Clostridium difficile Colonization and Infection in the Elderly and Associations with the Aging Intestinal Microbiome

A Dissertation Presented

By

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Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Sciences, Worcester

in partial fulfillment of the requirements for the degree of

DOCTOR OF BIOMEDICAL SCIENCES

March 14th 2018

REVIEWER PAGE

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Millennium PhD Program

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Acknowledgements

I want to fist thank my thesis advisor Dr. Beth McCormick. Through her thoughtful guidance of my career I have been able to expand into the complex field of human microbiome investigations. There is no doubt that her mentorship has made me a better scientist. Thank you to the members of my dissertation committee, Drs. Jeffrey A Bailey, Raffi Van Aroian, Melissa Clark and Read Pukkila-Worley. I have been fortunate to have a collaborative, insightful, and dedicated committee.

There are many others not part of my dissertation committee that have helped mold my development that I would like to thank. Thank you to the Dr. Edward Boyer who has been my mentor since my arrival here at UMass as new faculty over 6 years ago. His guidance has helped me to form lasting collaborations that have helped advance my career and his particular attention to my grant writing has made me an early success in procuring funding. A special thanks to Drs. Doyle V. Ward and Vanni Bucci, both have been instrumental in expanding my knowledge into microbiome sciences especially with analytical techniques and presentation of complex data. Without their help my the work presented here would have not been possible. I also want to thank Dr. Jennifer Tjia, who helped guide me in the world of geriatrics and pharmacoepidemiology.

Thank you to the Department of Emergency Medicine and in particular, Dr. Gregory Volturo, my Department Chair and Dr. Edwin Boudreaux, the Vice Chair of Research. Without both the financial and intellectual support of my department, along with Drs. Volturo and Boudreaux, this degree would not have been possible. Their dedication to the careers of physician-scientists should be modeled in all academic medical centers.

Last and not certainly not least, thank you to my family. My wife, Francesca, has been my rock, best friend, and one of my biggest supporters. Having gone through this same process, she has given me invaluable advice and kept my head on straight. Finally, thanks to my three amazing kids (Wesley, Lucas, and Caroline). Always love telling them I research poop and just watching their reactions. Everything I have accomplished in life has been made possible with their love and support.

Research reported in this manuscript was supported by the National Institute on Aging (NIA) of the National Institutes of Health (NIH) under award number R03AG056356-01, the Society for Academic Emergency Medicine (SAEM) under their Research Training Grant Award, Emergency Medicine Foundation (EMF) GEMSSTAR under award number RF2017-011 and finally through support funding from the Department of Emergency Medicine. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH, SAEM, or EMF.

Abstract

The widespread use of antibiotics has led to dramatic increases in the incidence and severity of *Clostridium difficile* infection (CDI). No group of patients suffers more from CDI than the elderly. Nursing homes (NH) represent the perfect storm of a vulnerable group of frail elders living in confined communities. Nursing home residents suffer from increased morbidity and mortality from CDI and corresponding high rates of *C. difficile* colonization. Upwards of 40 to 50% of CDI current cases originate from NHs and the prevalence of colonization rates remain high within these facilities, with as many as half of the residents being colonized with *C. difficile* at any given time. One factor that has become of increasing interest and a target of preventive strategies is the human intestinal microbiome. A healthy, diverse microbiome interacts with the host immune system and contributes to pathogen resistance. In this investigation, we first examine elder specific variables to determine if the associated risks of CDI differ by home living environment (nursing home versus community-dwelling). We then go on explore the relationships of NH environment, frailty, nutritional status, and residents' age with microbiome composition and potential metabolic function. Finally, we describe the *C. difficile* colonization patterns among elderly NH residents and the associated risk of colonization based on clinical variables and microbiome determinants. A better understanding of the microbiome's contribution to *C. difficile* colonization will provide the basis for informing rational interventions and public health policies to better combat CDI in the nursing home.

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The first two chapters of this work have been published. The references are below:

Chapter 1: Haran JP, Bradley E, Howe E, Wu X, Tjia J. Medication exposure and the risk of recurrent Clostridium difficile Infection in community dwelling older people vs. nursing home residents. J Am Geriatr Soc. 2018;66(2):333-38. PubMed PMID: 29120481.

Chapter 2: Haran JP, Bucci V, Dutta P, Ward D, McCormick B. The nursing home elder microbiome stability and associations with age, frailty, nutrition, and physical location. J Med Microbiol. 2018;67(1):40-51. doi: 10.1099/jmm.0.000640. PubMed PMID: 29134939

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INTRODUCTION

1. Overview

Clostridium difficile is an anaerobic, motile bacteria that is a member of the genus Clostridium and the Clostridiaceae family. This bacterium is ubiquitous in nature with a strong prevalence in soil. However *C. difficile* shows optimum growth on blood agar plates at human body temperatures. It is a Gram-positive organism that is rod shaped, pleomorphic, and occurs in pairs or short chains. [1] In 1935, *C. difficile* was first identified in the stool of healthy newborns. However it was not until four decades later, in 1978 when it was first identified as a causative agent in human cases of antibiotic-associated diarrhea. [2] It was not until March of 2003 when several hospitals in Quebec, Canada first noted a marked increase in the incidence of *Clostridium difficile*-associated diarrhea. [3] Since that time the prevalence and virulence of *Clostridium difficile* infections (CDI) in Western countries have been increasing [4, 5] and CDI is now the leading cause of gastroenterologic hospitalizations and associated deaths.[6]

C. difficile produces two major types of toxins, enterotoxin A and cytotoxin B. [7] These toxins can disrupt the cytoskeleton and signal transduction in the human intestine. When humans are exposed to these toxins they generally develop colonic inflammation leading to the major disease manifestation of diarrhea. Diarrheal symptoms range from a few days of watery stools to potential life-threatening pseudomembranous colitis. The toxins expressed by *C. difficile* are this organism's main virulence factor and it is believed that the presence of toxin in stool is a positive correlate of disease. [20]

C. difficile is only one causative mechanism of diarrhea after antibiotic exposure. Antibiotic-associated diarrhea (AAD), a common side effect of antibiotic administration, complicates between 5% to 39% of treatment regimens. [8] The frequency of AAD is influenced by antibiotic selection and by patient characteristics, including comorbidities and age. [9] *C. difficile* infection is responsible for 10-20% of all AAD cases and is thus an important part of AAD given it carries most of the morbidity and mortality associated with this disease. [8] It also makes diagnosing CDI in a patient with AAD critical to targeted therapy. Although virtually all antibiotics have been implicated in CDI, the cephalosporins, clindamycin, and broad-spectrum penicillins have higher associated risks of disease. [10] Prolonged courses of antibiotic treatment, administration of multiple antibiotics, patient age >65 years, or history of diarrhea following antibiotic use impart additional risk. [8, 11, 12] The great majority of patients who develop CDI do so within the first two weeks of antibiotic exposure, [13] but AAD can occur at any time, including up to 2–3 weeks following cessation of antibiotic therapy. [11]

What makes C. difficile particularly difficult to deal with is the fact that it is a spore forming bacterium. When under stress *C. difficile* produces spores which are able tolerate extreme conditions. [2] The major route of transmission, which is from person to person via the fecal-oral route, is attributed to the presence of these spores in the feces. These spores can contaminate any surface including toilets, bathing tubs, and medical equipment. These surfaces can then serve as a reservoir for transmission. In the hospital environment transmission mainly occurs via the hands of healthcare workers who come in contact with those surfaces harboring *C. difficile* spores. These spores are hardy and can live for long periods of time on surfaces, which is upwards of 5 months on solid surfaces. [14, 15] Accordingly, hospital policies presently focus

on environmental measures to control CDI by enforcing hand hygiene, contact precautions, and decontamination procedures after CDI is identified. [16] These interventions aimed at reducing *C. difficile* transmission have had only a minimal impact on stemming the alarming increase in *C. difficile* cases. Unfortunately, these heat-resistant spores survive in clinical environments even after routine cleaning procedures are performed. Once *C. difficile* spores are ingested they easily transit through the stomach unscathed, due to their acid-resistance properties. Once the spores become exposed to bile acids within the colon they then germinate and multiply into vegetative cells.

In summary, C. difficile is a very important nosocomial infection that is difficult to contain. with.. Clearly an important element in dealing with it is accurately diagnosing it.

Diagnosis of *Clostridium difficile* Infection

In order to diagnose a patient with CDI two conditions need to be met. [17] First, the patient must have diarrheal symptoms defined as three or more loose stools per day for two or more consecutive days. [11, 18] Second, there must be a stool test result positive for the presence of *C. difficile* toxins or colonoscopic findings demonstrating pseudomembranous colitis. Most patients are diagnosed by toxin assays rather than colonoscopy. The gold standard for the diagnosis of CDI is the cytotoxin assay test in tissue culture. [8] It is the most sensitive test, being able to detect as little as 10 pg of toxin. [19] However, due to its expensive nature, this diagnostic test has been abandoned by most clinical laboratories. Instead laboratories rely on either immunoassays or nucleic acid amplification test (NAAT) assays to detect *C. difficile* toxins presence. The enzyme immunoassay (EIA) has the advantage of detecting toxin antigen when performed on stool samples thus directly assessing toxin production. Toxin EIA is

unfortunately associated with widely varying sensitivities (50 to 99%) and specificities (70 to 100%) making its reliability questionable for an accurate diagnostic of CDI. [17] In 2010, the Society for Healthcare Epidemiology (SHEA) and the Infectious Diseases Society of American (IDSA) indicated that toxin EIAs were no longer sufficient as standalone diagnostic tests for CDI. [17]

The alternative, the EIA test, functions by detection of toxin genes by NAATs via polymerase chain reaction (PCR). The disadvantage of NAATs is that gene presence does not mean active toxin production. These assays have greater than 90% sensitivities and specificities, thus making them the current standard detection test of *C. difficile* presence for clinical laboratory use. [21] The significance of a positive NAAT test and EIA test is unclear. Since NAATs will detect colonization of *C. difficile* as well as infective states, there is a concern that NAATs testing is leading to over diagnosis and over treatment of CDI. [22] Very few well-controlled studies have established the clinical efficacy of using EIA and NAATs in parallel for the diagnosis of CDI. Nonetheless, several test algorithms have been developed to improve the rapid and accurate diagnosis of CDI (**Figure 1**). The European Society of Clinical Microbiology and Infectious Diseases (ESCMID) recommends the use of a two-step algorithm which starts with NAATs. When the NAAT is positive then continuation on to a secondary confirmatory step with toxin-EIA is necessary before a sample can be reported as positive for CDI. [23] Performance of this diagnostic approach still needs further evaluation and validation.

Recurrent *Clostridium difficile* Infection

One of the troubling complications that arises from *C. difficile* treatment is the high rate of recurrent infections. The increasing incidence of recurrent *Clostridium difficile* infection (rCDI) has been alarming with an almost 200% increase from 2001 to 2012. [24] Patients remain vulnerable to rCDI for months or even years after initial CDI treatment. [25] Recurrent infection is common, occurring in up to 22% of initial cases.[26] Recurrent CDI includes both relapse and reinfection with a new strain. [27] The relative frequency of relapse versus reinfection has not been well described. From limited published data, it is believed that anywhere from 33% to 75% of cases of rCDI can be attributed to infection by a new strain. [25] The Centers for Disease Control and Prevention (CDC) defines the window for rCDI to occur between 2 to 8 weeks after the last positive specimen was tested, however many believe this risk extends out to 1 year. [28] Since most patients do not undergo routine stool sample testing, other investigators have adopted a definition of recurrence that extends up to 90 days after completion of treatment. [29] It has however been shown that the risk may extend out to one year, especially when considering a patients risk of reinfection. [30] In fact it may take as long as 1-2 years for certain normal intestinal microbial species to recover after antimicrobial therapy. [31]

The exact mechanism for recurrence is unknown. The most common risk factors for recurrence are advanced age, comorbidities, use of antibiotics after CDI diagnosis, and gastric acid-suppressive therapy. [26, 32] Both of the acid reducing medications, histamine-2 receptor antagonists and proton pump inhibitors, have been linked to CDI. [33, 34] The judicious use of antibiotics and proton pump inhibitors may play an important role in the prevention of rCDI. [35] The roles of other medications such as corticosteroids are less clear as they have been shown to both increase and decrease the risk of incidence of and mortality from CDI and rCDI. [36-39]

Clostridium difficile and the Elderly

Extensive use of antibiotics has resulted in a dramatic increase in the magnitude of CDI especially among older adults. [4, 5] *Clostridium difficile* infection disproportionally affects the elderly population. [40, 41] The increased risk burden begins at age 65[42] and increases by 2% for each additional year of aging. [43] Not only are elders at increased risk of acquiring CDI but they also have higher rates of complications, recurrence, and death. [26] Recently, the burden of CDI has shifted with a predominance of elderly patients affected being from a nursing homes (NHs) and not the hospital setting. [40, 44] Upwards of 40 to 50% of CDI cases are now from NHs. [45, 46] Residing in an NH is also a significant predictor of increased CDI disease severity. [47, 48] The presence of frailer elders in the NH with multiple comorbidities, are associated with an increased risk of CDI; these risk factors alone, however, do not adequately or completely explain the increased risk.

Nursing homes provide two types of care to elders. The first is custodial care or care that is primarily non-medical in nature. This care is supportive of activities of daily living. The second type of care is skilled nursing care where elders require more intensive medical, nursing, and rehabilitative services typically after discharge from the hospital setting for an acute illness. Nursing homes typically provide both custodial care and skilled nursing care. Thus nursing homes can be referred to as both long-term care facilities (LTCFs) or skilled nursing facilities (SNFs). The short-stay and long-stay resident populations in facilities differ in burden of illness,[49] functional status,[49] and risk for bacterial colonization and infection. [50] However,

these groups of residents are sometimes mixed when reporting disease outcomes. Both groups share in an increased risk of both CDI and *C. difficile* colonization.

It remains a challenge to conduct research in the nursing home environment. Issues surrounding informed consent and the protection of human rights make it a challenge to conduct research among nursing homes elders. [51] Characteristics of the nursing homes, the staff, and the residents living there also present difficult hurdles in conducting elder research in this settings. Much time and effort are required for the investigator to get all three NH tiers (administrators, nursing, and residents) to buy in to participation in human subjects research to make any investigation successful. This does not even take into account the family and ancillary staff that can be essential to NH research success. Because of these hurdles, research focused on NH elders has been lacking, especially with studies involving primary data and specimen collection. These hurdles need to be cleared to drive investigations among NH elders. This is of critical importance given that NH elders now are at the center of the *C. difficile* epidemic.

The intestinal microbiome and *Clostridium difficile* colonization

One factor that has become of increasing contemporary interest and a target of preventive strategies is the human microbiome. The microbiome is a vast array of microbes that influence human health and disease. [52] The intestinal flora changes with age, especially as the presence of anaerobes decreases. [53, 54] Elders from NHs differ from community-dwelling counterparts in their microbiome composition with higher proportions of the phylum Bacteroidetes and lower proportions of other bacteria at the family and genus levels. [55]

C. difficile can be considered a member of the normal gut bacterial flora. It is not a dominant member and its growth is suppressed by the more dominant anaerobes present. In fact, the healthy microbiota of the large intestine is thought to act as a barrier effect in resisting colonization to *C. difficile*.[56] The intestinal microbiota is known to be intrinsically stable with a sub-network of bacterial groups implicated in protection against CDI. [57] The rates of *C. difficile* present in the microbiome are the highest in NHs, with 20% to 50% of residents affected compared with 1.6% in the general community and 9.5% in the outpatient settings. [53, 58] Colonization with *C. difficile* is a well-documented source of new CDI cases, however approaches to managing carriage as a means to prevent CDI are lacking. [59-61]

By altering the structure of the gut microbiome, antibiotics alter its function leading to a loss in resistance to growth of *C. difficile* and an increased risk of CDI and diarrhea. [62] CDI requires the right combination of favorable growth conditions resulting from microbiome community alterations (i.e. after antibiotic administration) and the presence of the bacterium *C. difficile* to lead to CDI. Certain disruptions in the microbiota allow *C. difficile* to proliferate and cause CDI while other microbiome states allow for colonization but resist CDI (disease). Understanding this "at-risk for developing CDI" microbiome in relationship to a microbiome that permits colonization without disease development and/or one that resists both colonization and infection could provide a novel target for preventative strategies.

One term used when describing dysfunction within the microbiome is dysbiosis. Dysbiosis is a microbial imbalance or maladaption inside or on the human body. This occurs if the normal microbiome becomes deranged when bacterial species known to associate with or cause disease are present or are more in abundance than what is typical of that environment.

These “bad bacteria” are typically dominated by "healthy bacteria” that carry out benign or beneficial functions for the human host. Dysbiosis can refer to this bacterial presence or can refer to a balance of bacteria that then associate with human disease. Dysbiosis can also refer to derangements in the normal metabolism of a healthy microbiome.

Microbiome Sciences

The intestinal microbiome plays a critical role in maintaining human health throughout life. The microbiome’s presence was not generally recognized until the late 1990s, but has an enormous impact on human health. Historically members of the microbiome were identified broadly by culture and *in situ* staining techniques. Thus, it was necessary to grow an organism in the lab to then identify and study it. These limited techniques led to an understanding of bacteria that were easily grown, but as a whole, represented a minority of the microbiome taxonomy composition. The development of culture-independent techniques, where DNA is directly extracted from a sample, made it possible to investigate the entirety of the microbiome.

DNA-based methods can generally be broken down into taxonomic and functional metagenomic diversity. [52] Taxonomic diversity refers to how many different organism types are present in the microbe community while functional metagenomics refers to describing the assigned biological tasks that members of this community have the potential to carry out. Currently microbiome-based investigations have analyzed community composition and metabolic profiles independently or focused on identifying statistical associations between these two data types. [63] Methods for integrating taxonomic and metabolic data are lagging but seem

to be at the forefront of the more recent novel analytical techniques developed and reported on in the literature.

Technological advances in high-throughput sequencing have facilitated culture-independent analysis of the microbiome that rely on either sequencing of the 16S ribosomal RNA or of the entire genome present. 16S ribosomal RNA (16S rRNA) is a critical component of cell function, is universal in bacteria, and is highly conserved between different species of bacteria. 16S rRNA has proven useful for bacterial identification due to its gene sequences containing hypervariable regions that can provide a species-specific signature sequence. [64] Comparisons of the bacterial 16S rRNA gene sequences has emerged as one preferred genetic technique for taxonomy comparisons. [64] In fact, the 16S rRNA technique is the most common sequencing approach used to analyze genomic data collected from the Human Microbiome Project. [65] 16S rRNA gene sequence analysis is superior to culture based techniques because it can better identify poorly described, rarely isolated strains, can lead to the recognition of novel pathogens, and can identify nonculturable bacteria.

There are several limitations to the 16S rRNA methodology of microbiome investigations. First, the annotation is based on putative association of the 16S rRNA gene with taxa defined as an operational taxonomic unit (OTU). An OTU is an operational definition used to classify groups of closely related sequences used for analysis at the phyla or genera level. It is unfortunately less precise at the species level. [66] So, specific genes are not directly sequenced, but rather predicted based on the OTUs, and this lack of direct gene identification is a potential limitation in exploring and understanding the microbiome.

An alternative to the16S rRNA technique is whole genome sequencing. Whole genome sequencing is a sequencing method that uses random primers to sequence overlapping regions of an entire genome. This technique will determine the complete DNA sequence of an entire microbiome, including bacterial, viral and archaea, at a single time. Whole genome sequencing has led to the term metagenomics. The metagenome refers to the collection of genes sequenced from one environmental sample that is analyzed in an analogous way to the study of single genomes. [67] One specific type of whole genome sequencing is referred to as shotgun metagenomics or whole metagenomic genome shotgun sequencing. Whole genome sequencing differs from 16S rRNA techniques in several key ways (**Table 1**). This approach randomly shears all the DNA from an environmental sample, sequences many short sequences, and reconstructs them into a consensus sequence (**Figure 2**). Shotgun metagenomics provides both information on the taxonomy of organisms present and the metabolic processes that are possible from the microbiota community. [68] Shotgun whole genome sequencing has multiple advantages when compared to the 16S method that include enhanced and improved detection accuracy of bacterial species, increased detection of diversity, and increased prediction of genes. [66] In addition, whole genome sequencing gives metabolic pathway information that is not attainable from the 16S rRNA approach. A major drawback to whole genome sequencing is the cost difference between the two techniques with 16S rRNA sequencing costing roughly 10 times less than whole genome sequencing. Whole genome sequencing also requires much more computer space to store the sequenced data. Nonetheless, we chose to use the shotgun metagenomic approach in this project due to the listed advantages above.

Metagenomic Analysis

The data obtained from metagenomic sequencing of a sample is enormous with fragmented data of upwards of 3 million genes from as many as 10,000 species that requires hundreds of gigabases of sequence data. [69] There are multiple steps that need to be taken to go from sample to data. The first steps after obtaining the data involve filtering the data for redundant, low-quality sequences. There are also programs available to remove eukaryotic genomic DNA sequences such as Eu-Detect. [70] The next step involves matching the sequences obtained from the sample to a reference library. Metagenomic Phylogenetic Analysis (MetaPhlAn) is a method based on unique clade-specific markers for estimating organismal relative abundances. [71] Alternatively, the Kyoto Encyclopedia of Genes and Genomes (KEGG) database is a collection of databases dealing with genomes, biological pathways, diseases, drugs, and chemical substances. [72] Thus, using MetaPhlAn will give taxonomy from whole genome sequencing and KEGG can be used to get functional metabolic diversity.

Once the genomic data is matched to a reference library, one of the first methods to explore the resultant data is to look at species diversity. The most common types of diversities are α and β-diversity. α-diversity is the biodiversity in a defined habitat or in the case of microbiome analysis the diversity within the sample. This asks the fundamental question of how many different species are detected in one sample. The Shannon index is a popular α-diversity index which quantifies the entropy or information content. [73] The Shannon index will perform well when approximating the microbial diversity of common taxa, however it may not be accurate when examining the numerous low abundant organisms present. [74]

β-diversity compares the diversity between habitats or samples. The question asked by β-diversity is how different is the microbiome composition from one sample to the other. Commonly, two- or three-dimensional plots are used to visually represent the observed β-diversity. Beta-diversity is a 'distance' measure of community similarity/dissimilarity. If two samples have very similar species composition, they will map close together (low dissimilarity) while samples with non-overlapping composition will map far apart (high dissimilarity). There forth, similar microbiomes from different samples will cluster together. Dissimilarity can be assessed on presence/absence of taxa (binary) or relative abundances (weighted). Distances can be measured with many different metrics. Two commonly used measures of β-diversity distances are Bray-Curtis or Jaccard. [75] The Jaccard similarity index is a way to compare populations by determining what percent of organisms identified were present in both samples. The Jaccard coefficient measures similarity between samples and is defined as the size of the intersection divided by the size of the union. The Jaccard distance is a metric distance, and measures the dissimilarity between samples. It is complementary to the Jaccard coefficient and is obtained by subtracting the Jaccard coefficient from 1. [76] Bray–Curtis dissimilarity is used to quantify the compositional dissimilarity between two samples. Bray–Curtis dissimilarity is often erroneously called a distance as it does not satisfy triangle inequality. [77] The choice between the binary and weighted methods can be made depending on what relevant information you are exploring. For example, if you want to think about bacterial species that may be protective against a disease process than binary methods may be more relevant. However, if you want to consider the impact of an intervention on the microbiome, then abundance methods may be preferred.

The distance measures from a large set of samples can be plotted in multi-dimensional space visualized in two- or three-dimensional scatterplots. Principal Component Analysis (PCA) is applied to the distance matrix to find the 2 or 3 dimensions (or principal coordinates) in which we can visualize the largest amount of distance variation (i.e. maximally spreads of the samples in space). The percentage of total variation explained by each axis is indicated on the axis. [78] When visualizing PCA using Jaccard one can start to explore if different subject specific variables associate with microbiome samples that cluster together. The non-parametric multivariate statistical test that is used to compare groups of objects is the permutational multivariate analysis of variance (PERMANOVA) test. The PERMANOVA tests the hypothesis that the centroids and dispersion of the groupings made by one covariate, as defined by measured space, are equivalent for all groups (**Figure 3**). A rejected null hypothesis means that either the centroid and/or the dispersion of the points is different between the groups. [79] The PERMANOVA tests is quite often the first step in describing associations with the microbiome between different variables.

Observations based on PCA plots and factors thought to be important in associating with microbiome composition, either by PERMANOVA analysis or by defining them *a priori*, can then be substantiated with modeling techniques. Modeling techniques will assess the clustering of microbiome points in space in relation to other covariates. The species abundances in a set of samples will hardly ever be normally distributed given the presence of multiple zeros for rarer microbiome species. Since the data is zero-inflated, there is a need for using either transformations or nonparametric statistics. [78] One popular and standard procedure that has been in use for long periods of time when analyzing proportional data in ecology is the arcsine

square root transformation. [80] The arcsine square root transformation is calculated as two times the arcsine of the square root of the proportion. Arcsine square root transformation enables the data to be analyzed as normally distributed thus allowing mixed-effect, logistic, and other modeling techniques to be applied. [81, 82]

Limited studies on the elderly NH microbiome: potential links to CDI

Clearly, the microbiome is important for gut health and CDI is all about gut health. Furthermore, elderly NH residents are amongst the most important targets of CDI. Therefore, looking at the microbiome of elderly NH patients is a critical part of studying CDI. To date there are a very limited number of studies among the elderly in the NH setting evaluating the intestinal microbiome. The largest intestinal microbiome investigations involved the ELDERMET cohort out of Ireland. The group studying this cohort has reported on correlations between diet, health status, and the microbiome composition of 371 elderly subjects living in different settings (NH and community). [83] This cohort contained 107 NH elders with longitudinal data and anywhere from 1 to 3 samples taken and analyzed. Here they noted microbiota temporal instability in both community-dwelling and NH elders, particularly in those with low initial microbiota diversity. Other published longitudinal elder metagenomic intestinal investigations have utilized patient numbers ranging from 1-20, [84-88] with elderly antibiotic administration and NH microbiome based investigations of less than 60 subjects. [55, 87] Important to note is that these investigations have not previously explored disease outcomes such as CDI or *C. difficile* colonization among NH elders and associations with the intestinal microbiome composition.

What we currently know is that elderly residents in NH care gain a defined population of bacteria that are associated with increased frailty. [89] The diet of long-term care elders is typically low in fiber with moderate to high fat content. [55] This diet is thought to associate with patterns in the intestinal microbiome without a proven cause and effect. [90] It is unclear if it is the environment, increased frailty, or diet of residents in NHs that affects the microbiome composition towards one of dysbiosis. Various dysbiotic changes in the intestinal microbiota are known to increase susceptibility to CDI. [57, 62, 91-93] These dysbiotic microbiomes have been shown in animal and human studies but have differed by human patient populations. This dysbiosis has a range of potential culprits from both specific bacterial species and metabolic functions, such as decreasing levels of secondary bile acids, glucose, and dipeptides to increases in primary bile acids and sugar alcohols. [91, 93] A dysbiotic profile for *C. difficile* colonization, especially in NH elderly which are at the highest risk of disease, has not yet been described.

The link noted above between CDI and antibiotics also fits in with this model for the microbiome being central to CDI in NH patients. It is well established that antibiotic exposure is a major risk factor for CDI. [4] Antibiotics have a profound and rapid effect on the gut microbiota, with a loss of diversity and a shift in bacterial community composition and function. [94] By altering the structure of the gut microbiome, antibiotics alter its function leading to a loss in resistance to growth of *C. difficile* and an increased risk of diarrhea. [62] Not all patients that are exposed to *C. difficile* go on to develop CDI when placed on antibiotics. This raises the question of what role the microbiome plays in increasing or decreasing CDI risk after antibiotic exposure. Other medications, such as acid reducing medications and corticosteroids, still remain controversial over their associations with CDI. Additionally, the effects of these medications in

elderly NH residents still remains poorly studied due to the difficulties in NH based research outlined above. It is also important to note that associated risks of these medications to CDI may differ with regards to *C. difficile* colonization. In fact, a better understanding of factors, both clinical and microbiome based, that increase the risk of *C. difficile* colonization would give a better understanding of this pathogen and novel targets for infection control.

2. Specific Aims

We have set out in a systematic manor to start to address the gaps in our knowledge in *C. difficile* infection and colonization among NH elders. We start off in **Chapter 1** by investigating the differences between elders from the community and those from the NH setting with regards to recurrent CDI. Here we explore if medication exposures from three important classes of medications (antibiotics, acid reducing medications, and corticosteroids) have different associated risks with recurrent CDI dependent on the home living environment (community or nursing home). Next we begin our investigation into the NH elder microbiome. To accomplish this we established a cohort of NH elders that in the end involved 91 residents from 4 different facilities. Early in this investigation we asked key questions about dysbiosis among NH elders reported in **Chapter 2**. These questions focus on what dysbiotic associations are observed among NH elders with regards to advancing age, frailty, malnutrition, and where they live in the facility. We had a unique situation in that the first NH facility we enrolled elders was segregated by floors with 2 floors in which residents intermingled and 2 others in which they were kept separate. Importantly all residents consumed the same diet, thus addressing this important confounder. Within this cohort of NH elders we also asked the question about whether there is variation in the microbiome composition if stool sampling was performed more frequently (every 3 days) or less frequently (every 30 days). We also explored whether pathogenic bacterial strains (*Escherichia coli*) are more similar from a phylogenic analysis among residents living on the same floor. Finally in **Chapter 3** we use the entire cohort of NH elders to investigate: 1) what is the prevalence and patterns of *C. difficile* colonization; 2) do elders living together have more similar microbiome profiles; 3) is the phylogeny *E. coli* grouped by floor and site; and 4) what species present in the microbiome can predict the presence of *C. difficile* in a sample thus

defining key bacterial species that together promote *C. difficile* colonization. The main Aims for each chapter are thus:

- Chapter 1: To determine if certain medications elders are exposed to have associated risks to recurrent CDI that differ by home environment.

- Chapter: To explore the associations of NH environment, frailty, nutritional status, and residents' age with microbiome composition and potential metabolic functions.

- Chapter 3: To examine the colonization patterns seen in NH elders, over time, and to identify clinical and microbiome based factors that are associated with colonization status.

Figure 1: Test algorithms for the diagnosis of Clostridium difficile infection. (Adapted from[22])

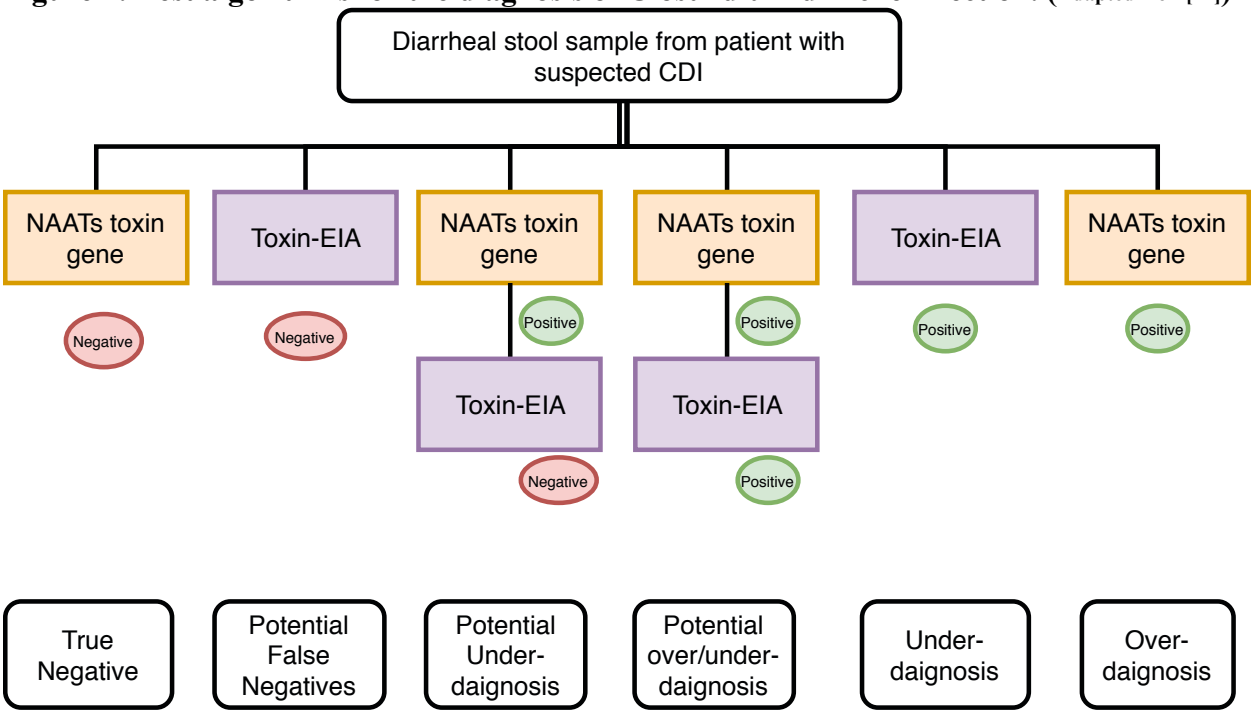


Figure 2: Whole genome shotgun sequencing technique for getting data from a sample

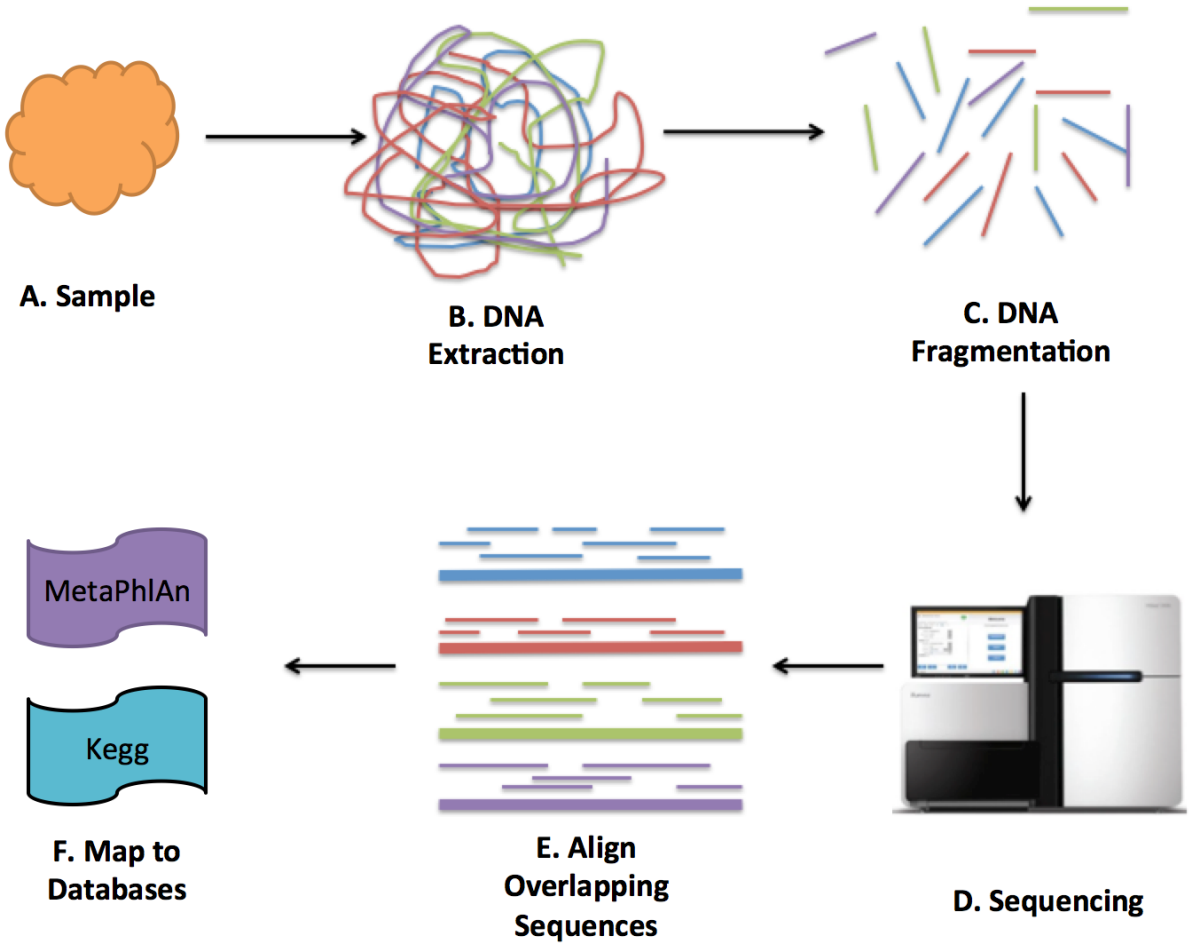
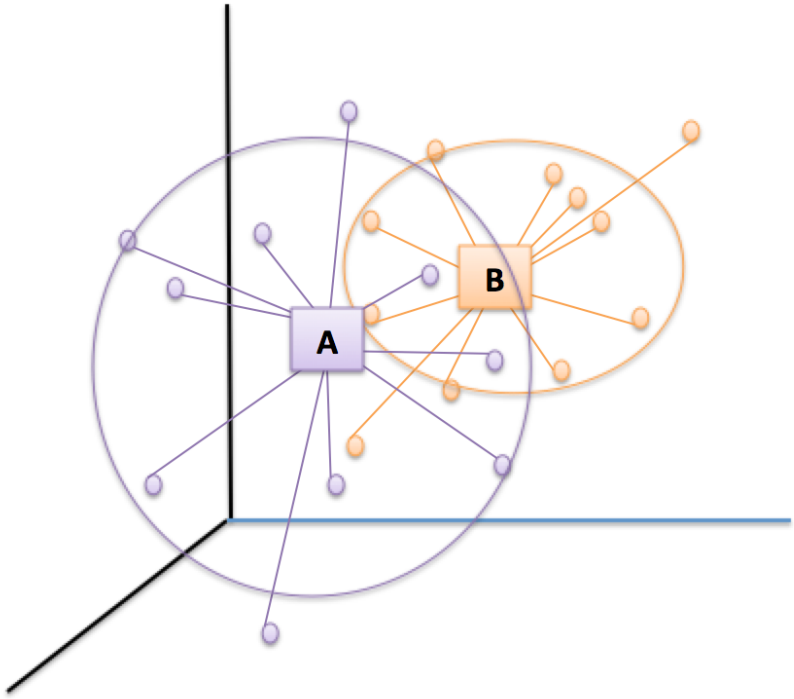


Figure 2 Legend: Steps above include: (A) obtaining a sample; (B) extracting the DNA; (C) fragmenting the DNA; (D) sequencing the genome; (E) aligning the sequences reads; and (F) mapping the reads to a database to obtain taxonomic or gene function data. Databases depicted here are Metagenomic Phylogenetic Analysis, MetaPhlAn and Kyoto Encyclopedia of Genes and Genomes, KEGG

Table 1: Comparison of 16S rRNA to whole genome sequencing techniques		
	16S rRNA	**Whole Genome Sequencing**
Sequencing target	Only the 16S rDNA	All genes present
Diversity	Less diversity identified	More diversity identified
Taxonomy quality	Better at identification at genera or phyla level	Superior at the species level
Virus, fungi, and protozoa identification	No	Yes
Data on metabolic pathways	No	Yes
Identify bacterial resistance	No	Yes
Data	<10 gigabytes	>>10 gigabytes
Costs	$10-15/sample	$100-200/sample

Figure 3: Representation of a Permutational multivariate analysis of variance (PERMANOVA) analysis of Jaccard distances between two groups A and B on a Principal Coordinates Analysis 3D graph



CHAPTER 1

Medication Exposure and the Risk of Recurrent Clostridium difficile Infection by Home Environment

ABSTRACT

Background: It is unclear how medication exposures differ in their association with recurrent *Clostridium difficile* infection (rCDI) between nursing home and community-dwelling elders. This study examines these exposures to determine if the risks of rCDI differ by home environment.

Methodology: This is a retrospective study of patients 65 years or older with CDI diagnosed by symptoms plus positive stool toxin testing from both the academic and community healthcare settings. Sociodemographic data, patient characteristics, and medications were extracted from the electronic medical record (EMR). We used separate extended Cox models conducted according to home environment to identify the association between medication use and risk of rCDI.

Results: Of the 616 CDI treated elders, 24.1% of community-dwelling and 28.1% of nursing home elders experienced recurrence within 1 year. Among community-dwelling elders, exposure to antibiotics and acid-reducing medications were associated with an increased risk of rCDI (1.6 and 2.5 times, respectively), however corticosteroid exposure reduced the risk of recurrence by 39%. Among nursing home elders, the risk of rCDI was 2.9 times higher with acid-reducing and 5.9 times higher with corticosteroid medication exposures.

Conclusion: Recurrent CDI risk was greater for acid-reducing medication than antibiotic use after initial CDI treatment, and these risks varied depending on the home environment. Additionally, corticosteroid use was associated with increased risk of recurrence in nursing home elders but decreased risk in community-dwelling elders.

1. INTRODUCTION

There has been a worldwide increase in the incidence of *Clostridium difficile* infection (CDI),[4] with annual direct costs associated with the CDI treatment estimated to be nearly $3.4 billion.[95] Both the prevalence and virulence of this opportunistic pathogen are rising, especially among elderly nursing home residents.[5, 40, 47] CDI is common among elders living in the nursing home environment, from which a large proportion of new CDI cases are presently coming.[96, 97]

The increasing incidence of recurrent *Clostridium difficile* infection (rCDI) has been alarming with almost a 200% increase from 2001 to 2012.[24] Patients remain vulnerable to rCDI for months or even years after initial CDI treatment.[25] Recurrent infection is common, occurring in up to 22% of initial cases.[26] The risk factors associated with rCDI are believed to be similar to primary CDI although rCDI has been less studied, especially among the elderly. Modifiable risk factors include antibiotics, gastric acid medication, and immunosuppressants such as corticosteroids.[35, 98, 99] We know that how the initial CDI case is treated may affect recurrence, however this has mostly been studied in the hospital setting. Since medication treatment after CDI is arguably the most common intervention to reduce rCDI, we focused on medications prescribed in both the hospital and outpatient settings.

Recurrent CDI includes both relapse and reinfection with a new strain.[27] Nursing home residents are at particularly high risk of rCDI since exposure to *Clostridium difficile* in nursing home elders is a magnitude of ten times higher than among community-dwelling elders.[100] There forth, nursing home elders may be at an increased risk of reinfection compared to community-dwelling elders. It is possible that different medication exposures may change the associated risk of reinfection after CDI treatment in this environment. Given this large difference

in environmental exposure, combined with the higher rates of CDI within a year after CDI treatment, we sought to examine the effect of medication use (i.e., antibiotics, antacids, corticosteroids) on rCDI and whether it varies by home environment. To accomplish this, we followed a cohort of incident CDI patients for 1 year to identify rCDI to determine the association of specific medication exposures in a cohort of elders with rCDI, stratified by home living status (community-dwelling versus nursing home). This study reports the incidence of rCDI among community-dwelling and nursing home elders and the association of rCDI with specific medication exposures.

2. METHODS

2.1. Study Setting and Population

This retrospective cohort study was approved by the institutional review board at the University of Massachusetts Medical School. The cohort of CDI positive elders (age ≥ 65 years) was identified by using the UMass Memorial Health Care System's (UMMHC) Theradoc Clinical Surveillance Software System (Premier Inc., Charlotte, NC). Using this system we constructed a cohort of elderly patients with incident *Clostridium difficile* enterocolitis between 2012 and 2014 who initially presented to both academic and community hospital settings. An incident case was defined as one positive *Clostridium difficile* toxin PCR and no evidence of CDI in Theradoc or electronic medical record (EMR) within the prior 60 days.[101] We confirmed that the incident case was done on a diarrheal stool sample and the patient was treated for a CDI. We excluded patients if there were no clinical visits recorded within a six-month window after CDI diagnosis or if there was no documentation of initial CDI treatment.

2.2. Data Collection

In order to reduce the potential for systematic error and to mitigate bias, we followed protocols for the optimal conduct of retrospective studies.[102] Prior to data abstraction activities, we *a priori* defined the pertinent predictor and outcome variables to be collected in a standardized manner. Trained abstractors used a standardized collection form to query the EMR to obtain longitudinal data pertaining to healthcare visits for up to 12 months after completing initial CDI treatment. Demographic data, including age at CDI diagnosis, sex, and race/ethnicity, were collected. The EMR was reviewed to obtain data on medication usage and the nature of subsequent healthcare visits. We calculated a Charlson comorbidity index (CCI) score to characterize the patient's medical comorbidities.[103, 104] Abstractors worked on different parts

of the chart and were blinded to outcome status and patient assignments to reduce information bias during patient record review.

2.3. Classification of Drug Exposures and rCDI Outcomes

We used the EMR to monitor the prescriptions given to patients during non-CDI related visits both within the hospital and outpatient settings at UMMHC. We specifically queried the EMR for new antibiotic prescriptions given within 6 months after initial CDI treatment, either prevalent corticosteroid use (i.e. treatment for rheumatoid arthritis) or incident corticosteroid use within 6 months (i.e. treatment for asthma exacerbation), and both prevalent and incident acid-reducing medication use (within 6 months after CDI), which included both proton pump inhibitors (PPIs) and H2 blocker medications. Antibiotic prescriptions taken for the treatment of CDI were not included as an antibiotic exposure (e.g. metronidazole or oral vancomycin). Given immunological senescence that accompanies age may be a major risk factor for rCDI[105] and this may be exacerbated by use of steroids in older adults, we included steroids in the drug exposures we monitored for after CDI treatment. Only after all study information was collected for all participants were rCDI outcome status assignments made by two independent physician reviewers (JH, EB). Where there was a disagreement between the reviewers a third independent adjudicator (XW) made the final determination.

2.4. Data Analysis

We used Chi-square tests to compare categorical variables, and the student's t-test for continuous variables, between patients with rCDI and those that were censored. Since the Cox model assumes that each covariate has a multiplicative effect on the hazards function that is constant over time, we tested this hypothesis among our variables of interest graphically with log-log plots of survival and on the basis of Schoenfeld residuals. After examining the

proportional hazards assumption for Cox proportional hazards model, we decided to use an extended Cox model which allowed for time-varying exposures[106] to identify association of predictors of interest with the outcome of rCDI. Clinically important factors included antibiotic exposure within 6 months of initial CDI treatment, acid-reducing medication use, and corticosteroids as the main variables of interest, in addition to patient demographics (including age, gender, and race), patient medical comorbidities (CCI score), and patient residence (nursing home versus community). The final adjusted model included both clinically important factors *a priori* and variables associated with the outcome at $p<.10$ in unadjusted analysis. Kaplan-Meier survival analysis was used to determine time to incident rCDI diagnosis. The logrank test was used to compare survival curves of time to incident rCDI diagnosis. Separate extended Cox model analyses were conducted according to home environment. Significance was set at .05 for all analysis.

We utilized multiple-imputation to address missing data in our data set, assuming data were missing at random. We conducted sensitivity analyses to examine the effect of patients lost to follow-up. Using best-case and worst-case scenarios, we constructed a logistic model predicting rCDI using initial patient demographics and treatment conditions. The models with the patients that were lost to follow-up were coded as either none having rCDI or all having rCDI. We used Stata, version 13.1 for all analyses (StataCorp LP, College Station, TX).

3. RESULTS

3.1. Characteristics of the Study Subjects

During the 3-year study period from 2012 – 2014, there were 863 incident cases of CDI. We excluded 84 (9.7%) patients who died during the initial index visit, 125 (14.5%) patients without follow-up EMR data, and 38 (4.4%) patients who only had EMR visit data during the initial treatment phase. The final cohort included 616 elders. Within the following year, 161 (26.1%) patients experienced recurrent CDI. The prevalence of rCDI among community-dwelling elders was 24.1% and among nursing home residents was 28.2%.

3.2. Risk Factors for Incident rCDI

Among patients with and without rCDI, there were no differences in baseline demographics or medical history with the exception of a higher prevalence of prior 6 month history of CDI treatment among those with rCDI (**Table 1.1**). Patients with rCDI had higher prevalence of exposure to an antibiotic within 6 months after initial treatment and higher prevalence of exposure to acid-reducing medications. The number of corticosteroid exposure events was similar between the two groups.

Extended Cox model analysis found a history of CDI within the previous 6 months, antibiotic and acid-reducing medication exposures to be significantly associated with reduced time to rCDI after adjusting for age, gender, home environment (nursing home versus community), medical comorbidities, and corticosteroid use. In adjusted analysis, antibiotic exposure within 6 months after completion of CDI treatment yielded an adjusted hazard ratio (aHR) of 2.62 (95% confidence interval [CI], 1.44 to 4.76) while acid-reducing medication exposure had an aHR of 4.68 (95% CI, 2.83 to 7.73). A history of CDI prior to the initial CDI episode as entrance into this cohort had an aHR of 1.52 (95% CI, 1.04 to 2.20). The significance

of demographic and treatment variables did not change in our sensitivity analysis using best-case and worst-case scenarios when including those patients lost to follow-up.

3.3. Risk Factors Stratified by Living Environment

We stratified our analysis by home environment to test the hypothesis that medication exposure carried different risk profiles for rCDI depending on whether the patient lived in a nursing home or in the community. Community-dwelling and nursing home elders did not significantly differ by age, gender, race, CCI score, or previous history of CDI nor did they differ in their rates of exposure to antibiotics, acid-reducing medications, or corticosteroids. We observed higher levels of exposure to antibiotics among those with rCDI compared to those without rCDI in the community (58.3% versus 28.5%, $p<0.001$) and in the nursing home (50.0% versus 30.1%, $p=0.038$). Similarly, elders with rCDI had greater exposure to acid-reducing medications compared to those without rCDI in the community (62.9% versus 30.8%, $p<0.001$) and nursing home (72.2% versus 24.1%, $p<0.001$). With regards to corticosteroid exposure, elders with rCDI had similar levels of exposure to elders without rCDI in the community (17.4% versus 17.4%, $p=.98$), but a higher level of exposure in the nursing home setting (19.4% versus 8.4%, $p=0.045$).

Stratifying the extended Cox model by home environment resulted in different HR for each medication exposure (**Table 1.2**). Among community-dwelling elders, exposure to antibiotics and acid-reducing medications was associated with an increased risk of rCDI (63% and 2.5 times higher than in those not exposed, respectfully), however corticosteroid exposure reduced the risk of recurrence by 39% (**Table 1.2**). Among nursing home residents, the HR for antibiotic exposure was not significant; however, the HR was 2.9 times higher with acid-reducing medication exposure and 5.9 times higher when exposed to corticosteroids. Medication

exposures demonstrated in the Kaplan-Meier survival curves varied significantly stratified by home environment (**Figure 1.1**).

4. DISCUSSION

Elders living in a nursing home had slightly higher, but non-significant, occurrence of rCDI. Medication exposures studied here were associated with an increased risk of rCDI however this associated risk differed by home environment. In the nursing home, the risk of rCDI was increased with exposure to acid-reducing medication and corticosteroids. In the community, antibiotic exposure within the first 6 months after treatment for CDI was associated with an increased risk of rCDI while corticosteroid exposure in this group was associated with a reduced rCDI risk. Our findings add to the literature by highlighting the possible importance of the patient's home environment when it comes to medication exposure and risk of rCDI. We feel these findings underscore the importance for clinicians of reducing any antibiotic or acid-reducing medication exposure among the elderly after CDI treatment, especially among NH residents.

Our findings of an association between medication exposures and an increased risk of rCDI are consistent with the literature. While the mechanism for recurrence is unknown, the most common risk factors include the use of antibiotics and gastric acid-suppressive therapy.[26, 32] Acid-reducing medications that include both PPIs and H2 blockers are well known to be associated with increased risk of CDI.[107] The exact relationship between acid-reducing medication use and incident rCDI remains elusive with no causative pathway having yet been demonstrated.[29, 108] Recent attention has turned to the human microbiome as a novel vehicle for predicting and preventing disease. The human colon harbors a vast array of microbes (the gut microbiota) that critically influence human physiology and have long been known to influence

human health and disease.[52] It has been observed that patients taking a PPI have changes in their microbiome that are in line with known changes that predispose patients to CDI and can potentially explain the increased risk of CDI in PPI users.[109]

The role of corticosteroids is much less clear because they have been shown to both increase and decrease the risk of incidence of and mortality from CDI.[36-39] Patients with chronic steroid use have been shown to have a threefold increased risk of CDI.[110] This, however, has been balanced with reports showing that when corticosteroids are used intermittently it can reduce the risk of CDI.[37] Here, we have demonstrated that corticosteroid exposure is associated with a reduction in the risk of rCDI in community-dwelling elders, however it is associated with an increased risk in nursing home elders. Corticosteroids significantly alter the microbiome of the intestine,[111] which might also lead to predisposing a patient to rCDI. Our findings of the associated increased risk of rCDI with corticosteroid exposure in NH residents needs further exploration, however from a clinical standpoint, avoiding corticosteroid use in NH elders after CDI treatment could possibly reduce the risk of rCDI.

Recurrent CDI includes both relapse of CDI with the same strain and reinfection that is a result of a new strain, which is clinically indistinguishable.[27] We believe that the role of the home environment in the different observed associations of rCDI to these medications may be due to the level of exposure to *C. difficile* spores in the environment causing reinfection. Rates of *C. difficile* present in the stool of elders are the highest in nursing homes with 20% to 50% of residents affected compared with 1.6% in the general community and 9.5% in the outpatient setting.[53, 58] Since corticosteroids and acid-reducing medications significantly alter the microbiome of the intestine, this might allow for *C. difficile* spores present in the environment to take hold and cause a recurrence of CDI within a year after initial CDI treatment.

4.1. Strengths and Limitations

One limitation of this current study is the retrospective design. Since patients needed follow-up data in the EMR to confirm recurrence, there is a possibility of misclassification of the outcome. This retrospective study did exclude some patients after the date of CDI diagnosis thus it is subject to a number of biases, most notably selection bias. Since we excluded patients that died before initial treatment completion, due to the fact that there was not a possibility for recurrence given the death, we may have introduced selection bias. However, there were similar numbers of deaths among each home environment group. To help mitigate this bias we developed a priori inclusion criteria. Additionally, medication exposures may have varied in magnitude given whether a patient took the prescription as written or at all. In addition, we included both incident and prevalent corticosteroid and acid-reducing medication use if a resident was taking the medication during entrance to the cohort and maintained on this medication throughout the study (i.e. corticosteroid treatment for rheumatoid arthritis). The type of corticosteroid exposure (chronic or intermittent) has been reported to differ with an associated increased risk of rCDI with intermittent use but a reduced risk of rCDI with chronic use.[36-39] Interestingly, all nursing home elders with corticosteroid exposures were incident. Finally, the study is limited by the small number of participants who were living in a nursing home. Further study in a larger population is warranted.

4.2. Conclusions

In conclusion, this paper's findings highlight differences in the associated risks of rCDI with antibiotic and acid-reducing medication use after initial CDI treatment that vary depending on the home environment. Avoiding both of these medications in the elderly patient in the 6 months after recovering from CDI may reduce that patient's risk of recurrence. Additionally, we

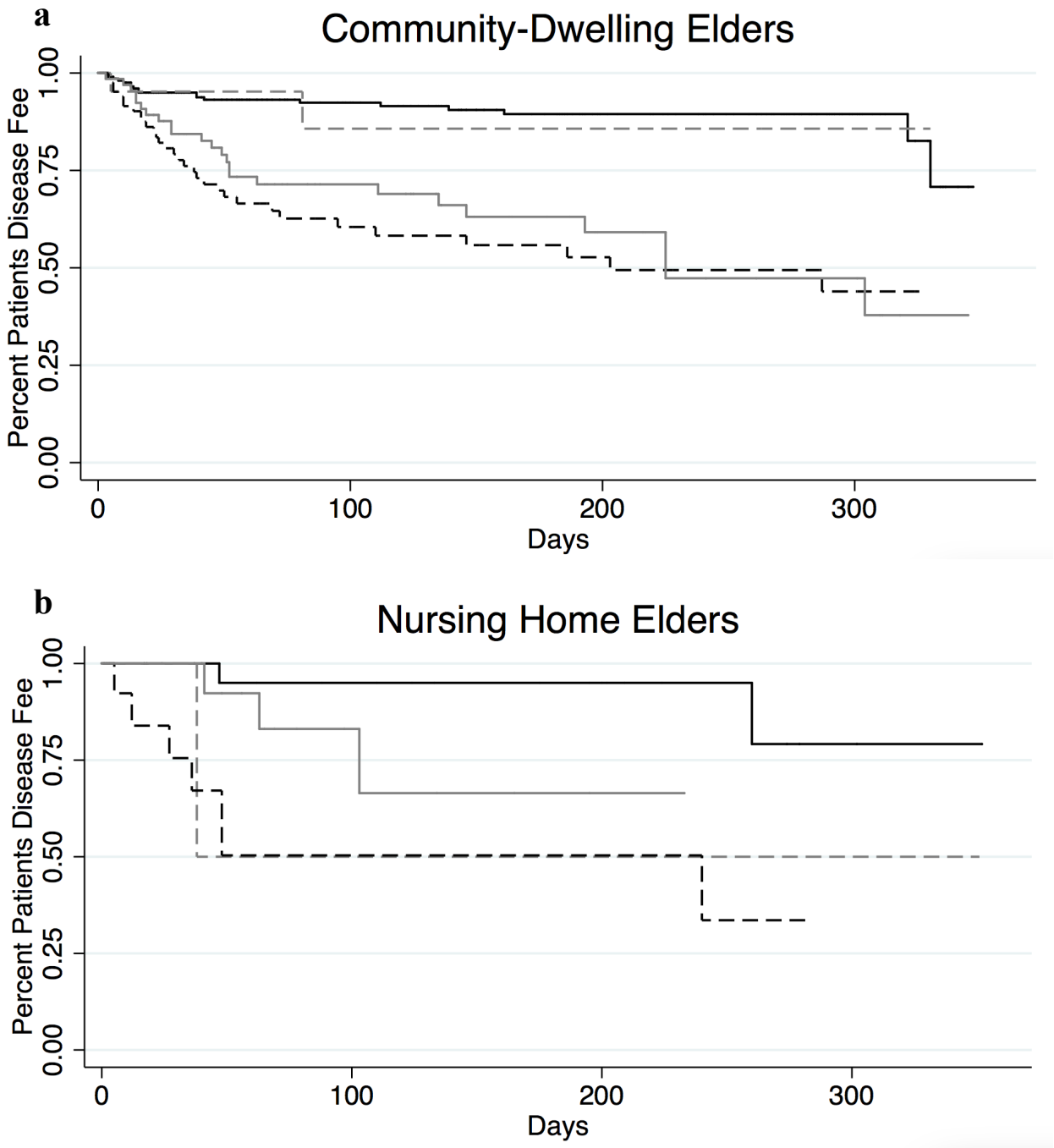
found that corticosteroid use was associated with an increased risk in nursing home elders and decreased risk in community-dwelling elders for rCDI, adding a further dimension to the controversy over steroid use after CDI. Studies are needed to confirm these finding of increased risk with corticosteroid use and also to determine what affects corticosteroids are having on the patient or their microbiome to increase this risk. Until that time, reducing corticosteroid exposure in nursing home patients recovering from a CDI may reduce the subsequent risk of recurrence.

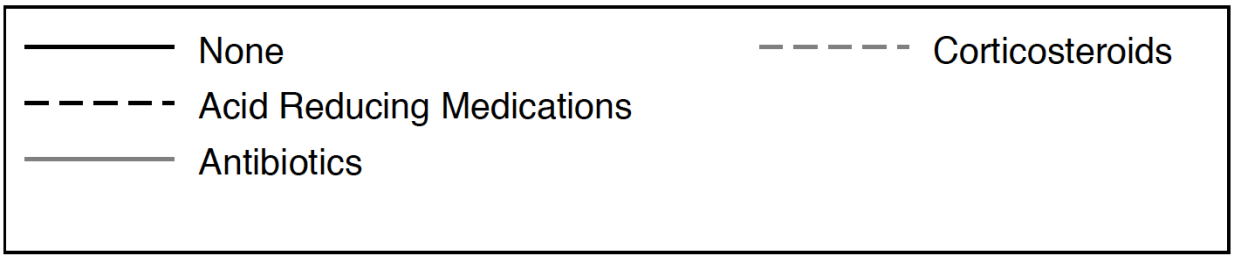
5. TABLES AND FIGURES

Table 1.1: Characteristics of the Baseline Population of 616 Older Adults at Risk for Recurrent *Clostridium difficile* Infection[a]			
Baseline Characteristics			
	No rCDI (n=455)	rCDI (n=161)	p value
Age (SD)	76.5 (8.0)	76.7 (8.4)	0.39
Female	262 (57.6)	99 (61.5)	0.39
White	413 (90.8)	141 (87.6)	0.25
Black	13 (2.9)	6 (3.7)	0.58
Hispanic	21 (4.6)	8 (5.0)	0.86
Nursing Home	83 (18.2)	36 (22.4)	0.26
CCI Score (SD)	2.7 (2.2)	2.7 (2.3)	0.60
Hx CDI			
< 6 months	61 (13.5)	61 (37.9)	0.001
> 6 months	28 (6.2)	39 (24.2)	0.07
Medication Exposures			
Antibiotics	132 (29.0)	94 (58.4)	<0.001
Acid Reducing	139 (30.5)	105 (65.2)	<0.001
Steroids	72 (15.8)	30 (18.6)	0.41
rCDI, recurrent *Clostridium difficile* Infection; SD, standard deviation; CCI, Charlson comorbidity index; Hx CDI, previous history of *Clostridium difficile* infection [a] Data are presented as n (percentage) unless otherwise indicated			

Table 1.2: Stratified Extended Cox Regression Model for Community-dwelling and Nursing Home Elders[a]				
	Community Elders		NH Elders	
	Hazard Ratio	p value	Hazard Ratio	p value
CCI Score	0.80 (0.71-0.90)	<0.001	1.12 (0.95-1.33)	0.18
Hx CDI <6 mo	1.46 (0.97-2.20)	0.07	2.49 (1.03-6.01)	0.043
Antibiotics	1.63 (1.00-2.67)	0.05	1.12 (0.47-2.68)	0.80
Acid Reducing	2.51 (1.72-3.78)	<0.001	2.98 (1.12-7.92)	0.029
Steroid	0.61 (0.38-0.98)	0.039	5.90 (1.66-20.98)	0.006
CCI, Charlson comorbidity index; Hx CDI, previous history of *Clostridium difficile* infection [a] Data are presented as HR (95% confidence intervals)				

Figure 1.1 Kaplan-Meier survival curves of time to incident diagnosis of recurrent Clostridium difficile Infection (rCDI) in patients with and without medication exposures stratified by n=497 community-dwelling (a) and n=119 nursing home (b) elders.




None
Corticosteroids
Acid Reducing Medications
Antibiotics

CHAPTER 2

The Nursing Home Elder Microbiome Stability and Associations with Age, Frailty, Nutrition, and Physical Location

ABSTRACT

Background: The microbiome from nursing home (NH) residents is marked by a loss in diversity that is associated with increased frailty. Our objective was to explore the associations of NH environment, frailty, nutritional status, and residents' age with microbiome composition and potential metabolic function.

Methodology: We conducted a prospective longitudinal cohort study of 23 residents 65 years or older from one NH that had 4 floors: 2 separate medical-intensive floors, and 2 floors with active elders over a 4 month period of time. Residents' were assessed using the Mini Nutritional Assessment tool and Clinical Frailty Scale. Bacterial composition and metabolic potential of resident stool samples was determined by metagenomic sequencing. We performed traditional unsupervised correspondence analysis and linear-mixed effect modeling regression to assess the bacteria and functional pathways significantly affected by age, clinical frailty, malnutrition, and floor location of residence. .

Results: Nursing home resident microbiomes demonstrated temporal stability (PERMANOVA p=0.001) and differing dysbiotic associations with increasing age, frailty, and malnutrition scores. In older residents, the abundance of microbiota-encoded genes and pathways related to essential amino acid, nitrogenous base, and vitamin B production declined. With increasing frailty, residents had lower abundances of butyrate producing organisms, which are associated

with increased health, and higher abundances of known dysbiotic species. Butyrate producing organisms declined and dysbiotic bacterial species increased with increasing malnutrition scores. Finally, the microbiome of residents living in proximity shared similar species as demonstrated by the phylogenetic tree for *E. coli*, where residents on the same floor had similar strains.

Conclusion: These findings support that a signature "nursing home" microbiota may exist that is affected by the residents age, frailty, nutritional status and physical location.

1. INTRODUCTION

In the US, nursing homes (NHs) provide custodial care to older adults that need both medical and non-medical assistance, such as support for activities of daily living. These NH elders are grouped together and share a diet that is typically low in fiber with moderate to high fat content [55]. Residents living in NHs suffer from a high prevalence of *Clostridium difficile* and multi-drug resistant organisms (MDROs) that both colonize the intestinal track and cause active disease. The prevalence of these pathogens colonizing the gut is so high that the NH is now considered a reservoir for introducing these pathogens into other healthcare settings [112, 113]. Unrelated individuals living together have more similar gut bacterial communities compared to individuals living in other households, suggesting a shared environment affects the similarity of the fecal microbiome [114]. The similarities of NH elders microbiome has not previously been explored, warranting further research to better understand the factors leading to the development of the NH as a reservoir for MDROs for the community at large [115].

A healthy, diverse intestinal microbiome interacts positively with the host immune system and contributes to pathogen resistance [116]. Older adults that enter a NH experience an overall decline in intestinal microbiome function [117] and a significant loss in diversity when compared to community-dwelling elders [55]. Elders from NHs differ from community-dwelling counterparts in their microbiome composition, with higher proportions of the phylum Bacteroidetes compared to phylum Firmicutes and lower genus Coprococcus and Roseburia abundances [55]. Most previous elder microbiome investigations have focused on comparing community-dwelling to NH elders. These studies have concluded that NH residents gain a nursing home-associated microbiome that is mostly a result of the NH provided diet along with the increasing individual frailty associated with elders that need NH services [89]. Given the

variability in diets of elders living in NH or community-dwelling environments included in these studies, their conclusions have strongly highlighted the associations between diet and microbiome composition. This still leaves ambiguity as to what occurs to a NH resident microbiome as they age and their health status evolves with increasing frailty and malnutrition.

Elders are commonly grouped together in sections of the NH by medical or mental (i.e. dementia units) needs. Geographical location is known to influence the microbiome composition, however the influence diet versus physical location plays is not well understood [118]. A better understanding of environmental influences on human microbial communities could be an important factor to consider when understanding disease etiologies especially among vulnerable NH elders. Accordingly, the "nursing home microbiota" remains poorly defined especially when it comes to other factors besides frailty and diet that may influence its composition such as increasing age, malnutrition, or physical location. In addition, the complex interplay between patient-level and environmental (facility)-level factors and their influence on the microbiome in this vulnerable population is poorly understood.

Accordingly, we set out to follow a cohort of elders from one NH to investigate the associations of age, frailty, malnutrition and physical location and observed dysbiosis and the stability of the microbiome over time. We performed these observations among NH elders consuming the same diet, however they lived in separate sections of the same NH facility with specific floors being isolated from others. Our findings contribute to the understanding of how patient-level factors, such as age, frailty, and malnutrition level, influence the microbiota composition while adding novel discoveries as to the associations that facility-level factors (i.e. floor location) have with microbiome composition.

2. METHODS

2.1. Study Setting and Population

This prospective cohort study was approved by the institutional review board at the University of Massachusetts Medical School. This cohort is NH residents ≥65 years of age who lived in one NH facility that contained 4 different floors. The 3rd floor was the facility's locked dementia unit where residents were not allowed off the floor unless for issues regarding medical care. The next floor (4th floor) was termed the medical care unit housing residents with chronic medical issues requiring a higher level of nursing care, their food and care limited to this location. The 5th and 6th floors housed higher functioning long-term care residents who all ate and engaged in activities in one central location. They were also allowed to travel off of the floor frequently into the community. We approached residents across all floors who had been living at that facility for ≥1 month and did not have any diarrheal illness or antimicrobial exposure within the preceding 4 weeks. All residents throughout the facility followed the same low-fiber diet across all floors prepared in one central kitchen that is typical for a nursing home diet. No patients suffered from dysphagia or had a feeding tube.

2.2. Data Collection

Data were collected over a 4 month period of time. We conducted baseline and end of study medical record abstraction for factors associated with key study outcomes. These factors included age, nutritional status, comorbidities, use of proton pump inhibitors, and frailty [55]. Prior history of hospitalizations, antibiotic exposures in the past year, and histories of *Clostridium difficile* infection or urinary tract infections were collected from the medical record. The key outcome variables were age, frailty status, malnutrition status and floor location. We obtained age, sex, race, and length of NH stay from the medical record. We categorized residents

into 4 age categories for analysis: 1) 65 to 74; 2) 75 to 84; 3) 85 to 94; and 4) ≥95 years of age. This was done to show relations with decade increases in age rather than by year. Frailty was categorized according to the validated and widely utilized Canadian Study of Health and Aging's (CSHA) 7-point Clinical Frailty Scale [119]. This has been previously validated in demonstrating signatures of frailty in the gut microbiota [120, 121]. We assessed nutritional status using the Mini Nutritional Assessment (MNA) tool [122-124]. Residents were categorized as normal, at risk, or malnourished based on the MNA survey administered by trained research staff to the residents or the nurse caring for the resident if mentally impaired. All residents were enrolled for a total of 4 months in which we monitored for any changes to their care.

2.3. Sample Collection and Processing

We collected 4 monthly stool samples from each resident. Additionally, from 6 residents we collected samples every 3 days for 2 weeks and then monthly for 4 months to investigate whether the microbiome varied over short or long sampling intervals. DNA was extracted from samples using the PowerMagTM Soil DNA Isolation Kit on an epMotion 5075 TMX liquid handling workstation according to manufacture protocols (MO BIO Laboratories, #27100-4-EP). Sequencing libraries were constructed using the Nextera XT DNA Library Prep Kit (Illumina, Inc., #FC-131-1096) and sequenced on a NextSeq 500 Sequencing System as 2 x 150 base pair-end reads.

2.4. Sequence Processing and Analysis

Shotgun metagenomic reads were first trimmed and filtered of host contamination using Trimmomatic [125] and Bowtie2 [126] as part of the KneadData pipeline (https://bitbucket.org/biobakery/kneaddata). Reads were then profiled for microbial species abundances using Metaphlan2 [127] and for abundance of Uniref genes, KEGG orthologues, and

of the corresponding functional pathways (Metacyc pathways, KEGG pathways, and KEGG modules) using the software pipeline HUMAnN2 [128] and in-house written scripts (available upon request). Normalized taxonomic, gene, and pathway abundances were then used for downstream statistical analysis in R (see below). Strain-level analysis of *Escherichia coli* present in metagenomic samples was performed using StrainPhlAn [129]. Reads were mapped against the MetaPhlan2 clade-specific marker gene database [130]. Reconstructed *E.coli*-specific consensus markers were derived from the mapping data. The reconstructed markers were then used to build a phylogeny of the strains. The tools cited, in turn, depend upon the following—for KneadData: Trimmomatic [125], Bowtie2 [131], SAMtools (https://github.com/samtools/); for StrainPhlan: MetaPhlan2 [130], MUSCLE [132], RAxML [133], blastn [134]. The phylogenetic tree was visualized with FigTree (http://tree.bio.ed.ac.uk/software/figtree/)

2.5. Data Analysis

We performed traditional unsupervised correspondence analysis (NMDS and unsupervised hierarchical clustering) to first determine samples similarity with respect to the above covariates of interest. Permutation Multivariate Analysis of Variance (PERMANOVA) was performed to evaluate inter- vs intra-individual variability in bacterial abundance. To determine the contribution of each covariate to changes in microbiome composition (including microbial and functional pathways abundances) we performed linear-mixed effect modeling regression after arcsine square root transformation [81] using the R package *lme4*. P-values were calculated using a Kenward-Roger Degrees of Freedom Approximation and the returned t-value from the regression modeling. Covariates with $p < 0.05$ were retained and used for downstream visualization and data interpretation.

3. RESULTS

3.1. Characteristics of the Study Subjects by Floor Location

Over a six-month period we enrolled and followed 23 NH elders collecting monthly samples for a total of 4 months. Residents of the medical floor (4th floor) and dementia unit (3rd floor) had higher frailty and malnutrition scores than those on the 5th and 6th floors (**Table 2.1**). Residents on the 4th floor were older with an average age of 96.4. There were no differences in how long the resident had been living on that floor (length of stay) nor with regards to CCI score. No residents were exposed to antimicrobials or hospitalized during the study period. The last antimicrobial exposure occurred three months' prior to enrollment in only one subject.

3.2. The Individual NH Microbiome Demonstrates Stability Over Short and Long Time-Interval Observations

There were 6 residents for whom we collected samples every 3 days for 2 weeks and then monthly afterwards for 4 months, resulting in a total of 10 samples per resident. We had one resident in which we were only able to obtain 8 samples with 2 missed time points during the 2-week sampling time period. The average age of this group was 82.7 (9.2) years, all living on the 5th and 6th floors with an average frailty score of 5.2 (0.4) and malnutrition score of 0.2 (0.4). None of these residents had any changes in medications or healthcare exposure over the study period. Microbiota compositional differences were greater between individuals than within individuals demonstrating fecal microbiota stability in NH elders (PERMANOVA – Jaccard distance p=0.001; **Figure 2.1a**). The microbiome composition between individuals varied as demonstrated at the order level in **Figure 2.1b**. These data indicate that, for long-stay NH residents, both 3-day and 30-day collection frequencies exhibit similar microbiome variation

within an individual, which remains stable over time as long as there are not any changes to their diet or changes in medications.

3.3. Distance Measures Demonstrate Grouping Patterns by Age, Frailty, and Malnutrition

We explored beta-diversity by principal component analysis (PCA) using Jaccard distances for a measure of community species dissimilarity among combinations of malnutrition scores, frailty scores, age categories and floor locations in **Figure 2.2**. Microbiota compositional differences were greater between the covariates of malnutrition scores, frailty scores, age categories and floor location than within them with a PERMANOVA – Jaccard distance $p<0.001$ for each of the covariates. First, with regards to age and frailty, we note not only a pattern of shifting of composition towards the right as the age category increases but also a clustering of groups by frailty score, **Figure 2.2a**. Comparing malnutrition and frailty, we see a similar pattern of shifting composition towards the top right. As malnutrition scores increased they maintain a clustering by frailty score, **Figure 2.2b**. However, when we explore PCA analysis by floor location, any discernable pattern seems to be mixed with floors 3 and 4 clustered towards the middle surrounded by resident samples from floors 5 and 6, **Figure 2.2c**. There seemed to be a difference between floors when residents from floors 5 and 6 were combined as if they were on the same floor. We explored combinations of each of the variables represented in **Figure 2.2a-c**. with ellipses that represent 75% confidence intervals. These ellipses demonstrate clustering of combinations of the grouping categorical variables. If there were less than 4 samples per grouping, we omitted the ellipses. From these groupings, certain patterns emerged. For example, in **Figure 2.2a**. residents in age category 3 shifted in microbiome composition to the right as their frailty score increases from 3 to 6. From this analysis, we noted clustering among the variables of malnutrition, frailty and age without any specific pattern to floor location,

suggesting the overall microbiome composition did not correlate with location as it did to malnutrition, frailty and age.

3.4. Mixed Effect Modeling Demonstrated Age, Malnutrition, Frailty, and Location Affected Bacterial Species Composition and Functional Pathways

After unsupervised correspondence analysis, we performed linear-mixed effect modeling regression to determine the bacterial species and contributed functional pathways that were significantly affected by the covariates of interest. Briefly, after arctangent-square root transformation of microbial species (and pathways) abundances, we performed linear-mixed effect modeling regression. We fit the model Abundance ~ 1 + Age + Malnutrition + Floor + Frailty + (1 | ID), where Age, Malnutrition, Floor and Frailty are the fixed effect and ID represent the random effect. Using this approach, we decoupled the effect of each of the modeled covariates towards the microbial (or pathways) abundance and assessed each covariate's statistical significance independently. Row-normalized abundances of significant Species and KEGG Pathways are displayed as hierarchical clustered heat-maps in **Figure 2.3a** and **Figure 2.3b**. The Species and KEGG Pathways depicted in **Figure 2.3** are statistically significant for at least one of the model fixed effects.

3.5. Dysbiosis with Increasing Age in both Bacterial Species and Metabolic Pathways

We observed correlation of species and pathway abundances with increasing age. *Akkermansia muciniphila,* a mucin-degrading bacterium known to decrease in the elderly [135], was significantly decreased in residents in age category 3 (p=0.018) and *Ruminococcus bromii*, a keystone species in degradation of starch, was elevated in age category 2 (p=0.012) and then decreased in older age categories. The bacterial species *Ruminococcus gnavus*, which has been associated with a dysbiotic microbiota [136], was more abundant in age category 2 (0.003) and

lower in older residents with the lowest abundances in age category 4 (0.015). Several bacterial species known to be butyrate producers [137] were elevated in older adults. Butyrate is known to contribute to the maintenance of the gut barrier functions, and has both immunomodulatory and anti-inflammatory properties [138]. Butyrate producing *Eubacterium siraeum* was significantly elevated in age category 4 (p=0.004) and *Roseburia intestinalis* was elevated in age category 2 (p= 0.012), 3 (p= 0.002), and 4 (p= 0.016).

Metabolic pathways involved in essential amino acid biosynthesis and metabolism were higher in younger residents and decreased over the subsequent age categories, **Figure 2.4**. This involved cysteine and methionine metabolism (ko00270; p= 0.017), histidine metabolism (ko00340; p= 0.020), valine, leucine and isoleucine biosynthesis (ko00290; p= 0.005), and lysine biosynthesis (ko00300; p< 0.001). In addition, nitrogenous base metabolism also decreased with increasing age as purine and pyrimidine metabolism decreased over age categories 2 (ko00230 and ko00240; p = 0.004, 0.010) and 3 (0.028, 0.010). Finally, vitamin metabolism was also lower in older residents. Vitamin B5, pantothenate and CoA biosynthesis, decreased in age categories 2 (ko00770; p= 0.013) and 3 (p= 0.004), and vitamin B1 thiamine metabolism was lowest in age category 3 (ko00730; p=0.022). Combined with the species data, these results indicate that the dysbiosis associated with aging included decreases in mucin and starch degradation, essential amino acid synthesis, and decreases in nitrogenous base and vitamin synthesis.

In conjunction with the observed butyrate-producing species being of higher abundance in older residents, we also noted that butyrate metabolism also rose with increasing age and was significantly elevated in age category 3 (ko00650; p= 0.031). Pyruvate metabolism increased over the age categories and was highest in age category 3 (ko00620; p= 0.019) while CoA biosynthesis increased in age category 2 (ko00770; p= 0.013) and 3 (p= 0.004). Butyrate is

synthesized via pyruvate and acetyl-coenzyme A (CoA) [139]. Taken together this signifies that butyrate producing organisms and butyrate production, a sign of increased intestinal health, increased with age.

3.6. Dysbiotic Patterns with Increasing Frailty

After adjusting for age, malnutrition and floor location, residents with lower frailty scores had higher abundances of butyrate-producing organisms in **Figure 2.5**, notably members of the Clostridium cluster XIVa [140] as well as Lachnospiraceae bacterium 5_1_63FAA [141]. Conversely the bacterial species *R. gnavus*, which is associated with a dysbiotic microbiota [136], was higher in residents with higher frailty scores peaking at a clinical frailty score of 7 ($p=0.009$). From a metabolic potential standpoint, residents with higher clinical frailty scores had higher abundances of lipopolysaccharide (LPS) biosynthesis (ko00540; $p=0.043$), peptidoglycan (PGN) biosynthesis (ko00550; $p= 0.029$) and sphingolipid metabolism (ko00600; $p=0.014$). The observed dysbiosis with increasing frailty included lower butyrate-producing organisms with increases in LPS and PGN biosynthesis as well as sphingolipid metabolism. Alterations in LPS, PGN, and sphingolipid synthesis and metabolism have all been linked to increased bowel inflammation [142, 143].

3.7. Malnutrition's Association with Dysbiotic and Opportunistic Organisms

For residents who were either at risk of malnutrition or scored as malnourished, we noted trends of higher abundances of organisms associated with a dysbiotic microbiome. We found increased abundances of *Citrobacter freundii* in malnourished residents ($p=0.020$). These bacteria serve as opportunistic and super-infectious agents in immunocompetent and compromised patients [144]. Additionally, *Enterococcus faecalis*, which causes life-threatening hospital associated infections in humans such as sepsis, urinary tract infections and meningitis

[145], was also elevated in malnourished residents (p=0.014). Similar to increasing frailty, the bacterial dysbiotic species *R. gnavus*, was lowest in non-malnourished residents (<0.001). Finally, the butyrate producing organism *R. intestinalis*, was less abundant in both residents at risk of malnutrition (p<0.001) and those that were malnourished (p<0.001). *Subdoligranulum*, a spore-forming butyrate producer [146] was also reduced in the malnourished (p=0.008). From a metabolic standpoint PGN biosynthesis was noted to be elevated in malnourished residents (ko00550; p= 0.008). Malnutrition associated with opportunistic dysbiotic bacterial species as well as lower butyrate producing organisms with increased inflammation was associated with higher PGN biosynthesis levels.

3.8. Residents from the Same Location Share Similar Bacterial Organisms

Specific bacterial species were found to be more abundant among residents located on different floors, **Figure 2.6a-e**. Residents living on the medical floor (4^{th} floor) had higher abundances of the dysbiotic bacterial species, *R. gnavus* (p<0.001), and organisms that can cause opportunistic infections such as *Clostridium bolteae* [147, 148] (p=0.038). Other bacterial species, such as *Coprococcus catus* and *Eubacterium ventriosum* were of higher abundances in residents on the dementia unit (3^{rd} floor) in comparison to the residents on other floors. Not much is known of these two bacterial species. The anti-inflammatory Lachnospiraceae bacterium 8_1_57FAA [149] was of greater abundance in residents living on floors with higher functioning elders (5^{th} floor; p=0.015 and 6^{th} floor; p=0.006). *Lactobacillus reuteri*, and anti-inflammatory bacterial species [150], was also higher in residents on the 5^{th} and 6^{th} floors (p=0.003 and p=0.006). Taking all of these associations together, it points towards elderly residents that live together share specific bacterial species.

Finally, we wondered if residents shared genetically similar strains of bacteria. Observing that strains are shared between NH residents could have implications with respect to transmission of infections. Thus, we constructed the genetic relationship of *Escherichia coli* carried by seven individual residents from metagenomic sequence data, **Figure 2.7**. *Escherichia coli* was chosen for strain-level analysis due to it being both a common bacterial species that colonizes the intestines and is a species known to cause disease (e.g., urinary tract infections). The phylogenic tree demonstrates that ambulatory residents on the 5th and 6th floors had *E. coli* strains sharing more similar phylogenetic relationships than patients on the 4th floor (medical floor). Interestingly two of the resident *E. coli* phylogeny intermingle (yellow and pink, **Figure 2.7**), suggesting a high-degree of strain similarity. These data suggest that residents that share common living areas had genetically related *Escherichia coli* strains compared to other residents living in a separate area of the same facility that did not intermingle. We also performed strain-level analysis on other common commensal bacterial species but did not note any such floor association patterns.

4. DISCUSSION

Nursing home residents demonstrated different dysbiotic associations with increasing age, frailty, and malnutrition scores. As the age of residents increased, the abundance of microbiota-encoded genes and pathways related to essential amino acid, nitrogenous base, and vitamin B production declined. With increasing frailty, residents had lower abundances of butyrate-producing organisms, higher abundances of known dysbiotic species, higher LPS and PGN biosynthesis, and higher sphingolipid metabolism. Alterations in LPS, PGN, and sphingolipid biosynthesis and metabolism have all been linked to increased bowel inflammation [142, 143]. Among residents who were at risk of or were malnourished, butyrate producing organisms declined and opportunistic and dysbiotic bacterial species increased along with PGN biosynthesis. Interestingly, when looking at physical location within the nursing home, residents living together shared similar bacterial species and had similar *E. coli* phylogeny. Taken together, this suggests that the "nursing home" microbiota is influenced by resident age, frailty, nutritional status and physical location.

With increasing resident age, we found that bacterial species previously observed to decline with age were reduced. Specifically, *A. muciniphila* and *R. bromii* decline as a likely result of changes in the diet of older adults. This dietary change favors growth of bacteria that are able to degrade mucins [135, 151] and metabolizing dietary plant polysaccharides [152]. Additionally, *R. gnavus*, a species known to decrease with age [153], was higher in residents aged 65-74 and exhibited lowest abundances in those 95 and older. In older NH residents, we also observed a dysbiotic decrease of metabolic pathways involved in essential amino acid, nucleotide, and vitamin B biosynthesis. Aging has been associated with a progressive loss of muscle mass (sarcopenia) which is linked to lower availability of essential amino acid [154,

155]. Purine and pyrimidine nucleotide biosynthesis modules are known to be globally decreased in inflammatory bowel diseases (IBD) patients [156] as well as the vitamin B complex [157]. Taken together, these metabolic pathways reflect an age-specific dysbiosis specific to chronic intestinal inflammation seen in the elderly.

With increasing frailty or malnourishment, we noted increased abundance of *R. gnavus* and decreased Lachnospiracae and Ruminococcaceae families. The abundance of butyrate-producing organisms also declined with increasing frailty and malnutrition. Similar dysbiotic patterns have been observed in the disease states of IBD [136, 158, 159] [137] as well as in systemic inflammatory disorders such as multiple sclerosis [160]. Butyrate is an essential metabolite in the human colon. It is the preferred energy source for the colonic epithelial cells and it contributes to the gut barrier maintenance as well as having both immunomodulatory and anti-inflammatory properties [138]. Our finding adds to the growing evidence that a dysbiotic gut microbiota, with reduced butyrate production, is linked to medical disorders and may be a target of dietary and probiotic interventions.

Lipopolysaccharides and PGN biosynthesis was increased in frail or malnourished residents. Both of these gut-microbiota derived molecules stimulate specific systemic inflammatory pathways that result in low-grade systemic inflammation [143]. LPS- and PGN-associated inflammation has been linked to central nervous system disorders (e.g. chronic fatigue syndrome and complex regional pain syndrome) [161], as well as colorectal carcinoma tumor progression [162], and obesity and obesity-related pathologies [163]. Additionally, patients who have had a stroke or cardiovascular disease have had a greater inflammatory gut profile with an increase in PGN-producing enzymes [164]. Besides LPS and PGN biosynthesis, residents with higher frailty scores also were enriched in genes for sphingolipid metabolism. Alterations of

sphingolipids metabolisms have been associated with IBD [157] and nonalcoholic fatty liver disease [165]. Changes in sphingolipid levels result in a variety of effects on the epithelial barrier integrity, immune cell targeting and signaling, and innate/adaptive immune responses [142]. Taken together, elderly nursing home residents with higher frailty scores associated with a dysbiotic microbiota that resembles an inflammatory gut profile.

The NH had 2 floors where the residents did not leave that location and 2 floors of higher functioning elders that intermingled and used common socialization areas. Importantly, all residents were provided the same diet. From the linear-mixed effect modeling, we noted that several bacterial species were uniquely associated with specific floors irrespective of age, frailty, and malnutrition. The dementia floor had greater abundances of *C. catus* and *E. ventriosum* whereas both the dementia and medical floor had higher abundances of dysbiotic bacterial species. *C. bolteae*, an organism that causes opportunistic infections [147, 148] as well as *R. gnavus*, which characterizes the dysbiosis seen in IBD patients [148, 159] and also causes opportunistic infections [166] were both present in residents living on the 3rd and 4th floors. Of note, residents from these floors had more frequent contact with the hospital setting. The healthier residents of the 5th and 6th floor, had both higher abundances of *L. bacterium*, a butyrate-producing organism [140, 141] as well as *L. reuteri*, a species used as a probiotic for its anti-inflammatory effects [150]. When we performed the phylogenetic tree analysis of *E. coli*, resident sequences from the 5th and 6th floors intermingled while the 4th floor residents were genetically separate. Taken together, the location of the resident in the NH had associations with both specific enriched bacteria organisms and genetically similar *E. coli* phylogeny.

4.1. Strengths and Limitations

This study had several notable strengths and limitations. One limitation of this study is that it reports data from only a single site, however this is balanced by the strengths that all study participants had the same diet, effectively removing this important covariate. To our knowledge all previous elder microbiome investigations did not account for variations in diet. This study is also limited in the number of residents enrolled. The limited sample size may affect the generalizability of our findings especially when it concerns elders with varying medical comorbidities. Other potentially confounding variables such as polypharmacy and specific classes of medications the residents were exposed to were not evaluated in this cohort. Finally, the physical location microbiome association findings may have been biased by the clustering of residents with similar medical conditions onto the same floor. Following up this investigation with a multi-center cohort study would strengthen the findings and further explore the dysbiosis associations with age and frailty. However, we report new findings with regards to malnutrition and physical location within a the nursing home among elders. Confirming our study's findings in multiple NH facilities and addressing how these dysbiotic patterns are associated with *C. difficile* or MDRO colonization would be key to improving the health and wellbeing of elders living in the NH setting.

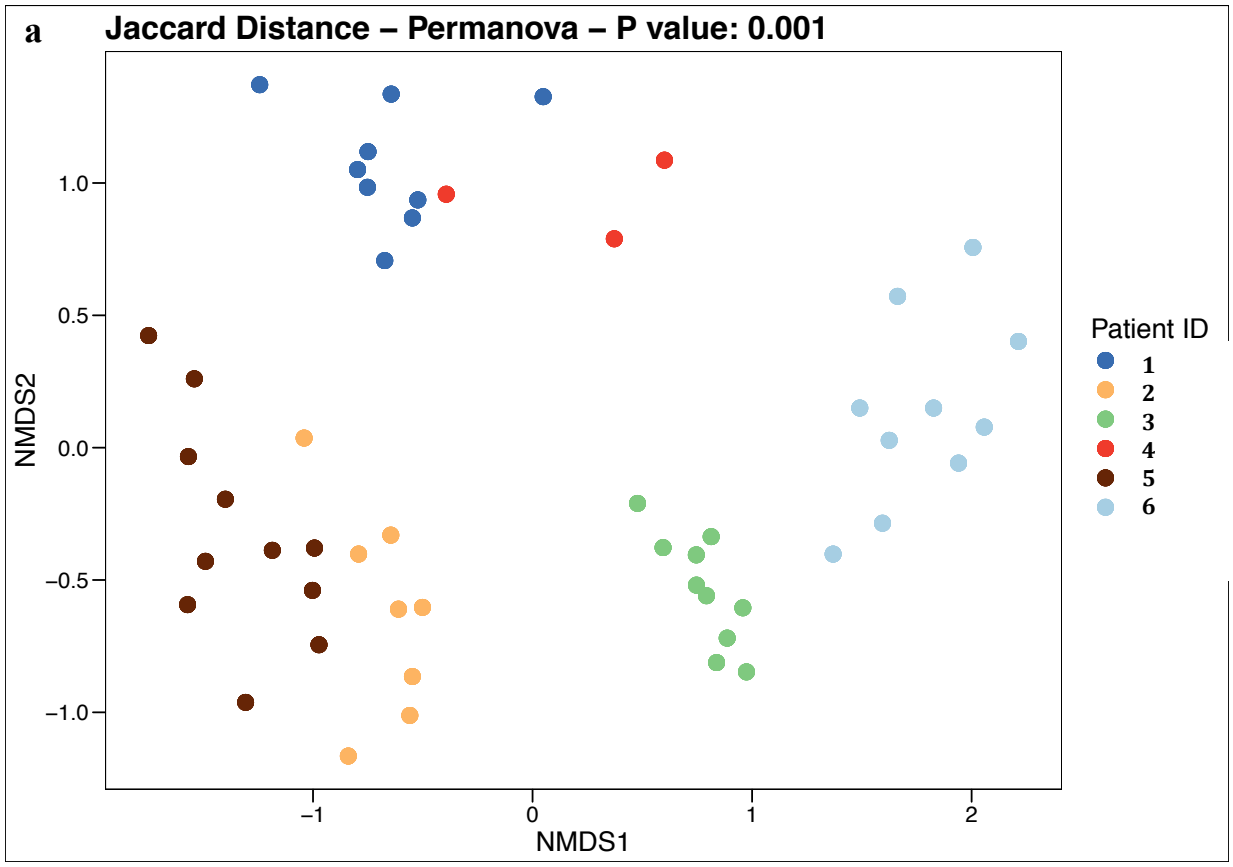
4.2. Conclusions

In conclusion, the dysbiosis of the NH elderly gut microbiome not only differs with increasing age, frailty, and nutrition, but also physical location within the NH. Further work is needed to see if introducing dietary changes or probiotics could affect these relationships with a goal of moving the microbiome away from being dysbiotic and supporting inflammation towards a healthier non-inflammatory profile that could help prevent disease.

5. TABLES AND FIGURES

Table 2.1: Characteristics of the Nursing Home Cohort by Floor of Residence					
Floor Number (n)	Third (3)	Fourth (4)	Fifth (7)	Sixth(8)	p-value
Age	82 (2.0)	96.4 (4.6)	89.9 (7.1)	86.3 (8.4)	0.036
Age Category	2 (0.0)	3.6 (0.5)	2.9 (0.9)	2.5 (0.8)	0.010
Length of Stay	23 (18.2)	14.6 (19.2)	22.9 (32.3)	17.9 (22.2)	0.93
CCI Score	2 (1.7)	1 (1.7)	1.3 (1.8)	1.0 (1.4)	0.82
Malnutrition	1 (0.0)	1.6 (0.5)	0.6 (0.5)	0.4 (0.5)	0.008
Frailty	6.7 (0.6)	6.8 (0.8)	4.9 (0.9)	4.9 (0.6)	0.053
Data presented as means (sd). CCI = Charlston Comorbidity Index, Length of Stay in months, Age Category 1= 65-74, 2 = 75-84, 3 = 85-94, and 4 = ≥95					

Figure 2.1. Individual variability of the microbiome over time



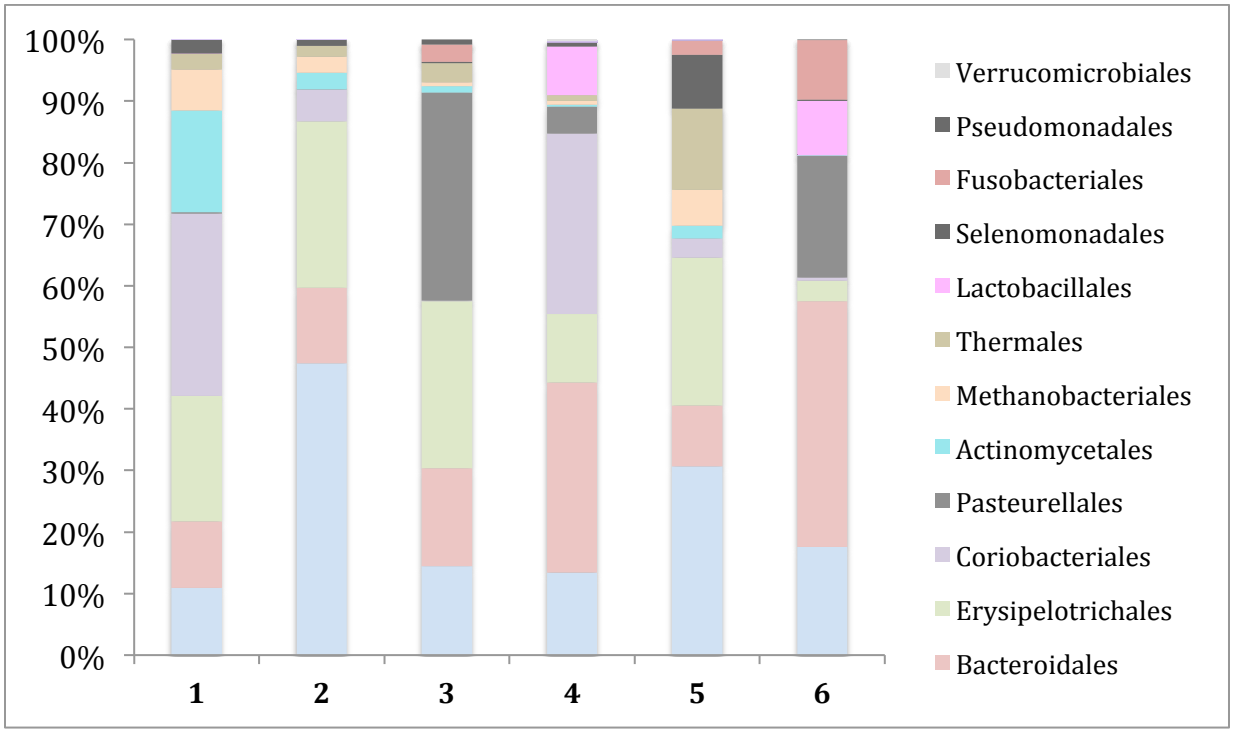
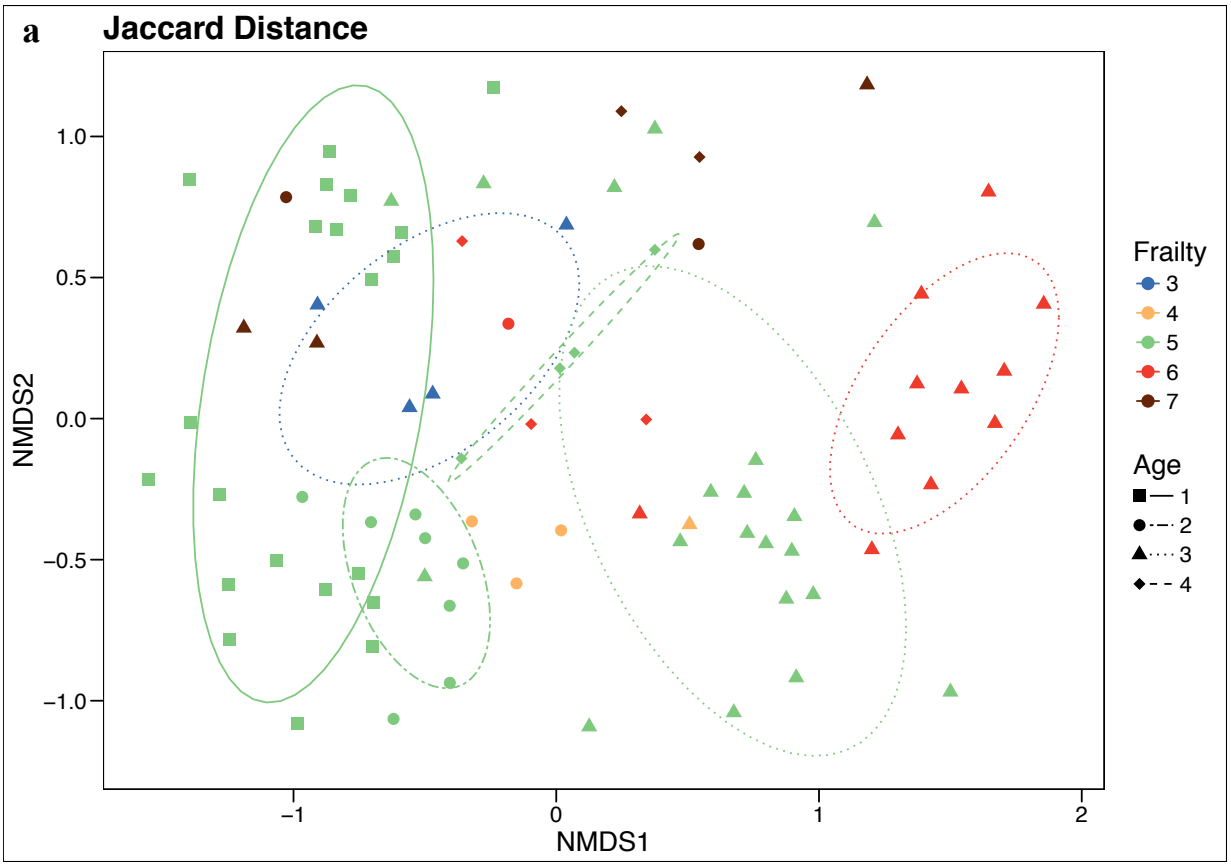
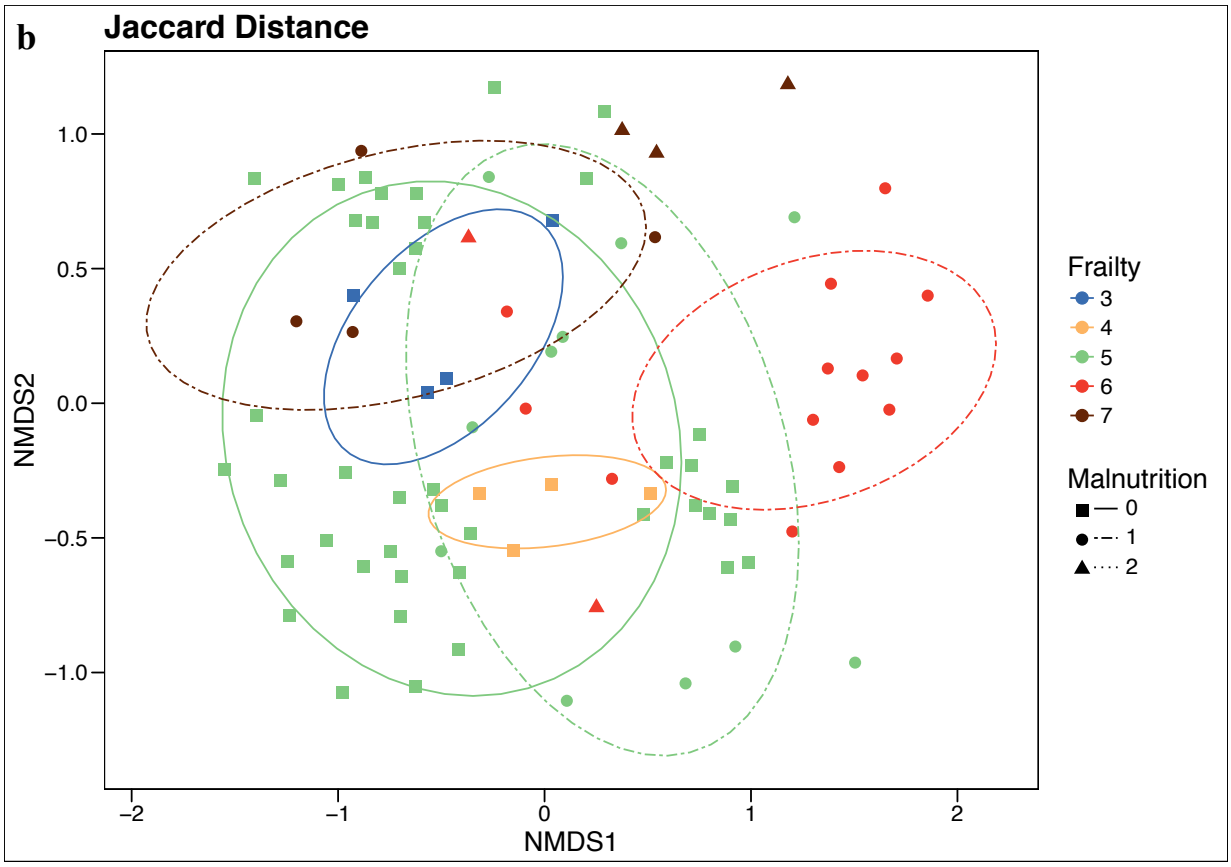


Figure 2.1 Legend: (a) A distance matrix of sample similarity from the presence or absence of bacterial species in each sample using the Jaccard Binary Index. Sample-to-sample distances are presented in a 2D Principal Component Analysis plot. Samples from 6 nursing home residents are colored by participant. No resident experienced any change in medications or a healthcare event over the sampling period. Each point represents a sample time point taken at 3 day and then 30 day intervals over a 4 month period of time. (b) The different community compositions among the six residents defined at the order level.

Figure 2.2 Binary jaccard index principal component analysis




b
Jaccard Distance
NMDS2
NMDS1
1.0
0.5
0.0
−0.5
−1.0
−2
−1
0
1
2
Frailty
3
4
5
6
7
Malnutrition
0
1
2

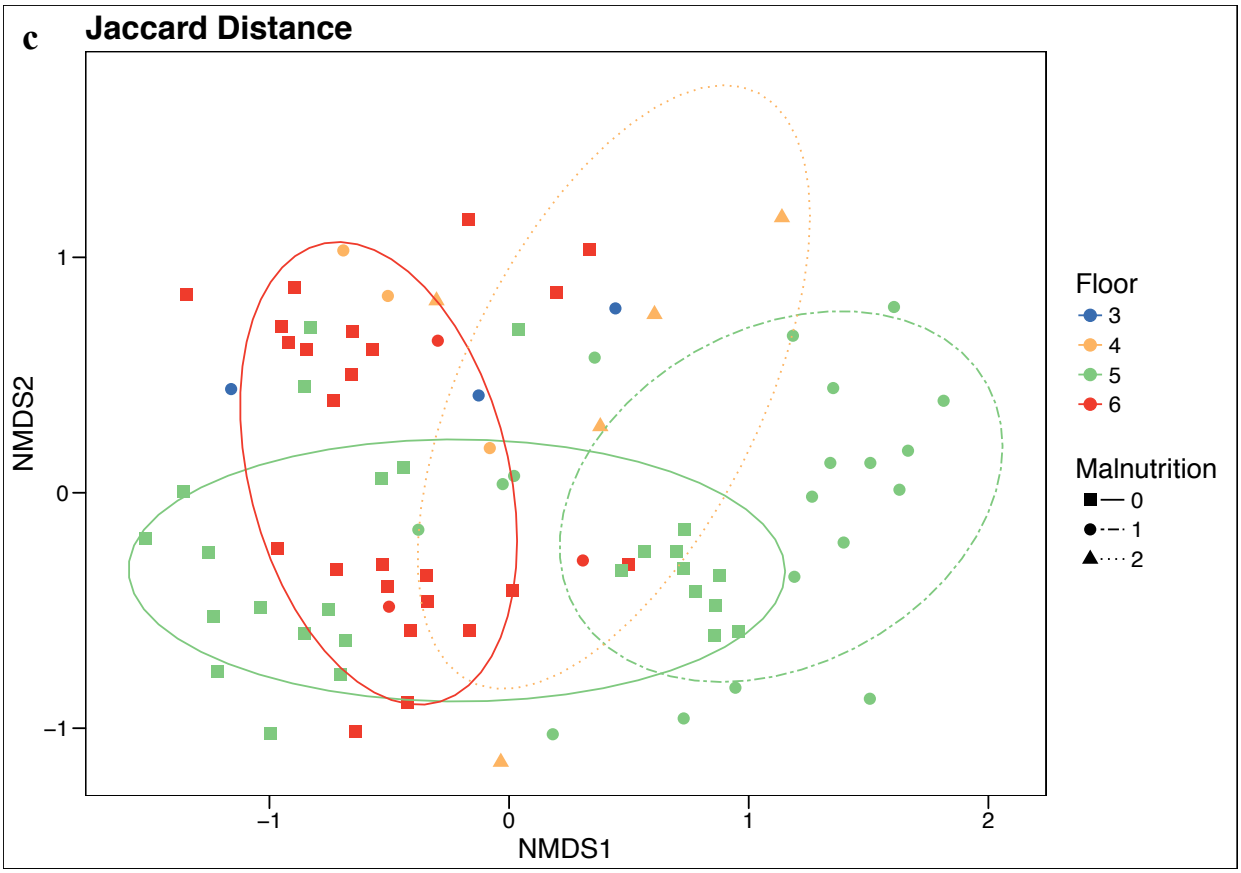

c
Jaccard Distance
NMDS2
NMDS1
1
0
−1
−1
0
1
2
Floor
3
4
5
6
Malnutrition
0
1
2

Figure 2.2 Legend: (a) Binary Jaccard Index principal component analysis by age category and frailty score. The Jaccard Binary Index compares similarity in presence or absence of bacterial species in the microbiome between samples. Residents are categorized into different age groups represented by different symbols where category 1 has residents 65 to 74 years, category 2 is 75 to 84, category 3 is 85 to 94 and category 4 is ≥95 years of age. Frailty scores are each colored differently. (b) Binary Jaccard Index principal component analysis by malnutrition and frailty score. Malnutrition scores are represented by different symbols where frailty scores are each colored differently. (c) Binary Jaccard Index principal component analysis by malnutrition and floor location. Malnutrition scores are represented by different symbols where each floor is colored differently. All the panels display ellipses with confidence interval of 75%. Ellipses are drawn for each combination of the grouping variables (e.g. Frailty-5 & Malnutrition 0). Ellipses cannot be drawn for group with less than 4 samples and are therefore omitted from the plots.

Figure 2.3. Heat map of the relative abundance of each gene type in each individual and hierarchical clustering depicting (A)species and (B) pathways

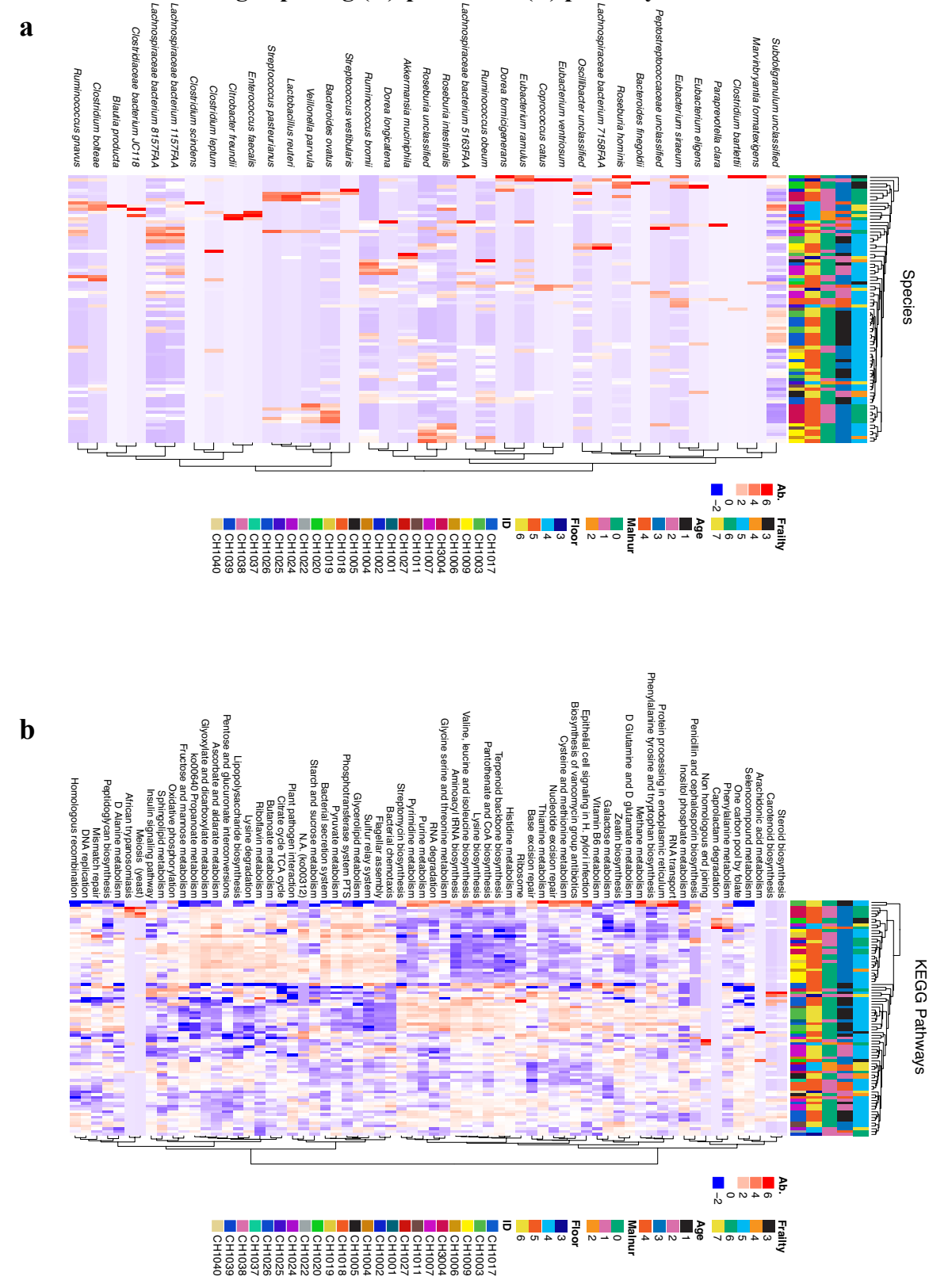


Figure 2.3 Legend: (a) The heatmap depicts the relative abundances of gene sequences assigned to each bacterial genus (y axis) across the 100 samples analyzed (x axis). The heatmap colors represent the relative abundances of the microbial genus assignments within each sample. Square colors shifted towards red to indicate higher abundance. The colored bars across the top of the graph depict the frailty score, age category, malnutrition score, floor location and finally individual resident from top to bottom. (b) This heatmap differs by depicting the relative abundances of gene sequences assigned to each kegg pathway (y axis) across the 100 samples analyzed (x axis).

Figure 2.4. Essential amino acids by age

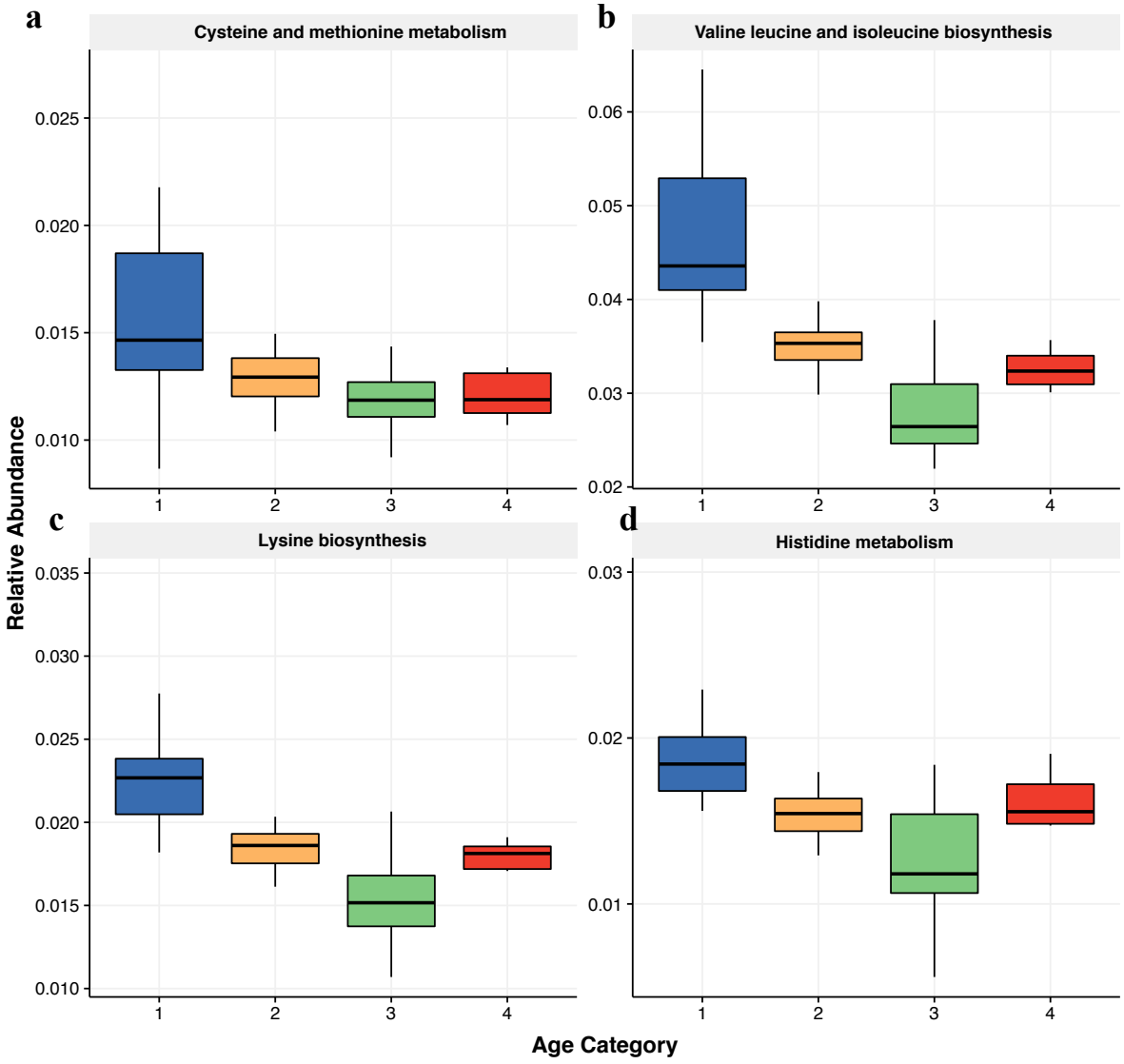


Figure 4 Legend: Relative abundances of essential amino acid pathways by age category: 1) 65 to 74; 2) 75 to 84; 3) 85 to 94; and 4) ≥95 years of age. Data presented as boxplots with the box being the first and third quartiles, the band inside the box is the median, and the wiskers represent the 95th percentiles. Each pathway depicted is as follows: (a), Cystine and methionine metabolism; (b), Valine, leucine and isoleucine biosynthesis; (c), Lysine biosynthesis; and (d), Histidine metabolism.

Figure 2.5. Butyrate-producing bacteria by frailty

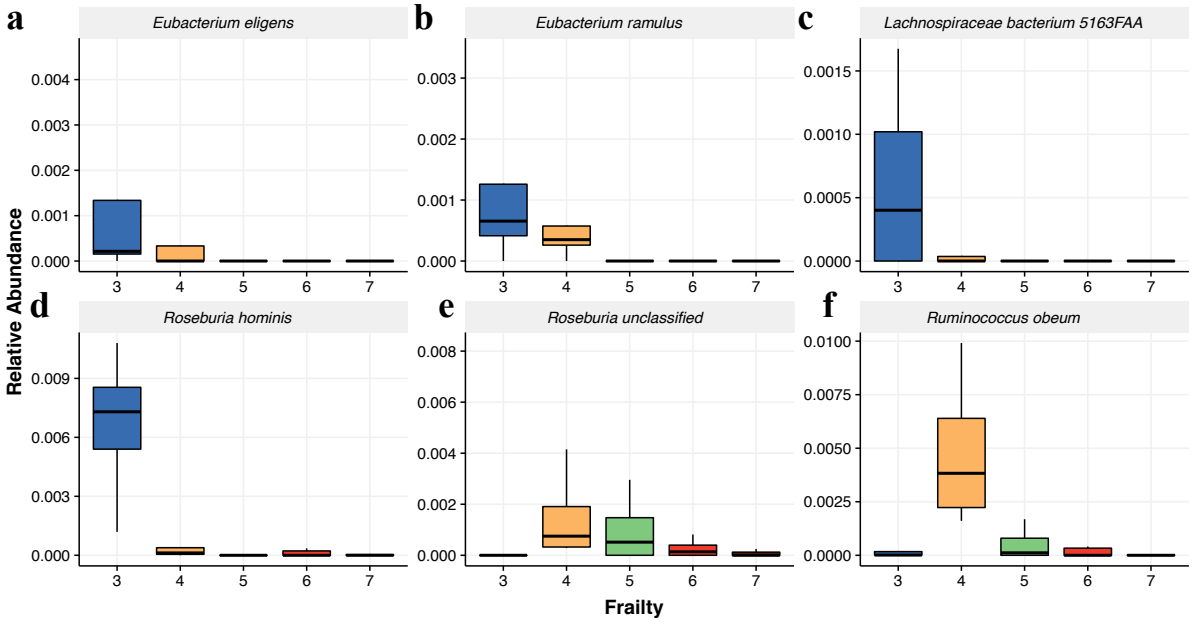


Figure 5 Legend: Relative abundances of butyrate-producing organisms by Clinical Frailty Score. Data presented as boxplots with the box being the first and third quartiles, the band inside the box is the median, and the wiskers represent the 95th percentiles. Each organism depicted is as follows: (a), *Eubacterium eligens*; (b), *Eubacterium ramulus*; (c), *Lachnospiraceae bacterium*; (d), *Roseburia hominis*; (e), *Roseburia unclassified;* (f), *Ruminococcus obeum*

Figure 2.6: Bacterial species by floor

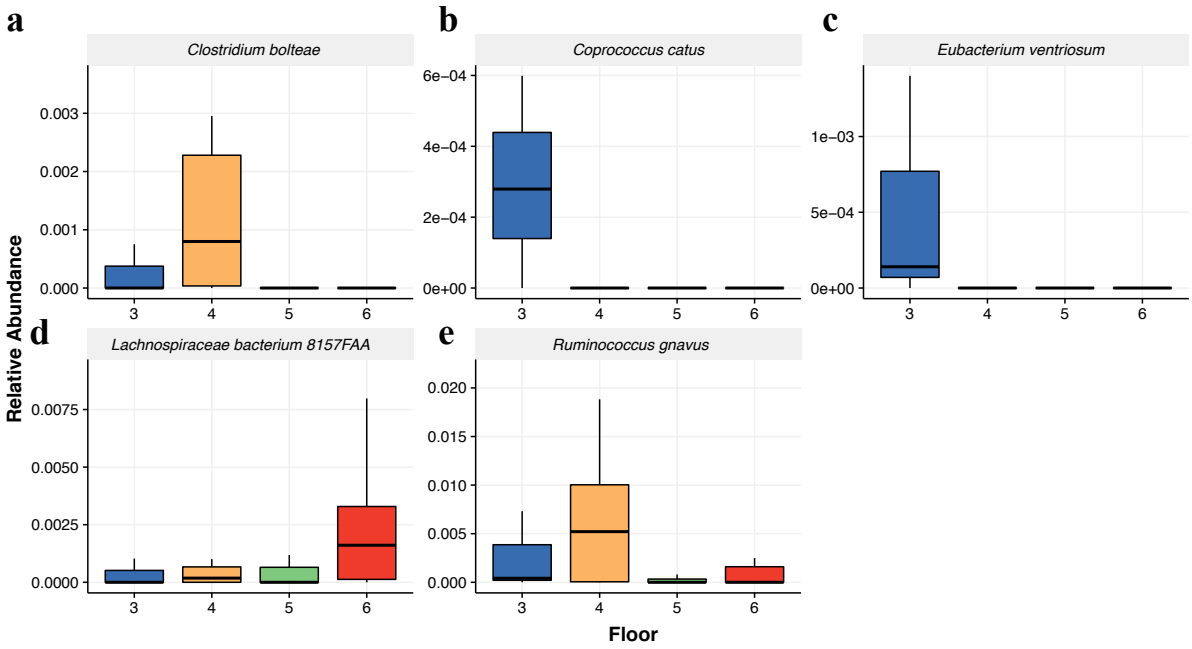


Figure 6 Legend: Relative abundances of organisms by floor in the nursing home. Data presented as boxplots with the box being the first and third quartiles, the band inside the box is the median, and the wiskers represent the 95th percentiles. The dots represent the outliers. Each organism depicted is as follows: (a), *Clostridium bolteae*; (b), *Coprococcus catus*; (c), *Eubacterium ventriosum*; (d), *Lachnospiraceae bacterium*; (e), *Ruminococcus gnavus*.

Figure 2.7. Phylogenetic tree of *Escherichia coli* by individual resident and floor

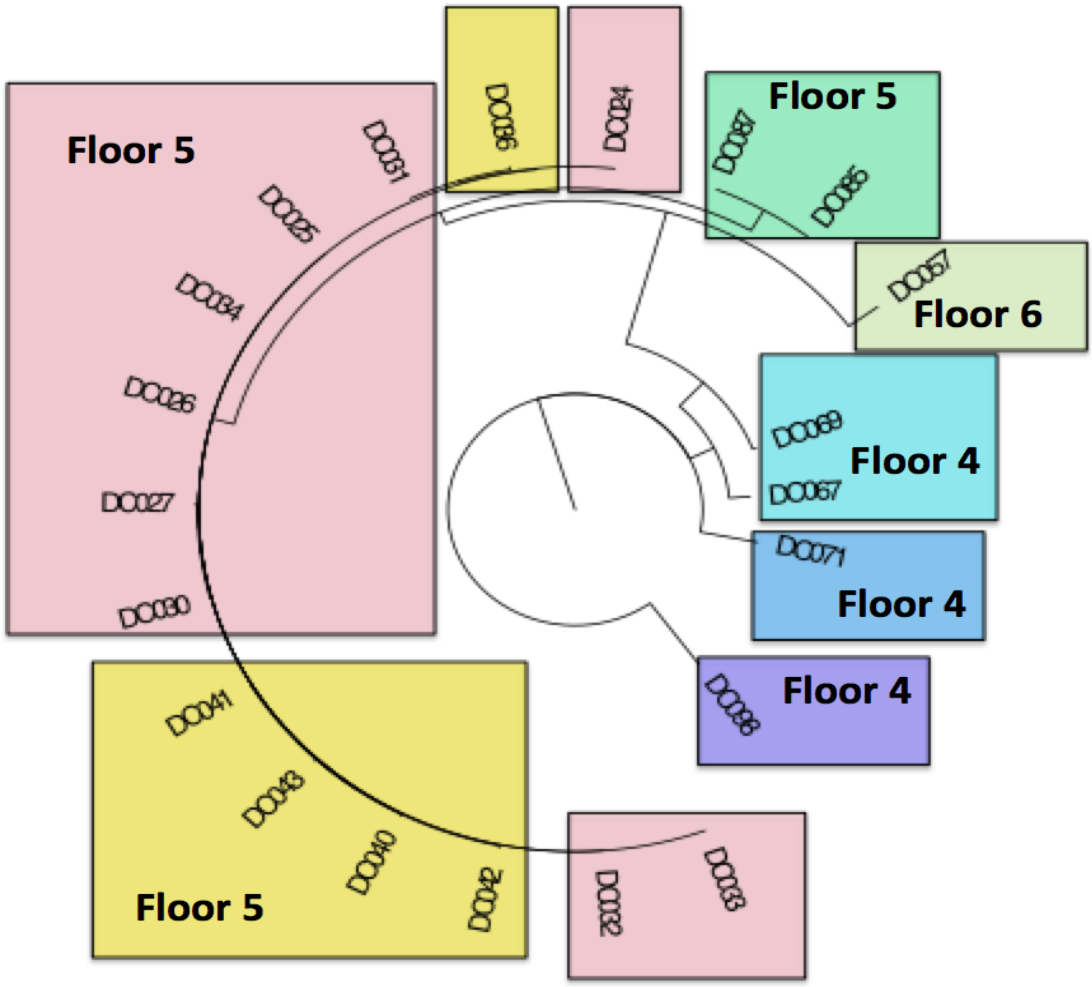


Figure 7 Legend: The phylogenic tree of *Escherichia coli* species from seven of the individual residents for whom sequence data was abundant enough to map. The identification numbers listed represent a unique sample time-point. Each individual resident is color coded and their floor location noted on the graph. The ambulatory residents on the 5th and 6th floors (pink/yellow/green colored) had more similar *Escherichia coli* phylogeny than patients on the 4th floor (blue/purple colored).

CHAPTER 3

Patterns of *Clostridium difficile* Colonization among Nursing Home Elders and the Associations with Clinical Characteristics and Microbiome Composition

ABSTRACT

Background: *Clostridium difficile* disproportionally affects the elderly nursing home (NH) population, and high prevalence rates of *C. difficile* colonization may be to blame. Our objective was to explore the colonization patterns seen in NH elders, over time, to identify clinical and microbiome based factors that are associated with colonization status.

Methodology: We constructed a cohort of NH residents ≥65 years of age that lived in four NH facilities in central Massachusetts from whom we collected stool samples for 4 sequential months. Key clinical variables were extracted from the medical record. *C. difficile* colonization was determined by real-time polymerase-chain reaction detection of Toxin genes tcdA and tcdB. Bacterial composition of resident stool samples was determined by metagenomic sequencing. We performed bivariate analysis followed by logistic regression to identify the association between clinical variables and colonization status. We used random forest machine learning to identify bacterial species that were predictive of colonization.

Results: We enrolled 91 NH elders who contributed 292 clinical samples. Of these, 34 (37.4%) of the residents had one time point in which *C. difficile* was detected and 16 (47.1%) had multiple stool samples where *C. difficile* was detected. Residents with daily acid reducing medication use were 76% less likely to be colonized with *C. difficile* than residents not taking

this type of medication (odds ratio [OR] of 0.24; 95% confidence interval [CI], 0.08 to 0.71). Microbiome composition was similar among elders living together, and the microbiomes of roommates closely approximated each other. Microbiome species with known relationships to *C. difficile* predicted colonization.

Conclusion: *C. difficile* colonization is common among NH elders and is associated with prior antibiotic exposures and inversely related to acid reducing medication use. The microbiome of NH elders is influenced by the environment in which they live and key intestinal bacterial species are predictive of *C. difficile* colonization.

1. INTRODUCTION

The elderly are disproportionally affected by the ongoing *Clostridium difficile* infection (CDI) epidemic.[40, 41] The rate of CDI is several folds higher in persons age 65 year and older [42] with an increase in risk of 2% for each year starting at 65.[43] The rate of CDI is 228/100,000 in those 65 and older compared to 40/100,000 in those aged 45-64 years and 11/100,000 in patients 15-45 years of age. Not only are elders at increased risk of acquiring CDI but they also have higher rates of complications, recurrence, and death.[26] Elders living in nursing homes are now the predominant group suffering from CDI.[40, 44] On average, 40 to 50% of new CDI cases come from elders living in nursing homes.[45, 46] Most NHs in the US have a structured infection prevention and control programs, [167] however environmental measures to control CDI, such as enforcing hand hygiene, contact precautions, and decontamination procedures only after CDI is identified have not been able to stem this CDI concern. [16]

One factor that has become of increasing contemporary interest and a target of preventive strategies is the human microbiome. The microbiome is a vast ecosystem of microbes that influence human health and disease.[52] The intestinal flora changes with age, especially as the presence of anaerobes decreases.[53, 54] Elders from nursing homes differ from community-dwelling counterparts in their microbiome composition with higher proportions of the phylum Bacteroidetes and lower proportions of other bacteria at the family and genus levels.[55] The microbiome of nursing home elders forms dysbiotic patterns with increasing age, frailty and malnutrition.[168] Microbial dysbiosis can be in the form of abnormal function or an association with disease. Connecting these dysbiotic patterns and attempting to correct them may serve as a means to prevent CDI.

Another target for CDI prevention is reducing the amount of *C. difficile* present in the environment. The rates of *C. difficile* present in the stool are the highest in nursing homes, with 20% to 50% of residents affected compared to 1.6% in the general community and 9.5% in the outpatient setting.[53, 58] Carriage of *C. difficile* is a well-documented source of new CDI cases from spread of the bacteria, however approaches to managing carriage as a means to prevent CDI are lacking.[59-61] The risks of *C. difficile* colonization in nursing home elders have been understudied to date.

A better understanding of *C. difficile* colonization in the nursing home and how it is associated with clinical factors and microbiome composition would provide a novel tool into combating the CDI epidemic. Accordingly, we set out to follow longitudinally a cohort of elders from multiple NH facilities to investigate: 1) the patterns and rates of *C. difficile* colonization; 2) the associations of *C. difficile* colonization to medication exposures and other clinical variables; and 3) the microbiome's association with *C. difficile* colonization status. Our findings contribute to the understanding of how the microbiome composition associates with *C. difficile* colonization as a potential target to reduce CDI burden in the elderly.

2. METHODS

2.1. Study Setting and Population

This prospective cohort study was approved by the institutional review board at the University of Massachusetts Medical School. This cohort is of NH residents ≥65 years of age who lived in one of four NH facilities in central Massachusetts. We approached residents who had been living in the facility for ≥1 month and did not have any diarrheal illness or antimicrobial exposure within the preceding 4 weeks. Our trained staff used a standardized Capacity for Informed Consent Instrument [169] that combines capacity assessment questions with observation. If the resident was deemed unable to provide consent, we contacted the healthcare proxy to obtain informed consent. Residents were enrolled for a minimum of 4 months. No patients suffered from dysphagia or had a feeding tube.

2.2. Data Collection

We conducted baseline and end of study medical record abstraction for factors associated with key study variables. Here our outcome was *C. difficile* colonization and our variables of interest included age, frailty, malnutrition, location and medication exposures. We used the medical records as the "gold standard" of information for resident's clinical information. Variables known to be associated with *C. difficile* infection were collected and included: previous hospital exposure, chronic dialysis, steroid or immunosuppressant medication, antibiotic use, and gastric acid suppressant use.[170, 171] Resident Characteristics included age and sex. Other factors we have previously reported on being associated with intestinal microbial dysbiosis included: nutritional status, comorbidities, medications, and frailty [55]. Prior history of hospitalizations, antibiotic exposures in the past year, and history of *C. difficile* infections were extracted from the medical record as well as basic demographic data including age, sex,

race, and length of NH stay. We categorized residents into 4 age categories for analysis: 1) 65 to 74; 2) 75 to 84; 3) 85 to 94; and 4) ≥95 years of age. Frailty was measured in two ways: (1) the validated and widely utilized Canadian Study of Health and Aging's (CSHA) 7-point Clinical Frailty Scale (CFS) [119]; and (2) the Edmonton Frailty Scale (EFS).[172] The CFS is a categorical scaling system containing 9 categories ranging from very fit to terminally ill. Since we were enrolling elders in the nursing home not on hospice, our scale ranged from 3 (managing well) to 8 (very severely frail). The EFS is a continuous scoring system combining general health questions, nutrition, functional performance, and cognition. These scoring systems have been previously validated for demonstrating signatures of frailty in the gut microbiota [120, 121]. We assessed nutritional status using the Mini Nutritional Assessment (MNA) tool [122-124]. Residents were categorized as normal, at risk, or malnourished based on the MNA survey administered by trained research staff to the residents or the nurse caring for the resident if mentally impaired. All residents enrolled were monitored during their involvement in the study for any changes to their care or for new exposures.

2.3. Sample Collection and Processing

We collected 4 monthly stool samples from each resident. DNA was extracted from samples using the PowerMagTM Soil DNA Isolation Kit on an epMotion 5075 TMX liquid handling workstation according to manufacture protocols (MO BIO Laboratories, #27100-4-EP). Sequencing libraries were constructed using the Nextera XT DNA Library Prep Kit (Illumina, Inc., #FC-131-1096) and sequenced on a NextSeq 500 Sequencing System as 2 x 150 base pair-end reads.

2.4. Detection *C. difficile* colonization as outcome

All samples were tested for *C. difficile* toxin genes to determine *C. difficile* colonization. This was done using real-time polymerase-chain reaction with AdvanSure RT-PCR kit (LG Life Science) for the simultaneous detection of tcdA and tcdB genes. The primers target sequences of the tcdA and tcdB genes based on TaqMan technology. We used the SLAN RTPCR detection system (LG Life Science) according to the manufacturer's instructions.[173] Each sample needed to be positive for both tcdA and tcdB genes to then be categorized as positive for *C. difficile*. We categorized residents into two outcome categories. The first being residents that had any stool sample positive for *C. difficile* and the second, residents that had multiple time points in which they had stool samples positive for colonization (multi-colonization).

2.5. Sequence Processing and Analysis

Shotgun metagenomic reads were first trimmed and filtered to remove sequencing adapters and host contamination using Trimmomatic [125] and Bowtie2 [126], respectively, as part of the KneadData pipeline (https://bitbucket.org/biobakery/kneaddata). Reads were then profiled for microbial species abundances using Metaphlan2 [127] and for abundance of Uniref genes, KEGG orthologues, and of the corresponding functional pathways (Metacyc pathways, KEGG pathways, and KEGG modules) using the software pipeline HUMAnN2 [128] and in-house written scripts (available upon request). Normalized taxonomic, gene, and pathway abundances were then used for downstream statistical analysis in R (see below). Strain-level analysis of *Escherichia coli* present in metagenomic samples was performed using StrainPhlAn [129]. Reads were mapped against the MetaPhlan2 clade-specific marker gene database [130]. Reconstructed *E.coli*-specific consensus markers were derived from the mapping data. The reconstructed markers were then used to build a phylogeny of the strains. The tools cited, in turn, depend upon the following tools—for KneadData: Trimmomatic [125], Bowtie2 [131],

SAMtools (https://github.com/samtools/); for StrainPhlan: MetaPhlan2 [130], MUSCLE [132], RAxML [133], blastn [134]. The phylogenetic tree was visualized with FigTree (http://tree.bio.ed.ac.uk/software/figtree/)

2.6. Data Analysis

We performed traditional unsupervised correspondence analysis (NMDS and unsupervised hierarchical clustering) to first determine samples similarity with respect to the above covariates of age, frailty, malnutrition, acid medication use, and physical location. Permutation Multivariate Analysis of Variance (PERMANOVA) was performed to evaluate inter- vs intra-individual variability in bacterial abundance. To determine the contribution of each covariate to changes in microbiome composition (including microbial and functional pathways abundances) we performed linear-mixed effect modeling regression after arcsine square root transformation [81] using the R package *lme4*. P-values were calculated using a Kenward-Roger Degrees of Freedom Approximation and the returned t-value from the regression modeling. Covariates with $p < 0.05$ were retained and used for downstream visualization and data interpretation.

We used multivariable logistic regression analysis to test whether clinical variables alone were associated with *C. difficile* colonization. To select the set of covariates for the multivariable model, we selected any covariates with a $p<0.20$ from our unadjusted bivariate analysis. We ran two models, first with the outcome of any colonization time point and then again with the outcome of multiple colonization time points. We included all acid reducing medications rather than proton pump inhibitors alone in the model given both were significantly associated with the outcome.

Finally we used random forests technique to examine a large ensemble of decision trees of bacterial species composition of the microbiome in predicting *C. difficile* colonization status. Variable importance plot was used with mean decrease accuracy to identify species important in predicting colonization and explored these species relationship (greater or lesser abundances) to colonization.

3. RESULTS

3.1. Characteristics of the Study Subjects

Over a seventeen-month period we enrolled and followed 91 NH elders from 4 different facilities collecting monthly samples, totaling 292 clinical samples. We obtained a complete stool sample collection from all time points among 44 (48.4%) of the residents enrolled. We were unable to obtain complete samples from the remaining residents due to a variety of reasons which are outlined in **Figure 3.1**. Reasons for incomplete sample collection included issues with the facility staff on a given floor, the resident choosing to withdraw from the study, resident death, nursing withdrawal of the resident, or if the resident moved out of the NH. Of note several facilities had delayed enrollment and were not able to get complete sample collection by the time of this study's completion. No residents included in this analysis were exposed to antimicrobials or were hospitalized during the study period. The last antimicrobial exposure occurred three months' prior to enrollment in only one subject. Of the residents enrolled, the average age was 84.4 years (SD 9.5), 19.8% were male, with 25 (27.5%) having been hospitalized and 29 (32.6%) having an antimicrobial exposure in the preceding year.

3.2. *Clostridium difficile* colonization and clinical associations

Of the 296 samples collected 56 (18.9%) were positive for *C. difficile.* Over the course of the study 34 (37.4%) residents had one time point in which *C. difficile* was detected in the stool. Out of these 18 (53.9%) had only one sample positive for *C. difficile* while 16 (47.1%) had multiple time points in which *C. difficile* was detected. The number and percentage of residents colonized by *C. difficile* varied by both the nursing home facility and floor/wing in which the elder lived (**Figure 3.2**). This ranged from 28.0% to 50.0% across the four sites and went as low as 12.5% on one floor to as high as 57.1% on another. Residents exposed to acid reducing

medications were 76% less likely to be colonized with *C. difficile* than residents not taking this type of medication (Odds Ratio [OR] of 0.24; 95% confidence interval [CI], 0.08 to 0.71). We did not see any differences in resident demographics or clinical scores (for both frailty and malnutrition) among colonized and non-colonized residents (**Table 3.1**). Of note, residents colonized with *C. difficile* had a non-statistically significant higher percentage of an antibiotic exposure within the preceding 6 months. The association of acid reducing medication use and colonization did not differ when looking at the second outcome of multi-colonization.

In our multivariable logistic regression, which included clinical covariates with a $p<0.20$, residents taking acid reducing medications had 83% reduced risk of *C. difficile* colonization compared to those not taking this class of medication (**Table 3.2**). Additionally, residents exposed to antibiotics in the preceding 6 months had over 3 times the risk of *C. difficile* colonization than those not exposed (OR 3.42; 95%CI 1.01 – 11.91). Only the significance of acid reducing medication use remained the same when the model was re-run with multi-colonization as the outcome.

3.3. Microbiome similarity among residents within the same facility

We explored beta-diversity by principal coordinate analysis (PCA) using Bray–Curtis Index principal component analysis. The Bray–Curtis dissimilarity is a compositional (abundance) dissimilarity that is bounded between 0 and 1, where 0 means the two sites have the same composition (that is they share all the species), and 1 means the two sites do not share any species. Bray–Curtis distances for a measure of community species dissimilarity among residents is broken down by nursing home site in **Figure 3.3**. We note a pattern of shifting of microbiome composition from each resident towards residents from the same facility. This is not a clear separation but residents from facilities seem to group together with some overlap. For example,

residents from site 4 (purple) cluster towards the middle with site 3 (green) and 2 (orange) around them as you move towards the periphery. Residents from site one (blue) seem to make up the majority of the periphery in 3D space. When we look to elders who were roommates we notice that residents that share the same room during the study period (larger color coded circles) tend to approximate each other (**Figure 3.4**). Microbiota compositional differences were greater between the covariates of both site and floor than within them with a PERMANOVA – Jaccard distance p=0.05 for site and p=0.001 for floor.

We explored whether residents share genetically similar strains of bacteria. Sharing strains between NH residents housed together in the same facility or on the same floor could have implications with respect to transmission of infections. We constructed the phylogenic tree of *Escherichia coli* species from 29 samples that mapped from18 individual residents from all 4 facilities using metagenomic sequence data, **Figure 3.5**. *Escherichia coli* was chosen for strain-level analysis due to it being both a common bacterial species that colonizes the intestines and is a species known to cause disease (i.e., urinary tract infections). The phylogenic tree demonstrates that residents at the same facility had *E. coli* strains sharing more similar phylogenetic relationships than residents living at other facilities. Additionally five of the residents with multiple samples mapping had *E. coli* phylogeny that did not change over time, suggesting they maintained that same strain over time. These data suggest that residents that live together in both the same facility and even on the same floor within that facility had genetically related *E. coli* strains compared to other residents living in a separate floors/facilities that did not intermingle.

3.4. *Clostridium difficile* colonization and the intestinal microbiome

By exploring beta-diversity by principal coordinate analysis (PCA) using Bray–Curtis Index, we were able to investigate if residents that are colonized at any time point or who are

colonized at multiple time points have similar microbiome diversity profiles (**Figure 3.6a. and 3.6b.**). Microbiota compositional differences were greater between the outcomes of *C. difficile* colonization and multi-*C. difficile* colonization than within them with a PERMANOVA – Jaccard distance p=0.002 and p=0.001 for each. First, with regards *C. difficile* colonization, we note a pattern of shifting of composition towards the bottom/base of the plot as the resident is categorized as a colonized resident, **Figure 3.6a**. Furthermore we see clearer delineation when looking at the outcome of multi-colonization, **Figure 3.6b**. In comparison we plotted the beta-diversity for residents on acid reducing medications and noted an inverse relationship (**Figure 3.7.**).

In order to select bacterial species that are predictive of the outcome of *C. difficile* colonization, we used random forests. Random forests technique is a machine leaning methodology for classification of variables by constructing multiple decision trees that predict in this case the outcome of colonization. The variable importance plot orders from greatest to least the bacterial species most predictive of colonization (**Figure 3.8.**). Among the most predictive are several species of the *Bacteroides* genus (**Figure 3.9.)** that have been previously reported in the literature as being associated with *C. difficile* colonization. *Ruminococcus gnavus,* a bacterial species that has been reported as being associated with dysbiotic microbiomes, and CDI was seen in higher abundances in *C. difficile* colonized residents. In addition to the members of the *Bacteroides* genus, *Akkermasnsia muciniphila* was present in lower abundances and *Bacteroidales bacterium ph8* was present in higher abundances in colonized samples. These species have been reported to have an inverse relationship to CDI in the literature (*A. muciniphila* higher in CDI and *B. bacterium* lower in CDI) (**Figure 3.10b-c**). Finally, there are several species identified as important in predicting *C. difficile* colonization that were at lower

abundances in colonized samples that have been reported to exhibit an inhibitory effect against *C. difficile* (**Figure 3.10d-e**).

4. DISCUSSION

Nursing home residents demonstrated high *C. difficile* prevalence in our study with colonized residents having clinical associations with recent antibiotic exposure and an inverse relation to acid reducing medication use (i.e. more non-colonized residents were daily acid reducing medication users). The nursing home environment had significant influences on intestinal microbiome composition with residents in the same facility having similar diversity patterns, roommates approximating the others' microbiome profile, and sharing of E. coli strains among residents at the same NH facilities. Finally, there is an intestinal microbiome composition, with key known species that have reported associations to CDI and colonization in the literature, which we identified as predicting *C. difficile* colonization. This gives a preliminary signature of susceptibility to being colonized with *C. difficile* in nursing home elders.

4.1. *Clostridium difficile* colonization is common among NH elders

Over one-third of residents enrolled in our study were colonized with *C. difficile* at least one point over the 4 months in which we collected samples with roughly half of the colonized residents having multiple samples positive for *C. difficile*. These residents with multiple positive samples represent a group of long-term colonized elders that could serve as reservoirs of *C. difficile* rather than those with only one sample positive, or transiently colonized elders. This ranged as low as 28% at one site to as high as 50% at another. Our findings are consistent with other *C. difficile* colonization studies in nursing homes that demonstrated a range of colonization from 20% to 50% of residents sampled.[53, 58]

4.2. Acid reducing medication are associated with a reduced risk of *Clostridium difficile* colonization

Acid-reducing medications, including both PPIs and H2 blockers classes of medications, have been known for quite some time to be associated with an increased risk of CDI.[107] A recent large systematic review and meta-analysis including 56 studies involving 366,683 patients demonstrated a pooled Odds Ratio of 1.99, CI: 1.73-2.30, $P < 0.001$.[174] This meta-analysis provided further evidence that PPI use is associated with an increased risk for development of CDI. However, whether this is causation or association is still up for debate given that the mechanism by which acid reducing therapy contributes to an increased risk of CDI is still unknown.[29, 108]

Many mechanisms by which acid reducing therapy causes CDI have been proposed. This started with the theory that the vegetative form of *C. difficile* has a better chance of survival when gastric conditions have a pH greater than 4. [175] This theory has recently been refuted by investigations in both mouse models and hospitalized patients where *C. difficile* spores were not affected by the acid gastric pH content. [176] Other mechanisms have been proposed. PPI therapy has been shown to affect the human colonic epithelium by decreasing the expression of human genes holding an important role in mucosal integrity, thus favoring the development of CDI. [177] With increasing attention to the intestinal microbiome and its relationship to CDI, long-term use of PPIs has been shown to decrease microbial diversity, a similar condition found in patients with CDI. [178] Besides diversity, differences among bacterial species composition in the intestines between PPI users and non-users are consistently associated with changes towards a less healthy gut microbiome and are in line with known changes in the microbiome that predispose individuals to CDI.[109, 179]

In our study, we found that residents taking acid reducing medications had a reduced risk of *C. difficile* colonization. There are few studies of *C. difficile* colonization in the elderly,

especially ones involving elders living in nursing homes. The systematic review noted above did not reveal any association with PPI and *C. difficile* in the elderly. [174] Among older hospitalized adults, treatment upon admission with PPIs has not been shown to be associated with *C. difficile* colonization. [180] The use of PPI was also shown not to be associated with *C. difficile* colonization in previous studies with a lower number of subjects, 68 long-term care elders, although a non-statistically significant higher percentage of colonized residents were on a PPI.[58] Our findings suggest that changes in the intestinal microbiome with acid reducing medication use may be associated with a less favorable environment for *C. difficile* colonization.

4.3. Importance of environment in shaping the intestinal microbiota

We are reporting here how the microbiome composition is more similar among residents living at the same facility compared to those at other facilities and how the microbiomes of roommates' microbiomes are similar to each other. We also demonstrated that resident's living together share similar *E. coli* strains by phylogenic tree analysis. Taken together, the location of the resident in the NH was associated with microbiome composition and bacterial phylogeny suggesting that there is sharing of bacteria among residents living together. The influence of environment in shaping the intestinal microbiome in the literature is becoming more apparent. One recent study of over 1,000 healthy individuals demonstrated that there are significant similarities in the compositions of the microbiomes of genetically unrelated individuals who share a household.[181] They go on to state that only about 20% of the inter-person microbiome variability is associated with factors related to diet and medications. Our findings echo these and highlight the importance of grouping vulnerable elders together. It also places an importance on environmental control as a means to control microbiome composition, preventing pathogen spread, and potentially preventing healthcare associated diseases. We are only starting to

understand how the NH environment influences microbiome composition. A key next step would be to manipulate the environment to influence microbiome composition towards one less likely to promote colonization. The exact methods to do this still need to be determined.

4.4. Microbiome composition and *Clostridium difficile* colonization

One of the more frequently cited combinations of microbial bacterial community compositions associated with CDI involve higher abundances of *Peptostreptococcaceae* and *Enterococcus*, with decreased population density of *Bacteroides*.[182] In this study we found that increases in *Bacteroides* species were associated with *C. difficile* colonization. This interesting inverted relationship of higher abundances of bacterial species associating with *C. difficile* colonization but lower abundances associating with infection, especially in the care of the *Bacteroides* species, has been known for some time.[183, 184] *Bacteroides* are abundant commensal members of the human intestinal microbiome. They are involved in key metabolic processes, including carbohydrate fermentation and polysaccharide production, and their ability to modulate surface polysaccharides helps them to evade host immune systems.[185] The mechanisms by which they promote colonization but resist infection are not known.

We also found other species associated with colonization here whose relationship to colonization had previously been reported. The presence of *Firmicutes* species, such as *Ruminococcus gnavus*, in significant quantities is associated with *C. difficile* colonization.[186] This species, however, has been shown to produce a trypsin-dependent antimicrobial substance against *C. difficile.*[187] *Ruminococcus gnavus* is also known for its association with a dysbiotic microbiota.[136] We found higher abundances of several of the *Ruminococcus* species in our *C. difficile* colonized residents.

We also found other bacterial species that are positively associated with CDI in the literature but inversely related to colonization here. For example, we noted that there were lower abundances of *Akkermansia muciniphila* in the *C. difficile* colonized residents where increased abundances of this species has been noted in CDI patients. [182] *Akkermansia* over-representation may reflect enteric mucosa inflammation in CDI with increased mucus production.[121] Another species positively associated with *C. difficile* colonization in our study but also seen in lower abundances in CDI was Bacteroidales. [182] Bacteroidales are known to produce butyrate, a short chain fatty acid that has been shown to promote colonic barrier strength at appropriate concentrations by increasing mucin production, decreasing colonic permeability, and thereby reducing the susceptibility of the colon to infections.[188] Finally species that have known inhibitory effects on *C. difficile* such as *Bacteroides ovatus*, [189] and Lachnospiraceae bacterium, [190] were in lower abundances.

Taken together the species identified here as important in predicting *C. difficile* colonization are known to associate with either a colonized or infected state and some key combination of these groups probably provide a suitable environment for *C. difficile* to take hold and grow without causing disease symptoms (i.e. toxin producing diarrhea). Interestingly, similar to our findings where acid reducing medication use reduced the risk of colonization but is reported in the literature to increase the risk of infection, the associations of specific bacterial species, in either increased or decreased abundances, reported in the literature with CDI we noticed an inverse association with *C. difficile* colonization (i.e. higher abundances in infection lower in colonization).

4.5. Strengths and Limitations

This study had several notable strengths and limitations. One limitation of this study is that it did not have 4 stool samples from each resident. This may have led to misclassification of the secondary outcome of multi-colonization in these residents. This is the largest longitudinal cohort of nursing home elders reporting microbiome composition. It is also the largest study to survey NH residents for *C. difficile* colonization. That being said this study is still limited in the number of residents enrolled. A more robust cohort would help us to take a much deeper look at the multiple levels of data and to better explore other classes of medications used less frequently by NH elders. There are potential confounding variables, specifically classes of medications the residents were taking (such as corticosteroids and immunosuppressants) that were not evaluated in this cohort due to the small number of residents on these drugs. Finally, the physical location microbiome association findings may have been biased by the clustering of residents with similar medical conditions onto the same floor. Following up this investigation with a cohort including larger numbers of residents from more facilities would strengthen the findings and further explore the dysbiosis associations with medication exposure in the elderly and further address how these dysbiotic patterns are associated with *C. difficile* colonization.

4.6. Conclusions

In conclusion, *C. difficile* colonization is common among NH elders with a large portion of these colonized residents harboring this pathogen over the course of months. We found that the NH elderly intestinal microbiome is influenced by the environment in which the elder lives but this did not seem to influence the *C. difficile* colonization state. *C. difficile* colonization state was associated with prior antibiotic exposures and inversely related to acid reducing medication use. Finally we found that the abundances of several key intestinal bacterial species were

associated with *C. difficile* colonization. Further work is needed to see if a microbiome based model could predict *C. difficile* colonization and then if the use of interventions to change the elder microbiome to one that favors colonization resistance could affect high rates of *C. difficile* colonization seen within the nursing home, thus preventing this disease.

5. TABLES AND FIGURES

Figure 3.1. Sample collection flow diagram

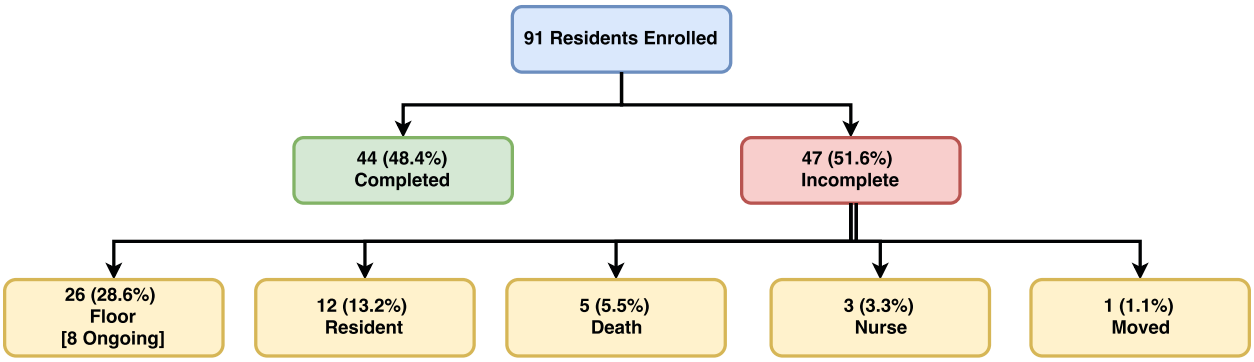


Figure Legend: This flow diagram outlines the reasons for incomplete sample collection. This included: Floor - issues with the facility staff on a given unit; Resident - resident's choosing to withdraw from the study; Death - resident death during study activities; Nurse - nursing withdrawal of the resident due to collection issues; and Moved - resident moved out of the facility. Of note several facilities had delayed enrollment and were not able to get complete sample collection by the time of this study's completion (Ongoing).

Figure 3.2. Breakdown of resident enrollment and colonization outcome by nursing home site

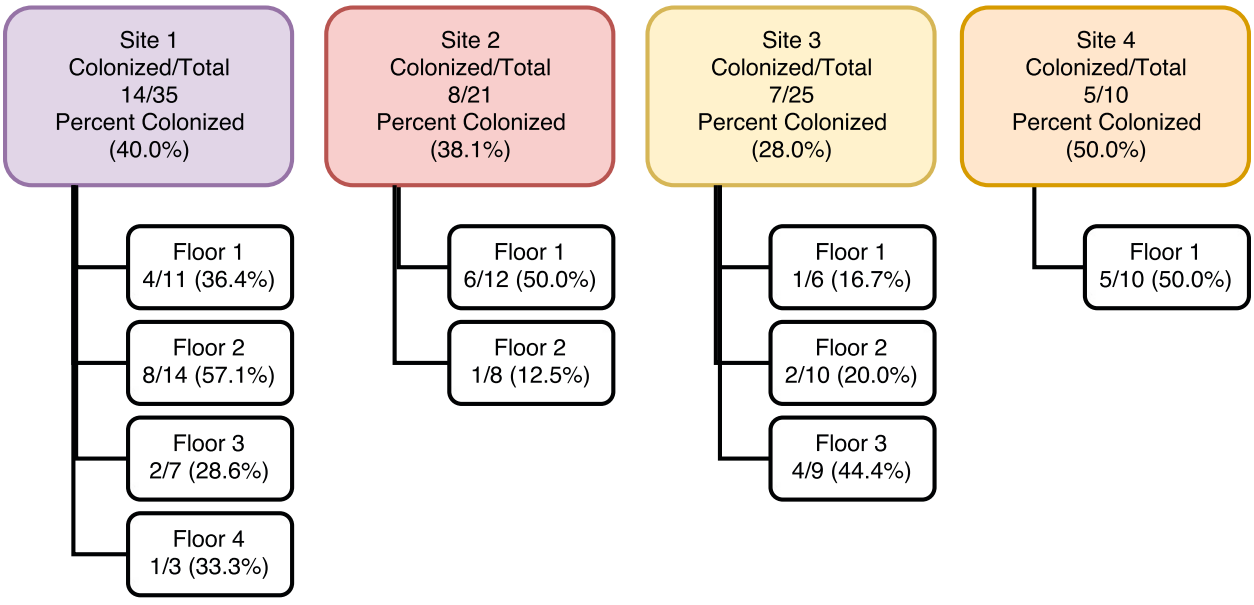


Figure 3.3. Bray-Curtis index principal component analysis of residents by facility site

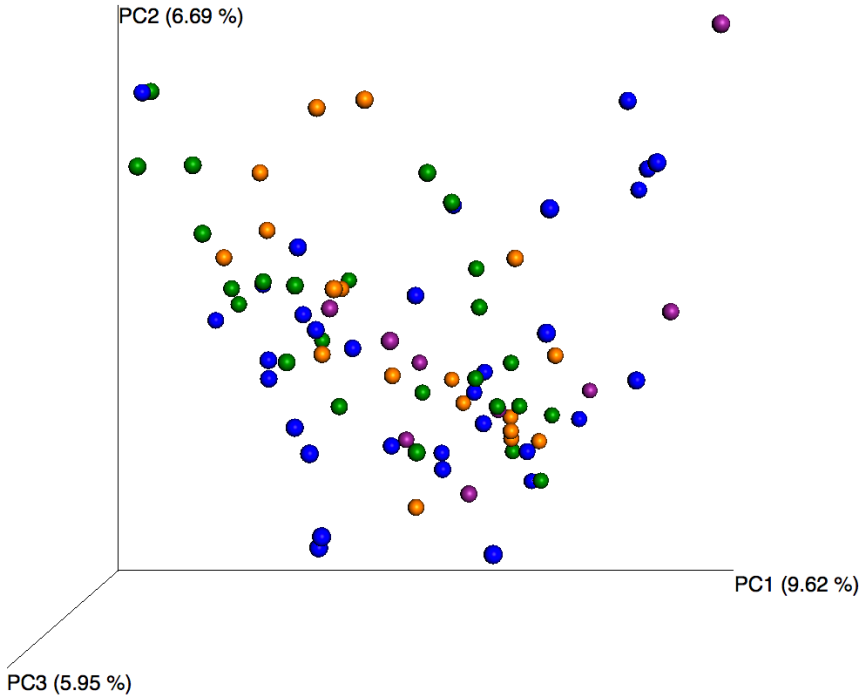


Figure 3.3 Legend: Bray–Curtis Index principal component analysis by facility location. The Bray–Curtis dissimilarity is bounded between 0 and 1, where 0 means the two sites have the same composition (that is they share all the species), and 1 means the two sites do not share any species. Each colored dot represents one of the four nursing home sites

Figure 3.4. Bray-Curtis index principal component analysis of residents linking roommates

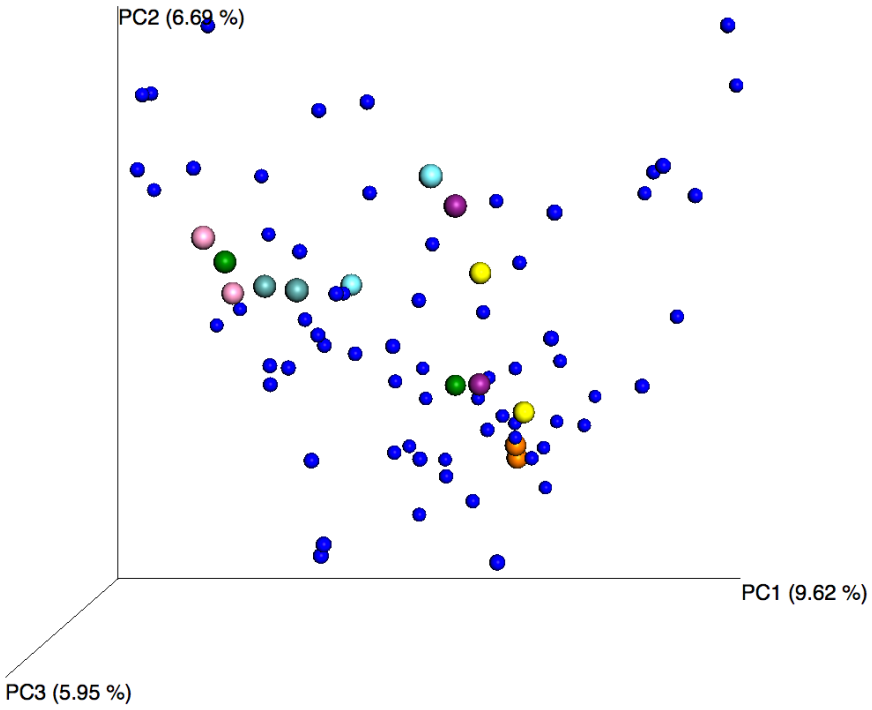


Figure 3.4 Legend: Bray–Curtis Index principal component analysis linking residents that were roommates during the study period. The Bray–Curtis dissimilarity is bounded between 0 and 1, where 0 means the two sites have the same composition (that is they share all the species), and 1 means the two sites do not share any species. Each dark blue dot represents a resident that did not have a roommate that was involved in the study. Each of the colored dots link the seven resident pairs that were giving stool samples during the same time period.

Figure 3.5. The phylogenic tree of *Escherichia coli* species from residents by facility

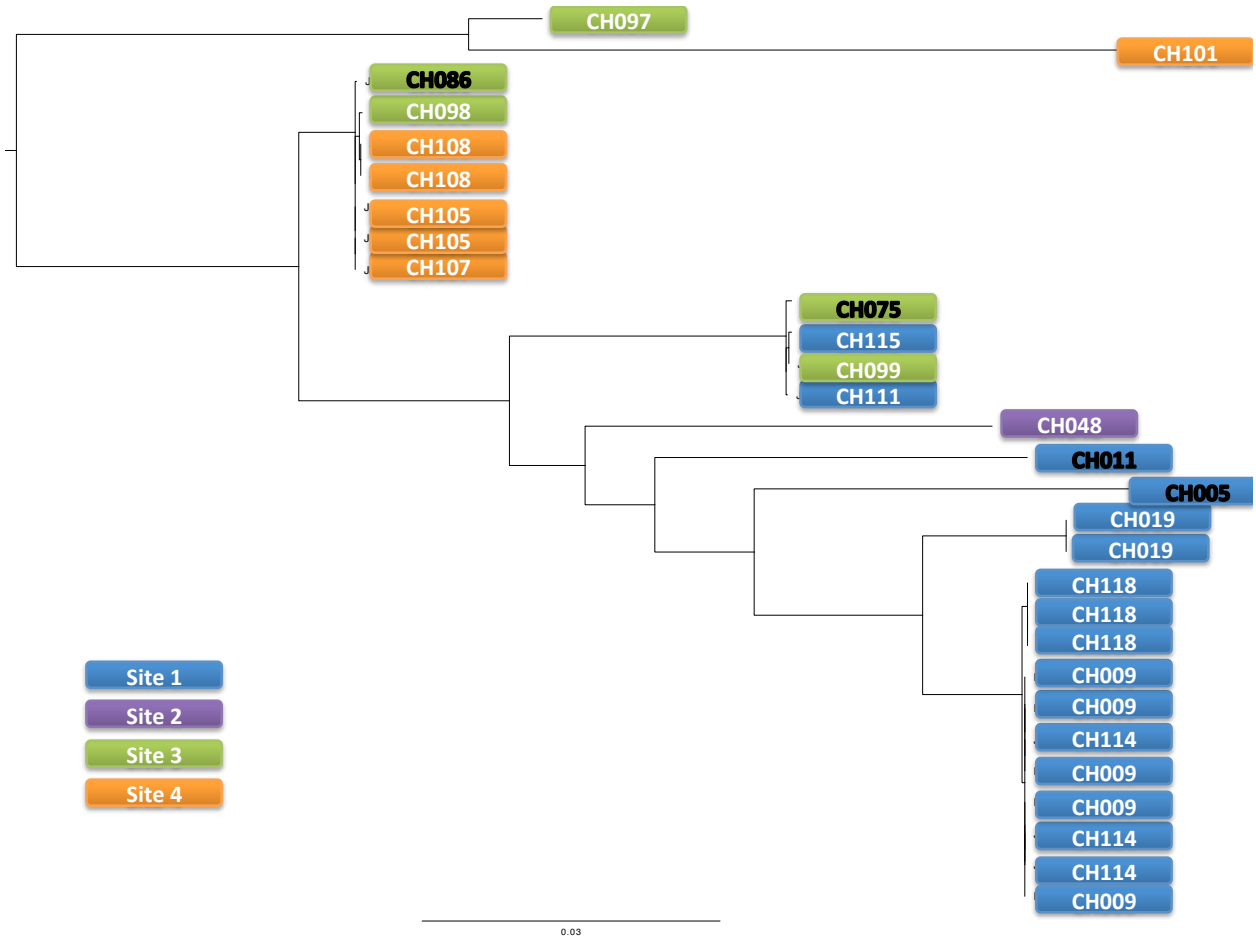


Figure 3.5 Legend: The phylogenic tree of *Escherichia coli* species from eighteen of the individual residents for whom sequence data was abundant enough to map. Each point is a unique sample time-point with identification numbers for each resident. Each sample is color coded for nursing home facility 1-4 (blue/purple/green/orange colored).

Figure 3.6. Bray-Curtis index principal component analysis of residents by *Clostridium difficile* colonization (a) and multi-colonization (b) outcomes

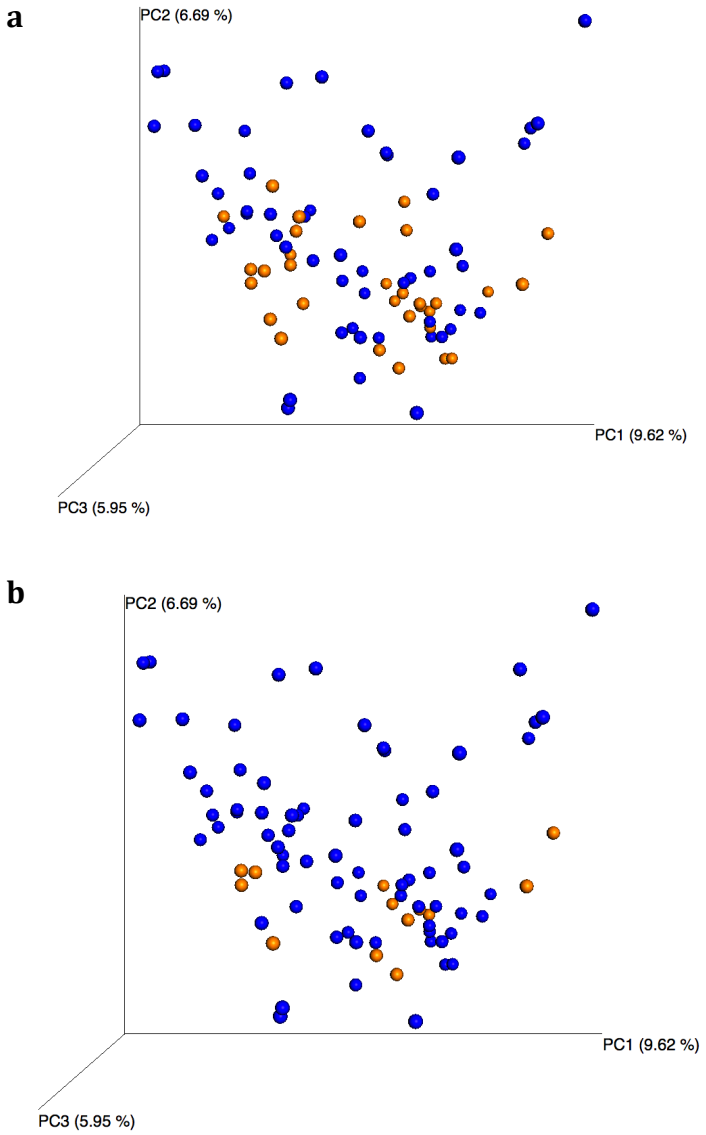


Figure 3.6 Legend: Bray–Curtis Index principal component analysis by *Clostridium difficile* colonization status. The Bray–Curtis dissimilarity is bounded between 0 and 1, where 0 means the two sites have the same composition (that is they share all the species), and 1 means the two sites do not share any species. The orange dots and the residents positive for *Clostridium difficile* colonization and the blue are controls. (a) Depicts the outcome on any sample being positive for *Clostridium difficile* while (b) depicts the outcome of multi-colonization.

Figure 3.7. Bray-Curtis index principal component analysis by acid reducing medication status

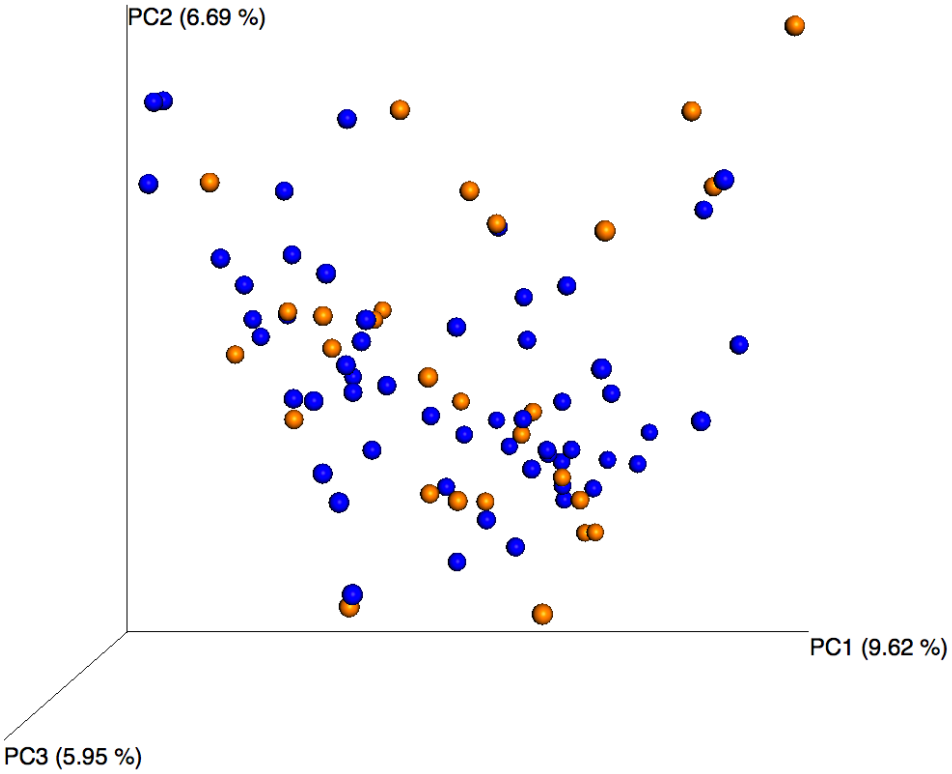


Figure 3.6 Legend: Bray–Curtis Index principal component analysis by acid reducing medication status. The Bray–Curtis dissimilarity is bounded between 0 and 1, where 0 means the two sites have the same composition (that is they share all the species), and 1 means the two sites do not share any species. Each dark blue dot represents a resident that was not taking either a proton pump inhibitor or an H2 blocker during the study period while the orange dots represent those that were.

Figure 3.8. Random forests plot of mean decrease accuracy in predicting Clostridium difficile colonization

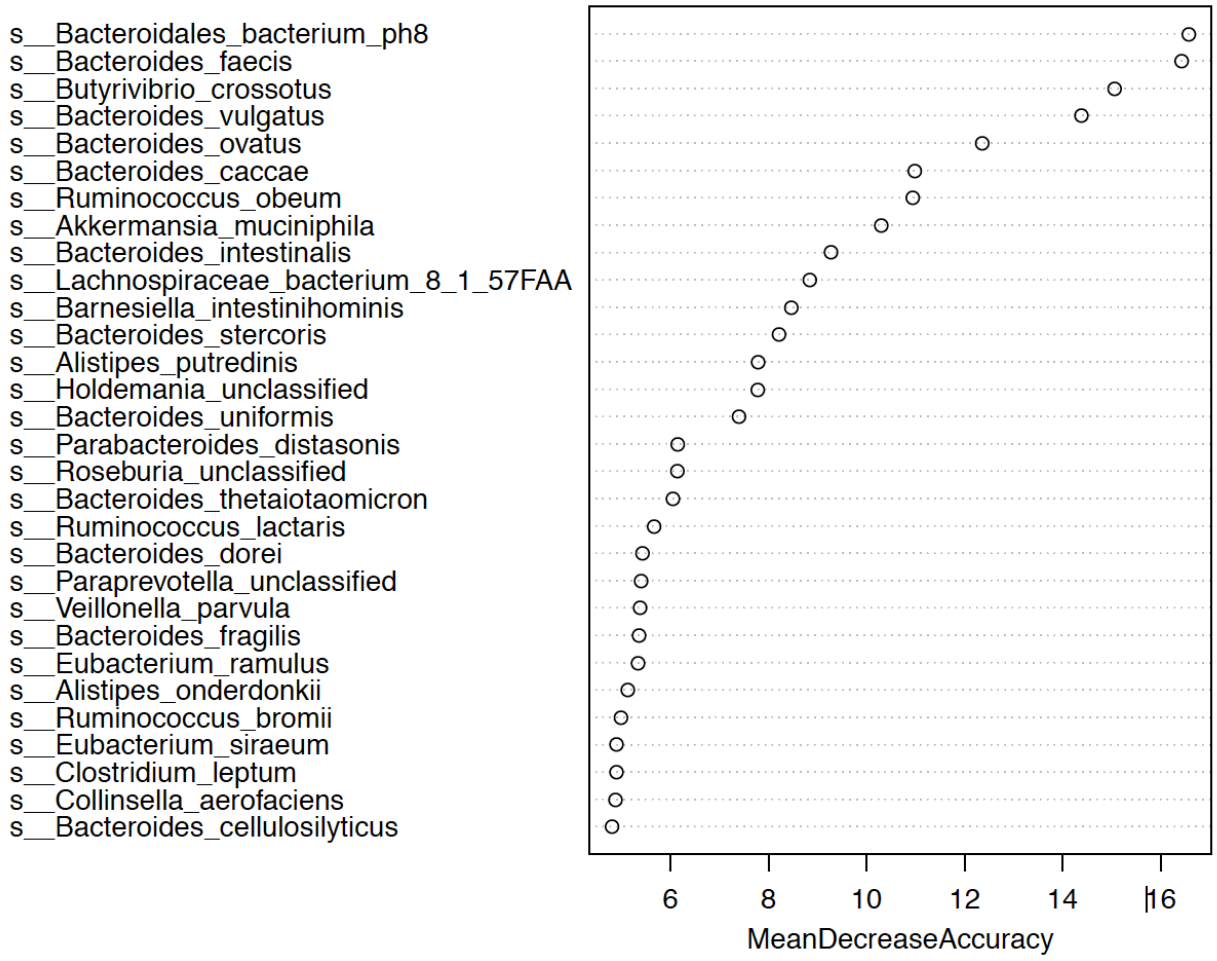


Figure 3.8 Legend: The variable importance plot of mean decrease accuracy ordering bacterial species most important in predicting colonization ordered from most top-to-bottom. The most important variables are at the top of the y-axis and an estimate of their importance is given by the position of the dot on the x-axis.

Figure 3.9. Plots of members of the *Bacteriodes* genus that appear towards the top of the random forests plot

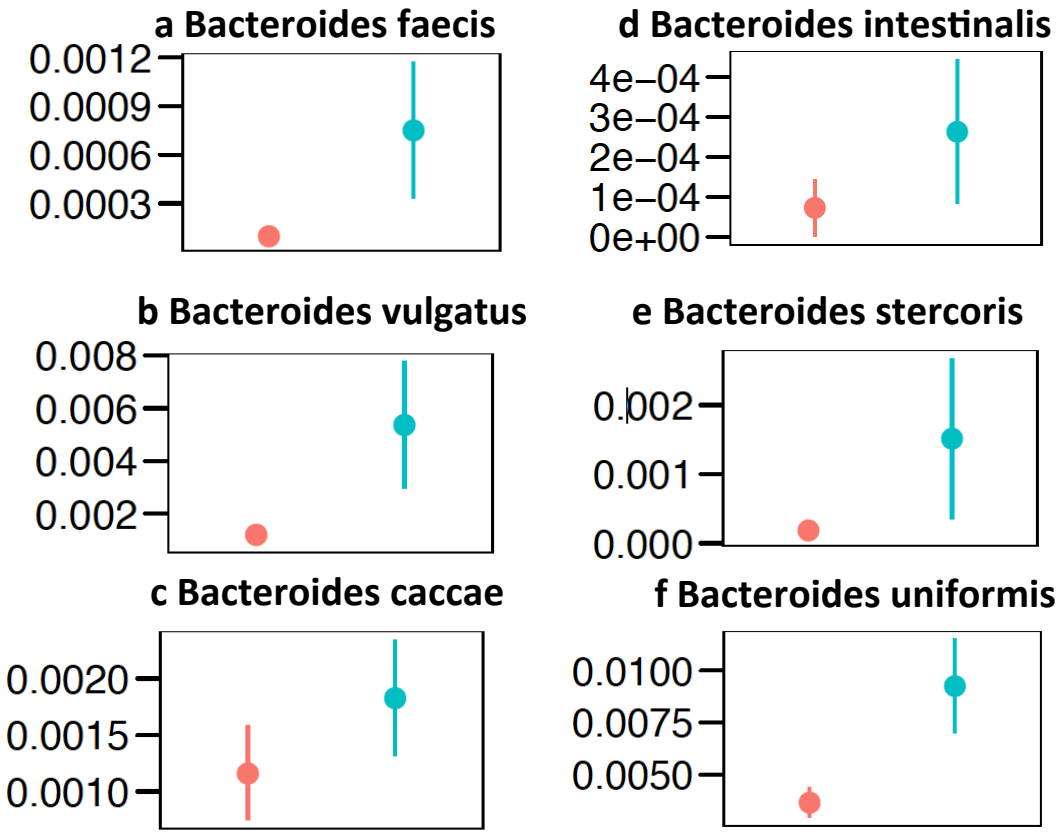


Figure 3.9 Legend: Members of the *Bacteriodes* genus whose increased abundacnes have been associated with Clostridium difficile colonization. Species listed starting with (a) the most important in predicting colonization by random forest to the least (f).

Figure 3.10. Plots of species identifies by the random forests plot as being predictive of Clostridium difficile colonization by either being in higher or lower abundances

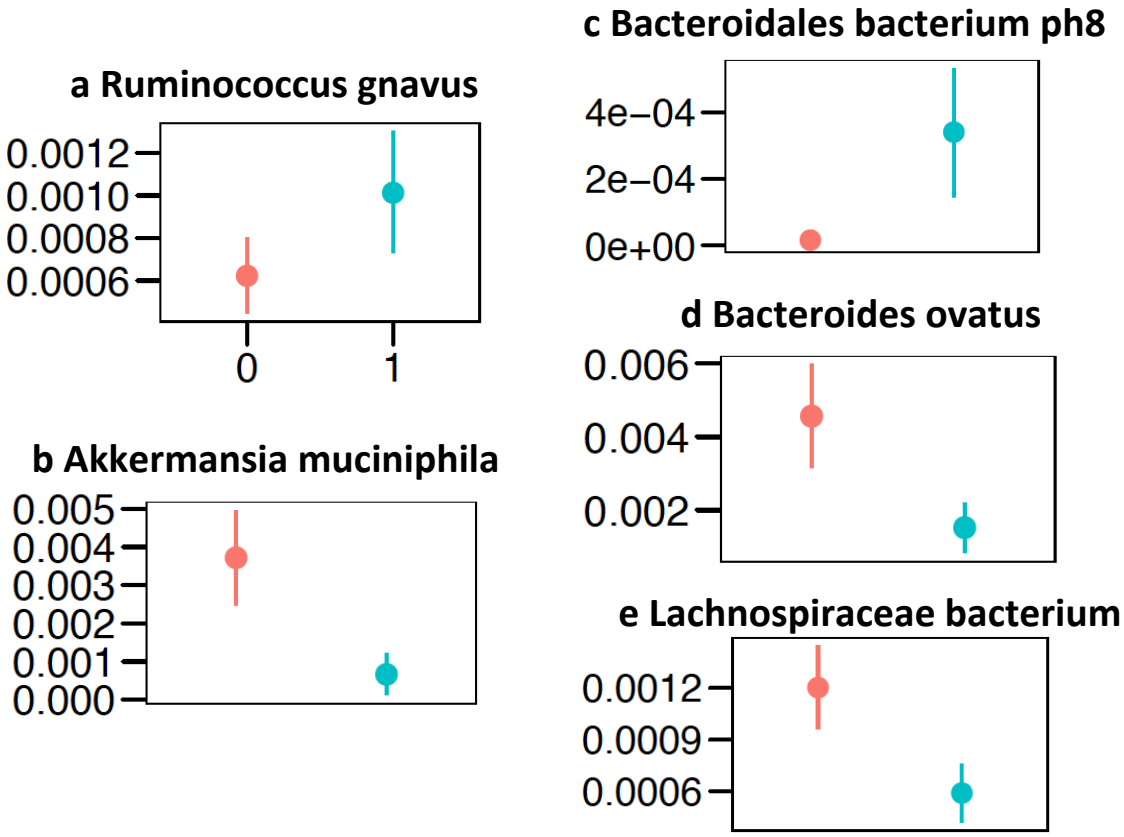


Figure 3.10 Legend: Bacterial species identified by random forests as being predictive of Clostridium difficile colonization by either their increased abundances (a,c) or their decreased abundances (b,d,e).

Table 3.1: Baseline clinical characteristics among residents that were colonized with Clostridium difficile at any time point and colonized at multiple time points compared to those residents that were not colonized

	Not Colonized (n=57)	Colonized (n=34)	Multi-Colonized (n=16)
Demographics			
Age (SD)	84.2 (9.5)	84.7 (9.7)	83.2 (10.5)
Age Category (SD)	2.5 (0.8)	2.6 (0.9)	2.5 (0.8)
Male	14 (24.6)	4 (11.8)	3 (18.8)
CCI (SD)	1.7 (1.6)	1.5 (1.7)	0.7 (0.8)
CCI >=2	30 (52.6)	13 (38.2)	3 (18.8)
Length of Stay (months)	22.9 (23.2)	27.6 (22.8)	18.1 (17.3)
Hospital 1yr	19 (33.3)	6 (17.6)	5 (31.3)
Antibiotics 1yr	16 (28.6)	12 (35.3)	5 (31.3)
Antibiotics 6mo	8 (14.0)	9 (26.5)	5 (31.3)
Clinical Scores			
Frailty CFS (SD)	6.3 (1.0)	6.0 (1.0)	5.9 (1.2)
Frailty Edmond (SD)	7.5 (3.2)	6.7 (3.4)	6.7 (3.9)
Malnutrition Score (SD)	19.7 (5.7)	20.8 (4.3)	20.8 (4.7)
Malnutrition Cat. (SD)	2.0 (0.8)	1.9 (0.7)	1.8 (0.7)
Medication Exposures			
Acid Reducing	24 (42.1)	5 (14.7)*	1 (6.3)*
PPI	18 (31.6)	4 (11.8)*	0 (0.0)*
Steroid	4 (7.0)	2 (5.9)	1 (6.3)
Immunosup	3 (5.3)	1 (2.9)	0 (0.0)

Data expressed as number (percentage) unless otherwise notes as mean (SD); SD, standard deviation; CCI, Charlson comorbidity index; yr, year; mo; months; CFS, Clinical Frailty Score; Cat, category; PPI, proton pump inhibitor; Immunosup, immunosuppressant medication; Polypharm, polypharamcy (5 or more daily medications)
*Denotes p value <0.05

Table 3.2: Factors significantly affecting the risk of *Clostridium difficile* colonization from multivariable logistic regression

	Colonization		Multi-Colonization	
	Odds Ratio	95% Confidence Intervals	Odds Ratio	95% Confidence Intervals
Frailty Edmond	0.79	0.48 – 1.28	0.51	0.24 – 1.01
Acid	0.17*	0.05 – 0.60	0.05*	0.01 – 0.56
Antibiotics 6mo	3.42*	1.01 – 11.91	2.59	0.63 – 10.71
Hospital 1yr	0.44	0.13 – 1.53	2.17	0.54 – 8.66

Acid, acid reducing medication use; yr, year; mo; months;
*Denotes p value <0.05

Conclusions

No group suffers more from *C. difficile* than the elderly, especially those living in nursing homes. Nursing homes represent the perfect storm of a vulnerable group of frail elders living in confined communities. This study has combined different data sources to demonstrate that *C. difficile* colonization and infection is high in the nursing home and that medication exposures are associated differently with *C. difficile* colonization and infection. One reason we believe this occurs is the contributions that the intestinal microbiome makes to either resist colonization or promote it. Microbiome composition is influenced by many factors in the elderly, such as age, frailty, and nutrition. One previously underappreciate factor, which we highlighted for its importance in shaping the bacteria that reside in the microbiome, is the living environment.

The **first chapter** findings highlight differences in the associated risks of rCDI, with antibiotic and acid-reducing medication use, after initial CDI treatment that vary depending on the home environment. One interesting finding was our discovery that corticosteroid exposure was associated with a reduction in the risk of rCDI in community-dwelling elders however is associated with an increased risk in nursing home elders. This finding mirrors the reported role of corticosteroids where it has been shown to both increase and decrease the risk of incidence of and mortality from CDI. [36-39] Corticosteroids have been shown to significantly alter the microbiome of the intestine. [111] This might lead to predisposing a patient to rCDI in the form of reinfection with a different strain rather than recurrence of the same strain. Our findings of the associated increased risk of rCDI with corticosteroid exposure in NH residents does need further exploration, however from a clinical standpoint, avoiding corticosteroid use in NH elders after CDI treatment could possibly reduce the risk of rCDI. With regards to acid reducing medications

and antibiotics, in the nursing home, the risk of rCDI was increased with exposure to acid-reducing medication but not antibiotics. In the community, antibiotic exposure within the first 6 months after treatment for CDI was associated with an increased risk of rCDI along with acid-reducing medication exposure. Our findings add to the literature by highlighting the possible importance of the patient's home environment when it comes to medication exposure and associated risks of rCDI.

In the **second chapter** we demonstrated different dysbiotic associations with increasing age, frailty, and malnutrition scores. As the age of residents increased, the abundance of microbiota-encoded genes and pathways related to essential amino acid, nitrogenous base, and vitamin B production declined. With increasing frailty, residents had lower abundances of butyrate-producing organisms, higher abundances of known dysbiotic species, higher LPS and PGN biosynthesis, and higher sphingolipid metabolism. Among residents who were at risk of or were malnourished, butyrate producing organisms declined and opportunistic and dysbiotic bacterial species increased along with PGN biosynthesis. Interestingly, when looking at physical location within the nursing home, residents living together shared similar microbiomes and had similar *E. coli* phylogeny shared between them. This highlights the different dysbiotic patterns that emerge when looking at key elder factors that influence elder health in the form of advancing age, frailty, and malnutrition. We concluded that the dysbiosis of the NH elderly gut microbiome not only differs with increasing age, frailty, and nutrition, but also physical location within the NH. Physical location might play a vital role in shaping the intestinal microbiome which has implications for housing frail elders together and reducing pathogenic bacterial spread.

In the **third and final chapter,** we go on to demonstrate that *C. difficile* colonization is common among NH elders from different facilities, is associated with prior antibiotic exposures, and inversely related to acid reducing medication use. We demonstrated that the microbiome of NH elders is influenced by the environment in which they live which mirrors recent reports in the literature where upwards of about 20% of the inter-person variability of the microbiome may be attributable to living environment. [181] Finally, we identified key intestinal bacterial species that are predictive of *C. difficile* colonization. The species identified here as important in predicting *C. difficile* colonization have been previously reported on as being well known to be associated with either a *C. difficile* colonized or infected state. We believe that some key combination of these groups probably provide a suitable environment for *C. difficile* to take hold and grow without causing symptomatic disease. Future work in this area would be to better define and then validate a microbiome mixture, either supportive or resistant to *C. difficile* colonization, which can then predict colonization risk. This would then inform a rational study design to intervene to change the microbiome from a dysbiotic one supporting colonization to one that can resist colonization.

This investigation also leaves many unanswered and intriguing questions. Why did corticosteroids lead to an increase in recurrence among only NH elders? Why are acid reducing medications associated with decreased risk of colonization? Why are bacterial species known to be associated with CDI in the literature shown here to have an inverse relationship to *C. difficile* colonization? Our belief is that the microbiome composition plays an integral role in balancing: 1) if *C. difficile* can even get established to colonize the gut; and 2) if *C. difficile* that has colonized the gut then starts to overgrow, produce toxin, and lead to symptoms. The medications

mentioned above probably play a role in disrupting the microbiome which has different effects with regards to colonization and infection (and differ if the elder lives in a NH or in the community). The best way to further investigate these associations and prove causation lies in first using in vivo or in vitro models to define mechanisms and second expanding this NH cohort with larger numbers and longer observation times to capture elders in the NH that then have incidental exposures to these medications and others that go on to develop either *C. difficile* colonization or infection.

As we continue to form a better understanding of the intestinal dysbiosis that occurs among nursing home elders and how this dysbiosis can be linked to *C. difficile* disease, we will be able to generate insights into novel infection prevention strategies using the aging microbiome to prevent *C. difficile* from taking hold in this population; a population that is key to combating this deadly disease epidemic.

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