UNDERSTANDING CARRIAGE AND TRANSMISSION OF *STREPTOCOCCUS PNEUMONIAE* THROUGH A COMMUNITY COHORT STUDY IN SOUTH AFRICA

Maimuna Carrim
0406728G

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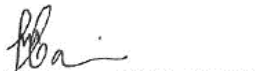
Supervisors: Dr Nicole Wolter and Prof Anne von Gottberg

1. DECLARATION

I, Maimuna Carrim declare that this thesis is my own, unaided work. It is being submitted for the Degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



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Maimuna Carrim

5th November 2025

2. DEDICATION

For my parents, whose faith never swayed,
Through every challenge, you quietly stayed.
Your love and wisdom lit my way,
Turning doubt to hope each day.
And when my daughter graced my days,
She fueled my will in countless ways.

3. PRESENTATIONS ARISING FROM THIS RESEARCH PROJECT

1. **Maimuna Carrim**, Stefano Tempia, Deus Thindwa, Neil A Martinson, Kathleen Kahn, Stefan Flasche, Orienka Hellferscee, Florette K Treurnicht, Meredith L McMorrow, Jocelyn Moyes, Thulisa Mkhencele, Azwifarwi Mathunjwa, Jackie Kleynhans, Limakatso Lebina, Katlego Mothlaoleng, Floidy Wafawanaka, Francesc Xavier Gómez-Olivé, Cheryl Cohen, Anne von Gottberg and Nicole Wolter for the PHIRST group. Understanding carriage and transmission of *Streptococcus pneumoniae* through a community cohort study in South Africa (Oral Presentation)
    - Clinical Microbiology and Infectious Diseases (CMID) Research Day, Clinical Microbiology and Infectious Diseases Department, University of the Witwatersrand, Johannesburg, 6th September 2019

2. **Maimuna Carrim**, Stefano Tempia, Deus Thindwa, Neil A Martinson, Kathleen Kahn, Stefan Flasche, Orienka Hellferscee, Florette K Treurnicht, Meredith L McMorrow, Jocelyn Moyes, Thulisa Mkhencele, Azwifarwi Mathunjwa, Jackie Kleynhans, Limakatso Lebina, Katlego Mothlaoleng, Floidy Wafawanaka, Francesc Xavier Gómez-Olivé, Cheryl Cohen, Anne von Gottberg and Nicole Wolter for the PHIRST group. Descriptive analysis of the carriage burden of the pneumococcus and an update on microbiome analysis (Oral Presentation)
    - PHIRST Principal Investigator's meeting, National Institute for Communicable Diseases, Johannesburg, 1st August 2019

3. **Maimuna Carrim**, Stefano Tempia, Deus Thindwa, Neil A Martinson, Kathleen Kahn, Stefan Flasche, Orienka Hellferscee, Florette K Treurnicht, Meredith L McMorrow, Jocelyn Moyes, Thulisa Mkhencele, Azwifarwi Mathunjwa, Jackie Kleynhans, Limakatso Lebina, Katlego Mothlaoleng, Floidy Wafawanaka, Francesc Xavier Gómez-Olivé, Cheryl Cohen, Anne von Gottberg and Nicole Wolter for the PHIRST group. Unmasking pneumococcal carriage over time in two communities in South Africa, 2016-2018: The PHIRST Study
    - NICD Monthly Scientific Forum, Johannesburg, 29th September 2021

4. **Maimuna Carrim**, Stefano Tempia, Deus Thindwa, Neil A Martinson, Kathleen Kahn, Stefan Flasche, Orienka Hellferscee, Florette K Treurnicht, Meredith L McMorrow, Jocelyn Moyes, Thulisa Mkhencele, Azwifarwi Mathunjwa, Jackie Kleynhans, Limakatso Lebina, Katlego Mothlaoleng, Floidy Wafawanaka, Francesc Xavier Gómez-Olivé, Cheryl Cohen, Anne von Gottberg and Nicole Wolter for the PHIRST group. Pneumococcal colonization in South African communities a decade after Pneumococcal Conjugate Vaccine (PCV) introduction, 2016-2018: The PHIRST Study (e-Poster with Spotlight talk)
    - 12th International Symposium for Pneumococcus and Pneumococcal Diseases (ISPPD), Toronto, Canada, 19th – 23rd June 2022

5. **Maimuna Carrim**, Wouter de Steenhuijsen Piters, Adam Fitch, Jocelyn Moyes, Jackie Kleynhans, Mei Ling Chu, Raiza Hasrat, Neil A Martinson, Kathleen Kahn, Thulisa Mkhencele, Azwifarwi Mathunjwa, Limakatso Lebina, Katlego Mothlaoleng, Floidy Wafawanaka, Francesc Xavier Gómez-Olivé, Cheryl Cohen, Anne von Gottberg, Debby Bogaert and Nicole Wolter for the PHIRST group. Changes in nasopharyngeal microbiota and *Streptococcus* genus during influenza infection in a community cohort study in South Africa (Poster Presentation)
    - 12th International Symposium for Pneumococcus and Pneumococcal Diseases (ISPPD), Toronto, Canada, 19th – 23rd June 2022

6. **Maimuna Carrim**, Stefano Tempia, Deus Thindwa, Neil A Martinson, Kathleen Kahn, Stefan Flasche, Orienka Hellferscee, Florette K Treurnicht, Meredith L McMorrow, Jocelyn Moyes, Thulisa Mkhencele, Azwifarwi Mathunjwa, Jackie Kleynhans, Limakatso Lebina, Katlego Mothlaoleng, Floidy Wafawanaka, Francesc Xavier Gómez-Olivé, Cheryl Cohen, Anne von Gottberg and Nicole Wolter for the PHIRST group. Unmasking pneumococcal carriage over time in two communities in South Africa, 2016-2018 (Poster Presentation)
    - Wits Faculty of Health Sciences Biennial Research Day, Johannesburg, 15th September 2022

7. **Maimuna Carrim**, Wouter de Steenhuijsen Piters, Adam Fitch, Jocelyn Moyes, Jackie Kleynhans, Mei Ling Chu, Raiza Hasrat, Neil A Martinson, Kathleen Kahn, Thulisa Mkhencele, Azwifarwi Mathunjwa, Limakatso Lebina, Katlego Mothlaoleng, Floidy Wafawanaka, Francesc Xavier Gómez-Olivé, Cheryl Cohen, Anne von Gottberg, Debby Bogaert and Nicole Wolter for the PHIRST group. Changes in nasopharyngeal microbiota and *Streptococcus* genus during influenza infection in a community cohort study in South Africa (Oral Presentation)
    - H3Africa African Microbiome Day, Virtual Conference, 19th September 2022

8. **Maimuna Carrim**, Wouter de Steenhuijsen Piters, Adam Fitch, Jocelyn Moyes, Jackie Kleynhans, Mei Ling Chu, Raiza Hasrat, Neil A Martinson, Kathleen Kahn, Thulisa Mkhencele, Azwifarwi Mathunjwa, Limakatso Lebina, Katlego Mothlaoleng, Floidy Wafawanaka, Francesc Xavier Gómez-Olivé, Cheryl Cohen, Anne von Gottberg, Debby Bogaert and Nicole Wolter for the PHIRST group. Changes in nasopharyngeal microbiota and *Streptococcus* genus during influenza infection in a community cohort study in South Africa (Oral Presentation)
    - H3Africa 2nd African Microbiome Day, Virtual Conference, 11th September 2023

4. PUBLICATIONS ARISING FROM THIS RESEARCH PROJECT

Data are from the prospective household cohort study of influenza, respiratory syncytial virus (RSV), and other respiratory pathogens community burden and transmission dynamics in South Africa (PHIRST) conducted over a three-year period (2016-2018) for which I was responsible for setting up all laboratory processes and contributing to testing and managing processing of all specimens. This includes but is not limited to, testing of specimens, overall management of testing, storage and laboratory database management.

In addition, I conducted all descriptive analyses and statistical analyses for the publications included in this thesis and drafted and revised the published manuscripts.

1. Carrim M, Tempia S, Thindwa D, Martinson NA, Kahn K, Flasche S, Hellferscee O, Treurnicht FK, McMorrow ML, Moyes J, Mkhencele T, Mathunjwa A, Kleynhans J, Lebina L, Mothlaoleng K, Wafawanaka F, Gómez-Olivé FX, Cohen C, von Gottberg A, Wolter N. Unmasking Pneumococcal Carriage in a High Human Immunodeficiency Virus (HIV) Prevalence Population in two Community Cohorts in South Africa, 2016-2018: The PHIRST Study. Clinical Infectious Diseases, 2023, 76(3), pages e710-e717.
2. Carrim, M, Kleynhans, J, Tempia, S, Hellferscee O, Treurnicht FK, McMorrow ML, Moyes J, Wafawanaka F, Cohen C, von Gottberg A and Wolter N. Temporal changes in pneumococcal density associated with influenza and RSV in a South African household cohort study, 2016-2018. Open Forum Infectious Diseases. 2025 May 31; 12(6):ofaf267. eCollection 2025 June.
3. Carrim M, Li K, de Steenhuijsen Piters W, Fitch A, Moyes J, Kleynhans J, Mei Chu ML, Hasrat R, Martinson NA, Kahn K, Mkhencele T, Mathunjwa A, Lebina L, Mothlaoleng K, Wafawanaka F, Gómez-Olivé FX, Cohen C, von Gottberg A, Bogaert D, Methè B and Wolter N. The invisible players: Nasopharyngeal Microbiota and *Streptococcus* genus during influenza infection in a South African community cohort study, 2017. (In preparation)

5. ETHICS CLEARANCE CERTIFICATE

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

R49 Ms M Carrim and Professor C Cohen

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M210367

NAME: (Principal Investigator)	Ms M Carrim and Professor C Cohen
DEPARTMENT:	School of Pathology Dept of Clinical Microbiology & Infectious Diseases Medical School University
PROJECT TITLE:	*Understanding carriage and transmission of Streptococcus pneumoniae through a community cohort study in South Africa*
DATE CONSIDERED:	2021/03/26
DECISION:	Approved unconditionally
CONDITIONS:	
NOTE:	If contact information regarding student study participants is required, please contact the Registrar's office - <Nicoleen.Potgieter@wits.ac.za>
SUPERVISOR:	Dr N Wolter and Professor A von Gottberg
APPROVED BY:	Dr CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	2021/08/19

This Clearance Certificate is valid for 5 years from the date of approval. An extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office secretariat on the 3rd floor, Phillip Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated from the research protocol as approved, I/we undertake to submit details to the Committee. **I agree to submit a yearly progress report**. When a funder requires annual re-certification, the application date will be one year after the date when the study was initially reviewed. In this case, the study was initially reviewed in **March** and therefore reports and re-certification will be due in the month of **March** each year. Unreported changes to the study may invalidate the clearance given by the HREC (Medical).

Signature of Principal Investigator

20-08-2021
Date

6. ABSTRACT

Pneumococcal carriage is shaped by host demographics, human immunodeficiency virus (HIV) status, viral co-infections, and microbial community dynamics, yet the longitudinal interplay of these factors remains underexplored in high HIV prevalence settings. Despite the substantial reductions in disease achieved through widespread pneumococcal conjugate vaccine (PCV) use, colonization and invasive disease continue. Gaps remain in understanding how colonization evolves over time, particularly during respiratory viral infections such as influenza and respiratory syncytial virus (RSV). This study investigates how demographic factors, HIV status, and viral co-infections influence pneumococcal colonization and density over time, and examines nasopharyngeal microbiome changes before, during, and after influenza infections to better understand microbial interactions that may modulate colonization.

We analyzed data from a prospective household observational cohort study of influenza, RSV and other respiratory pathogens community burden and transmission dynamics in South Africa (the PHIRST study) of 1684 individuals across 327 randomly selected households conducted in two communities in South Africa from 2016 – 2018. Nasopharyngeal (NP) swabs were collected twice-weekly. Pneumococcal detection and quantification, RSV and influenza virus detection were performed using real-time polymerase chain reaction (qPCR) and nasopharyngeal microbiome were profiled via 16S ribosomal ribonucleic acid (*rRNA*) gene sequencing (V4 region). Colonization episodes were defined using longitudinal qPCR data and analyzed with Markov models to estimate acquisition and clearance rates, adjusting for age, HIV status, and misclassification. Pneumococcal density was summarized as the median across episodes, with associations assessed via multivariable logistic regression. In viral co-infection analyses, changes in pneumococcal density before, during, and after RSV or influenza infection were evaluated using mixed-effects and linear regression models. For microbiome analyses, samples were selected from before, during, and after influenza episodes. Alpha and beta diversity metrics were compared using mixed-effects and regression models. Changes in dominant Operational Taxonomic Units (OTUs) were analyzed using additive log-ratio transformation and regression adjusted for host factors. Predictor-responder analysis was used to identify directional interactions between

OTUs. Analyses were conducted using Stata and R/RStudio, with significance set at P<0.05.

Pneumococcal colonization was detected in 98% (1,655/1,684) of participants. Younger children (<5 years of age) had higher odds of colonization (adjusted odds ratio (aOR) 14.1; 95% confidence interval (CI) 1.8–111.3) and longer episode durations (median 35.5 days, interquartile range (IQR) 17–88) than individuals aged ≥5 years (median 5.5 days, IQR 4–12). People living with HIV (PLWHIV) also had an increased odds of colonization (aOR 10.1; 95% CI 1.3–77.1). An increase in pneumococcal density was observed during RSV infection compared to the period before (log mean before vs. during: 9.3 vs. 10.2 copies/ml; P<0.01). Influenza infection was not associated with a significant mean increase in pneumococcal density (9.6 vs. 9.9 copies/ml; P=0.2), though high influenza viral load (cycle threshold (Ct) <25) was associated with increased pneumococcal density (coefficient 2.8; 95% CI 1.4–4.2). During RSV infection, underweight individuals were less likely to have an increase in pneumococcal density compared to individuals with a normal body mass index (coefficient -1.8; 95% CI -3.5 to -0.1; P=0.04). Influenza infection was associated with shifts in nasopharyngeal microbiome composition, particularly post-infection, with greater changes in younger individuals, females, and PLWHIV. Alpha diversity remained largely stable however older individuals showed a decrease before an influenza infection compared to after (coefficient=-1.03, P=0.01). Our results showed that beta diversity changed across all three periods. *Streptococcus* 5 was more abundant during influenza episodes and showed a temporal patter of medium-high-low relative abundance across the periods. Furthermore, we found that *Streptococcus* 5 abundance before infection predicted increases in both *Streptococcus* 5 and *Moraxella* 1 during infection, while *Moraxella* 1 increases during infection preceded declines in *Streptococcus* 5 and *Dolosigranulum pigrum* 4 after infection.

Although PCVs have reduced disease burden, children and PLWHIV remain key reservoirs for pneumococcal transmission. Our findings corroborate previous studies demonstrating that viral co-infections are associated with increased pneumococcal density and alterations in the nasopharyngeal microbiome, supporting the concept of a synergistic effect that may promote progression to invasive disease. These results show that respiratory viral infections, particularly RSV, are associated with increases in

pneumococcal colonization density. This suggests that vaccination against respiratory viruses, such as RSV, could play a role in reducing bacterial disease risk. Furthermore, observed microbiome shifts during viral infections may help explain changes in pneumococcal colonization patterns, offering insights into host-microbe interactions relevant to disease development.

7. ACKNOWLEDGEMENTS

“The journey to greatness is a testament to the strength of those who stand beside and uplift us.” – Maimuna Carrim

I begin with gratitude to Allah Almighty, the source of all wisdom and strength, whose divine guidance has illuminated my path throughout this difficult journey of pursuing a doctoral degree.

To my parents, your boundless love, sacrifices, and boundless support have been the cornerstone of my academic accomplishments. Your encouragement and resilience have been the driving force behind my perseverance in the face of challenges.

I would like to sincerely thank my supervisors, Prof. Anne von Gottberg and Dr. Nicole Wolter, for their invaluable support, guidance, and patience throughout my PhD journey. Your mentorship and understanding during challenging times have been greatly appreciated. Thank you for your continuous feedback and encouragement, which have been essential to my growth both academically and personally.

I extend my sincere appreciation to my mentors, whose guidance, mentorship, scholarly insights, and constructive feedback have shaped my academic growth and fueled my intellectual curiosity. I am grateful for the lessons learned and the skills acquired under your guidance.

I extend my thanks to the collaborators involved in this study for their intellectual input, and shared enthusiasm for the advancement of knowledge. The intellectual exchange and shared commitment to science have been instrumental in shaping the outcomes of this study.

To the study participants, I extend my heartfelt thanks for their willingness to participate in the study, without which the study would not be possible. Your contributions have been significant to science.

I am thankful for my work colleagues, whose shared commitment to science created a focused environment conducive to my productivity.

Thanks go to my sister and brother-in-law, whose understanding and moral support provided solace reminding me of the importance of familial bonds in the face of challenges. Your belief in my capabilities has been a source of strength. To my nephew and niece, your innocence and joy provided much-needed breaks from the intensity of academic activities. Your laughter echoed through the pages of my thesis, turning challenges into moments of delight.

To my husband, your unwavering support, patience, and understanding have been my pillars of strength. Your confidence in my abilities has propelled me to accomplish this milestone. Thank you for holding everything together with calm and love when I couldn’t, for being my anchor when life threw its curveballs, and for making me laugh when I couldn’t even think.

Finally, to my precious daughter, your arrival during the final stages of this journey became my greatest source of motivation. Your presence reminded me of the importance of perseverance and purpose. This is dedicated to you, with the hope that as you grow, you will always believe in your limitless potential and know that you can achieve anything you set your heart and mind to. You are my greatest inspiration, and I cannot wait to see the incredible things you will accomplish.

In acknowledging the multitude of individuals who have played pivotal roles in this journey, I realize that this achievement is a testament to the collective support of my loved ones, supervisors, mentors and collaborators.

8. TABLE OF CONTENTS

9. LIST OF FIGURES

10. LIST OF TABLES

11. LIST OF ABBREVIATIONS

alr – additive-log ratio transformation

aOR – Adjusted odd ratio

ATCC – American Type Culture Collection

BMI – Body Mass Index

CDC – United States Centers for Disease Control and Prevention

CI – Confidence interval

Ct – Cycle threshold

e.g. – Exempli gratia

HDSS – Health and demographic surveillance system

HIV – Human immunodeficiency virus

HREC – Human Research Ethics Committee

i.e. – id est (that is)

IPD – Invasive pneumococcal disease

IQR – Interquartile range

ISPPD – International Symposium for Pneumococci and Pneumococcal Diseases

LRTI – Lower respiratory tract infection

lytA – Autolysin gene

MDS – Multidimensional scaling plot

NICD – National Institute for Communicable Diseases

NP – Nasopharyngeal

OR – Odds ratio

OTU – Operational taxonomic units

PCV – Pneumococcal conjugate vaccine

PCR – Polymerase chain reaction

PHIRST – Prospective household observational cohort study of influenza, respiratory syncytial virus and other respiratory pathogens community burden and transmission dynamics in South Africa

PLWHIV – People living with HIV

qPCR – real-time polymerase chain reaction

RDP – Ribosomal Database Project

RedCap - Research Electronic Data Capture

RNaseP – Ribonuclease P gene

rRNA – ribosomal ribonucleic acid

RSV – Respiratory syncytial virus

RT-qPCR – Reverse-transcription real-time polymerase chain reaction

SARS – Severe acute respiratory syndrome

USA – United States of America

viz. – videre licet (namely)

WHO – World Health Organization

12. Chapter 1: Literature Review

12.1. Pneumonia – burden of disease and risk factors

Pneumonia, an inflammation affecting lung tissue, results in acute illness in both adults and children. Common symptoms include a cough with purulent sputum, pyrexia, and dyspnea (1). Despite numerous interventions and significant advancements in reducing the morbidity and mortality caused by pneumonia, lower respiratory tract infections (LRTI) rank fourth globally and in Africa as a cause of death, accounting for 2.6 million deaths in all age groups, globally, and 435,000 deaths in Africa in 2019 (2,3). In South Africa, LRTI accounted for 67 deaths per 100,000 individuals of all ages in 2019 (2,3). In 2008, approximately 18% of deaths in children aged under five were attributed to pneumonia worldwide (4). South Africa has a high annual incidence of hospitalization for LRTI estimated to be 2530-3173 per 100,000 children aged <5 years and 325-617 per 100,000 individuals aged ≥5 years in 2012 (5,6). Recent surveillance showed that 65 per 100,000 children <5 years were hospitalized with LRTI in Soweto in 2022 (7).

People living with human immunodeficiency virus (PLWHIV) have been shown to be at increased risk of hospitalization and death due to LRTI (5,6). Compared to HIV-uninfected children, those living with HIV have a 6.5 times higher odds of pneumonia hospitalization and a 5.9 times higher odds of death, with over 90% of pneumonia episodes and deaths in children living with HIV occurring in sub-Saharan Africa (8). In KwaZulu-Natal, HIV infection was present in 54% of adults hospitalized with pneumonia, and mortality among this group was 39.5%, illustrating the continued burden of HIV on pneumonia-related deaths (9). A multi-site cohort in sub-Saharan Africa found HIV-exposed uninfected children had higher hospitalizations and mortality risks compared to HIV-unexposed children (10). In a South African study of hospitalized infants less than 1 year old, infants living with HIV had four times higher risk of in-hospital mortality compared to HIV-uninfected infants (11). Similarly, in Kwa-Zulu Natal in South Africa, in 2007, they found that children living with HIV were more likely to develop severe pneumonia and have a higher mortality rate than HIV-uninfected children and children infected with multiple respiratory pathogens faced a higher risk of mortality than children infected with a single pathogen (12). However, the risk in adults especially those with poorly controlled HIV, remains a concern. Between 2009 and 2011, adults living with

HIV had a four to eight times higher incidence of hospitalization for influenza-associated LRTI than HIV-negative individuals in South Africa. These adults were also more likely to experience pneumococcal co-infection, longer hospital stays and increased mortality (13). This highlights that while pediatric HIV-related pneumonia may be declining, the adult burden particularly in those with untreated or advanced HIV remains high.

12.2. *Streptococcus pneumoniae*

Streptococcus pneumoniae is a fastidious, mucosal pathogen, which causes life-threatening diseases such as pneumonia, meningitis, and sepsis (14). There are over 100 serotypes but only a few are responsible for causing the majority of invasive pneumococcal disease (IPD). Asymptomatic colonization of *S. pneumoniae* occurs in the human nasopharynx and is often a beneficial immunogenic event. However, pneumococcal carriage is also a precursor for invasive disease (15,16). Thus, reducing pneumococcal carriage of invasive serotypes in the nasopharynx may reduce the potential for the development of IPD.

The 7-valent pneumococcal conjugate vaccine (PCV), targeting 7 serotypes (4, 6B, 9V, 14, 18C, 19F and 23F), was introduced into the South African routine infant immunization schedule in 2009 with a 2+1 schedule at 6, 14 and 36 weeks of age, and was replaced by the higher valency PCV13 (1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19Fand 23F) in 2011. With the introduction of PCV, both carriage rates and invasive disease caused by vaccine serotypes have declined globally, in vaccinated children (direct effect) as well as unvaccinated children and adults (due to an indirect effect due to decreased vaccine-serotype carriage and therefore transmission from vaccinated individuals) (17–19).

12.3. PCV effects on invasive disease and carriage

In South Africa, in children <2 years of age, IPD caused by PCV7 serotypes declined 89% (32.1 to 3.4 cases per 100,000 person-years), and in adults aged 25-44 years IPD caused by PCV7 serotypes declined 57% (3.7 to 1.6 cases per 100,000 person-years) from the baseline period (2005 – 2008) to early post-vaccine years (2011-2012) (20).

Non-vaccine serotypes (NVTs) did not change significantly in children <2 years however, in adults aged 25–44 years, NVTs increased by 78.5% and in individuals aged older than 64 years NVTs increased by 234.9% by 2019 (19).

Positive direct and indirect effects of PCV on colonization have been observed, with studies showing reductions in the carriage of vaccine serotypes in all age groups (21). In South Africa, from 2009 to 2011, carriage of PCV7 serotypes declined by 50% in children <2 years of age, 21% in children 2-5 years of age, 34% in children 6-12 years and 64% in individuals >12 years. However, colonization caused by non-PCV serotypes has increased post-PCV introduction indicating that non-vaccine serotypes have filled the ecological niche left vacant by vaccine-serotype strains in the nasopharynx (21–23).

Pneumococcal carriage rates differ by country both pre- and post-PCV. A meta-analysis evaluating the prevalence of pneumococcal carriage in 29 studies revealed that the point prevalence ranged from 0% to 39% in individuals aged 60 years and older (24). In children in Canada and Laos, 45% and 37% of children were pneumococcal carriers, respectively (25,26). In a cross-sectional study conducted in South Africa, they observed a 12% decrease in pneumococcal carriage of vaccine serotypes in children after PCV introduction (21). In longitudinal studies, pneumococcal carriage ranged from 25% to 86% (27–29). One such study in The Gambia showed that all children carried the pneumococcus at least once during their study of a year, conducted before routine immunization (29,30). Furthermore, in the longitudinal Drakenstein study in South Africa post-PCV, which followed infants from birth through 12 months of age with fortnightly sampling, 54% of infants were colonized with pneumococcus over this time period (31).

12.4. The pneumococcus and respiratory viral infections

The pneumococcus can reside in the nasopharynx of healthy individuals without causing disease (32). However, a compromised immune system or the existence of another respiratory pathogen can trigger the pneumococcus to proliferate and move from the nasopharynx to the lower respiratory tract or invade the bloodstream or meninges directly, resulting in IPD (15,16). The pneumococcal serotype is known to influence the ability of the pneumococcus to transition from a commensal to a pathogen. However,

other host and environmental factors such as immune suppression, age and microbiome composition have also been identified (33,34). Viral infections in particular are thought to play a role in this behavioral shift (35–37).

During the COVID-19 pandemic, both England and Hong Kong observed significant declines in IPD cases (38,39). In England, IPD incidence decreased by 30% in 2019-2020 compared to 2018-2019, primarily due to lockdowns and reduced social interactions (39). Similarly, Hong Kong reported a notable decrease in IPD and pneumococcal pneumonia hospitalizations compared to the previous five years (38). Despite these declines, rare coinfections of *S. pneumoniae* and severe acute respiratory syndrome (SARS) coronavirus were linked to high case-fatality rates, particularly among older adults (39).

Among other respiratory viruses extensively studied in association with the pneumococcus are respiratory syncytial virus (RSV) and influenza. RSV and the pneumococcus can act synergistically when co-infecting a host, leading to more severe respiratory illness and increased morbidity and mortality (37). RSV infection can also precede pneumococcal disease by compromising mucosal barriers and enhancing bacterial adherence and proliferation, thereby increasing the risk of progression from pneumococcal carriage to disease (35–37,40). This may be attributed to factors such as macrophage polarization, immune system impairment, or enhanced pneumococcal virulence due to RSV binding to the penicillin-binding protein, which subsequently increases bacterial adherence and infection of the respiratory epithelium (35,36,40).

The interaction of the pneumococcus with influenza virus elicits notable concerns, as the simultaneous presence of both organisms may worsen the prognosis for an infected individual, similar to RSV. The Spanish influenza pandemic of 1918 stands out as the most famous historical instance of viral-bacterial synergism in the respiratory tract where millions of people succumbed to secondary bacterial pneumonia following an initial influenza A virus infection (41). During the 1918 pandemic, there was a significant increase in pneumococcal pneumonia cases, which were a major contributor to the high mortality rate seen in the pandemic. A study found that experimental infection with

influenza in animal models resulted in an increase in pneumococcal load in the nasopharynx (42). This increase in pneumococcal load was accompanied by changes in the nasopharyngeal environment including increased inflammation and decreased bactericidal activity, which are likely contributors to the persistence and proliferation of pneumococci during colonization (43,44). Other studies found that natural influenza infections in humans were associated with increased odds of pneumococcal carriage (45–47). One of the primary ways in which respiratory viruses are believed to increase the likelihood of bacterial infections is by disrupting the airway-epithelial barrier which allows bacterial pathogens to adhere (48–50). Additionally, evidence indicates that influenza virus infection increases pneumococcal colonization by releasing host-derived nutrients (51). Given these findings, it is crucial to grasp the dynamics between influenza and RSV in relation to pneumococcal colonization and disease, and implement measures for the prevention and management of both infections.

Importantly, the interactions between pneumococcus and other respiratory pathogens are not uniformly synergistic**.** While certain viruses can enhance pneumococcal colonization or disease risk, other microbes in the nasopharynx, such as *Staphylococcus aureus* and *Corynebacterium* spp., may antagonize pneumococcal colonization (52–55). ogether, these findings illustrate that both viral–bacterial and bacterial–bacterial interactions contribute to the dynamic and context-dependent nature of microbial ecology in the upper respiratory tract. Understanding these dynamics is helpful for designing preventive strategies and clinical management approaches targeting both viral and bacterial pathogens.

12.5. The nasopharyngeal microbiota

The nasopharyngeal microbiome is heterogeneous, consisting of a network of harmless commensals and pathobionts, which are organisms that are commensals but have the potential to cause disease. The microbiome is thought to be at equilibrium when individuals are healthy. However, perturbations in the microbiome occur with changes in habitats including exposure to other organisms, which may lead to disequilibrium (54).

Colonization by *S. pneumoniae*, facilitated by its ability to form biofilms (structured communities encased in extracellular matrix) plays a crucial role in disease initiation. These biofilms enhance bacterial adherence and antibiotic resistance, promoting persistent asymptomatic colonization (56). During asymptomatic carriage, biofilm-associated pneumococci exhibit reduced virulence and invasiveness, allowing them to evade host immune responses (56). However, transitions to pathogenic states can occur under conditions of environmental change, triggered by factors like respiratory viruses, which alter the nasopharyngeal niche and promote biofilm dispersion and disease progression (56).

Research indicates that the composition of the microbiome in both children and adults correlates with pneumococcal carriage (57,58). For instance, the interplay and antagonistic relationship between *S. pneumoniae* and *Staphylococcus aureus* illustrates dynamic changes in microbiome composition within the nasopharynx (59–62). In another study, the antagonistic relationship between non-pathogenic *Corynebacterium* spp. and carriage of the pneumococcus has also been observed (33). The synergistic relationship of *S. pneumoniae* and *Haemophilus influenzae* has also been described indicating that co-colonization of *H. influenzae* with pneumococcus in the nasopharynx facilitates *S. pneumoniae* biofilm formation resulting in infections like otitis media (63,64) The interaction of the pneumococcus and influenza virus is one of the most well-documented synergistic bacterial-viral interactions where influenza infection can elevate pneumococcal colonization density and increase the risk of invasive pneumococcal pneumonia (65).

The nasopharyngeal microbiome is influenced by multiple host and environmental factors, including age. Infants and young children generally exhibit higher bacterial diversity and more dynamic microbial communities, whereas older children appear to show more stable microbial profiles though data in adults are less extensive (52,55,66). These age-related differences may interact with other host factors, such as vaccination status and HIV infection, to influence susceptibility to colonization by pathogens such as *S. pneumoniae* and modulate microbiome responses to viral infection.

Advances in molecular methods such as 16S *rRNA* gene sequencing have enabled comprehensive characterization of the nasopharyngeal microbiota. This approach targets conserved regions of the bacterial 16S *rRNA* gene to detect and identify bacterial taxa present in a sample, although it does not capture viral or fungal components of the microbiome. While 16S sequencing provides valuable insights into bacterial community structure, it has limitations in taxonomic resolution often distinguishing only at the genus level and may be influenced by biases in amplification and reference databases. Nonetheless, it remains a widely used tool for studying the composition and diversity of bacterial communities in the upper respiratory tract.

12.6. Motivation

The dynamics of pneumococcal carriage are influenced by a multitude of factors, including demographics, HIV status, and co-infections with respiratory viruses. Understanding the dynamics of pneumococcal carriage, particularly in the context of HIV status and respiratory viral infections, remains a critical area of research. While significant progress has been made in reducing IPD through vaccination programs, gaps persist in our knowledge of how pneumococcal carriage behaves over time in diverse populations, especially in regions with high HIV prevalence like South Africa. Current studies have largely focused on the immediate impacts of vaccines and have not sufficiently explored the longitudinal patterns of pneumococcal carriage in various demographic groups. This gap is particularly evident in the context of co-infections with respiratory viruses such as influenza and RSV, where the interactions and their impact on pneumococcal colonization density are not fully understood.

Furthermore, the nasopharyngeal microbiome's role in mediating pneumococcal behavior and its interaction with other microbial communities during viral infections is still an emerging field. Previous research has highlighted the importance of microbial interactions in disease progression, but there are limited longitudinal data capturing these dynamics in real-world settings. This study addresses these critical gaps by providing a comprehensive analysis of pneumococcal carriage over time, considering demographic characteristics and HIV status, and examining the influence of RSV and influenza co-infections on colonization density. Additionally, by characterizing the

nasopharyngeal microbiome and its changes in relation to pneumococcal carriage before, during, and after influenza infections, this research offers valuable insights into the microbial interactions that could inform future prevention and treatment strategies.

12.7. Aims and objectives

This study aims to determine the prevalence and describe the acquisition, transmission and clearance of *S. pneumoniae* strains in the nasopharynx, over time, in a community-based household cohort study in South Africa. This will be achieved by the following objectives:

1.7.1. To describe pneumococcal carriage in individuals over time by demographic characteristics and HIV status

1.7.2. To determine whether viral infection (influenza and RSV) is associated with an increase in nasopharyngeal pneumococcal colonization density

1.7.3. To describe the composition of the nasopharyngeal microbiome over time and to understand how the pneumococcal component changes in the nasopharyngeal microbiome before, during and after an influenza infection.

13. Chapter 2: Materials and methods

13.1. Existing study platform

13.1.1. Study design and population

The study was nested within a prospective household observational cohort study of influenza, RSV and other respiratory pathogens community burden and transmission dynamics in South Africa (the PHIRST study) (67,68).

13.1.2. The PHIRST Study

The PHIRST study was a household-level community cohort study in a rural community in Agincourt in Mpumalanga Province [a health and demographic surveillance system (HDSS)] and Klerksdorp an urban area in the Matlosana Municipality in the North West Province that was conducted over three consecutive years, from 2016-2018.
In the PHIRST study, 327 different households (1684 study participants) were enrolled over the 3 years (100 in 2016; 109 in 2017 and 118 in 2018), with an average number of 5 participants per household, and followed-up from May - October in 2016 (due to a late start in the first year of the study) and January-October in 2017 and 2018. Each household member was followed up twice weekly (Monday–Wednesday and Thursday–Saturday) for the year of enrolment.

13.1.3. Inclusion and exclusion criteria

Households were randomly selected. Households were required to have three or more individuals sharing at least four meals a week and >80% of individuals in the household consented to participate in the study to be eligible. In addition, individuals needed to have resided in the selected communities for at least one year before the commencement of the study and for the duration of the study. At least 50% of households selected were required to have at least one child aged <5 years in the household. Written informed consent (individuals ≥18 years), assent (children aged 7 – 17 years) and parent or guardian consent (children aged <18 years) was required to participate in the study.

Non-eligible households were households not randomly selected, households that did not consent to participate or households that had ≥1 household member that did not consent for inclusion in the study. Furthermore, a household was excluded if the head of the household was a minor or was unavailable to request permission for participation after 3 attempts. Households were further excluded if they moved from the study sites, refused sample collection, withdrew from the study or if members died or were unavailable for the study period resulting in a household having less than <3 members.

13.1.4. Data collection and management

At enrolment, structured interviews were conducted to collect baseline data on household and individual characteristics, including demographic information, presence of chronic medical conditions, and environmental factors. All data were entered into Research Electronic Data Capture (REDCap), a secure, web-based platform designed for managing research data. During the follow-up period, fieldworkers conducted twice-weekly visits to each household. At each visit, a structured questionnaire was administered to record any respiratory symptoms, episodes of absenteeism from work or school, and contact with the health system together with sample collection, regardless of symptom presence. Fieldworkers received annual training on the identification and documentation of respiratory symptoms to ensure data quality and consistency. Symptoms assessed included at least one of the following: fever (self-reported or tympanic temperature ≥38°C), cough, difficulty breathing, sore throat, nasal congestion, chest pain, muscle aches, headache, vomiting, or diarrhea. Fieldworkers were trained to administer these assessments consistently, and participants received grocery store vouchers valued at United States of America (USA) $2.00–2.50 per visit as compensation for their time and the discomfort associated with study activities.

13.1.5. Specimen collection

As part of the larger PHIRST study, nasopharyngeal (NP) flocked swabs were used, with pediatric swabs used for children and adult swabs used for older participants to ensure appropriate fit and sampling depth relative to nasopharyngeal size. The NP swabs were collected twice weekly from a total of 1,684 individuals (542 in 2016, 577 in 2017, and 565 in 2018) irrespective of symptoms and were placed in PrimeStore® Molecular

Transport Medium (Longhorn Vaccines and Diagnostics, Bethesda, USA). The NP swabs were shipped in cooler boxes to the National Institute for Communicable Diseases (NICD) in Johannesburg.

13.1.6. Laboratory testing for *S. pneumoniae*, RSV and influenza

Total nucleic acids from 200 µl NP samples were extracted using a MagNA Pure 96 system with the MagNA Pure 96 DNA and Viral NA Small Volume Kit and "Viral NA Plasma SV" protocol (Roche, Mannheim, Germany) and eluted to a volume of 100 µl. The human ribonuclease P (*RNAseP*) gene was used as a control to assess the quality of sample collection procedures. Thereafter, a quantitative, in-house, singleplex real-time PCR (qPCR) targeting the autolysin gene (*lytA*) was used to identify and quantify *S. pneumoniae* using universal cycling conditions (69). A cycle threshold (Ct) value of ≤40 for *S. pneumoniae* was considered positive, and no replicates were performed. A standard curve using serially diluted DNA extracts from a known starting quantity of American Type Culture Collection (ATCC) control (ATCC 49619) was included with every PCR run to calculate pneumococcal density (genomic copies/ml). The limit of detection of this assay was found to be <10 genomic copies/ml (69). DNA extraction and *lytA* qPCR were conducted under strict laboratory controls, including the use of negative extraction and PCR controls in each batch to monitor for contamination. While the extraction protocol used had been optimized for nucleic acid recovery from nasopharyngeal swabs, no comparison with alternative extraction methods was performed. For RSV and influenza detection, we performed reverse transcription real-time polymerase chain reaction (RT-qPCR) using the FTD Flu/RSV detection assay (Fast Track Diagnostics, Luxembourg), with RT-qPCR Ct-value used as a proxy for viral load (id. est. (i.e.), a low Ct-value implied a high viral load and vice versa). Influenza-positive samples were subtyped using the US Centers for Disease Control and Prevention (CDC) subtyping kit (available through the International Reagent Resource Program at https://www.internationalreagentresource.org). We defined an RSV or influenza virus infection as at least one RT-qPCR (Ct-value <37) NP swab positive for the respective virus, irrespective of symptoms.

13.2. Ethics

The PHIRST study protocol was registered on http://clinicaltrials.gov on 6 August 2015 (Reference NCT02519803). The primary study, the PHIRST study was approved by the University of the Witwatersrand Human Research Ethics Committee (HREC) (Reference 150808) and the USA Centers for Disease Control and Prevention's Institutional Review Board relied on the local review (#6840). This activity was reviewed by the CDC and was conducted consistent with applicable USA federal law and CDC policy. Approval of this study entitled, "Understanding carriage and transmission of *Streptococcus pneumoniae* through a community cohort study in South Africa" for laboratory testing for *S. pneumoniae* and microbiome testing (M210367) was obtained from the University of the HREC.

13.3. Candidate's contribution

For the overall study, the candidate conceptualized the study components relevant to pneumococcal carriage, selected samples, processed data, performed statistical analysis, and interpreted results. Laboratory testing for *S. pneumoniae*, RSV, and influenza was performed at the National Institute for Communicable Diseases (NICD). Testing was divided amongst NICD staff with the candidate testing a subset of samples and participating in quality control and data verification. For Chapter 3, the candidate conceptualized the study components relevant to pneumococcal carriage, selected samples, processed data, performed statistical analysis, and interpreted results. Markov modeling was performed by the London School of Tropical Hygiene and Medicine, with the candidate contributing to result interpretation. For Chapter 4, the candidate conceptualized the study components, conducted mixed-effects modeling, statistical analysis, and interpretation of results. For Chapter 5, the candidate conceptualized the microbiome study component, selected samples for microbiome analysis, and performed data processing, statistical analysis, and interpretation. DNA extraction and sequencing for microbiome analysis were performed by Rijksinstituut voor Volksgezondheid en Milieu, with the candidate participating in quality control and data verification. Microbiome data analysis was assisted by the University of Pittsburgh. All subsequent data analysis, visualization, and manuscript preparation were performed by the candidate.

14. Chapter 3: Unmasking pneumococcal carriage in a high HIV prevalence population in two community cohorts with a high prevalence of HIV in South Africa, 2016-2018: the PHIRST study

This chapter is based on a published manuscript.

14.1. Reference

Carrim M, Tempia S, Thindwa D, Martinson NA, Kahn K, Flasche S, Hellferscee O, Treurnicht FK, McMorrow ML, Moyes J, Mkhencele T, Mathunjwa A, Kleynhans J, Lebina L, Mothlaoleng K, Wafawanaka F, Gómez-Olivé FX, Cohen C, von Gottberg A, Wolter N. Unmasking Pneumococcal Carriage in a High Human Immunodeficiency Virus (HIV) Prevalence Population in two Community Cohorts in South Africa, 2016-2018: The PHIRST Study. Clinical Infectious Diseases, 2023, 76(3), pages e710-e717.

14.2. Introduction

While PCV has markedly decreased the incidence of IPD in South Africa, asymptomatic colonization of *S. pneumoniae* continues to pose a risk for transmission and subsequent disease, particularly in high HIV-prevalence areas. Pneumococcal colonization is a dynamic process, influenced by host factors such as age, immune status, and HIV infection, as well as environmental factors including household exposure and seasonality (70,71). Most available data on pneumococcal colonization come from cross-sectional studies, which provide snapshots of carriage prevalence at a single point in time (24–26). While useful, these studies cannot capture the temporal dynamic processes of acquisition, persistence, clearance or changes in bacterial load that occur over time. Longitudinal cohort studies, though fewer in number, have demonstrated that carriage duration varies by age and serotype and that colonization density may influence transmission potential and progression to disease (30,72–75).

In sub-Saharan Africa, particularly in South Africa where HIV prevalence remains high, understanding patterns of pneumococcal colonization is critical, as HIV-infected individuals have been shown to carry pneumococci more frequently and at higher densities, factors that may increase their risk of IPD and facilitate transmission within households (12,13,76). Despite the introduction of PCV and evidence of vaccine-induced shifts in serotype distribution, there remains limited longitudinal data on

colonization dynamics post-PCV introduction in high HIV prevalence settings, particularly regarding how HIV status and other demographic factors influence colonization duration and density. To bridge the knowledge gap in the longitudinal patterns of pneumococcal colonization post-PCV introduction, we aimed to determine factors associated with pneumococcal colonization, duration of colonization, and density in the nasopharynx over time, in a household community cohort study.

14.3. Materials and methods

14.3.1. Study design and laboratory testing

The study design, population studied and laboratory testing has been described in section 13.1.

Among the 1,684 individuals from the PHIRST study previously described in section 2.1.2., 79 (5%) were lost to follow-up (53 (67%) left the study sites, 21 (27%) withdrew prematurely and 5 (6%) died but remained in the analysis. Total nucleic acids were extracted (described in section 13.1.6) and qPCR was performed for the identification of *S. pneumoniae* as previously described (section 13.1.6)

14.3.2. Data analysis

Individuals with ≤10 follow-up visits when NP swabs were collected were excluded from this analysis. The proportion of pneumococcal colonization was defined as the number of individuals colonized with pneumococcus at least once during the period of twice-weekly swabbing. Pneumococcal colonization was stratified by year, site (Klerksdorp and Agincourt), sex, age group, and HIV infection status. We selected three time points corresponding to the first visit in May, July, and September each year reflecting pneumococcal colonization point prevalence in autumn, winter and spring, respectively. A Markov model was constructed to estimate clearance and acquisition events from the observed data thus consecutive episodes of colonization. The model adjusted for misclassification of samples based on probabilistic ascertainment as well as age and HIV status and is described in more detail elsewhere (77). Using the results obtained from the Markov model, the median number of episodes per person and the median

duration of a colonization episode were estimated by age group. We used the $\chi 2$ or Fisher exact test for comparison of categorical variables.

Household clustering was accounted for in the analysis using mixed-effects models with household included as a random effect, similar to the approach used in the microbiome chapter. This ensured that observations within the same household were not treated as independent, while still allowing us to assess individual-level associations.

A sub-analysis was implemented to assess factors associated with high pneumococcal colonization density. This was determined by calculating the median pneumococcal density over an episode and the median pneumococcal density, by individual, over all pneumococcal episodes. Based on evidence showing that pneumococcal pneumonia is associated with a colonization density ≥1,000 copies/ml, we categorized those with pneumococcal colonization into high- (median of ≥1,000 copies/ml) and low-density (median of <1,000 copies/ml) (65). Unconditional logistic regression was performed to assess factors associated with pneumococcal colonization and density of pneumococcal carriage. For multivariable models, we assessed all variables that were significant at $P<0.1$ on the univariate analysis or a priori (HIV status) and dropped non-significant factors ($P\geq0.05$) with manual backward elimination. We assessed pairwise interactions by the inclusion of product terms for all variables remaining in the final multivariable additive model. Carriage duration was left truncated and right censored at the first and last NP collection point for each year respectively. We performed the analysis using Stata version 14 (StataCorp LLC, College Station, Texas, USA). Statistical significance was assessed at $P<0.05$.

14.4. Results

In 327 households, 1,684 individuals were enrolled and followed up for 6-10 months (67). The study population comprised 60% (1,009/1,684) females. HIV status was known for 97% (1628/1684) of participants and 15% (249/1,684) were PLWHIV. Individuals aged <5, 5-14, 15-44, and ≥45 years comprised 17% (279/1,684), 32% (547/1,684) 35% (590/1,684), and 16% (268/1,684), respectively. Of PLWHIV with known CD4+ counts, 15/214 (7%) had a CD4+ count of less than 200 cells/mm3 (78). PCV vaccination

information was available for 78.5% (219/279) of participants aged <5 years and among those, 96.8% (212/219) of individuals were fully vaccinated for their age.

There were 122,113 potential follow-up visits during the study period; 105,783 (87%) follow-up visits were conducted, and 105,686 (99.9%) (23,693 in 2016, 41,679 in 2017, and 40,314 in 2018) NP specimens were collected and tested for S. *pneumoniae* and *RNAseP*. The proportion testing positive for *RNAseP* was 91% (93,456/102,379). We detected *S. pneumoniae* in 36.7% (38,829/105,686) of NP specimens (39.3% (9,310/23,693) in 2016, 37.6% (15,672/41,679) in 2017 and 34.3% (13,847/40,314)] in 2018). The seasonal point prevalence for the three years was 36.1% (578/1,599) in May (autumn), 40.7% (614/1,508) in July (winter) and 40.5% (645/1,591) in September (spring) (P=0.98).

14.4.1. Proportion of individuals with pneumococcal colonization

We assessed the proportion of individuals with pneumococcal colonization for 1,684 individuals over the three-year cohorts and found that 98% (1,655/1,684) of individuals had at least one colonization episode (Table 14.1). No significant difference in colonization was observed by sex or site. However, individuals enrolled in 2017 were 3.6 times (adjusted odds ratio (aOR) 3.6; 95% confidence interval (95% CI) 1.2-11.3) more likely to be colonized with pneumococcus at least once during the study period than individuals enrolled in 2016. Younger age (<5 years: (278/279; 99.6%), aOR 14.1, 95% CI 1.8-111.3 and 5-24 years: (811/820; 98.9%), aOR 4.8, 95% CI 1.9-11.9, compared to 25-44 years (303/317; 98.1%)) was associated with increased odds of colonization; PLWHIV were tenfold more likely to be colonized with pneumococcus at least once than HIV-uninfected individuals after adjusting for age (aOR 10.1; 95% CI 1.3-77.1).

14.4.2. Number of colonization episodes

After fitting the Markov model to the data, there were 1,625 participants, whose HIV status and information were available. Of these, 1,585 (97.5%) had at least 1 carriage episode. The median (interquartile range: IQR) number of colonization episodes per individual during the follow-up periods (6 to 10 months) was 5 episodes (3-7 episodes)

(Figure 14.1). The median (IQR) carriage duration for an episode was 7 days (4-19 days). Children aged <5 years had fewer colonization episodes than individuals aged ≥5 years (<5 years: 9 episodes vs. ≥5 years: 18 episodes; P<0.001) but their duration of carriage was longer (<5 years: median (IQR) 35.5 days (17-88 days) vs. ≥5 years: 5.5 days (4-12 days); P<0.001 (Figure 14.2).

14.4.3. Pneumococcal colonization density

Individual median pneumococcal colonization density, over an episode, was 619 DNA copies/ml (IQR 133.5-4,286.5). We observed that the median log pneumococcal density decreased with an increase in age (P<0.01) (Figure 14.3). Following categorization of pneumococcal density into low (<1,000 copies/ml) and high (≥1,000 copies/ml) loads, we found that high pneumococcal loads were associated with younger age (<1 year: aOR 25.4, 95% CI 7.4-87.6; 1-4 years: aOR 13.5, 95% CI 8.3-22.9; 5-14 years: aOR 3.1, 95% CI 2.1-4.4 vs. 45-65-year-olds). On univariate analysis PLWHIV had a lower odds of high pneumococcal load of OR 0.7, 95% CI 0.5-0.9 however after adjusting for age in the multivariable model, PLWHIV were at greater risk for high pneumococcal loads (aOR 1.7, 95% CI 1.2-2.4) (Table 14.2).

Table 14.1: Proportion of individuals colonized with pneumococcus at least once during the follow-up period, PHIRST study, South Africa, 2016-2018 (N=1684)

Characteristics	Proportion of pneumococcal colonization	Univariate Analysis		Multivariable Analysis**	
	n/N (%)	OR (95% CI)	P-value	aOR (95% CI)	P-value
Overall	1655/1684 (98.3)	-	-		
Year					
May-October 2016	529/542 (97.6)	Reference	N/A	Reference	N/A
January-October 2017	572/577 (99.1)	2.8 (1.0-7.9)	0.05	3.6 (1.2-11.3)	**0.03**
January-October 2018	554/565 (98.1)	1.2 (0.5-2.8)	0.61	1.5 (0.6-3.5)	0.38
Site					
Agincourt	841/849 (99.1)	2.7 (1.2-6.2)	**0.02**	2.0 (0.9-4.7)	0.11
Klerksdorp	814/835 (97.5)	Reference	N/A	Reference	N/A
Sex					
Male	660/675 (97.8)	Reference	N/A	-	-
Female	995/1009 (98.6)	1.6 (0.8-3.4)	0.20	-	-
Age group (years)					
<5	278/279 (99.6)	12.8 (1.7-98.3)	**0.01**	14.1 (1.8-111.3)	**0.01**
5-24	811/820 (98.9)	4.2 (1.8-9.8)	**<0.01**	4.8 (1.9-11.9)	**<0.01**
25-44	303/317 (95.6)	Reference	N/A	Reference	N/A
≥45	263/268 (98.1)	2.4 (0.9-6.8)	0.09	2.4 (0.8-7.0)	0.12
HIV status					
Negative	1354/1379 (98.2)	Reference	N/A	Reference	N/A
Positive	248/249 (99.6)	1.5 (-0.4-3.5)	0.14	10.1 (1.3-77.1)	**0.03**

*Bold font denotes statistical significance (P<0.05); OR, odds ratio; aOR, adjusted OR; N/A, not applicable. Only variables found to be statistically significant (P<0.1) in univariate analysis or included *a priori* were assessed in the multivariable model.

** Year, site and age group (P<0.1 on univariate analysis) were evaluated in the multivariable model. Only *a priori* variables and variables remaining significant in the final model are shown in the table.

Table 14.2: Comparison of characteristics of individuals with low and high pneumococcal colonization density, PHIRST study, South Africa, 2016-2018 (N=1585)

Characteristic	Median pneumococcal colonization density		Univariate Analysis		Multivariable Analysis**	
	Low pneumococcal density (<1000 copies/ml) n/N (%)	High pneumococcal density (≥1000 copies/ml) n/N (%)	OR (95% CI)	P-value	aOR (95% CI)	P-value
Overall	893/1585 (56.34)	692/1585 (43.7)	N/A	N/A	-	-
Year						
2016	281/514 (54.7)	233/514 (45.3)	Reference	N/A	-	-
2017	321/549 (58.5)	228/549 (41.5)	0.9 (0.7-1.1)	0.21	-	-
2018	291/522 (55.8)	231/522 (44.3)	0.9 (0.7-1.2)	0.73	-	-
Site						
Agincourt	433/813 (53.3)	380/813 (46.7)	1.3 (1.1-1.6)	**0.01**	-	-
Klerksdorp	460/772 (59.6)	312/772 (40.4)	Reference	N/A	-	-
Sex						
Male	313/626 (50.0)	313/626 (50.0)	1.5 (1.2-1.9)	**<0.01**	-	-
Female	580/959 (60.5)	379/959 (39.5)	Reference	N/A	-	-
Age group (years)						
<1	3/30 (10.0)	27/30 (90.0)	21.9 (6.4-75.3)	**<0.01**	25.4 (7.4-87.6)	**<0.01**
1-4	38/215 (17.7)	177/215 (82.3)	11.3 (7.1-18.2)	**<0.01**	13.5 (8.3-21.9)	**<0.01**
5-14	255/529 (48.2)	274/529 (51.8)	2.6 (1.8-3.7)	**<0.01**	3.1 (2.1-4.4)	**<0.01**
15-24	189/257 (73.5)	68/257 (26.5)	0.9 (0.6-1.3)	0.5	1.0 (0.6-1.5)	0.9
25-44	222/297 (74.8)	75/297 (25.3)	0.8 (0.5-1.2)	0.4	0.8 (0.5-1.2)	0.2
45-65	134/189 (70.9)	55/189 (29.1)	Reference	N/A	Reference	N/A
>65	52/68 (76.5)	16/68 (23.5)	0.7 (0.4-1.4)	0.4	0.8 (0.1-1.6)	0.6
HIV status						
Negative	737/1338 (55.1)	601/1338 (44.9)	Reference	N/A	Reference	N/A
Positive	156/247 (63.2)	91/247 (36.8)	0.7 (0.5-0.9)	**0.02**	1.7 (1.2-2.4)	**<0.01**

*Bold font denotes statistical significance (P<0.05); OR, odds ratio; aOR, adjusted OR; N/A, not applicable.
** Site, sex, age group and HIV status (P<0.1 on univariate analysis) were evaluated in the multivariable model. Only *a priori* variables and variables remaining significant in the final model are shown in the table

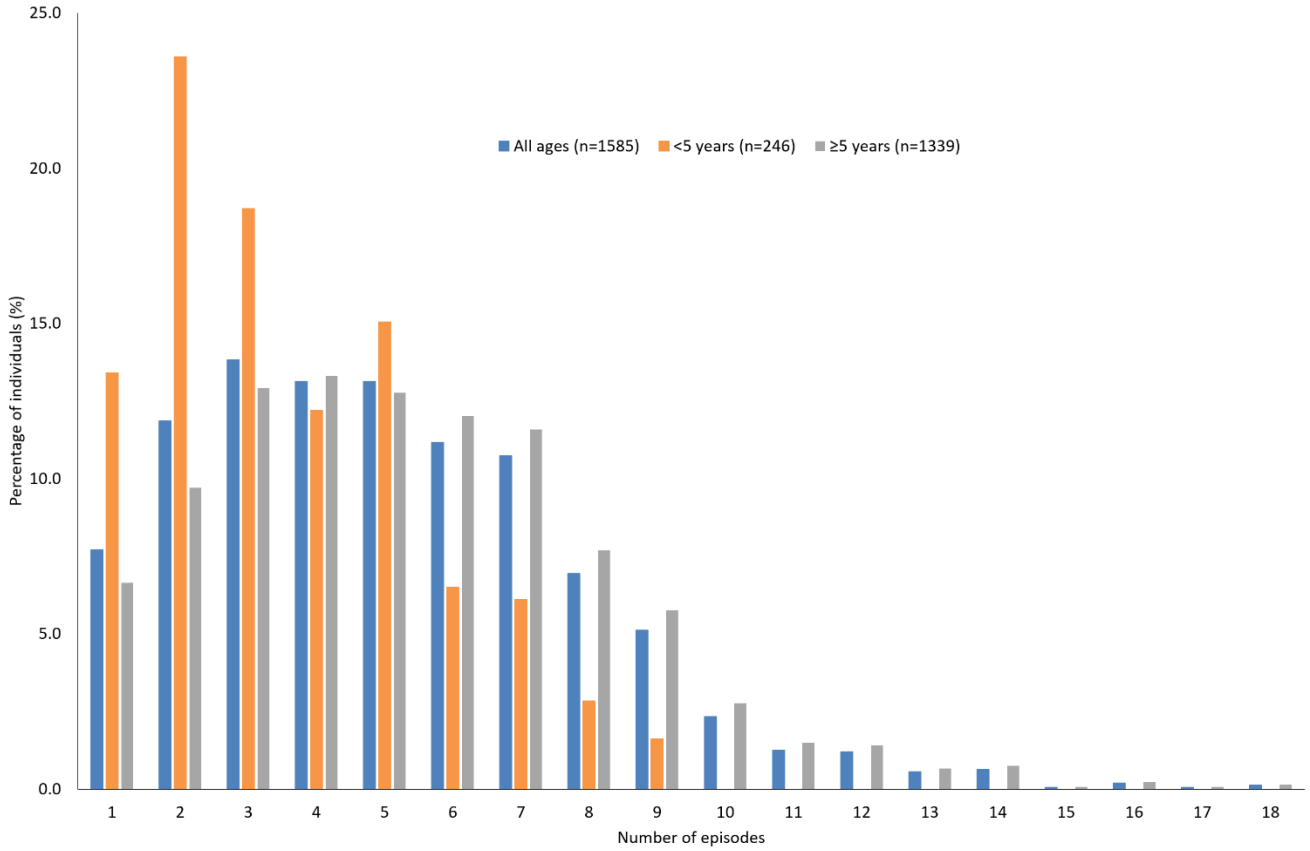


Figure 14.1: Distribution of the percentage of individuals that carried the pneumococcus at least once during the study period by number of pneumococcal colonization episodes and age group, PHIRST study, South Africa, 2016-2018 (N=1585)

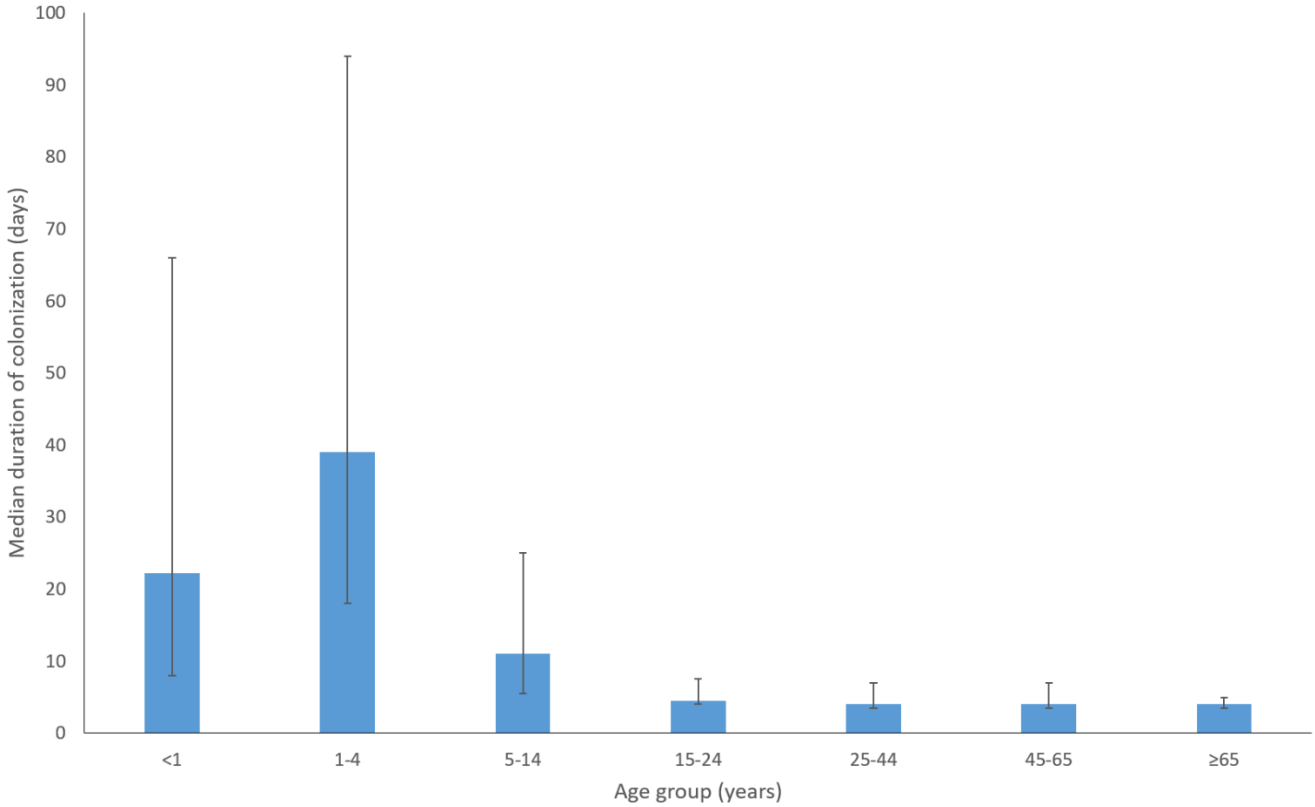


Figure 14.2: Median pneumococcal carriage duration by age group, with error bars indicating interquartile range, PHIRST study, South Africa, 2016-2018 (N=1585)

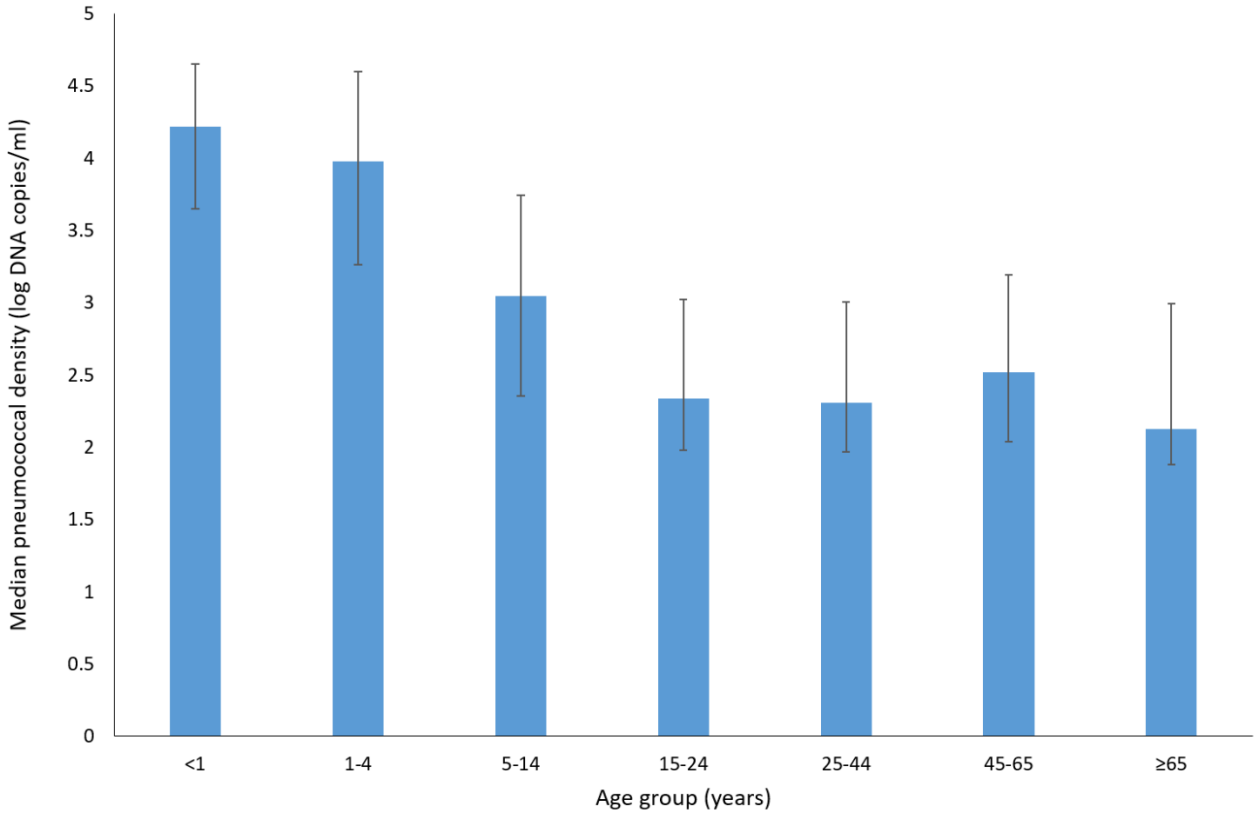


Figure 14.3: Median pneumococcal colonization density with interquartile range by age group, PHIRST study, South Africa, 2016-2018 (N=1585)

14.5. Discussion

Ten years after routine PCV introduction in settings with high vaccine coverage, we observed a high proportion of enrolled persons experienced at least one episode of pneumococcal colonization. While nearly everyone was colonized at some point during the three-year study period, younger children and PLWHIV were more likely to be colonized with the pneumococcus during the follow-up period. Furthermore, younger individuals (<5 years) had fewer episodes of pneumococcal carriage but were colonized with pneumococcus for a longer duration. Younger age (<5 years) and HIV infection were associated with high colonization densities (≥1,000 copies/ml).

Colonization point prevalence ranged from 36% in autumn to 41% in winter and spring. In a cross-sectional study, Pernica *et al.* showed a prevalence of 45% in Canadian children ≤18 years and Carr *et al.* showed that 37% of children in Vientiane (2013-2018) were pneumococcal carriers (25,26). In a meta-analysis of 29 studies, it was found that the point prevalence colonization ranged from 0% to 39% in individuals aged 60 years and older (24). In our study, 38% of all NP specimens tested were positive for *S. pneumoniae*. Althouse *et al.* reported similar results, 36% of swabs, longitudinally collected once a month for 6 months, from Navajo and White Mountain Apache families in the United States were positive for pneumococcus from 2006-2008 (79). A possible reason for similar results between our study and their study despite their less frequent sampling strategy could be due to the long carriage duration observed in our study. In other cohort studies, the detection rate in NP specimens ranged from 25% to 86% with the highest detection seen in The Gambia (27–29). Differences by study may be attributed to the age of the study populations enrolled, the number of individuals in a household, the frequency of specimen collection, and the method used to detect pneumococcus.

Some studies have shown a decrease in colonization and others reported that colonization remained the same after pneumococcal immunization. A longitudinal study in The Gambia showed that all children carried pneumococcus at least once when swabs were collected twice monthly for 6 months with a high proportion of colonization in both children and adults however this was before routine pneumococcal immunization

in that country (29,30). In Peru, 92% of children were colonized at least once where NP specimens were collected monthly for an average of 6 months (28). We also found a high proportion of colonized individuals. Pneumococcal colonization levels vary across studies, likely attributed to sampling strategy employed in the study, the population sampled, and immunization levels of the study population. In Peru, Nelson *et al.* have shown that carriage rates remained high and did not change after PCV7 introduction (28). In the United Kingdom, 58% of participants were colonized with pneumococcus after PCV introduction throughout the 10-month study period during which samples were collected monthly (80). Similar to the UK, in the Drakenstein study in South Africa, pneumococcal carriage was 54% in infants after PCV introduction over the study period (31).

Although colonization rates were high in all age groups, there were differences by age. Young children (<5 years of age) were more likely to be colonized with pneumococcus than older individuals (25-44-year-olds). This is not surprising as this association is well-documented, dating as far back as the 1950s (73,81,82). Recently, pneumococcal colonization rates decreasing with increasing age have been documented in The Gambia (30), Uganda (74), Kenya (75), the United Kingdom (80), Nigeria (83), and the USA (79). However, serotyping data are important to determine whether this decrease in colonization is due to vaccine serotypes. We observed that PLWHIV were more likely to be colonized with pneumococcus than HIV-uninfected individuals. Other studies from South Africa report adults living with HIV had a higher pneumococcal carriage prevalence than HIV-uninfected adults (84,85).

We found children were colonized with pneumococcus for longer periods than adults and as a corollary, the number of episodes differed by age, with children having fewer episodes than adults, similar to research findings by Masters *et al.* (73). A limitation to our study is that the data are left truncated and right censored; therefore, we are not certain how long the pneumococcus may have been carried before the first visit or after the last visit. Abdullahi *et al.* found that host characteristics affect the clearance of pneumococcus and that the clearance rate increased with age (86). In our study, the

median duration of an episode of carriage was over a month in children aged <5 years, similar to studies from Peru and Kenya (28,86).

Previous studies have shown the importance of high pneumococcal carriage density in the development of IPD and transmission of the pneumococcus between individuals (65,87). We found that young age and HIV infection were associated with a high pneumococcal density. Even with the decline in pneumococcal carriage of vaccine serotypes after PCV introduction in South Africa, research has shown the persistent colonization of vaccine serotypes 19F and 23F in South Africa (88). Our data coupled with previous data suggest that after PCV introduction, even in populations with high PCV coverage, children may still play an important role in transmitting pneumococcus (87,89). Even though disease has decreased in children after PCV introduction, children may still be at risk of disease if not immunized or if they have comorbidities (89). This highlights the possible importance of HIV infection in the transmission of pneumococcus, as it has been shown that PCV vaccine efficacy is lower in children living with HIV versus HIV-uninfected children (89). Serotyping of pneumococcus and mathematical modeling could describe this in greater detail to gain a better understanding of the transmission dynamics within the households and communities in South Africa.

A key strength of this study lies in its longitudinal, community-based design, which enabled detailed observation of pneumococcal acquisition and clearance in a real-world, post-PCV setting. Frequent sampling allowed detection of short-term carriage dynamics, while inclusion of diverse demographic and health characteristics, including HIV status, provided nuanced insights into host factors influencing colonization. Another strength of our study was that we unlikely missed episodes of colonization, which affords us the ability to gain a higher resolution into pneumococcus over time, an advantage over cross sectional or case-related studies. However, a limitation is that NP specimens were tested for pneumococcus using a qPCR, which has reduced specificity because of the genetic exchange that occurs between pneumococcus and *Streptococcus* spp. such as *S. pseudopneumoniae* and *S. mitis* (90). Tavares *et al.* showed that using the *lytA* gene with the SP2020 gene to identify *S. pneumoniae* improved specificity from 99.5% to 100% (91). We identified pneumococcus by targeting the *lytA* gene only, potentially

misidentifying 0.5% of *Streptococcus* spp. as *S. pneumoniae.* This limitation may influence interpretation of carriage prevalence, particularly in samples with low bacterial density. Although serotyping or complementary gene targets could further improve specificity, these data were not available in the current study. Nonetheless, the *lytA*-based assay used here has been validated in multiple studies and is generally considered robust for pneumococcal detection in epidemiological surveillance.

We observed a difference in the proportion of colonization in 2017 compared to 2016. This difference may be attributed to the difference in the duration of the follow-up period, in which participants were followed up for a longer period in 2017 compared with 2016 (ten vs six months), which may have underestimated the number of episodes identified in 2016. In addition, individuals who were lost to follow-up remained in the analysis, and we are unable to determine whether these individuals would have been colonized had they not withdrawn. Another limitation is serotyping data for this study were not available; hence, the number of pneumococcal episodes may be underestimated and the duration of carriage may be overestimated since the duration of colonization differs by serotype and multiple episodes of colonization with different serotypes could have been incorrectly classified as a single episode (75). We applied a Markov model to the follow-up data to help address this limitation.

NP swabs were size-appropriate for participants' ages, with pediatric swabs used for children and adult swabs used for older participants. Nonetheless, minor differences in swab tip size relative to nasopharyngeal anatomy could influence sample yield and bacterial load estimates. While this effect is likely minimal, it remains a potential source of variability when comparing colonization density across age groups. Comparing the pneumococcal density in the nasopharynx over time is complex because the bacterial load is dependent on the volume of nasal secretions, the quality of the specimen taken, and variation in sample collection technique used. However, in a publication in which the concentration of human DNA was used to control for this limitation, the authors found that flocked swabs are efficient at collecting a standard volume of sample from the nasopharynx (92).

Future studies could build on these findings by incorporating genomic characterization of pneumococcal isolates to explore strain-level transmission, serotype replacement, and antibiotic resistance patterns in greater depth. Extending follow-up beyond a single year and integrating host immunological data could further clarify how age, HIV infection, and vaccination shape long-term colonization dynamics in the post-PCV era.

In our study cohort, we found high levels of pneumococcal colonization across all age groups. Children aged <5 years carried pneumococcus for longer periods. PLWHIV and children <5 years were associated with high pneumococcal densities. These findings highlight that young children may potentially impact transmission and remain important in the transmission of pneumococcus even after PCV introduction.

15. Chapter 4: Temporal changes in nasopharyngeal pneumococcal colonization density associated with RSV and influenza infection in a South African household cohort study, 2016-2018

This chapter is based on a published manuscript.

15.1. Reference

Carrim, M, Kleynhans, J, Tempia, S, Hellferscee O, Treurnicht FK, McMorrow ML, Moyes J, Wafawanaka F, Cohen C, von Gottberg A and Wolter N. Temporal changes in pneumococcal density associated with influenza and RSV in a South African household cohort study, 2016-2018. Open Forum Infectious Diseases. 2025 May 31; 12(6):ofaf267. eCollection 2025 June.

15.2. Introduction

The interplay between respiratory viruses and bacterial pathogens is increasingly recognized as an important determinant of the burden and severity of pneumonia, particularly in settings with high infectious disease prevalence. Among the most significant interactions are those between RSV or influenza virus and *S. pneumoniae*, which have shown to influence colonization dynamics and the risk of disease (35,36,40). Viral infections impair epithelial barriers and immune responses, aiding pneumococcal colonization (35,36,40). Experimental and observational studies have demonstrated that both RSV and influenza can increase pneumococcal load in the upper respiratory tract, enhancing transmission potential and increasing the likelihood of progression to disease (41,42). Numerous studies have suggested that influenza and RSV can increase pneumococcal density in the nasopharynx, which in turn may elevate the risk of invasive pneumococcal disease (37,45–47,93). Most of this evidence, however, comes from high-income settings, where background risk factors differ substantially from those in low- and middle-income countries. By exploring this relationship in a diverse South African cohort, with a high disease and HIV burden, this study aims to provide insights that could inform public health strategies and improve clinical outcomes. We therefore aimed to determine whether RSV or influenza infection is associated with a temporal increase in pneumococcal nasopharyngeal colonization density and to determine factors associated with changes in pneumococcal density during a RSV or influenza infection episode in a household community cohort in South Africa.

15.3. Materials and methods

15.3.1. Study design and laboratory testing

The study design, population studied and laboratory testing have been described in section 13.1.

15.3.2. Data analysis

For this analysis, we included only those individuals who tested positive for influenza or RSV at least once during the study period and tested positive for pneumococcus before and during the viral infection. RT-qPCR Ct-value was used as a proxy for viral load (i.e., a low Ct-value implied a high viral load and vice versa). To assess whether viral load is associated with a change in pneumococcal density, we used the result of the swab with the lowest Ct-value during the infection to assess the viral load.

15.3.3. Statistical Analysis

We defined an RSV or influenza virus infection as at least one RT-qPCR (Ct-value <37) NP swab positive for the respective virus, irrespective of symptoms. For influenza, we considered an infection to be new when the individual tested positive for a different virus subtype or the same subtype more than 2 weeks after the last day of previous positivity; all other instances were considered the same infection. These criteria were used as individuals could test negative and then positive again due to fluctuations in viral load or specimen quality (68,94). For our analysis, we did not stratify RSV by subtype. A study interval was defined as all pneumococcal-positive swabs tested 2 weeks (4 visits) before a viral (RSV or influenza) infection (before) and all pneumococcal-positive swabs tested during a viral infection (during). The "after" period was defined as starting from the first RSV/influenza negative swab collected after the last RT-qPCR-positive swab. We calculated the mean across swabs collected 2 weeks (4 visits) before, during, and 2 weeks after infection for the 2-week time point. Similarly, for the 8-week time point, we used swabs from 8 weeks (16 visits) before, during, and after infection.

If an individual was positive for RSV and thereafter influenza or vice versa, each infection was regarded as a separate episode and included in the analysis for the respective virus. The duration of a viral infection in days was determined for each participant by multiplying the number of visits with positive results by 2.5 days since

swabs were collected every 2 to 3 days for both an RSV and influenza infection. Thereafter these data were used to determine the mean duration of the infection for each virus. Furthermore, we computed the mean pneumococcal density for each study interval per individual. For each participant, the mean pneumococcal density was first computed from the raw measurements. This mean density was then log-transformed using the natural logarithm (ln, base e) to calculate the logarithmic mean. Natural log transformation was used to stabilize variance and normalize the distribution of density values, ensuring that subsequent analyses were robust and comparable across participants. We compared the log mean pneumococcal density by time (during vs. before, during vs. after and after vs. before) in relation to an RSV or influenza infection using a t-test and a 95% confidence interval (CI). We determined the factors associated with a change in pneumococcal density (the difference in the log mean pneumococcal density before an RSV or influenza infection and the log mean pneumococcal density during the viral infection) in a multivariable linear regression model. We assessed all variables that were significant at $P<0.2$ on univariate linear regression analysis or a priori (age group, HIV status, RSV, and influenza) and dropped non-significant factors in the multivariable model ($P\geq0.05$) with manual backward elimination. We assessed pairwise interactions by the inclusion of product terms for all variables remaining in the final multivariable additive model. Furthermore, we accounted for clustering by study site (Klerksdorp or Agincourt) and within the household using mixed effects models. We performed the analysis using Stata version 14 (StataCorp LLC, College Station, Texas, USA), R Foundation for Statistical Computing (R Core Team, Vienna, Austria) and RStudio: integrated Development for R (RStudio Team, PBC, Boston, USA). Statistical significance was assessed at $P<0.05$.

15.4. Results

In the PHIRST study, 325 households were enrolled comprising 1658 individuals (Figure 15.1). With twice-weekly follow-ups, 105,686 samples were tested for *S. pneumoniae*, RSV, and influenza of which 38,829 (36.7%) were positive for the pneumococcus, 1002 (0.9%) were positive for RSV and 1265 (1.2%) were positive for influenza [597 (0.6%) influenza A, 665 (0.6%) influenza B, and 3 (<0.1%) influenza A and B]. During the study, 1079/1442 (74.8%) individuals were infected with RSV or influenza; 462/1442 (32.0%)

positive for RSV, 314/1442 (21.8%) positive for influenza A and 303/1442 (21.0%) positive for influenza B. Of the individuals positive for a virus, 46.7% (504/1079) [35.7% (165/462) RSV, 58.3% (183/314) influenza A and 51.5% (156/303) influenza B] had at least one symptom.

Among individuals positive for either RSV or influenza during the study period, 530 (49.1%) were colonized with the pneumococcus before and during the infection (Figure 15.1, Table 15.1) and included in our analysis. Among individuals colonized with the pneumococcus and infected with RSV or influenza, 55% (291/530) were female, 54% (286/530) were from the rural setting, 93% (472/509) were HIV-uninfected, and 84% (194/231) were fully vaccinated for their age (Table 15.1). Furthermore, 54.0% (286/530) [42.4% (98/231) RSV, 66.4% (97/146) influenza A, 59.5% (91/153) influenza B] of individuals reported at least one symptom during the infection.

The mean duration of a viral infection in individuals colonized with the pneumococcus was 7.8 days (95% CI 6.5 – 9.0) for RSV, 6 days (95% CI 5.5 – 6.8) for influenza A and 6.8 days (95% CI 5.8 – 7.8) for influenza B. When we looked at the mean pneumococcal density by days a sample was collected in relation to the first day positive, there was an increase in the density of pneumococcus at the first day in the viral infection and the density weaned thereafter (Figure 15.2A). However, for influenza virus A and B, we did not observe a clear increase before or on the first day positive. (Figure 15.2B, and 15.2C). We observed a significant increase in the log mean pneumococcal colonization density (expressed as log genomic copies/ml) before vs. during an RSV infection (log mean before vs. log mean during, 9.3 vs. 10.2; $P<0.01$) (Figure 15.3A). Conversely, there was no significant increase in the log mean pneumococcal density before and during an influenza infection (log mean before vs. log mean during, 9.6 vs. 9.9 genomic copies/ml; $P=0.2$) (Figure 15.3B). Furthermore, findings were similar when we analyzed influenza A and influenza B separately (influenza A infection [log mean before vs. log mean during, 9.5 vs. 9.8 genomic copies/ml; $P=0.3$], influenza B infection [log mean before vs. log mean during, 9.7 vs. 10.0 genomic copies/ml; $P=0.4$]) (Figure 15.3C and Figure 15.3D).

Two weeks following an RSV infection, pneumococcal density remained elevated compared to pre-viral infection levels (log mean before 9.3 [95% CI 8.9 – 9.8] vs. log mean 2 weeks after 10.3 [95% CI 9.9 – 10.7]; P<0.01). This elevation in pneumococcal density persisted 2 months after an RSV (log mean during 10.3 (95% CI 9.9 – 10.6) vs. log mean 2 months after 11.0 (95% CI 10.7 – 11.3); P<0.01).

When ascertaining factors associated with changes in pneumococcal carriage density during an RSV infection, we found that an increase in pneumococcal density was observed for individuals with a higher viral load (viral Ct-value <25 coefficient 2.5, 95% CI 1.1 – 3.9; P<0.01 and viral Ct-value 25-29 coefficient 1.1 95% CI 0.1 – 2.2; P=0.04) vs. viral Ct-value 30-34) (Table 15.2). Furthermore, underweight individuals were less likely to have an increase in density (coefficient -1.8, 95% CI -3.5 – -0.1; P=0.04) compared to individuals with a normal body mass index (BMI) (Table 15.2).

Even though there was no statistically significant increase in the pneumococcal density in the presence of influenza we found that a higher mean influenza viral load (low Ct-value) was associated with a significant change in pneumococcal density during an influenza infection compared to before an influenza infection (viral Ct-value <25 coefficient 2.8, 95% CI 1.4 – 4.2; P<0.01 and viral Ct-value 25-29 coefficient 1.2 95% CI 0.2 – 2.3; P=0.03) vs. viral Ct-value 30-34) (Table 15.3).

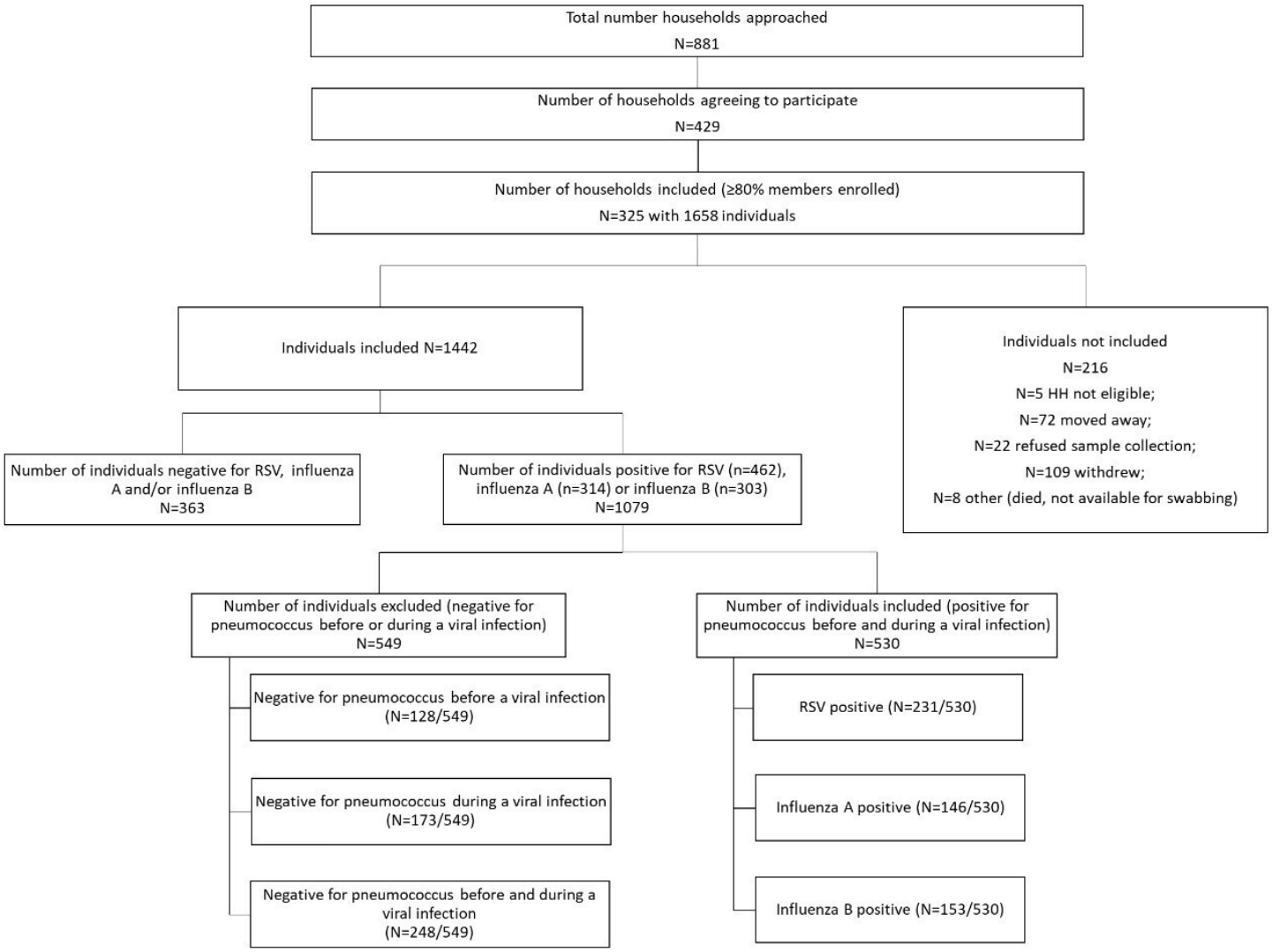


Figure 15.1: A flow chart depicting the number of participants enrolled, tested and included in the analysis – PHIRST study, South Africa, 2016-2018

Table 15.1: Demographic characteristics of all individuals enrolled in the PHIRST Study and individuals colonized with pneumococcus during an infection with RSV, influenza A or influenza B – PHIRST Study, South Africa, 2016-2018.

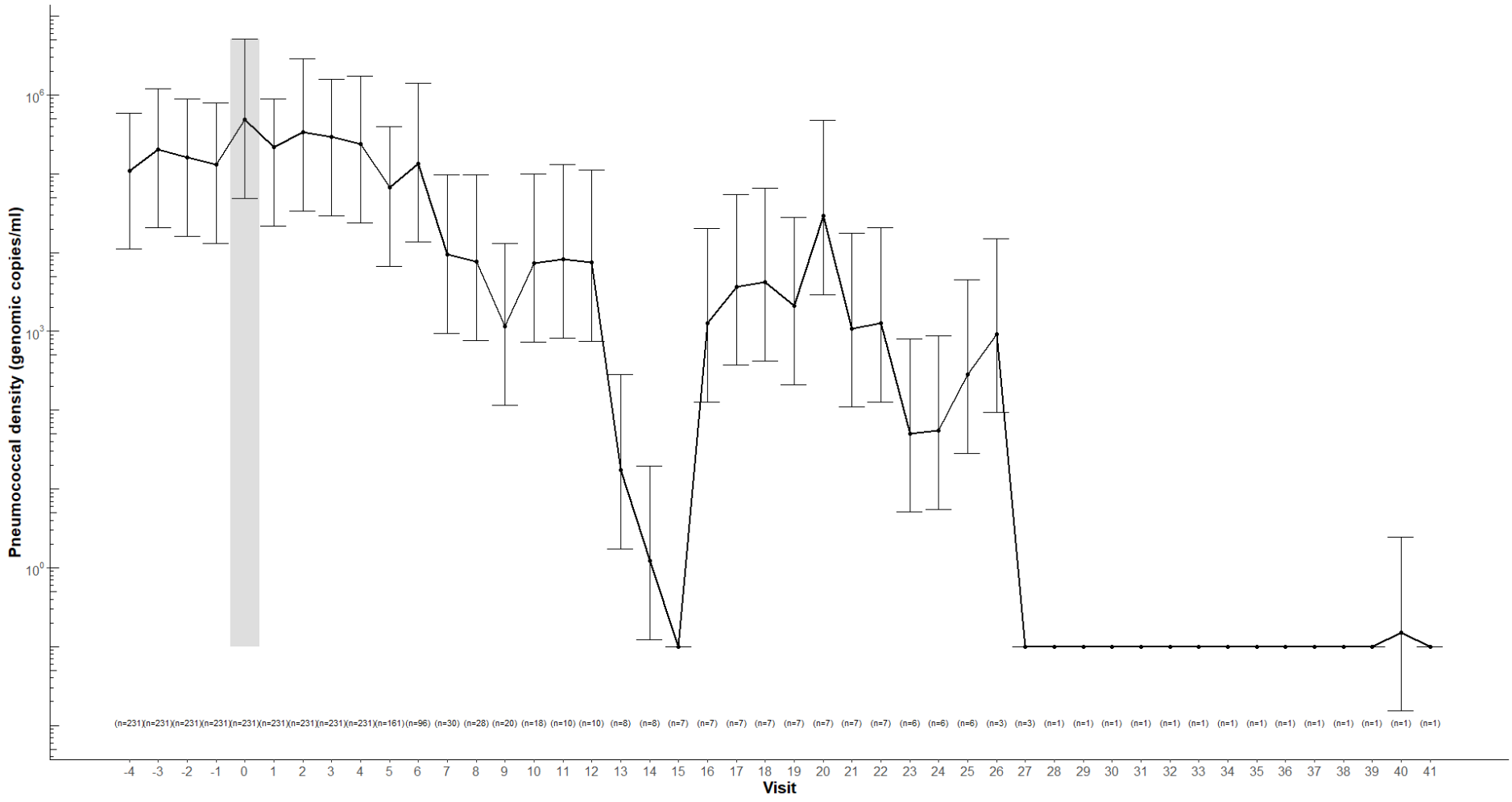
Characteristic	PHIRST Study Population n/N (%)	RSV and influenza infections among pneumococcal colonized individuals n/N (%)	RSV infections among pneumococcal colonized individuals n/N (%)	Influenza A infections among pneumococcal colonized individuals n/N (%)	Influenza B infections among pneumococcal colonized individuals n/N (%)
Year					
2016	542/1658 (33)	154/530 (29)	76/231 (33)	30/146 (20)	48/153 (31)
2017	558/1658 (34)	185/530 (35)	76/231 (33)	74/146 (51)	35/153 (23)
2018	558/1658 (34)	191/530 (36)	79/231 (34)	42/146 (29)	70/153 (46)
Age (years)					
<1	34/1658 (2)	19/530 (4)	10/231 (4)	5/146 (3)	4/153 (3)
1-4	240/1658 (15)	212/530 (40)	97/231 (42)	61/146 (42)	54/153 (35)
5-14	546/1658 (33)	216/530 (41)	92/231 (40)	51/146 (35)	73/153 (48)
15-24	264/1658 (16)	30/530 (6)	9/231 (4)	11/146 (8)	10/153 (7)
25-44	311/1658 (19)	29/530 (6)	11/231 (5)	9/146 (6)	9/153 (6)
>45	263/1658 (16)	24/530 (4)	12/231 (5)	9/146 (6)	3/153 (2)
Sex					
Female	991/1658 (60)	291/530 (55)	132/231 (57)	76/146 (48)	83/153 (54)
Male	667/1658 (40)	239/530 (45)	99/231 (43)	70/146 (52)	70/153 (46)

Site					
Rural	841/1658 (51)	286/530 (54)	140/231(61)	73/146 (50)	73/153 (48)
Urban	817/1658 (49)	244/530 (46)	91/231 (39)	73/146 (50)	80/153 (52)
HIV status					
Uninfected	1363/1609 (85)	472/509 (93)	206/222 (93)	135/141 (96)	131/146 (90)
PLWHIV	246/1609 (15)	37/509 (7)	16/222 (7)	6/141 (4)	15/146 (10)
PCV vaccination*					
Fully vaccinated	221/274 (81)	194/231 (84)	85/107 (79)	58/66 (88)	51/58 (88)
Partially vaccinated	7/274 (2)	4/231 (2)	2/107 (2)	0/66 (0)	2/58 (4)
Unknown	46/274 (16)	33/231 (14)	20/107 (19)	8/66 (12)	5/58 (8)
Chronic medical conditions[#]					
Yes	50/1658 (3)	11/530 (2)	5/231 (2)	1/146 (1)	5/153 (3)
No	1608/1658 (97)	519/530 (98)	226/231 (98)	145/146 (99)	148/153 (97)

* Individuals younger than 5 years with available data

Self-reported history of asthma, lung disease, heart disease, stroke, spinal epilepsy, organ transplant, immunosuppressive therapy, organ transplantation, cancer, liver disease, renal disease, or diabetes. Abbreviations: PHIRST – Prospective household cohort study of influenza, RSV, and other respiratory pathogens community burden and transmission dynamics in South Africa; RSV – Respiratory syncytial virus; PLWHIV – People living with HIV; PCV – pneumococcal conjugate vaccine.

A


Pneumococcal density (genomic copies/ml)
10^6
10^3
10^0
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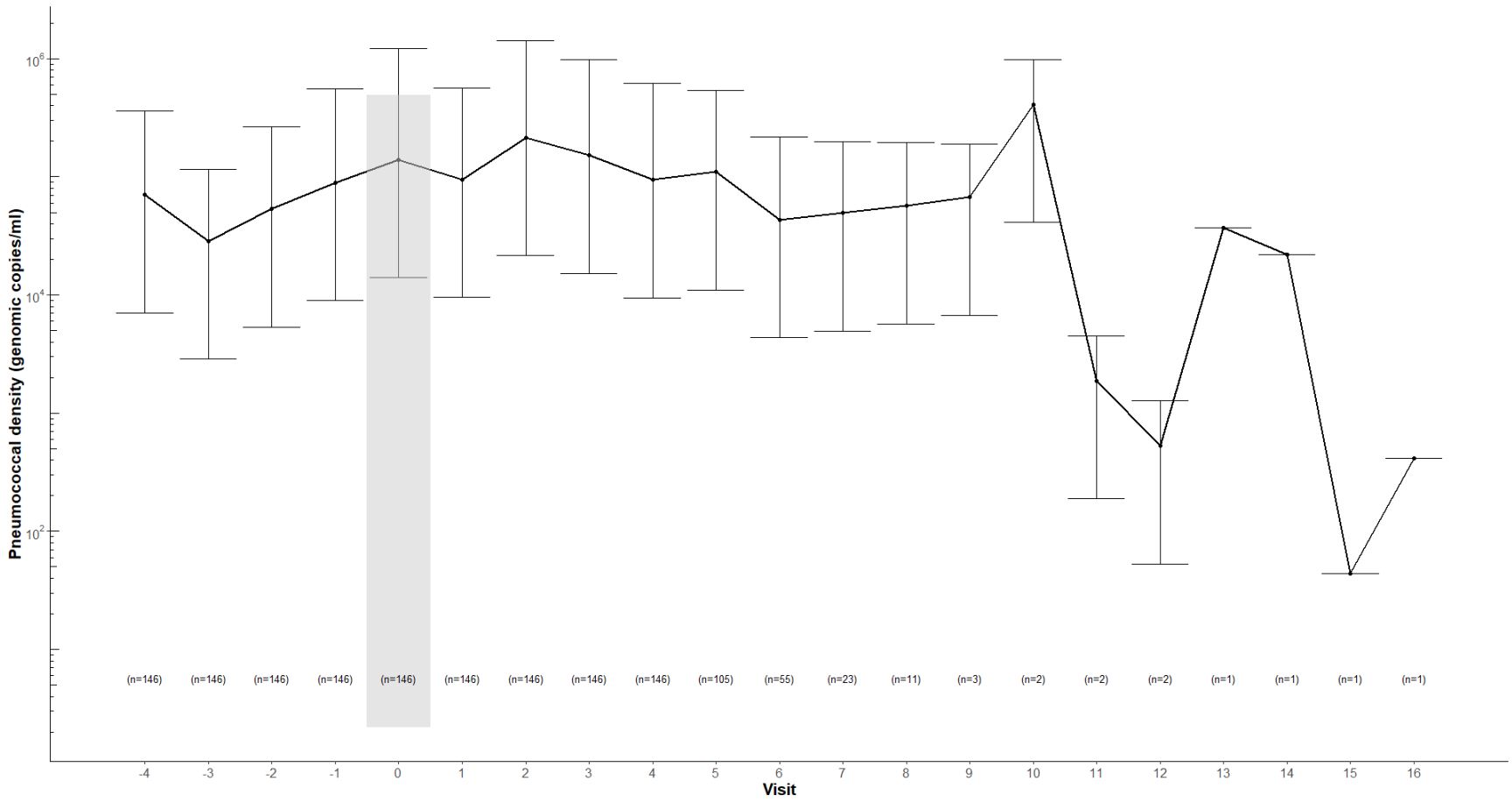
Pneumococcal density (genomic copies/ml)
10^6
10^4
10^2
(n=146) (n=146) (n=146) (n=146) (n=146) (n=146) (n=146) (n=146) (n=146) (n=105) (n=55) (n=23) (n=11) (n=3) (n=2) (n=2) (n=2) (n=1) (n=1) (n=1) (n=1)
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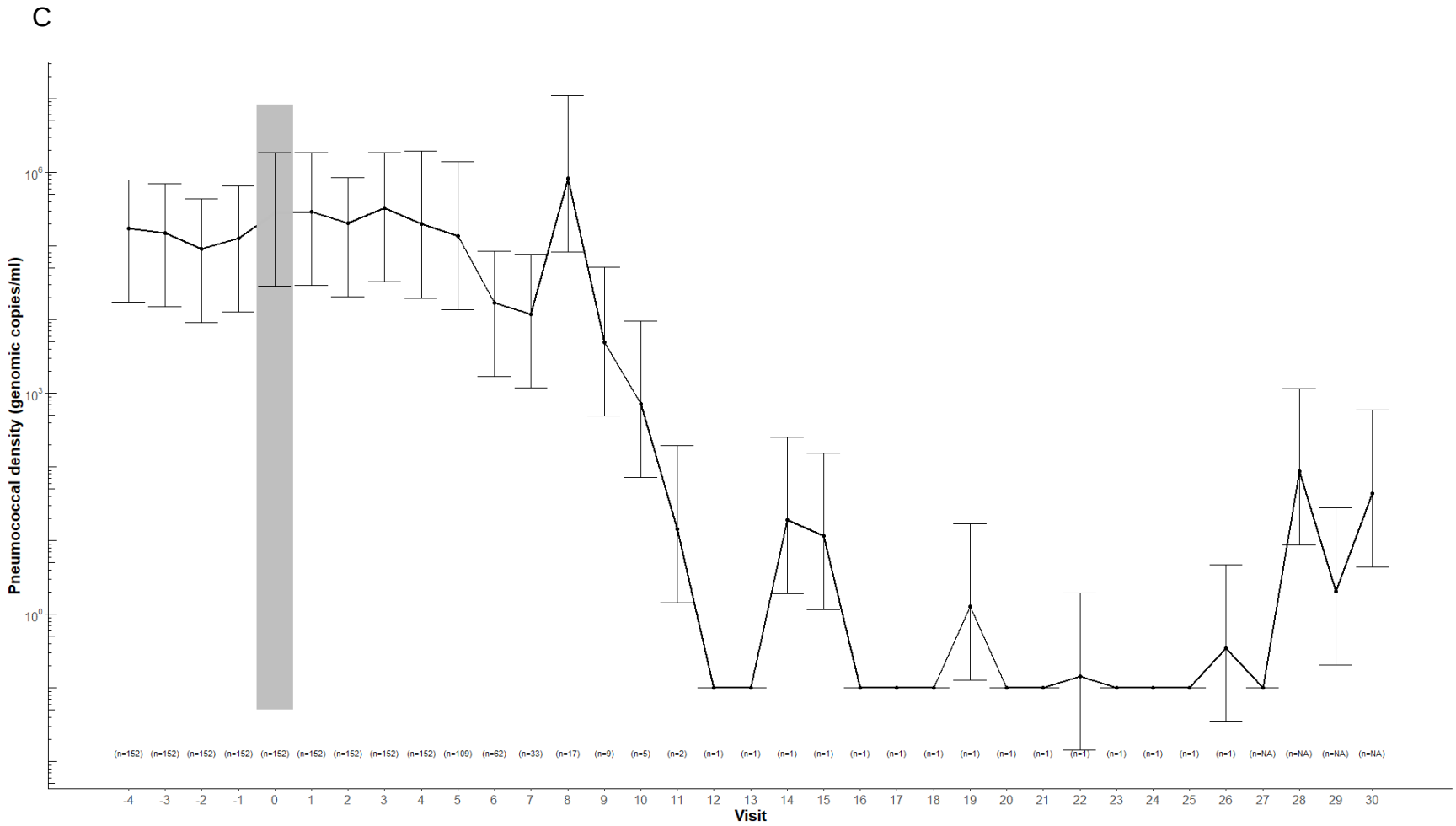


Figure 15.2: The mean pneumococcal density (genomic copies/ml) of all individuals with (A) RSV (N=231), (B) influenza A virus (N=146) (C) influenza B (N=152) by visit in relation to the first visit positive for the virus, The PHIRST Study, South Africa, 2016-2018 Error bars depict ±standard deviation per point. Grey shaded block indicates the first day of a respiratory virus episode.

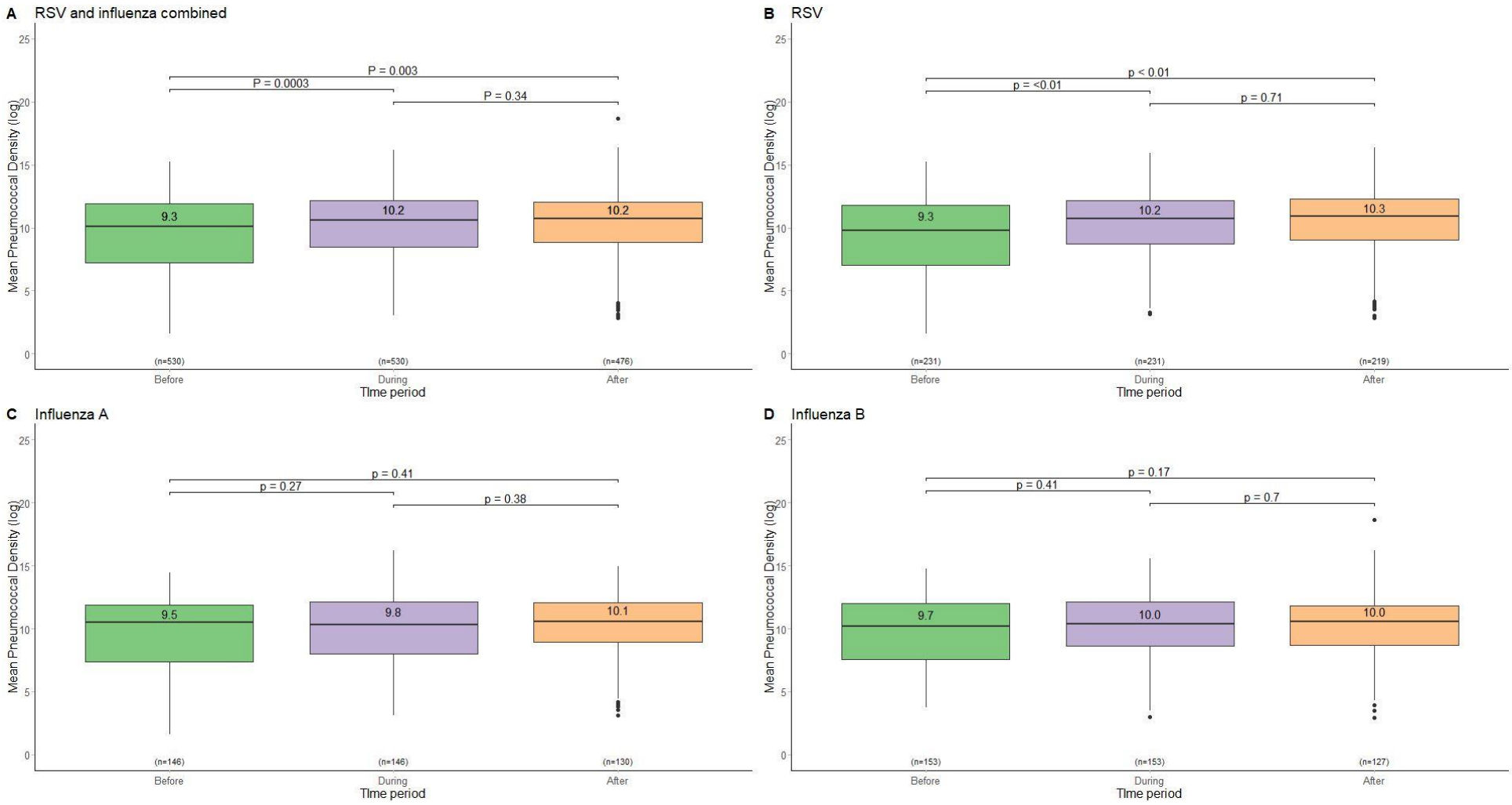


Figure 15.3: Box and whisker plots comparing the natural log mean pneumococcal density before, during and after an RSV or influenza infection, PHIRST Study, South Africa, 2016-2018. The panels show (A) RSV only (N=231), (B) Influenza A and B combined (N=299), (C) influenza A (N=146), (D) influenza B (N=153). Bars on the top of each figure show the comparison of log mean density by period and the corresponding t-test P-value.

Table 15.2: Factors associated with a change in pneumococcal carriage density before vs. during a RSV infection, PHIRST Study, South Africa, 2016-2018 (N=231)

Characteristic	Number of individuals [1]	Natural log mean density		Natural log mean density differences[4] (95% CI)	Univariate Regression Analysis		Multivariable Regression Analysis	
		Before	During		Coefficient (95% CI)	p-value	Coefficient (95% CI)	p-value
Enrollment Year								
2016	76	10.0	10.4	0.4 (-0.3 – 1.1)	Reference			
2017	76	9.2	10.5	1.3 (0.4 – 2.1)	0.9 (-0.3 – 2.0)	0.1		
2018	79	8.8	9.8	1.0 (0.1 – 1.8)	0.5 (-0.6 – 1.7)	0.4		
Age (years)								
<1	10	11.1	11.0	-0.1 (-1.7 – 1.5)	-1.0 (-3.2 – 1.2)	0.4	-1.9 (-4.3 – 0.5)	0.1
1-4	97	10.4	11.0	0.6 (-0.1 – 1.3)	-0.4 (-1.4 – 0.5)	0.4	-1.0 (-2.1 – 0.04)	0.06
5-14	92	9.0	10.0	1.0 (0.2 – 1.8)	Reference		Reference	
15-24	9	7.4	8.7	1.3 (-0.5 – 3.1)	0.4 (-1.9 – 2.8)	0.7	0.7 (-1.9 – 3.2)	0.6
25-44	11	6.2	7.9	1.7 (-0.1 – 3.4)	0.7 (-1.5 – 2.8)	0.5	0.6 (-1.8 – 2.9)	0.6
≥45	12	5.9	8.1	2.2 (-0.1 – 4.5)	1.2 (0.9 – 3.3)	0.3	1.1 (-1.3 – 3.5)	0.4
Sex								
Female	132	9.4	9.9	0.6 (0.1 – 1.3)	Reference			
Male	99	9.2	10.6	1.1 (0.4 – 1.9)	0.4 (-0.5 – 1.3)	0.4		
HIV Status								
Uninfected	206	9.4	10.3	0.9 (0.4 – 1.4)	Reference		Reference	

PLWHIV	16	7.6	8.8	1.2 (0.2 – 2.2)	0.1 (-1.4 – 1.6)	0.4	-0.8 (-2.8 – 1.2)	0.4
Chronic medical condition[2]								
Yes	5	9.6	12.1	2.5 (0.01 – 5.0)	1.6 (-1.40 – 4.7)	0.3		
No	226	9.3	10.2	0.8 (0.4 – 1.3)	Reference			
BMI								
Underweight	18	10.9	10.1	-0.8 (-2.7 – 1.0)	**-1.7 (-3.4 – -0.04)**	**0.04**	**-1.8 (-3.5 – -0.1)**	**0.04**
Normal	177	9.5	10.4	0.9 (0.4 – 1.4)	Reference		Reference	
Overweight	14	7.1	9.5	2.3 (0.01 – 4.6)	1.4 (-0.5 – 3.3)	0.1	0.3 (-1.1 – 2.3)	0.8
Obese	20	7.6	9.2	1.6 (0.2 – 3.1)	0.8 (-0.8 – 2.4)	0.3	0.1 (-0.6 – 2.0)	0.9
Symptoms[3]								
Yes	98	9.1	10.6	1.5 (0.8 – 2.3)	**1.1 (0.2 – 2.0)**	**0.02**	0.9 (-0.9 – 1.8)	0.07
No	133	9.5	9.9	0.4 (-0.2 – 1.0)	Reference		Reference	
RSV infection duration (days)								
<10	203	9.2	10.1	0.8 (0.4 – 1.3)	Reference			
≥10	28	10.0	11.2	1.1 (-0.2 – 2.4)	0.3 (-1.1 – 1.7)	0.7		
Mean Ct-value of the RSV virus								
<25	42	9.3	11.6	2.3 (1.2 – 3.4)	**2.1 (0.9 – 3.4)**	**0.01**	**2.5 (1.1 – 3.9)**	**<0.01**
25-29	94	9.3	10.4	1.1 (0.4 – 1.8)	0.9 (-0.09 – 1.9)	0.07	**1.1 (0.1 – 2.2)**	**0.04**
30-34	79	9.3	9.5	0.2 (-0.5 – 1.0)	Reference		Reference	
≥35	16	9.2	8.3	-0.9 (-2.8 – 0.9)	-1.2 (-3.0 – 0.6)	0.2	-0.6 (-2.5 – 1.4)	0.6

[1]Number of individuals colonized with pneumococcus and infected with influenza [2]Self-reported history of asthma, lung disease, heart disease, stroke, spinal epilepsy, organ transplant, immunosuppressive therapy, organ transplantation, cancer, liver disease, renal disease, or diabetes. [3]Symptoms included at least one of the following: fever (self-reported or measured tympanic temperature ≥38 °C), cough, difficulty breathing, sore throat, nasal congestion, chest pain, muscle aches, headache, vomiting or diarrhea. [4] Log mean density differences were

calculated by subtracting the log mean pneumococcal density before an RSV or influenza infection from the log mean pneumococcal density during an RSV or influenza infection. BMI calculated using the formula (weight in kilograms)/ (height in meters squared). We defined BMI categories as follows: underweight - age <18 years weight for age or BMI <-2 standard deviations of the World Health Organization (WHO) Child Growth Standards, age ≥18 years BMI <18.5kg/m2; overweight - age <18 years BMI >+1 and ≤+2 standard deviations of the WHO growth standards, age ≥18 years BMI ≥25 and <30kg/m2, obese – age <18 years BMI >+2 standard deviations of the WHO growth standards, age ≥18 years BMI ≥30 kg/m^2. Abbreviations: PHIRST - a prospective household cohort study of influenza, RSV, and other respiratory pathogens community burden and transmission dynamics in South Africa; BMI – Body Mass Index; PLWHIV – people living with HIV; CI – Confidence interval, Ct-value – Cycle-threshold value. Bold font denotes statistical significance (P<0.05). Only variables found to be statistically significant (P<0.2) in univariate analysis or included a priori (age group and HIV status) were assessed in the multivariable model. Only a priori variables and variables remaining significant in the final model are shown in the table.

Table 15.3: Factors associated with a change in pneumococcal carriage density before and during an influenza infection, PHIRST Study, South Africa, 2016-2018 (N=299)

Characteristic	Number of individuals[1]	Natural log mean density		Natural log mean density differences[4] (95% CI)	Univariate Regression Analysis		Multivariable Regression Analysis	
		Before	During		Coefficient (95% CI)	p-value	Coefficient (95% CI)	p-value
Year								
2016	78	9.7	9.9	0.2 (-0.7 – 1.1)	Reference			
2017	109	9.1	9.3	0.2 (-0.4 – 0.8)	0.1 (-1.0 – 1.0)	0.9		
2018	112	10.1	10.4	0.3 (-0.2 – 0.9)	0.1 (-0.9 – 1.1)	0.9		
Age (years)								
<1	9	11.5	11.2	-0.3 (-4.1 – 3.5)	-1.1 (-3.3 – 1.1)	0.3	-2.4 (-4.9 – 0.1)	0.06
1-4	115	10.3	10.6	0.3 (-0.3 – 0.9)	-0.5 (-1.4 – -0.3)	0.2	-1.0 (-2.0 – 0.02)	0.06
5-14	124	9.4	10.0	0.6 (0.4 – 1.2)	Reference		Reference	
15-24	21	8.9	8.5	0.4 (-1.7 – 0.8)	-1.0 (-2.5 – 0.5)	0.2	0.4 (-2.2 – 2.9)	0.8
25-44	18	7.7	7.1	-0.7 (-2.6 – 1.2)	-1.5 (-3.1 – 0.1)	0.06	0.9 (-1.4 – 3.2)	0.4
≥45	12	7.5	6.6	-0.9 (-3.2 – 1.3)	-1.7 (-3.6 – 1.2)	0.08	1.1 (-1.1 – 3.4)	0.3
Sex								
Female	159	9.6	9.8	0.2 (0.4 – 0.7)	Reference			
Male	140	9.6	10.0	0.4 (-0.2 – 9.4)	0.2 (-0.5 – 1.0)	0.5		
HIV Status								
Uninfected	266	9.7	10.0	0.3 (-0.1 – 0.7)	Reference		Reference	

PLWHIV	21	8.1	8.5	0.4 (-1.2 – 1.9)	0.1 (-1.4 – 1.6)	0.9	-0.9 (-2.9 – 1.1)	0.4
Chronic medical condition[2]								
Yes	6	8.7	11.4	2.7 (-0.2 – 0.6)	**2.4 (-0.2 – 5.0)**	**0.08**	2.1 (-1.1 – 5.4)	0.2
No	293	9.6	9.8	0.2 (-1.4 – 6.9)	Reference		Reference	
BMI								
Underweight	32	10.8	10.1	-0.7 (-1.9 – 0.4)	-1.0 (-2.2 – -0.3)	0.1		
Normal	215	9.7	10.0	0.3 (-0.1 – 0.8)	Reference			
Overweight	31	8.9	9.3	0.4(-0.8 – 1.6)	0.3 (-1.0 – 1.5)	0.7		
Obese	21	8.3	9.0	0.7 (-0.9 – 2.2)	0.2 (-1.2 – 1.7)	0.8		
Symptoms[3]								
Yes	188	9.7	10.3	0.4 (-0.01 – 0.9)	0.4 (-0.4 – 1.2)	0.3		
No	111	9.5	9.4	-0.1 (-0.7 – 0.6)	Reference			
Virus type								
Influenza A	146	9.5	9.8	0.3 (-0.2 – 0.8)	Reference			
Influenza B	153	9.7	9.9	0.2 (-0.3 – 0.8)	-0.04 (-0.8 – 0.7)	0.9		
Virus infection duration (days)								
<10	271	9.6	9.7	0.1 (-0.3 – 0.5)	Reference		Reference	
≥10	28	9.8	11.5	1.7 (0.6 – 2.7)	**1.5 (0.3 – 2.8)**	**0.02**	0.4 (-1.0 – 1.8)	0.6
Mean Ct-value of the virus								
<25	67	10.0	9.9	-0.1 (-0.8 – 0.7)	0.1 (-1.0 – 1.1)	0.9	**2.8 (1.4 – 4.2)**	**<0.01**
25-29	139	9.7	10.4	0.7 (0.1 – 1.3)	0.8 (-0.5 – 1.7)	0.07	**1.2 (0.2 – 2.3)**	**0.03**
30-34	83	9.2	9.1	-0.1 (-0.7 – 0.5)	Reference		Reference	

≥35	10	9.6	9.2	-0.4 (-2.9 – 0.9)	-0.3 (-2.4 – 1.8)	0.7	0.8 (-2.8 – 1.1)	0.4

[1]Number of individuals colonized with pneumococcus and infected with influenza [2]Self-reported history of asthma, lung disease, heart disease, stroke, spinal epilepsy, organ transplant, immunosuppressive therapy, organ transplantation, cancer, liver disease, renal disease, or diabetes. [3]Symptoms included at least one of the following: fever (self-reported or measured tympanic temperature ≥38 °C), cough, difficulty breathing, sore throat, nasal congestion, chest pain, muscle aches, headache, vomiting or diarrhea. [4] Log mean density differences were calculated by subtracting the log mean pneumococcal density before an RSV or influenza infection from the log mean pneumococcal density during an RSV or influenza infection. BMI calculated using the formula (weight in kilograms)/ (height in meters squared). We defined BMI categories as follows: underweight - age <18 years weight for age or BMI <-2 standard deviations of the World Health Organization (WHO) Child Growth Standards, age ≥18 years BMI <18.5kg/m2; overweight - age <18 years BMI >+1 and ≤+2 standard deviations of the WHO growth standards, age ≥18 years BMI ≥25 and <30kg/m2, obese – age <18 years BMI >+2 standard deviations of the WHO growth standards, age ≥18 years BMI ≥30 kg/m^2. Abbreviations: PHIRST - a prospective household cohort study of influenza, RSV, and other respiratory pathogens community burden and transmission dynamics in South Africa; BMI – Body Mass Index; PLWHIV – people living with HIV; CI – Confidence interval, Ct-value – Cycle-threshold value. Bold font denotes statistical significance (P<0.05). Only variables found to be statistically significant (P<0.2) in univariate analysis or included a priori (age group and HIV status) were assessed in the multivariable model. Only a priori variables and variables remaining significant in the final model are shown in the table.

15.5. Discussion

Previous research shows that respiratory viral infections such as RSV and influenza increase pneumococcal colonization density, but studies have predominantly been limited to hospitalized populations. Our household cohort study examined pneumococcal households, providing insights into how these viral infections influence pneumococcal colonization density over time.

We observed significant changes in the density of the pneumococcus in the nasopharynx before vs. during an RSV infection. Furthermore, we found that 2 weeks after an RSV infection, pneumococcal density remained elevated. Pneumococcal carriage density increased in individuals with higher viral loads during RSV and influenza infections compared to before the infections. Underweight individuals were less likely to have an increase in pneumococcal density in the presence of RSV compared to individuals with a normal BMI.

The demographic characteristics of individuals colonized with pneumococcus and infected with RSV or influenza were similar to those of the overall PHIRST study population. This suggests that differences in outcomes are less likely to be driven by demographic variability and more likely to reflect the factors under investigation.

A study in the USA showed that infection with common respiratory viruses such as enterovirus, RSV, adenovirus, rhinovirus and human coronavirus were associated with an increase in pneumococcal density (approximately 3 Ct-values lower in individuals with a virus vs. without a virus) while other viruses such as human parainfluenza virus, human metapneumovirus and influenza virus were not (95). Similar to our study, this supports the concept that pneumococcal density in an individual infected with a virus may be pathogen-specific (95). Additionally, to this study, Bennet *et al.* showed that *S. pneumoniae* carriage density was associated with RSV infection but not with influenza. Comparable to our results, in a Kenyan study, investigating children with acute respiratory infections found a positive correlation between RSV infections and increased pneumococcal nasopharyngeal carriage density (92). The increase in pneumococcal density in the presence of RSV was also shown in animal models as described by

Manna *et al.* (96). Miellet *et al.* observed that in their study population the pneumococcal density was 8-fold higher in individuals with influenza infection after influenza-like illness onset compared to influenza-negative controls (97). In our study, we found that there was an increase in the carriage density of pneumococcus during an influenza infection compared to before however, this increase was not statistically significant. For influenza, we may have been underpowered to detect a significant difference. Miellet *et al.* collected saliva from individuals greater than 60 years old with acute influenza-like illness which were tested on a multiplex ligation-dependent probe amplification assay for the detection of influenza. Variability across studies could also be attributed to the difference in the study population, study participants' age, the test used, the sample type tested and the time the study was conducted. The non-significant difference with an influenza infection and the significant difference with RSV infection may be pathogen-specific. We cannot ignore that the disparities in pneumococcal carriage density may be linked to other inherent differences among individuals and their immune response against viral infection (34,97). We further found that pneumococcal density remained elevated long after an RSV infection (i.e., up to 2 months), however, since we only tested for RSV and influenza we cannot exclude that the elevation might have been due to an infection with other respiratory viruses not tested.

Miellet *et al.* found that the synergistic relationship between respiratory viruses and pneumococcus may be related to host factors rather than the direct interaction of the virus with the pneumococcus (97). Thus, we investigated the factors associated with changes in pneumococcal density in the nasopharynx during viral infection. To our knowledge, no similar studies have looked at the factors associated with an increase in pneumococcal density in the presence of influenza or RSV in individuals irrespective of symptoms. We found a correlation between increased viral loads, as indicated by a lower Ct-value, with increased pneumococcal density. When a virus infects the respiratory epithelial cells, it can compromise the integrity of the epithelial layer, creating a favorable environment for bacterial colonization and proliferation (40,98–100). This compromised epithelial layer impedes the immune system's ability to effectively clear bacterial invaders, thereby fostering conditions conducive to bacterial growth and pneumococcal density increases (98). Influenza virus, in particular, has been shown to

deplete alveolar macrophages which impair pneumococcal clearance (101). Similarly, RSV infection can induce inflammation and mucosal damage facilitating bacterial adherence and colonization increasing pneumococcal carriage density (40). Moreover, the increased viral load likely exacerbates the disruption of the epithelial layer, increasing the host's susceptibility to increased bacterial colonization density. However, more research looking at factors associated with changes in pneumococcal density in the presence of a virus in healthy individuals over time is needed for meaningful comparisons to be made.

A limitation of our study is that we did not have controls; however, each participant served as their control since we looked at the data before, during, and after an infection in the same individual. DNA extraction in this study did not include an enzymatic lysis step, and alternative extraction methods were not explored. These factors may have influenced extraction efficiency and, consequently, the sensitivity of pneumococcal detection and quantification, potentially leading to underestimation of low-density carriage. However, the extraction protocol was optimized for downstream qPCR analysis, and detection was based on a validated *lytA* assay with established specificity for *S. pneumoniae*, supported by strict laboratory controls to minimize contamination. Therefore, while methodological factors cannot be completely excluded, they are unlikely to fully explain the observed low densities. Future studies could compare extraction approaches and incorporate enzymatic pre-treatment to enhance bacterial DNA yield and detection accuracy.

Another limitation is the use of qPCR for pneumococcus identification, which, while highly sensitive, can have specificity challenges due to genetic exchange with other *Streptococcus* spp., such as *S. pseudopneumoniae* and *S. mitis* (90). However, culture also has limitations, including potential misclassification due to nonviable or low-density carriage. While Tavares *et al.* reported that combining *lytA* and *SP2020* improved specificity from 99.5% to 100% (91), recent studies have raised concerns about the specificity of *SP2020*, as findings have not been consistently replicated across carriage studies. By exclusively targeting *lytA*, our approach may have led to minor misclassification. An alternative marker, *piaB*, has demonstrated higher specificity for

pneumococcal identification, and future studies should consider using a combination of genetic targets, including *piaB*, to enhance the accuracy of pneumococcal detection (102).

Pneumococcal pneumonia is associated with high colonization density, and previous studies have shown that increased pneumococcal carriage density plays a key role in the development of IPD and the transmission of pneumococcus between individuals (87,103,104). The observed increases in pneumococcal density during viral infections may also have implications for transmission and community spread (65,87). While our study was not designed to assess transmission directly, our findings contribute to the understanding of factors influencing pneumococcal density, which may, in turn, affect transmission dynamics. Furthermore, we did not serotype the pneumococcal-positive specimens, which limits our ability to explore serotype-specific relationships. Similarly, we did not differentiate between viral lineages, and it is possible that variation in RSV or influenza subtypes and lineages could influence pneumococcal density and its dynamics. Future analyses incorporating viral subtype and lineage information, along with serotyping, will be important to clarify how specific viral strains impact pneumococcal carriage and transmission.

Another constraint in our study was the omission of examination of pneumococcal density in the context of concurrent coinfection with multiple viruses. Thus, we cannot exclude that the change in pneumococcal density could be affected by the coinfection with other viruses besides RSV and influenza that were not tested for. Plausible mechanisms for the sustained increases in density include virus-induced epithelial damage, which can facilitate bacterial adherence and proliferation, and virus-mediated modulation of the host immune response, potentially reducing bacterial clearance. Future studies could investigate these mechanisms by including a broader panel of respiratory viruses, combined with longitudinal sampling and assessment of mucosal and systemic immune markers, to better understand the pathways through which viral infections affect pneumococcal density over time.

A further limitation of our study is that we did not analyze the data by RSV and influenza subtype, which may have influenced our findings. Different viral strains may have varying effects on pneumococcal carriage and density, and without strain identification, we could not assess these interactions. Data from the larger PHIRST study indicate that influenza A (H1N1) was the predominant circulating strain in 2016 and 2018, while A (H3N2) was dominant in 2017 (67). Additionally, seasonal variability in circulating influenza strains may have introduced unmeasured differences in pneumococcal dynamics, potentially masking strain-specific effects.

Nevertheless, the strength of our study, was the consistent sampling of individuals (twice weekly) irrespective of symptoms, enabling us to identify episodes of viral infection and explore the fluctuations in pneumococcal carriage density within individuals over time. The high-frequency sampling allowed identification of short-lived yet potentially biologically meaningful changes in bacterial density following a respiratory virus infection. Integration of detailed epidemiological, clinical, and microbiological data provided a comprehensive framework for exploring host and environmental factors influencing bacterial–viral interactions. The concurrent collection of viral and bacterial samples represents a major strength of this analysis, allowing precise temporal assessment of viral-associated changes in pneumococcal density. The use of qPCR-based quantification and mixed-effects modeling improved sensitivity and statistical rigor, enabling robust detection of transient within-person changes following influenza or RSV infection. Our study was a community cohort study in which individuals of all ages were enrolled from an urban and rural site which afforded us the ability to determine several factors associated with changes in pneumococcal density during an RSV or influenza infection and makes our results more generalizable in our setting. In addition, our study was a community-level household cohort study, unique to previous studies in which they primarily examined the relationship between *S. pneumoniae* carriage and viral co-detection among hospitalized individuals with acute respiratory illness.

It is important to note that bacterial-viral interactions in the nasopharynx can be both synergistic and antagonistic. In this study, we measured the overall changes in pneumococcal density following viral infection without separating the direction of

interaction. Future studies could investigate the specific mechanisms underlying these complex interactions. Future research should also investigate the immunological mechanisms underlying the observed post-RSV increases in pneumococcal density, particularly in individuals with differing HIV or nutritional status. Expanding analyses to include additional respiratory viruses and to model sequential or co-infections could clarify whether viral priming of bacterial proliferation is pathogen-specific or a broader phenomenon influencing transmission risk.

Our results emphasize the complex interplay of viral infections and microbial dynamics, displaying a distinct impact on pneumococcal density. This further highlights the unique dynamics of the pneumococcal response to viral infections particularly in individuals with high viral loads. The outcome of our research bears relevance to public health, offering insights that can inform vaccination strategies for both bacterial and viral infections such as the development of combination vaccines that provide broader protection against respiratory infections. Furthermore, vaccination strategies could be optimized to coincide with periods of heightened pneumococcal density following viral infections.

16. Chapter 5: The invisible players: nasopharyngeal microbiota and *Streptococcus* genus during influenza virus infection in a South African community cohort, 2017

16.1. Reference

Carrim M, Li K, de Steenhuijsen Piters W, Fitch A, Moyes J, Kleynhans J, Mei Chu ML, Hasrat R, Martinson NA, Kahn K, Mkhencele T, Mathunjwa A, Lebina L, Mothlaoleng K, Wafawanaka F, Gómez-Olivé FX, Cohen C, von Gottberg A, Bogaert D, Methè B and Wolter N. The invisible players: Nasopharyngeal Microbiota and Streptococcus genus during influenza infection in a South Africa community cohort study, 2017. (In preparation)

16.2. Introduction

LRTIs account for around 3 million deaths each year and remain a major cause of morbidity and mortality worldwide, especially affecting low- and middle-income countries where access to healthcare is limited (105). *S. pneumoniae* is the most common bacterial cause of these infections and a driver of childhood pneumonia, bacterial meningitis and sepsis, even in the post-PCV era (14–16). Although colonization of the upper respiratory tract by the pneumococcus is often asymptomatic, it is a necessary first step in the pathway towards disease. Understanding the factors that drive progression from asymptomatic colonization to disease is important for guiding prevention and treatment strategies. Respiratory viral infections, especially influenza, are recognized as contributors to this transition (41–43). Influenza can impair mucosal barriers, alter immune responses and increase the density of pneumococci in the nasopharynx (36). These effects are believed to arise not only from direct immune suppression but also from changes in the ecological balance of the nasopharyngeal microbiome which normally provides colonization resistance through competition of the micro-organisms for nutrients and space (106). However, most of what we know about these interactions comes from studies of hospitalized individuals or controlled experimental models. Such studies may not reflect the natural history of infection or microbiome dynamics in the general population.

Recent advances in high-throughput sequencing have enabled more detailed investigation of microbial community structure and its changes over time. Yet few studies have applied these methods in community-based settings, especially in high HIV-prevalence regions like South Africa where the burden of both viral and bacterial respiratory infections remains high. Furthermore, while it is known that influenza increases pneumococcal density, it is less clear how changes in *Streptococcus* spp. abundance relate to shifts in the broader microbial community.

We aimed to describe the changes that occur in the nasopharyngeal microbiome during influenza virus infection, in a longitudinal household cohort in South Africa.

16.3. Materials and methods

16.3.1. Study design and population

The study design, population studied and laboratory testing for influenza has been described in section 13.1

16.3.2. Microbiome sample selection and testing

16.3.2.1. Microbiome sample selection

Samples for microbiome testing were selected from the 2017 cohort of the PHIRST study. An influenza episode was defined as the period from the first to the last nasopharyngeal swab testing positive for influenza virus by qPCR. The study interval for sample selection included sample collected from two weeks before, during and two weeks after the influenza infection each episode, with one NP swab selected per week from before and after the episode and all NP swabs collected during the episode itself (reflecting higher sampling frequency).

For the selection of the weekly swabs, the first sample collected for that week was tested. If this sample was not collected, the second sample from the same week was selected for testing instead. Since sampling for microbiome analysis (repeated measures) over time for each individual was sporadic (as it was relative to an influenza episode) and at different time points, we grouped samples by periods relative to the influenza infection for statistical analysis. Thus, we created 3 periods (viz. before, during

and after). The ‘before period’ was defined as the average of up to two microbiome samples collected from the same individual in the two weeks preceding the onset of influenza infection, typically one sample per week. The ‘during period’ comprised an average of microbiome profiles of all samples collected during an influenza infection episode (i.e., influenza-positive samples from the same individual). The ‘after period’ was defined as the average of up to two microbiome samples collected from the same individual during the two weeks following the end of the influenza episode (i.e. after the last influenza-positive sample), typically corresponding to samples collected approximately one and two weeks post-infection. We averaged samples collected within the 2-week period prior to and after an influenza infection to better represent the individual's baseline microbiome and reduce the impact of transient fluctuations that may occur in a single sample. This approach allowed us to smooth out short-term variability and provide a more stable pre-infection microbial profile. We randomly selected 40/89 (44%) influenza-positive households (households with at least one individual positive for influenza) from the 2017 cohort. Within these households, 52 individuals were identified as having had influenza infections. Of these, nine (9/52, 17%) individuals were excluded due to insufficient samples (8 individuals only having one sample collected on day 7 in the after period and 1 individual having one sample collected on day 9). Therefore, there were 42 individuals from whom we included samples collected at three study intervals corresponding to the periods before, during, and after an influenza episode, irrespective of *S. pneumoniae* qPCR positivity. This resulted in 290 samples (a median of 6.5 samples per individual, range 4-12) from participants with complete sampling across all three time periods, enabling longitudinal and within-individual comparisons over time.

16.3.2.2. DNA isolation for microbiota analyses

Bacterial DNA was extracted from 200 µl of the NP using a phenol-based extraction followed by manual magnetic bead extraction using an Agowa Mag DNA extraction kit (LGC genomics, Berlin, Germany) and eluted into to a final volume of 50 µl of elution buffer, and stored at -20°C. Quantification of the DNA was achieved using a quantitative

qPCR targeting the 16S *rRNA* gene as previously described (104). This data was used to normalize the DNA before library preparation and sequencing the sample.

16.3.2.3. 16S *rRNA* sequencing and quality control

We characterized the bacterial composition of the microbiome using 16S *rRNA* sequencing targeting the V4-region by PCR using the primers, 515F (5'-GTG CCA GCM GCC GCG GTA S-3') and 806R (5'-GGA CTA CHV GGG TWT CTA AT-3') with Illumina adapters and sample-specific barcodes (Illumina Incorporation California, USA) (105,106). Negative extraction controls (consisting of uninoculated PrimeStore® MTM, processed alongside study samples), no template controls, and ZymoBIOMICS Microbial Community Standards (Zymo Research Corporation, California, USA) were included in each PCR assay and were sequenced simultaneously with the samples. The size of the amplified fragments was evaluated via agarose gel electrophoresis, and quantification was performed using the Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific, Massachusetts, USA). Equimolar pooling of barcoded amplicons was then carried out. The library was prepared as recommended by Illumina (Illumina Incorporation. California, USA). Sequencing was performed using an Illumina MiSeq instrument with the Illumina MiSeq reagent V2 or V3 (paired end, 500bp) kit (Illumina Incorporation, California, USA).

16.3.2.4. Bioinformatics processing and taxonomic assignment

After sequencing, we trimmed the raw reads with a quality score of ≤Q30 and a length threshold of 150 nucleotides using Sickle (version 1.33), and error-corrected using BayesHammer within SPAdes (version 3.8.1). The reads were assembled using PANDAseq (version 2.10). Chimeras were removed using the VSEARCH toolkit (version 2.0.3), thereafter VSEARCH abundance-based clustering was performed to obtain operational taxonomic units (OTUs) with 97% identity thresholds. OTUs were annotated using Ribosomal Database Project (RDP) classifier and the SILVA database (https://www.arb-silva.de/). OTUs were ranked by mean relative abundance across all samples from all individuals using custom R scripts (version 4.2.2), and the top 15 most

abundant OTUs were selected for further analysis. OTUs that shared the same genus-level classification were distinguished by numerical suffixes (e.g., *Moraxella 1*, *Moraxella 39*), which represent distinct sequence clusters within the genus that could not be resolved to species level. To account for potential contamination in these low biomass samples, we applied decontamination procedures using the decontam R package.

16.3.3. Statistical analysis

We performed statistical analyses using a paired-difference analyses comparing samples from the same individual across all period combinations: a) before and during influenza infection b) before and after influenza infection and c) during and after influenza infection (107). To account for repeated measures and potential clustering within households, mixed-effects models were fitted with random intercepts for individuals nested within households. This structure adjusted for within-subject and within-household correlations when comparing pneumococcal density during and after RSV infection. For comparisons, we first aggregated multiple samples within each period by averaging the transformed genus-level including relative abundances for each participant. This approach produced a single representative microbial profile per period per individual, allowing for standardized, within-individual comparisons across time. We then calculated pairwise differences between these averaged profiles to assess microbial changes across the infection timeline. We used three complementary methods (i) distribution-based methods (ii) distanced-based methods and (iii) abundance-based methods designed to each address different aspects of microbial community dynamics (108). This was supplemented with a predictor-responder analysis.

16.3.3.1. Distribution-based analysis: Alpha diversity measures

To assess within-sample microbial diversity, we used alpha diversity metrics, including the tail statistic (109). We applied linear mixed-effects regression models to estimate differences in alpha diversity between influenza periods, incorporating fixed effects a priori for age (as a continuous variable), sex, HIV status and site and a random effect

for individuals to account for repeated measures. A decrease in alpha diversity during or after influenza infection may reflect a loss of microbial richness or dominance by a few OTUs, potentially indicating a disrupted or less resilient microbial community. Conversely, an increase in diversity could signal recovery or rebalancing of the microbiota. In the models, a positive coefficient for a covariate indicates that individuals with that characteristic had higher alpha diversity compared to the reference group, while a negative coefficient suggests reduced diversity. This analysis enabled us to determine whether microbial richness and evenness varied across influenza periods and whether host characteristics were associated with those changes.

16.3.3.2. Distance-based analysis: Beta diversity measures

To explore differences in overall microbiome composition between samples, we calculated Manhattan distances between paired samples from the same individual across influenza infection periods. Manhattan distance was used to assess beta diversity, and results were robust to the choice of distance metric. The Manhattan distance was used as a distance-based measure of beta diversity to maintain consistency with previous analyses in similar datasets and to provide a straightforward interpretation of absolute abundance differences. While Bray–Curtis and Aitchison distances are also appropriate for compositional data, the results observed here were robust across transformations, and the conclusions are not expected to differ substantially by the choice of distance metric. This distanced-based method quantifies beta diversity, allowing us to assess community-wide changes in the structure of the microbiome between influenza infection periods. A larger distance indicates a greater shift in microbial composition, reflecting either a loss of OTUs, overgrowth of specific groups or broader reorganization of the community, while a smaller distance suggests greater stability in the microbiota. We then used linear regression models to evaluate whether changes in beta diversity were associated with host characteristics. This enabled us to determine whether overall microbiome structure shifted significantly across influenza infection time periods and whether specific host characteristics were associated with these shifts. To visualize these differences and assess within-individual

changes over time, multi-dimensional scaling (MDS) was performed on the dissimilarity matrix. The first two dimensions represent the primary axes of variation in microbial composition across all samples. Each point on the MDS plots represents a single sample, and the proximity between points reflects their similarity in overall microbial composition. Group centroids (i.e., the average microbial composition for each group) were calculated and plotted to indicate the average microbial community structure for each period.

16.3.3.3. Abundance-based analysis: Additive log-ratio transformation

We used abundance-based analyses to identify changes in specific OTUs within an individual over time. We applied the additive log-ratio (alr) transformation using RStudio (RStudio Team, PBC, Boston, USA) to relative abundances of OTUs, which enables independent and normally distributed comparisons by dividing the relative abundance of each of the top 15 most abundant OTUs by the total abundance of all OTUs followed by a log transformation, which enabled each OTU to be treated as a separate variable with normal distribution properties (109,110). This method reduces the risk of false associations due to the compositional nature of microbiome data. The top 15 OTUs were selected based on overall relative abundance across all samples. This approach prioritizes OTUs most likely to contribute to microbiome shifts in taxonomic dominance across the influenza infection timeline while reducing the influence of low-abundance, potentially spurious OTUs. To handle zero values, we replaced them with one-tenth of the lowest non-zero abundance. We used these transformed values to test for significant increases or decreases in individual OTUs between periods to understand shifts in taxonomic dominance across the influenza timeline. A positive change indicates an increase in abundance during the comparison period (e.g., during vs. before influenza), while a negative change reflects a decrease. Given the strong age-related variation in microbiome composition, all analyses of paired abundance differences were conducted using models that included age as a fixed effect. To further explore factors associated with changes in OTU abundance over time, we used multivariable linear regression models using RStudio (RStudio Team, PBC, Boston, USA) in which the

outcome was the within-individual change in OTU abundance between two periods. In these models, participant-level characteristics (sex, age, HIV status and study site) were included a priori as covariates to examine whether these factors were associated with microbiome changes. Rather than simply adjusting for these variables as confounders, the models were used to estimate their independent associations with microbiome shifts. In these models, a positive coefficient for a covariate suggests that individuals with that characteristic had an increase in the abundance of that OTU over time, and a negative coefficient indicates a decrease.

16.3.3.4. Predictor-responder analysis of OTU interactions

We conducted a predictor vs. responder analysis to explore directional relationships between specific OTUs over time (107). This method tests whether one OTU predicts changes in other OTU in subsequent periods (predictor role) or whether it responds to changes in other OTUs (responder role). For each pairwise model, we considered relationships significant only if both the predictor and responder models had P-values <0.025. This analysis was done using RStudio (RStudio Team, PBC, Boston, USA). A positive association between two OTUs suggests a potentially synergistic relationship, where an increase in one OUT is followed by an increase in another. A negative association, on the other hand, may reflect a competitive interaction, where an increase in one OTU is associated with a subsequent decrease in another, suggesting competition for space or resources. This analysis provides insight into potential ecological dynamics, such as dominance shifts or cooperative expansions, occurring in the microbiome during and after influenza infection. This analysis was used to infer potential ecological interactions by identifying potentially competitive or synergistic interactions between OTUs during influenza infection.

16.4. Results

From the 2017 cohort, 42 individuals from influenza-positive households with complete sample collection across the three defined time periods (before, during, and after influenza) were included, resulting in 290 samples for within-individual, longitudinal

analysis across the three defined periods. There were 94 samples collected before an influenza infection ('before period'), 114 samples collected during an influenza infection ('during period') and 82 samples collected after an influenza infection ('after period'). Among the individuals included, 53.5% (23/43) were female, 11.6% (5/43) were living with HIV, 62.8% (27/43) were aged ≥5 years, and 62.8% (27/43) were enrolled in Agincourt (rural site).

We observed a median of 43532 (interquartile range (IQR): 8670-81640) reads per sample that were binned into 1084 OTUs. These OTUs were represented by 16 phyla, of which the most common 4 phyla were Proteobacteria, Firmicutes, Actinobacteria and Bacteriodota. The top 15 OTUs identified (in relative abundance order) were *Moraxella* 1, *Haemophilus* 3, *Haemophilus* 2, *Corynebacterium* 7, *Dolosigranulum pigrum* 4, *Streptococcus* 5, *Staphylococcus* 6, *Corynebacterium* 9, *Neisseria meningitidis* 13, *Peptoniphilus* 20, *Ornithobacterium* 14, *Moraxella nonliquefaciens* 16, *Neisseriaceae* 12, *Ornithobacterium* 11, and *Haemophilus* 8.

16.4.1. Distribution-based analyses (alpha diversity)

Alpha diversity did not change significantly between influenza infection periods for most participant characteristics. However, in the before and after period comparison, age (as a continuous variable) was significantly associated with a decrease in alpha diversity (coefficient= –1.03, P=0.01), indicating that older individuals experienced a greater reduction in within-sample microbial diversity after the influenza infection episode. No significant associations were observed for sex, HIV status, or site across the three periods (Table 16.1).

Table 16.1: Estimated differences in alpha diversity (Tail Index) between influenza-associated periods by participant characteristics, PHIRST Study, 2017, South Africa.

Characteristic	Before-During		During-After		Before-After	
	Coefficient	P-value	Coefficient	P-value	Coefficient	P-value

Sex	-4.69	0.75	-17.19	0.16	-21.88	0.08
Age	-0.54	0.27	-0.49	0.21	-1.03	**0.01**
HIV status	-19.46	0.39	34.46	0.06	14.99	0.41
Site	8.75	0.55	-18.80	0.11	-10.04	0.39

Linear mixed-effects regression model estimates of changes in alpha diversity (as measured by Tail indexes) across influenza-associated periods (before–during, during–after, before–after) stratified by participant characteristics including sex, age, HIV status, and study site. Negative estimates indicate a decrease in alpha diversity across the specified interval. P-values reflect the significance of the association for each characteristic. Bold red font denotes significance. Significant assessed P<0.05.

16.4.2. Distanced-based analyses (beta-diversity)

Analysis of paired distances using MDS revealed significant differences in the beta-diversity across all three periods, with each comparison showing a P-value<0.01 (Figure 16.1). These findings indicate that the nasopharyngeal microbiome composition shifted in response to influenza infection over time. Changes in community composition between the before and during periods were more pronounced among individuals at the lower end of the age spectrum and those from the rural site (P=0.01 for age, P=0.04 for site), indicating greater microbial instability in these subgroups (data not shown). In the during-after period, differences were again linked to age (P=0.04) and were more marked in females (P=0.01). No significant associations were observed in the before–after comparison, suggesting a return to baseline.

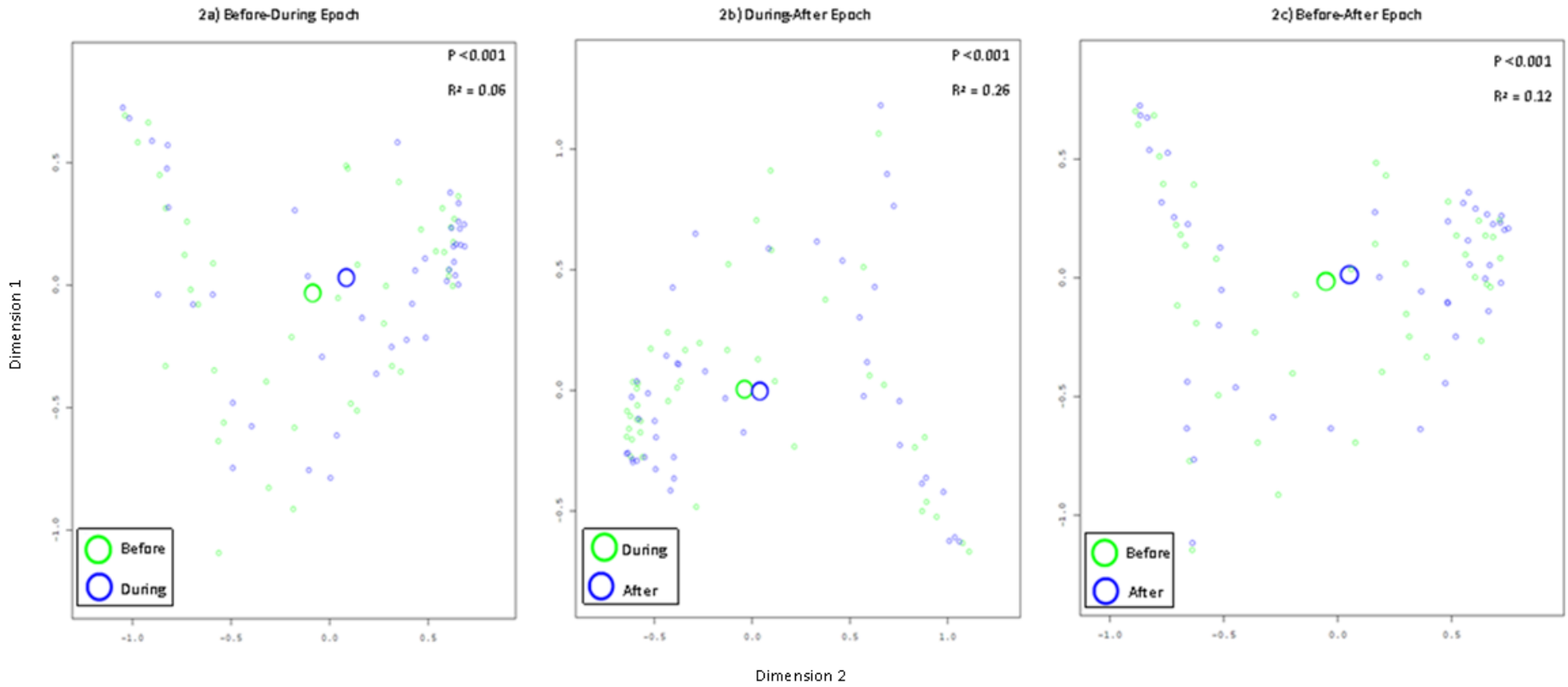


Figure 16.1: Multi-dimensional scaling plots of paired difference analysis showing distances in microbial composition within an individual comparing between two periods: (a) before-during, (b) during-after, and (c) before-after. Small circles represent individual samples, and large circles indicate group averages as shown in the legend, PHIRST Study, South Africa

16.4.3. Abundance-based analysis: alr

Analyses of paired differences in the abundance of OTUs, using the alr transformation, revealed no statistically significant changes in the abundance of the top 15 OTUs between the before and during influenza periods. However, there were notable shifts in the OTU rank across the periods, indicating temporal changes in community composition (Figure 16.2). While the rank abundance of OTUs changed in each period, *Moraxella* 1 remained the most abundant in all three periods. *Streptococcus* 5 was more abundant during influenza episodes than both before and after, and its abundance was also higher before infection than after, suggesting a temporal pattern of medium-high-low relative abundance across the periods.

Despite the lack of significant changes in the abundance of OTUs in the before and during period, several OTUs demonstrated significant changes in abundance in other period comparisons. Comparing the during and after periods, both *Moraxella nonliquefaciens* 16 ($P<0.01$) and *Ornithobacterium* 14 ($P=0.03$) were significantly more abundant during influenza infection. Comparing the before and after periods, significantly higher abundances before influenza infection were observed for *Ornithobacterium* 11 ($P=0.02$), *Ornithobacterium* 14 ($P<0.01$), *Moraxella nonliquefaciens* 16 ($P<0.01$), *Neisseria meningitidis* 13 ($P<0.01$), and *Moraxella* 39 ($P<0.01$), suggesting a decline in these OTUs following influenza infection. These findings suggest that while the microbiome remained relatively stable at the onset of infection, greater perturbations occurred following influenza infection, with specific OTUs declining in abundance, particularly *Neisseria meningitidis* 13 and *Moraxella*.

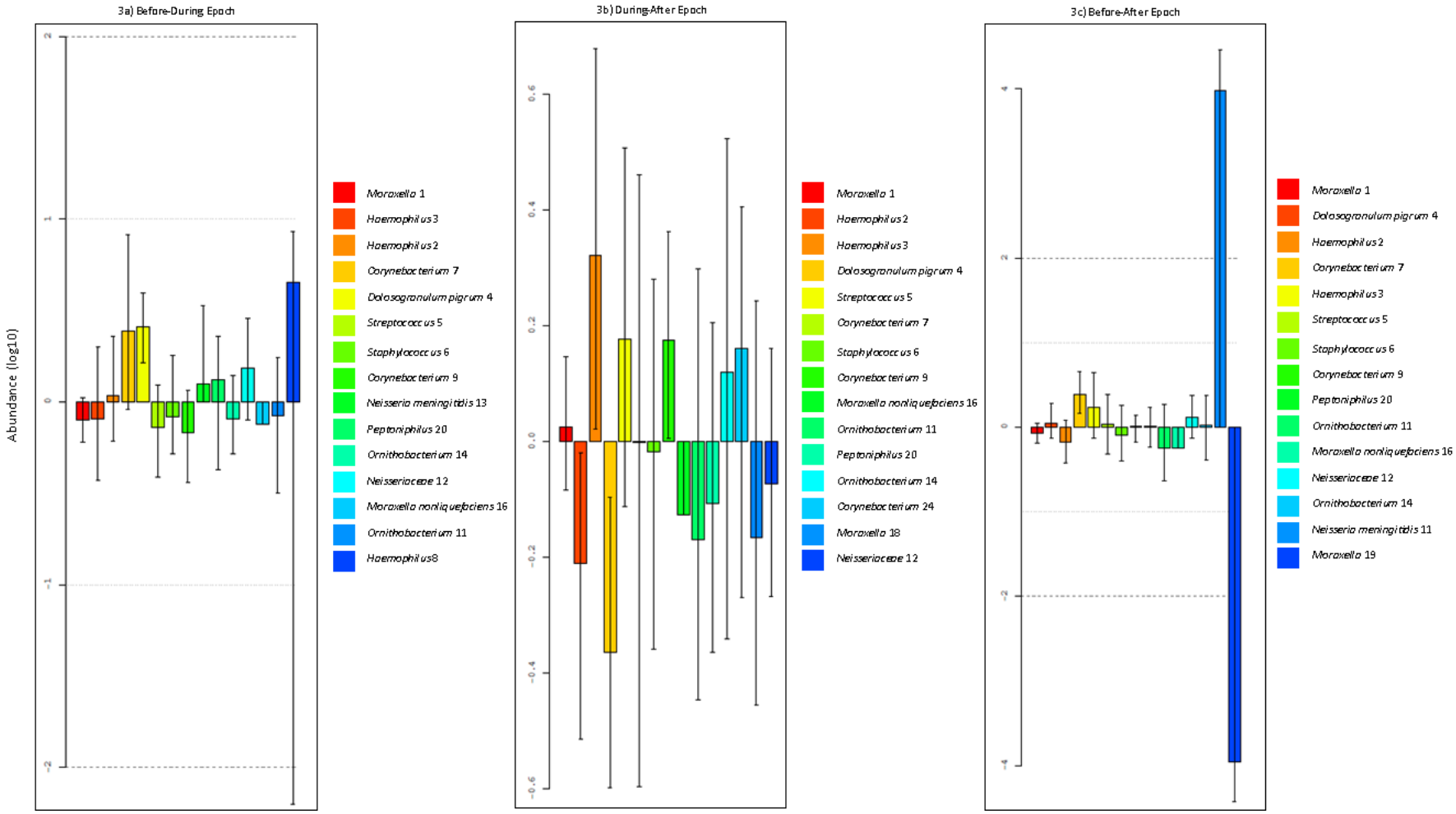


Figure 16.2: Changes in $\log_{10}$ abundance of dominant nasopharyngeal microbiome OTUs compared between influenza infection periods (before–during, during–after, before–after), ranked by most abundant OTUs, PHIRST Study, South Africa, 2017.

Box plots display within-subject differences in $\log_{10}$-transformed abundance for the top 15 bacterial OTU ranked by overall abundance across all samples within individuals (N=293). Each panel represents a pairwise comparison of influenza infection period: (a) before–during, (b) during–after, and (c) before–after. Boxes indicate the interquartile range (IQR), horizontal lines represent medians, and whiskers show 25th and 75th quartile range. Data include individuals with complete sampling across all three periods (N = 43). These comparisons illustrate temporal dynamics in the nasopharyngeal microbiome during influenza infection and recovery. Also note that the scales for each figure differ.

Although there were no statistically significant changes in abundance of the top 15 OTUs in the before and during period comparison, our analyses suggested non-significant shifts in certain OTUs. We therefore investigated whether participant characteristics were associated with the magnitude of change in abundance of OTUs between the before and during influenza infection period (Figure 16.3a). In this analysis, the outcome was the within-person change in abundance, and the covariates reflected participant-level exposures. *Moraxella* 1 decreased with an increase in age (coefficient=–0.06, P=0.02) and in PLWHIV (coefficient=3.17, P=0.01), indicating that these groups experienced a rise in *Moraxella* 1 abundance of this OTU from before to during infection. Living with HIV was positively associated with *Moraxella* 1 abundance in this period (coefficient=3.17, P=0.01), suggesting that PLWHIV were more likely to have an increase in *Moraxella* 1 abundance during infection compared to HIV-negative individuals. *Streptococcus* 5 showed an increase in females (coefficient=2.88, P=0.04), while *Corynebacterium* 9 (coefficient=–2.13, P=0.03) and *Peptoniphilus* 20 (coefficient=–1.6, P=0.02) decreased more in females, suggesting sex-related differences in microbiome responses to infection.

Interestingly, the direction of association between *Moraxella* 1 and HIV status reversed comparing the during and after periods (Figure 16.3b), where *Moraxella* 1 abundance decreased significantly in PLWHIV after influenza infection (coefficient=–4.57, P=0.01). No significant associations with HIV status were observed for this OTU comparing the before and after period (Figure 16.3c).

In the comparison of the during and after periods, sex, HIV status and study site were significantly associated with changes in the abundance of several OTUs (Figure 16.3b). PLWHIV had significantly increases in *Haemophilus* 2 (coefficient=5.58, P=0.03) and *Staphylococcus* 6 (coefficient= 3.16, P=0.01) compared to HIV-negative individuals. Both sex and HIV status were associated with increases in *Corynebacterium* 9 (female: coefficient= 2.75, P=0.01) and (PLWHIV: coefficient=3.16, P=0.04) were associated with higher abundance, suggesting increases in females and PLWHIV. In contrast, *Moraxella nonliquefaciens* 16 showed a significant decline in abundance among

PLWHIV (coefficient=–1.37, P=0.02), but was more abundant in participants from the rural site (Agincourt) compared to the urban site (Klerksdorp) (coefficient=0.88, P=0.02). Similarly, *Moraxella* 39 abundance increased more in females (coefficient=0.77, P=0.04) and participants from the rural site (coefficient=0.72, P=0.04), but decreased in PLWHIV (coefficient=–1.56, P=0.01).

In the before and after period comparison, living with HIV was significantly associated with reductions. PLWHIV showed greater declines in several OTUs after influenza infection compared to HIV-negative individuals, including *Staphylococcus* 6 (coefficient=–3.43, P=0.01), *Corynebacterium* 9 (coefficient=–4.06, P=0.01), *Neisseriaceae* 12 (coefficient=–3.69, P=0.02), and *Neisseria meningitidis* 13 (coefficient=–4.13, P=0.01) (Figure 16.3c). These results suggest that PLWHIV individuals may experience a greater loss of certain commensals following influenza, pointing to a potentially more disrupted microbial recovery phase in this group.

a)

	Sex (Female)	Age (range 0-91 years)	HIV status (PLWHIV)	Site (Urban site)
Moraxella 1		-0.06	3.17	
Haemophilus 3				
Haemophilus 2				
Corynebacterium 7				
Dolosogranulum pigrum				
Streptococcus 5	2.88			
Staphylococcus 6				
Corynebacterium 9	-2.13			
Neisseria meningitidis 13				
Peptoniphilus 20	-1.6			
Ornithobacterium 11				
Moraxella nonliquefaciens 16				
Neisseriaceae 12				
Ornithobacterium 14				
Haemophilus 8				

b)

	Sex (Female)	Age (range 0-91 years)	HIV status (PLWHIV)	Site (Urban site)
Moraxella 1			-4.57	
Haemophilus 2			5.58	
Haemophilus 3				
Dolosogranulum pigrum				
Streptococcus 5				
Corynebacterium 7				
Staphylococcus 6			-3.16	
Corynebacterium 9	2.75		-3.16	
Moraxella nonliquefaciens 16			-1.37	0.88
Ornithobacterium 11				
Peptoniphilus 20				
Ornithobacterium 14				
Corynebacterium 24				
Moraxella 18				
Moraxella 39	0.77		-1.56	0.72

	Sex (Female)	Age (range 0-91 years)	HIV status (PLWHIV)	Site (Urban site)
c) *Moraxella 1*				
Dolosogranulum pigrum				
Haemophilus 2				
Corynebacterium 7				
Haemophilus 3				
Streptococcus 5				
Staphylococcus 6			-3.43	
Corynebacterium 9			-4.06	
Peptoniphilus 20				
Ornithobacterium 11				
Moraxella nonliquefaciens 16				
Neisseriaceae 12			-3.69	
Ornithobacterium 14				
Neisseria meningitidis 13			-4.13	
Moraxella 39				

P-value heat map scale for OTU-wise comparisons across influenza infection periods

0.01	0.01
0.02	0.02
0.03	0.03
0.04	0.04
0.05	0.05
0.06	0.06

Figure 16.3: Results of the linear regression model showing significant paired-differences between abundance of OTUs assessed for the comparison of a) before and during periods, b) during and after periods c) before and after period, by sex (female), age (continuous variable), HIV status (PLWHIV) and site (Klerksdorp), PHIRST Study, South Africa.

Values in blocks depict the correlation coefficients. Colored blocks indicate P-values (darker colors indicate lower P-values). In addition, red color indicates a negative correlation and green a positive correlation. Sex, age, HIV status and site were included as co-variates in the model. OTUs are ordered by their relative abundance within each period, which may differ between panels due to temporal shifts in microbial community composition. Abbreviations: PLWHIV: people living with HIV.

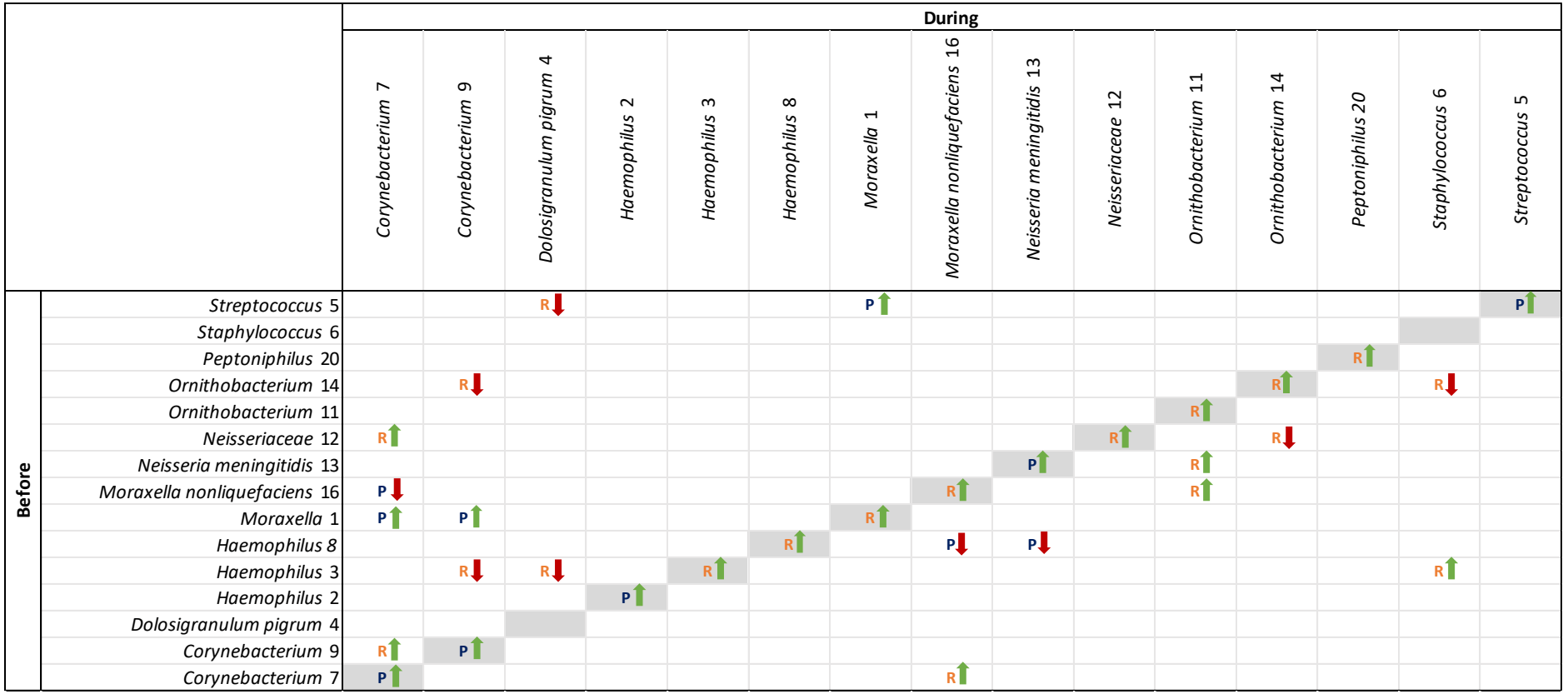
16.4.4. Predictor-responder analysis of OTU interactions

Numerous predictor-responder interactions between OTUs were observed across the different time periods, however, of particular interest were those involving *Streptococcus* 5, and whether these OTUs also participated in notable interactions with other OTUs within the microbial community. The relative abundance of *D. pigrum* 4 decreased during an influenza infection episode, in response to a preceding increase in *Streptococcus* 5, suggesting competitive interactions between the two OTUs. Furthermore, a higher relative abundance of *Streptococcus* 5 before an influenza episode predicted a higher relative abundance of *Moraxella* 1 and *Streptococcus* 5 during an influenza episode (Figure 16.4a).

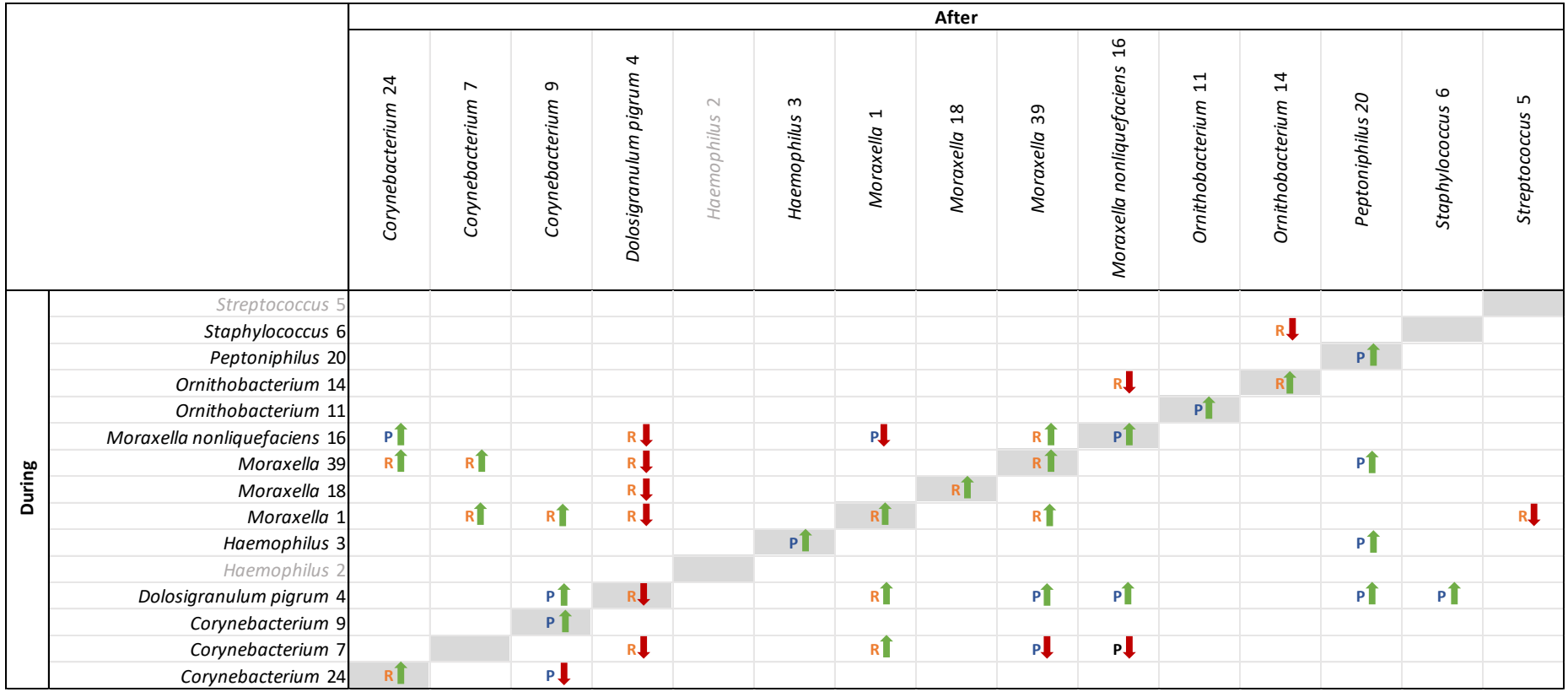
Comparing during and after periods, we found the relative abundance of *Streptococcus* 5 and *D. pigrum* 4 decreased in response to an increase in *Moraxella* 1. In addition, we found that the relative abundance of *Moraxella* 1 increased in response to the increasing relative abundance of *Corynebacterium* 7, *Corynebacterium* 9, *Moraxella* 1 and *Moraxella* 39 (Figure 16.4b).

We found that the relative abundance of *Streptococcus* 5 after an influenza episode decreased in response to an increase in *Moraxella* 1 before an influenza episode. We also found that an increase in *Streptococcus* 5 after an influenza episode predicted an increase in *Moraxella* 39 after an influenza episode (Figure 16.4c).

a)


During
Corynebacterium 7
Corynebacterium 9
Dolosigranulum pigrum 4
Haemophilus 2
Haemophilus 3
Haemophilus 8
Moraxella 1
Moraxella nonliquefaciens 16
Neisseria meningitidis 13
Neisseriaceae 12
Ornithobacterium 11
Ornithobacterium 14
Peptoniphilus 20
Staphylococcus 6
Streptococcus 5
Before
Streptococcus 5
Staphylococcus 6
Peptoniphilus 20
Ornithobacterium 14
Ornithobacterium 11
Neisseriaceae 12
Neisseria meningitidis 13
Moraxella nonliquefaciens 16
Moraxella 1
Haemophilus 8
Haemophilus 3
Haemophilus 2
Dolosigranulum pigrum 4
Corynebacterium 9
Corynebacterium 7

b)


After
Corynebacterium 24
Corynebacterium 7
Corynebacterium 9
Dolosigranulum pigrum 4
Haemophilus 2
Haemophilus 3
Moraxella 1
Moraxella 18
Moraxella 39
Moraxella nonliquefaciens 16
Ornithobacterium 11
Ornithobacterium 14
Peptoniphilus 20
Staphylococcus 6
Streptococcus 5
During
Streptococcus 5
Staphylococcus 6
Peptoniphilus 20
Ornithobacterium 14
Ornithobacterium 11
Moraxella nonliquefaciens 16
Moraxella 39
Moraxella 18
Moraxella 1
Haemophilus 3
Haemophilus 2
Dolosigranulum pigrum 4
Corynebacterium 9
Corynebacterium 7
Corynebacterium 24

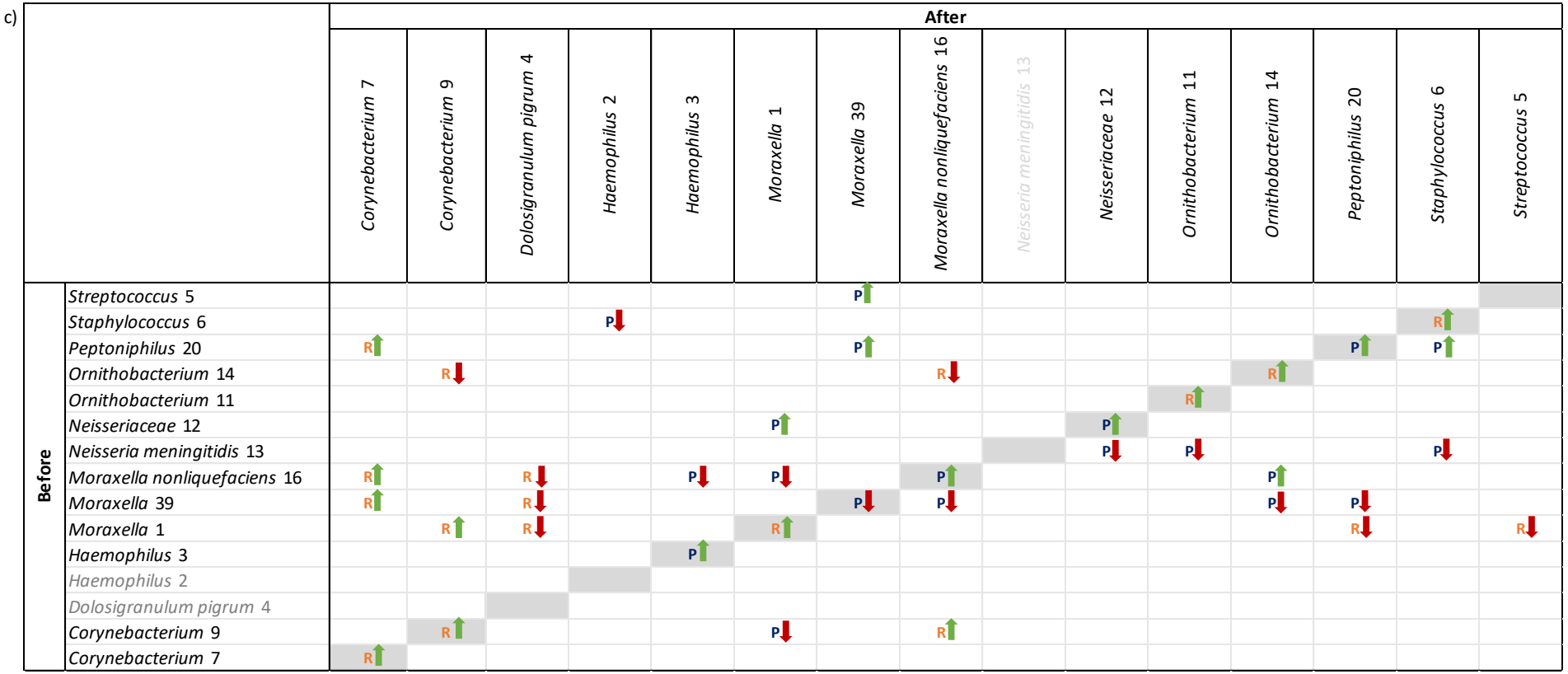


Figure 16.4: Predictor-responder modeling of microbial diversity and OTU dynamics between influenza infection periods. The summary matrix depicts statistically significant relationships of the 15 most common OTU by period a) before-during period, b) during-after period c) before-after period. The blue P's (predictor) represent the associations that predict an increase during the influenza-infection period and the orange R (responder) depicts when the relative abundance of OTU responds to a change by period. The green upward arrow represents a positive association and the red downward arrow represents a negative association. Greyed out OTUs were neither predictors nor responders.

16.5. Discussion

We identified a diverse microbial landscape in the nasopharynx. Our results demonstrate that while alpha diversity remained broadly stable across influenza infection periods, specific shifts in beta diversity in all periods and OTU-level abundance occurred, particularly among younger individuals, females, and PLWHIV. Despite no statistically significant changes overall in the abundance of the top 15 OTUs between the before and during periods, temporal patterns were observed. Several OTUs, including *Moraxella, Ornithobacterium*, and *Neisseria meningitidis* 13, declined in abundance following an influenza infection, particularly among PLWHIV. *Streptococcus* 5, followed a medium-high-low abundance pattern across the 3 periods (before-during-after). *Moraxella* 1 remained the most abundant OTU throughout but showed variation by age and HIV status, highlighting how the community of microbes changes over time and in different individuals. Predictor–responder analysis revealed directional relationships between key OTUs. We found that *Streptococcus* 5 abundance before infection predicted increases in both *Streptococcus* 5 and *Moraxella* 1 during infection, while *Moraxella* 1 increases during infection preceded declines in *Streptococcus* 5 and *D. pigrum* 4 after infection. These results suggest that while the microbiome stays fairly stable at the start of influenza infection, changes during recovery depend on individual factors, possibly due to differences in immune status, the ability of their microbiome to maintain its composition or environment exposures.

In our study, a diverse microbiota comprising OTUs from 16 bacterial phyla was identified, indicating a rich and complex microbial community in the nasopharynx. The samples prominently featured Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidota. This composition reflects the typical diversity and abundance of bacterial communities in the nasopharynx that has been observed in other studies (34,106,111–116). This further highlights these phyla as major players in the nasopharyngeal microbiome of children and adults with influenza infection.

Our study revealed that while alpha diversity in microbial communities remains stable during and after an influenza episode, there is a significant difference before and after

the episode, associated with age. The finding suggests that influenza infection acts as a disruptive event that alters the microbial community structure, leading to a new equilibrium state that persists even after the influenza resolves. This positive association may reflect age-related changes in immune development, environmental exposure, or cumulative microbial encounters that contribute to greater microbial diversity over time. The significant association with age, but not with sex, HIV status or site, highlights that age plays a crucial role in how microbial communities respond to influenza, with different age groups experiencing unique shifts in microbial diversity and stability as was previously reported (117–119). This could reflect a loss of protective microbial species or an imbalance in community composition, potentially reducing mucosal resilience and increasing vulnerability to secondary infections in older individuals.

Furthermore, beta diversity analyses indicated substantial shifts in the microbiome composition across all periods suggesting that an influenza infection significantly impacts microbial communities and indicates a substantial reconfiguration of the microbial community structure. Similar findings have been reported in studies examining respiratory infections, where significant changes in the nasopharyngeal microbiome composition were observed in the presence of a viral infection (106,116,120–122). In a study looking at perturbations in the microbiota in children infected with RSV compared to healthy controls, they found a distinct difference in controls vs. infected children (120). They further found an association between distinct microbial profiles and host inflammatory responses (120). Thus, in our study, these shifts are likely driven by the direct effects from the impact of the influenza infection on the microbiome, coupled with the host's immune response which can disrupt the microbial equilibrium and create a more hostile environment for certain microbial species while allowing others to proliferate (120,123). The persistent differences post-infection suggest a prolonged recovery period for the microbiome, which could have implications for susceptibility to secondary infections.

Notably, associations were observed between sex, age, HIV status, and site with alterations in specific OTUs between different periods, suggesting nuanced microbial responses influenced by individual characteristics. Age-related differences in microbiome response are a well-established phenomenon with older individuals often exhibiting reduced microbial diversity and altered immune responses compared to younger individuals (117–119). This age-associated decline in immune function, known as immunosenescence, impacts how the microbiome reacts to infections such as influenza. For instance, O'Toole *et al.* and Biagi *et al.* discuss how aging affects the microbiome, leading to decreased diversity and altered microbial interactions (117,119). In addition, sex-based differences in microbiome composition are influenced by hormonal variations and immune responses (124). Our findings show that sex significantly affects microbiome changes during the recovery phase of influenza infection. Markle *et al.* highlighted how sex hormones regulate immune function and microbiome composition, potentially impacting disease outcomes (124). We also found that study site was associated with microbiome diversity. Studies such as those by Yatsunenko *et al.* and Davenport *et al.* have demonstrated significant geographical differences in microbiome profiles, highlighting the importance of considering the local environment (118,125). Consistent with our results in which we found a positive association with pathobionts such as *Moraxella* and *Haemophilus*, previous research have also shown that PLWHIV often experience a reduction in microbial diversity and a shift in microbial community composition, with a higher prevalence of opportunistic pathogens and a decrease in beneficial commensal bacteria (126). We found that *Corynebacterium* was less abundant in PLWHIV during and after an influenza episode which is similar to a study in Botswana, where they found that HIV infection led to increased colonization by potentially pathogenic bacteria in the nasopharynx, while *Corynebacterium* were diminished (127). Furthermore, Li *et al.* found that PLWHIV had distinct microbiota profiles compared to their HIV-negative counterparts (128). BMI was not assessed in this analysis, nutritional status may play an important role in shaping host–microbe and virus–bacteria interactions in the nasopharynx. Variations in BMI could influence mucosal immunity or microbial community stability, potentially modifying

the microbiome's response to viral infections. Future studies incorporating BMI or other indicators of nutritional status could help elucidate these relationships.

Changes in relative abundance of OTUs between periods and shifts in rank abundance per OTU between periods were observed, with *Moraxella* 1 consistently the most abundant. *Streptococcus* 5 showed higher abundance during influenza episodes compared to both pre- and post-infection periods, with the highest levels observed during the episode itself. This temporal association suggests a potential effect of influenza infection on *Streptococcus* 5 proliferation. While changes in abundance before and after the episode could imply that other factors also contribute, the distinct peak during the influenza episode supports the interpretation that the virus infection is potentially a driver of this shift. Several mechanisms may underlie this association. Influenza infection can disrupt the mucosal barrier, impair mucociliary clearance, and alter local immune responses, creating a more permissive environment for *Streptococcus* 5 proliferation (36,99,100,129,130). Additionally, influenza may transiently suppress competing commensals, allowing *Streptococcus* 5 to expand, similar to what was found in this study. The observed patterns are consistent with previous studies showing similar microbiome changes during respiratory viral infections such as influenza, metapneumovirus, rhinovirus, and RSV (116,120).

Certain bacterial colonizers of the nasopharynx are associated with a higher risk of infections and diseases, whereas others offer protective benefits (66). We found that before influenza infection, an increase in *Streptococcus* 5 was associated with a decrease in *D. pigrum* 4 during the infection, and it also predicts higher levels of *Moraxella* 1 and *Streptococcus* 5 during an influenza infection. After the influenza episode, both *Streptococcus* 5 and *D. pigrum* 4 decrease as *Moraxella* 1 increases, which is influenced by higher levels of *Corynebacterium* and *Moraxella*. *Streptococcus* 5 decreases post-infection if there was an earlier increase in *Moraxella* 1, which also predicts a rise in *Moraxella* 39. This demonstrates how influenza infection can alter the nasopharyngeal microbiome, significantly promoting the expansion of *Streptococcus* species. Other studies have reported similar results showing increased levels of

pathogenic bacteria like *Streptococcus* and *Moraxella*, coupled with a decrease in beneficial commensals such as *D. pigrum* during a respiratory virus infection (55,131–133). A meta-analysis on the upper respiratory tract microbiome revealed associations between viral infections and increased densities of *Streptococcus* and *Moraxella*, suggesting these pathogens can proliferate when the microbiome is disturbed by a virus (131). Another study focusing on the nasopharyngeal microbiome in children found that viral infections, including RSV, were associated with a significant rise in *Moraxella* and a concurrent decline in beneficial commensals like *D. pigrum* (132,134). The antagonistic relationship between an increase in *Moraxella* and *Streptococcus* and a decrease in *Dolosigranulum* and *Corynebacterium* have been shown to be associated with respiratory tract illnesses such as pneumonia (132).

The temporal changes in nasopharyngeal microbial composition observed during and after influenza infection are consistent with evidence that respiratory viruses can modulate bacterial communities in the upper respiratory tract. Influenza infection can increase pneumococcal density and the risk of secondary bacterial infection by disrupting the epithelial barrier, enhancing bacterial adherence, and altering host immune responses (42,65,116). These synergistic effects may promote pneumococcal proliferation and influence microbiome stability during infection. Overall, influenza-driven perturbations appear to extend beyond single-pathogen effects to broader community-level changes within the nasopharynx.

The study has several limitations that need consideration. The sample size of 43 individuals, although providing valuable insights, may not sufficiently capture the full diversity of nasopharyngeal microbiomes, especially considering the high interpersonal variation that can exist even within populations sharing similar demographic and environmental characteristics. The selection of households from specific sites in South Africa could limit the generalizability of the findings. A limitation of this study is that the 16S *rRNA* sequencing data were analyzed at the OTU level, which restricts taxonomic resolution to the genus level. As a result, specific species identifications could not be confirmed. However, based on previous literature, several of the dominant OTUs

observed such as *Streptococcus* 5, *Haemophilus* 2 and *Moraxella* 1 likely correspond to common nasopharyngeal species such as *S. pneumoniae, H. influenzae,* and *M. catarrhalis*, which have been consistently reported in similar studies. A potential avenue for future work is to incorporate species-specific qPCR assays to complement 16S *rRNA* sequencing and confirm the taxonomic identity of key OTUs. This would enable more definitive linking of specific *Streptococcus* OTUs, particularly *Streptococcus 5,* to *S. pneumoniae*. Integrating these datasets in future studies could provide stronger evidence for the association between pneumococcal density dynamics and microbiome composition. A further limitation was the use of a period-based approach, where samples were categorized into discrete periods (before, during, and after influenza infection) rather than analyzed using continuous-time temporal models. While this strategy enabled clear comparisons of microbial abundance across infection stages using paired analyses, it may have overlooked more gradual or transient microbiome changes. Similarly, we did not perform formal cluster analysis to identify distinct microbial community profiles, which could have provided additional insights into patterns of compositional change and their associations with host characteristics. Both temporal modeling and clustering analysis were limited by variability in sampling intervals across participants, inconsistent numbers of repeated measures, and small sample sizes within key subgroups such as age or HIV status. These factors reduced the feasibility and interpretability of more complex modeling techniques. Future studies with larger, more evenly sampled cohorts and consistent longitudinal follow-up may benefit from applying time series and clustering methods to better characterize dynamic microbiome trajectories during infection. We did not recruit controls, however, each participant served as their own control since we looked at the data before, during and after an infection in the same individual. Additionally, we did not control for diet, antibiotic usage and environmental factors which could confound the results. Furthermore, our study was not designed to investigate mechanistic relationships between the human host and microbiome, nor did we assess the biological function of the microbiome, therefore, we are unable to establish causal relationships. In addition, our findings might not be applicable to populations with different demographic characteristics or health statuses.

This study's strengths lie in its integrative application of microbiome sequencing within a well-defined longitudinal influenza cohort, enabling characterization of nasopharyngeal microbial community shifts before, during, and after natural infection. The within-individual paired design and high sampling frequency minimized confounding by inter-individual variability and provided an opportunity to capture short-term microbiome changes associated with influenza infection. The use of 16S *rRNA* sequencing, combined with viral and bacterial data and analyzed using mixed-effects models to account for repeated measures, ensured robust statistical inference and offered insights into the temporal interplay between influenza and the nasopharyngeal microbiota.

Understanding microbial dynamics may have implications for clinical practice and therapeutic interventions. Managing the microbiome through probiotics or targeted antimicrobial treatments could help maintain a balanced microbiome during and after influenza infections, potentially reducing the risk of secondary infections and improving patient outcomes. Additionally, the identification of competitive relationships between bacterial species could inform the development of novel therapeutic strategies aimed at disrupting harmful microbial interactions and promoting beneficial ones. Our findings also suggest the potential for personalized approaches to managing influenza infections and its complications. Monitoring individual microbiome responses could help tailor treatments to maintain or restore a healthy microbial balance, while also informing diagnostics by identifying microbial signatures associated with increased susceptibility or delayed recovery. This approach acknowledges the specific microbial interactions and host factors present in each patient. Future research should focus on elucidating the mechanisms driving these microbiome shifts, exploring the role of the immune system in mediating these changes, and evaluating the efficacy of targeted microbiome interventions in clinical settings. Additionally, further work is needed to determine the functional consequences of influenza-associated microbiome shifts, including how disruptions in microbial balance affect pathogen susceptibility or recovery. Incorporating metagenomic or metatranscriptomic sequencing could help identify metabolic and immunological pathways involved, while parallel measurement of host immune markers

would provide mechanistic insight into host–microbiome interactions during viral infection.

Monitoring individual microbiome responses could help tailor treatments to maintain or restore a healthy microbial balance, while also informing diagnostics by identifying microbial signatures associated with increased susceptibility or delayed recovery. This approach acknowledges the specific microbial interactions and host factors present in each patient.

This study emphasizes the alterations of the microbiome in the presence of influenza infection and highlights the need for further investigation into the complex interactions between microbial communities and their host during and after viral infections.

17. Chapter 6: Conclusions

This body of work aimed to elucidate the dynamics of *S. pneumoniae* colonization in the nasopharynx over time within a community-based household cohort in South Africa, particularly in the context of demographic characteristics, HIV status, and co-infections with respiratory viruses such as RSV and influenza. The study provides valuable insights into the interactions between pneumococcus, the nasopharyngeal microbiome and viral infections, offering new perspectives on the progression from colonization to invasive disease.

Across the 3-year study, in which new cohorts were enrolled annually, 98% of individuals experienced at least one episode of pneumococcal colonization. Particularly, younger children and PLWHIV were more susceptible to colonization and showed higher colonization densities, aligning with previous studies that highlight the increased susceptibility of these groups to pneumococcal infections and progression to disease (12,73,81,82,87,89,135). These findings emphasize the continued risk for colonization of the pneumococci in these populations.

Our data demonstrated that respiratory viral infections, particularly RSV, significantly increased pneumococcal colonization density, supporting existing literature that shows how viral infections exacerbate bacterial carriage and predispose individuals to IPD (40,87,97,104). This finding is consistent with the work of Madhi *et al.*, who highlighted that viruses contribute to the pathogenesis of bacterial pneumonia (47)**.** Furthermore, the Waterlow *et al.* study, also using PHIRST data, reported a transient increase in influenza susceptibility following RSV infection, highlighting the complex temporal interplay between viruses in this setting (136). Our work complements this by showing that RSV not only predisposes individuals to influenza infection but may also exacerbate pneumococcal colonization, thereby compounding the risk for severe co-infections. Together, this suggests the importance of ensuring high uptake and effective implementation of existing strategies targeting both bacterial and viral pathogens, such as co-vaccination or combined prevention efforts in high-risk populations. This underscores the importance of integrating viral and bacterial surveillance in clinical settings to better predict and manage the risk of progression to IPD.

Cohen *et al.* provided the foundational epidemiological framework for our work, detailing RSV incidence and transmission patterns in both urban and rural South African communities (94). Their findings showed high rates of RSV transmission, particularly among young children, and revealed that household clustering played a significant role in community spread. Our work builds on this foundation by layering in pneumococcal carriage and density data, showing how RSV infections in these same settings can modulate bacterial colonization, particularly in children and PLWHIV. These findings support the importance of introducing RSV vaccines, especially in high-burden settings, where reducing RSV transmission could have downstream effects not only on viral illness but also on bacterial colonization and potentially disease.

The study identified a diverse nasopharyngeal microbial landscape and revealed dynamic shifts in its composition across influenza infection periods. Alpha diversity remained stable, although older individuals experienced a decrease in alpha diversity post-infection. Beta diversity changed significantly over time, indicating that influenza infection disrupted the microbial community. These shifts were associated in younger individuals, females and PLWHIV, who showed greater fluctuation in specific OTUs. Differential abundance analyses showed time-specific changes in *Streptococcus 5*, *Moraxella 1*, and *Ornithobacterium*, while participant characteristics such as age, sex, HIV status, and site were associated with the magnitude and direction of these changes. Notable shifts in microbial composition were observed during influenza episodes, particularly an increase in *Streptococcus 5* before influenza infection and a corresponding decrease in *D. pigrum 4*. Post influenza infection, both *Streptococcus 5* and *D. pigrum 4* decreased, while *Moraxella 1* increased. Thus indicating that respiratory viral infections, like influenza, can cause shifts in the nasopharyngeal microbiome, potentially facilitating bacterial colonization. These shifts are consistent with findings from other studies that suggest viral infections can disrupt the microbial balance in the nasopharynx, facilitating bacterial colonization (106,116,120–122). This observation may be key to understanding why some individuals with asymptomatic colonization progress to IPD, while others do not**.** Understanding these microbial shifts may help identify early microbial signatures of risk, offering opportunities to intervene through changing the

microbiome or other targeted strategies, to minimize disease progression or even prevent colonization from evolving into illness. Future studies should investigate how microbiome modulation, possibly through probiotics, could play a role in preventing the transition from colonization to disease.

These findings underscore the importance of considering both individual factors, such as age, HIV status and viral co-infections when assessing the risk of progression from asymptomatic colonization to IPD. Identifying individuals at elevated risk of developing disease requires a holistic approach that incorporates microbial dynamics, viral exposure, and host immune status. Although this study was limited by the absence of serotype data and the reliance on a single genetic target (*lytA*) for pneumococcal detection, the high-resolution, community-based data support and extend existing evidence that respiratory viruses, particularly RSV and influenza, play an important role in increasing pneumococcal density and influencing colonization dynamics. As RSV vaccines are introduced into immunization programs, understanding their broader impact on bacterial pathogens, including shifts in pneumococcal colonization and microbiome composition, becomes increasingly important. Monitoring these indirect effects, alongside vaccine uptake, could provide a better understanding of the interplay between pathogens. Ultimately, identifying those most vulnerable to severe outcomes from viral–bacterial co-infections may help inform targeted vaccination strategies, timely antiviral or antibiotic treatment and potentially the use of microbiome-modulating interventions such as probiotics.

To build on the findings of this research, future work should prioritize long-term, multi-cohort studies to assess the impact of newer vaccines on colonization dynamics, particularly in high-risk groups such as young children and PLWHIV. While several studies have begun to explore viral-bacterial interactions, more research is needed to elucidate the precise immunological and ecological mechanisms by which respiratory viruses such as RSV and influenza alter pneumococcal colonization and density. In addition, research should consider avenues such as probiotics or microbial transplantation to influence colonization dynamics. Although these approaches remain largely theoretical in this context and require foundational evidence. Additionally, the

epidemiology and pathogenic potential of emerging non-PCV serotypes, and the effect of antiviral treatments on bacterial carriage, require further investigation. Future studies should not only examine individual pathogen dynamics, but also consider how interventions targeting one infection may have unintended consequences or benefits on others. Therefore, integrated surveillance systems that capture multiple pathogens will be essential to fully assess the broader public health impact of vaccines and treatments. Genomic studies of circulating pneumococcal strains, combined with immunological analyses of host responses to co-infections, could enhance our understanding of pneumococcal transmission and virulence. Transmission studies focused on pathogen interplay within households, especially during viral outbreaks, are also needed. Lastly, clinical trials evaluating the efficacy of combination vaccines or co-administered antiviral and antibacterial therapies may offer innovative strategies for managing co-infections in vulnerable populations. Taken together, these future directions will be crucial for shaping more holistic and adaptive disease prevention frameworks in the context of evolving pathogen landscapes. Future work should also focus on multi-omic longitudinal studies that combine genomic, transcriptomic, and immunological profiling to reveal the mechanisms linking viral infection, microbial ecology, and bacterial transmission. Expanding such work to diverse populations and other geographic regions will be important to inform targeted prevention strategies and guide interventions aimed at interrupting the transition from colonization to disease.

Overall, this research contributes to a better understanding of the pneumococcal colonization dynamics in a real-world setting in a region with a high HIV prevalence and a significant burden of respiratory viral infections. These findings may help inform future research and provide useful context for vaccination strategies and public health responses in high-risk communities.

18. Chapter 7: References

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19. Appendices

Carrim M, Tempia S, Thindwa D, Martinson NA, Kahn K, Flasche S, et al. Unmasking pneumococcal carriage in a high HIV prevalence population in two community cohorts with a high prevalence of HIV in South Africa, 2016-2018: the PHIRST study. Clin Infect Dis. 2022; Access at: https://academic.oup.com/cid/article/76/3/e710/6611495

Carrim M, Kleynhans J, Tempia S, Hellferscee O, Treurnicht FK, McMorrow ML, et al. Temporal Changes in Nasopharyngeal Pneumococcal Colonization Density Associated With Respiratory Syncytial Virus and Influenza in a South African Household Cohort Study, 2016–2018. Open Forum Infect Dis. 2025;12(6):ofaf267. Access at: https://pmc.ncbi.nlm.nih.gov/articles/PMC12125651/

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

R49 Ms M Carrim and Professor C Cohen

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M210367

NAME: (Principal Investigator)	Ms M Carrim and Professor C Cohen
DEPARTMENT:	School of Pathology Dept of Clinical Microbiology & Infectious Diseases Medical School University
PROJECT TITLE:	*Understanding carriage and transmission of Streptococcus pneumoniae through a community cohort study in South Africa*
DATE CONSIDERED:	2021/03/26
DECISION:	Approved unconditionally
CONDITIONS:	
NOTE:	If contact information regarding student study participants is required, please contact the Registrar's office - <Nicoleen.Potgieter@wits.ac.za>
SUPERVISOR:	Dr N Wolter and Professor A von Gottberg
APPROVED BY:	CB Penny Dr CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	2021/08/19

This Clearance Certificate is valid for 5 years from the date of approval. An extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office secretariat on the 3rd floor, Phillip Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated from the research protocol as approved, I/we undertake to submit details to the Committee. **I agree to submit a yearly progress report**. When a funder requires annual re-certification, the application date will be one year after the date when the study was initially reviewed. In this case, the study was initially reviewed in **March** and therefore reports and re-certification will be due in the month of **March** each year. Unreported changes to the study may invalidate the clearance given by the HREC (Medical).

Signature of Principal Investigator

20-08-2021
Date