Characterization of the microbiome in a cohort of U.S. Veterans; implications for general and physical health, insomnia, and mental health

By

Christopher E. Stamper

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This thesis entitled:

Characterization of the microbiome in a cohort of U.S. Veterans; implications for general and physical health, insomnia, and mental health

written by Christopher E. Stamper

has been approved by the Department of Integrative Physiology

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Noah Fierer, Ph.D.

_____

Matthew B. McQueen, Sc.D.

_____

Pei-San Tsai, Ph.D.

_____

Christopher A. Lowry, Ph.D.

Date _____

The final copy of this thesis has been examined by the signatories, and we find that both the content and the form meet acceptable presentation standards of scholarly work in the above-mentioned discipline.

Abstract

Stamper, Christopher Edward (Ph.D., Integrative Physiology)

Characterization of the microbiome in a cohort of U.S. Veterans; implications for general and physical health, insomnia, and mental health

Dissertation directed by Associate Professor Christopher A. Lowry

The human microbiota is a term that is used to describe the microorganisms (archaea, bacteria, eukaryotes, and viruses) within the human body. The past decade has been characterized by a number of landmark surveys of the human microbiome such as The Human Microbiome Project. The main objective of these surveys was to generate a point of reference and large databases of comparators. Nevertheless, in-depth analyses of the human microbiome in association with validated measures of human general, physical, and mental health are lacking, especially the latter.

The main goal of this dissertation was to assess the skin, oral, and fecal microbiomes in association with validated measures of general health, physical health, insomnia, and mental health in a cohort of U.S. Veterans. We sought to study this select group of individuals with unique environmental exposures and health outcomes, to determine if there were identifiable microbial signatures associated with specific health measures. We collected microbiome samples from three anatomical sites (skin, oral, and fecal) from 188 U.S. Veterans. From our results, we detail the general characteristics of the sample types (skin, oral and fecal) as they relate to each other and as they relate to gender, race, and age. We then characterized the microbiome of all three sample types (skin, oral and fecal) as they relate to metadata collected in three main categories: general and physical health, insomnia, and mental health.

Overall, our results comparing the microbiome at the three anatomical sites with respect to gender, race, and age were consistent with previous studies of different cohorts with different demographics. Measures of general and physical health displayed similar relationships to the fecal microbiome; specifically, we observed increased alpha diversity of the fecal microbiome in association with "healthy" states. We found that severe insomnia symptoms and several measures of mental health were associated with microbial feature from the various sampling sites. Further analyses are needed to understand the biological basis of the associations between the skin, oral, and fecal microbiomes and measures of physical health, insomnia, mental health, and if microbiome-based interventions can be developed in order to improve these health outcomes.

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First and foremost, from the bottom of my heart, I must acknowledge and thank Dr. Christopher Lowry. Dr. Lowry and I have worked and grown together for ten years. I started in his lab as an ambitious undergraduate, while his lab was just a bunch of boxes. Since then we have published nine manuscripts, moved labs, and changed the whole scope of the Lowry lab's research program. The dissertation is a token of the last accomplishment. Dr. Lowry has provided unwavering financial, academic, professional, and personal support and mentorship for me. When I knocked on his office door and told him that I would like to change my dissertation focus from brain neurocircuitry to the microbiome, which we knew nothing about, he paused and said "OK". From there it has been an absolute whirlwind and a tremendous accomplishment that you are reading this dissertation. None of it would have been possible without Dr. Lowry's support and connections. Dr. Lowry, I see you as a second father and want you to know how much I appreciate everything that you have done for me.

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samples and provided me with all the information needed to build the capacity to process samples autonomously. This project has made the Fierer lab a great ally of the Lowry lab.

This project clearly would have never been possible without Lisa Brenner and Kelly Stearns-Yoder at the VA. Lisa is a true leader and pioneer at the VA in their effort to incorporate the microbiome into their healthcare initiatives. Lisa, Kelly, and their team secured the funding, recruited subjects, collected the samples, and collected metadata for this project. They provided an unprecedented amount of metadata for this project that have allowed us to analyze data that have never been related to the microbiome previously. We anticipate many high impact publications to be produced from the analyses done in this dissertation.

I personally would still be in the discipline of brain neurocircuitry if it were not for Andy Hoisington. I consider him my second mentor and without him advocating and making personal sacrifices for me to attend conferences and workshops, I would never have known the field of microbiology. Thank you Andy. I am truly grateful for everything that you have done for me.

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List of Abbreviations

BDI	Beck Depression Inventory
BMI	body-mass index
bp	base pair
CDC	Centers for Disease Control
COMIRB	Colorado Multiple Institutional Review Board
DNA	deoxyribonucleic acid
DSM-5	Diagnostic and Statistical Manual of Mental Disorders 5th Edition
ECHCS	Eastern Colorado Health Care System
EMR	electronic medical records
GI	gastrointestinal
HBV	hepatitis B virus
HCV	hepatitis C virus
HIPPA	Health Insurance Portability and Accountability Act
HIV	human immunodeficiency virus
HMP	Human Microbiome Project
HOME	Housing, Occupancy, Materials, and Environment
ISI	Insomnia Severity Index
MCS	Mental Component Summary
MDD	major depressive disorder
MDS	multidimensional scaling
MEQ	Morningness/ Eveningness Questionnaire
MIRECC	Mental Illness Research, Education, and Clinical Centers
MVM-CORE	Military and Veterans Microbiome Consortium for Research and Education
NHIS	National Health Interview Survey
NIH	National Institutes of Health
NSI	Neurobehavioral Symptom Inventory
OQ-45	45-Item Outcome Questionnaire
OSU-TBI-ID	Ohio State University Traumatic Brain Injury Identification
OTU	Operational Taxonomic Unit
PCL-5	PTSD Checklist for DSM-5
PCoA	principle coordinate analysis
PCR	polymerase chain reaction
PCS	Physical Component Summary
PERMANOVA	permutational analysis of variance
PHQ-9	Patient Health Questionaire-9
PTSD	Posttraumatic stress disorder
QIIME	Quantitative Insights Into Microbial Ecology

RA	relative abundance
RDP	Ribosomal Database Project
rRNA	ribosomal ribonucleic acid
SAD	seasonal affective disorder
SCID-5	Structured Clinical Interview for the DSM-5
SD	standard deviation
SES	socioeconomic status
SF-36	36-Item Short Form Health Survey
SPAQ	Seasonality Pattern Assessment Questionnaire
TBI	traumatic brain injury
UniFrac	unique fraction
V3	Version 3
VA	Veterans Affairs
VHA	Veterans Health Administration

Chapter 1. Introduction

The relationship between humans and microbes has been long and complicated with a rich history that included some of science's greatest historical figures and chance happenings. We have coevolved with our microscopic counterparts since the beginning of time and we are coming to understand that this relationship is mainly symbiotic. Humans serve as the host while we are still discovering all the benefits that our commensals provide to us. Arguably one of the most overlooked benefits of our relationship with microbes is the endosymbiotic hypothesis of mitochondria [1]. Although it is still being debated, the organelle that is the power house of every cell in the body most likely has a prokaryotic lineage that at some point in evolution was captured and incorporated into our eukaryotic existence.

Bacteria were first discovered by Antonie van Leewenhoek in 1683. Leewenhoek was a Dutch business man who owned a draper shop and became the "father of microbiology" because of a desire to observe the fiber quality of his drapes at a level of resolution that was beyond what was available at the time.[2] He began to master the art of glass lens fabrication and, through diligence, he developed one of the first microscopes. His obsession with perfecting the lenses culminated in making his microscopes capable of a magnification power of 270x.[3] His natural curiosity compelled him to turn the objective on his microscopes toward specimens other than his drapes, including pond water where he was the first to observe and document what he called "animalcules".[4] These "animalcules" later became known to be bacteria and protists and his work in the development of microscopes ultimately founded the field of microbiology.

In the late 19th century, the work of Louis Pasteur and Robert Koch determined that bacteria and other microorganisms were causing many diseases. Pasteur first demonstrated that microorganisms were in the air and played a role in spoilage.[5] He showed that by preventing air from contacting a nutrient-rich medium such as broth sanitized by boiling, one could prevent the

spoilage of the broth. However, if air was allowed to contact the broth, it would ferment and spoil. Koch added to the findings of Pasteur with his work with pathogens cultured from diseased animals and showed that they have the ability to harm a host. From these experiments, Koch was responsible for creating the first set of scientifically-based criteria to determine if a microorganism was responsible for causing a disease. The criteria for identification of microbial agents with virulence factors were deemed Koch's Postulates and were[6]:

1. The microorganism must be found in abundance in all organisms suffering from the disease, but should not be found in healthy organisms.
2. The microorganism must be isolated from a diseased organism and grown in pure culture.
3. The cultured microorganism should cause disease when introduced into a healthy organism.
4. The microorganism must be re-isolated from the inoculated diseased experimental host and identified as being identical to the original specific causative agent.

The work of Pasteur and Koch confirmed the "germ theory of disease."[7] This confirmation was an extraordinary conceptual leap from what had previously been proposed by Girolamo Fracastoro in 1546 that "clothes, linens, etc., which although not themselves corrupt, can nevertheless foster the essential seeds of the contagion and thus cause infection" to showing that very specific microscopic organisms were causing disease.[8] This leap was the key that allowed medicine to pass into a semi-modern realm. Furthermore, Koch's Postulates have withstood the test of time and are the current scientific theory of disease.[9]

The confirmation of the "germ theory of disease" was a scientific discovery that shifted the mind set and approach taken towards microbes by the medical field. During that time in

history, the findings of Koch and Pasteur sparked the initiation of much needed changes in medical practice to actually begin to comply with the Hippocratic Oath, "above all, do no harm."[10] In adapting aseptic techniques that were backed by scientific experiments, medicine began to come out of the dark ages and set the stage for transforming our relationship with the now perceived harmful microbes. However, steps toward attempting to totally eradicate these microbes from our existence were not put into action until Alexander Fleming's accidental happening upon benzylpenicillin in 1928.[11]

As the story goes, Fleming was a brilliant researcher, but did not keep the most sanitary lab. He was characterizing *Staphylococcus* and went on holiday for a month and upon his return he found that some of the cultures of *Staphylococcus* had grown fungi. However, instead of tossing them out, he examined them and noticed that there were no colonies of *Staphylococcus* in close proximity to the fungi, while colonies remained present further from the fungi. He concluded that the fungi were producing a substance that he called "mould juice" with a property of being a "bacteria killer."[12] Fleming cultured the fungi and found it to be *Penicillium*, a genus of Ascomycetous fungi, and changed the name of the antibacterial "mould juice" to penicillin in 1929. This was a major breakthrough in medicine and Sir Fleming was knighted and awarded the Nobel Prize in Physiology and Medicine in 1945 for his discovery. Other researchers, namely Ernst Boris Chain and Howard Florey, WWII, and funding from the US and British governments were the main driving forces for the mass production of penicillin and the development of antibiotics as we known them today. These developments changed the world of medicine and provided the medical community with a tool to effectively combat bacterial infection and inadvertently began an era of sanitization.[13]

The advent of antibiotics may have led medical professionals to believe that a large chunk of what was causing disease and illness in humans was solved. And for the most part this was true; humans were able to treat acute illnesses and infections caused by bacteria relatively effectively with antibiotics and antiseptic practices. However, fast forward to today and the story becomes a bit more complicated. Westernized societies, the societies with the "best" modern medical practices and sanitary guidelines, have been on a campaign against microbes for decades and are now experiencing rapid and unexplained rises in chronic inflammatory diseases.[14] These diseases span almost all the body systems and include atopic sensitization,[15–17] food allergies,[18] autoimmune diseases,[19] and inflammatory bowel disorders.[19] Researchers and medical practitioners are beginning to question the approach of eradicating and dramatically reducing the exposure to microorganisms that we have coevolved with for thousands of years. It has only recently been recognized that these "old friends" may have immunoregulatory properties that are necessary to keep our immune system in check. Evidence suggests that, if these interactions are not present, the immune system becomes hyperactive and proinflammatory, leading to chronic low-grade inflammation and an exaggerated immune (re)activity that may be contributing to the current rise in inflammatory diseases. This theory has been outlined in two prominent hypotheses, the "old friends" hypothesis[20] and the biodiversity hypothesis,[21] which propose that our immune system depends on the stimulation of environmental microbes for normal and balanced function. So again, the perception of our relationship with microbes is changing. The embracement of "good bacteria" or probiotics and a healthy gut microbiota are in full swing and so the journey continues.

Probiotics have a rich history that began even before antibiotics were discovered, when it was noticed and published that not all microbes were detrimental to health. In 1907, Ilya Ilyich

Mechnikov (who also went by the pen name Elie Metchnikoff) published *The prolongation of life, optimistic studies* where he discussed, in detail, his thoughts on the current scientific principles of how to prolong life and for reasons irrelevant to this dissertation Metchnikoff came to the conclusion that preserving the intestines or inhibiting putrefaction of the intestines was the key to extending life.[22] In *The prolongation of life* there is a chapter entitled "Lactic acid as inhibiting intestinal putrefaction" that might have been the first review ever published on the benefits of bacteria on human health nearly half a century before the term "probiotics" was coined by Werner Kollath in 1953.[23] He cites several examples of natural lactic acid fermentation processes in foods such as cabbage, cucumbers, apples, melons, bread, fish, and milk that preserve these foods in a way that is not toxic to humans. He also describes that these naturally preserving processes allow civilizations in regions of the world with long hard winters to sustain themselves. He concludes these arguments with the following statement "as lactic fermentation serves so well to arrest putrefaction in general why should it not be used for the same purpose within the digestive tube?" (p. 119).[22] He continues by reporting "research" showing that lactic acid bacteria consumed by humans shows evidence of preventing putrefaction of the digestive tube by measuring sulpho-conjugate ethers in urine. Finally, he arrives at his most cited observation that Bulgarian peasants were far outliving their wealthier counterparts because of their daily consumption of yogurt, mainly because the lactic acid bacteria in the yogurt, which later became to be known as *Lactobacillus bulgaricus*, were preventing putrefaction of the digestive tube and leading to a prolongation of life. This conclusion also influenced Metchnikoff to culture his own lactic acid bacilli and consume it every day for 8 year leading up to the publishing of his book and presumably an additional 8 years until his death in 1916. Yet the most profound statement within the whole chapter in my

opinion was “a reader who has little knowledge of such matters may be surprised by my recommendation to absorb large quantities of microbes as the general belief is that microbes are all harmful. This belief, however, is erroneous. There are many useful microbes, amongst which the lactic bacilli have an honourable place” (p. 128).[22]

If only Metchnikoff could see the world of probiotics today. Probiotics species have expanded far beyond *Lactobacillus* and never have we been exposed to such a volume of advertising recommending that we “absorb large quantities of microbes.” Based on a market report by Global Market Insights, Inc. the global probiotics market is projected to exceed $63 billion by 2022 and, according to a survey of Grants.gov [https://www.grants.gov, accessed April 14, 2018], probiotics are being used in clinical trials to treat obesity, inflammatory bowel disorders, and posttraumatic stress disorder (PTSD). The past decade has been filled with massive survey studies such as the Human Microbiome Project,[24] American Gut Project,[25] Belgian Flemish Gut Flora Project,[26] Dutch LifeLines DEEP study,[27] and many more. The main objective of these surveys was to generate a point of reference and large databases of comparators. The field is taking the necessary steps to characterize how our commensal bacteria influence our health.

The main goal of the current study and this dissertation was to achieve a similar end in a Veteran cohort. We sought to look at this select group of individuals with unique environmental exposures and health outcomes, to determine if there were identifiable microbial signatures associated with specific health measures. We collected microbiome samples from three anatomical sites, skin, oral, and fecal, from 188 United States of America Veterans. From our results, we detail in Chapter 3 the general characteristics of the sample types (skin, oral and fecal) as they relate to each other and as they relate to gender, race, and age. In Chapters 4, 5,

and 6 we characterize the microbiome of all three sample types (skin, oral and fecal) as they relate to metadata collected in three main categories: general health and physical health (Chapter 4), insomnia (Chapter 5), and mental health (Chapter 6). Finally, in Chapter 7 we state our conclusions with overall interpretations, limitations, and future directions.

Chapter 2. General materials and methods

2.1 Participants and study design

This observational study included a sample of 188 participants from a population of Veterans seeking care within the US Department of Veteran Affairs health care system. In summary, microbiome samples were collected from three body sites: skin, oral, and fecal. Several demographic, service history, physical health, and mental health measures were collected via medical records, surveys, questionnaires, and interviews. The metrics of physical and mental health were used to make associations to the microbiome at the three sampling sites. These associations are detailed in the successive chapters of this dissertation. All research and experimental practices associated with this dissertation were approved and performed in accordance with the Colorado Multiple Institutional Review Board (COMIRB; protocol number 15-1885) at the University of Colorado Anschutz Medical Campus. Additionally, the study was given exempt status from the University of Colorado Boulder IRB as data were de-identified and no further contact with participants was planned.

2.1.1 Recruitment of participants

The research team worked with providers within the Eastern Colorado Health Care System (ECHCS) to recruit potential participants. Specific recruitment strategies included: attending Veteran groups and events within the ECHCS, hosting regular recruitment/information booths inside the Veteran Health Administration (VHA), posting of recruitment flyers inside the VHA and in multiple locations throughout the community, attending community/regional events geared for Veterans and/or suicide prevention, posting study opportunities on the Rocky Mountain MIRECC website, and including research information in welcome letters to newly enrolling Veterans. In addition, health care professionals treating patients within the VHA system were informed about the study and assisted in recruitment of patients. Approximately 30% of Veterans choose not to seek care within the VHA. Recruitment efforts included strategies

to target Veterans in the community who are eligible to receive VHA care, but do not seek care within VHA system.

2.1.2 Screening and inclusion/ exclusion criteria

Screening

Individuals who responded to recruitment efforts participated in a brief screening process by telephone or in person. The screening process objective was to determine if participants were eligible to participate in the study based on the inclusion/ exclusion criteria. Screening also included a review of VHA medical records. Those who were eligible to participant were scheduled for a clinical visit.

Inclusion criteria

Veterans between ages 18-99 years of age at the time of enrollment

Capability of providing a signed and dated informed consent

Capability and willingness to provide skin, oral, and fecal samples

Exclusion criteria

Females who were pregnant

Positive test for human immunodeficiency virus (HIV), hepatitis B virus (HBV), or hepatitis C virus (HCV) per self-report or chart review

Unstable dietary history as defined by major changes in diet during the previous month, where the participant has eliminated or significantly increased a major food group in their diet

Any confirmed or suspected condition/state of immunosuppression or immunodeficiency

Major surgery of the GI tract, with the exception of cholecystectomy and appendectomy, in the past five years, including any major bowel resection at any time

Chronic dry mouth

Individuals exhibiting symptoms of acute disease or infection at the time of enrollment

2.1.3 Consent process

All members of the research team were trained in Colorado Multiple Institutional Review Board (COMIRB) procedures and were under the direct supervision of Lisa A. Brenner, PhD. Participants were informed of the nature of the study and potential risks and benefits were discussed in a private office at the ECHCS and participants were given the opportunity to ask questions about the study. The participants were then presented with an informed consent document and were asked to answer six questions to ensure the participants' ability to adequately provide informed consent. If participants were not able to adequately respond to the six questions, they were excluded from the study. The questions were:

1) Why is this study being done?
2) What is the study asking you to do?
3) What are the risks/side effects of being in the study?
4) What are the benefits of being in the study?
5) Is the study voluntary?
6) What do you do if you have questions or possible side effects?

Participants were provided with a copy of the consent form and the consent process was documented in the participant's electronic VHA medical record.

Participants also provided authorization to collect protected health information. A signed and dated copy of the release was provided to the participants. All data in electronic format were stored in password protected spreadsheets on the VHA shared drive, behind the VHA firewall and on a secure server and behind a firewall at University of Colorado Anschutz Medical Campus. Original signed informed consent forms and Health Insurance Portability and Accountability Act (HIPAA) authorization forms were stored in locked filing cabinets. Only

members of the research team listed on the COMIRB protocol were permitted access to the paper documents.

2.1.4 Compensation

Participants were compensated $35 for the completion of each study visit. Participants were also compensated $10 for providing skin, oral, and fecal samples.

2.1.5 Surveys

Participants completed several surveys and results were compiled into a metadata spreadsheet to examine associations between survey endpoints and the microbiome. Information about the surveys administered to participants can be found in the **Table 2.1**. Descriptions of the surveys and justification for administering them can be found in the Appendix (Chapter 8) of this dissertation.

Table 2.1 Survey information and details

Measure	Time (min)	Purpose
Baseline visit at ECHCS		
University of Washington Risk Assessment Protocol- Revised (UWRAP)[28]	5	Safety
Rocky Mountain MIRECC Demographics Form	5	Demographic Information
Structured Clinical Interview for DSM-5 Axis I Disorders (SCID-5)[29]	30	Mental health
Ohio State University TBI-ID (OSU-TBI-ID)[30]	25	TBI
PTSD Symptom Checklist 5 (PCL-5)[31]	5	PTSD
45-Item Outcome Questionnaire (OQ-45)[32]	10	Mental health
36-Item Short Form Health Survey (SF-36)[33]	10	General, physical and mental health
Harvard Food Frequency Questionnaire Booklet[34]	20	Diet information
Insomnia Severity Index (ISI)[35]	5	Sleep information
Beck Depression Inventory (BDI)[36]	5	Depression severity

Abbreviations: UWRAP, University of Washington Risk Assessment Protocol; MIRECC, Rocky Mountain Mental Illness Research, Education, and Clinical Center; DSM-5, Diagnostic and Statistical Manual of Mental Disorders; SCID-5, Structured Clinical Interview for the DSM-5; OSU-TBI-ID, Ohio State University Traumatic Brain Injury- Identification; PTSD, Posttraumatic stress disorder; PCL-5, PTSD Symptom Checklist 5; OQ-45, 45-Item Outcome Questionnaire; SF-36, 36-Item Short Form Health Survey; ISI, Insomnia Severity Index; BDI, Beck Depression Inventory.

During a clinical visit to the VHA, participants self-reported demographic data including age, gender, race, ethnicity, marital status, sexual orientation, education level, employee status, student status, currently homeless, numbers of times homeless, and ever homeless. Demographic characteristics about the Veteran cohort can be found in **Table 2.2**.

Table 2.2 Veteran cohort demographic characteristics

Variable	*N* (%) *or* Mean ± SD (range)
Total	188 (100%)
Age	47.0 ± 13.8 (24-77)
Age Groups	
20-29	22 (11.7%)
30-39	50 (26.6%)
40-49	25 (13.3%)
50-59	50 (26.6%)
60-69	36 (19.1%)
70+	5 (2.7%)
Gender	
Male	160 (85.1%)
Female	28 (14.9%)
Race	
Caucasian	120 (63.8%)
African American	40 (21.3%)
Asian	3 (1.6%)
Native American	5 (2.7%)
Multiracial	6 (3.2%)
Other	14 (7.4%)
Ethnicity	
Hispanic	25 (13.3%)
Non-Hispanic	163 (86.7%)
Marital Status	
Married	62 (33.0%)
Single	52 (27.7%)
Cohabitating	7 (3.7%)
Separated/Divorced	58 (30.8%)
Widowed	9 (4.8%)
Sexual Orientation	
Heterosexual	173 (92.0%)
Gay/Lesbian/Queer	9 (4.8%)
Bisexual	4 (2.1%)
Other	2 (1.1%)
Education Level	

No High School Degree	1 (0.6%)
High School Degree	23 (12.2%)
Some College	72 (38.3%)
Associate Degree	26 (13.8%)
Bachelor Degree	50 (26.6%)
Master's Degree	13 (6.9%)
Doctoral Degree	3 (1.6%)
Employment Status	
Employed Full-Time	48 (25.5%)
Employed Part-Time	17 (9.0%)
Unemployed Seeking Job	40 (21.3%)
Unemployed Not Seeking Job	34 (18.1%)
Retired	49 (26.1%)
Student Status	
Not in school	153 (81.4%)
Full-Time	27 (14.3%)
Part-Time	8 (4.3%)
Currently Homeless	
Yes	16 (8.5%)
No	172 (91.5%)
Number of Times Ever Homeless	1.3 ± 2.6 (0-20)
Ever Homeless	
Yes	92 (48.9%)
No	96 (51.0%)

Abbreviations: SD, standard deviation.

Participants provided service data including service branch, service type, months in each type of service, service era, number of deployments, and number of times in combat. The survey questionnaire responses for each category can be found in **Table 2.3**.

Table 2.3 Veteran cohort military service characteristics

Variable	*N* (%) *or* Mean ± SD (range)
Service Branch	
Army	91 (48.4%)
Air Force	36 (19.1%)
Navy	28 (14.9%)
Marines	25 (13.3%)
Coast Guard	1 (0.6%)
Multiple	7 (3.7%)
Service Type	
Active Duty (AD) only	134 (71.3%)
Reserve Duty (RD) only	4 (2.1%)
AD & RD	50 (26.6%)
Months on Active Duty (*n* = 184)	81.3 ± 67.1 (3-360)
Months on Reserve Duty[1] (*n* = 53)	63.1 ± 70.0 (2-372)
Service Era	
Post Korean	2 (1.1%)
Vietnam	17 (9.0%)
Post-Vietnam	44 (23.4%)
Desert Storm	18 (9.6%)
OEF/OIF/OND	63 (33.5%)
Multiple	44 (23.4%)
Year Separated from Service[1] (*n* = 179)	1997 ± 16 (1959-2017)
Deployed	
Yes	132 (70.2%)
No	56 (29.8%)
Number Times Deployed[2] (*n* = 132)	2.8 ± 2.4 (1-15)
Combat	
Yes	89 (47.3%)
No	99 (52.7%)
Number Times in Combat[2] (*n* = 89)	2.0 ± 1.3 (1-8)

[1]Some observations had missing data on variables of interest. Specifically, one observation for a Reserve Duty Veteran was missing data on months of service, and 9 observations were missing data for the year separated from service. [2]Continuous variables on the number of times deployed and the number of times in combat were only reported among those observations with any deployment or combat exposure. Abbreviations: SD, standard deviation; OEF/OIF/OND, Operation Enduring Freedom/ Operation Iraqi Freedom/ Operation New Dawn

Psychometrically sound measures to obtain data regarding general and physical health, insomnia, and mental health conditions of interest were administered and included the Beck

Depression Inventory (BDI), Insomnia Severity Index (ISI), 45-Item Outcome Questionnaire (OQ-45) Severity measures, 36-Item Short Form Health Survey (SF-36), PTSD Checklist 5 (PCL-5), traumatic brain injury (TBI) presence, moderate to severe brain injury presence, and number of TBIs. Summaries of the listed surveys and questionnaires can be found in **Tables 2.4 and 2.5**.

Table 2.4 Veteran cohort mental health characteristics with variables categorized and interpreted

Variable	*N* (%) *or* Mean ± SD (range)	Number of missing samples
Beck Depression Inventory[1]		2
Severe	28 (15.1%)	
Moderate	33 (17.7%)	
Mild	29 (15.6%)	
Minimal	96 (51.6%)	
Insomnia Severity Index[2]		3
Clinical Insomnia (Severe)	21 (11.3%)	
Clinical Insomnia (Moderate Severity)	44 (23.8%)	
Subthreshold Insomnia	63 (34.1%)	
No Clinically Significant Insomnia	57 (30.8%)	
OQ-45 Total Severity[2]		2
Clinically Significant	83 (44.6%)	
Not Clinically Significant	103 (55.4%)	
OQ-45 Symptom Distress Severity[3]		2
Clinically Significant	78 (41.9%)	
Not Clinically Significant	108 (58.1%)	
OQ-45 Interpersonal Relations Severity[4]		2
Clinically Significant	108 (58.1%)	
Not Clinically Significant	78 (41.9%)	
OQ-45 Social Role Severity[5]		2
Clinically Significant	69 (37.1%)	
Not Clinically Significant	117 (62.9%)	
SF-36 Physical Component Summary (PCS)[6]		7
Impaired Function	73 (40.3%)	
No Impaired Function	108 (59.7%)	
SF-36 Mental Component Summary (MCS)[6]		7
Impaired Function	93 (51.4%)	
No Impaired Function	88 (48.6%)	
PCL-5 Symptom Severity[7]		2
Significant	72 (38.7%)	

Not Significant	114 (61.3%)	
Traumatic brain injury		1
Yes	133 (71.1%)	
No	54 (28.9%)	
Moderate or severe brain injury[8]		
Yes	23 (12.2%)	
No	165 (87.8%)	
Number of traumatic brain injuries	2.50 ± 1.74 (1-11)	
Participants that reported experiencing a traumatic brain injury	133	

[1] Beck Depression Inventory: 0-13-Minimal, 14-19-Mild, 20-28-Moderate, 29-63 Severe; [2]OQ-45 Total Severity: >63 Clinically Significant; [3]OQ-45 Symptom Distress Severity: >36 Clinically Significant; [4]OQ-45 Interpersonal Relations Severity: >15 Clinically Significant; [5]OQ-45 Social Role Severity: >12 Clinically Significant; [6]SF-36 Physical and Mental Component Summary scores: >45 Average Function, <45 Impaired Function; [7]PCL-5 Symptom Severity: >33 Significant; [8]Moderate or Severe Traumatic Brain Injury: one or more times lost consciousness for over 30 minutes in lifetime. Abbreviations: SD, standard deviation, OQ-45, 45-Item Outcome Questionnaire; SF-36, 36-Item Short Form Health Survey; PCL-5, PTSD Checklist for DSM-5.

Table 2.5 Veteran cohort mental health characteristics without categorical assignments

Variable	*N* (%) *or* Mean ± SD (range)	Number of missing samples
Beck Depression Inventory	14.6 ± 13.0 (0-57)	2
Insomnia Severity Index	11.6± 7.3 (0-28)	3
OQ-45 Total Severity	58.1 ± 30.7 (0-148)	2
OQ-45 Symptom Distress Severity	32.7 ± 18.7 (0-90)	2
OQ-45 Interpersonal Relations Severity	15.7 ± 8.5 (0-38)	2
OQ-45 Social Role Severity	9.7 ± 5.6 (0-26)	2
SF-36 Physical Component Summary (PCS)	46.6 ± 9.9 (17.8-69.3)	7
SF-36 Mental Component Summary (MCS)	43.3 ± 12.8 (10.6-64.9)	7
PCL-5 Symptom Severity	26.7 ± 21.3 (0-80)	2
Traumatic brain injury		1
Yes	133 (71.1%)	
No	54 (28.9%)	
Moderate or severe brain injury[1]		
Yes	23 (12.2%)	
No	165 (87.8%)	

[1]Moderate or severe traumatic brain injury: one or more times lost consciousness for over 30 minutes in lifetime. Abbreviations: SD, standard deviation; OQ-45, 45-Item Outcome Questionnaire; SF-36, 36-Item Short Form Health Survey; PCS, Physical Component Summary, MCS, Mental Component Summary; PCL-5, PTSD Checklist for DSM-5.

2.2 Sample collection and preparation

Skin, oral, and fecal samples from each participant were collected with double tipped polyurethane swabs (BD BBL™ CultureSwab™ EZ II, Cat No. B220144, Fisher Scientific, Pittsburgh, PA, USA). Skin and oral swabs were collected by the participant during an inpatient evaluation and were stored at −80 ^{o}C within one hour of sampling. Skin samples were collected by swabbing the antecubital fossa (inner elbow). A detailed protocol for how the skin sample was acquired can be found in the appendix (section A2.1) of this dissertation. Oral samples were collected by swabbing the buccal mucosa (inner cheek). A detailed protocol for how the oral sample was acquired can be found in the appendix (section A2.2) of this dissertation. Participants either provided a fecal sample during the same clinical visit as when the skin and oral samples were collected or received a pre-packaged sampling kit for home use with instructions for sample

collection. A detailed protocol for how the fecal samples were acquired can be found in the appendix (section A2.3 and A2.4) of this dissertation. Fecal samples collected at home were mailed back to the Rocky Mountain MIRECC within 24 hours of collection. Upon receipt, all samples were stored at –80 ^{o}C. All samples were transported to the University of Colorado Boulder on dry ice or in portable freezers at –20 ^{o}C for molecular processing.

2.3 Molecular processing

Sample DNA was extracted using the PowerSoil DNA extraction kit (Cat No. 12955-4, Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Marker genes in isolated DNA were PCR-amplified using GoTaq Master Mix (Cat No. M5133, Promega, Madison, WI, USA) and 515 F (5'-GTGCCAGCMGCCGCGGTAA-3') 806 R (5'-GGACTACHVGGGTWTCTAAT-3') primer pair (Integrated DNA Technologies, Coralville, IA, USA) targeting the V4 hypervariable region of the 16S rRNA gene modified with a unique 12-base sequence identifier for each sample and the Illumina adapter as previously described in Caporaso et al., 2012.[37] The thermal cycling program consisted of an initial step at 94 °C for 3 min followed by 35 cycles (94 °C for 45 sec, 55 °C for 1 min, and 72 °C for 1.5 min), and a final extension at 72 °C for 10 min. Polymerase chain reactions (PCR) were run in duplicate and the products from the duplicate reactions were pooled and visualized on an agarose gel to ensure successful amplification. PCR products were cleaned and normalized using a SequalPrep Normalization Kit (Cat. No. A1051001, ThermoFisher, Waltham, MA, USA) following manufacturer's instructions. The normalized amplicon pool was sequenced on an Illumina MiSeq run by using V3 chemistry and 600 cycle, 2 x 300-bp paired-end sequencing. All library preparation and sequencing were conducted at the University of Colorado Boulder BioFrontiers Next-Gen Sequencing core facility, https://bficores.colorado.edu/sequencing-lab.

2.4 Computational analyses

2.4.1 Sequence processing

Raw sequences were trimmed, demultiplexed, merged, quality filtered (maxee value of 1 and singletons removed), and clustered into greater than or equal to 97% similar phylotypes using UPARSE v8.[38] Quality reports from the fastq_eestats2 command were used to determine the fixed-length (200 nucleotides for the forward read and 150 nucleotides for the reverse read) at which the raw sequences were trimmed as suggested by the developers of UPARSE. Merging criteria were adjusted as suggested by UPARSE developers for merging reads with long overlap. Taxonomy was assigned using the Ribosomal Database Project (RDP) classifier[39] trained on the Greengenes 13_8 16S rRNA gene database.[40] For downstream analysis rarefaction curves were examined by sample type and the following depths were chosen: skin, 2000; oral, 2000; fecal, 3000. The samples removed or not present as well as final sample sizes used for analysis are listed in **Table 2.**6.

Table 2.6 Final sample sizes and sources of missing or removed data

Sample type	Missing samples	Samples removed due to sampling depth	Final sample size
Skin	2	9	177
Oral	1	9	178
Fecal	25	2	161

2.4.2 Alpha and beta diversity

Alpha diversity

Analysis of alpha diversity was performed with the R package phyloseq (v 1.20.0)[41] and mctoolsr (v 0.1.1.1) (https://github.com/leffj/mctoolsr/). Stacked bar plots for a taxonomic level were created by using the summarize_taxa command and designating the desired taxonomic level in the mctoolsr package and then passing the output through the plot_taxa_bars command. The parameters of the summarize_taxa command were set to only plot the top ten taxa. Statistics on taxa driving differences between categories within a variable were generated by running the

summarize taxa command and designating the desired taxonomic level in the mctoolsr package and then passing the output through the taxa_summary_by_sample_type command. The parameters of the taxa_summary_by_sample_type command were set to filter out taxa that had a relative abundance less than 0.01 and to run Kruskal-Wallis statistics.

Beta diversity

Quantitative Insights Into Microbial Ecology (QIIME v 1.9.1) was used to generate weighted and unweighted UniFrac distance matrices to examine the microbial community structure.[42,43] Further analysis of beta diversity was performed with the R package phyloseq (v 1.20.0)[41] and mctoolsr (v 0.1.1.1) (https://github.com/leffj/mctoolsr/). Bacterial community composition differences were tested with permutational analysis of variance (PERMANOVA) performed with the vegan (v 2.4-4) package.[44] Both weighted and unweighted UniFrac distance matrices were analyzed by PERMANOVA and significant unweighted UniFrac *p*-values were only reported if weighted UniFrac *p*-values were not significant. All statistics and most graphics were created in R (v 3.4.1). Mean dissimilarity principal coordinate analysis plots were made by generating a distance matrix using either weighted or unweighted (depending on the variable) UniFrac distance metrics. The distance metric was collapsed into mean dissimilarity distances using the calculate_mean_dissimilarities command in the mctoolsr package. This distance matrix was then passed into the calc_ordination command in the mctoolsr package and finally plotted using the plot_ordination command in the phyloseq package. It should be noted that these plots are strictly for visualizing general trends in bacterial community structure between groups of samples. Interpretation of these plots beyond this is limited and we want to be explicit about this concept and stress that no statistical analyses were run on the mean dissimilarity PCoA analysis and plots.

Machine learning

Random forest analysis was done using the randomForest (v 4.6-12) package. Random forest parameters were set to create 1000 trees and run using the 10-fold cross-validation method.[45] Importance and proximity parameters were set to TRUE to generate importance values for the most predictive OTUs and to allow for the generation of a multidimensional scaling (MDS) plot of the results.

2.4.3 Initial analysis of target variables

The metadata file generated for this study had 177 total variables assessed or associated with each participant. Categorical variables with severely unbalanced sample sizes (i.e. yes: 95% of samples, no: 5% of samples) were removed (n = 63), unless the variable had previously been cited in other studies as a variable of significant interest to the field (i.e. age group, gender, race) or if the variable related to the major topics of this dissertation (measures of general and physical health, insomnia, and mental health). Finally, variables unrelated to the topics of this dissertation were removed (i.e. PCR primer plate number and barcode sequence) (n = 30). After the data were filtered based on these criteria, 84 target variables remained. To gain general insight into the microbial associations with these variables, a for-loop was generated in R (v3.4.1) and the following metrics were generated and assessed:

- Alpha diversity
    - Observed OTUs
        - Kruskal-Wallis test
        - Pairwise Wilcoxon rank-sum test
    - Shannon diversity
        - Kruskal-Wallis test
        - Pairwise Wilcoxon rank-sum test

- Beta diversity
    - Taxa driving differences at all taxonomic levels
        - Taxa filtered at a relative abundance of 0.01
        - Kruskal-Wallis test
    - Weighted UniFrac
        - PCoA plot
        - PERMANOVA
        - Permutational test of homoscedasticity
    - Unweighted UniFrac
        - PCoA plot
        - PERMANOVA
        - Permutational test of homoscedasticity

These results were compiled, separated into skin, oral, and fecal results, sorted into general and physical health, insomnia, or mental health categories, and evaluated for significance. Significant alpha and beta diversity were validated and results were reported. Specific taxa were not assessed if beta diversity results were not significant unless specified as "exploratory analysis".

2.4.4 Veteran health personal data

Height and weight data were collected from the participants during the inpatient visit. This information was used to generate a body-mass index (BMI) for each participant. The BMIs of the cohort were compiled into categories outlined by the Center for Disease Control (CDC). Compiled BMI data for the cohort can be found in **Table 2.7**. The analyses done in this dissertation excluded BMIs over 40.

Table 2.7 Body-mass index (BMI) categories and sample sizes

BMI Category	Number of participants
Underweight (< 18)	0
Healthy (19-24)	46
Overweight (25-29)	70
Obese (30-39)	57
Extremely Obese (> 40)	8
Missing BMI data	7

Abbreviations: BMI, body-mass index.

2.4.5 General graphics

Violin plots

Violin plots are similar to box-and-whisker plots but with added information about the distribution of samples within the distribution. The violin plots that are displayed in this dissertation have a box-and-whisker plot overlaid on the violin plot. The box-and-whisker plot shows the median and quartile information (for a more detailed description of box-and-whisker plots please refer to the *box-and-whisker plots* section immediately below). The violin plot is a kernel density estimation that represents the probability that a sample will fall into a specific region of the plot. Wider areas of the plots represent areas where there is a higher probability for a sample to fall and *vice versa* for narrower regions of the plot.

Box-and-whisker plots

In the box-and-whisker plots, the median is illustrated by a thick black line; bottoms and tops of boxes indicate the first and third quartiles, respectively; whiskers indicate the 1.5 interquartile range (IQR) beyond the upper and lower quartiles. Values outside the whiskers are indicated by black dots.

Chapter 3. Characterization of the skin, oral, and fecal microbiomes among a cohort of United States Military Veterans

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3.1 Introduction

In 2007, the United States National Institutes of Health (NIH) launched the Human Microbiome Project (HMP) as an extension of the Human Genome Project, in the spirit and full embracement of the human as a "supra-organism", comprised of both intrinsic (host-derived) and extrinsic (microbe-derived) genetic material.[46] The HMP set out to achieve the following: a) construct draft assemblies of reference genomes, b) obtain reference microbiome datasets, c) create a reference dataset of "normal healthy individuals", d) expand the project to create a global human microbiome diversity project. Initial efforts for objective c), above, included sequencing the microbiomes of 300 "healthy" human subjects at 5 major body sites (skin, oral, airways, gut, and vagina).[46] Since the conception of medicine and science, members of medical and scientific communities have been interested in diseased or allostatic states; however, in order to be able to understand the fundamentals of these maladaptive states a homeostatic comparison state is required. The value of the HMP in sequencing the microbiomes of 300 "healthy" human subjects is directly in line with that objective. In order to identify and characterize a state of dysbiosis, microbial imbalance or maladaptation, it was first necessary to develop a reference

dataset of a "healthy" cohort. The creation of this dataset was a tremendous endeavor, a major achievement, and has provided a reference for the comparison of contingents with potentially dysbiotic states against a "healthy" cohort. Researchers have started investigating the human microbiome, most often the gut microbiome, as it relates to clinical conditions ranging from obesity to PTSD.[47,48] Towards this end, efforts are needed to delineate configurations of microbial communities with a focus on how microbial communities contribute to disease onset and progression, as well as treatment response.

We examined the 16S rRNA gene sequencing results of skin, oral, and gut samples from 188 U.S. Veterans. This population is unique in terms of potential trauma and stressor-related exposures (e.g., combat, TBI), which have yet to be studied in terms of the human microbiome. Also of note is the health system in which this project has been initiated. As discussed in terms of the Million Veteran Program, "a mega-biobank to study genetic influences on health and disease" (p. 214),[49] the VHA possesses unique elements necessary for successful execution of genomic research including access to longitudinal patient information via electronic medical records (EMR). In the present study, in order to augment data available in EMRs, extensive data were collected from the participants via the administration of psychometrically sound measures regarding physical and mental health, as well as demographic/military history survey data (e.g., combat exposures, TBI).

In this chapter we characterize the skin, oral, and fecal microbiomes of the Veteran cohort. We first examined alpha and beta diversity measures and taxonomy of all the Veteran cohort sample types together, followed by a more in-depth evaluation of the alpha and beta diversity measures and taxonomy of individual sample types in relation to gender, race, and age.

3.2 Materials and methods

3.2.1 General procedures

Please refer to Chapter 2 of this dissertation for details on *Participants and study design*, *Sample collection and preparation*, *Molecular processing*, and *Computational analyses*.

3.3 Results

3.3.1 General characteristics of the Veteran cohort

This Veteran cohort had a rich and diverse set of demographic metrics including general demographics, and service history, in addition to measures of insomnia, TBI exposures, and interpretations of various physical and mental health surveys. A majority of the Veteran cohort were Caucasian (63.8%) and a majority of the cohort were males (85.1%). However, the cohort was relatively diverse from a demographic standpoint considering the population that the sample was drawn from including: African American (21%), Asian (1.6%), Native American (2.7%), multiracial (3.2%), and "other" (7.7%) participants. The cohort exhibited a wide range of ages (24-77 years; average age ± SD, 47.0 ± 13.8 years), and a vast majority pursued education beyond high school (~87%). Perhaps unique to this dataset, 8.5% of the sample reported current homelessness and 48% reported ever being homeless. Further details regarding the demographic characteristics of this sample are highlighted in **Table 2.2**. The military service history of this Veteran cohort was also diverse with all service branches represented, a wide range of time spent on Active Duty service (3-360 months), and service across multiple eras (general time of service, i.e. Vietnam era or Iraq/ Afghanistan era) reported. Further details regarding the service details of this sample are highlighted in **Table 2.3**. Of note, 83% (n = 156) of the sample met criteria for at least one mental health condition (current and/or lifetime) based on the SCID-5. For a summary of mental health characteristics refer to **Tables 2.4 and 2.5**.

3.3.2 Characteristics of skin, oral, and gut microbiomes of a Veteran cohort

The Shannon alpha diversity index, a measure of richness and evenness,[50] revealed that, among the three sample types, the fecal microbiome (median ± SD, 3.18 ± 0.64) and the skin

microbiome (median ± SD, 3.11 ± 0.73) had higher alpha diversity, relative to the oral microbiome (median ± SD, 2.15 ± 0.52) but were not different from each other (**Figure 3.1A**). Analysis of beta diversity, a measure of dissimilarity among samples,[51] assessed using weighted UniFrac, revealed that the three sample types clustered separately and harbored bacterial community structures that were different (PERMANOVA, $p < 0.01$) (**Figure 3.1B**).

The analysis of the Veteran skin microbiome, after processing for quality control, included 177 samples (mean ± SD, 9,885 ± 5,550 sequences/sample). The taxonomy of the skin microbiome in terms of relative abundance (RA) was dominated by three phyla, Firmicutes (mean RA ± SD, 44.9 ± 17.3%), Actinobacteria (mean RA ± SD, 23.9 ± 13.7%), and Proteobacteria (mean RA ± SD, 21.6 ± 16.0%), which, together, accounted for 90.4% of all taxa (**Figure 3.1C**). At the genus level, the dominant genera were *Corynebacterium* (mean RA ± SD, 16.5 ± 13.5%), *Staphylococcus* (mean RA ± SD, 16.3 ± 16.3%), and *Streptococcus* (mean RA ± SD, 10.0 ± 8.8%) (data not shown).

The analysis of the Veteran oral microbiome, after processing for quality control, included 178 samples (mean ± SD, 29,894 ± 30,046 sequences/sample), which was the highest mean number of sequences from the three sampling locations. The taxonomy of the oral microbiome consisted of mainly three phyla, namely Firmicutes (mean RA ± SD, 54.8 ± 17.7%), Proteobacteria (mean RA ± SD, 23.2 ± 17.7%), and Bacteroidetes (mean RA ± SD, 9.7 ± 7.3%) (**Figure 3.1C**), which, together, accounted for 87.7% of all taxa. The most prevalent genera from the oral samples were *Streptococcus* (mean RA ± SD, 40.0 ± 17.8%), *Haemophilus* (mean RA ± SD, 17.6 ± 15.9%), and *Veillonella* (mean RA ± SD, 7.2 ± 6.7%).

The Veteran fecal microbiome, after processing for quality control, included 161 samples (mean ± SD, 26,920 ± 27,070 sequences/sample). The lower sample size in this category was not

reflective of sequencing efforts, but a result of fewer Veterans returning fecal samples, which were in some cases collected at home and shipped back to the study site, compared to the other two sample types, which were collected during the clinical visit. Further details pertaining to sample sizes within the sample types can be found in **Table 2.6**. The three most abundant phyla in the fecal microbiome were Firmicutes (mean RA ± SD, 42.0 ± 20.7%), Bacteroidetes (mean RA ± SD, 37.8 ± 23.1%), and Proteobacteria (mean RA ± SD, 13.0 ± 21.1%), which, together, accounted for 92.8% of all taxa (**Figure 3.1C**). Genera that appeared most frequently in the fecal samples included *Bacteroides* (mean RA ± SD, 20.2 ± 17.3%), *Prevotella* (mean RA ± SD, 8.5 ± 14.5%), and *Escherichia* (mean RA ± SD, 7.9 ± 17.1%).

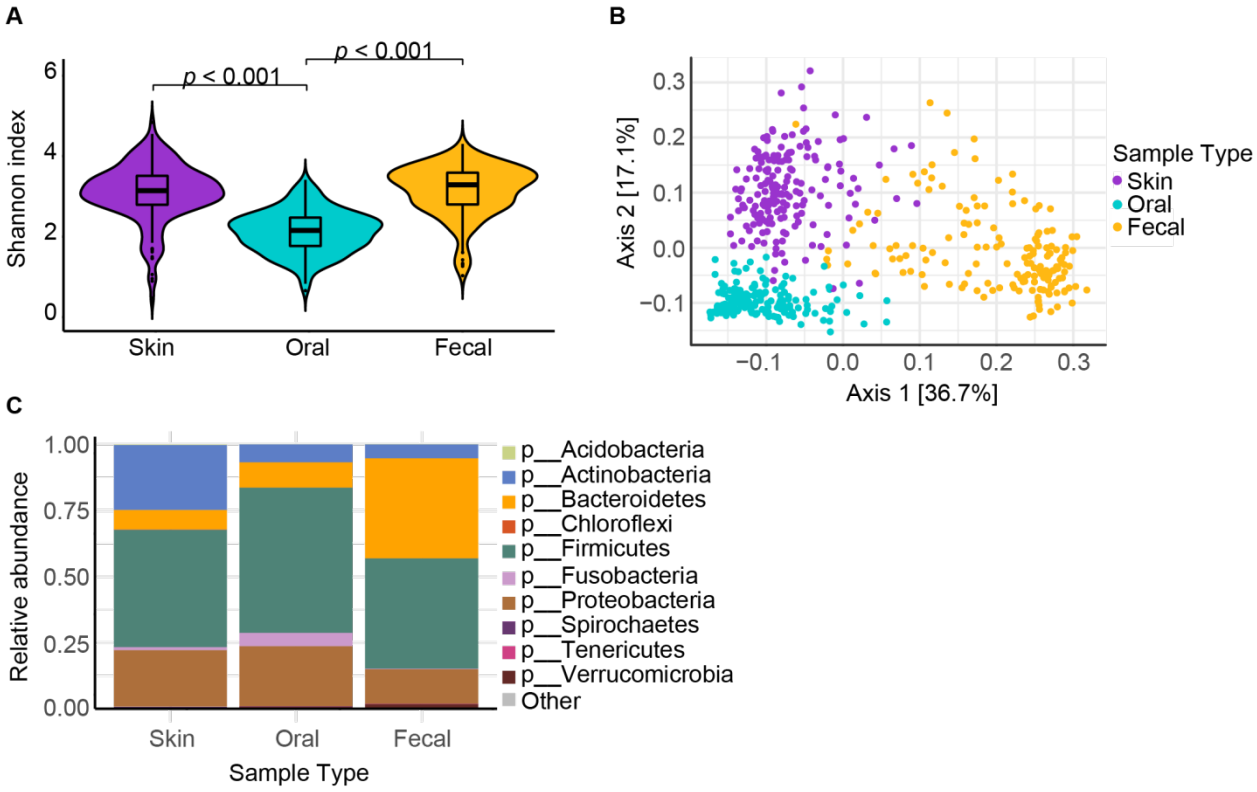

Figure 3.1 Alpha and beta diversity, and phylum level taxonomy, of skin, oral, and fecal microbiomes
A) Violin plot of Shannon diversity index between sample types with statistics reported based on Wilcoxon rank-sum test. B) Principal coordinate analysis plot from weighted UniFrac distance matrix. Principal coordinate axis 1 explained 36.7% of the variance and principal coordinate axis 2 explained 17.1% of the variance; together, principal coordinate axes 1 and 2 explained 53.8% of variance. C) Top taxa for all sample types at the phylum taxonomic level. Sample sizes: skin, 177; oral, 178; fecal, 161.

3.3.3 Microbial diversity characteristics by sample type in relation to gender, race, and age

Skin microbiome

Alpha diversity was higher in males than in females as measured by both observed OTUs (Wilcoxon rank-sum test; $p < 0.05$) and Shannon diversity (Wilcoxon rank-sum test; $p < 0.05$) (**Figure 3.2A**). Beta diversity analysis using unweighted UniFrac and further analysis by PERMANOVA of skin samples revealed differences in bacterial community structure based on gender ($p = 0.007$) (**Figure 3.2B**). Males showed greater relative abundance of the phyla Actinobacteria (Wilcoxon rank-sum test; $p < 0.01$) and Fusobacteria (Wilcoxon rank-sum test; $p < 0.05$) (**Figure 3.2C**). These phyla were the only phyla that showed differences in enrichment between genders (for more information on how analysis of the relative abundances for individual taxa were performed and how top taxa plots were generated see the *Alpha diversity* section of the *Computational analysis* segment of Chapter 2). Males showed increased relative abundance of the genus *Akkermansia* (Wilcoxon rank-sum test; $p < 0.05$), while females showed enrichment in the genera *Lactobacillus* (Wilcoxon rank-sum test; $p < 0.01$), *Oscillospira* (Wilcoxon rank-sum test; $p < 0.05$), and *Dialister* (Wilcoxon rank-sum test; $p < 0.05$).

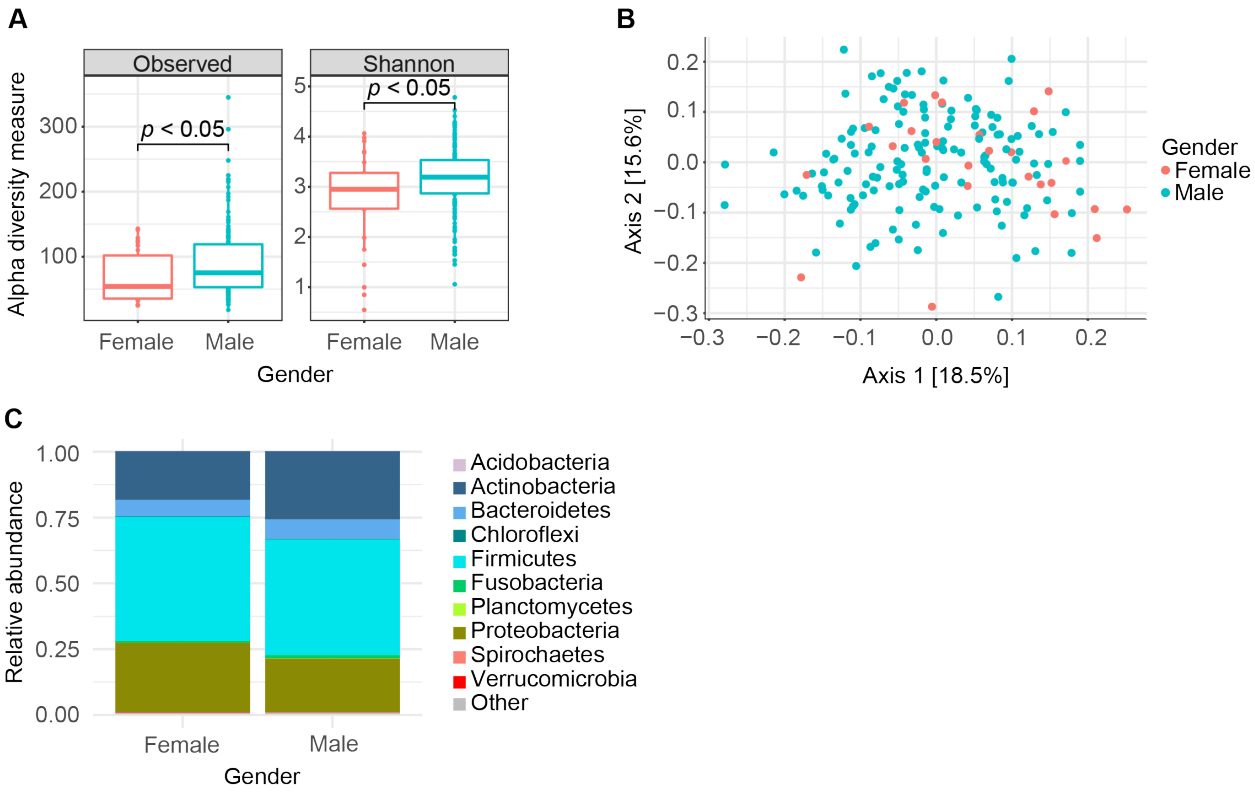

Figure 3.2 Alpha and beta diversity and top taxa plots for the skin microbiome based on gender
A) Boxplots of alpha diversity metrics, observed operational taxonomic units (OTUs) and Shannon diversity, for the skin microbiome, based on gender. B) Principal coordinate analysis plot of weighted Unifrac distance matrix for the skin microbiome, based on gender. Principal coordinate axis 1 explained 18.5% of the variance and principal coordinate axis 2 explained 15.6 % of the variance; together, principal coordinate axes 1 and 2 explained 34.1% of variance. C) Top 10 taxa at the phylum taxonomic level for the skin microbiome, based on gender. Sample sizes: female, 28; male, 158.

There were no differences in alpha diversity of skin microbiomes, as measured by observed OTUs or Shannon diversity, based on race, as determined based on analysis using Kruskal-Wallis test (**Figure 3.3A**). Beta diversity analysis using unweighted UniFrac and further analysis by PERMANOVA of skin samples revealed differences in bacterial community structure based on race ($p = 0.04$) (**Figure 3.3B**). In order to better visualize dissimilarity among races we collapsed the distance matrix into a mean dissimilarity distance matrix (for methods on how mean dissimilarity PCoA plots were created see the *Beta diversity* section of the *Computational analysis* section of Chapter 2). The resulting PCoA analysis and plot of the mean

dissimilarity unweighted UniFrac distance matrix shows each race as one data point (**Figure 3.3C**). Differences in the relative abundances of top taxa (i.e. taxa that had a relative abundance of 0.01 or greater) at the phylum taxonomic level yielded no differences based on race as determined by Kruskal-Wallis test (**Figure 3.3D**).

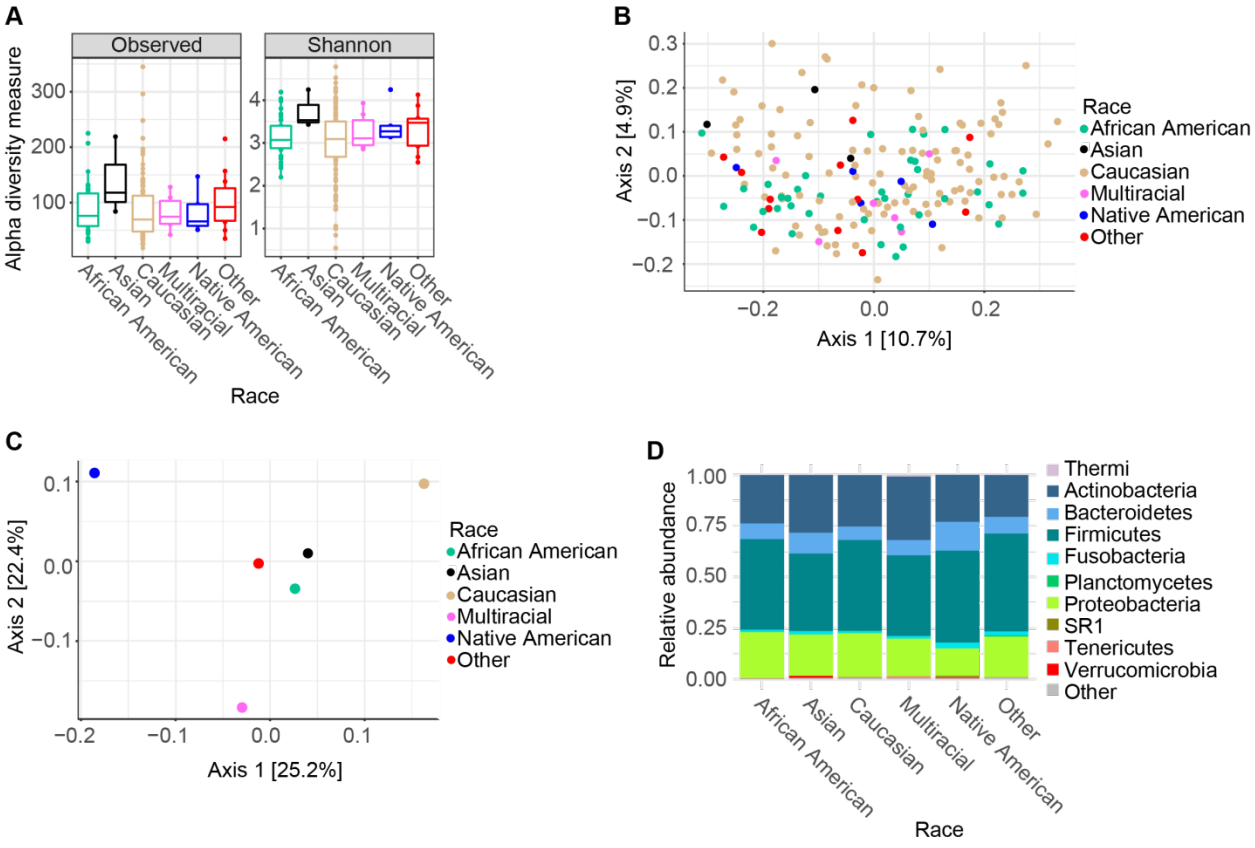

Figure 3.3 Alpha and beta diversity and top taxa plots for the skin microbiome based on race
A) Boxplots of alpha diversity metrics, observed operational taxonomic units (OTUs) and Shannon diversity, for the skin microbiome, based on race. B) Principal coordinate analysis plot of unweighted UniFrac distance matrix for the skin microbiome, based on race. Principal coordinate axis 1 explained 10.7% of the variance and principal coordinate axis 2 explained 4.9% of the variance; together, principal coordinate axes 1 and 2 explained 15.6% of variance. C) Principal coordinate analysis plot of unweighted Unifrac mean dissimilarity distance matrix for the skin microbiome, based on race. Principal coordinate axis 1 explained 25.2% of the variance and principal coordinate axis 2 explained 22.4% of the variance; together, principal coordinate axes 1 and 2 explained 47.6% of variance. D) Top 10 taxa at the phylum taxonomic level for the skin microbiome, based on race. Sample sizes: African American, 40; Asian, 3; Caucasian, 118; Multiracial, 6; Native American, 5; Other, 14.

There were no differences in alpha diversity of skin microbiomes, as measured by either observed OTUs or Shannon diversity, based on age, as determined by Kruskal-Wallis test (data not shown). Beta diversity analysis using weighted and unweighted UniFrac and further analysis by PERMANOVA of skin samples revealed no differences in bacterial community structure based on age (data not shown).

Oral microbiome

There were no differences in alpha diversity of oral microbiome samples based on gender or race as determined by Wilcoxon rank-sum test (data not shown). Beta diversity analysis using weighted and unweighted UniFrac and further analysis by PERMANOVA of oral samples revealed no differences in bacterial community structure based on gender or race (data not shown).

Alpha diversity varied among age groups, as measured by both observed OTUs and Shannon diversity, as determined by Wilcoxon rank-sum test (**Figure 3.4A**; **Table 3.1** (observed OTUs); **Table 3.2** (Shannon diversity)), with a general trend for alpha diversity to decline with age.

Table 3.1 Results matrix for pairwise comparisons of observed OTUs in the oral microbiome, based on age groups

Age group	20-29	30-39	40-49	50-59	60-69	70+
20-29	NA	NS	< 0.05	NS	< 0.01	< 0.01
30-39		NA	NS	NS	< 0.05	< 0.05
40-49			NA	NS	NS	< 0.01
50-59				NA	< 0.01	< 0.01
60-69					NA	NS
70+						NA

Statistics for pairwise comparisons were done with Wilcoxon rank-sum test. *P*-values are listed; NS, not significant. NA represents comparisons between an age group and itself. Blank cells would have been replicates of data already in the matrix. Age ranged from 24-77. Sample sizes: 20-29, 22; 30-39, 50; 40-49, 25; 50-59, 50; 60-69, 36; 70+, 5.

Table 3.2 Results matrix for pairwise comparisons of Shannon diversity in the oral microbiome, based on age groups

Age group	20-29	30-39	40-49	50-59	60-69	70+
20-29	NA	NS	< 0.05	NS	< 0.05	< 0.05
30-39		NA	< 0.05	NS	< 0.05	< 0.05
40-49			NA	NS	< 0.05	NS
50-59				NA	NS	< 0.05
60-69					NA	NS
70+						NA

Statistics for pairwise comparisons were done with Wilcoxon rank-sum test. *P*-values are listed; NS, not significant. NA represents comparisons between an age group and itself. Blank cells would have been replicates of data already in the matrix. Age ranged from 24-77. Sample sizes: 20-29, 22; 30-39, 50; 40-49, 25; 50-59, 50; 60-69, 36; 70+, 5.

Beta diversity analysis using unweighted UniFrac and further analysis by PERMANOVA of oral microbiome samples revealed a difference in bacterial community structure based on age group ($p = 0.002$) (**Figure 3.4B)**. Once again, the distance matrix was collapsed into a mean dissimilarity distance matrix based on age group (**Figure 3.4C**). PCoA of the mean dissimilarity matrix revealed that the older age groups (60-69 and 70+ years of age) separate from the younger age groups (< 60 years of age) (**Figure 3.4D**). The most abundant phyla in the oral microbiome were Firmicutes and Proteobacteria in all age groups. The relative abundances of selected low abundance phyla in the oral microbiome, including Actinobacteria, Bacteroidetes, and Fusobacteria, are shown for each age group in **Figure 3.4E**. No differences in specific taxa were observed at the phylum or genus level across age groups, based on Kruskal-Wallis test. It should be noted that sample sizes within age groups are not evenly distributed (**Table 3.3**).

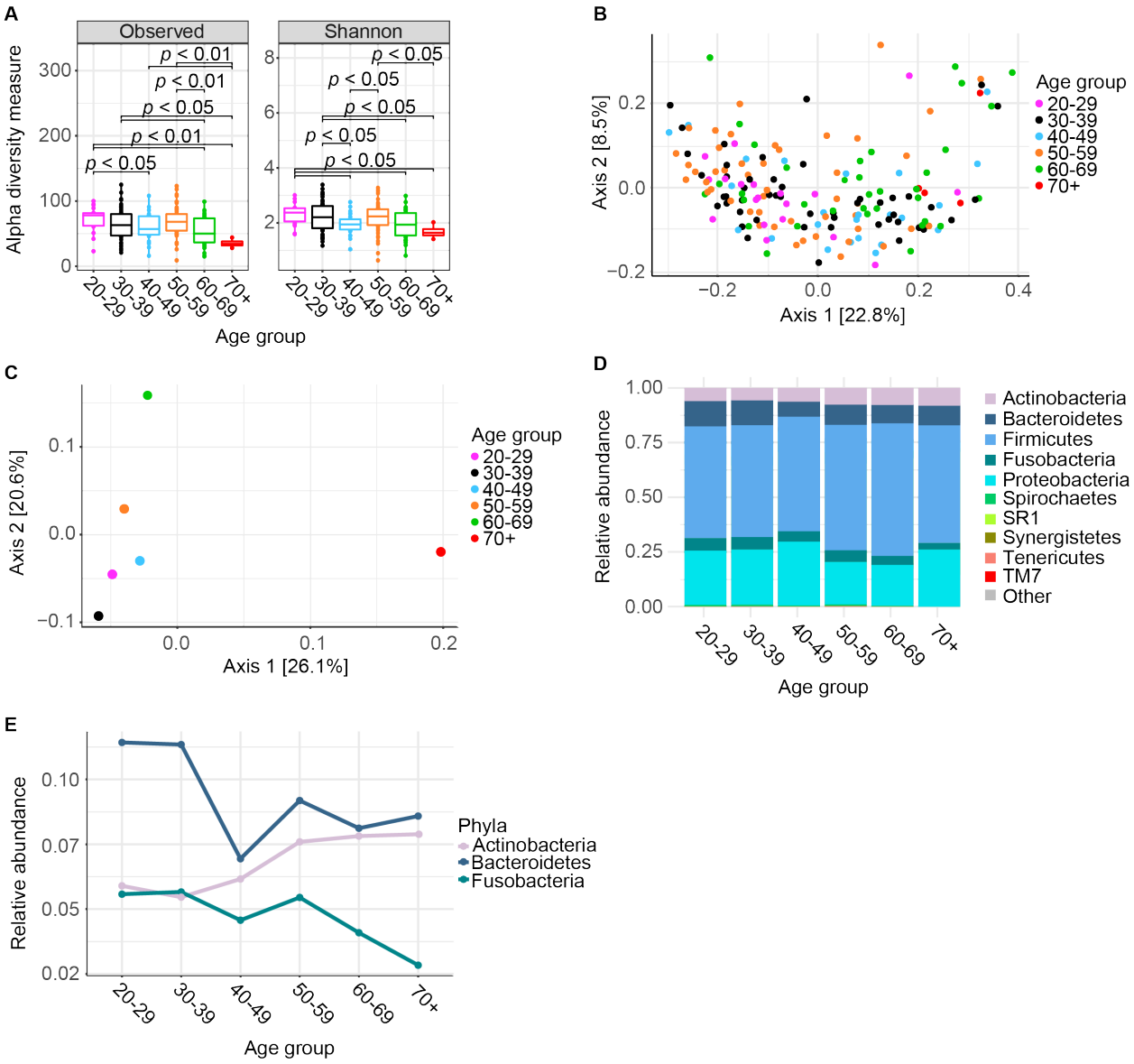

Figure 3.4 Alpha and beta diversity plots, top taxa plots, and relative abundance of low abundance phyla for the oral microbiome based on age group

A) Boxplots of alpha diversity metrics, observed operational taxonomic units (OTUs) and Shannon diversity, for the oral microbiome, based on age group. B) Principal coordinate analysis plots of unweighted UniFrac distance matrix for the oral microbiome, based on age group. Principal coordinate axis 1 explained 22.8% of the variance and principal coordinate axis 2 explained 8.5% of the variance; together, principal coordinate axes 1 and 2 explained 31.3% of variance. C) Principal coordinate analysis plot of unweighted UniFrac mean dissimilarity distance matrix for the oral microbiome, based on age group. Principal coordinate axis 1 explained 26.1% of the variance and principal coordinate axis 2 explained 20.6% of the variance; together, principal coordinate axes 1 and 2 explained 46.7% of variance. D) Top 10 taxa at the phylum taxonomic level for the oral microbiome, based on age group. E) Line and scatter plot of relative abundance in selected low abundance phyla for the oral microbiome based on age group. Sample sizes: 20-29, 22; 30-39, 50; 40-49, 25; 50-59, 50; 60-69, 36; 70+, 5.

Table 3.3 Sample sizes of oral microbiome samples by age group

Age Groups	
20-29	22 (11.7%)
30-39	50 (26.6%)
40-49	25 (13.3%)
50-59	50 (26.6%)
60-69	36 (19.1%)
70+	5 (2.7%)

Fecal microbiome

There were no differences in alpha diversity of fecal microbiome samples based on gender as determined by Wilcoxon rank-sum test (**Figure 3.5A**). Beta diversity analysis using unweighted UniFrac and further analysis by PERMANOVA of fecal samples revealed that the bacterial community structure differed between males and females ($p < 0.001$) (**Figure 3.5B**). The top ten taxa at the phylum and family level are shown in **Figure 3.5C** and **Figure 3.5D**, respectively. Analysis of the top taxa at the phylum taxonomic level, i.e., those taxa with relative abundances over 0.01, using Wilcoxon rank-sum test did not reveal any differences between males and females. However, analysis of the top taxa at the family taxonomic level, i.e., those taxa with relative abundances over 0.01, exhibited an enrichment in females in the family Lactobacillaceae (Wilcoxon rank-sum test, $p < 0.01$), while males show enrichment in the family Verrucomicrobiaceae (Wilcoxon rank-sum test, $p < 0.05$).

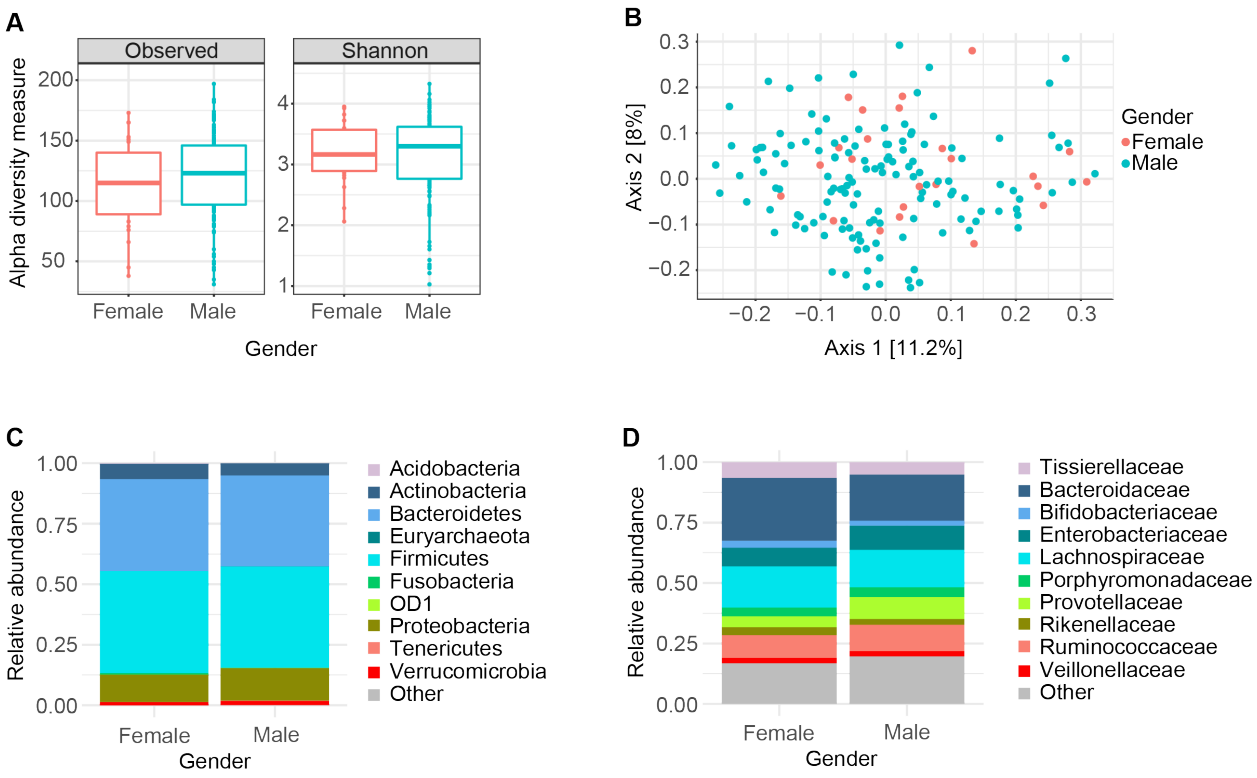

Figure 3.5 Alpha and beta diversity and top taxa plots for the fecal microbiome based on gender
A) Boxplots of alpha diversity metrics, observed operational taxonomic units (OTUs) and Shannon diversity, for the fecal microbiome, based on gender. B) Principal coordinate analysis plots of unweighted UniFrac distance matrix for the fecal microbiome, based on gender. Principal coordinate axis 1 explained 11.2% of the variance and principal coordinate axis 2 explained 8% of the variance; together, principal coordinate axes 1 and 2 explained 19.2% of variance. C) Top 10 taxa represented at the phylum taxonomic level for the fecal microbiome based on gender. D) Top 10 taxa represented at the family taxonomic level for the fecal microbiome based on gender. Sample sizes: female, 25; male, 138.

There were no differences in alpha diversity of fecal microbiome samples based on race as determined by Kruskal-Wallis test (**Figure 3.6A**). Analysis of beta diversity using unweighted UniFrac and further analysis by PERMANOVA of fecal samples revealed that differences in the bacterial community structure based on race approached statistical significance ($p = 0.07$) (**Figure 3.6B, C**). No differences were observed in the relative abundances of exploratory analysis of top taxa, i.e., those taxa with relative abundances over 0.01, at the phylum level (Kruskal-Wallis test, **Figure 3.6D**).

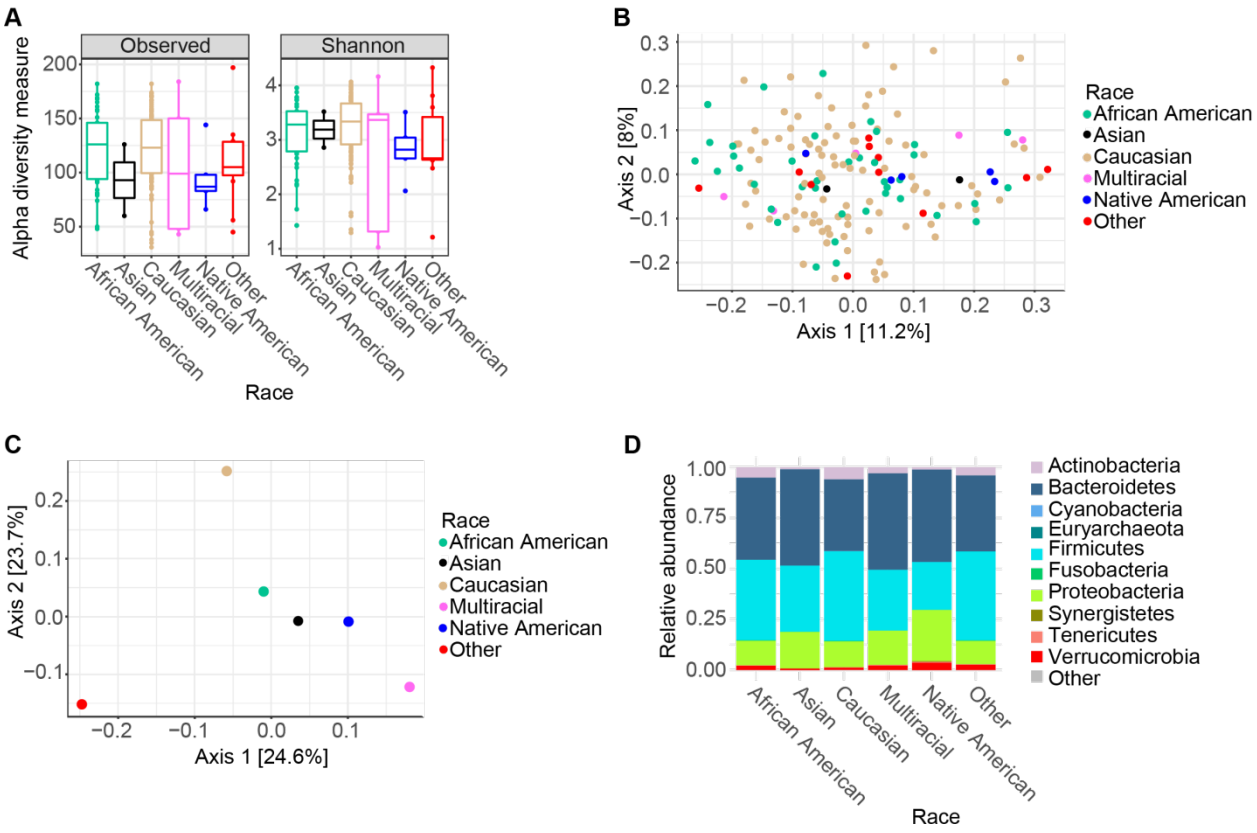

Figure 3.6 Alpha and beta diversity and top taxa plots for the fecal microbiome based on race
A) Boxplots of alpha diversity metrics, observed operational taxonomic units (OTUs) and Shannon diversity, for the fecal microbiome, based on race. B) Principal coordinate analysis plots of unweighted UniFrac distance matrix for the fecal microbiome, based on race. Principal coordinate axis 1 explained 11.2% of the variance and principal coordinate axis 2 explained 8% of the variance; together, principal coordinate axes 1 and 2 explained 19.2% of variance. C) Principal coordinate analysis plot of unweighted UniFrac mean dissimilarity distance matrix for fecal microbiome, based on race. Principal coordinate axis 1 explained 24.6% of the variance and principal coordinate axis 2 explained 23.7% of the variance; together, principal coordinate axes 1 and 2 explained 48.3% of variance. D) Top 10 taxa for race represented at the phylum taxonomic level for the fecal microbiome based on race. Sample sizes: African American, 39; Asian, 2; Caucasian, 100; Multiracial, 5; Native American, 5; Other, 12.

There were no differences in alpha diversity in the fecal microbiome based on age group as determined by Kruskal-Wallis test (**Figure 3.7A**). Analysis of beta diversity using unweighted UniFrac and further analysis by PERMANOVA of fecal samples revealed that the bacterial community structure differed among age groups ($p = 0.02$) (**Figure 3.7B**). PCoA analysis of the mean dissimilarity unweighted UniFrac distance matrix highlights differences in community structure between specific age groups (**Figure 3.7C**). The three youngest age groups (< 49 years

of age) were fairly distinct and the three older age groups (> 50 years of age) clustered closely together (**Figure 3.7C**). Analysis by Kruskal-Wallis test of the top phyla, i.e., those taxa with relative abundances over 0.01, yielded no differences based on age group (**Figure 3.7D**).

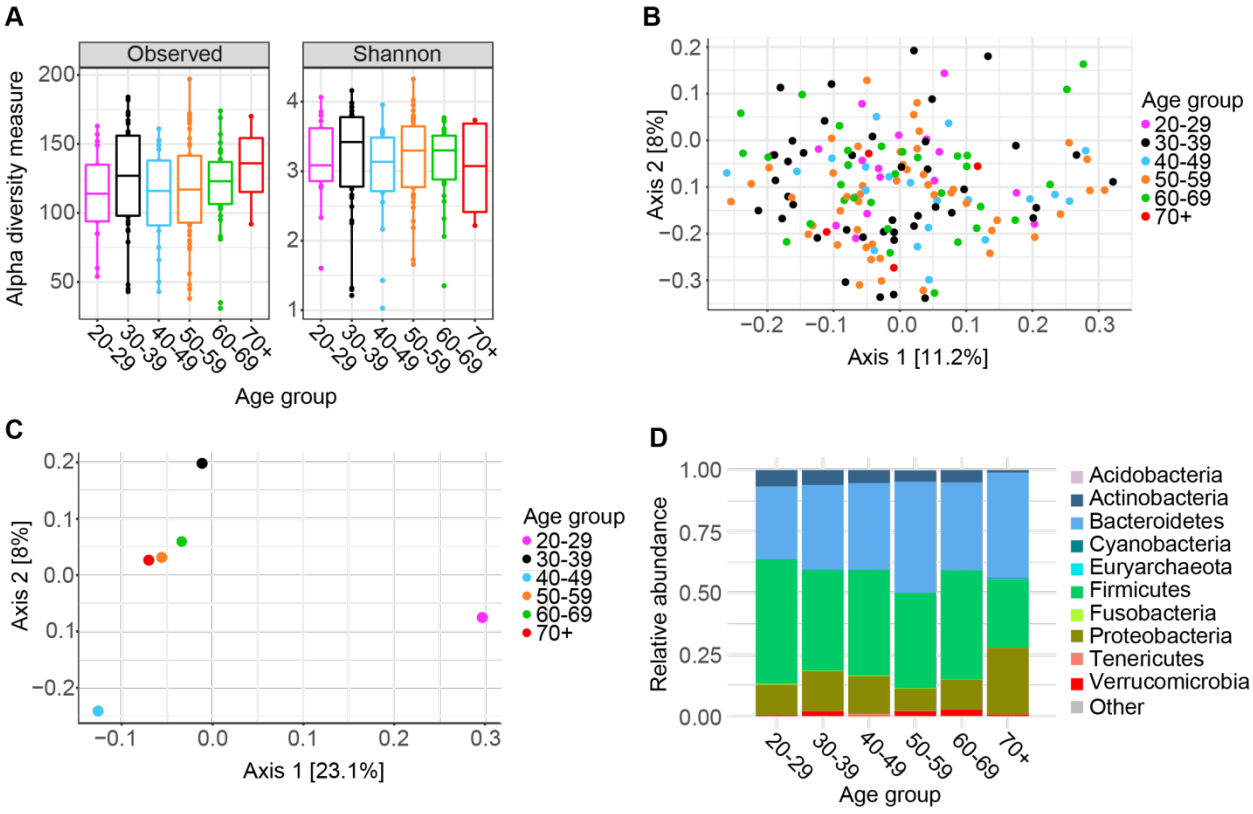

Figure 3.7 Alpha and beta diversity and top taxa plots for the fecal microbiome based on age group A) Boxplots of alpha diversity metrics, observed operational taxonomic units (OTUs) and Shannon diversity for the fecal microbiome, based on age group. B) Principal coordinate analysis plots of unweighted UniFrac distance matrix for the fecal microbiome, based on age group. Principal coordinate axis 1 explained 11.2% of the variance and principal coordinate axis 2 explained 8% of the variance; together, principal coordinate axes 1 and 2 explained 19.2% of variance. C) Principal coordinate analysis plot of unweighted UniFrac mean dissimilarity distance matrix for the fecal microbiome, based on age group. Principal coordinate axis 1 explained 23.1% of the variance and principal coordinate axis 2 explained 8% of the variance; together, principal coordinate axes 1 and 2 explained 19.2% of variance. D) Top 10 taxa for age group represented at the phylum taxonomic level for the fecal microbiome. Sample sizes: 20-29, 17; 30-39, 41; 40-49, 22; 50-59, 47; 60-69, 32; 70+, 4.

A summary table of the result of alpha and beta diversity based on gender, race, and age can found in **Table 3.4**.

Table 3.4 Table summary of alpha and beta diversity results based on comparisons with gender, race, and age

	Skin		Oral		Fecal	
Metric	Alpha	Beta	Alpha	Beta	Alpha	Beta
Gender	Observed OTUs and Shannon diversity; increased in males	Unweighted UniFrac				Unweighted UniFrac
Race		Unweighted UniFrac				Unweighted UniFrac
Age			Observed OTUs and Shannon diversity; decreased in 70+	Unweighted UniFrac		Unweighted UniFrac

[1]Table showing the results for the analysis of gender, race, and age as they relate to alpha and beta diversity of skin, oral, and fecal microbiomes. Green represents a significant *p*-value ($p < 0.05$), yellow represents a *p*-value approaching significance ($p \leq 0.10$), and red represents a *p*-value > 0.10. Abbreviations: OTU, operational taxonomic unit.

3.4 Discussion

Consistent with previous studies, we found robust differences in microbial community structure based on body site (i.e., skin, oral, and fecal microbiomes). We observed higher alpha diversity of the skin microbiome in males, relative to females, and differences in alpha diversity of the oral microbiome across age groups, with a decline in alpha diversity with age. Despite the diverse demographics of this Veteran cohort, analysis of beta diversity of the skin microbiome revealed differences based on gender and race. In contrast, analysis of beta diversity of the oral microbiome revealed differences based on age, while analysis of beta diversity of the fecal microbiome revealed differences based on gender and age. These findings are of potential interest in part as they identify specific variables that may be treated as covariates in analysis of the skin, oral, and fecal microbiomes in relation to other endpoints in this cohort of US Veterans, including general and physical health, insomnia, and mental health.

Skin microbiome

Despite the diverse demographics of this Veteran cohort, analysis of alpha diversity of the skin microbiome revealed differences based on gender, while analysis of beta diversity revealed differences based on gender and race. Males exhibited higher alpha diversity as shown by observed OTUs and Shannon diversity. Results of gender differences in alpha diversity in previous studies are conflicting, potentially due to non-standardized anatomical sampling locations. Nevertheless, a study sampling from the same anatomical location reported no effect of gender in Choa1, Phylogenetic distance, or Shannon diversity metrics.[52] Two studies of the skin microbiome reported that females had higher alpha diversity than males.[53,54] However, these studies did not sample from the same anatomical location as the samples collected from the Veteran cohort. Ross et al., (2017) sampled from several body sites, not including the antecubital fossa, and Fierer et al., (2006) sampled from the hand. A separate study of college dormitory rooms with passive settling plates showed that male-inhabited rooms had 67% more bacteria than female-inhabited rooms as determined by qPCR.[55] More studies are needed examining sex differences in skin bacteria with standardized anatomical sampling locations to further characterize sex differences in skin-associated alpha diversity metrics.

Consistent with previous studies, the most abundant taxa at the phylum level in skin microbiomes in this cohort were Firmicutes, Actinobacteria, and Proteobacteria.[56] Lower abundance phyla were also present, including Fusobacteria, which have previously been observed in forearm microbiomes, but not forehead microbiomes, of a subset (27%) of male and female participants.[56] Also consistent with previous studies, the most abundant taxa at the genus level in skin microbiomes in this cohort included *Corynebacterium*, *Staphylococcus*, and *Streptococcus*.[52,56–58] Results from this Veteran cohort are consistent with previous gender differences reported in the skin microbiome. For example, consistent with previous reports,[53,59]

males had higher relative abundance of the phylum, Actinobacteria, whereas females had higher relative abundance of the family Lactobacillaceae and genus *Lactobacillus* in skin samples.

Although there are few studies that specifically sampled the skin microbiome from the same anatomical location (antecubital fossa; inner elbow), as in our study, a number of studies have sampled the skin microbiome from the antecubital fossa, including Grice et al., (2008), Kong et al., (2012), Oh et al., (2012), Ying et al., (2015), and the Human Microbiome Project.[24,52,58,60–62] Results from this Veteran cohort are consistent with previous gender differences reported in the skin microbiome from samples taken from the antecubital fossa, especially when examining the data at the genus taxonomic level. The gender differences from the current study and results reported by Ying et al., (2015) showed the same gender-based enrichment and depletion patterns. In both studies, males showed enrichment in the genera *Anaerococcus* in the nares, *Corynebacterium* (1.6-fold enrichment in both studies), and *Streptococcus* (1.4-fold enrichment in the Veteran cohort, 3-fold enrichment in Ying et al., (2015)) in the antecubital fossa, while females showed enrichment in *Acinetobacter*, especially in skin samples from the back, and *Staphylococcus* in the nares. Although the enrichment patterns for the Veteran Cohort and Ying et al., (2015) were similar, the gender differences in these specific taxa in the Veteran cohort did not reach statistical significance.

Bacteria, like any living organism, are sensitive to environmental factors and there are important gender-driven physiological differences in skin characteristics that create an environment that favors certain bacterial community structures. Male skin is known to be thicker, produce more sebum (an "oily" secretion of mainly glycerides, free fatty acids, and esters from sebaceous glands[63]), produce more sweat during exercise, have higher surface pH, and lower intra-dermal pH (for review see[64]). Differences in pH, moisture levels, frequency of

moisture, and availability of different nutrients for energy production are well known environmental factors that lead to differences in bacterial community structures in different terrestrial ecosystems,[65–68] These same factors likely influence colonization of human skin by bacterial species, leading to differences in bacterial community structure observed between males and females.

Although we observed no differences in alpha diversity of the skin microbiome based on race, we did observe differences in beta diversity. Previous studies have found differences in alpha and beta diversity in the skin microbiome based on geographical location and by default different ethnic backgrounds.[57,69,70] However, to the best of our knowledge, this is the first study to demonstrate a difference in bacterial community structure based on race in the same geographical location. These results should be interpreted with caution because, of the 188 participants, 63.8% were Caucasian and some racial groups were highly underrepresented (i.e., 3 Asian, 6 Multiracial, and 5 Native American samples). This preliminary result needs to be reproduced in a sample population that is larger and more balanced than this current cohort.

Although previous studies have described age-related differences in the skin microbiome, we did not find age-related differences in this Veteran cohort. The lack of age-related differences in the skin microbiome in this Veteran cohort may be due to a restricted age range (24-77 years), which may exclude age ranges where the skin microbiome is known to change. These age ranges are in the first year of life[71,72] and around puberty in adolescents.[57,73] Veterans, by definition must be at least 18 years of age and the youngest participant in the Veteran cohort is 24 years of age. Since this cohort does not include participants within the age range that the microbiome is known to change it is not surprising that we did not see an effect of age in the skin microbiome.

Oral microbiome

Overall, the most abundant phyla in the oral microbiome were Firmicutes, Proteobacteria, and Bacteroidetes, while the most abundant genera were *Streptococcus* (whose members are the most abundant bacterial species in the mouth), *Haemophilus*, and *Veillonella*, consistent with previous studies.[62,74] In addition to effects of gender and race on the skin microbiome, we observed differences in the oral microbiome based on age, but not gender or race in this Veteran cohort. Although we did not find differences in specific taxa at the phylum or genus level across age groups, previous studies of microbiota in the oral cavity and atherosclerotic plaques of subjects with atherosclerosis demonstrating that higher levels of bacterial phyla seen in atherosclerotic plaques correlated with the bacterial phyla seen in the oral cavity suggest that changes in the microbiome of the oral cavity, particularly in older participants, may be important in general and physical health measures, discussed later in this dissertation.[75]

Fecal microbiome

Consistent with previous studies, the most abundant taxa in fecal microbiomes in this cohort included Firmicutes, Bacteroidetes, and Proteobacteria at the phylum level, and *Bacteroides*, *Prevotella*, and *Escherichia* at the genus level.[62,76] Although we observed no differences in alpha diversity of the fecal microbiome due to gender, race, or age group, we observed differences in the beta diversity of fecal microbiome in relation to gender and age in this Veteran cohort, while differences in relation to race approached statistical significance. We did not observe gender differences in the fecal microbiome community structure at the phylum level, but we did find increased Lactobacillaceae, and decreased Verrucomicrobiaceae,[77] in

females, relative to males, in line with previous studies.[78] Previous research has shown gender differences in the gut microbiome, albeit not in all studies. A study by Mueller et al., (2006) showed that males have increases in the genus *Bacteroides*, while another study by Haro et al., (2016) found the opposite result.[79,80] One study by Schnorr et al., (2014) showed profound gender differences in the gut microbiome in a hunter-gather population in Eastern Africa. However, these differences were mainly due to social hierarchies that created sex-related divergences in various aspects of lifestyle, including diet, which are likely not to be relevant to Western societies.[81] Further, the authors stated that these profound differences have not been documented in other human populations, and it is unlikely that similar differences would be observed in a modern westernized population. Yet, it remains possible that gender-driven dietary preference could lead to differences in bacterial community structure. Perhaps the difference between males and females in this Veteran cohort is driven by slight dietary differences. It should also be noted that the gender differences viewed in this Veteran cohort may be sensitive to unbalanced sample sizes (males, 85%; females 15%), in that the analysis of the collective community structure in a group with a lower sample size is more susceptible to be influenced by an outlying individual than analysis performed on a comparator group with a substantially larger sample size.[82]

Although analysis of the gut microbiome in this Veteran cohort found that differences in beta diversity of the gut microbiome in relation to race only approached statistical significance, previous studies have found differences. Gupta et al., (2017), published a comprehensive review of microbial studies with associations of the fecal microbiome and race/geography. This group noted that the racial/ geographical differences seen in fecal bacterial community composition may be most influenced by regional culinary differences between these populations.[76,83] Diet has

been shown to impact the gut microbiome and when diet is altered, an individual's gut microbiome is also altered.[76,81,84,85] In support of this notion, rural and isolated populations in Africa that share genetic lineage, but differ in dietary habits (fish- vs farming-based diets) show divergent gut microbiomes. The gut microbiomes of these African populations are more similar to individuals with their respective dietary habits, fish- or agriculture-based diets, in regions of the world thousands of miles apart than they are to each other.[86] Arguably, one of the most defining attributes separating cultures is dietary habits and gustatory preferences.

Finally, despite the fact that this Veteran cohort had a restricted age range, (24-77 years), we observed differences in beta diversity of the gut microbiome in relation to age group. It is well documented that the gut microbiome changes throughout the phases of life especially in the very early and late phases.[85,87,88] The microbiome changes substantially around the time that an infant transitions from breast feeding to a solid or semi-solid food diet,[89,90] where the microbiome transitions from a *Bifidobacterium*-dominant phenotype to an "adult phenotype" dominated by Bacteroidetes and Firmicutes.[91] The microbiome is relatively stable until the later stages in life when various physiological systems and processes decline in function such as gastrointestinal function and dental health.[92–94] A consistent finding with aging is an increase in the relative abundance of Proteobacteria, and a decrease in relative abundance of Actinobacteria (*Bifidobacterium*), but these changes are more pronounced in the extreme decades of life.[95]

3.5 Conclusions

This Veteran cohort has a rich and diverse range of metadata collected on demographics, armed forces service, physical health, and mental health metrics. The sample types exhibited differences in alpha and beta diversity metrics. Examining the sample types individually allowed for the identification of different bacterial community structures in relation to gender, race, and age group. Overall, our results were consistent with previous studies of different cohorts with

different demographics. This chapter aimed to characterize the skin, oral, and fecal microbiomes in a cohort of U.S. Veterans in relation to gender, race, and age. Now we will focus on how the microbiome is associated with various physiologic and behavioral states in this Veteran cohort.

Chapter 4. Association of general and physical health and the microbiome in a Veteran cohort

4.1 Introduction

Alexander Fleming's discovery of benzylpenicillin in 1928 changed the world of medicine and shaped how the medical community perceived the relationship that humans should have with bacteria and other microbes.[13] Especially at that time in history, this discovery was a major breakthrough in medicine and Sir Fleming was knighted and awarded the Nobel Prize in Physiology and Medicine in 1945 for his discovery. However, a new era is upon the medical community to revisit the relationship between microbes and humans. In a time when many non-communicable diseases (NCDs), principally chronic inflammation-related disorders, are on the rise,[14] scientists and physicians alike are turning to the microbiome for insights into how dysbioses of human microbiomes may be contributing to increasing rates of chronic low-grade inflammation in Western societies, and how the microbiome might be harnessed to improve outcomes in all disciplines of health.

Physical health, as it relates to the microbiome, is rather complex because there are single microbes that can be either detrimental or beneficial to physical health, while differences in the commensal microbial structure as a whole are being implicated in health and disease states of the host. There are virulent bacterial species and strains that well known to cause severe detriments to physical health such as *Mycobacterium tuberculosis*, *Clostridium botulinum*, *Salmonella*, and *Escherichia coli*. In contrast, probiotics such as *Lactobacillus* spp. and *Bifidobacterium* spp. can improve general health and wellbeing.[96–99]

An excellent example of the dichotomous relationship between microbes and physical health is that of *Clostridium difficile* (*C. diff*) infection. This microbe is highly virulent, causing severe and chronic diarrhea, and like its name implies, it is very difficult to eradicate. The

recommended treatment for *C. diff* infection is aggressive antibiotic treatment.[100] However, *C. diff* has a terribly high reinfection rate,[100] and the reason is because a stable microbiome is needed to outcompete *C. diff* in colonization. Unfortunately the antibiotics that kill *C. diff* also wipe out the rest of the commensal community allowing for *C. diff* to recolonize after cessation of the antibiotic treatment. One of the most effective treatments for *C. diff* is a fecal microbiome transplant where the patient receives the microbiome from a "healthy" individual in order compete with *C. diff*.[101] Clinicians have recognized that this treatment option is much more effective than traditional antibiotics regimens and have added fecal microbiome transplants to the most recent update of the *Clinical Practice Guidelines for Clostridium difficile Infection in Adults and Children*.[100]

Now that it is being fully embraced that there are clear interactions between the human microbiome and human health, researchers are trying to find clear links and microbial signatures of general and physical health. Several studies have targeted BMI to try and determine if there are relationships between physical health and important features of the microbiome, including alpha and beta diversity, and taxonomic composition. However, identifying concrete relationships between BMI and microbial diversity or composition has been difficult. Le Chatelier and colleagues (2013) showed that a subset of individuals (23%) with a low bacterial richness were characterized by increased overall adiposity, insulin resistance and dyslipidemia as well as an inflammatory phenotype when compared with individuals with high bacterial richness. However, other studies have reported no differences between obese and lean phenotypes in alpha diversity metrics.[24,85,102] Furthermore, no consistent associations between specific taxa and BMI have been found. Two taxa that received a lot of attention were Firmicutes and Bacteroidetes. Initially, it was shown that the Firmicutes/Bacteroidetes ratio increased with BMI.[47,103] However,

subsequent studies have shown the opposite or no relation of Firmicutes/Bacteroidetes ratio and BMI.[104–106]

In this Chapter, we explore how the microbiome is related to health measures collected in the Veteran Cohort. According to the World Health Organization (WHO), "Health is a state of complete physical, mental, and social well-being and not merely the absence of disease or infirmity."[107] As measures of health, we focused on analysis of BMI data and outcomes of the 36-Item Short Form Health Survey (SF-36). For the SF-36, we analyzed eight subdomains, including general health perception, which gauges the participant's views and expectations of their health. Also from the SF-36, we analyze the Physical Component Summary (PCS) score, which is a score that is determined by an algorithm that takes into account questions from all the subdomains of the SF-36 and is an overall measure of physical health. We use these measures to examine differences in alpha and beta diversity as well as differences in specific taxa that may provide insight into microbial signatures of general and physical health in this Veteran Cohort.

4.2 Materials and methods

4.2.1 General procedures

Please refer to Chapter 2 of this dissertation for details on *Participants and study design*, *Sample collection and preparation*, *Molecular processing*, and *Computational analyses*.

4.2.2 Short Form Health Survey (SF-36) metrics

We used the 36-Item Short Form Health Survey (SF-36)[108,109] to assess relationships between health and wellbeing and alpha diversity, beta diversity, and taxonomic composition of the fecal microbiome. In all cases, higher scores on the SF-36 reflect higher levels of functioning. We used the RAND scoring table to generate scores as a percentage of the total points possible for each specific area of functional health status, for a final score within each of the eight dimensions measured.[110] These dimensions of health and wellbeing were 1) physical functioning, 2) role limitations due to physical health problems, 3) role limitations role due to

emotional problems, 4) vitality (energy and fatigue), 5) general mental health (psychological health and wellbeing), 6) social functioning, 7) bodily pain, and 8) general health perceptions. For example, the general health perceptions metric is composed of five questions on the survey that are compiled into a composite general health score. The questions on the survey include the participant rating their health and addressing their view and expectations of their health. Below are the actual questions from the survey accompanied by the possible responses.

a) In general, would you say your health is

- 1 - Excellent
- 2 - Very good
- 3 - Good
- 4 - Fair
- 5 – Poor

How TRUE or FALSE is **each** of the following statements for you.

b) I seem to get sick a little easier than other people

c) I am as healthy as anybody I know

d) I expect my health to get worse

e) My health is excellent

Questions b-e above involve the following 5 responses:

- 1 - Definitely true
- 2 - Mostly true
- 3 - Don’t know
- 4 - Mostly false
- 5 - Definitely false

In addition to the scales above, we also analyzed the Physical Component Summary (PCS) score in relation to alpha and beta diversity, and taxonomic composition of the fecal microbiome. This metric is a comprehensive gauge of physical health that takes into account all questions that pertain to physical health in all of the eight subdomains of the SF-36.[111] This produces a single score that can be used as an overall measure of physical health. More information about how this metric is scored is in the *Scoring* section below.

Scoring

The metrics of the SF-36 are scored by converting responses into a score from 0-100, which represents the percentage of total possible scores achieved. For example, if there were 5 responses possible, the responses would be converted to percentage scores: 1 = 0, 2 = 25, 3 = 50, 4 = 75, 5 = 100. The percentage scores are ordered so that more favorable health states are assigned higher scores. In the example above, 1(rescore = 0) would be a less favorable health state and 5 (rescore = 100) would be a more favorable health state. The eight subdomains of the SF-36 are the mean of the rescored values of the questions included in the subdomain. Items left blank are not included in calculating subdomain scales. The PCS score is produced by an algorithm that is proprietary and no further information is available about what questions are used and how the score is actually generated.[111]

4.2.3 Microbiome results for skin and oral samples

In exploratory analysis of the microbiome data, only fecal microbiome associations were revealed as significant findings in metadata categories that were relevant to health, based on analysis of the eight subscales within the SF-36, or the PCS score from the SF-36. Therefore, no skin or oral microbiome results will be shown in this chapter. Furthermore, in exploratory

analysis of the microbiome data, only the general health perceptions scale of the SF-36 and the PCS score had associations with alpha diversity, beta diversity, or taxonomy of bacterial communities. Consequently, we focus in this chapter on analysis of general health and PCS scores.

4.2.4 Body-mass index designations

All BMI designations were gathered from information available from the Centers for Disease Control (CDC).

https://www.cdc.gov/healthyweight/assessing/bmi/adult_bmi/index.html

Healthy (BMI < 25.0) and not healthy (BMI ≥ 25.0) designations were determined by a BMI of 24.9 being listed as the upper limit for "normal or healthy" by the CDC and a BMI of 25.0 being listed as the lower limit of "overweight." Minimal (BMI < 25.0), moderate (BMI 25.0-29.9), and high (BMI ≥ 30.0) risk BMI categories were designated from weight status classifications of "normal or healthy" (BMI < 25.0), "overweight" (BMI 25.0-29.9), and "obese" (BMI ≥ 30) by the CDC in combination with the following statement on the CDC's BMI webpage (link above) "people who have obesity are at increased risk for many diseases and health conditions". The conditions listed by the CDC were: "all-causes of death (mortality)", high blood pressure (hypertension), high low density lipoprotein (LDL) cholesterol, low high density lipoprotein (HDL) cholesterol, or high levels of triglycerides (dyslipidemia), type 2 diabetes, coronary heart disease, stroke, gallbladder disease, osteoarthritis, sleep apnea and breathing problems, chronic inflammation and increased oxidative stress, some cancers (endometrial, breast, colon, kidney, gallbladder, and liver), low quality of life, mental illness such as clinical depression, anxiety, and other mental disorders, and bodily pain and difficulty with physical functioning.[112–117]

4.3 Results

4.3.1 BMI

Analysis of BMI based on gender, race, and age

In Chapter 3 of this dissertation, we showed that gender, race, and age were all factors that affected the fecal microbiome. Here, we examined these variables as they relate to BMI. There was no difference in BMI in male versus female participants, although this comparison approached statistical significance (Wilcoxon rank-sum test; $p = 0.09$) (**Figure 4.1C**). Kruskal-Wallis test failed to reveal differences among racial groups or age groups based on BMI (**Figure 4.1B, C**).

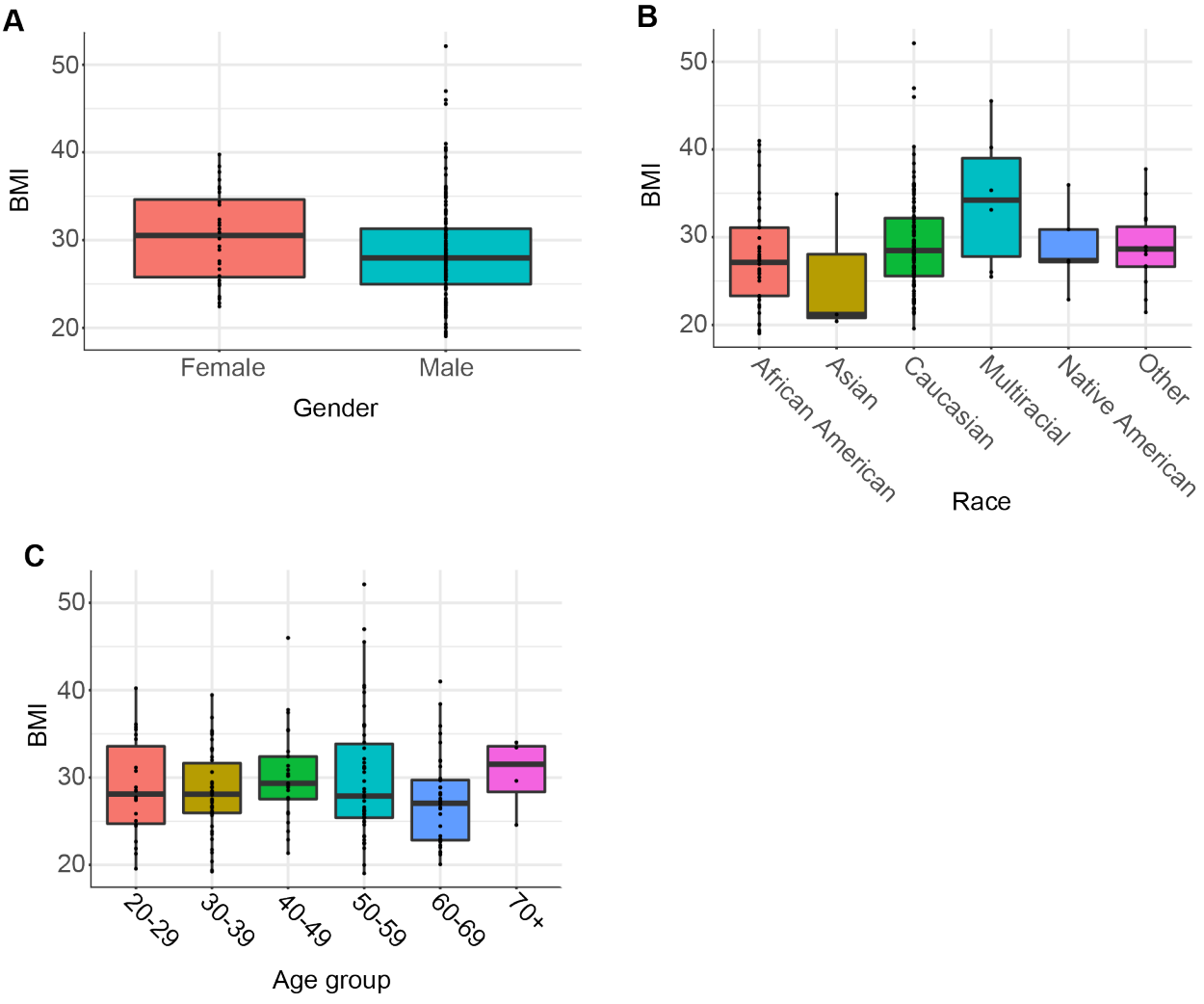

Figure 4.1 Body-mass index (BMI) relative to gender, race, and age group
A) Boxplots of BMI within gender. B) Boxplots of BMI within race categories. C) Boxplots of BMI within age groups. Abbreviations: BMI, body-mass index. Sample sizes: A) female, 25; male, 138. B) African American, 39; Asian, 2; Caucasian, 100; Multiracial, 5; Native American, 5; Other, 12. C) 20-29, 17; 30-39, 41; 40-49, 22; 50-59, 47; 60-69, 32; ≥70, 4.

Alpha and beta diversity in the fecal microbiome based on BMI

We performed several analyses on BMI designations as they associate with alpha and beta diversity. A summary table of results can be found in **Table 4.1**.

Table 4.1 Table of body-mass index (BMI) variables and their associations with alpha and beta diversity outcomes of skin, oral, and fecal microbiomes[1]

		Skin		Oral		Fecal	
Survey	Metric	Alpha	Beta	Alpha	Beta	Alpha	Beta
Body-mass index (BMI)	BMI correlation						
	BMI health categorization: healthy (<25), not healthy (≥25)						
	BMI risk categorization: minimal (<25), moderate (25-29.99), high (≥30)						
	BMI quartiles						

[1]Table showing the results for the initial analyses of BMI variables as they relate to alpha and beta diversity of skin, oral, and fecal microbiomes. Green represents a significant *p*-value ($p < 0.05$), yellow represents a *p*-value approaching significance ($p \leq 0.10$), and red represents a *p*-value > 0.10. Abbreviations: BMI, body-mass index.

Alpha diversity in the fecal microbiome was higher in the healthy BMI (BMI < 25) group measured by observed OTUs than the not healthy group (BMI ≥ 25) (Wilcoxon rank-sum test, $p = 0.05$) (**Figure 4.2A**). However, this result was not observed in Shannon diversity (**Figure 4.2A**). BMI was further divided into risk categories of minimal (BMI < 25.0), moderate (BMI 25-29.9), and high (BMI ≥ 30) risk for several diseases cited by the CDC to be associated with obesity (see Section 4.2.3, *Body-mass index designation*). Kruskal-Wallis test did not reveal any differences in observed OTUs or Shannon diversity based on BMI risk category (**Figure 4.2B**). In addition, there was no correlation between BMI and either alpha diversity metric, when including data from all participants ($n = 146$; **Figure 4.2C**). Principal coordinate analysis of weighted and unweighted UniFrac distance matrix and statistical assessment with PERMANOVA did not reveal differences in bacterial community structure for BMI divided into health categories (Healthy: BMI < 25; Not Healthy: BMI ≥ 25) or risk categories (Minimal risk,

Moderate risk, High risk) (data not shown). However, principal coordinate analysis of weighted UniFrac distance matrix and statistical assessment with PERMANOVA approached statistical significance in BMI divided into quartiles ($p = 0.1$; data not shown). Exploratory analysis of specific taxonomic differences between BMI quartiles with Kruskal-Wallis test at all taxonomic levels down to the genus level revealed only two taxa that were significantly different, *Ruminococcus* ($p < 0.05$), or approached significance, *Lactobacillus* ($p = 0.09$). Wilcoxon rank-sum test determined that the relative abundance *Ruminococcus* was lower in Quartile 2 as compared to Quartiles 1 and 4.

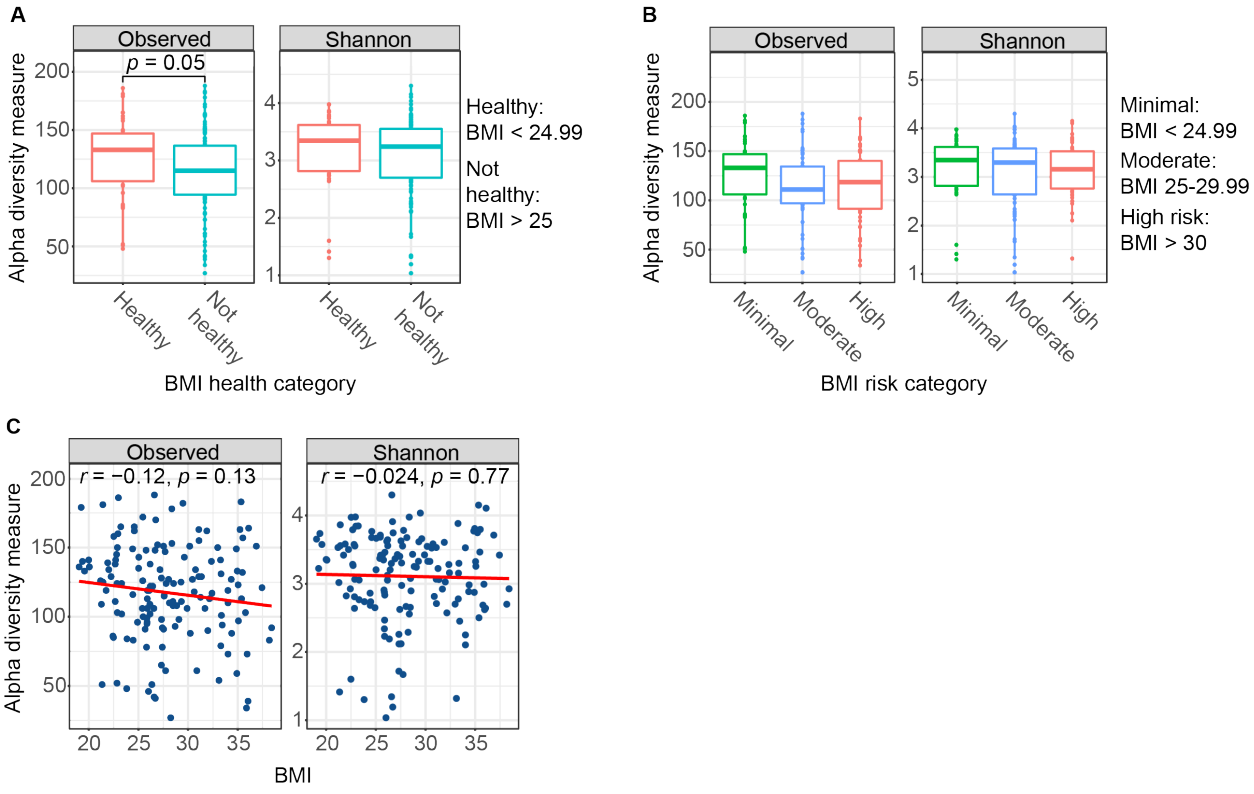

Figure 4.2 Alpha diversity plots in the fecal microbiome based on body-mass index (BMI) health and risk categories

A) Boxplot of observed operational taxonomic units (OTUs) and Shannon alpha diversity metrics in the fecal microbiome based on BMI categories of healthy (< 25.0) and not healthy (≥25.0) as designated by the Centers for Disease Control (CDC). B) Boxplots of observed OTUs and Shannon diversity metrics in the fecal microbiome based on BMI risk categories of several diseases mainly relating to cardiovascular health outlined by the CDC (see Section 4.2.3, *Body-mass index designations*). C) Scatter plots with regression lines of observed OTUs and Shannon alpha diversity metrics in the fecal microbiome based on BMI scores of all participants. Abbreviations: BMI, body-mass index; CDC, Centers for Disease Control;

OTU, operational taxonomic unit. Sample sizes: A) Healthy, 39; Not healthy, 107. B) Minimal risk, 48; Moderate risk, 39; High risk, 59.

Firmicutes/Bacteroidetes ratio

Previous studies have suggested a relationship between the Firmicutes/Bacteroidetes ratio and obesity, with an increased ratio in obese people compared to lean people, and a tendency for this ratio to decrease with weight loss (for reviews, see[118–120]). However, a number of studies have found no relationship.[105,121–125] Examination of the relationship between relative abundances of Firmicutes, Bacteroidetes, and the Firmicutes/Bacteroidetes ratio, relative to BMI showed no significant correlations (**Figure 4.3 A, B**).

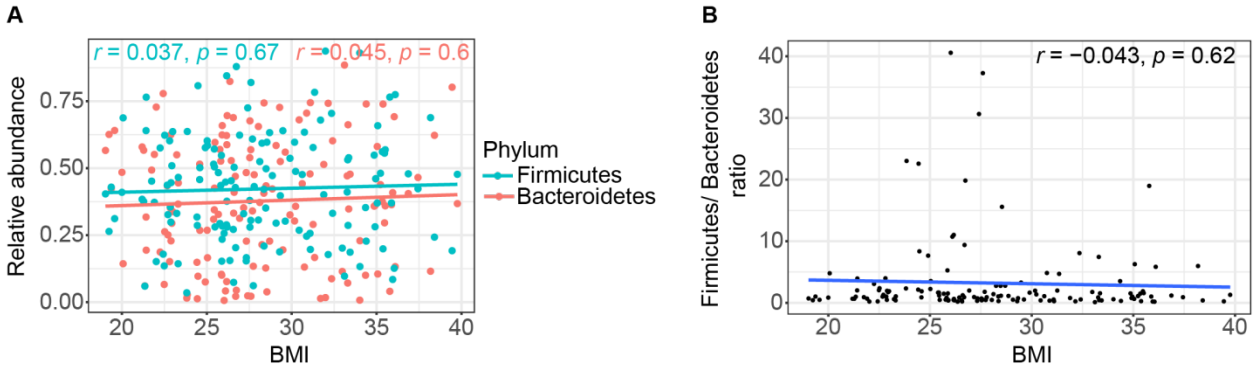

Figure 4.3 Relative abundances of Firmicutes and Bacteroidetes, and the Firmicutes/Bacteroidetes ratio, in the fecal microbiome relative to body-mass index (BMI)
A) Scatter plot and regression lines relating BMI scores to the relative abundance of Firmicutes (blue) and Bacteroidetes (red) in the fecal microbiome. B) Scatter plot and regression line relating BMI scores to the Firmicutes/Bacteroidetes ratio in the fecal microbiome. Abbreviations: BMI, body-mass index.

4.3.2 Short Form Health Survey (SF-36) metrics

General health and Physical Component Summary (PCS) scores based on BMI and age

We examined the association of general health and PCS scores to BMI and age group. General health scores displayed a negative relationship with BMI ($r = –0.21$, $p < 0.05$), but not with age ($r = –0.08$, $p = 0.27$) as determined using Pearson correlation (**Figure 4.4 A, B**). The PCS score exhibited negative relationships with both BMI ($r = –0.19$, $p < 0.05$) and age ($r = –$

0.3, $p < 0.001$) as determined using Pearson correlation (**Figure 4.4 C, D**). Physical function scores also displayed a negative relationship with BMI ($r = –0.21$, $p < 0.01$) as determined by Pearson correlation (data not shown).

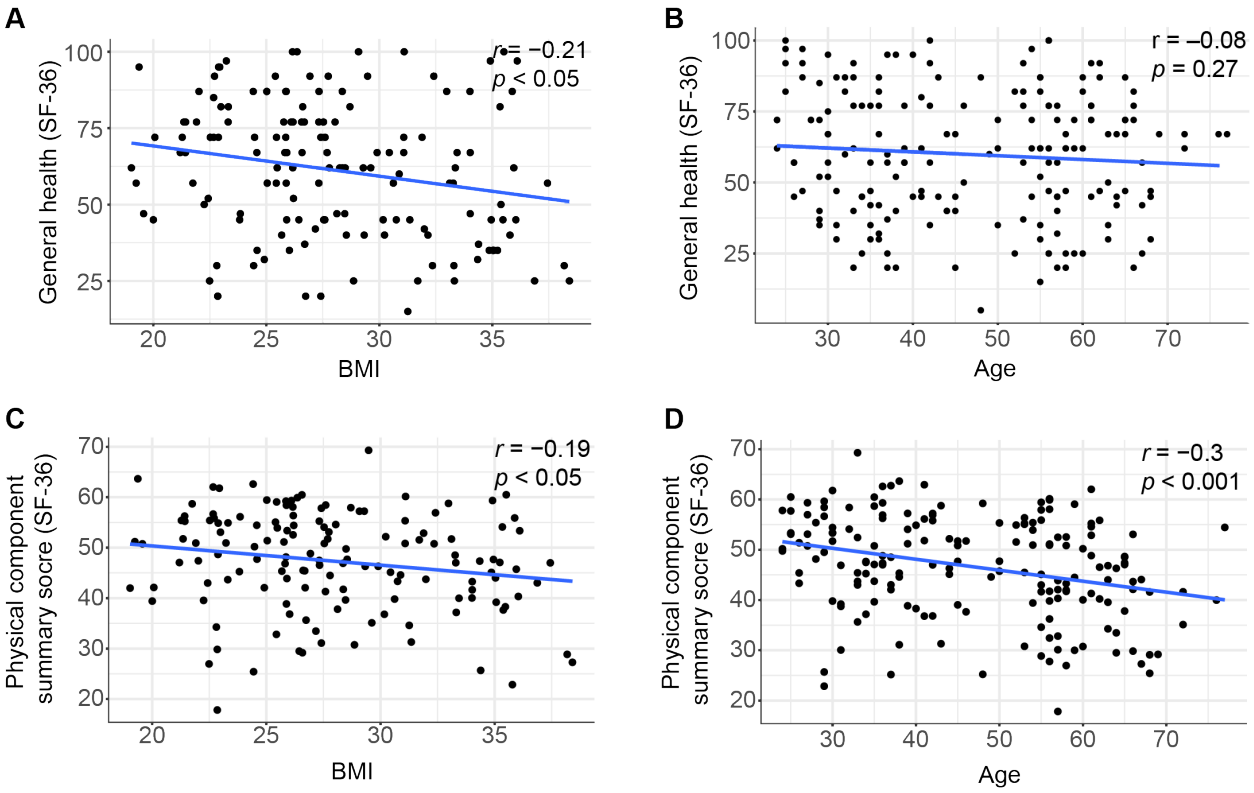

Figure 4.4 Relationships between body-mass index (BMI) or age and general health and Physical Component Summary (PCS) scores from the 36-item Short Form Health Survey (SF-36)
A) Scatter plot and regression line illustrating the relationship between BMI and general health scores of the SF-36. B) Scatter plot and regression line illustrating the relationship between age and the general health scores of the SF-36. C) Scatter plot and regression line illustrating the relationship between BMI and the PCS scores of the SF-36. D) Scatter plot and regression line illustrating the relationship between age and the PCS scores of the SF-36. Abbreviations: BMI, body-mass index; PCS, Physical Component Summary; SF-36, 36-Item Short Form Health Survey.

Analysis of alpha and beta diversity and top taxa in the fecal microbiome based on general health scores from the SF-36

We performed several analyses on the general and physical health variables within the SF-36 as they associate to alpha and beta diversity. Many of the results were null and a summary table of these results can be found in **Table 4.2**.

Table 4.2 Table of general and physical health variables within the 36-Item Short Form Health Survey (SF-36) and their associations with alpha and beta diversity outcomes of skin, oral, and fecal microbiomes[1]

		Skin		Oral		Fecal	
Survey	Metric	Alpha	Beta	Alpha	Beta	Alpha	Beta
36-Item Short Form Health Survey (SF-36)	Physical functioning score correlation						
	Physical functioning score quartiles						
	Role physical score correlation						
	Role physical score quartiles						
	General health score correlation						
	General health score quartiles						
	Bodily pain score correlation						
	Bodily pain score quartiles						
	Vitality score correlation						
	Vitality score quartiles						
	Social functioning score correlation						
	Social functioning score quartiles						
	Role-emotional score correlation						
	Role-emotional score quartiles						
	Mental health score correlation						
	Mental health score quartiles						
	Physical Component Summary (PCS) score correlation						
	Physical Component Summary (PCS) score health category: healthy (≥45), not healthy (<45)						
	Physical Component Summary (PCS) score quartiles						
	Mental Component Summary (MCS) score correlation						
	Mental Component Summary (MCS) score health category: healthy (≥45), not healthy (<45)						
	Mental Component Summary (MCS) score quartiles						

[1]Table showing the results for the initial analyses of general and physical health variables within the SF-36 as they relate to alpha and beta diversity of skin, oral, and fecal microbiomes. Green represents a significant *p*-value ($p < 0.05$), yellow represents a *p*-value approaching significance ($p \leq 0.10$), and red represents a *p*-value > 0.10. Abbreviations: SF-36, 36-Item Short Form Health Survey; PCS, Physical Component Summary; MCS, Mental Component Summary.

Analysis of the alpha diversity of the fecal microbiome by Kruskal-Wallis, based on analysis of quartiles of general health scores from the SF-36, revealed a difference in observed OTUs ($p < 0.05$) but not Shannon diversity. Further analysis with Wilcoxon rank-sum test

revealed higher observed OTUs in the uppermost quartile (representing greater general health), relative to the first and second quartile ($p < 0.05$) (**Figure 4.5A**). These results were mirrored by analysis, using Pearson correlation, of the relationship between the SF-36 general health scores and alpha diversity measures (**Figure 4.5B**). Observed OTUs increased with general health scores ($r = 0.15$, $p = 0.05$) and Shannon diversity showed no relationship with general health scores ($r = 0.1$, $p = 0.19$) (**Figure 4.5B**). Principal coordinate analysis of the weighted UniFrac distance matrix and further assessment with PERMANOVA revealed that the bacterial community structure differed among general health quartiles ($p < 0.01$) (**Figure 4.5C**). Analysis of specific taxa with Kruskal-Wallis test revealed that the phylum Actinobacteria ($p < 0.01$) differed among general health quartiles (**Figure 4.5D**). Actinobacteria were higher in Quartile 1 as compared to every other quartile (Wilcoxon rank-sum test; Q1, $p < 0.05$; Q3 & Q4, $p < 0.01$). Analysis of specific taxa at the class taxonomic level with the Kruskal-Wallis test revealed that Coriobacteriia (phylum: Actinobacteria) ($p < 0.05$) and Bacilli (phylum: Firmicutes) ($p < 0.05$) differed among the general health quartiles. Coriobacteriia were higher in Quartile 1 as compared to Quartile 3 (Wilcoxon rank-sum test; $p < 0.01$). Bacilli were higher in Quartile 1 as compared to all other Quartiles (Wilcoxon rank-sum test; Q2 & Q4, $p < 0.05$; Q3, $p < 0.01$). Analysis of specific taxa at the order taxonomic level using the Kruskal-Wallis test revealed Coriobacteriales (phylum: Actinobacteria) ($p < 0.05$) and Lactobacillales (phylum: Firmicutes) ($p < 0.05$) differed among the general health quartiles. Coriobacteriales were higher in Quartile 1 as compared to Quartile 3 (Wilcoxon rank-sum test; $p < 0.01$). Lactobacillales were higher in Quartile 1 as compared to Quartile 3 (Wilcoxon rank-sum test; $p < 0.01$). Analysis of specific taxa at the family taxonomic level with the Kruskal-Wallis test revealed Coriobacteriaceae (phylum: Actinobacteria) ($p < 0.05$) and Streptococcaceae (phylum: Firmicutes) ($p < 0.05$) differed among

the general health quartiles. Coriobacteriaceae were higher in Quartile 1 as compared to Quartile 3 (Wilcoxon rank-sum test; $p < 0.01$). Streptococcaceae were lower in Quartile 3 as compared to Quartile 1 (Wilcoxon rank-sum test; $p < 0.01$). No genera were determined to be different in the general health quartiles based on analysis using the Kruskal-Wallis.

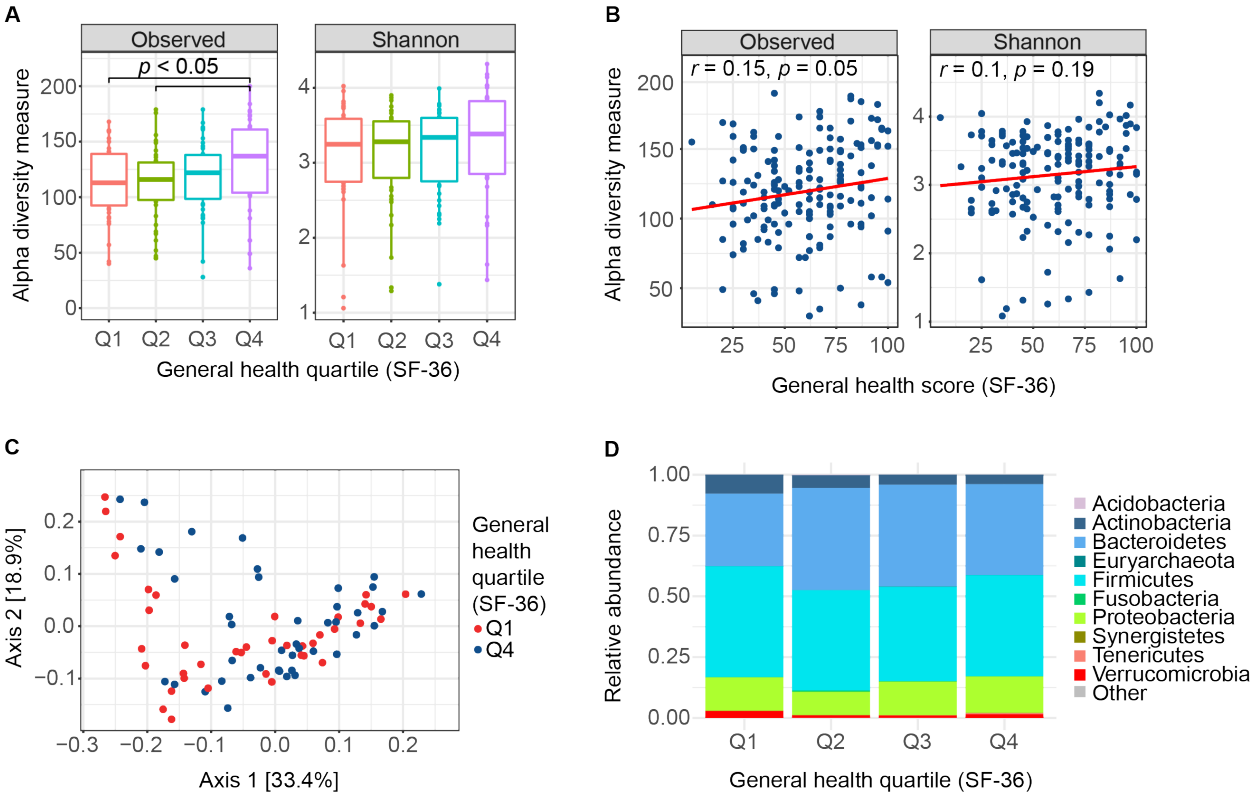

Figure 4.5 Alpha and beta diversity and top taxa plots for the fecal microbiome based on the general health metric of the 36-Item Short Form Health Survey (SF-36)
A) Boxplots of alpha diversity metrics, observed operational taxonomic units (OTUs) and Shannon diversity in the fecal microbiome based on quartiles of general health scores from the SF-36. B) Scatter plots with regression lines of alpha diversity metrics, observed OTUs and Shannon diversity, in the fecal microbiome based on general health scores from the SF-36. C) Principal coordinate analysis of weighted UniFrac distance matrix in the fecal microbiome based on general health scores of the SF-36. Only Quartiles 1 and 4 shown. Principal coordinate axis 1 explained 33.4% of the variance and principal coordinate axis 2 explained 18.9% of the variance; together, principal coordinate axes 1 and 2 explained 52.3% of variance. D) Top ten phyla in fecal microbiome based on general health quartiles. Abbreviations: SF-36, 36-Item Short Form Health Survey; OTU, operational taxonomic unit. Sample sizes: Q1, 40; Q2, 40; Q3, 40; Q4, 41.

Analysis of alpha and beta diversity and top taxa in the fecal microbiome based on the PCS scores of the SF-36

Alpha diversity was higher in healthy individuals (PCS score ≥ 45) than not healthy individuals (PCS score < 45) in observed OTUs (Wilcoxon rank-sum test, $p < 0.05$), but not in Shannon diversity (**Figure 4.6A**). Analysis of the relationship between PCS scores and alpha diversity using Pearson correlation revealed a positive correlation for observed OTUs ($r = 0.18$, $p < 0.05$) and a positive relationship that approached statistical significance for Shannon diversity ($r = 0.16$, $p = 0.051$) (**Figure 4.6B**). Principal coordinate analysis of the unweighted UniFrac distance matrix and further assessment with PERMANOVA revealed that the bacterial community structure differed among PCS health designation ($p < 0.05$; **Figure 4.6C**) and PCS quartiles ($p < 0.01$) (Q1-Q4; **Figure 4.6D**). Analysis of specific taxa by Wilcoxon rank-sum test based on PCS health designation revealed no differences at the phylum taxonomic level (**Figure 4.5E**). Analysis of specific taxa by Wilcoxon rank-sum test at the class taxonomic level revealed enrichment of Gammaproteobacteria (phylum: Proteobacteria) ($p < 0.05$) in not healthy individuals. Analysis of specific taxa by Wilcoxon rank-sum test at the order taxonomic level revealed enrichment of Enterobacteriales (phylum: Proteobacteria) ($p < 0.01$) in not healthy individuals. Analysis of specific taxa by Wilcoxon rank-sum test at the family taxonomic level revealed enrichment of Enterobacteriaceae (phylum: Proteobacteria) ($p < 0.01$) in not healthy individuals and Paraprevotellaceae (phylum: Bacteroidetes) ($p < 0.05$) and Ruminococcaceae (phylum: Firmicutes) ($p < 0.05$) in healthy individuals. Analysis of specific taxa by Wilcoxon rank-sum test at the genus taxonomic level revealed enrichment in *Escherichia* (phylum: Proteobacteria) ($p < 0.05$) in not healthy individuals and enrichment of *Faecalibacterium* (phylum: Firmicutes) ($p < 0.01$), *Roseburia* (phylum: Firmicutes) ($p < 0.05$), and *Coprococcus* (phylum: Firmicutes) ($p < 0.05$) in healthy individuals.

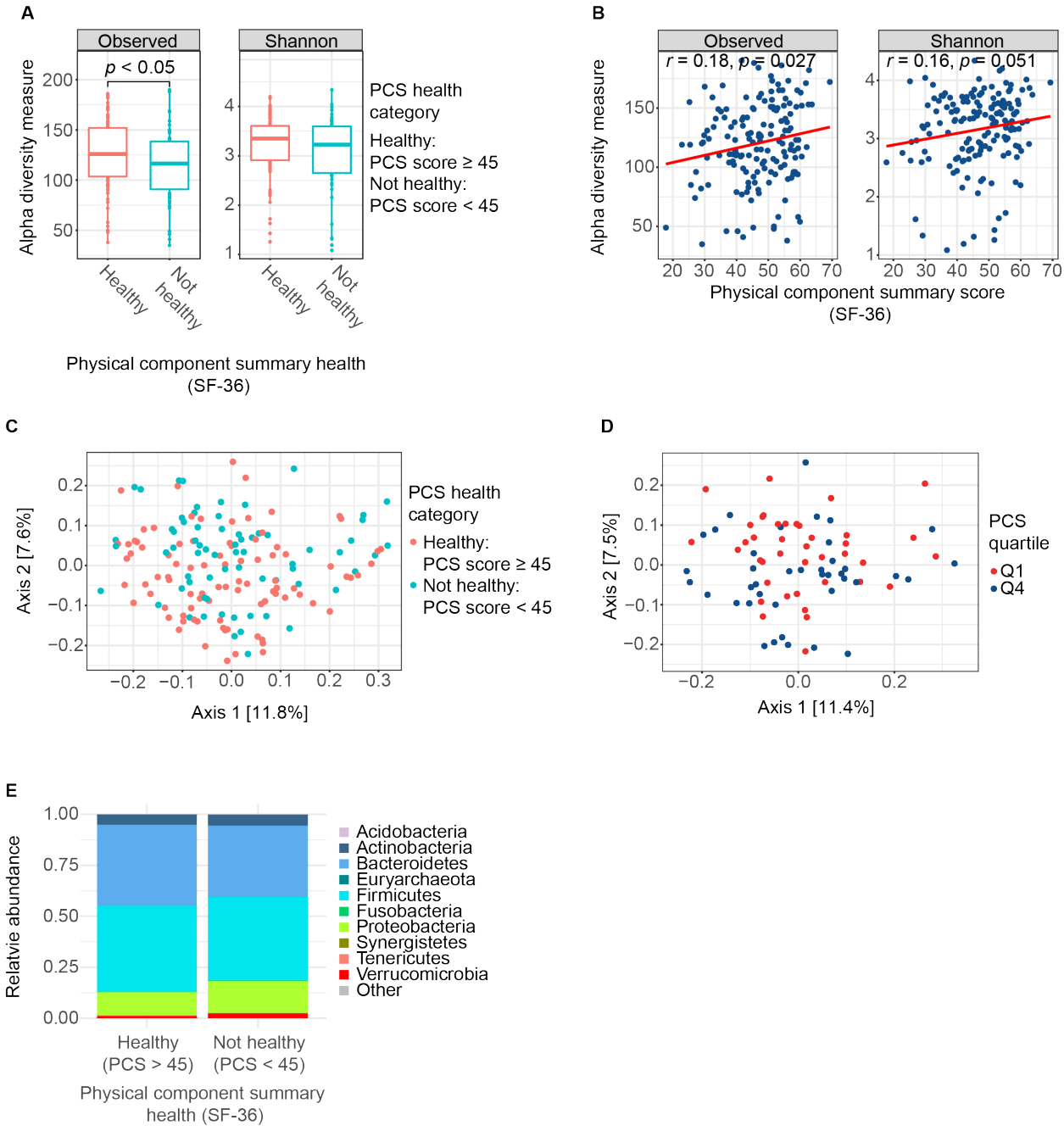

Figure 4.6 Alpha and beta diversity and top taxa plots in the fecal microbiome based on physical Component Summary (PCS) scores of the 36-Item Short Form Health Survey (SF-36)
A) Boxplots of alpha diversity metrics, observed operational taxonomic units (OTUs) and Shannon diversity, in the fecal microbiome based on the PCS metric of the SF-36. PCS scores were collapsed into healthy (PCS ≥ 45) and not healthy (PCS < 45) as designated by the developers of the survey. B) Scatter plots and regression lines of alpha diversity metrics, observed OTUs and Shannon diversity in the fecal microbiome based on PCS scores. C) Principal coordinate analysis of unweighted UniFrac distance matrix in the fecal microbiome, based on PCS health designation of healthy (PCS ≥ 45) and not healthy (PCS < 45). Principal coordinate axis 1 explained 11.8% of the variance and principal coordinate axis 2 explained 7.6 % of the variance; together, principal coordinate axes 1 and 2 explained 19.4% of variance.

D) Principal coordinate analysis of unweighted UniFrac distance matrix in the fecal microbiome, based on PCS quartiles. Analysis included quartiles 1- 4, but only quartiles 1 and 4 are shown. Principal coordinate axis 1 explained 11.4% of the variance and principal coordinate axis 2 explained 7.5 % of the variance; together, principal coordinate axes 1 and 2 explained 18.9% of variance. E) Top ten phyla in fecal microbiome based on the PCS health designation of healthy (PCS ≥ 45) and not healthy (PCS < 45). Abbreviations: OTUs, operational taxonomic units; PCS, Physical Component Summary; SF-36, 36-Item Short Form Health Survey. Sample sizes: A, E) Healthy, 91; Not healthy, 69. C, D) Q1, 40; Q2, 39; Q3, 39; Q4, 40.

Analysis of the relationship between PCS and general health scores of the SF-36 using Pearson correlation revealed that the two metrics are highly correlated ($r = 0.72$, $p < 0.001$). Analysis of the relationship between PCS and physical function scores of the SF-36 using Pearson correlation revealed that the two metrics are highly correlated ($r = 0.72$, $p < 0.001$). Analysis of the microbiome and physical function scores from the SF-36 revealed no differences in alpha or beta diversity.

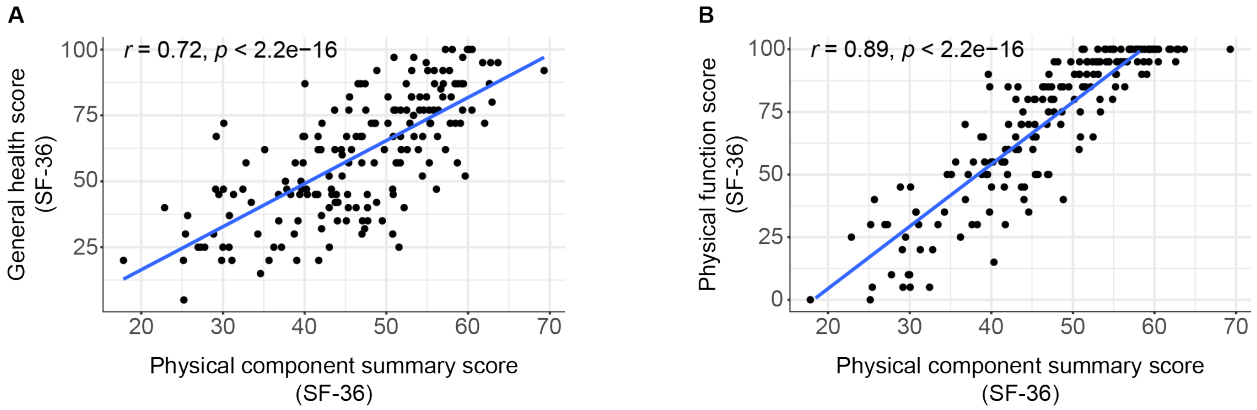

Figure 4.7 Correlation of physical Component Summary (PCS) score and general health score and physical function score from the SF-36
A) Graph illustrating the relationship between PCS and general health scores from the SF-36, using Pearson correlation. B) Graph illustrating the relationship between PCS and physical function scores from the SF-36, using Pearson correlation. Abbreviations: PCS, physical Component Summary; SF-36, 36-Item Short Form Health Survey.

4.4 Discussion

In this Veteran cohort, we found that BMI was not different based on gender, race, or age group. However, there was a relationship between BMI and measures of health. Higher BMI was correlated with lower health, as measured by the physical functioning, general health and PCS

scores from the SF-36, but not any other subscores from the SF-36. In line with these findings, general health scores and PCS scores as well as physical function scores and PCS scores were highly correlated with each other. As for how BMI and health measures from validated surveys were associated with the microbiome, observed OTUs were lower in all the health metrics that have been designated as "not healthy" (BMI ≥ 25, lower general health quartiles, and PCS scores < 45). Further, raw general health and PCS scores from the SF-36 were positively correlated with observed OTUs. Based on analysis of quartiles of general health scores, differences in general health scores were associated with differences in bacterial community structure. Finally, based on analysis of PCS score health designations (healthy ≥ 45; not healthy < 45) groups differed in bacterial community structure, with not healthy individuals displaying enrichment in several taxa from the Proteobacteria phylum while healthy individuals displayed enrichment in obligate anaerobic taxa belonging to the Firmicutes and Bacteroidetes phyla.

4.4.1 BMI in gender, race, and age groups

Gender

The National Center for Health Statistics reported that from 2011-2014 women showed higher rates of obesity (38.3%) than men (34.3%).[126] This Veteran cohort, as mentioned previously, has far fewer females (15% of sample) than males (85% of sample). In this gender biased sample, we were unable to detect differences in BMI in female and male participants. In this Veteran cohort, 54% of women and 41% of men were obese, higher than mean rates of reported nationally between 2011-2014, consistent with previous reports that obesity rates are higher in Veteran populations than non-Veteran populations.[127]

Race

Several studies have shown variability in BMI, body fat composition, body fat distribution, lean body mass, and overall adiposity among racial and ethnic groups.[128–130] A comprehensive review cited by the CDC reported that age-adjusted obesity rates among adults in the United States between 2011 and 2012 were: non-Hispanic blacks (48.1%), Hispanics (42.5%), non-Hispanic whites (34.5%), and non-Hispanic Asians (11.7%).[131] The obesity rates in this Veteran cohort based on race were: African American (22.8%; *n* = 39), Asian (50.0%; *n* = 2), Caucasian (36.3%; *n* = 100), Multiracial (50.0%; *n* = 5), Native American (40.0%; *n* = 5), and Other (22.2%; *n* = 12). Similar obesity rates were seen in Caucasian populations between the national survey and Veterans in this cohort, while African American obesity rates in this Veteran cohort (*n* = 39) were much lower than the national survey. The obesity rate in African Americans in this Veteran cohort were more similar to the obesity rate of African Americans specifically in Colorado (25-30%) as reported by the CDC.[132] Interpretation of these results outside of African American and Caucasian Veteran populations were limited because of sample size.

Age

Our data are similar to data reported by the CDC from the same study mentioned above examining obesity across the United States between 2011 and 2012. The CDC study reported obesity rates in the following age groups: 20-39 (32.3%), 40-59 (40.2%), and 60+ (37%).[131] Obesity rates in this Veteran cohort in the same age groups were: 20-39 (34.6%; *n* = 58), 40-59 (39.3%; *n* = 69), and 60+ (18.1%; *n* = 36). The obesity rates among the 20-39 and 40-59 age groups were comparable to the national survey, while obesity rates in the 60+ age group in this Veteran cohort was much lower than obesity rates for the same age group in the national survey. The CDC does not report on the age group of 60+ specifically in Colorado; however Colorado

has the lowest rate of obesity when compared to the rest of the continental US and US territories.[132]

4.4.2 Microbiome and BMI

Alpha diversity measured by observed OTUs was higher in individuals with a healthy BMI (BMI < 25) than a not healthy BMI (BMI ≥ 25). However, there were no differences in alpha diversity when categorizing BMI by risk category, and there was no correlation between BMI and alpha diversity measures using raw BMI scores. Previous studies have reported inconsistent findings in relation to the association between BMI or body weight the gut microbiome. Some studies have observed decreases in observed OTUs in obese individuals,[133–135] while other studies have reported no differences between obese and lean phenotypes in alpha diversity metrics.[24,85,102] Despite results of the transfer of an obesity phenotype to gnotobiotic mice by microbiome transplant from obese humans,[136] concrete associations between BMI and characteristics of the gut microbiome have yet to be established, suggesting that there are a number of confounding variables.

A meta-analysis of several studies relating BMI and obesity to the microbiome suggested that the use of a closed reference OTU picking scheme may be behind the lack of differences seen in alpha diversity.[137] The authors of the meta-analysis suggest that a closed reference OTU picking scheme may be throwing out OTUs that are not held within the database and therefore artificially decreasing the number of OTUs being observed within samples. The microbial data in this veteran cohort was analyzed using a UCLUST pipeline which utilizes a *de novo* approach to assigning OTUs.[138,139] This approach does not throw out OTUs and therefore could be a better assessment of true alpha diversity. This could also explain why our data show significant differences in observed OTUs between healthy and not healthy BMI categories.

We found no differences in bacterial community structure based on BMI divided into health categories or risk categories. A meta-analysis by Walters et al., (2014) of several studies[24,85,102,134,135] examining obesity and the microbiome did not detect differences in bacterial community structure between obese and lean phenotypes. The authors compiled data from several studies and used PCoA analysis to assess beta diversity or bacterial community structure between lean and obese phenotypes. Visual clustering of samples from lean or obese individuals was not immediately discernable and further analysis by PERMANOVA did not yield any significant results.[137] The authors of this meta-analysis were quick to disclose that there was very little standardization in the molecular techniques used to process the samples from the various studies, citing, specifically, that there were differences in extraction techniques, PCR primers, and sequencing platforms. These inconsistencies are not trivial and the field is divided on whether datasets with the disparities detailed above are indeed comparable.[140–143] Fortunately, this meta-analysis was not comparing studies to each other, but more looking into each of the studies to determine if there was consensus on associations between BMI and features of the fecal microbiome.

Previous studies have reported conflicting findings in regard to associations between specific taxa and BMI. One of the main associations of the microbiome and BMI that received a lot of attention was the ratio of the phyla Firmicutes to Bacteroidetes. These phyla are commonly the two most abundant phyla observed in the human gut and initial research published in high impact journals showed that high Firmicutes/Bacteroidetes ratios were associated with high BMI.[47,103] Since these initial findings, this observation has been corroborated in several studies[134,144–146] and disputed in several others.[104–106,147,148] In this Veteran cohort, we found no relationship between Firmicutes or Bacteroidetes or the Firmicutes/Bacteroidetes ratio and BMI.

Further analysis of taxonomy within BMI quartiles revealed that the only bacterial taxa that significantly differed among quartiles was the genus *Ruminococcus*. *Ruminococcus* was implicated by Arumugam et al., (2011) as one of the three purposed "enterotypes" that received much scrutiny,[149] and has been shown in a previous study to vary with BMI.[26] We observed that in the second quartile *Ruminococcus* was reduced relative to the other three quartiles, which showed similar relative abundances of *Ruminococcus*. It is difficult to determine the physiologic relevance of this finding. Further it has been shown that the relative abundance of the genus *Akkermansia*[26] exhibits a negative relationship with BMI, while *Lactobacillus* shows a positive relationship with BMI.[137] In this Veteran cohort *Akkermansia* did not differ in any of our divisions of BMI, but *Lactobacillus* was approaching statistical significance among BMI quartiles.

4.4.3 Associations between BMI or age and general health and PCS metrics of the SF-36

General health and PCS scores from the SF-36 survey displayed negative relationships with BMI in this Veteran cohort. BMI is a convenient and quick indirect measure of body fat and is correlated with several metabolic diseases (for full list please refer to the *Body-mass index designations* segment of the materials and methods section). However, BMI has been disputed in being a poor measure of actual body fat,[150–152] especially in athletes.[153,154] The results from this Veteran cohort follow the "normal" associations of BMI with general and physical health; i.e., increased BMI is correlated with decreased general and physical health. Similarly, PCS scores from the SF-36 decrease with increasing age. In contrast, general health showed no relationship to age. This could be because our sample has a limited age range, with only 5 participants 70 years of age or older.

4.4.4 Relationship between the general health metric of the SF-36 and the fecal microbiome

We observed increased alpha diversity in the gut microbiome in individuals with higher general health perception scores on the SF-36, as measured by observed OTUs, a measure of species richness, when general health was categorized by quartiles. Those in the highest quartile had increased alpha diversity relative to those in the first and second quartile. This finding was supported by a significant correlation between general health scores and observed OTUs, when assessing all individuals in the cohort. According to Ware et al., (1992), the general health dimension of the SF-36 can be interpreted such that individuals with low scores believe personal health is poor and likely to get worse, while individuals with high scores believe personal health is excellent. In both humans[133] and animal models,[134] increased alpha diversity is associated with increased measures of health. Although the mechanisms underlying this association are not clear, studies in animal models have demonstrated that stress exposure frequently decreases alpha diversity,[155,156] and stress-induced reductions of alpha diversity are associated with increased proliferation of gut pathogens, including pathobionts, such as *Helicobacter* spp., that are frequently present in the microbiome.[157–159] The stress-induced expansion of *Helicobacter* spp. is mediated by glucocorticoid actions on the glucocorticoid receptor, and therefore may be a direct outcome of stress exposures.[158,159]

In addition to these effects on alpha diversity, we observed differences in beta diversity in relation to general health perception scores, based on principal coordinate analysis of the weighted UniFrac distance matrix and further assessment with PERMANOVA. The bacterial community structure differed among the quartiles of the general health perception scores. Analysis using Wilcoxon rank-sum test revealed that the relative abundance of a number or

taxonomic groups within the phyla Actinobacteria and Firmicutes were increased in quartile 1 (representing lower general health perception) relative to quartile 3 (representing higher general health perception). For Actinobacteria, these included Actinobacteria (taxonomic level: phyla), Coriobacteriia (taxonomic level: class), Coriobacteriales (taxonomic level: order), and Coriobacteriaceae (taxonomic level: family). For Firmicutes, these included Bacilli (taxonomic level: class), Lactobacillales (taxonomic level: order), and Streptococcaceae (taxonomic level: family). These results are similar to previous research showing increased Actinobacteria and Firmicutes and decreased Bacteroidetes in obese individuals relative to lean individuals.[160] In addition, this group showed that increases in Actinobacteria and Firmicutes were associated with decreases in alpha diversity, which was also observed in this Veteran cohort.[160] Finally, metagenomics analysis of obese vs lean individuals showed that of the genes that were enriched in obese individuals 75% of them were derived from Actinobacteria and 25% were derived from Firmicutes.[160] We observed a negative relationship between general health and BMI, which may explain how these results align with results from obese individuals.

4.4.5 Relationship between PCS scores from the SF-36 and the fecal microbiome

Alpha diversity measured by observed OTUs was higher in healthy individuals (PCS ≥ 45) than in not healthy individuals (PCS < 45). Likewise, raw PCS scores showed a positive relationship with observed OTUs and a positive relationship that approached statistical significance with Shannon diversity. These data are consistent with previous research that has shown that alpha diversity of the gut microbiome is reduced in individuals with a number of gastrointestinal disease states, including Crohn's disease,[161–163] irritable bowel syndrome,[164–166] and colorectal cancer (for review see[167]), as well as systemic disorders such as systemic

inflammatory response syndrome [168] and metabolic disorders (type 1[169] and type 2,[120,170] as well as diabetes and metabolic syndrome.[114,171–173]

Principal coordinate analysis of the unweighted UniFrac distance matrix and further analysis by PERMANOVA determined that the bacterial community structure differed between healthy (PCS $\geq$ 45) and not healthy (PCS < 45) individuals. Further analysis, using Wilcoxon rank-sum test, of specific taxa, revealed that the relative abundance of a number or taxonomic groups within the Proteobacteria phylum were increased in individuals with PCS scores in the not healthy range. These taxa included Gammaproteobacteria (taxonomic level: class), Enterobacteriales (taxonomic level: order), Enterobacteriaceae (taxonomic level: family), and *Escherichia* (taxonomic level: genus).

Proteobacteria are gram-negative bacteria that express lipopolysaccharide (LPS, an endotoxin that acts on toll-like receptor 4 to induce a cellular inflammatory response) on the surface of their outer membrane. Severe endotoxemia can cause septic shock.[174] In a study by Karl and colleagues, those individuals who had higher relative abundances of Proteobacteria had increased gut permeability in response to a stressful long distance march.[175] These findings are supported by animal studies showing increased relative abundance of Proteobacteria in response to stress exposure.[157,176,177] Sustained low grade inflammation, potentially driven by LPS from increased Proteobacteria in the gut, is well known to be associated with the development of metabolic disorders (for review see[178]) and is also thought to be a risk factor in stress-related psychiatric disorders, including anxiety and affective disorders.[179] Studies have shown that 4 weeks of high fat diet consumption in mice increases Proteobacteria species in the gut and induces endotoxemia with blood concentrations of bacterially-derived LPS reaching levels that are 2-3 fold higher than concentrations found in mice given a control diet.[180] Further, 4 weeks of

subcutaneous injections of LPS, leading to a chronic endotoxemia, leads to the same metabolic complications as those found in mice maintained on a high fat diet.[180] In a follow-up study, endotoxemia and fecal concentrations of LPS were reduced in both control and high fat diet mice in response to antibiotic treatment. Severity of metabolic complications in high fat diet mice were also reduced in response to antibiotic administration.[181]

Proteobacteria are not only associated with metabolic disorders but several other disease states. The relative abundance of Gammaproteobacteria has been shown to be increased in inflammatory bowel diseases,[163,182,183] while the genus *Escherichia* has been shown to be increased in ileal Crohn's disease[184] and spontaneous colitis.[185] Enterobacteriaceae has been shown to be enriched following increased consumption of artificial sweeteners.[186] Increased relative abundance of Proteobacteria is frequently observed in various disease states and this has led some to label Proteobacteria as markers of dysbiosis of the gut microbiome.[176,177]

One proposed mechanism underlying the association between increased relative abundance of Proteobacteria, inflammatory bowel diseases and gut dysbiosis is the "oxygen hypothesis."[187] Rigottier-Gois (2013), noted that inflammation of the gastrointestinal (GI) tract leads to an overall decrease in obligate anaerobic taxa (Firmicutes and some Bacteroides) and increases in facultative aerobic taxa (Proteobacteria, specifically Enterobacteriaceae). The author proposes that, in an inflammatory state, the GI epithelium is damaged and therefore has impaired function. One of the functions of the GI epithelium is to deplete oxygen in the gut lumen through beta oxidation of fatty acids at the mucosal interface, which in turn creates an anaerobic environment.[188] In an inflammatory state, it is proposed that the GI epithelium may have a decreased capacity to perform beta oxidation of fatty acids and therefore leave the lumen of the GI tract in an aerobic state allowing facultative aerobic taxa like Enterobacteriaceae to bloom.[189]

This hypothesis is further supported by metagenomics analyses that show an overrepresentation of genes for encoding respiratory pathways in mice with chemically induced colitis.[190] Enrichment patterns of specific taxa in the healthy individuals (PCS scores ≥ 45) of this Veteran cohort are consistent with the "oxygen hypothesis". The bacterial taxa that were shown to be enriched in healthy individuals were Paraprevotellaceae (phylum: Bacteroidetes), Ruminococcaceae (phylum: Firmicutes), *Faecalibacterium* (phylum: Firmicutes), *Roseburia* (phylum: Firmicutes), and *Coprococcus* (phylum: Firmicutes). All of these taxa are obligate anaerobes.

4.5 Conclusions

Three measures of general and physical health, i.e., BMI, SF-36 general health perception, and SF-36 PCS score showed associations with features of the fecal microbiome in this Veteran cohort. All of these metrics displayed a similar relationship with alpha diversity, i.e., indicators of lower general and physical health status were associated with lower alpha diversity. Conversely, observed OTUs were increased in the "healthy" state, using all three metrics of general and physical health. BMI displayed no association with the Firmicutes/Bacteroides ratio. The lowest quartile of general health perception exhibited an overall enrichment in taxa within the Actinobacteria and Firmicutes phyla. This result may suggest that general health perception is influenced by an individual's perception of health based on weight because these taxa have previously been shown to be enriched in obese individuals. The PCS score on the other hand may be tapping into the inflammatory state of an individual, as "not healthy" (PCS < 45) individuals displayed enrichment in Proteobacteria taxa, while "healthy" (PCS ≥ 45) individuals displayed enrichment in obligate anaerobic taxa. These results align with the recently proposed "oxygen hypothesis" explaining patterns of taxonomic composition of the fecal microbiome in association with inflammation in the GI tract.

Chapter 5. Association of insomnia and the microbiome in a Veteran cohort

5.1 Introduction

Sleep is essential for normal cognitive function and humans spend roughly one-third of their existence sleeping or trying to fall asleep.[191] It is clear that even acute bouts of sleep deprivation and restriction lead to decreases in cognitive performance[192–194] and physical health.[195,196] It has also been shown that sleep irregularity in the form of reversed or misaligned circadian cycles as seen in shift workers leads to increases in various metabolic disorders such as type 2 diabetes, hypertension, and obesity.[196–198] As reviewed in the previous chapter of this dissertation, the microbiome is clearly linked to metabolic diseases, general health, and physical health.[87,120,133] Despite the several lines of evidence that sleep restriction is linked to same poor physical health outcomes that have been linked to the microbiome there are relatively few studies relating sleep and the microbiome.

Studies of the relationship between acute or chronic sleep restriction and the microbiome, although relatively few in number, have been done in humans and rodents. Two recent studies examining the association of acute sleep restriction (i.e., 4 hrs of sleep opportunity for two[199] or five days,[200] respectively) in humans show that the microbiome is relatively robust to changes under these conditions. These studies revealed no differences in alpha or beta diversity and very few specific taxa were altered in response to acute sleep restriction.[199,200] A study performed in rats showed acute sleep disruption of 5 days leads to changes in bacterial community structure, but only one relatively obscure taxa (OTU; *TM7a-3*) showed differential abundance between treatment groups.[200] A study done in mice of chronic sleep fragmentation (4 weeks) showed changes in the bacterial community structure with several taxa including probiotic-related taxa that were affected.[201] These studies collectively provide some evidence that the microbiome is

affected by sleep restriction and fragmentation. However, a comprehensive characterization of the microbiome as it relates to sleep, and sleep disorders is needed.

In this Veteran cohort, we conducted two surveys, to gauge insomnia symptom severity and presence of a current insomnia disorder, respectively. The first survey, the insomnia severity index (ISI) is a seven-item self-report survey that is validated as a metric to gauge the severity an individual's insomnia.[202,203] The second survey, the Structured Clinical Interview, fifth edition (SCID-5; Module H) is assessed by a trained professional in an interview as part of the Diagnostic and Statistical Manual of Mental Health Disorders, fifth edition (DSM-5; research version) gauged at determining the presence of a sleep disorder.[204] In our analysis of the SCID-5 metric, we focused on the presence or absence of an insomnia disorder. In this chapter, we sought to examine how these two metrics of insomnia related to the microbiome independently as well as comparing and contrasting the two metrics against each other to assess for similar or confounding results.

5.2 Materials and methods

5.2.1 General procedures

Please refer to Chapter 2 of this dissertation for details on *Participants and study design*, *Sample collection and preparation*, *Molecular processing*, and *Computational analyses*.

5.2.2 Insomnia severity index (ISI)

The ISI is a seven-item instrument assessing the nature and severity of insomnia symptoms, satisfaction with sleep, interference of sleep disturbance with daily functioning, and how distressing and noticeable the sleep impairment is.[203] The scale is considered a reliable and valid measure of insomnia.[202] The ISI is structured so that all questions have five total responses that are scored 0-4. The response scores represent worsening insomnia symptoms where 0 represents a lack of an insomnia symptoms and 4 represents a severe insomnia symptom. The cumulative ISI score is simply the sum of the response scores for the seven questions. The

developers of the ISI provide the following cutoffs for ISI scores: 0-7, no clinically significant insomnia; 8-14, subthreshold insomnia; 15-21, clinical insomnia (moderate severity), and 22-28, clinical insomnia (severe).[203] These categories can be condensed into clinical insomnia (ISI score ≥ 15) and subthreshold or no insomnia (ISI < 15).[203]

5.2.3 Structured Clinical Interview for DSM-5 (SCID-5, research version) Module H

The SCID-5 is a diagnostic interview that is administered by a trained professional used to determine metal health disorders as a component of the Diagnostic and Statistical Manual of Mental Disorders 5 (DSM-5). In this study, the research version of the DSM-5 was used and the interview was administered by trained research assistants. Module H of this interview is aimed at determining if an individual is suffering from sleep-wake disorders. The interview for this module aims to determine if an individual has a current (within the past 3 months) insomnia disorder, hypersomnolence disorder, or substance/ medication-induced sleep disorder. Each of these sleep related disorders are assessed independently and each receive a presence or absence designation. In this study, we only assessed insomnia disorder (presence/absence) as it related to the microbiome. Therefore, we will not detail the criteria for diagnosis of hypersomnolence disorder or substance/ medication-induced sleep disorder here. The interviewer asked a series of questions to determine if a current insomnia disorder was present based on the following criteria.

- The time of going to sleep and time of waking
- Difficulty initiating sleep
- Difficulty maintaining sleep, characterized by frequent awakenings or problems returning to sleep after awakening
    - These criteria exclude frequent toilet use
- Early morning awakening with inability to return to sleep

- Average total sleep time
    - Insomnia disorder was automatically assigned if average total sleep time was less than 6.5 hours

The criteria above are assessed not as specific questions to be explicitly asked in a standardized manner, but rather in a format similar to a visit with a clinical psychologist. The manual specifically outlines that if the patient/ participant has answered "yes" to a screening question in a written portion of the DSM-5 regarding major concerns about their sleeping habits, the interviewer should prompt the patient/ participant with the question "You've said that over the past 3 months a major concern of yours has been that you are not getting enough good sleep or feeling rested. Tell me about that?"

5.2.4 Microbiome results for skin and oral samples

In exploratory analysis of the microbiome data, only fecal microbiome associations were revealed as significant in relation to metadata categories that were relevant to insomnia. Therefore, no skin or oral microbiome results will be shown in this chapter.

5.3 Results

We performed several analyses on the ISI scores and SCID-5 current insomnia designation variables as they associate with alpha and beta diversity. A summary table of results can be found in **Table 5.1**.

Table 5.1 Table of insomnia variables and their associations with alpha and beta diversity outcomes of skin, oral, and fecal microbiomes[1]

		Skin		Oral		Fecal	
Survey	Metric	Alpha	Beta	Alpha	Beta	Alpha	Beta
Insomnia Severity Index (ISI)	ISI severity (severe vs not severe)						
	ISI severity clinical categories (clinical insomnia, not clinical insomnia)						
	ISI score correlation						
	ISI severity categories (not significant, subthreshold, moderate, severe)						
Structured Clinical Interview for the DSM-5 (SCID-5)	Current insomnia disorder						

[1]Table showing the results for the initial analyses of insomnia variables as they relate to alpha and beta diversity of skin, oral, and fecal microbiomes. Green represents a significant *p*-value ($p < 0.05$), yellow represents a *p*-value approaching significance ($p \leq 0.10$), and red represents a *p*-value > 0.10. Any *p*-value > 0.05 is not significant. Abbreviations: ISI, Insomnia Severity Index; DSM-5, Diagnostic and Statistics Manual of Mental Disorders 5th edition; SCID-5, Structured Clinical Interview for the DSM-5.

5.3.2 Analysis of alpha and beta diversity and top taxa in the fecal microbiome based on ISI categories and scores

Alpha diversity was lower in individuals with severe insomnia symptoms (ISI score ≥ 22) relative to individuals without severe insomnia symptoms (ISI score < 22) based on analysis using observed OTUs (Wilcoxon rank-sum test; $p < 0.05$), but not Shannon diversity (**Figure 5.1A**). We investigated if this difference was also evident when comparing clinical insomnia with subthreshold or no insomnia (clinical insomnia (moderate or severe) = ISI scores ≥15; subthreshold or no insomnia = ISI scores < 15) (**Figure 5.1B**). Alpha diversity approached statistical significance in individuals with clinical insomnia (ISI score ≥ 15) exhibiting fewer observed OTUs than individuals without clinical insomnia (ISI score < 15) (Wilcoxon rank-sum test; $p = 0.06$). Shannon diversity did not differ between individuals with or without clinical insomnia. Scatter plots of raw ISI scores exhibited a negative relationship that approached

statistical significance in observed OTUs ($r = –0.14$, $p = 0.08$), but no relationship was observed with Shannon diversity (**Figure 5.1C**). Principal coordinate analysis of the weighted UniFrac distance matrix and further assessment with PERMANOVA revealed that the bacterial community structure differed between individuals with severe insomnia symptoms from individuals without severe insomnia symptoms ($p < 0.05$; data not shown). Analysis of specific taxa between individuals with severe insomnia symptoms and individuals without severe insomnia symptoms by Wilcoxon rank-sum test at the phylum taxonomic level revealed no differences. Analysis of specific taxa by Wilcoxon rank-sum test at the class taxonomic level revealed enrichment of Bacilli (phylum: Firmicutes) ($p < 0.05$) in individuals with severe insomnia symptoms. Analysis of specific taxa by Wilcoxon rank-sum test at the order taxonomic level revealed enrichment of Lactobacillales (phylum: Firmicutes) ($p < 0.05$) in individuals with severe insomnia symptoms. Analysis of specific taxa by Wilcoxon rank-sum test at the family taxonomic level revealed enrichment of Lactobacillaceae (phylum: Firmicutes) ($p < 0.05$) in individuals with severe insomnia symptoms and Clostridiaceae (phylum: Firmicutes) ($p < 0.05$) in individuals without severe insomnia symptoms. Analysis of specific taxa by Wilcoxon rank-sum test at the genus taxonomic level revealed enrichment of *Lactobacillus* (phylum: Firmicutes) ($p < 0.05$) in individuals with severe insomnia symptoms and enrichment in *Faecalibacterium* (phylum: Proteobacteria) ($p < 0.05$) and *Sporobacterium* (phylum: Firmicutes) in individuals without severe insomnia symptoms.

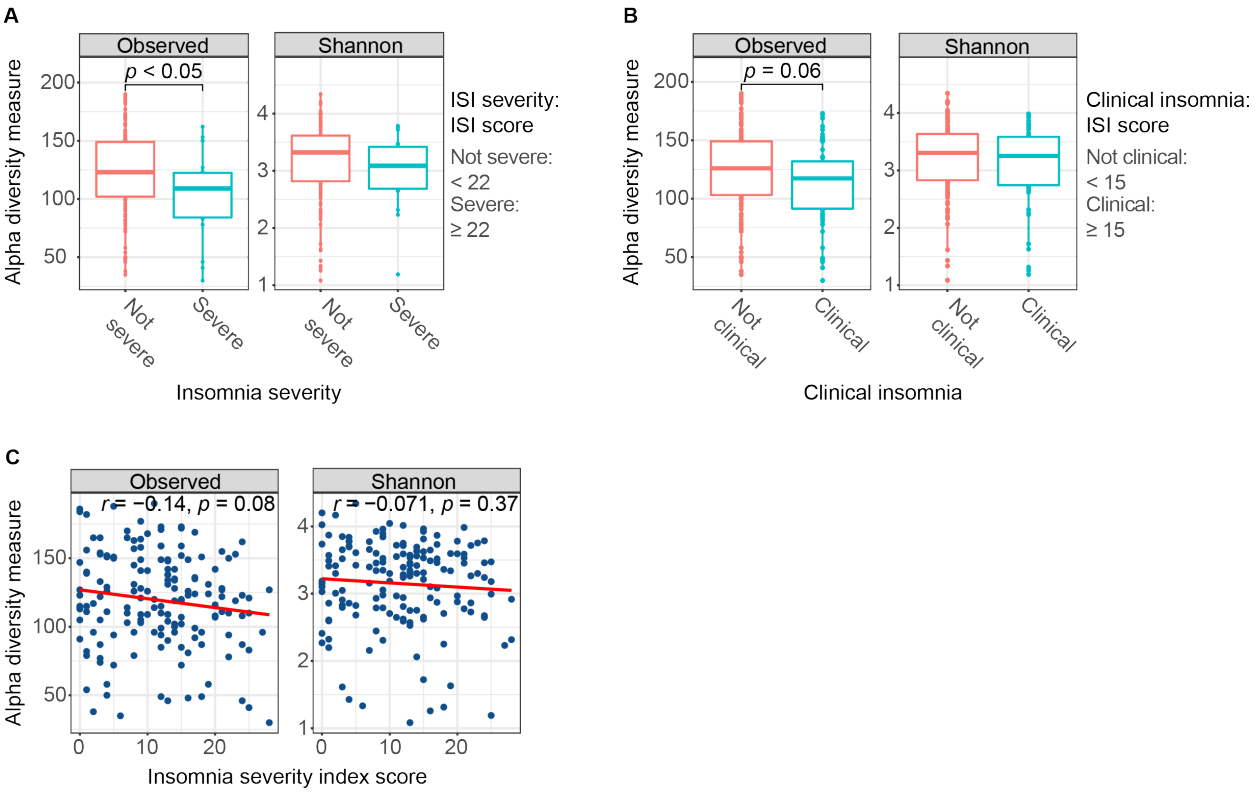

Figure 5.1 Alpha diversity in the fecal microbiome based on insomnia severity index (ISI) designations and scores

A) Boxplots of observed operational taxonomic units (OTUs) and Shannon diversity metrics within ISI designations of not severe (ISI score < 22) and severe clinical insomnia (ISI score ≥ 22), using ISI severity thresholds designated by the developers of the index. B) Boxplots of observed OTUs and Shannon diversity metrics within ISI designation of no clinical insomnia (ISI score < 15) and clinical insomnia (moderate or severe) (ISI score ≥ 15), using ISI clinical insomnia thresholds designated by the developers of the index. C) Scatter plots and regression lines of raw ISI scores versus observed OTUs and Shannon diversity metrics. Abbreviations: ISI, insomnia severity index, OTU, operational taxonomic unit. Sample sizes: A) no severe clinical insomnia, 143; severe clinical insomnia, 18. B) no clinical insomnia, 105; clinical insomnia (moderate or severe), 56.

5.3.3 Analysis of alpha and beta diversity in the fecal microbiome relative to a current insomnia disorder based on SCID-5

Analysis of alpha diversity, comparing individuals with and without a current insomnia disorder based on the SCID-5, approached statistical significance with more observed OTUs in individuals with a current diagnosis of insomnia (Wilcoxon rank-sum test; $p = 0.08$) (**Figure 5.2**). Principal coordinate analysis of weighted and unweighted UniFrac distance matrices

determined that the bacterial community structures were not different in individuals with and without a current insomnia disorder.

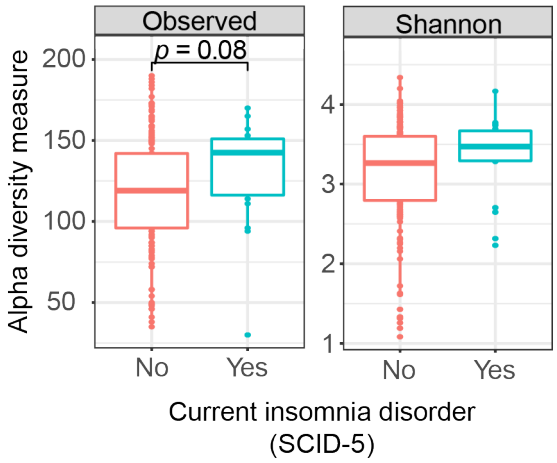

Figure 5.2 Alpha diversity in the fecal microbiome based on SCID-5 current insomnia disorder designations
Boxplots of observed operational taxonomic units (OTUs) and Shannon diversity metrics for individuals with and without a current insomnia disorder based on the SCID-5. Abbreviations: OTU, operational taxonomic unit; SCID-5, Structured Clinical Interview for the DSM-5. Sample sizes: No, 141; Yes, 18.

5.3.4 Exploratory analysis of sample sizes and beta diversity in the fecal microbiome comparing ISI severity categories relative to a current insomnia disorder based on SCID-5

A comparative table of sample sizes cross referencing ISI severity with SCID-5 presence or absence of a current insomnia disorder displayed conflicting categorization (**Table 5.1**). A survey of the sample sizes displayed that the highest number of individuals with the presence of a current insomnia disorder fell into the subthreshold ISI category (n = 8), while the lowest number of individuals with the presence of a current insomnia disorder fell into the severe ISI category (n = 3).

Table 5.2 Sample sizes cross referencing ISI severity designations with SCID-5 presence or absence of current insomnia disorder

	Not significant (0-7)	Subthreshold (8-14)	Moderate (15-21)	Severe (22-28)	Total
No	46	45	35	15	141
Yes	4	8	3	3	18
Total	50	53	38	18	159

Sample sizes of ISI severity designations (with ISI score ranges in parentheses) against SCID-5 current insomnia disorder designation.

Exploratory principal coordinate analysis of the unweighted UniFrac distance matrix and further examination with PERMANOVA determined that bacterial community structure based on ISI severity categories (no insomnia, subthreshold insomnia, moderate insomnia, or severe insomnia) approached statistical significance in determining that the bacterial community structure among the ISI severity categories differed ($p = 0.06$) (**Figure 5.3,** only ISI severity categories of subthreshold insomnia and severe insomnia are shown). This analysis was exploratory in the sense that these observations were based on low sample sizes and therefore many of the statistics were underpowered. Also, the beta diversity PCoA analysis of ISI severity designations were not fully supported by statistics because a permutational test of homoscedasticity produced a significant result meaning that there was unequal variance among groups. A visual examination of the PCoA plot comparing SCID-5 and ISI assessments of insomnia shows that, similar to the cross-referenced sample sizes, individuals with a current insomnia disorder clustered more closely with the subthreshold ISI severity individuals.

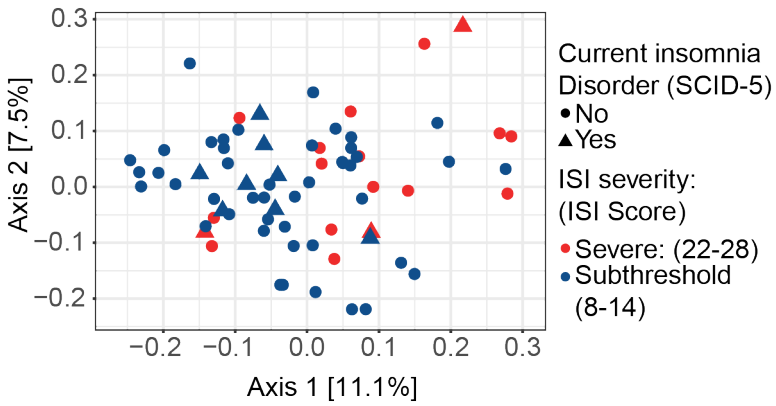

Figure 5.3 Beta diversity comparisons in the fecal microbiome based on insomnia severity index (ISI) designations in individuals with and without a current insomnia disorder based on SCID-5
Principal coordinate analysis of unweighted UniFrac distance matrix of ISI severity (all categories) with only ISI severity categories of severe (red, ISI score 22-28) and subthreshold (blue, ISI score 8-14) shown. A current insomnia disorder is designated by shape: No (circle) and Yes (triangle). Principal coordinate axis 1 explained 11.1% of the variance and principal coordinate axis 2 explained 7.5% of the variance; together, principal coordinate axes 1 and 2 explained 18.6% of variance. Abbreviations: ISI, insomnia severity index, OTU, operational taxonomic unit, SCID-5, Structured Clinical Interview for the DSM-5. Sample sizes: No current insomnia disorder and ISI severe (red circle), 15; Yes current insomnia disorder and ISI subthreshold (blue triangle), 8; No current insomnia disorder & ISI subthreshold (blue circle), 4; Yes current insomnia disorder and ISI severe (red triangle), 3.

5.4 Discussion

In this Veteran cohort, we determined that the fecal microbiome differed between the two metrics that were used to gauge insomnia, i.e., ISI score and SCID-5 current insomnia disorder presence or absence. Observed OTUs were lower in individuals with severe insomnia ISI scores (ISI score ≥ 22) compared to individuals without severe insomnia ISI scores (ISI score < 22), but, paradoxically, higher in individuals with the presence of a current diagnosis of insomnia disorder (SCID-5). Beta diversity as measured by PCoA of the unweighted UniFrac distance matrix determined that the bacterial community structure differed between individuals with and without severe ISI severity scores, while bacterial community structure was not changed by the presence or absence of a current insomnia disorder, although it should be emphasized that of, 159 individuals assessed for a diagnosis of insomnia using SCID-5, only 18 (11.3%) had a diagnosis of current insomnia, resulting in an extreme bias in the sample toward those without a

diagnosis of current insomnia. Analysis of specific taxa in the fecal microbiome showed that individuals with severe ISI severity scores were enriched in taxa from the Firmicutes phylum and specifically several Lactobacilli taxa. In contrast, individuals without severe ISI severity scores showed enrichment in the genus *Faecalibacterium*.

Alpha diversity, beta diversity, and top taxa were altered in individuals with severe insomnia ISI scores

This Veteran cohort displayed changes in alpha and beta diversity in individuals exhibiting severe insomnia ISI scores, relative to those without severe insomnia ISI scores. To our knowledge, this is the first report of reduced gut microbiome alpha diversity in individuals with severe insomnia. Two recent studies that assessed the effects of acute sleep restriction (4 hrs of sleep opportunity for two days[199] and five days,[200] respectively) on the gut microbiome did not detect changes in alpha and beta diversity of the gut microbiome, suggesting acute sleep restriction for two to five days may not be a long enough period of sleep restriction to induce detectable changes in alpha and beta diversity. However previous metabolomics studies of acute sleep restriction have detected changes in the host metabolome within a single day of sleep restriction.[205,206] The host metabolome is different from the gut metabolome and these results may be mediated by physiological factors of the host altered by acute sleep restriction rather than changes within the microbiome. There are important differences between our study and these studies of sleep restriction, as these studies performed a sleep restriction intervention and our surveys examined current insomnia symptoms or the presence of a current insomnia disorder.

The insomnia measures collected within this Veteran cohort may be more comparable to chronic models of sleep fragmentation. A study done in mice with a chronic sleep fragmentation

regimen (4 weeks) supports the notion that chronic sleep fragmentation is sufficient to detect changes in beta diversity. Poroyko et al., (2016) found that chronic sleep fragmentation (SF) leads to changes in bacterial community structure and other behavioral and physiological changes. Examination of beta diversity and specific taxa in SF mice mostly supported the results observed in severe ISI Veterans. Both SF mice and severe ISI Veterans showed differing bacterial community structures with a specific increase in Firmicutes. However, the increase observed in Firmicutes in SF mice was driven by Lachnospiraceae, while *Lactobacillus* taxa drove this finding in Veterans with severe ISI symptoms. Furthermore, SF mice showed a 4-fold decrease in *Lactobacillus* taxa. Poroyko et al., (2016) did note that the SF mice also experienced significant changes in food intake, insulin resistance, and inflammation, which could be influencing the discrepancies between the two studies. Poroyko et al., (2016) did not report alpha diversity metrics in the main manuscript or supplemental material, so no comparisons between alpha diversity could be made.

Faecalibacterium was depleted in Veterans with severe ISI scores. This genus consists of a single known species, *F. prausnitzii*, and is one of the most abundant commensal bacteria in the healthy human large intestine.[207] *F. prausnitzii* is known to be immunoregulatory[208] and has been implicated in protection against a number of inflammatory disorders. *Faecalibacterium* has been shown to be depleted in patients suffering from inflammatory bowel disease (IBD), i.e. ulcerative colitis and Crohn's disease, as well as irritable bowel syndrome, and colorectal cancer.[209–211] *Faecalibacterium* is an extremely oxygen sensitive (EOS) bacterium[208,212]; consistent with the oxygen hypothesis (outlined in Chapter 4) inflammatory bowel diseases may compromise the normal anaerobic environment of the lumen of the GI tract.[187] A recent characterization of *F. prausnizii* showed that most strains decreased the proinflammatory

cytokine, IL-8, increased the anti-inflammatory cytokine IL-10, and produced butyrate in *in vitro* assays.[208] The immunoregulatory properties and butyrate producing properties of this genus have led some to propose *F. prausnizii* as a next generation probiotic.[208] It is not known if *Faecalibacterium* is associated with insomnia; however, the depletion of *Faecalibacterium* in Veterans with severe ISI symptoms may infer other health complications beyond severe insomnia symptoms.

Alpha diversity and beta diversity were not altered in individuals with a current insomnia disorder based on the SCID-5

Alpha diversity as measured by observed OTUs approached statistical significance with individuals with a current insomnia disorder displaying more OTUs. The ability to detect differences in the gut microbiome in association with ISI severity scores, but not in association with a current diagnosis of insomnia may be due to differences in insomnia severity or differential use of sleep medications in these two groups. These results stimulated us to perform more in depth analyses to probe at the differences between the ISI and SCID-5 insomnia measures.

5.4.1 Comparison of the two insomnia measures

Associations between measures of insomnia, using either the ISI or SCID-5 surveys (both considered valid assessments of insomnia in clinical studies[202,213]), and alpha and beta diversity of the fecal microbiome were not in full alignment. One possibility underlying the different outcomes is that severity of insomnia is an important determinant of the association between insomnia and the gut microbiome, as those with severe insomnia showed differences in alpha diversity, beta diversity, and specific taxa, relative to those without severe insomnia, while the

SCID-5 diagnostic criteria do not. In support of this reasoning, cross-referencing sample sizes for ISI severity designations with SCID-5 current insomnia disorder designation shows only 3 of 18 individuals with a current insomnia disorder expressing severe ISI symptoms. Further, two-thirds of individuals with a current insomnia disorder (12 of 18) have either "not significant" or "subthreshold insomnia", symptoms, based on the ISI survey, which are both below the standard determined to be clinical insomnia.[203]

One potential factor that our analysis has not taken into account is whether or not participants were taking medications, for insomnia or other conditions. Falony et al., (2016) performed an extensive meta-analysis of the gut microbiome in the combined Belgian Flemish Gur Flora Project and Dutch LifeLines DEEP study with a combined sample size of 3948 to reveal covariates, biomarkers, and core microbiota. One of the main covariates explaining significant variation in the microbiome and the covariate with the largest effect size was medications.[26] The effect was influenced in a large part by antibiotics; however, contained within the "shortlist" of 13 medications that, independent of other covariates, influenced the community composition of the microbiome were benzodiazepines (top insomnia medication recommended by clinician guidelines[214]) as well as other medications that are not selectively insomnia medications but are frequently prescribed for insomnia, such as antidepressants, and anti-panic/anti-anxiety, as well as over-the-counter drugs that are frequently taken by individuals with insomnia, such as antihistamine medications.[214] The medications venlafaxine (antidepressant) and clonazepam (anti-panic and anxiolytic) were shown to specifically influence the relative abundance of the phyla Firmicutes and Bacteroides, respectively.[26] Antihistamines were shown to influence the bacterial community structure as a whole and drive specific increases in the

relative abundances of *Eggerthella* (phylum; Actinobacteria), *Flavonifractor* (Phylum; Clostridium), and *Parasutterella* (phylum; Proteobacteria).[26]

Although we currently do not have information on whether or not participants were taking sleep medications, it seems reasonable that if an individual was taking one of these medications, not only would their ISI symptoms decrease but they would also be preventing the detriments of chronic sleep restriction, which has been associated with dysbiosis of the gut microbiome[201] and increases in BMI and risk for metabolic disorders.[195–197] In other words, it is possible that individuals with a current diagnosis of insomnia disorder were also taking sleep medications, leading to decreases in ISI severity scores, increases in observed OTUs, and changes in bacterial community structure, resulting in those participants being more similar to participants in the subthreshold insomnia category, based on the ISI survey.

5.4.2 Limitations

A major limitation of this analysis was that we did not have information regarding current medications for this Veteran cohort, where many individuals, particularly those with a diagnosis of current insomnia, were likely to be taking medications. Medications were likely to lead to reductions in symptoms of insomnia leading to lower insomnia severity, and also have potential to directly impact the diversity and community structure of the gut microbiome.

5.5 Conclusions

These results, along with previous animal studies and recently published clinical studies of sleep restriction, support the conclusion that chronic sleep restriction or chronic sleep disruption is sufficient to observe changes in alpha and beta diversity of the gut microbiome. It is clear that the validated ISI and SCID-5 surveys are identifying different individuals within populations with insomnia-like symptoms. Comparison of the two metrics revealed that self-

reported severity of insomnia symptoms, as measured by the ISI, was a more sensitive measure in terms of associations with features of the gut microbiome.

Chapter 6. Association of mental health and skin, oral, and fecal microbiomes in a cohort of U.S. Veterans

6.1 Introduction

"No health without mental health" has been the defining mantra of the mental health field since this proclamation by the World Health Organization (WHO).[215] It is becoming more and more evident that the barrier separating physical health and mental health is a conceptual barrier and the two health states are intimately linked. Mental health disorders are a serious problem globally.[216] The Global Burden of Diseases, Injuries, and Risk Factors Study 2016, spanning from 1990 to 2016 reported that major depressive disorder ranked in the top-10 causes of illness in every country around the world with the exception of four countries.[217] Further, the globe has shown little improvement in mental health since 1990, while communicable diseases on the other hand have largely decreased during the same time period.[217] Mental health disorders have proven to be a persistent problem around the world. This realization lead the director of the NIMH in 2006, Thomas Insel, to propose that the field needs to raise the bar for developing novel strategies for prevention of mental health disorders.[218] Insel made the resonating statement that "psychiatry will need to develop strategies for prevention" of mental health disorders.

The concept that prevention of mental health disorders may be conceivable is nested in the well-established link between inflammation and depression. Individuals with depression that are otherwise healthy have increased systemic inflammatory markers and reduced immunoregulatory markers.[219] Proinflammatory cytokines have been found to be raised in the cerebrospinal fluid of a specific subset of depressed patients and raised expression of proinflammatory markers have been shown in the brains of deceased individuals who died from suicidal self-directed violence.[220,221] Cancer patients who were previously not depressed and receive treatment with the proinflammatory cytokine interferon-α develop symptoms of depression.[222] These symptoms can be alleviated by administering an antidepressant shown to be anti-inflammatory.[222,223] Further, individuals resistant to traditional antidepressant treatments and elevated baseline inflammatory markers respond to anti-inflammatory treatment targeting TNF signaling.[224] Several lines of evidence show that depression has clear links to inflammation.

One approach to prevention of mental health disorders is to identify risk factors for development of these disorders, and then to design interventions to alleviate this risk. Known risk factors for psychiatric disorders include genetic risk factors,[225] and environmental risk factors including adverse early life experience,[226] and major stressful life events.[227] There is increasing evidence that chronic low-grade inflammation may also be a risk factor for development of mental health disorders.[220,228,229] A major determinant of chronic low-grade inflammation is the level of exposure to immunoregulatory microorganisms during development.[230]

The biodiversity and "old friends" hypotheses suggest that decreased exposure to a diverse set of microbes, the microbes that we have been exposed to and co-evolved with since the beginning of time, may lead to improper regulation of the immune system.[20,231] Not only has

depression been a persistent global problem, but several other inflammatory disorders such as atopic sensitization,[15–17] autoimmune disorders,[19] including inflammatory bowel disease[19] have been rising in westernized civilizations and are becoming more prevalent in developing nations.[14] One theory behind this increase in inflammatory disorders is the high level of urbanization in westernized nations and the unprecedented rate of urbanization in developing nations.[232] Urban environments are known to harbor less microbial diversity than rural environments and green and blue spaces.[66,233,234] This prevents those living in urban environments from having frequent contact with environmental microbes that humans previously coexisted with and our immune systems learned to tolerate, which is the main theory behind the biodiversity and "old friends" hypothesis. An unfortunate and unintended consequence of this microbial deprivation is a dysregulated immune system with a bias toward a more proinflammatory response.[20,235,236] These hypotheses suggest that chronic low-grade inflammation may be a risk factor for development of stress-related psychiatric disorders where inflammation has been identified as a risk factor.[220] In this Chapter, we seek to examine if there are associations between mental health outcomes and features of skin, oral, and fecal microbiomes collected from this Veteran cohort.

6.2 Materials and methods

6.2.1 General procedures

Please refer to Chapter 2 of this dissertation for details on *Participants and study design*, *Sample collection and preparation*, *Molecular processing*, and *Computational analyses*.

6.2.2 Structured Clinical Interview for DSM-5 (SCID-5, research version)

The SCID-5 is a diagnostic interview that is administered by a trained professional used to determine metal health disorders as a component of the Diagnostic and Statistical Manual of Mental Disorders 5 (DSM-5). The following metrics were gathered from the SCID-5: ever homeless, currently homeless, current PTSD, lifetime PTSD, current MDD, lifetime MDD.

These metrics were gathered from the interviewer asking questions similar to the following and other relevant follow up questions:

"Have you ever been homeless and if so how many times?"

"Are you currently homeless?"

"Do you have a history of mental health, emotional, or behavioral problems?"

"Are you currently experiencing mental health, emotional, or behavioral problems?"

"What has your mood been like?"

"How have you been spending your free time?"

"Who do you spend your free time with?"

Based on the answers to these questions and follow-up questions the trained interviewer will determine the presence or absence of various mental health disorders.

6.2.3 Ohio State University Traumatic Brain Injury Identification Method (OSU-TBI-ID)

We determined the number of TBIs in each participant with the OSU TBI-ID interview. This is a structured interview developed using recommendations from Centers for Disease Control (CDC) for the detection of history of exposure to TBI. It was designed to elicit self-reports of TBI occurring over a person's lifetime in a 3-5 minute interview.

6.2.4 Beck depression inventory (BDI)

The BDI is a 21-question self-report survey to measure the severity of depression. The survey targets specific symptom categories of hopelessness, irritability, guilt, feelings of being punished, fatigue, weight loss, and lack of interest in sex. The following categories of BDI scores were created by the developers of the survey: minimal depression (0-13); mild depression (14-19), moderate depression (20-28), severe depression (29-63).[36]

6.2.5 45-Item Outcome Questionnaire (OQ-45)

The OQ-45 is a 45-item questionnaire that was designed to measure key areas of mental health functioning (symptom distress, interpersonal functioning, and social role). As such it is a validated and accepted tool for identifying, tracking, and measuring behavioral health treatment outcomes and was used as a measure of symptom distress

6.2.6 PTSD Checklist (PCL-5)

The PCL-5 is a 20-item self-report measure that assesses the 20 DSM-5 symptoms of PTSD. The PCL-5 is a quicker alternative to performing a full Clinician-Administered PTSD Scale (CAPS-5), which is the gold standard for PTSD diagnosis. The PCL-5 is good for screening individuals for PTSD, making a provisional diagnosis of PTSD, or monitoring symptom change during and after treatment.

6.2.7 Random forest analysis

Variables that had significant PERMANOVA results were run through random forest models in R (details on parameters for random forest models can be found in the *Computational analyses* section in Chapter 2 of this dissertation).

6.3 Results

We performed extensive analyses of the variables relating to mental health as they associate with alpha and beta diversity of skin, oral, and fecal microbiomes. Relatively speaking there were very few mental health variables that were significantly associated with skin, oral, and fecal microbiomes (**Table 6.1**). There were many limitations that were related to these results that are detailed in the limitations section of Chapter 7. In summary, many of the statistics were likely underpowered due to low sample sizes and a lack of age-, race-, gender-, and physical health-matched controls. Further all random forest models were unsuccessful in classify any of the target variables for reasons outlined in detail in the *Limitations* section of Chapter 7 of this dissertation.

Table 6.1 Table of mental health variables and their associations with alpha and beta diversity outcomes of skin, oral, and fecal microbiomes[1]

		Skin		Oral		Fecal	
Survey	Metric	Alpha	Beta	Alpha	Beta	Alpha	Beta
Structured Clinical Interview for DSM-5 (SCID-5)	Ever homeless						
	Currently homeless						
	Correlation with number of times homeless						
	Lifetime MDD						
	Current MDD						
	Lifetime persistent depressive disorder						
	Current persistent depressive disorder						
	Lifetime alcohol drinking problem						
	Current alcohol drinking problem						
	Lifetime PTSD						
	Current PTSD						
	PTSD symptom severity						
Ohio State University TBI Identification (OSU-TBI-ID)	TBI count correlation						
	TBI 2 or more vs 1 or none						
Beck Depression Inventory (BDI)	BDI score correlation						
	BDI severity categories (minimal, mild, moderate, severe)						
	BDI severity (severe vs not severe)						
45-Item Outcome Questionnaire (OQ-45)	Total score severity categories (not significant or significant)						
	Symptom distress score categories (not significant or significant)						
	Interpersonal relations score categories (not significant or significant)						
	Social role score categories (not significant or significant)						
PTSD Checklist for DSM-5 (PCL-5)	PCL-5 categories (not clinical or clinical)						

[1]Table showing the results for the initial analysis of metal health variables as they relate to alpha and beta diversity of skin, oral, and fecal microbiomes. Green represents a significant *p*-value ($p < 0.05$), yellow represents a *p*-value approaching significance (≤ 0.10), and red represents a *p*-value > 0.10. Abbreviations: DSM-5, Diagnostic and Statistical Manual of Mental Disorders 5th edition; SCID-5, Structured Clinical Interview for the DSM-5; MDD, major depressive disorder; PTSD, posttraumatic stress disorder; TBI, traumatic brain injury; BDI, Beck Depression Inventory; OQ-45, 45-Item Outcome Questionnaire; PCL-5, PTSD Checklist for DSM-5.

6.3.2 Skin microbiome

Ever homeless

Alpha diversity in the skin microbiome was higher in individuals who reported experiencing homelessness in their lifetime as measured by observed OTUs (Wilcoxon rank-sum test; $p < 0.05$), but not in Shannon diversity (**Figure 6.1**). Beta diversity analysis using weighted and unweighted UniFrac and further analysis by PERMANOVA of skin samples revealed no differences in bacterial community structure based on homelessness.

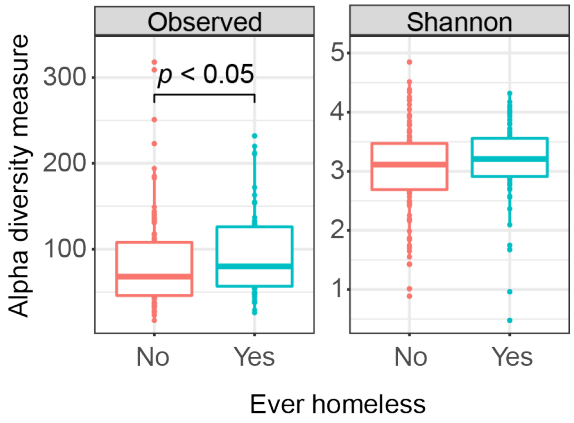

Figure 6.1 Alpha diversity boxplots in skin microbiome based on ever homeless
Boxplots of alpha diversity metrics in the skin microbiome based on ever experiencing homelessness. Sample size: No, 107; Yes, 70.

Posttraumatic stress disorder (PTSD)

Alpha diversity did not differ based on presence or absence of a diagnosis of PTSD. Beta diversity analysis using weighted UniFrac and further analysis by PERMANOVA of skin samples revealed differences in bacterial community structure based on PTSD assessed using the SCID-5. Both individuals with current PTSD ($p < 0.05$; No, $n = 131$; Yes, $n = 44$) and individuals who reported ever experiencing PTSD ($p < 0.05$; No, $n = 102$; Yes, $n = 73$) displayed

significant PERMANOVA results (data not shown). By default, individuals that currently are experiencing PTSD are also categorized as individuals who reported ever experiencing PTSD (denoted as lifetime PTSD from here forward). Therefore, current PSTD individuals make up 60% of the lifetime PTSD individuals. In addition, analysis of the specific taxa within current and lifetime PTSD determined that both categories showed the exact same enriched or depletion patterns. With the exception that lifetime PTSD displayed marginally more statistical power in each of the specific taxa. Because of these two factors specific taxa enrichment results will only be displayed for lifetime PTSD. No enrichment of any taxa was observed at the phylum taxonomic level based on lifetime PTSD. Lifetime PTSD was associated with greater relative abundance of the class Alphaproteobacteria (phylum: Proteobacteria) (Wilcoxon rank-sum test; $p < 0.05$). No enrichment of any taxa was observed at the order taxonomic level based on lifetime PTSD. Lifetime PTSD was associated with greater relative abundance of the family Micrococcaceae (phylum: Actinobacteria) (Wilcoxon rank-sum test; $p < 0.05$), but lower relative abundance of Corynebacteriaceae (phylum: Actinobacteria) (Wilcoxon rank-sum test; $p < 0.05$) and Lachnospiraceae (phylum: Firmicutes) (Wilcoxon rank-sum test; $p < 0.01$). Lifetime PTSD was associated with greater relative abundance of the genus *Corynebacterium* (phylum: Actinobacteria) (Wilcoxon rank-sum test; $p < 0.05$).

6.3.3 Oral microbiome

Currently homeless

Alpha diversity in the oral microbiome was higher in individuals who were currently homeless as measured by observed OTUs (Wilcoxon rank-sum test; $p = 0.05$), but not in Shannon diversity (**Figure 6.2**). Beta diversity analysis using weighted and unweighted UniFrac

and further analysis by PERMANOVA of oral samples revealed no differences in bacterial community structure based on homelessness.

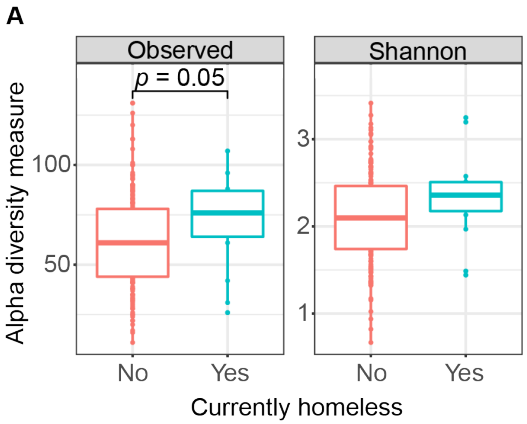

Figure 6.2 Alpha diversity boxplots in oral microbiome based on currently homeless
Boxplots of alpha diversity metrics in the oral microbiome based on currently experiencing homelessness. Sample size: No, 163; Yes, 15.

Traumatic brain injury

Alpha diversity in the oral microbiome approached significance in displaying a negative correlation with TBI count when all TBIs were included as measured by Shannon diversity (Pearson correlation; $r = -0.12$, $p < 0.10$), but not in observed OTUs (Pearson correlation; $r = -0.12$, $p = 0.11$) (**Figure 6.3A**). However, this relationship may be affected by the low number of participants with high TBIs (TBI > 6) and low alpha diversity measures. Alpha diversity in the oral microbiome was lower in individuals who had experienced two or more TBIs relative to individuals who had experienced one or no TBIs as measured by Shannon diversity (Wilcoxon rank-sum test; $p < 0.05$), but not in observed OTUs (**Figure 6.3C**). Beta diversity analysis using weighted and unweighted UniFrac and further analysis by PERMANOVA of oral samples revealed no differences in bacterial community structure based on TBI.

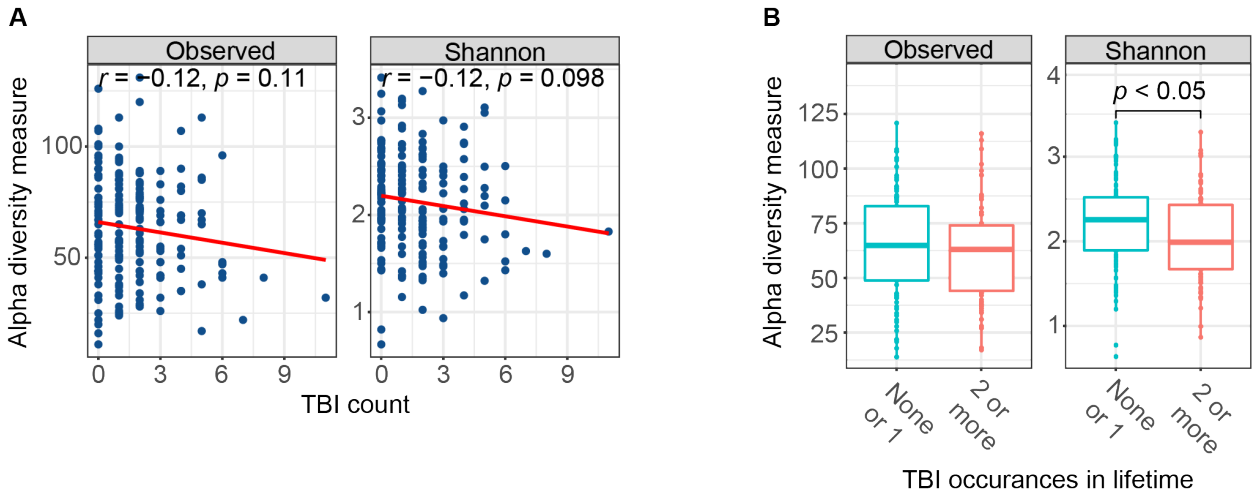

Figure 6.3 Alpha diversity in oral microbiome based on traumatic brain injury (TBI)
A) Scatter plots with regression lines of alpha diversity metrics in the oral microbiome based on total TBI count. B) Boxplots of alpha diversity metrics in the oral microbiome based on the grouping of one or no TBIs and two or more TBIs. Sample sizes: A, B) TBI count: 0, 50; 1, 43; 2, 38; 3, 18; 4, 12; 5, 8; 6, 5; 7, 1; 8, 1; 11, 1. C) None or 1, 93; 2 or more, 84.

6.3.4 Fecal microbiome

Lifetime major depressive disorder (MDD)

Alpha diversity in the fecal microbiome was higher in individuals who reported experiencing major depression during their lifetime as measured by observed OTUs (Wilcoxon rank-sum test; $p < 0.05$), and approached statistical significance as measured by Shannon diversity (Wilcoxon rank-sum test; $p = 0.07$) (**Figure 6.4**). Beta diversity analysis using weighted and unweighted UniFrac and further analysis by PERMANOVA of fecal samples revealed no differences in bacterial community structure based on reporting major depressive disorder (MDD) during an individual's lifetime.

Current major depressive disorder (MDD)

Alpha diversity in the fecal microbiome was not different based on current MDD. Beta diversity analysis using weighted UniFrac and further analysis by PERMANOVA of fecal

samples revealed a difference in bacterial community structure in individuals currently reporting MDD ($p < 0.05$; No, $n = 136$; Yes, $n = 23$). Further analysis of specific taxa revealed no differences at the phylum or class taxonomic levels. Individuals reporting current MDD showed greater relative abundance of the order Actinomycetales (phylum: Actinobacteria) (Wilcoxon rank-sum test; $p < 0.05$). Individuals reporting current MDD showed greater relative abundance of the family Prevotellaceae (phylum: Bacteroidetes), Tissierellaceae (phylum: Firmicutes), and Corynebacteriaceae (phylum: Actinobacteria), but lower in relative abundance of Bacteroidaceae (phylum: Bacteroidetes) (all, Wilcoxon rank-sum test; $p < 0.05$). Individuals reporting current MDD showed greater relative abundance of the genera *Prevotella* (phylum: Bacteroidetes), *Peptoniphilus* (phylum: Firmicutes), and *Anaerococcus* (Phylum: Firmicutes), but lower relative abundance of *Bacteroides* (Phylum: Bacteroidetes).

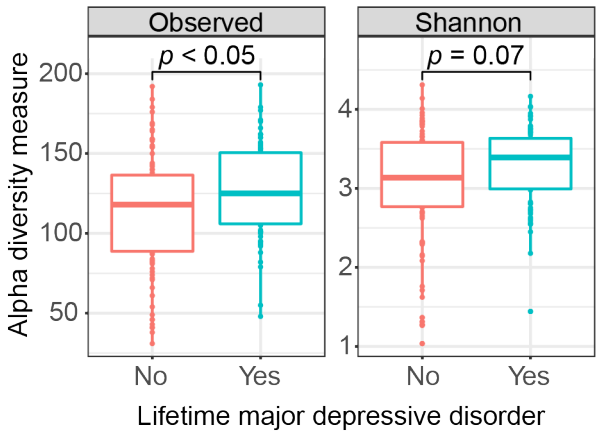

Figure 6.4 Alpha diversity boxplots in fecal microbiome based on reporting major depressive disorder during an individual's lifetime
Boxplots of alpha diversity metrics in the fecal microbiome based on reporting major depressive disorder during an individual's lifetime. Sample size: No, 88; Yes, 71.

6.4 Discussion

Extensive analyses were performed to elucidate associations of mental health variables and the skin, oral, and fecal microbiomes of a cohort of US Veterans. A majority of the mental health metrics were not associated with the microbiome. Veterans who had ever experienced

homelessness showed increased alpha diversity as measured by observed OTUs in the skin and oral microbiomes. Outcomes that are frequently associated with combat exposure (i.e., TBIs and PTSD, and MDD) showed associations with features of the oral microbiome, specifically, individuals with two or more TBIs had decreases in alpha diversity of the oral microbiome, as measure by Shannon diversity. Meanwhile, both current PTSD and lifetime PTSD were associated with changes in the bacterial community structure of the skin microbiome. Lifetime MDD was associated with an increase in alpha diversity of the fecal microbiome, as measured by observed OTUs, while current MDD was associated with altered bacterial community structure of the fecal microbiome.

6.4.1 Skin microbiome

Ever homeless

Veterans who had ever experienced homelessness showed an increase in alpha diversity of the skin microbiome as measured by observed OTUs. This effect may be related to increased relative exposure to the environment and therefore increased relative exposure to environmental bacteria. The outdoor environment harbors a high volume of biomass and diverse populations of microbes, relative to indoor environments,[237] while the environmental exposures are known to impact the skin microbiome.[21,238] In addition, it has been shown that populations that spend a significant proportion of their time interacting with their environment such as rural farming communities have significantly more alpha diversity in their skin microbiome as compared to urban non-farming populations.[239–241] Although this population of Veterans may not be living a rural farming lifestyle, they are likely to spend more time outdoors and interacting with the outdoor environment and the environmental microbes. Perhaps this population of Veterans also has higher alpha diversity because of this increased exposure to environmental bacteria.

Posttraumatic stress disorder (PTSD)

Analysis of the skin microbiome showed differences in the bacterial community structure within individuals who are currently or have ever experienced PTSD over their lifetime. Although the main focus of analysis of the microbiome as it relates to mental health has been within the gut, data suggest that the skin microbiome has potential to influence systemic immune programing,[238] which in turn has been associated with risk for development of mental health disorders, including PTSD.[20,235,242–245] Individuals with current PTSD and lifetime PTSD showed differences in the community structure within the skin microbiome with similar enrichment and depletion patterns in specific taxa, including increases in Alphaproteobacteria, Micrococcaceae, and *Corynebacterium* and decreases in Lachnospiraceae and Corynebacteriacae. It is unclear if differences in the skin microbiome of individuals with current or lifetime PTSD, relative to controls, represent a risk factor in development of PTSD, or represent differential exposures to physiologic or environmental factors that influence the composition of the skin microbiome. Addressing these questions will require further study.

6.4.2 Oral

Currently homeless

Individuals who were currently homeless showed an increase in alpha diversity in the oral microbiome, which may be an indicator of more exposure to outdoor environments, or oral associated plaque in this population. Plaque is mainly found on the teeth and gums around the teeth; however, there is a complex interplay and exchange of microbes between plaque and other surfaces and medium in the oral cavity.[246] Dental plaques are a heterogeneous and diverse community of microbes that form a cohesive film on various oral surfaces.[246] By definition, oral plaque possess all the qualities of a biofilm and this concept is widely accepted in the field.[247–249]

Oral biofilms (plaque) were found to be one of the most microbially diverse sites sampled in the human microbiome project.[24] Plaque is constantly shedding bacteria and has been shown to contribute to the microbiome of saliva and mucosal surfaces within the oral cavity.[246] Plaque has been shown to increase without daily mechanical removal.[250] It could be argued that this population may harbor more oral associated plaque and therefore higher alpha diversity because of an increase of these highly heterogeneous and diverse biofilms.

Traumatic brain injury (TBI)

Individuals with two or more TBIs in their lifetime exhibited lower alpha diversity of the oral microbiome as measured by Shannon diversity. Although not significant, the number of TBIs incurred by an individual was negatively associated with Shannon diversity when all TBIs were accounted for. In Chapter 4 of this dissertation we showed that decreases in alpha diversity in the fecal microbiome were associated with poor general and physical health as measured by BMI, general health scores from the SF-36, and the Physical Component Summary scores from the SF-36. However, few studies have examined general health implications of decreases in alpha diversity in the oral microbiome. Dysbiosis of the oral microbiome has been shown to be associated with oral health maladies such as periodontitis and dental caries.[251,252] Meanwhile, the need for ongoing dental follow-up and oral hygiene programs in the post-acute phase of rehabilitation care following TBI has been recognized.[253]

6.4.3 Fecal

Lifetime major depressive disorder (MDD)

Individuals who had experienced MDD at some point throughout their lifetime showed greater alpha diversity in the fecal microbiome. This result was contradictory to what other

studies have found. What is intriguing; however, is that these effects persisted once the microbiome was transferred into rats, which were not administered antidepressant medication. One remarkable detail about the Veterans with lifetime MDD is that they displayed an increase in alpha diversity relative to individuals that had never experienced MDD. It is unclear if this relationship is due to preexisting differences in alpha diversity, prior to onset of MDD, physiologic consequences of MDD, effects of antidepressant drugs, or lifestyle factors associated with the risk or consequences of having experienced MDD; these questions will require further study.

Current major depressive disorder (MDD)

Individuals in this Veteran cohort that reported current MDD as measured using the SCID-5 did not show differences in alpha diversity, but did show differences in bacterial community structure and differences in specific taxa, relative to individuals without current MDD. One study by Kelly et al., (2016) showed that participants with a current diagnosis of MDD had decreases in several measures of alpha diversity compared to non-depressed controls.[254] They then inoculated rats 3 days after a 28-day antibiotic regimen with the microbiome of a subset of depressed and control participants via fecal microbiome transplant. The behavioral phenotype of the rats based on sucrose preference test was that of the fecal microbiome transplant donor (depressed or control). Further, the microbiome of the rats from the MDD donors also displayed decreases in several metrics of alpha diversity compared to rats that received the microbiome from non-depressed control donors.[254] It is likely that the individuals with current MDD were also taking antidepressant medication because these subjects were recruited from "outpatient and inpatient psychiatric clinics". Lithium, SSRIs, and other

antidepressants are known to have antimicrobial effects.[255,256] In addition, Maier et al., (2018) performed an extensive meta-analysis of over 1000 drugs for their anti-commensal effects on the gut microbiome and determined that the anti-psychotic class of drugs (drug class: N05A) were highly significant in being anti-commensal.[257] These findings very well could be the explanation for the decrease in alpha diversity in individuals with current MDD observed by Kelly et al., (2016). Several studies have reported decreases in probiotic taxa such as *Lactobacillus,*[155,258,259] *Bifidobacterium,*[259] and *Faecalibacterium.*[260] Veterans with current MDD, relative to Veterans without current MDD, did not show any differences in these probiotic taxa. Previous studies have shown decreases in *Prevotella,*[254,260] while we observed an increase. One consistent finding with previous research was a decrease in *Bacteroides.*[260] Decreases in *Bacteroides* have previously been linked to metabolic disorders including diabetes and obesity.[144,261] Depression can be comorbid with metabolic disorders.[262] The individuals in this Veteran cohort with current MDD also displayed enrichment in two unique taxa, *Peptoniphilus* and *Anaerococcus*, both from the Firmicutes phylum. These genera are gram-positive anaerobic cocci that have been shown to be present in human clinical samples and are sensitive to common antibiotics.[263,264] However, based on a literature review it does not appear that these taxa are enriched or depleted in depression or other mental health disorders. It has also been noted that identification of these genera has been increasing in prevalence since the advent and widespread use of 16S ribosomal RNA gene PCR, and therefore, there may be an unknown primer bias towards these genera.[265]

6.5 Conclusions

These results provide insight into the complex relationship between the microbiome and mental health. However, further analyses need to be performed with age-, race-, gender-, and health-matched controls, matched antidepressant and other drug use, and larger sample sizes to

validate these findings. Homelessness influenced alpha diversity in both the skin and oral microbiome, which may be related to lifestyle factors that lead to more exposure the outdoors. Current and lifetime PTSD were associated with the bacterial community structure of the skin microbiome. Meanwhile, the oral microbiome showed lower alpha diversity in individuals with two or more TBIs. Major depressive disorder was associated with an increase in alpha diversity (lifetime MDD) and changes in beta diversity (current MDD) in the fecal microbiome. The increase in alpha diversity in the fecal microbiome based on lifetime MDD was a novel finding and is in need of further investigation. Examination of the enrichment and depletion patterns of specific taxa in current MDD showed both similarities and differences to results from previous studies as well as some novel taxa not previously associated with MDD.

Chapter 7. Conclusions

7.1 General characteristics of the skin, oral, and fecal microbiomes based on gender, race, and age in a Veteran cohort

This Veteran cohort has a rich and diverse range of metadata collected on demographics, armed forces service, general health, physical health, and mental health metrics. The associations observed in this Veteran cohort between features of the skin, oral, and fecal microbiomes and gender, race, and age reflect what has been previously shown in the literature. Overall, our results were consistent with previous studies of different cohorts with different demographics, which provided confidence in the data moving forward with more focused analyses.

Table 7.1 Table summary of gender, race, and age variables and their associations with alpha and beta diversity outcomes of skin, oral, and fecal microbiomes[1]

	Skin		Oral		Fecal	
Metric	Alpha	Beta	Alpha	Beta	Alpha	Beta
Gender	Observed OTUs and Shannon diversity; increased in males	Unweighted UniFrac				Unweighted UniFrac
Race		Unweighted UniFrac				Unweighted UniFrac
Age			Observed OTUs and Shannon diversity; decreased in 70+	Unweighted UniFrac		Unweighted UniFrac

[1]Table showing the summary of results for gender, race, and age as they relate to alpha and beta diversity of skin, oral, and fecal microbiomes. Green represents a significant *p*-value ($p < 0.05$), yellow represents a *p*-value approaching significance ($p \leq 0.10$), and red represents a *p*-value > 0.10. Abbreviations: OTU, operational taxonomic unit.

7.2 Association of general and physical health and the microbiome in a Veteran cohort

Three measures of general and physical health (BMI, SF-36 general health subdomain, and SF-36 PCS score) displayed similar relationships in the fecal microbiome in alpha diversity with increased observed OTUs in the "healthy" states. The SF-36 measures, but not BMI were

associated with changes in the fecal bacterial community structure. BMI displayed no association with the fecal Firmicutes/Bacteroidetes ratio. The SF-36 PCS score may be associated with the inflammatory state of an individual because "not healthy" (PCS < 45) individuals displayed enrichment in fecal Proteobacteria taxa, while "healthy" (PCS ≥ 45) individuals displayed enrichment in fecal obligate anaerobic taxa. These results align with the recently proposed "oxygen hypothesis" explaining patterns of taxonomic composition of the fecal microbiome in association with inflammation in the GI tract.

The SF-36 questions are based on the individual's perception of their health. Much of the previous research examining health and the microbiome have targeted specific disease states or a direct correlate of health such as BMI. Very few studies have examined softer metrics of health, such as frequency of exercise or even comparisons of professional athletes and non-professional athletes. To our knowledge, this is the first study to relate the general and physical health metrics of the SF-36 to the microbiome. Nor has any other study shown that an individual's perception of their general or physical health has the potential to provide insights into the fecal microbiome. Further investigation of general and physical health and perception of general and physical health as these variables relate to the microbiome are needed.

Table 7.2 Table summary of general and physical health variables and their associations with alpha and beta diversity outcomes of skin, oral, and fecal microbiomes[1]

		Skin		Oral		Fecal	
Survey	Metric	Alpha	Beta	Alpha	Beta	Alpha	Beta
Body-mass index (BMI)	BMI health categorization: healthy (<25), not healthy (≥25)					Observed OTUs; increased in healthy individuals	
	BMI quartiles						Weighted UniFrac
36-Item Short Form Health Survey (SF-36)	General health score correlation					Observed OTUS; increased with general health scores	
	General health score quartiles					Observed OTUs; increased in healthiest quartile	Weighted UniFrac
	Physical Component Summary (PCS) score correlation					Observed OTUS; increased with PCS scores	
	Physical Component Summary (PCS) score health category: healthy (≥45), not healthy (<45)					Observed OTUs; increased in healthy individuals	Unweighted UniFrac
	Physical Component Summary (PCS) score quartiles						Unweighted UniFrac

[1]Table showing the summary of results for general and physical health variables as they relate to alpha and beta diversity of skin, oral, and fecal microbiomes. Green represents a significant p-value ($p < 0.05$), yellow represents a p-value approaching significance ($p \leq 0.10$), and red represents a p-value > 0.10. Abbreviations: BMI, body-mass index; SF-36, 36-Item Short Form Health Survey; PCS, Physical Component Summary; OTU, operational taxonomic unit.

7.3 Association of insomnia and the microbiome in a Veteran cohort

Our results showed that the ISI and SCID-5 surveys are identifying different individuals within populations with insomnia-like symptoms. Comparison of the two metrics revealed that self-reported severity of insomnia symptoms, as measured by the ISI, was a more sensitive

measure in terms of associations with features of the gut microbiome. To the best of our knowledge, our result is the first report of decreased alpha diversity of the fecal microbiome in individuals with severe insomnia symptoms, as measured by the ISI. In addition, individuals with severe insomnia symptoms, as measured by the ISI, displayed changes in bacterial community structure of the fecal microbiome, which has also been displayed in mice exposed to chronic (4 weeks) sleep fragmentation.[201] Both SF mice and severe ISI Veterans with severe insomnia symptoms based on the ISI showed increases in Firmicutes. However, the increase observed in Firmicutes in SF mice was driven by Lachnospiraceae, while *Lactobacillus* taxa drove this finding in Veterans with severe ISI symptoms. Further investigation of the microbiome as it relates to insomnia is needed.

Table 7.3 Table summary of insomnia variables and their associations with alpha and beta diversity outcomes of skin, oral, and fecal microbiomes[1]

		Skin		Oral		Fecal	
Survey	Metric	Alpha	Beta	Alpha	Beta	Alpha	Beta
Insomnia Severity Index (ISI)	ISI severity (severe vs not severe)					Observed OTUs; decreased in severe ISI group	Weighted UniFrac
	ISI severity clinical categories (clinical insomnia, not clinical insomnia)					Observed OTUs; decreased in clinical ISI group	
	ISI score correlation					Observed OTUs; decreased with ISI scores	
	ISI severity categories (not significant, subthreshold, moderate, severe)						Unweighted UniFrac
Structured Clinical Interview for the DSM-5 (SCID-5)	Current insomnia disorder					Observed OTUs; increased in current insomnia group	

[1]Table showing the summary of results for the insomnia variables as they relate to alpha and beta diversity of skin, oral, and fecal microbiomes. Green represents a significant *p*-value ($p < 0.05$), yellow represents a *p*-value approaching significance ($p \leq 0.10$), and red represents a *p*-value > 0.10. Abbreviations: ISI, Insomnia Severity Index; DSM-5, Diagnostic and Statistical Manual of Mental Disorders 5th edition; SCID-5, Structured Clinical Interview for the DSM-5; OTU, operational taxonomic unit.

7.4 Association of mental health and skin, oral, and fecal microbiomes in a Veteran cohort

The number of mental health metrics collected within this Veteran cohort was exceptional. Most of the associations between features of the skin, oral, and fecal microbiomes and measures of mental health were determined to be null; however, there were some mental health measures that were associated with features of the skin and oral microbiomes. However, further analyses are need with age-, race-, gender-, and health-matched controls, matched antidepressant and other drug use, and larger sample sizes to validate these findings.

Current and lifetime PTSD were associated with the bacterial community structure of the skin microbiome. These changes in bacterial community structure may be related to behavioral changes known to be associated with PTSD. One of the core symptoms associated with PTSD is avoidance behavior and social isolation.[266] These types of behaviors could lead to an individual spending a majority of their time within their dwelling or confining themselves to a single room, essentially creating a microbial island with limited exposure to environmental bacteria, perhaps leading to unique microbial community structure presumably dominated by skin associated taxa.

Meanwhile, the oral microbiome showed lower alpha diversity in individuals with two or more TBIs, relative to individuals with one or no TBIs. Although the mechanisms underlying this association are not clear, it has been previously shown the single largest predictor of a reduction in alpha diversity was the presence of periodontitis.[267] A study determined that dental care and oral hygiene programs for individuals with TBI increased oral health and hygiene.[253]

Individuals reporting to have ever experienced MDD in their lifetime (lifetime MDD) showed increased alpha diversity of the fecal microbiome, as measured by Shannon diversity. We showed that increases in alpha diversity of the fecal microbiome were associated with increases in general and physical health in Chapter 4 of this dissertation. MDD is characterized by apathy, general discontent, and loss of interest or pleasure in activities. It is difficult to determine why these individuals exhibited increased fecal alpha diversity. One possibility is that antimicrobial activity of antidepressant drugs targets pathobionts that would otherwise proliferate and reduce alpha diversity.[255,256]

Individuals reporting current MDD showed changes in fecal bacterial community structure relative to individuals without current MDD. Investigation of patterns of enriched and depleted taxa in current MDD individuals did not align with patterns previously reported in the

literature.[254,268] MDD has a broad range of symptoms. In addition, MDD can have opposite effects on the same symptom such as appetite (both loss of appetite and excessive hunger are reported). Lithium, SSRIs, and other antidepressants are known to have antimicrobial effects,[255,256] and the whole class of anti-psychotic drugs (drug class: N05A) were highly significant in being anti-commensal.[257] Individual-to-individual dissimilarities in the medications prescribed, doses, and frequency of taking medications has a high potential to introduce variation in this population. Examining mental health from a big-picture perspective highlights the large amount of variability associated with this mental health disorders. It is likely that large sample sizes or tightly controlled studies, using age, gender, race, and medication matched controls, will be required to determine true associations between mental health disorders and the microbiome.

Table 7.4 Table summary of mental health measures and their associations with alpha and beta diversity outcomes of skin, oral, and fecal microbiomes[1]

		Skin		Oral		Fecal	
Survey	Metric	Alpha	Beta	Alpha	Beta	Alpha	Beta
Structured Clinical Interview for DSM-5 (SCID-5)	Ever homeless	Observed OTUs; increased in ever homeless group					
	Currently homeless			Observed OTUs; increased in currently homeless group			
	Lifetime MDD					Observed OTUS; increased in lifetime MDD group	
	Current MDD						Weighted UniFrac
	Lifetime PTSD		Weighted UniFrac				
	Current PTSD		Weighted UniFrac				
Ohio State University TBI Identification	TBI count correlation			Shannon diversity; decreased with TBI count			
	TBI 2 or more vs 1 or none			Shannon diversity; decreased in 2 or more TBI group			

[1]Table showing the summary of results for the metal health measures as they relate to alpha and beta diversity of skin, oral, and fecal microbiomes. Green represents a significant *p*-value ($p < 0.05$), yellow represents a *p*-value approaching significance ($p \leq 0.10$), and red represents a *p*-value > 0.10. Abbreviations: DSM-5, Diagnostic and Statistical Manual of Mental Disorders 5th edition; SCID-5, Structured Clinical Interview for the DSM-5; MDD, major depressive disorder; PTSD, posttraumatic stress disorder; TBI, traumatic brain injury; OTU, operational taxonomic unit.

7.5 Limitations

7.5.1 Sample population and sample size

This dissertation evaluated the skin, oral, and fecal microbiomes of a cohort of 188 U.S. Veterans enrolled in the Veteran Microbiome Study as part of the Military and Veteran

Microbiome Consortium for Research and Education (MVM-CoRE) initiative. This cohort was the first cohort among what are planned to be larger cohorts with larger sample sizes and additional endpoints. For this initial study, although the overall sample size is relatively high (*N* = 188), sample sizes for a number of endpoints were low and unbalanced. This population is unique in that these individuals are Veterans and the armed forces tend to attract certain demographics more than others. For example, this Veteran cohort included a low number of women (*n* = 28, 15% of sample population); however, this is higher than the percentage of women within the total Veteran population as of 2015 (9.4%).[269] Some racial groups were underrepresented, such as Native Americans, Asians, Multiracial, and "Other". Also, the oldest age group +70 was underrepresented. Many mental health measures were collected from this Veteran cohort. This had the advantage of examining the relationship between skin, oral, and fecal microbiomes with a large number of measures of mental health. However, some of the mental health measures had small sample sizes of participants exhibiting the condition or disorder. Because many of these variables were dichotomous (present or absent) this resulted in a high proportion of the sampling population in the "absent" category. For example, for the fecal microbiome of this Veteran cohort, 23 individuals reported current symptoms of MDD (14.5%) while 136 individuals did not. In future analyses, we plan to balance sample sizes with controls matched for variables such as gender, race, age, and BMI.

An unfortunate consequence of the highly unbalanced sample sizes in some of the outcomes studies was the implications for machine learning. A meta-analysis by Walters et al., (2014) details how random forest is sensitive to unbalanced sample sizes in classifying a category "whose composition is/are highly similar." Walters et al., (2014) was referring to classifying lean vs obese when referring to "highly similar" bacterial compositions, which is

arguably much less similar than some of the metadata categories of interest. This was confirmed empirically as, in our attempts to run random forest models on the highly unbalanced sample sizes, the models were not able to converge and therefore were not able to make any valid classifications.

Furthermore, we understand that we may not have reached statistical significance within many of the examined variables because of a lack of statistical power. Falony et al., (2016) performed a power analysis to determine the samples sizes need to reach statistical significance of bacterial community structure based on PERMANOVA between lean vs obese individuals. They determined that it would require 865 lean and 865 obese individuals (p-value = 0.05, power = 80%) to reach statistical significance in analysis of bacterial community structure based on PERMANOVA between lean vs obese individuals. This provides a general sample size for future studies of the association of BMI with skin, oral, and fecal microbiomes.

7.5.2 Effect size

We did not directly measure effect size; however, it was shown by Falony et al., (2016) that variables associated with "lifestyle" showed relatively low effect sizes of ~ 0.04. It is possible that effect sizes for between-group differences for most of our variables were similar to this or even lower. It should be noted that genome-wide association studies (GWAS) studies also have low effect sizes.[270] So, perhaps as has been demonstrated in GWAS studies, microbiome-related studies will be found to have low effect sizes in relation to measures of general, physical, and mental health, and results will need to be interpreted in this context.

7.6 Future directions

As part of the Veteran Microbiome Study, samples continue to be collected. In addition, the study is being expanded to include additional surveys listed in **Table 7.5**, as well as longitudinal sampling (currently under review by COMIRB).

Table 7.5 Future survey information and details

Measure	Time (min)	Purpose
Baseline Visit at ECHCS		
Neurobehavioral Symptom Inventory (NSI)[271]	5	TBI
Patient Health Questionnaire (PHQ)-9[272]	5	Mental health
Morningness/Eveningness Questionnaire (MEQ)[273]	5	Sleep
Seasonality Pattern Assessment Questionnaire (SPAQ)[274]	5	Mental health
National Health Interview Survey (NHIS) – Chronic Conditions[275]	5	Physical health
Housing, Occupancy, Materials and Environment (HOME) Survey[276]	5	Housing status/ mental health

Based on results from other studies, the importance of the Bristol stool chart survey has come to light.[26] The Bristol stool chart score has been highly related to the bacterial community structure of the microbiome.[26] This could also be an additional useful survey for future cohorts. Furthermore, a list of medications currently being taken should be added to the metadata variables. Medications have been shown to have a large effect size in other studies.[26] Although this large effect size may be largely influenced by antibiotics, a recent meta-analysis by Maier et al., (2018) showed that anti-psychotic medications and other related medications are anti-commensal. These are of particular relevance to this Veteran population and would be a useful addition to the metadata for analysis of this cohort and future cohorts.

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Appendix

A1 Survey descriptions

A1.1 University of Washington Risk Assessment Protocol-Revised (UWRAP)

The UWRAP was used to assess and address any potential risk associated with participating in this study. Participants were asked to articulate pre-test potential stressors (Pre-Assessment Risk Assessment Questions). Post-test administration, a debriefing checklist and protocol were initiated. Using results from the debriefing, members of the research team were trained to evaluate responses and access additional assistance if necessary. The UWRAP has been recommended by National Institute of Mental Health, and has been used for over 20 years in research with potentially high-risk patients.

A1.2 Rocky Mountain MIRECC Demographic Questionnaire

This survey gathered demographic information including participant age, gender, race/ethnicity, education, period of military service, and combat exposure.

A1.3 Structured Clinical Interview for DSM-5-TR Axis I Disorders, Research Version, Patient Edition with Psychotic Screen (SCID-I/P W/ PSY SCREEN)

The SCID interview is a reliable and valid semi-structured interview used to diagnose Axis I psychiatric disorders in clinical and research settings.

A1.4 Ohio State University TBI Identification Method (OSU-TBI-ID)

The OSU TBI-ID interview is a structured interview developed using recommendations from Centers for Disease Control (CDC) for the detection of history of exposure to TBI. It was designed to elicit self-reports of TBI occurring over a person's lifetime. This method has been validated in two studies including 103 and 119 participants, respectively, where the OSU TBI-ID

was found to have high inter-rater reliability. Our study used the OSU TBI-ID to identify whether a participant had experienced a moderate or severe TBI.

A1.5 PTSD Checklist 5 (PCL-5)

The PCL-*5* is a self-report measure that assesses PTSD symptom severity, based on the DSM-*5*. Each item is rated on a 5 point Likert scale. The PCL-5 can be used to screen for PTSD or monitor change in PTSD symptoms.

A1.6 Outcome Questionnaire (OQ-45)

The OQ-45 is a 45-item questionnaire that was designed to measure key areas of mental health functioning (symptom distress, interpersonal functioning, and social role). As such it is a validated and accepted tool for identifying, tracking, and measuring behavioral health treatment outcomes and will be the measure of symptom distress in this study. The measure possesses good psychometric properties when used with adult psychiatric patients.

A1.7 36-Item Short Form Health Survey (SF-36)

The SF-36 is a multi-purpose, short-form health survey with only 36 questions. It yields an 8-scale profile of functional health and well-being scores as well as psychometrically-based physical and mental health summary measures and a preference-based health utility index. The instrument was administered by a trained coordinator.

A1.8 Harvard Food Frequency Questionnaire 2007 Booklet (Harvard FFQ)

The Harvard FFQ is a comprehensive 101-item semi-quantitative food frequency questionnaire that includes questions on specific foods, diet types, and supplements. Analysis of the questionnaire provides a wide range of macro- and micronutrient quantities. A number of

studies have demonstrated fair to good validity and fair to good reproducibility depending upon nutrient of interest.

A1.9 The Insomnia Severity Index (ISI)

The ISI is a 7-item instrument assessing the nature and severity of insomnia symptoms, satisfaction with sleep, interference of sleep disturbance with daily functioning, and how distressing and noticeable the sleep impairment. The scale is considered a reliable and valid measure of insomnia. The ISI was used in our study to assess the presence of persistent insomnia complaints.

A1.10 PTSD Checklist for the DSM-5 (PCL-5)

The PCL-5 is a 20-item self-report measure that assesses the 20 DSM-5 symptoms of PTSD. The PCL-5 is a quicker alternative to performing a full Clinician-Administered PTSD Scale (CAPS-5), which is the gold standard for PTSD diagnosis. The PCL-5 is good for screening individuals for PTSD, making a provisional diagnosis of PTSD, or monitoring symptom change during and after treatment.

A1.11 Beck depression inventory (BDI)

The BDI is a 21-question self-report survey to measure the severity of depression. The survey targets specific symptom categories of hopelessness, irritability, guilt, feelings of being punished, fatigue, weight loss, lack of interest in sex.

A2 Sample collection protocols

A2.1 Skin sampling protocol

1. A research assistant would put on nitrile or latex gloves
2. The research assistant would hand the participant a sealed double tipped sampling swab

3. The research assistant would instruct the participant to open the collection tube to expose the swab
4. The participant was instructed to firmly swab their inner elbow for 10 seconds being careful to avoid contacting anything else with the swab
5. The participant was instructed to place the swabs back into the collection tube
6. The participant would hand the collection tube back to the research assistant
7. The research assistant wrote the date and time on the collection tube label
8. The research assistant would then place the sample into a freezer for storage at –80°C

A2.2 Oral sampling protocol

1. A research assistant would put on nitrile or latex gloves
2. The research assistant would hand the participant a sealed double tipped sampling swab
3. The research assistant would instruct the participant to open the collection tube to expose the swab
4. The participant was instructed to open their mouth and swab the inside of one cheek firmly for 10 seconds being careful to avoid contacting the teeth, gums, and tongue
5. The participant was instructed to place the swabs back into the collection tube
6. The participant would hand the collection tube back to the research assistant
7. The research assistant wrote the date and time on the collection tube label
8. The research assistant would then place the sample into a freezer for storage at –80 °C

A2.3 Fecal sampling protocol (in-clinic)

1. A research assistant would put on nitrile or latex gloves

2. The research assistant would hand a sampling kit to a participant, which contained the following instructions

How to collect your sample:

- Take the collection tube to the restroom with you to collect your stool sample.
- Once your stool movement is complete. Put on your gloves and wipe with toilet paper.
- Carefully holding the used toilet paper, open the tube with the swab inside with the other hand. Be careful not to touch the stem or cotton swab.
- Use the swab tip to collect a sample from the toilet paper. You only want to collect enough to color half the swabs brown. Do not fill the entire swab with your sample.
- Place the swab back into the tube
- Remove your gloves, flush the toilet and wash your hands after you have completed collecting the sample.

3. Upon the return of the participant, the research assistant would take the collection tube with soiled swab and write the date on the collection tube label

4. The research assistant would then place the sample into a freezer for storage at –80°C

A2.4 Fecal sampling protocol (out-of-clinic)

Below is the text from an instruction sheet that was provided with the fecal sampling clinic for out-of-clinic use.

Sampling Protocol for Gut Swab Microbial Sampling

- Please do not open the kits until after you are in the restroom. Handle the tube by the red cap only.
- You will not collect your entire stool sample. You will be using the swabs to get your sample from the "first use" toilet paper (the first wipe with the toilet paper you make after your stool movement is complete)
- Do not collect your sample until you are sure you can drop the sample in the mailbox on the same day.
- Place the cold pack provided in the freezer.

How to collect your sample:

1. Take the 2 collection tubes to the restroom with you to collect your stool sample.
2. Once your stool movement is complete. Put on your gloves and wipe with toilet paper.
3. Carefully holding the used toilet paper, open the tube with the swab inside with the other hand. Be careful not to touch the stem or cotton swab.

4. Use the swab tip to collect a sample from the toilet paper. You only want to collect enough to color half the swabs brown. Do not fill the entire swab with your sample.
5. Place the swab back into the tube.
6. Repeat steps 3-5 with the second tube.
7. Remove your gloves, flush the toilet and wash your hands after you have completed collecting the sample.

After you have collected your sample:

1. Record the date and time you collected your sample on the outside of the tube.
2. Put the tubes and the cold pack in the self-addressed envelope provided to you.
3. Seal the envelope.
4. Drop the envelope off at the nearest mailbox or post office.

If something out of the ordinary occurs, like you touch the swab to another surface or drop the swab on the floor, please notify Kelly Stearns-Yoder at 303-399-8020.

A3 Future Surveys

A3.1 Neurobehavioral Symptom Inventory (NSI)

The NSI is a widely-used measure of post concussive symptoms among military personnel and Veterans and is recommended by the VHA for screening and evaluation.

A3.2 Patient Health Questionnaire-9 (PHQ-9)

The PHQ-9 is a frequently used and psychometrically sound self-report measure of depression. It is free to use and has been validated and administered in many clinical settings.

A3.3 Morningness/Eveningness Questionnaire (MEQ)

The MEQ is a 19-item self-report instrument designed to assess chronotype (evening, intermediate, or morning) in individuals. Information about participant chronotype was assessed to determine whether certain chronotypes were more or less sensitive to conventional insomnia treatment.

A3.4 Seasonality Pattern Assessment Questionnaire (SPAQ)

The SPAQ is a research and screening tool that is widely-used in studies of seasonality to assess mood and behaviors relating to Seasonal Affective Disorder (SAD). The SPAQ evaluates severity of global seasonal changes, degree of functional impairment with those changes, and seasonal patterns of affective states.

A3.5 National Health Interview Survey – Chronic Conditions

The National Health Interview Survey (NHIS) has been used to monitor the health of the population of the nation since 1957. NHIS data on a broad range of health topics have been collected through personal household interviews for over 50 years through the US Census Bureau. Survey results have been instrumental in providing data to track health care access, and progress toward achieving national health objectives. Our study administered the NHIS to assess chronic conditions in our sample population.

A3.6 Housing, Occupancy, Materials, and Environment (HOME) Survey

The HOME survey determines if aspects of the built environment influence the mental well-being of individuals and their relative significance. Ultimately, the desired outcome would be to establish design guidelines that take into consideration factors of the built environment that correlate to diagnoses of depression, anxiety, and PTSD. Our study used the HOME survey to assess the living situation and mental health measures in our sample population.