blood cells so extracellular lung lining fluid may not accurately reflect the level of inflammasome activation if it is internalised within cells. Liao et al used whole lung samples from mice models to identify inflammasome components therefore detecting intracellular inflammasome activation, only using BAL samples to identify IL-1β and its receptor antagonist (Liao et al. 2015).

The data for Day 1 shows a higher concentration of ASC and caspase-1p10 in the samples from term infants than preterm infants (Figure 6-5). The effect narrowly loses statistical significance for ASC when corrected for protein concentration, however given the small numbers in this study, this is still suggestive of a difference between the two groups. Given the limitations previously discussed of using the inflammasome components as biomarkers of inflammation, it

remains unexpected to have higher levels of ASC and caspase-1p10 in the term infants group, suggesting increased inflammatory activity in the term over the preterm infants. TLR and NLRP3 mediated responses have been shown to affected by gestational age using cord monocytes obtained from preterm infants (Sharma et al. 2014; 2015). Delayed expression and activation of inflammasome proteins due to reduced expression within the lungs of preterm infants could explain the lower detection of inflammasome components. The caspase-1p10 result could also be explained by the protein concentration of most preterm sample being too low to detect caspase-1p10, a fact that would not be corrected by the adjustment for protein concentration, however, the pattern of higher ASC and capsase-1 levels together would be expected in a genuine effect suggesting the caspase-1p10 result is likely to be genuine. NLRP3 levels would be expected to mirror those of ASC and caspase-1 following NLRP3 inflammasome activation, however many other inflammasomes utilise ASC and caspase-1 (Lu et al. 2014). Activation of these inflammasomes could result in higher extracellular ASC and caspase-1 levels without a rise in NLRP3. Another explanation for the lower concentrations of ASC and caspase-1p10 in preterm compared to term BAL fluid could be due to consumption of these components within preterm lungs. Greater protease activity could degrade these products more quickly in preterm infants.

Overall the results of Western blotting for inflammasome components have shown that this methodology does not provide a good biomarker for CLD in preterm infants. The presence of inflammasome component within BAL samples from ventilated preterm and term infants corroborates the evidence of previous studies that inflammasomes are activated within the lungs of ventilated preterm infants (Cayabyab et al. 2003; Liao et al. 2015). However, the role of NLRP3 in CLD disease pathophysiology as identified by Liao et al could not be corroborated by my results.

6.11.2 Producing rASC

Recombinant proteins may differ from naturally occurring proteins in post-transciptional modifications. My recombinantly produced ASC lacked glycosylation and protein folding can be different in prokaryotic cells compared to eukaryotic cells (Overton 2014). My methodology, however, resulted in protein production at the correct molecular weight that bound anti-ASC antibody on Western blot. The bio-activity of the rASC was that expected from the experiments depleting ASC from BAL samples. The data, therefore, suggests that the protein that I purified was rASC which retained biological activity despite recombinant production.

6.11.3 Cell Stimulations

The cell stimulation experiments consistently demonstrated the effect of extracellular ASC causing an increase in IL-6 and IL-8 secretion. This result was surprising as published works

suggest that extracellular ASC acts as a prion like molecule and induces inflammasome formation when phagocytosed. This should result in the secretion of IL-1β, with an insignificant change in IL-6 and IL-8 concentration. The previous studies did not publish any data for IL-6 and IL-8 and it is unclear whether these cytokines were tested for in their experiments (Baroja-Mazo et al. 2014; Franklin et al. 2014; Franklin, Latz, and Schmidt 2018). I observed a pattern of cytokine induction inconsistent with extracellular ASC causing intracellular inflammasome activation, with no change in IL-1β secretion. An increase in IL-6 and IL-8 is suggestive of induction of the NF-KB pathway.

Activation of the NF-kB pathway results in the first of the two signals required for inflammasome activation. The lack of IL-1β secretion in response to ASC stimulation in my data suggests the second signal for inflammasome activation was not activated during cell stimulations. A549 and BEAS-2B cells have been shown to have the potential to undergo inflammasome activation (Y Wang et al. 2016; Peeters et al. 2013); however, the different cell lines used in the different studies may explain the difference in effect with the majority of studies using macrophage cell lines to study inflammasome activation and the effects of extracellular ASC.

Lung lining fluid from preterm infants is known to contain pro-inflammatory cytokines (Schneibel et al. 2013), LPS (Lohmann et al. 2014) and other compounds capable of inducing a pro-inflammatory effect. It is possible that the immunoprecipitation process co-precipitated other unintended constituents of BAL fluid. The reduction in IL-6 and IL-8 concentration following stimulation with ASC depleted BAL to was to levels of only around 10% of the IL-6 and IL-8 concentrations when stimulated with un-depleted BAL. This marked decrease is a larger effect than was expected given the multiple constituents of BAL known to induce IL-6 and IL-8 secretion, raising the possibility that other mechanisms of IL-6 and IL-8 secretion may have been affected by the immunoprecipitation process. Other pro-inflammatory components of BAL should still be inducing the NF-κB pathway despite ASC depletion. Each time ASC was immunoprecipitated from BAL fluid the success of the ASC depletion was confirmed by Western blotting of the immunoprecipitated prior to cell stimulation, but other co-precipitants were not specifically investigated.

ASC is known to form macromolecular complexes as part of inflammasomes and ASC specks (Kuri et al. 2017). It is not fully understood what happens to such complexes following cell death and release of these complexes extracellularly. Other compounds bound to ASC as part of macromolecular complexes are likely to be removed alongside ASC during immunoprecipitation. This could cause the reduction in IL-6 and IL-8 secretion seen following immunoprecipitation for ASC. The addition of rASC to immunodepleted BAL addressed this

issue. The pattern of cytokines induced by rASC was the same as the pattern of cytokines depleted by ASC immunoprecipitation suggesting the same molecule is involved.

The thiobarbiturate assay was used to ensure the rASC was not contaminated with LPS. From my work it is impossible to exclude contamination of rASC with LPS below detection levels of the thiobarbiturate assay.

Recently published work has demonstrated that ASC is susceptible to degradation through freeze-thaw cycles (Ulke-Lemée et al. 2018). It is likely that the rASC would also be suscepticle to similar degradation. After purification the rASC used in this work was divided into 20 µL aliquots and a new aliquot used for each stimulation experiment. The risk of degradation of rASC affecting the experiments is therefore minimal.

When designing the cell stimulations involving rASC it was impossible to know what physiological concentrations of extracellular ASC exist in the lung, or in BAL fluid to attempt to reproduce such conditions. The concentration chosen was based on the dose-response experiments and comparing the cytokine concentrations of IL-6 and IL-8 achieved with those achieved from BAL stimulation.

I have demonstrated a consistent pattern of extracellular ASC stimulating IL-6 and IL-8 production, a phenomenon previously not described, which was repeatable in two respiratory cell lines. Both IL-6 and IL-8 are powerful chemoattractants for neutrophils. It is possible that ASC signalling may play an important role in CLD development if by activating IL-6 and IL-8 production, ASC is contributing to the influx of neutrophils. ASC therefore may be a useful therapeutic target for preventing CLD and other pulmonary diseases associated with inflammation such as COPD.

6.11.4 Role of TLRs

Unfortunately attempts to further investigate the mechanism of action of ASC inducing IL-6 and IL-8 secretion were unsuccessful. A549 cells are known to express TLR2 and TLR4 (Guillot et al. 2004; Zhihong Zhang et al. 2005) and have been used in previous published studies involving transfection of plasmid DNA (Marcos-Vadillo and García-Sánchez 2016). Successful knockdown of cell surface TLR receptors would have allowed me to investigate further the method of activation of ASC on the A549 cells. Potential transfection inhibitors (e.g. antibiotics) were removed and cells were at optimum densities during attempted transfections. The modifications made to the protocol to try to overcome the difficulties of achieving successful transfection were changing the final lipofectamine/DNA concentrations, changing the length of time the cells were incubated with the lipofectamine/DNA complexes and changing proportions of lipofectamine to DNA ratio used to produce the lipofectamine/DNA complexes.

The cells repeatedly did not grow and began to decline in number and appearance once the selection antibiotic (Zeocin) was added to the media. The failure of the experiment was demonstrated by the cell's reaction to the addition of the selection antibiotic. This suggested that the cells were not resistant to Zeocin, implying they had not taken up or were not translating the plasmid. The failure of the transfection may be down to the protocol using volumes of extracted plasmid DNA rather than specific concentrations of plasmid DNA. The failure to quantify the DNA concentration of the plasmid DNA may have resulted in an inappropriate concentration of DNA being used despite varying the volume of plasmid DNA used to adjust the concentration. Other explanations could be that the plasmid DNA contained a high level of endotoxin which was harmful to the cells, however the phenol-chlorophorm DNA extraction was chosen specifically for the purity of the DNA obtained and to minimise carry over of contaminants such as LPS. Another possibility would be the if the passage number of the cells was excessively high, however following the initial difficulties cells from early stocks were revived to overcome this issue, but problems with the transfection failure persisted. Cells were always incubated in Optimem reduced serum media during the transfection process as per standard practice in our laboratory with all cell lines. Possibly the viability of cells was affected by the reduced serum concentration affecting the transfection efficiency.

6.11.5 Direction of further work

This study has identified a potentially important pathway warranting further investigation. Further work to verify my results would benefit from placing rASC through a step of endotoxin removal to reduce the chance of any low concentrations of LPS from affecting the results. Identification of the mechanism by which ASC induces IL-6 and IL-8 is an important next stage. Refining the TLR knockdown experiments that I was unable to complete to obtain successful knock down of TLR2 and TLR4 and observing the effect of this on cytokine secretion by respiratory epithelial cells would useful. Alternative approaches would be the use of function blocking antibodies to TLRs or using CRISPR (clusters of regularly interspaced short palindromic repeats) to edit the genes for TLR2 and TLR4 making them non-functioning.

A new technique has been published for measuring ASC concentration in biological samples (Ulke-Lemée et al. 2018). Using similar techniques to measure ASC concentration in BAL fluid or serum of preterm infants and comparing the results from infants with and without CLD would provide more evidence to the biological relevance of extracellular ASC.

Further experiments targeting other components in the NF-κB pathway would be needed to confirm this pathway was involved.

Imaging studies using fluorescently labelled ASC and fluorescently labelled TLRs would allow visualisation of co-location of ASC and a specific TLR if a TLR was implicated in binding extracellular ASC from the previous experiments.

6.12 Conclusion

The work in this chapter adds to the literature regarding the role of extracellular ASC in preterm infants. Previous studies have highlighted an important role for the NLRP3 inflammasome in CLD development (Liao et al. 2015). I aimed to demonstrate the pro-inflammatory potential of ASC in neonatal BAL fluid from preterm infants if inflammasome components could be detected in BAL fluid from preterm infants. I have shown that components of the inflammasome are detectable within BAL fluid from preterm and term ventilated infants, supporting the suggestion that inflammasome activation is occurring in the lungs of ventilated infants.

I did not detect increased levels of inflammasome components in BAL from infants who developed CLD however this should not contradict the work of Lieu et al, for the reasons given in Section 6.11.3, given the extracellular nature of BAL fluid.

Published work has shown that extracellular ASC retains pro-inflammatory potential when released from cells (Baroja-Mazo et al. 2014; Franklin et al. 2014). I aimed to examine the pro-inflammatory potential of ASC in neonatal BAL fluid from preterm infants. I have demonstrated that ASC present in BAL fluid from preterm infants has the potential to induce inflammatory effects in cells via IL-6 and IL-8. The effect of ASC inducing IL-6 and IL-8 secretion has been consistently found both by depleting ASC from BAL and using rASC to stimulate two respiratory epithelial cell lines in culture. The pattern of cytokine secretion induced by ASC is a new finding, not previously reported.

I aimed to determine the mechanism of action of the pro-inflammatory effect of extracellular ASC on respiratory epithelial cells in culture. I was unable to implicate the NF-κB pathway due to time constraints, but this would be an important next step in understanding how extracellular ASC is inducing a pro-inflammatory reaction in respiratory epithelial cells. Production of IL-6 and IL-8 is suggestive of activation of the NF-kB, likely via cell surface or cytoplasmic receptors.

IL-6 and IL-8 concentration were associated with a positive bacterial load in BAL and TA samples from preterm lungs of infants (Chapter 3 and Chapter 4). Previously the rises in NF-kB induced cytokines has been explained by TLR activation in response to bacterial infection. My work suggests that extracellular release of ASC may contribute to production of IL-6 and IL-8 within the lungs.

IL-6 and IL-8 are powerful neutrophil chemoattractants. Neutrophil chemotaxis into the lungs of preterm infants is known to be associated with CLD. The role of extracellular ASC that I have identified in causing IL-6 and IL-8 secretion from respiratory epithelial cells is a potential therapeutic target for preventing CLD in preterm infants. Further work is necessary to understand the mechanism of this effect before targeted therapeutic interventions can be developed. Further investigation is also warranted to identify whether ASC has a similar pro-inflammatory effect in other pathological states beyond the neonatal period.

Chapter 7: General Discussion

7 General Discussion

7.1 Introduction

This thesis has addressed important questions regarding early bacterial colonisation of preterm infants at risk of CLD, describing colonisation patterns and identifying associated immunological responses to bacterial colonisation of the airways. Chapter 3 analysed samples from the lower respiratory tract (TA) and lungs (BAL) demonstrating that the early bacterial colonisation of these mucosal surfaces is an infective, dysbiotic process in intubated preterm infants. Chapter 4 focused on upper airway bacterial colonisation of preterm infants showing changes over time and associations with mode of delivery that had previously not been demonstrated in preterm infants. Comparisons between bacterial colonisation between the 3 anatomical sites were made in Chapter 5. The final results chapter examined the role of extracellular ASC within BAL fluid from preterm infants looking at a potential mechanism by which the early bacterial colonisation of the airways may influence neonatal immunology and potentially the risk of developing CLD.

Bacterial colonisation of the lungs of healthy individuals, a concept once considered non-existent, is now well recognised and accepted (Sze, Hogg, and Sin 2014; Twigg et al. 2013). The introduction of culture independent techniques has transformed understanding of the complexity and variety of bacterial communities colonising many anatomical niches of human hosts.

Despite advances in obstetric and neonatal care preterm birth remains a significant issue and rates of CLD of prematurity remain static (Stoll et al. 2015). The results presented in this thesis focus on infants at risk of CLD due to their gestation at birth and need for mechanical ventilation. The very high rate of CLD in preterm infants recruited to this study is reflective of modern neonatal practice, only ventilating the most unwell and most extreme preterm infants. Infants needed to be ventilated at the time of recruitment to meet the inclusion criteria, resulting in exclusion of those preterm infants managed on non-invasive ventilation and those only briefly intubated. The interventions as part of this study are unlikely to have affected outcomes of recruited infants as previous work has shown BAL sampling, the only extra procedure undertaken by infants to be safe (B. Morrow and Argent 2001; Vyas et al. 2002) and the rate of moderate or severe CLD was higher in the NBT collection site (94.3%), where BAL samples were not taken, than the UHW collection site (65%).

7.2 Early bacterial colonisation of the respiratory tract of preterm infants

This is the first study to longitudinally examine the upper and lower respiratory tract bacterial colonisation in preterm infants. Low bacterial load was detected in NPA, BAL and TA samples

throughout the first month of life, with the lowest bacterial load detected in days 1-3 of life. The literature contains conflicting reports on whether bacterial colonisation occurs *in-utero* (DiGiulio et al. 2008; Collado et al. 2016; Perez-Muñoz et al. 2017). While the work in thesis does not address this issue directly, the data does not support evidence of widespread *in-utero* colonisation, given the low bacterial loads in the first few days of life, showing a rising trend after birth.

Sequencing was not possible on 78% of all samples from Cardiff and Bristol due to low bacterial loads. This is suggesting that colonisation respiratory tract is at very low levels in preterm infants or that respiratory colonisation may be delayed in preterm infants. Detection of bacterial DNA in BAL and TA samples of recruited infants was associated with a significantly higher concentrations of the pro-inflammatory cytokines IL-6 and IL-8. Peaks of IL-6 and IL-8 concentration in BAL samples from intubated preterm infants in the first few weeks of life are well recognised (S. Kotecha, Wilson, et al. 1996; Beeton et al. 2011; S. Kotecha et al. 1995), with one study suggesting the presence of predominant organisms associated with these peaks (Beeton et al. 2011). The data from this thesis corroborates this previous work but builds on this showing the changes over time in bacterial load associated with changes in IL-6 and IL-8 concentration. The association of bacterial load and specific dominant OTUs in BAL and TA samples with peaks in IL-6 and IL-8 concentration is suggestive of an infective process within the lungs of preterm infants in the first weeks of life. This is an example of probable dysbiosis in the airways and lungs of preterm infants, with this pattern of early bacterial colonisation likely to influence the later development of CLD.

This study does not answer the question of whether this infective dysbiotic process is a pathological state specific to ventilated preterm infants or whether it is witnessed in healthy term infants. A small number of BAL and TA samples were collected from ventilated term infants with no underlying lung disease (ventilated for abdominal surgery), however, no samples were successfully sequenced for bacterial DNA, suggesting low bacterial loads in normal term infants. Other studies have also identified the presence of dominant OTUs within respiratory samples from ventilated preterm infants (Lal et al. 2016; Mourani et al. 2011; Lohmann et al. 2014). My work is the first to present this data in combination with associated inflammatory cytokine profiles. It is possible that the lungs of healthy infants undergo an inflammatory reaction to early colonisation with dominant organisms. The only way to investigate this would be with invasive sampling from healthy term infants. Local innate immune response to early bacterial colonisation have been identified in well term infants as potentially being responsible for the benign skin lesions of erythema toxicum witnessed in many apparently healthy infants (Marchini et al. 2005). It is possible that the results of this study indicate a similar process occurring in the lungs of preterm infants. More likely, however, is that

bacterial colonisation is inducing a harmful inflammatory response which may contribute to CLD development (S. Kotecha, Wilson, et al. 1996; Davies, Maxwell, and Kotecha 2006).

Antibiotics may therefore have a role to play in reducing the risk of CLD development. The evidence presented in Chapter 3 and Chapter 4 showed that NPA, BAL and TA samples were less likely be successfully sequenced for bacterial DNA when the infant was receiving antibiotics. This suggests that antibiotics are effective at suppressing bacterial colonisation of the lungs in preterm infants and may, therefore, prevent CLD. Previous evidence suggests however that antibiotic use is associated with increased risk of CLD (Cantey et al. 2016), but it is likely the sickest infants receive more antibiotics. Current practice is to attempt to reduce antibiotic use in preterm infants due to the risk of NEC, effects on microbiota acquisition and concerns over emerging bacterial resistance to antibiotics (Goel et al. 2019). These results suggest that antibiotics in preterm infants may have a beneficial effect of reducing lung inflammation by suppressing bacterial load. All the preterm infants recruited to this study received antibiotics after birth which may have affected airway and lung colonisation. Concentrations of IL-6 and IL-8 peaked associated with detection of bacterial DNA during antibiotic therapy suggesting organisms not treated with first line antibiotics were responsible. Antibiotic resistance and organisms outside the spectrum of action of these antibiotics may be the cause of these episodes.

Reverse causality must be considered – that an increase in pro-inflammatory activity creates an environment allowing a bloom in bacteria. This is, however, unlikely due to the release of proteases into the lungs associated with the inflammatory response (Davies et al. 2010) and the fact that the innate immune system is designed primarily to destroy invading microorganisms.

Consistent with some other studies of the neonatal respiratory microbiome, the early respiratory colonisation of the upper and lower respiratory tract was dominated by Proteobacteria and Firmicutes (Lal et al. 2016; Lohmann et al. 2014; Wagner et al. 2017). Greater than 90% of NPA, BAL and TA samples were dominated by a single OTU (>50% of reads from single OTU). This is also consistent with the previously published works on neonatal respiratory colonisation of preterm infants which found the same dominant phyla in TA samples from preterm infants (Lal et al. 2016; Lohmann et al. 2014; Wagner et al. 2017).

Previous studies have described a difference in early bacterial colonisation of the gut between vaginally delivered and caesarean section delivered term infants (Rutayisire et al. 2016; Shao et al. 2019). My work was able to replicate this in preterm infants in both the upper and lower respiratory tract an increase in *Staphylococcus* was seen in caesarean section delivered infants. Vaginally delivered infants had a greater proportion of *Mycoplasma* and *Acinetobacter* in TA samples and of *Serratia* in NPA samples. These findings would be consistent with respiratory microbiota acquisition at birth being influenced by early exposure to either maternal vaginal

flora or skin organisms depending on the mode of delivery. The BAL samples showed no differences with delivery mode. This is likely to be the effect of a smaller sample size.

The longitudinal nature of this study enabled changes over time to be studied. The proportion of Firmicutes and Proteobacteria in the lower airways samples remained stable over the first month of life, however a progression from Firmicutes dominated profiles to more Proteobacteria dominated profiles was seen in NPA samples. This transition has previously been witnessed in the nasopharynx of term infants (Bosch et al. 2017) but this is the first time this has been witnessed in preterm infants.

NPA and TA samples were collected from two recruitment centres with statistically significant differences identified in average community structure between the two sites. Comparing the differences between the two sites showed differences which could not fully be explained by an outbreak of *Acinetobacter Baumannii* at one centre. This suggest that the environment, staffing and postnatal exposures on a neonatal unit are important in microbiota establishment. The evidence from the gut microbiota demonstrates influences of colonisation patterns over multiple diseases throughout the life course (Moran and Shanahan 2014; J. Kelly et al. 2016; W. Chen et al. 2012). Neonatologists and neonatal nurses, therefore, have a responsibility to help establish a "healthy microbiome" in preterm infants, which may include a healthy respiratory microbiome. The optimal microbiota is assumed to be that obtained through vaginal delivery, skin to skin contact with the mother and exclusive breast feeding without exposure to antibiotics. Most preterm infants do not have this pattern of early life experiences. With regard to the respiratory microbiome there is limited evidence that the early colonisation may impact CLD (Lal et al. 2016; Lohmann et al. 2014; Pammi et al. 2018; Wagner et al. 2017) and some evidence of early colonisation with known respiratory pathogens in the upper respiratory tract of healthy term infants can affect long term respiratory health (Biesbroek, Tsivtsivadze, et al. 2014). The role of oral probiotics in establishing an optimum gut microbiota with clinical affects in preterm infants is controversial (Costeloe et al. 2015). My work suggests that further work to assess the role of oral probiotics on the respiratory colonisation is warranted as potential respiratory pathogens were detected in smaller proportions in infants taking probiotics compared to those not on probiotics. A mechanism for a potential effect is possible through dendritic cell antigen presentation. Probiotics lead to dendritic cells producing the regulatory cytokines IL-10 and IL-12 affecting the T-helper cell population. Increased pathogen clearance from modified T-helper cells responses may then reduces carriage of respiratory pathogens (Forsythe 2011).

Comparing the BAL, TA and NPA samples types collected from preterm infants suggests that each sample type is detecting separate bacterial populations. Significant differences were detected in the community structure of the samples from each anatomical site as shown by the PERMANOVA analysis. Work in children and adult patients has also found differences

between the upper and lower airway microbiota (Bassis et al. 2014; Marsh et al. 2016) and between different sites within explanted lungs (Erb-Downward et al. 2011).

Alpha diversity indices showed no differences between the three sites in measures of evenness, showing the level of dominance of the most abundant OTU was similar in each location. Measures of richness did, however, show differences with NPA samples showing surprisingly overall lower numbers of OTUs per sample than samples from the lower airways. One reason for this may be the presence of an oral, un-cuffed ET tube separating the upper and lower airways. The ET tube colonisation may increase the number of OTUs detected on samples collected via the ET tube. The ET tube also results in no air flow through the nasopharynx during respiration, which may affect the number of organisms colonising the nasopharynx. Lower airway colonisation is thought to occur mainly through micro aspiration based on work in adults (Bassis et al. 2015). An ET tube may inhibit this process thus affecting colonisation of the lower airways.

7.3 Microbiota and innate immunity

Data from Chapter 3 demonstrated a relationship between bacterial colonisation and an innate immune response. A rise in IL-6 and IL-8 concentrations within BAL and TA samples was associated with successful amplification of the bacterial 16S rRNA gene. IL-6 and IL-8 are powerful neutrophil chemoattractants responsible for the influx of neutrophils to the lungs in the first week of life (Munshi et al. 1997; S. Kotecha et al. 1995). In Chapter 6, I presented data from my study of one branch of the innate immune system focusing on extracellular ASC. Formation of inflammasomes requires the adaptor molecule ASC. Following pyroptosis, ASC is released and there is evidence that it can act as a cell signalling molecule to propagate the immune response (Baroja-Mazo et al. 2014; Franklin et al. 2014). I was able to detect extracellular ASC within the cell free supernatant of neonatal BAL samples; this has not previously been reported.

Further work didn't find differences between inflammasome components in BAL supernatant comparing infants with RDS that resolved and infants who went on to develop CLD. When samples taken on day 1 of life from preterm and term infants were compared higher concentration of the inflammasome components ASC and Caspase 1p10 were detected BAL supernatant from term infants. This finding may indicate a delay in maturation of the preterm innate immune system which is known to occur with other aspects of the preterm innate immune system (Zasada et al. 2014).

Further work looking at the functional effects of extracellular ASC in BAL fluid suggested that ASC induces IL-6 and IL-8 secretion from respiratory epithelial cells in culture, a finding that was verified using recombinantly produced ASC. Previous work suggests that extracellular ASC acts by inflammasome activation and should induce IL-1β secretion from cells (Franklin et

al. 2014), so my findings are very interesting. Rises in IL-6 and IL-8 concentration were associated with detectable bacterial DNA in BAL and TA samples. IL-6 and IL-8 are released in response to NF-κB activation within alveolar and airway epithelial cells. Bacterial products including LPS are powerful activators of this pathway via cell surface receptors TLR2 and TLR4 (Qureshi et al. 1999). IL-6 and IL-8 concentrations from BAL fluid in the first week of life from preterm infants are predictive of the chance of developing CLD (S. Kotecha, Wilson, et al. 1996; S. Kotecha et al. 1995). Strategies to minimise the inflammatory response in the lungs of preterm infants are used to reduce the risk of infants developing CLD. Extracellular ASC is, therefore, potentially a therapeutic target for reducing the propagation of inflammation within the lungs of preterm infants especially as intervention would be at a higher level in the NF-κB pathway than is currently possible with corticosteroids.

7.4 Strengths and weaknesses

There are a number of strengths to this work. This is the first study to undertake serial sampling of the respiratory microbiota in preterm infants. This has allowed characterisation of the microbiota of ventilated preterm infants over time demonstrating peaks and troughs of bacterial load and the dynamic changes seen in the dominant organism in the airways of some infants. The study design has also enabled comparison of inflammatory profiles of preterm infants with bacterial load and with dominance of the microbiota by specific lung pathogens.

Corroboration between the dominant organism identified between the BAL and TA samples taken within 24 hours, but processed independently and sequenced on different runs suggests that the sequencing results are genuine.

There are some weaknesses. The clinical study recruited only intubated preterm infants, thus a select group were studied. The proportion of infants who went to develop CLD was, therefore, high and precluded being able to compare differences between infants who developed CLD compared to infants who did not develop CLD. The numbers of infants eligible for recruitment were also smaller than anticipated which has limited the power of the study to detect subtle changes in microbiota profiles. The contamination issues of samples from Newcastle prevented comparison with a third geographical location.

A large proportion of each sample type did not have detectable bacterial DNA. This is an important finding suggesting establishing the respiratory microbiota in preterm infants takes time. The potential reasons for the high rate of negative samples were discussed in detail in Chapter 3. This may represent true sterile samples or possibly the methodology used was not sensitive to detect the low levels of bacterial colonisation in the respiratory tract of preterm infants. Other studies using BAL samples have also had large proportions of samples unable to detect bacterial DNA. A recent study in children with CF was able to obtain sequence data for 66% of BAL samples from CF children but only 27% of BAL samples from disease controls

(Zemanick et al. 2017). The low rate of samples being positive for bacterial DNA has limited the power of the work in this thesis. The longitudinal nature of the study was designed to show how bacterial colonisation patterns change over time. The weakness of this is that some of the analysis and patterns contain different numbers of samples from each infant which may introduce a bias of repeated measures.

The threshold of DNA concentration postamplification for BAL samples to be considered have amplified successfully had to be lowered due to difficulty amplifying bacterial DNA. The result of this was lower DNA concentrations being put through for sequencing resulting in lower quality assurance scores for the sequencing run. Larger numbers of ambiguous bases had to be included for these samples in the bioinformatics processing. Despite this the OTUs could still be identified to genus level.

The work looking at extracellular ASC used BAL supernatant that had been stored for several years prior to analysis. These samples were those remaining from previous work on preterm infants, the number of samples available, particularly beyond the first two days of life limited the ability to interpret the results. Time constraints meant that I was unable to complete the ASC work and repeat the TLR knockdown experiments as planned.

7.5 Suggestions for further research

The work presented in this thesis has shown the peaks in inflammatory cytokines present in BAL fluid from preterm infants continue in modern cohorts of patients and are associated with episodic peaks in bacterial load in BAL samples. Modulation of this inflammatory response has been tried with corticosteroids associated with a significant burden of side effects. Other immunomodulatory drugs show potential with azithromycin an attractive prospect. Azithromycin would also have the benefit of antibiotic effect against some of the bacteria associated with the peaks in cytokines such as *Ureaplasma* and *Mycoplasma* species. This work is already underway by our group in the AZTEC trial that has started recruiting (ISRCTN-11650227 2019).

Further work to understand whether the concept of a “healthy microbiota” applies to respiratory colonisation in preterm infants would aid clinicians in creating an environment to support optimum colonisation. Strict adherence to antibiotic policies, encouraging skin to skin contact with parents and breast feeding are all potential methods of influencing respiratory colonisation with the aim of optimising long-term respiratory health.

Single organism specific PCRs are more sensitive than 16S rRNA sequencing for detecting specific organisms (Blaschke et al. 2013). Using the existing samples and doing specific PCRs for individual organisms including *Ureaplasma* species may validate my findings and test the sensitivity of the 16S rRNA sequencing to detect specific organisms.

Using DNA based studies to identify bacteria colonising the airways is complicated by the possibility of detecting non-viable organisms. Further studies should consider using RNA based technologies from respiratory samples in preterm infants. This would have the advantage of identifying viable bacteria residing in the airways. Transcriptomic studies from respiratory samples in preterm infants would shed further light on host-microbiome interactions in the lungs, and may also help understand the transcriptional profile of the preterm lung in infants developing CLD.

Metagenomic studies, where the whole genome of microorganisms are sequenced may provide more information on microbiota influences over the host. The only study that has attempted to analyse metagenomic data in from preterm airways utilised an assumed metagenomic profile based on organisms detected using 16S rRNA gene sequencing rather than conducting a metagenomic library from sequence data (Lal et al. 2018).

One weakness the work presented in this thesis is the lack of a control group of infants with healthy lungs or a significant number of infants without CLD to act as a comparison group. To fully understand the impact of early colonisation on CLD of prematurity then a control of healthy term infants and comparison group of infants without CLD would be needed. An international study comparing colonisation patterns in different climates would allow comparisons between units in different countries to look at geographical effects.

A clear difference was seen between the bacterial communities at the two recruitment centres. It is likely the different environments and the exposure to different staff may cause this however further work could seek to identify the source of the respiratory microbiota by pairing samples from the mother, environmental samples, and those from the clinical staff. Identification to strain level would be required for this. Identifying if the differences in clinical management, such as giving probiotics, have any significant effect on the airway microbiota would help shape future policies directed at enhancing the chances of optimum colonisation by microorganisms in the airways and lungs of preterm infants.

Further work building on the results in Chapter 6 would need to explore the mechanism behind extracellular ASC inducing IL-6 and IL-8 secretion. This could be done by refining the TLR knockdown experiments that I was unable to successfully complete. Alternative approaches could be to use function blocking antibodies to TLRs or using CRISPR to deactivate TLR encoding genes to identify if TLR activation is the mechanism of activation of the NF-κB pathway. Further knockdowns and or blocking antibody experiments of components further in the pathway would be needed to confirm the mechanism of action. Imaging studies using fluorescent light microscopy labelling extracellular ASC and staining for TLRs would provide further evidence of TLR binding.

If the suspected mechanism of action of extracellular ASC is confirmed, then the potential for a therapeutic intervention targeting extracellular ASC could then be explored to limit the inflammatory response within the lungs witnessed in infants at risk of developing CLD. Other applications for such a therapeutic intervention would include cryopyrin associated periodic syndrome, known to be associated with abnormal NLRP3 inflammasome activation and excessive IL-1β secretion (Haverkamp et al. 2014) and other inflammatory lung diseases where extracellular ASC has been shown to be involved in a pro-inflammatory response such as COPD (Franklin et al. 2014).

7.6 Summary of main findings

Despite limitations I have made a number of important observations.

1. Bacterial colonisation of the lower airways and lungs in preterm infants occurs as an infective process with peaks of pro-inflammatory cytokines IL-6 and IL-8 associated with detectable bacterial load.

2. Antibiotics play and important role in suppressing bacterial colonisation within the airways and lungs of preterm infants.

3. Bacterial Colonisation of the nasopharynx is affected by delivery mode in preterm infants, similarly to how previous studies have described the effect on term infants.

4. Bacterial colonisation patterns change over time in the nasopharynx of preterm infants with decreasing proportion of Firmicutes and increasing proportion of Proteobacteria.

5. NPA, BAL and TA sample from distinct niche's with differences in community structure.

6. Extracellular ASC is present in neonatal BAL fluid and remains biologically active extracellularly

7. Extracellular ASC from preterm BAL fluid induces IL-6 and IL-8 secretion on in-vitro testing of respiratory epithelial cells in culture

8. Recombinant ASC has the same effect of inducing IL-6 and IL-8 secretion of respiratory epithelial cells in culture.

Chapter 8: References

8 References

Aagaard, Kjersti, Jun Ma, Kathleen M Antony, Radhika Ganu, Joseph Petrosino, and James Versalovic. 2014. “The Placenta Harbors a Unique Microbiome.” *Science Translational Medicine* 6 (237): 237ra65.

Aaltonen, Riikka, Tero Vahlberg, Liisa Lehtonen, and Anna Alanen. 2006. “Ureaplasma Urealyticum: No Independent Role in the Pathogenesis of Bronchopulmonary Dysplasia.” *Acta Obstetricia et Gynecologica Scandinavica* 85 (11): 1354–59.

Adlerberth, I., and A Wold. 2009. “Establishment of the Gut Microbiota in Western Infants.” *Acta Paediatrica, International Journal of Paediatrics* 98: 229–38.

Adlerberth, I, F Jalil, B Carlsson, L Mellander, L Hanson, P Larsson, K Khalil, and A Wold. 1998. “High Turnover Rate of Escherichia Coli Strains in the Intestinal Flora of Infants in Pakistan.” *Epidemiology and Infection* 121: 587–98.

Adlerberth, I, Erika Lindberg, Nils Åberg, Bill Hesselmar, Robert Saalman, Inga Lisa Strannegård, and Agnes E. Wold. 2006. “Reduced Enterobacterial and Increased Staphylococcal Colonization of the Infantile Bowel: An Effect of Hygienic Lifestyle?” *Pediatric Research* 59 (1): 96–101.

Adlerberth, I, David P. Strachan, Paolo M. Matricardi, Siv Ahrné, Lia Orfei, Nils Åberg, Michael R. Perkin, et al. 2007. “Gut Microbiota and Development of Atopic Eczema in 3 European Birth Cohorts.” *Journal of Allergy and Clinical Immunology* 120 (2): 343–50.

Ahmed, Bushra, Michael J. Cox, Leah Cuthbertson, Phillip L. James, William O. C. Cookson, Jane C. Davies, Miriam F. Moffatt, and Andrew Bush. 2018. “Comparison of the Upper and Lower Airway Microbiota in Children with Chronic Lung Diseases.” Edited by Alexander Larcombe. *PLOS ONE* 13 (8): e0201156.

Aho, Velma T. E., Pedro A. B. Pereira, Tari Haahtela, Ruby Pawankar, Petri Auvinen, and Kaisa Koskinen. 2015. “The Microbiome of the Human Lower Airways: A next Generation Sequencing Perspective.” *World Allergy Organization Journal* 8 (1): 23.

Ahola, Terhi, Risto Lapatto, Kari O. Raivio, Bo Selander, Lennart Stigson, Baldvin Jonsson, Finn Jonsbo, et al. 2003. “N-Acetylcysteine Does Not Prevent Bronchopulmonary Dysplasia in Immature Infants: A Randomized Controlled Trial.” *Journal of Pediatrics* 143 (6): 713–19.

Alexopoulou, L, A C Holt, R Medzhitov, and R A Flavell. 2001. “Recognition of Double-Stranded RNA and Activation of NF-KappaB by Toll-like Receptor 3.” *Nature* 413 (6857): 732–38.

Ali, Ebtihal, Mohamed Abdel Wahed, Zahraa Alsalami, Hasnaa Abouseif, Tania Gottschalk, Rasheda Rabbani, Ryan Zarychanski, and Ahmed M Abou-Setta. 2016. “New Modalities to Deliver Surfactant in Premature Infants: A Systematic Review and Meta-Analysis.” *The Journal of Maternal-Fetal & Neonatal Medicine : The Official Journal of the European Association of Perinatal Medicine, the Federation of Asia and Oceania Perinatal Societies, the International Society of Perinatal Obstetricians*, March, 1–6.

Aliprantis, A O, R B Yang, M R Mark, S Suggett, B Devaux, J D Radolf, G R Klimpel, P Godowski, and A Zychlinsky. 1999. “Cell Activation and Apoptosis by Bacterial Lipoproteins through Toll-like Receptor-2.” *Science (New York, N.Y.)* 285 (5428): 736–39.

Allen, Irving C, Corey M Jania, Justin E Wilson, Erin M Tekeppe, Xiaoyang Hua, Willie J Brickey, Mildred Kwan, Beverly H Koller, Stephen L Tilley, and Jenny P-Y Ting. 2012. “Analysis of NLRP3 in the Development of Allergic Airway Disease in Mice.” *Journal of Immunology (Baltimore, Md. : 1950)* 188 (6): 2884–93.

Amann, Rudolf I, Wolfgang Ludwig, and K H Schleifer. 1995. “Phylogenetic Identification and in Situ Detection of Individual Microbial Cells without Cultivation.” *Microbiological Reviews* 59 (1): 143–69.

Ambalavanan, Namasivayam, Waldemar A Carlo, Carl T D’Angio, Scott A McDonald, Abhik Das, Diana Schendel, Poul Thorsen, and Rosemary D Higgins. 2009. “Cytokines Associated with Bronchopulmonary Dysplasia or Death in Extremely Low Birth Weight Infants.” *Pediatrics* 123 (4): 1132–41.

Anderson, Marti J. 2017. “Permutational Multivariate Analysis of Variance (PERMANOVA).” In *Wiley StatsRef: Statistics Reference Online*, 1–15. Chichester, UK: John Wiley & Sons, Ltd.

Apisarnthanarak, Anucha, Galit Holzmann-Pazgal, Aaron Hamvas, Margaret A Olsen, and Victoria J Fraser. 2003. “Ventilator-Associated Pneumonia in Extremely Preterm Neonates in a Neonatal Intensive Care Unit: Characteristics, Risk Factors, and Outcomes.” *Pediatrics* 112 (6 Pt 1): 1283–89.

Ather, Jennifer L, Rebecca A Martin, Ckless Karina, and Matthew E Poynter. 2013. “Inflammasome Activity in Non-Microbial Lung Inflammation.” *Journal of Enviromental Immunology and Toxicology* 1 (3): 108.

Attar, Mohammad Ali, and Steven M Donn. 2002. “Mechanisms of Ventilator-Induced Lung Injury in Premature Infants.” *Seminars in Neonatology : SN* 7 (5): 353–60.

Bacci, Giovanni, Patrizia Paganin, Loredana Lopez, Chiara Vanni, Claudia Dalmastri, Cristina Cantale, Loretta Daddiego, et al. 2016. “Pyrosequencing Unveils Cystic Fibrosis Lung Microbiome Differences Associated with a Severe Lung Function Decline.” *PloS One* 11 (6): e0156807.

Bagchi, A, R M Viscardi, V Taciak, J E Ensor, K McCrea, and J D Hasday. 1994. “Increased Activity of Interleukin-6 but Not Tumor Necrosis Factor-Alpha in Lung Lavage of Premature Infants Is Associated with the Development of Bronchopulmonary Dysplasia.” *Pediatric Research* 36 (2): 244–52.

Balbi, B, P Pignatti, M Corradi, P Baiardi, L Bianchi, G Brunetti, A Radaeli, et al. 2007. “Bronchoalveolar Lavage, Sputum and Exhaled Clinically Relevant Inflammatory Markers: Values in Healthy Adults.” *The European Respiratory Journal* 30 (4): 769–81.

Ballabh, Praveen, Maciej Simm, Jaishree Kumari, Alfred N Krauss, Ajey Jain, Peter A M Auld, and Susanna Cunningham-Rundles. 2003. “Lymphocyte Subpopulations in Bronchopulmonary Dysplasia.” *American Journal of Perinatology* 20 (8): 465–75.

Ballinger, Megan N, Marc Peters-Golden, and Bethany B Moore. 2011. “Impaired Neonatal Macrophage Phagocytosis Is Not Explained by Overproduction of Prostaglandin E2.” *Respiratory Research* 12 (1): 155.

Bals, R., and P.S. Hiemstra. 2004. “Innate Immunity in the Lung: How Epithelial Cells Fight against Respiratory Pathogens.” *European Respiratory Journal* 23 (2): 327–33.

Barbé, François, Todd Douglas, and Maya Saleh. 2014. “Advances in Nod-like Receptors (NLR) Biology.” *Cytokine & Growth Factor Reviews* 25 (July): 681–97.

Barker, D. 1986. “Infant Mortality, Childhood Nutrition, and Ischaemic Heart Disease in England and Wales.” *The Lancet* 327 (8489): 1077–81.

Baroja-Mazo, Alberto, Fatima Martín-Sánchez, Ana I Gomez, Carlos M Martínez, Joaquín Amores-Iniesta, Vincent Compan, Maria Barberà-Cremades, et al. 2014. “The NLRP3 Inflammasome Is Released as a Particulate Danger Signal That Amplifies the Inflammatory Response.” *Nature Immunology* 15 (8).

Barrett, Eoin, Colm Kerr, Kiera Murphy, Orla O'Sullivan, C Anthony Ryan, Eugene M Dempsey, Brendan P Murphy, et al. 2013. "The Individual-Specific and Diverse Nature of the Preterm Infant Microbiota." *Archives of Disease in Childhood. Fetal and Neonatal Edition* 98: F334-40.

Bartram, Ulrike, and Christian P. Speer. 2004. "The Role of Transforming Growth Factor β in Lung Development and Disease." *Chest* 125 (2): 754–65.

Bassis, Christine M, John R. Erb-Downward, Robert P Dickson, Christine M Freeman, Thomas M Schmidt, Vincent B Young, James M Beck, Jeffrey L Curtis, and Gary B. Huffnagle. 2015. "Analysis of the Upper Respiratory Tract Microbiotas as the Source of the Lung and Gastric Microbiotas in Healthy Individuals." *MBio* 6 (2): e00037-15.

Bassis, Christine M, Alice L Tang, Vincent B Young, and Melissa A Pynnonen. 2014. "The Nasal Cavity Microbiota of Healthy Adults." *Microbiome* 2 (January): 27.

Baud, Olivier, Laure Maury, Florence Lebail, Duksha Ramful, Fatima El Moussawi, Claire Nicaise, Véronique Zupan-Simunek, et al. 2016. "Effect of Early Low-Dose Hydrocortisone on Survival without Bronchopulmonary Dysplasia in Extremely Preterm Infants (PREMILOC): A Double-Blind, Placebo-Controlled, Multicentre, Randomised Trial." *Lancet (London, England)* 387 (10030): 1827–36.

Bauer, S, C J Kirschning, H Häcker, V Redecke, S Hausmann, S Akira, H Wagner, and G B Lipford. 2001. "Human TLR9 Confers Responsiveness to Bacterial DNA via Species-Specific CpG Motif Recognition." *Proceedings of the National Academy of Sciences of the United States of America* 98 (16): 9237–42.

Bauernfeind, Franz G, Gabor Horvath, Andrea Stutz, Emad S Alnemri, Kelly MacDonald, David Speert, Teresa Fernandes-Alnemri, et al. 2009. "Cutting Edge: NF-KappaB Activating Pattern Recognition and Cytokine Receptors License NLRP3 Inflammasome Activation by Regulating NLRP3 Expression." *Journal of Immunology (Baltimore, Md. : 1950)* 183 (2): 787–91.

Beck, James M, Vincent B Young, and Gary B Huffnagle. 2012. "The Microbiome of the Lung." *Translational Research : The Journal of Laboratory and Clinical Medicine* 160 (4): 258–66.

Beeton, M L, N C Maxwell, P L Davies, D Nuttall, E McGreal, M Chakraborty, O B Spiller, and S Kotecha. 2011. "Role of Pulmonary Infection in the Development of Chronic Lung Disease of Prematurity." *The European Respiratory Journal* 37 (6): 1424–30.

Belkaid, Yasmine, and Timothy W Hand. 2014. "Role of the Microbiota in Immunity and Inflammation." *Cell* 157 (1): 121–41.

Bettelheim, K A, and S M Lennox-King. 1976. "The Acquisition of Escherichia Coli by New-Born Babies." *Infection* 4 (3): 174–79.

Bhandari, V, M J Bizzarro, A Shetty, X Zhong, G P Page, H Zhang, L R Ment, and J R Gruen. 2006. "Familial and Genetic Susceptibility to Major Neonatal Morbidities in Preterm Twins." *Pediatrics* 117 (6): 1901–6.

Bhatt, A J, G S Pryhuber, H Huyck, R H Watkins, L A Metlay, and W M Maniscalco. 2001. "Disrupted Pulmonary Vasculature and Decreased Vascular Endothelial Growth Factor, Flt-1, and TIE-2 in Human Infants Dying with Bronchopulmonary Dysplasia." *American Journal of Respiratory and Critical Care Medicine* 164 (10 Pt 1): 1971–80.

Biasucci, Giacomo, Monica Rubini, Sara Riboni, Lorenzo Morelli, Elena Bessi, and Cristiana Retetangos. 2010. "Mode of Delivery Affects the Bacterial Community in the Newborn Gut." *Early Human Development* 86 (1): 13–15.

Biedermann, L, K Brulisauer, J Zeitz, P Frei, M Scharl, S R Vavricka, M Fried, M J Loessner, G Rogler, and M Schuppler. 2014. “Smoking Cessation Alters Intestinal Microbiota: Insights from Quantitative Investigations on Human Fecal Samples Using FISH.” *Inflamm Bowel Dis* 20 (9): 1496–1501.

Biesbroek, Giske, Astrid A T M Bosch, Xinhui Wang, Bart J F Keijser, Reinier H Veenhoven, Elisabeth a M Sanders, and Debby Bogaert. 2014. “The Impact of Breastfeeding on Nasopharyngeal Microbial Communities in Infants.” *American Journal of Respiratory and Critical Care Medicine* 190 (3): 298–308.

Biesbroek, Giske, Evgeni Tsivtsivadze, Elisabeth A M Sanders, Roy Montijn, Reinier H Veenhoven, Bart J F Keijser, and Debby Bogaert. 2014. “Early Respiratory Microbiota Composition Determines Bacterial Succession Patterns and Respiratory Health in Children.” *American Journal of Respiratory and Critical Care Medicine* 190 (11): 1283–92.

Biesbroek, Giske, Xinhui Wang, Bart J F Keijser, Rene M J Eijkemans, Krzysztof Trzciński, Nynke Y Rots, Reinier H Veenhoven, Elisabeth A M Sanders, and Debby Bogaert. 2014. “Conjugate Vaccine and Nasopharyngeal Microbiota in Healthy Children” 20 (2).

Bird, J J, D R Brown, A C Mullen, N H Moskowitz, M A Mahowald, J R Sider, T F Gajewski, C R Wang, and S L Reiner. 1998. “Helper T Cell Differentiation Is Controlled by the Cell Cycle.” *Immunity* 9: 229–37.

Bisgaard, H, M N Hermansen, F Buchvald, L Loland, L B Halkjaer, K Bonnelykke, M Brasholt, et al. 2007. “Childhood Asthma after Bacterial Colonization of the Airway in Neonates.” *N Engl J Med* 357 (15): 1487–95.

Blaschke, Anne J, Carrie L Byington, Krow Ampofo, Andrew T Pavia, Caroline Heyrend, Shelley C Rankin, Karin L McGowan, Mary C Harris, and Samir S Shah. 2013. “Species-Specific PCR Improves Detection of Bacterial Pathogens in Parapneumonic Empyema Compared with 16S PCR and Culture.” *The Pediatric Infectious Disease Journal* 32 (3): 302–3.

Blattner, F. R., G Plunkett, C A Bloch, N T Perna, V Burland, M Riley, J Collado-Vides, et al. 1997. “The Complete Genome Sequence of Escherichia Coli K-12.” *Science (New York, N.Y.)* 277 (5331): 1453–62.

Blencowe, Hannah, Anne C C Lee, Simon Cousens, Adil Bahalim, Rajesh Narwal, Nanbert Zhong, Doris Chou, et al. 2013. “Preterm Birth-Associated Neurodevelopmental Impairment Estimates at Regional and Global Levels for 2010.” *Pediatric Research* 74 Suppl 1 (December): 17–34.

Blic, J de, F Midulla, A Barbato, A Clement, I Dab, E Eber, C Green, et al. 2000. “Bronchoalveolar Lavage in Children. ERS Task Force on Bronchoalveolar Lavage in Children. European Respiratory Society.” *The European Respiratory Journal* 15 (1): 217–31.

Bonikos, D S, K G Bensch, S K Ludwin, and W H Northway. 1975. “Oxygen Toxicity in the Newborn.” *Lab Invest* 32: 619–35.

Bosch, Astrid A. T. M., Wouter A. A. de Steenhuijsen Piters, Marlies A. van Houten, Mei Ling J. N. Chu, Giske Biesbroek, Jolanda Kool, Paula Pernet, et al. 2017. “Maturation of the Infant Respiratory Microbiota, Environmental Drivers, and Health Consequences. A Prospective Cohort Study.” *American Journal of Respiratory and Critical Care Medicine* 196 (12): 1582–90.

Bosch, Astrid A.T.M., Evgeni Levin, Marlies A. van Houten, Raiza Hasrat, Gino Kalkman, Giske Biesbroek, Wouter A.A. de Steenhuijsen Piters, et al. 2016. “Development of Upper Respiratory Tract Microbiota in Infancy Is Affected by Mode of Delivery.” *EBioMedicine* 9: 336–45.

Bose, C L, C E L Dammann, and M M Laughon. 2008. “Bronchopulmonary Dysplasia and Inflammatory Biomarkers in the Premature Neonate.” *Archives of Disease in Childhood. Fetal and Neonatal Edition* 93 (6): F455-61.

Bose, Carl, Linda J Van Marter, Matthew Laughon, T Michael O’Shea, Elizabeth N Allred, Padmani Karna, Richard a Ehrenkranz, Kim Boggess, and Alan Leviton. 2009. “Fetal Growth Restriction and Chronic Lung Disease among Infants Born before the 28th Week of Gestation.” *Pediatrics* 124 (3): e450-8.

Boutin, Sébastien, Simon Y. Graeber, Michael Weitnauer, Jessica Panitz, Mirjam Stahl, Diana Clausznitzer, Lars Kaderali, et al. 2015. “Comparison of Microbiomes from Different Niches of Upper and Lower Airways in Children and Adolescents with Cystic Fibrosis.” Edited by Tom Coenye. *PLOS ONE* 10 (1): e0116029.

Bray, J Roger, and J T Curtis. 1957. “An Ordination of the Upland Forest Communities of Southern Wisconsin.” *Ecological Monographs* 27 (4): 325.

Brew, Nadine, Stuart B Hooper, Valerie Zahra, Megan Wallace, and Richard Harding. 2013. “Mechanical Ventilation Injury and Repair in Extremely and Very Preterm Lungs.” *PloS One* 8 (5): e63905.

Brotman, R M, X He, P Gajer, D Fadrosh, E Sharma, E F Mongodin, J Ravel, E D Glover, and J M Rath. 2014. “Association between Cigarette Smoking and the Vaginal Microbiota: A Pilot Study.” *BMC Infect Dis* 14: 471.

Brown, E R, A Stark, I Sosenko, E E Lawson, and M E Avery. 1978. “Bronchopulmonary Dysplasia: Possible Relationship to Pulmonary Edema.” *The Journal of Pediatrics* 92 (6): 982–84.

Brubaker, Sky W, Kevin S Bonham, Ivan Zanoni, and Jonathan C Kagan. 2015. *Innate Immune Pattern Recognition: A Cell Biological Perspective. Annual Review of Immunology*.

Buchmann, Kurt. 2014. “Evolution of Innate Immunity: Clues from Invertebrates via Fish to Mammals.” *Frontiers in Immunology* 5 (September): 459.

Burri, P H, and E R Weibel. 1971. “Morphometric Estimation of Pulmonary Diffusion Capacity. II. Effect of Po2 on the Growing Lung, Adaption of the Growing Rat Lung to Hypoxia and Hyperoxia.” *Respir Physiol* 11 (2): 247–64.

Cantey, Joseph B, Landon W Huffman, Abirami Subramanian, Anderson S Marshall, A Rebecca Ballard, Cassandra Lefevre, Malvika Sagar, Jessica E Pruszynski, and Lea H Mallett. 2016. “Antibiotic Exposure and Risk for Death or Bronchopulmonary Dysplasia in Very Low Birth Weight Infants.” *The Journal of Pediatrics* 181: 289-293.e1.

Carlson, Bruce. 2009. *Human Embryology and Developmental Biology*. 4th ed. Mosby.

Carmody, R J, and Y H Chen. 2007. “Nuclear Factor-KappaB: Activation and Regulation during Toll-like Receptor Signaling.” *Cell Mol Immunol* 4 (1): 31–41.

Case, Rebecca J, Yan Boucher, Ingela Dahllöf, Carola Holmström, W Ford Doolittle, and Staffan Kjelleberg. 2007. “Use of 16S RRNA and RpoB Genes as Molecular Markers for Microbial Ecology Studies.” *Applied and Environmental Microbiology* 73 (1): 278–88.

Cayabyab, R. G., C. A. Jones, K. Y. C. Kwong, C. Hendershott, C. Lecart, P. Minoo, and R. Ramanathan. 2003. “Interleukin-1β in the Bronchoalveolar Lavage Fluid of Premature Neonates: A Marker for Maternal Chorioamnionitis and Predictor of Adverse Neonatal Outcome.” *The Journal of Maternal-Fetal & Neonatal Medicine* 14 (3): 205–11.

Cenit, M C, V Matzaraki, E F Tigchelaar, and A Zhernakova. 2014. “Rapidly Expanding

Knowledge on the Role of the Gut Microbiome in Health and Disease." *Biochim Biophys Acta* 1842 (10): 1981–92.

Cernada, María, Marta Aguar, María Brugada, Antonio Gutiérrez, José Luis López, Marta Castell, and Máximo Vento. 2013. "Ventilator-Associated Pneumonia in Newborn Infants Diagnosed With an Invasive Bronchoalveolar Lavage Technique." *Pediatric Critical Care Medicine* 14 (1): 55–61.

Cernada, María, María Brugada, Sergio Golombek, and Máximo Vento. 2014. "Ventilator-Associated Pneumonia in Neonatal Patients: An Update." *Neonatology* 105 (2): 98–107.

Chakraborty, Mallinath, Eamon P McGreal, Philip L Davies, Mari A Nowell, Simon Jones, and Sailesh Kotecha. 2013. "Role of Interleukin-6, Its Receptor and Soluble Gp130 in Chronic Lung Disease of Prematurity." *Neonatology* 104 (3): 161–67.

Chakraborty, Mallinath, Eamon P McGreal, and Sailesh Kotecha. 2010. "Acute Lung Injury in Preterm Newborn Infants: Mechanisms and Management." *Paediatric Respiratory Reviews* 11 (3): 162–70; quiz 170.

Chakraborty, Mallinath, Eamon P McGreal, Andrew Williams, Philip L Davies, Wendy Powell, Salima Abdulla, Nikolai N Voitenok, et al. 2014. "Role of Serine Proteases in the Regulation of Interleukin-877 during the Development of Bronchopulmonary Dysplasia in Preterm Ventilated Infants." Edited by Oliver Eickelberg. *PloS One* 9 (12): e114524.

Chakravorty, Soumitesh, Danica Helb, Michele Burday, Nancy Connell, and David Alland. 2007. "A Detailed Analysis of 16S Ribosomal RNA Gene Segments for the Diagnosis of Pathogenic Bacteria." *Journal of Microbiological Methods* 69 (2): 330–39.

Chao, Anne. 1984. "Board of the Foundation of the Scandinavian Journal of Statistics Nonparametric Estimation of the Number of Classes in a Population Nonparametric Estimation of the Number of Classes in a Population." *Source: Scandinavian Journal of Statistics*. Vol. 11.

Chaput, Catherine, Leif Erik Sander, Norbert Suttorp, and Bastian Opitz. 2013. "NOD-like Receptors in Lung Diseases." *Frontiers in Immunology* 4 (November): 1–12.

Charlson, E S, K Bittinger, A R Haas, A S Fitzgerald, I Frank, A Yadav, F D Bushman, and R G Collman. 2011. "Topographical Continuity of Bacterial Populations in the Healthy Human Respiratory Tract." *Am J Respir Crit Care Med* 184 (8): 957–63.

Chen, Tong, Daisuke Noto, Yasunobu Hoshino, Miho Mizuno, and Sachiko Miyake. 2019. "Butyrate Suppresses Demyelination and Enhances Remyelination." *Journal of Neuroinflammation* 16 (1): 165.

Chen, Weiguang, Fanlong Liu, Zongxin Ling, Xiaojuan Tong, and Charlie Xiang. 2012. "Human Intestinal Lumen and Mucosa-Associated Microbiota in Patients with Colorectal Cancer." *PLoS ONE*.

Chess, Patricia R, Carl T D'Angio, Gloria S Pryhuber, and William M Maniscalco. 2006. "Pathogenesis of Bronchopulmonary Dysplasia." *Seminars in Perinatology* 30 (4): 171–78.

Chu, Derrick M, Jun Ma, Amanda L Prince, Kathleen M Antony, Maxim D Seferovic, and Kjersti M Aagaard. 2017. "Maturation of the Infant Microbiome Community Structure and Function across Multiple Body Sites and in Relation to Mode of Delivery." *Nature Medicine* 23 (3): 314-326 13.

Chu, Jessica, L Michael Thomas, Simon C Watkins, Luigi Franchi, Gabriel Núñez, and Russell D Salter. 2009. "Cholesterol-Dependent Cytolysins Induce Rapid Release of Mature IL-1beta from Murine Macrophages in a NLRP3 Inflammasome and Cathepsin B-Dependent

Manner." *Journal of Leukocyte Biology* 86 (5): 1227–38.

Coburn, Bryan, Pauline W Wang, Julio Diaz Caballero, Shawn T Clark, Vijaya Brahma, Sylva Donaldson, Yu Zhang, et al. 2015. "Lung Microbiota across Age and Disease Stage in Cystic Fibrosis." *Scientific Reports* 5 (January): 10241.

Cole, James R., Qiong Wang, Jordan A. Fish, Benli Chai, Donna M. McGarrell, Yanni Sun, C. Titus Brown, Andrea Porras-Alfaro, Cheryl R. Kuske, and James M. Tiedje. 2014. "Ribosomal Database Project: Data and Tools for High Throughput RRNA Analysis." *Nucleic Acids Research* 42 (D1): D633–42.

Collado, Maria Carmen, Samuli Rautava, Juhani Aakko, Erika Isolauri, and Seppo Salminen. 2016. "Human Gut Colonisation May Be Initiated in Utero by Distinct Microbial Communities in the Placenta and Amniotic Fluid." *Scientific Reports* 6 (October 2015): 23129.

Compan, Vincent, Fátima Martín-Sánchez, Alberto Baroja-Mazo, Gloria López-Castejón, Ana I Gomez, Alexei Verkhratsky, David Brough, and Pablo Pelegrín. 2014. "Apoptosis-Associated Speck-like Protein Containing a CARD Forms Specks but Does Not Activate Caspase-1 in the Absence of NLRP3 during Macrophage Swelling." *Journal of Immunology (Baltimore, Md. : 1950)*, December.

Connett, G J. 2000. "Bronchoalveolar Lavage." *Paediatr Respir Rev* 1 (1): 52–56.

Cools, F, D J Henderson-Smart, M Offringa, and L M Askie. 2009. "Elective High Frequency Oscillatory Ventilation versus Conventional Ventilation for Acute Pulmonary Dysfunction in Preterm Infants." *Cochrane Database Syst Rev*, no. 3: Cd000104.

Costello, Elizabeth K, Christian L Lauber, Micah Hamady, Noah Fierer, Jeffrey I Gordon, and Rob Knight. 2009. "Bacterial Community Variation in Human Body Habitats across Space and Time." *Science (New York, N.Y.)* 326 (5960): 1694–97.

Costeloe, Kate, Pollyanna Hardy, Edmund Juszczak, Mark Wilks, and Michael R Millar. 2015. "Bifidobacterium Breve BBG-001 in Very Preterm Infants: A Randomised Controlled Phase 3 Trial." *The Lancet*, November.

Costeloe, Kate, E M Hennessy, S Haider, F Stacey, N Marlow, and E S Draper. 2012. "Short Term Outcomes after Extreme Preterm Birth in England: Comparison of Two Birth Cohorts in 1995 and 2006 (the EPICure Studies)." *Bmj* 345: e7976.

Cotten, C. M., S. McDonald, B. Stoll, R. N. Goldberg, K. Poole, D. K. Benjamin, and National Institute for Child Health and Human Development Neonatal Research Network. 2006. "The Association of Third-Generation Cephalosporin Use and Invasive Candidiasis in Extremely Low Birth-Weight Infants." *PEDIATRICS* 118 (2): 717–22.

Cotten, C, Sarah Taylor, Barbara Stoll, Ronald N Goldberg, Nellie I Hansen, Pablo J Sánchez, Namasivayam Ambalavanan, Daniel K Benjamin, and NICHD Neonatal Research Network. 2009. "Prolonged Duration of Initial Empirical Antibiotic Treatment Is Associated with Increased Rates of Necrotizing Enterocolitis and Death for Extremely Low Birth Weight Infants." *Pediatrics* 123 (1): 58–66.

Couroucli, Xanthi I, Stephen E Welty, Patricia L Ramsay, Mary E Wearden, F Javier Fuentes-Garcia, Jiyuan Ni, Tamara N Jacobs, Jeffrey a Towbin, and Neil E Bowles. 2000. "Detection of Microorganisms in the Tracheal Aspirates of Preterm Infants by Polymerase Chain Reaction: Association of Adenovirus Infection with Bronchopulmonary Dysplasia." *Pediatric Research* 47 (2): 225–225.

Cox, Michael J, Martin Allgaier, Byron Taylor, Marshall S Baek, Yvonne J Huang, Rebecca a Daly,

Ulas Karaoz, et al. 2010. “Airway Microbiota and Pathogen Abundance in Age-Stratified Cystic Fibrosis Patients.” *PloS One* 5 (6): e11044.

D’Angio, C T, K Basavegowda, N E Avissar, J N Finkelstein, and R A Sinkin. 2002. “Comparison of Tracheal Aspirate and Bronchoalveolar Lavage Specimens from Premature Infants.” *Biol Neonate* 82 (3): 145–49.

D’Angio, C T, J N Finkelstein, M B Lomonaco, A Paxhia, S A Wright, R B Baggs, R H Notter, and R M Ryan. 1997. “Changes in Surfactant Protein Gene Expression in a Neonatal Rabbit Model of Hyperoxia-Induced Fibrosis.” *The American Journal of Physiology* 272 (4 Pt 1): L720-30.

Dargaville, P, M South, and P N McDougall. 1999. “Comparison of Two Methods of Diagnostic Lung Lavage in Ventilated Infants with Lung Disease.” *American Journal of Respiratory and Critical Care Medicine* 160 (3): 771–77.

Davies, Philip L., Nicola C. Maxwell, and Sailesh Kotecha. 2006. “The Role of Inflammation and Infection in the Development of Chronic Lung Disease of Prematurity.” *Advances in Experimental Medicine and Biology* 582: 101–10.

Davies, Philip L, O Brad Spiller, Michael L Beeton, Nicola C Maxwell, Eileen Remold-O’Donnell, and Sailesh Kotecha. 2010. “Relationship of Proteinases and Proteinase Inhibitors with Microbial Presence in Chronic Lung Disease of Prematurity.” *Thorax* 65 (3): 246–51.

Davis, Beckley K, Haitao Wen, and Jenny P-Y Ting. 2011. “The Inflammasome NLRs in Immunity, Inflammation, and Associated Diseases.” *Annual Review of Immunology* 29 (December 2010): 707–35.

Davis, J., W. N. Rosenfeld, S. E. Richter, R. Parad, I. H. Gewolb, A. R. Spitzer, W. A. Carlo, et al. 1997. “Safety and Pharmacokinetics of Multiple Doses of Recombinant Human CuZn Superoxide Dismutase Administered Intratracheally To.” *PEDIATRICS* 100 (1): 24–30.

Dickson, Robert P., John R. Erb-Downward, Christine M. Freeman, Lisa McCloskey, Nicole R Falkowski, Gary B Huffnagle, and Jeffrey L. Curtis. 2017. “Bacterial Topography of the Healthy Human Lower Respiratory Tract.” Edited by Jose C. Clemente. *MBio* 8 (1): e02287-16.

Dickson, Robert P, John R Erb-Downward, Christine M Freeman, Natalie Walker, Brittan S Scales, James M Beck, Fernando J Martinez, Jeffrey L Curtis, Vibha N Lama, and Gary B Huffnagle. 2014. “Changes in the Lung Microbiome Following Lung Transplantation Include the Emergence of Two Distinct Pseudomonas Species with Distinct Clinical Associations.” *PloS One* 9 (5): e97214.

Dickson, Robert P, John R Erb-Downward, and Gary B Huffnagle. 2013. “The Role of the Bacterial Microbiome in Lung Disease.” *Expert Review of Respiratory Medicine* 7 (3): 245–57.

Dickson, Robert P, John R Erb-Downward, Hallie C Prescott, Fernando J Martinez, Jeffrey L Curtis, Vibha N Lama, and Gary B Huffnagle. 2014. “Analysis of Culture-Dependent vs. Culture-Independent Techniques for the Identification of Bacteria in Clinically-Obtained Bronchoalveolar Lavage Fluid.” *Journal of Clinical Microbiology* 52 (July): 3605–13.

Dickson, Robert P, Fernando J Martinez, and Gary B Huffnagle. 2014. “The Role of the Microbiome in Exacerbations of Chronic Lung Diseases.” *Lancet* 384 (9944): 691–702.

DiGiulio, Daniel B, Maria Teresa Gervasi, Roberto Romero, Edi Vaisbuch, Shali Mazaki-Tovi, Juan Pedro Kusanovic, Kimberley S Seok, et al. 2010. “Microbial Invasion of the Amniotic Cavity in Pregnancies with Small-for-Gestational-Age Fetuses.” *J Perinat Med* 38 (5): 495–502.

DiGiulio, Daniel B, Roberto Romero, Harold P Amogan, Juan Pedro Kusanovic, Elisabeth M Bik, Francesca Gotsch, Chong Jai Kim, Offer Erez, Sam Edwin, and David a Relman. 2008. "Microbial Prevalence, Diversity and Abundance in Amniotic Fluid during Preterm Labor: A Molecular and Culture-Based Investigation." *PloS One* 3 (8): e3056.

Dolinay, Tamás, Young Sam Kim, Judie Howrylak, Gary M. Hunninghake, Chang Hyeok An, Laura Fredenburgh, Anthony F. Massaro, et al. 2012. "Inflammasome-Regulated Cytokines Are Critical Mediators of Acute Lung Injury." *American Journal of Respiratory and Critical Care Medicine* 185 (13): 1225–34.

Dominguez-Bello, Maria G, Elizabeth K Costello, Monica Contreras, Magda Magris, Glida Hidalgo, Noah Fierer, and Rob Knight. 2010. "Delivery Mode Shapes the Acquisition and Structure of the Initial Microbiota across Multiple Body Habitats in Newborns." *Proceedings of the National Academy of Sciences of the United States of America* 107 (26): 11971–75.

Donn, S M, and S K Sinha. 2006. "Minimising Ventilator Induced Lung Injury in Preterm Infants." *Arch Dis Child Fetal Neonatal Ed* 91 (3): F226-30.

Dostert, Catherine, Greta Guarda, Jackeline F Romero, Philippe Menu, Olaf Gross, Aubry Tardivel, Mario-Luca Suva, et al. 2009. "Malarial Hemozoin Is a Nalp3 Inflammasome Activating Danger Signal." *PloS One* 4 (8): e6510.

Doyle, Lex W, Richard A Ehrenkranz, and Henry L Halliday. 2014. "Late (> 7 Days) Postnatal Corticosteroids for Chronic Lung Disease in Preterm Infants." *The Cochrane Database of Systematic Reviews* 5 (5): CD001145.

Duncan, Joseph A, Daniel T Bergstralh, Yanhong Wang, Stephen B Willingham, Zhengmao Ye, Albert G Zimmermann, and Jenny Pan-Yun Ting. 2007. "Cryopyrin/NALP3 Binds ATP/DATP, Is an ATPase, and Requires ATP Binding to Mediate Inflammatory Signaling." *Proceedings of the National Academy of Sciences of the United States of America* 104 (19): 8041–46.

Edgar, Robert C. 2010. "Search and Clustering Orders of Magnitude Faster than BLAST." *Bioinformatics* 26 (19): 2460–61.

Elliott, Eric I., and Fayyaz S. Sutterwala. 2015. "Initiation and Perpetuation of NLRP3 Inflammasome Activation and Assembly." *Immunological Reviews* 265 (1): 35–52.

Eltom, Suffwan, Maria G Belvisi, Christopher S Stevenson, Sarah a Maher, Eric Dubuis, Kate a Fitzgerald, and Mark a Birrell. 2014. "Role of the Inflammasome-Caspase1/11-IL-1/18 Axis in Cigarette Smoke Driven Airway Inflammation: An Insight into the Pathogenesis of COPD." *PloS One* 9 (11): e112829.

Erb-Downward, John R, Deborah L Thompson, Meilan K Han, Christine M Freeman, Lisa McCloskey, Lindsay a Schmidt, Vincent B Young, et al. 2011. "Analysis of the Lung Microbiome in the 'Healthy' Smoker and in COPD." *PloS One* 6 (2): e16384.

EURO-PERISTAT. 2010. "European Perinatal Health Report." Vol. 2014.

Feldmeyer, Laurence, Martin Keller, Gisela Niklaus, Daniel Hohl, Sabine Werner, and Hans-Dietmar Beer. 2007. "The Inflammasome Mediates UVB-Induced Activation and Secretion of Interleukin-1beta by Keratinocytes." *Current Biology : CB* 17 (13): 1140–45.

Fernandes-Alnemri, T, J Wu, J-W Yu, P Datta, B Miller, W Jankowski, S Rosenberg, J Zhang, and E S Alnemri. 2007. "The Pyroptosome: A Supramolecular Assembly of ASC Dimers Mediating Inflammatory Cell Death via Caspase-1 Activation." *Cell Death and Differentiation* 14 (9): 1590–1604.

Ferreira, Paul J, T Jared Bunch, Kurt H Albertine, and David P Carlton. 2000. “Circulating Neutrophil Concentration and Respiratory Distress in Premature Infants.” *The Journal of Pediatrics* 136 (4): 466–72.

Ferrero-Miliani, L, O H Nielsen, P S Andersen, and S E Girardin. 2007. “Chronic Inflammation: Importance of NOD2 and NALP3 in Interleukin-1beta Generation.” *Clinical and Experimental Immunology* 147 (2): 227–35.

Fire, A, D Albertson, S W Harrison, and D G Moerman. 1991. “Production of Antisense RNA Leads to Effective and Specific Inhibition of Gene Expression in C. Elegans Muscle.” *Development (Cambridge, England)* 113 (2): 503–14.

Følsgaard, Nilofar V., Susanne Schjørring, Bo L. Chawes, Morten a. Rasmussen, Karen a. Krogfelt, Susanne Brix, and Hans Bisgaard. 2013. “Pathogenic Bacteria Colonizing the Airways in Asymptomatic Neonates Stimulates Topical Inflammatory Mediator Release.” *American Journal of Respiratory and Critical Care Medicine* 187 (6): 589–95.

Forsythe, Paul. 2011. “Probiotics and Lung Diseases.” *Chest* 139 (4): 901–8.

Franchi, Luigi, Tatjana Eigenbrod, and Gabriel Núñez. 2009. “Cutting Edge: TNF-Alpha Mediates Sensitization to ATP and Silica via the NLRP3 Inflammasome in the Absence of Microbial Stimulation.” *Journal of Immunology (Baltimore, Md. : 1950)* 183 (2): 792–96.

Franco, Marie-Laure, Paul Waszak, Gaëlle Banalec, Micheline Levame, Chantal Lafuma, Alain Harf, and Christophe Delacourt. 2002. “LPS-Induced Lung Injury in Neonatal Rats: Changes in Gelatinase Activities and Consequences on Lung Growth.” *American Journal of Physiology-Lung Cellular and Molecular Physiology* 282 (3): L491–500.

Franklin, Bernardo S, Lukas Bossaller, Dominic De Nardo, Jacqueline M Ratter, Andrea Stutz, Gudrun Engels, Christoph Brenker, et al. 2014. “The Adaptor ASC Has Extracellular and ‘prionoid’ Activities That Propagate Inflammation.” *Nature Immunology* 15 (8): 727–37.

Franklin, Bernardo S, Eicke Latz, and Florian Ingo Schmidt. 2018. “The Intra- and Extracellular Functions of ASC Specks.” *Immunological Reviews* 281 (1): 74–87.

Fryklund, B, K Tullus, B Berglund, and L G Burman. 1992. “Importance of the Environment and the Faecal Flora of Infants, Nursing Staff and Parents as Sources of Gram-Negative Bacteria Colonizing Newborns in Three Neonatal Wards.” *Infection* 20 (5): 253–57.

Fujioka, Kazumichi, Akio Shibata, Tomoyuki Yokota, Tsubasa Koda, Miwako Nagasaka, Mariko Yagi, Yasuhiro Takeshima, Hideto Yamada, Kazumoto Iijima, and Ichiro Morioka. 2014. “Association of a Vascular Endothelial Growth Factor Polymorphism with the Development of Bronchopulmonary Dysplasia in Japanese Premature Newborns.” *Sci Rep* 4 (January): 4459.

Fukumoto, Jutaro, Itsuko Fukumoto, Prasanna Tamarapu Parthasarathy, Ruan Cox, Bao Huynh, Gurukumar Kollongod Ramanathan, Rajan Babu Venugopal, Diane S Allen-Gipson, Richard F Lockey, and Narasaiah Kolliputi. 2013. “NLRP3 Deletion Protects from Hyperoxia-Induced Acute Lung Injury.” *American Journal of Physiology. Cell Physiology* 305 (2): C182-9.

Galluzzi, L, I Vitale, J M Abrams, E S Alnemri, E H Baehrecke, M V Blagosklonny, T M Dawson, et al. 2012. “Molecular Definitions of Cell Death Subroutines: Recommendations of the Nomenclature Committee on Cell Death 2012.” *Cell Death and Differentiation* 19 (1): 107–20.

Geuking, Markus B, Yasmin Köller, Sandra Rupp, and Kathy D McCoy. 2014. “The Interplay between the Gut Microbiota and the Immune System.” *Gut Microbes* 5 (3): 411–18.

Giard, Donald J., Stuart A. Aaronson, George J. Todaro, Paul Arnstein, John H. Kersey, Harvey Dosik, and Wade P. Parks. 1973. “In Vitro Cultivation of Human Tumors: Establishment of Cell Lines Derived From a Series of Solid Tumors2.” *JNCI: Journal of the National Cancer Institute* 51 (5): 1417–23.

Gille, Christian, Baerbel Spring, Lena Tewes, Christian F. Poets, and Thorsten Orlikowsky. 2006. “A New Method to Quantify Phagocytosis and Intracellular Degradation Using Green Fluorescent Protein-LabeledEscherichia Coli: Comparison of Cord Blood Macrophages and Peripheral Blood Macrophages of Healthy Adults.” *Cytometry Part A* 69A (3): 152–54.

Gilmore, Thomas. 2007. “NF-KB Target Genes » NF-KB Transcription Factors | Boston University.” 2007. http://www.bu.edu/nf-kb/gene-resources/target-genes/.

Gleeson, Kevin, Douglas F. Eggli, and Steven L. Maxwell. 1997. “Quantitative Aspiration during Sleep in Normal Subjects.” *Chest* 111 (5): 1266–72.

Goddard, Amanda F, Benjamin J Staudinger, Scot E Dowd, Amruta Joshi-Datar, Randall D Wolcott, Moira L Aitken, Corinne L Fligner, and Pradeep K Singh. 2012. “Direct Sampling of Cystic Fibrosis Lungs Indicates That DNA-Based Analyses of Upper-Airway Specimens Can Misrepresent Lung Microbiota.” *Proceedings of the National Academy of Sciences of the United States of America* 109 (34): 13769–74.

Goel, Nitin, Sudeep Shrestha, Rhian Smith, Akshay Mehta, Malini Ketty, Helen Muxworthy, Artur Abelian, et al. 2019. “Screening for Early Onset Neonatal Sepsis: NICE Guidance-Based Practice versus Projected Application of the Kaiser Permanente Sepsis Risk Calculator in the UK Population.” *Archives of Disease in Childhood - Fetal and Neonatal Edition*, July, fetalneonatal-2018-316777.

Goffau, Marcus C. de, Susanne Lager, Ulla Sovio, Francesca Gaccioli, Emma Cook, Sharon J. Peacock, Julian Parkhill, D. Stephen Charnock-Jones, and Gordon C. S. Smith. 2019. “Human Placenta Has No Microbiome but Can Contain Potential Pathogens.” *Nature* 572 (7769): 329–34.

Goldbach-Mansky, Raphaela, Natalie J. Dailey, Scott W. Canna, Ana Gelabert, Janet Jones, Benjamin I. Rubin, H. Jeffrey Kim, et al. 2006. “Neonatal-Onset Multisystem Inflammatory Disease Responsive to Interleukin-1β Inhibition.” *New England Journal of Medicine* 355 (6): 581–92.

Goldenberg, R L, J F Culhane, J D Iams, and R Romero. 2008. “Epidemiology and Causes of Preterm Birth.” *Lancet* 371 (9606): 75–84.

Goldenberg, R L, J C Hauth, and W W Andrews. 2000. “Intrauterine Infection and Preterm Delivery.” *The New England Journal of Medicine* 342 (20): 1500–1507.

Gonzalez, A, I R Sosenko, J Chandar, H Hummler, N Claure, and E Bancalari. 1996. “Influence of Infection on Patent Ductus Arteriosus and Chronic Lung Disease in Premature Infants Weighing 1000 Grams or Less.” *The Journal of Pediatrics* 128 (4): 470–78.

Goodwin, R A. 1983. “Apical Localization of Pulmonary Tuberculosis, Chronic Pulmonary Histoplasmosis, and Progressive Massive Fibrosis of the Lung.” *CHEST Journal* 83 (5): 801.

Grailer, Jamison J, Bethany A Canning, Miriam Kalbitz, Mikel D Haggadone, Rasika M Dhond, Anuska V Andjelkovic, Firas S Zetoune, and Peter A Ward. 2014. “Critical Role for the NLRP3 Inflammasome during Acute Lung Injury.” *Journal of Immunology (Baltimore, Md. : 1950)* 192 (12): 5974–83.

Gray, R, SR Bonellie, J Chalmers, I Greer, S Jarvis, and C Williams. 2007. “Social Inequalities in Preterm Birth in Scotland 1980-2003: Findings from an Area-Based Measure of

Deprivation." *BJOG: An International Journal of Obstetrics & Gynaecology* 115 (1): 82–90.

Greenberg, David, Noga Givon-Lavi, Arnon Broides, Irena Blancovich, Nechama Peled, and Ron Dagan. 2006. "The Contribution of Smoking and Exposure to Tobacco Smoke to Streptococcus Pneumoniae and Haemophilus Influenzae Carriage in Children and Their Mothers." *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America* 42 (7): 897–903.

Greenough, Anne. 2012. "Long Term Respiratory Outcomes of Very Premature Birth (<32 Weeks)." *Seminars in Fetal and Neonatal Medicine*.

Grice, Elizabeth A, and Julia A Segre. 2011. "The Skin Microbiome." *Nature Reviews. Microbiology* 9: 244–53.

Grigg, J, S Arnon, and M Silverman. 1992. "Fractional Processing of Sequential Bronchoalveolar Lavage Fluid from Intubated Babies." *Eur Respir J* 5 (6): 727–32.

Groneck, P, B Götze-Speer, M Oppermann, H Eiffert, and C P Speer. 1994. "Association of Pulmonary Inflammation and Increased Microvascular Permeability during the Development of Bronchopulmonary Dysplasia: A Sequential Analysis of Inflammatory Mediators in Respiratory Fluids of High-Risk Preterm Neonates." *Pediatrics* 93 (5): 712–18.

Groneck, P, M Oppermann, and C P Speer. 1993. "Levels of Complement Anaphylatoxin C5a in Pulmonary Effluent Fluid of Infants at Risk for Chronic Lung Disease and Effects of Dexamethasone Treatment." *Pediatric Research* 34 (5): 586–90.

Groneck, P, J Schmale, V Soditt, H Stützer, B Götze-Speer, and C P Speer. 2001. "Bronchoalveolar Inflammation Following Airway Infection in Preterm Infants with Chronic Lung Disease." *Pediatric Pulmonology* 31 (5): 331–38.

Gross, Olaf, Hendrik Poeck, Michael Bscheider, Catherine Dostert, Nicole Hannesschläger, Stefan Endres, Gunther Hartmann, et al. 2009. "Syk Kinase Signalling Couples to the Nlrp3 Inflammasome for Anti-Fungal Host Defence." *Nature* 459 (7245): 433–36.

Guillot, Loïc, Samir Medjane, Karine Le-Barillec, Viviane Balloy, Claire Danel, Michel Chignard, and Mustapha Si-Tahar. 2004. "Response of Human Pulmonary Epithelial Cells to Lipopolysaccharide Involves Toll-like Receptor 4 (TLR4)-Dependent Signaling Pathways: Evidence for an Intracellular Compartmentalization of TLR4." *The Journal of Biological Chemistry* 279 (4): 2712–18.

Haas, Brian J, Dirk Gevers, Ashlee M Earl, Mike Feldgarden, Doyle V Ward, Georgia Giannoukos, Dawn Ciulla, et al. 2011. "Chimeric 16S RRNA Sequence Formation and Detection in Sanger and 454-Pyrosequenced PCR Amplicons." *Genome Research* 21 (3): 494–504.

Hall, M, C B Cole, S L Smith, R Fuller, and C J Rolles. 1990. "Factors Influencing the Presence of Faecal Lactobacilli in Early Infancy." *Archives of Disease in Childhood* 65: 185–88.

Hall, T.A. 1999. "BioEdit: A User-Friendly Biological Sequence Alignment Editor and Analysis Program for Windows 95/98/NT." *Nucleic Acids Symposium Series*.

Hanna, Nazeeh, Pedro Vasquez, Petra Pham, Diane E. Heck, Jeffrey D. Laskin, Debra L. Laskin, and Barry Weinberger. 2005. "Mechanisms Underlying Reduced Apoptosis in Neonatal Neutrophils." *Pediatric Research* 57 (1): 56–62.

Hansen, Richard, Karen P Scott, Shoaib Khan, Jenny C Martin, Susan H Berry, Matthew Stevenson, Augusta Okpapi, Michael J Munro, and Georgina L Hold. 2015. "First-Pass Meconium Samples from Healthy Term Vaginally-Delivered Neonates: An Analysis of the Microbiota." *PloS One* 10 (7): e0133320.

Harju, K, V Glumoff, and M Hallman. 2001. “Ontogeny of Toll-like Receptors Tlr2 and Tlr4 in Mice.” *Pediatric Research* 49 (1): 81–83.

Harris, J Kirk, Mary Ann De Groote, Scott D Sagel, Edith T Zemanick, Robert Kapsner, Churee Penvari, Heidi Kaess, Robin R Deterding, Frank J Accurso, and Norman R Pace. 2007. “Molecular Identification of Bacteria in Bronchoalveolar Lavage Fluid from Children with Cystic Fibrosis.” *Proceedings of the National Academy of Sciences of the United States of America* 104 (51): 20529–33.

Harris, J, T A Merritt, C G Alexson, L Longfield, and J A Manning. 1982. “Parenteral Indomethacin for Closure of the Patent Ductus Arteriosus. Clinical Experience with 67 Preterm Infants.” *American Journal of Diseases of Children* 136 (11): 1005–8.

Haverkamp, M H, E van de Vosse, R Goldbach-Mansky, and S M Holland. 2014. “Impaired Cytokine Responses in Patients with Cryopyrin-Associated Periodic Syndrome (CAPS).” *Clinical and Experimental Immunology* 177 (3): 720–31.

Hayashi, F, K D Smith, A Ozinsky, T R Hawn, E C Yi, D R Goodlett, J K Eng, S Akira, D M Underhill, and A Aderem. 2001. “The Innate Immune Response to Bacterial Flagellin Is Mediated by Toll-like Receptor 5.” *Nature* 410 (6832): 1099–1103.

Hecht, J L, R N Fichorova, V F Tang, E N Allred, T F McElrath, and A Leviton. 2011. “Relationship Between Neonatal Blood Protein Concentrations and Placenta Histologic Characteristics in Extremely Low GA Newborns.” *Pediatr Res* 69 (1): 68–73.

Heijtz, R. D., S. Wang, F. Anuar, Y. Qian, B. Bjorkholm, A. Samuelsson, M. L. Hibberd, H. Forssberg, and S. Pettersson. 2011. “Normal Gut Microbiota Modulates Brain Development and Behavior.” *Proceedings of the National Academy of Sciences* 108 (7): 3047–52.

Heil, Florian, Hiroaki Hemmi, Hubertus Hochrein, Franziska Ampenberger, Carsten Kirschning, Shizuo Akira, Grayson Lipford, Hermann Wagner, and Stefan Bauer. 2004. “Species-Specific Recognition of Single-Stranded RNA via Toll-like Receptor 7 and 8.” *Science (New York, N.Y.)* 303 (5663): 1526–29.

Herlemann, Daniel Pr, Matthias Labrenz, Klaus Jürgens, Stefan Bertilsson, Joanna J Waniek, and Anders F Andersson. 2011. “Transitions in Bacterial Communities along the 2000 Km Salinity Gradient of the Baltic Sea.” *The ISME Journal* 5 (10): 1571–79.

Hill, M. O. 1973. “Diversity and Evenness: A Unifying Notation and Its Consequences.” *Ecology* 54 (2): 427–32.

Hilty, Markus, Conor Burke, Helder Pedro, Paul Cardenas, Andy Bush, Cara Bossley, Jane Davies, et al. 2010. “Disordered Microbial Communities in Asthmatic Airways.” *PloS One* 5 (1): e8578.

Hilty, Markus, Weihong Qi, Silvio D. Brugger, Laurence Frei, Philipp Agyeman, Pascal M. Frey, Suzanne Aebi, and Kathrin Mühlemann. 2012. “Nasopharyngeal Microbiota in Infants With Acute Otitis Media.” *The Journal of Infectious Diseases* 205 (7): 1048–55.

Hoffman, H M, A A Wanderer, and D H Broide. 2001. “Familial Cold Autoinflammatory Syndrome: Phenotype and Genotype of an Autosomal Dominant Periodic Fever.” *The Journal of Allergy and Clinical Immunology* 108 (4): 615–20.

Hogan, Deborah A., Sven D. Willger, Emily L. Dolben, Thomas H. Hampton, Bruce A. Stanton, Hilary G. Morrison, Mitchell L. Sogin, Julianna Czum, and Alix Ashare. 2016. “Analysis of Lung Microbiota in Bronchoalveolar Lavage, Protected Brush and Sputum Samples from Subjects with Mild-To-Moderate Cystic Fibrosis Lung Disease.” *Plos One* 11 (3): e0149998.

Honma, Yoko, Yukari Yada, Naoto Takahashi, Mariko Y Momoi, and Yoshikazu Nakamura. 2007. "Certain Type of Chronic Lung Disease of Newborns Is Associated with Ureaplasma Urealyticum Infection in Utero." *Pediatrics International : Official Journal of the Japan Pediatric Society* 49 (4): 479–84.

Hooper, Lora V, Dan R Littman, and Andrew J Macpherson. 2012. "Interactions between the Microbiota and the Immune System." *Science (New York, N.Y.)* 336 (6086): 1268–73.

Hornung, Veit, Franz Bauernfeind, Annett Halle, Eivind O Samstad, Hajime Kono, Kenneth L Rock, Katherine A Fitzgerald, and Eicke Latz. 2008. "Silica Crystals and Aluminum Salts Activate the NALP3 Inflammasome through Phagosomal Destabilization." *Nature Immunology* 9 (8): 847–56.

Huang, Yvonne J, Craig E Nelson, Eoin L Brodie, Todd Z Desantis, Marshall S Baek, Jane Liu, Tanja Woyke, et al. 2011. "Airway Microbiota and Bronchial Hyperresponsiveness in Patients with Suboptimally Controlled Asthma." *J Allergy Clin Immunol* 127 (2): 372–73.

Huffnagle, G B, R P Dickson, and N W Lukacs. 2017. "The Respiratory Tract Microbiome and Lung Inflammation: A Two-Way Street." *Mucosal Immunology* 10 (2): 299–306.

Hughes, Jennifer B, Jessica J Hellmann, Taylor H Ricketts, and B J Bohannan. 2001. "Counting the Uncountable: Statistical Approaches to Estimating Microbial Diversity." *Applied and Environmental Microbiology* 67 (10): 4399–4406.

Hysinger, Erik, Nicholas Friedman, Erik Jensen, Huayan Zhang, and Joseph Piccione. 2019. "Bronchoscopy in Neonates with Severe Bronchopulmonary Dysplasia in the NICU." *Journal of Perinatology* 39 (2): 263–68.

Iliodromiti, Zoe, Dimitrios Zygouris, Stavros Sifakis, Kalliopi I Pappa, Panagiotis Tsikouras, Nikolaos Salakos, Angelos Daniilidis, Charalambos Siristatidis, and Nikolaos Vrachnis. 2013. "Acute Lung Injury in Preterm Fetuses and Neonates: Mechanisms and Molecular Pathways." *The Journal of Maternal-Fetal & Neonatal Medicine : The Official Journal of the European Association of Perinatal Medicine, the Federation of Asia and Oceania Perinatal Societies, the International Society of Perinatal Obstetricians* 26 (17): 1696–1704.

Im, H., and A Ammit. 2014. "The NLRP3 Inflammasome: Role in Airway Inflammation." *Clinical and Experimental Allergy* 44: 160–72.

Imamura, Takashi, Maki Sato, Hayato Go, Kei Ogasawara, Yuji Kanai, Hajime Maeda, Mina Chishiki, et al. 2016. "The Microbiome of the Lower Respiratory Tract in Premature Infants with and without Severe Bronchopulmonary Dysplasia." *American Journal of Perinatology*, May 30, 2016.

ISRCTN-11650227. 2019. "Azithromycin Therapy for Chronic Lung Disease of Prematurity." 2019. https://www.isrctn.com/ISRCTN11650227?q=aztec&filters=&sort=&offset=1&totalResults=2&page=1&pageSize=10&searchType=basic-search.

Jacquot, Aurelien, Dorine Neveu, Fabien Aujoulat, Gregoire Mercier, Hélne Marchandin, Estelle Jumas-Bilak, and Jean Charles Picaud. 2011. "Dynamics and Clinical Evolution of Bacterial Gut Microflora in Extremely Premature Patients." *Journal of Pediatrics* 158: 390–96.

Jakkula, M, T D Le Cras, S Gebb, K P Hirth, R M Tuder, N F Voelkel, and S H Abman. 2000. "Inhibition of Angiogenesis Decreases Alveolarization in the Developing Rat Lung." *Am J Physiol Lung Cell Mol Physiol* 279 (3): L600-7.

Jeng, Suh Fang, Chyong Hsin Hsu, Po Nien Tsao, Hung Chieh Chou, Wang T. Lee, Hsin a. Kao, Han Yang Hung, Jui Hsing Chang, Nan Chang Chiu, and Wu Shiun Hsieh. 2008.

"Bronchopulmonary Dysplasia Predicts Adverse Developmental and Clinical Outcomes in Very-Low-Birthweight Infants." *Developmental Medicine and Child Neurology* 50 (1): 51–57.

Jenke, A. C., J. Postberg, B. Mariel, K. Hensel, D. Foell, J. Däbritz, and S. Wirth. 2013. "S100A12 and HBD2 Correlate with the Composition of the Fecal Microflora in ELBW Infants and Expansion of E. COLI Is Associated with NEC." *BioMed Research International* 2013 (i).

Jiménez, Esther, Leonides Fernández, María L Marín, Rocío Martín, Juan M Odriozola, Carmen Nueno-Palop, Arjan Narbad, Mónica Olivares, Jordi Xaus, and Juan M Rodríguez. 2005. "Isolation of Commensal Bacteria from Umbilical Cord Blood of Healthy Neonates Born by Cesarean Section." *Current Microbiology* 51 (4): 270–74.

Jobe, A. 2006. "The New BPD." *NeoReviews* 7 (10): e531–45.

Jobe, Alan. 1999. "The New BPD: An Arrest of Lung Development." *Pediatric Research* 46 (6): 641–43.

Jobe, Alan, and Eduardo Bancalari. 2001. "Bronchopulmonary Dysplasia." *American Journal of Respiratory and Critical Care Medicine* 163 (7): 1723–29.

Jones, Hannah E, Kathryn Harris, Malika Azizia, Lindsay Bank, Bernadette Carpenter, John C Hartley, Nigel Klein, and Donald Peebles. 2009. "Differing Prevalence and Diversity of Bacterial Species in Fetal Membranes from Very Preterm and Term Labor." *PloS One* 4 (12): e8205.

Jónsson, B, Y H Li, G Noack, A Brauner, and K Tullus. 2000. "Downregulatory Cytokines in Tracheobronchial Aspirate Fluid from Infants with Chronic Lung Disease of Prematurity." *Acta Paediatrica (Oslo, Norway : 1992)* 89 (11): 1375–80.

Jorth, Peter, Benjamin J. Staudinger, Xia Wu, Katherine B. Hisert, Hillary Hayden, Jayanthi Garudathri, Christopher L. Harding, et al. 2015. "Regional Isolation Drives Bacterial Diversification within Cystic Fibrosis Lungs." *Cell Host & Microbe*, 1–13.

Jost, T., C. Lacroix, C. Braegger, and C. Chassard. 2015. "Impact of Human Milk Bacteria and Oligosaccharides on Neonatal Gut Microbiota Establishment and Gut Health." *Nutrition Reviews* 73 (7): 426–37.

Jubinville, Eric, Marc Veillette, Julie Milot, François Maltais, André M Comeau, Roger C Levesque, and Caroline Duchaine. 2018. "Exacerbation Induces a Microbiota Shift in Sputa of COPD Patients." *PloS One* 13 (3): e0194355.

Juliana, Christine, Teresa Fernandes-Alnemri, Seokwon Kang, Andrew Farias, Fengsong Qin, and Emad S Alnemri. 2012. "Non-Transcriptional Priming and Deubiquitination Regulate NLRP3 Inflammasome Activation." *The Journal of Biological Chemistry* 287 (43): 36617–22.

Kadowaki, N, S Ho, S Antonenko, R W Malefyt, R A Kastelein, F Bazan, and Y J Liu. 2001. "Subsets of Human Dendritic Cell Precursors Express Different Toll-like Receptors and Respond to Different Microbial Antigens." *The Journal of Experimental Medicine* 194 (6): 863–69.

Kalliomäki, M, P Kirjavainen, E Eerola, P Kero, S Salminen, and E Isolauri. 2001. "Distinct Patterns of Neonatal Gut Microflora in Infants in Whom Atopy Was and Was Not Developing." *The Journal of Allergy and Clinical Immunology* 107 (1): 129–34.

Källman, J, J Schollin, C Schalèn, a Erlandsson, and E Kihlström. 1998. "Impaired Phagocytosis and Opsonisation towards Group B Streptococci in Preterm Neonates." *Archives of Disease in Childhood. Fetal and Neonatal Edition* 78 (1): F46–50.

Kanneganti, Thirumala-Devi, Mathilde Body-Malapel, Amal Amer, Jong-Hwan Park, Joel Whitfield, Luigi Franchi, Zenobia F Taraporewala, et al. 2006. "Critical Role for Cryopyrin/Nalp3 in Activation of Caspase-1 in Response to Viral Infection and Double-Stranded RNA." *The Journal of Biological Chemistry* 281 (48): 36560–68.

Kavvadia, V, A Greenough, G Dimitriou, and R Hooper. 2000. "Randomised Trial of Fluid Restriction in Ventilated Very Low Birthweight Infants." *Archives of Disease in Childhood. Fetal and Neonatal Edition* 83 (2): F91-6.

Kawai, Taro, and Shizuo Akira. 2007. "TLR Signaling." *Seminars in Immunology* 19 (1): 24–32.

Kayagaki, Nobuhiko, Søren Warming, Mohamed Lamkanfi, Lieselotte Vande Walle, Salina Louie, Jennifer Dong, Kim Newton, et al. 2011. "Non-Canonical Inflammasome Activation Targets Caspase-11." *Nature* 479 (7371): 117–21.

Kehrl, J H, A B Roberts, L M Wakefield, S Jakowlew, M B Sporn, and A S Fauci. 1986. "Transforming Growth Factor Beta Is an Important Immunomodulatory Protein for Human B Lymphocytes." *Journal of Immunology (Baltimore, Md. : 1950)* 137 (12): 3855–60.

Kelly, C A, C J Kotre, C Ward, D J Hendrick, and E H Walters. 1987. "Anatomical Distribution of Bronchoalveolar Lavage Fluid as Assessed by Digital Subtraction Radiography." *Thorax* 42 (8): 624–28.

Kelly, John, Yuliya Borre, Ciaran O' Brien, Elaine Patterson, Sahar El Aidy, Jennifer Deane, Paul J. Kennedy, et al. 2016. "Transferring the Blues: Depression-Associated Gut Microbiota Induces Neurobehavioural Changes in the Rat." *Journal of Psychiatric Research*.

Kent, Alison L, Ian M Wright, and Mohamed E Abdel-Latif. 2011. "Mortality and Adverse Neurologic Outcomes Are Greater in Preterm Male Infants."

Kinsella, John P, Anne Greenough, and Steven H Abman. 2006. "Bronchopulmonary Dysplasia." *Lancet* 367 (9520): 1421–31.

Klappenbach, Joel A, Paul R Saxman, James R Cole, and Thomas M Schmidt. 2001. "Rrndb: The Ribosomal RNA Operon Copy Number Database." *Nucleic Acids Research* 29 (1): 181–84.

Klindworth, Anna, Elmar Pruesse, Timmy Schweer, Jörg Peplies, Christian Quast, Matthias Horn, and Frank Oliver Glöckner. 2013. "Evaluation of General 16S Ribosomal RNA Gene PCR Primers for Classical and Next-Generation Sequencing-Based Diversity Studies." *Nucleic Acids Research* 41 (1): e1.

Klinger, G., I. Levy, L. Sirota, V. Boyko, L. Lerner-Geva, B. Reichman, and Israel Neonatal Network. 2010. "Outcome of Early-Onset Sepsis in a National Cohort of Very Low Birth Weight Infants." *PEDIATRICS* 125 (4): e736–40.

Knight, D B. 2001. "The Treatment of Patent Ductus Arteriosus in Preterm Infants. A Review and Overview of Randomized Trials." *Seminars in Neonatology : SN* 6 (1): 63–73.

Koenderman, Leo, Wim Buurman, and Mohamed R. Daha. 2014. "The Innate Immune Response." *Immunology Letters* 162 (2): 95–102.

Koksal, N, B Kayik, M Cetinkaya, H Ozkan, F Budak, S Kilic, Y Canitez, and B Oral. 2012. "Value of Serum and Bronchoalveolar Fluid Lavage Pro- and Anti-Inflammatory Cytokine Levels for Predicting Bronchopulmonary Dysplasia in Premature Infants." *Eur Cytokine Netw* 23 (2): 29–35.

Kolliputi, Narasaiah, Rahamthulla S Shaik, and Aaron B Waxman. 2010. "The Inflammasome Mediates Hyperoxia-Induced Alveolar Cell Permeability." *Journal of Immunology*

(Baltimore, Md. : 1950) 184 (10): 5819–26.

Kotecha, Sailesh. 1999. “Bronchoalveolar Lavage of Newborn Infants.” *Pediatric Pulmonology. Supplement* 18 (January): 122–24.

Kotecha, Sailesh, B Chan, N Azam, M Silverman, and R J Shaw. 1995. “Increase in Interleukin-8 and Soluble Intercellular Adhesion Molecule-1 in Bronchoalveolar Lavage Fluid from Premature Infants Who Develop Chronic Lung Disease.” *Archives of Disease in Childhood. Fetal and Neonatal Edition* 72 (2): F90-6.

Kotecha, Sailesh, R J Mildner, L R Prince, J R Vyas, A E Currie, R A Lawson, and M K B Whyte. 2003. “The Role of Neutrophil Apoptosis in the Resolution of Acute Lung Injury in Newborn Infants.” *Thorax* 58 (11): 961–67.

Kotecha, Sailesh, A Wangoo, M Silverman, and R J Shaw. 1996. “Increase in the Concentration of Transforming Growth Factor Beta-1 in Bronchoalveolar Lavage Fluid before Development of Chronic Lung Disease of Prematurity.” *The Journal of Pediatrics* 128 (4): 464–69.

Kotecha, Sailesh, L Wilson, A Wangoo, M Silverman, and R J Shaw. 1996. “Increase in Interleukin (IL)-1 Beta and IL-6 in Bronchoalveolar Lavage Fluid Obtained from Infants with Chronic Lung Disease of Prematurity.” *Pediatric Research* 40 (2): 250–56.

Kotecha, Sarah J, Martin O Edwards, W John Watkins, A John Henderson, Shantini Paranjothy, Frank D Dunstan, and Sailesh Kotecha. 2013. “Effect of Preterm Birth on Later FEV1: A Systematic Review and Meta-Analysis.” *Thorax* 68 (8): 760–66.

Kramer, Boris W, Suhas Kallapur, John Newnham, and Alan H Jobe. 2009. “Prenatal Inflammation and Lung Development.” *Seminars in Fetal & Neonatal Medicine* 14 (1): 2–7.

Kramer, Boris W, Susanne Kramer, Machiko Ikegami, and Alan H Jobe. 2002. “Injury, Inflammation, and Remodeling in Fetal Sheep Lung after Intra-Amniotic Endotoxin.” *American Journal of Physiology. Lung Cellular and Molecular Physiology* 283 (2): L452–59.

Kribs, Angela, Claudia Roll, Wolfgang Göpel, Christian Wieg, Peter Groneck, Reinhard Laux, Norbert Teig, et al. 2015. “Nonintubated Surfactant Application vs Conventional Therapy in Extremely Preterm Infants: A Randomized Clinical Trial.” *JAMA Pediatrics* 169 (8): 723–30.

Kuipers, Maria T., Hamid Aslami, John R. Janczy, Koenraad F. van der Sluijs, Alexander P. J. Vlaar, Esther K. Wolthuis, Goda Choi, et al. 2012. “Ventilator-Induced Lung Injury Is Mediated by the NLRP3 Inflammasome.” *Anesthesiology* 116 (5): 1104–15.

Kunig, Anette M, Vivek Balasubramaniam, Neil E Markham, Danielle Morgan, Greg Montgomery, Theresa R Grover, and Steven H Abman. 2005. “Recombinant Human VEGF Treatment Enhances Alveolarization after Hyperoxic Lung Injury in Neonatal Rats.” *American Journal of Physiology. Lung Cellular and Molecular Physiology* 289 (4): L529-35.

Kuri, Paola, Nicole L. Schieber, Thomas Thumberger, Joachim Wittbrodt, Yannick Schwab, and Maria Leptin. 2017. “Dynamics of in Vivo ASC Speck Formation.” *The Journal of Cell Biology* 216 (9): 2891–2909.

Lahra, Monica M, Philip J Beeby, and Heather E Jeffery. 2009. “Intrauterine Inflammation, Neonatal Sepsis, and Chronic Lung Disease: A 13-Year Hospital Cohort Study What’s Known on This Subject.”

Lal, Charitharth Vivek, Jegen Kandasamy, Kalsang Dolma, Manimaran Ramani, Ranjit Kumar, Landon Wilson, Zubair Aghai, et al. 2018. “Early Airway Microbial Metagenomic and

Metabolomic Signatures Are Associated with Development of Severe Bronchopulmonary Dysplasia." *American Journal of Physiology - Lung Cellular and Molecular Physiology* 315 (5): L810–15.

Lal, Charitharth Vivek, Colm Travers, Zubair H Aghai, Peter Eipers, Tamas Jilling, Brian Halloran, Waldemar A Carlo, et al. 2016. "The Airway Microbiome at Birth." *Scientific Reports* 6 (January): 31023.

Lambert, Laura, and Fiona J. Culley. 2017. "Innate Immunity to Respiratory Infection in Early Life." *Frontiers in Immunology* 8 (November).

Lamkanfi, Mohamed, and Vishva M Dixit. 2014. "Mechanisms and Functions of Inflammasomes." *Cell* 157 (5): 1013–22.

Lamkanfi, Mohamed, James L Mueller, Alberto C Vitari, Shahram Misaghi, Anna Fedorova, Kurt Deshayes, Wyne P Lee, Hal M Hoffman, and Vishva M Dixit. 2009. "Glyburide Inhibits the Cryopyrin/Nalp3 Inflammasome." *The Journal of Cell Biology* 187 (1): 61–70.

Latz, Eicke. 2010. "The Inflammasomes: Mechanisms of Activation and Function." *Current Opinion in Immunology* 22 (1): 28–33.

Laughon, Matthew, Elizabeth N Allred, Carl Bose, T Michael O'Shea, Linda J Van Marter, Richard Ehrenkranz, and Alan Leviton. 2009. "Patterns of Respiratory Disease during the First 2 Postnatal Weeks in Extremely Premature Infants." *Pediatrics* 123 (4): 1124–31.

Lavoie, Pascal M, Chandra Pham, and Kerry L Jang. 2008. "Heritability of Bronchopulmonary Dysplasia, Defined According to the Consensus Statement of the National Institutes of Health." *Pediatrics* 122 (3): 479–85.

Layne, Ennis. 1957. "[73] Spectrophotometric and Turbidimetric Methods for Measuring Proteins." *Methods in Enzymology* 3: 447–54.

Lecart, C, R Cayabyab, S Buckley, J Morrison, K Y Kwong, D Warburton, R Ramanathan, C A Jones, and P Minoo. 2000. "Bioactive Transforming Growth Factor-Beta in the Lungs of Extremely Low Birthweight Neonates Predicts the Need for Home Oxygen Supplementation." *Biology of the Neonate* 77 (4): 217–23.

Lederberg, J, and AT McCray. 2001. "'Ome Sweet 'Omics-- A Genealogical Treasury of Words." *Scientist* 15 (8).

Lee, Geun-Shik, Naeha Subramanian, Andrew I Kim, Ivona Aksentijevich, Raphaela Goldbach-Mansky, David B Sacks, Ronald N Germain, Daniel L Kastner, and Jae Jin Chae. 2012. "The Calcium-Sensing Receptor Regulates the NLRP3 Inflammasome through Ca2+ and CAMP." *Nature* 492 (7427): 123–27.

Lee, Suki M Y, Kin-Hang Kok, Martial Jaume, Timothy K W Cheung, Tsz-Fung Yip, Jimmy C C Lai, Yi Guan, Robert G Webster, Dong-Yan Jin, and J S Malik Peiris. 2014. "Toll-like Receptor 10 Is Involved in Induction of Innate Immune Responses to Influenza Virus Infection." *Proceedings of the National Academy of Sciences of the United States of America* 111: 3793–98.

Lee, Yun Kyung, Juscilene S Menezes, Yoshinori Umesaki, and Sarkis K Mazmanian. 2011. "Proinflammatory T-Cell Responses to Gut Microbiota Promote Experimental Autoimmune Encephalomyelitis." *Proceedings of the National Academy of Sciences of the United States of America* 108 Suppl (March): 4615–22.

Lemaitre, B, E Nicolas, L Michaut, J Reichhart, and J Hoffmann. 1996. "The Dorsoventral Regulatory Gene Cassette Controls the Potent Antifungal Response in Drosophila Adults." *Cell* 86 (6): 973–83.

Liao, Jie, Vishal S Kapadia, L Steven Brown, Naeun Cheong, Christopher Longoria, Dan Mija, Mrithyunjay Ramgopal, Julie Mirpuri, Donald C McCurnin, and Rashmin C Savani. 2015. "The NLRP3 Inflammasome Is Critically Involved in the Development of Bronchopulmonary Dysplasia." *Nature Communications* 6: 8977.

Lin, Su-Chang, Yu-Chih Lo, and Hao Wu. 2010. "Helical Assembly in the MyD88-IRAK4-IRAK2 Complex in TLR/IL-1R Signalling." *Nature* 465 (7300): 885–90.

Lindberg, Erika, I Adlerberth, Bill Hesselmar, Robert Saalman, Inga Lisa Strannegård, Nils Åberg, and Agnes E. Wold. 2004. "High Rate of Transfer of Staphylococcus Aureus from Parental Skin to Infant Gut Flora." *Journal of Clinical Microbiology* 42 (2): 530–34.

Ling, Zongxin, Xia Liu, Yueqiu Luo, Li Yuan, Karen E Nelson, Yuezhu Wang, Charlie Xiang, and Lanjuan Li. 2013. "Pyrosequencing Analysis of the Human Microbiota of Healthy Chinese Undergraduates." *BMC Genomics* 14 (January): 390.

Liu, Cindy M, Maliha Aziz, Sergey Kachur, Po-Ren Hsueh, Yu-Tsung Huang, Paul Keim, and Lance B Price. 2012. "BactQuant: An Enhanced Broad-Coverage Bacterial Quantitative Real-Time PCR Assay." *BMC Microbiology* 12 (1): 56.

Lohmann, Pablo, Ruth A Luna, Emily B Hollister, Sridevi Devaraj, Toni-Ann Mistretta, Stephen E Welty, and James Versalovic. 2014. "The Airway Microbiome of Intubated Premature Infants: Characteristics and Changes That Predict the Development of Bronchopulmonary Dysplasia." *Pediatric Research* 76 (3): 294–301.

Lowe, J, W J Watkins, M O Edwards, O B Spiller, E Jacqz-Aigrain, S J Kotecha, and S Kotecha. 2014. "Association between Pulmonary Ureaplasma Colonization and Bronchopulmonary Dysplasia in Preterm Infants: Updated Systematic Review and Meta-Analysis." *Pediatr Infect Dis J* 33 (7): 697–702.

Lu, Alvin, Venkat Giri Magupalli, Jianbin Ruan, Qian Yin, Maninjay K Atianand, Matthijn R Vos, Gunnar F Schröder, Katherine A Fitzgerald, Hao Wu, and Edward H Egelman. 2014. "Unified Polymerization Mechanism for the Assembly of ASC-Dependent Inflammasomes." *Cell* 156 (6): 1193–1206.

Mackay, Rose-Marie A., J. Paul Townsend, Jennifer Calvert, Mark Anthony, Andrew R. Wilkinson, Anthony D. Postle, Howard W. Clark, and David A. Todd. 2019. "Increased Surfactant Protein-D Levels in the Airways of Preterm Neonates with Sepsis Indicated Responses to Infectious Challenges." *Acta Paediatrica* 108 (5): 870–76.

Madan, J C, D C Koestler, B A Stanton, L Davidson, L A Moulton, M L Housman, J H Moore, et al. 2012. "Serial Analysis of the Gut and Respiratory Microbiome in Cystic Fibrosis in Infancy: Interaction between Intestinal and Respiratory Tracts and Impact of Nutritional Exposures." *MBio* 3 (4): e00251-12-e00251-12.

Magnan, A, J L Mege, J C Escallier, J Brisse, C Capo, M Reynaud, P Thomas, et al. 1996. "Balance between Alveolar Macrophage IL-6 and TGF-Beta in Lung-Transplant Recipients. Marseille and Montréal Lung Transplantation Group." *American Journal of Respiratory and Critical Care Medicine* 153 (4): 1431–36.

Maheshwari, Akhil, Nikolai N Voitenok, Svetlana Akalovich, Sadiq S Shaik, David A Randolph, Brian Sims, Rakesh P Patel, Cheryl R Killingsworth, Michael B Fallon, and Robin K Ohls. 2009. "Developmental Changes in Circulating IL-8/CXCL8 Isoforms in Neonates." *Cytokine* 46 (1): 12–16.

Mai, Volker, Christopher Michael Young, Maria Ukhanova, Xiaoyu Wang, Yijun Sun, George Casella, Douglas Theriaque, et al. 2011. "Fecal Microbiota in Premature Infants Prior to Necrotizing Enterocolitis." *PLoS ONE* 6 (6): 1–7.

Man, Si Ming, Lee J Hopkins, Eileen Nugent, Susan Cox, Ivo M Glück, Panagiotis Tourlomousis, John a Wright, Pietro Cicuta, Tom P Monie, and Clare E Bryant. 2014. "Inflammasome Activation Causes Dual Recruitment of NLRC4 and NLRP3 to the Same Macromolecular Complex." *Proceedings of the National Academy of Sciences of the United States of America* 111 (20): 7403–8.

Maniscalco, W M, R H Watkins, C T D'Angio, and R M Ryan. 1997. "Hyperoxic Injury Decreases Alveolar Epithelial Cell Expression of Vascular Endothelial Growth Factor (VEGF) in Neonatal Rabbit Lung." *American Journal of Respiratory Cell and Molecular Biology* 16 (5): 557–67.

Marchesi, Julian R., and Jacques Ravel. 2015. "The Vocabulary of Microbiome Research: A Proposal." *Microbiome* 3 (1): 31.

Marchini, Giovanna, Annika Nelson, Josefine Edner, Solbritt Lonne-Rahm, Anneli Stavréus-Evers, and Kjell Hultenby. 2005. "Erythema Toxicum Neonatorum Is an Innate Immune Response to Commensal Microbes Penetrated into the Skin of the Newborn Infant." *Pediatric Research* 58 (3): 613–16.

Marcos-Vadillo, Elena, and Asunción García-Sánchez. 2016. "Protocol for Lipid-Mediated Transient Transfection in A549 Epithelial Lung Cell Line." In *Methods in Molecular Biology (Clifton, N.J.)*, 1434:185–97.

Mariathasan, Sanjeev, David S Weiss, Kim Newton, Jacqueline McBride, Karen O'Rourke, Meron Roose-Girma, Wyne P Lee, Yvette Weinrauch, Denise M Monack, and Vishva M Dixit. 2006. "Cryopyrin Activates the Inflammasome in Response to Toxins and ATP." *Nature* 440 (7081): 228–32.

Marsh, R. L., M. Kaestli, A. B. Chang, M. J. Binks, C. E. Pope, L. R. Hoffman, and H. C. Smith-Vaughan. 2016. "The Microbiota in Bronchoalveolar Lavage from Young Children with Chronic Lung Disease Includes Taxa Present in Both the Oropharynx and Nasopharynx." *Microbiome* 4 (1): 37.

Marsland, Benjamin J, Aurélien Trompette, and Eva S Gollwitzer. 2015. "The Gut–Lung Axis in Respiratory Disease." *Annals of the American Thoracic Society* 12 (Supplement 2): S150–56.

Marter, Linda J Van, Olaf Dammann, Elizabeth N Allred, Alan Leviton, Marcello Pagano, Marianne Moore, and Camilia Martin. 2002. "Chorioamnionitis, Mechanical Ventilation, and Postnatal Sepsis as Modulators of Chronic Lung Disease in Preterm Infants." *The Journal of Pediatrics* 140 (2): 171–76.

Martinon, Fabio, Kimberly Burns, and Jürg Tschopp. 2002. "The Inflammasome: A Molecular Platform Triggering Activation of Inflammatory Caspases and Processing of ProIL-Beta." *Molecular Cell* 10 (2): 417–26.

Masumoto, J, S Taniguchi, K Ayukawa, H Sarvotham, T Kishino, N Niikawa, E Hidaka, T Katsuyama, T Higuchi, and J Sagara. 1999. "ASC, a Novel 22-KDa Protein, Aggregates during Apoptosis of Human Promyelocytic Leukemia HL-60 Cells." *The Journal of Biological Chemistry* 274 (48): 33835–38.

Matsumiya, Yoshiko, Naoki Kato, Kunitomo Watanabe, and Haru Kato. 2002. "Molecular Epidemiological Study of Vertical Transmission of Vaginal Lactobacillus Species from Mothers to Newborn Infants in Japanese, by Arbitrarily Primed Polymerase Chain Reaction." *Journal of Infection and Chemotherapy : Official Journal of the Japan Society of Chemotherapy* 8 (1): 43–49.

McClure, Ryan, and Paola Massari. 2014. "TLR-Dependent Human Mucosal Epithelial Cell

Responses to Microbial Pathogens." *Frontiers in Immunology* 5 (January): 386.

McGinn, Steven, and Ivo Glynne Gut. 2013. "DNA Sequencing - Spanning the Generations." *New Biotechnology* 30 (4): 366–72.

McMurdie, Paul, and Susan Holmes. 2014. "Waste Not, Want Not: Why Rarefying Microbiome Data Is Inadmissible." Edited by Alice Carolyn McHardy. *PLoS Computational Biology* 10 (4): e1003531.

McMurdie, Paul J., and Susan Holmes. 2013. "Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data." *PLoS ONE* 8 (4).

Means, T K, E Lien, A Yoshimura, S Wang, D T Golenbock, and M J Fenton. 1999. "The CD14 Ligands Lipoarabinomannan and Lipopolysaccharide Differ in Their Requirement for Toll-like Receptors." *Journal of Immunology (Baltimore, Md. : 1950)* 163 (12): 6748–55.

MedCalc. 2010. "MedCalc's Odds Ratio Calculator." 2010. https://www.medcalc.org/calc/odds_ratio.php.

Medzhitov, R, P Preston-Hurlburt, and C A Janeway. 1997. "A Human Homologue of the Drosophila Toll Protein Signals Activation of Adaptive Immunity." *Nature* 388 (6640): 394–97.

Metsalu, Tauno, and Jaak Vilo. 2015. "ClustVis: A Web Tool for Visualizing Clustering of Multivariate Data Using Principal Component Analysis and Heatmap." *Nucleic Acids Research* 43 (W1): W566–70.

Mika, Moana, Ines Mack, Insa Korten, Weihong Qi, Suzanne Aebi, Urs Frey, Philipp Latzin, and Markus Hilty. 2015. "Dynamics of the Nasal Microbiota in Infancy: A Prospective Cohort Study." *The Journal of Allergy and Clinical Immunology* 135 (4): 905-12.e11.

Mildner, R J, N Taub, J R Vyas, H M Killer, R K Firmin, D J Field, and S Kotecha. 2001. "Repeatability of Cellular Constituents and Cytokine Concentration in Fluid Obtained by Non-Bronchoscopic Bronchoalveolar Lavage of Infants Receiving Extracorporeal Oxygenation." *Thorax* 56 (12): 924–31.

Moran, C P, and F Shanahan. 2014. "Gut Microbiota and Obesity: Role in Aetiology and Potential Therapeutic Target." *Best Pract Res Clin Gastroenterol* 28 (4): 585–97.

Morgan, Xochitl C., and Curtis Huttenhower. 2012. "Chapter 12: Human Microbiome Analysis." Edited by Fran Lewitter and Maricel Kann. *PLoS Computational Biology* 8 (12): e1002808.

Morris, Alison, James M. Beck, Patrick D. Schloss, Thomas B. Campbell, Kristina Crothers, Jeffrey L. Curtis, Sonia C. Flores, et al. 2013. "Comparison of the Respiratory Microbiome in Healthy Nonsmokers and Smokers." *American Journal of Respiratory and Critical Care Medicine* 187 (10): 1067–75.

Morrow, Ardythe, Anne J Lagomarcino, Kurt R Schibler, Diana H Taft, Zhuoteng Yu, Bo Wang, Mekibib Altaye, et al. 2013. "Early Microbial and Metabolomic Signatures Predict Later Onset of Necrotizing Enterocolitis in Preterm Infants." *Microbiome* 1 (1): 13.

Morrow, B, and A Argent. 2001. "Risks and Complications of Nonbronchoscopic Bronchoalveolar Lavage in a Pediatric Intensive Care Unit." *Pediatric Pulmonology* 32 (5): 378–84.

Mourani, Peter M., J. Kirk Harris, Marci K. Sontag, Charles E. Robertson, and Steven H. Abman. 2011. "Molecular Identification of Bacteria in Tracheal Aspirate Fluid from Mechanically Ventilated Preterm Infants." *PLoS ONE* 6 (10): e25959.

Munshi, Upender K, Josie O Niu, Mustafa M Siddiq, and Lance A Parton. 1997. “Elevation of Interleukin-8 and Interleukin-6 Precedes the Influx of Neutrophils in Tracheal Aspirates from Preterm Infants Who Develop Bronchopulmonary Dysplasia.” *Pediatric Pulmonology* 24 (5): 331–36.

Muruve, Daniel, Virginie Pétrilli, Anne K Zaiss, Lindsay R White, Sharon a Clark, P Joel Ross, Robin J Parks, and Jurg Tschopp. 2008. “The Inflammasome Recognizes Cytosolic Microbial and Host DNA and Triggers an Innate Immune Response.” *Nature* 452 (7183): 103–7.

Muzio, M, D Bosisio, N Polentarutti, G D’amico, A Stoppacciaro, R Mancinelli, C van’t Veer, et al. 2000. “Differential Expression and Regulation of Toll-like Receptors (TLR) in Human Leukocytes: Selective Expression of TLR3 in Dendritic Cells.” *Journal of Immunology (Baltimore, Md. : 1950)* 164 (11): 5998–6004.

Muzzey, Dale, Eric A Evans, and Caroline Lieber. 2015. “Understanding the Basics of NGS: From Mechanism to Variant Calling.” *Current Genetic Medicine Reports* 3 (4): 158–65.

National Institute for Health and Clinical Excellence. 2012. “Neonatal Infection: Antibiotics for Prevention and Treatment | NICE Guideline [CG149].” NICE. 2012. http://www.nice.org.uk/guidance/cg149.

Normann, Erik, Thierry Lacaze-Masmonteil, Farah Eaton, Leslie Schwendimann, Pierre Gressens, and Bernard Thébaud. 2009. “A Novel Mouse Model of Ureaplasma-Induced Perinatal Inflammation: Effects on Lung and Brain Injury.” *Pediatric Research* 65 (4): 430–36.

Northway, W H, R B Moss, K B Carlisle, B R Parker, R L Popp, P T Pitlick, I Eichler, R L Lamm, and B W Brown. 1990. “Late Pulmonary Sequelae of Bronchopulmonary Dysplasia.” *The New England Journal of Medicine* 323: 1793–99.

Northway, W H, R C Rosan, and D Y Porter. 1967. “Pulmonary Disease Following Respirator Therapy of Hyaline-Membrane Disease. Bronchopulmonary Dysplasia.” *The New England Journal of Medicine* 276 (7): 357–68.

O’Sullivan, Brian P, and Steven D Freedman. 2009. “Cystic Fibrosis.” *Lancet* 373 (9678): 1891–1904.

Ogden, B E, S A Murphy, G C Saunders, D Pathak, and J D Johnson. 1984. “Neonatal Lung Neutrophils and Elastase/Proteinase Inhibitor Imbalance.” *The American Review of Respiratory Disease* 130 (5): 817–21.

Ohashi, K, V Burkart, S Flohé, and H Kolb. 2000. “Cutting Edge: Heat Shock Protein 60 Is a Putative Endogenous Ligand of the Toll-like Receptor-4 Complex.” *Journal of Immunology (Baltimore, Md. : 1950)* 164 (2): 558–61.

Oksanen, J, F Guillaume, M Friendly, R Kindt, P Legendre, D McGlinn, P Minchin, et al. 2016. “CRAN - Package Vegan.” 2016. https://cran.r-project.org/web/packages/vegan/index.html.

Olesen, Scott W., and Eric J. Alm. 2016. “Dysbiosis Is Not an Answer.” *Nature Microbiology* 1 (November): 16228.

Olsen, G J, and C R Woese. 1993. “Ribosomal RNA: A Key to Phylogeny.” *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology* 7 (1): 113–23.

Olszak, Torsten, Dingding An, Sebastian Zeissig, Miguel Pinilla Vera, Julia Richter, Andre Franke, Jonathan N Glickman, et al. 2012. “Microbial Exposure during Early Life Has Persistent

Effects on Natural Killer T Cell Function." *Science (New York, N.Y.)* 336 (6080): 489–93.

Onland, Wes, Martin Offringa, Filip Cools, Anne P De Jaegere, Karin Rademaker, Henry Blom, Eric Cavatorta, et al. 2011. "Systemic Hydrocortisone To Prevent Bronchopulmonary Dysplasia in Preterm Infants (the SToP-BPD Study); a Multicenter Randomized Placebo Controlled Trial." *BMC Pediatrics* 11 (January): 102.

Oosting, M., S.-C. Cheng, J. M. Bolscher, R. Vestering-Stenger, T. S. Plantinga, I. C. Verschueren, P. Arts, et al. 2014. "Human TLR10 Is an Anti-Inflammatory Pattern-Recognition Receptor." *Proceedings of the National Academy of Sciences* 111 (42): E4478-84.

Overton, Tim W. 2014. "Recombinant Protein Production in Bacterial Hosts." *Drug Discovery Today* 19 (5): 590–601.

Ozinsky, A, D M Underhill, J D Fontenot, A M Hajjar, K D Smith, C B Wilson, L Schroeder, and A Aderem. 2000. "The Repertoire for Pattern Recognition of Pathogens by the Innate Immune System Is Defined by Cooperation between Toll-like Receptors." *Proceedings of the National Academy of Sciences of the United States of America* 97 (25): 13766–71.

Paepe, Monique E. De, Chintan Patel, Amy Tsai, Sravanthi Gundavarapu, and Quanfu Mao. 2008. "Endoglin (CD105) Up-Regulation in Pulmonary Microvasculature of Ventilated Preterm Infants." *American Journal of Respiratory and Critical Care Medicine* 178 (2): 180–87.

Paganin, Patrizia, Ersilia Vita Fiscarelli, Vanessa Tuccio, Manuela Chiancianesi, Giovanni Bacci, Patrizia Morelli, Daniela Dolce, et al. 2015. "Changes in Cystic Fibrosis Airway Microbial Community Associated with a Severe Decline in Lung Function." *PloS One* 10 (4): e0124348.

Pammi, Mohan, Charitharth Vivek Lal, Brandie D. Wagner, Peter M. Mourani, Pablo Lohmann, Ruth Ann Luna, Amy Sisson, et al. 2018. "Airway Microbiome and Development of Bronchopulmonary Dysplasia in Preterm Infants: A Systematic Review." *Journal of Pediatrics* 204: 126-133.e2.

Pandey, Anubhav, Benu Dhawan, Vikas Gupta, Rama Chaudhry, and A. K. Deorari. 2007. "Clinical Significance of Airways Colonization with Ureaplasma Urealyticum in Premature (<34 Wk) Neonates." *Indian Journal of Medical Research* 125 (5): 679–84.

Parks, Donovan H., and Robert G. Beiko. 2010. "Identifying Biologically Relevant Differences between Metagenomic Communities." *Bioinformatics* 26 (6): 715–21.

Parton, L A, S S Strassberg, D Qian, P A Galvin-Parton, and I A Cristea. 2006. "The Genetic Basis for Bronchopulmonary Dysplasia." *Front Biosci* 11: 1854–60.

Patterson, A M, V Taciak, J Lovchik, R E Fox, A B Campbell, and R M Viscardi. 1998. "Ureaplasma Urealyticum Respiratory Tract Colonization Is Associated with an Increase in Interleukin 1-Beta and Tumor Necrosis Factor Alpha Relative to Interleukin 6 in Tracheal Aspirates of Preterm Infants." *The Pediatric Infectious Disease Journal* 17 (4): 321–28.

Payne, Matthew S., K. C W Goss, Gary J. Connett, Tanoj Kollamparambil, Julian P. Legg, Richard Thwaites, Mark Ashton, Victoria Puddy, Janet L. Peacock, and Kenneth D. Bruce. 2010. "Molecular Microbiological Characterization of Preterm Neonates at Risk of Bronchopulmonary Dysplasia." *Pediatric Research* 67 (4): 412–18.

Peden, D B, K VanDyke, A Ardekani, M D Mullett, D Z Myerberg, and C VanDyke. 1987. "Diminished Chemiluminescent Responses of Polymorphonuclear Leukocytes in Severely and Moderately Preterm Neonates." *The Journal of Pediatrics* 111 (6): 904–6.

Peeters, Paul M, Timothy N Perkins, Emiel F M Wouters, Brooke T Mossman, and Niki L

Reynaert. 2013. “Silica Induces NLRP3 Inflammasome Activation in Human Lung Epithelial Cells.” *Particle and Fibre Toxicology* 10 (February): 3.

Pei, Anna Y, William E Oberdorf, Carlos W Nossa, Ankush Agarwal, Pooja Chokshi, Erika Gerz, Zhida Jin, et al. 2010. “Diversity of 16S RRNA Genes within Individual Prokaryotic Genomes.” *Applied and Environmental Microbiology* 76 (12): 3886–97.

Penders, J, E E Stobberingh, C Thijs, H Adams, C Vink, R van Ree, and P A van den Brandt. 2006. “Molecular Fingerprinting of the Intestinal Microbiota of Infants in Whom Atopic Eczema Was or Was Not Developing.” *Clinical and Experimental Allergy : Journal of the British Society for Allergy and Clinical Immunology* 36 (12): 1602–8.

Penders, John, Carel Thijs, Cornelis Vink, Foekje F Stelma, Bianca Snijders, Ischa Kummeling, Piet van den Brandt, and Ellen E Stobberingh. 2006. “Factors Influencing the Composition of the Intestinal Microbiota in Early Infancy.” *Pediatrics* 118 (2): 511–21.

Peng, Luying, Zhenjuan He, Wei Chen, Ian R. Holzman, and Jing Lin. 2007. “Effects of Butyrate on Intestinal Barrier Function in a Caco-2 Cell Monolayer Model of Intestinal Barrier.” *Pediatric Research* 61 (1): 37–41.

Perez-Muñoz, Maria Elisa, Marie-Claire Arrieta, Amanda E. Ramer-Tait, and Jens Walter. 2017. “A Critical Assessment of the ‘Sterile Womb’ and ‘in Utero Colonization’ Hypotheses: Implications for Research on the Pioneer Infant Microbiome.” *Microbiome* 5 (1): 48.

Perez, Geovanny F, Marcos Pérez-Losada, Natalia Isaza, Mary C Rose, Anamaris M Colberg-Poley, and Gustavo Nino. 2017. “Nasopharyngeal Microbiome in Premature Infants and Stability during Rhinovirus Infection.” *Journal of Investigative Medicine : The Official Publication of the American Federation for Clinical Research* 65 (6): 984–90.

Picker, L J, and Eugene C Butcher. 1992. “Physiological and Molecular Mechanisms of Lymphocyte Homing.” *Annual Review of Immunology* 10: 561–91.

Placzek, M, and M Silverman. 1983. “Selective Placement of Bronchial Suction Catheters in Intubated Neonates.” *Arch Dis Child* 58 (10): 829–31.

Pohunek, P, H Pokorna, and I Striz. 1996. “Comparison of Cell Profiles in Separately Evaluated Fractions of Bronchoalveolar Lavage (BAL) Fluid in Children.” *Thorax* 51 (6): 615–18.

Poon, Chuen Yeow, Martin Oliver Edwards, and Sailesh Kotecha. 2013. “Long Term Cardiovascular Consequences of Chronic Lung Disease of Prematurity.” *Paediatric Respiratory Reviews* 14 (4): 242–49.

Pragman, Alexa A., Hyeun Bum Kim, Cavan S. Reilly, Christine Wendt, and Richard E. Isaacson. 2012. “The Lung Microbiome in Moderate and Severe Chronic Obstructive Pulmonary Disease.” *PLoS ONE* 7 (10).

Prevaes, Sabine M.P.J., Wouter A.A. de Steenhuijsen Piters, Karin M. de Winter-de Groot, Hettie M. Janssens, Gerdien A. Tramper-Stranders, Mei Ling J.N. Chu, Harm A. Tiddens, et al. 2017. “Concordance between Upper and Lower Airway Microbiota in Infants with Cystic Fibrosis.” *European Respiratory Journal* 49 (3): 1602235.

Price, M. N., P. S. Dehal, and A. P. Arkin. 2009. “FastTree: Computing Large Minimum Evolution Trees with Profiles Instead of a Distance Matrix.” *Molecular Biology and Evolution* 26 (7): 1641–50.

Prince, Lynne R, Nicola C Maxwell, Sharonjit K Gill, David H Dockrell, Ian Sabroe, Eamon P McGreal, Sailesh Kotecha, and Moira K Whyte. 2014. “Macrophage Phenotype Is Associated with Disease Severity in Preterm Infants with Chronic Lung Disease.” *PloS One* 9 (8): e103059.

Próchnicki, Tomasz, Matthew S Mangan, and Eicke Latz. 2016. “Recent Insights into the Molecular Mechanisms of the NLRP3 Inflammasome Activation.” *F1000Research* 5.

Proell, Martina, Motti Gerlic, Peter D Mace, John C Reed, and Stefan J Riedl. 2013. “The CARD Plays a Critical Role in ASC Foci Formation and Inflammasome Signalling.” *The Biochemical Journal* 449 (3): 613–21.

Prosch, S, U Lienicke, C Priemer, G Flunker, W F Seidel, D H Kruger, and R R Wauer. 2002. “Human Adenovirus and Human Cytomegalovirus Infections in Preterm Newborns: No Association with Bronchopulmonary Dysplasia.” *Pediatr Res* 52 (2): 219–24.

Quast, C., E. Pruesse, P. Yilmaz, J. Gerken, T. Schweer, P. Yarza, J. Peplies, and F. O. Glockner. 2013. “The SILVA Ribosomal RNA Gene Database Project: Improved Data Processing and Web-Based Tools.” *Nucleic Acids Research* 41 (D1): D590–96.

Qureshi, S T, L Larivière, G Leveque, S Clermont, K J Moore, P Gros, and D Malo. 1999. “Endotoxin-Tolerant Mice Have Mutations in Toll-like Receptor 4 (Tlr4).” *The Journal of Experimental Medicine* 189 (4): 615–25.

R&D-Systems. 2005. “DuoSet ELISA Development Systems Assay Principle: R&D Systems.” 2005. https://www.rndsystems.com/resources/technical/duoset-elisa-development-systems-assay-principle.

R Core Team. 2017. “R: Language and Environment for Statistical Computing.”

Rahman-Fink, N., B. L. Chawes, J. Thorsen, J. Stokholm, K. A. Krogfelt, S. Schjørring, M. Kragh, K. Bønnelykke, S. Brix, and H. Bisgaard. 2018. “Neonates Colonized with Pathogenic Bacteria in the Airways Have a Low-Grade Systemic Inflammation.” *Allergy* 73 (11): 2150–59.

Ramakrishnan, Vijay R, Leah M Feazel, Sarah A Gitomer, Diana Ir, Charles E Robertson, and Daniel N Frank. 2013. “The Microbiome of the Middle Meatus in Healthy Adults.” *PloS One* 8 (12): e85507.

Rand, I A Du, J Blaikley, R Booton, N Chaudhuri, V Gupta, S Khalid, S Mandal, et al. 2013. “British Thoracic Society Guideline for Diagnostic Flexible Bronchoscopy in Adults: Accredited by NICE.” *Thorax* 68 (Suppl 1): i1–44.

Ratjen, F, M Bredendiek, M Brendel, J Meltzer, and U Costabel. 1994. “Differential Cytology of Bronchoalveolar Lavage Fluid in Normal Children.” *Eur Respir J* 7 (10): 1865–70.

Reddel, Roger, Ke Yang, Johng Rhim, Douglas Brash, GerwinCurtis, Robert Su, John Lechner, Brenda Gerwin, Curtis Harris, and Paul Amstad. 1987. Immortalized human bronchial epitherial mesothelial cell lines, issued October 30, 1987.

Robbins, M., J. Trittmann, E. Martin, Kristina M. Reber, L. Nelin, and E. Shepherd. 2015. “Early Extubation Attempts Reduce Length of Stay in Extremely Preterm Infants Even If Re-Intubation Is Necessary.” *Journal of Neonatal-Perinatal Medicine* 8 (2): 91–97.

Rofael, Sylvia A.D., Timothy D. McHugh, Rachael Troughton, Joanne Beckmann, David Spratt, Neil Marlow, and John R. Hurst. 2019. “Airway Microbiome in Adult Survivors of Extremely Preterm Birth: The EPICure Study.” *European Respiratory Journal* 53 (1): 1801225.

Rogers, Geraint B, Dominick Shaw, Robyn L Marsh, Mary P Carroll, David J Serisier, and Kenneth D Bruce. 2015. “Respiratory Microbiota: Addressing Clinical Questions, Informing Clinical Practice.” *Thorax* 70 (1): 74–81.

Ronaghi, M, M Uhlén, and P Nyrén. 1998. “A Sequencing Method Based on Real-Time Pyrophosphate.” *Science (New York, N.Y.)* 281 (5375): 363, 365.

Rook, A H, J H Kehrl, L M Wakefield, A B Roberts, M B Sporn, D B Burlington, H C Lane, and A S Fauci. 1986. “Effects of Transforming Growth Factor Beta on the Functions of Natural Killer Cells: Depressed Cytolytic Activity and Blunting of Interferon Responsiveness.” *Journal of Immunology (Baltimore, Md. : 1950)* 136 (10): 3916–20.

Rossol, Manuela, Matthias Pierer, Nora Raulien, Dagmar Quandt, Undine Meusch, Kathrin Rothe, Kristin Schubert, et al. 2012. “Extracellular Ca2+ Is a Danger Signal Activating the NLRP3 Inflammasome through G Protein-Coupled Calcium Sensing Receptors.” *Nature Communications* 3 (January): 1329.

Rothberg, Jonathan M, Wolfgang Hinz, Todd M Rearick, Jonathan Schultz, William Mileski, Mel Davey, John H Leamon, et al. 2011. “An Integrated Semiconductor Device Enabling Non-Optical Genome Sequencing.” *Nature* 475 (7356): 348–52.

Rutayisire, Erigene, Kun Huang, Yehao Liu, and Fangbiao Tao. 2016. “The Mode of Delivery Affects the Diversity and Colonization Pattern of the Gut Microbiota during the First Year of Infants’ Life: A Systematic Review.” *BMC Gastroenterology* 16 (1): 86.

Ryan, Rita M., Qadeer Ahmed, and Satyan Lakshminrusimha. 2008. “Inflammatory Mediators in the Immunobiology of Bronchopulmonary Dysplasia.” *Clinical Reviews in Allergy & Immunology* 34 (2): 174–90.

Salter, Susannah J, Michael J Cox, Elena M Turek, Szymon T Calus, William O Cookson, Miriam F Moffatt, Paul Turner, Julian Parkhill, Nicholas J Loman, and Alan W Walker. 2014. “Reagent and Laboratory Contamination Can Critically Impact Sequence-Based Microbiome Analyses.” *BMC Biology* 12 (1): 87.

Salzman, Nita H, Dipankar Ghosh, Kenneth M Huttner, Yvonne Paterson, and Charles L Bevins. 2003. “Protection against Enteric Salmonellosis in Transgenic Mice Expressing a Human Intestinal Defensin.” *Nature* 422 (6931): 522–26.

Sanchez, P J. 1993. “Perinatal Transmission of Ureaplasma Urealyticum: Current Concepts Based on Review of the Literature.” *Clin Infect Dis* 17 Suppl 1: S107-11.

Sanger, F, S Nicklen, and A R Coulson. 1977. “DNA Sequencing with Chain-Terminating Inhibitors.” *Proceedings of the National Academy of Sciences of the United States of America* 74 (12): 5463–67.

Santos, Scott R, and Howard Ochman. 2004. “Identification and Phylogenetic Sorting of Bacterial Lineages with Universally Conserved Genes and Proteins.” *Environmental Microbiology* 6 (7): 754–59.

Sawyer, M H, D K Edwards, and S A Spector. 1987. “Cytomegalovirus Infection and Bronchopulmonary Dysplasia in Premature Infants.” *Am J Dis Child* 141 (3): 303–5.

Schlapbach, Luregn J, Maude Aebischer, Mark Adams, Giancarlo Natalucci, Jan Bonhoeffer, Philipp Latzin, Mathias Nelle, Hans Ulrich Bucher, Beatrice Latal, and Swiss Neonatal Network and Follow-Up Group. 2011. “Impact of Sepsis on Neurodevelopmental Outcome in a Swiss National Cohort of Extremely Premature Infants.” *Pediatrics* 128 (2): e348-57.

Schloss, Patrick D, Sarah L Westcott, Thomas Ryabin, Justine R Hall, Martin Hartmann, Emily B Hollister, Ryan a Lesniewski, et al. 2009. “Introducing Mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities.” *Applied and Environmental Microbiology* 75 (23): 7537–41.

Schmidlin, Patrick R, Patrick Fachinger, Gabrielo Tini, Sereina Graber, Burkhardt Seifert, Sylke Dombrowa, and Sarosh Irani. 2015. “Shared Microbiome in Gums and the Lung in an Outpatient Population.” *The Journal of Infection* 70 (3): 255–63.

Schmidt, Barbara, Robin S Roberts, Peter Davis, Lex W Doyle, Keith J Barrington, Arne Ohlsson, Alfonso Solimano, Win Tin, and Caffeine for Apnea of Prematurity Trial Group. 2006. "Caffeine Therapy for Apnea of Prematurity." *The New England Journal of Medicine* 354 (20): 2112–21.

Schneibel, K R, A M Fitzpatrick, X-D Ping, L A S Brown, and Theresa W Gauthier. 2013. "Inflammatory Mediator Patterns in Tracheal Aspirate and Their Association with Bronchopulmonary Dysplasia in Very Low Birth Weight Neonates." *Journal of Perinatology : Official Journal of the California Perinatal Association* 33 (5): 383–87.

Schneider, Caroline A, Wayne S Rasband, and Kevin W Eliceiri. 2012. "NIH Image to ImageJ: 25 Years of Image Analysis." *Nature Methods* 9 (7): 671–75.

Schroder, Kate, Vitaliya Sagulenko, Alina Zamoshnikova, Ayanthi A Richards, Jasmyn A Cridland, Katharine M Irvine, Katryn J Stacey, and Matthew J Sweet. 2012. "Acute Lipopolysaccharide Priming Boosts Inflammasome Activation Independently of Inflammasome Sensor Induction." *Immunobiology* 217 (12): 1325–29.

Schwandner, R, R Dziarski, H Wesche, M Rothe, and C J Kirschning. 1999. "Peptidoglycan- and Lipoteichoic Acid-Induced Cell Activation Is Mediated by Toll-like Receptor 2." *The Journal of Biological Chemistry* 274 (25): 17406–9.

Shamir, Raanan. 2016. "The Benefits of Breast Feeding." *Nestlé Nutrition Institute Workshop Series* 86 (January): 67–76.

Shanahan, Fergus, and Colin Hill. 2019. "Language, Numeracy and Logic in Microbiome Science." *Nature Reviews Gastroenterology & Hepatology* 16 (7): 387–88.

Shannon, C. E. 1948. "A Mathematical Theory of Communication." *Bell System Technical Journal* 27 (3): 379–423.

Shao, Yan, Samuel C. Forster, Evdokia Tsaliki, Kevin Vervier, Angela Strang, Nandi Simpson, Nitin Kumar, et al. 2019. "Stunted Microbiota and Opportunistic Pathogen Colonization in Caesarean-Section Birth." *Nature* 574 (7776): 117–21.

Sharma, Ashish, Roger Jen, Rollin Brant, Mihoko Ladd, Qing Huang, Amanda Skoll, Christof Senger, Stuart E Turvey, Nico Marr, and Pascal M Lavoie. 2014. "Hierarchical Maturation of Innate Immune Defences in Very Preterm Neonates." *Neonatology* 106 (1): 1–9.

Sharma, Ashish, Roger Jen, Bernard Kan, Abhinav Sharma, Elizabeth Marchant, Anthony Tang, Izabelle Gadawski, et al. 2015. "Impaired NLRP3 Inflammasome Activity during Fetal Development Regulates IL-1β Production in Human Monocytes." *European Journal of Immunology* 45 (1): 238–49.

Shendure, Jay, Gregory J Porreca, Nikos B Reppas, Xiaoxia Lin, John P McCutcheon, Abraham M Rosenbaum, Michael D Wang, Kun Zhang, Robi D Mitra, and George M Church. 2005. "Accurate Multiplex Polony Sequencing of an Evolved Bacterial Genome." *Science (New York, N.Y.)* 309 (5741): 1728–32.

Shennan, Andrew T, Michael S Dunn, Ame Ohlsson, Kathryn Lennox, and Elizabeth M Hoskins. 1988. "Abnormal Pulmonary Outcomes in Premature Infants: Prediction from Oxygen Requirement in the Neonatal Period." *Pediatrics* 82 (4): 527–32.

Siegrist, Claire-Anne, and Richard Aspinall. 2009. "B-Cell Responses to Vaccination at the Extremes of Age." *Nature Reviews. Immunology* 9 (3): 185–94.

Sime, Patricia J., Robert A. Marr, David Gauldie, Zhou Xing, Bryan R. Hewlett, Frank L. Graham, and Jack Gauldie. 1998. "Transfer of Tumor Necrosis Factor-α to Rat Lung Induces Severe Pulmonary Inflammation and Patchy Interstitial Fibrogenesis with Induction of

Transforming Growth Factor-B1 and Myofibroblasts." *The American Journal of Pathology* 153 (3): 825–32.

Simpson, E. H. 1949. "Measurement of Diversity." *Nature* 163 (4148): 688–688.

Sjögren, Y M, M C Jenmalm, M F Böttcher, B Björkstén, and E Sverremark-Ekström. 2009. "Altered Early Infant Gut Microbiota in Children Developing Allergy up to 5 Years of Age." *Clinical and Experimental Allergy : Journal of the British Society for Allergy and Clinical Immunology* 39 (4): 518–26.

Smalley, D M, and K Ley. 2005. "L-Selectin: Mechanisms and Physiological Significance of Ectodomain Cleavage." *Journal of Cellular and Molecular Medicine* 9 (2): 255–66.

Smith, Birgitte, Susan Bodé, Bodil L Petersen, Tim K Jensen, Christian Pipper, Julie Kloppenborg, Mette Boyé, Karen Angeliki Krogfelt, and Lars Mølbak. 2011. "Community Analysis of Bacteria Colonizing Intestinal Tissue of Neonates with Necrotizing Enterocolitis." *BMC Microbiology* 11 (1): 73.

Smith, Daniel J, Alison C Badrick, Martha Zakrzewski, Lutz Krause, Scott C Bell, Gregory J Anderson, and David W Reid. 2014. "Pyrosequencing Reveals Transient Cystic Fibrosis Lung Microbiome Changes with Intravenous Antibiotics." *The European Respiratory Journal* 44 (4): 922–30.

Snel, B, P Bork, and M A Huynen. 1999. "Genome Phylogeny Based on Gene Content." *Nature Genetics* 21 (1): 108–10.

Somborac-Bačura, Anita, Lada Rumora, Ruđer Novak, Dubravka Rašić, Jerka Dumić, Ivana Čepelak, and Tihana Žanić-Grubišić. 2018. "Differential Expression of HSPs and Activation of MAPKs in A549 Alveolar Epithelial Cells Exposed to Cigarette Smoke Extract." *Experimental Physiology*, September.

Soong, W J, and B T Hwang. 1991. "Selective Placement of Bronchial Suction Catheters in Intubated Full Term and Premature Neonates." *Zhonghua Yi Xue Za Zhi (Taipei)* 48 (1): 45–48.

Speer, Christian P. 2003. "Inflammation and Bronchopulmonary Dysplasia." *Seminars in Neonatology*.

Spencer, WG. 1936. *Celsus de Medicina*. *The American Journal of the Medical Sciences*. London: Heinemann.

Stackebrandt, Erko, and Jonas Ebers. 2006. "Taxonomic Parameters Revisited: Tanrished Gold Standards." *Microbiology Today* 33: 152–55.

Stahl, D A, D J Lane, G J Olsen, and N R Pace. 1984. "Analysis of Hydrothermal Vent-Associated Symbionts by Ribosomal RNA Sequences." *Science (New York, N.Y.)* 224 (4647): 409–11.

Staley, Christopher, Thomas Kaiser, Byron P Vaughn, Carolyn Graiziger, Matthew J Hamilton, Amanda J Kabage, Alexander Khoruts, and Michael J Sadowsky. 2019. "Durable Long-Term Bacterial Engraftment Following Encapsulated Fecal Microbiota Transplantation To Treat Clostridium Difficile Infection." *MBio* 10 (4).

Stearns, Jennifer C, Carla J Davidson, Suzanne McKeon, Fiona J Whelan, Michelle E Fontes, Anthony B Schryvers, Dawn M E Bowdish, James D Kellner, and Michael G Surette. 2015. "Culture and Molecular-Based Profiles Show Shifts in Bacterial Communities of the Upper Respiratory Tract That Occur with Age." *The ISME Journal* 9 (5): 1246–59.

Stewart, Ceri E., Elizabeth E. Torr, Nur H. Mohd Jamili, Cynthia Bosquillon, and Ian Sayers. 2012. "Evaluation of Differentiated Human Bronchial Epithelial Cell Culture Systems for

Asthma Research." *Journal of Allergy* 2012 (di): 1–11.

Stewart, Christopher J, Emma C L Marrs, Andrew Nelson, Clare Lanyon, John D Perry, Nicholas D Embleton, Stephen P Cummings, and Janet E Berrington. 2013. "Development of the Preterm Gut Microbiome in Twins at Risk of Necrotising Enterocolitis and Sepsis." *PloS One* 8 (8): e73465.

Stoll, Barbara J., Nellie I. Hansen, Edward F. Bell, Michele C. Walsh, Waldemar A. Carlo, Seetha Shankaran, Abbot R. Laptook, et al. 2015. "Trends in Care Practices, Morbidity, and Mortality of Extremely Preterm Neonates, 1993-2012." *JAMA* 314 (10): 1039.

Stoll, Barbara J, Nellie I Hansen, Edward F Bell, Seetha Shankaran, Abbot R Laptook, Michele C Walsh, Ellen C Hale, et al. 2010. "Neonatal Outcomes of Extremely Preterm Infants from the NICHD Neonatal Research Network." *Pediatrics* 126 (3): 443–56.

Stoscheck, Christa M. 1990. "Quantitation of Protein." In *Methods in Enzymology*, 182:50–68.

Stralin, K., B. Herrmann, G. Abdeldaim, P. Olcen, H. Holmberg, and P. Molling. 2014. "Comparison of Sputum and Nasopharyngeal Aspirate Samples and of the PCR Gene Targets LytA and Spn9802 for Quantitative PCR for Rapid Detection of Pneumococcal Pneumonia." *Journal of Clinical Microbiology* 52 (1): 83–89.

Stressmann, F A, G B Rogers, E R Klem, A K Lilley, S H Donaldson, T W Daniels, M P Carroll, et al. 2011. "Analysis of the Bacterial Communities Present in Lungs of Patients with Cystic Fibrosis from American and British Centers." *J Clin Microbiol* 49 (1): 281–91.

Strunk, Tobias, Andrew Currie, Peter Richmond, Karen Simmer, and David Burgner. 2011. "Innate Immunity in Human Newborn Infants: Prematurity Means More than Immaturity." *The Journal of Maternal-Fetal & Neonatal Medicine* 24 (1): 25–31.

Strunk, Tobias, Amy Prosser, Ofer Levy, Victoria Philbin, Karen Simmer, Dorota Doherty, Adrian Charles, Peter Richmond, David Burgner, and Andrew Currie. 2012. "Responsiveness of Human Monocytes to the Commensal Bacterium Staphylococcus Epidermidis Develops Late in Gestation." *Pediatric Research* 72 (1): 10–18.

Suau, A, R Bonnet, M Sutren, J J Godon, G R Gibson, M D Collins, and J Doré. 1999. "Direct Analysis of Genes Encoding 16S RRNA from Complex Communities Reveals Many Novel Molecular Species within the Human Gut." *Applied and Environmental Microbiology* 65 (11): 4799–4807.

Sureshbabu, Angara, Mansoor A Syed, Chandra Boddupalli, Madhav V Dhodapkar, Robert J Homer, Parviz Minoo, and Vineet Bhandari. 2015. "Conditional Overexpression of TGFß1 Promotes Pulmonary Inflammation, Apoptosis and Mortality via TGFßR2 in the Developing Mouse Lung." *Respiratory Research* 16 (1): 4.

Sze, Marc A, James C Hogg, and Don D Sin. 2014. "Bacterial Microbiome of Lungs in COPD." *International Journal of Chronic Obstructive Pulmonary Disease* 9 (January): 229–38.

Takeda, Kiyoshi, and Shizuo Akira. 2005. "Toll-like Receptors in Innate Immunity." *International Immunology* 17 (1): 1–14.

Takeda, Kiyoshi, Tsuneyasu Kaisho, and Shizuo Akira. 2003. "Toll-like Receptors." *Annual Review of Immunology* 21 (1): 335–76.

Takeuchi, O, T Kawai, P F Mühlradt, M Morr, J D Radolf, A Zychlinsky, K Takeda, and S Akira. 2001. "Discrimination of Bacterial Lipoproteins by Toll-like Receptor 6." *International Immunology* 13 (7): 933–40.

Takeuchi, Osamu, Shintaro Sato, Takao Horiuchi, Katsuaki Hoshino, Kiyoshi Takeda, Zhongyun

Dong, Robert L Modlin, and Shizuo Akira. 2002. “Cutting Edge: Role of Toll-like Receptor 1 in Mediating Immune Response to Microbial Lipoproteins.” *Journal of Immunology (Baltimore, Md. : 1950)* 169 (1): 10–14.

Tammela, O K, and M E Koivisto. 1992. “Fluid Restriction for Preventing Bronchopulmonary Dysplasia? Reduced Fluid Intake during the First Weeks of Life Improves the Outcome of Low-Birth-Weight Infants.” *Acta Paediatrica (Oslo, Norway : 1992)* 81 (3): 207–12.

Tanaka, Shigemitsu, Takako Kobayashi, Prapa Songjinda, Atsushi Tateyama, Mina Tsubouchi, Chikako Kiyohara, Taro Shirakawa, Kenji Sonomoto, and Jiro Nakayama. 2009. “Influence of Antibiotic Exposure in the Early Postnatal Period on the Development of Intestinal Microbiota.” *FEMS Immunology and Medical Microbiology* 56: 80–87.

Teo, Shu Mei, Danny Mok, Kym Pham, Merci Kusel, Michael Serralha, Niamh Troy, Barbara J. Holt, et al. 2015. “The Infant Nasopharyngeal Microbiome Impacts Severity of Lower Respiratory Infection and Risk of Asthma Development.” *Cell Host & Microbe* 17 (5): 704–15.

Tetzner, Reimo. 2009. “Prevention of PCR Cross-Contamination by UNG Treatment of Bisulfite-Treated DNA.” In , 357–70. Humana Press.

The Human Microbiome Project Consortium. 2012. “Structure, Function and Diversity of the Healthy Human Microbiome.” *Nature* 486 (7402): 207–14.

Thorpe, J E, R P Baughman, P T Frame, T A Wesseler, and J L Staneck. 1987. “Bronchoalveolar Lavage for Diagnosing Acute Bacterial Pneumonia.” *J Infect Dis* 155 (5): 855–61.

Tissier, Henry. 1900. *Recherches Sur La Flore Intestinale Des Nourrissons (État Normal et Pathologique).* Méd.--Paris.

Torow, N, B J Marsland, M W Hornef, and E S Gollwitzer. 2017. “Neonatal Mucosal Immunology.” *Mucosal Immunology* 10 (1): 5–17.

Tramper, Jacobus, Huayan Zhang, Elizabeth E. Foglia, Kevin C. Dysart, Michael A. Padula, Kaede V. Sullivan, and Erik A. Jensen. 2016. “The Association between Positive Tracheal Aspirate Cultures and Adverse Pulmonary Outcomes in Preterm Infants with Severe Bronchopulmonary Dysplasia.” *American Journal of Perinatology*, 2016.

Tran, Hai B, Martin D Lewis, Lor Wai Tan, Susan E Lester, Leonie M Baker, Jia Ng, Monica A Hamilton-Bruce, et al. 2012. “Immunolocalization of NLRP3 Inflammasome in Normal Murine Airway Epithelium and Changes Following Induction of Ovalbumin-Induced Airway Inflammation.” *Journal of Allergy* 2012: 819176.

Trembath, Andrea, and Matthew M Laughon. 2012. “Predictors of Bronchopulmonary Dysplasia.” *Clinics in Perinatology* 39 (3): 585–601.

Tsolia, M, M Psoma, S Gavrili, V Petrochilou, S Michalas, N Legakis, and Th Karpathios. 2003. “Group B Streptococcus Colonization of Greek Pregnant Women and Neonates: Prevalence, Risk Factors and Serotypes.” *Clinical Microbiology and Infection : The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases* 9 (8): 832–38.

Turunen, Riikka, Outi Vaarala, Irmeli Nupponen, Eero Kajantie, Sanna Siitonen, Aulikki Lano, Heikki Repo, and Sture Andersson. 2009. “Activation of T Cells in Preterm Infants with Respiratory Distress Syndrome.” *Neonatology* 96 (4): 248–58.

Twigg, Homer L, Alison Morris, Elodie Ghedin, Jeffrey L Curtis, Gary B Huffnagle, Kristina Crothers, Thomas B Campbell, et al. 2013. “Use of Bronchoalveolar Lavage to Assess the Respiratory Microbiome: Signal in the Noise.” *The Lancet. Respiratory Medicine* 1 (5):

354–56.

Twomey, Kate B., Mark Alston, Shi-Qi An, Oisin J. O'Connell, Yvonne McCarthy, David Swarbreck, Melanie Febrer, J. Maxwell Dow, Barry J. Plant, and Robert P. Ryan. 2013. "Microbiota and Metabolite Profiling Reveal Specific Alterations in Bacterial Community Structure and Environment in the Cystic Fibrosis Airway during Exacerbation." Edited by Willem van Schaik. *PLoS ONE* 8 (12): e82432.

Tzialla, C., A. Borghesi, G. F. Perotti, F. Garofoli, P. Manzoni, and M. Stronati. 2012. "Use and Misuse of Antibiotics in the Neonatal Intensive Care Unit." *The Journal of Maternal-Fetal & Neonatal Medicine* 25 (sup4): 27–29.

Ulke-Lemée, Annegret, Arthur Lau, Michelle C. Nelson, Matthew T. James, Daniel A. Muruve, and Justin A. MacDonald. 2018. "Quantification of Inflammasome Adaptor Protein ASC in Biological Samples by Multiple-Reaction Monitoring Mass Spectrometry." *Inflammation* 41 (4): 1396–1408.

Underhill, D M, A Ozinsky, A M Hajjar, A Stevens, C B Wilson, M Bassetti, and A Aderem. 1999. "The Toll-like Receptor 2 Is Recruited to Macrophage Phagosomes and Discriminates between Pathogens." *Nature* 401 (6755): 811–15.

Vergnano, Stefania, Esse Menson, Nigel Kennea, Nick Embleton, Alison Bedford Russell, Timothy Watts, Michael J Robinson, Andrew Collinson, and Paul T Heath. 2011. "Neonatal Infections in England: The NeonIN Surveillance Network." *Archives of Disease in Childhood. Fetal and Neonatal Edition* 96 (1): F9–14.

Větrovský, Tomáš, and Petr Baldrian. 2013. "The Variability of the 16S RRNA Gene in Bacterial Genomes and Its Consequences for Bacterial Community Analyses." *PloS One* 8 (2): e57923.

Vinolo, Marco A R, Elaine Hatanaka, Rafael H. Lambertucci, Philip Newsholme, and Rui Curi. 2009. "Effects of Short Chain Fatty Acids on Effector Mechanisms of Neutrophils." *Cell Biochemistry and Function* 27 (1): 48–55.

Vinolo, Marco A R, Hosana G Rodrigues, Elaine Hatanaka, Cristina B Hebeda, Sandra H P Farsky, and Rui Curi. 2009. "Short-Chain Fatty Acids Stimulate the Migration of Neutrophils to Inflammatory Sites." *Clinical Science (London, England : 1979)* 117 (9): 331–38.

Vissing, Nadja H, Bo L K Chawes, and Hans Bisgaard. 2013. "Increased Risk of Pneumonia and Bronchiolitis after Bacterial Colonization of the Airways as Neonates." *American Journal of Respiratory and Critical Care Medicine* 188 (10): 1246–52.

Vyas, R, Gareth Tudor, Andrew Rickett, and Sailesh Kotecha. 2002. "Serial Bronchoalveolar Lavage of Preterm Infants Is Not Associated with Chest Radiological Changes." *European Journal of Pediatrics* 161 (6): 313–18.

Wagner, Brandie D., Marci K. Sontag, J. Kirk Harris, Joshua I. Miller, Lindsey Morrow, Charles E. Robertson, Mark Stephens, Brenda B. Poindexter, Steven H. Abman, and Peter M. Mourani. 2017. "Airway Microbial Community Turnover Differs by BPD Severity in Ventilated Preterm Infants." *Plos One* 12 (1): e0170120.

Walker, A W, J Ince, S H Duncan, L M Webster, G Holtrop, X Ze, D Brown, et al. 2011. "Dominant and Diet-Responsive Groups of Bacteria within the Human Colonic Microbiota." *Isme J* 5 (2): 220–30.

Walsh, M. C., Qing Yao, Patricia Gettner, Ellen Hale, Monica Collins, Angelita Hensman, Ruth Everette, et al. 2004. "Impact of a Physiologic Definition on Bronchopulmonary Dysplasia Rates." *Pediatrics* 114 (5): 1305–11.

Wang, Hui, Krystal R St Julien, David K Stevenson, Thomas J Hoffmann, John S Witte, Laura C Lazzeroni, Mark A Krasnow, et al. 2013. “A Genome-Wide Association Study (GWAS) for Bronchopulmonary Dysplasia.” *Pediatrics* 132 (2): 290–97.

Wang, Y, H Kong, X Zeng, W Liu, Z Wang, X Yan, H Wang, and W Xie. 2016. “Activation of NLRP3 Inflammasome Enhances the Proliferation and Migration of A549 Lung Cancer Cells.” *Oncology Reports* 35 (4): 2053–64.

Wang, Yunwei, Jeanette D Hoenig, Kathryn J Malin, Sanaa Qamar, Elaine O Petrof, Jun Sun, Dionysios a Antonopoulos, Eugene B Chang, and Erika C Claud. 2009. “16S RRNA Gene-Based Analysis of Fecal Microbiota from Preterm Infants with and without Necrotizing Enterocolitis.” *The ISME Journal* 3 (8): 944–54.

Ward, C, M Duddridge, J Fenwick, S Williams, P V Gardiner, D J Hendrick, and E H Walters. 1992. “The Origin of Water and Urea Sampled at Bronchoalveolar Lavage in Asthmatic and Control Subjects.” *American Review of Respiratory Disease* 146 (2): 444–47.

Warner, Barbara B., and Aaron Hamvas. 2015. “Lungs, Microbes and the Developing Neonate.” *Neonatology* 107 (4): 337–43.

Wassenaar, T M, and P Panigrahi. 2014. “Is a Foetus Developing in a Sterile Environment?” *Letters in Applied Microbiology* 59 (6): 572–79.

Watts, Catherine L., and Margaret C. Bruce. 1995. “Comparison of Secretory Component for Immunoglobulin A with Albumin as Reference Proteins in Tracheal Aspirate from Preterm Infants.” *The Journal of Pediatrics* 127 (1): 113–22.

Weiss, Sophie, Zhenjiang Zech Xu, Shyamal Peddada, Amnon Amir, Kyle Bittinger, Antonio Gonzalez, Catherine Lozupone, et al. 2017. “Normalization and Microbial Differential Abundance Strategies Depend upon Data Characteristics.” *Microbiome* 5 (1): 27.

Westerbeek, Elisabeth, Anemone van den Berg, Harrie N. Lafeber, Jan Knol, Willem P F Fetter, and Ruurd M. van Elburg. 2006. “The Intestinal Bacterial Colonisation in Preterm Infants: A Review of the Literature.” *Clinical Nutrition* 25: 361–68.

Wickham, H. 2016. *Ggplot2:Elegant Graphics for Data Analysis*. Springer-Verlag, New York.

Wiersinga, Willem Joost, Stije J Leopold, Duncan R Cranendonk, and Tom van der Poll. 2014. “Host Innate Immune Responses to Sepsis.” *Virulence* 5 (1): 36–44.

Williams, Thomas C, and Amanda J Drake. 2015. “What a General Paediatrician Needs to Know about Early Life Programming.” *Archives of Disease in Childhood*, May.

Woese, C R, and G E Fox. 1977. “Phylogenetic Structure of the Prokaryotic Domain: The Primary Kingdoms.” *Proceedings of the National Academy of Sciences of the United States of America* 74 (11): 5088–90.

Wostmann, B S. 1981. “The Germfree Animal in Nutritional Studies.” *Annual Review of Nutrition* 1 (1): 257–79.

Wu, Hsin-Jung, Ivaylo I Ivanov, Jaime Darce, Kimie Hattori, Tatsuichiro Shima, Yoshinori Umesaki, Dan R Littman, Christophe Benoist, and Diane Mathis. 2010. “Gut-Residing Segmented Filamentous Bacteria Drive Autoimmune Arthritis via T Helper 17 Cells.” *Immunity* 32 (6): 815–27.

Wu, Jianbo, Zhibo Yan, David E Schwartz, Jingui Yu, Asrar B Malik, and Guochang Hu. 2013. “Activation of NLRP3 Inflammasome in Alveolar Macrophages Contributes to Mechanical Stretch-Induced Lung Inflammation and Injury.” *Journal of Immunology (Baltimore, Md. : 1950)* 190 (7): 3590–99.

Yan, Sen Rong, Gefei Qing, David M Byers, Andrew W Stadnyk, Walla Al-Hertani, and Robert Bortolussi. 2004. “Role of MyD88 in Diminished Tumor Necrosis Factor Alpha Production by Newborn Mononuclear Cells in Response to Lipopolysaccharide.” *Infection and Immunity* 72 (3): 1223–29.

Yang, Wen Chien, Chien Yi Chen, Hung Chieh Chou, Wu Shiun Hsieh, and Po Nien Tsao. 2015. “Angiogenic Factors in Cord Blood of Preterm Infants Predicts Subsequently Developing Bronchopulmonary Dysplasia” 56 (6): 382–85.

Yarza, Pablo, Pelin Yilmaz, Elmar Pruesse, Frank Oliver Glöckner, Wolfgang Ludwig, Karl-Heinz Schleifer, William B. Whitman, Jean Euzéby, Rudolf Amann, and Ramon Rosselló-Móra. 2014. “Uniting the Classification of Cultured and Uncultured Bacteria and Archaea Using 16S RRNA Gene Sequences.” *Nature Reviews Microbiology* 12 (9): 635–45.

Yatsunenko, Tanya, Federico E Rey, Mark J Manary, Indi Trehan, Maria Gloria Dominguez-Bello, Monica Contreras, Magda Magris, et al. 2012. “Human Gut Microbiome Viewed across Age and Geography.” *Nature* 486 (7402): 222–27.

Young, Robert P, Raewyn J Hopkins, and Benjamin Marsland. 2016. “The Gut-Liver-Lung Axis. Modulation of the Innate Immune Response and Its Possible Role in Chronic Obstructive Pulmonary Disease.” *American Journal of Respiratory Cell and Molecular Biology* 54 (2): 161–69.

Yu, Jiujiu, Hajime Nagasu, Tomohiko Murakami, Hai Hoang, Lori Broderick, Hal M Hoffman, and Tiffany Horng. 2014. “Inflammasome Activation Leads to Caspase-1-Dependent Mitochondrial Damage and Block of Mitophagy.” *Proceedings of the National Academy of Sciences of the United States of America* 111 (43): 15514–19.

Yun, Yeojun, Girish Srinivas, Sven Kuenzel, Miriam Linnenbrink, Safa Alnahas, Kenneth D. Bruce, Ulrich Steinhoff, John F. Baines, and Ulrich E. Schaible. 2014. “Environmentally Determined Differences in the Murine Lung Microbiota and Their Relation to Alveolar Architecture.” Edited by Yuanpu Peter Di. *PLoS ONE* 9 (12): e113466.

Zasada, Magdalena, Przemko Kwinta, Wojciech Durlak, Mirosław Bik-Multanowski, Anna Madetko-Talowska, and Jacek Józef Pietrzyk. 2014. “Development and Maturation of the Immune System in Preterm Neonates: Results from a Whole Genome Expression Study.” *BioMed Research International* 2014 (January): 498318.

Zasada, Magdalena, Marzena Lenart, Magdalena Rutkowska-Zapała, Małgorzata Stec, Ola Czyz, Nina Mól, Maciej Siedlar, and Przemko Kwinta. 2018. “Inflammasome Function in Monocyte Subsets and a Risk of Late-Onset Sepsis in Preterm Very Low Birth Weight Neonates.” *Minerva Pediatrica*, January.

Zeitlin, Jennifer, Marie-Josèphe Saurel-Cubizolles, Jaques De Mouzon, Lucile Rivera, Pierre-Yves Ancel, Béatrice Blondel, and Monique Kaminski. 2002. “Fetal Sex and Preterm Birth: Are Males at Greater Risk?” *Human Reproduction (Oxford, England)* 17 (10): 2762–68.

Zemanick, Edith T, Brandie D Wagner, Charles E Robertson, Richard C Ahrens, James F Chmiel, John P Clancy, Ronald L Gibson, et al. 2017. “Airway Microbiota across Age and Disease Spectrum in Cystic Fibrosis.” *The European Respiratory Journal* 50 (5).

Zhang, Yang, Gongjian Liu, Randal O Dull, David E Schwartz, and Guochang Hu. 2014. “Autophagy in Pulmonary Macrophages Mediates Lung Inflammatory Injury via NLRP3 Inflammasome Activation during Mechanical Ventilation.” *American Journal of Physiology. Lung Cellular and Molecular Physiology* 307 (2): L173-85.

Zhang, Zhigang, Jiawei Geng, Xiaodan Tang, Hong Fan, Jinchao Xu, Xiujun Wen, Zhanshan Sam Ma, and Peng Shi. 2014. “Spatial Heterogeneity and Co-Occurrence Patterns of Human

Mucosal-Associated Intestinal Microbiota." *The ISME Journal* 8 (4): 881–93.

Zhang, Zhihong, Rongyu Liu, Jacobien A. Noordhoek, and Henk F. Kauffman. 2005. "Interaction of Airway Epithelial Cells (A549) with Spores and Mycelium of Aspergillus Fumigatus." *Journal of Infection* 51 (5): 375–82.

Zhao, Jiangchao, Patrick D Schloss, Linda M Kalikin, Lisa Carmody, Bridget K Foster, Joseph F Petrosino, James D Cavalcoli, et al. 2012. "Decade-Long Bacterial Community Dynamics in Cystic Fibrosis Airways." *Proceedings of the National Academy of Sciences of the United States of America* 109 (15): 5809–14.

Zhao, Weiming, Li Ma, Cheng Cai, and Xiaohui Gong. 2019. "Caffeine Inhibits NLRP3 Inflammasome Activation by Suppressing MAPK/NF-KB and A2aR Signaling in LPS-Induced THP-1 Macrophages." *International Journal of Biological Sciences* 15 (8): 1571–81.

Zhou, Rongbin, Amir S Yazdi, Philippe Menu, and Jürg Tschopp. 2011. "A Role for Mitochondria in NLRP3 Inflammasome Activation." *Nature* 469 (7329): 221–25.

Appendix

9 Appendix

9.1 Appendix 1: Ethical approval for sample collection

Part of the research infrastructure for Wales funded by the National Institute for Social Care and Health Research, Welsh Government.
Yn rhan o seilwaith ymchwil Cymru a ariannir gan y Sefydliad Cenedlaethol ar gyfer Ymchwil Gofal Cymdeithasol ac Iechyd, Llywodraeth Cymru

NISCHR
Gwasanaeth Moeseg Ymchwil | RES | Research Ethics Service

Wales Research Ethics Committee 2
6th Floor Churchill House
17 Churchill Way
Cardiff
CF10 2TW

Telephone : 02920 376823
E-mail : carl.phillips@wales.nhs.uk
Website : www.nres.nhs.uk

1 August 2014

Prof Sailesh Kotecha
Department of Child Health
Cardiff University
University Hospital of Wales
CF14 4XW

Dear Prof Kotecha

Study title:	**The microbiome of the neonatal lung and its role in chronic lung disease of prematurity**
REC reference:	**14/WA/0190**
Protocol number:	**SPON 1330-14**
IRAS project ID:	**151514**

Thank you for your letter of the 24 July 2014, responding to the Committee's request for further information on the above research and for submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this opinion letter.

Should you wish to provide a substitute contact point, require further information, or wish to make a request to postpone publication, please contact the REC Manager, Mr Carl Phillips, carl.phillips@wales.nhs.uk.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation [as revised], subject to the conditions specified below.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

- Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.
- Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

- Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

- Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

- For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

- Sponsors are not required to notify the Committee of approvals from host organisations

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database within 6 weeks of recruitment of the first participant (for medical device studies, within the timeline determined by the current registration and publication trees).

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non clinical trials this is not currently mandatory.

If a sponsor wishes to contest the need for registration they should contact Catherine Blewett (catherineblewett@nhs.net), the HRA does not, however, expect exceptions to be made. Guidance on where to register is provided within IRAS.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	Version	Date
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only)		26 July 2014
IRAS Checklist XML [Checklist_04062014]		04 June 2014
Letter from sponsor [sponsors letter]	1	23 May 2014
Participant information sheet (PIS) [For Parents/Guardians - Postnatal Ward Babies]	2	05 July 2014
Participant information sheet (PIS) [inc Consent Form for Parents/Guardians - Postnatal Ward Babies]	3	24 July 2014

Participant information sheet (PIS) [For Parents/Guardians - Babies admitted to the NeoNatal Unit]	2	05 July 2014
Participant information sheet (PIS) [inc Consent Form for Parents/Guardians - Babies admitted to the NeoNatal Ward]	3	24 July 2014
REC Application Form [REC_Form_04062014]		23 May 2014
Referee's report or other scientific critique report [Scientific Review]	1	23 May 2014
Research protocol or project proposal	2	05 July 2014
Research protocol or project proposal	3	24 July 2014
Response to Request for Further Information		08 July 2014
Response to Request for Further Information		24 July 2014
Summary CV for Chief Investigator (CI) [S Kotecha]	1	14 May 2014
Summary CV for student [D J Gallagher]	1	01 May 2014
Summary CV for supervisor (student research) [S Kotecha]	1	14 May 2014

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors.

You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website: http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/

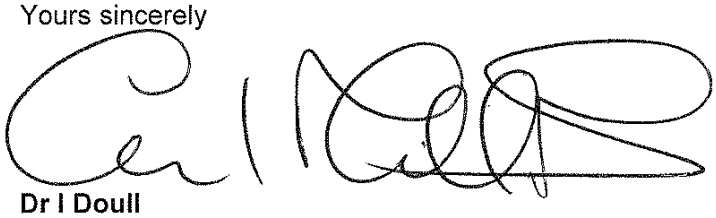
HRA Training

We are pleased to welcome researchers and R&D staff at our training days – see details at http://www.hra.nhs.uk/hra-training/

14/WA/0190	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project.

Yours sincerely



Dr I Doull
Chair, Wales Research Ethics Committee 2
Email:carl.phillips@wales.nhs.uk

Enclosures: "After ethical review – guidance for researchers" [SL-AR2]

Copied:- Prof S Kotecha, kotechas@cardiff.ac.uk

Dr D Gallacher, drgallacher@gmail.com

R&D office Cardiff University, , resgov@cardiff.ac.uk

R&D office Cardiff&Vale UHB, CAV_research.development@wales.nhs.uk

Lee.Hathaway@wales.nhs.uk

9.2 Appendix 2: Parent information sheet and consent form - Cardiff

Cardiff and Vale University Health Board | **Bwrdd Iechyd Prifysgol Caerdydd a'r Fro**

University Hospital of Wales
Ysbyty Athrofaol Cymru

Heath Park,
Cardiff,
CF14 4XW
Phone (029) 2074 7747
Fax (029) 2074 3838

Parc Y Mynydd Bychan,
Caerdydd,
CF14 4XW
Ffôn (029) 2074 7747
Ffacs (029) 2074 3838

Information sheet for Parents/Guardians – Postnatal Ward Babies

Principal Investigator: Prof Sailesh Kotecha, Consultant Neonatologist.

1. Study Title
The microbiome of the neonatal lung and its effect on chronic lung disease of prematurity

2. Invitation
You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Please take time to decide whether or not you wish to take part.

Thank you for reading this leaflet.

3. What is the purpose of the study?
Chronic Lung Disease of prematurity (CLD), which is also often called BPD (for bronchopulmonary dysplasia), is a common disease of premature babies. Babies with CLD require oxygen for prolonged periods and are sometimes discharged home on oxygen. Our research is trying to understand why some babies develop CLD.

Until the past few years, it was thought that the lungs of healthy babies (and adults) were sterile, i.e. contained no bacteria. New techniques, however, have shown that there are low levels of bacteria in all people's lungs (known as the microbiome of the lung). It is not known when babies acquire these bacteria, or what type of bacteria are present in healthy baby's lungs. Our research seeks learn more about the acquisition of this bacteria.

It is becoming clear that these bacteria may play a part in many lung diseases. Our research will also look into the role these bacteria may have in the development of chronic lung disease of prematurity.

4. Why have I been chosen?
We would like to invite 3 groups of babies to join the study:

Study group: Babies who have been born prematurely (At or before 32 weeks gestation) and require the assistance of a breathing machine to support their breathing

Control group of ventilated babies: Babies who have been born at term (i.e. at or after 37 weeks gestation) and who need help with a breathing machine to support breathing for non-respiratory reasons) commonly if they undergo surgery)

Control group of babies on the postnatal wards: Babies born at term without any complications. Only the nasal passage samples will be collected.

We would like to invite you to join the study because your baby has been delivered at or after 37 weeks gestation and is well on the postnatal wards.

5. Does my baby have to take part?
It is up to you to decide whether or not to consent for your baby to take part. If you do allow your baby to take part in the study, you will be given this information sheet to keep and be asked to sign a consent form. Even if you do decide to consent to your baby taking part, you are still free to withdraw your baby at any time without giving any reasons. A decision to withdraw at any time, or a decision not to take part, will not affect the care that your baby will receive.

6. What will happen to my baby if we take part?

For babies who are well and on the postnatal ward, we would like to collect samples of the fluid and mucous in the nasal passages, to act as a marker of what may be happening in the lungs. This would involve inserting a thin suction tube into both nostrils and suctioning any secretions that are present. These secretions will be analysed for any bacteria. We would like to suction the nasal passages daily during the first week of life, or for as long as you and your baby remain on the postnatal ward, whichever is sooner.

We would also like to collect daily stool samples from your baby's dirty nappy while he/she is in hospital.

7. Will this affect my baby's treatment?
The medical care of your baby will not be affected by this study. The information from this study will not be used to diagnose or treat your baby.

8. What will happen to the samples collected?
The samples will be transferred to our laboratories at Cardiff University. We shall analyse the fluid from the nasal passages and the stool in a number of ways:

1) We will use molecular biological techniques to identify any bacterial DNA present in the samples to identify what species of bacteria are present
2) We will measure markers of inflammation in the samples to help us identify if the bacteria are causing infection.
3) We will look for molecules that many germs may produce (metabolites) in the samples to see if blood tests can be used to identify any bacteria.

We shall analyse the samples in our laboratories at Cardiff University but some analyses may be conducted by commercial companies or other university laboratories which have expertise to analyse the samples. The samples will have a code and will not have any information about the baby.

The baby's DNA will be extracted alongside the bacterial DNA but it will not be used further in this study. With your permission any remaining samples, including lung fluid, stool, blood and DNA (baby and bacterial), may be stored for future research into chronic lung disease of prematurity. The samples will be anonymised before use in future studies and may be accessed by researchers in the UK and abroad, the research may include genetic (e.g. DNA), commercial and animal research. You may

withdraw your consent for the storage and future use of your baby's samples at any point. If you do withdraw your consent your baby's samples will not be used in any subsequent studies and will be destroyed according to locally approved practices. Any samples already distributed for use in research prior to the withdrawal of consent will continue to be used in that study and any samples remaining at the end of the study will be destroyed.

9. Is there any benefit to taking part in this study?
There will be no benefit to you or your baby from taking part in this study. The information from this study will not be used to diagnose or treat your baby. The study should improve our understanding the role of germs play in the development of lung disease in premature babies.

10. What are the risks of taking part?
Suctioning of the nasal passages is a procedure regularly performed on babies on the neonatal unit and babies admitted to paediatric wards. The procedure only takes 20 – 30 seconds and is only mildly uncomfortable for the babies.

11. What if something goes wrong?
If your baby is harmed by taking part in this research project, there are no special compensation arrangements. If your baby is harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you or your baby have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms should be available to you.

12. Will my baby's taking part be kept confidential?
All information which is collected about your baby during the course of the research will be kept strictly confidential. We will assign a number to each baby and use this to label the samples obtained for the study.

13. What will happen to the results of the study?
We will publish the results in reputable medical journals and present the data at scientific and medical meetings. Your baby's name and details will NOT be revealed at any stage. Please let us know if you would like a copy of the report.

14. Who is paying for the study?
The study is being funded by departmental funds.

15. Who had reviewed the study?
This study has been reviewed by the Wales Research Ethics Committee 2 and also by the Cardiff and Vale University Health Board.

16. Who can I contact for further information?
You may contact Dr David Gallacher or Professor Sailesh Kotecha by asking one of the staff on the neonatal unit or by telephoning 029 20743375 or by mail to: gallacherdj@cardiff.ac.uk .

Professor Sailesh Kotecha, Neonatal Unit, Heath Hospital, Heath Park, Cardiff CF14 4XN.

Thank you for taking time to read this information leaflet at this time. Please do not hesitate to ask Dr David Gallacher or Professor Sailesh Kotecha if you would like to discuss anything further.

Dr David Gallacher

Clinical Research Fellow

Professor Sailesh Kotecha

Consultant in Neonatal Medicine

Patient Identification Number for this study:

Patient Sticker

PARENT/GUARDIAN CONSENT FORM

Project Title: The Microbiome of the Neonatal Lung and its Effect on Chronic Lung Disease of Prematurity
Principle Investigators: Professor Sailesh Kotecha, Consultant Neonatologist
Contact Details: Neonatal Unit, 029 20 74 3375
This form should be read in conjunction with the Patient Information Leaflet (postnatal ward babies), version 4 dated 28th July 2016.

Please initial relevant boxes

1. I confirm that I have read and understand the information sheet dated 28th July 2016 (version 4 – post natal ward babies) for the above study and have had the opportunity to ask questions. ☐

2. I understand that my and my baby's 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. ☐

3. I understand that sections of any of my baby's medical notes may be looked at by the research individuals I give permission for these individuals to have access to my baby's records. ☐

4. I agree for my baby to take part in the above study. ☐

5. I understand that samples of my baby's nasal fluid and stool will be collected for this study. I understand that my baby's DNA will be extracted alongside the bacterial DNA but will not be used in this study. ☐

6. I give permission for any remaining samples (including my baby's DNA) to be used in future for chronic lung disease of prematurity research in the UK and abroad, which may include genetic (e.g. DNA), commercial or animal research. I understand I am free to withdraw my consent to future research at any point and that all samples will be destroyed as detailed in the information sheet.

Yes ☐ No ☐

_____	_____	_____
Name of Parent/Guardian	Date	Signature
_____	_____	_____
Name of Person taking consent (if different from researcher)	Date	Signature
_____	_____	_____
Researcher	Date	Signature

1 for patient; 1 for researcher; 1 to be kept with hospital notes

9.3 Appendix 3: Parent information sheet and consent form - Bristol

Information sheet for Parents/Guardians – Babies admitted to the neonatal unit

Principal Investigator: Dr R Wach, Consultant Neonatologist.

1. Study Title
The microbiome of the neonatal lung and its effect on chronic lung disease of prematurity

2. Invitation
You are being invited to take part in a research study being led by Cardiff University. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Please take time to decide whether or not you wish to take part.

Thank you for reading this leaflet.

3. What is the purpose of the study?
Chronic Lung Disease of prematurity (CLD), which is also often called BPD (for bronchopulmonary dysplasia), is a common disease of premature babies. Babies with CLD require oxygen for prolonged periods and are sometimes discharged home on oxygen. Our research is trying to understand why some babies develop CLD.

Until the past few years, it was thought that the lungs of healthy babies (and adults) were sterile, i.e. contained no bacteria. New techniques, however, have shown that there are low levels of bacteria in all people's lungs (known as the microbiome of the lung). It is not known when babies acquire these bacteria, or what type of bacteria are present in healthy baby's lungs. Our research seeks learn more about the acquisition of this bacteria.

It is becoming clear that these bacteria may play a part in many lung diseases. Our research will also look into the role these bacteria may have in the development of chronic lung disease of prematurity.

4. Why have I been chosen?
We would like to invite 3 groups of babies to join the study:

Study group: Babies who have been born prematurely (At or before 32 weeks gestation) and require the assistance of a breathing machine to support their breathing

Control group of ventilated babies: Babies who have been born at term (i.e. at or after 37 weeks gestation) and who need help with a breathing machine to support breathing for non-respiratory

reasons) commonly if they undergo surgery)

*We would like to invite you to join the study because your baby was born at 32 weeks gestation or less and needs a breathing machine to help his/her breathing **OR** your baby was born at or after 37 weeks gestation and requires a breathing machine to help his/her breathing*

5. Does my baby have to take part?
It is up to you to decide whether or not to consent for your baby to take part. If you do allow your baby to take part in the study, you will be given this information sheet to keep and be asked to sign a consent form. Even if you do decide to consent to your baby taking part, you are still free to withdraw your baby at any time without giving any reasons. A decision to withdraw at any time, or a decision not to take part, will not affect the care that your baby will receive.

6. What will happen to my baby if we take part?

Airway Fluid Samples
We would like to obtain airway fluid from your baby. All babies who are on a breathing machine regularly have the breathing tube sucked out to clear the secretions to prevent the tube from blocking. The secretions sucked out are usually thrown away. We would use these secretions for our study.

Samples will be taken only when the nurse or doctor caring for your baby feels the baby needs suctioning. There are no extra risks associated with collecting the secretions.

Nasal Fluid Samples
Babies on the neonatal unit often have the secretions from their nose suctioned out. When the nursing staff do this suctioning we would like to use the sample for our study.

We would like to collect nose fluid samples daily during the first week of life and then twice weekly until 4 weeks of age, while the baby remains on the neonatal unit, but only when it is being performed by the nursing staff as needed by the baby. Once the breathing tube is removed we would continue to samples from the nose.

Stool Samples
There is some evidence that the bacteria in the gut can influence the lung bacteria or microbiome. We would therefore like to analyse stool samples from your baby. We would aim to collect the first stool sample and one sample per week for the first month, or until your baby is discharged from the neonatal unit, whichever is sooner.

7. Will this affect my baby's treatment?
The medical care of your baby will not be affected by this study. The information from this study will not be used to diagnose or treat your baby.

8. Is there any benefit to taking part in this study?
There will be no benefit to you or your baby from taking part in this study. The information from this study will not be used to diagnose or treat your baby. The study should improve our understanding the role of germs play in the development of lung disease in premature babies.

9. What will happen to the samples collected?
The samples will be transferred to our laboratories at Cardiff University. We shall analyse the fluid from the airways in a number of ways:

4) We will use molecular biological techniques to identify any bacterial DNA present in the fluid

to identify what species of bacteria are present

5) We will measure markers of inflammation in the fluid to help us identify if the bacteria are causing infection.
6) We will look for molecules that many germs may produce (metabolites) in the lung fluid and blood to see if blood tests can be used to identify any bacteria.

We shall analyse the samples in our laboratories at Cardiff University but some analyses may be conducted by commercial companies or other university laboratories which have expertise to analyse the samples. The samples will have a code and will not have any information about the baby.

The baby's DNA will be extracted alongside the bacterial DNA but it will not be used further in this study. With your permission any remaining samples, including DNA (baby and bacterial), may be stored for future research into chronic lung disease of prematurity. The samples will be anonymised before use in future studies and may be accessed by researchers in the UK and abroad, the research may include genetic (e.g. DNA), commercial and animal research. You may withdraw your consent for the storage and future use of your baby's samples at any point. If you do withdraw your consent your baby's samples will not be used in any subsequent studies and will be destroyed according to locally approved practices. Any samples already distributed for use in research prior to the withdrawal of consent will continue to be used in that study and any samples remaining at the end of the study will be destroyed.

10. What are the risks of taking part?
Babies who receive mechanical ventilation are monitored closely for their heart rate and oxygen levels. The risks are the same as those of routine suctioning that the baby may have. Sometimes the babies may need extra oxygen, typically 5 – 10%, for 5 – 10 minutes and sometimes especially when the suction tube is placed the heart rate may drop for a few seconds (usually less than 30 seconds). We would monitor the baby throughout the procedure and stop it if the baby becomes unwell in any way.

11. What if something goes wrong?
If your baby is harmed by taking part in this research project, there are no special compensation arrangements. If your baby is harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you or your baby have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms should be available to you.

12. Will my baby's taking part be kept confidential?
All information which is collected about your baby during the course of the research will be kept strictly confidential. We will assign a number to each baby and use this to label the samples obtained for the study.

13. What will happen to the results of the study?
We will publish the results in reputable medical journals and present the data at scientific and medical meetings. Your baby's name and details will NOT be revealed at any stage. Please let us know if you would like a copy of the report.

14. Who is paying for the study?
The study is being funded by departmental funds.

15. Who had reviewed the study?
This study has been reviewed by the Wales Research Ethics Committee 2 and also by the North Bristol NHS Trust Research and Development committee.

16. Who can I contact for further information?
You may contact Dr Richard Wach by asking one of the staff on the neonatal unit, by telephone 0117 414 6800 or by email Richard.wach@nbt.nhs.uk . Alternatively, please contact Dr David Gallacher by email gallacherdj@cardiff.ac.uk or telephone 029 20 74 3375.

Thank you for taking time to read this information leaflet at this time. Please do not hesitate to ask Dr David Gallacher or Dr Richard Wach if you would like to discuss anything further.

Dr David Gallacher	Dr Richard Wach	Professor Sailesh Kotecha
Clinical Research Fellow	**Consultant Neonatologist**	**Consultant in Neonatal Medicine**

Patient Identification Number for this study:

Patient Sticker

PARENT/GUARDIAN CONSENT FORM

Project Title: The Microbiome of the Neonatal Lung and its Effect on Chronic Lung Disease of Prematurity
Principle Investigators: **Dr R Wach, Consultant Neonatologist**
Contact Details: Neonatal Unit, 0117 414 6800
This form should be read in conjunction with the Patient Information Leaflet (Neonatal Unit Patients), version no 4(Bristol) dated 4th March 2015.

Please initial relevant boxes

1. I confirm that I have read and understand the information sheet dated 4th March 2015 (version 4- babies admitted to the neonatal unit) for the above study and have had the opportunity to ask questions. ☐

2. I understand that my and my baby's 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. ☐

3. I understand that sections of any of my baby's medical notes and data collected during the study, may be looked at by individuals from Cardiff University, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

4. I agree for my baby to take part in the above study. ☐

5. I understand that samples of my baby's airway fluid will be collected for this study. I understand that my baby's DNA will be extracted alongside the bacterial DNA but will not be used in this study. ☐

6. I give permission for any remaining samples (including my baby's DNA) to be used in future for chronic lung disease of prematurity research in the UK and abroad, which may include genetic (e.g. DNA), commercial or animal research. I understand I am free to withdraw my consent to future research at any point and that all samples will be destroyed as detailed in the information sheet.

Yes ☐ No ☐

_____	_____	_____
Name of Parent/Guardian	Date	Signature
_____	_____	_____
Name of Person taking consent (if different from researcher)	Date	Signature
_____	_____	_____
Researcher	Date	Signature

1 for patient; 1 for researcher; 1 to be kept with hospital notes

9.4 Appendix 4: Sequences of primers used in 16 bacterial RNA gene sequencing

SA501	AATGATACGGCGACCACCGAGATCTACACATCGTACGTATGGTAATTGGCCTACGGGNGGCWGCAG
SA502	AATGATACGGCGACCACCGAGATCTACACACTATCTGTATGGTAATTGGCCTACGGGNGGCWGCAG
SA503	AATGATACGGCGACCACCGAGATCTACACTAGCGAGTTATGGTAATTGGCCTACGGGNGGCWGCAG
SA504	AATGATACGGCGACCACCGAGATCTACACCTGCGTGTTATGGTAATTGGCCTACGGGNGGCWGCAG
SA505	AATGATACGGCGACCACCGAGATCTACACTCATCGAGTATGGTAATTGGCCTACGGGNGGCWGCAG
SA506	AATGATACGGCGACCACCGAGATCTACACCGTGAGTGTATGGTAATTGGCCTACGGGNGGCWGCAG
SA507	AATGATACGGCGACCACCGAGATCTACACGGATATCTTATGGTAATTGGCCTACGGGNGGCWGCAG
SA508	AATGATACGGCGACCACCGAGATCTACACGACACCGTTATGGTAATTGGCCTACGGGNGGCWGCAG
SB501	AATGATACGGCGACCACCGAGATCTACACCTACTATATATGGTAATTGGCCTACGGGAGGCWGCAG
SB502	AATGATACGGCGACCACCGAGATCTACACCGTTACTATATGGTAATTGGCCTACGGGAGGCWGCAG
SB503	AATGATACGGCGACCACCGAGATCTACACAGAGTCACTATGGTAATTGGCCTACGGGAGGCWGCAG
SB504	AATGATACGGCGACCACCGAGATCTACACTACGAGACTATGGTAATTGGCCTACGGGAGGCWGCAG
SB505	AATGATACGGCGACCACCGAGATCTACACACGTCTCGTATGGTAATTGGCCTACGGGAGGCWGCAG
SB506	AATGATACGGCGACCACCGAGATCTACACTCGACGAGTATGGTAATTGGCCTACGGGAGGCWGCAG
SB507	AATGATACGGCGACCACCGAGATCTACACGATCGTGTTATGGTAATTGGCCTACGGGAGGCWGCAG
SB508	AATGATACGGCGACCACCGAGATCTACACGTCAGATATATGGTAATTGGCCTACGGGAGGCWGCAG
SA701	CAAGCAGAAGACGGCATACGAGATAACTCTCGAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
SA702	CAAGCAGAAGACGGCATACGAGATACTATGTCAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
SA703	CAAGCAGAAGACGGCATACGAGATAGTAGCGTAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
SA704	CAAGCAGAAGACGGCATACGAGATCAGTGAGTAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
SA705	CAAGCAGAAGACGGCATACGAGATCGTACTCAAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
SA706	CAAGCAGAAGACGGCATACGAGATCTACGCAGAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
SA707	CAAGCAGAAGACGGCATACGAGATGGAGACTAAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
SA708	CAAGCAGAAGACGGCATACGAGATGTCGCTCGAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
SA709	CAAGCAGAAGACGGCATACGAGATGTCGTAGTAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
SA710	CAAGCAGAAGACGGCATACGAGATTAGCAGACAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
SA711	CAAGCAGAAGACGGCATACGAGATTCATAGACAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
SA712	CAAGCAGAAGACGGCATACGAGATTCGCTATAAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT

9.5 Appendix 5: Syntax for Mothur v1.39.5

Where XXX.txt = name of stability file, YYY = max length of sequence to include and ZZZ = number of reads required to rarefy to even depth.

module load mothur/1.39.5

mothur

make.contigs(file=XXX.txt, processors=10)

summary.seqs(fasta=XXX.trim.contigs.fasta, processors=10)

screen.seqs(fasta=XXX.trim.contigs.fasta, group=dest2.contigs.groups, summary=XXX.trim.contigs.summary, maxambig=0, maxlength=YYY, processors=10)

count.groups(group=XXX.contigs.good.groups)

unique.seqs(fasta=XXX.trim.contigs.good.fasta)

count.seqs(name=XXX.trim.contigs.good.names, group=XXX.contigs.good.groups)

align.seqs(fasta=XXX.trim.contigs.good.unique.fasta, reference=silva.bacteria.fasta, processors=10)

summary.seqs(fasta=XXX.trim.contigs.good.unique.align, count=XXX.trim.contigs.good.count_table, processors=10)

screen.seqs(fasta=XXX.trim.contigs.good.unique.align, count=XXX.trim.contigs.good.count_table, summary=XXX.trim.contigs.good.unique.summary, start=6428, end=23444, maxhomop=6)

count.groups(count=XXX.trim.contigs.good.good.count_table)

filter.seqs(fasta=XXX.trim.contigs.good.unique.good.align, vertical=T, trump=.)

unique.seqs(fasta=XXX.trim.contigs.good.unique.good.filter.fasta, count=XXX.trim.contigs.good.good.count_table)

pre.cluster(fasta=XXX.trim.contigs.good.unique.good.filter.unique.fasta, count=XXX.trim.contigs.good.unique.good.filter.count_table, diffs=2, processors=10)

chimera.vsearch(fasta=XXX.trim.contigs.good.unique.good.filter.unique.precluster.fasta, count=XXX.trim.contigs.good.unique.good.filter.unique.precluster.count_table, dereplicate=t)

remove.seqs(fasta=XXX.trim.contigs.good.unique.good.filter.unique.precluster.fasta, accnos=XXX.trim.contigs.good.unique.good.filter.unique.precluster.denovo.vsearch.accnos)

split.abund(fasta=XXX.trim.contigs.good.unique.good.filter.unique.precluster.pick.fasta, count=XXX.trim.contigs.good.unique.good.filter.unique.precluster.denovo.vsearch.pick.count_table, cutoff=1)

count.groups(count=XXX.trim.contigs.good.unique.good.filter.unique.precluster.denovo.vsearch.pick.abund.count_table)

classify.seqs(fasta=XXX.trim.contigs.good.unique.good.filter.unique.precluster.pick.abund.fasta,
count=XXX.trim.contigs.good.unique.good.filter.unique.precluster.denovo.vsearch.pick.abund.count_table, reference=trainset14_032015.rdp.fasta,
taxonomy=trainset14_032015.rdp.tax, cutoff=80, method=wang, processors=10)

remove.lineage(fasta=XXX.trim.contigs.good.unique.good.filter.unique.precluster.pick.abund.fasta,
count=XXX.trim.contigs.good.unique.good.filter.unique.precluster.denovo.vsearch.pick.abund.count_table,
taxonomy=XXX.trim.contigs.good.unique.good.filter.unique.precluster.pick.abund.rdp.wang.taxonomy, taxon=Chloroplast-Mitochondria-unknown-Archaea-Eukaryota)

cluster.split(fasta=XXX.trim.contigs.good.unique.good.filter.unique.precluster.pick.abund.pick.fasta,
count=XXX.trim.contigs.good.unique.good.filter.unique.precluster.denovo.vsearch.pick.abund.pick.count_table,
taxonomy=XXX.trim.contigs.good.unique.good.filter.unique.precluster.pick.abund.rdp.wang.pick.taxonomy, splitmethod=classify, taxlevel=4, cutoff=0.03, processors=10)

make.shared(list=XXX.trim.contigs.good.unique.good.filter.unique.precluster.pick.abund.pick.opti_mcc.unique_list.list,
count=XXX.trim.contigs.good.unique.good.filter.unique.precluster.denovo.vsearch.pick.abund.pick.count_table, label=0.03)

classify.otu(list=XXX.trim.contigs.good.unique.good.filter.unique.precluster.pick.abund.pick.opti_mcc.unique_list.list,
count=XXX.trim.contigs.good.unique.good.filter.unique.precluster.denovo.vsearch.pick.abund.pick.count_table,
taxonomy=XXX.trim.contigs.good.unique.good.filter.unique.precluster.pick.abund.rdp.wang.pick.taxonomy, label=0.03)

count.groups(count=XXX.trim.contigs.good.unique.good.filter.unique.precluster.denovo.vsearch.pick.abund.pick.count_table)

sub.sample(shared=XXX.trim.contigs.good.unique.good.filter.unique.precluster.pick.abund.pick.opti_mcc.unique_list.shared, size=ZZZZ)

#Rename the files

dist.seqs(fasta=XXX.final.fasta, output=lt, processors=10)

quit

Use FastTree to generate phylogenetic tree:

fasttree -gtr -nt dest2.final.fasta > dest2.final.tre

#Reload Mothur:

collect.single(shared=XXX.final.subsample.shared, calc=chao-invsimpson-shannon-npshannon, freq=dest2)

summary.single(calc=nseqs-sobs-chao-ace-invsimpson-npshannon-coverage-shannon)

unifrac.weighted(tree=XXX.final.tre, name=XXX.final.names, group=XXX.final.groups, distance=square, processors=10, random=F, subsample=ZZZZ)

get.oturep(fasta=XXX.final.fasta, count=xx.trim.contigs.good.unique.good.filter.unique.precluster.denovo.vsearch.pick.abund.pick.count_table, list=XXX.final.list, label=0.03, method=abundance)

9.6 Appendix 6: Rscript for use in R-Studio with R v3.4.2

```
setwd("H:/My Documents/Data/Sequencing/TAF/FINAL ANALYSIS")
#Required packages
library(phyloseq)
library(vegan)
library(ggplot2)
#Data Import
data<-import_mothur(mothur_shared_file='TAF_formatted.txt')
tax = import_mothur(mothur_constaxonomy_file='TAF_Dec_2018_v1.taxonomy')
map<-import_qiime_sample_data('TAF_Metadata_Dec2018_v1.txt')
tree<-read_tree('RepOTU.final.tre')
merge<-merge_phyloseq(data,map,tax)
#Assign taxonomy classes
colnames(tax_table(merge))
colnames(tax_table(merge))=c("Kingdom","Phylum","Class","Order","Family","Genus")
colSums(otu_table(merge))
#Select Phylum data and export data to draw stacked bar charts in Excel and transform to relative
abduncance
merge_rel_rare = transform_sample_counts(merge, function(x) 100 * x/sum(x))
phylumonly <- tax_glom(merge_rel_rare, taxrank='Phylum')
otus <- otu_table(phylumonly)
write.csv(otus, file='TAF_otus-phylum.csv')
#Export genus data for stacked bar chart plotting
genusonly= tax_glom(merge, taxrank="Genus")
genus=otu_table(genusonly)
write.csv(genus, file= "TAF genus abundance.csv")
#Individual NMDS
rare.all.ord <- ordinate(merge, "NMDS", "bray", trymax=1000)
NMDS.rel.ord.figure = plot_ordination(merge, rare.all.ord, type="samples",color='BabyNo')
NMDS.rel.ord.figure + geom_polygon(aes(colour=BabyNo), fill="#e5e5e5", alpha=0.75)
theme_set(theme_bw())
#NMDS plot comparing recruitment sites
NMDS.rel.ord.figure.location = plot_ordination(merge, rare.all.ord, type="samples",color='Location')
NMDS.rel.ord.figure.location
#PERMANOVA
set.seed()
##Calculate bray curtis distance matrix
TAF_bray <- phyloseq::distance(merge, method = "bray")
## make a data frame from the sample_data
sampledf <- data.frame(sample_data(merge))
## Adonis test
adonis(TAF_bray ~ Location, data = sampledf)
## Homogeneity of dispersion test
beta <- betadisper(TAF_bray, sampledf$Location)
permutest(beta)
#Plot NMDS with centriods
scres=scores(rare.all.ord)
scres <- cbind(as.data.frame(scres), Site = sample_data(merge)$Location)
cent <- aggregate(cbind(NMDS1, NMDS2) ~ Site, data = scres, FUN = mean)
segs <- merge(scres, setNames(cent, c('Site','oNMDS1','oNMDS2')),
        by = 'Site', sort = FALSE)
```

```
ggplot(scres, aes(x = NMDS1, y = NMDS2, colour = Site)) +
  geom_segment(data = segs,
               mapping = aes(xend = oNMDS1, yend = oNMDS2)) + # spiders
  geom_point(data = cent, size = 5) +                         # centroids
  geom_point() +                                    # sample scores
  coord_fixed()
#Remove samples from babies with acientobacter infection
AcinNeg = subset_samples(merge, BabyNo!=10)

#Alpha diversity
alpha=estimate_richness(mergeMinusNC)
median(alpha$Observed)
quantile(alpha$Observed)
boxplot(alpha$Observed~sample_data(mergeMinusNC)$AntiB)

#Bee-swarm with box plot of cytokine concentration
Data= read.table("xxx.txt", header=TRUE)
library(beeswarm)
beeswarm(IL8~Sequencing_Pos,pch=16, pwcol = Location, log=TRUE, ylab="IL8 Concentration
(pg/ml)",main="IL8 Concentration in TA samples by Sequencing Result", labels =c("Negative",
"Positive"), xlab="Sequencing Result")
legend("bottomright", legend = c("NBT", "UHW"), title = "Location", pch = 16, col = 1:2)
bxplot(IL8 ~ Sequencing_Pos, add = TRUE)
wilcox.test(IL8~Sequencing_Pos)

#PERMNOVA for multiple groups
library("vegan")
## read in matrix of otu data otu=row and sample=col tab delimited
x=read.table(file.choose(), header=TRUE) #opens
## Generate correlation matrix but this can be skipped if a correlation matrix has been inputed e.g. a
##unifrac matrix
mat=data.matrix(x)
x = cor(mat,method= 'spearman')
head(x)
# reads in env data as csv file with headers
env=read.table(file.choose(), header=TRUE)
factors=env$DOL_Group
##change your_variable for your variable header
head(factors)
factors
##Identify which is larger, the positive or negative correlation
TF = abs(min(as.numeric(x))) < max(as.numeric(x))
#Replace ones for visualisation
if(TF){ x[x == 1] =  -max(as.numeric(x)) } else { x[x == 1] =  -min(as.numeric(x))}
pairwise.adonis <- function(x,factors, sim.method, p.adjust.m)
 {
library(vegan)
  co = as.matrix(combn(unique(factors),2))
  pairs = c()
  F.Model =c()
  R2 = c()
```

```
  p.value = c()
    for(elem in 1:ncol(co)){
    ad = adonis(x[factors %in% c(as.character(co[1,elem]),as.character(co[2,elem])),] ~
                factors[factors %in% c(as.character(co[1,elem]),as.character(co[2,elem]))] , method
=sim.method);
    pairs = c(pairs,paste(co[1,elem],'vs',co[2,elem]));
    F.Model =c(F.Model,ad$aov.tab[1,4]);
    R2 = c(R2,ad$aov.tab[1,5]);
    p.value = c(p.value,ad$aov.tab[1,6])
  }
  p.adjusted = p.adjust(p.value,method=p.adjust.m)
  pairw.res = data.frame(pairs,F.Model,R2,p.value,p.adjusted)
  return(pairw.res)
}
PW.Adonis=pairwise.adonis(x,factors,sim.method="bray",p.adjust.m = "fdr")
PW.Adonis
```

9.7 Appendix 7: Papers published during research

Gallacher, David J., and Sailesh Kotecha. 2016. “Respiratory Microbiome of New-Born Infants.” Frontiers in Pediatrics 4 (February): 10.

Gallacher, David J, Kylie Hart, and Sailesh Kotecha. 2016. “Common Respiratory Conditions of the Newborn.” Breathe (Sheffield, England) 12 (1): 30–42.

Cousins, Michael, Kylie Hart, David Gallacher, María Angélica Palomino, and Sailesh Kotecha. 2018. “Long-Term Respiratory Outcomes Following Preterm Birth.” Revista Médica Clínica Las Condes 29 (1): 87–97.

Manuscript submitted:

Gallacher, David, Emma Mitchell, Dagmar Alber, Richard Wach, Nigel Klein, Julian Marchesi and Sailesh Kotecha. 2019. “Dissimilarity of the gut-lung axis and dysbiosis of the lower airways in ventilated preterm infants.” Submitted to European Respiratory Journal.