

**Influence of the intestinal microbiota on the immune response to oral
poliovirus and rotavirus vaccines in a low-income community in south India**

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Oral vaccines have consistently underperformed in the low-income countries where they are needed most. This has formed a potent obstacle to several public health initiatives that rely on such vaccines, including the polio eradication endgame.

This thesis examines the hypothesis that the composition of the intestinal microbiota – including both pathogenic and commensal microbes – contributes to the impaired performance of oral vaccines in low-income countries. Based on a systematic review and meta-analysis, we observed a decrease in the likelihood of responding to oral poliovirus vaccine (OPV) among infants with concurrent enterovirus infections or diarrhoea. We subsequently tested for multiple bacterial, viral, and eukaryotic enteropathogens and sequenced the 16S rRNA gene (to characterise the total bacterial microbiota) among infants living in a semi-urban slum in south India who had received Rotarix (an oral rotavirus vaccine) and OPV at 6 and 10 weeks of age. We did not observe significant differences in bacterial microbiota composition according to seroconversion status for either vaccine. However, the presence of bacterial pathogens was positively correlated with Rotarix response and negatively correlated with OPV response, suggesting that distinct mechanisms may impact these vaccines.

The same methods were applied to samples from 6–11 month-old infants who had received OPV after a 3-day course of azithromycin or placebo. Once again, the association between bacterial microbiota composition and vaccine outcome was modest, although microbiota diversity was negatively correlated with vaccine poliovirus replication. As expected, viral pathogens were associated with a decrease in OPV immunogenicity. Moreover, recently acquired viral infections appeared to inhibit OPV response to a greater extent than persistent viruses.

Together, these findings have substantiated the inhibitory effect of viral pathogens on OPV response, while implicating bacterial pathogens as a potential risk factor for OPV failure in early infancy. Risk factors for rotavirus vaccine failure remain elusive.

Declaration of originality

I declare that the contents of this thesis are original. Any contributions made by others are appropriately acknowledged.

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Publications

The systematic review described in Chapter 2 has been published in the *Journal of Infectious Diseases*, while sections of Chapter 1 are adapted from a review paper published in *Expert Review of Vaccines*. Citation details for these and other first-author articles published during the course of this PhD are listed below. All articles are freely available online.

Parker EP (2014). Advances in enteric disease vaccines: from innovation to implementation.

Expert Rev Vaccines, 13: 317-9.

Parker EP, Kampmann B, Kang G, et al (2014). Influence of enteric infections on response to oral poliovirus vaccine: a systematic review and meta-analysis. J Infect Dis, 210: 853-64.

Parker EP, Molodecky NA, Pons-Salort M, et al (2015). Impact of inactivated poliovirus vaccine on mucosal immunity: implications for the polio eradication endgame. Expert Rev Vaccines, 14: 1113-23.

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The laboratory procedures described in the following pages involved a large number of researchers. Given the collaborative nature of this work, I have generally felt it appropriate to use the pronoun 'we' in relevant chapters. The extent of my involvement in the laboratory work is clarified below.

Chapters 4 and 5

The collection of samples and much of the laboratory work relevant to the primary outcomes of the clinical trial described in these chapters had been completed before I had any involvement in the project. I subsequently carried out approximately two-thirds of the nucleic acid extractions described in Chapter 4 (of which >750 were carried out overall), set up an equivalent proportion of the 105 TaqMan array cards, and prepared the two 16S rRNA libraries (including PCR, purification and quantification of amplicons, pooling of samples, and quantitative PCR of the pooled libraries). The preparation of 200-mg aliquots of faecal samples was carried out by Revathi R., the detection of Sabin viruses was conducted by Sidhartha Giri, the measurement of rotavirus shedding was carried out by Robin Lazarus, and the remainder of the nucleic acid extractions and TaqMan array card assays were performed by Angeline Metilda, Revathi R., and Ira Praharaj. Anna Zekavati and Emanuel Savage assisted in several steps of the 16S rRNA library preparation at Imperial's Biomedical Research Centre Genomics Facility in Hammersmith Hospital, London. The MiSeq runs were set up by Laurence Game and Katerina Rekopoulou at the Medical Research Council Clinical Sciences Centre Genomics Facility (also in Hammersmith Hospital). The additional extractions and TaqMan array card assays described in Chapter 5 were carried out by Ira Praharaj.

Chapter 6

All enteropathogen testing and other laboratory assays relevant to the primary outcomes of the clinical trial described in this chapter were performed by researchers in the Department of Gastrointestinal Sciences at the Christian Medical College, Vellore. I subsequently carried out all steps involved in preparation of the 16S rRNA library, including aliquoting of faecal samples and DNA extraction for the 280 samples included in this study. Quantitative PCR and sequencing were carried by members of the Medical Research Council Clinical Sciences Centre Genomics Facility.

Abstract	2
Declarations	3
Acknowledgements	4
Laboratory work	6
Contents	7
List of tables	11
List of figures	13
Selected abbreviations	15
Prologue	16
Chapter 1. Introduction	18
1.1. Background	18
1.2. Polio	19
1.2.1. Pathogenesis and clinical course	19
1.2.2. The polio endgame	21
1.2.3. Variation in oral poliovirus vaccine performance	24
1.3. Rotavirus	33
1.3.1. Classification and pathogenesis	33
1.3.2. Epidemiology	34
1.3.3. Rotavirus vaccines: development and implementation	36
1.3.4. Variation in rotavirus vaccine performance	37
1.4. Other oral vaccines	42
1.5. Explaining variation in oral vaccine performance	44
1.5.1. Transplacental antibodies	44
1.5.2. Breastfeeding	46
1.5.3. Host genetics	48
1.5.4. Malnutrition and micronutrient deficiencies	49
1.5.5. Concurrent enteropathogens	50
1.5.6. The bacterial microbiota	53
1.5.7. Environmental enteropathy	60
1.5.8. The infectious agent	61
1.6. Knowledge gaps and hypotheses	62
1.6.1. General versus specific effects	64
1.6.2. Objectives	65
1.7. Conclusions	65
Chapter 2. Influence of enteric infections on response to oral poliovirus vaccine: a systematic review and meta-analysis	66
2.1. Background	66
2.1.1. Hypotheses	66
2.2. Methods	67
2.2.1. Literature search	67
2.2.2. Screening of abstracts and titles	67
2.2.3. Search for additional studies	67

2.2.4. Inclusion and exclusion criteria	68
2.2.5. Data extraction	68
2.2.6. Statistical analysis	69
2.3. Results	71
2.3.1. Study selection	71
2.3.2. Concurrent non-polio enteroviruses	73
2.3.3. Concurrent diarrhoea	85
2.3.4. Other enteropathogens	88
2.3.5. Environmental enteropathy	89
2.4. Discussion	89
2.4.1. Limitations	92
2.4.2. Assessment of study quality	93
2.4.3. Conclusions	93
Chapter 3. Assessing the intestinal microbiota: laboratory and bioinformatic methods	95
3.1. Background	95
3.2. The TaqMan array card	95
3.2.1. Nucleic acid extraction	96
3.2.2. Enteropathogen detection	96
3.2.3. Interpretation and analysis	98
3.3. 16S rRNA gene sequencing	99
3.3.1. Nucleic acid extraction	99
3.3.2. Library preparation	99
3.3.3. Merging of paired-end reads	103
3.3.4. Sequence processing in Qiime	103
3.3.5. Interpretation and analysis	107
3.4. Mock community profile	111
3.5. Conclusions	112
Chapter 4. Influence of enteropathogens and the bacterial microbiota on response to oral rotavirus vaccine: a case-control study in south India	113
4.1. Background	113
4.1.1. Hypotheses	113
4.2. Vellore	114
4.3. Methods	115
4.3.1. Study population	115
4.3.2. Enteropathogen testing	116
4.3.3. 16S rRNA gene sequencing	119
4.3.4. Statistical analysis	120
4.4. Results	125
4.4.1. Oral rotavirus vaccine immunogenicity	125
4.4.2. Association between concurrent enteropathogens and seroconversion	125
4.4.3. Dose 1 rotavirus vaccine take	130
4.4.4. Association between concurrent enteropathogens and vaccine take	130
4.4.5. Association between bacterial microbiota composition and rotavirus vaccine outcome	133
4.4.6. Impact of probiotics on the bacterial microbiota	141

4.4.7. Negative controls	143
4.4.8. Comparison of technical replicates	143
4.4.9. Sensitivity analyses	145
4.5. Discussion	148
4.5.1. Limitations	152
4.5.2. Conclusions	154
Chapter 5. Influence of enteropathogens and the bacterial microbiota on response to oral poliovirus vaccine among infants in south India	155
5.1. Background	155
5.1.1. Hypotheses	155
5.2. Methods	155
5.2.1. Additional TaqMan array card assays	155
5.2.2. Statistical analysis	156
5.3. Results	159
5.3.1. Oral poliovirus vaccine immunogenicity	159
5.3.2. Association between concurrent enteropathogens and seroconversion	159
5.3.3. Oral poliovirus vaccine take	164
5.3.4. Association between concurrent enteropathogens and vaccine take	164
5.3.5. Attributable fraction of vaccine non-response associated with measured enteropathogens	169
5.3.6. Association between bacterial microbiota composition and oral poliovirus vaccine outcome	170
5.3.7. Sensitivity analyses	175
5.4. Discussion	179
5.4.1. Conclusions	183
Chapter 6. Influence of the intestinal microbiota on oral poliovirus vaccine response among infants in south India treated with azithromycin or placebo in a randomised controlled trial	184
6.1. Background	184
6.1.1. The EVI trial	184
6.1.2. Objectives	185
6.1.3. Hypotheses	186
6.2. Methods	186
6.2.1. Study population	186
6.2.2. 16S rRNA gene sequencing	188
6.2.3. Statistical analysis	189
6.3. Results	193
6.3.1. Microbiota at enrolment	193
6.3.2. Impact of azithromycin on the bacterial microbiota	193
6.3.3. Impact of non-intervention antibiotics on the bacterial microbiota	196
6.3.4. Association between bacterial microbiota composition and oral poliovirus vaccine outcome	201
6.3.5. Microbiota stability	206
6.3.6. Novel, persistent, and resolved infections	206
6.3.7. Random Forests	212
6.3.8. Multivariate logistic regression	214

6.3.9. Sensitivity analyses	214
6.3.10. Negative controls	215
6.4. Discussion	215
6.4.1. Conclusions	219
Chapter 7. Conclusions	221
7.1. Summary of findings	221
7.2. Limitations	222
7.3. The road ahead	224
7.3.1. Viral interference: a day-by-day perspective	224
7.3.2. Animal models, human microbes	225
7.3.3. Oral vaccine failure on a broader scale	226
Epilogue	227
References	228

Table 1.1. Seroprevalence after multiple doses of trivalent oral poliovirus vaccine among infants in the USA	26
Table 1.2. Per-dose protective efficacy of oral poliovirus vaccine against paralytic poliomyelitis attributable to type 1 poliovirus	30
Table 1.3. Efficacy of Rotarix against severe rotavirus-associated gastroenteritis (up to 1 year)	39
Table 2.1. Summary of eligible trials	72
Table 2.2. Summary of studies reporting the influence of enteric viruses on oral poliovirus vaccine response	76
Table 2.3. Subgroup and sensitivity analyses	82
Table 2.4. Summary of studies reporting the influence of diarrhoea on oral poliovirus vaccine response	86
Table 3.1. TaqMan array card targets	97
Table 4.1. Summary of study population	118
Table 4.2. Prevalence and abundance of enteropathogens by rotavirus seroconversion status	128
Table 4.3. Prevalence and abundance of enteropathogens by dose 1 oral rotavirus vaccine take	132
Table 4.4. Summary of alpha diversity analyses	136
Table 4.5. Summary of beta diversity analyses	138
Table 4.6. Summary of significant differences in taxon abundance	139
Table 4.7. Significant differences in taxon abundance among technical replicates	144
Table 4.8. Summary of sensitivity analyses	145
Table 5.1. Summary of study population	156
Table 5.2. Prevalence and abundance of enteropathogens by type 3 poliovirus seroconversion status	162
Table 5.3. Prevalence and abundance of enteropathogens by oral poliovirus vaccine take (≥ 1 Sabin)	167
Table 5.4. Summary of alpha diversity analyses	171
Table 5.5. Summary of beta diversity analyses	172
Table 5.6. Summary of significant differences in taxon abundance	173
Table 5.7. Multivariate logistic regression outputs	175
Table 5.8. Summary of sensitivity analyses	176
Table 6.1. Comparison of the zinc/probiotic supplementation trial and EVI trial	188
Table 6.2. Random Forest input variables	192

Table 6.3. Baseline associations between age, microbiota diversity and age, and pathogen burden	195
Table 6.4. Top 20 age-discriminatory operational taxonomic units	195
Table 6.5. Summary of alpha diversity analyses	198
Table 6.6. Summary of beta diversity analyses	199
Table 6.7. Influence of azithromycin on taxon abundance	200
Table 6.8. Summary of taxon abundance comparisons according to type 3 poliovirus seroconversion status	204
Table 6.9. Summary of taxon abundance comparisons according to type 3 poliovirus take	205
Table 6.10. Impact of resolved, novel, and persistent viral infections on oral poliovirus vaccine immunogenicity	208
Table 6.11. Impact of resolved, novel, and persistent infections on oral poliovirus vaccine immunogenicity: full and stratified analyses	209
Table 6.12. Impact of resolved, novel, and persistent infections on oral poliovirus vaccine take: full and stratified analyses	211
Table 6.13. Multivariate logistic regression outputs	214

Figure 1.1. Outcomes of poliovirus infection	21
Figure 1.2. Impaired immunogenicity of trivalent oral poliovirus vaccine in India versus the USA	27
Figure 1.3. The global roll-out of rotavirus vaccines in national immunisation programmes	37
Figure 2.1. Flow chart of study selection process	71
Figure 2.2. Forest plot of the impact of concurrent non-polio enterovirus infections on the odds of seroconversion per dose of oral poliovirus vaccine	80
Figure 2.3. Funnel plots	81
Figure 2.4. Forest plot of the impact of concurrent non-polio enterovirus infections on the odds of vaccine take per dose of oral poliovirus vaccine	84
Figure 2.5. Forest plot of the impact of concurrent diarrhoea on the odds of seroconversion per dose of oral poliovirus vaccine	87
Figure 2.6. Forest plot of the impact of concurrent diarrhoea on the odds of seroconversion following multiple doses of oral poliovirus vaccine	87
Figure 3.1. TaqMan array card amplification curves	98
Figure 3.2. 16S rRNA microbiota analysis pipeline	100
Figure 3.3. Amplification of the 16S rRNA gene V4 region	101
Figure 3.4. Purification of the pooled 16S rRNA gene amplicons via gel extraction	102
Figure 3.5. Random Forests: tree building and out-of-bag sample classification	110
Figure 3.6. Mock community composition	111
Figure 4.1. Zinc/probiotic supplementation trial design	116
Figure 4.2. Association between concurrent pathogens and rotavirus vaccine immunogenicity	127
Figure 4.3. Association between enterovirus subgroups and rotavirus vaccine immunogenicity	129
Figure 4.4. Rotavirus shedding at 6 weeks of age	130
Figure 4.5. Association between concurrent pathogens and dose 1 rotavirus vaccine take	131
Figure 4.6. Association between enterovirus subgroups and dose 1 vaccine take	133
Figure 4.7. Composition of the bacterial microbiota among vaccine recipients	134
Figure 4.8. Association between microbiota diversity and rotavirus vaccine response	135
Figure 4.9. Clustering of samples according to rotavirus vaccine response	137
Figure 4.10. Association between microbiota stability and rotavirus vaccine response	137
Figure 4.11. Pre-vaccination Proteobacteria abundance by rotavirus vaccine outcome	139
Figure 4.12. Predictive accuracy of Random Forest models	140

Figure 4.13. Highest ranking taxa by Random Forest importance score for prediction of rotavirus vaccine take	141
Figure 4.14. Impact of probiotic supplements on the bacterial microbiota	142
Figure 4.15. Highest ranking taxa by Random Forest importance score for prediction of probiotic treatment	143
Figure 4.16. Comparison of technical replicates	144
Figure 4.17. Impact of threshold cycle on the association between concurrent pathogens and rotavirus vaccine immunogenicity	146
Figure 4.18. Conceptual model of interactions between oral rotavirus and poliovirus vaccines administered in early infancy	150
Figure 5.1. Association between concurrent pathogens and oral poliovirus vaccine immunogenicity	161
Figure 5.2. Association between enterovirus subgroups and oral poliovirus vaccine immunogenicity	163
Figure 5.3. Shedding of Sabin serotypes at 10 weeks of age	164
Figure 5.4. Association between concurrent pathogens and dose 1 oral poliovirus vaccine take	166
Figure 5.5. Association between concurrent pathogens and dose 1 take of type 3 Sabin viruses	168
Figure 5.6. Population attributable fraction of oral poliovirus vaccine failure associated with concurrent infections	169
Figure 5.7. Association between microbiota diversity and oral poliovirus vaccine immunogenicity	171
Figure 5.8. Clustering of samples according to oral poliovirus vaccine response	172
Figure 5.9. Prediction of oral poliovirus vaccine response using Random Forests	174
Figure 5.10. Impact of threshold cycle on the association between concurrent pathogens and oral poliovirus vaccine immunogenicity	177
Figure 6.1. EVI trial design	187
Figure 6.2. Composition of the bacterial microbiota at enrolment	194
Figure 6.3. Impact of azithromycin on the bacterial microbiota	197
Figure 6.4. Association between microbiota composition and oral poliovirus vaccine response	202
Figure 6.5. Changes in taxon abundance associated with oral poliovirus vaccine response	203
Figure 6.6. Microbiota stability in relation to study arm and oral poliovirus vaccine response	206
Figure 6.7. Impact of resolved, novel, and persistent viral infections on oral poliovirus vaccine immunogenicity	207
Figure 6.8. Impact of resolved, novel, and persistent viral infections on oral poliovirus vaccine take	210
Figure 6.9. Predictive accurate of Random Forest models using different modules of data	212
Figure 6.10. Highest ranking variables by Random Forest importance score	213

AFP	–	acute flaccid paralysis
bOPV	–	bivalent oral poliovirus vaccine
bp	–	base pair
CI	–	confidence interval
cVDPV	–	circulating vaccine-derived poliovirus
EAEC	–	enteroaggregative <i>Escherichia coli</i>
ELISA	–	enzyme-linked immunosorbent assay
EPEC	–	enteropathogenic <i>E. coli</i>
EPI	–	Expanded Programme on Immunisation
ETEC	–	enterotoxigenic <i>E. coli</i>
EE	–	environmental enteropathy
GPEI	–	Global Polio Eradication Initiative
HBGA	–	histo-blood group antigen
IPV	–	inactivated poliovirus vaccine
LRT	–	likelihood ratio test
mOPV	–	monovalent oral poliovirus vaccine
NPEV	–	non-polio enterovirus
OPV	–	oral poliovirus vaccine
OR	–	odds ratio
PAF	–	population attributable fraction
SIA	–	supplementary immunisation activity
TAC	–	TaqMan array card
tOPV	–	trivalent oral poliovirus vaccine

In June 1959, the world's leading experts in attenuated poliovirus vaccines gathered at Georgetown University, Washington, D. C., to attend the First International Conference on Live Poliovirus Vaccines. Efforts to develop a safe and effective live vaccine to protect against polio had not been perturbed by the success of Jonas Salk's inactivated vaccine, licensed in 1955, and had recently broken out of the laboratory into large-scale field trials. In the previous year, various live vaccine candidates had been tested in the USA, Poland, Mexico, Singapore, Colombia, and Cuba, among others.

It was at the end of the third day of the conference that Stanley Plotkin from Philadelphia's Wistar Institute took the floor to present findings from a trial recently conducted in the Belgian Congo (now the Democratic Republic of Congo). Between August 1958 and April 1959, more than 45,000 doses of the lab's CHAT strain of attenuated type 1 poliovirus had been administered to children under 5 years of age (Plotkin and Koprowski, 1959). Among 340 children previously lacking immunity to type 1 poliovirus, 60% developed neutralising antibodies after being immunised with the virus. While by no means a failure, these findings fell somewhat short of the response rates observed in previous trials of the vaccine in Europe and North America. As noted by Plotkin:

"The low serologic response was surprising considering the results obtained with the CHAT strain both in small, carefully controlled groups [in the USA; Plotkin et al (1959)] and in field studies by Przesmycki et al [in Poland; Przesmycki et al (1959)], using exactly the same pool of virus material as was used in Léopoldville. These studies indicated a serological efficacy of 90 to 95% for the CHAT strain."

This was the first indication that the immunogenicity of live polio vaccines might be to some degree impaired in low-income, tropical countries. It would not be the last.

In the years following the 1959 conference, the attenuated strains developed by Albert Sabin at the Children's Hospital Research Foundation (Cincinnati, USA) edged out their competitors (including the CHAT strain) to become the first licensed live poliovirus vaccines. Monovalent vaccines targeting each of the three poliovirus serotypes were licensed in the USA in 1961 and 1962, and a trivalent vaccine combining all three serotypes was licensed in 1963. These vaccines have since formed the cornerstone of efforts to halt poliovirus transmission. Their use as part of the Global Polio Eradication Initiative has helped to bring about the reduction of polio from a global disease responsible for 350,000 cases of paralysis each year (the estimated incidence when the programme was launched in 1988) to a disease

on the brink of extinction. Just 74 wild poliovirus cases were documented in 2015 – the lowest number on record.

But the issue of impaired vaccine immunogenicity in low-income countries has not gone away. Vaccine trials in these regions have consistently documented seroconversion rates inferior to the near-complete serological protection observed in high-income countries (Patriarca et al, 1991). This has been a formidable obstacle to polio eradication efforts, but not an insurmountable one. In 2007, 874 wild poliovirus cases occurred in India – 66% of the world's total (WHO, 2016a). However, the use of oral poliovirus vaccine in routine immunisation alongside frequent rounds of mass immunisation (national or subnational campaigns targeting all children under 5 years of age) was sufficient to interrupt polio transmission, and India was declared free of the disease in 2012. It is likely that efforts on a similar scale will be required to halt transmission in the two countries that remain endemic for polio – Afghanistan and Pakistan – where the issue of impaired vaccine performance is compounded by political unrest, vaccine boycotts, and a recent surge in violence targeting vaccination workers.

The phenomenon of impaired vaccine efficacy in low-income countries is not unique to polio. Similar deficits have been observed for licensed oral vaccines targeting rotavirus (Madhi et al, 2010) and cholera (Su-Arehawaratana et al, 1992). One live-attenuated *Shigella* vaccine candidate developed by the Pasteur Institute was reactogenic and immunogenic when administered to North American adults; however, during trials in Bangladesh, the same vaccine elicited no adverse reactions, was not excreted, and failed to induce a serological response in any recipient (WHO, 2006; Levine et al, 2007).

In spite of several decades of research, we have failed to establish a clear understanding of the factors responsible for the impaired efficacy of oral vaccines in low-income countries. While it is conceivable that polio may be eradicated before these mechanisms are fully elucidated, this obstacle stands in the way of other oral vaccines, including those currently in use and those yet to be developed. Moreover, poliovirus has repeatedly demonstrated its potential to spread to regions previously clear of infection. Until eradication is achieved, there is an imperative to better understand the factors that shape our immune response to the virus, and potential ways this immunity might be enhanced.

I have spent the last 4 years studying the mechanisms influencing the immunogenicity of oral vaccines, with an emphasis on the impact of the commensal and pathogenic organisms that inhabit the gut in infancy. The results of these endeavours are presented in the pages that follow.

1.1. Background

Enteric pathogens represent a substantial threat to public health. This is a threat that takes many forms. Bacterial pathogens such as *Vibrio cholerae* and enterotoxigenic *Escherichia coli* (ETEC) colonise the mucosal surface of the small intestine before releasing toxins that induce diarrhoea, vomiting, and abdominal cramps. Rotavirus and several other enteric viruses invade enterocytes in the intestinal epithelium, inducing functional changes and cell damage that give rise to severe diarrhoea, vomiting, cramps, and fever. The ingestion of just ten cysts of the flagellate protozoan *Giardia lamblia* is sufficient to induce morphological changes and apoptosis of epithelial cells in the small intestine, resulting in diarrhoea that may last in excess of 6 weeks (Rendtorff, 1954). Together, enteropathogens account for several million cases of diarrhoea each year and approximately 800,000 deaths, the majority of which occur among children in low-income countries (Liu et al, 2012).

Not all symptoms are elicited in the gastrointestinal tract. Following replication in the intestinal mucosa, polioviruses and several other members of the Enterovirus genus can spread via the bloodstream to the central nervous system, where the destruction of nerve cells may give rise to paralysis. Enteroviruses may also cause encephalitis, hand-foot-and-mouth disease, myocarditis, and a range of other sequelae (Pons-Salort et al, 2015). In addition to the direct consequences of infection, repeated exposure to bacterial, viral, and eukaryotic enteropathogens is thought to contribute to the ‘vicious cycle’ of intestinal damage, malabsorption, and immune dysfunction at the heart of malnutrition and stunting among infants in low-income countries (Lang, 2010). Malnutrition, in turn, may contribute to more than half of deaths that occur among children under 5 years of age worldwide (Caulfield et al, 2004).

Oral vaccines are, in many respects, ideal tools for tackling enteric infections. These vaccines are easy to administer and cause minimal discomfort for the recipient. They have the capacity to induce local immunity in the intestinal mucosa (the primary site of enteropathogen replication), thereby protecting the vaccinated individual from infection following subsequent pathogen exposure and – by blocking onward transmission – enhancing the herd effects of vaccination. And they have the potential to be produced in large quantities at low cost – the price per dose of oral poliovirus vaccine (OPV) is currently less than US \$0.15, while inactivated poliovirus vaccine (IPV) costs approximately \$0.85 per

dose in countries eligible for Gavi support¹, and more than \$1.50 per dose elsewhere (UNICEF, 2014).

Yet oral vaccines have an Achilles' heel. They have consistently been shown to be less immunogenic in the low-income countries that experience the greatest burden of enteric disease. In this chapter, I will outline what is currently known regarding the scale of this phenomenon, focusing predominantly on polio and rotavirus vaccines given their central role in the chapters that follow. I will then review our understanding of the potential mechanisms that might account for the impaired performance of oral vaccines in low-income countries. In doing so, I will introduce the central concern of this thesis – the potential role of the intestinal microbiota in shaping our immune response to oral vaccines.

1.2. Polio

Despite the potential benefits of oral vaccines, they make up only a small portion of the global vaccine market in 2016. By far the most widely utilised is OPV, of which several billion doses are currently administered as part of routine and supplementary immunisation programmes each year. Although I have touched briefly on the history of OPV above, in this section I will expand on the nature of this vaccine, the pathogenesis of the disease it targets, and the available data regarding geographic variation in vaccine performance.

1.2.1. Pathogenesis and clinical course

Polioviruses are members of the genus Enterovirus in the family Picornaviridae – a group of small (~27-nm diameter), non-enveloped viruses that also includes rhinovirus (the infectious agent responsible for the common cold) and hepatovirus A (responsible for hepatitis A). Picornaviruses are made up of single-stranded, positive-sense RNA genomes approximately 7,500 nucleotides in length, which are surrounded by protein capsids with icosahedral symmetry. Polioviruses are stable at acidic pH (a prerequisite for gastrointestinal transit). They remain infectious at 4°C for several weeks and at 30°C for several days, but are inactivated by temperatures exceeding 55°C (Sutter et al, 2008).

Following ingestion, polioviruses attach to and enter cells via CD155 (also referred to as the poliovirus receptor) – a receptor that is not shared by other members of the Enterovirus genus. Replication occurs in both the nasopharynx and gastrointestinal tract. Although some

¹ The Gavi Alliance is a public–private partnership set up in 2000 with the aim of improving access to vaccines in the world's poorest countries.

uncertainty remains regarding the primary sites of viral replication, studies in humans and non-human primates point to the importance of mucosa-associated lymphoid tissues, including the tonsils, intestinal M cells, and Peyer's patches, as well as the lymph nodes that drain these tissues (Sabin and Ward, 1941; Bodian, 1955; Sicinski et al, 1990). Following replication at these sites, polioviruses may transfer via the bloodstream to other susceptible tissues, including the central nervous system, where replication in nerve fibres may lead to acute flaccid paralysis (AFP). Recent intramuscular injection or skeletal muscle injury may also predispose individuals to 'provocation poliomyelitis', wherein invasion of the nervous system occurs via retrograde axonal transport (Gromeier and Wimmer, 1998).

Although the consequences of poliovirus infection can be severe, the majority of infections are either asymptomatic (~72%) or resolve after a period of minor illness characterised by fever, nausea, malaise, vomiting, constipation, sore throat, or a combination of these symptoms (~24%) (Gelfand et al, 1957; Sutter et al, 2008). Nonparalytic poliomyelitis occurs in 1–4% of cases, wherein a period of fever and malaise is followed by the onset of aseptic meningitis characterised by severe headache, vomiting, and stiffness in the neck and back (Horstmann [1949], as cited by Sutter et al [2008]). Symptoms last for up to 10 days, and are typically followed by a swift and complete recovery (Sutter et al, 2008).

Paralytic poliomyelitis occurs in less than 1% of cases (Melnick and Ledinko, 1953). It is this form of the disease that is associated with iconic images of the 'iron lung' – the coffin-like structure developed in the late 1920s to provide artificial respiration for individuals whose paralysis has compromised their breathing. The clinical course of paralytic disease is a cruel one. After a period of minor illness, symptoms typically resolve for several days. This is followed by the sudden onset of fever accompanied by flaccid paralysis, usually beginning 7–28 days after infection (Sartwell, 1952). The extent of paralysis depends on the region and severity of nerve damage. Involvement of the spinal column (spinal poliomyelitis) accounts for approximately 79% of paralytic cases², and is characterised by paralysis of one or more limbs. Spinal poliomyelitis is generally asymmetric, more severe proximally than distally, and accompanied by the loss (partial or complete) of deep tendon reflexes. In 2% of paralytic cases, nerve damage occurs in the bulbar region of the brain stem (bulbar poliomyelitis), affecting muscles involved in swallowing, speech, and respiration, among others. In the remaining 19% of cases, both the spinal column and brain stem are affected (bulbosplinal poliomyelitis). The maximum extent of paralysis is typically reached within a few days of the onset of symptoms. Although the damaged nerve cells cannot be replaced, other muscles

² Based on documented cases of poliomyelitis in the USA between 1969 and 1979 (CDC, 2009).

may compensate to bring about a partial or complete recovery within 6 months. However, paralytic poliomyelitis is fatal in 2–5% of children and 15–30% of adults, and this fatality rate increases to 25–75% among individuals with bulbar involvement (CDC, 2009).

Notably, while clinical outcomes vary, infected individuals may contribute to poliovirus transmission regardless of whether or not they are symptomatic. Faecal shedding of viruses begins within several days of exposure, and reaches peak levels after 7 days (Onorato et al, 1991). In a study of 110 children infected with wild polioviruses in Louisiana, shedding lasted for 1–114 days, with a mean duration of 24 days (Gelfand et al, 1957). As noted above, polioviruses also replicate in the nasopharynx, and may be detected in pharyngeal secretions for 1–2 weeks (Onorato et al, 1991). Nasopharyngeal shedding is suspected to be the major route of poliovirus transmission in areas with good sanitation and hygiene (Salk and Salk, 1977). While it may also contribute to circulation elsewhere, faecal–oral transmission is thought to predominate in regions where wild poliovirus persists today.

Figure 1.1 provides a summary of the potential outcomes of poliovirus infection. Further details regarding the structure, pathology, and natural history of this virus can be found in several detailed reviews, among them Robertson (1993), Racaniello and Ren (1996), and Sutter et al (2008).

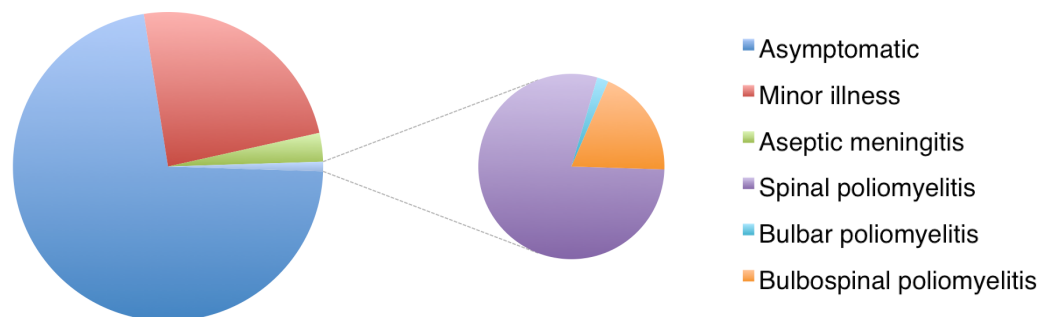


Figure 1.1. Outcomes of poliovirus infection.

1.2.2. The polio endgame

When the Global Polio Eradication Initiative (GPEI)³ was formed in 1988, polio was endemic in 125 countries. Thanks to the roll-out of OPV and IPV, the virus had largely been brought under control in industrialised countries – the UK had not seen a domestically acquired case of polio since 1984, while the USA had been clear of wild poliovirus cases since

³ The GPEI is a public–private partnership led by the WHO, Rotary International, the US Centers for Disease Control and Prevention, and UNICEF. It was launched following the 41st World Health Assembly, held in Geneva in May 1988.

1979. However, polio was thriving elsewhere: it has been estimated that during the 1970s and 1980s, between 200,000 and 400,000 cases of poliomyelitis occurred each year in India alone (John and Vashishtha, 2013).

For its first two decades, the GPEI relied predominantly on the use of trivalent OPV (tOPV) to combat poliovirus circulation. Each dose of this vaccine contains 10^6 median tissue culture infective doses (TCID₅₀)⁴ of attenuated type 1 virus, 10^5 TCID₅₀ of type 2 virus, and $10^{5.5}$ TCID₅₀ of type 3 virus – a ‘balanced’ formulation that was developed in the early 1960s to overcome the interference observed among vaccine strains when they are co-administered (with type 2 viruses often replicating at the expense of types 1 and/or 3) (Robertson et al, 1962; Morimoto, 2001). The vaccine strains in use today have not changed from those first developed by Albert Sabin at the Children’s Hospital Research Foundation, Cincinnati, in the late 1950s. For each serotype, the attenuated strain was developed by passaging wild polioviruses through kidney, testicular, and/or skin cells obtained from non-human primates (preceded for serotype 3 by intracerebral passage in rhesus macaques), leading to the accumulation of genetic changes that removed the neurovirulent potential of the virus while still allowing it to infect the gastrointestinal tract (Sutter et al, 2008). The WHO has been responsible for the custody and distribution of Sabin seed strains since 1973 (Cockburn, 1988).

As a live-attenuated vaccine, OPV induces an immune response analogous to that produced by wild viruses (Ghendon and Sanakoyeva, 1961). The vaccine polioviruses elicit both systemic humoral immunity, which prevents the transit of poliovirus to the central nervous system and thus protects against paralysis, and mucosal immunity, which limits poliovirus replication at the nasopharyngeal and intestinal mucosal surfaces (Onorato et al, 1991). In addition to its low cost and ease of administration, the ability of OPV to induce a mucosal immune response is a crucial advantage over IPV – while the latter is capable of inducing a strong systemic immune response that protects vaccinated individuals from paralysis, it induces only limited mucosal immunity, and is therefore less effective than OPV at preventing poliovirus replication and shedding following subsequent virus exposure⁵.

Although initial optimism that polio would not see the new millennium turned out to be misplaced, the GPEI has made huge steps towards achieving its goal, reducing the annual incidence of wild poliovirus cases by more than 99% since its launch. The last case of wild

⁴ The quantity of virus required to produce a cytopathic effect in 50% of cells in tissue culture.

⁵ It is worth noting that while IPV fails to induce a strong mucosal immune response on its own, it has recently become clear that the vaccine may boost mucosal immunity among individuals previously immunised with OPV (Jafari et al, 2014; John et al, 2014).

type 2 poliovirus occurred in northern India in 1999, while paralysis attributable to wild type 3 poliovirus has not been documented since November 2012 (the last case occurring in Yobe, Nigeria). As of early 2016, Afghanistan and Pakistan are the last remaining refuges of endemic poliovirus transmission.

The continued use of OPV is pivotal to the polio endgame. Currently, 145 countries use the vaccine in routine immunisation programmes (WHO, 2016c), with three doses typically administered at monthly intervals starting at 6 weeks of age. Routine immunisation also includes a birth dose of OPV in high-risk settings. To supplement these efforts, mass immunisation campaigns (also termed supplementary immunisation activities [SIAs]) have increasingly been used in populations with persistent poliovirus circulation or a heightened risk of re-introduction. SIAs are performed at either national or subnational level (targeting one or several districts) with the aim of immunising all children under 5 years of age in a window of several days. To enhance coverage, vaccines are administered both at fixed vaccination posts and in house-to-house visits. Since 2005, SIAs have frequently employed monovalent OPV (mOPV) and bivalent OPV (bOPV) targeting poliovirus types 1 and 3 in order to improve the protective efficacy against these serotypes (by reducing interference between the vaccine strains) (Sutter et al, 2010; Cochi and Linkins, 2012). These changes have proven critical to halting the spread of polio in regions where the virus is most resilient, such as India (John and Vashishtha, 2013) and Nigeria (Mangal et al, 2014).

In 2014, an estimated 2.3 billion doses of OPV were administered across more than 300 SIAs in 45 countries (Hagan et al, 2015). If these efforts are sustained, wild type 1 poliovirus may soon be consigned to the same fate as types 2 and 3. However, the polio endgame does not conclude with the eradication of wild polioviruses. On rare occasions, vaccine-associated paralytic poliomyelitis (VAPP) may occur among OPV recipients or their close contacts (Platt et al, 2014). There are an estimated 1–2 cases of VAPP per million primary immunisations with OPV (Nathanson and Kew, 2010). Moreover, like wild polioviruses, vaccine viruses have the potential to spread from person to person. While circulation is generally short-lived, sustained transmission chains may occur in underimmunised populations. In rare circumstances, the Sabin strains may lose their attenuating mutations, regaining transmissibility and the ability to cause paralysis (Burns et al, 2014). Such viruses are termed circulating vaccine-derived polioviruses (cVDPVs), and may have a similar clinical attack rate and severity to wild-type viruses (Jenkins et al, 2010). Between 2000 and 2013, outbreaks of cVDPV occurred in 18 different countries, causing more than 720 cases of poliomyelitis (Burns et al, 2014).

Given the risk of VAPP and cVDPVs, the use of OPV must be phased out from all immunisation activities if the eradication of polio is to be achieved (GPEI, 2013). Accordingly, we are presently in the midst of the sequential and globally synchronised withdrawal of the three strains of OPV. This began with the withdrawal of serotype 2 in April 2016 – a transition requiring the replacement of tOPV with bOPV in all routine and supplementary immunisation programmes – and will culminate (as currently scheduled) in the cessation of bOPV in 2019. To fill the gap in poliovirus immunity, the WHO Strategic Advisory Group of Experts (SAGE) on immunisation recommended that all countries introduce at least one dose of IPV into routine immunisation programmes by the end of 2015 (SAGE, 2013a). This deadline was met by all but 45 countries, with remaining countries formally committed to the introduction of IPV by the end of 2017 (WHO, 2016b).

Thus, the timely eradication of poliovirus could see the end of OPV use by the close of the decade. Until then, the widescale use of mOPV and bOPV in routine immunisation and SIAs remains intrinsic to the polio endgame.

1.2.3. Variation in oral poliovirus vaccine performance

As noted above, the impaired performance of OPV in low-income countries was first brought to light during the early field trials of this vaccine. The immunogenicity and efficacy of OPV has since been examined in numerous studies conducted across the globe. How have these studies measured OPV performance? And what have they taught us about the discrepancies in vaccine outcomes between high- and low-income countries?

i. Systemic immunity: kinetics, duration, and the microneutralisation assay

Following exposure to live polioviruses, there are several facets to the ensuing immune response, with both systemic and mucosal components. Systemic humoral immunity is by far the most commonly tested outcome in clinical trials of poliovirus vaccines, and is the primary focus in the chapters that follow. It is characterised initially by the production of poliovirus-specific IgM antibodies, which appear in serum within 1–3 days of viral exposure and persist for up to 3 months (Ogra and Karzon, 1971; Ogra et al, 1980). Serum IgA is detected in some individuals, and may persist at low levels (Ogra and Karzon, 1971; Herremans et al, 1999). However, IgG antibodies are the mainstay of the systemic immune response. These begin to appear in the 7 days following poliovirus exposure, and increase in titre over the next 4–5 weeks to become the predominant class of persistent antibody.

Systemic immunity following wild poliovirus infection may be long-lived, potentially lifelong – a study conducted among members of an isolated community in northern Alaska found that serum antibodies may persist for more than 40 years without subsequent re-infection (Paul et al, 1951). Serum antibodies have also been shown to persist for several years after OPV administration (Rousseau et al, 1973; Nishio et al, 1984; Tafuri et al, 2008). However, titres wane over time (Krugman et al, 1977), and subsequent re-immunisation may lead to the reappearance of IgM in serum and a boost in IgG titre (Ogra et al, 1980).

Clinical trials of OPV generally do not distinguish between different classes of serum antibody. Instead, neutralising antibodies (predominantly of class IgG) are measured using a standardised microneutralisation assay in which known quantities of polioviruses of a given serotype are incubated with serum samples at two-fold serial dilutions and subsequently tested for their capacity to cause a cytopathic effect in tissue culture – the most dilute sample in which viruses are neutralised is then recorded as the antibody titre (WHO, 1997). Notably, passive antibody administration has been shown to protect against poliomyelitis in humans and non-human primates, and the detection of antibodies at a titre of 1 in 8 or greater is considered a good correlate of protection (Hammon et al, 1954; Bodian and Nathanson, 1960).

Seroconversion following the administration of OPV is generally defined as a four-fold rise in neutralising antibody titre between pre- and post-vaccination samples or the detection of neutralising antibodies in individuals who were previously seronegative. In infants, the presence of passively acquired maternal antibodies, transferred to the foetus via the placenta, must also be taken into account. These antibodies decay with a half-life of approximately 28 days (Gelfand et al, 1960; Robertson, 1993). Accordingly, during immunogenicity studies in infants, pre-vaccination antibodies are assumed to decay at this rate and seroconversion is defined as a four-fold rise in titre over the expected level of maternal antibodies upon collection of the post-vaccination sample.

Notably, seroconversion is strongly correlated with the intestinal replication – or ‘take’ – of homotypic vaccine polioviruses, as inferred by the presence of these viruses in post-vaccination faecal samples (John, 1975; Onorato et al, 1991). In a recent trial of monovalent type 3 OPV (mOPV3) – a trial that I will return to in Chapter 6 of this thesis – seroconversion was observed in 85% of infants who shed type 3 poliovirus 7 days after vaccination and 10% of non-shedders. In light of this correlation, the replication of vaccine poliovirus has frequently been considered as an endpoint in clinical trials of OPV.

ii. Oral poliovirus vaccine immunogenicity

During the first trial of ‘balanced’ tOPV, a single dose of vaccine was offered to all residents of Prince Albert (a city in Saskatchewan, Canada) within an 8-day period starting on the 27th February 1961 (Robertson et al, 1962). In a subset of approximately 600 individuals, two-thirds of whom were infants or pre-adolescent children, antibody titres were measured in serum samples collected pre- and post-vaccination. The majority of vaccinees already harboured antibodies to one or more serotypes owing to the widespread use of IPV since 1955. However, among individuals without homotypic antibody pre-vaccination, 92% developed detectable antibodies to type 1 poliovirus (albeit at trace levels in 26%), while 100% developed antibodies to serotypes 2 and 3.

Subsequent trials in high-income countries have confirmed that tOPV is capable of inducing a robust systemic immune response, particularly after multiple doses (Table 1.1). Seroconversion rates have been considerably more variable, however, among studies in low-income countries. In a comprehensive review of OPV trials conducted in developing countries, Patriarca et al (1991) documented average seroconversion rates after three doses of tOPV of 73% for type 1 poliovirus (range, 36–99%), 90% for type 2 (range, 71–100%), and 70% for type 3 (range, 40–100%). The impaired performance of tOPV was not universal – one study in Sri Lanka, for example, recorded seroconversion rates in excess of 97% for all three serotypes after a course of three doses commencing at 3 months of age (EPI, 1989). On the other hand, in studies conducted in India (Choudhury et al, 1973; Pangi et al, 1977), Iran (Mokhtary et al, 1986), Kenya (Metselaar et al, 1977), Ghana (Bottiger et al, 1981), and Brazil (de Brito Bastos et al, 1974), seroconversion rates did not exceed 80% for any serotype.

Table 1.1. Seroprevalence after multiple doses of trivalent oral poliovirus vaccine among infants in the USA.

Age at vaccination (months)	Seroprevalence after two doses (%)			Seroprevalence after three doses (%)			Ref.
	PV1	PV2	PV3	PV1	PV2	PV3	
2, 4, 6	93	100	91	97	100	96	Hardy et al (1970)
	89	100	93	99	100	99	Cohen-Abbo et al (1995)
2, 4, 12	92	99	90	96	100	96	Hardy et al (1970)
	100	100	100	100	100	100	Faden et al (1990)
2, 4, 18	92	100	96	97	100	100	McBean et al (1988)
	95	100	90	96	100	98	Modlin et al (1997)

Adapted from a table published by Gindler et al (1995). Abbreviations: PV1, type 1 poliovirus; PV2, type 2 poliovirus; PV3, type 3 poliovirus.

Findings have been similar for mOPV and bOPV (though comparisons of the latter are undermined by the scarcity of relevant data from high-income countries). In a systematic review by Caceres and Sutter (2001), median homotypic seroconversion rates per dose of mOPV among studies in temperate countries – including the USA, Denmark, Hungary, and the Netherlands – were 95% for type 1 poliovirus (range, 90–100%), 98% for type 2 (range, 83–100%), and 94% for type 3 (range, 70–100%). Meanwhile, among studies in non-temperate countries – including Brazil, India, Mexico, Singapore, and Uganda – median seroconversion rates were 81% for serotype 1 (range, 53–89%), 89% for type 2 (range, 77–93%), and 72% for type 3 (range, 52–80%).

Figure 1.2 provides a comparison of seroconversion rates observed across successive doses of tOPV in Vellore, India (John, 1976), and Maryland, USA (McBean et al, 1988). These should be interpreted with caution given the notable differences in study design, but provide an illustration of the scale of the deficits observed in OPV immunogenicity between low- and high-income countries.

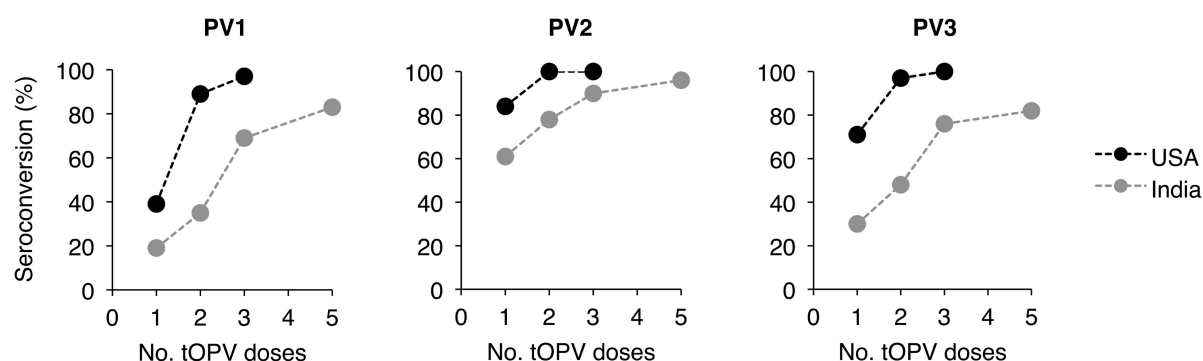


Figure 1.2. Impaired immunogenicity of trivalent oral poliovirus vaccine in India versus the USA.

Seroconversion rates for India are obtained from a report by John (1976), which combines results from several trials performed in Vellore between 1969 and 1976 (John and Jayabal, 1972; John, 1976; John et al, 1976). Up to five doses of tOPV were administered to infants aged between 6 weeks and 6 years at intervals of 4–8 weeks in these studies. For at least the first two doses of OPV, seroconversion rates are derived from homotypic seronegative infants, so maternal antibodies cannot account for the deficits in immunogenicity compared with US children. Immunogenicity data from the USA are obtained from a study by McBean et al (1988) in which three doses of tOPV were administered to infants in Maryland at 2, 4, and 18 months of age. Seroconversion rates following the second and third doses are based on the proportion of individuals with detectable antibodies at 18 and 20 months of age, respectively. These are interpreted here as seroconversion on the assumption that there was no exposure to live viruses aside from the administered vaccine, and that the persistence of maternal antibodies at 18 months of age was negligible. Abbreviations: PV1, type 1 poliovirus; PV2, type 2 poliovirus; PV3, type 3 poliovirus; tOPV: trivalent oral poliovirus vaccine.

iii. Mucosal immunity

In addition to humoral immunity, exposure to live polioviruses elicits a local response at the nasopharyngeal and intestinal mucosal surfaces, preventing replication at these sites following subsequent viral exposure (Hird and Grassly, 2012). The most widely adopted measure of poliovirus-specific mucosal protection involves administration of a challenge dose of OPV, wherein the subsequent shedding of vaccine polioviruses in nasopharyngeal and stool samples signifies viral replication in the nasopharynx and gut, respectively, while the absence of shedding can be viewed as an indicator of mucosal protection.

Although some uncertainty remains regarding the immune effectors responsible for poliovirus-specific mucosal immunity, secretory antibodies appear to play an important role. Following exposure to OPV, secretory IgA appears in the nasopharynx and duodenum after 1–3 weeks (Ogra et al, 1968). This rise in intestinal antibody coincides with the decline in viral excretion (Valtanen et al, 2000). Following viral clearance, the titre of poliovirus-specific IgA in stool samples has been shown to correlate with protection against subsequent intestinal replication (Onorato et al, 1991; Morimoto, 2001; Samoilovich et al, 2003), and a similar relationship has been observed between nasal IgA and nasopharyngeal shedding (Ogra and Karzon, 1971). A study performed among elderly individuals in the Netherlands also found that the presence of homotypic circulating IgA antibodies decreased the likelihood of viral excretion following challenge with mOPV of serotypes 1 or 3 (Buisman et al, 2008). However, the titre of secretory or serum IgA required for protection against poliovirus replication is unknown. Moreover, in contrast to serum neutralising antibodies, a standardised assay for the detection of poliovirus-specific IgA is currently lacking.

It is likely that the variation in OPV-induced humoral immunity observed in different geographic settings also extends to mucosal protection, particularly given that vaccine poliovirus replication is correlated both with seroconversion and the induction of secretory antibody responses. However, data to support this case are undermined by several factors. First, mucosal immunity to OPV has not been as extensively studied as humoral immunity, particularly in low-income countries (Hird and Grassly, 2012). Second, among challenge studies that have examined mucosal immunity, factors such as the nature of the challenge dose and the age at which it is administered have varied considerably (Kok et al, 1992; WHO, 1996; Jafari et al, 2014; John et al, 2014). The latter is particularly relevant – in contrast to humoral immunity, wherein seroconversion can be interpreted as an all-or-nothing response that is durable over many years, mucosal immunity wanes over time, enabling OPV-immunised individuals to be re-infected (Smith et al, 1976; Onorato et al, 1991). During a

retrospective analysis of AFP surveillance data in India, Grassly et al (2012) observed a marked increase in the odds of shedding challenge virus among individuals who had not received a dose of OPV for more than 6 months compared with those who had received a dose in the past 40 days, suggesting that significant waning of mucosal protection occurs within a year of vaccination. Finally, although failure to shed vaccine viruses following challenge is widely considered as an indicator of mucosal immunity, there are (as considered in greater detail below) a range of factors that may inhibit replication of vaccine polioviruses. Thus, if shedding after OPV challenge is observed to be lower in a certain population, it may be incorrect to infer that mucosal immunity must be higher in these individuals.

iv. Cell-mediated immunity

In addition to the induction of humoral immunity, OPV has been shown to elicit a cell-mediated immune response, including the production of poliovirus-specific CD4⁺ and CD8⁺ T cells (Katrak et al, 1991; Wahid et al, 2005). T cell responses display cross-reactivity among poliovirus serotypes, and may therefore confer a degree of heterotypic immunity (Katrak et al, 1991). However, in contrast to humoral immunity, cell-mediated responses do not appear to be a prerequisite for viral clearance. In particular, while B cell immunodeficiencies such as hypogammaglobulinaemia have consistently been linked with the long-term excretion of vaccine polioviruses after OPV administration (Martin et al, 2000; MacLennan et al, 2004), this is not the case for T cell immunodeficiencies such as HIV (Hennessey et al, 2005).

v. Efficacy

The efficacy of IPV was determined on the basis of one of the largest field trials in history, wherein approximately 400,000 individuals were randomly assigned to receive IPV or placebo, and an additional 200,000 vaccinated individuals were followed up alongside unvaccinated children (Francis et al [1955], as cited by Plotkin and Vidor [2008]). No comparable study has been performed for OPV (nor, indeed, would it have been ethical to do so given the availability of IPV). However, the efficacy of OPV has been determined in several studies by comparing the vaccination histories of paralytic poliomyelitis cases with those of controls. These have predominantly taken one of two forms: case-control studies following poliovirus outbreaks (e.g., Deming et al, 1992) and retrospective analyses of AFP databases in which poliomyelitis cases are compared with cases of non-polio AFP (e.g., Grassly

et al, 2006). A summary of per-dose protective efficacy estimates against type 1 poliovirus from a selection of these studies is provided in Table 1.2.

Table 1.2. Per-dose protective efficacy of oral poliovirus vaccine against paralytic poliomyelitis attributable to type 1 poliovirus.

Country	Method	Per-dose efficacy, % (95% CI)			Ref.
		mOPV	bOPV	tOPV	
Gambia	Outbreak-CC	-	-	68 (27–86)	Deming et al (1992)
Oman	Outbreak-CC	-	-	30 (-234–85)	Sutter et al (1991)
India	AFP-CC	-	-	13 (10–16)	Grassly et al (2006) ^a
India (Uttar Pradesh)	AFP-CC	30 (19–39)	-	11 (7–14)	Grassly et al (2007) ^a
Nigeria	AFP-CC	67 (39–82)	-	16 (10–21)	Jenkins et al (2008)
Afghanistan/Pakistan	AFP-CC	35 (16–49)	23 (10–35)	13 (6–19)	O'Reilly et al (2012)
Nigeria	AFP-CC	32 (26–38)	30 (20–38)	19 (16–23)	Mangal et al (2014)

Abbreviations: AFP-CC, case-control study conducted within acute flaccid paralysis surveillance database; bOPV, bivalent oral poliovirus vaccine; CI, confidence interval; mOPV, monovalent oral poliovirus vaccine; Outbreak-CC, case-control study conducted following outbreak of poliomyelitis; tOPV, trivalent oral poliovirus vaccine.

^a Data overlap.

Notably, data regarding the protective efficacy of OPV come almost exclusively from low-income countries. There is limited scope, therefore, to compare efficacy estimates between high- and low-income settings, though it would be reasonable to assume that discrepancies in OPV immunogenicity are reflected by similar disparities in efficacy given that humoral immunity is a well-established correlate of protection against poliomyelitis. Estimates derived from AFP surveillance databases do, however, provide insight into within-country variation in OPV efficacy that is pertinent both to the polio endgame and the topic of this thesis. During an analysis of more than 96,000 AFP cases recorded in India between 1997 and 2005, Grassly et al (2006) observed a decrease in the odds of paralytic poliomyelitis consistent with a constant probability of protection per dose of tOPV. While a per-dose efficacy of 13% was observed across the whole of India for type 1 poliovirus, when a separate analysis was performed to delineate OPV response in the northern states of Uttar Pradesh and Bihar – responsible for the majority of poliomyelitis cases in India since 1997 despite intensive vaccination efforts – per-dose efficacy estimates were 9% for Uttar Pradesh, 18% for Bihar, and 21% for the rest of India. Similar results were observed for protective efficacy against type 3 poliovirus. Thus, the authors concluded that the persistence of polio in India in 2005 was attributable, in part, to within-country variation in OPV efficacy. Mangal et al (2014) applied a similar approach when examining factors underlying the persistence of

poliomyelitis in Nigeria, revealing markedly lower protective efficacy of tOPV and mOPV in northern versus southern states.

vi. Inactivated poliovirus vaccine

Before moving on from polio, it is worth briefly considering the extent to which the geographic discrepancies in vaccine immunogenicity outlined above extend to IPV. When three doses of this vaccine are administered to infants in high-income countries at 2, 4, and 6 months of age, seroconversion to all three serotypes is consistently observed in more than 95% of individuals (Aristegui et al, 2003; Plotkin and Vidor, 2008; Tapiero et al, 2013). Seroconversion rates may fall short of these levels when IPV is administered in low-income countries using the Expanded Programme on Immunisation (EPI) schedule (6, 10, and 14 weeks of age) – one study adopting this schedule in Thailand, for example, documented seroconversion rates of 67%, 65%, and 94% for serotypes 1, 2, and 3, respectively (WHO, 1996). However, it is likely that these findings largely reflect differences in the timing and interval of IPV administration, and the influence of these factors on the titre of maternal antibodies at the time of vaccination. The presence of elevated titres of maternal antibodies (e.g., 1 in 64 or greater) has been shown to have a marked inhibitory effect on IPV immunogenicity (Kok et al, 1992; WHO, 1996). Accordingly, the earlier IPV is administered, the less time maternal antibodies will have had to decay, and the greater their inhibitory capacity. This notion is supported by a study conducted in Puerto Rico in which infants were randomly assigned to receive IPV at 6, 10, and 14 weeks of age or 2, 4, and 6 months of age – while seroconversion was observed in more than 99% of infants for all three serotypes using the latter schedule, the EPI schedule induced seroconversion in 86%, 86%, and 97% for serotypes 1, 2, and 3, respectively (Dayan et al, 2007).

Variation in maternal antibody titres among populations may therefore influence the immunogenicity of IPV during early infancy (as discussed below with reference to OPV). This variation has not been well quantified; however, it is feasible that titres will tend to be higher in countries where mothers have recently been exposed to circulating wild or vaccine polioviruses (SAGE, 2013b).

Once maternal antibody titres have decayed, IPV induces a robust systemic immune response irrespective of geographic setting, and has proven highly immunogenic in regions where the performance of OPV is compromised. This was recently illustrated during a study conducted in Uttar Pradesh, India – a state previously linked with impaired OPV efficacy

(Grassly et al, 2006) – in which children up to 10 years of age were randomised to receive IPV, bOPV, or no vaccine (Jafari et al, 2014). Seroconversion to type 1 poliovirus was observed in 83%, 98%, and 96% of IPV recipients and 14%, 13%, and 42% of bOPV recipients aged 6–11 months, 5 years, or 10 years, respectively. Similar seroconversion rates were observed among IPV recipients for serotypes 2 and 3 (82–100%). Thus, while the immunogenicity of IPV may be impeded by maternal antibodies in early infancy, it appears that the injected vaccine does not face the same barriers to vaccine performance in low-income countries observed for its oral counterpart.

vii. Seasonal variation in oral poliovirus vaccine performance

In addition to the geographic variation observed in the immunogenicity of OPV, marked seasonal variation in vaccine performance has been documented – a fact that will be important to bear in mind when considering the factors shaping oral vaccine performance below. In particular, among infants in the Gambia who received four doses of tOPV starting at 6 weeks of age, seroconversion rates for all three serotypes declined as the number of doses received during the rainy season increased (Cirne et al, 1995). Seroconversion to type 3 poliovirus was observed in 84% of infants who received all doses outside the rainy season compared with 51% of those who received three or more doses within the rainy season. Similar trends in vaccine efficacy were subsequently observed in a case-control study conducted among children in the Gambia paralysed during an outbreak of type 1 poliovirus (Deming et al, 1997), while a marked reduction in OPV take was observed among Indian children immunised between April and September (warmer months that coincide with the high season for enterovirus transmission) (Grassly et al, 2012).

viii. Conclusions

For more than half a century, OPV has been at the forefront of efforts to control poliovirus transmission owing to its low cost, ease of use, and ability to induce systemic and mucosal immunity. However, the immunogenicity of OPV in low-income countries has consistently been shown to fall short of the robust immune response observed in high-income countries. Moreover, within countries in which polio has proven most resilient, significant variation in OPV efficacy between regions has been implicated in sustaining viral circulation. This variation in the efficacy of OPV continues to stand in the way of the eradication endgame as we move ever closer to polio's extinction.

1.3. Rotavirus

As we prepare for the synchronised withdrawal of one oral vaccine, another is in the process of being rolled out across the globe. The target is rotavirus – a disease that infects the majority of the world’s infants before the end of their third year of life, and a leading cause of severe gastroenteritis in high- and low-income countries alike (Clark et al, 2008; Estes and Greenberg, 2013).

1.3.1. Classification and pathogenesis

The potential role of viruses in the aetiology of diarrhoeal illness did not come to light until 1972, when a viral agent was identified as the cause of an outbreak of acute gastroenteritis in the US city of Norwalk, Ohio (the virus was subsequently termed Norwalk virus) (Kapikian et al, 1972). Human rotaviruses were discovered soon after, following the use of electron microscopy to identify viral particles in duodenal samples obtained from infants with acute gastroenteritis (Bishop et al, 1973). These 70-nm icosahedral viruses, which make up a distinct genus in the family Reoviridae, consist of an outer capsid, an inner capsid, and an inner core surrounding a genome that consists of 11 segments of double-stranded RNA. Rotaviruses can be separated into seven serogroups (A to G, of which groups A, B, and C have been documented in humans) based on the cross-reactivity of antisera obtained from hyperimmunised animals. Serogroup determinants are predominantly located in the highly immunogenic inner capsid protein VP6 (Estes and Greenberg, 2013). Genetic reassortment can occur among members of the same serogroup, but not between groups (Yolken et al, 1988).

Rotavirus serogroup A accounts for the vast majority of the diarrhoeal disease burden in infants, and has been the focus of vaccine development efforts to date. As with other serogroups, group A rotaviruses can be divided into multiple serotypes based on the composition of the outer capsid proteins VP4 and VP7, which elicit serotype-specific antibody responses (as well as cross-neutralising antibodies in some instances). Rotaviruses can be distinguished according to G serotype (based on the glycoprotein VP7) and P serotype (based on the protease-sensitive protein VP4). However, owing to extensive cross-reactivity between P serotypes when assessed by monoclonal or polyclonal antisera, strains are now more commonly discriminated according to VP4 genotype (signified in square brackets), as determined by nucleic acid sequencing. More than 60 combinations of G and P serotypes have been shown to cause diarrhoeal disease. Although a small number of strains, including G1P[8], G2P[4], G3P[8], G4P[8], and G9P[8], account for the majority of cases globally

(Gentsch et al, 2005; Santos and Hoshino, 2005), other strains may be regionally important; for example, strain G8P[6] accounted for 42% of rotavirus cases observed during a study of children with acute diarrhoea in Blantyre, Malawi (Cunliffe et al, 1999). Moreover, the distribution of serotypes within a region is not static – between 1999 and 2007, the proportion of rotavirus cases attributed to serotype G9 by Australia's National Rotavirus Surveillance Program ranged annually from 7% to 75% (O'Ryan, 2009).

Rotaviruses are highly contagious, even in settings with high standards of hygiene and sanitation. Infected individuals may shed up to 100 billion viral particles per gram of stool (Flewett, 1983), while as few as 90 virions may be sufficient to establish a new infection in animal models (Payment and Morin, 1990)⁶. Transmission is predominantly faecal–oral, although spread via respiratory droplets has also been proposed (Gurwith et al, 1981). Following ingestion, viruses replicate primarily in mature epithelial cells near the tips of the small intestinal villi. In contrast to the gastroenteritis induced by numerous bacterial enteropathogens, diarrhoea occurs with limited intestinal inflammation. Based on studies conducted *in vitro* and in animal models, disease pathogenesis has been linked with several distinct mechanisms, including malabsorption induced by the destruction of epithelial cells and the disruption of epithelial cell function (Boshuizen et al, 2003), paracellular leakage caused by the disruption of tight junctions (Dickman et al, 2000), and the viral enterotoxin NSP4, which induces the secretion of chloride ions (and the consequent release of water) via a calcium-dependent signalling pathway (Ball et al, 2005). Viraemia may also occur, but has not been linked with any specific pathologic sequelae (Blutt et al, 2007). Additional details regarding the replication cycle and pathogenesis of rotavirus can be found in reviews by Estes and Greenberg (2013) and Desselberger (2014), among others.

1.3.2. Epidemiology

Among the myriad bacterial, viral, and eukaryotic diarrhoeal pathogens, rotavirus forms the greatest threat to human health. Exposure to the virus is universal – in the USA, evidence of prior rotavirus infection was apparent in more than 50% of infants aged 6–12 months and more than 90% of children aged 2–3 years prior to the introduction of rotavirus vaccination (Kapikian et al, 1978). Similarly, during a cohort study in Vellore, India, 81%, 96%, and 99% of

⁶ Although a minimum infectious dose of 100 virions is commonly cited for human infections, during a human challenge study by Ward et al (1986), infection was observed at a minimum dose of 9×10^{-1} focus forming units (ffu), with approximately 1.56×10^4 particles per ffu. No infections were observed at a dose of 9×10^{-2} or 9×10^{-3} ffu (each tested in seven volunteers). These findings suggest that a higher infectious dose ($\sim 10^4$ particles) is typically required to establish infection.

children had been infected by rotavirus by 1, 2, and 3 years of age, respectively (Gladstone et al, 2011).

Rotavirus infections are more likely to be severe between 3 and 24 months of age. Prior to 3 months, infections are typically mild or asymptomatic (Chrystie et al, 1978; Fischer et al, 2002), which may relate in part to a protective effect of passively acquired maternal antibodies (Xu et al, 2005) and breastfeeding (Jayashree et al, 1988a; Plenge-Bonig et al, 2010). The first exposure to rotavirus after 3 months of age is often associated with mild or severe diarrhoea (Velazquez et al, 1996). Although subsequent infections are more likely to be asymptomatic, re-infection rates are high – during the aforementioned cohort study in south India, 11% of infants had experienced five or more episodes of rotavirus by 3 years of age (Gladstone et al, 2011). Notably, the protective effect of prior rotavirus exposure may vary by setting – whereas two previous infections conferred complete protection against severe disease among infants in Mexico (Velazquez et al, 1996), the equivalent rate of protection in south India was 57% (Gladstone et al, 2011).

Prior to the introduction of rotavirus vaccination in the USA, the virus was responsible for an estimated 2.7 million cases of gastroenteritis each year, of which 55,000–70,000 resulted in hospitalisation (Payne et al, 2011). Rotavirus-associated gastroenteritis accounted for 20–60 deaths among children under 5 years of age, and approximately US \$1 billion in direct and indirect costs (Tucker et al, 1998). The per-capita disease burden is likely to be comparable in other high-income countries, although hospitalisation rates vary from approximately 250 per 100,000 children under 5 years of age each year in Spain (Visser et al, 1999) to more than 800 per 100,000 in Hungary and Australia (Ferson, 1996; Szucs et al, 1999).

In low-income countries, rotavirus-associated disease often ranks among the highest causes of infant mortality. Based on estimates from 2004, rotavirus was responsible for 475,000–580,000 deaths among children under 5 years of age, with more than half of these deaths occurring in just six countries – India, Pakistan, China, Nigeria, Ethiopia, and the Democratic Republic of Congo (Parashar et al, 2009). Estimated rotavirus-associated mortality rates ranged from less than 1 per 100,000 children under 5 years of age (observed in 50 countries) to 439 per 100,000 in Sierra Leone – a discrepancy attributable at least in part to variation in access to appropriate medical care, such as oral rehydration therapy.

Thus, while rotavirus infection in the first few years of life is experienced by nearly all children in both high- and low-income countries in the absence of vaccination, the consequences of infection vary significantly by geographic setting, with the majority of deaths occurring in a small number of countries in sub-Saharan Africa and the Indian subcontinent.

1.3.3. Rotavirus vaccines: development and implementation

It is remarkable to consider that by the time rotavirus was discovered in 1973, OPV had already been in use for more than a decade. As the significant disease burden of this novel pathogen became increasingly apparent, efforts to develop a vaccine accelerated swiftly, and human trials of rotavirus vaccine candidates began in the early 1980s. The first vaccine to be licensed, Rotashield, was a tetravalent vaccine consisting of a simian rotavirus strain (of serotype G3) alongside three simian–human reassortant strains (one each for serotypes G1, G2, and G4) containing human VP7 genes. After proving safe and efficacious during trials conducted in the USA (Bernstein et al, 1995; Rennels et al, 1996), Finland (Joensuu et al, 1997), and elsewhere (Clark et al, 2008), Rotashield was licensed in the USA in 1998. However, use of this vaccine would be short-lived. Less than a year after licensure, Rotashield was withdrawn after being linked with an increased risk of intussusception – a rare condition characterised by intestinal obstruction following the folding of one section of the bowel into another (Murphy et al, 2001).

Rotashield has since been replaced by RotaTeq (developed by Merck) and Rotarix (developed by GlaxoSmithKline), which were approved in the USA in 2006 and 2008, respectively. Like Rotashield, RotaTeq is an animal–human reassortant vaccine. It consists of four strains (one each for serotypes G1, G2, G3, and G4) containing human VP7 genes expressed by a bovine rotavirus (the Wistar Calf 3 [WC3] strain), and one strain in which WC3 is reassorted to express a human VP4 gene of genotype P[8]. Rotarix, on the other hand, comprises a single human rotavirus strain of serotype G1P[8] – the most common serotype of circulating wild-type rotavirus – obtained from a child in Cincinnati, USA, and passaged multiple times through monkey kidney cells (Bernstein et al, 1998)⁷. Three other vaccines – the Lanzhou lamb rotavirus vaccine, a lamb rotavirus strain licensed in China in 2000; Rotavin, a human strain licensed in Vietnam in 2007; and Rotavac, a naturally occurring human–bovine reassortant G9P[11] strain (termed 116E) licensed in India in 2014 – are also being used to combat rotavirus, but are currently licensed only for national markets.

In 2006, the WHO recommended that rotavirus vaccination be included in the routine immunisation programmes of all countries in Europe, North America, and Latin America based on the results of clinical trials confirming the safety and protective efficacy of Rotarix (in a two-dose series) and RotaTeq (in a three-dose series) in these regions (Ruiz-Palacios et

⁷ A nice coincidence: the Rotarix strain traces back to a child being treated during the 1988–1989 rotavirus season at the Cincinnati Children’s Hospital – the same hospital where Albert Sabin developed OPV several decades earlier (Cincinnati Children’s Hospital Medical Center, 2010).

al, 2006; Vesikari et al, 2006; WHO, 2007). These recommendations were subsequently expanded to include all regions following the completion of trials that confirmed the efficacy (albeit variable, as considered further below) of rotavirus vaccination in Asia and Africa (WHO, 2009; Armah et al, 2010; Madhi et al, 2010; Zaman et al, 2010). The implementation of rotavirus vaccines has expanded steadily since – an effort aided by funding from Gavi, which currently identifies rotavirus vaccination as a ‘cornerstone objective’ (Gavi, 2016). As of January 2016, 80 countries (including 37 countries eligible for Gavi support) have introduced rotavirus vaccination into their national routine immunisation programmes (Figure 1.3).

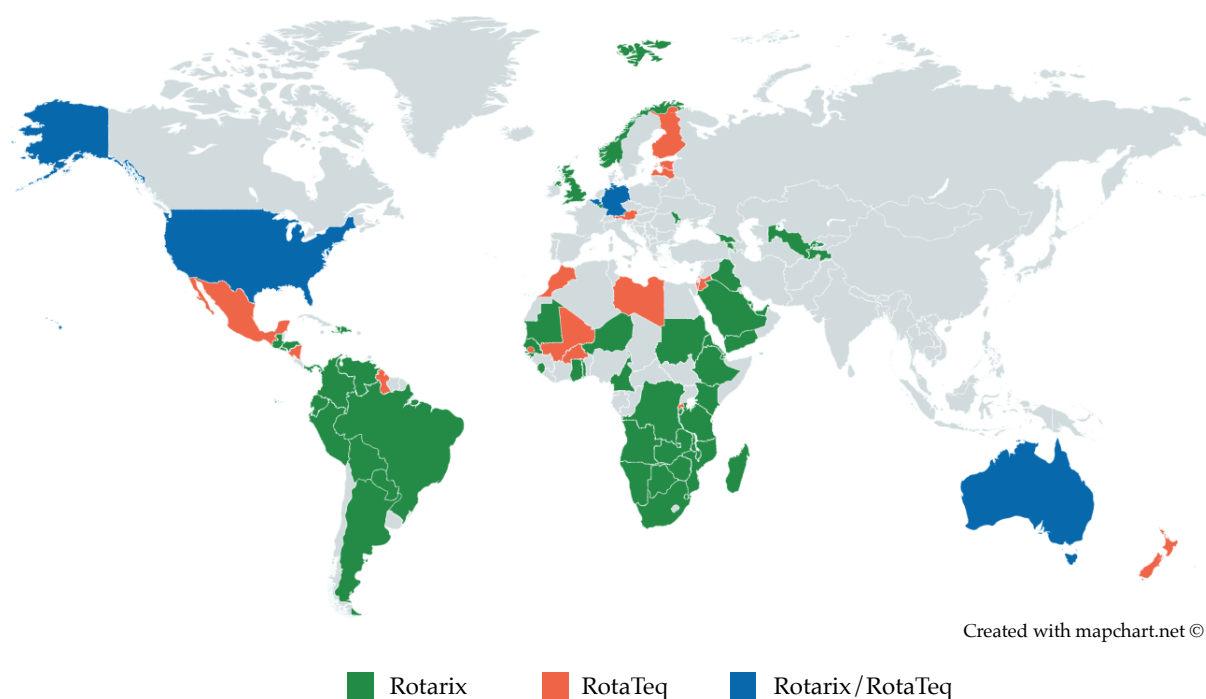


Figure 1.3. The global roll-out of rotavirus vaccines in national immunisation programmes. Data were obtained from PATH (2016). Rotavirus vaccination status is displayed as of January 2016. Among countries that have implemented vaccination schemes, 57 have introduced Rotarix (including 31 of the 37 Gavi-eligible countries), 19 have introduced RotaTeq, and four have introduced both vaccines. Vaccination has also been introduced in pilot or regional programmes in Canada, the Philippines, and Thailand.

1.3.4. Variation in rotavirus vaccine performance

i. Efficacy

Whereas much of our data regarding variation in the performance of OPV is based on immunogenicity, a primary endpoint of several placebo-controlled trials of rotavirus vaccines has been the protective efficacy of immunisation against rotavirus-associated gastroenteritis. In a systematic review and meta-analysis by Soares-Weiser et al (2012), which included 41

rotavirus vaccine trials overall, this was identified as an outcome in 21 trials of Rotarix and eight trials of RotaTeq. While the particulars vary from study to study, the following would be typical of these trials: infants are enrolled at the age of 6–12 weeks and administered a full course of either vaccine or placebo, with the former typically comprising two doses of Rotarix or three doses of RotaTeq, separated by an interval of 4–10 weeks; these individuals are then followed up for a period of up to 2 years of active surveillance involving regular (often weekly) meetings with trial staff; during any episodes of gastroenteritis in this surveillance period, severity is determined based on pre-defined criteria (usually the Vesikari severity score [Ruuska and Vesikari, 1990]) and stool samples are collected and assessed for the presence of rotavirus, generally by enzyme-linked immunosorbent assay (ELISA); finally, the relative risk of rotavirus-associated gastroenteritis in vaccine versus placebo recipients is used to calculate the protective efficacy of the vaccine⁸.

Estimates of the 1-year efficacy of Rotarix against severe rotavirus-associated gastroenteritis among the clinical trials reviewed by Soares-Weiser et al (2012) are provided in Table 1.3. Combining results across studies in low-mortality countries (WHO strata A and B)⁹, Rotarix had an efficacy of 86% (range, 74–97%) for up to 1 year of follow-up, and 85% (range, 78–96%) for up to 2 years. By contrast, in high-mortality countries (stratum E), the vaccine had an efficacy of 63% (range, 45–78%) for up to 1 year, and just 42% (range, 38–59%) for up to 2 years of follow-up, suggesting not only that Rotarix performance is severely impaired in these environments, but that protection wanes more swiftly. Comparable discrepancies in protective efficacy and duration of protection have been observed during clinical trials of RotaTeq, suggesting that similar barriers to vaccine performance affect both of these vaccines (Soares-Weiser et al, 2012).

Notably, despite containing only one strain of rotavirus (G1P[8]), Rotarix has been shown to confer both homotypic and heterotypic protection. Among infants in Malawi and South Africa, protection against severe rotavirus-associated gastroenteritis was equivalent for G1 versus non-G1 serotypes up to 1 year of age (Madhi et al, 2010), while efficacy in excess of 90% was observed for serotypes G1, G2, G3, and G9 up to 2 years of age among infants in several countries in Asia (Phua et al, 2009). However, in a multi-centre study performed across Latin America and Finland, Ruiz-Palacios et al (2006) observed a protective efficacy

⁸ Vaccine efficacy = (1 - relative risk) x 100

⁹ Based on the strata A to E defined by the WHO, wherein stratum A has very low mortality rates in children under 5 years of age (quintile 1), strata B and C have low mortality rates (quintiles 2 and 3), and strata D and E have high mortality rates (quintiles 4 and 5) (WHO, 2003).

Table 1.3. Efficacy of Rotarix against severe rotavirus-associated gastroenteritis (up to 1 year).

Study ^a	Location	Mortality group	Cases of severe RVGE (n/N)		RR (95% CI)	VE
			RV1	Placebo		
Bernstein et al (1999)	USA	A	2/108	9/107	0.22 (0.05–1.00)	78
Vesikari et al (2007)	Czech Republic, Finland, Germany, Italy, Spain	A	5/2572	60/1302	0.04 (0.02–0.10)	96
Phua et al (2009)	Hong Kong, Singapore, Taiwan	A	0/5263	15/5256	0.03 (0.00–0.54)	97
Salinas et al (2005)	Brazil, Mexico, Venezuela	B	27/1392	34/454	0.26 (0.16–0.42)	74
GlaxoSmithKline (2008)	Argentina, Brazil, Dominican Republic, Honduras, Panama	B	7/4211	19/2099	0.18 (0.08–0.44)	82
Ruiz-Palacios et al (2006)	Argentina, Brazil, Chile, Colombia, Dominican Republic, Finland, Honduras, Mexico, Nicaragua, Panama, Peru, Venezuela	A, B, D	12/9009	77/8858	0.15 (0.08–0.28)	85
Summary (strata A/B)					0.14 (0.07–0.26)	86
Madhi et al (2010)	Malawi	E	52/1182	47/591	0.55 (0.38–0.81)	45
Madhi et al (2010)	South Africa	E	16/2116	36/1050	0.22 (0.12–0.40)	78
Steele et al (2010)	South Africa	E	5/379	3/96	0.42 (0.10–1.74)	58
Summary (stratum E)					0.37 (0.18–0.75)	63

Data are from clinical trials included in a systematic review by Soares-Weiser et al (2012). Infection rates and risk ratios are those reported in Analysis 1.1 of this review. Abbreviations: CI, confidence interval; RR, risk ratio; RV1, Rotarix; RVGE, rotavirus-associated gastroenteritis; VE, vaccine efficacy.

^a Major publication, as cited by Soares-Weiser et al (2012), is indicated here.

for severe gastroenteritis of 91% against fully homotypic strains (G1P[8]), 87% against partly heterotypic strains (G3P[8], G4P[8], and G9P[8]), and 45% against fully heterotypic strains (G2P[4]). Thus, while Rotarix clearly confers a degree of heterotypic protection, this may be lower for viruses that differ from the vaccine at both G and P epitopes.

ii. Immunogenicity

In contrast to poliovirus, wherein the presence of neutralising antibodies in serum is an established correlate of protection against paralytic disease, the immunological correlates of protection against rotavirus remain equivocal (Clarke and Desselberger, 2015). The titres of

both serum and faecal IgA have been shown to reflect the level of rotavirus-specific IgA in the duodenum, and can therefore be viewed as markers of antibody responses at the intestinal mucosal surface (Hjelt et al, 1985; Grimwood et al, 1988). Elevated titres of serum neutralising antibodies (Chiba et al, 1986; Ward et al, 1992), serum IgA (Hjelt et al, 1987; O’Ryan et al, 1994), serum IgG (O’Ryan et al, 1994), and faecal IgA (Matson et al, 1993) have all been shown to correlate with protection against infection and/or severe gastroenteritis following natural rotavirus exposure, although symptomatic infection may still occur among individuals with high antibody titres (including serum IgA titres of 1 in 400 and serum IgG titres in excess of 1 in 6,400) (Velazquez et al, 2000). These findings likely reflect a failure to fully delineate the effectors involved in rotavirus immunity. Studies in animal models have highlighted the potential importance of cytotoxic T cells targeting conserved viral epitopes (Offit and Dudzik, 1989), while memory B cells expressing gut-homing markers have been shown to correlate weakly with protection in humans (Rojas et al, 2007).

Despite their equivocal value as protective correlates, serum antibody responses have been considered in a subset of vaccinees during several efficacy trials of Rotarix and RotaTeq. These efforts have focused predominantly on rotavirus-specific serum IgA (determined by ELISA), though several trials of RotaTeq have also examined serotype-specific neutralising antibodies (Armah et al, 2010; Zaman et al, 2010). To confuse matters, definitions of seroconversion have differed according to the vaccine being investigated – while a post-vaccination IgA concentration of ≥ 20 U/mL has been used to define seroconversion to Rotarix (Vesikari et al, 2007; Madhi et al, 2010), a three-fold increase between pre- and post-vaccination titres has been adopted in RotaTeq trials (Vesikari et al, 2006; Zaman et al, 2010).

Nonetheless, these immunogenicity studies have helped to corroborate the geographic trends in vaccine performance observed for efficacy endpoints. In a systematic review by Patel et al (2013), mean seroconversion rates following two doses of Rotarix were 87%, 74%, and 53% across studies conducted in countries with low (WHO stratum A), medium (stratum B), and high child mortality rates (strata D and E), respectively, and post-vaccination IgA concentrations followed a similar trend. Combining data across Rotarix and RotaTeq, the authors observed a significant correlation between post-vaccination IgA titre/concentration and efficacy (with an R^2 of 0.56). Moreover, lower IgA titres were associated with a greater drop-off in protective efficacy in the second year after vaccination (with post-vaccination concentrations of >90 U/mL generally indicative of sustained protection). The absence of serological and efficacy data from the same individual vaccinees precluded the examination of more precise thresholds of protection in this review. Overall, these findings

suggest that while serum IgA levels may not be a perfect correlate of protection, they provide a useful indicator of the marked discrepancies in vaccine performance between high- and low-income settings.

iii. Vaccine take

The link between the replication of vaccine viruses and seroconversion is less clearcut for oral rotavirus vaccines than for OPV (see section 1.2.3). I will refer to this outcome as ‘take’ in the following chapters, although rotavirus vaccine trials have often used this term to encompass either seroconversion or post-vaccination viral shedding (Salinas et al, 2005). Following challenge with live rotavirus, the shedding of higher quantities of rotavirus has been linked with greater increases in serum neutralising antibody titre (Ward et al, 1986), thus supporting the link between viral replication efficiency and immunogenicity. Similarly, during clinical trials of Rotarix, the presence of rotavirus antigens in stool samples collected approximately 1 week after vaccination has been observed in 50–80% of infants following the first dose of vaccine and 4–18% following the second dose (Cortese et al, 2009) – rates that are broadly consistent with dose-specific estimates of seroconversion (Vesikari et al, 2004; Salinas et al, 2005). Although few clinical trials of Rotarix have reported on vaccine take, it is reasonable to suspect that the geographic variation observed in the immunogenicity of this vaccine also applies to the replication efficiency of vaccine rotaviruses. By contrast, Yen et al (2011a) observed rotavirus antigen in the stool samples of just 21% of US infants within the 9 days following their first dose of RotaTeq. Given the high immunogenicity and efficacy of RotaTeq in this setting, it would appear that vaccine take is not as relevant an indicator of RotaTeq response as it is for OPV and Rotarix.

iv. Post-licensure effectiveness

The final form of data pertinent to the geographic variation in rotavirus vaccine performance is the effectiveness of Rotarix and RotaTeq in countries where they have been implemented in routine immunisation programmes. Increasingly, studies have been able to examine vaccine effectiveness by comparing the vaccination histories of individuals hospitalised for rotavirus-associated gastroenteritis with those of uninfected controls (Correia et al, 2010; Wang et al, 2010; Staat et al, 2011) – analogous to the case–control studies conducted within AFP surveillance databases for OPV. These post-licensure effectiveness studies have largely recapitulated the findings of large-scale studies of vaccine efficacy, while

also highlighting the protection conferred by a partial vaccination series. The receipt of a full series of RotaTeq has generally been shown to protect more than 90% of infants in high-income countries, while effectiveness in the range of 69–93% has been observed after a partial series (Patel et al, 2012b). By contrast, during a case–control study in Nicaragua, the same vaccine had an effectiveness of 44% after a full series (Patel et al, 2009a). Similarly, vaccine effectiveness after two doses of Rotarix has been estimated to be in excess of 97% among children in Spain, compared with 57% and 64% in South Africa and Malawi, respectively (Groome et al, 2014b; Bar-Zeev et al, 2015). As observed in vaccine efficacy trials, Rotarix has been shown to protect against both homotypic and heterotypic strains, although effectiveness appears to be lower and may wane more rapidly for heterotypic viruses (Correia et al, 2010; Patel et al, 2013b; Bar-Zeev et al, 2015). Data regarding the herd effects of immunisation (inferred based on the incidence of rotavirus-associated disease in children too old to be vaccinated) have been variable – whereas significant indirect protection after rotavirus vaccine introduction was recently documented in Moldova (Gheorghita et al, 2016) and Armenia (Sahakyan et al, 2016), such effects were absent in South Africa (Groome et al, 2016) and Zambia (Mpabalwani et al, 2016).

v. Conclusions

Overall, it is clear that oral rotavirus vaccines suffer from similar deficits in performance in low-income countries as OPV. Data from large-scale trials have highlighted severe discrepancies in the efficacy and immunogenicity of Rotarix and RotaTeq in low- versus high-income countries, and protection wanes more rapidly in the former. As rotavirus vaccines are rolled out in routine immunisation programmes across the globe, these deficits are likely to impede the control of rotavirus-associated disease in regions that endure its greatest burden.

1.4. Other oral vaccines

Before discussing the mechanisms that might account for geographic variation in oral vaccine performance, it is worth noting that this phenomenon is not restricted to vaccines targeting poliovirus and rotavirus. Indeed, several oral vaccines targeting bacterial enteropathogens have demonstrated similar trends in immunogenicity and/or efficacy.

As noted in the preface to this chapter, a live-attenuated oral *Shigella* vaccine candidate (referred to as SC602) was both reactogenic and immunogenic among adults in North

America, but displayed limited immunogenicity among children or adults in Bangladesh (WHO, 2006; Levine et al, 2007). Oral cholera vaccines may face similar obstacles in endemic settings. A single dose comprising 5×10^8 colony-forming units (CFU) of the live-attenuated cholera vaccine candidate CVD 103-HgR¹⁰ was sufficient to induce seroconversion (defined as a significant rise in vibriocidal antibodies) in more than 90% of North American adults (Levine et al, 1988; Kotloff et al, 1992); however, at the same dose, Su-Arehawaratana et al (1992) observed seroconversion in less than 50% of Thai soldiers during a series of studies conducted between 1988 and 1991¹¹. A study among Indonesian children 5–9 years of age documented similar deficiencies in immunogenicity following a single CVD 103-HgR dose at 5×10^8 CFU (with seroconversion in 16% of recipients), although the administration of an elevated dose (5×10^9 CFU) elicited seroconversion in 75–87% (Suharyono et al, 1992). While these findings suggest that increasing the dose of inoculum may partly overcome the barriers to CVD 103-HgR performance observed in low-income countries, estimates of vaccine efficacy when administered at elevated doses have produced mixed results (Richie et al, 2000; Calain et al, 2004).

CVD 103-HgR was licensed in Switzerland in 1991, and was subsequently approved in several other countries for use in individuals traveling to high-risk regions. However, the licensure process was never completed in the USA, and manufacture of the vaccine ceased in 2004. Two oral cholera vaccines are in use today – Dukoral, a monovalent vaccine comprising killed whole cells of *V. cholerae* serogroup O1 along with recombinant cholera toxin B subunit; and Shanchol, a bivalent vaccine containing killed whole cells of serogroups O1 and O139. Vaccination is largely restricted to emergency response (for example, following the recent cholera outbreak in Haiti [Ivers et al, 2012]), and the WHO has been maintaining a stockpile of the two vaccines for this purpose since 2012 (WHO, 2013). The degree to which Dukoral and Shanchol efficacy varies by geographic setting is unclear given that the use of these vaccines has largely been restricted to cholera-endemic countries or outbreak response. Notably, the vaccines have proven efficacious in both of these applications (Lucas et al, 2005; Shin et al, 2011; Luquero et al, 2014; Ivers et al, 2015), and Shanchol recently demonstrated a cumulative 5-year protective efficacy of 65% during a cluster-randomised trial in Kolkata, India, with no evidence of a significant drop-off in protection over time (Bhattacharya et al,

¹⁰ CVD 103-HgR consists of a strain of *V. cholerae* (classical Inaba 569B) in which the enzymatically active A subunit of cholera toxin has been deleted. The vaccine also contains a gene for mercury resistance, which serves as a marker for the modified strain.

¹¹ Intriguingly, the vaccine was more immunogenic among civilians, although the seroconversion rate in this group (63%) still fell short of the response rates observed in Europe and North America.

2013). It is worth noting, however, that during an immunogenicity study conducted among children up to 12 years of age, a vaccine similar in composition to Dukoral induced higher vibriocidal antibody titres in Swedish than in Nicaraguan individuals (Hallander et al, 2002).

In a review by Levine (2010), the live-attenuated *Salmonella* Typhi vaccine Ty21a, which is licensed in more than 50 countries, was highlighted as a “notable exception” to the trend of impaired oral vaccine performance in low-income countries on the basis of efficacy data from Chile, Egypt, and Indonesia. However, comparable data from industrialised countries are not available for this vaccine. Moreover, among the studies cited by Levine (2010), efficacy was considerably greater in Egypt (96% over 3 years of follow-up among children 6–7 years of age) than in Indonesia (42–53% over 30 months of follow-up among individuals 3–44 years of age), suggesting that important geographic variation in efficacy may still exist for this vaccine (Wahdan et al, 1982; Simanjuntak et al, 1991).

Overall, it is clear that oral vaccines targeting both viral and bacterial enteropathogens must contend with significant deficits in performance in low- versus high-income countries. These discrepancies present a clear obstacle not only to the effective use of available vaccines targeting poliovirus, rotavirus, and cholera, but to the development of novel oral vaccines targeting major enteric infections such as norovirus, astrovirus, *Shigella*, *Campylobacter*, and ETEC. Identifying the barriers to oral vaccine performance, and in due course the means to circumvent them, thus presents a crucial challenge to the control of enteric disease.

1.5. Explaining variation in oral vaccine performance

So far in this chapter, I have reviewed the considerable (and growing) body of evidence to suggest that oral vaccine performance is impeded in low-income countries. Efforts to elucidate the biological mechanisms responsible for this phenomenon date back to the early field trials of OPV. In the remainder of this chapter, I will summarise what is currently understood regarding the causes of variation in oral vaccine performance, and highlight the gaps in this understanding that the present thesis aims to address.

1.5.1. Transplacental antibodies

As noted above, newborn infants passively acquire serum antibodies from their mothers via the placenta. These antibodies, which are predominantly of class IgG (Cohen and Norins, 1968), are actively transported across the outer cell layer of the placenta via a specific receptor, termed the neonatal Fc receptor (Roopenian and Akilesh, 2007). IgG titres in cord

blood and infant sera have been shown to correlate closely with titres in maternal serum (Da Silva et al, 1958; Moon et al, 2016), while IgA and IgM antibodies are either absent or occur in trace amounts (Cohen and Norins, 1968). Following placental transfer, rotavirus- and poliovirus-specific antibodies decay with a half-life of 3–4 weeks (Gelfand et al, 1960; Robertson, 1993; Behrend et al, 2014; Moon et al, 2016). When acquired at a starting titre of 1 in 1,024 or greater, poliovirus-specific antibodies typically remain detectable for at least 9–12 months post birth (Da Silva et al, 1958).

As with so many of the issues that have remained central to the OPV research agenda, the potential impact of maternal antibodies on vaccine immunogenicity was highlighted during the First International Conference on Live Poliovirus Vaccines in 1959, when Konald Prem from the University of Minnesota described an impaired response to tOPV (in this case using strains developed at Lederle Laboratories) among newborn infants with elevated titres (>1 in 32) of passively acquired antibodies (Prem et al, 1959). This effect has consistently been demonstrated in field trials of tOPV since (Cirne et al, 1995; Asturias et al, 2007; Chandir et al, 2014; Estivariz et al, 2015), with the greatest inhibitory effect typically observed for serotype 3 (Asturias et al, 2007; Estivariz et al, 2015). Among infants in Brazil who received tOPV at 0, 6, 10, and 14 weeks of age, type 3 seroconversion was observed in 70% of individuals who had a baseline homotypic antibody titre of less than 1 in 64, but only 29% of those with a baseline titre of at least 1 in 256 (Cirne et al, 1995). Similarly, passively acquired serum antibodies have been observed to confer protection against natural rotavirus infection (Xu et al, 2005) and to reduce the immunogenicity of oral rotavirus vaccines, including Rotarix (Moon et al, 2016), RotaTeq (Becker-Dreps et al, 2015), and strain 116E (Appaiahgari et al, 2014).

The mechanisms by which maternal antibodies interfere with oral vaccine performance remain uncertain. Although serum IgG may passively transfer across the intestinal mucosa (termed transudation), it is unlikely to reach sufficient concentrations to directly inhibit the replication of vaccine viruses (Siegrist, 2003). Inhibition of serum antibody responses may instead occur during the downstream processing of antigens from replicating viruses. As highlighted by Siegrist (2003), these antigens may be exposed to maternal antibodies during transit to lymph nodes, masking them from B cell receptors. Antigen–antibody complexes are also readily taken up by antigen-presenting cells, preventing exposure to B cell receptors while still enabling T cell responses to be elicited (the latter have demonstrated a greater resistance to inhibition by maternal antibodies for parenteral vaccines [Gans et al, 1998]). In both cases, the degree of inhibition would be expected to correlate with the titre of passive

antibodies, which is consistent with observations for OPV (Cirne et al, 1995) and rotavirus vaccine (Appaiahgari et al, 2014).

Irrespective of the precise mechanism, the inhibitory influence of maternal antibodies on serological response to OPV and oral rotavirus vaccines is clear. The degree to which this contributes to geographic trends in vaccine performance is less certain. Although cross-sectional surveys of poliovirus- and rotavirus-specific maternal antibody titres have been carried out at individual sites (Chan et al, 2011; John et al, 2011), direct comparison of multiple sites using standardised methods are currently lacking for either disease (although such studies have been conducted for breastmilk, as detailed below). In the case of OPV, recent exposure to live viruses – either circulating wild viruses or vaccine viruses shed by OPV recipients – is likely to be more common among mothers in low-income countries (where OPV is widely implemented), which may boost the titre of serum antibodies that are passed on to newborns. Similarly, maternal exposure to natural rotavirus infection, and consequent boosting of serum antibody titres, will be greatest in high-transmission settings, enhancing the potential influence of passively acquired antibodies.

Discrepancies in vaccine performance by geographic setting may therefore be partly attributable to variation in the titre of passively acquired antibodies. However, there are several aspects of this phenomenon that maternal antibodies cannot explain, including: (i) the significant within-country variation in OPV efficacy observed during studies of AFP surveillance databases in India and Nigeria, wherein the majority of doses would have been received during SIAs (and thus after the waning of passively acquired antibodies) (Grassly et al, 2006; Mangal et al, 2014); (ii) the impaired immunogenicity of tOPV observed among Indian infants 3–12 months of age who were seronegative to all three types of poliovirus at enrolment (John and Jayabal, 1972)¹²; and (iii) the significant discrepancies in CVD 103-HgR immunogenicity among adults in high- versus low-income countries (section 1.4).

1.5.2. Breastfeeding

Breastmilk has the capacity to decrease the titre of vaccine viruses that reach the gut owing to the presence of neutralising antibodies (predominantly of class IgA) and of innate immune factors such as lactadherin and lactoferrin (Patel et al, 2009b). This may, in turn,

¹² After one dose of tOPV, seroconversion to serotypes 1, 2, and 3 was observed in 15%, 60%, and 30% of these infants, respectively – considerably lower than the seroconversion rates observed after one dose of tOPV among North American children with a high prevalence of pre-vaccination antibodies (McBean et al, 1988).

contribute to geographic variation in oral vaccine performance. Moon et al (2010) observed rotavirus-specific IgA and neutralising antibody titres to be significantly higher in breastmilk obtained from Indian mothers compared with mothers from the USA, with intermediate titres observed in samples from Vietnam and South Korea. The researchers observed similar discrepancies in the levels of lactoferrin, lactadherin, rotavirus-specific IgA, and neutralising antibodies in breastmilk obtained from Indian and South African women when compared with North American women (Moon et al, 2013). Although no analogous studies have recently been performed for poliovirus, Zaman et al (1993) documented significantly higher IgA titres specific to type 1 virus in breastmilk samples obtained from unvaccinated women from Pakistan compared with OPV-immunised women from Japan or IPV-immunised women from Sweden, and it is likely that similar discrepancies between endemic and non-endemic settings persist today.

In spite of these marked differences in antibody titres, findings regarding the impact of breastfeeding on vaccine performance are less than clearcut. In the case of OPV, Lepow et al (1961) documented a marked reduction in the excretion of vaccine viruses in breastfed versus non-breastfed neonates immunised shortly after birth. Other studies have observed similar trends when OPV is administered during the first few days of life (Sabin et al, 1963), suggesting that the elevated antibody titres in colostrum may interfere with the replication of vaccine polioviruses. Later in infancy, the picture is more ambiguous – while an increase in the duration of breastfeeding was associated with a decrease in seroconversion rate (significant for serotype 3) following four doses of tOPV among infants in Brazil, no significant differences in either shedding or seroconversion were observed following the administration of type 1 mOPV to breastfed versus non-breastfed infants in Uganda (Domok et al, 1974). Among Indian infants between 6 and 51 weeks of age, withholding breastfeeding for 3–6 hours before and after vaccination did not impact the immunogenicity after one or three doses of tOPV (compared with infants breastfed within the 30 minutes prior to each dose) (John et al, 1976). To confuse matters further, a recent trial in Bangladesh documented a significant increase in type 1 seroconversion in association with exclusive breastfeeding following the administration of tOPV at 6, 10, and 14 weeks of age (Estivariz et al, 2015), and is not alone in highlighting the potential benefits of breastfeeding for OPV response (Pickering et al, 1998; Haque et al, 2014).

Studies of oral rotavirus vaccines have similarly failed to establish a clear inhibitory (or beneficial) effect of breastfeeding on vaccine performance. While several trials have observed a reduction in immunogenicity among breastfed infants (Rennels, 1996), the effect has

typically been modest, and recent trials in India, Pakistan, and South Africa have failed to document a significant change in seroconversion rate in conjunction with the temporary withholding of breastfeeding immediately before and after vaccination (Groome et al, 2014a; Rongsen-Chandola et al, 2014; Ali et al, 2015).

It is possible that the conflicting findings of these studies reflect contradictory influences of breastfeeding on vaccine immunogenicity. On the one hand, IgA antibodies and innate immune factors may inhibit the replication of vaccine viruses – an effect that is likely to be particularly potent during the neonatal period given the elevated titres of antibodies in colostrum. However, breastfeeding may also prevent enteric infections (Bilenko et al, 2008), promote the maturation of the infant immune system (M'Rabet et al, 2008), and buffer vaccine viruses from gastric acid (Rennels, 1996), which together may alleviate or indeed reverse the inhibitory effects of breastmilk IgA.

1.5.3. Host genetics

The potential contribution of host genetics to variation in vaccine response has garnered increasing interest as sequencing technologies have become more widely available. In particular, candidate gene and genome-wide association studies have helped to uncover genetic variants that influence the performance of vaccines targeting measles, rubella, and hepatitis B, among others (Mentzer et al, 2015; Milet et al, 2016). An important role for genetic variation in shaping the immune response to OPV is supported by a study of twin pairs in the Gambia who received tOPV at 0, 1, 2, 3, and 9 months of age (Newport et al, 2004). Based on the titre of serotype 1 neutralising antibodies in monozygotic versus dizygotic twins at 5 months of age, the authors estimated that heritability may account for 60% of the variation in OPV response. It is worth noting, however, that factors influencing antibody titre after immunisation may not be the same as those influencing seroconversion, which is the primary outcome of interest in the chapters that follow.

A recent genome-wide association study was conducted among infants in Bangladesh to identify genetic variants that may contribute to variation in OPV response (Wojcik, 2013). This distinguished several suggestive associations for single-nucleotide polymorphisms on chromosome 7 (of which the top-ranking fell upstream of the gene *SHH*) and chromosome 14 (of which the top-ranking fell downstream of the gene *MAPK1IP1L*), and also highlighted the potential importance of genes involved in histone modification. However, these findings have not been independently validated and should thus be treated with caution.

Studies considering the impact of genetic variation on rotavirus vaccine response are currently lacking. However, recent findings suggest that variation in the phenotype of histo-blood group antigens (HBGAs) – previously linked with susceptibility to noroviruses and several other enteropathogens (Flores and Okhuysen, 2009) – may influence clinical outcomes following natural rotavirus exposure (Imbert-Marcille et al, 2014; Van Trang et al, 2014). Specifically, it appears that individuals with a non-secretor HBGA phenotype may be resistant to infection with P[8] rotavirus genotypes (Imbert-Marcille et al, 2014). The extent to which HBGA phenotype impacts the efficacy of oral rotavirus vaccines remains a topic of ongoing research, albeit one that is beyond the scope of the present thesis.

1.5.4. Malnutrition and micronutrient deficiencies

Malnutrition is common in the world's poorest countries, and as noted above, may contribute to more than half of deaths among children under 5 years of age worldwide (Caulfield et al, 2004). The detrimental effects of malnutrition may arise in part through abnormalities in immune function – including deficits in antibody production, complement formation, and thymic function, among others – which have been linked both with impaired growth (e.g., stunting and wasting) and deficiencies in specific micronutrients, such as zinc, iron, and vitamin A (Scrimshaw and SanGiovanni, 1997; Hughes and Kelly, 2006). In light of these immunological deficits, there has been much interest in the possible link between malnutrition and vaccine performance in low-income countries. To date, these efforts have failed to uncover a consistent link between the two (Savy et al, 2009; Prendergast, 2015). In the case of OPV, a recent study among infants in Bangladesh documented a significant decrease in type 3 poliovirus antibody titres in conjunction with malnutrition (as defined by a weight-for-age Z score of <-2) after at least three doses of OPV (Haque et al, 2014). However, other studies in India (Chopra et al, 1989) and Nigeria (Greenwood et al, 1986) have failed to uncover an association between weight and OPV immunogenicity. Similarly, supplementation with vitamin A did not significantly impact OPV seroconversion rates during trials in Bangladesh, Indonesia, and Ghana (Rahman et al, 1998; Semba et al, 1999; Newton et al, 2005), while the receipt of zinc supplements – which are known to have a significant therapeutic effect during the treatment of acute gastroenteritis (Patro et al, 2008) – had no effect on OPV seroconversion during a recent placebo-controlled trial in Pakistan (Habib et al, 2015).

Data regarding the potential impact of malnutrition on oral vaccines other than OPV are limited, although supplementation with zinc (but not vitamin A) significantly boosted

vibriocidal antibody response among recipients of CVD 103-HgR in Bangladesh (Albert et al, 2003). Data from a recent trial examining the impact of zinc and/or probiotic supplementation on the immunogenicity of Rotarix and OPV among infants in south India will be discussed further in Chapters 4 and 5 of this thesis (although the focus here will be on the association between the intestinal microbiota and vaccine outcome). Overall, while it remains plausible that malnutrition and micronutrient deficiencies may contribute to the impaired performance of oral vaccines in low-income countries, we currently lack robust data to support this notion.

1.5.5. Concurrent enteropathogens

Enteropathogen infections have long been recognised as a major cause of morbidity and mortality, and this burden is greatest in the low-income countries where oral vaccines are least immunogenic. Of the 7.6 million deaths among children under 5 years of age in 2010, diarrhoea accounted for approximately 10%, with the vast majority of these occurring in Africa and southeast Asia (Liu et al, 2012).

In addition to symptomatic disease, the pervasive nature of asymptomatic enteric infections in low-income countries has become increasingly apparent in recent years, placing the remarkable scale of enteropathogen transmission in a new light. This is perhaps best illustrated by the MAL-ED study – a multi-centre cohort study in which children from eight low- and middle-income countries in Africa, Asia, and South America were followed for the first 2 years of life (Lang, 2010; Platts-Mills et al, 2015). The prevalence of common bacterial, viral, and eukaryotic enteropathogens was assessed in stool samples collected during episodes of diarrhoea as well as in non-diarrhoeal surveillance samples (collected at monthly intervals for the first year life, and every 3 months thereafter). This approach revealed the burden of enteropathogens to be considerable irrespective of diarrhoea status (Platts-Mills et al, 2015). Indeed, at least one pathogen was identified in 65% of non-diarrhoeal stools, while two pathogens were present in 29%. Infection intensity varied considerably across the study sites – in Naushero Feroze, Pakistan, more than one pathogen per stool was observed by 2 months of age on average, and this rose to approximately two pathogens per surveillance sample by 1 year of age; by contrast, in Venda, South Africa, the mean number of pathogens per surveillance sample did not exceed one throughout the follow-up period. Considerable seasonal variation was also observed for several pathogens. Together, these findings, along with those of other recent studies that have sought to quantify the burden and aetiology of diarrhoeal disease (such as the Global Enteric Multicenter Study [Kotloff et al, 2013]),

highlight the intense and varied burden of enteropathogens experienced by infants in low-income countries from soon after birth.

Non-polio enteroviruses (NPEVs) were not considered by the MAL-ED study, but are an important player in the chapters that follow. Like diarrhoeal pathogens, NPEVs occur frequently among infants living in low-income countries. Among children enrolled in a polio vaccine trial in Kenya, NPEV prevalence ranged from less than 30% at 2–3 months of age to over 70% at 8–9 months (Kok et al, 1992). Although studies in high-income countries have occasionally documented comparably high NPEV infection rates (e.g., ~40% during an OPV trial conducted in Italy [Nardi et al, 1966]), the prevalence of these viruses tends to fall well short of the rates observed in low-income settings. Indeed, NPEVs were identified in only one of 203 children aged between 3 months and 5 years during the first trial of ‘balanced’ tOPV conducted in Canada in 1961 (Robertson et al, 1962).

Do NPEVs and other enteropathogens interfere with the performance of oral vaccines? As noted above, OPV response varies by season in tropical settings, and diminished vaccine take has been shown to coincide with the high season of enterovirus transmission in India (Grassly et al, 2012). Although these seasonal variations are consistent with an inhibitory effect of concurrent infections on OPV, direct evidence of enteropathogen interference is more conflicting. In the spring of 1958, a trial of OPV was conducted in Mexico in which a reduction in both seroconversion and vaccine take was observed in infants infected with NPEVs at the time of vaccination, suggesting that existing viruses may interfere with the establishment of vaccine polioviruses in the intestinal tract of OPV recipients (Benyesh-Melnick et al, 1959). These and other similar findings during early studies of OPV gave rise to a firm conviction in the inhibitory effect of enteric pathogens on vaccine response, and led Albert Sabin to conclude the following during the First International Conference on Live Poliovirus Vaccines (Sabin, 1959):

“It is obvious from these observations and from others to be reported here ... that the high incidence of naturally occurring enteric viruses among children living under poor conditions of sanitation and hygiene, especially in subtropical and tropical climates, presents a special problem in the successful utilization of live poliovirus vaccine under these conditions.”

In spite of this initial certainty, subsequent efforts to determine the impact of enteropathogens on OPV have yielded contradictory results – while several studies have supported the inhibitory effects of NPEVs (Japan Live Poliovaccine Research Commission,

1966; Triki et al, 1997), bacterial enteropathogens (Mahmoud et al, 1976), and concurrent diarrhoea (Posey et al, 1997), others have refuted these effects (John and Christopher, 1975; Kok et al, 1992). During a large field trial involving two doses of tOPV in Toluca, Mexico, Sabin himself observed seroconversion rates of 96%, 96%, and 72% for serotypes 1, 2, and 3, respectively, despite the presence of NPEVs in more than 50% of children in the study population before the trial commenced (Sabin et al, 1960). In light of these conflicting data, the relative contribution of NPEVs and other enteric infections to the phenomenon of impaired OPV immunogenicity in low-income countries remains uncertain.

Data regarding the impact of enteric infections on vaccines other than OPV are limited. During a placebo-controlled trial in Ecuador, treatment with albendazole (an anthelmintic) prompted an increase in seroconversion rates and vibriocidal antibody titres following a subsequent dose of CVD 103-HgR among children with ascariasis, highlighting the potential inhibitory effect of eukaryotic pathogens on oral vaccine immunogenicity (Cooper et al, 2000). Given that OPV has been co-administered alongside oral rotavirus vaccines in a number of trials, the potential interference between these attenuated viruses has garnered considerable interest (Patel et al, 2012a). Notably, while co-administration does not appear to inhibit the immunogenicity of OPV, rotavirus-specific seroconversion rates and antibody titres have consistently been lower when either Rotarix or RotaTeq are administered with OPV than without, particularly following the first dose of rotavirus vaccine (Rennels, 1996; Patel et al, 2012a). Thus, the continued use of OPV during routine immunisation in low-income countries (as opposed to the IPV-only schedules generally adopted in high-income countries) may contribute to the geographic discrepancies observed in rotavirus vaccine performance.

In addition to the influence of co-administered Sabin viruses, the potential inhibitory effect of natural enterovirus exposure on Rotarix outcome was highlighted during a recent study among infants in Bangladesh (Taniuchi et al, 2016)¹³. Herein, the quantity of enteroviruses at the time of immunisation was negatively correlated with the immunogenicity and efficacy of the rotavirus vaccine – an effect that was not observed for any other viral, bacterial, or eukaryotic enteropathogens. Since no association was observed between the quantity of Sabin polioviruses and Rotarix outcome, the inhibitory effect of enteroviruses in this study was ascribed principally to NPEVs. The degree to which these findings (published late in the write-up of this thesis) are representative of the risk factors for rotavirus vaccine failure in other low-income settings remains to be seen.

¹³ Notably, this study adopted the same PCR-based methods of enteropathogen detection adopted in Chapters 4, 5, and 6.

A central aim of the present thesis is to address the gaps in our current understanding of the relationship between enteropathogen infections and oral vaccine response, first by integrating data from past studies of OPV in a systematic review and meta-analysis (Chapter 2), and subsequently by using novel diagnostic methods to clarify the influence of enteropathogen exposure on the immunogenicity of OPV and Rotarix (Chapters 4, 5, and 6). Potential mechanisms that might account for an inhibitory effect of enteropathogens on oral vaccines will be considered during the discussion of these data.

1.5.6. The bacterial microbiota

The potential influence of the intestinal microbiota on oral vaccine response is not restricted to concurrent enteropathogens. Indeed, there is good reason to suspect that the microbiota as a whole (commensal and pathogenic) may play an important role in shaping our immune response to these vaccines, and it is this possibility that forms the second principal line of enquiry in the present thesis.

The intestinal microbiota is a diverse and crowded community comprising an estimated 100 trillion prokaryotes (the bacterial microbiota) (Whitman et al, 1998), an even greater number of bacteriophages and eukaryotic viruses (the virome), and an array of protists, yeasts, fungi, and helminths (the eukaryome). Although this thesis focuses predominantly on the bacterial microbiota, the potential contribution of the virome and eukaryome to geographic discrepancies in oral vaccine outcome should not be discounted. Indeed, both vary markedly by geographic setting (Holtz et al, 2014; Parfrey et al, 2014), and both have been shown to influence development of the mammalian immune system and intestinal homeostasis (Kernbauer et al, 2014; Lukes et al, 2015).

A more detailed introduction to the laboratory and bioinformatic methods adopted to assess the bacterial microbiota will be provided in Chapter 3. Here, I will restrict my focus to two key questions: what is known about the development of the bacterial microbiota in early infancy? And what might lead us to suspect that the composition of the microbiota contributes to the discrepancies in oral vaccine performance between high- and low-income countries?

i. Development of the bacterial microbiota

The timing of first microbial colonisation of the infant gut has been the subject of considerable debate in recent years (Rodriguez et al, 2015). While it was previously believed

that the foetus develops in an environment that is kept sterile by the placental barrier, this view has been challenged by the detection of bacteria in umbilical cord blood (Jimenez et al, 2005), amniotic fluid (Bearfield et al, 2002), and placental tissue (Aagaard et al, 2014) of healthy newborns. Genetically labeled *Enterococcus* strains administered orally to pregnant mice have been shown to appear in both amniotic fluid and meconium (the first stool produced following birth), suggesting that members of the maternal oral or gut microbiota may be transmitted directly to neonates during gestation (Jimenez et al, 2005; Jimenez et al, 2008). However, the extent of prenatal colonisation and health implications of this microbial exposure remain uncertain (Rodriguez et al, 2015).

Following any intrauterine exposure, the next major colonisation opportunity occurs during delivery. The meconium microbiota of neonates born via vaginal delivery has been shown to overlap in composition with the vaginal microbiota of their mothers (with major genera including *Prevotella*, *Lactobacillus*, and *Sneathia*), while that of neonates born by caesarean section more closely resembles the skin microbiota of mothers (including a predominance of *Staphylococcus*, *Acinetobacter*, and *Micrococcineae*) (Dominguez-Bello et al, 2010). These initial differences in composition according to delivery mode may form the basis of longer-term discrepancies in the bacterial microbiota, including a delay in Bacteroidetes colonisation and a decrease in Clostridia abundance among infants delivered by caesarean section (Salminen et al, 2004; Jakobsson et al, 2014). Contrary to this notion, however, several studies have observed the microbiota composition of infants born via vaginal delivery and caesarean section to converge over the first 6 months of life (Trosvik et al, 2010; Dogra et al, 2015).

Other important sources of early colonisation include exposure to the mother's faecal microbiota during delivery, which may enable transmission of Enterobacteriaceae, among other taxa (Rodriguez et al, 2015); contact with the mother's skin, enabling transfer of *Staphylococcus* species (prominent early colonisers that are swiftly outcompeted over the first few months of life) (Subramanian et al, 2014; Rodriguez et al, 2015); and breastmilk, which contains a complex bacterial community encompassing several hundred species, rich in the genera *Streptococcus*, *Staphylococcus*, *Weissella*, *Leuconostoc*, and *Lactococcus* (Hunt et al, 2011; Cabrera-Rubio et al, 2012).

Following these initial colonisation events, the gut microbiota remains in a state of near-constant flux and maturation for the first 2–3 years of life, with diversity increasing constantly (Koenig et al, 2011; Schloss et al, 2014; Subramanian et al, 2015). Factors known to influence this process include gestational age, diet, maternal health status, host genetics, and

(as detailed below) geographic location (Rodriguez et al, 2015). Overall, the maturation of the microbiota can be thought of as a process of ecological succession, with early colonisers that are predominantly facultative anaerobes (e.g., *Staphylococcus*, *Lactococcus*, and *Enterobacteriaceae*) fostering changes in the infant gut, including a decrease in redox potential, that enable colonisation by obligate anaerobes (e.g., *Bifidobacterium* and *Clostridium*) (Rodriguez et al, 2015). Gradual progressions are punctuated by major shifts in composition that coincide with changes in diet, antibiotic usage, and infection – as elegantly demonstrated by a 2.5-year case study in which faecal samples from a single infant were collected at regular intervals from birth onwards (Koenig et al, 2011). During this study, sequencing of the highly conserved 16S rRNA gene – encoding the 16S ribosomal RNA subunit – was used to determine the composition of the bacterial microbiota, while metagenomic analysis (encompassing composition and function) of a subset of samples was conducted via shotgun sequencing. This revealed an early microbiota (coinciding with exclusive breastfeeding) dominated by Firmicutes and enriched in genes involved in lactate utilisation. A shift in diet to include formula and solid foods at approximately 4 months of age was associated with an abrupt expansion of the phylum Bacteroidetes, while the cessation of breastfeeding and shift to an adult diet was associated with the establishment of a more stable microbial community capable of performing the core metabolic functions of the adult microbiome, including vitamin biosynthesis and the breakdown of polysaccharides. Other events, including a fever at 3 months of age and the treatment of an ear infection with cefdinir (a broad-spectrum oral cephalosporin antibiotic) at 1 year of age, were also associated with temporary shifts in community composition and functional profile.

The maturation of the infant microbiota culminates in a diverse and stable bacterial community after approximately 3 years (Yatsunenko et al, 2012). Estimates of the number of species present in a typical adult gut vary according to the depth of sampling and the definition of ‘species’ adopted: while a minimum of 160 species per individual is often cited based on a metagenomic study of European adults (Qin et al, 2010), with sufficient sequencing depth more than 1,500 operational taxonomic units (OTUs) – typically defined as sequences with at least 97% identity spanning one or more hypervariable regions of the 16S rRNA gene – have been observed in samples from healthy adults (Yatsunenko et al, 2012).

ii. Potential influence of the bacterial microbiota on oral vaccines

As noted above, the infant microbiota is in a state of profound flux for the first 2–3 years of life – a time encompassing the administration of oral vaccines during routine

immunisation and (in the case of OPV) SIAs. Several lines of reasoning suggest that the composition of the gut microbiota may contribute to geographic trends observed in oral vaccine performance. I shall tackle each of these in turn below.

(1) The bacterial microbiota shapes the development of the infant immune system

To date, much of our understanding regarding the relationship between the bacterial microbiota and the immune system comes from the study of germ-free animals or those colonised by defined microbial communities (termed gnotobiotic animals). Although this may be a somewhat blunt approach for assessing what is undoubtedly a complex set of interactions, it has nonetheless provided a clear indication of the importance of the gut microbiota to the development and function of the mammalian immune system. Germ-free mice harbour gross structural abnormalities in the gut-associated lymphoid tissue, including a decrease in the number of IgA-producing plasma cells, a reduction in the size of Peyer's patches, and a decrease in the number of intraepithelial lymphocytes (for a more complete account, refer to Smith et al [2007]). The activation of innate immune receptors (e.g., Toll-like receptors) by microbial signals has been shown to enhance epithelial cell proliferation and the repair of epithelial damage (Pull et al, 2005; Hooper et al, 2012). Specific bacterial taxa have also been shown to promote the expansion of distinct immune cell populations – for example, segmented filamentous bacteria trigger the accumulation of CD4⁺ Th17 cells in the small intestine (Ivanov et al, 2009), while members of the genus *Clostridium* have been shown to induce CD4⁺ regulatory T cells in the colonic mucosa (Atarashi et al, 2011). Microbial metabolites such as short-chain fatty acids may interact with host immune cells to produce similar immunomodulatory effects (Arpaia et al, 2013).

The reach of the gut microbiota goes beyond the intestinal mucosa (Hooper et al, 2012). Germ-free animals have fewer plasma cells in systemic circulation, fewer germinal centres, and diminished serum antibody responses to bacterial antigens (Smith et al, 2007). The risk of atopic disease has been linked with composition of the gut microbiota during critical windows of early childhood in both mice and humans (Russell et al, 2013; Arrieta et al, 2015). New interactions between the microbiota and the immune system are constantly being uncovered (e.g., Gomez de Agüero et al, 2016), highlighting the myriad ways in which the immune system has evolved to shape and respond to the body's microbial inhabitants. While these findings may be insufficient to directly tie the microbiota with oral vaccine response, they do establish a robust link between the composition of the gut microbiota and the maturation and functional capacity of the immune system in early infancy.

(2) The bacterial microbiota facilitates viral colonisation of the intestinal mucosa

In addition to its role in shaping the development of the mammalian immune system, the bacterial microbiota has been shown to facilitate the replication and persistence of a number of enteric viruses (Pfeiffer and Virgin, 2016). Again, relevant data regarding these ‘transkingdom interactions’ are derived largely from animal models, including germ-free, antibiotic-treated, and immunodeficient mice. In the case of poliovirus, depletion of the bacterial microbiota by antibiotics has been shown to inhibit viral replication and pathogenesis in mice (Kuss et al, 2011), while *in vitro* virus infectivity is enhanced by exposure to live bacteria (both Gram-negative and Gram-positive), killed bacteria, and bacterial surface polysaccharides such as lipopolysaccharide and peptidoglycan (Kuss et al, 2011). These polysaccharides have been shown to increase the thermal stability of virions as well as enhance attachment to the poliovirus receptor (Robinson et al, 2014), suggesting that polioviruses have evolved to exploit microbiota-derived signals in order to colonise the intestinal mucosa. Analogous effects have been reported for rotavirus, which exhibits reduced infectivity and pathogenicity in antibiotic-treated mice (Uchiyama et al, 2014). In spite of this, treated mice exhibited higher titres of rotavirus-specific serum IgG, serum IgA, and faecal IgA compared with untreated mice, highlighting the complexity of interactions between the microbiota and the immunogenicity of enteric viruses.

Thus, there is a growing body of evidence reflecting the importance of transkingdom interactions to enteric virus colonisation and infectivity. It is worth noting, however, that while microbiota-derived signals may be necessary for viral infection, it is by no means clear that variation in microbiota composition translates to variation in viral (and viral vaccine) outcomes, especially given that signals such as lipopolysaccharide and peptidoglycan would be readily available in the infant gut irrespective of geographic setting.

(3) Composition of the infant microbiota varies by geographic location

In order to account for the observed variation in oral vaccine performance between high- and low-income countries, it is necessary for the composition of the infant microbiota to vary geographically. Such variation has been amply demonstrated in several recent studies. By sequencing the V4 hypervariable region of the 16S rRNA gene in 531 individuals aged 0–83 years from the USA, Venezuela, and Malawi, Yatsunenکو et al (2012) observed considerable differences in microbiota composition according to location, with particularly marked separation between US individuals and those from Venezuela and Malawi. These

discrepancies were evident among adults and infants (under 6 months of age). Using the Random Forests machine-learning algorithm (discussed further in Chapter 3), the researchers identified 28 OTUs that discriminated US from non-US infants, and 23 OTUs that distinguished Venezuelan from Malawian infants (with several members of the family Enterococcaceae enriched in the former). Compositional differences were mirrored by discrepancies in the functional profile of the infant microbiome, as determined by shotgun sequencing of faecal samples. In particular, several genes involved in vitamin biosynthesis (especially vitamin B2) and carbohydrate metabolism were enriched among Malawian and Venezuelan compared with US infants, suggesting that functional differences in the microbiota may reflect discrepancies in diet and vitamin availability.

Several other studies have documented variation in microbiota composition when comparing individuals from different regions (De Filippo et al, 2010; Grzeskowiak et al, 2012; Nakayama et al, 2015). Using fluorescence *in situ* hybridisation and flow cytometry, Fallani et al (2010) highlighted the possible existence of a ‘geographic gradient’ across Europe in the composition of the faecal microbiota at 6 weeks of age, with northern countries characterised by the enrichment of the genus *Bifidobacterium* and several *Clostridium* species, and southern countries by the enrichment of *Bacteroides* and *Lactobacillus*, among others. Notably, the differences observed between countries exceeded the variation among samples due to delivery mode and feeding method (breast fed, formula fed, or mixed). While these and other similar findings are consistent with the presence of broad-scale latitudinal gradients in microbiota composition (Suzuki and Worobey, 2014), it has also been suggested that similarities in host lifestyle may link the bacterial microbiota of individuals from geographically disparate settings. Indeed, Pehrsson et al (2016) recently compared faecal microbiota data from individuals in Peru, El Salvador, Venezuela, Malawi, and the USA, and documented marked clustering of microbiota composition according to whether hosts had an ‘urban industrial’, ‘peri-urban industrialising’, ‘rural agriculturalist’, or ‘hunter–gatherer’ lifestyle.

Together, these findings highlight the substantial variation that occurs in microbiota composition according to geographic setting and lifestyle – variation that is undoubtedly sufficient to account for all or some of the discrepancies observed in oral vaccine performance.

(4) Preliminary data support a link between the microbiota and oral vaccine response

A recent study among infants in Bangladesh immunised at 0, 6, 10, and 14 weeks of age provides the first direct evidence of a potential link between the composition of the

microbiota and the immune response to OPV (Huda et al, 2014). The V4 region of the 16S rRNA gene was sequenced via Illumina Miseq to determine the diversity and composition of the microbiota in 48 infants at 6, 11, and 15 weeks of age. This revealed the relative abundance of the phylum Actinobacteria at 15 weeks to be positively correlated with poliovirus-specific IgG concentration in serum and CD4+ T cell stimulation index (also measured at 15 weeks). The quantity of several members of the genus *Bifidobacterium*, as determined by quantitative PCR, was also correlated with poliovirus-specific T cell response, highlighting the potential influence of these taxa on vaccine immunogenicity. It is worth noting, however, that the study did not adopt the standard microneutralisation assay during the measurement of OPV response (section 1.2.3), and may therefore have failed to capture discrepancies in microbiota composition that influence seroconversion to the vaccine. Moreover, overall microbiota diversity within samples (alpha diversity), compositional overlap between samples (beta diversity), and OTU-level differences in relative abundance were not reported in relation to OPV response¹⁴. While this study provides a preliminary validation of the potential role of the microbiota in shaping OPV immunogenicity, much uncertainty remains regarding the nuances and key players in this relationship.

Several other studies lend support to the hypothesised link between microbiota composition and oral vaccine response. Small bowel bacterial overgrowth, as measured by hydrogen breath test, was associated with a decrease in vibriocidal antibody titre following one dose of CVD 103-HgR among children in Chile (Lagos et al, 1999), while an increase in microbiota diversity was linked with a higher CD8+ T cell response (but not humoral response) among individuals who received four doses of the oral typhoid vaccine Ty21a (Eloe-Fadrosch et al, 2013), albeit among only six vaccinees. Owing to the increased availability of high-throughput sequencing technologies, it is reasonable to expect that numerous studies in the coming years will probe further into the influence of the microbiota on vaccine immunogenicity (both oral and parenteral).

iii. The bacterial microbiota as a marker for other risk factors of oral vaccine failure

In light of its impact on both the development of the infant immune system and the infectivity of viral pathogens, it is reasonable to hypothesise that the composition of the bacterial microbiota may directly influence the outcomes of oral immunisation. However, it is also possible that the intestinal flora might act as a diagnostic or prognostic marker for

¹⁴ See Chapter 3 for further details regarding these measures.

other risk factors of oral vaccine failure. In particular, significant shifts in the composition and diversity of the bacterial microbiota have been documented in conjunction with malnutrition (Smith et al, 2013; Dinh et al, 2016), current or recent diarrhoea (Subramanian et al, 2014), and chronic inflammation of the gut (Manichanh et al, 2006; Fava and Danese, 2011), among others. Given that the present thesis aims to characterise the association between the composition of the bacterial microbiota and oral vaccine outcome among infants in south India, as opposed to the mechanisms of vaccine failure in animal models or *in vitro* systems, it will be important to bear these potential indirect effects in mind during the chapters ahead.

1.5.7. Environmental enteropathy

At the intersection of the mucosal immune system, the intestinal microbiota, and enteropathogen exposure is a pervasive condition termed ‘environmental enteropathy’ (EE). This subclinical disorder was first recognised in the 1960s, when jejunal biopsies of individuals from Thailand (Sprinz et al, 1962), Pakistan (Lindenbaum et al, 1966), and several other developing countries revealed structural abnormalities of the intestinal architecture to be a frequent occurrence among asymptomatic individuals. EE is characterised by the flattening of intestinal villi, an increase in crypt depth, nutrient malabsorption, intestinal inflammation (including an influx of inflammatory cells into the lamina propria), increased gut permeability, and bacterial translocation across the gut wall, among other physiological and immunologic manifestations (Korpe and Petri, 2012; Prendergast and Kelly, 2012). Although the precise aetiology of this condition is unclear, exposure to enteropathogens is thought to be crucial. As noted by Korpe and Petri (2012):

“Constant exposure to fecal–oral contamination, and sustained episodes of self-limited infectious gastroenteritis, may lead to a perpetual state of small bowel injury. This injury could cause hyperstimulation of the mucosal immune system and result in the small bowel pathology that has been described.”

The characteristics of EE may begin to appear soon after birth, and thereby have the potential to overlap with the administration of oral vaccines during routine immunisation – in a study conducted in south India, finger-like villi were observed in small intestinal biopsies of stillborn foetuses; however, some degree of villous blunting was evident within the first 30 days of life in the majority of infants, while more severe manifestations of EE were apparent by 8 weeks of age (Chacko et al, 1969). Brown et al (2015) recapitulated the

features of EE by administering a cocktail of microbes (including a mixture of Bacteroidales species and *E. coli*) to malnourished mice, thereby implicating diet and microbial exposure in the aetiology of this condition. EE also displays a degree of seasonality – during a 3-year cohort study involving more than 200 adults in Zambia, marked seasonal variations were observed in villous height, gut permeability, and carbohydrate absorption (Kelly et al, 2004).

Owing to the pervasive nature of EE and its apparent link with chronic inflammation and other alterations of intestinal immune function, this condition has been hypothesised to play a key role in explaining the impaired response to oral vaccines in low-income settings (Serazin et al, 2010; Korpe and Petri, 2012; Keusch et al, 2014). A key difficulty faced in unravelling this potential association is how best to measure and define EE. Owing to the invasive nature of intestinal biopsies, studies of EE have increasingly adopted alternative means of identifying the subclinical condition from blood or stool samples, including markers of gut inflammation (e.g., faecal calprotectin, myeloperoxidase, and lactoferrin), microbial translocation (e.g., faecal neopterin and soluble CD14 in serum or plasma), and mucosal permeability (e.g., faecal α 1-antitrypsin and reg1B in serum). Although a recent study observed significant associations between several of these EE biomarkers and serum antibody titres to Rotarix and OPV among infants in Bangladesh (Naylor et al, 2015), individual markers were often not consistent in the direction of their association, either across the oral vaccines (e.g., reg1B, which was positively associated with Rotarix response but negatively associated with type 3 poliovirus response), or across different timepoints for the same vaccine (e.g., soluble CD14, which differed in the direction of its association with type 1 poliovirus response when measured at 6 and 18 weeks of age). The relative importance of EE in shaping the immune response to oral vaccines thus remains uncertain. Moreover, establishing the extent of this association is made all the more difficult by the inevitable overlap of EE with several other hypothesised causes of oral vaccine failure, including enteropathogen exposure and malnutrition.

1.5.8. The infectious agent

The nature of exposure to the targeted pathogen may have an important contribution to geographic variation in the protective efficacy of oral vaccines. As noted above, while Rotarix is capable of inducing both homotypic and heterotypic immunity, protection appears to be lower when individuals are exposed to viral strains that differ from the vaccine at both G and P epitopes (Ruiz-Palacios et al, 2006). Such exposure may be more frequent in low-income countries, where the circulation of unusual rotavirus strains is more common (Ramachandran

et al, 1996; Gentsch et al, 2005; Moyo et al, 2014). Variation in transmission intensity may also be relevant to vaccine response. Following a trial of Ty21a in Indonesia, Simanjuntak et al (1991) highlighted the possibility that an increase in the infectious inoculum (“more frequent inoculations of greater numbers of bacteria”) in this high-transmission setting may have contributed to the lower efficacy observed compared with earlier trials in Chile and Egypt. Similarly, an increase in the infectious load following extensive flooding was implicated during a large outbreak of type 1 paralytic poliomyelitis within a highly immunised population in South Africa between 1987 and 1988 (Schoub et al, 1992).

In the case of rotavirus, exposure to natural infection in the neonatal period is common, particularly in settings with a high transmission intensity. One study performed in an urban hospital in India observed rotavirus shedding in 36% of infants within the first 3 days of life (Jayashree et al, 1988b). In a review of Rotarix trials, Cunliffe et al (2014) documented baseline rotavirus-specific IgA seropositivity rates of 0–9% among studies conducted in Europe and North America and 0–26% among studies in Asia, Africa, and Latin America (given that transfer of IgA antibodies across the placenta is negligible, the presence of rotavirus-specific IgA in serum can be seen as indicator of prior viral exposure). Neonatal exposure has been linked with a reduction in the severity (but not frequency) of subsequent rotavirus infection (Bishop et al, 1983; Vethanayagam et al, 2004), albeit not in all studies (Banerjee et al, 2007). Notably, a reduction in vaccine immunogenicity among seropositive infants was recently observed during studies of Rotarix conducted in Zambia (Chilengi et al, 2016) and Bangladesh (Emperador et al, 2016), suggesting that the induction of rotavirus-specific immunity following neonatal viral exposure may contribute to the impaired immunogenicity of rotavirus vaccines in high-transmission settings.

1.6. Knowledge gaps and hypotheses

Significant deficits in the immunogenicity of oral vaccines targeting poliovirus, rotavirus, and several other enteropathogens have consistently been documented in settings where they might confer the greatest public health benefit. The biological mechanisms responsible for this phenomenon have not been fully elucidated, in part because a range of interconnected factors are undoubtedly involved. In the present thesis, I will focus on just one aspect of this broader tapestry – the influence of the intestinal microbiota. In particular, I will aim to answer two central questions. Do bacterial, viral, or eukaryotic enteropathogens influence the immunogenicity and replication efficiency of OPV or oral rotavirus vaccine? And is the composition of the commensal bacterial flora associated with response to these vaccines?

Although the potential inhibitory effect of concurrent enteropathogens on OPV has been considered in a number of previous trials (section 1.5.5), the influence of these infections (particularly non-viral infections) remains uncertain. Meanwhile, limited data are currently available regarding the potential effect of enteropathogens on the immunogenicity of oral rotavirus vaccines. I have attempted to clarify the impact of concurrent pathogens on the immunogenicity of OPV and oral rotavirus vaccine by exploiting recent advances in molecular diagnostics. Specifically, in Chapter 4 of this thesis, I will describe how TaqMan array cards – comprising 384-well arrays of singleplex real-time PCR assays targeting a multitude of common bacterial, viral, and eukaryotic enteropathogens (Liu et al, 2013) – were used to examine the influence of concurrent infections on response to Rotarix among infants in a low-income community in south India. The same approach was then extended to examine the impact of enteropathogens on OPV response, first within the same cohort of infants (Chapter 5) and subsequently among infants 6–11 months of age enrolled in a randomised, placebo-controlled trial examining the impact of oral azithromycin treatment on the immunogenicity of mOPV3 (Chapter 6). By comparing pathogen burden according to vaccine outcome in each of these study populations, I sought to test the hypothesis that the presence of enteropathogens at the time of immunisation inhibits the immunogenicity of oral vaccines targeting poliovirus and rotavirus, and thereby contributes to the geographic variation observed in the efficacy of these vaccines.

The potential influence of the bacterial microbiota on oral vaccine immunogenicity might take one of several forms. First, specific taxa may interact with vaccine strains in either an inhibitory or beneficial way (analogous to keystone species that impact their ecosystem in a manner disproportionate to their relative abundance). In the case of allergic atopy, for example, it was recently observed that a decrease in the relative abundance of four genera (*Faecalibacterium*, *Lachnospira*, *Rothia*, and *Veillonella*) at 3 months of age was associated with a significant increase in the risk of developing asthma later in childhood (Russell et al, 2013). While there are some data to suggest that members of the genus *Bifidobacterium* may have a beneficial effect on OPV response (Huda et al, 2014), further investigation of the potential impact of ‘keystone taxa’ on OPV and other oral vaccines is certainly warranted. In Chapters 4, 5, and 6, I will describe how sequencing of the 16S rRNA gene within the faecal samples of infants in south India was used to identify whether specific taxa differed in relative abundance according to the outcome of Rotarix or OPV. Owing to the relative novelty of this line of enquiry, there were limited existing data regarding the potential identity of such

discriminant taxa¹⁵. However, taking cues from the published literature on inflammatory bowel disease (IBD), we hypothesised *a priori* that failure to respond to the oral vaccines would be associated with an elevated abundance of Proteobacteria – a phylum containing numerous pathogenic species (including *Campylobacter*, *Shigella*, and the assorted pathotypes of diarrhoeagenic *E. coli*) that has consistently been implicated in the onset of Crohn’s disease and other chronic inflammatory conditions of the gut (Carvalho et al, 2012; Mukhopadhyaya et al, 2012).

In addition to the influence of specific taxa, the immune response to oral vaccines may be shaped by a broader shift (or ‘dysbiosis’¹⁶) of the gut’s microbial community. Such shifts have been documented in association with a broad range of pathologies, including IBD (Fava and Danese, 2011), obesity (Turnbaugh et al, 2009), diabetes (Larsen et al, 2010), and malnutrition (Smith et al, 2013), among others (Balder, 2012). It is reasonable to suspect that analogous broad-scale discrepancies in microbiota composition might also be apparent among infants at risk of oral vaccine failure. This possibility was tested – both in relation to oral rotavirus vaccine (Chapter 4) and OPV (Chapters 5 and 6) – by assessing the overall diversity and composition of the bacterial microbiota in faecal samples collected from infants in south India at the time immunisation. Specifically, we hypothesised that failure to respond to oral vaccination would be associated with a reduction in the diversity of the bacterial microbiota, as observed in conjunction with malnutrition (Subramanian et al, 2014), IBD (Manichanh et al, 2006), and acute gastroenteritis (Mai et al, 2006; Chang et al, 2008).

1.6.1. General versus specific effects

While the trend of impaired performance in low-income countries appears to be general to oral vaccines, the underlying mechanisms responsible for this phenomenon may not be. OPV and Rotarix are both composed of attenuated viruses that are capable of replicating and inducing an immune response in the intestinal mucosa, but target different cell types and elicit different immune effectors. It is reasonable to suspect that certain risk factors for vaccine failure may be shared across these oral vaccines, such as the inhibitory effect of passively acquired maternal antibodies, while others may be specific to a particular vaccine

¹⁵ The study by Huda et al (2014) was not published until after we had designed the 16S rRNA gene sequencing work described in Chapters 4 and 5.

¹⁶ The term ‘dysbiosis’ is somewhat nebulous, but is often used to describe a significant change in microbiota diversity or composition in conjunction with a particular pathology. I will refrain from using it further in this thesis owing to its lack of a replicable definition as a diagnostic entity.

or vaccine strain. For example, it may transpire that HBGA phenotype influences the infectivity of P[8] rotavirus genotypes, but not other attenuated strains of this virus. Thus, a secondary aim of this thesis is to determine the extent to which the association between the intestinal microbiota and vaccine outcome is shared across Rotarix and OPV.

1.6.2. Objectives

- i) To integrate published data regarding the impact of concurrent infections on OPV response via systematic review and meta-analysis (Chapter 2);
- ii) To introduce the laboratory and bioinformatic methods adopted in subsequent chapters for the assessment of (i) bacterial, viral, and eukaryotic enteropathogens, and (ii) the composition of the bacterial microbiota (Chapter 3);
- iii) To assess the impact of concurrent enteropathogens and the bacterial microbiota on the immunogenicity and take of Rotarix and tOPV doses co-administered to infants in south India at 6 and 10 weeks of age (Chapters 4 and 5);
- iv) To determine the proportion of failed vaccine seroconversions per dose of tOPV that can be attributed to enteropathogens in this setting (Chapter 5);
- v) To establish whether the composition of the bacterial microbiota influences the immunogenicity and take of mOPV3 administered to 6–11 month-old infants in south India (Chapter 6);
- vi) To determine whether novel, persistent, or recently resolved enteropathogens differ in the extent to which they interfere with mOPV3 response (Chapter 6).

1.7. Conclusions

The central issue of this thesis – why do oral vaccines underperform in low-income countries? – has existed for more than half a century. Despite considerable effort, we are still some way from defining the biological mechanisms at the heart of this phenomenon, and further still from establishing how best to circumvent it. My work set out to gain novel insight into this aged query by harnessing recent technological advances – both in the detection of multiple enteropathogens using novel diagnostic methods and in the profiling of the bacterial flora via high-throughput sequencing.

Before considering these novel techniques further, however, we shall first turn our gaze to what has gone before by applying a more traditional technique – the systematic review and meta-analysis.

2.1. Background

In 1959, Albert Sabin referred to enteric infections as a “special problem” facing the successful implementation of OPV in low-income countries based on the observation of diminished or delayed replication of vaccine polioviruses in individuals with concurrent viruses (Sabin, 1959). Not only are infections such as NPEVs more prevalent in regions where OPV is least immunogenic (Mangal et al, 2014), but within these regions, OPV outcomes are diminished during the high season of pathogen transmission (Grassly et al, 2012). In light of the inhibitory potential of enteropathogens, the WHO modified its immunisation policy in the mid-1980s to recommend that an additional OPV dose be given to children if they are immunised during an episode of gastroenteritis (WHO, 1986).

However, as highlighted in the previous chapter (section 1.5.5), data regarding the influence of enteric infections on OPV have yielded contradictory results – while several reports have emphasised the inhibitory influence of concurrent NPEVs (Japan Live Poliovaccine Research Commission, 1966; Triki et al, 1997), bacteria (Mahmoud et al, 1976), or diarrhoea (Posey et al, 1997), others have refuted these effects (Kok et al, 1992; John and Christopher, 1975; Sabin et al, 1960). The relative contribution of enteric infections to the impaired immunogenicity of OPV in low-income countries thus remains uncertain.

With this in mind, we carried out a systematic review and meta-analysis with the aim of clarifying the influence of enteric infections and diarrhoea on the odds of responding to OPV. The review also considered the potential effects of EE (the subclinical disorder associated with blunted intestinal villi, nutrient malabsorption, and intestinal inflammation described in section 1.5.7), which has been widely documented among individuals in low-income countries, and suggested as a possible cause of impaired oral vaccine performance (Korpe and Petri, 2012; Serazin et al, 2010).

2.1.1. Hypotheses

- i) The balance of evidence in the published literature supports the presence of an inhibitory effect of concurrent enteropathogens or diarrhoea on OPV response;
- ii) This inhibitory effect is reflected by a decline in the relative odds of seroconversion and vaccine take per dose of OPV in infected versus uninfected individuals.

2.2. Methods

2.2.1. Literature search

A literature search was carried out in October 2012 to identify published articles examining the influence of concurrent enteric infections, diarrhoea, or environmental enteropathy on response to OPV. Titles, key words, and abstracts in the citation databases PubMed and ISI Web of Knowledge were searched using the following term: “polio* and (oral or OPV) and (compet* or interfer* or diarrh* or entero* or enteric or coinfect* or bacter* or helminth* or parasit* or protozoa*) and (immunogen* or stool or excret* or shed* or sero* or serum or antibod* or efficac* or fec* or faec*)”. The asterisk symbols in this search term function as ‘wildcards’ enabling the matching of truncated words (e.g., “seroprevalence”, “seroconversion”, and “serology” would all be matched by the search term “sero*”).

2.2.2. Screening of abstracts and titles

PRISMA guidelines were followed throughout the study selection process (Moher et al, 2009). Following the removal of duplicates, the titles and abstracts of studies identified during the above search were evaluated for their relevance to the review topic. Articles were considered eligible for full-text review if their abstracts reported the delivery of OPV to human recipients alongside the assessment of: (i) concurrent diarrhoea; (ii) concurrent enteric infection; (iii) enterovirus excretion or faecal sample collection at or near the time of vaccination; (iv) EE; or (v) unspecified factors influencing or interfering with OPV response. Articles were excluded if they did not report on primary research or included fewer than ten OPV recipients. Among articles for which no abstracts were available, full-text copies were obtained if the title referred to OPV delivery alongside one of the five potential indicators of interference listed above. Eligible articles were obtained from electronic journals or from the holdings of the British Library (London, UK) or Wellcome Library (London, UK).

2.2.3. Search for additional studies

Additional studies were identified by scanning the text and bibliographies of articles being considered for full-text review. Articles were obtained if they were described as having examined the effect of non-polio enteric infections, diarrhoea, or EE on OPV response, or if their title referred to OPV and one of these potential sources of interference. Several complete conference proceedings were also scanned for potentially relevant studies (including the First International Conference on Live Poliovirus Vaccines [1959], the Second International

Conference on Live Poliovirus Vaccines [1960], and the Fourth Scientific Conference of the Institute of Poliomyelitis and Virus Encephalitis [1960]), as were the text and bibliographies of a number of relevant articles identified during the review of abstracts or known to the authors (Gerichter et al, 1978; Oduntan et al, 1978; Salk, 1979; Sabin, 1980; Tulchinsky et al, 1989; Patriarca et al, 1991; Fine and Carneiro, 1999; Grassly et al, 2007).

2.2.4. Inclusion and exclusion criteria

Studies were included in the review if they fulfilled the following criteria: (i) delivery of OPV in a prospective trial; (ii) reporting 0–7 days prior to OPV delivery of diarrhoea, NPEV excretion, other indicators of enteric co-infection, or markers of EE; and (iii) measurement of OPV response, including assessment of seroconversion or poliovirus-specific antibody titres within 8 weeks of OPV delivery, shedding of vaccine poliovirus between 1 and 4 weeks after immunisation (hereafter referred to as vaccine take), or intestinal immunity (including measurement of poliovirus-specific faecal IgA following vaccination or shedding of vaccine virus after OPV challenge). If the reporting of enteric infections spanned the 7 days preceding vaccination, but was not limited to this window, the study was included. Studies were excluded if: (i) they included only immunocompromised individuals; (ii) fewer than ten individuals were present in either the infected or control group; (iii) OPV outcomes were not presented according to the presence or absence of concurrent infection, diarrhoea, or markers of EE; or (iv) the reporting of concurrent enterovirus infection did not distinguish polioviruses from NPEVs. Conference abstracts were excluded if a complete report of the study was already included in the review. If multiple eligible reports of the same trial were encountered, the most comprehensive report was used for data extraction, although other reports were consulted for relevant details where necessary. Several reports presented data from separate trials of mOPV and tOPV (Urasawa, 1964b; Ramos Alvarez, 1966) or of tOPV trials conducted in different countries (Cirne et al, 1995) – these were considered as separate studies during the analysis. Publications in languages other than English were translated with the assistance of proficient speakers.

2.2.5. Data extraction

Data were extracted from eligible studies regarding the type, schedule, and potency of the administered OPV, the timing of sample collection or diarrhoea assessment, the collection method for faecal specimens, the laboratory methods used for the assessment of enteric

viruses, and the criteria and methods used to assess serological response. Results presented only in graphical form were digitised and data extracted using Plot Digitizer software (version 2.6.2). Where relevant, an effort was made to obtain supplementary details from authors of the included studies, although this was generally not feasible given that the majority of trials were conducted more than 30 years ago.

2.2.6. Statistical analysis

i. Random-effects meta-analyses

Meta-analyses were conducted to examine the effects of concurrent NPEV infection and diarrhoea on the odds of seroconversion per dose of OPV, and of NPEVs on the odds of vaccine take. Intestinal immunity was not used as a measure of OPV response in any of the eligible studies. Studies were included in the analyses if they reported the number or proportion of individuals demonstrating seroconversion or vaccine virus shedding after a given dose of OPV by serotype and according to the presence or absence of concurrent NPEVs (excluding poliovirus-infected individuals) or diarrhoea. In several studies (e.g., Dardanoni et al, 1962; Ingram et al, 1962), data were presented for individual vaccinees or groups of vaccinees; in these instances, OPV response data were grouped according to infection status at the time of vaccination. If data were presented as proportions, the number of responders was inferred by rounding to the nearest whole number. An additional analysis was conducted to compare the odds of seroconversion after multiple OPV doses in individuals presenting with diarrhoea at the time of one or more doses with those free of diarrhoea at all doses. Summary odds ratios (ORs) and 95% confidence intervals (CIs) were calculated separately for each serotype on a log scale using random-effects models (DerSimonian and Laird, 1986). Heterogeneity among studies was assessed using the χ^2 statistic. A continuity correction of 0.5 was used in trials with zero events in any comparison group.

An important decision before undertaking these analyses was whether to use fixed-effect or random-effects meta-analytic models. As noted by Borenstein et al (2010), fixed-effect models assume that all studies are estimating a common effect (the fixed effect), with variability arising only due to sampling variation among studies. By contrast, random-effects models assume that there are two sources of variation among studies – variation in the effect size being estimated, and random sampling variation in the estimation of these effects. Owing to the marked differences in the age and location of the study populations, and

discrepancies in the timing of sample collection and laboratory techniques adopted (as discussed further below), it was reasonable to expect that a distribution of effects would be captured by the included studies. Random-effects models were therefore deemed more appropriate for these analyses.

Following the estimation of serotype-specific effects, data across the three serotypes were combined in multi-level meta-analytic models based on a structural equation modelling approach (Cheung, 2014), incorporating study as a cluster effect. Overall ORs for the multi-level models were calculated using maximum likelihood estimation, with likelihood-based CIs. Stratified analyses and mixed-effects meta-regression were used to assess the impact of serotype, formulation (mOPV vs tOPV), and trial setting (low-, lower-middle-, or upper-middle-income vs high-income countries, as listed by the World Bank in April 2013) on effect size; the impact of each factor on model fit was assessed using the likelihood ratio test (LRT).

Potential publication bias was assessed using funnel plots and Egger's test (Egger et al, 1997). Sensitivity analyses were performed to include studies with at least five individuals in the infected and control groups (rather than ten), and to include trials in which poliovirus-infected individuals were not distinguished from NPEV-infected individuals. Analyses were conducted in the programming language R (using the packages *meta* and *rmeta* for serotype-specific models, and *metaSEM* for multi-level models) and using Review Manager software (RevMan 5.2).

ii. A note on multi-level models

The decision to use multi-level meta-analytic models in this study revolves around their capacity to reflect hierarchies in the structure of the data when estimating the overall OR across serotypes. Crucially, it would not be appropriate to treat data for separate poliovirus serotypes from an individual study as independent given that they were collected from the same group of vaccine recipients. By incorporating study as a 'cluster effect' within a multi-level modelling approach, it was possible to account for this pseudoreplication (analogous to including study as a random effect within a mixed-effects regression model). We used the R package *metaSEM* – which implements random-effects meta-analyses as structural equation models (as described by Cheung [2014]) – to fit the multi-level models described above owing to its ability to recapitulate serotype-specific OR estimates obtained using standard random-effects models (as implemented in the R packages *meta* and *rmeta*) as well as its capacity to compare the fit of nested models using the LRT.

2.3. Results

2.3.1. Study selection

The literature search led to the identification of 203 articles of potential relevance to the review. Among these articles, 28 fulfilled the criteria for inclusion in the review, reporting on 25 distinct trials of OPV. Figure 2.1 presents a flow chart of the study selection process, while a summary of the eligible studies is provided in Table 2.1.

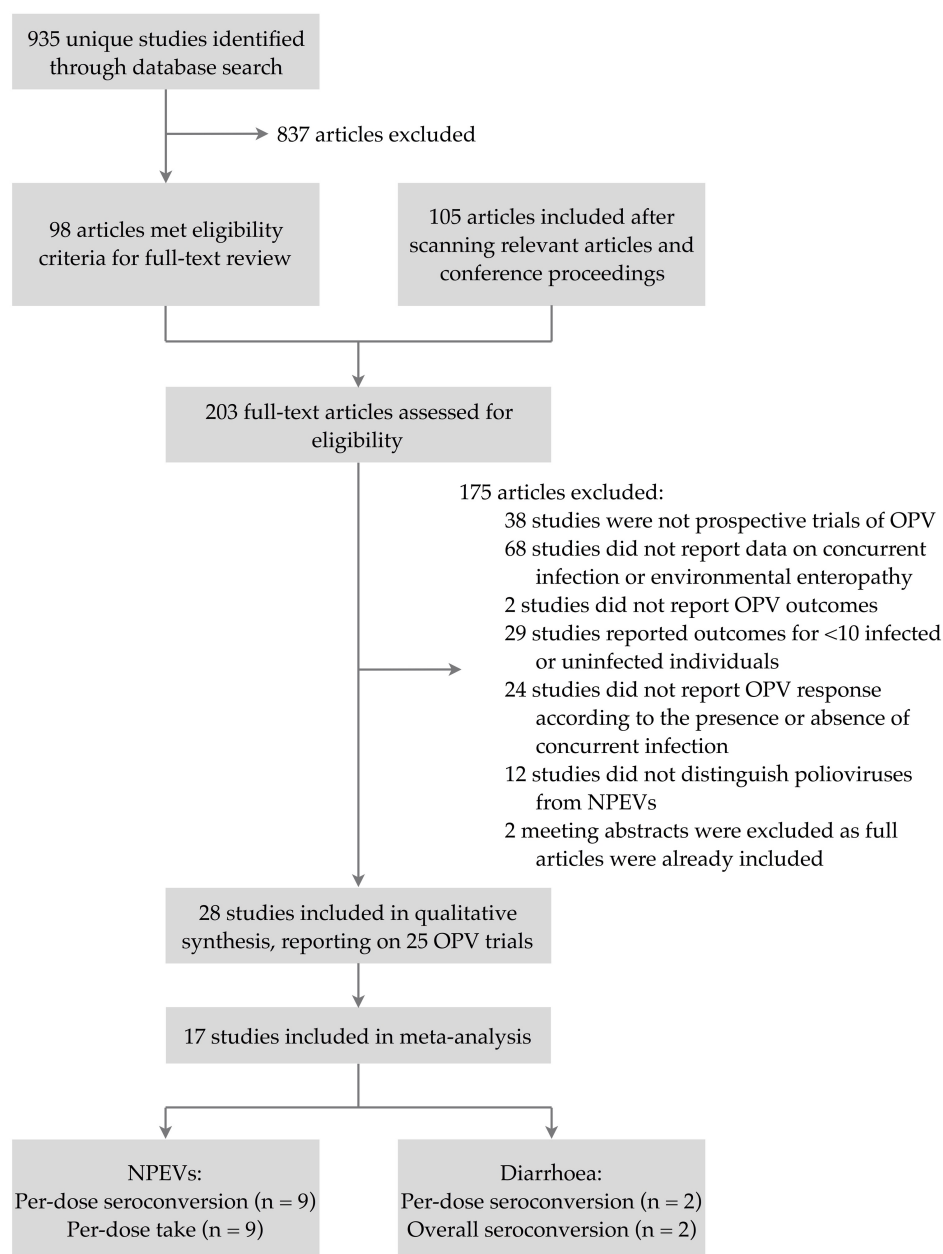


Figure 2.1. Flow chart of study selection process. A study by Swartz et al (1972b) was initially classified as eligible, but was subsequently re-classified based on details in a separate report by the authors (Swartz et al, 1972a), which clarified that the presence of NPEV infections in the week following (as opposed to preceding) OPV delivery had been used as an indicator of concurrent infection. Abbreviations: NPEVs, non-polio enteroviruses; OPV, oral poliovirus vaccine.

Table 2.1. Summary of eligible trials.

Study ^a	Country	IG ^b	Vaccine	Doses	Interval	N ^c	Age	Vaccination history	Interference measure						OPV response ^d	
									DI	NP	VI	BA	EU	EE	SER	GMT Take
Benyesh-Melnick et al, 1959	Mexico	UM	mOPV	3 ^e	3w	81	0–12y	-	-	✓	-	-	-	-	✓	✓
Fang-Cho, 1960	China	UM	tOPV	1	-	610	<7y	-	-	✓	-	-	-	-	✓	-
Levine and Goldblum, 1960	Israel	H	tOPV	≤2	4m	~500	0–4m	-	-	✓	-	-	-	-	-	✓
Voroshilova et al, 1960	Russia	UM	tOPV	2	4–6w	140	2m–15y	-	-	✓	-	-	-	-	-	✓
Domok et al, 1961	Hungary	H	tOPV	2	6w	160	3m–15y	IPV in most	-	✓	-	-	-	-	-	✓
Ramos-Alvarez, 1961	Mexico	UM	mOPV	3 ^e	3w	181	<3y	-	-	✓	-	-	-	-	✓	✓
Dardanoni et al, 1962	Italy	H	mOPV	1	-	~55	3m–2y	some IPV	-	✓	-	-	-	-	✓	-
Ingram et al, 1962	USA	H	mOPV	1	-	25	10w–8m	some IPV	-	✓	-	-	-	-	✓	✓
Paul et al, 1962	Costa Rica	UM	tOPV	2	1m	48	5–22m	some IPV	-	✓	-	-	-	-	✓	✓
Urasawa, 1964b	Japan	H	tOPV	1	-	NA	NA	-	-	✓	-	-	-	-	✓	-
Spano and Dardanoni, 1965	Italy	H	mOPV	3 ^e	1m	229	3m–5y	-	-	✓	-	-	-	-	-	✓
JLPRC, 1966 ^f	Japan	H	mOPV	3 ^e	4w	~5000	0–60y	some IPV	-	✓	-	-	-	-	✓	✓
Nardi et al, 1966 ^g	Italy	H	tOPV	1	-	197	1–10y	3 mOPV ^e	-	✓	-	-	-	-	✓	✓
Ramos-Alvarez, 1966	Mexico	UM	mOPV	3 ^e	4w	NA	7m–2y	-	-	✓	-	-	-	-	✓	✓
Ramos-Alvarez, 1966	Mexico	UM	tOPV	1	-	NA	7m–2y	-	-	✓	-	-	-	-	✓	-
Sureau et al, 1966	Algeria	UM	tOPV	3	4w	100	3m–3y	-	-	✓	-	-	-	-	-	✓
John and Christopher, 1975	India	LM	tOPV	2	8w	191	3m–5y	-	-	✓	-	-	-	-	✓	✓
Mahmoud et al, 1976	Egypt	LM	NA	1	-	24	3m–2y	-	✓	-	-	✓	-	-	-	✓
Faden and Duffy, 1992	USA	H	tOPV	≤3	2–8m	68	6–10w	-	-	✓	✓	-	-	-	✓	✓
Kok et al, 1992	Kenya	L	tOPV	3	2m	100	2–3m	-	✓	✓	-	-	-	-	✓	✓
Cirne et al, 1995	Gambia	L	tOPV	4	NA	1087	<6w	-	✓	-	-	-	-	-	✓	-
Myaux et al, 1996	Bangladesh	L	tOPV	3	4w	391	6–16w	-	✓	-	✓	-	-	-	✓	✓
Posay et al, 1997	Brazil	UM	tOPV	4	4–6w	1395	0 (birth)	-	✓	-	-	-	-	-	✓	-
Maldonado et al, 1997	Mexico	UM	tOPV	2	8w	237	6w–6m	-	-	✓	✓	✓	-	-	✓	✓
Triki et al, 1997	Tunisia	UM	tOPV	3	4w	121	3m	-	-	✓	✓	✓	✓	-	✓	-

See following page for table legend.

Legend to Table 2.1. Abbreviations: BA, enteric bacteria; DI, diarrhoea; EE, environmental enteropathy; EU, eukaryotic parasites; GMT, geometric mean titre; H, high; IG, income group; IPV, inactivated poliovirus vaccine; JLP RC, Japan Live Poliovaccine Research Commission; L, low; LM, lower middle; m, months; mOPV, monovalent oral poliovirus vaccine; NA, not reported; NP, non-polio enteroviruses; OPV, oral poliovirus vaccine; SER, seroconversion; tOPV, trivalent oral poliovirus vaccine; UM, upper middle; VI, other enteric viruses (non-enteroviruses); w, weeks; y, years.

- ^a Where multiple reports describing the same trial fulfilled the eligibility criteria for inclusion in the review, the most comprehensive report is indicated here.
- ^b Income status designated by the World Bank in April 2013.
- ^c The total number of OPV recipients recruited in each trial is indicated; however, the influence of enteric infections was often examined in a subset of the total study population.
- ^d Outcomes are listed if they were reported within the eligible timeframe (i.e., within 8 weeks of vaccination for seroconversion and within 4 weeks for vaccine take) and compared according to the presence or absence of infection at the time of vaccine delivery; other OPV outcomes may have been reported in each study, but not with respect to enteric infection or within the eligible timeframe.
- ^e Three doses of mOPV were delivered in the order type 1, type 3, type 2.
- ^f The study reported on a collaborative trial of mOPV involving 27 laboratories across Japan, commencing in May 1961; data from preceding eligible reports, using the same vaccine formulation and also commencing in May 1961 (by Takatsu [1962], Urasawa [1964a], and Urasawa [1964b]) were assumed to be included in or to overlap with this later and more comprehensive report in order to avoid the risk of duplicating data from individual vaccinees. However, the study by Urasawa (1964b) also reported on a trial of tOPV, which was included in the analysis separately.
- ^g Data regarding vaccine take were obtained from the report by Monaci et al (1965), which included a subset of the children under 5 years of age reported on by Nardi et al (1966) and an additional 70 vaccinees between 6 and 10 years of age.

2.3.2. Concurrent non-polio enteroviruses

i. Serological response

Sixteen eligible studies reported serological response to OPV according to the presence or absence of concurrent NPEVs (Table 2.2). Across nine studies eligible for inclusion in the meta-analysis (all involving delivery of Sabin vaccine), the presence of NPEVs had a significant inhibitory effect on seroconversion rates for type 1 poliovirus (OR 0.44, 95% CI 0.23–0.84), but not types 2 or 3 (OR 0.53, 95% CI 0.19–1.46 and OR 0.56, 95% CI 0.27–1.12, respectively; Figure 2.2). Overall, the reduction in per-dose seroconversion among NPEV-

infected individuals approached significance (summary OR 0.47, 95% CI 0.20–1.04). There was no evidence of marked publication bias (Egger's test, P values >0.05 for each serotype; Figure 2.3). Serotype did not significantly influence effect size (LRT, $P = 0.680$). Significant heterogeneity in ORs across studies was observed for each serotype (χ^2 , P values 0.002, <0.001 , and 0.001 for type 1, 2, and 3 data, respectively) and for the overall OR (χ^2 , $P <0.001$).

When the meta-analysis was stratified according to OPV formulation (mOPV vs tOPV), NPEV-associated interference was significant for each serotype following the administration of mOPV (summary OR 0.17, 95% CI 0.07–0.42) but not tOPV (summary OR 0.92, 95% CI 0.45–1.86; Table 2.3). The impact of formulation on effect size was significant (LRT, $P = 0.008$). A separate subgroup analysis revealed that interference was generally greater among studies conducted in low- or middle-income than high-income countries (Table 2.3), although the impact of trial setting was not significant (LRT, $P = 0.181$). A sensitivity analysis including studies with a minimum of five individuals in the infected and control groups did not alter the outcomes of this meta-analysis (Table 2.3).

ii. Impact of concurrent non-polio enteroviruses across multiple doses of oral poliovirus vaccine

A limited number of trials examined the influence of NPEV infections at the time of one or more doses on seroconversion rates after multiple doses of tOPV. Maldonado et al (1997) reported a significant decrease in type 1 seroconversion rate after two doses of tOPV among infants in Mexico presenting with concurrent NPEVs (including any infection identified in stool samples 1 week before, at the time of, or 1 week after OPV delivery) compared with those free of NPEV infections. By contrast, Kok et al (1992) did not observe a significant effect of concurrent NPEVs on the antibody levels obtained after three doses of tOPV among children in Kenya, while John and Christopher (1975) did not observe significant inhibition of seroconversion among NPEV-infected children in India who were seronegative to one or more poliovirus serotypes upon receipt of a second dose of tOPV. Heterogeneity in the reporting of these trials precluded the synthesis of data across studies.

iii. Concurrent enteroviruses

Studies were not included in the primary analysis if the reporting of concurrent enterovirus infection did not distinguish poliovirus infections (wild-type or vaccine-derived) from NPEVs. This specification was included to avoid the risk of biasing the results in favour of an interference effect, given the inter-serotype interference that is known to arise between

the vaccine poliovirus strains (Patriarca et al, 1991). However, we performed a sensitivity analysis to incorporate studies in which the reporting of data did not enable poliovirus-infected individuals to be excluded.

Following the inclusion of six additional studies (Campillo Sainz et al, 1962; Paul et al, 1962; Poliomyelitis Commission of the Western Region Ministry of Health, Nigeria, 1966; Peradze et al, 1968; Domok et al, 1974; Domok and Balayan, 1976), significant inhibition of per-dose seroconversion was evident for type 1 poliovirus (OR 0.51, 95% CI 0.34–0.75) but not for types 2 or 3 (OR 0.67, 95% CI 0.33–1.36 and OR 0.58, 95% CI 0.33–1.02, respectively). Inhibition of per-dose seroconversion was significant overall (summary OR 0.55, 95% CI 0.33–0.89; Table 2.3). Heterogeneity was significant for each poliovirus serotype (*P* values 0.012, <0.001, and 0.001 for type 1, 2, and 3 data, respectively) and for the overall OR (*P* <0.001). During meta-regression, effect size did not differ significantly according to poliovirus serotype (LRT, *P* = 0.434), trial setting (LRT, *P* = 0.303), or – in contrast to the primary analysis – formulation (mono-, bi-, or tri-valent OPV; LRT, *P* = 0.248). Inclusion of one extra study (Paul et al, 1962) in the analysis of vaccine take did not markedly change the results (Table 2.3). Since the primary focus of this review was to assess the impact of non-polio enteric infections on OPV response, poliovirus-infected individuals were still excluded from this sensitivity analysis where possible. The proportion of enteroviruses accounted for by polioviruses, where reported, varied from less than 10% (Poliomyelitis Commission of the Western Region Ministry of Health, Nigeria, 1966) to more than 50% (Campillo Sainz et al, 1962).

Table 2.2. Summary of studies reporting the influence of enteric viruses on oral poliovirus vaccine response.

Study	Virus detection			Measurement of take		Measurement of seroconversion				Take		Seroconversion									
	Timing, pre	Sample ^a	Cell type	SM	Other ^b	Sample	Timing, post	Min. dilution	Threshold ^c	Timing, post	Type	Strain	Dose (log10 TCID ₅₀)	IR	IT	UR	UT	IR	IT	UR	UT
Benyesh-Melnick et al, 1959 Fang-Cho, 1960	0d	swab	MK	NA	NA	swab	7,14,21d	N 1:10	NA	3w	NA	Sabin	NA	13	47	18	28	13	47	18	28
	0d	stool	NA	NA	NA	-	-	N 1:4	4-fold rise	1m	1	Sabin	5-5.5	-	-	-	-	35	60	58	69
											2	Sabin	5-5.5	-	-	-	-	47	54	56	63
Levine and Goldblum, 1960 ^d											3	Sabin	5-5.5	-	-	-	-	30	58	54	66
	0d	swab	MK	NA	NA	swab	7,14,21, 28d	-	-	-	1	NA	5.7-6.2	9	14	52	65	-	-	-	-
											2	NA	4.2-5.7	0	14	0	65	-	-	-	-
Voroshilova et al, 1960 ^e											3	NA	5.7-6.2	9	14	55	65	-	-	-	-
	0d	stool	MK, HeLa, KB, Detroit-6	yes	yes	stool	2x per w	-	-	-	1	Sabin	5	15	58	36	82	-	-	-	-
											2	Sabin	5	22	58	42	82	-	-	-	-
Domok et al, 1961 ^f											3	Sabin	5	15	58	24	82	-	-	-	-
	1-5d (3x)	stool	MK	yes	NA	stool	3,5,12, 15,22, 24d	-	-	-	1	Sabin	5	10	19	15	33	-	-	-	-
											2	Sabin	5	16	19	21	33	-	-	-	-
Ramos-Alvarez, 1961											3	Sabin	5	16	19	18	33	-	-	-	-
	0d	swab	HK	NA	NA	swab	NA	N NA	NA	NA	1	Sabin	5.6	2	10	13	18	4	10	16	18
											2	Sabin	5.7	3	10	8	10	5	10	9	10
Dardanoni et al, 1962											3	Sabin	5.9	0	11	8	19	1	11	10	19
	0d	swab	MK, HeLa	NA	yes	-	-	N 1:2 or 1:4	<2-≥2 or <4-≥4	1,2,4,6, 8.5w	1	Sabin	6-6.9	-	-	-	-	13	22	10	13
	0-7d (2x)	stool	MK	NA	NA	stool	2x per w for 6-8w	MI 1:4	4-fold rise	2-4w	1	Sabin	6	9	10	14	14	-	-	-	-
Paul et al, 1962 ^h											1	Led	5-5.5	6	20	17	28	5	20	12	28
	0-7d (2x)	swab	MK, HEp-2	NA	NA	swab	2x per w for ≥4w	N 1:4	<4-≥4	1m	2	Led	5-5.5	3	14	2	21	3	14	2	21
											3	Led	5-5.5	7	9	22	25	6	9	21	25

Table 2.2. Summary of studies reporting the influence of enteric viruses on oral poliovirus vaccine response (cont.).

Study	Virus detection				Measurement of take		Measurement of seroconversion				Dose (log10 TCID ₅₀)		Take				Seroconversion					
	Timing, pre				Sample	Timing, post OPV	Min.		Threshold ^c	Timing, post OPV			Type	Strain	IR	IT	UR	UT	IR	IT	UR	UT
	Sample ^a	OPV	Cell type	SM			Other ^b	Test			dilution	rise										
Urasawa, 1964 ^b	stool	0d	HeLa	NA	NA	-	-	N	1:4	4-fold	1m	1	Sabin	4.8–5.7	-	-	-	-	13	20	39	60
Spano and Dardanoni, 1965	stool/ swab	0d	MK, HeLa	NA	yes	stool/ swab	15d	-	-	-	-	2	Sabin	4.5–5.8	-	-	-	-	16	21	46	67
												3	Sabin	5.3–6.3	-	-	-	-	15	20	47	70
												1	NA	NA	3	14	3	19	-	-	-	-
JLPRC, 1966 ^j	stool	0,+7d	MK	NA	NA	stool	2,4w	N	1:4	<4–≥4	4,8w	1	Sabin	5.5	6	25	196	430	10	14	193	211
												2	Sabin	5.5	9	56	99	321	34	50	130	149
												3	Sabin	5.5	7	21	129	354	25	34	151	180
Nardi et al, 1966 ^k	stool	1–2d	MK, HA	NA	NA	stool	4,7,14, 21,28d	N	1:4	<4–≥4 or 4-fold rise	45–60d	1	Sabin	5.5	2	14	1	26	28	48	27	54
												2	Sabin	5.5	7	14	8	26	29	36	34	53
												3	Sabin	5.5	7	14	8	26	27	49	28	64
Ramos-Alvarez, 1966, mOPV ^l	swab	0d	HK	NA	NA	NA	NA	N	undiluted	negative to positive in undiluted serum	4–5w	1	Sabin	5.5	29	64	31	39	38	64	37	39
												2	Sabin	5.5	23	44	37	43	26	44	41	43
												3	Sabin	5.5	26	62	25	29	31	62	25	29
Ramos-Alvarez, 1966, tOPV ^l	swab	0d	HK	NA	NA	-	-	N	undiluted	negative to positive in undiluted serum	7–8w	1	Sabin	5.7	-	-	-	-	31	45	57	69
												2	Sabin	5–5.3	-	-	-	-	36	52	62	64
												3	Sabin	5.4	-	-	-	-	20	40	43	87
Sureau et al, 1966	stool	2d	KB	yes	NA	stool	8d	-	-	-	-	1	NA	NA	4	19	5	18	-	-	-	-
												2	NA	NA	3	19	5	18	-	-	-	-
												3	NA	NA	2	19	5	18	-	-	-	-

Table 2.2. Summary of studies reporting the influence of enteric viruses on oral poliovirus vaccine response (cont.).

Study	Virus detection				Measurement of take			Measurement of seroconversion				Take	Seroconversion								
	Sample ^a stool/ swab	Timing, pre OPV NA (weekly)	Cell type MK, HEp-2	SM yes	Other ^b NA	Sample stool/ swab	Timing, post OPV 1,2w	Min. Test dilution MN 1:10	Threshold ^c <10-≥10	Timing, post OPV 8w	Type		Strain	Dose (log10 TCID ₅₀)	IR	IT	UR	UT			
John and Christopher, 1975 ^m	stool/ swab	NA (weekly)	MK, HEp-2	yes	NA	stool/ swab	-	MN 1:10	<10-≥10	8w	1	Sabin 6	6	-	-	-	10	40	4	23	
											2	Sabin 5	5	-	-	-	-	31	44	10	26
											3	Sabin 5.5	5.5	-	-	-	-	13	50	7	26
Faden and Duffy, 1992	stool	0d	MK, HEp-2	no	yes	-	-	MN 1:10	>10	1m	1	Sabin 5.5-6.4	5.5-6.4	-	-	-	-	-	-	-	
											2	Sabin 4.5-5.5	4.5-5.5	-	-	-	-	-	-	-	-
											3	Sabin 5.2-6.2	5.2-6.2	-	-	-	-	-	-	-	-
Kok et al, 1992	stool	0d	NA	NA	NA	-	-	MN 1:8	≥8 after 3 doses	2m	1	Sabin 6	6	-	-	-	-	-	-	-	
											2	Sabin 5	5	-	-	-	-	-	-	-	-
											3	Sabin 5.5	5.5	-	-	-	-	-	-	-	-
Maldonado et al, 1997	stool	7,0,+7d	PMK, BGMK, RD, MRC-5	NA	yes	-	-	MN 1:8	<8-≥8 or 4-fold increase over mAb	8w	1	Sabin 6	6	-	-	-	-	-	-	-	
											2	Sabin 5	5	-	-	-	-	-	-	-	-
											3	Sabin 5.6	5.6	-	-	-	-	-	-	-	-
Triki et al, 1997	stool	0d	HEp-2C, RD	NA	yes	-	-	MN 1:4	<8-≥8 or 4-fold rise	1m	1	Sabin 6	6	-	-	-	-	-	-	-	
											2	Sabin 5	5	-	-	-	-	-	-	-	-
											3	Sabin 5.8	5.8	-	-	-	-	-	-	-	-

See following page for table legend.

Legend to Table 2.2. Abbreviations: 2x, two samples; 3x, three samples; d, days; IR, infected responders; IT, infected total; JLPRC, Japan Live Poliovaccine Research Commission; Led, Lederle; m, months; mAb, maternal antibody; MI, metabolic inhibition assay; MN, microneutralisation assay; mOPV, monovalent oral poliovirus vaccine; N, neutralisation assay; NA, not reported; OPV, oral poliovirus vaccine; SM, suckling mice; TCID₅₀, median tissue culture infective doses; tOPV, trivalent oral poliovirus vaccine; UR, uninfected responders; UT, uninfected total; w, weeks.

- ^a Studies reporting the use of rectal swabs are indicated as 'swab'. All other faecal sampling methods are listed as 'stool'.
- ^b Other tests included assays for haemagglutination/haemagglutination-inhibition, infection of suckling and adult cotton rats, ELISA, RNA–RNA hybridisation, and electron microscopy.
- ^c Antibody titres are expressed as the reciprocal of the minimum dilution producing virus neutralisation.
- ^d Dose-specific take data from this study were not included in the meta-analysis as the age range of NPEV-infected individuals (1–4 months) did not match that of uninfected individuals (3–4 months); the younger age of infected individuals may therefore be responsible for the differences observed between these groups.
- ^e Additional details were obtained from a report by Voroshilova et al (1960).
- ^f The total number of uninfected individuals was not reported directly by the authors; however, 33 children under 4 years of age (the age range in which the influence of NPEVs on OPV take was examined) lacked any virus during the pre-vaccination survey. This was therefore used as the total number of uninfected controls during the meta-analysis.
- ^g Any poliovirus excretion within the 4-week period after vaccine delivery (rather than the full study period of 10 weeks) was considered as indicative of vaccine take.
- ^h This study was not included in the primary analysis as eight individuals excreting type 3 poliovirus at the time of vaccination were not excluded from the data.
- ⁱ Methods were assumed to follow those described in a prior publication by the same author (Urasawa, 1964a).
- ^j The study reported outcomes relative to the presence or absence of NPEVs for vaccine take at 2 and 4 weeks after OPV delivery, and seroconversion 4 and 8 weeks after vaccination; in both cases, the 4-week data were available for more individuals, and were therefore used in the meta-analysis. The reporting of concurrent NPEVs in this study spanned the period immediately before and after vaccination.
- ^k Data regarding vaccine take were obtained from a report by Monaci et al (1965).
- ^l Additional details were obtained from a report by Sabin (1980).
- ^m Additional details were obtained from reports by John and Jayabal (1972) and John (1975). The presented data were summed across ditypic and tritypic seronegative individuals; data for monotypic seronegative individuals were not reported according to poliovirus serotype.

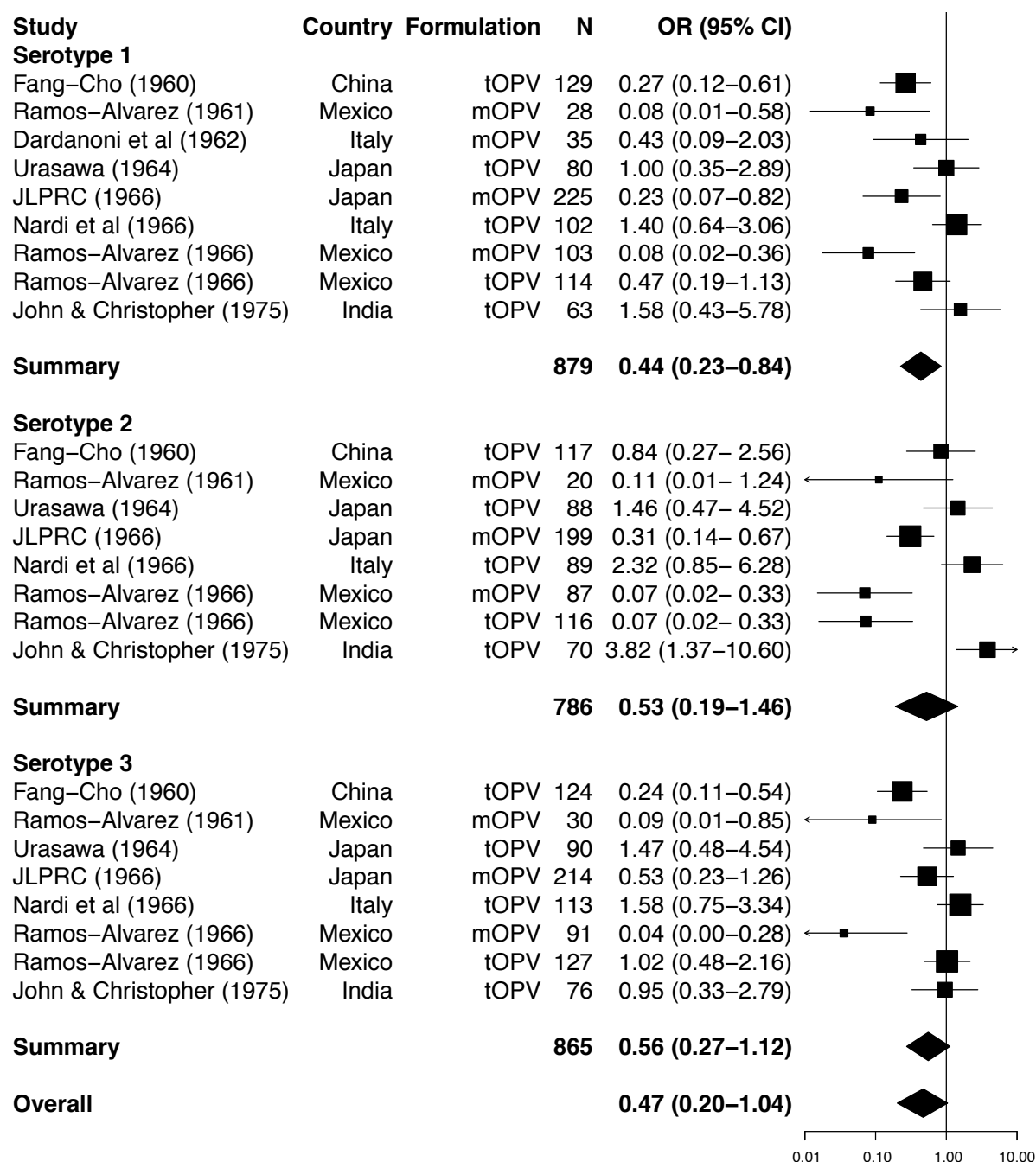


Figure 2.2. Forest plot of the impact of concurrent non-polio enterovirus infections on the odds of seroconversion per dose of oral poliovirus vaccine. ORs and 95% CIs, calculated using random-effects models, are presented for each serotype by boxes and black lines, with box area proportional to study weight. Summary ORs are indicated by diamonds, the width of which represents the 95% CI. Abbreviations: CI, confidence interval; JLPRC, Japan Live Poliovaccine Research Commission; mOPV, monovalent oral poliovirus vaccine; OR, odds ratio; tOPV, trivalent oral poliovirus vaccine.

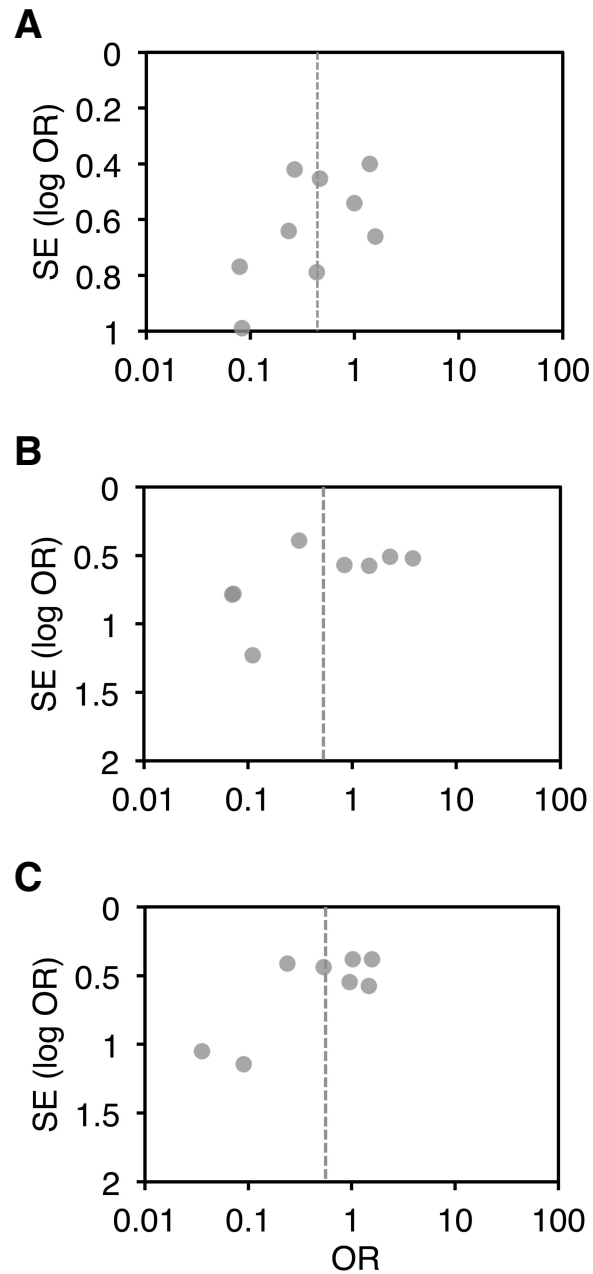


Figure 2.3. Funnel plots. Data are presented for (A) serotype 1, (B) serotype 2, and (C) serotype 3, and correspond to the forest plot depicted in Figure 2.2. Abbreviations: OR, odds ratio; SE (log OR), standard error of log odds ratio.

Table 2.3. Subgroup and sensitivity analyses.

		Seroconversion, OR (95% CI)					Vaccine take, OR (95% CI)				
		No. studies	Serotype 1	Serotype 2	Serotype 3	Overall	No. studies	Serotype 1	Serotype 2	Serotype 3	Overall
baseline		9	0.44 (0.23–0.84)	0.53 (0.19–1.46)	0.56 (0.27–1.12)	0.47 (0.20–1.04)	9	0.50 (0.28–0.89)	0.59 (0.30–1.13)	0.67 (0.26–1.71)	0.58 (0.26–1.24)
Stratification by vaccine formulation											
monovalent OPV		4	0.17 (0.07–0.44)	0.15 (0.03–0.66)	0.18 (0.05–0.66)	0.17 (0.07–0.42)	5	0.31 (0.15–0.64)	0.31 (0.13–0.70)	0.25 (0.06–1.04)	0.30 (0.13–0.69)
trivalent OPV		5	0.73 (0.38–1.41)	1.06 (0.37–3.03)	0.87 (0.39–1.94)	0.92 (0.45–1.86)	4	0.79 (0.37–1.68)	1.07 (0.48–2.38)	1.31 (0.41–4.15)	1.11 (0.48–2.63)
Stratification by income group											
low- or middle-income		5	0.31 (0.13–0.72)	0.33 (0.08–1.39)	0.34 (0.14–0.86)	0.31 (0.11–0.82)	4	0.32 (0.16–0.64)	0.31 (0.13–0.78)	0.28 (0.09–0.86)	0.29 (0.12–0.63)
high-income		4	0.68 (0.27–1.70)	0.99 (0.17–5.68)	1.07 (0.37–3.07)	0.80 (0.26–2.42)	5	0.83 (0.38–1.84)	1.09 (0.44–2.71)	1.91 (0.57–6.39)	1.17 (0.53–2.56)
Sensitivity analysis											
enterovirus co-infection (including some poliovirus)		15	0.51 (0.34–0.75)	0.67 (0.33–1.36)	0.58 (0.33–1.02)	0.55 (0.33–0.89)	10	0.44 (0.25–0.77)	0.65 (0.34–1.25)	0.67 (0.26–1.71)	0.55 (0.27–1.10)
n ≥5 in infected/ control group		9	0.44 (0.23–0.84)	0.53 (0.19–1.46)	0.56 (0.27–1.12)	0.47 (0.20–1.04)	10	0.48 (0.28–0.81)	0.59 (0.30–1.13)	0.63 (0.26–1.50)	0.54 (0.26–1.08)

Baseline data correspond with those presented in Figures 2.2 and 2.4. Abbreviations: CI, confidence interval; OPV, oral poliovirus vaccine; OR, odds ratio.

iv. Vaccine take

Nine studies reporting vaccine take according to the presence or absence of NPEVs were eligible for inclusion in the meta-analysis (Figure 2.4). Sabin vaccine was used in each study (where specified). As with serological response, a significant decrease in the odds of shedding in NPEV-infected individuals was observed for serotype 1 (OR 0.50, 95% CI 0.28–0.89) but not types 2 or 3 (OR 0.59, 95% CI 0.30–1.13 and OR 0.67, 95% CI 0.26–1.71, respectively). There was no evidence of marked publication bias (Egger's test, P values >0.05 for each serotype). Overall, the reduction in shedding among NPEV-infected individuals was not significant (summary OR 0.58, 95% CI 0.26–1.24). The impact of serotype was not significant following meta-regression (LRT, $P = 0.489$). Heterogeneity among studies was significant for serotypes 2 and 3 (χ^2 , P values 0.081, 0.015, and 0.001 for type 1, 2, and 3 data, respectively), and for the overall OR (χ^2 , $P < 0.001$). Again, stratification of the meta-analysis according to vaccine formulation revealed that NPEV-associated interference was greater for mOPV than tOPV (LRT, $P = 0.032$), and in low- or middle-income than high-income countries (LRT, $P = 0.019$; Table 2.3).

v. Impact of specific non-polio enteroviruses

Seven studies reported the influence of NPEVs on OPV take and/or seroconversion according to the presence of specific pathogens (Levine and Goldblum, 1960; Dardanoni et al, 1962; Ingram et al, 1962; Urasawa, 1964b; Spano and Dardanoni, 1965) or pathogen groups (Fang-Cho, 1960; John and Christopher, 1975). Although the numbers of individuals affected by particular NPEVs were generally small, the findings of four studies merit particular mention. Ingram et al (1962) documented vaccine response in a US nursery following the delivery of type 1 mOPV during an epidemic of echovirus 14 (a species B enterovirus). The virus did not inhibit seroconversion or vaccine take, although the study included just 25 vaccinees. Urasawa (1964b) reported an inhibitory effect of Coxsackie B5 virus (also a species B enterovirus) on both take and serological response following the delivery of mOPV to individuals in Japan; however, Coxsackie B5 virus did not inhibit serological response to tOPV (Figure 2.2). During a study in China involving one dose of tOPV, interference with seroconversion was similar for different subgroups of NPEVs (Coxsackie A, Coxsackie B, or echovirus/other viruses) (Fang-Cho, 1960). On the other hand, John and Christopher (1975) observed no interference with seroconversion among children in India irrespective of the subgroup of NPEV present at the time of tOPV administration. Overall, we observed no clear evidence to support the particular inhibitory effect of any specific NPEV or NPEV group.

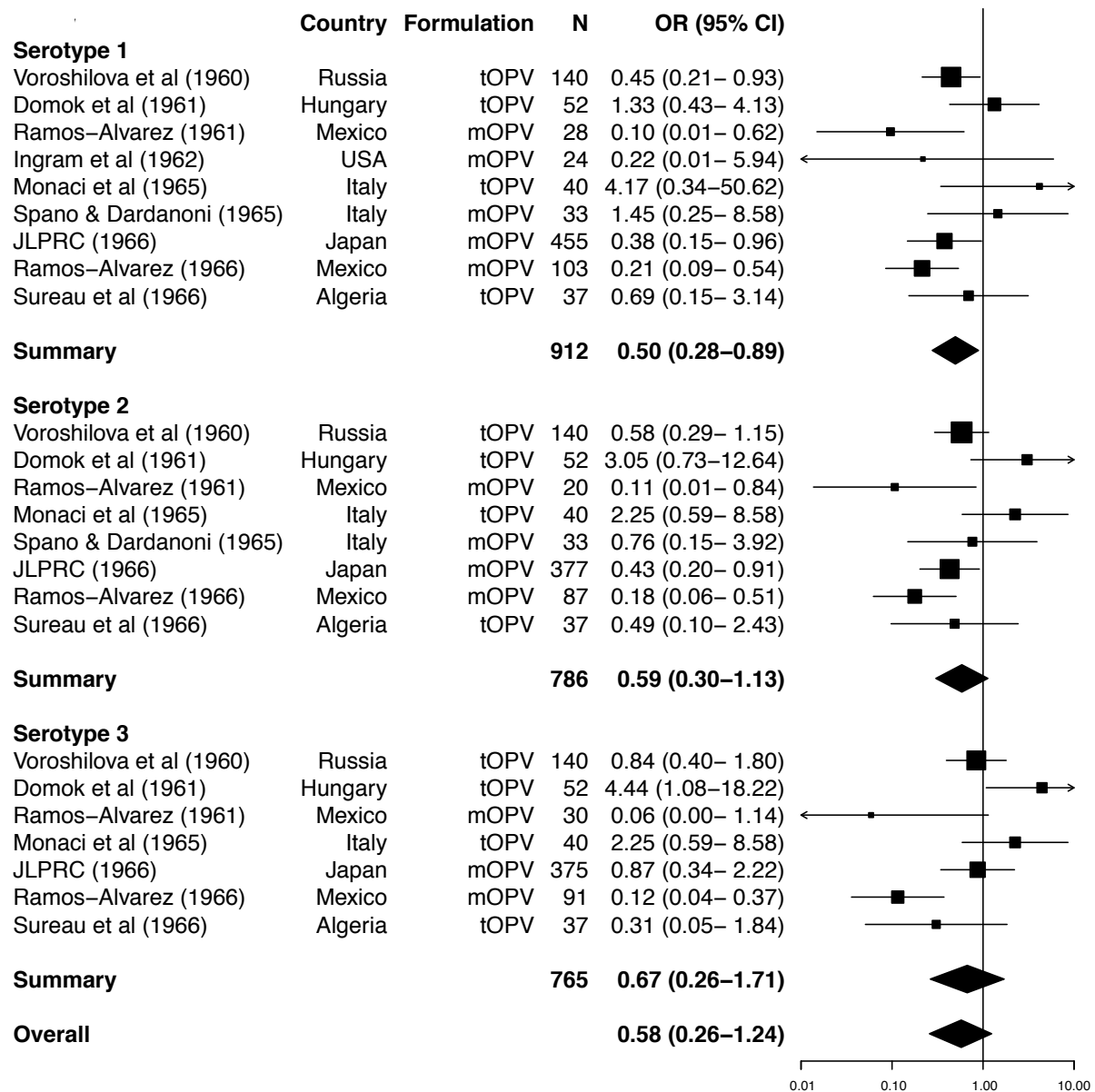


Figure 2.4. Forest plot of the impact of concurrent non-polio enterovirus infections on the odds of vaccine take per dose of oral poliovirus vaccine. See Figure 2.2 for additional details. Abbreviations: CI, confidence interval; JLPRC, Japan Live Poliovaccine Research Commission; mOPV, monovalent oral poliovirus vaccine; OR, odds ratio; tOPV, trivalent oral poliovirus vaccine.

2.3.3. Concurrent diarrhoea

i. Serological response

Four of the included trials considered the influence of concurrent diarrhoea on serological response to tOPV (Table 2.4), of which two were eligible for inclusion in the meta-analysis (Figure 2.5). Both studies involved the delivery of Sabin strains. Concurrent diarrhoea was associated with a significant decrease in per-dose seroconversion rates for serotypes 2 and 3 (OR 0.52, 95% CI 0.34–0.80 and OR 0.45, 95% CI 0.26–0.78, respectively), but not type 1 (OR 0.92, 95% CI 0.60–1.40). Overall, the impact of diarrhoea on dose-specific seroconversion was significant (summary OR 0.61, 95% CI 0.38–0.87). The difference in effect size among serotypes approached significance (LRT, $P = 0.067$). Heterogeneity among studies was not significant for any serotype (χ^2 , P values >0.05) or overall (χ^2 , $P = 0.207$).

We carried out a separate analysis to assess the impact of experiencing diarrhoea at the time of one or more doses on overall seroconversion after multiple tOPV doses (Figure 2.6). Overall, a significant decline in the odds of seroconversion was observed in children who experienced at least one concurrent diarrhoeal episode (summary OR 0.68, 95% CI 0.48–0.93). As with the dose-specific seroconversion data, this interference effect was significant for serotypes 2 and 3 (OR 0.47, 95% CI 0.28–0.79 and OR 0.59, 95% CI 0.44–0.79, respectively), but not type 1 (OR 1.05, 95% CI 0.71–1.55). Heterogeneity among studies was not significant for any serotype (χ^2 , P values >0.05) or overall (χ^2 , $P = 0.154$). Effect size differed significantly according to serotype (LRT, $P = 0.024$). It should be noted that two other studies did not observe a significant impact of concurrent diarrhoea on serological response after multiple doses of tOPV, but were not suitable for inclusion in this analysis (Kok et al, 1992; Myaux et al, 1996). There were insufficient studies to formally assess publication bias regarding diarrhoea-associated interference.

ii. Vaccine take

One trial, conducted in Egypt by Mahmoud et al (1976), examined the influence of concurrent diarrhoea on OPV take. Over a 3-week period after OPV delivery, vaccine shedding was observed in 1/10 (10%) children with diarrhoea at the time of immunisation and 9/14 (64%) of those without diarrhoea.

Table 2.4. Summary of studies reporting the influence of diarrhoea on response to oral poliovirus vaccine.

Study	Definition of diarrhoea	Measurement of take		Measurement of seroconversion				Dose (log ₁₀ TCID ₅₀)	Take				Per-dose seroconversion				Overall seroconversion			
		Sample	Timing	Test	Min. dilution	Threshold	Timing		Type	Strain	NA	DR	DT	NR	NT	DR	DT	NR	NT	
Mahmoud et al, 1976	NA	stool	7,14,21d	-	-	-	-	NA	NA	1	10	9	14	-	-	-	-	-	-	
Cirne et al, 1995 ^a	≥4 watery stools in the 24h preceding OPV delivery	-	-	MN	1:8	<8-≥8 or 4-fold rise over expected residual maternal antibody	>4w	1	Sabin	6-6.3	-	-	-	-	-	-	151	177	420	
								2	Sabin	4.7-5	-	-	-	-	-	-	164	177	484	
								3	Sabin	5.5-5.8	-	-	-	-	-	-	113	177	377	
Myaux et al, 1996	≥3 watery stools per d for <7d, and the absence of other complications	-	-	MN	1:16	<16-≥16 or 4-fold rise over expected residual maternal antibody	4w	1	Sabin	6	-	-	-	33	99	71	211	-	-	
								2	Sabin	5	-	-	-	40	98	116	209	-	-	
								3	Sabin	5.5	-	-	-	27	99	88	212	-	-	
Posey et al, 1997 ^a	≥3 watery stools in a 24h period in the 2w preceding OPV delivery ^b	-	-	MN	1:8	<8-≥8 or 4-fold rise over expected residual maternal antibody	4-6w	1	Sabin	6-6.3	-	-	-	11	32	196	490	72	84	
								2	Sabin	4.7-5	-	-	-	7	22	162	305	73	84	
								3	Sabin	5.5-5.8	-	-	-	4	36	186	598	45	84	

See Table 2.2 for additional details regarding column headings. Abbreviations: d, days; DR, diarrhoeal responders; DT, diarrhoeal total; h, hours; MN, microneutralisation assay; NA, not reported; NR, non-diarrhoeal responders; NT, non-diarrhoeal total; OPV, oral poliovirus vaccine; TCID₅₀, median tissue culture infective doses; w, weeks.

^a The reports by Cirne et al (1995) and Posey et al (1997) describe trials conducted in Brazil and the Gambia in which four different formulations of tOPV (with varying potencies) were randomly assigned to vaccinees. The effect of diarrhoea was comparable among the formulations, and data were therefore pooled across vaccine groups. Both trials are described in the report by Cirne et al (1995), with additional dose-specific data documented by Posey et al (1997) for the trial conducted in Brazil. For the study by Posey et al (1997), seroconversion rates for the OPV dose delivered at 6 weeks of age were used in this analysis; these data were available for the greatest number of infants. Diarrhoea-associated interference was also apparent for doses delivered at 10 and 14 weeks in this study, although the effect size was smaller.

^b In the report on this trial by Cirne et al (1995), which was used during the analysis of diarrhoea-associated interference over multiple doses of tOPV (Figure 2.6), diarrhoea was defined as ≥4 watery stools per day for at least 3 consecutive days in the 2 weeks preceding OPV delivery.

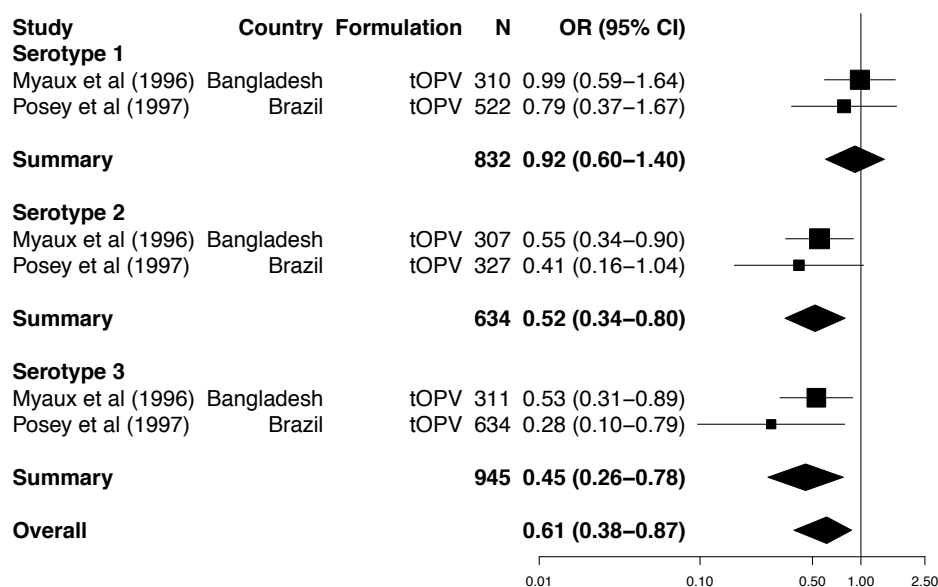


Figure 2.5. Forest plot of the impact of concurrent diarrhoea on the odds of seroconversion per dose of oral poliovirus vaccine. See Figure 2.2 for additional details. Abbreviations: CI, confidence interval; OR, odds ratio; tOPV, trivalent oral poliovirus vaccine.

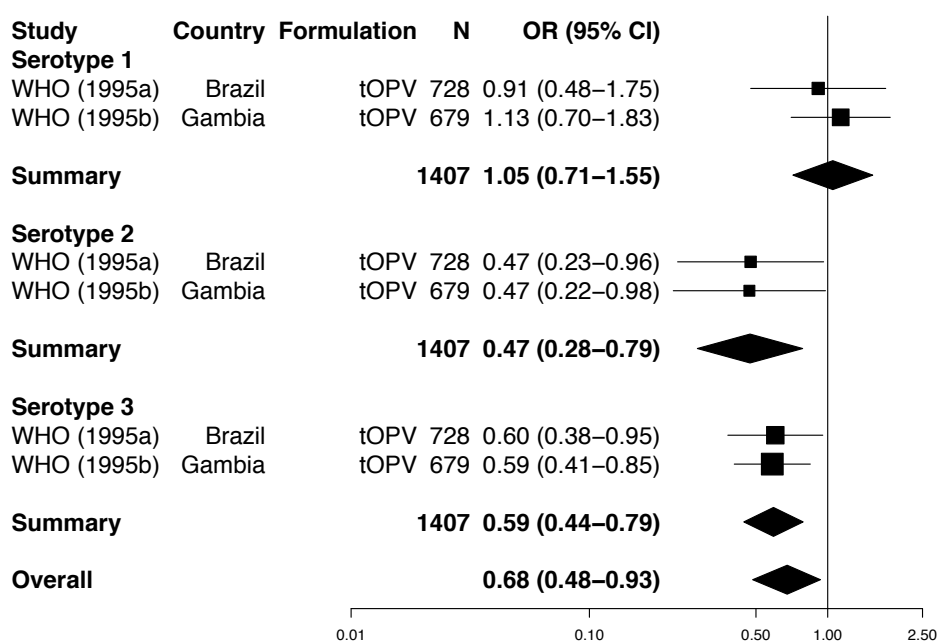


Figure 2.6. Forest plot of the impact of concurrent diarrhoea on the odds of seroconversion following multiple doses of oral poliovirus vaccine. See Figure 2.2 for additional details. Data were obtained from one article (Cirne et al, 1995), reporting on separate trials of trivalent OPV in Brazil (WHO 1995a) and the Gambia (WHO 1995b). The reporting of concurrent diarrhoea spanned the 2-week period preceding vaccination. Abbreviations: CI, confidence interval; OR, odds ratio; tOPV, trivalent oral poliovirus vaccine.

2.3.4. Other enteropathogens

We identified only five studies that reported the effect of enteric infections other than NPEVs on response to OPV (Myaux et al, 1996; Maldonado et al, 1997; Triki et al, 1997; Faden and Duffy, 1992; Mahmoud et al, 1976). Although several other studies used isolation techniques permissive to the detection of adenoviruses or reoviruses (e.g., John and Christopher, 1975; Spano and Dardanoni, 1965), either the infections were not observed in pre-vaccination samples or their impact on OPV response was not reported.

During a study in Bangladesh, Myaux et al (1996) examined rectal swabs for rotavirus antigen before delivery of the first of three tOPV doses. Among 11 individuals positive for rotavirus with complete follow-up data, lower seroconversion rates were observed to all three serotypes following one dose of OPV compared with uninfected individuals, although differences were not significant.

Maldonado et al (1997) examined the combined influence of concurrent viral pathogens (including NPEV, adenovirus 40/41, astrovirus, and rotavirus infections) and enteric bacteria on seroconversion rates across two doses of tOPV among infants in Mexico. Concurrent infections were generally more frequent among non-responders compared with responders at each dose, and were linked with a diminished response to serotypes 1 and 2 at the second dose. Antibody titres for serotypes 1 and 2 were significantly lower among infants with concurrent viral or bacterial infections compared with those lacking infections after the first dose of OPV, and for type 2 poliovirus after the second dose. Notably, in spite of the significant interference effects for type 1 and 2 poliovirus observed in this study, overall seroconversion rates for these serotypes after two doses of tOPV were 86% and 97%, respectively. Corresponding seroconversion rates for type 3 poliovirus were only 61% after two doses, though no significant interference effect for concurrent enteropathogens was observed for this serotype.

Triki et al (1997) measured the influence of enteric bacteria and eukaryotic parasites on serological response to tOPV among infants in Tunisia. Overall seroconversion rates were high in this study, with only 16 out of 114 individuals failing to seroconvert to all three serotypes after three doses. Among individuals with incomplete seroconversion, a significant increase in the frequency of eukaryotic parasites – including *Candida albicans*, *Candida tropicalis*, and *Giardia intestinalis* – was observed (5/16 [31%], compared with 12/98 [12%] in those with complete seroconversion). The prevalence of enteric bacteria, rotaviruses, and adenoviruses did not differ significantly between these groups. It should be noted, however, that stool samples were obtained both at the time of vaccination and during intervening

episodes of diarrhoea in this study; the reporting of enteropathogen prevalence therefore included, but was not limited to, infections coinciding with OPV delivery.

In a US study examining the influence of concurrent enteric and nasopharyngeal viral pathogens (including NPEV, adenovirus, and rotavirus infections) on response to OPV and/or enhanced-potency IPV, Faden and Duffy (1992) reported detectable antibodies to each poliovirus serotype in all participants after three vaccine doses, irrespective of immunisation schedule (OPV alone, IPV alone, or combined). Compared with uninfected controls, individuals with viral infections had similar serum neutralising antibody, nasopharyngeal neutralising antibody, and nasopharyngeal IgA titres following the delivery of tOPV doses at either 4 or 12 months of age, though a modest decrease in serum antibody titres was evident for the latter dose among infected individuals.

Finally, in the trial by Mahmoud et al (1976) described above, in which concurrent diarrhoea was observed to reduce OPV take among children in Egypt, each case of diarrhoea was attributed to *Shigella* infection.

Overall, while these findings provide some indication of a potential inhibitory effect of enteric bacteria or eukaryotic parasites on serological response to OPV, the extent of this interference remains uncertain owing to the small number and heterogeneous nature of the available studies.

2.3.5. Environmental enteropathy

No studies encountered in this review examined the influence of EE on OPV immunogenicity or take.

2.4. Discussion

The potential link between enteropathogen exposure and the impaired immunogenicity of OPV in low-income countries has been the subject of a varied and conflicted literature that dates back to the earliest days of the vaccine's implementation. This systematic review and meta-analysis was carried out with the aim of integrating the findings of this extensive body of work, and thereby determine which direction the balance of evidence leans when it comes to the impact of enteropathogens on OPV. Overall, the review supports the role of enteropathogens as a risk factor for impaired OPV response – for type 1 poliovirus, a significant reduction in the odds of both seroconversion and vaccine poliovirus replication

for a given dose of OPV was observed in NPEV-infected compared with -uninfected individuals, while concurrent diarrhoea was associated with a decrease in seroconversion for serotypes 2 and 3. Enteropathogens other than NPEVs may also interfere with the immune response to OPV, but have been considered by only a small number of studies to date.

Significant heterogeneity in the extent of NPEV-associated interference was observed among studies included in this review. Factors that may contribute to this include vaccine potency, age at vaccination, collection method for faecal samples, and viral isolation methods, which varied widely among studies. Of particular note is the starting dilution adopted during neutralisation assays, which has previously been shown to influence the seroconversion rates observed for tOPV (Patriarca et al, 1991) – this varied from the use of undiluted sera to 1 in 10 dilutions among the included studies.

Stratification of the analysis revealed that interference with both take and seroconversion was generally greater following the delivery of mOPV than tOPV, and in trials conducted in low- or middle-income as opposed to high-income countries. Trends were consistent across serotypes. These stratified findings should be interpreted with caution given the small number of studies in each subgroup, and the potential for individual studies with marked interference effects (e.g., Ramos-Alvarez, 1961) to exert a strong influence on a particular subgroup. Nonetheless, they raise the possibility that mOPV may be more susceptible than tOPV to the influence of enteric infections. This observation might reflect the dynamics of competition between enteroviruses coinciding in the gut – in particular, while an existing NPEV infection may have a marked inhibitory effect on a single attenuated vaccine strain in mOPV, the additive effect on top of the inter-serotype interference arising between poliovirus strains in tOPV may be smaller.

The disparity observed according to trial setting suggests that infants in low- or middle-income countries may be more susceptible to the inhibitory effect of concurrent NPEVs on OPV – potentially indicative of differences in the strains of circulating enteroviruses or the heightened prevalence of comorbidities such as acute malnutrition, which might influence the outcomes of NPEV-associated interference. One must also consider the possibility that infants in low-income countries with other risk factors for OPV non-response (including malnutrition and EE, among others) may simply be more susceptible to NPEV infections, and that the apparent association between NPEVs and impaired OPV response is not causal.

At present, we can only speculate as to the potential mechanisms that may account for the observed interference effects. Although NPEVs do not use the poliovirus receptor to access cells, following entry these viruses might compete for cellular factors required for

replication (i.e., direct interference at a cellular level). The potential for species C enteroviruses to infect the same cells as polioviruses is supported by the observation of recombinant strains in cVDPVs (Jegouic et al, 2009; Combelas et al, 2011). *In vitro* studies of viral co-infection have demonstrated a decline in poliovirus replication in cells infected with NPEVs (Hsiung, 1961), although this effect is absent for certain NPEV serotypes (Crowell, 1963). Interference by NPEVs or other enteric pathogens might also arise indirectly via the induction of non-specific innate antiviral immunity. Type I and III interferons have been shown to play an important role in the innate immune response to enterovirus infections (Deonarain et al, 2004; Lind et al, 2014; Reid and Charleston, 2014). As such, the induction of these innate immune mediators by an existing viral infection might inhibit the replication of an attenuated vaccine poliovirus. In the case of diarrhoea, interference may also involve a reduction in the gut's mucosal surface area (and hence access to poliovirus receptors) and an increase in the rate of gastrointestinal transit following OPV administration.

The inhibitory effect of NPEVs was more pronounced for type 1 poliovirus than types 2 or 3, while the opposite was true of diarrhoea-associated interference. Although the differences were generally not significant following meta-regression, these findings suggest that the extent of interference may differ among poliovirus serotypes, and that this specificity may vary according to the nature of the concurrent infection. The notion that Sabin polioviruses of serotype 1 may be more susceptible to interference by concurrent enteroviruses is also consistent with the need for an elevated potency of this serotype within the tOPV formulations currently in use.

The relative influence of individual NPEVs or NPEV groups on OPV response remains unclear based on the available evidence. While Urasawa (1964b) highlighted the potential inhibition of mOPV response by Coxsackie B5 virus, this pathogen did not interfere with seroconversion following tOPV delivery. Moreover, studies carried out in India (John and Christopher, 1975) and China (Fang-Cho, 1960) documented comparable seroconversion rates in individuals infected with Coxsackie A viruses, Coxsackie B viruses, and echoviruses at the time of tOPV delivery, albeit with small numbers of individuals in each group. It is worth noting that since the studies included in this review were published, the classification of enteroviruses has shifted to reflect the molecular properties of this group of viruses. Based on the sequence of the VP1 gene, human enteroviruses can be separated into four major clusters – species A through D (Oberste et al, 1999; Oberste and Pallansch, 2005). Although polioviruses are typically listed as a separate species owing to their distinct pathogenicity, they fall within the same genetic cluster as species C enteroviruses. It is tempting to

speculate that species C enteroviruses might therefore induce a greater interference effect on vaccine polioviruses. As noted above, recombination between species C viruses and OPV occurs relatively frequently, suggesting that the viruses have similar cell tropism (Jegouic et al, 2009; Combelas et al, 2011). However, whether or not these similarities give rise to an increased capacity for interference with OPV replication has not yet been assessed.

2.4.1. Limitations

Several limitations of the present review should be acknowledged. By considering the impact of enteric infections 0–7 days preceding OPV delivery, the study did not account for the possible effects of the stage of enterovirus infection on the outcomes of co-infection (e.g., whether infections have reached peak multiplication within the intestinal tract [Drozdov and Shirman, 1961]) or the potential influence of infections arising after OPV delivery (Voroshilova et al, 1960b; Swartz et al, 1972a; Swartz et al, 1972b)¹⁷. In addition, past exposure to enteropathogens might induce inflammatory changes and other alterations in innate immune function within the intestinal mucosa that affect OPV response even in the absence of concurrent infection, or give rise to a state of mucosal or systemic immune tolerance that diminishes OPV response. As noted in the previous chapter, EE has been linked with exposure to enteropathogens, and may contribute to the impaired immunogenicity of OPV in low-income settings (Korpe and Petri, 2012). However, we encountered no studies reporting on the association between markers of this subclinical condition and OPV response before October 2012. Findings published since the completion of this systematic review have thus far failed to observe a consistent association between EE biomarkers and oral vaccine outcome (Naylor et al, 2015).

Another important consideration is the general tendency of the included studies to focus on a specific group of enteric pathogens. Only two studies examined the influence of both viral and non-viral pathogens on OPV response (Triki et al, 1997; Maldonado et al, 1997), while the use of only monkey kidney cells for enterovirus detection by several trials would result in certain NPEV infections (e.g., several Coxsackie A viruses) going undetected within the study population (Lee et al, 1965). Given the pervasive nature of enteropathogens (Kotloff et al, 2013; Platts-Mills et al, 2015), it is likely that many individuals classified as ‘uninfected’ during the present analysis harboured undetected pathogens at the time of vaccination. This could result in an underestimate of the association between NPEV infection

¹⁷ The potential discrepancy between the inhibitory effect of novel, persistent, and recently resolved infections is an issue we shall consider in Chapter 6.

and vaccine non-response. Conversely, co-infection with other enteropathogens among individuals harbouring NPEVs may be common given the shared risk factors for pathogen exposure. An effect of these co-infections may be captured by the meta-analyses reported above, which would bias our findings in the opposite direction.

2.4.2. Assessment of study quality

An issue I faced during the assessment of eligible studies was how best to evaluate study quality or the risk of bias. For the analysis of NPEVs, for instance, the influence of infections on vaccine response was generally reported as a secondary outcome within a larger trial of OPV (e.g., Japan Live Poliovaccine Research Commission, 1966). Although several tools exist for the assessment of quality in observational studies (Sanderson et al, 2007), their applicability to the studies in this review was questionable. To use the example of the Newcastle–Ottawa quality assessment scale for cohort studies (Wells et al, 2014), criteria were either not applicable (e.g., “demonstration that outcome of interest was not present at start of study” cannot be applied to seroconversion given that this was defined based on the comparison of pre- and post-vaccination samples) or would have given the same score to all studies (e.g., for “selection of the non-exposed cohort” [i.e., individuals without NPEVs or diarrhoea], all studies would have been listed as “drawn from the same community as the exposed cohort”). The ability of such criteria to meaningfully discriminate the included studies according to their risk of bias is therefore equivocal.

This is not to say that meaningful differences in study quality were absent. While I considered the possibility of formulating a customised set of criteria with which to judge study quality, the inferences that could be made based on the application of an unvalidated tool would be limited. Accordingly, I opted to assess the risk of publication bias using funnel plots and Egger’s test, and to extract detailed information regarding study size, laboratory methods, and outcome definitions (presented in the tables above), thereby enabling readers to assess the relative validity of the included studies based on relevant aspects of their design.

2.4.3. Conclusions

As a whole, the present review supports the hypothesised link between enteropathogens and the impaired response to OPV in low-income countries. In particular, there is a sizeable

body of evidence from the 1960s to suggest that existing NPEVs have an inhibitory effect on the immunogenicity and take of vaccine polioviruses.

Of the 25 studies included in this review, it is interesting to note that none were performed in the last 15 years. Why the question has dropped out of fashion is not entirely clear, though this may relate to the costly, labour-intensive efforts required to detect enteric viruses using culture-based methods (when OPV was first introduced, the identification of enteroviruses required up to 2 weeks of cell culture), as well as a gradual shift away from the early conviction in the importance of concurrent infections as an inhibitor of OPV response (John and Christopher, 1975; Patriarca et al, 1991). Given recent advances in molecular diagnostics, the detection of enteropathogens is no longer the cumbersome task it once was. There is considerable potential, therefore, to return to the question of whether viral, bacterial, and eukaryotic enteropathogens contribute to the impaired immunogenicity of OPV in low-income countries. We also have the potential to expand our view beyond the role of specific pathogens and consider the broader influence of the intestinal microbiota, including both its commensal and pathogenic constituents.

It is these issues that form the foundation for the remainder of this thesis.

3.1. Background

The systematic review described above validates the inhibitory effect of enteropathogens on the immunogenicity of oral vaccines. However, the review also highlights notable gaps in our understanding of this phenomenon. While it appears that NPEVs are capable of interfering with the immunogenicity and take of OPV, these infections form only a portion of the pathogen spectrum to which infants living in low-income countries are exposed (Kotloff et al, 2013; Platts-Mills et al, 2015). Few studies have considered the potential influence of other viral, bacterial, or eukaryotic enteropathogens on OPV response. Meanwhile, as alluded to in Chapter 1, there are currently limited published data regarding the impact of enteropathogens on the performance of oral rotavirus vaccines.

In the remainder of this thesis, I will describe several lines of enquiry that have sought to clarify the relationship between the intestinal microbiota and oral vaccine immunogenicity. These analyses revolved around two distinct but related themes – the assessment of multiple enteropathogens via real-time PCR and the high-throughput sequencing of the 16S rRNA gene. Here, I will introduce the key laboratory and bioinformatic methods adopted in the chapters that follow. Specifically, I will provide an overview of: (i) the detection of enteropathogens via real-time PCR using TaqMan array cards (TACs); (ii) the preparation of the 16S rRNA gene amplicon libraries (of which four were sequenced during the course of this thesis); (iii) the bioinformatic analysis pipeline adopted for processing of the resulting 16S rRNA gene sequences; and (iv) the primary comparisons of interest during analysis of the bacterial microbiota data, including the prediction of vaccine outcome using the Random Forests machine-learning algorithm. Specific details on how these methods were applied to examine the relationship between the intestinal microbiota and oral vaccine immunogenicity are described in later chapters.

3.2. The TaqMan array card

The principle method of enteropathogen detection adopted in this thesis involved the use of TACs¹⁸. Each card consists of a 384-well array of singleplex real-time reverse transcription PCR (RT-PCR) assays. Originally developed for studies of gene expression

¹⁸ Other assays for the detection of specific enteropathogens (including Sabin polioviruses and rotavirus) are described where relevant in the following chapters.

(Lossos et al, 2004), TACs have since been customised for a wide range of purposes, including the detection of respiratory pathogens (Kodani et al, 2011) and potential bioterrorism agents (Rachwal et al, 2012). The cards used in the studies described below were specifically developed for the detection of enteropathogens by researchers at the University of Virginia (Charlottesville, USA), and have demonstrated high sensitivity and specificity for this purpose (Liu et al, 2013; Liu et al, 2014). Eight samples were assessed per card, with 48 discrete assays per sample spanning a comprehensive range of bacterial, viral, and eukaryotic enteropathogens. A full list of targets is provided in Table 3.1.

3.2.1. Nucleic acid extraction

Extraction of DNA and RNA was performed from 200 mg of stool using the QIAamp DNA Stool Mini Kit (Qiagen). The Qiagen extraction protocol was modified to include an initial bead-beating step of 2–3 minutes using 500- μ m glass beads (a process that has been shown to increase the yield of DNA from bacterial and eukaryotic enteropathogens [Halstead et al, 2013]), after which samples were heated to 90–95°C for 5 minutes. The final elution volume was 200 μ l, of which 100 μ l was transferred to a tube containing 100 μ l of RNA storage solution (AM7001, Ambion). The resulting total nucleic acid samples were stored at -70°C until testing. Extrinsic controls for DNA (10^6 copies of phocine herpesvirus [PhHV]) and RNA (10^7 copies of MS2 bacteriophage) were spiked into the lysis buffer at the start of the procedure to monitor extraction and amplification efficiency. A negative extraction control was included in each batch to monitor cross-contamination.

3.2.2. Enteropathogen detection

Procedures for the TAC assay have previously been described (Liu et al, 2013). Briefly, for each sample, we mixed 40 μ l of the extracted total nucleic acid with 50 μ l of AgPath-ID RT-PCR buffer, 4 μ l of AgPath-ID one-step enzyme mix, and 6 μ l of nuclease-free water. Reaction mixtures were added to each of the eight loading ports of the TAC (one per sample), which was centrifuged twice at 1,200 RPM for 1 minute. Cards were then sealed and the loading ports removed. RT-PCR was performed for the 384-well array on a QuantStudio 12K Flex system (Applied Biosystems). Cycle conditions were as follows: 45°C for 20 minutes; 95°C for 10 minutes; and 45 cycles of 95°C for 15 seconds and 60°C for 1 minute.

Diarrhoeagenic *E. coli* subgroups were examined using multiple targets and defined as follows: enteroaggregative *E. coli* (EAEC), *aatA* and/or *aaiC*; enteropathogenic *E. coli* (EPEC),

Table 3.1. TaqMan array card targets.

Target	Gene	Reference
Controls		
MS2 ^a	-	Rolfe et al (2007)
PhHV ^a	-	Liu et al (2013)
Hs99999901-s1	18S rRNA gene	Manufacturer control
Bacterial targets		
Bacteria (any)	16S rRNA gene	Rousselon et al (2004)
<i>Aeromonas</i>	Aerolysin	Liu et al (2013)
<i>Bacteroides fragilis</i>	EGBF	Modified from Merino et al (2011)
<i>Campylobacter</i>	Cpn60	Designed based on Hill et al (2006)
<i>Campylobacter jejuni/coli</i>	cadF	Cunningham et al (2010)
<i>Clostridium difficile</i>	tcdB	Liu et al (2013)
EAEC	aaiC	Boise et al (2008)
EAEC	aatA	Boise et al (2008)
EPEC	eae	Liu et al (2013)
EPEC	bfpA	Liu et al (2013)
ETEC	LT	Hidaka et al (2009)
ETEC	ST	Liu et al (2013)
<i>Helicobacter pylori</i>	ureC	Designed based on Shukla et al (2011)
<i>Mycobacterium tuberculosis</i>	IS6110	Halse et al (2010)
<i>Salmonella</i>	ttr	Malorny et al (2004)
<i>Shigella</i> / EIEC	ipaH	Vu et al (2004)
STEC	stx1	Liu et al (2013)
STEC	stx2	Hidaka et al (2009)
<i>Vibrio cholerae</i>	lyA	Liu et al (2013)
Eukaryotic targets		
<i>Ancylostoma</i>	Ribosomal gene	Basuni et al (2011)
<i>Ascaris</i>	Ribosomal gene	Modified from Wiria et al (2010)
<i>Cryptosporidium</i> ^a	Ribosomal gene	Liu et al (2013)
<i>Cryptosporidium</i> typing	Lib13	Hadfield et al (2011)
<i>Cyclospora</i>	Ribosomal gene	Verweij et al (2003)
<i>Enterocytozoon bieneusi</i>	Ribosomal gene	Verweij et al (2007)
<i>Entamoeba histolytica</i>	18S	Verweij et al (2004)
<i>Encephalitozoon intestinalis</i>	Ribosomal gene	Verweij et al (2007)
<i>Giardia</i>	18S	Verweij et al (2004)
<i>Giardia</i> typing	tpi	Modified from Almeida et al (2010)
<i>Isospora</i>	Ribosomal gene	ten Hove et al (2008)
<i>Necator</i>	Ribosomal gene	Basuni et al (2011)
<i>Strongyloides</i>	Ribosomal gene	Verweij et al (2009)
<i>Trichuris</i>	Ribosomal gene	Liu et al (2013)
Viral targets		
Adenovirus serotypes 40/41	Fiber gene	Jothikumar et al (2005)
Adenovirus	Hexon	Heim et al (2003)
Astrovirus	Capsid	Liu et al (2013)
Enterovirus	5' UTR	Modified from Oberste et al (2010)
Norovirus genogroup 1	ORF1-2	Modified from Kageyama et al (2003)
Norovirus genogroup 2	ORF1-2	Kageyama et al (2003)
Rotavirus ^a	NSP3	Zeng et al (2008)
Sapovirus	RdRp	Liu et al (2013)

Adapted from Grassly et al (2016). Abbreviations: EAEC, enteroaggregative *Escherichia coli*; EIEC, enteroinvasive *E. coli*; EPEC, enteropathogenic *E. coli*; ETEC, enterotoxigenic *E. coli*; LT, heat-labile enterotoxin; PhHV, phocine herpesvirus; ST, heat-stable enterotoxin; STEC, Shiga toxin-producing *E. coli*.

^a Assay carried out in duplicate for each sample.

eae and/or *bfpA*; ETEC, heat-stable enterotoxin and/or heat-labile enterotoxin; and Shiga toxin-producing *E. coli* (STEC), *stx1* and/or *stx2*. The presence of adenovirus was determined based on amplification of the pan-adenovirus or serotype 40/41 targets, while norovirus was defined as the detection of either genogroup 1 or genogroup 2 targets. The presence of *Campylobacter*, *Giardia*, and *Cryptosporidium* was determined based on the amplification of genus- or species-specific targets. Based on these criteria, we distinguished between 31 distinct enteropathogens in each sample.

3.2.3. Interpretation and analysis

Upon completion of the TAC assay, each of the 48 singleplex reactions per sample gave rise either to an exponential amplification curve or no amplification (Figure 3.1). Each TAC target has a fluorescence threshold predetermined by the assay developers. The threshold cycle (Ct) value for an amplification curve is the reaction cycle at which the curve crosses this threshold, wherein a lower Ct value signifies a higher copy number for the target in question. A Ct value of 35 was generally adopted as a uniform cut-off for pathogen detection based on previous standardisation of the TAC assay (Liu et al, 2013). We visually inspected the amplification curve of each RT-PCR assay with a Ct value of less than 35 to ensure that an exponential (S-shaped) reaction curve was apparent. If a non-exponential rise in fluorescence was observed (indicative of a gradual shift in the signal of the reaction dyes as opposed to amplification from template DNA), the reaction baseline (encompassing the period before the exponential rise in the amplification curve) was manually adjusted to correct this.

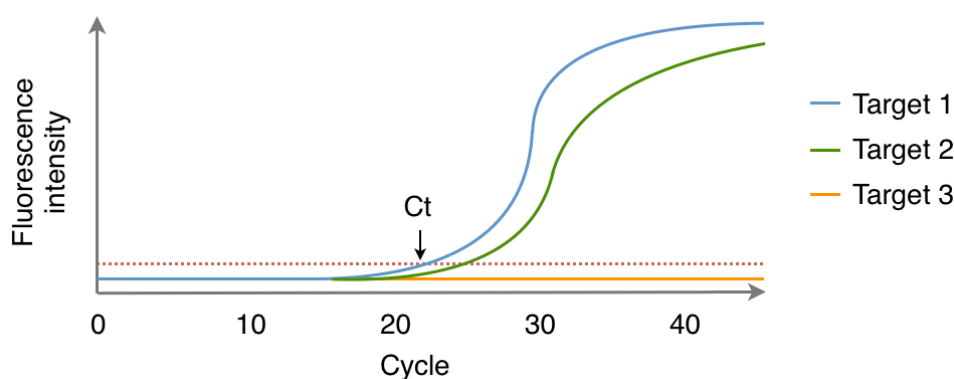


Figure 3.1. TaqMan array card amplification curves. Targets were considered positive if the Ct value – at which the amplification curve crosses the threshold (dotted) line – was less than 35, as exemplified by targets 1 and 2 in the example above. Abbreviation: Ct, threshold cycle.

3.3. 16S rRNA gene sequencing

A summary of the 16S rRNA gene sequencing pipeline adopted in the following chapters – from stool sample through to the preparation of a table of OTU counts with taxonomic assignments – is provided in Figure 3.2. Details regarding the specific steps of this pipeline are provided below.

3.3.1. Nucleic acid extraction

A separate extraction from stool samples was performed for sequencing of the 16S rRNA gene. Again, DNA was extracted from 200 mg of stool using the QIAamp DNA Stool Mini Kit. The Qiagen protocol was modified to include an incubation step at 37°C for 10 minutes with 1.67 µl of lysozyme (30 mg/ml), 13 µl of mutanolysin (11.7 U/µl), and 3 µl of lysostaphin (4.5 U/µl) – an enzyme cocktail that has been shown to improve taxonomic representativeness during studies of mock bacterial communities (Yuan et al, 2012). Incubation was carried out using a plate thermo-shaker (PST-60HL-4, Biosan) set at 250 RPM. Following incubation, we added 10 µl of proteinase K, 50 µl of sodium dodecyl sulphate (10%), and 20 µl of RNase A (1 mg/ml) to each sample prior to a second incubation step (this time in a water bath) at 70°C for 10 minutes. Samples were subsequently vortexed at high speed with 370 mg of 500-µm glass beads (added to samples at the start of the protocol) for 5 minutes. At the end of the extraction procedure, the final elution was performed using 50 µl of buffer AE. A negative control was included in each extraction batch.

3.3.2. Library preparation

The protocol for the preparation of the 16S rRNA gene V4 hypervariable region libraries was adapted by Dr Anna Zekavati (Imperial Biomedical Research Centre Genomics Facility, Hammersmith Hospital, London) from a protocol developed by Balamurugan Ramadass (Department of Gastrointestinal Sciences, Christian Medical College, Vellore) and colleagues (Dinh et al, 2016). Analogous methods targeting the 16S rRNA gene V4 region have been widely adopted during studies of acute malnutrition (Subramanian et al, 2014), geographic variation in microbiota composition (Yatsunenکو et al, 2012), and IBD (Davenport et al, 2014), among others. Before conducting the full experiments described in the following chapters, we validated the laboratory methods – from PCR through to sequencing – during a pilot study of 48 faecal DNA samples.

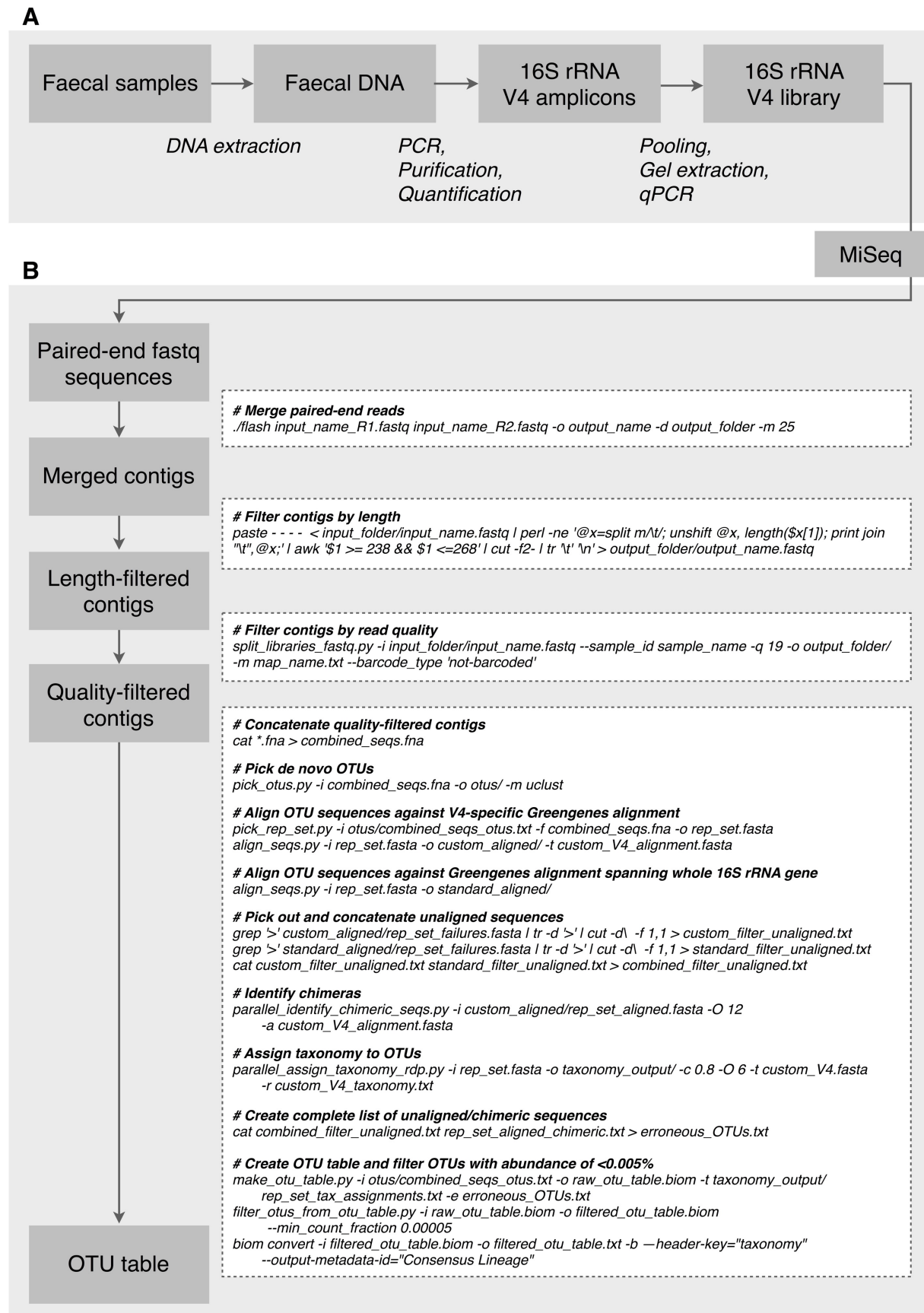


Figure 3.2. 16S rRNA microbiota analysis pipeline. The pipeline is separated into (A) laboratory steps and (B) bioinformatic steps. The Unix commands illustrate the key steps involved in preparation of the OTU table. Abbreviations: OTU, 97%-identity operational taxonomic unit; qPCR, quantitative PCR.

The V4 region of the 16S rRNA gene was amplified via PCR in triplicate for each sample using primers 515F (5'-GTGCCAGCAGCCGCGTAA-3') and 806R (5'-GGACTACCAGG-GTATCTAAT-3'). Primers also contained Illumina adapter sequences, primer pads (to adjust the overall melting temperature of the primer [Kozich et al, 2013]), and linker sequences (that are not complementary to the 16S rRNA gene at the corresponding site), as detailed by Caporaso et al (2012). Each reverse primer contained a unique 12-bp Golay barcode to enable multiplexed sequencing. The reaction mixture comprised 11 µl of distilled water, 10 µl of 2.5X HotMasterMix (5 Prime), 1 µl each of forward and reverse primers at 10 µM (Invitrogen), and 2 µl of template DNA. Cycle conditions were as follows: 95°C for 2 minutes; 20 cycles of 94°C for 30 seconds, 52°C for 45 seconds, and 65°C for 5 minutes; and a final extension of 65°C for 15 minutes (Dinh et al, 2016). Following PCR, products from the triplicate assays were combined, purified using the QIAquick PCR Purification Kit (Qiagen), visualised using gel electrophoresis (Figure 3.3), and quantified via Qubit fluorometer with broad-range assay reagents (Life Technologies). A no-template control was included in each batch of PCR and up to two of these controls were included in each sequencing run.

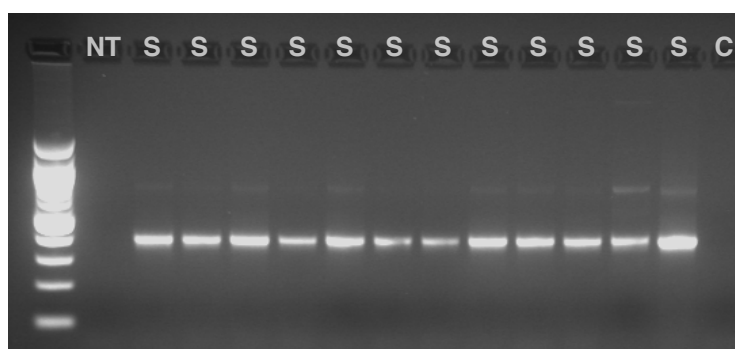


Figure 3.3. Amplification of the 16S rRNA gene V4 region. Purified PCR products were visualised under UV light on a 2% agarose/TAE gel containing ethidium bromide. The bands depicted in this gel are approximately 400 bp in length. Abbreviations: C, extraction control; NT, no-template control; S, faecal DNA sample.

PCR products to be sequenced in a given MiSeq run were pooled in equimolar quantities (300 ng per sample) and 1X TE buffer added to make up a total volume of 4 ml. Since extraction and no-template controls did not yield sufficient PCR product to be detected by Qubit, these products were added to the pool in a quantity equivalent to the lowest volume being added for a faecal DNA amplicon (personal communication, Dr Michael Cox, National Heart and Lung Institute, Imperial College London). 10 ml of 100% ethanol and 600 µl of 5 M NaCl were added to the pooled library, which was vortexed lightly and stored overnight at -20°C. The library was subsequently divided evenly into ten Eppendorf tubes, which were

centrifuged for 30 minutes at full speed (14,000 g). Following the removal of supernatant, the pellets were washed with 800 µl of 70% ethanol, centrifuged for 5 minutes at full speed, allowed to air-dry for 10 minutes, then resuspended in 20 µl of EB buffer (Qiagen) and pooled. 100 µl of this pool was subsequently run on a 1.5% agarose gel and the band excised then purified using the QIAquick Gel Extraction Kit (Qiagen; Figure 3.4).

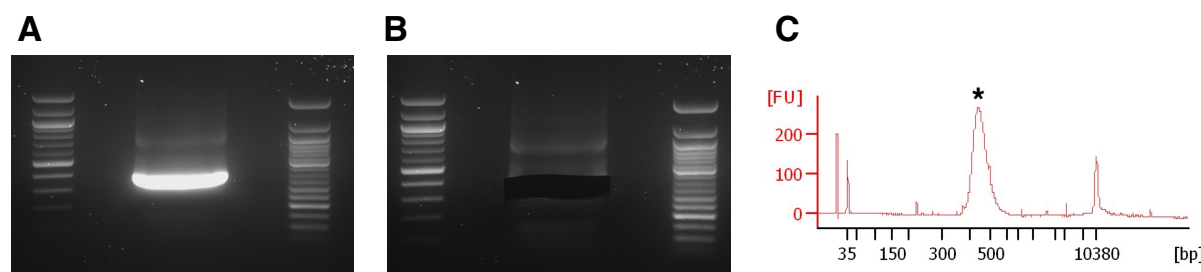


Figure 3.4. Purification of the pooled 16S rRNA gene amplicons via gel extraction. (A) The pooled library was visualised under UV on a 1.5% agarose/TAE gel containing ethidium bromide. (B) After the band had been cut out using a scalpel, the same gel was imaged to verify that the band had been accurately excised. (C) The size distribution of the library was verified using a High Sensitivity DNA Kit on a 2100 BioAnalyzer or equivalent approach. The peak corresponding to the 16S rRNA gene amplicon library is signalled by an asterisk. Abbreviations: bp, base pairs; FU, fluorescence units.

The eluted library was quantified using the KAPA Library Quantification Kit (KK4824, KAPA Biosystems) on a 7500 Fast real-time PCR system (Applied Biosystems) and diluted to a concentration of 2 nM¹⁹. The size distribution of amplicons was verified using a High Sensitivity DNA Kit on an Agilent 2100 BioAnalyzer (Figure 3.4C) or (for the library described in Chapter 6) using a D1000 ScreenTape on an Agilent 2200 TapeStation. The library was then sequenced via Illumina MiSeq, following the 151 × 151 base pair (bp) protocol described by Caporaso et al (2012). This method includes the spiking of libraries with phi X (bacteriophage) control DNA to enable calculation of sequencing error rate and to improve the accuracy of base calls, which is impaired during the sequencing of amplicons owing to the limited diversity observed among sequences (Caporaso et al, 2012). For each run, the final mixture consisted of 60% library DNA and 40% phi X DNA.

¹⁹ We initially quantified the pooled library via Qubit. Although successfully applied during the pilot study, this approach subsequently led to a failed MiSeq run in which all of the reads mapped to the phi X control genome, suggesting severe under-representation of the 16S rRNA gene amplicons. The use of quantitative PCR resolved this issue. Thus, it appears that Qubit had failed to accurately quantify the yield of adapter-ligated amplicons within the eluted library. Despite much consideration of the issue, the reasons for this discrepancy elude me.

3.3.3. Merging of paired-end reads

Sequence data were automatically demultiplexed by the MiSeq analysis software. The sequencing output thus consisted of two 'fastq' files for each sample – one containing 151-bp forward reads (into the V4 region from primer 515F) and the other containing 151-bp reverse reads (from primer 806R). Each sequence, in turn, has three key pieces of information associated with it: (i) a sequence identifier, containing information such as the instrument name, run ID, and the coordinates of the cluster within the MiSeq flow cell (which are used to match paired reads); (ii) the string of base calls; and (iii) a corresponding string of quality (or 'phred') scores, which signal the probability that the base call was erroneous (Illumina, 2014). For example, the character '?', indicating a quality score of 30, signals an error probability of 0.001. The fastq format is illustrated as follows:

```
@M01823:172:000000000-A8EYD:1:1101:12988:1798 1:N:0:41    sequence identifier
TACGTAGGGTGCAAGCGTTATCCGGAATTATTGGGCGTA...           base calls
+
CCCCCFFCCFCDDGGGGGGGGGGHGGGGGHHHHHHHGG...           quality scores
```

Forward and reverse reads from each sample were assembled into contiguous sequences (or 'contigs') using FLASH – an algorithm implemented via the Unix command line that assembles paired reads and retains a quality score for each base (Magoc and Salzberg, 2011). A minimum overlap size of 25 bp was specified during this assembly process – a conservative estimate given that the median length of the assembled reads was 253 bp, indicating a typical overlap between paired reads of 49 bp. A length-filtering step was then performed to exclude assembled reads outside of the target range (253 ± 15 bp) using a customised Unix script (adapted from code published by Ziemann [2012]; Figure 3.2).

3.3.4. Sequence processing in Qiime

Assembled sequences were analysed using Qiime (MacQiime version 1.8.0) – a suite of bioinformatic processing tools written in the programming language Python that are tailored to the analysis of microbiota data (Caporaso et al, 2010b). Several commands in the bioinformatic pipeline were adapted from a shell script published by Nelson et al (2014). Qiime commands were implemented via the Unix command line using customised shell scripts. Unless otherwise specified, default parameter settings were applied when implementing the commands described below.

i. Quality filtering

Quality filtering was performed using the Qiime function *split_libraries_fastq.py*. We applied the filtering criteria previously validated by Bokulich et al (2013), but with an elevated quality score of ≤ 19 to define low-quality base calls (corresponding to an error probability of >0.01). These criteria retain contigs with: (i) no ambiguous base call (i.e., calls that could not be assigned as A, C, G, or T); (ii) no more than three consecutive low-quality bases; and (iii) a minimum number of consecutive high-quality bases spanning at least 75% of the total sequence length.

ii. OTU picking

After quality filtering, we clustered sequences into OTUs. A threshold of $\geq 97\%$ nucleotide identity is typically adopted to define OTUs in sequence-based microbiota analyses (Navas-Molina et al, 2013). However, there are several distinct approaches with which to group sequences into these 97%-identity clusters. During ‘closed-reference’ OTU picking, sequences are matched to a reference database (e.g., the Greengenes database of 16S rRNA gene sequences [DeSantis et al, 2006a]) and any sequences without a match in the database are discarded. This approach is computationally efficient and enables OTUs to be directly compared from study to study if the same database is used. By discarding sequences without a match, however, potentially relevant data are lost. The use of ‘open-reference’ OTU picking makes up for this drawback by initially matching sequences with a reference database, then grouping any remaining sequences into novel clusters (or ‘*de novo* OTUs’).

In our study, however, we adopted a *de novo* OTU-picking strategy. This approach assigns contigs into clusters based solely on their sequence identity (i.e., without use of a reference database). Although computationally intensive, *de novo* OTU selection benefits from using all available sequence data and avoids the tendency of reference-based approaches to overestimate the number of OTUs present in a set of sequences (which we observed while testing the bioinformatics pipeline). In particular, databases such as Greengenes contain reference OTUs with a given level of similarity (e.g., 97%) across the entire 16S rRNA gene (~1,500 bp). However, within the V4 region targeted in our study, reference OTUs may have $>99\%$ sequence identity. As a result, sequences that would fall within the same OTU during *de novo* selection have the potential to be assigned to separate OTUs by reference-based methods. Other researchers have documented similar issues with reference-based methods of OTU selection (Nelson et al, 2014; Westcott and Schloss, 2015).

To pick *de novo* OTUs, we initially concatenated the contigs from different samples, then clustered these sequences using the ‘uclust’ algorithm (via the Qiime command *pick_otus.py*). This algorithm is described in full by Edgar (2010). Briefly, sequences are initially sorted by abundance²⁰. The most abundant sequence then becomes the ‘seed’ of the first OTU. For each subsequent sequence, the existing database of seeds is examined to determine if there is a match of $\geq 97\%$ (using the algorithm ‘usearch’). If a match is found, the sequence is assigned to that OTU (for this reason, uclust is referred to as a ‘greedy’ algorithm). If there is no match at a 97% threshold, the sequence becomes a new seed. In each iteration of this algorithm, the search of the seed database ceases as soon as a match is found. However, seed sequences are sorted based on the number of unique ‘words’ (in our case, 12-bp sequences) they share with the query sequence, such that the first match is typically the most similar sequence in the existing database (Edgar, 2011). At the end of this process, all sequences are assigned to OTUs that fulfil the following criteria: (i) all sequences within an individual OTU have $\geq 97\%$ identity with the seed sequence; and (ii) all seed sequences have $< 97\%$ identity.

iii. Alignment, chimera checking, and taxonomy assignment

Having assigned all sequences to OTUs, reference sequences from each OTU were selected using the function *pick_rep_set.py*. We then generated a multiple alignment of these sequences using PyNAST (DeSantis et al, 2006b; Caporaso et al, 2010a) and identified potential chimeras using the algorithm ChimeraSlayer (Haas et al, 2011). These steps were achieved by implementing the functions *align_seqs.py* and *parallel_identify_chimeric_seqs.py*, respectively. Chimeras are artefacts of PCR that contain more than one parent sequence. Although they may be formed by a range of mechanisms, the majority are thought to arise following incomplete extension from a primer during the PCR amplification cycle, which enables partially extended strands to prime extension from a heterologous site during subsequent cycles (Smyth et al, 2010). The removal of chimeric sequences is crucial to avoid the artificial inflation of bacterial diversity estimates during microbiota analyses.

To assign a taxonomic classification to each OTU, we used the naïve Bayesian Ribosomal Database Project (RDP) classifier developed by Wang et al (2007), as implemented via the function *parallel_assign_taxonomy_rdp.py*. The RDP classifier makes taxonomic assignments from kingdom through to species level and provides a confidence estimate for each assignment. We adopted a minimum confidence threshold of 0.8. The rank of classification

²⁰ This is a modification of the algorithm originally described by Edgar (2010), wherein sequences are sorted by length as opposed to abundance.

(kingdom, phylum, etc.) assigned by this approach may vary from OTU to OTU – thus, while some sequences can be assigned a species-level classification above the confidence threshold of 0.8, others can be assigned only as far as, for example, class or order.

Notably, the use of reference sequences corresponding to the 16S rRNA hypervariable region of interest has previously been shown to improve the depth and confidence of taxonomic classification during studies of the bacterial microbiota (Werner et al, 2012). We therefore used customised reference files comprising the V4 region of the Greengenes 97%-identity OTU sequences (version 2013/08) during alignment, chimera checking, and taxonomic assignment. A V4-specific alignment was initially generated by identifying the locations of the primers 515F and 806R in the NAST-aligned Greengenes reference file, trimming the alignment 15-bp upstream and downstream of the primer locations using the function *pcr.seqs* in *mothur*²¹ (Schloss et al, 2009), and removing common gaps using the function *filter.seqs* (analogous to the approach adopted by Nelson et al [2014] to generate reference files specific to the V4–V5 region of the 16S rRNA gene). The resulting file was used as a basis for alignment and chimera checking in the pipeline outlined above. Alignment characters were removed to generate an unaligned set of V4 sequences, which was used to retrain the RDP classifier during taxonomic classification.

iv. Phylogenetic tree

To prepare a phylogenetic tree of the V4 sequences, we created a new alignment of OTU reference sequences by implementing the Qiime function *align_seqs.py* with default settings (using a Greengenes alignment spanning the entire 16S rRNA gene as a reference instead of the V4-specific file described above). It was then possible to filter this alignment while applying the ‘Lane mask’ (Lane, 1991) by implementing the function *filter_alignment.py* with default parameter settings. The filtering of alignments involves the removal of gap characters that are common to all sequences. Crucially, by applying the Lane mask during this process, it is also possible to filter out nucleotide positions that demonstrate too much variability to be useful when generating a phylogeny (in practice, the Lane mask comprises a series of 0s and 1s, wherein 1s correspond to the positions in the 16S rRNA gene alignment that should be ignored when generating a phylogenetic tree). After filtering the alignment in this way, a phylogenetic tree was prepared using FastTree (Price et al, 2009) – the default tree-building algorithm implemented by the Qiime function *make_phylogeny.py*.

²¹ Like Qiime, *mothur* is a package of bioinformatics analysis tools tailored for the analysis of microbiota sequence data.

v. OTU table

To conclude the bioinformatics pipeline, an OTU table was constructed using the Qiime functions *make_otu_table.py* and *biom convert*. This table contains a column for each sample and a row for each OTU, with each cell indicating the number of assembled contigs for a given sample that were assigned to the corresponding OTU. Unaligned or chimeric sequences were removed during the construction of this table. OTUs representing <0.005% of sequences were also removed by applying the function *filter_otus_from_otu_table.py*, as recommended by Bokulich et al (2013).

3.3.5. Interpretation and analysis

After processing sequences via the pipeline outlined above, our analyses centred around detecting discrepancies in the diversity and composition of the bacterial microbiota according to vaccine response. In each analysis, the OTU table was randomly subsampled – a process termed ‘rarefaction’ – to standardise sequencing depth across samples. Rarefied versions of the filtered OTU table were generated using the Qiime function *multiple_rarefactions.py*. Specific details regarding statistical analyses are described in the following chapters; however, the key outcomes of interest are introduced briefly below.

i. Alpha diversity

For the assessment of alpha (within-sample) diversity, we determined the OTU count (a measure of taxonomic richness) and Shannon index (a composite of richness and evenness) using the Qiime functions *multiple_rarefactions.py*, *alpha_diversity.py*, and *collate_alpha.py*. Mean OTU count and Shannon index for each sample across ten independent rarefactions at a given depth served as the input for analyses.

ii. Beta diversity

Weighted and unweighted Unifrac distances were used to assess beta diversity (divergence between microbial communities). As noted by Lozupone et al (2007), the Unifrac distance between two samples is a measure of “the fraction of the branch length in a phylogenetic tree that leads to descendants in either, but not both, of the two communities”. Accordingly, the greater the overlap in composition between two samples, the lower the Unifrac distance between them. By comparing distances across multiple samples, it is

possible to assess the degree to which the overall composition of the bacterial microbiota clusters according to an outcome of interest (e.g., vaccine response). Unifrac distances can either be weighted by the relative abundance of the bacterial taxa (weighted Unifrac) or not (unweighted Unifrac) – whereas weighted Unifrac distances are influenced primarily by changes in the most abundant taxa within the samples being compared, unweighted distances capture changes in the presence/absence of low-abundance taxa (Chen et al, 2012).

We used the Qiime function *beta_diversity.py* to calculate weighted and unweighted Unifrac distances between samples in the analyses described below. The phylogenetic tree generated by the function *make_phylogeny.py* (see above) served as the basis of these calculations. Unifrac distances were visualised using principal coordinates analysis (via the function *principal_coordinates.py*). The clustering of samples according to outcomes of interest was then assessed using the adonis function – a non-parametric permutation-based test (implemented in the R package *vegan*) that partitions sums of squares within a distance matrix between variables and residuals in a manner equivalent to multivariate analysis of variance (Oksanen et al, 2015).

iii. Taxon relative abundance

To distinguish whether individual taxa discriminated individuals according to vaccine outcome (as suggested in the ‘keystone taxa’ hypothesis outlined in section 1.6), we examined whether phylum-, class-, and OTU-level differences in relative abundance distinguished responders from non-responders. To account for the highly skewed distribution of taxon relative abundances observed across samples, we performed these analyses using non-parametric tests, including a bootstrapped *t* statistic and Wilcoxon’s rank sum test (as described in relevant sections of the chapters that follow). To reduce the risk of type I error given the multitude of taxa present in samples, *P* values for each set of comparisons (phylum-, class-, or OTU-level) were adjusted via Benjamini–Hochberg false discovery rate (FDR) correction (Benjamini and Hochberg, 1995).

iv. Random Forests

The Random Forests machine-learning algorithm has increasingly been applied in studies of the gut microbiota as a means of assessing the degree to which individuals can be discriminated according to an outcome of interest based on the composition of their bacterial flora (e.g., Yatsunenko et al, 2012; Subramanian et al, 2014). First described by Breiman

(2001), Random Forests functions by building multiple classification trees from a set of input variables (in our case, 1,000 trees were generated for each analysis from an input table containing the relative abundances of OTUs). Each tree is generated from a subset of samples equivalent in size to the original dataset but picked at random with replacement (i.e., a bootstrap sample). As such, approximately a third of samples are left out for each tree (referred to as the 'out-of-bag' samples). For categorical outcome variables, trees are then built as follows:

- i) A subset of the M available variables (of size $\sqrt{M}$ by default) is selected (e.g., out of 135 OTU abundances, 12 are chosen at random);
- ii) The variable that enables the best possible separation of samples according to the outcome of interest (e.g., responders vs non-responders) is selected²² – this variable is used to split the first node of the tree;
- iii) Steps (i) and (ii) are repeated for each daughter node;
- iv) This process continues until all nodes contain either one individual or one class of individual (e.g., vaccine responders).

A benefit of Random Forests is that the available data do not need to be divided into training and validation subsets. Instead, each tree is used to predict the class of out-of-bag samples. At the end of the run, each sample will have been left out of approximately a third of the trees in the forest (a result of the bootstrap sampling that occurs before each tree is generated). Thus, in a forest of 1,000 trees, there will be in excess of 300 'votes' for the class of each sample. The class that receives the majority of these votes is then assigned as the predicted outcome of the sample (Breiman and Cutler, 2004). A summary of the tree-building process and out-of-bag classification procedure is provided in Figure 3.5.

We focused on two key outputs of the Random Forest algorithm. First, we calculated the overall out-of-bag classification accuracy of the forest (i.e., the percentage of samples that were assigned to the correct class). For each analysis, this value was compared to the baseline accuracy of the model, wherein all samples are assigned to the majority class. Second, we considered the estimated 'importance' of each variable, calculated as follows:

- i) For a given tree, the values of the variable are randomly permuted in the out-of-bag samples;
- ii) Each out-of-bag sample is run down the classification tree to obtain a predicted class;

²² Random Forests applies the Gini splitting rule, which attempts to isolate the majority class from the remainder of the data at each node in the classification tree.

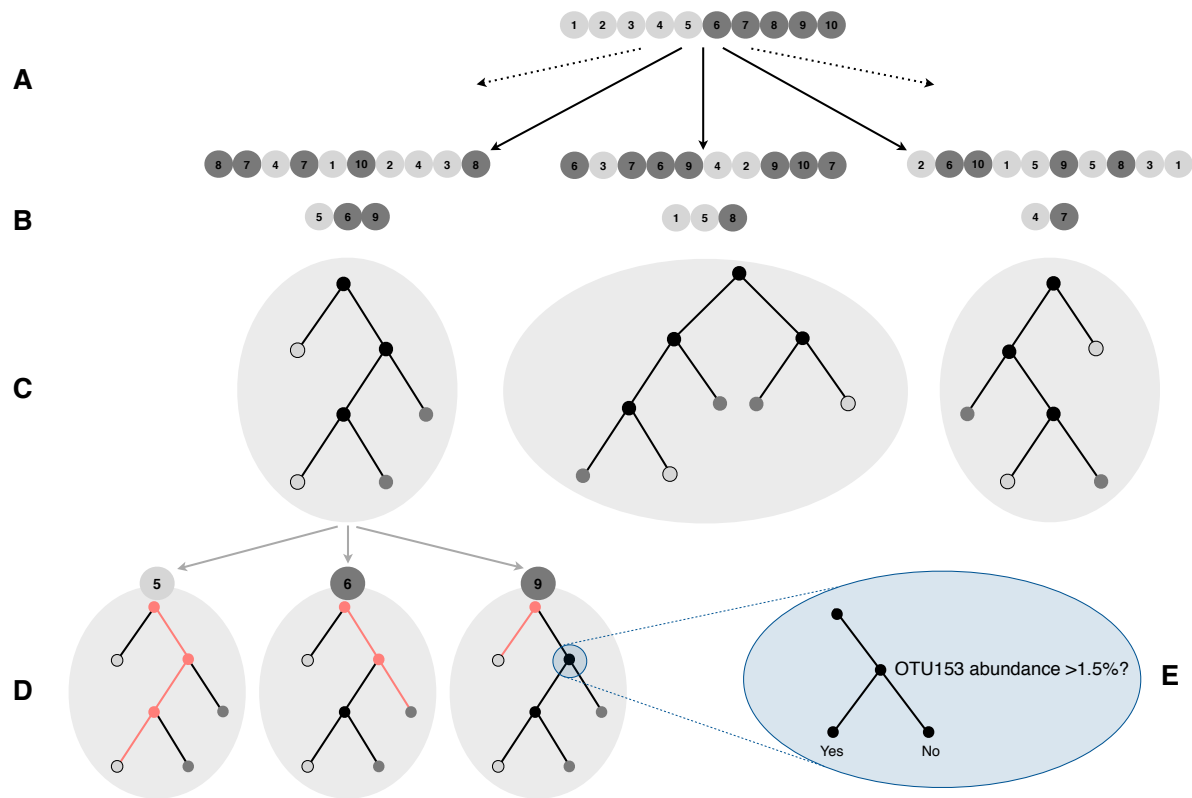


Figure 3.5. Random Forests: tree building and out-of-bag sample classification. (A) The Random Forests algorithm functions by selecting multiple bootstrap samples of the input data. (B) In each of these bootstrap samples, approximately a third of the input samples are left ‘out-of-bag’. (C) Classification trees are generated for each bootstrap sample, as described in the main text. During this process, a subset of the potential predictors is considered at each node. (D) The out-of-bag samples are then run down each tree; for a given sample, the class that receives the majority of votes across the forest is its predicted outcome. (E) Each node of the tree corresponds to a threshold in one of the input variables (in our case, the relative abundance of an OTU). Abbreviation: OTU, 97%-identity operational taxonomic unit.

- iii) The proportion of votes for the correct class in the permuted out-of-bag dataset is subtracted from the proportion of votes for the correct class in the untouched data;
- iv) This process is repeated for each tree in the forest, enabling the mean decrease in classification accuracy to be calculated (Breiman and Cutler, 2004).

Crucially, important variables will give rise to a greater decrease in the predictive accuracy of the tree when permuted in this way.

Random Forests has been shown to confer high predictive accuracy when applied to a variety of different data forms (Gislason et al, 2006; Cutler et al, 2007), including microbiota data (Subramanian et al, 2014). The algorithm is capable of handling ‘wide’ data (containing more predictors than observations), making it well suited to the analysis of OTU

abundances. Moreover, in contrast to the analyses of alpha diversity, beta diversity, and relative taxon abundance described above, classification trees have the capacity to capture complex interactions among the input variables.

3.4. Mock community profile

In the final MiSeq run of this thesis (described in Chapter 6), we sequenced PCR products amplified from two mock communities obtained from BEI Resources (Manassas, USA): HM-782D, comprising genomic DNA derived from a community of 20 bacterial strains with an even rRNA operon count per organism (BEI Resources, 2012a); and HM-783D, comprising genomic DNA from the same community but with an uneven ('staggered') rRNA operon count per organism (BEI Resources, 2012b).

The sequencing data obtained from these communities were analysed via the pipeline described above. We restricted our attention to the mock community with an even rRNA operon count per organism during the ensuing analyses. Among the 16,860 sequences obtained from this sample after quality filtering, we excluded OTUs with a relative abundance of <0.1%, then matched the remaining OTUs ($n = 18$) to their corresponding taxa in the mock community based on genus-level taxonomic classifications or (where the mock community contained multiple strains in the same genus) using BLAST (Johnson et al, 2008).

The relative abundances of these taxa were generally consistent with their expected values (Figure 3.6). Although we observed no sequences corresponding to the strain *Propionibacterium acnes* and under-representation of strains in the genera *Enterococcus*, *Deinococcus*, and *Rhodobacter*, these findings are consistent with the biases documented in previous studies using the primers 515F and 806R (Nelson et al, 2014).

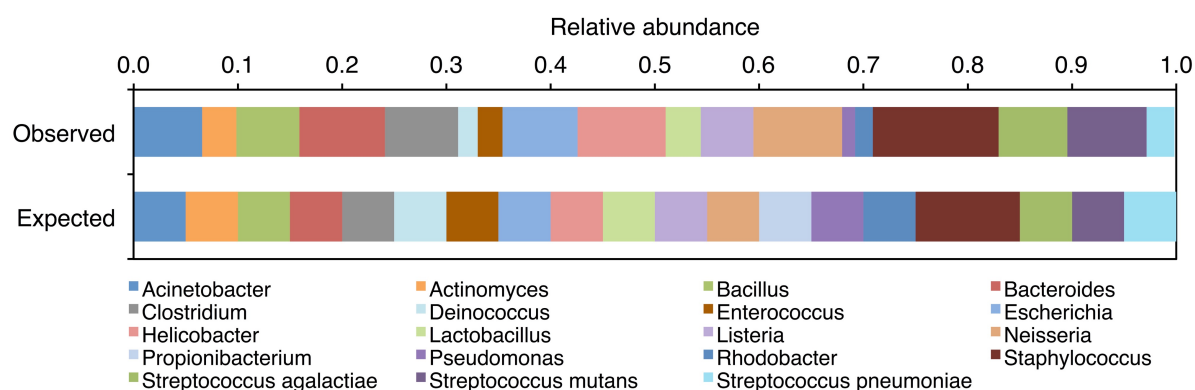


Figure 3.6. Mock community composition. Data are displayed for the mock community with an even rRNA operon count per organism (HM-782D, BEI Resources).

3.5. Conclusions

Recent advances in molecular diagnostics and high-throughput sequencing have enabled the composition of the intestinal microbiota – including both pathogenic microbes and the commensal bacterial flora as a whole – to be characterised with a sensitivity and depth that was not possible when OPV was first developed. These advances, in turn, provide us with novel opportunities for understanding the potential contribution of the intestinal microbiota to the impaired performance of oral vaccines in low-income settings.

In this chapter, I have introduced the laboratory and bioinformatic methods that form the foundation of the investigations that follow; in the next, I will describe how they were put to use to characterise the intestinal flora of infants who received Rotarix and tOPV as part of a clinical trial in south India.

Chapter 4: Influence of enteropathogens and the bacterial microbiota on response to oral rotavirus vaccine: a case–control study in south India

4.1. Background

During the course of the systematic review described in Chapter 2, a clinical trial was being carried out in Vellore, India, led by researchers at the Christian Medical College (ClinicalTrials.gov identifier NCT01616693). The primary aim of this study was to assess the impact of zinc and/or probiotic supplementation on the immunogenicity of Rotarix and tOPV when co-administered to infants at 6 and 10 weeks of age. Crucially, stool samples were collected on the day of each vaccine dose and stored at -70°C. These samples provided us with an opportunity to conduct a nested case–control study comparing the pre-vaccination intestinal microbiota – including the burden of bacterial, viral, and eukaryotic enteropathogens as well as the overall composition of the bacterial flora (assessed via 16S rRNA gene sequencing) – according to whether or not infants responded to the oral vaccines. In doing so, we could test the hypothesis that the composition of the intestinal microbiota is associated with the immunogenicity and take of oral rotavirus vaccine and tOPV.

In this chapter, I will present the results of these endeavours with respect to Rotarix response. These analyses will then be extended to consider OPV outcome in Chapter 5. The influence of probiotic supplements on the composition of the bacterial microbiota, which was considered as a secondary objective, will also be briefly described. Where applicable, findings are reported according to STROBE guidelines (von Elm et al, 2007).

4.1.1. Hypotheses

i. Enteropathogen burden

- i) The presence and number of enteropathogens (bacterial, viral, or eukaryotic) at the time of immunisation is associated with a significant reduction in the likelihood of responding to Rotarix and tOPV (based either on seroconversion or take);
- ii) The replication of vaccine polioviruses is negatively correlated with the replication of vaccine rotaviruses.

ii. Bacterial microbiota

- i) Failure to respond to Rotarix and tOPV (based either on seroconversion or take) is associated with a decrease in the diversity of the bacterial microbiota;

- ii) Oral vaccine responders have a bacterial microbiota composition that is distinct from non-responders, as inferred by Unifrac distances and the discriminatory capacity of Random Forest models;
- iii) These shifts are driven in part by an elevated abundance and diversity of Proteobacteria among non-responders compared with responders.

4.2. Vellore

Before describing the methods adopted in this study, a brief tangent is warranted to consider the setting in which the trial took place. Vellore is a city with a population of approximately 40,000 located in Tamil Nadu, a state in the southern-most tip of the Indian subcontinent. Temperatures range from 18°C in December to 44°C in May (Kattula et al, 2014), and the city experiences a mean annual rainfall of approximately 1,000 mm (by comparison, London has a mean annual rainfall of less than 600 mm [Met Office, 2016]). Rainfall is at its peak between August and November (the northeast monsoon season).

The clinical trial took place among residents of Chinnallapuram, a densely populated semi-urban slum area in the southwest of Vellore. Within this and adjacent areas, the most common occupation is the production of 'beedis' – hand-made cigarettes consisting of tobacco wrapped in dried leaves (Kattula et al, 2014). Homes in Chinnallapuram are predominantly built from permanent materials such as bricks and concrete, although 'kutcha' houses (made from mud brick, thatch, or other low-quality materials) are not uncommon (Sarkar et al, 2013). Residents have access to a local urban health clinic, and a government hospital is located approximately 5 km away. Also within a few kilometres of the area is the Christian Medical College, a 2,500-bed hospital in the centre of Vellore, as well as its two outreach centres – the Low Cost Effective Care Unit and the Department of Community Health and Development. Infants living in Chinnallapuram and adjacent areas are known to experience a considerable burden of gastrointestinal disease – within a cohort of 176 children, Sarkar et al (2013) documented 4.0 episodes of gastrointestinal illness per child-year in the first year of life, and 2.5 episodes in the second year. Stunting, defined as a height-for-age Z score of <-2, was observed in approximately 30% of children at 2 years of age. Caesarean section accounts for 6–18% of births (Gladstone et al, 2008; Kattula et al, 2014).

Several factors may contribute to the high incidence of enteropathogens observed among infants in Vellore. High rates of rotavirus shedding have been documented in neonatal nurseries at the Christian Medical College, likely via perinatal transmission or exposure to contaminated surfaces (Ramani et al, 2008). Microbial contamination of municipally supplied

water is ubiquitous (Brick et al, 2004), and is enhanced by household storage practices, wherein water is routinely kept for up to several weeks in wide-mouthed containers and consumed without additional treatment. Close contact with animals, poor hand hygiene practices, overcrowding, and lack of access to functioning toilets may also enhance the likelihood of enteropathogen exposure (Collinet-Adler et al, 2015; Kattula et al, 2015).

The central role of Vellore in the history of polio research is also worth highlighting. In particular, studies led by Professor Thekkekara Jacob John in the 1970s were among the key works to shed light on the impaired immunogenicity of OPV in low-income, tropical settings (John, 1976). These studies notably did not support the inhibitory effect of NPEVs on OPV response, as is evident in Figure 2.2 (John and Christopher, 1975).

4.3. Methods

4.3.1. Study population

Samples were obtained from a clinical trial that set out to assess the impact of daily supplements of zinc and/or probiotics on the immunogenicity of oral rotavirus vaccine and tOPV in early infancy. At the time of writing, a full report of the trial is pending (Lazarus et al, manuscript in preparation). In brief, between 18 July 2012 and 22 February 2013 a total of 620 infants living in Chinnallapuram were recruited to the trial at 5 weeks of age. Participants were assigned at random to receive one of four treatment regimens: (i) supplements of zinc (5 mg of zinc sulphate heptahydrate) and probiotics (*Lactobacillus rhamnosus* GG capsules containing 10^{10} organisms) administered daily from 5 to 11 weeks of age; (ii) zinc supplements and probiotic placebo; (iii) probiotic supplements and zinc placebo; and (iv) zinc placebo and probiotic placebo. All participants received Rotarix at 6 and 10 weeks of age and tOPV at 6, 10, and 14 weeks of age. Infants also received routine vaccines according to the national schedule in India, including tOPV at birth. Serum anti-rotavirus VP6 IgA antibodies were measured at 6 and 14 weeks of age using an antibody-sandwich enzyme immunoassay (Ward et al, 2004). Response to Rotarix was defined as a four-fold increase in serum anti-VP6 IgA titre²³ or detection of antibodies at ≥ 20 U/ml in previously seronegative infants. Anti-poliovirus neutralising antibody titres were assessed at the same timepoints using the standardised microneutralisation method (WHO, 1997). Response to tOPV was defined as an increase in poliovirus serotype 3 neutralising antibody titre from $<1:8$ to $\geq 1:8$ or a four-fold rise in titre, assuming a decay in pre-vaccination titre

²³ Strictly speaking, these antibody measures are concentrations rather than titres; however, for simplicity I will use the term 'titres' when referring to both rotavirus and poliovirus antibody levels.

with a half-life of 28 days. Stool samples were collected on the day of and 4 and/or 7 days after the 6- and 10-week vaccine doses. All study procedures were completed by 25 April 2013. Key features of the trial design are highlighted in Figure 4.1. The study was supported by funding from the Department of Biotechnology (India) and PATH.

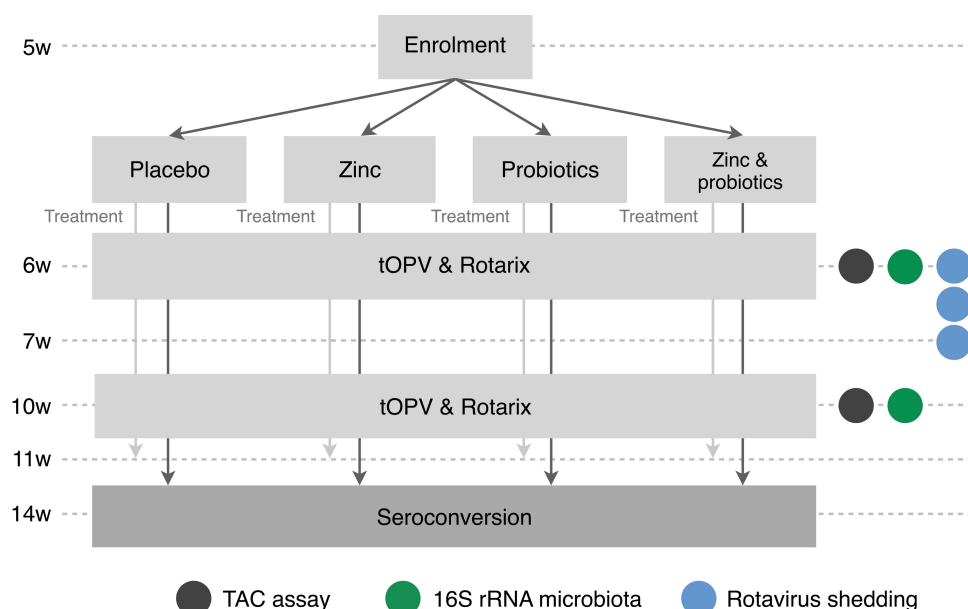


Figure 4.1. Zinc/probiotic supplementation trial design. The additional laboratory assays undertaken as part of the nested case-control study described in this chapter are indicated. Abbreviations: TAC, Taqman array card; tOPV, trivalent oral poliovirus vaccine; w, weeks.

4.3.2. Enteropathogen testing

Enteropathogen testing was carried out in the Department of Gastrointestinal Sciences at the Christian Medical College, Vellore. My role in the laboratory work is clarified above (p6).

i. Primary outcome and sample size calculation

Following completion of the trial, we devised a nested case-control study to assess the impact of concurrent enteropathogens on oral rotavirus vaccine immunogenicity. Our initial objective was to examine the impact of harbouring ≥ 1 enteropathogen at either 6 or 10 weeks of age on the odds of seroconverting to rotavirus. However, since the sensitive detection of multiple enteropathogens via TACs had not previously been conducted in this study population, we used an interim analysis of enteropathogen prevalence to inform final sample size calculations. Herein, we selected 112 individuals (14 who seroconverted to rotavirus and 14 who failed to seroconvert from each of the four study arms) and performed the extraction and TAC assay for the 6- and 10-week stool samples of this subset (according to the methods described in Chapter 3). Without unblinding study arm or vaccine outcome, the prevalence

of enteropathogens at 6 and 10 weeks of age was determined. Five individuals were excluded at each timepoint owing to insufficient sample availability or the absence of RNA targets (including MS2) in the TAC assay. The interim analysis revealed a high prevalence of EAEC (67/107 [63%] at 6 weeks and 70/107 [65%] at 10 weeks) and enteroviruses (74/107 [69%] at 6 weeks and 78/107 [73%] at 10 weeks) – we subsequently confirmed that Sabin viruses accounted for the majority of the latter, as described below. Rotavirus infections were observed in 3/107 (3%) 6-week samples and 1/107 (1%) 10-week samples. Infections other than EAEC, enterovirus, and rotavirus were observed in 48/107 (45%) and 44/107 (41%) of 6- and 10-week samples, respectively, and were present during at least one of the two doses in 68/105 (65%) individuals. By contrast, when enteroviruses and EAEC were included, ≥ 1 enteropathogen was observed at 6 or 10 weeks in 104/105 (99%) infants.

In light of the high prevalence of EAEC and enteroviruses, as well as the limited association between EAEC and diarrhoea in previous studies using analogous methods of pathogen detection (Taniuchi et al, 2013; Platts-Mills et al, 2014), we decided to omit these pathogens from the primary outcome analyses. Rotavirus infections were also excluded given that – in contrast to the hypothesised inhibitory effect of enteropathogens – one would expect natural rotavirus exposure or shedding of vaccine rotaviruses to be positively correlated with rotavirus seroconversion. We estimated that the inclusion of at least 150 infants who seroconverted to rotavirus (hereafter referred to as ‘responders’) and 150 infants who failed to seroconvert (‘non-responders’) would provide 80% power to detect a reduction in the odds of rotavirus seroconversion (with an OR of ≤ 0.5 and an α of 0.05) in conjunction with the presence of ≥ 1 enteropathogen (excluding EAEC, enterovirus, and rotavirus) at 6 or 10 weeks of age, assuming an overall infection rate of 65% in a study population comprising 50% responders and 50% non-responders (Dupont and Plummer, 1990).

ii. Eligibility criteria and TaqMan array card assay

Trial participants were considered eligible for the study if they received supplements or placebo, received both doses of Rotarix and tOPV, and provided paired serum samples ($n = 563$). To meet sample size requirements, we included all eligible infants who seroconverted to rotavirus, subject to constraints in sample availability ($n = 162$, of whom 52 received zinc and probiotics, 35 received zinc alone, 39 received probiotics alone, and 36 received placebo). For each arm, we selected an approximately equivalent number of non-responders at random (to account for the potential confounding of study arm with infection status and seroconversion). The final study population is summarised in Table 4.1. Infants were

Table 4.1. Summary of study population.

Treatment group	Trial population (per protocol)		No. in enteropathogen subset		No. in 16S rRNA microbiota subset	
	Total	Rotarix responders (%)	Total	Rotarix responders (%)	Total	Rotarix responders (%)
Zinc/probiotics	137	54 (39.4)	105	52 (49.5)	38	19 (50.0)
Zinc only	143	40 (28.0)	69	35 (50.7)	-	-
Probiotics only	136	42 (30.9)	78	39 (50.0)	69	35 (50.7)
Placebo	135	37 (27.4)	73	36 (49.3)	63	31 (49.2)
Overall	551	173 (31.4)	325	162 (49.8)	170	85 (50.0)

Five individuals in the enteropathogen study were excluded from the final analyses as they did not complete the study per protocol – two received zinc and probiotics (of whom one seroconverted to rotavirus); one received zinc alone (a responder); and two received probiotics alone (of whom one seroconverted to rotavirus). Two infants were excluded from the microbiota analyses (both non-responders who received probiotics alone) – one did not complete the study per protocol, while the other was excluded owing to a clerical error that led to the incorrect samples being extracted. Protocol deviations included the receipt of OPV outside of the study clinic, failure to visit the study clinic within the pre-specified window period, and the omission of ≥ 6 days of test article (supplement/placebo) consumption.

excluded if either the 6- or 10-week stool sample lacked sufficient material for nucleic acid extraction (< 200 mg)²⁴. Pre-vaccination samples for the 6- and 10-week vaccine doses were tested for each individual. In each sample, the presence of 31 enteropathogens was assessed using TACs, as described in Chapter 3.

Samples were considered eligible for the analysis if they were positive for PhHV and at least one RNA target (MS2 or other). The extraction and assay were repeated for samples that failed to meet these criteria. If repetition failed to yield an eligible assay, the sample was excluded from the analyses. A Ct value of 35 was used as a uniform cut-off for pathogen detection. If a pathogen target was present at a Ct of < 35 in the negative extraction control, the extraction batch was repeated. The order in which samples were extracted and analysed was randomised, and the nature of samples (responder vs non-responder, 6- vs 10-week sample, and study arm) was blinded. A no-template control was assessed approximately once per week to monitor laboratory contamination.

²⁴ Since the preparation of 200-mg aliquots, nucleic acid extraction, and TAC assays were carried out in tandem, there were several instances in which it became apparent that the 6- or 10-week stool sample collected from an infant lacked sufficient material for nucleic acid extraction only after the TAC assay for the corresponding pre-vaccination sample had been completed. In these instances, the completed assays were retained in the final analyses.

iii. Detection of Sabin polioviruses among enterovirus-positive samples

In addition to the assessment of enteropathogens using TACs, we tested for the presence of Sabin polioviruses in all samples positive for the pan-enterovirus target during the TAC assay. This was achieved using a multiplex real-time one-step RT-PCR assay for simultaneous detection of all three Sabin types that has previously been described (Taniuchi et al, 2015). Viral RNA was extracted from a 20% stool suspension in minimum essential medium using the QIAxtractor (Qiagen) – an automated nucleic acid purification system – with Vx reagents. Positive controls for each serotype, a negative control, and a no-template control were included in each run.

iv. Detection of rotavirus shedding

To examine the impact of the intestinal microbiota on Rotarix replication (or ‘take’), we quantified viral shedding in stool samples collected pre-vaccination and 4 and 7 days after administration of the 6-week dose of Rotarix using a real-time RT-PCR assay targeting the rotavirus VP6 gene. Infants were included in this analysis if their 6-week pre-vaccination sample had been successfully assayed via TAC. RNA was extracted from 200 µl of 10% stool suspension using the QIAxtractor (Qiagen) with Vx reagents. Reverse transcription was performed with random primers (Pd(N)6 hexamers; Invitrogen) using Moloney murine leukaemia virus reverse transcriptase (Superscript II MMLV RT; Invitrogen). Real-time PCR was then conducted on an ABI PRISM 7500 thermal cycler (Applied Biosystems) using the primers 5'-GACGGVGCRACTACATGGT-3' and 5'-GTCCAATTCATNCCTGGTG-3' (Sigma–Aldrich), which amplify a 379-bp region of the VP6 gene, and the probe 5'-(FAM)-CCACC-RAAYATGACRCCAGC-NGTA-NFQ-MGB-3' (Applied Biosystems) (Iturriza Gomara et al, 2002; EuroRotaNet, 2009). For calibration of the assay, a standard curve was generated from a ten-fold dilution series representing between 10^1 and 10^8 copies of a plasmid containing the VP6 gene of a rotavirus G1P[8] strain. Rotavirus shedding was defined as the presence of >100 viral copies per reaction either 4 or 7 days after vaccination. Infants shedding above this threshold on the day of vaccination were excluded.

4.3.3. 16S rRNA gene sequencing

In a subset of 170 infants (85 infants who seroconverted to rotavirus and 85 who failed to seroconvert), we assessed the composition of the bacterial microbiota by sequencing the V4 hypervariable region of the 16S rRNA gene in stool samples collected prior to the 6- and

10-week vaccine doses (340 samples in total). This sample size was not informed by a specific power calculation, but represented a pragmatic optimum given the trade-off between increased multiplexing and decreased sequencing depth (with the aim of achieving >2,000 sequences per sample). Infants were considered for the microbiota subset if eligible TAC assays (positive for PhHV and at least one RNA target) had been performed for both 6- and 10-week samples and if sufficient stool material for DNA extraction was available at both timepoints. During selection of the microbiota subset, we included all eligible individuals who received only placebos and all who received probiotics with zinc placebo. The remaining individuals were selected at random from recipients of both zinc and probiotics. This enabled us to assess the impact of probiotic supplementation on the microbiota as a secondary objective.

The laboratory procedures and bioinformatic analysis pipeline for sequencing of the 16S rRNA gene V4 region are described in Chapter 3. DNA extraction was carried out in the Department of Gastrointestinal Sciences at the Christian Medical College, Vellore. Samples were then transferred to the UK, where library preparation was carried out in the Imperial Biomedical Research Centre Genomics Facility at Hammersmith Hospital, London. Completed libraries were submitted to the Medical Research Council Clinical Sciences Centre Genomics Facility at Hammersmith Hospital for sequencing via Illumina MiSeq.

The order of samples for nucleic acid extraction, PCR, and sequencing was randomised. Samples were split across two MiSeq runs, each containing 190 samples (180 samples, eight extraction controls, and two no-template controls in the first run; 160 samples, six extraction controls, two no-template controls, and 22 technical replicates in the second). Technical replicates included sequencing replicates (using the same PCR product; $n = 8$); PCR replicates (using different reverse primers on the same MiSeq run; $n = 5$); replicates of both PCR and sequencing (using different reverse primers on different MiSeq runs; $n = 5$); and amplicons obtained from template DNA extracted without enzyme incubation (included for exploratory purposes only; $n = 5$).

4.3.4. Statistical analysis

i. Enteropathogen burden

The primary outcome of this study was the association between rotavirus seroconversion (as a categorical dependent variable) and the presence of ≥ 1 enteropathogen (excluding EAEC, enterovirus, and rotavirus) at 6 or 10 weeks of age, as determined via

logistic regression. Individuals were excluded from the analysis if they did not complete the study per protocol. As secondary outcomes, we compared the prevalence of individual pathogens, pathogen groups (bacterial, viral, eukaryotic, or any), mixed infections (defined as >1 enteropathogen), and concurrent diarrhoea (defined as ≥ 3 loose stools in a 24-hour period within the 7 days preceding vaccination) according to oral rotavirus vaccine response (serological responders vs non-responders and shedders vs non-shedders) at each dose using the χ^2 test or Fisher's exact test (the latter applied if there were <5 infected or uninfected individuals in either of the groups being compared). For prevalence estimates, 95% CIs were calculated using the Clopper–Pearson exact method (Clopper and Pearson, 1934). Rotavirus infections were excluded from analyses of mixed infections, pathogen groups, and pathogen count. To examine the impact of pathogen abundance on vaccine outcome, we compared Ct values for TAC targets present in at least 1% of the study population according to vaccine outcome using Wilcoxon's rank sum test; a one-unit decrease in Ct corresponds to a two-fold increase in target copy number. Wilcoxon's rank sum test was also used to compare the total pathogen count at each dose according to vaccine outcome. *P* values of 0.05 were considered significant. For comparisons of prevalence or abundance for individual TAC targets present in at least 1% of the study population, *P* values were adjusted via Benjamini–Hochberg FDR correction.

NPEVs were defined as the presence of an enterovirus in the absence of any Sabin viruses – a definition that assumes equal sensitivity of the pan-enterovirus and Sabin-specific assays. We compared NPEV prevalence at each dose according to rotavirus vaccine outcome using the χ^2 test. Since our assays did not allow distinction of samples positive for both Sabin viruses and NPEVs from those positive for just one of these viruses, infants harbouring any Sabin virus were excluded from comparisons of NPEV prevalence.

The association between rotavirus seroconversion and rotavirus take was assessed using the χ^2 test. To provide a preliminary indication of the impact of poliovirus replication following the birth dose of tOPV on the take of Sabin viruses administered at 6 weeks of age in this study population, we compared the shedding of enteroviruses (and of each Sabin serotype) at 10 weeks of age (i.e., 4 weeks after vaccination) according to whether ≥ 1 Sabin serotype was present in pre-vaccination samples (also using the χ^2 test)²⁵.

Across the 6- and 10-week doses, we assessed the cumulative impact of concurrent infections on the odds of seroconversion among individuals with eligible samples (positive

²⁵ A more in-depth analysis of factors impacting the immunogenicity and take of OPV in this study population is described in Chapter 5.

for PhHV and at least one RNA target at both doses) via logistic regression. For each pathogen group (bacterial, viral, eukaryotic, or any), we assessed the number of doses in which ≥ 1 infection was present (0, 1, or 2) as a categorical explanatory variable. As with the primary outcome analysis outlined above, we excluded EAEC, enterovirus, and rotavirus infections in these logistic regression models. Owing to the limited number of individuals infected with ≥ 1 viruses (excluding enterovirus) or ≥ 1 eukaryote at both doses (six and two, respectively), we combined across infants infected at one or both doses for these pathogen groups. All analyses were carried out in the programming language R.

ii. Bacterial microbiota: alpha diversity, beta diversity, and relative taxon abundance

After processing sequences via the bioinformatic pipeline described in Chapter 3, we observed a minimum of 3,726 sequences per sample. To standardise sequencing depth across samples, all comparisons were carried out at a depth of 3,500 sequences per sample. Our primary outcome during assessment of the bacterial microbiota was the association between within-sample diversity (OTU count and Shannon index as continuous dependent variables) and rotavirus vaccine response (seroconversion or shedding), as determined via linear regression. MiSeq run was included as a covariate to account for potential run-to-run variation. Separate analyses were performed to examine the association between rotavirus vaccine response and the number of OTUs within the phylum Proteobacteria at each dose. These phylum-specific OTU counts were determined using the Qiime functions *filter_taxa_from_otu_table.py* and *alpha_diversity.py*.

To assess divergence between bacterial communities (beta diversity) according to vaccine outcome, we calculated weighted and unweighted Unifrac distances between samples in ten rarefied OTU tables containing 3,500 sequences per sample. Mean Unifrac distances were visualised using principal coordinates analysis. The clustering of samples according to vaccine outcome and individual was assessed using the *adonis* function in the R package *vegan*, with 9,999 permutations stratified by MiSeq run. Unifrac distances between the 6- and 10-week samples from each individual were also used as an indicator of microbiota stability. These distances were compared according to vaccine outcome using Wilcoxon's rank sum test.

To identify potential phylum-, class-, and OTU-level differences in relative taxon abundance according to vaccine outcome, we used a non-parametric test based on a bootstrapped *t* statistic (implemented in the R package *permute*). For each taxon, a

distribution of 9,999 t statistics was generated by randomly permuting the outcome assigned to each sample. The non-parametric P value was then determined by calculating the proportion of permuted t statistics greater than or equal to the observed t statistic. Permutations were stratified by MiSeq run. Taxa were assessed if they were present in at least 2% of samples being compared. Since failure to respond to Rotarix was hypothesised *a priori* to be associated with an elevated abundance of Proteobacteria, P values of <0.05 (phylum- and class-level) or <0.005 (OTU-level) were considered significant for comparisons of this phylum in pre-vaccination samples. For all other taxa, P values for each level of comparison (phylum, class, or OTU) were adjusted via Benjamini–Hochberg FDR correction, and adjusted P values of <0.05 considered significant.

We prioritised the placebo-only and probiotics-only study arms in our 16S rRNA gene sequencing study to enable the impact of probiotic supplementation on the microbiota to be assessed as a secondary objective. Accordingly, we repeated the aforementioned analyses of alpha diversity, beta diversity, and relative taxon abundance to compare the composition of the bacterial microbiota between recipients of placebo alone ($n = 63$) with those who received probiotics with zinc placebo ($n = 69$).

iii. Random Forests

An overview of the Random Forests machine-learning algorithm is provided in Chapter 3. We fitted Random Forest models to discriminate infants according to: (i) rotavirus seroconversion; (ii) rotavirus take; and (iii) receipt of probiotics (probiotics only vs placebo only)²⁶. Separate analyses were carried out for 6- and 10-week samples. Models were implemented in the R package *randomForest*, with 1,000 trees per forest and all other parameters at their default values. A table of OTU relative abundances (determined at a depth of 3,500 sequences per sample) served as input, and OTUs were included as predictors if they were present in at least 2% of the samples being compared. For each comparison, mean out-of-bag classification accuracy and variable importance scores were determined over 100 iterations of the algorithm (analogous to the approach adopted by Subramanian et al [2014]). Significance of the model fit was assessed by generating 999 null models in which class labels were randomly assigned to the input variables and determining the proportion of null models with a classification accuracy greater than the mean accuracy of the fitted model.

²⁶ Since the *L. rhamnosus* GG probiotic strain appeared to correspond to a single OTU, we fitted separate Random Forest models either with or without this OTU in order to clarify the impact of probiotic supplementation on taxa other than the administered strain.

Since the tree-building process in Random Forests is tailored towards isolating the majority class, the algorithm has a tendency to perform poorly if classes are imbalanced (e.g., if the number of non-shedders greatly outweighs the number of shedders) (Chen et al, 2004). Under-sampling of the majority class has been shown to improve classification accuracy of minority class members when applying several machine-learning algorithms (Drummond and Holte, 2003), including Random Forests (Chen et al, 2004). Accordingly, if the majority class contained more than 60% of observations (as was the case in the analysis of vaccine take), we performed a separate analysis in which a random subset of samples from the majority class equal in size to the minority class was selected for each of the 100 iterations of the Random Forest algorithm.

iv. Variation between MiSeq runs

In the 13 samples sequenced in both MiSeq runs, we compared the number of OTUs, Shannon index, and phylum-, class-, and OTU-level relative abundances between replicates at a depth of 3,500 sequences per sample using Wilcoxon's signed-rank test. During comparisons of taxon abundance, *P* values for each set of comparisons (phylum-, class-, or OTU-level) were adjusted via Benjamini-Hochberg FDR correction. The clustering of sequencing replicates based on weighted and unweighted Unifrac distances was visualised using principal coordinates analysis, and the proportion of variance explained by MiSeq run and technical replicate assessed using the adonis function.

v. Sensitivity analyses

For comparisons of vaccine outcome with respect to enteropathogen burden, we performed sensitivity analyses in which: (i) lower Ct cut-offs (30, 25, and 20) were used to assess the presence or absence of TAC targets; (ii) samples lacking amplification of MS2 were excluded (to examine the impact of variation in the extraction efficiency of RNA targets); (iii) individuals positive for rotavirus-specific IgA at baseline were excluded (to account for the potential impact of prior rotavirus exposure on the outcomes of concurrent infection); and (iv) infants who experienced a robust response to vaccination (defined as both a four-fold rise in serum IgA titre and a post-vaccination titre of >90 U/ml) were compared with those who were seronegative post-vaccination (titre <20 U/ml) or experienced a fall in antibody titre between pre- and post-vaccination samples. A post-vaccination IgA titre of >90 U/ml has previously been suggested as an indicator of sustained protection against

severe rotavirus-associated gastroenteritis (Patel et al, 2013a), and was therefore used as to define a robust serological response here.

In each sensitivity analysis, we repeated the primary outcome comparison (considering the impact of harbouring ≥ 1 enteropathogen at 6 or 10 weeks on the odds of rotavirus seroconversion). We also compared the proportion of responders and non-responders infected with EAEC, ≥ 1 bacteria other than EAEC, enteroviruses, ≥ 1 virus other than enterovirus, ≥ 1 eukaryote, ≥ 1 infection of any kind (either including or excluding EAEC and enterovirus), or mixed infections at 6 weeks of age, 10 weeks of age, or at both timepoints. To examine the potential influence of treatment group on the primary outcome comparison, we fitted separate logistic regression models in which we added interaction terms between enteropathogen infection status and (i) zinc supplementation (zinc vs no zinc); (ii) probiotic supplementation (probiotics vs no probiotics); and (iii) study arm.

The same sensitivity analyses were performed (where applicable) to analyses of the bacterial microbiota. In each case, we examined discrepancies in alpha diversity (number of OTUs and Shannon index) and beta diversity (cluster significance based on adonis) according to seroconversion status at a depth of 3,500 sequences per sample.

4.4. Results

4.4.1. Oral rotavirus vaccine immunogenicity

Out of 551 individuals who completed the study per protocol, 173 (31%) seroconverted to rotavirus. Supplementation with either zinc or probiotics alone did not significantly improve rotavirus seroconversion rates compared with placebo (χ^2 , P values >0.05 ; Table 4.1); however, a modest but significant increase in seroconversion rate was observed among infants who received both supplements compared with those who received neither (χ^2 , $P = 0.036$) (Lazarus et al, manuscript in preparation).

4.4.2. Association between concurrent enteropathogens and seroconversion

i. Primary outcome

We assessed the presence of enteropathogens using TACs in a total 325 infants (Table 4.1), of whom 320 completed the trial per protocol. Among per-protocol individuals, we obtained eligible TAC assays (positive for PhHV and at least one RNA target) for 6-week samples in 313 infants, 10-week samples in 316 infants, and 6- and 10-week samples in 309

infants (including 155 [50%] responders and 154 [50%] non-responders). In infants with eligible assays at both timepoints, pathogen exposure did not significantly influence the odds of rotavirus seroconversion: ≥ 1 infection (excluding EAEC, enterovirus, and rotavirus) was observed at either 6 or 10 weeks of age in 70/154 (45%) non-responders and 78/155 (50%) responders (OR 1.22, 95% CI 0.78–1.90).

ii. Secondary outcomes

The prevalence of enteropathogens present in at least 1% of the study population at the time of each vaccine dose is displayed in Figures 4.2A and 4.2B by rotavirus seroconversion status (see Table 4.2 for the frequency and abundance of all TAC targets). EAEC and enteroviruses were the predominant infections at each timepoint. EAEC was identified in 150/313 (48%) 6-week samples and 180/316 (57%) 10-week samples, and was present in a similar proportion of responders and non-responders at each dose (χ^2 , P values >0.05). Enteroviruses were observed in 217/313 (69%) 6-week samples and 237/316 (75%) 10-week samples. The majority of enterovirus-positive samples (155/217 [71%] at 6 weeks and 176/235 [75%] at 10 weeks²⁷) contained one or more Sabin serotypes, reflecting the routine administration of OPV in this population, including at birth. The prevalence of enteroviruses, Sabin polioviruses, and NPEVs did not differ significantly between responders and non-responders at either dose (χ^2 , P values >0.05 ; Figure 4.3). However, enterovirus abundance was greater among responders than non-responders at 6 weeks of age (Ct, 29.5 ± 4.5 vs 31.0 ± 4.1 , Wilcoxon's rank sum, FDR-corrected $P = 0.042$; Table 4.2).

Other enteropathogens were generally more common in responders than non-responders at 6 weeks of age (Figure 4.2A), although no individual comparisons of prevalence or abundance were significant after FDR adjustment (Table 4.2). Combining across pathogens, ≥ 1 infection (excluding EAEC, enterovirus, and rotavirus) was observed in 56/156 (36%) responders and 37/157 (24%) non-responders at 6 weeks (χ^2 , $P = 0.017$). This discrepancy can be attributed primarily to bacterial pathogens, which were more common in responders than non-responders (40/156 [26%] vs 21/157 [13%]; χ^2 , $P = 0.006$), albeit only when EAEC was excluded (Figure 4.2A). These differences were no longer apparent at 10 weeks of age (Figure 4.2B). The proportion of individuals infected with ≥ 1 viral or ≥ 1 eukaryotic enteropathogen did not differ significantly between responders and non-responders at either dose.

²⁷ The detection of Sabin viruses was not performed for two 10-week samples that were positive for enterovirus.

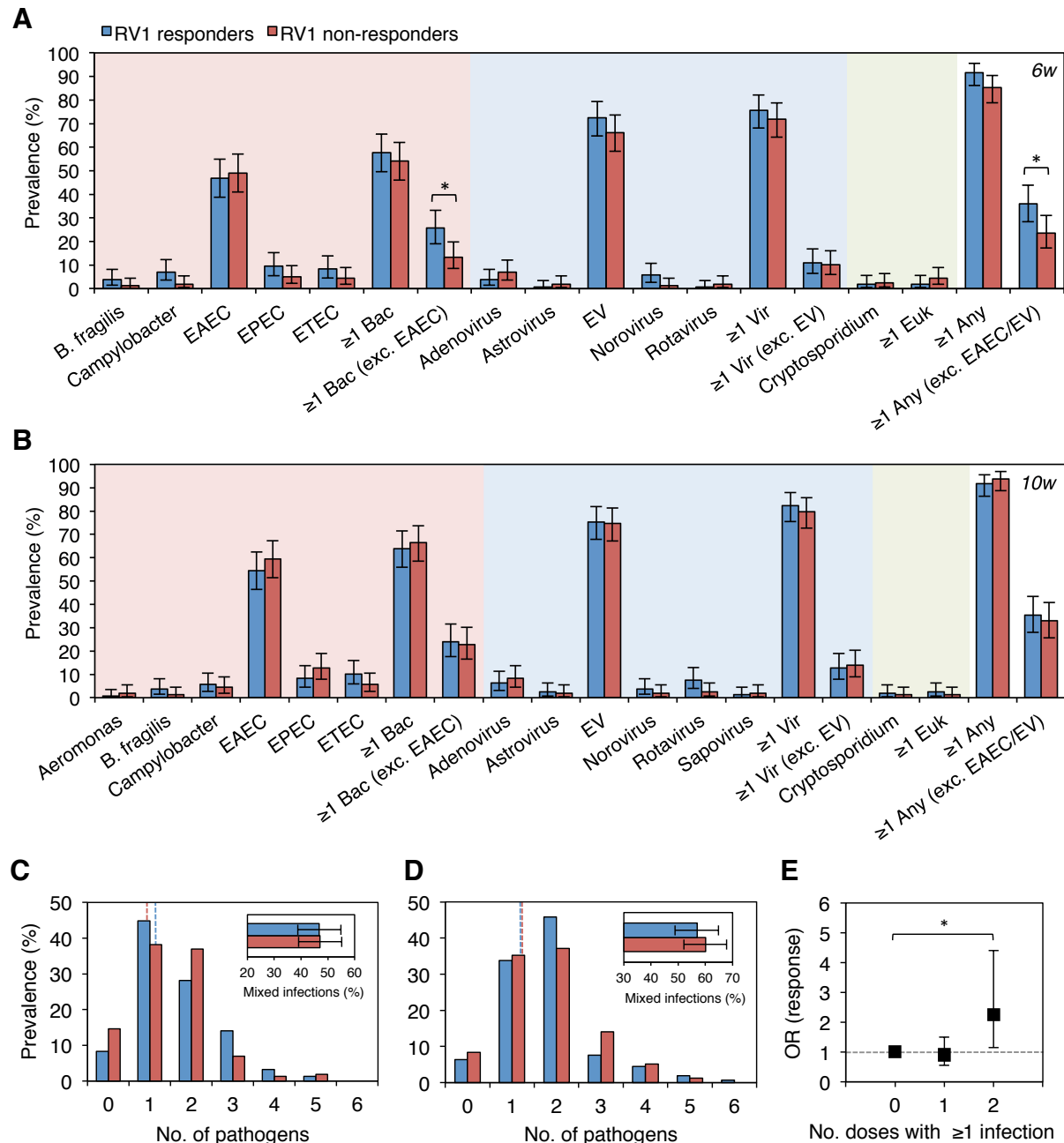


Figure 4.2. Association between concurrent pathogens and rotavirus vaccine immunogenicity. The prevalence of concurrent pathogens is displayed for Rotarix doses administered at (A) 6 weeks and (B) 10 weeks of age. Pathogens present in at least 1% of the study population are included. (C, D) The number of pathogens and prevalence of mixed infections are displayed at (C) 6 weeks and (D) 10 weeks of age. Mean pathogen counts are indicated by dotted lines. Rotaviruses were excluded from analyses of pathogen groups, mixed infections, and pathogen count. (E) Compared with individuals clear of infection at both doses, an increase in the odds of seroconversion was evident among individuals with ≥ 1 infection at both doses. ORs and 95% CIs are indicated. EAEC, enteroaggregative *Escherichia coli*; EPEC, enteropathogenic *E. coli*; ETEC, enterotoxigenic *E. coli*; EV, enterovirus; Euk, eukaryote; OR, odds ratio; RV1, Rotarix; Vir, virus; w, weeks.

Table 4.2. Prevalence and abundance of enteropathogens by rotavirus seroconversion status.

Target	6 weeks						10 weeks					
	Prevalence, n (%)			Abundance, mean Ct $\pm$ s.d.			Prevalence, n (%)			Abundance, mean Ct $\pm$ s.d.		
	sero+ (n = 156)	sero- (n = 157)	P	FDR	P	FDR	sero+ (n = 158)	sero- (n = 158)	P	FDR	sero+ (n = 158)	sero- (n = 158)
<i>Aeromonas</i>	1 (0.6)	0 (0.0)	0.498				1 (0.6)	3 (1.9)	0.623	0.962	35.0 $\pm$ 0.0	35.0 $\pm$ 0.2
<i>Bacteroides fragilis</i>	6 (3.8)	2 (1.3)	0.173	0.369	0.149	0.296	6 (3.8)	2 (1.3)	0.283	0.919	34.7 $\pm$ 1.5	34.9 $\pm$ 1.0
<i>Campylobacter</i> ^a	11 (7.1)	3 (1.9)	0.031	0.193	0.029	0.120	9 (5.7)	7 (4.4)	0.608	0.962	34.5 $\pm$ 2.3	34.6 $\pm$ 1.9
<i>Clostridium difficile</i>	0 (0.0)	3 (1.9)	0.248				0 (0.0)	2 (1.3)	0.498			
EAEC ^a	73 (46.8)	77 (49.0)	0.690	0.759	0.371	0.408	86 (54.4)	94 (59.5)	0.363	0.945	29.8 $\pm$ 6.2	28.5 $\pm$ 6.5
EPEC ^a	15 (9.6)	8 (5.1)	0.125	0.369	0.129	0.296	13 (8.2)	20 (12.7)	0.198	0.857	34.4 $\pm$ 2.4	34.1 $\pm$ 2.8
ETEC ^a	13 (8.3)	7 (4.5)	0.161	0.369	0.162	0.296	16 (10.1)	9 (5.7)	0.145	0.857	34.2 $\pm$ 2.8	34.5 $\pm$ 2.5
<i>Salmonella</i>	0 (0.0)	1 (0.6)	1.000				1 (0.6)	0 (0.0)	1.000			
<i>Shigella</i> / EIEC	0 (0.0)	0 (0.0)	1.000				1 (0.6)	0 (0.0)	1.000			
STEC ^a	0 (0.0)	3 (1.9)	0.248				1 (0.6)	2 (1.3)	1.000			
<i>Vibrio cholerae</i>	2 (1.3)	1 (0.6)	0.623				0 (0.0)	0 (0.0)	1.000			
Adenovirus ^a	6 (3.8)	11 (7.0)	0.217	0.369	0.231	0.363	10 (6.3)	13 (8.2)	0.516	0.962	34.6 $\pm$ 2.2	34.8 $\pm$ 0.7
Astrovirus	1 (0.6)	3 (1.9)	0.623	0.759	0.315	0.396	4 (2.5)	3 (1.9)	1.000	1.000	34.9 $\pm$ 1.1	34.8 $\pm$ 1.3
Enterovirus	113 (72.4)	104 (66.2)	0.235	0.369	0.004	0.042	119 (75.3)	118 (74.7)	0.897	1.000	29.5 $\pm$ 4.3	29.3 $\pm$ 4.4
Norovirus ^a	9 (5.8)	2 (1.3)	0.035	0.193	0.033	0.120	6 (3.8)	3 (1.9)	0.666	0.962	34.7 $\pm$ 1.7	34.8 $\pm$ 1.4
Rotavirus ^a	1 (0.6)	3 (1.9)	0.623	0.759	0.324	0.396	12 (7.6)	4 (2.5)	0.069	0.857	34.8 $\pm$ 1.1	35.0 $\pm$ 0.4
Sapovirus	1 (0.6)	0 (0.0)	0.498				2 (1.3)	3 (1.9)	1.000	1.000	35.0 $\pm$ 0.2	35.0 $\pm$ 0.3
<i>Cryptosporidium</i> ^a	3 (1.9)	4 (2.5)	1.000	1.000	0.715	0.715	3 (1.9)	2 (1.3)	1.000	1.000	35.0 $\pm$ 0.3	34.9 $\pm$ 1.4
<i>Giardia</i> ^a	0 (0.0)	3 (1.9)	0.248				1 (0.6)	1 (0.6)	1.000			

FDR correction and comparisons of abundance were carried out for pathogens present in at least 1% of the study population. Abbreviations: Ct, threshold cycle; EAEC, enteroaggregative *Escherichia coli*; EIEC, enteroinvasive *E. coli*; EPEC, enteropathogenic *E. coli*; ETEC, enterotoxigenic *E. coli*; FDR, adjusted by Benjamini-Hochberg false discovery rate correction; sero+, responders; sero-, non-responder; STEC, Shiga toxin-producing *E. coli*.

^a For enteropathogens assessed using multiple targets and /or in duplicate (see Table 3.1), the minimum Ct value was used for comparisons of pathogen abundance.

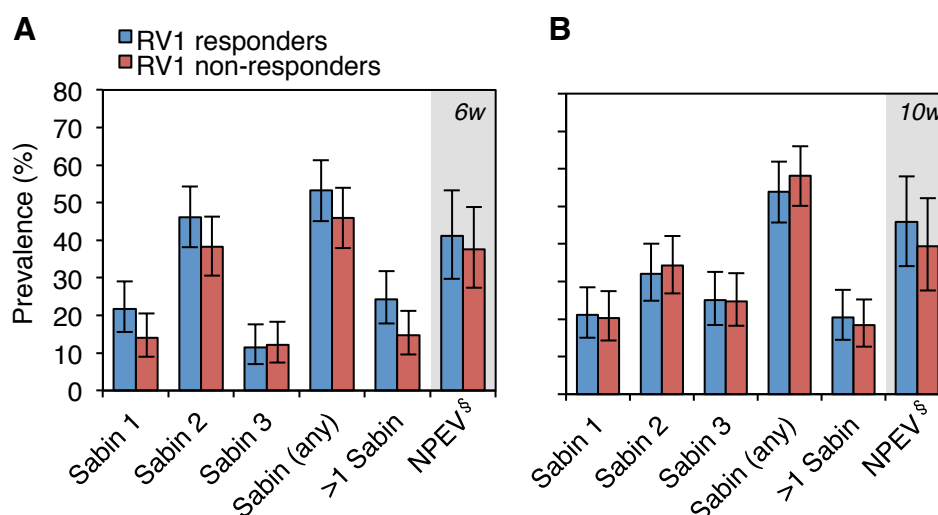


Figure 4.3. Association between enterovirus subgroups and rotavirus vaccine immunogenicity. Data are displayed for Rotarix doses administered at (A) 6 weeks and (B) 10 weeks of age. § comparison of enterovirus prevalence among infants negative for all Sabin serotypes. Abbreviation: NPEV, non-polio enterovirus; RV1, Rotarix; w, weeks.

We observed up to six pathogens per sample in this study. Mixed infections were observed in a similar proportion of rotavirus vaccine responders and non-responders at 6 weeks (73/156 [47%] vs 74/157 [47%]; χ^2 , $P = 0.862$) and 10 weeks (90/158 [57%] vs 95/158 [60%]; χ^2 , $P = 0.568$). Likewise, total pathogen count did not differ significantly between responders and non-responders at either dose (Wilcoxon's rank sum, P values >0.05 ; Figures 4.2C and 4.2D).

The infections observed in this study were largely subclinical. Concurrent diarrhoea was documented in $<5\%$ of individuals at both 6 and 10 weeks of age. This proportion did not differ significantly between responders and non-responders at either 6 weeks (6/173 [3%] vs 12/378 [3%]; χ^2 , $P = 0.857$) or 10 weeks (7/173 [4%] vs 17/378 [4%]; χ^2 , $P = 0.810$).

iii. Pathogen prevalence over successive Rotarix doses

Compared with individuals clear of infections at both 6 and 10 weeks, we observed a significant increase in the odds of seroconversion when ≥ 1 concurrent infection was present at both doses (OR 2.25, 95% CI 1.15–4.41; Figure 4.2E) – an effect that was absent among individuals infected during only one of the two vaccine doses (OR 0.91, 95% CI 0.55–1.50). A similar trend was apparent among infants harbouring bacterial infections at one or both doses (OR 1.32, 95% CI 0.76–2.31 and OR 1.98, 95% CI 0.93–4.23, respectively). The presence of ≥ 1 virus or ≥ 1 eukaryote during at least one dose did not significantly impact the odds of seroconversion (OR 0.82, 95% CI 0.48–1.41 and OR 0.85, 95% CI 0.28–2.58, respectively).

4.4.3. Dose 1 rotavirus vaccine take

Among the 313 per-protocol infants in which 6-week pre-vaccination samples were successfully assayed via TAC, the measurement of rotavirus shedding on the day of and 4 and 7 days after vaccination was carried out in 288 (92%) – stool samples were lacking at one or more timepoint for the remaining individuals. Rotavirus shedding at >100 copies per reaction on day 0 was observed in 10/288 (3%) individuals. In the remaining infants, we observed shedding at up to 4.2 million copies per reaction in the week following immunisation, although shedding at >1,000 copies per reaction was rare (Figure 4.4B). Shedding at >100 copies per reaction at either day 4 or 7 was observed in 66/278 (24%) infants. Rotavirus seroconversion was observed in 46/66 (80%) shedders and 95/212 (45%) non-shedders (χ^2 , $P < 0.001$).

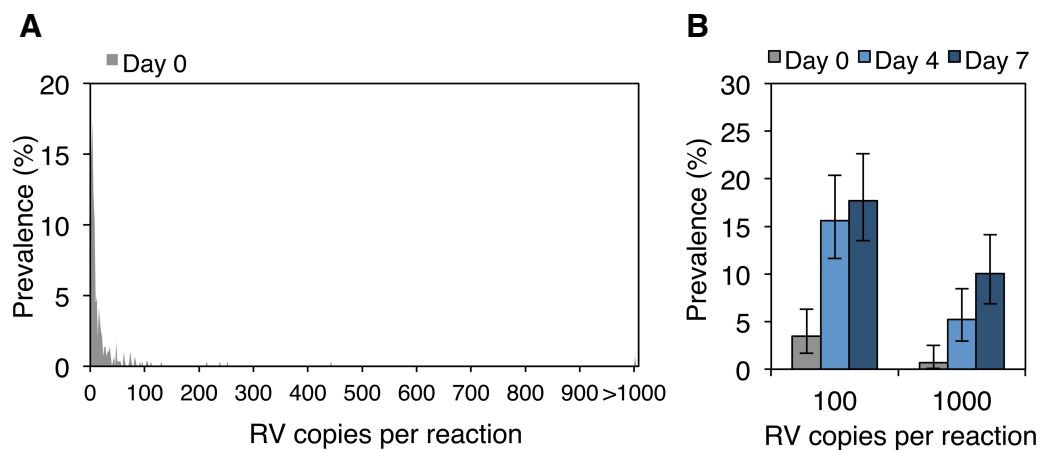


Figure 4.4. Rotavirus shedding at 6 weeks of age. (A) Rotavirus shedding in pre-vaccination samples. (B) The proportion of individuals shedding on the day of and 4 and 7 days after the first dose of Rotarix. Abbreviation: RV, rotavirus.

4.4.4. Association between concurrent enteropathogens and vaccine take

After adjusting for multiple comparisons, we observed no association between the prevalence of enteropathogens at 6 weeks of age and dose 1 Rotarix take (Figure 4.5A; Table 4.3). Pathogen count was comparable in shedders and non-shedders (1.7 ± 0.9 [mean $\pm$ s.d.] vs 1.5 ± 1.0 ; Wilcoxon's rank sum, $P = 0.282$), as was the prevalence of mixed infections (35/66 [53%] vs 100/212 [47%]; χ^2 , $P = 0.405$). However, enterovirus abundance was significantly greater in shedders than non-shedders (Ct, 28.8 ± 4.3 vs 30.5 ± 4.3 ; Wilcoxon's rank sum, FDR-corrected $P = 0.046$; Table 4.3) – an effect that appears to be attributable in part to a greater prevalence of Sabin viruses in the pre-vaccination samples of shedders (Figure 4.6A). By contrast, at 10 weeks of age, enteroviruses were observed in 39/65 (60%) and 166/210 (79%) rotavirus shedders and non-shedders, respectively (χ^2 , FDR-corrected $P = 0.014$). This

discrepancy encompasses differences in the prevalence of Sabin viruses (present in 29/64 [45%] and 122/209 [58%] shedders and non-shedders, respectively; χ^2 , $P = 0.066$) as well as NPEVs (9/35 [26%] vs 43/87 [49%]; χ^2 , $P = 0.017$; Figure 4.6B). We also observed a significant reduction in the shedding of Sabin viruses at 10 weeks of age among individuals harbouring ≥ 1 Sabin serotype at 6 weeks, potentially indicative of interference with the take of OPV given at 6 weeks of age by poliovirus replication following the birth dose of OPV (Figure 4.6C). Aside from a degree of residual rotavirus shedding, no other discrepancies in the prevalence of TAC targets were evident among rotavirus shedders versus non-shedders at 10 weeks of age (Figure 4.5B; Table 4.3). Diarrhoea in the week preceding vaccination was observed in 3/66 [5%] and 8/212 [4%] of shedders and non-shedders, respectively (Fisher's test, $P = 0.726$).

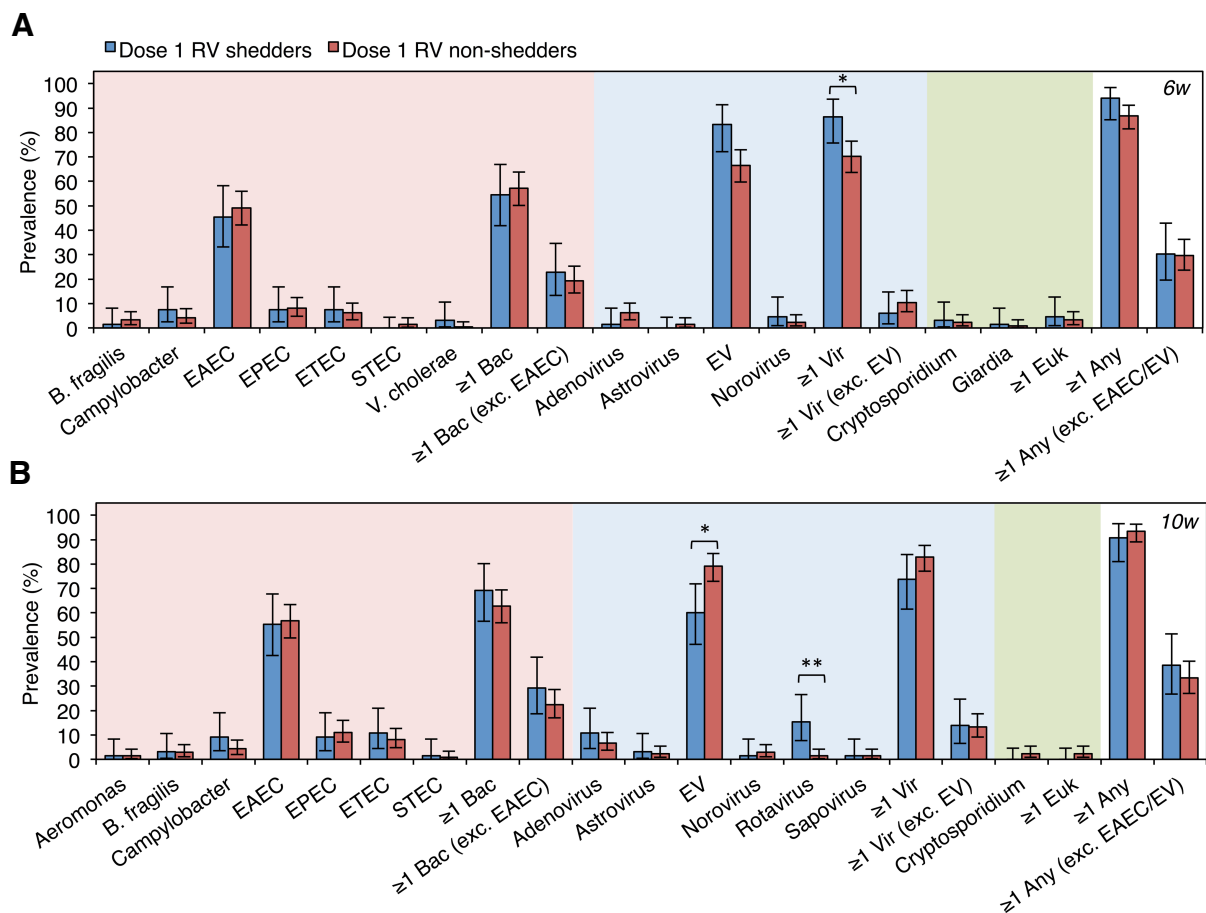


Figure 4.5. Association between concurrent pathogens and dose 1 rotavirus vaccine take. Data are displayed for samples collected at (A) 6 weeks and (B) 10 weeks of age. Pathogens present in at least 1% of the study population are included. Rotaviruses were excluded from analyses of pathogen groups, mixed infections, and pathogen count. * $P < 0.05$; ** $P < 0.005$. Abbreviations: Bac, bacteria; EAEC, enteroaggregative *Escherichia coli*; EPEC, enteropathogenic *E. coli*; ETEC, enterotoxigenic *E. coli*; Euk, eukaryote; EV, enterovirus; RV, rotavirus; STEC, Shiga toxin-producing *E. coli*; Vir, virus; w, weeks.

Table 4.3. Prevalence and abundance of enteropathogens by dose 1 oral rotavirus vaccine take.

Target	6 weeks						10 weeks									
	Prevalence, n (%)			Abundance, mean Ct ± s.d.			Prevalence, n (%)			Abundance, mean Ct ± s.d.						
	shed+ (n = 66)	shed- (n = 212)	P	FDR P	shed+ (n = 66)	shed- (n = 212)	P	FDR P	shed+ (n = 65)	shed- (n = 210)	P	FDR P	shed+ (n = 65)	shed- (n=210)	P	FDR P
<i>Aeromonas</i>	0 (0.0)	1 (0.5)	1.000						1 (1.5)	3 (1.4)	1.000	1.000	35.0 ± 0.0	35.0 ± 0.1	0.962	0.962
<i>Bacteroides fragilis</i>	1 (1.5)	7 (3.3)	0.685	0.890	34.9 ± 0.7	34.8 ± 1.2	0.453	0.736	2 (3.1)	6 (2.9)	1.000	1.000	34.9 ± 0.8	34.8 ± 1.5	0.944	0.962
<i>Campylobacter</i> ^a	5 (7.6)	9 (4.2)	0.280	0.890	34.5 ± 2.0	34.8 ± 1.2	0.265	0.706	6 (9.2)	9 (4.3)	0.125	0.583	34.2 ± 2.9	34.7 ± 1.8	0.119	0.554
<i>Clostridium difficile</i>	0 (0.0)	1 (0.5)	1.000						0 (0.0)	0 (0.0)	1.000					
EAEC ^a	30 (45.5)	104 (49.1)	0.609	0.890	30.3 ± 6.5	30.6 ± 6.3	0.813	0.880	36 (55.4)	119 (56.7)	0.887	1.000	28.3 ± 7.1	29.4 ± 6.2	0.360	0.720
EPEC ^a	5 (7.6)	17 (8.0)	0.907	1.000	34.4 ± 2.5	34.5 ± 2.1	0.925	0.925	6 (9.2)	23 (11.0)	0.693	1.000	34.3 ± 2.4	34.2 ± 2.5	0.688	0.962
ETEC ^a	5 (7.6)	13 (6.1)	0.677	0.890	34.5 ± 2.2	34.6 ± 1.9	0.672	0.880	7 (10.8)	17 (8.1)	0.504	1.000	34.1 ± 3.0	34.3 ± 2.7	0.508	0.889
<i>Salmonella</i>	0 (0.0)	1 (0.5)	1.000						1 (1.5)	0 (0.0)	0.236					
<i>Shigella</i> / EIEC	0 (0.0)	0 (0.0)	1.000						0 (0.0)	1 (0.5)	1.000					
STEC ^a	0 (0.0)	3 (1.4)	1.000	1.000	35.0 ± 0.0	34.9 ± 1.0	0.335	0.706	1 (1.5)	2 (1.0)	0.556	1.000	34.9 ± 0.5	34.9 ± 0.6	0.695	0.962
<i>Vibrio cholerae</i>	2 (3.0)	1 (0.5)	0.141	0.865	35.0 ± 0.1	35.0 ± 0.4	0.082	0.533	0 (0.0)	0 (0.0)	1.000					
Adenovirus ^a	1 (1.5)	13 (6.1)	0.200	0.865	35.0 ± 0.0	34.8 ± 1.1	0.128	0.555	7 (10.8)	14 (6.7)	0.276	0.968	34.7 ± 1.6	34.7 ± 1.8	0.313	0.720
Astrovirus	0 (0.0)	3 (1.4)	1.000	1.000	35.0 ± 0.0	34.8 ± 1.6	0.335	0.706	2 (3.1)	5 (2.4)	0.670	1.000	34.8 ± 1.5	34.8 ± 1.2	0.759	0.962
Enterovirus	55 (83.3)	141 (66.5)	0.009	0.115	28.8 ± 4.3	30.5 ± 4.3	0.004	0.046	39 (60.0)	166 (79.0)	0.002	0.014	30.3 ± 4.8	29.3 ± 4.2	0.100	0.554
Norovirus ^a	3 (4.5)	5 (2.4)	0.400	0.890	34.9 ± 0.3	34.8 ± 1.7	0.380	0.706	1 (1.5)	6 (2.9)	1.000	1.000	34.8 ± 1.2	34.8 ± 1.5	0.563	0.720
Rotavirus ^a	1 (1.5)	1 (0.5)	0.419						10 (15.4)	3 (1.4)	<0.001	0.001	34.7 ± 1.1	34.9 ± 0.8	<0.001	<0.001
Sapovirus	0 (0.0)	1 (0.5)	1.000						1 (1.5)	3 (1.4)	1.000	1.000	35.0 ± 0.0	35.0 ± 0.3	0.962	0.962
<i>Cryptosporidium</i> ^a	2 (3.0)	5 (2.4)	0.672	0.890	34.8 ± 1.7	34.9 ± 1.2	0.764	0.880	0 (0.0)	5 (2.4)	0.595	1.000	35.0 ± 0.0	34.9 ± 1.2	0.212	0.720
<i>Giardia</i> ^a	1 (1.5)	2 (0.9)	0.558	0.890	35.0 ± 0.2	34.9 ± 0.7	0.706	0.880	0 (0.0)	1 (0.5)	1.000					

FDR correction and comparisons of abundance were carried out for pathogens present in at least 1% of the study population. Abbreviations: Ct, threshold cycle; EAEC, enteroaggregative *Escherichia coli*; EIEC, enteroinvasive *E. coli*; EPEC, enteropathogenic *E. coli*; ETEC, enterotoxigenic *E. coli*; FDR, adjusted by Benjamini-Hochberg false discovery rate correction; shed+, shedders; shed-, non-shedders; STEC, Shiga toxin-producing *E. coli*.

^a For enteropathogens assessed using multiple targets and/or in duplicate (see Table 3.1), the minimum Ct value was used for comparisons of pathogen abundance.

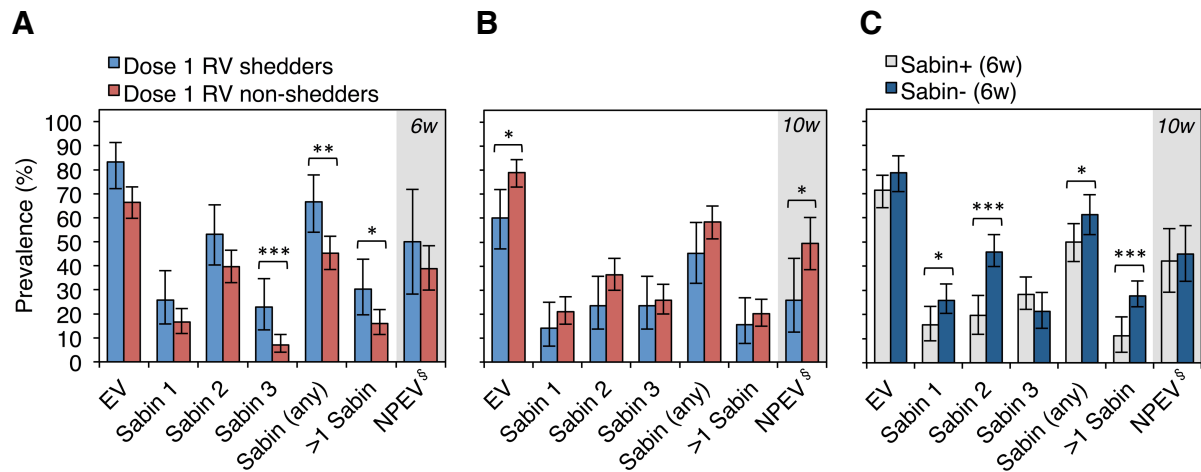


Figure 4.6. Association between enterovirus subgroups and dose 1 vaccine take. Viral shedding is displayed at (A) 6 weeks and (B) 10 weeks of age according to the shedding of rotavirus in the week following the first dose of Rotarix. (C) Viral shedding at 10 weeks of age is displayed according to whether or not ≥ 1 Sabin serotype was present at 6 weeks of age. § comparison of enterovirus prevalence among infants negative for all Sabin serotypes; * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$. Abbreviations: EV, enterovirus; NPEV, non-polio enterovirus; RV, rotavirus; Sabin+, positive for ≥ 1 Sabin serotype; Sabin-, negative for all Sabin serotypes; w, weeks.

4.4.5. Association between bacterial microbiota composition and rotavirus vaccine outcome

i. Composition of the bacterial microbiota

We sequenced the V4 region of the 16S rRNA gene in 340 samples from 170 individuals (of which two were excluded from the final analyses, as detailed in Table 4.1). After quality filtering, we obtained $25,254 \pm 13,091$ (mean $\pm$ s.d.) sequences per sample, encompassing 153 97%-identity OTUs. The overall composition of the bacterial microbiota was broadly similar at 6 and 10 weeks of age (Figure 4.7A), and a small number of taxa were dominant across samples (Figure 4.7B).

ii. Alpha diversity

We observed no significant differences in OTU count – across all taxa (Figure 4.8A) or within the phylum Proteobacteria (Figure 4.8B) – or Shannon index (Figure 4.8C) between rotavirus vaccine responders and non-responders at either dose (linear regression, P values > 0.05). However, individuals who shed rotavirus after the 6-week Rotarix dose harboured a greater number of OTUs at the time of vaccination (linear regression, $P = 0.007$; Figure 4.8D) – a discrepancy that was still evident among these individuals at 10 weeks of age (linear

regression, $P = 0.027$). This observation was attributable in part to a greater number of OTUs in the phylum Proteobacteria among shedders (Figure 4.8E). No significant differences in Shannon index were observed between shedders and non-shedders at either 6 or 10 weeks of age (P values >0.05 ; Figure 4.8F). A summary of alpha diversity analyses is provided in Table 4.4.

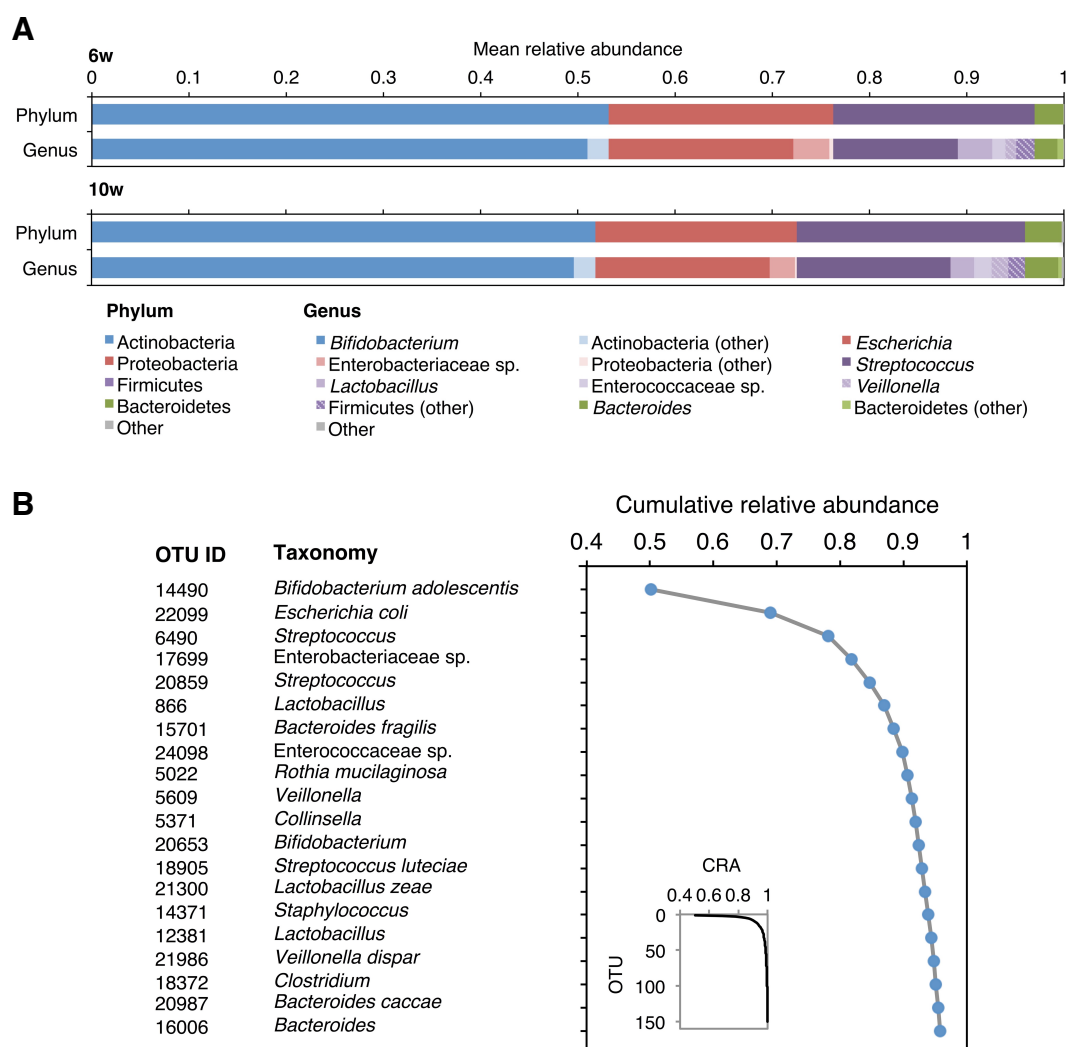


Figure 4.7. Composition of the bacterial microbiota among vaccine recipients. (A) Phylum- and genus-level composition of the bacterial microbiota at 6 and 10 weeks of age. (B) Top 20 OTUs based on mean relative abundance among 6-week samples. The abundance distribution of all OTUs is displayed in the inset. When ranked by decreasing mean relative abundance, 18 OTUs accounted for $>95\%$ of the observed microbiota in these samples. Abbreviations: CRA, cumulative relative abundance; OTU, 97%-identity operational taxonomic unit; w, weeks.

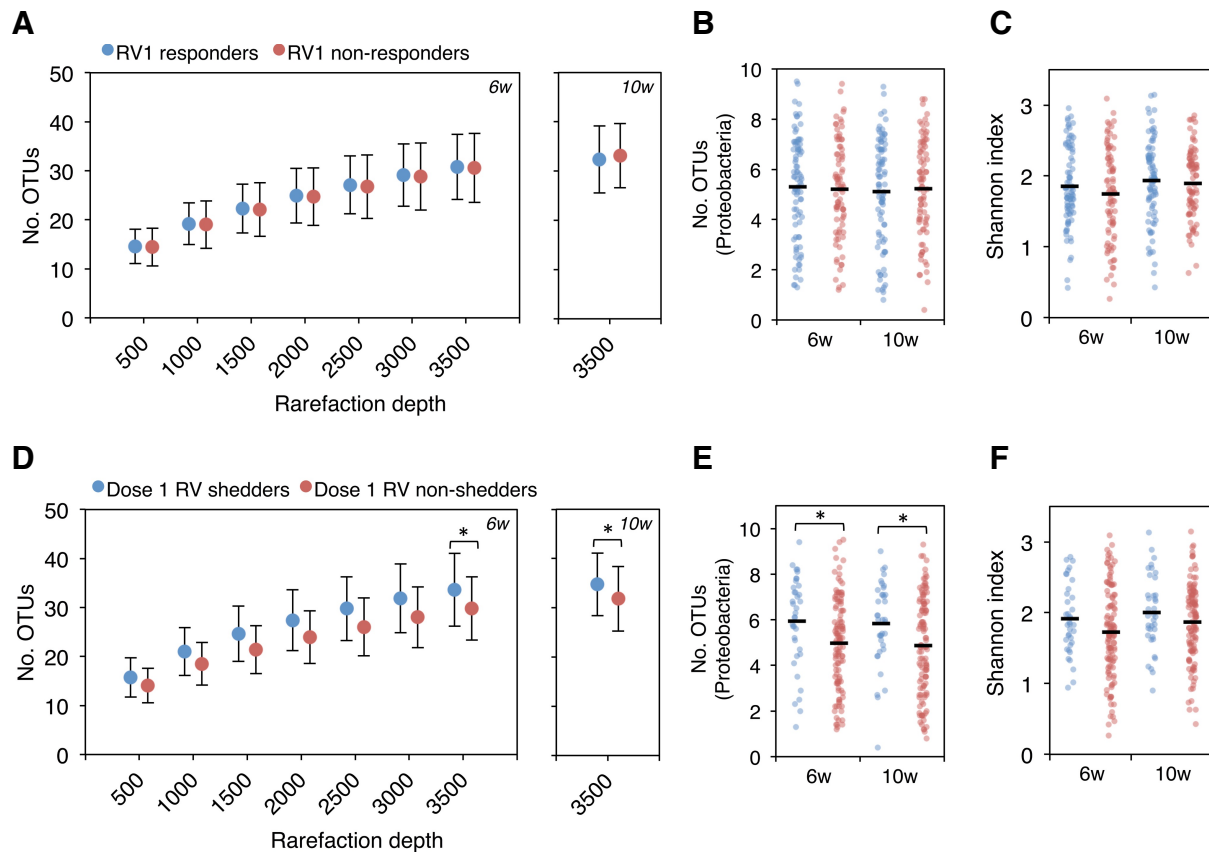


Figure 4.8. Association between microbiota diversity and rotavirus vaccine response. (A) Mean OTU count ($\pm$ s.d.) at multiple rarefaction depths, (B) OTU count within the phylum Proteobacteria, and (C) Shannon index according to rotavirus seroconversion status. Proteobacteria-specific OTU counts and Shannon index were calculated at a depth of 3,500 sequences per sample. Mean values for these measures in each group are indicated. (D, E, F) The same comparisons are displayed with respect to Rotarix take following the first (6-week) dose of vaccine. * $P < 0.05$. Abbreviations: OTU, 97%-identity operational taxonomic unit; RV, rotavirus; RV1, Rotarix; w, weeks.

Table 4.4. Summary of alpha diversity analyses.

Comparison	Age (w)	N ^a	N ^b	Number of OTUs			Number of OTUs (Proteobacteria)			Shannon index		
				Effect size	SE	P	Effect size	SE	P	Effect size	SE	P
MiSeq run	-	13	13	-	-	0.152	-	-	-	-	-	<0.001
RV1 seroconversion	6	85	83	0.078	1.066	0.942	0.026	0.313	0.935	0.072	0.092	0.436
	10	85	83	-0.612	1.029	0.553	-0.054	0.312	0.863	0.020	0.082	0.804
Dose 1 RV1 take	6	37	116	3.558	1.298	0.007	0.841	0.387	0.031	0.111	0.115	0.338
	10	37	116	2.759	1.234	0.027	0.896	0.387	0.022	0.164	0.101	0.105
Probiotics ^c	6	67	63	2.206	1.173	0.062	-	-	-	0.110	0.107	0.307
	10	67	63	1.105	1.178	0.350	-	-	-	-0.021	0.094	0.826
Sensitivity analyses												
RV1 seroconversion (titre >90 U/ml)	6	36	69	0.454	1.404	0.747	-	-	-	0.098	0.128	0.446
	10	36	69	0.159	1.380	0.909	-	-	-	0.016	0.111	0.887
RV1 seroconversion (IgA-)	6	67	57	0.017	1.228	0.989	-	-	-	-0.004	0.104	0.968
	10	67	57	-0.772	1.198	0.520	-	-	-	0.008	0.095	0.937

With the exception of MiSeq run, which was assessed via Wilcoxon's signed-rank test, the impact of each factor on the specified measure of alpha diversity was assessed via linear regression, with MiSeq run included as a covariate. Abbreviations: IgA-, analysis restricted to individuals negative for rotavirus-specific IgA at baseline; OTU, 97%-identity operational taxonomic unit; RV, rotavirus; RV1, Rotarix; SE, standard error; w, weeks.

^a Number of responders/shedders/probiotics recipients/Miseq run 1 samples.

^b Number of non-responders/non-shedders/placebo recipients/Miseq run 2 samples.

^c Compares probiotics-only with placebo recipients.

iii. Beta diversity

We observed no significant difference in bacterial community composition according to seroconversion status based on unweighted or weighted Unifrac distances (adonis, P values >0.05; Figure 4.9). Although a modest shift in composition was evident in the pre-vaccination microbiota according to whether or not infants shed rotavirus after the 6-week Rotarix dose based on unweighted Unifrac (adonis, $P = 0.032$; Figure 4.9C), this accounted for only a small portion of the variance among samples ($R^2 = 0.012$).

The 6- and 10-week samples of each individual tended to cluster together based on unweighted Unifrac (adonis, $R^2 = 0.635$ and $P < 0.001$; Figure 4.10A) and weighted Unifrac (adonis, $R^2 = 0.644$ and $P < 0.001$). When within-subject Unifrac distances were used as a measure of microbiota stability, we observed no significant differences in stability according to seroconversion status (Wilcoxon's rank sum, P values 0.889 and 0.557 for unweighted and weighted Unifrac, respectively; Figure 4.10B) or dose 1 take (Wilcoxon's rank sum, P values 0.644 and 0.527 for unweighted and weighted Unifrac, respectively; Figure 4.10C). A summary of beta diversity analyses is provided in Table 4.5.

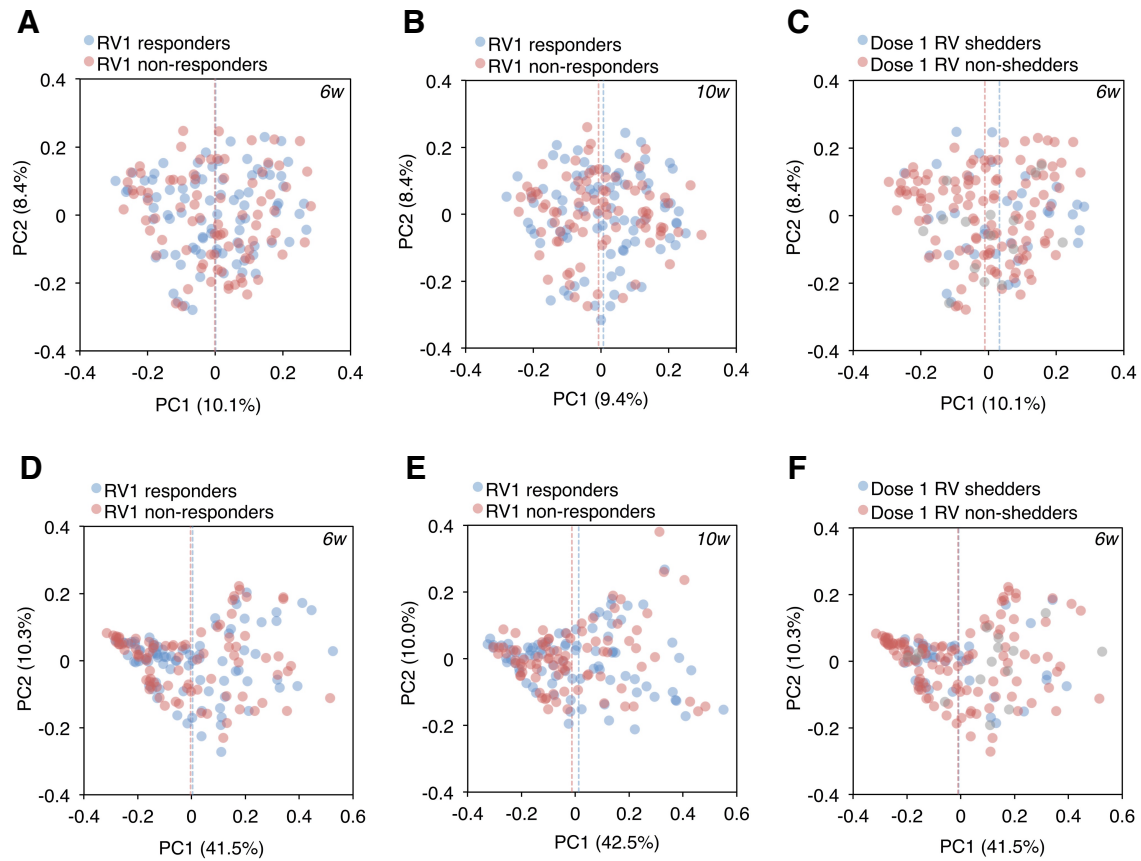


Figure 4.9. Clustering of samples according to rotavirus vaccine response. Unweighted Unifrac distances between samples, visualised via principal coordinates analysis, are displayed at (A) 6 weeks and (B) 10 weeks according to seroconversion status, and (C) according to rotavirus take following the 6-week vaccine dose. Mean values for principal coordinate 1 are indicated by dotted lines. (D, E, F) The same comparisons are displayed for weighted Unifrac distances. Abbreviations: PC, principal coordinate; RV, rotavirus; RV1, Rotarix.

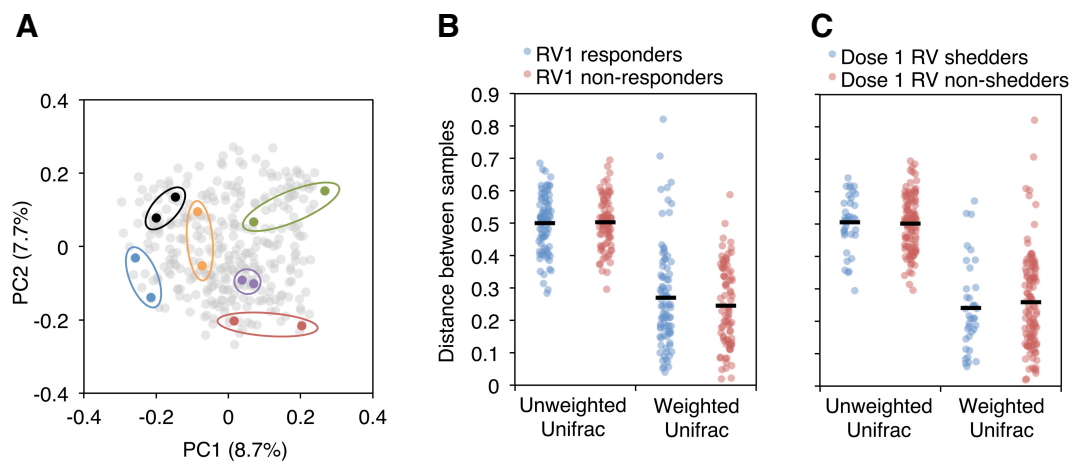


Figure 4.10. Association between microbiota stability and rotavirus vaccine response. (A) Clustering of 6- and 10-week samples within each individual based on unweighted Unifrac distances. Six sample pairs are highlighted. (B, C) Within-subject Unifrac distances, used as an indicator of microbiota stability, are displayed according to (B) seroconversion status and (C) vaccine take. Mean values in each group are indicated. Abbreviations: PC, principal coordinate; RV, rotavirus; RV1, Rotarix.

Table 4.5. Summary of beta diversity analyses.

Comparison	Age (w)	Unweighted Unifrac		Weighted Unifrac	
		R ²	P	R ²	P
MiSeq run ^a	-	0.017	0.992	0.006	0.946
Technical replicate ID ^a	-	0.858	<0.001	0.987	<0.001
Study ID	-	0.635	<0.001	0.644	<0.001
RV1 seroconversion	6	0.006	0.605	0.003	0.733
	10	0.005	0.815	0.004	0.533
Dose 1 RV1 take	6	0.012	0.032	0.015	0.149
	10	0.015	0.003	0.006	0.452
Probiotics ^b	6	0.012	0.021	0.008	0.322
	10	0.013	0.014	0.003	0.741
Sensitivity analyses					
RV1 seroconversion (titre >90 U/ml)	6	0.007	0.920	0.003	0.814
	10	0.007	0.868	0.008	0.423
RV1 seroconversion (IgA-)	6	0.007	0.758	0.009	0.348
	10	0.006	0.863	0.003	0.718

See Table 4.4 for details regarding the number of infants included in each comparison. The impact of each factor on the specified measure of beta diversity was assessed using the function *adonis* in the R package *vegan*; 9,999 permutations were included in each comparison. Abbreviations: IgA-, analysis restricted to individuals negative for rotavirus-specific IgA at baseline; OTU, 97%-identity operational taxonomic unit; RV1, Rotarix; w, weeks.

^a Comparison includes 13 samples sequenced in both the first and second MiSeq runs.

^b Compares probiotics-only with placebo recipients.

iv. Taxon relative abundance

No significant phylum-, class-, or OTU-level differences in taxon relative abundance were observed according to rotavirus seroconversion status – either within the phylum Proteobacteria (Figure 4.11A) or across all taxa (non-parametric *t* test, FDR-corrected *P* values >0.05). Similar results were observed with respect to vaccine take (Figure 4.11B) – one OTU in the phylum Proteobacteria (family Enterobacteriaceae) was significantly more abundant in the pre-vaccination samples of shedders compared with non-shedders (Table 4.6); however, no other phylum-, class-, or OTU-level differences in abundance distinguished these infants. At 10 weeks of age, an increase in the relative abundance of Bacteroidetes and Verrucomicrobia (specifically the classes Bacteroidia and Verrucomicrobiae) was observed among infants who shed rotavirus following the 6-week Rotarix dose compared with non-shedders (Table 4.6). These findings were driven in part by the enrichment of an OTU classified as *Parabacteroides distasonis* among shedders (Table 4.6).

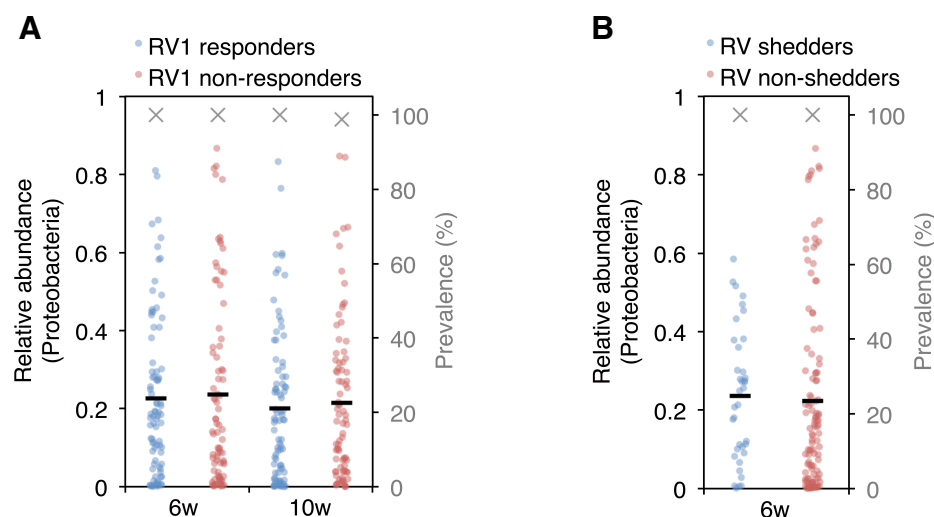


Figure 4.11. Pre-vaccination Proteobacteria abundance by rotavirus vaccine response. Results are displayed according to (A) seroconversion status and (B) dose 1 vaccine take. Mean relative abundance for each group is indicated by a horizontal line, while prevalence is indicated by a cross. Abbreviation: RV, rotavirus; RV1, Rotarix; w, weeks.

Table 4.6. Summary of significant differences in taxon abundance.

Level	Taxonomic classification	OTU ID	Age (w)	Relative abundance (%) in group 1 (mean $\pm$ s.d.)	Relative abundance (%) in group 2 (mean $\pm$ s.d.)	<i>P</i>	FDR <i>P</i>
RV1 seroconversion							
NS							
Dose 1 RV1 take				<i>Non-shedders</i>	<i>Shedders</i>		
OTU	Enterobacteriaceae sp.	745	6	0.003 $\pm$ 0.010	0.010 $\pm$ 0.018	0.004 ^a	-
P	Verrucomicrobia		10	0.007 $\pm$ 0.053	0.600 $\pm$ 3.064	0.003	0.019
P	Bacteroidetes		10	2.730 $\pm$ 8.129	7.434 $\pm$ 14.917	0.009	0.027
C	Verrucomicrobiae		10	0.007 $\pm$ 0.053	0.600 $\pm$ 3.064	0.002	0.019
C	Bacteroidia		10	2.730 $\pm$ 8.129	7.434 $\pm$ 14.917	0.008	0.046
OTU	<i>Parabacteroides distasonis</i>	23512	10	0.011 $\pm$ 0.074	0.431 $\pm$ 1.242	0.0003	0.039
Probiotics				<i>Placebo</i>	<i>Probiotics</i>		
OTU	<i>Lactobacillus zeae</i>	21300	6	0.045 $\pm$ 0.182	0.659 $\pm$ 1.008	0.0001	0.014
OTU	<i>Lactobacillus zeae</i>	21300	10	0.116 $\pm$ 0.315	0.912 $\pm$ 1.540	0.0001	0.013
OTU	<i>Actinomyces</i>	21955	10	0.0005 $\pm$ 0.004	0.013 $\pm$ 0.032	0.0002	0.013

Groups were compared using a non-parametric *t* test stratified by MiSeq run. Abbreviations: C, class; FDR, adjusted by Benjamini–Hochberg false discovery rate correction; NS, no significant differences; OTU, 97%-identity operational taxonomic unit; P, phylum; RV1, Rotarix; w, weeks.

^a *P* value was not adjusted as taxon was within the phylum Proteobacteria, hypothesised *a priori* to be associated with vaccine outcome.

Random Forest models based on OTU abundance data failed to accurately distinguish infants according to rotavirus seroconversion status (Figure 4.12A). Across 100 iterations of the algorithm, Rotarix response was predicted with a mean accuracy of 43.4% and 45.7% based on OTU abundances at 6 and 10 weeks, respectively (baseline accuracy: 50.6%). The models conferred no significant improvement in predictive accuracy compared with null models in which outcomes were randomly assigned to the input data (P values >0.05). When using the full dataset (comprising 37 shedders and 116 non-shedders), Random Forests similarly failed to distinguish infants according to vaccine take following the 6-week dose of Rotarix (Figure 4.12B; P values >0.05), with the majority of shedders ($>93\%$ on average) being misclassified as non-shedders using both 6- and 10-week data. However, the algorithm performed somewhat better when a balanced dataset comprising all shedders and 37 randomly selected non-shedders was used for each iteration of the Random Forest algorithm. This led to a predictive accuracy of 61.4% and 60.9% for 6- and 10-week data, respectively (baseline accuracy: 50.0%; P values 0.038 and 0.040; Figure 4.12C). Variables with mean importance scores of >0.001 for the latter models are displayed in Figure 4.13.

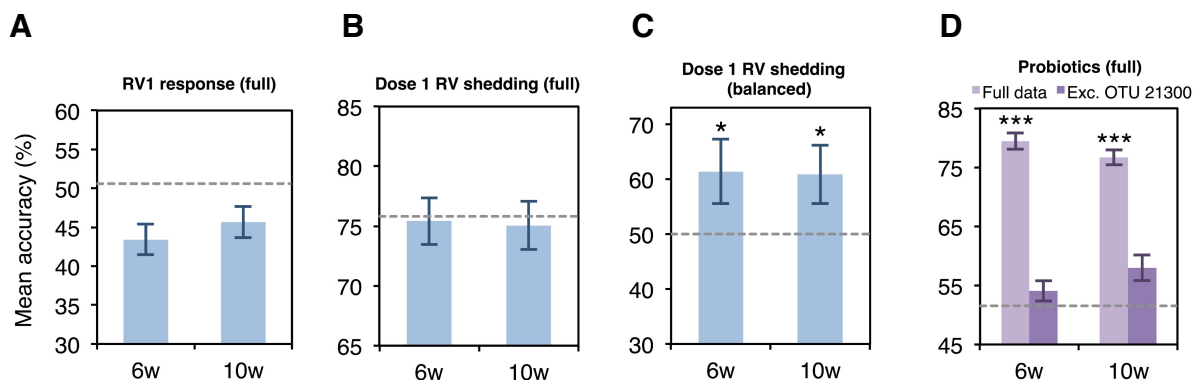


Figure 4.12. Predictive accuracy of Random Forest models. Mean accuracy ($\pm$ s.d.) across 100 iterations of the Random Forest algorithm are displayed for models predicting (A) seroconversion status, (B) dose 1 vaccine take (full data), (C) dose 1 vaccine take using balanced data (including 37 shedders and 37 non-shedders in each iteration of the algorithm), and (D) receipt of probiotics. Dotted lines indicate baseline accuracy, wherein all individuals are assigned to the majority class. OTU 21300 corresponds to the *Lactobacillus* strain that was enriched among probiotics recipients (section 4.4.6). * $P < 0.05$; *** $P < 0.0005$. Abbreviations: OTU, 97%-identity operational taxonomic unit; RV, rotavirus; RV1, Rotarix; w, weeks.

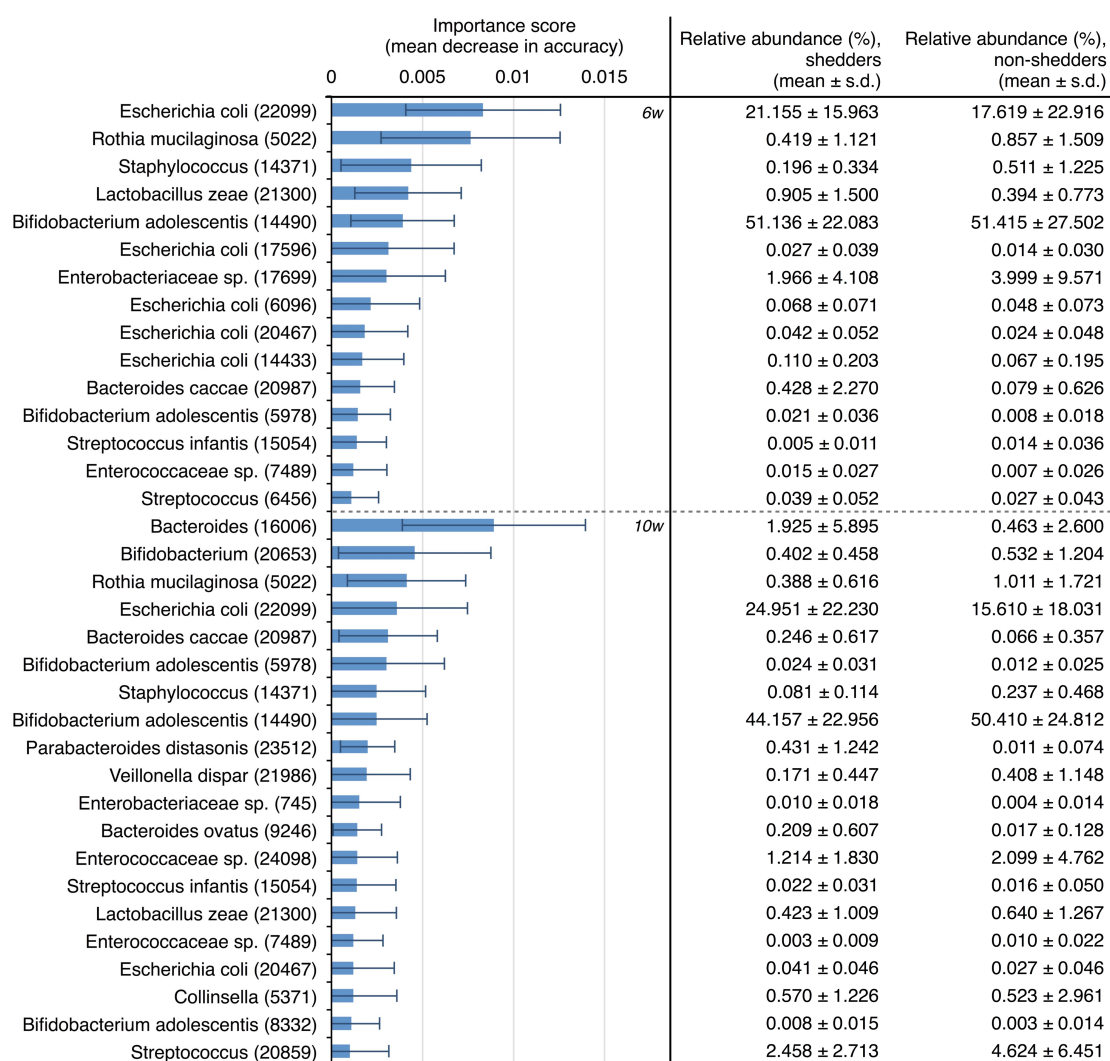


Figure 4.13. Highest ranking taxa by Random Forest importance score for prediction of rotavirus vaccine take. Data are displayed for models predicting dose 1 vaccine take using balanced data (Figure 4.12C). Mean importance scores ($\pm$ s.d.) were calculated over 100 iterations of the Random Forest algorithm. Separate analyses were carried out for 6- and 10-week samples. Abbreviation: w, weeks.

4.4.6. Impact of probiotics on the bacterial microbiota

The influence of probiotic supplementation on the bacterial microbiota was modest. A single OTU, classified as *Lactobacillus zeae*, was significantly more abundant among probiotics compared with placebo recipients at 6 weeks (relative abundance, $0.659 \pm 1.008\%$ [mean $\pm$ s.d.] vs $0.045 \pm 0.182\%$; non-parametric t test, FDR-corrected $P = 0.014$; Table 4.6; Figure 4.14A) and 10 weeks (relative abundance, $0.912 \pm 1.540\%$ vs $0.116 \pm 0.315\%$; non-parametric t test, FDR-corrected $P = 0.013$). Given the clear discrepancies in the prevalence and abundance of this OTU according to treatment group, it is reasonable to conclude that it corresponds to the probiotic strain (the Greengenes database used for taxonomic assignment in this study does not contain a specific sequence for *L. rhamnosus*). An OTU in the genus *Actinomyces* was also enriched in probiotics recipients at 10 weeks of age (Table 4.6).

However, no other phylum-, class-, or OTU-level differences in taxon relative abundance distinguished probiotics from placebo recipients at either timepoint (non-parametric t test, FDR-corrected P values >0.05).

Receipt of probiotics was not associated with a significant change in OTU count or Shannon index (Table 4.4; Figure 4.14B). A shift in microbiota composition among recipients of probiotics was apparent based on unweighted Unifrac distances (adonis, P values 0.021 and 0.014 at 6 and 10 weeks, respectively; Figure 4.14C), but accounted for only a small proportion of variance among samples (R^2 , 0.012 and 0.013 at 6 and 10 weeks, respectively; Table 4.5). No significant clustering was apparent when using weighted Unifrac distances (adonis, P values >0.05 at both doses).

The outputs of Random Forests were consistent with these findings (Figure 4.12D). Models predicted the receipt of probiotics with a mean accuracy of 79.4% and 76.7% at 6 and 10 weeks, respectively (baseline accuracy: 51.5%) – a significant improvement in fit compared with null models (P values <0.001). However, when separate models were fitted in which the OTU corresponding to the probiotic strain was excluded, this predictive capacity was abrogated (mean accuracy, 54.1% and 58.0% at 6 and 10 weeks, respectively; P values >0.05). In line with these findings, the probiotic strain had a mean importance score that far outweighed that of any other OTUs when models were fitted using full data (Figure 4.15).

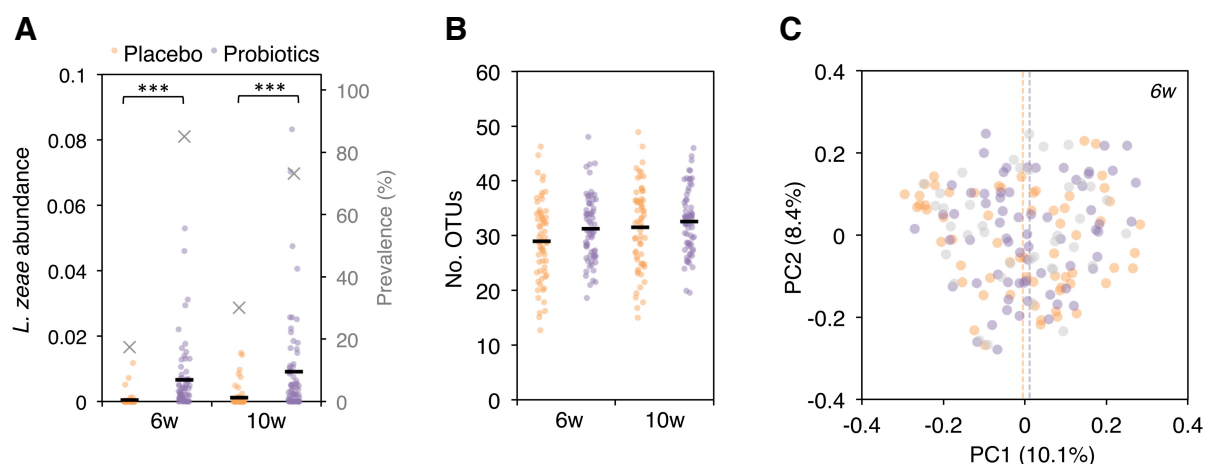


Figure 4.14. Impact of probiotic supplements on the bacterial microbiota. (A) Receipt of probiotics resulted in enrichment of a single OTU (classified as *L. zeae*). Mean relative abundance of this OTU in each group is indicated by a horizontal line, while prevalence is indicated by a cross. (B) OTU count according to treatment group. Mean values in each group are indicated. (C) Clustering of samples according to treatment group. Unweighted Unifrac distances between 6-week samples, visualised via principal coordinates analysis, are displayed. Mean values for principal coordinate 1 in each group are indicated by dotted lines. *** $P < 0.0005$ prior to FDR adjustment. Abbreviations: OTU, 97%-identity operational taxonomic unit; PC, principal coordinate; w, weeks.

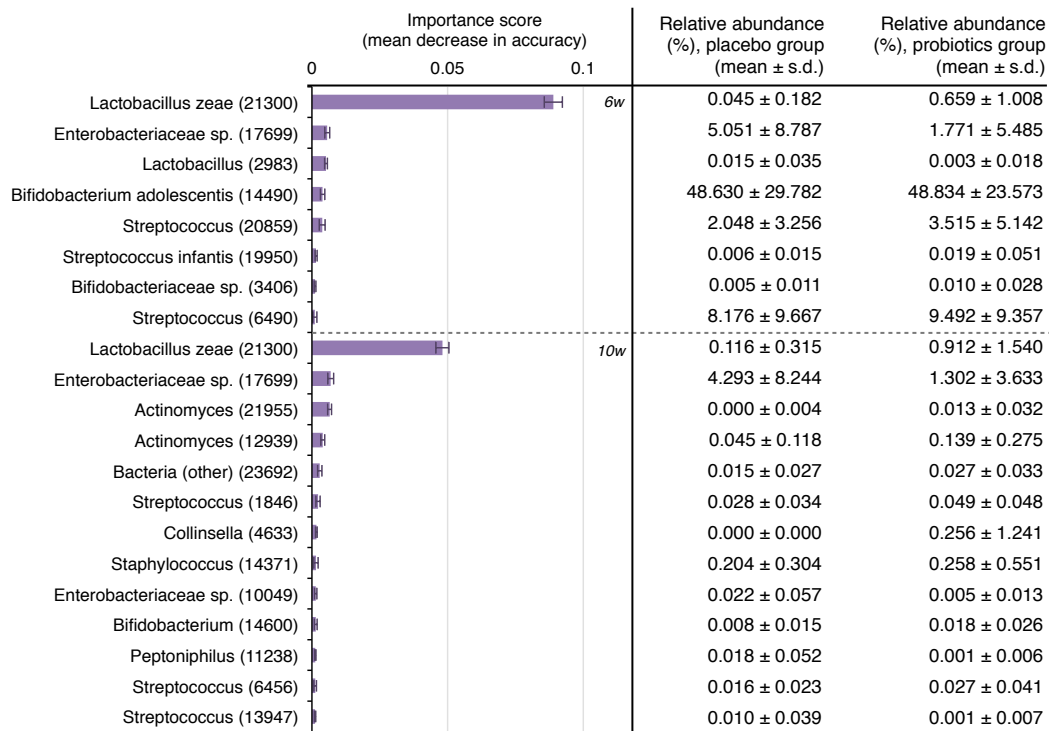


Figure 4.15. Highest ranking taxa by Random Forest importance score for prediction of probiotic treatment. Mean importance scores ($\pm$ s.d.) were calculated across 100 iterations of the Random Forest algorithm. Separate analyses were carried out for 6- and 10-week samples. Abbreviation: w, weeks.

4.4.7. Negative controls

Combining across the 14 extraction controls and four no-template controls included in the two sequencing runs, we obtained a total of 58 sequences at the end of the analysis pipeline (4.1 sequences per control sample on average), suggesting that any contamination of laboratory reagents or cross-contamination during sample handling was negligible. Among these sequences, 53 (91%) corresponded to the five most abundant OTUs among faecal samples.

4.4.8. Comparison of technical replicates

Thirteen samples were sequenced in both MiSeq runs. Measures of alpha diversity were generally consistent among these technical replicates (Figures 4.16A and 4.16B), although a decrease in Shannon index was evident among samples in the second MiSeq run when assessed by Wilcoxon's signed-rank test ($P < 0.001$). No significant shift in beta diversity was evident according to MiSeq run (Table 4.5). As would be expected, replicability was generally higher among samples in which the same PCR product was sequenced than those in which PCR was repeated (Figures 4.16C and 4.16D). Technical replicate ID accounted for the majority of variance among these 26 samples based on Unifrac distances (adonis, R^2 , 0.858 and 0.987 for unweighted and weighted distances, respectively). Significant differences in

relative abundance of the phyla Actinobacteria and Firmicutes were evident according to MiSeq run, although no OTU-level differences were apparent after FDR correction (Table 4.7). While these observations point to the potential influence of run-to-run variation on our findings, the effect appears to be modest.

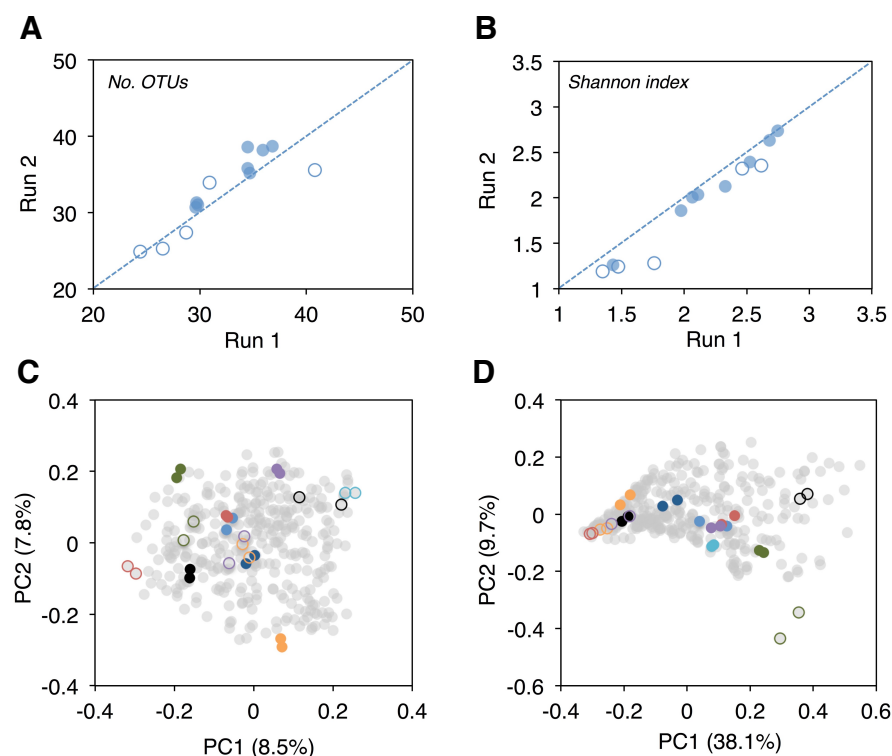


Figure 4.16. Comparison of technical replicates. Concordance between technical replicates is shown for (A) number of OTUs, (B) Shannon index, (C) unweighted Unifrac distances, and (D) weighted Unifrac distances. The same PCR product was re-sequenced in eight of these samples (closed circles), while PCR was repeated for the remaining five (open circles). Abbreviations: OTU, 97%-identity operational taxonomic unit; PC, principal coordinate.

Table 4.7. Significant differences in taxon abundance among technical replicates.

Taxonomic classification	Relative abundance (%) in run 1 (mean $\pm$ s.d.)	Relative abundance (%) in run 2 (mean $\pm$ s.d.)	<i>P</i>	FDR <i>P</i>
Phylum				
Actinobacteria	49.877 $\pm$ 26.893	54.543 $\pm$ 27.722	0.0002	0.001
Firmicutes	18.081 $\pm$ 11.156	15.114 $\pm$ 11.027	0.0007	0.002
Class				
Bacilli	14.244 $\pm$ 12.690	11.490 $\pm$ 11.783	0.0002	0.001
Actinobacteria	46.022 $\pm$ 28.969	49.600 $\pm$ 30.454	0.0002	0.001
OTU				
NS				

Phylum-, class-, and OTU-level differences in abundance among the 13 samples included in both MiSeq runs were assessed using Wilcoxon's signed-rank test. Abbreviations: FDR, adjusted by Benjamini-Hochberg false discovery rate correction; NS, no significant differences; OTU, 97%-identity operational taxonomic unit.

4.4.9. Sensitivity analyses

i. Threshold cycle

When lower Ct cut-off values were adopted for pathogen detection (corresponding to higher copy numbers of the RNA/DNA targets), the primary outcome was unchanged (Table 4.8). We observed a significantly greater prevalence of enteroviruses in Rotarix responders than non-responders at 6 weeks of age (Ct 30, 89/156 [57%] vs 57/157 [36%]; χ^2 , $P < 0.001$; Figure 4.17A) – a finding consistent with the analyses of pathogen abundance described above (Table 4.2) – and a decrease in the prevalence of EAEC at this timepoint (Ct 20, 10/156 [6%] vs 23/157 [15%]; χ^2 , $P = 0.018$). Other associations were largely unchanged (Figure 4.17), although the increased proportion of responders infected with ≥ 1 pathogen at both doses observed using a Ct cut-off of 35 was no longer significant at lower thresholds.

ii. Antibody titre

Among per-protocol infants included in the enteropathogen survey, 67 experienced a four-fold rise in rotavirus-specific serum IgA titre and had a post-vaccination titre in excess of 90 U/ml. When we compared these individuals to those who were seronegative post-vaccination or experienced a fall in antibody titre, associations between TAC targets and vaccine outcome were similar to those observed using standard seroconversion criteria (Table 4.8) – these ‘robust responders’ were more likely than non-responders to harbour ≥ 1 bacterial enteropathogen (excluding EAEC) at 6 weeks of age (17/67 [25%] vs 17/136 [13%]; χ^2 , $P = 0.021$), and a borderline-significant increase in the likelihood of being infected with ≥ 1

Table 4.8. Summary of sensitivity analyses.

Sensitivity analysis	Proportion with ≥ 1 pathogen at 6 or 10 weeks, n/N (%)		OR (95% CI)
	Responders	Non-responders	
Primary outcome	78/155 (67.8)	70/154 (45.5)	1.22 (0.78–1.90)
Ct 30	48/155 (31.0)	47/154 (30.5)	1.02 (0.63–1.66)
Ct 25	27/155 (17.4)	26/154 (16.9)	1.04 (0.57–1.88)
Ct 20	7/155 (4.5)	4/154 (2.6)	1.77 (0.51–6.19)
IgA titre >90 U/ml	33/67 (49.3)	58/133 (43.6)	1.26 (0.70–2.26)
MS2+	54/128 (42.1)	59/121 (48.8)	1.30 (0.79–2.15)
IgA-	51/110 (46.4)	57/114 (50.0)	1.16 (0.68–1.95)

Abbreviations: CI, confidence interval; Ct, threshold cycle; IgA-, analysis restricted to infants negative for rotavirus-specific IgA at baseline; MS2+, analysis restricted to infants with MS2-positive samples at 6 and 10 weeks of age; OR, odds ratio.

enteropathogen at both doses (largely driven by bacterial infections) was observed in these individuals (13/67 [19%] vs 35/133 [10%]; χ^2 , $P = 0.056$). No significant associations were observed between viral or eukaryotic TAC targets and vaccine outcome as defined in this way. Similarly, comparisons of alpha and beta diversity did not reveal any significant discrepancies in the composition or diversity of the bacterial microbiota among robust responders versus non-responders (Tables 4.4 and 4.5).

iii. Treatment group interactions

In the primary outcome analysis, we assessed the impact of harbouring ≥ 1 enteropathogen at either 6 or 10 weeks on the odds of rotavirus seroconversion via logistic regression. We fitted separate logistic regression models to examine potential interactions between enteropathogen infection status and treatment group (zinc supplementation, probiotic supplementation, or study arm). No significant interaction terms were observed (data not shown), suggesting that the association between concurrent infections and rotavirus seroconversion was not influenced by treatment group.

iv. Other sensitivity analyses

The observed associations between concurrent enteropathogens and rotavirus seroconversion status were largely unchanged by excluding samples negative for MS2 (the extrinsic RNA control) or excluding individuals who were positive for rotavirus-specific serum IgA at baseline (observed in 167/551 [30%] infants who completed the trial per protocol) (Table 4.8). In both instances, responders were significantly more likely than non-responders to harbour ≥ 1 enteropathogen (excluding EAEC and enterovirus) at both doses (χ^2 , P values < 0.05) – an effect attributable primarily to discrepancies in the prevalence of bacterial infections, which were significantly more common among responders at 6 weeks of age (χ^2 , P values < 0.05). When considering MS2-positive samples, non-responders were more likely than responders to harbour EAEC at both doses (50/128 [39%] vs 33/121 [27%]; χ^2 , $P = 0.049$) – a result that was also apparent in the primary analysis when using a Ct cut-off of 30 (Figure 4.17C). Likewise, comparisons of alpha and beta diversity were largely unaltered by the exclusion of infants positive for rotavirus-specific serum IgA at baseline (Tables 4.4 and 4.5).

4.5. Discussion

Throughout infancy, individuals living in tropical, low-income countries are exposed to a diverse array of enteropathogens. In Chapter 2, the inhibitory potential of these pathogens on the replication efficiency and immunogenicity of OPV was highlighted during a systematic review and meta-analysis. Among infants in south India with a considerable burden of enteropathogens, we did not observe a similar inhibitory effect of concurrent infections on the immunogenicity of Rotarix doses administered at 6 and 10 weeks of age. Indeed, individuals harbouring ≥ 1 bacterial infection at the time of both doses were more likely to respond serologically to this vaccine.

The potential for concurrent infection with bacterial enteropathogens to have a beneficial impact on vaccine immunogenicity is not without precedent. Oh et al (2014) recently demonstrated that sensing of the bacterial microbiota via the pattern recognition receptor TLR5 significantly improved the immunogenicity of inactivated influenza vaccine in mice, while double-mutant heat-labile enterotoxin derived from ETEC enhanced the mucosal immune response observed during a Phase I clinical trial of an oral inactivated ETEC vaccine candidate (Lundgren et al, 2014). The induction of Toll-like receptor signalling and other inflammatory mediators by concurrent enteropathogens may have had a similar adjuvant effect in our study. However, given that rotavirus vaccine immunogenicity is considerably greater in high-income countries, where infants experience a low burden of enteropathogens, we consider such a mechanism to be unlikely.

An alternative explanation for the elevated burden of bacterial pathogens among Rotarix responders relates to the replication of co-administered vaccine polioviruses. Rotavirus shedding following the 6-week vaccine dose was negatively correlated with OPV take – an observation consistent with an inhibitory effect of OPV on the replication of vaccine rotaviruses (Patel et al, 2012a). Given their potential to interfere with OPV take, the presence of concurrent bacteria may have enhanced the immunogenicity of Rotarix in this study population by blocking the replication of co-administered Sabin viruses. Notably, the discrepancy in bacterial enteropathogen burden according to rotavirus vaccine outcome was most pronounced for the first (6-week) vaccine dose, which is also when the inhibitory effect of OPV on Rotarix appears to be greatest (Patel et al, 2012a). In Chapter 5, I will expand further on the association between concurrent enteropathogens and response to tOPV in these infants, which may further elucidate this possibility.

It is worth noting that the association between bacterial pathogens and rotavirus seroconversion was contingent on the exclusion of EAEC, which we chose to omit from

primary comparisons because of its very high prevalence and limited association with diarrhoea in previous studies using similar methods of pathogen detection (Taniuchi et al, 2013; Platts-Mills et al, 2014). When lower Ct cut-off values were adopted (corresponding to an elevated copy number), we observed a significantly greater prevalence of EAEC in non-responders at 6 weeks of age – a finding consistent with an inhibitory effect of EAEC on rotavirus vaccine immunogenicity that appears to contradict the trend observed for other bacterial enteropathogens. However, we did not observe any significant differences in the abundance of EAEC when Ct values were directly compared according to seroconversion status or vaccine take. Overall, our findings highlight several possible associations of modest effect size between bacterial enteropathogens and rotavirus vaccine response, though it appears unlikely that bacterial infections form a major driving force behind the diminished immunogenicity of Rotarix observed in this study population.

Rotarix responders exhibited a greater burden of enteroviruses (and more specifically Sabin viruses) at 6 weeks of age – an effect that was clearest when considering vaccine take. These enteroviruses can be attributed primarily to the residual replication of Sabin viruses administered at birth, although secondary transmission of vaccine polioviruses later in infancy cannot be excluded. Thus, it appears that infants who experienced robust replication of one or more Sabin viruses following their birth dose of tOPV were more likely to respond to Rotarix during this study. Again, this observation may relate to the replication of Sabin viruses co-administered with Rotarix. OPV take was significantly lower among infants positive for Sabin viruses at 6 weeks of age, which may reflect either an inhibitory effect of the replicating Sabin viruses on the 6-week OPV dose, or the existence of poliovirus-specific mucosal protection among individuals who experienced extensive replication following the birth dose of tOPV. By either mechanism, existing polioviruses may have enhanced Rotarix response by inhibiting the replication of co-administered OPV. Factors influencing the take of Sabin viruses administered at birth are unclear, although maternal antibodies (including IgG transferred across the placenta and IgA in colostrum) are likely to be important (Sabin et al, 1963). A conceptual model outlining the potential interactions between OPV and rotavirus vaccine doses administered in early infancy is provided in Figure 4.18.

At 10 weeks of age, an increased prevalence of enteroviruses was evident among individuals who failed to shed rotavirus following the 6-week vaccine dose. Notably, both Sabin viruses and NPEVs appear to have contributed to this discrepancy. While an elevated prevalence of Sabin viruses among rotavirus non-shedders would fit neatly within the conceptual model described above, the negative association between Rotarix take and

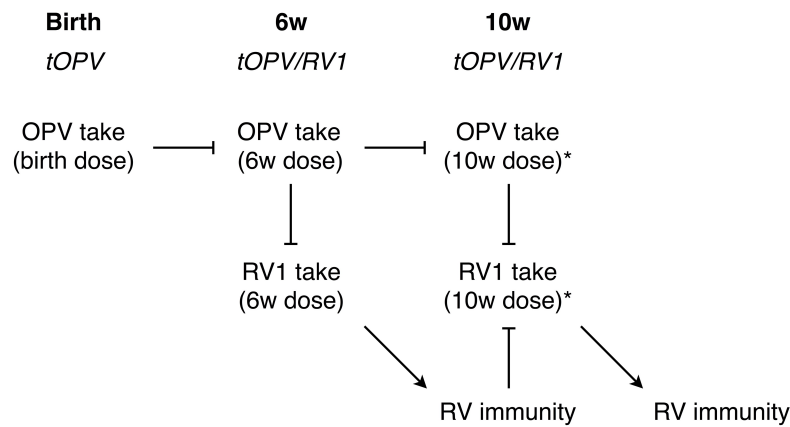


Figure 4.18. Conceptual model of interactions between oral rotavirus and poliovirus vaccines administered in early infancy. * outcome not measured in this study. Abbreviations: OPV, oral poliovirus vaccine; RV, rotavirus; RV1, Rotarix; tOPV, trivalent oral poliovirus vaccine; w, weeks.

subsequent NPEV infection is more surprising. The potential for intestinal viruses to interfere with one another is one of the central tenets of this thesis. Thus, it is certainly possible that replicating vaccine rotaviruses may have an inhibitory effect on NPEVs, or that NPEVs arising after vaccine delivery may interfere with Rotarix take – a notion validated by the apparent inhibitory effects of NPEVs on Rotarix recently documented among infants in Bangladesh (Taniuchi et al, 2016). However, it is also important to recognise the risk of over-interpreting these findings given key limitations in how they were attained. In particular, we did not target NPEVs with a specific diagnostic assay, but defined these viruses as the presence of an enterovirus detected via TAC in the absence of any Sabin serotypes. Since this approach did not allow us to identify samples positive for both Sabin viruses and NPEVs, we excluded infants positive for the former when assessing the impact of NPEVs on vaccine response. Not only did this approach limit the NPEV-specific analyses to a subset of infants that may not be representative of the broader population, but in light of the extensive shedding of Sabin polioviruses, it essentially entailed omitting half of the study population when assessing the potential impact of NPEVs (at least one Sabin serotype was identified in 50% and 56% of infants at 6 and 10 weeks of age, respectively). No other viral TAC targets (assessed via specific assays in every sample) differed according to rotavirus shedding status, and we observed no significant association between NPEV prevalence and seroconversion status.

Approximately 30% of the infants in this study were positive for rotavirus-specific serum IgA at 6 weeks of age, indicative of high exposure to rotavirus during the neonatal period. The exclusion of seropositive infants from the analyses did not influence the associations observed between concurrent enteropathogens and vaccine outcome.

Discrepancies in pathogen burden were similarly unchanged when we adopted an elevated threshold antibody titre to define seroconversion. Thus, it is reasonable to conclude that the presence of enteropathogens did not represent a risk factor for failure to achieve a robust antibody response following two doses of Rotarix in these infants.

To what extent are these findings generalisable to other low-income settings linked with impaired oral vaccine response? Based on the findings of the MAL-ED study (see section 1.5.5), the enteropathogen burden experienced by infants in Vellore appears to be typical of that endured in other developing countries (Platts-Mills et al, 2015). However, at a seroconversion rate of 31% after two doses, the immunogenicity of Rotarix in this study was poor even by the standards of low-income settings, where seroconversion rates of approximately 50% are typical (Patel et al, 2013a). Reasons for the poor immunogenicity of Rotarix in these infants are unclear, especially given that a previous trial of this vaccine in India yielded a seroconversion rate of 58% after two doses at 8 and 12 weeks of age (Narang et al, 2009). Based on the findings presented here, we can merely conclude that (with the possible exception of co-administered vaccine polioviruses) concurrent infections do not appear to account for discrepancies in rotavirus vaccine performance among these infants. Notably, these results are broadly consistent with recent findings from Bangladesh, where an increase in the quantity of enteroviruses (but no other enteropathogens) was linked with a reduction in the odds of rotavirus seroconversion (Taniuchi et al, 2016).

Turning our attention to the bacterial flora as a whole, we observed no consistent discrepancies in microbiota composition, diversity, or stability according to rotavirus seroconversion status. Referring back to the 'keystone taxa' hypothesis outlined in Chapter 1, we did not identify any OTUs that differed significantly in abundance between responders and non-responders (either in the phylum Proteobacteria or across other taxa). Findings were more intriguing with respect to vaccine take. At both 6 and 10 weeks of age, the number of OTUs was significantly lower among infants who failed to shed rotavirus after the first dose of Rotarix (a result consistent with the hypothesised association between microbiota diversity and oral vaccine outcome), and a shift in overall microbiota composition according to shedding status was apparent based on unweighted Unifrac distances. In each instance, however, the size of the observed discrepancies was modest. Likewise, Random Forest models based on OTU abundance data conferred only a small increase in predictive accuracy for rotavirus shedding status compared with baseline. Thus, our findings suggest that the bacterial microbiota – in as much as it was captured by the sampling method, extraction

techniques, primers, and sequencing methods adopted – is not a key factor underpinning the variation in rotavirus vaccine response observed in this study population.

These findings certainly do not preclude an important role for the bacterial microbiota in shaping broader geographic trends in rotavirus vaccine immunogenicity. If the comparison of infants from Malawi, Venezuela, and the USA is representative of the geographic variation present in microbiota composition (Yatsunenکو et al, 2012), it is likely that the taxonomic structure and functional profile of the gut flora among infants in this study population differ considerably from those of infants living in high-income countries. Given the poor immunogenicity of Rotarix in this trial, it is possible that all infants harboured a bacterial community structure inhibitory to vaccine virus replication, with insufficient variation among individuals to distinguish compositional differences pertinent to Rotarix failure. Future studies on a broader geographic and sociodemographic scale may help to test this possibility.

The administration of probiotics had a minimal impact on the bacterial microbiota of these infants. This is perhaps best illustrated by the failure of Random Forest models to accurately distinguish between probiotics and placebo recipients when the OTU corresponding to the *L. rhamnosus* GG probiotic strain was omitted. Despite the daily administration of capsules containing 10^{10} organisms, the enriched OTU accounted for a mean relative abundance of <1% among probiotics recipients at both 6 and 10 weeks of age. Since these samples were collected within the window of supplement administration, it is unclear whether the presence of this strain represents successful colonisation of the intestinal microbiota or passive transit.

4.5.1. Limitations

A number of limitations relating to technical details of the laboratory work involved in this study merit brief consideration. First, amplification of the extrinsic RNA control MS2 was not observed in a portion of samples run on the TACs (including approximately 10% of samples included in the final analysis). By contrast, the extrinsic DNA control PhHV was recovered in all samples. Although inconsistency in MS2 amplification is potentially indicative of suboptimal extraction and amplification efficiency for RNA targets, based on recent correspondence with researchers in the Department of Gastrointestinal Sciences at the Christian Medical College, it transpires that the use of a new batch of MS2 in subsequent projects led to much more consistent recovery of the RNA control (personal communication,

Dr Ira Prahara). It is therefore likely that the issue lay in the stability of the MS2 rather than a problem with the extraction protocol. Nonetheless, we conducted a sensitivity analysis in which we excluded samples lacking amplification of MS2. This did not alter the primary outcomes of the study.

A more significant drawback concerns the 16S rRNA gene V4-specific primers. The primer sequences published by Caporaso et al (2012) – 5'-GTGCCAGCMGCCGCGGTAA-3' (515F) and 5'-GGACTACHVGGGTWTCTAAT-3' (806R) – are degenerate at several positions, such that a mixture of similar oligonucleotides is added during PCR. For example, the letter M corresponds to inclusion of the nucleotides C or A at the relevant site in the primer, while H corresponds to A, C, or T. The degeneracy of these primers enables a greater number of bacterial 16S rRNA gene sequences to be targeted within a given DNA sample. In the present study, however, we used the best available non-degenerate variants of these primers. Thus, while our 515F primer sequence has 88,946 perfect matches within the V4-specific Greengenes reference file used in the analyses described above, the degenerate variants of the primer used by Caporaso et al (2012) have 90,495 perfect matches. The corresponding 806R primers have 71,778 versus 88,473 matches, respectively. While the use of non-degenerate primers may have reduced the taxonomic coverage of our sequencing pipeline, these biases would have impacted all samples equally, and therefore do not undermine any observed discrepancies in relative abundance or diversity in our analyses. It is also worth noting that these constraints play out within the context of biases in coverage and amplification efficiency that exist regardless of whether or not degenerate primers are used (Hong et al, 2009; Wang and Qian, 2009). During a study of mock bacterial communities using the degenerate V4 primer sequences described above, Nelson et al (2014) documented the under-representation of several taxa (e.g., members of the genera *Propionibacterium* and *Pseudomonas*) and corresponding over-representation of others (e.g., *Helicobacter* species) – similar biases were apparent during the sequencing of mock bacterial communities using our non-degenerate primers, as described above (section 3.4). The existing biases are likely only to have been exacerbated in our study by the use of non-degenerate primers.

The comparison of technical replicates revealed a degree of variation according to MiSeq run that cannot be discounted. The sources of this variation are unclear given that the protocol, personnel, and sequencing facility were standardised across the two runs, but may include differences in reagent lot and sequencing error rate, among others. Similar batch effects have been documented in previous studies involving high-throughput sequencing of the 16S rRNA gene, particularly those using pyrosequencing (Ge et al, 2014; Mutlu et al,

2014). Although replicability appears to be higher when using MiSeq (Lluch et al, 2015), the potential influence of batch effects is often not reported in studies that collate data from multiple sequencing runs, and I suspect that the issue may be more widespread than is currently appreciated or reported. Notably, we accounted for potential batch effects during the design of this study by randomising the order in which samples were assayed, and during statistical analyses via the inclusion of MiSeq run as a covariate in regression models and a constraint in permutation-based tests. It is therefore unlikely that the observed run-to-run variation substantially altered the outcomes of this study. Given the opportunity to repeat the investigation, however, I would have obtained a greater number of barcoded reverse primers in order to increase the multiplexing capacity of the sequencing pipeline and thereby included all samples on a single run²⁸.

Finally, comparisons of the bacterial microbiota with respect to probiotic treatment group were conducted within a subset of infants enriched in Rotarix responders (owing to the selection criteria of this nested case-control study), and thus not entirely representative of the underlying study population. However, considering that microbiota composition did not differ significantly according to seroconversion status in any of the comparisons described above, it is unlikely that the selection criteria introduced any major bias into our findings regarding the impact of *L. rhamnosus* GG administration on the bacterial flora.

4.5.2. Conclusions

There are currently limited published data regarding the impact of the intestinal microbiota on response to oral rotavirus vaccine. Overall, our findings corroborate the potential inhibitory effect of co-administered vaccine poliovirus on Rotarix response. Contrary to expectations, we observed a greater burden of enteropathogens among individuals who seroconverted to rotavirus – an effect that may well have been mediated by an inhibitory influence of these infections on OPV take. This possibility will be considered further in the following chapter. Although we observed no significant differences in the composition of the bacterial microbiota according to vaccine immunogenicity, a similar investigation on a broader geographic scale may yet reveal an important role for the bacterial (or indeed viral) microbiota in shaping rotavirus vaccine response.

²⁸ This is the approach adopted during the library preparation described in Chapter 6, wherein 295 samples were included on a single MiSeq run.

5.1. Background

The findings presented in the previous chapter raised the possibility that concurrent bacterial enteropathogens may have a beneficial impact – albeit a modest one – on the immunogenicity of Rotarix doses administered to infants in south India at 6 and 10 weeks of age. Was this effect mediated by an inhibitory effect of bacterial infections on the replication of vaccine polioviruses co-administered with Rotarix? Here, we shall explore this notion further by examining the association between the intestinal microbiota and OPV outcome within the same study population. Again, both the burden of enteropathogens and the composition of the bacterial microbiota will be considered. As noted in the systematic review described in Chapter 2, few prior studies have reported on the association between non-viral pathogens and response to OPV, while the potential impact of the bacterial microbiota on either seroconversion or vaccine take has not been examined to date. The laboratory and statistical methods involved in these analyses have been outlined in detail above, and will not be repeated here. However, several relevant additions and amendments to the methods are described below.

5.1.1. Hypotheses

The primary hypotheses of this study are outlined in the previous chapter (section 4.1.1). In brief, our principal objective was to test the notion that failure to respond to OPV (based either on type 3 poliovirus seroconversion or the take of Sabin viruses) is associated with an increase in the prevalence and number of enteropathogens at the time of immunisation. We also hypothesised that failure to respond to OPV is associated with a decrease in the diversity of the bacterial microbiota, and a shift in taxonomic composition (driven in part by an elevated relative abundance of Proteobacteria) based on Unifrac distances and the discriminatory capacity of Random Forest models.

5.2. Methods

5.2.1. Additional TaqMan array card assays

The subset of infants recruited to our primary enteropathogen survey included just 62 individuals who failed to seroconvert to type 3 poliovirus. To increase our power to detect

the influence of enteropathogens on OPV immunogenicity, we extended the study population to include all other OPV non-responders with sufficient 6- and 10-week pre-vaccination stool samples (n = 30). Nucleic acid extraction and TAC assays were conducted for these samples using the methods described in Chapters 3 and 4 (albeit without randomisation by OPV outcome). Thus, our final study population encompassed 263 OPV responders and 92 non-responders (a ratio of approximately 3:1), while the 16S rRNA gene sequencing subset comprised 138 responders and 32 non-responders (a ratio of approximately 4:1; Table 5.1).

Table 5.1. Summary of study population.

Treatment group	Trial population (per protocol)		No. in enteropathogen subset		No. in 16S rRNA microbiota subset	
	Total	PV3 responders (%)	Total	PV3 responders (%)	Total	PV3 responders (%)
Zinc/probiotics	137	108 (78.8)	109	85 (78.0)	38	29 (76.3)
Zinc only	143	116 (81.1)	80	56 (70.0)	-	-
Probiotics only	136	112 (82.4)	83	61 (73.5)	69	55 (79.7)
Placebo	135	112 (83.0)	83	61 (73.5)	63	54 (85.7)
Overall	551	448 (81.3)	355	263 (74.1)	170	138 (81.2)

Six infants in the enteropathogen subset were excluded from the final analyses as they did not complete the study per protocol, while two infants were excluded from the microbiota analyses. See Table 4.1 for additional details. Abbreviation: PV3, type 3 poliovirus.

5.2.2. Statistical analysis

i. Enteropathogen burden

In line with the assessment of rotavirus vaccine response, the primary outcome of interest in this study was the association between type 3 poliovirus seroconversion (as a categorical dependent variable) and the presence of ≥ 1 enteropathogen (excluding EAEC and enterovirus) at 6 or 10 weeks of age, as determined via logistic regression. Type 3 Sabin viruses have generally proven to be the least immunogenic component of tOPV (Patriarca et al, 1991). Response to this serotype was therefore used as a marker of OPV immunogenicity in the present trial. Seroconversion was defined as an increase in poliovirus serotype 3 neutralising antibody titre from $<1:8$ to $\geq 1:8$ or a four-fold rise in titre between 6 and 14 weeks of age, assuming a decay in pre-vaccination titre with a half-life of 28 days. Secondary outcomes followed those described in section 4.3.4. Rotavirus infections were no longer excluded from analyses of pathogen groups or mixed infections as – in contrast to Rotarix

outcome – we did not anticipate these infections to be positively correlated with OPV response. However, we excluded enteroviruses from analyses of mixed infections and total pathogen count owing to the high prevalence of Sabin viruses among enterovirus-positive samples and the likely confounding of Sabin virus shedding with OPV outcome measures. The association between rotavirus seroconversion and type 3 poliovirus seroconversion was assessed using the χ^2 test.

Although the shedding of vaccine polioviruses in the week following immunisation was not assessed, we used the shedding of Sabin viruses at 10 weeks of age as an indicator of vaccine take following the 6-week dose of tOPV. Specifically, we examined the influence of the intestinal microbiota on: (i) the shedding of any Sabin serotype at 10 weeks of age; and (ii) the shedding of type 3 Sabin viruses at 10 weeks of age. Infants were considered eligible for the analysis of OPV take if their 10-week sample was positive for PhHV and at least one RNA target during the TAC assay, and was successfully assayed for Sabin serotypes if positive for enterovirus. Individuals were excluded if they did not meet these criteria.

ii. Population attributable fraction of vaccine non-response associated with enteropathogens

We extended our analyses of OPV outcomes by estimating the fraction of vaccine failure that can be attributed to the presence of concurrent pathogens. First, to determine per-dose seroconversion rates in infected versus uninfected individuals, we calculated the expected numbers of responders and non-responders in the trial population as a whole infected at: neither 6 nor 10 weeks (E_0^R for responders and E_0^{NR} for non-responders); at one of the two doses (E_1^R and E_1^{NR}); and at both doses (E_2^R and E_2^{NR}). These estimates were obtained by multiplying the observed numbers of responders in groups E_0 , E_1 , and E_2 within the TAC subset by 1.79 (448/250) and the observed numbers of non-responders by 1.21 (103/85) to account for the sampling proportions in our enteropathogen survey²⁹. EAEC and enterovirus infections were excluded at each dose. Assuming a constant probability of seroconversion per OPV dose for infected (p_i) and uninfected individuals (p_u), we then calculated (using the Solver function in Microsoft Excel) the values of p_i and p_u that would maximise the likelihood:

$$L(p_u, p_i \mid E_0^R, E_1^R, E_2^R) = P(E_0^R \mid n_0, p_0) * P(E_1^R \mid n_1, p_1) * P(E_2^R \mid n_2, p_2)$$

where p_0 (the probability of seroconversion in infants infected at neither dose) = $1 - (1 - p_u)^2$, p_1 (the probability of seroconversion in infants infected at one dose) = $1 - (1 - p_u)(1 - p_i)$,

²⁹ Overall, we successfully assayed the 6- and 10-week samples of 250 of the 448 per-protocol OPV responders and 85 out of the 103 non-responders.

and p_2 (the probability of seroconversion in infants infected at both doses) = $1 - (1 - p_i)^2$; n_0 , n_1 , and n_2 are the estimated numbers of individuals infected at neither, one, or both doses in the trial population; and the conditional probabilities are given by the binomial probability density function.

Per-dose seroconversion rates were then used to estimate the population attributable fraction (PAF) of failed type 3 poliovirus seroconversions associated with concurrent enteropathogens under different burdens of infection, as follows:

$$\text{PAF} = \frac{(\text{overall risk of non-response}) - (\text{risk of non-response among uninfected})}{(\text{overall risk of non-response})} = \frac{I_t^d - (1 - p_u)^d}{I_t^d}$$

where I_t (the per-dose non-response rate in the whole population) = $(1 - f_i)(1 - p_u) + f_i(1 - p_i)$, f_i is the proportion of the population infected per dose, and d is the number of doses administered. We estimated f_i for this study population at 6 weeks of age by multiplying the observed numbers of infected and uninfected responders within the TAC subset by 1.78 (448/252) and the observed numbers of non-responders by 1.17 (103/88) to account for the sampling proportions of the 6-week TAC assays (as described above).

iii. Bacterial microbiota

The OTU table generated following the 16S rRNA gene sequencing pipeline described in the previous chapter served as the input for analyses of the bacterial microbiota. Once again, our primary outcome for these analyses was the association between within-sample diversity (OTU count and Shannon index as continuous dependent variables) and vaccine response (type 3 poliovirus seroconversion after the 6- and 10-week doses or shedding after the 6-week dose), as determined via linear regression. Secondary outcomes included comparisons of OTU count within the phylum Proteobacteria, sample clustering based on Unifrac distances, microbiota stability, and taxon relative abundance according to OPV outcome, as described in section 4.3.4. We also tested the capacity of Random Forest models to discriminate infants according to type 3 poliovirus seroconversion and OPV take.

To determine whether OTU count and the presence of ≥ 1 bacterial enteropathogen at 6 weeks of age were independently associated with the odds of OPV response (seroconversion after the 6- and 10-week doses or shedding of any Sabin viruses after the 6-week dose), we compared the effect size and significance of these explanatory variables in univariate versus multivariate logistic regression models (with OPV outcome as the dependent variable). No other variables were adjusted for in these multivariate models.

iv. Sensitivity analyses

Sensitivity analyses were carried out in which: (i) lower Ct cut-offs (30, 25, and 20) were used for the assessment of TAC targets; (ii) samples lacking amplification of MS2 were excluded; (iii) individuals positive for serum type 3 poliovirus antibodies at baseline were excluded (thus removing any infants with maternal antibodies or who had responded to the tOPV dose administered at birth); and (iv) comparisons were stratified by rotavirus seroconversion status (to account for the enrichment of the study population with Rotarix responders). Among infants lacking type 3 poliovirus antibodies at 6 weeks of age, we compared individuals who failed to respond with those who developed antibody titres of $\geq 1:256$, thus enabling us to assess the impact of the intestinal microbiota on the extent of the serum antibody response (or relative immunogenicity) achieved by immunisation. Outcomes of interest during sensitivity analyses followed those described in section 4.3.4.

5.3. Results

5.3.1. Oral poliovirus vaccine immunogenicity

After tOPV doses administered at 6 and 10 weeks of age, 448 (81%) of the 551 infants who completed the study per protocol seroconverted to type 3 poliovirus (Lazarus et al, manuscript in preparation). No significant differences in immunogenicity were observed across the study arms (χ^2 , P values >0.05 for all comparisons). Seroconversion to rotavirus was observed in 147/448 (33%) individuals who responded to OPV and 26/103 (25%) of those who did not (χ^2 , $P = 0.136$).

5.3.2. Association between concurrent enteropathogens and seroconversion

i. Primary outcome

Among individuals who completed the study per protocol, we obtained eligible TAC assays for 6-week samples in 340 infants, 10-week samples in 343 infants, and 6- and 10-week samples in 335 infants (including 85 [25%] infants who seroconverted to type 3 poliovirus and 250 [75%] non-responders). Exposure to ≥ 1 enteropathogen (excluding EAEC and enterovirus) at 6 or 10 weeks of age was observed in 51/85 (60%) non-responders and 120/250 (48%) responders (OR 0.62, 95% CI 0.37–1.01).

ii. Secondary outcomes

The prevalence of concurrent enteropathogens according to seroconversion status is indicated in Figures 5.1A and 5.1B for 6- and 10-week samples, respectively. Comparisons of pathogen abundance are summarised in Table 5.2. At 6 weeks of age, non-responders were more likely to harbour ≥ 1 enteropathogen (excluding EAEC and enterovirus) than infants who seroconverted (38/88 [43%] vs 68/252 [27%]; χ^2 , $P = 0.005$). Although no individual pathogens differed significantly in prevalence or abundance according to seroconversion status (Table 5.2), this discrepancy was driven predominantly by bacterial infections, which were significantly more common in non-responders than responders overall (29/88 [33%] vs 42/252 [17%]; χ^2 , $P = 0.001$), albeit only when EAEC was excluded. At 10 weeks of age, the proportion of infants infected with ≥ 1 pathogen no longer differed significantly between non-responders and responders (36/87 [41%] vs 93/256 [36%]; χ^2 , $P = 0.401$); however, non-responders were still more likely to harbour ≥ 1 bacterial infection (28/87 [32%] vs 55/256 [21%]; χ^2 , $P = 0.044$). The proportion of individuals infected with ≥ 1 viral or ≥ 1 eukaryotic enteropathogen did not differ significantly between the two groups at either dose.

Although the proportion of individuals shedding enteroviruses at either 6 or 10 weeks of age did not differ according to seroconversion status, responders were more likely than non-responders to harbour ≥ 1 Sabin serotype at 6 weeks of age (130/252 [52%] vs 31/88 [35%]; χ^2 , $P = 0.008$; Figure 5.2A) – a discrepancy attributable primarily to type 2 Sabin viruses, which were shed by 115/252 (46%) responders and 21/88 (24%) non-responders (χ^2 , $P < 0.001$). As expected, the replication of type 3 Sabin viruses after the 6-week dose of tOPV was positively correlated with seroconversion – shedding of this serotype 4 weeks after immunisation (i.e., at 10 weeks of age) was observed in 76/254 (30%) responders and 3/87 (3%) non-responders (χ^2 , $P < 0.001$; Figure 5.2B). The prevalence of NPEVs (based on exclusion of Sabin polioviruses) was greater among non-responders than responders at 6 weeks (28/57 [49%] vs 44/122 [36%]) and 10 weeks (30/56 [54%] vs 42/101 [42%]); however, these differences were not statistically significant (χ^2 , P values 0.097 and 0.149, respectively).

Mixed infections, defined as the presence of >1 enteropathogen (excluding enterovirus) were more common in non-responders than responders at 6 weeks (26/88 [30%] vs 38/252 [15%]; χ^2 , $P = 0.003$; Figure 5.1C) and 10 weeks (30/87 [34%] vs 57/256 [22%]; χ^2 , $P = 0.024$; Figure 5.1D). The total number of pathogens (excluding enterovirus) did not differ significantly between non-responders and responders at 6 weeks (1.1 ± 1.1 [mean $\pm$ s.d.] vs 0.8 ± 0.8 ; Wilcoxon's rank sum, $P = 0.081$) or 10 weeks (1.3 ± 1.2 vs 1.0 ± 0.9 ; Wilcoxon's rank sum, $P = 0.189$).

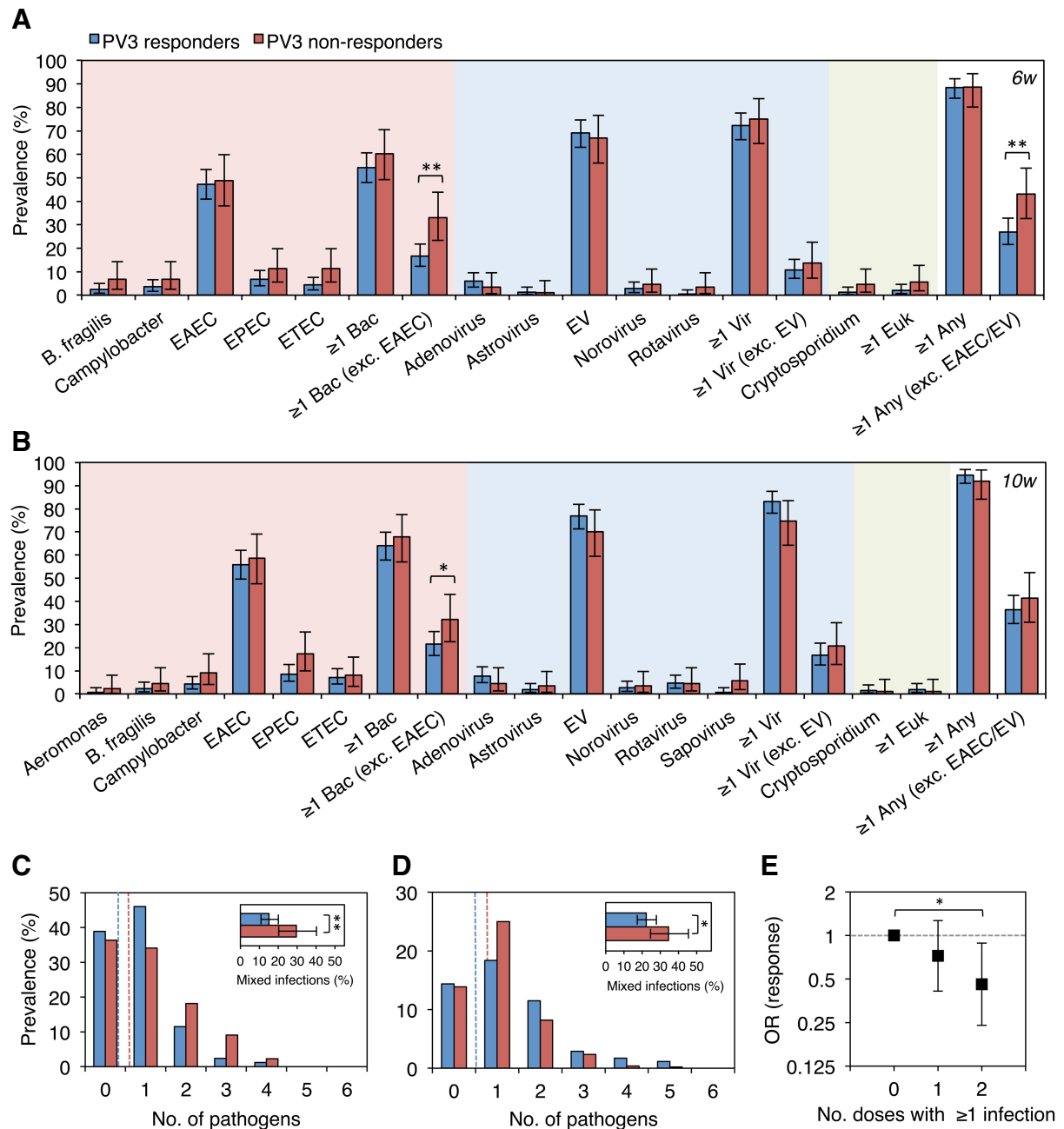


Figure 5.1. Association between concurrent pathogens and oral poliovirus vaccine immunogenicity. The prevalence of concurrent pathogens is displayed for tOPV doses administered at (A) 6 weeks and (B) 10 weeks of age. Pathogens present in at least 1% of the study population are included. (C, D) The number of pathogens and prevalence of mixed infections are displayed at (C) 6 weeks and (D) 10 weeks of age. Enteroviruses were excluded from these analyses. Mean pathogen counts are indicated by dotted lines. (E) Compared with individuals clear of infection at both doses, a decrease in the odds of seroconversion was evident among individuals with ≥ 1 infection at both doses. ORs and 95% CIs are indicated. EAEC and enterovirus infections were excluded. * $P < 0.05$; ** $P < 0.005$. Abbreviations: Bac, bacteria; EAEC, enteroaggregative *Escherichia coli*; EPEC, enteropathogenic *E. coli*; ETEC, enterotoxigenic *E. coli*; EV, enterovirus; Euk, eukaryote; OR, odds ratio; PV3, type 3 poliovirus; Vir, virus; w, weeks.

Table 5.2. Prevalence and abundance of enteropathogens by type 3 poliovirus seroconversion status.

Target	6 weeks						10 weeks					
	Prevalence, n (%)			Abundance, mean Ct $\pm$ s.d.			Prevalence, n (%)		Abundance, mean Ct $\pm$ s.d.			
	sero+ (n = 252)	sero- (n = 88)	P	FDR P	sero+ (n = 252)	sero- (n = 88)	P	FDR P	sero+ (n = 256)	sero- (n = 87)	P	FDR P
<i>Aeromonas</i>	1 (0.4)	0 (0.0)	1.000		34.9 $\pm$ 0.6	34.4 $\pm$ 2.6	0.046	0.163	35.0 $\pm$ 0.0	35.0 $\pm$ 0.2	0.251	0.705
<i>Bacteroides fragilis</i>	6 (2.4)	6 (6.8)	0.052	0.201					34.8 $\pm$ 1.4	34.7 $\pm$ 1.9	0.284	0.705
<i>Campylobacter</i> ^a	9 (3.6)	6 (6.8)	0.202	0.370	34.8 $\pm$ 1.1	34.6 $\pm$ 1.8	0.198	0.385	34.6 $\pm$ 2.1	34.1 $\pm$ 3.3	0.083	0.360
<i>Clostridium difficile</i>	3 (1.2)	0 (0.0)	0.571									
EAEC ^a	119 (47.2)	43 (48.9)	0.791	0.870	30.5 $\pm$ 6.4	29.9 $\pm$ 6.2	0.695	0.850	29.3 $\pm$ 6.3	28.7 $\pm$ 6.5	0.516	0.838
EPEC ^a	17 (6.7)	10 (11.4)	0.168	0.369	34.5 $\pm$ 2.2	34.5 $\pm$ 1.9	0.210	0.385	34.4 $\pm$ 2.1	33.7 $\pm$ 3.6	0.025	0.165
ETEC ^a	11 (4.4)	10 (11.4)	0.019	0.201	34.7 $\pm$ 1.7	34.2 $\pm$ 2.6	0.019	0.131	34.4 $\pm$ 2.5	34.3 $\pm$ 2.5	0.767	0.915
<i>Salmonella</i>	1 (0.4)	1 (1.1)	0.208									
<i>Shigella</i> / EIEC	0 (0.0)	0 (0.0)	1.000									
STEC ^a	3 (1.2)	0 (0.0)	0.571									
<i>Vibrio cholerae</i>	2 (0.8)	1 (1.1)	1.000									
Adenovirus ^a	15 (6.0)	3 (3.4)	0.580	0.797	34.8 $\pm$ 1.1	34.8 $\pm$ 1.6	0.362	0.564	34.7 $\pm$ 1.7	34.8 $\pm$ 1.3	0.325	0.705
Astrovirus	3 (1.2)	1 (1.1)	1.000	1.000	34.9 $\pm$ 1.1	34.8 $\pm$ 1.9	0.978	0.978	34.9 $\pm$ 1.2	34.8 $\pm$ 1.0	0.434	0.806
Enterovirus	174 (69.0)	59 (67.0)	0.728	0.870	30.2 $\pm$ 4.5	30.0 $\pm$ 4.6	0.936	0.978	29.4 $\pm$ 4.2	29.2 $\pm$ 4.9	0.849	0.920
Norovirus ^a	7 (2.8)	4 (4.5)	0.484	0.761	34.8 $\pm$ 1.3	34.5 $\pm$ 2.5	0.410	0.564	34.8 $\pm$ 1.4	34.5 $\pm$ 2.8	0.710	0.915
Rotavirus ^a	1 (0.4)	3 (3.4)	0.055	0.201	35.0 $\pm$ 0.1	34.9 $\pm$ 0.9	0.024	0.131	34.9 $\pm$ 0.9	34.9 $\pm$ 0.4	0.966	0.966
Sapovirus	1 (0.4)	1 (1.1)	0.451						35.0 $\pm$ 0.2	34.8 $\pm$ 1.3	0.005	0.061
<i>Cryptosporidium</i> ^a	3 (1.2)	4 (4.5)	0.077	0.211	34.9 $\pm$ 1.0	34.8 $\pm$ 1.7	0.050	0.163	34.9 $\pm$ 1.1	35.0 $\pm$ 0.2	0.775	0.915
<i>Giardia</i> ^a	2 (0.8)	1 (1.1)	1.000									

FDR correction and comparisons of abundance were carried out for pathogens present in at least 1% of the study population. Abbreviations: Ct, threshold cycle; EAEC, enteroaggregative *Escherichia coli*; EIEC, enteroinvasive *E. coli*; EPEC, enteropathogenic *E. coli*; ETEC, enterotoxigenic *E. coli*; FDR, adjusted by Benjamini-Hochberg false discovery rate correction; sero+, responders; sero-, non-responder; STEC, Shiga toxin-producing *E. coli*.

^a For enteropathogens assessed using multiple targets and/or in duplicate (see Table 3.1), the minimum Ct value was used for comparisons of pathogen abundance.

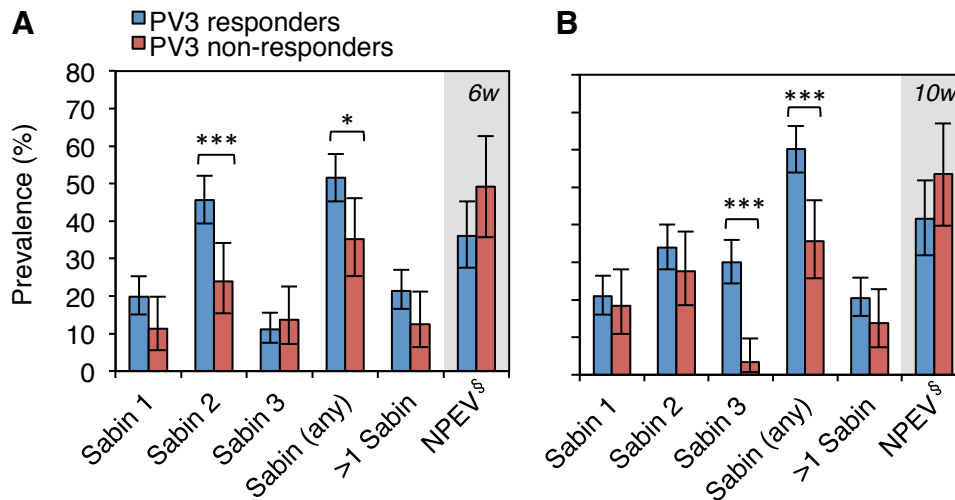


Figure 5.2. Association between enterovirus subgroups and oral poliovirus vaccine immunogenicity. Data are displayed for tOPV doses administered at (A) 6 weeks and (B) 10 weeks of age. § comparison of enterovirus prevalence among infants negative for all Sabin serotypes; * $P < 0.05$; *** $P < 0.0005$. Abbreviations: NPEV, non-polio enterovirus; PV3, type 3 poliovirus; w, weeks.

Among per-protocol infants in the study population as a whole, the proportion of individuals with diarrhoea in the 7 days preceding vaccination did not differ according to type 3 poliovirus seroconversion status – concurrent diarrhoea was observed in 17/448 (4%) responders and 1/103 (1%) non-responders at 6 weeks (Fisher’s test, $P = 0.219$), while the corresponding rates at 10 weeks of age were 20/448 (5%) and 4/103 (4%), respectively (Fisher’s test, $P = 1.000$).

iii. Pathogen prevalence over successive oral poliovirus vaccine doses

Compared with individuals free of infections at both 6 and 10 weeks of age, a decrease in the odds of OPV response was evident with each additional dose in which ≥ 1 concurrent infection (excluding EAEC and enterovirus) was present (OR 0.72, 95% CI 0.41–1.27 and OR 0.46, 95% CI 0.24–0.89 for infants infected at one and two doses, respectively; Figure 5.1E). A similar trend was evident when analyses were restricted to bacterial infections (OR 0.76, 95% CI 0.41–1.40 and OR 0.30, 95% CI 0.15–0.61, respectively). The presence of ≥ 1 virus or ≥ 1 eukaryote during at least one dose did not significantly impact the odds of seroconversion (OR 0.76, 95% CI 0.44–1.32 and OR 0.53, 95% CI 0.17–1.66, respectively).

5.3.3. Oral poliovirus vaccine take

Shedding of ≥ 1 Sabin serotype was observed in 184/341 (54%) individuals in the enteropathogen subset with eligible 10-week samples³⁰, while shedding of type 3 Sabin viruses was observed in 79/341 (23%). Among shedders of ≥ 1 serotype, Sabin viruses of types 1, 2, and 3 were observed in 69 (38%), 110 (60%), and 79 (43%) individuals, respectively (Figure 5.3A). The proportion of infants shedding Sabin viruses of serotypes 1 and 2 at 10 weeks of age did not differ according to type 3 Sabin virus shedding status (χ^2 , P values >0.05 ; Figure 5.3B).

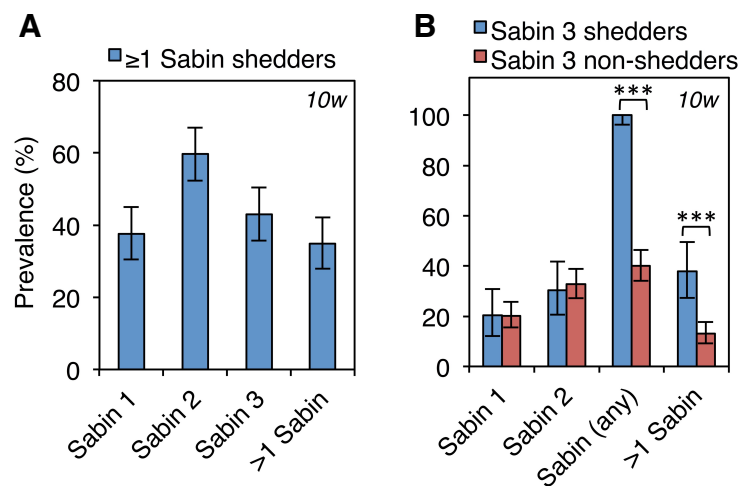


Figure 5.3. Shedding of Sabin serotypes at 10 weeks of age. (A) Prevalence of Sabin virus shedding by serotype at 10 weeks of age among shedders of ≥ 1 serotype ($n = 184$). (B) Prevalence of Sabin virus shedding according to type 3 virus shedding status. *** $P < 0.0005$. Abbreviation: w, weeks.

5.3.4. Association between concurrent enteropathogens and vaccine take

Eligible 6-week TAC assays were available for 333/341 (98%) infants included in the analysis of OPV take. In line with the results observed for vaccine immunogenicity, individuals who failed to shed ≥ 1 Sabin serotype were more likely than shedders to harbour ≥ 1 bacterial pathogen (excluding EAEC) in pre-vaccination samples (43/155 [28%] vs 25/178 [14%]; χ^2 , $P = 0.002$; Figure 5.4). This discrepancy was individually significant for EPEC (95/155 [14%] vs 5/178 [3%]; χ^2 , FDR-corrected $P = 0.002$) and was still apparent across bacterial pathogens when EAEC was included (22/155 [61%] vs 90/178 [51%]; χ^2 , $P = 0.049$). We observed no significant differences in the prevalence of viral pathogens (including Sabin serotypes and NPEVs; Figure 5.4E) or eukaryotes among 6-week samples according to shedding status. However, mixed infections were more common in non-shedders than

³⁰ As noted in Chapter 4, the detection of Sabin viruses was not performed for two infants with enterovirus-positive 10-week TAC assays. These infants were excluded from analyses of OPV take.

shedders (39/155 [25%] vs 23/178 [13%]; χ^2 , $P = 0.004$; Figure 5.4C) and the total number of pathogens was greater in these individuals (1.0 ± 1.0 [mean $\pm$ s.d.] vs 0.7 ± 0.8 ; Wilcoxon's rank sum, $P = 0.008$). At 10 weeks of age (i.e., concurrent with the measurement of Sabin viruses), non-shedders continued to harbour a greater burden of enteropathogens (including an increased prevalence of viral infections [excluding enterovirus] that was not apparent at 6 weeks of age; Figures 5.4B and 5.4D). However, the discrepancy in bacterial infection prevalence was no longer significant at this timepoint (Figure 5.4B). Comparisons of prevalence and abundance for individual TAC targets according to shedding status are summarised in Table 5.3.

When analyses were restricted to type 3 Sabin viruses, we observed no significant association in the prevalence of bacterial, viral, or eukaryotic infections at 6 weeks of age according to shedding status (Figure 5.5), although a borderline-significant increase in the likelihood of harbouring ≥ 1 bacterial pathogen (excluding EAEC) was observed in non-shedders compared with shedders (58/256 [23%] vs 10/77 [13%]; χ^2 , $P = 0.065$). Shedders were more likely than non-shedders to harbour type 2 Sabin viruses at 6 weeks of age (39/77 [51%] vs 95/256 [37%]; χ^2 , $P = 0.034$; Figure 5.5E), and were less likely to be infected with type 3 Sabin viruses (4/77 [5%] vs 35/256 [14%]; Fisher's test, $P = 0.044$). At 10 weeks of age, we observed no significant discrepancies in pathogen burden (aside from an elevated prevalence of enteroviruses) according to shedding status (Figures 5.5B and 5.5D). Moreover, aside from a decreased abundance of enteroviruses at 10 weeks of age among infants who failed to shed type 3 Sabin virus (Ct, 26.8 ± 2.8 vs 30.1 ± 4.5 in shedders and non-shedders, respectively; Wilcoxon's rank sum, FDR-corrected $P < 0.001$), we observed no significant difference in the abundance of TAC targets at either 6 or 10 weeks of age according to serotype 3 shedding status (FDR-corrected P values > 0.05 ; data not shown).

The proportion of infants with diarrhoea in the week preceding the 6-week OPV dose did not differ significantly according to the shedding of Sabin viruses (7/184 [4%] vs 4/157 [3%] in shedders and non-shedders of ≥ 1 serotype, respectively, and 2/79 [3%] vs 9/262 [3%] in shedders and non-shedders of serotype 3, respectively; Fisher's test, P values > 0.05).

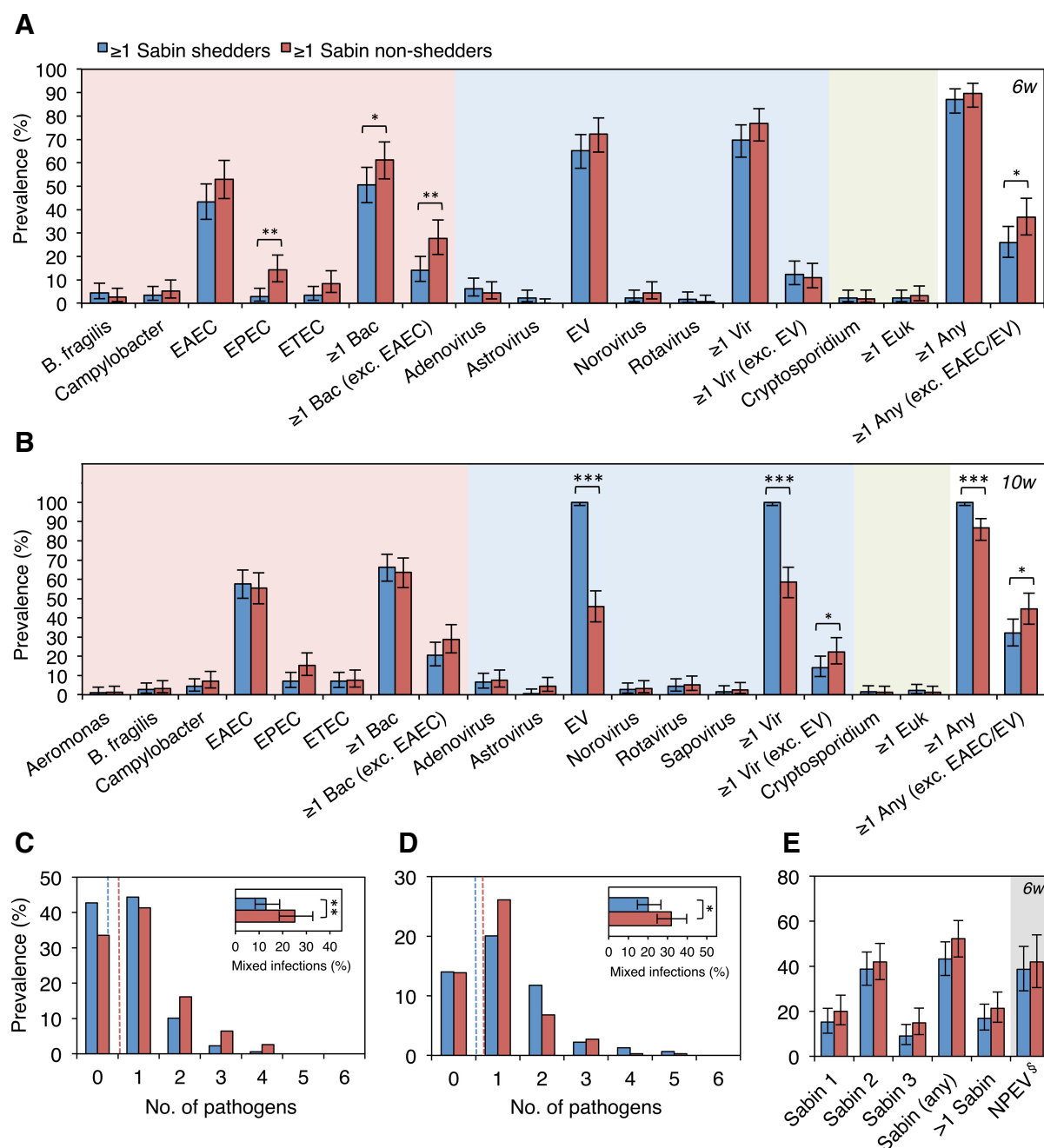


Figure 5.4. Association between concurrent pathogens and dose 1 oral poliovirus vaccine take. Pathogen prevalence is displayed for samples collected at (A) 6 weeks and (B) 10 weeks of age according to the shedding of ≥ 1 Sabin serotype at 10 weeks of age. Pathogens present in at least 1% of the study population are included. (C, D) The number of pathogens and prevalence of mixed infections are displayed at (C) 6 weeks and (D) 10 weeks of age. Mean pathogen counts are indicated by dotted lines. Enteroviruses were excluded from these analyses. (E) Prevalence of enterovirus subgroups at 6 weeks of age according to the shedding of ≥ 1 Sabin serotype 4 weeks later. [§] comparison of enterovirus prevalence among infants negative for all Sabin serotypes; * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$. Abbreviations: Bac, bacteria; EAEC, enteroaggregative *Escherichia coli*; EPEC, enteropathogenic *E. coli*; ETEC, enterotoxigenic *E. coli*; Euk, eukaryote; EV, enterovirus; NPEV, non-polio enterovirus; Vir, virus; w, weeks.

Table 5.3. Prevalence and abundance of enteropathogens by oral poliovirus vaccine take (≥ 1 Sabin).

Target	6 weeks						10 weeks					
	Prevalence, n (%)			Abundance, mean Ct $\pm$ s.d.			Prevalence, n (%)		Abundance, mean Ct $\pm$ s.d.			
	shed+ (n = 178)	shed- (n = 155)	P	shed+ (n = 178)	shed- (n = 155)	P	shed+ (n = 184)	shed- (n = 157)	P	shed+ (n = 184)	shed- (n = 157)	P
<i>Aeromonas</i>	0 (0.0)	1 (0.6)	0.465				2 (1.1)	2 (1.3)	1.000	35.0 $\pm$ 0.0	35.0 $\pm$ 0.2	0.866
<i>Bacteroides fragilis</i>	8 (4.5)	4 (2.6)	0.393	34.7 $\pm$ 1.4	34.8 $\pm$ 1.6	0.355	5 (2.7)	5 (3.2)	0.799	34.8 $\pm$ 1.4	34.8 $\pm$ 1.7	0.799
<i>Campylobacter</i> ^a	6 (3.4)	8 (5.2)	0.417	34.8 $\pm$ 1.4	34.8 $\pm$ 1.2	0.415	8 (4.3)	11 (7.0)	0.286	34.7 $\pm$ 1.7	34.3 $\pm$ 3.1	0.269
<i>Clostridium difficile</i>	1 (0.6)	2 (1.3)	0.600				1 (0.5)	1 (0.6)	1.000			0.875
EAEC ^a	77 (43.3)	82 (52.9)	0.079	30.5 $\pm$ 6.4	30.0 $\pm$ 6.3	0.240	106 (57.6)	87 (55.4)	0.684	29.3 $\pm$ 6.4	29.1 $\pm$ 6.4	0.966
EPEC ^a	5 (2.8)	22 (14.2)	<0.001	34.8 $\pm$ 0.9	34.1 $\pm$ 2.9	<0.001	13 (7.1)	24 (15.3)	0.015	34.5 $\pm$ 2.1	34.0 $\pm$ 2.1	0.017
ETEC ^a	6 (3.4)	13 (8.4)	0.049	34.7 $\pm$ 1.7	34.4 $\pm$ 2.2	0.050	13 (7.1)	12 (7.6)	0.838	34.4 $\pm$ 2.4	34.3 $\pm$ 2.7	0.834
<i>Salmonella</i>	1 (0.6)	1 (0.6)	1.000				1 (0.5)	1 (0.6)	1.000			0.938
<i>Shigella</i> / EIEC	0 (0.0)	0 (0.0)	1.000				0 (0.0)	1 (0.6)	0.460			
STEC ^a	1 (0.6)	2 (1.3)	0.600				3 (1.6)	0 (0.0)	0.253			
<i>Vibrio cholerae</i>	0 (0.0)	3 (1.9)	0.100				0 (0.0)	1 (0.6)	0.460			
Adenovirus ^a	11 (6.2)	7 (4.5)	0.503	34.7 $\pm$ 1.6	34.9 $\pm$ 0.4	0.473	12 (6.5)	12 (7.6)	0.687	34.8 $\pm$ 1.2	34.6 $\pm$ 2.0	0.639
Astrovirus	4 (2.2)	0 (0.0)	0.126	34.7 $\pm$ 1.9	35.0 $\pm$ 0.0	0.061	1 (0.5)	7 (4.5)	0.026	35.0 $\pm$ 0.2	34.7 $\pm$ 1.6	0.017
Enterovirus	116 (65.2)	112 (72.3)	0.165	30.3 $\pm$ 4.5	30.0 $\pm$ 4.5	0.446	184 (100.0)	72 (45.9)	<0.001	27.5 $\pm$ 3.1	31.5 $\pm$ 4.7	<0.001
Norovirus ^a	4 (2.2)	7 (4.5)	0.358	34.8 $\pm$ 1.1	34.6 $\pm$ 2.2	0.246	5 (2.7)	5 (3.2)	0.799	34.7 $\pm$ 1.9	34.7 $\pm$ 1.7	0.802
Rotavirus ^a	3 (1.7)	1 (0.6)	0.626	35.0 $\pm$ 0.2	34.9 $\pm$ 0.6	0.392	8 (4.3)	8 (5.1)	0.745	34.9 $\pm$ 0.7	34.9 $\pm$ 0.9	0.751
Sapovirus	0 (0.0)	2 (1.3)	0.216				3 (1.6)	4 (2.5)	0.708	35.0 $\pm$ 0.3	34.9 $\pm$ 1.0	0.548
<i>Cryptosporidium</i> ^a	4 (2.2)	3 (1.9)	1.000	34.8 $\pm$ 1.6	34.9 $\pm$ 0.6	0.831	3 (1.6)	2 (1.3)	1.000	35.0 $\pm$ 0.2	34.9 $\pm$ 1.4	0.799
<i>Giardia</i> ^a	0 (0.0)	2 (1.3)	0.216				1 (0.5)	1 (0.6)	1.000			0.938

FDR correction and comparisons of abundance were carried out for pathogens present in at least 1% of the study population. Abbreviations: Ct, threshold cycle; EAEC, enteroaggregative *Escherichia coli*; EIEC, enteroinvasive *E. coli*; EPEC, enteropathogenic *E. coli*; ETEC, enterotoxigenic *E. coli*; FDR, adjusted by Benjamini-Hochberg false discovery rate correction; shed+, shedders; shed-, non-shedders; STEC, Shiga toxin-producing *E. coli*.

^a For enteropathogens assessed using multiple targets and/or in duplicate (see Table 3.1), the minimum Ct value was used for comparisons of pathogen abundance.

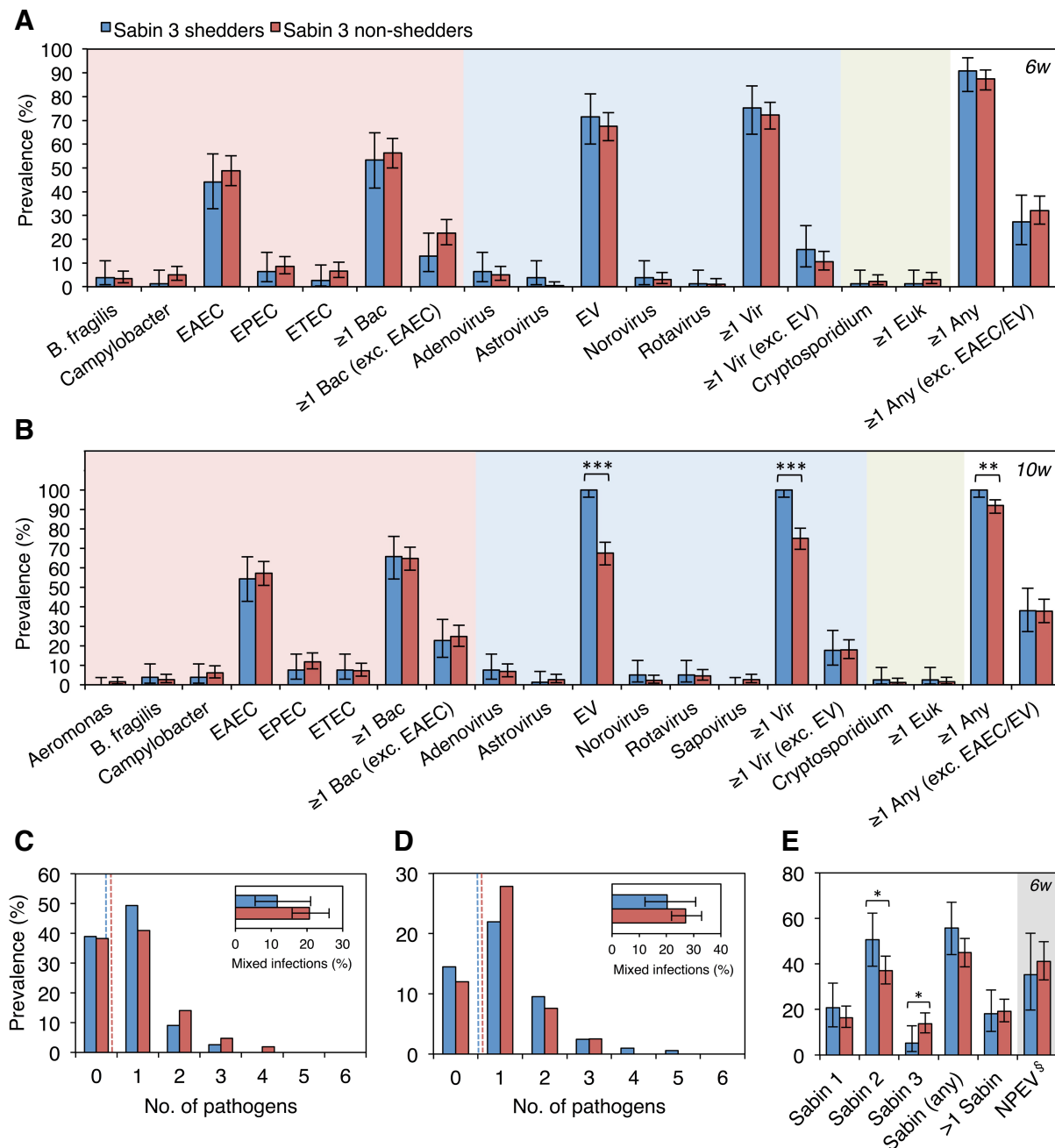


Figure 5.5. Association between concurrent pathogens and dose 1 take of type 3 Sabin viruses. Pathogen prevalence is displayed for samples collected at (A) 6 weeks and (B) 10 weeks of age according to the shedding of type 3 Sabin viruses at 10 weeks of age. (C, D) The number of pathogens and prevalence of mixed infections are displayed at (C) 6 weeks and (D) 10 weeks of age. Mean pathogen counts are indicated by dotted lines. Enteroviruses were excluded from these analyses. (E) Prevalence of enterovirus subgroups at 6 weeks of age according to the shedding of type 3 Sabin viruses 4 weeks later. $\S$ comparison of enterovirus prevalence among infants negative for all Sabin serotypes; * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$. Abbreviations: Bac, bacteria; EAEC, enteroaggregative *Escherichia coli*; EPEC, enteropathogenic *E. coli*; ETEC, enterotoxigenic *E. coli*; Euk, eukaryote; EV, enterovirus; NPEV, non-polio enterovirus; Vir, virus; w, weeks.

5.3.5. Attributable fraction of vaccine non-response associated with measured enteropathogens

If we assume our study population to be representative of the 103 non-responders and 448 responders included in the trial as a whole, our data are consistent with a per-dose type 3 poliovirus seroconversion rate of 61.6% among uninfected individuals and 48.0% among individuals infected with ≥ 1 enteropathogen (excluding EAEC and enterovirus). In a population experiencing a 30% chance of infection at the time of OPV delivery (the estimated prevalence of infections at 6 weeks of age in this study population), this disparity in immunogenicity would result in 9.5% of failed type 3 poliovirus seroconversions being attributable to concurrent infection per OPV dose. The corresponding proportion would rise to 26.0% after three doses. At an infection rate of 50%, the PAF of vaccine non-response associated with concurrent infection would increase to 14.9% per OPV dose and 38.5% after three doses (Figure 5.6).

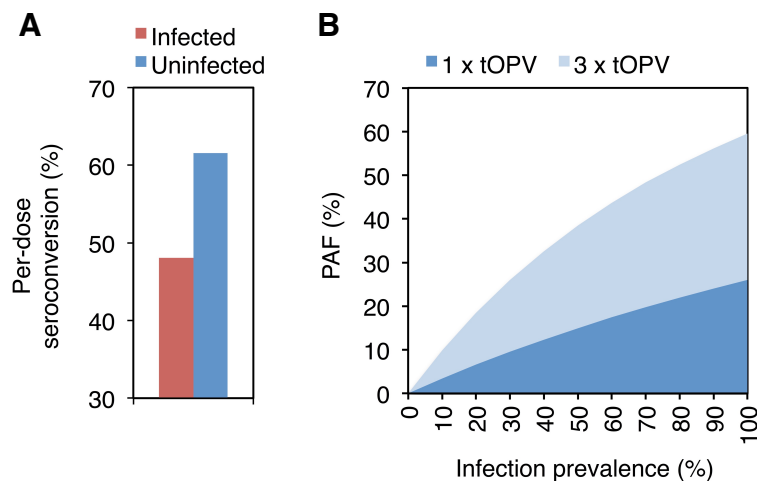


Figure 5.6. Population attributable fraction of oral poliovirus vaccine failure associated with concurrent infections. (A) Estimated per-dose seroconversion rates in infected versus uninfected individuals in this study population. (B) PAF of failed type 3 poliovirus seroconversions under different burdens of infection. Abbreviations: PAF, population attributable fraction; tOPV, trivalent oral poliovirus vaccine.

5.3.6. Association between bacterial microbiota composition and oral poliovirus vaccine outcome

i. Alpha diversity

Among the 168 per-protocol infants included in the 16S rRNA gene sequencing subset, 136 (81%) seroconverted to type 3 poliovirus, 99 (59%) shed ≥ 1 Sabin virus of any serotype at 10 weeks of age, and 41 (24%) shed type 3 Sabin virus at 10 weeks of age³¹. The baseline composition of the bacterial microbiota in these infants is described above (section 4.4.5). Comparisons of microbiota diversity according to OPV outcome are summarised in Table 5.4. In contrast to the results observed for rotavirus vaccine, type 3 poliovirus seroconversion was associated with a modest but significant decrease in the number of OTUs present at the time of vaccination, although this discrepancy was only apparent at 6 weeks of age (linear regression, P values 0.005 and 0.688 for 6- and 10-week comparisons, respectively; Figure 5.7). Similarly, OTU count was negatively correlated with the take of Sabin viruses following the 6-week dose of tOPV – a result that was significant when comparing infants who shed ≥ 1 Sabin serotype at 10 weeks of age with those who did not (Table 5.4). Comparisons of OTU count within the phylum Proteobacteria according to OPV outcome were generally not significant (Table 5.4), although the diversity of this phylum at 6 weeks of age was negatively correlated with the subsequent shedding of type 3 Sabin viruses (linear regression, $P = 0.011$). No significant differences in Shannon index were observed according to either seroconversion status or OPV take (Table 5.4).

ii. Beta diversity

No significant clustering of bacterial community composition was evident according to OPV outcome (seroconversion or take) based on unweighted or weighted Unifrac distances (adonis, P values >0.05 ; Table 5.5; Figure 5.8). Similarly, no significant difference in microbiota community turnover (as inferred by the Unifrac distances between 6- and 10-week samples of each individual) were observed according to serological response to OPV, shedding of type 3 Sabin viruses, or shedding of ≥ 1 Sabin serotype (Wilcoxon's rank sum, P values >0.05 ; data not shown).

³¹ Detection of Sabin serotypes was not performed for one individual in the microbiota subset who was positive for enterovirus (via TAC assay) at 10 weeks of age. Accordingly, the analyses of OPV take in this section include 167 per-protocol infants.

Table 5.4. Summary of alpha diversity analyses.

Comparison	Age (w)	N ^a	N ^b	Number of OTUs			Number of OTUs (Proteobacteria)			Shannon index		
				Effect Size	SE	P	Effect Size	SE	P	Effect Size	SE	P
PV3 seroconversion	6	136	32	-3.763	1.314	0.005	-0.537	0.393	0.173	-0.098	0.117	0.402
	10	136	32	-0.526	1.310	0.688	-0.072	0.397	0.856	-0.023	0.104	0.824
Dose 1 OPV take (≥1 Sabin)	6	99	68	-2.729	1.057	0.011	-0.238	0.316	0.453	-0.173	0.093	0.064
	10	99	68	0.264	1.045	0.801	-0.202	0.318	0.527	-0.044	0.084	0.601
Dose 1 OPV take (Sabin 3)	6	41	126	-2.157	1.219	0.079	-0.846	0.355	0.018	-0.126	0.107	0.239
	10	41	126	0.556	1.197	0.643	-0.064	0.365	0.861	-0.074	0.096	0.443
Sensitivity analyses												
PV3 seroconversion (≥1:256 vs <1:8, sero- at baseline)	6	39	13	-4.265	2.285	0.068	-	-	-	-0.073	0.197	0.712
	10	39	13	-3.131	1.927	0.111	-	-	-	-0.041	0.166	0.804
PV3 seroconversion (RV1 responders)	6	73	12	-4.420	2.031	0.032	-	-	-	-0.204	0.168	0.229
	10	73	12	-2.301	2.119	0.281	-	-	-	-0.297	0.175	0.094
PV3 seroconversion (RV1 non-responders)	6	63	20	-3.437	1.790	0.058	-	-	-	-0.051	0.167	0.760
	10	63	20	0.839	1.687	0.620	-	-	-	0.155	0.126	0.222
PV3 seroconversion (sero- at baseline)	6	72	13	-5.603	2.194	0.013	-	-	-	-0.176	0.188	0.350
	10	72	13	-3.752	2.151	0.085	-	-	-	-0.104	0.169	0.538

The impact of each factor on the specified measure of alpha diversity was assessed via linear regression, with MiSeq run included as a covariate. Abbreviations: OTU, 97%-identity operational taxonomic unit; OPV, oral poliovirus vaccine; PV3, type 3 poliovirus; RV1, Rotarix; SE, standard error; sero-, seronegative; w, weeks.

^a Number of responders/shedders.

^b Number of non-responders/non-shedders.

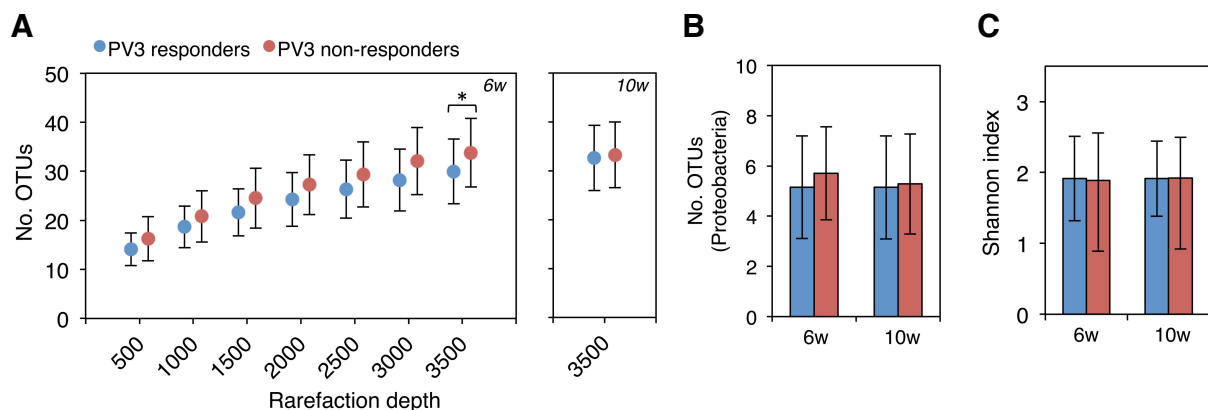


Figure 5.7. Association between microbiota diversity and oral poliovirus vaccine immunogenicity.

(A) Number of OTUs, (B) OTU count within the phylum Proteobacteria, and (C) Shannon index according to type 3 poliovirus seroconversion status. Mean values ($\pm$ s.d.) are displayed. Proteobacteria-specific OTU counts and Shannon index were calculated at a depth of 3,500 sequences per sample. * $P < 0.05$. Abbreviations: OTU, 97%-identity operational taxonomic unit; PV3, type 3 poliovirus; w, weeks.

Table 5.5. Summary of beta diversity analyses.

Comparison	Age (w)	Unweighted Unifrac		Weighted Unifrac	
		R ²	P	R ²	P
PV3 seroconversion	6	0.007	0.213	0.009	0.173
	10	0.004	0.589	0.001	0.941
Dose 1 OPV take (≥ 1 Sabin)	6	0.007	0.172	0.005	0.470
	10	0.005	0.611	0.009	0.202
Dose 1 OPV take (Sabin 3)	6	0.007	0.289	0.003	0.636
	10	0.005	0.696	0.005	0.408
Sensitivity analyses					
PV3 seroconversion ($\geq 1:256$ vs $<1:8$, sero- at baseline)	6	0.017	0.712	0.029	0.202
	10	0.019	0.537	0.010	0.686
PV3 seroconversion (RV1 responders)	6	0.018	0.035	0.020	0.163
	10	0.014	0.229	0.010	0.404
PV3 seroconversion (RV1 non-responders)	6	0.014	0.264	0.007	0.630
	10	0.012	0.426	0.002	0.915
PV3 seroconversion (sero- at baseline)	6	0.012	0.467	0.006	0.678
	10	0.013	0.296	0.006	0.629

See Table 5.4 for details regarding the number of infants included in each comparison. The impact of each factor on the specified measure of beta diversity was assessed using the function *adonis* in the R package *vegan*; 9,999 permutations were included in each comparison. Abbreviations: OPV, oral poliovirus vaccine; PV3, type 3 poliovirus; RV1, Rotarix; sero-, seronegative; w, weeks.

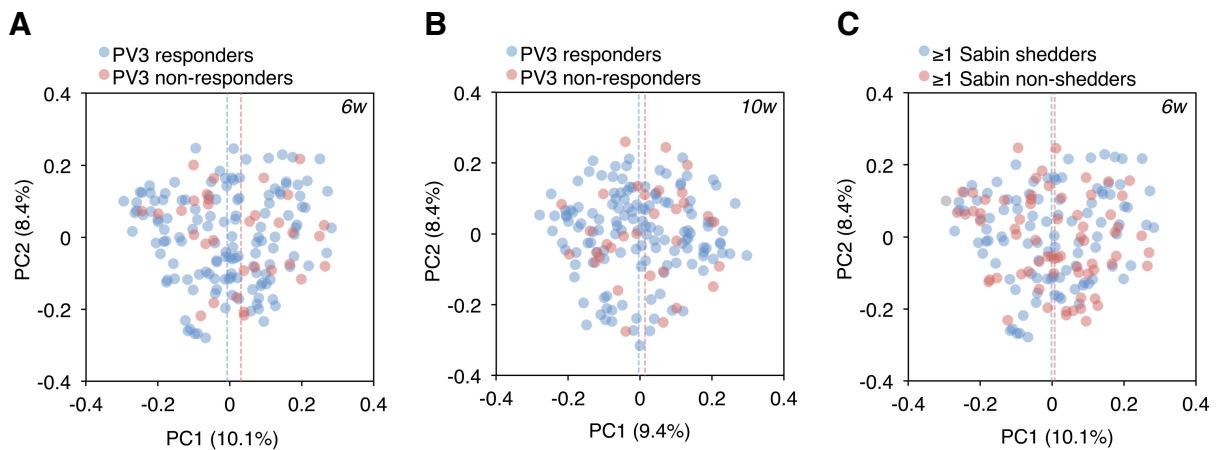


Figure 5.8. Clustering of samples according to oral poliovirus vaccine response. Unweighted Unifrac distances between samples, visualised via principal coordinates analysis, are displayed at (A) 6 weeks and (B) 10 weeks according to seroconversion status, and (C) according to OPV take following the 6-week vaccine dose (≥ 1 Sabin serotype). Mean values for principal coordinate 1 are indicated by dotted lines. Abbreviations: PC, principal coordinate; PV3, type 3 poliovirus; w, weeks.

iii. Taxon relative abundance

Comparisons of relative taxon abundance according to OPV outcome are summarised in Table 5.6. At 6 weeks of age, we observed a significant increase in the relative abundance of bacteria in the classes Betaproteobacteria and Epsilonproteobacteria among OPV non-responders. However, these discrepancies were driven by a small number of individuals – Betaproteobacteria were observed in 3/32 (9%) non-responders and 4/134 (3%) responders, while Epsilonproteobacteria were observed in 3/32 (9%) and 2/134 (1%) individuals in these groups, respectively. We observed no other phylum-, class-, or OTU-level differences in taxon relative abundance at 6 or 10 weeks of age according to poliovirus seroconversion status – either within the phylum Proteobacteria or across all other taxa. Similarly, no significant differences in relative abundance were observed at either 6 or 10 weeks of age according to the take of Sabin viruses (either of ≥ 1 Sabin serotype or of type 3) following the 6-week dose of tOPV (non-parametric t test, P values >0.05).

Table 5.6. Summary of significant differences in taxon abundance.

Level	Taxonomic classification	OTU ID	Age (w)	Relative abundance (%) in group 1 (mean $\pm$ s.d.)	Relative abundance (%) in group 2 (mean $\pm$ s.d.)	P	FDR P
PV3 seroconversion				<i>Non-responders</i>	<i>Responders</i>		
C	Epsilonproteobacteria		6	0.584 $\pm$ 2.026	0.006 $\pm$ 0.066	0.006 ^a	-
C	Betaproteobacteria		6	0.456 $\pm$ 2.198	0.006 $\pm$ 0.061	0.009 ^a	-
C	Verrucomicrobiae		6	0.079 $\pm$ 0.353	0.001 $\pm$ 0.007	0.013	0.050
Dose 1 OPV take (≥ 1 Sabin)							
NS							
Dose 1 OPV take (Sabin 3)							
NS							

Groups were compared using a non-parametric t test stratified by MiSeq run. Abbreviations: C, class; FDR, adjusted by Benjamini–Hochberg false discovery rate correction; NS, no significant differences; OPV, oral poliovirus vaccine; OTU, 97%-identity operational taxonomic unit; PV3, type 3 poliovirus; w, weeks.

^a P value was not adjusted as the OTU was within the phylum Proteobacteria, hypothesised *a priori* to be associated with vaccine outcome.

iv. Random Forests

Consistent with the lack of significant discrepancies in microbiota composition based on relative taxon abundance or Unifrac distances, Random Forests failed to accurately discriminate infants according to type 3 poliovirus seroconversion status or OPV take (Figure 5.9), and no significant improvement in classification accuracy was observed compared with

null models (P values >0.05). Indeed, across 100 iterations of the Random Forests algorithm using OTU relative abundances at either 6 or 10 weeks of age as input variables, the 32 OPV non-responders were misclassified as responders in every instance, reflecting the tendency of the tree-building algorithm to assign individuals to the majority class when there is a lack of discriminatory power in the predictors. The random under-sampling of responders to match the number of non-responders during each iteration of the Random Forest algorithm did not improve the predictive accuracy of the models (Figure 5.9B). This was also the case when under-sampling was used to generate balanced datasets with respect to the shedding of serotype 3 Sabin viruses (Figure 5.9E).

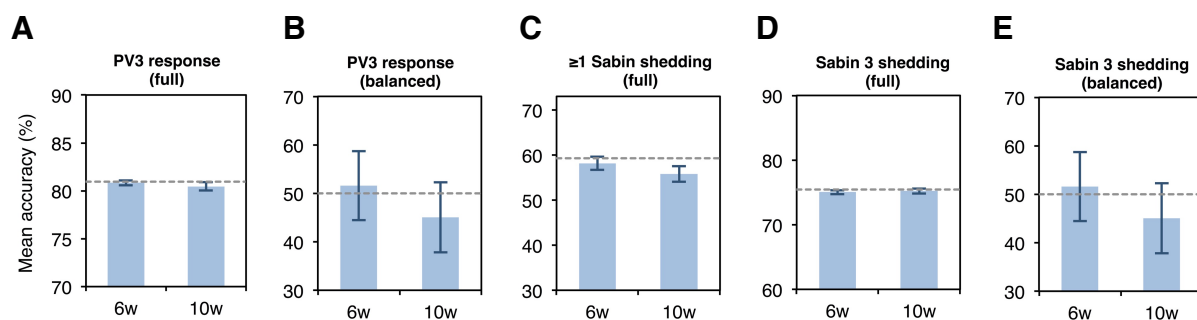


Figure 5.9. Prediction of oral poliovirus vaccine response using Random Forests. Estimates of mean accuracy ($\pm$ s.d.) based on 100 iterations of the Random Forests algorithm are displayed for models predicting (A) seroconversion status (full data), (B) seroconversion status using balanced data (including 32 responders and 32 non-responders in each iteration of the algorithm), (C) shedding of ≥ 1 Sabin serotype at 10 weeks of age; (D) shedding of serotype 3 Sabin viruses at 10 weeks of age (full data), and (E) shedding of serotype 3 Sabin viruses using balanced data (including 41 shedders and 41 non-shedders in each iteration of the algorithm). Dotted lines indicate baseline accuracy, wherein all individuals are assigned to the majority class. Abbreviations: PV3, type 3 poliovirus; w, weeks.

v. Multivariate logistic regression

In the primary analyses, OPV outcome was associated with both the presence of concurrent bacterial pathogens (excluding EAEC) and the number of OTUs at 6 weeks of age (comparisons that were significant when considering either seroconversion or the shedding of any Sabin serotype). To determine whether these variables were independently associated with OPV outcome, we compared the results of univariate logistic regression models (including each predictor separately) with multivariate models including both predictors (Table 5.7). Unfortunately, these analyses were constrained by the incomplete overlap of the microbiota subset with the enteropathogen dataset – the associations between bacterial

enteropathogens and OPV outcome were only significant when considering the TAC data as a whole. However, the association between OTU count and OPV outcome (either seroconversion or take) was significant and had a consistent effect size in both univariate and multivariate analyses, suggesting that the link between microbiota diversity and vaccine response was independent of the presence of bacterial enteropathogens detected via TAC.

Table 5.7. Multivariate logistic regression outputs.

Variable	Age (w)	N	Univariate		Multivariate	
			OR (95% CI)	<i>P</i>	OR (95% CI)	<i>P</i>
Model 1: PV3 seroconversion, full data						
≥1 Bac (exc. EAEC)	6	340	0.41 (0.23–0.71)	0.001	-	-
Model 2: PV3 seroconversion, microbiota subset						
≥1 Bac (exc. EAEC)	6	168	0.65 (0.25–1.70)	0.382	0.79 (0.29–2.11)	0.635
OTU count	6	168	0.92 (0.86–0.98)	0.006	0.92 (0.86–0.98)	0.007
Model 3: Dose 1 OPV take (≥1 Sabin), full data						
≥1 Bac (exc. EAEC)	6	333	0.43 (0.25–0.74)	0.002	-	-
Model 4: Dose 1 OPV take (≥1 Sabin), microbiota subset						
≥1 Bac (exc. EAEC)	6	167	0.53 (0.24–1.21)	0.133	0.61 (0.27–1.41)	0.250
OTU count	6	167	0.94 (0.90–0.99)	0.012	0.94 (0.90–0.99)	0.019

ORs signify the change in the relative odds of responding to OPV in conjunction with: (i) the presence versus absence of concurrent infection; or (ii) the addition of one OTU. Aside from those listed, no other variables were included in the multivariate models. Abbreviations: Bac, bacteria; CI, confidence interval; EAEC, enteroaggregative *Escherichia coli*; OR, odds ratio; OTU, 97%-identity operational taxonomic unit; PV3, type 3 poliovirus; w, weeks.

5.3.7. Sensitivity analyses

i. Threshold cycle

When Ct values of 30, 25, or 20 (as opposed to 35) were adopted as universal thresholds for pathogen detection, a more marked decrease in the odds of type 3 poliovirus seroconversion was apparent in conjunction with the presence of ≥1 enteropathogen during at least one of the 6- or 10-week tOPV doses (Table 5.8). Indeed, in contrast to the primary outcome analysis, the decrease in vaccine immunogenicity among infected infants was statistically significant at each of these thresholds. The discrepancies in the prevalence of bacterial enteropathogens at 6 and 10 weeks of age according to seroconversion status observed in the primary analyses were generally apparent at more stringent cut-offs (Figure 5.10). Moreover, at a Ct cut-off of 25, non-responders were more likely than responders to harbour ≥1 viral pathogen (excluding enterovirus) at 6 weeks of age (6/88 [7%] vs 4/252 [2%]; Fisher's test, $P = 0.022$).

Table 5.8. Summary of sensitivity analyses.

Sensitivity analysis	Proportion with ≥ 1 pathogen at 6 or 10 weeks, n/N (%)		OR (95% CI)
	Responders	Non-responders	
Primary outcome	120/250 (48.0)	51/85 (60.0)	0.62 (0.37–1.01)
Ct 30	71/250 (28.4)	37/85 (43.5)	0.51 (0.31–0.86)
Ct 25	39/250 (15.6)	22/85 (25.9)	0.53 (0.29–0.96)
Ct 20	6/250 (2.4)	9/85 (10.6)	0.21 (0.07–0.60)
$\geq 1:256$ vs $<1:8$, sero-	31/64 (48.4)	22/38 (57.9)	0.68 (0.30–1.53)
RV1 responders	66/131 (50.4)	17/24 (70.8)	0.42 (0.16–1.08)
RV1 non-responders	54/119 (45.4)	34/61 (55.7)	0.66 (0.35–1.23)
MS2+	95/202 (47.0)	40/72 (55.6)	0.71 (0.41–1.22)
sero-	63/139 (45.3)	22/38 (57.9)	0.60 (0.29–1.25)

Abbreviations: CI, confidence interval; Ct, threshold cycle; sero-, seronegative at baseline; MS2+, analysis restricted to infants with MS2-positive samples at 6 and 10 weeks of age; OR, odds ratio; RV1, Rotarix.

ii. Antibody titre

Among per-protocol infants in the enteropathogen survey who lacked type 3 poliovirus antibodies at enrolment ($n = 104$), 64 achieved a post-vaccination titre of $\geq 1:256$. When these ‘robust responders’ were compared with infants who failed to seroconvert (and were also seronegative at baseline), the effect size of the primary outcome was consistent with that observed using standard seroconversion criteria (Table 5.8), albeit with broader CIs. The increased prevalence of bacterial pathogens, mixed infections, and infections of any kind (excluding enterovirus and EAEC) among non-responders observed in the primary analyses were still apparent (data not shown), and the scale of the discrepancies were comparable to those observed using standard seroconversion criteria. Comparisons of alpha diversity and beta diversity were similarly unchanged when comparing robust responders with non-responders (Tables 5.4 and 5.5).

iii. Stratification by rotavirus seroconversion status

To account for the potential introduction of bias via the enrichment of our study population with Rotarix responders, we examined the impact of stratifying the analysis by rotavirus seroconversion status. Irrespective of rotavirus vaccine outcome, the presence of ≥ 1 enteropathogen at either 6 or 10 weeks of age was associated with a reduction in the odds of type 3 poliovirus seroconversion, albeit non-significant (Table 5.8). Dose-specific

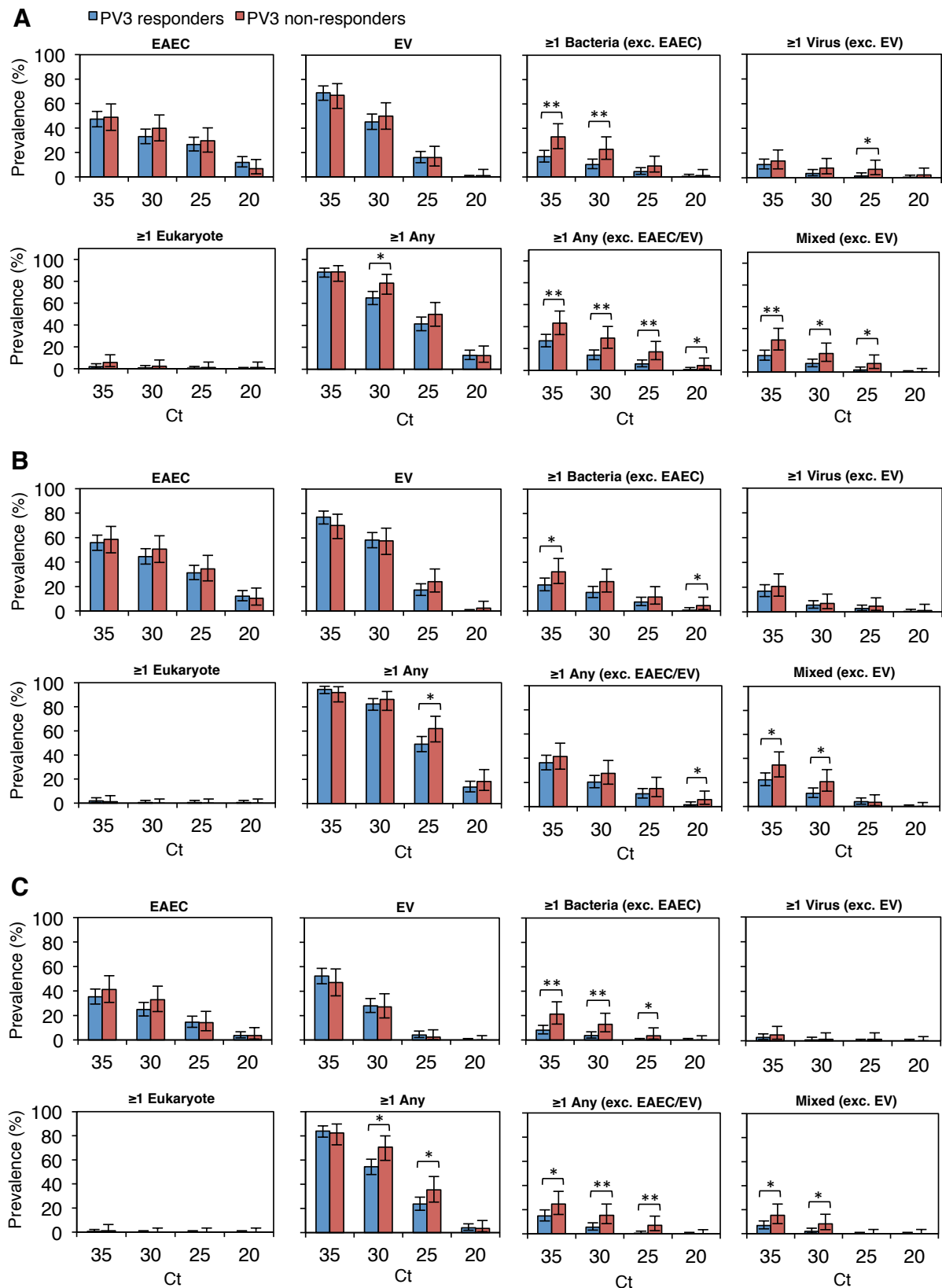


Figure 5.10. Impact of threshold cycle on the association between concurrent pathogens and oral poliovirus vaccine immunogenicity. The proportion of infants infected with the specified pathogen or pathogen group at (A) 6 weeks, (B) 10 weeks, and (C) both timepoints is displayed. * $P < 0.05$; ** $P < 0.005$. Abbreviations: Ct, threshold cycle; EAEC, enteroaggregative *Escherichia coli*; EV, enterovirus; PV3, type 3 poliovirus.

discrepancies in pathogen burden were also robust to Rotarix outcome. In particular, at 6 weeks of age, the increased prevalence of bacterial infections among infants who failed to seroconvert to type 3 poliovirus was consistent across Rotarix responders (12/24 [50%] vs 28/132 [21%]; χ^2 , $P = 0.003$) and non-responders (17/64 [27%] vs 14/120 [12%]; χ^2 , $P = 0.010$). Other discrepancies observed in the primary analyses were generally conserved across Rotarix responders and non-responders, although tests were not always statistically significant within these stratified comparisons (data not shown). Comparisons of alpha and beta diversity were largely unaffected in this sensitivity analysis (Tables 5.4 and 5.5).

iv. Treatment group interactions

In the primary analysis, we observed a borderline-significant decrease in the odds of poliovirus seroconversion when ≥ 1 concurrent infection was present during at least one dose of tOPV (Table 5.8). When we added interaction terms between enteropathogen infection status (presence vs absence of ≥ 1 concurrent infection at 6 or 10 weeks of age) and either zinc supplementation (zinc vs no zinc), probiotic supplementation (probiotics vs no probiotics), or study arm, no significant interactions were observed (data not shown).

v. Other sensitivity analyses

Associations between enteropathogen infection and type 3 poliovirus seroconversion status were largely unchanged by the exclusion of samples lacking MS2 (Table 5.8). Dose-specific comparisons of pathogen prevalence were generally consistent with the primary analyses, though some significant discrepancies became borderline-significant in the sensitivity analysis and vice versa (data not shown). Similarly, the negative correlation between concurrent enteropathogens and seroconversion was still apparent when individuals with type 3 poliovirus antibodies at enrolment (6 weeks of age) were excluded (such antibodies were observed in 264/551 [48%] infants in this study population). Although the reduction in the odds of seroconversion among infants infected during at least one OPV dose was not significant (Table 5.8), non-responders were more likely than responders to harbour ≥ 1 enteropathogen (excluding EAEC and enterovirus) at both doses (11/38 [29%] vs 16/139 [12%]; χ^2 , $P = 0.008$) – a discrepancy primarily attributable to an increased prevalence of bacterial infection (excluding EAEC) in these infants at 6 weeks (14/39 [36%] vs 19/140 [14%]; χ^2 , $P = 0.001$), 10 weeks (14/38 [36%] vs 27/141 [19%]; χ^2 , $P = 0.027$), or both timepoints (10/38 [26%] vs 9/139 [6%]; χ^2 , $P < 0.001$).

5.4. Discussion

The findings presented above provide clear support for the notion that enteric infections have the capacity to inhibit OPV response. During vaccine doses administered at 6 and 10 weeks of age, a higher burden of enteropathogens was observed in infants who failed to respond serologically to type 3 poliovirus, and an increase in the odds of non-response was apparent with each additional dose in which ≥ 1 enteropathogen was present.

A key novelty of this study was the assessment of multiple enteropathogens in each sample. As highlighted in the systematic review described in Chapter 2, most previous studies concerning the influence of enteric infections on OPV have focused on the role of NPEVs. By contrast, we used TACs to assess 31 putative viral, bacterial, and eukaryotic infections, thus providing a comprehensive view of the pathogen burden present within each sample. This approach revealed a greater prevalence of mixed infections among OPV non-responders, while also implicating concurrent bacterial infections as a potential determinant of oral vaccine response in early infancy – at both 6 and 10 weeks of age, non-responders were significantly more likely than responders to harbour ≥ 1 bacterial infection (excluding EAEC), while bacterial pathogens were negatively correlated with the replication of Sabin viruses administered at 6 weeks of age. Overall, our findings were consistent with per-dose seroconversion rates for type 3 poliovirus of 48% and 62% among infected and uninfected infants, respectively – a discrepancy that would result in approximately 10% of failed seroconversions per dose of tOPV being attributable to concurrent enteropathogens at the infection rates observed in this study population.

A challenge with the use of PCR-based pathogen detection is the effective distinction of clinically relevant infections from low-level infections of equivocal significance. The sensitivity of TACs has been shown to markedly exceed that of conventional enteropathogen assays (such as culture, antigen detection, and microscopy), particularly for bacterial targets (Liu et al, 2013). When TAC assays were used to compare pathogen burden in diarrhoeal versus pre-diarrhoeal stool samples, Platts-Mills et al (2014) observed significant associations between infection and disease only when pathogen quantity was considered. In the present study, the abundance of individual enteropathogens tended to be comparable between responders and non-responders. However, we also performed a semi-quantitative sensitivity analysis in which more stringent Ct cut-offs were adopted for pathogen detection. This revealed a more marked decrease in the odds of poliovirus seroconversion in conjunction with the presence of high copy-number pathogen infections. Moreover, the elevated burden of enteropathogens among non-responders was no longer contingent on the exclusion of

EAEC when lower Ct cut-offs were applied. Together, these findings suggest that infections of greater severity may have a more pronounced inhibitory effect on OPV outcome.

Although bacterial enteropathogens have previously been linked with a reduction in the titre of antibodies induced by OPV (Maldonado et al, 1997; Taniuchi et al, 2016), this is the first study to document a significant decrease in seroconversion rate in conjunction with bacterial infections during early infancy. Moreover, since the majority of infections in this study occurred in the absence of diarrhoea, our findings implicate subclinical bacterial infections as a potential source of interference with OPV. The inhibitory effect of individual enteropathogens was generally not significant after correcting for multiple comparisons. However, whether considering seroconversion or vaccine take, ETEC, EPEC, and *Campylobacter* were consistently more prevalent among OPV non-responders, resulting in a significant interference effect when combining across bacterial enteropathogens. Given that the mechanisms of pathogenesis vary widely across these infections, their consistent influence on OPV (if we assume a causal relationship) is somewhat surprising. For instance, whereas ETEC adheres to the intestinal mucosa and elicits symptoms via the release of heat-labile and heat-stable enterotoxins, EPEC attaches to the intestinal epithelium and induces characteristic lesions (termed ‘attaching and effacing lesions’) via the destruction of microvilli and the formation of dense plaques of actin (Hodges and Gill, 2010). In spite of their different modes of attack, each of these pathogens has potent immunomodulatory properties (Bahrami et al, 2011), and it is certainly plausible that they might upregulate common innate immune mediators (e.g., type I or type III interferons [Bierne et al, 2012; Long et al, 2014]) that inhibit the replication of vaccine polioviruses, even in the absence of overt diarrhoeal symptoms.

It is also possible, however, that the observed associations might arise in the absence of a direct inhibitory effect of bacterial enteropathogens on OPV response. As noted above (section 2.4), malnutrition, EE, and other putative risk factors for OPV non-response are likely to be correlated with (or indeed caused by) early enteropathogen exposure, and it may well be these factors that are responsible for OPV non-response in this study population. This scenario would undermine estimates of the fraction of OPV non-response attributable to enteropathogens given that these assume a causal relationship to exist between concurrent infections and vaccine failure.

The elevated prevalence of type 2 Sabin viruses among responders at 6 weeks of age – evident when considering either seroconversion or the take of type 3 Sabin viruses – is in keeping with previous studies of sequential tOPV administration. In particular, Morimoto

(2001) elegantly demonstrated that Sabin viruses of serotypes 1 and 2 tend to replicate at the expense of serotype 3 following primary exposure to tOPV. The ensuing serotype-specific mucosal immune response – likely mediated by secretory IgA – prevents the replication of these serotypes on subsequent tOPV exposure, enabling type 3 Sabin viruses to replicate. Similarly, in the present study, the observation of type 2 Sabin viruses at 6 weeks of age is likely to be indicative of replication following the birth dose of tOPV, or possibly community exposure.

We cannot rule out the possibility that the birth dose of tOPV induced seroconversion to type 3 poliovirus in a portion of infants in this study (though a recent study in India documented type 3 poliovirus seroconversion in just 4% of infants following the administration of tOPV at birth [Sutter et al, 2010]). In such infants, one would not expect the antibody titre to decay between 6 and 14 weeks of age (as is the case for passively acquired maternal antibodies), thereby invalidating the criteria we used to define poliovirus seroconversion. However, when we excluded infants with type 3 poliovirus-specific antibodies at baseline (encompassing both maternal antibodies as well as those induced by tOPV at birth), the discrepancies in pathogen burden between responders and non-responders were still apparent. Thus, prior tOPV exposure does not appear to have impacted the primary outcomes of this study.

In contrast to the results of the systematic and meta-analysis reported above, we did not observe a significant inhibitory effect of NPEVs on OPV response in this study population. However, this may simply reflect a lack of power to detect such an effect given the limitations in the methods of NPEV detection adopted (as discussed in detail in section 4.5). These limitations notwithstanding, at both 6 and 10 weeks of age, NPEVs were more common among infants who failed to seroconvert to type 3 poliovirus, and it is possible that a significant interference effect (and a greater PAF of vaccine non-response associated with concurrent infection) would have been observed had a more specific diagnostic assay for these viruses been adopted. The interaction between NPEVs and OPV response is one that we shall return to in the following chapter.

What are the implications of these analyses for the conceptual model linking OPV and oral rotavirus vaccine outcome outlined in the previous chapter (Figure 4.18)? To briefly recap, we hypothesised that the increased burden of bacterial enteropathogens among Rotarix responders might reflect an inhibitory effect of these pathogens on OPV response, and a consequent reduction in interference between vaccine polioviruses and Rotarix. The findings presented in this chapter highlight an association between bacterial pathogens and

OPV response of equal scale but opposite direction to that observed for Rotarix (indeed, Figures 4.2A and 5.1A form neat mirror images), and are thus consistent with the proposed model.

Once again, we did not observe any strong associations between the composition of the bacterial microbiota and oral vaccine outcome: no significant shift in community structure was evident based on unweighted or weighted Unifrac distances; Random Forest models failed to accurately discriminate infants according to seroconversion status or vaccine take; and aside from a handful of class-level associations driven by a small number of individuals, no significant discrepancies in taxon relative abundance were observed with respect to OPV response. However, contrary to the hypothesised association between microbiota diversity and oral vaccine outcome, the number of OTUs present at 6 weeks of age was negatively correlated with OPV response (either seroconversion or take) – a result that is (again) contradictory to the relationship observed for Rotarix. Notably, the negative association between OTU count and poliovirus seroconversion remained significant when this variable was included alongside bacterial pathogen infection status during multivariate logistic regression. Although we cannot rule out confounding of OTU count with an alternative risk factor for vaccine failure, these findings raise the possibility that an increase in microbiota diversity may inhibit the immunogenicity of vaccine polioviruses.

As noted in the previous chapter, the restricted geographic and sociodemographic diversity of infants in this study population may have undermined our capacity to identify compositional differences in the bacterial microbiota that contribute to the discrepancies in oral vaccine immunogenicity between low- and high-income settings. It is also important to recognise that the microbiota subset was selected so as to balance the number of Rotarix responders and non-responders, and thus included just 32 infants who failed to seroconvert to type 3 poliovirus after two doses. A more in-depth analysis of the potential impact of the bacterial microbiota on OPV response will be described in the following chapter.

In contrast to the poor response observed for oral rotavirus vaccine, the seroconversion rate for type 3 poliovirus in this study population – at 81% after two tOPV doses – was high. Patriarca et al (1991) reported an average seroconversion rate for this serotype of 70% (range, 40–100%) after three doses of tOPV among studies conducted in developing countries, consistent with an average per-dose response rate of <35%. The extent to which our findings are representative of factors influencing OPV immunogenicity in other tropical, low-income settings is therefore uncertain. Nonetheless, we estimated the PAF of OPV non-response associated with different burdens of enteric disease. Among infants in Vellore, our results

suggest that upwards of 25% of failed type 3 poliovirus seroconversions after three tOPV doses may be attributable to the enteropathogens measured in this study. In Afghanistan and Pakistan, where poliovirus remains endemic, the burden of enteropathogens in early infancy is likely to exceed that observed in the present study, as evidenced by the recent MAL-ED cohort study (Platts-Mills et al, 2015). At a per-dose infection rate of 50%, we estimate the PAF of OPV non-response associated with enteropathogens to be approximately 40% after three doses.

5.4.1. Conclusions

Overall, this study supports the link between concurrent enteric infections and impaired response to OPV, and emphasises the key role of enteropathogens as a barrier to the optimal use of OPV in regions where poliovirus transmission continues. By examining multiple bacterial, viral, and eukaryotic infections, our findings are among the first to demonstrate this association in the context of the diverse array of pathogens to which infants living in tropical, low-income countries are exposed.

Chapter 6: Influence of the intestinal microbiota on oral poliovirus vaccine response among infants in south India treated with azithromycin or placebo in a randomised controlled trial

6.1. Background

Before continuing with the final research chapter of this thesis, it is worth briefly taking stock of where we stand. So far, I have presented findings from a systematic review and meta-analysis that supported the inhibitory effect of concurrent enteropathogens – including NPEVs and diarrhoea – on the immunogenicity and take of OPV. In the previous chapter, we extended these findings by testing for a diverse array of bacterial, viral, and eukaryotic enteropathogens among infants in south India who received tOPV at 6 and 10 weeks of age. In doing so, we observed a marked increase in enteropathogen burden among OPV non-responders driven primarily by asymptomatic bacterial infections. Overall, our findings suggested that approximately 10% of failed type 3 poliovirus seroconversions per dose of tOPV might be attributable to enteric co-infections in this study population.

Findings with regard to Rotarix, which we examined in the same group of infants, were more ambiguous. I do not intend to recap them here, but suffice it to say that we did not observe a significant inhibitory effect of enteropathogens on the immunogenicity or take of this vaccine, nor did we observe any marked discrepancies in the taxonomic composition of the 16S rRNA microbiota according to vaccine outcome. Although additional exploration of the factors that influence oral rotavirus vaccine response is certainly warranted, such work is beyond the scope of the present thesis. Our attention in the remaining pages lies solely with OPV.

6.1.1. The EVI trial

On 5th August 2014, enrolment commenced for the Enteric infection and Vaccine Immunogenicity (EVI) study. Conducted across 14 blocks in Vellore, this trial set out to examine the impact of a 3-day course of oral azithromycin on the immune response following a subsequent dose of mOPV3 among 6–11 month-old infants who lacked immunity to type 3 poliovirus. By diminishing the burden of enteropathogens prior to vaccination, it was hoped that an increase in mOPV3 immunogenicity might be observed in azithromycin compared with placebo recipients. The primary outcomes of the EVI trial were recently published (Grassly et al, 2016). Notably, while antibiotic treatment significantly reduced the prevalence of bacterial enteropathogens and the levels of several faecal

biomarkers of intestinal inflammation (including calprotectin, myeloperoxidase, and α 1-antitrypsin), it did not significantly impact OPV immunogenicity – among children who completed the trial per protocol, seroconversion was observed in 175/348 (50%) azithromycin recipients and 192/357 (54%) placebo recipients.

In contrast to the findings presented in the previous chapter, the prevalence of bacterial enteropathogens did not differ significantly according to vaccine outcome in the EVI trial, suggesting that the determinants of OPV immunogenicity in early infancy may differ from those in older children. However, seroconversion was negatively impacted by the presence of viral pathogens (particularly enteroviruses, of which the vast majority can be assumed to be NPEVs in light of the low prevalence of Sabin polioviruses [$<2\%$] among enterovirus-positive samples obtained on the day of vaccination). Immunogenicity was negatively correlated with age in this study population (seroconversion was observed in 227/408 [56%] infants aged 6–7 months and 140/297 [47%] aged 8–11 months; Fisher’s test, $P = 0.027$). After adjusting for age, the inhibitory effect of viral pathogen count on the odds of seroconversion remained significant.

6.1.2. Objectives

The samples collected during the EVI study provided us with an opportunity to probe further into the relationship between the intestinal microbiota and oral vaccine outcome. First, by assessing the 16S rRNA microbiota in a subset of trial participants, we sought to determine the impact of azithromycin on the bacterial microbiota, and of the bacterial microbiota on the immune response to mOPV3. Although the latter was considered in the previous chapter, our sequencing data encompassed only 32 infants who failed to respond to OPV, and may therefore have lacked sufficient power to detect discrepancies in microbiota composition pertinent to vaccine outcome. Furthermore, considering that the gut’s bacterial community is in a constant state of maturation for the first 2 years of life (Yatsunen et al, 2012; Subramanian et al, 2015), the impact of microbiota composition on oral vaccine outcome at 6–11 months of age (a window in which OPV is regularly administered during SIAs) may well differ from that at 1–2 months.

The second line of enquiry in this chapter concerns the turnover in the gut’s microbial residents. Since stool samples were collected on the day of vaccination and 14 days earlier, it was possible to assess how changes in pathogen prevalence in the two weeks preceding vaccination impacted response to OPV. Specifically, did novel enteropathogen infections

inhibit mOPV3 response to a greater extent than persistent infections (e.g., owing to the transient up-regulation of innate immune effectors [Juhela et al, 1999])? And how did the recent resolution of an enteric infection influence vaccine response?

Finally, we sought to make inroads into the challenge of integrating different forms of data when assessing the determinants of oral vaccine immunogenicity. The data collected as part of the EVI trial are diverse and extensive, encompassing anthropometric measurements, sociodemographic variables, antibiotic usage, enteropathogen prevalence, and (following the additional assays described below) the composition of the bacterial microbiota. Using the Random Forest machine-learning techniques introduced in Chapter 3, we assessed whether combining these different forms of data enhanced our capacity to predict mOPV3 outcome.

6.1.3. Hypotheses

- i) Treatment with azithromycin depletes the diversity of the bacterial microbiota;
- ii) mOPV3 non-responders have a bacterial microbiota composition that is distinct from responders, as inferred by discrepancies in alpha diversity, sample clustering based on Unifrac distances, and the discriminatory capacity of Random Forest models;
- iii) Novel enteropathogen infections inhibit mOPV3 response to a greater extent than persistent infections;
- iv) Combining different modules of data (enteropathogen prevalence, 16S rRNA microbiota composition, anthropometry, etc.) increases the capacity of Random Forest models to discriminate infants according to mOPV3 outcome.

6.2. Methods

6.2.1. Study population

A full description of the study design and laboratory methods adopted during the EVI trial was recently published by Grassly et al (2016). Briefly, the trial was performed among 6–11 month-old infants in Vellore. Individuals were initially screened for the presence of serum neutralising antibodies to type 3 poliovirus and considered eligible for the study if they lacked detectable antibodies at a dilution of 1 in 8. At enrolment, infants were randomised 1:1 to receive a 3-day course of azithromycin (a broad-spectrum bacteriostatic macrolide antibiotic administered once daily at a dose of 10 mg/kg) or placebo, starting on study day 0. Two weeks later (day 14), all participants were given a single dose of mOPV3 containing at least $10^{5.8}$ median cell culture infective doses of Leon 12a,b strain type 3

poliovirus. The primary outcome was seroconversion to type 3 poliovirus, defined as the detection of neutralising antibodies at a dilution of 1 in 8 or higher in serum samples collected 3 weeks after immunisation (day 35). Stool samples collected on days 0 and 14 (before treatment and immunisation, respectively) were tested for the presence of enteropathogens using TACs (see Table 3.1 for a list of targets). In a subset of individuals, including 291 infants who completed the trial per protocol, biomarkers of intestinal inflammation (calprotectin, myeloperoxidase, neopterin, and α 1-antitrypsin) were tested in stool samples collected at enrolment (day 0) and pre-vaccination (day 14). In the same subset, shedding of type 3 poliovirus was assessed 7 days after vaccination (day 21) as a measure of OPV take. Key features of the EVI trial are summarised in Figure 6.1, and a comparison of this study with the zinc/probiotic supplementation trial described in Chapters 4 and 5 is provided in Table 6.1. Methods for the extraction of nucleic acids and set-up of TACs followed those described in Chapter 3. However, to place an emphasis on high copy-number infections, a Ct value of 30 was adopted as a universal threshold for pathogen detection. For consistency with the primary analyses, the same cut-off was applied where relevant in the present study. The trial was funded by the Bill & Melinda Gates Foundation.

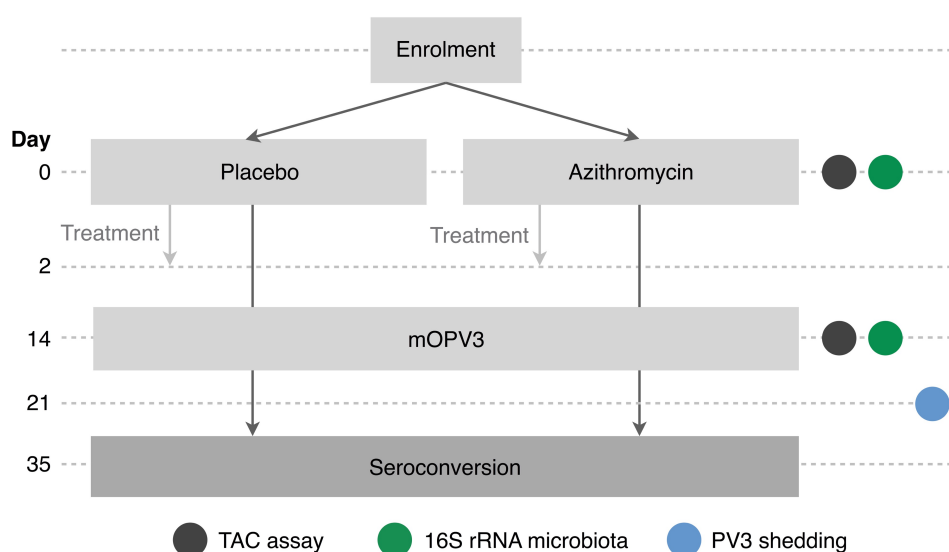


Figure 6.1. EVI trial design. Assays of enteropathogen burden and bacterial microbiota composition that form the basis of this chapter's analyses are indicated. Abbreviations: TAC, TaqMan array card; mOPV3, monovalent type 3 oral poliovirus vaccine; PV3, type 3 poliovirus.

Table 6.1. Comparison of the zinc/probiotic supplementation trial and EVI trial.

Feature	Zinc/probiotic supplementation trial	EVI trial
Setting	Vellore, India	Vellore, India
Age at enrolment	5 weeks	6–11 months
Enrolment criteria (baseline antibody titre)	- Negative for type 3 poliovirus antibodies	
Treatment	Zinc and probiotics, probiotics, zinc, or placebo; daily from 5–11 weeks	Azithromycin or placebo; 3-day course starting on study day 0
Oral vaccines	Rotarix and tOPV at 6 and 10 weeks	mOPV3 on study day 14
Primary outcome	Seroconversion between 6 and 14 weeks	Seroconversion between study days 0 and 35
Measurement of enteropathogens/ 16S rRNA microbiota	6 and 10 weeks	Study days 0 and 14

Abbreviations: mOPV3, type 3 oral poliovirus vaccine; tOPV, trivalent oral poliovirus vaccine.

6.2.2. 16S rRNA gene sequencing

A subset of 120 infants (60 per study arm) out of the first 300 individuals enrolled in the study were selected at random for assessment of the bacterial microbiota. For each individual, we assessed stool samples collected before treatment (day 0) and pre-vaccination (day 14). We also assessed samples collected from 40 adults cohabiting with trial participants. DNA extraction, amplification of the 16S rRNA gene V4 region, and library preparation was performed as previously described (section 3.3)³². Extraction and PCR for infant samples was performed in batches of 24, with each batch containing the day-0 and -14 samples of 12 infants. Individuals were randomly assigned to extraction batches. All extractions were blinded with regard to study arm and outcome. For a small number of samples (18/240 infant samples and 10/40 adult samples), the initial PCR did not yield sufficient product for sequencing (<15 ng/μl), likely due to the presence of PCR inhibitors after DNA extraction (Schrader et al, 2012). PCR was repeated for these samples, but with the inclusion of 0.2 μl of bovine serum albumin (BSA) in the 25-μl reaction mixture. In all cases, PCR inhibition was overcome by the addition of BSA. Pooled amplicons were sequenced in a single MiSeq run containing 240 infant samples, 40 adult samples, 12 no-template extraction controls, and one no-template PCR control. We also sequenced PCR products amplified from two mock communities, as described in section 3.4.

³² In contrast to the 16S rRNA microbiota analyses described in Chapters 4 and 5, library preparation was conducted in Imperial College London's Medical School Building at St Mary's Hospital.

Contig assembly, quality filtering, OTU picking, and other sequence processing steps followed the methods described above (section 3.3). Since adults accounted for a minority (40/280) of the stool DNA samples included in the MiSeq run, we divided the OTU table into infant and adult samples before removing OTUs that represented <0.005% of sequences, thus reducing the risk of erroneously excluding low-abundance OTUs specific to adult samples. These infant- and adult-specific OTU tables were generated using the Qiime command *filter_samples_from_otu_table.py*. After filtering by abundance, the OTU tables were merged using the command *merge_otu_tables.py*.

6.2.3. Statistical analysis

i. Bacterial microbiota

After sequence processing, we observed a minimum of 7,708 sequences per sample. All analyses were performed at a rarefaction depth of 7,500 sequences per sample. We examined the association between 16S rRNA microbiota composition and the following variables: seroconversion, vaccine take (as inferred by the shedding of type 3 polioviruses 7 days after immunisation), study arm, and exposure to non-intervention antibiotics. All analyses of OPV outcome and antibiotic exposure were restricted to individuals who completed the study per protocol. Consistent with previous chapters, our primary outcome was the association between alpha diversity (OTU count and Shannon index) and oral vaccine outcome³³. For the assessment of diversity within and between samples, we generated ten separate rarefactions at a depth of 7,500 sequences per sample and calculated mean values for OTU count, Shannon index, and between-sample Unifrac distances (unweighted and weighted). OTU count and Shannon index were compared according to OPV outcome and antibiotic exposure (as categorical explanatory variables) via linear regression, with age included as a covariate, and between infant and adult samples using Student's *t* test. Unweighted and weighted Unifrac distances were visualised using principal coordinates analysis, and cluster significance assessed using the *adonis* function with 9,999 permutations. Age was included prior to OPV outcome or antibiotic exposure when implementing *adonis*, and significance determined based on sequential sums of squares.

Once again, we used Unifrac distance between day-0 and -14 samples from each infant as a measure of microbiota stability. Clustering of samples by individual was assessed using

³³ Owing to the lack of significant discrepancies in Proteobacteria-specific OTU count in the analyses described above, this was no longer included as a separate measure of alpha diversity.

adonis, and within- versus between-subject distances were compared using Wilcoxon's rank sum test (wherein between-subject distances comprised the mean Unifrac distance of day-14 samples obtained from other individuals for a given day-0 sample). Within-subject distances were compared according to study arm and OPV outcome using Wilcoxon's rank sum test.

Phylum-, class-, and OTU-level discrepancies in taxon relative abundance were assessed according to OPV outcome and antibiotic exposure using Wilcoxon's rank sum test³⁴. To account for multiple comparisons, *P* values were adjusted by Benjamini–Hochberg FDR correction (applied separately at each taxonomic rank for a given comparison). OTUs associated with study arm or vaccine outcome were visualised using Interactive Tree of Life (Letunic and Bork, 2007).

Adult samples were included in this study to provide a population-specific baseline of the mature bacterial microbiota. For each infant sample, we calculated the mean Unifrac distance (both unweighted and weighted) of the microbiota community from all non-cohabiting adults. This distance was used as an indicator of relative microbiota maturity ('microbiota age'), and assessed in relation to study arm and OPV outcome via linear regression, with age as a covariate.

Associations between infant age, microbiota diversity and age, and the number of enteropathogens (calculated separately for bacteria, viruses, and eukaryotes) were assessed in day-0 samples using Spearman's rank correlation coefficient (ρ). This metric was also used to identify OTUs that showed monotonic changes in relative abundance with increasing age at enrolment. To assess whether multiple antibiotic exposures had an additive effect on microbiota diversity, we compared day-14 OTU count and Shannon index among azithromycin recipients according to exposure to any non-intervention antibiotic. The potential association between non-intervention antibiotic use and study arm was assessed using the χ^2 test. All analyses were performed in the programming language R, and associations with a *P* value of <0.05 were considered statistically significant.

ii. Novel, persistent, and resolved infections

To examine how changes in pathogen burden in the 14 days preceding immunisation influenced OPV outcome, we classified enteropathogens as absent (not detected on day 0 or

³⁴ This differs from the permutation-based *t* test used to compare taxon abundance in previous chapters, wherein permutations were stratified by MiSeq run. Since all samples from the EVI trial were included in a single run, stratification was not necessary, and the use of Wilcoxon's rank sum test was therefore appropriate (and more computationally efficient).

14), resolved (present on day 0, absent on day 14), novel (absent on day 0, present on day 14), or persistent (present on days 0 and 14) – hereafter referred to as ‘infection subclasses’. For each enteropathogen and pathogen group (≥ 1 virus, ≥ 1 virus excluding enterovirus, ≥ 1 bacteria, ≥ 1 bacteria excluding EAEC, and ≥ 1 eukaryote), the impact of resolved, novel, and persistent infections on vaccine outcome (seroconversion or take as categorical dependent variables) was assessed via logistic regression, with age included as a covariate³⁵. For individual pathogens, we fitted both univariate models (including infection subclasses for each enteropathogen in turn) and multivariate models (including infection subclasses for all pathogens), and adjusted the resulting *P* values via Benjamini–Hochberg FDR correction. Aside from age, no other variables were included in the multivariate models. Infection subclasses were excluded from these analyses if they contained fewer than ten infants. Finally, for viral enteropathogens, we directly compared seroconversion and take according to the presence of novel versus persistent infections using the χ^2 test. If a significant discrepancy was observed for a given pathogen, we determined whether this was associated with a difference in pathogen abundance at day 14 by comparing (using Wilcoxon’s rank sum test) the Ct values of the TAC target between infants with novel versus persistent infections.

iii. Random Forests

The Random Forest machine-learning algorithm was used to classify infants according to study arm, seroconversion status, and vaccine take using four modules of data: (i) baseline characteristics (demography, growth, and antibiotic usage, among others); (ii) concurrent pathogens; (iii) 16S rRNA microbiota composition; and (iv) all of the above. A summary of the variables included in each module is provided in Table 6.2. Classification accuracy, variable importance, and model significance were assessed as described above (section 4.3.4).

iv. Multivariate logistic regression

To assess whether Shannon index and viral infection status (presence of ≥ 1 virus on day 14) were independently associated with the odds of OPV response (seroconversion or shedding), we compared the effect size and significance of these explanatory variables in univariate versus multivariate logistic regression models. Age was included as a covariate in all univariate and multivariate models. No other variables were adjusted for.

³⁵ During comparisons of pathogen groups, persistent infections encompassed the presence of any infection at days 0 and 14. Thus, the replacement of one pathogen with another (i.e., one resolved infection and one novel infection) would be categorised as a persistent infection.

Table 6.2. Random Forest input variables.**Module 1: Baseline characteristics**

Age (months) on day of vaccination
 Date of vaccination
 Sex
 Religion (Christian/Hindu/Muslim)
 Mother's education (illiterate/primary/middle/secondary/university graduate)
 House type (concrete/tiled/thatched)
 Weight-for-age Z score
 Height-for-age Z score
 Currently breastfed (yes/no)
 Number of OPV doses received prior to enrolment
 Receipt of antibiotics in the month before enrolment (yes/no)
 Receipt of non-intervention antibiotics between days 0 and 14 (yes/no)
 Concurrent diarrhoea on day 14 (yes/no)
 Study arm^a

Module 2: Concurrent pathogens

Ct values for each TAC target served as input for the models. Targets were excluded if they were absent in all individuals. Where pathogens were assessed using multiple targets (see Table 3.1), the targets were included as separate predictors. Samples were assigned a value of 35 for a given target if no amplification was observed below this threshold.

Module 3: 16S rRNA microbiota

An OTU table comprising 7,500 sequences per sample served as input for the models. OTUs were included if they were present in at least 2% of individuals.

Abbreviations: Ct, threshold cycle; OPV, oral poliovirus vaccine; OTU, 97%-identity operational taxonomic unit; TAC, TaqMan array card.

^a Excluded when study arm was the output of the Random Forest models.

v. Sensitivity analyses

Sensitivity analyses were carried out in which: (i) comparisons were stratified by study arm; (ii) non-responders were compared with individuals who developed post-vaccination antibody titres of $\geq 1:256$; (iii) the association between study arm and microbiota composition was assessed among infants with no exposure to non-intervention antibiotics; and (iv) the association between seroconversion and microbiota composition was assessed among infants with no antibiotic exposure (azithromycin or other). In each instance, we compared alpha diversity (number of OTUs and Shannon index) and beta diversity (sample clustering based on unweighted and weighted Unifrac distances) according to vaccine outcome or study arm in pre-vaccination (day-14) samples. Sensitivity analyses (i) and (ii) were also performed for comparisons of infection subclasses. Herein, we assessed the influence of resolved, novel, and persistent infections with ≥ 1 virus, ≥ 1 virus excluding enterovirus, ≥ 1 bacteria, ≥ 1 bacteria excluding EAEC, or ≥ 1 eukaryote on the odds of seroconversion or vaccine take. If fewer than ten individuals lacked infections at days 0 and 14 in a given comparison, resolved infections were used as the reference group in logistic regression models.

6.3. Results

6.3.1. Microbiota at enrolment

We sequenced the 16S rRNA gene V4 region in 240 infant samples and 40 adult samples in this study. After contig assembly and quality filtering, we obtained $14,685 \pm 2,386$ (mean $\pm$ s.d.) sequences per sample, spanning 821 97%-identity OTUs (of which 555 [68%] were unique to adult samples). At enrolment, the infant microbiota was characterised by a small number of dominant taxa, evident at both genus level (Figure 6.2A) and OTU level (Figure 6.2B). Whereas the top 20 OTUs among infants had a cumulative mean relative abundance of 90.2%, the equivalent proportion in adults was 67.8% (Figure 6.2C).

Infant samples were significantly less diverse than adult samples (72.2 ± 14.7 vs 249.8 ± 57.2 for OTU count and 2.72 ± 0.51 vs 4.59 ± 0.63 for Shannon index; Student's *t* test, *P* values <0.001 ; Figure 6.2D), and clustered separately from adult samples based on unweighted and weighted Unifrac distances (adonis, *R*² of 0.297 and 0.375 for unweighted and weighted Unifrac, respectively; *P* values <0.001 ; Figure 6.2E). With increasing age, we observed a significant increase in the number of OTUs and Shannon index (Figure 6.2D; Table 6.3), and a decrease in Unifrac distance from adult samples (increase in 'microbiota age'), reflecting a shift in composition towards a more 'adult-like' microbiota between 6 and 11 months of age (Figure 6.2F; Table 6.3)³⁶. The highest-ranking age-discriminatory OTUs among infants, as determined by Spearman's ρ , are summarised in Table 6.4.

A correlation matrix summarising the associations between age, microbiota composition, and pathogen burden at enrolment is provided in Table 6.3. Alongside the patterns described above, this matrix highlights the significant shifts in pathogen burden that occurred with increasing age in this study population. The number of bacterial pathogens, in turn, was associated with an increase in both diversity and maturity of the microbiota.

6.3.2. Impact of azithromycin on the bacterial microbiota

Of the 120 infants included in the microbiota subset, 114 completed the trial per protocol³⁷. No significant differences in alpha diversity, beta diversity, taxon relative abundance, or microbiota age distinguished azithromycin from placebo recipients at day 0 (Figure 6.3; Tables 6.5 and 6.6). The receipt of a 3-day course of azithromycin resulted in a

³⁶ Two adult samples were excluded from calculations of infant microbiota age as they had substantially lower diversity and clustered separately from the remaining adult samples (Figures 6.2D and 6.2E).

³⁷ Protocol deviations are described by Grassly et al (2016).

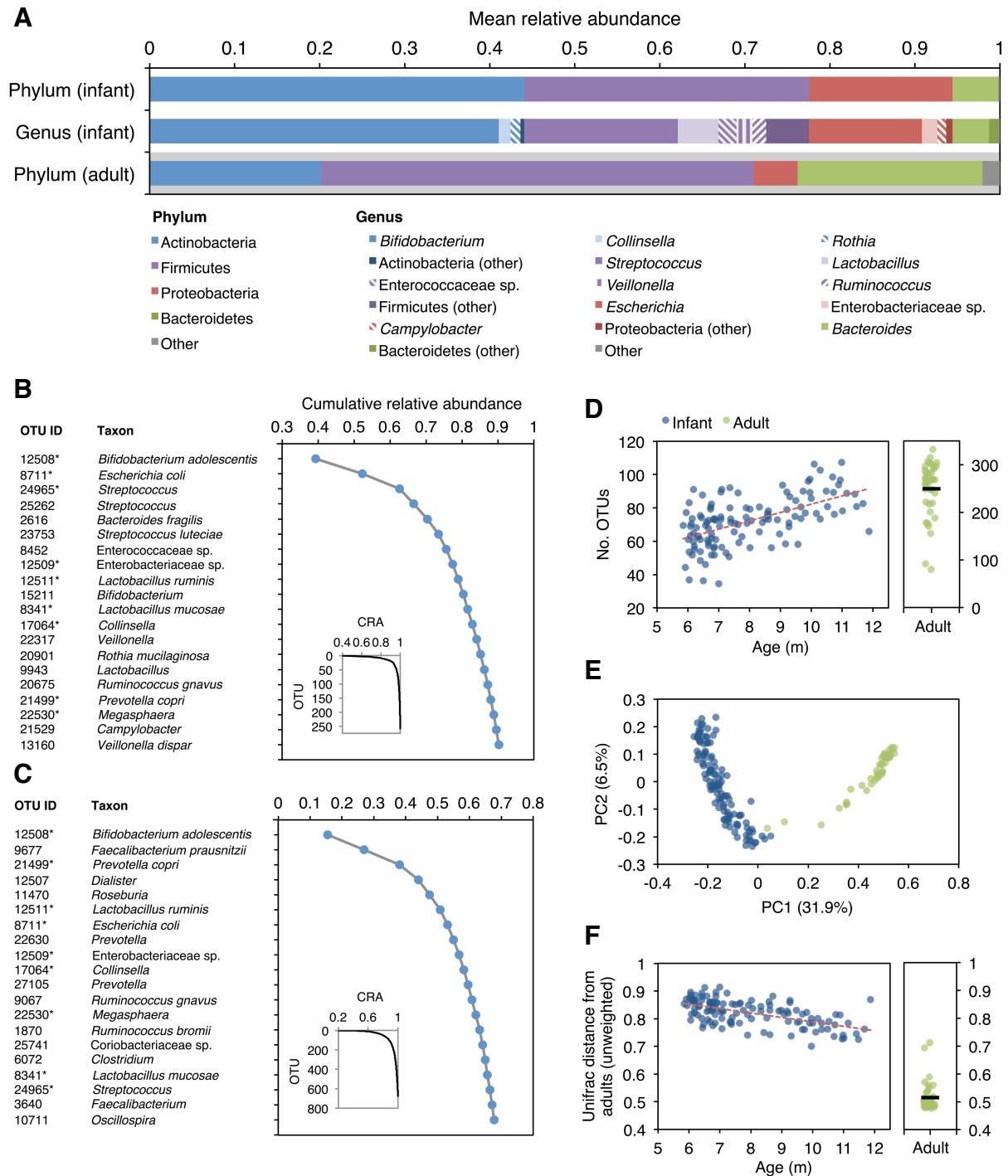


Figure 6.2. Composition of the bacterial microbiota at enrolment. (A) Mean relative taxon abundance at genus level (infants) and phylum level (infants and adults). (B) Top 20 OTUs based on mean relative abundance among day-0 infant samples ($n = 120$). (C) Top 20 OTUs among adult samples ($n = 40$). OTUs present in both (B) and (C) are indicated with an asterisk. The abundance distribution of all OTUs in these samples is displayed in the inset. (D) Number of OTUs by age. (E) Microbiota maturity, as determined by mean unweighted Unifrac distance from non-cohabiting adults, by age. Abbreviations: CRA, cumulative relative abundance; m, months; OTU, 97%-identity operational taxonomic unit; PC, principal coordinate.

Table 6.3. Baseline associations between age, microbiota diversity and age, and pathogen burden.

	Alpha diversity		Unifrac distance from adults		Number of pathogens		
	No. OTUs	Shannon	MD-U	MD-W	Bacteria	Viruses	Eukaryotes
Age (m)	0.56 (<0.001)	0.48 (<0.001)	-0.56 (<0.001)	-0.42 (<0.001)	0.24 (0.009)		
No. OTUs		0.63 (<0.001)	-0.74 (<0.001)	-0.48 (<0.001)	0.38 (<0.001)		
Shannon			-0.52 (<0.001)	-0.69 (<0.001)	0.22 (0.015)		
MD-U				0.56 (<0.001)	-0.36 (<0.001)		
MD-W							
Bacteria							0.23 (0.013)
Viruses							0.26 (0.004)
Eukaryotes							

Analyses were performed on day-0 samples for the 120 infants included in the microbiota subset. Spearman's ρ and associated P values are displayed for correlations with a P value of <0.05 . Abbreviations: m, months; MD-U, mean distance (unweighted Unifrac); MD-W, mean distance (weighted Unifrac); OTU, 97%-identity operational taxonomic unit.

Table 6.4. Top 20 age-discriminatory operational taxonomic units.

Taxonomy	OTU ID	Prevalence (%)		Relative abundance (mean % $\pm$ s.d.)		ρ	P	FDR P
		<8 m (n = 70)	≥ 8 m (n = 50)	<8 m (n = 70)	≥ 8 m (n = 50)			
<i>Faecalibacterium prausnitzii</i> *	9677	25.7	66.0	0.057 $\pm$ 0.154	0.844 $\pm$ 2.054	0.49	1.16E-08	2.85E-06
<i>Lactobacillus ruminis</i>	12511	30.0	62.0	0.871 $\pm$ 5.516	2.693 $\pm$ 5.754	0.44	5.12E-07	4.20E-05
<i>Ruminococcaceae</i> sp.*	19836	8.6	44.0	0.045 $\pm$ 0.289	0.067 $\pm$ 0.129	0.44	4.83E-07	4.20E-05
<i>Faecalibacterium</i> *	3866	4.3	30.0	0.001 $\pm$ 0.003	0.021 $\pm$ 0.054	0.42	1.75E-06	0.0001
<i>Dorea formicigenerans</i> *	13285	17.1	52.0	0.096 $\pm$ 0.360	0.207 $\pm$ 0.382	0.41	3.00E-06	0.0001
<i>Faecalibacterium</i> *	5200	8.6	32.0	0.003 $\pm$ 0.010	0.017 $\pm$ 0.043	0.37	3.26E-05	0.0011
<i>Coprococcus</i> *	18159	7.1	36.0	0.062 $\pm$ 0.370	0.257 $\pm$ 0.709	0.37	2.84E-05	0.0011
<i>Ruminococcus gnavus</i> *	9067	11.4	38.0	0.120 $\pm$ 0.566	0.615 $\pm$ 1.532	0.35	8.86E-05	0.0025
<i>Blautia</i> *	15230	7.1	34.0	0.093 $\pm$ 0.495	0.113 $\pm$ 0.301	0.35	9.28E-05	0.0025
<i>Lachnospiraceae</i> sp.*	15571	7.1	28.0	0.026 $\pm$ 0.135	0.042 $\pm$ 0.122	0.35	0.0001	0.0025
<i>Blautia</i> *	3067	17.1	44.0	0.022 $\pm$ 0.068	0.466 $\pm$ 1.135	0.34	0.0001	0.0031
<i>Lactobacillus</i>	2346	7.1	30.0	0.011 $\pm$ 0.074	0.035 $\pm$ 0.128	0.33	0.0002	0.0045
<i>Lactobacillus</i>	2549	7.1	28.0	0.038 $\pm$ 0.282	0.054 $\pm$ 0.120	0.33	0.0003	0.0047
<i>Streptococcus infantis</i>	5074	61.4	34.0	0.022 $\pm$ 0.031	0.008 $\pm$ 0.015	-0.33	0.0003	0.0047
<i>Bifidobacterium adolescentis</i>	19111	7.1	28.0	0.001 $\pm$ 0.005	0.016 $\pm$ 0.039	0.32	0.0004	0.0063
<i>Ruminococcus</i> *	4023	5.7	26.0	0.155 $\pm$ 1.033	0.379 $\pm$ 2.078	0.31	0.0005	0.0077
<i>Lachnospiraceae</i> sp.*	23320	4.3	20.0	0.004 $\pm$ 0.020	0.015 $\pm$ 0.048	0.31	0.0005	0.0079
<i>Leuconostocaceae</i> sp.	15235	32.9	58.0	0.018 $\pm$ 0.051	0.027 $\pm$ 0.050	0.30	0.0008	0.0115
<i>Streptococcus</i>	23667	11.4	32.0	0.004 $\pm$ 0.012	0.014 $\pm$ 0.029	0.30	0.0009	0.0121
<i>Lactobacillus ruminis</i>	19280	8.6	28.0	0.005 $\pm$ 0.018	0.015 $\pm$ 0.037	0.30	0.0010	0.0123

OTUs that showed monotonic changes in relative abundance with increasing age were identified based on Spearman's ρ . The top 20 age-discriminatory taxa are displayed above. * OTU belongs to the class Clostridia. Abbreviations: FDR, adjusted by Benjamini-Hochberg false discovery rate correction; m, months; OTU, 97%-identity operational taxonomic unit.

significant decrease in the number of OTUs observed on day 14 and a borderline-significant decrease in Shannon index (Figure 6.3A; Table 6.5). A shift in taxonomic composition was apparent based on unweighted and weighted Unifrac distances (adonis, P values <0.05), albeit with modest effect size (R^2 of 0.020 and 0.022 for unweighted and weighted distances, respectively; Figures 6.3B and 6.3C; Table 6.6). These changes can be attributed primarily to phylum-, class-, and OTU-level decreases in the relative abundance of Proteobacteria and Verrucomicrobia among individuals treated with azithromycin (Figures 6.3D and 6.3E; Table 6.7). Within the latter phylum, all sequences mapped to a single OTU, classified as *Akkermansia muciniphila*. Microbiota age, as inferred by mean Unifrac distance from adult samples, was comparable in azithromycin and placebo recipients at day 14 (0.826 ± 0.042 [mean $\pm$ s.d.] vs 0.821 ± 0.051 for unweighted distances and 0.598 ± 0.041 vs 0.593 ± 0.042 for weighted distances; linear regression, P values 0.577 and 0.543, respectively).

6.3.3. Impact of non-intervention antibiotics on the bacterial microbiota

The use of antibiotics other than the study intervention in this population was common. Among infants who completed the study per protocol, 185/705 (26%) took oral antibiotics in the month before enrolment (which was delayed until after infants had stopped taking antibiotics), while 78/705 (11%) took antibiotics other than the intervention between days 0 and 14. Pre-enrolment antibiotics, where known, included amoxicillin ($n = 40$), co-trimoxazole ($n = 25$), and cefixime ($n = 21$), among numerous others. Prior exposure to antibiotics was associated with a significant decline in the number of OTUs at day 0 (Table 6.5). However, this discrepancy was no longer apparent at day 14. In contrast to the effect of azithromycin, we did not observe any significant clustering of samples according to pre-enrolment antibiotic exposure based on unweighted or weighted Unifrac (Table 6.6). Similarly, there were no significant phylum-, class-, or OTU-level differences in taxon relative abundance at enrolment according to antibiotic exposure status (Wilcoxon's rank sum, FDR-corrected P values >0.05).

The proportion of infants exposed to non-intervention antibiotics did not differ significantly according to study arm before enrolment (89/348 [26%] vs 96/357 [27%] in recipients of azithromycin and placebo, respectively; χ^2 , $P = 0.592$) or between days 0 and 14 (37/348 [11%] vs 41/357 [11%]; χ^2 , $P = 0.718$). Among azithromycin recipients included in the microbiota subset, exposure to any non-intervention antibiotics (either before enrolment or between days 0 and 14) was associated with a reduction in OTU count at day 14, albeit non-significant (Table 6.5).

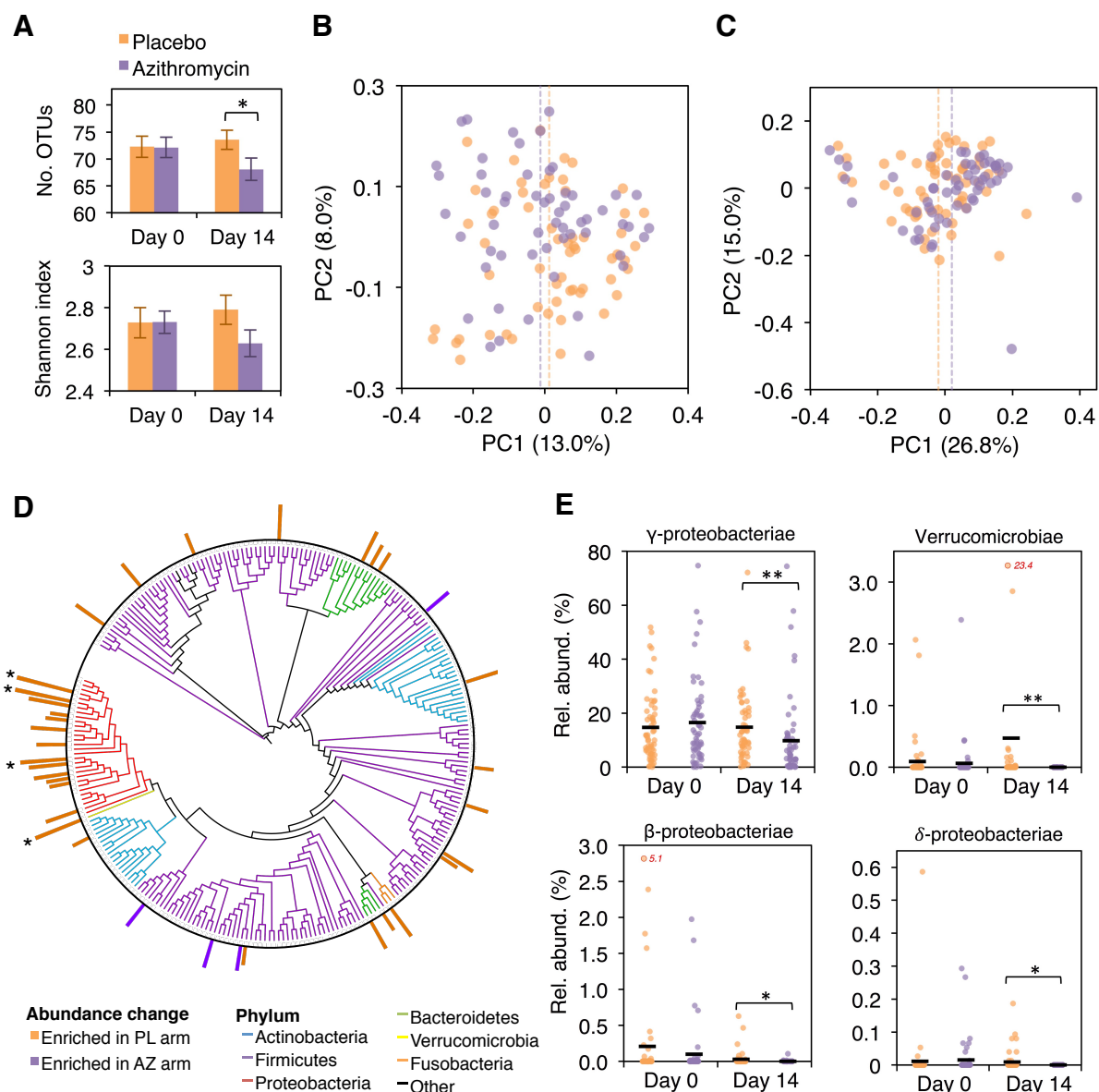


Figure 6.3. Impact of azithromycin on the bacterial microbiota. (A) OTU count and Shannon index (mean $\pm$ standard error) according to study arm. (B) Unweighted and (C) weighted Unifrac distances between day-14 infant samples, visualised via principal coordinates analysis. Mean values for principal coordinate 1 are indicated by dotted lines. (D) OTU-level differences in relative taxon abundance at day 14 according to study arm. Bars display P values on a negative $\log_{10}$ scale. Comparisons with a P value of <0.05 prior to FDR correction are indicated. The tree was constructed from *de novo* OTU sequences spanning the V4 region of the 16S rRNA gene. Owing to the use of a relatively short (and hypervariable) segment of the 16S rRNA gene for tree construction, phyla do not always separate into discrete lineages. (E) Class-level differences in taxon abundance according to study arm. Values beyond the scale of the y-axis are indicated in red. * $P < 0.05$ (after FDR correction for abundance comparisons); ** FDR-corrected $P < 0.005$. Abbreviations: AZ, azithromycin; OTU, 97%-identity operational taxonomic unit; Rel. abund., relative abundance; PC, principal coordinate; PL, placebo.

Table 6.5. Summary of alpha diversity analyses.

Comparison	Group	Day	N ^a	N ^b	Number of OTUs				Shannon index					
					Mean ± s.d. ^a	Mean ± s.d. ^b	Effect size	SE	P	Mean ± s.d. ^a	Mean ± s.d. ^b	Effect size	SE	P
Age	All	0	-	-	-	5.053	0.664	<0.001	-	-	0.138	0.024	<0.001	
Azithromycin	All	0	58	56	72.3 ± 14.9	72.1 ± 14.1	0.165	2.217	0.941	2.73 ± 0.55	2.73 ± 0.41	0.011	0.081	0.893
	All	14	58	56	73.6 ± 13.7	68.1 ± 15.4	-5.234	2.329	0.027	2.79 ± 0.54	2.63 ± 0.48	-0.155	0.090	0.087
	AB-	14	37	43	74.6 ± 15.2	69.7 ± 15.6	-5.164	2.912	0.081	2.85 ± 0.54	2.64 ± 0.47	-0.214	0.106	0.048
Pre-enrolment antibiotics	All	0	86	28	74.0 ± 13.9	66.7 ± 14.9	-6.623	2.498	0.009	2.77 ± 0.49	2.60 ± 0.47	-0.151	0.093	0.106
	All	14	86	28	71.4 ± 15.3	69.3 ± 13.1	-1.441	2.764	0.603	2.72 ± 0.51	2.70 ± 0.52	0.000	0.106	0.997
Any non-intervention antibiotics	AZ	14	43	13	69.7 ± 15.6	62.7 ± 14.2	-4.917	3.876	0.210	2.64 ± 0.47	2.58 ± 0.54	-0.019	0.141	0.892
PV3 seroconversion (≥1:8 vs <1:8)	All	0	62	52	71.5 ± 14.7	73.0 ± 14.3	0.288	2.231	0.898	2.76 ± 0.48	2.69 ± 0.49	-0.106	0.081	0.192
	All	14	62	52	68.8 ± 14.1	73.4 ± 15.3	3.492	2.373	0.144	2.62 ± 0.47	2.82 ± 0.55	0.173	0.090	0.057
	PL	14	29	29	71.9 ± 13.7	75.2 ± 13.7	2.212	3.258	0.500	2.72 ± 0.52	2.86 ± 0.55	0.119	0.136	0.386
	AZ	14	33	23	66.0 ± 14.0	71.1 ± 17.2	4.063	3.319	0.226	2.54 ± 0.41	2.76 ± 0.55	0.206	0.117	0.084
	PL/AB-	14	21	16	71.3 ± 14.8	79.0 ± 15.0	5.761	4.515	0.211	2.72 ± 0.58	3.03 ± 0.45	0.257	0.173	0.146
PV3 seroconversion (≥1:256 vs <1:8)	All	14	23	52	71.0 ± 15.0	73.4 ± 15.3	0.693	3.290	0.834	2.68 ± 0.44	2.82 ± 0.55	0.102	0.120	0.400
PV3 take	All	0	42	33	69.9 ± 14.9	73.2 ± 15.1	2.654	2.917	0.366	2.66 ± 0.45	2.70 ± 0.44	0.024	0.099	0.805
	All	14	42	33	65.4 ± 14.5	72.0 ± 16.4	5.941	2.969	0.049	2.57 ± 0.50	2.84 ± 0.59	0.250	0.120	0.040
	PL	14	22	15	67.3 ± 13.6	76.4 ± 13.3	9.137	3.720	0.019	2.62 ± 0.54	2.96 ± 0.62	0.341	0.183	0.072
	AZ	14	20	19	63.3 ± 15.5	68.3 ± 18.0	3.437	4.469	0.447	2.52 ± 0.46	2.73 ± 0.57	0.186	0.159	0.250

The impact of each factor on the specified measure of alpha diversity was assessed via linear regression. Age was included as a covariate in all analyses of antibiotic groups or OPV outcome. Abbreviations: AB-, excludes individuals who received oral antibiotics other than the study intervention; AZ, azithromycin arm; OTU, 97%-identity taxonomic unit; PL, placebo arm; PV3, type 3 poliovirus; SE, standard error.

^a Placebo recipients/untreated individuals/responders/shedders.

^b Antibiotic recipients/non-responders/non-shedders.

Table 6.6. Summary of beta diversity analyses.

Comparison	Group	Day	Unweighted Unifrac		Weighted Unifrac	
			R ²	P	R ²	P
Age	All	0	0.049	<0.001	0.034	0.003
Study ID	All	-	0.676	<0.001	0.651	<0.001
Azithromycin	All	0	0.006	0.901	0.003	0.935
	All	14	0.020	<0.001	0.022	0.024
	AB-	14	0.025	0.003	0.041	0.006
Pre-enrolment antibiotics	All	0	0.012	0.070	0.008	0.477
	All	14	0.011	0.165	0.015	0.116
Any non-intervention antibiotics	AZ	14	0.017	0.485	0.029	0.143
PV3 seroconversion ($\geq 1:8$ vs $< 1:8$)	All	0	0.010	0.185	0.011	0.254
	All	14	0.013	0.031	0.008	0.465
	PL	14	0.021	0.141	0.012	0.682
	AZ	14	0.019	0.295	0.009	0.772
	PL/AB-	14	0.031	0.260	0.012	0.914
PV3 seroconversion ($\geq 1:256$ vs $< 1:8$)	All	14	0.015	0.252	0.017	0.234
PV3 take	All	0	0.015	0.207	0.010	0.575
	All	14	0.025	0.005	0.016	0.301
	PL	14	0.034	0.137	0.024	0.523
	AZ	14	0.035	0.110	0.019	0.604

See Table 6.5 for details regarding the number of infants included in each group. The impact of each factor on the specified measure of beta diversity was assessed using the function *adonis* in the R package *vegan*; 9,999 permutations were included in each comparison. Abbreviations: AB-, excludes individuals who received oral antibiotics other than the study intervention; AZ, azithromycin arm; PL, placebo arm; PV3, type 3 poliovirus.

Table 6.7. Influence of azithromycin on taxon abundance.

Taxonomic classification	OTU ID	Prevalence (%)		Relative abundance (mean % ± s.d.)		P	FDR P
		PL (n = 58)	AZ (n = 56)	PL (n = 58)	AZ (n = 56)		
Phylum (all)							
Proteobacteria	-	100.0	98.2	15.889 ± 13.207	10.200 ± 15.401	0.0001	0.0008
Verrucomicrobia	-	20.7	0.0	0.474 ± 3.092	0.000 ± 0.000	0.0004	0.0012
Fusobacteria	-	37.9	21.4	0.151 ± 0.856	0.745 ± 4.609	0.0509	0.1189
Bacteroidetes	-	89.7	83.9	5.163 ± 10.268	3.497 ± 7.518	0.1075	0.1882
Actinobacteria	-	100.0	100.0	41.017 ± 17.551	44.353 ± 20.885	0.2114	0.2959
Firmicutes	-	100.0	100.0	37.275 ± 19.016	41.177 ± 20.596	0.3268	0.3813
Other	-	79.3	80.4	0.031 ± 0.031	0.028 ± 0.025	0.7529	0.7529
Class (P value <0.05 prior to FDR adjustment)							
Gammaproteobacteria	-	100.0	96.4	14.801 ± 13.270	9.812 ± 15.365	0.0002	0.0025
Verrucomicrobiae	-	20.7	0.0	0.474 ± 3.092	0.000 ± 0.000	0.0004	0.0025
Deltaproteobacteria	-	13.8	0.0	0.009 ± 0.031	0.000 ± 0.000	0.0042	0.0197
Betaproteobacteria	-	24.1	7.1	0.030 ± 0.106	0.003 ± 0.014	0.0096	0.0335
OTU (P value <0.05 prior to FDR adjustment)							
Escherichia coli	8711	100.0	96.4	12.087 ± 12.457	7.309 ± 13.258	0.0001	0.0133
Escherichia coli	154	74.1	42.9	0.047 ± 0.046	0.022 ± 0.046	0.0001	0.0133
Akkermansia muciniphila	15064	20.7	0.0	0.474 ± 3.092	0.000 ± 0.000	0.0004	0.0295
Enterobacteriaceae sp.	4144	58.6	30.4	0.020 ± 0.027	0.006 ± 0.012	0.0005	0.0329
Escherichia coli	23059	75.9	53.6	0.044 ± 0.046	0.024 ± 0.040	0.0012	0.0582
Escherichia coli	22660	67.2	42.9	0.031 ± 0.036	0.014 ± 0.020	0.0019	0.0796
Campylobacter	21529	19.0	1.8	1.037 ± 3.858	0.001 ± 0.005	0.0029	0.0868
Escherichia coli	17709	91.4	69.6	0.205 ± 0.304	0.118 ± 0.221	0.0035	0.0868
Streptococcus	9412	27.6	7.1	0.009 ± 0.019	0.002 ± 0.008	0.0036	0.0868
Lactobacillus mucosae	8341	34.5	12.5	1.044 ± 3.871	0.023 ± 0.124	0.0038	0.0868
Bilophila	8458	13.8	0.0	0.009 ± 0.031	0.000 ± 0.000	0.0042	0.0868
Megasphaera	6777	13.8	0.0	0.007 ± 0.021	0.000 ± 0.000	0.0042	0.0868
Fusobacterium	13808	20.7	3.6	0.020 ± 0.095	0.000 ± 0.002	0.0047	0.0868
Bifidobacterium	19019	39.7	14.3	0.010 ± 0.017	0.005 ± 0.013	0.0049	0.0868
Bacteroides fragilis	2616	75.9	62.5	3.258 ± 8.058	0.962 ± 2.839	0.0059	0.0969
Sutterella	23966	12.1	0.0	0.018 ± 0.088	0.000 ± 0.000	0.0077	0.1129
Parabacteroides gordonii	22351	12.1	0.0	0.017 ± 0.092	0.000 ± 0.000	0.0077	0.1129
Dorea	17630	6.9	25.0	0.028 ± 0.190	0.042 ± 0.127	0.0088	0.1199
Peptostreptococcus	18269	15.5	1.8	0.345 ± 1.839	0.000 ± 0.002	0.0092	0.1199
Lactobacillales sp.	15744	70.7	82.1	0.042 ± 0.069	0.068 ± 0.069	0.0105	0.1261
Clostridiaceae	3647	13.8	33.9	0.005 ± 0.015	0.043 ± 0.120	0.0107	0.1261
Ruminococcus	12123	10.3	28.6	0.005 ± 0.028	0.008 ± 0.021	0.0172	0.1936
Escherichia coli	16451	39.7	19.6	0.015 ± 0.031	0.006 ± 0.018	0.0222	0.2393
Campylobacter	2226	8.6	0.0	0.010 ± 0.036	0.000 ± 0.000	0.0259	0.2530
Bacteroides	14039	19.0	5.4	0.009 ± 0.029	0.001 ± 0.006	0.0259	0.2530
Veillonellaceae sp.	1769	15.5	3.6	0.017 ± 0.067	0.000 ± 0.002	0.0265	0.2530
Streptococcus	24037	65.5	44.6	0.035 ± 0.047	0.024 ± 0.048	0.0276	0.2538
Escherichia coli	22987	43.1	25.0	0.014 ± 0.024	0.007 ± 0.017	0.0314	0.2780
Bacteroides ovatus	19772	36.2	17.9	0.091 ± 0.319	0.066 ± 0.288	0.0403	0.3421
Alloiococcus	19997	22.4	8.9	0.005 ± 0.013	0.002 ± 0.006	0.0456	0.3421
Ruminococcus bromii	1870	6.9	0.0	0.163 ± 0.943	0.000 ± 0.000	0.0474	0.3421
Coriobacteriaceae sp.	20404	6.9	0.0	0.044 ± 0.266	0.000 ± 0.000	0.0474	0.3421
Megasphaera	22530	22.4	8.9	0.510 ± 1.577	0.235 ± 1.523	0.0479	0.3421
Parabacteroides distasonis	3968	24.1	10.7	0.075 ± 0.327	0.043 ± 0.303	0.0487	0.3421
Proteus	2389	13.8	3.6	0.029 ± 0.116	0.001 ± 0.005	0.0489	0.3421

No phylum-, class-, or OTU-level differences distinguished azithromycin from placebo recipients at enrolment (FDR-corrected *P* values >0.05). Abbreviations: AZ, azithromycin arm; FDR, adjusted by Benjamini–Hochberg false discovery rate correction; OTU, 97%-identity operational taxonomic unit; PL, placebo arm.

6.3.4. Association between bacterial microbiota composition and oral poliovirus vaccine outcome

At enrolment (14 days prior to mOPV3 administration), we observed no significant differences in alpha diversity, beta diversity, or relative taxon abundance among individuals who seroconverted to type 3 poliovirus compared with those who did not (Figure 6.4A; Tables 6.5 and 6.6). In pre-vaccination samples (day 14), an increase in OTU count and Shannon index was apparent in non-responders compared with responders; however, these discrepancies were not statistically significant (linear regression, *P* values 0.144 and 0.057, respectively; Figure 6.4A; Table 6.5). A modest shift in pre-vaccination community composition according to seroconversion status was evident based on unweighted but not weighted Unifrac (adonis, *P* values 0.031 and 0.465, respectively; Figure 6.4B; Table 6.6), albeit accounting for only a small proportion of variance among samples ($R^2 = 0.013$). Although we observed no significant phylum-, class-, or OTU-level differences in taxon abundance according to seroconversion status (Wilcoxon's rank sum, FDR-corrected *P* values >0.05), several OTUs belonging to the class Clostridia were enriched in the day-14 samples of infants who failed to seroconvert (*P* values <0.05 prior to FDR adjustment; Figure 6.5; Table 6.8). We also observed a decrease in unweighted Unifrac distance from adults (or increase in microbiota age) in non-responders compared with responders on day 14 (0.811 ± 0.050 [mean $\pm$ s.d.] vs 0.833 ± 0.042 for unweighted distances and 0.587 ± 0.044 vs 0.601 ± 0.038 for weighted distances; linear regression, *P* values 0.011 and 0.098, respectively; Figure 6.4C). This discrepancy was absent on day 0 (linear regression, *P* values 0.323 and 0.220, respectively).

Similar results were obtained when comparing individuals according to vaccine take. On day 14, we observed a significant increase in OTU count and Shannon index among non-shedders compared with shedders (Figure 6.4D; Table 6.5), significant clustering of samples was evident based on unweighted but not weighted Unifrac distances (Figure 6.4E; Table 6.6), and non-shedders had a more adult-like microbiota than shedders (0.806 ± 0.052 vs 0.838 ± 0.046 for unweighted distances and 0.583 ± 0.048 vs 0.607 ± 0.038 for weighted distances; linear regression, *P* values 0.002 and 0.014, respectively; Figure 6.4F). The class Clostridia was also enriched in non-shedders compared with shedders on day 14 (relative abundance, $11.6 \pm 10.2\%$ vs $5.6 \pm 7.7\%$; Wilcoxon's rank sum, FDR-corrected *P* = 0.044; Table 6.9).

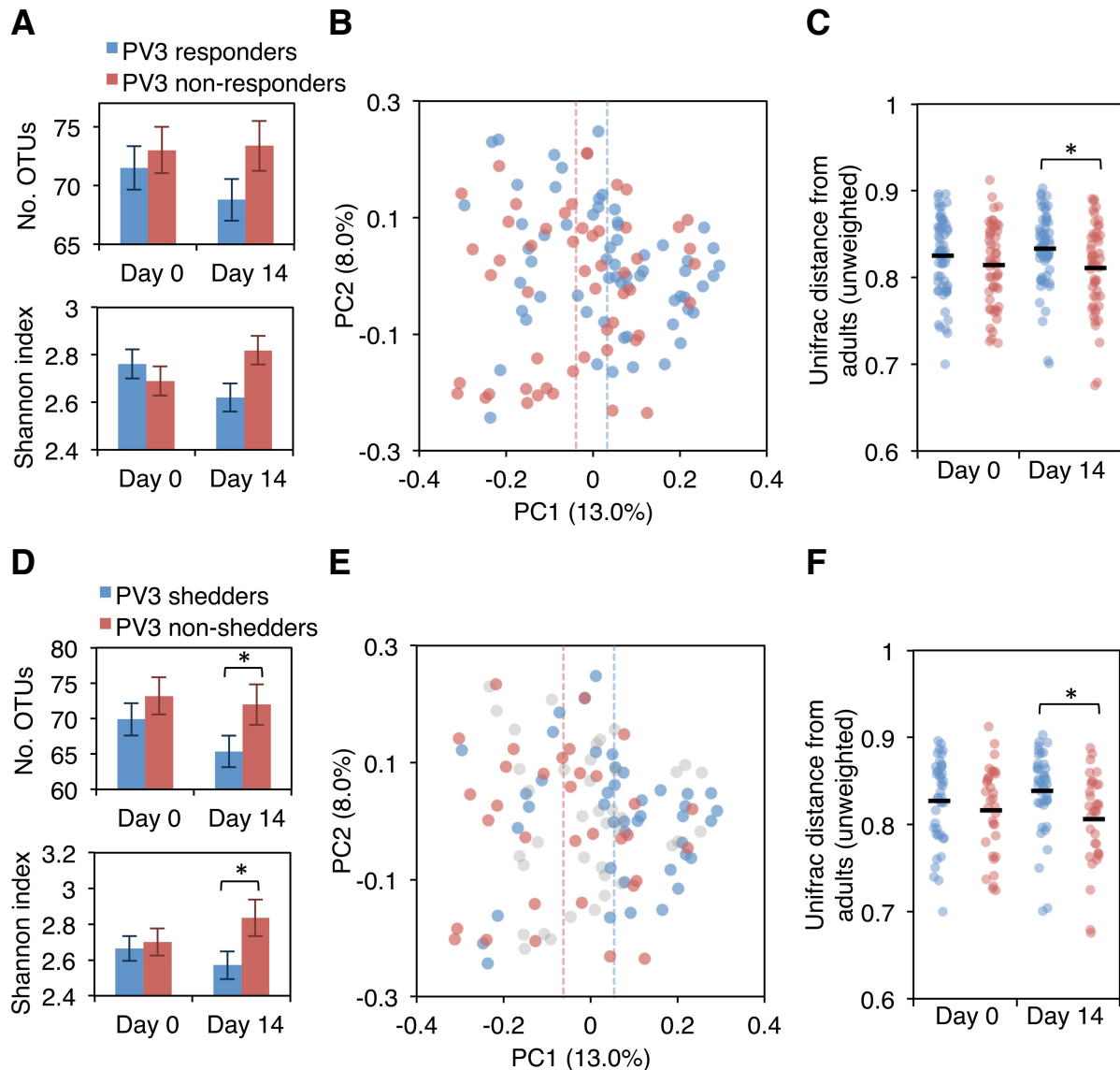


Figure 6.4. Association between microbiota composition and oral poliovirus vaccine response. (A) OTU count and Shannon index (mean $\pm$ standard error) according to type 3 poliovirus seroconversion status. (B) Unweighted Unifrac distances between day-14 (pre-vaccination) samples, visualised via principal coordinates analysis. Mean values for principal coordinate 1 are indicated by dotted lines. (C) Relative microbiota maturity, as inferred by mean unweighted Unifrac distance from adult samples, according to seroconversion status. (D, E, F) The same comparisons are displayed with respect to vaccine take. * $P < 0.05$. Abbreviations: OTU, 97%-identity operational taxonomic unit; PC, principal coordinate; PV3, type 3 poliovirus.

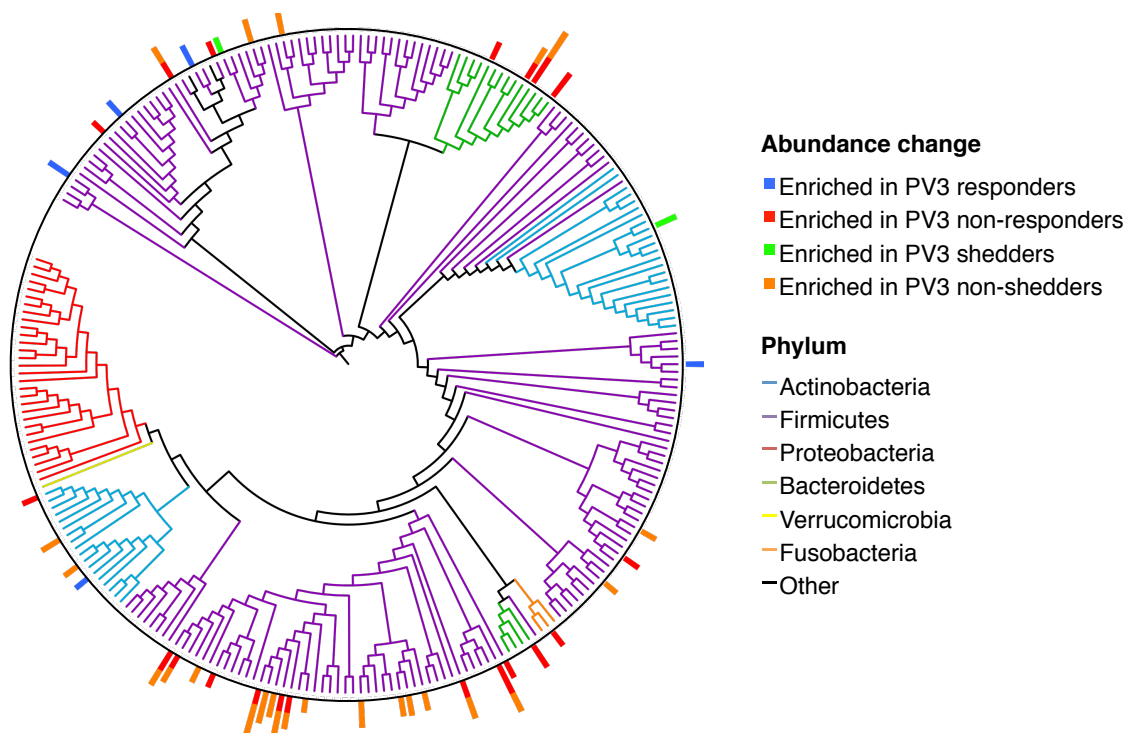


Figure 6.5. Changes in taxon abundance associated with oral poliovirus vaccine response. Bars display P values on a negative $\log_{10}$ scale. Comparisons with a P value of <0.05 prior to FDR correction are indicated. Abbreviation: PV3, type 3 poliovirus.

Table 6.8. Summary of taxon abundance comparisons according to type 3 poliovirus seroconversion status.

		Prevalence (%)		Relative abundance (mean % $\pm$ s.d.)			
Taxonomic classification	OTU ID	sero+ (n = 62)	sero- (n = 52)	sero+ (n = 62)	sero- (n = 52)	<i>P</i>	FDR <i>P</i>
Phylum (all)							
Fusobacteria	-	22.6	38.5	0.010 $\pm$ 0.027	0.958 $\pm$ 4.840	0.0457	0.2985
Bacteroidetes	-	80.6	94.2	4.821 $\pm$ 10.722	3.777 $\pm$ 6.498	0.0853	0.2985
Other	-	83.9	75.0	0.032 $\pm$ 0.026	0.027 $\pm$ 0.031	0.1749	0.4081
Proteobacteria	-	98.4	100.0	13.296 $\pm$ 16.585	12.854 $\pm$ 11.819	0.4493	0.7862
Firmicutes	-	100.0	100.0	38.633 $\pm$ 19.072	39.858 $\pm$ 20.838	0.7848	0.9660
Verrucomicrobia	-	11.3	9.6	0.385 $\pm$ 2.976	0.070 $\pm$ 0.398	0.8517	0.9660
Actinobacteria	-	100.0	100.0	42.824 $\pm$ 18.644	42.455 $\pm$ 20.126	0.9660	0.9660
Class (<i>P</i> value <0.05 prior to FDR adjustment)							
Fusobacteria	-	22.6	38.5	0.010 $\pm$ 0.027	0.958 $\pm$ 4.840	0.0457	0.2985
OTU (<i>P</i> value <0.05 prior to FDR adjustment)							
Ruminococcaceae sp.*	19836	12.9	40.4	0.054 $\pm$ 0.207	0.190 $\pm$ 0.648	0.0017	0.2271
<i>Bacteroides caccae</i>	13096	9.7	34.6	0.206 $\pm$ 1.099	0.084 $\pm$ 0.215	0.0023	0.2271
<i>Bacteroides</i>	13617	17.7	42.3	0.107 $\pm$ 0.528	0.397 $\pm$ 1.164	0.0027	0.2271
<i>Parabacteroides distasonis</i>	3968	8.1	28.8	0.025 $\pm$ 0.150	0.099 $\pm$ 0.435	0.0052	0.3228
<i>Streptococcus</i>	25262	100.0	98.1	5.025 $\pm$ 4.876	3.732 $\pm$ 4.925	0.0069	0.3414
<i>Streptococcus</i>	18919	71.0	46.2	0.032 $\pm$ 0.039	0.021 $\pm$ 0.037	0.0097	0.3503
<i>Coprococcus</i> *	22483	4.8	21.2	0.012 $\pm$ 0.079	0.013 $\pm$ 0.039	0.0099	0.3503
<i>Lactobacillus</i>	11591	27.4	9.6	0.023 $\pm$ 0.078	0.019 $\pm$ 0.112	0.0194	0.4005
<i>Streptococcus infantis</i>	5074	53.2	32.7	0.017 $\pm$ 0.025	0.007 $\pm$ 0.014	0.0196	0.4005
<i>Bacteroides uniformis</i>	1419	17.7	36.5	0.108 $\pm$ 0.514	0.116 $\pm$ 0.385	0.0209	0.4005
<i>Coprococcus</i> *	9446	4.8	17.3	0.007 $\pm$ 0.044	0.259 $\pm$ 0.853	0.0260	0.4005
<i>Collinsella</i>	4136	1.6	11.5	0.001 $\pm$ 0.005	0.028 $\pm$ 0.119	0.0277	0.4005
<i>Megasphaera</i> *	19218	1.6	11.5	0.000 $\pm$ 0.003	0.148 $\pm$ 0.704	0.0277	0.4005
Lachnospiraceae sp.*	13505	32.3	51.9	0.019 $\pm$ 0.045	0.047 $\pm$ 0.106	0.0277	0.4005
<i>Streptococcus</i>	2401	25.8	44.2	0.010 $\pm$ 0.022	0.033 $\pm$ 0.072	0.0281	0.4005
<i>Bacteroides ovatus</i>	19772	19.4	36.5	0.085 $\pm$ 0.381	0.072 $\pm$ 0.174	0.0283	0.4005
<i>Clostridium</i> *	23959	1.6	11.5	0.000 $\pm$ 0.003	0.034 $\pm$ 0.162	0.0287	0.4005
Lachnospiraceae sp.*	26629	16.1	34.6	0.008 $\pm$ 0.035	0.011 $\pm$ 0.026	0.0295	0.4005
Fusobacteriaceae sp.	12902	11.3	26.9	0.005 $\pm$ 0.018	0.805 $\pm$ 4.779	0.0307	0.4005
<i>Actinomycetales</i>	15867	48.4	30.8	0.012 $\pm$ 0.017	0.006 $\pm$ 0.009	0.0376	0.4481
Lachnospiraceae sp.*	23320	9.7	23.1	0.003 $\pm$ 0.009	0.017 $\pm$ 0.044	0.0379	0.4481
Lachnospiraceae sp.*	1106	4.8	17.3	0.007 $\pm$ 0.046	0.007 $\pm$ 0.017	0.0408	0.4498
<i>Streptococcus</i>	17841	71.0	76.9	0.061 $\pm$ 0.109	0.086 $\pm$ 0.097	0.0454	0.4498
<i>Coprococcus</i> *	18159	17.7	32.7	0.231 $\pm$ 1.190	0.396 $\pm$ 1.210	0.0468	0.4498
<i>Streptococcus</i>	25593	33.9	51.9	0.015 $\pm$ 0.028	0.026 $\pm$ 0.039	0.0469	0.4498
Clostridiaceae sp.*	3647	16.1	32.7	0.026 $\pm$ 0.108	0.020 $\pm$ 0.051	0.0497	0.4498

* OTU belongs to the class Clostridia. Abbreviations: FDR, adjusted by Benjamini–Hochberg false discovery rate correction; OTU, 97%-identity operational taxonomic unit; sero+, responders; sero-, non-responders.

Table 6.9. Summary of taxon abundance comparisons according to type 3 poliovirus take.

Taxonomic classification	OTU ID	Prevalence (%)		Relative abundance (mean % $\pm$ s.d.)		<i>P</i>	FDR <i>P</i>
		shed+ (n = 42)	shed- (n = 33)	shed+ (n = 42)	shed- (n = 33)		
Phylum (all)							
Bacteroidetes	-	78.6	100.0	4.383 $\pm$ 9.604	4.408 $\pm$ 7.486	0.0734	0.4600
Verrucomicrobia	-	19.0	6.1	0.578 $\pm$ 3.614	0.096 $\pm$ 0.498	0.1314	0.4600
Proteobacteria	-	97.6	100.0	15.236 $\pm$ 18.064	11.557 $\pm$ 12.988	0.3615	0.5913
Actinobacteria	-	100.0	100.0	41.777 $\pm$ 20.422	44.672 $\pm$ 17.791	0.4226	0.5913
Other	-	81.0	75.8	0.028 $\pm$ 0.024	0.026 $\pm$ 0.032	0.4980	0.5913
Fusobacteria	-	19.0	27.3	0.032 $\pm$ 0.150	0.009 $\pm$ 0.022	0.5068	0.5913
Firmicutes	-	100.0	100.0	37.966 $\pm$ 21.086	39.233 $\pm$ 20.560	0.7462	0.7462
Class (<i>P</i> value <0.05 prior to FDR adjustment)							
Clostridia	-	100.0	100.0	5.646 $\pm$ 7.668	11.558 $\pm$ 10.208	0.0031	0.0435
OTU (<i>P</i> value <0.05 prior to FDR adjustment)							
<i>Ruminococcus gnavus</i> *	9067	14.3	45.5	0.067 $\pm$ 0.216	1.532 $\pm$ 2.522	0.0010	0.1544
<i>Lachnospiraceae</i> sp.*	23320	7.1	36.4	0.002 $\pm$ 0.007	0.021 $\pm$ 0.041	0.0013	0.1544
<i>Bacteroides caccae</i>	13096	7.1	36.4	0.073 $\pm$ 0.452	0.339 $\pm$ 1.421	0.0019	0.1544
<i>Lachnospiraceae</i> sp.*	5031	2.4	24.2	0.000 $\pm$ 0.002	0.017 $\pm$ 0.047	0.0037	0.2279
<i>Ruminococcus gnavus</i> *	20675	66.7	84.8	1.370 $\pm$ 6.785	1.895 $\pm$ 1.895	0.0056	0.2574
<i>Lachnospiraceae</i> sp.*	26629	14.3	42.4	0.009 $\pm$ 0.042	0.016 $\pm$ 0.031	0.0069	0.2574
<i>Streptococcus</i>	23667	19.0	45.5	0.005 $\pm$ 0.016	0.017 $\pm$ 0.027	0.0084	0.2574
<i>Clostridium</i> *	23959	0.0	15.2	0.000 $\pm$ 0.000	0.052 $\pm$ 0.202	0.0099	0.2574
<i>Bifidobacterium adolescentis</i>	25246	42.9	15.2	0.014 $\pm$ 0.026	0.003 $\pm$ 0.009	0.0100	0.2574
<i>Streptococcus</i>	25916	7.1	30.3	0.008 $\pm$ 0.043	0.009 $\pm$ 0.020	0.0110	0.2574
<i>Eggerthella lenta</i>	3636	35.7	63.6	0.046 $\pm$ 0.104	0.107 $\pm$ 0.150	0.0115	0.2574
<i>Dorea formicigenerans</i> *	6937	4.8	24.2	0.002 $\pm$ 0.010	0.011 $\pm$ 0.026	0.0147	0.2917
<i>Ruminococcaceae</i> sp.*	19836	14.3	39.4	0.037 $\pm$ 0.144	0.202 $\pm$ 0.728	0.0154	0.2917
<i>Dorea formicigenerans</i> *	13285	23.8	45.5	0.094 $\pm$ 0.335	0.376 $\pm$ 0.715	0.0171	0.3008
<i>Lachnospiraceae</i> sp.*	18150	9.5	30.3	0.005 $\pm$ 0.019	0.021 $\pm$ 0.057	0.0223	0.3499
<i>Bacteroides ovatus</i>	19772	16.7	36.4	0.009 $\pm$ 0.024	0.126 $\pm$ 0.364	0.0245	0.3499
<i>Faecalibacterium prausnitzii</i> *	9677	26.2	48.5	0.254 $\pm$ 0.890	1.139 $\pm$ 3.346	0.0257	0.3499
<i>Streptococcus</i>	2401	21.4	42.4	0.006 $\pm$ 0.015	0.037 $\pm$ 0.085	0.0257	0.3499
<i>Coprococcus</i> *	22483	4.8	21.2	0.001 $\pm$ 0.005	0.017 $\pm$ 0.048	0.0269	0.3499
<i>Rothia</i>	20305	28.6	54.5	0.006 $\pm$ 0.011	0.015 $\pm$ 0.020	0.0284	0.3513
<i>Dialister</i> *	13671	4.8	21.2	0.001 $\pm$ 0.005	0.010 $\pm$ 0.032	0.0325	0.3682
<i>Lachnospiraceae</i> sp.*	13505	31.0	54.5	0.015 $\pm$ 0.036	0.056 $\pm$ 0.127	0.0335	0.3682
<i>Coprococcus</i> *	18159	19.0	39.4	0.136 $\pm$ 0.651	0.460 $\pm$ 1.341	0.0343	0.3682
<i>Streptococcus</i>	24037	61.9	45.5	0.036 $\pm$ 0.052	0.014 $\pm$ 0.026	0.0392	0.4035
<i>Finegoldia</i> *	16551	9.5	27.3	0.002 $\pm$ 0.005	0.029 $\pm$ 0.137	0.0419	0.4138
<i>Ruminococcus bromii</i> *	1870	0.0	9.1	0.000 $\pm$ 0.000	0.285 $\pm$ 1.245	0.0494	0.4697

* OTU belongs to the class Clostridia. Abbreviations: FDR, adjusted by Benjamini–Hochberg false discovery rate correction; OTU, 97%-identity operational taxonomic unit; shed+, shedders; shed-, non-shedders.

6.3.5. Microbiota stability

Significant clustering of samples was observed within individuals (adonis, R^2 of 0.676 and 0.651 for unweighted and weighted Unifrac distances, respectively; P values <0.001), and within-subject distances were significantly smaller than between-subject distances (Wilcoxon's rank sum, P values <0.001 for both unweighted and weighted Unifrac; Figure 6.6A). An increase in community turnover between days 0 and 14 was observed in azithromycin compared with placebo recipients based on unweighted Unifrac (0.485 ± 0.074 [mean $\pm$ s.d.] vs 0.446 ± 0.076 ; Wilcoxon's rank sum, $P = 0.005$; Figure 6.6B) but not weighted Unifrac (0.230 ± 0.105 vs 0.218 ± 0.101 ; $P = 0.694$). Microbiota stability did not differ significantly according to OPV outcome (seroconversion or shedding) based on unweighted or weighted distances (Wilcoxon's rank sum, P values >0.05 ; Figures 6.6C and 6.6D).

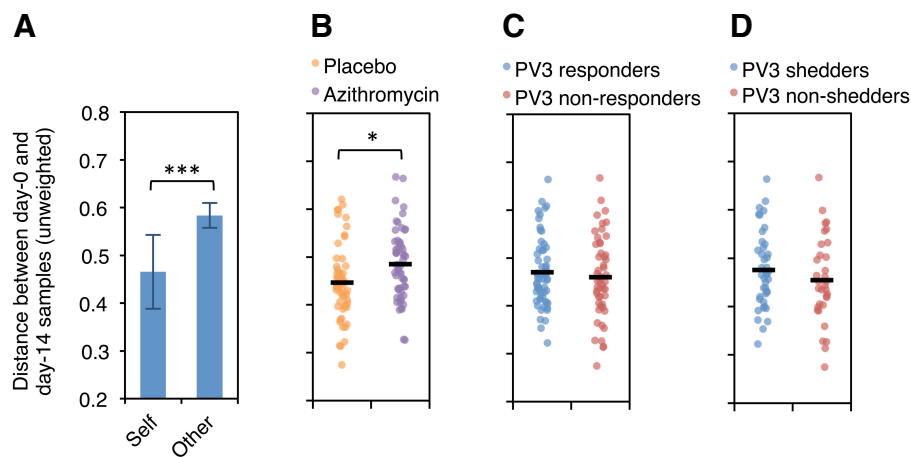


Figure 6.6. Microbiota stability in relation to study arm and oral poliovirus vaccine response.

(A) Within-subject unweighted Unifrac distances were significantly smaller than between-subject distances. Mean values ($\pm$ s.d.) are indicated. (B, C, D) Within-subject unweighted Unifrac distances, used as an indicator of microbiota stability, are displayed according to (B) study arm, (C) seroconversion status, and (D) vaccine take. Mean values in each group are indicated. * $P < 0.05$; *** $P < 0.0005$. Abbreviation: PV3, type 3 poliovirus.

6.3.6. Novel, persistent, and resolved infections

i. Seroconversion

After adjusting for age, we observed a significant reduction in the odds of seroconversion among infants harbouring either novel or persistent infections with ≥ 1 virus on day 14 compared with infants who were uninfected on days 0 and 14 (OR 0.43, 95% CI 0.27–0.67 and OR 0.59, 95% CI 0.40–0.88; P values <0.001 and 0.009, respectively), but not in those with recently resolved infections (OR 1.08, 95% CI 0.69–1.69; $P = 0.748$; Figure 6.7A). Findings with respect to individual viral pathogens are summarised in Table 6.10. Notably, a

significant decrease in the odds of seroconversion was detected among individuals with novel enteroviruses (adjusted OR 0.33, 95% CI 0.21–0.53; FDR-corrected $P < 0.001$) but not persistent enterovirus infections (adjusted OR 0.65, 95% CI 0.42–1.02; FDR-corrected $P = 0.502$; Figure 6.7B). When comparing these infection subclasses directly, we observed a lower seroconversion rate among infants with novel as opposed to persistent enteroviruses (44/127 [35%] vs 63/129 [49%]; χ^2 , $P = 0.021$) – a finding that was not significant for viruses as a whole (χ^2 , $P = 0.128$). The Ct values of persistent versus novel enterovirus infections did not differ significantly at day 14 (27.0 ± 2.2 [mean $\pm$ s.d.] vs 27.2 ± 2.1 ; Wilcoxon’s rank sum, $P = 0.475$), suggesting that the discrepancy in seroconversion rate between these groups was not driven by pathogen copy number.

Across other viral enteropathogens, the odds of seroconversion did not differ significantly among infants with novel, persistent, or recently resolved infections (logistic regression, FDR-corrected P values > 0.05 ; Table 6.10). This was also the case when combining all viral pathogens aside from enteroviruses (logistic regression, P values > 0.05 ; Figure 6.7C). Contrary to the results observed for enterovirus, in no instance did the seroconversion rate differ significantly between individuals with novel as opposed to persistent viral infections (χ^2 , P values > 0.05). Immunogenicity was not impacted by the presence of novel, persistent, or recently resolved infections for any individual bacterial or eukaryotic pathogens (logistic regression, FDR-corrected P values > 0.05 for all univariate and multivariate analyses) or when combining across the pathogens in these groups (logistic regression, P values > 0.05 ; Table 6.11).

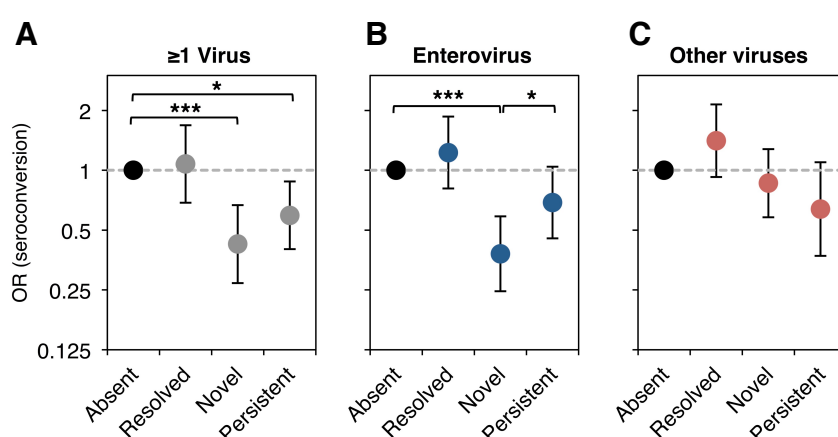


Figure 6.7. Impact of resolved, novel, and persistent viral infections on oral poliovirus vaccine immunogenicity. The impact of infection subclasses on the odds of seroconversion are displayed for (A) ≥ 1 virus, (B) enteroviruses, and (C) ≥ 1 virus (excluding enterovirus). ORs and 95% CIs from univariate logistic regression models are shown. * $P < 0.05$; *** $P < 0.0005$. Abbreviation: OR, odds ratio.

Table 6.10. Impact of resolved, novel, and persistent viral infections on oral poliovirus vaccine immunogenicity.

Pathogen		Responders		Univariate			Multivariate			<i>P</i> Nv vs Pe
		N	(%)	OR (95% CI)	<i>P</i>	FDR <i>P</i>	OR (95% CI)	<i>P</i>	FDR <i>P</i>	
Adenovirus	Absent	507	269 (53.1)							
	Resolved	74	45 (60.8)	1.37 (0.83–2.27)	0.215	0.621	1.60 (0.92–2.79)	0.096	0.503	
	Novel	88	40 (45.5)	0.74 (0.47–1.16)	0.191	0.621	0.72 (0.44–1.20)	0.209	0.636	
	Persistent	35	13 (37.1)	0.54 (0.26–1.09)	0.087	0.616	0.44 (0.21–0.95)	0.035	0.438	0.401
Astrovirus	Absent	684	355 (51.9)							
	Resolved	10	5 (50.0)	1.08 (0.30–3.83)	0.904	0.960	0.90 (0.24–3.37)	0.874	0.941	
	Novel	10	7 (70.0)	2.29 (0.58–9.04)	0.235	0.621	2.92 (0.69–12.43)	0.147	0.611	
	Persistent	0	-	-	-	-	-	-	-	-
Enterovirus	Absent	313	178 (56.9)							
	Resolved	135	82 (60.7)	1.23 (0.81–1.86)	0.334	0.677	1.26 (0.80–1.97)	0.319	0.677	
	Novel	127	44 (34.6)	0.38 (0.25–0.59)	<0.001	<0.001	0.33 (0.21–0.53)	<0.001	<0.001	
	Persistent	129	63 (48.8)	0.69 (0.45–1.04)	0.078	0.502	0.65 (0.42–1.02)	0.060	0.502	0.021
Norovirus	Absent	604	318 (52.6)							
	Resolved	39	22 (56.4)	1.19 (0.62–2.29)	0.608	0.866	1.12 (0.55–2.27)	0.763	0.866	
	Novel	46	20 (43.5)	0.69 (0.38–1.27)	0.234	0.677	0.72 (0.37–1.38)	0.322	0.677	
	Persistent	15	7 (46.7)	0.67 (0.24–1.89)	0.453	0.805	0.69 (0.23–2.04)	0.499	0.805	0.829
Rotavirus	Absent	685	362 (52.8)							
	Resolved	6	2 (33.3)	-	-	-	-	-	-	
	Novel	12	3 (25.0)	0.33 (0.09–1.25)	0.103	0.611	0.36 (0.09–1.50)	0.160	0.611	
	Persistent	1	0 (0.0)	-	-	-	-	-	-	-
Sapovirus	Absent	671	348 (51.9)							
	Resolved	15	8 (53.3)	1.08 (0.38–3.02)	0.886	0.857	0.83 (0.50–4.36)	0.734	0.857	
	Novel	16	9 (56.2)	1.29 (0.47–3.54)	0.619	0.805	1.48 (0.27–2.50)	0.479	0.805	
	Persistent	2	2 (100.0)	-	-	-	-	-	-	-

Infection subclasses of all bacterial, viral, and eukaryotic enteropathogens were included in the FDR correction process. Age was included as a covariate in all logistic regression models (univariate and multivariate). In addition to the variables included above, the multivariate model also included infection subclasses of *B. fragilis*, *Campylobacter*, *Clostridium difficile*, EAEC, EPEC, ETEC, STEC, *Shigella*/EIEC, *Salmonella*, *Cryptosporidium*, and *Giardia*. Across these enteropathogens, no infection subclass was associated with the odds of seroconversion (FDR-corrected *P* values >0.05). Abbreviations: CI, confidence interval; FDR, adjusted by Benjamini–Hochberg false discovery rate correction; Nv, novel; OR, odds ratio; Pe, persistent.

Table 6.11. Impact of resolved, novel, and persistent infections on oral poliovirus vaccine immunogenicity: full and stratified analyses.

Pathogen group	Full				Placebo arm				Azithromycin arm			
	Responders (%)		OR (95% CI)		Responders (%)		OR (95% CI)		Responders (%)		OR (95% CI)	
	N			P	N			P	N			P
≥1 Virus ^a	186	Absent	112 (60.2)		104	64 (61.5)			82	48 (58.5)		
	144	Resolved	88 (61.1)	0.748	72	40 (55.6)	0.79 (0.43–1.45)	0.445	72	48 (66.7)	1.55 (0.79–3.02)	0.202
	139	Novel	55 (39.6)	<0.001	69	29 (42.0)	0.44 (0.23–0.82)	0.009	70	26 (37.1)	0.43 (0.22–0.83)	0.011
	235	Persistent	112 (47.7)	0.009	112	59 (52.7)	0.68 (0.40–1.18)	0.173	123	53 (43.1)	0.53 (0.30–0.95)	0.032
≥1 Virus (exc. EV) ^b	388	Absent	204 (52.6)		197	106 (53.8)			191	98 (51.3)		
	119	Resolved	72 (60.5)	0.111	60	36 (60.0)	1.30 (0.72–2.34)	0.388	59	36 (61.0)	1.54 (0.84–2.81)	0.159
	134	Novel	65 (48.5)	0.459	66	35 (53.0)	1.00 (0.57–1.75)	0.988	68	30 (44.1)	0.74 (0.42–1.30)	0.298
	63	Persistent	26 (41.2)	0.105	34	15 (44.1)	0.68 (0.33–1.41)	0.301	29	11 (37.9)	0.59 (0.26–1.32)	0.200
≥1 Bacteria	34	Absent	22 (64.7)		11	8 (72.7)			23	14 (60.9)		
	128	Resolved	69 (53.9)	0.386	29	17 (58.6)	0.60 (0.13–2.76)	0.510	99	52 (52.5)	0.78 (0.31–1.99)	0.609
	45	Novel	24 (53.3)	0.380	29	18 (62.1)	0.66 (0.14–3.05)	0.596	16	6 (37.5)	0.40 (0.11–1.51)	0.178
	497	Persistent	252 (50.7)	0.216	288	149 (51.7)	0.45 (0.12–1.74)	0.246	209	103 (49.3)	0.71 (0.29–1.74)	0.458
≥1 Bacteria (exc. EAEC)	130	Absent	72 (55.4)		58	35 (60.3)			72	37 (51.4)		
	173	Resolved	85 (49.1)	0.515	59	30 (50.8)	0.71 (0.34–1.49)	0.372	114	55 (48.2)	1.02 (0.56–1.87)	0.950
	107	Novel	53 (49.5)	0.447	65	34 (52.3)	0.74 (0.36–1.51)	0.402	42	19 (45.2)	0.83 (0.38–1.78)	0.626
	294	Persistent	157 (53.4)	0.784	175	93 (53.1)	0.82 (0.44–1.53)	0.541	119	64 (53.8)	1.32 (0.72–2.41)	0.376
≥1 Eukaryote	583	Absent	313 (53.7)		301	166 (55.1)			282	147 (52.1)		
	26	Resolved	11 (42.3)	0.276	14	4 (28.6)	0.33 (0.10–1.08)	0.067	12	7 (58.3)	1.31 (0.40–4.24)	0.657
	29	Novel	14 (48.3)	0.566	10	4 (40.0)	0.54 (0.15–1.96)	0.349	19	10 (52.6)	1.02 (0.40–2.60)	0.969
	66	Persistent	29 (43.9)	0.291	32	18 (56.3)	1.17 (0.55–2.49)	0.675	34	11 (32.3)	0.49 (0.23–1.05)	0.066

Abbreviations: CI, confidence interval; EAEC, enteroaggregative *Escherichia coli*; EV, enterovirus; OR, odds ratio.

^a χ^2 (novel vs persistent infections), *P* values 0.128, 0.164, and 0.419 for full study population, placebo recipients, and azithromycin recipients, respectively.

^b χ^2 (novel vs persistent infections), *P* values 0.342, 0.398, and 0.572 for full study population, placebo recipients, and azithromycin recipients, respectively.

ii. Vaccine take

Analyses of infection subclass in relation to vaccine take were constrained by the fact that type 3 poliovirus shedding was assessed in only a subset of per-protocol infants with available TAC data (291/704). As such, infection subclasses often lacked sufficient individuals for their impact to be assessed. Nonetheless, we observed a reduction in the odds of vaccine take among infants who experienced either novel or persistent infections with ≥ 1 virus (OR 0.50, 95% CI 0.24–1.04 and OR 0.53, 95% CI 0.28–1.00, respectively; Figure 6.8A); however, in contrast to the analyses of seroconversion status, this effect was significant only for the latter (P values 0.062 and 0.050, respectively). Among individual viral pathogens (including enteroviruses; Figure 6.8B), no infection subclass significantly impacted the odds of vaccine take after adjusting for multiple comparisons (logistic regression, FDR-corrected P values >0.05 for all univariate and multivariate analyses; data not shown). When combining across viruses other than enterovirus, the resolution of infections between days 0 and 14 was associated with an increase in the odds of vaccine take (OR 2.46, 95% CI 1.20–5.06; $P = 0.014$; Figure 6.8C). OPV take was not affected by resolved, novel, or persistent bacterial infections (logistic regression, FDR-corrected P values >0.05 for univariate and multivariate analyses of individual pathogens and P values >0.05 when combining across bacteria; Table 6.12). However, we observed a significant decrease in the odds of vaccine take among infants persistently infected with ≥ 1 eukaryote (OR 0.30, 95% CI 0.11–0.74; $P = 0.009$; Table 6.12) – a result consistent with the lower prevalence of *Giardia* observed in shedders compared with non-shedders during the primary outcome analyses (Grassly et al, 2016).

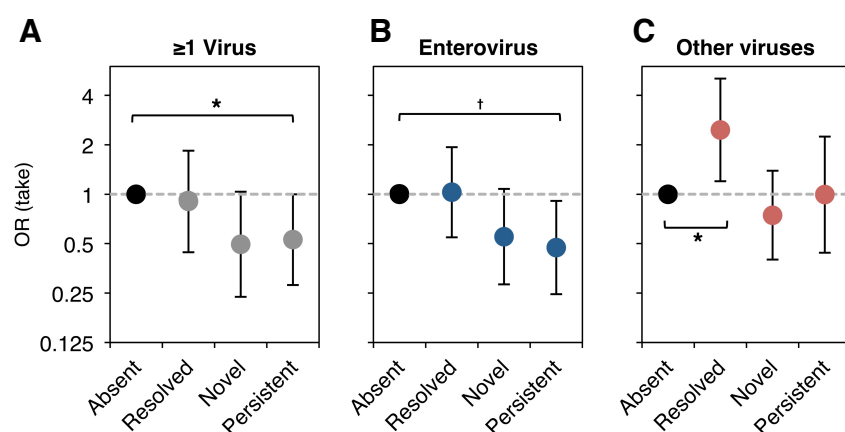


Figure 6.8. Impact of resolved, novel, and persistent viral infections on oral poliovirus vaccine take. The impact of infection subclasses on the odds of vaccine take are displayed for (A) ≥ 1 virus, (B) enteroviruses, and (C) ≥ 1 virus (excluding enterovirus). ORs and 95% CIs from univariate logistic regression models are shown. * $P < 0.05$; †, $P < 0.05$ prior to FDR adjustment. Abbreviation: OR, odds ratio.

Table 6.12. Impact of resolved, novel, and persistent infections on oral poliovirus vaccine take: full and stratified analyses.

Pathogen group	Full				Placebo arm				Azithromycin arm			
	Shedders (%)		OR (95% CI)		Shedders (%)		OR (95% CI)		Shedders (%)		OR (95% CI)	
	N			P	N			P	N			P
≥1 Virus ^a	67	Absent	42 (62.7)		34	20 (58.9)			33	22 (66.7)		
	65	Resolved	38 (58.5)	0.90 (0.44–1.84)	34	18 (52.9)	0.81 (0.30–2.15)	0.666	31	20 (64.5)	1.02 (0.36–2.93)	0.968
	55	Novel	26 (47.3)	0.50 (0.24–1.04)	31	14 (45.2)	0.50 (0.18–1.37)	0.176	24	12 (50.0)	0.49 (0.17–1.46)	0.202
	104	Persistent	50 (48.1)	0.53 (0.28–1.00)	49	31 (63.3)	1.14 (0.45–2.85)	0.783	55	19 (34.5)	0.26 (0.10–0.65)	0.004
≥1 Virus (exc. EV) ^b	161	Absent	83 (51.6)		79	41 (51.9)			82	42 (52.5)		
	47	Resolved	34 (72.3)	2.46 (1.20–5.06)	26	19 (73.1)	2.55 (0.94–6.89)	0.066	21	15 (71.4)	2.37 (0.83–6.77)	0.108
	55	Novel	25 (45.5)	0.74 (0.40–1.39)	30	16 (53.3)	1.06 (0.45–2.49)	0.902	25	9 (36.0)	0.49 (0.19–1.26)	0.137
	28	Persistent	14 (50.0)	0.99 (0.44–2.25)	13	7 (53.8)	1.08 (0.32–3.59)	0.904	15	7 (46.7)	0.92 (0.30–2.80)	0.876
≥1 Bacteria	12	Absent	8 (66.7)		2	2 (100.0)			10	6 (60.0)		
	53	Resolved	28 (52.8)	0.67 (0.18–2.53)	15	10 (66.7)		baseline	38	18 (47.4)	0.67 (0.16–2.81)	0.585
	16	Novel	10 (62.5)	0.90 (0.18–4.36)	7	6 (85.7)	-	-	9	4 (44.4)	-	-
	210	Persistent	110 (52.4)	0.64 (0.19–2.22)	124	65 (52.4)	0.51 (0.16–1.62)	0.255	86	45 (52.3)	0.80 (0.21–3.08)	0.747
≥1 Bacteria (exc. EAEC)	50	Absent	27 (54.0)		19	12 (63.2)			31	15 (48.4)		
	66	Resolved	34 (51.5)	1.05 (0.50–2.23)	26	14 (53.8)	0.83 (0.24–2.88)	0.773	40	20 (50.0)	1.19 (0.46–3.09)	0.723
	47	Novel	26 (55.3)	1.02 (0.46–2.29)	28	16 (57.1)	0.70 (0.21–2.35)	0.565	19	10 (52.6)	1.22 (0.38–3.84)	0.740
	128	Persistent	69 (53.9)	1.22 (0.62–2.40)	75	41 (54.7)	0.91 (0.31–2.66)	0.864	53	28 (52.8)	1.36 (0.55–3.39)	0.504
≥1 Eukaryote	247	Absent	142 (57.5)		131	74 (56.5)			116	68 (58.6)		
	5	Resolved	2 (40.0)	-	3	2 (66.7)	-	-	2	0 (0.0)	-	-
	11	Novel	5 (45.5)	0.65 (0.19–2.20)	5	3 (60.0)	-	-	6	2 (33.3)	-	-
	28	Persistent	7 (25.0)	0.30 (0.12–0.74)	9	4 (44.4)	-	-	19	3 (15.8)	0.14 (0.04–0.52)	0.003

Abbreviations: CI, confidence interval; EAEC, enteroaggregative *Escherichia coli*; EV, enterovirus; OR, odds ratio.

^a χ^2 (novel vs persistent infections), *P* values 0.923, 0.112, and 0.196 for full study population, placebo recipients, and azithromycin recipients, respectively.

^b χ^2 (novel vs persistent infections), *P* values 0.695, 0.975, and 0.505 for full study population, placebo recipients, and azithromycin recipients, respectively.

6.3.7. Random Forests

The accuracy of Random Forest models for the prediction of study arm and OPV outcome is displayed in Figure 6.9 for different modules of input data. The use of either concurrent pathogen or microbiota data obtained from day-14 samples enabled the prediction of study arm with 61.2% and 67.5% accuracy, respectively (baseline accuracy: 50.9%) – a significant improvement in fit compared with null models (P values <0.05 ; Figure 6.9A). Prediction of seroconversion status was poor for all models (Figure 6.9B). Although a significant improvement in model fit was observed when 16S rRNA microbiota composition was used to distinguish type 3 poliovirus shedders from non-shedders, the increase in accuracy was negligible (61.6% vs 56.0% for fitted and baseline models, respectively; Figure 6.9C). In no instance did the combination of data across different modules substantially improve the predictive accuracy of the Random Forest models. Variables with the highest importance scores for the combined models are displayed in Figure 6.10. Despite the poor predictive capacity of the models, enterovirus was consistently identified as the most important enteropathogen when predicting OPV response.

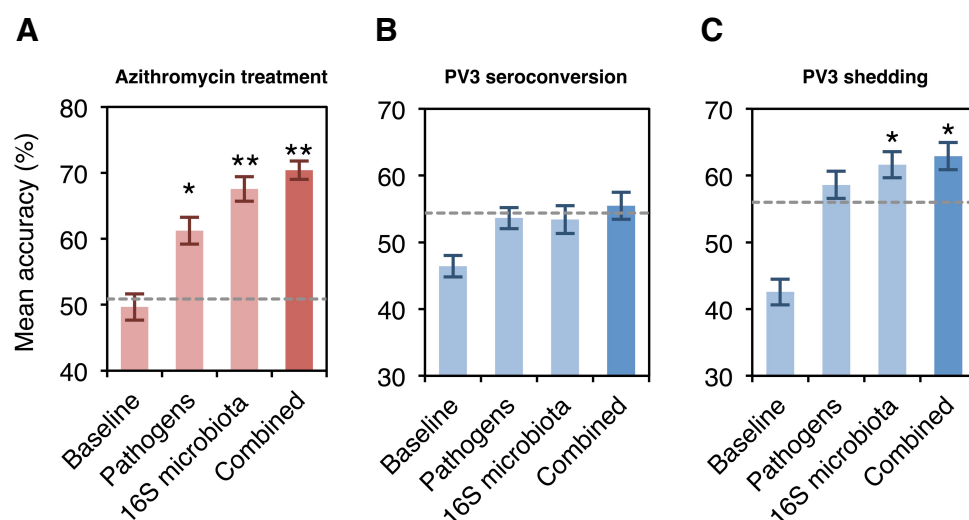


Figure 6.9. Predictive accurate of Random Forest models using different modules of data. Mean accuracy ($\pm$ s.d.) across 100 iterations of the Random Forest algorithm is displayed for models predicting (A) azithromycin treatment, (B) seroconversion status, and (C) vaccine take. See Table 6.2 for a list of variables included in each module. Dotted lines indicate baseline accuracy, wherein all individuals are assigned to the majority class. * $P < 0.05$; ** $P < 0.005$. Abbreviation: PV3, type 3 poliovirus.

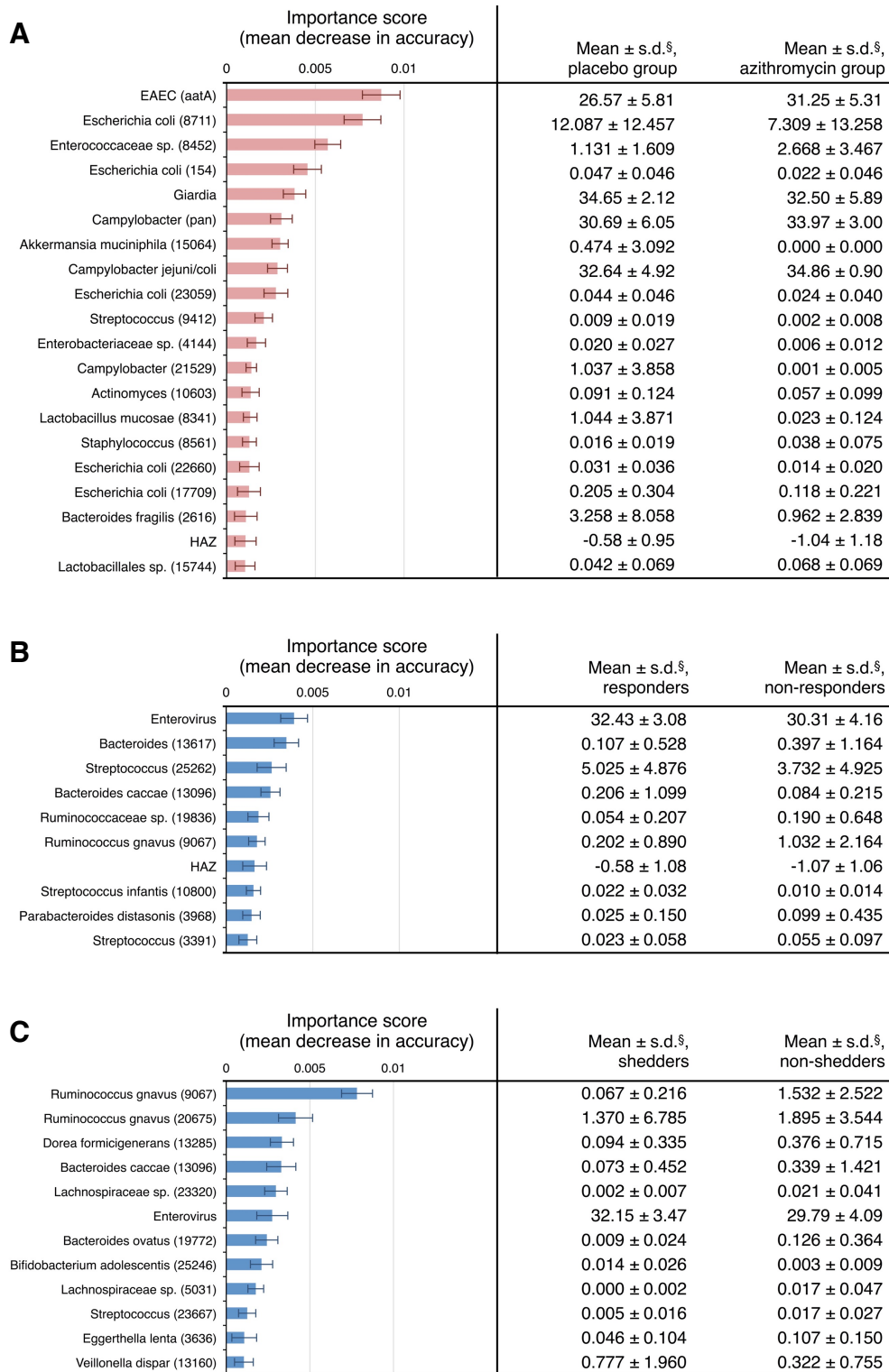


Figure 6.10. Highest ranking variables by Random Forest importance score. Data are displayed for models predicting (A) azithromycin treatment, (B) seroconversion status, and (C) vaccine take using variables from all modules of data (Table 6.2). Mean importance scores ($\pm$ s.d.) were calculated over 100 iterations of the Random Forest algorithm. § Values correspond to TAC Ct for enteropathogens and relative abundance (%) for OTUs. Abbreviations: EAEC, enteroaggregative *Escherichia coli*; HAZ, height-for-age Z score.

6.3.8. Multivariate logistic regression

During the analyses of microbiota composition described above, the Shannon index of day-14 samples demonstrated the strongest association with OPV outcome (Table 6.5). When Shannon index and viral pathogen infection status on day 14 were included as predictors of OPV response in multivariate logistic regression models, the associations for the former were partially abrogated (Table 6.13), suggesting that the observed link between Shannon index and OPV outcome may reflect a degree of confounding between microbiota diversity and viral infection status.

Table 6.13. Multivariate logistic regression outputs.

Table 6.15. Multivariate logistic regression outputs.						
Variable	Day	N	Univariate		Multivariate	
			OR (95% CI)	P	OR (95% CI)	P
Model 1: PV3 seroconversion, full data						
≥1 Virus	14	704	0.51 (0.38–0.69)	<0.001		
Model 2: PV3 seroconversion, microbiota subset						
≥1 Virus	14	114	0.44 (0.21–0.96)	0.039	0.48 (0.22–1.06)	0.068
Shannon index			0.46 (0.20–1.03)	0.059	0.50 (0.22–1.15)	0.105
Model 3: PV3 take, full data						
≥1 Virus	14	291	0.54 (0.34–0.88)	0.013		
Model 4: PV3 take, microbiota subset						
≥1 Virus	14	75	0.27 (0.10–0.76)	0.013	0.32 (0.11–0.90)	0.031
Shannon index			0.37 (0.14–0.97)	0.044	0.45 (0.17–1.23)	0.118

Age was included as a covariate in all models (univariate and multivariate). No other variables were adjusted for. ORs signify the change in the relative odds of responding to OPV in conjunction with: (i) the presence versus absence of concurrent infection; or (ii) a 1-unit change in Shannon index. Abbreviations: CI, confidence interval; OR, odds ratio; OTU, 97%-identity operational taxonomic unit; PV3, type 3 poliovirus.

6.3.9. Sensitivity analyses

i. Stratification by study arm

When analyses were stratified by study arm, discrepancies in alpha and beta diversity according to OPV outcome were largely consistent among placebo and azithromycin recipients, although comparisons were generally not statistically significant (Tables 6.5 and 6.6). We performed similar stratified analyses when considering the impact of resolved, novel, and persistent infections on OPV outcome (Tables 6.11 and 6.12). This revealed that the reduction in the odds of type 3 poliovirus shedding in conjunction with persistent viral infections was apparent only in azithromycin recipients (Table 6.12). By contrast, the reduced odds of seroconversion among infants with novel viral infections was evident in both study arms (Table 6.11).

ii. Antibody titre

Among per-protocol infants included in the microbiota subset, 23/114 (20%) achieved a post-vaccination antibody titre of $\geq 1:256$. When these 'robust responders' were compared to infants who failed to seroconvert, we observed no significant discrepancies in alpha diversity (Table 6.5), and no significant clustering of samples based on unweighted or weighed Unifrac distances (Table 6.6). ORs associated with resolved, novel, and persistent infections for pathogen groups were generally consistent with those observed in the primary analysis, though in no instance did infection subclass significantly impact the odds of seroconversion within this subset of the study population (data not shown).

iii. Exclusion of infants exposed to non-intervention antibiotics

The observed impact of azithromycin on alpha and beta diversity was consistent when infants who received antibiotics other than the study intervention were excluded (Tables 6.5 and 6.6). In a related analysis, we excluded infants who received any antibiotics (azithromycin or other), and examined the associated between microbiota diversity and seroconversion status. As with the primary analyses, an increased OTU count and Shannon index was apparent in non-responders compared with responders, albeit not statistically significant (Table 6.5). No significant clustering of samples according to seroconversion status was evident based unweighted or weighted Unifrac distances (Table 6.6).

6.3.10. Negative controls

Across the 12 extraction controls and single no-template control included in the MiSeq run, we obtained five sequences in total (0.4 sequences per sample on average), suggesting that any contamination of laboratory reagents or cross-contamination during library preparation was negligible.

6.4. Discussion

Antibiotic treatment had a marked effect on the composition of the bacterial microbiota in this study population. Although tangential to the primary aims of this thesis, these novel findings warrant brief consideration. Azithromycin has previously been shown to inhibit growth of numerous Gram-negative and Gram-positive bacteria, and is widely used to treat infections of the respiratory tract, urogenital tract, and skin (Parnham et al, 2014).

Therapeutic activity also extends to bacterial enteropathogens, as highlighted in previous analyses of this study population (Grassly et al, 2016), and the drug has proven effective for the treatment of travellers' diarrhoea (Adachi et al, 2003). Antibacterial mechanisms are varied, encompassing the inhibition of protein synthesis via binding of the 50S subunit of the prokaryotic ribosome, the disruption of quorum sensing (the mechanism by which bacteria regulate gene expression in response to fluctuations in population density), and interference with adherence to host cells, among others (Kano and Rubin, 2010; Parnham et al, 2014). The macrolide antibiotic also appears to have immunomodulatory effects independent of its antibacterial activity, including the suppression of pro-inflammatory cytokines such as TNF α (Cigana et al, 2007; Tong et al, 2011).

Although the antimicrobial effects of azithromycin have been extensively studied in relation to pathogenic bacteria, less is known regarding the impact of this drug on the bacterial microbiota as a whole. We assessed 16S rRNA microbiota composition among 6–11 month-old infants before and 12 days after the completion of a 3-day course of azithromycin or placebo. Antibiotic treatment led to a decline in the number of OTUs by approximately 10% and a modest but significant shift in community composition based on unweighted or weighted Unifrac distances. These changes were driven primarily by the azithromycin-induced depletion of Proteobacteria (including several OTUs classified as *E. coli*) and Verrucomicrobia (including a single OTU classified as *A. muciniphila*). Given the broad-spectrum activity of azithromycin, it is likely that absolute decreases in bacterial abundance occurred across other phyla in these infants. However, the sequencing pipeline adopted in this study only enabled us to capture changes in the relative abundance of bacterial taxa at a given sequencing depth. It is important, therefore, to frame any conclusions from this work accordingly – while azithromycin depleted the abundance of OTUs in the phyla Proteobacteria and Verrucomicrobia to a greater extent than other taxa, we cannot preclude a broader impact of the antibiotic on the infant microbiota.

Notably, these results appear to contradict recent findings from Finland regarding the impact of macrolide antibiotics on microbiota composition. Among children 2–7 years of age, Korpela et al (2016) observed a significant decrease in the relative abundance of Actinobacteria and a corresponding increase in Proteobacteria and Bacteroidetes among children exposed to azithromycin or clarithromycin within the preceding 6 months. The discord between these studies can likely be attributed to differences in setting (India vs Finland), age (6–11 months vs 2–7 years), and time since antibiotic exposure (12 days vs up to 6 months). A follow-up study exploring the longer-term effects of azithromycin in this study

population is a potential avenue of future investigation, albeit beyond the scope of the present thesis.

The azithromycin-induced depletion of *A. muciniphila* (the only OTU observed within the phylum Verrucomicrobia in this study) is also at odds with previous findings. An increased abundance of this mucin-degrading species of Gram-negative bacteria has been observed following the treatment of adults with a range of non-macrolide antibiotic regimens (Dubourg et al, 2013). Similar findings have been documented following the treatment of mice with the veterinary macrolide tylosin, suggesting that *A. muciniphila* may be an opportunistic microbe that thrives in response to ecosystem disturbance (Nobel et al, 2015). However, while the strain was observed in the day-14 stool samples of approximately 20% of infants who received placebo in the present study, it was absent from the post-treatment samples of all azithromycin recipients.

Discrepancies in microbiota composition with respect to OPV response were more modest than those induced by azithromycin. As observed among recipients of tOPV at 6 weeks of age in the previous chapter, vaccine outcome was negatively correlated with diversity of the bacterial microbiota at the time of immunisation (discrepancies were significant for comparisons of OPV take but not seroconversion status). In line with these changes in microbiota diversity on day 14, we observed a significant shift in taxonomic composition with respect to vaccine outcome (seroconversion and take) based on unweighted Unifrac distances, albeit accounting for <3% of the variation among samples. By contrast, no differences in microbiota diversity or composition were observed 14 days prior to immunisation.

Taken together, these findings suggest that a transient shift in 16S rRNA microbiota composition, including an increase in microbial diversity, may represent a significant risk factor for OPV non-response. Whether these shifts in composition are causally related to OPV outcome, or simply correlate with other risk factors for vaccine failure, remains uncertain. Notably, the results of multivariate logistic regression revealed a degree of confounding between microbiota diversity and viral pathogen infection status at day 14. We did not identify any OTUs that differed significantly in relative abundance with respect to seroconversion status or vaccine take, thus providing further evidence to refute the hypothesis that 'keystone taxa' might inhibit OPV response (see section 1.6). Among OTUs with the largest discrepancies in abundance according to seroconversion status or vaccine take, several taxa in the class Clostridia were observed to be negatively correlated with vaccine outcome. However, in contrast to the analyses of alpha and beta diversity described

above, we did not adjust for age during comparisons of taxon relative abundance (a constraint of the non-parametric tests adopted for these analyses). Given that older infants were less likely to respond to OPV in this study population (Grassly et al, 2016), and that OTUs in the class Clostridia were among the highest-ranking age-discriminatory taxa, the observed discrepancies in taxon relative abundance may simply reflect the confounding of these variables with age. Equally, one cannot preclude the possibility that shifts in microbiota composition are among the factors responsible for age-associated changes in OPV immunogenicity.

Our analyses of the microbiota also included measures of stability and maturity. Whereas azithromycin treatment resulted in a significant decline in microbiota stability (based on within-subject unweighted Unifrac distances), the degree of microbial community turnover in the 14 days preceding vaccination did not significantly influence OPV outcome – a finding consistent with observations for oral rotavirus vaccine and tOPV in the preceding chapters. We assessed microbiota maturity among infant samples by measuring their mean Unifrac distance from adult samples. Although not as nuanced, perhaps, as the microbiota-for-age Z score recently applied among infants in Bangladesh based on the outputs of a Random Forests regression model (Subramanian et al, 2014), this approach nonetheless captured the shift towards a more adult-like microbiota with increasing age in this study population. After adjusting for age, microbiota maturity on the day of vaccination was negatively correlated with OPV outcome (both seroconversion and shedding). However, maturity index was closely correlated with OTU count and Shannon index at enrolment. Since the discrepancies in microbiota maturity were absent on day 0, the differences observed in day-14 samples are more likely to be indicative of the transient increase in diversity among vaccine non-responders than an accelerated maturation of the bacterial microbiota in these individuals.

Turning our attention away from the bacterial microbiota, we observed a reduction in OPV immunogenicity in infants with novel as opposed to persistent enterovirus infections. This finding should be interpreted with a degree of caution given its modest effect size (seroconversion was observed in 35% and 49% of infants with novel and persistent enterovirus infections, respectively), and the fact that the discrepancy was absent when considering vaccine take. These caveats notwithstanding, the possibility that recently acquired viral infections may have an enhanced inhibitory impact on OPV response is plausible. Viral infections initiate a cascade of innate immune effectors that typically peak within several days of exposure (Van Reeth, 2000; Duan et al, 2014). For example, following

the infection of peripheral blood leukocytes with either Coxsackie B4 virus or type 1 poliovirus, the production of pro-inflammatory cytokines, including TNF α and IL-1 β , has been shown to peak within 24 hours (Juhela et al, 1999). Similarly, the antiviral cytokine IFN α reaches maximum levels in serum within 2 days of the onset of symptomatic rotavirus infection (De Boissieu et al, 1993). If the induction of innate antiviral immunity is responsible for the inhibitory effect of enteroviruses on OPV immunogenicity, the timescale of viral exposure may well be pertinent to the outcomes of co-infection.

Our final line of enquiry was to assess the added value of combining different types of data when predicting study arm or OPV outcome using Random Forests. The use of either TAC or microbiota data alone essentially recapitulated the outcomes of the primary analyses described above – models incorporating these variables had a modest accuracy when predicting azithromycin treatment, but limited predictive capacity for OPV outcome. In no instance did the combination of data regarding baseline characteristics (including sociodemographic and anthropometric data), pathogen infection status, and OTU relative abundances substantially improve the predictive capacity of the Random Forest models compared with the most accurate individual module of data. It is possible that alternative statistical or machine-learning approaches may have enabled the more productive integration of these data, or that the inclusion of a greater number of infants may have improved the accuracy of our Random Forest models (of the 705 infants who completed the trial per protocol, our models encompassed 75 individuals for the prediction of OPV take and 114 for the prediction of study arm and seroconversion status). As high-throughput technologies become more readily available, how to make best use of the resulting data represents a considerable bioinformatic and statistical challenge. The use of machine-learning approaches such as Random Forests undoubtedly holds significant potential as we seek novel insights within these ever-expanding datasets. When used on an exploratory basis in the present study, however, such insights were not forthcoming.

6.4.1. Conclusions

The intestinal microbiota is in a constant state of flux throughout infancy. This flux encompasses a continual expansion in the diversity of the bacterial microbiota, and exposure to a diverse array of bacterial, viral, and eukaryotic enteropathogens. The inhibitory effect of concurrent viral infections (particularly NPEVs) on OPV has become increasingly clear throughout this thesis, and was confirmed in the EVI study. The influence of the bacterial microbiota is more ambiguous, although we have now observed a modest increase in

microbial diversity among OPV non-responders in south India at 6 weeks of age (in Chapter 5) and at 6–11 months of age (above). As expected, azithromycin exposure had a considerable impact on composition of the gut's microbial inhabitants – evident both in the prevalence and abundance of bacterial enteropathogens and in the composition and diversity of the bacterial microbiota as a whole.

Perhaps the most interesting finding to emerge from the present chapter, however, is the importance of recent changes in microbiota composition to oral vaccine outcome. Failure to respond to OPV was associated with a transient increase in diversity of the bacterial microbiota, and novel enteroviruses appeared to have a greater inhibitory impact on vaccine immunogenicity than persistent infections. Although these findings do not preclude a role for more entrenched risk factors of OPV failure, such as the chronic inflammation associated with EE, they suggest that the likelihood of responding to oral vaccination may fluctuate from week to week. The gut's microbial residents – particularly viral enteropathogens – appear to play a key role in shaping these short-term fluctuations.

This primary aim of this thesis was to characterise the association between the composition of the intestinal microbiota at the time of immunisation and the ensuing response to live-attenuated oral vaccines targeting poliovirus and rotavirus. In doing so, we hoped to provide some insight into the biological mechanisms that undermine the performance of oral vaccines in the low-income countries where they are needed most.

7.1. Summary of findings

There are two distinct facets of the intestinal microbiota that we considered in the research described above: (i) the burden of bacterial, viral, and eukaryotic enteropathogens; and (ii) the composition of the bacterial flora as a whole (including the diversity and relative abundance of bacterial taxa, irrespective of their pathogenicity). With respect to the former, our findings have helped to substantiate the inhibitory effect of enteropathogens (particularly viral infections) on OPV. In Chapter 2, I presented the findings of a systematic review and meta-analysis considering the impact of enteric infections on the immunogenicity and take of OPV. Within a somewhat conflicted literature, we observed a reduction in the odds of responding to a given dose of OPV among individuals with NPEVs or diarrhoea at the time of immunisation.

We subsequently extended these findings by exploiting recent advances in molecular diagnostics (introduced in Chapter 3) to assess the burden of 31 distinct enteropathogens among infants living in south India. In Chapters 4 and 5, we compared the burden of pathogens according to the immunogenicity and take of Rotarix and tOPV doses administered at 6 and 10 weeks of age. Enteropathogens did not have a significant inhibitory effect on Rotarix outcome (indeed, a greater prevalence of pathogens was observed at 6 weeks of age among infants who seroconverted to rotavirus). However, we observed a modest but significant increase in the burden of pathogens – and specifically of asymptomatic bacterial infections – among infants who failed to seroconvert to OPV. The potential for asymptomatic bacteria to interfere with OPV administered during routine immunisation constitutes a novel outcome of this thesis. Moreover, if we assume the observed enteric infections to be causally related to the impairment of OPV response, our findings were consistent with approximately 10% of failed seroconversions per dose of tOPV being attributable to concurrent pathogens in this study population.

During a subsequent trial assessing the impact of azithromycin treatment on the immunogenicity of mOPV3 among 6–11 month-old infants, no discrepancy in the prevalence of bacterial enteropathogens was observed according to vaccine outcome (Grassly et al, 2016). However, viral infections were significantly more common among infants who failed to seroconvert to type 3 poliovirus. In Chapter 6, we extended the primary outcome analyses of this trial by discriminating between novel, persistent, and recently resolved infections. This revealed that novel enteroviruses (present on the day of immunisation but absent 14 days earlier) had a more dramatic inhibitory effect on OPV immunogenicity than persistent infections. Although the same effect was not apparent when considering vaccine take, and should thus be interpreted with a degree of caution, the notion that viral interference may fade in the window after an infection is acquired is an intriguing possibility.

Our results with respect to the 16S rRNA microbiota should – in my opinion – be considered negative findings. Although microbiota diversity at the time of immunisation was positively correlated with the take of Rotarix (Chapter 4), and negatively correlated with the take and/or immunogenicity of OPV (Chapters 5 and 6), the effect size was modest in each instance. No significant associations were observed between the relative abundance of individual taxa and vaccine outcome – or to use the terminology introduced in Chapter 1, we did not identify any ‘keystone taxa’ associated with vaccine response. Likewise, machine-learning algorithms were incapable of accurately discriminating responders from non-responders for either vaccine based on the OTU abundance data generated in these studies. By contrast, significant discrepancies in taxon relative abundance were observed in conjunction with exposure to either *L. rhamnosus* GG (Chapter 4) or azithromycin (Chapter 6), confirming that the sequencing pipeline and bioinformatic methods adopted in this thesis were capable of distinguishing clinically relevant shifts in the composition of the bacterial microbiota.

7.2. Limitations

Specific limitations relating to the methods adopted in this thesis have been discussed in detail in the preceding chapters. Here, I will expand briefly on the broader limitations of this work. In doing so, I hope to provide better context to the key findings outlined above.

First, it is important to acknowledge constraints in the extent to which we have accurately captured the diversity and functional capacity of the intestinal microbiota in this study. Bacteriophages were not considered, nor were many of the diverse array of eukaryotic

viruses, yeasts, fungi, and helminths that constitute the intestinal microbiota. Meanwhile, despite the use of an amplicon sequencing method that has been widely adopted in recent studies of the bacterial microbiota (Caporaso et al, 2012; Subramanian et al, 2014), we cannot discount biases introduced by the incomplete coverage of the primers adopted (Nelson et al, 2014), and by the inability to discriminate between distinct bacterial strains when sequencing only the V4 region of the 16S rRNA gene. Indeed, the extraction techniques, primers, and sequencing methods should all be seen as caveats to the findings presented in this thesis. It is certainly possible that the use of an alternative pipeline – such as profiling of the metagenome via shotgun sequencing (Smith et al, 2013), assessment of the enteric virome (Norman et al, 2015), or even the selection of a different hypervariable region of the 16S rRNA gene for amplicon-based sequencing – might have highlighted discrepancies in microbiota composition pertinent to the impaired performance of oral vaccines. Given the increased accessibility and cost-effectiveness of these methods, it is likely that numerous studies will address this possibility in the coming years. In spite of the predominantly negative findings reported here, I would not be surprised if the commensal intestinal flora is yet found to play an important role in shaping the immune response to oral vaccines.

In the latter chapters of this thesis, we explored potential associations between the composition of the intestinal microbiota and oral vaccine outcome by conducting nested case-control studies among infants who had participated in clinical trials of Rotarix and OPV in south India. A benefit of this approach is that it enables the risk factors for oral vaccine failure to be studied within the very setting in which it manifests itself as a public health problem. The poor immunogenicity of Rotarix in the study population described in Chapters 4 and 5 (at 31% after two doses) is a testament to this. However, there are limits to the mechanistic insights that can be garnered from such an approach. Although the increased burden of bacterial enteropathogens observed at 6 and 10 weeks of age among infants who failed to seroconvert to type 3 poliovirus may reflect a direct inhibitory effect of these infections on the take of attenuated vaccine polioviruses, the infections might also be indicative of an alternative underlying risk factor for OPV failure, such as acute malnutrition or EE. Exploring oral vaccine failure using animal models may help to delineate the key mechanisms responsible for this phenomenon. This is a possibility that I will consider further below.

The practical implications of this work are somewhat modest, at least for the foreseeable future. While our findings are among the first to implicate bacterial enteropathogens as a risk factor for OPV failure, the logical intervention arising from this observation – to treat infants

with antibiotics prior to immunisation – did not bring about any improvement in OPV immunogenicity when tested among infants in south India (Grassly et al, 2016). Equivalent interventions targeting enteric viruses are presently lacking. However, the findings presented in Chapter 6 raise the possibility that a transient reduction in viral exposure prior to immunisation might be sufficient to boost the efficacy of oral vaccination. The ongoing Sanitation Hygiene Infant Nutrition Efficacy (SHINE) trial in Zimbabwe – examining the impact of water, sanitation, and hygiene and/or infant feeding interventions on stunting and anaemia (with OPV and rotavirus vaccine immunogenicity considered as secondary outcomes) – may provide some insight here (Prendergast et al, 2015).

Finally, following the rapid proliferation of next-generation sequencing and the packaging of bioinformatic tools into open-source pipelines such as Qiime, we are currently in a position where our ability to characterise the intestinal microbiota is more advanced than our capacity to augment it in a predictable manner. Indeed, it would be reasonable to ask the following of the work described above: if shifts in the composition of the bacterial microbiota were to be identified as a direct cause of oral vaccine failure, what could we do about it? Faecal microbiota transplantation has been used as an intervention for IBD (Anderson et al, 2012) and recurrent *Clostridium difficile* (Gough et al, 2011), though it is hard to envision this being applied as a means of boosting the efficacy of oral vaccines during infancy. Meanwhile, as described in Chapters 3 and 4, supplementation with a single probiotic strain did not substantially augment the immunogenicity of Rotarix and tOPV among infants in south India (Lazarus et al, manuscript in preparation). However, future ‘microbiota-directed therapeutics’ may enable us to mould the intestinal flora in a more sophisticated manner – for example, by augmenting the infant diet to foster the growth of specific bacterial taxa that are conducive to the development of the mucosal immune system (Blanton et al, 2016) or by administering defined mixtures of bacteria (‘probiotic cocktails’) that achieve a similar aim (Lemon et al, 2012).

7.3. The road ahead

7.3.1. Viral interference: a day-by-day perspective

In this thesis, we used snapshots of the intestinal microbiota obtained on the day of immunisation as a means of assessing the potential inhibitory effect of enteropathogens on Rotarix and OPV. However, this approach may fail to capture the dynamic interplay between infections that co-occur in the infant gut. Assessing the abundance of enteropathogens and

vaccine viruses in faecal samples at multiple timepoints in the days following immunisation may provide a more intimate perspective of these interactions. Notably, such an approach was adopted by Albert Sabin during the early studies of viral interference among OPV recipients, albeit in a small number of individuals (Sabin, 1959). The manifestations of viral interference in these cases were varied – while the presence of a concurrent enteric virus completely suppressed the replication of vaccine polioviruses in some instances, in others interference was characterised only by a delay in poliovirus replication or lower quantities of shedding. I would advocate the application of a similar approach not only to better characterise the association between bacterial, viral, and eukaryotic enteropathogens and vaccine virus replication, but to delineate the relationship between attenuated vaccine polioviruses and rotaviruses when these strains are co-administered. Specifically, is the replication efficiency of Rotarix impacted by the quantity and duration of Sabin virus shedding? And, as posited in Chapter 4, does the suppression of Sabin virus replication by existing enteropathogens enhance the replication of vaccine rotaviruses?

7.3.2. Animal models, human microbes

As noted above, observing an association between the composition of the intestinal microbiota and the outcome of oral immunisation does not imply a causal relationship between the two. Animal models have the capacity to shed light on this issue. Given that intestinal poliovirus carriage in mice requires transgenic expression of the human poliovirus receptor alongside knockout of the alpha/beta interferon receptor gene (Ohka et al, 2007), rotavirus is likely to be a more profitable basis for such enquiries (Little and Shadduck, 1982). In particular, one could assess the impact of innate immune stimulation on rotavirus carriage in murine models by orally administering poly I:C (a synthetic mimic of double-stranded RNA), lipopolysaccharide, or other pathogen-associated molecular patterns (PAMPs) prior to viral inoculation. In doing so, we might gain insight into the impact of PAMP-triggered immunity on rotavirus replication efficiency, as well as the extent to which this effect is conserved across different pattern recognition receptor ligands. Moreover, in line with the enhanced inhibitory effect of novel versus persistent infection observed with respect to OPV seroconversion in Chapter 6, one could use this approach to assess how the timing of PAMP exposure relative to viral inoculation impacts rotavirus take.

In a similar vein, murine models with a ‘humanised microbiota’ (attained by transplanting faecal samples into germ-free mice) have increasingly been used to validate causal relationships between the composition of the intestinal microbiota and disease states

such as kwashiorkor (Smith et al, 2013) and IBD (Nagao-Kitamoto et al, 2016). It would be interesting to apply a similar system to the issue of oral vaccine failure. In particular, does transplanting the faecal microbiota of infants who fail to respond to oral vaccination impede the take of rotavirus in humanised mouse models? Such an approach might also enable the identification of specific taxa that either halt or facilitate rotavirus replication and thereby inform the design of future microbiota-directed therapeutics.

7.3.3. Oral vaccine failure on a broader scale

With the exception of the systematic review described in Chapter 2, the findings presented above relate to a specific geographic setting, thus limiting the extent to which we can comment on the mechanisms responsible for broader geographic trends in oral vaccine performance. Indeed, as highlighted above (section 4.5), it is possible that all of the infants included in this work harboured a bacterial microbiota inhibitory to the replication of vaccine viruses. Future work examining the phenomenon of oral vaccine failure on a broader geographic and sociodemographic scale is warranted. Although the use of OPV is largely restricted to low-income settings, it would be feasible to perform a study considering multiple risk factors for rotavirus vaccine failure (microbiota composition, maternal antibody titres, HBGA phenotype, etc.) in tandem across infants in high- and low-income countries. Given that one would expect to observe marked discrepancies in vaccine immunogenicity between these settings, such an approach would provide an effective basis for delineating factors that undermine oral vaccine efficacy in low-income countries.

While conducting a multi-centre study such as this, the inclusion of an arm receiving IPV alone (as opposed to a mixed schedule of OPV and IPV) during routine immunisation in low-income trial sites would be beneficial. As well as enhancing comparability with IPV-immunised infants in high-income settings, this would enable the influence of OPV on rotavirus vaccine outcome to be examined as a secondary objectives. In doing so, the trial would provide insight into the implications of the impending withdrawal of OPV for rotavirus vaccination efforts.

We are in the midst of a period of profound change in the use of oral vaccines. As we prepare for the synchronised withdrawal of OPV – a vaccine that has formed the foundation of global efforts to eradicate poliomyelitis – the global roll-out of oral vaccines targeting rotavirus continues. It is possible that polio’s eradication will be achieved in spite of the impaired immunogenicity of OPV in endemic settings, though much uncharted terrain lies ahead. On the other hand, the impaired performance of oral rotavirus vaccines in low-income countries is likely to gain ever-greater prominence as immunisation efforts expand.

Based on the findings presented in this thesis, it appears increasingly certain that enteric viruses present an obstacle to the successful implementation of OPV in low-income countries. Bacterial enteropathogens may also impede the immunogenicity of this vaccine in early infancy. However, aside from the potential inhibitory effect of co-administered vaccine polioviruses, we failed to identify risk factors associated with the impaired performance of oral rotavirus vaccine among infants in south India. There is a pressing mandate to rectify this state of affairs as we attempt to combat a disease that is responsible for upwards of 500,000 deaths among young infants each year.

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