Influenza and the Respiratory Microbiome

by

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Dedication

To my parents, brothers, and friends who supported me over the years. You made this happen.

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Table of Contents

Dedication ii

Acknowledgements iii

List of Tables viii

List of Figures x

List of Abbreviation xiii

Abstract xvi

Chapter 1 Introduction 1

1.1 Influenza Virus 1
1.2 The Human Microbiome 2
1.3 The Bi-directional Relationship between Influenza and the Microbiome 3
1.4 Dissertation Aims 4
1.5 References 6

Chapter 2 The Role of Respiratory Viruses in the Etiology of Bacterial Pneumonia: an Ecological Perspective 10

2.1 Abstract 10
2.2 Introduction 11
2.3 Methods 12
2.4 Bacterial Selection in the Upper Respiratory Tract 13
2.5 Bacterial selection in the lower respiratory tract 14
2.6 Risk Factors of Bacterial Pneumonia 15
2.7 Temporal Associations between Viruses and Bacteria 16
2.8 Respiratory Virus Alters Asymptomatic Carriage of Known Bacterial Pathogens 17

2.9 The Missing Link between Bacterial Carriage Structure and Pneumonia 19
2.10 Mechanisms of Interaction Suggest Virus Can Alter Bacterial Selection in the Lower Respiratory Tract 20
2.11 Conclusions 22
2.12 References 30
Chapter 3 The Respiratory Microbiome and Susceptibility to Influenza Virus Infection 41
3.1 Author summary 41
3.2 Abstract 41
3.3 Introduction 42
3.4 Results 44
3.4.1 Study population 44
3.4.2 Bacterial community type associated with lower susceptibility to influenza virus infection 46
3.4.3 Resistance of bacterial community structure to perturbation by influenza virus infection . 47
3.4.4.... Notable characteristics of community type inversely associated with influenza virus infection 48
3.5 Discussion 49
3.6 Methods 51
3.6.1 Study population and sample collection 52
3.6.2 Ethics statement 53
3.6.3 RNA extraction and RT-PCR 53
3.6.4 DNA extraction and 16S rRNA sequencing 53
3.6.5 Oligotyping and community typing 54
3.6.6 Generalized linear mixed effects models 55
3.6.7 Markov chain analysis 56
3.7 References 81
Chapter 4 The Respiratory Microbiota on Influenza Symptomology and Viral Shedding 85
4.1 Author Summary 85
4.2 Abstract 85
4.3 Introduction 86
4.4 Methods 87
4.4.1 Study Population 87
4.4.2 Laboratory Assays 88
4.4.3 Microbiota Characterization 88

4.4.4 Influenza Shedding and Symptom Data 89
4.4.5 Statistical Analysis 89
4.5 Results 90
4.5.1 Study population 90
4.5.2 Bacterial community prior to infection on symptomology and viral shedding 92
4.5.3 Bacterial community prior to infection on time to infection 92
4.5.4 The role of community diversity 93
4.5.5 The role of individual taxa 93
4.5.6 Sensitivity analysis 94
4.6 Discussion 94
4.7 References 122
Chapter 5 Summary and Conclusions 125
5.1 Lessons Learned 125
5.1.1 Building Leadership Skills 125
5.1.2 Building Research Skills 126
5.1.3 Surrounding Yourself with Good People 128
5.1.4 Learning from Hardships 129
5.2 Challenges 130
5.2.1 Challenges with Systematic Bias 130
5.2.2 Challenges in Working with Microbiome Data 132
5.2.3 Challenges due to Sample Size 133
5.3 Future Directions 133
5.3.1 A Numbers Game 133
5.3.2 Investigating Viral Shedding 134
5.3.3 Functional Potential 135
5.3.4 Host-Pathogen-Microbiome Interactions 136
5.3.5 Interactions between Respiratory Viruses 136
5.4 Conclusions 137
5.5 References 140

List of Tables

Table 2.1 Known interactions and potential mechanisms for observed associations between primary bacterial colonizers of the nasopharynx. 24

Table 2.2 Temporal associations between respiratory viruses and *S. pneumoniae*, the United States. 25

Table 2.3 Mechanisms of synergistic virus-bacteria interaction. 26

Table 3.1 Characteristics of 537 household contacts of influenza cases from 144 households, Managua, Nicaragua, 2012-2014, by baseline community type. 57

Table 3.2 Characteristics of 71 secondary cases from 48 households, Managua, Nicaragua, 2012-2014, by baseline community type. 58

Table 4.1 Summary of models used to investigate the relationship between bacterial community types and various symptom and viral shedding outcomes. 97

Table 4.2 Characteristics of 124 secondary influenza cases from 70 households, Managua, Nicaragua, 2012-2014, by bacterial community type. 98

Table 4.3 Generalized linear mixed effects models examining associations between bacterial community types and the development of symptoms and viral shedding. 99

Table 4.4 Generalized estimating equation accelerated failure time models assess the impact of alpha diversity on outcomes associated with community type 5. 100

Table 4.5 Models assessing the impact individual oligotypes on outcomes associated with community types. 101

Table 4.6 Sensitivity analysis: influenza-associated illness period does not exclude symptoms if fever recurs ≥3 days after fever alleviation..... 103

Table 4.7 Sensitivity analysis: influenza-associated illness period only considers ILI symptoms. 104

Table 4.8 Sensitivity analysis: all ARI symptoms during follow up contribute to influenza-associated illness period..... 105

List of Figures

Figure 2.1 Rate of hospitalization for pneumonia; the United States, 2007-2009..... 27

Figure 2.2 Network of interactions between virus and bacteria in the upper respiratory tract..... 28

Figure 3.1 Graphical Abstract..... 59

Figure 3.2 Model fit of negative log models by number of Dirichlet components. 60

Figure 3.3 Principal coordinates analysis of nose/throat samples assigned to community types. 61

Figure 3.4 Taxa composition of community types renormalized to the 15 oligotypes that account for >50% of the difference between community types. 1,405 samples from 717 study participants residing in 144 households in Managua, Nicaragua, 2012-2014..... 62

Figure 3.5 Susceptibility to influenza virus infection following household exposure varies by bacterial community type..... 63

Figure 3.6 Influenza susceptibility model, additionally adjusting for undefined community type. 64

Figure 3.7 Transitions between in nose/throat bacterial community type over time among the 513 study participants with enrollment and follow up microbiota data..... 65

Figure 3.8 Change in nose/throat bacterial community type over time among the 513 study participants with complete sample pairs, all transition rates. 66

Figure 3.9 Resistance to change in community type by selected variables among the 484 study participants with defined community types at enrollment and follow up..... 67

Figure 3.10 Community resistance model, additionally adjusting for undefined community type 68
Figure 3.11 Community resistance model, additionally adjusting for days of follow up..... 69
Figure 3.12 Alpha and beta diversity and taxa composition of the nose/throat microbiome by bacterial community type..... 70
Figure 3.13 Chao 1 index and non-binary Jaccard distance of the nose/throat microbiome by bacterial community type..... 71
Figure 3.14 Generalized linear mixed effects model estimating odds of influenza virus infection. 72
Figure 3.15 Taxa composition of community types, all oligotypes..... 73
Figure 3.16 Change in relative abundance of selected oligotypes associated with influenza virus infection. 80
Figure 4.1 Principal coordinates analysis of nose/throat samples assigned to community types. 106
Figure 4.2 Model fit of negative log models by number of Dirichlet components using the first and last samples of all study participants (n=1,405 samples)..... 107
Figure 4.3 Characteristics of bacterial community types based on first and last nose/throat samples of 717 study participants from 144 households, Managua, Nicaragua, 2012-2014..... 108
Figure 4.4 Diversity by community type types based on first and last nose/throat samples of 717 study participants from 144 households, Managua, Nicaragua, 2012-2014, using alternative metrics.ad..... 109
Figure 4.5 Relative abundance of oligotypes by community type..... 110

Figure 4.6 Accelerated failure time models estimating acceleration factor and 95% confidence interval for symptom and shedding durations. 117
Figure 4.7 Accelerated failure time models estimating acceleration factor and 95% confidence interval for serial interval and time to shedding onset. 118
Figure 5.1 Potential systematic bias at various stages of microbiome study 138
Figure 5.2 Established pipeline for microbiome studies 139

List of Abbreviation

AF	Acceleration factor
AFT	Accelerated failure time
ARI	Acute respiratory infection
CDC	Centers for Disease Control and Prevention
CI	Confidence interval
CT	Community type
DNA	Deoxyribonucleic acid
EPIC	Etiology of Pneumonia in the Community
EV	Enterovirus
FARI	Febrile acute respiratory infection
GEE	Generalized estimating equation
GLME	Generalized linear mixed effects
HAdV	Human adenovirus
HAI	Hemagglutinin inhibition
HBoV	Human bocavirus
HCoV	Human coronavirus
HI	*Haemophilus influenzae*
HMPV	Human metapneumovirus
HOMD	Human Oral Microbiome Database

HPeV	Human parechovirus
HRV	Human rhinovirus
IAV	Influenza A virus
IFN	Interferon
ILI	Influenza-like illness
IPD	Invasive pneumococcal disease
IQR	Interquartile range
IV	Influenza virus
LRT	Lower respiratory tract
MC	*Moraxella catarrhalis*
MED	Minimum Entropy Decomposition
NTHi	Nontypeable *Haemophilus influenzae*
OP	Odd ratio
PcV	Picovirus
PIV	Parainfluenza virus
PRR	Pathogen recognition receptor
QIIME	Quantitative Insights Into Microbial Ecology
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RSV	Respiratory syncytial virus
RT-PCR	Reverse-transcription polymerase chain reaction
SA	*Staphylococcus aureus*

SMRT	Single Molecule, Real-Time Sequencer
SP	*Streptococcus pneumoniae*
TLR	Toll-like receptor
URT	Upper respiratory tract
US	United States
WUPyV	WU polyomavirus

Abstract

Despite the availability of vaccines, influenza causes approximately 3-5 million cases of severe illness and 400,000 deaths each year. Prevention efforts might potentially be strengthened by harnessing the host microbiome, which plays an important role in maintaining human health by promoting host immunity and colonization resistance. Although vaccines are the best available means of prevention, vaccine effectiveness has been low to moderate in recent years and vaccine coverage remains low, especially in low- to middle-income countries. Exploring the relationship between influenza virus and the respiratory microbiome may contribute to alternative strategies of prevention.

This dissertation explores the relationship between influenza virus and the nose/throat microbiome. In chapter 2, we describe our current understanding of respiratory virus-bacteria interactions using systematic and targeted literature searches. We explore whether respiratory viruses can place selective pressures on bacteria in the upper respiratory tract. Further, as colonization in the upper respiratory tract is a necessary precursor for many respiratory pathogens, we explore whether virus-associated changes in the upper respiratory tract microbiome can influence the etiology of bacterial pneumonia. We found strong biological support for a link between respiratory viruses, the upper respiratory tract microbiome, and bacterial pneumonia. However, we found a lack of longitudinal studies among human populations that examined all three components.

To address this knowledge gap, we used a household transmission study of influenza in Nicaragua to explore potential relationships between influenza and the nose/throat microbiome. In

chapter 3, we examine whether the respiratory microbiome mediates susceptibility to influenza virus infection and characterize structural changes to the nose/throat microbiome during influenza virus infection. We used Dirichlet multinomial mixture models to assign nose/throat samples to bacterial community types and generalized linear mixed effects models which account for clustering by household. We found a single community type associated with decreased susceptibility to influenza. Further, we found high rates of change in the microbiome structure following influenza virus infection as well as among household contacts who were never infected with influenza during follow up.

In chapter 4, we use secondary cases from the Nicaraguan household transmission study to investigate whether the respiratory microbiome impacts influenza symptomology and viral shedding. We used generalized linear mixed effects models to examine the presentation of symptoms and viral shedding. Further, we used accelerated failure time models with a generalized estimating equation approach to examine time-to-event outcomes including symptom duration, shedding duration, and time to infection. The duration of symptoms varied by bacterial community type both prior to and during influenza virus infection. Further, a community type with low diversity was associated with shorter duration of viral shedding and delayed time to infection among secondary cases. The results of these various analyses suggest the respiratory microbiome may be a potential target for reducing influenza risk, household transmission, and disease severity. In the final chapter, I review the skills I learned and the challenges I encountered during the dissertation process. Finally, I review future research directions that focus on deciphering the complex dynamics between the host, pathogen, and microbiome.

Chapter 1 Introduction

This dissertation explores the relationship between influenza virus and the respiratory microbiome. In this chapter, I discuss the global impact of influenza and the importance of identifying unknown risk factors and developing new strategies for prevention. With increasing support for the role of the host microbiome on human health, I explore the relationship between influenza and the respiratory microbiome. Further, I propose the respiratory microbiome may be a potential target for future interventions.

1.1 Influenza Virus

Influenza is a negative-stranded RNA virus from the *Orthomyxoviridae* family [1]. As a highly contagious and rapidly evolving pathogen [2], influenza virus is estimated to cause 3-5 million cases of severe illness [3] and 400,000 deaths [4] each year. Animal experiments [5] and human challenge studies [6] provide highly valuable information about influenza pathology and factors that impact viral shedding and symptomology. However, results from these studies may not accurately represent true influenza dynamics in human populations. Household studies have played an important role in filling this gap by allowing researchers to examine the natural progression of infections among household contacts exposed to influenza virus [7–9]. These studies have been useful for identifying risk factors of influenza and for evaluating potential interventions.

Currently, the most well-known risk factors include young and old age, chronic conditions, and immunodeficiencies [10] and common strategies for prevention have included vaccines,

antivirals, facemasks, and hand hygiene [8,11,12]. Murine studies suggest the host microbiome may also mediate influenza virus infection, but this relationship has not yet been explored in human populations. Although influenza vaccines are the best available means of protection, vaccine effectiveness has been low to moderate in recent years [13,14] and vaccine coverage remains low, especially in low- and middle-income countries [15]. Exploring the relationship between the host microbiome and influenza virus may contribute to supplementary strategies of prevention.

1.2 The Human Microbiome

The human body is colonized by a dynamic microbial community consisting of bacteria, virus, archaea, and fungi. These microbes and their genetic content is collectively termed the microbiome. In recent years, research has shown the microbiome plays an important role in maintaining human health by shaping systemic and local host immunity [16–20] and preventing pathogen colonization or expansion (i.e. colonization resistance) [21–24]. As a relatively new field, one of the primary goals of microbiome research is to understand what constitutes a "healthy" microbiome. This involves examining the abundance of individual taxa as well as differences in the overall microbial community structure through clustering algorithms [25,26] and diversity metrics [27]. In addition to structural differences, exploring functional characteristics of the microbiome through metagenomics, metatranscriptomics, proteomics, and metabolomics can provide insight to which microbial activities and metabolites may contribute to health [28].

An important topic in microbiome research is ecological stability [29], defined as the capacity of an ecosystem to avoid perturbation (i.e. resistance) or return to the original state after perturbation (i.e. resilience). Perturbations can cause disease by promoting an expansion of opportunistic pathogens or through a loss of beneficial commensals [30]. An improved

understanding of factors that perturb the microbiome could lead to synbiotic interventions that prevent the microbiome from transitioning into diseased states (i.e. improve resistance) or promote a rapid return to a healthy state following perturbation (i.e. improve resilience). In addition to lifestyle and environmental factors, host immunological factors play a crucial role in the development and stability of the microbiome [27,30,31]. Although not yet fully understood, this relationship involves a complex network of interactions between host epithelial cells, innate and adaptive immune cells, and the microbiome. A thorough summary of biological mechanisms described in the literature is in an earlier review by Levy *et al.* [30]. Infection by an exogenous pathogen can perturb the microbiome by stimulating a host immune response and disrupting regular cross-talks between the host and microbiome. Perturbations by pathogens such as influenza virus can lead to secondary infections, associated with increased severity and mortality [32,33].

1.3 The Bi-directional Relationship between Influenza and the Microbiome

The relationship between influenza virus and the microbiome is believed to be bi-directional. First, the microbiome may influence influenza virus infection. Although not yet examined in human populations, murine studies suggest the microbiome can influence influenza virus infection through immunomodulation [34,35]. Mice treated with antibiotics prior to inoculation with influenza expressed impaired macrophage responses to type I and type II interferons [35] and defective T-cell and B-cell responses [34]. It is still unclear which attributes of the microbiome may be driving this relationship. However, recent randomized controlled trials report substantial reductions in the incidence of respiratory tract infections among newborns given synbiotic treatment compared to placebo [36,37]. Further exploration in human populations could enhance synbiotic approaches for preventing influenza virus infection.

Second, influenza virus may influence the microbiome. Man *et al.* fittingly describes the upper respiratory tract as the "gatekeeper to respiratory health" [38] as colonization at this site is a necessary precursor of respiratory infection for certain bacterial pathogens [39,40]. Respiratory viruses such as influenza can perturb the microbiome, enhancing the acquisition [41,42] and overgrowth [43,44] of opportunistic pathogens in the upper respiratory tract. This perturbation increases risk of invasive disease [20,21], potentially through more frequent microaspiration into the lung or migration to the middle ear [22]. However, no longitudinal studies among human populations have yet examined how influenza virus infection alters the microbiome. As the majority of influenza deaths are attributed to secondary bacterial infections caused by common bacterial residents of the upper respiratory tract [32], characterizing these changes in the microbiome would be a first step towards designing synbiotic methods for improving microbiome resilience and reducing disease severity.

1.4 Dissertation Aims

The overall goal of this dissertation is to explore the relationship between influenza virus and the upper respiratory tract microbiome. In chapter 2, we review the role of respiratory viruses in the etiology of bacterial pneumonia. Through systematic and targeted literature searches, we examine whether respiratory viruses alter the bacterial community structure in the upper respiratory tract. Further, we investigate whether virus-related changes in the upper respiratory tract determine the etiology of bacterial pneumonia.

In chapter 3, we use a household transmission study to examine whether the nose/throat microbiome is associated with susceptibility to influenza virus infection. Further, we explore whether influenza virus infection alters the bacterial community structure. Our unique study design

allows us to characterize the microbiome prior to influenza virus infection and to explore characteristics that may contribute to reduced influenza susceptibility.

In chapter 4, we use the previously mentioned household study to examine whether the nose/throat microbiome impacts influenza symptomology or viral shedding. Using daily symptom diaries and laboratory test results over follow up, we examine whether the microbiome both prior to and during influenza virus infection is associated with differences in symptomology and viral shedding.

The last chapter provides a reflective summary of the dissertation process. I discuss the skills I gained, the challenges I encountered, and the lessons I learned from working at the intersection of epidemiology and microbial ecology. Lastly, I discuss future research directions primarily focused on deciphering the complex dynamics between the host, pathogen, and microbiome.

1.5 References

1. Lamb RA. Structure and Replication. In: Webster RG, Monto AS, Braciale TJ, Lamb RA, eds. Textbook of Influenza. Wiley, 2013.
2. Monto AS, Webster RG. Influenza pandemics: History and lessons learned. In: Webster RG, Monto AS, Braciale TJ, Lamb RA, eds. Textbook of Influenza. Wiley, 2013.
3. World Health Organization. Influenza. Available at: http://www.who.int/immunization/topics/influenza/en/. Accessed 17 February 2017.
4. Iuliano AD, Roguski KM, Chang HH, et al. Estimates of global seasonal influenza-associated respiratory mortality: a modelling study. The Lancet **2017**; 0. Available at: http://www.thelancet.com/journals/lancet/article/PIIS0140-6736(17)33293-2/abstract. Accessed 1 March 2018.
5. Fukuyama S, Kawaoka Y. The pathogenesis of influenza virus infections: the contributions of virus and host factors. Curr Opin Immunol **2011**; 23:481–486.
6. Carrat F, Vergu E, Ferguson NM, et al. Time lines of infection and disease in human influenza: a review of volunteer challenge studies. Am J Epidemiol **2008**; 167:775–785.
7. Cowling BJ, Fang VJ, Riley S, Peiris JSM, Leung GM. Estimation of the serial interval of influenza. Epidemiol Camb Mass **2009**; 20:344–347.
8. Ohmit SE, Petrie JG, Malosh RE, et al. Influenza Vaccine Effectiveness in the Community and the Household. Clin Infect Dis **2013**; 56:1363–1369.
9. Ng S, Lopez R, Kuan G, et al. The Timeline of Influenza Virus Shedding in Children and Adults in a Household Transmission Study of Influenza in Managua, Nicaragua. Pediatr Infect Dis J **2016**; 35:583–586.
10. Whitley RJ, Monto AS. Prevention and Treatment of Influenza in High-Risk Groups: Children, Pregnant Women, Immunocompromised Hosts, and Nursing Home Residents. J Infect Dis **2006**; 194:S133–S138.
11. Lee VJ, Yap J, Cook AR, et al. Oseltamivir Ring Prophylaxis for Containment of 2009 H1N1 Influenza Outbreaks. N Engl J Med **2010**; 362:2166–2174.
12. Cowling BJ, Chan K-H, Fang VJ, et al. Facemasks and hand hygiene to prevent influenza transmission in households: a cluster randomized trial. Ann Intern Med **2009**; 151:437–446.
13. Paules CI, Sullivan SG, Subbarao K, Fauci AS. Chasing Seasonal Influenza — The Need for a Universal Influenza Vaccine. N Engl J Med **2018**; 378:7–9.
14. Belongia EA, Simpson MD, King JP, et al. Variable influenza vaccine effectiveness by subtype: a systematic review and meta-analysis of test-negative design studies. Lancet Infect Dis **2016**; 16:942–951.

15. WHO Global Influenza Programme. Seasonal Influenza Vaccine Use in Low and Middle Income Countries in the Tropics and Subtropics. A systematic review. Geneva, Switzerland: World Health Organization, 2015. Available at: http://apps.who.int/iris/bitstream/10665/188785/1/9789241565097_eng.pdf.

16. Mazmanian SK, Liu CH, Tzianabos AO, Kasper DL. An Immunomodulatory Molecule of Symbiotic Bacteria Directs Maturation of the Host Immune System. Cell **2005**; 122:107–118.

17. Beura LK, Hamilton SE, Bi K, et al. Recapitulating adult human immune traits in laboratory mice by normalizing environment. Nature **2016**; 532:512–516.

18. Dasgupta S, Erturk-Hasdemir D, Ochoa-Reparaz J, Reinecker H-C, Kasper DL. Plasmacytoid Dendritic Cells Mediate Anti-inflammatory Responses to a Gut Commensal Molecule via Both Innate and Adaptive Mechanisms. Cell Host Microbe **2014**; 15:413–423.

19. Ivanov II, Atarashi K, Manel N, et al. Induction of Intestinal Th17 Cells by Segmented Filamentous Bacteria. Cell **2009**; 139:485–498.

20. Atarashi K, Tanoue T, Shima T, et al. Induction of Colonic Regulatory T Cells by Indigenous Clostridium Species. Science **2011**; 331:337–341.

21. Buffie CG, Pamer EG. Microbiota-mediated colonization resistance against intestinal pathogens. Nat Rev Immunol **2013**; 13:790.

22. Candela M, Perna F, Carnevali P, et al. Interaction of probiotic Lactobacillus and Bifidobacterium strains with human intestinal epithelial cells: Adhesion properties, competition against enteropathogens and modulation of IL-8 production. Int J Food Microbiol **2008**; 125:286–292.

23. Fukuda S, Toh H, Hase K, et al. Bifidobacteria can protect from enteropathogenic infection through production of acetate. Nature **2011**; 469:543–547.

24. Abt MC, Pamer EG. Commensal bacteria mediated defenses against pathogens. Curr Opin Immunol **2014**; 29:16–22.

25. Holmes I, Harris K, Quince C. Dirichlet multinomial mixtures: generative models for microbial metagenomics. PloS One **2012**; 7:e30126.

26. Koren O, Knights D, Gonzalez A, et al. A guide to enterotypes across the human body: meta-analysis of microbial community structures in human microbiome datasets. PLoS Comput Biol **2013**; 9:e1002863.

27. The Human Microbiome Project Consortium. Structure, Function and Diversity of the Healthy Human Microbiome. Nature **2012**; 486:207–214.

28. Rojo D, Méndez-García C, Raczkowska BA, et al. Exploring the human microbiome from multiple perspectives: factors altering its composition and function. FEMS Microbiol Rev **2017**; 41:453–478.

29. Begon M, Townsend CR, Harper JL. Ecology: From Individuals to Ecosystems. 4th ed. Malden, MA, USA: Blackwell Publishing, 2006.

30. Levy M, Blacher E, Elinav E. Microbiome, metabolites and host immunity. Curr Opin Microbiol **2017**; 35:8–15.

31. Blekhman R, Goodrich JK, Huang K, et al. Host genetic variation impacts microbiome composition across human body sites. Genome Biol **2015**; 16:191.

32. Morens DM, Taubenberger JK, Fauci AS. Predominant Role of Bacterial Pneumonia as a Cause of Death in Pandemic Influenza: Implications for Pandemic Influenza Preparedness. J Infect Dis **2008**; 198:962–970.

33. Brundage JF. Interactions between influenza and bacterial respiratory pathogens: implications for pandemic preparedness. Lancet Infect Dis **2006**; 6:303–312.

34. Ichinohe T, Pang IK, Kumamoto Y, et al. Microbiota regulates immune defense against respiratory tract influenza A virus infection. Proc Natl Acad Sci U S A **2011**; 108:5354.

35. Abt MC, Osborne LC, Monticelli LA, et al. Commensal bacteria calibrate the activation threshold of innate antiviral immunity. Immunity **2012**; 37:158–170.

36. Luoto R, Ruuskanen O, Waris M, Kalliomäki M, Salminen S, Isolauri E. Prebiotic and probiotic supplementation prevents rhinovirus infections in preterm infants: A randomized, placebo-controlled trial. J Allergy Clin Immunol **2014**; 133:405–413.

37. Panigrahi P, Parida S, Nanda NC, et al. A randomized synbiotic trial to prevent sepsis among infants in rural India. Nature **2017**; advance online publication. Available at: http://www.nature.com/nature/journal/vaop/ncurrent/full/nature23480.html?foxtrotcallback=true. Accessed 21 August 2017.

38. Man WH, de Steenhuijsen Piters WAA, Bogaert D. The microbiota of the respiratory tract: gatekeeper to respiratory health. Nat Rev Microbiol **2017**; 15:259–270.

39. Bogaert D, De Groot R, Hermans PWM. Streptococcus pneumoniae colonisation: the key to pneumococcal disease. Lancet Infect Dis **2004**; 4:144–154.

40. Simell B, Auranen K, Käyhty H, et al. The fundamental link between pneumococcal carriage and disease. Expert Rev Vaccines **2012**; 11:841–855.

41. Grijalva CG, Griffin MR, Edwards KM, et al. The Role of Influenza and Parainfluenza Infections in Nasopharyngeal Pneumococcal Acquisition Among Young Children. Clin Infect Dis **2014**; 58:1369–1376.

42. de Lastours V, Malosh R, Ramadugu K, et al. Co-colonization by Streptococcus pneumoniae and Staphylococcus aureus in the throat during acute respiratory illnesses. Epidemiol Infect **2016**; :1–13.

43. McCullers JA, McAuley JL, Browall S, Iverson AR, Boyd KL, Henriques Normark B. Influenza enhances susceptibility to natural acquisition of and disease due to Streptococcus pneumoniae in ferrets. J Infect Dis **2010**; 202:1287–1295.

44. Fan RR, Howard LM, Griffin MR, et al. Nasopharyngeal Pneumococcal Density and Evolution of Acute Respiratory Illnesses in Young Children, Peru, 2009-2011. Emerg Infect Dis **2016**; 22:1996–1999.

Chapter 2 The Role of Respiratory Viruses in the Etiology of Bacterial Pneumonia: an Ecological Perspective

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2.1 Abstract

Pneumonia is the leading cause of death among children less than five years old worldwide. A wide range of viral, bacterial, and fungal agents can cause pneumonia: although viruses are the most common etiologic agent, the severity of clinical symptoms associated with bacterial pneumonia and increasing antibiotic resistance make bacterial pneumonia a major public health concern. Bacterial pneumonia can follow upper respiratory viral infection and complicate lower respiratory viral infection. Secondary bacterial pneumonia is a major cause of influenza-related deaths. In this review, we evaluate the following hypotheses: 1) Respiratory viruses influence the etiology of pneumonia by altering bacterial community structure in the upper respiratory tract (URT); and, 2) Respiratory viruses promote or inhibit colonization of the lower respiratory tract (LRT) by certain bacterial species residing in the URT. We conducted a systematic review of the literature to examine temporal associations between respiratory viruses and bacteria and a targeted review to identify potential mechanisms of interactions. We conclude that viruses both alter the bacterial community in the URT and promote bacterial colonization of the LRT. However, it is

uncertain whether changes in the URT bacterial community play a substantial role in pneumonia etiology. The exception is *Streptococcus pneumoniae* where a strong link between viral co-infection, increased carriage, and pneumococcal pneumonia has been established.

2.2 Introduction

Pneumonia is the leading cause of death in children under five worldwide, responsible for one million deaths each year [1]. The burden is greatest in developing countries, at an estimated 0.22 episodes per child-year, but remains a major public health concern even among developed countries where there are an estimated 0.015 episodes per child-year [2]. In the United States, pneumonia is second only to newborn infant births as the most common reason for hospital admissions (36 cases per 10,000 persons [3]) and causes nearly 50,000 deaths each year [4].

A wide range of viral, bacterial, and fungal agents can cause pneumonia when aspirated into the lungs. The Centers for Disease Control and Prevention (CDC) Etiology of Pneumonia in the Community (EPIC) study identified viruses as the most commonly identified etiologic agent in children and adults hospitalized with pneumonia. An etiologic agent was detected in 81% of 2,222 children under 18 years of age: 66% had one or more viral pathogens, 8% one or more bacterial pathogens, and 7% both bacterial and viral pathogens [5]. Among 2,259 adults, an etiologic agent was detected in 38%: 23% had one or more viral pathogens, 11% one or more bacterial pathogens, and 3% both bacterial and viral pathogens [6]. However, virtually all these serious pneumonia cases were treated with antibiotics, as secondary bacterial infection can complicate lower respiratory viral infection. Therefore, even in cases determined to be pneumonia solely of viral etiology, bacteria interactions of virus and bacteria may play some role.

The large proportion of pneumonia cases without a detected pathogen underscores the limitations of current surveillance and detection methods and how they frame our understanding of pneumonia etiology (Appendix 2.1). EPIC study results suggest we may not be detecting the full panel of pathogens in cases we currently define as viral pneumonia, nor considering the potential role of bacteria on the pathogenic potential of viruses. Bacterial causes of pneumonia are associated with more severe clinical symptoms and increasing antibiotic resistance complicates treatment [2,7–10], making bacterial causes of pneumonia a major concern.

In this review, we examine two hypotheses that argue the etiology of bacterial pneumonia is a consequence of ecologic selection influenced by the interaction of respiratory viruses and bacteria within the host: 1) Respiratory viruses influence the etiology of pneumonia by altering bacterial carriage structure in the upper respiratory tract (URT); and, 2) Respiratory viruses promote or inhibit colonization of the lower respiratory tract (LRT) by certain bacterial species residing in the URT. We begin by describing the normal processes of bacterial selection in the upper and lower respiratory tracts and then present evidence on how these processes can potentially be altered by respiratory viruses.

2.3 Methods

We conducted a systematic literature search in PubMed for studies published between January 1, 1990 and December 9, 2015. We restricted studies to those conducted in the United States to minimize potential geographic variation of associations. The following search string was used: *(bacteria[All Fields] OR bacterial[All Fields]) AND (virus[All Fields] OR viral[All Fields]) AND (lower respiratory tract infection[All Fields] OR LRTI[All Fields] OR lower respiratory tract[All Fields] OR LRT[All Fields] OR lower respiratory infection[All Fields] OR LRI[All*

Fields] OR pneumonia[All Fields] OR bronchitis[All Fields]) AND ("1990/01/1"[PDAT] : "2015/12/09"[PDAT]) AND United States[All Fields] AND (time[All Fields] OR temporal[All Fields] OR season[All Fields]).* Among 464 articles written in English, exclusions were made based on titles, abstracts, and full articles. We excluded reviews, *in vivo* and *in vitro* experiments, and studies of immunocompromised populations. 9 articles were retrieved from the literature search and 3 additional studies were selected from the reference list of retrieved articles.

2.4 Bacterial Selection in the Upper Respiratory Tract

Bacterial pneumonia is primarily caused by the commensal bacteria normally residing in the URT [11,12]. The most common causes of bacterial pneumonia for children under five years of age are *S. pneumoniae*, followed by *H. influenzae* and *S. aureus* [11], although this varies over time and space. From a rudimentary ecological perspective, the human respiratory tract can be defined as an ecosystem with two distinct niches: the URT, characterized by regular asymptomatic carriage of commensal bacteria, and the LRT, which is inhabited at a low abundance by bacteria in healthy individuals [13]. During the first year after birth, the nasopharynx is rapidly colonized [14] and URT carriage is established via ongoing synergistic and antagonistic interactions among commensal bacteria [15]. Though pneumonia is an infection of the lungs, microbial selection in the URT may play an important role in etiology as bacterial strains in the URT can be readily aspirated into the LRT. For example, URT carriage is believed to be a necessary precursor of pneumonia due to *S. pneumoniae* [16,17].

Numerous epidemiologic studies describe synergistic and antagonistic relationships among various commensal bacteria [18–33] and, although the exact biological mechanisms remain unclear, *in vivo* and *in vitro* experiments suggest potential mechanisms involve either direct

interaction between bacterial species or indirect interactions via the host immune system (Table 2.1). A number of population studies suggest *S. pneumoniae* carriage is positively associated with *H. influenzae* [18–27] and *M. catarrhalis* [18,23–28] carriage but negatively associated with *S. aureus* [19,20,24–27,29–32]. Furthermore, *S. aureus* carriage is generally negatively associated with *H. influenzae* and *M. catarrhalis* [19,24,31] carriage while *H. influenzae* and *M. catarrhalis* are believed to be positively associated [18,22,24,33]. Nevertheless, our understanding is limited, as the dynamics of niche competition likely consist of complex relationships between multiple species [31,34] and strains [15,35], further influenced by host and environmental factors [22,36]. As carriage is an important precursor of respiratory infections for certain bacterial species [12], unraveling the complex system of bacterial interactions that determine URT microbiota may be key factor for understanding the etiology of pneumonia.

2.5 Bacterial selection in the lower respiratory tract

Lung microbiome studies suggest that bacteria colonizing the LRT overlap with those found in the URT, but that the abundance of organisms is quite low [37], and their role in pneumonia etiology has yet to be explored. To colonize the LRT, an organism must overcome mucociliary clearance and phagocytosis by resident alveolar macrophages, neutrophils, and monocyte-derived macrophages [38,39], but many URT pathogens have developed strategies to overcome these barriers. *H. influenzae*, *Mycoplasma pneumoniae*, and *Bordetella pertussis* resist mucociliary clearance by impairing ciliary function. *Streptococcus pyogenes, Streptococcus agalactiae, H. influenzae, Neisseria meningitidis, and S. pneumoniae* possess capsules which resist phagocytosis [38]. *S. pneumoniae*, the leading cause of pneumonia [40], is characterized by over 90 serotypes differentiated by variations in the bacterial polysaccharide capsule [41,42] and

associated with different propensities of invasive potential [43]. In addition to protecting against phagocytosis, the capsule prevents clearance by mucous secretion and restricts autolysis [44]. Other species, including *S. aureus,* release anti-opsonizing proteins and possess surface protein A to evade phagocytosis. Furthermore, *S. aureus* secretes leukotoxins that lyse leukocytes and express superantigens that hinder immune response (reviewed by Naber *et al*. [45]).

The crucial role these various mechanisms play in determining respiratory disease is demonstrated by contrasting *M. catarrhalis* with *S. pneumoniae.* Similar to *S. pneumoniae*, *M. catarrhalis* is a primary carriage species estimated to colonize between 31% to 50% of children under 2 years in the US [46] and frequently causes URT infections, such as acute otitis media. However, unlike *S. pneumoniae*, *M. catarrhalis* rarely causes pneumonia [47], suggesting that differences in mechanisms of pathogenicity may be the explanation.

2.6 Risk Factors of Bacterial Pneumonia

Various other factors -- including underlying medical conditions and smoking -- can increase the risk of pneumonia by compromising pulmonary clearance mechanisms and the host immune response [48], potentially influencing the selection of pathogens in both the upper and lower respiratory tracts. Age plays a major role in pneumonia risk. In developed countries such as the US, the risk of pneumonia is highest in individuals who are 65 years or over (Figure 2.1) [49]. The elevated risk in the elderly is likely due to impaired host defenses and an increase in comorbidities -- heart failure, liver disease, and underlying lung disease -- that increase risk of aspiration pneumonia that can occur from dysphagia and gastroesophageal reflux disease (reviewed by Akgün *et al*. [50]). In developing countries, the burden of pneumonia is greatest in young children [2] due to their inability to physically remove and immunologically deal with

bacterial pathogens (reviewed by Siegrist [51]). Very young children also have the greatest prevalence in the nasopharynx of common bacterial pneumonia pathogens: *S. pneumoniae, H. influenzae,* and *M. catarrhalis* [23,52]. Increased carriage may be an important risk factor for pneumonia if the URT bacterial community structure is a determinant of pneumonia etiology. Unfortunately, the majority of carriage studies have been conducted among children under five years of age, which limits our ability to establish the role of nasopharyngeal carriage in other age groups.

Regardless of age, viral infection is an important risk factor for bacterial pneumonia. Viruses can lead to rapid, drastic increases in morbidity and mortality in all age groups as seen in historic influenza epidemics and pandemics [53], making it a major public health concern.

2.7 Temporal Associations between Viruses and Bacteria

The 1918 Spanish flu pandemic resulted in approximately 50 million deaths worldwide: most of the deaths were caused by secondary bacterial pneumonia [54,55]. During the 2009 H1N1 pandemic, bacterial co-infection was detected in 18% to 34% of influenza cases (reviewed by Chertow and Memoli [8]) with vulnerability peaking approximately one week after influenza infection [56]. The association of viral infection and bacterial pneumonia is not limited to influenza, although that interaction has been most studied: adenovirus, human metapneumovirus, respiratory syncytial virus (RSV), and other viruses have been temporally associated with an increased risk of pneumococcal pneumonia and invasive pneumococcal disease (IPD), defined as the isolation of *S. pneumoniae* from a normally sterile site, in the United States (Table 2.2) [57–67]. The majority of US studies suggest strong associations between *S. pneumoniae* infections (both pneumonia and IPD) and influenza virus and RSV, with potential effect modification by age.

Temporal associations with other viruses are less supported and limited to IPD. We did not find any studies in the US that examined temporal associations between viruses and bacterial species other than *S. pneumoniae*. Six studies conducted in other developed countries examined temporal associations between respiratory viruses and IPD [59,68–72]. Three out of five studies that examined influenza virus found associations with IPD in England and Wales, The Netherlands, and Sweden [69,70,72]. Among four studies that examined RSV in other countries, two indicated associations with IPD in all age groups [69,70], one found an association only among children [59], and the last observed an association only in individuals 2 years or older [68].

Temporal associations provide evidence of virus-bacterial interactions, but do not necessarily prove these interactions exist. Many viral infections are seasonal, as is pneumonia infection, so the temporal associations may merely reflect the influence of other seasonal phenomena, environmental or host, that are shared by both viral infection and pneumonia [73]. However, evidence for true virus-bacterial interactions are supported by population studies that estimate a high prevalence of viral co-infection during pneumonia [5,6] and animal models which suggest increased susceptibility to pneumonia and increased disease severity during viral co-infection [93]. In the US, approximately 47% of children and 19% of adults with bacterial pneumonia are co-infected with one or more viruses [5,6]. Further, vaccination for *S. pneumoniae* reduced pneumonia associated with RSV, influenza A, and PIV types 1-3 [74]. Influenza vaccine probe studies may provide additional insight to the burden of influenza co-infection on bacterial pneumonia.

2.8 Respiratory Virus Alters Asymptomatic Carriage of Known Bacterial Pathogens

Consistent with our first hypothesis, viral infection frequently has been associated with carriage of common pneumonia pathogens. In a cross-sectional analysis of Aboriginal and non-Aboriginal children in Western Australia, Jacoby *et al*. observed positive associations between rhinovirus and *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* and a positive association between adenovirus and *M. catarrhalis* in the nasopharynx [18]. In a US study, children with a viral URT co-infection not associated with otitis media had a higher prevalence of nontypeable *H. influenzae* and *M. catarrhalis* relative to healthy children. Furthermore, children with viral co-infection associated with acute otitis media had an increased prevalence of *S. pneumoniae*, nontypeable *H. influenzae*, and *M. catarrhalis* but a decreased prevalence of α-hemolytic *Streptococci* [75]. van den Bergh *et al*. assessed the prevalence of 20 respiratory viruses and the main commensal bacteria in the nasopharynx of 433 healthy Dutch children aged 6 to 24 months. In their study, rhinovirus was positively associated with *S. pneumoniae* and *H. influenzae*, RSV was positively associated with *H. influenzae*, coronaviruses and adenovirus were positively associated with *M. catarrhalis*, and influenza virus was positively associated *S. aureus* (Figure 2.2) [26]. However, as the associations found in the above-mentioned studies are based on cross-sectional analyses, we cannot determine whether viruses influenced carriage structure, bacterial carriage influenced host susceptibility to viruses, or if bidirectional interactions occurred. Prospective studies are required to resolve this temporal ambiguity.

Although the impact of the host microbiota on viral infections is an important consideration (reviewed by Wilks *et al*. [76]), the majority of *in vivo* experiments pertaining to virus-bacterial interactions in the URT focus on the role of viruses on the host microbiota. The results of these studies suggest viruses can alter carriage structure by promoting the colonization of certain commensals. In both animal models and human adults, infection with influenza A virus showed

increased colonization by *S. pneumoniae* and *H. influenzae* in the URT [77–81]. Similarly, infecting rats and chinchillas with RSV led to increased colonization by nontypeable *H. influenzae* [82,83]. Collectively, epidemiologic studies and laboratory experiments suggest the introduction of a virus to the URT niche can substantially alter the bacterial community present [26].

2.9 The Missing Link between Bacterial Carriage Structure and Pneumonia

While there is substantial evidence that viral infection influences the URT bacterial community, whether these changes are reflected in the LRT and ultimately in pneumonia etiology is unclear, which weakens our first hypothesis (i.e. respiratory viruses can influence the etiology of pneumonia by altering bacterial carriage structure in the URT). Studies that examine the joint effects of viral co-infection, bacterial carriage, and bacterial pneumonia would provide one strategy for filling this gap. However, we found only two such studies. In a South African hospital-based surveillance study of severe acute respiratory illness, 969 nasopharyngeal-oropharyngeal specimens were tested for *S. pneumoniae* and a panel of respiratory viruses. A high pneumococcal colonization density in the nasopharynx and oropharynx was associated with both respiratory virus co-infection and pneumococcal pneumonia [84]. A second hospital-based case-control study compared nasopharyngeal carriage among 274 radiologically confirmed cases of pneumonia, 276 cases of other LRT infections, and 350 controls in Vietnam. Their findings for *S. pneumoniae* were similar to that of the South African study. However, the investigators also studied *H. influenzae* and *M. catarrhalis* and found no clear association between viral co-infection, nasopharyngeal bacterial load, and pneumonia for these species [85]. As noted above, *M. catarrhalis* rarely causes pneumonia, but *H. influenzae* is second only to *S. pneumoniae*. While there appears to be a persuasive argument for a link between viral co-infection, carriage, and pneumonia for *S.*

pneumoniae, whether or why the interaction is not true for other URT bacteria needs further exploration. In particular, studies that can directly test if viral infection led to bacterial colonization or overgrowth by a potential pathogen -- which led to bacterial pneumonia by that pathogen, are in order. In conclusion, there is no definitive answer to our first hypothesis. Epidemiologic studies and experiments indicate viruses alter the bacterial community in the URT, but they do not yet adequately address whether these changes in the URT bacterial community play a significant role in pneumonia etiology.

2.10 Mechanisms of Interaction Suggest Virus Can Alter Bacterial Selection in the Lower Respiratory Tract

There are several studies that support our second hypothesis, that respiratory viruses can promote bacterial colonization of the LRT by certain commensals in the URT. Viruses interact with bacteria and the host at various stages along the pathologic pathway to promote bacterial pneumonia (Table 2.3). For example, virus can increase shedding of URT bacteria into the LRT: *in vitro* biofilm and murine studies suggest influenza A virus infection can lead to the dispersion of *S. pneumoniae* biofilms, releasing virulent pneumococci for subsequent secondary infections in the LRT [86,87]. When in a biofilm, *S. pneumoniae* is less virulent; capsule polysaccharide and pneumolysin production are reduced and synthesis of the bacterial adhesin phosphorylcholine increased [88,89].

Viral infections also can promote bacterial adhesion to host cells [90–92]. Influenza and parainfluenza (PIV) promote bacterial adhesion with respiratory epithelium cells by cleaving sialic acid and exposing receptors on host cell oligosaccharide chains [93,94]. *In vitro* and *in vivo* experiments suggest free sialic acid released by viral neuraminidase can behave as signaling

molecules promoting pneumococcal biofilm formation, nasopharyngeal colonization, and bacterial spread to the lungs [95]. Free sialic acid is believed to play a role in invasion by nontypeable *H. influenza* as it is an important component of the biofilm matrix and incorporated into the bacterial capsular polysaccharide to evade host defense mechanisms [105. Though literature is scarce, the relationship may be bilateral as bacterial neuraminidase can promote virus survival during treatment with neuraminidase inhibitors [96]. In addition, viruses can promote bacterial adhesion by upregulating cell surface receptors for pathogenic bacteria. For example, RSV and PIV-3 infection can lead to upregulation of receptors ICAM-1, CEACAM1, and PAF-r to promote binding of nontypeable *H. influenz*ae and *S. pneumoniae* to epithelial cells [97].

Respiratory viral infection can damage and impede the repair of respiratory epithelial cells leading to reduced mucociliary clearance. Consequently, bacteria can more easily enter the lungs to cause pneumonia [98]. Many of the virus-bacteria interaction mechanisms involve viral compromise of the innate immune system. These include impairment and depletion of resident alveolar macrophages [99–101] and neutrophils, which are necessary for bacterial clearance, mediated by induction of type I interferons (IFN) [102] and desensitization to Toll-like receptor (TLR) ligands [103]. Detailed descriptions of potential biological pathways involved in these mechanisms are discussed in earlier reviews by Robinson *et al*. and McCullers [9,56]. Lastly, excessive inflammation in the lungs due to virus-initiated exacerbation of inflammatory mediators, cytokines and chemokines, can cause tissue damage [104], which increases susceptibility to secondary bacterial infections.

Despite the considerable literature on potential mechanisms of viral-bacterial interactions that may lead to pneumonia, most studies are limited to experiments conducted in animal models using select viral and bacterial strains, which may not reflect what is occurring in human

populations. Furthermore, the interactions between virus and bacteria are undoubtedly far more complex than identified in animal models, and likely consists of a complex web of interactions between different viruses and bacteria with viruses similar to that described in the URT [12,26]. Even after considering these limitations, the overwhelming evidence for the existence of multiple biological mechanisms under various conditions supports our second hypothesis that respiratory viruses can alter bacterial selection in the LRT and is an important factor in pneumonia etiology.

2.11 Conclusions

In this review, we discussed how the respiratory tract is an ecosystem with two niches, the URT and the LRT; each with ecological and microbial pressures that determine bacterial selection. We hypothesized that viruses influence bacterial selection in the URT leading to colonization of the LRT and sometimes pneumonia. There appears to be a complex network of interactions among viruses and bacteria in the URT that responds to viral introduction by altering what bacteria are present or modifying their relative abundance. For a least one species, *S. pneumoniae,* viruses can increase nasopharyngeal carriage density and increase risk of pneumococcal pneumonia. Whether this is true for other URT bacteria that cause pneumonia is uncertain. We also proposed that bacterial selection in the LRT could be altered by viral infection. The LRT is normally inhabited by low density of microbes, a state maintained by local host defenses and bacterial mechanisms of evasion. *In vitro* and *in vivo* studies suggest viruses can promote entry and colonization of the LRT for select bacterial species via a range of biological mechanisms including URT biofilm dispersion, increased bacterial adhesion to host epithelial cell by upregulation of cell receptors, reduced pulmonary clearance, impairment of multiple components of the innate immune response, and changes in inflammatory response. Though there are limitations in interpreting the results of

experiments, evidence of numerous mechanisms observed under various conditions strongly suggest that viruses also play an important role in the selection of bacteria in the LRT and pneumonia etiology.

The greatest difficulty in addressing our hypotheses was our inability to determine the relative contributions of URT bacterial community structure and local host defenses on bacterial selection into the LRT. In the simplest case, how much is the risk of pneumonia following viral infection attributable to the presence of a known bacterial pneumonia pathogen (such as *S. pneumoniae*) in the URT? To determine this, studies must examine time-dependent carriage of bacteria, species-specific pneumonia outcomes, and the effects of viral co-infection among other known risk factors – which, to the best of our knowledge, do not currently exist. Nonetheless, the literature strongly supports the presence of an interaction between viral infection and secondary bacterial pneumonia; the failure to fully understand the mechanisms should act as a spur for future studies while continuing current efforts to reduce the worldwide burden of pneumonia.

Table 2.1 Known interactions and potential mechanisms for observed associations between primary bacterial colonizers of the nasopharynx.

Organism 1	**Organism 2**	**Interaction[a]**	**Potential Mechanisms[b]**
S. pneumoniae	*S. aureus*	Antagonism [19,20,24–27,29–32]	Hydrogen peroxide production [105] Catalase [106] Pilus [107] Immune-mediated competition [108,109]
S. pneumoniae	*H. influenzae*	Synergism [18–27,110]	Provision of nutrients [15] Production of β-lactamase [111] Formation of biofilms [111] Phosphorychlorine expression [12]
S. pneumoniae	*H. influenzae*	Antagonism [31]	Hydrogen peroxide production [112] Catalase [112] Desialylation [113] Immune-mediated competition [15,114,115]
S. pneumoniae	*M. catarrhalis*	Synergism [18,23–28]	Passive antibiotic protection [116,117]
S. pneumoniae	*M. catarrhalis*	Antagonism	Hydrogen peroxide production [112]
S. aureus	*H. influenzae*	Synergism [22]	Provision of nutrients [15]
S. aureus	*H. influenzae*	Antagonism [19,24,31]	
S. aureus	*M. catarrhalis*	Antagonism [24]	
H. influenzae	*M. catarrhalis*	Synergism [18,22,24,33]	Outer membrane vesicles [118]

[a]Epidemiologic studies
[b]*In vitro* and *in vivo* experiments

Table 2.2 Temporal associations between respiratory viruses and *S. pneumoniae*, the United States.

Study	Virus	Outcome	Age Group	Temporal Association
Kim *et al.* [57]	HAdV	IPD	All	Yes
	IV			Yes
	PcV			No
	PIV			No
	RSV			Yes
	All except IV			Yes
Talbot *et al.* [58]	IV	IPD	All	Yes
	RSV			Yes
Ampofo *et al.* [60]	HAdV	IPD	<18 years	No
	HMPV			Yes
	IV			Yes
	PIV			No
	RSV			Yes
Murdoch and Jennings [61]	IV	IPD	All	Yes
	PIV1			No
	PIV2			No
	PIV3			Yes
	RSV			Yes, only in <5 years
Nelson *et al.* [62]	IV	IPD	All	Yes
Walter *et al.* [63]	IV	Pneumonia	All	Yes
Zhou *et al*. [119]	IV	Pneumonia	All	Varies by season
	RSV			Varies by season
Weinberger *et al.* 2012 [63]	2009 H1N1 season	Pneumonia	All	Yes
Shrestha *et al.* [64]	Influenza seasons	Pneumonia	All	Yes
Fleming-Dutra *et al.* [65]	2009 H1N1 season	Pneumonia	All	Yes
Weinberger *et al.* 2014 [66]	RSV	Pneumonia	<7 years	Yes
Weinberger *et al.* 2015 [67]	RSV	Pneumonia	<1 years	Yes
			1 to <2 years	Yes
	IV		<1 years	No
			1 to <2 years	Yes

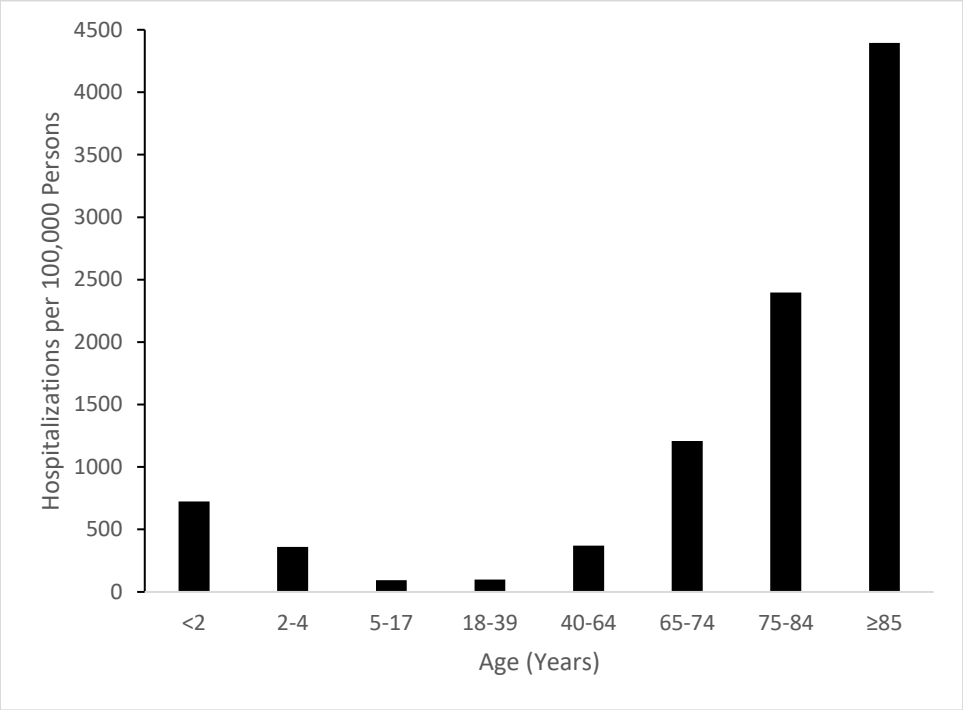
Abbreviations: HAdV, human adenovirus; HMPV, human metapneumovirus; IV, influenza virus; PcV, picornavirus; PIV, parainfluenza virus; RSV, respiratory syncytial virus; IPD, invasive pneumococcal disease.

Table 2.3 Mechanisms of synergistic virus-bacteria interaction.

Mechanism	**Virus**	**Bacteria**
Biofilm dispersion	IAV	*S. pneumoniae* [86,87]
Increased expression of cell surface receptors	HAdV	*S. pneumoniae* [120]
	IAV	*S. pneumoniae* [121]
	PIV	*H. influenzae* [97,122]
		S. pneumoniae [97,122]
	RSV	*H. influenzae* [97,122]
		S. pneumoniae [97,122]
Direct binding of virus and bacteria	RSV	*S. pneumoniae* [123,124]
Damaged and inhibited repair of respiratory epithelium cells	IAV	*S. aureus* [125]
		S. pneumoniae [98]
Decreased mucociliary velocity	IAV	*S. pneumoniae* [126]
Viral neuraminidase	IAV	*S. pneumoniae* [93,94]
Impairment of leukocytes (i.e. neutrophils) response	IAV	*S. aureus* [127]
		S. pneumoniae [102,103,128,129]
	RSV	*S. pneumoniae* [130]
Impairment of alveolar macrophage response	IAV	*S. aureus* [99,131–133]
Impairment of monocytes	IAV	*S. aureus* [127]
	RSV	*M. catarrhalis* [134]
		NTHi [134]
		S. pneumoniae [134]
Reduced natural killer cell recruitment	IAV	*S. aureus* [135]
Exacerbation of inflammatory mediators and tissue damage	HMPV	*S. pneumoniae* [136]
	IAV	*S. pneumoniae* [137–140]

Abbreviations: HAdV, adenovirus; IAV, influenza A virus; HMPV, human metapneumovirus; NTHi, nontypeable *Haemophilus influenzae*; PIV, parainfluenza virus; RSV, respiratory syncytial virus.

Figure 2.1 Rate of hospitalization for pneumonia; the United States, 2007-2009.



Adapted from Griffin *et al.* [49]

Figure 2.2 Network of interactions between virus and bacteria in the upper respiratory tract.

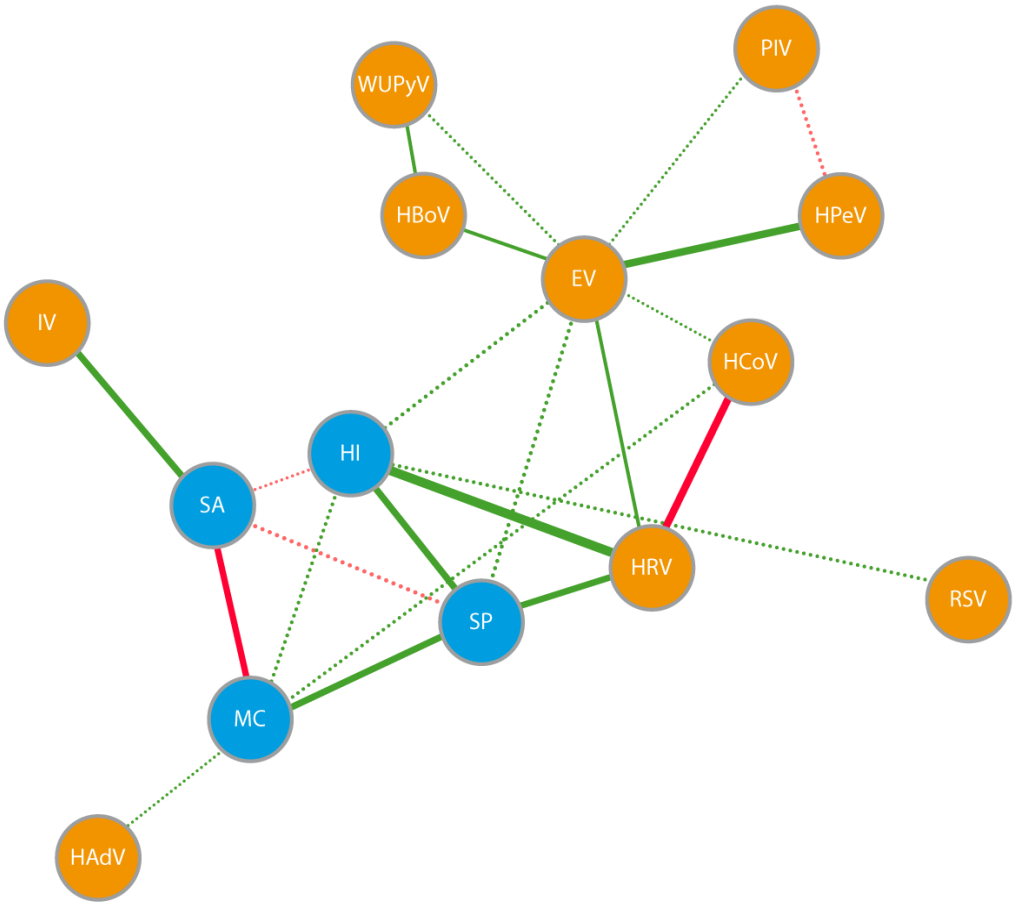


Figure 1A in van den Bergh *et al*. [26] used under the Creative Commons Attribution License. Green lines indicate synergistic associations and red lines indicate antagonistic associations. Solid lines indicate associations with p-values <0.01 and dashed lines indicate associations with p-values between 0.01 and 0.05 for associations between species.

Abbreviations: EV, enterovirus; HI, *Haemophilus influenzae*; HAdV, human adenovirus; HBoV, bocavirus; HCoV, human coronavirus; HPeV, human parechovirus; HRV, human rhinovirus; IV, influenza virus; MC, *M. catarrhalis*; PIV, parainfluenza virus; RSV, respiratory syncytial virus; SA, *Staphylococcus aureus*; SP, *Streptococcus pneumoniae*, WUPyV, WU polyomavirus. doi:10.1371/journal.pone.0047711.g00

Appendix 2.1 Challenges in Determining the Etiology of Pneumonia.

Even in countries where pneumonia surveillance is routinely conducted such as the US, no information on microbial etiology is recorded for approximately 65 to 85% of hospitalized pneumonia cases [11,12]. Severely ill patients often are not included in surveillance, organisms on the causal pathway may have been cleared by the time that the patient presents clinically or prior to testing because of rapid treatment with antibiotics when pneumonia is suspected, and autopsies are infrequently done on the elderly. To optimally determine etiology, direct sampling via bronchoalveolar lavage is required, but usually detection of causal agents is conducted on blood, sputum and urine because of ease of collection, ethical issues and costs. Bacteremia is observed in only 7% to 13% of adult pneumonia cases and 1% to 5% in child pneumonia cases, sputum can potentially be contaminated by bacteria in the URT and is difficult to obtain from children, and blood and urine antigen assays require further validation or are limited to adults and specific to only a few pathogens (e.g. *S. pneumoniae* and Legionella species) (reviewed by Murdoch et al. [13]). Although modern molecular biologic techniques make it feasible to conduct untargeted screens for all bacterial, viral and fungal species present, it is still difficult to distinguish between infection, colonization or contamination [14]. Continued efforts are needed to develop more accurate methods to determine the etiology of pneumonia, and thus maximize treatment and prevention efforts.

2.12 References

1. Liu L, Johnson HL, Cousens S, et al. Global, regional, and national causes of child mortality: an updated systematic analysis for 2010 with time trends since 2000. The Lancet **2012**; 379:2151–2161.

2. Rudan I, O'Brien KL, Nair H, et al. Epidemiology and etiology of childhood pneumonia in 2010: estimates of incidence, severe morbidity, mortality, underlying risk factors and causative pathogens for 192 countries. J Glob Health **2013**; 3. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3700032/. Accessed 17 June 2015.

3. Pfuntner A, Wier LM, Stocks C. Most Frequent Conditions in U.S. Hospitals, 2011. HCUP Statistical Brief #162. Rockville, MD: Agency for Healthcare Research and Quality, 2013. Available at: http://www .hcupus. ahrq .gov/reports/statbriefs/sb162.pdf. Accessed 12 April 2015.

4. Murphy SL, Kochanek KD, Jiaquan X, Heron M. Deaths: Final Data for 2012. Natl Vital Stat Rep **2015**; 63. Available at: http://www.cdc.gov/nchs/data/nvsr/nvsr63/nvsr63_09.pdf.

5. Jain S, Williams DJ, Arnold SR, et al. Community-Acquired Pneumonia Requiring Hospitalization among U.S. Children. N Engl J Med **2015**; 372:835–845.

6. Jain S, Self WH, Wunderink RG, et al. Community-Acquired Pneumonia Requiring Hospitalization among U.S. Adults. N Engl J Med **2015**; 373:415–427.

7. Beadling C, Slifka MK. How do viral infections predispose patients to bacterial infections? Curr Opin Infect Dis **2004**; 17:185–191.

8. Chertow DS, Memoli MJ. Bacterial coinfection in influenza: a grand rounds review. JAMA **2013**; 309:275–282.

9. McCullers JA. Insights into the interaction between influenza virus and pneumococcus. Clin Microbiol Rev **2006**; 19:571–582.

10. WHO. Antimicrobial resistance: global report on surveillance. World Health Organization, 2014.

11. Rudan I, Boschi-Pinto C, Biloglav Z, Mulholland K, Campbell H. Epidemiology and etiology of childhood pneumonia. Epidemiol Etiol Neumonía En Niñez **2008**; 86:408–416.

12. Bosch AATM, Biesbroek G, Trzcinski K, Sanders EAM, Bogaert D. Viral and Bacterial Interactions in the Upper Respiratory Tract. PLoS Pathog **2013**; 9:e1003057.

13. Beck JM, Young VB, Huffnagle GB. The microbiome of the lung. Transl Res J Lab Clin Med **2012**; 160:258–266.

14. Faden H, Duffy L, Wasielewski R, et al. Relationship between Nasopharyngeal Colonization and the Development of Otitis Media in Children. J Infect Dis **1997**; 175:1440–1445.

15. Margolis E, Yates A, Levin BR. The ecology of nasal colonization of Streptococcus pneumoniae, Haemophilus influenzae and Staphylococcus aureus: the role of competition and interactions with host's immune response. BMC Microbiol **2010**; 10:59.

16. Bogaert D, De Groot R, Hermans PWM. Streptococcus pneumoniae colonisation: the key to pneumococcal disease. Lancet Infect Dis **2004**; 4:144–154.

17. Simell B, Auranen K, Käyhty H, et al. The fundamental link between pneumococcal carriage and disease. Expert Rev Vaccines **2012**; 11:841–855.

18. Jacoby P, Watson K, Bowman J, et al. Modelling the co-occurrence of Streptococcus pneumoniae with other bacterial and viral pathogens in the upper respiratory tract. Vaccine **2007**; 25:2458–2464.

19. Shiri T, Nunes MC, Adrian PV, Van Niekerk N, Klugman KP, Madhi SA. Interrelationship of Streptococcus pneumoniae, Haemophilus influenzae and Staphylococcus aureus colonization within and between pneumococcal-vaccine naïve mother-child dyads. BMC Infect Dis **2013**; 13:483.

20. Chien Y-W, Vidal JE, Grijalva CG, et al. Density interactions among Streptococcus pneumoniae, Haemophilus influenzae and Staphylococcus aureus in the nasopharynx of young Peruvian children. Pediatr Infect Dis J **2013**; 32:72–77.

21. Abdullahi O, Karani A, Tigoi CC, et al. The Prevalence and Risk Factors for Pneumococcal Colonization of the Nasopharynx among Children in Kilifi District, Kenya. PLoS ONE **2012**; 7:e30787.

22. Jourdain S, Smeesters PR, Denis O, et al. Differences in nasopharyngeal bacterial carriage in preschool children from different socio-economic origins. Clin Microbiol Infect **2011**; 17:907–914.

23. Mackenzie GA, Leach AJ, Carapetis JR, Fisher J, Morris PS. Epidemiology of nasopharyngeal carriage of respiratory bacterial pathogens in children and adults: cross-sectional surveys in a population with high rates of pneumococcal disease. BMC Infect Dis **2010**; 10:304.

24. Bae S, Yu J-Y, Lee K, Lee S, Park B, Kang Y. Nasal colonization by four potential respiratory bacteria in healthy children attending kindergarten or elementary school in Seoul, Korea. J Med Microbiol **2012**; 61:678–685.

25. Tsai M-H, Huang S-H, Chen C-L, et al. Pathogenic Bacterial Nasopharyngeal Colonization and Its Impact on Respiratory Diseases in the First Year of Life: The PATCH Birth Cohort Study. Pediatr Infect Dis J **2015**; 34:652–658.

26. van den Bergh MR, Biesbroek G, Rossen JWA, et al. Associations between Pathogens in the Upper Respiratory Tract of Young Children: Interplay between Viruses and Bacteria. PLoS ONE **2012**; 7:e47711.

27. Kwambana BA, Barer MR, Bottomley C, Adegbola RA, Antonio M. Early acquisition and high nasopharyngeal co-colonisation by Streptococcus pneumoniae and three respiratory pathogens amongst Gambian new-borns and infants. BMC Infect Dis **2011**; 11:175.

28. Friedel V, Zilora S, Bogaard D, Casey JR, Pichichero ME. Five-year prospective study of paediatric acute otitis media in Rochester, NY: modelling analysis of the risk of pneumococcal colonization in the nasopharynx and infection. Epidemiol Infect **2014**; 142:2186–2194.

29. Bogaert D, van Belkum A, Sluijter M, et al. Colonisation by Streptococcus pneumoniae and Staphylococcus aureus in healthy children. The Lancet **2004**; 363:1871–1872.

30. Regev-Yochay G, Dagan R, Raz M, et al. Association between carriage of Streptococcus pneumoniae and Staphylococcus aureus in Children. JAMA **2004**; 292:716–720.

31. Pettigrew MM, Gent JF, Revai K, Patel JA, Chonmaitree T. Microbial Interactions during Upper Respiratory Tract Infections. Emerg Infect Dis **2008**; 14:1584–1591.

32. Žemlicková H, Urbášková P, Adámková V, Motlová J, Lebedová V, Procházka B. Characteristics of Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis and Staphylococcus aureus isolated from the nasopharynx of healthy children attending day-care centres in the Czech Republic. Epidemiol Infect **2006**; 134:1179.

33. Verhaegh SJC, Snippe ML, Levy F, et al. Colonization of healthy children by Moraxella catarrhalis is characterized by genotype heterogeneity, virulence gene diversity and co-colonization with Haemophilus influenzae. Microbiology **2011**; 157:169–178.

34. Pettigrew MM, Laufer AS, Gent JF, Kong Y, Fennie KP, Metlay JP. Upper Respiratory Tract Microbial Communities, Acute Otitis Media Pathogens, and Antibiotic Use in Healthy and Sick Children. Appl Environ Microbiol **2012**; 78:6262–6270.

35. Auranen K, Mehtälä J, Tanskanen A, S Kaltoft M. Between-strain competition in acquisition and clearance of pneumococcal carriage--epidemiologic evidence from a longitudinal study of day-care children. Am J Epidemiol **2010**; 171:169–176.

36. Adegbola RA, DeAntonio R, Hill PC, et al. Carriage of Streptococcus pneumoniae and Other Respiratory Bacterial Pathogens in Low and Lower-Middle Income Countries: A Systematic Review and Meta-Analysis. PLoS ONE **2014**; 9:e103293.

37. Beck JM, Schloss PD, Venkataraman A, et al. Multi-center Comparison of Lung and Oral Microbiomes of HIV-infected and HIV-uninfected Individuals. Am J Respir Crit Care Med **2015**;

38. McAdam AJ, Milner DA, Sharpe AH. Infectious Diseases. In: Robbins and Cotran Pathologic Basis of Disease. Philadelphia, PA: Elsevier/Saunders, 2015: 341–402.

39. Smith AM, McCullers JA, Adler FR. Mathematical model of a three-stage innate immune response to a pneumococcal lung infection. J Theor Biol **2011**; 276:106–116.

40. O'Brien KL, Wolfson LJ, Watt JP, et al. Burden of disease caused by Streptococcus pneumoniae in children younger than 5 years: global estimates. The Lancet **2009**; 374:893–902.

41. Henrichsen J. Six newly recognized types of Streptococcus pneumoniae. J Clin Microbiol **1995**; 33:2759–2762.

42. Park IH, Pritchard DG, Cartee R, Brandao A, Brandileone MCC, Nahm MH. Discovery of a new capsular serotype (6C) within serogroup 6 of Streptococcus pneumoniae. J Clin Microbiol **2007**; 45:1225–1233.

43. Babl FE, Pelton SI, Theodore S, Klein JO. Constancy of Distribution of Serogroups of Invasive Pneumococcal Isolates Among Children: Experience during 4 Decades. Clin Infect Dis **2001**; 32:1155–1161.

44. van der Poll T, Opal SM. Pathogenesis, treatment, and prevention of pneumococcal pneumonia. The Lancet **2009**; 374:1543–1556.

45. Naber CK. Staphylococcus aureus Bacteremia: Epidemiology, Pathophysiology, and Management Strategies. Clin Infect Dis **2009**; 48:S231–S237.

46. Xu Q, Almudervar A, Casey JR, Pichichero ME. Nasopharyngeal Bacterial Interactions in Children. Emerg Infect Dis **2012**; 18:1738–1745.

47. Murphy TF, Parameswaran GI. Moraxella catarrhalis, a Human Respiratory Tract Pathogen. Clin Infect Dis **2009**; 49:124–131.

48. Musher DM. Infections Caused by Streptococcus pneumoniae: Clinical Spectrum, Pathogenesis, Immunity, and Treatment. Clin Infect Dis **1992**; 14:801–807.

49. Griffin MR, Zhu Y, Moore MR, Whitney CG, Grijalva CG. U.S. Hospitalizations for Pneumonia after a Decade of Pneumococcal Vaccination. N Engl J Med **2013**; 369:155–163.

50. Akgün KM, Crothers K, Pisani M. Epidemiology and management of common pulmonary diseases in older persons. J Gerontol A Biol Sci Med Sci **2012**; 67:276–291.

51. Siegrist C-A. Neonatal and early life vaccinology. Vaccine **2001**; 19:3331–3346.

52. García-Rodríguez JÁ, Martínez MJF. Dynamics of nasopharyngeal colonization by potential respiratory pathogens. J Antimicrob Chemother **2002**; 50:59–74.

53. Brundage JF. Interactions between influenza and bacterial respiratory pathogens: implications for pandemic preparedness. Lancet Infect Dis **2006**; 6:303–312.

54. Taubenberger JK, Reid AH, Fanning TG. The 1918 influenza virus: A killer comes into view. Virology **2000**; 274:241–245.

55. Morens DM, Taubenberger JK, Fauci AS. Predominant Role of Bacterial Pneumonia as a Cause of Death in Pandemic Influenza: Implications for Pandemic Influenza Preparedness. J Infect Dis **2008**; 198:962–970.

56. Robinson KM, Kolls JK, Alcorn JF. The immunology of influenza virus-associated bacterial pneumonia. Curr Opin Immunol **2015**; 34:59–67.

57. Kim PE, Musher DM, Glezen WP, Barradas MCR, Nahm WK, Wright CE. Association of Invasive Pneumococcal Disease with Season, Atmospheric Conditions, Air Pollution, and the Isolation of Respiratory Viruses. Clin Infect Dis **1996**; 22:100–106.

58. Talbot TR, Poehling KA, Hartert TV, et al. Seasonality of invasive pneumococcal disease: temporal relation to documented influenza and respiratory syncytial viral circulation. Am J Med **2005**; 118:285–291.

59. Watson M, Gilmour R, Menzies R, Ferson M, McIntyre P, Network NSWP. The Association of Respiratory Viruses, Temperature, and Other Climatic Parameters with the Incidence of Invasive Pneumococcal Disease in Sydney, Australia. Clin Infect Dis **2006**; 42:211–215.

60. Ampofo K, Bender J, Sheng X, et al. Seasonal Invasive Pneumococcal Disease in Children: Role of Preceding Respiratory Viral Infection. Pediatrics **2008**; 122:229–237.

61. Murdoch DR, Jennings LC. Association of respiratory virus activity and environmental factors with the incidence of invasive pneumococcal disease. J Infect **2009**; 58:37–46.

62. Nelson GE, Gershman KA, Swerdlow DL, Beall BW, Moore MR. Invasive Pneumococcal Disease and Pandemic (H1N1) 2009, Denver, Colorado, USA. Emerg Infect Dis **2012**; 18:208–216.

63. Weinberger DM, Simonsen L, Jordan R, Steiner C, Miller M, Viboud C. Impact of the 2009 Influenza Pandemic on Pneumococcal Pneumonia Hospitalizations in the United States. J Infect Dis **2012**; 205:458–465.

64. Shrestha S, Foxman B, Weinberger DM, Steiner C, Viboud C, Rohani P. Identifying the Interaction Between Influenza and Pneumococcal Pneumonia Using Incidence Data. Sci Transl Med **2013**; 5:191ra84-191ra84.

65. Fleming-Dutra KE, Taylor T, Link-Gelles R, et al. Effect of the 2009 Influenza A(H1N1) Pandemic on Invasive Pneumococcal Pneumonia. J Infect Dis **2013**; 207:1135–1143.

66. Weinberger DM, Grant LR, Steiner CA, et al. Seasonal drivers of pneumococcal disease incidence: impact of bacterial carriage and viral activity. Clin Infect Dis Off Publ Infect Dis Soc Am **2014**; 58:188–194.

67. Weinberger DM, Klugman KP, Steiner CA, Simonsen L, Viboud C. Association between respiratory syncytial virus activity and pneumococcal disease in infants: a time series analysis of US hospitalization data. PLoS Med **2015**; 12:e1001776.

68. Stensballe LG, Hjuler T, Andersen A, et al. Hospitalization for respiratory syncytial virus infection and invasive pneumococcal disease in Danish children aged <2 years: a population-based cohort study. Clin Infect Dis Off Publ Infect Dis Soc Am **2008**; 46:1165–1171.

69. Nicoli EJ, Trotter CL, Turner KME, Colijn C, Waight P, Miller E. Influenza and RSV make a modest contribution to invasive pneumococcal disease incidence in the UK. J Infect **2013**; 66:512–520.

70. Jansen AGSC, Sanders E a. M, VAN DER Ende A, VAN Loon AM, Hoes AW, Hak E. Invasive pneumococcal and meningococcal disease: association with influenza virus and respiratory syncytial virus activity? Epidemiol Infect **2008**; 136:1448–1454.

71. Toschke AM, Arenz S, von Kries R, et al. No temporal association between influenza outbreaks and invasive pneumococcal infections. Arch Dis Child **2008**; 93:218–220.

72. Grabowska K, Högberg L, Penttinen P, Svensson A, Ekdahl K. Occurrence of invasive pneumococcal disease and number of excess cases due to influenza. BMC Infect Dis **2006**; 6:58.

73. Feikin DR, Scott JAG, Gessner BD. Use of vaccines as probes to define disease burden. The Lancet **2014**; 383:1762–1770.

74. Madhi SA, Klugman KP, The Vaccine Trialist Group. A role for Streptococcus pneumoniae in virus-associated pneumonia. Nat Med **2004**; 10:811–813.

75. Friedel V, Chang A, Wills J, Vargas R, Xu Q, Pichichero ME. Impact of Respiratory Viral Infections on α-Hemolytic Streptococci and Otopathogens in the Nasopharynx of Young Children: Pediatr Infect Dis J **2013**; 32:27–31.

76. Wilks J, Beilinson H, Golovkina TV. Dual role of commensal bacteria in viral infections. Immunol Rev **2013**; 255:222–229.

77. McCullers JA, McAuley JL, Browall S, Iverson AR, Boyd KL, Henriques Normark B. Influenza enhances susceptibility to natural acquisition of and disease due to Streptococcus pneumoniae in ferrets. J Infect Dis **2010**; 202:1287–1295.

78. Diavatopoulos DA, Short KR, Price JT, et al. Influenza A virus facilitates Streptococcus pneumoniae transmission and disease. FASEB J **2010**; 24:1789–1798.

79. Wadowsky RM, Mietzner SM, Skoner DP, Doyle WJ, Fireman P. Effect of experimental influenza A virus infection on isolation of Streptococcus pneumoniae and other aerobic bacteria from the oropharynges of allergic and nonallergic adult subjects. Infect Immun **1995**; 63:1153.

80. Wren JT, Blevins LK, Pang B, et al. Influenza A virus alters pneumococcal nasal colonization and middle ear infection independently of phase variation. Infect Immun **2014**; 82:4802–4812.

81. Hirano T, Kurono Y, Ichimiya I, Suzuki M, Mogi G. Effects of influenza A virus on lectin-binding patterns in murine nasopharyngeal mucosa and on bacterial colonization. Otolaryngol Head Neck Surg **1999**; 121:616–621.

82. McGillivary G, Mason KM, Jurcisek JA, Peeples ME, Bakaletz LO. Respiratory syncytial virus-induced dysregulation of expression of a mucosal beta-defensin augments colonization of the upper airway by non-typeable Haemophilus influenzae. Cell Microbiol **2009**; 11:1399–1408.

83. Patel J, Faden H, Sharma S, Ogra PL. Effect of respiratory syncytial virus on adherence, colonization and immunity of non-typable Haemophilus influenzae: implications for otitis media. Int J Pediatr Otorhinolaryngol **1992**; 23:15–23.

84. Wolter N, Tempia S, Cohen C, et al. High Nasopharyngeal Pneumococcal Density, Increased by Viral Coinfection, Is Associated With Invasive Pneumococcal Pneumonia. J Infect Dis **2014**; 210:1649–1657.

85. Vu HTT, Yoshida LM, Suzuki M, et al. Association between nasopharyngeal load of Streptococcus pneumoniae, viral coinfection, and radiologically confirmed pneumonia in Vietnamese children. Pediatr Infect Dis J **2011**; 30:11–18.

86. Marks LR, Davidson BA, Knight PR, Hakansson AP. Interkingdom Signaling Induces Streptococcus pneumoniae Biofilm Dispersion and Transition from Asymptomatic Colonization to Disease. mBio **2013**; 4:e00438-13-e00438-13.

87. Pettigrew MM, Marks LR, Kong Y, Gent JF, Roche-Hakansson H, Hakansson AP. Dynamic changes in the Streptococcus pneumoniae transcriptome during transition from biofilm formation to invasive disease upon influenza A virus infection. Infect Immun **2014**; 82:4607–4619.

88. Chao Y, Marks LR, Pettigrew MM, Hakansson AP. Streptococcus pneumoniae biofilm formation and dispersion during colonization and disease. Front Cell Infect Microbiol **2014**; 4:194.

89. Sanchez CJ, Kumar N, Lizcano A, et al. Streptococcus pneumoniae in Biofilms Are Unable to Cause Invasive Disease Due to Altered Virulence Determinant Production. PLoS ONE **2011**; 6:e28738.

90. Selinger DS, Reed WP, McLaren LC. Model for studying bacterial adherence to epithelial cells infected with viruses. Infect Immun **1981**; 32:941–944.

91. Davison VE, Sanford BA. Adherence of staphylococcus aureus to influenza A virus-infected Madin-Darby canine kidney cell cultures. Infect Immun **1981**; 32:118.

92. George RC, Broadbent DA, Drasar BS. The effect of influenza virus on the adherence of Haemophilus influenzae to human cells in tissue culture. Br J Exp Pathol **1983**; 64:655–659.

93. McCullers JA, Bartmess KC. Role of Neuraminidase in Lethal Synergism between Influenza Virus and Streptococcus pneumoniae. J Infect Dis **2003**; 187:1000–1009.

94. Peltola VT, Murti KG, McCullers JA. Influenza virus neuraminidase contributes to secondary bacterial pneumonia. J Infect Dis **2005**; 192:249–257.

95. Trappetti C, Kadioglu A, Carter M, et al. Sialic acid: a preventable signal for pneumococcal biofilm formation, colonization, and invasion of the host. J Infect Dis **2009**; 199:1497–1505.

96. Nishikawa T, Shimizu K, Tanaka T, et al. Bacterial Neuraminidase Rescues Influenza Virus Replication from Inhibition by a Neuraminidase Inhibitor. PLoS ONE **2012**; 7:e45371.

97. Avadhanula V, Rodriguez CA, DeVincenzo JP, et al. Respiratory Viruses Augment the Adhesion of Bacterial Pathogens to Respiratory Epithelium in a Viral Species- and Cell Type-Dependent Manner. J Virol **2006**; 80:1629–1636.

98. Kash JC, Walters K-A, Davis AS, et al. Lethal Synergism of 2009 Pandemic H1N1 Influenza Virus and Streptococcus pneumoniae Coinfection Is Associated with Loss of Murine Lung Repair Responses. mBio **2011**; 2:e00172-11-e00172-11.

99. Nickerson CL, Jakab GJ. Pulmonary antibacterial defenses during mild and severe influenza virus infection. Infect Immun **1990**; 58:2809–2814.

100. Kleinerman ES, Daniels CA, Polisson RP, Snyderman R. Effect of virus infection on the inflammatory response. Depression of macrophage accumulation in influenza-infected mice. Am J Pathol **1976**; 85:373–382.

101. Astry CL, Jakab GJ. Influenza virus-induced immune complexes suppress alveolar macrophage phagocytosis. J Virol **1984**; 50:287–292.

102. Shahangian A, Chow EK, Tian X, et al. Type I IFNs mediate development of postinfluenza bacterial pneumonia in mice. J Clin Invest **2009**; 119:1910–1920.

103. Didierlaurent A, Goulding J, Patel S, et al. Sustained desensitization to bacterial Toll-like receptor ligands after resolutionof respiratory influenza infection. J Exp Med **2008**; 205:323–329.

104. Sun J, Madan R, Karp CL, Braciale TJ. Effector T cells control lung inflammation during acute influenza virus infection by producing IL-10. Nat Med **2009**; 15:277–284.

105. Regev-Yochay G, Trzcinski K, Thompson CM, Malley R, Lipsitch M. Interference between Streptococcus pneumoniae and Staphylococcus aureus: In Vitro Hydrogen Peroxide-Mediated Killing by Streptococcus pneumoniae. J Bacteriol **2006**; 188:4996–5001.

106. Park B, Nizet V, Liu GY. Role of Staphylococcus aureus catalase in niche competition against Streptococcus pneumoniae. J Bacteriol **2008**; 190:2275–2278.

107. Regev-Yochay G, Lipsitch M, Basset A, et al. The Pneumococcal Pilus Predicts the Absence of Staphylococcus aureus Co-Colonization in Pneumococcal Carriers. Clin Infect Dis **2009**; 48:760–763.

108. Lijek RS, Weiser JN. Co-infection subverts mucosal immunity in the upper respiratory tract. Curr Opin Immunol **2012**; 24:417–423.

109. Lijek RS, Luque SL, Liu Q, Parker D, Bae T, Weiser JN. Protection from the acquisition of Staphylococcus aureus nasal carriage by cross-reactive antibody to a pneumococcal dehydrogenase. Proc Natl Acad Sci **2012**; 109:13823–13828.

110. Odutola A, Antonio M, Owolabi O, et al. Comparison of the Prevalence of Common Bacterial Pathogens in the Oropharynx and Nasopharynx of Gambian Infants. PLoS ONE **2013**; 8:e75558.

111. Weimer KED, Juneau RA, Murrah KA, et al. Divergent mechanisms for passive pneumococcal resistance to β-lactam antibiotics in the presence of Haemophilus influenzae. J Infect Dis **2011**; 203:549–555.

112. Pericone CD, Overweg K, Hermans PW, Weiser JN. Inhibitory and bactericidal effects of hydrogen peroxide production by Streptococcus pneumoniae on other inhabitants of the upper respiratory tract. Infect Immun **2000**; 68:3990–3997.

113. Shakhnovich EA, King SJ, Weiser JN. Neuraminidase Expressed by Streptococcus pneumoniae Desialylates the Lipopolysaccharide of Neisseria meningitidis and Haemophilus influenzae: a Paradigm for Interbacterial Competition among Pathogens of the Human Respiratory Tract. Infect Immun **2002**; 70:7161–7164.

114. Lysenko ES, Ratner AJ, Nelson AL, Weiser JN. The role of innate immune responses in the outcome of interspecies competition for colonization of mucosal surfaces. PLoS Pathog **2005**; 1:e1.

115. Weiser JN, Shchepetov M, Chong ST. Decoration of lipopolysaccharide with phosphorylcholine: a phase-variable characteristic of Haemophilus influenzae. Infect Immun **1997**; 65:943–950.

116. Budhani RK, Struthers JK. Interaction of Streptococcus pneumoniae and Moraxella catarrhalis: investigation of the indirect pathogenic role of beta-lactamase-producing moraxellae by use of a continuous-culture biofilm system. Antimicrob Agents Chemother **1998**; 42:2521–2526.

117. Perez AC, Pang B, King LB, et al. Residence of Streptococcus pneumoniae and Moraxella catarrhalis within polymicrobial biofilm promotes antibiotic resistance and bacterial persistence in vivo. Pathog Dis **2014**; 70:280–288.

118. Tong TT, Mörgelin M, Forsgren A, Riesbeck K. Haemophilus influenzae Survival during Complement-Mediated Attacks Is Promoted by Moraxella catarrhalis Outer Membrane Vesicles. J Infect Dis **2007**; 195:1661–1670.

119. Zhou H, Haber M, Ray S, Farley MM, Panozzo CA, Klugman KP. Invasive pneumococcal pneumonia and respiratory virus co-infections. Emerg Infect Dis **2012**; 18:294–297.

120. Håkansson A, Kidd A, Wadell G, Sabharwal H, Svanborg C. Adenovirus infection enhances in vitro adherence of Streptococcus pneumoniae. Infect Immun **1994**; 62:2707–2714.

121. McCullers JA, Rehg JE. Lethal synergism between influenza virus and Streptococcus pneumoniae: characterization of a mouse model and the role of platelet-activating factor receptor. J Infect Dis **2002**; 186:341–350.

122. Avadhanula V, Wang Y, Portner A, Adderson E. Nontypeable Haemophilus influenzae and Streptococcus pneumoniae bind respiratory syncytial virus glycoprotein. J Med Microbiol **2007**; 56:1133–1137.

123. Smith CM, Sandrini S, Datta S, et al. Respiratory Syncytial Virus Increases the Virulence of Streptococcus pneumoniae by Binding to Penicillin Binding Protein 1a. A New Paradigm in Respiratory Infection. Am J Respir Crit Care Med **2014**; 190:196–207.

124. Hament J-M, Aerts PC, Fleer A, et al. Direct binding of respiratory syncytial virus to pneumococci: a phenomenon that enhances both pneumococcal adherence to human epithelial cells and pneumococcal invasiveness in a murine model. Pediatr Res **2005**; 58:1198–1203.

125. Lee M-H, Arrecubieta C, Martin FJ, Prince A, Borczuk AC, Lowy FD. A postinfluenza model of Staphylococcus aureus pneumonia. J Infect Dis **2010**; 201:508–515.

126. Pittet LA, Hall-Stoodley L, Rutkowski MR, Harmsen AG. Influenza Virus Infection Decreases Tracheal Mucociliary Velocity and Clearance of Streptococcus pneumoniae. Am J Respir Cell Mol Biol **2010**; 42:450–460.

127. Abramson JS, Mills EL, Giebink GS, Quie PG. Depression of monocyte and polymorphonuclear leukocyte oxidative metabolism and bactericidal capacity by influenza A virus. Infect Immun **1982**; 35:350.

128. McNamee LA, Harmsen AG. Both Influenza-Induced Neutrophil Dysfunction and Neutrophil-Independent Mechanisms Contribute to Increased Susceptibility to a Secondary Streptococcus pneumoniae Infection. Infect Immun **2006**; 74:6707–6721.

129. Cobb NK, Byron MJ, Abrams DB, Shields PG. Novel Nicotine Delivery Systems and Public Health: The Rise of the “E-Cigarette”. Am J Public Health **2010**; 100:2340–2342.

130. Stark JM, Stark MA, Colasurdo GN, LeVine AM. Decreased bacterial clearance from the lungs of mice following primary respiratory syncytial virus infection. J Med Virol **2006**; 78:829–838.

131. Jakab G. Immune impairment of alveolar macrophage phagocytosis during influenza virus pneumonia. Am Rev Respir Dis **1982**; 126:778–782.

132. Ghoneim HE, Thomas PG, McCullers JA. Depletion of Alveolar Macrophages during Influenza Infection Facilitates Bacterial Superinfections. J Immunol **2013**; 191:1250–1259.

133. Sun K, Metzger DW. Inhibition of pulmonary antibacterial defense by interferon-γ during recovery from influenza infection. Nat Med **2008**; 14:558–564.

134. Raza MW, Blackwell CC, Elton RA, Weir DM. Bactericidal activity of a monocytic cell line (THP-1) against common respiratory tract bacterial pathogens is depressed after infection with respiratory syncytial virus. J Med Microbiol **2000**; 49:227–233.

135. Small C-L, Shaler CR, McCormick S, et al. Influenza Infection Leads to Increased Susceptibility to Subsequent Bacterial Superinfection by Impairing NK Cell Responses in the Lung. J Immunol **2010**; 184:2048–2056.

136. Kukavica-Ibrulj I, Hamelin M-È, Prince GA, et al. Infection with Human Metapneumovirus Predisposes Mice to Severe Pneumococcal Pneumonia. J Virol **2009**; 83:1341–1349.

137. McAuley JL, Hornung F, Boyd KL, et al. Expression of the 1918 Influenza A Virus PB1-F2 Enhances the Pathogenesis of Viral and Secondary Bacterial Pneumonia. Cell Host Microbe **2007**; 2:240–249.

138. Smith MW, Schmidt JE, Rehg JE, Orihuela CJ, McCullers JA. Induction of Pro- and Anti-inflammatory Molecules in a Mouse Model of Pneumococcal Pneumonia after Influenza. Comp Med **2007**; 57:82.

139. van der Sluijs KF, van Elden LJR, Nijhuis M, et al. IL-10 Is an Important Mediator of the Enhanced Susceptibility to Pneumococcal Pneumonia after Influenza Infection. J Immunol **2004**; 172:7603–7609.

140. Li W, Moltedo B, Moran TM. Type I Interferon Induction during Influenza Virus Infection Increases Susceptibility to Secondary Streptococcus pneumoniae Infection by Negative Regulation of T Cells. J Virol **2012**; 86:12304–12312.

Chapter 3 The Respiratory Microbiome and Susceptibility to Influenza Virus Infection

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3.1 Author summary

Microbiome research has transformed our understanding of microbes and human health. Resident bacteria can protect the host from pathogens by shaping immunological responses. These new insights suggest the microbiome could be a target for preventing influenza virus infection, a major cause of illness and death worldwide. In this study, we explore the relationship between the nose/throat microbiome and influenza virus among Nicaraguan households. Household members were enrolled immediately after one member was diagnosed with influenza virus infection. This study design allowed us to identify an association between the microbiome structure and influenza susceptibility. Further, this association may be due differences in the abundance of *Veillonella*. We also explored whether influenza virus infection altered the microbiome structure and found short-term changes were common among both secondary cases and household members who remained uninfected. Age played a major role in influenza susceptibility and in short-terms changes in the microbiome. Although much work is needed, our findings suggest strategies that appropriately modify the microbiome might be useful in preventing influenza virus infections.

3.2 Abstract

Influenza is a major cause of morbidity and mortality worldwide. However, vaccine effectiveness has been low to moderate in recent years and vaccine coverage remains low, especially in low- and middle-income countries. Supplementary strategies for prevention should be explored. The epithelial cells of the respiratory tract are a primary target for influenza virus infection and replication. They also are enveloped by complex bacterial communities, a respiratory microbiome, which plays a critical role in shaping host immunity. Using a household transmission study, we examined whether influenza susceptibility was associated with the nose/throat microbiome structure among participants exposed to influenza in the household. We also described changes in the nose/throat microbiome following influenza virus infection. We identified a single bacterial community type associated with decreased susceptibility to influenza using a mixed model accounting for household clustering of secondary cases, community types, and other risk factors. The community type – characterized by a low abundance of *Veillonella* – was rare and transitory among young children but a prevalent and stable community type among adults. We found high rates of change in the microbiome structure following influenza virus infection as well as among household contacts who were never infected with influenza during follow up. Further, age strongly influenced susceptibility to influenza and short-term dynamics of the nose/throat microbiome. These results suggest that the nose/throat microbiome might mediate influenza risk and may be a target for future interventions.

3.3 Introduction

Influenza is a major contributor of human illness and death worldwide, estimated to cause 3-5 million cases of severe illness [1] and 400,000 deaths during interpandemic years [2]. Vaccination is the best available means of influenza prevention. However, low vaccine

effectiveness has been low to moderate in recent year [3,4]. Further, vaccine coverage remains low, especially in low- and middle-income countries [5]. With increasing support for the microbiome shaping host immunity [6–8], exploring whether these effects extend to influenza risk could contribute to supplementary methods of prevention.

We hypothesized that the nose/throat microbiome is an unrecognized factor associated with susceptibility to influenza virus. Murine and human studies support this assertion. Compared to controls, mice treated with oral antibiotics exhibited enhanced degeneration of the bronchiole epithelial layer and increased risk of death following intranasal infection with influenza virus [7]. In two separate randomized controlled trials, newborns fed prebiotics and probiotics had significantly lower incidence of respiratory tract infections compared to placebo [9,10]. These studies suggest the manipulation of the microbiome, either through disruption or supplementation, can alter risk of respiratory tract infections.

The epithelial cells of the upper and lower respiratory tracts are the primary targets for influenza virus infection and replication [11]. However, these cells are enveloped by complex bacterial communities that may directly or indirectly interact with influenza virus to mediate risk of infection. Commensal bacteria may prevent infection by regulating innate and adaptive host immune responses [6,7], but immune response to infection also might stimulate changes to the microbiome [12–14]. Alternatively, or in addition, infection may directly act on the microbiome [15,16]. A human experimental trial has shown intranasal administration of live attenuated influenza vaccine can alter the nasal microbiota of young adults by increasing taxa richness [17].

Further, influenza-related changes in the bacterial community structure might explain the enhanced risk of bacterial pneumonia and otitis media following influenza virus infection [18–21]. The most commonly detected causative organisms of bacterial pneumonia and otitis media

increase in abundance in the upper respiratory tract following respiratory virus infection [22,23]. We previously showed that adults in the US with influenza virus infection expressed increased nose/throat carriage of *Streptococcus pneumoniae* and *Staphylococcus aureus* [22]. Similarly, other studies have observed an increase in pneumococcal density following rhinovirus infection [23] and changes in the microbiota during rhinovirus and respiratory syncytial virus infection [24]. Increased carriage elevates risk of invasive disease [25,26], potentially through more frequent microaspiration into the lung or migration to the middle ear [27]. However, an association between the nose/throat microbiome and influenza risk has not been demonstrated in human populations.

In this study, we used data from a longitudinal household transmission study of influenza to assess the relationship between the nose/throat microbiota and susceptibility to influenza virus infection and to determine whether influenza virus infection alters the bacterial community structure using an untargeted 16S rRNA (V4) taxonomic screen. We classified the nose/throat microbiota into five community types using the Dirichlet multinomial mixture method [28] (Graphical abstract available in Figure 3.1).

3.4 Results

3.4.1 Study population

Among the 537 household contacts from 144 households enrolled in the Nicaraguan Household Transmission Study during 2012 to 2014, 61 were children ≤5 years of age (median: 2 years; interquartile range (IQR): 1-4), 163 were children 6-17 years of age (median: 10 years; IQR: 8-14), and 313 were adults (median: 33 years; IQR: 24-43). At least one tobacco smoker resided in 51% of all households and 29% of all participants lived in crowded households, defined as households with, on average, >3 persons per bedroom. Household contacts were rarely vaccinated

against influenza (5%). Few used antibiotics (<1% two weeks prior to enrollment and <1% during follow up) or oseltamivir (6% during follow up) (Table 3.1). 71 secondary cases in 48 households were identified using real-time reverse transcription polymerase chain reaction (RT-PCR) on all nose/throat samples longitudinally collected during follow up. Fourteen out of the 48 households had >1 secondary case (29%), suggesting clustering of cases by household. Most secondary cases were older children and young adults (median: 13.0 years; IQR: 6, 23) and had at least one symptom of an acute respiratory infection (79%) (Table 3.2).

We identified 5 bacterial community types in the nose/throat using Dirichlet multinomial mixture modeling [28] fit to 16S rRNA sequencing data from the first and last available nose/throat samples of each study participant (Figure 3.2: model fit by Dirichlet components; Figure 3.3: principal coordinates analysis). 97% of all sequenced samples were assigned to a community type. Among household contacts without influenza virus infection at time of enrollment, 19 first samples and 13 second samples were assigned to an undefined community type. Fifty percent of the difference between the single-community type model and the five-community type model was attributed to 15 out of the total 230 oligotypes (Figure 3.4).

The prevalence of community types differed significantly by age. Most notably, community type 4 was rare among young children and became more prevalent with age (0-5 years: 4.9%, 6-17 year: 11.0%, adults: 19.8%; $\chi 2$-test, $p<0.004$) (Table 3.1). We observed similar results when restricting our analysis to household contacts who remained uninfected during follow up (Figure 3.5A). The prevalence of community type 4 increased with age (0-5 years: 7%, 6-17 years: 11%, adults: 20%; $\chi 2$ test, $p=0.013$). Young children were primarily colonized by community type 5, which was less common among older age groups (0-5 years: 50%, 6-17 years: 6%, adults: 7%; $\chi 2$-test, $p<0.001$).

3.4.2 Bacterial community type associated with lower susceptibility to influenza virus infection

To investigate the relationship between the bacterial community structure and influenza susceptibility, we first estimated secondary attack rates by bacterial community type. We observed a lower secondary attack rate among household contacts with baseline community type 4 compared to all other community types (6.0% vs. 10.3-16.4%; χ^2-test, p=0.064). Similar patterns were observed after stratifying by age (Figure 3.5B).

We used a generalized linear mixed effects model to examine the relationship between community types and influenza susceptibility after adjusting for age, a smoker in the household, household crowding, and clustering by household. Our decision to account for clustering of predictors and the outcome by household was supported by an intra-class correlation of 0.21. This indicates that 21% of the total variance was due to clustering. Household contacts with baseline community type 4 had a lower odds of influenza virus infection (odds ratio (OR): 0.27; 95% CI: 0.07, 1.03). Further, young children were most likely to acquire influenza virus (OR: 4.71; 95% CI: 1.63, 13.64), followed by older children (OR: 2.89; 95% CI: 1.46, 5.74) (Fig 3.5C). We were inadequately powered for influenza type/subtype-specific models; however, no household contacts with community type 4 at baseline (n=83) were infected with H3N2, the most commonly identified subtype in this population (52% of all secondary infections).

3.4.3 Resistance of bacterial community structure to perturbation by influenza virus infection

To characterize the ability of the bacterial community to resist perturbation by influenza virus (termed resistance), we compared changes in bacterial communities over time by influenza virus infection status. We restricted our analysis to household contacts with microbiota data at enrollment and follow up (n=513). The median days of follow up was 9.0 (IQR: 9.0, 10.0). In Figure 3.7, we used Markov chain plots to represent short-term dynamics of the nose/throat microbiota among household contacts. Arrow width and number indicate the magnitude of the transition rates between community types. Persistence was estimated as the proportion of household contacts that remained within a given community type over follow up. Circle size represents the prevalence of each community type at baseline. Among household contacts who remained uninfected, 62% remained in community type 4, compared to 40% among secondary cases. Further, among the uninfected there were 3 transitions to community type 4 with rates >10% compared to only 1 among secondary cases. Resistance among uninfected household contacts with community type 4 increased with age (0-5 years: 0%, 6-17 years: 43%, adults: 70%; Fisher exact test, p=0.018) (Figure 3.7B). We were inadequately powered for a similar analysis among secondary cases.

We used a generalized linear mixed effects model to examine whether community resistance was associated with influenza virus infection, after adjusting for baseline community type, age, a smoker in the household, household crowding, and clustering by household (Figure 3.9). We did not find an association between community persistence and influenza virus infection. However, we found resistance was lowest among children 6-17 years old (OR: 1.67; 95% CI: 1.07,

2.61) and potentially among those with community type 3 at baseline (OR: 1.61; 95% CI: 0.93, 2.80).

3.4.4 Notable characteristics of community type inversely associated with influenza virus infection

To gain insight into why household contacts with community type 4 at baseline had a lower odds of influenza virus infection, even after adjusting for known risk factors, we explored whether community type 4 differed in bacterial diversity and taxa composition from all others. Bray-Curtis dissimilarity and Shannon diversity were significantly different between community type 4 and each of the other 4 community types (Wilcox rank-sum tests, all comparisons $p<0.001$) (Figure 3.12). However, community type 4 was not notably distinct from other community types (Bray-Curtis median: 0.57 vs. 0.47-0.76; Shannon diversity median: 3.43 vs. 2.56-3.58). We observed similar results with other alternative diversity metrics including the non-binary Jaccard distance and the Chao1 index (Figure 3.13; exception: Chao1 between community types 3 and 4, $p=0.135$). To further explore whether community diversity influenced the relationship between community types and influenza susceptibility, we reran a generalized linear mixed effects model using Shannon diversity as our primary predictor. Although not statistically significant, as diversity increased, so did influenza susceptibility (OR: 1.76; 95% CI: 0.83, 3.72) (Figure 3.14).

Next, we explored whether community type 4 differed from others in taxa composition. The most noticeable difference was a lack of *Veillonella dispar / atypica / parvula / rogosae*, the oligotype contributing most to between-community variation (9.6%) (Figure 3.4 and Figure 3.15). To explore whether *Veillonella* and other oligotypes were associated with influenza susceptibility, we reran our models using the $\log_{10}$-transformed relative abundance of 15 oligotypes that

contributed most to between-community variation. After adjusting for age, a smoker in the household, household crowding, and clustering by household, a 10-fold increase in the relative abundance of *Veillonella dispar / atypica / parvula / rogosae* was associated with a 2-fold increased odds of influenza (OR: 2.04; 95% CI: 0.95, 4.41) (Figure 3.16).

3.5 Discussion

To our knowledge, this is the first human population study to prospectively explore the relationship between the nose/throat microbiome and influenza virus infection. We demonstrate the nose/throat bacterial community structure prior to infection is associated with susceptibility to influenza virus infection. Further, this relationship may be attributed to differences in taxa composition, most notably in the relative abundance of *Veillonella*, gram-negative anaerobes commonly found in the oral cavity. Although little is known about the interaction between *Veillonella* and influenza virus, *Veillonella dispar* in the nasal cavity was negatively correlated with influenza H1-specific antibody titers among healthy individuals inoculated with live attenuated influenza vaccine [17]. Generally unable to ferment sugars, *Veillonella* adhere to lactic acid-producing bacteria in the oral cavity for carbon and energy, making it an important component of multispecies bacterial networks [29,30]. Together this suggests *Veillonella* could modulate influenza susceptibility directly or indirectly by influencing the bacterial community structure within the nose and throat.

Although the exact mechanisms that might account for the association between the respiratory microbiome and influenza susceptibility remain unclear, the few studies that have examined this in murine models indicate the association is likely mediated by immunomodulation. Antibiotic-treated mice exhibited impaired innate and adaptive immune responses, potentially due

impaired macrophage responses to type I and type II interferons (IFN) and a lack of bacterial lipopolysaccharides that stimulate Toll-like receptors and other pattern recognition receptors (PRRs) [6,7]. As the primary site for influenza virus infection and replication [11], the respiratory epithelium plays a critical role in the host immune response. Recognition of influenza virus by PRRs on epithelial cells and sentinel cells (i.e. dendritic cells and macrophages) leads to the activation of antiviral pathways including the secretion of IFN I and the expression of inflammasome-dependent cytokines [31]. Taken together with our current understanding of host immunity, our results highlight the importance of examining host-microbiome interactions at the primary site of infection in addition to the potential systemic effects of the gut microbiome on host immunity [32] and respiratory infections (i.e. the gut-lung axis) [33]. Further work is needed to explore whether these mechanisms are taxon-specific or driven by other factors at the bacterial community level. For example, metabolomic screening could be used to characterize functional differences between bacterial communities.

Most studies that have characterized the upper respiratory tract microbiome are limited to infants [24,34,35]. Here, we demonstrate age effects both the prevalence and resistance of nose/throat bacterial communities in a population with both children and adults. Interestingly, the community type associated with decreased susceptibility to influenza became more prevalent and resistant to perturbation with age. Over the short period of follow-up (median: 9.0 days, IQR: 9.0-10.0), the nose/throat microbiome changed frequently among study participants. We found the microbiome structure changed substantially for both influenza cases and household contacts who remained uninfected during follow up. This high degree of change among uninfected household contacts could represent a response to influenza exposure in the household. Characterizing the microbiome among individuals without household influenza exposure could help us examine this

hypothesis. Nonetheless, preliminary findings from our Markov chain analysis suggest community dynamics may differ by influenza status. This is consistent with studies demonstrating an increased colonization of some bacterial species in the upper respiratory tract following respiratory virus infection [22,23]. However, additional studies that compare by influenza type/subtype, in more defined groups of interests, such as young children, and with multiple longitudinal samples per participant are required to more completely explore this finding.

Individuals can be infected with influenza virus (i.e. ≥4-fold increase in hemagglutinin inhibition antibody titer) and not shed virus [36]. Our results are limited to those with detectable influenza virus shedding. Future studies should evaluate the effects of the respiratory microbiome on viral shedding and symptomology. In addition, influenza transmission could be affected by pre-existing immunity of household contacts. Although vaccination rates were low in our study population, pre-existing immunity from previous infections might potentially confound or modify associations between the microbiome and influenza.

While much work is needed to translate these results into potential clinical and public health applications, our findings contribute to a growing literature suggesting that it may be possible to manipulate the microbiome and decrease risk of disease [9,10]. Influenza virus is a major cause of severe illness and death each year [1,2]. However, vaccine effectiveness varies by year [4] and there still much debate on the use of antivirals for prophylaxis, especially for preventing asymptomatic infections and transmission [37]. Our findings suggest synbiotic treatment may be a possible alternative.

3.6 Methods

3.6.1 Study population and sample collection

The Nicaraguan Household Transmission Study of Influenza is an ongoing prospective case-ascertained study conducted among urban households in Managua, Nicaragua. Patients attending the Health Center Sócrates Flores Vivas were screened for study eligibility. Index cases of influenza were identified as patients with a positive QuickVue Influenza A+B rapid test, symptom onset of an acute respiratory infection within the past 48 hours, and living with at least one other household member. Symptoms of acute respiratory infection included fever or feverishness with cough, sore throat, or runny nose.

Index cases and household members were invited to participate and clinical, sociodemographic, and household data were collected at time of enrollment. Participants were followed for up to 13 days through 5 home visits conducted at 2-3 day intervals. At each home visit, oropharyngeal and nasal swabs were collected, combined, and stored at 4ºC in viral transport media. All samples were transported to the National Virology Laboratory at the Nicaraguan Ministry of Health within 48 hours of collection and stored at -80ºC. A symptom diary was collected for all participants during follow up.

A total of 168 households were enrolled for follow up during 2012-2014. Households were excluded from analysis if a suspected index case was negative for influenza virus by real-time reverse-transcription polymerase chain reaction (RT-PCR) at time of enrollment. Two household contacts were excluded from analysis due to missing influenza virus infection status at time of enrollment. The remaining participants consisted of 144 index cases of influenza positive by RT-PCR, 537 household contacts influenza negative by RT-PCR at time of enrollment, and 36 household contacts who were RT-PCR positive for influenza virus on the first day of follow up.

3.6.2 Ethics statement

Written informed consent was obtained from adult participants and from parents or legal guardians of participants under 18 years of age. In addition, verbal assent was obtained from children over 5 years of age. The study was approved by Institutional Review Boards at the University of Michigan, the Nicaraguan Ministry of Health, and the University of California at Berkeley.

3.6.3 RNA extraction and RT-PCR

Total RNA was extracted from all available nasal/oropharyngeal samples using the QIAmp Viral Mini Kit (QIAGEN, Hilden, Germany) per manufacturer's instructions at the National Virology Laboratory in Nicaragua. Samples were tested for influenza virus by RT-PCR using standard protocols validated by the Centers for Disease Control and Prevention [38].

3.6.4 DNA extraction and 16S rRNA sequencing

Total DNA was extracted from a pair of samples from each study participant: the first sample collected at time of enrollment and the second sample collected at the last day of follow up (median days between samples: 9.0 days, IQR: 9.0-10.0). Among the 717 total study participants, five first samples and 19 second samples were not available for DNA extraction. DNA was extracted using the QIAmp DNA Mini Kit and an enzyme cocktail composed of cell lysis solution (Promega, Madison, USA), lysozyme, mutanolysin, RNase A, and lysostaphin (Sigma-Aldrich, St. Lious, USA) in 22.5:4.5:1.125:1.125:1 parts, respectively. 100 µL of sample was incubated at 37°C for 30 minutes with 80 µL of the enzyme cocktail. After adding 25 µL proteinase K and 200 µL of Buffer AL, samples were vortexed and incubated at 56°C for 30 minutes. Samples

were washed with 200 µL of 100% ethanol, 500 uL of Buffer AW1, and then 500 uL of Buffer AW2. To maximize DNA yield, DNA was eluted twice with 100 uL of Buffer AE and stored at -80°C.

The V4 hypervariable region of the 16S rRNA gene was amplified and sequenced at the University of Michigan Microbial Systems Laboratories using Illumina MiSeq V2 chemistry 2x250 (Illumina, San Diego, CA) and a validated dual-indexing method [39]. Briefly, primers consisted of an Illumina adapter, an 8-nt index sequence, a 10-nt pad sequence, a 2-nt linker, and the V4-specific F515/R806 primer [40]. Amplicons were purified and pooled in equimolar concentrations. A mock community of 21 species (Catalog No. HM-782D, BEI Resources, Manassas, VA) or a mock community of 10 species (Catalog No. D6300, Zymo Research, Irvine, CA) was included by the Microbial Systems Laboratories to assess sequencing error rates. For every 96-well plate submitted for amplification and sequencing (90 study samples), we included two aliquots of an in-house mock community consisting of *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Haemophilus influenzae*, and *Moraxella catarrhalis* and two aliquots of an oropharyngeal control sample. These internal controls were randomly assigned to plate wells and used to assess systematic variation in sequencing. All samples were sequenced in duplicate, demultiplexed, and quality filtered.

3.6.5 Oligotyping and community typing

We used mothur v1.38.1 [41] to align and perform quality filtering on raw sequences using the mothur standard operating procedures (https://www.mothur.org/wiki/MiSeq_SOP, accessed November 18, 2016). Sequences were converted to the appropriate oligotyping format as previously described [42]. We used the Minimum Entropy Decomposition (MED) algorithm [43]

with default parameters (-M: 13779.0, -V: 3 nt) to cluster sequences into oligotypes. Briefly, the algorithm identifies variable nucleotide positions and uses Shannon entropy to partition sequences into nodes. The process is iterative and continues to decompose parent nodes into child nodes until there are no discernable entropy peaks. Oligotyping has previously been used to examine within-genus variations in the microbiota [42,44–46] and provides increased resolution relative to conventional distance-based clustering methods.

After excluding five samples with less than 1,000 reads, our dataset consisted of 1,405 samples with a total of 61,784,957 sequences decomposed into 230 oligotypes. To assign taxonomy, we searched representative sequences of each oligotype against the Human Oral Microbiome Database (HOMD) v14.51 [47] using blastn v2.2.23 [48].

We used Dirichlet multinomial mixture models [28] in R v3.3.2 [49] and the DirichletMultinomial v1.16.0 package [50] to assign all samples to 5 community types. We determined the number of community types by comparing the Laplace approximation of the negative log models and identifying the point at which an increase in Dirichlet components resulted in minor reductions in model fit (Figure 3.2). Samples were assigned to community types with the greatest posterior probability. 96.8% of all samples had a posterior probability of 90% or higher. To minimize misclassification, samples were assigned as having an undefined community type if the posterior probability was less than 90%. Each community type contained between 12.9-24.4% of all samples (n=181-343) and 3.2% of all samples (n=45) were undefined.

3.6.6 Generalized linear mixed effects models

To examine the association between baseline community types and susceptibility of influenza virus infection, we used a generalized linear mixed effects model estimating the odds of

infection after adjusting for community type (relative to community type 1), age (relative to adults), a smoker in the household, household crowding, and clustering by household. Household crowding was defined as having, on average, more than three household members per bedroom. The model was adapted to examine the effects of alpha diversity and individual oligotypes on susceptibility of influenza virus infection. We examined the 15 oligotypes that contributed most to variation between community types (oligotypes listed in Figure 3.4). Relative abundance values were $\log_{10}$-transformed in consideration of the constant sum constraint, which is a characteristic of compositional data [51].

To examine the effect of influenza virus infection on microbiota resistance, we used a generalized linear mixed effects model estimating the odds of any change in community type over follow up after adjusting for baseline community type (relative to community type 1), age (relative to adults), a smoker in the household, household crowding (average of >3 persons per bedroom), and clustering by household. All generalized linear mixed effects models were created using R v3.3.2 [49] and the lme4 package [52].

3.6.7 Markov chain analysis

We estimated community transition rates over time using methods described previously [53]. Briefly, we restricted our dataset to household contacts with complete nose/throat sample pairs (i.e. microbiota data at enrollment and at follow up). Community transition rates were calculated as Markov chain probabilities. Analysis was repeated after stratifying by influenza status and age.

Table 3.1 Characteristics of 537 household contacts of influenza cases from 144 households, Managua, Nicaragua, 2012-2014, by baseline community type.

Characteristic	All[a] (n=537)	Community Type 1 (n=131)	Community Type 2 (n=122)	Community Type 3 (n=120)	Community Type 4 (n=83)	Community Type 5 (n=58)
	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)
Age (years)						
0-5	61 (11)	9 (7)	14 (11)	3 (3)	3 (4)	26 (45)
6-17	163 (30)	51 (39)	40 (33)	38 (32)	18 (22)	9 (16)
≥18	313 (58)	71 (54)	68 (56)	79 (66)	62 (75)	23 (40)
Female	347 (65)	85 (65)	86 (70)	85 (71)	44 (53)	35 (60)
Influenza infection	71 (13)	20 (15)	20 (16)	15 (13)	5 (6)	6 (10)
Influenza vaccination[b]	27 (5)	8 (6)	6 (5)	8 (7)	1 (1)	3 (5)
Smoker in household	245 (51)	54 (46)	55 (51)	56 (52)	43 (59)	27 (49)
>3 persons per bedroom in the household	156 (29)	40 (31)	34 (28)	36 (30)	22 (27)	18 (31)
Antibiotic use <2 weeks prior	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (2)
Antibiotic use during follow up	4 (1)	2 (2)	0 (0)	1 (1)	0 (0)	1 (2)
Oseltamivir use during follow up	33 (6)	4 (3)	14 (12)	5 (4)	2 (2)	5 (9)

[a]Community types were defined using Dirichlet multinomial mixture method (see Methods). Includes household contacts with undefined community types.
[b]Prior to enrollment and in same year as index case

Table 3.2 Characteristics of 71 secondary cases from 48 households, Managua, Nicaragua, 2012-2014, by baseline community type.

Characteristic	All[a] (n=71)	Community Type 1 (n=20)	Community Type 2 (n=20)	Community Type 3 (n=15)	Community Type 4 (n=5)	Community Type 5 (n=6)
	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)
Age (years)						
0-5	15 (21)	3 (15)	5 (25)	1 (7)	0 (0)	4 (67)
6-17	30 (42)	10 (50)	8 (40)	7 (47)	3 (60)	1 (17)
≥18	26 (37)	7 (35)	7 (35)	7 (47)	2 (40)	1 (17)
Female	43 (61)	11 (55)	15 (75)	9 (60)	3 (60)	3 (50)
Influenza vaccination[b]	2 (3)	0 (0)	1 (5)	1 (8)	0 (0)	0 (0)
Smoker in household	29 (52)	7 (39)	9 (53)	6 (43)	3 (100)	3 (50)
>3 persons per bedroom in the household	25 (35)	8 (40)	8 (40)	4 (27)	2 (40)	1 (17)
Antibiotic use <2 weeks prior	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Antibiotic use during follow up	1 (1)	0 (0)	0 (0)	1 (7)	0 (0)	0 (0)
Oseltamivir use during follow up	9 (13)	2 (10)	4 (20)	2 (13)	0 (0)	1 (17)
ARI symptom	56 (79)	14 (70)	17 (85)	13 (87)	5 (100)	4 (67)
Fever	37 (52)	9 (45)	12 (60)	6 (40)	4 (80)	3 (50)
Cough	43 (61)	8 (40)	16 (80)	11 (73)	4 (80)	3 (50)
Sore throat	29 (41)	7 (35)	11 (55)	7 (47)	1 (20)	2 (33)
Runny nose	42 (59)	9 (45)	15 (75)	10 (67)	3 (60)	3 (50)

Abbreviations: ARI, acute respiratory infection

[a]Community types were defined using Dirichlet multinomial mixture method (see Methods). Includes secondary cases with undefined community types

[b]Prior to enrollment and in same year as index case

Figure 3.1 Graphical Abstract

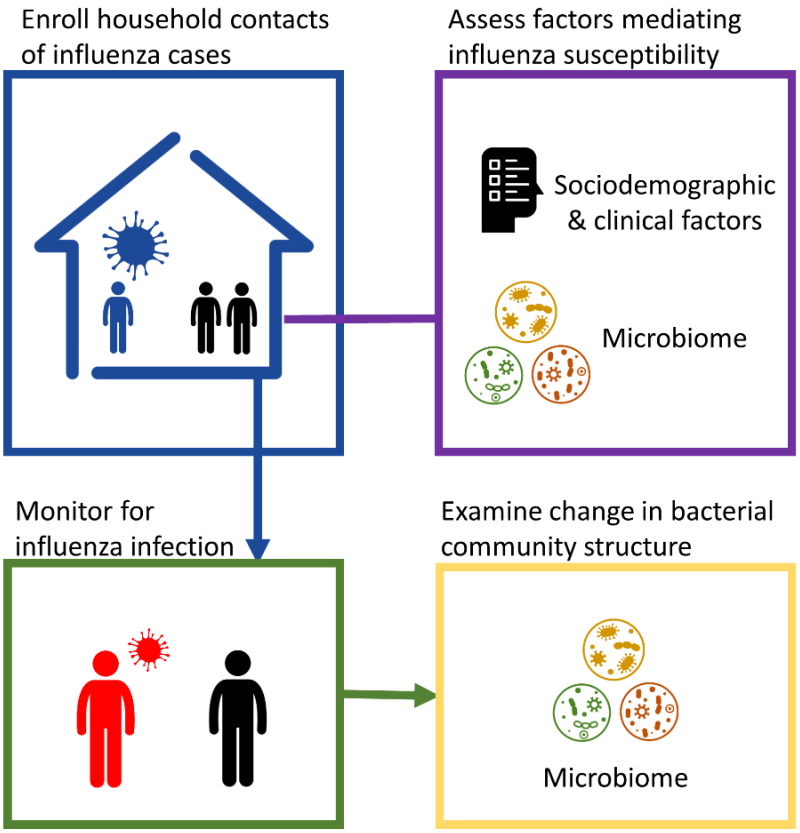
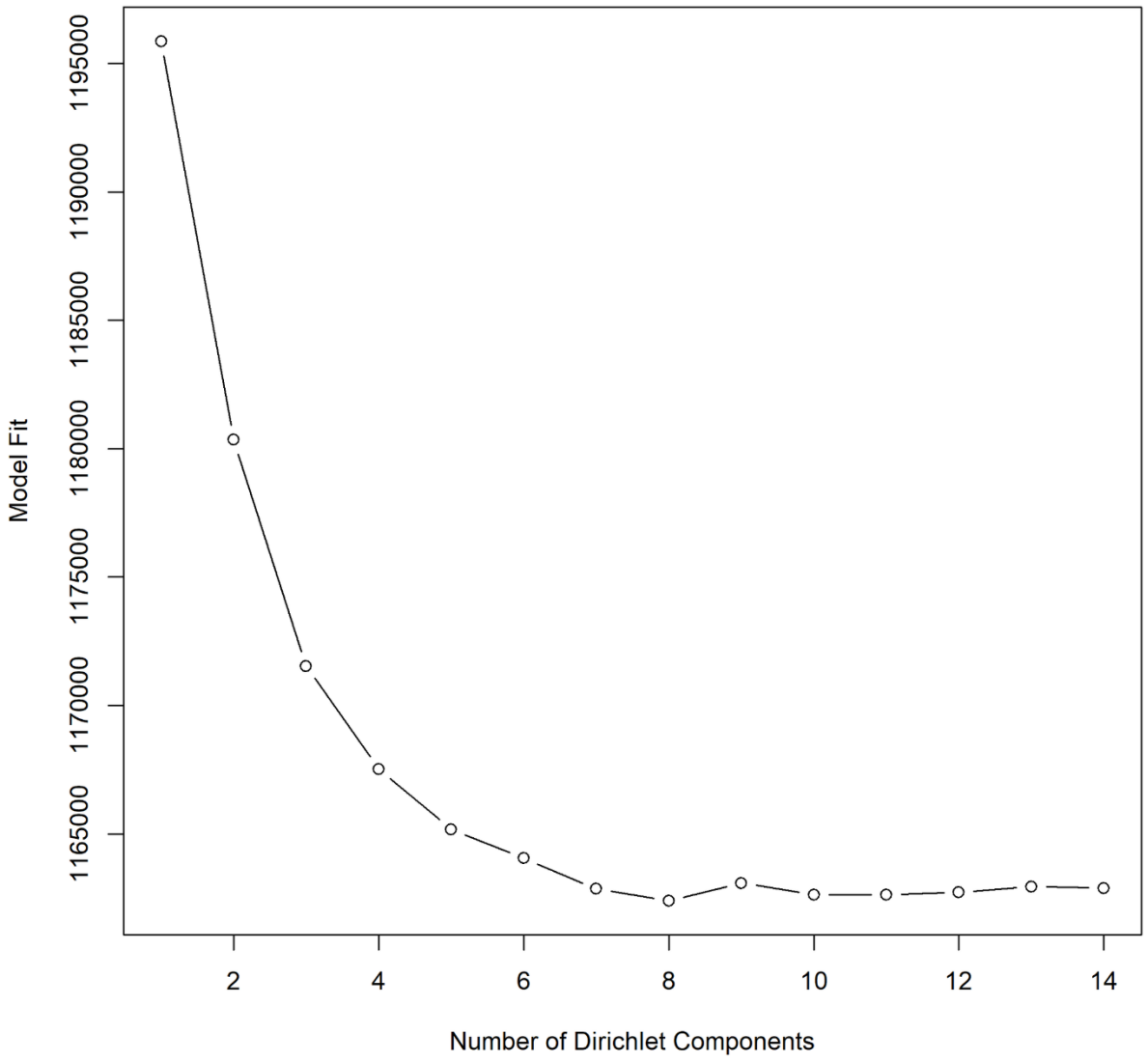
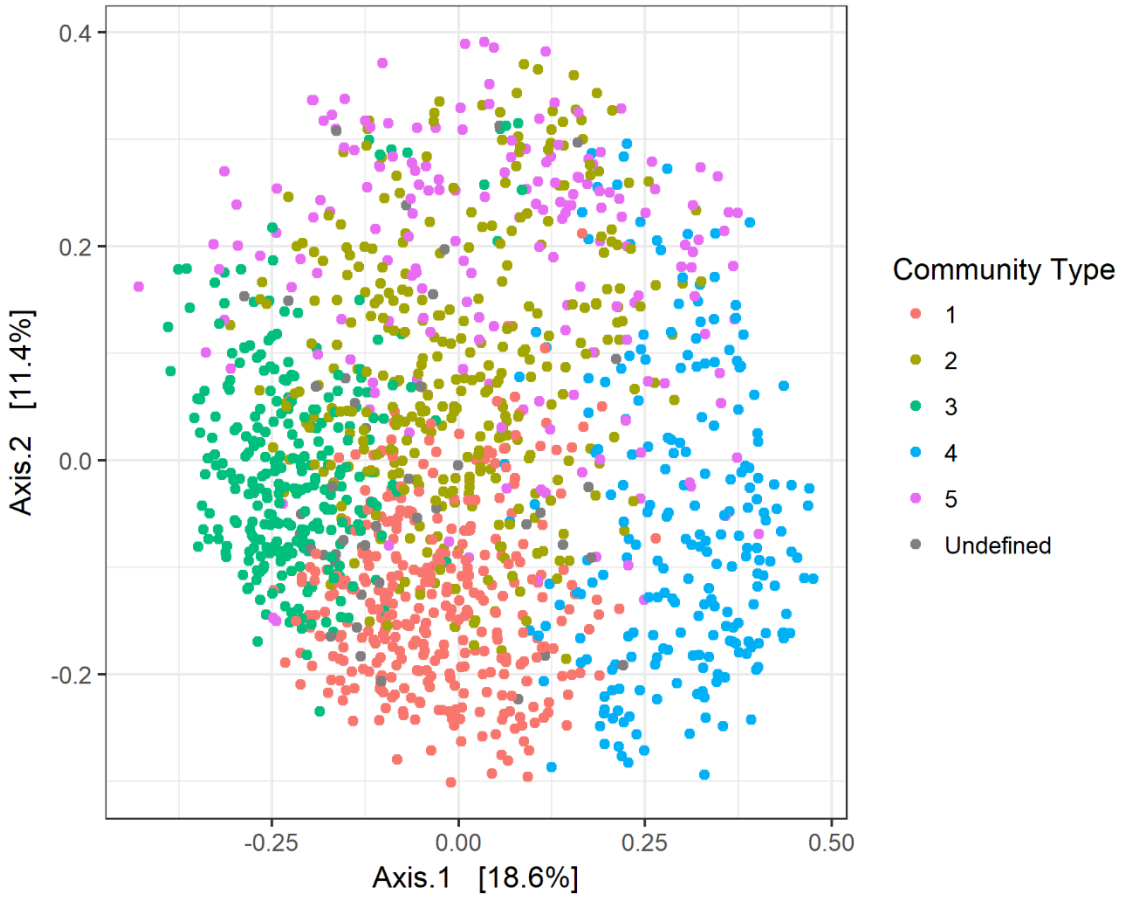


Figure 3.2 Model fit of negative log models by number of Dirichlet components.



We determined the number of community types by estimating the Laplace approximation of the negative log models and identifying the point at which an increase in Dirichlet components resulted in minor reductions in model fit.

Figure 3.3 Principal coordinates analysis of nose/throat samples assigned to community types.



1,405 nose/throat samples from 717 study participants residing in 144 households in Managua, Nicaragua, 2012-2014. Based on Bray-Curtis dissimilarity.

Figure 3.4 Taxa composition of community types renormalized to the 15 oligotypes that account for >50% of the difference between community types. 1,405 samples from 717 study participants residing in 144 households in Managua, Nicaragua, 2012-2014.

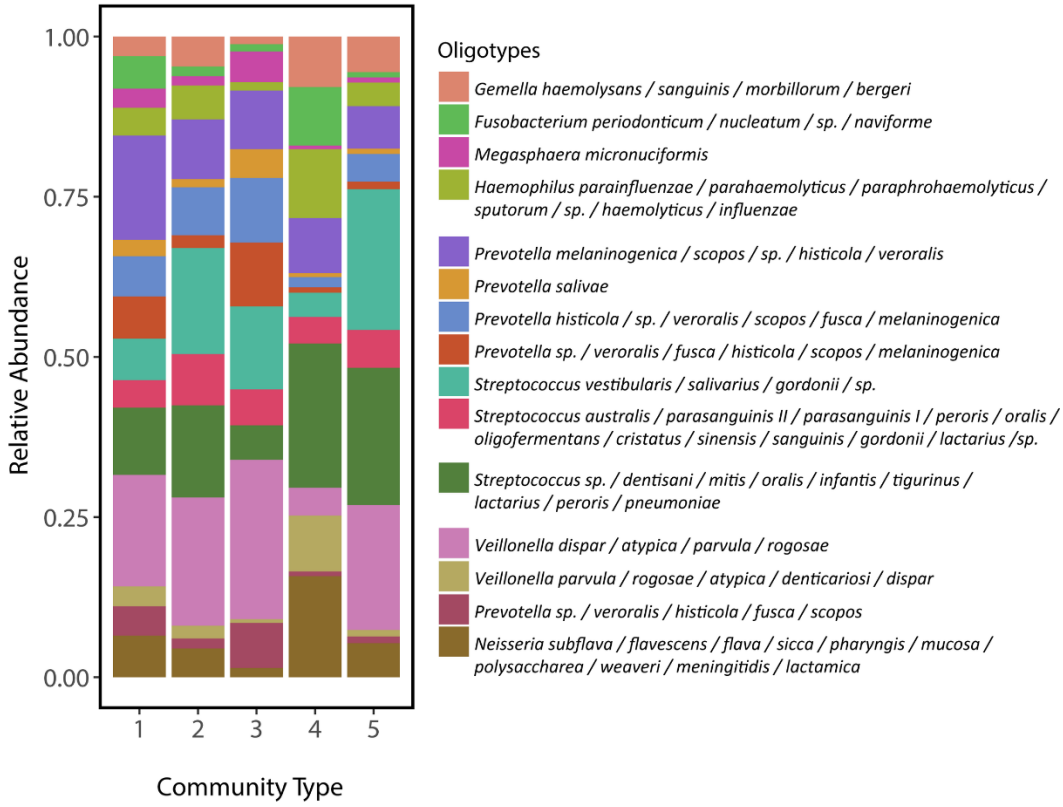
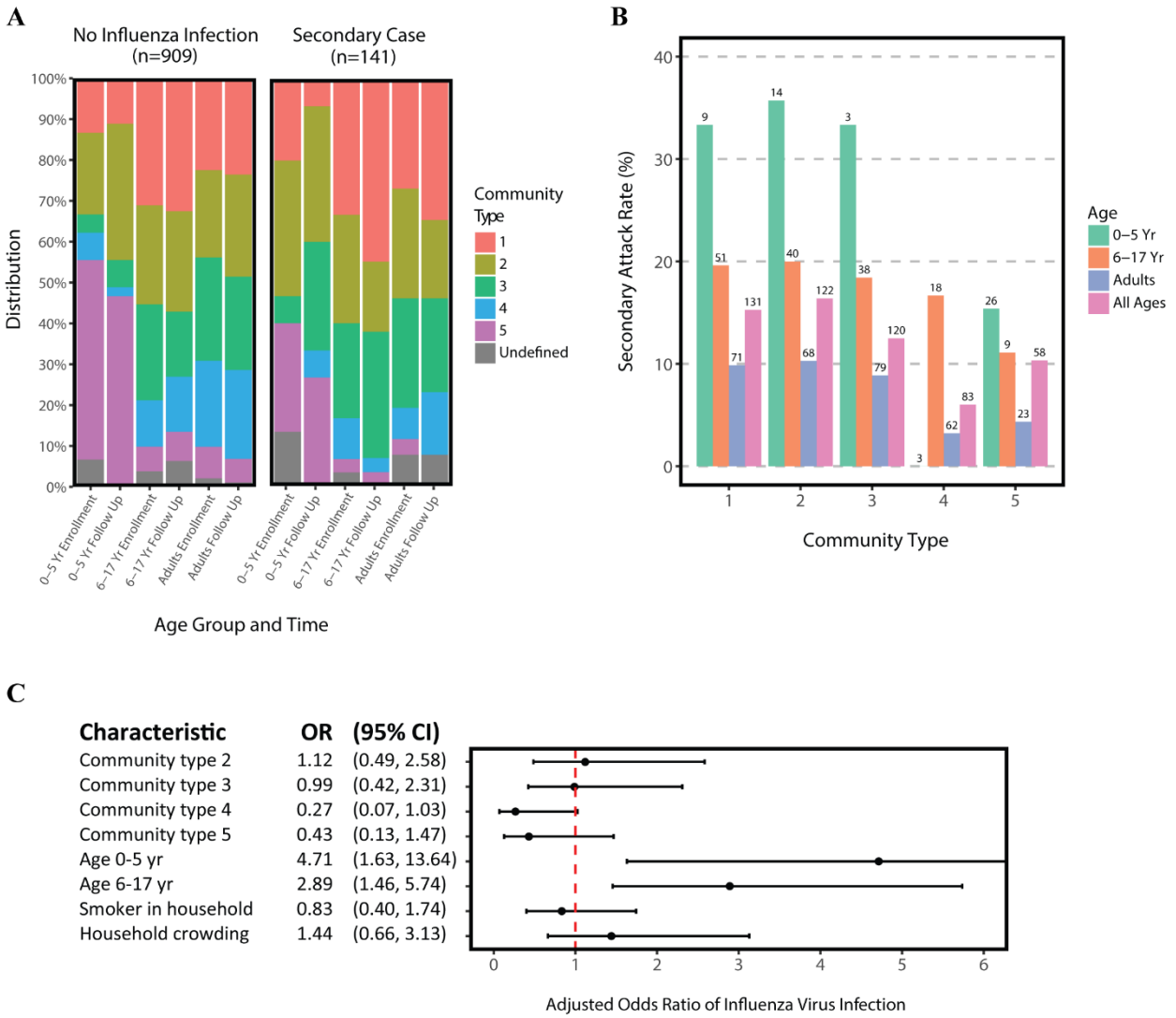
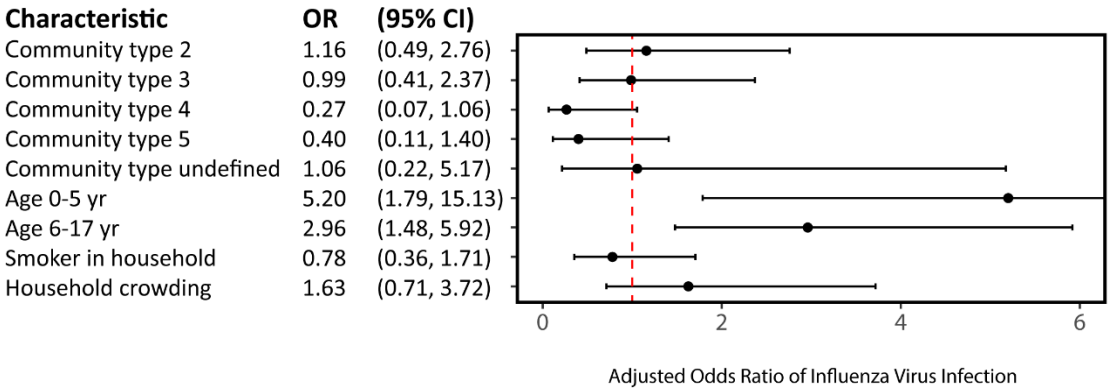


Figure 3.5 Susceptibility to influenza virus infection following household exposure varies by bacterial community type.



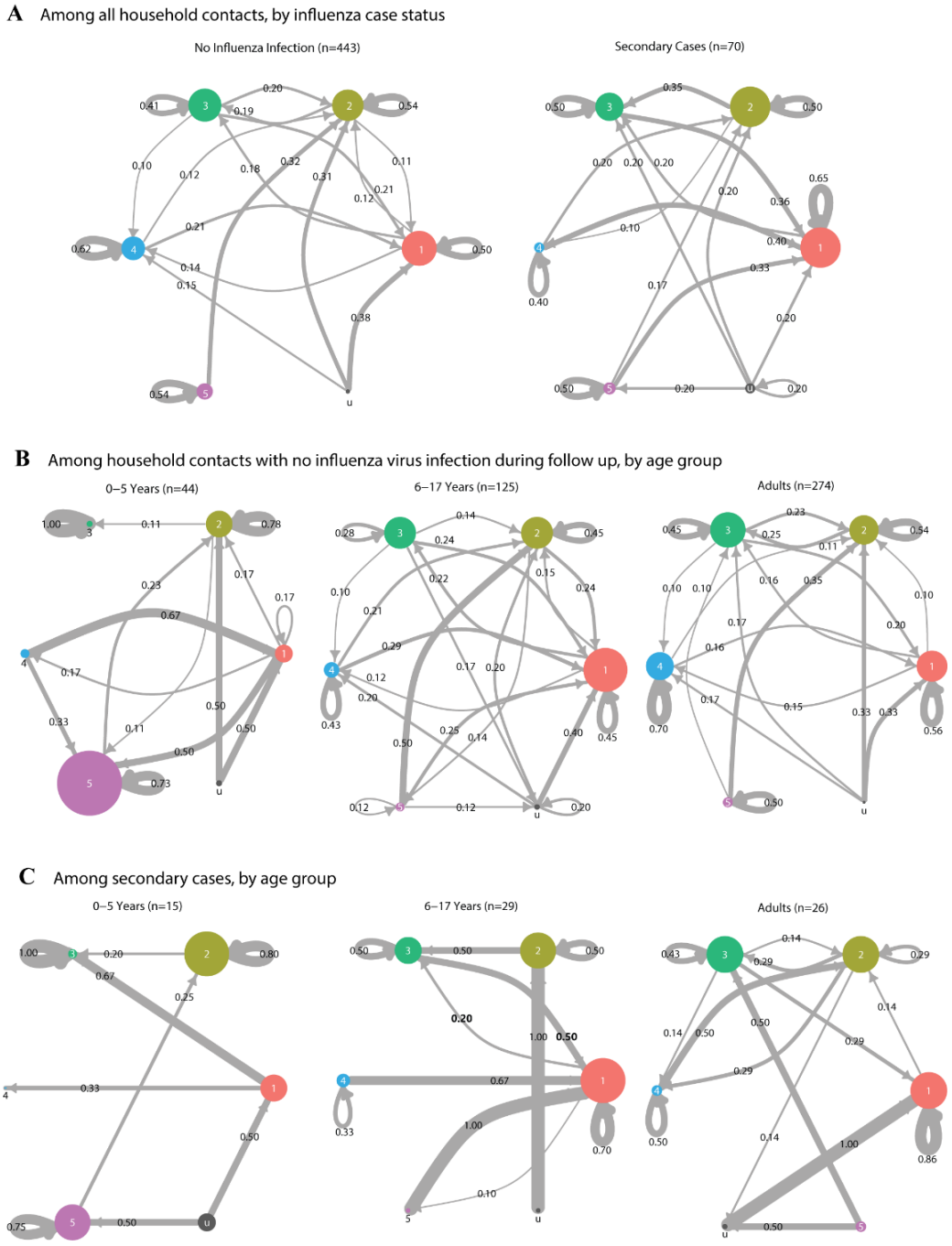
537 household contacts of influenza cases residing in 144 households in Managua, Nicaragua, 2012-2014. (A) Distribution of community types by age, time, and whether acquired influenza by time of follow-up. (B) Secondary attack rates by baseline community type and age. Numbers represent sample size for each group. (C) Generalized linear mixed effects model estimating odds of influenza virus infection after adjusting for community type (relative to community type 1), age (relative to adults), a smoker in the household, household crowding (average of >3 persons per bedroom), and clustering by household. Model additionally adjusting for undefined community type did not show any notable change in estimates (Figure 3.6).

Figure 3.6 Influenza susceptibility model, additionally adjusting for undefined community type.



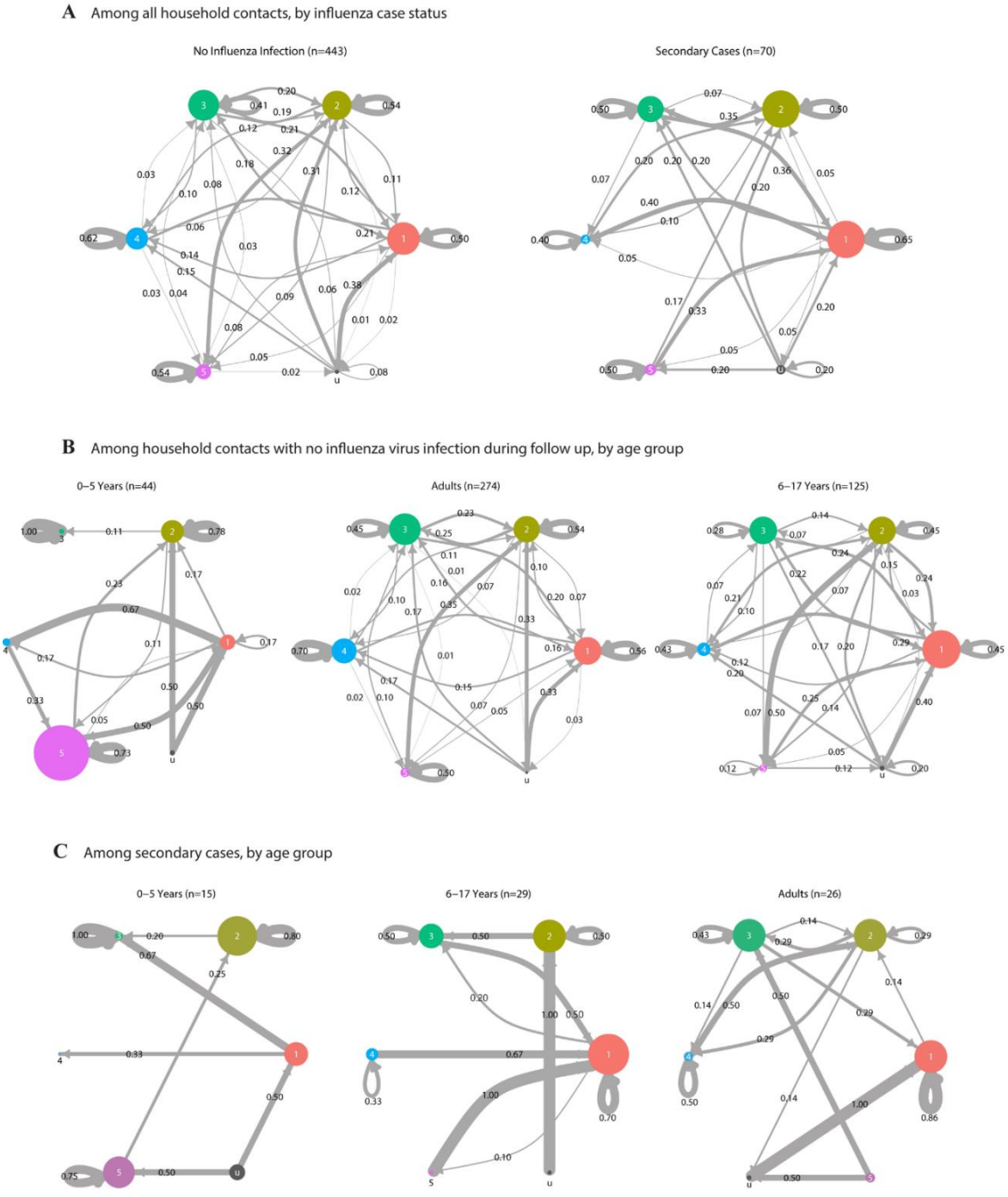
Generalized linear mixed effects model estimating odds of influenza virus infection after adjusting for community type (relative to community type 1), age (relative to adults), a smoker in the household, household crowding (average of >3 persons per bedroom), and clustering by household. 537 household contacts of influenza cases residing in 144 households in Managua, Nicaragua, 2012-2014. No notable changes in estimates compared to model excluding household contacts with an undefined community type (Figure 3.5C).

Figure 3.7 Transitions between in nose/throat bacterial community type over time among the 513 study participants with enrollment and follow up microbiota data.



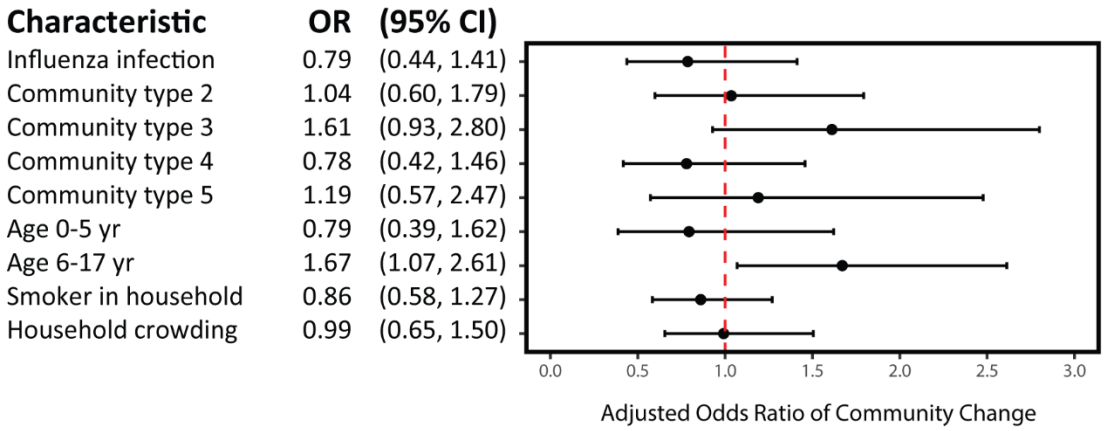
Household contacts of persons with influenza residing in 144 households in Managua, Nicaragua, 2012-2014. (A) By influenza status over follow up. (B) By age, among 443 household contacts who never developed influenza virus infection during follow up. (C) By age, among 70 secondary cases. Circles represent community types and circle size is proportional to prevalence of baseline community types. Community type u corresponds to samples with an undefined community type. Transition rates between community types were estimated as Markov chain probabilities and are shown numerically. Transitions rates <0.10 were removed for simplicity. Complete figures are available in Figure 3.8.

Figure 3.8 Change in nose/throat bacterial community type over time among the 513 study participants with complete sample pairs, all transition rates.



Household contacts of persons with influenza residing in 144 households in Managua, Nicaragua, 2012-2014. (A) By influenza status over follow up. (B) By age among 443 household contacts who never developed influenza infection during follow up. (C) By age among 70 secondary cases. Circles represent community types and circle size is proportional to prevalence of baseline community types. Community type u corresponds to undefined community type. Transition rates between community types were estimated as Markov chain probabilities and are shown numerically.

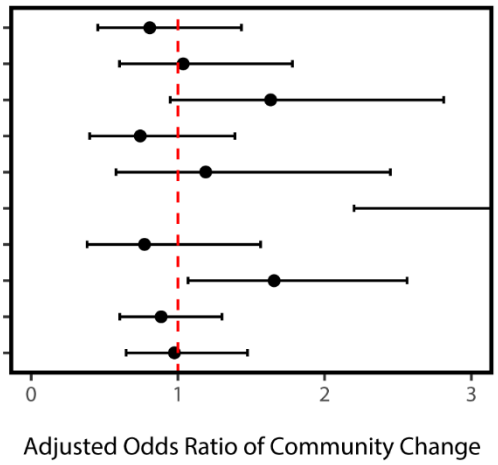
Figure 3.9 Resistance to change in community type by selected variables among the 484 study participants with defined community types at enrollment and follow up.



Household contacts of persons with influenza residing in 141 households in Managua, Nicaragua, 2012-2014. Mixed effects model estimating odds of change in community type after adjusting for influenza virus infection, baseline community type (relative to community type 1), age (relative to adults), a smoker in the household, household crowding (average of >3 persons per bedroom), and clustering by household. Household contacts with an undefined community type were excluded from analysis. Models additionally adjusting for days of follow up (Figure 3.10) and undefined community type (Figure 3.11) did not notably change point estimates.

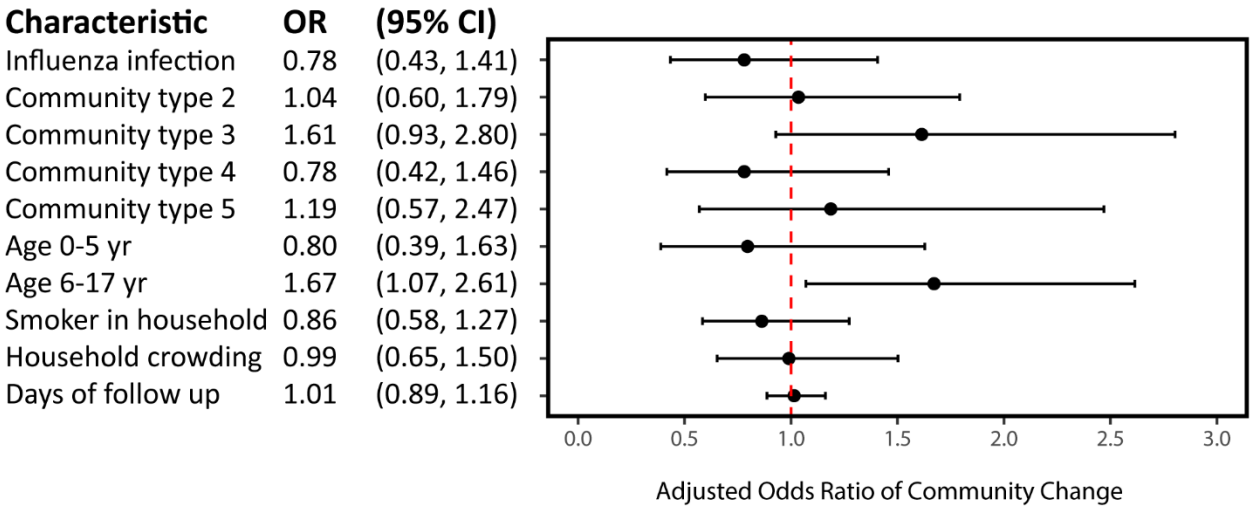
Figure 3.10 Community resistance model, additionally adjusting for undefined community type

Characteristic	OR	(95% CI)
Influenza infection	0.81	(0.50, 1.25)
Community type 2	1.04	(0.45, 1.43)
Community type 3	1.63	(0.60, 1.78)
Community type 4	0.74	(0.95, 2.81)
Community type 5	1.19	(0.40, 1.39)
Community type undefined	17.51	(2.20, 139.29)
Age 0-5 yr	0.77	(0.38, 1.56)
Age 6-17 yr	1.66	(1.07, 2.56)
Smoker in household	0.89	(0.60, 1.30)
Household crowding	0.98	(0.65, 1.47)



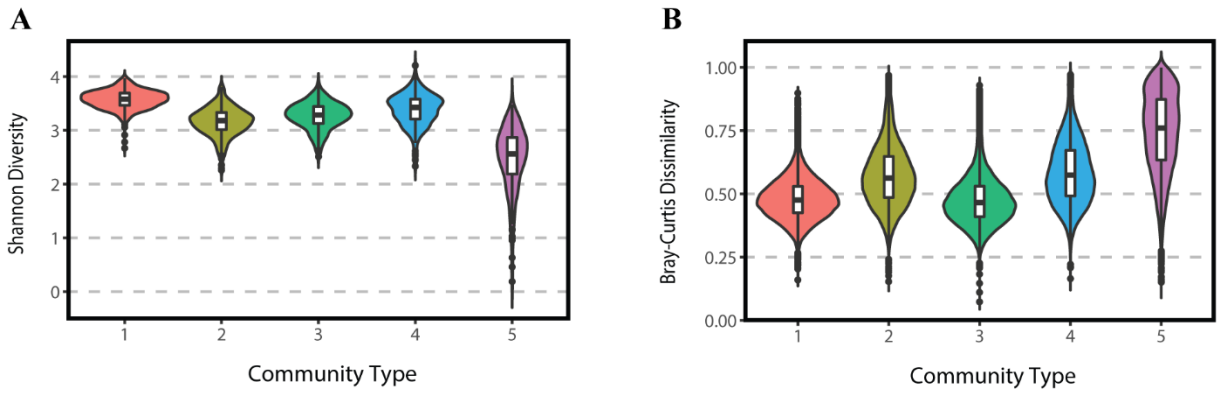
513 household contacts of persons with influenza residing in 144 households in Managua, Nicaragua, 2012-2014. Mixed effects model estimating odds of change in community type after adjusting for influenza virus infection, baseline community type (relative to community type 1), age (relative to adults), a smoker in the household, household crowding (average of >3 persons per bedroom), and clustering by household. No notable changes in estimates compared to model excluding household contacts with an undefined community type (Figure 3.9).

Figure 3.11 Community resistance model, additionally adjusting for days of follow up.



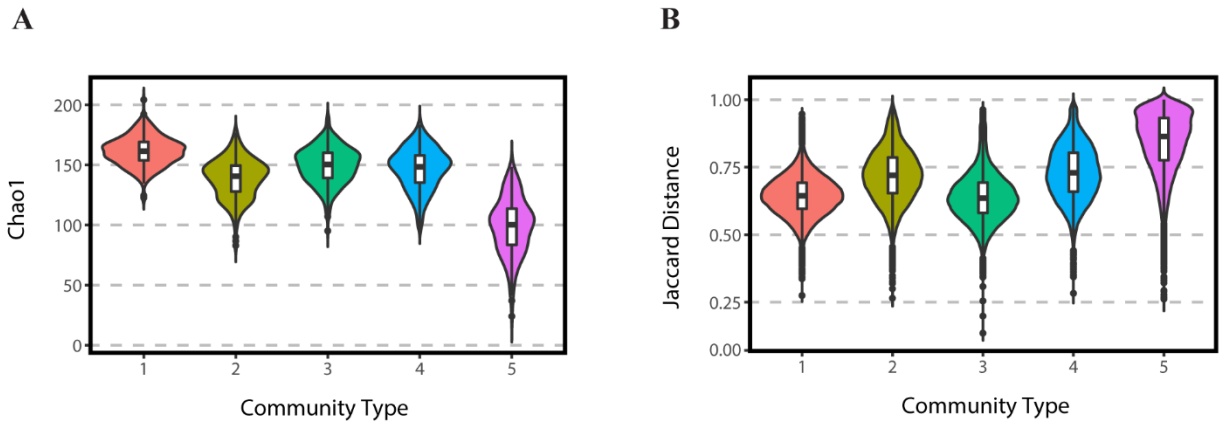
484 household contacts of persons with influenza residing in 141 households in Managua, Nicaragua, 2012-2014. Mixed effects model estimating odds of change in community type after adjusting for influenza virus infection, baseline community type (relative to community type 1), age (relative to adults), a smoker in the household, household crowding (average of >3 persons per bedroom), clustering by household, and days of follow up. Household contacts with an undefined community type were excluded from analysis. No notable changes in estimates compared to model without days of follow up (Figure 3.9).

Figure 3.12 Alpha and beta diversity and taxa composition of the nose/throat microbiome by bacterial community type.



1,405 samples from 717 study participants residing in 144 households in Managua, Nicaragua, 2012-2014. (A) Shannon diversity. (B) Bray-Curtis dissimilarity.

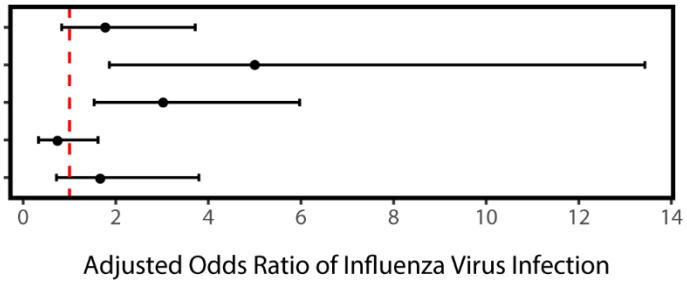
Figure 3.13 Chao 1 index and non-binary Jaccard distance of the nose/throat microbiome by bacterial community type.



1,405 samples from 717 study participants residing in 144 households in Managua, Nicaragua, 2012-2014. (A) Chao1. (B) Non-binary Jaccard distance.

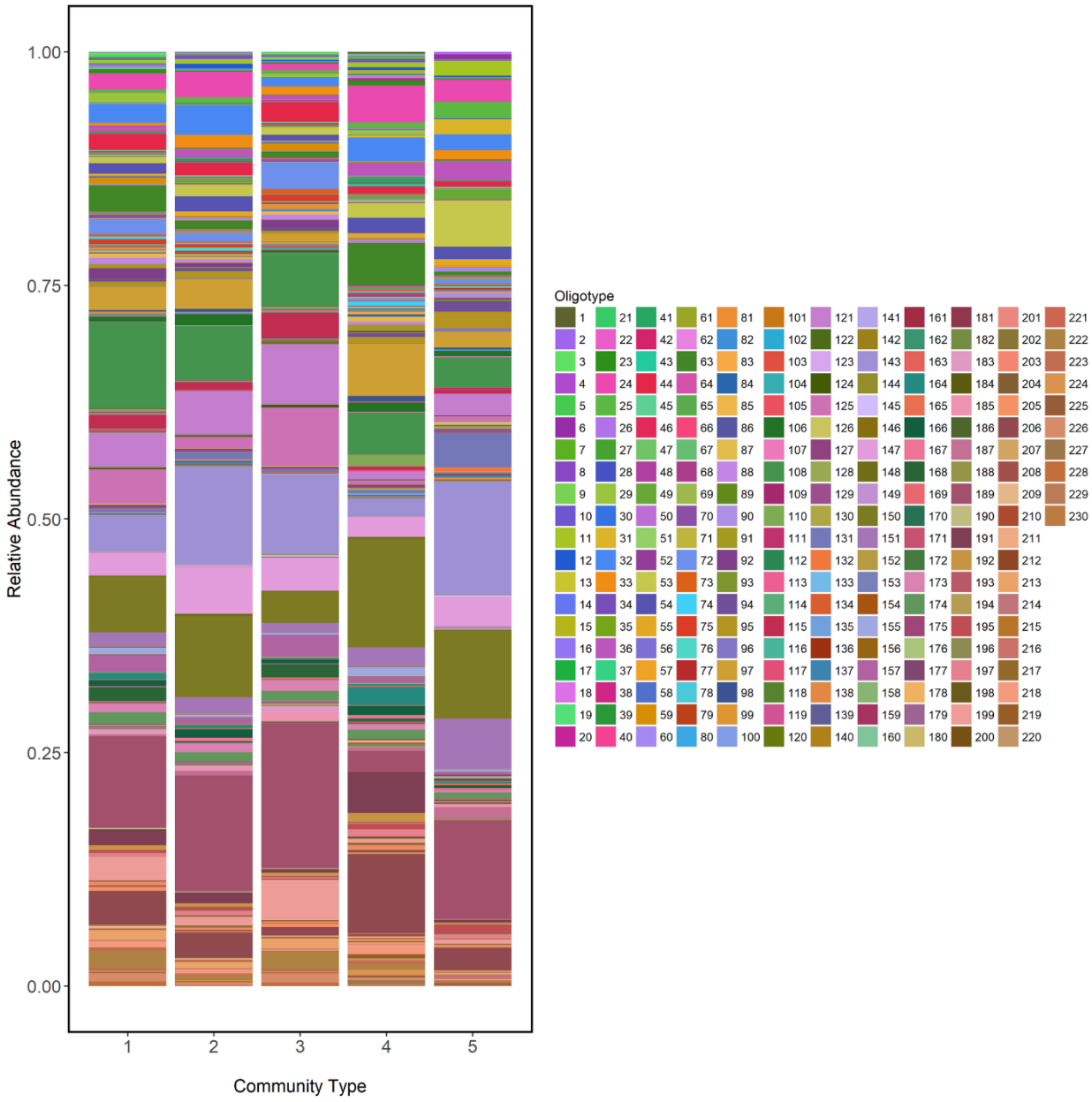
Figure 3.14 Generalized linear mixed effects model estimating odds of influenza virus infection.

Characteristic	OR	(95% CI)
Shannon diversity	1.76	(0.83, 3.72)
Age 0-5 yr	5.00	(1.86, 13.43)
Age 6-17 yr	3.02	(1.53, 5.97)
Smoker in household	0.73	(0.33, 1.62)
Household crowding	1.65	(0.72, 3.79)



Model adjusts for Shannon diversity, age (relative to adults), a smoker in the household, household crowding (average of >3 persons per bedroom), and clustering by household.

Figure 3.15 Taxa composition of community types, all oligotypes.



A total of 1,405 samples from 717 study participants residing in 144 households in Managua, Nicaragua, 2012-2014.

Legend

Oligotype	Taxonomy
1	*Filifactor alocis*
2	Unclassified
3	*Peptostreptococcaceae [XI][G-1] [Eubacterium] sulci / Peptostreptococcaceae [XI][G-1] [Eubacterium] infirmum*
4	Unclassified
5	*Oribacterium parvum / Oribacterium sinus / Oribacterium asaccharolyticum*
6	Unclassified
7	*Corynebacterium matruchotii / Corynebacterium diphtheriae*
8	*Oribacterium sinus / Oribacterium parvum*
9	*Oribacterium asaccharolyticum / Oribacterium parvum*
10	Unclassified
11	*Streptococcus pneumoniae / Streptococcus tigurinus / Streptococcus dentisani / Streptococcus sp. / Streptococcus oralis / Streptococcus mitis / Streptococcus infantis / Streptococcus peroris / Streptococcus lactarius*
12	*Streptococcus sanguinis / Streptococcus oligofermentans / Streptococcus sinensis / Streptococcus cristatus / Streptococcus australis / Streptococcus parasanguinis II / Streptococcus sp. / Streptococcus gordonii / Streptococcus parasanguinis I / Streptococcus pneumoniae / Streptococcus oralis / Streptococcus intermedius / Streptococcus mitis*
13	*Streptococcus mutans*
14	*Streptococcus vestibularis / Streptococcus salivarius*
15	*Leptotrichia sp.*
16	*Leptotrichia sp.*
17	*Leptotrichia sp.*
18	*Leptotrichia sp.*
19	*Leptotrichia sp.*
20	*Leptotrichia sp.*
21	*Bacillus subtilis*
22	*Listeria monocytogenes*
23	*Peptostreptococcus stomatis / Peptostreptococcus anaerobius*
24	*Gemella haemolysans / Gemella sanguinis / Gemella morbillorum / Gemella bergeri*
25	*Dolosigranulum pigrum*
26	*Bergeyella sp.*
27	*Leptotrichia shahii / Leptotrichia sp. / Leptotrichia hongkongensis*
28	Unclassified
29	*Alloprevotella tannerae*
30	*Alloprevotella tannerae*
31	*Rothia mucilaginosa*
32	*Rothia mucilaginosa*
33	*Rothia mucilaginosa*
34	*Rothia aeria / Rothia dentocariosa*
35	*Rothia dentocariosa / Rothia aeria*
36	Unclassified
37	Unclassified
38	*Oribacterium asaccharolyticum*
39	Unclassified
40	*Ruminococcaceae [G-1] sp.*
41	*Capnocytophaga leadbetteri / Capnocytophaga sp. / Capnocytophaga ochracea*
42	Unclassified
43	*Porphyromonas endodontalis / Porphyromonas sp.*
44	*Actinomyces graevenitzii*

45	*Actinomyces lingnae [NVP] / Actinomyces sp. / Actinomyces odontolyticus / Actinomyces meyeri / Actinomyces cardiffensis*
46	*Actinomyces lingnae [NVP] / Actinomyces sp. / Actinomyces odontolyticus / Actinomyces meyeri*
47	*Actinomyces sp. / Actinomyces oris / Actinomyces naeslundii / Actinomyces johnsonii / Actinomyces viscosus / Actinomyces radicidentis / Actinomyces meyeri*
48	*Butyrivibrio sp.*
49	Unclassified
50	*SR1 [G-1] sp.*
51	*SR1 [G-1] sp.*
52	*Abiotrophia defectiva*
53	*Staphylococcus caprae / Staphylococcus epidermidis / Staphylococcus aureus / Staphylococcus warneri*
54	*Granulicatella adiacens / Enterococcus italicus / Enterococcus faecalis*
55	*Granulicatella elegans*
56	*Lactobacillus gasseri / Lactobacillus johnsonii*
57	*Escherichia coli*
58	*Ruminococcaceae [G-2] sp.*
59	*Leptotrichia sp.*
60	*Unclassified*
61	Unclassified
62	Unclassified
63	*Fusobacterium periodonticum / Fusobacterium nucleatum subsp. animalis / Fusobacterium sp. / Fusobacterium nucleatum subsp. vincentii / Fusobacterium nucleatum subsp. polymorphum / Fusobacterium naviforme / Fusobacterium nucleatum subsp. nucleatum*
64	*Fusobacterium sp. / Fusobacterium nucleatum subsp. polymorphum / Fusobacterium nucleatum subsp. nucleatum / Fusobacterium naviforme / Fusobacterium nucleatum subsp. vincentii / Fusobacterium nucleatum subsp. animalis / Fusobacterium periodonticum*
65	*Peptococcus sp.*
66	*Fusobacterium nucleatum subsp. animalis / Fusobacterium naviforme / Fusobacterium nucleatum subsp. vincentii / Fusobacterium periodonticum / Fusobacterium sp. / Fusobacterium nucleatum subsp. nucleatum / Fusobacterium nucleatum subsp. polymorphum*
67	*Fusobacterium sp. / Fusobacterium nucleatum subsp. animalis / Fusobacterium nucleatum subsp. polymorphum / Fusobacterium nucleatum subsp. nucleatum / Fusobacterium periodonticum / Fusobacterium nucleatum subsp. vincentii / Fusobacterium naviforme*
68	*Fusobacterium periodonticum / Fusobacterium nucleatum subsp. animalis / Fusobacterium sp. / Fusobacterium nucleatum subsp. vincentii / Fusobacterium nucleatum subsp. polymorphum / Fusobacterium naviforme / Fusobacterium nucleatum subsp. nucleatum*
69	*Fusobacterium necrophorum / Fusobacterium gonidiaformans*
70	*Atopobium parvulum / Atopobium sp. / Atopobium rimae*
71	*Catonella morbi / Catonella sp.*
72	*Megasphaera micronuciformis*
73	*Lachnospiraceae [G-2] sp.*
74	*Lachnoanaerobaculum umeaense / Lachnoanaerobaculum sp.*
75	*Lachnoanaerobaculum orale / Lachnoanaerobaculum saburreum*
76	*Veillonella dispar / Veillonella atypica / Veillonella parvula / Veillonella rogosae*
77	Unclassified
78	*Treponema denticola / Treponema putidum / Treponema sp.*
79	*Veillonella dispar / Veillonella atypica / Veillonella parvula / Veillonella rogosae / Veillonella denticariosi*
80	*Selenomonas sputigena / Selenomonas sp.*
81	*Selenomonas sp.*
82	*Mitsuokella sp.*
83	*Aggregatibacter sp. / Aggregatibacter segnis / Haemophilus haemolyticus / Haemophilus sp. / Haemophilus influenzae / Haemophilus aegyptius*
84	*Haemophilus pittmaniae / Aggregatibacter sp. / Aggregatibacter aphrophilus / Aggregatibacter paraphrophilus*

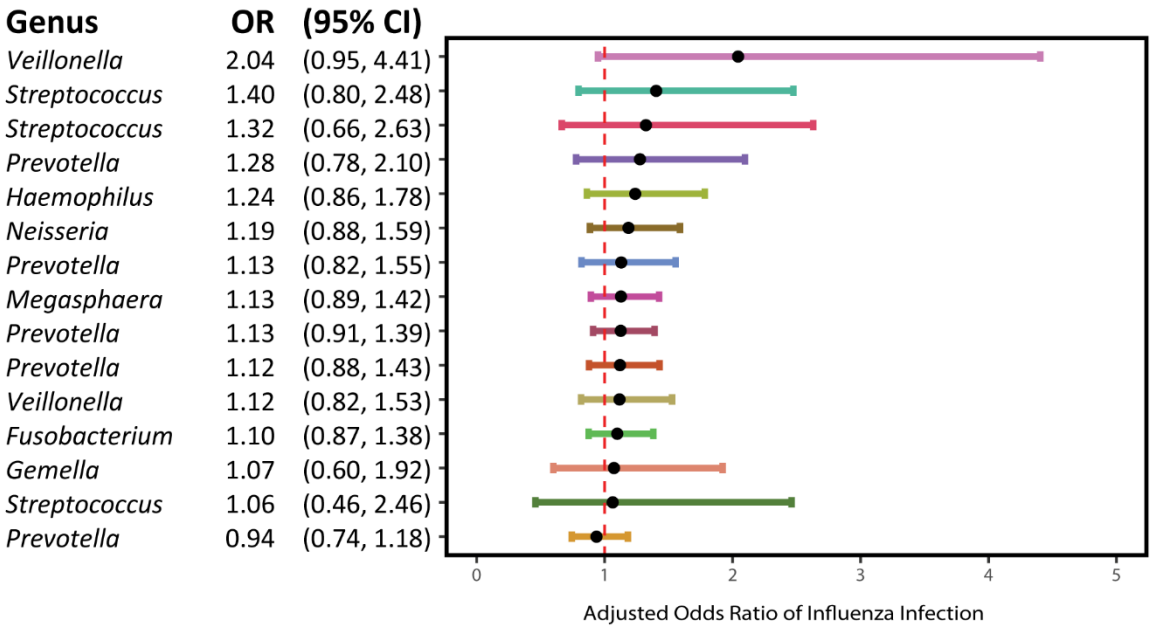
85	*Mogibacterium neglectum / Mogibacterium pumilum / Mogibacterium diversum / Mogibacterium vescum / Mogibacterium timidum*
86	Unclassified
87	*Stomatobaculum sp.*
88	*Campylobacter concisus / Campylobacter curvus*
89	*Helicobacter pylori*
90	*Pseudomonas aeruginosa / Pseudomonas otitidis / Pseudomonas sp.*
91	*Alloprevotella sp.*
92	*Alloprevotella sp.*
93	*Prevotella sp.*
94	*Haemophilus aegyptius / Haemophilus influenzae / Haemophilus sp. / Haemophilus haemolyticus / Aggregatibacter sp. / Aggregatibacter segnis*
95	*Haemophilus sp. / Haemophilus haemolyticus / Haemophilus influenzae / Haemophilus aegyptius / Aggregatibacter sp. / Aggregatibacter segnis / Haemophilus parainfluenzae*
96	*Haemophilus sp. / Haemophilus haemolyticus / Haemophilus influenzae / Haemophilus aegyptius / Aggregatibacter sp. / Aggregatibacter segnis*
97	*Haemophilus parainfluenzae / Haemophilus parahaemolyticus / Haemophilus paraphrohaemolyticus / Haemophilus sputorum / Haemophilus sp. / Haemophilus haemolyticus / Haemophilus influenzae*
98	*Haemophilus parahaemolyticus / Haemophilus sputorum / Haemophilus paraphrohaemolyticus / Haemophilus parainfluenzae*
99	*Prevotella sp. / Prevotella oulorum*
100	*Prevotella sp. / Prevotella oulorum*
101	*Prevotella oulorum / Prevotella sp.*
102	*Prevotella sp. / Prevotella oulorum*
103	*Prevotella sp. / Prevotella veroralis / Prevotella histicola / Prevotella fusca / Prevotella scopos*
104	*Prevotella veroralis / Prevotella sp. / Prevotella scopos / Prevotella fusca / Prevotella histicola / Prevotella melaninogenica*
105	*Prevotella veroralis / Prevotella sp. / Prevotella scopos / Prevotella fusca / Prevotella histicola*
106	*Neisseria pharyngis / Neisseria sicca / Neisseria mucosa / Neisseria flava / Neisseria subflava / Neisseria flavescens / Neisseria polysaccharea / Neisseria lactamica / Neisseria meningitidis / Neisseria gonorrhoeae / Neisseria oralis*
107	*Kingella denitrificans / Neisseria elongata / Neisseria weaveri / Kingella sp. / Eikenella corrodens / Eikenella sp.*
108	*Prevotella melaninogenica / Prevotella scopos / Prevotella sp. / Prevotella histicola / Prevotella veroralis*
109	*Prevotella sp. / Prevotella melaninogenica / Prevotella histicola / Prevotella veroralis / Prevotella scopos / Prevotella fusca*
110	*Prevotella melaninogenica / Prevotella scopos / Prevotella sp. / Prevotella histicola / Prevotella veroralis / Prevotella fusca*
111	*Prevotella sp. / Prevotella scopos / Prevotella histicola / Prevotella melaninogenica / Prevotella veroralis / Prevotella fusca*
112	*Prevotella melaninogenica / Prevotella sp. / Prevotella histicola / Prevotella scopos / Prevotella veroralis / Prevotella fusca*
113	*Prevotella melaninogenica / Prevotella scopos / Prevotella sp. / Prevotella histicola / Prevotella veroralis / Prevotella fusca*
114	*Prevotella histicola / Prevotella sp. / Prevotella veroralis / Prevotella fusca / Prevotella scopos / Prevotella melaninogenica*
115	*Prevotella salivae*
116	*Prevotella salivae*
117	*Prevotella sp.*
118	*Prevotella sp.*
119	*Prevotella sp. / Prevotella histicola / Prevotella veroralis / Prevotella scopos / Prevotella fusca / Prevotella melaninogenica*
120	*Prevotella sp.*
121	*Prevotella histicola / Prevotella sp. / Prevotella veroralis / Prevotella scopos / Prevotella fusca / Prevotella melaninogenica*

122	*Prevotella histicola / Prevotella scopos / Prevotella sp. / Prevotella melaninogenica / Prevotella veroralis / Prevotella fusca*
123	*Prevotella histicola / Prevotella melaninogenica / Prevotella sp. / Prevotella scopos / Prevotella veroralis*
124	*Prevotella sp. / Prevotella scopos / Prevotella veroralis / Prevotella histicola / Prevotella fusca / Prevotella melaninogenica*
125	*Prevotella sp. / Prevotella veroralis / Prevotella fusca / Prevotella histicola / Prevotella scopos / Prevotella melaninogenica*
126	*Prevotella sp. / Prevotella scopos / Prevotella veroralis / Prevotella histicola / Prevotella fusca / Prevotella melaninogenica*
127	*Prevotella sp. / Prevotella scopos / Prevotella melaninogenica / Prevotella veroralis / Prevotella histicola / Prevotella fusca*
128	*Leptotrichia sp.*
129	*Leptotrichia sp.*
130	*Capnocytophaga sputigena / Capnocytophaga sp.*
131	*Moraxella catarrhalis*
132	*Moraxella catarrhalis*
133	*Prevotella sp.*
134	*Prevotella sp.*
135	*Prevotella sp.*
136	*Porphyromonas sp.*
137	*Porphyromonas sp.*
138	*Streptococcus constellatus / Streptococcus intermedius / Streptococcus anginosus / Streptococcus gordonii / Streptococcus sp.*
139	*Streptococcus anginosus / Streptococcus constellatus / Streptococcus intermedius*
140	*Streptococcus pyogenes / Streptococcus agalactiae*
141	*Prevotella histicola / Prevotella sp. / Prevotella veroralis / Prevotella fusca / Prevotella scopos / Prevotella melaninogenica*
142	*Streptococcus sp. / Streptococcus gordonii / Streptococcus oligofermentans / Streptococcus sinensis / Streptococcus cristatus / Streptococcus parasanguinis II / Streptococcus parasanguinis I / Streptococcus australis / Streptococcus sanguinis / Streptococcus intermedius / Streptococcus salivarius / Streptococcus mitis / Streptococcus oralis*
143	*Streptococcus vestibularis / Streptococcus salivarius / Streptococcus gordonii / Streptococcus sp.*
144	*Streptococcus vestibularis / Streptococcus salivarius / Streptococcus oligofermentans / Streptococcus sinensis / Streptococcus cristatus*
145	*Streptococcus vestibularis / Streptococcus salivarius*
146	*Streptococcus vestibularis / Streptococcus salivarius*
147	*Streptococcus australis / Streptococcus parasanguinis II / Streptococcus parasanguinis I / Streptococcus sp. / Streptococcus oligofermentans / Streptococcus cristatus / Streptococcus sinensis / Streptococcus sanguinis / Streptococcus gordonii / Streptococcus lactarius / Streptococcus peroris / Streptococcus oralis*
148	*Streptococcus sinensis / Streptococcus oligofermentans / Streptococcus cristatus / Streptococcus australis / Streptococcus parasanguinis II / Streptococcus sp. / Streptococcus gordonii / Streptococcus sanguinis / Streptococcus parasanguinis I / Streptococcus oralis / Streptococcus mitis / Streptococcus infantis*
149	*Streptococcus agalactiae / Streptococcus pyogenes*
150	*Streptococcus sp. / Streptococcus dentisani / Streptococcus mitis / Streptococcus oralis / Streptococcus infantis / Streptococcus tigurinus / Streptococcus lactarius / Streptococcus peroris / Streptococcus pneumoniae*
151	*Streptococcus peroris / Streptococcus lactarius / Streptococcus sp. / Streptococcus tigurinus / Streptococcus infantis / Streptococcus dentisani / Streptococcus oralis / Streptococcus mitis*
152	*Streptococcus sp. / Streptococcus dentisani / Streptococcus mitis / Streptococcus oralis / Streptococcus infantis / Streptococcus tigurinus / Streptococcus lactarius / Streptococcus peroris / Streptococcus pneumoniae*
153	*Leptotrichia sp.*
154	*Leptotrichia sp.*
155	*Leptotrichia sp.*
156	*Leptotrichia sp.*
157	*Leptotrichia sp. / Leptotrichia wadei*
158	*Leptotrichia sp. / Leptotrichia wadei*

159	Unclassified
160	Unclassified
161	Unclassified
162	*Alloprevotella tannerae*
163	*Alloprevotella tannerae*
164	*Porphyromonas pasteri / Porphyromonas sp. / Porphyromonas catoniae*
165	*Porphyromonas sp. / Porphyromonas pasteri / Porphyromonas catoniae*
166	*Rothia mucilaginosa*
167	*Rothia mucilaginosa*
168	*Alloprevotella rava*
169	*Alloprevotella rava*
170	*Alloprevotella rava*
171	*Campylobacter rectus / Campylobacter showae / Campylobacter gracilis*
172	*Campylobacter rectus / Campylobacter showae / Campylobacter gracilis*
173	*Actinomyces sp. / Actinomyces odontolyticus / Actinomyces meyeri / Actinomyces cardiffensis / Actinomyces lingnae [NVP] / Actinomyces georgiae*
174	*Actinomyces sp. / Actinomyces odontolyticus / Actinomyces meyeri / Actinomyces cardiffensis / Actinomyces lingnae [NVP] / Actinomyces georgiae / Actinomyces gerencseriae / Actinomyces massiliensis*
175	*Enterococcus faecalis / Enterococcus durans / Enterococcus saccharolyticus / Enterococcus casseliflavus / Enterococcus italicus / Granulicatella adiacens*
176	*Bacillus anthracis / Lysinibacillus fusiformis*
177	*Capnocytophaga granulosa / Capnocytophaga sp. / Capnocytophaga gingivalis*
178	*Capnocytophaga gingivalis / Capnocytophaga granulosa / Capnocytophaga sp.*
179	*Stomatobaculum longum / Stomatobaculum sp.*
180	*Stomatobaculum longum / Stomatobaculum sp.*
181	*Fusobacterium nucleatum subsp. vincentii / Fusobacterium naviforme / Fusobacterium sp. / Fusobacterium nucleatum subsp. nucleatum / Fusobacterium nucleatum subsp. animalis / Fusobacterium nucleatum subsp. polymorphum / Fusobacterium periodonticum*
182	*Fusobacterium nucleatum subsp. vincentii / Fusobacterium naviforme / Fusobacterium nucleatum subsp. animalis / Fusobacterium nucleatum subsp. nucleatum / Fusobacterium sp. / Fusobacterium nucleatum subsp. polymorphum / Fusobacterium periodonticum*
183	*Atopobium parvulum / Atopobium rimae / Atopobium sp.*
184	*Atopobium parvulum / Atopobium rimae / Atopobium sp.*
185	*Veillonella sp.*
186	*Veillonella sp.*
187	*Veillonella sp.*
188	*Veillonella sp.*
189	*Veillonella dispar / Veillonella atypica / Veillonella parvula / Veillonella rogosae*
190	*Veillonella dispar / Veillonella atypica / Veillonella parvula*
191	*Veillonella parvula / Veillonella rogosae / Veillonella atypica / Veillonella denticariosi / Veillonella dispar*
192	*Veillonella rogosae / Veillonella parvula / Veillonella atypica / Veillonella denticariosi / Veillonella dispar*
193	*Parvimonas micra / Parvimonas sp.*
194	*Parvimonas micra / Parvimonas sp.*
195	*Alloprevotella sp.*
196	*Alloprevotella sp.*
197	*Haemophilus parahaemolyticus / Haemophilus sputorum / Haemophilus paraphrohaemolyticus / Haemophilus parainfluenzae*
198	*Haemophilus parahaemolyticus / Haemophilus sputorum / Haemophilus paraphrohaemolyticus / Haemophilus parainfluenzae*
199	*Prevotella sp. / Prevotella veroralis / Prevotella histicola / Prevotella fusca / Prevotella scopos*
200	*Prevotella sp. / Prevotella veroralis / Prevotella histicola / Prevotella fusca / Prevotella scopos*
201	*Bordetella pertussis / Achromobacter xylosoxidans*
202	*Lautropia mirabilis*

203	*Neisseria subflava / Neisseria flavescens / Neisseria flava / Neisseria sicca / Neisseria pharyngis / Neisseria mucosa / Neisseria polysaccharea / Neisseria weaveri*
204	*Neisseria subflava / Neisseria flavescens / Neisseria flava / Neisseria sicca / Neisseria pharyngis / Neisseria mucosa*
205	*Solobacterium moorei*
206	*Neisseria subflava / Neisseria flavescens / Neisseria flava / Neisseria sicca / Neisseria pharyngis / Neisseria mucosa / Neisseria polysaccharea / Neisseria weaveri / Neisseria meningitidis / Neisseria lactamica*
207	*Neisseria subflava / Neisseria flavescens / Neisseria flava / Neisseria sicca / Neisseria pharyngis / Neisseria mucosa / Neisseria polysaccharea / Neisseria weaveri*
208	*Neisseria subflava / Neisseria flavescens / Neisseria flava / Neisseria sicca / Neisseria pharyngis / Neisseria mucosa / Neisseria polysaccharea / Neisseria weaveri*
209	*Prevotella melaninogenica / Prevotella scopos / Prevotella sp. / Prevotella histicola / Prevotella veroralis / Prevotella fusca*
210	*Prevotella melaninogenica / Prevotella scopos / Prevotella sp. / Prevotella histicola / Prevotella veroralis*
211	*Prevotella oris*
212	*Prevotella denticola / Prevotella multiformis*
213	*Leptotrichia sp.*
214	*Neisseria lactamica / Neisseria sicca / Neisseria flava / Neisseria polysaccharea / Neisseria pharyngis / Neisseria mucosa / Neisseria meningitidis / Neisseria oralis / Neisseria subflava / Neisseria bacilliformis / Neisseria gonorrhoeae / Neisseria flavescens*
215	*Neisseria meningitidis / Neisseria polysaccharea / Neisseria flava / Neisseria gonorrhoeae / Neisseria sicca / Neisseria pharyngis / Neisseria mucosa / Neisseria lactamica / Neisseria flavescens / Neisseria subflava*
216	Unclassified
217	*Acinetobacter baumannii / Acinetobacter sp.*
218	*Prevotella nanceiensis*
219	Unclassified
220	*Prevotella nanceiensis*
221	*Prevotella shahii / Prevotella sp.*
222	*Prevotella pallens*
223	*Prevotella pallens*
224	*Prevotella intermedia*
225	*Prevotella nigrescens*
226	*Prevotella pallens*
227	*Prevotella aurantiaca*
228	*Prevotella aurantiaca*
229	*Prevotella aurantiaca*
230	*Prevotella aurantiaca / Prevotella pallens*

Figure 3.16 Change in relative abundance of selected oligotypes associated with influenza virus infection.



Odds ratio of 2 corresponds to a two-fold increase in odds of influenza virus infection per 10-fold increase in relative abundance. Results of separate generalized linear mixed effects models estimating the odds of influenza virus infection by selected taxa (log_{10}-transformed relative abundance). Each model is adjusted for age (relative to adults), a smoker in the household, household crowding (average of >3 persons per bedroom), and clustering by household. Colors correspond to oligotypes shown in Figure 3.4. Duplicate genus names represent different oligotypes.

3.7 References

1. World Health Organization. Influenza. Available at: http://www.who.int/immunization/topics/influenza/en/. Accessed 17 February 2017.
2. Iuliano AD, Roguski KM, Chang HH, et al. Estimates of global seasonal influenza-associated respiratory mortality: a modelling study. The Lancet **2017**; 0. Available at: http://www.thelancet.com/journals/lancet/article/PIIS0140-6736(17)33293-2/abstract. Accessed 1 March 2018.
3. Paules CI, Sullivan SG, Subbarao K, Fauci AS. Chasing Seasonal Influenza — The Need for a Universal Influenza Vaccine. N Engl J Med **2018**; 378:7–9.
4. Belongia EA, Simpson MD, King JP, et al. Variable influenza vaccine effectiveness by subtype: a systematic review and meta-analysis of test-negative design studies. Lancet Infect Dis **2016**; 16:942–951.
5. WHO Global Influenza Programme. Seasonal Influenza Vaccine Use in Low and Middle Income Countries in the Tropics and Subtropics. A systematic review. Geneva, Switzerland: World Health Organization, 2015. Available at: http://apps.who.int/iris/bitstream/10665/188785/1/9789241565097_eng.pdf.
6. Ichinohe T, Pang IK, Kumamoto Y, et al. Microbiota regulates immune defense against respiratory tract influenza A virus infection. Proc Natl Acad Sci U S A **2011**; 108:5354.
7. Abt MC, Osborne LC, Monticelli LA, et al. Commensal bacteria calibrate the activation threshold of innate antiviral immunity. Immunity **2012**; 37:158–170.
8. Levy M, Blacher E, Elinav E. Microbiome, metabolites and host immunity. Curr Opin Microbiol **2017**; 35:8–15.
9. Luoto R, Ruuskanen O, Waris M, Kalliomäki M, Salminen S, Isolauri E. Prebiotic and probiotic supplementation prevents rhinovirus infections in preterm infants: A randomized, placebo-controlled trial. J Allergy Clin Immunol **2014**; 133:405–413.
10. Panigrahi P, Parida S, Nanda NC, et al. A randomized synbiotic trial to prevent sepsis among infants in rural India. Nature **2017**; advance online publication. Available at: http://www.nature.com/nature/journal/vaop/ncurrent/full/nature23480.html?foxtrotcallback=true. Accessed 21 August 2017.
11. Russell RJ, Gamblin SJ, Skehel JJ. Influenza glycoproteins: hemagglutinin and neuraminidase. In: Webster RG, Monto AS, Braciale TJ, Lamb RA, eds. Textbook of Influenza. Wiley, 2013.
12. Lee KH, Gordon A, Foxman B. The role of respiratory viruses in the etiology of bacterial pneumonia: An ecological perspective. Evol Med Public Health **2016**; 2016:95–109.

13. de Steenhuijsen Piters WAA, Heinonen S, Hasrat R, et al. Nasopharyngeal Microbiota, Host Transcriptome, and Disease Severity in Children with Respiratory Syncytial Virus Infection. Am J Respir Crit Care Med **2016**; 194:1104–1115.

14. Lynch SV. Viruses and Microbiome Alterations. Ann Am Thorac Soc **2014**; 11:S57–S60.

15. Marks LR, Davidson BA, Knight PR, Hakansson AP. Interkingdom Signaling Induces Streptococcus pneumoniae Biofilm Dispersion and Transition from Asymptomatic Colonization to Disease. mBio **2013**; 4:e00438-13-e00438-13.

16. Hament J-M, Aerts PC, Fleer A, et al. Direct binding of respiratory syncytial virus to pneumococci: a phenomenon that enhances both pneumococcal adherence to human epithelial cells and pneumococcal invasiveness in a murine model. Pediatr Res **2005**; 58:1198–1203.

17. Salk HM, Simon WL, Lambert ND, et al. Taxa of the Nasal Microbiome Are Associated with Influenza-Specific IgA Response to Live Attenuated Influenza Vaccine. PloS One **2016**; 11:e0162803.

18. Wolter N, Tempia S, Cohen C, et al. High Nasopharyngeal Pneumococcal Density, Increased by Viral Coinfection, Is Associated With Invasive Pneumococcal Pneumonia. J Infect Dis **2014**; 210:1649–1657.

19. Shrestha S, Foxman B, Weinberger DM, Steiner C, Viboud C, Rohani P. Identifying the Interaction Between Influenza and Pneumococcal Pneumonia Using Incidence Data. Sci Transl Med **2013**; 5:191ra84-191ra84.

20. Vesa S, Kleemola M, Blomqvist S, Takala A, Kilpi T, Hovi T. Epidemiology of documented viral respiratory infections and acute otitis media in a cohort of children followed from two to twenty-four months of age. Pediatr Infect Dis J **2001**; 20:574–581.

21. Morens DM, Taubenberger JK, Fauci AS. Predominant Role of Bacterial Pneumonia as a Cause of Death in Pandemic Influenza: Implications for Pandemic Influenza Preparedness. J Infect Dis **2008**; 198:962–970.

22. de Lastours V, Malosh R, Ramadugu K, et al. Co-colonization by Streptococcus pneumoniae and Staphylococcus aureus in the throat during acute respiratory illnesses. Epidemiol Infect **2016**; :1–13.

23. Fan RR, Howard LM, Griffin MR, et al. Nasopharyngeal Pneumococcal Density and Evolution of Acute Respiratory Illnesses in Young Children, Peru, 2009-2011. Emerg Infect Dis **2016**; 22:1996–1999.

24. Rosas-Salazar C, Shilts MH, Tovchigrechko A, et al. Differences in the Nasopharyngeal Microbiome During Acute Respiratory Tract Infection With Human Rhinovirus and Respiratory Syncytial Virus in Infancy. J Infect Dis **2016**; 214:1924–1928.

25. Bogaert D, De Groot R, Hermans PWM. Streptococcus pneumoniae colonisation: the key to pneumococcal disease. Lancet Infect Dis **2004**; 4:144–154.

26. Wertheim HFL, Melles DC, Vos MC, et al. The role of nasal carriage in Staphylococcus aureus infections. Lancet Infect Dis **2005**; 5:751–762.

27. Krone CL, van de Groep K, Trzciński K, Sanders EAM, Bogaert D. Immunosenescence and pneumococcal disease: an imbalance in host-pathogen interactions. Lancet Respir Med **2014**; 2:141–153.

28. Holmes I, Harris K, Quince C. Dirichlet multinomial mixtures: generative models for microbial metagenomics. PloS One **2012**; 7:e30126.

29. Kolenbrander PE. Intergeneric Coaggregation Among Human Oral Bacteria and Ecology of Dental Plaque. Annu Rev Microbiol **1988**; 42:627–656.

30. Kolenbrander P. The Genus Veillonella. In: Dr MDP, Falkow S, Rosenberg E, Schleifer K-H, Stackebrandt E, eds. The Prokaryotes. Springer US, 2006: 1022–1040. Available at: http://link.springer.com.proxy.lib.umich.edu/referenceworkentry/10.1007/0-387-30744-3_36. Accessed 20 June 2017.

31. Iwasaki A, Pillai PS. Innate immunity to influenza virus infection. Nat Rev Immunol **2014**; 14:315–328.

32. Clarke TB, Davis KM, Lysenko ES, Zhou AY, Yu Y, Weiser JN. Recognition of Peptidoglycan from the Microbiota by Nod1 Enhances Systemic Innate Immunity. Nat Med **2010**; 16:228–231.

33. Schuijt TJ, Lankelma JM, Scicluna BP, et al. The gut microbiota plays a protective role in the host defence against pneumococcal pneumonia. Gut **2016**; 65:575–583.

34. Laufer AS, Metlay JP, Gent JF, Fennie KP, Kong Y, Pettigrew MM. Microbial communities of the upper respiratory tract and otitis media in children. mBio **2011**; 2:e00245-00210.

35. Teo SM, Mok D, Pham K, et al. The infant airway microbiome in health and disease impacts later asthma development. Cell Host Microbe **2015**; 17:704–715.

36. Zambon M. Influenza surveillance and laboratory diagnosis. In: Webster RG, Monto AS, Braciale TJ, Lamb RA, eds. Textbook of Influenza. Wiley, 2013.

37. Jefferson T, Jones M, Doshi P, Spencer EA, Onakpoya I, Heneghan CJ. Oseltamivir for influenza in adults and children: systematic review of clinical study reports and summary of regulatory comments. BMJ **2014**; 348:g2545.

38. Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team, Dawood FS, Jain S, et al. Emergence of a novel swine-origin influenza A (H1N1) virus in humans. N Engl J Med **2009**; 360:2605–2615.

39. Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. Development of a Dual-Index Sequencing Strategy and Curation Pipeline for Analyzing Amplicon Sequence Data on the MiSeq Illumina Sequencing Platform. Appl Environ Microbiol **2013**; 79:5112–5120.

40. Caporaso JG, Lauber CL, Walters WA, et al. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proc Natl Acad Sci **2011**; 108:4516–4522.

41. Schloss PD, Westcott SL, Ryabin T, et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol **2009**; 75:7537–7541.

42. Berry MA, White JD, Davis TW, et al. Are Oligotypes Meaningful Ecological and Phylogenetic Units? A Case Study of Microcystis in Freshwater Lakes. Front Microbiol **2017**; 8:365.

43. Eren AM, Morrison HG, Lescault PJ, Reveillaud J, Vineis JH, Sogin ML. Minimum entropy decomposition: unsupervised oligotyping for sensitive partitioning of high-throughput marker gene sequences. ISME J **2015**; 9:968–979.

44. Eren AM, Zozaya M, Taylor CM, Dowd SE, Martin DH, Ferris MJ. Exploring the Diversity of Gardnerella vaginalis in the Genitourinary Tract Microbiota of Monogamous Couples Through Subtle Nucleotide Variation. PLOS ONE **2011**; 6:e26732.

45. Eren AM, Borisy GG, Huse SM, Mark Welch JL. Oligotyping analysis of the human oral microbiome. Proc Natl Acad Sci U S A **2014**; 111:E2875–E2884.

46. Eren AM, Sogin ML, Morrison HG, et al. A single genus in the gut microbiome reflects host preference and specificity. ISME J **2015**; 9:90–100.

47. Chen T, Yu W-H, Izard J, Baranova OV, Lakshmanan A, Dewhirst FE. The Human Oral Microbiome Database: a web accessible resource for investigating oral microbe taxonomic and genomic information. Database J Biol Databases Curation **2010**; 2010:baq013.

48. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol **1990**; 215:403–410.

49. R Core Team. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing, 2017. Available at: https://www.R-project.org/.

50. Morgan M. DirichletMultinomial: Dirichlet-Multinomial Mixture Model Machine Learning for Microbiome Data. 2017.

51. Aitchison J. The Statistical Analysis of Compositional Data. J R Stat Soc Ser B Methodol **1982**; 44:139–177.

52. Bates D, Maechler M, Bolker B, Walker S. Fitting Linear Mixed-Effects Models Using lme4. J Stat Softw **2015**; 67:1–48.

53. DiGiulio DB, Callahan BJ, McMurdie PJ, et al. Temporal and spatial variation of the human microbiota during pregnancy. Proc Natl Acad Sci U S A **2015**; 112:11060–11065.

Chapter 4 The Respiratory Microbiota on Influenza Symptomology and Viral Shedding

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4.1 Author Summary

Influenza virus infection varies considerably in symptomology and viral shedding. We found influenza symptom duration, viral shedding duration, and time to infection was significantly associated with differences in the nose/throat microbiota prior to infection.

4.2 Abstract

The aim of this study was to examine whether the nose/throat microbiota influences influenza symptoms and shedding. Exploring this relationship could lead to alternative methods for reducing influenza severity and transmission. Household index cases of influenza virus infection were identified at a primary healthcare center. An index case was defined as the first household member with symptom onset of a febrile acute respiratory infection and detectable influenza virus. Household contacts of index cases were followed for up to 13 days. A secondary case was defined as a household contact with detectable influenza virus or a >4-fold change in hemagglutinin inhibition antibody titers. We characterized the nose/throat microbiota of secondary cases at time of enrollment and compared symptomology and viral shedding by bacterial community types. We identified 124 secondary cases of influenza. The durations of fever and

cough varied by bacterial community type. Further, a community type with low diversity was associated with shorter duration of viral shedding and delayed time to infection. We demonstrate influenza symptomology and viral shedding are associated with differences in the nose/throat microbiota. Further work is needed to identify underlying factors that contribute to the associations we observed at the community type level.

4.3 Introduction

The clinical manifestation of influenza virus infection varies considerably, ranging from asymptomatic infections to severe illness and death [1]. Among the estimated 90 million new cases of influenza that occurred in young children in 2008, 20 million had acute respiratory infections (ARI), 1 million had severe ARIs, and 28,000-111,500 cases resulted in death [2]. Influenza cases also vary in degrees of viral shedding [3–6], which likely reflects infectiousness [3]. A meta-analysis of challenge studies estimated the average duration of shedding among young adults to be around 5 days after inoculation [3]. However, viral shedding is often undetected during infections [3,4] and longer durations of shedding have been observed in more symptomatic cases [7] and in young children [4,8].

This heterogeneity in influenza illness and infectiousness is largely attributed to the host immune response, which impacts pathogenicity and viral replication. Increasingly, the microbiome is recognized as an important mediator of host immunity [9–12], spurring epidemiologic studies to examine whether the microbiome impacts the risk and severity of infectious diseases [13–15]. To our knowledge, no epidemiologic study has examined whether the microbiome impacts symptoms or viral shedding during influenza virus infection. Identifying these links would lay

groundwork for synbiotic approaches to reducing influenza severity and transmission. Here, we aim to fill this gap using data from a household transmission study in Nicaragua.

4.4 Methods

4.4.1 Study Population

This analysis uses data and samples collected from the Nicaraguan Household Transmission Study was conducted in Managua, Nicaragua, between 2012-2014. Household index cases of influenza virus infection were identified at a primary healthcare center using the following criteria: 1) a positive QuickVue Influenza A + B rapid diagnostic test, 2) symptom onset of an febrile acute respiratory infection (FARI; fever or feverishness with a rhinorrhea, sore throat, and/or cough) within the past 48 hours, 3) residing in a household with at least one other member (household contact), and 4) no household contacts with influenza symptoms in the two weeks prior to symptom onset in the index case.

Index cases and household contacts were invited for study participation and monitored through up to 5 home visits, conducted at 2-3 day intervals. Nasal and oropharyngeal swabs were collected and combined at each visit. Blood samples were collected at enrollment and 30-45 days later. A secondary case was defined as a household contact with a positive real-time reverse transcription polymerase chain reaction (RT-PCR) result or a >4-fold change in hemagglutination inhibition (HAI) antibody titers specific to the subtype/type identified in the index case.

A written informed consent or proxy consent was obtained for all participants. Verbal assent was obtained from children ≥5 years. The study was approved by the Institutional Review Boards at the University of Michigan and the Nicaraguan Ministry of Health.

4.4.2 Laboratory Assays

Influenza type/subtype-specific RT-PCR was conducted on all samples using validated Centers for Disease Control and Prevention protocols [16]. Influenza type/subtype-specific HAI titers were measured using validated World Health Organization protocols [17].

4.4.3 Microbiota Characterization

Detailed methods used for microbiota characterization are available in Appendix 4.1. Briefly, DNA was extracted from the first and last nasal/oropharyngeal sample collected from all index cases and household contacts. The V4 hypervariable region of the 16S rRNA gene was amplified and sequenced on an Illumina MiSeq System using a validated dual-indexing method [18]. Following alignment and quality filtering in mothur v1.38.1 [19] and oligotyping to assign reads to taxonomic units [20], Dirichlet multinomial mixture models [21] were used to assign all nasal/oropharyngeal samples to 5 bacterial community types (Figure 4.1). Each community type represents a group of samples with similar compositions. We determined the number of community types by estimating the Laplace approximation of the negative log models and identifying the point at which an increase in Dirichlet components resulted in minor reductions in model fit (Figure 4.2). Taxonomy was assigned using the Human Oral Microbiome Database v14.51 [22] and blastn v2.2.23 [23].

We estimated the diversity of community types using data on samples from all study participants (n=1,405 samples). β-diversity, representing within-group dissimilarity of samples, was estimated using Bray-Curtis dissimilarity and Jaccard distance. α-diversity, representing within-sample community diversity, was estimated using Shannon diversity index and Chao1

index. Shannon diversity accounts for both richness and evenness of taxa while Chao1 only accounts for richness.

4.4.4 Influenza Shedding and Symptom Data

Household contacts with ≥1 positive RT-PCR result during follow were defined as secondary cases with viral shedding. Shedding duration was estimated as the time between the first positive RT-PCR result and a negative RT-PCR result.

Study participants completed a daily symptom diary documenting the presence of the following symptoms: fever or feverishness, rhinorrhea, sore throat, and cough. To reduce potential bias from symptoms unrelated to influenza virus infection, we defined an influenza-associated illness period for each participant using symptom onset and alleviation dates. Illness onset was defined as the earliest date of any symptom. However, symptoms were excluded if they were alleviated >1 day prior to onset of viral shedding. Illness alleviation was defined as the date on which all symptoms were alleviated. Any recurring symptoms were excluded if the symptom recurred ≥3 days after viral shedding cessation or if fever recurred ≥3 days after fever alleviation. The duration of each symptom was estimated within the defined illness period. Febrile acute respiratory illness (FARI) was defined as the presence of fever plus rhinorrhea, sore throat, and/or cough and influenza-like illness (ILI) was defined as fever plus sore throat and/or cough.

4.4.5 Statistical Analysis

Generalized linear mixed effects models were used to examine the association between nasal/oropharyngeal bacterial community types and the presentation of symptoms and presentation of viral shedding among secondary cases, after accounting for clustering by household. Clustered

accelerated failure time (AFT) models using a generalized estimating equation (GEE) approach were used to examine the relationship between community types and symptom duration, viral shedding duration, the serial interval (defined as time between onset of symptoms between an index case and a secondary case), and time to shedding onset. Time to shedding onset was based on symptom onset dates of index cases. Survival time was parameterized as a Weibull distribution in all AFT models [24].

For any statistically significant effect estimates observed at the community type level, we further explored whether outcomes were associated with the relative abundance of 15 oligotypes that contributed to >50% of the difference between community types. We ran single-oligotype models using $\log_{10}$-transformed relative abundance in consideration of the constant sum constraint [25] and the Benjamin-Hochberg method to correct for multiple testing. We also reran models using α-diversity metrics to explore whether community diversity contributed to associations at the community type level.

We adjusted for age in models estimating viral shedding outcomes and adjusted for age and sex in models estimating symptom outcomes. We adjusted for age, a smoker in the household, and household crowding for our models estimating time to shedding onset. We additionally adjusted for sex for our model estimating the serial interval. A summary of our models is available in Table 4.1. All statistical analyses were conducted using R version 3.4.2 [26].

4.5 Results

4.5.1 Study population

A total of 144 index cases and 573 household contacts were enrolled in the Nicaraguan Household Transmission Study during 2012-2014. 160 secondary infections were identified by

RT-PCR over a ≤13-day follow up period or a ≥4-fold increase in HAI titer specific to the influenza type/subtype of the household index case 30-45 days after enrollment. Analysis was conducted on 124 secondary cases after excluding 36 household contacts with a positive RT-PCR result at the first home visit. Among secondary cases, 71 were positive for influenza by RT-PCR (57%) and 92 (74%) were positive by HAI during follow up.

We assigned 1,405 nose/throat samples from all study participants to 5 bacterial community types. Bray-Curtis dissimilarity and Shannon diversity of community types were significantly different between all community types ($p<0.001$). However, community type 5 was the most heterogeneous type and had the lowest community diversity compared to all other community types (Figures 4.3A & 4.3B). Results were similar when using Jaccard distance and Chao1 as alternative diversity metrics (Figure 4.4). The taxa composition of each community type, renormalized to 15 oligotypes that contributed to >50% of the difference between community types, is depicted in Figure 4.3C. The complete taxa composition is available in Figure 4.5.

Half of all secondary cases were adults (48%) and most infections were symptomatic (61%) (Table 4.2). Thirty-six secondary cases experienced FARIs (29%), including 34 with ILI (27%). 41% of households had more than 1 secondary case, suggesting clustering of secondary cases by household. Compared to secondary infections without viral shedding (n=53), most secondary infections with viral shedding (n=71) were among younger household contacts (mean: 16.7 years vs. 25.2 years, t-test, $p=0.001$), more likely to be symptomatic (75% vs. 43% with ≥1 symptom, $\chi 2$ test, $p<0.001$), and more likely to result in FARI (42% vs. 6%, $\chi 2$ test, $p<0.001$).

4.5.2 Bacterial community prior to infection on symptomology and viral shedding

The duration of symptoms differed significantly by community type among secondary cases, after adjusting for age, sex, and clustering by household. Cough persisted longer for community type 2 (AF: 1.23; 95% CI: 1.15, 4.32), community type 3 (AF: 1.34; 95% CI: 1.26, 4.35), and community type 4 (AF: 1.71; 95% CI: 1.42, 5.17) compared to community type 1 (Figure 4.6). Further, fever persisted longer for community type 3 (AF: 1.75; 95% CI: 1.27, 5.97) and community type 4 (AF: 1.76, 95% CI: 1.04, 2.98). Although not statistically significant, we also observed longer fever for community type 2 (AF: 1.46; 95% CI: 0.93, 2.30). We found no associations between community types and the duration of rhinorrhea or sore throat. An alternate interpretation of these results is that the durations of cough and fever were attenuated among secondary cases with community type 1. We did not find any significant associations between community types and the presentation of symptoms, after adjusting for sex, age, and clustering by household (Table 4.3). However, we observed notably larger odds of FARI (adjusted odds ratio (OR): 4.12; 95% CI: 0.86, 19.74) and cough (OR: 3.67; 95% CI: 0.91, 14.78).

Shedding duration was 47% shorter among secondary cases with community type 5 (AF: 0.53; 95% CI: 0.32, 0.90), after adjusting for age and clustering by household (Figure 4.6). We found no associations between community types and the presence of viral shedding (Table 4.3).

4.5.3 Bacterial community prior to infection on time to infection

We examined whether community types were associated with time to infection using two different proxy measures, serial interval and time to shedding onset, after adjusting for age, sex, a smoker in the household, household crowding, and clustering by household. Compared to community type 1, the serial interval was longer among secondary cases with community type 5

(AF: 1.85; 95% CI: 1.03, 3.34) (Figure 4.7). Further, shedding onset was delayed among secondary cases with community type 5 (AF: 1.43; 95% CI: 1.01, 2.02).

4.5.4 The role of community diversity

We explored whether α-diversity influenced associations at the community type level. We found no associations between community diversity and symptom durations (Table 4.4). However, Shannon diversity was associated with longer shedding duration (AF: 1.62; 95% CI: 1.25, 2.10) and Chao1 was associated with earlier time to shedding onset (AF: 0.995; 95% CI: 0.990, 0.998). The serial interval was negatively associated with Shannon diversity (AF: 0.72; 95% CI: 0.53, 0.97) and Chao1 (AF: 0.992; 95% CI: 0.986, 0.998). These results support associations found with community type 5, which was substantially less diverse than other community types.

4.5.5 The role of individual taxa

To explore the role of individual taxa on associations at the community type level, we examined whether the relative abundance of select oligotypes impacted symptomology and viral shedding. We considered the 15 oligotypes that contributed to >50% of the difference between community types and used the Benjamin-Hochberg method to correct for multiple testing (Table 4.5).

The duration of fever was negatively associated with *Veillonella parvula / rogosae / atypica / denticariosi / dispar* (AF: 0.66; 95% CI: 0.50, 0.86). Shedding duration was positively associated with the abundance *of Fusobacterium* (AF: 1.14; 95% CI: 7%, 22%), *Neisseria* (AF: 1.16; 95% CI: 1.06, 1.27), and *Haemophilus* (AF: 1.13; 95% CI: 1.04, 1.23). Shedding duration was negatively associated with the abundance of *Streptococcus vestibularis / salivarius / gordonii*

/ sp (AF: 0.61; 95% CI: 0.48, 0.77). *Fusobacterium* (AF: 0.89; 95% CI: 0.83, 0.95) and *Neisseria* (AF: 0.87; 95% CI: 0.79, 0.95) were also associated with a shorter serial interval.

4.5.6 Sensitivity analysis

To investigate whether the criteria used to define illness periods affected our results, we reran our models with three sets of modified criteria: illness period does not exclude symptoms if fever recurs ≥3 days after fever alleviation, illness period only considers ILI symptoms, and all symptoms during follow up contribute to illness period. Model estimates either remained the same or there were minor differences that did not affect our overall conclusions (Tables 4.6-4.8).

4.6 Discussion

We explored whether the nose/throat microbiota influenced symptomology and viral shedding among secondary cases identified by RT-PCR or >4-fold increase in HAI titers. We found the bacterial community structure prior to influenza virus infection is associated with the duration of symptoms and duration of viral shedding. We also examined whether the bacterial community structure predicted earlier infection by considering the serial interval and time to shedding onset. We found secondary cases with a less diverse community type become infected earlier. Interestingly, secondary cases with this community type also had a shorter period of viral shedding. In addition to low diversity, these associations may also be driven by the abundance of specific oligotypes, such as *Fusobacterium* and *Neisseria*. Further investigations are needed to explore factors that may contribute to the associations we observed at the community type level. Metagenomics and metabolomic screening could assess functional differences between community types.

Murine experiments support our findings of a relationship between the microbiome and influenza symptoms and viral shedding. Mice treated with antibiotics prior to inoculation with influenza virus expressed enhanced disease severity and increased risk of death [12]. Among mice with microbiomes disrupted by antibiotics, macrophages expressed defective responses to type I and type II IFNs [12] and exhibited defective T-cell and B-cell responses linked to reduced priming of inflammasome-dependent cytokines [10]. These impairments resulted in higher viral replication [10,12]. However, these studies did not characterize the microbiota using an untargeted 16S rRNA taxonomic screen, making it difficult to connect our epidemiologic findings with specific biological mechanisms. One mechanism of protection described in mice involves Toll-like receptor 2 signaling by *Staphylococcus aureus*, which results in the recruitment of alveolar macrophages [27]. In our study, *Staphylococcus* represented only 1 of 230 total oligotypes and contributed <0.02% of the relative abundance in each community type. This suggests our finding were independent of *Staphylococcus*. Future studies should examine both aspects to better characterize the relationship between the host microbiome, host immunity, and influenza virus infection.

Our study has several potential limitations. First, any criteria used to define an influenza-associated illness period is subject to misclassification. However, sensitivity analysis indicates our criteria did not meaningfully impact our results. Second, our estimates for serial intervals and time to shedding onset assume the index case is the source of infection. Mathematical models [28] that allow secondary cases to contribute to transmission should be used in more adequately powered studies. Third, viral shedding may influence symptomology [7]. However, we were inadequately powered to examine the relationship between the microbiota and symptomology among secondary cases by shedding status. As the primary aim of this study was to explore whether differences in

the respiratory microbiota influenced symptomology and viral shedding, we did not control for multiple testing by symptom. Lastly, due to limited sample size we were unable to examine potential differences by influenza type.

In conclusion, our study demonstrates influenza symptomology and viral shedding may be influenced by differences in the nose/throat bacterial community structure. By extension, the microbiota may influence influenza transmission, which is likely dependent on both the duration and level of viral shedding as well as the presence of symptoms. Current methods for reducing influenza transmission and disease severity involve reducing exposure to the virus, vaccination, and antiviral treatment. However, supplementary strategies should be explored to reduce the 3-5 million cases of severe illness [29] and 400,000 deaths [30] estimated to occur each year. Randomized synbiotic studies have shown drastic reductions in respiratory tract infections [13,14]. As synbiotic trials are limited to newborns, more work is needed to understand their impact among different age groups and communities. Nevertheless, with week-long costs of synbiotic treatment estimated to be around $1 per person [13], synbiotics may be a simple, cost-efficient means for reducing influenza virus transmission and the burden of disease, especially in low- and middle-income countries. In summary, our findings contribute to the growing support for the role of the microbiome on human health and the potential for microbiome-related strategies in reducing the global burden of infectious diseases.

Table 4.1 Summary of models used to investigate the relationship between bacterial community types and various symptom and viral shedding outcomes.

Model	Type	Community type	Age	Sex	Smoker in household	Crowding
Symptom presentation	GLME	Yes	Yes	Yes	No	No
Symptom duration	GEE AFT	Yes	Yes	Yes	No	No
Shedding duration	GEE AFT	Yes	Yes	No	Yes	Yes
Serial interval	GEE AFT	Yes	Yes	Yes	Yes	Yes
Time to shedding onset	GEE AFT	Yes	Yes	No	Yes	Yes

Abbreviations: GLME, generalized linear mixed effects; GEE, generalized estimating equation; AFT, accelerated failure time.

Models additionally controlled for clustering by household and are not specific to influenza type/subtype. Columns to the right of the bold vertical line indicate independent variables included in the model. Models with statistically significant associations at the community type level were rerun using $\log_{10}$-transformed relative abundance of 15 oligotypes that contributed to 50% of difference between community types. Models were also rerun using α-diversity metrics.

Table 4.2 Characteristics of 124 secondary influenza cases from 70 households, Managua, Nicaragua, 2012-2014, by bacterial community type.

Characteristics	All (n=124[a])	Community Type 1 (n=35)	Community Type 2 (n=31)	Community Type 3 (n=30)	Community Type 4 (n=14)	Community Type 5 (n=7)
	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)
Influenza type/subtype (RT-PCR)						
H1N1	12 (10)	2 (6)	3 (10)	2 (7)	4 (29)	0 (0)
H3N2	37 (30)	12 (34)	9 (29)	9 (30)	0 (0)	5 (71)
B	21 (17)	6 (17)	7 (23)	4 (13)	1 (7)	1 (14)
Co-infection	1 (1)	0 (0)	1 (3)	0 (0)	0 (0)	0 (0)
None	53 (43)	15 (43)	11 (35)	15 (50)	9 (64)	1 (14)
Influenza type/subtype (HAI)						
H1N1	18 (15)	4 (11)	5 (16)	4 (13)	4 (29)	0 (0)
H3N2	48 (39)	13 (43)	8 (26)	15 (50)	6 (43)	1 (0)
B	26 (21)	6 (17)	8 (26)	7 (23)	3 (21)	1 (14)
Co-infection	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
None	23 (19)	7 (20)	7 (23)	4 (13)	1 (7)	2 (29)
Missing	9 (7)	3 (9)	3 (10)	0 (0)	0 (0)	3 (43)
Age (years)						
0-5	19 (15)	5 (14)	6 (19)	1 (3)	0 (0)	5 (71)
6-17	45 (36)	16 (46)	10 (32)	13 (43)	4 (29)	1 (14)
≥18	60 (48)	14 (40)	15 (48)	16 (53)	10 (71)	1 (14)
Female	80 (65)	20 (57)	23 (74)	19 (63)	10 (71)	4 (57)
Influenza vaccination[b]	6 (5)	1 (3)	3 (10)	2 (7)	0 (0)	0 (0)
Smoker in household	59 (54)	16 (52)	14 (52)	17 (61)	6 (55)	4 (57)
Number of symptoms						
0	48 (39)	12 (34)	12 (39)	13 (43)	4 (29)	3 (43)
1	20 (16)	10 (29)	2 (6)	6 (20)	2 (14)	0 (0)
2	16 (13)	5 (14)	3 (10)	5 (17)	0 (0)	2 (29)
3	20 (16)	5 (14)	6 (19)	2 (7)	6 (43)	1 (14)
4	20 (16)	3 (9)	8 (29)	4 (13)	2 (14)	1 (14)
FARI[c]	36 (29)	7 (20)	11 (35)	6 (20)	6 (43)	3 (43)
ILI[d]	34 (27)	7 (20)	11 (35)	6 (20)	6 (43)	2 (29)
Symptoms						
Fever	44 (35)	11 (31)	12 (39)	8 (27)	7 (50)	3 (43)
Rhinorrhea	53 (43)	14 (40)	16 (52)	10 (33)	7 (50)	3 (43)
Sore throat	35 (28)	8 (23)	12 (39)	6 (20)	5 (36)	2 (29)
Cough	60 (48)	14 (40)	18 (58)	14 (47)	9 (64)	3 (43)

Abbreviations: RT-PCR, real-time reverse transcription polymerase chain reaction; HAI, hemagglutination inhibition; ARI, acute respiratory infection; ILI, influenza-like illness.

[a]Secondary cases were defined as household contacts of index cases with a positive RT-PCR result for influenza or >4-fold change in HAI titer during follow up.

Includes secondary cases with undefined community types

[b]Prior to enrollment and in same year as index case

[c]Fever/feverishness with rhinorrhea, sore throat or cough

[d]Fever/feverishness with sore throat or cough

Table 4.3 Generalized linear mixed effects models examining associations between bacterial community types and the development of symptoms and viral shedding.

Model	Community Type 2	Community Type 3	Community Type 4	Community Type 5	Age 0-5 Years	Age 6-17 Years	Female
			Odd Ratio (95% Confidence Interval)				
≥1 symptom	0.57 (0.13, 2.57)	0.62 (0.16, 2.45)	1.83 (0.30, 11.40)	0.18 (0.01, 3.03)	6.36 (0.74, 55.04)	2.04 (0.60, 6.93)	1.48 (0.45, 4.84)
FARI	2.31 (0.63, 8.45)	1.21 (0.31, 4.74)	4.12 (0.86, 19.74)	1.84 (0.23, 14.62)	3.70 (0.65, 21.06)	2.06 (0.68, 6.23)	2.06 (0.68, 5.71)
Fever	1.32 (0.45, 3.82)	0.84 (0.27, 2.58)	2.59 (0.67, 9.45)	1.28 (0.19, 8.71)	2.26 (0.54, 9.45)	2.12 (0.81, 5.55)	1.99 (0.76, 5.20)
Rhinorrhea	1.52 (0.49, 4.71)	0.94 (0.30, 2.92)	2.09 (0.51, 8.52)	0.32 (0.03, 3.15)	8.65 (1.38, 54.37)	0.75 (0.17, 3.33)	1.37 (0.35, 5.45)
Sore throat	2.32 (0.67, 7.97)	0.82 (0.23, 2.92)	1.87 (0.43, 8.16)	1.65 (0.19, 14.32)	0.81 (0.17, 3.92)	1.31 (0.46, 3.74)	1.69 (0.59, 4.79)
Cough	2.06 (0.72, 5.87)	1.52 (0.53, 4.34)	3.67 (0.91, 14.78)	0.68 (0.10, 4.70)	3.63 (0.81, 16.24)	2.37 (0.91, 6.16)	1.72 (0.66, 4.43)
Viral shedding	1.58 (0.49, 5.13)	0.85 (0.27, 2.64)	0.53 (0.12, 2.32)	3.76 (0.28, 49.87)	3.12 (0.68, 14.32)	2.91 (1.06, 8.01)	-

Abbreviations: FARI, febrile acute respiratory illness

Among 124 secondary cases from 70 households, Managua, Nicaragua, 2012-2014.

Table 4.4 Generalized estimating equation accelerated failure time models assess the impact of alpha diversity on outcomes associated with community type 5.

Outcome	**Shannon Diversity**	**Chao1 Index**
	Acceleration Factor (95% Confidence Interval)	
Duration of fever	0.80 (0.31, 2.07)	1.00 (0.99, 1.01)
Duration of cough	1.39 (0.56, 3.49)	1.00 (0.99, 1.02)
Duration of shedding	1.62 (1.25, 2.10)	1.01 (0.998, 1.01)
Serial interval	0.72 (0.53, 0.97)	0.992 (0.986, 0.998)
Time to shedding onset	0.84 (0.67, 1.06)	0.995 (0.990, 0.999)

Among 124 secondary cases from 70 households, Managua, Nicaragua, 2012-2014.

Table 4.5 Models assessing the impact individual oligotypes on outcomes associated with community types.

Oligotype	Fever duration GEE AFT		Cough duration GEE AFT		Shedding duration GEE AFT	
	AF (95% CI)	q-value[a]	AF (95% CI)	q-value[a]	AF (95% CI)	q-value[a]
Veillonella 1	1.20 (0.82, 1.75)	0.409	0.81 (0.47, 1.40)	0.759	0.82 (0.66, 1.02)	0.201
Streptococcus 1	1.34 (0.83, 2.17)	0.409	0.69 (0.39, 1.22)	0.621	0.61 (0.48, 0.77)	<0.001
Fusobacterium	0.89 (0.64, 1.23)	0.772	1.33 (1.02, 1.72)	0.255	1.14 (1.07, 1.22)	<0.001
Streptococcus 2	1.04 (0.51, 2.09)	0.912	0.63 (0.28, 1.40)	0.621	1.26 (0.85, 1.86)	0.337
Prevotella 1	1.05 (0.89, 1.25)	0.730	0.84 (0.64, 1.09)	0.621	0.86 (0.69, 1.07)	0.267
Gemella	0.64 (0.42, 0.99)	0.200	0.85 (0.51, 1.42)	0.782	1.17 (0.87, 1.57)	0.372
Neisseria	0.85 (0.65, 1.11)	0.730	1.37 (1.03, 1.82)	0.292	1.16 (1.06, 1.27)	0.005
Haemophilus	0.64 (0.42, 0.98)	0.285	1.27 (0.78, 2.06)	0.694	1.13 (1.04, 1.23)	0.020
Prevotella 2	1.12 (0.96, 1.31)	0.409	0.92 (0.73, 1.14)	0.759	1.04 (0.93, 1.17)	0.513
Prevotella 3	1.23 (1.03, 1.48)	0.200	1.06 (0.84, 1.33)	0.782	1.04 (0.90, 1.21)	0.579
Prevotella 4	1.10 (0.71, 1.70)	0.772	1.42 (0.88, 2.29)	0.621	1.22 (0.96, 1.54)	0.220
Streptococcus 3	1.22 (0.74, 2.01)	0.648	0.79 (0.35, 1.78)	0.782	0.60 (0.38, 0.94)	0.078
Megasphaera	1.02 (0.91, 1.14)	0.772	0.98 (0.86, 1.13)	0.844	0.97 (0.88, 1.06)	0.513
Prevotella 5	1.09 (0.91, 1.31)	0.648	0.97 (0.79, 1.19)	0.844	1.09 (0.97, 1.22)	0.262
Veillonella 2	0.66 (0.50, 0.86)	0.030	0.83 (0.62, 1.12)	0.621	1.01 (0.96, 1.27)	0.267

Oligotype	Serial Interval GEE AFT		Time to shedding onset GEE AFT	
	AF (95% CI)	q-value[a]	AF (95% CI)	q-value[a]
Veillonella 1	1.31 (0.98, 1.74)	0.255	1.16 (0.90, 1.50)	0.501
Streptococcus 1	1.02 (0.74, 1.40)	0.920	0.90 (0.71, 1.15)	0.612
Fusobacterium	0.89 (0.83, 0.95)	0.015	0.97 (0.92, 1.02)	0.501
Streptococcus 2	1.07 (0.79, 1.44)	0.773	0.95 (0.62, 1.45)	0.884
Prevotella 1	0.97 (0.79, 1.20)	0.840	0.95 (0.89, 1.02)	0.501
Gemella	1.31 (0.91, 1.89)	0.362	1.21 (0.86, 1.69)	0.501
Neisseria	0.87 (0.79, 0.95)	0.015	0.97 (0.89, 1.04)	0.587
Haemophilus	0.93 (0.68, 1.26)	0.773	1.01 (0.95, 1.07)	0.884
Prevotella 2	0.92 (0.83, 1.01)	0.255	0.94 (0.87, 1.00)	0.435
Prevotella 3	1.07 (0.92, 1.25)	0.554	0.99 (0.92, 1.06)	0.884
Prevotella 4	0.81 (0.63, 1.03)	0.384	0.89 (0.78, 1.01)	0.435
Streptococcus 3	0.88 (0.66, 1.19)	0.255	0.83 (0.62, 1.10)	0.501
Megasphaera	1.07 (0.96, 1.18)	0.409	1.00 (0.93, 1.08)	0.997
Prevotella 5	0.94 (0.72, 1.22)	0.773	0.95 (0.89, 1.01)	0.435
Veillonella 2	0.86 (0.66, 1.12)	0.435	0.97 (0.87, 1.07)	0.712

[a]Corrected for multiple testing using the Benjamin-Hochberg method.
Abbreviations: GEE, generalized estimating equation; AFT, accelerated failure time; AF, acceleration factor; CI, confidence interval.

Models used $\log_{10}$-transformed relative abundance of 15 oligotypes that contributed 50% of difference between community types. Benjamin-Hochberg method to correct for multiple testing. Among 124 secondary cases from 70 households, Managua, Nicaragua, 2012-2014.

Legend

Oligotype	Full taxonomic classification
Veillonella 1	*Veillonella dispar / atypica / parvula / rogosae*
Streptococcus 1	*Streptococcus vestibularis / salivarius / gordonii / sp*
Fusobacterium	*Fusobacterium periodonticum / nucleatum subsp. animalis / sp. / nucleatum subsp. Vincentii / nucleatum subsp. polymorphum / naviforme / nucleatum subsp. nucleatum*
Streptococcus 2	*Streptococcus sp. / dentisani / mitis / oralis / infantis / tigurinus / lactarius / peroris / pneumoniae*
Prevotella 1	*Prevotella histicola / sp. / veroralis / scopos / fusca / melaninogenica*
Gemella	*Gemella haemolysans / sanguinis / morbillorum / bergeri*
Neisseria	*Neisseria subflava / flavescens / flava / sicca / pharynges / mucosa / polysaccharea / weaver / meningitidis / lactamica*
Haemophilus	*Haemophilus parainfluenzae / parahaemolyticuss / paraphrohaemolyticus / sputorum / sp. / haemolyticus / influenzae*
Prevotella 2	*Prevotella sp. / veroralis / histicola / fusca / scopos*
Prevotella 3	*Prevotella sp. / veroralis / fusca / histicola / scopos / melaninogenica*
Prevotella 4	*Prevotella melaninogenica / scopos / sp. / histicola / veroralis*
Streptococcus 3	*Streptococcus australis / parasanguinis II / parasanguinis I/ sp. / oligofermentans / cristatus / sinensis / sanguinis / gordonii / lactarius / peroris / oralis*
Megasphaera	*Megasphaera micronuciformis*
Prevotella 5	*Prevotella salivae*
Veillonella 2	*Veillonella parvula / rogosae / atypica / denticariosi / dispar*

Table 4.6 Sensitivity analysis: influenza-associated illness period does not exclude symptoms if fever recurs ≥3 days after fever alleviation.

Model	CT 2	CT 3	CT 4	CT 5	Age 0-5 Yr	Age 6-17 Yr	Female
GLME	**Odds Ratio (95% Confidence Interval)**						
≥1 symptom	0.57 (0.13, 2.57)	0.62 (0.16, 2.45)	1.83 (0.30, 11.40)	0.18 (0.01, 3.03)	6.36 (0.74, 55.04)	2.03 (0.60, 6.93)	1.48 (0.45, 4.84)
ARI	2.31 (0.63, 8.44)	1.21 (0.31, 4.74)	4.12 (0.86, 19.74)	1.84 (0.23, 14.62)	3.70 (0.65, 21.06)	2.06 (0.68, 6.23)	1.87 (0.61, 5.71)
Fever	1.32 (0.45, 3.82)	0.84 (0.27, 2.58)	2.58 (0.67, 9.95)	1.28 (0.19, 8.71)	2.26 (0.54, 9.45)	2.12 (0.81, 5.55)	1.99 (0.76, 5.20)
Rhinorrhea	1.52 (0.49, 4.71)	0.94 (0.30, 2.92)	2.09 (0.51, 8.52)	0.32 (0.03, 3.15)	8.65 (1.38, 54.34)	1.39 (0.51, 3.85)	1.26 (0.47, 3.39)
Sore throat	2.32 (0.67, 7.97)	0.82 (0.23, 2.92)	1.87 (0.43, 8.16)	1.65 (0.19, 14.31)	0.81 (0.17, 3.92)	1.31 (0.46, 3.75)	1.69 (0.59, 4.79)
Cough	2.06 (0.72, 5.87)	1.52 (0.53, 4.34)	3.67 (0.91, 14.78)	0.68 (0.10, 4.70)	3.63 (0.81, 16.24)	2.37 (0.91, 6.16)	1.72 (0.66, 4.43)
AFT GEE[b]							
Fever	1.94 (1.16, 3.22)	2.54 (1.15, 5.59)	1.56 (0.87, 2.77)	0.88 (0.37, 2.10)	1.23 (0.50, 3.00)	0.77 (0.44, 1.34)	0.79 (0.37, 1.69)
Sore throat[c]	0.88 (0.62, 1.25)	0.72 (0.47, 1.12)	1.00 (0.47, 2.35)	0.79 (0.50, 1.26)	-	0.72 (0.43, 1.20)	0.52 (0.21, 1.27)
Cough	2.23 (1.15, 4.32)	2.34 (1.26, 4.35)	2.71 (1.42, 5.17)	1.63 (0.48, 5.46)	1.59 (0.48, 5.29)	0.99 (0.62, 1.57)	1.05 (0.56, 1.99)
Rhinorrhea	1.39 (0.61, 3.13)	1.26 (0.59, 2.68)	1.25 (0.53, 2.96)	0.56 (0.09, 3.54)	3.30 (1.23, 8.91)	1.33 (0.76, 2.31)	1.14 (0.55, 2.37)

Model	CT 2	CT 3	CT 4	CT 5	Age 0-5 Years	Age 6-17 Years	Female	Smoker in Household	Household Crowding
GEE AFT	**Acceleration Factor (95% Confidence Interval)**								
Serial interval	0.94 (0.62, 1.42)	0.96 (0.58, 1.57)	0.64 (0.38, 1.07)	1.85 (1.03, 3.34)	0.70 (0.46, 1.08)	1.04 (0.74, 1.47)	1.13 (0.77, 1.65)	0.99 (0.68, 1.44)	0.99 (0.65, 1.50)

Abbreviations: CT, community type; GLME, generalized linear mixed effects; GEE, generalized estimating equation; AFT, accelerated failure time.

Table 4.7 Sensitivity analysis: influenza-associated illness period only considers ILI symptoms.

Model	CT 2	CT 3	CT 4	CT 5	Age 0-5 Yr	Age 6-17 Yr	Female
GLME	**Odd Ratio (95% Confidence Interval)**						
≥1 symptom	0.57 (0.13, 2.57)	0.62 (0.16, 2.45)	1.83 (0.30, 11.40)	0.18 (0.01, 3.03)	6.36 (0.74, 55.04)	2.04 (0.60, 6.93)	1.48 (0.45, 4.84)
ARI	2.31 (0.63, 8.45)	1.21 (0.31, 4.74)	4.12 (0.86, 19.74)	1.84 (0.23, 14.62)	3.70 (0.65, 21.06)	2.06 (0.68, 6.23)	1.87 (0.61, 5.71)
Fever	1.32 (0.45, 3.82)	0.84 (0.27, 2.58)	2.58 (0.67, 9.95)	1.28 (0.19, 8.71)	2.25 (0.54, 9.45)	2.12 (0.81, 5.55)	1.99 (0.76, 5.20)
Runny nose	1.52 (0.49, 4.71)	0.94 (0.30, 2.92)	2.09 (0.51, 8.52)	0.32 (0.03, 3.15)	8.65 (1.38, 54.34)	1.40 (0.51, 3.85)	1.26 (0.47, 3.39)
Sore throat	2.32 (0.67, 7.97)	0.82 (0.23, 2.92)	1.87 (0.43, 8.16)	1..65 (0.19, 14.31)	0.81 (0.17, 3.92)	1.31 (0.46, 3.75)	1.69 (0.59, 4.78)
Cough	2.06 (0.72, 5.87)	1.52 (0.53, 4.34)	3.67 (0.91, 14.78)	0.68 (0.10, 4.70)	3.63 (0.81, 16.24)	2.37 (0.91, 6.16)	1.72 (0.66, 4.43)
GEE AFT	**Acceleration Factor (95% Confidence Interval)**						
Fever	1.46 (0.93, 2.30)	2.75 (1.27, 5.97)	1.76 (1.04, 2.98)	0.82 (0.35, 1.92)	1.71 (0.78, 3.75)	0.99 (0.61, 1.60)	0.89 (0.43, 1.83)
Runny nose	1.16 (0.51, 2.64)	1.23 (0.58, 2.60)	1.27 (0.54, 2.97)	0.78 (0.18, 3.33)	3.78 (1.24, 11.52)	1.45 (0.83, 2.52)	1.21 (0.59, 2.47)
Sore throat[c]	0.88 (0.62, 1.25)	0.72 (0.47, 1.12)	1.00 (0.42, 2.35)	0.79 (0.50, 1.26)	-	0.72 (0.43, 1.20)	0.52 (0.21, 1.27)
Cough	1.97 (1.09, 3.56)	2.37 (1.29, 4.38)	2.78 (1.45, 5.33)	1.55 (0.48, 4.95)	1.83 (0.59, 5.71)	1.06 (0.67, 1.68)	1.08 (0.58, 2.01)

Model	CT 2	CT 3	CT 4	CT 5	Age 0-5 Yr	Age 6-17 Yr	Female	Smoker in Household	Household Crowding
GEE AFT	**Acceleration Factor (95% Confidence Interval)**								
Serial interval	0.97 (0.67, 1.42)	0.97 (0.60, 1.58)	0.63 (0.38, 1.58)	1.19 (0.83, 1.79)	0.58 (0.39, 0.88)	1.02 (0.73, 1.43)	1.11 (0.76, 1.61)	0.93 (0.64, 1.34)	1.01 (0.70, 1.47)

Abbreviations: CT, community type; GLME, generalized linear mixed effects; GEE, generalized estimating equation; AFT, accelerated failure time.

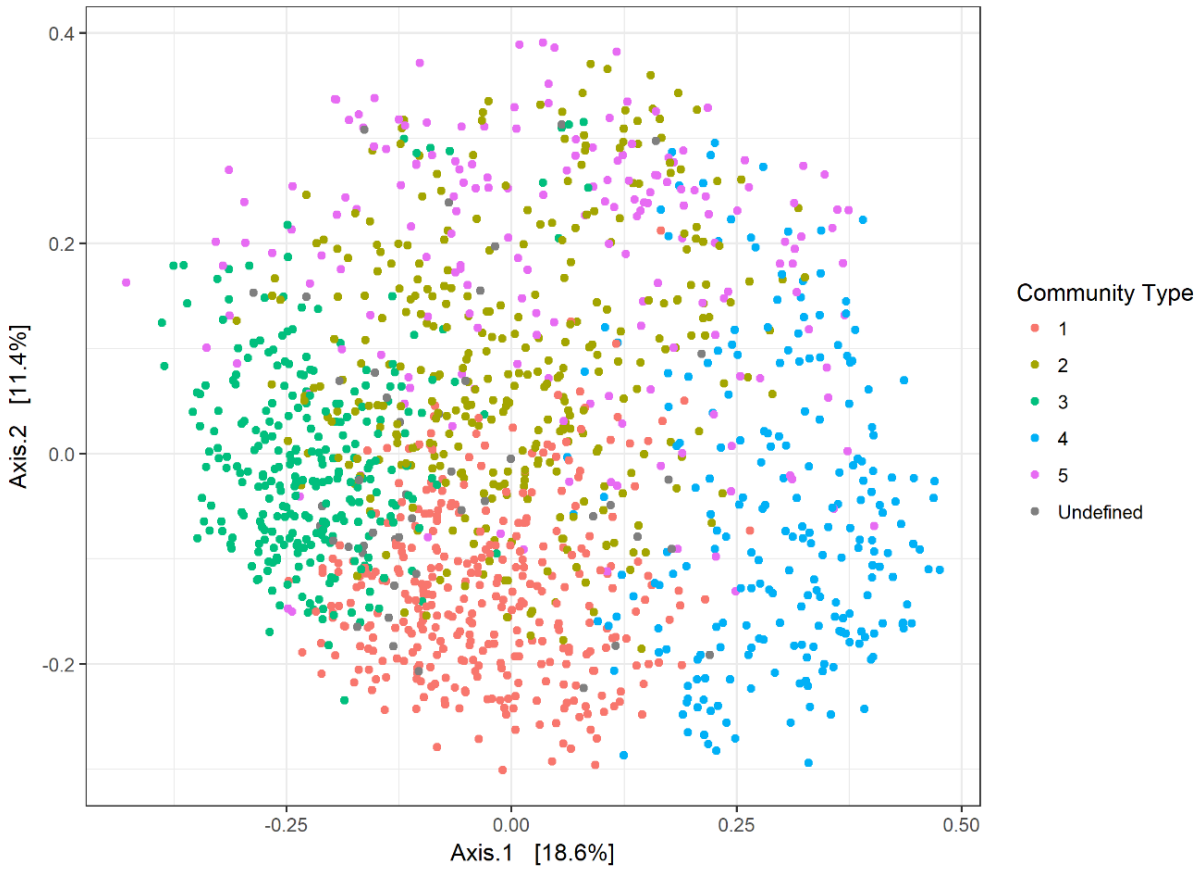
Table 4.8 Sensitivity analysis: all ARI symptoms during follow up contribute to influenza-associated illness period.

Model	CT 2	CT 3	CT 4	CT 5	Age 0-5 Yr	Age 6-17 Yr	Female
GLME	**Odds Ratio (95% Confidence Interval)**						
≥1 symptom	0.59 (0.13, 2.60)	0.52 (0.13, 2.06)	1.46 (0.24, 8.89)	0.17 (0.01, 2.82)	4.99 (0.60, 41.30)	1.73 (0.51, 5.85)	1.56 (0.48, 5.05)
ARI	1.87 (0.54, 6.48)	0.98 (0.26, 3.69)	3.24 (0.71, 14.75)	1.59 (0.21, 12.39)	3.20 (0.59, 17.34)	1.83 (0.62, 5.38)	1.94 (0.64, 5.84)
Fever	1.30 (0.45, 3.74)	0.71 (0.23, 2.17)	2.10 (0.55, 8.08)	1.18 (0.17, 8.10)	1.85 (0.45, 7.69)	1.78 (0.69, 4.65)	2.09 (0.80, 5.46)
Rhinorrhea	1.52 (0.49, 4.71)	0.94 (0.30, 2.92)	2.09 (0.51, 8.52)	0.32 (0.03, 3.15)	8.65 (1.38, 54.34)	1.40 (0.51, 3.85)	1.26 (0.47, 3.39)
Sore throat	2.08 (0.57, 7.57)	0.85 (0.24, 3.01)	1.54 (0.34, 7.06)	1.48 (0.15, 14.41)	0.70 (0.14, 3.55)	1.06 (0.36, 3.14)	1.94 (0.65, 5.80)
Cough	2.06 (0.72, 5.87)	1.52 (0.53, 4.34)	3.67 (0.91, 14.78)	0.68 (0.10, 4.70)	3.63 (0.81, 16.24)	2.37 (0.91, 6.16)	1.72 (0.66, 4.43)
GEE AFT	**Acceleration Factor (95% Confidence Interval)**						
Fever	1.93 (1.15, 3.22)	2.72 (1.27, 5.83)	1.69 (0.99, 2.88)	0.91 (0.38, 2.19)	1.37 (0.58, 3.23)	0.83 (0.49, 1.43)	0.82 (0.40, 1.69)
Sore throat[c]	9.26 (0.65, 1.32)	6.91 (0.48, 1.00)	1.09 (0.46, 2.61)	0.82 (0.52, 1.29)	-	0.76 (0.47, 1.22)	0.48 (0.21, 1.12)
Cough	2.34 (1.33, 4.13)	2.47 (1.40, 4.37)	2.60 (1.38, 4.89)	1.68 (0.51, 5.52)	1.42 (0.47, 4.28)	0.91 (0.59, 1.39)	1.05 (0.57, 1.91)
Rhinorrhea	1.37 (0.61, 3.09)	1.26 (0.60, 2.64)	1.27 (0.53, 3.01)	0.80 (0.18, 3.62)	3.43 (1.12, 10.48)	1.35 (0.77, 2.35)	1.17 (0.56, 2.47)

Model	CT 2	CT 3	CT 4	CT 5	Age 0-5 Yr	Age 6-17 Yr	Female	Smoker in Household	Household Crowding
GEE AFT	**Acceleration Factor (95% Confidence Interval)**								
Serial interval	0.91 (0.66, 1.25)	0.94 (0.57, 1.54)	0.69 (0.40, 1.17)	1.19 (0.82, 1.74)	0.69 (0.45, 1.06)	1.24 (0.82, 1.87)	1.09 (0.75, 1.59)	0.97 (0.67, 1.40)	0.88 (0.59, 1.32)

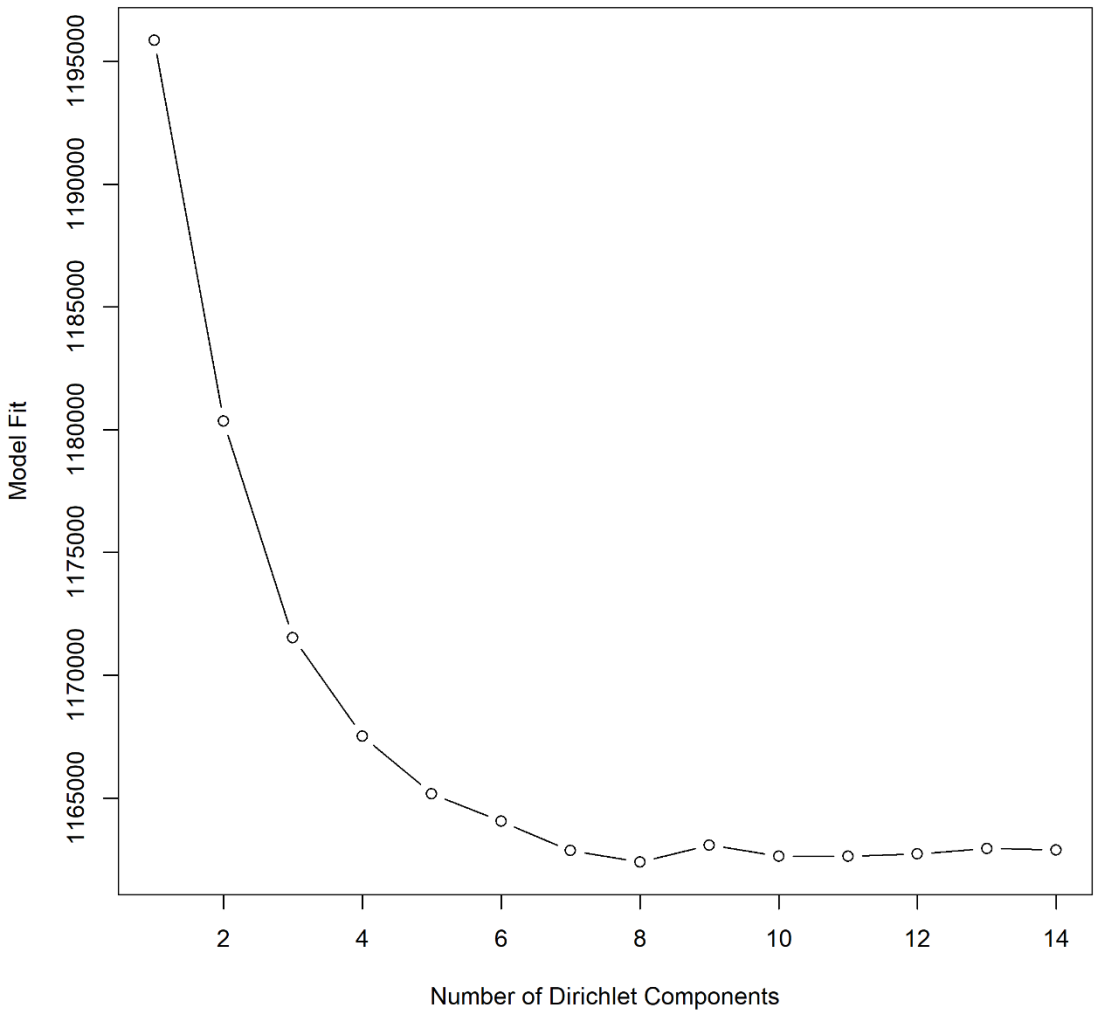
Abbreviations: CT, community type; GLME, generalized linear mixed effects; GEE, generalized estimating equation; AFT, accelerated failure time.

Figure 4.1 Principal coordinates analysis of nose/throat samples assigned to community types.



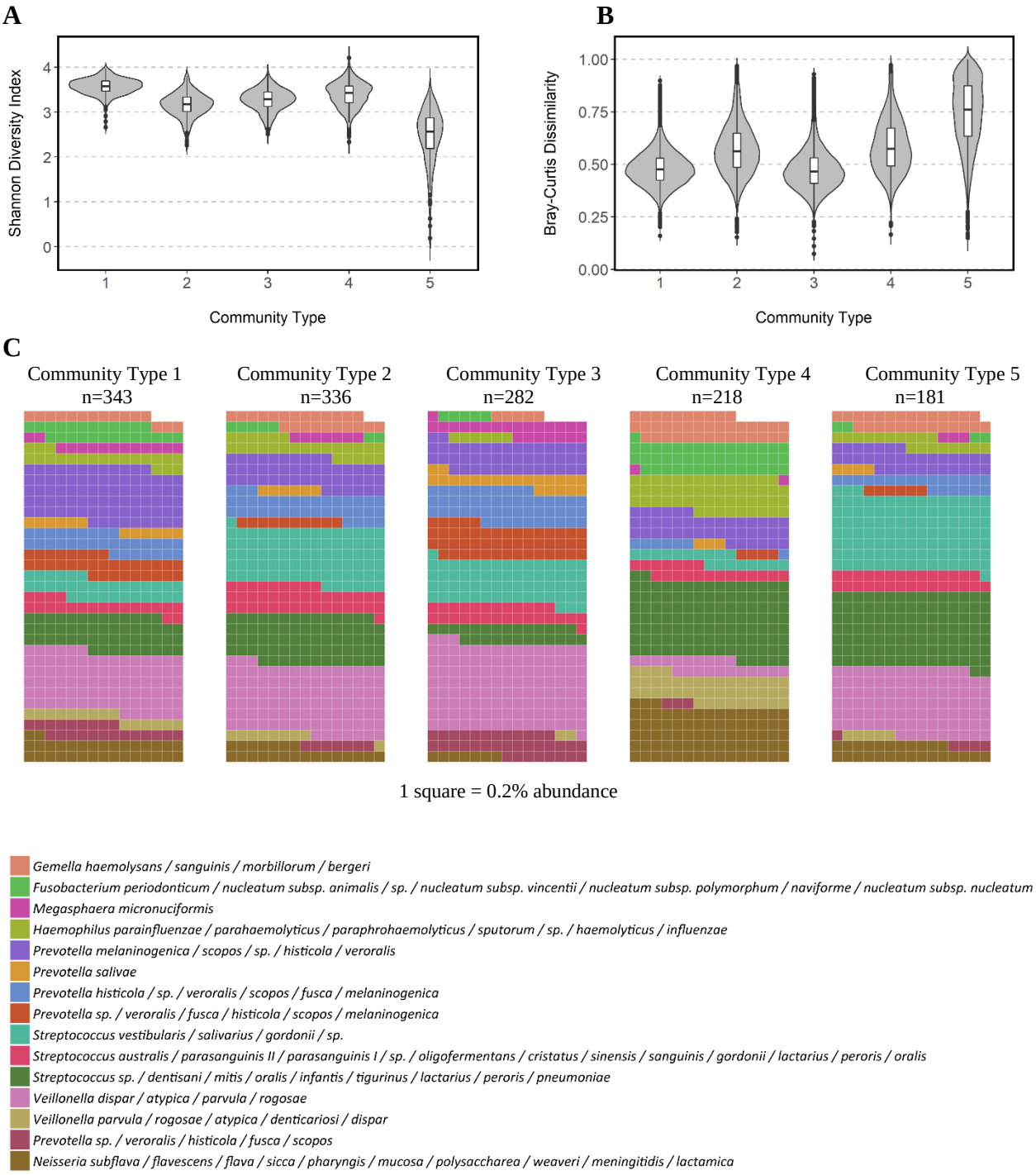
1,405 nose/throat samples from 717 study participants residing in 144 households in Managua, Nicaragua, 2012-2014. Based on Bray-Curtis dissimilarity.

Figure 4.2 Model fit of negative log models by number of Dirichlet components using the first and last samples of all study participants (n=1,405 samples).



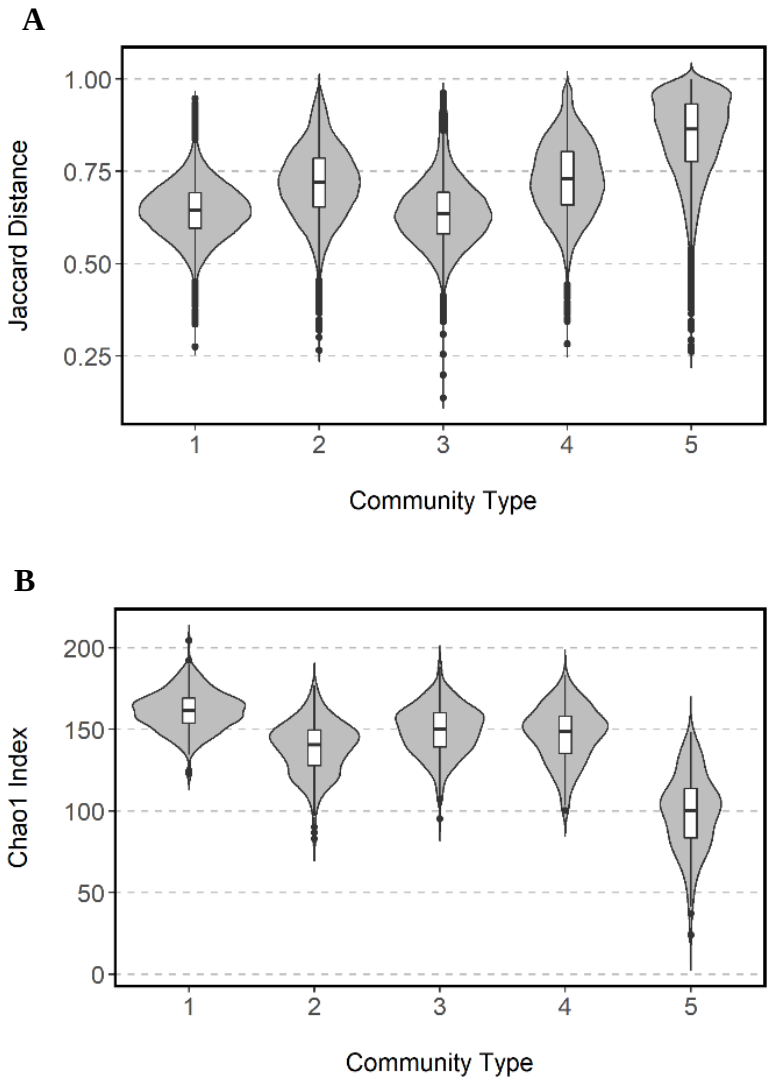
We determined the number of community types by estimating the Laplace approximation of the negative log models and identifying the point at which an increase in Dirichlet components resulted in minor reductions in model fit.

Figure 4.3 Characteristics of bacterial community types based on first and last nose/throat samples of 717 study participants from 144 households, Managua, Nicaragua, 2012-2014.



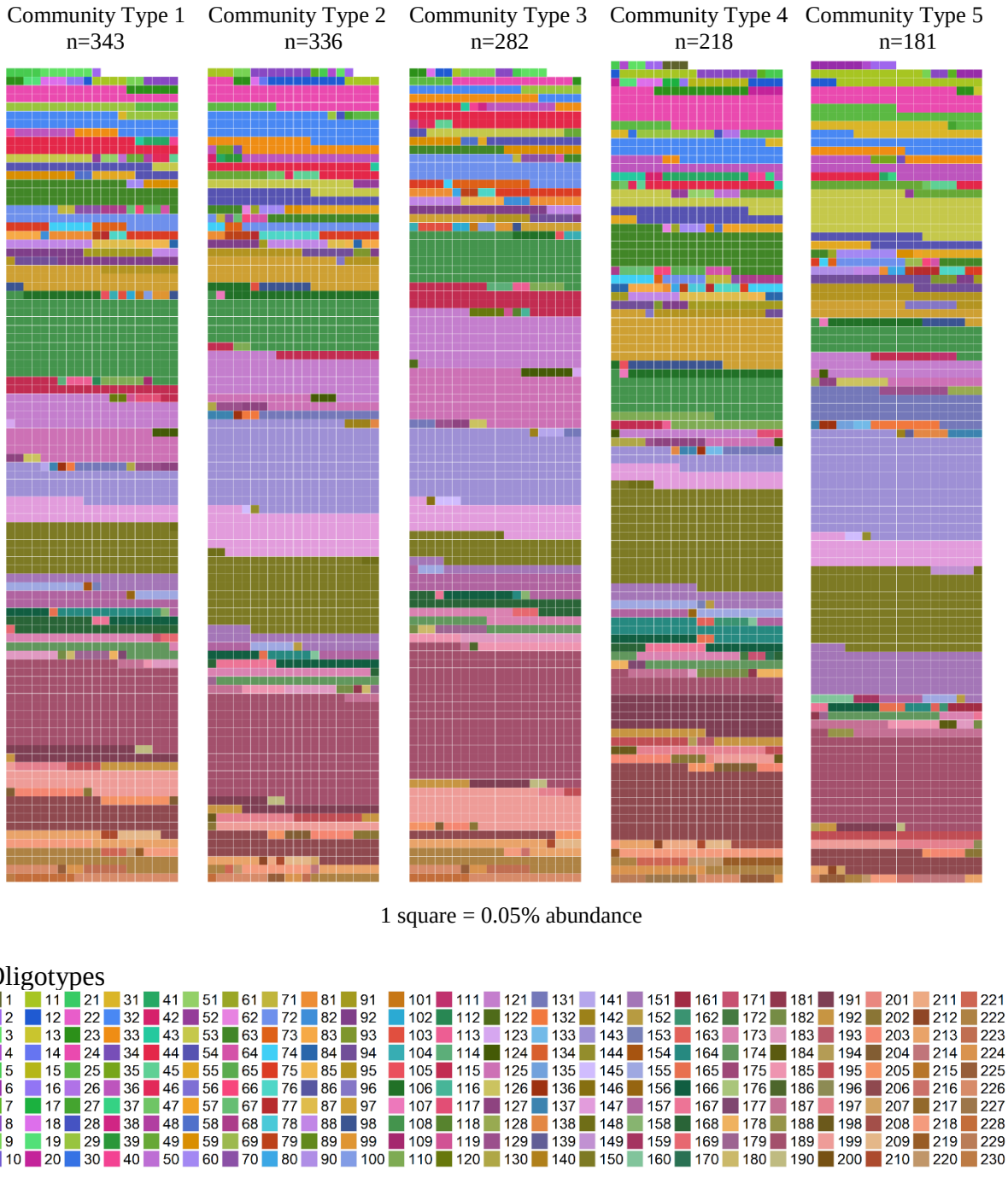
(A) Shannon diversity. (B) Bray-Curtis dissimilarity by community type. Each violin plot contains a box plot with a kernel density estimation on each side depicting the distribution of data. (C) Relative abundance renormalized to 15 oligotypes that contributed to 50% of difference between community types. Each square represents 0.2% relative abundance.

Figure 4.4 Diversity by community type types based on first and last nose/throat samples of 717 study participants from 144 households, Managua, Nicaragua, 2012-2014, using alternative metrics.



(A) Jaccard distance. (B) Chao1 index. Each violin plot contains a box plot with a kernel density estimation on each side depicting the distribution of data.

Figure 4.5 Relative abundance of oligotypes by community type.



Based on first and last nose/throat samples of 717 study participants from 144 households, Managua, Nicaragua, 2012-2014. Each square represents 0.05% relative abundance.

Legend

Oligotype	Taxonomy
1	*Filifactor alocis*
2	Unclassified
3	*Peptostreptococcaceae [XI][G-1] [Eubacterium] sulci / Peptostreptococcaceae [XI][G-1] [Eubacterium] infirmum*
4	Unclassified
5	*Oribacterium parvum / Oribacterium sinus / Oribacterium asaccharolyticum*
6	Unclassified
7	*Corynebacterium matruchotii / Corynebacterium diphtheriae*
8	*Oribacterium sinus / Oribacterium parvum*
9	*Oribacterium asaccharolyticum / Oribacterium parvum*
10	Unclassified
11	*Streptococcus pneumoniae / Streptococcus tigurinus / Streptococcus dentisani / Streptococcus sp. / Streptococcus oralis / Streptococcus mitis / Streptococcus infantis / Streptococcus peroris / Streptococcus lactarius*
12	*Streptococcus sanguinis / Streptococcus oligofermentans / Streptococcus sinensis / Streptococcus cristatus / Streptococcus australis / Streptococcus parasanguinis II / Streptococcus sp. / Streptococcus gordonii / Streptococcus parasanguinis I / Streptococcus pneumoniae / Streptococcus oralis / Streptococcus intermedius / Streptococcus mitis*
13	*Streptococcus mutans*
14	*Streptococcus vestibularis / Streptococcus salivarius*
15	*Leptotrichia sp.*
16	*Leptotrichia sp.*
17	*Leptotrichia sp.*
18	*Leptotrichia sp.*
19	*Leptotrichia sp.*
20	*Leptotrichia sp.*
21	*Bacillus subtilis*
22	*Listeria monocytogenes*
23	*Peptostreptococcus stomatis / Peptostreptococcus anaerobius*
24	*Gemella haemolysans / Gemella sanguinis / Gemella morbillorum / Gemella bergeri*
25	*Dolosigranulum pigrum*
26	*Bergeyella sp.*
27	*Leptotrichia shahii / Leptotrichia sp. / Leptotrichia hongkongensis*
28	Unclassified
29	*Alloprevotella tannerae*
30	*Alloprevotella tannerae*
31	*Rothia mucilaginosa*
32	*Rothia mucilaginosa*
33	*Rothia mucilaginosa*
34	*Rothia aeria / Rothia dentocariosa*
35	*Rothia dentocariosa / Rothia aeria*
36	Unclassified
37	Unclassified
38	*Oribacterium asaccharolyticum*
39	Unclassified
40	*Ruminococcaceae [G-1] sp.*
41	*Capnocytophaga leadbetteri / Capnocytophaga sp. / Capnocytophaga ochracea*
42	Unclassified
43	*Porphyromonas endodontalis / Porphyromonas sp.*
44	*Actinomyces graevenitzii*

45	*Actinomyces lingnae [NVP] / Actinomyces sp. / Actinomyces odontolyticus / Actinomyces meyeri / Actinomyces cardiffensis*
46	*Actinomyces lingnae [NVP] / Actinomyces sp. / Actinomyces odontolyticus / Actinomyces meyeri*
47	*Actinomyces sp. / Actinomyces oris / Actinomyces naeslundii / Actinomyces johnsonii / Actinomyces viscosus / Actinomyces radicidentis / Actinomyces meyeri*
48	*Butyrivibrio sp.*
49	Unclassified
50	*SR1 [G-1] sp.*
51	*SR1 [G-1] sp.*
52	*Abiotrophia defectiva*
53	*Staphylococcus caprae / Staphylococcus epidermidis / Staphylococcus aureus / Staphylococcus warneri*
54	*Granulicatella adiacens / Enterococcus italicus / Enterococcus faecalis*
55	*Granulicatella elegans*
56	*Lactobacillus gasseri / Lactobacillus johnsonii*
57	*Escherichia coli*
58	*Ruminococcaceae [G-2] sp.*
59	*Leptotrichia sp.*
60	*Unclassified*
61	Unclassified
62	Unclassified
63	*Fusobacterium periodonticum / Fusobacterium nucleatum subsp. animalis / Fusobacterium sp. / Fusobacterium nucleatum subsp. vincentii / Fusobacterium nucleatum subsp. polymorphum / Fusobacterium naviforme / Fusobacterium nucleatum subsp. nucleatum*
64	*Fusobacterium sp. / Fusobacterium nucleatum subsp. polymorphum / Fusobacterium nucleatum subsp. nucleatum / Fusobacterium naviforme / Fusobacterium nucleatum subsp. vincentii / Fusobacterium nucleatum subsp. animalis / Fusobacterium periodonticum*
65	*Peptococcus sp.*
66	*Fusobacterium nucleatum subsp. animalis / Fusobacterium naviforme / Fusobacterium nucleatum subsp. vincentii / Fusobacterium periodonticum / Fusobacterium sp. / Fusobacterium nucleatum subsp. nucleatum / Fusobacterium nucleatum subsp. polymorphum*
67	*Fusobacterium sp. / Fusobacterium nucleatum subsp. animalis / Fusobacterium nucleatum subsp. polymorphum / Fusobacterium nucleatum subsp. nucleatum / Fusobacterium periodonticum / Fusobacterium nucleatum subsp. vincentii / Fusobacterium naviforme*
68	*Fusobacterium periodonticum / Fusobacterium nucleatum subsp. animalis / Fusobacterium sp. / Fusobacterium nucleatum subsp. vincentii / Fusobacterium nucleatum subsp. polymorphum / Fusobacterium naviforme / Fusobacterium nucleatum subsp. nucleatum*
69	*Fusobacterium necrophorum / Fusobacterium gonidiaformans*
70	*Atopobium parvulum / Atopobium sp. / Atopobium rimae*
71	*Catonella morbi / Catonella sp.*
72	*Megasphaera micronuciformis*
73	*Lachnospiraceae [G-2] sp.*
74	*Lachnoanaerobaculum umeaense / Lachnoanaerobaculum sp.*
75	*Lachnoanaerobaculum orale / Lachnoanaerobaculum saburreum*
76	*Veillonella dispar / Veillonella atypica / Veillonella parvula / Veillonella rogosae*
77	Unclassified
78	*Treponema denticola / Treponema putidum / Treponema sp.*
79	*Veillonella dispar / Veillonella atypica / Veillonella parvula / Veillonella rogosae / Veillonella denticariosi*
80	*Selenomonas sputigena / Selenomonas sp.*
81	*Selenomonas sp.*
82	*Mitsuokella sp.*
83	*Aggregatibacter sp. / Aggregatibacter segnis / Haemophilus haemolyticus / Haemophilus sp. / Haemophilus influenzae / Haemophilus aegyptius*
84	*Haemophilus pittmaniae / Aggregatibacter sp. / Aggregatibacter aphrophilus / Aggregatibacter paraphrophilus*

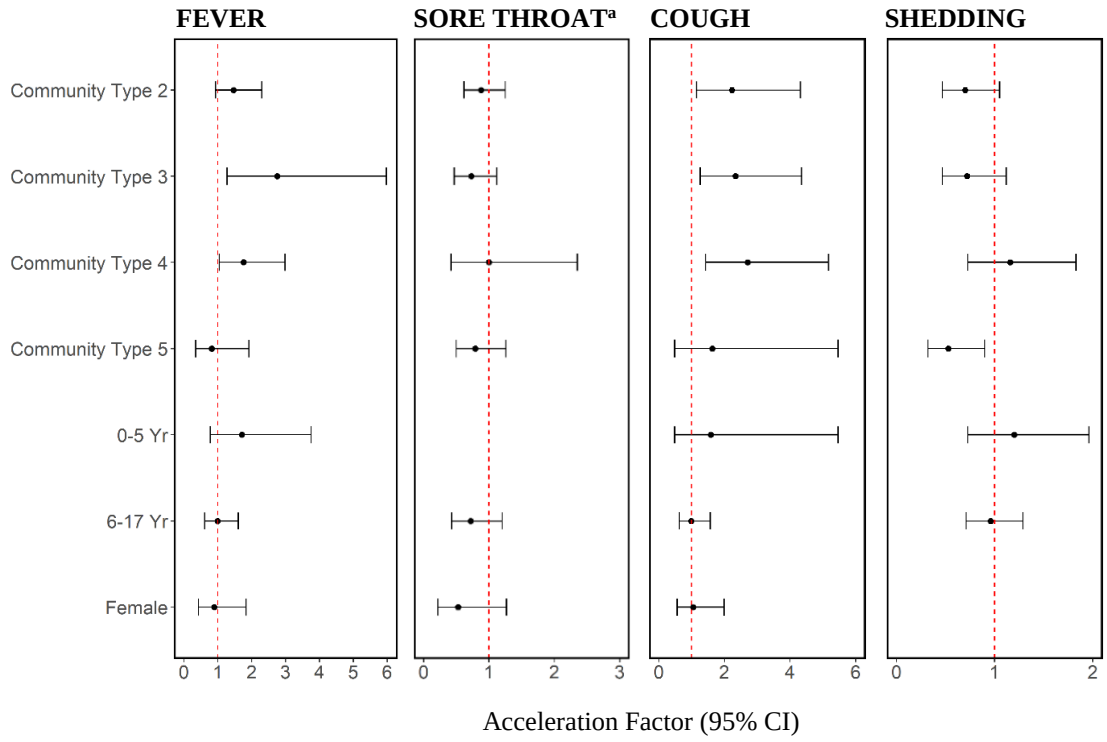
85	*Mogibacterium neglectum / Mogibacterium pumilum / Mogibacterium diversum / Mogibacterium vescum / Mogibacterium timidum*
86	Unclassified
87	*Stomatobaculum sp.*
88	*Campylobacter concisus / Campylobacter curvus*
89	*Helicobacter pylori*
90	*Pseudomonas aeruginosa / Pseudomonas otitidis / Pseudomonas sp.*
91	*Alloprevotella sp.*
92	*Alloprevotella sp.*
93	*Prevotella sp.*
94	*Haemophilus aegyptius / Haemophilus influenzae / Haemophilus sp. / Haemophilus haemolyticus / Aggregatibacter sp. / Aggregatibacter segnis*
95	*Haemophilus sp. / Haemophilus haemolyticus / Haemophilus influenzae / Haemophilus aegyptius / Aggregatibacter sp. / Aggregatibacter segnis / Haemophilus parainfluenzae*
96	*Haemophilus sp. / Haemophilus haemolyticus / Haemophilus influenzae / Haemophilus aegyptius / Aggregatibacter sp. / Aggregatibacter segnis*
97	*Haemophilus parainfluenzae / Haemophilus parahaemolyticus / Haemophilus paraphrohaemolyticus / Haemophilus sputorum / Haemophilus sp. / Haemophilus haemolyticus / Haemophilus influenzae*
98	*Haemophilus parahaemolyticus / Haemophilus sputorum / Haemophilus paraphrohaemolyticus / Haemophilus parainfluenzae*
99	*Prevotella sp. / Prevotella oulorum*
100	*Prevotella sp. / Prevotella oulorum*
101	*Prevotella oulorum / Prevotella sp.*
102	*Prevotella sp. / Prevotella oulorum*
103	*Prevotella sp. / Prevotella veroralis / Prevotella histicola / Prevotella fusca / Prevotella scopos*
104	*Prevotella veroralis / Prevotella sp. / Prevotella scopos / Prevotella fusca / Prevotella histicola / Prevotella melaninogenica*
105	*Prevotella veroralis / Prevotella sp. / Prevotella scopos / Prevotella fusca / Prevotella histicola*
106	*Neisseria pharyngis / Neisseria sicca / Neisseria mucosa / Neisseria flava / Neisseria subflava / Neisseria flavescens / Neisseria polysaccharea / Neisseria lactamica / Neisseria meningitidis / Neisseria gonorrhoeae / Neisseria oralis*
107	*Kingella denitrificans / Neisseria elongata / Neisseria weaveri / Kingella sp. / Eikenella corrodens / Eikenella sp.*
108	*Prevotella melaninogenica / Prevotella scopos / Prevotella sp. / Prevotella histicola / Prevotella veroralis*
109	*Prevotella sp. / Prevotella melaninogenica / Prevotella histicola / Prevotella veroralis / Prevotella scopos / Prevotella fusca*
110	*Prevotella melaninogenica / Prevotella scopos / Prevotella sp. / Prevotella histicola / Prevotella veroralis / Prevotella fusca*
111	*Prevotella sp. / Prevotella scopos / Prevotella histicola / Prevotella melaninogenica / Prevotella veroralis / Prevotella fusca*
112	*Prevotella melaninogenica / Prevotella sp. / Prevotella histicola / Prevotella scopos / Prevotella veroralis / Prevotella fusca*
113	*Prevotella melaninogenica / Prevotella scopos / Prevotella sp. / Prevotella histicola / Prevotella veroralis / Prevotella fusca*
114	*Prevotella histicola / Prevotella sp. / Prevotella veroralis / Prevotella fusca / Prevotella scopos / Prevotella melaninogenica*
115	*Prevotella salivae*
116	*Prevotella salivae*
117	*Prevotella sp.*
118	*Prevotella sp.*
119	*Prevotella sp. / Prevotella histicola / Prevotella veroralis / Prevotella scopos / Prevotella fusca / Prevotella melaninogenica*
120	*Prevotella sp.*
121	*Prevotella histicola / Prevotella sp. / Prevotella veroralis / Prevotella scopos / Prevotella fusca / Prevotella melaninogenica*

122	*Prevotella histicola / Prevotella scopos / Prevotella sp. / Prevotella melaninogenica / Prevotella veroralis / Prevotella fusca*
123	*Prevotella histicola / Prevotella melaninogenica / Prevotella sp. / Prevotella scopos / Prevotella veroralis*
124	*Prevotella sp. / Prevotella scopos / Prevotella veroralis / Prevotella histicola / Prevotella fusca / Prevotella melaninogenica*
125	*Prevotella sp. / Prevotella veroralis / Prevotella fusca / Prevotella histicola / Prevotella scopos / Prevotella melaninogenica*
126	*Prevotella sp. / Prevotella scopos / Prevotella veroralis / Prevotella histicola / Prevotella fusca / Prevotella melaninogenica*
127	*Prevotella sp. / Prevotella scopos / Prevotella melaninogenica / Prevotella veroralis / Prevotella histicola / Prevotella fusca*
128	*Leptotrichia sp.*
129	*Leptotrichia sp.*
130	*Capnocytophaga sputigena / Capnocytophaga sp.*
131	*Moraxella catarrhalis*
132	*Moraxella catarrhalis*
133	*Prevotella sp.*
134	*Prevotella sp.*
135	*Prevotella sp.*
136	*Porphyromonas sp.*
137	*Porphyromonas sp.*
138	*Streptococcus constellatus / Streptococcus intermedius / Streptococcus anginosus / Streptococcus gordonii / Streptococcus sp.*
139	*Streptococcus anginosus / Streptococcus constellatus / Streptococcus intermedius*
140	*Streptococcus pyogenes / Streptococcus agalactiae*
141	*Prevotella histicola / Prevotella sp. / Prevotella veroralis / Prevotella fusca / Prevotella scopos / Prevotella melaninogenica*
142	*Streptococcus sp. / Streptococcus gordonii / Streptococcus oligofermentans / Streptococcus sinensis / Streptococcus cristatus / Streptococcus parasanguinis II / Streptococcus parasanguinis I / Streptococcus australis / Streptococcus sanguinis / Streptococcus intermedius / Streptococcus salivarius / Streptococcus mitis / Streptococcus oralis*
143	*Streptococcus vestibularis / Streptococcus salivarius / Streptococcus gordonii / Streptococcus sp.*
144	*Streptococcus vestibularis / Streptococcus salivarius / Streptococcus oligofermentans / Streptococcus sinensis / Streptococcus cristatus*
145	*Streptococcus vestibularis / Streptococcus salivarius*
146	*Streptococcus vestibularis / Streptococcus salivarius*
147	*Streptococcus australis / Streptococcus parasanguinis II / Streptococcus parasanguinis I / Streptococcus sp. / Streptococcus oligofermentans / Streptococcus cristatus / Streptococcus sinensis / Streptococcus sanguinis / Streptococcus gordonii / Streptococcus lactarius / Streptococcus peroris / Streptococcus oralis*
148	*Streptococcus sinensis / Streptococcus oligofermentans / Streptococcus cristatus / Streptococcus australis / Streptococcus parasanguinis II / Streptococcus sp. / Streptococcus gordonii / Streptococcus sanguinis / Streptococcus parasanguinis I / Streptococcus oralis / Streptococcus mitis / Streptococcus infantis*
149	*Streptococcus agalactiae / Streptococcus pyogenes*
150	*Streptococcus sp. / Streptococcus dentisani / Streptococcus mitis / Streptococcus oralis / Streptococcus infantis / Streptococcus tigurinus / Streptococcus lactarius / Streptococcus peroris / Streptococcus pneumoniae*
151	*Streptococcus peroris / Streptococcus lactarius / Streptococcus sp. / Streptococcus tigurinus / Streptococcus infantis / Streptococcus dentisani / Streptococcus oralis / Streptococcus mitis*
152	*Streptococcus sp. / Streptococcus dentisani / Streptococcus mitis / Streptococcus oralis / Streptococcus infantis / Streptococcus tigurinus / Streptococcus lactarius / Streptococcus peroris / Streptococcus pneumoniae*
153	*Leptotrichia sp.*
154	*Leptotrichia sp.*
155	*Leptotrichia sp.*
156	*Leptotrichia sp.*
157	*Leptotrichia sp. / Leptotrichia wadei*
158	*Leptotrichia sp. / Leptotrichia wadei*

159	Unclassified
160	Unclassified
161	Unclassified
162	*Alloprevotella tannerae*
163	*Alloprevotella tannerae*
164	*Porphyromonas pasteri / Porphyromonas sp. / Porphyromonas catoniae*
165	*Porphyromonas sp. / Porphyromonas pasteri / Porphyromonas catoniae*
166	*Rothia mucilaginosa*
167	*Rothia mucilaginosa*
168	*Alloprevotella rava*
169	*Alloprevotella rava*
170	*Alloprevotella rava*
171	*Campylobacter rectus / Campylobacter showae / Campylobacter gracilis*
172	*Campylobacter rectus / Campylobacter showae / Campylobacter gracilis*
173	*Actinomyces sp. / Actinomyces odontolyticus / Actinomyces meyeri / Actinomyces cardiffensis / Actinomyces lingnae [NVP] / Actinomyces georgiae*
174	*Actinomyces sp. / Actinomyces odontolyticus / Actinomyces meyeri / Actinomyces cardiffensis / Actinomyces lingnae [NVP] / Actinomyces georgiae / Actinomyces gerencseriae / Actinomyces massiliensis*
175	*Enterococcus faecalis / Enterococcus durans / Enterococcus saccharolyticus / Enterococcus casseliflavus / Enterococcus italicus / Granulicatella adiacens*
176	*Bacillus anthracis / Lysinibacillus fusiformis*
177	*Capnocytophaga granulosa / Capnocytophaga sp. / Capnocytophaga gingivalis*
178	*Capnocytophaga gingivalis / Capnocytophaga granulosa / Capnocytophaga sp.*
179	*Stomatobaculum longum / Stomatobaculum sp.*
180	*Stomatobaculum longum / Stomatobaculum sp.*
181	*Fusobacterium nucleatum subsp. vincentii / Fusobacterium naviforme / Fusobacterium sp. / Fusobacterium nucleatum subsp. nucleatum / Fusobacterium nucleatum subsp. animalis / Fusobacterium nucleatum subsp. polymorphum / Fusobacterium periodonticum*
182	*Fusobacterium nucleatum subsp. vincentii / Fusobacterium naviforme / Fusobacterium nucleatum subsp. animalis / Fusobacterium nucleatum subsp. nucleatum / Fusobacterium sp. / Fusobacterium nucleatum subsp. polymorphum / Fusobacterium periodonticum*
183	*Atopobium parvulum / Atopobium rimae / Atopobium sp.*
184	*Atopobium parvulum / Atopobium rimae / Atopobium sp.*
185	*Veillonella sp.*
186	*Veillonella sp.*
187	*Veillonella sp.*
188	*Veillonella sp.*
189	*Veillonella dispar / Veillonella atypica / Veillonella parvula / Veillonella rogosae*
190	*Veillonella dispar / Veillonella atypica / Veillonella parvula*
191	*Veillonella parvula / Veillonella rogosae / Veillonella atypica / Veillonella denticariosi / Veillonella dispar*
192	*Veillonella rogosae / Veillonella parvula / Veillonella atypica / Veillonella denticariosi / Veillonella dispar*
193	*Parvimonas micra / Parvimonas sp.*
194	*Parvimonas micra / Parvimonas sp.*
195	*Alloprevotella sp.*
196	*Alloprevotella sp.*
197	*Haemophilus parahaemolyticus / Haemophilus sputorum / Haemophilus paraphrohaemolyticus / Haemophilus parainfluenzae*
198	*Haemophilus parahaemolyticus / Haemophilus sputorum / Haemophilus paraphrohaemolyticus / Haemophilus parainfluenzae*
199	*Prevotella sp. / Prevotella veroralis / Prevotella histicola / Prevotella fusca / Prevotella scopos*
200	*Prevotella sp. / Prevotella veroralis / Prevotella histicola / Prevotella fusca / Prevotella scopos*
201	*Bordetella pertussis / Achromobacter xylosoxidans*
202	*Lautropia mirabilis*

203	*Neisseria subflava / Neisseria flavescens / Neisseria flava / Neisseria sicca / Neisseria pharyngis / Neisseria mucosa / Neisseria polysaccharea / Neisseria weaveri*
204	*Neisseria subflava / Neisseria flavescens / Neisseria flava / Neisseria sicca / Neisseria pharyngis / Neisseria mucosa*
205	*Solobacterium moorei*
206	*Neisseria subflava / Neisseria flavescens / Neisseria flava / Neisseria sicca / Neisseria pharyngis / Neisseria mucosa / Neisseria polysaccharea / Neisseria weaveri / Neisseria meningitidis / Neisseria lactamica*
207	*Neisseria subflava / Neisseria flavescens / Neisseria flava / Neisseria sicca / Neisseria pharyngis / Neisseria mucosa / Neisseria polysaccharea / Neisseria weaveri*
208	*Neisseria subflava / Neisseria flavescens / Neisseria flava / Neisseria sicca / Neisseria pharyngis / Neisseria mucosa / Neisseria polysaccharea / Neisseria weaveri*
209	*Prevotella melaninogenica / Prevotella scopos / Prevotella sp. / Prevotella histicola / Prevotella veroralis / Prevotella fusca*
210	*Prevotella melaninogenica / Prevotella scopos / Prevotella sp. / Prevotella histicola / Prevotella veroralis*
211	*Prevotella oris*
212	*Prevotella denticola / Prevotella multiformis*
213	*Leptotrichia sp.*
214	*Neisseria lactamica / Neisseria sicca / Neisseria flava / Neisseria polysaccharea / Neisseria pharyngis / Neisseria mucosa / Neisseria meningitidis / Neisseria oralis / Neisseria subflava / Neisseria bacilliformis / Neisseria gonorrhoeae / Neisseria flavescens*
215	*Neisseria meningitidis / Neisseria polysaccharea / Neisseria flava / Neisseria gonorrhoeae / Neisseria sicca / Neisseria pharyngis / Neisseria mucosa / Neisseria lactamica / Neisseria flavescens / Neisseria subflava*
216	Unclassified
217	*Acinetobacter baumannii / Acinetobacter sp.*
218	*Prevotella nanceiensis*
219	Unclassified
220	*Prevotella nanceiensis*
221	*Prevotella shahii / Prevotella sp.*
222	*Prevotella pallens*
223	*Prevotella pallens*
224	*Prevotella intermedia*
225	*Prevotella nigrescens*
226	*Prevotella pallens*
227	*Prevotella aurantiaca*
228	*Prevotella aurantiaca*
229	*Prevotella aurantiaca*
230	*Prevotella aurantiaca / Prevotella pallens*

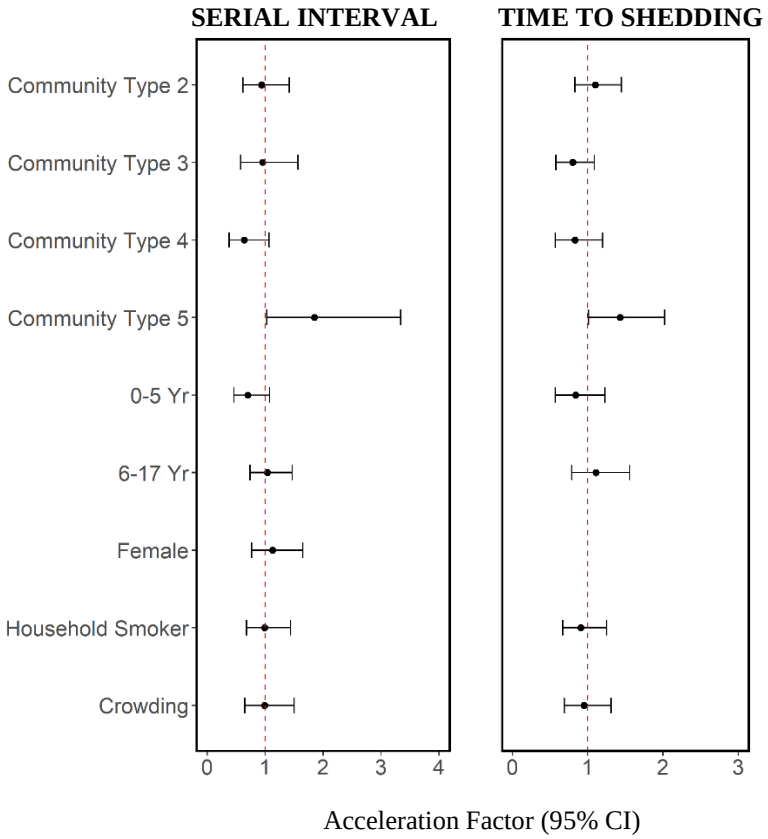
Figure 4.6 Accelerated failure time models estimating acceleration factor and 95% confidence interval for symptom and shedding durations.



Among 124 secondary cases from 70 households, Managua, Nicaragua, 2012-2014. Acceleration factors estimate the relative change in survival time. Models are not specific to influenza type/subtype.

[a]0-5 years removed from model as very few experienced a sore throat and all with sore throat were right censored.

Figure 4.7 Accelerated failure time models estimating acceleration factor and 95% confidence interval for serial interval and time to shedding onset.



Among 124 secondary cases from 70 households, Managua, Nicaragua, 2012-2014. Acceleration factors estimate the relative change in survival time. Models are not specific to influenza type/subtype**.**

Appendix 4.1 Materials and Methods for Microbiota Data

DNA extraction

Total DNA was extracted from a pair of samples from each study participant: the first sample collected at time of enrollment and the second sample collected at the last day of follow up (median days between samples: 9.0 days, IQR: 9.0-10.0). Study participants included all index cases and household contacts. Among the 717 total study participants, five first samples and 19 second samples were not available for DNA extraction. DNA was extracted using the QIAmp DNA Mini Kit and an enzyme cocktail composed of cell lysis solution (Promega, Madison, USA), lysozyme, mutanolysin, RNase A, and lysostaphin (Sigma-Aldrich, St. Lious, USA) in 22.5:4.5:1.125:1.125:1 parts, respectively. 100 µL of sample was incubated at 37°C for 30 minutes with 80 µL of the enzyme cocktail. After adding 25 µL proteinase K and 200 µL of Buffer AL, samples were vortexed and incubated at 56°C for 30 minutes. Samples were washed with 200 µL of 100% ethanol, 500 uL of Buffer AW1, and then 500 uL of Buffer AW2. To maximize DNA yield, DNA was eluted twice with 100 uL of Buffer AE and stored at -80°C.

16S rRNA sequencing

The V4 hypervariable region of the 16S rRNA gene was sequenced at the University of Michigan Microbial Systems Laboratories using Illumina MiSeq V2 chemistry 2x250 (Illumina, San Diego, CA) and a validated dual-indexing method [18]. Briefly, primers consisted of an Illumina adapter, an 8-nt index sequence, a 10-nt pad sequence, a 2-nt linker, and the V4-specific F515/R806 primer [31]. Amplicons were purified and pooled in equimolar concentrations. A mock community of 21 species (Catalog No. HM-782D, BEI Resources, Manassas, VA) or a mock community of 10 species (Catalog No. D6300, Zymo Research, Irvine, CA) was included by the Microbial Systems Laboratories to assess sequencing error rates. For every 96-well plate submitted

for amplification and sequencing (90 study samples), we included two aliquots of an in-house mock community consisting of *Streptococcus pneumoniae, Streptococcus pyogenes, Staphylococcus aureus, Haemophilus influenzae,* and *Moraxella catarrhalis* and two aliquots of an oropharyngeal control sample. These internal controls were randomly assigned to plate wells and used to assess systematic variation in sequencing. All samples were sequenced in duplicate, demultiplexed, and quality filtered.

Oligotyping

We used mothur v1.38.1 [19] to align and perform quality filtering on raw sequences using the mothur standard operating procedures (https://www.mothur.org/wiki/MiSeq_SOP, accessed November 18, 2016). Sequences were converted to the appropriate oligotyping format as previously described [32]. We used the Minimum Entropy Decomposition (MED) algorithm [33] with default parameters (-M: 13779.0, -V: 3 nt) to cluster sequences into oligotypes. Briefly, the algorithm identifies variable nucleotide positions and uses Shannon entropy to partition sequences into nodes. The process is iterative and continues to decompose parent nodes into child nodes until there are no discernable entropy peaks. Oligotyping has previously been used to examine within-genus variations in the microbiota [32,34–36] and provides increased resolution relative to conventional distance-based clustering methods. After excluding five samples with less than 1,000 reads, our dataset consisted of 1,405 samples with a total of 61,784,957 sequences decomposed into 230 oligotypes. To assign taxonomy, we searched representative sequences of each oligotype against the Human Oral Microbiome Database (HOMD) v14.51 [9] using blastn v2.2.23 [10].

Community typing

We used Dirichlet multinomial mixture models [11] in R v3.3.2 [12] and the DirichletMultinomial v1.16.0 package [37] to assign all samples to 5 community types. This method has frequently been used as a method of dimension reduction in microbiome studies [38,39]. We determined the number of community types by comparing the Laplace approximation of the negative log models and identifying the point at which an increase in Dirichlet components resulted in minor reductions in model fit (Figure 4.2). Samples were assigned to community types with the greatest posterior probability. 96.8% of all samples had a posterior probability of 90% or higher. To minimize misclassification, samples were assigned as having an undefined community type if the posterior probability was less than 90%. Each community type contained between 12.9-24.4% of all samples (n=181-343) and 3.2% of all samples (n=45) were undefined. Principal coordinates analysis of nose/throat samples assigned to community types is depicted in Figure 4.1.

4.7 References

1. Widdowson M-A, Monto AS. Epidemiology of influenza. In: Webster RG, Monto AS, Braciale TJ, Lamb RA, eds. Textbook of Influenza. Wiley, 2013.
2. Nair H, Brooks WA, Katz M, et al. Global burden of respiratory infections due to seasonal influenza in young children: a systematic review and meta-analysis. The Lancet **2011**; 378:1917–1930.
3. Carrat F, Vergu E, Ferguson NM, et al. Time lines of infection and disease in human influenza: a review of volunteer challenge studies. Am J Epidemiol **2008**; 167:775–785.
4. Ng S, Lopez R, Kuan G, et al. The Timeline of Influenza Virus Shedding in Children and Adults in a Household Transmission Study of Influenza in Managua, Nicaragua. Pediatr Infect Dis J **2016**; 35:583–586.
5. Cowling BJ, Chan KH, Fang VJ, et al. Comparative epidemiology of pandemic and seasonal influenza A in households. N Engl J Med **2010**; 362:2175–2184.
6. Ferguson NM, Cummings DAT, Cauchemez S, et al. Strategies for containing an emerging influenza pandemic in Southeast Asia. Nature **2005**; 437:209.
7. Ip DKM, Lau LLH, Leung NHL, et al. Viral Shedding and Transmission Potential of Asymptomatic and Paucisymptomatic Influenza Virus Infections in the Community. Clin Infect Dis Off Publ Infect Dis Soc Am **2017**; 64:736–742.
8. Loeb M, Singh PK, Fox J, et al. Longitudinal study of influenza molecular viral shedding in Hutterite communities. J Infect Dis **2012**; 206:1078–1084.
9. Ivanov II, Atarashi K, Manel N, et al. Induction of Intestinal Th17 Cells by Segmented Filamentous Bacteria. Cell **2009**; 139:485–498.
10. Ichinohe T, Pang IK, Kumamoto Y, et al. Microbiota regulates immune defense against respiratory tract influenza A virus infection. Proc Natl Acad Sci U S A **2011**; 108:5354.
11. Shaw MH, Kamada N, Kim Y-G, Núñez G. Microbiota-induced IL-1β, but not IL-6, is critical for the development of steady-state TH17 cells in the intestine. J Exp Med **2012**; 209:251–258.
12. Abt MC, Osborne LC, Monticelli LA, et al. Commensal bacteria calibrate the activation threshold of innate antiviral immunity. Immunity **2012**; 37:158–170.
13. Panigrahi P, Parida S, Nanda NC, et al. A randomized synbiotic trial to prevent sepsis among infants in rural India. Nature **2017**; advance online publication. Available at: http://www.nature.com/nature/journal/vaop/ncurrent/full/nature23480.html?foxtrotcallback=true. Accessed 21 August 2017.

14. Luoto R, Ruuskanen O, Waris M, Kalliomäki M, Salminen S, Isolauri E. Prebiotic and probiotic supplementation prevents rhinovirus infections in preterm infants: A randomized, placebo-controlled trial. J Allergy Clin Immunol **2014**; 133:405–413.

15. Schuijt TJ, Lankelma JM, Scicluna BP, et al. The gut microbiota plays a protective role in the host defence against pneumococcal pneumonia. Gut **2016**; 65:575–583.

16. Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team, Dawood FS, Jain S, et al. Emergence of a novel swine-origin influenza A (H1N1) virus in humans. N Engl J Med **2009**; 360:2605–2615.

17. World Health Organization. WHO Manual on Animal Influenza Diagnosis and Surveillance. Geneva, Switzerland: World Health Organization, 2002.

18. Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. Development of a Dual-Index Sequencing Strategy and Curation Pipeline for Analyzing Amplicon Sequence Data on the MiSeq Illumina Sequencing Platform. Appl Environ Microbiol **2013**; 79:5112–5120.

19. Schloss PD, Westcott SL, Ryabin T, et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol **2009**; 75:7537–7541.

20. Eren AM, Morrison HG, Lescault PJ, Reveillaud J, Vineis JH, Sogin ML. Minimum entropy decomposition: unsupervised oligotyping for sensitive partitioning of high-throughput marker gene sequences. ISME J **2015**; 9:968–979.

21. Holmes I, Harris K, Quince C. Dirichlet multinomial mixtures: generative models for microbial metagenomics. PloS One **2012**; 7:e30126.

22. Chen T, Yu W-H, Izard J, Baranova OV, Lakshmanan A, Dewhirst FE. The Human Oral Microbiome Database: a web accessible resource for investigating oral microbe taxonomic and genomic information. Database J Biol Databases Curation **2010**; 2010:baq013.

23. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol **1990**; 215:403–410.

24. Cowling BJ, Fang VJ, Riley S, Peiris JSM, Leung GM. Estimation of the serial interval of influenza. Epidemiol Camb Mass **2009**; 20:344–347.

25. Aitchison J. The Statistical Analysis of Compositional Data. J R Stat Soc Ser B Methodol **1982**; 44:139–177.

26. R Core Team. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing, 2017. Available at: https://www.R-project.org/.

27. Wang J, Li F, Sun R, et al. Bacterial colonization dampens influenza-mediated acute lung injury via induction of M2 alveolar macrophages. Nat Commun **2013**; 4. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3715851/. Accessed 16 March 2018.

28. Cauchemez S, Donnelly CA, Reed C, et al. Household transmission of 2009 pandemic influenza A (H1N1) virus in the United States. N Engl J Med **2009**; 361:2619–2627.

29. World Health Organization. Influenza. Available at: http://www.who.int/immunization/topics/influenza/en/. Accessed 17 February 2017.

30. Iuliano AD, Roguski KM, Chang HH, et al. Estimates of global seasonal influenza-associated respiratory mortality: a modelling study. The Lancet **2017**; 0. Available at: http://www.thelancet.com/journals/lancet/article/PIIS0140-6736(17)33293-2/abstract. Accessed 1 March 2018.

31. Caporaso JG, Lauber CL, Walters WA, et al. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proc Natl Acad Sci **2011**; 108:4516–4522.

32. Berry MA, White JD, Davis TW, et al. Are Oligotypes Meaningful Ecological and Phylogenetic Units? A Case Study of Microcystis in Freshwater Lakes. Front Microbiol **2017**; 8:365.

33. Eren AM, Morrison HG, Lescault PJ, Reveillaud J, Vineis JH, Sogin ML. Minimum entropy decomposition: unsupervised oligotyping for sensitive partitioning of high-throughput marker gene sequences. ISME J **2015**; 9:968–979.

34. Eren AM, Zozaya M, Taylor CM, Dowd SE, Martin DH, Ferris MJ. Exploring the Diversity of Gardnerella vaginalis in the Genitourinary Tract Microbiota of Monogamous Couples Through Subtle Nucleotide Variation. PLOS ONE **2011**; 6:e26732.

35. Eren AM, Borisy GG, Huse SM, Mark Welch JL. Oligotyping analysis of the human oral microbiome. Proc Natl Acad Sci U S A **2014**; 111:E2875–E2884.

36. Eren AM, Sogin ML, Morrison HG, et al. A single genus in the gut microbiome reflects host preference and specificity. ISME J **2015**; 9:90–100.

37. Morgan M. DirichletMultinomial: Dirichlet-Multinomial Mixture Model Machine Learning for Microbiome Data. 2017.

38. Donahue Carlson R, Sheth AN, Read TD, et al. The Female Genital Tract Microbiome Is Associated With Vaginal Antiretroviral Drug Concentrations in Human Immunodeficiency Virus-Infected Women on Antiretroviral Therapy. J Infect Dis **2017**; 216:990–999.

39. Chen C-H, Lin Y-L, Chen K-H, et al. Bacterial diversity among four healthcare-associated institutes in Taiwan. Sci Rep **2017**; 7. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5557925/. Accessed 16 March 2018.

Chapter 5 Summary and Conclusions

While publications serve as a public measure of scientific achievement, they often overlook the underlying and difficult process that is required to reach these achievements. As a doctoral student, my personal measure of achievement was based on the experiences that shaped me into a more capable and innovative researcher. In this chapter, I review the major lessons and challenges I experienced during the dissertation process. In addition, I discuss future research directions which may contribute to a better understanding of host-pathogen-microbiome dynamics.

5.1 Lessons Learned

5.1.1 Building Leadership Skills

This study provided me with numerous opportunities for growth as a project manager and research supervisor. I took on several new responsibilities that I once took for granted. As a lab assistant, my main concern was in learning how to efficiently run lab protocols. As a project lead, I was responsible for developing these protocols and for asking the "what", "why", and "who" questions, in addition to "how". I spent considerable time conducting literature reviews to optimize our protocols and in managing logistics. This transition into a leadership position was particularly difficult due to significant turnover of our lab team during my first semester, leaving few individuals with experience in managerial tasks.

I was responsible for supervising and mentoring graduate and undergraduate students, many of whom had little to no experience in the lab. My initial mentoring approach was to make myself completely accessible, as many of my previous mentors had done before. I quickly learned

that this was impossible for a doctoral student with a full load of courses, teaching a course, conducting research, and studying for the competency exam. I learned to create a strict schedule to keep myself on track while maximizing my availability to students. More importantly, I realized my initial mentoring approach might be hindering critical thinking. While simply giving the answer to a student's question may have been the easiest solution, I found the best long-term solution was to help students reach the answers themselves through active discussion and encouragement.

5.1.2 Building Research Skills

The multi-disciplinary nature of my dissertation project required me to develop a new set of research skills through courses at various departments, mentors and colleagues with specific expertise, and self-education. One of the goals of this project was to develop a bioinformatic and analytic pipeline, which could be used in future microbiome studies. Although our research group had prior experience with microbiome analysis, we used the QIIME program, conventional distance-based taxonomic units, and statistical models that only examined one taxon at a time. My first decision was to use mothur, software developed here at the University of Michigan. This decision doubled my work load as I attempted to learn this software on top of a unix-based coding language and a high-performance computer cluster. However, I feel this decision allowed our group to be proficient in two of the mostly commonly used microbiome programs. It took us two years to establish a pipeline which involves quality filtering of raw sequences in mothur or QIIME, oligotyping to create taxonomic units with improved resolution, and Dirichlet multinomial mixture modeling for community typing. As a team, we established an annotated pipeline which can be

used to train new students in microbiome analysis and serve as a foundation for new and improved methods as they are continually developed.

In addition to bioinformatics, I learned new statistical models that took into account clustered and longitudinal data. Mixed effects models were used to examine continuous and binomial outcomes while accounting for clustering by household. Accelerated failure time (AFT) models were used to examine time-to-event outcomes while accounting for interval and right censored data. AFT models were further expanded using a generalized estimating equation approach to account for clustering by household. I was only able to discover and apply these unique methods through the guidance of my committee members.

I have made significant efforts in learning the core concepts of immunology and using this knowledge to interpret epidemiologic results. As the association between influenza virus infection and the microbiome is likely mediated through the host immune response, I took courses to untangle the jumble of immunology acronyms and pathways that were discussed in various microbiome studies and animal experiments. Although my knowledge is still limited, it allows me to better understand the underlying biological mechanisms that may be driving what I observed in epidemiologic studies. Further, it spurs new research questions that I hope to investigate in the near future.

Lastly, I learned to develop strategies for scientific writing. Although I enjoyed sharing my results through presentations, I struggled with writing them down on paper. In the lab, I was taught to be extremely detail-oriented as minor measurement errors could lead to failed experiments. Instinctively, I was applying the same strategy in writing, which resulted in countless revisions of a single paragraph and, in some cases, a single sentence. By the end of the day, the paragraph had somehow remained unchanged. I have learned to fight this impulse and to begin the writing process

with an outline of unrefined statements. This approach helped me focus my time and energy into developing a clear story rather than contemplate trivial details, which could be improved at a later time. In addition, I identified unique strategies that personally worked for me, such as writing through dictation and learning to give my writing time to "settle" especially for sections that were particularly challenging.

5.1.3 Surrounding Yourself with Good People

One of the most important lessons I learned during the PhD program was to surround myself with good people who shared my passion and interests. I would not have reached this point without compatible and supportive mentors. Dr. Betsy Foxman is an expert in infectious disease epidemiology and microbial ecology, with years of experience in training doctoral students. Dr. Aubree Gordon is an incredible resource in influenza epidemiology and continues to be a role model in global health. My co-advisors helped me establish a foundation needed to explore the relationship between influenza virus and the host microbiome.

I learned the importance of identifying my weaknesses and finding mentors who could help me overcome them. Dr. Kerby Shedden is one of the few statisticians who has explored numerous approaches to analyzing microbiome data in epidemiologic studies. Dr. Marie Griffin is an infectious disease epidemiologist with clinical expertise in respiratory infections. Dr. Sophia Ng is an expert in household studies and influenza transmission modeling. This multi-disciplinary group of mentors provided me with invaluable advice, critiques, and perspectives in analyzing complex data.

I also learned to rely on fellow students who shared my interests in integrating molecular techniques to infectious disease epidemiology. This project has often felt like an expedition in

uncharted territories, largely due to my personal inexperience in microbiome analysis and the lack of any established methods in the literature. As a team, we worked together to explore and decipher new methods. This ultimately led to a bioinformatic and analytic pipeline, which we now use for other microbiome studies.

5.1.4 Learning from Hardships

Two hardships stand out the most. The first was when my initial dissertation project was terminated. I started the PhD program with a specific study in mind and had received permission to use archival samples from the principal investigator, former project lead, and study collaborators. I spent considerable time exploring potential research questions that could be answered using this study design. However, the project fell apart due to miscommunication and other factors that are still unknown to me. The main lesson I learned from this experience was the importance of bouncing back after difficult circumstances and learning to keep moving forward as there will always be other important research questions that both fascinate me and can contribute to improving public health.

A second notable hardship was as a research supervisor. An important component of my dissertation project involved using qPCR to quantify the absolute abundance of select bacterial species. I had trained one of my research assistants in qPCR in hopes of having her run plates while I away in Nicaragua for a summer research trip. Unfortunately, the majority of qPCR runs had poor efficiencies and had to be scrapped. This experience taught me the importance of quality control checks throughout any research process and for providing adequate support my research assistants.

5.2 Challenges

5.2.1 Challenges with Systematic Bias

One of the greatest challenges in my dissertation project was in learning and dealing with systematic bias that was specific to microbiome studies. In theory, microbiome data should represent a random sample of the bacterial community from which a specimen is collected. However, this is not achievable with current techniques and bias can be introduced at various stages of the study (Figure 5.1). A more realistic goal was to identify where bias could be introduced, to reduce bias when possible, and to standardized bias when it is unavoidable.

I learned bias can be introduced at the earliest stages of data collection, specifically during sample collection and storage. First, the microbiome differs by body site, even in areas of close proximity [1]. Site-specific differences can overwhelm and/or bias the effects of the primary exposure of interest (e.g. influenza virus infection). Nasal, oropharyngeal, and nasopharyngeal samples have all been used to characterize the upper respiratory tract bacterial community. However, differences in the community structure between these sites have been reported [2,3]. Second, bias can arise from differences in collection methodology and storage conditions [4,5]. For example, communities sampled through swabs may differ from those sampled as aspirates. Further, certain storage solutions may differentially impact DNA integrity and recovery. In our study, we combined oropharyngeal and nasal swabs into one sample and stored them at -80°C in universal transport media. Although potential bias may have been introduced as a result of combining samples from two different sites, this bias was minimized by using consistent methods across all study participants and limiting freeze/thaw cycles. Although sample collection and storage were already completed prior to my dissertation project, understanding how our protocols could affect our results and comparability between studies influenced how I interpreted our data.

DNA extraction methods can influence DNA yield and bacterial community structure representation [6]. For example, DNA is more difficult to extract from gram positive bacteria due to their thick cell walls. To efficiently lyse bacterial cells, I used the Qiagen® DNA Mini Kit with an extra enzyme cocktail, which included cell lysis solution, lysozyme, mutanolysin, RNase A, and lysostaphin. Further, I personally conducted all DNA extractions to reduce potential inter-personnel variability.

Sequencing bias is unavoidable due to limitations in our current technologies. The most common approach for characterizing the microbiota is to sequence the 16S rRNA gene and to quantify taxa based on differences in the sequence. Although the optimal approach is to sequence the entire gene using platforms such as the PacBio Single Molecule, Real-Time Sequencer (SMRT), it is not yet an affordable option especially for large population studies that examine hundreds to thousands of samples. Instead, most studies only sequence 1 to 3 of the total 9 hypervariable regions, which greatly influences the results. Primer choice considerably influences abundance estimations [7]. Further, studies have reported region-specific differences in estimation of community richness and in identification of select taxa [8,9]. Unfortunately, there is still no consensus in the literature on which region most accurately depicts the oral microbiome. We sequenced the V4 hypervariable region in our study. This decision was largely based on 1) affordability and timeliness of V4 sequencing at the University of Michigan Microbial Systems Laboratory, 2) a publicly available and widely-used mothur standard operating procedure that was based on the V4 region, and 3) an increased potential for comparability of our results with prior and future microbiome studies.

5.2.2 Challenges in Working with Microbiome Data

I encountered many challenges in working with complex microbiome data. Microbiome data is compositional as sequencing data can only be used to estimate the relative abundance. In addition, a single sample can contain hundreds of different taxonomic units, each representing a potential variable for consideration in analysis. As there are no established methods in the literature, we spent considerable time exploring different approaches that would allow us to combine microbiome data and epidemiologic models. Our earliest approach involved using models with log-transformed relative abundances of the most common taxa [10]. We expanded this by exploring additional methods that involved identifying key taxa (e.g. lasso, elastic net, Bayesian variable selection, and random forest), using principle components analysis, and identifying bacterial community types using clustering algorithms (e.g. hierarchical clustering, Partitioning Around Medoids, Dirichlet multinomial mixture models).

Microbiome data are difficult to interpret. Statistical differences in beta diversity and alpha diversity do not necessarily represent biological significance. It is not clear how much diversity constitutes health. Further, diversity is not the answer to our research questions. Rather it should spur us to the identify the underlying factors that influence both community diversity and our outcome of interest [11]. The same challenge exists when examining the relative abundance of specific taxon and our outcomes of interest. In my dissertation, I found associations between bacterial community types and influenza susceptibility, shedding, and symptomology. Although community typing is an excellent method for dimension reduction and allows us to examine the relationship between the overall bacterial community structure and our outcomes of interest, it is difficult to identify specific characteristics that uniquely define these community types.

5.2.3 Challenges due to Sample Size

Sample size was a recurring challenge in this project. Although the Nicaraguan Household Transmission Study is one of the largest of its kind, our budget only allowed us to characterize the microbiome at two points in time for each study participant enrolled in 2012-2014. Further, there is no consensus in the literature on how to determine power for microbiome studies. Crude estimates could have been made if we had more information on how much the upper respiratory tract microbiome varied between individuals and over time. However, little data were available with exception to cross-sectional data from healthy adults in the Human Microbiome Project [1]. The few longitudinal studies that characterized the upper respiratory tract microbiome were not published until recently and are restricted to infants [12,13]. To our surprise, we found the nose/throat microbiome varied substantially even among healthy individuals. This high degree of heterogeneity in our population limited our analysis. While this was a novel and important finding, it also motivated me to explore news methods for exploring longitudinal data analysis.

5.3 Future Directions

5.3.1 A Numbers Game

As previously mentioned, a major challenge in this study was sample size. The full Nicaraguan household transmission study was conducted during 2012-2017 and included up to 5 archival nose/throat samples per participant. Expanding microbiome analysis to all 6 years of the study may permit the use of models that were previously underpowered with our smaller subset. For example, applying an individual-based transmission hazard model [14] would allow us to examine whether the nose/throat microbiome influences the serial interval after accounting for

community infection risk and household transmission chains. We could also assess whether the microbiome of infected individuals contributes to the serial interval.

As samples were collected at 2-3 day intervals, expanding our analysis to all samples would allow us to longitudinally characterize changes in the upper respiratory tract microbiome during influenza virus infection. In addition, we could use these results to estimate periods of enhanced risk for secondary bacterial infections and examine which factors may mediate risk. To the best of my knowledge, no study has examined this using microbiome data. With the caveat that all or a majority of study participants were exposed to influenza virus in the household, we could also examine the stability of the nose/throat microbiome among healthy individuals and explore which factors may contribute to microbiome stability. For example, we could investigate whether asymptomatic viral infections (non-influenza viruses) can influence the microbiome structure. Lastly, we could compare these households to households without any influenza exposure (no index case) to assess whether household influenza exposure itself can perturb the microbiome.

5.3.2 Investigating Viral Shedding

Influenza virus infection can be detected through hemagglutinin inhibition (HAI) antibody assays [15] or RT-PCR [16]. There are advantages and disadvantage to each method. HI assays are considered the gold standard but require blood samples and provide less information on time of infection, RT-PCR requires easily obtainable nose or throat samples and can be conducted relatively rapidly. However, RT-PCR only captures individuals with detectable viral shedding.

Preliminary results from our study suggest the relationship between the microbiome and susceptibility to influenza virus infection may differ by viral shedding status. Our original analysis only used RT-PCR to detect secondary infections as we did not yet have complete HI data for all

our study participants. Several months after submitting our results for publication, we had HI titers for all study participants who contributed blood samples. Due to curiosity, we decided to rerun our analysis using all secondary infections detected by RT-PCR or HAI. Although community type 4 was associated with reduced susceptibility to infection with viral shedding, community type 5 was associated with reduced susceptibility to all influenza virus infections (both with shedding and without viral shedding). Further investigation is needed to decipher this difference and I plan to submit a brief report to address this in the near future.

5.3.3 Functional Potential

In my dissertation, we examined the bacterial community structure of the nose and throat using 16S rRNA sequencing. However, the microbiome is more than just the composition of microbes in a given habitat but encompasses microbial genes and activity. The functional potential of the microbiome may be as or more important than the microbial composition itself. In the future, I hope to use various -omic tools to decipher how microbial genes (metatranscriptomics), proteins (metaproteomics), and metabolites (metabolomics) may influence human diseases. I would specifically focus on genes, proteins, and metabolites that influence innate and adaptive host immunity. For example, we could assess whether influenza risk, symptomology, or viral shedding is associated with differences in the production of flagellin and lipoproteins that stimulate Toll-like receptors [17]. We could also examine differences in microbial metabolites such as butyrate which regulate macrophage function [18] and tryptophan which affect innate lymphoid cells [19].

5.3.4 Host-Pathogen-Microbiome Interactions

Respiratory infections involve a complex network of interactions between the invading pathogen, the microbiome, and host immunity. I hope to conduct research that aims at elucidating these dynamics. In our study, we saw the relationship between the nose/throat microbiome and influenza virus infection may vary by influenza subtype/type. For example, H3N2 was never detected by RT-PCR in any household contact with community type 4. Although the study lacked the power for subtype-specific analysis, it made me consider how much strain variation may influence the relationship between the microbiome and infection. We could explore subtype-specific associations if we expand our study period to include all 6 years of the household transmission study. In addition, we could conduct whole genome sequencing on circulating influenza strains to identify genes that may potentially mediate or modify the association between the microbiome and influenza virus infection. I would also like to use host transcriptomics to characterize microbiome-associated immunomodulation during influenza virus infection. With direct measurements of the host immune response, we could learn more about the underlying biological mechanisms that link the microbiome to influenza susceptibility, viral shedding, and severity.

5.3.5 Interactions between Respiratory Viruses

I would like to explore whether interactions between respiratory viruses are mediated through the microbiome. A recent study reported interference between respiratory syncytial virus and human RSV infection in infants [20]. While there are likely direct mechanisms of interaction between these respiratory viruses, I am interested in investigating whether virus-virus interactions are also mediated by the microbiome. As the relationship between host immunity and the

microbiome is bi-directional, infection with one respiratory virus may lead to perturbations of the microbiome. These perturbations could lead to microbiome-related immunomodulation and alter susceptibility to other viruses. Murine experiments have shown influenza virus infection can lead to intestinal injury. After inoculation with influenza, lung-derived T-cells are recruited into the gut where they produce IFN-γ. IFN-γ then disrupts the gut microbiome, leading to an increased production of IL-15 and Th17 cells. Th17 promotes inflammation and neutrophil recruitment, which contributes to intestinal injury [21]. Interestingly, we also see similar microbiome-related upregulation of interferons and neutrophils during human RSV infections [22]. This suggests microbiome-related immunomodulation may contribute to altered susceptibility to other viruses. However, no study has yet investigated whether microbiome-related immunomodulation may contribute to viral interference. These types of studies could help in predicting downstream consequences of public health interventions. For example, if an RSV vaccine is introduced, should we expect changes in incidence of other respiratory viruses and can we develop synbiotic interventions to attenuate this risk?

5.4 Conclusions

In conclusion, this dissertation examined three potential relationships between influenza virus and the respiratory microbiome. We found the respiratory microbiome is associated with susceptibility to influenza virus infection. Further, our analysis suggests community dynamics may differ by influenza status. Lastly, we observed associations between the microbiome and the development and duration of influenza symptoms as well as the duration of viral shedding. Our findings indicate the respiratory microbiome may be a potential target for reducing influenza risk, household transmission, and disease severity.

Figure 5.1 Potential systematic bias at various stages of microbiome study

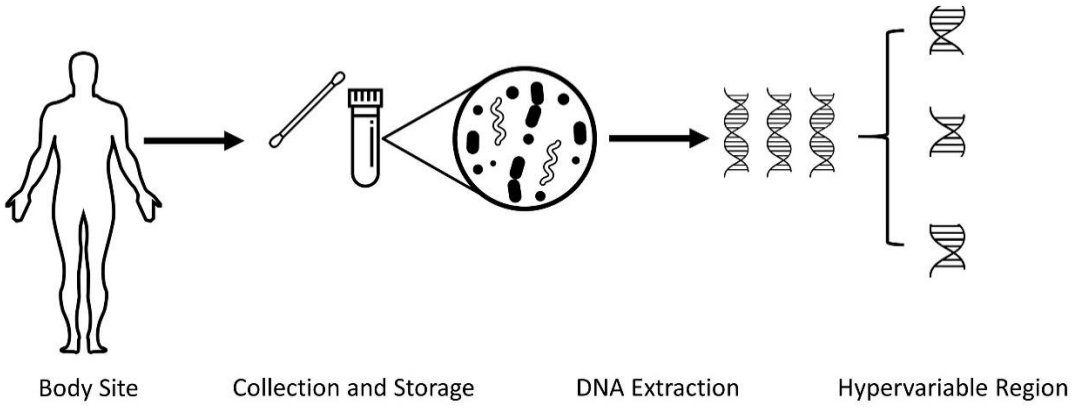
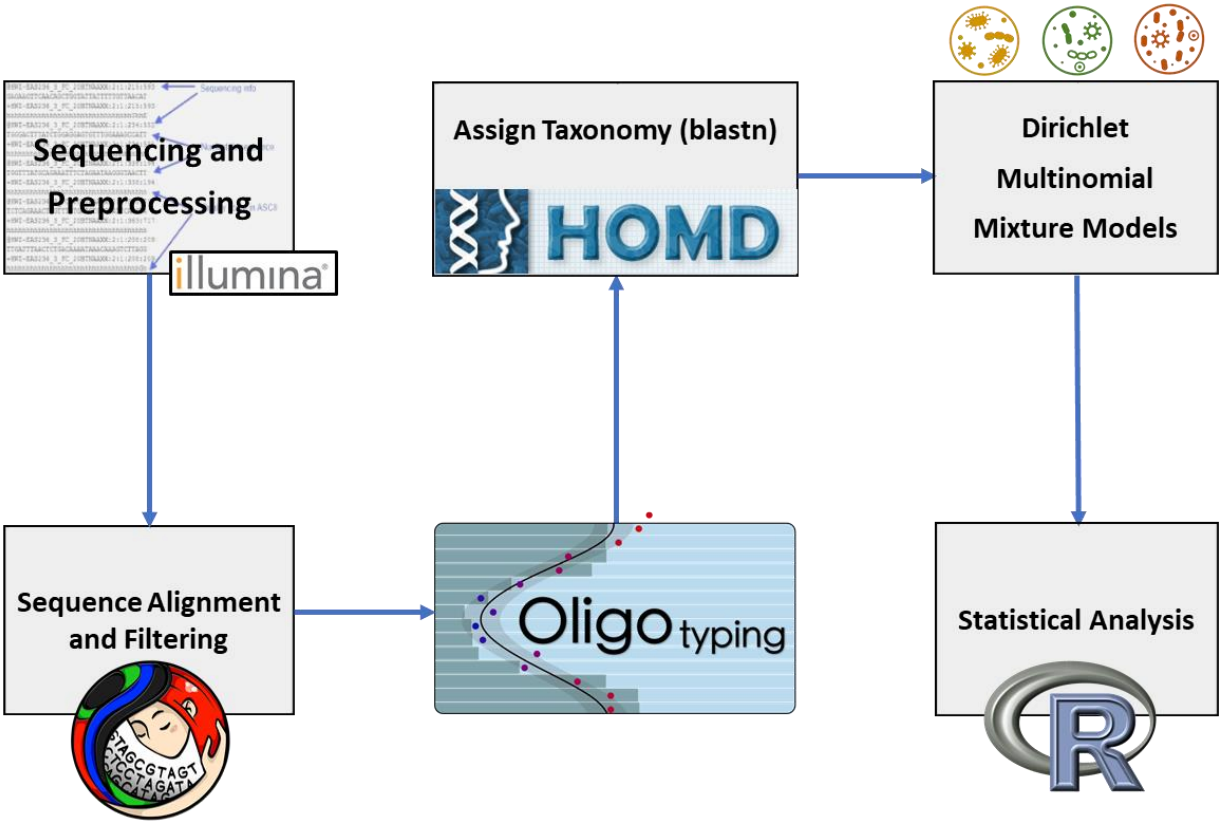


Figure 5.2 Established pipeline for microbiome studies



5.5 References

1. The Human Microbiome Project Consortium. Structure, Function and Diversity of the Healthy Human Microbiome. Nature **2012**; 486:207–214.
2. Boeck ID, Wittouck S, Wuyts S, et al. Comparing the Healthy Nose and Nasopharynx Microbiota Reveals Continuity As Well As Niche-Specificity. Front Microbiol **2017**; 8. Available at: https://www-ncbi-nlm-nih-gov.proxy.lib.umich.edu/pmc/articles/PMC5712567/. Accessed 26 January 2018.
3. Schenck LP, Surette MG, Bowdish DME. Composition and immunological significance of the upper respiratory tract microbiota. FEBS Lett **2016**; 590:3705–3720.
4. Goodrich JK, Di Rienzi SC, Poole AC, et al. Conducting a Microbiome Study. Cell **2014**; 158:250–262.
5. Luo T, Srinivasan U, Ramadugu K, et al. Effects of Specimen Collection Methodologies and Storage Conditions on the Short-Term Stability of Oral Microbiome Taxonomy. Appl Environ Microbiol **2016**; 82:5519–5529.
6. Yuan S, Cohen DB, Ravel J, Abdo Z, Forney LJ. Evaluation of Methods for the Extraction and Purification of DNA from the Human Microbiome. PLOS ONE **2012**; 7:e33865.
7. Tremblay J, Singh K, Fern A, et al. Primer and platform effects on 16S rRNA tag sequencing. Front Microbiol **2015**; 6. Available at: http://journal.frontiersin.org/Article/10.3389/fmicb.2015.00771/abstract. Accessed 8 September 2015.
8. Kim M, Morrison M, Yu Z. Evaluation of different partial 16S rRNA gene sequence regions for phylogenetic analysis of microbiomes. J Microbiol Methods **2011**; 84:81–87.
9. Chakravorty S, Helb D, Burday M, Connell N, Alland D. A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. J Microbiol Methods **2007**; 69:330–339.
10. Foxman B, Luo T, Srinivasan U, et al. The effects of family, dentition, and dental caries on the salivary microbiome. Ann Epidemiol **2016**; 26:348–354.
11. Shade A. Diversity is the question, not the answer. ISME J **2017**; 11:1–6.
12. Bosch AATM, Levin E, van Houten MA, et al. Development of Upper Respiratory Tract Microbiota in Infancy is Affected by Mode of Delivery. EBioMedicine **2016**; 9:336–345.
13. Teo SM, Mok D, Pham K, et al. The infant airway microbiome in health and disease impacts later asthma development. Cell Host Microbe **2015**; 17:704–715.
14. Cauchemez S, Donnelly CA, Reed C, et al. Household transmission of 2009 pandemic influenza A (H1N1) virus in the United States. N Engl J Med **2009**; 361:2619–2627.

15. World Health Organization. WHO Manual on Animal Influenza Diagnosis and Surveillance. Geneva, Switzerland: World Health Organization, 2002.

16. Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team, Dawood FS, Jain S, et al. Emergence of a novel swine-origin influenza A (H1N1) virus in humans. N Engl J Med **2009**; 360:2605–2615.

17. Vijay-Kumar M, Aitken JD, Carvalho FA, et al. Metabolic syndrome and altered gut microbiota in mice lacking Toll-like receptor 5. Science **2010**; 328:228–231.

18. Chang PV, Hao L, Offermanns S, Medzhitov R. The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition. Proc Natl Acad Sci U S A **2014**; 111:2247–2252.

19. Zelante T, Iannitti RG, Cunha C, et al. Tryptophan catabolites from microbiota engage aryl hydrocarbon receptor and balance mucosal reactivity via interleukin-22. Immunity **2013**; 39:372–385.

20. Achten NB, Wu P, Bont L, et al. Interference Between Respiratory Syncytial Virus and Human Rhinovirus Infection in Infancy. J Infect Dis **2017**; 215:1102–1106.

21. Wang J, Li F, Wei H, Lian Z-X, Sun R, Tian Z. Respiratory influenza virus infection induces intestinal immune injury via microbiota-mediated Th17 cell–dependent inflammation. J Exp Med **2014**; 211:2397–2410.

22. de Steenhuijsen Piters WAA, Heinonen S, Hasrat R, et al. Nasopharyngeal Microbiota, Host Transcriptome, and Disease Severity in Children with Respiratory Syncytial Virus Infection. Am J Respir Crit Care Med **2016**; 194:1104–1115.