INFANT NASOPHARYNGEAL MICROBIAL ECOLOGY AND THE PNEUMOCOCCAL CONJUGATE VACCINE

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The nasopharynx is an important reservoir of microbes some of which can cause serious mucosal and invasive disease. The impact of the 7-Valent Pneumococcal Conjugate Vaccine (PCV-7) on pharyngeal microbial ecology is not well characterized. The aim of this study is characterize the infant nasopharyngeal microbiome and subsequently to address the hypothesis that PCV-7 vaccination influences its development. Nasopharyngeal (NP) swabs were collected from neonates at birth, bi-weekly from zero to six months and bi-monthly from six to twelve months. High co-carriage of *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis* was confirmed by species-specific PCR. Molecular-fingerprinting of the infant NP microbial communities combined with partial 16S rRNA gene showed that microbes are acquired rapidly after birth and that there are distinct microbial profiles over time and across individuals. Bar-coded 454 pyrosequencing of the 16S rRNA gene showed over 800 operational taxonomic units (genera). *Streptococcus* was the only genus present in all the NP communities. Six genera including *Streptococcus, Haemophilus, Moraxella* and *Staphylococcus* comprised over 80% of the infant NP microbiome. Most genera had low relative abundance (<1%) and their variable accounted for much of the diversity between individuals. Although early PCV-7 intervention did not appear to alter the abundance of *Streptococcus*, overall, the abundance of microbes was less turbulent among infants vaccinated early, before four months. Early intervention also appeared to alter the interactions between *Streptococcus* and several OTUs. *Streptococcu*s was negatively correlated with *Staphylococcus, Corynebacterium* and *Pseudomonas*; this could play a major role in replacement. Preliminary data suggests that PCV-7 intervention may alter the development of the microbiome and microbial interactions within the nasopharynx. The long term implications of this finding for PCV-7 vaccination are yet to be determined, but continued surveillance of replacement in carriage and disease is necessary as immunisation with vaccines covering more serotypes are implemented.

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Statement of work personally performed

All the nasopharyngeal swabs (NP) were collected from the subjects by trained field nurses. I supervised the collection of the NP swabs collected in the prospective study and was responsible for the quarterly training of the nurses and field workers. I performed all the laboratory bench work for the data presented in Chapters, 3, 4 and 5. For the data presented in Chapter 6, I prepared the nucleic acids for 454-pyrosequencing which was conducted at the Genome Centre, at the University of Washington in St. Louis, USA. The Genome Centre also provided statistical support for identification of significant changes occurring after vaccination. I received statistical support from the Medical Research Council, The Gambia unit for the Generalised Estimating Equations (GEE) and random effects regression modelling presented in Chapter 3. Also, statistical support was sought for the binning and aligning of terminal restriction fragments based on hierarchical clustering and the parametric survival time regression models used to examine the impact of genetic and environmental factors presented in Chapter 5. I independently performed all other statistical analyses presented in this study.

Peer-Reviewed publications from this study

1. Kwambana BA, Mohammed NI, Jeffries D, Barer M, Adegbola RA, Antonio M. Differential effects of frozen storage on the molecular detection of bacterial taxa that inhabit the nasopharynx. BMC Clin Pathol. 2011;11:2.
2. Kwambana BA, Barer MR, Bottomley C, Adegbola RA, Antonio M. Early acquisition and high nasopharyngeal co-colonisation by Streptococcus pneumoniae and three respiratory pathogens amongst Gambian new-borns and infants. BMC Infect Dis. 2011;11:175.

Abbreviations

6-FAM	6 - Carboxyfluorescein
ALRI	Acute Lower Respiratory Tract Infection
ALRI	Acute Lower Respiratory Tract Infections
ALRTI	Acute Lower Respiratory Tract Infections
AOM	Acute Otitis Media
ARDRA	Amplified Ribosomal Deoxyribonucleic Acid Restriction Analysis
ARISA	Amplified Ribosomal Intergenic Spacer Analysis
AURTI	Acute Upper Respiratory Tract Infections
BLAST	Basic Local Alignment Search Tool
bp	Base Pair
CD	Crohn's Disease
CI	Confidence Interval
CMV	Cytomegalovirus
COPD	Chronic Obstructive Pulmonary Disorder
DGGE	Denaturation Gradient Gel Electorphoresis
DHPLC	Denaturing High-Performance Liquid Chromatography
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
EBV	Epstein-Bar Virus
EDTA	Ethylenediaminetetraacetic Acid
EPI	Expanded Programme On Immunization
EV	Early Vaccination
FISH	Fluorescent *In Situ* Hybridization
FU	Fluorescence Units
GI	Gastrointestinal
GIT	Gastrointestinal Tract
HHV	Human Herpesvirus
Hib	Haemophilus Influenzae Type B
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HMP	Human Microbiome Project
HOMD	Human Oral Microbiome
HPIV-3	Human Parainfluenzae Virus 3
HR	Hazard Ratio
IBD	Invasive Bacterial Disease
ID	Identification
IPD	Invasive Pneumococcal Disease
IPTG	Isopropyl-B-D-Thiogalactopyranoside
LB	Luria Broth
LRT	Lower Respiratory Tract
LRTI	Lower Respiratory Tract Infections
LV	Late Vaccination
MetaHit	Metagenomics Of The Human Intestinal Tract
MiCA 3	Microbial Community Analysis Iii

mL	Millilitres
MLST	Multilocus Sequence Typing
mM	Millimolar
MRC	Medical Research Council
NA	Not Applicaple
NEC	Necrotising Enterocolitis
ng	Nanogram
NIH	National Institutes Of Health
nM	Nanomolar
NP	Nasopharyngeal
NPS	Nasopharyngeal Swab
NT	Non Typeable Pneumococci
NVT	Non Vaccine Serotypes
OM	Otitis Media
OPIMA	Operational Interrogation Of Microbiome Array
OR	Odds Ratio
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PCV	Pneumococcal Polysaccharide-Diptheria CRM_{197} Protein Conjugate Vaccine
PMN	Polymorphonuclear Leucocytes
PPS	Pneumococcal Polysaccharide Vaccine
RDP	Ribosomal Database Project
RNA	Ribonucleic Acid
RNAP	Ribonucleic Acid Polymerase
rpm	Revolution Per Minute
rRNA	Ribosomal Ribonucleic Acid
RSV	Respiratory Syncytial Virus
RT	Respriatory Tract
RTI	Respiratory Tract Infection
SCC	Scientific Co-Ordinating Committee
ß	Beta
ssRNA	Small Subunit Ribosomal Ribonucleic Acid
STGG	Skim Milk-Tryptone-Glucose-Glycerol
SVT	Sibanor Vaccine Trial
TAE	Tris-Acetate-EDTA
TGGE	Temperature Gradient Gel Electrophoresis
T-RFLP	Terminal Restriction Fragment Length Polymorphisms
UK	United Kingdom
UPMGA	Unweighted Pair Group Method With Arithmetic Mean
URT	Upper Respiratory Tract
URTI	Upper Respiratory Tract Infections
USA	United States Of America
VAT	Vaccine Associated Serotypes
VT	Vaccine serotypes
WGA	Whole genome amplification
WGS	Whole genome sequencing

WHO	World health organisation
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
α	alpha
µg	Microgram
µL	Microlitre

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1. Introduction

1.1. Human Microbial Ecology

The human body can be viewed as a micro-ecosystem in which diverse microbes occupy a wide range of microenvironments ranging from areas of high acidity (stomach), to high ionic strength (skin)[1]. Mucosal surfaces are the commonest site of microbial colonization; these are the mucosae of the respiratory, digestive tract, urogenital tract, eye conjunctiva, the inner ear and exocrine gland ducts which together provide a surface area of 400m^2 [2]. The term 'human microbiome' describes the microbial communities and their components that colonize the human body including the skin and mucosal surfaces [3].

There are an estimated 100 trillion bacterial cells in the human body, 10 times the average number of human cells [1]. Microbial inhabitants of the human body are highly heterogeneous, represent up to 100 000 different species and only a small fraction of the commensal microorganisms are pathogenic, such as *Streptococcus pneumoniae*, *Staphylococcus aureus, Moraxella catarrhalis* and *Haemophilus influenzae* in the upper respiratory tract. Microbial genes outnumber human genes by a factor of 100. Hence, it would hardly be surprising to find that the microbiome not only plays an important role in disease, but also in the maintenance of health by enhancing or complementing host physiology [4]. Host-microbe interactions are key components of normal human physiology; essential in the regulation of the inflammatory response, development of the immune system, metabolic function and nutrient processing, uptake and storage and inhibition of pathogens [5]. An illustration

of host-microbe mutualism is found in the colonic production of butyrate. Butyrate is produced through the degradation of polysaccharides which depends on the intricate metabolic activities of both *Bifidobacterium* and *Eubacteria [6].*

Furthermore, it appears that some infections are polymicrobial (more than one microbe acting synergistically or sequentially to cause an infection) while other diseases are associated with disruptions or changes in normal microbial ecology [7, 8]. There is tantalising evidence of associations between the microbiome and several diseases such as necrotising enterocolitis (NEC) [9, 10], atopic eczema [11], obesity [12-14], Crohn's disease [15-17], chronic obstructive pulmonary disease (COPD) [18], cystic fibrosis [19, 20], type II diabetes [21, 22], oral cancer [23], bacterial vaginosis [24-26] and periodontal disease [27] amongst many others. NEC is an acquired gastrointestinal (GI) disease which affects up to 10% of babies born less than 1.5Kg with up to 28% case mortality and there is currently no known causative pathogen. However, there is compelling evidence that decreased GI microbial diversity, increased Gammaproteobacteria dominance and prolonged antibiotic treatment are associated with NEC aetiology in preterm infants [10].

The significance of the human microbiome in relation to health and disease has come to the forefront of microbiology with several hundred million dollars poured into multi-centre international projects initiated in the last decade. These include the National Institutes of Health (NIH) Human Microbiome Project (HMP) [28], the Canadian Institutes of health Research (CIHR) Canadian Microbiome Initiative (http://www.cihr-irsc.gc.ac) and Metagenomics of the Human Intestinal Tract (MetaHit) (http://www.metahit.eu). Several factors associated with modern-lifestyle

may have the capacity to influence microbial ecology; these include hygiene, nutrition, living conditions, medications, genetic background, geographic location, stress, alcohol consumption, smoking and day-care attendance [29]. We are only beginning to unveil the host and microbial, genetic and environmental interplays which govern the healthy and diseased states [30, 31].

1.2 The Upper Respiratory Tract (URT)

The respiratory tract respiratory tract (RT) is divided into two sections, the upper (URT) and lower (LRT) respiratory tracts. The URT comprises the nose, pharynx and larynx and is constantly exposed to microbes (Figure 1.1). Approximately 10^4 viable microbial cells are inhaled everyday [32], and not surprisingly, the URT is densely colonized by microorganisms [29, 33, 34]. Despite significant advances in the understanding of the microbial communities that inhabit the mucosae of the human body, there are significant gaps in our depth of understanding of the microbiota of the respiratory tract (RT). The bulk of published data on the nasopharyngeal microbial communities have been biased towards analyses of bacterial pathogens such as *S. pneumoniae, H. influenzae, S. aureus, M. catarrhalis* and *Neisseria meningitidis* [35-37]. Although these inhabitants of the URT have been studied extensively, the full spectrum of microorganisms present in the URT, the functions they play, their interactions and the scale of the communities are yet to be comprehensively described. Furthermore, relatively little is known about the viral, archaeal, fungal and possibly the protozoan inhabitants of the URT and the functional roles they play which may influence health and disease.

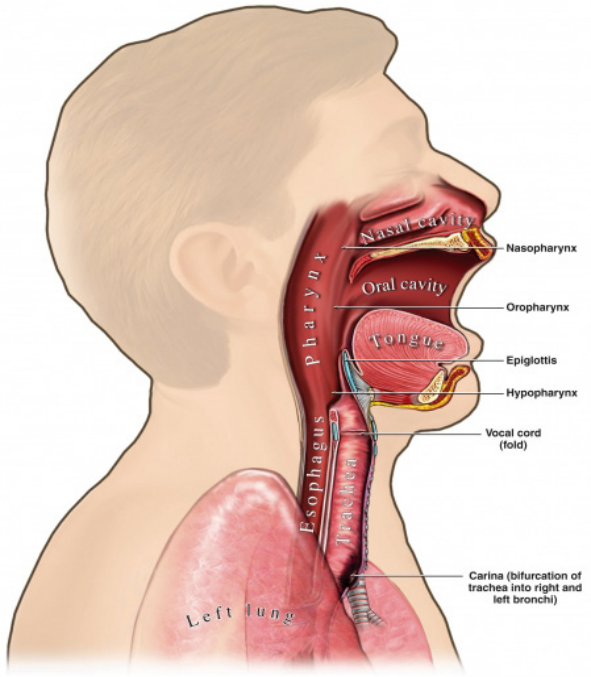


Figure 1.1.The anatomy of the upper respiratory tract (URT) showing the pharynx (nasopharynx and oropharynx).

1.1.1. Significance of the nasopharyngeal microbiota

The nasopharynx (Figure 1.1) is an important reservoir of commensal and pathogenic microbes which can migrate to and cause disease in other compartments such as the sinus, middle ear, lungs and blood [38]. Invasive bacterial disease (IBD) such as pneumonia, meningitis and bacteraemia contribute to the disparity in childhood mortality between developing and developed countries [39, 40]. Nasopharyngeal carriage is thought to be the main source of transmission of bacterial pathogens across individuals [41-43]. Although the specific mechanisms are poorly understood, attachment to the nasopharyngeal epithelial surface is thought to be an essential step in the development of mucosal and invasive disease [44, 45].

At least 60 different species have been cultured from the nasopharyngeal cavities of healthy individuals, representing five phyla, *Firmicutes, Proteobacteria, Bacteroides, Fusobacteria* and *Actinobacteria* [33, 46]. Cultured species of the nasopharyngeal

microbiota include viridans group streptococci, *Streptococcus pneumoniae*, coagulase-negative staphylococci, coryneform bacteria, *Haemophilus influenzae, Staphylococcus aureus, Moraxella catarrhalis,* and Enterobacteriaceae. As has been the case with other mucosal surfaces, the diversity and richness of microbes found to colonize the nasopharynx is expected to increase exponentially with the application of culture-independent tools. Pyrosequencing allows high depth of coverage and detection of low abundance taxonomic groups which would be difficult to achieve with Sanger sequencing. 59 distinct phylotypes were identified with culture-independent 16S rRNA gene-based clone library analysis, as opposed to 28 species isolated using culture-based analysis [47]. With the application of pyrosequencing analyses, at least 700 species have been described from the oral cavity [48, 49]. Similarly, the human intestinal microbiota has been shown to comprise over 1000 bacterial species by pyrosequencing [50]. Hence, in order to gain in-depth knowledge of the specific host-microbe and microbe-microbe interactions that influence the development and composition of the URT microbiota, application of pyrosequencing technology is necessary.

1.1.2. Nasopharyngeal Microbial Interactions

Co-colonisers of the nasopharynx may have synergistic or competitive relations. There is a plethora of evidence of complex competitive relationships between *S. pneumoniae* and other respiratory pathogens that are co-colonisers of the nasopharyngeal mucosa [6, 7]. Respiratory co-infection in a murine model resulted in rapid *H. influenzae*-induced clearance of *S. pneumoniae* which was dependent on neutrophil recruitment [51]. Contrary to this report, a few studies have also found a positive relation between *H. influenzae* and *S. pneumoniae* colonization in the nasopharynx [35, 52-55].

A few studies have also reported a negative association between *S. pneumoniae* and *S. aureus* [56-58] which was not found HIV infected infants (206) and after adjustment for other confounding factors [44]. Most of these studies have been culture or polymerase chain reaction (PCR) based studies, limited to specific pathogenic colonizers of the nasopharynx and used pairwise associations which may not account for other microbe-microbe and microbe-host interactions. Indeed, a reductionist view (studies of a few species) may skew our understanding of the complex and dynamic inter-species interactions that occur in this microbial reservoir. The importance of a holistic view on microbial community interactions is highlighted by Pettigrew et. al's study of bacterial interactions during upper respiratory tract infections. This group found a negative association between *S. pneumoniae* and *H. influenzae* colonization; however, if *M. catarrhalis* was present, these microbes had a positive correlation, suggesting that microbial interactions are complex. It is unclear what microbial interactions and relations exist amongst the pathogenic and commensal, high abundance and low abundance components of the nasopharyngeal microbiota. Application of culture-independent tools will be essential to elucidate the complex relations between microbes inhabiting the nasopharynx, including viral components. A recent *in-vitro* study demonstrated a significant positive correlation between Rhinovirus infection and colonization with bacterial respiratory pathogens including *S. aureus* [59]. Viral infection of nasal epithelial cells significantly increased adhesion of the bacterial pathogens by the induction of the adhesion and receptor molecule expression on the epithelial surface. Virus-associated respiratory epithelial cell susceptibility to bacterial attachment and infections has been reported for rhinovirus, adenovirus, respiratory syncytial virus (RSV), human parainfluenzae virus 3 (HPIV-3)

and influenzae [60-62]. Although existing data is tantalizing, the specific relations and interactions between microbes and their competitors are poorly understood, particularly the anaerobes, fastidious and uncultured bacteria. It is unclear how the microbial associations differ with host genetic background, co-infection with other microbes and the niche assayed. Microbe-microbe and host-microbial relations are complex and interlinked and it will require the most high-through-put and technologies to unravel them.

1.2. Microbe-Host Interactions

1.2.1. Immune modulation

For survival and proliferation, microbes have to establish themselves in a microbial community competing for resources, nutrients and other growth factors [51, 63]. Within an environment of polymicrobial immune stimulation, persisting microbes must also contend with multilayered defences which constitute a barrage of bacteriostatic proteins, bacteriocidal factors, secretory immunoglobulins and a mucociliary clearance system [64]. Hence, microorganisms, particularly those that have co-evolved with the human host have developed several strategies and virulence factors to evade the mucosal immune responses, reviewed in [32, 65, 66], summarised in Table 1.1 The innate and adaptive components of immune defence are interlinked and concomitantly repel harmful microbes while maintaining tolerance to the commensal microbes (mucosal tolerance)[5, 44].

Table 1.1. Clearance Subversion Strategies and Mechanisms of Respiratory Microbes [32, 66]

Strategies	Mechanisms
Impede mucociliary clearance	• Induce excessive mucous production • Distort and reduce ciliary beat • Impair ion transport • Damage epithelial surface
Neutralization of IgA	• Secretion of IgA protease that cleave IgA
Impair effector cell function	• Inhibit neutrophil phagocytosis and chemotaxis • Impair macrophage viability • Activate suppressor T-cells and impede lymphocyte cytokine production
Adherence to epithelial surface	• Damage epithelial surface
Evade immune surveillance	• Polymorphic surface antigens • Biofilm formation • Production of polysaccharide capsules to prevent phagocytosis • Endocytosis

Vaughan et al. investigated the mucosal immune responses to two closely related obligate colonizers of the URT mucosa, *Neisseria lactamica* and *N. meningitidis* in tonsil tissue. In contrast to its pathogenic relative, *N. lactamica* does not induce the development of mucosal T and B cells in young individuals, maintaining immunological ignorance in the host which may facilitate the longer and more frequent colonization observed for by *N. lactamica* strains [67]. Genetic, phenotypic and biochemical variations among members of the same species influence the microbe's capacity to evade host immune pressure and thrive in the microenvironment. Weinberger et al. demonstrated that there is a link between prevalence of nasopharyngeal colonization and pneumococcal polysaccharide biochemistry and structure [68]. It will be

interesting to compare immune responses to low abundance and high abundance taxa in the nasopharynx and investigate any correlates with cell biochemistry.

There are distinct age-dependent differences in immune function which may dictate the outcome of encounters with microbial invaders; immediate clearance, commensal carriage, transient colonization or less often harmful invasion. Although not fully characterized, the mucosal adaptive and innate immune function of the very old and the very young differ from that in adults between less than 60 years old. In the very young, the major immune deficiencies include delayed immunoglobulin production, decreased levels of complement components and impairment of polymorphonuclear leucocytes (PMN). Although antigen-independent immune development commences in-utero, as early as the ten-week gestation period, antigen dependent immunity develops after birth [69]. At birth the infant mucosa rapidly comes in contact with numerous microorganisms. Age-dependent immunologic responses should extend beyond the investigation of a small subset of microbes. The full spectrum of microorganisms that initially colonize the nasopharynx and the subsequent succession of microbes are yet to be elucidated.

1.2.2. Age distribution

Several studies have shown that there are distinct age-related patterns of microbial carriage in the nasopharynx [29]. *S. pneumoniae, H. influenzae* and *M. catarrhalis* are generally detected at high frequency in young children compared to adults. Conversely, coagulase-negative staphylococci and diptheroids are observed at lower frequency in young children compared to adults [46]. The microbiota in the nasal cavity of healthy children is distinguished by high carriage of Gram negative microbes which are found at low frequencies in healthy adolescents and adults [70, 71]. T-RFLP

analysis of the gut microbiome of two infants showed initial colonization with *Enterobacteriaceae*, *Veillonella, Enterococcus, Streptococcus, Staphylococcus* and *Bacteroidetes*. However, after weaning, *Enterobacteriaceae* decreased and clostridia increased [72]. Culture-independent applications will elucidate the ecological, taxonomic and phylogenetic age-dependent profiles of the nasopharyngeal microbiome.

1.2.3. Pre- and Post-natal Factors

An intact amniotic membrane keeps the intrauterine foetus sterile; however, microbial colonization of mucosal surfaces occurs rapidly after birth in neonates [73, 74]. Within hours, bacteria can be detected in the faeces and oral cavity of a newborn [74]. However, in the nasopharynx, it is yet unclear when initial colonization occurs and by which microorganisms, the roles they play and the factors that influence acquisition and elimination of the microbes. Evidence from the GI tract (GIT) suggests that type of birth (vaginal versus caesarean section), gestation age and hospitalisation affect the early development of the microbiome. [75-77]. Molecular studies of the development of the (GIT) microbiome suggest that vaginally born neonates are initially colonized by the mother's faecal, skin and vaginal microbiota; the same strains of bacteria such as *Escherichia coli*, are detected in the baby and in the mothers' faeces. Furthermore, the microbiota of the gastric content of newborns was similar to the mothers' vaginal and cervical microbiota. Neonates delivered by caesarean section are exposed to bacteria from the environment, medical equipment, other infants and healthcare workers delaying the development of the native GIT microbiota, which is predominantly anaerobic.

1.2.4. Microbial Dysbiosis and Aetiology of Disease

Interestingly, the microbial ecology of the GI tract has been implicated in the aetiology of autoimmune diseases such as CD. Joosens et al's investigation of the faecal microbiota of CD patients showed that a dysbiosis involving *Dialister invisus, Bifidobacterium adolescentis, Ruminococcus gnavus* and a *Clostridium* XIVa taxa is involved in the pathogenesis of CD [17]. In recent years, an astounding link between a high-fat diet, obesity and the gut microbiome has been uncovered. Breakthrough culture-independent studies of the distal gut microbiota of obese and lean individuals demonstrated that obesity is associated with the capacity to harvest energy from food which in turn is associated with reduced abundance of gut *Bacteriodetes* compared to *Firmicutes*, reduced bacterial diversity, and various other changes in bacterial gene expression [14, 78]. However, the findings of this study still need to be replicated and verified in counter studies.

Interactions between the nasopharyngeal microbial ecology and the pathogenesis of several infections such as sinusitis, otitis media (OM), tonsillitis and pneumonia have been studied, primarily by culture-based techniques [79, 80]. Anh *et al.* showed that children with pneumonia and acute bronchitis had high rates of intense colonization ($\geq 10^6$ colony-forming units/mL) compared to healthy controls [81]. Children with intense colonization with any bacterial respiratory pathogen were significantly more likely to develop pneumonia, potentially as a direct result of the vertical spread of pathogens from the nasopharynx to the LRT. In addition, at least 90% of the children who developed acute lower respiratory tract infections (ALRTI) had a preceding viral acute URTI (AURTI) associated with increased bacterial colonization [81]. In another study, bacterial respiratory pathogens were detected at least double the frequency in

individuals with long-standing cough compared with health individuals from all age groups [82]. Few studies suggest that anaerobic bacteria are absent in nasopharynx of healthy children and their presence is strongly associated with infections such as otitis media [33, 83]. Virus infections may increase attachment and infection by bacterial pathogens [81, 84]. Investigations of associations between disruption of the normal upper airway ecology and aetiology of disease need to be conducted and may guide treatment and prevention strategies.

1.2.1. Biogeography and Spatial Distribution

Specific families and genera occupy the same microenvironments across individuals. Hence, it appears that microorganisms that have coevolved with the humans and are organized in site specific or 'biogeographical' communities. A recent pyrosequencing enabled study of 9 healthy adults sampled at 27 sites showed that there are systematic changes in microbial composition across different compartments amongst different subjects [85]. Distinct microbial distribution patterns have also been shown in proximal and contiguous oral surfaces (tongue dorsum, saliva, supragingival and subgingival plaque) amongst children [86, 87]. It is yet unclear if there are any distinct distribution patterns in the nasopharyngeal mucosae. Kaieda et al. found that there were no differences in the distribution of *S. pneumoniae, M. catarrhalis* and *H. influenzae* between different loci of the nasopharynx sampled from the left and right eustachian tubes [88]. However, a major limitation of this study is that it was culture-based and only targeted three pathogenic microbes. Hence, culture-independent studies are necessary to understand the organization of microbes (diversity, richness and abundance) across the nasopharynx. Culture-based studies have provided evidence that microbial distribution is different across the nasopharyngeal,

oropharyngeal and nasal mucosae [79, 89]. Various facets of a microenvironment such as salinity, pH, immune constitution, physiology and location influence the ecology of microbial communities. There is also evidence of intrapersonal variability in species diversity, richness and abundance in different microenvironments across the human body. However, the microbial species and strains harboured may be as distinct as fingerprints amongst different individuals [1], this needs further investigation in the nasopharynx.

1.2.2. Genetic Background and Genetics

One of the major goals of the NIH HMP is to determine if there is a human core microbiome of genes or species shared by individuals [28, 90]. Although there is limited comprehensive data, available reports suggest that the genomic and functional constitution of the URT microbiome markedly vary with geographic location. Wolf *et al.* showed that Gram-negative bacilli had nasopharyngeal prevalence rates >50% among Brazilian and Angolan children, in sharp contrast to 4% among Dutch children ($p<0.0001$). Not surprisingly, Gram-negative bacilli are a common cause of pneumonia in Brazil and Angola as in many other non-industrialized countries [91, 92]. With the exception of *S. aureus*, the prevalence of respiratory pathogens is higher amongst Aboriginal than non-Aboriginal children in Australia [93, 94]. Nasopharyngeal carriage of respiratory pathogens among Chinese and Vietnamese children < 5years in Hong Kong was significantly different after adjusting for age, smoking and socio-economic conditions. There is a significant interaction between the human leukocyte antigen (HLA) DR8 chromosomal loci and increased susceptibility to *H. influenzae* type b (Hib) disease among Alaskan Eskimo children [95]. Interestingly, ethnic and racial factors are also important determinants of colonization and pathogenesis at other mucosal

surfaces such as the vagina which cannot be attributed to socio-demographic, hygiene and health and sexual behaviour alone [96]. This finding suggests that host genetic factors are important factors in pathogenesis, and potentially microbial colonization and community composition. Worldwide nasopharyngeal carriage profiles for *H. influenzae, M. catarrhalis* and *S. pneumoniae* also show remarkable geographic variations [29, 97, 98]. None the less, different nasopharyngeal carriage rates have been reported from the same region [35, 55, 93, 99] and even proximal day-care centres [100, 101] suggesting that there may be several factors influencing prevalence. It is important to determine if and how the genetic background influence the nasopharyngeal microbiota and how this may correlate with susceptibility to disease.

1.3. Influence of Environment Factors on the URT Microbiome

However, several activities and other factors such as hygiene, overcrowding, contact with other children (number of siblings and day-care attendance), sleeping position, and maternal educational level are all factors that influence microbial communities of the URT mucosae [55, 102]. However, the relative importance and interaction of each of these factors with regards to the URT microbiome are yet to be fully explored.

1.3.1. Breastfeeding and Diet

Balanced nutrition is essential for normal immune development and function which are essential in the regulation of autochthonous microbial communities and the prevention of pathogenesis [103]. The type of feeding given to young infants modulates the mucosal microbial communities by two mechanism; 1) influencing immune development; 2) transmitting microbes from the mother or environment to the child. In early life, breastfeeding not only provides all the essential nutrients required for normal development, but breast milk also contains several anti-

inflammatory factors, growth factors, nucleotides, cytokines, macrophages, and granulocytes which may confer antibacterial and antiviral benefits [104]. Although the specific mechanisms of protection are poorly understood, breastfeeding appears to decreases carriage of respiratory pathogens and reduces the incidence of acute OM in young children [105, 106]. It needs to be investigated if breast milk stimulated mucosal secretion of IgA plays a role in protection against environmental microbes [107, 108] . In addition to promoting immune maturation, human milk is also an important source of bacteria. The epithelial surface on and surrounding the nipple and the milk ducts are thought to be the bacterial reservoirs. Human milk has a bacterial load of approximately 1 million cells per mL detectable by culture in healthy mothers. The most abundant microbes in breast milk appear to be *Bifidobacterium, Lactobacillus, Staphylococcus, Bacteroides, Enterococcus, Streptococcus,* diptheroids, *Micrococcus*, *PropionibacteriaClostridium* and *Enterococcus* species [103, 109]. Interestingly, breast milk may also be a source of pathogens such as *Mycoplasma* and *Norcadia* species. Amongst HIV-1 positive women, breast milk can also transmit infectious viruses such as human herpesvirus 6 (HHV-6), HHV-7, HHV-8, cytomegalovirus (CMV), Epstein-Bar virus (EBV) and HIV-1 as well as fungal potential pathogens such as *Candida* and *Aspergillus* [110]. Although there is limited data on the direct effect of type of feeding on the development of the nasopharyngeal microbiome, few culture-independent studies have highlighted the importance of breastfeeding on the development of the GIT microbiome, with specific emphasis on *Bifidobacterium spp.* is relatively well reported [111].

1.3.2. Seasonal Effects

Interestingly, nasopharyngeal carriage of respiratory pathogens has been postulated to have a seasonal component [112, 113]. Few studies have shown seasonal variations in the URT carriage of microbial species or specific serotypes e.g. pneumococcal serotype 1 in Gambia, West Africa [112]. In Western Australia there was marked seasonal variation in the carriage of *S. pneumoniae, M. catarrhalis* and *H. influenzae* among non-Aborignal< 30 months. Among non-Aboriginal children, *M. catarrhalis, S. pneumoniae* and *H. influenzae* carriage rates were significantly lower in the summer compared with other seasons. In contrast, *S. aureus* carriage was significantly higher in summer compared to winter. Only *H. influenzae* showed seasonal variability among Aboriginal children [55]. Likewise, Hendley *et al.*[113] showed that nasopharyngeal carriage of respiratory pathogens was highest in the winter season (85%) compared to summer and fall (56%). In Costa Rica the incidence of *H. influenzae* and *S. pneumoniae* in the middle ear fluid of children with OM was highest during the rainy season when there is also increased morbidity associated with respiratory pathogens [114].

1.3.3. Antibiotics

Since the development of antimicrobials such as β-lactams and aminoglycosides in the early 1940s, morbidity and mortality from microbial infections has been dramatically reduced, establishing antimicrobial therapy as a spectacular medical advancement of the 21st Century [115, 116]. Antimicrobials can be bacteriostatic or bactericidal and are typically indiscriminate or non-specific, targeting a narrow or broad spectrum of microbes. The URT micro-ecosystems may be altered by antimicrobial therapy [117]; however, the effects appear to be dependent upon the class of antibiotic, dosage and duration of treatment. Ghaffar*et al.'s* study of the effect of high dosages of amoxicillin

and azithromyicin on the nasopharyngeal bacteria suggested that antibiotic therapy alters the competitive balance between microbes, particularly ά-haemolytic streptococci and *S. pneumoniae* [118]. Brook and Gober's comparison of the long term effects of two types of antibiotics, amoxycilin-clavulanate and cefdinir on the nasopharyngeal microbiota in children with AOM demonstrated that the two treatment regimens have differential selective activity against components of the nasopharyngeal microbiota. Amoxicillin-clavulanate with a broader spectrum of activity also showed more extensive depletion of both pathogens and microbes with pathogen interfering potential [119]. This effect was also observed at least two months after antibiotic treatment concomitant with rapid reacquisition of pathogenic bacteria. Dethlefsen *et al.* applied 16S rRNA-based pyrosequencing and Sanger sequencing to investigate the effects of cirpoflaxin on the distal gut microbiota of three healthy individuals. This group demonstrated that ciprofloxacin treatment decreased overall taxonomic diversity, richness and balance by altering the abundance of approximately 30% of gut bacterial taxa. Although most taxa returned to normal abundance 6-months post treatment, several taxa failed to return to pre-treatment levels during the course of follow-up [120]. A study carried out among 629 children with respiratory tract infections prior to and following antimicrobial therapy showed that the effects of treatment on the nasopharyngeal microbiota differed based on the class of antibiotic administered. β-lactam and macrolide antimicrobials exerted selective pressure against sensitive strains, increasing the resistant populations [117].

1.4. Sampling and Analysis

Although molecular tools avert considerable bias introduced by selective cultivation and have revolutionized our understanding of microbial ecology. PCR-based

applications have several pitfalls which can occur at every stage of sample processing [121]. Bias can be introduced during sample collection and transport, sample storage, cell lysis, nucleic acid extraction, PCR amplification, and other downstream applications [122, 123]. One of the advantages of molecular applications is that samples can be stored and analyzed even after the loss of viability of microbial cells. This is of great importance in regions where real time analysis, cultivation and proper storage of microbial cells are unachievable. PCR reactions are reliant upon the specific annealing of selected primer pairs to the targeted microbial gene sequence. There are several potential pitfalls and sources of bias at every step of a PCR reaction that may produce artefacts and inaccuracies in the final amplicons produced. Consequently, molecular techniques that are reliant upon PCR are invariably affected by the quality of the nucleic acid amplification [121]. The steps of a typical PCR reaction and the associated pitfalls are outlined in Table 1.2. The solutions suggested only reduce the presence of contaminants and artefacts.

Table 1.2. Drawbacks in PCR-based investigations of complex microbial communities [121]

PCR Step	Problem	Solution
Sample Collection	• Contamination of sample • Delayed processing and inappropriate storage of organisms (e.g. anaerobes and highly fastidious species) • Presence of excess nucleases	• Appropriate use of sterile technique • Prompt processing of samples and storage in appropriate conditions • Use of nuclease inhibitors
DNA Extraction	Insufficient and preferential disruption of cells • Over processing and fragmentation of nucleic acid	• Use of peptidoglycan cell wall disruptors (e.g. lysozyme, lysostaphin and proteinase K). • Avoiding excessive chemical and mechanical disruption of cell
Amplification	• Inhibition of PCR by contaminants by impurities and proteins • Differential PCR amplification • Formation of chimeric sequences • Introduction of nucleic acid deletions, substitutions and insertions • Non-specific spurious amplification	• Sufficient purification of template nucleic • Use of broad range but gene specific primers or probes • Use of high fidelity polymerase • Reduce the number of PCR cycles
Storage	• Nuclease degradation of nucleic acids	• Storage of DNA in appropriate conditions *e.g.* -70^{o}C

1.4.1. Metagenomic Applications

Metagenomics refers to the collective culture-independent functional and/or sequence-based study of microbial genomes (termed the metagenome) contained in animal hosts, plants and environmental niches [124]. This includes shotgun-sequencing

of microbial genomes or the sequencing of rRNA genes from heterogeneous microbial communities. The initial expansion in culture-independent applications was facilitated by developments in nucleic acid amplification methods, molecular cloning, Sanger DNA-sequencing and gene expression technologies. Most recently, major advancements in next-generation DNA-sequencing tools with the capacity to handle mega meta-data sets previously unimaginable and increased appreciation of the importance of complex microbial communities in health and disease have brought culture-independent approaches to the forefront of molecular microbiology and microbial ecology [3]. Various culture-independent applications reviewed here are outlined in Figure 1.2.

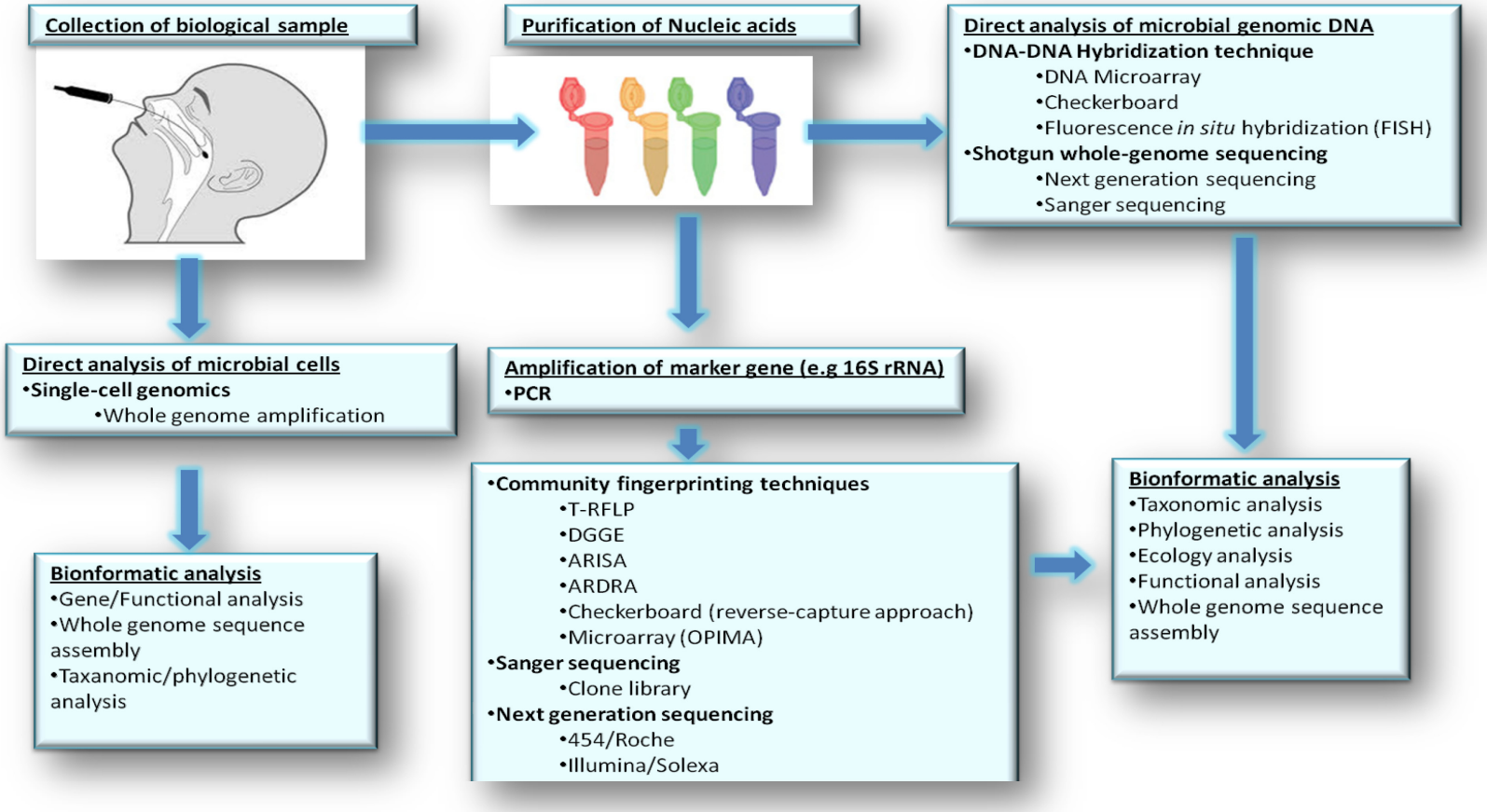


Figure 1.2. Workflow for culture-independent analyses highlighting some of the most commonly used applications

1.4.2. Small subunit ribosomal RNA (SS rRNA) genes

The usefulness of a marker gene used in the identification and classification of organisms depends on the extent to which it is conserved and constrained in function [121]. Ideally, a marker gene should have conserved and variable regions. Among prokaryotes, one candidate marker gene is the *rpo*B gene encoding the β-subunit of RNA polymerase (RNAP). RNAP is essential in DNA transcription and regulation of gene expression in all living organisms and synthesizes mRNA, rRNA and tRNA in bacteria [125]. This gene has been used in the phylogenetic analysis of *Mycobacterium sp., Staphylococcus sp.* and *Enterobacteriacea* amongst other studies [126, 127]. Other candidate phylogenetic markers are the superoxide dismutase A (*soda*) gene [128, 129] and *rec*A gene which encodes a DNA-dependent ATPase that binds to single-stranded DNA [130]. However, a major limitation of these phylogenetic markers in culture-independent applications is the lack of extensive gene-specific reference databases against which sequences obtained from mixed communities can be classified using the Basic Local Alignment Search Tool (BLAST).

At present, the most important and extensively used phylogenetic markers are the ribosomal RNA genes. Ribosomes are composed of protein and RNA moieties, which are 16S (~1600 bp), 23S (~3000 bp) and 5S (~120 bp) rRNA. Ribosomal genes are ubiquitously distributed and evolve gradually [131, 132]. These evolutionarily and functionally homologous RNAs are essential components of the protein-synthesis machinery in all organisms. The 16S rRNA gene contains conserved regions flanking hypervariable regions. There are a total of nine hypervariable regions, between 50 bp and 100 bp. There are numerous broad range oligonucleotides that target the

conserved regions of the 16S rRNA gene flanking one or more hypervariable regions and facilitate identification down to species level (Figure 1.3) [3, 133] .

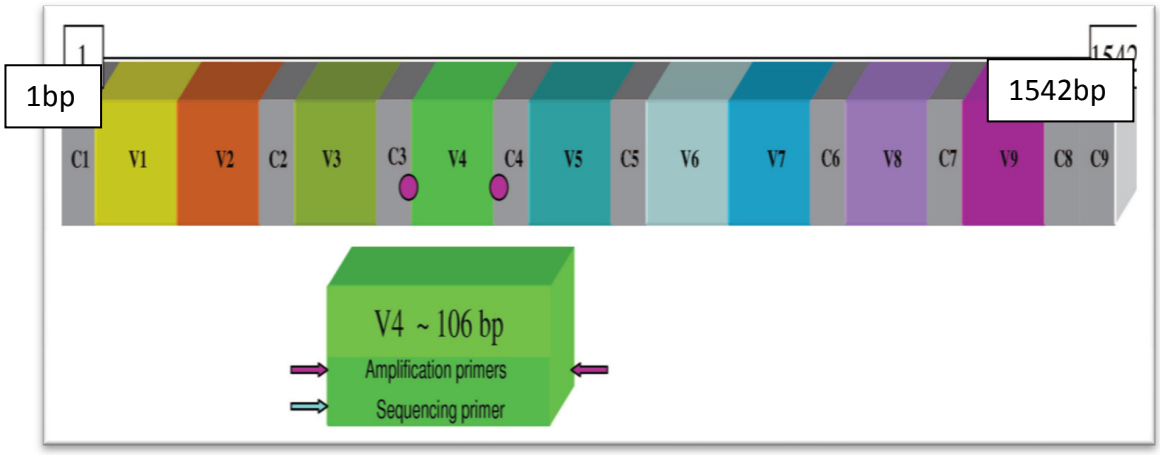


Figure 1.3. Conserved and hypervariable regions in the 16S rRNA gene. Shown in gray are the interspersed conserved regions (C1–C9) flanking the hypervariable regions (V1–V9). Oligonucleotides for DNA amplification can be designed to target the conserved regions flanking one or more hypervariable regions. The pink circles and arrows represent potential primer-binding sites amplifying the V4 region. Adapted from [3].

It is important to note that there are currently no truly 'universal' oligonucleotides; however, there are broad range oligonucleotides which target 16S rRNA genes from a wide spectrum of microbes, with different specificities for bacteria or archaea [131]. Even though the 16S rRNA gene has conserved regions, the absolutely conserved stretches are between one and four base pairs long across the gene[134]. Hence, the region of the 16S rRNA gene applied may result in variable representation of taxonomic groups [3]. A drawback of 16S rRNA gene-based analysis is that gene copy numbers can vary by an order of magnitude between different taxonomic groups which can bias estimates of community composition.

Carl Woese *et. al*. showed that 16S rRNA gene-based studies provided the initial comprehensive phylogeny of prokaryotes establishing that there are two distinct prokaryotic lines of decent, 'bacteria' and 'archaea' [135, 136]. In recent years, the acquisition of 16S rRNA nucleotide signatures from complex microbial communities has shown that more than 80% of gut bacteria and 99% of bacteria from some environments are uncultured or recalcitrant to standard laboratory cultivation [137, 138]. This phenomenon explained the "great plate count anomaly" [139], which refers to discrepancies between bacterial population estimates based on microscopy and culture plate counts. The depth with which we can analyse complex microbial communities depends on the resolution and selectivity of the technique applied. For instance, in the gut, *Enterobacteriaceae* such as *E. coli* were previously thought to be the dominant gut bacteria. However, these microbes account for less than 1% of the gut microbiota, but their abundance was overestimated because they are readily cultivated and detectable at low abundance [90].

PCR amplification of 16S rDNA directly from a sample or after reverse transcription of rRNA is the primary method of obtaining rRNA sequence data from mixed microbial communities. It is astounding that two decades ago, when 16S rRNA gene-based phylogeny was first proposed, there were twelve recognized bacterial phyla [136]. Currently, there are fifty-two phyla, most of which have few or no cultured members, and as such, 90% of cultured bacteria are clustered within four phyla [140]. Typically, >95% pairwise sequence identity distinguishes genera, while >97% distinguishes species based on the 16S rRNA gene. The power of 16S rRNA-based analysis is largely attributed to mega reference databases for 16S rRNA gene-based analysis of which the largest are the Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu/index.jsp)

which uses Bergey's taxonomy [141], Greengenes (http://greengenes.lbl.gov) which offers a wide array of taxonomic schemes [142], and ARB-SILVA (http://www.arb-silva.de/) which also offers several taxonomic schemes [143]. 16S rRNA gene sequences can be deposited and accessed on GENBANK (http://www.ncbi.nlm.nih.gov/genbank/) and there are also specialised reference databases such as the Human Oral Microbiome (HOMD) database (www.homd.org). RDP-II has seen major expansion since its inception just over a decade ago. Release 8.1 in 2003 had 16 300 prokaryotic sequences, and by release 10.3 in 2008 RDP maintained 677 000 sequences, of which 95% were bacterial and 5% were archaeal small subunit rRNA sequences. By release 10.24 in January 2011, the number of sequences maintained had doubled to 1.5 million rRNA sequences, of which 96% were bacterial [144, 145].

1.4.3. PCR-based Community Profiling

Fingerprinting approaches facilitate the high throughput study of the diversity and dynamics of microbial communities as well as the correlates of the microbiota with health and disease. Most fingerprinting techniques have been used extensively in environmental microbiology studies and more recently to study the human microbiome. Community fingerprinting has the capacity to produce both taxonomic and phylogenetic data from a large number of samples fairly quickly, with comparatively low resource demands [90]. Several community fingerprinting techniques have been developed such as Temperature gradient gel electrophoresis (TGGE), amplified ribosomal intergenic spacer analysis (ARISA) denaturing gradient gel electrophoresis (DGGE), amplified rDNA restriction analysis (ARDRA), denaturing high-performance liquid chromatography (DHPLC), Ibis T5000 and terminal restriction

fragment length polymorphism (T-RFLP) amongst many others. However, of these, T-RFLP and DGGE are the most extensively used [146]. Although each community fingerprinting approach is distinct, they all exploit the biochemical properties of nucleic acids and are often used in combination with DNA sequencing.

DHPLC, TGGE and DGGE separate amplified DNA fragments of marker genes such as 16S rRNA on a linear gradient of denaturants and/or temperature [146, 147]. Fragment sequences with different G+C content have different degrees of melting and thus different migration patterns in the gel or matrix used. In DGGE and TGGE fragments of interest can be isolated and sequenced to obtain taxonomic and phylogenetic data. Of these tools, DGGE has been used most extensively in investigations of the saliva, vaginal, oral and gut microbiome [148, 149]. DGGE produces community profiles that reflect the structure, complexity and relative abundance of taxonomic groups present in a community fairly rapidly; however, this tool has poor reproducibility between assays. In addition, long fragments (>500 bp) may not be resolved on the gel which can result in underestimation of diversity. Furthermore, the use of degenerate oligonucleotides which increases the spectrum may produce multiple bands for one taxonomic group and thus, overestimate diversity. Perhaps the most important limitation of DGGE is that low abundance taxa may not be detectable on the gel, even with the most sensitive staining techniques which poses a challenge for the analysis of very complex microbial communities [147].

T-RFLP is a cost effective, reproducible, throughput, comparative community profiling tool, ideal for ecology and phylogenetic investigations of complex microbial communities. This technique exploits the polymorphisms in marker genes which give

rise to variable restriction sites across different taxonomic groups (e.g. species, genera – phyla) [150, 151]. Variations in microbial diversity and abundance result in distinct 'fingerprints' or profiles for each community [152]. In T-RFLP, marker genes from mixed microbial communities are amplified with fluorescently labelled oligonucleotides (5'end, 3'end or both). Amplicons are digested with one or more endonucleases and the resultant labelled terminal restriction fragments (T-RFs) are separated and sized by electrophoresis. Automated capillary electrophoresis has greatly enhanced the accuracy, throughput and utility of T-RFLP. Numerous fluorescent labels are available which makes pooling samples and multiplex T-RFLP possible. Singh *et. al.* developed a bacterial, fungal and archaeal multiplex assay using the 16S and 18S rRNA genes which has been applied in soil culture-independents investigations [153, 154].

Putative taxonomic assignments of the T-RFs can be done by analysing them in T-RFLP databases of known organisms for the marker gene used; this can be done on Microbial Community Analysis III (MiCA 3) accessible on (http://mica.ibest.uidaho.edu/trflp.php). Clone library DNA sequencing can be conducted in parallel to the T-RFLP analysis. Cloned sequences are identified by BLAST analysis then digested in silico to predicted the T-RFs and match them with the T-RFs found in the corresponding microbial community [155]. Several open source 16S rRNA gene T-RFLP analysis packages are available; T-REX [156], TRiFLE [157] and TAP T-RFLP available on RDP (http://wdcm.nig.ac.jp/RDP/trflp/). In addition several DNA sequence in silico digest tools are available (http://molbiol-tools.ca/Restriction_endonuclease.htm).

Since its development in 1997, T-RFLP has been used extensively to characterize the associations between microbial ecology and the aetiology of diseases such as bacterial vaginosis [96, 158], Crohn's disease [159, 160], ulcerative colitis [150, 161], periodontal disease [162, 163], atopic eczema [11] and gastric cancer [164]. T-RFLP has also been used to profile differences in the microbiota associated with antibiotic treatment [165, 166], different GIT diseases [159], different mucosal surfaces [167], diet [168-170], age [72, 171] and surgical treatment [172, 173].

The inability to directly generate sequence data makes it difficult to identify the microbes represented by the T-RFs in a sample. T-RFs of the same length can represent members of different taxonomic groups and equally members of the same taxonomic group can have different T-RF lengths. This not only makes identification of microbes difficult, but also makes estimation of diversity in a community problematic. Although parallel clone library analysis may improve identification of microbes, low abundance taxa are difficult to identify [147]. Recently, 16S rRNA gene 454-pyrosequencing has also been used in conjunction with T-RFLP to investigate the effects of metronidazole and clarithromycin treatment on the throat and gut microbiota [165]. Single-stranded amplicons may be formed which give rise to pseudo-terminal restriction fragments and overestimation of diversity. None the less, T-RFLP is an effective comparative profiling tool useful for tracking changes in the microbiota across space and time.

1.4.4. DNA-DNA Hybridization Applications

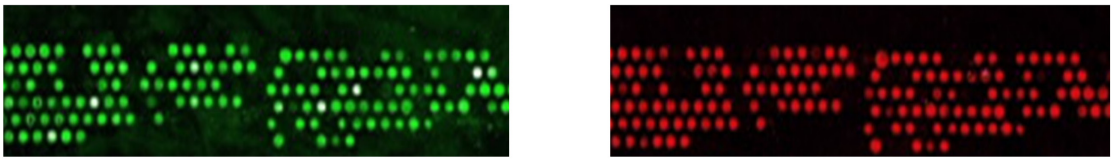
DNA-DNA re-association kinetics provides a platform for estimating genomic relationships between microorganisms. There are several techniques which utilize DNA-DNA hybridization to determine diversity in microbial communities, these include

Fluorescent *in situ* hybridization (FISH), DNA microarray and checkerboard approaches. FISH has been applied in studies of the gut microbiome and its associations with disease [174, 175] while Checkerboard approaches have been applied extensively in studies of periodontal disease [176, 177]. The major limitation of these techniques is that they are generally limited to the identification of genes or organisms from defined taxa for which probes or oligonucleotides are available.

There are several DNA microarray platforms which provide high throughput platforms for phylogenetic and functional analysis [90]. Phylogenetic oligonucleotide arrays are imprinted with signature sequences of specific organisms or alternately a library of sequences from a microbial community. Signature sequences are usually 16S rRNA genes of prokaryotes. Palmer *et. al*. employed a 16S rRNA oligonucleotide microarray to investigate the development of the human intestinal microbiota in infants [178].

I worked on the development of an operational interrogation of microbiome array (OPIMA), which should facilitate the classification of cloned fragments into taxonomic groups. Amplicons derived from cloned 16S rDNA fragments were arrayed as probes then interrogated with tester oligos with various (phylum, family, genus or species) levels of specificity. Up to 36,000 cloned fragments could be accommodated by printing multiple arrays. In a pilot run of this system, 2800 16S rRNA amplicons from 4 induced sputum filtrates and a plaque sample were printed on glass aldehyde microarray slides. The Genisphere 3DNA Capture System™ was used to label bacterial 16S rRNA gene probes which were subsequently hybridized with the printed amplicon testers (Figure 1.4). Hybridizations with high sensitivity were obtained and this system

appears reproducible and produces analyzable signals which hold great promise for rapid affordable community analysis.



A B

Figure 1.4. Array scans of 16S rDNA testers interrogated with a broad-range 16S rDNA probe 338F labeled with Cy3 (A) and Cy5 (B). Probes were labeled using the 3DNA Capture System (Genisphere, USA).

Although microarray technology is relatively cheap and suitable for large scale community analysis, there are still significant challenges to be resolved. A major limitation of DNA microarray technology is that only DNA sequences printed on the chip can be detected, hence previously uncharacterized microbes or microbes with divergent sequences to that on the chip can be missed. The human microbiome is composed of highly heterogeneous groups of organisms with complex genomic relationships. Cross-hybridization is inevitable and the extent to which this factor may skew microarray specificity, sensitivity and quantification is unknown. Furthermore, detailed sequence and taxonomic information offered by DNA sequencing technologies is unparalleled by microarray analysis.

Further studies need to be carried out in order for the full potential of this high throughput tool in culture-independent studies to be realized.

1.4.5. Sanger sequencing and cloning

At the forefront of DNA sequencing technologies was Fred Sanger who 30 years ago developed di-deoxy chain termination sequencing [179, 180] and placed genomics at

the forefront of biology. Sanger sequencing relies upon DNA synthesis on a single strand with the random incorporation of chain terminators [181]. Sanger sequencing is often combined with cloning in culture-independent applications[182]. Cloning provides an efficient and robust mechanism of separating and amplifying individual DNA fragments from a mixed library. *In vivo* cloning systems utilize described DNA vectors (plasmids and phage) and suitable hosts (commonly *E. coli* and *Saccharomyces cerevisiae*). There are numerous commercially available systems that have been specifically designed to accommodate different types and sizes of fragments for functional and/or genomic studies. Vectors with large insert capacities are ideal for genome organization studies of uncultured members of the human microbiome. A large culture-independent study demonstrating that obesity is linked to a gut microbiota with the capacity for increased energy harvest from food [1, 30] sequenced 5088 bacterial 16S rRNA genes from clone libraries. In order to study an association between microbial dysbiosis and mucosal inflammation in IBD patients, Walker *et. al*. generated a remarkable ten thousand full-length 16S rRNA sequences from 29 biopsy samples. Partial sequencing of the 16S rRNA gene from clone libraries has large economic and technical demands, which make it impractical to apply in studies of the microbiome with large sample sizes [31].

1.4.6. Next Generation Sequencing - Pyrosequencing

Although culture-independent data is still primarily based on Sanger sequencing methods, parallel, high throughput sequencing technologies are now at the forefront of culture-independent studies. There are several next generation sequencing platforms with quite different chemistries [3]. The leading platforms are Illumina/Solexa, Applied Biosystems (SOLiD) and Roche/454 Life Sciences

pyrosequencing amongst many others at different developmental stages [3]. The major advantages of pyrosequencing over Sanger sequencing in culture-independent applications are that cloning is not necessary and that that per base sequencing costs are much lower and continuing to drop. The development of 454 pyrosequencing preceded the other next generation sequencing platforms and has been extensively applied in culture-independent studies. With the current Genome Sequencer FLX System with long-read GS FLX Titanium chemistry read lengths of 400 – 600 bases are achieved. This is unparalleled by other next generation sequencing platforms and a great improvement from the ~100 bp reads of the pioneer GS 20 instrument. Roche reports read lengths approaching 1000 bp with the upcoming GS FLX series chemistry. Current 454-sequencing platforms can generate in excess of 400 Mb of sequence data per run.

454 pyrosequencing can be conducted directly on genomic DNA fragments (300 – 800 bp) or PCR products. A library of single stranded DNA fragments or PCR products with adapter sequences on both the 5'-end and 3'-end is generated. Each fragment is attached to a capture-bead and emulsified with PCR reagents in an oil-water mixture. Amplicons attached to the capture beads are sequenced by synthesis in a PicoTiterPlate device. Nucleotides are flowed through the PicoTiterPlate device in sequence, and when a complementary nucleotide is flowed into a well the polymerase adds the nucleotide in a reaction which generates chemiluminescent signal which is recorded by the CCD camera in the instrument (Figure 1.5).

The strength of the signal generated is directly proportional to the number of nucleotide incorporated at each nucleotide flow. The workflow of 454 pyrosequencing

is shown below. The 454 pyrosequencing sample preparation and post sequencing analysis are discussed in more detail in section 2.0. 454 pyrosequencing has huge computational power and demands high through put efficient bioinformatics pipelines for the verification, trimming, aligning, binning, and identification of microbial taxa from mixed communities. Phylogenetic classification can be carried out on RDP II, Greengenes and ARB-Silva. An important advancement in pyrosequencing is multiplexing made possible by barcoding of DNA fragments of PCR products, hence, several-fold more sequences can be generated per pyrosequencing run.

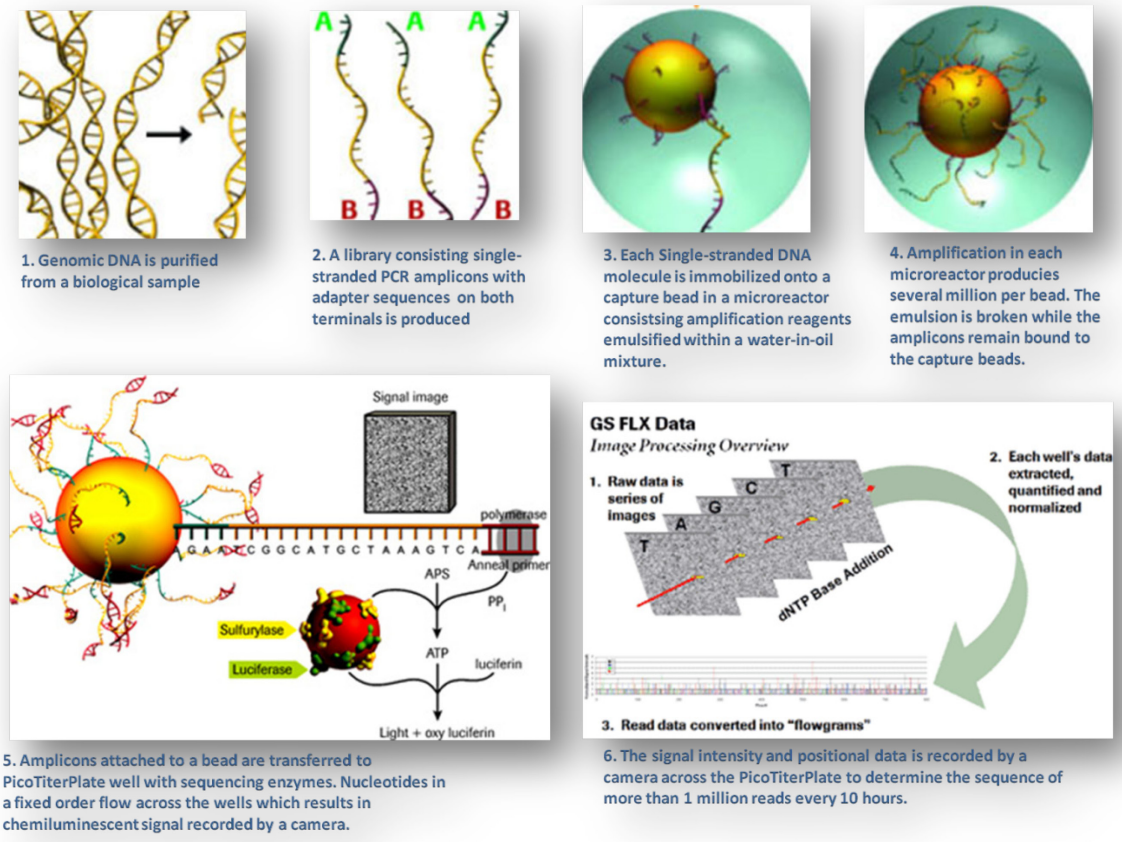


Figure 1.5. 454/Roche Pyrosequencing (One Fragment = One Bead = One Read) workflow for PCR products. Adapted from (http://454.com/products-solutions/how-it-works/index.asp)

There are over 300 peer-reviewed culture-independent and microbial diversity publications which utilized 454 pyrosequencing technology accessible on (http://www.ncbi.nlm.nih.gov/pubmed/). Some of the most recent applications in human microbiome studies include investigations of the vaginal microbiome in HIV-seropositive and –seronegative women [183, 184], chronic wound microbiota [185, 186], energy harvest of gut microbiota and its association with obesity [13, 14], differences in saliva microbiota across space and time [187], viruses in faecal microbiota [188], the pharyngeal microbiota in cigarette smokers and non-smokers [189] and the association between mode of delivery and early development of the gut microbiome [190]. 454-sequencing has also been applied in studies of associations between microbial ecology and the aetiology of various diseases [26, 191, 192].

The use of high fidelity DNA polymerase (high accuracy, proofreading and thermostability) is critical in PCR amplification prior to 454 pyrosequencing analysis. The error-rates of pyrosequencing are less than 0.1%, comparable to Sanger sequencing. Errors can be further reduced by removing any reads with ambiguous sequences, reads with mismatches in the primer sequences and short read lengths (e.g. <300 bp with the GS FLX system) [3]. A study of bacterial diversity within the subgingival crevice showed that culture-independent techniques can produce more than double the resolution of cultivation-based techniques.

1.4.7. New Frontiers

1.4.7.1. Whole-Genome Sequencing (WGS) and Single-Cell Genomics

Genomes of strains classified in the same species by 16S rRNA gene analysis (>97% sequence similarity) can have high heterogeneity and plasticity amongst different strains.

Substantial variations in the genomes of the same species have been demonstrated for *E. coli [193], Helicobacter pylori [194]* and *Haemophilus influenzae [195]*. For instance, 20% of the genomes of six *Streptococcus agalactiae* strains analysed were either partially shared or strain specific genes [196]. Variations in the "pan genome" (core genome present in all strains and genes found in one or a subset of strains of the same species) is not captured by 16S rRNA gene-based community analyses. Comprehensive characterization of the "pan-genome" (microbial evolution and population structure) has significant implications in the design of vaccines and treatment strategies against pathogens, particularly those that are components of microbiota.

Variations in genomes not represented by marker genes and problems of primer bias, oligonucleotides binding site polymorphisms, and low depth of sampling associated with marker gene analyses may be overcome by whole genome sequencing of the members of a mixed microbial community. Sequencing of microbial genomes is a tool that not only provides comprehensive taxonomic and phylogenetic information about a microbe, but also to its functional (biochemical and metabolic) properties within a mixed community [3]. WGS sequencing can be conducted using Sanger sequencing (clone library) and next generation sequencing approaches. A major limitation of WGS sequencing is the requirement for large amounts of in-put Genomic DNA. Whole genome amplification (WGA) can be used to circumvent this limitation. WGA based on ph29 polymerase-mediated multiple displacement amplification has been developed to generate billion-fold amplification of DNA from femtogram amounts, even from a single microbial cell [197]. Contamination with host genetic material is an important consideration in WGS. Annotation of genomes of taxonomic groups without previously

sequenced genomes is a major challenge and large amounts of nucleic acids are required for WGS [3]. Although still under development, WGA and single-cell genomics hold great promise for investigations of low abundance and uncultured commensal and pathogenic components of the human microbiome.

1.5. The pneumococcal Conjugate Vaccine

Conjugate vaccines against selected clinically significant microbes have been applied successfully in different parts of the world [198]. Specifically, the seven-valent pneumococcal polysaccharide-diptheria CRM_{197} protein conjugate vaccine (PCV-7) elicits mucosal immunity, possibly due to the induction of opsonizing IgA antibodies [199]; markedly reducing the carriage of vaccine pneumococcal serotypes, subsequently decreasing the incidence of vaccine serotype invasive disease and antimicrobial resistance PCV-7 is a remarkable public health success story [200]. However, PCV-7 only protects against seven of the 94 known serotypes of *S. pneumoniae* which are frequently involved in paediatric infections (serotype 4, 6B, 14, 18C, 19F and 23F). Hence, the long-term effectiveness of PCV-7 depends not only on a reduction in the incidence of vaccine serotype disease but also the emergence of serotype or species replacement disease.

The issue of serotype and disease replacement disease is of particular interest within the Gambian context where infant pneumococcal nasopharyngeal carriage rates can exceed 90% and PCV-7 vaccine coverage is 29% (57% including vaccine-related serotypes) [201]. Several bacterial species, including pathogens such as *H. influenzae, M. cattarrhalis, N. meningiditis* and *S. aureus* are commensals of the nasopharynx, sharing a niche with *S. pneumoniae*. Development of infections caused by all these

pathogens can often be linked with preceding pharyngeal colonization which is in turn believed to be an important source of horizontal spread within a community.

In order to understand the full ecological impact of PCV-7 vaccination which may influence replacement disease; it is necessary to carry out in depth unbiased analysis of the microbial components which inhabit the nasopharynx. Hence, 16S rRNA-based culture-independent approaches will be applied in the characterization of the infant nasopharyngeal microbiome, which, to my knowledge has never been done at the depth embarked here. New important perspectives on the acquisition, development, magnitude and composition of the nasopharyngeal microbiome will be elucidated which have important implications for pathogenicity and polymicrobial disease

1.6. Aims and Objectives

The primary aim of this study was to characterize the impact of PCV-7 vaccination on the development and composition of the infant nasopharyngeal microbiome using culture-independent approaches, specifically 454-deep pyrosequencing. This study sets out to begin to address important questions of scientific and public health concern surrounding the long-term effectiveness of PCV-7:

1. Does PCV-7 vaccination significantly alter the composition and development of the infant nasopharyngeal microbiome?
2. Is the niche left vacant by the elimination of PCV-7 serotypes replenished by non-Streptococcal species?
3. Does PCV-7 alter microbial interactions within the infant nasopharynx.

However, the set out goals necessitated the basal characterization of the microbiome during infancy, which was the secondary aim of this study.

1. Characterize the development and composition of the nasopharyngeal microbiome from birth to one year among Gambian infants not exposed to PCV-7 using molecular fingerprinting approaches.
2. Determine the carriage of *S. pneumoniae* and three other respiratory pathogens from birth to one year among PCV-naïve infants.
3. Investigate the effects of frozen storage of nasopharyngeal specimens on microbial communities.

The long-term effectiveness of vaccine strategies that prevent mucosal colonization could be challenged by the demonstration of an association between PCV-7 usage and an increase in the carriage of bacteria within the nasopharynx. Such a finding could be additional support for the development of disease-related protein-based vaccines which do not alter colonization but prevent invasive disease. In a country where national disease surveillance is inadequate, findings from this study could give Gambian public health bodies, healthcare providers and the government useful insights into pathogens that could be of concern in the future. This study will provide new insights into the effects of restricted-valency polysaccharide vaccination on the infant nasopharyngeal microbiome, which may or may not contraindicate the long-term efficacy of this vaccination strategy.

2. Materials and Methods

2.1. Study Population

Villages in the Western Division, The Gambia covering an area of approximately 90km^2 were selected for this study (see Figure 2.1). The western Division is located in the middle of the Gambia and representative of rural areas in the region [202].

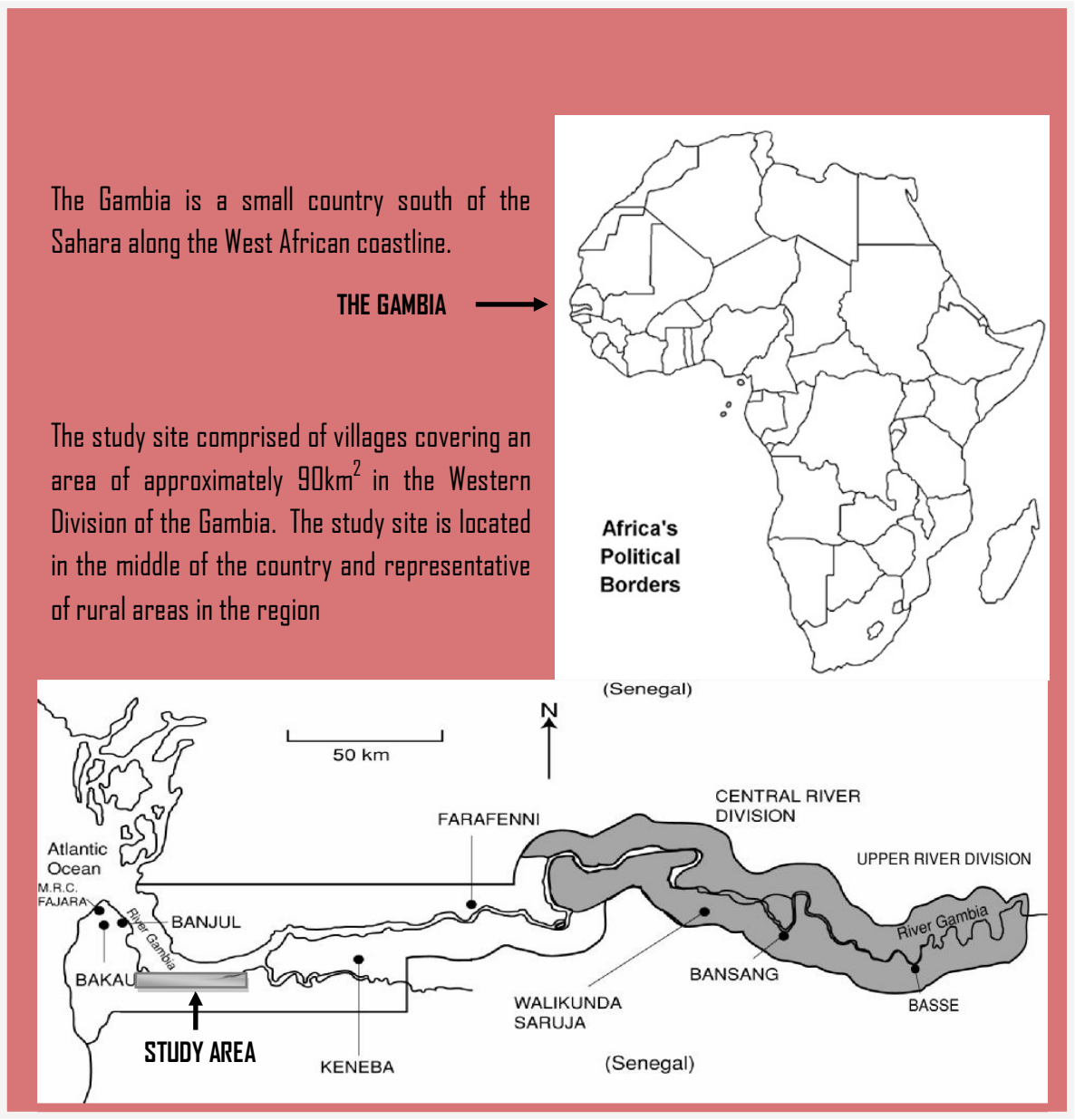


Figure 2.1 Map of Africa and the Gambia, highlighting the study area

HIV prevalence is 2% and the majority of villagers belong to the Mandinka, Jola and Fula ethnic groups although there are several other ethnic groups [202]. Most of the villagers are subsistence farmers growing maize, millet and groundnuts. There are approximately 55 villages in the Western Division see Figure 2.2. Study participants were recruited from villages with 100 to 700 persons and at least 3km from the major highway transecting the Division.

2.1.1. The Prospective Study: Effect of Vaccination

For the prospective study, subjects were recruited from 27 villages with estimated birth rates between three and twenty-six per year. The villages were split into 3 groups of 9 villages with estimated population sizes of 2000 (see Figure 2.3) and birth rates of approximately eighty per year. Group I and II villages had to be at least 1km from Group III villages where PCV-7 had been trialed. Trained village reporters in each village recorded and reported pregnancies, births, deaths and other serious events to the field team. Recruitment of subjects was carried out between November 2008 and April 2009. To avert recruiting bias, participant were enrolled on a roll-in basis, whereby infants born in any of the participating villages and granted parental consent were included in the study. Subject recruitment continued until all the groups had at least the targeted thirty infants. This resulted in over recruitment in Group I (33 infants) and group III (39 infants). Group I did not receive PCV-7 doses and were born in PCV-7 naïve villages. PCV-7 vaccine trials were conducted in Group III villages prior to the prospective study; hence a large proportion of the children and adults had received at least one dose of the vaccine [201, 203]. Group II and III infants received three doses of PCV-7 at 2, 3 and 4 months see Figure 2.4. NP swabs were collected within seven days of birth, then biweekly for the first six months and subsequently

bimonthly up to one year (n=17), see Figure 2.4. All infants received Expanded Programme on Immunization (EPI) vaccines according to the national schedule.

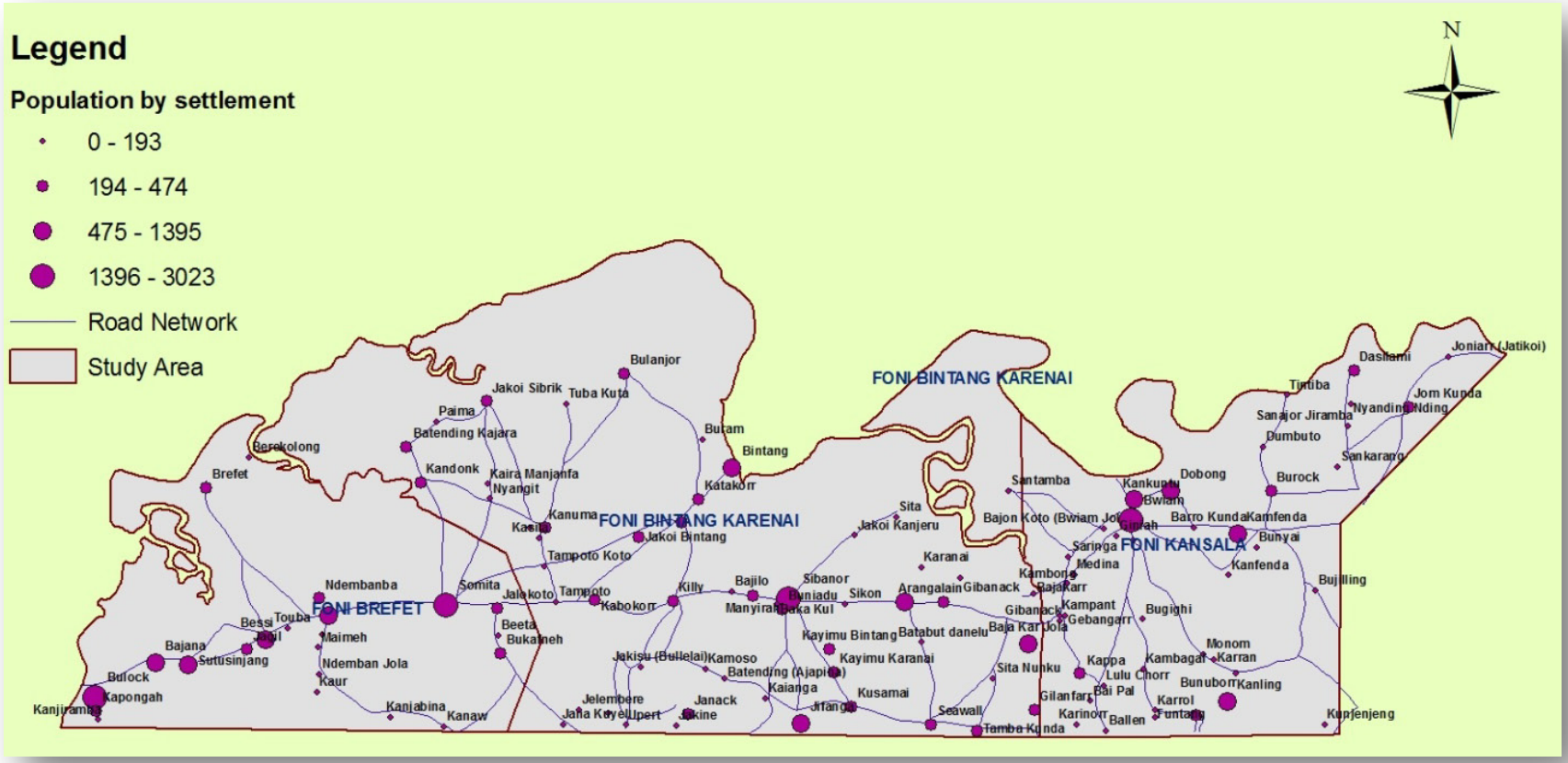


Figure 2.2 Map of the villages in the Western Division of the Gambia showing population size

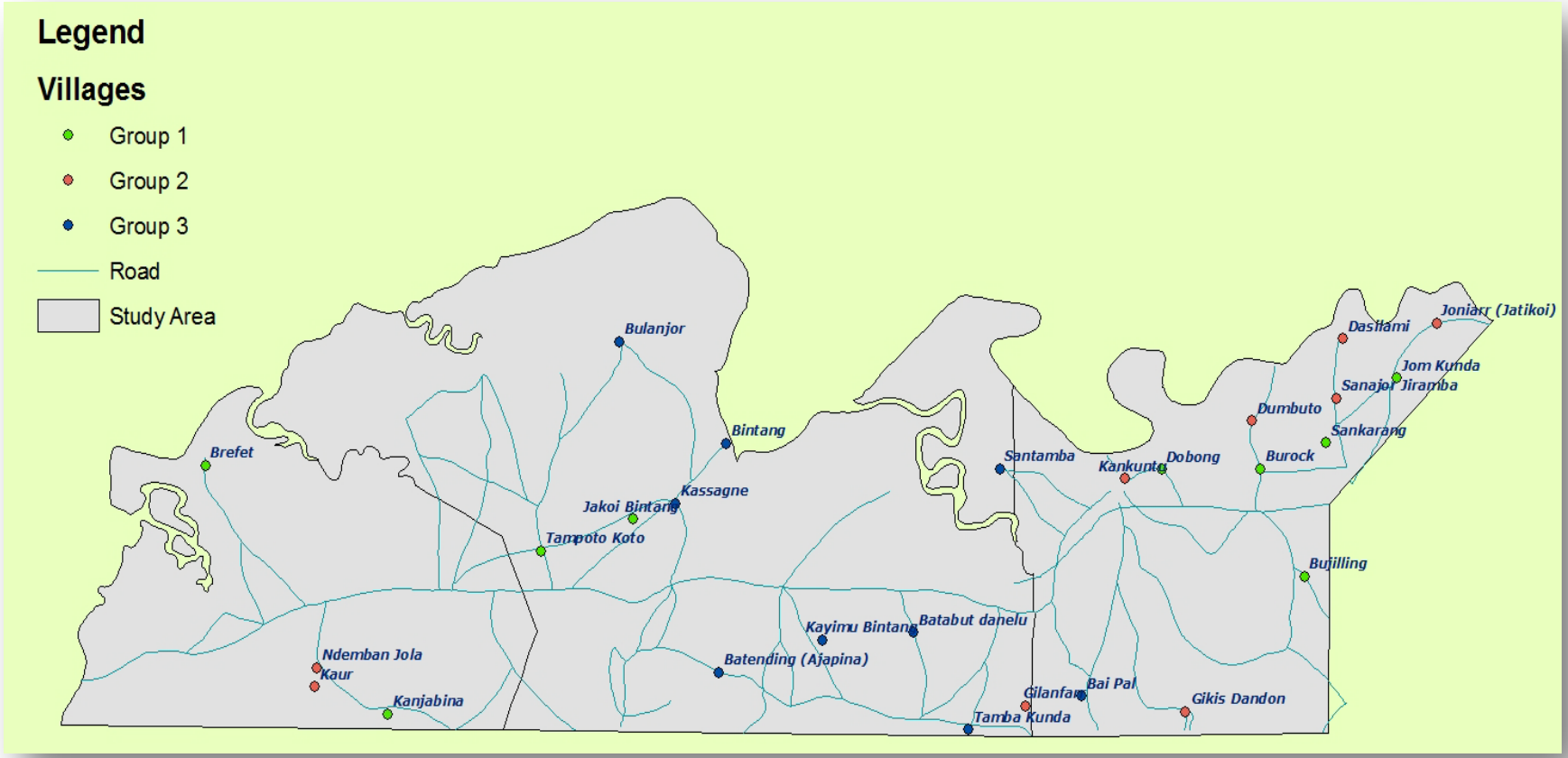
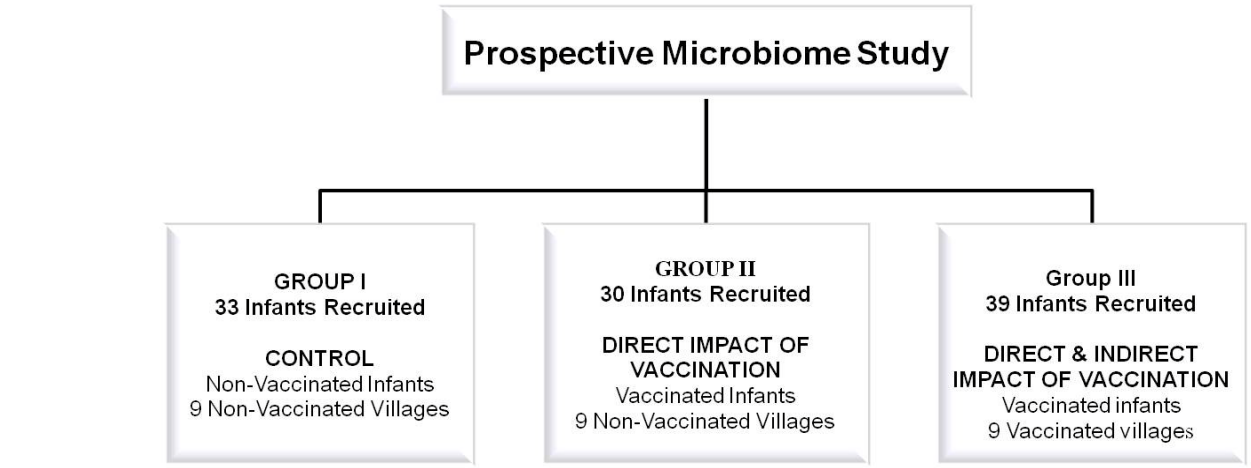


Figure 2.3 Map of the 27 villages included in the prospective study showing the study groups.



	Group I	Group II	Group III
SAMPLE COLLECTION SCHEDULE			
NPS Sample A	<7 days after birth	<7 days after birth	<7 days after birth
NPS Samples B to N (First 6 months)	Bi-weekly (13 times)	Bi-weekly (13 times)	Bi-weekly (13 times)
NPS Samples O to Q (Last 6months)	Bi-monthly (3 times)	Bi-monthly (3 times)	Bi-monthly (3 times)
VACCINATION SCHEDULE			
PCV7 Dose 1	NONE	8 Weeks (sample E)	8 Weeks (sample E)
PCV7 Dose 2	NONE	12 Weeks (sample G)	12 Weeks (sample G)
PCV7 Dose 3	NONE	16 Weeks (sample I)	16 Weeks (sample I)

Figure 2.4 Summary of the prospective longitudinal study design investigating the impact of PCV-7 vaccination on the nasopharyngeal microbiome.

102 infants were recruited, 52 male and 50 female and follow-up was completed in April of 2010. 3 infants died of post-natal complications, prior to PCV-7 vaccination. 2 infants dropped out of the study, 1 was lost to follow-up and 96 (94%) completed the study. If the scheduled 17 NP swabs had been collected from all the infants, 1734 samples would have been collected, however, 1595 (92%) were collected.

2.1.2. The Retrospective Study: Development of the Microbiome

For the retrospective phase of the study, thirty of 236 infants who participated in a longitudinal investigation of *S. pneumoniae* carriage between 2004 and 2006 were selected [201]. The infants were from thirteen villages in the Western Division and sample collection was conducted prior to the pneumococcal conjugate vaccine trials and the subsequent introduction of PCV-7 in August of 2009. The first NP swabs were collected within six days of birth, (avg. = 3 days) and follow up collections were carried out approximately bi-weekly for the first 6 months and then bi-monthly for another 6 months.

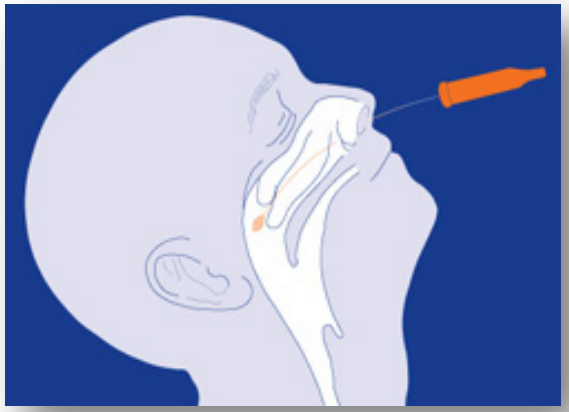
A total of 499 NP swabs were collected from the thirty infants throughout the one year follow up. The average birth weight was 3Kg (range 2.1 - 4Kg). [201]. 37% (11/30) were female and 63% (19/30) were male. All the infants in this study were breastfed; the infants were exclusively breastfed for a minimum of 57 days and a maximum of 201 days. Mixed feeding which encompassed other foods and water were introduced at variable time periods when the infants were between 2 months and 6 months of age. NP swabs were collected soon after birth, then biweekly for the first six months and bimonthly up to one year. The expected number of NP swabs per infant was 17 however; the average number of NP swaps obtained was 16.6 /infant (range16 -17). Each subject had the first NP swabs collected within a week of birth, and had the last swab collected within two weeks of the first birthday. The NP swabs were collected bi-weekly for the first 6 months and then bi-monthly for another 6 months.

Approval to conduct this study was sought from the MRC, The UK, The Gambia Scientific Coordinating Committee and Joint MRC & Gambian Government Ethics Committee.

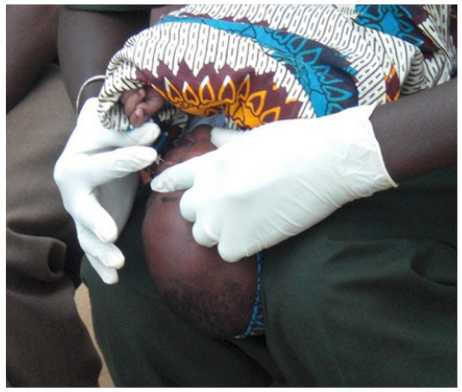
Sensitization (explanation of the study goals) was conducted and permission from village heads was sought prior to the commencement of the study. At the time of sampling, risk factor information was collected through interviews conducted by trained field workers (see appendix).

2.1. Nasopharyngeal Sampling Process

Nasopharyngeal swabs were collected using sterile calcium alginate fibre tipped swabs with aluminium shafts (Fisher Brand ®, USA). All NP swabs were collected by trained field nurses following an outlined protocol using sterile technique. The head of the subject was tilted slightly backward and the NP swab was passed directly backwards, parallel to the floor of the nasopharynx. It was insured that the NP swab passed without resistance until reaching the posterior pharynx which is about half the distance from the nostril to the ear lobe, see Figure 2.5. If resistance was encountered, the swab would be discarded and a new swab was passed from the other nostril. Once the swab was in the nasopharynx, it was left in position for five seconds to saturate the tip before removing it slowly, again avoiding resistance. The NP swab was immediately inoculated into a vial containing 1mL of chilled Skim milk–tryptone–glucose-glycerol (STGG) transport medium. The vials were kept on ice, transported to the MRC Laboratories site in Fajara and stored at -70^{o}C within eight hours of collection.



Trained field nurse prepares baby B, removes any excess mucus from nose and calms baby.



Nurse collects baby B's NPS sample by carefully inserting the swab in the posterior nasopharynx, waiting 5 seconds and then rotating it 360 degrees before careful removal and immediate placement in STGG

Field worker collects meta data from baby B's mother (or guardian). The baby is also weighed and her height is measured.

Figure 2.5 Nasopharyngeal swabs collection. The head of the infant is tilted slightly backward and the NP swab passed directly backwards, parallel to the floor of the nasopharynx until reaching the posterior pharynx which is about half the distance from the nostril to the ear lobe.

2.2. Culture detection of *S. pneumoniae*

S. pneumoniae was isolated by sub-culturing NP swabs onto gentamicin (5µg/mL) sheep blood agar overnight at 37^{o}C at 5% CO_2. Pneumococci were identified by morphological characteristics and optochin sensitivity and serotyped using the latex agglutination technique [201].

2.3. DNA Extraction

2.3.1. DNA Isolation for T-RFLP Analysis

For DNA extraction, the NP swabs were thawed on ice, vortexed and 100µL collected and centrifuged at *5 000 x g* for 10 minutes. The pellet was resuspended in 20 mg/mL lysozyme in lysis buffer (20mM Tris-Cl, pH 8.0, 2mM sodium EDTA, 1.2% Triton® X-100). DNA was extracted using the DNeasy Blood & Tissue Kit®, (QIAGEN, UK), following manufacturer's Gram Positive protocol. DNA was eluted in 100 µL of elution buffer and stored at -20^{o}C. Extractions were done in batches of 24 including controls which were tested for the presence of nucleic acids by spectrophotometry and 16S rRNA gene amplification.

2.3.2. DNA Isolation for Effect of Frozen-storage Analysis

Twelve NP swabs were collected and duplicates prior to and post freezing were analyzed for each sample, giving a total of 48 samples DNA extractions carried out. 100µL of each NP swab sample were conducted using the UltraClean® Microbial DNA Isolation Kit (Mo-Bio, USA) following manufacturer's protocol. For direct analysis, DNA was extracted from NP swabs kept on ice within 4 hours of collection. For frozen analysis, DNA was extracted from the NP swabs frozen at -70^{o}C for 30 days and gently thawed on ice. DNA was eluted in 50 µL of elution buffer (MD5) and stored at -20^{o}C.

Duplicate extractions were carried out for both fresh and frozen analyses to control for variability associated with the extraction process.

2.3.3. DNA Isolation for 454 Pyrosequencing

1595 NP swabs in STGG were collected from 102 infants and stored at -70^{o}C. NP swabs were gently thawed on ice and gently vortexed for five seconds. DNA was extracted from of each NP swab by transferring 250µL of the swab solution to the PowerBead® tubes and following manufacture's protocol. DNA was eluted in 100µL of the kit elution buffer and immediately stored at -20^{o}C. DNA isolations were carried out in batches of 24 including one extraction control to which 250µL of sterile DNAse free water was added instead of NP swab. DNA isolations were done in accordance with the Human Microbiome Consortium manual of procedure available at (http://www.hmpdacc.org/doc/sops/sampling/manual_of_procedures.pdf)

2.4. PCR Detection of Respiratory Pathogens

PCR reactions were carried out in 25 µL reaction volumes consisting 2.5µL (~10ng measured by nanodrop) of purified genomic DNA, 1x Green GoTaq® reaction buffer with 1.5mM $MgCl_2$, 1.0U Go*Taq* Polymerase (Promega, UK), each deoxynucleoside triphosphate at 0.2mM (dNTP) (QIAGEN, UK). The gene targets and primer information specific for each microbe as described below. PCR controls were purified genomic DNA (20ng/µL) of the following clinical isolates- *S. pneumoniae, S. aureus, H. influenzae* type B, NtHi, *M. catarrhalis, Pseudomonas aeruginosa, Escherichia coli,* Group A streptococcus, Group B streptococcus, *N. meningitidis, Citrobacter freundii, Shigella flexneri* and coagulase negative staphylococcus. Thermal cycling was performed in the Gradient Palm–Cycler™ (Corbett Life Sciences, UK) as follows, 95^{o}C for 2 minutes,

followed by 35 cycles of 95^{o}C for 30 seconds, 60^{o}C for 30 seconds and 72^{o}C for 45 seconds and a final extension of 72^{o}C for 10 minutes.

Detection of *S. pneumoniae* was carried out using primers that target the capsular polysaccharide synthesis gene locus A, (*cpsA),*cpsAf:5'-GCAGTACAGCAGTTTGTTGGACTGACC-3' and cpsAr:5'-GAATATTTTCATTATCAGTCCCAGTC-3' [204]. *M. catarrhalis* detection was done using primers that target the outer membrane protein *copB* gene, copBf:5'-GTGAGTGCCGCTTTTACAACC-3' and copBr:5'-TGTATCGCCTGCCAAGACAA-3' [205]. *S. aureus* was detected using primers that target the Thermonuclease (Tnase) encoding gene (*nuc*), nucf:5' -GCGATTGATGGTGATACGGTT-3'5 and nucr:5'-AGCCAAGCCTTGACGAACTAAAGC-3' [206]. *H. influenzae* detection was done using primers that target the Outer membrane lipoprotein P2 (*OmpP2),* ompP2f:5'-GGTGCATTCGCAGCTTCAG-3' and ompP2r:5'GATTGCGTAATGCACCGTGTT-3'[207]. Capsule detection and typing of *H. influenzae* type B were carried using the oligonucleotides described by Howie *et al*. [208]. 16S rRNA-gene PCR detection was carried out on NP swabs that did not yield any of the pathogens assayed using the following primers: 338f-5'-ACTCCTACGGGNGGCNGCA-3' and 1046r-5'-CACGAGCTGACGACANCCATGCANCACC-3'.

5 µL of PCR product were loaded on 2% (w/v) agarose gels stained with ethidium bromide and analyzed by gel electrophoresis in 1x TAE buffer (40 mM Tris, 20 mM of glacial acetic acid, 1 mM EDTA, pH 8.0) for 60 min at 100 V. Gel images were recorded and the sizes of the PCR products were confirmed by comparison with the molecular

size standard HyperLadder II (Bioline, UK). DNA spiking tests were done by adding 10ng of *S. pneumoniae* purified genomic DNA to the reaction mixes described above.

2.5. Primer Selection of 16S rRNA Analysis

Primers were selected that had the highest proportion of target sequences when interrogated against all available 16S rRNA genes on RDP Release 10 using the Probe Match Program (http://rdp.cme.msu.edu/probematch), 0 mismatches were allowed to show the best possible outcomes. Commonly used 16S rRNA gene broad range primers and in-house primers were compared *in silico* Table 2.1. Primers with the highest proportion of matches were optimized for T-RFLP analysis using various bacterial isolates (data not shown), confirming results.

Table 2-1 Prevalence of commonly used 16S rRNA gene universal primer targets in the Ribosomal Database Project Release 10

Primer Name	Primer Sequence (5' - 3')	Number of sequences with target sequence (0 mismatches)	Sequence Hits/Total Searched
338F	ACTCCTACGGGNGGCNGCA	884403	71.4
27F	AGAGTTTGATMCTGGCTCAG	151771	12.3
8F	AGAGTTTGATCMTGGCTCAG	151723	12.3
63F	AGGCCTAACACATGCAAGTC	72315	5.8
1406R	ACGGGCGGTGTGTRC	346664	28.0
1046R	CACGAGCTGACGACANCCATGCANCACC	611169	49.4
1494R	TACGGYTACCTTGTTACGAC	64363	5.2

* The widest selection of sequences (1237963) were enterrogated against the primers

*0 mismatches allowed only in the Probe Match Program on RDP Release 10

2.6. Terminal Restriction Fragment Length (T-RFLP) Analysis

16S rDNA amplification yielding a fragment ~700bp was performed in 50 µL reaction volumes consisting 5 µL of DNA, 1x Green GoTaq reaction buffer with 1.5mM $MgCl_2$,

1.0U Go*Taq* Polymerase (Promega, UK), each deoxynucleoside triphosphate at 0.2mM of each deoxynucleoside triphosphate (dNTP) (QIAGEN, UK), 0.5µM of 6-carboxyfluorescein labeled forward primer 338F-[6-FAM] (5'-ACT CCT ACG GGN GGC NGC A-3') (Applied Biosystems, UK) and 0.25µM 1046R (Table 2.1). Amplification was carried out by initial denaturation of 94^{o}C for 1 minute, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 58°C for 1 minute, and extension at 72°C for 2 minutes. Final extension was done at 72°C for 10 minutes. Duplicate PCR products were pooled and from 50 µL of PCR product was purified using MultiScreen Ultracel®-10 Kit (Millipore, UK) following manufacturers protocol. Purified DNA was stored at -20°C and was checked by 1.5% agarose gel electrophoresis. PCR duplicate amplifications were done for each DNA sample and pooled to reduce bias associated with PCR. Of the 483 samples from 29 subjects assayed, sufficient amplicons could not be generated from 50 NP swabs for T-RFLP analysis. Hence, T-RFLP profiles were generated and analyzed for 433 samples corresponding to 29 subjects.

Approximately 100ng of DNA were digested with 5U of restriction enzyme ALUI (NEB, UK) for 3 hours at 37°C, followed by 20 minutes at 65°C. Approximately equal amounts of PCR product from each sample were analyzed to reduce the effects of fingerprinting different amounts of DNA. Digested amplicons were cleaned using SureClean (Bioline, UK) and eluted in 10µL 10 mM Tris-Cl, pH 8.5 (QIAGEN, UK). 1µL (~10ng) of purified digest, 0.5 µL of GeneScan-600Liz and 8.5µL of Hi-Di™ formamide (Applied Biosystems, UK) were mixed followed by DNA denaturation at 95°C for 3 minutes. Fragments were separated by size by capillary electrophoresis on a 3130*xl* Genetic Analyzer (Applied Biosystems, UK) and visualized by excitation of the 6-FAM label

attached to the 5' terminal fragment by the following protocol: Oven temperature 60 1C, injection voltage 1.6 kV, injection time 15 s, running voltage 15 kV and running time 2,500 s. The workflow of T-RFLP analysis is outlined in Figure 2.6.

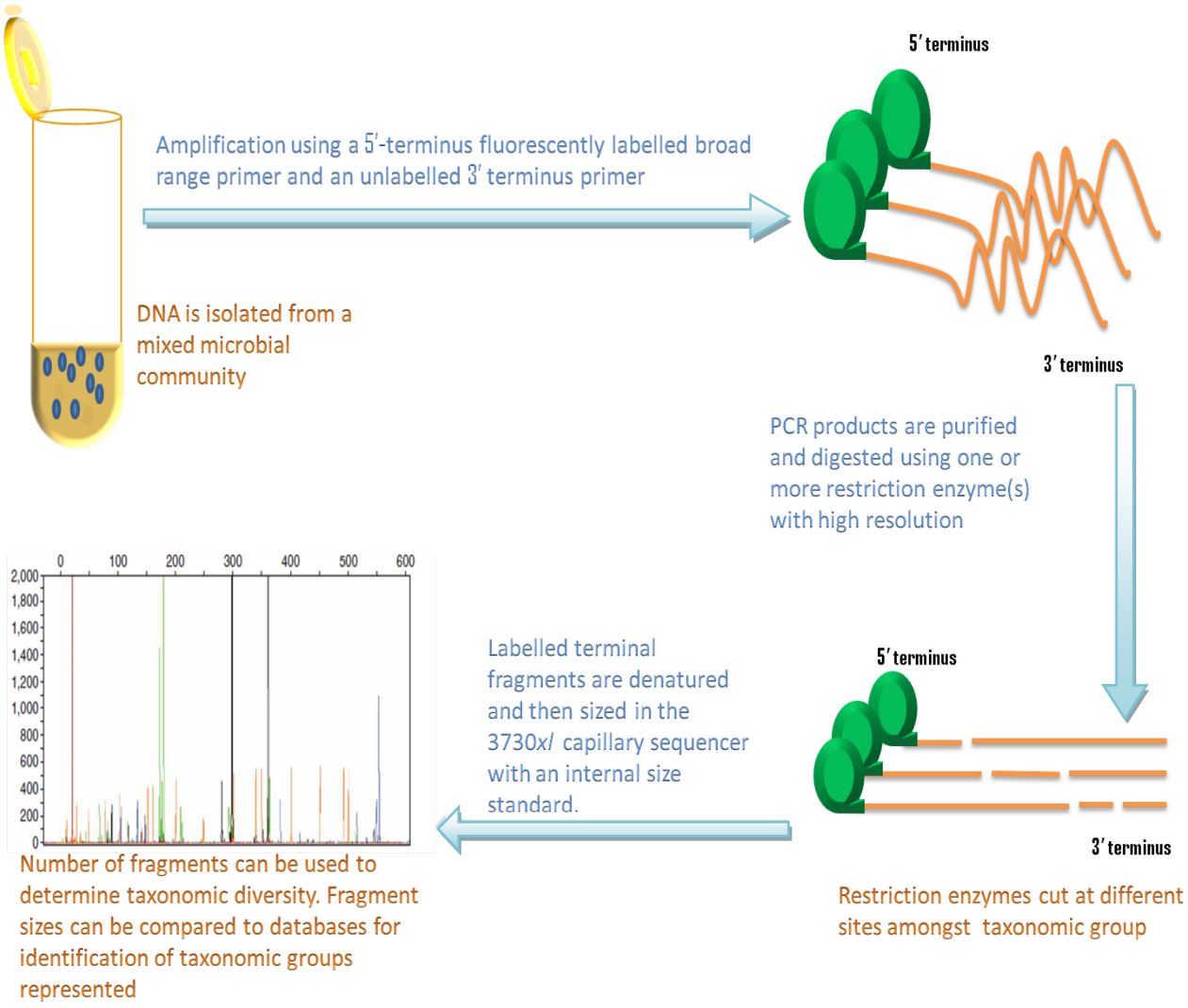


Figure 2.6 Workflow of Terminal Restriction Fragment Length Polymorphism Analysis

2.7. Reference Nasopharyngeal Clone Library

Cloning and partial sequencing of 16S rRNA genes amplified from DNA extracted from the NP swabs was performed to confirm identities of bacterial species corresponding to the dominant terminal restriction fragments (OTUs). NP samples from 6 subjects

were selected for clone analysis. DNA extracts of the NP swabs were used as template for the amplification of 16S rRNA gene fragments, using unlabelled universal primers 338F and 1046R as described above. Purified 16S rDNA fragments were directly cloned using the pGEM®-TEasy Vector System following manufacturers protocol (Promega, UK). Putative positive colonies were selected by blue-white screening and cultured overnight in 100µg/mL ampicillin LB broth. Inserts were amplified and sequenced using M13/pUC primers M13/pUCF (5'CCCAGTCACGACGTTGTAAAACG-3'), M13/pUCR (5'AGCGGATAACAATTTCACACAGG-3'), (Promega, UK) following manufacturers protocol. Raw sequences were edited using Lasergene SeqMan (DNASTAR, UK). Partial 16S rRNA gene sequences were aligned against GenBank database sequences using the nucleotide BLAST system at NCBI (www.ncbi.nlm.nih.gov). Sequences were screened for the presence of chimeras with Mallard chimera-checking tool prior to submission [209]. Sequences defined as unknown or uncultured bacteria were also aligned against sequenced bacterial genomes (genomic BLAST at NCBI) and also analyzed in the Ribosomal Database Project, Release 10, SeqMatch, for classification. Partial 16S rRNA gene sequences with > 99% maximum identity were assigned the same name as the species hit, and sequences with 97–99% maximum identity were classed in the same genus[164]. 165 of the 242 sequences are available on GenBank HM179296-HM179460 http://www.ncbi.nlm.nih.gov/Genbank. The *in silico* digestion of sequences was carried out using WebCutter 2.0 (http://rna.lundberg.gu.se/cutter2/) and matched with corresponding OTUs.

2.8. 454 GS FLX Titanium Pyrosequencing

2.8.1. DNA amplification and sequencing

The purified genomic DNA was amplified using oligonucleotides that target the V1-V3 (Window 1) and V3-V5 (Window 2) hypervariable regions of the bacterial 16S rRNA gene. V1-27F: 5'-*BA*-AGAGTTTGATCCTGGCTCAG-3' and V3-534R: 5'-*AA*-*X*-ATTACCGCGGCTGCTGG-3') for Window 1. V3-537F: 5'-*BA*-CCTACGGGAGGCAGCAG-3' and V5-926R: 5'-*AA*-*X*-CCGTCAATTCMTTTRAGT-3') for Window 2. *BA* denotes the 'B' adapter sequence, *AA* denotes the 'A' adapter sequence and *X* the unique 5 to 10 nucleotide long barcode uniquely identifying samples amplified in each 96-well plate, the sequences are included in the appendix section. Each reaction contained 2µM of the forward primer, 2µM of the bacroded reverse primer unique for each sample, 2µL of genomic DNA, 2µL of 10X AccuPrime PCR Buffer II and 0.15µL of Accuprime high fidelity Taq polymerase, the volume was brought to 20µL using RNAse /DNAse free water. The positive controls for all runs were purified *Anaerotruncus colhominis* DNA and negative controls were RNAse/DNAse free water. Reactions were briefly spun down at 2000g to collect samples at the bottom of the wells.

PCR reactions were carried out under the following conditions: initial denaturation at 95°C for 2 min; the 30 cycles of denaturation at 95°C for 20 sec, annealing at 50°C (Window 2) or 56°C (Window 1) for 30 sec, and elongation at 72°C for 5 min 20 sec. The amplified products were checked by E-gel electrophoresis systems (Invitrogen, USA) and purified using AmPure beads (Agencourt, USA) following manufacturers specifications). The DNA was eluted in 25µL 1x Low TE, pH 8.0 and quantified using the SYBR-Green Quantification system following manufacturer's specifications. The 454 deep sequencing pipeline is outlined in Figure 2.7.

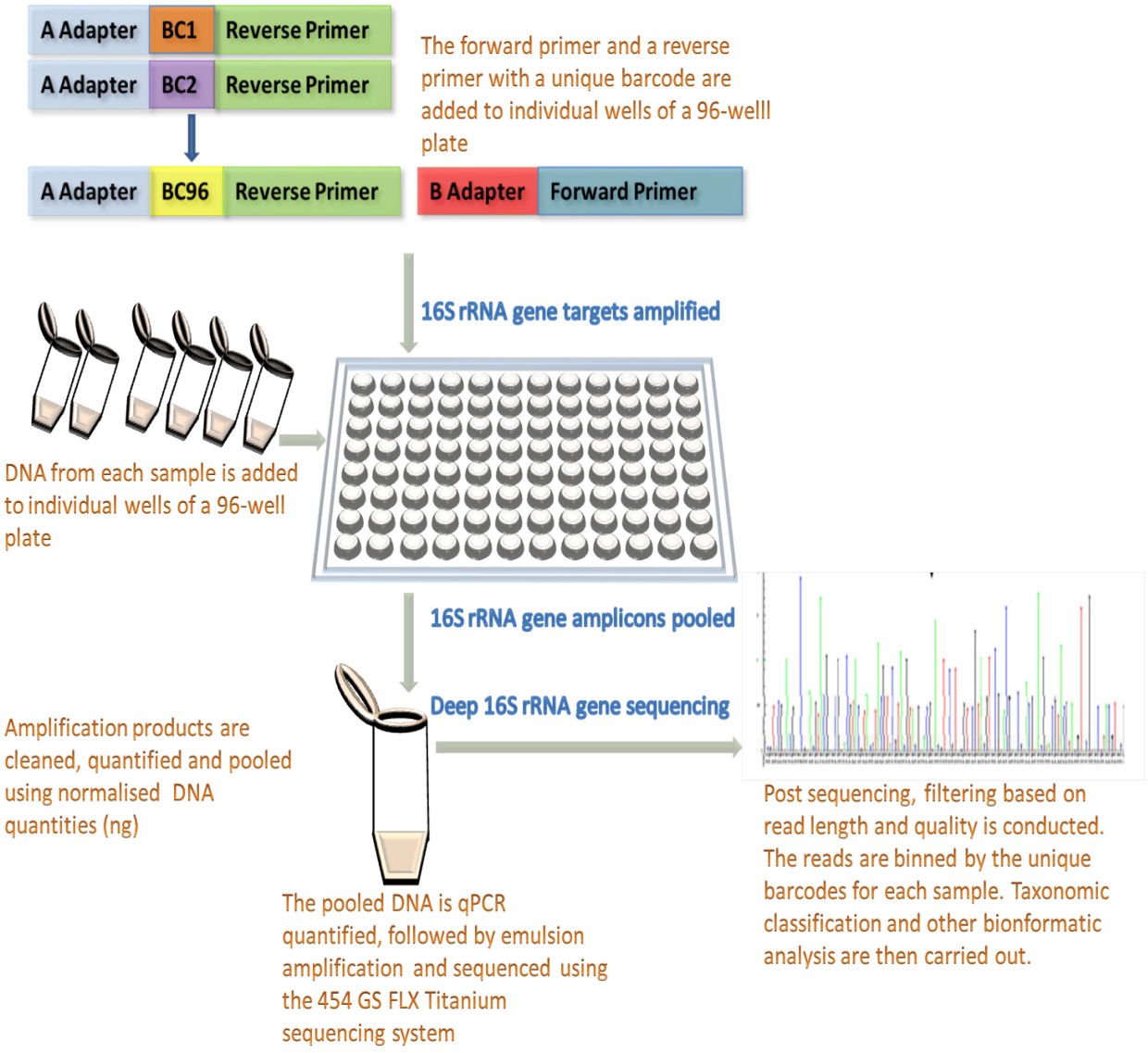


Figure 2.7 Workflow of the 16S rRNA gene 454 deep sequencing analysis

$$\text{Volume added to pool (µL)} = \frac{(Y/2) \times (M)}{T}$$

Y is the total volume (µl) of the sample
M is the concentration in ng/ul of the sample with the lowest concentration in the pool
T is the concentration (ng/uL) of the sample

The DNA was pooled (normalized) using the formula above. A minimum transfer volume of 1µL was applied; hence, dilutions were necessary in some instances where DNA concentration was very high. The maximum transfer volume was 25 µL and

samples that failed to achieve this were not included in the pool and subjected to re-amplification. The minElute DNA purification kit (Qiagen, USA) was used to purify the pooled DNA following manufacturer's protocol and DNA was stored at – 20°C until used. 454 GS FLX Titanium Sequencing (Roche, USA) was carried out at the Genome Centre, (University of Washington in St. Louis, MO, USA) following manufacturer's specification.

2.8.2. Bioinformatic Analysis

The reads from were filtered based on read length (< 300bp discarded) and read quality (>1 ambiguous base calls (Ns) discarded). Reads without the Adapter sequences were also removed. Reads from the same samples were binned based on barcode and then the barcode, adapter and primer sequences at both terminals were trimmed. Alignment and taxonomic (down to Genus) classification of the reads was carried out on the Ribosomal Database Project (Release 10) using a 0.5 filter.

2.9. Statistical analysis

2.9.1. PCR Detection of Pathogens

Results of the organism specific PCR assays were recorded as presence/absence outcomes and all the statistical analyses were carried out in STATA release 11 (StataCorp LP, USA). The frequency of detection for each of the microbes in the 497 NP samples, *S. pneumoniae, H. influenzae, S. aureus* and *M. catarrhalis* was reported as proportions of NP swabs in 16 age strata ranging from (1, 2, 4, 6,8 weeks up to 26 weeks and then 34, 42 and 54 weeks based on the actual timing of NP swab collection from the subjects. Logistic regression was used to determine associations between carriage and genetic, microbial and environmental factors. Sixteen to seventeen samples were collected per infant; hence, to account for correlations of response

variables from the same infant, subject identity was entered as a random effect. Log likelihood Ratio Tests were performed to determine the contribution of each factor to the model. Kaplan-Meier survivor function was used to conducted time to first detection for each of the microbes.

2.9.2. T-RFLP Analysis

Hierarchical clustering of the T-RFLP profiles was conducted on BioNumerics v6.5 software (Applied Maths Inc, USA). Unweighted Pair Group Method with Arithmetic Mean (UPGMA) distances was used to determine relationships between profiles. Random intercept modelling was used to determine the relationship of age and sex with richness of the microbiome (number of OTUs detected per sample) with a random effect for subject. The rates (per year) of acquisition and loss for each OTU were calculated as the total number of events (acquisitions or losses) divided by the person years of follow-up. Parametric survival time regression models assuming Weibull distribution and multiple acquisitions and losses per subject were used to examine associations with genetic and environmental factors, correcting for the confounding effect of age. Adjustment for multiple testing was performed using Benjamini and Hochberg method with a p value of 0.05 for false discovery rate [210]. Richness was measured as the number of distinct OTUs per sample [211].

2.9.3. Effect of frozen-storage analysis

The electropherograms were analyzed with GeneMapper® Software v4.0 (Applied Biosystems).The advanced peak detection algorithm was used and fragment sizes were determined by the Local Southern method and fluorescence signal was normalized for all the samples analysed. A fixed detection threshold of 100 fluorescence units (FU) was used to reduce inclusion of noise peaks in the analysis [155, 212]. Terminal

restriction fragments (T-RFs) that differed by ±1 bp in different profiles was binned in the same operational taxonomic unit (OTU). T-RFs between 50 bp and 600 bp were considered in the analysis to obtain 5' terminal fragments within the linear range of the GS-600Liz (Applied Biosystems). Composition was calculated as the total number of distinct OTUs detected in a sample. The relative abundance of each OTU was determined by expressing the fluorescence units as a proportion of the sum of relative fluorescence signal for each sample [169]. Random effects model allowing for the within subject correlation was used to investigate the effect of freezing on composition and total RFUs per subject adjusting for age and sex. A random effects logistic regression model was used to study the association of detection of each OTU with the type of sample (frozen versus fresh) adjusting for age and sex when appropriate. Only OTUs detected in both primary and frozen samples were included and correction for multiple testing was done using Holm's method.

2.9.4. 454 deep sequencing

Analysis was done using a Genome Center in-house pipeline on the linux operating system. The data for each sample were standardized and collapsed at family level**.** Taxa (Genus or Family) counts within each sample were converted to proportions of the total (reads/1000) for the NP community**.** For each subject, the genera with total number of adjusted reads of 100/1000 were selected to avoid spurious results due to very small number fluctuations. To determine if significant changes occurred in the abundance of OTUs (Genera) during the early vaccination period, the change in adjusted reads between weeks 1-17 when the early vaccination was conducted and weeks 18-27 was calculated and the Wilcoxon test was used to test if the changes

between early and late weeks were significant among early and late vaccination infants.

3. Differential effects of frozen storage on the molecular detection of bacterial taxa that inhabit the nasopharynx

3.1. Introduction

Nasopharyngeal swabbing is an important diagnostic and epidemiological surveillance tool used to detect several pathogens in one of the most clinically relevant microbial reservoirs in the human body [213, 214]. Although detection of microbes in nasopharyngeal (NP) swabs stored in skim milk-tryptone-glucose-glycerin (STGG) is often employed in epidemiology and diagnostic research [215], few studies have investigated the potential bias introduced during sample storage, which usually involves deep freezing at -20^{o}C or -70^{o}C [55, 213, 216, 217]. Furthermore, these studies have focused on the effects of frozen storage on the culture-based detection of specific pathogens such as *S. pneumoniae*. However, the impact of freezing on the molecular culture-independent detection of the microbial communities inhabiting the nasopharynx is yet to be fully understood.

With the widespread application of vaccines and antibiotics against respiratory pathogens, serotype and species replacement is becoming increasingly important. The occurrence of species replacement depends on the ecological events that occur during the replenishment of the niche left vacant following vaccination or antibiotic treatment. Hence, extensive microbial ecology studies will be necessary to fully understand these events and identify microbes that play a significant role in replenishment. Furthermore, the competitive relations between bacterial types inhabiting the nasopharynx which may alter predisposition to disease will be

elucidated by broad comparative community analyses. This reinforces the need to broaden the scope of upper airway microbiology studies [57, 199, 218, 219]. However, only a small fraction of bacterial composition can be cultivated by standard laboratory techniques [1, 220].

Hence, culture-dependent applications grossly underestimate species richness, relative abundance and composition, limiting their utility in the comprehensive analysis of microbial communities [121]. To address various aspects of diverse microbial communities, microbiologists have employed an extensive array of culture-independent molecular tools which are often based on PCR and target the rRNA gene cluster [31, 140]. Although molecular tools avert considerable bias introduced by selective cultivation and have revolutionized our understanding of microbial ecology, they also have several limitations. Common to all PCR-based applications are several pitfalls which can occur at every stage of sample processing [121]. Bias can be introduced during sample collection and transport, sample storage, cell lysis, nucleic acid extraction, PCR amplification, and other downstream applications [3, 121-123, 221, 222]. One of the advantages of molecular applications is that samples can be stored and analysed even after the loss of viability of microbial cells. For logistic and technical reasons, frozen storage often precedes the molecular analysis of biological samples. However, it is becoming increasingly evident that the deep freezing process can have adverse effects on microbial composition and the detection of specific bacterial strains [216, 223, 224].

Prior to a study characterizing the development of the infant nasopharyngeal microbiome, we set out to validate an effect of frozen storage of NP swabs on the molecular detection of bacterial taxa using 16S rDNA based-Terminal restriction fragment length polymorphism (T-RFLP). T-RFLP is a PCR based DNA fingerprinting tool that has been applied in community analyses across different ecosystems [212]. To our knowledge this is the first investigation of an effect of freezing on the 16S rRNA gene-based profiling of microbial communities of the infant nasopharynx.

3.2. Results

Duplicate NP DNA extracts from each of the twelve infants were analysed within hours of collection and after thirty days of frozen storage of the NP swabs at -70^{o}C in STGG. Hence, a total of 48 nucleic acid samples were analysed by 16S rDNA T-RFLP (Table 3.1). Terminal restriction fragments (T-RFs) were not generated for seven samples, six frozen and one fresh sample due to poor amplification. Hence, 96% and 75% of samples analysed fresh and after frozen storage were successfully analysed by T-RFLP respectively. A total of 42 distinct OTUs were detected in the NP swabs, 86%, 74%, and 62% were detected in fresh analysis only, after frozen storage only and in both analyses respectively.

Table 3-1. Description of samples collected for this study

Subject	Sex	NP Swabs Collected	Fresh DNA Extracts	Days Frozen	Frozen DNA Extracts	Age at Collection (Weeks)	Fresh DNA Extracts	95% CI (Poisson)	After Frozen Storage	95% CI (Poisson)
1	M	1	2	30	2	16.9	12.0	7.7,17.9	14.5	9.7,20.8
2	M	1	2	30	2	16.6	3.5	1.4,7.2	11.5	7.3,17.3
3	F	1	2	30	2	16.4	8.0	4.6,13.0	1.5	0.3,4.4
4	F	1	2	30	2	16.1	13.5	8.9,19.6	0	0,1.8*
5	F	1	2	30	2	16.1	9.5	5.7,14.8	0	0,1.8*
6	F	1	2	30	2	16.0	10.0	6.1,15.4	1.0	0.1,3.6
7	M	1	2	30	2	26.6	6.5	3.5,11.1	3.5	1.4,7.2
8	F	1	2	30	2	26.6	3.5	1.4,7.2	5.5	2.7,9.8
9	F	1	2	30	2	26.3	6.5	3.5,11.1	5.0	2.4,9.2
10	F	1	2	30	2	25.9	10.0	6.1,15.4	9.0	5.3,14.2
11	M	1	2	30	2	26.1	14.0	9.3,20.2	12.0	7.7,17.9
12	M	1	2	30	2	26.1	11.5	7.3,17.3	12.0	7.7,17.9

(*) one-sided, 97.5% confidence interval (CI)

3.2.1. Effect on bacterial composition

Bacterial composition in frozen and fresh analysis was measured by the total number of distinct OTUs detected from NP swabs before and after freezing, which ranged from 0 to 14.5 OTUs per sample. The mean number of OTUs was 6.3 per sample for analysis post freezing and 9.0 per sample for fresh analysis. Results from a random effects model showed that there was a significant association between subjects and the effect of frozen storage on composition ($p = 0.0083$). The model also indicated significant interaction between the sex of the subject and the effect of freezing on composition. Before freezing the mean number of OTUs for males and females were quite close, 9.5 and 8.7 respectively, however after freezing these were 10.7 and 3.1 respectively, see Figure 3.1. The difference in composition pre and post freezing was significant for female ($p = 0.0014$) but not for male infants ($p=0.56$).

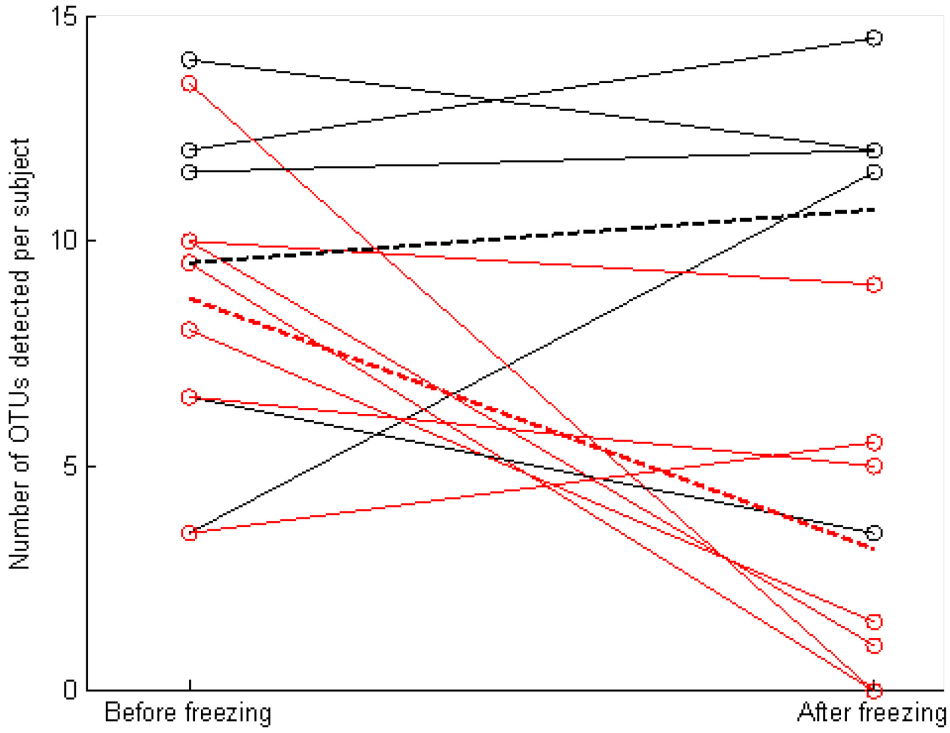


Figure 3.1 Effects of frozen storage on the composition of bacterial OTUs found in the infant nasopharynx. The composition (mean number of OTUs) per subject against 16S rRNA-based T-RFLP before and after freezing at -70 Degrees Celsius dichotomized by gender. Red lines represent females, and dotted lines show the mean change. The difference in composition pre and post freezing was significant for female (p = 0.0014) but not for male infants (p=0.56).

3.2.2. Effect on odds of detection

The effect of freezing NP swabs on the odds of detecting bacterial OTUs was analysed using a random effects logistic regression model, adjusting for sex. The odds of detecting most OTUs did not change with freezing; however, the detection of four taxa was significantly reduced at the 5% level by freezing, see Table 3.2. Bioinformatics analysis of these T-RFs based on fragment size matched them with specific bacterial taxa as follows; *Firmicutes* (91 bp), *Pseudomonas sp* (309 bp), *Moraxellaceae* (147 bp), and *Haemophilus sp/Burkholderia sp*. (518 bp) (see Table 3.1). After correcting p-

values for multiple testing using Holm's method none remained significant, thus the results should be interpreted with caution.

Table 3-2. Effect of frozen storage on the detection of bacterial OTUs by 16S rRNA-based T-RFLP

OTU (bp)	Log odds of detection	p value	95% CI	Closest Bacterial Match (>97% Sequence Similarity)
87	1.81	0.25	-1.25, 4.86	
91	**-3.04**	**0.01**	**-5.43, -0.65**	*Firmicutes*
114	**-2.00**	**0.04**	**-3.95, -0.05**	*Moraxella sp.*
116	2.63*	0.36	-2.96, 8.22	*Marinomonas sp.*
133, 215	2.63*	0.36	-2.96, 8.22	*Flavobactriaceae*
155	1.34	0.20	-0.69, 3.38	
216	-1.18	0.15	-2.80, 0.43	*Corynebacterium propinquum, Rothia mucilaginosa*
273	-2.44	0.26	-6.66, 1.79	*Flavobacteriaceae*
294	0.94*	0.52	-1.89, 3.76	
309	**-2.77**	**0.03**	**-5.34, -0.21**	*Pseudomonas sp.*
392	-2.21	0.06	-4.52, 0.11	*Moraxellaceae*
492	-0.67	0.57	-2.99, 1.66	*Clostridiales Incertae Sedis XI*
502	2.63*	0.36	-2.96, 8.22	
504	-2.39	0.25	-6.49, 1.70	*Haemophilus influenzae*
518	**-2.59**	**0.01**	**-4.58, -0.61**	*Haemophilus influenzae, Burkholderia fungorum, Comamonadaceae*
520	-0.36	0.68	-2.02, 1.31	*Staphylococcus sp.*
521	-1.70	0.11	-3.80, 0.40	

A random effects logistic regression model adjusting for sex was used. * Indicates OTUs for which sex adjustment was not done. Bold indicates significant results at the 5% level after correcting for multiple testing using Holm's method none of them appeared significant.

3.2.3. Effect on abundance

The effect of freezing on the relative abundance of OTUs was investigated. For more than 70% of the OTUs, there was less than a 0.5% change in relative abundance after freezing, see Figure 3.2. 28% of the OTUs had 0.6% to 18.6% shifts in relative abundance post deep frozen storage, with half showing an increase and the other half showing a decrease in relative abundance. The relative distributions of OTUs were compared for male and female infants as well as before and after freezing, see Figure 3.3. The relative distribution of bacterial taxa was comparable between male and

female infants as well as before and after freezing. The relative proportions of some major taxonomic groups including *Haemophilus sp., Staphylococcus sp. Moraxella sp.,* and *Firmicutes* were comparable before and after freezing for both sexes, see Figure 3.3. However, the relative proportions of some OTUs including the OTU 392bp, the 309bp OTU *(Pseudomonas sp),* the 216bp OTU (*Rothia sp),* the 278bp OTU (*Acinetobacter sp.)* and OTUs with relative abundance less than 1% showed some change before and after freezing amongst the infants.

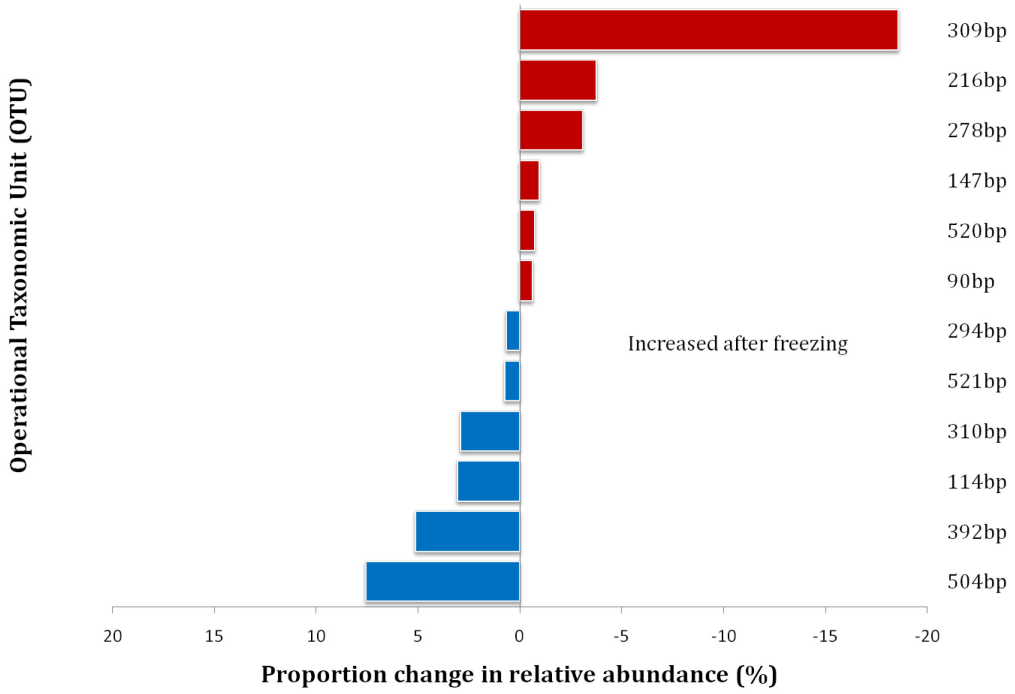


Figure 3.2. Bar graph showing proportion change in relative abundance of bacterial OTUs after frozen storage of NP swabs at -70°C analyzed by 16S rRNA T-RFLP. The difference in relative abundance before and after frozen storage was expressed as a proportion of the relative abundance for direct analysis. Most of the 42 OTUs detected showed minimal (<0.5%) change and OTUs that had proportion change >0.5% are shown here.

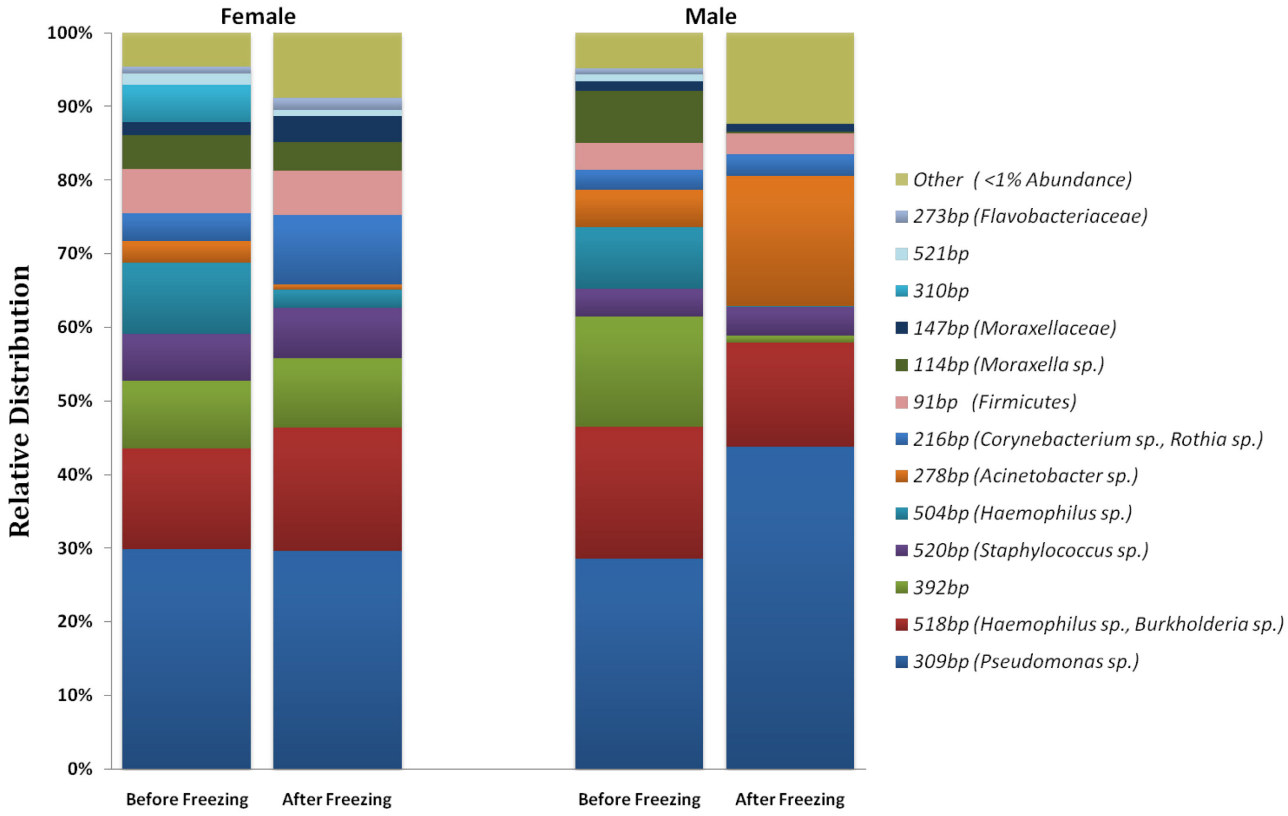


Figure 3.3. Bar Graph showing the relative distribution of the bacterial OTUs detected before and after frozen storage of NP swabs at -70°C amongst male and female infants by 16S rRNA-based T-RFLP. Partial 16S rRNA gene sequences from infant nasopharyngeal clone libraries were BLASTED to identify the microbes (>97% sequence similarity) and *in silico* T-RFLP analysis was used to match them to the OTUs.

3.3. Discussion

We report preliminary data showing that deep freezing NP swabs in STGG medium at -70°C may have a modest effect on the fingerprint of bacterial communities and a differential effect on the detection of bacterial OTUs found in the infant nasopharynx. Although the relative proportions of some OTUs changed, overall, the relative distributions were comparable before and after frozen storage (Figure 3.3). Previous studies which investigated the effects of frozen storage on the detection of various microbes in biological samples have shown minimal or no significant effects [213, 223, 225-227].

Abdullahi *et al*.(2007) reported that the recovery of *S. pneumoniae* from fresh and frozen NP swabs in STGG was consistent, but there were differences in the serotype distributions [223]. This could be attributed to a differential capacity to survive the freezing process among *S. pneumoniae* serotypes. In another culture-based study, there was no effect of freezing on the recovery of *S. pneumoniae* and *S. aureus* from milk samples frozen at -20^{o}C [225, 226], but there was an increase in the detection of coagulase negative staphylococci and a decrease in the recovery of *Escherichia coli* and *Actinomyces pyogenes* [226]. Likewise, a recent 16S rRNA gene-based study of Black Band Disease showed that frozen storage increased the proportion of Proteobacteria phylotypes while direct analysis promoted the detection of cyanobacterial and sulfur-oxidizing bacteria [224].

These reports suggest that there may be differential survival capacities to frozen storage among bacterial taxa from the same community. As such, the deep freezing and thawing processes may alter the odds of detection and relative abundance of some, but not all OTUs in a biological sample. In this study, frozen storage significantly altered the odds of detecting a small proportion (<10%) of the bacterial OTUs found (Table 3.1) and the relative abundance of a couple of OTUs changed by more than 5% after frozen storage (Figure 3.2). This could be explained by DNA degradation amongst some bacterial taxa; Suomalainen and colleagues demonstrated that the freezing process results in the disintegration of the *Flavobacterium columnare* cell wall, associated with the release of large quantities of DNAse, and proteases[216]. There is further evidence that the structure and stability of bacterial cells influence cryo-preservation of nucleic acids [228].

Interestingly, the observed decrease in the number of OTUs found after frozen storage was significant among female (p = 0.0014) but not among male infants (p=0.56) (Figure 3.1). This preliminary data suggests that an effect of freezing on microbial detection may also be differential across sexes. Interestingly, several OTUs detected in both sexes in fresh NP swabs were not detected or were detected at much lower frequencies among female infants post frozen storage. Although sex has been shown to be an important factor in colonization by various bacterial pathogens [229, 230], this finding has not been reported elsewhere to our knowledge. It is unclear how an effect of deep freezing on bacterial detection could be differential depending on the sex of a subject, which necessitates further investigations.

Frozen storage of biological samples is necessary for archiving and often done for logistic purposes where real-time processing of samples is not practicable. With the widespread use of vaccines targeting commensals of the respiratory mucosae, it is essential to effectively monitor non-vaccine serotypes and species replacement disease [200, 231]. Tracking intra-species serotype replacement and/or switching has overshadowed comprehensive research into the long term effects of vaccination on the microbiome, which may influence health, predisposition to disease and the pathogenesis of various infections [232, 233]. Loss or increased detection of particular taxa due to deep frozen storage may have a bearing on microbial ecology and species replacement surveillance. Furthermore, the intricate competitive relations between bacterial phylotypes may not be fully understood if deep freezing alters the fingerprint of microbial communities, albeit modestly [57, 199, 218, 219].

In this study, we investigated the effect of frozen storage on NP swabs stored in STGG from 12 infants. However, the sample size and study design limit the validity of the findings. Broad investigations of different biological specimens, storage media, storage duration and microbial detection tools are needed to validate these findings. An effect of frozen storage on microbial detection using culture-based and culture-independent approaches needs to be studied. Finally, further investigation is needed to determine the precise mechanisms by which shifts in microbial community structure occur following frozen storage.

3.4. Conclusions

The potential effects of frozen storage on the composition and relative abundance of microbial populations should not be overlooked with the widespread use of molecular applications in microbial ecology studies.

4. Early acquisition and high nasopharyngeal co-colonization by *S. pneumoniae* and three respiratory pathogens

4.1. Introduction

An astounding 83% of childhood deaths (< 5 years) between 1970 and 2009 occurred in Asia and sub-Saharan Africa, sharply contrasted with less than 1% which occurred in high-income nations [234]. Invasive bacterial disease (IBD) such as pneumonia, meningitis and bacteraemia contribute to the disparity in childhood mortality in developing and developed countries [39, 40]. 18% of the estimated eight million childhood deaths (<5 years) which occurred worldwide in 2008 were attributed to pneumonia, making it the single commonest cause of death in the under five year olds [235].

Approximately 50% of the severe pneumonia cases in developing countries are attributed to *S. pneumoniae* and *H. influenzae type b* (Hib) in areas where the vaccine is not widely available [236]. Non-typeable strains of *H. influenzae* (NtHi) are associated with otitis media (OM), community acquired pneumonia and other IBD among vulnerable populations [237-239]. OM is the most common bacterial infectious disease amongst children [240] and up to 20% of acute OM episodes are caused by *M. catarrhalis* [241]. In the Gambia, pneumococcal nasopharyngeal carriage occurs rapidly after birth and carriage exceeds 80% in infants [201]. Not surprisingly, *S. pneumoniae* is also the leading cause of IBD [242], with the highest prevalence occurring in infancy. *S. pneumoniae* and *Staphylococcus aureus* accounted for 45.2%

and 18.3% respectively of bacteraemia cases in a study amongst hospitalised patients in the Gambia with a median age of 2 years (range 2 months to 80 years) [243].

The nasopharynx is an important reservoir of commensal and pathogenic microbes which can migrate to the sinuses, middle ear and lower respiratory tract and invade the blood system. Nasopharyngeal carriage is thought to be the main source of transmission of pathogens across individuals [41, 42]. Although the specific mechanisms are poorly understood, attachment to the nasopharyngeal epithelial surface is thought to be an essential step in the development of mucosal and invasive disease [44, 244]. The epidemiology, transmission and nasopharyngeal carriage of *S. pneumoniae* have been studied in the Gambia [42, 201, 243, 245]. However co-carriage of *S. pneumoniae* with *H. influenzae, S. aureus* and *M. catarrhalis* has not been described in West Africa.

The seven-valent pneumococcal polysaccharide-diphtheria CRM_{197} protein conjugate vaccine (PCV-7) markedly reduces the carriage of vaccine serotypes and decreases the incidence of vaccine serotype invasive disease, making it a remarkable public health success story [200]. However, the long term effectiveness of PCV-7 also depends on the emergence of serotype and species replacement disease as the vacant niche is replenished by non-vaccine pneumococcal serotypes or possibly other respiratory pathogens that share the nasopharyngeal niche [199, 246].

This study set out to determine the co-carriage of *H. influenzae, S. aureus* and *M. catarrhalis* with *S. pneumoniae* in the nasopharynx amongst PCV-7 naïve infants using PCR-based methods.

4.2. Results

Species specific PCR was applied to detect four pathogens in 498 NP swabs collected from 30 children in the first year of life (n= 16.6NP swabs/infant). At least one pathogen was detected in 473 (95%) of the nasopharyngeal swabs. The presence of bacteria was confirmed by 16S rRNA gene PCR for 15 (3%) of the nasopharyngeal swabs that were negative for all four pathogens. Amplification of spiked NP swab DNA contraindicated the presence of inhibitors in 5 (2%) of the NP swabs which were negative for any of the pathogens and the 16S rRNA gene (2%).

4.2.1. Carriage of respiratory pathogens

During infancy, the average carriage rates accounting for repeated measures for *S. pneumoniae* was 78% (95CI: 76%, 83%), *M. catarrhalis* 71% (95CI: 67%, 75%), *H. influenzae* 70% (95CI: 65%, 74%) and *S. aureus* 20% (95CI: 16%, 24%). The proportion of encapsulated *H. influenzae* was 7% (95CI: 4.6%, 9.2%); (Hib) had a total proportion of 0.7% (95CI: 0.5%, 0.9%) in the NP swabs assayed. Carriage of both *S. pneumoniae* and *H. influenzae* was below 30% in the first week but increased rapidly as the infants got older, exceeding 90% between 15 and 19 weeks, shown in Figure 4.1. *M. catarrhalis* carriage was 57% in the first week and increased gradually to above 80% by 21 weeks. In contrast, *S. aureus* carriage was 50% in the first week; however carriage rapidly fell to 10% by 9 weeks and remained low thereafter (Figure 4.1).

4.2.2. High co-carriage of *S. pneumoniae* and three respiratory pathogens

Of the 391 NP swabs in which *S. pneumoniae* was found, it was the only pathogen detected in 25 (6%) and it was detected with at least one other pathogen in 366 NP swabs (94%). *S. pneumoniae* was found with one, two and three pathogens in 102

(26%), 228 (58%) and 36 (9%) 391 NP swabs respectively. During infancy, the average carriage prevalence for each of the pathogens was as follows; *S. pneumoniae* 78% (95CI: 76%, 83%), *M. catarrhalis* 71% (95CI: 67%, 75%), *H. influenzae* 70% (95CI: 65%, 74%) and *S. aureus* 20% (95CI: 16%, 24%). Carriage of both *S. pneumoniae* and *H. influenzae* was below 30% in the first week but increased rapidly as the infants got older, exceeding 90% between 15 and 19 weeks respectively (Figure 4.1). *M. catarrhalis* carriage was 57% in the first week and increased gradually to above 80% by 21 weeks. In contrast, *S. aureus* carriage was 50% in the first week and rapidly fell to 10% by 9 weeks and remained low thereafter (Figure 4.1). The average prevalence of encapsulated *H. influenzae* was 7% (95CI: 4.6%, 9.2%) and Hib had average prevalence of 0.7% (95CI: 0.5%, 0.9%) in the NP swabs assayed (Figure 4.1).

4.2.3. Early acquisition of *S. pneumoniae* and three respiratory pathogens

The average times to first detection of *S. pneumoniae*, *H. influenzae, M. catarrhalis* and *S. aureus* were 5, 7, 3 and 14 weeks respectively. By 129 days, 142 days and 149 days all the infants had carried *M. catarrhalis*, *S. pneumoniae* and *H. influenzae* at least once respectively (Figure 4.2). Within the first 30 days, more than 75% of the infants had acquired *S. aureus* at least once, (Figure 4.2); however, this pathogen was not detected in any of the NP swabs from 5 (17%) of the infants. 25 (83%) of the samples collected soon after birth (<6 days) had at least one pathogen detected, see Figure 4.3.

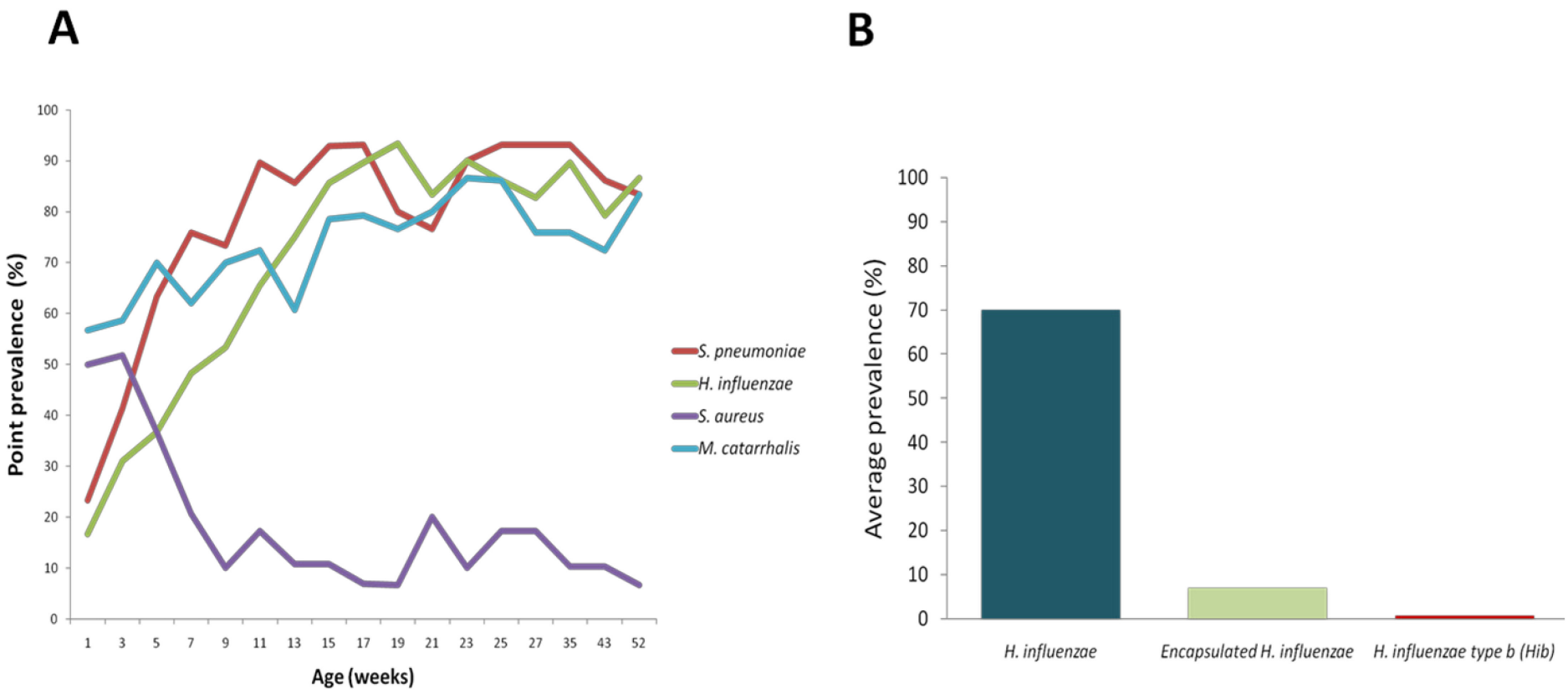


Figure 4.1. Carriage of *S. pneumoniae* and three respiratory pathogens in the first year of life. The point prevalence of *S. pneumoniae* and three respiratory in the nasopharynx among 30 infants followed-up from birth to one year (A). The average prevalence of *H. influenzae*, encapsulated *H. influenzae* and Hib in the nasopharynx among 30 infants followed-up from birth to one year (B).

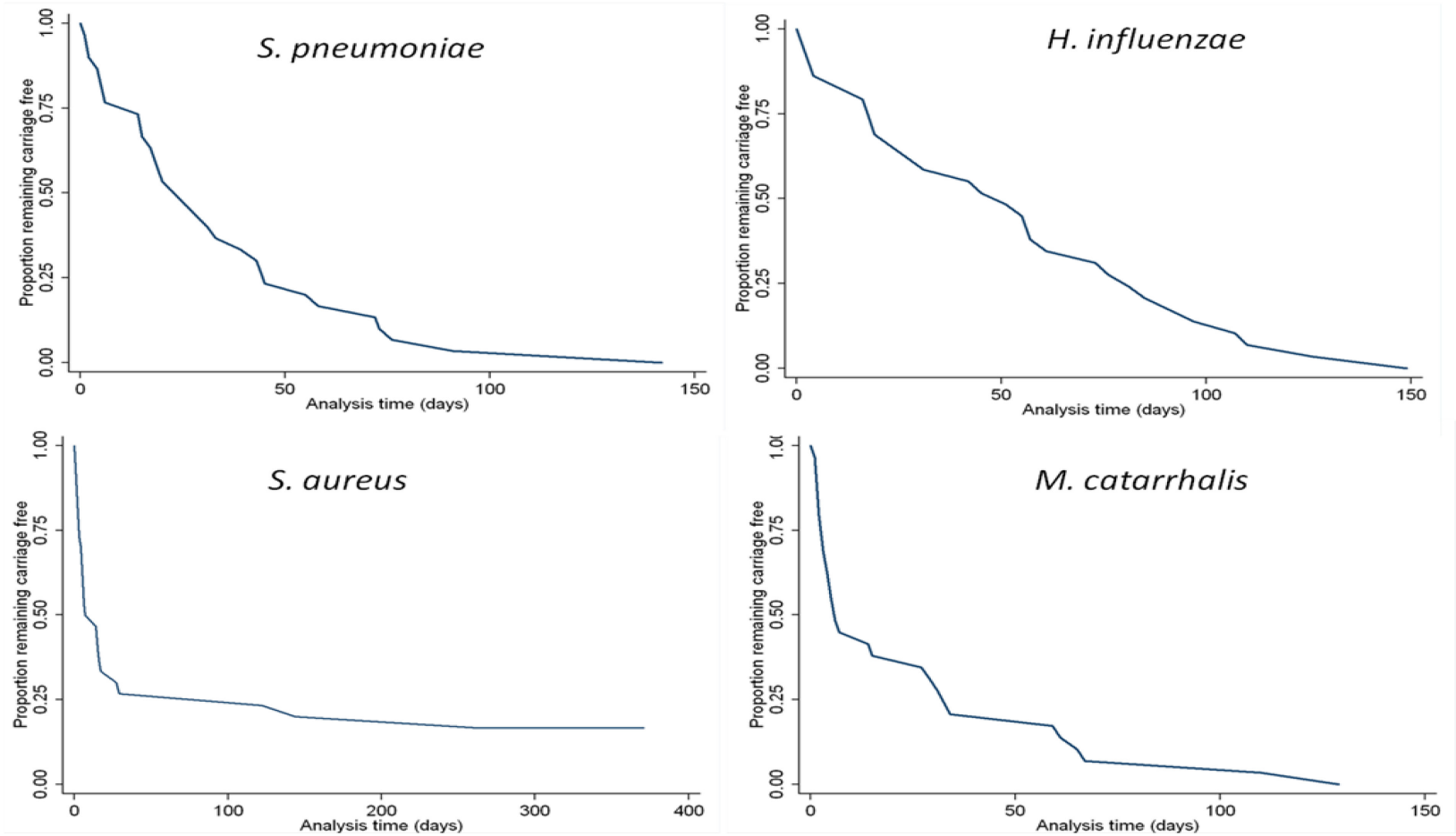


Figure 4.2. Survival curve for time to first detection of *S. pneumoniae* and three respiratory pathogens among 30 infants followed-up from birth to one year.

4.2.4. Associations between *S. pneumoniae* and three respiratory pathogens

Logistic regression modelling was used to determine the univariate and adjusted associations between *S. pneumoniae* carriage and the other respiratory pathogens. The adjusted model included the following confounding factors; age, ethnic group, sex, antibiotic treatment, type of feeding and weight [29, 201]. There was a significant positive interaction between colonization with *S. pneumoniae* and *H. influenzae* in univariate analysis (OR 5.03; 95% CI 3.02, 8.39; p<0.01) and the adjusted analysis OR 2.02; 95% CI 1.04, 3.91; p=0.04). A significant positive association was found between *S. pneumoniae* and *M. catarrhalis* in univariate analysis (OR 2.20; 95% CI 1.29; p<0.01) but not in the adjusted analysis (OR 1.40; 95% CI 0.72, 2.71; p=0.33). A significant negative correlation was found between *S. pneumoniae* and *S. aureus*, (OR 0.53; 95% CI 0.30, 0.94; p=0.03) but not after the adjusted analysis (OR 1.33; 95% CI 0.59, 3.02; p=033).

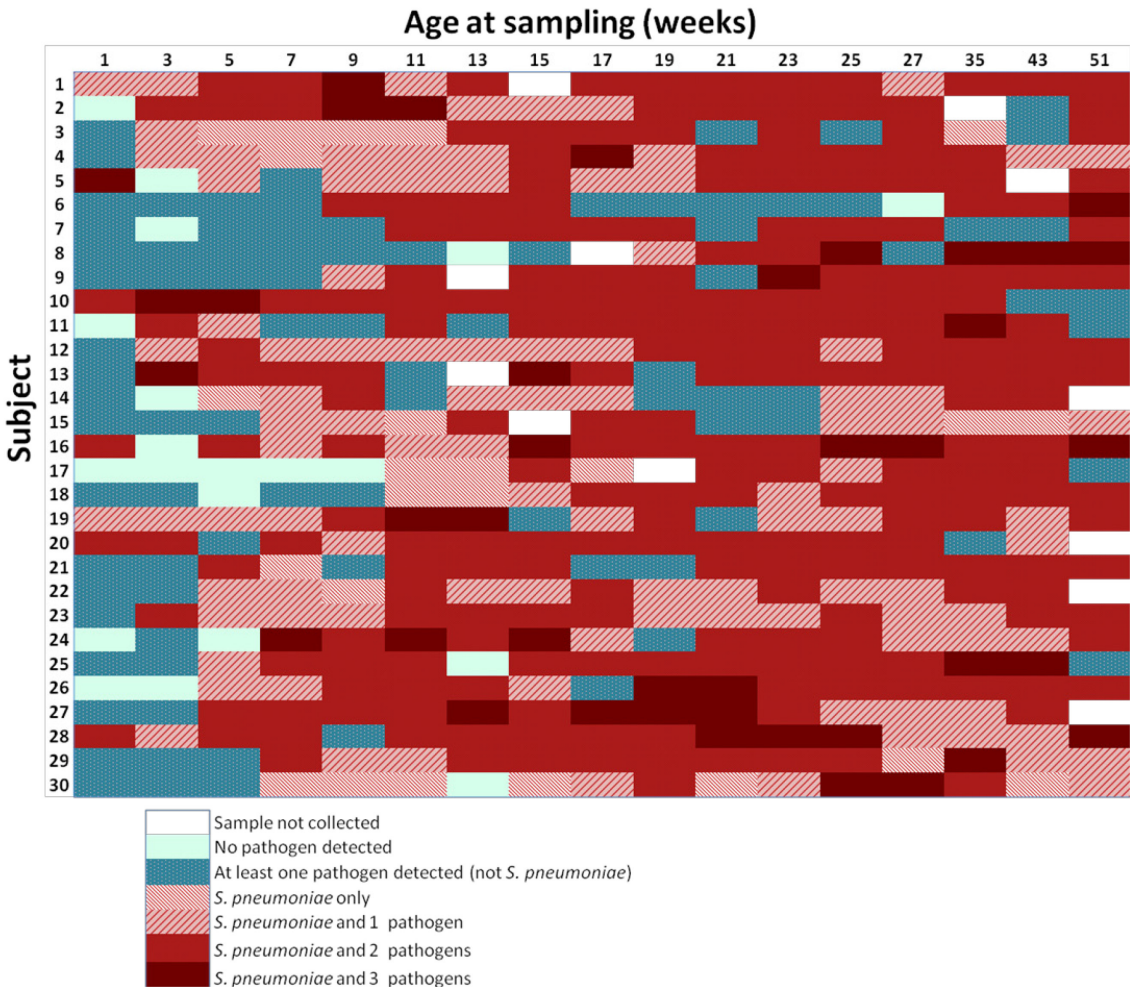


Figure 4.3. Heatmap showing co-carriage of *S. pneumoniae* with three respiratory pathogens in the nasopharynx among 30 infants followed up from birth to one year.

4.3. Discussion

For the first time in West Africa, the nasopharyngeal carriage and co-occurrence of *S. pneumoniae* with respiratory pathogens, *H. influenzae, S. aureus* and *M. catarrhalis* detected by PCR amongst PCV naive infants is described. Nine out of ten times, S. *pneumoniae* was co-carried with at least one other pathogen, most often *H. influenzae* and or *M. catarrhalis* (Figure 4.3). Multiple colonisation of the nasopharynx may have important clinical significance with regards to biofilm formation, polymicrobial infections and antibiotic resistance [55, 247].

Here we report very early onset of colonisation similar to reports from high risk populations (Figure 4.1 and Figure 4.2) [55, 248]. 83% of the nasopharyngeal samples collected within a week of birth had between one and four pathogens detected (Figure 4.3) and the time to first acquisition was less than 8 weeks for of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis*. In a culture-based study, Watson *et al*. [55] showed that by 6 months, 72%, 69% and 60% of rural Aboriginal infants from Western Australia had acquired *M. catarrhalis, S. pneumoniae and H. influenzae* respectively. In another culture-based study, *M. catarrhalis* colonization was reported to be as high as 100% amongst 60-day old Aboriginal infants [93].

This finding is comparable to data from the highlands of Papua New Guinea, by 3 months all infants had acquired *S. pneumoniae* [248]; whereas reports from other regions suggest much longer times to first acquisition for *S. pneumoniae*; 50% colonization by 8 weeks in Bangladesh [249], 34% colonization by 6 months in Finland [250] and average time to first detection of 6 months in the United States [41]. However, as these studies were culture-based, it is yet to be determined if the application of molecular tools would result in earlier detection of *S. pneumoniae* and the other pathogens.

Consistent with findings amongst Aboriginal and non-Aboriginal infants in Western Australia [55], *S. pneumoniae, H. influenzae* and *M. catarrhalis* point prevalence (at each age at sampling) increased in the first few months of life. *S. pneumoniae* carriage exceeded 80% by 12 weeks, which was also reported in the larger cohort of 236 infants [201]. Although *S. aureus* had the highest point prevalence amongst the new-borns

(>50%) (Figure 4.1) and was acquired very rapidly by most infants (Figure 4.2), the point prevalence declined to less than 20% by 9 weeks, similar to the report from Western Australian [55]. High nasopharyngeal carriage of *S. aureus* amongst the Gambian neonates may be linked with the high burden invasive *S. aureus* disease in this age group. *S. aureus* was the most frequently isolated pathogen amongst Gambian neonates (less than three months old) with serious infections excluding meningitis [251]. This finding has important implications for *S. aureus* vaccine development and scheduling.

The introduction of the Hib polysaccharide-tetanus toxoid conjugate vaccine in 1997 saw Hib carriage among Gambian children under five years drop from 12% to 0.25% ($p<0.01$) [252-254]. In this study, Hib was found in 0.7% (95CI 0.5%, 0.9%) of the NP swabs and most *H. influenzae* detected was non-typeable (Figure 4.1). Low levels of carriage may explain why Hib is still responsible for a small proportion of IBD amongst Gambian infants despite widespread vaccination [208]. Low levels of Hib carriage in the Gambia may provide a "boosting" effect within the communities [255], preventing higher levels of re-emergence disease. Continued Hib vaccination as well as surveillance of Hib carriage and disease are of great importance in this region.

There is evidence of complex relationships between pneumococci and other respiratory pathogens that co-colonise the nasal and pharyngeal mucosae [102, 256, 257]. In this study, we found that *S. pneumoniae* had a positive association with *H. influenzae* and *M. catarrhalis* consistent with previous reports [53, 55]. The association between *S. pneumoniae* and *H. influenzae* remained significant after

adjusting for confounding factors (age and sex). In contrast, the negative association between *S. pneumoniae* and *S. aureus* was reversed after adjusting for confounding factors. Although there is evidence of direct interference mechanisms between *S. aureus* and *S. pneumoniae* [258], host immune function may play an important role in modulating the association between these two pathogens [53].

The average prevalence of *S. pneumoniae* amongst the infants using *cPSA* gene-based PCR detection was 78% (95CI: 76%, 83%), very close to 79% (95CI: 75%, 82%) which was previously determined by culture for the same group of infants. *S. pneumoniae* was found in 36 (34%) of the 106 culture-negative samples, however, it was not detected in 34 (9%) of the 392 culture-positive samples [201]. The *cPSA* primers used do not detect all pneumococcal serotypes which could be accountable for the failed detections [259]. Broth-enrichment prior to molecular analysis has been shown to improve the molecular detection of low-density *S. pneumoniae* carriage [260]; however, it needs to be determined if broth-enrichment would give comparable enhancement for the other pathogens assayed. The detection of *S. pneumoniae* in the culture-negatives could be associated with the specificity of the *cPSA* gene primers used; hence, the inclusion of an alternate gene locus such as the autolysin gene (l*ytA)* which has been shown to have high specificity could be used to verify the data presented here [261]. The samples were stored at -70°C for up to five years prior to the molecular analysis. Kwambana *et. al.*, provided preliminary evidence that deep frozen-storage of NP swabs in STGG may have differential effects on the detection of some bacterial taxonomic groups [262]. Hence, real time processing of nasopharyngeal swabs may be considered in future studies. The limits of detection

were not determined during this study, which could have important implications for the detection of carriage below the threshold. However, in a quantitative PCR assay (unpublished data) using the same primers, amplification bands on agarose gels were visible up to 10 000 genome copies/uL of purified DNA for all four organisms. However, the pathogens were detectable up to 10 genome copies/uL of purified DNA with quantitative PCR which suggests that this method should be adopted in future assays.

S. aureus had the lowest average prevalence among the infants (<20%). The low rates of detection could be in part explained by the use of lysozyme for the extraction of nucleic acids. Lysozyme does not effectively degrade the *S. aureus* cell wall whereas the inclusion of lysostaphin has been shown to significantly improve the PCR-based detection of *S. aureus* [263]. An important limitation of using culture-independent molecular detection of pathogens is that important antibiotic resistance data cannot be determined.

4.4. Conclusion

It is apparent that *S. pneumoniae, H. influenzae, M. catarrhalis* and *S. aureus* are co-colonisers of the nasopharynx and our results may reflect both synergistic and competitive relations. These pathogens display rapid acquisition in neonates, high carriage and co-occurrence in the nasopharyngeal mucosae during infancy. This has important potential implications for polymicrobial infections, vaccine scheduling and strategies. The relations between these microbes, other colonizers of the nasopharynx and the host immune system are complex [6, 7]. Although it is yet unclear how space and resources are shared amongst co-colonizers, it seems likely that these pathogens carried at high rates play central roles in modulating microbial ecology in the nasopharynx. (PCV-7) which markedly reduces carriage of vaccine and vaccine associated serotypes (56%) in the Gambia is in widespread use [200]. A protein vaccine which may eliminate mucosal carriage of both *S. pneumoniae* and *H. influenzae* is currently under trials [264, 265]. The effect of these vaccines on nasopharyngeal microbial ecology are yet to be described but the maintenance of a healthy ecological balance and prevention of species replacement disease may be of paramount importance in the success of these vaccines [6]. Concerns that treatment and prevention strategies which eliminate key components of the microbial communities and alter the microbial ecology may have long term adverse effects are valid and require more attention [5].

5. Community fingerprinting of the nasopharyngeal microbiome among Gambia infants

5.1 Introduction

Despite significant advances in understanding the microbial communities that inhabit the various mucosal surfaces of the human body, there are significant gaps in our knowledge regarding the pharynx, which includes several clinically significant areas [1]. The pharyngeal mucosa connects the nasal and oral cavities and is divided regionally into the nasopharynx and oropharynx [266]. While there appears to be overlap with the extensively studied oral microbiome [49] and the occurrence of respiratory pathogens such as *Streptococcus pneumoniae, Haemophilus influenzae* and *Moraxella catarrhalis* in the normal pharyngeal microbiota, their significance for health and disease is yet to be comprehensively described.

Microbes colonizing the pharynx can invade the blood stream or migrate to contiguous surfaces of upper and lower respiratory tract (LRT) [267]. Consequently, pharyngeal carriage of respiratory pathogens is a risk factor for the development of otitis media (OM), pneumonia, meningitis and bacteraemia [246, 268]. Droplet secretions from the nasopharynx are also an important mechanism for the horizontal transfer of microbes [269]. Hence, the microbial ecology of the pharynx is closely linked with both the pathogenesis and transmission of respiratory pathogens that are the leading causes of morbidity and mortality among children less than five in developing countries [234].

The development of the nasopharyngeal microbiota in infants amongst communities with a high burden of disease caused by respiratory pathogens is poorly described. It

is essential to understand the full spectrum of nasopharyngeal colonisers as a basis to subsequently investigate the complex interactions and relations between microbial ecology and pathogenesis [84, 270] and, a more comprehensive impact evaluation of drug or vaccine interventions. Nasopharyngeal colonization by S. *pneumoniae* is high (>90%) amongst Gambian infants and the burden of disease caused by respiratory pathogens is several-fold higher than in industrialised nations [243, 251, 271]. Here we describe work characterizing the development and composition of the nasopharyngeal microbiome from birth through the first year of life among Gambian infants using 16S rRNA gene-based T-RFLP community fingerprinting.

5.2 Results

To survey the composition of the infant nasopharyngeal microbiome we used 16S rRNA gene-based partial sequencing combined with a comparative community fingerprinting tool, T-RFLP. 483 NP swabs were collected from 29 neonates (11 female) from birth to one year (mean = 16.6 NP swabs) summarised in Table 5.1. 16S rRNA PCR yield was low (<20ng) for 10.3% of the 483 NP swabs. However, *S. pneumoniae* was detected by culture in 84% of the NP swabs for which amplification yield was low. Hence, T-RFLP statistical analysis was conducted on 90% of the NP swabs collected.

5.2.1. Composition of the nasopharyngeal microbiota

To 351 high-quality clone sequences (~700bp) were obtained from a reference library constructed from amplified 16S rRNA gene fragments. The taxonomic assignment of the 16S rRNA gene sequences was conducted on the RDP-10 Classifier using a 90% confidence filter (Table 5.1). The sequences represented seven phyla, of which

Proteobacteria and *Firmicutes* were predominant (Figure 5.1A). Members of the *Moraxella*, *Streptococcus, Pseudomonas, Haemophilus, Corynebacterium*, *Ornithobacterium* and *Suttonella* accounted for >95% of the sequenced clones. The 16S rRNA gene sequences obtained were subjected to *in silico* digestion to determine the expected 5'-terminal fragments and then matched with the T-RFLP OTUs (Figure 5.1B). The OTUs with >1% abundance that were not assigned identities by clone sequences were putatively classified using the MiCA 3 software and the RDP 10 database.

Table 5.1. Characteristics of study participants

Subject	No. of Swabs profiled	Age at first sampling	Ethnicity	Sex	Birth weight	Antibiotic Courses[a]	Chest infections[a]
1	16	1	Jola	Female	3.5	0	0
2	14	1	Jola	Male	2.6	0	0
3	16	4	Jola	Male	3.5	0	0
4	14	6	Mandinka	Male	3	1	0
5	16	2	Mandinka	Female	2.1	3	0
6	15	2	Other[b]	Male	2.7	2	0
7	16	3	Other[b]	Male	3	1	0
8	13	2	Mandinka	Male	3.2	2	0
9	16	2	Mandinka	Male	2.3	1	0
10	15	2	Mandinka	Female	2.8	1	0
11	16	1	Mandinka	Female	-	0	0
12	17	3	Mandinka	Male	3.2	1	2
13	16	5	Mandinka	Male	3.4	2	0
14	16	4	Jola	Male	3	1	0
15	14	1	Jola	Male	3.2	2	1
16	16	6	Jola	Male	3.2	0	0
17	16	7	Fula	Female	3.4	0	0
18	16	6	Jola	Male	2.7	1	0
19	13	4	Jola	Female	2.9	0	0
20	11	2	Jola	Male	3.5	0	0
21	17	3	Mandinka	Female	2.5	0	0
22	16	5	Mandinka	Male	2.7	1	2
23	14	2	Jola	Male	3.3	2	0
24	17	3	Jola	Male	4	3	1
25	6	3	Fula	Female	3	0	0
26	14	3	Jola	Female	2.6	1	0
27	16	6	Jola	Male	3.2	0	0
28	15	2	Mandinka	Female	3.5	0	0
29	16	1	Jola	Female	3	1	0

[a] Reported within two weeks of nasopharyngeal swabbing

[b] Other ethnicities includes Serer and Serahule

Table 5.2. Bacterial taxa detected by partial 16S rRNA gene sequencing of a nasopharyngeal library

Phylum	Family	Genus	Species*	No. of sequences	Sequence Relative abundance (%)	OTU size (bp)
Fusobacteria	*Fusobacteriaceae*	*Fusobacterium*	*F. nucleatum*	2	**0.6**	**374**
Actinobacteria	*Corynebacteriaceae*	*Corynebacterium*	*C. accolens*	27	**7.7**	**110, 216**
	Micrococcaceae	*Rothia*	*R. mucilaginosa*	10	**2.8**	**216**
Chlamydiae	*Chlamydiaceae*	*Chlamydophila*	*C. pneumoniae*	2	**0.6**	**180**
Bacteroidetes	*Prevotellaceae*	*Prevotella*		1	**0.3**	**-**
	Porphyromonadaceae	*Porphyromonas*		2	**0.6**	**388**
Flavobacteria	*Flavobacteriaceae*	*Chryseobacterium*		1	**0.3**	**132**
	Flavobacteriaceae	*Cloacibacterium*	*C. normanense*	2	**0.6**	**132**
	Flavobacteriaceae	*Ornithobacterium*	*O. rhinotracheale*	22	**6.8**	**132**
	Flavobacteriaceae				**0.6**	**273**
				2		
Firmicutes	*Staphylococcaceae*	*Staphylococcus*	*S. aureus* *S. epidermis*	14	**4**	**520**
	Bacillaceae	*Geobacillus*		1	**0.3**	**91**
	Bacillaceae	*Bacillus*		2	**0.6**	**91**
	Carnobacteriaceae	*Dolosigranulum*		5	**1.4**	**91**
	Streptococcaceae	*Streptococcus*	*S. pneumoniae* *S. peroris*	48	**13.7**	**91**
	Veillonellaceae	*Veillonella*		2	**0.6**	**91**
	Clostridales Incertae Sedis XI	*Parvimonas*		1	**0.3**	**91**
	Clostridiales Incertae Sedis XI	*Helcococcus*		8	**2.3**	**492**
	Peptostreptococcaceae	*Peptostreptococcus*		3	**0.9**	**492**
Betaproteobacteria	*Burkholderiaceae*	*Burkholderia*	*B. cepacia*	12	**3.4**	**518**
Gammaproteobacteria	*Comamonadaceae*	*Diaphorobacter*		1	**0.3**	**518**
	Oceanospirillaceae	*Marinomonas*		3	**0.9**	**116**
	Pseudomonadaceae	*Pseudomonas*	*P. aeruginosa* *P. putida*	39	**11.1**	**309**
	Moraxellaceae	*Acinetobacter*	*A, junii*	2	**0.6**	**278**

	Moraxellaceae	*Moraxella*	*M. catarrhalis* *M. lincolnii* *M. equi* *M. nonliqeifaciens*	80	**22.8**	**114, 147, 392**
	Pasteurellaceae	*Actinobacillus*		2	**0.6**	**278**
	Pasteurellaceae	*Haemophilus*	*H. influenzae* *H. haemolyticus* *H. parainfluenzae*	33	**9.4**	**518, 504**
	Cardiobacteriaceae	*Suttonella*	*S. indologenes*	22	**6.3**	**147**
Unclassified Bacteria				2	**0.6**	**271**

*Species shows the microbes with the highest percentage match to the 16S rRNA gene clone library sequences above, similarity above 97%.

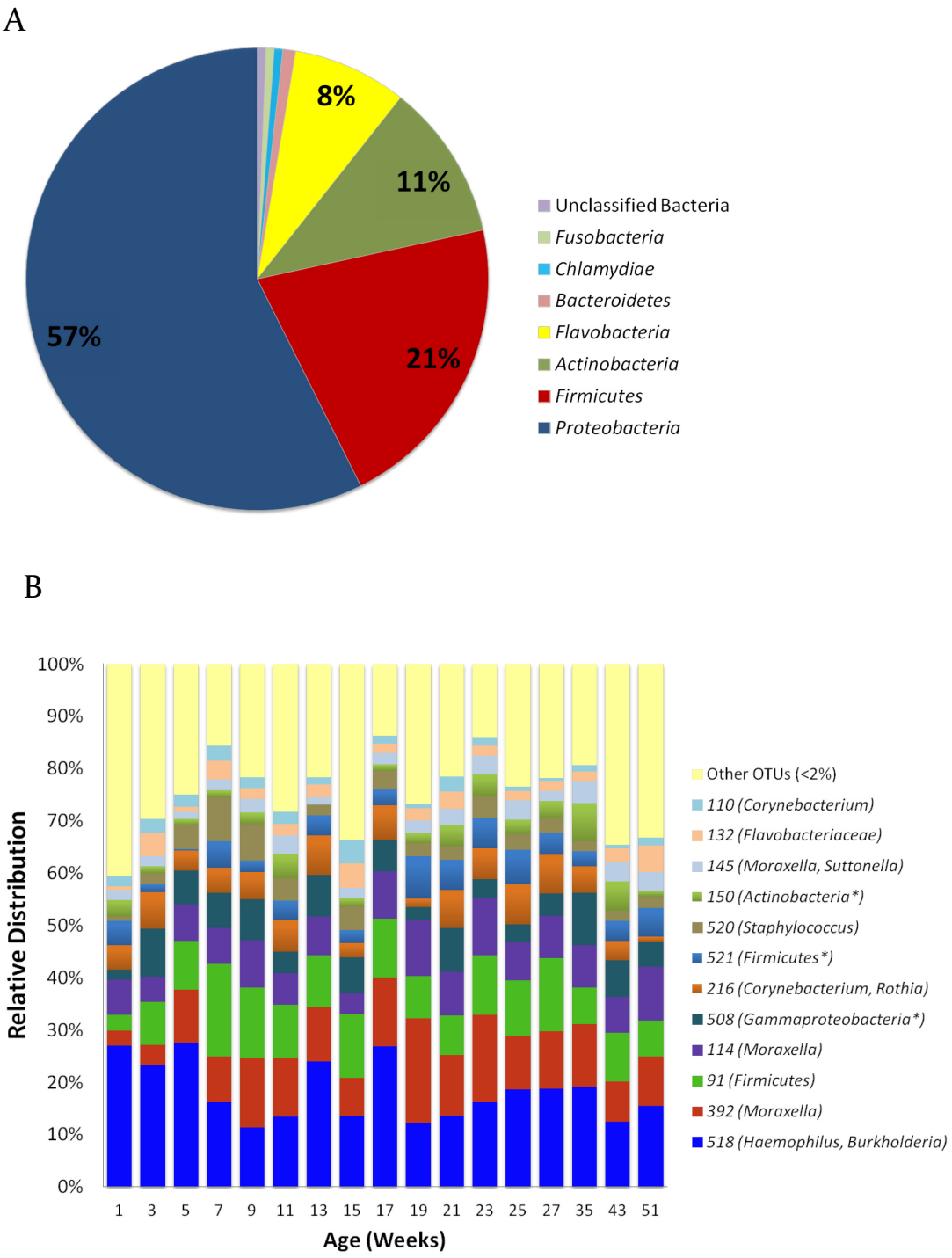


Figure 5.1. The relative distribution of the bacterial taxa in the infant nasopharynx by 16S rRNA gene partial sequencing (A) and T-RFLP analysis (B). 351 high-quality sequences (~700bp) were derived from an infant nasopharyngeal microbiome clone library and classified on the RDP 10 Classifier. The relative distribution of the most abundant bacterial OTUs (>1%) detected by T-RFLP in the nasopharynx from birth to one year. *Putative taxonomic assignments of OTUs were carried out on MiCA3 software using RDP 10 for OTUs with >1% abundance that were not identified by partial sequencing.

Heatmap analysis of the T-RFLP profiles showed that there were two types of community fingerprints amongst the infants. The first and most common community fingerprint found among 24 infants was characterized by the presence of the dominant OTUs in most or all the NP communities. The dominant OTUs were 91bp (*Firmicutes*), 114 (*Moraxella*), 392bp (*Moraxellaceae*), 518bp (*Haemophilus*) and 520bp *(Staphylococcus)* (Figure 5.2). The second community fingerprint found among five infants 1, 2, 11, 17 and 22 was generally characterized the absence of the dominant OTUs (Figure 5.2).

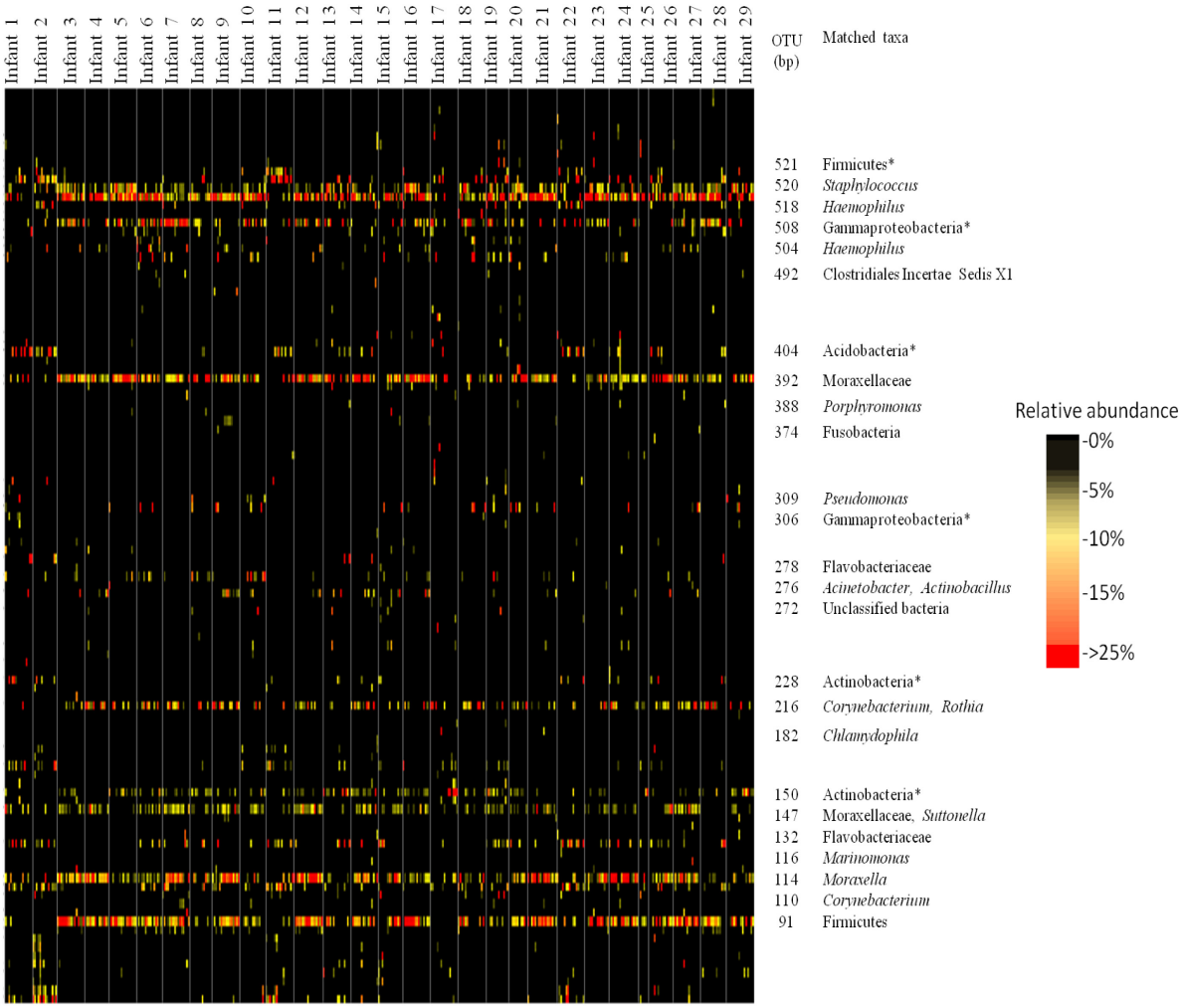


Figure 5.2. Heatmap showing the fingerprints and relative abundance of OTUs found amongst Gambian infants from birth to twelve months. The vertical sections denote community fingerprints from the same infant arranged in order of age at sampling along the horizontal axis with the earliest samples on the left. Taxonomic assignments based on partial sequencing of a nasopharyngeal clone library are presented. *Putative taxonomic assignment for OTUs with >1% abundance that were not identified by partial sequencing was carried out using MiCA3 software and the RDP 10 database

5.2.2. Development of the nasopharyngeal microbiome

To determine the stability of community fingerprints within and across individuals, T-RFLP profiles were sorted according to their clustering with the UPGMA index as a distance matrix (Figure 5.3). Community profiles from NP specimens collected consecutively did not consistently cluster together and there was also no consistent clustering of profiles by age at sampling. Interestingly, there were two distinct clusters, one representing the profiles from infants lacking the dominant OTUs and the other, the profiles from infants with the dominant OTUs.

Bacterial OTUs were detected in all the swabs collected within 7 days of birth (mean= 4.5 OTUs/NP swab) (Figure 5.4). The effect of age on OTU richness was examined using a random intercept model with a random effect for subjects. When the infants were older than one month they had 0.73 (95% CI: 0.13-1.32) more OTUs per NP swabs than before one month (p=0.016). After the rapid initial colonization, OTU richness did not continue to increase linearly, but instead varied around six OTUs per NP swab throughout the first year (Figure 5.4). The variability in OTU richness made it difficult to capture the trends over time amongst all the infants in a single model.

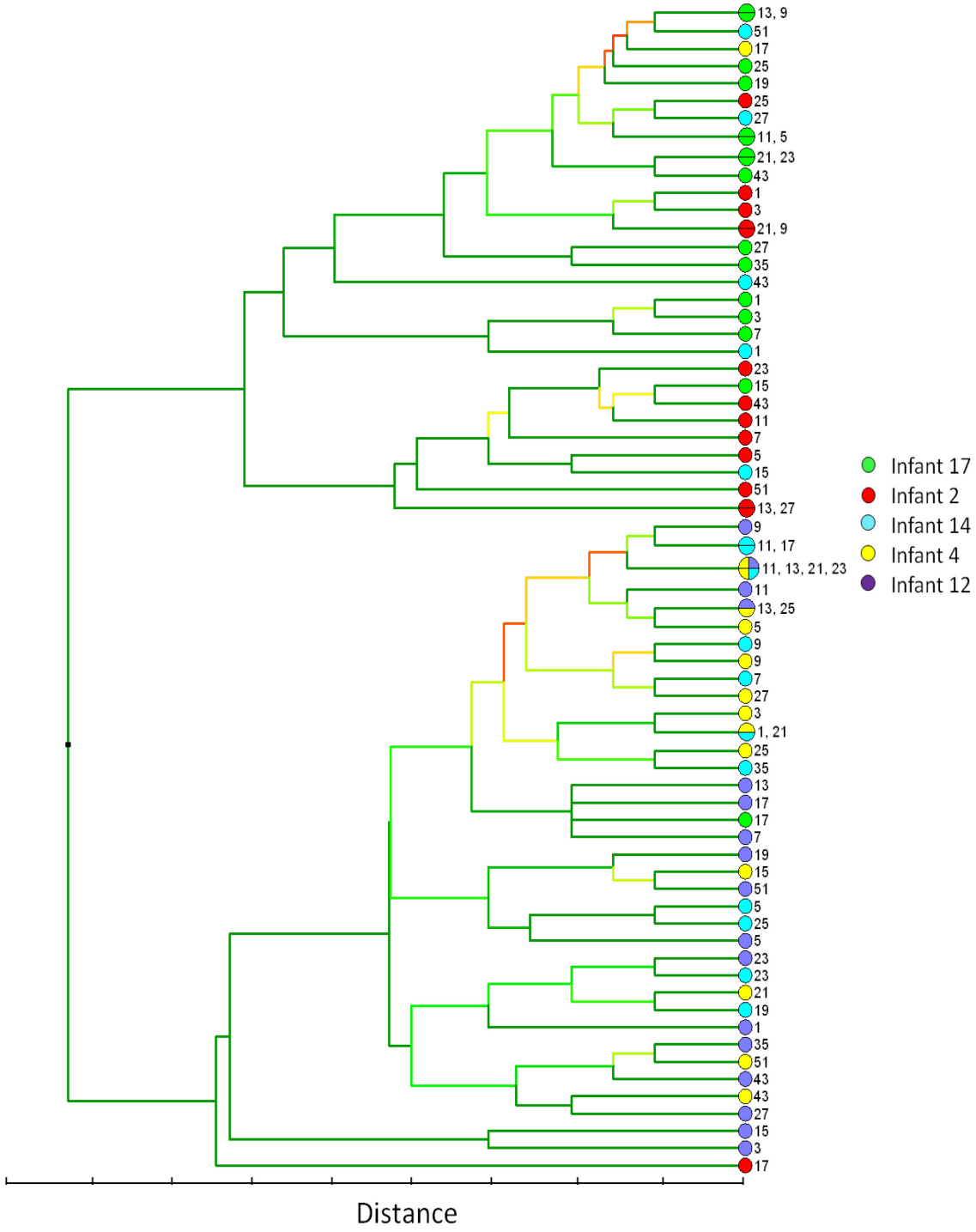
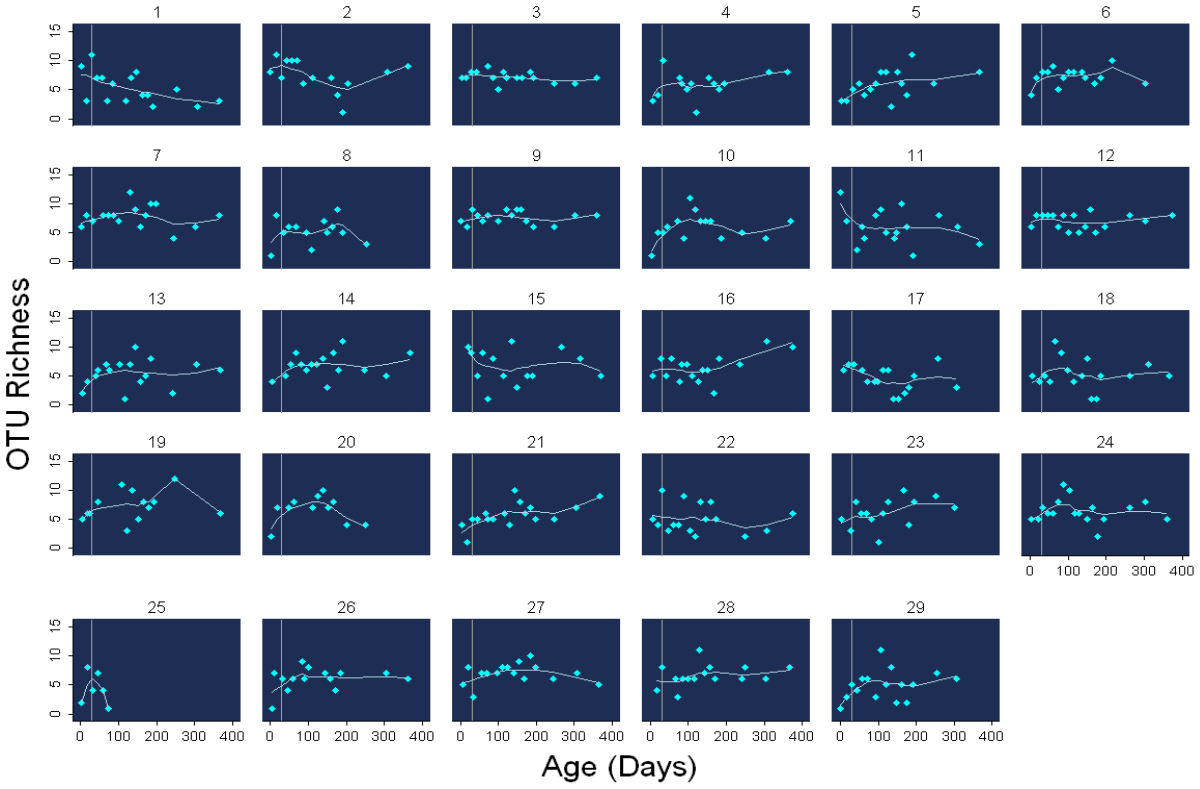


Figure 5.3. Cluster analyses of nasopharyngeal T-RFLP profiles using the UPGMA algorithms. To determine the stability of community fingerprints within and across individuals, T-RFLP profiles were sorted according to their clustering with the UPGMA index as a distance matrix. However, profiles from five infants are included here because of space.

5.2.3. Effect of host and environmental factors

The average number of OTUs associated with NP swabs from male infants was 6.52 (n=273 NP swabs) and female infants 5.73 (n=160 NP swabs). Likewise male infants showed on average 0.78 (95% CI: 0.17-1.39) more OTUs per sample compared to females (p=0.012) after adjusting for age. The rates of acquisition and loss for each OTU (>2% abundance, n=35) were calculated as the total number of events (acquisitions or losses) divided by the person years of follow-up. There was a direct relation between rates of acquisition and loss of OTUs (correlation coefficient: 0.56, p<0.001).

Associations of factors such as weight at birth and at each visit, type of feeding (exclusive breastfeeding vs. mixed feeding) and antibiotic usage with rates of acquisition and loss were examined using parametric survival time regression models. The birth weight ranged from 2.1Kg to 4.0Kg (Table 5.1) and had a significant association with the rates of loss of 95% of the OTUs (>2% abundance). Weight was significantly associated with the rates of acquisition and loss of 60% and 95% of the OTUs respectively. Type of feed had significant interaction with the rates of acquisition and loss of 60% and 34% of the OTUs. 59% of the infants had at least one NP swab collected within two weeks of antibiotic treatment. Antibiotic treatment was significantly associated with the rates of loss of 5.6% of the OTUs.



5.4. The number of distinct OTUs detected at different sampling time points with lowess smoothers for each infant in the first year of life. There was a significant difference in OTU richness between infants older and younger than one month of age (p=0.016) where older infants had on average 0.73 (95% CI 0.13-1.32) more OTUs detected.

5.3. Discussion

The nasopharynx is a reservoir of bacterial pathogens which contribute to high morbidity and mortality among children under five in West Africa [52, 97, 272, 273]. Comprehensive understanding of the breadth of the nasopharyngeal microbiome is necessary to fully characterize the role of microbial ecology in disease and health, particularly in high risk communities. In this study, we have begun to unravel the themes that govern the nasopharyngeal microbiome as a reference for subsequent studies examining the impact of drug and vaccine interventions on its development and ecology.

A striking finding of this study is that there are two distinct microbial community fingerprints amongst Gambian infants which represent a high risk population. The first and most common fingerprint found amongst 83% of the infants is characterized by the heavy presence of *Moraxella, Haemophilus, Staphylococcus* and *Firmicutes* (including *Streptococcus),* which genera all include respiratory pathogens [97, 234] (Figure 5.2). The second community fingerprint found among 17% of the infants was characterized by the lack of these dominant OTUs. Although it is difficult to determine the clinical significance of the microbial community fingerprint described here given the small sample size, there may be some indications regarding its importance.

More than half (67%) of the infants from the first group received at least one course of antibiotics during the twelve months of follow-up (Table 5.1). In contrast, 20% (1/5) of the infants from the second group received antibiotic treatment. Furthermore, three out of four of the infants who developed chest infections during infancy belonged to group 1. Hence, high co-carriage of OTUs that include bacterial pathogens may be an important risk factor for developing infections during infancy. Another possibility is that the less common OTUs carried by infants from the second group have protective effects against carriage of the dominant OTUs. For instance, carriage of *Dolosigranulum* and *Corynebacterium* in the nasal cavity was shown to be protective against colonization by *S. pneumoniae* and the development of otitis media [274].

Another important finding was the early bacterial colonization of the nasopharynx; several bacterial OTUs were detected in swabs collected within days of birth (Figure Figure 5.4). A recent study demonstrated that bacterial nasopharyngeal colonization

may occur within minutes of birth [190]. After the first month, OTU richness varied around six OTUs per NP community amongst the infants (Figure 5.2). This suggests that there may be a normal range for OTU richness and this feature might reflect the proposal that the mucosal immune system controls the magnitude of colonisation at any given point [64].

The infant nasopharynx comprises a few bacterial phyla which colonise early and form the backbone of the microbiome amongst most infants. Numerous low abundance and transient phyla may account for the bulk of the difference between and within individuals (Figure 5.2). It is possible that the infant local nasopharyngeal immune system selectively "tolerates" the high abundance taxonomic groups and eliminates the rare OTUs [64]. High rates of acquisition were directly associated with high rates of loss (correlation coefficient: 0.56, $p<0.001$). This finding could be an indication that there is rapid turnover of bacterial taxa in the nasopharynx, as OTUs are acquired continuously acquired and eliminated. Another possibility is that bacterial taxa reported as absent were present but below the detection threshold of the analytical approach used. None the less, there appears to be a continuous succession of bacterial taxa in the infant nasopharynx.

There was no evidence of clustering among NP swabs collected consecutively from the same infant which suggests that the infant nasopharyngeal microbiome is in a constant state of flux, with continuous succession of microbes [267]. The most successful colonizers may compete for space, resources, growth factors and nutrients more effectively, outcompeting low abundance taxa [51].

At least sixty different species have been cultured from the pharyngeal mucosae of healthy individuals, representing five phyla; *Firmicutes, Proteobacteria, Bacteroidetes*, *Fusobacteria* and *Actinobacteria* [33, 46, 55]. Community fingerprinting identified over a hundred taxonomic units in the nasopharyngeal mucosa of the infants representing seven phyla and twenty-seven genera (Table 5.2). Taxonomic groups which are rarely described in culture-based community studies were amongst the most abundant microbes detected, i.e. *Ornithobacterium, Cloacibacterium*, *Rothia*, *Dolosigranulun*, Clostridiales Incertae Sedis XI, and *Suttonella* (Figure 5.4). This finding suggests that the spectrum of organisms that colonize the nasopharynx could be several fold-higher than described in culture-based investigations.

Laufer et al. surveyed the nasal microbiome among 108 children presenting with upper respiratory tract infections (URTI) [274]. Similar to the findings of this study, Proteobacteria was the predominant phylum and *Moraxellaceae* (25.6%), *Streptococcus* (17.9%) *Corynebacterium* (7.04%), *Haemophilus* (4.66%) and *Staphylococcus* (3.84%) were the most abundant genera found (Figure 5.1). These findings suggest that the nasal microbiome amongst children with URTI has wide overlap with the infant nasopharyngeal microbiome. In contrast, the oral microbiome amongst children and adults appears to bear a distinct fingerprint. *Veillonellaceae, Prevotella, Neisseraceae* and *Actinobacteria,* found at low abundance (<1%) in the nasopharynx are amongst the most abundant taxa in saliva [275-279]. Unlike the oral cavity where anaerobes such as *Porphyromonas, Prevotella* and *Fusobacterium* represent a significant part of the indigenous microbiota [83], anaerobes comprise a small proportion (<2%) of the nasopharyngeal microbiome. Moraxellaceae, which was

the most abundant taxonomic group in the nasopharynx, had <5% abundance in the saliva of children >3 years [280]. However, the infant oral microbiome which is currently poorly characterised may show wider overlap with the infant nasopharyngeal microbiome.

Consistent with previous findings [29], factors that were shown to influence the carriage of OTUs were age, sex, weight, ethnicity, type of feeding and antibiotic treatment. There are distinct age-dependent differences in immune function which may dictate the outcome of encounters with microbes ranging from immediate clearance, transient colonisation, commensal carriage to harmful invasion [69]. Gender has been shown to influence the carriage of some nasopharyngeal colonisers such as *S. pneumoniae, S. aureus* and *H. influenzae* [281]. Although these differences may be attributable to behavioural and physiological differences, immune function may play an important role. Female adaptive and innate immune responses mounted to pathogen challenges are more robust than in males [282]. This may explain why male infants showed on average higher OTU richness compared to females, even after adjusting for age. It is also possible that the differences observed were in part attributable to the deep frozen storage of the NP swabs in -70^{o}C [283].

OTUs tended to have broad specificities, representing a wide spectrum of related bacterial taxa, hence the total species diversity is most probably underestimated by T-RFLP and sequencing alone. It would be useful to validate some of the findings of this study using a culture-based approach as some of the bacterial taxa detected may represent dead organisms. T-RFLP profiles could not be generated for 10% of the NP

swabs due to poor amplification of the 16S rRNA gene, possibly due to DNA degradation during frozen storage [283]. However, this basal characterization of the infant nasopharyngeal microbiota forms the basis for further investigations of the microbiome in high risk populations. The source of the nasopharyngeal microbiome, the immune correlates of colonization and the impact of genetic factors, antibiotic usage, vaccination and other environmental stresses on the microbiome need further exploration.

5.4. Conclusion

The impact of drug and vaccine interventions on nasopharyngeal microbial ecology is not well understood, particularly in the very young amongst whom the carriage and burden of disease by respiratory pathogens is highest. This necessitated the basal characterization of the nasopharyngeal microbiome conducted out in this study, which provides a platform for further studies investigating the source, ecology, function and metabolic activity of the components of the nasopharyngeal microbiota. The results of this study show that respiratory pathogens co-colonize a milieu with numerous microbes in a constant state of flux which may play a role in maintaining health or pathogenesis. Further explorations using more advanced culture-independent tools such as next-generation sequencing technologies and high density microarrays are needed to unveil the components and functions of the nasopharyngeal microbiome.

6. Temporal effects of PCV-7 vaccination on the ecology of the nasopharyngeal microbiome

6.1. Introduction

The large burden of *S. pneumoniae* disease worldwide has necessitated the development and implementation of effective vaccine strategies. However, *S. pneumoniae* has high antigenic diversity of the polysaccharide capsule [284] and 6-11 of the 94 serotypes are associated with over 70% of paediatric *S. pneumoniae* IBD globally [285]. The development of efficacious vaccines against *S. pneumoniae* has been challenging and it has not been possible to construct a conjugate vaccine covering all *S. pneumoniae* serotypes for technical reasons. Hence, all currently licensed polysaccharide-targeted conjugate vaccines are of limited valency, covering a subset of circulating serotypes [239]. One approach to increase coverage beyond a given number of serotypes is the development of pneumococcal vaccines based on common, conserved pneumococcal proteins. GlaxoSmithKline Biologicals has developed such a pneumococcal candidate vaccine containing pneumococcal proteins that are highly conserved among *S. pneumoniae* strains: pneumolysin toxoid (dPly) and histidine triad protein D (PhtD) [264]. This vaccine is currently undergoing clinical trial in children in The Gambia.

Currently, three pneumococcal conjugate vaccines containing 7, 10 and 13 pneumococcal serotypes respectively are available for use in children less than five. The 7-valent pneumococcal polysaccharide-diphtheria CRM_{197} protein conjugate vaccine (PCV-7) now part of the routine immunization programmes in many countries have been available since 2000 and are particularly efficacious in infants. Widespread

use of PCV-7 markedly reduces carriage of vaccine serotypes amongst both vaccinated and unvaccinated individuals [286-288].

7-Valent	4	6B	9V	14	18C	19F	23F						
10-Valent	4	6B	9V	14	18C	19F	23F	1	5	7F			
13-Valent	4	6B	9V	14	18C	19F	23F	1	5	7F	3	6A	19A

Figure 6.1. The pneumococcal conjugate vaccine serotypes

PCV-7 was introduced in the routine immunization programme in the Gambia in August 2009 and PCV-13 was introduced in June 2011. Although the PCVs are a remarkable public health success story, their long term success is threatened by the emergence of serotype replacement, serotype switching and species replacement disease [200]. Few studies have reported changes in the epidemiology of bacterial diseases associated with widespread use of PCV-7 [218, 289, 290]; however, it is unclear how these changes are related to nasopharyngeal microbial colonisation. The issue of replacement disease is of particular interest within the Gambian context where infant pneumococcal nasopharyngeal carriage rates can exceed 90% and PCV-7 could eradicate approximately 63% of circulating serotypes [201, 291].

Although serotype replacement associated with the PCV-7 has been reported, the impact of vaccination on the nasopharyngeal microbiome which may influence replacement disease has not been determined. This study sets out to begin to address a question of public health concern regarding the long term effects of PCV-7 on nasopharyngeal microbial ecology; does PCV-7 vaccination significantly alter the

composition and development of the infant nasopharyngeal microbiome among infants and neonates? This study begins to answer this question by the culture-independent approach employing deep sequencing tools.

6.1. Results

6.1.1. Study infants

Twenty-two out of 102 infants recruited at birth and followed for a year were selected for the 454-pyrosequencing analysis. Seventeen NP swabs were collected from each infant within seven days of birth, biweekly for the first six months and then bimonthly for another six months (n = 374 NP swabs). The infants were divided into two groups, the early vaccination group (EV) and the late vaccination group (LV) (Table 6-1). The EV group comprised 12 infants recruited from villages with previous exposure to PCV-7. The EV group of infants received three doses of the PCV-7 at two, three and four months. The LV group comprised 10 infants recruited from PCV-7 vaccine naïve villages. The LV group of infants did not receive PCV-7 until they were at least eight months old. The mean birth weight was 3.25Kg (range 2.5 – 4.2Kg) and the infants had between one and eight siblings (mean = 2.8). All the infants were breastfed and a mixed diet including water was introduced between 1 week and six months (mean=4.5 months). 9 (75%) of the infants from the EV group had at least one sibling who had received the PCV-7 and 7 (58%) had PCV-7 vaccinated mothers. In contrast, none of the LV group infants had a vaccinated mother or sibling. Overall 32% of the infants received at least one course of antibiotic treatment within two weeks of NP sampling. Of the seven infants who had at least one chest infection, 71% were from the LV

group. Three infants (14%) had at least one episode of ear discharge during the first year of life.

6.1.1. 454-pyrosequencing analysis

Over 2.2 million sequences were obtained from 93% of the 374 NP swabs collected from 22 infants. 7 % of the NP swabs had poor amplification of the 16S rRNA gene. A mean of 6371 (95CI 6074-6668) clean sequences $\geq$ 300bp were obtained per sample with an average read length of 463bp. 814 operational taxonomic units (OTUs) were classified using the Ribosomal Database Project (RDP) classifier and represented over 30 Bacterial phyla. Of these, 83% were classified at the genus and subgenus level. 11.1% were classified at the family and subfamily level and 1.5% at the phylum and subphylum level. More than 90% of the 438 NP communities had at least 5 of the following 6 organisms, *Streptococcus, Haemophilus, Moraxella, Pseudomonas, Corynebacterium* and *Staphylococcus*.

6.1.1. Effect of early PCV-7 intervention on composition

Proteobacteria, Firmicutes, Actinobacteria and *Bacteroidetes* were the dominant phyla among both EV and LV infants (Figure 6-2). However, the proportion of *Actinobacteria* was 7% among EV infants, six-fold higher than among LV infants. In contrast, the proportion of other phyla with individual abundance less than 1% abundance was 12%, six-fold higher among LV compared to EV infants (Figure 2). The relative distribution of genera per phylum was comparable among EV and LV infants. *Firmicutes, Actinobacteria* and *Bacteroidete*s were dominated (50% - 90%) by *Streptococcus, Corynebacterium* and *Ornithobacterium* respectively. *Moraxella, Pseudomonas* and

Haemophilus made up >80% of the *Proteobacteria* (Figure 6-2). Only *Streptococcus* was present in all the NP swabs (Figure 6-3).

Table 6-1. Characteristics of the study infants

		Early Vaccination		Late Vaccination		Total	
Characteristic	**Variable**	**n**	**%**	**n**	**%**	**n**	**%**
Sex	Male	9	(75)	3	30	12	55
	Female	2	17	7	70	9	41
Type of birth	Vaginal	12	100	10	100	22	100
	Caesarean	0	0	0	0	0	0
Ethnicity	Mandinka	4	33	3	30	7	32
	Fula	7	58	5	50	12	55
	Jola	1	8	2	20	3	14
Vaccinated mother	Yes	7	58	0	0	7	32
	No	5	42	10	100	15	68
Vaccinated sibling(s)	Yes	9	75	0	0	9	41
	No	3	25	10	100	13	59
Place of birth	Home	11	92	4	40	15	68
	Health centre	1	8	2	20	3	14
	Hospital	0	0	4	40	4	18
***Antibiotic Treatment**	Yes	2	17	5	50	7	32
	No	10	83	5	50	15	68
***Chest infection**	Yes	6	50	2	20	8	36
	No	6	50	8	80	14	64

*Reported within two weeks of NP swab collection

Pseudomonas had abundance below 1% in the first 5 weeks, but the abundance rose sharply thereafter. Conversely, *Staphylococcus* made up ~30% of the microbiome soon after birth, but rapidly declined to <1% by 13 weeks among both EV and LV infants. There were also notable differences the relative distribution of *Shewanella, Dolosigranulum, Ornithobacterium,* and *Lactococcus* during the first 27 weeks (Figure 6-3). Overall, the relative abundance of the most abundant OTUs appeared more stable among EV infants, compared to LV infants for which larger fluctuations were observed throughout the first 27 weeks.

In order to confirm the statistical significance of these trends, the OTU abundance in the early weeks (1-17 weeks) and the late weeks (18-26 weeks) was compared for each subject using the Wilcoxon signed rank test (Figure 4). The abundance of *Staphylococcus* and *Corynebacterium* decreased significantly during the late weeks among both groups of infants, however, *Dolosigranulum* and *Sphingomonas* decreased significantly among LV infants only. Likewise, *Streptococcus, Lactococcus* and *Aeromonas* and five other OTUs increased significantly after the early weeks among LV but not EV infants. Overall, there were more significant changes in OTU abundance amongst LV infants between early and late weeks.

6.1.2. Effect of PCV-7 on *Streptococcus*

Among LV infants, the abundance of *Streptococcus* was below 5% in the first week; this gradually increased and fluctuated about 20% and 60% after 7 weeks. In contrast among EV infants, *Streptococcus* fluctuated between 30% and 50% and the vaccine doses administered at 7, 11 and 13 weeks did not appear to cause a decline in the abundance of *Streptococcus* (Figure 6-3). Although the abundance of *Streptococcus* declined by 20% between weeks 21 and 25 among EV infants, there was a similar decline observed among LV infants who had not received PCV-7.

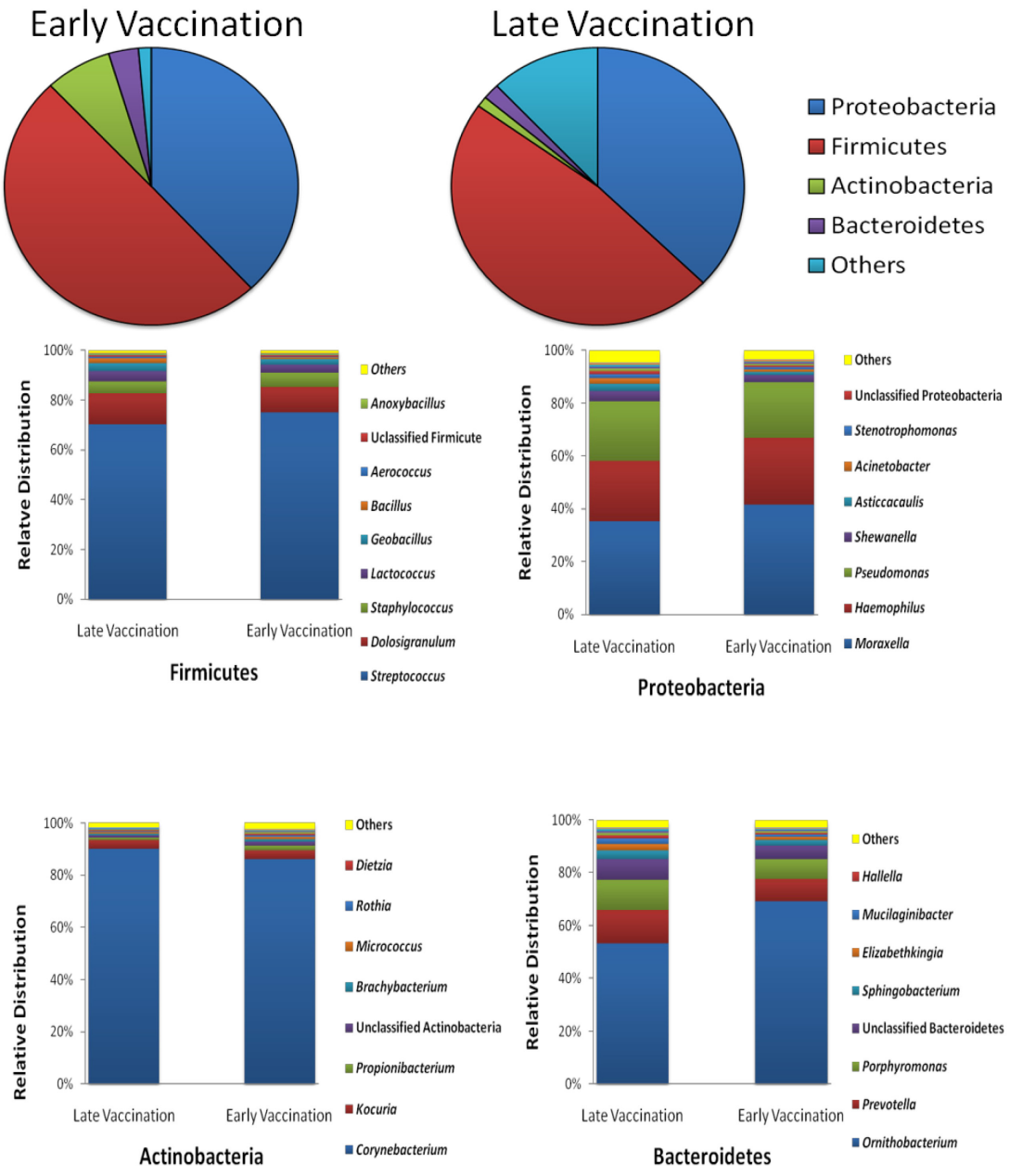


Figure 6.2. The relative distribution of the most abundant Phyla and Genera among early and late vaccination infants. NP swabs collected biweekly from the infants from birth to twenty-seven weeks were analysed using 16S-based 454-pyrosequencing, this included NP samples from twelve infants vaccinated early (<4 months) and ten infants vaccinated late (>8 months)

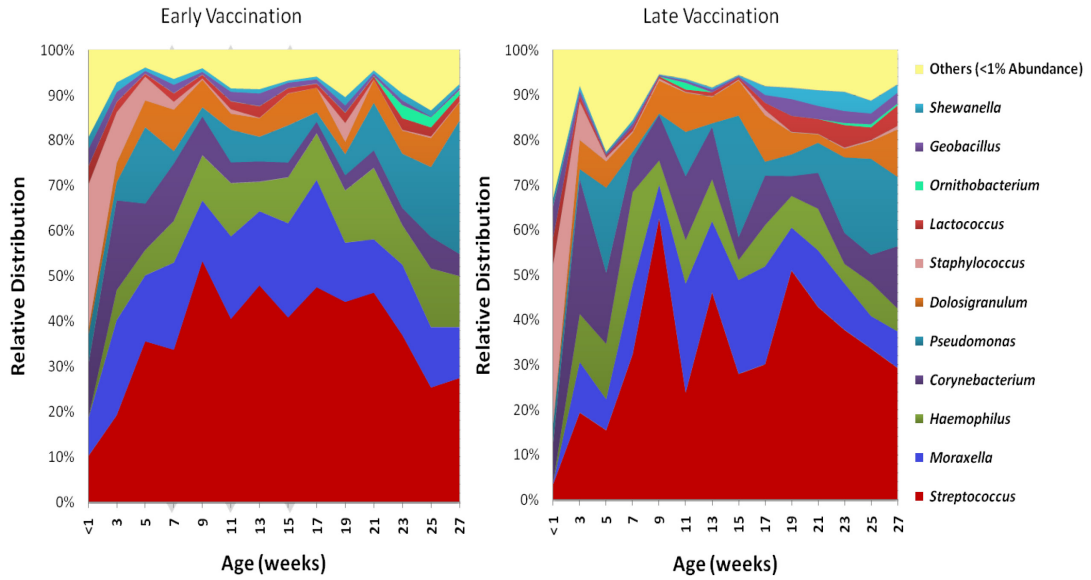


Figure 6.3. The relative distribution of the OTUs found in the NP microbiome among early and late vaccination infants throughout the first 27 weeks of life.

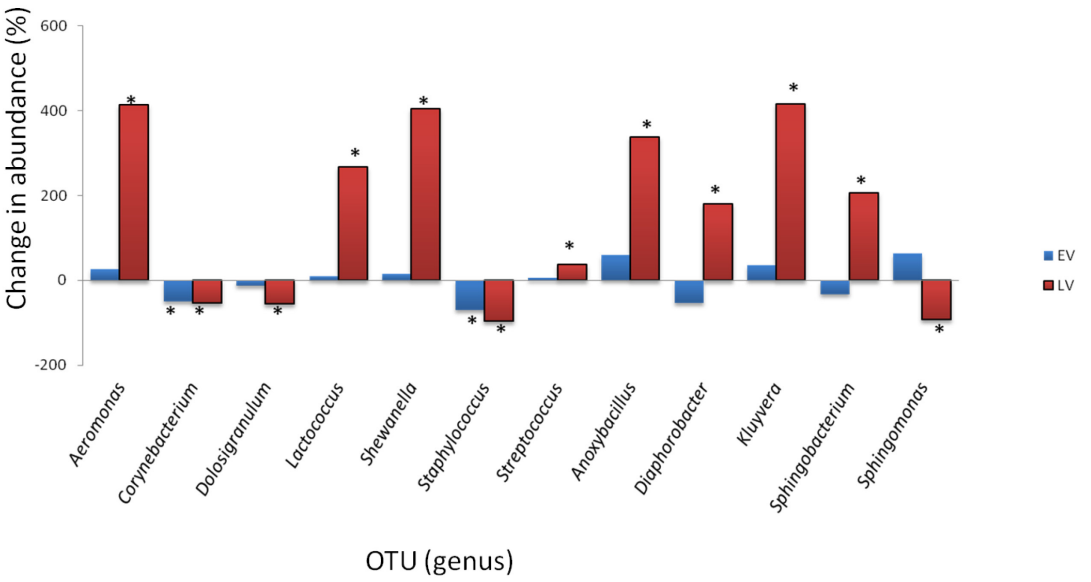


Figure 6.4. The change in OTU abundance between weeks 1-17 (early vaccination period) and weeks 18 -26 among EV and LV infants. The mean abundance of each OTU during weeks 1-17 and weeks 18-26 were computed for each subject. The Wilcoxon test was used to determine significant changes between the two time periods. OTUs for which no significant changes were found were excluded from the graph. * Above the bar represents a significant change ($p<0.05$). OTUs with less than 1 read per 1000 reads were excluded from the analysis to avoid spurious results due to very small number fluctuations.

6.1.1. Effect of PCV-7 on microbial ecology

There was no difference in OTU richness (no. of OTUs found per NP swabs), 53.3 (95CI 49.3 – 57.2) and 51.5 (95CI 48.0 – 55.2) among EV and LV infants respectively. OTU richness varied about 50 throughout the first year amongst most infants; however, there were sharp spikes in OTU richness, over 200 OTUs per NP swab between weeks 17 and 30 amongst the infants (Figure 6-5). However, after week 30, OTU richness remained below 125 OTUs per NP swab among both LV and EV infants. Although not significant, it appears that there was an increase in both the Shannon (p=0.084) and Simpson α diversity indices (p=0.105) between the early and late weeks among LV infants (Figure 6-6). The increase in diversity was coupled with a decrease in Dominance among LV infants (Figure 6-6). Overall, all diversity indices were comparable in late and early weeks amongst EV infants. In both groups of infants, nasopharyngeal communities were dominated by a few OTUs and >99% of the OTUs detected represented less than 1 in 1000 sequences per NP swab (Figure 6-7).

Table 6-2. Pairwise correlations of OTUs with *Streptococcus* among LV and EV infants

			Late Vaccination		Early Vaccination	
16S rRNA Region	OTU (Genus)	*Proportion/1000 sequences*	Coeffecient	p value	Coeffecient	p value
V1-V3	*Moraxella*	143.7	-0.2	1.00	**-0.4**	**<0.001**
	Haemophilus	89.8	-0.3	1.00	0.0	1.00
	Corynebacterium	86.8	**-0.4**	**<0.001**	**-0.3**	**<0.001**
	Pseudomonas	80.3	**-0.3**	**0.00**	**-0.3**	**<0.001**
	Dolosigranulum	55.7	**-2.9**	**0.02**	**-0.4**	**<0.001**
	Staphylococcus	26.3	-0.2	1.00	**-0.3**	**0.01**
	Lactococcus	17.4	-0.3	0.06	**0.3**	**<0.001**
	Ornithobacterium	15.5	-0.1	1.00	-0.1	1.00
	Geobacillus	13.3	-0.2	0.61	-0.2	0.16
	Shewanella	12.1	-0.2	0.14	**-0.3**	**<0.001**
	Asticcacaulis	6.3	-0.1	1.00	-0.1	1.00
	Bacillus	5.8	-0.2	1.00	-0.1	1.00

***Only OTUs with mean abundance greater than 5/1000 sequences per NP swab were included in this analysis.**

6.1.1. Effect of PCV-7 on microbial competition

Streptococcus had negative correlations with all OTUs with mean abundance greater than 1 in 1000 sequences except for *Haemophilus* among EV infants (Table 6-2). Significant correlations were found between *Streptococcus* and several OTUs, *Corynebacterium, Pseudomonas, Dolosigranulum, Staphylococcus, Moraxella, Lactococcus, Shewanella* and *Ornithobacterium* among EV infants (Table 6-2). However, among LV infants, only the correlations with the first three organisms were significant. *Shewanella, Lactococcus, Geobacillus* and *Aeromonas* were the only OTUs with consistent strong correlations over time amongst the infants (Table 6-3).

To visualise the competitive relations between *Streptococcus* and *Corynebacterium, Pseudomonas, Dolosigranulum, Staphylococcus* and *Lactococcus,* the relative abundance was plotted against time for each infant, trends from 8 infants presented in Figure 6-8. The abundance of *Streptococcus* fluctuated frequently between 0% and 90% throughout infancy and the deeps in abundance were usually accompanied by spikes in the abundance of all or at least one of the competing OTUs, particularly *Pseudomonas*.

Table 6-3. OTUs that were consistently correlated over time among all the infants

Genera	Min. corr.	Max. corr.	p value	No. weeks
Shewanella & Aeromonas	0.93	>0.99	<0.01	11
Shewanella &Lactococcus	0.70	0.99	<0.01	16
Shewanella & Geobacillus	0.70	0.98	<0.01	14
Aeromonas & Shewanella	0.91	0.99	<0.01	14
Aeromonas & Lactococcus	0.64	0.98	0.05	11
Aeromonas & Geobacillus	0.65	0.98	<0.01	11
Lactococcus & Shewanella	0.77	0.99	<0.01	10
Lactococcus & Aeromonas	0.72	0.97	<0.01	16
Lactococcus & Geobacillus	0.57	0.99	<0.01	11
Geobacillus & Shewanella	0.74	0.98	<0.01	14
Geobacillus &Aeromonas	0.72	0.98	<0.01	14
Geobacillus & Lactococcus	0.59	0.99	<0.01	10

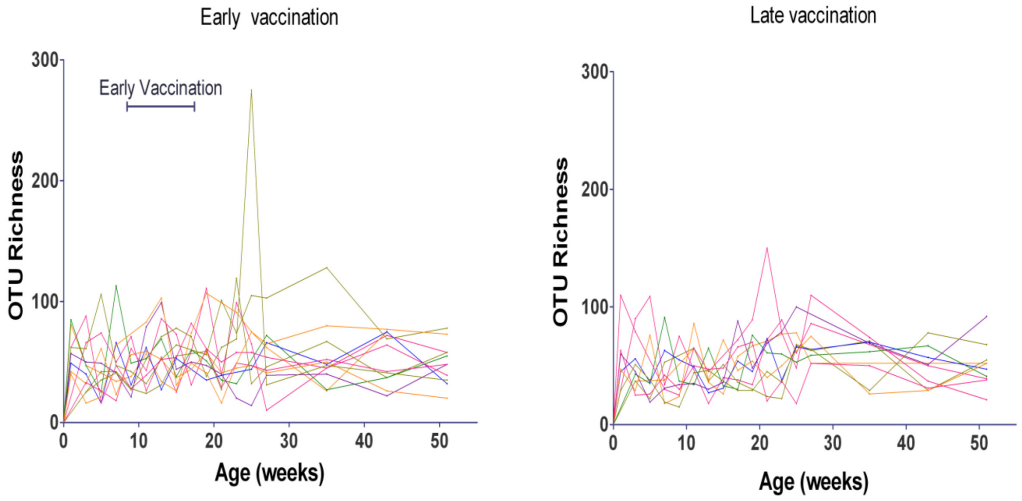


Figure 6.5. OTU richness amongst EV and LV infants throughout the year of follow-up.

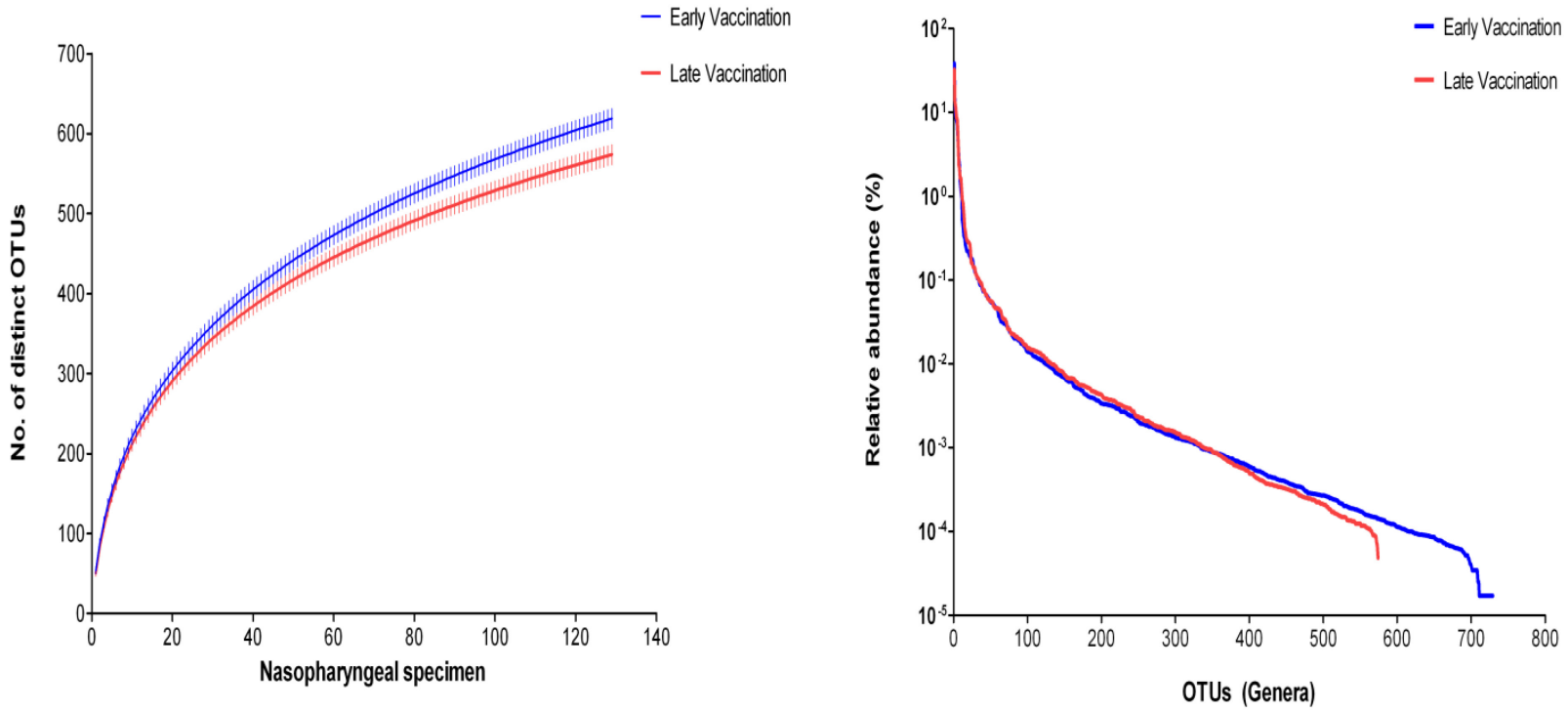


Figure 6.6. Rarefaction analysis and rank abundance for OTU detection in the nasopharynx among EV and LV infants.

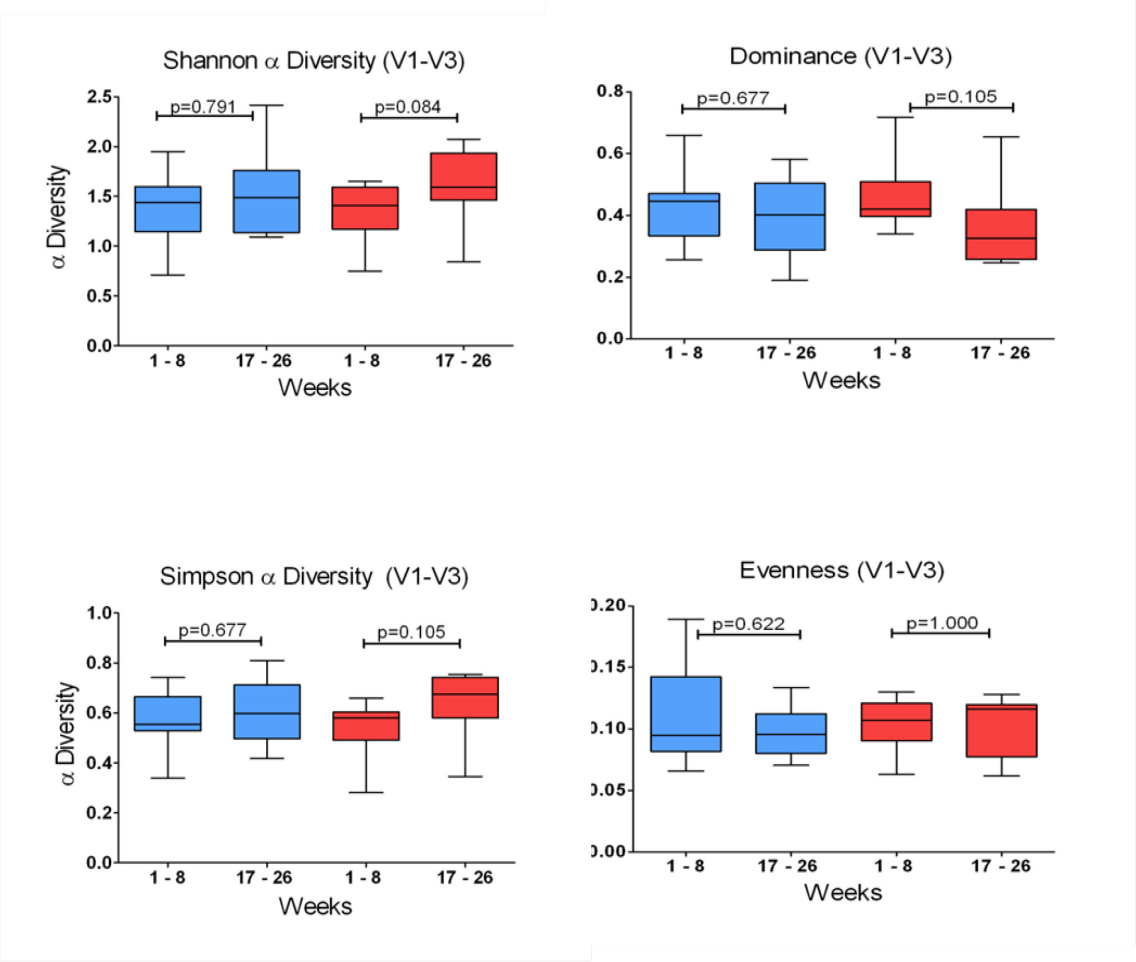


Figure 6.7. Diversity indices amongst EV and LV infants during and after the early vaccination period.

6.2. Discussion

PCV-7 eliminates a subset of pneumococcal serotypes (vaccine serotypes) from the normal nasopharyngeal microbiota but the indirect effects of immunization on nasopharyngeal microbial ecology are poorly understood. There is growing concern that treatment and prevention strategies which alter the normal balance of microbial communities could have temporal or pervasive adverse effects on the host [28]. Eradication of vaccine serotypes creates a vacant ecological niche and replacement with non-vaccine serotypes invariably occurs following PCV vaccination [292-301]. A

striking element of serotype replacement is that it is mostly due to the expansion of co-colonizing non-vaccine serotypes as opposed to de novo emergence of novel pneumococcal clones [292]. However, it is yet unclear if replacement with pre-existing microbes other than *S. pneumoniae* occurs after vaccination. Hence, to determine if replacement with other microbial taxa occurred amongst the EV infants, we compared the relative distribution in the first 17 weeks to the 8 weeks after vaccination of EV infants.

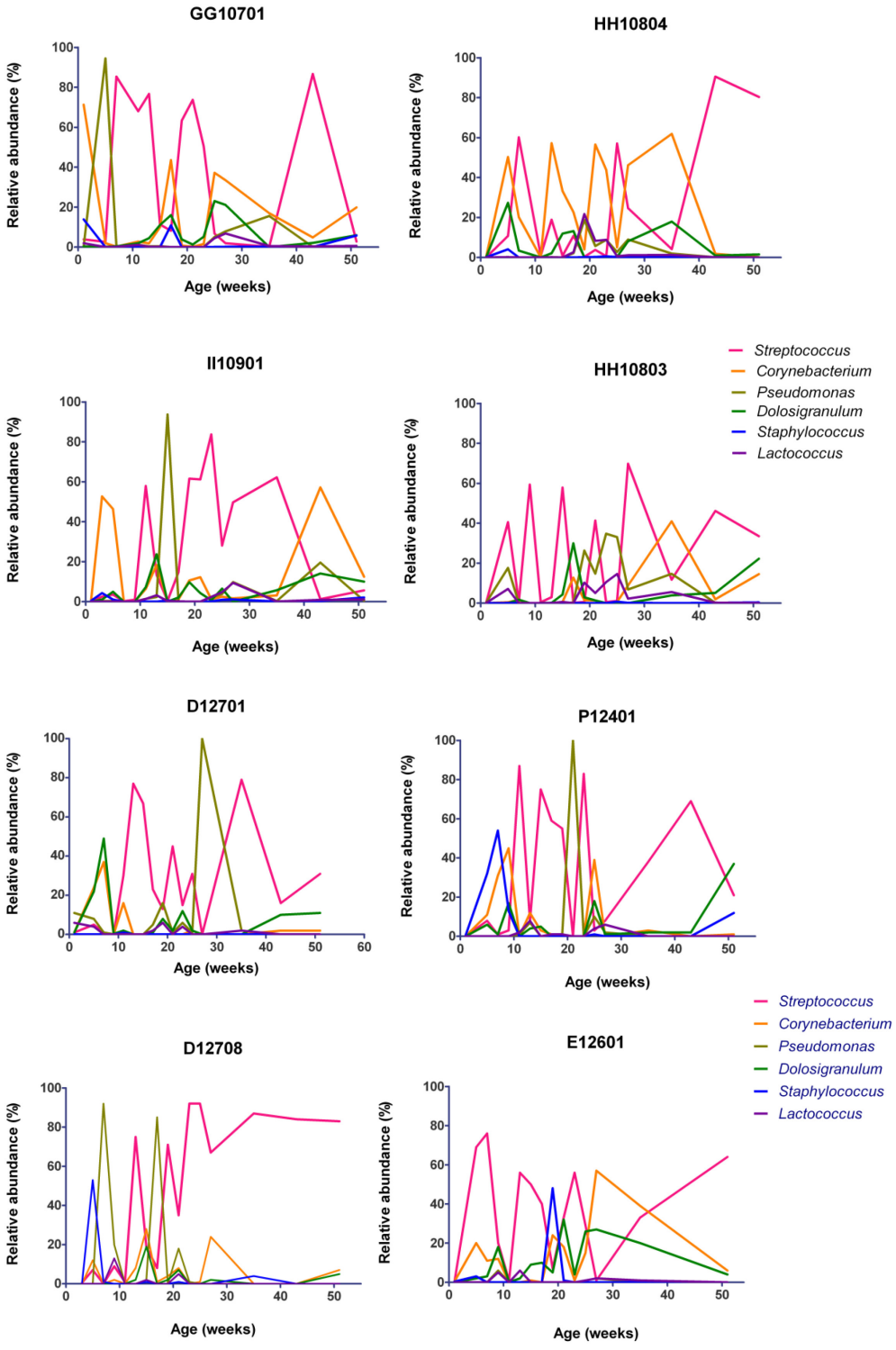


Figure 6.8. Taxonomic competition among microbes negatively correlated with *Streptococcus* in the first year of life among Gambian infants. EV infants are (D12701, P12401, D12708, E12601) and LV infants are (GG10701, HH10804, II10901, HH10803).

6.2.1. Impact of PCV-7 vaccination on the microbial distribution

Interestingly, there was no significant increase in the abundance of any of the OTUs found amongst EV infants (Figures 6-3 and 6-4). On the contrary, significant increases in the abundance of OTUs were found among the infants that had not been vaccinated (LV group) during the 8 weeks after the early vaccination period. *Shewanella, Aeromonas, Lactococcus* and *Geobacillus* which had strong positive correlations with each other over time and negatively correlated with *Streptococcus* were amongst the OTUs that increased significantly among LV infants only (Tables 6-2 and 6-3). Likewise, the abundance of *Dolosigranulum* and *Sphingomonas* decreased significantly among LV group infants only. Consistent with a recent 16S rRNA gene-based pyrosequencing study conducted among children in the Netherlands, Proteobacteria were the predominant phylum (64%) in the nasopharyngeal microbiota. Firmicutes, Bacteroides, Actinobacteria and Fusobacteria were the other phyla found [23]. Likewise, *Moraxella, Haemophilus, Streptococcus, Dolosigranulum* and *Corynebacterium* were the dominant phyla [23]. These findings suggest that there may be a large overlap in the nasopharyngeal microbiome

Hence, in this study, there was no evidence to support that PCV-7 directly results in increased abundance of OTUs co-colonizing the nasopharynx during the weeks ensuing vaccination. Currently, most of the evidence to support species replacement associated with PCV is derived from investigations conducted in sick children. Revai *et al.* found that among children (<5 years) with acute otitis media (AOM), vaccinated children had higher nasopharyngeal carriage of *M. catarrhalis* than unvaccinated and under immunized children (< 3 doses) (p=0.006) [219]. The rates of *H. influenzae*

colonization during AOM episodes also increased although not statistically significant (p=0.17). Several studies have found an increase in the proportion of AOM attributed to non-vaccine types of *S. pneumoniae*, non-typeable *H. influenzae (*NTHi) and *M. catarrhalis* associated with PCV-7 immunization [302-306]. However, it is important to note that the normal balance of the nasopharyngeal microbiota may be perturbed during infections of the middle ear and respiratory tract, usually characterised by an increase in the abundance of pathogens [34, 35, 80, 81, 229].

In this study, there were no significant changes found in the abundance of *Haemophilus* and *Moraxella* OTUs. Although the abundance of the *Staphylococcus* OTU declined significantly in the 8 weeks post vaccination amongst EV infants, a significant decline was also observed amongst LV infants (Figure 4). This suggests that the decline in *Staphylococcus* may be attributable to other factors other than PCV-7 vaccination [307]. The increases in the carriage of pathogenic bacteria associated with PCV-7 vaccination are not adequately demonstrated in healthy individuals.

PCV vaccination does not necessarily result in reduced pneumococcal carriage because replacement with non-vaccine serotypes invariably occurs after vaccination. Since unambiguous species level identification was not achievable using the ~400bp 16S rRNA gene sequences, the trends in *Streptococcus* were examined and showed no significant change associated with vaccination (Figure 3). Therefore, any replacement that occurred may have been primarily driven by non-vaccine *S. pneumoniae* serotypes or other Streptococci. This reinforces the need for typing of *S. pneumoniae* carried by the infants using highly sensitive techniques such as PCR, multilocus sequence typing

(MLST) [308] and microarray analysis which can detect multiple serotype carriage including low abundance capsular types [309]. Changes in abundance that occurred at the species or subspecies level could have been missed.

6.2.2 Impact of vaccination on microbial associations

Despite the lack of evidence to support the occurrence of OTU replacement among PCV-7 vaccinated infants, there was evidence suggesting that vaccination in communities with high immunologic pressure affects the early development of the microbiome. Overall, more significant changes in OTU distribution occurred among LV infants compared to EV infants in the weeks following vaccination (Figure 6-4). Furthermore, α diversity appeared to remain stable among EV infants but increased among LV infants (Figure 6-6). Hence, it is possible that the elimination of pneumococcal vaccine serotypes from the nasopharyngeal microbiota perturbed the increase in α diversity and the rise in abundance of some OTUs which occurred in the late-intervention group.

Although the reason for this finding is unclear, one plausible hypothesis is that the elimination of PCV-7 serotypes from the microbiome has an effect on microbial interactions. Co-colonizers of the nasopharynx may have synergistic or competitive relations; there is a plethora of *in vivo* and *in vitro* evidence of complex relations between pneumococci and other respiratory pathogens that are co-colonizers of the nasopharyngeal mucosa *[35, 80, 256, 258, 270, 310-313]*. There is even more compelling evidence that PCV-7 serotypes may actually have distinct correlations with

other microbes such as *S. aureus* which are not displaced by non-vaccine serotypes. *[102]*. There was compelling evidence of competition occurring between *Streptococcus* and *Staphylococcus, Lactococcus, Pseudomonas* and *Corynebacterium* (Table 6-2). Declines in *Streptococcus* abundance were concurrent with increases in the abundance of at least one or all of these microbes, particularly *Pseudomonas, Staphylococcus* and *Corynebacterium* (Figure 8). However, it is important to note that EV infants were predominantly male while LV infants were predominantly female (Table 6.1), hence any of the differences seen could be due to sex which has been shown to be an important factor in the carriage of microbes [29].

In the Gambia, *S. pneumoniae* is acquired rapidly after birth and carriage can exceed 90% among infants ≥3 months old and PCV-7 may eliminate up to 63% of circulating serotypes (including vaccine associated serotypes) [201, 202, 291, 298]. Hence, it is probable that the elimination of PCV-7 serotypes from nasopharyngeal carriage alters the microbial interactions and the composition and ecology of the nasopharyngeal microbiome in the weeks following vaccination. Data from this study supports this hypothesis as the correlations between the *Streptococci* were different in the LV and EV group, whereby more significant correlations with *Streptococcus* were found amongst the vaccinated infants (Table 2).

Although the microbial interactions and ecology of the development of the nasopharyngeal microbiome appeared to have some distinctions amongst EV and LV infants, the major question is whether these differences harbour clinical significance and have any implications for the long term effectiveness of PCV-7. Interestingly, five

of the seven infants that had at least one episode of a chest infection during the year of follow-up were from the LV group which may be evidence of the protective effect of PCV-7. *Corynebacterium, Pseudomonas, Lactococcus* and *Staphylococcus* comprise ~30% of the microbiome among the infants has a significant negative correlation with *Streptococcus*. Although there was no evidence of replacement in this preliminary analysis, the concern is that these genera all of which include pathogenic species could replace the niche left vacant by PCV-7 serotypes is still valid. *Pseudomonas* which can constitute >80% of the microbiome among the infants in Gambia (Figure 6-8) has naturally high antibiotic resistance and is associated with chronic lower respiratory tract infections, particularly among immuno-compromised individuals in other parts of the world [314, 315]. It is unclear what the burden of *Pseudomonas* disease is in this region which may warrant more attention. This issue becomes even more important with the application of PCVs with greater valency such as PCV-13 (Figure 1) [316, 317] which will eliminate even more pneumococcal serotypes from the nasopharynx and leave a larger niche to fill. Of even greater concern is the implementation of pneumococcal protein vaccines that could eliminate nasopharyngeal carriage of *S. pneumoniae* and *H. influenzae* which will have dramatic effects on nasopharyngeal microbial ecology. Currently, a protein vaccine, the 10-Valent Pneumococcal Nontypeable *Haemophilus Influenzae* Protein D Conjugate Vaccine which may eliminate mucosal carriage of both *S. pneumoniae* and *H. influenzae* is currently under trial the Gambia [264, 265]. These findings suggest that *Corynebacterium, Pseudomonas* and *Staphylococcus* could be important in replacement following selective or complete elimination of *S. pneumoniae*.

6.2.3 Limitations

The findings of this study need to be verified by the inclusion of data from more subjects. Although 102 infants were recruited for this study, data from 22 is presented here. The preliminary analysis compared infants from PCV-7 naïve communities and infants from communities where the herd effect is at play. The importance of maternal antibodies [307, 318, 319] and the break in transmission of vaccine serotypes [320] suggests that *S. pneumoniae* populations among the two groups could have been different from birth. Hence, to determine the direct impact of vaccination on the nasopharyngeal microbiome, analysis of NP swabs collected from the thirty infants from PCV-7 naïve village who received three doses of PCV-7 is of great importance. Furthermore, there appeared to be a sex bias among the infants from the different groups, hence, analysis of the larger cohort of 102 infants could even out some of the sex distribution. As suggested above, the application of sensitive molecular serotyping techniques will be essential to understand the effects of PCV-7 vaccination on microbial ecology. There were differences in analysis in the two regions of the 16S rRNA gene which may be attributed to primer/amplification bias (Figure 6-2 and Figure 6-3). Variations in genomes not represented by 16S rRNA gene analysis (e.g. capsular type) and the problems of primer/amplification bias may be circumvented by WGS which is part of the future plans for this project [3]. Confirmation of the low abundance taxa by WGS and culture will also further validate the preliminary data reported here. It will also be interesting to determine the sources of different components of the microbiome, particularly in early life and to investigate the correlates of immune development and the development of the microbiota.

6.3. Conclusion

Preliminary data suggests that PCV-7 intervention within the first few months of life may not result in replacement by non Streptococcal microbes within weeks of vaccination. However, PCV-7 may temporarily alter the development of the microbiota and microbial interactions within the nasopharynx. The implications of this preliminary finding on the long-term effect of PCV-7 are yet to be determined, but continued surveillance of replacement in carriage and disease is necessary.

7. Concluding remarks and future pursuits

7.1 Concluding remarks

Although several studies have set out to characterize nasopharyngeal colonisation by respiratory pathogens (a necessary precursor to disease development for many lower respiratory tract infections), the full spectrum of organisms that share this ecological niche is poorly understood. Most investigations of nasopharyngeal microbial communities have had a narrow focus on respiratory pathogens and the few broad studies have been culture-based which invariably excludes microbes that have yet to be propagated in axenic culture.

Working towards a holistic view of the nasopharyngeal microbiome is important for several reasons:

(1) to describe the normal ecological balance in the nasopharynx and elucidate the ecological events that may drive harmful invasion,
(2) to characterize complex microbial interactions which occur in the nasopharynx and the significant host factors that influence the microbiome and
(3) to understand how drug and vaccine interventions may tilt the ecological balance of the nasopharyngeal microbiome.

Previous investigations of microbial interactions within the nasopharynx which focused on a few microbes may have inadequately represented the complex microbial interactions that occur in the nasopharyngeal mucosa [35, 80, 258, 270, 311, 321-323]. Evidence that competitive relations between microbes can be reversed in the presence

of other taxa [35] and that microbial interactions can exist between bacterial components [35] all highlight the importance of a holistic characterization of the infant nasopharyngeal microbiome.

7.1.1. Composition of the infant nasopharyngeal microbiome

Representatives of five phyla and sixty species have been cultured from the normal nasopharyngeal microbiota [33, 46, 55, 324]. However, 16S rRNA gene-based culture-independent analyses in this study showed that members of at least thirty phlya and 900 genera are found in the infant nasopharynx. The number of genera found per NP swab ranged from nineteen to 311 and this provides evidence that numerous organisms can co-colonize the nasopharynx simultaneously. Although previous studies had shown that *Streptococcus, Moraxella, Haemophilus* and *Corynebacterium* are the most frequently detected microbes in the nasopharynx, their relative abundance was unclear. Overall, it appears that genera that include recognised pathogenic species make up between 60% and 80% of the microbiome throughout infancy. These microbes form the backbone of the infant nasopharyngeal microbiome while there are numerous low abundance and transient microbes which account for most of the diversity within and across individuals. This is illustrated in Figure 7.1.

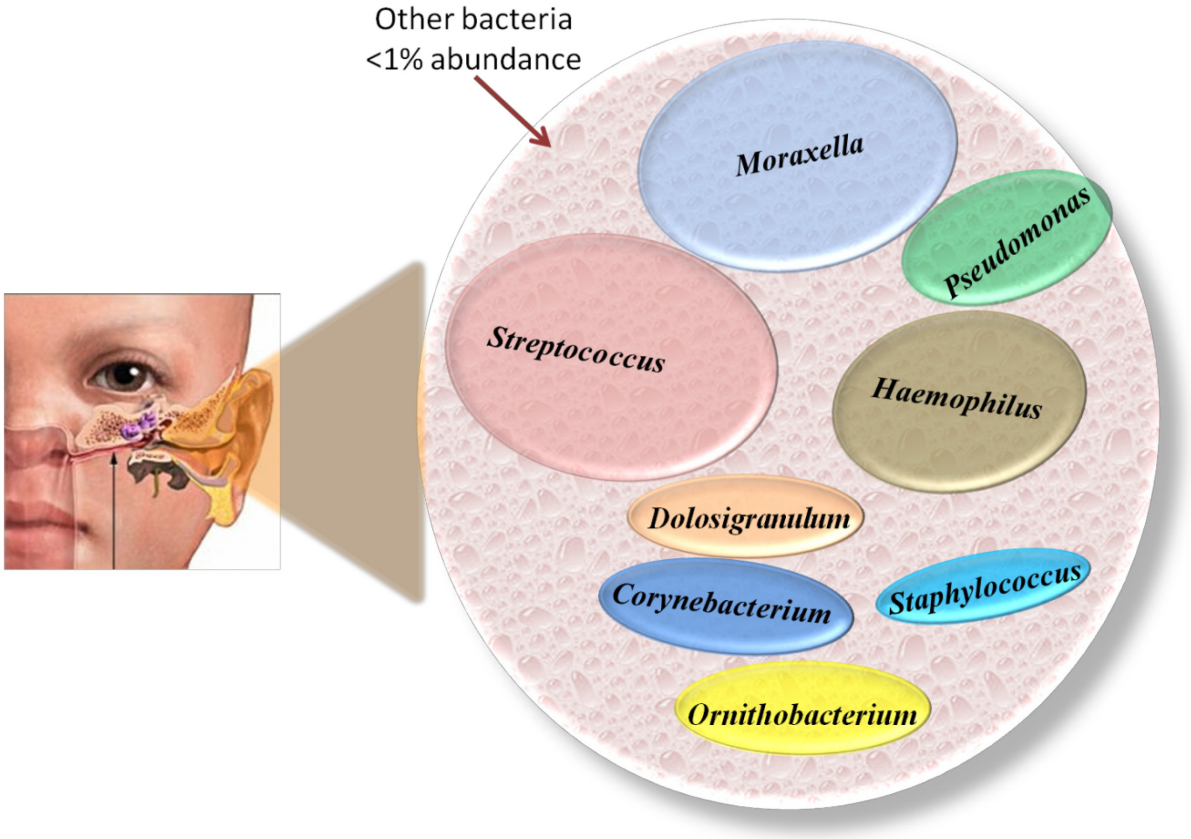


Figure 7.1. Schematic of the infant nasopharyngeal microbiome. The size of each labelled oval represents the relative abundance of each genus. Genera with abundance (<1%) are represented by the pink background .

S. pneumoniae, M. catarrhalis and *H. influenzae* were found in >70% of the NP swabs collected from the infants. The high carriage of these respiratory pathogens is consistent with the high burden of invasive disease. *S. aureus* had high prevalence among neonates (>50%), however, the carriage decreased to below 10% within a few months. 454-sequencing showed that *Staphylococcus* is not only highly prevalent in new-borns, but also comprises 32% of the microbiome which may contribute to the high burden of staphylococcal neonatal sepsis in the Gambia [243, 251]. Taxonomic groups that are not described in culture-based community studies were amongst the most abundant microbes (>1% abundance) found including *Ornithobacterium,*

Shewanella and *Dolosigranulun.* The infant nasopharyngeal microbiome is characterized by the high abundance of *Proteobacteria, Firmicutes* and *Actinobacteria*. The infant nasopharynx appears to have a unique distribution of microbes, distinct from the GI tract and the nasal and oral cavities to which it is proximal and contiguous.

7.1.1. Development of the nasopharyngeal microbiome

Bacterial colonization was detected in the nasopharynx among all the new-borns which supports previous findings that colonization occurs rapidly after birth [190]. By two months, three-quarters of the infants had carried *S. pneumoniae*, *S. aureus, M. catarrhalis,* and *H. influenzae* at least once. This has important implications for vaccine scheduling, particularly for PCV-7 which is first administered at two months under the EPI programme when most infants have already been exposed to *S. pneumoniae*. However, this may be less important in areas with high vaccine coverage and herd immunity is present reducing circulation and transmission of vaccine serotypes [325]. Furthermore, maternal antibodies may provide protection for infants that are born to vaccinated mothers [318, 326].

The proportion of low abundance taxa (<1%) and diversity were highest amongst neonates, this suggests that soon after birth, colonisation by a wider spectrum of microbes is tolerated. However, as the infants got older high abundance taxa appeared to out-compete low abundance taxa. This trend may be attributed to effective evasion of host immune defences and competition for space, resources, growth factors and nutrients [44, 51]. It is also plausible that the infant local nasopharyngeal immune system selectively “tolerates” the high abundance taxonomic groups and eliminates

the rare taxonomic groups; immune tolerance is reviewed elsewhere [64]. Age was found to be the most important risk factor influencing the carriage of respiratory pathogens and the rates of acquisition and loss of bacterial taxonomic groups within the nasopharynx. Other factors that have strong correlations with the microbiota are sex, type of feeding, weight, antibiotic usage and ethnicity. Sex, weight and type of feeding probably have direct immune correlates which influence microbial colonization and elimination.

There was high intra-subject diversity as the microbial community fingerprints from the same subjects were distinct at every time point. High rates of acquisition had a strong correlation with high rates of loss may be an indication that bacterial taxonomic groups which are acquired quickly tend also to be lost quickly. However, it is equally likely that the microbes reported as absent were present but below the detection limit of the analytical approach. Either way, there evidence suggesting that the nasopharyngeal microbiome is dynamic characterized by continuous succession of bacterial taxa.

7.1.2. Impact of vaccination on the nasopharyngeal microbiome

The primary objective of this study was to determine the impact of PCV-7 vaccination on the infant nasopharyngeal microbiome using a 16S rRNA gene-based 454-pyrosequencing approach. Preliminary analysis was conducted by comparing twelve infants from communities with widespread PCV-7 vaccination that were immunized at two, three and four months (EV group) and ten infants from PCV-7 naïve communities that were immunized after eight months (LV group). The relative abundance of several taxa changed significantly in the first six months among LV infants but not EV infants.

Furthermore, an increase in the proportion of low abundance taxa (<1%) and a corresponding increase in α diversity indices was observed among LV but not EV infants. This may be in part explained by the two-fold increase in significant negative interactions between Streptococci and other taxa observed among EV infants. These preliminary findings suggest that early PCV-7 intervention may alter microbial interactions and the development of the microbiome in the nasopharynx during infancy. However, there was no evidence of replacement with non-Streptococcal microbes within weeks of vaccination. For future work, it will be beneficial to measure the overall levels of colonisation and determine how these may vary within and between individuals. The clinical implications of this finding on the long-term effect of PCV-7 are yet to be determined, but continued surveillance of replacement in carriage and disease is necessary.

7.1.3. Importance of deep analysis

Overall the characterisation of the nasopharyngeal microbiome using 16S rRNA gene-based T-RFLP combined with partial 16S rRNA sequencing and 454-pyrosequencing showed similar trends. The most abundant microbes identified by the former were confirmed by the latter. However, 454 pyrosequencing had the added advantage of separating out the different components represented by the OTUs found in the T-RFLP. This is particularly important for the *Firmicutes* cluster which could not be separated out using the primer and restriction enzyme pair used. Furthermore, over 800 OTUs were identified by 454-pyrosequencing from 376 samples as opposed to just over 100 with T-RFLP on 433 samples. This becomes particularly important when low abundance microbes (<0.01% abundance) are of interest in a study (Figure 7.1). Future studies

could employ the metagenomic approach, whereby all the genomic material from a clinical sample is directly analysed using next-generation sequencing tools. This approach would draw a more comprehensive picture of the nasopharyngeal microbiome and its ecology (including bacterial, viral, fungal and archaeal components) and avoid the selective bias introduced by using 16S rRNA gene amplification approaches.

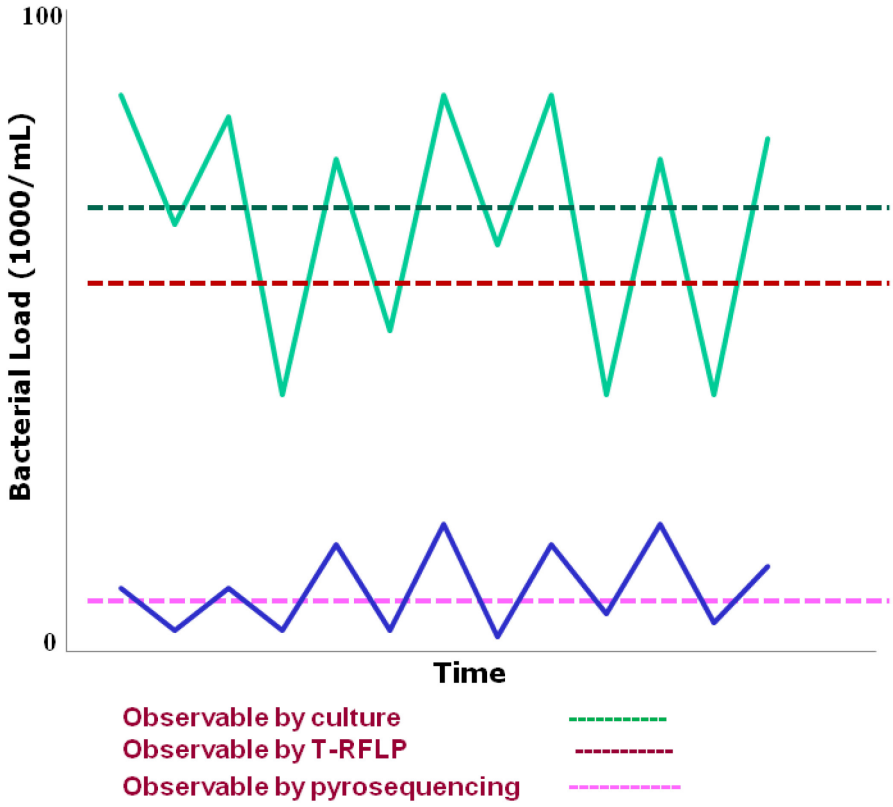


Figure 7.2. The importance of deep analysis for measuring changes in microbial ecology associated with PCV-7. The bold green line represents a high abundance taxon while the bold blue line represents a low abundance taxon.

7.2. Ongoing studies

7.2.1. Completion of culture-independent analysis

In total, 1595 NP swabs were collected from 102 infants in the study investigating the impact of PCV-7 vaccination on the development of the nasopharyngeal microbiome. Here data from 374 NP swabs collected from 22 infants is presented and this preliminary data suggest that PCV-7 vaccination may alter the development of the nasopharyngeal microbiome. Hence, analysis of the full dataset is crucial to validate the preliminary findings and will also allow for regression analyses adjusting for confounding factors such as age, sex, and ethnicity, type of feeding and antibiotic usage which influence the nasopharyngeal microbiome. Secondly, in the preliminary analysis were compared infants from PCV-7 naïve communities with infants from communities where the herd effect is at play. The importance of maternal antibodies [307, 318, 319] and the break in transmission of vaccine serotypes [320] suggest that *S. pneumoniae* populations among the two groups of infants was different from birth. Hence, to determine the direct impact of vaccination on the nasopharyngeal microbiome, analysis of NP swabs collected from the thirty infants from PCV-7 naïve village who received three doses of PCV-7 is of great importance. 454-sequencing analysis of the full sample-set (1595 NP swabs) is currently underway at the Genome Centre at Washington University in St. Louis Missouri, USA and is expected to be complete by June 2011.

7.2.2. Characterisation of *S. pneumoniae* carriage and serotypes

Several studies have shown that replacement of vaccine and vaccine associated serotypes with non-vaccine serotypes invariably occurs following PCV-7 vaccination.

Some studies have shown that serotype replacement occurs immediately, while others have shown that replacement occurs after several months. In order to comprehensively characterize the effects of vaccination on microbial ecology within the nasopharynx, it is essential to understand what the trends in *S. pneumoniae* populations and determine their magnitude and time frame. A limitation of the 16S rRNA gene-based culture-independent analysis is that it was not possible to confidently classify *S. pneumoniae* species within the *Streptococcus* genus.

Although there are several techniques available for serotyping which include latex agglutination, Quellung typing and multiplex PCR, microarray serotyping may have the highest sensitivity with the capacity to detect multiple serotypes including low relative abundance capsular types. The microarray approach will be used to detect and serotype *S. pneumoniae* in the 374 NP swabs on which culture-independent analysis was done to begin with. This work will be conducted at St. Georges University, University of London, UK and is expected to be complete by August 2011.

7.2.3. Co-occurrence of *S. pneumoniae* with pathogens

Species specific PCR will be used to determine the carriage of *S. pneumoniae, S. aureus* and *H. influenzae* in the NP swabs collected from all 102 children from birth to eight months as described in Chapter 2. Although I did not find evidence of a strong interaction between vaccine intervention and the carriage of *Moraxella, Staphylococcus* and *Haemophilus,* there could be significant associations occurring at species level missed by culture-independent analyses. Evidence that PCV-7 serotypes have strong interactions with *S. aureus* highlights the importance of carrying out species level analysis [102, 313, 321, 323, 327]. Multiplex specific real-time PCR will

be used to determine the bacterial loads for *S. pneumoniae* and each of the pathogens in the NP swabs among the 22 infants among which the preliminary culture-independent analysis was conducted. This will provide a solid platform for comparing the quantitative PCR and culture-independent data. This work is being conducted at the MRC Unit in The Gambia.

7.3. Future pursuits

7.3.1. Whole Genome Sequencing (WGS)

There are variations in genomes amongst closely related taxa not represented by the 16S rRNA marker gene. There are also issues with primer bias introduced by differential oligonucleotide binding associated with PCR based approaches. These limitations can be overcome by WGS sequencing of the members of a mixed microbial community. WGS not only provides comprehensive taxonomic and phylogenetic information about the microbes present, but also has the potential to provide information about the possible functional properties of the microbes [3]. Hence, future work from this study will include WGS of a subset of NP swabs collected from the infants. In addition to providing confirmation for the presence of the very low relative abundance taxa ($\leq$0.01%), WGS will add to the collection of sequenced genomes of upper airway microbes. WGS has the added advantage of facilitating the sequencing of fungal, archaeal and viral genomes which may form part of the microbiome among infants.

7.3.2. Immune Correlates

Blood and breast milk were not collected from the infants and their mothers respectively for immunological analysis. In retrospect, this would have greatly enhanced this study. The factors that appear to exert the greatest influence on the development of the microbiome such as age, sex, weight and type of feeding all have strong correlations with immune function. There are distinct differences in immune function which dictate the outcome of encounters with microbial invaders; immediate clearance, commensal carriage, transient colonization or less often harmful invasion. However, the relative importance of various components of the innate and adaptive immune responses in driving the microbiota within an environment of polymicrobial stimulation is yet to be understood. To begin with, the correlates between bacterial carriage and IgG class antibodies which exhibit a highly effective complement-dependent bactericidal activity and enhance neutrophil-mediated opsonophagocytosis against some respiratory pathogens will be determined [328, 329]. Subsequently, Th17-type responses which mobilizes phagocytes and promote resistance against extracellular bacterial and fungal pathogens in epithelial mucosae could be investigated [330]. These investigations may begin to provide clues to why nasopharyngeal carriage of *S. aureus* is initially very high but declines with age while *H. influenzae* and *S. pneumoniae* increase with age during infancy [331].

7.3.3. Deeper and longer look into the microbiome

A very important question not addressed in this study is the source of the nasopharyngeal microbiome. There is compelling evidence that vaginally born babies initially acquire bacteria from the mother's vagina. However, the early succession of

microbes which gives rise to distinct community profiles in different parts of the body has not been characterized. Hence, future studies could include prenatal and post natal sampling of mothers, collection of breast milk, sampling of siblings, household contacts and livestock. Analysis of viral, archaeal, fungal and possibly protozoan inhabitants of the nasopharyngeal microbiome will also be important. Archaeal sequences were detected in the nasopharynx by 454-pyrosequencing and a pilot study of the 18S rRNA gene study showed that fungi can be detected in the nasopharyngeal specimen.

Although I have started work characterizing the nasopharyngeal microbiome among infants, the oral, skin and GI microbiomes among infants, particularly in Africa, have not been extensively described. Furthermore, it is important to determine what happens after the first year. For instance, there is evidence that *S. pneumoniae* and *H. influenzae* carriage declines *while S. aureus* carriage increases with age in older children. Hence, it is plausible that significant changes in the microbiome may occur later in life. This is particularly important with respect to the effect of vaccination on the development of the nasopharyngeal microbiome. Preliminary data suggests that there are temporal changes in the microbiome associated with vaccination; however, the duration, long term implications and clinical significance of these changes are yet to be determined. Hence, continued follow-up of the infants that participated in this study is of great importance.

7.4. Closing remark

It is essential to understand the nasopharynx as a milieu with numerous symbiotic, commensal and pathogenic microbes in a constant state of flux which may influence

disease outcome. The source of the nasopharyngeal microbiome and the impact of genetic factors, antibiotic usage, vaccination and other environmental stresses on the development of the nasopharyngeal microbiome warrant further exploration.

I am proud to have been amongst the first to pick at the tip of the iceberg i.e. the nasopharyngeal microbiome which is somewhat of an ecological enigma.

8. Appendix

MRC Medical Research Council

Gambia Community Pneumococcal Studies

Sibanor Nasopharyngeal Microbiome Study
Form 014 - Birth Report Sheet

Village Name: |__|__|__|__|__|__|__|__|__|__|__|__| **Infant ID:** |__|__||__|__||__|__||__|

Name of Compound Head: First |__|__|__|__|__|__|__|__| **Last** |__|__|__|__|__|__|__|__|

Date of Birth: |__|__|/|__|__|/|__|__|__|__| **Place of Birth:** |__| (1=Hospital, 2=Health centre, 3=Home/TBA)

Type of Birth: |__| (1= Vaginal, 2=Caesarean section) **Age of mother:** |__|__|

Term of Baby: |__| (1=Full term, 2=Premature)

No. of older siblings (same mother): |__|__| **Sex of Baby:** |__| (M=Male, F=Female)

Is the mother of the child vaccinated with PCV7? _____ **If yes, please fill in the table below.**

MOTHER	DATE DOSE 1	DATE DOSE 2	DATE DOSE 3

Are any of the child's siblings vaccinated with PCV7? _____ **If yes, please fill in the table below.**

SIBLING NO. (Oldest = 1)	MRC SVT ID	DATE DOSE 1	DATE DOSE 2	DATE DOSE 3

What is the ethnic group of this child |__|
(1=Mandinka, 2=Jola, 3=Fula, 4=Wollof, 5=Sarahule 6=Senegalese, 7=Other Specify.....)

Mother's Name: First |__|__|__|__|__|__|__|__| **Last** |__|__|__|__|__|__|__|__|

Father's Name: First |__|__|__|__|__|__|__|__| **Last** |__|__|__|__|__|__|__|__|

Child's Birth Weight: |__|__|.|__| (Kg)

Name of Reporter: First |__|__|__|__|__|__|__|__| **Last** |__|__|__|__|__|__|__|__|

Date of Report |__|__|/|__|__|/|__|__|__|__|

Gambia Community Pneumococcal Studies
Sibanor Nasopharyngeal Microbiome Study
Form 011: Risk factor Data Questionnaire (New Births)

MRC Medical Research Council

1101 Child's StudyID **Sample serial ID:** [ ]
(eg A for First sample, B for Second sample, C for Third sample etc)

1102 Mother's First Name: **Last Name:**
1102b HoC First name: **Last Name:**

1103a Expected Collection Date:
1103 b Date of interview: |__|__|/|__|__|/|__|__|__|__| **Interviewer's ID code** |__|__|__|

1104 Child's First Name |__|__|__|__|__|__|__|__|__|__|	Last Name |__|__|__|__|__|__|__|__|__|__|
1105 Date of Birth:	
1106 Gender	(M=Male, F=Female)
1107 What is the ethnic group of this child	(1=Mandinka, 2=Fula, 3=Fula, 4=Wollof, 5=Sarahule, 6=Senegalese, 7=Other Specify:
1108 Weight: |__|__|.|__| (Kg)	
1109 Height: |__|__|__| cm	

1110 Relation of interviewee to this child |__|
(1=Mother, 2=Father, 3=Grandmother, 4=other blood relatives, 5= other adult)

1111 Is this child currently breastfed? |__|
(0=No, 1=Mixed, 2=Breast milk with water, 3=Exclusive breast feeding)

1112 Is this child currently receiving any other feed apart from breast milk? |__| (0=No, 1=Yes)

1113 Has the child had any antibiotics in the last two weeks? ***(Check health card)*** |__|
(0=No, 1=Yes, 8=Not applicable, 9=Not known)

1114 If yes to question 1113, what were the antibiotics from most recent prescription within the last two weeks?

First antibiotic	Second antibiotic
|__|__|	|__|__|
(1115)	**(1116)**

(01=Co-trimoxazole (Septrin), 02=Amoxycillin, 03=Penicillin, 04=Ampicillin, 05=Chloramphenicol, 06=Erythromycin, 07=Gentamicin, 08=Cloxacillin, 10=Tetracycline, 11=Nitrofurantoin, 12=other, specify _____ _____ 88=not applicable 99=Not known)

1117 Have you travelled with the baby out of this village in the last two weeks? |__| (0=No, 1=Yes)
If yes, where did you go? |__|

(1=Other Foni village, 2=Basse, 3=FF, 4=SK, 5=FK, 6=BJL, 7=BK, 8=Senegal village, 10=Senegal town, 11=Other country, specify..... 9=Not known)

1018 Has the child had any ear discharge visible in the last two weeks? (observe) |__|
(1=Right, 2=Left, 3=Both, 4=None, 5=child was not at home, 9=Not known)

1019 Has the child had any chest infection in the last two weeks? |__| (0=No, 1=Yes)

1020 Was nasopharyngeal swab taken? |__| (0=No, 1=Yes)

1021 If no to question 1020, why? |__| **(Attach consent form)**
(1=consent was not given, 2=child was not at home, 3=Other, (specify:.....)

Gambia Community Pneumococcal Studies
Sibanor Nasopharyngeal Microbiome Study
(Form 003: Subject Status Form)

0300	Child's StudyID	D12701
0301	Child's Name	BABOCARR JOBARTEH
0302	Mother's Name	SALLY SANYANG
0303	Head of Compound Name	FODAY JOBARTEH
0304	Child's Date of Birth	12/12/2008
0305	Dose	8-WEEK

			(0=No, 1=Yes 9=Don't Know)	Duration (In months)
0306	**Medical history** Does your child have any of the following conditions? (Please enter the number/s against condition /s that apply.)	0. None 1. Heart disease 2. Diabetes 3. Asthma 4. Renal Disease 5. Liver Disease 6. Other Please specify- ……	|__| |__| |__| |__| |__| |__| |__|	|__|__| |__|__| |__|__| |__|__| |__|__| |__|__|
0307	What medication is your child currently taking?	…… …… ……		
0308	Has your child ever had any allergic reaction/s to any food, drink, drugs etc..	0=No 1=Yes 9=Not known	|__|	
0309	If yes to Q.0308, please state details of allergic reaction and to what substance	…… ……		

0310	a. Pulse Rate b. Respiratory Rate	(per minute) (per minute)	_____ _____
0311	**General Examination** Does the child have any of the following symptoms on examination? (Please enter the number/s against symptom/s that apply.)	0. None 1. Lymphadenopathy 2. Pallor 3. Jaundice 4. Cyanosis 5. Hepatomegaly 6. Splenomegaly	___ ___ ___ ___ ___ ___
0312	**Details of Vaccine administered** Vaccine code administered to subject	Please state or attach code	___
0313	Vaccine dose number	1. First dose 2. Second dose 3. Third dose	___
0314	Date of Vaccine administration	__/__/____	

MEDICAL RESEARCH COUNCIL (MRC)/GAMBIAN COMMUNITY

SIBANOR NASOPHARYNGEAL MICROBIOME STUDY

INFORMATION SHEET TO BE EXPLAINED TO PARENTS (NPS ONLY)

Pneumonia, an infection of the lungs and meningitis an infection of the brain, are both serious diseases in The Gambia causing many deaths and much serious illness. These conditions are most frequent in young children but can also affect adults. The germs that cause pneumonia, meningitis and many other diseases are often found in the throats of babies who are quite healthy, but the germs can spread from these healthy individuals to babies who may then become seriously sick.

We would want to know when and how babies acquire these germs in their throats after they are born. This information can help us to determine the best time points to give extra protection against disease causing germs. Each baby in this study will have swabs collected from his/her throat using a small stick with wool on the end. The schedule for collection will be one every two weeks starting from within 7 days of birth up to the age of 6 months, and then every two months up to the age of 12 months.

We will place a thin cotton-tipped soft plastic swab into one of the nostrils to collect some secretions from the throat. This procedure is not painful and is not associated with any risk of injury. It will only cause mild and temporary discomfort to the person.

Samples and germs collected from this study will be analyzed for germs and stored in our freezers at the MRC laboratories and may be used for future studies.

You and your family can withdraw from this study at any time. Participation in the study is entirely voluntary and will not interfere with standard healthcare that you and your family would normally receive or with their routine vaccination.

Do you have any questions about the study?

Do you agree to join the study? If you agree the consent form will be read to you before you sign the form.

The field assistant will countersign the consent form to indicate the compound heads understood the explanation and freely gave their consent

Contact information: If you require further information about the study, please contact the following people at the addresses shown below:

Dr Richard Adegbola

MRC Laboratories, Fajara

P O Box 273 Banjul. The Gambia

Phone: 4494491

Brenda A. Kwambana

MRC Laboratories

Phone: 7239782

Information sheet for babies scheduled for swab collection

MEDICAL RESEARCH COUNCIL (MRC)/GAMBIAN COMMUNITY

SIBANOR NASOPHARYNGEAL MICROBIOME STUDY MRC

INFORMATION SHEET TO BE EXPLAINED TO PARENTS

Pneumonia, an infection of the lungs and meningitis an infection of the brain, are both serious diseases in The Gambia causing many deaths and much serious illness. These conditions are most frequent in young children but can also affect adults. The germs that cause pneumonia, meningitis and many other diseases are often found in the throats of babies who are quite healthy, but the germs can spread from healthy individuals to babies who may then become seriously sick. MRC is working with the Government of The Gambia on the evaluation of a vaccine (PCV7) to prevent some kinds of pneumonia and meningitis. A successful trial of this pneumonia vaccine was concluded in URD and CRD last year.

We would want to know when and how babies acquire disease causing germs in their throats after they are born. We also want to know whether or not there would be changes in the types of germs in the throat when the vaccine PCV7 is given to babies. This information can help us to reaffirm the proven effectiveness of the vaccine and the best time points to give extra protection against other disease causing germs. Each baby in this study will receive three doses of the PCV7 vaccine at age 2 months, 3 months and 4 months. Each child will also have swabs collected from his/her throat using a small stick with wool on the end. The schedule for collection will be one every two weeks starting from within 7 days of birth up to the age of 6 months, and then every two months up to the age of 12 months.

We will place a thin cotton-tipped soft plastic swab into one of the nostrils to collect some secretions from the throat. This procedure is not painful and is not associated with any risk of injury. It will only cause mild and temporary discomfort to the person. Samples and germs collected from this study will be analyzed for germs and stored in our freezers at the MRC laboratories and may be used for future studies.

You and your family can withdraw from this study at any time. Participation in the study is entirely voluntary and will not interfere with standard healthcare that you and your family would normally receive or with their routine vaccination.

Do you have any questions about the study?

Do you agree to join the study? If you agree the consent form will be read to you before you sign the form.

The field assistant will countersign the consent form to indicate the compound heads understood the explanation and freely gave their consent

Contact information: If you require further information about the study, please contact the following people at the addresses shown below:

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P O Box 273 Banjul. The Gambia

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Brenda A. Kwambana

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Phone: 7239782

Information sheet for babies scheduled for (PCV7) vaccination and swab collection

MEDICAL RESEARCH COUNCIL (MRC)/GAMBIAN INFANT NASOPHARYNGEAL MICROBIOME DEVLOPMENT STUDY

CONSENT FORM 1

Consent form for subjects who will be vaccinated and repeated nasopharyngeal samples collected

I understand I have been asked to permit my child to take part in a study in which a vaccine that prevents pneumonia will be given. I understand that if I agree to permit my child to take part in the trial my baby will receive 3 injection.. It has been explained to me that these vaccines have been given to many thousands of people and are safe but that they may cause a little pain where they are injected and a little fever. I understand that if I agree for my child to be vaccinated then swabs may be collected from his/her throats using a small stick with wool on the end one every two weeks starting from soon after birth up to the age of 6 months, and then every two months up to the age of 12 months. I understand that participation in this study is entirely voluntary and will not interfere with standard healthcare that my family would normally receive or with their routine vaccination. The study doctor and nurses will provide free healthcare services to all study participants during the study period. I understand that I or any member of my family can leave the study at any point without this interfering with their access to healthcare service if they become sick in any way.

Samples and germs collected from this study will be stored in our freezers at MRC laboratories and may be used for future studies. I have had an opportunity to ask the MRC field worker who explained the trial to me and answers to any questions that I had about the trial.

I agree that my child can join the study.

Name.....

Signature or thumbprint.....

Subject ID of child.....

_____

I, ..., confirm that I have explained the nature of the

trial to..... as set out in the study protocols, that s/he understood what I said, had an opportunity to ask questions and freely gave his/her consent for him/her and/or* his/her family to join the trial.

NAME OF FIELD WORKER.....

SIGNATURE.....

DATE |__|__|/|__|__|/|__|__|__|__|

and/or* = delete as appropriate

Contact information: If you require further information about the study, please contact the following people at the addresses shown below:

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Phone: 496580 (home)

Fax: 495919

Dr Uzochukwu Egere

MRC Laboratories

s Phone: 7820343.

MEDICAL RESEARCH COUNCIL (MRC)/GAMBIAN INFANT NASOPHARYNGEAL MICROBIOME DEVLOPMENT STUDY

CONSENT FORM 2

Consent form for subjects who will have repeated nasopharyngeal samples collected

I understand I have been asked to permit my baby to take part in a study in which is looking at the germs that live in my child's nose. I understand that my child will have swabs collected from his/her throat using a small stick with wool on the end. The schedule for collection will be one every two weeks starting from soon after birth up to the age of 6 months, and then every two months up to the age of 12 months.

I understand that participation in this study is entirely voluntary and will not interfere with standard healthcare that my family would normally receive. I understand that I or any member of my family can leave the study at any point without this interfering with their access to healthcare service if they become sick in any way.

The study doctor and nurses will provide free healthcare services to all study participants during the study period.

Samples and germs collected from this study will be stored in our freezers at MRC laboratories and may be used for future studies. I have had an opportunity to ask the MRC field worker who explained the study to me and answers to any questions that I had about the study.

I agree that my child can join the study.

Name.....

Signature or thumbprint.....

Subject ID of child.....

_____

I, ..., confirm that I have explained the nature of the

study to..... as set out in the study protocols, that s/he understood what I said, had an opportunity to ask questions and freely gave his/her consent for him/her and/or* his/her family to join the trial.

NAME OF FIELD WORKER.....

SIGNATURE.....

DATE |__|__|/|__|__|/|__|__|__|__|

and/or* = delete as appropriate

Contact information: If you require further information about the study, please contact the following people at the addresses shown below:

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