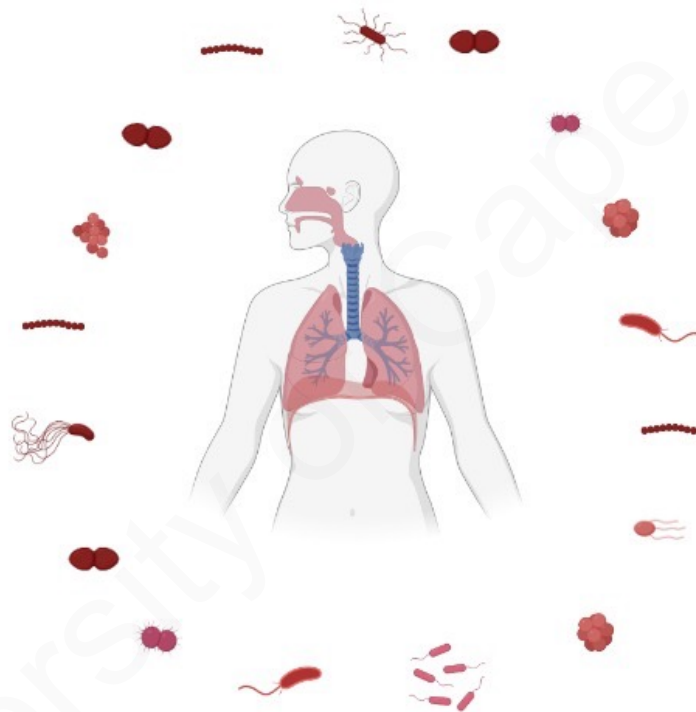




The effect of long-term azithromycin treatment on the respiratory microbiota of HIV-infected children with chronic lung disease



Regina Esinam Abotsi

Thesis submitted for the degree of DOCTOR OF PHILOSOPHY
in the Department of Molecular and Cell Biology
UNIVERSITY OF CAPE TOWN
April 2022

The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.

The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only. Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.

DECLARATION

I, Regina Esinam Abotsi hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

Signature:

Signed by candidate

Date: 8th April 2022

DECLARATION: INCLUSION OF PUBLICATIONS

I confirm that I have been granted permission by the University of Cape Town's Doctoral Degrees Board to include the following publications in my PhD thesis, and where co-authorships are involved, my co-authors have agreed that I may include the publications:

1. Abotsi RE, et al, Prevalence and antimicrobial resistance profiles of respiratory microbial flora in African children with HIV-associated chronic lung disease. *BMC Infect Dis* 2021 21:216. <https://doi.org/10.1186/s12879-021-05904-3>
2. Abotsi RE et al, The impact of long-term azithromycin on antibiotic resistance in HIV-associated chronic lung disease. *ERJ Open Res* 2022; 8: 00491-2021
<https://dx.doi.org/10.1183/23120541.00491-2021>
3. Abotsi RE et al. Long-term azithromycin treatment of children with HIV-associated chronic lung disease reduces sputum bacterial load and diversity. *Microbiome* (submitted and under review, preprint <https://dx.doi.org/10.21203/rs.3.rs-1386887/v1>)

SIGNATURE:

STUDENT NAME: Regina Esinam Abotsi

DATE: 8th April 2022

STUDENT NUMBER: ABTREG001

Supervisor

Dr. Felix Sizwe Dube (BSc, PhD)

Faculty of Health Sciences

Institute of Infectious Disease and Molecular Medicine

Department of Molecular and Cell Biology

University of Cape Town, South Africa

Co-supervisor

Professor Mark Patrick Nicol

MBBCh, MMed (Med Microbiol), SA FCPath (Microbiol), PhD

Faculty of Health and Medical Sciences

School of Biomedical Sciences

University of Western Australia, Australia

&

Institute of Infectious Disease and Molecular Medicine

Department of Pathology

Division of Medical Microbiology

University of Cape Town, South Africa.

To my family and friends with love!

ABSTRACT

Background: HIV-associated chronic lung disease (HCLD), predominantly obliterative bronchiolitis, affects more than 30% of perinatally HIV-infected children in Sub-Saharan Africa. There are currently no treatment guidelines for HCLD. A recent clinical trial demonstrated that long-term azithromycin therapy reduced the frequency of acute respiratory exacerbations and all-cause hospitalisations in HCLD patients. However, our understanding of the microbial determinants of HCLD and how azithromycin therapy modulates these determinants is limited. This study investigated the carriage and antibiotic resistance of common potentially pathogenic bacteria in the respiratory tract of children living with HIV (CLWH) with (CLD+) and without HCLD (CLD-). It also evaluated the impact of long-term azithromycin therapy on the prevalence and antibiotic resistance of selected bacterial species in the respiratory tract of CLD+ CLWH. Thirdly, the study assessed the effect of azithromycin on the sputum bacteriome of participants. Finally, the persistence of the bacteriological impact of azithromycin, six months post-intervention, was also investigated.

Methods: This study was nested within the Bronchopulmonary Function in Response to Azithromycin Treatment for Chronic Lung Disease in HIV-Infected Children (BREATHE) trial, a multi-site, double-blind, placebo-controlled, individually randomised trial (ClinicalTrials.gov Identifier: NCT02426112) that investigated the efficacy of azithromycin therapy in children and adolescents aged 6 to 19 years with HCLD (defined as forced-expiratory volume per second (FEV1) z -score < -1 without reversibility post-bronchodilation with salbutamol) in Zimbabwe and Malawi. Paired nasopharyngeal swabs and sputa were collected at baseline, 48 and 72 weeks from participants randomised to receive 48 weeks of once-weekly oral azithromycin or placebo and followed post-intervention until 72 weeks from baseline. Baseline samples were also collected from a comparison group of CLD- participants frequency matched for site, age (6-12 and 13-19 years), and duration of antiretroviral therapy use (6 months to < 2 years and ≥ 2 years). All samples were cultured for *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Haemophilus influenzae* and *Moraxella catarrhalis* and other Gram-negative bacilli. Antibiotic susceptibility testing was conducted on the isolated bacteria using the disk diffusion method. Total bacterial load and bacteriome profiles of the CLD+ sputum samples were determined using 16S rDNA quantitative (q)PCR and V4 region amplicon sequencing. Risk factors for bacterial carriage were identified using questionnaires and analysed using multivariate logistic regression. Bacterial carriage over the three visits, associations between clinical and socio-demographic factors,

and bacteriome diversity were determined by mixed effect models with participants as a random effect. Antibiotic resistance between arms was compared using Fisher's exact test.

Results: Between June 2016 and September 2018, a total of 410 participants (336 CLD+, 74 CLD-) were enrolled (median age, 15 years [IQR = 13–18]). *S. pneumoniae* and *M. catarrhalis* carriage in the nasopharyngeal swabs were higher in CLD+ than in CLD- children: 46% (154/336) vs 26% (19/74), $p = 0.008$; and 14% (49/336) vs 3% (2/74), $p = 0.012$, respectively. *S. pneumoniae* isolates from the nasopharyngeal swabs of CLD+ children were more likely to be non-susceptible to penicillin than those from CLD- children (36% [53/144] vs 11% [2/18], $p = 0.036$). Overall, methicillin-resistant *S. aureus* was uncommon [4% (7/195)]. In multivariate analysis, key factors associated with nasopharyngeal bacterial carriage included having HCLD (*S. pneumoniae*: adjusted odds ratio (aOR) 2 [95% Confidence Interval (CI) 1.1–3.9]), younger age (*S. pneumoniae*: aOR 3.2 [1.8–5.8]), HIV viral load suppression (*S. pneumoniae*: aOR 0.6 [0.4–1.0], *S. aureus*: 0.5 [0.3–0.9]), stunting (*S. pneumoniae*: aOR 1.6 [1.1–2.6]) and male sex (*S. aureus*: aOR 1.7 [1.0–2.9]). Key factors associated with sputum bacterial carriage included the Zimbabwean study site (*S. pneumoniae*: aOR 3.1 [1.4–7.3], *S. aureus*: 2.1 [1.1–4.2]), being on ART for a longer period (*S. pneumoniae*: aOR 0.3 [0.1–0.8]), and sampling in hot compared to rainy season (*S. pneumoniae*: aOR 2.3 [1.2–4.4]).

Of the 347 (174 azithromycin, 173 placebo) CLD+ participants included in the main trial (median age 15 years [IQR = 13–18], females 49%), nasopharyngeal carriage was significantly lower in the azithromycin ($n = 159$) compared to placebo ($n = 153$) arm for *S. pneumoniae* (18% vs 41%, $p < 0.001$), *H. influenzae* (7% vs 16%, $p = 0.01$), and *M. catarrhalis* (4% vs 11%, $p = 0.02$) at 48 weeks. *S. pneumoniae* resistance to azithromycin (62% [18/29] vs 13% [8/63], $p < 0.0001$) or tetracycline (60% [18/29] vs 21% [13/63], $p < 0.0001$) were also higher in the azithromycin arm at 48 weeks. Carriage of *S. aureus* resistant to azithromycin (91% [31/34] vs 3% [1/31], $p < 0.0001$), tetracycline (35% [12/34] vs 13% [4/31], $p = 0.05$) or clindamycin (79% [27/34] vs 3% [1/31], $p < 0.0001$) was also significantly higher in the azithromycin arm at 48 weeks and persisted at 72 weeks. Similar findings were observed for sputa. Placebo allocation was associated with decreased likelihood of azithromycin-resistant *S. pneumoniae* or *S. aureus* carriage in the nasopharyngeal swabs or sputum.

Forty-eight weeks of azithromycin reduced sputum bacterial load vs placebo arm (16S rDNA copies/ μ l in $\log_{10}$, mean difference and CI of azithromycin vs placebo -0.54 [-0.71; -0.36]). Shannon alpha diversity remained stable in the azithromycin arm but declined in the placebo arm between baseline

and 48 weeks (3.03 vs 2.80, $p = 0.04$, Wilcoxon paired test). Bacterial community structure changed in the azithromycin arm at 48 weeks compared with baseline (PERMANOVA test $p = 0.003$) but resolved at 72 weeks. The relative abundances of genera previously associated with HCLD decreased in the azithromycin arm at 48 weeks compared with baseline, including *Haemophilus* (17.9% vs 25.8%, $p < 0.05$, ANCOM $\omega = 32$) and *Moraxella* (1% vs 1.9%, 47, $p < 0.05$, ANCOM $\omega = 47$). This reduction was sustained at 72 weeks relative to baseline. Lung function (FEV1z-score) was negatively associated with bacterial load (linear mixed effect model coefficient, [CI]: -0.09 [-0.16; -0.02]) and positively associated with Shannon diversity (0.19 [0.12; 0.27]). The relative abundance of *Neisseria* (coefficient, [standard error]: (2.85, [0.7], $q = 0.01$), and *Haemophilus* (-6.1, [1.2], $q < 0.001$) were positively and negatively associated with FEV1z, respectively. An increase in the relative abundance of *Streptococcus* from baseline to 48 weeks was associated with improvement in FEV1z-score (3.2 [1.11], $q = 0.01$), whilst an increase in *Moraxella* was associated with a decline in FEV1z-score (-2.74 [0.74], $q = 0.002$).

Conclusions: CLD+ children were more likely to carry *M. catarrhalis* and *S. pneumoniae*, including penicillin-non-susceptible *S. pneumoniae* strains, than CLD- children. Long-term azithromycin reduced carriage of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* but promoted antibiotic resistance in *S. pneumoniae* and *S. aureus*; antibiotic-resistant *S. aureus* persisted at 72 weeks, six months after treatment completion. Azithromycin also reduced total bacterial load and modulated the sputum bacteriome away from potentially pathogenic taxa, which may explain the beneficial effect of azithromycin in reducing acute pulmonary exacerbations in this population. The risk of drug resistance should be considered during long-term azithromycin use and the clinical significance of this resistance and the benefits of azithromycin use beyond 48 weeks needs further investigation.

ACKNOWLEDGEMENTS

I am incredibly grateful to my supervisors, Dr. Felix Sizwe Dube and Prof Mark Patrick Nicol, for their unwavering support and mentorship throughout my PhD journey. I have acquired much knowledge in academic research and navigating interpersonal interactions in research collaborations. I am very grateful to you both for the investment you made in me and for the research networks, the environment, and opportunities for professional growth. Thank you!

I wish to acknowledge the personal financial support I received from the Swedish International Development Cooperation Agency (SIDA) through the Organisation of Women in Science for the developing world (OWSD) PhD Fellowship, The Boehringer Ingelheim Fonds (BIF) travel grant, Margaret McNamara Education Grants and L'Oréal UNESCO For Women in Science Fellowship. This journey would not have been possible without these organisations. I am equally grateful to the Global Health and Vaccination Research (GLOBVAC) Programme of the Medical Research Council of Norway and National Institutes of Health Common Fund, through the Office of Strategic Coordination/Office of the NIH Director, National Institute of Environmental Health Sciences and National Human Genome Institute of Health of the National Institutes of Health (H3Africa awards grant numbers U54HG009824 and 1U01AI110466) for funding this study.

I am grateful to the following individuals for their technical support and expertise: Dr. Shantelle Claassen-Weitz, for your selflessness in training me in 16S rRNA gene amplicon library preparation and sequencing, for conducting the decontamination step and for guidance on data extraction template design and data visualisation of chapter two when you should have been completing your own thesis; Dr. Kilaza Samson Mwaikono for your kind assistance in bioinformatic steps; Yao Xia for always being available to assist with the microbiome analysis; Prof. Sugnet Lubbe for providing the R script for the *in-silico* decontamination step, and Prof Rob Hebderman and Dr. Brenda Kwambana-Adams of MPRU for providing feedback on my work. I would also like to thank Ms Adelaide Mensah and Mrs Liesl Nicol for their assistance during the study protocol development and Ms. Namhla Madini, information specialist from University of Cape Town Library for guiding the literature search. I am also grateful to Dr. John Osei for editorial review of chapter three.

I would also like to extend my sincere gratitude to the entire BREATHE study team, especially Dr. Grace McHugh and Dr. Lucky Ngwira, the BREATHE study coordinators, and Dr. Andrea Rehman

and Dr. Victoria Simms, the study statisticians. I have acquired much statistical knowledge from you. I am grateful to the principal investigators of the BREATHE trial, Prof Jon Odland and Prof Rashida Ferrand for the opportunity to be part of such a collaborative team. Prof Ferrand, I am very grateful for your mentorship and leadership of the BREATHE trial. You inspire me as a woman in science involved in translational research. I also wish to thank the BREATHE trial participants and their families. Without you, this research would not have been possible. I hope that the findings from this work improve your quality of life. I am also grateful to Prof Trond Flægstad of the Arctic University of Norway for your financial support and collaborative opportunities. I also acknowledge facilities provided by the University of Cape Town's ICTS High Performance Computing team: <http://hpc.uct.ac.za> for computations performed.

I am grateful to the staff and students of Division of Medical Microbiology, Department of Molecular and Cell Biology and the Institute of Infectious Diseases and Molecular Medicine for the rigorous academic environment and training opportunities provided to me. I am particularly grateful to Prof Adrian Brink for his support and Ms. Charmaine Barthus for her training and assistance in the lab and regular motherly encouragement. A further extension of my gratitude goes to Ms. Slindile Mbhele and Mrs Pamela Jordan for your kind support, Dr. Widaad Zemanay for being a prompt “fixer” of any problem that I bring to you, Ms. Rasheeda, Dr. Fadheela Patel (for your kindness and listening ear), Dr. Lynthia Paul, Wendy Blose and Samantha Africa just to mention a few. I am also grateful to the Dube lab, especially Gillian Ndhlovu, for being my sounding board, trouble-shooting buddy, and a true friend.

On a personal note, I will thank my family for their love, encouragement, prayers, and support- my parents, Robert and Antoinette, and my brothers, Wisdom, Edwin, Leo, Wonder and their wives: Sandra, Hawa and Mataji. My nieces and nephews whom I love very much Edem, Elorm, Nutifafa, Selassie, Dzigbordi, Kafui, Dzidzor and my very own namesake Esinam. I would also like to thank Prof John and Mrs Hannah Akokpari for being my parents here in Cape Town and providing a home away from home. To Mowbray SDA Church members, I am grateful for the social and spiritual support. I am grateful to Mowbray SDA Church members for their social and spiritual support. I am equally grateful to my friends Mary-Ann Archer, Leeza Felicity Grimet, Nyambura Shawa, Adutwumwaa Quaye, Beatrice Broni, The Okunola family, Luntu Quntana, and Faith Lutomia emotional, spiritual and social support. To Yaw Afriyie, my steadfast partner throughout this journey. Thank you for being my rock and life coach during this challenging period of my life. Finally, to God without whom none of these will exist, I am exceedingly grateful.

TABLE OF CONTENTS

<i>DECLARATION</i>	<i>iii</i>
<i>DECLARATION: INCLUSION OF PUBLICATIONS</i>	<i>iv</i>
<i>ABSTRACT</i>	<i>vii</i>
<i>ACKNOWLEDGEMENTS</i>	<i>x</i>
<i>TABLE OF CONTENTS</i>	<i>xii</i>
<i>ABBREVIATIONS</i>	<i>xvi</i>
<i>List of Figures</i>	<i>xvii</i>
<i>List of Tables</i>	<i>xix</i>
<i>List of Appendices</i>	<i>xxi</i>
Chapter 1	1
<i>General Introduction</i>	<i>1</i>
<i>1.1 Background and Rationale</i>	<i>2</i>
<i>1.2 Outline of the thesis</i>	<i>10</i>
<i>1.3 References</i>	<i>12</i>
Chapter 2	15
<i>The impact of macrolides on the human respiratory microbiota: a systematic review</i>	<i>15</i>
<i>2.0 Abstract</i>	<i>16</i>
<i>2.1 Background</i>	<i>17</i>
<i>2.2 Materials and Methods</i>	<i>19</i>
2.2.1 Search terms, strategy, and databases	<i>19</i>
2.2.2 Screening of Studies	<i>19</i>
2.2.3 Outcomes	<i>20</i>
2.2.4 Study Selection Criteria	<i>20</i>
2.2.5 Data Extraction and Synthesis	<i>21</i>
<i>2.3 Results</i>	<i>22</i>
2.3.1 Results of the search and screening	<i>22</i>
2.3.2 Characteristics of included studies	<i>24</i>
2.3.3 Effect of macrolides on the prevalence and resistance of selected bacterial species	<i>46</i>
2.3.4 Effect of macrolides on the carriage of antibiotic resistance genes	<i>51</i>
2.3.5 Persistence of the bacteriological effects of macrolides post-intervention	<i>51</i>
2.3.6 Effects of macrolides on the total bacterial load of respiratory samples	<i>52</i>
2.3.7 Effects of macrolides on alpha (within-sample) diversity	<i>53</i>
2.3.8 Effects of macrolides on beta (between-sample) diversity and differentially abundant taxa	<i>53</i>
<i>2.4 Discussion</i>	<i>59</i>
<i>2.5 Conclusion</i>	<i>64</i>
<i>2.6 Declarations</i>	<i>65</i>
2.6.1. Deviations from the protocol	<i>65</i>
2.6.2 Availability of data and materials	<i>65</i>
2.6.3 Competing interests	<i>65</i>
2.6.4 Funding	<i>65</i>

2.6.5 Author contributions	66
2.6.6 Additional files	66
2.7 References	66
Chapter 3	75
<i>Prevalence and antimicrobial resistance profiles of respiratory microbial flora in African children with HIV-associated chronic lung disease</i>	75
3.0 Abstract	77
3.1 Background	78
3.2 Materials and Methods	78
3.2.1 Study design, settings, and participants	78
3.2.2 Sample collection, transportation, and processing	79
3.2.3 Bacterial culture, identification, and antimicrobial susceptibility testing	79
3.2.4 Statistical Analysis	80
3.3 Results	81
3.3.1 Participant clinical and socio-demographic characteristics	81
3.3.2 Prevalence of bacterial carriage	82
3.3.3 Risk factors associated with carriage of bacteria	83
3.3.4 Antibiotic and multidrug resistance of isolates	90
3.4 Discussion	91
3.5 Conclusion	96
3.6 Declarations	96
3.6.1 Ethical approval and consent to participate	96
3.6.2 Consent for publication	96
3.6.3 Availability of data and materials	96
3.6.4 Competing interests	96
3.6.5 Funding	97
3.6.6 Author contributions	97
3.6.7 Additional files	97
3.7 References	98
Chapter 4	102
<i>The impact of long-term azithromycin on antibiotic resistance in HIV-associated chronic lung disease</i>	102
4.0 Abstract	104
4.1 Background	105
4.2 Materials and Methods	106
4.2.1 Participants and intervention	106
4.2.2 Sample collection	106
4.2.3 Study outcomes	107
4.2.4 Statistical analyses	107
4.2.5 Ethical approval and consent to participate	107
4.3 Results	108
4.4 Discussion	115
4.5 Conclusion	119
4.6 Declarations	119
4.6.1 Contributors	119
4.6.2 Data sharing	120
4.6.3 Funding	120
4.7 References	120

Chapter 5	123
<i>Sputum bacterial load and bacterial composition correlate with lung function and are altered by long-term azithromycin treatment in children with HIV-associated chronic lung disease</i>	<i>123</i>
5.0 Abstract	125
5.1 Background	126
5.2 Materials and Methods.....	127
5.2.1 Study design, participants, setting, eligibility, and intervention	127
5.2.2 Study outcomes	128
5.2.3 Sample and data collection	128
5.2.4 Nucleic acid extraction and purification.....	129
5.2.5 16S rDNA library preparation and amplicon sequencing	129
5.2.6 Bioinformatics analysis of 16s rDNA metagenomic data	129
5.2.7 Quality control and <i>in silico</i> correction of contamination	130
5.2.8 Statistical analysis	131
5.3 Results	133
5.3.1 Participant characteristics	133
5.3.2 Number of samples	133
5.3.3 Azithromycin reduced sputum bacterial load among participants in the intervention arm.....	135
5.3.4 Association between azithromycin treatment, clinical and socio-demographic characteristics, and sputum alpha diversity	141
5.3.5 Azithromycin treatment resulted in changes in the overall bacteriome profile.....	146
5.3.6 Composition of taxa within the sputum bacteriome of participants	152
5.3.7 Concordance between differential abundance testing methods.....	157
5.3.8 Associations between the relative abundance of bacterial genera and clinical measures	162
5.4 Discussion	163
5.5 Conclusion.....	166
5.6 Declarations	166
5.6.1 Ethical approval and consent to participate.....	166
5.6.2 Consent for publication	167
5.6.3 Availability of data and material	167
5.6.4 Competing interests	167
5.6.5 Funding.....	167
5.6.6 Author contributions.....	168
5.7 References	168
Chapter 6	171
<i>General conclusions and future outlook</i>	<i>171</i>
6.1 General Conclusions	172
6.2 Limitations.....	174
6.3 Future Outlook	175
APPENDICES	177
<i>APPENDIX A: Chapter Two Supplementary Materials</i>	<i>177</i>
Protocol for the systematic review published in Prospero	177
<i>APPENDIX B: Chapter Three Supplementary Materials</i>	<i>186</i>
<i>APPENDIX C: Chapter Four Supplementary Materials</i>	<i>188</i>
<i>APPENDIX D: Chapter Five Supplementary Materials.....</i>	<i>219</i>
<i>Section 1. Detailed description of methods – differentially abundant taxa and SIMPER analysis</i>	<i>219</i>
<i>Section 2. Supplementary results</i>	<i>223</i>
2.1 Results of quality control steps.....	223

2.2 Results of differential abundance of taxa testing	237
2.2.1 Azithromycin and Placebo	237
2.2.2. Azithromycin arm only.....	237
2.2.3 Placebo arm only	238
<i>References</i>	239
<i>APPENDIX E: Publications, Conference presentations and statement by the candidate</i>	240
<i>APPENDIX F: Ethics and Biosafety clearance</i>	246
<i>APPENDIX G: Turnitin Report</i>	250

ABBREVIATIONS

ART	Antiretroviral therapy
AST	Antimicrobial susceptibility testing
ASV	Amplicon Sequence variant
AZM	Azithromycin
BP	Between-plate repeat
BR	Between-run repeat
BREATHE	The Bronchopulmonary Function in Response to Azithromycin Treatment for Chronic Lung Disease in HIV-Infected Children trial
CLA	Clarithromycin
CI	Confidence Interval
CLD	HIV-associated chronic lung disease
CLD+	Participants with HIV-associated chronic lung disease
CLD-	Participants without HIV-associated chronic lung disease
CLWH	Children living with HIV
DIR	Dirithromycin
DNA	Deoxyribonucleic acid
e.g.	Exempli gratia
ERY	Erythromycin
Etc	Et cetera
GNB	Other Gram-negative bacilli other than <i>Haemophilus influenzae</i>
HCLD	HIV-associated chronic lung disease
HI	<i>Haemophilus influenzae</i>
HIV	Human Immunodeficiency Virus
IDM	Institute of Infectious Disease and Molecular Medicine
IPD	Invasive Pneumococcal Disease
MALDI-TOF MS	Matrix-Assisted Laser Desorption/Ionization-Time-of-Flight mass spectrometry
MC	<i>Moraxella catarrhalis</i>
MCB	Department of Molecular and Cell Biology
NGS	Next-Generation Sequencing
NP	Nasopharyngeal
OTU	Operational Taxonomic Unit
PCoA	Principal Component Analysis
qPCR	Quantitative Polymerase Chain Reaction
PERMANOVA	permutational multivariate analysis of variance
rDNA	Ribosomal Deoxyribonucleic acid
RR	Risk ratio
ROX	Roxithromycin
rpm	Revolutions per minute
SA	<i>Staphylococcus aureus</i>
SP	<i>Streptococcus pneumoniae</i>
UNICEF	United Nations International Children's Emergency Fund
UCT	University of Cape Town
μl	Microliter
WHO	World Health Organisation
%	percent
°C	Degree(s) Celsius

List of Figures

Chapter 2

Figure 2. 1. PRISMA Flow-chart.	23
Figure 2. 2. Flow diagram showing the breakdown of the included studies.	24
Figure 2. 3. World map showing the total number of studies that administered macrolides in each country.	27
Figure 2. 4. Effect of macrolide use on the prevalence and resistance of selected bacteria.	47
Figure 2. 5. Effect of macrolide use on the prevalence and resistance of bacteria stratified by type of macrolide.	49
Figure 2. 6. Effect of macrolide use on the prevalence and resistance of selected bacteria stratified by sample source.	50

Chapter 3

Figure 3. 1. Barplot comparing the antibiotic resistance of selected bacteria.	90
---	----

Chapter 4

Figure 4. 1. Number of samples collected at each visit flowchart.	109
Figure 4. 2. Antibiotic resistance profiles of <i>Streptococcus pneumoniae</i> isolates.	111
Figure 4. 3. Antibiotic resistance profiles of <i>Staphylococcus aureus</i> isolates.	112

Chapter 5

Figure 5. 1. Flow chart of the number of samples collected from each study site at each visit and the numbers included and excluded from final analysis.	135
Figure 5. 2. Boxplot of bacterial load between trial arms at each visit (A) and between visits within AZM arm only (B) and Placebo arm only (C).	136
Figure 5. 3. Boxplot of Shannon alpha diversity index between trial arms at each visit (A) and between study visits in AZM (B) and Placebo (C) arms.	145
Figure 5. 4. Violin boxplot comparing two beta diversity metrics between samples collected from participants in the AZM arms at baseline and 48 weeks, 48 and 72 weeks and baseline and 72 weeks. PERMANOVA test used.	147
Figure 5. 5. Violin boxplot comparing two beta diversity metrics between samples collected from participants in the Placebo arms at baseline and 48 weeks, 48 and 72 weeks and baseline and 72 weeks. PERMANOVA test used.	148
Figure 5. 6. Violin boxplot comparing two beta diversity metrics between trial arms at 0, 48 and 72 weeks.	149
Figure 5. 7. Principal Coordinates Analysis of Aitchison (A) and Bray-Curtis (B) [on unrarefied ASV counts] distance matrixes between trial arms at each visit.	150
Figure 5. 8. Violin boxplot comparing the median within-participant change in Bray-Curtis and Aitchison distance of participants with and without adverse event.	152
Figure 5. 9. Line plot of the mean relative abundances of the top phyla and genus in all samples.	153
Figure 5. 10. Barplot of the relative abundances of the top 10 most prevalent phyla in all samples.	154
Figure 5. 11. Barplot of the relative abundances of the top 12 most prevalent genera in all samples.	155
Figure 5. 12. Analysis of Composition of Microbiomes (ANCOM2) differential abundance testing for samples at each visit and between trial arms.	157
Figure 5. 13. Heatmap displaying the q values of the genera detected as differentially abundant between AZM and placebo arms at 48 weeks by 10 statistical methods.	158
Figure 5. 14. Heatmap displaying the q values of the genera detected as differentially abundant within the AZM arm between baseline and 48-week samples by 10 methods.	159
Figure 5. 15. Heatmap displaying the q values of the genera detected as differentially abundant within the AZM arm between 48- and 72-week samples by 10 methods.	160

Appendix C – Supplementary figures from Chapter 4

Appendix C. Figure S 1. Antibiotic resistance profiles of <i>Haemophilus influenzae</i> isolated from the nasopharyngeal (NP) and sputum samples at baseline, 48 weeks, and 72 weeks.	188
--	-----

Appendix C. Figure S 2. Antibiotic resistance profiles of <i>Moraxella catarrhalis</i> isolated from the nasopharyngeal (NP) and sputum samples at baseline, 48 weeks, and 72 weeks.....	189
--	-----

Appendix D – Supplementary figures from Chapter 5

Appendix D. Figure S. 1. A bar plot of the taxa and their relative abundance of the extraction and sequencing mock controls compared to manufacturer profiles.	224
Appendix D. Figure S. 2. A scatterplot showing the correlation between samples repeated within a run (WR, n = 74) and between runs (BR, n = 28).	225
Appendix D. Figure S. 3. A scatterplot showing the spread of biological samples (n = 960) and the negative controls (primestore, n = 43).	226
Appendix D. Figure S. 4. Ordination plots of showing the spread of biological samples (n = 960) and the negative controls (primestore, n = 43) coloured by their 16S copies.	227
Appendix D. Figure S. 5. Ordination plots showing the spread of biological samples (n = 960) and the negative controls (primestore, n = 43) coloured by their number of reads.	228
Appendix D. Figure S. 6. Ordination plots showing the spread of biological samples (n = 960) and the negative controls (primestore, n = 43) coloured by the age of the participant.....	228
Appendix D. Figure S. 7. Rarefaction curves showing number of ASVs detected and 16S copies of samples.....	229
Appendix D. Figure S. 8. Rarefaction curves showing number of ASVs detected and number of reads of samples.....	229
Appendix D. Figure S. 9. Bar plot showing the profiles of biological samples with < 100 16S copies (n = 2) in comparison to Primestores profiles (n = 43).	230
Appendix D. Figure S. 10. Bar plot showing the profiles of biological samples with > 100 to < 1000 16S copies (n = 10) in comparison to Primestores profiles (n = 43).	231
Appendix D. Figure S. 11. Ordination plots showing the profiles of a subset of biological samples with low 16S copies and the negative controls.	231
Appendix D. Figure S. 12. Ordination plots showing the profiles of a subset of biological samples with low reads and the negative controls.	231
Appendix D. Figure S. 13. Ordination plots showing the spread of biological samples (n = 960) and the negative controls (primestore, n = 43) coloured by the run in which the sample was processed.	232
Appendix D. Figure S. 14. Ordination plots showing the spread of biological samples (n = 960) and the negative controls (primestore, n = 43) coloured by the country of sampling.	233
Appendix D. Figure S. 15. Ordination plots showing the spread of biological samples (n = 960) and the negative controls (primestore, n = 43) coloured by visit.	234
Appendix D. Figure S. 16. Ordination plots showing the spread of biological samples (n = 960) and the negative controls (primestore, n = 43) coloured by the age at sampling.....	234
Appendix D. Figure S. 17. Output from decontamination analysis using the DECONTAM R package.	235

List of Tables

Chapter 2

Table 2. 1. Search terms.....	19
Table 2. 2. Eligibility criteria.....	20
Table 2. 3. Summary of study characteristics	25
Table 2. 4. Studies that administered macrolides for short-term (≤ 14 days)	29
Table 2. 5. Studies that administered macrolides for long-term (> 28 days).....	39
Table 2. 6. Summary of microbiome-based studies	52
Table 2. 7. Studies that administered macrolides and investigated their effect on the respiratory microbiome using next-generation sequencing	55
Table 2. 8. Differentially abundant taxa detected between pre- and post-treatment samples from the azithromycin arm post-treatment	58

Chapter 3

Table 3. 1. Baseline characteristics of study participants	81
Table 3. 2. Prevalence of bacterial isolates among study participants	82
Table 3. 3. Univariate and multivariate analysis of factors associated with nasopharyngeal <i>S. pneumoniae</i> and <i>S. aureus</i> colonisation.....	84
Table 3. 4. Univariate and multivariate analysis of factors associated with sputum <i>S. pneumoniae</i> and <i>S. aureus</i> colonisation.....	85
Table 3. 5. Univariate and multivariate analysis of factors associated with nasopharyngeal <i>H. influenzae</i> and <i>M. catarrhalis</i> colonisation.....	87
Table 3. 6. Univariate and multivariate analysis of factors associated with sputum <i>H. influenzae</i> and <i>M. catarrhalis</i> colonisation.....	88

Chapter 4

Table 4. 1. Baseline characteristics of study participants	108
Table 4. 2. Nasopharyngeal and sputum bacterial carriage at baseline, 48 weeks, and 72 weeks in participants	114

Chapter 5

Table 5. 1. Characteristics of study participants at baseline.....	134
Table 5. 2. The association between bacterial load (16S rDNA copies) and selected variables using linear mixed effects modelling.....	138
Table 5. 3. The association between Shannon diversity indices and selected variables using linear mixed effects modelling.....	141
Table 5. 4. Contributions of top genera to overall dissimilarity between azithromycin and placebo arms at 48 weeks and, within the azithromycin arm, between baseline and 48-week samples- SIMPER analysis.	161
Table 5. 5. Linear regression analysis of within-participant change in FEV1z-score and FVCz-score and within-participant change in relative abundance of selected genera (outcome) between visits.....	162
Table 5. 6. Univariate linear regression analysis of within-participant Aitchison distance (outcome) and within-participant change in lung function metrics (FVCz-score and FEV1z-score) between visits.	163

Appendix A – Supplementary tables from Chapter 2

Appendix A. Table S 1. Definitions and abbreviations.....	184
Appendix A. Table S 2. Final search.....	184

Appendix B – Supplementary tables from Chapter 3

Appendix B. Table S 1. Semi-quantitative bacterial load distribution of isolates	186
--	-----

<i>Appendix B. Table S 2. Gram-negative bacilli other than H. influenzae isolated from respiratory samples.....</i>	<i>186</i>
<i>Appendix B. Table S 3. Co-carriage of bacteria in nasopharyngeal swabs and sputa</i>	<i>187</i>

Appendix C – Supplementary tables from Chapter 4

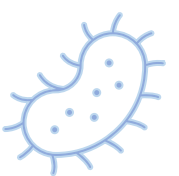
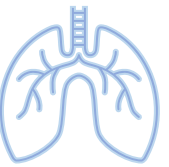
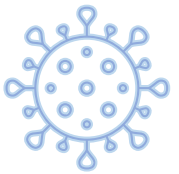
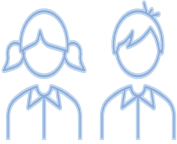
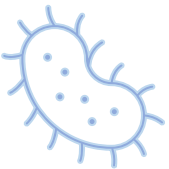
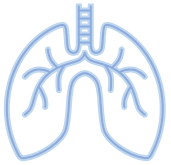
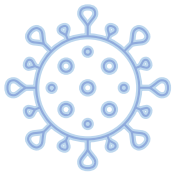
<i>Appendix C. Table S 1. Antibiotic resistance of bacteria isolated from the nasopharyngeal swabs collected at baseline, 48 weeks and 72 weeks from participants taking azithromycin or placebo.....</i>	<i>190</i>
<i>Appendix C. Table S 2. Antibiotic resistance of bacteria isolated from the sputa collected at baseline, 48 weeks and 72 weeks from participants taking azithromycin or placebo.</i>	<i>192</i>
<i>Appendix C. Table S 3. Nasopharyngeal and sputum bacterial carriage at baseline, 48 weeks and 72 weeks in participants taking azithromycin, by adherence to azithromycin.</i>	<i>193</i>
<i>Appendix C. Table S 4. Antibiotic resistance of nasopharyngeal bacterial isolates at baseline, 48 weeks and 72 weeks in participants taking azithromycin, by adherence to azithromycin.</i>	<i>194</i>
<i>Appendix C. Table S 5. Sputum carriage and antibiotic resistance at baseline, 48 weeks and 72 weeks in participants taking azithromycin, by adherence to azithromycin.</i>	<i>195</i>
<i>Appendix C. Table S 6. Factors associated with carriage of Streptococcus pneumoniae isolates at 48 weeks and 72 weeks in trial participants.</i>	<i>196</i>
<i>Appendix C. Table S 7. Factors associated with carriage of Staphylococcus aureus isolates at 48 weeks and 72 weeks in trial participants.</i>	<i>202</i>
<i>Appendix C. Table S 8. Factors associated with carriage of azithromycin resistant Streptococcus pneumoniae isolates at 48 weeks and 72 weeks in trial participants.</i>	<i>208</i>
<i>Appendix C. Table S 9. Factors associated with carriage of azithromycin resistant Staphylococcus aureus isolates at 48 weeks and 72 weeks in trial participants.</i>	<i>214</i>

Appendix D – Supplementary tables from Chapter 5

<i>Appendix D. Table S 1. The taxonomy of the ASVs in the extraction and sequencing control.....</i>	<i>224</i>
<i>Appendix D. Table S 2. List of 70 ASVs detected by the DECONTAM R package as potential contaminants based on comparison between biological samples and negative controls</i>	<i>236</i>
<i>Appendix D. Table S 3. Results of differential abundance testing of bacterial taxa from azithromycin and placebo samples from 48 weeks using 10 methods.....</i>	<i>237</i>
<i>Appendix D. Table S 4. Results of differential abundance testing of bacterial taxa from azithromycin and placebo samples from 72 weeks using Deseq2.</i>	<i>237</i>
<i>Appendix D. Table S 5. Results of differential abundance testing of bacterial taxa from azithromycin and placebo samples from 72 weeks using Ancom-II.</i>	<i>237</i>
<i>Appendix D. Table S 6. Results of differential abundance testing of bacterial taxa from samples from the azithromycin arm at baseline and 48 weeks using 10 methods.</i>	<i>237</i>
<i>Appendix D. Table S 7. Results of differential abundance testing of bacterial taxa from samples from the azithromycin arm at 48 and 72 weeks using 10 methods.....</i>	<i>238</i>
<i>Appendix D. Table S 8. Results of differential abundance testing of bacterial taxa from placebo samples from 48 and 72 weeks using Deseq2.</i>	<i>238</i>
<i>Appendix D. Table S 9. Results of differential abundance testing of bacterial taxa from placebo samples from baseline and 72 weeks using Deseq2.</i>	<i>238</i>

List of Appendices

<i>Appendix A. Chapter Two Supplementary Materials.....</i>	<i>177</i>
<i>Appendix B. Chapter Three Supplementary Materials.....</i>	<i>186</i>
<i>Appendix C. Chapter Four Supplementary Materials</i>	<i>188</i>
<i>Appendix D. Chapter Five Supplementary Materials.....</i>	<i>219</i>
<i>Appendix E. Publications, Conference presentations and statement by the candidate</i>	<i>240</i>
<i>Appendix F. Ethics and Biosafety clearance</i>	<i>246</i>
<i>Appendix G. Turnitin Report.....</i>	<i>250</i>



Chapter 1

General Introduction

1.1 Background and Rationale

According to the United Nations International Children's Emergency Fund (UNICEF) global 2020 estimates, more than 85% of the 3 million children under 20 years living with HIV (CLWH) reside in Sub-Saharan Africa [1]. Interventions such as early diagnosis of HIV infection, cotrimoxazole prophylaxis, better access to highly active antiretroviral therapy (ART) and childhood immunisation have improved the survival of perinatally HIV-infected children [2]. These strategies have also played a significant role in reducing the incidence of acute opportunistic infections. At the same time, there has been an increase in chronic conditions in CLWH, including growth failure, cardiac and lung diseases [2].

Chronic lung disease is the most common manifestation of HIV infection in children [3] and accounts for more than 50% of all HIV-associated mortality in children and adolescents [4]. These chronic lung diseases include tuberculosis, lymphocytic interstitial pneumonitis, bronchiectasis and chronic aspiration pneumonia [3]. Recently, a novel type of chronic lung disease, constrictive obliterative bronchiolitis (OB), was detected among this population in Malawi [5], Zimbabwe [6, 7] and South Africa [8, 9]. The prevalence of OB is estimated to be 30% [6, 7] and is not responsive to ART [6, 10].

Although the pathogenesis is not completely understood, HIV-associated OB is speculated to arise from interactions of immune-mediated mechanisms and processes such as infection or ischaemia, leading to tissue injury followed by aberrant fibro-proliferative remodelling in the small airways [11]. Specifically, an interplay between the chronic immune dysregulation associated with HIV [12] and the recurrence of respiratory tract infections [13] in this population is implicated.

The human microbiome or microbiota refers to the community of microorganisms – bacteria, viruses, archaea, fungi, and protozoa – found on skin and mucous membranes of cavities exposed to the exterior, such as the gastrointestinal, genitourinary and respiratory tracts and secretory glands such as the biliary, sebaceous, and mammary [14]. The bacteriome or bacteriota refers to the bacterial component of the microbiome or microbiota commonly profiled using 16S rRNA amplicon metagenomic sequencing [14]. The microbiome plays important metabolic and physiological functions in the human body [15]. The gastrointestinal microbiome, for instance, influences energy metabolism, adiposity/obesity, blood pressure control, glucose homeostasis, clotting risk, immune and cognitive function and even behaviour [15].

The human microbiome can be described and compared between body sites by metrics such as alpha and beta diversity. Alpha diversity refers to the diversity within a specific body site usually expressed by the number of species (richness) and how evenly distributed they are at the site (evenness). This is an important metric in human microbiome studies used to explore the correlation between specific health and disease states and the microbiome. Beta diversity measures the diversity between two ecosystems using the number of species change between the ecosystems. The use of antibiotics affects the human microbiome typically resulting in reduction in species richness or alpha diversity (a phenomenon known as dysbiosis). This reduction in alpha diversity especially in the gut has been associated with various disease conditions such as irritable bowel syndrome and Crohn's disease [16]. The lung previously thought to be sterile has now being demonstrated to have a microbiome dominated by bacterial genera *Prevotella*, *Streptococcus*, and *Veillonella* [17]. Specific signatures of these microbiome is associated with chronic lung conditions such as asthma, chronic obstructive pulmonary disease, cystic fibrosis and bronchiectasis [17].

The development or progression of HIV-associated OB may therefore also arise from alterations within the lower respiratory tract microbiota. This is plausible as microbes and their products play an essential role in maintaining immune homeostasis [18, 19]. As such, any change in their composition, diversity, or relative abundance may be associated with dysregulated immune system with consequences in HIV-associated OB. Indeed, recent data have shown clear differences between the lung microbiota of HIV-infected and uninfected individuals lending credence to this hypothesis [20–22]. More convincingly, a recent study [23] of a cohort of HIV-associated chronic lung disease patients (with OB as the predominant cause) in Zimbabwe revealed that distinct lung microbiome signatures were associated with the disease. Children and adolescents with sputum microbiota dominated by *Haemophilus*, *Moraxella*, or *Neisseria* (HMN) genera were at 1.5 times higher risk of HIV-associated OB than those with *Streptococcus* or *Prevotella*-dominant microbiota (risk ratio = 1.48, $p = 0.035$). Further investigation revealed that cell-free products of HMN sputum microbiota induced features of epithelial disruption and inflammatory gene expression *in vitro*, indicating the enhanced pathogenic potential of this OB-associated microbiota [23]. The bacterial pathogens previously implicated in pulmonary infections in HIV include *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Haemophilus influenzae* and *Moraxella catarrhalis* [3, 4]. These respiratory bacterial pathogens may play critical roles in the development and/or progression of HIV-associated OB however, our understanding of these roles is limited.

S. pneumoniae (pneumococcus), a natural component of the upper respiratory tract microbiota particularly the nasopharynx, is a leading cause of bacterial pneumonia globally [24]. HIV-infected persons are > 100 times more likely to develop pneumococcal disease than age-matched uninfected adults [25, 26]. Pneumococcus was responsible for 100,000 deaths among HIV-infected children in the year 2000. This mortality rate was dramatically reduced to 25, 000 in 2015 as a result of massive uptake of pneumococcal vaccination, especially in African countries [27]. Despite this reduction in the number of deaths, recurrent pneumococcal infections among HIV-infected patients remain high with rate of 8-25% [28] within a year after the initial episode [29]. This is speculated to be a result of the immune impairment in HIV that fails to mount optimal responses to the pneumococcal vaccines in cases where immunisation is offered [28]. Even after prolonged ART use, immune recovery remains only partial [30, 31]. Evidence suggests that delayed ART initiation and/or initiation at a low CD4 nadir count results in only partial immune recovery even after prolonged use (>7 years) [32]. In sub-Saharan Africa ART uptake remains sub-optimal (< 60%) [33] and therapy is initiated later rather than sooner [34]. Consequently, HIV-infected patients remain at increased risk of pneumococcal infections warranting further research into this phenomenon.

Although, *S. pneumoniae* is susceptible to common antibiotics, there is a steady increase in antibiotic resistance [24]. Globally, between 14% to 42% of *S. pneumoniae* isolates are resistant to penicillins, 0% to 30% to cephalosporins, 20% to 40% to macrolides, 26% to tetracyclines, 25% to 45% to cotrimoxazole and 22% to clindamycin [24]. Fluoroquinolone resistance in *S. pneumoniae* remains low (0% to 2%) [24]. In a systematic review of antimicrobial resistance in Africa, resistance of *S. pneumoniae* isolates is estimated to be [median (interquartile range)] 17.4% (3.4–70) to penicillins, 6.6% (0.6–19.8) to cephalosporins, 11.5% (2.7–21.5) to macrolides, 34.4% (18.8–65.4) to tetracyclines, 90.4% (71.4–98.6) to cotrimoxazole, 6.1% (0–13.2) clindamycin and 13.5% (4.1–70.1) to ciprofloxacin [35].

H. influenzae, another component of the upper respiratory tract microbiota, is a leading cause of bacterial pneumonia especially in children [27]. It also causes meningitis, septicaemia, cellulitis and osteomyelitis [27]. Altogether, these infections caused 363 000 deaths and 8.1 million total episodes in HIV-uninfected children in 2000. As a result of Hib vaccination, this figure reduced by about 150 000 in 2008 and even further to about 30 000 in 2015 [27]. Overall, *H. influenzae* infections were responsible for 2% of all-cause child mortality under five [36]. Among HIV-infected children, *H. influenzae* infections cause 8100 deaths annually [27]. Despite this improvement, *H. influenzae* infections still represent a major cause of morbidity and mortality particularly in HIV-infected children

whose defective immune systems fail to protect against encapsulated bacteria [37]. Furthermore, the immune systems of HIV-infected children mount lower response to *Haemophilus influenzae* type b (Hib) vaccine with an estimated risk of vaccine failure of 35.1-fold greater (95% CI 14.6–84.6) than HIV uninfected children [37]. In a systematic review of antimicrobial resistance in Africa, resistance of *H. influenzae* isolates is estimated to be [median (interquartile range)] 100 % (76.6–100) to ampicillins, 22.2% (0–39.5) to amoxicillin clavulanate, 20.4% (0–57.1) to ceftriaxone, 25% (11.1–25) to tetracyclines, 83.8% (0–100) to cotrimoxazole, and 14.6 (3.1–79.2% to ciprofloxacin [35].

Although, Pneumococcal Conjugate Vaccine (PCV) and Hib vaccines have reduced invasive diseases caused by *S. pneumoniae* and *H. influenzae*, these vaccines were introduced later into national immunization programs in sub-Saharan Africa [27]. As such older HIV-infected children and adolescent were not covered by these immunisation programs. For children who did receive, vaccine effectiveness and herd immunity were substantially limited compared to HIV-uninfected children [38]. As such this population remains susceptible to infections from these bacteria. Currently in Africa, unlike in developed countries [28], this population of older HIV-infected children do not receive PCV and Hib vaccinations that may confer the much-needed protection.

S. aureus, particularly methicillin-resistant *Staphylococcus aureus* (MRSA) is a major opportunistic pathogen in HIV-infected individuals who are 6-18 times more susceptible than uninfected people [39]. It causes pneumonia, bacteraemia, otitis media and abscess in these individuals [39]. The frequency with which HIV-infected individuals comes into contact with healthcare environment increases their risk of exposure to MRSA [39]. The increased susceptibility of HIV-infected children to these bacteria may suggests a role in chronic lung diseases. In a systematic review of antimicrobial resistance in Africa, resistance of *S. aureus* isolates were estimated to be [median (interquartile range)] 90.4% (80.8–96.4) to penicillins, 28.1% (2.7–54.2) to cephalosporins, 33.9% (13–46.4) to macrolides, 44.8% (27.7–58.7) to tetracyclines, 66.1% (42.8–84.3) to cotrimoxazole, 11.7% (0–47.4) to clindamycin, 10.4% (4.6–33.8) to cefoxitin (methicillin resistance) and 21.1% (8.5–33.7) to ciprofloxacin [35].

M. catarrhalis was once thought to be a non-pathogenic resident in the respiratory tract microbiota [40]. Recent studies have highlighted its pathogenic roles especially in the context of HIV where it causes lower respiratory tract infections [41, 42]. It is the third most common cause of acute otitis media after *S. pneumoniae* and *H. influenzae* [43]. It also causes sinusitis and the second most common cause of acute exacerbation of COPD after non-typeable *H. influenzae* [43]. In a meta-analysis of antimicrobial resistance in bacteria isolated in paediatric acute otitis media globally, *M. catarrhalis*

isolates were 88% and 87% resistant to amoxicillin, and penicillins due to widespread production of beta lactamase enzymes [44]. In Ethiopia, *M. catarrhalis* isolates recovered from the pharynx of HIV-infected children were resistant to penicillin (94.6%), erythromycin (86.5%), clindamycin (73%) and cotrimoxazole (78.4%) [45]. In Ghana, *M. catarrhalis* isolates recovered from the nasopharynx of HIV-infected children were 80.6% resistant to Ampicillin, 60% to cotrimoxazole, 42.6% to meropenem, 23% to chloramphenicol, 17% to cefuroxime, 17% to tetracycline, 15% to ciprofloxacin, 8.5% to ceftriaxone and 2.1% to gentamicin [46].

Currently, there are no cure or evidence-based treatment guidelines for HIV-associated OB. However, azithromycin, a macrolide (more accurately an azalide) antibiotic, has been suggested as a possible treatment [47]. Azithromycin is prescribed to more than 40 million patients annually [48]. It clears bacterial infections through the inhibition of bacterial protein synthesis by targeting the 50S subunit of the sensitive bacterial ribosome. Apart from its effectiveness in resolving bacterial infections, azithromycin also possess potent immunomodulatory properties [49], improve phagocytic competency [50] especially of defective alveolar macrophages [51], improve ciliary function, interfere with biofilm formation by *Pseudomonas aeruginosa*, mitigate airway mucus hypersecretion, and promote pulmonary epithelial cell healing [52]. These properties make it useful in chronic lung diseases [53] such as chronic obstructive pulmonary disease (COPD) [54], diffuse pan bronchiolitis [55], bronchiolitis obliterans related to lung transplantation [56], cystic fibrosis [57], non-cystic bronchiectasis [58] and asthma [59]. Although the evidence is not strong for all chronic lung diseases, azithromycin can improve lung function and survival, and reduce the frequency of acute pulmonary exacerbations, antibiotic administrations and hospitalisations [52]. Our recent clinical trial [Bronchopulmonary Function in Response to Azithromycin Treatment for Chronic Lung Disease in HIV-infected Children (BREATHE)] demonstrated that long-term azithromycin therapy reduced the frequency of acute respiratory exacerbations and all-cause hospitalisations in OB patients [60]. However, our understanding of how azithromycin therapy modulates the bacterial determinants implicated in HIV-associated OB is limited.

Azithromycin use is associated with reduction in the carriage but increase in antibiotic resistance [61, 62] in respiratory bacteria. These two phenomena were observed in common respiratory pathogens, including *S. pneumoniae*, *S. aureus*, *H. influenzae* and *M. catarrhalis* [61, 63–68]. The induction of macrolide resistance in *S. aureus* was particularly worrisome in that it resulted in the cessation of azithromycin therapy in a cystic fibrosis centre in Europe [67]. This antibiotic resistance phenomenon is more common with azithromycin than other macrolides because of the slow hepatic clearance that

allows it to remain at sub-inhibitory concentrations in tissues for several weeks [64, 65, 69]. This pharmacokinetic property of azithromycin makes it an effective “selector” of macrolide resistance.

There are multiple mechanisms underlining antibiotic resistance to azithromycin. The most relevant mechanism is the alteration of the target site by methylation or pseudouridylation of the 23S ribosomal subunit (related to the presence of *erm* genes carried on mobile genetic elements including plasmids and transposons) or by mutations in *rrl* alleles of the 23S rRNA gene, which blocks azithromycin binding to this subunit [48, 70, 71]. Another mechanism of resistance is the overexpression of efflux pumps that decrease the concentration of the drug in bacterial cells [48]. This mechanism is widespread in the bacteria *Pseudomonas aeruginosa*. Mutations in other ribosomal proteins such as L4 (encoded by the *rplD* gene) and L22 (encoded by the *rplV* gene) also results in azithromycin resistance in particular, *Enterobacteriaceae* [71]. Secretion of hydrolytic enzymes by resistant bacteria that modifies the structure of azithromycin is another mechanism of resistance. In *Enterobacteriaceae*, these enzymes include two esterases encoded by *ere* (A, B) and four different phosphotransferases encoded by *mph* (A, B, D, and E) gene [70, 71].

Some studies have shown that antibiotic resistance levels return to baseline after months of cessation of azithromycin [72, 73]. Adherence to long-term azithromycin therapy has been observed to reduce the occurrence of antibiotic resistance [62]. The extent to which long-term azithromycin therapy in OB will influence the development of macrolide resistance in this population of older HIV-infected children in sub-Saharan Africa is currently unknown. It is also undetermined whether macrolide resistance will persist, and for how long after the cessation of azithromycin. This information is relevant in this patient population that requires regular visits to healthcare centres for HIV care. There is a valid risk of the transmission of antibiotic-resistant bacteria to untreated persons both in the healthcare centres and the community. The resistant isolates in this population can also result in drug-resistant infections that will be complicated and more expensive to treat.

Azithromycin therapy is also associated with alterations in the respiratory microbiota characterised by loss of bacterial diversity and relative abundance [74–76]. In some chronic lung diseases, these changes were associated with the improvement or deterioration of the condition [74, 75]. However, it is unknown what the effect of long-term azithromycin therapy on the respiratory microbiome is, especially in the context of HIV, where dysbiosis has already been reported [20, 21]. It is also unknown how long-term azithromycin therapy will affect the distinct microbiota signatures associated with OB

and how this may mediate the response to treatment and lung function. Finally, the persistence of any alterations of the respiratory microbiota by azithromycin in this disease context is also unknown.

Notwithstanding the efforts to eradicate mother-to-child transmission, over 300,000 infants worldwide were infected with HIV in 2020 [1]. More than 80% of these new infections occur in sub-Saharan Africa [1]. With increased access to antiretroviral and cotrimoxazole prophylaxis, a higher number of these children will survive into adolescence. As these children grow older, chronic lung diseases, specifically OB, will become the new epidemic in the fight against HIV/AIDS in sub-Saharan Africa, especially as it is unresponsive to ART. Therefore, there is an urgent need to find evidence-based and effective therapies to manage this chronic lung disease. Azithromycin, currently the only tested drug for HIV-associated OB, may become the first-line drug for managing this condition among these children. Therefore, this thesis examines the impact of long-term azithromycin therapy on the respiratory microbiome and any associated antibiotic resistance in African children with HIV-associated OB or chronic lung disease.

This study was nested within a multi-site, double-blind, placebo-controlled, individually randomised trial, the Bronchopulmonary Function in Response to Azithromycin Treatment for Chronic Lung Disease in HIV-infected Children (BREATHE) trial [47, 60] (clinicaltrials.gov identifier NCT02426112). The trial protocol [47] and its main findings [60] have previously been described. Briefly, children and adolescents aged 6–19 years with perinatally-acquired HIV infection taking ART for a minimum of six months were recruited from outpatient HIV clinics in Blantyre, Malawi and Harare, Zimbabwe. HIV-associated chronic lung disease was defined as forced expiratory volume in 1 second (FEV1) z-score (FEV1z) less than -1.0 with no reversibility, i.e., < 12% improvement in FEV1z-score after 200 µg of salbutamol inhalation. FEV1z-score was calculated using the African American module of the Global Lung Function Initiative 2012 reference equations [77] validated among children in Zimbabwe by our team [78]. Exclusion criteria included acute respiratory symptoms, tuberculosis, pregnancy, hypersensitivity to azithromycin, prolonged QTc interval, impaired hearing, or renal clearance.

Participants were individually randomised to receive 48 weeks of once-weekly, weight-based doses of oral azithromycin (10 to 19.9 kg, 250 mg; 20 to 29.9 kg, 500 mg; 30 to 39.9 kg, 750 mg; and 40 kg or more, 1250 mg) [47]. Placebo arm participants received identical tablets without the active drug. This was followed by a six-month treatment-free period (to 72 weeks from the start of the intervention) for both trial arms. All trial participants were analysed at 48 weeks and a subset at 72 weeks. A comparison

group of perinatally HIV-infected children without chronic lung disease (FEV1 z-score > 0) was recruited at the same time as enrolment of trial participants using frequency matching for site, age (6–12 and 13–19 years) and the duration of ART use (6 months to < 2 years and two or more years). These children had no heart or apparent lung disease and reported no respiratory symptoms. Socio-demographic data and clinical history were obtained through an interviewer-administered questionnaire.

Although, HIV-associated OB was not confirmed in the trial participants by additional diagnostics such as high-resolution computation tomography scans, our previous study in the same cohort (both in Zimbabwe [6] and Malawi[5]), which was later confirmed among adolescents with perinatal HIV infection in South Africa [10], revealed HIV-associated OB as the predominant chronic lung disease (91%) in this population. Consequently, throughout this thesis, HIV-associated OB and HIV-associated chronic lung disease (HCLD) have been used interchangeably.

In the parent trial, there was no significant difference in the primary endpoint – mean difference in FEV1 z-score using intention-to-treat analysis – between the azithromycin and placebo arm for participants seen at 48 weeks. There was also no difference in the secondary outcome of growth (weight-for-age z-score). However, long-term azithromycin therapy reduced the frequency of acute respiratory exacerbations and all-cause hospitalisations in OB patients [60].

In this thesis, the following questions were addressed:

1. What is the effect of macrolide administration on the diversity and composition of the respiratory microbiome and total bacterial loads in general, and on the prevalence and antibiotic resistance of common potentially pathogenic respiratory bacteria [namely: *S. pneumoniae*, *S. aureus*, *H. influenzae*, *M. catarrhalis* and *P. aeruginosa*] in particular, in patients in any setting regardless of age, disease, or duration of treatment? How long did this effect persist post-administration?
2. What is the prevalence, antibiotic susceptibility profiles and factors associated with the carriage of *S. pneumoniae*, *S. aureus*, *H. influenzae*, *M. catarrhalis*, and other Gram-negative bacilli in the respiratory tract of children with HIV-associated chronic lung disease compared to age-, site- and duration of ART-matched HIV-infected children without HIV-associated chronic lung disease?

3. What is the impact of long-term azithromycin therapy on the prevalence and antibiotic susceptibility profiles of *S. pneumoniae*, *S. aureus*, *H. influenzae* and *M. catarrhalis*, recovered from the respiratory tract of participants in the azithromycin arm compared to placebo?
4. What is the impact of long-term azithromycin therapy on the bacteriome diversity and composition of the lower respiratory tract using sputum bacteriome as a proxy? Is there an association between sputum bacteriome profiles and lung function?

1.2 Outline of the thesis

Macrolides, including azithromycin, are widely used to manage both acute and chronic respiratory conditions. However, the effects of these antibiotics on the respiratory microbiota and associated antibiotic resistance have not been systematically analysed. Chapter 2 is an in-depth multi-indication systematic literature review of the impact of macrolide use on the composition and diversity of the respiratory microbiome and the prevalence and associated antibiotic resistance in common respiratory bacteria in children and adults, in any setting regardless of disease or duration of treatment.

Chapter 3 is a case-control study that explores whether there are differences in the bacterial flora in HIV-infected children and adolescents living with (cases) and without chronic lung disease (controls) established on ART in Zimbabwe and Malawi. It compares the prevalence, and antibiotic susceptibility of *S. pneumoniae*, *S. aureus*, *H. influenzae*, *M. catarrhalis*, and other Gram-negative bacilli isolated from nasopharynx and sputum of cases and controls. The methods used were bacterial culture for carriage and disk diffusion for antibiotic susceptibility testing. Risk factors for bacterial carriage were identified using questionnaires and multivariate logistic regression analysis.

Chapter 4 is original research that describes the impact of long-term azithromycin therapy on the prevalence, and antibiotic susceptibility of *S. pneumoniae*, *S. aureus*, *H. influenzae*, *M. catarrhalis*, and other Gram-negative bacilli recovered from the nasopharynx and sputum of children and adolescents with HIV-associated chronic lung diseases established on ART randomised to azithromycin or placebo. The study sites were in Zimbabwe and Malawi. The methods used were bacterial culture for prevalence and disk diffusion for antibiotic susceptibility testing. Risk factors for carriage of macrolide-resistant bacteria were identified using questionnaires and generalised logistic mixed effect modelling.

Chapter 5 is original research that utilised quantitative polymerase chain reaction (qPCR) and 16S rDNA amplicon metagenomic sequencing to determine the effect of long-term azithromycin therapy on the total bacterial load and bacteriome composition and diversity of sputa collected from children and adolescents with HIV-associated chronic lung diseases established on ART, randomised to azithromycin or placebo. The bacteriome profiles at baseline and end of therapy (48 weeks) were compared to assess the effect of treatment on the sputum bacteriome used as a proxy for the lung bacteriome. The bacteriome profile at 48 weeks was also compared with that at six months post-intervention (72 weeks) and the baseline profiles to assess whether bacteriome profiles returned to pre-treatment levels after therapy. The influence of socio-demographic and clinical factors on the study participants' sputum bacteriome was assessed using questionnaires, Permutation Multivariate Analysis of Variance (PERMANOVA), linear regression and linear mixed effect modelling. Finally, we evaluated the effect of treatment on bacterial genera associated with HIV-associated chronic lung diseases.

Chapter 6 is a brief chapter that summarises each chapter's significant findings and principal conclusions and the limitations of the technical approaches used. Recommendations for further research and planned future work are also outlined.

Appendix A contains the supporting information for chapter 2, including the systematic review protocol published in PROSPERO, definition of terms and the number of records retrieved from each database after literature search.

Appendix B contains the supplementary tables and figures for chapter 3.

Appendix C contains the supplementary tables and figures for chapter 4.

Appendix D contains the supplementary information for chapter 5, including a detailed description and results of quality control steps, supplementary tables, and figures.

Appendix E is the list of other publications produced during this PhD but not included in this thesis, conference presentations and the candidate's contribution to these bodies of work.

Appendix F contains ethics and biosafety certificates.

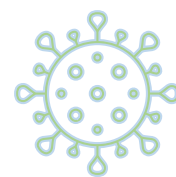
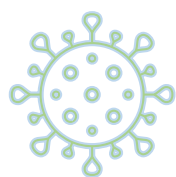
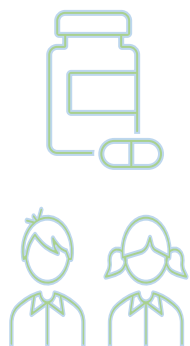
Appendix G contains the summary of the plagiarism (Turnitin™) report.

1.3 References

1. UNICEF Global and regional trends in HIV. Available from: <https://data.unicef.org/topic/hiv/aids/global-regional-trends/> accessed on 7th April 2022.
2. Lowenthal ED, Bakeera-Kitaka S, Marukutira T, Chapman J, Goldrath K, Ferrand RA. Perinatally acquired HIV infection in adolescents from sub-Saharan Africa: a review of emerging challenges. *Lancet Infect. Dis.* 2014; 14: 627–639.
3. Githinji L, Zar HJ. Respiratory complications in children and adolescents with human immunodeficiency virus. *Pediatr. Clin. North Am.* 2021; 68: 131–145.
4. Weber HC, Gie RP, Cotton MF. The challenge of chronic lung disease in HIV-infected children and adolescents. *J. Int. AIDS Soc.* 2013; 16: 18633.
5. Mwalukomo T, Rylance SJ, Webb EL, Anderson S, O'Hare B, Oosterhout V, J J, Ferrand RA, Corbett EL, Rylance J. Clinical characteristics and lung function in older children vertically infected with human immunodeficiency virus in Malawi. *J. Pediatr. Infect. Dis. Soc.* 2016; 5: 161–169.
6. Ferrand RA, Desai SR, Hopkins C, Elston CM, Copley SJ, Nathoo K, Ndhlovu CE, Munyati S, Barker RD, Miller RF, Bandason T, Wells AU, Corbett EL. Chronic lung disease in adolescents with delayed diagnosis of vertically acquired HIV infection. *Clin. Infect. Dis.* 2012; 55: 145–152.
7. McHugh G, Rehman AM, Simms V, Gonzalez-Martinez C, Bandason T, Dauya E, Moyo B, Mujuru H, Rylance J, Sovershaeva E, Weiss HA, Kranzer K, Odland J, Ferrand RA. Chronic lung disease in children and adolescents with HIV: a case-control study. *Trop Med Int Health.* 2020; 25: 590–599.
8. Githinji LN, Gray DM, Hlengwa S, Myer L, Zar HJ. Lung function in South African adolescents infected perinatally with HIV and treated long-term with antiretroviral therapy. *Ann. Am. Thorac. Soc.* 2017; 14: 722–729.
9. Barrera CA, du Plessis A-M, Otero HJ, Mahtab S, Githinji LN, Zar HJ, Zhu X, Andronikou S. Quantitative CT analysis for bronchiolitis obliterans in perinatally HIV-infected adolescents—comparison with controls and lung function data. *Eur. Radiol.* 2020; 30: 4358–4368.
10. du Plessis AM, Andronikou S, Machemedze T, Griffith-Richards S, Myer L, Mahtab S, Zar HJ. High-resolution computed tomography features of lung disease in perinatally HIV-infected adolescents on combined antiretroviral therapy. *Pediatr Pulmonol* 2019; 54: 1765–1773.
11. Barker AF, Bergeron A, Rom WN, Hertz MI. Obliterative Bronchiolitis. *N. Engl. J. Med.* 2014; 370: 1820–1828.
12. Paiardini M, Müller-Trutwin M. HIV-associated chronic immune activation. *Immunol. Rev.* 2013; 254: 78–101.
13. Feldman C, Anderson R. Bacterial respiratory infections complicating human immunodeficiency virus. *Semin. Respir. Crit. Care Med.* 2016; 37: 214–229.
14. Requena T, Velasco M. The human microbiome in sickness and in health. *Rev. Clinica Esp. Engl. Ed.* 2021; 221: 233–240.
15. Dominguez-Bello MG, Godoy-Vitorino F, Knight R, Blaser MJ. Role of the microbiome in human development. *Gut* 2019; 68: 1108–1114.
16. Young VB. The role of the microbiome in human health and disease: an introduction for clinicians. *BMJ* 2017; 356: j831.
17. Whiteside SA, McGinniss JE, Collman RG. The lung microbiome: progress and promise. *J. Clin. Invest.* 2021; 131: e150473.
18. Peterson CT, Sharma V, Elmén L, Peterson SN. Immune homeostasis, dysbiosis and therapeutic modulation of the gut microbiota. *Clin. Exp. Immunol.* 2015; 179: 363–377.
19. Levy M, Kolodziejczyk AA, Thaïss CA, Elinav E. Dysbiosis and the immune system. *Nat. Rev. Immunol.* 2017; 17: 219–232.
20. Twigg HL 3rd, Knox KS, Zhou J, Crothers KA, Nelson DE, Toh E, Day RB, Lin H, Gao X, Dong Q, Mi D, Katz BP, Sodergren E, Weinstock GM. Effect of advanced HIV infection on the respiratory microbiome. *Am. J. Respir. Crit. Care Med.* 2016; 194: 226–235.
21. Twigg HL, Weinstock GM, Knox KS. Lung microbiome in human immunodeficiency virus infection. *Transl. Res.* 2017; 179: 97–107.
22. Beck JM, Schloss PD, Venkataraman A, Twigg H 3rd, Jablonski KA, Bushman FD, Campbell TB, Charlson ES, Collman RG, Crothers K, Curtis JL, Drews KL, Flores SC, Fontenot AP, Foulkes MA, Frank I, Ghedin E, Huang L, Lynch SV, Morris A, Palmer BE, Schmidt TM, Sodergren E, Weinstock GM, Young VB. Multicenter comparison of lung and oral microbiomes of HIV-infected and HIV-uninfected individuals. *Am. J. Respir. Crit. Care Med.* 2015; 192: 1335–1344.
23. Bhadriraju S, Fadrosch DW, Shenoy MK, Lin DL, Lynch KV, McCauley K, Ferrand RA, Majonga ED, McHugh G, Huang L, Lynch SV, Metcalfe JZ. Distinct lung microbiota associate with HIV-associated chronic lung disease in children. *Sci. Rep.* 2020; 10: 16186.
24. Cherazard R, Epstein M, Doan T-L, Salim T, Bharti S, Smith MA. Antimicrobial resistant *Streptococcus pneumoniae*: prevalence, mechanisms, and clinical implications. *Am. J. Ther.* 2017; 24: e361–e369.
25. Marcus JL, Baxter R, Leyden WA, Muthulingam D, Yee A, Horberg MA, Klein DB, Towner WJ, Chao CR, Quesenberry CP, Silverberg MJ. Invasive pneumococcal disease among HIV-Infected and HIV-Uninfected adults in a large integrated healthcare system. *AIDS Patient Care STDs* 2016; 30(10): 463–470.
26. Chowers M, Regev-Yochay G, Mor O, Cohen-Poradosu R, Riesenber K, Zimhony O, Chemtob D, Stein M, Dagan R, Levy I. Invasive pneumococcal disease (IPD) in HIV infected patients in Israel since the introduction of pneumococcal conjugated vaccines (PCV): Analysis of a nationwide surveillance study, 2009–2014. *Hum. Vaccines Immunother.* 2017; 13(1): 216–219.

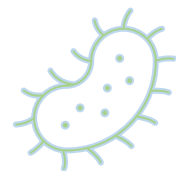
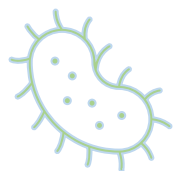
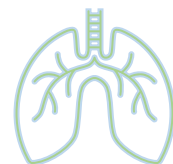
27. Wahl B, O'Brien KL, Greenbaum A, Majumder A, Liu L, Chu Y, Lukšić I, Nair H, McAllister DA, Campbell H, Rudan I, Black R, Knoll MD. Burden of *Streptococcus pneumoniae* and *Haemophilus influenzae* type b disease in children in the era of conjugate vaccines: global, regional, and national estimates for 2000–15. *Lancet Glob. Health* 2018; 6(7): e744–e757.
28. Lee KY, Tsai MS, Kuo KC, Tsai JC, Sun HY, Cheng AC, Chang SY, Lee CH, Hung CC. Pneumococcal vaccination among HIV-infected adult patients in the era of combination antiretroviral therapy. *Hum. Vaccines Immunother.* 2014; 10(12): 3700–3710.
29. Gilks CF, Ojoo SA, Ojoo JC, Brindle RJ, Paul J, Batchelor BIF, Kimari JN, Newnham R, Bwayo J, Plummer FA, Warrell DA. Invasive pneumococcal disease in a cohort of predominantly HIV-1 infected female sex-workers in Nairobi, Kenya. *Lancet* 1996; 347(9003): 718–723.
30. Iwajomo OH, Moons P, Nkhata R, Mzinza D, Ogunniyi AD, Williams NA, Heyderman RS, Finn A. Delayed reconstitution of B cell immunity to pneumococcus in HIV-infected Malawian children on antiretroviral therapy. *J. Infect.* 2015; 70(6): 616–623.
31. Sepako E, Glennie SJ, Jambo KC, Mzinza D, Iwajomo OH, Banda D, Van Oosterhout JJ, Williams NA, Gordon SB, Heyderman RS. Incomplete recovery of pneumococcal CD4 T cell immunity after initiation of antiretroviral therapy in HIV-infected Malawian adults. *PLoS One*. 2014;9(6): e100640.
32. Le T, Wright EJ, Smith DM, He W, Catano G, Okulicz JF, Young JA, Clark RA, Richman DD, Little SJ, Ahuja SK. Enhanced CD4+ T-cell recovery with earlier HIV-1 antiretroviral therapy. *N Engl J Med.* 2013;368(3):218–230.
33. Global HIV and AIDS statistics. Available from: <https://www.avert.org/global-hiv-and-aids-statistics> accessed on 6th April 2022.
34. Desmonde S, Tanser F, Vreeman R, Takassi E, Edmonds A, Lumbiganon P, Pinto J, Malateste K, McGowan C, Kariminia A, Yotebieng M, Dicko F, Yiannoutsos C, Mubiana-Mbewe M, Wools-Kaloustian K, Davies MA, Leroy V. Access to antiretroviral therapy in HIV-infected children aged 0–19 years in the International Epidemiology Databases to Evaluate AIDS (IeDEA) Global Cohort Consortium, 2004–2015: A prospective cohort study. *PLoS Med.* 2018; 15(5): e1002565.
35. Tadesse BT, Ashley EA, Ongarello S, Havumaki J, Wijegoonewardena M, González JJ, Dittich S. Antimicrobial resistance in Africa: a systematic review. *BMC Infect. Dis.* 2017; 17: 616.
36. World Health Organisation. Immun. Vaccines Biol. 2013 Available from: https://www.who.int/immunization/monitoring_surveillance/burden/estimates/Pneumo_hib/en/, accessed 7th March 2022.
37. Madhi SA, Kuwanda L, Saarinen L, Cutland C, Mothupi R, Käyhty H, Klugman KP. Immunogenicity and effectiveness of *Haemophilus influenzae* type b conjugate vaccine in HIV infected and uninfected African children. *Vaccine* 2005; 23(48–49): 5517–5525.
38. Vardanjani HM, Borna H, Ahmadi A. Effectiveness of pneumococcal conjugate vaccination against invasive pneumococcal disease among children with and those without HIV infection: A systematic review and meta-analysis. *BMC Infect. Dis.* 2019; 19(1): 685.
39. Hidron A, Moanna, M.D. A, Russell Kempker, M.D., David Rimland, M.D. Methicillin-resistant *Staphylococcus aureus* in HIV-infected patients. *Infect. Drug Resist.* 2010; 3:73–86.
40. Bernhard S, Spaniol V, Aebi C. Molecular pathogenesis of infections caused by *Moraxella catarrhalis* in children. *Swiss Med Wkly.* 2012;142: w13694
41. Manfredi R, Nanetti A, Valentini R, Chiodo F. *Moraxella catarrhalis* pneumonia during HIV disease. *J. Chemother.* 2000; 12(5):406–411.
42. Raj CJT, Shankar EM, Rothan HA, Rao UA. Molecular characterization of clinical isolates of *Moraxella catarrhalis* by randomly amplified polymorphic DNA fingerprinting. *J. Mol. Microbiol. Biotechnol.* 2014; 24(4):270–278.
43. Goldstein EJC, Murphy TF, Parameswaran GL. *Moraxella catarrhalis*, a human respiratory tract pathogen. *Clin. Infect. Dis.* 2009; 49: 124–131.
44. Mather MW, Drinnan M, Perry JD, Powell S, Wilson JA, Powell J. A systematic review and meta-analysis of antimicrobial resistance in paediatric acute otitis media. *Int. J. Pediatr. Otorhinolaryngol.* 2019; 123: 102–109.
45. Mulu W, Yizengaw E, Alemu M, Mekonnen D, Hailu D, Ketemaw K, Abera B, Kibret M. Pharyngeal colonization and drug resistance profiles of *Moraxella catarrhalis*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Haemophilus influenzae* among HIV infected children attending ART Clinic of Felegehiwot Referral Hospital, Ethiopia. *PLoS One* 2018; 13: e0196722–e0196722.
46. Sampene-Donkor E, Badoe EV, Annan JA, Nii-Trebi N. Colonisation of antibiotic resistant bacteria in a cohort of HIV infected children in Ghana. *Pan Afr Med J* 2017/04/30 ed. 2017; 26: 60–60.
47. Gonzalez-Martinez C, Kranzer K, McHugh G, Corbett EL, Mujuru H, Nicol MP, Rowland-Jones S, Rehman AM, Gutteberg TJ, Flaegstad T, Odland JO, Ferrand RA. Azithromycin versus placebo for the treatment of HIV-associated chronic lung disease in children and adolescents (BREATHE trial): study protocol for a randomised controlled trial. *Trials.* 2017; 18: 622–622.
48. Heidary M, Ebrahimi Samangani A, Kargari A, Kiani Nejad A, Yashmi I, Motahar M, Taki E, Khoshnood S. Mechanism of action, resistance, synergism, and clinical implications of azithromycin. *J. Clin. Lab. Anal.* 2022; 36: e24427.
49. Zimmermann P, Ziesenitz VC, Curtis N, Ritz N. The immunomodulatory effects of macrolides—a systematic review of the underlying mechanisms. *Front. Immunol.* 2018; 9: 302.
50. Hodge S, Reynolds PN. Low-dose azithromycin improves phagocytosis of bacteria by both alveolar and monocyte-derived macrophages in chronic obstructive pulmonary disease subjects. *Respirol. Carlton Vic* 2012; 17: 802–807.
51. Berenson CS, Kruzel RL, Eberhardt E, Sethi S. Phagocytic dysfunction of human alveolar macrophages and severity of chronic obstructive pulmonary disease. *J. Infect. Dis.* 2013; 208: 2036–2045.
52. Alchakaki A, Cramer C, Patterson A, Soubani AO. Which patients with respiratory disease need long-term azithromycin? *Cleve. Clin. J. Med.* 2017; 84: 755–758.
53. Smith D, Du Rand IA, Addy C, Collyns T, Hart S, Mitchelmore P, Rahman N, Saggi R. British Thoracic Society guideline for the use of long-term macrolides in adults with respiratory disease. *BMJ Open Respir. Res.* 2020; 7: e000489.
54. Albert RK, Connett J, Bailey WC, Casaburi R, Cooper JADJ, Criner GJ, Curtis JL, Dransfield MT, Han MK, Lazarus SC, Make B, Marchetti N, Martinez FJ, Madinger NE, McEvoy C, Niewoehner DE, Porsasz J, Price CS, Reilly J, Scanlon PD, Sciruba FC, Scharf SM, Washko GR, Woodruff PG, Anthonisen NR. Azithromycin for prevention of exacerbations of COPD. *N. Engl. J. Med.* 2011; 365: 689–698.

55. Weng D, Wu Q, Chen X-Q, Du Y-K, Chen T, Li H, Tang D-L, Li Q-H, Zhang Y, Lu L-Q, Zhou N-Y, Song J-C, Wang C, Li H-P. Azithromycin treats diffuse panbronchiolitis by targeting T cells via inhibition of mTOR pathway. *Biomed. Pharmacother.* 2019; 110: 440–448.
56. Arjuna A, Olson MT, Walia R, Bremner RM, Smith MA, Mohanakumar T. An update on current treatment strategies for managing bronchiolitis obliterans syndrome after lung transplantation. *Expert Rev. Respir. Med.* 2021; 15: 339–350.
57. Mayer-Hamblett N, Retsch-Bogart G, Kloster M, Accurso F, Rosenfeld M, Albers G, Black P, Brown P, Cairns A, Davis SD, Graff GR, Kerby GS, Orenstein D, Buckingham R, Ramsey BW. Azithromycin for early *Pseudomonas* infection in cystic fibrosis: the OPTIMIZE randomized trial. *Am. J. Respir. Crit. Care Med.* 2018; 198: 1177–1187.
58. Lee E, Sol IS, Kim JD, Yang H-J, Min TK, Jang GC, Hwang YH, Cho H-J, Suh DI, Kim K, Kim HS, Kim YH, Woo SI, Lee YJ, Jung S, Jeon YH. Long-term macrolide treatment for non-cystic fibrosis bronchiectasis in children: a meta-analysis. *Sci. Rep.* 2021; 11: 24287.
59. Undela K, Goldsmith L, Kew KM, Ferrara G. Macrolides versus placebo for chronic asthma. *Cochrane Database Syst. Rev.* 2021; 11: CD002997.
60. Ferrand RA, McHugh G, Rehman AM, Mujuru H, Simms V, Majonga ED, Nicol MP, Flaegstad T, Gutteberg TJ, Gonzalez-Martinez C, Corbett EL, Rowland-Jones SL, Kranzer K, Weiss HA, Odland JO, BREATHE Trial Group. Effect of once-weekly azithromycin vs placebo in children with HIV-associated chronic lung disease: the BREATHE randomized clinical trial. *JAMA Netw. Open* 2020; 3: e2028484.
61. Taylor SP, Sellers E, Taylor BT. Azithromycin for the Prevention of COPD Exacerbations: The Good, Bad, and Ugly. *Am. J. Med.* 2015; 128: 1362.e1-1362.e6.
62. Hare KM, Grimwood K, Chang AB, Chatfield MD, Valery PC, Leach AJ, Smith-Vaughan HC, Morris PS, Byrnes CA, Torzillo PJ, Cheng AC. Nasopharyngeal carriage and macrolide resistance in Indigenous children with bronchiectasis randomized to long-term azithromycin or placebo. *Eur. J. Clin. Microbiol. Infect. Dis.* 2015; 34: 2275–2285.
63. Emiralioğlu N, Öztürk Z, Yalçın E, Doğru D, Özçelik U, Kiper N. Long term azithromycin therapy in patients with cystic fibrosis. *Turk. J. Pediatr.* 2016; 58: 34.
64. Greenberg D, Givon-Lavi N, Sharf AZ, Vardy D, Dagan R. The association between antibiotic use in the community and nasopharyngeal carriage of antibiotic-resistant *Streptococcus pneumoniae* in Bedouin children. *Pediatr. Infect. Dis. J.* 2008; 27: 776–782.
65. Kastner U, Guggenbichler JP. Influence of macrolide antibiotics on promotion of resistance in the oral flora of children. *Infection* 2001; 29: 251–256.
66. Hansen CR, Pressler T, Hoiby N, Johansen HK. Long-term, low-dose azithromycin treatment reduces the incidence but increases macrolide resistance in *Staphylococcus aureus* in Danish CF patients. *J. Cyst. Fibros.* 2009; 8: 58–62.
67. Phaff SJ, Tiddens HAWM, Verbrugh HA, Ott A. Macrolide resistance of *Staphylococcus aureus* and *Haemophilus* species associated with long-term azithromycin use in cystic fibrosis. *J. Antimicrob. Chemother.* 2006; 57: 741–746.
68. Grimwood K, Bell SC, Chang AB. Antimicrobial treatment of non-cystic fibrosis bronchiectasis. *Expert Rev. Anti Infect. Ther.* 2014; 12: 1277–1296.
69. Baquero F, Baquero-Artigao G, Cantón R, García-Rey C. Antibiotic consumption and resistance selection in *Streptococcus pneumoniae*. *J. Antimicrob. Chemother.* 2002; 50: 27–38.
70. Gomes C, Martínez-Puchol S, Palma N, Horna G, Ruiz-Roldán L, Pons MJ, Ruiz J. Macrolide resistance mechanisms in *Enterobacteriaceae*: focus on azithromycin. *Crit. Rev. Microbiol.* 2017; 43: 1–30.
71. Gomes C, Ruiz-Roldán L, Mateu J, Ochoa TJ, Ruiz J. Azithromycin resistance levels and mechanisms in *Escherichia coli*. *Sci. Rep.* 2019; 9: 6089.
72. Bojang E, Jafari J, Perreten V, Hart J, Harding-Esch EM, Sillah A, Mabey DCW, Holland MJ, Bailey RL, Roca A, Burr SE. Short-term increase in prevalence of nasopharyngeal carriage of macrolide-resistant *Staphylococcus aureus* following mass drug administration with azithromycin for trachoma control. *BMC Microbiol.* 2017; 17: 75.
73. Haug S, Lakew T, Habtemariam G, Alemayehu W, Cevallos V, Zhou Z, House J, Ray K, Porco T, Rutar T, Keenan J, Lietman TM, Gaynor BD. The decline of pneumococcal resistance after cessation of mass antibiotic distributions for trachoma. *Clin. Infect. Dis.* 2010; 51: 571–574.
74. Slater M, Rivett DW, Williams L, Martin M, Harrison T, Sayers I, Bruce KD, Shaw D. The impact of azithromycin therapy on the airway microbiota in asthma. *Thorax* 2014; 69: 673–674.
75. Segal LN, Clemente JC, Wu BG, Wikoff WR, Gao Z, Li Y, Ko JP, Rom WN, Blaser MJ, Weiden MD. Randomised, double-blind, placebo-controlled trial with azithromycin selects for anti-inflammatory microbial metabolites in the emphysematous lung. *Thorax* 2017; 72: 13–22.
76. Lopes Dos Santos Santiago G, Brusselle G, Dauwe K, Deschaght P, Verhofstede C, Vaneechoutte D, Deschepper E, Jordens P, Joos G, Vaneechoutte M. Influence of chronic azithromycin treatment on the composition of the oropharyngeal microbial community in patients with severe asthma. *BMC Microbiol.* 2017; 17: 109.
77. Quanjer PH, Stanojevic S, Cole TJ, Baur X, Hall GL, Culver BH, Enright PL, Hankinson JL, Ip MSM, Zheng J, Stocks J. Multi-ethnic reference values for spirometry for the 3–95-yr age range: the global lung function 2012 equations. *Eur. Respir. J.* 2012; 40: 1324–1343.
78. Madanhire T, Ferrand RA, Attia EF, Sibanda EN, Rusakaniko S, Rehman AM. Validation of the global lung initiative 2012 multi-ethnic spirometric reference equations in healthy urban Zimbabwean 7–13-year-old school children: a cross-sectional observational study. *BMC Pulm. Med.* 2020; 20: 56–56.



Chapter 2

The impact of macrolides on the human respiratory microbiota: a systematic review



2.0 Abstract

Background: Macrolides, antibiotics widely used for respiratory conditions, can promote antibiotic resistance and alter the respiratory microbiota. However, there are no recent, comprehensive systematic reviews on the effect of macrolide use on the prevalence of clinically relevant bacteria, associated antibiotic resistance and the respiratory microbiome more broadly. We conducted a systematic review to investigate these effects of macrolides in humans regardless of age, clinical setting, disease or duration of treatment.

Methods: We searched The Cochrane Library, Medline via PubMed, Web of Science, Scopus, and Academic Search Premier, African Wide Information, Health Source Academic/Nursing and CINAHL via EBSCOhost from inception through to 25th January 2022. We included studies that reported the effect of macrolide use on the prevalence, bacterial load, and macrolide resistance of clinically relevant respiratory bacteria (*Streptococcus pneumoniae*, *Staphylococcus aureus*, *Haemophilus influenzae*, *Moraxella catarrhalis* and *Pseudomonas aeruginosa*) and/or respiratory microbiome diversity and composition using culture-dependent or independent techniques. The literature search, title, abstract and full-text screening, and data extraction were independently conducted by two reviewers.

Results: From the 6052 records retrieved, 122 eligible studies were included. Twelve studies assessed the effect of macrolide use on the respiratory microbiome using next-generation sequencing, while 110 studies used culture or PCR. Of these 110 studies, 65 and 45 administered macrolides for ≤ 14 days (short-term) or ≥ 28 days (long-term), respectively, mainly for respiratory conditions such as cystic fibrosis or asthma. Short-term macrolide administration commonly eradicated the respiratory carriage of *S. pneumoniae*, *S. aureus*, *H. influenzae* and *M. catarrhalis*, with minimal macrolide resistance. The treatment did not affect bacterial carriage or resistance in long-term studies. Few long-term studies (8/48) assessed the persistence of the bacteriological effects of macrolides post-intervention with conflicting findings. In the microbiome studies, macrolide use did not affect within-sample bacterial diversity but reduced between-sample diversity of samples from end-of-treatment compared to baseline. Macrolides reduced the relative abundance of the *Moraxella* genus while increasing the relative abundance of *Streptococcus* and macrolide resistance genes in the respiratory resistome.

Conclusions: In most studies, short-term macrolide use eradicated the respiratory carriage of *S. pneumoniae*, *S. aureus*, *H. influenzae* and *M. catarrhalis*. Consequently, only few instances of macrolide resistance were reported after treatment. Bacterial carriage and macrolide resistance remained unchanged in most long-term macrolide studies. Macrolide use altered the composition of the respiratory microbiome. The persistence of the bacteriological effects of macrolides post-intervention remains poorly characterised and warrants further investigation.

Keywords: *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Haemophilus influenzae*, *Moraxella catarrhalis*, azithromycin, antibiotic resistance, microbiome

2.1 Background

Macrolides remain the most prescribed antibiotics in the United States of America [1] and the third most consumed antibiotic globally [2]. This class of antibiotics includes azithromycin, clarithromycin, erythromycin, roxithromycin and telithromycin. Macrolides are often used for treating respiratory, urinary, and sexually transmitted bacterial infections [3]. Azithromycin has been employed in mass drug administration to control trachoma in endemic countries, including Malawi [4], Tanzania [5], Niger [6] and The Gambia [7]. A report of the potential of azithromycin to reduce childhood mortality in sub-Saharan Africa has led to proposals to introduce mass administration in developing countries to improve child survival [8].

Apart from their effectiveness in resolving bacterial infections, macrolides also possess potent immunomodulatory properties [9], improve phagocytic competency [10], especially of defective alveolar macrophages [11], improve ciliary function, interfere with biofilm formation by *Pseudomonas aeruginosa*, mitigate airway mucus hypersecretion, and promote pulmonary epithelial cell healing [12]. These properties make them useful in chronic lung diseases (CLD) [13] such as chronic obstructive pulmonary disease (COPD) [14], diffuse pan bronchiolitis [15], bronchiolitis obliterans related to lung transplantation [16], cystic fibrosis [17], non-cystic bronchiectasis [18] and asthma [71–73][19]. Although the evidence is not strong for all CLDs, macrolides can improve lung function and survival, and reduce the frequency of acute pulmonary exacerbations, antibiotic administrations and hospitalisations [12].

The use of macrolides can select for antibiotic resistance in both commensal and pathogenic bacteria in the respiratory tract [20,21]. For example, in a meta-analysis [22] of six randomised controlled trials of CLD patients who received long-term azithromycin (> 28 days), the risk of bacterial resistance increased by 2.7-fold (risk ratio [RR], 2.69 [95% confidence interval {95% CI}, 1.249, 5.211]) in the azithromycin arm compared to placebo at the end of treatment. In contrast, the intervention reduced the risk of bacterial colonisation by half (RR, 0.551 [95% CI, 0.460, 0.658]) compared to placebo at the end of treatment [22]. None of the trials included in this meta-analysis reported on the persistence of the bacteriological effect of azithromycin post-intervention [22]. Macrolides can increase the overall respiratory resistome in patients [21] and resistance at the community level through person-to-person transmission [23].

In addition to directly promoting antibiotic resistance, macrolides can perturb the delicate balance of bacterial communities in the respiratory microbiome by selecting for specific bacterial taxa [21,24–26]. These perturbations can subsequently lead to alterations in the functional activity of the respiratory microbiome [25]. Although an association between these microbial perturbations and acute exacerbations has been observed in CLDs, particularly chronic obstructive pulmonary disease (COPD) [27], the effect of these perturbations on host health is incompletely understood [28].

There are limited reviews focusing on the effect of macrolides on the human respiratory microbiome [22,23,29]. These include a narrative review [23] on macrolide use and antibiotic resistance, a multi-indication systematic review on the adverse effects of long-term use of macrolides in CLDs in any patients group [30], and the effect of macrolides on the respiratory microbiome in children with bronchiolitis [29]. None of these reviews provided a comprehensive review on the effect of macrolide use on the prevalence of clinically relevant respiratory bacteria, antibiotic resistance, and the respiratory microbiome composition. Therefore, we conducted a systematic review of primary studies to investigate the effect of macrolide use on clinically relevant respiratory bacteria, the composition and diversity of the respiratory microbiome, and associated antibiotic resistance in children and adults in any setting regardless of disease or duration of treatment.

2.2 Materials and Methods

2.2.1 Search terms, strategy, and databases

This systematic review was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines [31]. The review protocol ([CRD4202018477](https://doi.org/10.1111/CRD4.202018477)) is available from the International Prospective Register of Systematic Reviews (PROSPERO) [32] and Appendix A. We comprehensively searched the following databases according to the search strategy outlined in Table 2.1: The Cochrane Library, Medline via PubMed, ISI Web of Science (Web of Knowledge), Scopus, Academic Search Premier, African Wide Information, Health Source Academic/Nursing and CINAHL via EBSCOhost. No filter was applied to any of the database searches except for Web of Science and Scopus, where MEDLINE was excluded as this was already searched using PubMed. Both MeSH terms and free text words were used to identify eligible studies. The reference list of included studies and relevant review articles were screened to further identify eligible studies. Searches were conducted on 15th May 2020 by Regina Esinam Abotsi (REA) and Gillian Ndhlovu (GN) together with an experienced librarian/information specialist. The search was re-run on 25th January 2022 before the final analysis, and additional eligible studies were included. All citations were exported into Endnote X9TM (Clarivate, Cologne, Germany).

Table 2. 1. Search terms

(Azithromycin OR clarithromycin OR erythromycin OR Telithromycin OR Roxithromycin OR troleandomycin
AND
Respiratory OR sputum OR nasopharyngeal OR Broncho-alveolar OR nasal OR lung OR nares OR oropharyngeal OR bronchial OR pharyngeal OR throat
AND
Streptococcus pneumoniae OR Staphylococcus aureus OR Haemophilus influenzae OR Moraxella catarrhalis OR flora OR microflora OR microbiota OR microbiome OR microbiomics OR metagenome OR metagenomics OR resistome
AND
Human OR Humans

2.2.2 Screening of Studies

We used CovidenceTM (www.covidence.org, Melbourne, Australia), a web-based software platform for conducting systematic reviews, to facilitate the screening and selection of eligible studies. Retrieved records were successively deduplicated using Endnote X9TM and CovidenceTM. Two authors

(REA and GN), independently and in duplicate, screened titles and abstracts of retrieved papers according to the eligibility criteria. The full-text articles of all potentially eligible records were retrieved and independently assessed for eligibility by both reviewers. All disagreements were resolved by consensus. Reasons for the exclusion of full-text articles were recorded and included in Figure 2.1.

2.2.3 Outcomes

The primary outcomes were measures of the effect of macrolide administration on the a) prevalence and/or bacterial loads of *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Haemophilus influenzae*, *Moraxella catarrhalis* and *Pseudomonas aeruginosa* in the respiratory tract, b) alpha and beta diversity of the respiratory microbiome, c) presence/absence and relative abundances of major bacterial taxa in the respiratory microbiome, d) percentage of macrolide resistance, or detection and prevalence of previously undetected antibiotic resistance genes in each of the bacteria mentioned above at baseline, e) resistome of the respiratory tract (abundance of resistance genes relative to its species composition), and f) duration of effect on the respiratory resistome and microbiome. The secondary outcomes were measures of the effect of specific type of macrolide, sample type, doses, routes of administration, frequency, and duration of macrolide therapy on the above outcomes (a to f).

2.2.4 Study Selection Criteria

We included studies that met the eligibility criteria specified in Table 2.2 and terms are defined in Appendix A Table S1.

Table 2. 2. Eligibility criteria

PICOS strategy	Inclusion criteria (all required)	Exclusion criteria (any)
Population	Participants of any age group or health status or setting exposed to any macrolides for any duration Participants provided any respiratory samples before and after intervention	Participants without macrolide exposure Participants provided only either pre- or post-intervention respiratory samples
Intervention	Macrolides in any dosage form and route of administration for any duration	Non-macrolide antibiotic
Comparator	Placebo, untreated controls, baseline samples from the population, controls treated with another antibiotic	Controls with previous macrolide exposure within four weeks of enrolment except in randomised trials where this was balanced between arms
Outcome	Prevalence, bacterial loads and/or antibiotic resistance in <i>Streptococcus pneumoniae</i> , <i>Staphylococcus aureus</i> , <i>Haemophilus influenzae</i> , <i>Moraxella catarrhalis</i> or	Outcome measures from samples collected from other parts of the body

	<i>Pseudomonas aeruginosa</i> recovered from the respiratory tract of both intervention and comparator groups OR Total and/or specific bacterial loads and/or alpha diversity and/or beta diversity and/or resistome and/or differentially abundant taxa information of the respiratory microbiome of both intervention and comparator groups	
		Animal studies, Cross-sectional studies Case studies/reports/series, conference abstracts Non-primary literature (including editorials, commentaries, clinical guidelines, reviews, dissertations) No access to abstract and/or full text Non-English articles with no available translations
Study design	Interventional and/or comparative studies (randomised controlled trials, cohort studies, case-control studies)	

2.2.5 Data Extraction and Synthesis

Data extraction and accuracy checks were conducted by REA and GN. The data extracted included: study characteristics (first author, publication year, study design, country, sample size, sample type, study aims and objectives), disease under study if any, outcome measures (prevalence, bacterial loads and percentage antibiotic resistance at baseline and endpoint in the case of specific bacterial species and measures of bacterial diversity (alpha and beta diversity), presence or absence of major bacterial taxa and their relative abundances in microbiome studies). REA and GN extracted the data and reviewed each other’s work for accuracy. For effective data synthesis, the articles were divided into multi-level categories. Firstly, all studies were separated into either a microbiome or non-microbiome group. The microbiome group comprised of studies that used next-generation sequencing. The non-microbiome group included articles that used bacterial culture or polymerase chain reaction (PCR). Secondly, the non-microbiome group was further divided into studies that administered macrolides for the short-term (≤ 14 days) or long-term (> 28 days). These two sub-group were further divided into those that administered azithromycin only (AZM), studies that investigated a single non-azithromycin macrolides such as clarithromycin, dirithromycin, erythromycin, josamycin, roxithromycin and telithromycin (Others), and/or studies that investigated more than one macrolide simultaneously (Multiple macrolides). In many studies with short-term macrolide administration, bacterial eradication at the end of treatment was used as a proxy for a reduction in prevalence. Meta-analysis and some secondary outcome analyses were not possible as there was substantial heterogeneity between eligible studies with regards to the outcomes of interest, case, and antibiotic exposure definitions.

2.3 Results

2.3.1 Results of the search and screening

A total of 6052 records were retrieved, with 4426 remaining after removing duplicates. Of these, 4056 were excluded at the title and abstract screening stage because of ineligibility, and the remaining 370 full-text articles were assessed for eligibility. After full-text review, 122 studies (110 used culture- and/or PCR-based [non-microbiome] and 12 used next-generation sequencing [microbiome-based]) met the inclusion criteria and were incorporated into the data synthesis. The non-microbiome studies comprised 65 studies that administered macrolides short-term (≤ 14 days) and 45 studies that administered macrolides long-term (> 28 days); no studies administered macrolides from 15 days to < 28 days. Three of the 12 microbiome-based studies also reported on specific bacterial carriage and resistance [21,33,34] and hence were also included in the non-microbiome-based long-term studies (totalling 48 studies). Figure 2.1 shows the PRISMA flowchart with the steps followed leading to the final subset of papers included in the analysis. Figure 2.2 shows the breakdown of the included studies.

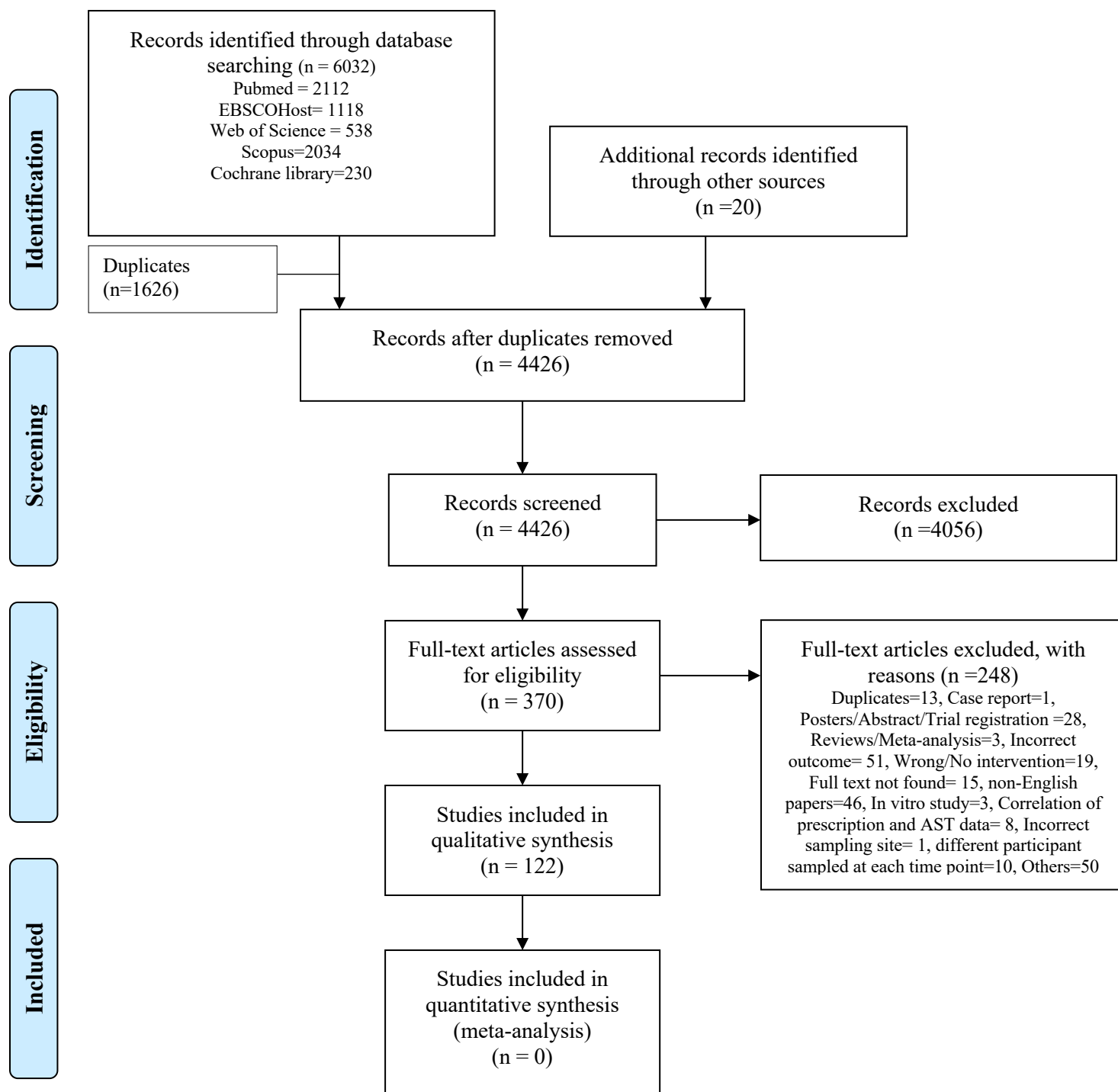


Figure 2. 1. PRISMA Flow-chart.

Flow diagram showing the selection of studies according to preferred reporting items for systematic reviews and meta-analysis (PRISMA) guidelines.

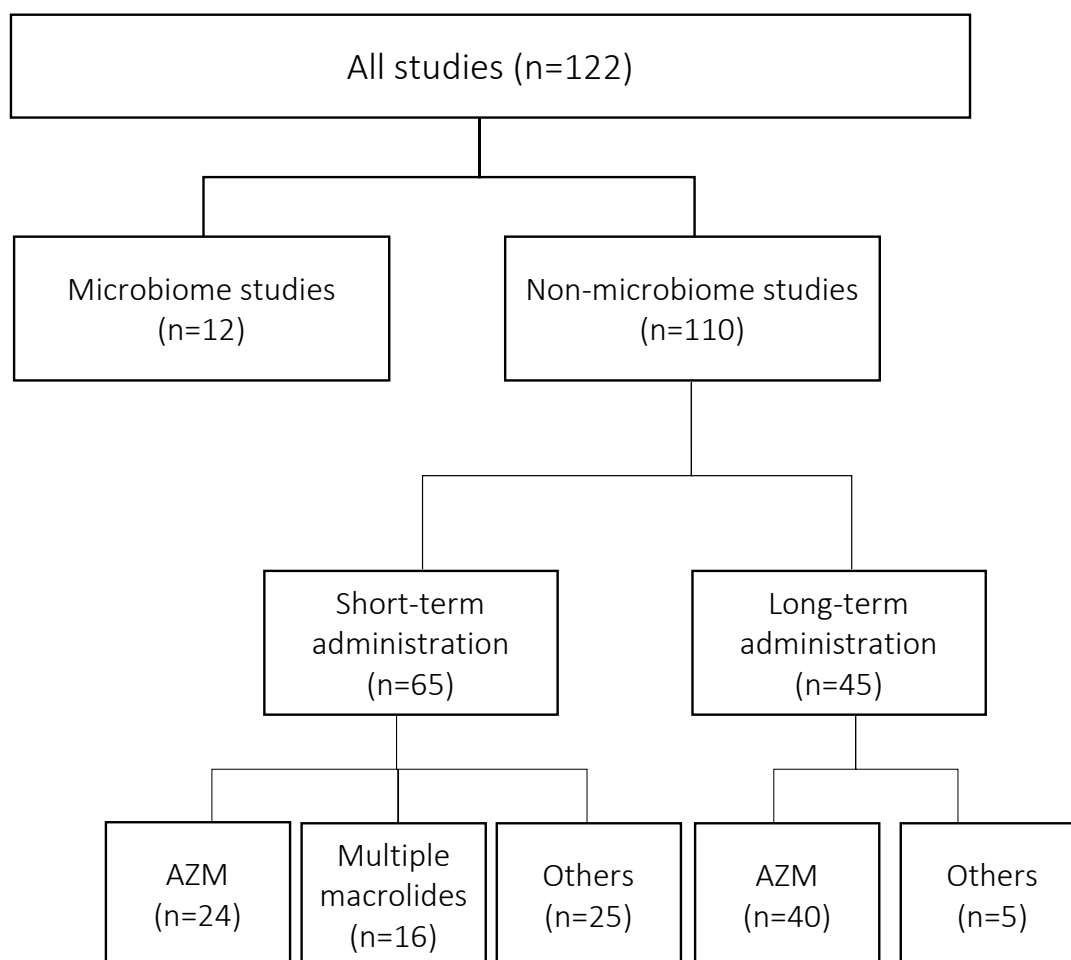


Figure 2. 2. Flow diagram showing the breakdown of the included studies.

Short-term administration refers to studies that administered macrolides for ≤ 14 days. Long-term administration refers to studies that administered macrolides for > 28 days. AZM = azithromycin. Multiple macrolides = studies that investigated more than one macrolide simultaneously. Others = studies that investigated a single non-azithromycin macrolides – clarithromycin, dirithromycin, erythromycin, josamycin, roxithromycin and telithromycin. Three of the microbiome studies also reported on specific bacterial prevalence and antibiotic resistance, but these were not included in the long-term non-microbiome studies in this figure.

2.3.2 Characteristics of included studies

Table 2.3 summarises the characteristics of all included studies. These studies were published from 1977 to 2021. Most of the studies included > 50 participants in the macrolide arm (68/122), only adults (79/122) and both sexes (121/122). The most common study designs were randomised, placebo-controlled clinical trials (44/122), randomised, drug comparative clinical trials (42/122), and non-randomised, comparative trials (10/122). Most of the studies were double-blinded (62/122), and about half were multicentred (60/122). Azithromycin was the most administered macrolide (74/122), followed by clarithromycin (15/122). Sputum was the most common sample type (56/122). One

hundred and seven (107/122) studies used culture to assess bacterial carriage, and 79/122 studies involved no post-intervention assessment.

Table 2. 3. Summary of study characteristics

Characteristics	Macrolide duration ≤ 14 days (Short-term) (n = 65)	Macrolide duration > 28 days (Long-term) [‡] (n = 45)	Microbiome studies (n = 12)
No. of participants (macrolide group only)			
≤ 50	27	29	12
51-100	19	10	0
101-600	19	12	0
Age groups			
Infants (≥ 1 year)	0	0	1
Children (1 to 12 years)	7	8	0
Adolescents (13 to 18 years)	0	0	0
Adults (≤ 18 years)	39	29	11
More than one category	19	11	0
Sex			
Male only	1	0	0
Female only	0	0	0
Both	64	48	12
Health and Disease state			
¹ Lower respiratory tract infections	40	0	0
² Upper respiratory tract infections	14	0	0
No respiratory tract infections/ “Healthy”	8	0	0
Both Upper and lower respiratory tract infections	3	0	0
Cystic fibrosis	0	14	1
Non-Cystic Fibrosis bronchiectasis	0	7	2
Asthma	0	4	3
Chronic Obstructive Pulmonary Disease	0	10	2
Other conditions	0	13	4
Study design			
Randomised, drug comparative clinical trial	35	7	0
Randomised, placebo-controlled clinical trial	6	29	9
Randomised, comparative and double dummy	7	1	0
Non-randomised, comparative trial	10	0	0
Non-randomised, non-comparative trial	5	0	0
Retrospective cohort	0	5	1
Retrospective case-control	0	1	0
Prospective cohort study	0	1	2
³ Others	2	4	0
Blinding			
Unblinded	33	13	3
Double-blinded	24	30	8
Single-blinded	7	5	1
Triple-blinded	1	0	0
*Number of centres			
Single centre	29	25	10
Multicentre	35	23	2
Type of Macrolide			
Azithromycin	24	41	9
Clarithromycin	13	1	1
Erythromycin	7	5	2

Roxithromycin	3	0	0
More than one macrolide	18	1	0
Sample types			
Sputum	34	18	4
Nasopharyngeal swabs	12	5	1
Oropharyngeal (throat) swabs	4	4	2
Nasal swabs	4	3	2
Multiple sample types	10	16	0
Bronchoalveolar lavage	0	1	3
Tracheal aspirate	1	1	0
Maxillary sinus aspirate	1	0	0
Sampling time points			
Baseline, end of treatment and post-treatment	38	7	1
Baseline and end of treatment	27	41	11
Last post-intervention assessment			
None	27	41	11
≥ 2 weeks	14	0	0
3 to 8 weeks	20	2	1
9 to 24 weeks	4	4	0
variable (4 weeks) to 52 weeks	0	1	0
Prevalence of bacteria and antibiotic resistance assessment			
Method of bacterial detection (prevalence)			
Culture	63	44	0
Culture and molecular methods (PCR)	1	1	1
Not determined	1	3	11
Method of antibiotic resistance detection			
Disk diffusion	16	7	0
Espilometric test	7	4	0
Dilution technique	7	4	0
PCR	0	4	1
Combination of methods	20	7	1
Not determined	15	22	10

Short-term refers to studies that administered macrolides for ≤ 14 days. Long-term refers to studies that administered macrolides for > 28 days. Microbiome refers to studies that used next generation 16S rDNA amplicon sequencing to characterise the respiratory microbiome. [¶]Three studies from the microbiome studies also reported on bacterial carriage and resistance hence the breakdown sums to 48 instead of 45. ¹This includes community-acquired pneumonia, acute exacerbations of chronic lung diseases such as chronic bronchitis, asthma, and COPD. ²This include otitis media, laryngitis, and tonsillitis. ³Includes open-label follow-up of a randomised placebo-controlled trial (1) and blinded, multidose, within-patient clinical comparison with an attendant placebo reference group. ^{*}Information for one short term study was unavailable.

The countries where the studies were conducted are shown in Figure 2.3A. Short-term studies (n = 65) were located in 30 countries, 23 of which were high-income, five were upper-middle-income, and two were lower-middle-income (World Bank 2021-2022 classification accessed on 10th January 2022; Figure 2.3B). Eleven studies included multinational sites. Only one African country was represented—South Africa (Figure 2.3B). Long-term studies were similarly mainly conducted in high-income countries, Figure 2.3C, with none of the studies conducted in Africa, South America or in a low-income setting. A number of studies investigated participants from the same cohort— BIS study (Valery [35] and Hare [36]); AMAZES study (Gibson [37], Taylor 2019 [21] and Shukla [38]); COLUMBUS study (Uzun [39], Djamin [40] and Djamin [41]); Saiman [42] and Saiman [43]; BLESS study (Taylor 2018 [44], Rogers [24] and Choo [34]) (Table 2.5).

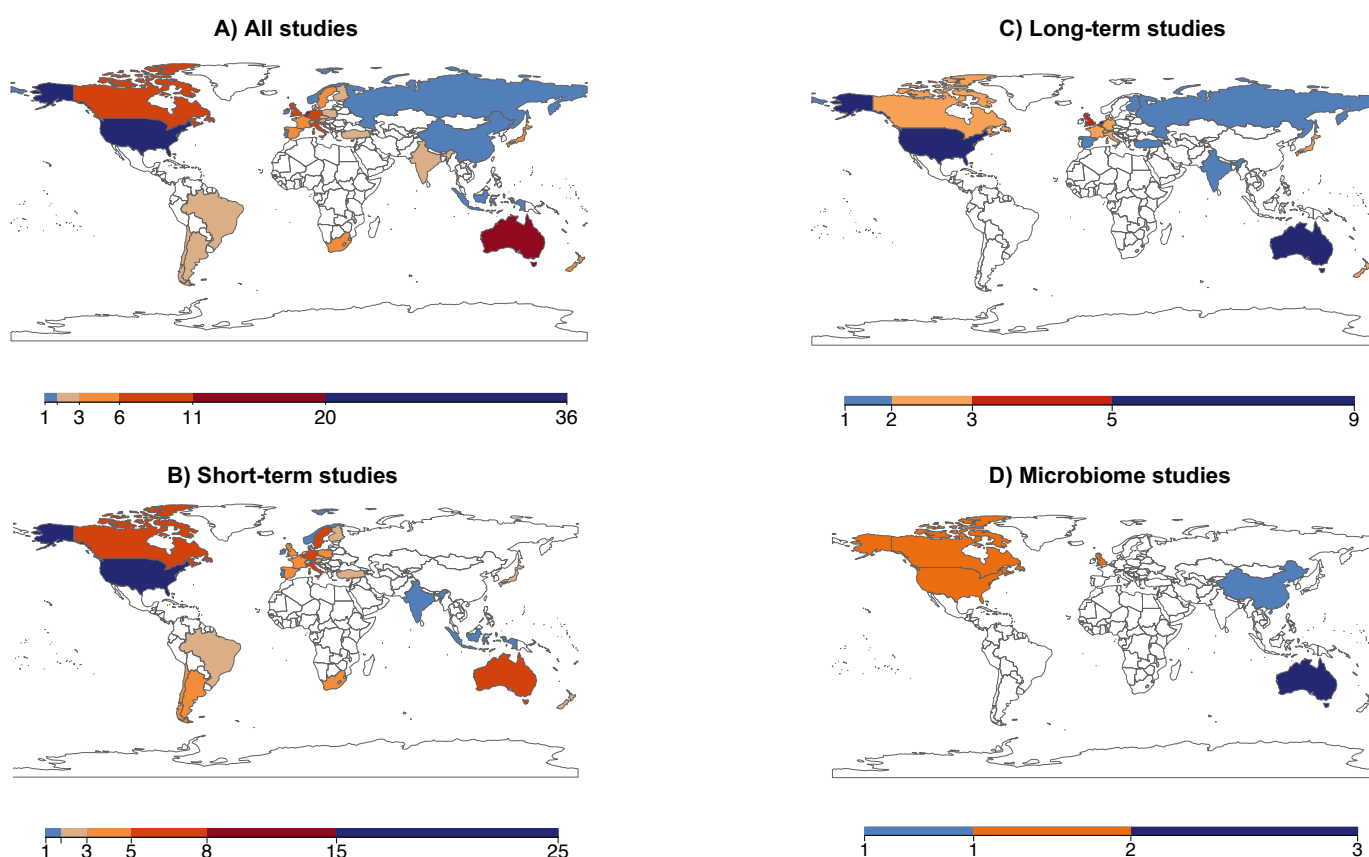


Figure 2. 3. World map showing the total number of studies that administered macrolides in each country.

All studies, those that administered macrolides for ≤ 14 days (short-term) and > 28 days (long-term) are shown in figures A, B and C, respectively. The number of studies per country that administered macrolides and characterised their effect on the respiratory microbiome using next-generation sequencing is shown in figure D.

Ten studies (10/122) screened for only *S. pneumoniae*, two studies (2/122) screened only *H. influenzae* and four (4/122) screened for only *S. aureus* (Table 2.4 and 2.5). All the other studies investigated more than one of the following bacteria –*S. pneumoniae*, *S. aureus*, *H. influenzae*, *M. catarrhalis* and *P. aeruginosa* (PA). Three long-term studies (3/48) screened for antibiotic resistance genes only. One long-term study (1/48) screened for both antibiotic resistance genes and bacterial species. None of the short-term studies evaluated antibiotic resistance genes. Among long-term studies, the shortest duration of macrolide treatment was 31 days and the longest seven years. Although more than half of short-term studies conducted post-intervention microbiological investigation (38/65, Table 2.4), most long-term studies (40/48) did not conduct this evaluation (Table 2.5). In most long-term studies (39/48), additional antibiotics were used throughout the study to manage acute respiratory exacerbations of COPD, asthma or cystic fibrosis in both arms of the trial. This was more common in

the placebo arm than the macrolide arm due to the reduced frequency of acute respiratory exacerbations in the latter.

All 12 studies that characterised the effect of macrolides on the respiratory microbiome (Table 2.6) were conducted in high-income countries in Europe, North America and Oceania and one upper-middle-income country, Figure 2.3D. Only one study assessed the persistence of macrolide effect on the respiratory microbiome after treatment (four weeks post-intervention). All the studies used 16S rDNA amplicon sequencing, however, the variable region sequenced differed (Table 2.7). All studies included clinically stable participants with no acute exacerbation at the time of enrolment, except one study that included infants having their first episode of respiratory syncytial virus bronchiolitis. Only two studies reported treatment compliance or adherence. Participants in nine studies were on concomitant corticosteroid treatment.

Table 2. 4. Studies that administered macrolides for short-term (≤ 14 days)

First author, year	Country	Study design	Disease of focus	Age group	Number of participants in macrolide group	Type of macrolide	Macrolide dosage and duration	Sample type	Sampling point-simplified	Bacteria screened*	Effect of macrolide on bacterial carriage	Effect of macrolide on resistance	Post-intervention follow-up microbiological investigation
Bacharier LB, 2015[45]	USA	Multicentre, double-blinded, randomised, placebo-controlled trial	Severe lower respiratory tract illnesses	Children	41	AZM	Oral, 12 mg/kg/day (with a maximum of 500mg/day), once daily for 5 days	Deep oropharyngeal swabs and nasal swabs/nasal secretions	Pre- and end of treatment	HI, HP, MC, SA, SP, PA	Not mentioned	SA was the common AZM-resistant bacterium but information not clear	Not conducted
Correa, 1996[46]	Chile	Single-centre, randomised, comparative trial	Acute Maxillary Sinusitis	Adolescents and Adults	15	AZM	Oral, 500mg, once daily for 3 days	Maxillary sinus aspirate	Pre-, end and post-treatment	SP, Haemophilus spp, Moraxella spp	Significant reduction in carriage or complete eradication of SP, HI, and MC	None reported	Significant reduction in carriage or complete eradication of SP, HI and MC sustained
Morris, 2010[47]	Australia	Multicentre, triple-blinded (Participant-blinded, care provider-blinded, assessor-blinded), randomised, comparative trial	Otitis media	Infants and Children	165	AZM	Oral, 30 mg/kg, once off	Nasal swabs	Pre- and end of treatment	SP and HI	Significant reduction in carriage of SP and HI but not different from control group	Increased in SP	Not conducted
Morita J, 2000[48]	USA	Single-centre, non-randomised, non-comparative trial	Invasive Group A streptococcal infections (GAS)	Children	300	AZM	Oral, 12 mg/kg/day, once off	Nasopharyngeal and oropharyngeal swabs	Pre-, end and post-treatment	group A streptococci spp and SP	Significant reduction in carriage of SP.	Significant increase in SP resistance	Significant reduction in carriage of SP and increase in resistance sustained at day 32
Ghaffar F, 2001[49]	USA	Single-centre, randomised, comparative trial	Acute otitis media	Children	68	AZM	Oral, 10mg/kg/day on the first day and 5mg/kg/day for the remaining 4 days	Nasopharyngeal specimens	Pre-, end and post-treatment	SP and HI	Significant reduction in carriage of SP and HI.	Increase in resistance in SP and HI	Significant reduction in carriage of SP and HI and increased in resistance observed at end of treatment sustained at post-treatment.
Leach AJ, 1997[50]	Australia	Single-centre, non-randomised, non-comparative trial	Trachoma	Children and adolescents	79	AZM	Oral, 20 mg/kg, once off	Nasopharyngeal swabs	Pre- and post-treatment	SP	Significant reduction in carriage of SP.	Significant increase in SP resistance	The carriage rate of SP at 6 months after AZM treatment was significantly higher than that before treatment.

McCallum 2013[51]	Australia	Dual-centre, double-blinded, randomised, placebo-controlled trial	Bronchiolitis	Children	50	AZM	Oral, 30 mg/kg, once off	Nasopharyngeal swabs	Pre- and end of treatment	SP, HI, MC, SA	Significant reduction in carriage of SP, SA, HI and MC	Not conducted	Not conducted
Olszewska-Sosinska 2016[52]	Poland	Single-centre, non-randomised, non-comparative trial	Adenotonsillar hypertrophy	Children	36	AZM	Oral, 20 mg/kg, once daily for 3 days	Nasopharyngeal swabs (one tonsillar swab and one adenoid swabs) and excised tissue	Pre-, end and post-treatment	HI	Reduction in the prevalence of HI.	None	The proportion of the persistent HI strains was similar in both groups
Ghaffar F, 2002[53]	USA	Single-centre, randomised, comparative, case control trial	Acute otitis media	Children	60	AZM	Oral, 10mg/kg/day on the first day and 5mg/kg/day for the remaining 4 days	Nasopharyngeal, oral, and anterior nares swabs	Pre-, end and post-treatment	SA, HI, SP	Significant reduction in carriage of SP and HI.	Increase in resistance in SP and HI	Significant reduction in carriage of SP and HI and increased in resistance observed at end of treatment sustained at post-treatment.
Ghaffar, 1999[54]	USA	Single-centre, randomised, comparative trial	Acute otitis media	Infants and Children	53	AZM	Oral, 10mg/kg/day on the first day and 5mg/kg/day for the remaining 4 days	Nasopharyngeal swabs	Pre-, end and post-treatment	alpha-haemolytic pneumococci	Significant reduction in carriage of SP.	increase in SP resistance	Significant reduction in carriage of SP and increase in resistance observed at end of therapy sustained at post-therapy.
Balmes P, 1991[55]	France	Multicentre, randomised, comparative trial	Acute bronchitis and acute exacerbations of chronic bronchitis (AEBC)	Adults	48	AZM	Oral, single dose of 500mg on day 1 followed by a single dose of 250mg on days 2–5	Sputum	Pre- and end of treatment	HI, HP, SP, SA, Streptococcus spp.	Eradication of baseline SP, SA, and HI.	None	Not conducted
Daniel, 1991[56]	Austria, Belgium, Denmark, France, Finland, The Netherlands, and Norway	Multicentre, randomised, comparative trial	Acute respiratory tract infections	Adults	218 AZM, 88 ERY	AZM	Oral, 250mg twice daily on day 1 followed by 250mg once daily for days 2-5 (AZM vs ERY) and Oral, 500mg once a day on day 1 followed by 250mg once daily for days 2-5 (AZM vs amoxicillin)	Sputum	Pre- and end of treatment	HI, SP, MC, and SA	Significant reduction in carriage or eradication of SP, SA, HI, and MC.	None reported	Not conducted
Dark, 1993[57]	USA	Multicentre, open, randomised, comparative trial	Lower respiratory	Adults	64	AZM	Oral, single dose of 500mg on day 1 followed by a	Sputum	Pre- and end of treatment	HI, SP, and MC	Significant reduction in carriage or	None reported	Not conducted

			tract infection and COPD			single dose of 250mg on days 2– 5				eradication of SP, HI, and MC.		
Davies, 1989[58]	The Netherlan ds	Single-centre, open, non- randomised, non- comparative trial	Acute exacerbation of chronic bronchitis	Adults	21	AZM Oral, single dose of 500mg on day 1 followed by a single dose of 250mg on days 2– 5	Sputum	Pre-, during, end and post- treatment	HI, PA, SA, SP, MC	Complete eradication of SP and MC during and after treatment. No effect on HI.	increased in MIC for HI isolates	reduction in prevalence of HI not seen at end of treatment. Insignificant increase in SP and MC reacquisition at 25 days post- treatment.
DeAbate, 2000[59]	USA	Multicentre, double-blinded, randomised, comparative trial	Acute exacerbation of chronic bronchitis	Adults	126	AZM Oral, single dose of 500mg on day 1 followed by a single dose of 250mg on days 2– 5	Sputum	Pre-, during, end and post- treatment	HI, SP, MC, SA, and HP	Significant reduction in carriage or complete eradication of SP, SA, HI, and MC	Increase resistance in SP and HI	The effectiveness of eradicating HI, HP and SP observed at the end of therapy decrease at post- treatment (i.e. 14- 21 days post- therapy)
Galova, 1996[60]	Slovak Republic	Multicentre, randomised, comparative trial	Community- acquired respiratory infections	Children	70	AZM Oral, 10mg/kg/day on the first day and 5mg/kg/day for the remaining 4 days	Sputum	Pre- and end of treatment	HI, SP, SA, and MC	Significant reduction in carriage or complete eradication of SP, SA, HI and MC	None reported	Not conducted
Gris, 1996[61]	Belgium	Multicentre, double-blinded, randomised, comparative trial	Acute bronchitis, acute infectious exacerbations of chronic bronchitis (AIECBs) or pneumonia	Adults	10	AZM Oral, 500mg, once daily for 3 days	Sputum	Pre- and end of treatment	SP, SA, HI, MC, and Moraxella spp.	None on SP, SA, HI, and MC	Not reported	Not conducted
Hoepelman, 1997[62]	The Netherlan ds	Multicentre, double-blinded, randomised, comparative trial	Acute exacerbation of chronic bronchitis	Adults	60	AZM Oral, 500mg, once daily for 3 days	Sputum	Pre- and end of treatment	HI, SP, MC, HP, SA, PA	Significant reduction in carriage of SP, HI, and MC.	None. Not reported for SA.	Not conducted
Shigeru K, 2014[63]	Japan	Multicentre, non- randomised, non- comparative trial	Community- acquired pneumonia	Adolesce nts and Adults	33	AZM Intravenous, 500mg once daily, followed by Oral, 500mg, once daily for 7 to 10 days	Sputum	Pre-, during, end and post- treatment	SP, HI, MC	Significant reduction in carriage of SP, HI, and MC.	No endpoint info	The suppressive effect of each of the antibiotic treatments lasts for the duration the study including posttreatment follow-up visits (days 15 and 29)
Uzun O, 1994[64]	Turkey	Single-centre, open, non- randomised, non- comparative trial	Community- acquired pneumococca l pneumonia	Adults	25	AZM Oral, single dose of 1000mg on day 1 followed by a single dose of	Sputum	Pre-, end and post- treatment	SP	Significant reduction in carriage of SP.	None reported	Significant reduction in carriage of SP and no resistance persisted.

						500mg on days 2–5							
Whitlock, 1995[65]	USA	Multicentre, investigator-blinded, randomised, comparative trial	Acute exacerbations of chronic obstructive pulmonary disease	Adults	21	AZM	Oral, single dose of 500mg on day 1 followed by a single dose of 250mg on days 2–5	Sputum	Pre-, during, end and post-treatment	SP, HI, MC, SA	Significant reduction in carriage or complete eradication of SP, SA, HI, and MC	None but not clearly reported	Recurrences at day 28 were observed in three AZM recipients
Yanagihara K, 2009[66]	Japan	Multicentre, open, non-randomised, non-comparative trial	Community-acquired pneumonia	Adults	59	AZM	Oral, 500mg, once daily for 3 days	Sputum	Pre-, end and post-treatment	SP, SA, MC, HI	Significant reduction in carriage of SP, HI, and MC.	Not provided for end of treatment	Not reported
Zachariah, 1996[67]	Great Britain and Ireland	Single-centre, double-blinded, randomised, comparative and placebo-controlled trial	Acute bronchitis, acute infectious exacerbations of chronic bronchitis (AIECBs), or community-acquired pneumonia,	Adolescents and Adults	82	AZM	Oral, 500mg, once daily for 3 days	Sputum	Pre- and end of treatment	SP, HI, MC, SA	Complete eradication of SP, SA, HI, and MC.	None	Not conducted
Zervos M, 2007[68]	United States of America and European countries (specific countries not mentioned)	Multicentre, third-party blinded, randomised, comparative trial	Acute exacerbations of chronic bronchitis	Adults	46	AZM	Oral, 500mg, once daily for 3 days	Sputum	Pre-, end and post-treatment	SP, HI, MC, HP	Significant reduction in carriage or complete eradication of SP, HI, and MC	None on SP, HI or MC	Significant reduction in carriage or complete eradication of SP, HI sustained at post-therapy. The prevalence of MC started increasing post-end of therapy in AZM.
Malhotra-Kumar, 2007[69]	Belgium	Single-centre, double-blinded, randomised, comparative and placebo-controlled trial	None	Adults	148	AZM, CLA	Oral, 500mg, once daily for 3 (AZM) days and Oral, 500mg twice daily for 7(CLA) days	Pharyngeal swabs	Pre-, end and post-treatment	Streptococci	Not reported	Significant increase in SP resistance, higher in AZM than CLA. Selection of resistance genes.	Increased resistance persisted post therapy (day 180)
Rizzato G, 1995[70]	Italy	Single-centre, open, randomised, comparative trial	Community-acquired pneumonia	Adults	40	AZM, CLA	Oral, 500mg, once daily for 3 (AZM) days and Oral, 250mg twice daily for ≥ 8(CLA) days	Sputum	Pre- and end of treatment	HI, HP, and Pneumococcus sp.	No endpoint information	None	Not conducted
Soepandi, 1998[71]	Indonesia	Single-centre, open, randomised, comparative trial	Acute lower respiratory tract infections	Adults	34	AZM, CLA	Oral, 500mg, once daily for 3 (AZM) days and Oral, 500mg twice daily for 10(CLA) days	Sputum	Pre- and end of treatment	SP, SA	Significant reduction in carriage of SP and SA.	None	Not conducted
Swanson, 2005[72]	US, Argentina, Brazil, Costa	Multicentre, double-blinded, randomised, comparative and	Acute exacerbations of chronic bronchitis.	Adults	116	AZM, CLA	Oral, 500mg, once daily for 3 (AZM) days and Oral,	Sputum	Pre-, end and post-treatment	HI, SP and MC	Significant reduction in carriage of SP, HI, and MC.	Not reported (few isolates at end of treatment to be detected)	Significant reduction in carriage of SP, HI

	Rica, India, Chile, Canada, and South Africa	placebo-controlled trial					500mg twice daily for 10(CLA) days							and MC sustained at post-treatment.
King, 2002[73]	England	Multicentre, open-labelled to GP, lab investigator-blinded, randomised, comparative trial	Respiratory tract infections	Adults	102	AZM, CLA	Oral, 500mg, once daily for 3 (AZM) days and Oral, 500mg twice daily for 7(CLA) days	Throat swabs	Pre-, end and post-treatment	SP, HI, MC, SA	Increase in the number of all bacterial isolates at end of treatment	Although resistance increased in both macrolides, baseline resistance was already high	Decrease in number of all bacterial isolates after 3 and 5- 6 weeks and decrease in the number with <i>MefA/E</i> and <i>ermA/M</i> genes at 3 and 5-6 weeks after treatment	
Cazzola M, 1999[74]	Italy	Single-centre, single-blinded, randomised, comparative trial	Acute bacterial exacerbations of chronic bronchitis (ABECB)	Adults	80	AZM, DIR	Oral, 500mg, once daily for 3 (AZM) and 5 (DIR) days	Sputum	Pre-, end and post-treatment	SP, SA, MC, HI, HP,	Significant reduction in carriage of SP, SA, HI, and MC compared to baseline.	None reported	the frequency of colonisation remained lower than at baseline at post and late-post therapy	
Laurent K, 1996[75]	Argentina, Austria, Belgium, and Italy	Multicentre, open, randomised, comparative trial	Acute lower respiratory tract infections	Adults	81	AZM, ROX	Oral, 500mg, once daily for 3 (AZM) days and Oral, 300mg twice daily for 10(Roxithromycin) days	Sputum	Pre-, during and end of treatment	HI, HP, SP, MC, and SA.	Significant reduction in carriage of SP, SA, HI, and MC	None	Not conducted	
Muller, 1996[76]	Germany	Multicentre, open, randomised, comparative trial	Acute upper respiratory tract infections, including otitis media, sinusitis, and pharyngitis/tonsillitis	Adults	431	AZM, ROX	Oral, 500mg, once daily for 3 (AZM) days and Oral, 150mg twice daily for 10(Roxithromycin) days	Throat or nasopharyngeal swabs or samples of exudate	Pre-, end and post-treatment	SP, HI, MC, SA	Significant reduction in carriage of SP, SA, and MC	None	Significant reduction in carriage of SP, SA, and MC sustained	
Brook I, 2006[77]	USA	Single-centre, non-randomised, comparative trial	Acute maxillary sinusitis	Adults	57	AZM, TEL	Oral, 500mg, once daily for 3 days for AZM, Oral, 800mg, once daily for 5 days for TEL	Nasopharyngeal swabs	Pre- and end of treatment	SP, SA, MC, HI	Significant reduction in carriage or eradication of SP, SA, HI, and MC in both groups but TEL was superior.	Non-significant increase in SP resistance	Not conducted	
Mueller O, 1993[78]	Multiple European settings (specific countries not mentioned)	Multicentre, randomised, comparative trial	Acute upper respiratory tract infections (i.e., otitis media, sinusitis,	Adolescents and Adults	357	AZM, CLA	Oral, 500mg, once daily for 3 (AZM) days and Oral, 250mg once daily for 7(CLA) days	Throat or nasopharyngeal swabs or exudates	Pre- and end of treatment	SA, HI, SP, HP, MC	Significant reduction in carriage of SP, SA, HI, and MC	One SA resistant in AZM, none in CLA group	Not conducted	

			pharyngitis or tonsillitis)										
Matute A, 2002[79]	The Netherlands	Single-centre, double-blinded, randomised, comparative and placebo-controlled trial	None	Adults	12	AZM, CLA	Oral, 500mg, once daily for 3 (AZM) days and Oral, 1000mg twice daily for 7(CLA) days	Throat washes	Pre-, end and post-treatment	Streptococcus spp.	None effect on bacterial carriage	No effect on SA or SP or any bacterial	None effect on bacterial carriage or resistance post-therapy
Kastner 2001[80]	Germany	Multicentre, open, randomised, comparative trial	Upper and Lower respiratory tract infections	Infants, Children	197	AZM, ERY, CLA, ROX, josamycin	Oral, ERY (40 mg/kg, in three daily doses), roxithromycin (8 mg/kg, once daily) and josamycin (40 mg/kg, in three daily doses, CLA (15 mg/kg, in two daily doses) and AZM (10 mg/kg, once daily). All for three days	Throat swabs	Pre-, end and post-treatment	Staphylococci, Streptococci	Not clear	Increase resistance in SP and SA	Increased resistance persisted in the AZM group but reduced in the other groups at 6 weeks.
Neu HC, 1993[81]	USA	Multicentre, double-blinded, randomised, comparative trial	Community acquired lower respiratory tract infections (acute bacterial exacerbation of chronic bronchitis or asthmatic bronchitis and bacterial pneumonia)	Adolescents and Adults	76	CLA	Oral, 500mg, thrice daily for 7 to 14 days	Bronchopulmonary secretions	Pre-, end and post-treatment	SP, HI, MC	Complete eradication of SP and MC. Significant reduction in carriage of HI.	None	Observation at end of treatment sustained.
Hopkins, 1999[82]	USA and Canada	Multicentre, double-blinded, randomised, comparative trial	Community-acquired pneumonia	Adolescents and Adults	156	CLA	Oral, 500mg, twice daily for 7 to 10 days	Freshly expectorated or induced sputum specimens or samples of lower respiratory tract secretions	Pre-, end and post-treatment	SP, HI, MC, SA	Significant reduction in carriage or complete eradication of SP, SA, HI, and MC	Few bacterial isolates at end of treatment and resistance information not reported.	Few bacterial isolates at end of treatment and resistance information not reported.
Sundberg, 1994[83]	Sweden	Single-centre, open, non-randomised, non-comparative trial	Otitis media	Children and adolescents	30	CLA	Oral, 7-5 mg/kg twice daily for 7days	Nasopharyngeal swabs	Pre-, during and end of treatment	SP, HI, MC, SA	Complete eradication of SP, and MC after 3 days of treatment. Significant reduction a in carriage and load of HI.	Non-significant increase in HI resistance (only one isolate). None in the others	Not conducted

Anzueto A, 1997[84]	USA	Multicentre, investigator-blinded, randomised, comparative trial	Acute bacterial exacerbations of chronic bronchitis (ABECB)	Adults	365	CLA	Oral, 500mg, once daily for 10 days	Sputum	Pre- and end of treatment	HI, HP, MC, SA, SP	Significant reduction in carriage or eradication of SP, SA, HI, and MC.	None reported	Not conducted
Anzueto, 1998[85]	USA	Multicentre, investigator-blinded, randomised, comparative trial	Chronic bronchitis, COPD, or severe/complicated AECB	Adults	328	CLA	Oral, 500mg, twice daily for 10 days	Sputum	Pre-, during, end and post-treatment	HI, HP, MC, SA, SP, PA	Significant reduction in carriage or eradication of SP, SA, HI, MC, and PA.	increase in resistance to penicillin and CLA but not timepoint of isolates not clearly stated	At 3-5 weeks post-therapy, bacterial eradication remained high (96%)
DeAbate CA, 1999[86]	USA	Multicentre, double-blinded, randomised, comparative trial	Acute bacterial exacerbations of chronic bronchitis	Adults	125	CLA	Oral, 500mg, twice daily for 10 days	Sputum	Pre-, during, end and post-treatment	SP, SA, MC, HI, HP	Significant reduction in carriage of SP, SA, HI, and MC.	No significant effect	Significant reduction in carriage of SP, SA, HI, and MC sustained at post-treatment
Guay DRP, 1993[87]	USA	Multicentre (pooled), double-blinded, randomised, comparative trial	Asthmatic bronchitis or acute bacterial exacerbations of chronic bronchitis and chronic bronchial asthma	Adults	60	CLA	Oral, 500mg, twice daily for 14 days	Sputum	Pre- and end of treatment	HI, SA, SP, HP	Significant reduction in carriage of HI.	Not clear	Not conducted
Moola, 1999[88]	Australia, Canada, Czech Republic, Germany, Italy, Israel, New Zealand, Poland, South Africa, Spain, and Sweden	Multicentre, double-blinded, randomised, comparative and placebo-controlled trial	Community-acquired pneumonia	Adults	47	CLA	Oral, 500mg, twice daily for 10 days	Sputum	Pre-, during, end and post-treatment	SP, HI, MC, SA	Significant reduction in carriage of SP, SA, HI, and MC	None reported	Significant reduction in carriage of SP, SA, HI, and MC was sustained.
Notario G, 1993[89]	USA	Multicentre, double-blinded, randomised, comparative trial	Bacterial bronchitis (asthmatic bronchitis or acute bacterial exacerbation of chronic bronchitis in all studies, and chronic bronchial	Adolescents and Adults	192	CLA	Oral, 500mg, twice daily for 14 days	Sputum	Pre- and end of treatment	HI, HP, SP, MC, SA	Significant reduction in carriage of SP, SA, HI, and MC	Timepoint for resistance of SP, SA, HI not mentioned. No effect on MC.	Not conducted

Study														Outcome
Author, Year [Ref]	Country	Study Design	Condition	Population	n	Intervention	Comparator	Sample	Timing	Pathogen	Primary Outcome	Secondary Outcome	Notes	
Tran JQ, 2000[90]	USA	Single-centre, open, randomised, comparative trial	Chronic bronchitis	Adults	10	CLA	Oral, 500mg, once daily for 10 days	Sputum	Pre-, during, end and post-treatment	Haemophilus spp., MC, SP	Significant reduction in carriage of HI and MC.	Not provided		The suppressive effect of each of the antibiotic treatments lasts for the duration the study
Langan, 1999[91]	Belgium, Canada, Czech Republic, France, Germany, Poland, Portugal, Spain, and the UK	Multicentre, double-blinded, randomised, comparative trial	Acute bacterial exacerbations of chronic bronchitis (ABECB)	Adults	104	CLA	Oral, 500mg, twice daily for 10 days	Sputum (expectorated)	Pre-, end and post-treatment	HI, HP, SP, MC, and SA.	Significant reduction in carriage of SP, SA, HI, and MC	None for HI and MC. No endpoint info for SP		Significant reduction in carriage of SP, SA, HI, and MC was sustained.
Berg, 2004[92]	The Netherlands	Single-centre, double-blinded, randomised, placebo-controlled trial	Patients scheduled for a coronary artery bypass graft surgery	Adults	148	CLA	Oral, 500mg, once daily for 14 days	Throat and nose	Pre-, end and post-treatment	SA (nasal); SP, Haemophilus spp, MC (throat swabs)	Significant reduction in carriage SA. No effect on HP. SP and MC prevalence were low at baseline and throughout study hence effect of treatment not clear	Significant increase in HP resistance. Significant rise in macrolide resistance in overall oral flora. Significant rise in streptococci <i>ermB</i> and <i>staphylococci ermC</i> . More than 90% of the macrolide-resistant streptococci contained both macrolide resistance genes and Tn916/ Tn1545 fragments.		Increase in oral flora overall macrolide resistance and prevalence of <i>ermB</i> and <i>ermC</i> genes persisted 8 weeks post-treatment.
Yildirim I, 2008[93]	Turkey	Single-centre, open, randomised, comparative trial	Group A beta-haemolytic streptococcal tonsilitis	Children and adolescents	68	CLA	Oral, 20 mg/kg/day, twice daily for 10 days	Throat specimens	Pre- and end of treatment	group A beta- and alpha haemolytic streptococci, SA	Significant increase in carriage of SA.	Only data on beta lactamase production for SA isolates was provided		Not conducted
Cazzola, 1997[94]	Italy	Single-centre, open, non-randomised, non-comparative trial	Acute exacerbation of severe chronic obstructive pulmonary disease	Adults	20	DIR	Oral, 500mg, once daily for 5 days	Respiratory secretions	Pre-, end and post-treatment	SP, SA, HI, MC, PA	Reduction in the prevalence of HI. Details not clear for the other SP, SA, MC, and PA.	None reported		Bacterial colonisation elimination was sustained for the duration of the study including post-therapy
Palva T, 1986[95]	Finland	Single-centre, non-randomised, non-comparative trial	Chronic secretory otitis media	Children and adolescents	78	ERY	Oral, 50mg/kg, twice daily for 7 to 8 days	Adenoid tissue and nasopharyngeal swabs	Pre-, end and post-treatment	SA, SP, HI, MC	Significant reduction in carriage of SP and MC.	None		The suppression of bacterial colonisation, especially M catarrhalis and S pneumoniae,

													observed at surgery from baseline, was mostly reverted one month after surgery to baseline levels
Lipsky, BA 1992[96]	USA	Single-centre, non-randomised, comparative trial	Skin and soft tissue infections	Adults	16	ERY	Oral, 400mg, four times daily for 10 days	Nasal swabs	Pre-, end and post-treatment	SA	Significant reduction in carriage of SA.	Not conducted	ERY eradication of SA did not persist at < 20 days follow up.
Trollfors B, 1978[97]	Sweden	Multicentre, non-randomised, comparative trial	Whooping cough/pertussis	Infants, Children, Adolescents, Adults	76	ERY	Oral, 40–50mg/kg, thrice daily for 10 days	Nasopharyngeal	Pre-, during, end and post-treatment	MC, HI, SA	Significant reduction in carriage of HI and MC.	None reported	The suppressive effect of each of the antibiotic treatments lasts for the duration the study
Schalen, 1992[98]	Sweden	Single-centre, double-blinded, randomised, placebo-controlled trial	Laryngitis	Adults	48	ERY	Oral, 500mg, twice daily for 5 days	Nasopharyngeal swabs	Pre-, end and post-treatment	HI, MC	Significant reduction in carriage of MC. No effect on HI.	None	After two weeks the respective isolation rates did not differ
Schalen, 1993[99]	Sweden	Single-centre, double-blinded, randomised, placebo-controlled trial	Acute laryngitis	Adults	48	ERY	Oral, 500mg, twice daily for 5 days	Nasopharyngeal swabs	Pre-, end and post-treatment	SP, HI, MC	Significant reduction in carriage of SP, HI, and MC.	None	There was no difference between the two groups in the recovery rate of M catarrhalis after 2 weeks
Darelid, 1993[100]	Sweden	Multicentre, open, randomised, no-treatment control trial	Cough persisting for more than 10 days	Infants and Children	41	ERY	Oral, 50mg/kg, once daily for 7 days	Nasopharyngeal swabs	Pre- and end of treatment	MC, SP, HI	Significant reduction in carriage or eradication of SP, HI, and MC.	None reported	Not conducted
Butzler JP, 1979[101]	Belgium	Non-randomised, comparative trial	Upper and lower respiratory tract infections	Adults	76	ERY	Oral, 1–2g of ERY stearate 2–4 times/day and 1g of ERY ethylsuccinate, 4 times/day	Sputum	Pre- and end of treatment	SP and HI	Significant reduction in carriage or eradication of SP and HI in both groups. ERY stearate did not affect HI levels.	None reported	Not conducted
Wasilewski 1999[102]	USA	Multicentre, double-blinded, randomised, comparative and placebo-controlled trial	Acute exacerbations of chronic bronchitis.	Adolescents and Adults	444	ERY and DIR	Oral, 250mg, four times daily for 7 (ERY) days and Oral, 500mg once daily for 5(DIR) days	Sputum	Pre-, during, end and post-treatment	HI, SP, MC, SA, HP, and streptococcus group A	Significant reduction in carriage of SP, SA, HI and MC in both groups	Not reported	Significant reduction in carriage of SP, SA, HI, and MC in both groups observed at end of treatment was sustained
Wilson, 1977[103]	USA	Single-centre, double-blinded, randomised, comparative and placebo-controlled trial	SA carriage	Adults	48	ERY and Josamicin	Oral, 1g of ERY or 1.5g Josamicin four times daily for 7 days	Nasal swabs	Pre-, during and end of treatment	SA	Significant reduction in carriage rates and total counts of SA by both macrolides.	No effect on SA	Not conducted

Wilson, 1979[104]	USA	Single-centre, double-blinded, randomised, comparative and placebo-controlled trial	SA carriage	Adults	87	ERY and ROX	Oral, 1g of ERY or 1.5g rosamycin four times daily for 7 days	Nasal swabs	Pre-, during, end and post-treatment	SA	Significant reduction in carriage of SA.	No effect on SA	Levels or counts of SA rebound to placebo levels 29 days after therapy
Karalus NC, 1994[105]	Australia and New Zealand	Multicentre, investigator-blinded, randomised, comparative trial	Lower respiratory tract infections	Adolescents and Adults	41	ROX	Oral, 150mg, twice daily for 7 to 14 days	Sputum	Pre- and end of treatment	SP, MC, HI	No effect on SP, reduction in the carriage of HI and MC	Few bacterial isolates at end of treatment and resistance information not reported.	Not conducted
Salvarezza CR, 1998[106]	Argentina	Multicentre, open, randomised, comparative trial	Community-acquired pneumonia	Adults	30	ROX	Oral, 300mg, once daily for 8 to 10 days	Sputum	Pre- and end of treatment	SP, SA, MC, HI, PA	No endpoint information	None	Not conducted
Zeluff, 1988[107]	South Africa	Single-centre, double-blinded, randomised, comparative trial	Acute pneumococcal pneumonia	Adults	46	ROX	Oral, 150mg, twice daily for 7 to 14 days	Sputum	Pre- and end of treatment	SP	Significant reduction in carriage of SP.	None	Not conducted
Brook, 2005[108]	USA	Single-centre, non-randomised, comparative trial	Acute maxillary sinusitis	Adults	25	TEL	Oral, 800mg, once daily for 5 days	Nasopharyngeal swabs	Pre- and end of treatment	alpha-haemolytic streptococci, SP, HI, and MC	Significant reduction in carriage of SP, HI, and MC.	None	Not conducted
Tellier G, 2004[109]	Argentina, Brazil, Canada, Chile, Germany, South Africa, Spain, UK, and USA	Multicentre, double-blinded, randomised, comparative trial	Community-acquired pneumonia	Adults	119	TEL, CLA	Oral, 800mg, once daily for 5 and 10 (TEL) days and Oral, 500mg twice daily for 10 (CLA) days	Sputum	Pre-, during, end and post-treatment	SP, MC, and HI	Significant reduction in carriage of SP, HI, and MC.	None reported	Significant reduction in carriage of SP, HI and MC persisted

*Bacteria of interest screened for: HI = *Haemophilus influenzae*, HP = *Haemophilus parainfluenzae*, MC = *Moraxella catarrhalis*, SA = *Staphylococcus aureus*, SP = *Streptococcus pneumoniae*
 AZM = Azithromycin, ERY= Erythromycin, CLA = Clarithromycin, DIR = Dirithromycin, ROX = Roxithromycin, TEL = Telithromycin

Table 2. 5. Studies that administered macrolides for long-term (> 28 days)

First author, year, Study name, reference	Country	Study Design	Disease of focus	Age group	Number of participants	Type of macrolide	Macrolide dosage and duration	Sample type	Sampling time points	Bacteria or genes screened for	Effect of macrolide on bacterial carriage	Effect of macrolide on resistance	Post-intervention follow-up microbiological investigation
van Heck,2019[110]	Belgium	Single-centre, double-blind, placebo-controlled, randomised	Chronic lung allograft dysfunction	Adults	68 AZM, 120 placebo	AZM	Oral, 1000mg loading dose and 250mg maintenance dose every other day for 31 days	Bronchoalveolar lavage	Pre-, end and post-treatment	SA, SP, HI, MC, PA	Significant reduction in positive cultures in AZM compared to placebo at 3 months.	Not investigated	No difference between groups at 6-month post-intervention follow up
Valery 2013, BIS[35]	Australia, New Zealand	Multicentre, double-blind, placebo-controlled, randomised	Non-cystic-fibrosis bronchiectasis or chronic suppurative lung disease	Children	45 AZM, 44 placebo	AZM	Oral, 30 mg/kg; maximum 600 mg once weekly for 24 months	Deep nasal swabs	Pre- and end of treatment	SA, SP, HI, MC	Lower carriage of HI and MC in the AZM group compared with placebo. No effect on SP and SA	Significant increase in AZM-resistant SA and SP in AZM group compared to placebo	Not conducted
Albert, 2011[14]	USA	Multicentre, double-blind, placebo-controlled, randomised	COPD	Adults	495 AZM, 502 placebo	AZM	Oral, 250mg, daily for 12 months	Deep nasopharyngeal swabs	Pre-, during and end of treatment	SA, SP, HI, MC	MC prevalence reduced in the AZM group. No difference between arms at the end of treatment for SA, SP, HI.	AZM resistance in SA, SP and MC increased in the AZM group compared to placebo. The incidence of AZM resistance bacteria was also higher in the AZM group.	Not conducted
Maniakas , 2021[111]	Canada	Single-centre, double-blind, placebo-controlled, randomised	Refractory chronic rhinosinusitis	Adults	24 AZM, 24 placebo	AZM	AZM, Oral 250 mg thrice weekly for 16 weeks	ethmoid bulla swabs	Pre- and end of treatment	SA, SP, HI, MC, PA	Lower prevalence of SA, PA, and HI at end of treatment	Reduced	Not conducted
Shukla, 2021, AMAZE S[38]	Australia	Multicentre, double-blind, placebo-controlled, randomised, parallel-group	Asthma	Adults	109 AZM, 103 placebo	AZM	AZM, Oral 500 mg thrice weekly for 48 weeks	Induced sputum	Pre- and end of treatment	HI	AZM resulted in a significant reduction in sputum HI	Reduced	Not conducted
** Taylor 2019, (AMAZE S)[21]	Australia	Multicentre, randomised, double-blind, placebo-controlled trial	Asthma	Adults	27 AZM, 34 placebo	AZM	Oral, 500mg, thrice weekly for 48 weeks	Induced sputum	Pre- and end of treatment	SA, SP, HI, MC, PA, <i>tetW</i> , <i>mel</i> , <i>msrE</i> , <i>tetM</i> , <i>ermF</i> , <i>mefA</i> , and <i>ermB</i>	Significant reduction in total bacteria isolates at end of treatment	Increase in macrolide and tetracycline resistance genes	Not conducted
Videler, 2011, MACS[112]	Netherlands, Finland, Belgium, UK, Croatia	Multicentre, double-blind, placebo-controlled, randomised	Chronic rhinosinusitis	Adults	29 AZM, 31 placebo	AZM	Oral, 500mg for 3 days in the first week, followed by 500 mg per week for the next 11 weeks	Nasal swabs	Pre- and end of treatment	SA, SP, HI, MC, PA	AZM caused a significant decrease in SP and a non-significant reduction of SA and HI carriage at the end of treatment.	Resistance was negligible and did not differ in SA and SP between groups. Resistance was not	Not conducted

													detected in any of the other bacteria
Gray GC, 1998[113]	USA	Prospective, (investigator) single-blinded, randomised, comparative, parallel group trial	Prophylaxis against acute respiratory disease	Adults	366 AZM, 366 Benzathine and 370 no treatment	AZM	Oral, 500mg once weekly 9 weeks	Nasopharyngeal (aspirate or swab where the aspirate was inadequate) and throat	Pre- and end of treatment	SP	AZM was not protective against SP colonisation compared to the no treatment group	No resistance isolates of SP recovered	Not conducted
Hare 2015, BIS[36]	Australia, New Zealand	Multicentre, double-blind, placebo-controlled, randomised	Bronchiectasis	Children	21 AZM, 17 placebo (Australian), 20 AZM, 20 placebo (New Zealand)	AZM	Oral, 30 mg/kg; maximum 600 mg once weekly for 24 months	Nasopharyngeal swabs	Pre-, during, end and post-treatment	SA, SP, HI, MC	End of treatment, carriage of HI and MC was substantially lower in the AZM group compared to placebo.	Significant increase in AZM-resistant SA and SP in AZM group compared to placebo	No more difference between groups at post-intervention follow up
Marchiso, 1996[114]	Italy	Single-blind, comparative, randomized study	Recurrent Acute Otitis Media	Infants and Children	75 AZM (10mg/kg/week), 9 AZM (5mg/kg/week), 75 Amoxicillin	AZM	Oral 5 or 10mg/kg depending on group, once weekly for 6 months	Nasopharyngeal swabs	Pre- and end of treatment	SP, HI, MC	No effect on bacterial prevalence in any group.	No effect on resistance in any group.	Not conducted
Guchev, 2004[115]	Russia	Open-label, prospective, comparative, multi-arm, randomised, clinical trial.	Prophylaxis for community-acquired pneumonia	Children, adolescents, and adults	548 long-term AZM, 507 once off AZM and 678 no treatment	AZM	Oral, 500mg once weekly 8 weeks 0r 1500mg once off	Nasopharyngeal and throat swabs	Pre-, end and post-treatment	SP	No difference between the groups	A dramatic increase in the prevalence of macrolide and lincosamide resistance at end of treatment in both AZM groups	Prevalence of macrolide and lincosamide resistance at end of treatment in both AZM groups remained high at week 21
Brusselle, 2013, AZISAS T[116]	Belgium	Multicentre, double-blind, placebo-controlled, randomised	Asthma	Adults	23 AZM, 23 placebo	AZM	Oral, 250mg daily for 5 days and then 250mg thrice weekly for 26 weeks.	Oropharyngeal swabs	Pre-, during, end and post-treatment	Streptococci	Not reported	Significant increase in macrolide-resistant streptococci in AZM arm compared to placebo and baseline samples from AZM arm	AZM-resistant <i>streptococci</i> decreased after the 4-week washout period but remained higher in AZM compared to placebo group.
Putnam, 2000[117]	USA	Prospective, multi-arm, randomised, chemoprophylaxis	Prophylaxis for Streptococcal infection	Adults	362 AZM, 374 Benzathine penicillin and 372 no treatment	AZM	Oral, 500mg once weekly for 63 days	Pharyngeal swabs	Pre- and end of treatment	SP	There was no difference between groups in terms of SP carriage at end of treatment	There was no difference between groups in terms of resistance at end of treatment	Not conducted

		(comparative) trial											
Altenburg, 2013, BAT[118]	Netherlands	Multi-centre, double-blind, placebo-controlled, randomised	Non-cystic bronchiectasis	Adults	43 AZM, 40 placebo	AZM	Oral, 250 mg daily for 12 months	Sputum	Pre-, during and end of treatment	SA, SP, HI, MC, PA	No difference between arms at the end of treatment	Significant increase in AZM-resistant SP, HI, MC, and SA in AZM group compared to placebo during treatment and exacerbations.	Not conducted
Berkhof, 2013[119]	Netherlands	Single-centre, double-blind, placebo-controlled, randomised, parallel-group	COPD and chronic cough	Adults	42 AZM and 42 placebo	AZM	Oral, 250 mg thrice weekly for 12 weeks	Sputum	Pre- and end of treatment	SA, SP, HI, MC, PA	Reduction of bacterial carriage in AZM group at the end of treatment.	One patient with AZM-resistant HI in AZM arm at the end of treatment	Not conducted
**Brill, 2015[33]	UK	Single-centre, single-blind, placebo-controlled, randomised	COPD	Adults	25 AZM, 24 placebo	AZM	Oral, 250 mg thrice weekly for 13 weeks	Sputum	Pre- and end of treatment	SA, SP, HI, MC, PA	No difference between arms at the end of treatment	AZM was associated with a significant increase in the MIC of isolates cultured in sputum of AZM patients compared with placebo	Not conducted
Diego, 2013[120]	Spain	Open-label, single-centre, single-blind, randomised	Non-cystic bronchiectasis	Adults	16 AZM, 14 placebo	AZM	Oral, 250 mg thrice weekly for 3 months	Sputum	Pre- and end of treatment	SA, SP, HI, MC, PA	Eradication of HI and PA in AZM group but not in placebo.	None reported	Not conducted
Djamin 2019, COLUMBUS[41]	Netherlands	Single-centre, double-blind, placebo-controlled, randomised	COPD	Adults	47 AZM, 45 placebo	AZM	Oral, 500 mg thrice weekly for 12 months	Sputum	Pre-, during and end of treatment	SA, SP, HI, MC, PA	During follow-up, fewer patients in the AZM group had positive sputum cultures with newly acquired respiratory pathogens compared with those in the placebo group	At the end of treatment, AZM-resistant bacteria were higher in placebo compared to AZM group	Not conducted
Kobbernael, 2020, BESTCLIA[121]	Denmark, Germany, Netherlands, Switzerland, and UK	Multicentre, double-blind, placebo-controlled, randomised Phase 3	Primary ciliary dyskinesia	Adults and Children	49 AZM, 41 placebo	AZM	Oral, 250 mg or 500 mg (weight ≥40 kg) thrice weekly for 6 months	Sputum	Pre-, during and end of treatment	SA, SP, HI, MC, PA	At the end of the treatment, the prevalence of HI and MC reduced in the AZM group. A higher proportion of the AZM group remained culture-negative throughout intervention compared to placebo.	Non-significant increase in carriage of AZM-resistant HI and SP at the end of treatment in AZM arm compared to placebo	Not conducted
Milito, 2019[122]	Italy	Multicentre, double-blind, placebo-controlled, randomised, parallel-group	Chronic infection-related pulmonary diseases, including bronchiectasis, COPD, and asthma	Adults	44 AZM, 45 placebo	AZM	Oral, 250 mg on three consecutive days a week for 2 years	Sputum	Pre-, during and end of treatment	SA, SP, HI, MC, PA	No difference between arms at the end of treatment	No difference between the two groups at the end of treatment	Not conducted

Phaff 2006[123]	The Netherlands	Retrospective cohort study	Cystic fibrosis	Infants, Children, Adolescents, and adults	155	AZM	Oral, AZM 250-500mg, thrice weekly, for 397 (range 5–1827) days	Sputum	Pre- and during treatment	HI, SA, and PA	Significant decrease in SA and HI.	Significant increase in SA and HI resistance.	Not conducted
Simpson, 2014[124]	Australia	Single-centre, double-blind, placebo-controlled, randomised	COPD	Adults	15 AZM, 15 placebo	AZM	Oral, 250 mg daily for 12 weeks	Sputum	Pre- and end of treatment	SA, SP, HI, MC, PA	No identifiable pathogen was recovered apart from PA	Not investigated	Not conducted
Steinkamp, 2008[125]	Germany and Switzerland	Multicentre, double-blind, placebo-controlled, randomised, parallel-group	Cystic fibrosis	Adults and Children	21 AZM, 17 placebo	AZM	Oral, weekly, for 8 weeks (20-29 kg: 500 mg, 30-39 kg: 750 mg, 40-49 kg: 1000 mg and > 50 kg: 1250 mg)	Sputum	Pre- and end of treatment	SA, SP, HI, MC, PA	At the end of treatment, SA prevalence increased in placebo compared to the AZM group. Non-significant reduction in PA prevalence in AZM group.	Not investigated	Not conducted
Uzun, 2014, COLUMBUS[39]	Netherlands	Single-centre, double-blind, placebo-controlled randomised	COPD	Adults	47 AZM, 45 placebo	AZM	Oral, 500 mg thrice weekly for 12 months	Sputum	Pre-, during and end of treatment	SA, SP, HI, MC, PA	End of treatment, fewer patients in the AZM group had positive sputum cultures with new respiratory pathogens compared with those in the placebo group	At the end of treatment, AZM-resistant bacteria were higher in placebo compared to AZM group.	Not conducted
Vermeersch, 2019, BACE[126]	Belgium	Multicentre, double-blind, placebo-controlled, randomised	COPD	Adults	147 AZM, 154 placebo	AZM	Oral, loading dose of 500 mg once daily for 3 days and maintenance dose of 250 mg every 2 days for 3 months	Sputum	Pre-, during, end and post-treatment	SA, SP, HI, MC, PA	No difference between arms at the end of treatment	No difference between the two groups at the end of treatment	No difference between groups at 6-month post-intervention follow up
Wolter, 2002[127]	Australia	Dual-centre, double-blind, placebo-controlled, randomised	Cystic fibrosis	Adults	30 AZM, 30 placebo	AZM	Oral, 250 mg daily for 3 months	Sputum	Pre-, during and end of treatment	SA, SP, HI, MC, PA	No difference between arms at the end of treatment	No difference between the two groups at the end of treatment	Not conducted
Wong, 2012, EMBRACE[128]	New Zealand	Multicentre, double-blind, placebo-controlled, randomised	Non-cystic bronchiectasis	Adults	71 AZM, 70 placebo	AZM	Oral, 500 mg thrice weekly for 6 months	Sputum	Pre- and end of treatment	SA, SP, HI, MC, PA	No difference between arms at the end of treatment	Macrolide resistance testing was not routinely undertaken, but two (4%) patients in the AZM group developed macrolide-resistant SP at 6 months	Not conducted
Hansen 2009[129]	Denmark	Retrospective cohort study	Cystic fibrosis	Children, Adolescents, and adults	70 patients	AZM	Oral, patients > 40 kg (250 mg every day), in patients < 40 kg 250 mg every other day. Smaller children received half the	Sputum and endo-laryngeal suction sample	Pre- and during treatment	SA, SP, HI, MC	Significant decrease in SP, SA, and HI.	Significant increase in SA resistance. Increase in MC resistance was not significant	Not conducted

							normal dose as syrup. Once daily or every other day based on weight for 4 years (range 0.7–5.1 years).						
Saiman,2010[42]	USA and Canada	Multicentre, double-blind, placebo-controlled, randomised	Cystic fibrosis	Children	131 AZM, 129 placebo	AZM	Oral, 250 mg or 500 mg (weight > 36 kg) thrice weekly for 168 days	Sputum and oropharyngeal swabs	Pre-, during and end of treatment	SA, SP, HI, MC, PA	No difference between arms at the end of treatment	Significantly higher incidence of macrolide-resistant SA and HI in AZM compared to the placebo group.	Not conducted
Saiman, 2012[43]	USA and Canada	Open-label, follow-on study	Cystic fibrosis	Children	146 AZM	AZM	Oral, 250 mg or 500 mg (weight > 36 kg) thrice weekly for 168 days	Sputum and oropharyngeal swabs	Pre-, during and end of treatment	SA, SP, HI, MC, PA	In the AZM–AZM group, there were no significant changes in emergent pathogens between the placebo-controlled and open-label trial phases. In the Placebo-AZM group, there was a non-significant reduction in treatment-emergent bacteria.	In the placebo–AZM group, macrolide-resistant SA increased in the open-label phase when compared to the placebo-controlled trial.	Not conducted
Clement, 2006[130]	France	Multicentre, double-blind, placebo-controlled, randomised	Cystic fibrosis	Adults and Children	40 AZM, 42 placebo	AZM	Oral, 250 mg or 500 mg (weight ≥ 40 kg) thrice weekly for 12 months	Sputum and throat swabs	Pre-, during and end of treatment	SA, SP, HI, MC, PA	No difference between arms at the end of treatment	Not investigated	Not conducted
Emiralioglu, 2016[131]	Turkey	Retrospective cohort study	Cystic fibrosis	Children and adolescents	24 patients	AZM	Oral, 10 mg/kg, twice weekly for at least 6 months	Sputum and throat swabs	Pre- and during treatment	SA and PA	Increased in the carriage of SA	Increase in SA resistance	Not conducted
Saiman, 2003[132]	USA	Multicentre, double-blind, placebo-controlled, randomised	Cystic fibrosis	Adults and Children	87 AZM, 98 placebo	AZM	Oral, 250 mg or 500 mg (weight ≥ 40 kg) thrice weekly for 168 days	Sputum and throat swabs	Pre- and end of treatment	SA, SP, HI, MC, PA	No difference between arms at the end of treatment	No difference between the two groups at the end of treatment	Not conducted
Samson 2016[133]	France	Retrospective cohort study	Cystic fibrosis	Children	68 patients	AZM	Oral, AZM 250-500mg, thrice weekly, for > 12 months	Sputum and throat swabs	Pre-, during and end of treatment	SA, PA	Non-significant increase in PA	Significant increase in SA resistance.	Not conducted
Tramper-Stranders 2007[134]	The Netherlands	Retrospective cohort study	Cystic fibrosis	Children and adolescents	100 patients	AZM	Oral, 5-10mg/kg, once daily for 3.5 years (SD, 1.6; range 0.9–7.0 years).	Sputum or oropharyngeal swab if sputum cannot be expectorated	Pre-, during and end of treatment	SA, PA	Non-significant reduction in SA. Non-significant increase in PA.	Significant increase in SA resistance.	Not conducted
McCormack, 2007[135]	Australia	Multi-centre, double-blind, randomised, comparative,	Cystic fibrosis	Adults and Children	103 AZM daily, 105 AZM weekly	AZM	Oral, 250 mg daily or 1200 mg weekly for 6 months	Sputum samples, throat swabs for S.	Pre-, during and end of treatment	SA, SP, HI, MC, PA	No difference between arms at the end of treatment	A non-significant increase in AZM resistant SA but not different between the two AZM group.	Not conducted

<i>pneumoniae</i>													
Equi, 2002[136]	UK	Dual-centre, double-blind, placebo-controlled, randomised, crossover	Cystic fibrosis	Children	20 AZM then placebo, 21 Placebo then AZM	AZM	Oral, 250mg or 500mg (weight > 40kg) daily for 6 months (1-month run-in, 6 months each cycle (AZM/placebo), 2-month washout period)	Sputum, cough swab	Pre-, during and end of treatment	SA, SP, HI, MC, PA	No difference between arms at the end of treatment	Not investigated	Not conducted
Kabra, 2010[137]	India	Single-centre, randomised, comparative,	Cystic fibrosis	Children	24 AZM (5 mg/kg/day), 23 AZM (15 mg/kg/day)	AZM	Oral, 5 mg/kg/day or 15 mg/kg/ day daily for 6 months	Sputum, deep throat swabs, cough swabs	Pre-, during, end and post-treatment	SA, SP, HI, MC, PA	No difference between arms measured during exacerbation.	All the PA isolates were resistant to AZM.	No difference between groups at exacerbations during follow-up
Gibson, 2017, AMAZE S[37]	Australia	Multicentre, double-blind, placebo-controlled, randomised, parallel-group	Asthma	Adults	213 AZM, 207 placebo	AZM	Oral, 500mg, thrice weekly for 48 weeks	Sputum, nose, and throat swabs	Pre- and end of treatment	SA, SP, HI, MC, PA	No difference between arms at the end of treatment	Non-significant increase in AZM resistant bacteria in AZM compared to placebo	Not conducted
Djamin, 2020, COLUMBUS[40]	The Netherlands	Single-centre, double-blind, placebo-controlled, randomised	COPD	Adults	44 AZM, 36 placebo	AZM	AZM, Oral 500 mg thrice weekly for 12months	Throat swabs	Pre-, during and end of treatment	<i>MefA</i> , <i>ermF</i> and <i>ermB</i>	Not studied	Difference in prevalence of <i>ermB</i> increased over time in the AZM compared to placebo. Number of patients that lost <i>ermB</i> gene were higher in the placebo compared to AZM group. The relative gene abundance of <i>mefA</i> , <i>ermF</i> and <i>ermB</i> genes significantly increased in the AZM arm compared to placebo by 12 months.	Not conducted
Blasi, 2010[138]	Italy	Multicentre, open-label, randomized, uncontrolled, pilot Phase II, trial	COPD and tracheostomy	Adults	11 AZM, 11 standard care	AZM	Oral, 500 mg thrice weekly for 6 months	Tracheal aspirate	Pre-, during and end of treatment	SA, SP, HI, MC, PA	No difference between arms at the end of treatment except for PA which reduced in AZM arm.	ERY-resistant SP strain identified in one patient in the AZM group after 6 months of treatment.	Only <i>P. aeruginosa</i> persisted at 3 and 6 months of follow up
Aberg, 2001[139]	USA	Single-centre, randomised, comparative trial	Advanced HIV disease (Mycobacterium avium)	Adults	22 participants at baseline, 21 at 6 weeks and 18 at 12 weeks	AZM, CLA	Oral, AZM 500mg, twice daily CLA 1200mg once	Nasopharyngeal, anterior nares, and throat	Pre-, during and end of treatment	SP and HI (nasopharynx); and SA (anterior nares)	Non-significant reduction in SP and SA. HI result not clear	Non-significant reduction in SP and SA resistance. No effect on HI.	Not conducted

			Complex Prophylaxis)				weekly for 12 weeks						
Iino, 2003[140]	Japan	Prospective study	Otitis media with effusion (OME) and chronic bronchitis	Children	171 (73 cases, 98 controls)	CLA	Oral, 5-8mg/kg/day, twice daily for 8 to 15 weeks	Nasopharyngeal swabs	Pre- and end of treatment	SA, SP, HI, MC, PA	Significant decrease in SA and MC. Non-significant decrease in SP and HI. Non-significant increase in PA.	Non-significant increase in SP resistance.	Not conducted
** Choo, 2018 (BLESS)[34]	Australia	Single centre, randomised, double-blind, placebo-controlled trial	non-cystic fibrosis bronchiectasis	Adults	43 ERY, 41 placebo	ERY	Oral, 400mg, twice daily for 48 weeks	Oropharyngeal swabs	Pre- and end of treatment	<i>erm(A)</i> , <i>erm(B)</i> , <i>erm(C)</i> , <i>erm(F)</i> , <i>mef(A/E)</i> , and <i>msrA</i>	Not studied	Abundance of <i>erm(B)</i> and <i>mef(A/E)</i> gene copies within carriers who had received ERY increased significantly	Not conducted
Vowels, BR, 1996[141]	USA	blinded, multidose, within-patient clinical comparison with an attendant placebo reference group	None	Adults	179 ERY, 46 placebo	ERY	Topical ERY 0.2g (2%, w/v) twice daily for 6 weeks	Specimens from the forehead, scapular back and anterior nares	Pre-, end and post-treatment	Staphylococci	There was no difference in detection rate Staphylococci between the two arms of the study at end of treatment.	Significant increase in the carriage of ERY resistance genes	Carriage of ERY genes began to decrease at week 3 post-intervention and reached baseline levels by 6 weeks post-intervention
Seemungal, 2008[142]	United Kingdom	Single-centre, randomized, double-blind, placebo-controlled trial	Chronic obstructive pulmonary disease (COPD)	Adults	53 ERY, 56 placebo	ERY	Oral ERY, 250mg twice daily for one year	Sputum	Pre-, during and end of treatment	SP, HI, MC	No difference in detection rate for any organism between the two arms of the study at any of the follow-up time points	No significant difference between the two groups at the end of treatment	Not conducted
Taylor, 2018 (BLESS)[143]	Australia	Single-centre, randomised, double-blind, placebo controlled trial	Non-CF bronchiectasis	Adults	63	ERY	Oral, 400mg, twice daily for 12 months	Sputum	Pre- and end of treatment	<i>ermA</i> , <i>ermB</i> , <i>ermC</i> , <i>16S</i> , <i>smpB</i> , <i>hmrM</i>	Not studied	Increase in macrolide resistance genes	Not conducted
Fujii, 1995[144]	Japan	Retrospective case control study	Diffuse pan-bronchiolitis	Adults	28	ERY	Oral, 200mg, thrice daily for at least 12 months	Sputum and bronchoalveolar lavage (BAL)	Pre- and end of treatment	SP, HI, PA	Non-significant reduction in SP and PA. Significant reduction in HI.	None	Not conducted

*Bacteria of interest screened for: HI = *Haemophilus influenzae*, HP = *Haemophilus parainfluenzae*, MC = *Moraxella catarrhalis*, SA = *Staphylococcus aureus*, SP = *Streptococcus pneumoniae*. MIC = minimum inhibitory concentration, AZM = azithromycin, ERY = erythromycin, CLA = Clarithromycin ** same study captured under microbiome table

2.3.3 Effect of macrolides on the prevalence and resistance of selected bacterial species

Of the 65 short-term studies, 51 reported *S. pneumoniae* prevalence. Among these, *S. pneumoniae* was completely eradicated in 30 studies, the prevalence was reduced or unchanged in nine and 12 studies respectively after treatment, Figure 2.4. Increased macrolide resistance was reported in only five of the 21 studies where *S. pneumoniae* was not eradicated at end of treatment. In contrast, long-term macrolide use did not affect the prevalence (35/38) or macrolide resistance of *S. pneumoniae* (24/30) in most studies that tested and reported this result, Figure 2.4.

Thirty-two short-term studies reported *S. aureus* prevalence. In 15 of these studies, *S. aureus* was completely eradicated at the end of treatment whilst the prevalence was reduced in five. *S. aureus* prevalence remained unchanged in 11 studies but increased in one study. There was no effect on macrolide resistance in *S. aureus* in 29 of the 30 studies in which resistance was tested and reported, Figure 2.4. Again, long-term macrolide use did not affect the prevalence (29/36) of *S. aureus*, but the prevalence of macrolide-resistant *S. aureus* increased in 12/27 of the studies in which resistance was tested and reported.

Of the 65 short-term studies, 48 reported *H. influenzae* prevalence. Among these 48 studies, the prevalence of *H. influenzae* was reduced in 21, remained unchanged in 13 and completely eradicated in 14 at the end of treatment. The prevalence of macrolide-resistant *H. influenzae* remained unchanged in 38 out of 40 studies that investigated macrolide resistance. Long-term macrolide use reduced the prevalence of *H. influenzae* in 10/36 studies with no effect in 26 studies, Figure 2.4. Of the 26 long-term studies in which resistance was tested and reported, only four observed increased macrolide resistance in *H. influenzae* after treatment.

Short-term macrolide administration also completely eradicated *M. catarrhalis* in a majority of the studies (22/39), reduced its prevalence in 5/39 studies with no report of macrolide resistance in any of the 33 studies that investigated this, Figure 2.4. Additionally, long-term macrolide use did not affect the prevalence (25/32) or macrolide resistance (20/22) of *M. catarrhalis* in most studies.

The effect of short-term macrolide use on *P. aeruginosa* prevalence was not investigated or reported in most studies (60/65). In most long-term macrolide studies, the prevalence (29/31) and macrolide resistance (19/20) of *P. aeruginosa* remained unchanged, Figure 2.4.

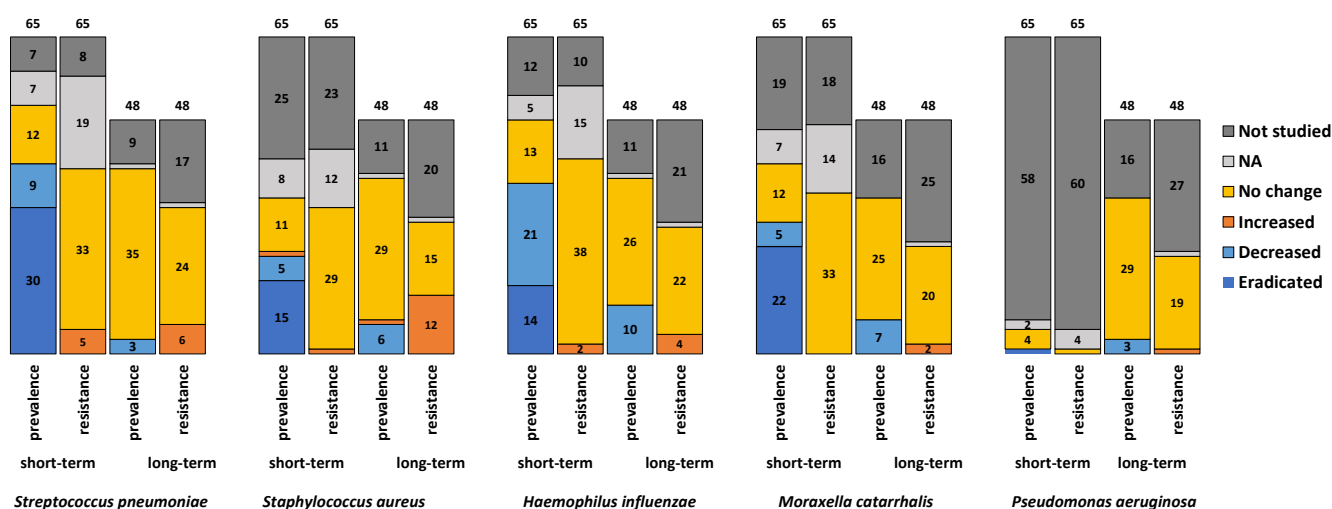
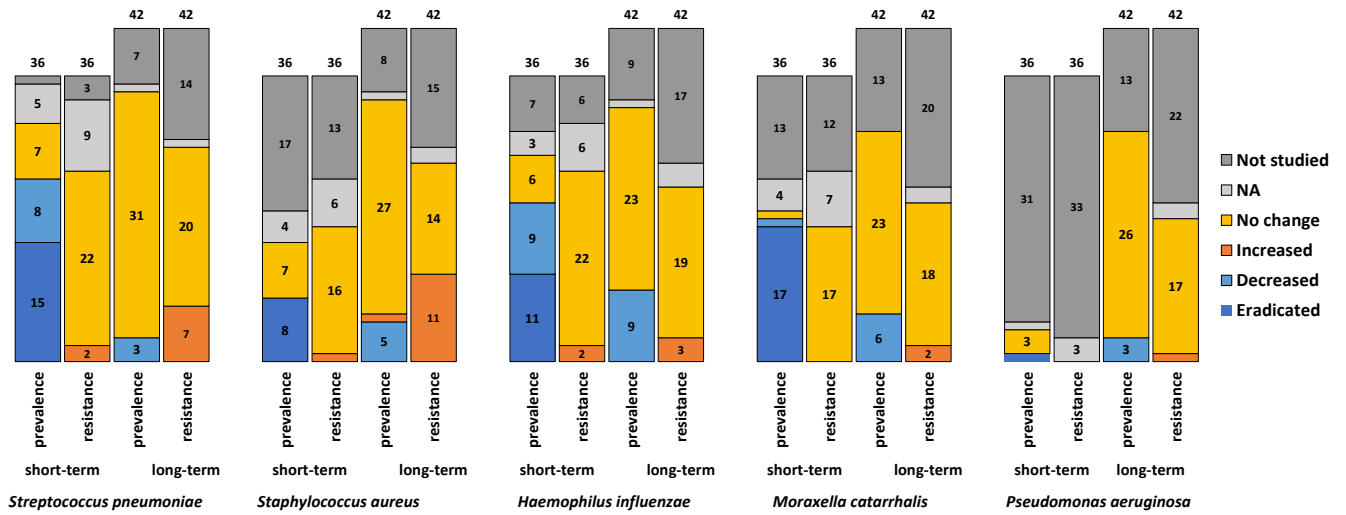


Figure 2. 4. Effect of macrolide use on the prevalence and resistance of selected bacteria.

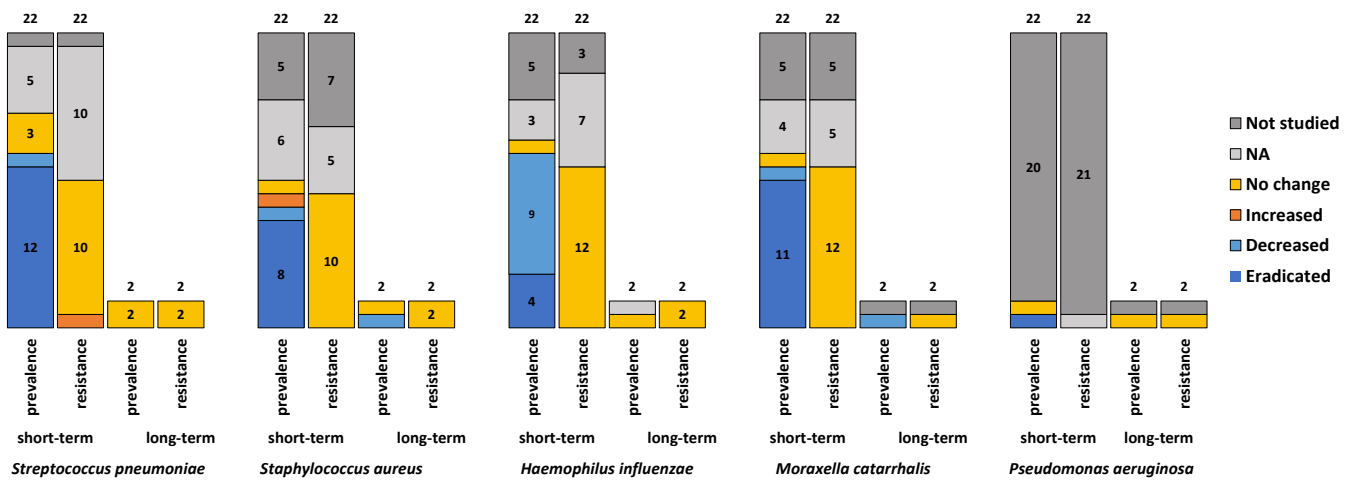
The bar labelled “prevalence” displays data on the carriage of each bacterium after macrolide treatment. The bar labelled “resistance” displays data on macrolide resistance in each bacteria after macrolide treatment. “Not studied” refers to studies that did not investigate prevalence or macrolide resistance of each bacteria. “NA” refers to studies where the prevalence or resistance of that specific bacteria was not determined, or was not clearly stated, or not reported. “No change” indicates studies where the prevalence and macrolide resistance of each bacterium remained unchanged before and after macrolide administration. “Increased” refers to studies where the prevalence or macrolide resistance in that bacteria increased after macrolide treatment. “Decreased” refers to studies where the prevalence or macrolide resistance in that bacteria decreased after macrolide treatment. “Eradicated” refers to studies where that specific bacteria was not recovered or detected after macrolide treatment. The data presented in each bar represent the number of studies reporting each category after treatment. Short-term = studies that administered macrolides for ≤ 14 days, long-term = studies that administered macrolides for > 28 days. Data labels for categories less than two studies were not shown. The total number of included studies is indicated on top of each bar.

When stratified according to the macrolide administered, we observed a similar trend in prevalence and resistance across studies (Figure 2.5). Interestingly, while some studies of long-term azithromycin treatment reported an increase in macrolide resistance post-intervention in all five bacterial species (Figure 2.5A), this was not observed for clarithromycin treatment, Figure 2.5B. Only one long-term study of erythromycin reported an increase in macrolide resistance but only in *S. aureus*, Figure 2.5C. Roxithromycin was administered only in short-term studies and no increased in macrolide resistance was reported, Figure 2.5D.

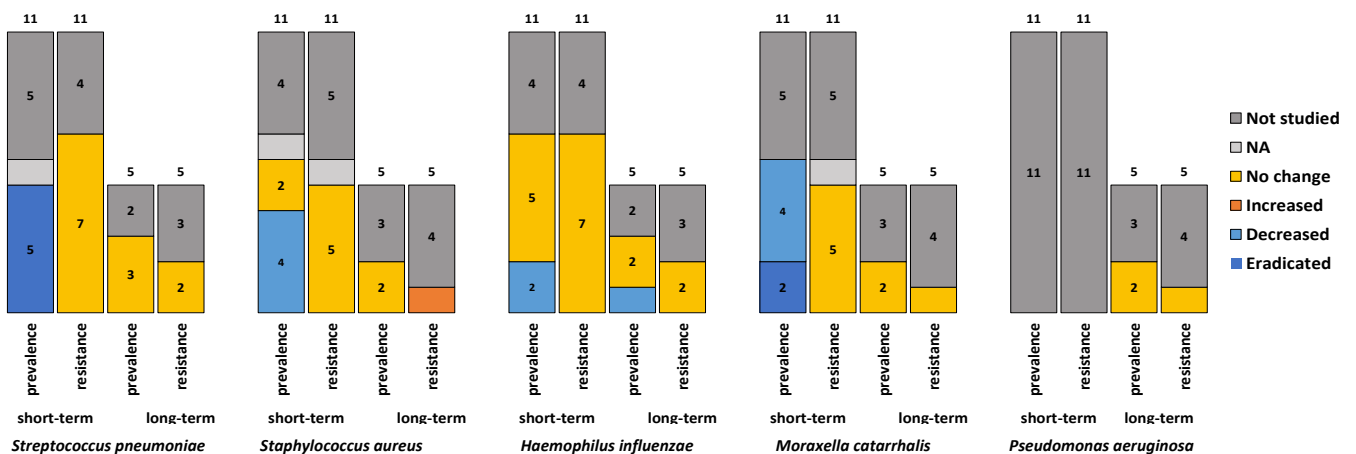
A. Azithromycin



B. Clarithromycin



C. Erythromycin



D. Roxithromycin

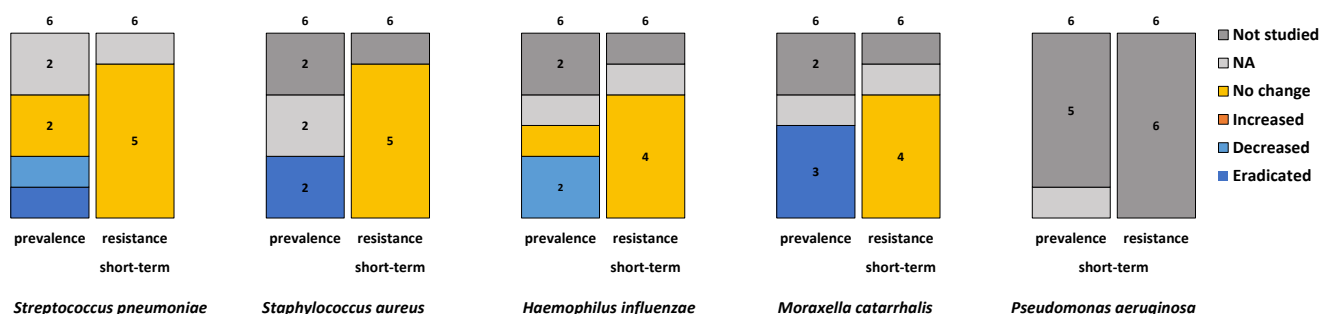
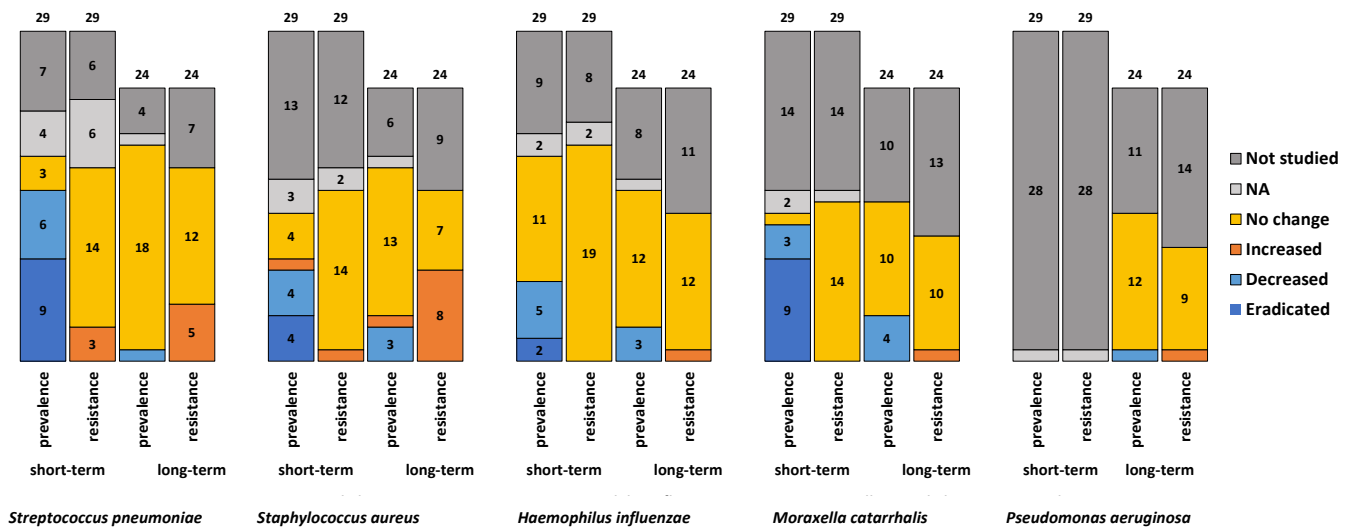


Figure 2. 5. Effect of macrolide use on the prevalence and resistance of bacteria stratified by type of macrolide.

The bar labelled “prevalence” displays data on the carriage of each bacterium after macrolide treatment. The bar labelled “resistance” displays data on macrolide resistance in each bacteria after macrolide treatment. “Not studied” refers to studies that did not investigate prevalence or macrolide resistance of each bacteria. “NA” refers to studies where the prevalence or resistance of that specific bacteria was not determined, or was not clearly stated, or not reported. “No change” indicates studies where the prevalence and macrolide resistance of each bacterium remained unchanged before and after macrolide administration. “Increased” refers to studies where the prevalence or macrolide resistance in that bacteria increased after macrolide treatment. “Decreased” refers to studies where the prevalence or macrolide resistance in that bacteria decreased after macrolide treatment. “Eradicated” refers to studies where that specific bacteria was not recovered or detected after macrolide treatment. The data presented in each bar represent the number of studies reporting each category after treatment. Short-term = studies that administered macrolides for ≤ 14 days, long-term = studies that administered macrolides for > 28 days. Data labels for categories less than two studies were not shown. The figures display studies that administered A) Azithromycin, B) Clarithromycin, C) Erythromycin, and D) Roxithromycin. The other macrolides were not displayed as less than five studies investigated each one of them. The total number of included studies is indicated on top of each bar. Some studies were counted twice because they included two macrolides. Roxithromycin was administered in only short-term studies.

The pattern of the effect of macrolides on bacterial prevalence and resistance remained similar when the studies were categorised based on the sample type tested. Short-term macrolide administration rarely reduced bacterial prevalence in studies that investigated samples from the upper respiratory tract (mainly nasal, nasopharyngeal and oropharyngeal swabs), Figure 2.6A, compared to studies that included samples from the lower respiratory tract (mainly sputum), Figure 2.6B. The number of studies that reported an increase in macrolide resistance after intervention did not differ based on sample type.

A. Samples from the upper respiratory tract



B. Samples from the lower respiratory tract

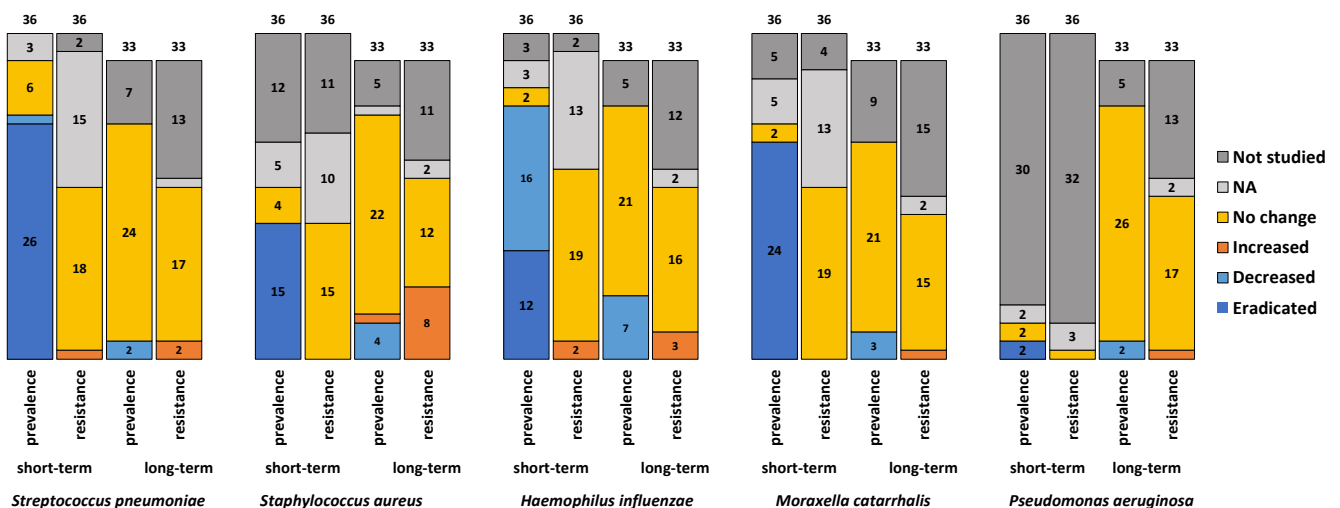


Figure 2. 6. Effect of macrolide use on the prevalence and resistance of selected bacteria stratified by sample source.

Samples were from the upper (A) and lower respiratory tract (B). Samples from the upper respiratory tract include nasal, maxillary sinus, nasopharyngeal, oropharyngeal and pharyngeal swabs. Samples from the lower respiratory tract include sputum and bronchopulmonary secretions. The bar labelled “prevalence” displays data on the carriage of each bacterium after macrolide treatment. The bar labelled “resistance” displays data on macrolide resistance in each bacteria after macrolide treatment. “Not studied” refers to studies that did not investigate prevalence or macrolide resistance of each bacteria. “NA” refers to studies where the prevalence or resistance of that specific bacteria was not determined, or was not clearly stated, or not reported. “No change” indicates studies where the prevalence and macrolide resistance of each bacterium remained unchanged before and after macrolide administration. “Increased” refers to studies where the prevalence or macrolide resistance in that bacteria increased after macrolide treatment. “Decreased” refers to studies where the prevalence or macrolide resistance in that bacteria decreased after macrolide treatment. “Eradicated” refers to studies where that specific bacteria was not recovered or detected after macrolide treatment. The data presented in each bar represent the number of studies reporting each category after treatment. Short-term = studies that administered macrolides for ≤ 14 days, long-term = studies that administered macrolides for > 28 days. Data labels for categories less than two

studies were not shown. The total number of included studies is indicated on top of each bar. Some studies counted twice because they included samples from both the upper and lower respiratory tract.

2.3.4 Effect of macrolides on the carriage of antibiotic resistance genes

Five [21, 34, 40, 141, 143] studies investigated the effect of long-term macrolide treatment on resistance genes (Table 2.5). In one study, there was no difference in prevalence of macrolide resistance genes *mefA*, *ermF* and *ermB* between azithromycin and placebo arms at the end of treatment, but the prevalence of *ermB* increased significantly over time in the azithromycin group [40]. The relative gene abundance of *mefA*, *ermF* and *ermB* genes also significantly increased in the azithromycin arm compared to placebo at the end of the intervention (12 months) [40]. In the second study which utilised shotgun metagenomics sequencing to characterised the respiratory resistome, macrolide (*mel*, *msrE*, *ermF*, *mefA*, and *ermB*) and tetracycline (*tetW*, *tetM*) resistance genes increased in the azithromycin arm but not placebo after 48 weeks of treatment [21]. In two of the studies that administered erythromycin, there was an increase in macrolide resistance genes in the active arm compared to placebo [141, 143]. In the final study, erythromycin did not increase the prevalence of macrolide resistance genes (*ermA*, *ermB*, *ermC*, *ermF*, *mefA/E*, and *msrA*) after 48-week treatment [34]. However, in the erythromycin arm, relative to placebo, the abundance of *ermB* and *mefA/E* gene copies increased significantly [34].

2.3.5 Persistence of the bacteriological effects of macrolides post-intervention

Only eight [36, 110, 115, 116, 126, 137, 138, 141] out of the 48 long-term studies ($n = 7$ for azithromycin and $n = 1$ for erythromycin) conducted a post-intervention assessment of bacterial carriage and resistance (Table 2.5). In three of these studies that administered azithromycin and placebo, there was no difference between the trial arms in terms of bacterial carriage and resistance six months post-intervention (2 studies) [110, 126] and > 30 days and ≤ 12 months post-intervention (one study) [36]. In the fourth study, although azithromycin-resistant streptococci decreased in prevalence in the azithromycin arm at four weeks post-intervention relative to the end of treatment levels, resistance remained significantly higher in the azithromycin arm compared to the placebo arm [116]. The fifth study [138] observed no difference in the bacterial carriage and antibiotic resistance between the azithromycin group and the standard care only group at the 3- and 6-month post-intervention follow-up. The seven study [137] compared two dosages (5 mg/kg/day or 15 mg/kg/ day daily for 6 months) of azithromycin. No difference between the two groups was observe in bacterial carriage and macrolide resistance at 6 and 12 months follow up. The final study of long-term

azithromycin treatment observed a non-significant reduction in macrolide resistance compared to end of treatment levels at 13 weeks post-intervention [115]. In the erythromycin long-term studies [141], the increase in macrolide-resistant genes in the active arm observed at the end of treatment decreased at three weeks post-intervention and reached baseline levels by six weeks post-intervention.

2.3.6 Effects of macrolides on the total bacterial load of respiratory samples

Of the 12 microbiome studies, seven did not investigate or report the effect of macrolides on the total bacterial load of the respiratory samples collected. Of the five that assessed this, four [21, 33, 149, 153] did not observe any difference between trial arms after the intervention or within arms over time (Tables 2.6 and 2.7). However, one study [147] reported an increase in the bronchoalveolar lavage total bacterial load at the end of treatment but only in the placebo arm. One study [153] observed that in non-cystic fibrosis bronchiectasis patients with non-*P. aeruginosa*-dominated airway infection, there was no change in either total bacterial or *H. influenzae* load. However, *P. aeruginosa* load increased significantly in the sputum collected from the erythromycin arm at the end of treatment compared to baseline [153].

Table 2. 6. Summary of microbiome-based studies

First author, year, reference	Macrolide	Sample type	Total bacterial load	Variable region sequenced	Alpha diversity after treatment ^a	Beta diversity after treatment ^b	Differentially abundant bacterial taxa detected	Test for differential abundant testing
Acosta, 2021, [145]	AZM	Sputum	Not investigated	V3-V4	—	—	yes	DESeq2
Brill, 2015, [33]	AZM	Sputum	—	—	Not investigated	Not investigated	Not investigated	Not investigated
Renteria, 2020*, [151]	AZM	Sinonasal	Not investigated	V3	—	changed	yes	DESeq2
Santiago, 2017, [150]	AZM	Oropharyngeal	Not investigated	V1-V2	Not investigated	—	yes	Boruta feature selection
Segal, 2017, [149]	AZM	Bronchoalveolar lavage	—	V4	↓	changed	yes	Linear discriminant analysis effect size
Slater, 2014, [148]	AZM	Saline washings of the right upper lobe	Not investigated	V4-V6	—	Not investigated	yes	Not tested statistically
Spence, 2019**, [147]	AZM	Bronchoalveolar lavage	—	V4	—	Not investigated	yes	Kruskal-Wallis test
Taylor, 2019, [21]	AZM	Sputum	—	V1-V3	↓	changed	yes	Linear discriminant analysis effect size
Zhou, 2016, [146]	AZM	Nasal lavage	Not investigated	V1-V3	—	changed	yes	Metastats is a statistical method

								based on Fisher exact test
Chen, 2021, [152]	Clarithromycin	Nasopharyngeal	Not investigated	V4	—	—	Not investigated	Not investigated
Choo, 2018, [34]	Erythromycin	Oropharyngeal	Not investigated	V1-V3	—	changed	yes	DESeq2
Rogers, 2014, [153]	Erythromycin	Sputum	—	V1-V3	—	changed	yes	SIMPER

↓ decreased in macrolide arm, — unchanged. ^aAlpha diversity refers to within-sample diversity measured using either Shannon, Simpson or Faith's diversity index. ^bBeta diversity refers to diversity between pre and post-treatment samples from the macrolide group or between the macrolide and placebo group at the end of treatment. *Renteria 2020 reported a decrease in alpha diversity in the placebo arm at the end of treatment. **Spence 2019 reported an increase in the bronchoalveolar lavage total bacterial load in the placebo arm at the end of treatment. AZM = azithromycin.

2.3.7 Effects of macrolides on alpha (within-sample) diversity

Of the ten studies that reported alpha diversity, nine [reported Shannon alpha diversity metric and one reported Faith phylogenetic diversity metric [21]. There were conflicting reports on the effect of macrolides on the alpha diversity of pre- and post-treatment samples from the treated groups (Tables 2.6 and 2.7). Five azithromycin studies [145, 147, 147, 148, 151] observed no change in Shannon diversity [measures both richness (number of unique taxa) and evenness (how evenly distributed these taxa are)] in post-treatment compared to pre-treatment samples from the azithromycin arm. In contrast, two studies [21, 149] reported a reduction in alpha diversity in post-treatment compared to pre-treatment samples collected from the azithromycin arm. Interestingly, one study [151] observed a reduction in alpha diversity in samples from the placebo arm compared to azithromycin at the end of treatment. However, there was no difference in the pre- and post-treatment samples from the azithromycin arm in this study [151]. In two studies that administered erythromycin [34, 153], alpha diversity (Shannon diversity, Faith and observed richness) at the end of treatment remained unchanged. The only study that administered clarithromycin [146] also observed no change in alpha diversity at the end of treatment.

2.3.8 Effects of macrolides on beta (between-sample) diversity and differentially abundant taxa

Two of the azithromycin studies [33, 146] did not report on beta diversity metrics and one [148] did not test this statistically (Tables 2.6 and 2.7). Six studies evaluated the effect of azithromycin on beta diversity between pre- and post-treatment samples. Of these, four, which investigated sinonasal swabs

[151], bronchoalveolar lavage samples [149], nasal lavage [146] and sputum samples [21], observed significant changes in the microbiome composition of the samples between the two timepoints. Another observed a non-significant change in oropharyngeal samples [150] while one did not observe changes in the microbiome composition of the sputum [145] (Tables 2.6 and 2.7). The two studies that investigated erythromycin also noted changes in the beta diversity of sputum [153] and oropharyngeal [34] samples between the erythromycin and placebo arms at the end of treatment. They also noted changes in the microbiome composition of the samples collected before and after treatment from the erythromycin arm [34, 153]. Finally, the study of clarithromycin [146] treatment observed no changes in beta diversity of the nasopharyngeal samples collected pre- and post-treatment.

Table 2. 7. Studies that administered macrolides and investigated their effect on the respiratory microbiome using next-generation sequencing

First author, year	Country	Study Design	Disease of focus	Age group	Number of participants	Macrolide, dosage, and duration	Sample type	Sampling Timepoints	Microbiome methods	Effect on total bacterial load by 16S rDNA qPCR	Effect on alpha diversity	Effect on beta diversity	Differentially abundant taxa between arms
Acosta, 2021 [145]	Canada	Single centre, retrospective cohort study	Cystic fibrosis	Adults	38	AZM, NA	Sputum	Pre-, during and end of treatment	16S rDNA, V3-V4 region, Illumina MiSeq	Not studied	No statistically significant difference in Shannon diversity between samples from different visits	No difference between pre and post samples	<i>Haemophilus</i> and <i>Stenotrophomonas</i> increased
Zhou 2016[146]	USA	Single centre, randomised, double-blind, placebo-controlled trial	Respiratory syncytial virus (RSV) bronchiolitis	Infants	19 AZM, 20 placebo	AZM, 10 mg/kg and 5 mg/kg daily for week 1 and 2 respectively	Nasal lavage samples	Pre- and end of treatment	V1-V3 region of 16S rDNA gene, pyrosequencing	Not studied	Richness and Shannon diversity did not differ between the two groups	Significantly differed between pre and post samples in both AZM and placebo and between the two groups at end of treatment	<i>Dolosigranulum</i> and <i>Corynebacterium</i> increased while <i>Streptococcus</i> decreased in Placebo. <i>Moraxella</i> significantly decreased in AZM post samples
Spence, 2020[147]	Australia	Single centre, randomised, double-blind, placebo-controlled trial	chronic lung allograft dysfunction	Adults	32 AZM, 37 placebo	AZM, 250 mg, thrice weekly for 2 years (from discharge post-transplant)	Bronchoalveolar lavage samples	Pre- and end of treatment	16S rDNA, V4 region, Illumina MiSeq	Bacterial burden increased in placebo patients ($p = 0.020$)	Shannon and evenness did not differ between arms at any visit or between pre- and post-treatment sample from the same arm.	Not studied	Decrease in relative abundance of <i>Pseudomonas</i> ($p = 0.049$), accompanied by an increase in relative abundance of <i>Streptococcus</i> ($p = 0.045$), <i>Lactobacillales</i> ($p = 0.032$), and <i>Gemellaceae</i> ($p = 0.036$) in the AZM group
Slater 2014[148]	Belgium	Single centre, prospective cohort study	Asthma	Adults	5	AZM, 250mg, daily for 6 weeks	Saline washings of the right upper lobe	Pre- and end of treatment	530F and 1100R primers FLX amplicon pyrosequencing	Not studied	Richness and Shannon diversity not statistically different between the pre and post samples although they reduced in post-samples	Not tested statistically	<i>Veillonella</i> and <i>Streptococcus</i> increased slightly. <i>Prevotella</i> , <i>Staphylococcus</i> , <i>Haemophilus</i> , <i>Pseudomonas</i> (not detected), <i>Nisseriae</i> , <i>Fusobacterium</i> , <i>Actinobacillus</i> (one patient) decreased
Segal, 2017[149]	UK	Single centre, randomised, double-blind, placebo-controlled trial	COPD (emphysema)	Adults	10 AZM, 10 placebo	AZM, 250mg, daily for 8 weeks	Bronchoalveolar lavage samples	Pre- and end of treatment	16S rDNA, V4 region, Illumina MiSeq	No significant difference between arms	Reduced Shannon diversity within AZM but not placebo. Between arm comparison at endpoint not conducted.	Baseline β -diversity was not similar to post-AZM β -diversity. β -diversity changes between arms post-treatment not reported.	<i>Acidimicrobiales</i> , <i>Bifidobacterium</i> and <i>Bradyrhizobiaceae</i> increased in active arm and <i>Tissierellaceae</i> , <i>Cytophaga</i> , <i>Flectobacillus</i> , <i>Neisseria</i> , <i>Ralstonia</i> and <i>Rhodospirillaceae</i> decreased

Lopes Dos Santos Santiago 2017[150]	Belgium	Multicentre, randomised, double-blind, placebo-controlled trial	Asthma	Adults	32 AZM, 37 placebo	AZM, 250mg, once daily for 5 days, followed by thrice weekly for 25 weeks	Oropharyngeal samples	Pre-, during, end and post-treatment	V1-V2 region of 16S rDNA gene, pyrosequencing	Not studied	Not studied	Non-significant difference in pre- and post-AZM arm samples nor between AZM and placebo samples after treatment.	<i>Streptococcus salivarius</i> increased while <i>Actinomyces sp.</i> , <i>Leptotrichia spp.</i> , <i>Leptotrichia hofstadii</i> and <i>Fusobacterium nucleatum</i> decreased in treated arm compared to placebo. <i>Firmicutes</i> , <i>Veillonella</i> , <i>Pseudomonadales</i> increased and <i>Fusobacteria</i> , <i>Leptotrichia wadei</i> decreased.
Brill, 2015[33]	UK	Single-centre, single-blind, placebo controlled, randomised controlled trial	COPD	Adults	25 AZM, 24 placebo	AZM, 250mg, thrice weekly for 13 weeks	Spontaneously expectorated sputum	Pre- and end of treatment	16S rDNA qPCR only	No significant difference between arms	Not studied	Not studied	Not studied
Taylor 2019, (AMAZES) [21]	USA	Multicentre, randomised, double-blind, placebo-controlled trial	Asthma	Adults	27 AZM, 34 placebo	AZM, 500mg, thrice weekly for 48 weeks	Induced sputum	Pre- and end of treatment	V1-V3 region 16S rDNA on Illumina Miseq, Shotgun meta genomics sequencing on Illumina HiSeq 400	No significant difference between arms	Faith phylogenetic diversity reduced in AZM arm but not placebo. Comparison between groups at endpoint was not conducted.	Significant difference in the AZM group but not placebo. β -diversity comparisons between arms post-treatment not conducted.	<i>S. pneumoniae</i> , <i>S. aureus</i> , <i>P. aeruginosa</i> , and <i>M. catarrhalis</i> bacterial loads were not significantly affected by AZM between visit nor between arms at 48 weeks. <i>Firmicutes</i> , <i>Veillonella</i> , <i>Pseudomonadales</i> increased and <i>Peptostreptococcus</i> , <i>Aggregatibacter</i> , <i>Peptococcus</i> , <i>Ruminococcaceae</i> , <i>Xanthomonadaceae</i> , <i>Pasteurellaceae</i> , <i>Gammaproteobacteria</i> decreased
Renteria, 2020[151]	Australia	Single centre, randomised, double-blind, placebo-controlled trial	Refractory chronic rhinosinusitis	Adults	19 AZM, 17 placebo	AZM, Oral 250 mg thrice weekly for 4 months	Sinonasal swab	Pre- and end of treatment	16S rDNA, V3 region, Illumina MiSeq	Not studied	Reduced inverse Simpson alpha diversity in the placebo arm compared to AZM at end of treatment. No difference in Shannon or inverse Simpson diversity between pre and post samples from AZM	Differences in beta-diversity between pre- and post-treatment samples from AZM arm only and difference between the arms at end of treatment	Decrease in <i>Staphylococcus</i> in AZM but not placebo at end of treatment but increased in <i>Corynebacterium spp</i> and <i>Enterobacter spp</i> . Decreased in <i>Haemophilus aegyptius</i> and <i>Veillonella spp</i> . In AZM compared to placebo at endpoint. <i>Bacteroides acidifaciens</i> , <i>Corynebacterium accolens</i> , <i>Fusobacterium OTU</i> ,

													<i>Enterobacteriaceae</i> enriched in AZM arm but not placebo at endpoint
Chen, 2021[152]	China	Single centre, prospective, self-controlled study	Refractory Chronic Sinusitis	Adults	18	CLA, Oral, 250 mg, once daily for 12 weeks	Nasopharyngeal swab	Pre-, during and end of treatment	16S rDNA, V4 region, Illumina HiSeq2500	Not studied	No effect on Shannon diversity	None	NA
Choo, 2018 (BLESS)[34]	Australia	Single centre, randomised, double-blind, placebo-controlled trial	non-cystic fibrosis bronchiectasis	Adults	43 Erythromycin, 41 placebo	ERY, 400mg, twice daily for 48 weeks	Oropharyngeal swabs	Pre- and end of treatment	16S rDNA, V1-V3 region, Illumina MiSeq	No significant difference between arms or within arms	Simpson's Evenness reduced in both arms but there were no significant differences in Shannon, Faith and observed richness between the arms at endpoint	There were differences between arms at 48 weeks. Beta diversity between pre- and post-samples from the same arm was not studied.	<i>Haemophilus</i> increased. <i>Actinomyces</i> , <i>Streptococcus</i> reduced. qPCR shows no difference for <i>A. odontolyticus</i> , <i>H. parainfluenzae</i> , and <i>S. pseudopneumoniae</i> . <i>H. influenzae</i> and <i>S. pneumoniae</i> bacterial loads did not differ between arms.
Rogers, 2014 (BLESS)[153]	USA	Single centre, randomised, double-blind, placebo-controlled trial	non-cystic fibrosis bronchiectasis	Adults	44 Erythromycin, 42 placebo	ERY, 400mg, twice daily for 48 weeks	spontaneously expectorated sputum	Pre- and end of treatment	16S rDNA, V1-V3 region, 454 pyrosequencing	In participants with non- <i>P. aeruginosa</i> -dominated airway infection, we noted no change in either total bacterial or <i>H. influenzae</i> load, but <i>P. aeruginosa</i> load increased significantly	No statistically significant difference between samples from different visits (richness and Shannon) within a group or between the two groups.	Significant difference in the pre- and post-erythromycin arm samples but not placebo. Significant difference in non- <i>P. aeruginosa</i> -dominated subgroup treated with erythromycin compared to placebo post-treatment.	No change in either total bacterial or <i>H. influenzae</i> load, but <i>P. aeruginosa</i> load increased significantly in participants with non- <i>P. aeruginosa</i> -dominated airway infection. In patients with non- <i>P. aeruginosa</i> -dominated infections in the erythromycin group, the relative abundance of <i>H. influenzae</i> decreased significantly between baseline and week 48

AZM = azithromycin, ERY= erythromycin, CLA = Clarithromycin, NA = Not available

There was broad agreement between studies on taxa that were differentially abundant (including *Moraxella*, and *Pseudomonas*) in response to macrolide treatment and the direction of change, Table 2.8. However, it was challenging to compare observations across studies as each study reported differentially abundant taxa at different taxonomic ranks. Also, different methods including Deseq2, linear discriminant analysis effect size and metastat based on varying underlying statistical testing were used to determine differential abundant taxa. This can be a source of bias. At the phylum level, the direction of change in the relative abundances of Firmicutes (e.g. *Streptococcus* [34, 147, 148], *Veillonella* [21, 148, 150, 151]) and Proteobacteria (e.g. *Haemophilus* [34, 145, 148]) after azithromycin use were conflicting across studies. However, the relative abundances of the phyla *Actinomycetota*, Bacteroidetes and Fusobacteria were consistently reduced in the studies while Actinobacteria consistently increased (Table 2.8). In one study of erythromycin treatment [34], *Haemophilus* increased in relative abundance while the *Actinomyces* and *Streptococcus* genera decreased in relative abundance in the erythromycin arm at the end of treatment. In the second study of erythromycin treatment [153], the relative abundance of *H. influenzae* decreased significantly while the relative abundance of *P. aeruginosa* increased between baseline and week 48 samples in patients with non-*P. aeruginosa*-dominated infections in the erythromycin group. No differentially abundant taxa were detected in the study that administered clarithromycin [152].

Table 2. 8. Differentially abundant taxa detected between pre- and post-treatment samples from the azithromycin arm post-treatment

Taxa that decreased in relative abundance in the azithromycin arm at the end of treatment				Taxa that increased in relative abundance in the azithromycin arm at the end of treatment			
Taxa reported	Taxonomical rank of reported taxa	Phylum of reported taxa	Number of studies	Taxa reported	Taxonomical rank of reported taxa	Phylum of reported taxa	Number of studies
<i>Actinomyces</i>	Genus	Actinomycetota	2	<i>Acidimicrobiales</i>	Order	Actinobacteria	1
<i>Cytophaga</i>	Genus	Bacteroidetes	1	<i>Bifidobacterium</i>	Genus	Actinobacteria	1
<i>Flectobacillus</i>	Genus	Bacteroidetes	1	<i>Corynebacterium</i>	Genus	Actinobacteria	2
<i>Tissierellaceae</i>	Family	Firmicutes	1	<i>Streptococcus</i>	Genus	Firmicutes	2
<i>Ruminococcaceae</i>	Family	Firmicutes	1	<i>Lactobacillales</i>	Order	Firmicutes	1
<i>Peptostreptococcus</i>	Genus	Firmicutes	1	<i>Gemellaceae</i>	Family	Firmicutes	1
<i>Peptococcus</i>	Genus	Firmicutes	1	<i>Firmicutes</i>	Phylum	Firmicutes	2
<i>Leptotrichia wadei</i>	Species	Firmicutes	1	<i>Veillonella</i>	Phylum	Firmicutes	3
<i>Leptotrichia</i>	Genus	Fusobacteria	1	<i>Streptococcus salivarius</i>	Species	Firmicutes	1
<i>Fusobacteria</i>	Genus	Fusobacteria	1	<i>Dolosigranulum</i>	Genus	Firmicutes	1

<i>Leptotrichia hofstadii</i>	Species	Fusobacteria	1	<i>Bradyrhizobiaceae</i>	Family	Proteobacteria	1
<i>Fusobacterium nucleatum</i>	Species	Fusobacteria	1	<i>Pseudomonadales</i>	Order	Proteobacteria	2
<i>Gammaproteobacteria</i>	Class	Proteobacteria	1	<i>Haemophilus</i>	Genus	Proteobacteria	2
<i>Rhodospirillaceae</i>	Family	Proteobacteria	1	<i>Stenotrophomonas</i>	Genus	Proteobacteria	1
<i>Xanthomonadaceae</i>	Family	Proteobacteria	1				
<i>Pasteurellaceae</i>	Family	Proteobacteria	1				
<i>Pseudomonas</i>	Genus	Proteobacteria	2				
<i>Neisseria</i>	Genus	Proteobacteria	1				
<i>Ralstonia</i>	Genus	Proteobacteria	1				
<i>Aggregatibacter</i>	Genus	Proteobacteria	1				
<i>Moraxella</i>	Genus	Proteobacteria	1				
<i>Streptococcus</i>	Genus	Firmicutes	2				
<i>Veillonella</i>	Phylum	Firmicutes	1				
<i>Haemophilus</i>	Genus	Proteobacteria	1				
<i>Haemophilus aegyptius</i>	Species	Proteobacteria	1				

2.4 Discussion

In this multi-indication review, 122 eligible articles out of 6052 retrieved from the literature search were included in the final data synthesis. Of these, 110 studies used culture and/or PCR to detect five bacteria of interest – *S. pneumoniae*, *S. aureus*, *H. influenzae*, *M. catarrhalis* and *P. aeruginosa*. Out of these 110 studies, 65 administered macrolides for ≤ 14 days (short-term studies) whilst 45 studies administered macrolides for ≥ 28 days (long-term studies). The remainder 12/122 studies used next-generation sequencing, specifically 16S rDNA amplicon sequencing to characterise the effect of macrolide use on the respiratory microbiota. The studies were conducted in 33 countries in Europe, North America, Asia, Oceania, and Africa. Majority of the studies were conducted in high-income countries in Europe, North America, and Oceania and only one short-term (≤ 14 days) study was conducted in Africa. *S. pneumoniae*, *S. aureus*, *H. influenzae* and *M. catarrhalis* isolates were completely eradicated at the end of treatment in about half of short-term studies that tested them. Consequently, increased macrolide resistance at the end of treatment in these bacteria was reported in only a few of these studies. In contrast, the prevalence and associated macrolide resistance in these four bacteria were unaffected in the long-term studies. Despite the minimal effect on the prevalence

of macrolide-resistant bacteria, long-term macrolide administration increased the prevalence and abundance of macrolide resistance genes in the respiratory resistome. The very few long-term studies (8/48) that assessed the persistence of these bacteriological effects post-intervention showed variable persistence of macrolide resistance. Additionally, macrolides did not commonly affect alpha diversity (except in two studies [21, 149] where this was reduced) but alter the beta diversity of post-treatment samples compared to pre-treatment in most studies. Although there was broad agreement on the taxa that were differentially abundant post-intervention, the direction of change for some taxa differed between the studies. For instance, the relative abundance of *Streptococcus* [34, 147, 148], *Veillonella* [21, 148, 150, 151], *Haemophilus* [34, 145, 148] increased after macrolide use in some studies while decreasing in others.

The ability of short-term macrolide treatment to reduce the prevalence of or frequently completely eradicate *S. pneumoniae*, *S. aureus*, *H. influenzae* and *M. catarrhalis* was expected [154]. Macrolides have antibacterial activity against these bacteria [154]. They also enhance the phagocytic competency of macrophages which further eliminate these bacteria from the respiratory tract [10]. Interestingly, long-term administration of macrolides did not affect the respiratory carriage of these bacteria in most studies when end-of-treatment samples from the macrolide and placebo arms were compared. Most long-term studies were conducted in patients with chronic lung diseases who experienced acute respiratory exacerbations throughout the study period. These acute exacerbations were treated with additional non-macrolide antibiotics in both the macrolide and placebo arm. The frequency of the acute exacerbations varied between arms (higher in placebo) and across studies. This is a valid source of bias which may explained the lack of effect on bacterial prevalence observed. A more accurate method of assessing the true impact of long-term macrolide use on the respiratory microbiota will involve comparison between trial arms of the bacterial prevalence from multiple temporally separated samples throughout the study. This comparison must include samples collected immediately before and after acute exacerbations to account for the bias introduced by the use of additional non-macrolide antibiotics.

Short-term macrolide administration less frequently reduced bacterial prevalence in studies that investigated samples from the upper respiratory tract than those that included samples from the lower respiratory tract. This may be explained by the accumulation of macrolides, especially azithromycin in high concentrations in the lungs compared to the plasma [155] and upper respiratory tract tissues [156]. This high concentration can eliminate bacteria including those that have low-level macrolide

resistance [155]. This is the proposed explanation for the efficacy of azithromycin in eliminating *S. pneumoniae* strains with *in vitro* resistance to other macrolides [155].

Interestingly, macrolide use did not affect the total bacterial load in any of the studies that investigated this but differentially affected the bacterial loads of specific bacteria, including *P. aeruginosa* and *H. influenzae* in one study [24]. This may be due to replacement by macrolide-resistant bacteria resulting in the total bacterial burden being unaffected. Also, pre-existing or subsequent development of macrolide resistance and poor compliance in long-term studies may explain the overall lack of effect on the bacterial load. Finally, the high level of heterogeneity in the sample types investigated across the studies may also explain the conflicting findings relating to bacterial loads.

The infrequent detection of macrolide resistance after both short and long-term macrolide administration was unexpected. This finding may be partly explained by the large number of short-term studies in which macrolide use eradicated the bacteria of interest. For long-term studies, the reason for this observation is unclear. Macrolides can promote resistance not only to themselves but to other antibiotics such as tetracyclines, lincosamides, streptogramins and even penicillins [23]. The modification of target site that affect the binding of other antibiotics (e.g. lincosamides, streptogramins) [157–159] and the colocation of genes that confer resistance to other antibiotics (tetracyclines, penicillins) explains this phenomenon [157,158]. However, most of the studies did not report whether there was resistance to antibiotics other than macrolides. Moreover, very few studies assessed the molecular mechanisms underlying the macrolide resistance observed [21,40,143]. Only one study investigated the effect of macrolides on the respiratory resistome using shotgun metagenomic sequencing [21]. More of such studies are warranted to provide a deeper insight into how macrolides affect the antibiotic resistance burden of the entire respiratory microbiota and not only in specific bacteria.

An increase in macrolide resistance was observed in a subset of studies of long-term azithromycin treatment but not in studies of clarithromycin or erythromycin treatment, consistent with previous findings [80,160]. Azithromycin, but not clarithromycin or erythromycin, has an extended half-life (due to slow hepatic clearance) with prolonged sub-inhibitory concentrations in plasma [156,160]. This promotes the development of antibiotic resistance in resident bacteria [156,160] and may explain the disproportionate increase in macrolide resistance observed with azithromycin but not the other macrolides. However, the comparison between macrolides was likely underpowered as fewer studies administered long-term clarithromycin and erythromycin compared to azithromycin.

Few long-term studies (8/48) assessed the persistence of bacteriological effects post-intervention. Given the relatively modest and variable effect of long-term macrolide treatment on bacterial prevalence and resistance, there may be limited interest in additional studies that assess persistence of bacteriological effect. High fitness cost associated with macrolide resistance genes to the bacteria prevents the persistence of macrolide-resistant bacteria in the absence of antibiotic pressure [36]. Variability regarding the resolution of macrolide resistance post-intervention may be explained by the differences in the dose and duration of macrolides used in the studies, the sampling timepoint for assessment, the indication for treatment and baseline bacterial prevalence and resistance levels.

There were inconsistencies in the effect of macrolides on within-sample (alpha) diversity. These discrepancies may be explained by differences in alpha diversity metrics reported (Shannon, Faith, Simpson and inverse Simpson), study design (drug, dose, duration, sample type), biological (underlying disease) or technical differences in testing (16S rDNA region targeted [161], sequencing platform [162], and reference database [163,164]). One study [151] observed a reduction in alpha diversity in the placebo arm compared to azithromycin but no difference in the pre- and post-treatment samples from the active arm. This could be explained by the higher frequency of acute exacerbations in CLDs in the placebo arm with consequent use of additional medications such as other antibiotics and corticosteroids that may have affected the microbiome.

In general, macrolides, particularly azithromycin, differentially affected the relative abundance of a number of bacterial taxa which resulted in an increased similarity of the microbiome profiles within post-treatment samples compared to pre-treatment samples. Overall, the direction of change in bacterial taxa such as *Moraxella* and *Pseudomonas* genera was identical in most studies. However, there were inconsistencies in the direction of change for some selected taxa such as the *Streptococcus* genus [26,34,147]. *Streptococcus* is an important genus in respiratory tract infections [165]. *S. pneumoniae*, a species belonging to this genus, is the most common bacterial cause of pneumonia globally [166]. The inconsistency in the direction of change after long-term macrolide use may be explained by the differences in the pre-existing macrolide resistance levels in this specific bacterial genus, the macrolide type (azithromycin vs erythromycin) and limiting factors such as poor adherence associated with long-term treatments. The heterogeneity in the differential abundance testing methods used and the different variable regions of the 16S gene sequenced across the studies may also explain the inconsistencies in the direction of change in the bacterial genera. Moreover, different species within the genus exist in a competitive relationship (e.g. *Streptococcus oralis* vs *Streptococcus mutans*) [167] and this may also explain the inconsistencies in the direction of change. As all of the studies

utilised 16S rDNA metagenomic sequencing, the resolution of taxa is limited to the genus level. Shotgun metagenomic sequencing methods or pathogen-specific qPCR can resolve bacterial identities beyond the genus taxonomical level and shed light on this discrepancy.

Moraxella is another important genus in respiratory infections and chronic lung disease [165,168]. It contains the species *M. catarrhalis* which is a common cause of respiratory infections and acute pulmonary exacerbation of COPD [168,169]. This species and the overall genus are commonly susceptible to macrolides [29,170] and this explains the consistent reduction in the relative abundance of this genus observed after macrolide treatment across the studies. *Haemophilus* is also an important genus in respiratory infections and chronic lung disease [165,169]. It contains the species *H. influenzae* which is also common causes of respiratory infections [169]. Although these species and the overall genus tend to be susceptible to macrolides [171], the rapid development of macrolide resistance after exposure is common [36,172]. This may explain the increase in the relative abundance of this genus observed after macrolide treatment in some of the studies. Another important genus in respiratory infections is *Pseudomonas* [173] which contain *P. aeruginosa*, a common pathogen in chronic lung infections, especially cystic fibrosis and non-cystic bronchiectasis [24,174]. The *Pseudomonas* genus is not susceptible to macrolides [17] and therefore, a reduction in its relative abundance after macrolide use observed in some studies was quite unexpected. However, macrolides, especially azithromycin, reduce biofilm formation in *P. aeruginosa* which promotes the effectiveness of other co-administered antibiotics and innate immune responses [175]. This may account for the reduction in the relative abundance observed.

Research gaps/Implications for research

None of the studies on the long-term administration of macrolides was conducted in Africa. This may be because long-term macrolide administration was mainly used for chronic lung diseases such as cystic fibrosis, non-CF bronchiectasis, asthma, and COPD, which are more commonly investigated in high-income countries [176]. However, prescription of macrolides for conditions such as non-CF bronchiectasis and asthma is increasing in Africa [176]. Long-term azithromycin therapy for a recently-detected HIV-associated obliterative bronchiolitis has been recently studied [177]. There is a need for further studies in Africa and other low and middle-income settings to provide context-specific information on antibiotic resistance to aid local antibiotic stewardship efforts.

None of the studies investigated the effect of macrolides on other components of the microbiome such as the virome, mycome and phageome. Azithromycin, for instance, has antiviral properties [178] and

may have an effect on the virome. Few studies reported on the persistence of antibiotic resistance post-intervention, with those that did limited by few participants or variable time points for post-intervention sampling within a study. Macrolide use can promote resistance to other antibiotics such as tetracyclines, lincosamides and increase penicillin resistance. However, most studies did not investigate resistance to other classes of antibiotics.

Most microbiome studies utilised short-read 16S rDNA sequencing which is limited to genus level taxonomic resolution [179]. Shotgun metagenomic sequencing methods or pathogen-specific qPCR can resolve bacterial identities at the species level [21].

Strength and limitations

There are several strengths of this systematic review. Firstly, it provides an up-to-date summary of the effects of both short- and long-term macrolide use on the respiratory microflora and antibiotic resistance regardless of age, disease condition or clinical setting. It also evaluates the effect of macrolide use on the respiratory microbiome. Furthermore, this review highlights the knowledge gaps in the persistence of the bacteriological effects associated with macrolide use.

There are also a number of limitations to this review. Firstly, the specific details of macrolide use on bacterial carriage were not always reported in the articles or in their supplementary materials. Additional antibiotics used during interventions were not always clearly reported. Conclusions were drawn from multiple study designs and not only randomised controlled trials and this may bias findings. Some of the *a priori* subgroup analyses including dose were not conducted due to the high level of heterogeneity among the studies resulting in few studies within subgroups. Most studies used the disk diffusion method to assess antibiotic resistance hence any change in minimum inhibitory concentration that did not result in change in category was missed.

2.5 Conclusion

Short-term macrolide use commonly reduced the respiratory carriage of *S. pneumoniae*, *S. aureus*, *H. influenzae* and *M. catarrhalis* but with minimal effect on macrolide resistance due to complete eradication of baseline isolates in most studies. Long-term macrolide use did not affect the prevalence or macrolide resistance of *S. pneumoniae*, *S. aureus*, *H. influenzae* and *M. catarrhalis* in most studies. Moreover, macrolides did not affect the total bacterial load and had a limited impact on within-sample

(alpha) diversity. Macrolides differentially affected bacterial taxa resulting in changes in between-sample (beta) diversity and differential abundance of key taxa. The persistence of the effect of macrolides post-intervention, on bacterial prevalence, resistance, and composition and diversity of the respiratory microbiota remains to be rigorously characterised.

2.6 Declarations

2.6.1. Deviations from the protocol

Search conducted up to 25th January 2022 instead of 15th May 2020. We included studies where other antibiotics were administered to participants up to 2 weeks provided this was balanced between the trial arms. Some of the subgroup analyses were not conducted due to the high degree of heterogeneity in the included articles.

2.6.2 Availability of data and materials

The datasets used and analysed during the current study are reported in this thesis and in the Appendix A.

2.6.3 Competing interests

The authors declare that they have no competing interests.

2.6.4 Funding

This study was funded by the Global Health and Vaccination Research (GLOBVAC) Programme of the Medical Research Council of Norway. REA acknowledges the financial support of the Swedish International Development Cooperation Agency (SIDA) through Organisation of Women in Science for the developing world (OWSD) PhD Fellowship, Margaret McNamara Education Grants and L'Oréal UNESCO For Women in Science Fellowship. GONN acknowledges the University of Cape Town Vice Chancellor's Research Scholarship for their financial assistance toward her Doctoral degree. MN is supported by an Australian National Health and Medical Research Council Investigator Grant [APP1174455]. FSD is supported by the National Research Foundation of South Africa (112160), Future Leaders – African Independent Research (FLAIR) Fellowship, the National Institute for Health Research (NIHR) Global Health Research Unit on Mucosal Pathogens using UK aid from

the UK Government, the University of Cape Town, and the Allergy Society of South Africa (ALLSA). The funders had no role in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

2.6.5 Author contributions

REA, FSD and MN conceived the study and REA developed the protocol. REA and GONN conducted the literature search, screening and data extraction. REA conducted the data analysis and synthesis and wrote the first draft of the manuscript. All author reviewed the manuscript.

2.6.6 Additional files

Appendix A Table S1. Search terms

Appendix A Table S2. Definitions and abbreviations

Appendix A Table S3. Final search results

2.7 References

1. Durkin MJ, Jafarzadeh SR, Hsueh K, Sallah YH, Munshi KD, Henderson RR, et al. Outpatient antibiotic prescription trends in the United States: a national cohort study. *Infect Control Hosp Epidemiol*. 2018; 39:584–9.
2. Van Boeckel TP, Gandra S, Ashok A, Caudron Q, Grenfell BT, Levin SA, et al. Global antibiotic consumption 2000 to 2010: an analysis of national pharmaceutical sales data. *Lancet Infect Dis*. 2014; 14:742–50.
3. Lenz KD, Klosterman KE, Mukundan H, Kubicek-Sutherland JZ. Macrolides: from toxins to therapeutics. *Toxins*. 2021; 13:347.
4. Hart JD, Samikwa L, Sikina F, Kalua K, Keenan JD, Lietman TM, et al. Effects of biannual azithromycin mass drug administration on malaria in Malawian children: a cluster-randomized trial. *Am J Trop Med Hyg*. 2020; 103:1329–34.
5. Bloch EM, Coles CL, Kasubi M, Weaver J, Mrango Z, Munoz B, et al. Biannual treatment of preschool children with single dose azithromycin to reduce mortality: impact on azithromycin resistance in the MORDOR trial in Tanzania. *Am J Trop Med Hyg*. 2020; 103:1301–7.
6. O'Brien KS, Arzika AM, Maliki R, Manzo F, Mamkara AK, Lebas E, et al. Biannual azithromycin distribution and child mortality among malnourished children: A subgroup analysis of the MORDOR cluster-randomized trial in Niger. *PLOS Med*. 2020;17:e1003285.
7. Burr SE, Milne S, Jafali J, Bojang E, Rajasekhar M, Hart J, et al. Mass administration of azithromycin and *Streptococcus pneumoniae* carriage: cross-sectional surveys in the Gambia. *Bull World Health Organ*. 2014; 92:490–498.
8. Keenan JD, Bailey RL, West SK, Arzika AM, Hart J, Weaver J, et al. Azithromycin to reduce childhood mortality in Sub-Saharan Africa. *N Engl J Med*. 2018; 378:1583–92.
9. Zimmermann P, Ziesenitz VC, Curtis N, Ritz N. The immunomodulatory effects of macrolides—a systematic review of the underlying mechanisms. *Front Immunol*. 2018; 9:302.
10. Hodge S, Reynolds PN. Low-dose azithromycin improves phagocytosis of bacteria by both alveolar and monocyte-derived macrophages in chronic obstructive pulmonary disease subjects. *Respirol Carlton Vic*. 2012; 17:802–7.
11. Berenson CS, Kruzel RL, Eberhardt E, Sethi S. Phagocytic dysfunction of human alveolar macrophages and severity of chronic obstructive pulmonary disease. *J Infect Dis*. 2013; 208:2036–45.
12. Alchakaki A, Cramer C, Patterson A, Soubani AO. Which patients with respiratory disease need long-term azithromycin? *Cleve Clin J Med*. 2017; 84:755–8.

13. Smith D, Du Rand IA, Addy C, Collyns T, Hart S, Mitchelmore P, et al. British Thoracic Society guideline for the use of long-term macrolides in adults with respiratory disease. *BMJ Open Respir Res.* 2020;7: e000489.
14. Albert RK, Connett J, Bailey WC, Casaburi R, Cooper JADJ, Criner GJ, et al. Azithromycin for prevention of exacerbations of COPD. *N Engl J Med.* 2011; 365:689–98.
15. Weng D, Wu Q, Chen X-Q, Du Y-K, Chen T, Li H, et al. Azithromycin treats diffuse panbronchiolitis by targeting T cells via inhibition of mTOR pathway. *Biomed Pharmacother.* 2019; 110:440–8.
16. Arjuna A, Olson MT, Walia R, Bremner RM, Smith MA, Mohanakumar T. An update on current treatment strategies for managing bronchiolitis obliterans syndrome after lung transplantation. *Expert Rev Respir Med.* 2021; 15:339–50.
17. Mayer-Hamblett N, Retsch-Bogart G, Kloster M, Accurso F, Rosenfeld M, Albers G, et al. Azithromycin for Early Pseudomonas Infection in Cystic Fibrosis. The OPTIMIZE Randomized Trial. *Am J Respir Crit Care Med.* 2018; 198:1177–87.
18. Lee E, Sol IS, Kim JD, Yang H-J, Min TK, Jang GC, et al. Long-term macrolide treatment for non-cystic fibrosis bronchiectasis in children: a meta-analysis. *Sci Rep.* 2021; 11:24287.
19. Undela K, Goldsmith L, Kew KM, Ferrara G. Macrolides versus placebo for chronic asthma. *Cochrane Database Syst Rev.* 2021;11:CD002997.
20. Samson C, Tamalet A, Thien HV, Taytard J, Perisson C, Nathan N, et al. Long-term effects of azithromycin in patients with cystic fibrosis. *Respir Med.* 2016; 117:1–6.
21. Taylor SL, Leong LEX, Mobegi FM, Choo JM, Wesselingh S, Yang IA, et al. Long-term azithromycin reduces *Haemophilus influenzae* and increases antibiotic resistance in severe asthma. *Am J Respir Crit Care Med.* 2019; 200:309–317.
22. Li H, Liu D-H, Chen L-L, Zhao Q, Yu Y-Z, Ding J-J, et al. Meta-analysis of the adverse effects of long-term azithromycin use in patients with chronic lung diseases. *Antimicrob Agents Chemother.* 2014;58:511–7.
23. Serisier DJ. Risks of population antimicrobial resistance associated with chronic macrolide use for inflammatory airway diseases. *Lancet Respir Med.* 2013; 1:262–74.
24. Rogers GB, Bruce KD, Martin ML, Burr LD, Serisier DJ. The effect of long-term macrolide treatment on respiratory microbiota composition in non-cystic fibrosis bronchiectasis: an analysis from the randomised, double-blind, placebo-controlled BLESS trial. *Lancet Respir Med.* 2014; 2:988–96.
25. Segal LN, Clemente JC, Wu BG, Wikoff WR, Gao Z, Li Y, et al. Randomised, double-blind, placebo-controlled trial with azithromycin selects for anti-inflammatory microbial metabolites in the emphysematous lung. *Thorax.* 2017; 72:13–22.
26. Slater M, Rivett DW, Williams L, Martin M, Harrison T, Sayers I, et al. The impact of azithromycin therapy on the airway microbiota in asthma. *Thorax.* 2014; 69:673–4.
27. Huang YJ, Sethi S, Murphy T, Nariya S, Boushey HA, Lynch SV. Airway microbiome dynamics in exacerbations of chronic obstructive pulmonary disease. *J Clin Microbiol.* 2014; 52:2813–23.
28. Dickson RP, Morris A. Macrolides, inflammation and the lung microbiome: untangling the web of causality. *Thorax.* 2017; 72:10–2.
29. Zhang Y, Dai J, Jian H, Lin J. Effects of macrolides on airway microbiome and cytokine of children with bronchiolitis: A systematic review and meta-analysis of randomized controlled trials. *Microbiol Immunol.* 2019; 63:343–9.
30. Hansen MP, Scott AM, McCullough A, Thorning S, Aronson JK, Beller EM, et al. Adverse events in people taking macrolide antibiotics versus placebo for any indication. *Cochrane Database Syst Rev.* 2019;1:CD011825.
31. Moher D, Liberati A, Tetzlaff J, Altman DG, Altman D, Antes G, et al. Preferred reporting items for systematic reviews and meta-analyses: The PRISMA statement. *PLoS Med.* 2009;6: e1000097.
32. Abotsi RE, Ndhlovu GON, Nicol MP, Dube FS. Systematic review of the impact of macrolide use on the human respiratory bacteriome. PROSPERO [Internet]. 2020;CRD42020187477. Available from: https://www.crd.york.ac.uk/prospere/display_record.php?ID=CRD42020187477 accessed on 7th October 2022.
33. Brill SE, Law M, El-Emir E, Allinson JP, James P, Maddox V, et al. Effects of different antibiotic classes on airway bacteria in stable COPD using culture and molecular techniques: a randomised controlled trial. *Thorax.* 2015; 70:930–8.
34. Choo JM, Abell GCJ, Thomson R, Morgan L, Waterer G, Gordon DL, et al. Impact of long-term erythromycin therapy on the oropharyngeal microbiome and resistance gene reservoir in non-cystic fibrosis bronchiectasis. *mSphere.* 2018; 3: e00103-18.
35. Valery PC, Morris PS, Byrnes CA, Grimwood K, Torzillo PJ, Bauert PA, et al. Long-term azithromycin for Indigenous children with non-cystic-fibrosis bronchiectasis or chronic suppurative lung disease (Bronchiectasis Intervention Study): a multicentre, double-blind, randomised controlled trial. *Lancet Respir Med.* 2013; 1:610–20.
36. Hare KM, Grimwood K, Chang AB, Chatfield MD, Valery PC, Leach AJ, et al. Nasopharyngeal carriage and macrolide resistance in indigenous children with bronchiectasis randomized to long-term azithromycin or placebo. *Eur J Clin Microbiol Infect Dis.* 2015; 34:2275–85.

37. Gibson PG, Yang IA, Upham JW, Reynolds PN, Hodge S, James AL, et al. Effect of azithromycin on asthma exacerbations and quality of life in adults with persistent uncontrolled asthma (AMAZES): a randomised, double-blind, placebo-controlled trial. *Lancet*. 2017;390:659–68.
38. Shukla SD, Taylor SL, Gibson PG, Barker D, Upham JW, Yang IA, et al. Add-on azithromycin reduces sputum cytokines in non-eosinophilic asthma: an AMAZES sub-study. *Thorax*. 2021; 76:733–6.
39. Uzun S, Djamin RS, Kluytmans JAJW, Mulder PGH, van't Veer NE, Ermens AAM, et al. Azithromycin maintenance treatment in patients with frequent exacerbations of chronic obstructive pulmonary disease (COLUMBUS): a randomised, double-blind, placebo-controlled trial. *Lancet Respir Med*. 2014; 2:361–8.
40. Djamin RS, Talman S, Schrauwen EJA, von Wintersdorff CJH, Wolffs PF, Savelkoul PHM, et al. Prevalence and abundance of selected genes conferring macrolide resistance genes in COPD patients during maintenance treatment with azithromycin. *Antimicrob Resist Infect Control*. 2020; 9:116.
41. Djamin RS, Bafadhel M, Uzun S, Russell REK, Ermens AAM, Kerstens R, et al. Blood eosinophil count and GOLD stage predict response to maintenance azithromycin treatment in COPD patients with frequent exacerbations. *Respir Med*. 2019; 154:27–33.
42. Saiman L, Anstead M, Mayer-Hamblett N, Lands LC, Kloster M, Hocevar-Trnka J, et al. Effect of azithromycin on pulmonary function in patients with cystic fibrosis uninfected with *Pseudomonas aeruginosa*: a randomized controlled trial. *JAMA*. 2010; 303:1707–15.
43. Saiman L, Mayer-Hamblett N, Anstead M, Lands LC, Kloster M, Goss CH, et al. Open-label, follow-on study of azithromycin in pediatric patients with CF uninfected with *Pseudomonas aeruginosa*. *Pediatr Pulmonol*. 2012; 47:641–8.
44. Taylor SL, Leong LEX, Mobegi FM, Choo JM, Burr LD, Wesselingh S, et al. Understanding the impact of antibiotic therapies on the respiratory tract resistome: a novel pooled-template metagenomic sequencing strategy. *Multidiscip Respir Med*. 2018; 13:30.
45. Bacharier LB, Guilbert TW, Mauger DT, Boehmer S, Beigelman A, Fitzpatrick AM, et al. Early administration of azithromycin and prevention of severe lower respiratory tract illnesses in preschool children with a history of such illnesses: a randomized clinical trial. *JAMA*. 2015; 314:2034–44.
46. Correa A, Cabeza Y, Corssen C. Acute maxillary sinusitis. *J Int Med Res*. 1996; 24:151–4.
47. Morris PS, Gadil G, McCallum GB, Wilson CA, Smith-Vaughan HC, Torzillo P, et al. Single-dose azithromycin versus seven days of amoxicillin in the treatment of acute otitis media in Aboriginal children (AATAAC): a double blind, randomised controlled trial. *Med J Aust*. 2010; 192:24–9.
48. Morita JY, Kahn E, Thompson T, Laclaire L, Beall B, Gherardi G, et al. Impact of azithromycin on oropharyngeal carriage of group A *Streptococcus* and nasopharyngeal carriage of macrolide-resistant *Streptococcus pneumoniae*. *Pediatr Infect J*. 2000; 19:41–6.
49. Ghaffar F, Muniz LS, Katz K. Effects of amoxicillin/clavulanate or azithromycin on nasopharyngeal carriage of *Streptococcus pneumoniae* and *Haemophilus influenzae* in children with acute otitis media. *Clin Infect Dis*. 2001; 32:176–176.
50. Leach AJ, Shelby-James TM, Mayo M, Gratten M, Laming AC, Currie BJ, et al. A prospective study of the impact of community-based azithromycin treatment of trachoma on carriage and resistance of *Streptococcus pneumoniae*. *Clin Infect Dis*. 1997; 24:356–62.
51. McCallum GB, Morris PS, Chatfield MD, MacLennan C, White AV, Sloots TP, et al. A single dose of azithromycin does not improve clinical outcomes of children hospitalised with bronchiolitis: a randomised, placebo-controlled trial. *PLoS One*. 2013;8: e74316.
52. Olszewska-Sosinska O, Zielnik-Jurkiewicz B, Stepinska M, Antos-Bielska M, Lau-Dworak M, Kozłowska K, et al. Persistence of non-typeable *Haemophilus influenzae* in the pharynx of children with adenotonsillar hypertrophy after treatment with azithromycin. *Pathog Dis*. 2016;74: ftv106.
53. Ghaffar F, Muniz LS, Katz K, Smith JL, Shouse T, Davis P, et al. Effects of large dosages of amoxicillin/clavulanate or azithromycin on nasopharyngeal carriage of *Streptococcus pneumoniae*, *Haemophilus influenzae*, non-pneumococcal alpha-hemolytic streptococci, and *Staphylococcus aureus* in children with acute otitis media. *Clin Infect Dis*. 2002; 34:1301–9.
54. Ghaffar F, Friedland IR, Katz K, Muniz LS, Smith JL, Davis P, et al. Increased carriage of resistant non-pneumococcal alpha-hemolytic streptococci after antibiotic therapy. *J Pediatr*. 1999; 135:618–23.
55. Balmes P, Clerc G, Dupont B, Labram C, Pariente R, Poirier R. Comparative study of azithromycin and amoxicillin/clavulanic acid in the treatment of lower respiratory tract infections. *Eur J Clin Microbiol Infect Dis*. 1991; 10:437–9.
56. Daniel R. Simplified treatment of acute lower respiratory tract infection with azithromycin: a comparison with erythromycin and amoxicillin. European Azithromycin Study Group. *J Int Med Res*. 1991; 19:373–83.
57. Dark D. Azithromycin versus cefaclor in the treatment of acute exacerbations of chronic obstructive pulmonary disease. *Curr Ther Res - Clin Exp*. 1993; 53:203–211.
58. Davies BI, Maesen FPV, Gubbeldmans R. Azithromycin (CP-62,993) in acute exacerbations of chronic bronchitis: An open clinical, microbiological and pharmacokinetic study. *J Antimicrob Chemother*. 1989; 23:743–51.
59. DeAbate CA, Mathew CP, Warner JH, Heyd A, Church D. The safety and efficacy of short course (5-day) moxifloxacin vs azithromycin in the treatment of patients with acute exacerbation of chronic bronchitis. *Respir Med*. 2000; 94:1029–1037.

60. Galova K, Sufliarska S, Kukova Z, Danisovicova A, Hrachova I, Grausova S, et al. Multicenter randomized study of two once daily regimens in the initial management of community-acquired respiratory tract infections in 163 children: azithromycin versus cefitibuten. *Chemotherapy*. 1996; 42:231-234.
61. Gris P. Once-daily, 3-day azithromycin versus a three-times-daily, 10-day course of co-amoxiclav in the treatment of adults with lower respiratory tract infections: results of a randomized, double-blind comparative study. *J Antimicrob Chemother*. 1996;37 Suppl C:93-101.
62. Hoepelman IM, Möllers MJ, van Schie MH, Greefhorst AP, Schlösser NJ, Sinninghe Damsté EJ, et al. A short (3-day) course of azithromycin tablets versus a 10-day course of amoxycillin-clavulanic acid (co-amoxiclav) in the treatment of adults with lower respiratory tract infections and effects on long-term outcome. *Int J Antimicrob Agents*. 1997; 9:141-146.
63. Shigeru K, Kazuhiro T, Jun-ichi K, Jiro F, Yoshihito N, Akira W, et al. Contradiction between in vitro and clinical outcome: Intravenous followed by oral azithromycin therapy demonstrated clinical efficacy in macrolide-resistant pneumococcal pneumonia. *J Infect Chemother*. 2014; 20:199-207.
64. Uzun O, Hayran M, Akova M, Gur D, Akalin HE. Efficacy of a three-day course of azithromycin in the treatment of community-acquired pneumococcal pneumonia. Preliminary report. *J Chemother*. 1994; 6:53-7.
65. Whitlock W. Multicenter comparison of azithromycin and amoxicillin/clavulanate in the treatment of patients with acute exacerbations of chronic obstructive pulmonary disease. *Curr Ther Res*. 1995; 56:985-95.
66. Yanagihara K, Izumikawa K, Higa F, Tateyama M, Tokimatsu I, Hiramatsu K, et al. Efficacy of azithromycin in the treatment of community-acquired pneumonia, including patients with macrolide-resistant *Streptococcus pneumoniae* infection. *Intern Med*. 2009; 48:527-35.
67. Zachariah J. A randomized, comparative study to evaluate the efficacy and tolerability of a 3-day course of azithromycin versus a 10-day course of co-amoxiclav as treatment of adult patients with lower respiratory tract infections. *J Antimicrob Chemother*. 1996;37 Suppl C:103-13.
68. Zervos M, Martinez FJ, Amsden GW, Rothermel CD, Treadway G. Efficacy and safety of 3-day azithromycin versus 5-day moxifloxacin for the treatment of acute bacterial exacerbations of chronic bronchitis. *Int J Antimicrob Agents*. 2007; 29:56-61.
69. Malhotra-Kumar S, Lammens C, Coenen S, Van Herck K, Goossens H. Effect of azithromycin and clarithromycin therapy on pharyngeal carriage of macrolide-resistant streptococci in healthy volunteers: a randomised, double-blind, placebo-controlled study. *Lancet*. 2007; 369:482-90.
70. Rizzato G, Montemurro L, Fraioli P, Montanari G, Fanti D, Pozzoli R, et al. Efficacy of a three-day course of azithromycin in moderately severe community-acquired pneumonia. *Eur Respir J*. 1995; 8:398-402.
71. Soepandi P, Mangunegoro H, Yunus F, Gunawan J. The pattern of micro-organisms and the efficacy of new macrolide in acute lower respiratory tract infections. *Respirology*. 1998; 3:113-7.
72. Swanson RN, Lainez-Ventosilla A, De Salvo MC, Dunne MW, Amsden GW. Once-daily azithromycin for 3 days compared with clarithromycin for 10 days for acute exacerbation of chronic bronchitis: a multicenter, double-blind, randomized study. *Respir Med*. 2005; 4:31-9.
73. King A, Bathgate T, Phillips I. Erythromycin susceptibility of viridans streptococci from the normal throat flora of patients treated with azithromycin or clarithromycin. *Clin Microbiol Infect*. 2002; 8:85-92.
74. Cazzola M, Vinciguerra A, Di Perna F, Califano C, Calderaro F, Salzillo A, et al. Comparative study of dirithromycin and azithromycin in the treatment of acute bacterial exacerbations of chronic bronchitis. *J Chemother*. 1999; 11:119-25.
75. Laurent K. Efficacy, safety and tolerability of azithromycin versus roxithromycin in the treatment of acute lower respiratory tract infections. *J Antimicrob Chemother*. 1996;37 Suppl C:115-24.
76. Muller O. An open comparative study of azithromycin and roxithromycin in the treatment of acute upper respiratory tract infections. *J Antimicrob Chemother*. 1996;37 Suppl C:83-92.
77. Brook I, Hausfeld JN. Effect of telithromycin and azithromycin on nasopharyngeal bacterial flora in patients with acute maxillary sinusitis. *Arch Otolaryngol Head Neck Surg*. 2006; 132:442-5.
78. Mueller O. Comparison of azithromycin versus clarithromycin in the treatment of patients with upper respiratory tract infections. *J Antimicrob Chemother*. 1993; 31:137-46.
79. Matute A, Schurink C, Krijnen R, Florijn A, Rozenberg-Arska M, Hoepelman I. Double-blind, placebo-controlled study comparing the effect of azithromycin with clarithromycin on oropharyngeal and bowel microflora in volunteers. *Eur J Clin Microbiol Infect Dis*. 2002; 21:427-31.
80. Kastner U, Guggenbichler JP. Influence of macrolide antibiotics on promotion of resistance in the oral flora of children. *Infection*. 2001; 29:251-6.
81. Neu HC, Chick TW. Efficacy and safety of clarithromycin compared to cefixime as outpatient treatment of lower respiratory tract infections. *Chest*. 1993; 104:1393-9.

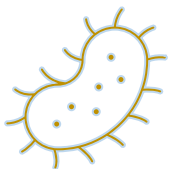
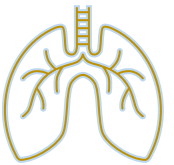
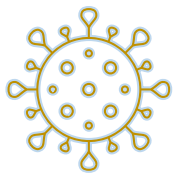
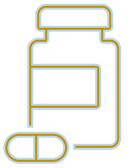
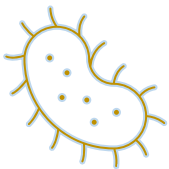
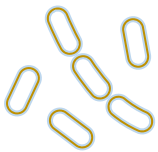
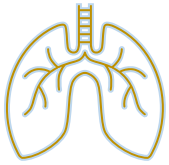
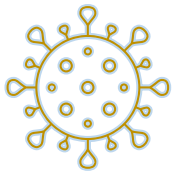
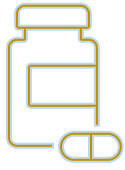
82. Hopkins DW, Trovafloxacin Study G. Trovafloxacin vs clarithromycin in patients with ambulatory community-acquired pneumonia. *Drugs*. 1999; 58:312–4.
83. Sundberg L, Cederberg A. Penetration of clarithromycin and its 14-hydroxy metabolite into middle ear effusion in children with secretory otitis media. *J Antimicrob Chemother*. 1994; 33:299–307.
84. Anzueto A, Niederman MS, Haverstock DC, Tillotson GS. Efficacy of ciprofloxacin and clarithromycin in acute bacterial exacerbations of complicated chronic bronchitis: interim analysis. Bronchitis Study Group. *Clin Ther*. 1997; 19:989–1001.
85. Anzueto A, Niederman MS, Tillotson GS. Etiology, susceptibility, and treatment of acute bacterial exacerbations of complicated chronic bronchitis in the primary care setting: Ciprofloxacin 750 mg BID versus clarithromycin 500 mg BID. *Clin Ther*. 1998; 20:885–900.
86. DeAbate CA, Myers D, Henry D, Upchurch J, Lecara G, Giguere G, et al. Efficacy and tolerability of once-daily grepafloxacin compared with clarithromycin in the treatment of acute bacterial exacerbations of chronic bronchitis. *Clin Drug Investig*. 1999; 18:243–53.
87. Guay DRP, Siepmann N, Tanaka SK, Devcich K, Craft JC. Comparative safety and efficacy of clarithromycin and 3 oral cephalosporins in the treatment of outpatients with bacterial bronchitis due to *Haemophilus influenzae*. *Drug Investig*. 1993; 6:33–41.
88. Moola S, Hagberg L, Churchyard GA, Dylewski JS, Sedani S, Staley H, et al. A multicenter study of grepafloxacin and clarithromycin in the treatment of patients with community-acquired pneumonia. *Chest*. 1999; 116:974–83.
89. Notario G, Siepmann N, Devcich K, Guay DRP. Comparative safety and efficacy of clarithromycin and three oral cephalosporins in the treatment of outpatients with bacterial bronchitis. *Curr Ther Res*. 1993; 54:628–40.
90. Tran JQ, Ballow CH, Forrest A, Hyatt JM, Sands MF, Peloquin CA, et al. Comparison of the abilities of grepafloxacin and clarithromycin to eradicate potential bacterial pathogens from the sputa of patients with chronic bronchitis: influence of pharmacokinetic and pharmacodynamic variables. *J Antimicrob Chemother*. 2000; 45:9–17.
91. Langan CE, Zuck P, Vogel F, McIvor A, Peirzchala W, Smakal M, et al. Randomized, double-blind study of short-course (5 day) grepafloxacin versus 10 day clarithromycin in patients with acute bacterial exacerbations of chronic bronchitis. *J Antimicrob Chemother*. 1999; 44:515–23.
92. Berg HF, Tjhi JHT, Scheffer G-J, Peeters MF, van Keulen PHJ, Kluytmans JAJW, et al. Emergence and persistence of macrolide resistance in oropharyngeal flora and elimination of nasal carriage of *Staphylococcus aureus* after therapy with slow-release clarithromycin: a randomized, double-blind, placebo-controlled study. *Antimicrob Agents Chemother*. 2004; 48:4183–8.
93. Yildirim I, Ceyhan M, Gur D, Kaymakoglu I. Comparison of the effect of benzathine penicillin G, clarithromycin, cefprozil and amoxicillin/clavulanate on the bacteriological response and throat flora in group A beta hemolytic streptococcal tonsillopharyngitis. *Turk J Pediatr*. 2008; 50:120–5.
94. Cazzola M, Caputi M, Santangelo G, Diu Vinciguerra A, Perna FD, Polverino M. A five-day course of dirithromycin in the treatment of acute exacerbation of severe chronic obstructive pulmonary disease. *J Chemother*. 1997; 9:279–84.
95. Palva T, Malmberg H, Lehtinen T. Effect of erythromycin on adenoid bacteria. *Acta Otolaryngol*. 1986; 101:348–52.
96. Lipsky BA, Pecoraro RE, Ahroni JH, Peugeot RL. Immediate and long-term efficacy of systemic antibiotics for eradicating nasal colonization with *Staphylococcus aureus*. *Eur J Clin Microbiol Infect Dis*. 1992; 11:43–7.
97. Trollfors B. Effect of erythromycin and amoxycillin on *Bordetella pertussis* in the nasopharynx. *Infection*. 1978; 6:228–30.
98. Schalen L, Eliasson I, Fex S, Kamme C, Schalen C. Acute laryngitis in adults: results of erythromycin treatment. *Acta Otolaryngol Suppl*. 1992; 492:55–7.
99. Schalen L, Kamme C, Eliasson I, Schalen C. Erythromycin in acute laryngitis in adults. *Ann Otol Rhinol Laryngol*. 1993; 102:209–14.
100. Darelid J, Lofgren S, Malmvall BE. Erythromycin treatment is beneficial for longstanding *Moraxella catarrhalis* associated cough in children. *Scand J Infect Dis*. 1993; 25:323–9.
101. Butzler JP, Vanhoof R, Clumeck N, de Mol P, Vanderlinden MP, Yourassowsky E. Clinical and pharmacological evaluation of different preparations of oral erythromycin. *Chemotherapy*. 1979; 25:367–72.
102. Wasilewski MM, Johns D, Sides GD. Five-day dirithromycin therapy is as effective as seven-day erythromycin therapy for acute exacerbations of chronic bronchitis. *J Antimicrob Chemother*. 1999; 43:541–8.
103. Wilson SZ, Martin RR, Putman M. In vivo effects of josamycin, erythromycin, and placebo therapy on nasal carriage of *Staphylococcus aureus*. *Antimicrob Agents Chemother*. 1977; 11:407–10.
104. Wilson SZ, Martin RR, Putman M, Greenberg SB, Wallace RJ, Jemsek JG. Quantitative nasal cultures from carriers of *Staphylococcus aureus*: effects of oral therapy with erythromycin, rosamicin, and placebo. *Antimicrob Agents Chemother*. 1979; 15:379–83.

105. Karalus NC, Garrett JE, Lang SDR, Scott WG, Leng RA, Kostalas GN, et al. A clinical and economic comparison of ft Roxithromycin 150mg twice daily vs Amoxicillin 500mg/Clavulanic Acid 125mg three times daily for the treatment of lower reespiratory tract infections in general practice. *Drug Investig.* 1994; 8:179–90.
106. Salvarezza CR, Mingrone H, Fachinelli H, Kijanczuk S. Comparison of roxithromycin with cefixime in the treatment of adults with community-acquired pneumonia. *J Antimicrob Chemother.* 1998;41 Suppl B:75–80.
107. Zeluff BJ, Lowe P, Koornhof HJ, Gentry LO. Evaluation of roxithromycin (RU-965) versus cephradine in pneumococcal pneumonia. *Eur J Clin Microbiol Infect Dis.* 1988; 7:69–71.
108. Brook I, Hausfeld JN. Recovery of interfering bacteria in the nasopharynx following antimicrobial therapy of acute maxillary sinusitis with telithromycin or amoxicillin-clavulanate. *Antimicrob Agents Chemother.* 2005; 49:4793–4.
109. Tellier G, Niederman MS, Nusrat R, Patel M, Lavin B. Clinical and bacteriological efficacy and safety of 5- and 7-day regimens of telithromycin once daily compared with a 10 day regimen of clarithromycin twice daily in patients with mild to moderate community-acquired pneumonia. *J Antimicrob Chemother.* 2004; 54:515–23.
110. Van Herck A, Frick AE, Schaevers V, Vranckx A, Verbeke EK, Vanaudenaerde BM, et al. Azithromycin and early allograft function after lung transplantation: A randomized, controlled trial. *J Heart Lung Transplant.* 2019; 38:252–9.
111. Maniakas A, Asmar M-H, Renteria AE, Nayan S, Alromaih S, Endam LM, et al. Azithromycin in high-risk, refractory chronic rhinosinusitis after endoscopic sinus surgery and corticosteroid irrigations: a double-blind, randomized, placebo-controlled trial. *Int Forum Allergy Rhinol.* 2021; 11:747–54.
112. Videler WJ, Badia L, Harvey RJ, Gane S, Georgalas C, van der Meulen FW, et al. Lack of efficacy of long-term, low-dose azithromycin in chronic rhinosinusitis: a randomized controlled trial. *Allergy.* 2011; 66:1457–68.
113. Gray GC, McPhate DC, Leinonen M, Cassell GH, Deperalta EP, Putnam SD, et al. Weekly oral azithromycin as prophylaxis for agents causing acute respiratory disease. *Clin Infect Dis.* 1998; 26:103–10.
114. Marchisio P, Principi N, Sala E, Lanzoni L, Sorella S, Massimini A. Comparative study of once-weekly azithromycin and once-daily amoxicillin treatments in prevention of recurrent acute otitis media in children. *Antimicrob Agents Chemother.* 1996; 40:2732–6.
115. Guchev IA, Gray GC, Klochkov OI. Two regimens of azithromycin prophylaxis against community-acquired respiratory and skin/soft-tissue infections among military trainees. *Clin Infect Dis.* 2004; 38:1095–101.
116. Brusselle GG, Vanderstichele C, Jordens P, Deman R, Slabbynck H, Ringoet V, et al. Azithromycin for prevention of exacerbations in severe asthma (AZISAST): a multicentre randomised double-blind placebo-controlled trial. *Thorax.* 2013; 68:322–9.
117. Putnam SD, Gray GC, Biedenbach DJ, Jones RN. Pharyngeal colonization prevalence rates for *Streptococcus pyogenes* and *Streptococcus pneumoniae* in a respiratory chemoprophylaxis intervention study using azithromycin. *Clin Microbiol Infect.* 2000; 6:2–8.
118. Altenburg J, de Graaff CS, Stienstra Y, Sloos JH, van Haren EHJ, Koppers RJH, et al. Effect of azithromycin maintenance treatment on infectious exacerbations among patients with non-cystic fibrosis bronchiectasis: the BAT randomized controlled trial. *JAMA.* 2013; 309:1251–9.
119. Berkhof FF, Doornwaard-ten Hertog NE, Uil SM, Kerstjens HAM, van den Berg JWK. Azithromycin and cough-specific health status in patients with chronic obstructive pulmonary disease and chronic cough: a randomised controlled trial. *Respir Res.* 2013; 14:125.
120. Diego AD, Milara J, Martinez-Moragón E, Palop M, León M, Cortijo J. Effects of long-term azithromycin therapy on airway oxidative stress markers in non-cystic fibrosis bronchiectasis. *Respirol Carlton Vic.* 2013; 18:1056–62.
121. Kobbernagel HE, Buchvald FF, Haarman EG, Casaulta C, Collins SA, Hogg C, et al. Efficacy and safety of azithromycin maintenance therapy in primary ciliary dyskinesia (BESTCILIA): a multicentre, double-blind, randomised, placebo-controlled phase 3 trial. *Lancet Respir Med.* 2020; 8:493–505.
122. Milito C, Pulvirenti F, Cinetto F, Lougaris V, Soresina A, Pecoraro A, et al. Double-blind, placebo-controlled, randomized trial on low-dose azithromycin prophylaxis in patients with primary antibody deficiencies. *J Allergy Clin Immunol.* 2019; 144:584–593.e7.
123. Phaff SJ, Tiddens HAWM, Verbrugh HA, Ott A. Macrolide resistance of *Staphylococcus aureus* and *Haemophilus* species associated with long-term azithromycin use in cystic fibrosis. *J Antimicrob Chemother.* 2006; 57:741–6.
124. Simpson JL, Powell H, Baines KJ, Milne D, Coxson HO, Hansbro PM, et al. The effect of azithromycin in adults with stable neutrophilic COPD: a double blind randomised, placebo-controlled trial. *PloS One.* 2014; 9: e105609.
125. Steinkamp G, Schmitt-Grohe S, Döring G, Staab D, Pfründer D, Beck G, et al. Once-weekly azithromycin in cystic fibrosis with chronic *Pseudomonas aeruginosa* infection. *Respir Med.* 2008; 102:1643–53.
126. Vermeersch K, Belmans A, Bogaerts K, Gyselinck I, Cardinaels N, Gabrovská M, et al. Treatment failure and hospital readmissions in severe COPD exacerbations treated with azithromycin versus placebo - a post-hoc analysis of the BACE randomized controlled trial. *Respir Res.* 2019; 20:237.

127. Wolter J, Seeney S, Bell S, Bowler S, Masel P, McCormack J. Effect of long-term treatment with azithromycin on disease parameters in cystic fibrosis: a randomised trial. *Thorax*. 2002; 57:212–6.
128. Wong C, Jayaram L, Karalus N, Eaton T, Tong C, Hockey H, et al. Azithromycin for prevention of exacerbations in non-cystic fibrosis bronchiectasis (EMBRACE): a randomised, double-blind, placebo-controlled trial. *The Lancet*. 2012; 380:660–7.
129. Hansen CR, Pressler T, Hoiby N, Johansen HK. Long-term, low-dose azithromycin treatment reduces the incidence but increases macrolide resistance in *Staphylococcus aureus* in Danish CF patients. *J Cyst Fibros*. 2009; 8:58–62.
130. Clement A, Tamalet A, Leroux E, Ravilly S, Fauroux B, Jais J-P. Long term effects of azithromycin in patients with cystic fibrosis: A double blind, placebo controlled trial. *Thorax*. 2006; 61:895–902.
131. Emiralioğlu N, Öztürk Z, Yalçın E, Doğru D, Özçelik U, Kiper N. Long term azithromycin therapy in patients with cystic fibrosis. *Turk J Pediatr*. 2016; 58:34–40.
132. Saiman L, Marshall BC, Mayer-Hamblett N, Burns JL, Quittner AL, Cibene DA, et al. Azithromycin in patients with cystic fibrosis chronically infected with *Pseudomonas aeruginosa*: a randomized controlled trial. *JAMA*. 2003; 290:1749–56.
133. Samson C, Tamalet A, Thien HV, Taytard J, Perisson C, Nathan N, et al. Long-term effects of azithromycin in patients with cystic fibrosis. *Respir Med*. 2016; 117:1–6.
134. Tramper-Stranders GA, Wolfs TFW, Fleer A, Kimpen JLL, van der Ent CK. Maintenance azithromycin treatment in pediatric patients with cystic fibrosis: long-term outcomes related to macrolide resistance and pulmonary function. *Pediatr Infect Dis J*. 2007; 26:8–12.
135. McCormack J, Bell S, Senini S, Walmsley K, Patel K, Wainwright C, et al. Daily versus weekly azithromycin in cystic fibrosis patients. *Eur Respir J*. 2007; 30:487–95.
136. Equi A, Balfour-Lynn IM, Bush A, Rosenthal M. Long term azithromycin in children with cystic fibrosis: a randomised, placebo-controlled crossover trial. *Lancet*. 2002; 360:978–84.
137. Kabra SK, Pawaiya R, Lodha R, Kapil A, Kabra M, Vani AS, et al. Long-term daily high and low doses of azithromycin in children with cystic fibrosis: a randomized controlled trial. *J Cyst Fibros*. 2010; 9:17–23.
138. Blasi F, Bonardi D, Aliberti S, Tarsia P, Confalonieri M, Amir O, et al. Long-term azithromycin use in patients with chronic obstructive pulmonary disease and tracheostomy. *Pulm Pharmacol Ther*. 2010; 23:200–7.
139. Aberg JA, Wong MK, Flamm R, Notario GF, Jacobson MA. Presence of macrolide resistance in respiratory flora of HIV-Infected patients receiving either clarithromycin or azithromycin for *Mycobacterium avium* complex prophylaxis. *HIV Clin Trials*. 2001; 2:453–9.
140. Iino Y, Sasaki Y, Miyazawa T, Kodera K. Nasopharyngeal flora and drug susceptibility in children with macrolide therapy. *The Laryngoscope*. 2010; 113:1780–5.
141. Vowels BR, Feingold DS, Sloughfy C, Foglia AN, Konnikov N, Ordoukhanian E, et al. Effects of topical erythromycin on ecology of aerobic cutaneous bacterial flora. *Antimicrob Agents Chemother*. 1996; 40:2598–604.
142. Seemungal TAR, Wilkinson TMA, Hurst JR, Perera WR, Sapsford RJ, Wedzicha JA. Long-term erythromycin therapy is associated with decreased chronic obstructive pulmonary disease exacerbations. *Am J Respir Crit Care Med*. 2008; 178:1139–47.
143. Taylor SL, Leong LEX, Mobegi FM, Choo JM, Burr LD, Wesselingh S, et al. Understanding the impact of antibiotic therapies on the respiratory tract resistome: a novel pooled-template metagenomic sequencing strategy. *Multidiscip Respir Med*. 2018; 13:30.
144. Fujii T, Kadota J, Kawakami K, Iida K, Shirai R, Kaseda M, et al. Long term effect of erythromycin therapy in patients with chronic *Pseudomonas aeruginosa* infection. *Thorax*. 1995; 50:1246–52.
145. Acosta N, Thornton CS, Surette MG, Somayaji R, Rossi L, Rabin HR, et al. Azithromycin and the microbiota of cystic fibrosis sputum. *BMC Microbiol*. 2021; 21:96.
146. Zhou Y, Bacharier LB, Isaacson-Schmid M, Baty J, Schechtman KB, Sajol G, et al. Azithromycin therapy during respiratory syncytial virus bronchiolitis: upper airway microbiome alterations and subsequent recurrent wheeze. *J Allergy Clin Immunol*. 2016; 138:1215–1219.e5.
147. Spence CD, Vanaudenaerde B, Einarsson GG, McDonough J, Lee AJ, Johnston E, et al. Influence of azithromycin and allograft rejection on the post-lung transplant microbiota. *J Heart Lung Transplant*. 2020; 39:176–83.
148. Slater M, Rivett DW, Williams L, Martin M, Harrison T, Sayers I, et al. The impact of azithromycin therapy on the airway microbiota in asthma. *Thorax*. 2014; 69:673–4.
149. Segal LN, Clemente JC, Wu BG, Wikoff WR, Gao Z, Li Y, et al. Randomised, double-blind, placebo-controlled trial with azithromycin selects for anti-inflammatory microbial metabolites in the emphysematous lung. *Thorax*. 2017; 72:13–22.
150. Lopes dos Santos Santiago G, Brusselle G, Dauwe K, Deschaght P, Verhofstede C, Vanechoutte D, et al. Influence of chronic azithromycin treatment on the composition of the oropharyngeal microbial community in patients with severe asthma. *BMC Microbiol*. 2017; 17:109.

151. Renteria AE, Maniakas A, Mfunu LE, Asmar M, Gonzalez E, Desrosiers M. Low-dose and long-term azithromycin significantly decreases *Staphylococcus aureus* in the microbiome of refractory CRS patients. *Int Forum Allergy Rhinol*. 2021; 11:93–105.
152. Chen H, Zhou B, Huang Q, Li C, Wu Y, Huang Z, et al. Efficacy and safety of long-term low-dose clarithromycin in patients with refractory chronic sinusitis after endoscopic sinus surgery: a prospective clinical trial. *Ear Nose Throat J*. 2021; 1455613211032020.
153. Rogers GB, Bruce KD, Martin ML, Burr LD, Serisier DJ. The effect of long-term macrolide treatment on respiratory microbiota composition in non-cystic fibrosis bronchiectasis: an analysis from the randomised, double-blind, placebo-controlled BLESS trial. *Lancet Respir Med*. 2014; 2:988–96.
154. Dinos GP. The macrolide antibiotic renaissance: The present and future of macrolide antibiotics. *Br J Pharmacol*. 2017; 174:2967–83.
155. Kobuchi S, Fujita A, Kato A, Kobayashi H, Ito Y, Sakaeda T. Pharmacokinetics and lung distribution of macrolide antibiotics in sepsis model rats. *Xenobiotica*. 2020; 50:552–8.
156. Davidson RJ. In vitro activity and pharmacodynamic/pharmacokinetic parameters of clarithromycin and azithromycin: why they matter in the treatment of respiratory tract infections. *Infect Drug Resist*. 2019; 12:585–96.
157. Chancey ST, Agrawal S, Schroeder MR, Farley MM, Tettelin H, Stephens DS. Composite mobile genetic elements disseminating macrolide resistance in *Streptococcus pneumoniae*. *Front Microbiol*. 2015; 6.
158. De Vries LE, Christensen H, Skov RL, Aarestrup FM, Agersø Y. Diversity of the tetracycline resistance gene tet (M) and identification of Tn 916- and Tn 5801-like (Tn 6014) transposons in *Staphylococcus aureus* from humans and animals. *J Antimicrob Chemother*. 2009; 64:490–500.
159. Schroeder MR, Stephens DS. Macrolide Resistance in *Streptococcus pneumoniae*. *Front Cell Infect Microbiol*. 2016; 6:98.
160. Eisenblätter M, Klaus C, Pletz MWR, Orawa H, Hahn H, Wagner J, et al. Influence of azithromycin and clarithromycin on macrolide susceptibility of viridans streptococci from the oral cavity of healthy volunteers. *Eur J Clin Microbiol Infect Dis*. 2008; 27:1087–92.
161. Soriano-Lerma A, Pérez-Carrasco V, Sánchez-Marañón M, Ortiz-González M, Sánchez-Martín V, Gijón J, et al. Influence of 16S rRNA target region on the outcome of microbiome studies in soil and saliva samples. *Sci Rep*. 2020; 10:13637.
162. Clooney AG, Fouhy F, Sleator RD, O’ Driscoll A, Stanton C, Cotter PD, et al. Comparing Apples and Oranges?: Next Generation Sequencing and Its Impact on Microbiome Analysis. *PLoS ONE*. 2016; 11: e0148028.
163. Faner R, Sibila O, Agustí A, Bernasconi E, Chalmers JD, Huffnagle GB, et al. The microbiome in respiratory medicine: current challenges and future perspectives. *Eur Respir J*. 2017; 49.
164. Ramakodi MP. Influence of 16S rRNA reference databases in amplicon-based environmental microbiome research. *Biotechnol Lett* [Internet]. 2022; Available from: <https://doi.org/10.1007/s10529-022-03233-2>
165. Kelly MS, Surette MG, Smieja M, Pernica JM, Rossi L, Luinstra K, et al. The nasopharyngeal microbiota of children with respiratory infections in Botswana. *Pediatr Infect Dis J*. 2017;36: e211–8.
166. Lanks CW, Musani AI, Hsia DW. Community-acquired pneumonia and hospital-acquired pneumonia. *Med Clin North Am*. 2019; 103:487–501.
167. Thurnheer T, Belibasakis GN. *Streptococcus oralis* maintains homeostasis in oral biofilms by antagonizing the cariogenic pathogen *Streptococcus mutans*. *Mol Oral Microbiol*. 2018; 33:234–9.
168. Perez AC, Murphy TF. A *Moraxella catarrhalis* vaccine to protect against otitis media and exacerbations of COPD: An update on current progress and challenges. *Hum Vaccines Immunother*. 2017; 13:2322–31.
169. Riesbeck K. Complement evasion by the human respiratory tract pathogens *Haemophilus influenzae* and *Moraxella catarrhalis*. *FEBS Lett*. 2020; 594:2586–97.
170. Lei W-T, Lin HH, Tsai M-C, Hung H-H, Cheng Y-J, Liu S-J, et al. The effects of macrolides in children with reactive airway disease: a systematic review and meta-analysis of randomized controlled trials. *Drug Des Devel Ther*. 2018; 12:3825–45.
171. Zhang Z, Chen M, Yu Y, Pan S, Liu Y. Antimicrobial susceptibility among *Streptococcus pneumoniae* and *Haemophilus influenzae* collected globally between 2015 and 2017 as part of the Tigecycline Evaluation and Surveillance Trial (TEST). *Infect Drug Resist*. 2019; 12:1209–20.
172. Zhou M, Fu P, Fang C, Shang S, Hua C, Jing C, et al. Antimicrobial resistance of *Haemophilus influenzae* isolates from pediatric hospitals in Mainland China: Report from the ISPED program, 2017-2019. *Indian J Med Microbiol*. 2021; 39:434–8.
173. Ahmed B, Cox MJ, Cuthbertson L, James PL, Cookson WOC, Davies JC, et al. Comparison of the upper and lower airway microbiota in children with chronic lung diseases. *PloS One*. 2018; 13: e0201156–e0201156.
174. Vidaillac C, Chotirmall SH. *Pseudomonas aeruginosa* in bronchiectasis: infection, inflammation, and therapies. *Expert Rev Respir Med*. 2021; 15: 649–62.

175. Elsheredy A, El-Soudany I, Elsherbini E, Metwally D, Ghazal A. Effect of azithromycin and phenylalanine-arginine beta-naphthylamide on quorum sensing and virulence factors in clinical isolates of *Pseudomonas aeruginosa*. *Iran J Microbiol*. 2021; 13: 37–49.
176. Soriano JB, Kendrick PJ, Paulson KR, Gupta V, Abrams EM, Adedoyin RA, et al. Prevalence and attributable health burden of chronic respiratory diseases, 1990–2017: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet Respir Med*. 2020; 8:585–96.
177. Ferrand RA, McHugh G, Rehman AM, Mujuru H, Simms V, Majonga ED, et al. Effect of Once-Weekly Azithromycin vs Placebo in Children With HIV-Associated Chronic Lung Disease: The BREATHE Randomized Clinical Trial. *JAMA Netw Open*. 2020; 3: e2028484.
178. Oliver ME, Hinks TSC. Azithromycin in viral infections. *Rev Med Virol*. 2021; 31: e2163.
179. Sillanpää S, Kramna L, Oikarinen S, Sipilä M, Rautiainen M, Aittoniemi J et al. Next-generation sequencing combined with specific PCR assays to determine the bacterial 16S rRNA gene profiles of middle ear fluid collected from children with acute otitis media. *mSphere*. 2017; 2: e00006-17.



Chapter 3

Prevalence and antimicrobial resistance profiles of respiratory microbial flora in African children with HIV-associated chronic lung disease

Prevalence and antimicrobial resistance profiles of respiratory microbial flora in African children with HIV-associated chronic lung disease

Regina E. Abotsi^{1,2*}, Mark P. Nicol³, Grace McHugh⁴, Victoria Simms⁵, Andrea M. Rehman⁵, Charmaine Barthus⁶, Slindile Mbhele⁶, Brewster W. Moyo⁷, Lucky G. Ngwira^{7,8}, Hilda Mujuru⁹, Beauty Makamure⁴, Justin Mayini⁴, Jon Ø Odland^{10,11,12}, Rashida A. Ferrand^{4,13}, Felix S. Dube¹

Affiliations

1. Department of Molecular and Cell Biology & Institute of Infectious Diseases and Molecular Medicine, University of Cape Town, Cape Town, South Africa
2. Department of Pharmaceutical Microbiology, School of Pharmacy, University of Health and Allied Sciences, Ho, Ghana
3. Division of Infection and Immunity, School of Biomedical Sciences, Faculty of Health and Medical Sciences, University of Western Australia, Perth, Australia
4. Biomedical Research and Training Institute, Harare, Zimbabwe
5. MRC International Statistics & Epidemiology Group, London School of Hygiene and Tropical Medicine, London, UK
6. Division of Medical Microbiology, University of Cape Town, Cape Town, South Africa
7. Malawi-Liverpool Wellcome Trust Clinical Research Programme, Blantyre, Malawi
8. Liverpool School of Tropical Medicine, Liverpool, UK
9. Department of Paediatrics, University of Zimbabwe, Harare, Zimbabwe
10. Department of Community Medicine, University of Tromsø, Tromsø, Norway
11. International Research Laboratory for Reproductive Ecotoxicology (IL RET), The National Research University Higher School of Economics, Moscow, Russia
12. School of Health Systems and Public Health, Faculty of Health Sciences, University of Pretoria, South Africa
13. Clinical Research Department, London School of Hygiene and Tropical Medicine, London, United Kingdom

3.0 Abstract

Background: HIV-associated chronic lung disease (CLD) is common among children living with HIV (CLWH) in sub-Saharan Africa, including those on antiretroviral therapy (ART). However, the pathogenesis of CLD and its possible association with microbial determinants remain poorly understood. We investigated the prevalence and antibiotic susceptibility of *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Haemophilus influenzae* and *Moraxella catarrhalis* among CLWH (established on ART) who had CLD (CLD+), or not (CLD-) in Zimbabwe and Malawi.

Methods: Nasopharyngeal swabs and sputa were collected from CLD+ CLWH (defined as forced-expiratory volume per second z-score < -1 without reversibility post-bronchodilation with salbutamol), at baseline as part of a randomised, placebo-controlled trial of azithromycin (BREATHE trial - NCT02426112), and from age- and sex-matched CLD- CLWH. Samples were cultured, and antibiotic susceptibility testing was conducted using disk diffusion. Risk factors for bacterial carriage were identified using questionnaires and analysed using multivariate logistic regression.

Results: A total of 410 participants (336 CLD+, 74 CLD-) were enrolled (median age, 15 years [IQR = 13–18]). *S. pneumoniae* and *M. catarrhalis* carriage in nasopharyngeal swabs were higher in CLD+ than in CLD- children: 46% (154/336) vs 26% (19/74), $p = 0.008$; and 14% (49/336) vs 3% (2/74), $p = 0.012$, respectively. *S. pneumoniae* isolates from the nasopharyngeal swabs of CLD+ children were more likely to be non-susceptible to penicillin than those from CLD- children (36% [53/144] vs 11% [2/18], $p = 0.036$). Methicillin-resistant *S. aureus* was uncommon [4% (7/195)]. In multivariate analysis, key factors associated with NP bacterial carriage included having CLD (*S. pneumoniae*: adjusted odds ratio (aOR) 2 [95% CI 1.1–3.9]), younger age (*S. pneumoniae*: aOR 3.2 [1.8–5.8]), viral load suppression (*S. pneumoniae*: aOR 0.6 [0.4–1.0], *S. aureus*: 0.5 [0.3–0.9]), stunting (*S. pneumoniae*: aOR 1.6 [1.1–2.6]) and male sex (*S. aureus*: aOR 1.7 [1.0–2.9]). Sputum bacterial carriage was similar in both groups (50%) and was associated with Zimbabwean site (*S. pneumoniae*: aOR 3.1 [1.4–7.3], *S. aureus*: 2.1 [1.1–4.2]), being on ART for a longer period (*S. pneumoniae*: aOR 0.3 [0.1–0.8]), and hot compared to rainy season (*S. pneumoniae*: aOR 2.3 [1.2–4.4]).

Conclusions: CLD+ CLWH were more likely to be colonised by *M. catarrhalis* and *S. pneumoniae*, including penicillin-non-susceptible *S. pneumoniae* strains, than CLD- CLWH. The role of these bacteria in CLD pathogenesis, including the risk of acute exacerbations, should be further studied.

Keywords: *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Moraxella catarrhalis*, *Haemophilus influenzae*, antibiotic resistance, children, HIV, chronic lung disease.

3.1 Background

More than 85% of the 3.8 million children living with HIV (CLWH) worldwide reside in sub-Saharan Africa [1]. Substantial scale-up of antiretroviral therapy (ART) programmes and increased access to cotrimoxazole prophylaxis have remarkably improved the survival of perinatally HIV-infected children, many whom would have died in infancy in the absence of these interventions [2]. As these children grow older, complications of long-standing HIV infection are becoming increasingly evident, including delayed growth and cardiorespiratory diseases [3].

Respiratory diseases, in particular, are responsible for more than 50% of all HIV-related mortality in sub-Saharan Africa [4]. Recent studies in Malawi [5], Zimbabwe [6–8] and South Africa [9] have estimated 30% prevalence of chronic lung disease (CLD) among CLWH. The typical clinical picture is that of hypoxia, tachypnoea, chronic cough, reduced exercise tolerance and impaired lung function [6, 10]; high resolution computed tomography findings are consistent with constrictive obliterative bronchiolitis [11].

Although the pathogenesis of HIV-associated CLD is incompletely understood, we and others have postulated that it may be a sequela of recurrent respiratory tract infections and dysregulated inflammation associated with HIV infection [12, 13]. Bacteria previously implicated in forms of HIV-associated CLDs such as bronchiectasis in individuals living with HIV include *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Haemophilus influenzae* and *Moraxella catarrhalis* [14, 15]; however, their role in HIV-associated CLD pathogenesis is unclear.

We investigated the prevalence, bacterial load and antibiotic susceptibility of *S. pneumoniae*, *S. aureus*, *H. influenzae*, *M. catarrhalis* and other Gram-negative bacilli (GNB) recovered from the nasopharyngeal swabs and sputa of CLWH (established on ART) who had CLD (CLD+) or not (CLD). We also investigated the risk factors for the carriage of these bacteria.

3.2 Materials and Methods

3.2.1 Study design, settings, and participants

This is a case-control study nested within the BREATHE study – a multi-site, double-blind, placebo-controlled, individually randomised trial that investigated the efficacy of azithromycin therapy in CLD

(ClinicalTrials.gov Identifier: NCT02426112) [12]. The study setting, population, and procedures of the trial are described elsewhere [10, 16]. Briefly, perinatally HIV-infected children aged 6–19 years with CLD (CLD+) who had been receiving ART for a minimum of six months were enrolled from outpatient HIV clinics in Blantyre, Malawi and Harare, Zimbabwe, from June 2016 through August 2018. CLD was defined as forced expiratory volume in 1 second (FEV₁) z-score less than -1.0 with no reversibility (< 12% improvement in FEV₁ after 200 µg of inhaled salbutamol). The justification for this definition is provided elsewhere [10]. A comparison group (CLD-) of perinatally HIV-infected children without CLD (FEV₁ z-score > 0) was recruited at the same time as enrolment of trial participants using frequency matching for site, age (6–12 and 13–19 years) and duration of ART use (6 months to < 2 years and ≥ 2 years). These children had no heart or apparent lung disease and reported no respiratory symptoms. Socio-demographic data and clinical history were obtained through an interviewer-administered questionnaire.

3.2.2 Sample collection, transportation, and processing

NP swabs were obtained at baseline using nylon flocked swabs (Copan Italia, Brescia, Italy) by study staff. Sputum was subsequently obtained (expectorated or induced where necessary). Samples were immediately stored in 1 mL of skim milk, tryptone, glucose, and glycerine (STGG) medium, placed on ice and transported to the laboratory within an one hour where they were frozen down to at -80°C. The samples were transported on dry ice with temperature monitoring to Cape Town, South Africa, for batch processing.

3.2.3 Bacterial culture, identification, and antimicrobial susceptibility testing

Nasopharyngeal swabs and sputum samples were thawed to 22°C and vortexed for 30 seconds. A 10µl volume of each sample was inoculated onto Bacitracin-heated blood agar (BHB), Columbia with Gentamicin agar (Colgent), Mannitol salt agar (MSA) and 2% sheep blood agar (BA) (NHLS Greenpoint Media, Cape Town, South Africa). The BHB, Colgent and BA were incubated in 5% carbon dioxide at 37°C for 48 hours, whilst the MSA was incubated in ambient air at 37°C for 48 hours. Alpha haemolytic colonies on Colgent, which were susceptible to optochin, were presumptively identified as *S. pneumoniae*. Colourless, medium-sized colonies on BHB with a strict requirement for factor X (Hemin) and/or V (NAD⁺) were presumed to be *H. influenzae* or *Haemophilus parainfluenzae*, respectively. Non-haemolytic grey to white colonies on BA that tested positive for the push test were identified as *M. catarrhalis*. DNase-positive colonies from MSA were identified as *S. aureus*. Colonies, other than *M. catarrhalis* and *H. influenzae*, from BA and BHB that grew on

MacConkey agar were presumptively identified as ‘other’ gram-negative bacilli (GNB) by colony morphology and Gram staining. The species identities of these GNBs were confirmed using Matrix-Assisted Laser Desorption/Ionization-Time-of-Flight mass spectrometry (MALDI-TOF MS, Lancet laboratories, Cape Town, South Africa). Bacterial load was semi-quantitatively assessed as follows: an aliquot of each sample was streaked for single colonies. Growth in one, two, three or all quadrants of a culture plate was assigned the labels 1, 2, 3 and 4, respectively. Antimicrobial susceptibility testing was conducted using the Kirby-Bauer disk diffusion method. The antibiotics tested for each pathogen were as follows: *S. pneumoniae* (oxacillin, cotrimoxazole, azithromycin and tetracycline), *S. aureus* (cotrimoxazole, azithromycin, tetracycline, clindamycin, penicillin, and cefoxitin), *H. influenzae* (cotrimoxazole, azithromycin, tetracycline, ampicillin, amoxicillin-clavulanate, and cefuroxime) and *M. catarrhalis* (amoxicillin-clavulanate, cotrimoxazole, azithromycin and tetracycline). *S. aureus* susceptibility to cefoxitin was tested as a surrogate for methicillin resistance. Antimicrobial susceptibility testing was conducted and interpreted according to the 2018 Clinical and Laboratory Standards Institute guidelines and breakpoints, respectively [17].

3.2.4 Statistical Analysis

R version 3.6.0 (R Foundation for Statistical Computing, Vienna, Austria; <https://www.r-project.org/>) was used to conduct statistical analyses. The 1990 British growth reference curves[18] were used to generate height-for-age and weight-for-age *z*-scores. The lung function *z*-scores were calculated using global lung function initiative GLI/ERS reference equations and the African American module [19]. Our team validated this among children in Zimbabwe and the findings were published elsewhere [20]. Comparison of categorical data, including semi-quantitative bacterial load distribution, was performed by Fisher’s exact test or Chi-square test where appropriate. Univariate and multivariate analyses of association with carriage of each bacterial species were performed using logistic regression and presented as odds ratios (OR) and adjusted ORs with a 95% confidence interval (CI), respectively. The following variables were selected *a priori* to investigate the risk factors for carriage of each bacterium: CLD, sex, age category (6–12, 13–16, 17–19 years), study site (Zimbabwe or Malawi), the season of sample collection (Rainy season: December to April, Cool season: May to August, Hot season: September to November), HIV viral load, previous TB treatment and ART regimen and duration. Other variables identified as independent predictors of carriage in univariate analysis ($p < 0.25$) were also included in the adjusted model for that species. The following were excluded from the multivariate model because of co-linearity: Baseline BMI-for-age *z*-score and weight-for-age (colinear with height-for-age), and CD4 count (colinear with viral load suppression).

3.3 Results

3.3.1 Participant clinical and socio-demographic characteristics

The study included 336 CLD+ and 74 CLD- participants, median (IQR) age 15 (13–18) years (Table 3.1). The median FEV1 *z*-score for CLD+ and CLD- children was -1.96 (IQR -2.46, -1.47) and 0.52 (IQR 0.23, 0.79), $p < 0.001$ respectively. The CLD+ group was more stunted (50% vs 30% [$p < 0.001$]) and underweight (52% vs 19% [$p < 0.001$]) than the CLD- group, and a higher proportion of CLD+ had been previously treated for tuberculosis (29% vs 12% [$p = 0.003$]). Overall, 90% were taking cotrimoxazole prophylaxis. Both groups had been on ART for a median of 6.5 years but more CLD+ participants were on a protease inhibitor-based (second line) ART regimen than CLD- participants (26% vs 11% [$p < 0.05$]). Virologic suppression was similar between groups (CLD+: 56% vs CLD-: 66% [$p = 0.12$]). None of the participants reported smoking.

Table 3. 1. Baseline characteristics of study participants

Variable		CLD+	CLD-	<i>p</i>
Socio-demographic				
Site	Zimbabwe	72% (241/336)	74% (55/74)	0.770
Sex	Female	49% (166/336)	62% (46/74)	0.054
Age	Median (IQR)	15 (13–18)	16 (12–18)	0.939
Anthropometric				
BMI-for-age <i>z</i> -score ²	Median (IQR)	-1.114 (-1.85, -0.28)	-0.32 (-0.83, -0.58)	< 0.001
Height-for-age <i>z</i> -score ^a	< -2 (Stunted)	50% (168/336)	30% (22/74)	0.002
Weight-for-age <i>z</i> -score ^a	< -2 (Underweight)	52% (176/336)	19% (14/74)	< 0.001
Current drug use				
Cotrimoxazole (CTX)		91% (304/334)	86% (60/70)	0.188
Antiretroviral regimen	^λ NNRTI-based regimen -1 st line	74% (247/335)	89% (65/73)	0.006
	[§] PI-based regimen-2 nd line	26% (88/335)	11% (8/73)	
HIV clinical parameters				
CD4 count categories	> 500 cells/mm ³	62% (208/336)	67% (49/73)	0.770
	200 - 500 cells/mm ³	28% (94/336)	25% (18/73)	
	< 200 cells/mm ³	10% (34/336)	8% (6/73)	
Viral Load (VL) suppression	VL < 1000 copies/mL	56% (187/336)	66% (49/74)	0.119
Age at ART initiation	Median (IQR)	8 (6-12)	9 (5-11)	0.667
Years spent on ART	Median (IQR)	6 (4-9)	7 (4-9)	0.571
Respiratory status				
Previously treated for TB, % (N)		29% (97/335)	12% (9/73)	0.003
FEV1 <i>z</i> -score	Median (IQR)	-1.96 (-2.46, -1.47)	0.52 (0.23, 0.79)	< 0.001
Admitted for chest problems in last 12 months		2% (6/336)	1% (1/74)	1
Asthma		3% (11/336)	0% (0/74)	0.227
MRC dyspnoea Score	1	55% (185/336)	78% (59/73)	0.001
	2	35% (120/336)	16% (12/73)	
	3	6% (19/336)	3% (2/73)	
	4	3% (10/336)	0% (0/73)	
	5	1% (2/336)	0% (0/73)	

Participants with missing responses are excluded from that variable. Only one participant (from the CLD group) uses an inhaler.^λ
 Nonnucleoside Reverse Transcriptase Inhibitor, [§] Protease inhibitor

3.3.2 Prevalence of bacterial carriage

In the CLD+ group, 67% (226/336) of the nasopharyngeal swabs had at least one of the four bacterial species (*S. pneumoniae*, *S. aureus*, *H. influenzae* and *M. catarrhalis*) compared to 39% (29/74) of swabs from the CLD- group ($p < 0.001$). Both *S. pneumoniae* (46% vs 26% [adjusted $p = 0.008$]), and *M. catarrhalis* (14% vs 3% [adjusted $p = 0.012$]) were more prevalent in the CLD+ group (Table 3.2). Four hundred sputa from 326 CLD+ and 74 CLD- participants were collected. Ninety-nine percent of the sputa (397/400) were spontaneously expectorated, and three sputa from CLD+ were induced. At least one bacterial species was isolated from the sputa of half of the participants in each group, with no difference by CLD status (Table 3.2). There was no difference in semi-quantitative loads of any bacteria from either nasopharyngeal swabs or sputum between the two groups (Appendix B. Table S1).

Table 3. 2. Prevalence of bacterial isolates among study participants

Bacterial species	Nasopharyngeal swabs			Sputum samples		
	CLD+	CLD-	<i>p</i>	CLD+	CLD-	<i>p</i>
	(n = 336)	(n = 74)		(n = 326)	(n = 74)	
	No. of isolates (%)			No. of isolates (%)		
<i>S. pneumoniae</i>	154 (46%)	19 (26%)	0.008	83 (25%)	17 (23%)	1
<i>S. aureus</i>	77 (23%)	9 (12%)	0.164	93 (29%)	21 (28%)	1
<i>H. influenzae</i>	40 (12%)	4 (5%)	0.576	12 (4%)	2 (3%)	1
<i>M. catarrhalis</i>	49 (15%)	2 (3%)	0.012	30 (9%)	4 (5%)	1
≥ 1 bacterial species	226 (67%)	29 (39%)	< 0.001	161(49%)	37 (50%)	1

Fisher's exact test was used for comparison. Statistical significance: p -value < 0.05 . p -values adjusted using the Bonferroni method.

Thirteen different GNBs from the nasopharyngeal swabs and sputa were identified by the MALDI-TOF MS platform. More GNBs were recovered from the CLD+ than in CLD- group (Appendix B. Table S2). Among bacteria isolated from nasopharyngeal swabs and sputa, there were statistically significant co-carriage relationships between *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* carriage (Appendix B. Table S3). These co-carriage relationships were independent of age or site.

3.3.3 Risk factors associated with carriage of bacteria

In multivariate analysis, participants on ART for two or more years were less likely to carry *S. pneumoniae* in both nasopharyngeal swab and sputum (Tables 3.3 and 3.4). Risk factors associated with *S. pneumoniae* carriage in the nasopharyngeal swabs were having CLD (adjusted OR: 2.0 [1.06–3.89], $p = 0.036$), younger age (e.g. being 6–12 years old at the time of sampling compared to 17–19 years (adjusted OR: 3.2 [1.76–5.85], $p > 0.001$), and stunting (height-for-age z -score < -2) (adjusted OR: 1.6 [1.05–2.58], $p = 0.03$). Participants with suppressed viral load (< 1000 copies/mL) (adjusted OR: 0.6 [0.38–0.95], $p = 0.032$), were less likely to carry *S. pneumoniae* in their nasopharyngeal swabs (Table 3.3). Participants in Zimbabwe (adjusted OR: 3.1 [1.43–7.34], $p = 0.006$), sample collected in hot season (adjusted OR: 2.3 [1.22–4.4], $p = 0.036$) and previous tuberculosis treatment (adjusted OR: 1.8 [1.02–3.17], $p = 0.043$) were associated with sputum carriage of *S. pneumoniae* (Table 3.4). Male participants had increased odds of carrying *S. aureus* in their nasopharyngeal swabs (adjusted OR: 1.7 [1.01–2.92], $p = 0.048$) whilst participants with suppressed viral loads (< 1000 copies/mL) were less likely to carry *S. aureus* in their nasopharyngeal swabs (adjusted OR: 0.5 [0.32–0.91], $p = 0.021$) (Table 3.3). For sputa, participants from Zimbabwe had higher odds of carrying *S. aureus* (adjusted OR: 2.1 [1.05–4.2], $p = 0.038$) than those from Malawi (Table 3.4). With regards to *H. influenzae* (Table 3.5 and 3.6), participants from Zimbabwe (adjusted OR: 3.9 [1.47–11.74], $p = 0.009$), those aged 13 to 16 years at sampling (adjusted OR: 3.6 [1.46–10.22], $p = 0.031$), and those that had MRC dyspnoea score > 1 (adjusted OR: 2.6 [1.16–5.75], $p = 0.02$) were more likely to carry *H. influenzae* in their nasopharyngeal swabs (Table 3.5). No other variable tested was associated with sputum *H. influenzae* carriage (Table 3.6). Sampling in the hot and cool seasons (May to November) compared to the rainy season (adjusted OR: 3.1 [1.25–8.08], $p = 0.036$), participants whose ages were less than 17 to 19 years (adjusted OR: 4 [1.39–13.22], $p = 0.039$), and participants who had MRC dyspnoea score > 1 (adjusted OR: 2.4 [1.06–5.43], $p = 0.036$), were more likely to carry *M. catarrhalis* in their nasopharyngeal swabs. In contrast, participants who had used ART for two or more years (adjusted OR: 0.3 [0.07–0.93], $p = 0.008$) were less likely to carry *M. catarrhalis* in their nasopharyngeal swabs (Table 3.5). No other variable tested was associated with sputum *M. catarrhalis* carriage (Table 3.6).

Table 3. 3. Univariate and multivariate analysis of factors associated with nasopharyngeal *S. pneumoniae* and *S. aureus* colonisation.

Variable	No. observations (n = 410) [§]	<i>Streptococcus pneumoniae</i>				<i>Staphylococcus aureus</i>						
		No. isolates (n = 173) [¶]	OR [95% CI]	<i>p</i>	Adjusted OR [95% CI]	<i>p</i>	No. isolates (n = 86) ^λ	OR [95% CI]	<i>p</i>	Adjusted OR [95% CI]	<i>p</i>	
Group												
	CLD-	18% (74)	11% (19)	Reference		Reference		10% (9)	Reference		Reference	
	CLD+	82% (336)	89% (154)	2.5 [1.4-4.4]	0.002	2.0[1.1-3.9]	0.04	90% (77)	2.2 [1.1-4.8]	0.04	1.8 [0.8-4.5]	0.17
Study site												
	Malawi	28% (114)	32% (56)	Reference		Reference		31% (27)	Reference		Reference	
	Zimbabwe	72% (296)	68% (117)	0.7 [0.4-1.1]	0.08	1.3 [0.7-2.4]	0.42	69% (59)	0.8 [0.5-1.4]	0.40	0.7 [0.4-1.5]	0.39
Sex												
	Female	52% (212)	54% (94)	Reference		Reference		41% (35)	Reference		Reference	
	Male	48% (198)	46% (79)	0.8 [0.6-1.2]	0.36	0.7 [0.4-1.0]	0.07	59% (51)	1.8 [1.1-2.9]	0.02	1.7 [1.0-2.9]	0.05
Season of sample collection												
	Dec - Apr - Rainy season	36% (149)	32% (56)	Reference		Reference		36% (31)	Reference		Reference	
	May - Aug - Cool season	39% (160)	43% (74)	1.4 [0.9-2.3]	0.30	1.7 [1.0-2.9]	0.13	29% (25)	0.7 [0.4-1.3]	0.03	0.9 [0.5-1.6]	0.11
	Sep - Nov - Hot season	25% (101)	25% (43)	1.2 [0.7-2.1]		1.3 [0.7-2.3]		35% (30)	1.6 [0.9-2.9]		1.7 [0.9-3.2]	
Baseline age category												
	17–19 years	32% (131)	20% (35)	Reference		Reference		28% (24)	Reference		Reference	
	13–16 years	41% (168)	47% (81)	2.6 [1.6-4.2]	< 0.001	2.2 [1.3-3.8]	< 0.001	43% (37)	1.3 [0.7-2.3]	0.66	1.1 [0.6-2.1]	0.83
	6–12 years	27% (111)	33% (57)	2.9 [1.7-5.0]		3.2 [1.8-5.8]		29% (25)	1.3 [0.7-2.4]		1.2 [0.6-2.5]	
Number of years on ART												
	6 months – < 2 years	9% (35)	14% (23)	Reference		Reference		7% (6)	Reference		Reference	
	2 – < 4 years	18% (72)	18% (30)	0.4 [0.2-0.9]	0.02	0.4 [0.2-1.0]	0.04	18% (15)	1.3 [0.5-3.9]	0.94	1.2 [0.4-3.9]	0.78
	4 – < 6 years	20% (81)	22% (36)	0.4 [0.2-0.9]		0.4 [0.2-1.0]		22% (18)	1.4 [0.5-4.1]		1.4 [0.5-4.4]	
	6 years+	53% (214)	47% (78)	0.3 [0.1-0.6]		0.3 [0.1-0.7]		53% (44)	1.3 [0.5-3.5]		1.0 [0.4-3.0]	
CD4 count												
	> 500	63% (257)	62% (108)	Reference				63% (54)	Reference			
	200-500	27% (112)	24% (42)	0.8 [0.5-1.3]	0.09			30% (26)	1.1 [0.7-1.9]	0.55		
	< 200	10% (40)	13% (23)	1.9 [1.0-3.7]				7% (6)	0.7 [0.2-1.6]			
Baseline viral load												
	Unsuppressed	42% (174)	46% (80)	Reference		Reference		55% (47)	Reference		Reference	
	Suppressed	58% (236)	54% (93)	0.8[0.5-1.1]	0.18	0.6 [0.4-1.0]	0.03	45% (39)	0.5 [0.3-0.9]	0.01	0.5 [0.3-0.9]	0.02
ART regimen												
	PI-based regimen-2 nd line	24% (98)	25% (44)	Reference		Reference		33% (28)	Reference		Reference	
	NNRTI-based regimen-1 st line	76% (312)	75% (129)	0.9 [0.6-1.4]	0.55	0.8 [0.5-1.4]	0.44	67% (58)	0.6 [0.3-1.0]	0.03	0.6 [0.3-1.1]	0.07
Ever treated for TB												
	No	74% (302)	70% (120)	Reference		Reference		71% (61)	Reference		Reference	
	Yes	26% (106)	30% (52)	1.5 [0.9-2.3]	0.10	1.5 [0.9-2.5]	0.12	29% (25)	1.2 [0.7-2.1]	0.46	0.9 [0.5-1.7]	0.83

Baseline weight- for-age z-score											
Not underweight	54% (220)	49% (85)	Reference				49% (42)	Reference			
Underweight	46% (190)	51% (88)	1.4 [0.9-2.0]	0.12			51% (44)	1.3 [0.8-2.1]	0.31		
Baseline height-for-age z-score											
Not stunted	54% (220)	45% (78)	Reference			Reference	52% (45)	Reference		Reference	
Stunted	46% (190)	55% (95)	1.8 [1.2-2.7]	0.003		1.6 [1.1-2.6]	0.03	48% (41)	1.1 [0.7-1.7]	0.78	0.8 [0.5-1.3] 0.39
Ever repeated a grade											
No	46% (183)	41% (69)	Reference					42% (36)	Reference		
Yes	54% (218)	59% (100)	1.4 [0.9-2.1]	0.10				58% (49)	1.2 [0.7-1.9]	0.49	
MRC dyspnoea score											
1 or 0	60% (244)	53% (91)	Reference			Reference		56% (48)	Reference		Reference
>1	40% (165)	47% (81)	1.6 [1.1-2.4]	0.02		1.3 [0.7-2.2]	0.36	44% (37)	1.2 [0.7-1.9]	0.50	1.0 [0.5-1.8] 0.94

§ Missing values: Number of years on ART (8), CD4 count (1), Ever treated for TB (2), Ever repeated a grade (9) and MRC dyspnoea score (1). º Number of years on ART (n = 167), ART regimen (n = 172), Ever treated for TB (n = 172), Ever repeated a grade (n = 169), MRC dyspnoea score (n = 172). ²n = 83, 85 and 85 for the number of years on ART, ever repeated a grade and MRC dyspnoea score. Variables with two levels where a level is less than 10% of total observations are not tested for associations. These included current school attendance and taking cotrimoxazole. Variables that have values in the adjusted odd ratios column were included in the multivariate model for that bacterium. Variables with *p* values < 0.25 were included in the multivariate model except where they are colinear with another variable within the model. Weight-for-age was colinear with height-for-age and hence excluded from the model. Ever repeated a grade was excluded because of co-linearity with MRC score. CD4 count is colinear with viral load suppression. Clinically relevant variables (*a priori*-defined) that were included in the multivariate model regardless of significance were group, age, sex, site, season of sample collection, number of years on ART, baseline viral load, ART regimen, ever treated for TB, baseline height-for-age z-score and MRC dyspnoea score.

Table 3. 4. Univariate and multivariate analysis of factors associated with sputum *S. pneumoniae* and *S. aureus* colonisation.

Variable	No. observations (n = 400) §	<i>Streptococcus pneumoniae</i>						<i>Staphylococcus aureus</i>					
		No. isolates (n = 100)¶	OR [95% CI]	<i>p</i>	Adjusted OR [95% CI]	<i>p</i>	No. isolates (n = 114)⋀	OR [95% CI]	<i>p</i>	Adjusted OR [95% CI]	<i>p</i>		
Group													
	CLD-	18% (74)	17% (17)	Reference		Reference		18% (21)	Reference		Reference		
	CLD+	82% (326)	83% (83)	1.2 [0.6-2.1]	0.66	1.3 [0.6-2.6]	0.52	82% (93)	1.0 [0.6-1.8]	0.98	1.0 [0.5-1.8]	0.92	
Study site													
	Malawi	27% (108)	13% (13)	Reference		Reference		18% (21)	Reference		Reference		
	Zimbabwe	73% (292)	87% (87)	3.1 [1.7-6.1]	< 0.001	3.1 [1.4-7.3]	0.01	82% (93)	1.9 [1.2-3.4]	0.02	2.1 [1.1-4.2]	0.04	
Sex													
	Female	52% (207)	52% (52)	Reference		Reference		50% (57)	Reference		Reference		
	Male	48% (193)	48% (48)	1 [0.6-1.6]	0.95	0.9 [0.6-1.5]	0.73	50% (57)	1.1 [0.7-1.7]	0.66	1.1 [0.7-1.8]	0.57	
Season of sample collection													
	Dec - Apr - Rainy	36% (143)	26% (26)	Reference		Reference		32% (37)	Reference		Reference		
	May - Aug - Cool	39% (157)	41% (41)	1.6 [0.9-2.8]	0.03	1.6 [0.9-3.0]	0.04	37% (42)	1.1 [0.6-1.8]	0.25	1.1 [0.6-1.8]	0.27	

	Sep - Nov - Hot	25% (100)	33% (33)	2.2 [1.2-4.1]		2.3 [1.2-4.4]		31% (35)	1.5 [0.9-2.7]		1.6 [0.9-2.8]	
Baseline age category												
	17–19 years	33% (130)	38% (38)	Reference		Reference		39% (45)	Reference		Reference	
	13–16 years	40% (161)	39% (39)	0.8 [0.5-1.3]	0.34	0.8 [0.5-1.4]	0.38	38% (43)	0.7 [0.4-1.1]	0.15	0.7 [0.4-1.2]	0.25
	6–12 years	27% (109)	23% (23)	0.7 [0.4-1.2]		0.6 [0.3-1.2]		23% (26)	0.6 [0.3-1.0]		0.6 [0.3-1.1]	
Number of years on ART												
	6 months – < 2 years	9% (35)	12% (12)	Reference		Reference		9% (10)	Reference		Reference	
	2 – < 4 years	18% (71)	21% (21)	0.8 [0.3-1.9]		0.6 [0.2-1.5]		17% (19)	0.9 [0.4-2.3]		0.7 [0.3-1.8]	
	4 – < 6 years	20% (79)	19% (19)	0.6 [0.3-1.5]	0.39	0.4 [0.2-1.0]	0.04	23% (26)	1.2 [0.5-3.0]	0.83	0.9 [0.4-2.4]	0.57
	6 years+	53% (208)	47% (47)	0.6 [0.3-1.2]		0.3 [0.1-0.8]		51% (58)	1 [0.5-2.2]		0.6 [0.3-1.6]	
CD4 count												
	> 500	63% (251)	63% (63)	Reference				59% (67)	Reference			
	200-500	28% (110)	26% (26)	0.9 [0.5-1.6]	0.81			32% (36)	1.3 [0.8-2.2]	0.51		
	< 200	10% (38)	11% (11)	1.2 [0.6-2.5]				10% (11)	1.1 [0.5-2.3]			
Baseline viral load												
	Unsuppressed	42% (169)	35% (35)	Reference				47% (54)	Reference		Reference	
	Suppressed	58% (231)	65% (65)	1.5 [0.9-2.4]	0.09	1.4 [0.8-2.3]	0.22	53% (60)	0.8 [0.5-1.2]	0.19	0.7 [0.4-1.1]	0.09
ART regimen												
	PI-based regimen-2 nd line	24% (98)	27% (27)	Reference		Reference		25% (28)	Reference		Reference	
	NNRTI-based regimen-1 st line	76% (302)	73% (73)	0.9 [0.5-1.5]	0.57	1.1 [0.6-2.1]	0.75	75% (86)	1 [0.6-1.7]	0.947	1.2 [0.7-2.2]	0.54
Ever treated for TB												
	No	74% (294)	65% (65)	Reference		Reference		72% (82)	Reference		Reference	
	Yes	26% (104)	35% (35)	1.8 [1.1-2.9]	0.02	1.8 [1.0-3.2]	0.04	28% (32)	1.2 [0.7-1.9]	0.58	1.0 [0.6-1.8]	0.92
Baseline weight- for-age z-score												
	Not underweight	54% (217)	61% (61)	Reference				54% (61)	Reference			
	Underweight	46% (183)	39% (39)	0.7 [0.4-1.1]	0.12			46% (53)	1.0 [0.7-1.6]	0.85		
Baseline height-for-age z-score												
	Not stunted	55% (218)	59% (59)	Reference		Reference		58% (66)	Reference		Reference	
	Stunted	46% (182)	41% (41)	0.8 [0.5-1.2]	0.3	0.9 [0.5-1.5]	0.64	42% (48)	0.8 [0.5-1.3]	0.39	0.8 [0.5-1.3]	0.46
Ever repeated a grade												
	No	46% (180)	49% (49)	Reference				47% (54)	Reference			
	Yes	54% (211)	51% (50)	0.8 [0.5-1.3]	0.43			53% (60)	0.9 [0.6-1.4]	0.74		
MRC dyspnoea score												
	1 or 0	60% (240)	68% (67)	Reference		Reference		65% (73)	Reference		Reference	
	>1	40% (159)	32% (32)	0.7 [0.4-1.0]	0.079	0.9 [0.5-1.7]	0.86	35% (40)	0.8 [0.5-1.2]	0.25	1.1 [0.6-2.0]	0.67

[§] Missing values: Number of years on ART (7), CD4 count (1), Ever treated for TB (2), Ever repeated a grade (9) and MRC dyspnoea score (1). [°] n = 99 for the number of years on ART, ever repeated a grade, MRC dyspnoea score. ^λ n = 113 for the number of years on ART and MRC dyspnoea score. Variables with two levels where a level is less than 10% of total observations were not tested for associations. These included current school attendance and taking cotrimoxazole. Variables that have values in the adjusted odd ratios column were included in the multivariate model for that bacterium. Variables with *p* values < 0.25 were included in the multivariate model except where they were colinear with another variable within the model. Weight-for-age was colinear with height-for-age and hence excluded from the model. Ever repeated a grade was excluded because of co-linearity with MRC score. CD4 count is colinear with viral load suppression. Clinically relevant variables (*a priori*) that were included in the multivariate model regardless of significance were group, age, sex, site, season of sample collection, number of years on ART, baseline viral load, ART regimen, ever treated for TB, baseline height-for-age z-score and MRC dyspnoea score.

Table 3. 5. Univariate and multivariate analysis of factors associated with nasopharyngeal *H. influenzae* and *M. catarrhalis* colonisation.

Variable	No. observations (n = 410) §	<i>Haemophilus influenzae</i>					<i>Moraxella catarrhalis</i>					
		No. isolates (n = 44) σ	OR [95% CI]	<i>p</i>	Adjusted OR [95% CI]	<i>p</i>	No. isolates (n = 51) ξ	OR [95% CI]	<i>p</i>	Adjusted OR [95% CI]	<i>p</i>	
Group												
	CLD-	18% (74)	9% (4)	Reference		Reference		4% (2)	Reference		Reference	
	CLD+	82% (336)	91% (40)	2.4 [0.9-8.1]	0.11	1.5 [0.5-5.7]	0.49	96% (49)	6.2 [1.9-38.2]	0.01	4.0 [1.1-26.2]	0.08
Study site												
	Malawi	28% (114)	23% (10)	Reference		Reference		49% (25)	Reference		Reference	
	Zimbabwe	72% (296)	77% (34)	1.4 [0.7-3.0]	0.43	3.9 [1.5-11.7]	0.01	51% (26)	0.3 [0.2-0.6]	< 0.001	0.6 [0.3-1.4]	0.27
Sex												
	Female	52% (212)	61% (27)	Reference		Reference		51% (26)	Reference		Reference	
	Male	48% (198)	39% (17)	0.6 [0.3-1.2]	0.18	0.6 [0.3-1.2]	0.13	49% (25)	1.0 [0.6-1.9]	0.91	1.0 [0.5-1.9]	0.90
Season of sample collection												
	Dec - Apr - Rainy season	36% (149)	30% (13)	Reference		Reference		20% (10)	Reference		Reference	
	May - Aug - Cool season	39% (160)	50% (22)	1.7 [0.8-3.5]	0.29	1.9 [0.8-4.4]	0.24	47% (24)	2.5 [1.2-5.6]	0.03	2.7 [1.2-6.7]	0.04
	Sep - Nov - hot season	25% (101)	20% (9)	1.0 [0.4-2.5]		1.0 [0.4-2.7]		33% (17)	2.8 [1.3-6.7]		3.1 [1.3-8.1]	
Baseline age category												
	17–19 years	32% (131)	16% (7)	Reference		Reference		10% (5)	Reference		Reference	
	13–16 years	41% (168)	61% (27)	3.4 [1.5-8.7]	0.01	3.6 [1.5-10.2]	0.03	55% (28)	5.0 [2.1-15.2]	0.004	3.5 [1.3-11.1]	0.04
	6–12 years	27% (111)	23% (10)	1.8 [0.7-5.0]		2.4 [0.8-7.7]		35% (18)	4.9 [1.9-15.2]		4.0 [1.4-13.2]	
Number of years on ART												
	6 months – < 2 years	9% (35)	15% (6)	Reference		Reference		22% (11)	Reference		Reference	
	2 – < 4 years	18% (72)	22% (9)	0.7 [0.2-2.2]	0.40	0.6 [0.2-2]	0.22	12% (6)	0.2 [0.1-0.6]	0.01	0.3 [0.1-0.8]	0.09
	4 – < 6 years	20% (81)	20% (8)	0.5 [0.2-1.7]		0.4 [0.1-1.4]		16% (8)	0.2 [0.1-0.7]		0.3 [0.1-0.9]	
	6 years+	53% (214)	44% (18)	0.4 [0.2-1.3]		0.3 [0.1-1.0]		50% (25)	0.3 [0.1-0.7]		0.3 [0.1-0.9]	
CD4 count												
	> 500	63% (257)	55% (24)	Reference				55% (28)	Reference			
	200-500	27% (112)	20% (9)	0.9 [0.4-1.8]	0.003			31% (16)	1.4 [0.7-2.6]	0.4		
	< 200	10% (40)	25% (11)	3.7 [1.6-8.2]				14% (7)	1.7 [0.7-4.1]			
Baseline viral load												
	Unsuppressed	42% (174)	50% (22)	Reference		Reference		47% (24)	Reference		Reference	
	Suppressed	58% (236)	50% (22)	0.7 [0.4-1.3]	0.28	0.6 [0.3-1.2]	0.13	53% (27)	0.8 [0.5-1.5]	0.48	0.6 [0.3-1.2]	0.14
ART regimen												
	PI-based regimen-2nd line	24% (98)	27% (12)	Reference		Reference		29% (15)	Reference		Reference	
	NNRTI-based regimen-1st line	76% (312)	73% (32)	0.8 [0.4-1.9]	0.74	0.9 [0.4-2.2]	0.83	71% (36)	0.8 [0.4-1.5]	0.43	0.6 [0.3-1.5]	0.27
Ever treated for TB												

	No	74% (302)	61% (27)	Reference		Reference		70% (35)	Reference		Reference	
	Yes	26% (106)	39% (17)	2.0 [1.0-3.7]	0.05	2.1 [1.0-4.6]	0.06	30% (15)	1.3 [0.6-2.4]	0.49	1.2 [0.5-2.6]	0.63
Baseline weight- for-age z-score												
	Not underweight	54% (220)	55% (24)	Reference				37% (19)	Reference			
	Underweight	46% (190)	45% (20)	1.0 [0.5-1.8]	0.90			63% (32)	2.1 [1.2-4]	0.01		
Baseline height-for-age z-score												
	Not stunted	54% (220)	57% (25)	Reference		Reference		37% (19)	Reference		Reference	
	Stunted	46% (190)	43% (19)	0.9 [0.5-1.6]	0.66	0.7 [0.3-1.5]	0.40	63% (32)	2.1 [1.2-4]	0.01	1.5 [0.8-3.0]	0.25
Ever repeated a grade												
	No	46% (183)	36% (15)	Reference				28% (14)	Reference			
	Yes	54% (218)	64% (27)	1.6 [0.8-3.2]	0.18			72% (36)	2.4 [1.3-4.7]	0.01		
MRC dyspnoea score												
	1 or 0	60% (244)	50% (22)	Reference		Reference		33% (17)	Reference		Reference	
	>1	40% (165)	50% (22)	1.6 [0.8-2.9]	0.17	2.6 [1.2-5.8]	0.02	67% (34)	3.5 [1.9-6.6]	< 0.001	2.4 [1.1-5.4]	0.04

§ Missing values: Number of years on ART (8), CD4 count (1), Ever treated for TB (2), Ever repeated a grade (9) and MRC dyspnoea score (1). ^σ n = 41 and 42 for the number of years on ART and Ever repeated a grade respectively ^ξ n = 50 for the number of years on ART, ever treated for TB & ever repeated a grade. Variables with two levels where a level is less than 10% of total observations were not tested for associations. These included current school attendance and taking cotrimoxazole. Variables that have values in the adjusted odd ratios column were included in the multivariate model for that bacterium. Variables with *p* values < 0.25 were included in the multivariate model except where they are colinear with another variable within the model. Weight-for-age was colinear with height-for-age and hence excluded from the model. Ever repeated a grade was excluded because of co-linearity with MRC score. CD4 count is colinear with viral load suppression. Clinically relevant variables (*a priori*-defined) that were included in the multivariate model regardless of significance were group, age, sex, site, season of sample collection, number of years on ART, baseline viral load, ART regimen, ever treated for TB, baseline height-for-age z-score and MRC dyspnoea score.

Table 3. 6. Univariate and multivariate analysis of factors associated with sputum *H. influenzae* and *M. catarrhalis* colonisation.

Variable	No. observations (n = 400) §	<i>Haemophilus influenzae</i>						<i>Moraxella catarrhalis</i>			
		No. isolates (n = 14)σ	OR [95% CI]	<i>p</i>	Adjusted OR [95% CI]	<i>p</i>	# isolates (n = 34)ξ	OR [95% CI]	<i>p</i>	Adjusted OR [95% CI]	<i>p</i>
Group											
	CLD-	18% (74)	14% (2)	Reference		Reference		12% (4)	Reference		Reference
	CLD+	82% (326)	86% (12)	1.4 [0.4-9.0]	0.68	0.8 [0.1-6.6]	0.82	88% (30)	1.8 [0.7-6.1]	0.30	1.4 [0.5-5.0] 0.6
Study site											
	Malawi	27% (108)	43% (6)	Reference		Reference		38% (13)	Reference		Reference
	Zimbabwe	73% (292)	57% (8)	0.5 [0.2-1.5]	0.18	1.1 [0.2-7.7]	0.88	62% (21)	0.6 [0.3-1.2]	0.13	0.6 [0.2-1.7] 0.36
Sex											
	Female	52% (207)	57% (8)	Reference		Reference		59% (20)	Reference		Reference
	Male	48% (193)	43% (6)	0.8 [0.3-2.3]	0.68	1.5 [0.4-5.8]	0.53	41% (14)	0.7 [0.4-1.5]	0.39	0.6 [0.3-1.3] 0.18
Season of sample collection											
	Dec - Apr - Rainy	36% (143)	50% (7)	Reference		Reference		32% (11)	Reference		Reference

	May - Aug - Cool	39% (157)	43% (6)	0.8 [0.2-2.4]	0.32	0.9 [0.2-3.7]	0.42	38% (13)	1.1 [0.5-2.6]	0.81	1.0 [0.4-2.6]	0.65
	Sep - Nov - hot	25% (100)	7% (1)	0.2 [0.01-1.1]		0.2 [0.0-1.6]		29% (10)	1.3 [0.5-3.3]		1.5 [0.6-3.9]	
Baseline age category												
	17–19 years	33% (130)	14% (2)	Reference	0.32	Reference	0.24	24% (8)	Reference	0.16	Reference	0.39
	13–16 years	40% (161)	57% (8)	3.3 [0.8-22.4]		6.1 [0.9-119.9]		56% (19)	2.0 [0.9-5.1]		1.6 [0.6-4.0]	
	6–12 years	27% (109)	29% (4)	2.4 [0.5-17.8]		6.7 [0.9-140.2]		21% (7)	1.0 [0.4-3.0]		0.9 [0.3-2.6]	
Number of years on ART												
	6 months – < 2 years	9% (35)	9% (1)	Reference	0.58	Reference	0.68	15% (5)	Reference	0.38	Reference	0.39
	2 – < 4 years	18% (71)	9% (1)	0.5 [0.0-12.5]		0.3 [0.0-8.6]		24% (8)	0.8 [0.2-2.7]		0.8 [0.2-2.9]	
	4 – < 6 years	20% (79)	36% (4)	1.8 [0.3-36.2]		1.3 [0.1-27.4]		18% (6)	0.5 [0.1-1.8]		0.5 [0.1-2.0]	
	6 years+	53% (208)	45% (5)	0.8 [0.1-16.3]		0.8 [0.1-16.4]		42% (14)	0.4 [0.2-1.4]		0.4 [0.1-1.4]	
CD4 count												
	> 500	63% (251)	43% (6)	Reference	0.01			62% (21)	Reference	0.9		
	200-500	28% (110)	21% (3)	1.1 [0.2-4.4]				26% (9)	1.0 [0.4-2.1]			
	< 200	10% (38)	36% (5)	6.2 [1.7-21.7]				12% (4)	1.3 [0.4-3.6]			
Baseline viral load												
	Unsuppressed	42% (169)	64% (9)	Reference	0.1	Reference	0.13	47% (16)	Reference	0.55	Reference	0.64
	Suppressed	58% (231)	36% (5)	0.4 [0.1-1.2]		0.4 [0.1-1.3]		53% (18)	0.8 [0.4-1.7]		0.8 [0.4-1.8]	
ART regimen												
	PI-based regimen-2nd line	24% (98)	14% (2)	Reference	0.19	Reference	0.23	29% (10)	Reference	0.66	Reference	0.49
	NNRTI-based regimen-1st line	76% (302)	86% (12)	3.9 [0.8-72.1]		3.9 [0.6-77.4]		71% (24)	0.8 [0.4-2.0]		0.7 [0.3-1.9]	
Ever treated for TB												
	No	74% (294)	64% (9)	Reference	0.41	Reference	0.09	76% (26)	Reference	0.72	Reference	0.68
	Yes	26% (104)	36% (5)	1.6 [0.5-4.7]		3.2 [0.8-12.3]		24% (8)	0.9 [0.4-1.9]		0.8 [0.3-2.0]	
Baseline weight- for-age z-score												
	Not underweight	54% (217)	50% (7)	Reference	0.75			32% (11)	Reference	0.01		
	Underweight	46% (183)	50% (7)	1.2 [0.4-3.6]				68% (23)	2.7 [1.3-5.9]			
Baseline height-for-age z-score												
	Not stunted	55% (218)	57% (8)	Reference	0.84	Reference	0.65	41% (14)	Reference	0.11	Reference	0.17
	Stunted	46% (182)	43% (6)	0.9 [0.3-2.6]		1.4 [0.4-5.3]		59% (20)	1.8 [0.9-3.7]		1.7 [0.8-3.9]	
Ever repeated a grade												
	No	46% (180)	50% (7)	Reference	0.76			39% (13)	Reference	0.43		
	Yes	54% (211)	50% (7)	0.9 [0.3-2.5]				61% (20)	1.4 [0.7-2.9]			
MRC dyspnoea score												
	1 or 0	60% (240)	57% (8)	Reference	0.82	Reference	0.80	56% (19)	Reference	0.60	Reference	0.79
	>1	40% (159)	43% (6)	1.1 [0.4-3.3]		0.8 [0.1-4.4]		44% (15)	1.2 [0.6-2.5]		0.9 [0.3-2.2]	

§ Missing values: Number of years on ART (7), CD4 count (1), Ever treated for TB (2), Ever repeated a grade (9) and MRC dyspnoea score (1). ^σ n = 11 for the number of years on ART. ^ξ n = 33 for the number of years on ART & ever repeated a grade. Variables with two levels where a level is less than 10% of total observations were not tested for associations. This included current school attendance and taking cotrimoxazole. Variables that have values in the adjusted odd ratios column were included in the multivariate model for that bacterium. Variables with *p* values < 0.25 were included in the multivariate model except where they are colinear with another variable within the model. Weight-for-age was colinear with Height-for-age and hence excluded from the model. Ever repeated a grade was excluded because of co-linearity with MRC score. CD4 count is colinear with viral load suppression. Clinically relevant variables (*a priori*-defined) that were included in the multivariate model regardless of significance were group, age, sex, site, season of sample collection, number of years on ART, baseline viral load, ART regimen, ever treated for TB, baseline height-for-age z-score and MRC dyspnoea score.

3.3.4 Antibiotic and multidrug resistance of isolates

Some *S. pneumoniae* isolates failed to grow after initial isolation hence antibiotic susceptibility was conducted on 147/154 and 18/19 isolates from nasopharyngeal swabs and 75/83 and 15/17 isolates from sputa of CLD+ and CLD- participants, respectively. The proportion of nasopharyngeal swab isolates non-susceptible to penicillin was 32% (55/173), and that for sputa was 22% (20/90). A total of 29% (75/263) of all *S. pneumoniae* isolates, regardless of sample type, were penicillin non-susceptible. Penicillin non-susceptibility among *S. pneumoniae* was more common in the CLD+ participants (36% [53/147] vs 10% [2/19] $p = 0.036$). There were no other statistically significant differences in the antibiotic resistance profiles of *S. pneumoniae*, *S. aureus*, *H. influenzae*, and *M. catarrhalis* isolated from any sample type from CLD+ vs CLD- participants. For all isolates recovered from both nasopharyngeal swabs and sputa, there were generally low levels of resistance to azithromycin (*S. pneumoniae* = 16% [27/166]; *S. aureus* = 8% [17/195]) and tetracycline (*S. pneumoniae* = 18% [45/253]; *S. aureus* = 20% [39/195]; *H. influenzae* = 10% [6/58] and *M. catarrhalis* = 15% [12/81]) and moderate to very high cotrimoxazole resistance (*S. pneumoniae* = 95% [240/255]; *S. aureus* = 67% [130/195], *H. influenzae* = 95% [55/58] and *M. catarrhalis* = 48% [38/81]) (Figure 3.1). Methicillin-resistant *S. aureus* was uncommon (4%, 7/195). β -lactamase production by *M. catarrhalis* was widespread (93%, 76/82) but not different between the two groups. Antibiotic susceptibility profiles did not differ between sputum or nasopharyngeal swab isolates for any bacterial species (Figure 3.1).

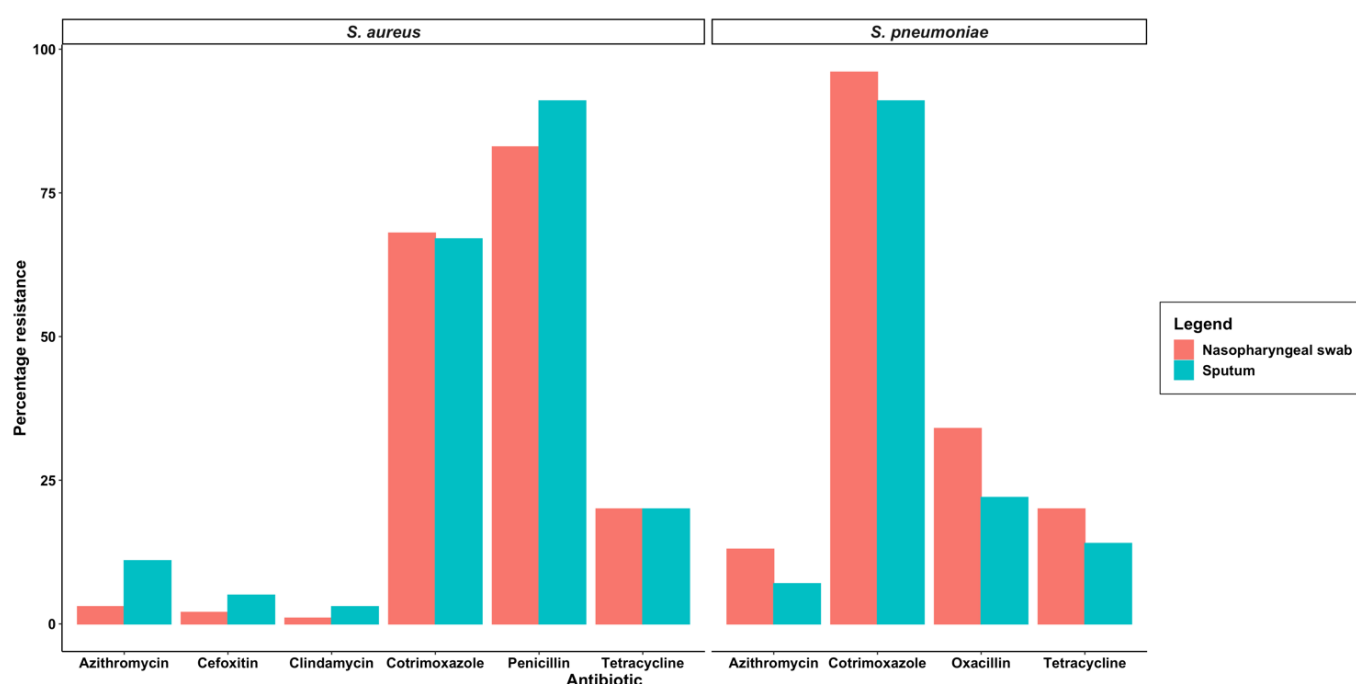


Figure 3. 1. Barplot comparing the antibiotic resistance of selected bacteria

Staphylococcus aureus [n = 85 for nasopharyngeal swab isolates, n = 110 for sputum isolates] and *Streptococcus pneumoniae* [n = 165 nasopharyngeal swab isolates, n = 90 or sputum isolates] isolates were recovered from the nasopharyngeal swabs and sputa. Only the antibiotics tested for each bacterium are shown.

3.4 Discussion

The main finding of our study was that CLD+ CLWH were more likely to carry *S. pneumoniae* and *M. catarrhalis* in their nasopharyngeal swabs than their CLD- counterparts, whereas participants with a high MRC dyspnoea score (reflecting respiratory disability) were more likely to carry *M. catarrhalis* and *H. influenzae*. In addition, age, HIV viral load, duration of ART, the season of sample collection, site and nutritional status were associated with bacterial carriage among study participants. More extended periods on ART or suppressed viral load were associated with reduced carriage for several bacterial species. The observed differences between the CLD+ and CLD- groups were more striking in nasopharyngeal swabs compared to sputa. Antimicrobial susceptibility patterns were similar between the CLD+ and CLD- groups, apart from *S. pneumoniae* penicillin non-susceptibility, which was higher in the CLD+ group.

Studies of bacterial carriage among CLWH with CLD but without acute infection are limited. Masekela *et al.* [14] observed that among CLWH aged 6–14 years (mean 6.9 years), diagnosed with HIV-related bronchiectasis in South Africa, the most prevalent bacteria, from cumulative induced sputum samples collected over one year, were *H. influenzae* (30%) and *H. parainfluenzae* (21%) followed by *Pseudomonas aeruginosa* (2%) and *S. aureus* (1%) [14]. Similarly, Verwey *et al.* [21] also found *H. influenzae* to be the most frequent bacterial species (38%) in respiratory samples (mainly sputum) of 52 CLWH with non-CF bronchiectasis [median age, 11.4 years (interquartile range 7.7–12.5)]. Samples were collected over two years and the prevalence of the other relevant bacterial species were *S. pneumoniae* (12%), *M. catarrhalis* (13%) and *S. aureus* (11%) [21]. Ferrand *et al.* [6] also found *H. influenzae* (n = 6) to be the most frequent bacteria recovered from 18 expectorated sputa from Zimbabwean CLWH (mean age, 14 ± 2.6 years) with CLD and without acute respiratory infection [6].

Although the most prevalent bacterial species that we identified were similar to those detected in previous studies using sputum samples, the prevalence differed substantially. *S. aureus* (29%) followed by *S. pneumoniae* (25%), then *M. catarrhalis* (9%) were the most common bacteria isolated from our CLD+ subjects. *H. influenzae* was infrequently detected (4%). These differences in study results may be explained by several factors, including sample type (lower respiratory tract samples vs NP swabs), clinical state of participants (acute exacerbation vs stable) and age. The studies by

Masekela [14] and Verwey [21] both included samples collected during acute exacerbations and intervening periods.

Differences in age between the studies may also explain the differences in the bacterial carriage; participants in the studies by Masekela [14] and Verwey [21] were younger than those included in our study (Masekela: mean 6.9 years (range 6–14 years) [14], Verwey: median 9.1 years (IQR 7.2–12.1) [21], Ferrand: mean 14 (SD $\pm$ 2.6) years [6]) and 15 (IQR: 13–18) in our study). Moreover, Masekela [14] investigated induced sputum, while Verwey [21] included expectorated and induced sputum, bronchoalveolar lavage and tracheal samples. In Ferrand's [6] study and ours, the samples collected were expectorated sputum for CLD+ subjects. Finally, both Masekela [14] and Verwey [21] studies also included multiple samples from the same participants – analysis did not adjust for multiple sampling from the same participant.

In our study, CLD+ participants were more stunted and underweight than their CLD- counterparts (Table 3.1). A meta-analysis showed that the prevalence of nasopharyngeal *S. pneumoniae* carriage is higher in malnourished children than their counterparts who were not malnourished [22]. This study also found that stunted and underweight children were also more likely to carry *S. pneumoniae* than children with normal weight and height, a finding that is consistent with our results. Malnutrition causes immune changes such as atrophy of the thymus, impairment of complement activity and immunoglobulin responses to encapsulated bacteria and a reduction in immunoglobulin A secretion [22].

The prevalence of *S. pneumoniae* in nasopharyngeal swabs of CLD- CLWH (26%) is comparable to similar studies of CLWH in Ghana [27.1%, mean age was 5.8 ± 3.3 years with 59.3% falling within 9 to 15 years old range] [23] and India [27.8%, median age: 6 years, IQR:4,9 with 57% falling within 12 to 17 years old range] [24]. However, this prevalence is higher than that observed in CLWH from Cambodia [17.6%, median age: 7 years, IQR = 5–9 years] [25] and Ethiopia [10.3%, median age: 11 years (range was 6–16 years)] [26], and lower than CLWH from Zambia [51%, median age: 5.1 years (IQR = 2.8 to 8.7 years)] [27] and Tanzania [81%, mean age is 6.39, SD = 3.18] [28]. The participants in these other studies were not reported to have CLD. These differences in bacterial prevalence between studies may be related to sampling site (pharyngeal vs nasopharyngeal) [26], age of participants (younger children have higher carriage prevalence) [27–29], and method of detection; PCR is associated with increased detection of *S. pneumoniae* strains compared to culture [28].

While we did not record vaccination history, the majority of our study participants are not expected to have been vaccinated against pneumococcal disease as these vaccines were introduced in both countries around 2012 [30, 31] when our study participants would have been too old to be eligible for immunisation. Pneumococcal vaccines have been shown to reduce both carriage and disease through individual protection of the vaccinated, which in turn disrupts transmission [32]. Hence, the high *S. pneumoniae* carriage in our settings might also be attributed to the lack of pneumococcal vaccination. *S. pneumoniae* serotype replacement may also account for the high *S. pneumoniae* carriage however, the relevance of this to the current study is unclear as serotyping was not conducted.

The prevalence of *S. aureus* in NP of CLD+ CLWH (23%) is comparable to similar studies conducted among CLWH in Ethiopia (29-31%) [26, 33] and India (24%) [34]. However, the prevalence in CLD- participants (12%) was lower in our study. These discrepancies in bacterial prevalence between studies may be explained by the differences in sampling sites (nasopharyngeal vs nasal swab) or age. The anterior nares are the natural niche for *S. aureus* and may be expected to be more frequently colonised than the NP [35].

Zambian [27] and Indian [34] studies of CLWH reported a much higher prevalence of *H. influenzae* (29% and 26% respectively) than either of our groups [12% (CLD+) and 5% (CLD-)]. This higher prevalence may be due to the younger age of the study participants (median ages were 5.1 years and 6.5 years for the Zambian and Indian studies, respectively, compared to the median 15 years for our study. Nasopharyngeal *H. influenzae* carriage declines with increasing age [29, 36].

The prevalence of *M. catarrhalis* in the nasopharyngeal swabs of CLD- CLWH (3%) was much lower than similar studies conducted among CLWH in Ethiopia (12.3%, median age is 11 years) [26] and Ghana (39.8%, median age is 5.8 years) [37] but comparable to a Cambodian study (6%, median age is 7.0 years) [25]. Again, age differences may explain these findings. Nasopharyngeal *M. catarrhalis* carriage declines with increasing age from about 60% at 1–2 years to about 12% at 7–14 years [36]. *M. catarrhalis* is implicated in acute exacerbations of chronic bronchitis [38] and chronic obstructive pulmonary disease (COPD) [39], and therefore, our finding of higher nasopharyngeal carriage in CLD+ participants (14%) vs CLD- participants (3%) is of interest. Sputum *M. catarrhalis* carriage was also higher in CLD+ (9%) vs CLD- CLWH (5%), but this difference was not statistically significant. Whether *M. catarrhalis* carriage plays a role in the pathogenesis of CLD or is a consequence of CLD requires further study.

The colonisation of the nasopharynx by multiple bacterial species may have important clinical consequences, including biofilm formation, polymicrobial infections and antibiotic resistance [40, 41]. We found strong positive associations between nasopharyngeal carriage of *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis*. While some previous reports observed similar positive associations [34, 36, 42–45], others found the opposite [46–48]. Possible reasons for the varying observations include differences in participants' age, vaccination schedules, year of study, host-genetic background, antibiotic use, and socio-economic status of the countries of study (low income compared to high-income status).

Interestingly, the differences we observed in nasopharyngeal bacterial carriage between CLD+ and CLD- groups were not mirrored in the sputum, despite the belief that the source of bacteria in the lower respiratory tract is mainly from the upper airways through micro-aspiration [49]. Evidence from studies suggests that the oropharyngeal samples rather than the nasopharyngeal mirror the lung microbiota (sputum) [50]. This may explain the discrepancies in nasopharyngeal and sputa bacterial carriage we observed.

We also found that ART for more than two years reduced the odds of pneumococcal carriage in both nasopharyngeal swabs and sputa. This is consistent with a report by Nicoletti et al. [51], who found that consistent use of the same ART for a year or more was negatively associated with the risk of nasopharyngeal pneumococcal colonisation in adults living with HIV. Incomplete recovery of B cell function was noted in children who were on ART for less than a year [52] and was associated with high nasopharyngeal pneumococcal carriage [52].

Increased odds of nasopharyngeal pneumococcal carriage in hot, dry seasons compared to rainy seasons is consistent with previous findings in Malawi [53] and other parts of Africa (Kenya [54] and Gambia [55]). Similar observations have also been made in Thailand [56] and the United States of America [57]. One reason for this observation is increased school attendance by children during the hot season, which may increase transmission. Furthermore, a study in Niger revealed that airborne dust and high temperatures were risk factors for invasive pneumococcal disease [56]. Dust exposure attenuates phagocyte-mediated bacterial killing whilst the high temperatures promote *S. pneumoniae* autolysis, accompanied by the release of the toxin pneumolysin [58]. Our finding that pneumococcal carriage was higher in the hot, dry seasons may further explain the higher incidence of invasive pneumococcal disease (IPD) among patients living with and without HIV irrespective of age in Malawi during these seasons [59].

The reason for the association between male sex and *S. aureus* carriage, also reported previously [60], is incompletely understood. The potential reasons include poor hand hygiene and participation in contact sports by males [60]. Furthermore, physiological factors, including sex hormones, regulation of the immune system and bacterial virulence have also been suggested [60].

Our finding of higher prevalence of penicillin-non-susceptible *S. pneumoniae* in the nasopharyngeal swabs of CLD+ participants is unsurprising. These participants were more likely to have been previously treated with antibiotics for acute exacerbations and were also more likely to have been treated for tuberculosis. Indeed, previous tuberculosis treatment was associated with increased odds of nasopharyngeal *S. pneumoniae* carriage in our multivariate model. A recent exposure to antibiotics is the strongest risk factor for nasopharyngeal carriage and spread of resistant *S. pneumoniae* [61]. Again, pneumococcal vaccination may be beneficial in this population. This is because vaccine serotypes are more likely to be resistant and therefore, a reduction in the carriage of vaccine serotypes resulting from prior immunisation can reduce antimicrobial resistance [62].

All four bacterial species tested exhibited moderate to high levels of resistance to cotrimoxazole. This was expected as 90% were receiving cotrimoxazole prophylaxis. Cotrimoxazole reduces morbidity and mortality not only in patients living with HIV but also in their family members who are living without the infection [63], despite high background resistance in respiratory pathogens [27]. This positive effect may result from a reduction in systemic and intestinal inflammation via the modulation of the gut microbiome and immune and epithelial cell activation [64].

The strengths of our study include the enrolment of age-matched CLD- participants from the same area as the CLD+ group as a comparison group. We also included participants from two sites, which increased generalisability. Limitations of our study include the use of culture alone, without PCR, which may be more sensitive for detection of bacteria and would allow more precise quantitation. Also, we did not conduct sputum quality assessment which could have informed on the degree of upper respiratory tract contamination. The relatively small sample size of the comparator CLD- group may have reduced statistical power. Also, the definition of CLD used may inadvertently include participants who may have normal lung function. However, our previous studies have shown that FEV1 z-score is an objective measure that correlates well with CLD based on radiological analysis (high-resolution computed tomography) [6, 65, 66]. Also, within the main trial under which this study was nested, we anticipated reduced efficacy of azithromycin in participants with advanced disease and therefore, did not restrict enrolment to more severely ill, symptomatic children.

3.5 Conclusion

In conclusion, CLD+ CLWH were more likely to be colonised in the nasopharynx by *M. catarrhalis* and *S. pneumoniae*, including penicillin-resistant *S. pneumoniae* strains. The role of these bacteria in CLD pathogenesis, including the risk of acute exacerbations, should be further investigated.

3.6 Declarations

3.6.1 Ethical approval and consent to participate

The parent study (BREATHE) was approved by the Human Research and Ethics Committee of the University of Cape Town - UCT HREC (HREC/REF: 754/2015), London School of Hygiene and Tropical Medicine Ethics Committee (reference 8818), the Harare Central Hospital Ethics Committee and Medical Research Council of Zimbabwe (reference MRCZ/A/1946), the College of Medicine Research Ethics Committee Malawi (reference P.04/15/1719) and by the Regional Committee for Medical and Health Research Ethics, Northern Norway (reference 2015/1650). The University of Oxford waived approval. Additional ethical approval was received for this sub-study from the UCT HREC (HREC/REF: 092/2019). No additional data was collected other than that approved in the parent study. Written informed consent and assent were given by guardians and participants, respectively. Participants who were 18 years old and above at the time of enrolment consented independently. All data obtained and generated during the study were kept confidential. This research was conducted in accordance with the Declaration of Helsinki.

3.6.2 Consent for publication

Not applicable.

3.6.3 Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request and ethical approval.

3.6.4 Competing interests

The authors declare that they have no competing interests.

3.6.5 Funding

This study was funded by the Global Health and Vaccination Research (GLOBVAC) Programme of the Medical Research Council of Norway. REA acknowledges the financial support of the Swedish International Development Cooperation Agency (SIDA) through the Organisation of Women in Science for the developing world (OWSD) PhD Fellowship, Margaret McNamara Education Grants and L'Oréal UNESCO For Women in Science Fellowship. MN is supported by an Australian National Health and Medical Research Council Investigator Grant [APP1174455]. AMR is additionally funded by the UK Medical Research Council (MRC) and the UK Department for International Development (DFID) under the MRC/DFID Concordat agreement which is also part of the EDCTP2 programme supported by the European Union, Grant Ref: MR/R010161/1. RAF is funded by the Wellcome Trust. FSD is supported by the National Research Foundation of South Africa (112160), Future Leaders – African Independent Research (FLAIR) Fellowship, the National Institute for Health Research (NIHR) Global Health Research Unit on Mucosal Pathogens using UK aid from the UK Government, the University of Cape Town, and the Allergy Society of South Africa (ALLSA). The funders had no role in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

3.6.6 Author contributions

REA conducted the antimicrobial susceptibility testing, data analysis and drafted the first draft of the manuscript. MPN designed the study and co-supervised the study. GM coordinated sample collection in Zimbabwe. VS and AMR guided the direction of the statistical analysis, reporting of results and interpretation of the data. CB conducted microbiological cultures, bacterial recovery, and identification. SM is the data manager in South Africa. BWM is the data manager and LGN is the study coordinator in Malawi. HM provides clinical support for participants. BM and JM were responsible for sample processing in Zimbabwe. JOO and RAF conceived and led the parent BREATHE study. JOO secured funding from GLOBVAC on behalf of the consortium. FSD supervised this study. All authors contributed to the manuscript, and all authors read and approved the final manuscript.

3.6.7 Additional files

Supplementary Table: S1. Semi-quantitative bacterial load distribution of isolates. This table compares the distribution of the semi-quantitative bacterial loads of *S. pneumoniae*, *S. aureus* and *H. influenzae*, isolated from the respiratory samples of CLWH with or without chronic lung disease.

Supplementary Table: S2. Gram-negative bacilli other than *H. influenzae* isolated from respiratory samples. This table shows the identities of Gram-negative bacilli other than *H. influenzae*, isolated from the respiratory samples of CLWH identified using Matrix-Assisted Laser Desorption/Ionisation-Time-of-Flight mass spectrometry.

Supplementary Table: S3. Co-carriage of bacteria in nasopharyngeal swabs and sputa. This table shows the co-carriage relationship between *S. pneumoniae*, *S. aureus* and *H. influenzae*, isolated from respiratory samples of CLWH with or without chronic lung disease.

3.7 References

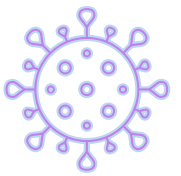
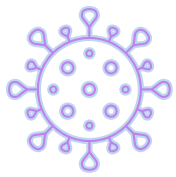
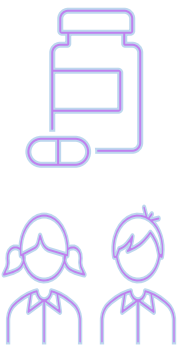
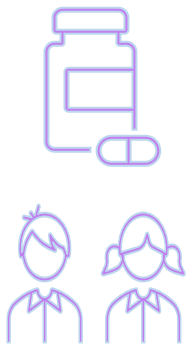
1. HIV Statistics - Global and Regional Trends - UNICEF DATA. <https://data.unicef.org/topic/hivaids/global-regional-trends/>. Accessed 1 Apr 2020.
2. Peacock-Villada E, Richardson BA, John-Stewart GC. Post-HAART outcomes in pediatric populations: comparison of resource-limited and developed countries. *Pediatrics*. 2011; 127: e423-41.
3. McHugh G, Rylance J, Mujuru H, Nathoo K, Chonzi P, Dauya E, et al. Chronic morbidity among older children and adolescents at diagnosis of HIV Infection. *J Acquir Immune Defic Syndr*. 2016; 73: 275–81.
4. Cox JA, Lukande RL, Lucas S, Nelson AM, Van Marck E, Colebunders R. Autopsy causes of death in HIV-positive individuals in sub-Saharan Africa and correlation with clinical diagnoses. *AIDS Rev*. 2010; 12: 183–94.
5. Mwalukomo T, Rylance SJ, Webb EL, Anderson S, O'Hare B, Oosterhout V, et al. Clinical characteristics and lung function in older children vertically infected with Human Immunodeficiency Virus in Malawi. *J Pediatric Infect Dis Soc*. 2016; 5: 161–9. doi:10.1093/jpids/piv045.
6. Ferrand RA, Desai SR, Hopkins C, Elston CM, Copley SJ, Nathoo K, et al. Chronic lung disease in adolescents with delayed diagnosis of vertically acquired HIV infection. *Clin Infect Dis*. 2012; 55: 145–52.
7. Desai SR, Nair A, Rylance J, Mujuru H, Nathoo K, McHugh G, et al. Human Immunodeficiency Virus-associated chronic lung disease in children and adolescents in Zimbabwe: chest radiographic and high-resolution computed tomographic findings. *Clin Infect Dis*. 2018; 66: 274–81.
8. Rylance J, McHugh G, Metcalfe J, Mujuru H, Nathoo K, Wilmore S, et al. Chronic lung disease in HIV-infected children established on antiretroviral therapy. *Aids*. 2016; 30: 2795–803.
9. du Plessis AM, Andronikou S, Machededze T, Griffith-Richards S, Myer L, Mahtab S, et al. High-resolution computed tomography features of lung disease in perinatally HIV-infected adolescents on combined antiretroviral therapy. *Pediatr Pulmonol*. 2019; 54: 1765–73.
10. McHugh G, Rehman AM, Simms V, Gonzalez-Martinez C, Bandason T, Dauya E, et al. Chronic lung disease in children and adolescents with HIV: a case-control study. *Trop Med Int Heal*. 2020; 25: 590–9.
11. Githinji LN, Gray DM, Zar HJ. Lung function in HIV-infected children and adolescents. *Pneumonia*. 2018; 10: 6.
12. Gonzalez-Martinez C, Kranzer K, McHugh G, Corbett EL, Mujuru H, Nicol MP, et al. Azithromycin versus placebo for the treatment of HIV-associated chronic lung disease in children and adolescents (BREATHE trial): study protocol for a randomised controlled trial. *Trials*. 2017; 18: 622.
13. Fitzpatrick ME, Nouraie M, Gingo MR, Camp D, Kessinger CJ, Sincebaugh JB, et al. Novel relationships of markers of monocyte activation and endothelial dysfunction with pulmonary dysfunction in HIV-infected persons. *Aids*. 2016; 30: 1327–39.
14. Masekela R, Anderson R, Moodley T, Kitchin OP, Risenga SM, Becker PJ, et al. HIV-related bronchiectasis in children: an emerging spectre in high tuberculosis burden areas. *Int J Tuberc Lung Dis*. 2012; 16: 114–9.
15. Zar HJ. Chronic lung disease in human immunodeficiency virus (HIV) infected children. *Pediatr Pulmonol*. 2008; 43: 1–10.
16. Rehman AM, Ferrand R, Allen E, Simms V, McHugh G, Weiss HA. Exclusion of enrolled participants in randomised controlled

trials: what to do with ineligible participants? *BMJ Open*. 2020; 10: e039546.

17. CLSI. Performance standards for antimicrobial susceptibility testing. 28th Editi. Wayne, PA, USA: Clinical Laboratory Standards Institute; 2018.
18. Cole TJ, Freeman J V, Preece MA. British 1990 growth reference centiles for weight, height, body mass index and head circumference fitted by maximum penalized likelihood. *Stat Med*. 1998; 17: 407–29.
19. Quanjer PH, Stanojevic S, Cole TJ, Baur X, Hall GL, Culver BH, et al. Multi-ethnic reference values for spirometry for the 3–95-yr age range: the global lung function 2012 equations. *Eur Respir J*. 2012; 40: 1324–43.
20. Madanhire T, Ferrand RA, Attia EF, Sibanda EN, Rusakaniko S, Rehman AM. Validation of the global lung initiative 2012 multi-ethnic spirometric reference equations in healthy urban Zimbabwean 7–13 year-old school children: a cross-sectional observational study. *BMC Pulm Med*. 2020; 20: 56.
21. Verwey C, Velaphi S, Khan R. Bacteria isolated from the airways of paediatric patients with bronchiectasis according to HIV status. *South African Med J*. 2017; 107: 435.
22. Smith HC, German E, Ferreira DM, Rylance J. Nasopharyngeal colonisation with *Streptococcus pneumoniae* in malnourished children: A systematic review and meta-analysis of prevalence. *Trans R Soc Trop Med Hyg*. 2019; 113: 227–33.
23. Donkor ES, Annan JA, Badoe E V, Dayie NT, Labi AK, Slotved HC. Pneumococcal carriage among HIV infected children in Accra, Ghana. *BMC Infect Dis*. 2017; 17: 133.
24. Bhattacharya SD, Niyogi SK, Bhattacharyya S, Fitzwater S, Chauhan N, Sudar A, et al. High rates of colonization with drug resistant *Haemophilus influenzae* type B and *Streptococcus pneumoniae* in unvaccinated HIV infected children from West Bengal. *Indian J Pediatr*. 2011; 78: 423–9.
25. Krmery V, Sokolova J, Kulkova N, Liskova A, Shahum A, Benca G. Nasopharyngeal bacterial colonisation in HIV-positive children in Cambodia. *Trop Med Int Heal*. 2013; 18: 1267–8.
26. Mulu W, Yizengaw E, Alemu M, Mekonnen D, Hailu D, Ketemaw K, et al. Pharyngeal colonization and drug resistance profiles of *Moraxella catarrhalis*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Haemophilus influenzae* among HIV infected children attending ART clinic of Felegehiwot Referral Hospital, Ethiopia. *PLoS One*. 2018; 13: e0196722.
27. Mwenya DM, Charalambous BM, Phillips PP, Mwansa JC, Batt SL, Nunn AJ, et al. Impact of cotrimoxazole on carriage and antibiotic resistance of *Streptococcus pneumoniae* and *Haemophilus influenzae* in HIV-infected children in Zambia. *Antimicrob Agents Chemother*. 2010; 54: 3756–62.
28. Anthony L, Meehan A, Amos B, Mtove G, Mjema J, Malahiyo R, et al. Nasopharyngeal carriage of *Streptococcus pneumoniae*: prevalence and risk factors in HIV-positive children in Tanzania. *Int J Infect Dis*. 2012; 16: e753–7.
29. Abdullahi O, Nyiro J, Lewa P, Slack M, Scott JA. The descriptive epidemiology of *Streptococcus pneumoniae* and *Haemophilus influenzae* nasopharyngeal carriage in children and adults in Kilifi district, Kenya. *Pediatr Infect Dis J*. 2008; 27: 59–64.
30. Pneumococcal vaccine launched in Zimbabwe. <https://www.gavi.org/news/media-room/pneumococcal-vaccine-launched-zimbabwe>. Accessed 25 Apr 2020.
31. Pneumococcal vaccines introduced in Malawi. <https://www.gavi.org/pneumococcal-vaccines-introduced-malawi>. Accessed 25 Apr 2020.
32. Madhi SA, Nunes MC. The potential impact of pneumococcal conjugate vaccine in Africa: Considerations and early lessons learned from the South African experience. *Hum Vaccin Immunother*. 2016; 12 :314–25.
33. Gebremedhn G, Gebremariam TT, Wasihun AG, Dejene TA, Saravanan M. Prevalence and risk factors of methicillin-resistant *Staphylococcus aureus* colonization among HIV patients in Mekelle, Northern Ethiopia. *Springerplus*. 2016; 5: 877.
34. Bhattacharya SD, Niyogi SK, Bhattacharyya S, Arya BK, Chauhan N, Mandal S. Associations between potential bacterial pathogens in the nasopharynx of HIV infected children. *Indian J Pediatr*. 2012; 79: 1447–53.
35. Krismer B, Weidenmaier C, Zipperer A, Peschel A. The commensal lifestyle of *Staphylococcus aureus* and its interactions with the nasal microbiota. *Nat Rev Microbiol*. 2017; 15: 675–87.
36. Kovács E, Sahin-Tóth J, Tóthpál A, van der Linden M, Tirczka T, Dobay O. Co-carriage of *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis* among three different age categories of children in Hungary. *PLoS One*. 2020; 15: e0229021.
37. Sampane-Donkor E, Badoe E V, Annan JA, Nii-Trebi N. Colonisation of antibiotic resistant bacteria in a cohort of HIV infected children in Ghana. *Pan Afr Med J*. 2017; 26: 60.
38. Read RC. Infection in acute exacerbations of chronic bronchitis: a clinical perspective. *Respir Med*. 1999; 93: 845–50.
39. George LM, Haigh RD, Mistry V, Haldar K, Barer MR, Oggioni MR, et al. Sputum *Moraxella catarrhalis* strains exhibit diversity within and between COPD subjects. *Int J Chron Obs Pulmon Dis*. 2018; 13: 3663–7.
40. Krishnamurthy A, McGrath J, Cripps AW, Kyd JM. The incidence of *Streptococcus pneumoniae* otitis media is affected by the polymicrobial environment particularly *Moraxella catarrhalis* in a mouse nasal colonisation model. *Microbes Infect*. 2009; 11:

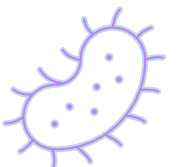
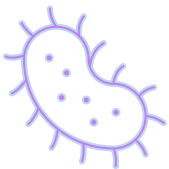
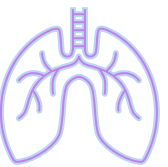
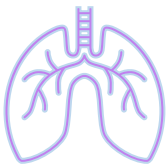
41. Watson K, Carville K, Bowman J, Jacoby P, Riley T V, Leach AJ, et al. Upper respiratory tract bacterial carriage in Aboriginal and non-Aboriginal children in a semi-arid area of Western Australia. *Pediatr Infect Dis J*. 2006; 25: 782–90.
42. McNally LM, Jeena PM, Gajee K, Sturm AW, Tomkins AM, Coovadia HM, et al. Lack of association between the nasopharyngeal carriage of *Streptococcus pneumoniae* and *Staphylococcus aureus* in HIV-1-infected South African children. *J Infect Dis*. 2006; 194: 385–90.
43. Kwambana BA, Barer MR, Bottomley C, Adegbola RA, Antonio M. Early acquisition and high nasopharyngeal co-colonisation by *Streptococcus pneumoniae* and three respiratory pathogens amongst Gambian new-borns and infants. *BMC Infect Dis*. 2011; 11: 175.
44. Jacoby P, Watson K, Bowman J, Taylor A, Riley T V, Smith DW, et al. Modelling the co-occurrence of *Streptococcus pneumoniae* with other bacterial and viral pathogens in the upper respiratory tract. *Vaccine*. 2007; 25: 2458–64.
45. Bae S, Yu JY, Lee K, Lee S, Park B, Kang Y. Nasal colonization by four potential respiratory bacteria in healthy children attending kindergarten or elementary school in Seoul, Korea. *J Med Microbiol*. 2012; 61 Pt 5: 678–85.
46. Pettigrew MM, Gent JF, Revai K, Patel JA, Chonmaitree T. Microbial interactions during upper respiratory tract infections. *Emerg Infect Dis*. 2008; 14: 1584–91.
47. Dunne EM, Smith-Vaughan HC, Robins-Browne RM, Mulholland EK, Satzke C. Nasopharyngeal microbial interactions in the era of pneumococcal conjugate vaccination. *Vaccine*. 2013; 31: 2333–42.
48. Dahlblom V, Söderström M. Bacterial interactions in the nasopharynx - effects of host factors in children attending day-care centers. *J Infect Public Heal*. 2012; 5: 133–9.
49. Hare KM, Grimwood K, Leach AJ, Smith-Vaughan H, Torzillo PJ, Morris PS, et al. Respiratory bacterial pathogens in the nasopharynx and lower airways of Australian indigenous children with bronchiectasis. *J Pediatr*. 2010; 157: 1001–5.
50. Liu HY, Zhang SY, Yang WY, Su XF, He Y, Zhou HW, et al. Oropharyngeal and sputum microbiomes are similar following exacerbation of chronic obstructive pulmonary disease. *Front Microbiol*. 2017; 8: 1163.
51. Nicoletti C, Brandileone MC, Guerra ML, Levin AS. Prevalence, serotypes, and risk factors for pneumococcal carriage among HIV-infected adults. *Diagn Microbiol Infect Dis*. 2007; 57: 259–65.
52. Iwajomo OH, Moons P, Nkhata R, Mzinza D, Ogunniyi AD, Williams NA, et al. Delayed reconstitution of B cell immunity to pneumococcus in HIV-infected Malawian children on antiretroviral therapy. *J Infect*. 2015; 70: 616–23.
53. Heinsbroek E, Tafatatha T, Phiri A, Swarthout TD, Alaerts M, Crampin AC, et al. Pneumococcal carriage in households in Karonga District, Malawi, before and after introduction of 13-valent pneumococcal conjugate vaccination. *Vaccine*. 2018; 36: 7369–76.
54. Abdullahi O, Karani A, Tigoi CC, Mugo D, Kungu S, Wanjiru E, et al. The prevalence and risk factors for pneumococcal colonization of the nasopharynx among children in Kilifi District, Kenya. *PLoS One*. 2012; 7: e30787.
55. Bojang A, Jafali J, Egere UE, Hill PC, Antonio M, Jeffries D, et al. Seasonality of pneumococcal nasopharyngeal carriage in rural Gambia determined within the context of a cluster randomized pneumococcal vaccine trial. *PLoS One*. 2015; 10: e0129649.
56. Numminen E, Chewapreecha C, Turner C, Goldblatt D, Nosten F, Bentley SD, et al. Climate induces seasonality in pneumococcal transmission. *Sci Rep*. 2015; 5: 11344.
57. Weinberger DM, Grant LR, Steiner CA, Weatherholtz R, Santosham M, Viboud C, et al. Seasonal drivers of pneumococcal disease incidence: impact of bacterial carriage and viral activity. *Clin Infect Dis*. 2014; 58: 188–94.
58. Jusot JF, Neill DR, Waters EM, Bangert M, Collins M, Bricio Moreno L, et al. Airborne dust and high temperatures are risk factors for invasive bacterial disease. *J Allergy Clin Immunol*. 2017; 139: 977-986.e2.
59. Everett DB, Mukaka M, Denis B, Gordon SB, Carrol ED, van Oosterhout JJ, et al. Ten years of surveillance for invasive *Streptococcus pneumoniae* during the era of antiretroviral scale-up and cotrimoxazole prophylaxis in Malawi. *PLoS One*. 2011; 6: e17765.
60. Humphreys H, Fitzpatrick F, Harvey BJ. Gender differences in rates of carriage and bloodstream infection caused by methicillin-resistant *Staphylococcus aureus*: Are they real, do they matter and why? *Clin Infect Dis*. 2015; 61: 1708–14.
61. Canet JJ, Garau J. Importance of dose and duration of beta-lactam therapy in nasopharyngeal colonization with resistant pneumococci. *J Antimicrob Chemother*. 2002; 50 Suppl S: 39–43.
62. Tin Tin Htar M, van Den Biggelaar AHJ, Sings H, Ferreira G, Moffatt M, Hall-Murray C, et al. The impact of routine childhood immunization with higher-valent pneumococcal conjugate vaccines on antimicrobial-resistant pneumococcal diseases and carriage: a systematic literature review. *Expert Rev Vaccines*. 2019; 18: 1069–89.
63. Mermin J, Lule J, Ekwaru JP, Downing R, Hughes P, Bunnell R, et al. Cotrimoxazole prophylaxis by HIV-infected persons in Uganda reduces morbidity and mortality among HIV-uninfected family members. *Aids*. 2005; 19: 1035–42.
64. Bourke CD, Gough EK, Pimundu G, Shonhai A, Berejena C, Terry L, et al. Cotrimoxazole reduces systemic inflammation in HIV infection by altering the gut microbiome and immune activation. *Sci Transl Med*. 2019; 11.

65. Desai SR, Nair A, Rylance J, Mujuru H, Nathoo K, McHugh G, et al. Human Immunodeficiency Virus-associated chronic lung disease in children and adolescents in Zimbabwe: chest radiographic and high-resolution computed tomographic findings. *Clin Infect Dis*. 2018; 66:274–81.
66. Mwalukomo T, Rylance SJ, Webb EL, Anderson S, O'Hare B, van Oosterhout JJ, et al. Clinical Characteristics and Lung Function in Older Children Vertically Infected With Human Immunodeficiency Virus in Malawi. *J Pediatric Infect Dis Soc*. 2016; 5: 161–9.



Chapter 4

The impact of long-term azithromycin on antibiotic resistance in HIV-associated chronic lung disease



The impact of long-term azithromycin on antibiotic resistance in HIV-associated chronic lung disease

Regina E. Abotsi^{1,2}, Mark P. Nicol³, Grace McHugh⁴, Victoria Simms^{4,5}, Andrea M. Rehman⁵, Charmaine Barthus⁶, Lucky G. Ngwira^{7,8}, Brenda Kwambana-Adams⁹, Robert S Heyderman⁹, Jon Ø Odland^{10,11,12}, Rashida A Ferrand^{4,13}, Felix S. Dube¹ and The BREATHE study team

Affiliations

1. Department of Molecular and Cell Biology & Institute of Infectious Diseases and Molecular Medicine, University of Cape Town, Cape Town, South Africa
2. Department of Pharmaceutical Microbiology, School of Pharmacy, University of Health and Allied Sciences, Ho, Ghana
3. Division of Infection and Immunity, School of Biomedical Sciences, Faculty of Health and Medical Sciences, University of Western Australia, Perth, Australia
4. Biomedical Research and Training Institute, Harare, Zimbabwe
5. International Statistics and Epidemiology Group, London School of Hygiene and Tropical Medicine, London, United Kingdom
6. Division of Medical Microbiology, University of Cape Town, Cape Town, South Africa
7. Malawi-Liverpool Wellcome Trust Clinical Research Programme, Blantyre, Malawi.
8. Liverpool School of Tropical Medicine, Liverpool, United Kingdom
9. NIHR Global Health Research Unit on Mucosal Pathogens, Research Department of Infection, Division of Infection and Immunity, University College London, London, United Kingdom
10. Department of Community Medicine, University of Tromsø, Tromsø, Norway
11. International Research Laboratory for Reproductive Ecotoxicology (IL RET), The National Research University Higher School of Economics, Moscow, Russia
12. School of Health Systems and Public Health, Faculty of Health Sciences, University of Pretoria, Pretoria, South Africa
13. Clinical Research Department, London School of Hygiene and Tropical Medicine, London, United Kingdom

4.0 Abstract

Background: Selection for resistance to azithromycin and other antibiotics such as tetracyclines and lincosamides remains a concern with long-term azithromycin use to treat chronic lung diseases (CLD). We investigated the impact of 48 weeks of azithromycin on the carriage and antibiotic resistance of common respiratory bacteria among children with HIV-associated CLD.

Methods: Nasopharyngeal swabs and sputa were collected at baseline, 48 and 72 weeks from participants with HIV-associated CLD randomised to receive weekly azithromycin or placebo for 48 weeks and followed post-intervention until 72 weeks. The primary outcomes were prevalence and antibiotic resistance of *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Haemophilus influenzae*, and *Moraxella catarrhalis* at these time points. Mixed-effects logistic regression and Fisher's exact test were used to compare carriage and resistance, respectively.

Results: Of 347 (174 azithromycin, 173 placebo) participants (median age 15 years [IQR = 13–18], females 49%), nasopharyngeal carriage was significantly lower in the azithromycin (n = 159) compared to placebo (n = 153) arm for *S. pneumoniae* (18% vs 41%, $p < 0.001$), *H. influenzae* (7% vs 16%, $p = 0.01$), and *M. catarrhalis* (4% vs 11%, $p = 0.02$); *S. pneumoniae* resistance to azithromycin (62% [18/29] vs 13% [8/63], $p < 0.0001$) or tetracycline (60% [18/29] vs 21% [13/63], $p < 0.0001$) were higher in the azithromycin arm. Carriage of *S. aureus* resistant to azithromycin (91% [31/34] vs 3% [1/31], $p < 0.0001$), tetracycline (35% [12/34] vs 13% [4/31], $p = 0.05$) and clindamycin (79% [27/34] vs 3% [1/31], $p < 0.0001$) was also significantly higher in the azithromycin arm and persisted at 72 weeks. Similar findings were observed for sputa.

Conclusions: The persistence of antibiotic resistance and its clinical relevance for future infectious episodes requiring treatment needs further investigation.

Keywords: HIV, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Haemophilus influenzae*, *Moraxella catarrhalis*, children

Summary: Forty-eight weeks of azithromycin reduced carriage of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* but promoted antibiotic resistance in *S. pneumoniae* and *S. aureus*; antibiotic-resistant *S. aureus* persisted at 72 weeks from randomisation.

4.1 Background

Long-term azithromycin therapy is commonly used to manage chronic lung diseases (CLD), including cystic fibrosis [1], non-cystic bronchiectasis [2], chronic obstructive pulmonary disease (COPD) [3] and asthma [4]. Evidence suggests that azithromycin may improve lung function and survival and reduce the frequency of acute pulmonary exacerbations, antibiotic administration, and hospitalisation in some of these CLDs. These beneficial effects may be mediated by the dual antimicrobial and immunomodulatory properties of azithromycin [3].

However, azithromycin use can promote antibiotic resistance in both commensal and potentially pathogenic bacteria in the respiratory tract [1–4], which can be further spread to untreated individuals. Azithromycin can also promote resistance to other antibiotics, including tetracyclines, lincosamides and streptogramins [5]. This is mediated by the alteration of drug targets shared by other antibiotics (lincosamides and Streptogramins) and co-location of resistance genes on the same mobile genetic elements (tetracyclines) [5]. In a meta-analysis of CLD patients (COPD, cystic fibrosis, bronchiectasis, and asthma) who received long-term azithromycin, the risk of bacterial resistance increased 2.7-fold whilst bacterial colonisation risk was halved in the azithromycin group compared to placebo [6]. None of the trials included in this meta-analysis reported on the persistence of the microbiological effect post-intervention, and none included African children or patients with HIV-related CLD. Two later studies that investigated the persistence of the azithromycin's microbiological effect post-intervention could not provide robust conclusions due to few participants [2, 7] (mean of 25 per trial arm), short follow-up period (4 weeks for one study) [7] and variable timepoints for post-intervention samples [2].

Children with HIV are at high risk of respiratory tract infections. Recent studies have shown that these children also have a high prevalence of CLD, despite antiretroviral therapy (ART) [8]. The clinical presentation is typically chronic cough, reduced exercise tolerance, hypoxia, and reduced lung function [8]. Up to a third of these children with HIV included in African studies have CLD, with constrictive obliterative bronchiolitis being the dominant cause [8]. As there are currently no treatment guidelines, we conducted a double-blind, placebo-controlled randomised trial that showed that 48 weeks of once-weekly azithromycin reduced acute respiratory exacerbations and all-cause hospitalisations in African children with HIV-associated CLD [9]. The microbiological sequelae of long-term azithromycin have not been previously investigated in a paediatric population with HIV-associated CLD. To address the potential for selection of antibiotic-resistant bacteria in this patient population, we investigated the

impact of long-term azithromycin therapy on the carriage and antibiotic resistance of *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Haemophilus influenzae* and *Moraxella catarrhalis* at the 48 weeks (end of therapy) and 72 weeks from randomisation. We also assessed factors associated with carriage and resistance.

4.2 Materials and Methods

4.2.1 Participants and intervention

The main results of the BREATHE trial (ClinicalTrials.gov Identifier: NCT02426112) [9] are reported elsewhere. Briefly, individuals with HIV-associated CLD aged 6–19 years and taking ART for at least six months were enrolled from HIV outpatient clinics in Harare, Zimbabwe and Blantyre, Malawi. CLD was defined as forced expiratory volume in 1 second (FEV₁) z-score less than -1.0 with no reversibility [$< 12\%$ improvement in FEV₁ after 200 µg of salbutamol inhaled using a spacer]. Participants were individually randomised to receive weight-based dosing of oral azithromycin (10 to 19.9 kg, 250 mg; 20 to 29.9 kg, 500 mg; 30 to 39.9 kg, 750 mg; and 40 kg or more, 1250 mg) or placebo once-weekly up to 48 weeks, with follow-up for a further 24 weeks post-intervention. Adherence to trial medication was defined as not missing, on average, more than two of the 12 dispensed doses, as assessed by pill count, splitting time in the study into four 12-week periods, according to visit and study medication dispensing schedule [9].

4.2.2 Sample collection

Nasopharyngeal swabs and sputa were collected from participants at baseline, 48- and 72-week visits. Details of sample collection, handling and transport, culture, and antibiotic susceptibility testing for *S. pneumoniae*, *S. aureus*, *H. influenzae* and *M. catarrhalis* have been previously described [10]. Briefly, susceptibility to azithromycin, tetracycline and cotrimoxazole was conducted for all four bacterial species using the Kirby-Bauer disk diffusion method. Additionally, antimicrobial susceptibility testing was done for *S. pneumoniae* to oxacillin, *S. aureus* to clindamycin, cefoxitin, and penicillin, *H. influenzae* to amoxicillin-clavulanate, cefuroxime, and ampicillin, and *M. catarrhalis* to amoxicillin-clavulanate. Inducible clindamycin resistance was assessed as previously reported [11]. SA susceptibility to cefoxitin was tested as a surrogate for methicillin resistance. Antimicrobial susceptibility testing was conducted in accordance with the 2018 Clinical and Laboratory Standards Institute guidelines and breakpoints [12].

4.2.3 Study outcomes

The primary outcomes for this study were the difference in the prevalence of *S. pneumoniae*, *S. aureus*, *H. influenzae* and *M. catarrhalis* and their azithromycin-resistant strains between the trial arms at azithromycin cessation (48 weeks). Secondary outcomes included the differences in the carriage and azithromycin resistance between the trial arms at 72 weeks. We also investigated the effect of long-term azithromycin on resistance to the additional antibiotics tested at both 48 and 72 weeks. The clinical and socio-demographic factors associated with the carriage of bacteria and azithromycin-resistant isolates at 48 and 72 weeks; also assessed was the effect of adherence to azithromycin on carriage and resistance (in the azithromycin arm only).

4.2.4 Statistical analyses

Statistical analyses were conducted in R software version 3.6.2 (R Foundation for Statistical Computing, Vienna, Austria; <https://www.r-project.org/>). Bacterial carriage and antibiotic resistance were reported as percentages of total samples cultured and tested for resistance for each trial arm. Fisher's exact test and Mann-Whitney test were used to compare proportions and continuous variables, respectively. Comparisons of bacterial carriage between trial arms at all visits were conducted using mixed-effects logistic regression models, including a random effect for participants, and reported as adjusted odds ratios with 95% confidence intervals in Stata version 15 (StataCorp, Texas, USA). Models were adjusted for site, sex, age category, HIV viral load at baseline, the season of sampling and visit. A trial arm by time interaction term was included in the models to compare trial arms at 48 and 72 weeks, without a trial arm main effect term [13].

4.2.5 Ethical approval and consent to participate

Approval for the main trial was obtained from local regulatory bodies at the study sites and the research ethics committees of the London School of Hygiene and Tropical Medicine, the University of Cape Town, and the Medical and Health Research in Norway. This sub-study was approved by the Human Research Ethics Committee of the University of Cape Town (HREC/REF: 092/2019). Guardians and participants under 18 years, respectively, provided written informed consent and age-appropriate assent. Older participants (≥ 18 years) consented independently.

4.3 Results

Between June 15, 2016, and September 4, 2018, 347 participants (173 azithromycin and 174 placebo arm) were enrolled and randomised to azithromycin or placebo (Figure 4.1). Eleven participants who were excluded from the main trial because they were later found not to meet the inclusion criteria for FEV₁ were included in this report. This is because we assessed the effect of long-term azithromycin on the respiratory bacteria and antibiotic resistance irrespective of FEV₁z-scores. At baseline, participants had a median age of 15 years [IQR = 13–18] with slightly more males than females (51% [177/347] vs 49% [170/347]). Most participants were on long-term cotrimoxazole prophylaxis (90% [313/347]) (Table 4.1). NP and sputum samples were obtained from about 97% of all participants at baseline and 48 weeks and 86% at 72 weeks (Figure 4.1).

Table 4. 1. Baseline characteristics of study participants

Characteristics	Details	Azithromycin arm n = 173	Placebo arm n = 174
Demographic Characteristics			
Age in years	Median (IQR)	15 (13 – 17)	16 (13 – 18)
Sex	Female: No. (%)	80 (46)	90 (52)
Currently in school	No. (%) ^a	146 (85)	139 (80)
Site	Zimbabwe: No. (%)	120 (69)	121 (70)
	Malawi: No. (%)	53 (31)	53 (30)
HIV Characteristics			
Age in years at diagnosis	Median (IQR)	7 (4 – 10)	8 (5 – 11)
Duration on ART in years	Median (IQR)	6 (4 – 9)	6 (4 – 8)
Age at ART initiation	Median (IQR)	8 (5 – 11)	9 (7 – 12)
HIV viral load log 10 copies/mL	Median (IQR)	2.5 (1.6- 4.0)	2.7 (1.7- 4.1)
HIV viral load suppression	< 1000 copies/mL: No. (%)	102 (59)	94 (54)
CD4 cell count /mm ³	Median (IQR)	601 (417 – 784)	550 (325 – 779)
CD4 count categories	200+ cells/mm ³ : No. (%)	157 (91)	156 (90)
	< 200 cells/mm ³ : No. (%)	16 (9)	18 (10)
Antiretroviral regimen	[†] NNRTI, No. (%) ^a	127 (73)	131 (75)
	[§] PI, No. (%) ^a	46 (27)	42 (25)
Lung function			
FEV ₁ z-score	Median (IQR)	-1.94 (-2.5, -1.4)	-2.0 (-2.4, -1.5)
Clinical Characteristics			
Weight-for-age z-score	Median (IQR)	-2.2 (-3.0, -1.4)	-1.9 (-2.8, -1.2)

Underweight	No. (%) ^b	98 (57)	83 (48)
Height-for-age z-score	Median (IQR)	-2.1 (-3.0, -1.4)	-1.9 (-2.6, -1.3)
Stunted	No. (%) ^b	95 (55)	80 (46)
History of TB	No. (%) ^a	58 (34)	39 (22)
Admitted for chest problems in last year	No. (%)	3 (2)	3 (2)
Current cough	No. (%) ^a	13 (8)	18 (10)
Coughing up sputum	No. (N) ^c	7 (43)	17 (46)
MRC dyspnoea Score	1	89 (51)	96 (55)
	2	64 (37)	62 (36)
	3	12 (7)	11 (6)
	4	7 (4)	4 (2)
	5	1 (1)	1 (1)
Cotrimoxazole prophylaxis	No. (%)	157 (92) ^a	156 (90)

^a Missing value: Currently attending school – n = 1 Azithromycin arm; Antiretroviral regimen, n = 1, Placebo arm; History of TB– n = 1, Azithromycin arm Current cough– n = 1, Azithromycin arm; Cotrimoxazole prophylaxis– n = 2, Azithromycin arm, ^b z-score < -2, ^c Only asked for those with current cough, [§] Protease inhibitor FEV1: Forced Expiratory Volume in one Second; Participants with missing responses are excluded from that variable. [†]Non-nucleoside Reverse Transcriptase Inhibitor, No. = number of participants, IQR = interquartile range, TB = tuberculosis, MRC = Medical Research Council.

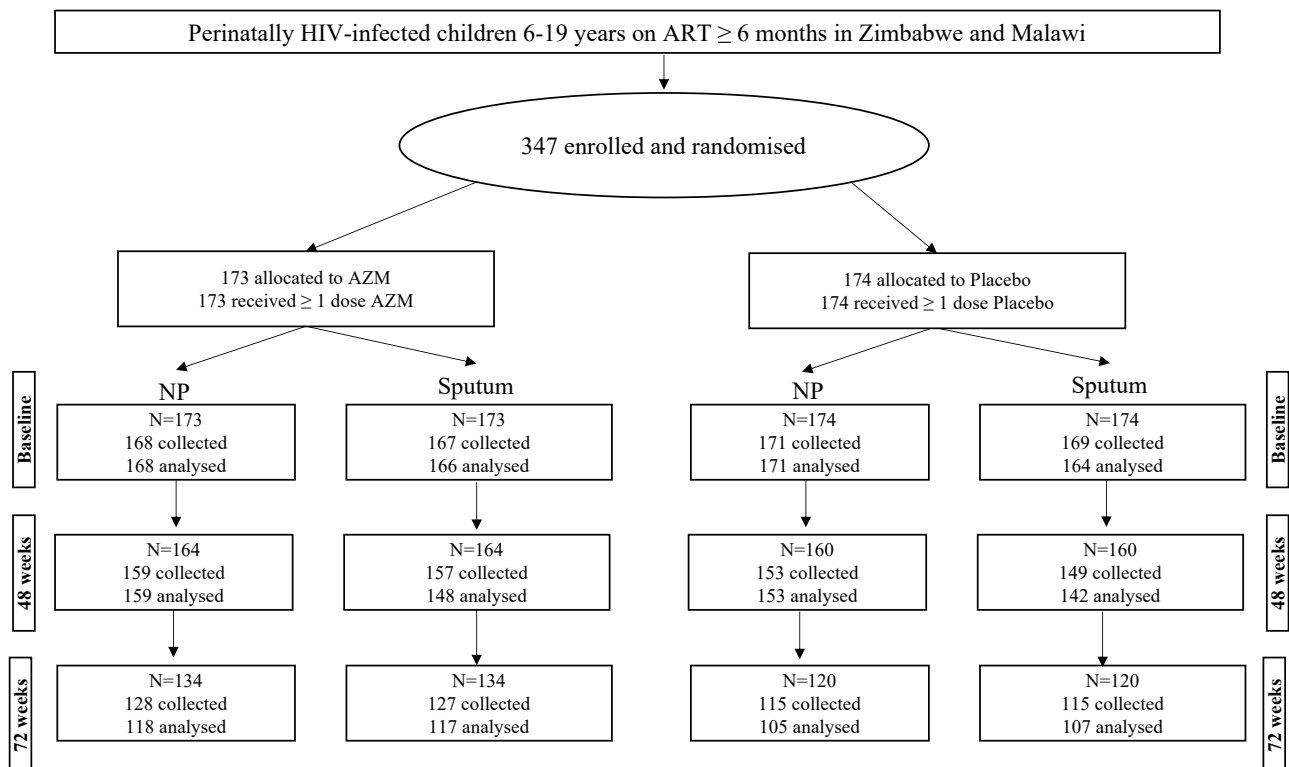


Figure 4. 1. Number of samples collected at each visit flowchart

Number of samples collected or analysed not equal to number of participants due to missingness, withdrawal, loss to follow-up, insufficiency or in the case of sputum, induction failure.

Participants randomised to receive azithromycin were less likely to carry *S. pneumoniae* (adjusted OR, aOR, 0.2, [0.1–0.4], $p < 0.0001$), *H. influenzae* (aOR 0.3, [0.1–0.8], $p = 0.01$) or *M. catarrhalis* (aOR 0.2, [0.1–0.8], $p = 0.02$) (Table 4.2) in their nasopharyngeal swabs at 48 weeks than placebo. The carriage of any of the four species was lower in the azithromycin arm at 48 weeks (aOR 0.4, [0.2–0.7], $p = 0.001$), but not at 72 weeks, compared to placebo (Table 4.2). These observations were mirrored in the sputum samples (Table 4.2).

At 48 weeks, the proportion of *S. pneumoniae* (62% [18/29] vs 13% [8/63], $p < 0.0001$) or *S. aureus* (91% [31/34] vs 3% [1/31], $p < 0.0001$) isolates from the nasopharyngeal swabs that were azithromycin-resistant was higher in the azithromycin arm than placebo (Figure 4.2 and 4.3). However, at 72 weeks, there were no differences in the proportion of azithromycin-resistant strains, except for azithromycin-resistant *S. aureus* (45% [9/20] in the azithromycin arm vs 4% [1/26] in the placebo arm, $p < 0.001$) (Figure 4.3). Overall azithromycin resistance (resistance in any of the four bacterial species) was higher in the azithromycin arm at 48 weeks (30% [45/154] vs 7% [10/146], $p < 0.0001$) and remained so at 72 weeks (17% [17/102] vs 7% [7/90], $p < 0.0001$) relative to placebo. The observations above were mirrored in sputum samples (Figures 4.2 and Figure 4.3).

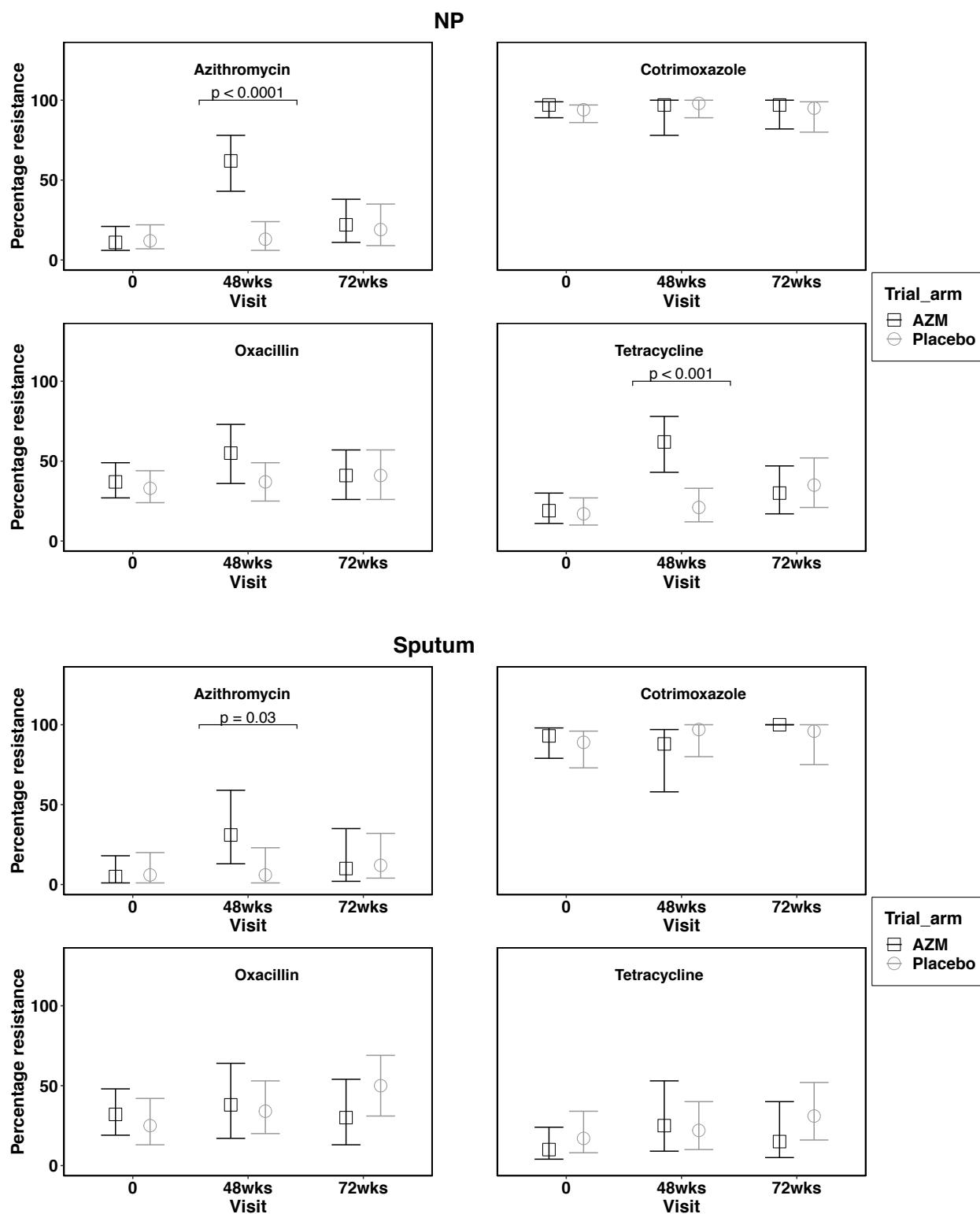


Figure 4. 2. Antibiotic resistance profiles of *Streptococcus pneumoniae* isolates.

Isolates were recovered from the nasopharyngeal (NP) and sputum samples at baseline (0 week), 48 weeks and 72 weeks. The x- and y-axes are sampling timepoints and percentage resistance, respectively. Number of isolates from NP at each timepoint for azithromycin (AZM) vs placebo were– baseline (71 vs 81), 48 weeks (29 vs 63), 72 weeks (37 vs 37). In the sputum, baseline (41 vs 36), 48 weeks (16 vs 32), 72 weeks (20 vs 26). Oxacillin was used as a surrogate for penicillin.

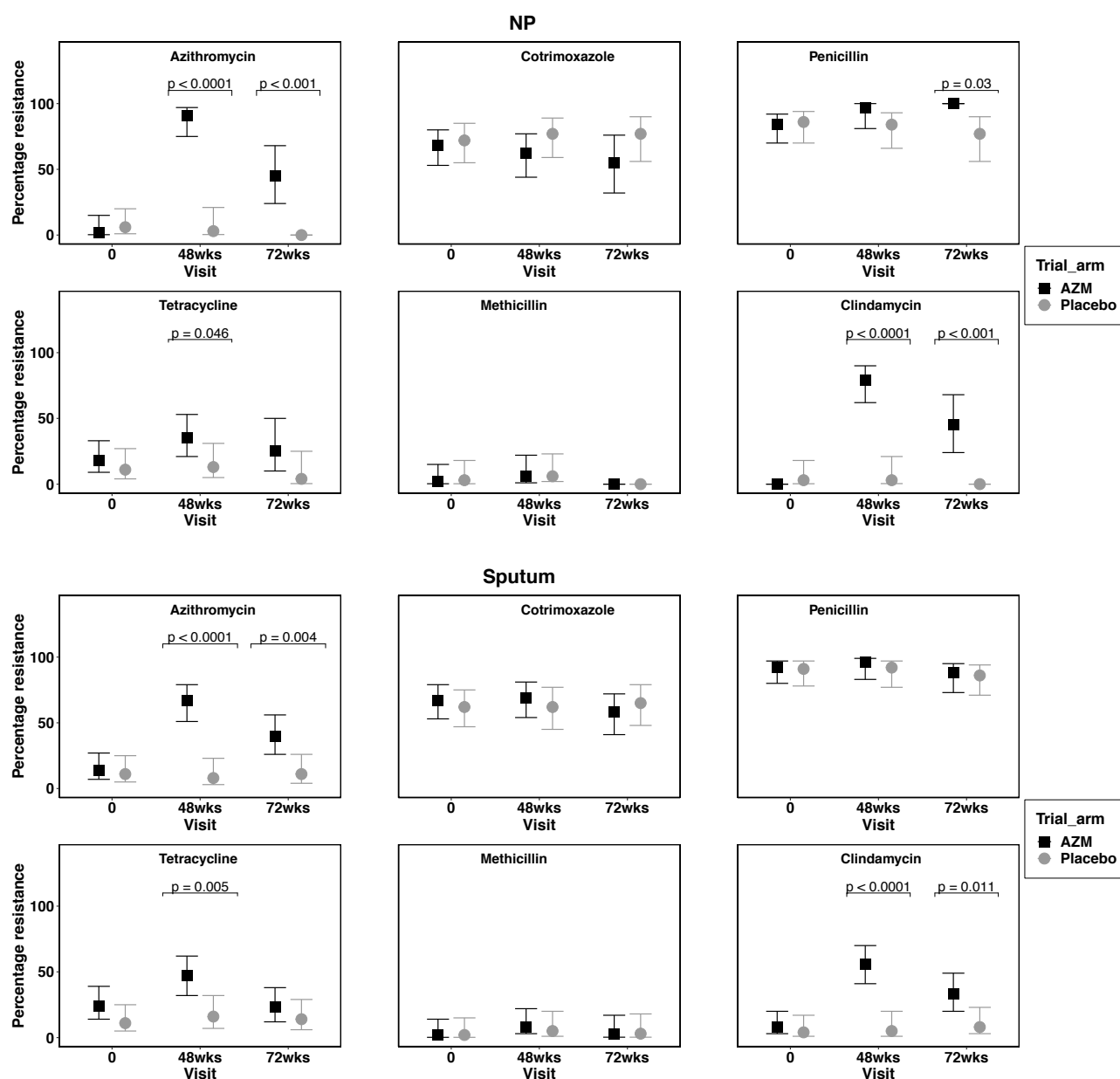


Figure 4. 3. Antibiotic resistance profiles of *Staphylococcus aureus* isolates.

Isolates were recovered from the nasopharyngeal and sputum samples at baseline (0 week), 48 weeks and 72 weeks. The x- and y-axes are sampling timepoints and percentage resistance, respectively. Number of isolates from NP at each timepoint for azithromycin (AZM) vs placebo were – baseline (44 vs 36), 48 weeks (34 vs 31), 72 weeks (20 vs 26). In the sputum, baseline (49 vs 45), 48 weeks (45 vs 37), 72 weeks (40 vs 37).

The proportion of *S. pneumoniae* and *S. aureus* in the nasopharyngeal swabs that was tetracycline-resistant was also higher at 48 weeks in the azithromycin arm than placebo (Figure 4.2 and Figure 4.3, Appendix C Table S1). Clindamycin resistance in *S. aureus* was higher in the azithromycin arm vs placebo at 48 weeks (79% [27/34] vs 3% [1/31], $p < 0.0001$) and remained higher at 72 weeks in the

azithromycin arm compared to placebo (45% [9/20] vs 0% [0/26], $p < 0.0001$) (Figure 4.3, Appendix C Table S1). Carriage of methicillin-resistant *S. aureus* remained low (6%) throughout the study in both trial arms (Figure 4.3, Appendix C Table S1). Penicillin (oxacillin) resistance in *S. pneumoniae* was moderate and did not differ between trial arms however, penicillin-resistant *S. aureus* was higher in azithromycin than placebo at 72 weeks (Appendix C Table S1). Cotrimoxazole resistance levels in *S. aureus* remained high (~69%), with no clear changes throughout the study period (Appendix C Table S1). There was no difference between the trial arms with *H. influenzae* and *M. catarrhalis* susceptibility to azithromycin, tetracycline, amoxicillin-clavulanate and cotrimoxazole (Appendix C Table S1, Appendix C Figure S1–S2). There was a universal *H. influenzae* susceptibility to cefuroxime and ampicillin (Appendix C Table S1, Appendix C Figure S1). The above observations were mirrored in sputum samples (Figure 4.3, Appendix C Table S2, Appendix C Figure S1–S2) except for tetracycline-resistant *S. pneumoniae*, which did not differ between trial arms at 48 weeks. Also, unlike in the NP, penicillin resistance in sputum *S. aureus* was similar between trial arms at all visits (Figure 4.3, Appendix C Table S2).

Adherence to azithromycin was associated with a reduced likelihood of sputum *S. aureus* and overall bacterial carriage at 48 weeks (Appendix C Table S3). Adherence was not associated with the carriage of resistant bacteria (Appendix C Table S4 and S5). The main factors associated with *S. pneumoniae* carriage were male sex (NP only) and placebo allocation (NP and sputum) at 48 weeks and age at 72 weeks (Appendix C Table S6). Similarly, factors associated with nasopharyngeal and sputum *S. aureus* carriage were non-adherence to azithromycin (nasopharyngeal swabs and sputum, Appendix C Table S7), viral load suppression (sputum) and season of sampling (Appendix C Table S7). Placebo allocation was associated with decreased likelihood of azithromycin-resistant *S. pneumoniae* and *S. aureus* carriage in the nasopharyngeal swab or sputum (Appendix C Table S8 and S9).

Table 4. 2. Nasopharyngeal and sputum bacterial carriage at baseline, 48 weeks, and 72 weeks in participants

NASOPHARYNGEAL SWABS										
	Baseline		48 weeks				72 weeks			
	AZM (n = 168) ¹	Placebo (n = 171) ¹	AZM (n = 159) ²	Placebo (n = 153) ²	aOR (95%CI)	<i>p</i> values	AZM (n = 118) ³	Placebo (n = 105) ³	aOR (95%CI)	<i>p</i> values
<i>Streptococcus pneumoniae</i>	74 (44%)	83 (49%)	29 (18%)	64 (41%)	0.2 [0.1-0.4]	< 0.0001	41 (35%)	38 (36%)	0.9 [0.4-1.7]	0.681
<i>Staphylococcus aureus</i>	45 (27%)	36 (21%)	34 (21%)	31 (20%)	1.1 [0.5-2.2]	0.882	23 (19%)	26 (25%)	0.7 [0.3-1.7]	0.433
<i>Haemophilus influenzae</i>	21 (13%)	18 (11%)	11 (7%)	24 (16%)	0.3 [0.1-0.8]	0.011	13 (11%)	10 (10%)	1.0 [0.4-2.8]	0.964
<i>Moraxella catarrhalis</i>	26 (15%)	21 (12%)	7 (4%)	17 (11%)	0.3 [0.1-0.8]	0.015	9 (8%)	7 (7%)	0.9 [0.3-2.9]	0.848
Bacterial carriage (any)	107 (64%)	112 (68%)	63 (43%)	86 (60%)	0.4 [0.2-0.7]	0.001	61 (53%)	63 (60%)	0.6 [0.3-1.2]	0.135
SPUTUM										
	Baseline		48 weeks				72 weeks			
	AZM (n = 166) ⁴	Placebo (n = 164) ⁴	AZM (n = 148) ⁵	Placebo (n = 143) ⁵	aOR (95%CI)	<i>p</i> values	AZM (n = 116) ⁶	Placebo (n = 106) ⁶	aOR (95%CI)	<i>p</i> values
<i>Streptococcus pneumoniae</i>	43 (26%)	38 (23%)	17 (11%)	34 (24%)	0.4 [0.2 -0.8]	0.008	20 (17%)	26 (25%)	0.6 [0.3-1.2]	0.122
<i>Staphylococcus aureus</i>	51 (31%)	46 (28%)	46 (31%)	37 (26%)	1.4 [0.7-2.8]	0.323	40 (34%)	37 (34%)	1.1 [0.5-2.3]	0.788
<i>Haemophilus influenzae</i>	4 (2%)	7 (4%)	3 (2%)	19 (13%)	0.1 [0.0-0.4]	0.002	6 (5%)	9 (8%)	0.5 [0.1-1.6]	0.221
<i>Moraxella catarrhalis</i>	15 (9%)	15 (9%)	4 (3%)	15 (10%)	0.2 [0.1-0.7]	0.008	7 (6%)	7 (7%)	0.9 [0.3-2.8]	0.882
Bacterial carriage (any)	82 (49%)	79 (48%)	62 (42%)	77 (54%)	0.6 [0.3-1.0]	0.056	56 (48%)	60 (57%)	0.8 [0.4-1.4]	0.395

¹Samples not collected from five and three participants in azithromycin (AZM) and placebo group at baseline respectively. ²Samples not collected from five and seven participants in AZM and placebo group at 48 weeks respectively. ³Samples not collected from 16 and 15 participants in AZM and placebo group at 72 weeks, respectively. ⁴Sputum samples were not collected from seven and 10 participants in AZM and placebo group at baseline, respectively. ⁵Sputum samples were not collected from 16 and 17 participants in AZM and placebo group at 48 weeks, respectively. ⁶Sputum samples were not collected from 18 and 14 participants in AZM and placebo group at 72 weeks, respectively. aOR = adjusted Odd Ratio

4.4 Discussion

Our study shows that HIV-infected children with CLD established on ART and long-term prophylaxis, who received 48 weeks of azithromycin therapy, were significantly less likely to carry *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* in their nasopharyngeal swabs or sputum than placebo at the end of treatment. However, these participants were more likely to carry azithromycin and tetracycline-resistant *S. pneumoniae* and *S. aureus* at 48 weeks. Azithromycin-, clindamycin- and tetracycline-resistant *S. aureus* remained more prevalent in participants in the azithromycin arm at 72 weeks.

Our finding of reduced nasopharyngeal and sputum carriage of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* in response to long-term azithromycin therapy is consistent with previous studies [2, 6]. Similarly, the lack of an effect of long-term azithromycin therapy on *S. aureus* carriage observed in our study was also reported in studies conducted in children with chronic suppurative lung disease in Australia and New Zealand [2], and cystic fibrosis in The Netherlands [14] and USA [3]. The reduction in *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* prevalence in our study may be related to the almost universal susceptibility of these isolates to azithromycin at baseline or the consequence of improved alveolar macrophage phagocytic activity [15]. Phagocytosis is generally reduced in chronic lung conditions, especially COPD, but improves with azithromycin therapy [15]. A survey conducted from 2015 to 2018, the same period as our study, reported a high *S. pneumoniae* carriage among children and adults in Malawi regardless of pneumococcal vaccination and HIV status [16]. Our finding that azithromycin reduced *S. pneumoniae* carriage may have important implications in reducing the risk of invasive *S. pneumoniae* disease and transmission. The lack of difference between trial arms in the carriage of *S. pneumoniae*, *H. influenzae* or *M. catarrhalis* at 72 weeks highlights that the antimicrobial effects of long-term azithromycin therapy may not be long-lasting. This is corroborated by reports from a randomised controlled trial of azithromycin among Indigenous Australian, Maori and Pacific Island children aged 1–8 years with chronic suppurative lung disease [2].

Long-term azithromycin therapy selected for azithromycin-resistant *S. pneumoniae* at the end of treatment. This is consistent with other studies that used azithromycin for long- [1–3, 14, 17] and short-term [11, 18, 19] treatment periods. Azithromycin resistance occurs soon after exposure, as evidenced by the recovery of resistant strains after a single azithromycin course or dose [11]. Previous studies revealed clonal expansion of existing resistant strains of *S. pneumoniae* following exposure to

azithromycin [19]. Studies that involved mass azithromycin administration for trachoma also noted an increase in resistant *S. pneumoniae*, which resolved over time [20, 21]. Since the genetic changes that confer macrolide resistance may reduce the fitness of *S. pneumoniae* strains (their ability to compete with drug-susceptible strains), resistant *S. pneumoniae* strains were likely to have been displaced by susceptible strains once the antibiotic selective pressure was removed [2]. Azithromycin use did not select for penicillin-resistant *S. pneumoniae* in our study. Although we did not determine minimum inhibitory concentrations for the isolates, this non-selection of penicillin-resistant *S. pneumoniae* is consistent with previous studies that administered azithromycin for both short [18] and long-term periods [2]. This is important as penicillins remain the first-line treatment for pneumococcal infections in sub-Saharan Africa.

Although the proportion of azithromycin-resistant *S. aureus* declined at 72 weeks, azithromycin-resistant *S. aureus* isolates remained significantly more common in the azithromycin arm at this visit. In a cystic fibrosis clinic in the Netherlands, the occurrence of azithromycin-resistant *S. aureus* was common enough to warrant discontinuation of long-term azithromycin therapy among cystic fibrosis patients [1]. In contrast, in Denmark, low prevalence of *S. aureus* in cystic fibrosis patients at baseline with a further reduction during treatment with azithromycin made increased macrolide resistance in *S. aureus* clinically insignificant [17]. It is possible that the baseline prevalence of *S. aureus* may influence the effect of long-term azithromycin therapy on resistance. Additionally, the differences in strength and frequency of dosing of azithromycin across studies may also contribute to the variations in the observed patterns of resistance. *S. aureus* is an important pathogen in HIV-infected patients where it causes bacteraemia leading to endocarditis [22]. Staphylococcal bacteraemia is about 17 times more likely to occur in a hospitalised HIV-infected adult than in an HIV-negative patient [22]. Therefore, this increase in azithromycin-resistant *S. aureus*, which persisted beyond six months post-intervention in our setting, is concerning.

The increase in tetracycline (*S. pneumoniae* and *S. aureus*) and clindamycin (*S. aureus* only) resistance at 48 weeks is not surprising since mobile genetic elements that mediate resistance to macrolides co-carry genes that confer resistance to other antibiotics [23]. Of note is the conjugative transposon Tn916-like element that carries *tet* (tetracycline resistance) and *erm* genes [23]. The *erm* gene product modifies the bacterial ribosomal target site of macrolides, lincosamides (clindamycin) and streptogramin antibiotics, thereby conferring resistance. These transposons are found in both *S. pneumoniae* [23] and *S. aureus* [24] hence the concurrent increase in resistance to macrolides, lincosamides and tetracyclines with azithromycin therapy in our study is expected [23]. These mobile

genetic elements with multiple resistance genes can be shared between bacterial species and across genera, increasing the overall burden of drug resistance [25].

S. pneumoniae is the most common bacterial cause of community-acquired pneumonia globally [26]. Macrolide-resistant *S. pneumoniae* is a major concern in the United States and other parts of the world as macrolides have been the first drug of choice in monotherapy for adults for decades [27]. There have been multiple reports of clinical failures resulting from macrolide-resistant *S. pneumoniae* when patients were treated with macrolides [28]. However, the clinical relevance of macrolide-resistant *S. pneumoniae* based on antimicrobial susceptibility testing has remained controversial [28]. This is fuelled by observations that although macrolide-resistant *S. pneumoniae*, as determined by antimicrobial susceptibility testing, has been on the rise, an increase in clinical failures due to these isolates has not been observed as frequently as expected (the so-called “*in vitro in vivo*” paradox) [27]. One explanation for this discrepancy is that the current *in vitro* resistance testing methods may not accurately model the action of an antibiotic in the body during an infection [26]. During inflammation, for instance, the level of azithromycin increases substantially because of intracellular accumulation within macrophages and leukocytes at the site of infection [26]. Furthermore, the antimicrobial susceptibility testing method does not account for the potent immunomodulatory properties of macrolides, which influence the resolution of respiratory infections [26].

Although macrolides are not the first-line treatment for *S. pneumoniae* and *S. aureus* respiratory infections in Malawi and Zimbabwe (penicillin and tetracyclines are used in Malawi and cotrimoxazole and amoxicillin in Zimbabwe), macrolides are indicated where there is treatment failure following amoxicillin use or in combination with ceftriaxone for severe illness (Malawi). They are also indicated in cases with penicillin allergy or atypical pneumonia in both countries. For *S. aureus* infections (including those at other body sites, such as skin and soft tissue infections), cloxacillin is the first-line antibiotic; however, clindamycin is indicated in penicillin hypersensitivity. Therefore, the clinical relevance of azithromycin-resistant *S. pneumoniae* and *S. aureus* and clindamycin-resistant *S. aureus* among study participants and their contacts should be further investigated.

To address concerns about the transmission of azithromycin-resistant *S. aureus* to untreated individuals, Tramper-Stranders *et al.* 2007 [29] compared the prevalence of azithromycin-resistant *S. aureus* among cystic fibrosis patients on long-term azithromycin therapy and their household contacts. They found that although there was a higher azithromycin-resistant *S. aureus* carriage among the cystic fibrosis patients (69.6%), carriage among household contact (9.6%) was similar to the general

population (6.3%) of the Netherlands. Furthermore, the transmission of resistant isolates from the patients to their household contacts could not be proved by genotyping, except for one household. This finding suggests that although the potential risk of transmission of azithromycin-resistant *S. aureus* exists, the probability is low. However, this observation may be quite different in an African household.

Taylor *et al.* [30, 4] showed that azithromycin's ability to reduce acute respiratory exacerbations is mediated by its capacity to reduce airway *H. influenzae* load, which is mirrored in our study. Our finding that azithromycin-resistant *H. influenzae* and *M. catarrhalis* strains were uncommon and similar between trial arms at all visits is consistent with previous clinical trials of long-term azithromycin therapy [2,3]. However, other studies of long-term azithromycin have observed an increase in azithromycin-resistant *H. influenzae* [1,31] and *M. catarrhalis* [3,31]. The discordance across studies in the observed patterns of *H. influenzae* and *M. catarrhalis* resistance may be explained by the differences in the dose and duration of azithromycin used. Also, relatively few isolates of both bacterial species were identified in many studies (including our own) and thus, these studies may have been underpowered to detect resistance in these species.

Huang *et al.* [32] observed that acute respiratory exacerbations were associated with an increase in the relative abundance of the bacterial phylum Proteobacteria in patients with COPD. Antibiotic treatment reduced this relative abundance and was associated with the resolution of symptoms. Broader changes in the airway microbiome and not only in the specific bacterial species we measured may modulate the effect of azithromycin in HIV-associated chronic lung disease. However, microbial dysbiosis may occur in other microbial niches, including the gut, following long-term azithromycin treatment, and the local and systemic consequences of such dysbiosis require further study.

Given concerns around the emergence of resistance with long-term azithromycin therapy, newer macrolide-based drugs that have potent immunomodulatory activity without resistance-promoting antibacterial effects should be considered. Hodge *et al.* [33] investigated two new macrolides with limited antibacterial activity against *S. pneumoniae*, *S. aureus*, *M. catarrhalis* and *H. influenzae*. They found that these drugs improve macrophage phagocytosis of bacteria and therefore reduce bacterial carriage and associated acute exacerbations of CLD [33].

The strength of our study is that it was a double-blind, individually randomised, placebo-controlled, multicentre study among children with HIV-associated CLD in sub-Saharan Africa, a previously

under-studied population. The study also analysed the persistence of the microbiological effect of long-term azithromycin therapy six months post-intervention. Furthermore, we also demonstrated that long-term azithromycin promoted resistance to other antibiotics (tetracycline and clindamycin). Study limitations include the lack of genomic investigation to delineate antibiotic resistance mechanisms and determine *S. pneumoniae* and *H. influenzae* serotypes. Furthermore, 27% of participants' outcomes could not be assessed at 72 weeks due to withdrawal, death, and loss to follow-up. However, our findings at this later time point are consistent with previous findings [2]. We could not determine whether or when *S. aureus* resistance to azithromycin, tetracycline and clindamycin resolved because our study concluded at 72 weeks from randomisation. Another limitation is that the study was not adequately powered to conclude whether azithromycin induced resistance in *H. influenzae* and *M. catarrhalis* as the number of isolates recovered from trial participants was low. We did not perform a quantitative assessment of minimum inhibitory concentrations of antibiotics, which may have identified increases in minimum inhibitory concentrations, which did not lead to changes in the resistance category.

4.5 Conclusion

Long-term azithromycin therapy reduced the prevalence of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* in African children with HIV-associated CLD while increasing the carriage of azithromycin and tetracycline-resistant *S. pneumoniae* and SA at 48 weeks. Inducible clindamycin resistance also increased in *S. aureus* at 48 weeks. However, these microbiological effects resolved six months post-intervention, except for azithromycin, tetracycline, and clindamycin resistance in *S. aureus*. Further studies are needed to determine whether resistance in *S. aureus* persists beyond 72 weeks. The benefit of reduced acute exacerbations of HIV-associated CLD shown in this study [9] should be weighed against the largely temporary acquisition of antimicrobial resistance to azithromycin and other antibiotics.

4.6 Declarations

4.6.1 Contributors

MPN, FSD and RAF conceived the study. REA conducted laboratory experiments and wrote the first manuscript draft, supervised by MPN and FSD. CB assisted with laboratory experiments. REA conducted the statistical analysis, supported by AMR and VS. GM and LN coordinated the trial in

Zimbabwe and Malawi. BW and RH reviewed the manuscript. JOO and RAF are trial PIs. All authors contributed to the manuscript, and all authors have read and approved the final version.

4.6.2 Data sharing

All de-identified data can be accessed at dryad.org (<https://doi.org/10.5061/dryad.5dv41ns71>) and analysis codes are available at Zenodo.org (<https://doi.org/10.5281/zenodo.5712334>).

4.6.3 Funding

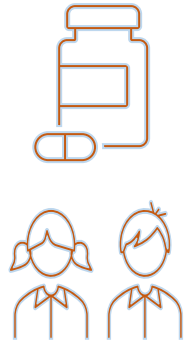
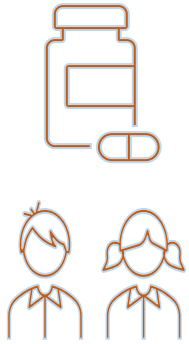
The Global Health and Vaccination Research (GLOBVAC) Programme of the Medical Research Council of Norway funded this study. This work was also supported by the National Institute for Health Research (NIHR) Global Health Research Unit on Mucosal Pathogens using UK aid from the UK Government (Project number 16/136/46). RSH is an NIHR Senior Investigator. The views expressed are those of the authors and not necessarily those of the NIHR, the Department of Health and Social Care. MN is supported by an Australian National Health and Medical Research Council Investigator Grant [APP1174455]. REA is funded by the L'Oréal UNESCO For Women in Science Fellowship, Margaret McNamara Education Grants, and the Swedish International Development Cooperation Agency (SIDA) through the Organisation of Women in Science for the developing world (OWSD) PhD Fellowship. FSD is supported by the National Research Foundation of South Africa (112160), Future Leaders – African Independent Research (FLAIR) Fellowship and the National Institute for Health Research (NIHR) Global Health Research Unit on Mucosal Pathogens (MPRU) using UK aid from the UK Government and the University of Cape Town. RAF is funded by the Wellcome Trust. AMR is supported by the UK Medical Research Council (MRC) and the UK Department for International Development (DFID) under the MRC/DFID Concordat agreement, which is also part of the EDCTP2 Programme supported by the European Union, Grant Ref: MR/R010161/1.

4.7 References

1. Phaff SJ, Tiddens HAWM, Verbrugh HA, Ott A. Macrolide resistance of *Staphylococcus aureus* and *Haemophilus* species associated with long-term azithromycin use in cystic fibrosis. *J. Antimicrob. Chemother.* 2006; 57: 741–746.
2. Hare KM, Grimwood K, Chang AB, Chatfield MD, Valery PC, Leach AJ, Smith-Vaughan HC, Morris PS, Byrnes CA, Torzillo PJ, Cheng AC. Nasopharyngeal carriage and macrolide resistance in indigenous children with bronchiectasis randomized to long-term azithromycin or placebo. *Eur. J. Clin. Microbiol. Infect. Dis.* 2015; 34: 2275–2285.
3. Albert RK, Connett J, Bailey WC, Casaburi R, Cooper JADJ, Criner GJ, Curtis JL, Dransfield MT, Han MK, Lazarus SC, Make B, Marchetti N, Martinez FJ, Madinger NE, McEvoy C, Niewoehner DE, Porsasz J, Price CS, Reilly J, Scanlon PD, Sciurba FC, Scharf SM, Washko GR, Woodruff PG, Anthonisen NR. Azithromycin for prevention of exacerbations of COPD. *N. Engl. J. Med.* 2011; 365: 689–698.

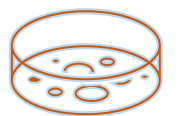
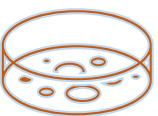
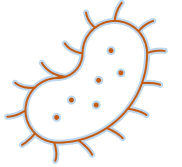
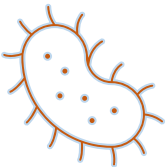
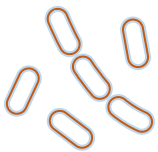
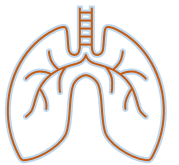
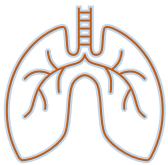
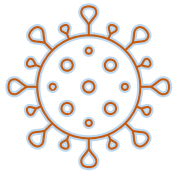
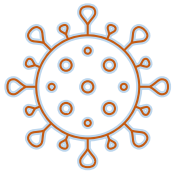
4. Taylor SL, Leong LEX, Mobegi FM, Choo JM, Wesselingh S, Yang IA, Upham JW, Reynolds PN, Hodge S, James AL, Jenkins C, Peters MJ, Baraket M, Marks GB, Gibson PG, Rogers GB, Simpson JL. Long-term azithromycin reduces *Haemophilus influenzae* and increases antibiotic resistance in severe asthma. *Am. J. Respir. Crit. Care Med.* 2019; 200: 309–317.
5. Serisier DJ. Risks of population antimicrobial resistance associated with chronic macrolide use for inflammatory airway diseases. *Lancet Respir Med.* 2013; 1(3):262–74.
6. Li H, Liu D-H, Chen L-L, Zhao Q, Yu Y-Z, Ding J-J, Miao L-Y, Xiao Y-L, Cai H-R, Zhang D-P, Guo Y-B, Xie C-M. Meta-analysis of the adverse effects of long-term azithromycin use in patients with chronic lung diseases. *Antimicrob. Agents Chemother.* 2014; 58: 511–517.
7. Brusselle GG, Vanderstichele C, Jordens P, Deman R, Slabbynck H, Ringoet V, Verleden G, Demedts IK, Verhamme K, Delporte A, Demeyere B, Claeys G, Boelens J, Padalko E, Verschakelen J, Van Maele G, Deschepper E, Joos GFP. Azithromycin for prevention of exacerbations in severe asthma (AZISAST): a multicentre randomised double-blind placebo-controlled trial. *Thorax* 2013; 68: 322–329.
8. Ferrand RA, Desai SR, Hopkins C, Elston CM, Copley SJ, Nathoo K, Ndhlovu CE, Munyati S, Barker RD, Miller RF, Bandason T, Wells AU, Corbett EL. Chronic lung disease in adolescents with delayed diagnosis of vertically acquired HIV infection. *Clin. Infect. Dis.* 2012; 55: 145–152.
9. Ferrand RA, McHugh G, Rehman AM, Mujuru H, Simms V, Majonga ED, Nicol MP, Flaegstad T, Gutteberg TJ, Gonzalez-Martinez C, Corbett EL, Rowland-Jones SL, Kranzer K, Weiss HA, Odland JO, BREATHE Trial Group. Effect of once-weekly azithromycin vs placebo in children with HIV-associated chronic lung disease: The BREATHE randomized clinical trial. *JAMA Netw. Open* 2020; 3: e2028484.
10. Abotsi RE, Nicol MP, McHugh G, Simms V, Rehman AM, Barthus C, Mbhele S, Moyo BW, Ngwira LG, Mujuru H, Makamure B, Mayini J, Odland JO, Ferrand RA, Dube FS. Prevalence and antimicrobial resistance profiles of respiratory microbial flora in African children with HIV-associated chronic lung disease. *BMC Infect. Dis.* 2021; 21: 216.
11. Bojang E, Jafali J, Perreten V, Hart J, Harding-Esch EM, Sillah A, Mabey DCW, Holland MJ, Bailey RL, Roca A, Burr SE. Short-term increase in prevalence of nasopharyngeal carriage of macrolide-resistant *Staphylococcus aureus* following mass drug administration with azithromycin for trachoma control. *BMC Microbiol.* 2017; 17: 75.
12. CLSI. Performance standards for antimicrobial susceptibility testing. 28th Editi. Wayne: Clinical Laboratory Standards Institute; 2018.
13. Twisk J, Bosman L, Hoekstra T, Rijnhart J, Welten M, Heymans M. Different ways to estimate treatment effects in randomised controlled trials. *Contemp. Clin. Trials Commun.* 2018; 10: 80–85.
14. Tramper-Stranders GA, Wolfs TFW, Fleer A, Kimpen JLL, van der Ent CK. Maintenance azithromycin treatment in pediatric patients with cystic fibrosis: long-term outcomes related to macrolide resistance and pulmonary function. *Pediatr. Infect. Dis. J.* 2007; 26: 8–12.
15. Hodge S, Reynolds PN. Low-dose azithromycin improves phagocytosis of bacteria by both alveolar and monocyte-derived macrophages in chronic obstructive pulmonary disease subjects. *Respirol. Carlton Vic* 2012; 17: 802–807.
16. Swarthout TD, Fronterre C, Lourenço J, Obolski U, Gori A, Bar-Zeev N, Everett D, Kamng'ona AW, Mwalukomo TS, Mataya AA, Mwansambo C, Banda M, Gupta S, Diggle P, French N, Heyderman RS. High residual carriage of vaccine-serotype *Streptococcus pneumoniae* after introduction of pneumococcal conjugate vaccine in Malawi. *Nat. Commun.* 2020; 11: 2222.
17. Hansen CR, Pressler T, Hoiby N, Johansen HK. Long-term, low-dose azithromycin treatment reduces the incidence but increases macrolide resistance in *Staphylococcus aureus* in Danish CF patients. *J. Cyst. Fibros.* 2009; 8: 58–62.
18. Skalet AH, Cevallos V, Ayele B, Gebre T, Zhou Z, Jorgensen JH, Zerihun M, Habte D, Assefa Y, Emerson PM, Gaynor BD, Porco TC, Lietman TM, Keenan JD. Antibiotic selection pressure and macrolide resistance in Nasopharyngeal *Streptococcus pneumoniae*: A cluster-randomized clinical trial. *PLoS Med.* 2010; 7: 12.
19. Keenan JD, Klugman KP, McGee L, Vidal JE, Chochua S, Hawkins P, Cevallos V, Gebre T, Tadesse Z, Emerson PM, Jorgensen JH, Gaynor BD, Lietman TM. Evidence for clonal expansion after antibiotic selection pressure: pneumococcal multilocus sequence types before and after mass azithromycin treatments. *J. Infect. Dis.* 2015; 211: 988–994.
20. Leach AJ, Shelby-James TM, Mayo M, Gratten M, Laming AC, Currie BJ, Mathews JD. A prospective study of the impact of community-based azithromycin treatment of trachoma on carriage and resistance of *Streptococcus pneumoniae*. *Clin. Infect. Dis.* 1997; 24: 356–362.
21. Haug S, Lakew T, Habtemariam G, Alemayehu W, Cevallos V, Zhou Z, House J, Ray K, Porco T, Rutar T, Keenan J, Lietman TM, Gaynor BD. The decline of pneumococcal resistance after cessation of mass antibiotic distributions for trachoma. *Clin. Infect. Dis.* 2010; 51: 571–574.
22. Senthilkumar A, Kumar S, Sheagren JN. Increased incidence of *Staphylococcus aureus* bacteremia in hospitalized patients with acquired immunodeficiency syndrome. *Clin. Infect. Dis.* 2001; 33: 1412–1416.
23. Chancey ST, Agrawal S, Schroeder MR, Farley MM, Tettelin H, Stephens DS. Composite mobile genetic elements disseminating macrolide resistance in *Streptococcus pneumoniae*. *Front. Microbiol.* 2015; 6: 26.

24. De Vries LE, Christensen H, Skov RL, Aarestrup FM, Agersø Y. Diversity of the tetracycline resistance gene tet (M) and identification of Tn 916- and Tn 5801-like (Tn 6014) transposons in *Staphylococcus aureus* from humans and animals. *J. Antimicrob. Chemother.* 2009; 64: 3.
25. Schroeder MR, Stephens DS. Macrolide resistance in *Streptococcus pneumoniae*. *Front. Cell. Infect. Microbiol.* 2016; 6.
26. Hoban DJ, Zhanel GG. Clinical implications of macrolide resistance in community-acquired respiratory tract infections. *Expert Rev. Anti Infect. Ther.* 2006; 4: 973–980.
27. Nuermberger E, Bishai WR. The Clinical significance of macrolide-resistant *Streptococcus pneumoniae*: It's all relative. *Clin. Infect. Dis.* 2004; 38: 99–103.
28. Klugman KP, Lonks JR. Hidden epidemic of macrolide-resistant pneumococci. *Emerg. Infect. Dis.* 2005; 11: 802–807.
29. Tramper-Stranders GA, van der Ent CK, Gerritsen SAM, Fleer A, Kimpfen JLL, Wolfs TFW. Macrolide-resistant *Staphylococcus aureus* colonization in cystic fibrosis patients: is there transmission to household contacts? *J. Antimicrob. Chemother.* 2007; 60: 665–668.
30. Taylor SL, Ivey KL, Gibson PG, Simpson JL, Rogers GB; AMAZES Study Research Group. Airway abundance of *Haemophilus influenzae* predicts response to azithromycin in adults with persistent uncontrolled asthma. *Eur Respir J.* 2020;56(4):2000194.
31. Altenburg J, de Graaff CS, Stienstra Y, Sloos JH, van Haren EH, Koppers RJ, van der Werf TS, Boersma WG. Effect of azithromycin maintenance treatment on infectious exacerbations among patients with non-cystic fibrosis bronchiectasis: the BAT randomized controlled trial. *JAMA.* 2013;309(12):1251-9.
32. Huang YJ, Sethi S, Murphy T, Nariya S, Boushey HA, Lynch SV. Airway microbiome dynamics in exacerbations of chronic obstructive pulmonary disease. *J Clin Microbiol.* 2014;52(8):2813-23.
33. Hodge S, Tran HB, Hamon R, Roscioli E, Hodge G, Jersmann H, Ween M, Reynolds PN, Yeung A, Treiberg J, Wilbert S. Nonantibiotic macrolides restore airway macrophage phagocytic function with potential anti-inflammatory effects in chronic lung diseases. *Am. J. Physiol.-Lung Cell. Mol. Physiol.* 2017; 312: L678–L687.



Chapter 5

**Sputum bacterial load and
bacterial composition correlate
with lung function and are altered
by long-term azithromycin
treatment in children with HIV-
associated chronic lung disease**



Sputum bacterial load and bacterial composition correlate with lung function and are altered by long-term azithromycin treatment in children with HIV-associated chronic lung disease

Regina E. Abotsi^{1,2}, Felix S. Dube¹, Andrea M. Rehman³, Shantelle Claassen-Weitz⁴, Yao Xia⁵, Victoria Simms^{3,9}, Kilaza S. Mwaikono^{6,7}, Sugnet Gardner-Lubbe⁸, Grace McHugh⁸, Lucky G. Ngwira^{10,11}, Brenda Kwambana-Adams¹², Robert S Heyderman¹², Jon Ø Odland¹³, Rashida A Ferrand^{9,14}, Mark P. Nicol^{4,5} and The BREATHE study team

Affiliations

1. Department of Molecular and Cell Biology & Institute of Infectious Diseases and Molecular Medicine, University of Cape Town, Cape Town, South Africa
2. Department of Pharmaceutical Microbiology, School of Pharmacy, University of Health and Allied Sciences, Ho, Ghana
3. International Statistics and Epidemiology Group, London School of Hygiene and Tropical Medicine, London, United Kingdom
4. Division of Medical Microbiology, Department of Pathology, Faculty of Health Sciences, University of Cape Town, Cape Town, South Africa
5. Division of Infection and Immunity, School of Biomedical Sciences, Faculty of Health and Medical Sciences, University of Western Australia, Perth, Australia
6. Computational Biology Group and H3ABioNet, Department of Integrative Biomedical Sciences, University of Cape Town, Cape Town, South Africa
7. Department of Science and Laboratory Technology, Dar es Salaam Institute of Technology, Dar es Salaam, Tanzania
8. Department of Statistics and Actuarial Science, Faculty of Economic and Management Sciences, Stellenbosch University, Stellenbosch, South Africa
9. Biomedical Research and Training Institute, Harare, Zimbabwe
10. Malawi-Liverpool Wellcome Trust Clinical Research Programme, Blantyre, Malawi
11. Department of Clinical Sciences, Liverpool School of Tropical Medicine, Liverpool, United Kingdom
12. NIHR Global Health Research Unit on Mucosal Pathogens, Research Department of Infection, Division of Infection and Immunity, University College London, London, United Kingdom
13. Department of Community Medicine, University of Tromsø, Tromsø, Norway & International Research Laboratory for Reproductive Ecotoxicology (IL RET), The National Research University Higher School of Economics, Moscow, Russia & School of Health Systems and Public Health, Faculty of Health Sciences, University of Pretoria, Pretoria, South Africa
14. Clinical Research Department, London School of Hygiene and Tropical Medicine, London, United Kingdom

5.0 Abstract

Background: Long-term azithromycin treatment reduces the frequency of acute respiratory exacerbation in children and adolescents with HIV-associated chronic lung disease (HCLD). However, the impact of this treatment on the respiratory bacteriome is unknown.

Methods: African children with HCLD (defined as forced expiratory volume in 1-second z-score (FEV1z) less than -1.0 with no reversibility) were enrolled in a placebo-controlled trial of once-weekly azithromycin given for 48 weeks (BREATHE trial). Sputum samples were collected at baseline, 48 weeks (end of treatment) and 72 weeks (six months post-intervention in participants who reached this timepoint before trial conclusion). Sputum bacterial load and bacteriome profiles were determined using 16S rDNA qPCR and V4 gene amplicon sequencing. The primary outcomes were within-participant and within-arm (azithromycin vs placebo) changes in the sputum bacteriome measured across baseline, 48 weeks, and 72 weeks. Associations between clinical or socio-demographic factors and bacteriome profiles were also assessed using linear regression.

Results: In total, 347 participants (median age: 15.3 years, interquartile range [12.7–17.7]) were enrolled and randomised to azithromycin (173) or placebo (174). After 48 weeks, participants in the azithromycin arm had reduced sputum bacterial load vs placebo arm (16S rDNA copies/ μ l in log₁₀, mean difference and 95% confidence interval [CI] of azithromycin vs Placebo -0.54 [-0.71; -0.36]). Shannon alpha diversity remained stable in the azithromycin arm but declined in the placebo arm between baseline and 48 weeks (3.03 vs 2.80, $p = 0.04$, Wilcoxon paired test). Bacterial community structure changed in the azithromycin arm at 48 weeks compared with baseline (PERMANOVA test $p = 0.003$) but resolved at 72 weeks. The relative abundances of genera previously associated with HCLD decreased in the azithromycin arm at 48 weeks compared with baseline, including *Haemophilus* (17.9% vs 25.8%, $p < 0.05$, ANCOM $\omega = 32$) and *Moraxella* (1% vs 1.9%, 47, $p < 0.05$, ANCOM $\omega = 47$). This reduction was sustained at 72 weeks relative to baseline. Lung function (FEV1z) was negatively associated with bacterial load (linear mixed effect model coefficient, [CI]: -0.09 [-0.16; -0.02]) and positively associated with Shannon diversity (0.19 [0.12; 0.27]). The relative abundance of *Neisseria* (coefficient, [standard error]: (2.85, [0.7], $q = 0.01$), and *Haemophilus* (-6.1, [1.2], $q < 0.001$) were positively and negatively associated with FEV1z, respectively. An increase in the relative abundance of *Streptococcus* from baseline to 48 weeks was associated with improvement in FEV1z-

score (3.2 [1.11], $q = 0.01$) whilst an increase in *Moraxella* was associated with a decline in FEV1z-score (-2.74 [0.74], $q = 0.002$).

Conclusions: Azithromycin treatment preserved sputum bacterial diversity and reduced the relative abundances of the HCLD-associated genera *Haemophilus* and *Moraxella*. These bacteriological effects were associated with improved lung function and may account for reduced respiratory exacerbations associated with azithromycin treatment in children with HCLD.

Keywords: Africa, bacteriome, microbiome, HIV, *Haemophilus*, *Moraxella*, children, adolescents, FEV1z-score

5.1 Background

Macrolides, including azithromycin, are commonly used to manage chronic lung diseases (CLD), including cystic fibrosis [1], chronic obstructive pulmonary disease (COPD) [2], asthma [3], post-transplantation obliterative bronchiolitis [4] and bronchiectasis [5]. Long-term use of azithromycin is associated with improved lung function and survival; and reduced frequency of acute respiratory exacerbation, antibiotic administration and hospitalisation in CLDs [6]. We have previously shown that long-term azithromycin reduced the frequency of acute respiratory exacerbations and all-cause hospitalisation in children and adolescents with HIV-associated CLD (HCLD) [7].

Mechanisms for these beneficial effects may include a combination of the antimicrobial, anti-inflammatory and immunomodulatory effects of macrolides [6]. The direct antibacterial effect of azithromycin on pathogens may be enhanced by improved phagocytic competency of alveolar macrophages, which tend to be defective in CLD [8,9]. Additional benefits of azithromycin in CLDs include antiviral activity, improved ciliary function, interference with *Pseudomonas aeruginosa* biofilm formation, mitigation of airway mucus hypersecretion and promotion of pulmonary epithelial cell healing [6].

We previously observed that a *Haemophilus*, *Moraxella*, or *Neisseria* (HMN)-dominant sputum bacteriome was associated with a 1.5-fold increased risk of HCLD, compared to a *Streptococcus* or *Prevotella*-dominant bacteriome [10]. We also showed an association between HCLD and respiratory carriage of *Streptococcus pneumoniae* and *Moraxella catarrhalis* [11]. The therapeutic effect of azithromycin in COPD may be mediated by changes in the lung bacteriome along with associated

changes in microbial metabolites that interact with the host immune system [2]. In COPD, Huang *et al.* [12] observed that acute exacerbations were associated with an increase in the relative abundance of the phylum Proteobacteria (HMN belong to this phylum). Antibiotic treatment reduced this relative abundance and resulted in the resolution of symptoms. Although similar mechanisms may apply in HCLD, the effect of azithromycin on the respiratory microbiome in HCLD has not been studied. An insight into how azithromycin affects the HCLD respiratory microbiome and how this may mediate its beneficial effects will contribute towards the understanding of the pathogenesis of HCLD and facilitate targeted therapeutics.

In this study, we determined the impact of long-term azithromycin treatment (weekly treatment for 48 weeks) on the diversity and composition of the sputum bacteriome of Zimbabwean and Malawian children and adolescents with HCLD. We also measured the persistence of bacteriome alterations six months post-treatment, which has not been previously investigated in other CLDs. Further, we assessed the association between bacteriome alterations and key clinical outcomes, including reduction in acute pulmonary exacerbations, all-cause hospitalisation, and lung function. Finally, we investigated whether azithromycin altered the airway bacteriome in favour of HCLD-protective taxa.

5.2 Materials and Methods

5.2.1 Study design, participants, setting, eligibility, and intervention

This was a sub-study of the Bronchopulmonary Function in Response to Azithromycin Treatment for Chronic Lung Disease in HIV-infected Children (BREATHE) trial [7,13] (Clinicaltrials.gov identifier: NCT02426112). The trial protocol [13] and its main findings [7] have been previously described. Briefly, children and adolescents aged 6–19 years with perinatally-acquired HIV infection taking antiretroviral therapy (ART) for a minimum of six months were recruited from outpatient HIV clinics in Blantyre, Malawi and Harare, Zimbabwe. HCLD was defined as forced expiratory volume in 1-second (FEV1) *z*-score (FEV1*z*) less than -1.0 with no reversibility, i.e., < 12% improvement in FEV1*z*-score after 200 µg of salbutamol inhalation. FEV1*z*-score was calculated using the African American module of the Global Lung Function Initiative 2012 reference equations [14] validated among children in Zimbabwe by our team [15]. Exclusion criteria included acute respiratory symptoms, tuberculosis, pregnancy, hypersensitivity to azithromycin, prolonged QTc interval, impaired hearing, or renal clearance. Participants were randomised to receive 48 weeks of once-weekly, weight-based doses of oral azithromycin [13]. Placebo arm participants received identical

tablets without the active drug. For both trial arms, this was followed by a six-month treatment-free period (to 72 weeks). Participant adherence was defined as “*not missing, on average, more than two of the 12 dispensed doses, as assessed by pill count, splitting time in the study into four 12-week periods, as per visit and study medication dispensing schedule*” [7]. All trial participants were analysed at 48 weeks. In this sub-study, only participants with sputum samples available from at least one of the three visits (baseline, 48 and 72 weeks) were included. Hospitalisation was defined as a period of stay in a hospital more than 24 hours. Acute respiratory exacerbation was defined as new or worsening respiratory symptoms as assessed by a physician.

5.2.2 Study outcomes

For this sub-study comparing azithromycin and placebo arms, the primary outcomes were within-participant change in bacteriome profiles (as defined by Aitchison distance, Bray-Curtis index, and differentially abundant taxa) of the sputum samples from baseline to 48-week, 48 to 72-week, and from baseline to 72-week study visits. Secondary outcomes were 1) differences in sputum bacterial load and bacteriome at all visits between azithromycin and placebo arms; 2) associations between alpha and beta diversity metrics and selected covariates including drug adherence, age, sex, site, season of sampling, duration of ART, lung function, MRC dyspnoea score [16] and acute respiratory exacerbation; 3) differences in within-participant sputum beta diversity of participants who developed an adverse event during the study (acute respiratory exacerbation, hospitalisation or receipt of additional antibiotics other than study drug or cotrimoxazole) compared to those that did not; and 4) the differentially abundant taxa driving changes and differences in bacteriome profiles between trial arms and visits.

5.2.3 Sample and data collection

Sputa were collected from participants at baseline, 48 weeks (end of treatment) and 72 weeks (six months post-intervention in participants who reached this time point before trial conclusion). For participants who could not expectorate spontaneously, sputum was induced with hypertonic saline. Samples were stored in PrimeStore® Molecular Transport medium [Primestore] (Longhorn Vaccines & Diagnostics LLC, Bethesda, USA) immediately after collection, transported on ice, and stored at -80°C at the study centres. This was followed by batch shipment on dry ice to Cape Town, South Africa, where samples were stored at -80°C until further processing. Clinical and socio-demographic data were collected at each visit using questionnaires administered by a study nurse and from participant hospital records.

5.2.4 Nucleic acid extraction and purification

Sputum samples stored at -80°C were thawed, vortexed for 10 seconds, and 450 µl transferred into ZR BashingBead™ Lysis Tubes® containing 0.5mm beads (catalogue no. ZR S6002–50, Zymo Research Corp., Irvine, CA, United States). Mechanical lysis was conducted at 50 Hz for 5 minutes using the TissueLyser LTTM® (Qiagen, FRITSCH GmbH, Idar-Oberstein, Germany). The lysate was centrifuged at 10000 rpm (10,640 g) (Eppendorf F-45-30-11, Merck KGaA, Darmstadt, Germany) for 2 minutes and 250 µl of the supernatant was loaded onto a QIA Symphony® SP instrument (Qiagen, Hombrechtikon, Switzerland). Nucleic acid was purified using the DSP Virus/Pathogen Mini Kit® (catalogue no. 937036, Qiagen GmbH, Hilden, Germany) and eluted into 70 µl. We included bacterial mock community cells (catalogue no. ZR D6300, Zymo Research Corp., Irvine, CA, United States) as template extraction controls on each of the four plates of the three sequencing runs. We also included neat Primestore and Milli-Q® ultrapure water (exposed to UV for 30 minutes before use) (Millipore Sigma, Burlington, MA, USA) as no-template extraction controls.

5.2.5 16S rDNA library preparation and amplicon sequencing

The total bacterial load (16S rDNA gene copies) of each extracted sample was determined using a previously described qPCR [17]. Gene copy numbers were extrapolated from standard curves derived from *Escherichia coli* strain JM109 DNA (catalogue no. ZR E2006, Zymo Research Corp., Irvine, CA, United States), using genome size (4.60E+06) and the number of 16S rDNA gene copies (seven). For bacteriome analysis, the V4 hypervariable region of the 16S rDNA gene underwent two-step amplification as previously described [18] with the following modifications: 5 µl of DNA was used as the template for each step of the PCR with a final concentration of 8 pM loaded on the Illumina Miseq. We also included a bacterial mock community DNA standard (catalogue no. ZR D6305, Zymo Research Corp., Irvine, CA, United States) and no-template extraction controls on all plates in each run. Randomly selected samples were repeated within or between sequencing runs to assess within or between-run reproducibility. Samples from the same participant were all processed on the same plate to avoid batch effects. Each run included a balanced number of samples from both trial arms and both study sites.

5.2.6 Bioinformatics analysis of 16s rDNA metagenomic data

After the quality of demultiplexed paired-end reads was deemed acceptable via FastQC [19] and MultiQC [20], the DADA2 pipeline [21] (wrapped in the Nextflow algorithm [22]) was used to filter

and trim reads, infer amplicon sequence variants (ASVs), and assign taxonomy to ASVs. Default parameters were applied for all DADA2 functions unless expressly mentioned. Briefly, forward reads were trimmed and truncated at 24 and 248 bases, and reverse reads were trimmed and truncated at 25 and 235 bases. The minimum read length after trimming and truncation was set to 250 bases. Sequencing reads were dereplicated and pooled, and ASVs were inferred for each sample via the DADA2 sample inference algorithm and the estimated error model. Denoised sequences were generated by merging forward and reverse reads with overlap length set to 20 and allowing for no mismatches in the overlap region. Chimeric sequences were identified by precisely reconstructing them by combining a left-segment and a right-segment of more abundant sequences. Then, they were removed from the ASV table of denoised merged sequences. Taxonomy was assigned to each ASV using the SILVA version 138 [23] species classifier implementation for DADA2 [21]. Phylogenetic alignment was performed using *AlignSeqs* in the DECIPHER [24] package, while a neighbour-joining phylogenetic tree was constructed using functions in the PHANGORN [25] package. The 16S rDNA gene sequencing datasets were submitted to the National Centre for Biotechnology Information (NCBI) Sequence Read Archive (SRA) repository (accession number [PRJNA769290](https://www.ncbi.nlm.nih.gov/sra/PRJNA769290)).

5.2.7 Quality control and *in silico* correction of contamination

Both experimental work and data analysis plan were completed before unblinding of study investigators. We used a previously published *in silico* quality control approach to minimise experimental error as outlined below [26]. Spurious ASVs, defined as ASVs with < 5 reads across all sequenced biological specimens and no-template control, were removed. We assessed nucleic acid extraction and sequencing efficiency by comparing the mock bacterial community extraction and sequencing controls to the manufacturer profiles. Next, we assessed reproducibility within and between runs by comparing bacteriome profiles of within- and between-run repeats. To determine whether the bacteriome profile of the low biomass samples was background noise from the storage medium (Primestore) or amplicon contamination, we assessed clustering of these samples with the no-template controls (Primestore) on a log-ratio biplot and Principal Coordinates Analysis (PCoA) plot. Next, biological specimens with 16S rDNA gene copy numbers $\leq 500/\mu\text{l}$ were removed, as we and others have shown that these low biomass specimens produce poorly reproducible sequencing profiles [26,27]. We used the *isContaminant* function embedded in the DECONTAM [28] package in R to identify potential contaminants using sequence data from biological specimens and no-template controls (Primestore). The maximum proportions of each identified contaminant in the no-template controls were subtracted from the biological specimens. Detailed quality control and decontamination

steps and results are described in the supplementary material (Appendix D Table S1 and S2, Appendix D Figure S1–S17).

5.2.8 Statistical analysis

Statistical analysis was performed using R statistical software version 4.0.4 (R Foundation for Statistical Computing, Vienna, Austria; <https://www.r-project.org/>). Categorical variables were reported as frequency distributions and continuous variables as medians with interquartile ranges (IQRs) if non-parametric or means with standard deviations (SD) if otherwise. Normality distribution of continuous variables was assessed using the Shapiro Wilk test. Comparing continuous outcomes between trial arms was done using Mann-Whitney U or Student t-test where appropriate. Fisher's exact test or Chi-square test were used to compare categorical variables between arms where appropriate. We used the Wilcoxon signed-rank matched-pairs test to compare continuous outcomes between visits within trial arms. Correction for multiple testing was with Benjamin/Hochberg (cut off = 0.05). Amplicon Sequence Variants (ASV) were merged to the genus level and 0.5% prevalence filtering was applied to samples before analysis. The number of ASVs in a single sample represented bacterial taxonomic richness. Shannon's index [29] was used to characterise richness and evenness (alpha diversity).

Beta diversity was determined for intra-individual differences in samples from different visits or inter-individual differences in samples collected at the same visit. Beta diversity was determined using two distances for validation – Euclidean distances of centred log-ratio (CLR) transformed ASV counts (Aitchison distance) after applying a pseudo count of one and by Bray-Curtis dissimilarity index [30] on unrarefied ASV counts, all implemented using *distance* functions in the PHYLOSEQ R package. PCoA plots were used to visualised beta diversity. We used Permutational multivariate analysis of variance (PERMANOVA) [31] implemented using the *adonis2* function in the VEGAN [32] R package to compare beta diversity between visits or trial arms. PERMANOVA tests were adjusted for age, sex, site, ART regimen, MRC dyspnoea score, FEV1z, cotrimoxazole prophylaxis, previous tuberculosis treatment and previous admission for chest problems within 12 months prior to enrolment. Permutational Analyses of Multivariate Dispersions (PERMDISP) (implemented using *betadisper* function in VEGAN) was used to assess the homogeneity of dispersion between visits and trial arms.

Differentially abundant taxa were identified using ANCOM2 [33] on unrarefied data with False Discovery Rate (FDR) Benjamini/Hochberg correction (cut off = 0.05). Nine other methods for detecting differentially abundant taxa were used for comparison and reported in the supplementary

material (Wilcoxon signed-rank test on data after the following normalisation methods—total sum scaling, variance stabilising transformation, and centred log transformation after applying a pseudocount of one, Aldex2 [34], Deseq2 [35], ANCOM-BC [36], Corncob [37], MaasLin2 [38] with total sum scaling and log transformation, MaasLin2 [38] with centred log transformation after applying a pseudo count of one). The relative contribution of each taxon to overall dissimilarity was measured using SIMPER analysis on the Bray-Curtis distances between samples.

To determine the association between sputum bacterial load ($\log_{10}$ transformed 16S rDNA gene copies) or alpha diversity (Shannon index) and clinical and socio-demographic factors, we used linear mixed-effect models (LME) via the LME4 and LMERTTEST R packages. For each LME, we included visit and the interaction term visit-versus-trial-arm [39] as fixed effects and the individual participant identifier as a random effect. We included in a stepwise manner, adherence, age, site, sex, season of sampling, Medical Research Council (MRC) dyspnoea score, body-mass-index (BMI), weight-for-age z-score, height-for-age z-score, BMI-for-age z-score, ART regimen, duration of ART, HIV viral load suppression, cotrimoxazole prophylaxis, previous tuberculosis treatment or previous hospitalisation for chest problems one year prior to study enrolment, forced vital capacity (FVC), forced vital capacity z-score (FVCz), FEV1z, FEV1 percentage predicted (FEVpcpred), FVC percentage predicted (FVCpcpred) and FEV1/FVCz as covariates in the models. Continuous outcome variables included in the models were log-transformed where necessary to satisfy model assumptions. Adherence to AZM, age, site, sex, season of sampling, MRC dyspnoea score, HIV viral load suppression (baseline values were used as values at 72 weeks were unavailable), cotrimoxazole prophylaxis, previous tuberculosis treatment or previous hospitalisation for chest problems one year prior to study enrolment, ART regimen and duration were selected *a priori* to be adjusted for. Any other variable identified as an independent predictor in the univariate analysis ($p < 0.05$) was also included in the multivariate LME. The following were excluded from the multivariate model because of co-linearity: BMI-for-age z-score and weight-for-age z-score (colinear with height-for-age z-score), and CD4 count (colinear with viral load suppression). We also assessed the association between bacterial genera and FEV1z, FVCz and adverse events (hospitalisation, additional antibiotic use and acute respiratory exacerbation during intervention) using a linear mixed-effect model implemented with MaasLin2 [38]. FEV1z, visit, interaction between trial arm and visit were included as fixed effects and participant as a random effect with ASV counts total sum scaled and converted to 100% and without log transformation. We also used linear regression implemented with MaasLin2 [38] to investigate the association between the within-participant change in FEV1z-score and FVCz-score over time in each trial arm (fixed effects) and (i) within-participant change in the percentage relative abundance of the five bacterial genera of

interest (*Streptococcus*, *Prevotella*, *Haemophilus*, *Neisseria* and *Moraxella*), (ii) within-participant (between visit) beta diversity (Aitchison distance). The results of the regressions were reported as beta coefficients and standard error of the models.

5.3 Results

5.3.1 Participant characteristics

The BREATHE trial took place between June 2016 and September 2019. The socio-demographic and clinical data have previously been published [7]. Briefly, 347 children and adolescents were randomised, 173 to the azithromycin group and 174 to the placebo arm. The median age of the participants at baseline was 15.3 years, interquartile range [12.7–17.7] and 49% (170/347) were female. The participants in the azithromycin arm were younger and had been more frequently treated for tuberculosis than the placebo arm. Other characteristics did not significantly differ between arms. Almost all participants (90%, 313/347) received long-term cotrimoxazole prophylaxis. The baseline clinical, socio-demographic and microbiological features of the participants are presented in Table 5.1. During the intervention, fewer participants in the azithromycin arm experienced acute respiratory exacerbations (16, 9.2%) and hospitalisations (2, 1.2%) compared to placebo (exacerbations: 30, 17.2%, hospitalisation, 9, 5.2%) [7].

5.3.2 Number of samples

Ninety-nine per cent of all samples (869/875) were spontaneously expectorated, while the remainder was induced (3 samples in each study arm). Nineteen per cent of participants (66/347) were not followed up at 72 weeks due to logistical limitations. Details of the number of samples collected from each study site and at each visit and the numbers included and excluded are summarised in Figure 5.1.

Table 5. 1. Characteristics of study participants at baseline.

Characteristics	Azithromycin arm n = 173	Placebo arm n = 174
Demographic Characteristics		
Age in years, Median (IQR)	15 (13 – 17)	16 (13 – 18)
Female Sex, No. (%)	80 (46)	90 (52)
Currently in school, No. (%) ^a	146 (85)	139 (80)
Site: Zimbabwe, No. (%)	120 (69)	121 (70)
HIV Characteristics		
Duration on ART in years, Median (IQR)	6 (4 – 9)	6 (4 – 8)
HIV viral load suppression, < 1000 copies/mL, No. (%)	102 (59)	94 (54)
CD4 count category, > 200 cells/ul, No. (%)	157 (91)	156 (90)
Antiretroviral regimen NNRTI, No. (%) ^a	127 (73)	131 (75)
Lung function		
FEV1 z-score, Median (IQR)	-1.94 (-2.5, -1.4)	-2.0 (-2.4, -1.5)
Clinical Characteristics		
Underweight, No. (%) ^b	98 (57)	83 (48)
Stunted, No. (%) ^b	95 (55)	80 (46)
History of TB, No. (%) ^a	58 (34)	39 (22)
Admitted for chest problems in last year, No. (%)	3 (2)	3 (2)
MRC dyspnoea Score		
1	89 (51)	96 (55)
2	64 (37)	62 (36)
3	12 (7)	11 (6)
4	7 (4)	4 (2)
5	1 (1)	1 (1)
Cotrimoxazole prophylaxis	157 (92) ^a	156 (90)
^dSputum culture		
<i>Streptococcus pneumoniae</i> , No. (%)	43 (26)	38 (23)
<i>Staphylococcus aureus</i> , No. (%)	51 (31)	46 (28)
<i>Haemophilus influenzae</i> , No. (%)	4 (2)	7 (4)
<i>Moraxella catarrhalis</i> , No. (%)	15 (9)	15 (9)
Any of the four bacteria above, No. (%)	82 (49)	79 (48)
Sputum total bacterial load		
Sputum bacterial count (16S copy numbers in log ₁₀), Median (IQR)	5.0 (4.5 – 5.5)	4.9 (4.4 – 5.5)
Sputum bacteriome alpha diversity		
Shannon diversity, Median (IQR)	3.0 (2.5 – 3.3)	3.0 (2.4 – 3.4)
Observed Taxon richness, Median (IQR)	29 (22 – 35)	29 (23 – 36)
^eSputum bacteriome within-arm beta diversity		
Aitchison distance, Median (IQR)	34.2 (30.9 – 37.5)	35.0 (32.0 – 38.3)
Bray-Curtis index, Median (IQR)	0.7 (0.6 – 0.8)	0.71 (0.6 – 0.8)

^aMissing value: Currently attending school – n = 1 Azithromycin arm; Antiretroviral regimen, n = 1, Placebo arm; History of TB– n = 1, Azithromycin arm; Cotrimoxazole prophylaxis– n = 2, Azithromycin arm, ^b z-score < -2, ^cOnly asked for those with current cough, FEV1: Forced Expiratory Volume in one Second; Participants with missing responses are excluded from that variable. ^dNumber of sputum samples is 166 (Azithromycin) and 164 (Placebo). IQR: Interquartile range, NNRTI: Non-nucleoside Reverse Transcriptase Inhibitor. CLR = centred-log ratio. ^eCompared using PERMANOVA test.

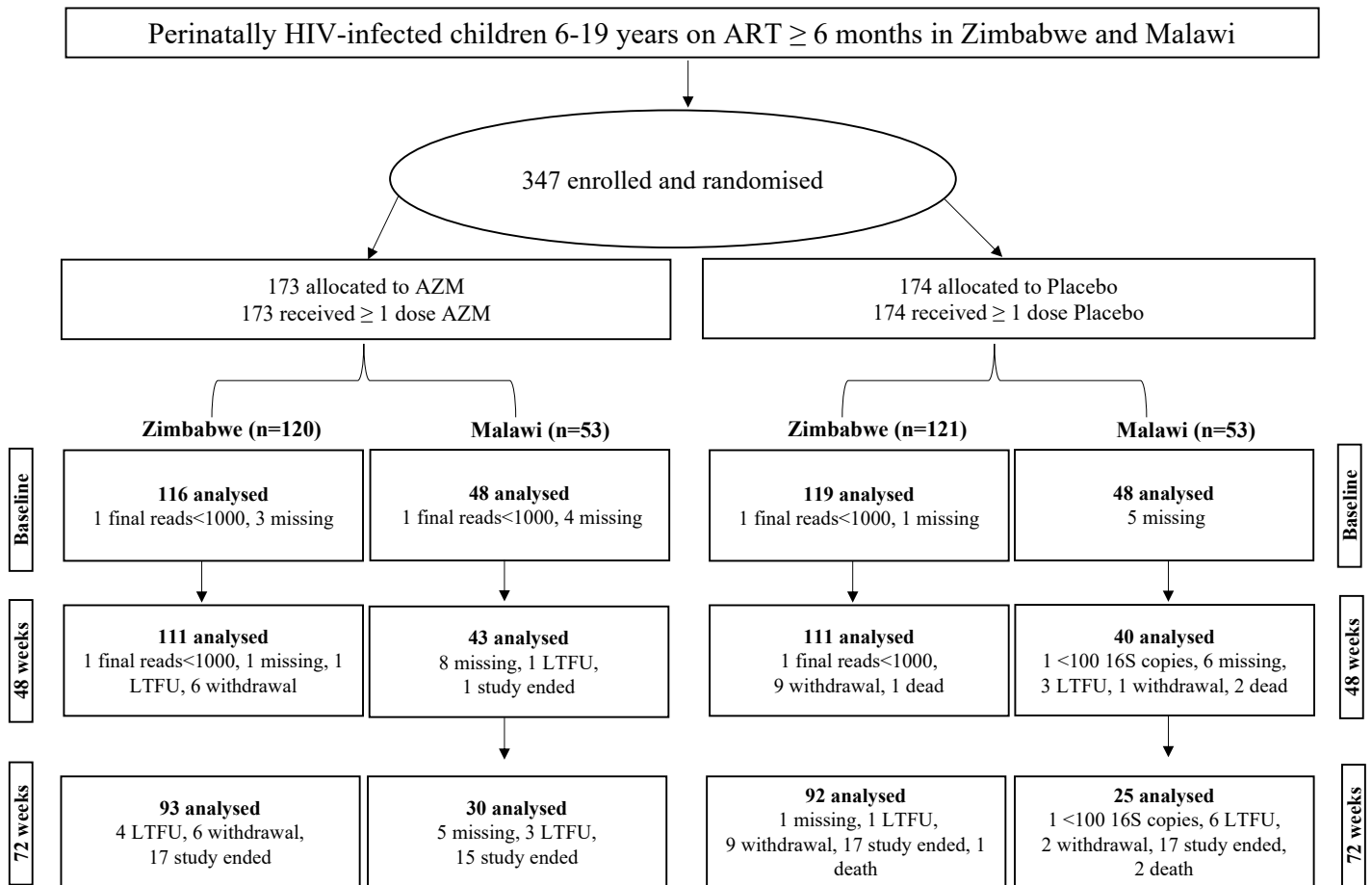


Figure 5. 1. Flow chart of the number of samples collected from each study site at each visit and the numbers included and excluded from final analysis.

LTFU = Loss to follow up. 16S copies = 16S rDNA copies

5.3.3 Azithromycin reduced sputum bacterial load among participants in the intervention arm

The median bacterial load ($\log_{10}$ 16S rDNA gene copies/ul) of the 875 samples was similar between the two arms at baseline [median (IQR) azithromycin, 5.0 (4.45–5.45) vs placebo 4.93 (4.41–5.54), $p = 0.89$] but was lower in azithromycin than placebo at both the 48-week [median (IQR) azithromycin, 4.65 (4.08–5.21) vs placebo 5.27 (4.57–5.78), $p < 0.0001$] and 72-week visits [median (IQR) azithromycin, 4.92 (4.46–5.48) vs placebo 5.26 (4.71–5.79), $p = 0.019$] (Figure 5.2A). Within the azithromycin arm, 16S rDNA gene copy numbers at 48 weeks 4.65 (4.08–5.21) were lower than at baseline (5.0 (4.45–5.45), $p < 0.001$) and at 72 weeks (4.92 (4.46–5.48), $p = 0.003$) (Figure 5.2B). Bacterial load remained similar across visits in the placebo arm (Figure 5.2C).

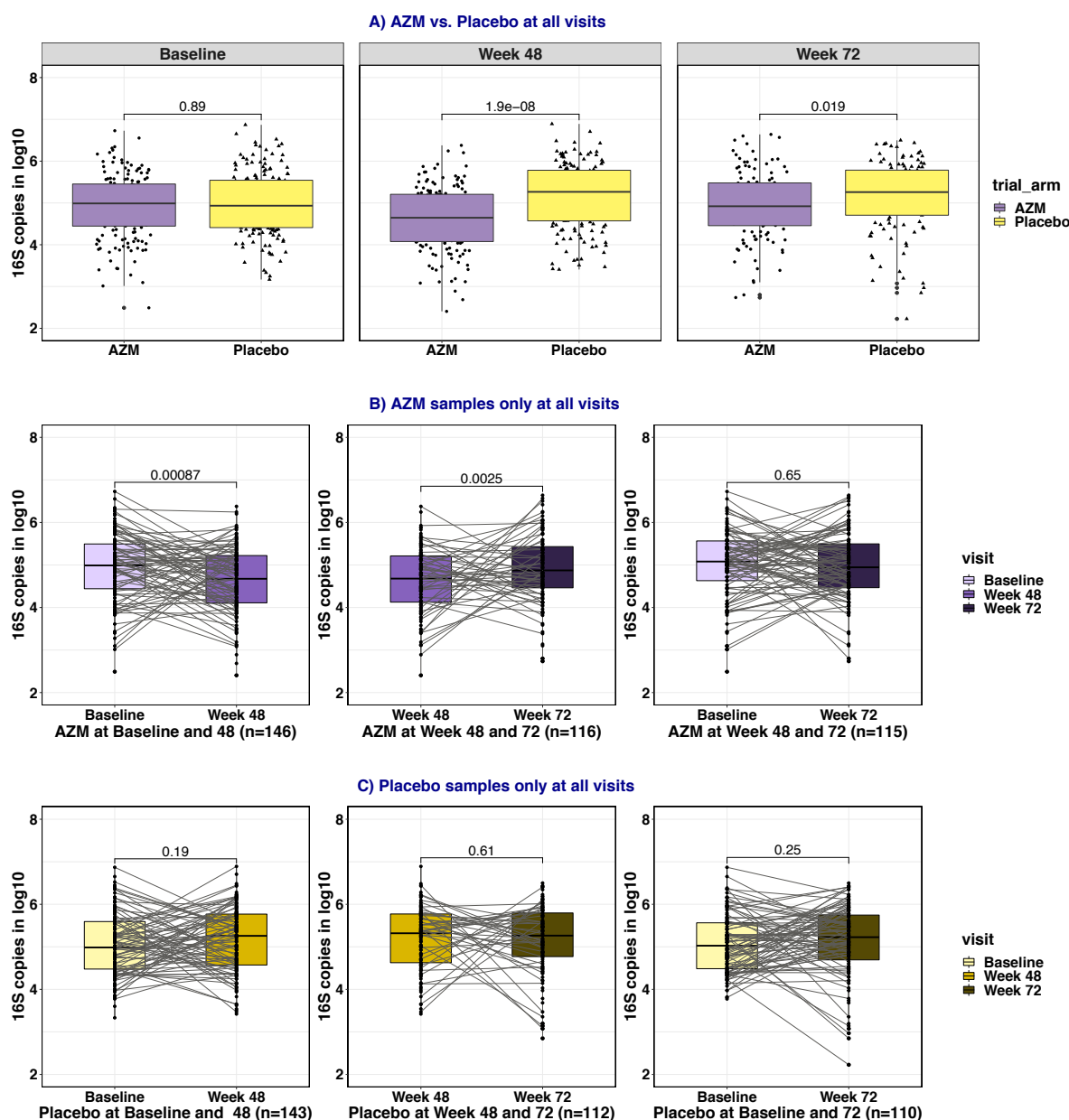


Figure 5. 2. Boxplot of bacterial load between trial arms at each visit (A) and between visits within AZM arm only (B) and Placebo arm only (C).

16S rRNA gene copy number, used as proxy for bacterial load, was measured with qPCR. The between arm comparisons were implemented using Wilcoxon signed rank test for unpaired samples while within-arm comparisons used Wilcoxon signed rank test for paired samples. The values on top of the horizontal bars are *p* values.

We used linear mixed-effect modelling (LME), Table 5.2, to explore associations between bacterial load (16S rDNA copies) and clinical and socio-demographic characteristics. Although bacterial load remained constant in the placebo arm, it declined significantly at 48 weeks compared to baseline in the azithromycin arm (adjusted beta coefficient and 95% confidence interval: $-0.5 \log_{10}$ copies per μl $[-0.63; -0.29]$). Participants from the Zimbabwean study site, on average, had a higher bacterial load

than those from Malawi ($0.3 \log_{10}$ copies per μl [0.11; 0.49]) at any visit. Better lung function was associated with a lower bacterial load. Specifically, a one unit increase in FEV1z-score was associated with $0.1 \log_{10}$ copies per μl [0.02- 0.2] decrease in bacterial load at any visit. Participants who had received treatment for tuberculosis before enrolment had higher bacterial loads ($0.2 \log_{10}$ copies per μl [0.03–0.32]) at any visit than those who were not previously treated.

Table 5. 2. The association between bacterial load (16S rDNA copies) and selected variables using linear mixed effects modelling.

Variable	Levels	^a Number of observations (n = 875)	Participants (n = 346)	¹ Co-efficient (95% CI)	¹ p value	² Adjusted Co-efficient (95% CI)	² p value
Visit	Placebo at Week 48	150 (17.1%)	150 (43.4%)			Reference	
	AZM at Week 48	154 (17.6%)	154 (44.5%)	-0.48 [-0.65; -0.32]	< 0.0001	-0.46 [-0.63; -0.29]	< 0.0001
	Placebo at Week 72	117 (13.4%)	117 (33.8%)			Reference	
	AZM at Week 72	123 (14.1%)	123 (35.5%)	-0.17 [-0.35; 0.02]	0.08	-0.19 [-0.38; 0.0]	0.051
Adherence	Adherent	661 (75.5%)	246 (71.1%)			Reference	
	Non-adherent	214 (24.5%)	214 (61.8%)	-0.22 [-0.37; -0.07]	0.004	-0.1 [-0.26; 0.06]	0.24
Site	Malawi	233 (26.6%)	106 (30.6%)			Reference	
	Zimbabwe	642 (73.4%)	240 (69.4%)	0.38 [0.24; 0.52]	< 0.0001	0.3 [0.11; 0.49]	0.003
Age in years		779 (89%)	346 (100%)	0.02 [0; 0.04]	0.05		
Sex	Female	420 (48%)	170 (49.1%)			Reference	
	Male	455 (52%)	176 (50.9%)	-0.06 [-0.19; 0.08]	0.41	-0.08 [-0.21; 0.05]	0.23
Season of sampling	May-Oct-Dry	465 (53.1%)	295 (85.3%)			Reference	
	Nov-Apr-Rainy	409 (46.7%)	277 (80.1%)	0.02 [-0.07; 0.12]	0.60	0.04 [-0.06; 0.13]	0.41
MRC dyspnoea score at Baseline	1	479 (54.7%)	184 (53.2%)			Reference	
	2	316 (36.1%)	126 (36.4%)	-0.2 [-0.34; -0.06]		-0.07 [-0.22; 0.09]	
	3	53 (6.1%)	23 (6.6%)	-0.28 [-0.55; 0]		-0.04 [-0.36; 0.28]	
	4	23 (2.6%)	11 (3.2%)	-0.36 [-0.75; 0.04]		-0.18 [-0.62; 0.27]	
	5	4 (0.5%)	2 (0.6%)	0.12 [-0.81; 1.05]		-0.79 [-2.29; 0.71]	0.76
FEV1z		862 (98.5%)	346 (100%)	-0.11 [-0.18; -0.04]	0.003	-0.09 [-0.16; -0.02]	0.02
Forced Vital Capacity (FVC)		852 (97.4%)	345 (99.7%)	0.02 [-0.08; 0.12]	0.76		
FVCz		852 (97.4%)	345 (99.7%)	-0.08 [-0.14; -0.02]	0.01		
FEV1/FVCz		852 (97.4%)	345 (99.7%)	-0.03 [-0.08; 0.02]	0.28		
% Predicted FVC		852 (97.4%)	345 (99.7%)	-0.01 [-0.01; 0]	0.01		

% Predicted FEV		862 (98.5%)	346 (100%)	-0.01 [-0.01; 0]	0.003		
BMI-for-age z-score		868 (99.2%)	346 (100%)	-0.03 [-0.09; 0.02]	0.21		
Weight-for-age z-score		868 (99.2%)	346 (100%)	-0.01 [-0.06; 0.03]	0.61		
Height-for-age z-score		868 (99.2%)	346 (100%)	0.02 [-0.04; 0.07]	0.55		
CD4 at baseline		868 (99.2%)	346 (100%)	0 [0; 0]	0.14		
CD4 at all visits		631 (72.1%)	346 (100%)	0 [0; 0]	0.04		
Viral load at all visits		676 (77.3%)	343 (99.1%)	0 [0; 0]	0.89		
Viral load suppression at baseline	Suppressed	495 (56.5%)	193 (55.8%)	Reference			
	Unsuppressed (≥ 1000 copies/ mL)	377 (43.0%)	151 (43.6%)	0.09 [-0.04; 0.22]	0.18	0.1 [-0.04; 0.23]	0.16
Weight-for-age z-score	Not underweight	407 (46.5%)	166 (48%)	Reference			
	Underweight	468 (53.5%)	180 (52%)	-0.04 [-0.17; 0.09]	0.54		
Height-for-age z-score	Not stunted	434 (49.6%)	171 (49.4%)	Reference			
	stunted	441 (50.4%)	175(50.6%)	-0.02 [-0.15; 0.12]	0.81	0.01 [-0.12; 0.15]	0.84
Acute exacerbation during intervention	No	752 (85.9%)	300 (86.7%)	Reference			
	Yes	123 (14.1%)	46 (13.3%)	0.15 [-0.04; 0.34]	0.13		
Hospitalised during intervention	No	852 (97.4%)	336 (97.1%)	Reference			
	Yes	23 (2.6%)	10 (2.9%)	0.14 [-0.27; 0.54]	0.5		
Additional antibiotics during intervention	No	843 (96.3%)	334 (96.5%)	Reference			
	Yes	32 (3.7%)	12 (3.5%)	-0.2 [-0.55; 0.15]	0.27		
³ Any events during intervention	No	724 (82.7%)	288 (83.2%)	Reference			
	Yes	151 (17.3%)	58 (16.8%)	0.1 [-0.08; 0.27]	0.28		
Cotrimoxazole prophylaxis at Baseline	No	88 (10.1%)	32 (9.2%)	Reference			
	Yes	783 (89.5%)	312 (90.2%)	-0.19 [-0.41; 0.04]	0.10	-0.14 [-0.35; 0.07]	0.20
Age group at Baseline	17–19 years	258 (29.5%)	103 (29.8%)	Reference			
	13–16 years	374 (42.7%)	149 (43.1%)	-0.05 [-0.21; 0.10]	0.02	-0.01 [-0.25; 0.24]	0.55

	10–12 years	165 (18.9%)	63 (18.2%)	-0.3 [-0.49; -0.11]		-0.14 [-0.34; 0.06]	
	6–9 years	78 (8.9%)	31 (9%)	-0.01 [-0.34; 0.15]		-0.01 [-0.16; 0.15]	
Ever admitted for chest problems in the past year before enrolment	No	863 (98.6%)	340 (98.3%)		Reference		
	Yes	12 (1.4%)	6 (1.7)	-0.08 [-0.62; 0.46]	0.76	-0.33 [-0.97; 0.3]	0.33
Ever treated for tuberculosis before enrolment	No	609 (69.8%)	248 (71.9%)		Reference		
	Yes	263 (30.2%)	97 (28.1%)	0.27 [0.13; 0.41]	< 0.0001	0.17 [0.03; 0.32]	0.02
Duration of ART at Baseline	6 months –2 years	80 (9.1%)	33 (9.5%)		Reference		
	2 – < 4 years	141 (16.1%)	58 (16.8%)	0.16 [-0.11; 0.43]		-0.04 [-0.3; 0.23]	
	4 – < 6 years	187 (21.4%)	72 (20.8%)	0.12 [-0.14; 0.38]	0.70	-0.05 [-0.3; 0.21]	
	6 years+	442 (50.5%)	172 (49.7%)	0.11 [-0.13; 0.34]		-0.11 [-0.35; 0.12]	0.71

Abbreviations: forced vital capacity (FVC), forced vital capacity z-score (FVCz), forced expiratory volume in 1 second (FEV1) z-score (FEV1z), FEV1 percentage predicted (FEV1pcpred), FVC percentage predicted (FVCpcpred) and FEV1/FVCz ratio of FEV1 and FVC z-score, Body mass index (BMI). ^aThe difference between the number of observations and the total (875) represents the number of missing observations for that variable. ¹The estimate of coefficient with 95% confidence intervals and p values were obtained from univariate linear mixed effect model with participant included as a random effect and each variable and trial arm: visit interaction term as explanatory variables and log₁₀ 16S rDNA copies of the sputum samples as the dependent variable. ²The estimate of coefficient with 95% confidence intervals and p values were obtained from multivariate linear mixed effect model with participant included as a random effect, trial arm, visit and trial arm: visit interaction term and all variables that have values under the “Adjusted Coefficient” column as explanatory variables and log₁₀ 16S rDNA copies of the sputum samples as the dependent variable. FEV1/FVCz, FVCz, FVCpcpred, FEV1pcpred were excluded from the final model because of collinearity. Viral load and CD4 counts were excluded from the final model because data was not collected at 72 weeks, the values at baseline were used instead. ³Any event refers to either acute respiratory exacerbation, additional antibiotics other than interventional drug or cotrimoxazole, or hospitalisation during the intervention.

5.3.4 Association between azithromycin treatment, clinical and socio-demographic characteristics, and sputum alpha diversity

The median number of sequences per sample was 36,456 (IQR: 31,392–42,086), which was reduced to 16,969 (IQR: 13,302–19,902) with a total of 1,665 ASVs, after quality control and *in-silico* decontamination steps. We used linear mixed-effect modelling (LME), Table 5.3, to explore associations between within-sample diversity (Shannon diversity index) and clinical and socio-demographic characteristics. Although the Shannon diversity in the azithromycin arm remained unchanged (Figure 5.3B), it was significantly higher than placebo at 48 weeks (0.25 [0.07; 0.42]) and 72 weeks (0.2 [0.01; 0.40]). Participants from the Zimbabwean study site, on average, also had a higher Shannon diversity index than those from Malawi (0.27 [0.06; 0.47]) at any visit. Also, participants with an MRC dyspnoea score of two had on average 0.26 units [0.1; 0.42] higher Shannon diversity index (higher sputum bacteriome alpha diversity) than participants with a score of one. Participants with higher FEV1z-score at any visit had a more diverse sputum bacteriome (higher Shannon index) compared to those with lower scores (0.19 [0.12; 0.27]). Finally, participants who were previously treated for tuberculosis before enrolment had, on average, 0.2 units [-0.34; -0.04] lower Shannon diversity index at any visit than those who were never treated.

Table 5.3. The association between Shannon diversity indices and selected variables using linear mixed effects modelling.

Variable	Levels	^a Number of observations (n = 875)	Participants (n = 346)	¹ Co-efficient (95% CI)	¹ p value	² Adjusted Co-efficient (95% CI)	² p value
Visit	Placebo at Week 48	150 (17.1%)	150 (43.4%)			Reference	
	AZM at Week 48	154 (17.6%)	154 (44.5%)	0.28 [0.11; 0.45]	0.001	0.25 [0.07; 0.42]	0.01
	Placebo at Week 72	117 (13.4%)	117 (33.8%)			Reference	
	AZM at Week 72	123 (14.1%)	123 (35.5%)	0.20 [0.01; 0.39]	0.04	0.2 [0.01; 0.40]	0.04
Adherence	Adherent	661 (75.5%)	246 (71.1%)			Reference	
	Non-adherent	214 (24.5%)	214 (61.8%)	0.02 [-0.15; 0.18]	0.85	0.07 [-0.1; 0.24]	0.42
Site	Malawi	233 (26.6%)	106 (30.6%)			Reference	
	Zimbabwe	642 (73.4%)	240 (69.4%)	0.07 [-0.09; 0.23]	0.39	0.27 [0.06; 0.47]	0.01
Age in years		779 (89%)	346 (100%)	-0.02 [-0.04; 0]	0.09		
Sex	Female	420 (48%)	170 (49.1%)			Reference	

	Male	455 (52%)	176 (50.9%)	0.14 [-0.01; 0.28]	0.06	0.13 [-0.01; 0.27]	0.07
Season of sampling	May-Oct-Dry	465 (53.1%)	295 (85.3%)	Reference			
	Nov-Apr-Rainy	409 (46.7%)	277 (80.1%)	-0.06 [-0.16; 0.03]	0.20	-0.09 [-0.19; 0.01]	0.07
MRC dyspnoea score at Baseline	1	479 (54.7%)	184 (53.2%)	Reference			
	2	316 (36.1%)	126 (36.4%)	0.14 [-0.01; 0.29]	0.04	0.26 [0.1; 0.42]	
	3	53 (6.1%)	23 (6.6%)	0.03 [-0.26; 0.33]		0.16 [-0.18; 0.49]	
	4	23 (2.6%)	11 (3.2%)	-0.11 [-0.53; 0.31]		0.25 [-0.21; 0.71]	
	5	4 (0.5%)	2 (0.6%)	-1.21 [-2.21; -0.21]		0.52 [-1.05; 2.09]	0.04
FEV1z		862 (98.5%)	346 (100%)	0.21 [0.14; 0.29]	< 0.001	0.19 [0.12; 0.27]	<0.001
Forced Vital Capacity (FVC)		852 (97.4%)	345 (99.7%)	0.05 [-0.06; 0.16]	0.37		
FVCz		852 (97.4%)	345 (99.7%)	0.1 [0.03; 0.16]	0.004		
FEV1/FVCz		852 (97.4%)	345 (99.7%)	0.12 [0.06; 0.18]	< 0.001		
% Predicted FVC		852 (97.4%)	345 (99.7%)	0.01 [0; 0.01]	0.003		
% Predicted FEV		862 (98.5%)	346 (100%)	0.02 [0.01; 0.02]	< 0.001		
BMI-for-age z-score		868 (99.2%)	346 (100%)	0.03 [-0.03; 0.08]	0.35		
Weight-for-age z-score		868 (99.2%)	346 (100%)	0.02 [-0.03; 0.07]	0.47		
Height-for-age z-score		868 (99.2%)	346 (100%)	0 [-0.06; 0.06]	0.98		
CD4 at baseline		868 (99.2%)	346 (100%)	0 [0; 0]	0.06		
CD4 at all visits		631 (72.1%)	346 (100%)	0 [0; 0]	0.01		
Viral load at all visits		676 (77.3%)	343 (99.1%)	0 [0; 0]	0.04		
Viral load suppression at baseline	Suppressed	495 (56.5%)	193 (55.8%)	Reference			
	Unsuppressed (≥ 1000 copies/ mL)	377 (43.0%)	151 (43.6%)	-0.11 [-0.26; 0.03]	0.12	-0.06 [-0.2; 0.08]	0.40
Weight-for-age z-score	Not underweight	407 (46.5%)	166 (48%)	Reference			
	Underweight	468 (53.5%)	180 (52%)	0.04 [-0.1; 0.18]	0.59		
Height-for-age z-score	Not stunted	434 (49.6%)	171 (49.4%)	Reference			
	stunted	441 (50.4%)	175(50.6%)	0.04 [-0.1; 0.18]	0.57	0.09 [-0.05; 0.23]	0.23

Acute exacerbation during intervention	No	752 (85.9%)	300 (86.7%)	Reference		
	Yes	123 (14.1%)	46 (13.3%)	-0.22 [-0.42; -0.01]	0.04	
Hospitalised during intervention	No	852 (97.4%)	336 (97.1%)	Reference		
	Yes	23 (2.6%)	10 (2.9%)	-0.28 [-0.72; 0.15]	0.21	
Additional antibiotics during intervention	No	843 (96.3%)	334 (96.5%)	Reference		
	Yes	32 (3.7%)	12 (3.5%)	-0.1 [-0.49; 0.28]	0.60	
³ Any events during intervention	No	724 (82.7%)	288 (83.2%)	Reference		
	Yes	151 (17.3%)	58 (16.8%)	-0.23 [-0.41; -0.04]	0.02	-0.16 [-0.35; 0.03] 0.11
Cotrimoxazole prophylaxis at Baseline	No	88 (10.1%)	32 (9.2%)	Reference		
	Yes	783 (89.5%)	312 (90.2%)	-0.1 [-0.34; 0.15]	0.44	-0.13 [-0.36; 0.09] 0.26
Oxygen Saturation	Normal	840 (96.0%)	345 (99.7%)	Reference		
	Abnormal	26 (3.0%)	26 (7.5%)	-0.27 [-0.56; 0.01]	0.06	
Heart rate	Normal	810 (92.6%)	340 (98.3%)	Reference		
	Abnormal	56 (6.4%)	44 (12.7%)	-0.22 [-0.44; -0.01]	0.04	-0.1 [-0.32; 0.11] 0.36
Age group at Baseline	17–19 years	258 (29.5%)	103 (29.8%)	Reference		
	13–16 years	374 (42.7%)	149 (43.1%)	-0.02 [-0.02; 0.18]	0.24	0.03 [-0.13; 0.2]
	10–12 years	165 (18.9%)	63 (18.2%)	0.19[-0.2; 0.40]		0.17 [-0.04; 0.38] 0.47
	6–9 years	78 (8.9%)	31 (9%)	0.13 [-0.14; 0.40]		0.01 [-0.24; 0.27]
Ever admitted for chest problems in the past year before enrolment	No	863 (98.6%)	340 (98.3%)	Reference		
	Yes	12 (1.4%)	6 (1.7)	-0.14 [-0.72; 0.44]	0.64	0.5 [-0.16; 1.17] 0.16
Ever treated for tuberculosis before enrolment	No	609 (69.8%)	248 (71.9%)	Reference		
	Yes	263 (30.2%)	97 (28.1%)	-0.24 [-0.39; -0.08]	0.003	-0.19 [-0.34; -0.04] 0.02
Duration of ART at Baseline	6 months –2 years	80 (9.1%)	33 (9.5%)	Reference		
	2 – < 4 years	141 (16.1%)	58 (16.8%)	0.07 [-0.22; 0.35]	0.87	0.18 [-0.1; 0.46] 0.63

4 – < 6 years	187 (21.4%)	72 (20.8%)	-0.01 [-0.28; 0.27]	0.11 [-0.16; 0.37]
6 years+	442 (50.5%)	172 (49.7%)	-0.02 [-0.27; 0.23]	0.14 [-0.1; 0.39]

Abbreviations: AZM = azithromycin, forced vital capacity (FVC), forced vital capacity z-score (FVCz), forced expiratory volume in 1 second (FEV1) z-score (FEV1z), FEV1 percentage predicted (FEV1pcpred), FVC percentage predicted (FVCpcpred) and FEV1/FVCz ratio of FEV1 and FVC z-score, Body mass index (BMI). ^aThe difference between the number of observations and the total (875) represent number of missing observations for that variable. ¹The estimate of coefficient with 95% confidence intervals and p values were obtained from linear mixed effect model with participant included as a random effect and each variable and trial arm: visit interaction term as explanatory variables and Shannon indices of the sputum samples as the dependent variable. ²The estimate of coefficient with 95% confidence intervals and p values were obtained from multivariate linear mixed effect model with participant included as a random effect, trial arm, visit and trial arm: visit interaction term and all variables that have values under the “Adjusted Coefficient” column as explanatory variables and Shannon indices of the sputum samples as the dependent variable. FEV1/FVCz, FVCz, FVCpcpred, FEV1pcpred were excluded from the final model because of collinearity. Viral load and CD4 counts were excluded from the final model because data was not collected at 72 weeks, the values at baseline were used instead. ³Any event refers to either acute respiratory exacerbation, additional antibiotics other than interventional drug or cotrimoxazole, or hospitalisation during intervention.

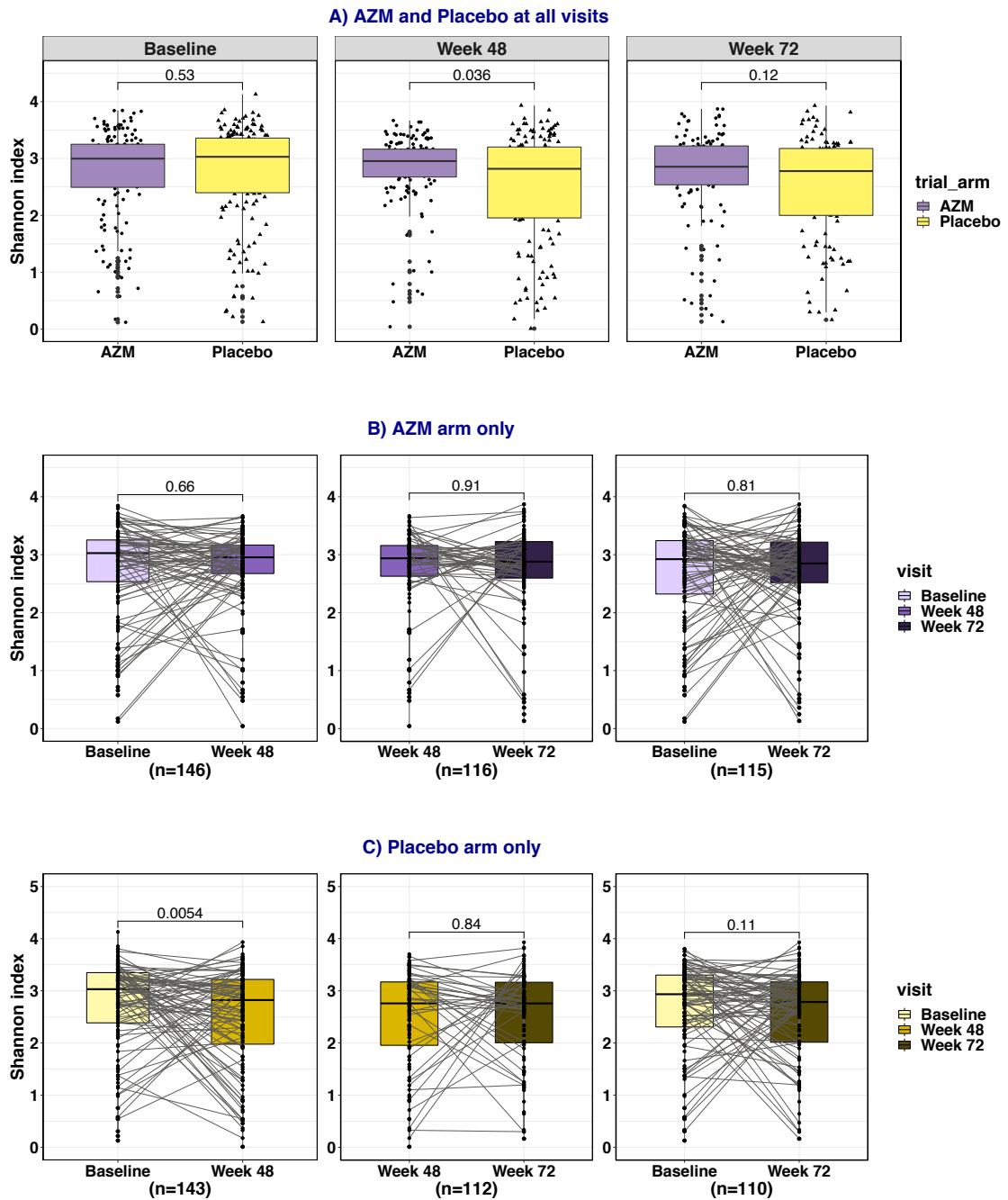


Figure 5. 3. Boxplot of Shannon alpha diversity index between trial arms at each visit (A) and between study visits in AZM (B) and Placebo (C) arms.

The between trial comparisons were implemented using Wilcoxon signed rank test for unpaired samples while within-trial comparisons used Wilcoxon signed rank test for paired samples. AZM = azithromycin. The values on top of the horizontal bars are p values.

5.3.5 Azithromycin treatment resulted in changes in the overall bacteriome profile

Beta diversity among samples from participants in the azithromycin arm between baseline and 48 weeks [PERMANOVA test, on Aitchison distance $p = 0.004$, Bray-Curtis, $p = 0.002$], or between 48 weeks and 72 weeks [Aitchison distance, $p = 0.004$, Bray-Curtis, $p = 0.002$], was greater than between samples within the same visit, showing within-participant changes in overall community structure across visits, Figure 5.4. There was no difference in the dispersion of samples between visits (PERMDISP, $p > 0.05$). In contrast, no difference in beta diversity between visits, compared to within visits, was observed within the placebo arm (Figure 5.5). At baseline, between-arm beta diversity did not differ significantly from within arm beta diversity in either study arm [PERMANOVA test on Aitchison distance, ($p = 0.76$), Bray-Curtis, ($p = 0.97$), Figure 5.6A, B, Figure 5.7A, B]. However, at 48 weeks, overall bacterial composition differed between the two trial arms, with greater diversity between arms than within arms [PERMANOVA test on Aitchison distance, ($p = 0.003$), Bray-Curtis, ($p = 0.003$) Figure 5.6C, D and Figure 5.7. A dispersion effect was absent when considering Aitchison distance (PERMDISP $p = 0.85$). However, it was present when considering Bray-Curtis distance (PERMDISP $p = 0.002$), with greater dispersion in the placebo than in the azithromycin arm (Figure 5.7). The difference in overall bacterial composition was not evident at the 72-week visits [PERMANOVA test on Aitchison distance, ($p = 0.14$), Bray-Curtis, ($p = 0.2$), Figure 5.6E, F and Figure 5.7].

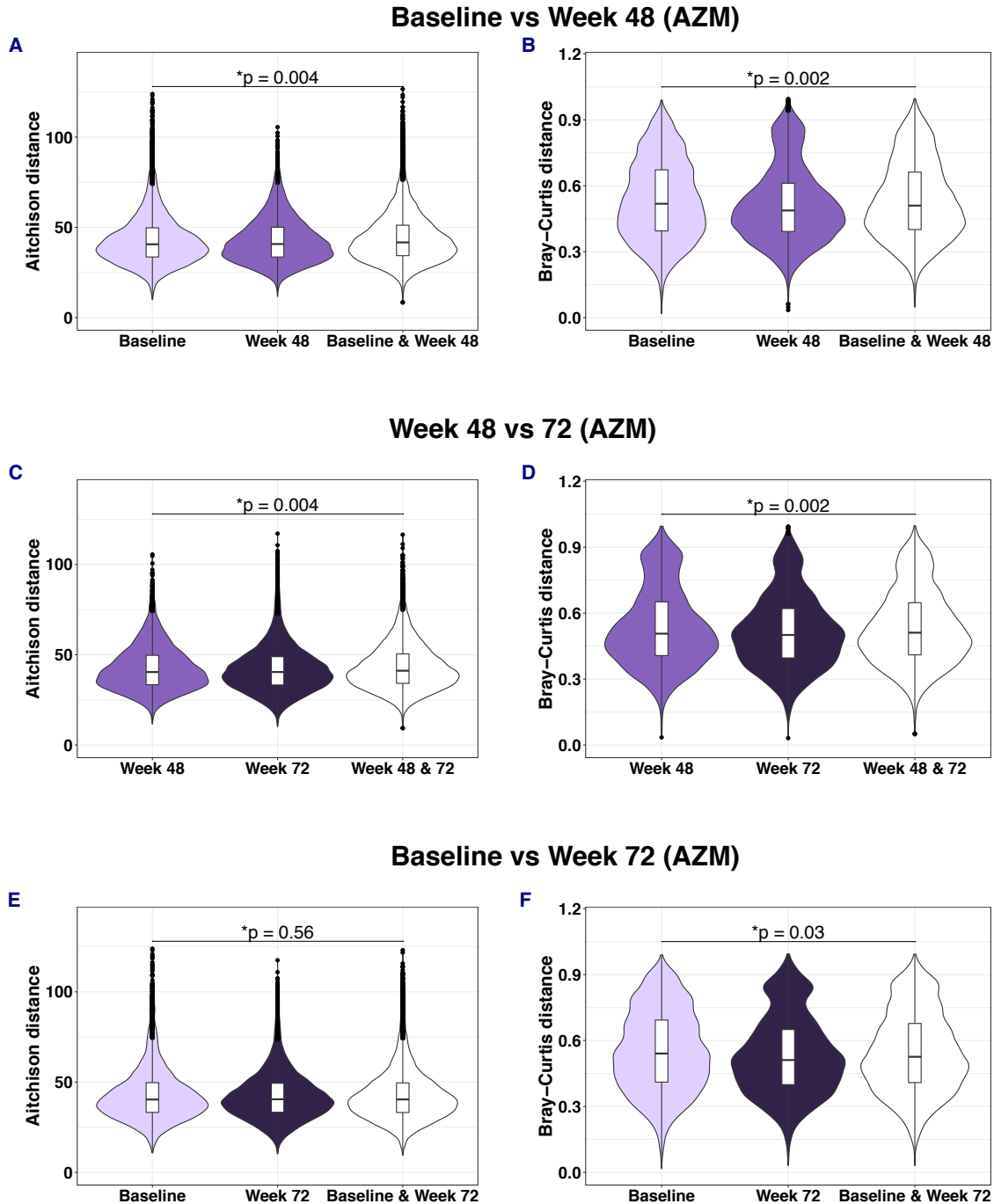


Figure 5. 4. Violin boxplot comparing two beta diversity metrics between samples collected from participants in the AZM arms at baseline and 48 weeks, 48 and 72 weeks and baseline and 72 weeks. PERMANOVA test used.

A) Comparison of samples from baseline and 48 weeks using Aitchison distance. B) Comparison of samples from baseline and 48 weeks using Bray-Curtis distance on unrarefied ASV counts. C) Comparison of samples from 48- and 72-weeks using Aitchison distance. D) Comparison of samples from 48- and 72-weeks using Bray-Curtis distance on unrarefied ASV counts. E) Comparison of samples from baseline and 72 weeks using Aitchison distance. F) Comparison of samples from baseline and 72 weeks using Bray-Curtis distance on unrarefied ASV counts. **p* values were adjusted using BH correction. The first two violin boxplots of each figure show the distribution of the within group distances in the samples from the two visits. The third violin boxplot of each figure shows the distribution of the between group distance between the two visits. The horizontal line in the middle of the box is the median. The box presents interquartile range. The whiskers show 95% confidence interval. The shape of the violin display frequencies of values. AZM = azithromycin.

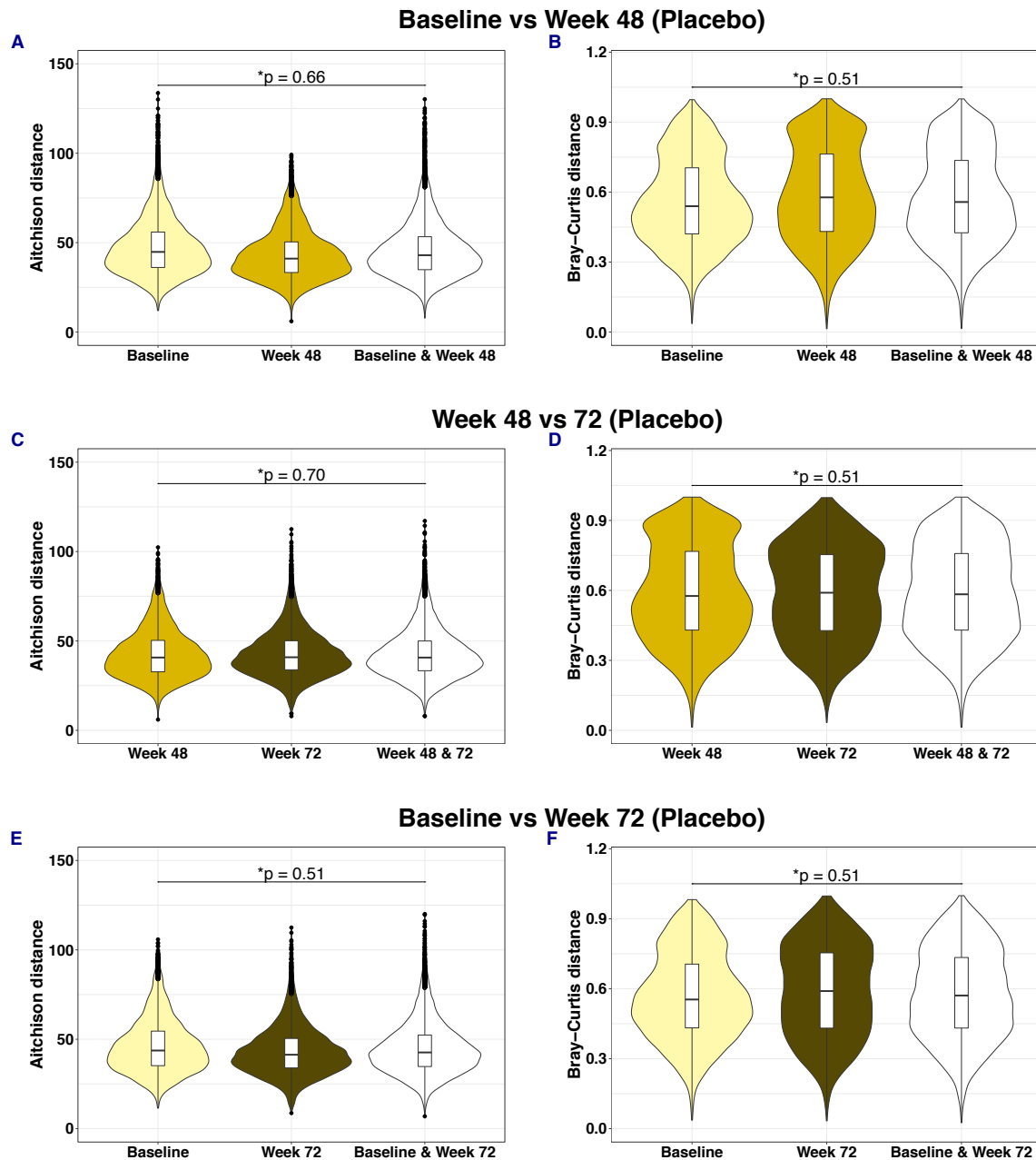


Figure 5. 5. Violin boxplot comparing two beta diversity metrics between samples collected from participants in the Placebo arms at baseline and 48 weeks, 48 and 72 weeks and baseline and 72 weeks. PERMANOVA test used.

A) Comparison of samples from baseline and 48 weeks using Aitchison distance. B) Comparison of samples from baseline and 48 weeks using Bray-Curtis distance on unrarefied ASV counts. C) Comparison of samples from 48- and 72-weeks using Aitchison distance. D) Comparison of samples from 48- and 72-weeks using Bray-Curtis distance on unrarefied ASV counts. E) Comparison of samples from baseline and 72 weeks using Aitchison distance. F) Comparison of samples from baseline and 72 weeks using Bray-Curtis distance on unrarefied ASV counts. *p values were adjusted using BH correction. The first two violin boxplots of each figure show the distribution of the within group distances in the samples from the two visits. The third violin boxplots of each figure show the distribution of the between group distance between the two visits. The horizontal line in the middle of the box is the median. The box presents interquartile range. The whiskers show 95% confidence interval. The shape of the violin display frequencies of values. AZM= azithromycin.

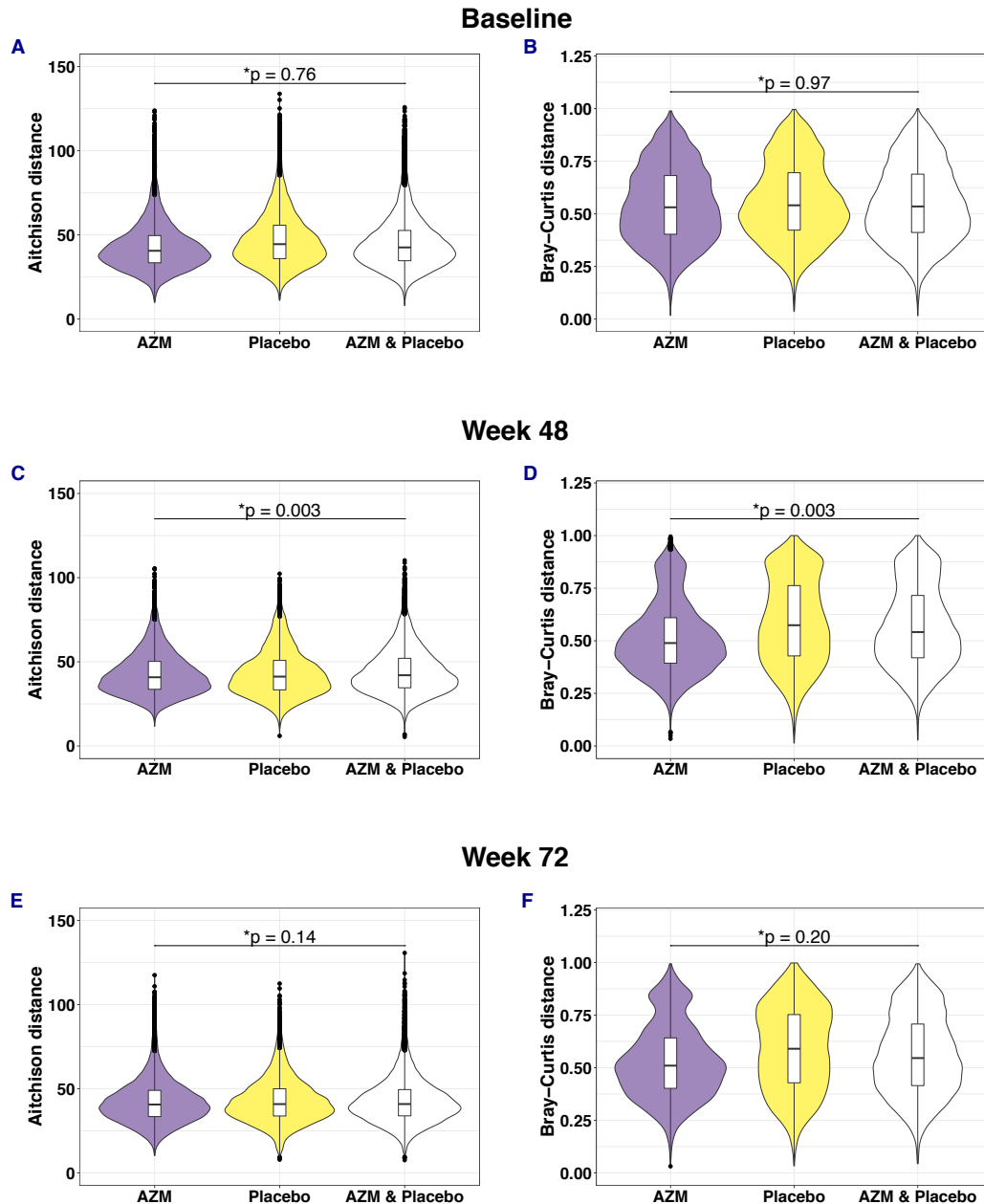
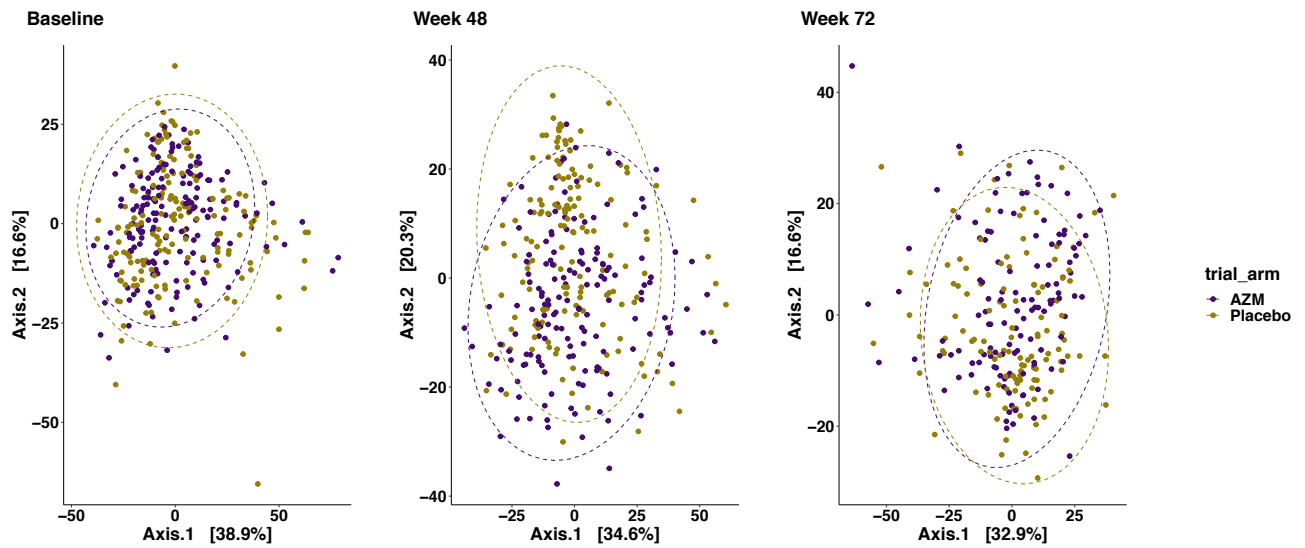


Figure 5. 6. Violin boxplot comparing two beta diversity metrics between trial arms at 0, 48 and 72 weeks.

A) Comparison of AZM and Placebo at baseline using Aitchison distance. B) Comparison of AZM and Placebo at baseline using Bray-Curtis distance on unrarefied ASV counts. C) Comparison of AZM and Placebo at 48 weeks using Aitchison distance. D) Comparison of AZM and Placebo at 48 weeks using Bray-Curtis distance on unrarefied ASV counts. E) Comparison of AZM and Placebo at 72 weeks using Aitchison distance. F) Comparison of AZM and Placebo at 72 weeks using Bray-Curtis distance on unrarefied ASV counts. *p values were adjusted using BH correction. The first two violin boxplots of each figure show the distribution of the within group distances in the AZM and Placebo arms respectively. The third violin boxplots of each figure show the distribution of the between group distance between AZM and Placebo arms. The horizontal line in the middle of the box is the median. The box presents interquartile range. The whiskers show 95% confidence interval. The shape of the violin display frequencies of values. AZM = azithromycin.

A) Aitchison distance



B) Bray-Curtis distance

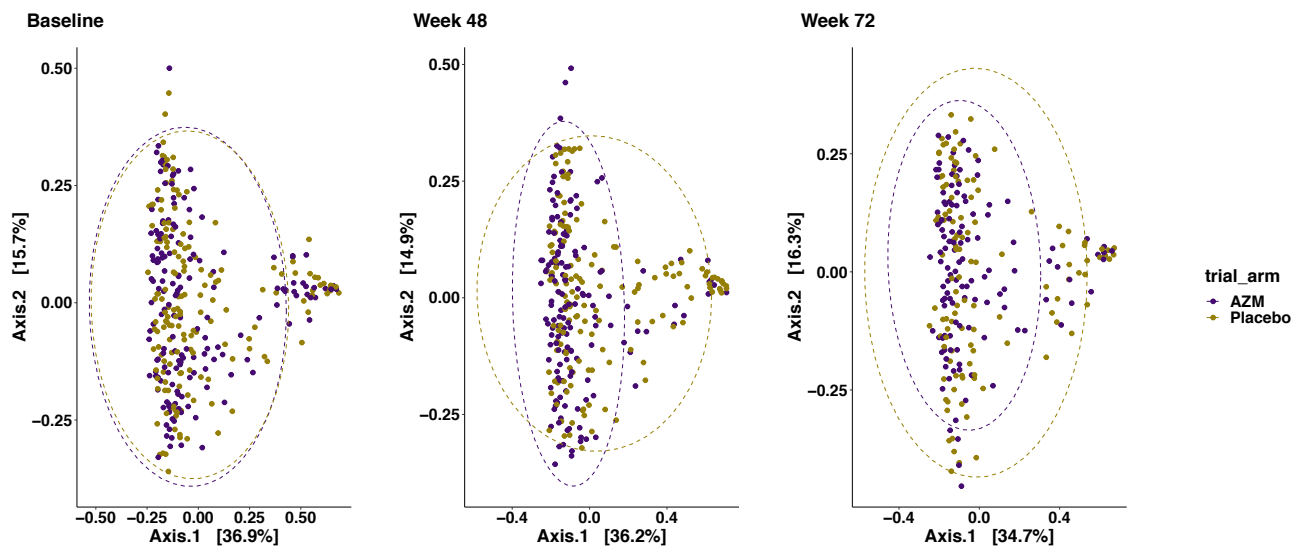


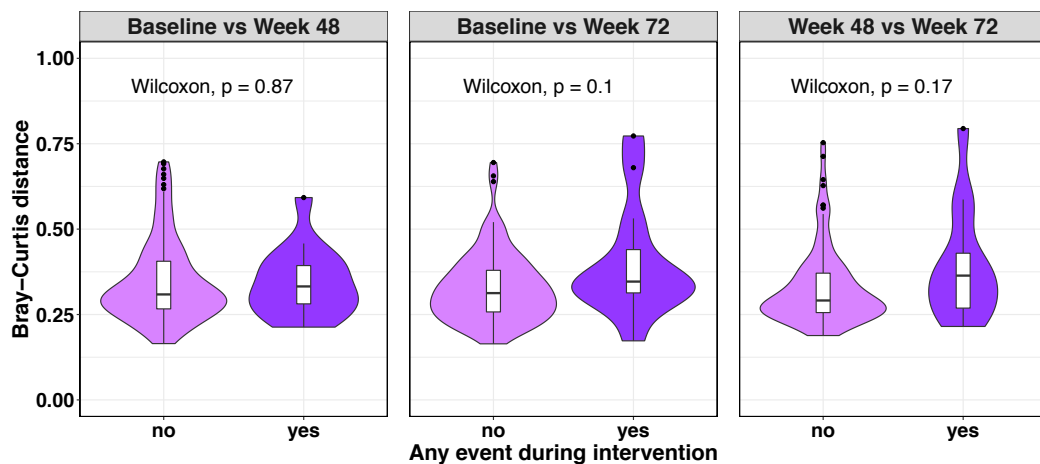
Figure 5. 7. Principal Coordinates Analysis of Aitchison (A) and Bray-Curtis (B) [on unrarefied ASV counts] distance matrixes between trial arms at each visit.

The confidence ellipses define the region that contains 95% of all samples that can be drawn from the underlying “t” distribution for each arm.

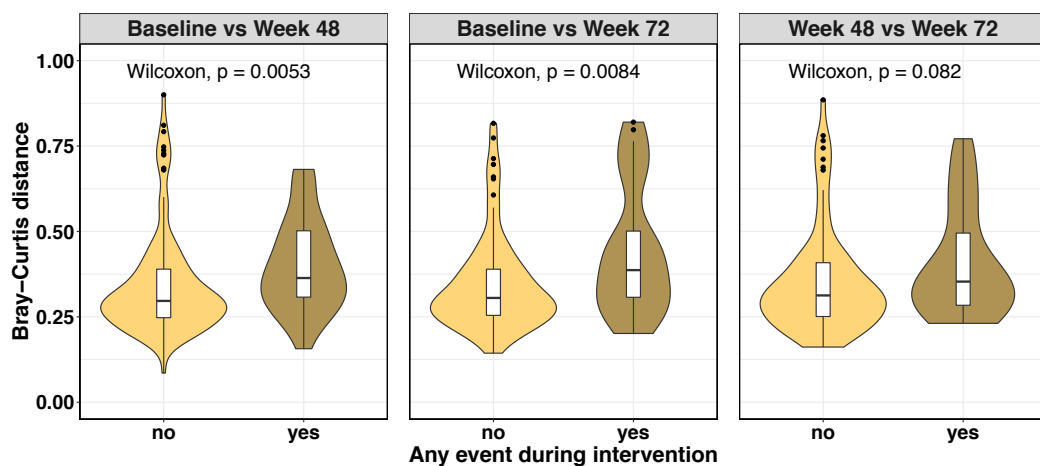
Within the placebo arm, the median within-participant Bray-Curtis distance between samples from participants who had an adverse event (acute exacerbation of respiratory illness, hospitalisation or required additional antibiotics, $n = 35$) was higher than between samples from participants without an adverse effect ($n = 115$), when comparing baseline to 48 weeks (Kruskal Wallis test, $p = 0.005$), or

baseline to 72 weeks (Kruskal Wallis test, $p = 0.008$) (Figure 5.8B). Within the azithromycin arm, fewer participants developed an adverse event ($n = 16$). There were no significant differences in within-participant Bray-Curtis distance across any of the time points when compared with participants who did or did not have an adverse event ($n = 138$), Figure 5.8A. Also, there was no significant difference between within-participant Aitchison distance between samples from participants who had an adverse event and those that did not in either azithromycin (Figure 5.8C) or placebo arm (Figure 5.8D).

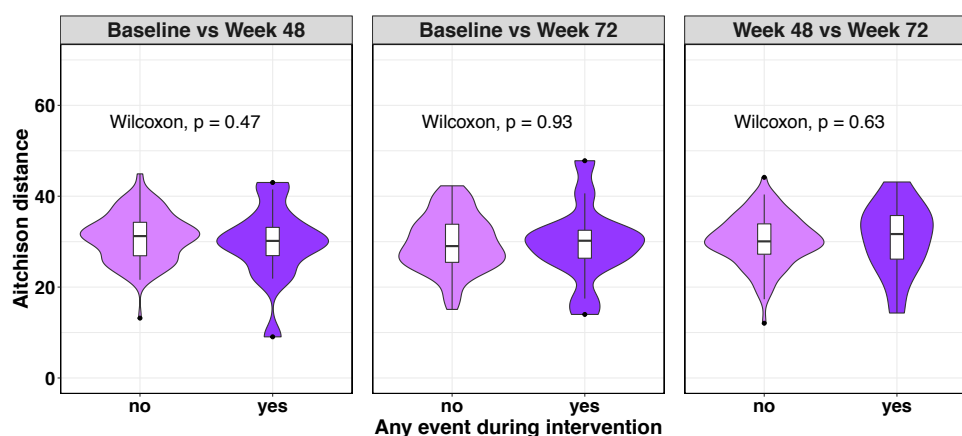
A) AZM arm only



B) Placebo arm only



C) AZM arm only



D) Placebo arm only

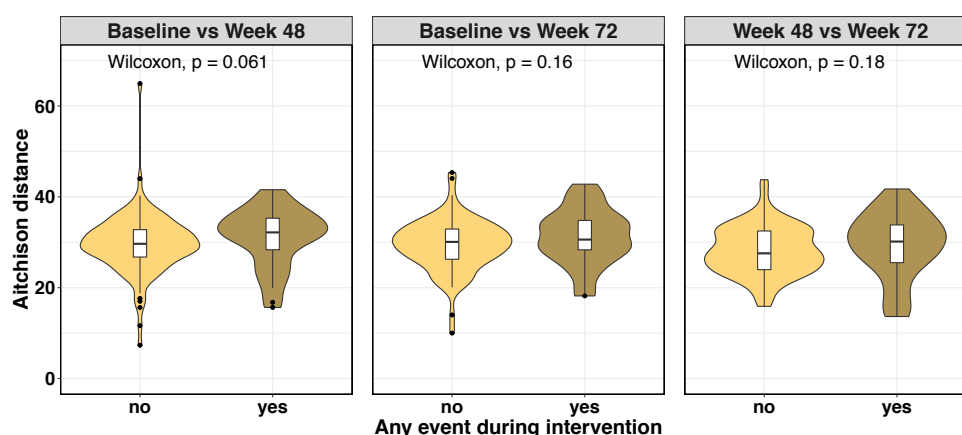


Figure 5. 8. Violin boxplot comparing the median within-participant change in Bray-Curtis and Aitchison distance of participants with and without adverse event.

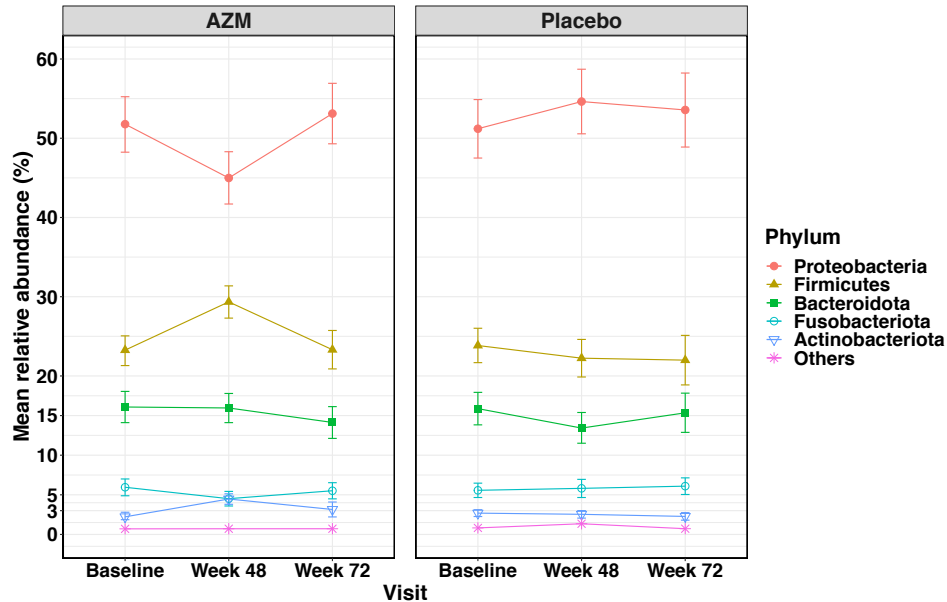
The Bray-Curtis and Aitchison distances were calculated from CLR transformed ASV counts from baseline to 48 weeks, baseline to 72 weeks and 48 weeks to 72 weeks and medians compared between participants who developed acute exacerbation, hospitalised or were administered additional antibiotics (yes, $n = 35$ for placebo, $n = 16$ for azithromycin) with those who did not (no, $n = 115$ for placebo, $n = 138$), in the AZM arm (Bray-Curtis = A & Aitchison = C) and Placebo arm (Bray-Curtis = B & Aitchison = D). Wilcoxon test was used for all comparisons. The horizontal line in the middle of the box is the median. The box presents the interquartile range. The whiskers show 95% confidence interval. The shape of the violin display frequencies of values. AZM = azithromycin.

5.3.6 Composition of taxa within the sputum bacteriome of participants

Five phyla accounted for 99.2% of the mean relative abundances of all bacteria, Proteobacteria (53.4%), Firmicutes (23.4%), Bacteroidetes (14.2%), Fusobacteria (5.2%) and Actinobacteria (3.0%)

(Figure 5.9A, 5.10). At the genus level, the 10 most abundant genera at baseline included *Haemophilus* (25.5%), *Neisseria* (18.8%), *Streptococcus* (15.8%), *Prevotella* (9.5%), *Veillonella* (3.9%), *Fusobacterium* (3.2%), *Alloprevotella* (2.9%), *Porphyromonas* (2.7%), *Moraxella* (2.5%) and *Leptotrichia* (2.3%) (Figure 5.9B, Figure 5.11).

A) Phyla



B) Genera

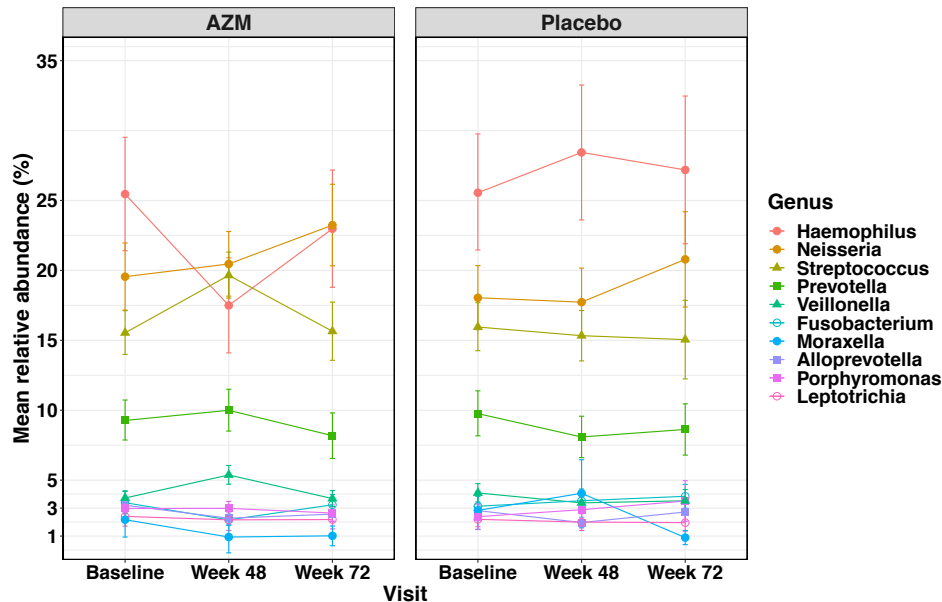


Figure 5. 9. Line plot of the mean relative abundances of the top phyla and genus in all samples.

Top five phyla (mean relative abundance $\geq 2\%$) shown in A) and top 10 genera (mean relative abundance $\geq 1\%$) shown in B) in all samples at all visits and between trial arms. The shape of the data points and the line colour represents the phylum in A. The shape of the data points for genus corresponds to the phylum it belongs to in A while the line colours indicate the genus in B. AZM = azithromycin.

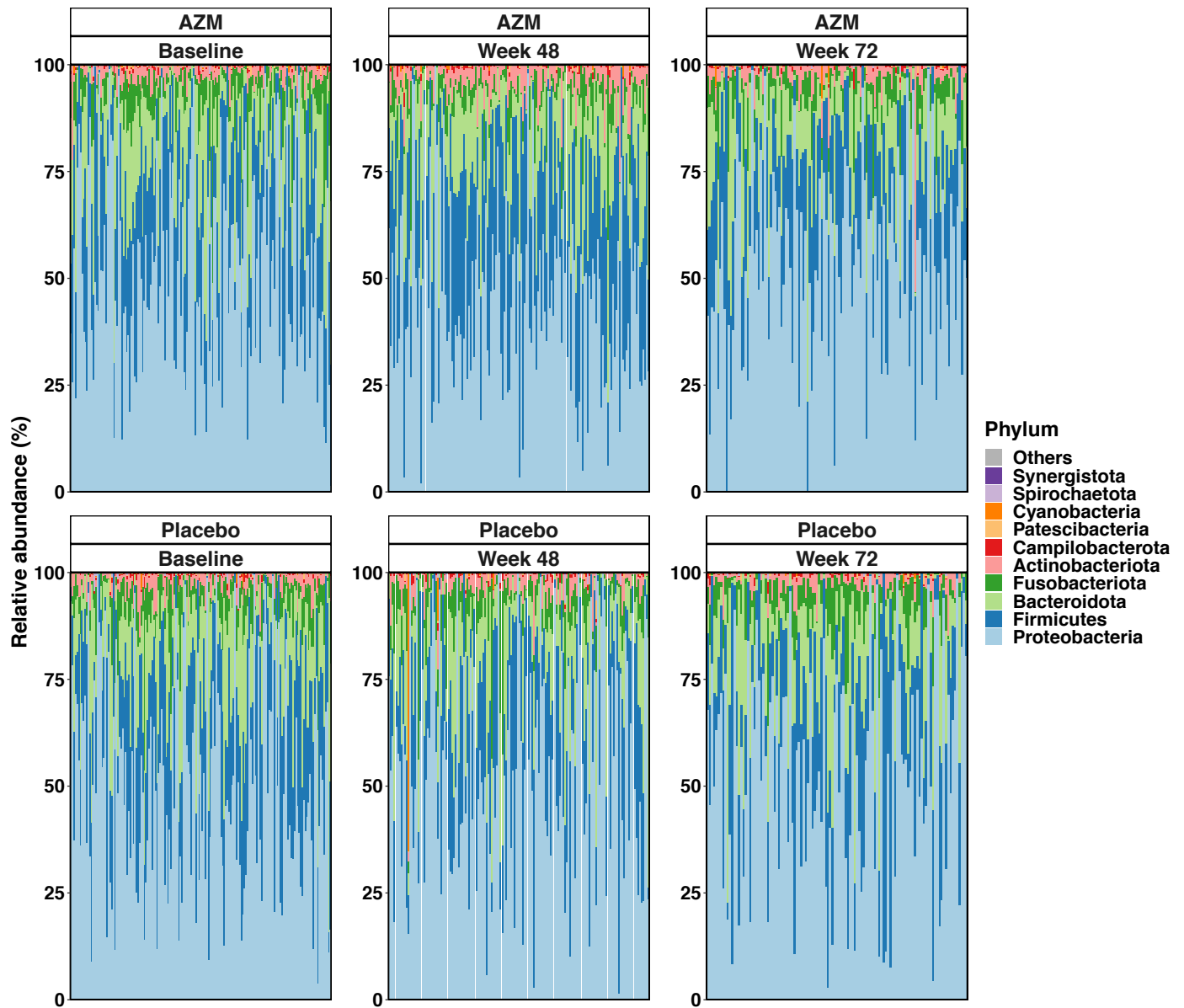


Figure 5. 10. Barplot of the relative abundances of the top 10 most prevalent phyla in all samples.

Upper right, middle, and left panels show samples from participants in the AZM arm at baseline, 48 weeks and 72 weeks. The lower right, middle and lower left panels show samples from participants in the Placebo arm at baseline, 48 weeks and 72 weeks. “Others” refers all phyla that are not included in the top 10. AZM = azithromycin.

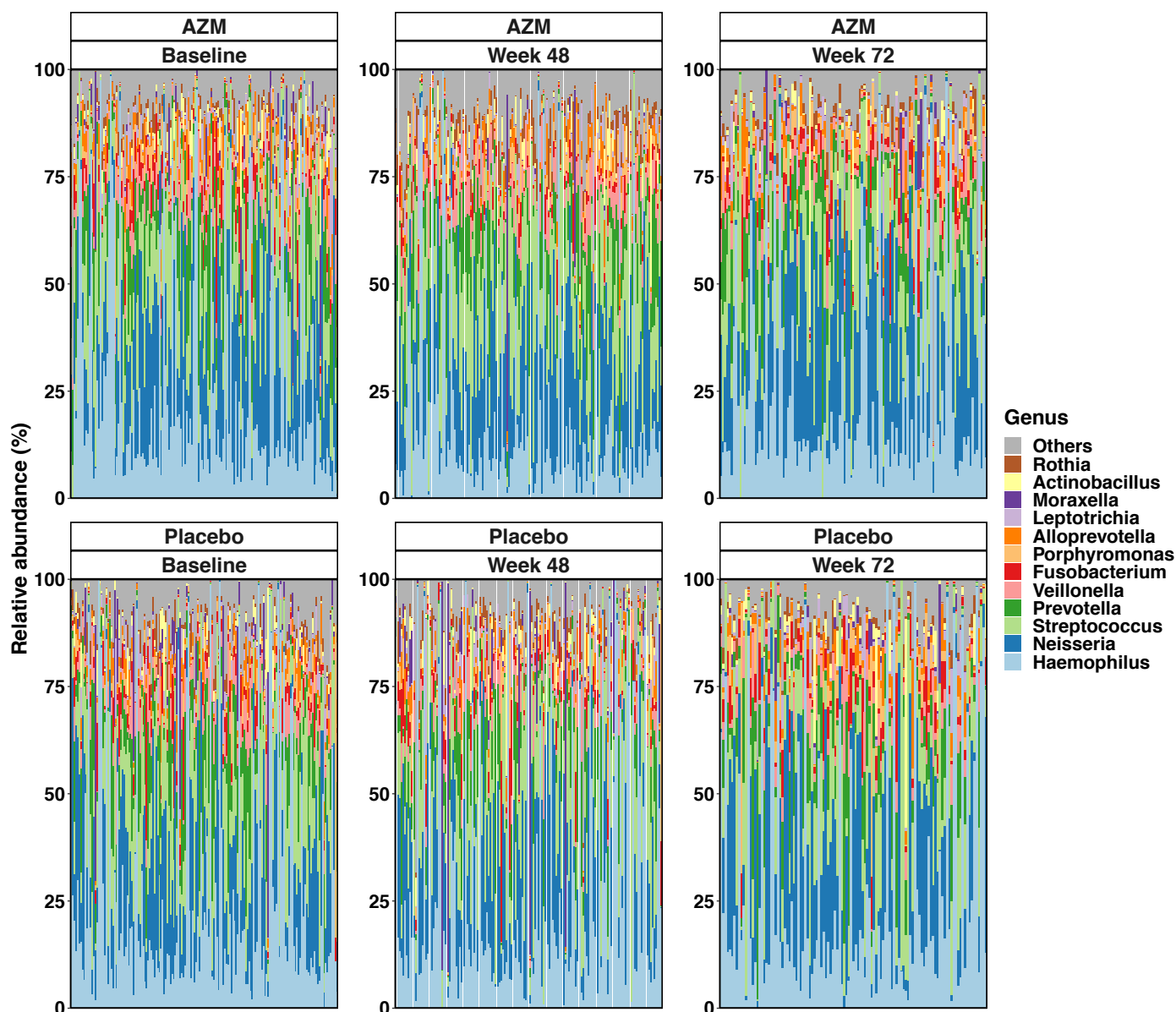


Figure 5. 11. Barplot of the relative abundances of the top 12 most prevalent genera in all samples.

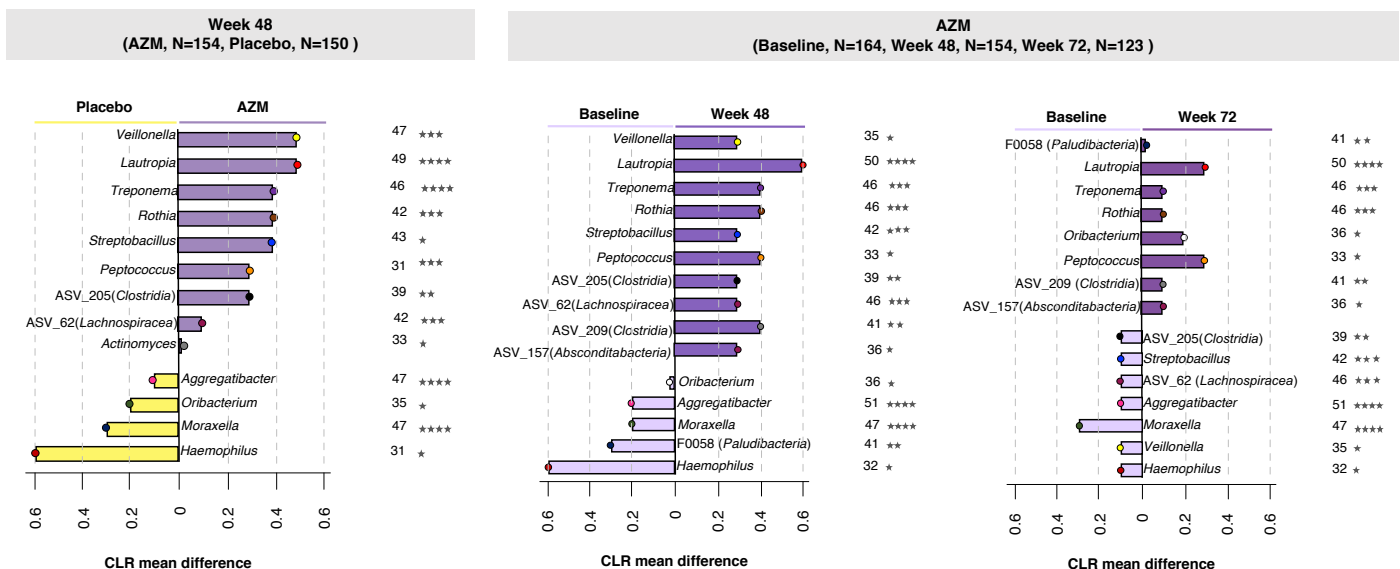
Upper right, middle, and left panels show samples from participants in the AZM arm at baseline, 48 weeks, and 72 weeks. The lower right, middle and lower left panels show samples from participants in the Placebo arm at baseline, 48 weeks, and 72 weeks. “Others” refers all other genera that are not included in the top 12. AZM = azithromycin.

To detect which genera were driving the changes in beta diversity observed between the trial arms at 48 weeks, we tested for differentially abundant taxa using ANCOM2. At baseline, no taxon was differentially abundant between the trial arms. At 48 weeks, 13 genera were differentially abundant between trial arms (Figure 5.12). *Moraxella*, *Haemophilus*, *Treponema*, *Aggregatibacter*, *Streptobacillus*, *Peptococcus*, ASV 205 (*Clostridia*_UCG-014) and ASV-62 (*Lachnospiraceae*) had

significantly lower relative abundance in the azithromycin arm compared to placebo (Figure 5.12). In contrast, *Veillonella*, *Rothia*, *Lautropia*, *Actinomyces* and *Oribacterium* had increased in relative abundance in the azithromycin arm (Figure 5.12A). Fifteen genera were detected to be differentially abundant in the samples from participants in the azithromycin arm between baseline and 48 weeks (Figure 5.12A). There was a high degree of concordance between those genera detected as differentially abundant between azithromycin and placebo arms at 48 weeks and those detected as differentially abundant between the baseline and 48 weeks samples in the azithromycin arm (12 out of 16 genera were concordant, all with the same direction of effect) Figure 5.12A.

When comparing baseline with 72 weeks, many of the same genera (*Lautropia*, *Treponema*, *Rothia*, *Peptococcus*, ASV_209 *Clostridia*, ASV_157 *Absconditabacteria*, *Aggregatibacter*, *Moraxella*, *Haemophilus*) that were differentially abundant when comparing baseline with 48 weeks remained differentially abundant, with the same direction of effect. However, the magnitude of the difference (estimated by CLR mean difference) was generally reduced (Figure 5.12A). Interestingly, several genera (*Veillonella*, *Oribacterium*, *Streptobacillus*, ASV_205 *Clostridia*, ASV_62 *Lachnospiraceae*) showed opposite directions of effect when comparing baseline to 48 weeks with baseline to 72 weeks.

A)



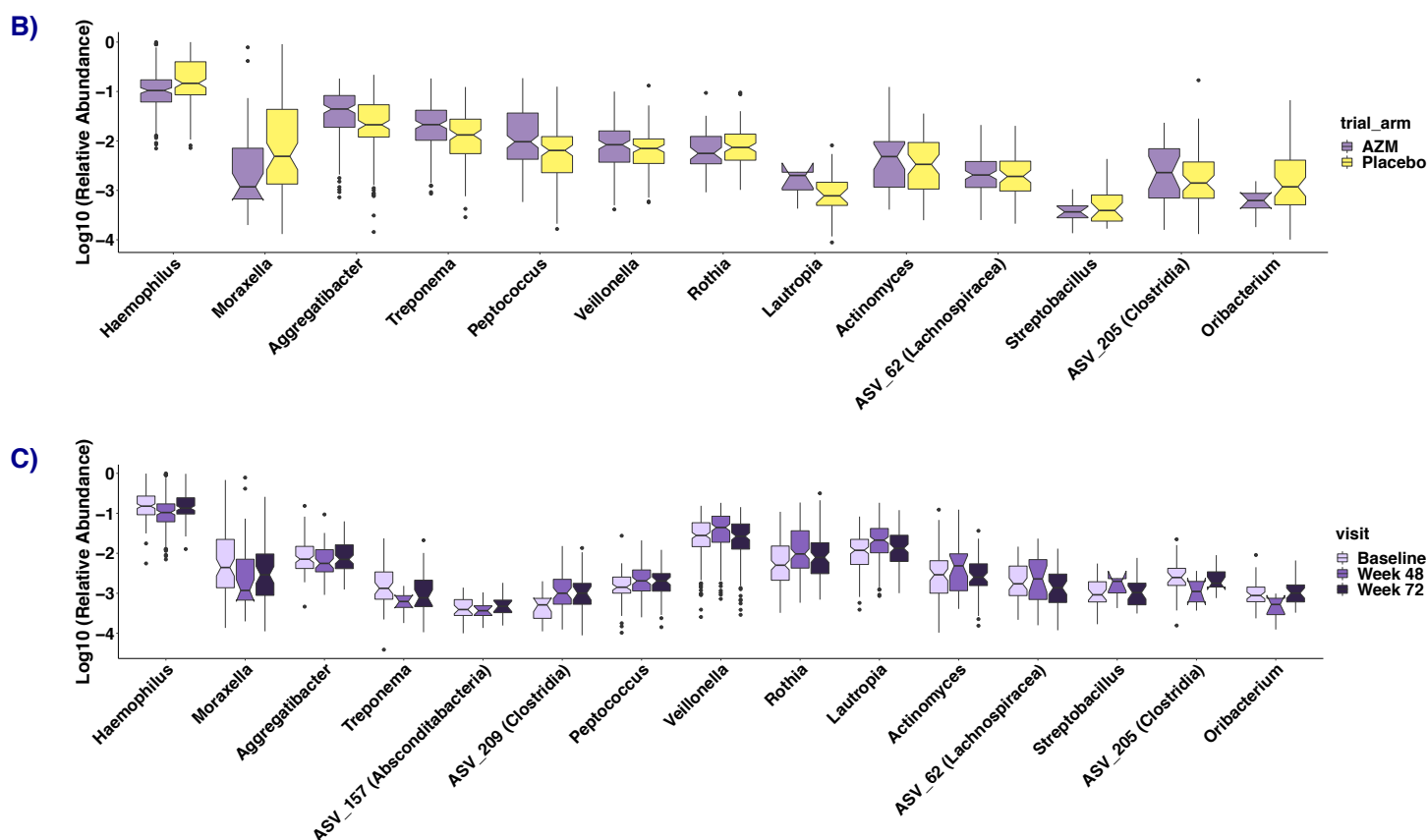


Figure 5. 12. Analysis of Composition of Microbiomes (ANCOM2) differential abundance testing for samples at each visit and between trial arms.

Taxa identified as differentially abundant between trial arms at 48 weeks (A, left panel), between AZM arm at Baseline and 48 weeks (A, middle panel) and between AZM arm at 48 and 72 weeks (A, right panel). Associations were considered significant using an ANCOM detection level ≥ 0.6 . Stars on the right of each bar indicate ANCOM detection levels: levels ≥ 0.6 are presented by 1 star, ≥ 0.7 by 2 stars, ≥ 0.8 by 3 stars and ≥ 0.9 by 4 stars. The raw W statistic for each comparison is shown left of the stars. The horizontal length of each bar indicates the mean centered log-ratio (CLR) difference in relative abundance. B) Boxplots of $\log_{10}$ relative abundances of bacterial genera detected as differentially abundant by ANCOM2 by trial arm at 48 weeks (B) and within AZM arm at Baseline, 48 and 72 weeks (C). AZM = azithromycin.

5.3.7 Concordance between differential abundance testing methods.

We used other statistical methods (Wilcoxon signed-rank test on data after the following normalisation methods—total sum scaling, variance stabilising transformation, and centred log transformation after applying a pseudocount of one, Aldex2 [34], Deseq2 [35], ANCOM-BC [36], Corncob [37], MaasLin2 [38] with total sum scaling and log transformation, MaasLin2 [38] with centred log transformation after applying a pseudo count of one) to compare the differential abundance results from ANCOM2, which generally showed strong concordance (Figure 5.13 to 5:15, Appendix D Table S3-S9). At 48 weeks, five genera were detected as differentially abundant between study arms by all methods (*Lautropia*, *Moraxella*, *Rothia*, *Treponema* and *Veillonella*) (Figure 5.13). *Lautropia*, *Moraxella*,

Treponema, *Oribacterium*, *F0058*, and *ASV 209* were detected as differentially abundant between the baseline and 48-week samples from participants in the azithromycin arm by all methods (Figure 5.14). *Moraxella* was the only genus detected as differentially abundant between the 48 and 72-week samples from participants in the azithromycin arm by all methods (Figure 5.15). There was also a high level of agreement between the differential abundance testing and the SIMPER analysis of the contribution of taxa to overall dissimilarity (Bray Curtis distance) Table 5.4.

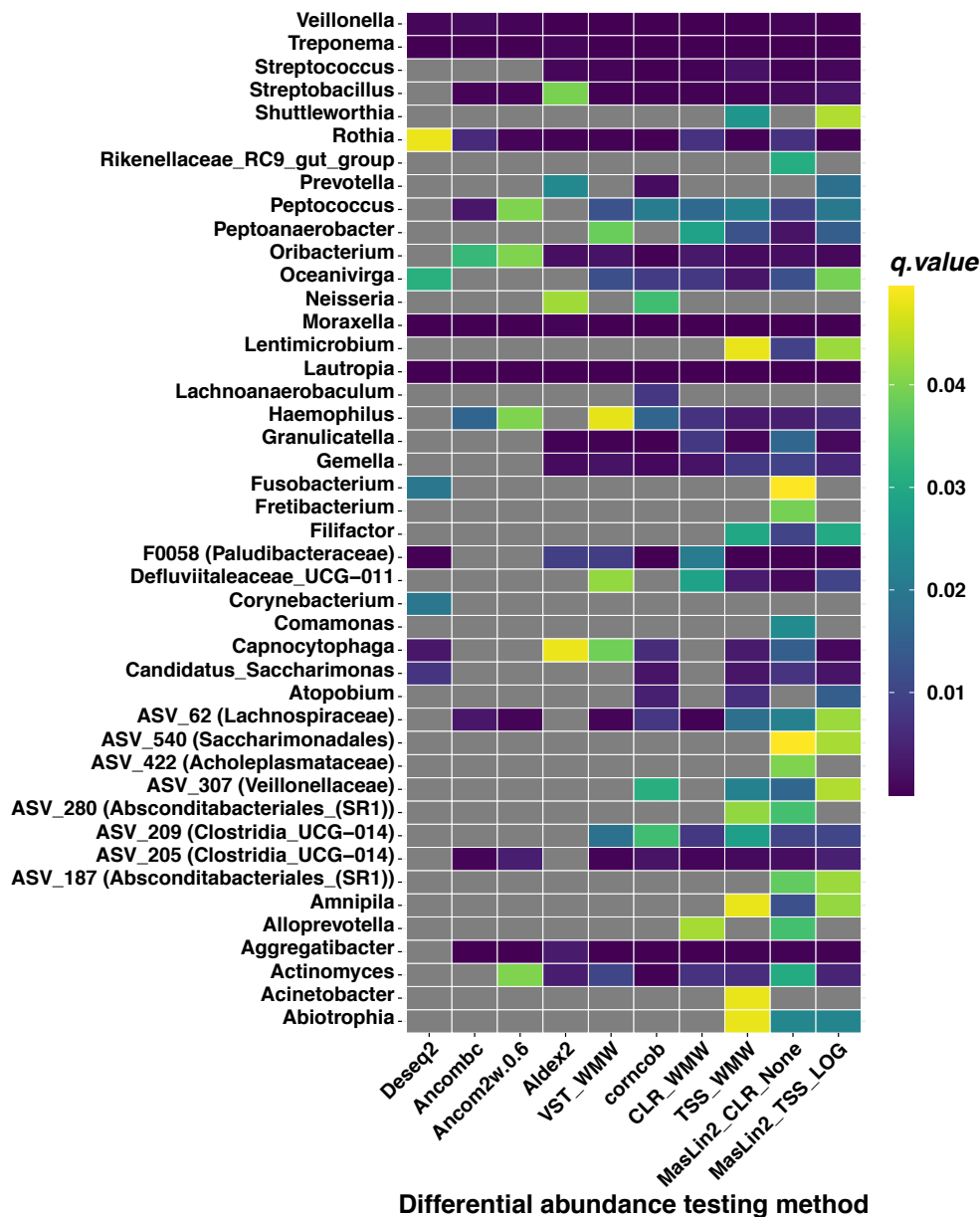


Figure 5. 13. Heatmap displaying the q values of the genera detected as differentially abundant between AZM and placebo arms at 48 weeks by 10 statistical methods.

For ANCOM2, taxa with w 0.6, 0.7, 0.8 and 0.9 were assigned q value of 0.01, 0.001, 0.0001 and 0.00001 respectively. Five genera were detected as differentially abundant by all methods (*Lautropia*, *Moraxella*, *Rothia*, *Treponema* and *Veillonella*).

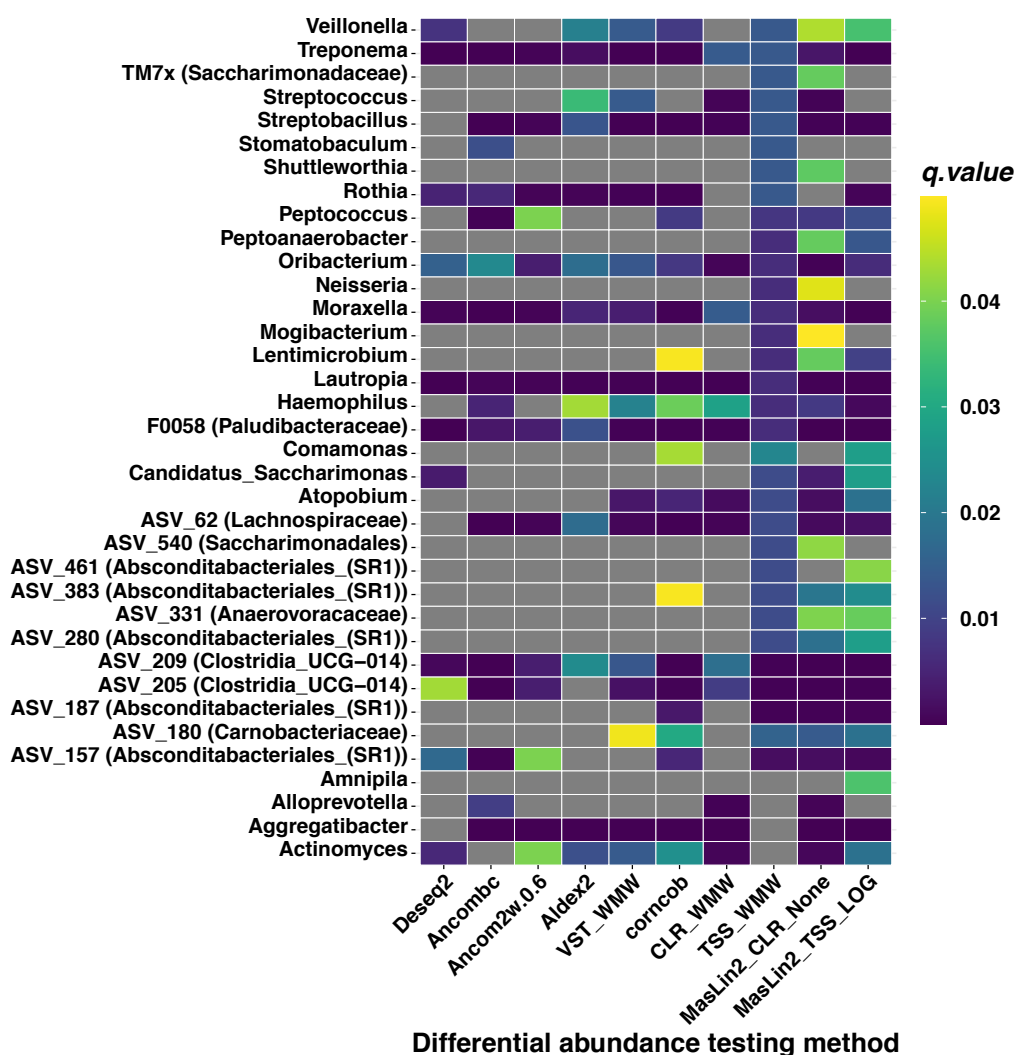


Figure 5. 14. Heatmap displaying the q values of the genera detected as differentially abundant within the AZM arm between baseline and 48-week samples by 10 methods.

For ANCOM2, taxa with w 0.6, 0.7, 0.8 and 0.9 were assigned q value of 0.01, 0.001, 0.0001 and 0.00001 respectively. *Lautropia*, *Moraxella*, *Treponema*, *Oribacterium*, F0058, and ASV 209 were detected as differentially abundant by all methods.

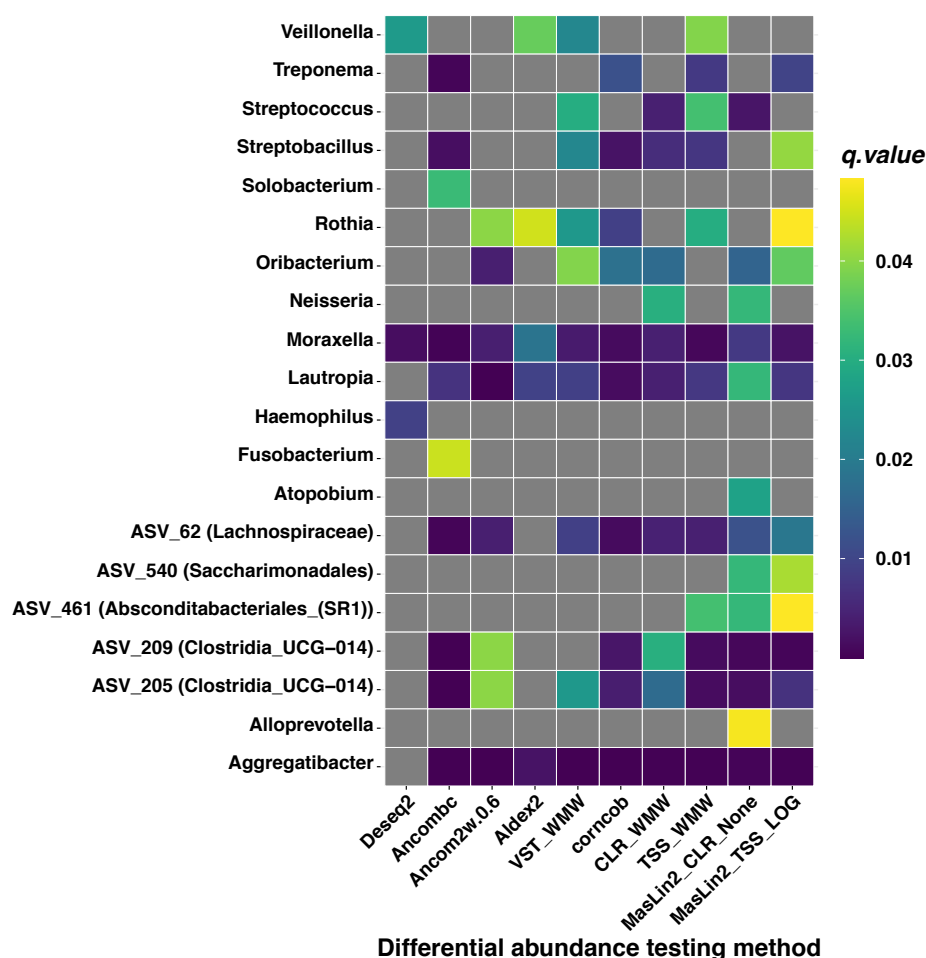


Figure 5. 15. Heatmap displaying the q values of the genera detected as differentially abundant within the AZM arm between 48- and 72-week samples by 10 methods.

For ANCOM2, taxa with w 0.6, 0.7, 0.8 and 0.9 were assigned q value of 0.01, 0.001, 0.0001 and 0.00001 respectively. Only *Moraxella* was detected as differentially abundant by all methods. AZM = azithromycin.

Table 5. 4. Contributions of top genera to overall dissimilarity between azithromycin and placebo arms at 48 weeks and, within the azithromycin arm, between baseline and 48-week samples- SIMPER analysis.

Genus	Azithromycin and Placebo at 48 weeks						Baseline and 48 weeks in the azithromycin arm					
	Average contribution to overall dissimilarity.	Standard deviation of contribution.	Mean abundance in AZM arm	Mean abundance in Placebo arm	Ordered cumulative contribution	*p	Average contribution to overall dissimilarity.	Standard deviation of contribution.	Mean abundance at Baseline	Mean abundance at 48 weeks	Ordered cumulative contribution	*p
<i>Haemophilus</i>	12.8	14.2	28.4	17.5	23.6	0.003	11.4	13.2	25.8	17.9	22.6	0.59
<i>Neisseria</i>	8.4	6.3	17.7	20.5	39.2	0.15	8.1	6.2	19.1	20.3	38.7	0.01
<i>Streptococcus</i>	6.2	4.8	15.3	19.6	50.7	0.002	5.8	4.8	15.9	19.4	50.2	0.004
<i>Prevotella</i>	5	4.4	8.1	10	59.9	0.07	5	4.3	9.4	10.1	60.1	0.73
<i>Moraxella</i>	2.4	8	4.1	0.9	64.4	< 0.0001	1.4	5	1.9	1	62.9	0.17
<i>Veillonella</i>	2.3	1.9	3.4	5.4	68.7	< 0.0001	2.1	1.8	3.8	5.3	67.2	< 0.001
<i>Porphyromonas</i>	1.8	2.7	2.9	3	72	0.10	1.7	2.2	3.1	3.1	70.5	0.06
<i>Fusobacterium</i>	1.7	2.4	3.5	2.2	75.2	0.10	1.8	2.5	3.6	2.2	74	0.002
<i>Leptotrichia</i>	1.5	2.6	2	2.2	78	< 0.0001	1.5	2.5	2	2.2	76.9	< 0.0001
<i>Actinobacillus</i>	1.4	2.5	1.8	1.7	80.6	0.22	1.3	2.2	1.6	1.7	79.4	0.06
<i>Lautropia</i>	1.4	2	0.8	2.9	83.3	0.01	1.4	2	1.1	2.9	82.3	0.88
<i>Rothia</i>	1.3	1.4	1.6	2.8	85.7	0.73	1.2	1.4	1.4	2.8	84.8	0.57
<i>Alloprevotella</i>	1.3	1.3	2	2.3	88.1	0.83	1.6	1.5	3.1	2.2	87.9	0.64
<i>Actinomyces</i>	0.6	0.8	0.7	1.1	89.1	< 0.001						
<i>Granulicatella</i>	0.6	0.5	1.1	1.5	90.2	< 0.001						
<i>Gemella</i>	0.6	0.6	1	1.3	91.2	< 0.001						
<i>Aggregatibacter</i>	0.4	0.7	0.7	0.4	92.1	0.99						

Contributions by genus were assessed by similarity of percentages (SIMPER) analysis of Bray-Curtis distance. Average dissimilarity is a measure of dissimilarity accounted for by each genus between the sputum bacteriome composition between trial arms at 48 weeks or, within the azithromycin arm, between baseline and 48 week visits. Contribution (%) is the percentage of total dissimilarity that the contribution of each genus accounts for, calculated as the mean contribution divided by mean dissimilarity across samples. Cumulative (%) is percentage of dissimilarity that is accounted for by all genera included in the model to this point. Mean abundance is the mean relative abundance of each genus. Only the taxa accounting for 92% of dissimilarity are shown. *p values for comparison of mean abundance between AZM and placebo at 48 weeks by Wilcoxon signed-rank test with BH correction.

5.3.8 Associations between the relative abundance of bacterial genera and clinical measures

We used a linear mixed effect model to identify possible associations between lung function (FEV1z-score, FVCz-score) and bacterial genera. The relative abundance of *Neisseria* was positively associated with FEV1z-score (coefficient, 2.85, standard error, 0.7, $q = 0.01$) while that of *Haemophilus* was negatively associated (coefficient, -6.1, standard error, 1.2, $q < 0.001$). In separate models, the adverse events of hospitalisation, additional antibiotic use and acute respiratory exacerbation during intervention were not associated with any of the five bacterial genera previously identified as associated with HCLD (*Streptococcus*, *Prevotella*, *Haemophilus*, *Moraxella* and *Neisseria*) [10].

We next used a univariate linear regression effect model to study whether change in lung function between visits over the study period was associated with changes in the relative abundances of the five bacterial genera of interest or with change in within-participant beta diversity over time. In the azithromycin arm, a within-participant increase in FEV1z-score was positively associated with within-participant increase in the relative abundance of *Streptococcus* (coefficient [standard error], 3.2 [1.11], $q = 0.01$, Table 5.4). In contrast, an increase in FEV1z-score was negatively associated with a within-participant increase in the relative abundance of *Moraxella* (-2.74 [0.74], $q = 0.002$, Table 5.4). Furthermore, in the azithromycin arm, an increase in within-participant Aitchison distance was positively associated with within-participant increase in both FEV1z-score (1.05 [0.45], $p = 0.02$, Table 5.6) and FVCz-score (0.95 [0.42], $p = 0.02$, Table 5.6).

Table 5. 5. Linear regression analysis of within-participant change in FEV1z-score and FVCz-score and within-participant change in relative abundance of selected genera (outcome) between visits.

Within-participant change in genus	Trial arm	Within-participant change in FEV1z-score				Within-participant change in FVCz-score			
		coef	stderr	pval	qval	coef	stderr	pval	qval
<i>Streptococcus</i>	AZM	3.22	1.11	0.004	0.01	2.66	1.04	0.01	0.08
	Placebo	-0.79	1.46	0.59	0.82	-0.42	1.2	0.72	0.93
<i>Prevotella</i>	AZM	0.1	0.86	0.91	0.97	0.59	0.78	0.45	0.63
	Placebo	-1.54	1.08	0.16	0.82	-1.56	0.87	0.07	0.51
<i>Haemophilus</i>	AZM	-0.92	1.95	0.64	0.89	-1.12	1.82	0.54	0.63
	Placebo	-0.76	2.86	0.79	0.9	0.15	2.3	0.95	0.95
<i>Moraxella</i>	AZM	-2.74	0.74	0.0003	0.002	-1.28	0.7	0.07	0.25
	Placebo	0.18	1.42	0.9	0.9	0.92	1.11	0.41	0.93
<i>Neisseria</i>	AZM	0.05	1.43	0.97	0.97	-0.34	1.33	0.8	0.8
	Placebo	1.5	1.83	0.41	0.82	2.66	1.04	0.01	0.08

Associations were tested with MaAsLin2 using a linear regression model with FEV1z-score or FVCz-score and trial arm as fixed effects and within-participant change in relative abundance of selected genera as outcome. Statistical significance was corrected for multiple testing using Benjamini/Hochberg correction. Columns correspond to the within-participant change in genus, trial arm, the coefficient estimate (coef) and standard error from the model (stderr), nominal *p*-value (pval), and FDR corrected *p*-value (qval). Number of samples in azithromycin (AZM) and placebo arms are 377 and 365, respectively.

Table 5. 6. Univariate linear regression analysis of within-participant Aitchison distance (outcome) and within-participant change in lung function metrics (FVCz-score and FEV1z-score) between visits.

Trial arm	Within-participant change in FEV1z-score			Within-participant change in FVCz-score		
	coef	stderr	pval	coef	stderr	pval
AZM	1.05	0.45	0.02	0.95	0.42	0.02
Placebo	0.3	0.57	0.6	-0.71	0.46	0.13

Associations were tested with MaAsLin2 using a linear regression model with FEV1z-score or FVCz-score and trial arm as fixed effects and within-participant change in beta diversity measured using Aitchison's distance as outcome. Statistical significance was corrected for multiple testing using Benjamini/Hochberg correction. Columns correspond to the within-participant change in genus, trial arm, the coefficient estimate (coef) and standard error from the model (stderr), nominal *p*-value (pval). Number of samples in azithromycin (AZM) and placebo arms are 377 and 365 respectively.

5.4 Discussion

We investigated the impact of long-term azithromycin treatment on the diversity and composition of the sputum bacteriome of children and adolescents with HCLD and the persistence of this effect six months post-intervention. Azithromycin treatment was associated with reduced bacterial load and maintenance of within-sample (alpha) diversity compared with placebo. In turn, low bacterial load and high alpha diversity were associated with better lung function measures. Treatment with azithromycin was associated with a reduction in the relative abundance of several potentially pathogenic taxa at 48 weeks, including *Haemophilus* and *Moraxella*. These changes persisted but were less marked at 72 weeks. The relative abundance of *Haemophilus* was negatively associated with lung function (FEV1z-score) and an increase in the relative abundance of *Moraxella* over time was associated with a decline in lung function. In contrast, the bacterial genera *Neisseria* and *Streptococcus* were associated with improved lung function measures.

The reduction in the total sputum bacterial load after 48 weeks of azithromycin treatment is expected, given the antibacterial activity and immunomodulatory effects of azithromycin [8,40], and may have contributed to the reduction in the frequency of acute respiratory exacerbations in the azithromycin arm [7]. The difference in the bacterial load between the arms at 48 weeks represents an approximately 3-fold reduction in bacterial 16S rDNA gene copies and may be biologically relevant. Sputum bacterial

load returned to baseline levels by 72 weeks. Interestingly, other studies of long-term administration of azithromycin did not observe any effect on total bacterial burden measured by 16 rDNA gene copy numbers [2,3,41] or quantitative culture [41]. The reasons for the inconsistent findings may include differences in dosage, duration and frequency of azithromycin treatment, varying sample types (bronchoalveolar lavage vs sputum), HIV-status, prior antibiotic exposure, smoking status, age and variations between different CLDs (COPD [2,41], asthma [3], cystic fibrosis [1]). For instance, azithromycin would likely have less effect on bacterial load if the dominant taxa, e.g., *Pseudomonas* or *Staphylococcus spp* (in cystic fibrosis), are resistant to azithromycin.

Low alpha diversity is a signal for dysbiotic bacterial communities in many diseases [42,43]. We observed a decline in alpha diversity in the placebo over time, but not the azithromycin arm. High alpha diversity (Shannon index) was associated with enhanced lung function (FEV1z-score and FVCz-score). Our findings are consistent with those of Rogers *et al.* [44], who showed that lower alpha diversity of the sputum bacteriome was associated with lower FEV1z-score in adult participants with non-cystic fibrosis bronchiectasis treated with twice-daily erythromycin for 12-months.

In our study, low alpha diversity was also associated with the development of adverse events in the placebo arm. The decrease in alpha diversity observed in the placebo arm at 48 weeks may, in part, have been a consequence of the additional antibiotic exposure due to the more frequent acute respiratory exacerbations and hospitalisations occurring in this arm. This additional antibiotic exposure may also explain our observation that the median within-participant change in the bacteriome profiles of placebo arm participants who experienced adverse events during the trial was greater than those that did not. Other studies have described changes in the microbial profiles associated with the treatment of acute exacerbations of chronic obstructive pulmonary disease [12].

Interestingly, azithromycin did not affect Shannon diversity (a measure of both species richness and evenness) of the sputum bacteriome at any visit. This observation is consistent with the findings of Acosta *et al.* [1], in which two years of azithromycin treatment did not affect the alpha diversity of cystic fibrosis sputum bacteriome [1]. However, this contrasts Segal *et al.* [2], who observed a reduction in Shannon alpha diversity in emphysema patients treated with azithromycin.

Bray-Curtis within-group dissimilarity was lower than between-group dissimilarity when comparing the 48-week samples in the azithromycin arm with baseline samples in the azithromycin arm or the 48-week samples in the placebo arm. Our findings are consistent with previous studies in asthma [3], COPD [2] and bronchiolitis [45] participants. In contrast, Acosta *et al.* [1] did not observe any effect

of azithromycin on the beta diversity of the sputum bacteriome of cystic fibrosis participants. The more prolonged duration of treatment may explain this difference in findings – two years compared to 48 weeks or less in the other studies. Over this period, the effect of azithromycin may diminish due to macrolide resistance or poor drug adherence. In our study, beta diversity (within-participant Aitchison distance) in the azithromycin arm between study visits was also associated with improvement in lung function measures over the same period, suggesting that the azithromycin-mediated change in composition of the sputum bacteriome may contribute to improved lung function in this population.

We identified 13 differentially abundant taxa including *Moraxella*, *Haemophilus*, *Treponema* (reduced) and *Rothia*, *Veillonella* and *Lautropia* (enriched) in the azithromycin compared to placebo arms at 48 weeks. The high degree of concordance (12/13) between these taxa and those identified as differentially abundant between the baseline and 48-week samples in the azithromycin arm indicates that this finding is unlikely to represent a false discovery. Many of the same taxa were identified by multiple different methods for differential abundance testing. These data are supported by our previous analysis of sputum bacterial culture from this cohort, which showed a reduction in the prevalence of *Moraxella catarrhalis* and *Haemophilus influenzae* at 48 weeks [46] compared with baseline.

In a separate cross-sectional study [10], we identified an increased risk of HCLD in children with sputum microbiota dominated by *Haemophilus*, *Moraxella*, or *Neisseria* (HMN) compared with microbiota dominated by *Prevotella* or *Streptococcus*. We also showed that cell-free products of HMN sputum microbiota induced epithelial disruption and inflammatory responses in vitro [10]. Our findings from the present study support the central role of *Haemophilus* and *Moraxella* in HCLD. Improvement in FEV1z-score was negatively associated with *Moraxella* relative abundance. Interestingly, the reduced relative abundance of *Haemophilus* and *Moraxella* at 48 weeks in the azithromycin arm persisted, but with reduced magnitude, at 72 weeks. Further work is required to explore the persistent effect of azithromycin on these taxa beyond 72 weeks and associated changes in lung function. The relative abundance of *Streptococcus* was positively associated with FEV1z, which is consistent with our earlier finding study [10] that this genus may be protective in HCLD. However, in the current study, the relative abundance of *Neisseria* was positively associated with FEV1z, in contrast to our previous findings.

Our study has several strengths. It is the first study to assess the effect of long-term azithromycin treatment on the sputum bacteriome of African children with HIV-associated chronic lung disease. The double-blind, randomised, placebo-controlled design allows a more direct inference of causal

relationships. It is also the first study to analyse the persistence of azithromycin effect on the sputum bacteriome six months after treatment cessation using this study design. Several factors limit our study. 16S rDNA amplicon sequencing cannot resolve taxonomy to the species level, which may be necessary, for example, given the widely varying pathogenicity of *Streptococcus* species. The use of sputum to assess the lower airway microbiota is limited by contamination from the upper airways. However, previous studies have demonstrated acceptable concordance between the sputum and bronchoalveolar lavage bacteriome [47,48] in asthma [48] and cystic fibrosis participants [47]. Also, we did not conduct sputum quality assessment which could have provided additional information on the degree of upper respiratory tract contamination. Finally, the 16S RNA qPCR that we used is subject to bias due to the varying copy number of 16S rDNA genes in bacteria.

5.5 Conclusion

In conclusion, 48 weeks of once-weekly azithromycin reduced the total bacterial load and preserved within-sample bacterial diversity of children and adolescents with HCLD. These features were associated with better lung function measures. Our results confirm and extend previous findings from a separate cohort that some species within the genera, *Haemophilus* and *Moraxella*, are likely play a central role in the pathogenesis of HCLD whilst some species of the *Streptococcus* genus may be protective. Modulation of the airway microbiota, with a targeted reduction in disease-associated taxa, may be a strategy to ameliorate disease in people with HCLD.

5.6 Declarations

5.6.1 Ethical approval and consent to participate

The trial was approved by the London School of Hygiene and Tropical Medicine Ethics Committee, the Regional Committee for Medical and Health Research Ethics, University of Cape Town Human Research Ethics Committee, and each local regulatory board before project commencement. This sub-study was approved by the Human Research and Ethics Committee of the University of Cape Town - (HREC/REF: 092/2019). Written informed consent and assent were provided by guardians and participants under 18 years, respectively. Older participants (≥ 18 years) consented independently at enrolment. All data obtained and generated during the study were kept confidential. This research was conducted in accordance with the Declaration of Helsinki.

5.6.2 Consent for publication

Not applicable

5.6.3 Availability of data and material

The 16S amplicon sequences, anonymised clinical, laboratory and socio-demographic data and analysis codes that support the findings of this study are available in Sequence Read Archive (accession number [PRJNA769290](#)) and GitHub repository https://github.com/ReginaEsinamAbotsi/BREATHE_Sputum_bacteriome_analysis.

5.6.4 Competing interests

The authors declare that they have no competing interests.

5.6.5 Funding

The Global Health and Vaccination Research (GLOBVAC) Programme of the Medical Research Council of Norway funded this study. 16S sequencing was partially supported through the National Institutes of Health Common Fund, through the Office of Strategic Coordination/Office of the NIH Director, National Institute of Environmental Health Sciences and National Human Genome Institute of Health of the National Institutes of Health (H3Africa awards grant numbers U54HG009824 and 1U01AI110466). REA is funded by L'Oréal UNESCO For Women in Science Fellowship, Margaret McNamara Education Grants and the Swedish International Development Cooperation Agency (SIDA) through the Organisation of Women in Science for the developing world (OWSD) PhD Fellowship. FSD is supported by the National Research Foundation of South Africa (112160), Future Leaders – African Independent Research (FLAIR) Fellowship and the National Institute for Health Research (NIHR) Global Health Research Unit on Mucosal Pathogens (MPRU) using UK aid from the UK Government and the University of Cape Town. RAF is funded by the Wellcome Trust (206316_z_17_Z). AMR is supported by the UK Medical Research Council (MRC) and the UK Department for International Development (DFID) under the MRC/DFID Concordat agreement, which is also part of the EDCTP2 Programme supported by the European Union, Grant Ref: MR/R010161/1. RSH is an NIHR Senior Investigator. The views expressed are those of the authors and not necessarily those of the NIHR, the Department of Health and Social Care. Mark Nicol is supported by an Australian National Health and Medical Research Council Investigator Grant [APP1174455]. The funders had no role in the design of the study and collection, analysis, and interpretation of data and in writing this manuscript and the decision to publish this report.

5.6.6 Author contributions

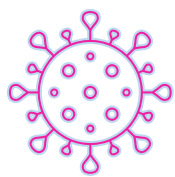
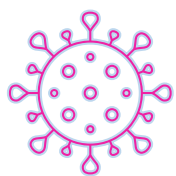
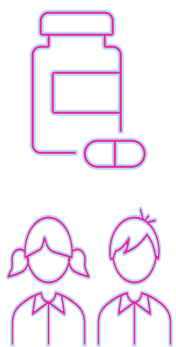
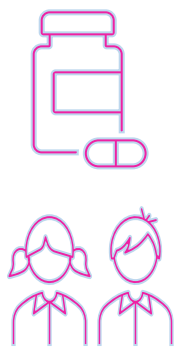
MPN and RAF conceived the study. REA conducted all laboratory experiments (DNA extraction, library preparation and sequencing), data analysis and wrote the first draft of the manuscript supervised by MPN and FSD. YX assisted REA with bioinformatics and statistical analysis of the data. SK conducted data preprocessing and bioinformatics analysis to generate Biom files from raw sequences. SCW assisted with laboratory workflow, supervised the laboratory experiments, and performed the in-silico quality control and decontamination steps. SGL provided the R scripts for in-silico quality control and decontamination steps. GM coordinated sample collection in Zimbabwe. LGN is the study coordinator in Malawi. AMR and VS are the trial statisticians, and they guided the reporting of results and interpretation of the data. BW and RH reviewed the manuscript. JOO and RAF are trial PIs. JOO secured funding from GLOBVAC on behalf of the consortium. All authors contributed to the manuscript, and all authors read and approved the final version.

5.7 References

1. Acosta N, Thornton CS, Surette MG, Somayaji R, Rossi L, Rabin HR, et al. Azithromycin and the microbiota of cystic fibrosis sputum. *BMC Microbiol.* 2021; 21:96.
2. Segal LN, Clemente JC, Wu BG, Wikoff WR, Gao Z, Li Y, et al. Randomised, double-blind, placebo-controlled trial with azithromycin selects for anti-inflammatory microbial metabolites in the emphysematous lung. *Thorax.* 2017; 72:13–22.
3. Taylor SL, Leong LEX, Mobegi FM, Choo JM, Wesselingh S, Yang IA, et al. Long-term azithromycin reduces *Haemophilus influenzae* and increases antibiotic resistance in severe asthma. *Am J Respir Crit Care Med.* 2019; 200:309–17.
4. Spence CD, Vanaudenaerde B, Einarsson GG, McDonough J, Lee AJ, Johnston E, et al. Influence of azithromycin and allograft rejection on the post-lung transplant microbiota. *J Heart Lung Transplant.* 2020; 39:176–83.
5. Valery PC, Morris PS, Byrnes CA, Grimwood K, Torzillo PJ, Bauert PA, et al. Long-term azithromycin for Indigenous children with non-cystic-fibrosis bronchiectasis or chronic suppurative lung disease (Bronchiectasis Intervention Study): a multicentre, double-blind, randomised controlled trial. *Lancet Respir Med.* 2013; 1:610–20.
6. Alchakaki A, Cramer C, Patterson A, Soubani AO. Which patients with respiratory disease need long-term azithromycin? *Cleve Clin J Med.* 2017; 84:755–8.
7. Ferrand RA, McHugh G, Rehman AM, Mujuru H, Simms V, Majonga ED, et al. Effect of once-weekly azithromycin vs placebo in children with HIV-associated chronic lung disease: the BREATHE randomized clinical trial. *JAMA Netw Open.* 2020;3:e2028484.
8. Hodge S, Reynolds PN. Low-dose azithromycin improves phagocytosis of bacteria by both alveolar and monocyte-derived macrophages in chronic obstructive pulmonary disease subjects. *Respirol Carlton Vic.* 2012; 17:802–7.
9. Berenson CS, Kruzel RL, Eberhardt E, Sethi S. Phagocytic dysfunction of human alveolar macrophages and severity of chronic obstructive pulmonary disease. *J Infect Dis.* 2013; 208:2036–45.
10. Bhadriraju S, Fadrosch DW, Shenoy MK, Lin DL, Lynch KV, McCauley K, et al. Distinct lung microbiota associate with HIV-associated chronic lung disease in children. *Sci Rep.* 2020; 10:16186.
11. Abotsi RE, Nicol MP, McHugh G, Simms V, Rehman AM, Barthus C, et al. Prevalence and antimicrobial resistance profiles of respiratory microbial flora in African children with HIV-associated chronic lung disease. *BMC Infect Dis.* 2021; 21:216.
12. Huang YJ, Sethi S, Murphy T, Nariya S, Boushey HA, Lynch SV. Airway microbiome dynamics in exacerbations of chronic obstructive pulmonary disease. *J Clin Microbiol.* 2014; 52:2813–23.

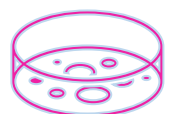
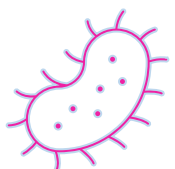
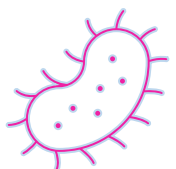
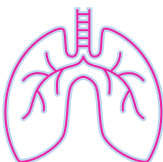
13. Gonzalez-Martinez C, Kranzer K, McHugh G, Corbett EL, Mujuru H, Nicol MP, et al. Azithromycin versus placebo for the treatment of HIV-associated chronic lung disease in children and adolescents (BREATHE trial): study protocol for a randomised controlled trial. *Trials*. 2017/12/29 ed. 2017; 18:622–622.
14. Quanjer PH, Stanojevic S, Cole TJ, Baur X, Hall GL, Culver BH, et al. Multi-ethnic reference values for spirometry for the 3–95-yr age range: the global lung function 2012 equations. *Eur Respir J*. 2012; 40:1324–43.
15. Madanhire T, Ferrand RA, Attia EF, Sibanda EN, Rusakaniko S, Rehman AM. Validation of the global lung initiative 2012 multi-ethnic spirometric reference equations in healthy urban Zimbabwean 7–13-year-old school children: a cross-sectional observational study. *BMC Pulm Med*. 2020; 20:56–56.
16. Fletcher CM, Elmes PC, Fairbairn AS, Wood CH. Significance of respiratory symptoms and the diagnosis of chronic bronchitis in a working population. *Br Med J*. 1959; 2:257.
17. Bogaert D, Keijsers B, Huse S, Rossen J, Veenhoven R, van Gils E, et al. Variability and diversity of nasopharyngeal microbiota in children: a metagenomic analysis. *PLoS ONE*. 2011;6: e17035.
18. Claassen-Weitz S, Gardner-Lubbe S, Nicol P, Botha G, Mounaud S, Shankar J, et al. HIV-exposure, early life feeding practices and delivery mode impacts on faecal bacterial profiles in a South African birth cohort. *Sci Rep*. 2018; 8:5078.
19. Andrews S. FASTQC A Quality control tool for high throughput sequence data. Babraham Inst. 2015
20. Ewels P, Magnusson M, Lundin S, Käller M. MultiQC: Summarize analysis results for multiple tools and samples in a single report. *Bioinformatics*. 2016; 32:3047–8.
21. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat Methods*. 2016; 13:581–3.
22. Di Tommaso P, Chatzou M, Floden EW, Barja PP, Palumbo E, Notredame C. Nextflow enables reproducible computational workflows. *Nat Biotechnol*. 2017; 35:316–9.
23. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res*. 2013;41: D590–6.
24. Wright ES. Using DECIPHER v2. 0 to analyze big biological sequence data in R. *R J*. 2016;8.
25. Schliep KP. phangorn: phylogenetic analysis in R. *Bioinformatics*. 2011; 27:592–3.
26. Claassen-Weitz S, Gardner-Lubbe S, Mwaikono KS, du Toit E, Zar HJ, Nicol MP. Optimizing 16S rRNA gene profile analysis from low biomass nasopharyngeal and induced sputum specimens. *BMC Microbiol*. 2020; 20:113–113.
27. Schneeberger PHH, Prescod J, Levy L, Hwang D, Martinu T, Coburn B. Microbiota analysis optimization for human bronchoalveolar lavage fluid. *Microbiome*. 2019; 7:141.
28. Davis NM, Proctor DM, Holmes SP, Relman DA, Callahan BJ. Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. *Microbiome*. 2018; 6:226.
29. Shannon CE. A mathematical theory of communication. *Bell Syst Tech J*. 1948; 27:379–423.
30. Bray JR, Curtis JT. An ordination of the upland forest communities of southern Wisconsin. *Ecol Monogr*. 1957; 27:325–49.
31. Anderson MJ. Permutational multivariate analysis of variance (PERMANOVA). *Wiley Statsref Stat Ref Online*. 2014;1–15.
32. Jari Oksanen FGB, Friendly M, Kindt R, Legendre P, McGlinn D, Minchin PR, et al. Vegan: community ecology package. R Package Version. 2018;2.
33. Kaul A, Mandal S, Davidov O, Peddada SD. Analysis of microbiome data in the presence of excess zeros. *Front Microbiol*. 2017; 8:2114.
34. Fernandes AD, Reid JN, Macklaim JM, McMurrough TA, Edgell DR, Gloor GB. Unifying the analysis of high-throughput sequencing datasets: characterizing RNA-seq, 16S rRNA gene sequencing and selective growth experiments by compositional data analysis. *Microbiome*. 2014; 2:15.
35. Love M, Anders S, Huber W. Differential analysis of count data—the DESeq2 package. *Genome Biol*. 2014; 15:10–1186.
36. Lin H, Peddada SD. Analysis of compositions of microbiomes with bias correction. *Nat Commun*. 2020; 11:3514.
37. Martin BD, Witten D, Willis AD. Modeling microbial abundances and dysbiosis with beta-binomial regression. *Ann Appl Stat*. 2020; 14:94.
38. Mallick H, Rahnavard A, McIver LJ, Ma S, Zhang Y, Nguyen LH, et al. Multivariable association discovery in population-scale meta-omics studies. *PLOS Comput Biol*. 2021;17: e1009442.
39. Twisk J, Bosman L, Hoekstra T, Rijnhart J, Welten M, Heymans M. Different ways to estimate treatment effects in randomised controlled trials. *Contemp Clin Trials Commun*. 2018; 10:80–5.
40. Hodge S, Hodge G, Jersmann H, Matthews G, Ahern J, Holmes M, et al. Azithromycin improves macrophage phagocytic function and expression of mannose receptor in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 2008; 178:139–48.

41. Brill SE, Law M, El-Emir E, Allinson JP, James P, Maddox V, et al. Effects of different antibiotic classes on airway bacteria in stable COPD using culture and molecular techniques: a randomised controlled trial. *Thorax*. 2015; 70:930–8.
42. Wypych TP, Wickramasinghe LC, Marsland BJ. The influence of the microbiome on respiratory health. *Nat Immunol*. 2019; 20:1279–90.
43. Kitsios GD, Yang H, Yang L, Qin S, Fitch A, Wang X-H, et al. Respiratory tract dysbiosis is associated with worse outcomes in mechanically ventilated patients. *Am J Respir Crit Care Med*. 2020; 202:1666–77.
44. Rogers GB, van der Gast CJ, Cuthbertson L, Thomson SK, Bruce KD, Martin ML, et al. Clinical measures of disease in adult non-CF bronchiectasis correlate with airway microbiota composition. *Thorax*. 2013; 68:731–7.
45. Zhou Y, Bacharier LB, Isaacson-Schmid M, Baty J, Schechtman KB, Sajol G, et al. Azithromycin therapy during respiratory syncytial virus bronchiolitis: upper airway microbiome alterations and subsequent recurrent wheeze. *J Allergy Clin Immunol*. 2016; 138:1215-1219.e5.
46. Abotsi RE, Nicol MP, McHugh G, Simms V, Rehman AM, Barthus C, et al. The impact of long-term azithromycin on antibiotic resistance in HIV-associated chronic lung disease. *ERJ Open Res*. 2022; 8:00491–2021.
47. Hogan DA, Willger SD, Dolben EL, Hampton TH, Stanton BA, Morrison HG, et al. Analysis of lung microbiota in bronchoalveolar lavage, protected brush and sputum samples from subjects with mild-to-moderate cystic fibrosis lung disease. *PloS One*. 2016;11: e0149998–e0149998.
48. Durack J, Huang YJ, Nariya S, Christian LS, Ansel KM, Beigelman A, et al. Bacterial biogeography of adult airways in atopic asthma. *Microbiome*. 2018; 6:104–104.



Chapter 6

General conclusions and future outlook



6.1 General Conclusions

Obliterative bronchiolitis (OB) is a novel form of HIV-associated chronic lung disease (HCLD) that affects more than 30% of perinatally HIV-infected children in Sub-Saharan Africa. Although there are currently no treatment guidelines for OB, long-term azithromycin has been evaluated as a possible management option in the Bronchopulmonary Function in Response to Azithromycin Treatment for Chronic Lung Disease in HIV-infected Children (BREATHE) clinical trial. The trial demonstrated that although long-term azithromycin therapy did not improve lung function as measured by FEV1 z-score (primary outcome of the trial), it reduced the frequency of acute respiratory exacerbations and all-cause hospitalisations in OB patients. This thesis focused on addressing two main information gaps regarding OB: the respiratory bacterial determinants of the condition using a case-control study and the impact and duration of long-term azithromycin treatment on these bacterial determinants using a multicentre, individually randomised, double-blind, placebo-controlled trial. Both culture-dependent and culture-independent techniques, specifically next-generation sequencing, were used to explore the respiratory bacteriome. Logistic and linear mixed-effect modelling were also employed to assess clinical and socio-demographic factors that influence the respiratory bacteriome in HCLD. As HIV-associated OB is the predominant HCLD (91%) in this cohort of patients based on our previous findings, the term was used interchangeably with HCLD throughout this thesis.

The first chapter of this thesis provided a background, study rationale and identified gaps this project sought to fill. The research questions answered in this thesis were stated. This chapter also outlined the structure of the thesis.

In the second chapter, a multi-indication systematic review of the scientific literature on the effect of macrolide administration on the human respiratory microbiota and associated antibiotic resistance was conducted. Existing gaps in the knowledge of the impact of macrolides on the human respiratory microbiota were identified. The results revealed that macrolide use for less than two weeks eradicated the respiratory carriage of *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Haemophilus influenzae*, and *Moraxella catarrhalis* without increasing macrolide resistance in most studies. Also, bacterial carriage and macrolide resistance remain unchanged in most studies where macrolides were administered for more than four weeks. Macrolide use altered the respiratory bacteriome. These findings suggest that the use of long-term azithromycin therapy for HCLD may alter the respiratory bacteriome but without significant effects on antibiotic resistance in *S. pneumoniae*, *S. aureus*, *H. influenzae*, and *M. catarrhalis*. The knowledge gaps identified included limited research on the

respiratory microbiota in general and the effect of antibiotic use on the respiratory tract. Of the limited studies on the respiratory microbiota, a vast majority were in Western countries, with African populations poorly represented. Rigorous studies on the persistence of the effect of macrolide use on the respiratory microbiota were also non-existent.

The third chapter is a published original research that adopted a case-control study design to compare the respiratory carriage and antibiotic resistance of common potentially pathogenic bacterial species in HIV-infected children with and without HCLD. The bacterial species studied included *S. pneumoniae*, *S. aureus*, *H. influenzae*, and *M. catarrhalis*. This study revealed that children with HCLD were more likely to carry *M. catarrhalis* and *S. pneumoniae*, including penicillin-non-susceptible *S. pneumoniae* strains, than HIV-infected children without OB. This finding suggests that an effective treatment for HCLD may involve a reduction in the respiratory carriage of these bacteria. It provided a rationale for the investigation and the medical use of azithromycin, which reduces respiratory carriage of bacteria, for HCLD. This finding also highlights the need for further investigations into the role of these bacteria in HCLD pathogenesis, including the risk of acute exacerbations.

Chapter four contains a published original research that describes the effect of long-term azithromycin treatment on selected bacterial species and associated antibiotic resistance in children and adolescents with HCLD. Using bacterial culture and disk diffusion, it was demonstrated that long-term azithromycin reduced the respiratory carriage of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* but promoted antibiotic resistance in *S. pneumoniae* and *S. aureus*; antibiotic-resistant *S. aureus* persisted at 72 weeks from randomisation. Adherence to the study drug, age and viral load suppression influenced bacterial carriage and antibiotic resistance. This finding further supported the idea that an effective therapy for HCLD may involve reducing the carriage of *S. pneumoniae* and *M. catarrhalis* in the respiratory tract. It also confirms that azithromycin, currently the only evidence-based therapy for HCLD, may be used for the clinical management of the condition without major concerns regarding the persistence of widespread antibiotic resistance post-intervention. A major strength of this study is the multicentre, individually randomised, double-blind, placebo-controlled design which allowed for causal inferences. The six-month follow-up allowed the persistence of the microbiological effect of azithromycin to be studied. However, the study was limited by the lack of follow-up beyond six months to ascertain whether and when antibiotic resistance in *S. aureus* resolved to baseline levels. The risk of antibiotic resistance should be considered during long-term azithromycin use and the clinical

significance of this resistance and the benefits of azithromycin use beyond 48 weeks needs investigation.

The final experimental chapter of this thesis (Chapter 5) investigated the effect of long-term azithromycin on the sputum bacteriome of children and adolescents with HCLD enrolled in the randomised, placebo-controlled trial. 16S rDNA quantitative PCR revealed that long-term azithromycin treatment reduced sputum bacterial loads, which persisted six months post-intervention. The reduction in bacterial load was associated with better lung function (high FEV1 *z*-score). Sequencing the V4 region of the 16S rDNA gene of sputum bacterial communities revealed that long-term azithromycin maintained within-sample diversity. The relative abundances of genera associated with HCLD – *Haemophilus* and *Moraxella* – were reduced in the azithromycin arm, consistent with findings from the previous chapter. Their reduction was associated with better lung function (increase in FEV1 *z*-score). These findings may explain the beneficial effect of azithromycin in reducing acute pulmonary exacerbations and all-cause hospitalisations in this population. It also supports the use of azithromycin for the clinical management of HCLD.

In summary, this thesis provides a detailed description of the bacterial determinants of OB and how they are affected by long-term azithromycin treatment in children and adolescents with HIV-associated OB in sub-Saharan Africa. Both culture-dependent and culture-independent techniques were employed. We identified factors associated with respiratory carriage of common potentially pathogenic respiratory bacteria and antibiotic resistance. The findings presented in this thesis clearly demonstrate the need for further investigation of the microbiological aspects of OB pathogenesis.

6.2 Limitations

The limitations of the techniques used in the work presented in this thesis include the following:

1. The use of only semi-quantitative techniques to describe differences in bacterial carriage between CLD+ and CLD- participants. The accuracy of this technique is limited, and the absolute bacterial loads could not be determined. Additional studies using quantitative PCR can overcome this limitation.
2. The use of culture-dependent technique to describe the prevalence of the five bacteria of interest is limited by bacterial viability. PCR may be more sensitive.
3. The use of disk diffusion to measure antibiotic resistance fails to detect any increase in resistance that did not result in a change in resistance category. The mechanisms underlying

antibiotic resistance are also not described by this methodology. Minimum inhibitory concentration using broth or agar dilution along with whole genome sequencing can overcome this limitation.

4. 16S rDNA amplicon sequencing to describe the sputum bacteriome is limited by resolution to genus and not species or strain level. Shotgun metagenomic sequencing can overcome this limitation and enable metabolic function and antibiotic resistance gene profiling.
5. True causal relationships between bacteriome profiles and clinical outcomes could not be distinguished from associations. It is possible, although unlikely, that changes in the bacteriome profiles are an epiphenomenon and unrelated to the clinical outcomes measured.
6. This study evaluated older children and our observations may be different in younger children who have more growth and poorer hygiene.
7. As this study was conducted in a cohort not immunised against *S. pneumoniae*, the results may not apply to similar but vaccinated cohorts.

6.3 Future Outlook

In addition to future work mentioned in each research chapter, additional areas of future investigation will include:

1. Whole genome sequencing of *S. pneumoniae* and *S. aureus* to characterise:
 - a. Mechanisms underlying antimicrobial resistance: key in understanding transmissibility and treatment strategies.
 - b. Serotypes and lineages of *S. pneumoniae*: provide data that will advise vaccine formulation strategies.
 - c. Population structure of *S. pneumoniae* and *S. aureus*: will improve preparedness and response to future outbreaks of infections caused by antibiotic-resistant bacteria.
2. Quantitative screening of more bacterial targets and common respiratory viruses to investigate their possible role in the pathogenesis of OB.
3. Characterising the metagenome of the nasopharyngeal and sputum of CLD+ and CLD- to detect additional metagenomic signatures associated with OB.
4. Characterising the metagenome of the nasopharyngeal and sputum CLD+ in both trial arms at each visit to investigate the effect of long-term azithromycin treatment on the broader metagenome.

5. Characterising the respiratory metabolome of the trial participants to detect metabolomic signatures associated with OB and how long-term azithromycin affects these signatures.

APPENDICES

APPENDIX A: Chapter Two Supplementary Materials

Appendix A. Chapter Two Supplementary Materials

Protocol for the systematic review published in Prospero

To enable PROSPERO to focus on COVID-19 submissions, this registration record has undergone basic automated checks for eligibility and is published exactly as submitted. PROSPERO has never provided peer review, and usual checking by the PROSPERO team does not endorse content. Therefore, automatically published records should be treated as any other PROSPERO registration. Further detail is provided [here](#).

Citation

Regina Esinam Abotsi, Gillian O.N Ndhlovu, Adelaide Mensah, Mark Nicol, Felix Dube. Systematic review of the impact of macrolide use on the human respiratory bacteriome. PROSPERO 2020 CRD42020187477 Available from: https://www.crd.york.ac.uk/prospéro/display_record.php?ID=CRD42020187477

Review question

1. What are the effects of macrolide antibiotics on the respiratory bacteriome with regards to:
 - a) the composition (presence/absence and relative abundances of specific taxa) of respiratory bacteriome and the prevalence of specific potential pathogens (*Streptococcus pneumoniae*, *Staphylococcus aureus*, *Haemophilus influenzae* and *Moraxella catarrhalis*)?
 - b) antibiotic resistance at the community level (resistome) and within specific pathogens of clinical importance in respiratory tract infections (*Streptococcus pneumoniae*, *Staphylococcus aureus*, *Haemophilus influenzae* and *Moraxella catarrhalis*)?
2. Does the dose, route of administration, frequency and duration of macrolide treatment moderate the effect of the macrolides on the respiratory bacteriome? If yes, what are these moderations and their clinical implications?
3. What is the duration of effect of macrolides on the respiratory bacteriome and resistome after cessation of therapy?

Searches

A comprehensive literature search will be undertaken using the following databases according to the search strategy outlined in (Table 1):

- MEDLINE via PubMed,
- EMBASE via Scopus,
- ISI Web of Science (Web of Knowledge),
- Academic Search Premier, African Wide Information, Health Source Academic/Nursing and CINAHL via EBSCOhost, and
- The Cochrane Library

No filter will be applied to any database searches except for Web of Science and EMBASE where MEDLINE will be excluded. Both MeSH terms and free text words will be used to identify eligible studies. The reference list of included studies and relevant review articles will be screened in order to identify eligible studies further. Searches of the various electronic databases will be conducted on 15th May 2020 by REA together with an experienced librarian. Unpublished studies will be sought. Searches will be re-run before final analysis, and any further studies will be identified and retrieved for inclusion. All citations will be exported into Endnote X9 and screened for eligibility using Covidence software (www.covidence.org).

Types of study to be included

Inclusion:

We will include studies that meet all the following criteria:

- i. administered a macrolide at any dosage and route to humans regardless of health status.
- ii. collected respiratory samples in the form of nasal, nasopharyngeal or oropharyngeal swabs, bronchoalveolar lavage, sputum or lung aspirates, endo-laryngeal suction samples, throat swabs.
- iii. measured the prevalence/bacterial loads and antibiotic resistance of SSHM from the respiratory tract OR bacterial diversity and resistome of respiratory samples collected using either culture-dependent or independent techniques.
- iv. included baseline and follow up samples of participants.

Exclusion:

- i. Studies conducted on animals.
- ii. Studies that looked at the effect of macrolide use on SSHM recovered from other body sites, e.g. blood rather than the respiratory tract.
- iii. Studies not published in English and translations are unavailable
- iv. Reviews, editorials, commentaries, conference abstracts/papers, case studies/reports and cross-sectional studies
- v. Controls were also exposed to macrolides less than 4 weeks before enrolment into the study

Condition or domain being studied

The effect of macrolide administration on the human respiratory bacteriome.

Participants/population

Inclusion: Any participants of any age group in any care setting receiving a macrolide antibiotic.

Exclusion: Participants of any age or care setting not receiving a macrolide antibiotic or receiving a macrolide in combination with other antibiotics.

Intervention(s), exposure(s)

Macrolide antibiotic is defined here as erythromycin, clarithromycin, azithromycin, telithromycin, roxithromycin and troleandomycin

Inclusion: Exposure to a macrolide at any dose via any route of administration for any duration for any indication.

Comparator(s)/control

Study participants who have not been exposed to macrolides in the preceding four weeks before enrolment into the study as controls.

Inclusion: Interventional or comparative studies, appropriate comparators will be participants who received a placebo. In the case of longitudinal studies with multiple timepoint sampling, participants' respiratory bacteriome composition at baseline (before exposure to macrolide treatment) would be the appropriate comparator.

Context

Inclusion:

Studies of any patient of any age in any health care setting who is indicated for macrolide treatment OR studies that investigated the effect of macrolide exposure on the respiratory bacteriome of healthy people OR studies that assessed the effectiveness or adverse effects of macrolides in healthy or sick individuals.

Exclusion:

Studies that merely investigated macrolide resistance in specific bacteria isolated from the respiratory bacteriome or investigated the respiratory resistome without clear information on prior macrolide exposure will be excluded.

Main outcome(s)

Measures of the effect of macrolide administration on the:

- a) prevalence and/or bacterial loads of *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Haemophilus influenzae* and *Moraxella catarrhalis* in the respiratory tract
- b) alpha and beta diversity of the respiratory tract bacteriome
- c) presence/absence and relative abundances of major bacterial taxa in the respiratory bacteriome
- d) percentage of macrolide resistance and/or detection and prevalence of previously undetected antibiotic resistance genes in each of the bacteria mentioned above
- e) resistome of the respiratory tract (abundance of resistance genes relative to its species composition)

Measures of effect

Prevalence of SSHM, percentage of antibiotic resistant isolates, alpha diversity measures

Additional outcome(s)

Measures of the effect of specific doses, routes of administration, frequency and duration of macrolide therapy on the

- a. prevalence and/or bacterial loads of *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Haemophilus influenzae* and *Moraxella catarrhalis* in the respiratory tract.
- b. alpha and beta diversity of the respiratory tract bacteriome.
- c. presence/absence and relative abundances of major bacterial taxa in the respiratory bacteriome
- d. percentage macrolide susceptibility and/or detection and prevalence of antibiotic resistance genes undetected in the baseline samples in each of the bacteria above.
- e. resistome of the respiratory tract (abundance of resistance genes relative to its species composition)
- f. duration of effect on the respiratory resistome and bacteriome.

Measures of effect

Prevalence of SSHM, percentage of antibiotic resistant isolates, alpha diversity measures

Data extraction (selection and coding)

Covidence (www.covidence.org), a web-based software platform for conducting systematic reviews will be used to facilitate the screening and selection of eligible studies. Retrieved records will be imported into ENDNOTE then uploaded to Covidence. The software will conduct deduplication of the records. Three authors (REA, AM and GN) will independently and in duplicate screen titles and abstracts of retrieved papers according to the eligibility criteria outlined above. The full-text articles of any potentially eligible records will be retrieved, and the review authors will independently and in duplicate assess the full-text articles for eligibility. All disagreements will be resolved by consensus or a simple majority vote where necessary. Reasons for exclusion of full-text articles will be recorded and presented in a table. Data extraction will be conducted by REA while AM and GN will review for accuracy. Authors will be contacted for data not reported in articles.

The extracted data will include:

- study characteristics (first author, publication year, study design, country, sample size, sample type, study

aims and objectives),

- exposure/case definition,
- disease under study if any,
- definition of the comparison group,
- fully adjusted outcome measure (prevalence, bacterial loads and percentage antibiotic resistance at baseline and endpoint in the case of specific bacterial isolates and measures of bacterial diversity (alpha and beta diversity), presence and absence of major bacterial taxa, their relative abundances
- confounders adjusted for.

Risk of bias (quality) assessment

Two reviewers will assess the risk of bias of included randomised trials using the Cochrane Risk of Bias (RoB) tool and non-randomised studies using the Newcastle-Ottawa Scale (NOS) criteria. In cases of disagreement, the third reviewer will adjudicate.

Strategy for data synthesis

The findings of this review will be presented as a narrative (descriptive) synthesis. A meta-analysis may not be possible as the research questions are quite broad, and we expect there to be substantial heterogeneity between eligible studies with regards to the outcomes of interest, case and antibiotic exposure definition.

Analysis of subgroups or subsets

If the data permits, the effect of macrolides on the composition of the respiratory bacteriome will be assessed using the following subgrouping:

- Type of macrolides (for example specific types of macrolides may be associated with different effects on the composition of the respiratory bacteriome)
- Macrolide dose
- Macrolide treatment duration
- Underlying disease being treated
- Participants previous exposure to macrolides or other antibiotics
- Effects on the upper and lower respiratory tract
- Community-level effects (presence/absence and relative abundances of bacterial taxa)
- Effect on the prevalence and resistance in *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Haemophilus influenzae* and *Moraxella catarrhalis*
- Age/sex-dependent effects
- Effects on antibiotic resistance to other antibiotic classes including tetracyclines and lincosamides

Contact details for further information

Regina Esinam Abotsi
abtreg001@myuct.ac.za

Organisational affiliation of the review

None

Review team members and their organisational affiliations

Ms Regina Esinam Abotsi. Institute of Infectious Diseases and Molecular Medicine and Department of Molecular and Cell Biology, University of Cape Town. AND Department of Pharmaceutical Microbiology, University of Health and Allied Sciences, Ghana
Miss Gillian O.N Ndhlovu. Department of Molecular and Cell Biology, University of Cape Town
Miss Adelaide Mensah. Department of Molecular and Cell Biology, University of Cape Town AND Department of Pharmaceutics, University of Health and Allied Sciences, Ghana
Professor Mark Nicol. Institute of Infectious Diseases and Molecular Medicine, University of Cape Town South Africa AND School of Biomedical Sciences, University of Western Australia
Dr Felix Dube. Institute of Infectious Diseases and Molecular Medicine and Department of Molecular and Cell Biology, University of Cape Town.

Type and method of review

Intervention, Narrative synthesis, Systematic review

Anticipated or actual start date

15 May 2020

Anticipated completion date

31 December 2021

Funding sources/sponsors

None

Conflicts of interest

None known

Language

English

Country

Australia, Ghana, South Africa

Stage of review

Review Ongoing

Subject index terms status

Subject indexing assigned by CRD

Subject index terms

MeSH headings have not been applied to this record

Date of registration in PROSPERO

05 July 2020

Date of first submission

21 May 2020

Details of any existing review of the same topic by the same authors

No previous reviews on the topic by the same authors.

Stage of review at time of this submission

Stage	Started	Completed
Preliminary searches	Yes	Yes
Piloting of the study selection process	Yes	Yes
Formal screening of search results against eligibility criteria	No	No
Data extraction	No	No
Risk of bias (quality) assessment	No	No
Data analysis	No	No

The record owner confirms that the information they have supplied for this submission is accurate and complete and they understand that deliberate provision of inaccurate information or omission of data may be construed as scientific misconduct.

The record owner confirms that they will update the status of the review when it is completed and will add publication details in due course.

Versions
05 July 2020

Appendix A. Table S 1. Definitions and abbreviations.

Term	Definition
Macrolides	This refers to Erythromycin, Clarithromycin, Azithromycin, Telithromycin, Roxithromycin, Troleandomycin, Dirithromycin, Josamycin
Respiratory bacteriome	This includes the upper and lower respiratory tract bacterial communities, as well as specific respiratory pathogens of interest (<i>Streptococcus pneumoniae</i> , <i>Staphylococcus aureus</i> , <i>Haemophilus influenzae</i> and <i>Moraxella catarrhalis</i>), collected from the following samples: nasopharyngeal, nasal, throat swabs, oropharyngeal swabs, lung aspirates sputum and broncho-alveolar lavage samples.
Infants	any child below one year
Children	any child from one to 12 years
Adolescent	Individuals from 13 to 18 years
Adults	an individual above 18 years
Setting	hospital, clinics, nursing homes, community
Antibiotic resistance	resistance in specific respiratory pathogens determined by disk diffusion, agar dilution or broth dilution, molecular detection of antibiotic resistance genes and characterisation of resistance genes at the community level (resistome).
SP	<i>Streptococcus pneumoniae</i>
SA	<i>Staphylococcus aureus</i>
HI	<i>Haemophilus influenzae</i>
MC	<i>Moraxella catarrhalis</i>

Appendix A. Table S 2. Final search.

Search terms	Filters	Comments	Hits
Pubmed			
((Erythromycin [Mesh] OR Azithromycin OR erythromycin OR clarithromycin OR Telithromycin OR Roxithromycin OR troleandomycin) AND (Respiratory System [Mesh] OR Sputum [Mesh] OR Respiratory OR sputum OR nasopharyngeal OR Broncho-alveolar OR nasal OR lung OR nares OR oropharyngeal OR bronchial OR pharyngeal OR throat) AND (<i>Streptococcus pneumoniae</i> [Mesh] OR <i>Staphylococcus aureus</i> [Mesh] OR Microbiota [Mesh] OR <i>Haemophilus influenzae</i> [Mesh] OR <i>Moraxella catarrhalis</i> [Mesh] OR Metagenome [Mesh] OR Metagenomics [Mesh] OR <i>Streptococcus pneumoniae</i> OR <i>Staphylococcus aureus</i> OR <i>Haemophilus influenzae</i> OR <i>Moraxella catarrhalis</i> OR microbiota OR flora OR microflora OR microbiota OR microbiome OR microbiomics OR metagenome OR metagenomics OR resistome))) NOT ((Animals[MeSH Terms]) NOT Humans[MeSH Terms])	none		2112
Web of Science			
(Azithromycin OR erythromycin OR clarithromycin OR Telithromycin OR Roxithromycin OR troleandomycin) AND (Respiratory OR sputum OR nasopharyngeal OR Broncho-alveolar OR nasal OR lung OR nares OR oropharyngeal OR bronchial OR pharyngeal OR throat) AND (<i>Streptococcus pneumoniae</i> OR <i>Staphylococcus aureus</i> OR <i>Haemophilus influenzae</i> OR <i>Moraxella catarrhalis</i> OR microbiota OR flora OR microflora OR microbiota OR microbiome OR microbiomics OR metagenome OR metagenomics OR resistome) NOT (Animals NOT Humans)	Exclude Medline	All databases , Medline = 3752 Web of Science core collection = 3599 (382) Biological abstract = 2613(223) SciELO citation Index = 51(12) KCI-Korean Journal Database = 29(17)	538

		Russian Science Citation Index = 22 (16) Zoological record = 3 (0)	
EBSCOhost			
(Azithromycin OR erythromycin OR clarithromycin OR Telithromycin OR Roxithromycin OR troleandomycin) AND (Respiratory OR sputum OR nasopharyngeal OR Broncho-alveolar OR nasal OR lung OR nares OR oropharyngeal OR bronchial OR pharyngeal OR throat) AND (<i>Streptococcus pneumoniae</i> OR <i>Staphylococcus aureus</i> OR <i>Haemophilus influenzae</i> OR <i>Moraxella catarrhalis</i> OR microbiota OR flora OR microflora OR microbiome OR microbiomics OR metagenome OR metagenomics OR resistome) NOT (Animals NOT Humans)	NONE	Resource: Research Databases was selected Academic Search Premier = 749 African Wide information = 161 CINAHL = 208 Health Source information = 76	1037 (1118 including duplicates)
SCOPUS			
(TITLE-ABS-KEY ((azithromycin OR erythromycin OR clarithromycin OR telithromycin OR roxithromycin OR troleandomycin) AND (respiratory OR sputum OR nasopharyngeal OR broncho-alveolar OR nasal OR lung OR nares OR oropharyngeal OR bronchial OR pharyngeal OR throat) AND ((<i>Streptococcus</i> AND <i>pneumoniae</i>) OR (<i>Staphylococcus</i> AND <i>aureus</i>) OR (<i>Haemophilus</i> AND <i>influenzae</i>) OR (<i>Moraxella</i> AND <i>catarrhalis</i>) OR microbiota OR flora OR microflora OR microbiota OR microbiome OR microbiomics OR metagenome OR metagenomics OR resistome)) not INDEX (medline)) AND NOT (TITLE-ABS-KEY (animals AND NOT humans))	Medline excluded		2034
The Cochrane library			
(Azithromycin OR erythromycin OR clarithromycin OR Telithromycin OR Roxithromycin OR troleandomycin) AND (Respiratory OR sputum OR nasopharyngeal OR Broncho-alveolar OR nasal OR lung OR nares OR oropharyngeal OR bronchial OR pharyngeal OR throat) AND (<i>Streptococcus pneumoniae</i> OR <i>Staphylococcus aureus</i> OR <i>Haemophilus influenzae</i> OR <i>Moraxella catarrhalis</i> OR microbiota OR flora OR microflora OR microbiota OR microbiome OR microbiomics OR metagenome OR metagenomics OR resistome)			230
Total number of records	6032		

Search was conducted with an experienced librarian on 25th January 2022

APPENDIX B: Chapter Three Supplementary Materials

Appendix B. Chapter Three Supplementary Materials

Appendix B. Table S 2. Semi-quantitative bacterial load distribution of isolates

Nasopharyngeal swabs									
Agar plate quadrants with growth	<i>Streptococcus pneumoniae</i>			<i>Staphylococcus aureus</i>			<i>Haemophilus influenzae</i>		
	CLD+ (n = 154)	CLD– (n =19)	<i>p</i>	CLD+ (n = 77)	Non-CLD– (n = 9)	<i>p</i>	CLD+ (n = 40)	CLD– (n = 4)	<i>p</i>
1	38 (24.7%)	10 (52.6%)	0.288	28 (36.4%)	2 (22.2%)	1	17 (42.5%)	1 (25%)	1
2	98 (63.6%)	8 (42.1%)		43 (55.8%)	6 (66.7%)		21 (52.5%)	3 (75%)	
3	17 (11.0%)	1 (5.3%)		4 (5.2%)	1 (11.1%)		2 (5%)	0 (0%)	
4	1 (0.6%)	0 (0%)		2 (2.6%)	0 (0%)		0 (0%)	0 (0%)	
Sputa									
Agar plate quadrants with growth	<i>Streptococcus pneumoniae</i>			<i>Staphylococcus aureus</i>			<i>Haemophilus influenzae</i>		
	CLD+ (n = 83)	CLD– (n = 17)	<i>p</i>	CLD+ (n = 93)	CLD– (n = 21)	<i>p</i>	CLD+ (n = 12)	non-CLD– (n = 2)	<i>p</i>
1	0 (0%)	0 (0%)	0.067	46 (49.5%)	10 (47.6%)	0.790	3 (25%)	0 (0%)	1
2	54 (65.1%)	11 (64.7%)		42 (45.2%)	10 (47.6%)		8 (66.7%)	2 (100%)	
3	25 (30.1%)	2 (11.8%)		5 (5.4%)	0 (0%)		1 (8.3%)	0 (0%)	
4	4 (4.8%)	4 (23.5%)		0 (0%)	1 (4.8%)		0 (0%)	0 (0%)	

1, 2, 3, 4 = growth in one, two, three and all quadrants on the agar plate. Proportions were compared using Fishers exact test and corrected for multiple testing using Bonferroni method. MC and GNB values too small for comparison.

Appendix B. Table S 3. Gram-negative bacilli other than *H. influenzae* isolated from respiratory samples

Nasopharyngeal swabs			Sputum		
Bacteria name	Number of isolates		Bacteria name	Number of isolates	
	CLD+* (n = 17)	CLD- ** (n = 1)		CLD+ (n = 7)	CLD- *** (n = 5)
§unidentified bacillus	1	1	§unidentified bacillus	2	2
<i>Proteus mirabilis</i>	4	0	<i>Proteus mirabilis</i>	1	0
<i>Enterobacter cloacae</i>	4	0	<i>Pantoea (Enterobacter) agglomerans</i>	2	1
<i>Acinetobacter ursingii</i>	2	0	<i>Acinetobacter species</i>	1	0
<i>Pseudomonas syringae</i> complex	1	0	<i>Psychrobacter faecalis</i>	1	0
<i>Serratia marcescens</i>	2	0	<i>Siccibacter turicensis</i>	0	2
<i>Morganella morganii</i>	1	0			
<i>Leclercia decarboxylata</i>	1	0			
<i>Raoultella planticola</i>	1	0			

*No Gram-negative bacilli other than *H. influenzae* was isolated from NP samples from participants from Malawi. *20 isolates from 19 unique participants, 3 isolates failed to grow. **non-CLD NP results: 2 isolates from 2 unique participants, one isolate failed to grow, and the other was a coliform bacillus. *** 6 isolates from 6 unique participants but 1 failed to grow. §isolate identity not in MALDI-TOF MS registry.

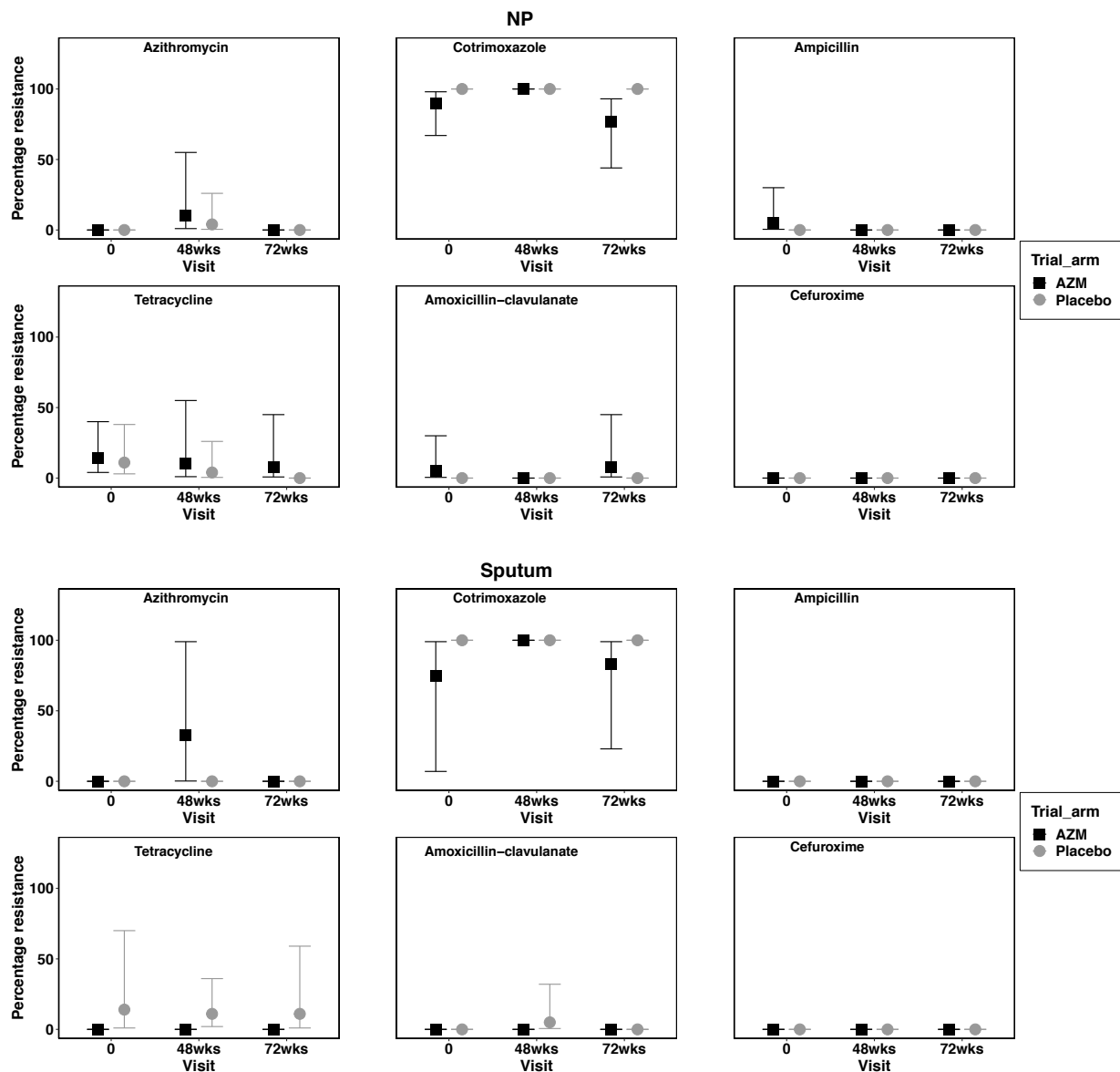
Appendix B. Table S 4. Co-carriage of bacteria in nasopharyngeal swabs and sputa

	Nasopharyngeal swabs (n = 410)								
	<i>S. aureus</i>			<i>H. influenzae</i>			<i>M. catarrhalis</i>		
	*Expected	*Observed	OR (95% CI)	Expected	Observed	OR (95% CI)	Expected	Observed	OR (95% CI)
<i>S. pneumoniae</i>	36 (8.9%)	32 (7.8%)	0.8 (0.5 – 1.2)	19 (4.5%)	37 (9.0%)	9.5 (4.3 – 24.2)	22 (5.2%)	40 (9.8%)	5.4 (2.7 – 11.6)
<i>S. aureus</i>				9 (2.3%)	5 (1.2%)	0.5 (0.2 – 1.1)	11 (2.6%)	6 (1.5%)	0.4 (0.2 – 1.0)
<i>H. influenzae</i>							5 (1.3%)	16 (3.9%)	6.4 (3.0 – 13.7)
Sputa (n = 400)									
<i>S. pneumoniae</i>	29 (7.1%)	38 (9.5%)	1.6 (1.0 – 2.6)	4 (0.8%)	5 (1.3%)	2.1 (0.6 – 6.7)	9 (2.1%)	18 (4.5%)	5.0 (2.3 – 11.0)
<i>S. aureus</i>				4 (1%)	4 (1%)	1.2 (0.3 – 3.7)	10 (2.4%)	11 (2.8%)	1.3 (0.6 – 2.8)
<i>H. influenzae</i>							1 (0.3%)	6 (1.5%)	9.3 (2.8 – 29.0)

*Chi square test used to generate the expected and observed frequencies. Associations were adjusted for age and site using logistic regression. ¹Bold text indicates statistical significance: *p*-value < 0.05

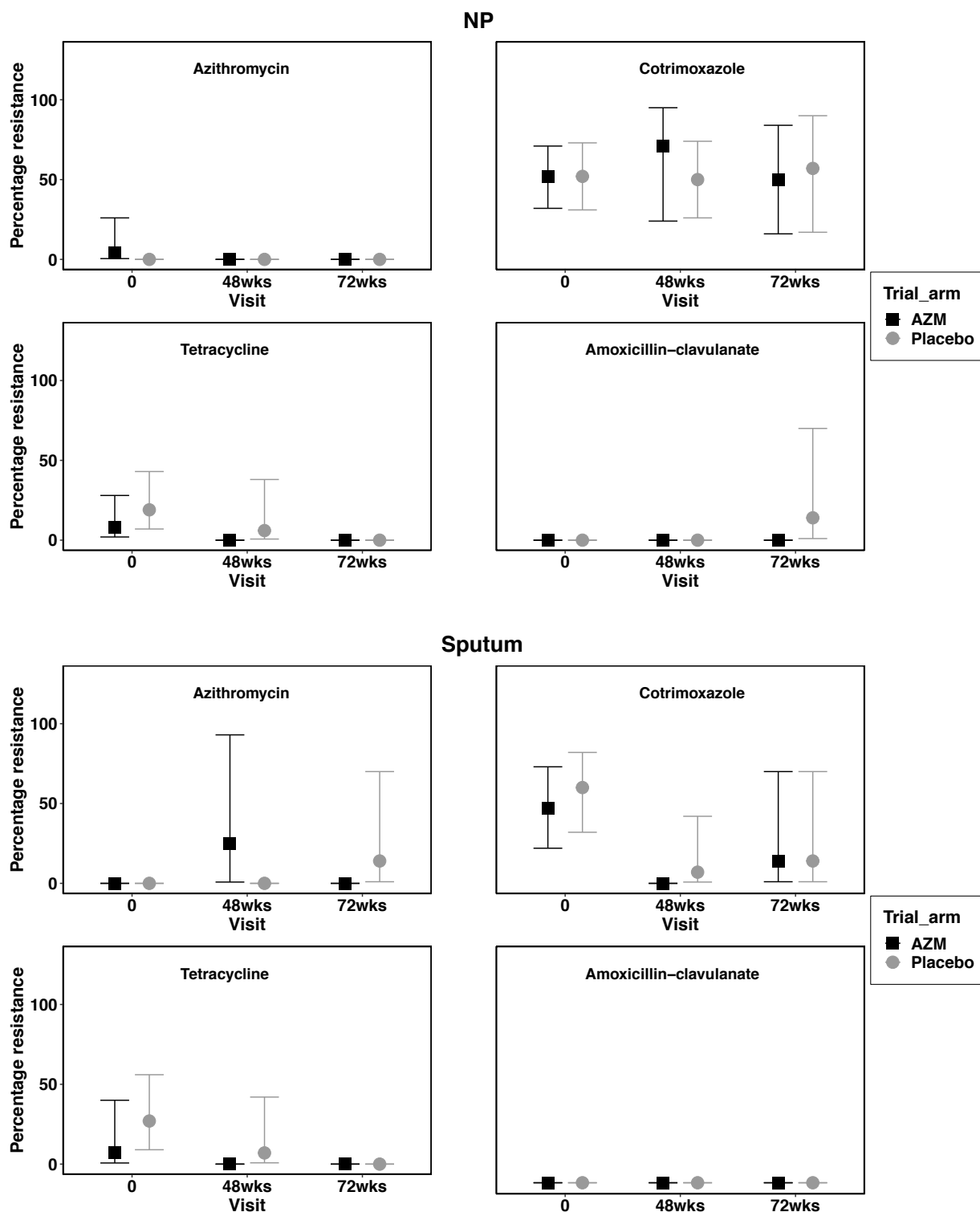
APPENDIX C: Chapter Four Supplementary Materials

Appendix C. Chapter Four Supplementary Materials



Appendix C. Figure S 1. Antibiotic resistance profiles of *Haemophilus influenzae* isolated from the nasopharyngeal (NP) and sputum samples at baseline, 48 weeks, and 72 weeks.

The x- and y-axes are sampling timepoints and percentage resistance respectively. Number of isolates from NP at each timepoint for azithromycin vs placebo were – baseline (21 vs 18), 48 weeks (10 vs 24), 72 weeks (13 vs 8). In the sputum, baseline (4 vs 7), 48 weeks (3 vs 19), 72 weeks (6 vs 9).



Appendix C. Figure S 2. Antibiotic resistance profiles of *Moraxella catarrhalis* isolated from the nasopharyngeal (NP) and sputum samples at baseline, 48 weeks, and 72 weeks.

The x- and y-axes are sampling timepoints and percentage resistance respectively. Number of isolates from NP at each timepoint for azithromycin vs placebo were– baseline (25 vs 21), 48 weeks (7 vs 16), 72 weeks (8 vs 7). In the sputum, baseline (15 vs 15), 48 weeks (4 vs 14), 72 weeks (7 vs 7).

Appendix C. Table S 1. Antibiotic resistance of bacteria isolated from the nasopharyngeal swabs collected at baseline, 48 weeks and 72 weeks from participants taking azithromycin or placebo.

Carriage	Baseline			48 weeks			72 weeks		
	Azithromycin (n = 168) ¹	Placebo (n = 171) ¹	<i>p</i> values	Azithromycin (n = 159) ²	Placebo (n = 153) ²	<i>p</i> values	Azithromycin (n = 118) ³	Placebo (n = 105) ³	<i>p</i> values
<i>Streptococcus pneumoniae (SP)</i>									
Azithromycin resistant SP	8/71 (11%)	10/81 (12%)	1	18/29 (62%)	8/63 (13%)	< 0.001	8/37 (22%)	7/37 (19%)	1
Tetracycline resistant SP	13/70 (19%)	14/81 (17%)	0.835	18/29 (60%)	13/63 (21%)	< 0.001	11/37 (30%)	13/37 (35%)	0.804
Cotrimoxazole resistant SP	68/70 (97%)	76/81 (93%)	0.451	28/29 (97%)	62/63 (98%)	0.533	36/37 (97%)	35/37 (95%)	1
⁴ Penicillin non-susceptible SP	26/70 (37%)	27/81 (33%)	0.733	16/29 (55%)	23/63 (37%)	0.114	15/37 (41%)	15/37 (41%)	1
<i>Staphylococcus aureus (SA)</i>									
Azithromycin resistant SA	1/44 (2%)	2/36 (6%)	0.586	31/34 (91%)	1/31 (3%)	< 0.001	9/20 (45%)	0/26 (0%)	< 0.001
Tetracycline resistant SA	8/44 (18%)	4/36 (11%)	0.532	12/34 (35%)	4/31 (13%)	0.046	5/20 (25%)	1/26 (4%)	0.072
Cotrimoxazole resistant SA	30/44 (68%)	26/36 (72%)	0.808	21/34 (62%)	24/31 (77%)	0.192	11/20 (55%)	20/26 (77%)	0.204
Penicillin resistant SA	37/44 (84%)	31/36 (86%)	1	33/34 (97%)	26/31 (84%)	0.095	20/20 (100%)	20/26 (77%)	0.029
Methicillin resistant SA	1/44 (2%)	1/36 (3%)	1	2/34 (6%)	2/31 (6%)	1	0/20 (0%)	0/26 (0%)	1
Clindamycin resistant SA	0/44 (0%)	1/36 (3%)	0.45	27/34 (79%)	1/31 (3%)	< 0.001	9/20 (45%)	0/26 (0%)	< 0.001
<i>Haemophilus influenzae (HI)</i>									
Azithromycin resistant HI	0/21 (0%)	0/18 (0%)	NA	1/10 (10%)	1/24 (4%)	NA	0/13 (0%)	0/8 (0%)	NA
Tetracycline resistant HI	3/21 (14%)	2/18 (11%)	1	1/10 (10%)	1/24 (4%)	0.508	1/13 (8%)	0/9 (0%)	1
Cotrimoxazole resistant HI	19/21 (90%)	18/18 (100%)	0.490	10/10 (100%)	24/24 (100%)	1	10/13 (77%)	9/9 (100%)	0.240
Ampicillin resistant HI	1/21 (5%)	0/18 (0%)	1	0/10 (0%)	0/24 (0%)	1	0/13 (0%)	0/9 (0%)	1
Amoxicillin-clavulanate resistant HI	1/21 (5%)	0/18 (0%)	1	0/10 (0%)	0/24 (0%)	1	1/13 (8%)	0/9 (0%)	1
Cefuroxime resistant HI	0/21 (0%)	0/18 (0%)	NA	0/10 (0%)	0/24 (0%)	NA	0/13 (0%)	0/9 (0%)	NA
<i>Moraxella catarrhalis (MC)</i>									
Azithromycin resistant MC	1/25 (4%)	0/21 (0%)	1	0/7 (0%)	0/16 (0%)	1	0/8 (0%)	0/7 (0%)	1

Tetracycline resistant MC	2/25 (8%)	4/21 (19%)	0.390	0/7 (0%)	1/16 (6%)	1	0/8 (0%)	0/7 (0%)	1
Cotrimoxazole resistant MC	13/25 (52%)	11/21 (52%)	1	5/7 (71%)	8/16 (50%)	0.405	4/8 (50%)	4/7 (57%)	1
Amoxicillin-clavulanate resistant MC	0/25 (0%)	0/21 (0%)	1	0/7 (0%)	0/16 (0%)	1	0/8 (0%)	1/7 (14%)	0.467
All four bacteria									
Macrolide resistant (any)	10 (6%)	12 (7%)	0.826	45 (30%)	10 (7%)	< 0.001	17 (15%)	7 (7%)	0.011
Tetracycline resistant (any)	24 (14%)	23 (14%)	0.742	30 (20%)	18 (13%)	< 0.001	16 (14%)	13(12%)	0.392

¹Samples not collected from five and three participants in AZM and placebo group at baseline respectively. ²Samples not collected from five and seven participants in AZM and placebo group at 48 weeks respectively. ³Samples not collected from 16 and 15 participants in AZM and placebo group at 72 weeks respectively. ⁴Oxacillin used as surrogate for penicillin. Fishers exact test used to compare proportions.

Appendix C. Table S 2. Antibiotic resistance of bacteria isolated from the sputa collected at baseline, 48 weeks and 72 weeks from participants taking azithromycin or placebo.

Carriage	Baseline			48 weeks			72 weeks		
	AZM (n = 166) ¹	Placebo (n = 164) ¹	p values	AZM (n = 148) ²	Placebo (n = 143) ²	p values	AZM (n = 116) ³	Placebo (n = 106) ³	p values
<i>Streptococcus pneumoniae</i> (SP)									
Azithromycin resistant SP	2/41 (5%)	2/36 (6%)	1	5/16 (31%)	2/32 (6%)	0.033	2/20 (10%)	3/26 (12%)	1
Tetracycline resistant SP	4/41 (10%)	6/35 (17%)	0.499	4/16 (25%)	7/32 (22%)	1	3/20 (15%)	8/26 (31%)	0.302
Cotrimoxazole resistant SP	38/41 (93%)	32/36 (89%)	0.699	14/16 (88%)	31/32 (97%)	0.254	20/20 (100%)	25/26 (96%)	1
⁴ Penicillin non-susceptible SP	13/41 (32%)	9/36 (25%)	0.616	6/16 (38%)	11/32 (34%)	1	6/20 (30%)	13/26 (50%)	0.232
<i>Staphylococcus aureus</i> (SA)									
Azithromycin resistant SA	7/49 (14%)	5/45 (11%)	0.762	30/45 (67%)	3/37 (8%)	< 0.001	16/40 (40%)	4/37 (11%)	0.004
Tetracycline resistant SA	12/49 (24%)	5/45 (11%)	0.113	21/45 (47%)	6/37 (16%)	0.005	9/40 (23%)	5/37 (14%)	0.382
Cotrimoxazole resistant SA	33/49 (67%)	28/45 (62%)	0.668	31/45 (69%)	23/37 (62%)	0.641	23/40 (58%)	24/37 (65%)	0.641
Penicillin resistant SA	45/49 (92%)	41/45 (91%)	1	43/45 (96%)	34/37 (92%)	0.654	35/40 (86%)	32/37 (86%)	1
Methicillin resistant SA	1/49 (2%)	1/45 (2%)	1	4/45 (8%)	2/37 (5%)	0.685	1/40 (3%)	1/37 (3%)	1
Clindamycin resistant SA	4/49 (8%)	2/45 (4%)	0.679	25/45 (56%)	2/37 (5%)	< 0.001	13/40 (33%)	3/37 (8%)	0.011
<i>Haemophilus influenzae</i> (HI)									
Azithromycin resistant HI	0/4 (0%)	0/7 (0%)	1	1/3 (33%)	0/19 (0%)	0.136	0/6 (0%)	0/9 (0%)	1
Tetracycline resistant HI	0/4 (0%)	1/7 (14%)	1	0/3 (0%)	2/19 (11%)	1	0/6 (0%)	1/9 (11%)	1
Cotrimoxazole resistant HI	3/4 (75%)	7/7 (100%)	0.364	3/3 (100%)	19/19 (100%)	1	5/6 (83%)	9/9 (100%)	0.4
Ampicillin resistant HI	0/4 (0%)	0/7 (0%)	NA	0/3 (0%)	0/19 (0%)	NA	0/6 (0%)	0/9 (0%)	NA
Amoxicillin-clavulanate resistant HI	0/4 (0%)	0/7 (0%)	NA	0/3 (0%)	1/19 (5%)	1	0/6 (0%)	0/9 (0%)	NA
Cefuroxime resistant HI	0/4 (0%)	0/7 (0%)	NA	0/3 (0%)	0/19 (0%)	NA	0/6 (0%)	0/9 (0%)	NA
<i>Moraxella catarrhalis</i> (MC)									
Azithromycin resistant MC	0/15 (0%)	0/15 (0%)	1	1/4 (25%)	0/14 (0%)	0.222	0/7 (0%)	1/7 (14%)	1
Tetracycline resistant MC	1/15 (7%)	4/15 (27%)	0.330	0/4 (0%)	1/14 (7%)	1	0/7 (0%)	0/7 (0%)	1
Cotrimoxazole resistant MC	7/15 (47%)	9/15 (60%)	0.715	0/4 (0%)	1/14 (7%)	1	1/7% (14%)	1/7 (14%)	1
Amoxicillin-clavulanate resistant MC	0/15 (0%)	0/15 (0%)	NA	0/4 (0%)	0/14 (0%)	NA	0/7 (0%)	0/7 (0%)	NA
All four bacteria									
Macrolide resistant (any)	9 (5%)	7 (4%)	0.795	36 (24%)	5 (3%)	< 0.001	18 (16%)	8 (8%)	0.025
Tetracycline resistant (any)	17 (10%)	16 (10%)	1	25 (17%)	15 (10%)	0.008	12 (10%)	13 (12%)	1

¹Samples not collected from seven and 10 participants in AZM and placebo group at baseline respectively. ²Samples not collected from 16 and 17 participants in AZM and placebo group at 48 weeks respectively. ³Samples not collected from 18 and 14 participants in AZM and placebo group at 72 weeks respectively. ⁴Oxacillin used as surrogate for penicillin. Fishers exact test used to compare proportions.

Appendix C. Table S 3. Nasopharyngeal and sputum bacterial carriage at baseline, 48 weeks and 72 weeks in participants taking azithromycin, by adherence to azithromycin.

NASOPHARYNGEAL SWABS													
	Baseline (n=168) ¹			48 weeks (n=159) ²					72 weeks (n=118) ³				
	Total (n=168)	Adherent (n=124) ¹	Non- adherent (n=44) ¹	Total (n=159)	Adherent (n=122) ²	Non- adherent (n=37)	aOR (95%CI)	P value	Total (n=118)	Adherent (n=94) ³	Non- adherent (n=24) ³	aOR (95%CI)	P value
<i>Streptococcus pneumoniae</i>	74 (44%)	58 (47%)	16 (36%)	29 (18%)	22 (18%)	7 (19%)	1.5 [0.4-5.2]	0.529	41 (35%)	34 (36%)	7 (29%)	1.4 [0.4-4.8]	0.554
<i>Staphylococcus aureus</i>	45 (27%)	33 (27%)	12 (27%)	34 (21%)	20 (16%)	14 (38%)	0.4 [0.1-1.2]	0.609	23 (19%)	19 (20%)	4 (17%)	2.2 [0.5-9.6]	0.293
<i>Haemophilus influenzae</i>	21 (13%)	15 (12%)	6 (14%)	11 (7%)	8 (7%)	3 (8%)	0.8 [0.2-4.0]	0.814	13 (11%)	10 (11%)	3 (13%)	0.9 [0.2-4.5]	0.975
<i>Moraxella catarrhalis</i>	26 (15%)	18 (15%)	8 (18%)	7 (4%)	4 (3%)	3 (8%)	0.6 [0.1-3.4]	0.537	9 (8%)	4 (4%)	5 (21%)	0.2 [0.0-0.98]	0.048
Bacterial carriage (any)	107 (64%)	78 (61%)	29 (63%)	63 (43%)	45 (35%)	18 (49%)	1.1 [0.4-2.6]	0.912	61 (53%)	49 (48%)	12 (39%)	1.7 [0.6-5.1]	0.310
SPUTUM													
	Baseline (n=166) ¹			48 weeks (n=148) ²					72 weeks (n=116) ³				
	Total (n=166) ⁴	Adherent (n=122) ⁴	Non- adherent (n=44) ⁴	Total (n=148) ⁵	Adherent (n=114) ⁵	Non- adherent (n=34) ⁵	aOR (95%CI)	P values	Total (n=117) ⁶	Adherent (n=93) ⁶	Non- adherent (n=24) ⁶	aOR (95%CI)	P values
<i>Streptococcus pneumoniae</i>	43 (26%)	36 (30%)	7 (16%)	17 (11%)	13 (11%)	4 (12%)	0.6 [0.2-2.6]	0.491	20 (17%)	16 (17%)	4 (17%)	0.8 [0.2-3.1]	0.750
<i>Staphylococcus aureus</i>	51 (31%)	40 (33%)	11 (25%)	46 (31%)	29 (25%)	17 (50%)	0.2 [0.1-0.6]	0.004	40 (34%)	32 (34%)	8 (33%)	1.0 [0.3-3.6]	0.972
<i>Haemophilus influenzae</i>	4 (2%)	2 (2%)	2 (5%)	3 (2%)	2 (2%)	1 (3%)	0.6 [0.0-14.2]	0.756	6 (5%)	5 (5%)	1 (4%)	5.4 [0.3-83.7]	0.229
<i>Moraxella catarrhalis</i>	15 (9%)	11 (9%)	4 (9%)	4 (3%)	2 (2%)	2 (6%)	0.3 [0.0-2.6]	0.257	7 (6%)	5 (5%)	2 (8%)	0.9 [0.1-6.7]	0.896
Bacterial carriage (any)	82 (49%)	60 (47%)	22 (48%)	62 (42%)	41 (32%)	21 (57%)	0.2 [0.1-0.6]	0.003	56 (48%)	45 (44%)	11 (35%)	1.3 [0.4-4.0]	0.634

¹Samples not collected from three and two participants in adherent and non-adherent group at baseline respectively. ²Samples not collected from five participants in adherent at 48 weeks. ³Samples not collected from nine and seven participants in adherent and non-adherent group at 72 weeks respectively. ⁴Samples not collected from five and two participants in adherent and non-adherent group at baseline respectively. ⁵Samples not collected from 13 and three participants in adherent and non-adherent groups at 48 weeks respectively. ⁶Samples not collected from 11 and seven participants in adherent and non-adherent group at 72 weeks respectively. aOR = adjusted Odds Ratio. Comparisons of bacterial carriage between trial arms at all visits were conducted using mixed-effects logistic regression models, including a random effect for participants. Models were adjusted for site, sex, age category, HIV viral load at baseline, the season of sampling and visit. A trial arm by time interaction term was included in models to compare trial arms at 48 and 72 weeks, without a trial arm main effect term.

Appendix C. Table S 4. Antibiotic resistance of nasopharyngeal bacterial isolates at baseline, 48 weeks and 72 weeks in participants taking azithromycin, by adherence to azithromycin.

	Baseline (n=168) ¹				48 weeks(n=159) ²				72 weeks (n=118) ³			
	Total (n=168)	Adherent (n=122) ¹	Non- adherent (n=44) ¹	P values	Total (n=159)	Adherent (n=122) ²	Non- adherent (n=37)	P values	Total (n=118)	Adherent (n=94) ³	Non- adherent (n=24) ³	P valu es
<i>Streptococcus pneumoniae (SP)</i>												
Azithromycin resistant SP	8/71 (11%)	6/56 (11%)	2/15 (13%)	0·673	18/29 (62%)	14/22 (64%)	4/7 (57%)	1	8/37 (22%)	7/30 (23%)	1/7 (14%)	1
Tetracycline resistant SP	13/70 (19%)	8/55 (15%)	5/15 (33%)	0·133	18/29 (62%)	14/22 (64%)	4/7 (57%)	1	11/37 (30%)	9/30 (30%)	2/7 (29%)	1
Cotrimoxazole resistant SP	68/70 (97%)	53/55 (96%)	15/15 (100%)	1	28/29 (97%)	21/22 (95%)	7/7 (100%)	1	36/37 (97%)	29/30 (97%)	7/7 (100%)	1
⁴ Penicillin non-susceptible SP	26/70 (37%)	20/55 (36%)	6/15 (40%)	1	16/29 (55%)	12/22 (54%)	4/7 (57%)	1	15/37(41%)	12/30(40%))	3/7 (43%)	1
<i>Staphylococcus aureus (SA)</i>												
Azithromycin resistant SA	1/44 (2%)	1/33 (3%)	0/11 (0%)	1	31/34 (91%)	18/20 (90%)	13/14 (93%)	1	9/20 (45%)	7/17 (41%)	2/3 (67%)	0·566
Tetracycline resistant SA	8/44 (18%)	6/33 (18%)	2/11 (18%)	1	12/34 (35%)	6/20 (30%)	6/14 (43%)	0·487	5/20 (25%)	4/17 (24%)	1/3 (33%)	1
Cotrimoxazole resistant SA	30/44 (68%)	22/33 (67%)	8/11 (72%)	1	21/34 (62%)	12/20 (60%)	9/14 (64%)	1	11/20 (55%)	9/17 (53%)	2/3 (67%)	1
Penicillin resistant SA	37/44 (84%)	28/33 (84%)	9/11 (82%)	1	33/34 (97%)	20/20 (100%)	13/14 (93%)	0·412	20/20 (100%)	17/17 (100%)	3/3 (100%)	1
Methicillin resistant SA	1/44 (2%)	1/33 (3%)	0/11 (0%)	1	2/34 (6%)	1/20 (5%)	1/14 (7%)	1	0/20 (0%)	0/17 (0%)	0/3 (0%)	1
Clindamycin resistant SA	0/44 (0%)	0/33 (0%)	0/11 (0%)	1	27/34 (79%)	17/20 (85%)	10/14 (71%)	0·4099	9/20 (45%)	7/17 (41%)	2/3 (67%)	0·566
<i>Haemophilus influenzae (HI)</i>												
Azithromycin resistant HI	0/21 (0%)	0/15 (0%)	0/6 (0%)	NA	1/10 (10%)	1/7 (14%)	0/3 (0%)	NA	0/13 (0%)	0/10 (0%)	0/3 (0%)	NA
Tetracycline resistant HI	3/21 (14%)	3/15 (20%)	0/6 (0%)	0·526	1/10 (10%)	1/7 (14%)	0/3 (0%)	1	1/13 (8%)	1/10 (10%)	0/3 (0%)	1
Cotrimoxazole resistant HI	19/21 (90%)	14/15 (93%)	5/6 (83%)	0·5	10/10 (100%)	7/7 (100%)	3/3 (100%)	1	10/13 (77%)	7/10 (70%)	3/3 (100%)	0·528
Ampicillin resistant HI	1/21 (5%)	1/15 (67%)	0/6 (0%)	1	0/10 (0%)	0/7 (0%)	0/3 (0%)	1	0/13 (0%)	0/10 (0%)	0/3 (0%)	1
Amoxicillin-clavulanate resistant HI	1/21 (5%)	1/15 (7%)	0/6 (0%)	1	0/10 (0%)	0/7 (0%)	0/3 (0%)	1	1/13 (8%)	1/10 (10%)	0/3 (0%)	1
Cefuroxime resistant HI	0/21 (0%)	0/15 (0%)	0/6 (0%)	NA	0/10 (0%)	0/7 (0%)	0/3 (0%)	NA	0/13 (0%)	1/10 (10%)	0/3 (0%)	NA
<i>Moraxella catarrhalis (MC)</i>												
Azithromycin resistant MC	1/25 (4%)	1/17 (6%)	0/8 (0%)	1	0/7 (0%)	0/4 (0%)	0/3 (0%)	1	0/8 (0%)	0/3(0%)	0/5 (0%)	1
Tetracycline resistant MC	2/25 (8%)	0/17 (0%)	2/8 (25%)	0·093	0/7 (0%)	0/4 (0%)	0/3 (0%)	1	0/8 (0%)	0/3 (0%)	0/5 (0%)	1
Cotrimoxazole resistant MC	13/25 (52%)	7/17 (41%)	6/8 (75%)	0·202	5/7 (71%)	3/4 (75%)	2/3 (67%)	1	4/8 (50%)	2/3 (67%)	2/5 (40%)	1

Amoxicillin-clavulanate resistant MC	0/25 (0%)	0/17 (0%)	0/8 (0%)	1	0/7 (0%)	0/4 (0%)	0/3 (0%)	1	0/8 (0%)	0/3 (0%)	0/5 (0%)	1
All four bacteria												
Macrolide resistant (any)	10 (6%)	8 (6%)	2 (7%)	1	45 (30%)	30 (68%)	15 (83%)	0·348	17 (15%)	14 (32%)	3 (27%)	1
Tetracycline resistant (any)	24 (14%)	17 (22%)	7 (25%)	0·797	30 (20%)	21 (48%)	9 (50%)	1	16 (14%)	13 (30%)	3 (27%)	1

¹Samples not collected from three and two participants in adherent and non-adherent group at baseline respectively. ²Samples not collected from five participants in adherent at 48 weeks. ³Samples not collected from nine and seven participants in adherent and non-adherent group at 72 weeks respectively. ⁴Oxacillin used as surrogate for penicillin. NA is Not applicable. Fishers exact test used to compare proportions.

Appendix C. Table S 5. Sputum carriage and antibiotic resistance at baseline, 48 weeks and 72 weeks in participants taking azithromycin, by adherence to azithromycin.

Carriage	Baseline (n=166) ¹				48 weeks				72 weeks			
	Total (n=166) ¹	Adherent (n=122) ¹	Non-adherent (n=44) ¹	P values	Total (n=148) ²	Adherent (n=114) ²	Non-adherent (n=34) ²	P values	Total (n=117) ³	Adherent (n=93) ³	Non-adherent (n=24) ³	P values
<i>Streptococcus pneumoniae (SP)</i>												
Azithromycin resistant SP	2/41 (5%)	2/34 (6%)	0/7 (0%)	1	5/16 (31%)	4/12 (33%)	1/4 (25%)	1	2/20 (10%)	2/16 (13%)	0/4 (0%)	1
Tetracycline resistant SP	4/41 (10%)	4/34 (12%)	0/7 (0%)	1	4/16 (25%)	3/12 (25%)	1/4 (25%)	1	3/20 (15%)	1/16 (6%)	2/4 (50%)	0·088
Cotrimoxazole resistant SP	38/41 (93%)	31/34 (91%)	7/7 (100%)	1	14/16 (88%)	10/12 (83%)	4/4 (100%)	1	20/20 (100%)	16/16 (100%)	4/4 (100%)	1
⁴ Penicillin non-susceptible SP	13/41 (32%)	12/34 (35%)	1/7 (14%)	0·399	6/16 (38%)	4/12 (33%)	2/4 (50%)	0·604	6/20 (30%)	5/16 (31%)	1/4 (25%)	1
<i>Staphylococcus aureus (SA)</i>												
Azithromycin resistant SA	7/49 (14%)	5/38 (13%)	2/11 (18%)	0·647	30/45 (67%)	17/29 (59%)	13/16 (81%)	0·189	16/40 (40%)	13/32 (41%)	3/8 (38%)	1
Tetracycline resistant SA	12/49 (24%)	9/38 (24%)	3/11 (27%)	1	21/45 (47%)	15/29 (52%)	6/16 (38%)	0·533	9/40 (23%)	8/32 (25%)	1/8 (13%)	0·655
Cotrimoxazole resistant SA	33/49 (67%)	27/38 (71%)	6/11 (54%)	0·466	31/45 (69%)	21/29 (72%)	10/16 (63%)	0·519	23/40 (58%)	18/32 (56%)	5/8 (63%)	1
Penicillin resistant SA	45/49 (92%)	36/38 (95%)	9/11 (81%)	0·214	43/45 (96%)	27/29 (93%)	16/16 (100%)	0·531	35/40 (86%)	27/32 (84%)	8/8 (100%)	0·563
Methicillin resistant SA	1/49 (2%)	1/38 (3%)	0/11 (0%)	1	4/45 (8%)	2/29 (7%)	2/16 (13%)	0·608	1/40 (3%)	1/32 (3%)	0/8 (0%)	1
Clindamycin resistant SA	4/49 (8%)	3/38 (8%)	1/11 (9%)	1	25/45 (56%)	14/29 (48%)	11/16 (69%)	0·224	13/40 (33%)	11/32 (34%)	2/8 (25%)	1
<i>Haemophilus influenzae (HI)</i>												
Azithromycin resistant HI	0/4 (0%)	0/2 (0%)	0/2 (0%)	1	1/3 (33%)	1/2 (50%)	0/1 (0%)	1	0/6 (0%)	0/5 (0%)	0/1 (0%)	1
Tetracycline resistant HI	0/4 (0%)	0/2 (0%)	0/2 (0%)	1	0/3 (0%)	0/2 (0%)	0/1 (0%)	1	0/6 (0%)	0/5 (0%)	0/1 (0%)	1
Cotrimoxazole resistant HI	3/4 (75%)	2/2 (100%)	1/2 (50%)	1	3/3 (100%)	2/2 (100%)	1/1 (100%)	1	5/6 (83%)	4/5 (80%)	1/1 (100%)	1

Ampicillin resistant HI	0/4 (0%)	0/2 (0%)	0/2 (0%)	NA	0/3 (0%)	0/2 (0%)	0/1 (0%)	NA	0/6 (0%)	0/5 (0%)	0/1 (0%)	NA
Amoxicillin-clavulanate resistant HI	0/4 (0%)	0/2 (0%)	0/2 (0%)	1	0/3 (0%)	0/2 (0%)	0/1 (0%)	1	0/6 (0%)	0/5 (0%)	0/1 (0%)	1
Cefuroxime resistant HI	0/4 (0%)	0/2 (0%)	0/2 (0%)	NA	0/3 (0%)	0/2 (0%)	0/1 (0%)	NA	0/6 (0%)	0/5 (0%)	0/1 (0%)	NA
<i>Moraxella catarrhalis</i> (MC)												
Azithromycin resistant MC	0/15 (0%)	0/11 (0%)	0/4 (0%)	1	1/4 (25%)	1/2 (50%)	0/2 (0%)	1	0/7 (0%)	0/5 (0%)	0/2 (0%)	1
Tetracycline resistant MC	1/15 (7%)	0/11 (0%)	1/4 (25%)	0·267	0/4 (0%)	0/2 (0%)	0/2 (0%)	1	0/7 (0%)	0/5 (0%)	0/2 (0%)	1
Cotrimoxazole resistant MC	7/15 (47%)	6/11 (55%)	1/4 (25%)	0·569	0/4 (0%)	0/2 (0%)	0/2 (0%)	1	1/7% (14%)	1/5 (20%)	0/2 (0%)	1
Amoxicillin-clavulanate resistant MC	0/15 (0%)	0/11 (0%)	0/4 (0%)	NA	0/4 (0%)	0/2 (0%)	0/2 (0%)	NA	0/7 (0%)	0/5 (0%)	0/2 (0%)	NA
All four bacteria												
Macrolide resistant (any)	9 (5%)	7 (12%)	2 (9%)	1	36 (24%)	22 (55%)	14 (70%)	0·402	18 (16%)	15 (33%)	3 (27%)	1
Tetracycline resistant (any)	17 (10%)	13 (22%)	4 (18%)	1	25 (17%)	18 (45%)	7 (35%)	0·581	12 (10%)	9 (20%)	3 (27%)	0·686

¹Samples not collected from three and two participants in adherent and non-adherent group at baseline respectively. ²Samples not collected from five participants in adherent at 48 weeks. ³Samples not collected from nine and seven participants in adherent and non-adherent group at 72 weeks respectively. ⁴Oxacillin used as surrogate for penicillin. NA is Not applicable. Fishers exact test used to compare proportions.

Appendix C. Table S 6. Factors associated with carriage of *Streptococcus pneumoniae* isolates at 48 weeks and 72 weeks in trial participants.

NASOPHARYNGEAL SWABS												
Variable	No. observations (n = 312)#	No. isolates (n = 93)*	48 weeks				No. observations (n =223)§	No. isolates (n = 79)ω	72 weeks			
			Univariate analysis		Multivariate analysis				Univariate analysis		Multivariate analysis	
			Adjusted Odds ratio, 95% CI	<i>p</i> value	Adjusted Odds ratio, 95% CI	<i>p</i> value			Adjusted Odds ratio, 95% CI	<i>p</i> value	Adjusted Odds ratio, 95% CI	<i>p</i> value
Site												
Malawi	31% (96)	41% (38)	Reference		Reference		20% (44)	23% (18)	Reference		Reference	
Zimbabwe	69% (216)	59% (55)	0.52 [0.31–0.87]	0.012	0.55 [0.22–1.35]	0.195	80% (179)	77% (61)	0.75 [0.38–1.48]	0.397	1.05 [0.32–3.44]	0.936
Sex												
Female	48% (151)	57% (53)	Reference		Reference		45% (100)	52% (41)	Reference		Reference	
Male	52% (161)	43% (40)	0.61 [0.37–0.99]	0.049	0.52 [0.28–0.96]	0.038	55% (123)	48% (38)	0.64 [0.37–1.12]	0.118	0.62 [0.32–1.18]	0.148
Season of sampling												
May-Oct-Dry	58% (180)	56% (52)	Reference		Reference		37% (82)	35% (28)	Reference		Reference	
Nov-Apr-Rainy	42% (132)	44% (41)	1.11 [0.68–1.81]	0.679	0.97 [0.53–1.75]	0.922	63% (141)	65% (51)	1.09 [0.62–1.95]	0.761	0.91 [0.47–1.75]	0.768

Weight-for-age z-score												
Not underweight	51% (158)	49% (45)	Reference		Reference		47% (104)	51% (40)	Reference		Reference	
underweight	49% (149)	51% (46)	1.12 [0.69–1.83]	0.647	1.56 [0.85–2.91]	0.158	53% (117)	49% (39)	0.8 [0.46–1.39]	0.428	0.92 [0.47–1.79]	0.795
Age at baseline												
6–9 years	9% (28)	10% (9)	Reference		Reference		11% (24)	19% (15)	Reference		Reference	
1012 years	20% (61)	22% (20)	1.03 [0.4–2.76]	0.33	1.12 [0.37–3.49]	0.24	18% (41)	15% (12)	0.25 [0.08–0.7]	0.04	0.28 [0.08–0.86]	0.1
13–16 years	43% (133)	47% (44)	1.04 [0.45–2.6]		1.14 [0.43–3.18]		43% (96)	42% (33)	0.31 [0.12–0.78]		0.31 [0.11–0.84]	
17–19 years	29% (90)	22% (20)	0.6 [0.24–1.58]		0.55 [0.19–1.63]		28% (62)	24% (19)	0.27 [0.1–0.7]		0.29 [0.09–0.84]	
Viral load suppression at baseline												
Suppressed (<1000 copies/mL)	58% (177)	53% (48)	Reference		Reference		60% (130)	57% (45)	Reference		Reference	
Unsuppressed (>1000 copies/mL)	42% (130)	47% (43)	1.33 [0.81–2.18]	0.259	1.51 [0.82–2.78]	0.186	40% (88)	43% (34)	1.19 [0.68–2.08]	0.545	1.5 [0.78–2.91]	0.227
Adherence and Trial arm												
Adherent AZM	39% (122)	24% (22)	Reference		Reference		42% (94)	43% (34)	Reference		Reference	
Adherent Placebo	37% (114)	49% (46)	3.07 [1.71–5.65]	0.0002	4.1 [2.14–8.14]	< 0.0001	38% (94)	38% (30)	0.98 [0.53–1.81]	0.92	1.01 [0.52–1.99]	0.85
Not Adherent AZM	12% (37)	8% (7)	1.06 [0.39–2.63]		0.53 [0.14–1.71]		11% (24)	9% (7)	0.73 [0.26–1.87]		0.6 [0.17–1.91]	
Not Adherent Placebo	13% (39)	19% (18)	3.9 [1.79–8.57]		2.93 [1.15–7.48]		9% (21)	10% (8)	1.09 [0.39–2.85]		0.86 [0.28–2.54]	
MRC dyspnoea score												
< 2	65% (204)	57% (53)	Reference		Reference		71% (159)	65% (51)	Reference		Reference	
2 and above	35% (108)	43% (40)	1.68 [1.01–2.76]	0.043	1.07 [0.46–2.42]	0.877	29% (64)	36% (28)	1.65 [0.9–2.99]	0.101	1.81 [0.69–4.85]	0.227
ART regimen												
ATV/LPV/PI	26% (81)	29% (27)	Reference		Reference		31% (70)	32% (25)	Reference		Reference	
EFV/NVP	74% (230)	71% (65)	0.79 [0.46–1.37]	0.39	0.51 [0.26–1]	0.049	69% (153)	68% (54)	0.98 [0.55–1.79]	0.951	1.06 [0.54–2.08]	0.876
Duration of ART at baseline												
6 months – < 2 years	10% (31)	15% (13)	Reference		Reference		8% (18)	13% (10)	Reference		Reference	

2 – < 4 years	18% (53)	21% (18)	0.71 [0.29–1.79]	0.21	0.84 [0.29–2.41]	0.35	16% (35)	17% (13)	0.47 [0.14–1.49]	0.34	0.71 [0.19–2.62]	0.58
4 – < 6 years	21% (64)	21% (18)	0.54 [0.22–1.34]		0.58 [0.21–1.61]		21% (46)	19% (15)	0.39 [0.12–1.17]		0.44 [0.13–1.5]	
6 years+	51% (154)	44% (38)	0.45 [0.2–1.03]		0.48 [0.19–1.25]		55% (119)	51% (40)	0.41 [0.14–1.1]		0.6 [0.19–1.9]	
Adherence to study drug												
Adherent	76% (236)	73% (68)	Reference				80% (178)	81% (64)	Reference			
Not Adherent	24% (76)	27% (25)	1.21 [0.69–2.1]	0.499			20% (45)	19% (15)	0.89 [0.44–1.76]	0.743		
Trial arm												
Azithromycin	51% (159)	31% (29)	Reference				53% (118)	52% (41)	Reference			
Placebo	49% (153)	69% (64)	3.22 [1.94–5.45]	< 0.0001			47% (105)	48% (38)	1.07 [0.61–1.85]	0.822		
BMI-for-age z-score												
Wasted	20% (62)	16% (15)	Reference				24% (54)	23% (18)	Reference			
Normal	80% (245)	84% (76)	1.41 [0.76–2.75]	0.295			76% (167)	77% (61)	1.15 [0.61–2.23]	0.67		
Height-for-age z-score												
Not stunted	46% (140)	52% (47)	Reference				43% (94)	41% (32)	Reference			
Stunted	54% (167)	48% (44)	0.71 [0.43–1.16]	0.168			57% (127)	59% (47)	1.14 [0.65–2]	0.649		

#Missing values: Weight-for-age *z*-score (5), Viral load suppression at baseline (5), ART regimen (1), Duration of ART at baseline (10), BMI-for-age *z*-score (5), Height-for-age *z*-score (5). *Missing values: Weight-for-age *z*-score (2), Viral load suppression at baseline (2), Duration of ART at baseline (1), BMI-for-age *z*-score (2), Height-for-age *z*-score (2). §Missing values: Weight-for-age *z*-score (2), Viral load suppression at baseline (5), Duration of ART at baseline (5), BMI-for-age *z*-score (2), Height-for-age *z*-score (2). ωMissing values: Duration of ART at baseline (1). ™Missing values: Season of sampling (1), Weight-for-age *z*-score (5), Viral load suppression at baseline (5), ART regimen (1), Duration of ART at baseline (9), BMI-for-age *z*-score (5), Height-for-age *z*-score (5). ØMissing values: Viral load suppression at baseline (1), Duration of ART at baseline (2). ΨMissing values: Season of sampling (2), Weight-for-age *z*-score (1), Viral load suppression at baseline (5), Duration of ART at baseline (6), BMI-for-age *z*-score (1), Height-for-age *z*-score (1). ⚡Missing values: Weight-for-age *z*-score (1), Viral load suppression at baseline (1), Duration of ART at baseline (4), BMI-for-age *z*-score (1), Height-for-age *z*-score (1). BMI-for-age *z*-score and Height-for-age *z*-score were not included in the multivariate analysis because of collinearity. Adherence to study drug and Trial arm were colinear with Adherence and Trial arm hence excluded from multivariate analysis. Statistical model applied is generalised logistic regression.

Appendix C Table S 6. Factors associated with carriage of *Streptococcus pneumoniae* isolates at 48 weeks and 72 weeks in trial participants cont'd.

SPUTUM												
Variable	No. observations (n = 290)§	No. isolates (n = 51)θ	48 weeks				No. observations (n = 224)Ψ	No. isolates (n = 46)ℳ	72 weeks			
			Univariate analysis		Multivariate analysis				Univariate analysis		Multivariate analysis	
			Adjusted Odds ratio, 95% CI	p value	Adjusted Odds ratio, 95% CI	p value			Adjusted Odds ratio, 95% CI	p value	Adjusted Odds ratio, 95% CI	p value
Site												
Malawi	29% (83)	35% (18)	Reference		Reference		25% (56)	30% (14)	Reference		Reference	
Zimbabwe	71% (207)	65% (33)	0.68 [0.36–1.32]	0.247	0.74 [0.24–2.25]	0.593	75% (168)	70% (32)	0.67 [0.33–1.41]	0.28	0.5 [0.12–1.91]	0.315
Sex												
Female	49% (142)	51% (26)	Reference		Reference		46% (104)	48% (22)	Reference		Reference	
Male	51% (148)	49% (25)	0.91 [0.49–1.66]	0.751	0.92 [0.43–1.97]	0.826	54% (120)	52% (24)	0.95 [0.5–1.83]	0.881	0.63 [0.29–1.38]	0.253
Season of sampling												
May-Oct-Dry	58% (167)	57% (29)	Reference		Reference		37% (82)	41% (19)	Reference		Reference	
Nov-Apr-Rainy	42% (122)	43% (22)	1.04 [0.56–1.9]	0.908	0.89 [0.43–1.81]	0.747	63% (140)	59% (27)	0.79 [0.41–1.55]	0.491	0.85 [0.4–1.86]	0.678
Weight-for-age z-score												
Not underweight	53% (150)	51% (26)	Reference		Reference		50% (111)	42% (19)	Reference		Reference	
underweight	47% (135)	49% (25)	1.08 [0.59–1.99]	0.794	1.18 [0.56–2.52]	0.66	50% (112)	58% (26)	1.5 [0.78–2.94]	0.23	1.42 [0.64–3.24]	0.391
Age at baseline												
6–9 years	9% (27)	10% (5)	Reference		Reference		10% (23)	11% (5)	Reference		Reference	
1012 years	19% (56)	16% (8)	0.73 [0.22–2.66]	0.88	0.86 [0.22–3.66]	0.53	20% (44)	11% (5)	0.46 [0.11–1.85]	0.41	0.5 [0.1–2.34]	0.7
13–16 years	43% (125)	47% (24)	1.05 [0.38–3.37]		1.74 [0.56–6.35]		44% (98)	50% (23)	1.12 [0.39–3.68]		0.97 [0.3–3.67]	
17–19 years	28% (82)	27% (14)	0.91 [0.31–3.06]		1.31 [0.38–5.07]		26% (59)	28% (13)	1.04 [0.34–3.62]		0.87 [0.23–3.52]	

Viral load suppression at baseline												
Suppressed (< 1000 copies/mL)	57% (162)	50% (25)	Reference		Reference		58% (127)	49% (22)	Reference		Reference	
Unsuppressed (>1000 copies/mL)	43% (123)	50% (25)	1.4 [0.76–2.59]	0.283	1.55 [0.74–3.26]	0.241	42% (92)	51% (23)	1.6 [0.83–3.11]	0.163	1.63 [0.75–3.57]	0.217
Adherence and Trial arm												
Adherent AZM	39% (114)	25% (13)	Reference		Reference		42% (93)	35% (16)	Reference		Reference	
Adherent Placebo	36% (105)	57% (29)	2.96 [1.47–6.26]	0.012	3.82 [1.76–8.82]	0.002	38% (84)	46% (21)	1.58 [0.77–3.33]	0.61	1.62 [0.72–3.74]	0.56
Not Adherent AZM	11% (34)	8% (4)	1.04 [0.28–3.18]		0.73 [0.14–2.8]		11% (24)	9% (4)	0.95 [0.25–2.94]		0.81 [0.16–3.24]	
Not Adherent Placebo	13% (37)	10% (5)	1.21 [0.37–3.5]		0.77 [0.18–2.77]		10% (23)	11% (5)	1.4 [0.41–4.15]		1.65 [0.43–5.8]	
MRC dyspnoea score												
< 2	66% (192)	59% (30)	Reference		Reference		68% (153)	67% (31)	Reference		Reference	
2 and above	34% (98)	41% (21)	1.47 [0.78–2.73]	0.221	1.73 [0.63–4.69]	0.283	32% (71)	33% (15)	1.06 [0.52–2.1]	0.86	0.5 [0.13–1.7]	0.294
ART regimen												
ATV/LPV/PI	25% (73)	25% (13)	Reference		Reference		30% (68)	30% (14)	Reference		Reference	
EFV/NVP	75% (216)	75% (38)	0.99 [0.5–2.03]	0.967	0.72 [0.32–1.7]	0.446	70% (156)	70% (32)	0.96 [0.48–1.99]	0.906	0.85 [0.38–1.97]	0.697
Duration of ART at baseline												
6 months –2 years	9% (27)	20% (10)	Reference		Reference		9% (19)	14% (6)	Reference		Reference	
2 – < 4 years	17% (49)	14% (7)	0.28 [0.09–0.86]	0.026	0.26 [0.07–0.88]	0.014	16% (34)	14% (6)	0.48 [0.13–1.81]	0.47	0.5 [0.11–2.19]	0.45
4 – < 6 years	22% (61)	27% (13)	0.46 [0.17–1.26]		0.33 [0.1–1.05]		23% (50)	26% (11)	0.61 [0.19–2.07]		0.65 [0.17–2.49]	
6 years+	51% (144)	39% (19)	0.26 [0.1–0.66]		0.17 [0.06–0.51]		53% (115)	45% (19)	0.43 [0.15–1.36]		0.39 [0.11–1.43]	
Adherence to study drug												
Adherent	76% (219)	82% (42)	Reference				79% (177)	80% (37)	Reference			
Not Adherent	24% (71)	18% (9)	0.61 [0.27–1.28]	0.214			21% (47)	20% (9)	0.91 [0.39–1.99]	0.828		

Trial arm							
Azithromycin	51% (148)	33% (17)	Reference		52% (117)	43% (20)	Reference
Placebo	49% (142)	67% (34)	2.43 [1.3–4.67]	0.006	48% (107)	57% (26)	1.56 [0.81–3.03] 0.183
BMI-for-age z-score							
Wasted	20% (56)	24% (12)	Reference		22% (48)	20% (9)	Reference
Normal	80% (229)	76% (39)	0.75 [0.37–1.61]	0.443	78% (175)	80% (36)	1.14 [0.52–2.7] 0.754
Height-for-age z-score							
Not stunted	44% (125)	47% (24)	Reference		42% (94)	49% (22)	Reference
Stunted	56% (160)	53% (27)	0.85 [0.47–1.58]	0.612	58% (129)	51% (23)	0.69 [0.36–1.34] 0.27

#Missing values: Weight-for-age z-score (5), Viral load suppression at baseline (5), ART regimen (1), Duration of ART at baseline (10), BMI-for-age z-score (5), Height-for-age z-score (5). *Missing values: Weight-for-age z-score (2), Viral load suppression at baseline (2), Duration of ART at baseline (1), BMI-for-age z-score (2), Height-for-age z-score (2). §Missing values: Weight-for-age z-score (2), Viral load suppression at baseline (5), Duration of ART at baseline (5), BMI-for-age z-score (2), Height-for-age z-score (2). ωMissing values: Duration of ART at baseline (1). ℑMissing values: Season of sampling (1), Weight-for-age z-score (5), Viral load suppression at baseline (5), ART regimen (1), Duration of ART at baseline (9), BMI-for-age z-score (5), Height-for-age z-score (5). θMissing values: Viral load suppression at baseline (1), Duration of ART at baseline (2). ΨMissing values: Season of sampling (2), Weight-for-age z-score (1), Viral load suppression at baseline (5), Duration of ART at baseline (6), BMI-for-age z-score (1), Height-for-age z-score (1). ℳMissing values: Weight-for-age z-score (1), Viral load suppression at baseline (1), Duration of ART at baseline (4), BMI-for-age z-score (1), Height-for-age z-score (1). BMI-for-age z-score and Height-for-age z-score were not included in the multivariate analysis because of colinearity. Adherence to study drug and Trial arm were colinear with Adherence and Trial arm hence excluded from multivariate analysis. Statistical model applied is generalised logistic regression.

Appendix C. Table S 7. Factors associated with carriage of *Staphylococcus aureus* isolates at 48 weeks and 72 weeks in trial participants.

NASOPHARYNGEAL SWABS												
Variable	No. observations (n = 312)#	No. isolates (n = 65)*	48 weeks				No. observations (n = 223)§	No. isolates (n = 49)ω	72 weeks			
			Univariate analysis		Multivariate analysis				Univariate analysis		Multivariate analysis	
			Odds ratio, 95% CI	<i>p</i> value	Adjusted Odds ratio, 95% CI	<i>p</i> value			Odds ratio, 95% CI	<i>p</i> value	Adjusted Odds ratio, 95% CI	<i>p</i> value
Site												
Malawi	31% (96)	45% (29)	Reference		Reference		20% (44)	20% (10)	Reference		Reference	
Zimbabwe	69% (216)	55% (36)	0.46 [0.26–0.81]	0.007	0.65 [0.24–1.7]	0.38	80% (179)	80% (39)	0.95 [0.44–2.17]	0.893	0.81 [0.19–3.28]	0.77
Sex												
Female	48% (151)	52% (34)	Reference		Reference		45% (100)	45% (22)	Reference		Reference	
Male	52% (161)	48% (31)	0.82 [0.47–1.42]	0.479	0.79 [0.41–1.53]	0.484	55% (123)	55% (27)	1 [0.53–1.9]	0.993	0.97 [0.46–2.06]	0.931
Season of sampling												
May-Oct-Dry	58% (180)	43% (28)	Reference		Reference		37% (82)	31% (15)	Reference		Reference	
Nov-Apr-Rainy	42% (132)	57% (37)	2.11 [1.22–3.7]	0.008	1.71 [0.91–3.21]	0.095	63% (141)	69% (34)	1.42 [0.73–2.87]	0.313	1.16 [0.55–2.51]	0.71
Weight-for-age z-score												
Not underweight	51% (158)	52% (32)	Reference		Reference		47% (104)	51% (25)	Reference		Reference	
underweight	49% (149)	48% (30)	0.99 [0.57–1.74]	0.979	0.89 [0.46–1.73]	0.735	53% (117)	49% (24)	0.82 [0.43–1.54]	0.529	0.69 [0.32–1.49]	0.347
Age at baseline												
6–9 years	9% (28)	8% (5)	Reference		Reference		11% (24)	8% (4)	Reference		Reference	
1012 years	20% (61)	22% (14)	1.37 [0.46–4.66]	0.29	1.18 [0.34–4.44]	0.48	18% (41)	18% (9)	1.41 [0.4–5.75]	0.22	1.52 [0.39–6.72]	0.17
13–16 years	43% (133)	51% (33)	1.52 [0.57–4.8]		1.32 [0.45–4.49]		43% (96)	55% (27)	1.96 [0.67–7.19]		1.79 [0.56–7.01]	
17–19 years	29% (90)	20% (13)	0.78 [0.26–2.63]		0.7 [0.21–2.56]		28% (62)	18% (9)	0.85 [0.25–3.41]		0.66 [0.17–2.93]	
Viral load suppression at baseline												
Suppressed (< 1000 copies/mL)	58% (177)	47% (30)	Reference		Reference		60% (130)	53% (26)	Reference		Reference	

Unsuppressed (>1000 copies/mL)	42% (130)	53% (34)	1.74 [1–3.03]	0.051	1.52 [0.79–2.94]	0.213	40% (88)	47% (23)	1.42 [0.74–2.69]	0.288	2.06 [0.98–4.42]	0.059
Adherence and Trial arm												
Adherent AZM	39% (122)	31% (20)	Reference		Reference		42% (94)	39% (19)	Reference		Reference	
Adherent Placebo	37% (114)	34% (22)	1.22 [0.62–2.39]	0.05	1.13 [0.55–2.35]	0.53	38% (94)	47% (23)	1.49 [0.74–3.01]	0.44	1.61 [0.76–3.48]	0.45
Not Adherent AZM	12% (37)	22% (14)	3.1 [1.36–7.06]		2 [0.74–5.25]		11% (24)	8% (4)	0.79 [0.21–2.39]		0.79 [0.18–2.92]	
Not Adherent Placebo	13% (39)	14% (9)	1.53 [0.61–3.64]		0.96 [0.3–2.78]		9% (21)	6% (3)	0.66 [0.14–2.21]		0.66 [0.13–2.48]	
MRC dyspnoea score												
< 2	65% (204)	55% (36)	Reference		Reference		71% (159)	71% (35)	Reference		Reference	
2 and above	35% (108)	45% (29)	1.71 [0.98–2.99]	0.058	0.99 [0.38–2.48]	0.989	29% (64)	29% (14)	0.99 [0.48–1.97]	0.982	0.8 [0.24–2.47]	0.71
ART regimen												
ATV/LPV/PI	26% (81)	23% (15)	Reference		Reference		31% (70)	33% (16)	Reference		Reference	
EFV/NVP	74% (230)	77% (50)	1.22 [0.66–2.39]	0.54	1.16 [0.55–2.57]	0.702	69% (153)	67% (33)	0.93 [0.48–1.86]	0.829	0.85 [0.4–1.82]	0.667
Duration of ART at baseline												
6 months –2 years	10% (31)	5% (3)	Reference		Reference		8% (18)	6% (3)	Reference		Reference	
2 – < 4 years	18% (53)	21% (13)	3.03 [0.88–14.12]	0.38	4.96 [1.16–34.68]	0.25	16% (35)	15% (7)	1.25 [0.3–6.45]	0.93	1.59 [0.33–9.21]	0.93
4 – < 6 years	21% (64)	25% (15)	2.86 [0.85–13.1]		4.37 [1.07–29.89]		21% (46)	23% (11)	1.57 [0.42–7.68]		1.67 [0.39–8.88]	
6 years+	51% (154)	49% (30)	2.26 [0.74–9.88]		3.5 [0.9–23.23]		55% (119)	55% (26)	1.4 [0.42–6.36]		1.56 [0.4–7.87]	
Adherence to study drug												
Adherent	76% (236)	65% (42)	Reference				80% (178)	86% (42)	Reference			
Not Adherent	24% (76)	35% (23)	2 [1.1–3.61]	0.021			20% (45)	14% (7)	0.6 [0.23–1.36]	0.248		
Trial arm												
Azithromycin	51% (159)	52% (34)	Reference				53% (118)	47% (23)	Reference			
Placebo	49% (153)	48% (31)	0.93 [0.54–1.61]	0.807			47% (105)	53% (26)	1.36 [0.72–2.58]	0.344		
BMI-for-age z-score												
Wasted	20% (62)	21% (13)	Reference				24% (54)	27% (13)	Reference			

Normal	80% (245)	79% (49)	0.94 [0.48–1.93]	0.865	76% (167)	73% (36)	0.87 [0.43–1.84]	0.699
Height-for-age z-score								
Not stunted	46% (140)	48% (30)	Reference		43% (94)	45% (22)	Reference	
Stunted	54% (167)	52% (32)	0.87 [0.5–1.52]	0.622	57% (127)	55% (27)	0.88 [0.47–1.69]	0.704

#Missing values: Weight-for-age *z*-score (5), Viral load suppression at baseline (5), ART regimen (1), Duration of ART at baseline (10), BMI-for-age *z*-score (5), Height-for-age *z*-score (5). *Missing values: Weight-for-age *z*-score (3), Viral load suppression at baseline (1), Duration of ART at baseline (4), BMI-for-age *z*-score (3), Height-for-age *z*-score (3). §Missing values: Weight-for-age *z*-score (2), Viral load suppression at baseline (5), Duration of ART at baseline (5), BMI-for-age *z*-score (2), Height-for-age *z*-score (2). ωMissing values: Duration of ART at baseline (2). ™Missing values: Season of sampling (1), Weight-for-age *z*-score (5), Viral load suppression at baseline (5), ART regimen (1), Duration of ART at baseline (9), BMI-for-age *z*-score (5), Height-for-age *z*-score (5). θMissing values: Weight-for-age *z*-score (1), Viral load suppression at baseline (1), Duration of ART at baseline (2), BMI-for-age *z*-score (1), Height-for-age *z*-score (1). ΨMissing values: Season of sampling (2), Weight-for-age *z*-score (1), Viral load suppression at baseline (5), Duration of ART at baseline (6), BMI-for-age *z*-score (1), Height-for-age *z*-score (1). ∕Missing values: Season of sampling (1), Viral load suppression at baseline (2), Duration of ART at baseline (2). BMI-for-age *z*-score and Height-for-age *z*-score were not included in the multivariate analysis because of colinearity. Adherence to study drug and Trial arm were colinear with Adherence and Trial arm hence excluded from multivariate analysis. Statistical model applied is generalised logistic regression.

Appendix C Table S 7. Factors associated with carriage of *Staphylococcus aureus* isolates at 48 weeks and 72 weeks in trial participants cont’d.

SPUTUM												
Variable	No. observations (n = 290)ϑ	No. isolates (n = 83)ϑ	48 weeks				No. observations (n = 224)Ψ	No. isolates (n = 77)ϑ	72 weeks			
			Univariate analysis		Multivariate analysis				Univariate analysis		Multivariate analysis	
			Odds ratio, 95% CI	p value	Adjusted Odds ratio, 95% CI	p value			Odds ratio, 95% CI	p value	Adjusted Odds ratio, 95% CI	p value
Site												
Malawi	29% (83)	32% (27)	Reference		Reference		25% (56)	25% (19)	Reference		Reference	
Zimbabwe	71% (207)	67% (56)	0.77 [0.44–1.35]	0.352	1.24 [0.49–3.15]	0.652	75% (168)	75% (58)	1.03 [0.55–1.97]	0.935	0.66 [0.21–2]	0.467
Sex												
Female	49% (142)	46% (38)	Reference		Reference		46% (104)	42% (32)	Reference		Reference	
Male	51% (148)	54% (45)	1.2 [0.72–2]	0.493	1.18 [0.64–2.18]	0.593	54% (120)	58% (45)	1.35 [0.78–2.37]	0.291	1.3 [0.68–2.5]	0.424
Season of sampling												
May-Oct-Dry	58% (167)	64% (53)	Reference		Reference		37% (82)	36% (27)	Reference		Reference	
Nov-Apr-Rainy	42% (122)	36% (30)	0.69 [0.41–1.17]	0.172	0.59 [0.32–1.06]	0.083	63% (140)	64% (49)	1.11 [0.63–1.98]	0.729	1.1 [0.58–2.11]	0.777
Weight-for-age z-score												
Not underweight	53% (150)	50% (41)	Reference		Reference		50% (111)	48% (37)	Reference		Reference	
underweight	47% (135)	50% (41)	1.16 [0.69–1.94]	0.572	0.92 [0.5–1.68]	0.784	50% (112)	52% (40)	1.11 [0.64–1.93]	0.709	0.81 [0.41–1.59]	0.545
Age at baseline												
6–9 years	9% (27)	11% (9)	Reference		Reference		10% (23)	5% (4)	Reference		Reference	
10–12 years	19% (56)	16% (13)	0.6 [0.22–1.7]	0.68	0.62 [0.2–1.96]	0.73	20% (44)	21% (16)	2.71 [0.84–10.61]	0.38	2.6 [0.72–11.18]	0.56
13–16 years	43% (125)	42% (35)	0.78 [0.33–1.96]		0.8 [0.3–2.22]		44% (98)	45% (35)	2.64 [0.91–9.65]		2.19 [0.69–8.58]	
17–19 years	28% (82)	31% (26)	0.93 [0.37–2.42]		1.02 [0.37–2.94]		26% (59)	29% (22)	2.82 [0.92–10.7]		2.37 [0.69–9.74]	
Viral load suppression at baseline												

Suppressed (<1000 copies/mL)	57% (162)	45% (37)	Reference		Reference		58% (127)	57% (43)	Reference		Reference	
Unsuppressed (>1000 copies/mL)	43% (123)	55% (45)	1.95 [1.16–3.29]	0.012	1.94 [1.07–3.57]	0.031	42% (92)	43% (32)	1.04 [0.59–1.83]	0.887	1.2 [0.62–2.3]	0.59
Adherence and Trial arm												
Adherent AZM	39% (114)	35% (29)	Reference		Reference		42% (93)	42% (32)	Reference		Reference	
Adherent Placebo	36% (105)	29% (24)	0.87 [0.46–1.61]	0.018	0.86 [0.44–1.68]	0.02	38% (84)	34% (26)	0.85 [0.45–1.6]	0.52	0.75 [0.38–1.49]	0.33
Not Adherent AZM	11% (34)	20% (17)	2.93 [1.33–6.53]		3.49 [1.37–9.15]		11% (24)	10% (8)	0.95 [0.35–2.42]		0.94 [0.3–2.77]	
Not Adherent Placebo	13% (37)	16% (13)	1.59 [0.7–3.49]		2 [0.77–5.16]		10% (23)	14% (11)	1.75 [0.69–4.43]		2.1 [0.73–6.17]	
MRC dyspnoea score												
< 2	66% (192)	60% (50)	Reference		Reference		68% (153)	74% (57)	Reference		Reference	
2 and above	34% (98)	40% (33)	1.44 [0.85–2.44]	0.175	1.7 [0.74–3.93]	0.208	32% (71)	26% (20)	0.66 [0.35–1.21]	0.184	0.41 [0.14–1.14]	0.099
ART regimen												
ATV/LPV/PI	25% (73)	24% (20)	Reference		Reference		30% (68)	27% (21)	Reference		Reference	
EFV/NVP	75% (216)	76% (63)	1.09 [0.61–2.01]	0.773	0.95 [0.48–1.91]	0.887	70% (156)	73% (56)	1.25 [0.69–2.34]	0.468	1.31 [0.66–2.65]	0.45
Duration of ART at baseline												
6 months –2 years	9% (27)	6% (5)	Reference		Reference		9% (19)	7% (5)	Reference		Reference	
2 – < 4 years	17% (49)	22% (18)	2.55 [0.87–8.67]	0.4	3.28 [1–12.35]	0.17	16% (34)	21% (16)	2.49 [0.76–9.13]	0.21	2.19 [0.59–9.04]	0.44
4 – < 6 years	22% (61)	22% (18)	1.84 [0.64–6.17]		2.15 [0.66–8.07]		23% (50)	17% (13)	0.98 [0.31–3.52]		0.98 [0.28–3.78]	
6 years+	51% (144)	49% (40)	1.69 [0.64–5.32]		1.6 [0.53–5.64]		53% (115)	55% (41)	1.55 [0.55–5.08]		1.42 [0.44–5.12]	
Adherence to study drug												
Adherent	76% (219)	64% (53)	Reference				79% (177)	75% (58)	Reference			
Not Adherent	24% (71)	36% (30)	2.29 [1.3–4.03]	0.004			21% (47)	25% (19)	1.39 [0.71–2.69]	0.327		
Trial arm												
Azithromycin	51% (148)	55% (46)	Reference				52% (117)	52% (40)	Reference			
Placebo	49% (142)	45% (37)	0.78 [0.47–1.3]	0.344			48% (107)	48% (37)	1.02 [0.58–1.77]	0.951		
BMI-for-age z-score												

Wasted	20% (56)	23% (19)	Reference		22% (48)	18% (14)	Reference	
Normal	80% (229)	77% (63)	0.74 [0.4–1.4]	0.343	78% (175)	82% (63)	1.37 [0.69–2.81]	0.379
Height-for-age z-score								
Not stunted	44% (125)	51% (42)	Reference		42% (94)	45% (35)	Reference	
Stunted	56% (160)	49% (40)	0.66 [0.39–1.1]	0.113	58% (129)	55% (42)	0.81 [0.47–1.42]	0.469

#Missing values: Weight-for-age z-score (5), Viral load suppression at baseline (5), ART regimen (1), Duration of ART at baseline (10), BMI-for-age z-score (5), Height-for-age z-score (5). *Missing values: Weight-for-age z-score (3), Viral load suppression at baseline (1), Duration of ART at baseline (4), BMI-for-age z-score (3), Height-for-age z-score (3). §Missing values: Weight-for-age z-score (2), Viral load suppression at baseline (5), Duration of ART at baseline (5), BMI-for-age z-score (2), Height-for-age z-score (2). ωMissing values: Duration of ART at baseline (2). ™Missing values: Season of sampling (1), Weight-for-age z-score (5), Viral load suppression at baseline (5), ART regimen (1), Duration of ART at baseline (9), BMI-for-age z-score (5), Height-for-age z-score (5). θMissing values: Weight-for-age z-score (1), Viral load suppression at baseline (1), Duration of ART at baseline (2), BMI-for-age z-score (1), Height-for-age z-score (1). ΨMissing values: Season of sampling (2), Weight-for-age z-score (1), Viral load suppression at baseline (5), Duration of ART at baseline (6), BMI-for-age z-score (1), Height-for-age z-score (1). ∕Missing values: Season of sampling (1), Viral load suppression at baseline (2), Duration of ART at baseline (2). BMI-for-age z-score and Height-for-age z-score were not included in the multivariate analysis because of collinearity. Adherence to study drug and Trial arm were colinear with Adherence and Trial arm hence excluded from multivariate analysis. Statistical model applied is generalised logistic regression.

Appendix C. Table S 8. Factors associated with carriage of azithromycin resistant *Streptococcus pneumoniae* isolates at 48 weeks and 72 weeks in trial participants.

NASOPHARYNGEAL SWABS													
Variable	No. observations (n=312) #	No. resistant isolates (n=26) *	48 weeks				No. observations (n=223) §	No. resistant isolates (n=15) ω	72 weeks				
			Univariate analysis		Multivariate analysis				Univariate analysis		Multivariate analysis		
			Adjusted Odds ratio, 95% CI	<i>p</i> value	Adjusted Odds ratio, 95% CI	<i>p</i> value			Adjusted Odds ratio, 95% CI	<i>p</i> value	Adjusted Odds ratio, 95% CI	<i>p</i> value	
Site													
Malawi	31% (96)	54% (14)	Reference		Reference		20% (44)	40% (6)	Reference		Reference		
Zimbabwe	69% (216)	46% (12)	0.49 [0.19–1.23]	0.128	0.12 [0.01–1.44]	0.115	80% (179)	60% (9)	0.38 [0.11–1.33]	0.121	0.06 [0–1.26]	0.091	
Sex													
Female	48% (151)	73% (19)	Reference		Reference		45% (100)	53% (8)	Reference		Reference		
Male	52% (161)	27% (7)	0.37 [0.13–0.96]	0.048	0.19 [0.02–1.06]	0.073	55% (123)	47% (7)	0.91 [0.28–2.84]	0.863	1.46 [0.29–8.13]	0.646	
Season of sampling													
May-Oct-Dry	58% (180)	54% (14)	Reference		Reference		37% (82)	33% (5)	Reference		Reference		
Nov-Apr-Rainy	42% (132)	46% (12)	1.16 [0.46–2.9]	0.745	1.48 [0.27–8.98]	0.656	63% (141)	67% (10)	1.19 [0.37–4.23]	0.776	0.47 [0.06–3.14]	0.446	
Weight-for-age <i>z</i> -score													
Not underweight	51% (158)	36% (9)	Reference		Reference		47% (104)	47% (7)	Reference		Reference		
underweight	49% (149)	64% (16)	2.07 [0.81–5.55]	0.133	2.43 [0.37–19.71]	0.371	53% (117)	53% (8)	1.1 [0.35–3.53]	0.863	0.78 [0.11–5.31]	0.795	
Age at baseline													
6–9 years	9% (28)	0% (0)	Reference		Reference		11% (24)	13% (2)	Reference		Reference		
10–12 years	20% (61)	27% (7)	22908745.07 [0–3.7E+85]	NA	68564812.43 [0–NA]	0.69	18% (41)	27% (4)	3 [0.47–25.62]	0.29	7.41 [0.49–192.04]	0.23	
13–16 years	43% (133)	62% (16)	24311321.3 [0–NA]		48087742.57 [0–NA]		43% (96)	53% (8)	2.09 [0.44–15.3]		6.74 [0.64–124.62]		
208													

17–19 years	29% (90)	12% (3)	7977152.3 [0–1.28143335973616E+85]		14669776.62 [0–NA]		28% (62)	7% (1)	0.38 [0.02–4.36]		0.6 [0.01–17.84]	
Viral load suppression at baseline												
Suppressed (<1000 copies/mL)	58% (177)	50% (12)	Reference		Reference		60% (130)	60% (9)	Reference		Reference	
Unsuppressed (>1000 copies/mL)	42% (130)	50% (12)	1.2 [0.47–3.08]	0.702	0.82 [0.16–4.14]	0.807	40% (88)	40% (6)	0.85 [0.25–2.65]	0.777	1.22 [0.18–8.73]	0.838
Adherence and Trial arm												
Adherent AZM	39% (122)	54% (14)	Reference		Reference		42% (94)	47% (7)	Reference		Reference	
Adherent Placebo	37% (114)	23% (6)	0.09 [0.02–0.28]	0.0002	0.07 [0.01–0.41]	0.01	38% (94)	40% (6)	0.86 [0.24–2.97]	0.89	2.24 [0.34–18.33]	0.18
Not Adherent AZM	12% (37)	15% (4)	0.76 [0.13–4.69]		0.02 [0–0.55]		11% (24)	7% (1)	0.55 [0.03–4.03]		0.8 [0.02–17.56]	
Not Adherent Placebo	13% (39)	8% (2)	0.07 [0.01–0.33]		0.01 [0–0.09]		9% (21)	6% (1)	0.47 [0.02–3.35]		0.03 [0–0.71]	
MRC dyspnoea score												
< 2	65% (204)	50% (13)	Reference		Reference		71% (159)	47% (7)	Reference		Reference	
2 and above	35% (108)	50% (13)	1.44 [0.58–3.63]	0.429	0.9 [0.08–9.67]	0.932	29% (64)	53% (8)	2.41 [0.76–7.83]	0.135	2.51 [0.12–56.6]	0.536
ART regimen												
ATV/LPV/PI	26% (81)	31% (8)	Reference		Reference		31% (70)	53% (8)	Reference		Reference	
EFV/NVP	74% (230)	69% (18)	0.86 [0.32–2.41]	0.769	1.49 [0.21–11.92]	0.692	69% (153)	47% (7)	0.27 [0.08–0.88]	0.03	0.05 [0.01–0.34]	0.005
Duration of ART at baseline												
6 months –2 years	10% (31)	13% (3)	Reference		Reference		8% (18)	20% (3)	Reference		Reference	
2 – < 4 years	18% (53)	33% (8)	2.67 [0.58–15.02]	0.36	4.4 [0.28–88.31]	0.74	16% (35)	20% (3)	0.78 [0.11–5.38]	0.8	3.75 [0.22–96.05]	0.4
4 – < 6 years	21% (64)	21% (5)	1.28 [0.25–7.48]		1.5 [0.11–20.26]		21% (46)	20% (3)	0.58 [0.09–3.92]		1.05 [0.05–23.23]	
6 years+	51% (154)	33% (8)	0.92 [0.22–4.81]		1.94 [0.15–28.02]		55% (119)	40% (6)	0.47 [0.1–2.62]		0.35 [0.02–5.3]	

Cotrimoxazole prophylaxis												
No	10% (31)	8% (2)	Reference		Reference		12% (27)	13% (2)	Reference	Reference		
Yes	90% (279)	92% (24)	1.2 [0.26–8.58]	0.83	0.34 [0.03–4.62]	0.382	88% (196)	87% (13)	0.47 [0.08–3.67]	0.415	0.28 [0.01–6.09]	0.39
Adherence to study drug												
Adherent	76% (236)	77% (20)	Reference				80% (178)	87% (13)	Reference			
Not Adherent	24% (76)	23% (6)	0.74 [0.24–2.06]	0.58			20% (45)	13% (2)	0.54 [0.08–2.32]	0.459		
Trial arm												
Azithromycin	51% (159)	69% (18)	Reference				53% (118)	53% (8)	Reference			
Placebo	49% (153)	31% (8)	0.09 [0.03–0.25]	< 0.0001			47% (105)	47% (7)	0.85 [0.26–2.65]	0.773		
BMI-for-age z-score												
Wasted	20% (62)	16% (4)	Reference				24% (54)	7% (1)	Reference			
Normal	80% (245)	84% (21)	1.07 [0.32–4.19]	0.916			76% (167)	93% (14)	5.21 [0.93–98.19]	0.125		
Height-for-age z-score												
Not stunted	46% (140)	56% (14)					43% (94)	47% (7)				
Stunted	54% (167)	44% (11)					57% (127)	53% (8)				

#Missing values: Weight-for-age z-score (5), Viral load suppression at baseline (5), ART regimen (1), Duration of ART at baseline (10), Cotrimoxazole prophylaxis (2), BMI-for-age z-score (5), Height-for-age z-score (5).

*Missing values: Weight-for-age z-score (1), Viral load suppression at baseline (2), Duration of ART at baseline (2), BMI-for-age z-score (1), Height-for-age z-score (1). §Missing values: Weight-for-age z-score (2), Viral load suppression at baseline (5), Duration of ART at baseline (5), BMI-for-age z-score (2), Height-for-age z-score (2). ωMissing values: None missing. ℑMissing values: Season of sampling (1), Weight-for-age z-score (5), Viral load suppression at baseline (5), ART regimen (1), Duration of ART at baseline (9), Cotrimoxazole prophylaxis (2), BMI-for-age z-score (5), Height-for-age z-score (5). θMissing values: Duration of ART at baseline (1), Cotrimoxazole prophylaxis (1). ΨMissing values: Season of sampling (2), Weight-for-age z-score (1), Viral load suppression at baseline (5), Duration of ART at baseline (6), Cotrimoxazole prophylaxis (1), BMI-for-age z-score (1), Height-for-age z-score (1). ℵMissing values: None missing. BMI-for-age z-score and Height-for-age z-score were not included in the multivariate analysis because of colinearity. Adherence to study drug and Trial arm were colinear with Adherence and Trial arm hence excluded from multivariate analysis. *The number of observations was too few for model convergence for AZM-resistant SP at both 48 and 72 weeks. Statistical model applied is generalised logistic regression.

Appendix C Table S 8. Factors associated with carriage of azithromycin resistant *Streptococcus pneumoniae* isolates at 48 weeks and 72 weeks in trial participants cont'd.

*SPUTUM								
Variable	No. observations (n = 290) ³	No. resistant isolates (n = 7) ⁶	48 weeks		No. observations (n = 224) ⁵	No. resistant isolates (n = 5) ⁴	72 weeks	
			Univariate analysis				Univariate analysis	
			Adjusted Odds ratio, 95% CI	p value			Adjusted Odds ratio, 95% CI	p value
Site								
Malawi	29% (83)	86% (6)	Reference		25% (56)	60% (3)	Reference	
Zimbabwe	71% (207)	14% (1)	0.06 [0–0.41]		75% (168)	40% (2)	0.24 [0.03–1.66]	0.15
Sex								
Female	49% (142)	71% (5)	Reference		46% (104)	80% (4)	Reference	
Male	51% (148)	29% (2)	0.31 [0.04–1.64]		54% (120)	20% (1)	0.2 [0.01–1.47]	0.16
Season of sampling								
May-Oct-Dry	58% (167)	86% (6)	Reference		37% (82)	60% (3)	Reference	
Nov-Apr-Rainy	42% (122)	14% (1)	0.19 [0.01–1.27]		63% (140)	40% (2)	0.43 [0.05–2.84]	0.379
Weight-for-age z-score								
Not underweight	53% (150)	71% (5)	Reference		50% (111)	20% (1)	Reference	
underweight	47% (135)	29% (2)	0.38 [0.05–1.99]		50% (112)	80% (4)	3.27 [0.44–67.09]	0.308
Age at baseline								
6–9 years	9% (27)	0% (0)	Reference		10% (23)	0% (0)	Reference	
10–12 years	19% (56)	43% (3)	69389275.89 [0–NA]		20% (44)	20% (1)	28912198.6 [0–NA]	NA
13–16 years	43% (125)	29% (2)	11564879.31 [0–NA]		44% (98)	40% (2)	11014170.9 [0–NA]	
17–19 years	28% (82)	29% (2)	21027053.3 [0–NA]		26% (59)	40% (2)	21027053.53 [0–NA]	
Viral load suppression at baseline								
Suppressed (< 1000 copies/mL)	57% (162)	43% (3)	Reference		58% (127)	60% (3)	Reference	

Unsuppressed (>1000 copies/mL)	43% (123)	57% (4)	1.33 [0.26–7.5]	0.728	42% (92)	40% (2)	0.6 [0.07–4.02]	0.601
Adherence and Trial arm								
Adherent AZM	39% (114)	57% (4)	Reference		42% (93)	40% (2)	Reference	
Adherent Placebo	36% (105)	29% (2)	0.16 [0.02–0.97]	0.29	38% (84)	40% (2)	0.74 [0.08–6.76]	0.94
Not Adherent AZM	11% (34)	14% (1)	0.67 [0.03–7.45]		11% (24)	0% (0)	0 [NA–5.4E+145]	
Not Adherent Placebo	13% (37)	0% (0)	0 [NA–1.0E+126]		10% (23)	20% (1)	1.75 [0.07–23.69]	
MRC dyspnoea score								
< 2	66% (192)	29% (2)	Reference		68% (153)	60% (3)	Reference	
2 and above	34% (98)	71% (5)	4.33 [0.82–32.96]	0.102	32% (71)	40% (2)	1.44 [0.17–9.71]	0.71
ART regimen								
ATV/LPV/PI	25% (73)	0% (0)	Reference		30% (68)	60% (3)	Reference	
EFV/NVP	75% (216)	100% (7)	75881451.83 [0–NA]	0.995	70% (156)	40% (2)	0.24 [0.03–1.66]	0.15
Duration of ART at baseline								
6 months –2 years	9% (27)	33% (2)	Reference		9% (19)	20% (1)	Reference	
2 – < 4 years	17% (49)	17% (1)	0.67 [0.03–8.68]	0.7	16% (34)	0% (0)	0 [NA–4.5E+196]	1
4 – < 6 years	22% (61)	33% (2)	0.89 [0.09–8.88]		23% (50)	0% (0)	0 [NA–3.9E+282]	
6 years+	51% (144)	17% (1)	0.24 [0.01–2.81]		53% (115)	80% (4)	1.33 [0.15–29.4]	
Cotrimoxazole prophylaxis								
No	10% (30)	0% (0)	Reference		11% (25)	20% (1)	Reference	
Yes	90% (258)	100% (6)	6899158.76 [0–NA]	0.994	89% (198)	80% (4)	0.32 [0.03–7.21]	0.364
Adherence to study drug								
Adherent	76% (219)	86% (6)	Reference		79% (177)	80% (4)	Reference	
Not Adherent	24% (71)	14% (1)	0.69 [0.03–4.88]	0.745	21% (47)	20% (1)	1.03 [0.05–8.26]	0.979
Trial arm								
Azithromycin	51% (148)	71% (5)	Reference		52% (117)	40% (2)	Reference	
Placebo	49% (142)	29% (2)	0.15 [0.02–0.79]	0.034	48% (107)	60% (3)	1.17 [0.18–9.64]	0.868
BMI-for-age z-score								
Wasted	20% (56)	29% (2)	Reference		22% (48)	0% (0)	Reference	
Normal	80% (229)	71% (5)	0.7 [0.13–5.48]	0.701	78% (175)	100% (5)	1.9E+8 [0–NA]	0.994

Height-for-age z-score				
Not stunted	44% (125)	29% (2)	42% (94)	100% (5)
Stunted	56% (160)	71% (5)	58% (129)	0% (0)

#Missing values: Weight-for-age z-score (5), Viral load suppression at baseline (5), ART regimen (1), Duration of ART at baseline (10), Cotrimoxazole prophylaxis (2), BMI-for-age z-score (5), Height-for-age z-score (5). *Missing values: Weight-for-age z-score (1), Viral load suppression at baseline (2), Duration of ART at baseline (2), BMI-for-age z-score (1), Height-for-age z-score (1). §Missing values: Weight-for-age z-score (2), Viral load suppression at baseline (5), Duration of ART at baseline (5), BMI-for-age z-score (2), Height-for-age z-score (2). ωMissing values: None missing. ℑMissing values: Season of sampling (1), Weight-for-age z-score (5), Viral load suppression at baseline (5), ART regimen (1), Duration of ART at baseline (9), Cotrimoxazole prophylaxis (2), BMI-for-age z-score (5), Height-for-age z-score (5). ϑMissing values: Duration of ART at baseline (1), Cotrimoxazole prophylaxis (1). ΨMissing values: Season of sampling (2), Weight-for-age z-score (1), Viral load suppression at baseline (5), Duration of ART at baseline (6), Cotrimoxazole prophylaxis (1), BMI-for-age z-score (1), Height-for-age z-score (1). ℵMissing values: None missing. BMI-for-age z-score and Height-for-age z-score were not included in the multivariate analysis because of colinearity. Adherence to study drug and Trial arm were colinear with Adherence and Trial arm hence excluded from multivariate analysis. *The number of observations was too few for model convergence for AZM-resistant SP at both 48 and 72 weeks. Statistical model applied is generalised logistic regression.

Appendix C. Table S 9. Factors associated with carriage of azithromycin resistant *Staphylococcus aureus* isolates at 48 weeks and 72 weeks in trial participants.

*NASOPHARYNGEAL SWABS								
Variable	No. observations (n = 312)#	No. resistant isolates (n = 32)*	48 weeks		No. observations (n = 223)§	No. resistant isolates (n = 9)ω	72 weeks	
			Univariate analysis				Univariate analysis	
			Adjusted Odds ratio, 95% CI	<i>p</i> value			Adjusted Odds ratio, 95% CI	<i>p</i> value
Site								
Malawi	31% (96)	66% (21)	Reference		20% (44)	44% (4)	Reference	
Zimbabwe	69% (216)	34% (11)	0.17 [0.05–0.48]	0.001	80% (179)	56% (5)	0.2 [0.04–1.01]	0.048
Sex								
Female	48% (151)	53% (17)	Reference		45% (100)	56% (5)	Reference	
Male	52% (161)	47% (15)	0.94 [0.35–2.49]	0.897	55% (123)	44% (4)	0.68 [0.15–2.97]	0.606
Season of sampling								
May-Oct-Dry	58% (180)	44% (14)	Reference		37% (82)	33% (3)	Reference	
Nov-Apr-Rainy	42% (132)	56% (18)	0.95 [0.35–2.54]	0.914	63% (141)	67% (6)	0.85 [0.19–4.57]	0.833
Weight-for-age z-score								
Not underweight	51% (158)	41% (13)	Reference		47% (104)	67% (6)	Reference	
underweight	49% (149)	59% (19)	2.52 [0.92–7.22]	0.076	53% (117)	33% (3)	0.53 [0.1–2.32]	0.413
Age at baseline								
6–9 years	9% (28)	9% (3)	Reference		11% (24)	11% (1)	Reference	
10–12 years	20% (61)	28% (9)	1.2 [0.13–9.91]	0.37	18% (41)	11% (1)	0.43 [0.01–13.33]	0.93
13–16 years	43% (133)	50% (16)	0.63 [0.08–4.26]		43% (96)	56% (5)	0.71 [0.07–16.13]	
17–19 years	29% (90)	13% (4)	0.3 [0.03–2.46]		28% (62)	22% (2)	1 [0.06–26.86]	
Viral load suppression at baseline								
Suppressed (< 1000 copies/mL)	58% (177)	58% (18)	Reference		60% (130)	56% (5)	Reference	
Unsuppressed (> 1000 copies/mL)	42% (130)	42% (13)	0.41 [0.15–1.11]	0.085	40% (88)	44% (4)	0.94 [0.2–4.11]	0.935
Adherence and Trial arm								
Adherent AZM	39% (122)	56% (18)	Reference		42% (94)	78% (7)	Reference	
Adherent Placebo	37% (114)	0% (0)	0 [0–1.3E+39]	0.005	38% (94)	0% (0)	0 [NA–1.7E+138]	0.89
Not Adherent AZM	12% (37)	41% (13)	1.44 [0.13–33.05]		11% (24)	22% (2)	2.86 [0.23–68.91]	

Not Adherent Placebo	13% (39)	3% (1)	0.01 [0–0.12]		9% (21)	0% (0)	0 [NA–Inf]	
MRC dyspnoea score								
< 2	65% (204)	44% (14)	Reference		71% (159)	56% (5)	Reference	
2 and above	35% (108)	56% (18)	2.57 [0.95–7.21]	0.066	29% (64)	44% (4)	2.16 [0.46–9.87]	0.315
ART regimen								
ATV/LPV/PI	26% (81)	16% (5)	Reference		31% (70)	11% (1)	Reference	
EFV/NVP	74% (230)	84% (27)	2.35 [0.72–8.47]	0.166	69% (153)	89% (8)	4.87 [0.77–95.34]	0.155
Duration of ART at baseline								
6 months –2 years	10% (31)	7% (2)	Reference		8% (18)	11% (1)	Reference	
2 – < 4 years	18% (53)	33% (10)	1.67 [0.06–24.88]	0.13	16% (35)	22% (2)	0.8 [0.04–23.23]	0.76
4 – < 6 years	21% (64)	23% (7)	0.44 [0.02–5.56]		21% (46)	11% (1)	0.22 [0.01–7.24]	
6 years+	51% (154)	37% (11)	0.29 [0.01–3.36]		55% (119)	56% (5)	0.53 [0.04–12.67]	
Cotrimoxazole prophylaxis								
No	10% (31)	6% (2)	Reference		12% (27)	11% (1)	Reference	
Yes	90% (279)	94% (29)	1.45 [0.22–11.62]	0.695	88% (196)	89% (8)	0.97 [0.12–20.34]	0.979
Adherence to study drug								
Adherent	76% (236)	56% (18)	Reference		80% (178)	78% (7)	Reference	
Not Adherent	24% (76)	44% (14)	2.07 [0.74–6.01]	0.168	20% (45)	22% (2)	2.36 [0.29–14.86]	0.372
Trial arm								
Azithromycin	51% (159)	97% (31)	Reference		53% (118)	100% (9)	Reference	
Placebo	49% (153)	3% (1)	0 [0–0.02]	< 0.0001	47% (105)	0% (0)	0 [NA–1.4E+115]	0.995
BMI-for-age z-score								
Wasted	20% (62)	22% (7)	Reference		24% (54)	22% (2)	Reference	
Normal	80% (245)	78% (25)	0.89 [0.25–3.07]	0.856	76% (167)	78% (7)	1.3 [0.26–9.69]	0.769
Height-for-age z-score								
Not stunted	46% (140)	59% (19)			43% (94)	44% (4)		
Stunted	54% (167)	41% (13)			57% (127)	56% (5)		

#Missing values: Weight-for-age z-score (5), Viral load suppression at baseline (5), ART regimen (1), Duration of ART at baseline (10), Cotrimoxazole prophylaxis (2), BMI-for-age z-score (5), Height-for-age z-score (5). *Missing values: Viral load suppression at baseline (1), Duration of ART at baseline (2), Cotrimoxazole prophylaxis (1). §Missing values: Weight-for-age z-score (2), Viral load suppression at baseline (5), Duration of ART at baseline (5), BMI-for-age z-score (2), Height-for-age z-score (2). ωMissing values: None missing. §Missing values: Season of sampling (1), Weight-for-age z-score (5), Viral load suppression at baseline (5), ART regimen (1), Duration of ART at baseline (9), Cotrimoxazole prophylaxis (2), BMI-for-age z-score (5), Height-for-age z-score (5). θMissing values: Duration of ART at baseline (1). ΨMissing values: Season of sampling (2), Weight-for-age z-score (1), Viral load suppression at baseline (5), Duration of ART at baseline (6),

Cotrimoxazole prophylaxis (1), BMI-for-age z-score (1), Height-for-age z-score (1). $\mathcal{M}$ Missing values: Viral load suppression at baseline (1). BMI-for-age z-score and Height-for-age z-score were not included in the multivariate analysis because of colinearity. Adherence to study drug and Trial arm were colinear with Adherence and Trial arm hence excluded from multivariate analysis. *The number of observations was too few for model convergence for AZM-resistant SP at both 48 and 72 weeks. Statistical model applied is generalised logistic regression.

Appendix C Table S 9. Factors associated with carriage of azithromycin resistant *Staphylococcus aureus* isolates at 48 weeks and 72 weeks in trial participants cont'd.

SPUTUM												
Variable	No. observations (n = 290) $\mathfrak{S}$	No. resistant isolates (n = 33) $\mathfrak{H}$	48 weeks				No. observations (n = 224) Ψ	No. resistant isolates (n = 20) $\mathcal{M}$	72 weeks			
			Univariate analysis		Multivariate analysis				Univariate analysis		Multivariate analysis	
			Adjusted Odds ratio, 95% CI	<i>p</i> value	Adjusted Odds ratio, 95% CI	<i>p</i> value			Adjusted Odds ratio, 95% CI	<i>p</i> value	Adjusted Odds ratio, 95% CI	<i>p</i> value
Site												
Malawi	29% (83)	64% (21)	Reference		Reference		25% (56)	30% (6)	Reference		Reference	
Zimbabwe	71% (207)	36% (12)	0.06 [0.02–0.19]	< 0.0001	0 [0–0.2]	0.042	75% (168)	70% (14)	0.69 [0.22–2.26]	0.522	0.42 [0.01–10.3]	0.599
Sex												
Female	49% (142)	45% (15)	Reference		Reference		46% (104)	50% (10)	Reference		Reference	
Male	51% (148)	56% (18)	0.98 [0.4–2.39]	0.96	0.53 [0.03–7]	0.627	54% (120)	50% (10)	0.63 [0.22–1.77]	0.375	3.34 [0.68–22.85]	0.169
Season of sampling												
May-Oct-Dry	58% (167)	64% (21)	Reference		Reference		37% (82)	45% (9)	Reference		Reference	
Nov-Apr-Rainy	42% (122)	36% (12)	1.08 [0.42–2.7]	0.877	0.03 [0–0.71]	0.065	63% (140)	55% (11)	0.56 [0.2–1.62]	0.282	1.25 [0.27–6.17]	0.773
Weight-for-age z-score												
Not underweight	53% (150)	48% (16)	Reference		Reference		50% (111)	70% (14)	Reference		Reference	
underweight	47% (135)	52% (17)	1.06 [0.44–2.59]	0.893	0.12 [0–1.65]	0.135	50% (112)	30% (6)	0.29 [0.09–0.83]	0.026	0.11 [0.02–0.65]	0.02
Age at baseline												
6–9 years	9% (27)	15% (5)	Reference		Reference		10% (23)	5% (1)	Reference		Reference	
10–12 years	19% (56)	24% (8)	1.6 [0.27–10.01]	0.071	0.18 [0–17.9]	0.41	20% (44)	30% (6)	1.8 [0.18–41.12]	0.42	0.03 [0–2.81]	0.3
13–16 years	43% (125)	42% (14)	0.53 [0.11–2.35]		1.17 [0.03–63.22]		44% (98)	50% (10)	1.2 [0.13–25.87]		0.29 [0.01–16.6]	

17–19 years	28% (82)	18% (6)	0.24 [0.04–1.17]		0.03 [0–3.91]		26% (59)	15% (3)	0.47 [0.04–11.3]		0.1 [0–7.65]	
Viral load suppression at baseline												
Suppressed (< 1000 copies/mL)	57% (162)	48% (16)	Reference		Reference		58% (127)	68% (13)	Reference		Reference	
Unsuppressed (>1000 copies/mL)	43% (123)	52% (17)	0.76 [0.31–1.85]	0.544	2.5 [0.16–66.17]	0.531	42% (92)	32% (6)	0.53 [0.17–1.55]	0.262	0.37 [0.05–2.09]	0.277
Adherence and Trial arm												
Adherent AZM	39% (114)	52% (17)	Reference		Reference		42% (93)	65% (13)	Reference		Reference	
Adherent Placebo	36% (105)	3% (1)	0.03 [0–0.18]	0.0001	0 [0–0.02]	0.085	38% (84)	10% (2)	0.12 [0.02–0.51]	0.056	0.08 [0–0.62]	0.24
Not Adherent AZM	11% (34)	39% (13)	3.06 [0.78–15.5]		0.14 [0–2.94]		11% (24)	15% (3)	0.88 [0.16–4.23]		0.59 [0.02–9.91]	
Not Adherent Placebo	13% (37)	6% (2)	0.13 [0.02–0.59]		0 [0–0.02]		10% (23)	10% (2)	0.32 [0.04–1.52]		1.14 [0.08–13.01]	
MRC dyspnoea score												
< 2	66% (192)	33% (11)	Reference		Reference		68% (153)	75% (15)	Reference		Reference	
2 and above	34% (98)	67% (22)	7.8 [2.95–22.23]	< 0.0001	175.77 [1.27–202838.5]	0.082	32% (71)	25% (5)	0.93 [0.27–2.89]	0.908	0.38 [0.01–6.05]	0.524
ART regimen												
ATV/LPV/PI	25% (73)	12% (4)	Reference		Reference		30% (68)	25% (5)	Reference		Reference	
EFV/NVP	75% (216)	88% (29)	3.52 [1.14–13.35]	0.041	18.43 [1.15–705.81]	0.065	70% (156)	75% (15)	1.17 [0.38–4.07]	0.791	1.39 [0.27–8.06]	0.697
Duration of ART at baseline												
6 months –2 years	9% (27)	13% (4)	Reference		Reference		9% (19)	20% (4)	Reference		Reference	
2 – < 4 years	17% (49)	22% (7)	0.18 [0.01–1.5]	0.3	0 [0–0.47]	0.36	16% (34)	20% (4)	0.08 [0–0.76]	0.12	0.01 [0–0.42]	0.1
4 – < 6 years	22% (61)	25% (8)	0.2 [0.01–1.69]		0.02 [0–1.76]		23% (50)	20% (4)	0.11 [0–1.05]		0.03 [0–0.6]	
6 years+	51% (144)	41% (13)	0.12 [0.01–0.91]		0.02 [0–2.45]		53% (115)	40% (8)	0.06 [0–0.48]		0.01 [0–0.25]	
Cotrimoxazole prophylaxis												
No	10% (30)	9% (3)	Reference		Reference		11% (25)	5% (1)	Reference		Reference	

Yes	90% (258)	91% (30)	1.14 [0.26–5.87]	0.868	0.03 [0–6.24]	0.239	89% (198)	95% (19)	2.24 [0.35–43.68]	0.47	1.63 [0.1–50.05]	0.742
Adherence to study drug												
Adherent	76% (219)	55% (18)	Reference				79% (177)	75% (15)	Reference			
Not Adherent	24% (71)	45% (15)	2.08 [0.83–5.32]	0.119			21% (47)	25% (5)	1.02 [0.29–3.2]	0.969		
Trial arm												
Azithromycin	51% (148)	91% (30)	Reference				52% (117)	80% (16)	Reference			
Placebo	49% (142)	9% (3)	0.04 [0.01–0.15]	< 0.0001			48% (107)	20% (4)	0.18 [0.05–0.57]	0.006		
BMI-for-age z-score												
Wasted	20% (56)	24% (8)	Reference				22% (48)	25% (5)	Reference			
Normal	80% (229)	76% (25)	0.93 [0.33–2.71]	0.89			78% (175)	75% (15)	0.56 [0.17–2.06]	0.362		
Height-for-age z-score												
Not stunted	44% (125)	58% (19)					42% (94)	45% (9)				
Stunted	56% (160)	42% (14)					58% (129)	55% (11)				

#Missing values: Weight-for-age z-score (5), Viral load suppression at baseline (5), ART regimen (1), Duration of ART at baseline (10), Cotrimoxazole prophylaxis (2), BMI-for-age z-score (5), Height-for-age z-score (5). *Missing values: Viral load suppression at baseline (1), Duration of ART at baseline (2), Cotrimoxazole prophylaxis (1). §Missing values: Weight-for-age z-score (2), Viral load suppression at baseline (5), Duration of ART at baseline (5), BMI-for-age z-score (2), Height-for-age z-score (2). ωMissing values: None missing. ℑMissing values: Season of sampling (1), Weight-for-age z-score (5), Viral load suppression at baseline (5), ART regimen (1), Duration of ART at baseline (9), Cotrimoxazole prophylaxis (2), BMI-for-age z-score (5), Height-for-age z-score (5). θMissing values: Duration of ART at baseline (1). ΨMissing values: Season of sampling (2), Weight-for-age z-score (1), Viral load suppression at baseline (5), Duration of ART at baseline (6), Cotrimoxazole prophylaxis (1), BMI-for-age z-score (1), Height-for-age z-score (1). ∕Missing values: Viral load suppression at baseline (1). BMI-for-age z-score and Height-for-age z-score were not included in the multivariate analysis because of collinearity. Adherence to study drug and Trial arm were colinear with Adherence and Trial arm hence excluded from multivariate analysis. *The number of observations was too few for model convergence for AZM-resistant SP at both 48 and 72 weeks. Statistical model applied is generalised logistic regression.

APPENDIX D: Chapter Five Supplementary Materials

Appendix D. Chapter Five Supplementary Materials

Section 1. Detailed description of methods – differentially abundant taxa and SIMPER analysis

Differentially abundant taxa were identified using ANCOM2 [1] on unrarefied data with False Discovery Rate (FDR) Benjamini/Hochberg correction (cut off = 0.05). Nine other methods for detecting differentially abundant taxa were used for comparison and reported below (Wilcoxon signed-rank test on data after the following normalisation methods—total sum scaling, variance stabilising transformation, and centred log transformation after applying a pseudo count of one, Aldex2 [2], Deseq2 [3], Ancombc [4], Corncob [5], MaasLin2 [6] with total sum scaling and log transformation, MaasLin2 [6] with centred log transformation after applying a pseudo count of one. The relative contribution of each taxon to overall dissimilarity was measured using SIMPER analysis on the Bray-Curtis distances between samples. The input for all the methods is a phyloseq object merged at the genus level and 0.5% prevalence filtered. No rarefaction was applied to the feature tables used in the analysis below. In the first three methods, we applied the Wilcoxon test on ASV counts normalised using three different methods to determine how different normalisations will affect the number of genera found to be differentially abundant.

a. Mann–Whitney–Wilcoxon test on Total sum scaled ASV counts

The ASV counts, previously merged at genus level and 0.5% prevalence filtered, were converted to relative abundances using total sum scaling equation – $x/\text{sum}(x)$, where x is ASV counts implemented using the *transform_sample_counts* function embedded in the phyloseq package. The resulting relative abundances of each genus in the AZM and Placebo arms were then compared using the Wilcoxon test. Comparison of samples from different visits but within the same trial arm were made by Wilcoxon test for paired samples. The p values were then adjusted for multiple testing using the Benjamin-Hochberg method to produce the q values. The false discovery rate was set to 0.05; hence only genera with q values < 0.05 were deemed differentially abundant.

b. Mann–Whitney–Wilcoxon test on variance stabilising transformed (VST) ASV counts

A pseudocount of one was applied to all ASVs before conversion from phyloseq to Deseq2 object. This was necessary to calculate log geometric means when the *estimateSizeFactors* function embedded in Deseq2 is later applied. The phyloseq object was then converted to a Deseq2 object using the *phyloseq_to_deseq2* function so that VST normalisation can be conducted using a function from this package. Size factors (*estimateSizeFactors*) and dispersions (*estimateDispersions*) were estimated before variance stabilising transformation was applied to the data (*getVarianceStabilizedData*). The resulting variance stabilising transformed (VST) ASVs count of each genus of AZM and Placebo arms were then compared using the Wilcoxon test. Comparison of samples from different visits but within the same trial arm were made by Wilcoxon test for paired samples. The *p* values were then adjusted for multiple testing using Benjamin-Hochberg method to produce the *q* values. The false discovery rate was set to 0.05; hence only genera with *q* values < 0.05 were deemed differentially abundant.

c. Mann–Whitney–Wilcoxon test on centre log ratio transformed (CLR) ASV counts

Here a pseudocount of one was again applied to allow the calculation of log geometric means. The ASV counts were then CLR transformed using the *transform_sample_counts* function embedded in the phyloseq package with the function $x/\exp(\text{mean}(\log(x)))$, *x* is ASV counts. The resulting log ratios of each ASV within the genera of AZM and Placebo arms were then compared using the Wilcoxon test. Comparison of samples from different visits but within the same trial arm were made by Wilcoxon test for paired samples. The *p* values were then adjusted for multiple testing using the Benjamin-Hochberg method to produce the *q* values. The false discovery rate was set to 0.05; hence only genera with *q* values < 0.05 were deemed differentially abundant.

d. DESeq2

This analysis is based on negative binomial distribution and uses of VST normalisation. It does not account for the compositional nature of the data. The phyloseq object was converted to deseql2 using the function *phyloseq_to_deseq2*. The DEseq function was applied to this object with Wald test, local *fitType* and poscounts option for *sfType*. The *lfcShrink* function was applied with *coef* = 2 and *type* = “*apeglm*”. The *p* values were then adjusted for multiple testing using the Benjamin-Hochberg method to produce the *q* values. The false discovery rate was set to 0.05; hence only genera with *q* values < 0.05 were deemed differentially abundant.

e. Analysis of compositions of microbiomes with bias correction (ANCOM-BC)

The *ancombc* function in the ANCOMBC R package v 1.0.5 was applied to the genus-agglomerated and 0.5% prevalence-filtered ASV counts with the following options: *p* value adjustment was Benjamin-Hochberg, library cut =1000, structural zeros= TRUE, neg_lb= TRUE, conserve=TRUE, global=TRUE. All other options were left as default. The false discovery rate was set to 0.05; hence only genera with *q* values < 0.05 were deemed differentially abundant.

f. Aldex2

A data frame of the genera counts, and corresponding sample metadata were passed to the *aldex* function in ALDEx2 R package v 1.22.0 setting the denom parameter to “iqlr”. All other parameters were set to default. Both Wilcoxon (ALDEx2 Wilcoxon) and t-test (ALDEx2 t-test) were used for testing differences in genera relative abundances between AZM and Placebo or samples from different visits. The false discovery rate was set to 0.05. The function returned Benjamini-Hochberg (BH) FDR-corrected *p* values.

g. Corncob

The genus-merged and 0.5% prevalence filtered phyloseq object was passed to the *differentialTest* function of the corncob R package version 0.2.0. We selected the Wald test for significance testing and false discovery rate to Benjamini-Hochberg. All other options were set to default. Since the false discovery rate was set to 0.05; only genera with *q* values < 0.05 were deemed differentially abundant.

h. MaAsLin 2 - Normalisation = TSS, transformation= LOG, fixed effects= trial_arm

A data frame of the genera counts, and corresponding sample metadata were passed to the *Maaslin2* function in MaAsLin2 R package v 1.4.0, setting the minimum prevalence to 0.0 because 0.5% prevalence filtered was already applied to the counts. Maximum significance was set to 0.05, standardise was set to FALSE and fixed effect was set as trial arm or visit. All other parameters were set to default. Since the false discovery rate was set to 0.05; only genera with *q* values < 0.05 were deemed differentially abundant.

i. MaAsLin 2 Normalisation = NONE, transformation= NONE, fixed effects= trial_arm

A data frame of the genera ASV counts that have previously been centred-log-ratio transformed and corresponding sample metadata were passed to the Maaslin2 function in MaAsLin2 R package v 1.4.0 setting the minimum prevalence to 0.0 because 0.5% prevalence filtered was already applied to the counts. Maximum significance was set to 0.05, standardise was set to FALSE and fixed effect was set trial arm or visit. Normalisation and transformation were set to “None”. All other parameters were set to default. Since the false discovery rate was set to 0.05; only genera with q values < 0.05 were deemed differentially abundant.

j. ANCOM-II

The genus-merged, 0.5% prevalence filtered ASV count table was inputted through the ANCOM-II[1] (<https://github.com/FrederickHuangLin/ANCOM>) `feature_table_pre_process` function which identified outlier and structural zeros. The trial arm or visit was specified as the group and main variable. The resulting feature table was then passed through the ANCOM function, and p -values were FDR-corrected using the BH method (alpha set at 0.05). W statistics greater than or equal to 60% of the total number of genera tested were considered significant.

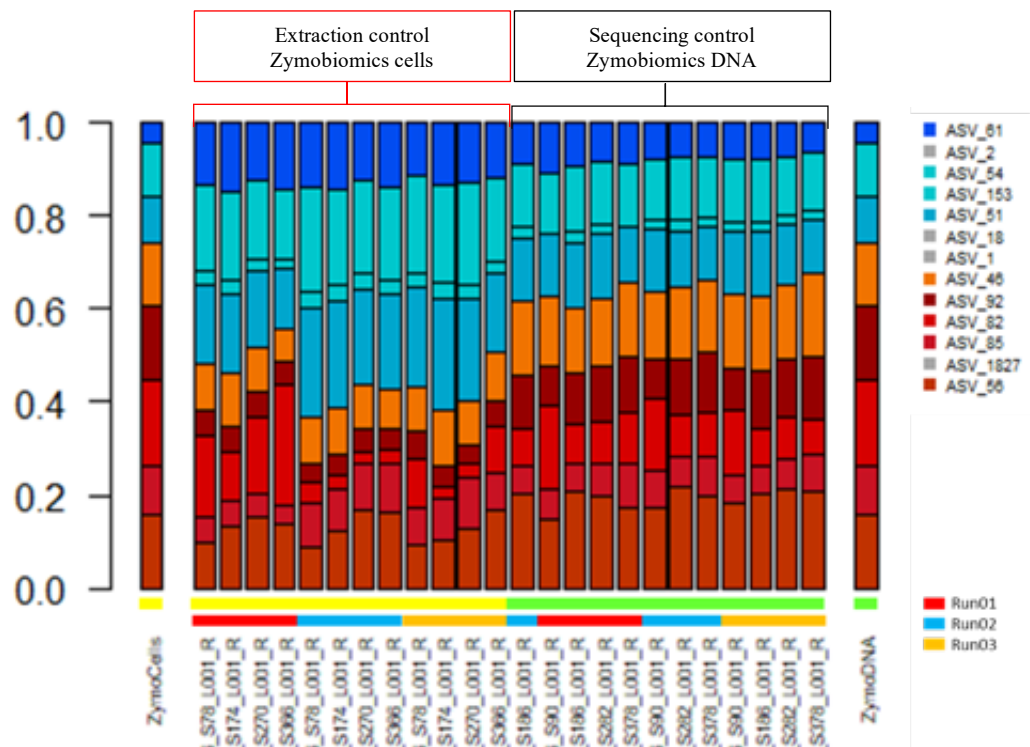
Section 2. Supplementary results

2.1 Results of quality control steps

A total of 1152 samples (78 biological samples belonging to a comparison group, 12 Zymobiomics extraction controls, 12 Zymobiomics sequencing control, 101 biological repeats, 43 non-template (negative or Primestore®) controls and 906 biological samples included in the main trial) including controls were processed in three runs of 384 samples each. To ensure that the extraction step and sequencing steps are validated, we included Zymobiomics mock community extraction controls (cells) (catalogue no. ZR D6300, Zymo Research Corp., Irvine, CA, United States) and sequencing controls (DNA) (catalogue no. ZR D6305, Zymo Research Corp., Irvine, CA, United States). We also repeated samples within plates and between plates in the same and between runs to assess reproducibility. This action is to ensure there are no differences in bacteriome profiles introduced by batch effects. Furthermore, we compared profiles from biological samples with non-template control (in this case, Primestore, which was used as a storage medium of the samples) to assess background contaminating profiles. We then assess whether samples with low biomass (low 16S copies) clustered with the negative controls on a log-ratio biplot, suggesting background contamination rather than true biological signal. Also, we assess clustering of samples based on age, run number, study site (country), visit/timepoint, number of reads and 16S copy numbers, which may introduce bias in our analysis. Finally, we used the *isContaminant* function within the DECONTAM R package to determine which ASVs are likely to be contaminants based on the Primestore profiles. This section of the supplementary material contains the results of each of these analyses.

a. Extraction and Sequencing Controls

The Zymobiomics mock community extraction controls (cells) (catalogue no. ZR D6300, Zymo Research Corp., Irvine, CA, United States) and sequencing controls (DNA) (catalogue no. ZR D6305, Zymo Research Corp., Irvine, CA, United States) were comparable to the theoretical compositions provided by the manufacturer (Appendix D Figure S1, Appendix D Table S1). However, the DNA profiles were more comparable than the cells highlighting a slight bias in the extraction step probably from the lysis step. The mock communities were included on each plate in each run, resulting in 12 samples of the extraction controls and 12 of the sequencing controls.



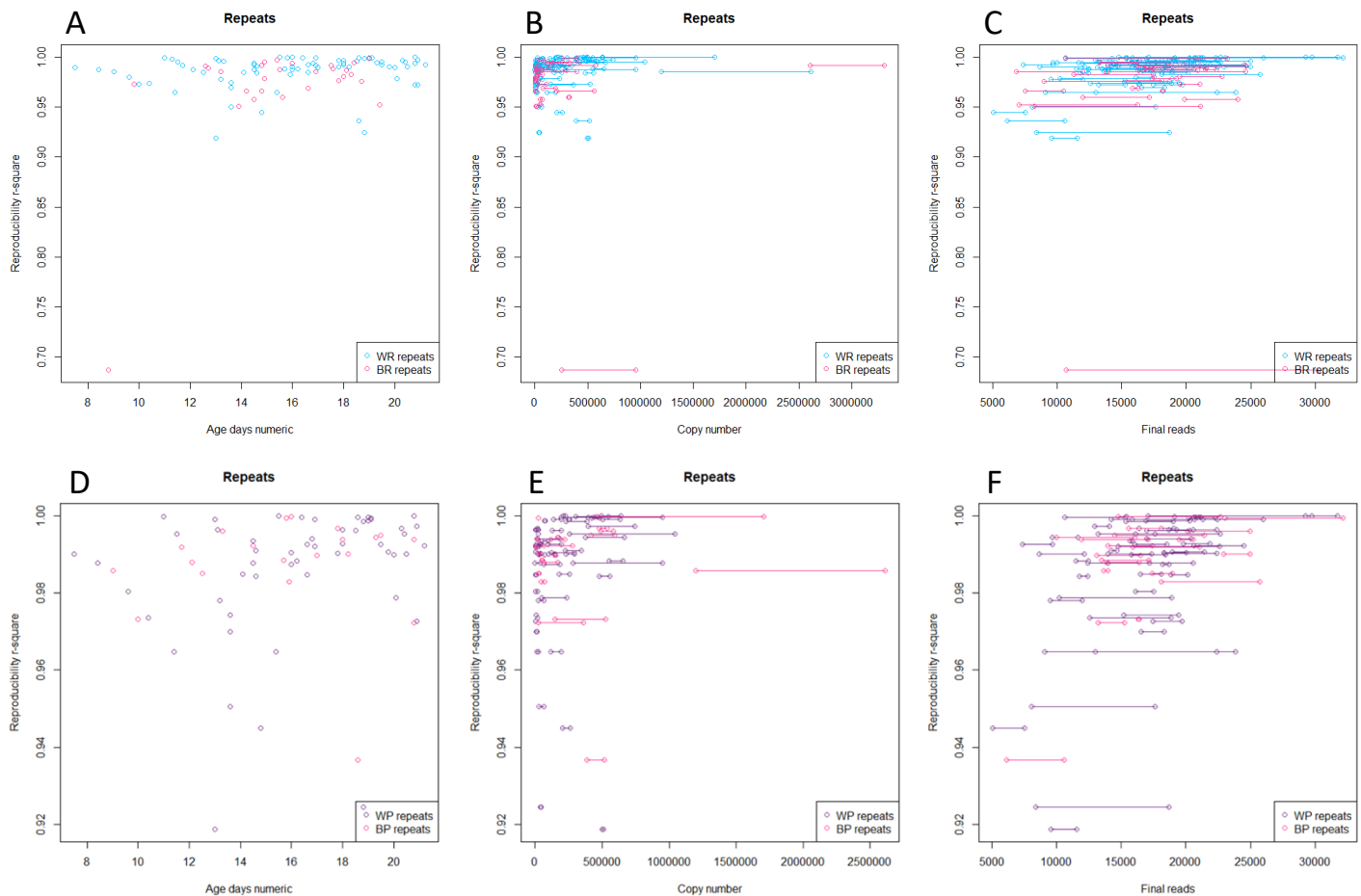
Appendix D. Figure S. 1. A bar plot of the taxa and their relative abundance of the extraction and sequencing mock controls compared to manufacturer profiles.

Appendix D. Table S 1. The taxonomy of the ASVs in the extraction and sequencing control.

	Kingdom	Phylum	Class	Order	Family	Genus
ASV_61	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas
ASV_2	Bacteria	Proteobacteria	Gammaproteobacteria	Pasteurellales	Pasteurellaceae	Haemophilus
ASV_54	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	Salmonella
ASV_153	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	Salmonella
ASV_51	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	Escherichia/ Shigella
ASV_18	Bacteria	Proteobacteria	Gammaproteobacteria	Burkholderiales	Neisseriaceae	Neisseria
ASV_1	Bacteria	Proteobacteria	Gammaproteobacteria	Burkholderiales	Neisseriaceae	Neisseria
ASV_46	Bacteria	Firmicutes	Bacilli	Staphylococcales	Staphylococcaceae	Staphylococcus
ASV_92	Bacteria	Firmicutes	Bacilli	Lactobacillales	Listeriaceae	Listeria
ASV_82	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus
ASV_85	Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Enterococcus
ASV_1827	Bacteria	Firmicutes	Bacilli	Bacillales	NA	NA
ASV_56	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus

b. Reproducibility within and between the three runs

One hundred one (101) biological specimens were repeated within the three runs, with one sample repeated thrice. Of these, 74 were repeated in the same run (WR) while 28 were repeated between runs (BR). Reproducibility, as measured by R^2 , was very high (> 0.9). Only one between run repeat had an R^2 value < 0.9 (0.69). Regarding reproducibility, no sample was excluded based on age, 16S copies, or number of reads as none of these affected reproducibility Appendix D Figure S2 (A, B and C). Out of the 74 WR repeats, 54 were repeated on the same plate to assess intra-plate reproducibility, while 20 specimens were repeated between plates in the same run to assess inter-plate reproducibility within the same run. Reproducibility for all 74 samples was very high (> 0.91) hence no sample was excluded (Appendix D Figure S2, D, E and F).

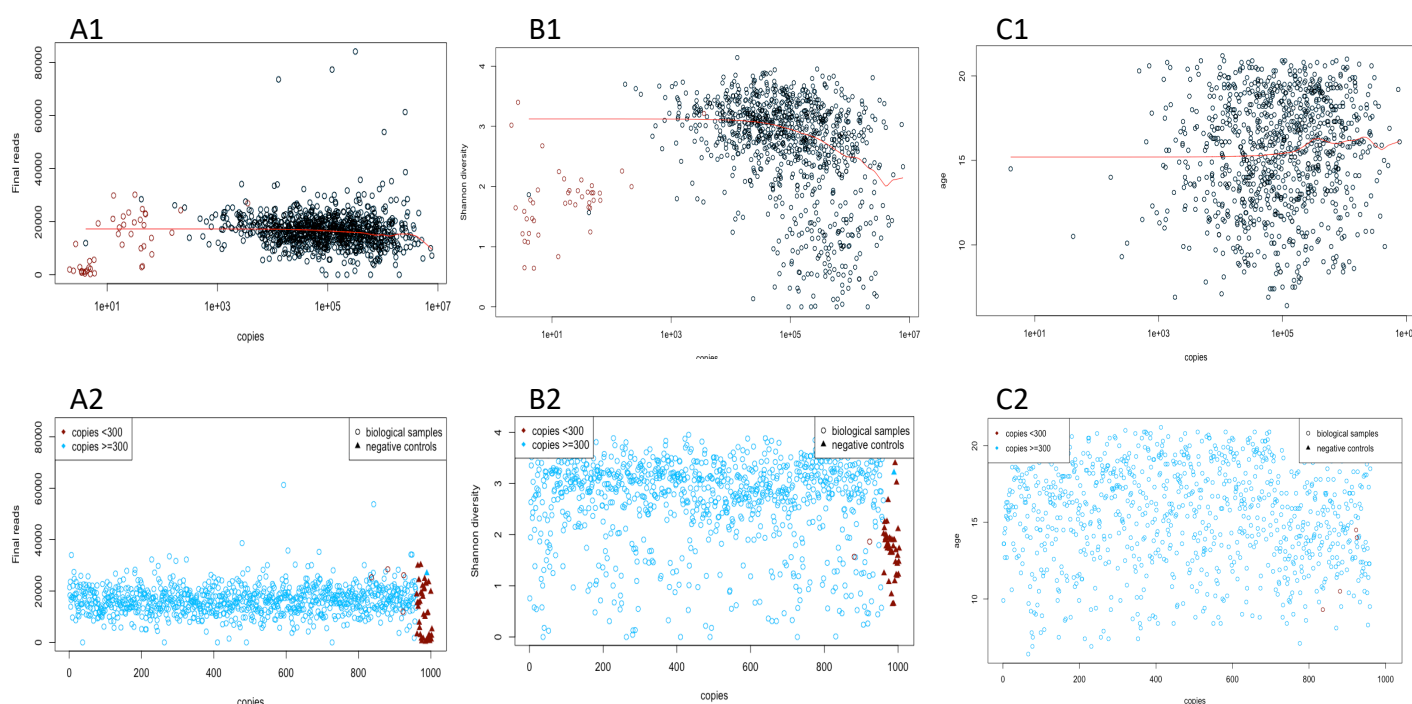


Appendix D. Figure S. 2. A scatterplot showing the correlation between samples repeated within a run (WR, $n = 74$) and between runs (BR, $n = 28$).

A) shows reproducibility in relation to age of the participants, B) shows reproducibility in relation to 16S copy numbers and C) shows reproducibility in relation to number of final reads. The second row of the figure shows samples repeated within the same plate in the same run (WP, $n=54$) and between plates in the same run (BP, $n=20$). D) shows reproducibility in relation to age of the participants, E) shows reproducibility in relation to 16S copy numbers and F) shows reproducibility in relation to number of final reads.

c. Relationship between the biological samples and negative controls (Primestore) profiles

We included 43 Primestores (sample storage media) as negative controls across the three runs (Run 1= 13, Run2=16, Run3 = 14). The Primestores samples used were made up of two different batches used in sample storage to better account for batch-to-batch variations in background profiles. The number of biological samples analysed was 960. There was a total of 3219 ASVs that had greater than zero reads. Our previous experience and that of others[7,8] has shown that age is positively correlated with biomass (16S copies). This also correlates with the number of reads and sometimes with alpha diversity. We aimed to assess this in our data. We found no correlation between the final reads and 16S copies ($r=0.05$) Appendix D Figure S3 (A1 and A2) and none between age and 16S copies ($r=0.07$) Appendix D Figure S3 (C1 and C2). However, we detected a slight negative correlation between the Shannon diversity index and 16S copies ($r= -0.26$) Appendix D Figure S3 (B1 and B2).

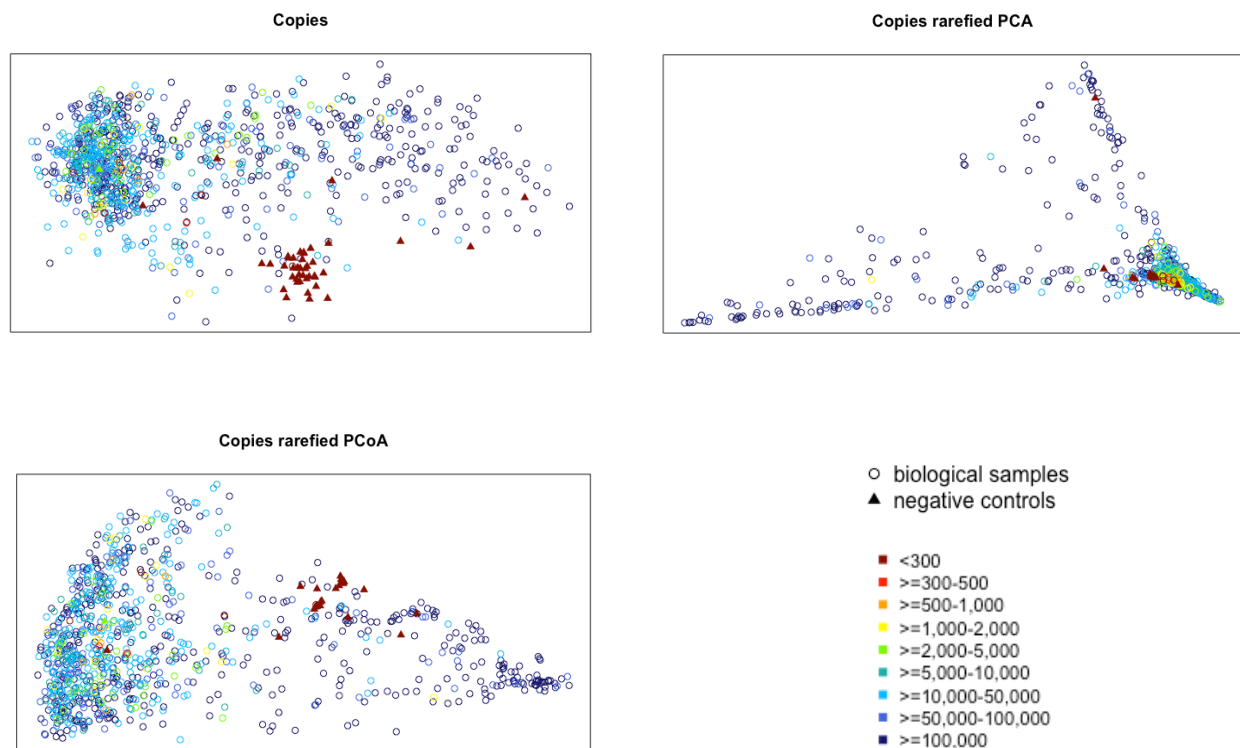


Appendix D. Figure S. 3. A scatterplot showing the spread of biological samples (n = 960) and the negative controls (primestore, n = 43).

16S copies vs final number of reads (A1 and A2), Shannon alpha diversity index (B1 and B2) and age of participant in years (C1 and C2).

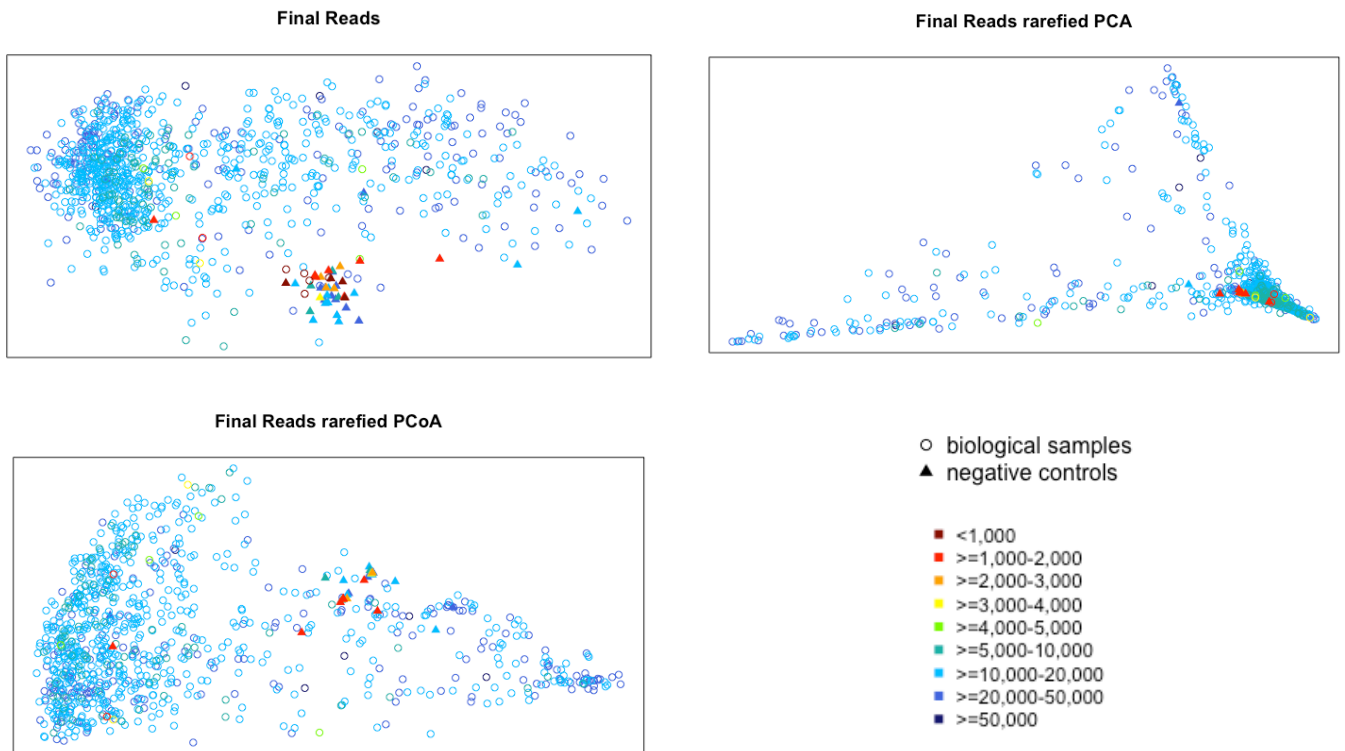
Next, we investigated whether our low biomass specimens (< 500 / μ l copies, n = 4) shared bacteriome profiles with our negative controls (cluster together on ordination plots). We observed that this was not the case, Appendix D Figure S4. However, we did excluded specimens with < 100 16S gene copies

(n = 2). Our further analysis revealed that samples with low read counts (< 1000 reads) may cluster with negative controls indicating similar bacteriome profiles, Appendix D Figure S5. We therefore excluded these samples as well (read counts were 0 – 4, n = 5). As we and others have shown that these low biomass specimens produced poor reproducible sequencing profiles[7,8]. Four Primestores had < 100 16S copies (n = 4) while a large number had > 1000 reads (39 of the 43). Specimens collected at younger ages do not seem to cluster with negative controls, Appendix D Figure S6. We assessed the relationship between the number of ASVs detected and the 16S copies and number of reads. We observed that the number of ASVs detected negatively correlated with low 16S copies (< 500), Appendix D Figure S7 and low number of reads, Appendix D Figure S8. This observation further supports our exclusion of biological samples with < 500 copies and/or < 1000 reads.



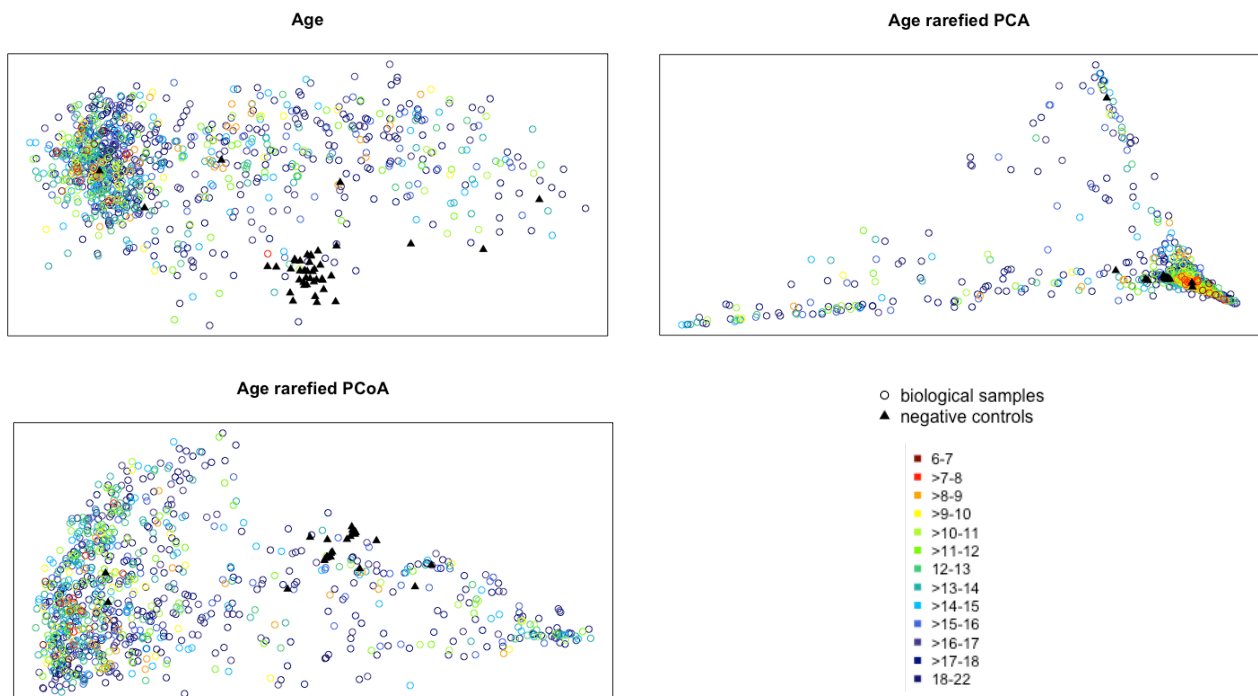
Appendix D. Figure S. 4. Ordination plots of showing the spread of biological samples (n = 960) and the negative controls (primestore, n = 43) coloured by their 16S copies.

Low biomass samples (low 16S copy numbers) did not seem to cluster with negative controls. Only four (4) biological samples had < 300 16S copies, the remaining red data points represent Primestores.



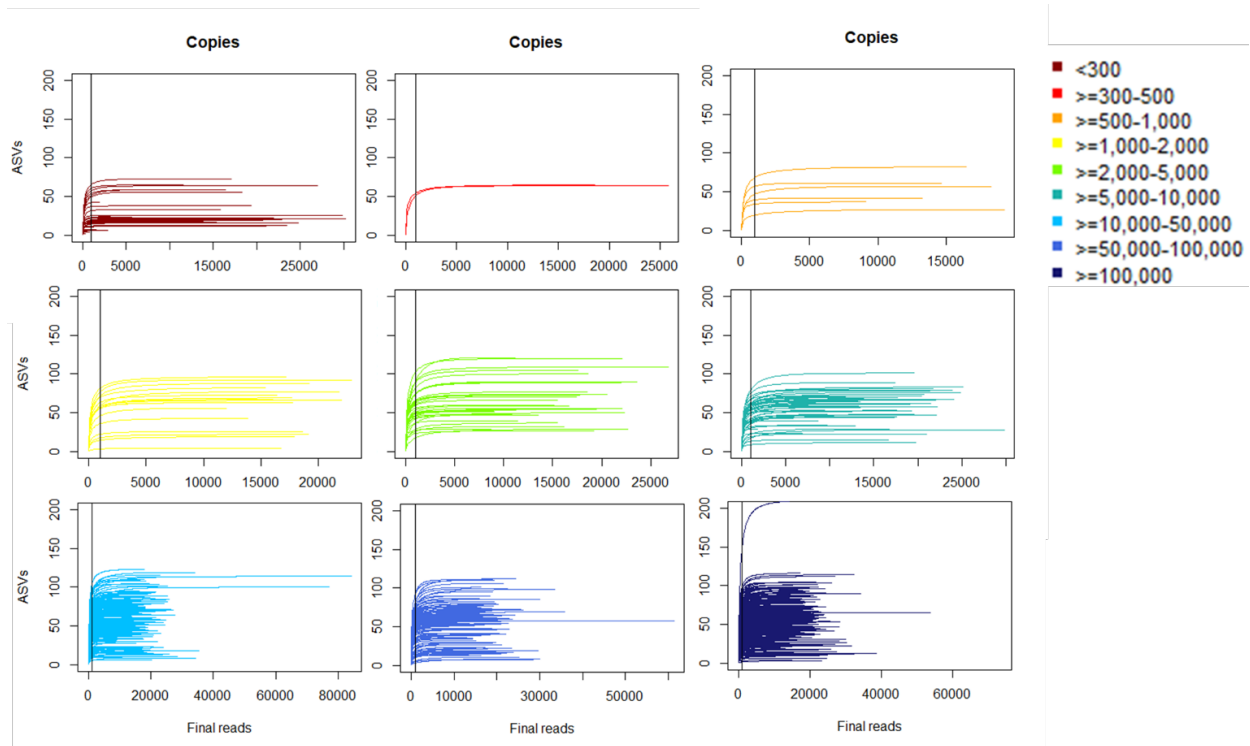
Appendix D. Figure S. 5. Ordination plots showing the spread of biological samples (n = 960) and the negative controls (primstore, n = 43) coloured by their number of reads.

Low read count samples (< 1,000 reads) may cluster with negative controls



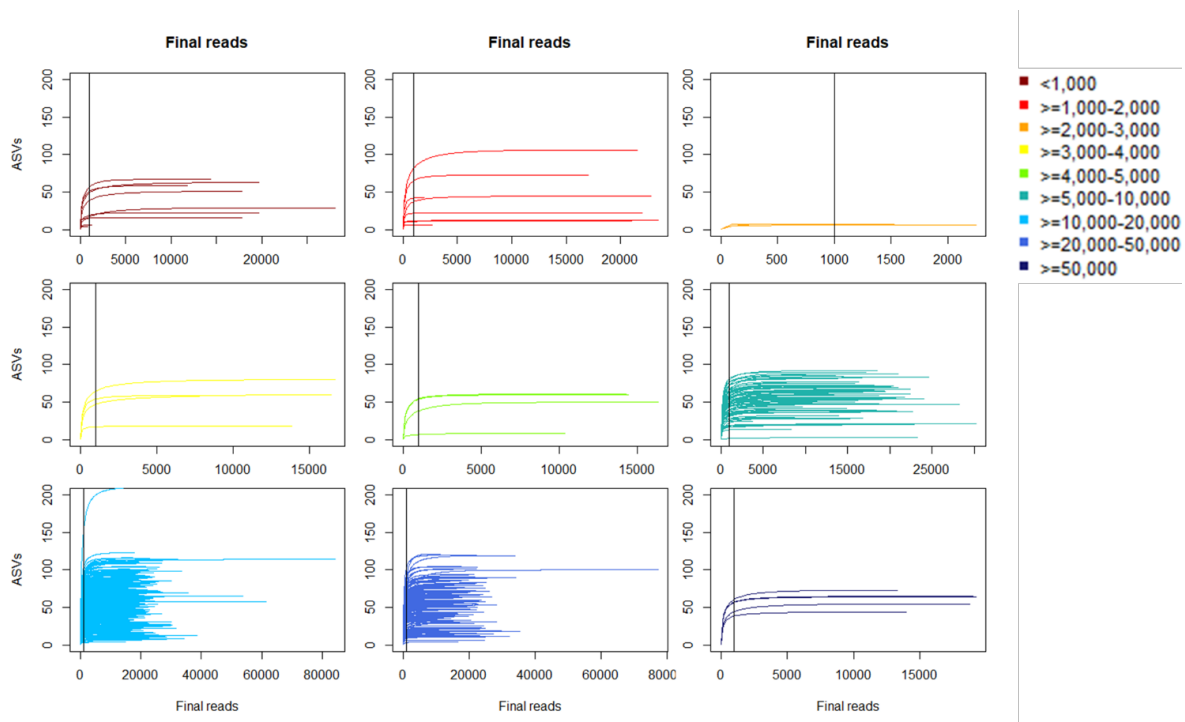
Appendix D. Figure S. 6. Ordination plots showing the spread of biological samples (n = 960) and the negative controls (primstore, n = 43) coloured by the age of the participant.

Specimens collected at younger ages do not seem to cluster with negative controls.



Appendix D. Figure S. 7. Rarefaction curves showing number of ASVs detected and 16S copies of samples.

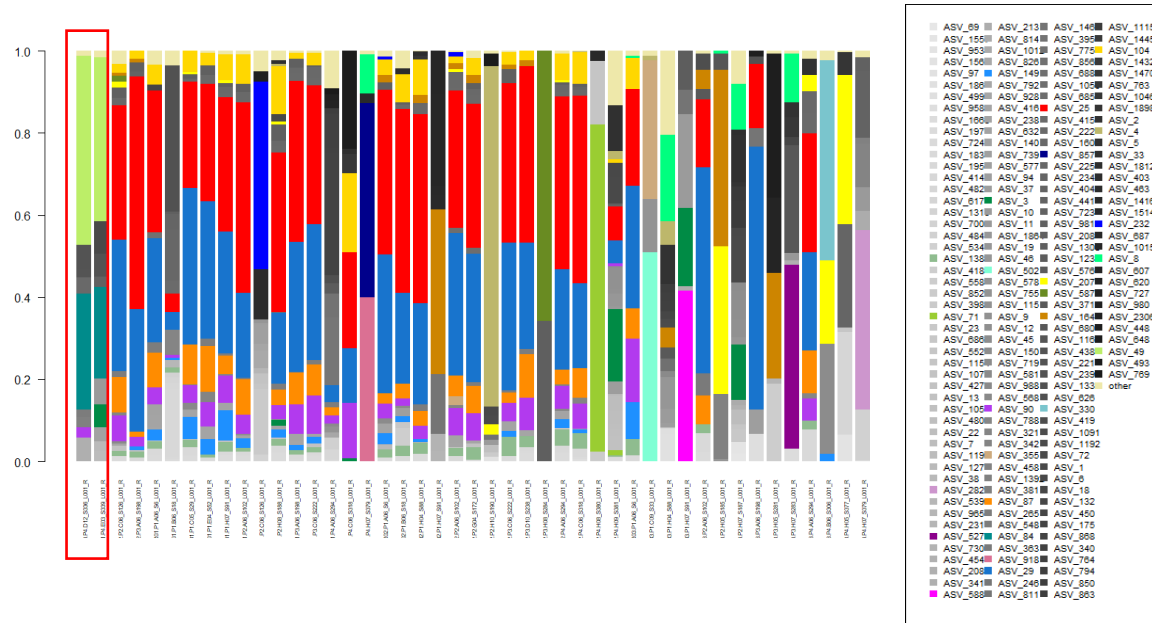
The number of ASVs detected negatively correlated with low 16S copies (< 500).



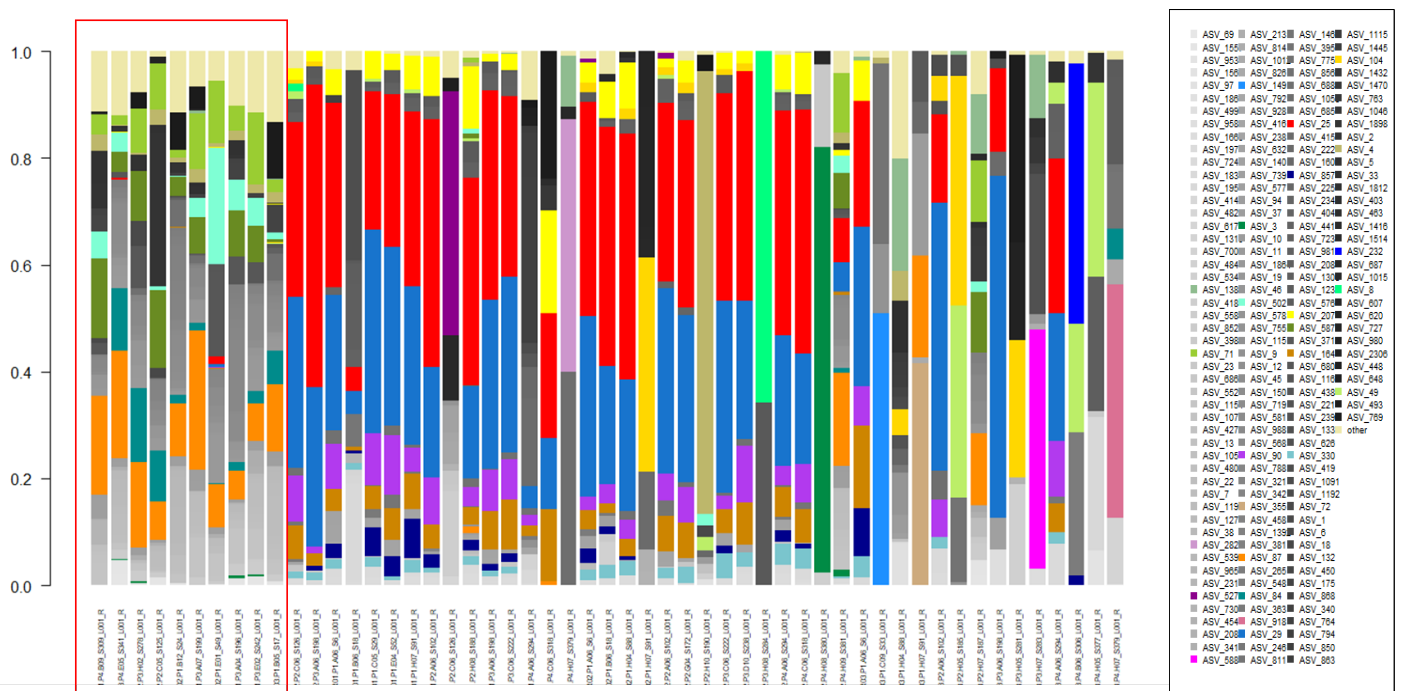
Appendix D. Figure S. 8. Rarefaction curves showing number of ASVs detected and number of reads of samples.

The number of ASVs detected negatively correlated with low read counts (< 1000).

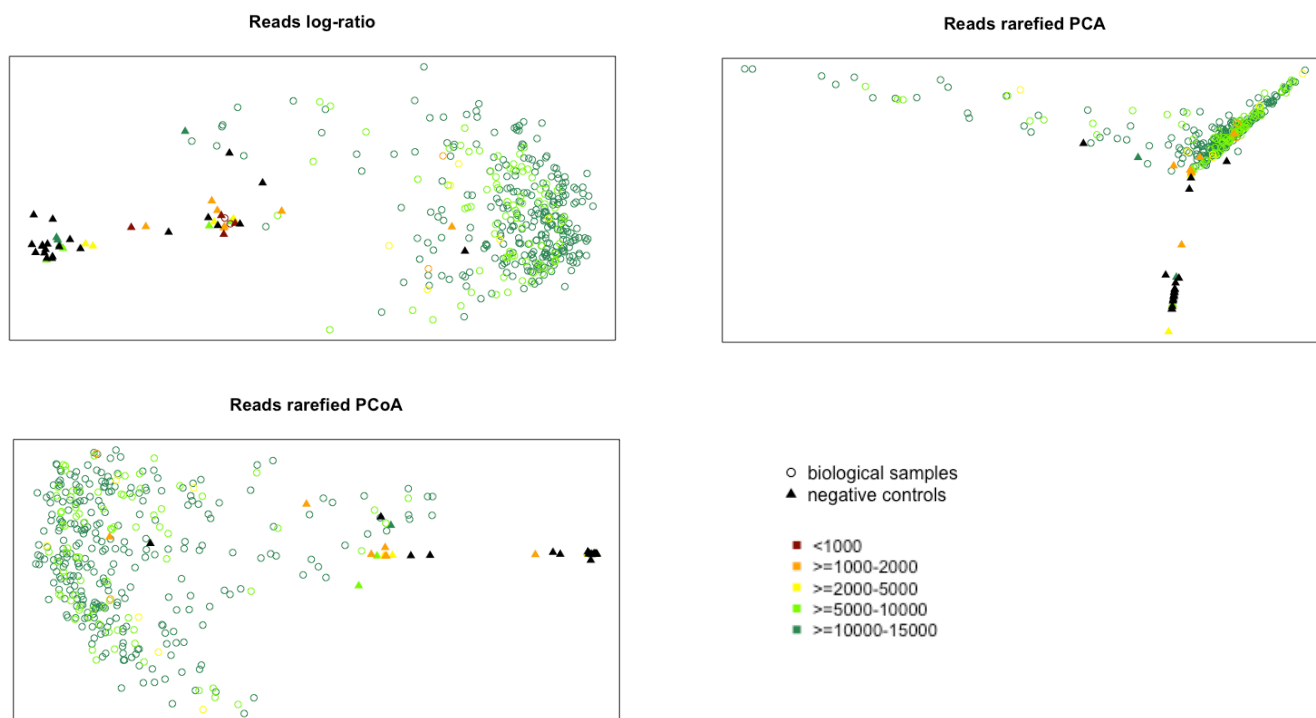
Next, we used a bar plot to visualise the ASV profiles of the two samples with less than 100 16S copies, Appendix D Figure S9 and the ten samples with > 100 to < 1000 16S copies, Appendix D Figure S10 in comparison to the primestore samples to further assess similarity in profiles. Finally, we constructed an ordination plots (log-ratio biplot, PCA and PCoA) on the subset of biological samples with low 16S copies, Appendix D Figure S11 and low read counts, Appendix D Figure S12 to assess w|



Appendix D. Figure S. 9. Bar plot showing the profiles of biological samples with < 100 16S copies (n = 2) in comparison to Primestores profiles (n = 43).

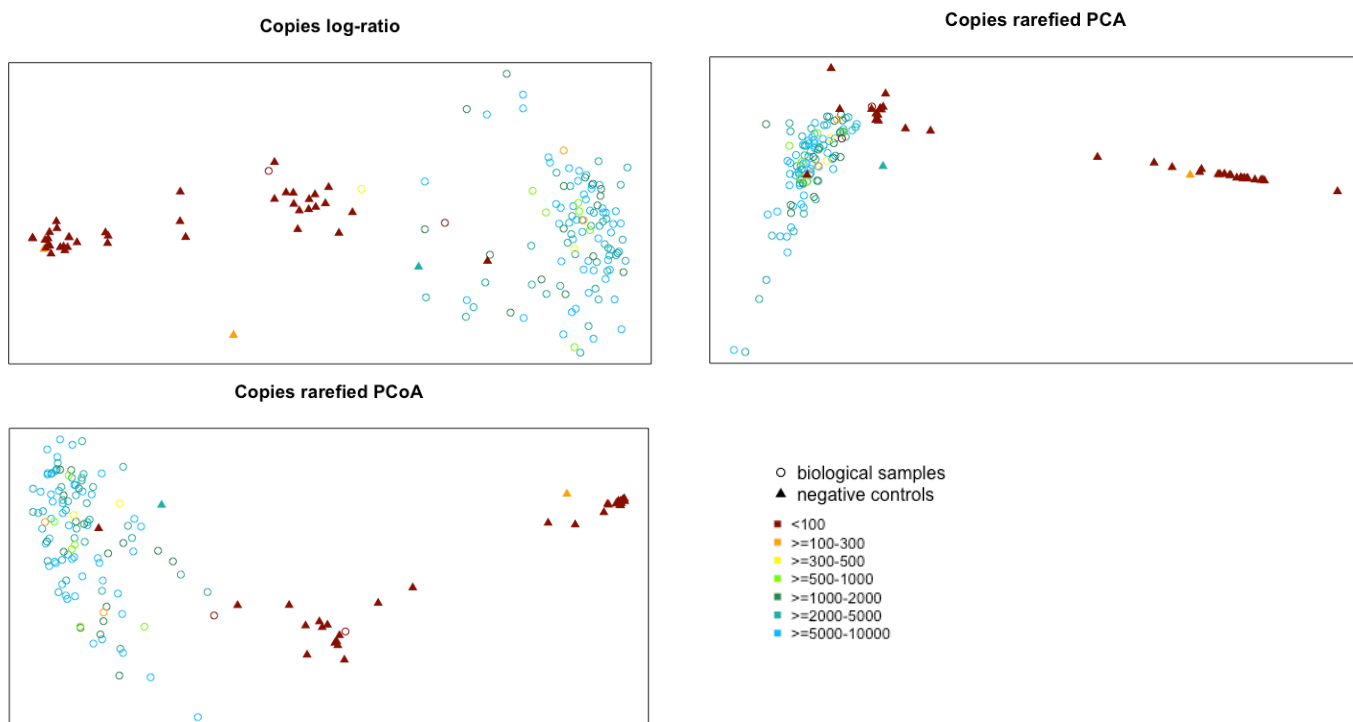


Appendix D. Figure S. 10. Bar plot showing the profiles of biological samples with > 100 to < 1000 16S copies (n = 10) in comparison to Primestores profiles (n = 43).



Appendix D. Figure S. 11. Ordination plots showing the profiles of a subset of biological samples with low 16S copies and the negative controls.

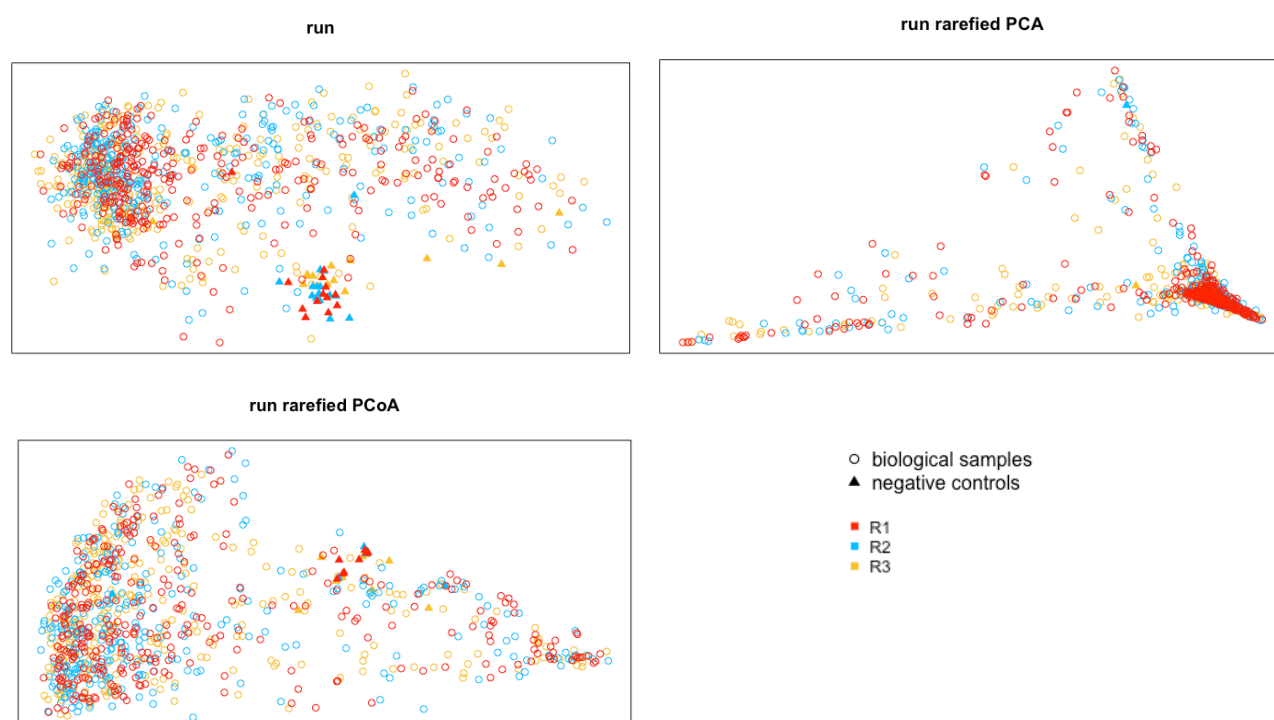
There is a separation between the biological samples with low 16S copies and the negative controls (Primestore, n = 43).



Appendix D. Figure S. 12. Ordination plots showing the profiles of a subset of biological samples with low reads and the negative controls.

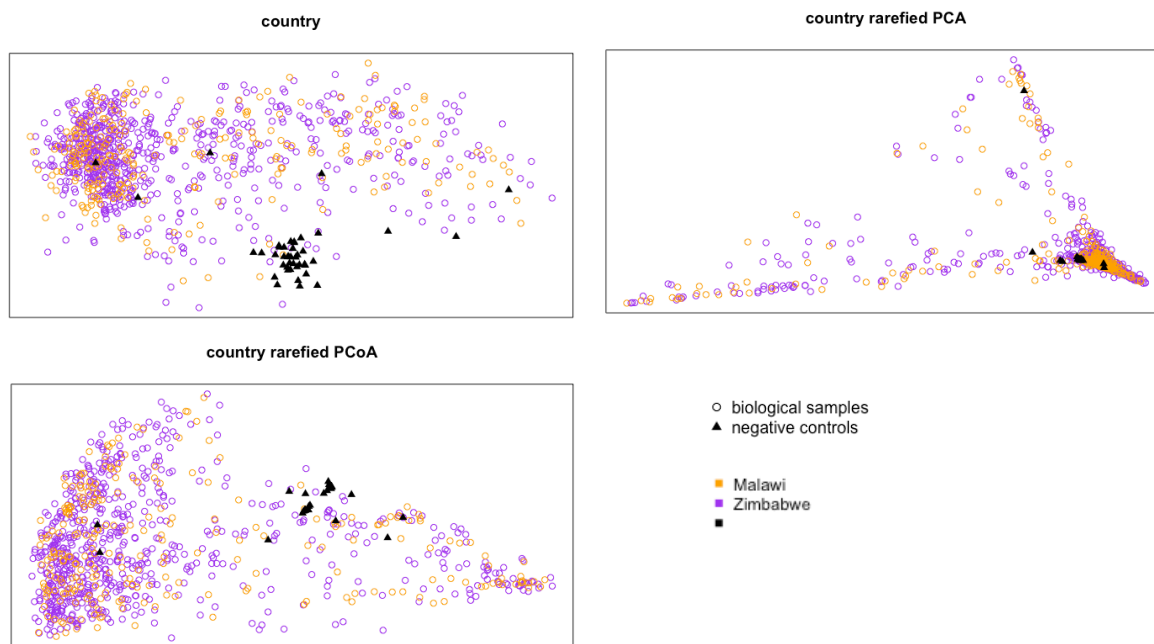
There is a separation between the biological samples with low reads and the negative controls (primestore, n = 43).

We continue to explore whether our specimens cluster together based on a) which of the three runs, the samples were processed in (run 1, 2 or 3), b) the country of origin of the sample (Zimbabwe or Malawi), c) the sampling time point or visit (baseline, 12 months (48 weeks) and 18 months (72 weeks)), d) sampling method (expectoration vs induction) and their relationship with negative controls, e) age especially for younger ages (6-10 years and 11 to 19 years). We did not observe any clustering pattern based on run numbers (Appendix D Figure S13), country (study site) from which the samples were collected (Appendix D Figure S14) or the visit or timepoint at which the sample was collected, Appendix D Figure S15. Specimens collected at younger ages do not seem to cluster with negative controls, Appendix D Figure S16. We detected no clustering based on any of these variables nor with negative controls. Hence no sample was excluded on the basis on any of these criteria.



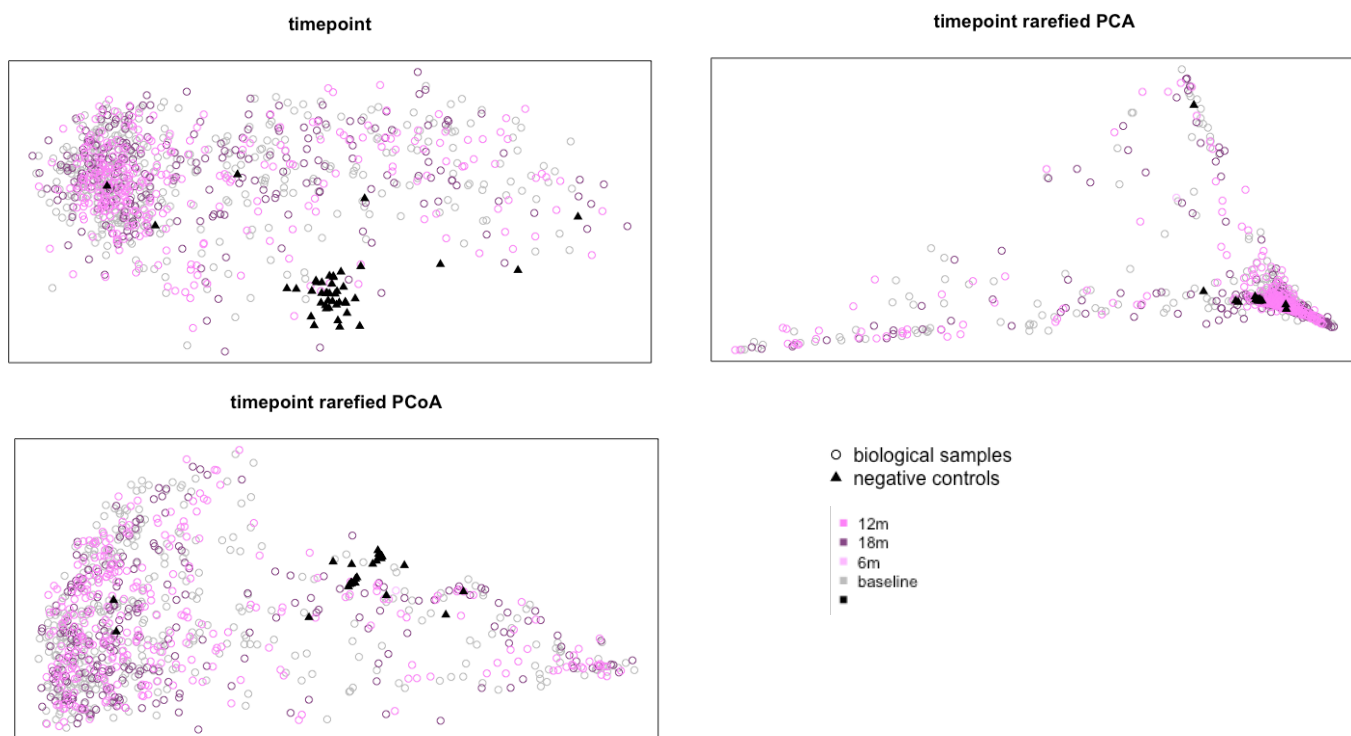
Appendix D. Figure S. 13. Ordination plots showing the spread of biological samples (n = 960) and the negative controls (primestore, n = 43) coloured by the run in which the sample was processed.

No clustering patterns based on run numbers.



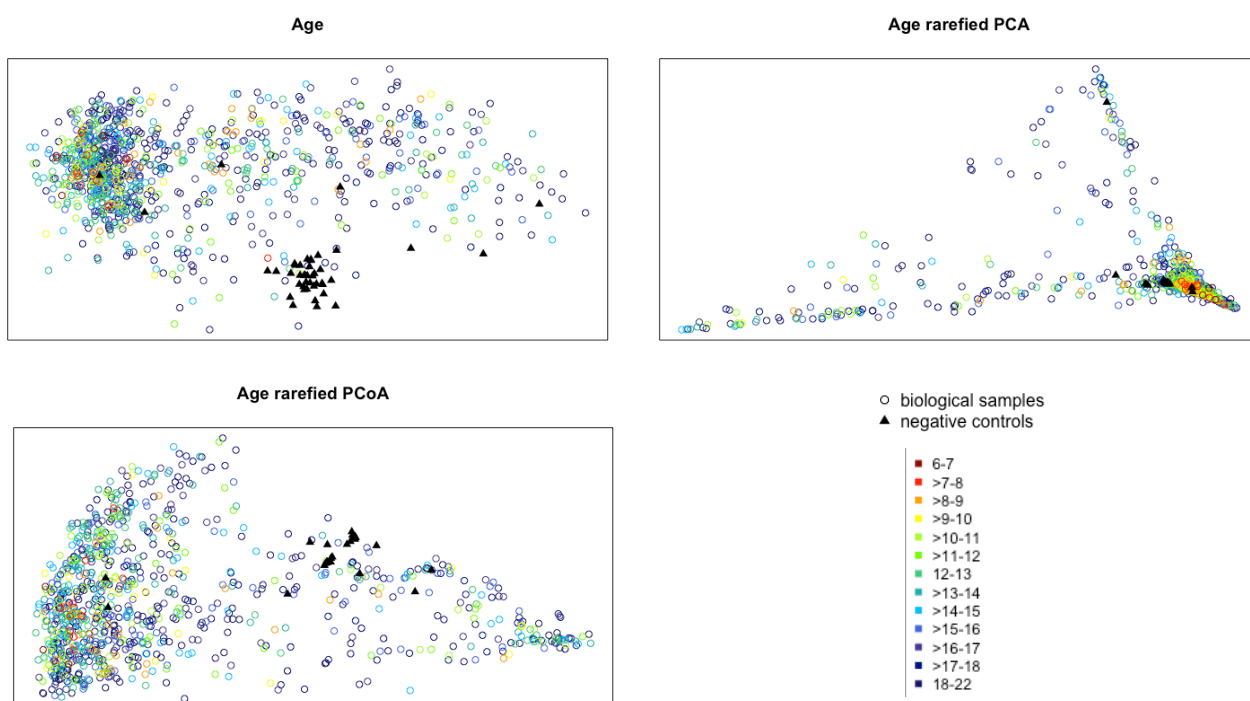
Appendix D. Figure S. 14. Ordination plots showing the spread of biological samples (n = 960) and the negative controls (primestore, n = 43) coloured by the country of sampling.

No clustering patterns based on country.



Appendix D. Figure S. 15. Ordination plots showing the spread of biological samples (n = 960) and the negative controls (primstore, n = 43) coloured by visit.

No clustering patterns based on visit.



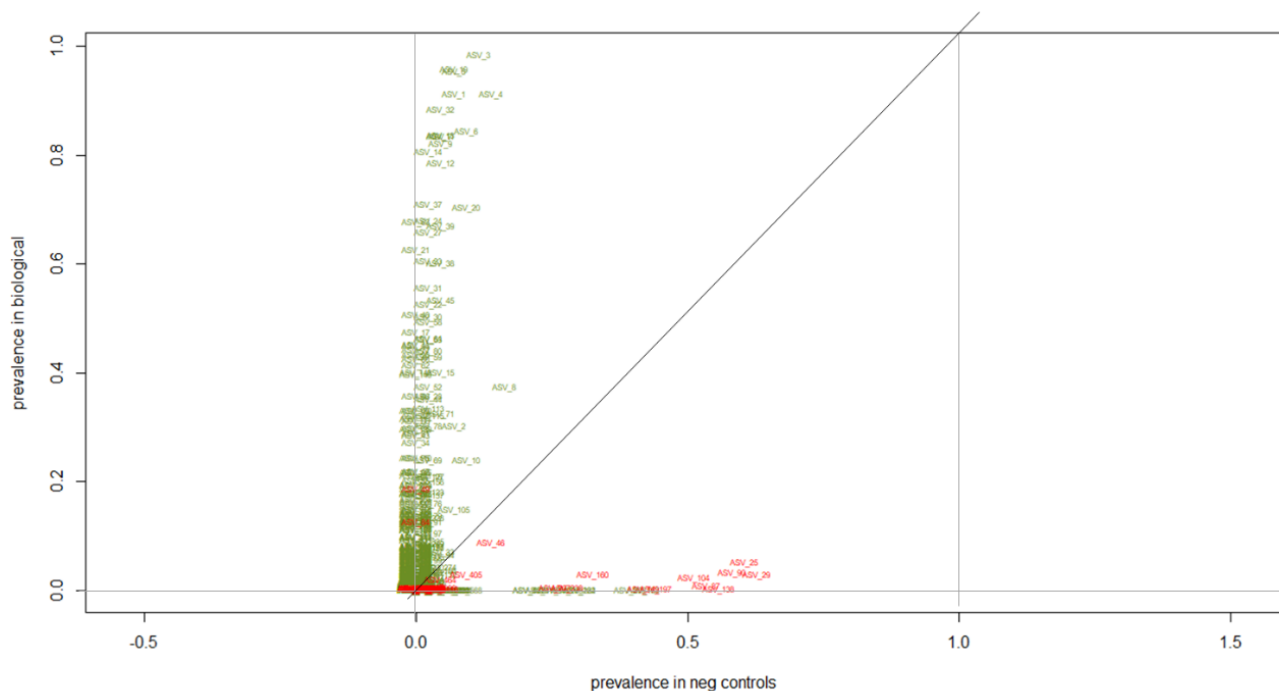
Appendix D. Figure S. 16. Ordination plots showing the spread of biological samples (n = 960) and the negative controls (primstore, n = 43) coloured by the age at sampling.

No clustering patterns based on age.

d. In silico correction of contamination and spurious ASVs

The number of biological samples remaining is 953 after excluding 7 samples ($2 < 100$ copies and $5 < 1000$ reads). The analysis using the DECONTAM R package was conducted on 43 Primestores (negative controls) and 953 biological samples. (Appendix D Figure S17). The exclusion of the seven biological samples reduced the initial number of ASVs with reads greater than zero from 3219 to 3216. A threshold of 0.4 was set for the *iscontaminant* function and 70 ASVs were returned as contaminants and were consequently removed. The number of ASVs used subsequently (> 0 reads and none from negative controls if occurring only here) was 2829.

Spurious ASVs defined as having ≤ 10 reads were identified from these 2829 and removed. These were 1161 ASVs representing 41% of the 2829 ASVs analysed here. They also represent $< 0.001\%$ of the profile of any given sample. Only 7 of these 1161 ASVs were detected in > 1 sample (i.e. 2 samples). 1668 ASVs remain from 953 biological samples for downstream analyses (15 ASVs are unclassified at phylum level). Of these 953 samples, 78 samples were from a comparison group not enrolled in the trial hence only 875 samples are reported in this paper.



Appendix D. Figure S. 17. Output from decontamination analysis using the DECONTAM R package.

The contaminants ASVs are shown in red at the bottom right and non-contaminants in green at the top left of the plot. A total of 70 ASVs have been identified as potential contaminants and removed.

Appendix D. Table S 2. List of 70 ASVs detected by the DECONTAM R package as potential contaminants based on comparison between biological samples and negative controls.

ASV	Genus	ASV	Genus	ASV	Genus
ASV_1344	<i>Actinomyces</i>	ASV_238	<i>Psychrobacillus</i>	ASV_160	<i>Sphingomonas</i>
ASV_1299	<i>Actinomyces</i>	ASV_739	<i>Aerococcus</i>	ASV_207	<i>Ralstonia</i>
ASV_197	<i>Mycobacterium</i>	ASV_82	<i>Lactobacillus</i>	ASV_1317	<i>Brachymonas</i>
ASV_1520	<i>Tropheryma</i>	ASV_1084	<i>Lactobacillus</i>	ASV_522	<i>Neisseria</i>
ASV_138	<i>Yonghaparkia</i>	ASV_46	<i>Staphylococcus</i>	ASV_1152	NA
ASV_558	<i>Kocuria</i>	ASV_1748	NA	ASV_104	<i>Methyloversatilis</i>
ASV_398	<i>Micrococcus</i>	ASV_1227	<i>Selenomonas</i>	ASV_338	<i>Klebsiella</i>
ASV_600	<i>Rothia</i>	ASV_841	<i>Dialister</i>	ASV_357	<i>Actinobacillus</i>
ASV_758	<i>Alloprevotella</i>	ASV_612	<i>Veillonella</i>	ASV_424	<i>Actinobacillus</i>
ASV_789	<i>Alloprevotella</i>	ASV_1072	<i>Veillonella</i>	ASV_1416	<i>Acinetobacter</i>
ASV_483	<i>Prevotella</i>	ASV_584	<i>Leptotrichia</i>	ASV_1085	<i>Moraxella</i>
ASV_510	<i>Prevotella</i>	ASV_825	<i>Leptotrichia</i>	ASV_317	<i>Moraxella</i>
ASV_658	<i>Prevotella</i>	ASV_1086	<i>Leptotrichia</i>	ASV_446	<i>Moraxella</i>
ASV_800	<i>Prevotella</i>	ASV_1142	<i>Leptotrichia</i>	ASV_1348	<i>Moraxella</i>
ASV_1296	<i>Prevotella</i>	ASV_1984	<i>Oceanivirga</i>	ASV_320	<i>Pseudomonas</i>
ASV_1376	<i>Prevotella</i>	ASV_1322	TM7x	ASV_1208	<i>Pseudomonas</i>
ASV_1410	<i>Prevotella</i>	ASV_1654	NA	ASV_405	<i>Pseudoxanthomonas</i>
ASV_1701	<i>Prevotella</i>	ASV_90	<i>Bosea</i>	ASV_49	<i>Stenotrophomonas</i>
ASV_946	<i>Capnocytophaga</i>	ASV_87	*ANPR	ASV_1270	<i>Treponema</i>
ASV_1184	<i>Bergeyella</i>	ASV_464	ANPR	ASV_1342	<i>Treponema</i>
ASV_1353	<i>Lentimicrobium</i>	ASV_84	<i>Bradyrhizobium</i>	ASV_1371	<i>Treponema</i>
ASV_728	<i>Campylobacter</i>	ASV_29	<i>Paracoccus</i>	ASV_1790	<i>Treponema</i>
ASV_735	NA	ASV_25	<i>Blastomonas</i>		
ASV_149	<i>Bacillus</i>	ASV_579	<i>Novosphingobium</i>		

*ANPR= Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium

2.2 Results of differential abundance of taxa testing

2.2.1 Azithromycin and Placebo

a. AZM and Placebo at baseline

No taxon was found to be differentially abundant by any of the methods.

b. AZM and Placebo at 48 weeks

Appendix D. Table S 3. Results of differential abundance testing of bacterial taxa from azithromycin and placebo samples from 48 weeks using 10 methods.

Results of all the methods are capture in Table S3 located at <https://figshare.com/s/6a4af26f8f696718de38> or <https://doi.org/10.25375/uct.19172150>

c. AZM and Placebo at 72 weeks.

Treponema was detected by Ancom2 and *Lautropia* by Deseq2 as differentially abundant taxa. None of the other methods detected a differentially abundant taxon.

Appendix D. Table S 4. Results of differential abundance testing of bacterial taxa from azithromycin and placebo samples from 72 weeks using Deseq2.

Genus	baseMean	log2FoldChange	lfcSE	p value	Adjusted p value
<i>Lautropia</i>	149.33	-1.42	0.35	1.44E-06	0.0002

Appendix D. Table S 5. Results of differential abundance testing of bacterial taxa from azithromycin and placebo samples from 72 weeks using Ancom-II.

Genus	W	detected_0.9	detected_0.8	detected_0.7	detected_0.6
<i>Treponema</i>	41	FALSE	TRUE	TRUE	TRUE

2.2.2. Azithromycin arm only

a. Azithromycin at baseline and 48 weeks

Appendix D. Table S 6. Results of differential abundance testing of bacterial taxa from samples from the azithromycin arm at baseline and 48 weeks using 10 methods.

Results of all the methods are capture in Table S6 located at <https://figshare.com/s/087eb661dba977926963> or <https://doi.org/10.25375/uct.19172162>

b. Azithromycin at 48 and 72 weeks

Appendix D. Table S 7. Results of differential abundance testing of bacterial taxa from samples from the azithromycin arm at 48 and 72 weeks using 10 methods.

Results of all the methods are capture in Table S7 located at <https://figshare.com/s/378daaaed6e97aa00085> or <https://doi.org/10.25375/uct.19172165>

c. Azithromycin at baseline and 72 weeks

No taxon was found to be differentially abundant by any of the methods.

2.2.3 Placebo arm only

a. Placebo at baseline and 48 weeks

No taxon was found to be differentially abundant by any of the methods.

b. Placebo at 48 and 72 weeks

Moraxella was detected as differentially abundant by Deseq2. None of the other methods detected a differentially abundant taxon.

Appendix D. Table S 8. Results of differential abundance testing of bacterial taxa from placebo samples from 48 and 72 weeks using Deseq2.

Genus	baseMean	log2FoldChange	lfcSE	p value	Adjusted p value
<i>Moraxella</i>	242.26	-9.05E-06	0.001	9.54E-07	1.31E-04

c. Placebo at baseline and 72 weeks

Moraxella detected by Deseq2. None of the other methods detected a differentially abundant taxon.

Appendix D. Table S 9. Results of differential abundance testing of bacterial taxa from placebo samples from baseline and 72 weeks using Deseq2.

Genus	baseMean	log2FoldChange	lfcSE	p value	Adjusted p value
<i>Moraxella</i>	231.49	-2.42	0.59	3.82E-05	5.20E-03

References

1. Kaul A, Mandal S, Davidov O, Peddada SD. Analysis of microbiome data in the presence of excess zeros. *Front Microbiol.* 2017; 8:2114.
2. Fernandes AD, Reid JN, Macklaim JM, McMurrough TA, Edgell DR, Gloor GB. Unifying the analysis of high-throughput sequencing datasets: characterizing RNA-seq, 16S rRNA gene sequencing and selective growth experiments by compositional data analysis. *Microbiome.* 2014; 2:15.
3. Love M, Anders S, Huber W. Differential analysis of count data—the DESeq2 package. *Genome Biol.* 2014; 15:10–1186.
4. Lin H, Peddada SD. Analysis of compositions of microbiomes with bias correction. *Nat Commun.* 2020; 11:3514.
5. Martin BD, Witten D, Willis AD. Modeling microbial abundances and dysbiosis with beta-binomial regression. *Ann Appl Stat.* 2020; 14:94.
6. Mallick H, Rahnavard A, McIver LJ, Ma S, Zhang Y, Nguyen LH, et al. Multivariable association discovery in population-scale meta-omics studies. *PLOS Comput Biol.* 2021; 17: e1009442.
7. Schneeberger PHH, Prescod J, Levy L, Hwang D, Martinu T, Coburn B. Microbiota analysis optimization for human bronchoalveolar lavage fluid. *Microbiome.* 2019; 7:141.
8. Claassen-Weitz S, Gardner-Lubbe S, Mwaikono KS, du Toit E, Zar HJ, Nicol MP. Optimizing 16S rRNA gene profile analysis from low biomass nasopharyngeal and induced sputum specimens. *BMC Microbiol.* 2020; 20:113–113.

APPENDIX E: Publications, Conference presentations and statement by the candidate

Appendix E. Publications, Conference presentations and statement by the candidate

Publications produced during PhD but not included in this thesis

1. Abotsi RE*, Allahi I*, et al, Human Microbiota Research in Africa: A Systematic Review Reveals Gaps and Priorities for Future Research. Microbiome December 2021

*co-first authors

Statement by the candidate: The candidate contributed toward study conceptualization, conducted the literature search and screening, extracted and analysed the data, and wrote the draft of the manuscript.

2. Abotsi RE et al, Impact of Long-Term Azithromycin Therapy on Carriage and Resistance of Respiratory Bacteria Among Children with HIV-Associated Chronic Lung Disease: A Randomised Controlled Trial. Preprint SSRN Electronic Journal <http://dx.doi.org/10.2139/ssrn.3863359>

Statement by the candidate: The candidate contributed toward study conceptualization, conducted the laboratory experiments, all statistical analysis and wrote all versions of the manuscript.

3. Ndhlovu G, Abotsi RE, et al, Molecular Epidemiology of *Staphylococcus aureus* in African Children from Rural and Urban Communities with Atopic Dermatitis. BMC Infect Dis 2021 21:348 <https://doi.org/10.1186/s12879-021-06044-4>

Statement by the candidate: The candidate contributed toward study conceptualization and co-supervised this study. The candidate also assisted with experiments, and validation of statistical analysis, writing and critical reviewed of the manuscript.

4. Ferrand R...Abotsi RE et al. Once-weekly azithromycin versus placebo in children with HIV-associated chronic lung disease (BREATHE): a randomised, double-blinded, Phase 3

trial, JAMA Netw Open. 2020;3(12): e2028484.
<https://doi.org/10.1001/jamanetworkopen.2020.28484>

Statement by the candidate: The candidate critically reviewed the manuscript.

Conference posters and presentations

1. Mushunje P, Dube FS, Nicol M, **Abotsi RE** et al, Prevalence and colonization density of respiratory microbes in children with and without HIV-associated chronic lung disease: a case-control study. 12th International Symposium on Pneumococci and Pneumococcal Diseases (ISPPD-12), 19–23 June 2022, Toronto, Canada, *Oral poster presentation*.

Statement by the candidate: The candidate is the co-supervisor of the student on the project (first author) and contributed toward study conceptualization, assisted with the laboratory experiments, statistical analysis, abstract writing and poster design for the conference.

2. **Abotsi RE**, Dube FS Nicol M et al, Sputum Bacteriome in Children with HIV-associated Chronic Lung Disease Correlate with Lung Function and are altered by Long-Term Azithromycin Treatment, ASM Microbe June 9 – 13, 2022, Washington DC, *Oral poster presentation*.

Statement by the candidate: The candidate contributed toward study conceptualization, conducted the all laboratory experiments, all statistical analysis, designed the poster and will be presenting the poster at the conference.

3. **Abotsi RE**, Nicol M, Dube FS et al, Impact of long-term azithromycin therapy on carriage and antibiotic resistance of respiratory bacteria among children with HIV-associated chronic lung disease: a randomised controlled trial, 12th World Congress of the World Society for Paediatric Infectious Diseases (WSPID), February 22–24, 2022- *Oral presentation*.

Statement by the candidate: The candidate contributed toward study conceptualization, conducted the laboratory experiments, all statistical analysis, designed the slides and presented the paper at the conference.

4. **Abotsi RE**, et al Old solutions for new problems, a public engagement poster November 2021, <https://twitter.com/UCTIDM/status/1462664834093301760?s=20>

Statement by the candidate: The candidate conceptualized and wrote the first draft of the public engagement piece.

5. **Abotsi RE**, Nicol M, Dube FS et al, Impact of long-term azithromycin therapy on respiratory microbes amongst African children with HIV-associated chronic lung disease: a randomised controlled trial, 46th Annual UCT Department of Medicine Research Symposium, 6–7 October 2021- *Oral presentation*.

Statement by the candidate: The candidate contributed toward study conceptualization, conducted the laboratory experiments, all statistical analysis, designed the slides and presented the paper at the conference.

6. **Abotsi RE**, Nicol M, Dube FS et al, The impact of azithromycin therapy on antimicrobial resistance in chronic lung disease MCB Research Day 2021 organised by University of Cape Town Departmental of Molecular and Cell Biology, 20–21 September, 2021- *Oral presentation*.

Statement by the candidate: The candidate contributed toward study conceptualization, conducted the laboratory experiments, all statistical analysis, designed the slides and presented the paper at the conference. The candidate won the best presentation award.

7. **Abotsi RE**, Nicol M, Dube FS et al, Long-term azithromycin increases antimicrobial resistance of *Streptococcus pneumoniae* and *Staphylococcus aureus* in African children with HIV-associated chronic lung disease, 5th NIHR-MPRU Annual Conference 2021, 6–7 May 2021 - *Oral presentation*.

Statement by the candidate: The candidate contributed toward study conceptualization, conducted the laboratory experiments, all statistical analysis, designed the slides and presented the paper at the conference.

8. **Abotsi RE**, Nicol M, Dube FS et al, Impact of azithromycin therapy on antimicrobial resistance. Zim-LSHTM Research Partnership Research Dissemination Meeting, Monday 30th November 2020 - *Oral presentation*.

Statement by the candidate: The candidate contributed toward study conceptualization, conducted the laboratory experiments, all statistical analysis, designed the slides and presented the paper at the conference.

9. **Abotsi RE**, Nicol M, Dube FS et al, Pneumococcal carriage in African children with HIV-associated chronic lung disease. MCB Research Day 2020 organised by University of Cape Town Departmental of Molecular and Cell Biology - *Oral presentation*.

Statement by the candidate: The candidate contributed toward study conceptualization, conducted the laboratory experiments, all statistical analysis, designed the slides and presented the paper at the conference.

10. **Abotsi RE**, Nicol M, Dube FS et al, Pneumococcal carriage in African children with HIV-associated chronic lung disease. 13th Annual Science Postgraduate Symposium, 16–17 September 2020 organised by University of Cape Town and University of Stellenbosch - *Oral presentation*.

Statement by the candidate: The candidate contributed toward study conceptualization, conducted the laboratory experiments, all statistical analysis, designed the slides and presented the paper at the conference. The candidate won the best presentation award.

11. Ndhlovu G, **Abotsi RE**, et al, The epidemiology and population structure of *Staphylococcus aureus* in paediatric atopic dermatitis in rural and urban South African: a case-control study. Int J Infect Dis 101, supp 1:413, 2020 <https://doi.org/10.1016/j.ijid.2020.09.1083> - *Poster presentation*.

Statement by the candidate: The candidate contributed toward study conceptualization and co-supervised this study. The candidate also assisted with experiments, and validation of statistical analysis, and critical reviewed of the poster.

12. Ndhlovu G, **Abotsi RE**, et al, The epidemiology and population structure of *Staphylococcus aureus* in paediatric atopic dermatitis in rural and urban South African: a case-control study. FIDSSA 2019. Johannesburg, South Africa: S Afr J Infect Dis, 2019 - *Oral presentation*.

Statement by the candidate: The candidate contributed toward study conceptualization and co-supervised this study. The candidate also assisted with experiments, and validation of statistical analysis, and critical reviewed the slides.

13. Dube F, Lo SW, Chaguza C, **Abotsi RE** et al, Genome-based approach to characterise pneumococcal carriage dynamics post PCV-13 implementation in an African birth cohort: The Drakenstein Child Health Study. FIDSSA 2019. Johannesburg, South Africa: S Afr J Infect Dis, 2019 - *Oral presentation*.

Statement by the candidate: The candidate critically reviewed the abstract presented at this conference.

14. Allahi I, **Abotsi RE** et al, Human Microbiota Research in Africa: A Systematic Survey of the Literature. ISCBAFRICA ASBCB November 2019, Kwame Nkrumah University of science and Technology, Ghana- *Poster presentation*.

Statement by the candidate: The candidate contributed toward study conceptualization, conducted the literature search and screening, extracted, and analysed the data, and co-wrote the abstract.

15. Malebo R, Ndhlovu G, **Abotsi RE**, Dube F, The colonisation dynamics of *Staphylococcus aureus* in the respiratory tract of HIV-infected children with chronic lung diseases. Infectious Disease and Immunology Research Day, 11th November 2019. University of Cape Town, South Africa- *Oral presentation*.

Statement by the candidate: The candidate conceptualised and supervised this study, trained and assisted with laboratory experiments, guided data analysis, thesis write-up and design and review of poster for presentation.

16. Mushunje P, Mokupi R, **Abotsi RE**, Dube F, *Streptococcus pneumonia* colonisation dynamics in children on long-term azithromycin therapy for chronic lung diseases. Molecular and Cell

Biology Research Day, 29th August 2019. University of Cape Town, South Africa - *Oral presentation.*

Statement by the candidate: The candidate conceptualised and supervised this study, trained and assisted with laboratory experiments, guided data analysis, thesis write-up, design and review of poster for presentation.

17. Ndidi L, **Abotsi RE**, Dube F, Characterisation of *Moraxella catarrhalis* isolated from the respiratory tract of HIV-infected children with chronic lung disease. Molecular and Cell Biology Research Day, 29th August 2019. University of Cape Town, South Africa - *Oral presentation.*

Statement by the candidate: The candidate conceptualised and supervised this study, trained and assisted with laboratory experiments, guided data analysis, thesis write-up, design and review of poster for presentation.

18. Ndhlovu G, **Abotsi RE**, et al, Epidemiology and population structure of *Staphylococcus aureus* in paediatric atopic dermatitis: A case-control study. Molecular and Cell Biology Research Day, 29th August 2019. University of Cape Town, South Africa - *Oral presentation.*

Statement by the candidate: The candidate contributed toward study conceptualization and co-supervised this study. The candidate also assisted with experiments, and validation of statistical analysis, and critical reviewed of the slides.

19. **Abotsi RE** et al. The contribution of the microbiome to vaso-occlusive crises in sickle cell disease patients. EMBL-EBI Metagenomics Bioinformatics Workshop. 2-5 October 2017 Hinxton, Cambridge, UK - *Poster presentation.*

Statement by the candidate: The candidate conceptualised the study, designed the poster and presented it at the workshop.

APPENDIX F: Ethics and Biosafety clearance

Appendix F. Ethics and Biosafety clearance



UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee



Room E53-46 Old Main Building
Grootes Schuur Hospital
Observatory 7925
Telephone [021] 406 6492
Email: sumayah.arietdien@uct.ac.za
Website: www.health.uct.ac.za/fhs/research/humanethics/forms

27 May 2019

HREC REF: 092/2019

Dr F Dube
Division of Molecular & Cell Biology
Room 227
University Avenue-Upper Campus

Dear Dr Dube

PROJECT TITLE: THE EFFECT OF LONG-TERM AZITHROMYCIN TREATMENT ON THE RESPIRATORY MICROBIOTA OF HIV-INFECTED CHILDREN WITH AND WITHOUT CHRONIC LUNG DISEASE V1.0 DATED 28TH NOVEMBER 2018. (SUB-STUDY LINKED TO 754/2015) (PHD CANDIDATE: MS RE ABOTSI)

Thank you for your response letter dated 27 March 2019, addressing the issues raised by the Human Research Ethics Committee (HREC).

It is a pleasure to inform you that the HREC has **formally approved** the above-mentioned study.

Approval is granted for one year until the 30 May 2020.

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.

(Forms can be found on our website: www.health.uct.ac.za/fhs/research/humanethics/forms)

We acknowledge that the student: Ms Regina Abotsi will also be involved in this study.

Please quote the HREC REF in all your correspondence.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please note that for all studies approved by the HREC, the principal investigator **must** obtain appropriate Institutional approval, where necessary, before the research may occur.

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, FHS HUMAN RESEARCH ETHICS COMMITTEE

Federal Wide Assurance Number: FWA00001637.



21 SEP 2020

FHS017: Annual Progress Report / Renewal**Record Reviews/Audits/Collection of Biological Specimens/Repositories/Databases/Registries**

HREC office use only (FWA00001637; IRB00001938)			
This serves as notification of annual approval, including any documentation described below.			
☒ Approved	Annual progress report	Approved until/next renewal date	30.5.21
☐ Not approved	See attached comments		
Signature Chairperson of the HREC/ Designee		Date Signed	21/9/20

Note: Please note that incomplete submissions will not be reviewed.
Please email this form and supporting documents (if applicable) in a combined pdf-file to hrec-enquiries@uct.ac.za.

Please clarify your plan for research-related activities during COVID-19 lockdown

Principal Investigator to complete the following:**1. Protocol information**

Date (when submitting this form)	6th April 2020		
HREC REF Number	092/2019	Current Ethics Approval was granted until	30th May 2020
Protocol title	The Effect of Long-Term Azithromycin Treatment on the Respiratory Microbiota of HIV-infected Children with and without Chronic Lung Disease V1.0 Dated 28 th November 2018 (Sub-Study linked to 754/2015) (PhD Candidature: Ms. RE Abotsi)		
Principal Investigator	Dr. Felix Dube		
Department / Office Internal Mail Address	Department of Molecular and Cell Biology University of Cape Town 22 University Avenue North Molecular Biology Building Second floor, Room 227 Upper Campus, Rondebosch, 7700 Email: sizwe.dube@uct.ac.za Tel: +27 021 650 3861		
1.1 Does this protocol receive US Federal funding?			☐ Yes ☒ No

2. Protocol status (tick ✓)

☒	Research-related activities are ongoing
☐	Data collection is complete, data analysis only
Please indicate (in the block below) the titles and HREC reference numbers of any projects currently making use of the Database/registry/repository.	

3. Protocol summary

Total number of records or specimens collected, reviewed or stored since the original approval	1978
Total number of records or specimens collected, reviewed or stored since last progress report	0



UNIVERSITY OF CAPE TOWN
UNIVERSITEIT VAN KAPSTAD

HUMAN RESEARCH
ETHICS COMMITTEE

10 JUN 2021

HEALTH SCIENCES FACULTY
UNIVERSITY OF CAPE TOWN

FACULTY OF HEALTH SCIENCES

Human Research Ethics Committee



FHS017: Annual Progress Report / Renewal

Record Reviews/Audits/Collection of Biological Specimens/Repositories/Databases/Registries

HREC office use only (FWA00001637; IRB00001938)			
This serves as notification of annual approval, including any documentation described below.			
☒ Approved	Annual progress report	Approved until/next renewal date	30.06.2022
☐ Not approved	See attached comments		
Signature Chairperson of the HREC/ Designee			Date Signed 10/6/21

Note: Please note that incomplete submissions will not be reviewed.
Please email this form and supporting documents (if applicable) in a combined pdf-file to hrec-enquiries@uct.ac.za.

Please clarify your plan for research-related activities during COVID-19 lockdown

Principal Investigator to complete the following:

1. Protocol information

Date (when submitting this form)	8th June 2021		
HREC REF Number	092/2019	Current Ethics Approval was granted until	30 th May 2021
Protocol title	The Effect of Long-Term Azithromycin Treatment on the Respiratory Microbiota of HIV-infected Children with and without Chronic Lung Disease V1.0 Dated 28 th November 2018 (Sub-Study linked to 754/2015) (PhD Candidature: Ms. RE Abotsi)		
Principal Investigator	Dr. Felix Dube		
Department / Office Internal Mail Address	Department of Molecular and Cell Biology University of Cape Town 22 University Avenue North Molecular Biology Building Second floor, Room 227 Upper Campus, Rondebosch, 7700 Email: sizwe.dube@uct.ac.za Tel: +27 021 650 3861		
1.1 Does this protocol receive US Federal funding?			☐ Yes ☒ No

2. Protocol status (tick ✓)

☒	Research-related activities are ongoing
☐	Data collection is complete, data analysis only
Please indicate (in the block below) the titles and HREC reference numbers of any projects currently making use of the Database/registry/repository.	

3. Protocol summary

Total number of records or specimens collected, reviewed or stored since the original approval	1978
Total number of records or specimens collected, reviewed or stored since last progress report	0



Research Office

Office of Research Integrity

Research Office
Allan Cormack House
2 Rhodes Avenue
Mowbray
7700

Tel: +27 (0) 21 650 1256

E-mail: research.integrity@uct.ac.za

Institutional Biosafety Committee (IBC)

15 December 2020

IBC REFERENCE NUMBER: IBC050-2020

Dr Felix Dube
Department of Molecular & Cell Biology
Molecular Biology Building
Faculty of Science
University of Cape Town

Sent via email: sizwe.dube@uct.ac.za

Project Title: The effect of long-term azithromycin treatment on the respiratory microbiota of HIV-infected children with chronic lung disease (sub-study of the BREATHe study, HREC 754/2015)

Dear Dr Dube,

The IBC considered your application, as per the above title, at a quorate meeting on 10 December 2020. I am pleased to inform you that your application has been granted **approval**.

Approval duration:

Approval is granted for one (1) year, as of the approval date of the study 10 December 2020, and may be extended to a maximum of three (3) years pending the submission of a satisfactory progress report and renewal request, as stipulated below.

Submission of reports:

Please submit a progress report and application for project renewal to the IBC on or before the 30th of September 2021. The IBC reviews all annual reports and requests for project renewals at the end of each calendar year (not project year), hence some projects may need to submit a report early for the first cycle. It is also up to each investigator to ensure that they comply with funder requirements for annual re-approvals. Please submit a standard final report if the study is completed within the approval period.

Amendments:

Any amendments to the protocol, including changes to the PI, must be approved by the IBC, via the Faculty Biosafety Committee which reviewed the initial application.

This study may not start until all necessary approvals are in place. It is the responsibility of the investigators to ensure they are clear as to what approvals, permits, permissions etc are required.

Please feel free to contact Werner Van Der Ross (werner.vanderross@uct.ac.za) should you have any queries. Please quote the IBC reference number in all correspondence.

Yours sincerely,

A handwritten signature in purple ink that reads "A. Williamson".

Professor Anna-Lise Williamson, PhD

Chair: Institutional Biosafety Committee
University of Cape Town

APPENDIX G: Turnitin Report

Appendix G. Turnitin Report

Chapter one, two and six were run through the Turnitin software and the summary of the report is shown below. Chapter three, four and five were not included because of they have already been published.

abtreg001:Non-published_sections_thesis.docx			
ORIGINALITY REPORT			
15%	12%	11%	3%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS
PRIMARY SOURCES			
1	Regina E. Abotsi, Mark P. Nicol, Grace McHugh, Victoria Simms et al. "The impact of long-term azithromycin on antibiotic resistance in HIV-associated chronic lung disease", ERJ Open Research	1%	
Internet Source			
2	www.ncbi.nlm.nih.gov	1%	
Internet Source			
3	Regina E. Abotsi, Felix S. Dube, Andrea M. Rehman, Shantelle Claassen-Weitz et al. "Sputum bacterial load and bacterial composition correlate with lung function and are altered by long term azithromycin treatment in children with HIV-associated chronic lung disease", Research Square Platform LLC, 2022	1%	
Publication			
4	worldwidescience.org	1%	
Internet Source			
5	www.science.gov	1%	
Internet Source			