HEALTHY AGING AND THE MICROBIOTA

CHARACTERIZING AGE-RELATED CHANGES AND PROTECTIVE FEATURES IN THE UPPER RESPIRATORY TRACT MICROBIOTA ASSOCIATED WITH HEALTHY AGING AND CHRONIC INFLAMMATION

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A Thesis Submitted to the Department of Medicine and School of Graduate Studies in Partial Fulfilment of the requirements for the Degree Master of Science

Master of Science (2022) McMaster University
(Medicine) Hamilton, ON

TITLE:	Characterizing Age-Related Changes and Protective Features in the Upper Respiratory Tract Microbiota Associated with Healthy Aging and Chronic Inflammation
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NUMBER OF PAGES:	xiii, 130

Abstract

Background: The upper respiratory tract (URT) is composed of an array of microbes that can contribute to or protect against upper respiratory infections. Older adults (>65 years) are at greatest risk of acquiring respiratory tract infections, particularly pneumococcal infections, resulting in over 7,000 deaths per year in older Canadians. The increased risk of infection may be linked to diversity, high variability, and a decrease in commensal bacteria residing in the URT microbiota. The impact local inflammation has on microbial communities in the nasal cavity is not well defined. Previous research in our laboratory identified 5 dominant microbial profiles in the anterior nares URT microbiota of healthy adults. We also started building a strain collection of commensal bacteria isolated from the URT that may provide protection against pathogenic bacteria, such as *S. pneumoniae*. Our aim is to characterize microbial changes in the URT microbiota with age-associated changes such as specific health conditions and inflammation to determine which microbes are protective against respiratory infections.
Methods: To approach this, we collected nasal samples from 178 community-dwelling adults (18-102 years) throughout 2020 – 2022. Participants provided an additional nasal sample if they were symptomatic during cold/flu season. We collected 99 nasal samples, and 18 nasal fluid samples from 46 adults, over 3 visitations, diagnosed with either osteoarthritis or rheumatoid arthritis, to measure how local inflammatory markers (GM-CSF, Fractalkine, IL-6, IL-1β and TNF) change the nasal microbiota. The samples were processed with 16S rRNA gene sequencing, and bacterial load was measured using qPCR to determine the total 16S rRNA abundance. Phenotypic and liquid assays determine commensal microbes that exhibit resistance to *S. pneumoniae* colonization.
Results: We identified 7 dominant microbial profiles in the aging nasal cavity, which differed statistically with bacterial load and seasonality. We observed higher movement between these profiles within individuals who experienced a respiratory infection. We identified 8 profiles in an inflamed nasal cavity. The composition of these profiles was associated with changes in DMARDS treatment for adults living with rheumatoid arthritis. The difference between microbial profiles in the nasal cavity is due to higher variation and a diverse microbiota.
Conclusion: In addition to observing compositional changes with bacterial load, seasonality, and inflammation, we identified protective microbial strains residing in the nasal cavity. This allows us to begin identifying secondary metabolites with inhibitory activity against *S. pneumoniae* in the URT microbiota and non – harmful properties to the host, to develop novel drug therapeutics to reduce the risk of pneumococcal infections in older Canadians.

Dedication

This thesis is dedicated to my parents, Anna Wozniak-Boron and Slawomir Boron, and grandparents, Babcia Wanda & Dana and Dziadek Romek.

Mom & Dad; Thank you for encouraging my curiousity in science since I was young and for continuously supporting my ambitious career choices. I love you.

Babcia Wanda; Thank you for watching over me throughout my undergraduate and graduate career. I know you are smiling down on me from the heavens. I love you.

Babcia Dana; Thank you for teaching me strength, to reach for the stars and not to ever give up. I love you.

Dziadek Romek; Thank you for teaching me the meaning of hard work, resilience, courage and how to be independent. I love you.

♥

Acknowledgements

To my supervisor, Dr. Dawn M.E. Bowdish: Thank you for your patience, guidance, and support throughout this project. Thank you for letting me use my curiosity to explore various avenues of this project, and for molding me into an innovative, ambitious, and hard-working early career scientist. Your care for your students is remarkable, and learning from your leadership, team-management, and decision-making skills, whilst cultivating a safe and healthy working environment throughout the duration of the pandemic was exceptional.

To my committee members, Dr. Michael Surette, and Dr. Martin Kolb: Thank you for providing me with the resources I required to make this project a success. Your insight and expertise are greatly appreciated.

To my external examiner, Dr. Jeremy Hirota: Thank you for your curiosity and for serving as an external examiner on my defence. Your questions are always welcomed!

To my lab mates: Thank you for your support, constructive criticism, feedback, knowledge, and challenging questions throughout my master's degree that shaped me into the scientist I am today. Your help never goes unnoticed and many of you inspire me to continuously push back the boundaries of science. Although our coffee runs, scientific discussions and brainstorming sessions were limited due to restrictions, I am grateful for the fun moments we did share and to have worked alongside you all.

To my University of Windsor mentors, Dr. Tanya Noel and Dr. Mika Tomac: Thank you for teaching me the research skills I needed to make this degree a success. Your support meant the world to me.

To my dear friends: I have been grateful to have completed my master's degree with you all by my side and have made friendships for a lifetime. You all inspire me to be a better scientist. To Taylor Bendig: Thank you for pushing me throughout the hardest of times. Your strength inspires me. To Amanda Bakke: Your friendship is a gift throughout this degree, and your motivation inspires me. To Chantelle Castanier: I am forever grateful for having you in my life. Thank you for showing up for me when I couldn't show up for myself. To Clare Edwards & Jenna Benoit: Thank you both for believing in me, for brainstorming sessions and for continuously teaching me new things. To Ruby Hoyland & Molly Hooks: Thank you for your friendship, the laughs and encouragement throughout my degrees. I am so grateful to have you both in my life, near or far.

This journey has not been an easy one, and I have learned many skills (both science and non-science related) that I will remember for a lifetime. Research is not for the faint of heart, and I am proud of my commitment and resilience that I have shown throughout this degree. Thank you all, from the bottom of my heart.

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List of Abbreviations

The following abbreviations were used in additions to those commonly accepted for units of measure and quantities.

ANOVA	analysis of variance
ASV	amplicon sequence variants
BSA	bovine serum albumin
BHI	brain-heart infusion
BGC	biosynthetic gene cluster
CAP	community acquired pneumonia
CRP	c – reactive protein
CRS	chronic rhinosinusitis
DADA2	divisive amplicon denoising algorithm 2
DNA	deoxyribonucleic acid
DMARDS	disease – modifying antirheumatic drugs
DMSO	dimethyl sulfoxide
ESR	erythrocyte sedimentation rate
H_2O_2	hydrogen peroxide
HRV	Human rhinovirus
IL	interleukin
ILI	influenza-like-infection
LPS	lipopolysaccharide
LRT	lower respiratory tract
LTCH	long term care homes
MALDI-ToF MS	matrix – assisted laser desorption/ionization of flight time mass spectrometry
MLF	mucosal lining fluid
NO	nitric oxide
OA	osteoarthritis
OD	optical density
OTU	operational taxonomic unit
PBS	phosphate buffer saline
PCA	principal component analysis
PCoA	principal coordinate analysis
PCR	polymerase chain reaction
PERMANOVA	permutational multivariate analysis of variance
RA	rheumatoid arthritis
ROS	reactive oxygen species
RSV	respiratory syncytial virus

TLR	toll-like receptor
TNF	tumor necrosis factor
URI	upper respiratory infection(s)
URT	upper respiratory tract
ZOI	zone of inhibition

Declaration of Academic Achievement

In accordance with the Guide for the Preparation of Theses at McMaster University, this thesis document is an accurate summary of the research that I have conducted during my time as a master's student. This thesis was written entirely by me with editing input provided by Dr. Dawn Bowdish. Details of my contributions, contributions of other scientists at McMaster University and collaborators are described below:

Chapter 3: I recruited and collected participant samples from the community with the help of Dr. Dawn M.E. Bowdish, Jessica G. Wallace, Braeden Cowborough, Alisa Nykolayeva and Allison E. Kennedy during 2020 – 2021 and Alex Chan, Kayla Zhang, and Sussan Kianpour during 2021 – 2022. Participants were also recruited from collaborators including Drs. Stephanie Atkinson, Nathan Hambly, Maggie Larché, Mark Larché, Monica Maly and Param Nair. I performed all experiments, processed samples, and analyzed and interpreted all the data with the following exceptions: Michelle Shah performed DNA extraction and sent samples for 16S rRNA sequencing at the McMaster Sequencing Facility. Laura Rossi performed DNA amplification and processed the 16S rRNA sequencing data through DADA. I used Jake Szamosi's exploratory graphics package for sample filtering and preliminary analyses, and Dr. Fiona J. Whelan provided me with a portion of code to identify clusters within the microbiota. Dr. David Bulir analyzed the infection nasal samples during the 2020 – 2021 cold/flu season (Table 1, Appendix A). Dr. Steve Bernier and Blerina Kadiu cultured the nasal and saliva samples in September – October 2020. A total of 99 isolates were

identified from the upper respiratory tract of adults and 49 of these isolates were identified from Blerina Kadiu's MSc thesis work, which are included in this thesis.

Chapter 4: Mohammed Malik and Dr. Dawn Bowdish recruited study participants with knee – osteoarthritis and rheumatoid arthritis. Patient consent, sample processing, and metadata collection was done by Dr. Dessi Loukov, Mohammed Malik, Dr. Allison E. Kennedy and Barbara Baker. Drs. Maggie Larche and Raja Bobba recruited rheumatoid arthritis patients throughout the study and provided clinical care. Dr. Dawn Bowdish and Dr. Dessi Loukov recruited and acquired participant consent for healthy age – and sex – matched controls. Michelle Shah performed DNA extraction and sent samples for 16S rRNA sequencing at the McMaster Sequencing Facility. Laura Rossi performed DNA amplification and processed the 16S rRNA sequencing data through DADA2. I analyzed and interpreted all the data.

Chapter 1: Introduction

1.1 Aging

Aging is a complex biological process. It can be defined as, "the time-related deterioration of the physiological functions necessary for survival and fertility"[1] and is accompanied by a wide variety of molecular and cellular damage. Chronological age is an imperfect measure of a person's aging trajectory as there is considerable variation at which people age. Aging is heterogenous, and not all adults age equivalently. Some 80-year-old adults can have similar or better physical and mental health than a 30-year-old individual[2].

The World Health Organization (WHO) defines healthy aging as, "the process of developing and maintaining the functional ability that enables wellbeing in older age"[3,4] and defines functional ability as, "having the capabilities to enable people to be and do what they value"[5]. This refers to the ability to meet their basic needs, make decisions, be mobile, build and maintain relationships and contribute to society. Multiple factors influence healthy aging including intrinsic capabilities (i.e., mental, and physical health of an individual) and environmental factors (i.e., residence, community, and society)[2]. Respiratory infections can also impact an adult's healthy aging trajectory and their quality of life.

1.2 Respiratory infections and aging

Respiratory infections are a leading cause of morbidity in older adults[6]. Pneumonia and influenza are Canada's 6th leading cause of death and one of the top ten reasons for emergency department visits in Canada[7]. Respiratory infections in older adults can lead to short- and long-term health consequences.

Short-term consequences range from uncomfortable symptoms (e.g., runny nose, fever, chills, body aches, headache, indigestion) to increasingly complicated infections, such as meningitis[6,8]. Long-term health consequences include increased risk of heart attacks and dementia, hospitalizations, decreased physical and mental health[6,7,9].

The aging population is increasingly growing in Canada. Currently, adults aged 60 years or more outnumber children under 5 years of age and by 2050, there will be twice as many adults over 60 years of age than children under 5[10]. Older adults are more vulnerable to respiratory diseases than younger adults, including acute upper respiratory infections, influenza and pneumonia, chronic lower respiratory diseases, and other lung diseases[11]. In 2020, these respiratory diseases caused 23,255 deaths in Canada[11]. Therefore, there are an increasing number of adult's at risk for acquiring respiratory infection, and some adults have better immune responses to respiratory infections than others[12,13].

1.3 Structure, organization, and immunology of the upper respiratory tract

The upper respiratory tract (URT) microbiota is consistently exposed to the external environment and plays an important role in protecting the host from environmental particles and pathogenic bacteria[14,15]. The URT is composed of several compartments which are lined with different types of epithelium, highlighted in Fig 1.1[6]. The anterior nares are lined with non-keratinized skin-like epithelium, changing into stratified squamous epithelial cells without microvilli and then to transitional epithelium with short microvilli before transitioning to the

mid-turbinates[16,17]. The mid-turbinates, also known as middle meatus, are lined with pseudostratified columnar epithelium[18].

Inhaled air via the nasal cavity is humidified, filtered, and warmed prior to reaching the lower respiratory tract (LRT), where gas exchange takes place[14]. The microvilli trap large particles (> 3 mm) from the external environment, and smaller particles (0.5 – 3 mm) including bacteria, are captured by the mucus layering the entire nasal cavity[19]. The ciliated columnar epithelial cells in the respiratory tract protect the host by removing the microbes, microparticles and excess mucus with a sweeping upwards motion[20]. This process is known as mucociliary clearance.

Host immune factors in the URT include the mucus layer, antimicrobial peptides (AMPs) and reactive oxygen species (ROS) as defence mechanisms against harmful bacteria[21]. The mucus layer contains antimicrobial compounds and initiates immune priming in the URT. Immune priming is defined as, "a process by which a host improves its immune defences following an initial pathogenic exposure", ultimately leading to better protection after another infection by the same (or different) harmful bacteria[22]. Commensal bacteria with immunomodulatory properties can prime a host's immune response to ensure quick and efficient defense against pathogenic bacteria[23,24]. The epithelial cells in the nasal cavity secrete AMPs including lysozymes, lactoferrin, and ROS such as hydrogen peroxide (HO) and nitric oxide (NO)[25]. For example, NO diffuses into the cells and destroys intracellular compounds, and activates protein kinase G and guanylyl cyclase to increases the frequency of ciliary beating and mucociliary

clearance in the nasal cavity[26]. AMPs are one of the host's first line of defense in the innate immune system and kills or slows the spread of pathogens[27]. They may also provide the basis for designing novel therapeutic treatments[27]. For example, *Streptococcus pneumoniae* produces hydrogen peroxide, which causes DNA damage to *Staphylococcus aureus*[28,29]. *S. aureus* can sometimes overcome *S. pneumoniae* killing strategies by producing enzymes such as catalase to protect against the ROS[30]. This interaction is also an example of antagonism, defined as the inhibition of one bacteria against another[31,32].

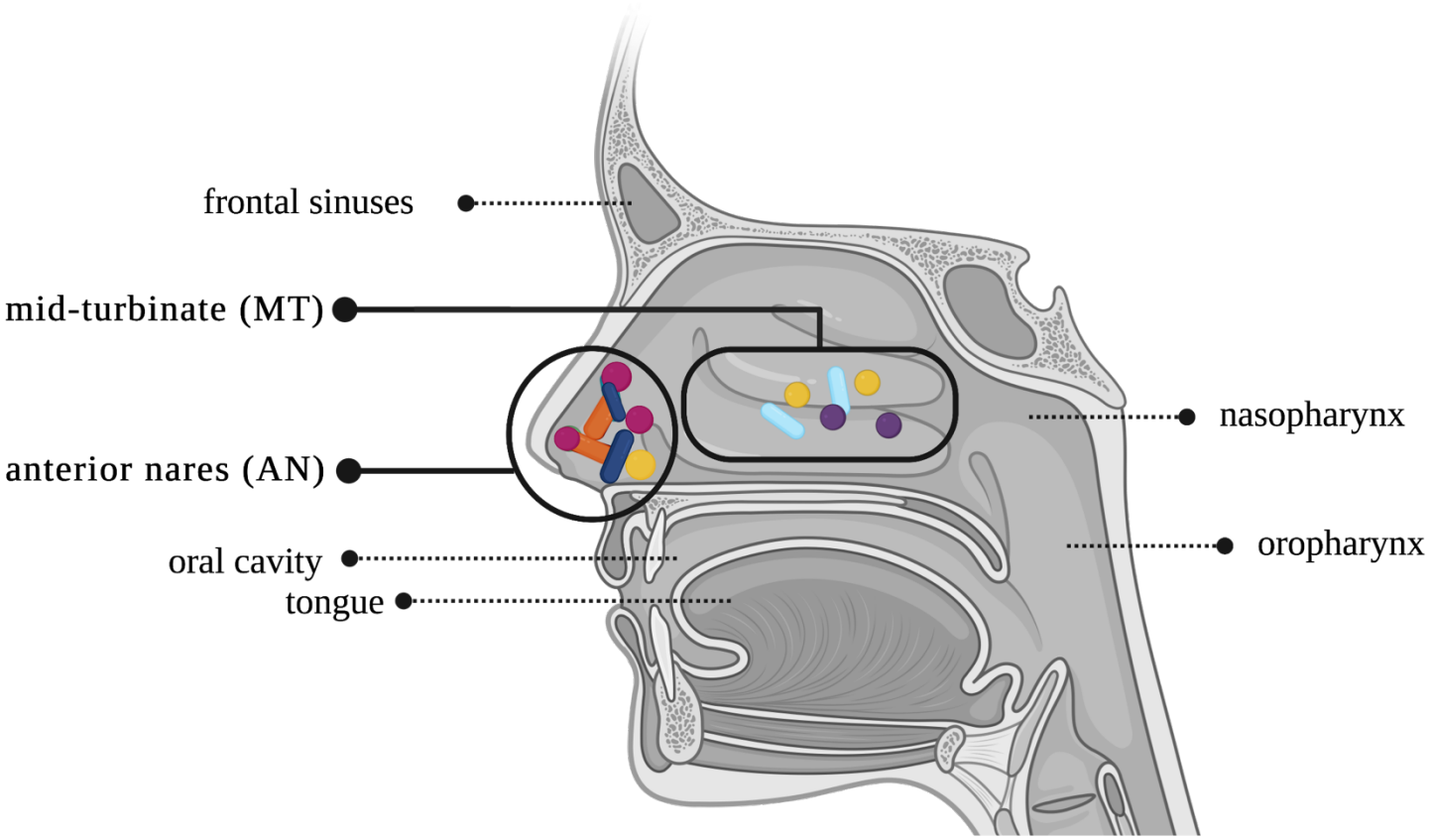


Figure 1.1: The structure of the URT is divided into several components: sinuses, middle turbinates, anterior nares, nasopharynx, oropharynx, and oral cavity. The sampling sites of interest for the purpose of our study are the anterior nares, middle turbinates, and the oral cavity. Adapted from Tai J et al.[33]

1.3.1 Age-related changes in the nasal mucosa

Increasing age is associated with physiological changes in the URT. These changes include less effective filtering and decreased mucociliary clearance due to the slower beating of epithelial cilia resulting in increased mucosal secretion[34]. The host's anatomy, temperature, pH, humidity, oxygen concentration and nutrients (carbohydrates, sugars, free-fatty acids) can all affect the growth conditions of the distinct niches within the nasal cavity, including the anterior nares and mid-turbinates[35]. The nasal mucosa is a growth restrictive food source for bacteria because it does not contain high amounts of essential nutrients, such as amino acids and nucleotides. Some bacteria are better at nutrient acquisition than others, thereby indirectly starving its competitors from essential nutrients such as iron[36]. These physiological and environmental changes can affect commensal bacteria's ability to colonize, interact and compete for space with potential pathogens[37]. Herein I characterized the microbiota of the mid-turbinates in community-dwelling adults, and the anterior nares microbiota of adults diagnosed with osteoarthritis and rheumatoid arthritis.

1.4 Age – related microbial changes in the URT microbiota: health and disease

The composition of the infant URT microbiota is driven by multiple host and environmental factors such as mode of birth, age, infection, antibiotics, and smoking. At birth, the method of delivery (either vaginal or caesarean) determines the composition of the infant's nasopharyngeal microbiota[38]. During the first week of life, the development of a healthy URT microbiota (e.g., nares and nasopharynx) has higher diversity including the abundance of *Staphylococcus aureus*, then by *Corynebacterium pseudodiphteriticum/propinquum*,

Dolosigranulum pigrum, and *Moraxella catarrhalis*/*nonliquefaciens*. *S. pneumoniae* and/or *Haemophilus influenzae*[39]. URT microbiota profiles characterized by higher abundance of *Corynebacterium* spp., and *Dolosigranulum* spp., early in life and *Moraxella* spp., at 4-6 months of age are associated with a stable microbial community and respiratory health[40,41]. A stable microbial community is resistant to pathogen overgrowth and more resilient against infections[35]. An unstable microbial community is less resistant to pathogen overgrowth and is more susceptible to infection and inflammation[35]. For example, children carrying *H. influenzae* and *S. pneumoniae* have a higher risk of acquiring bronchiolitis and respiratory viruses (e.g., enterovirus, influenza, respiratory syncytial virus (RSV))[42,43].

The microbial communities can vary within different niches in the URT of healthy adults. The nasal microbiota of healthy adults is quite diverse and remains stable, with some fluctuations in the presence of infection. The anterior nares are inhabited with a high abundance of *Staphylococcus* spp., *Cutibacterium* spp., and *Corynebacterium* spp. *Moraxella* spp., *Dolosigranulum* spp., and *Streptococcus* spp., have also been seen to inhabit the anterior nares[44,45]. The nasopharyngeal microbial community composition has higher abundance of commensal microbes than the anterior nares because it not constantly exposed to the external environment[18]. The nasopharyngeal microbiota has some similarities to the microbiota of the anterior nares. *Staphylococcus* spp., *Corynebacterium* spp., *Moraxella* spp., commonly colonize both locations but *Dolosigranulum* spp.,

Haemophilus spp., and *Streptococcus* spp, are more commonly found in the nasopharynx[46].

There are fewer longitudinal studies examining the role of the nasal microbiota in health and disease in older adults. The dominant genera within specific nasal niches in older adults are defined but determining if chronological age and chronic inflammation (i.e., inflamm-aging) affect diversity, compositional changes and stability in the nasal microbiota needs to be further elucidated.

Minor differences occur within the nasal cavity of nursing home residents. A smaller study determined the microbial community composition between nursing home and community-dwelling adults are similar at different body sites (i.e., anterior nares, posterior pharynx (throat) and subclavian (neck region) skin). They did find a decreased abundance of *Proteobacteria* (*Pseudomonas, Acinetobacter, Burkholderia* taxa) in nursing home resident's anterior nares and skin microbiota's, suggesting the health care environment is changing the URT microbiota, compared to the community[47]. These differences could also exist because nursing home residents are more frail than community-dwelling adults, suggesting frailty changes the nasal microbiota[48].

α-diversity measures (i.e., Observed ASVs, Shannon and Simpson indexes) the diversity within a sample. This is important to measure the species richness and evenness within an individual and can be compared to other individuals. Observed ASVs measures the number of ASVs within a sample. Shannon index estimates species richness and evenness in a sample, and Simpson index quantifies the variation in the proportion of species in the community in a

sample[131]. Previous research in our laboratory determined that there are no α-diversity differences in the anterior nares microbiota between nursing home residents and community-dwelling adults[47]. Diversity, measured by the Shannon index, increases in elderly patients with pneumonia compared to the healthy older adults, suggesting a-diversity is associated with pneumococcal disease[49].

Inter-individual variation in the nasal microbiota is measured using β-diversity metrics (i.e., Bray-Curtis dissimilarity). Inter-individual variation in the upper respiratory tract microbiota can change with age and disease. Children attending daycare develop frequent respiratory infections leading to a decline in the compositional variation between niches, such as the oropharynx and nasopharyngeal, measured by Bray-Curtis dissimilarities[50]. Contrary to the smaller study described above[47], our laboratory found the microbial composition of the anterior nares in older adults living in long-term care homes (LTCH) changes towards a microbial community resembling the oropharynx, as measured in a PCoA analysis using Bray-Curtis dissimilarities. This suggests the URT microbial structure of older adults is homogenous and dependent on inter-individual microbial variations, compared to community-dwelling adults[51]. Residents of LTCH do not have significant changes in the anterior nares microbial composition associated with chronological age, sex, various comorbidities, vaccination status and residency[51].

It is unclear how other age-related changes (e.g., inflammation, frailty, medications, and vaccinations) impact the URT microbiota and contribute to an increased risk of respiratory infection in older adults. By studying adults who do

and do not acquire respiratory infections, we aim to uncover some protective features in the upper respiratory tract (URT), in the context of the microbiota. A protective feature against respiratory infections can include beneficial bacteria that inhibit the growth of pathogenic bacteria, such as *Streptococcus pneumoniae*. In this thesis, we focus on understanding the nasal cavity microbiota and its associations to healthy aging and respiratory infections.

1.5 Microbiology of the URT

The microbiota is composed of commensal and pathogenic bacteria. Commensal bacteria are defined as harmless or beneficial organisms, that can induce protective responses to prevent colonization and invasion by pathogens[52]. Pathogenic bacteria (e.g., *Streptococcus pneumoniae*) can cause infectious disease and have harmful effects to the host[53]. Pathogens can reside in the URT microbiota without causing harm, and can cause disease if a perturbation (e.g., viral co-infection, wound or immunodeficiency) occurs in the host[54].

The nasal cavity is a challenging environment for microbes to colonize, due to the scarcity in nutrients including glucose, iron, amino acids, and nucleotides[30,36]. These nutrients are essential for bacteria to inhabit and grow in a small environmental niche. The anterior nares are the most acidic and high-salinity portion of the nasal cavity, making conditions even more unfavourable. The most robust and adaptable microbial species can colonize the anterior nares, and other regions within the nasal cavity[30].

1.6 Microbe-microbe interactions within the URT microbiome

Colonization resistance is the ability of the healthy URT microbiota (i.e., commensals) to prevent the colonization of pathogens and maintain homeostatic conditions within the host's nasal epithelial cells and tissues[55]. Microbes use direct (i.e., releasing inhibitory compounds) and indirect (i.e., nutrient competition) colonization resistance as protective features against pathogenic bacteria. Direct colonization resistance involves cell contact and bacterial cells releasing toxins, into its target pathogen[31]. For example, *Corynebacterium* inhibits the growth of *S. pneumoniae* by using triacylglycerol (TAG) lipase, LipS1, hydrolyzing skin and nostril surface TAGs and releasing oleic acid, amongst other free fatty acids (FFA) which inhibits pneumococcus[56,57]. Co-colonization of *Dolosigranulum pigrum* and *Corynebacterium pseudodiphtheriticum* effectively inhibit the growth of *S. pneumoniae*, suggesting this is due to nutrient competition in the nasal cavity and, as of yet, undefined antimicrobial compounds[58]. Indirect colonization resistance occurs when a microbe deprives its target pathogen from accessing nutrients through fast absorption, or sequestering important nutrients[36]. For example, *Corynebacterium propinquum* releases siderophores that can sequester iron from the environment and inhibit the growth of *Staphylococcus* spp., that require iron for growth[36,59]. This interaction is species specific and *S. aureus* is unaffected by *C. propinquum*'s siderophores, which may be due to *S. aureus* having similar siderophores[60].

1.6.1 Streptococcus pneumoniae

S. pneumoniae is a gram positive, extracellular, pathogen that can survive in both aerobic and anaerobic environments[61]. It is a leading cause of community

acquired pneumonia (CAP) in older adults and has a complex relationship with its host [37]. It asymptomatically colonizes the upper respiratory tract microbiota, which is called carriage. Increased transmission of this pathogen can result in micro-aspiration to the lungs, thereby increasing the risk of deathly infections such as pneumonia. Colonization can cause infectious diseases when host and bacterial factors, such as attachment to host nasal epithelial cells, mucosal immune mechanisms and mucociliary clearance, are ineffective. This allows invasion of the bacteria via horizontal dissemination because the endothelial barriers are breached to sterile sites including the bloodstream leading to bacteremia, the lungs causing invasive pneumococcal disease, the middle ear space causing acute otitis media and cerebral spinal fluid leading to meningitis[62,63]. High carriage rates, genetic adaptability, and its ability to shift from a commensal to pathogen all play a role in its ability to cause infectious disease[29]. Age impacts the carriage rates of *S. pneumoniae* in the nasal cavity. The nasal carriage rates of *S. pneumoniae* in children aged 2-6 years old is between 46.4 to 68.6%, whereas healthy adults have a lower carriage rate between 5% to <20%[64,65]. Healthy adults carriage rates are also affected by contact with younger children and frequent, or current, antibiotic usage[29,66]. Comparatively, older adults >65 years of age have carriage rates <10 % of *S. pneumoniae* and are still acquiring respiratory infections, which suggests colonization is transient and proceeds to infection quickly[67].

S. pneumoniae is transmitted through direct contact with carriers, mucus secretions, shedding and viral co-infection (e.g., influenza)[68]. Pneumonia is

responsible for 10 – 20% of deaths in adults, and up to 50% in high-risk groups, including the older population and LTCH residents[66]. The nasal microbiota could play a key role in preventative measures against these deathly infections. We aim to isolate commensal microbes with anti-pneumococcal activity that reside within the nasal cavity of healthy adults and begin to identify the secondary metabolites responsible for this activity by using comprehensive databases. This allows us to take the first step towards developing nasal probiotics to protect older adults from acquiring respiratory infections, such as pneumonia.

1.6.2 Respiratory tract virome

In addition to the human microbiome, the human body is also a host to a large amount and complex diversity of viruses, defined as the "virome".These include bacteriophages that infect bacteria, viruses that infect human cells and other cellular microorganisms (i.e., archaea)[69,70]. The dynamics of the virome across age have not well characterized, but some studies conclude the respiratory tract virome is typically stable within a healthy adult and has high inter-individual variation that is correlated with host variables including diet, age or disease states[71,72]. Large viral communities populate the healthy human lung and respiratory tract, including sputum, nasopharyngeal swabs and bronchoalveolar lavage samples[71]. Human rhinovirus (HRV) is a global endemic viruses and adults have 1-2 rhinovirus infection each year[73]. It may cause severe lower respiratory tract disease in older adults and is a predisposing factor for secondary bacterial infections including otitis media, sinusitis and pneumonia[74]. Recent data determines the nasal microbiota may influence the viral load, host immune

response and clinical symptoms during HRV infection, although the role of the nasal microbiota plays in HRV infection requires further understanding[73].

1.7 Inflammation and the URT microbiota

Inflammation is a defense mechanism to protect against inflammatory stimuli including exposure to pathogens, allergens, toxins and irritants[75]. Chronic inflammation can cause damage to healthy tissues over time, leading to major degenerative diseases in older adults. Aging phenotypes strongly associated with chronic inflammation include changes in body composition (body-mass index) and energy imbalance[76,77]. Proposed mechanisms for age-associated chronic inflammation includes impaired cellular functions resulting in excess ROS, accumulation of senescent cells and dysregulated immune pathways[76,78].

"Inflammaging" or age-associated inflammation is a low-grade chronic proinflammatory state that increases with age[79,80]. Inflammaging is characterized by increases in pro-inflammatory cytokines in the circulation and tissues. The cytokines tumor necrosis factor (TNF), interleukin-6 (IL-6), interleukin-1b (IL-1b) and the acute phase C-reactive protein (CRP) are the most commonly measured. These cytokines are specific measures of inflammation and are shown to increase with age[81]. CRP is non-specific measure of inflammation and is produced by the liver in response to IL-6 and is commonly used in clinical practice[82]. Higher than age-average levels of inflammatory markers are frequently associated with late-life health issues and premature mortality presumably due to increased cellular and molecular damage[83,84].

Most of our knowledge of how inflammation impacts the microbiota comes from studies in the gut. The gut microbiota plays a role in age-associated inflammation. Microbial dysbiosis contributes to intestinal permeability, translocation of bacterial products, and decreased macrophage function, at least in mice models[83]. Microbial dysbiosis is defined as changes in the proportion of beneficial to pathogenic microbes or increase in proinflammatory microbes[85].

Specifically, age-related dysbiosis is likely more correlated with biological age than chronological age[86]. The gut microbiota is largely shaped by intestinal physiology and intestinal physiology changes with age, such as a decrease in mucin production, creating a thinner and discontinuous layer which can result in an inflammatory response in the gut[87,88]. Physiological changes in the URT mucosa, such as effective mucociliarly clearance, could alter the composition of the URT microbiota and cause an inflammatory response.

Age-associated inflammation impacts the composition of the gut microbiota and TNF is a driver of these changes. In the fecal microbiota of TNF knock-out (KO) and wild type (WT) mice, the Bray-Curtis distances are larger between young and old WT mice, compared to young and old TNF KO mice[83]. There is more dissimilarity between young and old WT mice, indicating TNF is a driving factor of microbial dysbiosis in aged mice models[83].

More specifically, the relative abundance of colitis-promoting microbes in the gut, *Erysipelotrichaceae*, increases with TNF and IL-6[89]. Changes in the abundance of these microbes is likely dependent on inflammation, rather than age. These microbes could change because they have better adaptation to survive

changes in the local redox and nutrient environments, or there is a loss of specific microbial niches during inflammatory responses[90].

With the knowledge we have with studies in the gut microbiota, we hypothesize changes in the URT microbiota could be due to inflammation, rather than age. Currently, the relationship between age-associated inflammation and the nasal microbiota is unclear and characterizing the changes chronic inflammatory conditions have on the URT microbiota aid us in understanding this relationship.

Chronic inflammatory conditions of the respiratory tract (e.g., chronic rhinosinusitis, asthma, and cystic fibrosis) alter the microbial community composition and diversity in the nasal cavity and are correlated with microbial dysbiosis[91]. For example, chronic rhinosinusitis is defined as persistent inflammation of the paranasal sinuses and nasal cavity that lasts longer than 12 weeks, and is correlated with microbial dysbiosis[39,85,92]. Patients with chronic rhinosinusitis have decreased diversity in their URT microbiota which may be due to an outgrowth of anaerobic bacteria, and a relative increase in *Corynebacterium tuberculostearicum*[93]. This bacterium was associated with symptom severity of disease, and its impact on sinus epithelial responses and pathogenic potential was confirmed with the use of mice models, thereby indicating this bacterium contributes to the development of chronic rhinosinusitis[93]. Adults with combined asthma and allergic rhinitis (AR) have a decrease in species richness and evenness, measured by Shannon Index, in their nasal microbial composition, increasing the risk of immune-mediated diseases[41,94]. Specifically, increased relative abundance of *Cyanobacteria, Pedobacter*, *Jeotgalicoccus*, and

Janthinobacterium were associated with poor disease control[94]. These results suggest that alterations to the URT microbiota could result in autoimmune diseases by enhancing respiratory tract inflammatory responses.

Changes in microbial communities have also been shown to occur in diseases characterized by systemic inflammation such as rheumatoid arthritis and osteoarthritis, however these studies have been primarily focused on the gut microbiome[95,96]. We hypothesize that some of the microbial communities changes in the URT microbiota may not necessarily be due to chronological age but rather the inflammation that accompanies aging. To test this hypothesis, we characterized the cytokine milieu of the URT of adults living with osteoarthritis, compared to healthy controls, and assessed whether there were changes in the URT microbiota that correlated with nasal inflammation. We also characterized the composition of the URT microbiota in patients with rheumatoid arthritis and assessed whether the microbiota changed with response to disease – modifying antirheumatic drug (DMARDS) treatment and reductions in clinical markers of inflammation (erythroid sedimentation rate (ESR) and CRP levels).

1.8 Rationale, Hypothesis and Aims

Older adults greater than 65 years of age have the highest risk for acquiring respiratory infection and can lead to death[1,37]. We aim to characterize age-related changes, identify protective features of the nasal microbiota, and further clarify how age-associated inflammation changes microbial diversity and community composition. The nasal microbiota is understudied in the aging population and can inhabit protective microbes to reduce the risk for respiratory infections in older adults. The URT microbiota undergoes changes in the composition and host-microbe interactions from birth to middle aged adults, and these changes need to be further characterized within the aging population. Using 16S sequencing analysis, we will be able to investigate the role of chronological age and systemic inflammation in the nasal microbiota by identifying changes in microbial composition and diversity. Additionally, we are identifying specific amplicon sequence variants (ASVs) in adult's nasal cavity that do not receive a respiratory infection during cold/flu season, to determine if they are protective. Using culture-dependent microbiology techniques, we will complement these findings by identifying isolates with growth inhibiting properties against primary pathogens, such as *S. pneumoniae*, in the oral and nasal cavities. These characterizations will allow us to further understand age-associated changes and protective features in the URT microbiota, to improve the quality of life in older Canadians.[97]

Hypothesis: Increased chronological age and local inflammation leads to decreased diversity and increased inter-individual variation in the microbial composition of the mid-turbinate microbiota.

Aims:

1. Characterize chronological age, health, and seasonal changes in the mid-turbinate microbiota, and protective features against respiratory infections. (Chapter 3)
2. Investigate microbial contributions to colonization resistance by characterizing inhibitory activity of commensal microbes of the URT against *Streptococcus pneumoniae*. (Chapter 3)
3. Characterize age-associated inflammatory changes in adults' anterior nares microbiota diagnosed with chronic inflammatory conditions (OA and RA). (Chapter 4)

Chapter 2. Materials & Methods

2.1 Ethics Statement

The use of human-associated microbes for culture-dependent work was approved by the Hamilton Integrated Research Ethics Board and written informed consent was obtained from all participants before sample collection (Aging nasal microbiome participants: #11379, osteoarthritis controls: #1949, osteoarthritis participants: #15-021 and rheumatoid arthritis participants: #14-528T).

2.2 Characterizing age, season, and infection changes in the mid turbinate microbiota

We are conducting a longitudinal study from 2020-2023 to assess age, season, and infection changes in the URT microbiota (Figure 2.1). We recruited community-dwelling participants from Hamilton, ON and the Greater Toronto Area between August and December 2020. Participants completed a questionnaire to collect data on age, sex, transplant history, long-term health conditions, smoking status, recent infection, antibiotic use, vaccination history (*S. pneumoniae* and COVID-19), contact with children, and frailty. Participants self-collected a nasal swab and saliva sample during Baseline sample collection (September – December 2020). A nasal swab and a nasosorp sample were collected during Baseline sample collection the following year (September – December 2021) to determine how local nasal cytokine levels impact the nasal microbiota. Participants were instructed to provide a nasal swab if displayed any symptoms of infection during the cold/flu season between September – May 2020-2022. Lastly, a nasal swab was provided during the follow-up sample collection period (June – August 2021 and 2022). A PCR panel of viruses and bacteria (rhino/enterovirus, influenza A & B, respiratory syncytial (RSV) A & B,

parainfluenza 1 – 3, adenovirus, metapneumovirus, SARS – CoV – 2, coronavirus, bocavirus and *S. pneumoniae*) was used to analyze the infection samples (n = 12) collected during the 2020-2021 cold/flu season by Dr. David Bulir's laboratory to determine if the infections were bacterial or viral.

Sampling of the mid-turbinates was performed by self-administering a nasal swab (Copan FLOQSwabs; Copan Diagnostics Inc., Murrieta, CA) of the right and left mid-turbinate. Participants were taught to obtain nasal swabs from the sample site via pamplets, an in-person demonstration, and videos. Swabs were submerged in transport medium (Copan UTM-RT 330C 3mL LOT #2107368; Copan Diagnostics Inc., Murrieta, CA) and stored at -80°C until processing. Saliva was collected with a salivette (Sarstedt Inc., Thermo Fisher Scientific., Toronto, ON) and was kept chilled on ice until elution by centrifugation (1400 rpm for 5 minutes), aliquoted (0.350 µL) into microcentrifuge tubes and stored at -80°C. Nasosorp samples were collected by inserting a nasosorp (Nasosorption FX, LOT # HMD-20-15340; Hunt Developments (UK) Ltd, West Sussex, UK) against the cartilage of one nostril for 60 seconds. The nasosorp was inserted into the capsule and stored at -80°C.

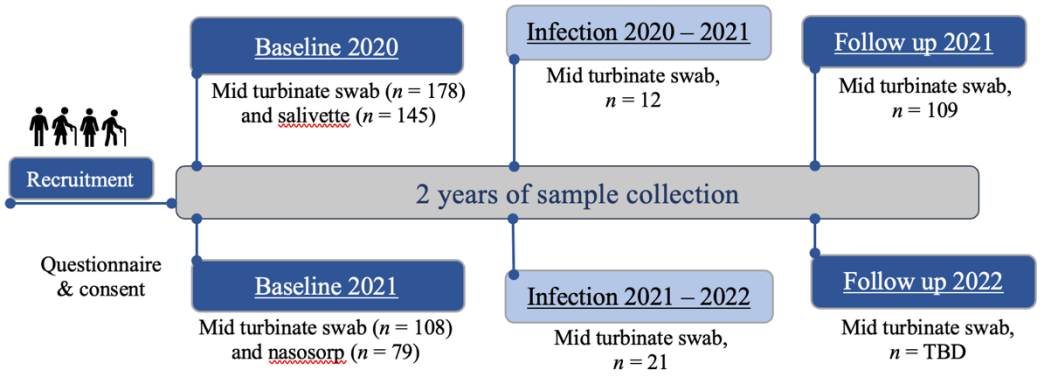


Figure 2.1 Longitudinal study timeline for mid turbinate sample collection. Samples were collected between 2020 – 2022. Baseline samples were collected during the Fall season (September – December), Infection samples were collected if a participant had cold/flu like symptoms and follow-up samples were collected during the Spring/Summer season (April – August). Age ranges from 18 – 102 years. Community-dwelling adults and residents in long term care homes were recruited.

2.3 Characterizing age and inflammation changes in the anterior nares microbiota

To determine how local nasal inflammation impacts changes in the nasal microbiota, we collected anterior nares swabs and nasosorp samples from women (51-84 years of age), with knee osteoarthritis in 2015 (Figure 1.2). Sampling of anterior nares was performed using self-administered nasal swabs (Copan eSwab, 481C; FisherScientific, Mississauga, CAN) and nasosorps (Nasosorption FX, LOT # HMD-20-15340; Hunt Developments (UK) Ltd, West Sussex, UK). A healthy age- and sex-matched control was recruited for each osteoarthritis participant. Participants consented to provide a nasal swab and nasosorp sample when they were recruited for a strengthening intervention exercise study as per methods described[98]. Samples were collected to measure elevated local nasal inflammation by measuring pro-inflammatory cytokines. The nasal fluid was eluted from the nasosorps as per the "20.2 Mucosal lining fluid elution from

synthetic absorptive matrix" protocol in the "Bowdish SARS-CoV-2 Level 2+ SOP" binder. Cytokine concentrations were measured using a customized immunological multiplex assay (MILLIPLEXâ). Cytokine concentrations (GM-CSF, fractalkine, IFNg, IL-10, IL-1b, IL-6 and TNF) were measured in pg/mL with the xPONENT MAGPIX program from Milliporeâ. Limits of detection were determined by the program and are as follows: fractalkine (<19.54 pg/mL), IFNg (<0.42 pg/mL), IL-10 (<0.71 pg/mL), IL-16 (<0.08 pg/mL) and TNF (<0.21 pg/mL). 98% of samples had IFNg and IL-10 concentrations at, or below the level of detection therefore these concentrations were excluded from further analyses. The 5 remaining cytokine concentrations (GM-CSF, fractalkine, IL-1b, IL-6 and TNF) were normalized by log transformation.

We conducted a clinical study with rheumatoid arthritis participants in 2015 – 2016, over 6 months of DMARD treatment to determine if decreasing inflammation would impact the composition of the URT microbiota. Rheumatoid arthritis participants (18 years +) were recruited, and consent was acquired by a clinical research nurse following their scheduled visit with Dr. Maggie Larché or Dr. Raja Bobba. Eligibility of rheumatoid arthritis participants included diagnosis by the 2010 American College of Rheumatology criteria, and not received DMARDs, nonsteroidal anti-inflammatory drugs (NSAIDs) or oral corticosteroids. Anterior nares swab (Copan eSwab, 481C; FisherScientific, Mississauga, CAN) samples were collected from patients at 3 timepoints: diagnosis (baseline), 3 – month post diagnosis (visit 1) and 6 – months post-diagnosis (visit 2) (Figure 2.2). Clinical erythroid sedimentation rate (ESR) and

C-reactive protein (CRP) measurements were performed on same day. Patient medications were documented at each visit. A health and immune questionnaire (Appendix B) data on long-term health conditions, smoking status, clinical blood work, medications, vaccination history, and contact with children was collected. Each rheumatoid arthritis patient was matched with a healthy control of the same sex within 1 – 2 years of their age, without any chronic conditions, joint disorder, or arthritis.

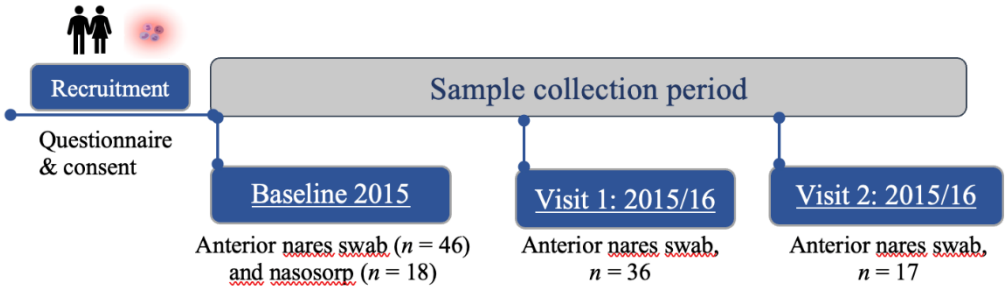


Figure 2.2 Longitudinal study timeline for rheumatoid arthritis and osteoarthritis sample collection. Samples were collected between 2015 – 2016. Baseline samples were collected at the first appointment, visit 1 and 2 samples were collected 2-8 months post – treatment. 26/46 Baseline samples were collected from participants with osteoarthritis (n = 20 had rheumatoid arthritis). 14/36 Visit 1 samples were collected from participants with osteoarthritis (n = 22 had rheumatoid arthritis). All visit 2 samples were collected from participants with rheumatoid arthritis. Participants with osteoarthritis were female and ages range from 51-84. Participants with rheumatoid arthritis have ages range from 29 – 73.

Lastly, we conducted an aging nasal microbiome study from 2015 – 2017 to assess compositional changes in the URT microbiota with host immune factors (Figure 2.3). I used this dataset to establish the composition of the anterior nares microbiota, and to expand the control group for the inflammatory condition (osteoarthritis/rheumatoid arthritis) dataset. We recruited community – dwelling

participants (18+ years) from Hamilton, ON between August – July 2015-2016 and administered a health and immune questionnaire, and written consent prior to sample collection. A health and immune questionnaire were administered prior to sample collection. It collected data on age, sex, transplant history, long-term health conditions, smoking status, recent infection, antibiotic use, vaccination (*S. pneumoniae*) history, contact with children, and frailty to determine how these host factors alter the composition of the URT microbiota. Participants were asked to provide a nasal swab and saliva sample before cold and flu season (Baseline, September – January 2015-2016), if they have any symptoms of infection (September – May 2015-2016) and at the end of cold and flu season (Follow-up, May – October 2016). Sampling of anterior nares was performed using self-administered nasal swabs (Copan eSwab, 481C; FisherScientific, Mississauga, CAN). Participants were taught to obtain nasal swabs from the sample site. Swabs were submerged in transport medium (Copan eSwab transport system liquid amies LOT #1803092; FisherScientific, Mississauga, CAN) and cryopreserved at -80°C until they were processed and sequenced. Saliva was collected with an oral swab (Oracol +, Malvern Medical Developments Ltd., Worcester UK) and was kept chilled until elution by centrifugation (1400 rpm for 5 minutes), aliquoted (0.350 µL) into microcentrifuge tubes and cryopreserved at -80°C until further use. Nasosorp samples were collected (n = 55) during the baseline sample collection in 2015 to determine how immune factors impact the composition of the anterior nares region. These samples were used as healthy

controls for individuals with osteoarthritis that donated nasosorp samples. The nasosorps were collected and processed as described above.

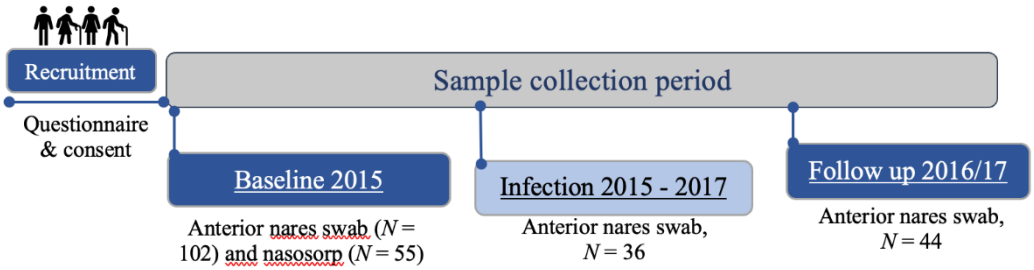


Figure 2.3 Longitudinal study timeline for anterior nares sample collection. Samples were collected between 2015 – 2017. Baseline samples were collected during the Fall/Winter season (September – January), infection samples were collected if a participant had cold/flu like symptoms and follow-up samples were collected 5-12 months after the Baseline sample. Age ranges from 20 – 100 years. Community dwelling adults were recruited.

2.4 Culture – independent methods

2.4.1 16S rRNA gene sequencing

DNA isolation was performed using the bead beating method as previously described[99]. 800mL of 200mM of monobasic NaPO4 (pH = 8) and 100mL of guandine thiocyanate, EDTA and N- laurylosarokine are added to a 2 mL plastic container containing 0.2 g of 0.1 mm glass beads. 300mL of the UTM media and the tip of the nasal swab or 300uL of the saliva sample are aliquoted into the container for mechanical lysis for 3 min at 3000 rpm to homogenize the sample. Enzymatic lysis was performed by adding 50mL lysozyme (100mg/mL) and 10mL RNase A (10mg/mL in H_2O) was mixed and incubated in 37°C between 1 to 1.5 hours. Afterwards, 25uL of 25% SDS, 25uL of Proteinase K and 62.5mL of 5M NaCl was added. The samples were mixed and incubated in 65°C between 0.5 to 1.5 hours. A phenol – chloroform extraction was performed, to separate proteins from the DNA. The samples were centrifuged at 13,500g for 5

min, 900mL of both phenol-chloroform-isoamyl alcohol and the supernatant are added to the samples and centrifuged at 13,000g for 10 min. The DNA was purified through a column by adding the supernatant to 200mL of DNA binding buffer and mixed prior to transferring it to a DNA column and centrifuged at 12,000 g for 30 sec. The DNA was washed twice by adding 200mL of wash buffer to the column and spun at 12,000 g for 30 sec. Lastly, the columns are placed into a new 1.75mL Eppendorf tubes by adding 50mL of sterile DNase/RNase free ddH_2O, incubating at room temperature for 5 min prior to eluting the DNA into the tubes by centrifuging the columns at 12,000 g for 1 min. Finally, the DNA was quantified using a spectrophotometer and was stored in a -20°C freezer in preparation for amplification and sequencing.

DNA amplification was performed using polymerase chain reaction (PCR) at the 16S rRNA variable region 3 (V3) and variable region 4 (V4). The anterior nares nasal swab samples were amplified at the V3 region using Illumina adapted forward and reverse primers for 30 cycles (94°C for 30 sec, 50°C for 30 sec and 72°C for 30 sec, with a final extension of 72°C for 10 minutes) as previously described[100]. The mid turbinate nasal swabs samples and saliva samples were amplified at the V3 – V4 region using Illumina adapted forward and reverse primers for 30 cycles (94°C for 30 sec, 50°C for 30 sec and 72°C for 40 sec, with a final extension of 72°C for 10 minutes)[100]. The resulting PCR products were normalized using the SequalPrep normalization kit and pooled for sequencing using the Illumina MiSeq sequencing platform at the Farncombe Genomics Facility at McMaster University, Hamilton ON. Single read analyses were

performed on the R2 read to compare the V3 region data of both the nasal and saliva samples prior to filtering and trimming adapter sequences with CutAdapt[101]. Further processing and filtering of low-quality reads was performed with the Divisive Amplicon Denoising Algorithm 2 (DADA2) pipeline, determining error rates and sequence variants with machine-learning[101,102]. The sequence variants were organized into amplicon sequence variants (ASVs), generating an ASV table. Bimeras were removed and taxonomy was assigned using the DADA2 implementation using the SILVA database (v.1.3.2) prior to data analysis[99].

2.4.2 16S rRNA data analysis, cluster organization and statistical analysis

The mid turbinate, anterior nares and saliva microbiota analysis was completed using R (v.4.0.2) in R Studio. The data was organized using dplyr (v.1.0.0)[103], tidyr (v.1.1.0)[104], tidyverse[105], microbiome (v.1.12.0)[106], and phyloseq (v.1.32.0)[107] packages. The data was cleaned by removing samples with a read depth less than 4000. The mid-turbinate nasal samples were rarefied at 4073 sequencing depth, and the samples have a sequencing depth ranging from 4073 to 254,930 reads. The anterior nares samples were rarefied at 4117 sequencing depth, and the samples have a sequencing depth ranging from 4117 to 245,412 reads. a-diversity metrics included Shannon and Simpson Indices and the total number of unique ASVs (i.e., Observed ASVs) in each sample. Statistical significance was assessed by using a linear mixed-effect model with the lme4 package (v 1.1-30)[108]. Age, sex, bacterial load, number of inflammatory conditions, number of medications, sampling time, smoking status, vitamin D consumption, pneumococcal vaccination status, pre-frailty assessment and contact

with children are fixed effects and the participant ID is the random effect to account for 1-3 samples donated per each participant. The Bonferroni test was used for multiple-comparisons correction because several independent variables were included in the linear mixed-model. Post-hoc analyses were performed using the emmeans (v.1.7.5)[109] package. b-diversity was assessed using the Bray-Curtis dissimilarity metric and visualized by Principal Coordinates Analysis (PCoA) using the phyloseq (v.1.32.0)[107] and ggplot2 (v.3.3.2)[110] packages and statistical significance was determined by PERMANOVA using the adonis function in the vegan package (v 2.6-2)[111].

To determine the optimal number of clusters in the mid turbinate and anterior nares microbiota, I used the package NbClust (v.3.0)[112]. This package uses 30 indices to determine the optimal number of clusters. Bray-Curtis Dissimilarities were used to measure the distance between each sample in the dendrogram, with the hierarchical clustering method. For the purpose of this analysis, a "cluster" is defined as any group of 3 or more individuals that have the same dominant ASV. With the exception of the mixed cluster, the clusters contain microbial communities that are typically dominated by a single ASV. This definition was determined after hierarchical clustering was performed on the dataset. Python programming software (v.3.8) was used to measure the dissimilarities within and between each cluster. A dendrogram and taxonomic bar plot was used to visualize the clusters, both at the genus and ASV level. These plots were combined using the patchwork (v.1.1.1)[113] package. A differential abundance analysis was performed on the mid turbinate and anterior nares

baseline samples, to determine if ASVs were associated with the variables listed above. A Principal Components Analysis (PCA) was performed on individuals that donated nasosorp samples, and Principal Components (PC) 1 and 2 were used in a differential abundance analysis, as continuous variables, to determine if nasal cytokine concentrations were associated with protective ASVs in the nasal cavity. This analysis is further outlined in Chapter 4. The DESeq2 (v.3.0)[114] package was used for all differential abundance analyses.

2.5 Bacterial load and *S. pneumoniae* quantification

2.5.1 16S rRNA gene quantification

Total bacterial loads in the mid turbinate and anterior nares samples were determined using quantitative PCR (qPCR) of the bacterial 16S rRNA gene using optimized concentrations of the forward and reverse primers (Table 1.2). qPCR was performed using extracted purified genomic DNA (1 µL) from all nasal and oral samples, SsoFastä EvaGreenâ Supermix, #1725204 (10µL), forward and reverse primers (0.1 µL), BSA (bovine serum albumin) (10mg/mL, 1µL) and distilled H_2O (7.8 µL). A negative control with no genomic DNA was included with each PCR run. Additional negative controls included genomic DNA extracted from the UTM media, the UTM media and a sterile nasal swab. A 5-fold dilution series with purified genomic DNA of *Escherichia coli* (*E. coli*) between 0.001 ng to 100 ng was used to create a standard curve. Amplification of each sample and negative control was performed in duplicate. The PCR amplification ran for 42 cycles (98°C for 2 minutes, 98°C for 5 sec, 60°C for 5 sec and a melt curve set to 0.5°C increments for 5 seconds each from 65°C to 95°C).

2.5.2 lytA gene quantification

The presence of *S. pneumoniae* in the nasal samples was determined by quantifying the *lytA* gene signal via PCR using pre-optimized concentrations of the forward and reverse primers. qPCR was performed using extracted purified genomic DNA (1 µL) from all mid turbinate samples, SsoFastä EvaGreenâ Supermix, #1725204 (10µL), forward and reverse primers (0.1 µL), BSA (10mg/mL, 1µL) and distilled H_2O (7.8 µL) to amplify the *lytA* gene. A negative control without genomic DNA was included with each PCR run. Additional negative controls included:

a. Genomic DNA extracted from the UTM media
b. A self-administered Copan FLOQSwabs submerged in UTM media
c. Whole blood human DNA from a participant.

Positive controls included:

d. Collecting a nasal sample from a participant with a Copan FLOQSwab, submerging UTM media and inoculating the UTM media with a single colony of *S. pneumoniae* (P1121)
e. Inoculating a single colony of *S. pneumoniae* (P1121) into a 3mL UTM tube without a sterile Copan FLOQSwab.

A 5-fold dilution series with purified genomic DNA of *S. pneumoniae* between 0.001 ng to 100 ng was used to standardize values. Each sample and negative control was performed in duplicate. The PCR amplification ran for 39 cycles (98°C for 2 minutes, 98°C for 5 sec, 60°C for 15 sec and a melt curve set to 0.5°C increments for 5 seconds each from 65°C to 95°C).

The bacterial load (16S rRNA copies) and *lytA* copies in the nasal samples was quantified by using the Ct values and standard curve from real-time qPCR. The equation was $10^{(Ct-b)/m}$. The geometric mean of duplicate technical replicates was calculated to determine the bacterial load (16S rRNA) and amount of *S. pneumoniae* (*lytA).*

Table 2.1 qPCR primers

Gene or region	Forward primer	Reverse primer
16S rRNA	926f (5'-AAACTCAAAKGAATTGACGG -3'; 100µM)	1062r (5'-CTCACRRCACGAGCTGAC-3'; 100µM)
lytA	MS87 (5'-ACG CAA TCT AGC AGA TGA AGC-3'; 100µM)	MS88 (3'-TGT TTG GTT GGT TAT TCG TGC-3'; 100µM)
V3	341F (CCTACGGGAGGCAGCAG)	518R (ATTACCGCGGCTGCTGG)
V3-V4	341F (CCTACGGGAGGCAGCAG)	806R (GGACTACNVGGGTWTCTAAT)

2.6 Culture – dependent methods

2.6.1 Culturing commensal microbes from nasal and oral samples

50µL of the saliva was diluted to 10^{-1}, 10^{-2}, and 10^{-3} concentration. 100 µL of the UTM media and saliva samples were pipetted separately onto BHI3+ (supplements listed as per previous protocol[29]) and CBA agar. Glass beads were used to evenly spread the liquid onto the plates and were grown in an anaerobic environment in ANOXOMAT jars (10% CO_2, 5% H_2 and 85% N_2) for 72 hours at 37^{o}C. Pure cultures were obtained by picking single colonies and identifying the microorganisms using a Matrix-assisted laser desorption/ionization of flight time mass spectrometry (MALDI-TOF MS). Duplicate or triplicate glycerol stocks were made with pure bacterial colonies grown in agar, suspended with a sterile

swab in 1 mL 15% glycerol and BHI3+[29]. Microbial glycerol stocks were stored at -80°C.

2.6.2 Liquid and phenotypic assays with commensal microbes against Streptococcus pneumoniae

Isolates were streaked to pure colonies in duplicate BHI plates and grown in aerobic (5% CO_2) and anaerobic conditions in ANOXOMAT jars as described above for 24 and 72 hours, respectively, at 37°C. Isolates were inoculated in either a 96 well plate with 150 µL of BHI3+ broth or on BHI3+ agar plates[115] (depending on the preferred condition) and are incubated aerobically for 24 hours 37°C, under 5% CO_2. Isolated strains of *S. pneumoniae* (P1121, P1547, P1542 and P2140) are grown on BHI agar supplemented with 1000 U/ml catalase to test for genetic variation. Each *S. pneumoniae* strain was inoculated in 3 mL of BHI broth, incubated until an OD_{600} of 0.3 to 0.5, measured with a spectrophotometer (Nanodrop 2000c, Thermo scientific), was reached and spread with a sterile swab on BHI agar to create lawns. The isolates were inoculated with a pin replicator on the *S. pneumoniae* lawns and were incubated aerobically for 24 hours at 37°C, under 5% CO_2 and anaerobically for 24 hour and 72 hours at 37°C, 0% O_2, 10% CO_2, 5% H_2 and 85% N_2. *S. lugdenensis* (GC 1736) was used as a positive control for the phenotypic assays because previous studies in the Surette lab have indicated growth inhibition with P1121[115]. Zones of inhibitions (ZOI) were photographed and measured with a ruler, starting from the edge of the colony, to determine growth inhibition of *S. pneumoniae* with isolates. Isolates displaying clear and consecutive zones of inhibition against the 5 strains of *S. pneumoniae* are prioritized based on interest, size, reproducible activity, and different growth

conditions. This is outlined in more detail in Chapter 5. Similarly, the growth inhibition of metabolic extracts was tested with liquid and phenotypic assays. *S. pneumoniae* (P1121) was grown aerobically for 24H, inoculated in liquid and diluted to OD_{600} 0.05. Four different concentrations (1 – 4%) of the metabolite extract were tested to determine their minimal inhibitory concentration (MIC). Each well contains 1µL of 1000U/mL catalase to prevent the production of H_2O_2, 1uL of *S. pneumoniae* (OD_{600} 0.05), 1 - 4µL of the metabolite extract and the remainder contains BHI broth (94 – 97 µL). Each metabolite concentration was repeated in triplicate. The negative control included different concentrations of DMSO (1 – 4%), BHI, *S. pneumoniae* and catalase to determine cell toxicity and a positive control with BHI, *S. pneumoniae* and catalase is included to determine the growth of *S. pneumoniae* overtime. The 96 – well plate assay is sealed with a transparent membrane and incubated at 37°C for 24H. A Synergy H1 Hybrid plate reader is used to determine the growth inhibition measurements at a single 24H timepoint read at OD_{600}. For phenotypic assays with metabolite extracts, *S. pneumoniae* is grown aerobically and spread evenly on BHI agar plates to create lawns. Once dry, 2mm blank paper disks (6mm diameter; Becton, Dickinson and Company, Le Pont de Claix, France, LOT #0056080) are placed on top of the lawns and 60µL of the metabolite is pipetted onto the disk, 20µL at a time to allow for absorption. DMSO is used as a negative control. Assays are incubated between 24 to 48 hours at 37°C and images are captured using the BIOMIC V3 microbiology system. Isolates were sequenced with Illumina whole – genome sequencing at McMaster's sequencing facility. Isolates were analyzed for

secondary metabolite biosynthetic gene clusters (BGCs) and bacteriocins that may encode anti - pneumococcal activity using antiSMASH[50].

2.6.3 Metabolite extraction from commensal microbial fermentation

Isolates of interest are used to extract metabolites to concentrate the compounds being released and test its ability to inhibit *S. pneumoniae*. A dense lawn was created by spreading the microbial culture of interest and incubated for 24 – 48 H for optimal growing conditions (anaerobic or aerobically). A metal spatula was used to cut the fermented agar in small pieces and transferred into a 50 mL Corningä Falconä conical centrifuge tube #352070, approximately 20 mL of methanol was added to begin the extraction process, and the tubes were left on a spinning wheel for 24 hours. Afterwards, the mixture of fermented agar and methanol (methanol extractions) was collected into glass tubes (16mm X 100 mm) using coffee filters and a glass funnel to prevent agar from falling into the tubes. Next, each tube was weighed and balanced, using methanol, prior to using the SP Genevac (HT Series 3i) for evaporation. Once dry, approximately 500 – 750 µL of dimethyl sulfoxide (DMSO) was added per tube. The extractions were vortexed for 2 minutes at 15,000 rpm, sat at room temperature (37°C) for 24 – 36 hours, and vortexed once more. The DMSO – containing extractions were transferred into 2mL microcentrifuge tubes, spun at 10,000 rpm for 2 to 3 minutes. Once completely dissolved, the extractions were transferred into a clean microcentrifuge tube and labelled accordingly. The extractions were stored at -4°C or -20°C until used for liquid and/or agar assays to test their growth inhibition against *S. pneumoniae.*

Chapter 3: Age, sampling time and bacterial load changes in the URT microbiota and protective features against respiratory infections

3.1 Introduction

Most of our knowledge about how the microbiota changes in late life comes from studies of the gut microbiota. These studies show that α- diversity (measured with Observed ASVs and the Chao Index) is lower in younger healthy adults, compared to healthy long-lived adults (i.e., centenarians). Long-lived adults have a greater community richness in their gut microbiota and are enriched with several potentially beneficial bacteria including members of *Clostridium*, *Bacteroides*, Ruminococcaceae, *Akkermansia* and Christensenellaceae, compared to healthy adults[116]. These bacteria play a role in producing short-chain fatty acids for healthy gut homeostasis and immunomodulation, indicating there is a link between healthy aging and the gut microbiota[117,118].

Inter-individual variation increases with age[116,117]. A PCoA analysis with Bray-Curtis distances determined dissimilarities within the healthy older adults and centenarians are larger than healthy younger adults[119]. The microbial composition also differs between age groups. Healthy young and older adults have more similar compositions, whereas significant differences exist between healthy young adults and centenarians, and healthy older adults and centenarians[119].

Less is known about how the URT changes with age in healthy adults, however in a cross-sectional study, α- diversity does not significantly decrease within the oropharynx of healthy young adults, compared to healthy older

adults[49]. The species richness significantly decreases within older pneumonia patients, compared to their healthy controls, suggesting *Streptococcus* spp. dominate the oropharynx during infection. Another study determined inter-individual variation within the nasopharyngeal microbiota, measured by Bray-Curtis dissimilarities, is lower in frail older adults residing in long-term care homes (LTCH), compared to healthy young adults[51].

Studies of the skin microbiome have determined changes in bacterial species with age. *Cutibacterium* and *Staphylococcus*, common skin commensal bacterium, decrease in abundance within older women's skin (cheek, forehead, and forearm) microbiota aged 60-76 years of age, compared to younger adults 21-37 years of age[120]. *Corynebacterium*, a commensal bacterium with anti-pneumococcal properties, increases in abundance within older women's skin (cheek and forehead) microbiota[120]. Determining how these bacterial species change in the nasal cavity with age and their protective features against respiratory infections requires further assessment.

There are limited studies determining the role bacterial load plays in the aging URT microbiota. Bacteria load is the amount of bacteria present within a sample and can determine how empty or full an environmental niche is, impacting the ability of commensal or pathogenic bacteria to colonize, thereby influencing microbial diversity and composition[121]. A cross-sectional study determined young healthy adults have a lower bacterial load (measured in pg μl^{-1}) compared to healthy older adults in the oropharyngeal microbiota[49]. This could be due to increased inflammation in the aging population and inflammation-induced

acidification of the local environment of the oropharyngeal niche, changing the pH and resulting in an outgrowth of acidophilic species such as lactobacilli[49,122]. The same study determined older pneumonia patients had a higher bacterial load, compared to their healthy controls, determining bacterial overgrowth is associated with disease[49]. The role bacterial load has on infection risk of developing respiratory illnesses within the nasal microbiota of the aging population needs to be further elucidated with longitudinal studies.

Most adults acquire 2 to 3 bacterial or viral infections per year, and older adults have an increased susceptibility to infection[123]. The duration and severity of these infections vary and environmental factors such as indoor crowding, attendance at work (e.g., health care settings) and school, poor ventilation, cold air and increased humidity influence susceptibility to infection[124].

Seasonality is an important driving factor of the compositional variation in the anterior nares microbiota[125]. α- diversity (measured by Shannon, Simpson and Observed ASVs) is highest in the Autumn and lowest in the Summer months[126]. Autumn supported a more diverse bacterial community, Winter and Spring months had sub-optimal growing conditions for bacteria and Summer was the least preferred season for bacterial growth[126]. The same study determined the four seasons diverged into 4 clusters, as determined with a β-diversity analysis using a PCoA with Bray Curtis distances and accounts for 22.5% of the variation at the 1st axes[126]. Autumn and Winter seasons were significantly different, whereas Spring and Summer clusters overlapped one another[126]. This suggests bacterial communities diverge during the more extreme temperatures in Winter and

Summer months, and moderate temperatures during Spring and Autumn months have different bacterial communities.

Localized factors such as temperature and humidity influence the microbial composition in the anterior nares microbiota because it is constantly exposed to the external environment[18]. The air quality of the external environment impacts the microbiology of the indoor environment, therefore adult's work environment (e.g., schools, hospitals, retirement homes) can impact their microbial composition because commensal and pathogenic bacteria are associated with different dust sizes[127,126].

Seasonal changes in the microbiota of the anterior nares include varying abundance of 3 prevalent genera. The relative abundance of *Staphylococcus* decreases from 23.3% in the Fall to 7% in the Summer, whereas *Corynebacterium* and *Moraxella* exhibit minor decreases[126]. *Corynebacterium* and *Moraxella* have a relative abundance of 21.5% and 15%, respectively, in the Fall which decreases to a total of 17% for both bacteria in the Summer.

In addition to investigating how seasonality impacts the URT microbiota, understanding association and recovery dynamics during and after an influenza-like-illness (ILI) is also important to define features of the microbiota that can aid in protection against respiratory illnesses, such as pneumonia[128]. An ILI is defined as cold/flu symptoms that are associated to an illness caused by respiratory bacterial or viral pathogens, including but not exclusive to the influenza virus[129].

A longitudinal study determined α- diversity (measured with Faith's Phylogenetic diversity) was higher in the adult nasopharyngeal microbiota 14-

days after ILI, compared to controls and acute ILI, and there was low evenness (measured with Simpson's evenness) in the nasopharyngeal microbiota 14-days after ILI[128]. There was higher inter-individual variation (measured with unweighted, generalized and weighted UniFrac distances) within adults nasopharyngeal microbiota from acute-ILI and at 14 days after ILI, compared to controls which could indicate a potential unstable environmental niche during ILI[128].

The same study determined *Corynebacterium* had a negative association with acute-ILI whereas *Haemophilus, Gemella* and *Porphyromonas* showed a positive association[128]. *Corynebacterium*, *Haemophilus* and *Gemella* were not associated with other factors such as medication, smoking or other health conditions (e.g., cardiac disease, diabetes, kidney transplant, autoimmune disease, and cancer)[128]. *Corynebacterium* increased in abundance after recovery from acute-ILI in adults >60 years of age, suggesting it could have a life-long key role associated with a healthy URT microbiota[128]. Future longitudinal studies exploring *Corynebacterium* mechanistic role in the healthy URT microbiome are required.

A cross-sectional study determined the microbial composition between infected and non-infected adults is significantly different, as measured with a PCoA analysis using Bray Curtis distances[130]. They had also stated that the nasopharyngeal microbiota is stable without disturbances or perturbances associated with influenza infections. The healthy microbiota is disturbed with infections and can cause transient ecological niches for pathogenic bacteria, such as *Pseudomonas*, to colonize[130]. Future longitudinal studies are required to further

investigate how age-related changes (e.g., co-morbidities, medications, frailty) play a role in the URT microbiota and if these features influence susceptibility to infection within the aging population. With this knowledge, we hypothesized there would be a decrease in α-diversity, compositional and ASVs differences in the mid-turbinate microbiota of individuals who did versus did not have an ILI, at the baseline timepoint.

In this chapter, we investigated whether 12 variables (age, sex, bacterial load, time of sample collection, inflammatory conditions, number of medications, vitamin D supplementation, smoking, pneumococcal vaccination status, contact with children, pre-frailty self-assessment score and ILI), impacted the diversity, composition and specific ASVs in the mid-turbinate microbiota.

To determine whether age, sex or other variables changed diversity and compositional changes in the URT microbiota, α- and β- diversity measurements were used in a linear model. α- diversity was measured using the Shannon Index, which quantifies the number of species in a sample (i.e., "species richness") and the Simpson Index which quantifies the variation in the proportion of species in the community (i.e., "species evenness")[131]. A low diversity index indicates the microbiota is dominated by fewer bacterial species, and a high diversity index suggests the microbiota is dominated by an even abundance of various genera[132]. Using both indices' accounts for these differences in measurements. The reasoning for including these variables in our analysis are described below.

Since studies of the gut microbiota have demonstrated that there are sex differences in the composition of the microbiome and sex hormones have been

linked to increased variability between females, we assessed whether there were sex differences in the mid-turbinate microbiota. Males have an increased abundance of *Bacteroides* and *Prevotella* compared to females, which could be due to the contribution of gonadal hormones in males[133]. A mouse study indicates that within periods of declining ovarian hormones in females, microbial communities in the gut have higher variability between mice relative to males[134]. To determine whether sex differences occur in the nasal microbiota, we measure if diversity metrics and ASVs are correlated with sex.

There are limited studies identifying the impact inflammatory conditions (or comorbidities) and medications have on the URT microbiota. A recent study investigating the nasopharyngeal microbiota determined there is a negative association between *Porphyromonas*, a potential pathogenic bacterium, and co-morbidities and respiratory diseases, whereas *Dolosigranulum*, a commensal bacteria, was negatively associated with a number of medications[128]. With this knowledge, we hypothesized there would be compositional and ASV differences between the mid-turbinate microbiota of adults that had more inflammatory conditions, compared to those that had none, and adults that had a higher number of medications, compared to those that had none.

Vitamin D supplementation is believed to alter the composition of the gut microbiota by increasing the expression of the antimicrobial peptide LL-37[135]. Increases in this antimicrobial peptide are associated with reduction in susceptible species and increased abundance of resistant species[136]. In addition, insufficient vitamin D intake frequently coexists with chronic inflammatory status within

older adults[137]. Supplementation with vitamin D is believed to reduce respiratory infection through a variety of immune stimulating pathways including induction of LL-37 which might influence the composition of the microbiota. LL-37 is expressed in the epithelial layers of the lung, therefore we hypothesize that vitamin D supplementation might influence the mid-turbinate microbiota[138].

Smoking decreases mucociliary clearance in the URT and LRT, leading to pathogen colonization and increased infection risk[139,140]. Previous research indicates that smokers have an increased diversity and likely to carry pathogenic bacteria (*S. pneumoniae*, *Streptococcus pyogenes*, *H. influenzae*, and *M. catarrhalis*) in their URT microbiota, compared to non-smokers[140].

Pneumococcal vaccination alters carriage of pathogenic bacteria including *S. aureus* and *H. influenzae*[64], therefore we hypothesized that diversity measurements and particular ASVs would differ between individuals who received pneumococcal vaccination and those that did not receive the pneumococcal vaccination.

Contact with children was tested because adults and older adults who have frequent contact with children (i.e., in work environments, or through volunteering) and/or grandchildren have a higher likelihood of having an infection due to the increased rate of bacterial transmission and colonization[124]. Healthy children's (0-2 years of age) nasal microbiota have a higher α- diversity and different microbial composition, compared to healthy adults[40]. Children's nasal microbiota are typically dominated by *Dolosigranulum*, *Moraxella* and *Corynebacterium* genera and nasal microbiome profiles dominated with *H.*

influenza and *S. pneumoniae* are associated with respiratory illnesses and increased susceptibility to bronchiolitis[141,142]. We hypothesized that individuals who had frequent contact with children would have an increased microbial diversity and change in composition, compared to adults that were not in frequent contact with children.

Studies assessing frailty in the gut microbiota determined there was a strong negative association between frailty (measured with the Rockwood Frailty Index) and α- diversity (measured with Shannon, Simpson indices and Observed OTUs)[143]. Specifically, *Eggerthella dolichum* and *E. lenta* are positively associated with frailty and are known pathogens, associated with gastrointestinal disease (e.g., Crohn's disease, irritable bowel syndrome)[144]. *Faecalibacterium prausnitzii* can have an anti-inflammatory effect on the gut in mice and were found to be negatively associated with frailty in older adults[143,145]. There are limited studies of assessing frailty in the nasal microbiota of the aging population. A recent study determined frailty is significantly associated with occurrence of and hospitalization due to pneumonia, whereas pre-frailty was only associated with the occurrence of pneumonia in community-dwelling older adults[146]. With this knowledge, we hypothesized that pre-frailty self-assessments would be associated with compositional differences in the mid-turbinate microbiota, and have negative associations with commensal bacteria, such as *Corynebacterium*, amongst others.

3.2 Results

3.2.1 Characteristics of the study population

We recruited a total of 270 community-dwelling adults from the Greater Hamilton and Toronto regions. A total of 428 mid-turbinate samples were collected throughout 2020 – 2021. A total of 33/428 (7.7%) mid-turbinate samples were provided when participants experienced cold/flu like symptoms, during cold/flu (C&F) season and are named ILI samples. A total of 395/428 (92.3%) mid-turbinate samples were provided during baseline or follow-up sample collection periods, outlined in Fig 2.1 (Chapter 2). Exclusion criteria were individuals who were less than 18 years of age. Baseline demographic and health information was collected with our health and immune questionnaire. The 428 samples were analyzed by 16S-based sequencing and 16S rRNA – quantitative PCR (qPCR).

3.2.2 The composition of the adult mid-turbinate microbiota

There is high inter-individual variability in the composition of the mid-turbinate microbiota of community-dwelling adults during different sampling times (Fig 3.1A). To understand the role of the mid-turbinate microbiota in respiratory infections and seasonal changes in older adults, we performed a hierarchical cluster analysis at the ASV level with mid-turbinate microbiota samples from Fall 2020, Spring/Summer 2021 and Fall 2021 to group similar microbial communities together, named clusters. The dendrogram (Fig 3.1D) measures the distance between each individual sample using the Bray – Curtis Dissimilarity. Individuals that have branches closer together in the dendrogram

are more similar in their microbial composition, compared to individuals whose branches are farther apart.

The adult nasal microbiota can be defined into 7 clusters by the most abundant ASVs, and are named Coryne1, Coryne2, Coryne3, Coryne4, Mixed, Staph1 and Staph2 (Table 3.1). The *Moraxella* cluster (Fig 3.1A) was removed from further analyses because it only contains 2 samples and did not meet the criteria of a cluster. *Corynebacterium* (51%)*, Staphylococcus* (28%)*, Streptococcus* (3.6%)*, Moraxella* (2.7%)*, Blautia* (1%) and *Dolosigranulum* (0.7%) are top 6 most dominant genera that have the highest relative abundance within the 7 clusters (Fig 3.2A).

The dominant taxon (defined as >35% abundance in >70% or more samples within each cluster) and number of samples within each cluster are defined as follows: the largest cluster is dominated by *Staphylococcus* spp. (Staph2; *n* = 109 [28%]), and the next largest cluster is dominated by *Corynebacterium* spp. (Coryne1; *n* = 97 [25%]). The subsequent clusters are dominated by various strains of *Staphylococcus* and *Corynebacterium* spp., named Coryne2 (*n* = 30 [7.7%]), Coryne3 (*n* = 55 [14%]), Coryne4 (*n* = 17 [4.4%]), Staph2 (*n* = 41 [10.5%]) and one mixed group of species (*n* = 39 [10%]). Most of the dominant species are represented by a single ASV. As an example, Coryne1 consists primarily of ASV 1, 3 and 9, whereas Coryne2 contains ASV 3 and 6. The mixed group is not dominated by a single ASV and contains multiple ASV from *Streptococcus, Corynebacterium, Moraxella,* and *Staphylococcus* genera (Fig 3.1B+C).

To determine the inter-individual variation amongst the 7 clusters, a PCoA analysis showed the samples within the clusters are significantly dispersed from one another across multiple axes (PERMANOVA, p = **0.001**, R^2 = 0.496). This separation explains 37.3% of the inter-individual variation amongst the adult mid-turbinate microbiota (Supplementary fig 3.1). We also assessed the similarities between the samples within each cluster, to ensure the samples within the same cluster were more similar to one another compared to samples within other clusters. With the exception of the mixed cluster, the samples within the same cluster have a dissimilarity closer to 0, compared to samples within other clusters, and was measured using Bray-Curtis distances (Fig 3.2B).

There are statistically significant differences in sex, bacterial load, season, and vitamin D consumption between individuals within the clusters (Table 3.1). There were no statistically significant differences in age, weight, height, smokers, contact with children, individuals who had ILI's, pre-frailty scores, non-inflammatory conditions and inflammatory conditions, number of medications and anti-inflammatory medications between individuals within each cluster (Table 3.1).

Table 3.1 Participant characteristics organized by cluster membership

Characteristic	**Coryne1**, N = 97[1]	**Coryne2**, N = 30[1]	**Coryne3**, N = 52[1]	**Coryne4**, N = 17[1]	**Mixed**, N = 39[1]	**Staph1**, N = 41[1]	**Staph2**, N = 105[1]
Age (years)	56.0 (17.2)	48.0 (16.1)	51.4 (18.4)	51.5 (15.3)	54.9 (18.7)	51.9 (19.3)	52.0 (16.5)
Sex*							
F	71 / 97 (73%)	17 / 30 (57%)	27 / 52 (52%)	15 / 17 (88%)	24 / 39 (62%)	31 / 41 (76%)	79 / 105 (75%)
M	26 / 97 (27%)	13 / 30 (43%)	25 / 52 (48%)	2 / 17 (12%)	15 / 39 (38%)	10 / 41 (24%)	26 / 105 (25%)
Weight (kgs)	74.9 (18.4)	73.9 (15.1)	74.2 (14.3)	75.6 (17.6)	73.9 (16.2)	71.6 (15.0)	75.7 (17.5)
Height (m)	1.7 (0.1)	1.7 (0.1)	1.7 (0.1)	1.7 (0.1)	1.7 (0.1)	1.7 (0.1)	1.7 (0.1)
Bacterial load*	5.4 (1.1)	5.3 (0.7)	5.3 (1.2)	5.0 (1.1)	4.9 (0.8)	5.4 (1.0)	5.0 (0.9)
Season***							
Fall	59 / 97 (61%)	14 / 30 (47%)	31 / 52 (60%)	14 / 17 (82%)	25 / 39 (64%)	14 / 41 (34%)	61 / 105 (58%)
Spring	17 / 97 (18%)	5 / 30 (17%)	9 / 52 (17%)	0 / 17 (0%)	2 / 39 (5.1%)	9 / 41 (22%)	17 / 105 (16%)
Summer	21 / 97 (22%)	11 / 30 (37%)	12 / 52 (23%)	3 / 17 (18%)	12 / 39 (31%)	18 / 41 (44%)	27 / 105 (26%)
Smoking							
N	70 / 97 (72%)	18 / 30 (60%)	43 / 52 (83%)	14 / 17 (82%)	24 / 39 (62%)	32 / 41 (78%)	69 / 104 (66%)
Y	27 / 97 (28%)	12 / 30 (40%)	9 / 52 (17%)	3 / 17 (18%)	15 / 39 (38%)	9 / 41 (22%)	35 / 104 (34%)
Contact with children							
N	51 / 97 (53%)	14 / 30 (47%)	24 / 52 (46%)	8 / 17 (47%)	20 / 39 (51%)	24 / 41 (59%)	47 / 105 (45%)
Y	46 / 97 (47%)	16 / 30 (53%)	28 / 52 (54%)	9 / 17 (53%)	19 / 39 (49%)	17 / 41 (41%)	58 / 105 (55%)
Influenza like infection							
N	89 / 97 (92%)	28 / 30 (93%)	47 / 52 (90%)	16 / 17 (94%)	33 / 39 (85%)	40 / 41 (98%)	98 / 105 (93%)
Y	8 / 97 (8.2%)	2 / 30 (6.7%)	5 / 52 (9.6%)	1 / 17 (5.9%)	6 / 39 (15%)	1 / 41 (2.4%)	7 / 105 (6.7%)
Pre-frailty score							
0	71 / 97 (73%)	27 / 30 (90%)	40 / 52 (77%)	17 / 17 (100%)	29 / 39 (74%)	35 / 41 (85%)	75 / 105 (71%)
1	20 / 97 (21%)	3 / 30 (10%)	10 / 52 (19%)	0 / 17 (0%)	6 / 39 (15%)	5 / 41 (12%)	19 / 105 (18%)
2	3 / 97 (3.1%)	0 / 30 (0%)	1 / 52 (1.9%)	0 / 17 (0%)	1 / 39 (2.6%)	0 / 41 (0%)	6 / 105 (5.7%)
3	3 / 97 (3.1%)	0 / 30 (0%)	1 / 52 (1.9%)	0 / 17 (0%)	3 / 39 (7.7%)	1 / 41 (2.4%)	5 / 105 (4.8%)

Characteristic	Coryne1, N = 97[1]	Coryne2, N = 30[1]	Coryne3, N = 52[1]	Coryne4, N = 17[1]	Mixed, N = 39[1]	Staph1, N = 41[1]	Staph2, N = 105[1]
Non-inflammatory conditions							
0	55 / 97 (57%)	19 / 30 (63%)	32 / 51 (63%)	11 / 17 (65%)	27 / 39 (69%)	20 / 41 (49%)	56 / 105 (53%)
1	28 / 97 (29%)	10 / 30 (33%)	8 / 51 (16%)	4 / 17 (24%)	7 / 39 (18%)	11 / 41 (27%)	32 / 105 (30%)
2	7 / 97 (7.2%)	1 / 30 (3.3%)	11 / 51 (22%)	0 / 17 (0%)	3 / 39 (7.7%)	6 / 41 (15%)	9 / 105 (8.6%)
3	1 / 97 (1.0%)	0 / 30 (0%)	0 / 51 (0%)	1 / 17 (5.9%)	2 / 39 (5.1%)	3 / 41 (7.3%)	3 / 105 (2.9%)
4	3 / 97 (3.1%)	0 / 30 (0%)	0 / 51 (0%)	0 / 17 (0%)	0 / 39 (0%)	1 / 41 (2.4%)	4 / 105 (3.8%)
5	3 / 97 (3.1%)	0 / 30 (0%)	0 / 51 (0%)	1 / 17 (5.9%)	0 / 39 (0%)	0 / 41 (0%)	1 / 105 (1.0%)
Inflammatory conditions							
0	55 / 97 (57%)	21 / 30 (70%)	35 / 51 (69%)	12 / 17 (71%)	21 / 39 (54%)	20 / 41 (49%)	55 / 105 (52%)
1	23 / 97 (24%)	8 / 30 (27%)	9 / 51 (18%)	1 / 17 (5.9%)	12 / 39 (31%)	13 / 41 (32%)	23 / 105 (22%)
2+	19 / 97 (20%)	1 / 30 (3.3%)	7 / 51 (14%)	4 / 17 (24%)	6 / 39 (15%)	8 / 41 (20%)	27 / 105 (26%)
Vitamin D consumption*							
N	39 / 95 (41%)	19 / 29 (66%)	35 / 52 (67%)	3 / 17 (18%)	19 / 39 (49%)	16 / 40 (40%)	43 / 102 (42%)
Y	56 / 95 (59%)	10 / 29 (34%)	17 / 52 (33%)	14 / 17 (82%)	20 / 39 (51%)	24 / 40 (60%)	59 / 102 (58%)
Number of medications	1.7 (2.6)	0.8 (1.9)	1.2 (1.5)	1.1 (1.0)	2.2 (2.9)	2.2 (2.8)	2.4 (3.4)
Anti-inflammatory medications							
0	79 / 97 (81%)	26 / 30 (87%)	43 / 52 (83%)	14 / 17 (82%)	31 / 38 (82%)	29 / 40 (72%)	83 / 105 (79%)
1	13 / 97 (13%)	3 / 30 (10%)	3 / 52 (5.8%)	1 / 17 (5.9%)	6 / 38 (16%)	7 / 40 (18%)	13 / 105 (12%)
2	3 / 97 (3.1%)	1 / 30 (3.3%)	4 / 52 (7.7%)	1 / 17 (5.9%)	1 / 38 (2.6%)	2 / 40 (5.0%)	1 / 105 (1.0%)
3	1 / 97 (1.0%)	0 / 30 (0%)	0 / 52 (0%)	1 / 17 (5.9%)	0 / 38 (0%)	2 / 40 (5.0%)	6 / 105 (5.7%)
4	1 / 97 (1.0%)	0 / 30 (0%)	1 / 52 (1.9%)	0 / 17 (0%)	0 / 38 (0%)	0 / 40 (0%)	1 / 105 (1.0%)
5	0 / 97 (0%)	0 / 30 (0%)	1 / 52 (1.9%)	0 / 17 (0%)	0 / 38 (0%)	0 / 40 (0%)	0 / 105 (0%)
7	0 / 97 (0%)	0 / 30 (0%)	0 / 52 (0%)	0 / 17 (0%)	0 / 38 (0%)	0 / 40 (0%)	1 / 105 (1.0%)

[1] Mean (SD); n / N (%)

P – values < 0.05 are indicated with a (*)

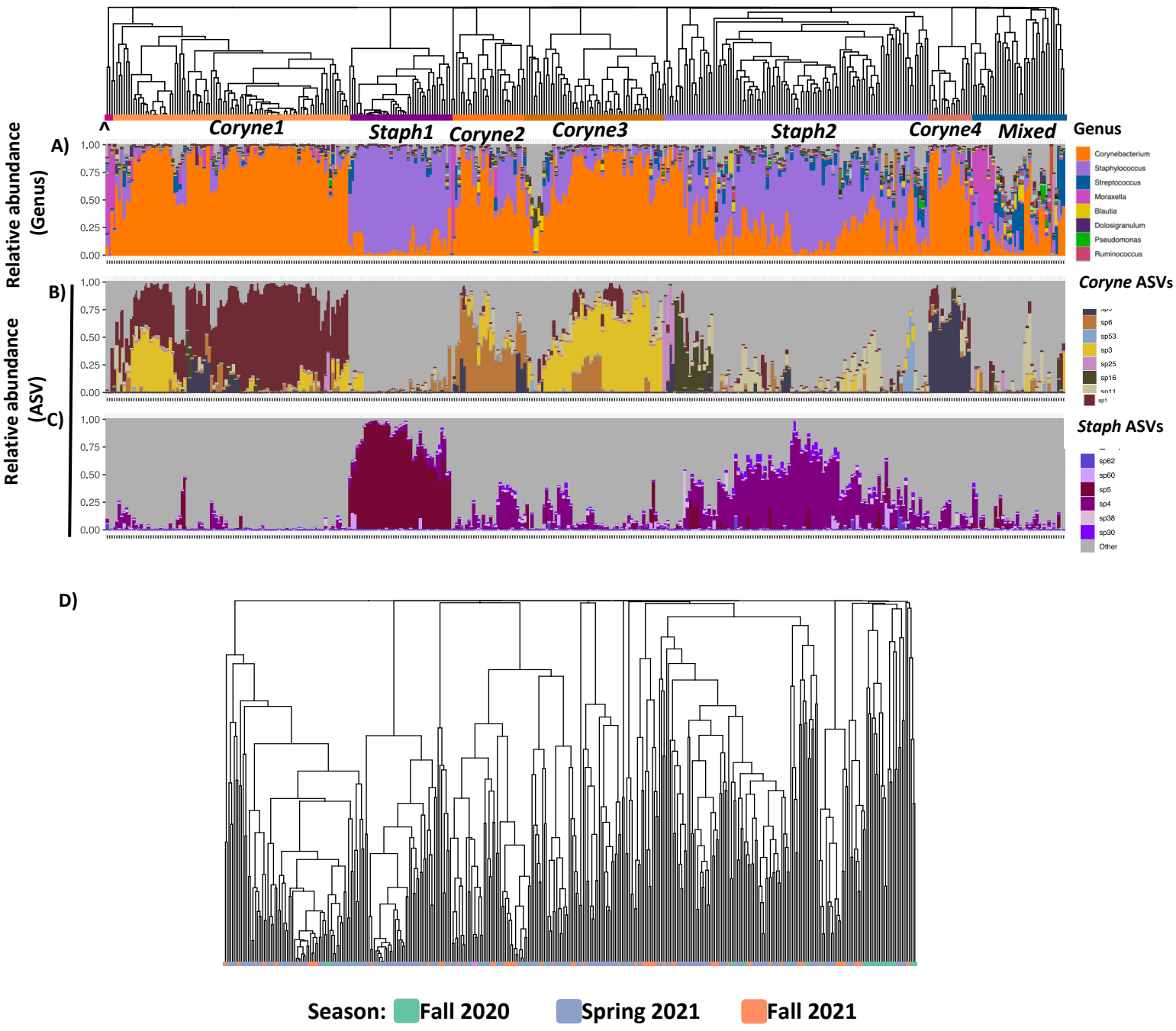


Figure 3.1A-D: The taxonomic composition of the nasal microbiota is defined into 7 clusters. The differences between each individual (n = 395) are measured using Bray-Curtis dissimilarities, at the ASV level. Nasal samples were collected between Fall 2020-Fall 2021. **(B-C)** The taxonomic bar plot is visualized with the top 20 dominant genera. Taxonomic bar plots visualize the most dominant *Corynebacterium* and *Staphylococcus* ASVs within the nasal cavity, within each cluster. **(D)** A dendrogram coloured by the season nasal samples were collected shows the samples collected within each season are unevenly dispersed within their microbial communities.

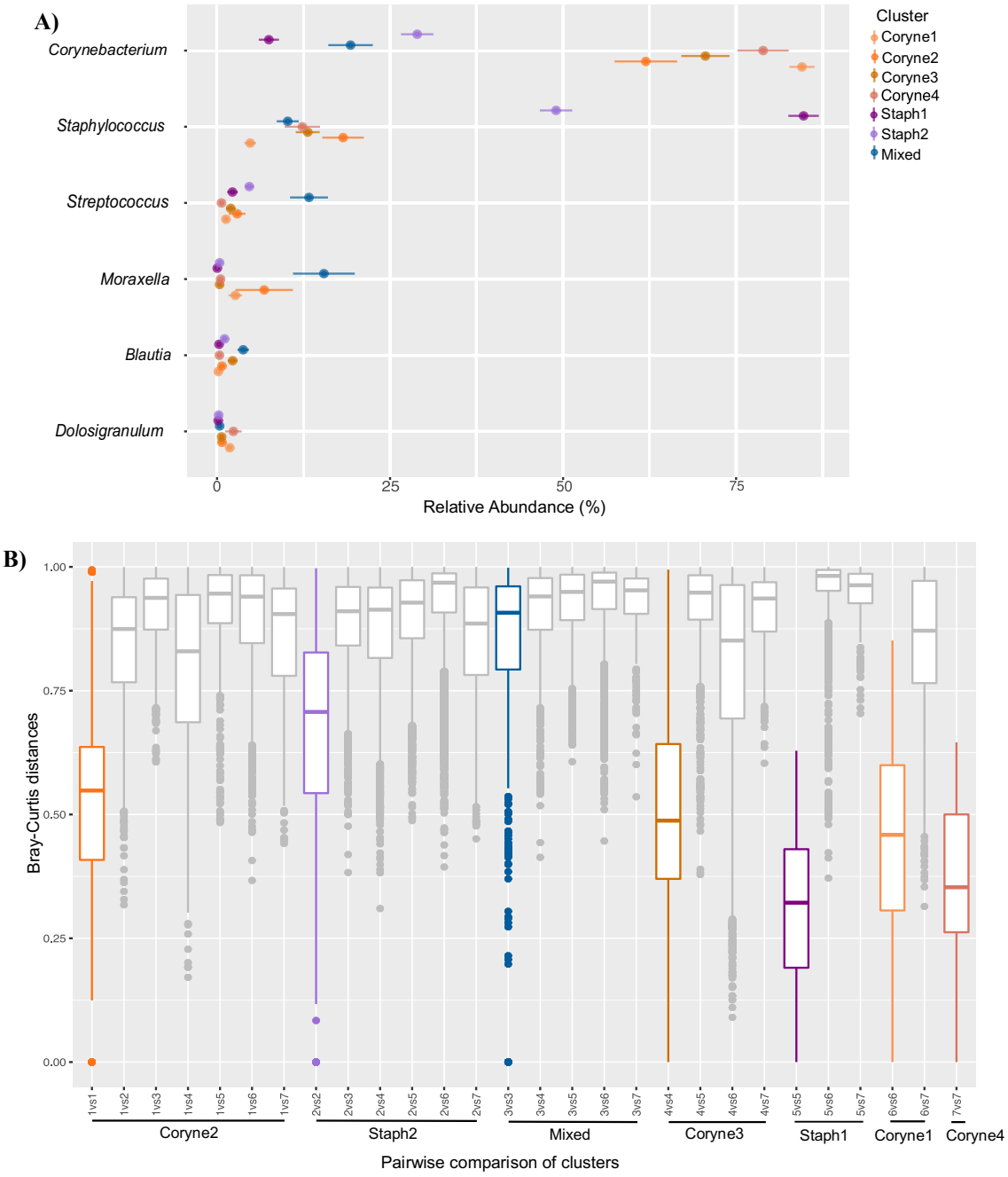


Figure 3.2A-B: (A) Relative abundances of the 6 dominant genera in all mid turbinate samples. Cluster colouring is as follow: Coryne1 (light orange), Coryne2 (orange), Coryne3 (dark orange), Coryne4 (salmon orange), Staph1 (violet), Staph2 (lavender), Mixed (blue). (B) Samples within a cluster are more similar to one another than between clusters. The 7 clusters are significantly dispersed, and statistical significance was tested using a permutational multivariate ANOVA [PERMANOVA] ($p = 0.001$, $R2 = 0.496$). An analysis of similarity (ANOSIM) determined the similarities between the clusters are significant and suggests there is more similarity within the clusters than there are between the clusters ($p = 0.001$, $R = 0.761$). Bray Curtis distances measured between each mid-turbinate sample for a pairwise comparison between clusters.

3.2.4 α-diversity, bacterial load, seasons, and vitamin D supplementation differ with cluster membership

To determine if the 7 clusters are associated with age-related, health, season, and bacterial load changes and diversity measures, a linear model was used to measure associations between the numerical variables and chi-square tests were performed for categorical variables. The 7 clusters were significantly associated with α - diversity, bacterial load, seasons, and vitamin D consumption. The remaining variables (age, sex, inflammatory conditions, number of medications, smoking, pneumococcal vaccination status, contact with children, pre-frailty self-assessment score and ILI) did not have a significant association with the mid-turbinate clusters. The α - diversity measures used for this linear model included Shannon and Simpson Index's and the observed number of ASVs within a sample.

Intra-individual variation of the mid-turbinate microbiota, across all 3 diversity measurements, fluctuates amongst the clusters (Fig 3.3A-C). The individuals belonging to Coryne1 cluster have significantly higher diversity measurements compared to individuals belonging to the Mixed and Staph2 clusters. These individuals have significantly lower diversity, compared to individuals belonging in the Staph1 cluster. The Mixed cluster is the most diverse (Shannon Index) and has the highest number of observed ASVs which aligns well with the fact that it is not dominant with specific ASVs. Individuals belonging to the Mixed cluster have significantly higher diversity than individuals in the Staph1 cluster, and individuals in the Staph2 cluster have significantly higher diversity compared to individuals in the Staph1 cluster. Bacterial load has a

significant main effect amongst the clusters overall, but none of the clusters were significantly different than one another (Fig 3.3D). Of the 19 metadata variables tested, 3 (sex, season, and vitamin D consumption) had a significant association with cluster type. Post-hoc analyses were performed on the 3 significant variables to determine which cluster membership was significant (Table 3.3). Females have a higher proportion in the Coryne3 cluster compared to males. Seasonality has a significant difference in the distribution of clusters, and mid-turbinate samples collected in Fall 2020 have a higher proportion in the Mixed cluster. Individuals that do not take vitamin D supplementation have a higher proportion in the Coryne3 cluster. Due to high variation (i.e., inter-individuality) between community-dwelling adults within the mid-turbinate microbiota, there are no age, frailty, inflammatory conditions, and number of medications associated with the 7 clusters found in the mid-turbinate microbiota. The associations between cluster membership and the metadata variables are summarized in Table 3.2.

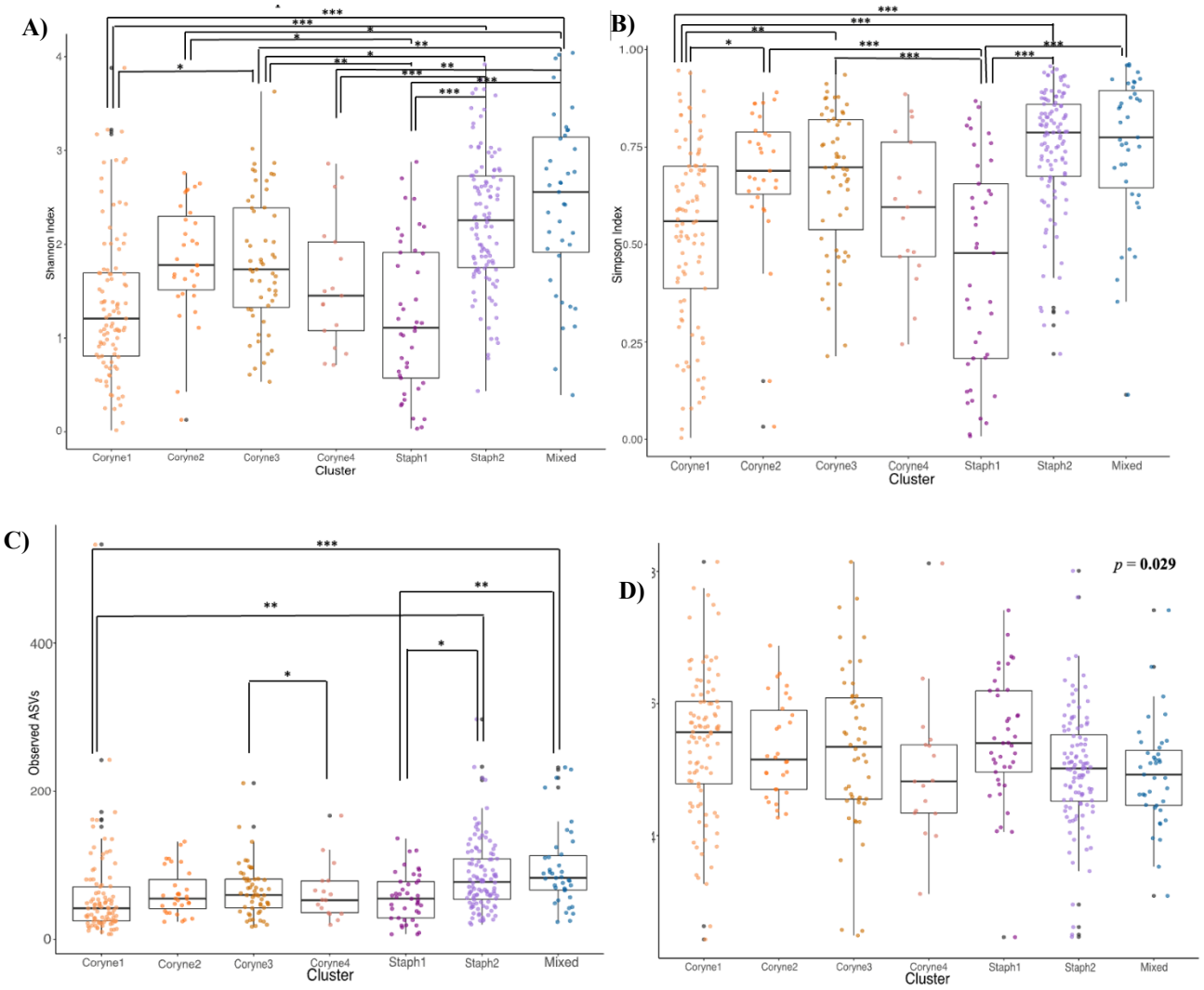


Figure 3.3A-D: a - diversity and bacterial load measured across cluster number in the mid turbinate samples. a - diversity was measured using Shannon index ($p < \mathbf{2.2^{-16}}$), Simpson Index ($p < \mathbf{2.13^{-15}}$) and observed ASVs ($p < \mathbf{6.344^{-5}}$). The bacterial load was calculated by using quantitative PCR and was expressed in $\log_{10}$ values (16S ribosomal RNA gene copies/mL of UTM media) ($p = \mathbf{0.029}$). Statistical significance was tested using a linear mixed - effect model, controlling for participant ID as a random effect.

Table 3.2. Cluster membership changes with sex, bacterial load, seasons, and Vitamin D supplementation

Variable	p-value	Pairwise comparison by cluster membership	Post-hoc by cluster membership
Age	0.281	NA	NA
Sex	**0.01**	**Coryne3**	**0.048**
Weight (kgs)	0.675	NA	NA
Height (m)	0.925	NA	NA
BMI	0.905	NA	NA
Bacterial load	**0.029**	**NS**	**NS**
Non-inflammatory conditions	0.381	NA	NA
Pre-frailty phenotype	0.183	NA	NA
Medications	0.110	NA	NA
Anti-inflammatory medications	0.920	NA	NA
Sampling time	**0.000185**	**Fall 2020 vs Mixed**	**0.0032**
Smoking	0.101	NA	NA
E-cigarette usage	0.068	NA	NA
Marijuana usage	0.941	NA	NA
Contact with children	0.85	NA	NA
ILI	0.536	NA	NA
Current usage of antibiotics	0.236	NA	NA
Inflammatory conditions	0.146	NA	NA
Vitamin D supplementation	**0.001**	**Coryne3**	**0.017**

NA is present when a post-hoc analysis was not applicable
NS means non-significant values

3.2.5 Bacterial load and seasonal changes impact the diversity, composition and specific ASVs in the adult mid-turbinate microbiota

To determine if the 12 variables are associated with diversity and compositional changes in the URT microbiota, a- and b- diversity measurements were used in a linear model. Shannon and Simpson Indexes were used for a-measurements. The Shannon Index is more sensitive to species richness, the number of species in a sample, whereas the Simpson index is more sensitive to

species evenness, and accounts for the proportion of species in a sample[131]. A low diversity index indicates the microbiota is dominated by fewer bacteria, and a high diversity index suggests the microbiota is dominated by an even abundance of various genera[132]. Using both indices' accounts for these differences in measurements. b – diversity was measured with the Bray-Curtis dissimilarity metric to measure how similar an individual's microbial composition is to another, on a scale from 0 to 1. A measurement of 0 indicates high similarity between individuals, and a measurement at, or closer to, 1 indicates that the communities are completely different between individuals[147].

Contrary to our hypotheses, there are little age-related changes in the mid-turbinate microbiota. Age, sex, inflammatory conditions, number of medications, vitamin D supplementation, smoking, pneumococcal vaccination status, contact with children and the pre-frailty self-assessment phenotype were not significantly correlated with a- and b- diversity (supplementary table 3.1-3.2). Bacterial load was negatively correlated with a - diversity measurements, indicated lower species richness and evenness is present with a higher bacterial load (Fig 3.4 A-B). This suggests that adults with a fuller niche could have fewer, but more dominant commensal microbes residing in their mid-turbinate microbiota, preventing pathogenic bacteria from colonizing in the mid-turbinate microbiota.

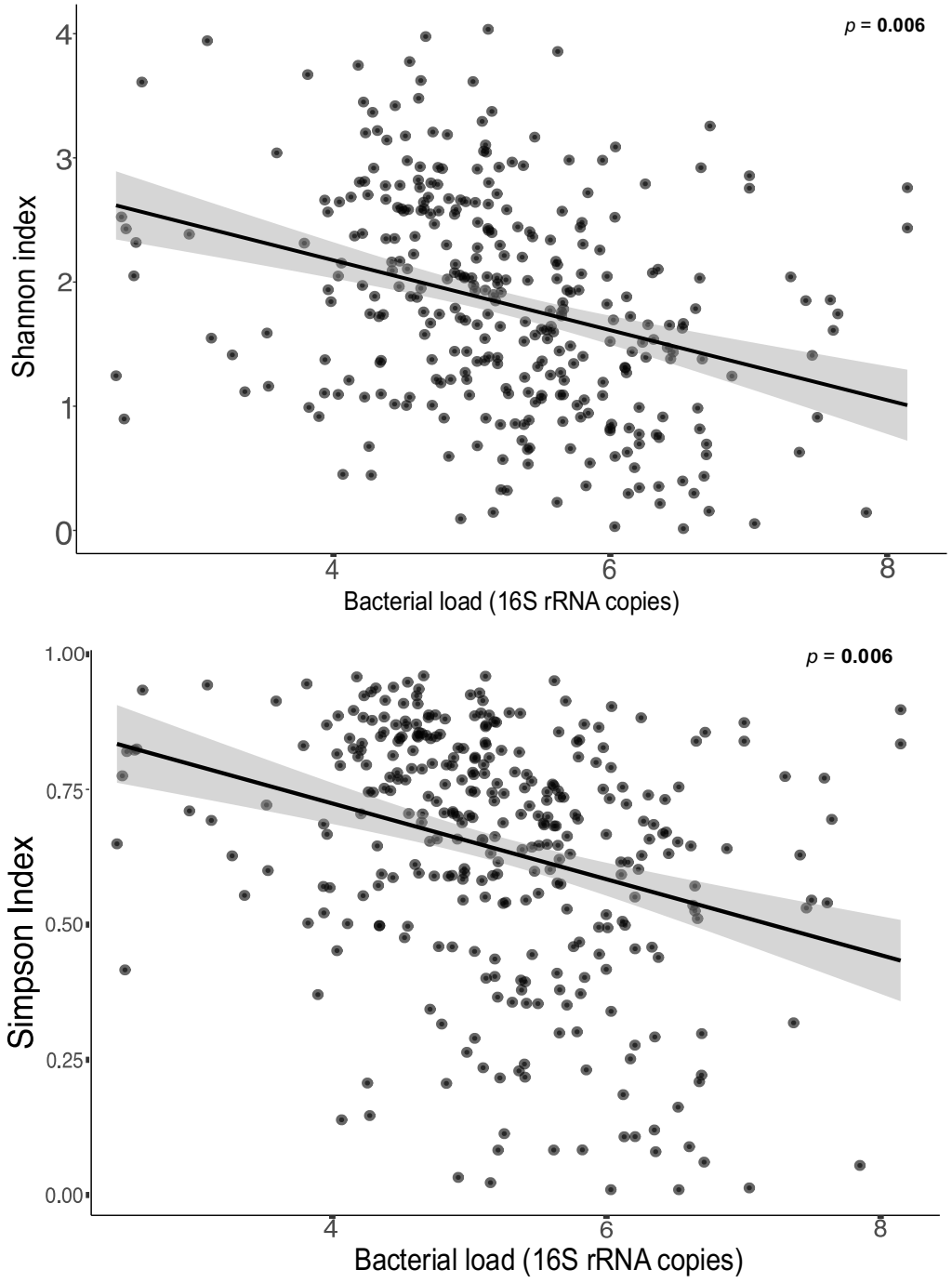


Figure 3.4A-B: Bacterial load and a - diversity are negatively correlated in the mid turbinate cavity. The bacterial load was calculated by using quantitative PCR and was expressed in $\log_{10}$ values (16S ribosomal RNA gene copies/mL of UTM media). a - diversity was measured using Shannon index (p = **0.006**, A) and Simpson index (p = **0.006**, B). Nasal samples were collected during Fall 2020, Spring and Summer 2021, and statistical significance was tested using a linear mixed - effect model, controlling for participant ID as a random effect. p – values were adjusted using the Benjamini & Hochberg (1995) (BH) method.

b-diversity significantly differs by sampling timepoints and bacterial load in the mid-turbinate microbiota. (Fig 3.5A-B). Each dot on the plot represents one nasal sample. A distance-based redundancy analysis (dbRDA) is a constrained ordination that was performed on sampling timepoints and bacterial load. In Fig 3.5A, the arrows indicate the magnitude of the effect size that Spring 2021 and Fall 2021 have on the data. Sampling timepoints represents 2.6% of the variation, which is determined with the first two constrained axes, CAP1 and CAP2 (Fig 3.5A). Pairwise comparison testing determined the mid-turbinate microbiota significantly differs between Fall 2020 and Fall 2021 samples (p = **0.025**, R^2 = 0.007), not between Fall 2020 and Spring 2021, or Spring 2021 and Fall 2021. This determines the composition of the mid-turbinate microbiota between Fall 2020 and Fall 2021 sampling timepoints has dissimilarity between individuals, leading to an increase in inter-individual variation between these samples. This could be due to behavioural changes in government lockdown between these two timepoints, including the frequency adults went to work (e.g., healthcare, schools, businesses), interacting with children, attended social gatherings and personal hygiene[128,148].

Bacterial load is another factor that drives the inter-individual variation in the mid-turbinate microbiota. The dbRDA analysis determined that bacterial load represents 1% of the variation in the data, determined by the first axis, CAP1 (Fig 3.5B). The arrow indicates the magnitude of the effect size is driven by high bacterial load (>6 16S rRNA copies (log)). Sampling timepoint is significantly correlated with bacterial load (Fig 3.5C). Bacterial load is significantly higher in Spring/Summer 2021 and Fall 2021 samples, compared to Fall 2020 (p < **0.0001**, p = **0.001**, respectively). This suggests that adults with a higher bacterial load in the Spring/Summer could have dominant commensal bacteria that colonize the mid-turbinate microbiota resulting in a fuller niche.

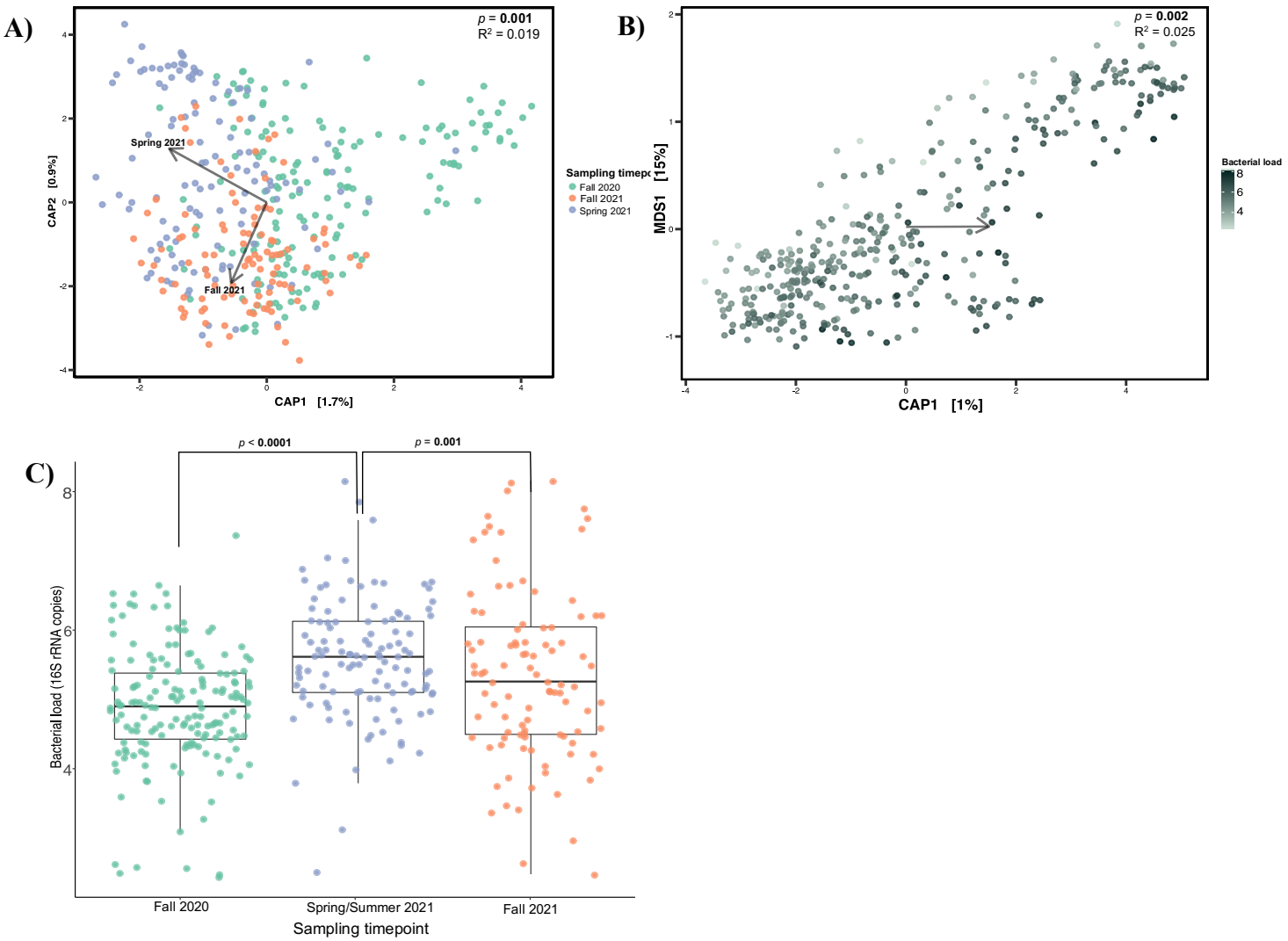


Figure 3.5A-C: The URT microbiota differs between sampling timepoint and bacterial load. (A) dbRDA between individuals using Bray – Curtis Dissimilarities at the ASV level. Each point represents one nasal sample. Sampling timepoints are as follows: Fall 2020 (Sept-Dec), Spring/Summer 2021 (May-Aug) and Fall 2021 (Sept-Dec). Statistical significance was tested using a permutational multivariate ANOVA [PERMANOVA] (p = **0.001**, R^2 = 0.019). Pairwise comparison testing determined significance between Fall 2020 and Fall 2021 (p = **0.025**). Fall 2020 vs Spring 2021 mid-turbinate samples were not significant (p = 0.219) and Spring 2021 vs Fall 2021 mid-turbinate samples were not significant (p = 0.137). (**(B)** dbRDA analysis between individuals using Bray-Curtis dissimilarities at the ASV level. Bacterial load was measured as described above. Each point represents 1 individual. Statistical significance was tested using a permutational multivariate ANOVA [PERMANOVA] (p = **0.002**, R^2 = 0.025). **(C) Bacterial load is significantly higher in Spring/Summer 2021, compared to Fall 2020.** The bacterial load was calculated by using quantitative PCR and was expressed in $\log_{10}$ values (16S ribosomal RNA gene copies/mL of UTM media) (p = **3.217^{-8}**). Sampling timepoints are the same as described above. The bacterial load within samples collected in Fall 2020 was statistically lower than Fall 2021, and Spring/Summer 2021 (p = **0.001**, p < **0.0001**). Statistical significance was tested using a linear mixed - effect model, controlling for participant ID as a random effect and pairwise comparisons were performed.

To determine if particular ASVs are positively or negatively correlated with the 12 variables, differential abundance (DA) testing was performed. Significant ASVs were correlated with bacterial load at each sampling timepoint: Fall 2020, Spring/Summer 2021 and Fall 2021. A total of 3 ASVs are associated with bacterial load in Fall 2020, 14 ASVs are associated with bacterial load in the Spring/Summer 2021, which also happens to have the highest bacterial load (Fig 3.3b), and only 1 ASV is associated with bacterial load in Fall 2021 (Table 3.1). Neither of the ASVs are the same across all 3 sampling timepoints.

The mean abundance is the average abundance of the ASV across all samples. In this analysis, a high abundance is defined as >20. The Log_2 fold change is the effect size estimate, and this value indicates how much the ASV has changed with bacterial load, within each sampling timepoint season. The values are reported on a logarithmic scale to the base 2. A positive value indicates the ASV is increasing in abundance with bacterial load, and a negative value indicates the ASV is decreasing in abundance with bacterial load. There are 7/18 [38.8%] ASVs over the 3 sampling timepoints that increase in abundance with increasing bacterial load, and 11/18 [61.1%] ASVs over the 3 sampling timepoints that are inversely correlated with bacterial load (i.e. decrease in abundance with increasing bacterial load).

The *Corynebacterium* taxon (sp1) is the most dominant ASV in the mid-turbinate microbiota (high mean abundance 29904.94) and is positively associated with an increasing bacterial load in Spring 2021. Similarly, *Moraxella nonliquefaciens* (sp27) has a high mean abundance (9169.13) in the mid-turbinate microbiota and is positively associated with an increasing bacterial load in Spring/Summer 2021. Although *M. nonliquefaciens* is increased in children with acute sinusitis[149], it is usually non-pathogenic and frequently colonizes the URT

microbiota. A protective mechanism of this taxon against respiratory infections in adults is currently unidentified. Comparatively, *Staphylococcus aureus* (sp5), a commensal bacterium that colonizes the URT microbiota asymptomatically[150], increase with increasing bacterial load in the mid-turbinate microbiota, whereas *Cutibacterium acnes*, decreases with decreasing bacterial load. These ASVs are independent of one another. A summary of all 18 differentially abundant ASVs that positively/negatively correlate with bacterial load across the 3 sampling timepoints are summarized in Table 3.1.

Table 3.3 Differentially abundant ASVs are positively/negatively correlated with increasing bacterial load (16S rRNA gene copies/mL of UTM media) during sampling time points in the mid-turbinate microbiota

Bacteria	ASV	Mean abundance	Change in mean abundance of bacteria relative to increasing bacterial load (Log_2 fold change)	p-value	p-adjusted value (BH method)
Sampling time point: Fall 2020					
Ralstonia insidiosa	sp223	48.05	0.419	**3.22^{-6}**	**0.0002**
Cutibacterium acnes	sp102	107.59	-0.713	**0.001**	**0.02**
Staphylococcus aureus	sp5	13477.70	1.937	**0.0005**	**0.01**
Sampling time point: Spring/Summer 2021					
Kocuria carniphila	sp342	22.07	-0.455	**0.003**	**0.03**
Staphylococcus haemolyticus	sp126	22.88	-0.222	**0.005**	**0.04**
Abiotrophia	sp92	24.17	-0.975	**0.005**	**0.04**
Pseudomonas	sp129	26.42	-0.642	**0.003**	**0.03**
Paracoccus	sp181	43.67	-0.505	**0.0007**	**0.01**
Caulobacter vibroides	sp142	45.66	-0.374	**0.0006**	**0.01**
Corynebacterium	sp270	51.66	-1.727	**0.003**	**0.03**
Corynebacterium	sp383	59.80	0.245	**0.0001**	**0.006**
Lechevalieria	sp198	64.09	-0.156	**6.17^{-7}**	**5.09^{-5}**
Corynebacterium kroppenstedtii	sp100	149.63	0.413	**0.0008**	**0.01**
Staphylococcus xylosus	sp149	193.66	-0.252	**0.0009**	**0.01**
Dolosigranulum pigrum	sp35	607.76	0.551	**0.0009**	**0.01**
Moraxella nonliquefaciens	sp27	9169.13	5.186	**0.0007**	**0.01**
Corynebacterium	sp1	29904.94	2.015	**1.09^{-7}**	**1.81^{-5}**
Sampling time point: Fall 2022					
Rothia mucilaginosa	sp52	77.68	-2.32^{-6}	**0.0003**	**0.013**

3.2.6 Higher bacterial load could decrease infection risk to influenza-like-infections in the URT microbiota

To determine if there were features of the mid-turbinate microbiota that contributed to increased risk to infection, diversity, bacterial load and ASVs were assessed between individuals who did and did not have an ILI. Using baseline mid-turbinate samples from Fall 2020, our results show that adults who did not have an ILI infection had a significantly higher bacterial

load, than those who did (Fig 3.7). Bacterial load in the Fall, can impact an individual's susceptibility to infection in the C&F season. a- and b- diversity measurements (Shannon, Simpson, Bray-Curtis dissimilarity) at the baseline collection did not differ between those who did or did not acquire an ILI during the 2020 C&F season. A summary of these findings is summarized in Table 3.4.

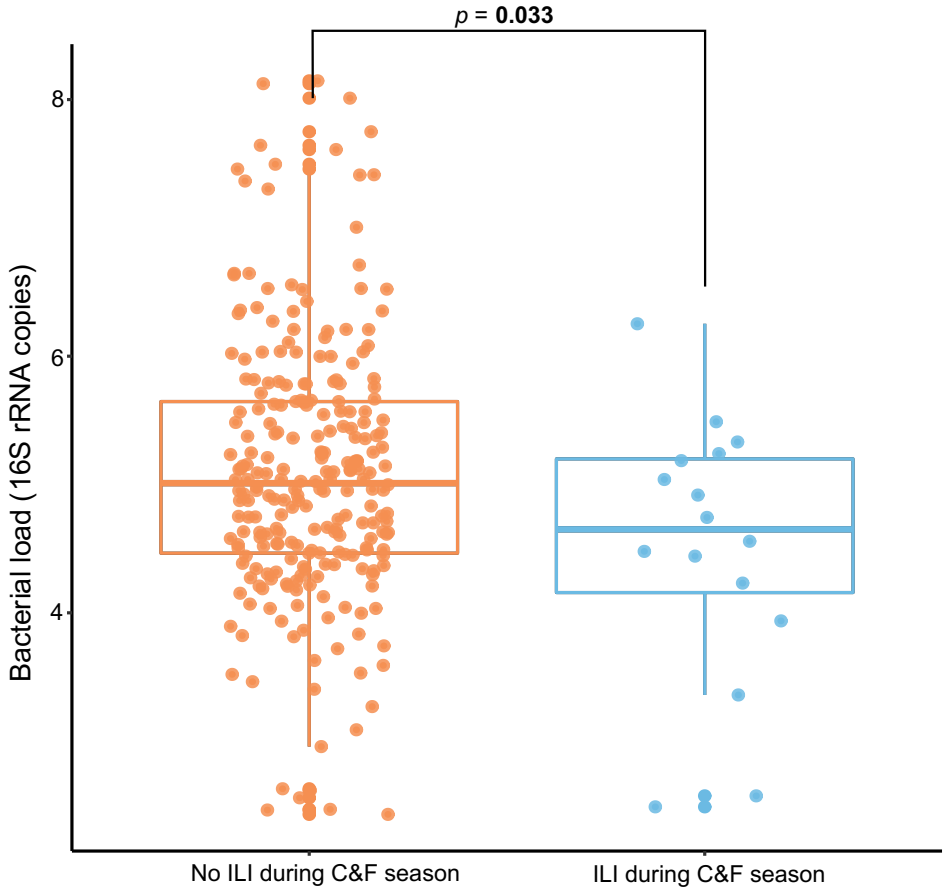


Figure 3.6: Bacterial load differs between adults who acquired an ILI and those who did not. The bacterial load was calculated by using quantitative PCR and was expressed in $\log_{10}$ values (16S ribosomal RNA gene copies/mL of UTM media) (p = **0.033**). Samples were collected at baseline, Fall 2020. Statistical significance was tested using a linear mixed - effect model.

Table 3.4. Diversity measures and bacterial load between adults who acquired an ILI and those who did not.

Variable	*n*		a – diversity			b – diversity	Bacterial load
			Shannon	Simpson	Observed ASVs		
Baseline 2020 (non ILI) – Baseline 2020 (ILI)	Non – ILI	246	$p = 0.091$	$p = 0.061$	$p = 0.916$	$P = 0.341$ $R^2 = 0.004$	**$p = 0.032$***
	ILI	17					

Although there were no consistent changes within the nasal microbiota on a whole-community-level (i.e., global scale) between individuals who did and did not have an ILI, there are 4 particular ASVs that are significantly correlated with individuals who did have an ILI. These results indicate that there are minor, significant decreases within ASVs that belong to the *M. catarrhalis, Corynebacterium segmentosum*, *Staphylococcus aureus* and an ASV in the *Corynebacterium* genera that could not be identified at the species level, have minor, significant decreases within the nasal microbiota of adults during pre-C&F season (Fall 2020). All these ASVs have a high mean abundance <100. The fold change is the effect size estimate of each ASV. A negative fold change indicates the abundance of the ASV decreases within adult's nasal microbiota during pre-C&F, who later had an ILI during C&F season, compared to adults who did not have an ILI. The effect sizes are quite small within these samples. For example, *S. aureus* has a high mean abundance amongst all samples and decreases by 1.45 times (calculated as $2^{0.54}$) compared to adults who did not have an ILI during C&F season. The effect sizes and significance for the remainder of the ASVs are summarized in Table 3.5.

Table 3.5. Differentially abundant ASVs decrease during pre-C&F season in the mid-turbinate microbiota, within adult's that acquire an ILI during C&F season

Taxon	ASV	Mean abundance	Change in mean abundance of bacteria (Log_2 fold change)	p-value	p-adjusted value
Sampling timepoint: Fall 2020					
Moraxella catarrhalis	sp15	111.42	-0.086	**1.53^{-11}**	**2.55^{-10}**
Corynebacterium segmentosum	sp16	125.89	-0.073	**1.09^{-8}**	**1.56^{-7}**
Corynebacterium	sp9	1107.65	-0.21	**5.25^{-8}**	**6.56^{-7}**
Staphylococcus aureus	sp5	3606.51	-0.54	**5.65^{-5}**	**5.65^{-4}**

It is evident that the sampling timepoints throughout our longitudinal study period have a significant association with the 7 microbiota clusters and on the composition of the mid-turbinate microbiota. To visualize the cluster movement between sampling timepoints during 2020-2021 (Fall 2020, Spring/Summer 2021, Fall 2021) within the mid-turbinate microbiota, a Sankey diagram was used to show the flow from one sampling time point to the next (Fig. 3.8). To assess the cluster movement (i.e., stability) within individuals who did and did not have an ILI during C&F season, the percentage was calculated within each possible type of movement (Table 3.9). The following 4 groups were identified within individuals who did have an ILI: no cluster change after ILI (1), change in cluster after ILI and return to baseline (2), change in cluster after ILI and no return to baseline (3), cluster change after ILI and remains in the same cluster (4). The following 5 groups were identified within individuals who did not have an ILI across seasons: no cluster change throughout seasons (1), any cluster change throughout seasons (2), cluster change in Spring 2021 and return to baseline (3), cluster change in Spring 2021 and remains in the same cluster (4), no cluster change until Fall 2021 (5).

The mid-turbinate microbiota of adults is defined into the 7 clusters identified above. There are a total of 117 individuals that donated at least one sample after Fall 2020 (in Spring 2021 or Fall 2021, or both). There were 100 individuals that did not have an ILI, and 17 individuals did have an ILI. The number of individuals within each cluster at Fall 2020 are as follows: Coryne1 = 20 (17.1%), Coryne2 = 9 (7.7%), Coryne3 = 15 (12.8%), Coryne4 = 5 (4.3%), Mixed = 20 (17.1%), Staph1 = 14 (11.9%), Staph2 = 29 (24.8%). There were 8/117 (6.8%) individuals that did not donate a sample in Spring 2021 and 66/117 (56.4%) individuals that did not donate a sample in Fall 2021. Adults who only donated 1 sample in either Fall 2020, Spring 2021 or Fall 2021 were removed, because temporal stability could not be assessed in their nasal microbiotas.

There were 20 adults who belonged in the Mixed cluster during Fall 2020, and only 3/20 (15%) of these individuals, remained in the Mixed cluster by Spring 2021. The movement from *Corynebacterium* to a *Staphylococcus* cluster is comparable to movement from *Staphylococcus* to *Corynebacterium*. 10/117 (8.55%) of individuals move from a *Corynebacterium* to *Staphylococcus* cluster and 11/117 (9.4%) of individuals move from a *Staphylococcus* to *Corynebacterium* cluster. Neither of these 21 individuals had an ILI during the cold/flu season. This could suggest that belonging in a cluster dominant with either *Corynebacterium* or *Staphylococcus* would result in a lower susceptibility to infection.

There were 9/17 (53%) adults who had an ILI during 2020-2021, changed clusters and did not return to their baseline cluster type. Only 1 adult changed cluster after an ILI and returned to their baseline sample, and 4/19 (24%) adults did not change clusters after an ILI. Almost 50% of adults who did not have an ILI, did not change clusters throughout the season and 33% did change clusters at 2 or all 3 timepoints. There is a trend associated within adults

who did not have an ILI remaining within the same cluster, compared to adults who did have an ILI and did change clusters. The dynamics of the URT microbiota are challenging to categorize, and this analysis depicts the association between cluster movement in the URT and susceptibility to infection during C&F season.

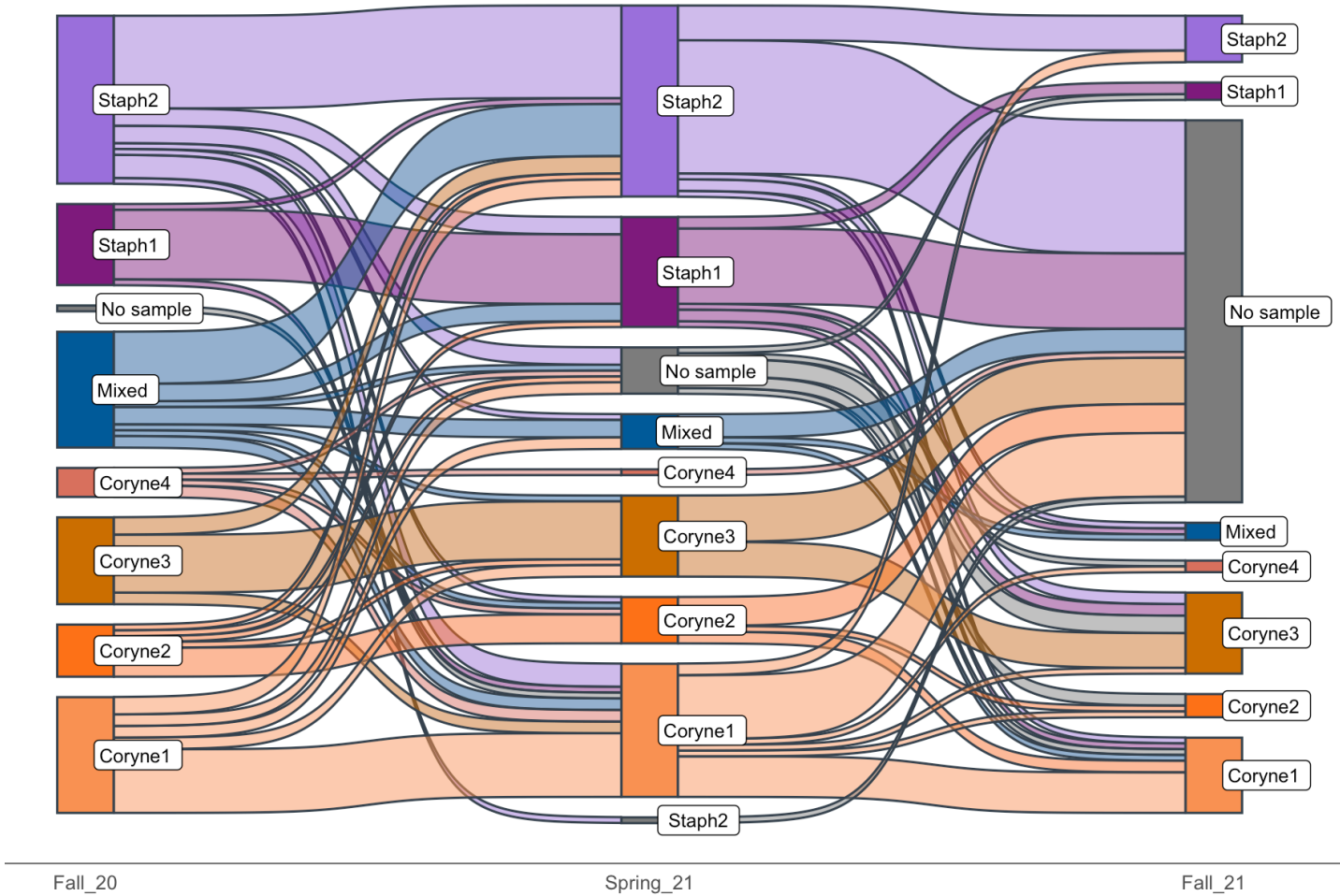


Figure 3.7: Cluster movement across 2020-2021 between individuals who did and did not acquire a respiratory infection. Individuals who did and did not have an ILI are organized in 7 clusters in the mid-turbinate microbiota and assessed for cluster movement over the course of the Fall 2020 – Spring 2021 sampling season. The 3 stages within the diagram are labelled by chronological sampling time along the x axis (Fall 2020, Spring 2021 and Fall 2021). The height of the nodes and thickness of the links connecting the nodes is proportionate to the number of samples within each cluster. Each cluster link is coloured the same legend as previous (Coryne1 = light orange, Coryne2 = orange etc). Individuals who did not provide a sample in Spring 2021 and Fall 2021 are labelled as "no sample" in gray.

Table 3.6 Mid-turbinate microbiota cluster movement

Variable	*n*, (%)
Individuals who did have an ILI (*n* = 17)	
No cluster change after ILI	4 (24%)
Change in cluster after ILI, return to baseline	1 (5.8%)
Change in cluster after ILI, no return to baseline	9 (53%)
Cluster change after ILI, remains the same	3 (17.6%)
Individuals who did not have an ILI (*n* = 100)	
No cluster change throughout seasons	49 (49%)
Cluster change throughout seasons	33 (33%)
Cluster change in Spring 2021, return to baseline	4 (4%)
Cluster change in Spring 2021, remains the same	9 (9%)
No cluster change until Fall 2021	5 (5%)

3.2.7 Investigation into host URT microbes with anti-pneumococcal activity

Due to the exploratory nature of 16S rRNA sequencing data, we need culture dependent work to complement our findings. Culture dependent work is used to isolate and screen isolates and understand their physiological importance within niche environments such as the nasal cavity[151]. We have started to build a strain collection of microbes that reside in the upper respiratory tract (anterior nares and mid-turbinates) and assess their inhibitory activity against *S. pneumoniae*. The primary pathogen, *S. pneumoniae* is used as a model organism to determine if resident microbes have anti-pneumococcal properties in the nasal cavity. The strain collection included 99 isolates, of various strains, that were tested against *S. pneumoniae* to determine if they had growth inhibition (Table 3.7). Older adults have a lower carriage rate of *S. pneumoniae,* whereas susceptibility to pneumococcal infections increase with age [8,152]. Novel drug therapies or nasal probiotics have not yet been developed to reduce pneumococcal infection in older adults, and we began to explore this preventative measure.

Of the 99 isolated microbes, 5 displayed anti – pneumococcal activity on solid agar assays (Fig 3.9). *A. odontolyticus* (GC1783) and *C. avidum* (GC1776) were cultured from adults' oral (56 years of age) and nasal (76 years of age) cavities, respectively, during the Fall 2020 sample collection period. *A. odontolyticus* is an opportunistic pathogen that is associated with acute otitis media infections in the inner ear, commonly seen in children[153]. *C. avidum* is a known commensal, commonly colonizing mucosal niches such as the nasal cavity, as opposed to dry, exposed niches[154]. These microbes were cultured from adults who did not have a respiratory infection during C&F season 2020 – 2021. Additionally, *N. mucosa* (GC1494) and *S. oralis* (GC1490) were cultured from the Surette laboratory's respiratory microbiota collection and screened by Blerina Kadiu (MSc). The demographic information of the participants are unknown (Table 3.8). *N.mucosa* is an opportunistic pathogen, colonizing mucosal surfaces in the URT (oral and nasal cavities), and can be the cause of bacteremia in immunocompromised individuals[53]. *S. oralis* is part of the Mitis group family, and commonly resides in the oral cavity. It releases H_2O_2 as a cytotoxin to reduce the growth of oral commensals such as *S. mutans*[53], as a competitive mechanism for nutrients and space within environmental niches.

These 5 strains are mostly benign to human health and less aggressive to the host than pathogenic bacteria, such as *Pseudomonas* and *Bacillus* species. The 5 chosen strains are commonly present in the healthy URT microbiome[155]. They are more likely to implement inhibitory mechanisms that are beneficial or neutral, not harmful, to the host.

The 5 strains were sequenced using whole-genome sequencing (WGS), and the genomes were assembled by the Surette laboratory. To begin browsing the secondary metabolite responsible for the anti-pneumococcal activity, an exploratory overview of biosynthetic gene cluster (BGC) predictions was assessed by using the antiSMASH database. There were 18 BGCs

identified amongst the 5 strains (Table 3.9). The most common BGC prediction was a saccharide found in *C. avidum* (GC1776), *A. odontolyticus* (GC1783), *S. oralis* (GC1490) and *N. mucosa* (GC1494) strains, followed by a cluster containing a halogenase (an enzyme) found in *C. avidum* (GC1776) and *A. odontolyticus* (GC1783) strains. There has been a rapid increase in identifying halogenated biomolecules from secondary metabolites[156]. For example, agrochemicals, detergents, polymers, and pharmaceuticals contain halogenated products[157,156]. These 5 strains lead us closer to identifying a secondary metabolite that is responsible for its pneumococcal activity, not harmful to the host or microbiome, and is a step towards future drug development. The remainder of the BGCs and the region they were found, frequency and similarity to other BGCs are summarized in Table 3.9.

Table 3.7. Summary of isolates in the URT microbiota and their inhibitory activity

Genus	Isolates (*n*)	Inhibitory activity (*n*)
Actinomyces	8	1
Bacillus	14	14
Bifidobacterium	1	0
Campylobacter	1	0
Corynebacterium	8	0
Cutibacterium	10	8
Dolosigranulum	1	0
Finegoldia	1	0
Fusobacterium	1	0
Gemella	3	0
Granulicatella	2	0
Neisseria	1	1
Prevotella	3	0
Pseudomonas	6	6
Selenomonas	1	0
Staphylococcus	22	20
Streptococcus	3	1
Veillonella	10	0

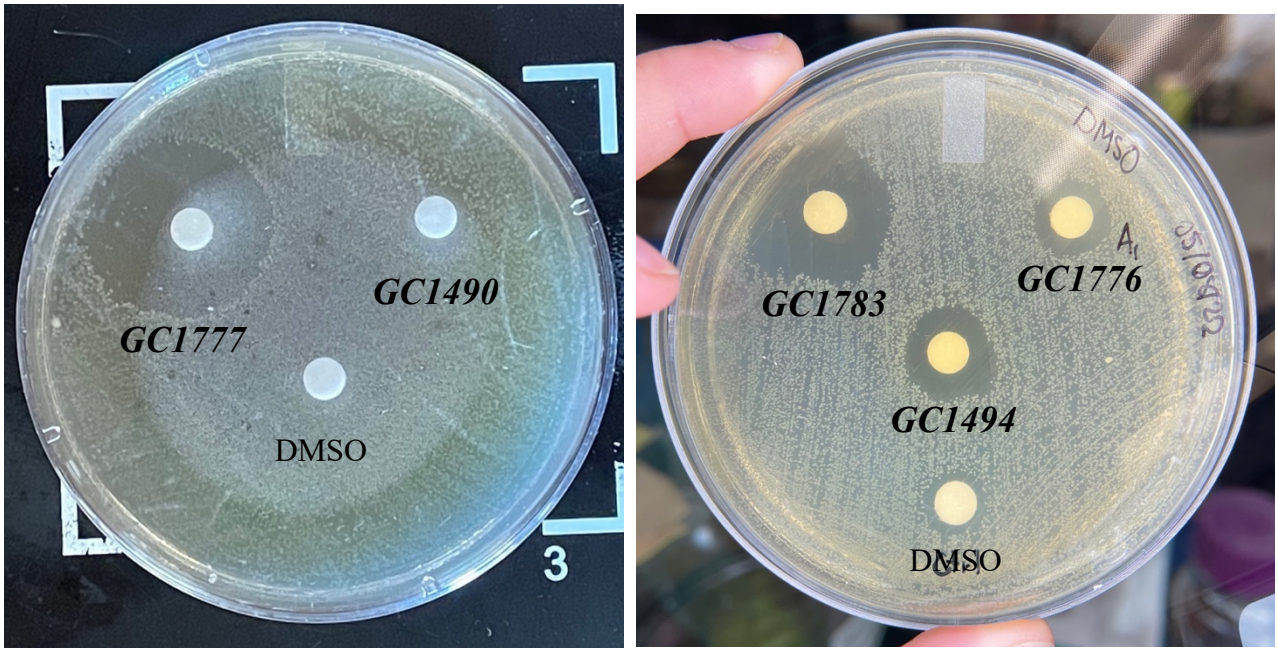


Figure 3.8: Commensal bacteria within the URT microbiota display anti-pneumococcal activity. Anti – pneumococcal activity of 5 crude extracts, isolated from the nasal cavity on a lawn of *S. pneumoniae* (P1121) and incubated in aerobic conditions for up to 48 hours. The isolates are as follows from left to right, top to bottom: GC1777 (*C. acnes)*, GC1490 (*S. oralis*), GC1783 (*A. odontolyticus*), GC 1776 (*C. avidum*) and GC1494 (*N. mucosa*). Dimethyl sulfoxide (DMSO) was used as a negative control.

Table 3.8. Strains chosen for growth inhibition testing and participant demographics

GC Number	Genus, Species	Zone of inhibition (cm)	Participant age & sex
GC1494	*Neisseria mucosa*	3	Unknown
GC1490	*Streptococcus oralis*	2.2	Unknown
GC1776	*Cutibacterium avidum*	2	76, female
GC1777	*Cutibacterium acnes*	3.2	76, female
GC1783	*Actinomyces odontolyticus*	2	56, male

Table 3.9. Biosynthetic gene cluster types identified within the 5 strains.

Cluster type	Most similar known cluster and the % of similarity to that cluster type	Bacterial species & GC #
Chlorizidine A	NRP + Polyketide: Modular type I, 7%	*C. avidum* (GC1776)
Formicamycins A-M	Polyketide, 4%	*S. oralis* (GC1490)
Exopolysaccharide	Saccharide, 6 and 10%	*C. avidum* (GC1776), *S.oralis* (GC1490)
Halogenated[+]	NA	*C. avidum* (GC1776), *A. odontolyticus* (GC1783)
Hserlactone	NA	*N. mucosa* (GC1494)
K53 capsular polysaccharide	Saccharide, 15%	*N. mucosa* (GC1494)
Molybdenum cofactor	Other, 11%	*S. oralis* (GC1490)
O&K-antigen	Saccharide, 3%	*N. mucosa* (GC1494)
Phosphonoglycan	Saccharide, 7 and 9%	*A. odontolyticus* (GC1783)
Polysaccharide	Saccharide, 11%	*A. odontolyticus* (GC1783)
Fatty acid	NA	*C. avidum* (GC1776), *A. odontolyticus* (GC1783), *N. mucosa* (GC1494)
Saccharide	NA	*C. avidum* (GC1776), *A. odontolyticus* (GC1783), *S. oralis* (GC1490), *N. mucosa* (GC1494)
RiPP-like[+++]	NA	*S. oralis* (GC1490), *N. mucosa* (GC1494)
Sch-47554/47555	Polyketide, 3%	*A. odontolyticus* (GC1783)
Streptovaricin	Polyketide, 4%	*A. odontolyticus* (GC1783)
T3PKS[++]	NA	*S. oralis* (GC1490)
Terpene	NA	*N. mucosa* (GC1494)
TP-1161	RiPP: Thiopeptide, 8%	*S. oralis* (GC1490)

[+]Cluster containing a halogenase and thus potentially generating a halogenated product

[++] Type III PKS, with region on contig edge

[+++] Other unspecified ribosomally synthesised and post-translationally modified peptide product (RiPP) cluster

3.3 Discussion

We characterized the mid-turbinate microbiota of community-dwelling adults in the Greater Hamilton Region, ON by sequencing 16S rRNA from nasal samples and performing qPCR to determine the total bacterial load within a given sample. We hypothesized increased chronological age leads to decreased diversity and increased inter-individual variation in the mid-turbinate microbiota. From these findings, we have identified that chronological age is not the driving factor for significant compositional changes in the URT microbiota. To identify relationships between the 12 factors listed above (Table 3.2) and compositional changes in the mid-turbinate microbiota, we assessed correlations between diversity measurements and ASVs with each of the factors. Bacterial load and seasonal changes between 2020-2021 are the two factors that have a significant relationship with microbial compositional composition and diversity in the mid-turbinate microbiota.

α-diversity measured the species richness and evenness within one sample, using Shannon and Simpson Index and the observed number of ASVs. b-diversity measured the similarity between individuals' microbial composition, using Bray-Curtis distances. Contrary to my hypothesis, we found the variability in the mid-turbinate microbiota does not change with chronological age, sex, frailty, inflammatory conditions, medication usage, vitamin D supplementation, smoking, contact with children and the presence of an ILI during C&F season. We expected larger inter-individual variation in community composition within older adults, as seen previously in the nasopharyngeal microbiota of frail older adults residing in LTCH[51], and the gut microbiome's of centenarians[117,116]. Bacterial load and seasonal changes are the two driving factors responsible for the inter-individual variation within the mid-turbinate microbiota and the heterogeneity in aging.

Bacterial load is defined as the number of bacteria (quantified by qPCR concentration) in each sample. The microbial community composition in the mid-turbinate cavity is correlated with bacterial load. Bacterial load is negatively associated with alpha diversity measurements, Shannon, and Simpson Indexes. An increasing bacterial load correlates with a lower species richness and evenness within the nasal microbial community. This indicates some adults have host immune factors (such as ROS, iron, carbohydrates, sugar and free-fatty acids) that support the growth of protective and commensal bacteria in their nasal cavity and could protect them from respiratory infection.

Individuals who provided a mid-turbinate sample during Spring 2021 had a significantly higher bacterial load, compared to Fall 2020, and Fall 2021. This could be associated with less humidity in the Fall season (Sept – Dec), resulting in a drier environmental niche within the nasal cavity both indoors and outdoors[158,159]. The Spring season has increased humidity in the air, especially after dry Winter months, and could result in a fuller niche with an increased abundance in diverse microbes. We hypothesized that an increase in bacterial load is positively associated with an increase in dominant ASVs within the nasal microbiota, such as *Corynebacterium, Staphylococcus* and *Streptococcus*. Out of the 18 ASVs correlated with bacterial load, 7/18 [38.8%] have a positive correlation with bacterial load in the mid-turbinate microbiota. These ASVs include *S. aureus, C. kroppenstedtii, D. pigrum, M. nonliquefaciens* and members of the *Corynebacterium* genera that could not be defined at the species level. This is contrary to data from a recent study in our lab[48], which did not find an increase in dominant taxa with an increasing bacterial load. Instead, they found decreasing microbial load was associated with an increase in other commensal bacteria within the frail older adult's microbiome. We hypothesize that commensal bacteria in the adult nasal microbiota belonging to

Corynebacterium, Staphylococcus, Dolosigranulum and *Cutibacterium* genera prime the immune system and are correlated with higher bacterial load in the mid-turbinate microbiota, leading to a fuller niche, and decreasing the ability of pathogenic bacteria to colonize. The higher bacterial load in the Fall could in turn, reduce the susceptibility to respiratory infection during C&F season[159].

We identified the nasal microbiota of community-dwelling adults could be grouped into 7 distinct clusters based on dominant microbial communities and ASVs. To determine if there were relationships between cluster membership and the host factors listed above, we assessed correlations between the 7 clusters and each of the factors. Vitamin D supplementation and sex differences (males vs females) are associated with the Coryne3 cluster. There are significantly more adults with *Corynebacterium* dominant profiles that do not supplement with Vitamin D, compared to those who do supplement with vitamin D. Contrary to our hypothesis, this could indicate the absence of antimicrobial peptides induced by vitamin D does not have an impact on beneficial commensal bacteria in the nasal cavity, such as *Corynebacterium*. There are a significantly higher number of females in the Coryne3 cluster, compared to males indicating *Corynebacterium* bacteria are dominant in females.

Identifying one microbe that is associated with protection from infection in the nasal microbiota of older adults is challenging to define. Our data suggests that preventative features in the URT microbiota against acquiring respiratory infections include the presence of 4 ASVs (*M. catarrhalis, Corynebacterium segmentosum*, *Staphylococcus aureus* and *Corynebacterium* (species level unidentified)). A larger sample size of adults who did versus did not acquire a respiratory infection is required to confirm these findings.

To determine if nasal microbiota stability influenced acquiring respiratory infections, we assessed the cluster movement between seasons during 2020-2021 between adults who did and did not acquire an ILI during C&F season. These data indicate 9/17 [53%] of adults that acquired respiratory infections, changed clusters after the infection and did not return to their original cluster membership. There was no pattern between which cluster adults moved to after the occurrence of a respiratory infection, and the cluster movement was unique to the individual. These results are consistent with previous findings in our laboratory, which determined the nasal microbiota of frail nursing home residents lacks stability in the event of an ILI[48].

Of the 100 adults who did not acquire a respiratory infection, 49% did not change clusters throughout seasons during 2020-2021. This could indicate that a more stable microbiota (i.e., less cluster movement) is attributed to less incidence of respiratory infections. Our sample size for the number of adults that acquired respiratory infections was small, and we are unable to make strong conclusions on the lack of stability in the adult nasal microbiota influencing respiratory infection risk.

The commensal bacterium *C. acnes* was one of the 5 strains that demonstrated anti-pneumococcal activity in the URT microbiota and is found to decrease with increasing bacterial load. *C. acnes* is a well-known commensal bacterium that colonizes the sebaceous regions of human skin, and is abundant across healthy people, composing 92% of the sebaceous skin microbiome[160,161]. It can kill *S. aureus* through the production of propionic acid and could limit *S. aureus* colonization on the skin[160]. There were no ASVs that were significantly associated with the other host factors in the adult mid-turbinate microbiota. The remaining 4 strains were isolated from the URT of older adults, that did not have an ILI during C&F season. These findings suggest individuals who did not have an ILI during C&F season could carry commensal

microbes with protective features such as growth inhibition against the primary pathogen, *S. pneumoniae*. We began to explore the secondary metabolites responsible for this activity using the antiSMASH database, leading us to believe that preventative measures against ILI requires a mixture of beneficial microbes, not a single, most beneficial microbe. *C. acnes* has is a well-known commensal bacterium, and individuals have a unique mixture and abundance of *C. acnes* strains[160]. This isolate is important to include for the development of nasal probiotics to prevent respiratory infections in older adults. Future work is required to identify if *Corynebacterium* strains also have anti-pneumococcal activity that can be included in preventative therapies against ILIs, because they are an important beneficial commensal inhabiting the nasal microbiota.

Chapter 4: The role of chronic inflammatory conditions in the URT microbiota

4.1 Introduction

4.1.1 Chronic inflammatory changes in the URT microbiota

As previously mentioned, age-associated inflammation impacts the microbiota in mucosal surfaces such as the gut[83]. The mucosal surface of the nasal cavity also undergoes physiological changes[19] and with this knowledge, we hypothesize inflammation could be a driving factor for the compositional, diversity and bacterial load changes occurring in the nasal microbiota. As stated beforehand, chronic inflammatory conditions (e.g., chronic rhinosinusitis, asthma, COPD) are associated with microbial dysbiosis[91]. Inflammation is a common underlying feature of all these conditions; therefore, we hypothesize that inflammation could also be an important correlate of dysbiosis in the nasal microbiota.

Within this chapter, we investigated the composition and diversity changes in the anterior nares microbiota of adults who are diagnosed with either osteoarthritis or rheumatoid arthritis and their age- and sex-matched controls, collected from community-dwelling adults. Consistent with our chapter 3 results, we determined chronological age does not correlate with the composition of the nasal microbiota. We found inter-individuality drives the variation in the anterior nares microbiota of individuals living with osteoarthritis and the anterior nares microbiota of individuals with rheumatoid arthritis changes in response to DMARD treatment and reduction in erythroid sedimentation rates. These results allow us to further understand the relationship between chronic inflammation and the URT microbiota to aid us in identifying causal relationships with these changes in the future.

4.2 Results

4.2.1 Characteristics of the study population

We recruited a total of 26 adults with osteoarthritis and 20 adults with rheumatoid arthritis in collaboration with Drs. Maggie Larché and Raja Bobba. We also recruited 92 healthy adults from the Greater Hamilton region. A total of 99 anterior nares samples were collected from adults diagnosed with osteoarthritis or rheumatoid arthritis over multiple visitation periods, outlined in Fig 2.2 (Chapter 2). A total of 113 anterior nares samples were collected from community-dwelling adults in the Greater Hamilton region as healthy controls, outlined in Fig 2.3 (Chapter 2). During the baseline sample collection period, 18 nasosorps were collected from adults diagnosed with osteoarthritis to measure nasal inflammation and 19 venous blood samples were collected from adults diagnosed with rheumatoid arthritis, to measure systemic inflammation. ESR and CRP measurements were collected as part of their clinical care. Demographic and health information was collected at the baseline sampling timepoint with the health questionnaire. The 99 anterior nares samples were analyzed by 16S rRNA based sequencing and total bacterial load was assessed by amplifying the 16S rRNA gene using qPCR.

4.2.2 The composition of the anterior nares microbiota of adults with and without chronic inflammatory conditions (osteoarthritis and rheumatoid arthritis)

There is high variability in the anterior nares' microbiota of adults living with osteoarthritis or rheumatoid arthritis. (Fig 4.5). To characterize the composition of the anterior nares microbiota of adults who have chronic inflammatory conditions, we performed hierarchical clustering to identify clusters, as described in chapter 3. The dendrogram (Fig 4.5F) measures the distance between each individual's sample using Bray-Curtis Dissimilarities, and individuals are coloured by the presence of a chronic inflammatory condition (OA vs RA) or no inflammatory

conditions (Control). This visualization shows the high inter-individual variation within these microbial communities. We hypothesized that individuals with either osteoarthritis or rheumatoid arthritis would have higher similarity, compared to individuals without inflammatory conditions, but they do not. Individuals with either of these chronic inflammatory conditions do not cluster together based on similar microbial communities, suggesting there is inter-individuality in the anterior nares microbiota of individuals with different chronic inflammatory conditions. Individuals with and without osteoarthritis and rheumatoid arthritis were combined to define clusters in the anterior nares microbiota.

In this analysis, a cluster is defined as 3 or more individuals that contain the same dominant ASV within their sample. The samples in these clusters were provided at the baseline sampling timepoint, to avoid replicates of the same individual at multiple timepoints. Two of these clusters contain only 2 samples (identified by a '^' symbol) and were removed from further analyses, because they did not fall under the definition of a cluster.

The 8 clusters are named: Coryne1, Coryne2, Coryne3, Mixed, Morax/Dolo, Staph1, Staph 2 and Strep (Table 4.2). These 8 clusters are significantly dispersed from one another and explains 30% of the inter-individual within the anterior nares adult microbiota (supplementary Fig 4.1). Sex has a significant main effect amongst cluster membership overall (Table 4.2). Post-hoc analyses were performed and determined none of the clusters were significantly correlated with either sex (male or female) (Supplementary table 4.8).

Cluster membership was not statistically significant different with age, the presence of a chronic inflammatory condition, the type of chronic inflammatory condition, bacterial load, season, DMARD treatment, sampling time and GM-CSF, Fractalkine, IL-1b, IL-6, TNF concentrations in the anterior nares microbiota (Table 4.2).

The dendrogram (Fig 4.5) measures the distance between each individual sample at the baseline timepoint using Bray – Curtis Dissimilarities. Individuals that have branches closer together in the dendrogram have a high similarity in their microbial composition, compared to individuals whose branches are farther apart. The taxonomic bar plots below the dendrogram indicate the relative abundance of the dominant genera within the samples (Fig 4.5A), and the dominant ASVs within each cluster (Fig 4.5B-E). The largest cluster is the Mixed cluster (*n* = 34 [28%]), which is dominated by multiple ASVs belonging to *Corynebacterium, Staphylococcus, Cutibacterium* and *Dolosigranulum* genera. The next largest cluster is dominated by *Moraxella* and *Dolosigranulum* spp. (*n* = 22 [18%]). The remainder of clusters are dominated by various ASVs of *Corynebacterium, Staphylococcus* and *Streptococcus* named Coryne1 (*n* = 13 [10.7%]), Coryne2 (*n* = 8 [6.6%]), Coryne3 (*n* = 6 [4.9%]), Staph1 (*n* = 19 [15.6%]), Staph2 (*n* = 14 [11.5%]) and Strep (*n* = 6 [4.9%]). Each cluster is mostly dominated by a single ASV. For example, Coryne1 is dominated by ASV 2, whereas Coryne2 is dominated by ASV 5. Staph1 is dominated by ASV 3 and Staph2 is dominated by ASV 8. *Corynebacterium* (63%), *Staphylococcus* (48-65%), *Cutibacterium* (19%), *Dolosigranulum* (38%), *Pseudomonas* (>11%), *Moraxella* (18%) and *Streptococcus* (40.5%) are the top 7 genera that have the highest relative abundance within the 8 clusters (Fig 4.2A). With the exception of the Mixed cluster, a pairwise comparison of the 8 clusters indicates that samples within the same cluster have higher similarity to one another (dissimilarity closer to 0) compared to samples belonging in other clusters (Fig 4.2B).

Intra-individual variation of the anterior nares microbiota was measured by 3 diversity measurements and differed by cluster membership (Fig 4.3A-D). Samples in the Mixed cluster have higher diversity than the Moraxella/Dolosigranulum cluster, due to containing a various

number of ASVs in their anterior nares microbiota (Fig 4.3A). The Strep cluster has higher diversity than the Moraxella/Dolosigranulum cluster and the Staph2 cluster. This indicates adults within this cluster carry a higher diversity of ASVs belonging to *Streptococcus* than *Staphylococcus* (Fig 4.3C).

Table 4.1 Participant characteristics organized by cluster membership in adults living with and without osteoarthritis and rheumatoid arthritis

Characteristic	**Coryne1**, N = 13[1]	**Coryne2**, N = 8[1]	**Coryne3**, N = 6[1]	**Mixed**, N = 34[1]	**Morax/Dolo**, N = 22[1]	**Staph1**, N = 19[1]	**Staph2**, N = 14[1]	**Strep**, N = 6[1]
Age (years)	65.4 (12.4)	50.5 (10.7)	59.8 (17.3)	51.1 (16.0)	61.5 (16.7)	56.5 (15.4)	58.2 (18.7)	60.7 (5.6)
Sex**								
F	13 / 13 (100%)	8 / 8 (100%)	2 / 6 (33%)	24 / 34 (71%)	15 / 22 (68%)	18 / 19 (95%)	11 / 14 (79%)	5 / 6 (83%)
M	0 / 13 (0%)	0 / 8 (0%)	4 / 6 (67%)	10 / 34 (29%)	7 / 22 (32%)	1 / 19 (5.3%)	3 / 14 (21%)	1 / 6 (17%)
ConditionType								
Inflammatory	6 / 13 (46%)	4 / 8 (50%)	1 / 6 (17%)	10 / 34 (29%)	5 / 22 (23%)	8 / 19 (42%)	3 / 14 (21%)	4 / 6 (67%)
Non-inflammatory	7 / 13 (54%)	4 / 8 (50%)	5 / 6 (83%)	24 / 34 (71%)	17 / 22 (77%)	11 / 19 (58%)	11 / 14 (79%)	2 / 6 (33%)
Condition								
Control	7 / 13 (54%)	4 / 8 (50%)	5 / 6 (83%)	24 / 34 (71%)	17 / 22 (77%)	11 / 19 (58%)	11 / 14 (79%)	2 / 6 (33%)
Osteoarthritis	6 / 13 (46%)	1 / 8 (12%)	0 / 6 (0%)	7 / 34 (21%)	5 / 22 (23%)	4 / 19 (21%)	1 / 14 (7.1%)	3 / 6 (50%)
Rheumatoid arthritis	0 / 13 (0%)	3 / 8 (38%)	1 / 6 (17%)	3 / 34 (8.8%)	0 / 22 (0%)	4 / 19 (21%)	2 / 14 (14%)	1 / 6 (17%)
Bacterial load	4.9 (0.8)	4.9 (0.6)	4.9 (0.9)	5.0 (0.6)	5.2 (1.0)	4.9 (0.9)	4.9 (0.6)	4.5 (0.8)
Season								
Fall	4 / 13 (31%)	3 / 8 (38%)	3 / 6 (50%)	13 / 34 (38%)	10 / 22 (45%)	8 / 19 (42%)	9 / 14 (64%)	3 / 6 (50%)
Spring	0 / 13 (0%)	2 / 8 (25%)	0 / 6 (0%)	2 / 34 (5.9%)	2 / 22 (9.1%)	2 / 19 (11%)	0 / 14 (0%)	0 / 6 (0%)
Summer	8 / 13 (62%)	3 / 8 (38%)	1 / 6 (17%)	11 / 34 (32%)	7 / 22 (32%)	8 / 19 (42%)	2 / 14 (14%)	3 / 6 (50%)
Winter	1 / 13 (7.7%)	0 / 8 (0%)	2 / 6 (33%)	8 / 34 (24%)	3 / 22 (14%)	1 / 19 (5.3%)	3 / 14 (21%)	0 / 6 (0%)
Sampling time								
Baseline	6 / 13 (46%)	4 / 8 (50%)	1 / 6 (17%)	10 / 34 (29%)	5 / 22 (23%)	8 / 19 (42%)	3 / 14 (21%)	4 / 6 (67%)
Baseline Control	7 / 13 (54%)	4 / 8 (50%)	5 / 6 (83%)	24 / 34 (71%)	17 / 22 (77%)	11 / 19 (58%)	11 / 14 (79%)	2 / 6 (33%)
Osteoarthritis specific parameters								
GM-CSF (pg/mL)	1.7 (2.1)	NA (NA)	-1.1 (2.1)	0.4 (1.8)	0.8 (2.3)	0.2 (3.5)	0.4 (2.1)	2.2 (1.0)
Fractalkine (pg/mL)	3.0 (2.8)	NA (NA)	3.9 (1.1)	2.5 (2.3)	3.3 (2.0)	3.0 (2.1)	2.5 (2.4)	4.4 (0.8)
IL-1B (pg/mL)	0.9 (2.8)	NA (NA)	-0.4 (2.5)	0.1 (1.3)	0.5 (0.8)	0.0 (3.7)	1.0 (1.6)	0.4 (1.0)
IL-6 (pg/mL)	1.6 (1.3)	NA (NA)	1.1 (1.5)	1.2 (1.6)	1.0 (1.4)	2.0 (0.9)	1.6 (0.9)	2.4 (1.6)
TNF-α (pg/mL)	1.5 (1.6)	NA (NA)	0.0 (0.5)	0.4 (0.9)	0.8 (1.4)	1.9 (1.3)	0.6 (0.9)	1.3 (1.4)
Rheumatoid arthritis specific parameters								
DMARD Treatment								
N	13 / 13 (100%)	5 / 8 (62%)	5 / 6 (83%)	32 / 34 (94%)	22 / 22 (100%)	15 / 19 (79%)	12 / 14 (86%)	5 / 6 (83%)
Y	0 / 13 (0%)	3 / 8 (38%)	1 / 6 (17%)	2 / 34 (5.9%)	0 / 22 (0%)	4 / 19 (21%)	2 / 14 (14%)	1 / 6 (17%)
ESR (mm/hr)	NA (NA)	24.3 (22.4)	44.0 (NA)	17.3 (20.5)	NA (NA)	32.2 (35.9)	37.0 (5.7)	13.0 (NA)
CRP (mm/hr)	3.9 (1.2)	62.3 (73.4)	29.3 (59.6)	7.8 (8.4)	4.4 (4.5)	5.7 (3.2)	7.5 (8.8)	13.1 (17.2)

[1] Mean (SD); n / N (%)

P – values < 0.05 are indicated with a (**)

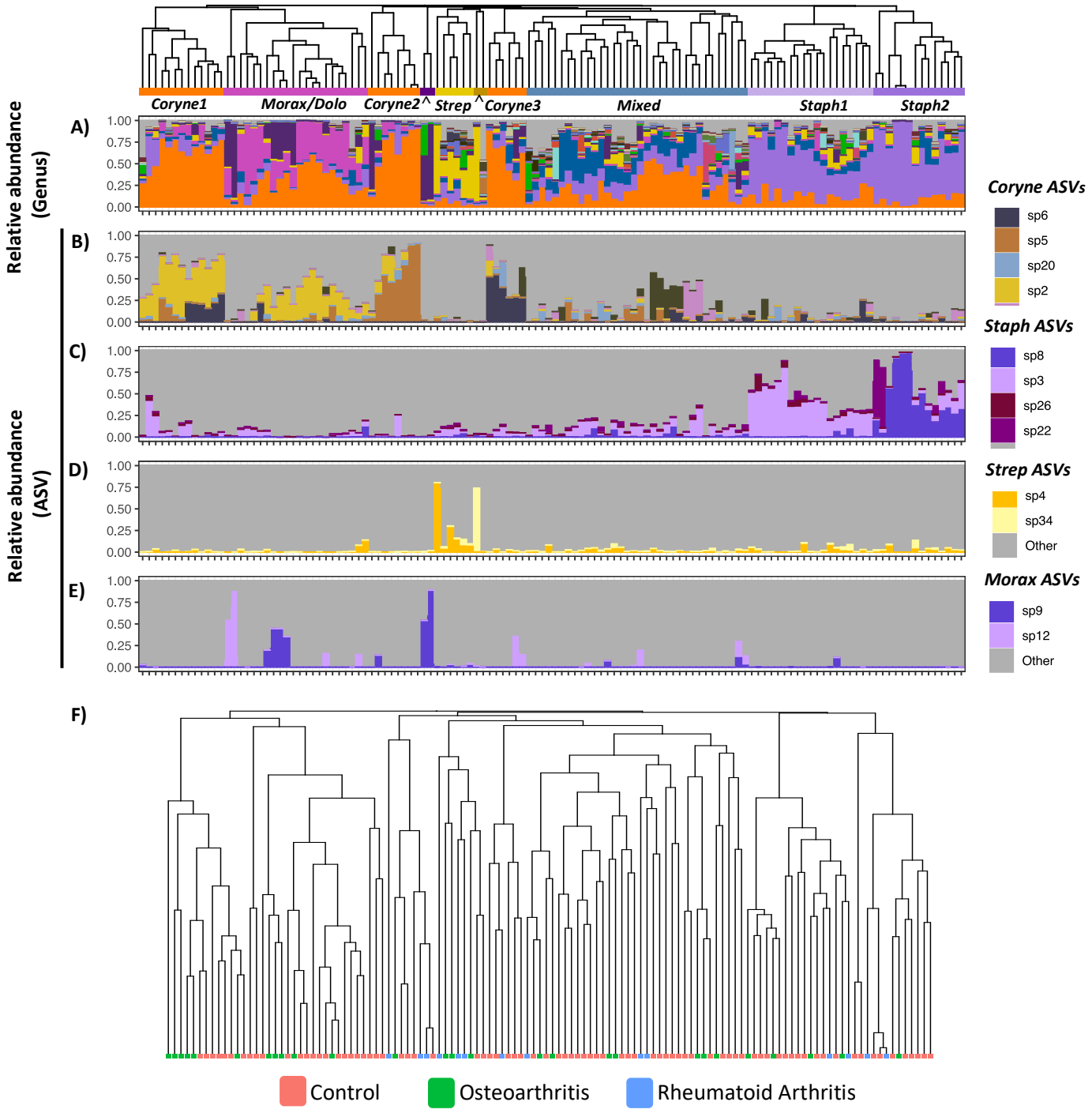


Figure 4.1 A-F: The nasal microbiota of adults living with or without osteoarthritis and rheumatoid arthritis is organized into 8 clusters. The differences between samples (n = 126) are measured using Bray-Curtis dissimilarities at the ASV level. **(A)** The taxonomic bar plot is visualized with the 20 dominant genera. The 10 clusters are significantly dispersed ($p < \mathbf{2.2^{-16}}$, ANOVA) and are clustered differently from one another (p = **0.001**, PERMANOVA). An analysis of similarity (ANOSIM) determined the similarities between the clusters are significant and suggests there is more similarity within the clusters than there are between the clusters (R = 0.774, p = **0.001**). **(B-E)** *Corynebacterium*, *Staphylococcus, Streptococcus* and *Moraxella* ASV taxonomic bar plots are visualized beneath the Genus taxonomic bar plot, displaying the dominant ASVs present within each cluster. (F) The dendrogram of adults nasal microbiota's living with osteoarthritis or rheumatoid arthritis and healthy controls, coloured accordingly.

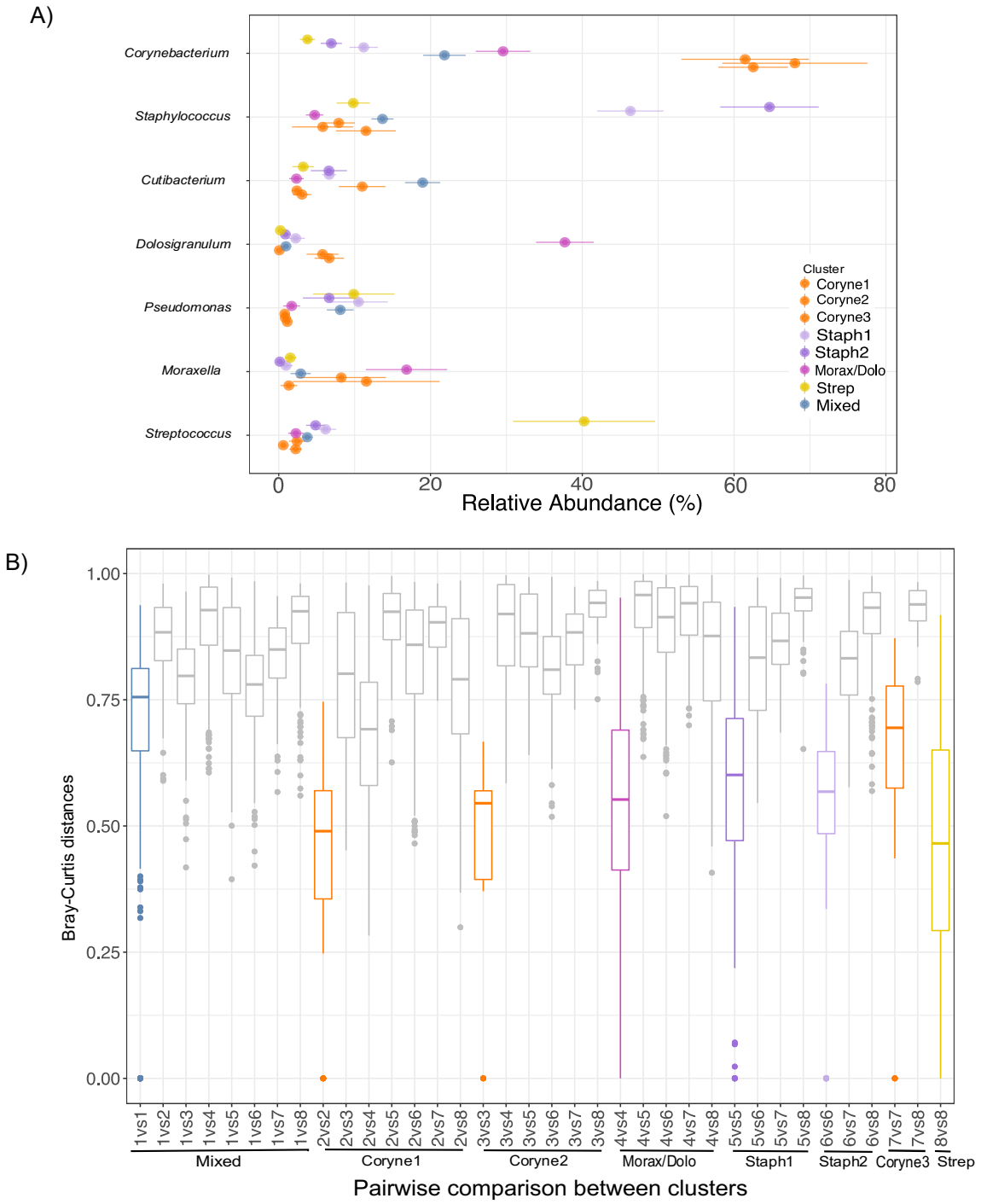


Figure 4.2A-B: (A) Relative abundances (%) of the 7 dominant genera in all mid turbinate samples coloured by cluster. Cluster colouring is as follow: Coryne1-3 (orange), Staph1 (lavender), Staph2 (purple), Morax/Dolo (pink), Strep (yellow) and Mixed (blue). **(B) Bray Curtis distances measured for a pairwise comparison between clusters.** Cluster number corresponds to the following cluster organization: 1 = Mixed (blue), 2 = Coryne1 (orange), 3 = Coryne2 (orange), 4 = Morax/Dolo (pink), 5 = Staph2 (purple), 6 = Staph1 (light purple), 7 = Coryne3 (orange) 8 = Strep (yellow).

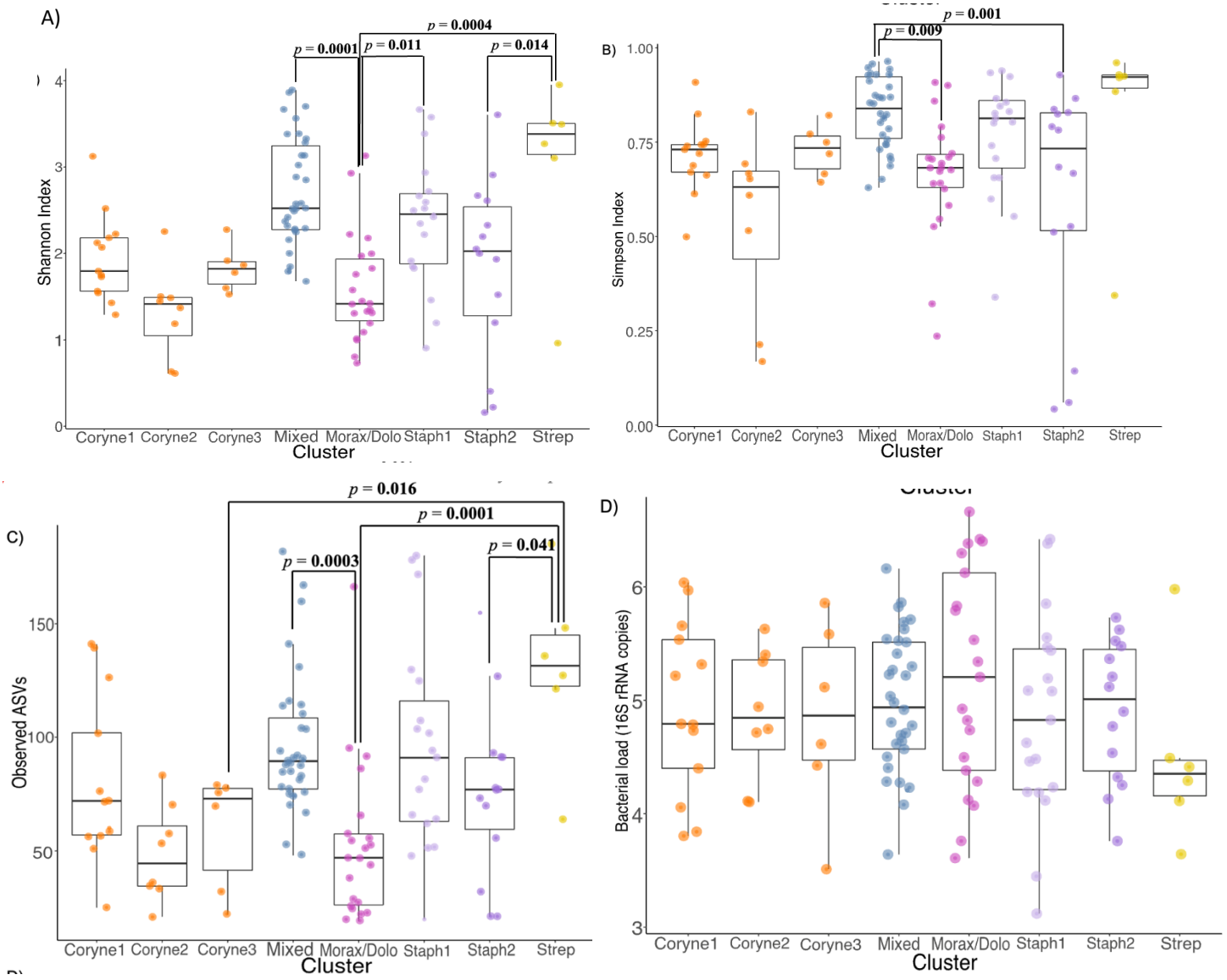


Figure 4.3A-D: a - diversity and bacterial load are correlated with cluster membership. (A-C) a - diversity was measured using Shannon index ($p < \mathbf{2.2^{-16}}$) and Simpson Index ($p = \mathbf{0.034}$). Observed ASVs were significantly different amongst the clusters ($p < \mathbf{2.2^{-16}}$). **(D)** The bacterial load was calculated by using quantitative PCR and was expressed in $\log_{10}$ values (16S ribosomal RNA gene copies/mL of UTM media) ($p = 0.617$, ns). Statistical significance was tested using a linear mixed - effect model, controlling for participant ID as a random effect. Cluster colouring is as follow: Coryne1-3 (orange), Staph1 (lavender), Staph2 (purple), Morax/Dolo (pink), Strep (yellow) and Mixed (blue).

4.2.3 Chronic inflammation changes the microbial composition of adults living with rheumatoid arthritis

In order to determine whether intra- and inter-individuality differ between adults living with osteoarthritis, rheumatoid arthritis and healthy controls, I quantified a and b-diversity measurements. Using Shannon, Simpson indices and Observed ASVs to measure a-diversity, I determined there were slight changes in intra-individual variation within the nasal microbiota of adults living with osteoarthritis and rheumatoid arthritis (Fig 4.4A-C). Adults living with osteoarthritis have a higher Simpson index in their nasal microbiota, compared to adults living with rheumatoid arthritis. This indicates the nasal microbiota of adults living with osteoarthritis are dominated by an even abundance of various bacteria, whereas the nasal microbiota of adults living with rheumatoid arthritis is dominated by fewer bacterial species. There were no significant changes in the nasal microbiota of adults living with osteoarthritis or rheumatoid arthritis as measured by the Shannon index and Observed ASVs. Other host factors including, age, sex, and bacterial load (16S rRNA copies per sample as measured by qPCR concentration) were also not correlated with a-diversity measurements (Shannon, Simpson indices and Observed ASVs) in the nasal microbiota (supplementary table 4.1).

Using a distance-based redundancy analysis (dbRDA) to assess constrained ordinations on how inflammatory conditions impact the nasal microbiota, we determined the nasal microbiota changes in adults with rheumatoid arthritis, not osteoarthritis, measured with Bray-Curtis dissimilarities at the ASV level (Fig 4.4D). This ordination explains 2.7% of the variation in the nasal microbiota and suggests systemic chronic inflammation (measured by rheumatoid arthritis) drives the inter-individual variation in the nasal microbiota (p = **0.017**). Other host factors including age, sex and bacterial load did not impact the composition of the nasal

microbiota in adults living with osteoarthritis and rheumatoid arthritis, measured by b-diversity (supplementary table 4.2).

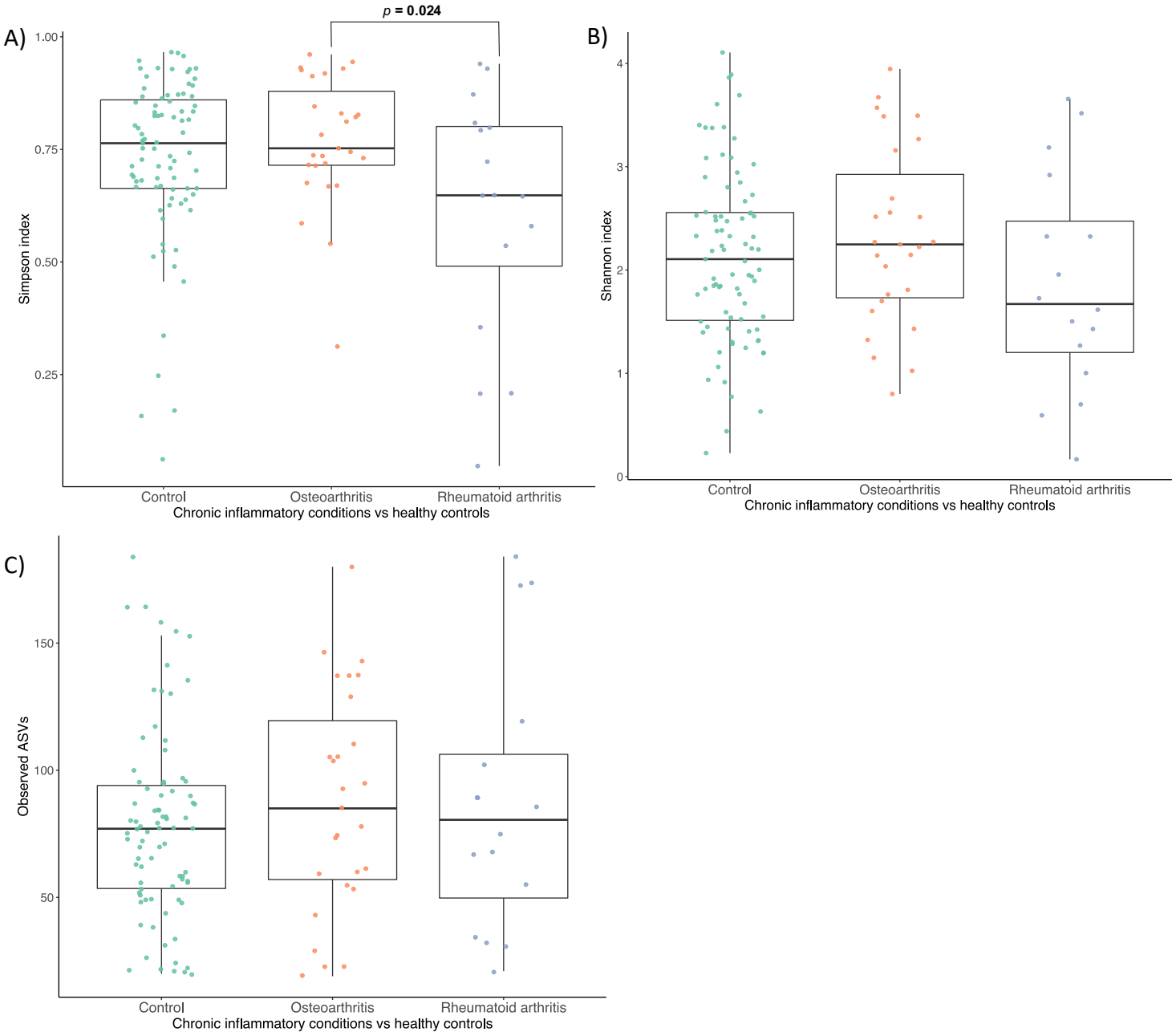


Figure 4.4A-C: a-diversity is higher in adults living osteoarthritis, compared to adults living with rheumatoid arthritis. α-diversity is measured between adults with osteoarthritis (n = 27), rheumatoid arthritis (n = 16) and age and sex matched controls (n = 83). Each point is an individual. Nasal samples were collected at baseline. Statistical testing was performed using a linear model. (A) Simpson index was statistically different between adults living with osteoarthritis versus rheumatoid arthritis (p = 0.024). (B-C) Shannon index and Observed ASVs did not have statistical significance between adults living with osteoarthritis, rheumatoid arthritis, or healthy controls.

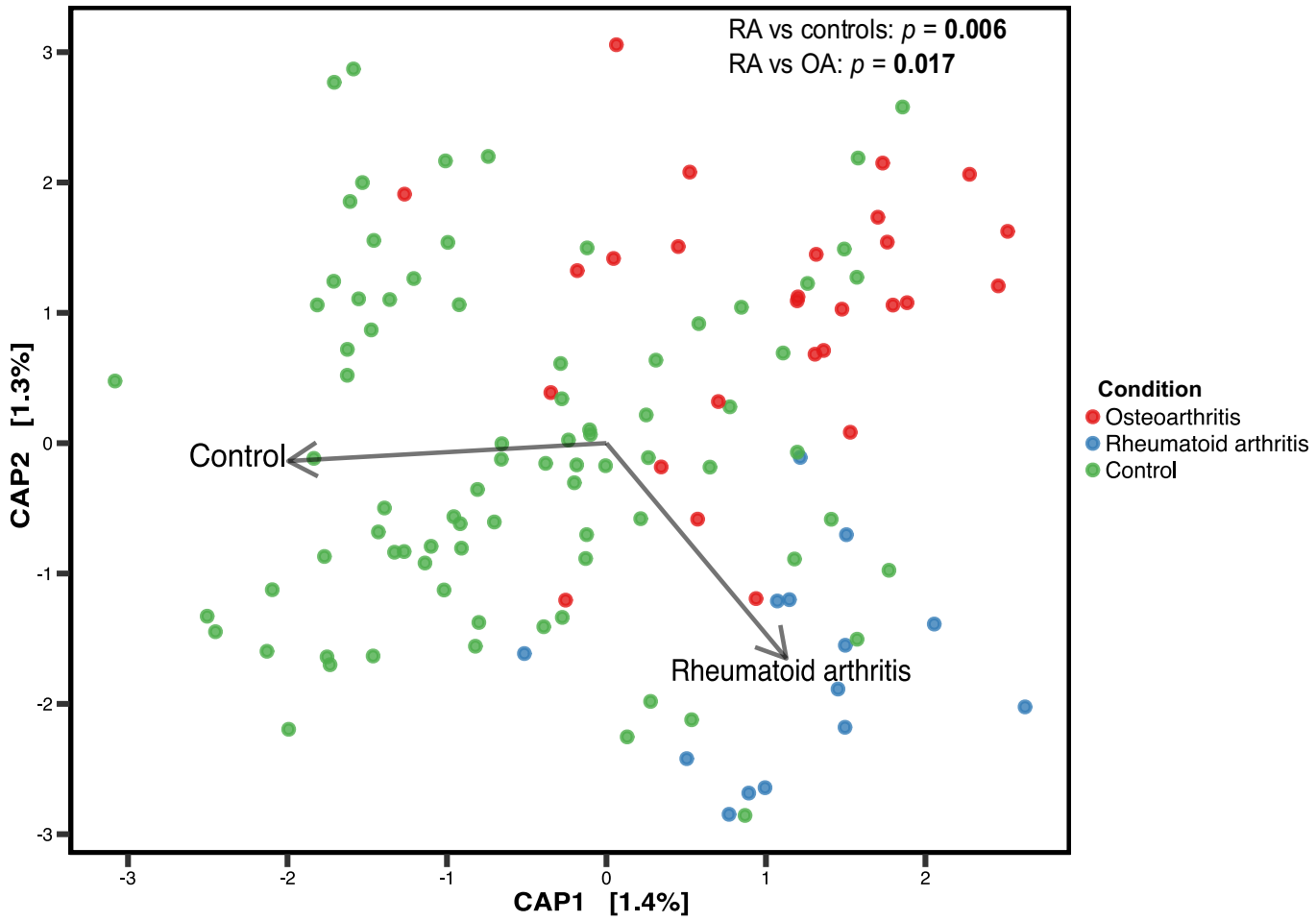


Figure 4.5: Adults with rheumatoid arthritis have different nasal microbial compositions compared to adults living with osteoarthritis. b-diversity is measured between adults with osteoarthritis (n = 27), rheumatoid arthritis (n = 16) and age and sex matched controls (n = 83). Each point is an individual. Nasal samples were collected at baseline. Statistical testing was performed using PERMANOVA for comparisons between groups, RA vs controls: p = **0.006**, RA vs OA: p = **0.017** and OA vs controls: p = 0.39.

4.2.4 Response status to DMARD treatment changes the nasal microbiota of adults living with rheumatoid arthritis

To test the hypothesis that the nasal microbiota changes with systemic inflammation, I measured changes in composition that correlate with changing ESR, a clinical measure of inflammation and of treatment success. Elevated ESR values are defined as >28 mm/hr in males and females[162]. Using this range, I categorized individuals with rheumatoid arthritis into three statuses: Unaffected by DMARDS treatment (their ESR values remain <28 mm/hr at the baseline timepoint, and they did not change during subsequent visitations), non-responsive to DMARDS treatment (their ESR values were >28 mm/hr at each sample timepoint, and did not decrease) and responsive to DMARDS treatment (ESR values at baseline were >28 mm/hr and decreased to <28 mm/hr with subsequent visitations). Bray-Curtis dissimilarities were measured between baseline and visit 2 anterior nares samples. The β-diversity, as measured by Bray – Curtis Dissimilarity, is higher in adults living with rheumatoid arthritis who are non-responsive to DMARD treatment (high levels of inflammation) and responsive to DMARD treatment (lower levels of inflammation) (p = **0.026**, Fig 4.6).

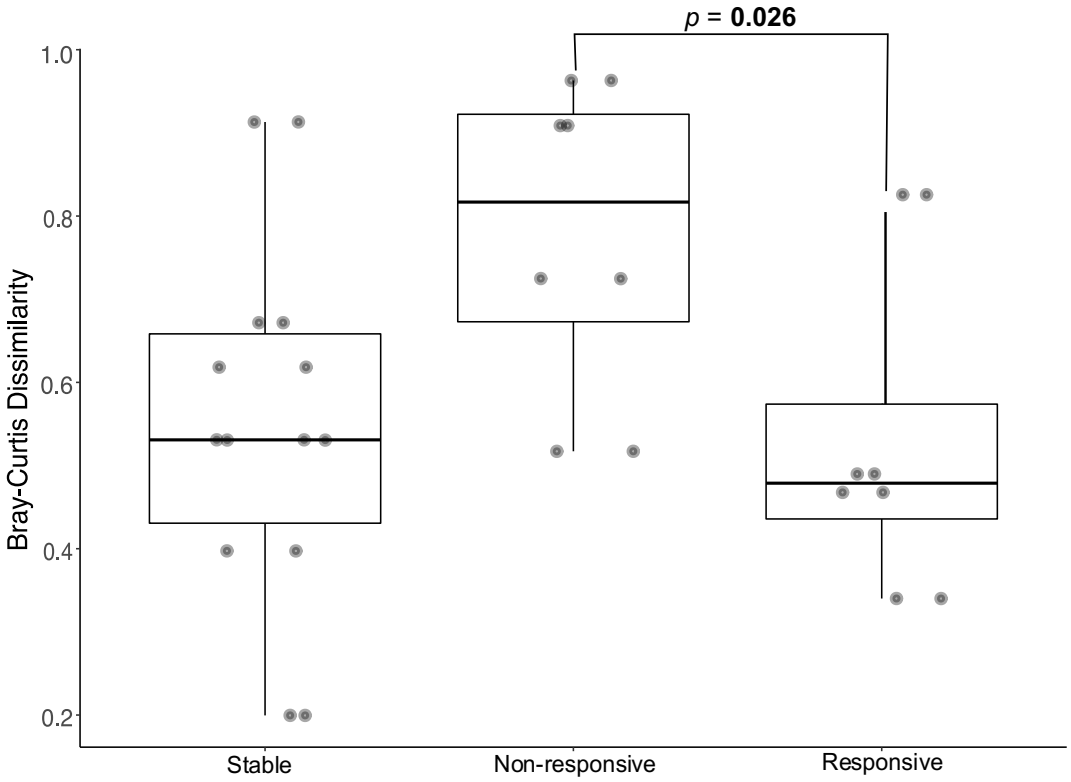


Figure 4.6: Adults that are non-responsive to DMARDS treatment have higher dissimilarity compared to adults that are responsive to DMARDS treatment. Bray-Curtis dissimilarity was measured at baseline and at a second visit when the participants had been on new therapies for at least 6 months. Adults who were unaffected by DMARDS treatment (i.e., they had no change in their ESR values, and the values remained <28 mm/hr, n = 14) did not have significantly different dissimilarities between timepoints. Adults who were non-responsive to DMARDS treatment (n = 8, their ESR values remain >28 mm/hr at each visitation) have significantly different dissimilarities between timepoints, compared to adults who were responsive to DMARDS treatment (n = 8, ESR values at baseline were >28 mm/hr and decreased to <28 mm/hr with subsequent visitations) (p = 0.026). Statistical testing was performed using a Kruskal-Wallis test.

4.2.5 Bacterial load changes within the nasal cavity of adults living with osteoarthritis

To determine if bacterial load is different between osteoarthritis, rheumatoid arthritis, and healthy control groups, we performed qPCR to measure 16S rRNA gene copies within each nasal sample at each sampling timepoint (Baseline, Visit 1 and Visit 2). Adults with osteoarthritis have a significantly lower mean bacterial load compared to healthy controls (p = **0.01**, Fig 4.7). Adults with rheumatoid arthritis also have a trend towards a lower mean bacterial load compared to healthy controls, but the difference is not significant (p = 0.248). When we investigated changes in the nasal microbiota over sampling timepoint, we determined bacterial load is significantly lower for adults with osteoarthritis at baseline compared to their healthy controls at baseline (p = **0.025**, supplementary fig 4.1). There is no significant difference between in adults living with osteoarthritis during their visit 1 and visit 2 sampling timepoints compared to their healthy controls (p = 0.999 and 0.792, respectively, supplementary table 4.3). Adults with osteoarthritis have the highest chronic inflammation at baseline, and we hypothesize that bacterial load could correlate with increased local inflammation.

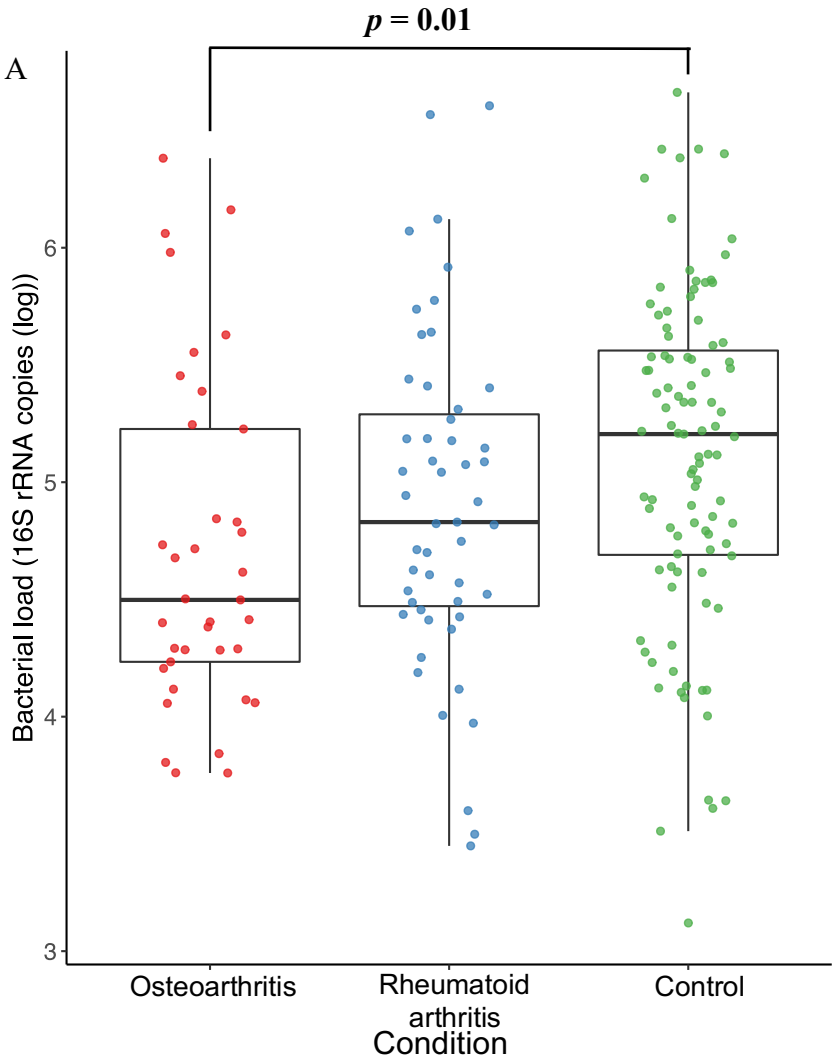


Figure 4.7: Bacterial load is lower in adults living with osteoarthritis, not rheumatoid arthritis, compared to healthy controls. The bacterial load was calculated by using quantitative PCR and was expressed in $\log_{10}$ values (16S ribosomal RNA gene copies/mL of UTM media). (A) Conditions include OA (n = 37), RA (n = 51) and Controls (n = 102). Statistical significance was tested using a linear mixed - effect model, controlling for participant ID as a random effect. (B) Sampling timepoints include baseline OA (n = 27) and baseline control (n = 54). Significance between timepoints were tested using post-hoc comparisons (Baseline OA vs baseline controls, p = 0.025)

4.2.6 Local inflammatory cytokines increase in the nasal cavity of adults with osteoarthritis

To determine if adults with osteoarthritis have higher local inflammation compared to healthy controls, t-test analyses are performed between adults living with osteoarthritis, and healthy controls (n = 18, n = 53, respectively). Amongst the 5 cytokines, GM-CSF, Fractalkine, IL-6 and TNFα have a significantly higher mean concentration within the nasal cavity of adults with osteoarthritis compared to healthy controls (Fig 4.8A-E). IL-1b did not have a significantly higher mean concentration within adults living with osteoarthritis compared to healthy controls (Fig 4.8A). The mean concentration of IL-6 of adults living with osteoarthritis appears to be slightly lower than healthy individuals, but the first to third IQR (interquartile range) of the concentrations within each osteoarthritis sample is higher than that of healthy individuals (Fig 4.8B). The limit of detection is defined with a grey dashed line for IL-6 (-2.53 pg/mL), Fractalkine (2.97 pg/mL) and TNFα (-1.56 pg/mL). A limit of detection is not defined for cytokines GM-CSF and IL-1b.

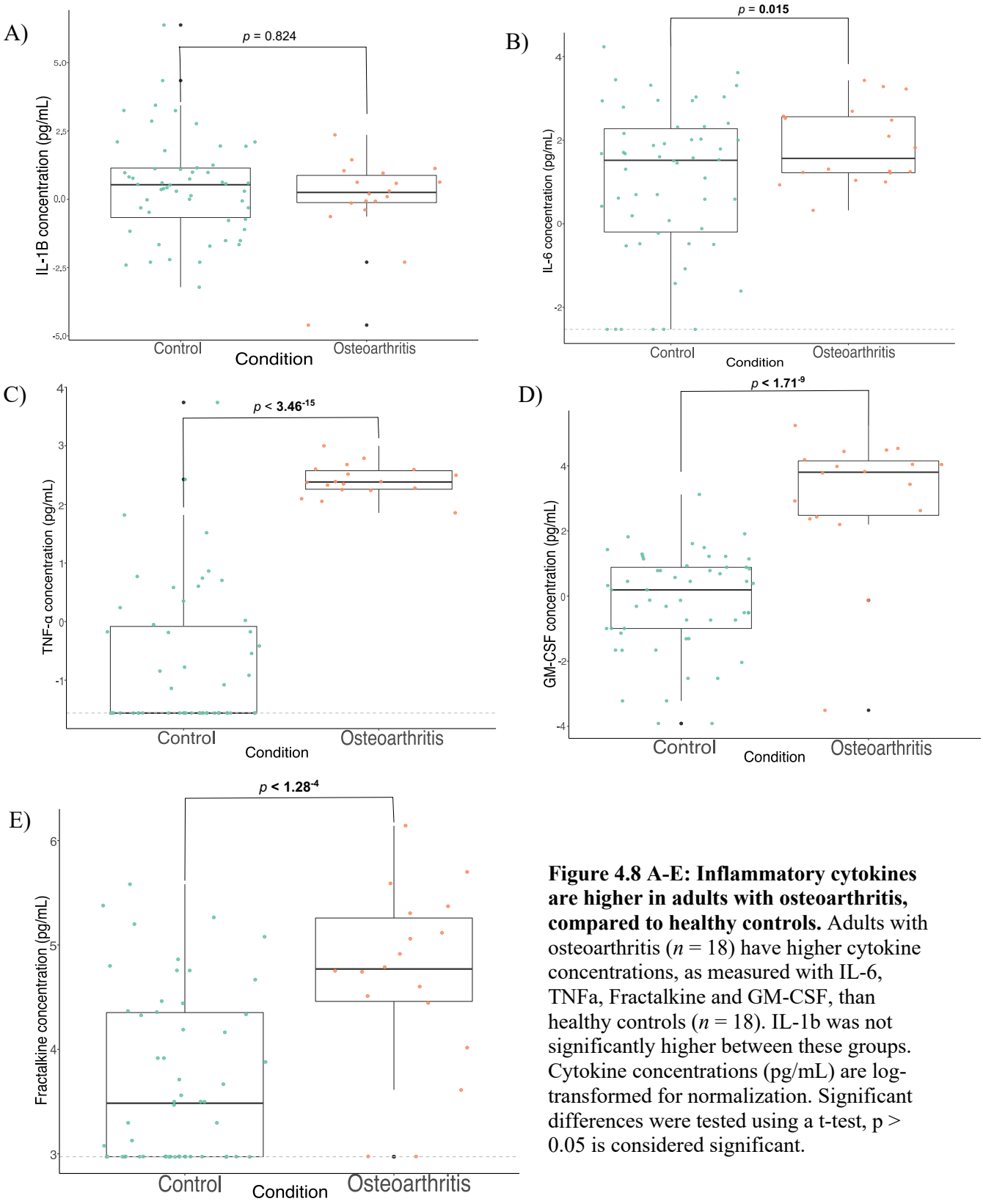


Figure 4.8 A-E: Inflammatory cytokines are higher in adults with osteoarthritis, compared to healthy controls. Adults with osteoarthritis (n = 18) have higher cytokine concentrations, as measured with IL-6, TNFa, Fractalkine and GM-CSF, than healthy controls (n = 18). IL-1b was not significantly higher between these groups. Cytokine concentrations (pg/mL) are log-transformed for normalization. Significant differences were tested using a t-test, p > 0.05 is considered significant.

To determine if local inflammation changes the composition of the anterior nares microbiota, we measured the linear relationship between Bray-Curtis Dissimilarities and the 5 cytokines. Our results indicate local inflammation does not change the composition of the anterior nares microbiota of adults living with osteoarthritis, compared to healthy adults (supplementary table 4.1).

To determine if particular ASVs change with inflammation in the anterior nares microbiota, we first performed a Principal Components Analysis (PCA). A PCA is a dimension reduction technique and is a linear combination of variables which explains the highest possible amount of variance within the dataset. A PCA is performed between adults with osteoarthritis and healthy controls. The variance is extracted from the data, converting the original variable data into linearly uncorrelated variables, named principal components (PC)[163]. The two orthogonal linear combinations of the cytokine concentrations, PC1 and PC2, capture 78% of the covariance in the dataset for adults living with osteoarthritis and their healthy controls (Fig 4.9). The PCR loadings (coloured in red, labelled by respective cytokine) show the relative contribution of each cytokine on PC1 and PC2. Based on the length of the arrows, these loadings have a reasonably fair and equal contribution to the dataset. The PC1 and PC2 components will be used in a generalized linear model as independent variables as a summary of the variation of these 5 cytokines, interacting with the condition (OA vs healthy controls) to determine which ASVs are correlated with local nasal cytokines. Since there were no change between b-diversity and the 5 cytokines in adults living with osteoarthritis, we hypothesize there will be minor changes in particular ASVs within the anterior nares microbiota of adults living with osteoarthritis and their healthy controls.

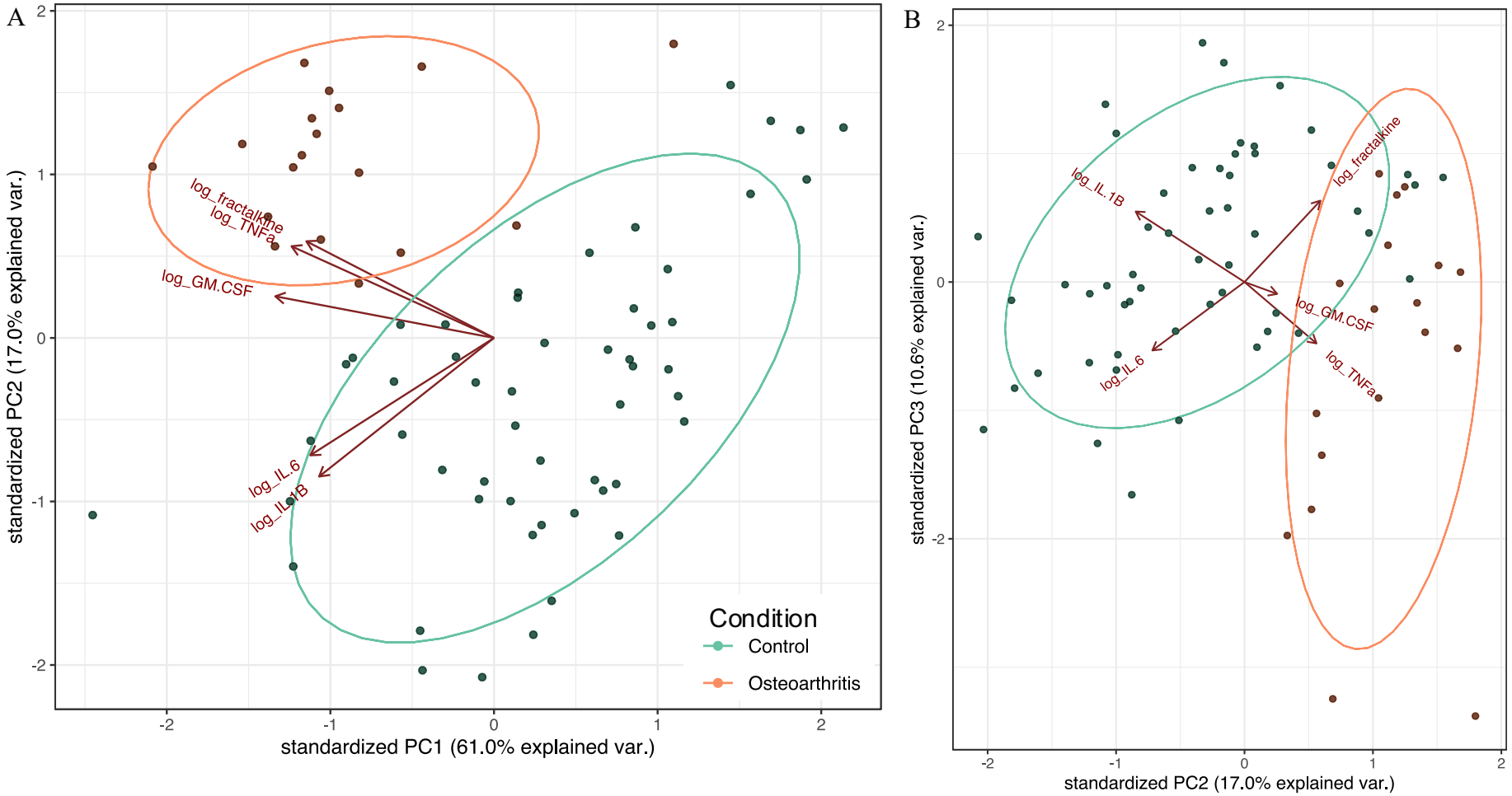


Figure 4.9A-B: Local nasal inflammation drives 78% of the variance in the nasal microbiota of adults with and without osteoarthritis. Distances were measured using Aitchison's distance. Each point represents one individual. Adults living with osteoarthritis are coloured in orange and healthy controls are coloured in green. Nasosorps were collected at the baseline timepoint. The PCA loadings are indicate with a red line, followed by the appropriately labelled cytokine (IL-6, IL-1b, TNFa, Fractalkine, GM-CSF). Cytokines concentrations were log transformed for normalization. The length and direction of the arrow shows the magnitude of effect the markers have on the dataset.

4.3 Discussion

Herein we have characterized intra- and inter-individual changes in the anterior nares microbiota of individuals with chronic inflammatory conditions (rheumatoid arthritis and osteoarthritis). We hypothesized that inflammation due to osteoarthritis and rheumatoid arthritis conditions results in a decrease in protective bacteria (*Corynebacterium*, *Staphylococcus*, *Dolosigranulum*) in the anterior nares microbiota. We measured these changes using a- (Shannon, Simpson indices and Observed ASVs) and b-diversity measurements (Bray-Curtis dissimilarity) to determine how host factors including age, sex, inflammatory markers (GM-CSF, Fractalkine, IL-1β, IL-6, TNF) and bacterial load, impact the composition of the anterior nares microbiota.

Consistent with our data in chapter 3, chronological age and sex do not drive the variation in the anterior nares microbiota of individuals with rheumatoid arthritis and osteoarthritis (supplementary table 4.1-4.2). This is consistent with previous data which stated a- and b-diversity measurements and differentially abundant taxa do not correlate with age and sex within the oral microbiota of individuals with rheumatoid arthritis and osteoarthritis[164]. There were only 5 taxa that significantly negatively correlated with age (i.e., *Cutibacterium acnes* and *Caulobacterales*) but these taxa do not belong to the identified differentially abundant taxa among rheumatoid arthritis, osteoarthritis, and healthy individuals[164].

Contrary to our data in chapter 3, bacterial load and sampling timepoint do not drive the diversity and compositional changes in the anterior nares microbiota. This indicates the species richness and evenness within the microbial community of the anterior nares is not affected by the amount of bacteria present in the nostril region of rheumatoid arthritis and osteoarthritis individuals. This could be due to the anterior nares microbiota having a large ‘Mixed’ cluster composed of a diverse array of microbes belonging to *Corynebacterium*, *Staphylococcus*,

Streptococcus, *Cutibacterium*, *Moraxella* and *Lactobacillus* genera, which is likely because the anterior nares are constantly exposed to the external environment, allowing various commensal or pathogenic microbes to colonize.

Sampling timepoint (Baseline, Visit 1 and Visit 2) between individuals with chronic conditions and controls does not impact changes in the anterior nares microbiota. We hypothesized that a-diversity would increase and there would be more similarity (b-diversity) between individuals with chronic inflammation and controls over the course of visitations, because inflammation would decrease over time. Individuals with osteoarthritis were performing knee-bearing exercises and individuals with rheumatoid arthritis were taking DMARD treatment to reduce chronic inflammation. The reason for this could be due to a lower retention rate with each subsequent visitation, which is a limitation of this study.

The inter-individual variation in the anterior nares microbiota of individuals with rheumatoid arthritis and osteoarthritis is largely driven by dominant microbial communities, characterized by 8 clusters. These clusters are dominated by bacteria belonging to *Corynebacterium*, *Moraxella/Dolosigranulum*, *Staphlyococcus* and one large 'Mixed' cluster containing diverse microbial communities. These clusters are significantly different from one another and explain 30% of the variation in the anterior nares microbiota, according to the first 2 axes in a PCoA analysis. Specifically, the second largest cluster is dominated by a mix of *Moraxella* and *Dolosigranulum* bacteria, which was not previously identified in the mid-turbinate microbiota clusters defined in chapter 3. Previous studies have indicated *Dolosigranulum pigrum* is negatively correlated with lower respiratory tract infection (lower RTI) in young children, whereas certain species of *Moraxella* (e.g., *M. nonliquefaciens*) is prevalent in children with acute sinusitis in the nasal microbiota[165]. This suggests species of

Moraxella could be pro-inflammatory microbes in the nasal microbiota of rheumatoid arthritis and osteoarthritis individuals.

There were no compositional changes (measured by β-diversity) in the anterior nares microbiota between individuals who do and do not have an inflammatory condition, regardless of the disease type. When investigating further, we determined the composition of the anterior nares is significantly different between individuals with rheumatoid arthritis and osteoarthritis, and between rheumatoid arthritis and healthy controls, but not between osteoarthritis and healthy controls. This suggests that disease type, rather than inflammation, could drive the variation in anterior nares microbiota. It is challenging to disentangle the aspect of disease versus inflammation within these individuals, because the measure of inflammation varies between the two groups. Individuals with osteoarthritis have measures of nasal inflammation with specific inflammatory cytokines (GM-CSF, Fractalkine, IL-1B, IL-6 and TNF) which were significantly higher than healthy controls. Individuals with rheumatoid arthritis have measures of systemic inflammation with non-specific inflammatory markers (ESR and CRP levels). Individuals with osteoarthritis could not have significant changes in their anterior nares microbiota, compared to healthy controls, because the inflammation was localized to the knee and was not affecting the nasal cavity as greatly as one would predict. Further investigation to measure local and systemic inflammation changes in the nasal microbiota within osteoarthritis and rheumatoid arthritis patients with the same measure of inflammation (inflammatory cytokines or ESR and CRP levels) is required. This would further uncover the role inflammation plays in the nasal microbiota and determine if these changes exist due to disease type or inflammation.

Individuals with rheumatoid arthritis have microbial compositional changes in their anterior nares microbiota, measured by ESR values. Anterior nares samples were collected over

the course of a 6–8-month treatment with DMARDS, with the intention to reduce their inflammation (measured by ESR and CRP levels) by the end of treatment. The microbial composition, as measured by Bray-Curtis dissimilarity, is significantly higher in adults that were non-responsive to DMARDS treatment, compared to adults who were responsive to DMARDS treatment. β-diversity was not significantly different in adults with RA that were unaffected, or stable, with DMARDS treatment, because their ESR values did not change. These results suggest that the baseline microbiota of adults living with rheumatoid arthritis could predict the stability of the anterior nares microbiota on DMARDS treatment, and adults with higher systemic inflammation have more dissimilarity in the anterior nares microbiota, compared to adults with lower systemic inflammation. Future work is required to determine which ASVs are associated with changes in non-responders and responders.

A previous study indicated the lung microbiota (measured by BAL fluid) of early-untreated rheumatoid arthritis individuals have a significantly different microbial compositions (measured by b-diversity, weighted UniFrac) compared to their healthy controls[166]. The lung microbiota in rheumatoid arthritis patients also have 40% less OTUs compared to healthy controls[166]. Together with our data, this suggests the inflammatory response in rheumatoid arthritis individuals impacts the respiratory microbiota community. Specifically, mucosal inflammation in the nasal cavity could impact the stability of the nasal microbiota in adults living with rheumatoid arthritis on DMARD medication and be a potential driver of microbial dysbiosis.

Future directions of this work include increasing the sample size of individuals with rheumatoid arthritis and osteoarthritis, to determine if there are specific ASVs that change with chronic inflammatory conditions and disease severity. Additionally, including nasal cytokine measurements for individuals with rheumatoid arthritis will allow future research to define which

inflammatory markers, such as IL-6, TNF, IL-10, are associated with protective or pathogenic ASVs, and how inflammation impacts microbe-microbe relationships in the niche nasal microbiota.

The type of inflammatory condition changes the bacterial load in the anterior nares microbiota. Individuals with osteoarthritis have a lower bacterial load in the anterior nares microbiota, compared to healthy controls, and this only occurs at the baseline sample timepoint. This indicates osteoarthritis individuals with the highest inflammation, have a higher bacterial load and a fuller niche. Although there is a lack of studies within the nasal microbiome of individuals with osteoarthritis, we hypothesize this is due to proinflammatory microbes colonizing the anterior nares microbiota. Previous studies determined there is an association between proinflammatory microbes (e.g., *Prevotella* and *Porphyromonas gingivalis*) in the lung microbiome of individuals with other inflammatory conditions (e.g., COPD)[167]. Individuals with rheumatoid arthritis did not have a significantly lower bacterial load compared to healthy controls, suggesting the anterior nares niche is emptier but could still allow for pro-inflammatory microbes to colonize. Future work to determine the specific ASVs that are correlated with a change in bacterial load within osteoarthritis individuals are required.

Chapter 5: Discussion

5.1 Discussion

The URT microbiota of community-dwelling adults contains a complex array of microbes and is highly variable [51]. In our work, I have focused on the mid-turbinate (Chapter 3) and anterior nares (Chapter 4) microbiota in adults 18 to 102 years of age. Using culture-independent techniques, we characterized the nasal microbiota and determined chronic inflammation, not age, has an impact on microbial composition in the nasal microbiota. We also identified possible ASVs and isolates in the mid-turbinate microbiota that could protect against acquiring a respiratory infection during cold & flu season.

Using hierarchical clustering of 16S rRNA sequencing data, we identified 7 distinct clusters in the mid-turbinate microbiota. With the exception of the first cluster (Coryne1), the dominant ASV in one cluster is not frequently found in a sample with a dominant ASV from another cluster, which could be due to intra-species competition within the nasal microbiota, as suggested by previous research in our laboratory[48]. There are 8 *Corynebacterium* ASVs and 6 *Staphylococcus* ASVs that are dominant within the mid-turbinate microbiota of community-dwelling adults. We found there are few age-related changes in the mid-turbinate microbiota. Participant's sex, sampling time, and vitamin D supplementation differed significantly by specific clusters (Coryne3 & Mixed) in the mid-turbinate microbiota, suggesting changes in abundance of *Corynebacterium* are influenced by these variables. Interestingly, *Dolosigranulum* and *Moraxella* have low abundance in the mid-turbinate microbiota of community-dwelling adults, but they are abundant and associated with acute respiratory illnesses in the nasal microbiota of children[38,50]. This may suggest healthy adults lose *Dolosigranulum* and *Moraxella*

species overtime, or these bacteria are more likely associated with respiratory illnesses, rather than protection in the nasal microbiota.

Bacterial load (measured as the number of 16S rRNA gene copies per sample) had an overall significant association with clusters, and a small, but significant effect on the microbial composition (measured with Bray-Curtis dissimilarity). Sampling timepoint (Fall 2020, Spring/Summer 2021, Fall 2021) also had a significant correlation with the Mixed cluster, and drives the variation in the mid-turbinate microbiota. Although we are not able to detect seasonal differences with only 1 year of sampling timepoints, this variation in the mid-turbinate microbiota could be due to various viruses (e.g., rhinovirus, enterovirus, SARS-CoV-2) and their variants circulating year-to-year. We found 18 ASVs are correlated with bacterial load between the 3 sampling timepoints. This suggests the diversity changes between seasons and there is less intra-species competition between commensal bacteria (e.g., *Staphylococcus*, *Corynebacterium*, *Dolosigranulum* species) during post-cold & flu season, compared to pre-cold & flu season. Determining if these taxa are correlated with the stability and recovery of the nasal microbiota after a respiratory infection is important for future work.

Previous studies in our laboratory have indicated that frailty is associated with increased inter-individual variability[83,143] and a lack of stability in the nasal microbiota[146]. Although frailty is a better indicator of infection risk than age[168], we found that frailty is not correlated with changes in microbial composition. This is likely because we sampled the nasal microbiota of community-dwelling adults, rather than adults living in LTCH. We also measured frailty using a self-assessment score, whereas studies in the gut microbiota have measured frailty using the Fried frailty index to assess physiological and mental health, which has higher accuracy[143]. The aging immune system is also highly variable because adults have various immune responses

throughout their lifetime to diseases (e.g., respiratory infection, cardiovascular, cancer) and inflammatory mediators in circulation are known to increase with age and impact the composition of the gut microbiota[83]. With this knowledge, we hypothesized the number of inflammatory conditions would change the composition of the mid-turbinate microbiota. Our results show the number of inflammatory conditions does not impact the mid-turbinate microbiota composition or diversity. Most adults that had an inflammatory condition were taking immune-modulatory drugs to reduce inflammation, which could have impacted the level of inflammation present in the nasal cavity and not have observed changes.

To determine protective features in the nasal microbiota against respiratory infection, we used a combination of a differential abundance analysis and growth inhibition assays. In our analysis, we found 4 ASVs belonging to *Moraxella*, *Corynebacterium,* and *Staphylococcus* genera that were decreased in the nasal microbiota of adults who later acquired a respiratory infection. This suggests these commensal microbes could have anti-microbial activity that protected the remainder of adults who did not acquire an ILI during cold and flu season. To compliment these findings, we isolated microbes from the mid-turbinate and oral cavity of adults >50 years of age and found 5 strains with anti-pneumococcal activity, determined using growth inhibition assays in liquid and agar. Using high-performance liquid chromatography (HPLC), future work is being completed to further purify the 5 isolates we found and *Corynebacterium* strains to determine the anti-microbial peptides responsible for inhibiting the growth of *S. pneumoniae*.

Our study has collected mid-turbinate samples during Summer 2022, and is currently collecting mid-turbinate swabs, mucosal lining fluid samples and mid-turbinate scrapings from approximately 50 to 75 community-dwelling adults across the age range (20-80+ years of age)

during Fall 2022. By the end of the sample collection (December 2022), we will be able to thoroughly assess seasonal, stability and recovery dynamics post-cold & flu season in the nasal microbiota for the duration of 2 full years. The additional samples being collected during Fall 2022 will be used to investigate how the nasal mucosa supports the diverse microbial communities to get a fulsome understanding of how the nasal microbiome changes with age.

Although we observed the number of inflammatory conditions is not correlated with the composition of the mid-turbinate microbiota (Chapter 3), we wanted to investigate the role chronic inflammation has on the nasal microbiota. To do this, we collected anterior nares samples from individuals with chronic inflammatory conditions (osteoarthritis and rheumatoid arthritis) and without chronic inflammatory conditions over multiple visits. We identified 8 distinct clusters in the anterior nares microbiota. Consistent with results in the midturbinate microbiota (chapter 3), the ASVs belonging in the clusters do not frequently co-exist. Contrary to the midturbinate microbiota (chapter 3), there is a large *Moraxella/Dolosigranulum* cluster present in the anterior nares microbiota. This cluster contains individuals who have osteoarthritis and rheumatoid arthritis and individuals who do not have a chronic inflammatory condition; therefore, it is unclear whether this cluster is correlated with the presence of chronic inflammation or not. As previously mentioned, these microbes are associated with disease in children's nasal microbiota[38,50], leading us to hypothesize that bacteria belonging to the *Moraxella* and/or *Dolosigranulum* genera are associated with nasal inflammation in adults.

We also found that the anterior nares microbiota of patients with rheumatoid arthritis is driven by their response to treatment and reduction in ESR, not CRP levels. Rheumatoid arthritis patients with higher inflammation have differing microbial compositions in their anterior nares microbiota, compared to patients with lower inflammation. Previous studies found microbial

dysbiosis of the oral microbiota in individuals with rheumatoid arthritis, including an increased abundance of *Porphyromonas gingivalis*, which promotes a dysbiotic state by manipulating host complement and toll-like receptor (TLR)-2 signaling, uncoupling bacterial clearance from inflammation[169,170]. Our results suggest microbial dysbiosis occurs in the nasal microbiota of patients with rheumatoid arthritis, but future work is required to identify the bacteria promoting this dysbiosis.

The cytokine milieu of the nasal cavity of adults living with osteoarthritis does not appear to change in the composition of the anterior nares microbiota composition, measured with β-diversity. Future work is required to determine if there are specific ASVs correlated with nasal inflammation in adults with chronic inflammatory conditions.

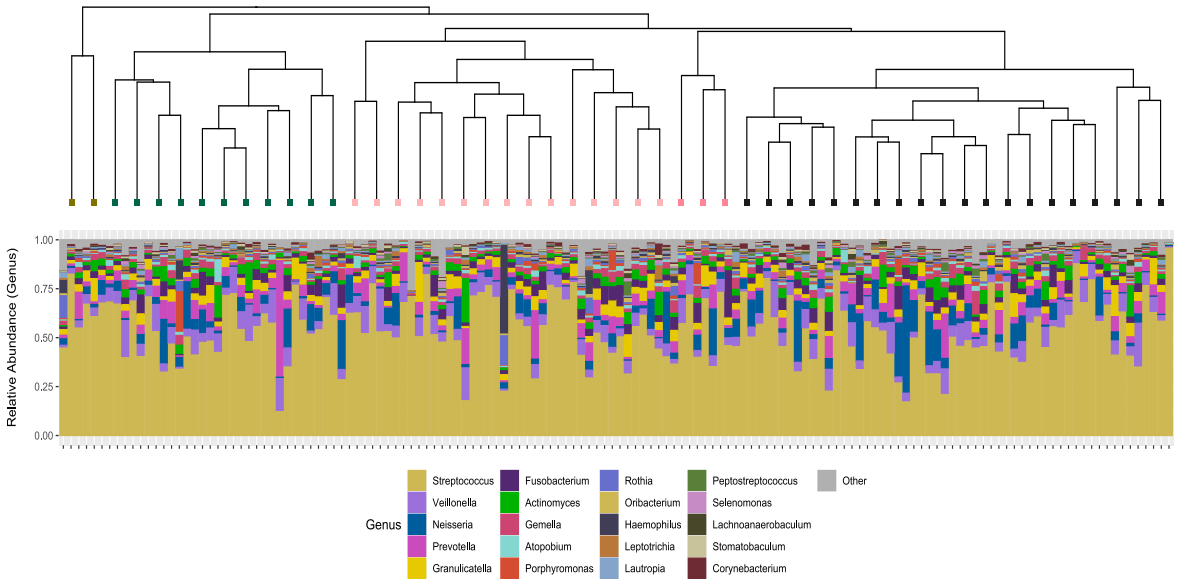
5.2 Conclusion

We have characterized the mid-turbinate microbiota of community-dwelling adults and the anterior nares microbiota of adults with and without chronic inflammatory conditions. As a measure of systemic inflammation. We have determined chronological age, sex, frailty, and other health factors (i.e., medication, inflammatory conditions, smoking) do not change the composition and diversity of the mid-turbinate microbiota in adults. We also determined that chronic inflammatory conditions, such as rheumatoid arthritis, change the composition of the anterior nares microbiota. It allows us to further explore the impact age-associated inflammation has on the nasal microbiota. With the limited cold and flu samples we had, we begun to uncover particular ASVs that are associated with acquiring a respiratory illness during cold and flu season. Understanding these changes *in vivo* provided us with the means to identify commensal microbes, isolated from the URT, with growth inhibitory activity against primary pathogen, *Streptococcus pneumoniae*, *in vitro*. Respiratory illnesses continue to be a burden for older adults, and greatly impacts their quality of life. Respiratory infections exacerbate symptoms of

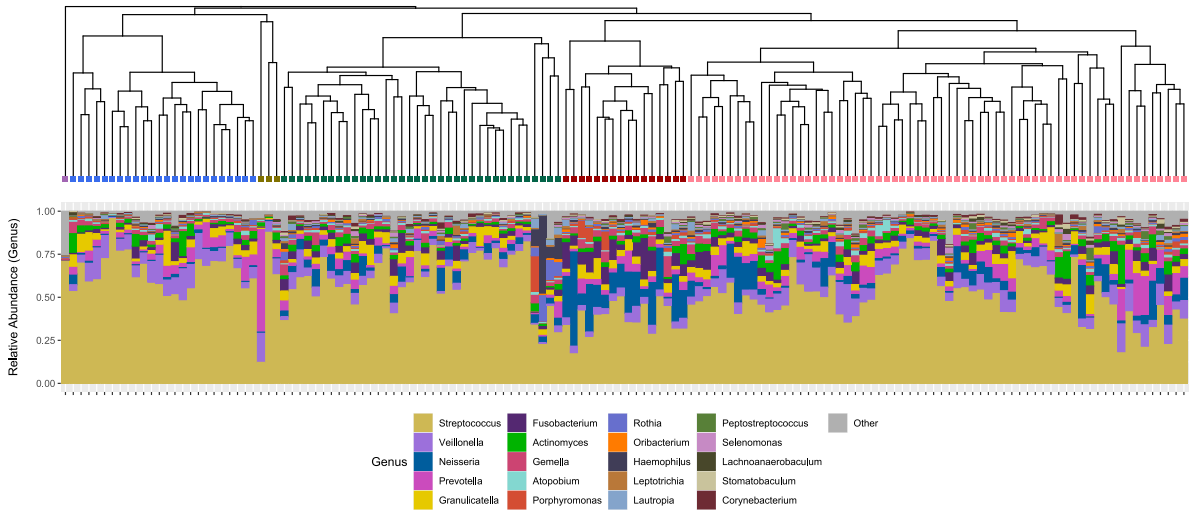
previous health conditions (i.e., cardiovascular diseases), frailty and mortality rates. Developing preventative measures against respiratory infection in older adults is of utmost importance, and we propose the nasal microbiota has important protective features.

Appendices

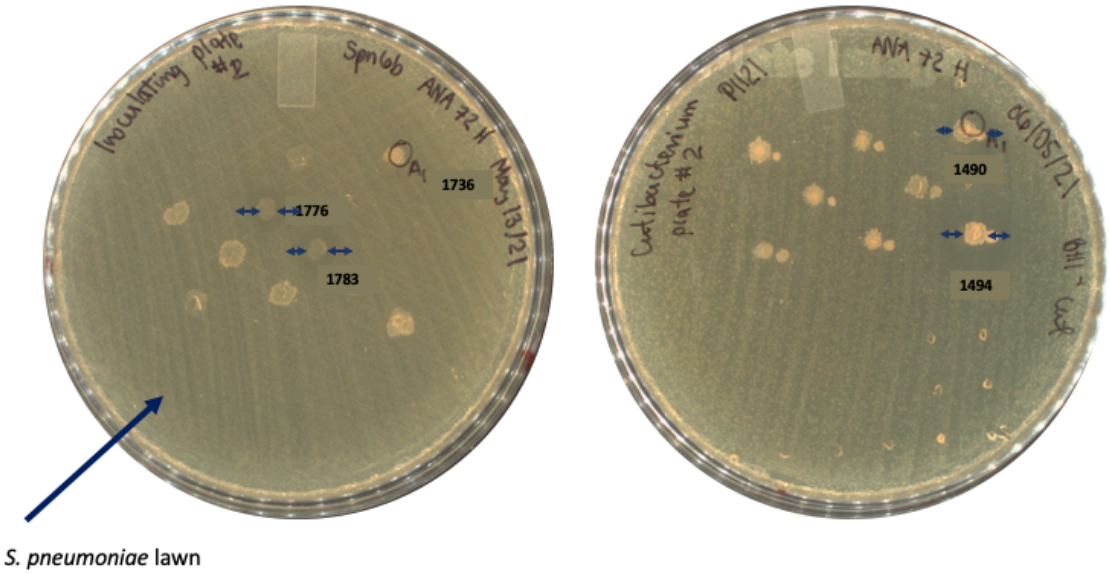
Appendix A



Supplemental Figure 3.3. The taxonomic composition of the oral microbiota (n = 51, oracol swabs) is organized into clusters using Bray – Curtis dissimilarities between ASVs. The taxonomic bar plot is visualized with the top 20 dominant genera. The 5 clusters are significantly dispersed (p = **< 2.2^{-16}**, ANOVA) and clustered from one another (p = **0.001**, PERMANOVA). *Streptococci* genera are predominant in the oral cavity microbiota.



Supplemental Figure 3.4. The taxonomic composition of the oral microbiota (n = 145, salivette samples) is organized into 6 clusters using Bray – Curtis dissimilarities between ASVs. The taxonomic bar plot is visualized with the top 20 dominant genera. The 6 clusters are significantly dispersed (p = **< 2.2^{-16}**, ANOVA) and are clustered differently from one another (p = **0.001**, PERMANOVA). An analysis of similarity (ANOSIM) determined the similarities between the clusters are significant and suggests there is more similarity within the clusters than there are between the clusters (R = 0. 7645, p = **0.001**).



Supplemental Figure 3.5. Anti – pneumococcal activity of single colonies, isolated from the upper respiratory tract nasal cavity. The isolates were stamped on 2 different genetic strains of *S. pneumoniae*, Spn6b (left) and P1121 (right), incubated in anaerobic conditions for 48 – 72 hours and photographed. GC1783 (*A. odontolyticus*, 1 cm zone of clearing) was isolated from a 56-year-old male, GC 1776 (*C. avidum*, 0.8 cm zone of clearing) was isolated from a 76-year-old female and the participant demographic information for GC1494 (*N. mucosa*, 1.2 cm zone of clearing) and GC1490 (*S. oralis*, 0.8 cm zone of clearing) are unknown.

Supplemental Table 3.1 α – diversity measures associated with host factors in the nasal microbiota.

Variable	Levels	*n*	a – diversity		
			Shannon	**Simpson**	**Observed ASVs**
Age	NA	390*	$p = 0.555$	$p = 0.788$	$p = 0.163$
Bacterial load	NA	390*	$\mathbf{p = 0.006}$***	$\mathbf{p = 0.006}$***	$p = 0.289$
Seasons	Fall	221	$p = 0.383$	$p = 0.377$	$p = 0.388$
	Spring	61			
	Summer	107			
Sex	Female	266	$p = 0.253$	$p = 0.363$	$p = 0.117$
	Male	118			
Inflammatory conditions	0	220	$p = 0.172$	$p = 0.210$	$p = 0.183$
	1	89			
	2+	74			
Number of medications	0	140	$p = 0.122$	$p = 0.302$	$p = 0.255$
	1	117			
	2	41			
	3+	84			
Vitamin D supplementation	Yes	177	$p = 0.966$	$p = 0.803$	$p = 0.286$
	No	200			
Smoking	Yes	273	$p = 0.084$	$p = 0.087$	$p = 0.370$
	No	110			
S. pneumoniae vaccination status	Yes	71	$p = 0.971$	$p = 0.571$	$p = 0.535$
	No	177			
	Unknown+	88			
Contact with children	Yes	195	$p = 0.351$	$p = 0.378$	$p = 0.308$
	No	189			
Pre-frailty score	NA	390*	$p = 0.274$	$P = 0.119$	$P = 0.289$
ILI during 2020 – 2022 cold/flu seasons	Yes	30	$p = 0.672$	$p = 0.542$	$p = 0.569$
	No	356			

* Total number of samples in the analysis after removing samples below a sequencing depth of 4000.

+Unknown responses were removed from the analysis as an exclusion factor.

Supplemental Table 3.2. β – diversity measures associated with host factors in the mid-turbinate microbiota.

Variable	Levels	n	b – diversity (Bray – Curtis dissimilarity)
Age	NA	166*	$p = 0.826$ $R^2 = 0.356$
Bacterial load	NA	166*	**$p = 0.002$**** **$R^2 = 0.025$**
Seasons	Fall	166	**$p = 0.001$**** **$R^2 = 0.019$**
	Spring	61	
	Summer	109	
Sex	Female	117	$p = 0.213$ $R^2 = 0.00077$
	Male	52	
Inflammatory conditions	0	85	$p = 0.325$ $R^2 = 0.013$
	1	46	
	2+	38	
Number of medications	0	59	$p = 0.424$ $R^2 = 0.079$
	1	41	
	2	23	
	3+	53	
Vitamin D consumption	Yes	97	$p = 0.507$ $R^2 = 0.005$
	No	72	
Smoking	Yes	56	$p = 0.654$ $R^2 = 0.005$
	No	113	
Pneumococcal vaccination status	Yes	36	$p = 0.246$ $R^2 = 0.014$
	No	95	
	Unknown+	38	
Contact with children	Yes	87	$p = 0.155$ $R^2 = 0.008$
	No	82	
Pre-frailty phenotype	NA	169	$P = 0.321$ $R^2 = 0.007$
ILI during 2020-2022 cold/flu seasons	Yes	17	$p = 0.245$ $R^2 = 0.007$
	No	152	

* Total number of samples in the analysis after removing samples below a sequencing depth of 4000.

+Unknown responses were removed from the analysis as an exclusion factor.

Supplemental Table 3.3. Pathogen identification results for ILI samples during the 2020 – 2021 cold/flu season.

PIV 1	**PIV 2**	**PIV 3**	**adenovirus**	**MPV**	**SARS - CoV - 2**	***Streptococcus pneumoniae***	**corona-boca**
Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative

Study ID	Date of infection	MS2 (Internal Control)	Rhino/Enterovirus	Influenza A	Influenza B	RSV A and B
W292	15-Oct-20	24.77	15.97	Negative	Negative	Negative
WS028	12-Dec-20	25.54	Negative	Negative	Negative	Negative
WS030	01-Feb-21	25.08	Negative	Negative	Negative	Negative
WS031	01-Oct-20	25.08	Negative	Negative	Negative	Negative
WS040	24-Dec-20	25.32	Negative	Negative	Negative	Negative
WS132	10-Dec-20	24.97	30.55	Negative	Negative	Negative
WS143	09-Nov-20	25.06	28.54	Negative	Negative	Negative
WS194	05-Oct-20	24.73	15.36	Negative	Negative	Negative
WS352	02-Dec-20	24.84	24.33	Negative	Negative	Negative
WS586	29-Sep-20	25.82	23.75	Negative	Negative	Negative
WS600	21-Jan-21	24.5	Negative	Negative	Negative	Negative
WS618	02-Jan-21	25.08	Negative	Negative	Negative	Negative

Supplemental Table 3.4. Diversity metrics associated with host factors in the oral microbiota (oracol swab samples).

Variable	Number of samples at baseline + follow-up (*n*)	a– diversity (Shannon, Simpson, Observed ASVs)	b– diversity (Bray – Curtis dissimilarity)
Age & bacterial load (continuous)	51	Shannon: $p = 0.851$, $p = 0.353$ Simpson: $p = 0.707$, $p = 0.327$ Observed: $p = 0.691$, $p = 0.944$	NA
Seasons	Fall = 51 (Baseline)	NA	NA
Sex (Female, Male)	73, 48	Shannon: $p = 0.974$ Simpson: $p = 0.392$ Observed: $p = 1.91^{-9}$***	$p = 0.14$ $R^2 = 0.017$
Inflammatory conditions (0, 1, 2+)	90, 24, 7	Shannon: $p = 0.876$ Simpson: $p = 0.963639$ Observed: $p = 0.486$	$p = 0.558$ $R^2 = 0.02237$
Number of medications	0 = 53, 1= 30, 2 = 10, 3 = 6, 4 =7, 5 = 7, 6 = 2, 8 = 1, 11 = 2, 17 = 1	Shannon: $p = 0.492$ Simpson: $p = 0.194$ Observed: $p = 0.862$	$p = 0.859$ $R^2 = 0.09611$
Smoking (Yes, No)	13, 108	Shannon: $p = 0.822$ Simpson: $p = 0.613$ Observed: $p = 0.353$	$p = 0.092$ $R^2 = 0.01778$
S. pneumoniae vaccination status (Yes, No, Unknown)	32, 79, 9	Shannon: $p = 0.6397$ Simpson: $p = 0.301$ Observed: $p = 0.771$	$p = 0.072$ $R^2 = 0.04575$
Contact with children (Yes, No)	60, 60	Shannon: $p = 0.288$ Simpson: $p = 0.082$ Observed: $p = 0.393$	$p = 0.584$ $R^2 = 0.02213$
Frailty	N – 0 = 108 N – 1 = 9 Y – 0 = 2 Y – 2 = 1 Y – 3 = 1	Shannon: $p = 0.569$ Simpson: $p = 0.880$ Observed: $p = 0.699$	$p = 0.976$ $R^2 = 0.03612$
Infected during cold/flu season (Yes, No)	38, 83	Shannon: $p = 0.752$ Simpson: $p = 0.612$ Observed: $p = 0.898$	$p = 0.199$ $R^2 = 0.01489$

Supplemental Table 3.5. Diversity metrics correlated with host factors in the oral microbiota (salivette samples).

Variable	Number of samples at baseline + follow-up (*n*)	a– diversity (Shannon, Simpson, Observed ASVs)	b– diversity (Bray – Curtis dissimilarity)
Age & bacterial load (continuous)	145	Shannon: $p = 0.395$, $p = 0.858$ Simpson: $p = 0.689$, $p = 0.406$ Observed : $p = 0.042$*, $p = 0.518$	NA
Seasons	Fall = 106 (Baseline) Summer = 39 (Baseline)	NA	P = 0.318 $R^2 = 0.00749$
Sex (Female, Male)	109, 36	Shannon: $p = 0.501$ Simpson: $p = 0.453$ Observed: $p = 0.967$	$p = 0.901$ $R^2 = 0.00409$
Inflammatory conditions (0, 1, 2+)	77, 36, 32	Shannon: $p = 0.682$ Simpson: $p = 0.625$ Observed: $p = 0.701$	$p =$ **0.037** $R^2 = 0.0213$
Number of medications	0 = 55, 1= 35, 2 = 16, 3 = 9, 4 = 11, 5 = 2, 6 = 6, 7 = 3, 8 = 1, 9 = 1, 11 = 2, 17 = 1	Shannon: $p = 0.876$ Simpson: $p = 0.729$ Observed: $p = 0.602$	$p = 0.285$ $R^2 = 0.104$
Vitamin D consumption (Yes, No)	65, 80	Shannon: $p = 0.539$ Simpson: $p = 0.910$ Observed: $p = 0.622$	$p = 0.141$ $R^2 = 0.00966$
Smoking (Yes, No)	96, 49	Shannon: $p = 0.359$ Simpson: $p = 0.519$ Observed: $p = 0.528$	$p =$ **0.044** $R^2 = 0.01264$
S. pneumoniae vaccination status (Yes, No, Unknown*)	26, 89, 30	Shannon: $p = 0.550$ Simpson: $p = 0.518$ Observed: $p = 0.511$	$p =$ **0.048** $R^2 = 0.02117$
Contact with children (Yes, No)	75, 70	Shannon: $p = 0.595$ Simpson: $p = 0.705$ Observed: $p = 0.897$	$p = 0.311$ $R^2 = 0.00761$
Frailty	N – 0 = 117 N – 1 = 16 N – 2 = 4 N – 3 = 5 Y – 0 = 2 Y – 1 = 1	Shannon: $p = 0.080$ Simpson: $p = 0.317$ Observed: $p = 0.286$	$p = 0.099$ $R^2 = 0.04365$
Infected during cold/flu season (Yes, No)	8, 137	Shannon: $p = 0.594$ Simpson: $p = 0.689$ Observed: $p = 0.772$	$p = 0.199$ $R^2 = 0.00927$

*Unknown responses were removed from the analysis as an exclusion factor.

Supplemental Table 3.6. Post-hoc comparisons with cluster associations and bacterial load

Variable	Sex	p-value
Coryne1 - Coryne2	F	0.999
Coryne1 - Coryne3	F	0.999
Coryne1 - Coryne4	F	0.604
Coryne1 - Mixed	F	0.316
Coryne1 - Staph1	F	0.999
Coryne1 - Staph2	F	0.088
Coryne2 - Coryne3	F	1
Coryne2 - Coryne4	F	0.832
Coryne2 - Mixed	F	0.726
Coryne2 - Staph1	F	0.999
Coryne2 - Staph2	F	0.637
Coryne3 - Coryne4	F	0.793
Coryne3 - Mixed	F	0.625
Coryne3 - Staph1	F	0.999
Coryne3 - Staph2	F	0.467
Coryne4 - Mixed	F	0.999
Coryne4 - Staph1	F	0.647
Coryne4 - Staph2	F	0.999
Mixed - Staph1	F	0.444
Mixed - Staph2	F	0.999
Staph1 - Staph2	F	0.273
Coryne1 - Coryne2	M	0.999
Coryne1 - Coryne3	M	0.999
Coryne1 - Coryne4	M	0.604
Coryne1 - Mixed	M	0.316
Coryne1 - Staph1	M	0.999
Coryne1 - Staph2	M	0.088
Coryne2 - Coryne3	M	1
Coryne2 - Coryne4	M	0.832
Coryne2 - Mixed	M	0.726
Coryne2 - Staph1	M	0.999
Coryne2 - Staph2	M	0.637
Coryne3 - Coryne4	M	0.793
Coryne3 - Mixed	M	0.625
Coryne3 - Staph1	M	0.999
Coryne3 - Staph2	M	0.467
Coryne4 - Mixed	M	0.999
Coryne4 - Staph1	M	0.647
Coryne4 - Staph2	M	0.999
Mixed - Staph1	M	0.444
Mixed - Staph2	M	0.999
Staph1 - Staph2	M	0.273

Appendix B

Supplemental Table 4.1. Chronic inflammatory conditions and host factors are not correlated with α-diversity measures in the nasal microbiota of adults with and without osteoarthritis and rheumatoid arthritis.

Variable	Levels	n	α – diversity		
			Shannon	**Simpson**	**Observed ASVs**
Age	NA	190	$p = 0.899$	$p = 0.314$	$p = 0.559$
Bacterial load	NA	190	$p = 0.058$	$p = 0.395$	$p = 0.323$
Sex	Female	155	$p = 0.053$	$p = 0.117$	$p = 0.281$
	Male	35			
Condition	Osteoarthritis	27	$p = 0.447$	$p = 0.117$	$p = 0.349$
	Rheumatoid arthritis	14			
	Healthy controls	81			
Sampling timepoint	Baseline	43	$p = 0.999$	$p = 0.997$	$p = 0.612$
	Baseline control	83			
	Visit1	27	$p = 0.998$	$p = 0.999$	$p = 0.999$
	Visit1 control	12			
	Visit2	18	$p = 0.995$	$p = 0.999$	$p = 0.999$
	Visit2 control	7			

Supplemental Table 4.2 β – diversity measurements associated with chronic inflammatory changes in the nasal microbiota.

Variable	Levels	n	b – diversity (Bray – Curtis dissimilarity)	
Age	NA	126	$p = 0.13$ $R^2 = 0.02$	
Bacterial load	NA	126	$p = 0.517$ $R^2 = 0.007$	
Sex	Female	100	$p = 0.192$ $R^2 = 0.009$	
	Male	26		
Inflammatory conditions	Yes	83	$p = 0.347$ $R^2 = 0.009$	
	No	43		
Condition	OA	27	OA vs Control	$p = 0.397$ $R^2 = 0.009$
	RA	16	RA vs OA	**$p = 0.017$** $R^2 = 0.051$
	Control	83	RA vs Control	**$p = 0.006$** $R^2 = 0.021$

Supplemental Table 4.3. Bacterial load changes with inflammatory conditions in the nasal microbiota.

Variable	Levels	*n*	Bacterial load	
Age	NA	190	$p = 0.279$	
Sex (Female, Male)	Female	155	$p = 0.809$	
	Male	35		
Inflammatory state	Yes	102	**$p = 0.007$**	
	No	88		
Condition	OA	37	OA vs Control	**$p = 0.01$**
	RA	51	RA vs Control	$p = 0.248$
	Control	102	OA vs RA	$p = 0.361$

Supplemental Table 4.4. Bacterial load changes during sampling timepoints in the nasal cavity of adults with osteoarthritis, compared to healthy controls.

Variable	Levels	*n*	Bacterial load
Sampling timepoint	Baseline	27	**$p = 0.025$**
	Baseline control	54	
	Visit 1	8	$p = 0.999$
	Visit 1 control	12	
	Visit 2	2	$p = 0.792$
	Visit 2 control	7	

Supplemental Table 4.5. Microbial composition, measured by Bray-Curtis Dissimilarities, does not change with local inflammatory cytokines in the nasal microbiota of adults with osteoarthritis.

Cytokine	p-value	R^2
GM-CSF	0.183	0.017
Fractalkine	0.501	0.016
IL-6	0.425	0.014
IL-1b	0.75	0.011
TNF	0.056	0.021

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