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An Investigation to Corroborate Volatile and Biological Profiles of Human Odor for Forensic Subject Identification

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AN INVESTIGATION TO CORROBORATE VOLATILE AND BIOLOGICAL PROFILES OF HUMAN ODOR FOR FORENSIC SUBJECT IDENTIFICATION

A dissertation submitted in partial fulfillment of

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in

BIOCHEMISTRY

by

Chantrell Janae Gladys Frazier

2022

To: Dean Michael R. Heithaus
College of Arts, Sciences and Education

This dissertation, written by Chantrell Janae Gladys Frazier, and entitled An Investigation to Corroborate Volatile and Biological Profiles of Human Odor for Forensic Subject Identification, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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Date of Defense: March 30, 2022

The dissertation of Chantrell Janae Gladys Frazier is approved.

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Andrés G. Gil
Vice President for Research and Economic Development
and Dean of the University Graduate School

Florida International University, 2022

DEDICATION

This research is dedicated to my village. I distinctively dedicate this to my mom, Ayanna McBride, who motivated me through this entire process with unconditional motherly love. My father, Dr. Charlie Frazier, who set the example for pursuing a doctorate. My Aunt Jessie M. Perry and Grandmother Mema for all the daily prayers and support. Also, my uncle Ken Perry for never letting me forget that I needed to hurry up and graduate but also encouraging me to set an example for my brothers and sisters. Dr. Jada Frazier, you understood my mental struggles and never let me give up on the task at hand. My Grandparents Dr. Charlie Frazier and Dr. Jacquelyn Frazier, thank you for your love and support. Lastly, I dedicate this to my cousins and family friends -- it's so many of you that I can't personally name- you all have encouraged me in some shape or form to remain strong and finish, and that's exactly what I've done!

IN MEMORY

During the execution of this research many loved ones were loss. This research was finished in memory of all the family members I lost during this journey: Jessie M. Perry (My Heart/Aunt) and cousins: Nancy Miller, Michelle Jenkins, and Jackie and Pat Creal.

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ABSTRACT OF THE DISSERTATION

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Hands are an integral part in transferring complex microbial communities to and from our surroundings. As a result, hands are significant in provenance investigations as specific microbiota can be deposited on everyday objects through touch interactions. Skin microbiome, including bacteria, fungi, and viruses, are unique to each person, and this 'uniqueness' can be exploited and applied to forensic identification. Skin microbiota and volatile organic compounds (VOCs) are closely related due to specific bacteria breaking down non-volatile organic compounds to volatile organic compounds that are characteristically present in human scent. However, analyses of microbiota from touch interactions have proven to be difficult due to the low levels of genomic DNA (gDNA) that can be collected and analyzed with downstream techniques.

Headspace Solid Phase Microextraction Gas Chromatography Mass Spectrometry (HS-SPME-GCMS) is commonly used to conduct VOC analyses of air and water samples. However, previous studies have utilized HS-SPME-GCMS as a forensic approach to examine VOCs exuded from various regions of the body as a form of identification.

Furthermore, the human microbiome has been closely studied in relation to health and disease, but more recently been examined as new potential forensic tool. The objective of this research is to analyze samples collected from subject's palms to determine the relationship between the bacterial microbiota profile and the VOC profile as it relates to the classification and discrimination of individuals. Palmar sweat samples and epithelial swabs were simultaneously collected for VOC and microbiome analysis, respectively. Supervised linear regression models (PLS-DA, OPLS-DA, and LDA) were evaluated as a tool for the prediction and discrimination of subject gender. The amplified DNA targeting the V1-V2 16S rDNA region was initially screened via Length Heterogeneity Polymerase Chain Reaction (LH-PCR) to obtain an initial assessment of the bacterial community's diversity. Amplicons were sequenced with Next Generation Sequencing (NGS) technology and further analyzed with bioinformatics, which identified well known skin bacterium on the genus level *Staphylococcus, Cutibacterium, Enhydrobacter, Streptococcus, Lawsonella, Fusobacterium, and Micrococcus* known to contribute to human odor volatiles. The combination of human odor and bacterial microbiome analysis could lead to the utilization of odor as a novel biometric for forensic identification where other physical and trace evidence may be lacking.

TABLE OF CONTENTS

LIST OF TABLES

TABLE PAGE

LIST OF FIGURES

FIGURE PAGE

LIST OF ABBREVIATIONS

AED	Atomic Emission Detector
bp	Base Pair
CE	Capillary Electrophoresis
DI	Direct Immersion
DNA	Deoxyribonucleic Acid
DVB/CAR/PDMS	Divinylbenzene/Carboxen/Polydimethylsiloxane
ECD	Electron-Capture Detector
FID	Flame Ionization Detector
GCMS	Gas Chromatography-Mass Spectrometry
HS	Headspace
IMS	Ion Mobility Spectrometry
ITS	Internal Transcribe Spacer
K-9	Canine
LCN	Low Copy Number
LDA	Linear Discriminant Analysis
LHPCR	Length Heterogeneity
LOOCV	Leave One Out Cross Validation
LSU	Large Subunit
LTDNA	Low Template DNA
MHC	Major Histocompatibility Complex
mRNA	Messenger RNA
NGS	Next Generation Sequencing

OPLS-DA	Orthogonal-Projections Latent Structures Discriminant Analysis
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PLS-DA	Partial Least Squares- Discriminant Analysis
rRNA	Ribosomal Ribonucleic Acid
SPME	Solid Phase Microextraction
spp	Species
SSU	Small Subunit
STR	Short Tandem Repeat
STU	Scent Transfer Unit
SWGDOG	Scientific Working Group on Dog and Orthogonal Detection Guidelines
TIC	Total Ion Chromatogram
TOFMS	Time-Of-Flight Mass Spectrometry
VOCs	Volatile Organic Compounds

1 INTRODUCTION

During violent and property crimes, there is often an exchange of biological and chemical material by the perpetrator or victim to the crime scene. This very exchange of materials between an object and/or a person or two persons is in accordance with *Locard's Exchange Principal* **(Saferstein, 2011)**. Various biological material that is often collected and analyzed to connect the suspect to the crime includes fingernails, blood, hair, saliva, and other bodily fluids. Downstream DNA and chemical analyses are performed to curate various forms of evidence. However, evidence (human DNA, fingerprints, etc.) collected at the crime scene is often degraded or found only in trace amounts **(Leary, 2018)**. Invisible or limited biological samples and/or DNA that amounts to less than 100pg is referred to as Trace DNA **(Gill, 2001).** This presents legal issues because a partial or non-interpretable human profile from trace samples is not always probative **(Leary, 2018)**. Therefore, many law enforcement agencies have shifted to include another form of trace evidence that is often overlooked yet always present: human scent.

However, the presentation of collected human scent as a form of forensic evidence has been difficult in the view of admissibility within the court of law. The admissibility criteria for expert evidence have been established by the Frye (1923) and Daubert (1993) standards **(DeMatteo et. al, 2019).** Human scent, comprised of Volatile Organic Compounds (VOCs), can be recognized by the human and animal olfactory systems. The investigation of VOCs has mainly been contributed by *Canis familiaris,* also known as the common dog or canines (K-9), most commonly used for the detection of a wide variety of hidden substances that include but are not limited to: explosives, narcotics, mass storage devices, firearms, search & rescue, medical detection, pests, ignitable liquid residues,

conservation, invasive species, agriculture, currency, and tracking/trailing. **(Jenkins et. al, 2018)**. In comparison to its animal counterpart, instrumental analysis of the chemical signatures presented by human scent has been limited because of sensitivity limitations. The correlation of the specific human odor signatures a canine may alert to when indicating a positive match to a subject has been limited within scientific literature **(Prada et. al, 2015-Human scent evidence book)**. As a result, there has been a forensic community effort to increase the scientific knowledge that supports what the K-9 has detected via standardized procedures and guidelines provided by the Scientific Working Group on Dog and Orthogonal Detection Guidelines (SWGDOG). This study seeks to contribute to the scientific literature to increase the probative value of human scent as a corroborating biometric for forensic evidence when other physical evidence is lacking.

The deposition of human odor is not only dispersed into the air or exchanged with the surrounding environment by our individual encompassing VOC cloud but can also be transferred to secondary objects through touch interactions **(Filetti et. al, 2019)**. Our hands are an integral part in transferring complex microbial communities to and from our surroundings. We eat, open doors, and touch multiple surfaces with our hands daily, leaving behind traces of our individual microbiomes and VOCs. The skin microbiome, including but not limited to bacteria, fungi, and viruses, is characteristic to each individual and can be utilized and applied for forensic identification. Skin microbiota and VOCs are intrinsically linked due to microbial metabolic transformation of non-volatile organic compounds to volatile organic compounds. Therefore, this study investigated the biological (microbiota) and chemical (VOCs) signatures of collected human scent samples.

Previous research has assessed demographical (ethnicities/race, age, and gender) information assessed from volatile and biological profiles to aid in the forensic identification of individuals. The limitations from instrumentation sensitivity, sample collection (SPME), and sample quality (trace DNA) have posed a challenge to the advancement of human scent knowledge. Many methodologies have been developed to increase the opportunity to generate a profile by increasing the number of cycles during amplification or selection of proper extraction kits to aid in maximizing the quantity of DNA that is input prior to downstream analysis **(Oorschot et al.)**. Specifically, from very minute samples such as the low copy number (LCN) or low template DNA (LTDNA). The developed methodologies aimed to increase information obtained from human scent volatile profiles data, has demonstrated the significance on a pictorial (stacked bar graphs) and unsupervised dimensional analysis (PCA) level. However, there is the need to transition the evaluation of volatile profiles for statistical significance with supervised methodologies and algorithms. This study engages and explores said biological methodologies such as PCR, capillary electrophoresis (fragment analysis), next generation sequencing, and chemical methodologies (HS-SPME-GCMS).

The concatenation of this combined data with supervised dimensional reduction techniques and machine learning algorithm via software such as Primer-E, JMP, and MetaboAnalyst should advance the scientific literature forensic identification. Previous research on trace DNA analysis, microbiome analysis, and VOC analysis, individually, have proven to be challenging and limited on the demographical information they can provide. *Thus, the goal of this dissertation research was to analyze samples collected from*

the subject's palms to determine the relationship between the individual's bacterial DNA profile and VOC profile for potential use as forensic identification.

2 LITERATURE REVIEW

2.1 Forensic Human Identification

According to Sollberger, the philosophical perspective of "identity" is that it's a predicate, which functions as an identifier to distinguish and differentiate one object from another object **(Sollberger et al., 2013)**. "Human "identification" and, more specifically, the biological aspects of human identity, are grounded in the well-defined and statistically verifiable sciences of biology, chemistry, and physics" **(Thompson et al., 2006)**. The application of well-established scientific research, tools, and methodologies to aid in the resolution of criminal and civil disputes is known as forensic science **(Kabir et al., 2021)**. Its influence on the criminal justice system has increased due to cross-disciplinary nature of fields and the capability to provide insight on what may have occurred at a crime scene. Over the last 30 years various forms of evidence (DNA, fingerprints, etc.) have been collected and analyzed to convict, acquit, or identify individuals.

2.2 Phenotypic Traits for Identification

Phenotypic traits: gender, race/ethnicity, and age have been the leading characteristics to identifying an individual. Within the past decade these observable traits have undergone societal influences outside of the inherited biological traits they were originally termed after. These influences have led for the terms' "sex" and "gender" to be clarified because both are in place of each other in research **(Short et al., 2013)**. The term "sex" differentiates males and females biologically because of their reproductive organs and functions **(Ryan, B., 2007)**. Moreover, sex, contains sex-specific variation in chromosomes, as women contain two X chromosomes, while males have and X and Y

chromosome **(Short et al., 2013)**. The social interaction and discussion of gender from a cultural and institutional lens allows for the terminology to be created and redefined in a continuously evolving cycle **(West et al., 1987)**. Therefore, individuals throughout this study self-identified their gender as no genetic information was collected to verify their sex. From this moment forward, the word gender will represent the participants' self-identification as either male or female.

Race and ethnicity are also phenotypic traits whose terms originated with a biological foundation. Specifically, within health-related research, race is notated as a combination of not only biological indicators, but personal and group identity influences as well **(Ford et al, 2005)**. This term is commonly implemented when specific racial groups are disproportionately or beneficially impacted by health, political, and social situations. Ethnicity, in contrast, is commonly referenced to a specific group of individuals who have a shared cultural identification that includes origin, language, tradition, and religion **(Ford et al, 2005)**. Within this study participants were classified into three racial (African American, Caucasian, and Hispanic) groups but were allowed to indicate their ethnic background or association. Overall, gender and race/ethnicity are not as simplistic as their terminology origination but are a major portion of human forensic identification. They have been the focal point of methodologies presented in court to assess their admissibility of evidence presented in that context.

2.3 Forensic Admissibility

The current tools utilized within forensics for the identification of humans, biological fluids, and other various forms of evidence have undergone a scrutinized process to become admissible in the court of law. There are two major court decisions that have established the admissibility criteria for expert evidence and witnesses. In *Frye v. United States* (1923), the United States Court of Appeals for the District of Columbia Circuit held that proffered expert evidence must be based on generally accepted scientific methods **(DeMatteo, 2019)**. This became known as the "general acceptance" rule and remained unchallenged for seventy years.

In accordance with the establishment of Federal Rules of Evidence (FRE) - specifically, FRE 702- in 1975, the *Frye standard* was replaced following the decision of *Daubert v. Merrell Dow Pharmaceuticals, Inc* (1993). In the *Daubert* ruling, four criteria were established to decide whether the proffered evidence (a) was derived from methodology that has or can be tested empirically, (b) has been subjected to peer review and publication, (c) has a known or documented potential rate of error, and (d) has achieved general acceptance in its relevant scientific community **(DeMatteo, 2019)**.

With most states adopting the *Daubert standard* and other modified versions, some psychologists feel as if the promising nature of *Daubert* has been unfulfilled. However, a study conducted by Gatowski et al. in 2001 contradicted that theory reporting percentages of 88%, 91%, 92%, and 93% from judges that believed in the utility of the Daubert criteria: recognizing the value of testing, considering the error rate of testing procedures, peer review and publication, and considering general acceptance, respectively **(Gatowski et al.,**

2001). Understanding the criteria required for newer evidence to be accepted in the court of law lays the foundation for exploring newer biometrics for forensic identification.

2.4 Current Biometrics for Forensic Identification

2.4.1 DNA

DNA is commonly known for its storage of genetic information. A single nucleotide of DNA is comprised of three components: sugar (deoxyribose), nitrogenous base (Adenine, Guanine, Thymine, or Cytosine), and a phosphate group (Figure 2-1). The sequence of DNA naturally follows a 5' to 3'directionality, as nucleotides bind through a phosphodiester bond between the 5' phosphate group and the 3' deoxyribose sugar with natural pairings of purines to pyrimidines (Adenine to Thymine and Guanine to Cytosine) (Figure 2-2). The basic unit of measurement for sizing DNA is a base pair (bp). The emergence of DNA has led to its use as a powerful tool to identify perpetrators of unspeakable crimes and to exonerate innocent individuals accused of similarly heinous actions **(Leary, 2017)**. The value of DNA as a form of forensic evidence has grown exponentially since its initial application.

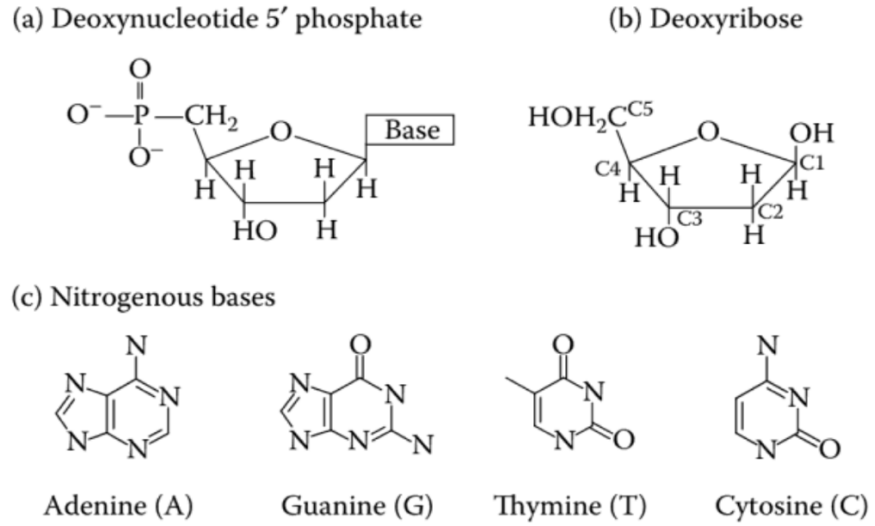

Figure 2-1: Molecular structure of DNA components (A) Deoxynucleotide 5' Phosphate (B) Deoxyribose (Sugar) (C) Nitrogenous bases. (Thompson et al., 2006).

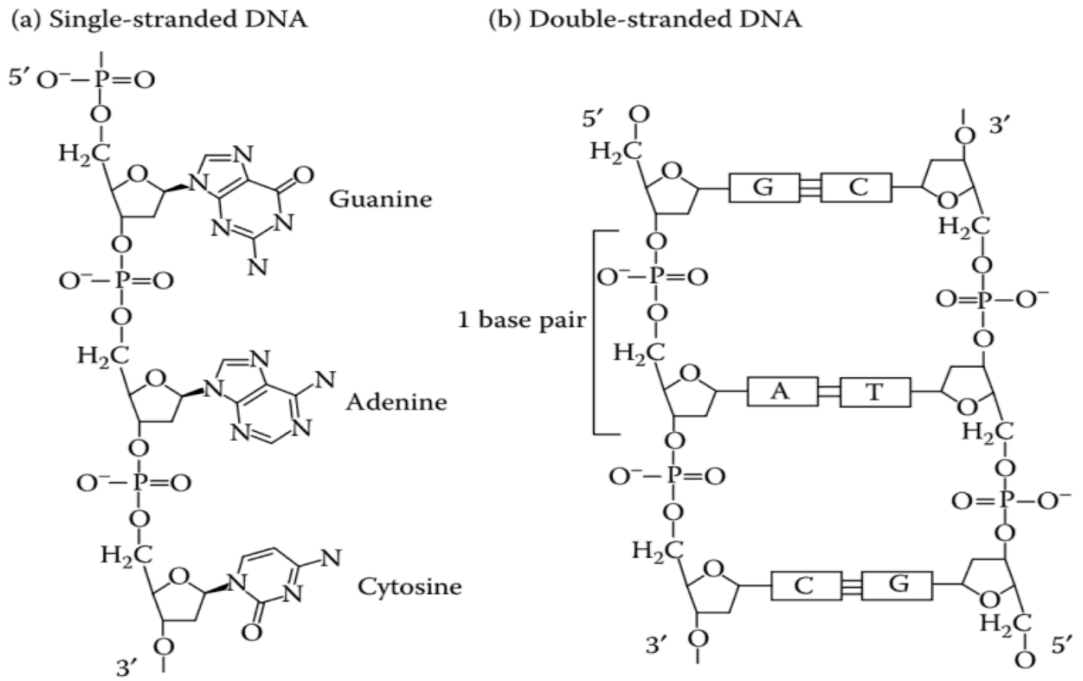

Figure 2-2:Molecular structure of (A) single-stranded DNA and (B) double-stranded DNA (Thompson et al., 2006).

The utilization of DNA for identification purposes dates to 1985 in the U.K. with paternity testing for an immigration case. It was later applied to criminal cases in 1988 for successful prosecution of a rapist after a 17-year-old kid was wrongly implicated. The idea of DNA profiling was established by Alex Jeffreys who discovered that certain regions of DNA were highly variable between individuals **(Thompson et al., 2006)**. Jeffrey's methods to analyze the polymorphic regions of DNA required large amounts of good quality DNA. Within forensics good quality DNA is often rare at a crime scene due to environmental factors or perpetrators attempt to obscure evidence. Therefore, to combat the requirement of high-quality DNA, short tandem repeats (STRs) within various loci within the human genome have become the focal point in DNA analysis. STRs allow for degraded or poor-quality DNA to be analyzed in small quantities.

2.4.2 Fingerprints

In addition to the growth of DNA as evidence, came blood spatter, ballistics, and fingerprint analysis. Fingerprinting became a more idealistic approach for forensics because of its uniqueness. Uniqueness is a characteristic often sought after in the scientific community to bring novel or clarifying information to a well-known topic. Through this ideology of individualism, we can exploit the uniqueness of multiple acceptable forensic tools, such as, fingerprinting.

A fingerprint is an impression or mark made by an individual's fingertips or thumbs; it is often used in forensics as an identification tool to assess who may have been present during the act of a crime. Despite DNA profiling being the most developed entity in identifying an individual, fingerprints have become the frontrunner as the most

established form of forensic evidence in its use to certainly identify an individual **(Kapoor et al. 2015)**. The papillary ridge patterns that are often examined by an expert analyst is known to not only remain topologically unchanged from birth for an individual but differ between individuals as well as from one finger to another **(Wang et al., 2017)**. These papillary ridge patterns are denoted as arches, loops, whorls, and composites (Figure 2-3).

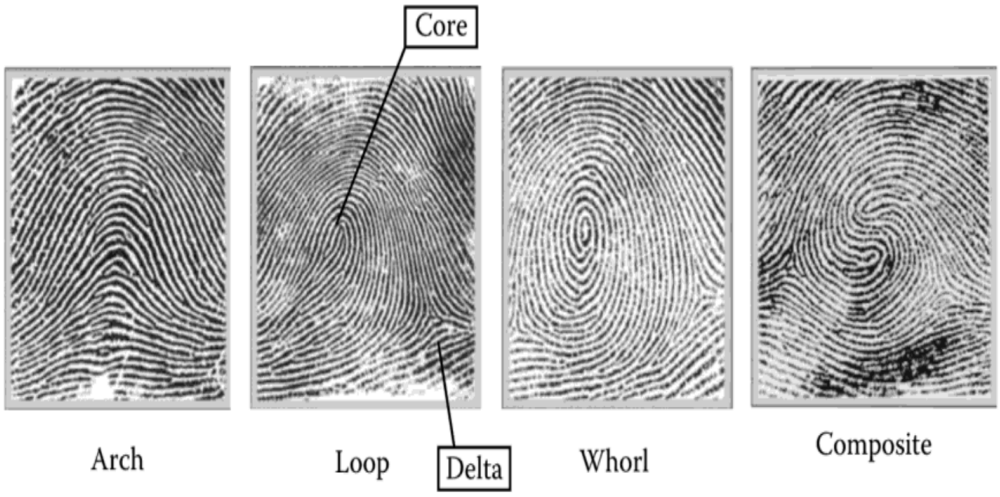

Figure 2-3:Common Papillary ridge patterns in Fingerprints (Thompson et al., 2006).

Generally, three types of fingerprint evidence are common at crime scenes, including impression (or indented) fingerprints, visible (or patent) fingerprints, and latent fingerprints **(Lee & Gaensslen, 2001)**. Many methods have been optimized for the various forms of fingerprints. Although, the two-dimensional reproduction of fingerprints specifically those invisible to the naked eye (latent), has posed the biggest complications within this area of forensic because of the perspiration, oils, or other contaminants that coat the surface of the ridges when a finger touches an item **(Kaushal & Kaushal, 2011)**. Developing methods for latent prints such as small particle reagent (SPR), black powder, and Cyanoacrylate fuming (super glue) have been proven to increase the visibility of latent prints in various

environments **(Madkour et al., 2017)**. Although, advanced technology has increased the visibility of a latent print, the print may be fragile to the point collection could further damage the print. The fragility of current biometrics discussed have led to their classifications as trace samples.

2.4.3 Trace samples

Trace evidence can include traces of DNA, various biological fluids (e.g., semen, saliva, blood), clothes, textiles, hair, or other fibers that can inform investigators about who was present at a crime scene and provide further information on what occurred **(Bouslimani et al., 2016)** Within trace DNA samples often the ability to obtain meaningful profiles is plagued with low copy number (LCN) or low template DNA (LTDNA) following extraction. Many methodologies have been developed to increase the opportunity to generate a profile by increasing the number of cycles during amplification or selection of proper extraction kits to aid in maximizing the quantity of DNA that's input prior to downstream analysis **(Oorschot et al., 2010)**. However, even with well-established protocols for evaluation of trace samples, the identification of the perpetrator or victim is often hindered. The impact trace samples has had on the forensic community has pushed the narrative to include newer biometrics for evidence. A form of evidence that has gained traction over the years that can address the scarcity of physical evidence is human odor. Examining human odor from a combined biological and chemical aspect could increase its probative value within the forensic science field.

2.5 Chemical Analysis of Human Odor

2.5.1 Volatile Organic Compounds

Volatiles are organic compounds (VOCs), often with high vapor pressures, are emitted into the environment as gases. VOCs can be exuded from many organic entities: explosives, drugs, agriculture, and the human body **(Colon-Crespo et al., 2017)**. Within the Furton lab VOCs have been examined for identification, disease detection, biomarkers, its emanation from various specimens, fabrics that trap and release VOCs the best, and their stability and reproducibility over time **(Furton & Myers, 2001; Curran et al., 2005, 2007, 2010; Colon-Crespo et al., 2017; Brown et al., 2013; Prada et al., 2014; Caraballo et al., 2016)**. One of the major advantages of VOCs from a sampling perspective is that they are readily available and can be obtained noninvasively from an individual as often as the researcher likes with little or no discomfort **(Filipiak et al., 2016)**. To date, there are 1,849 volatile organic compounds identified in correlation to humans. Of these 1,849 compounds, only 504 are found in bodily secretions **(Filipiak et al., 2016)**. Many compound classes: acids, alcohols, aldehydes, hydrocarbons, esters, and ketones are present in human emanations **(Curran et al., 2007)**. However, there is minimal research on how these VOCs are dependent upon the microbial metabolic contribution, altered by stress, age, time of day, gender, activity, disease status, or the transport to the site of their detection. Moreover, the information in relation to the metabolic pathway that leads to the production or degradation of VOCs is largely missing **(Filipiak et al., 2016)**. Overall, the established chemical profile curated from the abundance and presence of VOCs that are

particular to an individual can envision human odor as a biometric measurement **(Brown et al., 2013)**.

2.5.2 Human Odor

The qualitative and quantitative evaluations of human odor are distinctly different; as chemical signatures are qualitatively similar amongst individuals but quantitatively the abundance of said signatures make them characteristic of the individual they are extracted **(Brown et al., 2013)**. Host genetics, surrounding environments, and physiological secretions have a major impact on the volatile organic compounds (VOCs) that are released from the body; human odor is described as this gaseous surrounding cloud of VOCs fluctuated by our body temperatures and current state of health **(Prada et al., 2014)**. Curran et al. (2005) established three categorical distinctions of odor: (1) The primary odor of a person contains constituents that are stable over time regardless of diet or environmental factors, (2) Secondary odor contains constituents that are present due to diet and environmental factors, and (3) Tertiary odor contains constituents that are present because of the influence of outside sources (i.e., lotions, soaps, perfumes) **(Curran et al., 2005a)**. Secretions from the feet and axillary (armpit) area of the body have been the major contributors to the scientific investigation conducted on human odor **(Curran et al., 2007)**. However, the instrumental evaluations have shifted focus to other various body regions, such as the forearms and hands, as the skin volatiles released from those areas may contribute to the definition of general whole-body scent **(Prada et al.,2014)**.

2.5.3 Hand Odor

Human scent transfers into the environment at different rates and volumes dependent upon the exposure in which the body region is releasing them. One region that constantly interacts with humans, objects, and varying environments because they are the most exposed is the hand. Previous studies have examined and optimized hand odor as a valuable form of forensic evidence amongst various factors such as collection and instrumental analysis. From the collection perspective hand odor has been evaluated with contact vs non-contact methodologies. The contact collection category includes the following: the touching of a sorbent material by an individual, the direct swiping of an individual's body regions, as well as placing a sorbent material in contact with an item that has been in contact with an individual **(Prada et al., 2011)**.

In a 2011 study conducted by DeGreff et al., various porous materials: Rayon, Polyester, Dukal gauze, Johnson & Johnson gauze, and cotton and non-porous materials: glass beads and steel bars often used in VOC contact studies were analyzed to understand which material was best for collection purposes to suggest the need for standardization in VOC collection with said materials **(DeGreff et al., 2011; Caraballo et al., 2016)**. The noncontact collection category includes the collection of scent by placing a sorbent material near an individual for a specified time, as well as utilizing the Scent Transfer Unit (STU-100) as a collection device **(Prada et al., 2011)**. From an instrumental perspective hand odor has been examined primarily through the analytical method Gas Chromatography-Mass Spectrometry (GCMS) combined with sorbent-based sampling technique known as Solid Phase Microextraction (SPME).

2.6 Analytical Analysis of Human Hand Odor

2.6.1 Headspace Solid Phase Microextraction (HS-SPME)

The general analytical process of evaluating any kind of sample starts with the methodology chosen to collect the sample of interest, followed by the preparation, separation, and instrumental analysis. As previously discussed, the forensic community has been plagued by the fragility and insensitivity to analyze trace samples. Therefore, sample preparation has become one of the most important steps prior to instrumental analysis. Sample preparation is a combination of many processes that are not limited to but include purification, separation, pre-concentration, and derivatization. Commonly, this requires that the sample undergo large amounts of solvents and transfers, leading to potential error in analysis, loss of analyte when working with trace samples, and ultimately can be very time consuming. Many microextraction techniques have been developed to address these sample preparation issues by decreasing the required amount of volume needed, simplicity of the extraction matrix, and the possibility to automate their extraction procedures **(Sajid, 2017).**

In 1990, J. Pawliszyn developed solid phase microextraction (SPME) as one of the techniques to address these issues **(Arthur et al., 1990; Zhang et al., 1993).** Since then, SPME has garnered popularity amongst many fields of analytical chemistry because of its ability to pre-concentrate, extract analyte at a reduced volume, and improve the stability of sample during transport and storage (**Eckert et al., 2018).** Even with SPME having a fraction of sorbent capacity in comparison to other sorbent collection techniques, its solid phase composition holds practical and analytical advantages. Harper et al. (2000) listed sensitivity on small samples, reduced sampling time, and minimal equipment required as

some of SPME's advantages (**Harper et al., 2000)**. On the other hand, SPME has also faced criticism for its fragility during sample extraction; while the diminished capacity of the SPME fiber is a gift for certain samples, it can be a curse for others. The schematic of a SPME fiber is comprised of a silica-fused fiber, septum piercing needle, adjustable needle depth gauge, and plunger (Figure 2-4).

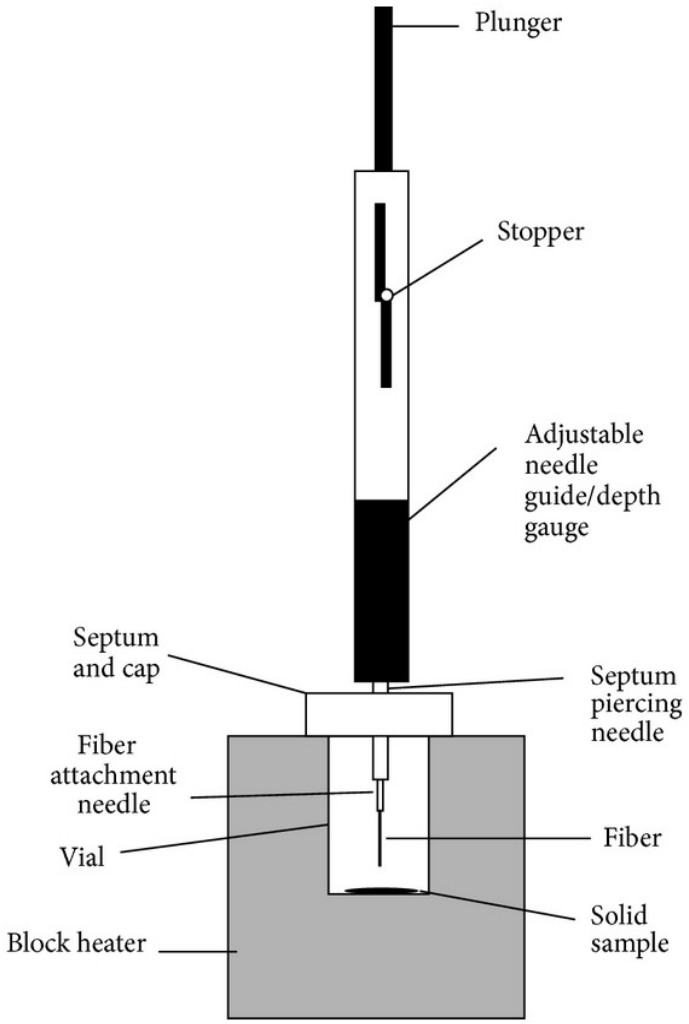

Figure 2-4:Schematic of SPME fiber device inside of a sampling vial. (Moein et al)

Scientific literature has notated that SPME can be utilized in one of three different modes: (1) direct immersion (DI-SPME), (2) headspace (HS-SPME), and (3) membrane protected SPME (Figure 2-5) **(Jalili et al., 2021; Moein et al., 2014; Sajid et al., 2017; Pawliszyn et al., 2012).**

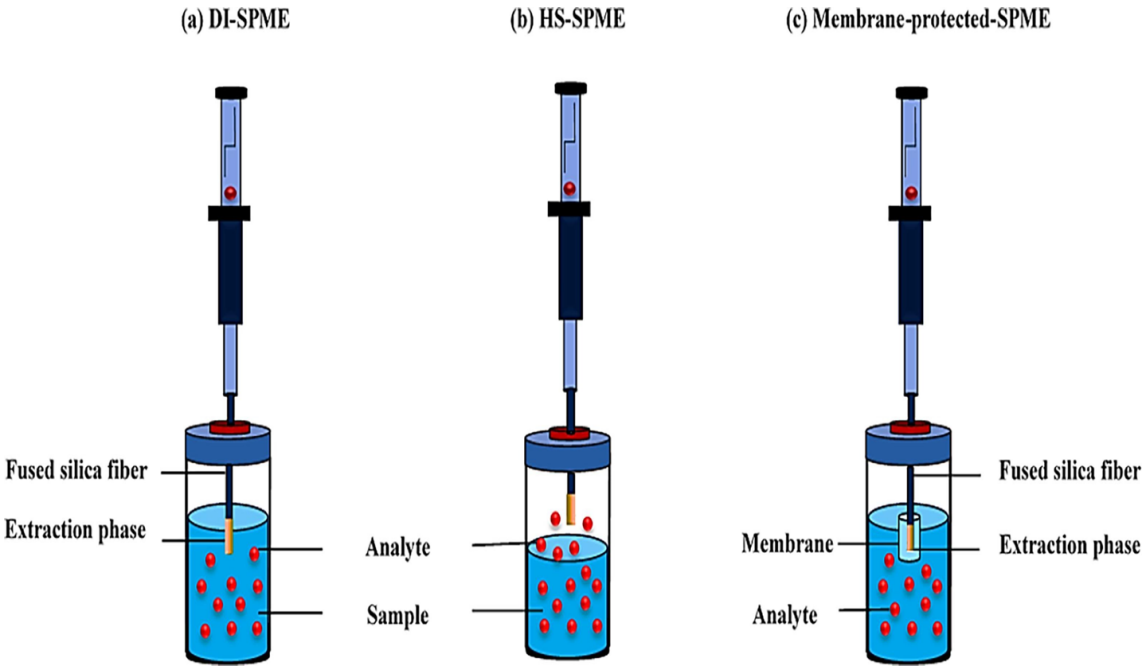

Figure 2-5:Sampling modes of SPME (A) DI-SPME (B)HS-SPME (C)Membrane-protected SPME (Jalili et al., 2021)

Within the DI-SPME mode the silica-fused fiber is directly immersed into the analyte being sampled and there is a direct exchange with the fiber matrix **(Jalili et al., 2021)**. DI-SPME is best implemented for pure aqueous samples in which the target analyte is either low emitting volatiles or non-volatiles (proteins and fatty acids). The downfalls of DI-SPME are that the longevity of the fiber is diminished because of direct exposure to the sample matrix and contamination is more likely to occur during transfer between samples if not properly cleaned.

When the analyte of interest cannot be interacted with due to its complex origination, solid nature, or emission of trace amounts of volatiles, HS-SPME mode is often utilized. HS-SPME has the capability to pre-concentrate volatiles and semi-volatiles without agitation to the sample matrix and extract the equilibrated headspace above the sample. Although, HS-SPME mode allows quick turnover for repeated sampling, increased analyte concentrations, and extended life span of the fiber, it eliminates the ability to extract non- and low-volatiles. Therefore, other methodologies (e.g., solvent extraction) must be implemented if the non-volatile information is required. With the pros and cons of both DI-SPME and HS-SPME, there was a gap for microextraction of complicated samples (e.g., ecological, biological, etc.) that non- or low-emitting volatiles were of interest. Six years later after the initial development of the SPME fiber, J. Pawliszyn and his co-workers took the first approach in closing this gap by developing a SPME that was protected with a hollow cellulose membrane, allowing for direct immersion into complex samples, avoiding fiber damage **(Sajid, 2017)**. The mode that best adapts to the samples of interest within this study, HS-SPME was chosen as the appropriate mode to engage.

The process of exposing a fiber requires for the plunger to be engaged in a downward motion from a pre-set adjusted depth of the gauge and locking the plunger by rotating the stopper into place. The same process can be done in the reverse to retract the fiber from the analyte being sampled. The stepwise process of transferring a HS-SPME fiber from extraction to analytical instrumental analysis via GCMS is illustrated in figure 2-6.

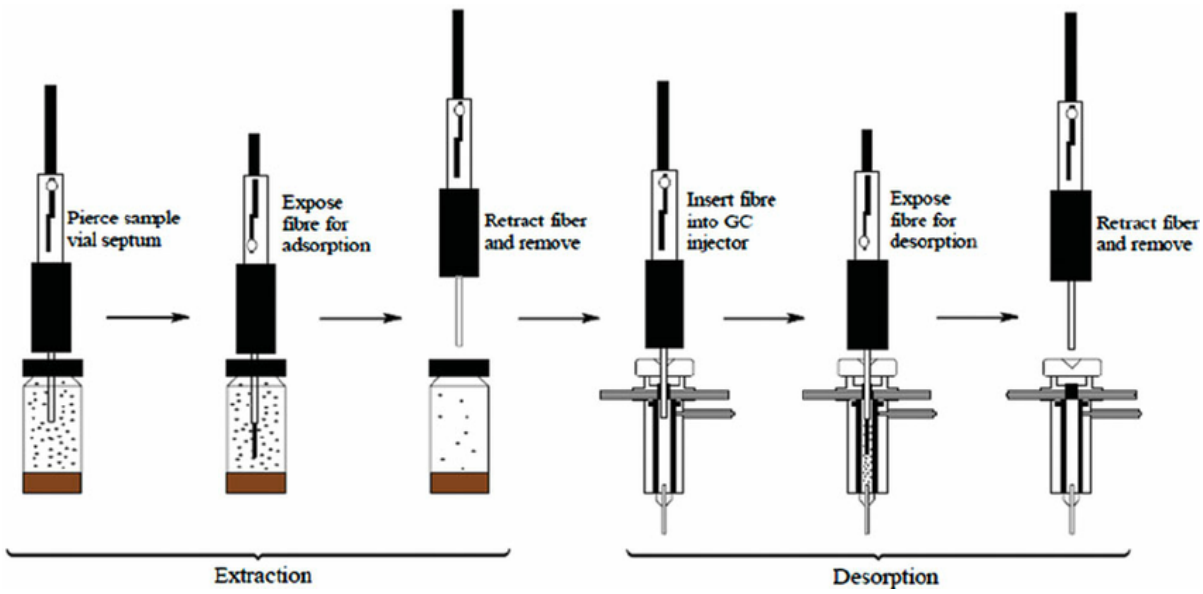

Figure 2-6: Process of SPME fiber from headspace sampling to GCMS injection. (Ahmed et al., 2015)

HS-SPME extraction process begins with an equilibration period of the analyte headspace. The equilibrated headspace is now within the extraction phase in which the fiber is exposed for a pre-determined time. After sufficient analyte has transferred onto the SPME matrix it can be directly injected for qualitative and quantitative analysis. There is a direct proportional sample calculation between the amount extracted and the original concentration of the analyte. Sample concentration calculations have been explained with two equations in the *Handbook of SPME* written by Pawliszyn. SPME extraction and collection from small volumes of sample, the amount of an analyte extracted from a sample is given by Equation (1), where n represents the extracted analyte amount, C_0 represents the analyte initial concentration, V_s and V_f are volumes of the sample and fiber, respectively, and K_{fs} represents the distribution coefficient of the sample and fiber:

$$n = \frac{C_0 K_{fs} V_s V_f}{K_{fs} V_f + V_s}$$

Equation 1:Total amount of analyte extracted from small volume sample (Pawliszyn et al., 2012)

(**Pawliszyn et al., 2012)**. Equation (1) simplifies to equation (2) when the sample size is larger than the capacity of the fiber and the sample concentration can be calculated as denoted in equation (2) independent of sample volume:

$$n = C_0 K_{fs} V_f$$

Equation 2: Total amount of analyte extracted from large volume sample (Pawliszyn et al., 2012)

Proper selection of the fiber matrix is extremely important because various matrices target specific analytes: volatiles, semi-volatiles, non-volatiles (proteins and fatty acids), and flavor, amine, and nitro-aromatic compounds based on their polarity and molecular weight (Table 1) **(Selection guide for Supelco, n.d.)**.

*Table 1: Supleco SPME fiber selection guide for analyte type, polarity, and molecular weight (**Selection guide for Supelco, n.d.**)*

Supleco Fiber Selection Guide		
Analyte Type	Molecular Weight (MW)	Recommended Fiber
Gases and low molecular weight compounds	30-225	75/85 µm CAR/PDMS
Volatiles	60-275	100 µm PDMS
Volatiles, amines and nitro-aromatic compounds	50-300	65 µm PDMS/DVB
Polar semi-volatiles	80-300	85 µm polyacrylate
Non-polar high molecular weight compounds	125-600	7 µm PDMS
Non-polar semi-volatiles	80-500	30 µm PDMS
Alcohols and polar compounds	40-275	Carbowax (PEG)
Flavor compounds: volatiles and semi-volatiles	40-275	50/30 µm DVB/CAR on PDMS on a StableFlex fiber
Trace compound analysis	40-275	50/30 µm DVB/CAR on PDMS on a 2 cm StableFlex fiber
Amines and polar compounds	HPLC use only	60 µm PDMS/DVB
CAR: Carboxen PDMS: Polydimethylsiloxane DVB: divinylbenzene		

The 50/30 µm Divinylbenzene/Carboxen on Polydimethylsiloxane fiber (DVB/CAR/PDMS) has been established and optimized for hand odor collection because of its selectivity to detect volatiles and semi-volatiles of trace samples, which is similar to the hand odor samples within this study **(Curran et al., 2005a; Brown et al., 2013)**. The ability to qualitatively and quantitatively identify compounds correlated to individuals through direct sampling of the hand and/or secondary transfer of objects they have handled via headspace analysis has been made possible with the advancement of analytical technologies, contributing to the scientific basis for the use of odor as a forensic biometric **(Schoon et al., 2009)**

2.6.2 Gas Chromatography Mass Spectrometry

Credited as "The father of chromatography" Mikhail Semenovich Tswett (or Tsvett) was led in the early 1900's to discover the fundamentals of chromatography because of his research in the field of botany **(Gal et al., 2019).** Chromatography is the analytical process that engages two phases (solid and mobile) to separate chemical or complex mixtures into their own individual components that can be identified against a known standard. For the chromatography process to initiate, the mixture has to be carried by the first phase (mobile phase) on to a second phase (stationary phase). The mobile phase can be either liquid or gas, while the solid phase is comprised of a porous solid. Often the decision regarding which mobile phase is best to use is dependent upon the type of mixture that will be analyzed. There are various types of chromatography: liquid (paper, thin layer), gas, ion-exchange, size exclusion, and affinity that can provide information on the simplest mixtures to the most complex. Although the analytical techniques follow the same basic principles of chromatography, the basis in which they separate the components of the mixture are different. Liquid, size exclusion, ion-exchange, affinity, and gas chromatography separate based on the molecule's polarity, size, ionic charge, binding affinity, and boiling point, respectively.

Gas chromatography (GC) is seen as the golden standard of the forensic community for analysis of trace, drugs, fire, lubricants, paint, ignitable liquids, and explosive samples. **(Bridge et al., 2019; Lu et al., 2007; Kabir et al., 2021)**. The mixed volatility of human odor compounds makes gas chromatography the best analytical technique to implement within this study. It simplifies the analytical process following sample preparation with SPME and can be transferred directly from the sample into the GC without any additional

modifications **(Moein et al., 2014)**. The GC is comprised of four components (injector port, carrier gas, column, and detector) responsible for the separation of a volatile mixtures. The injector port is simple in its assembly (Figure 2-7) but is the focal starting point of sample introduction into the GC.

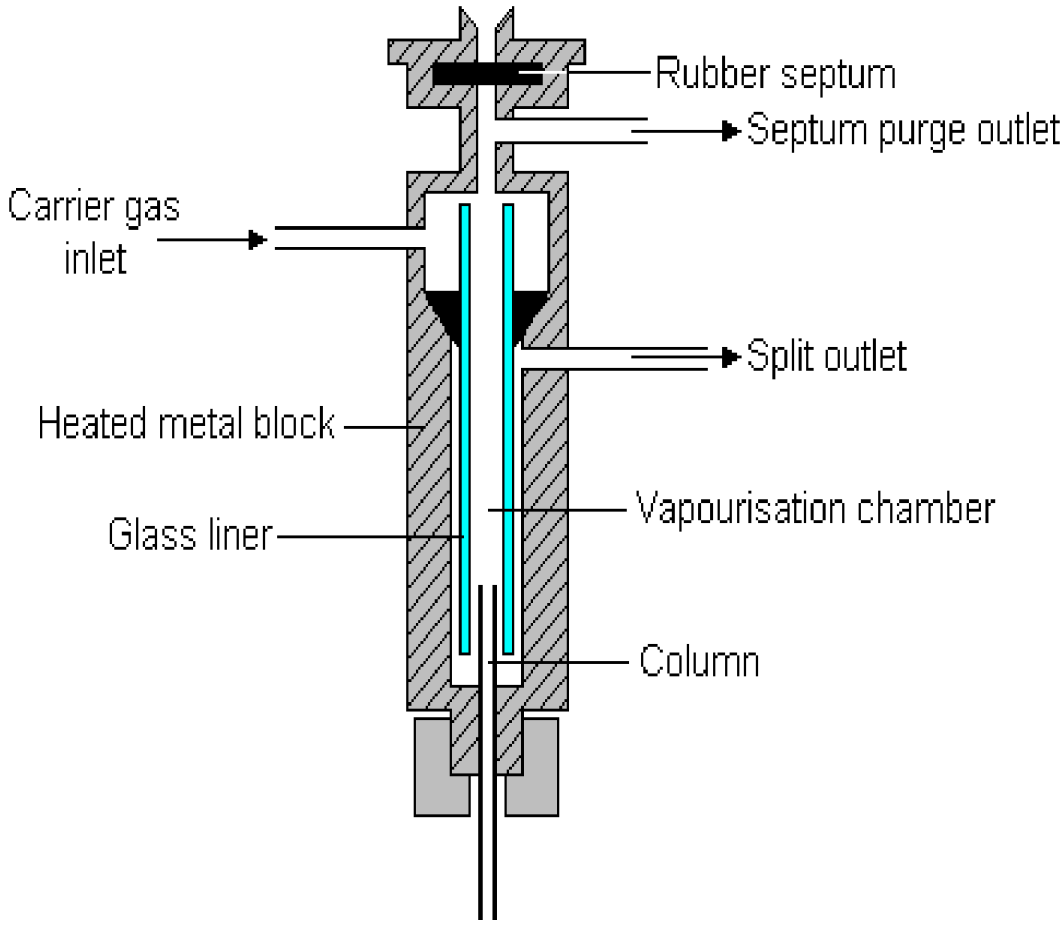

Figure 2-7:Schematic of GC injector port (Deepak, 2014)

Typically, the ending chromatographic results are often only as good as the prepared sample that is injected. Upon injection the sample is instantly vaporized and directed to the column. Therefore, sample concentration is very important to understand prior to injection because the analytical method the GC operates from can be programmed to operate in either a split or splitless mode.

Split mode is often utilized when the concentration of the analyte is high. An established split ratio instructs the GC of opening the split valve, partitioning the initial gas flow rate into two parts: the rate of column flow (1mL/min) and the combined flow rate of the septum purge and the split vent. The split valve is perpendicular to the route of the vaporized sample to the column and because the portioned flow rate is the highest in the split vent, the majority of the sample goes into the split vent and not onto the column (Figure 2-8).

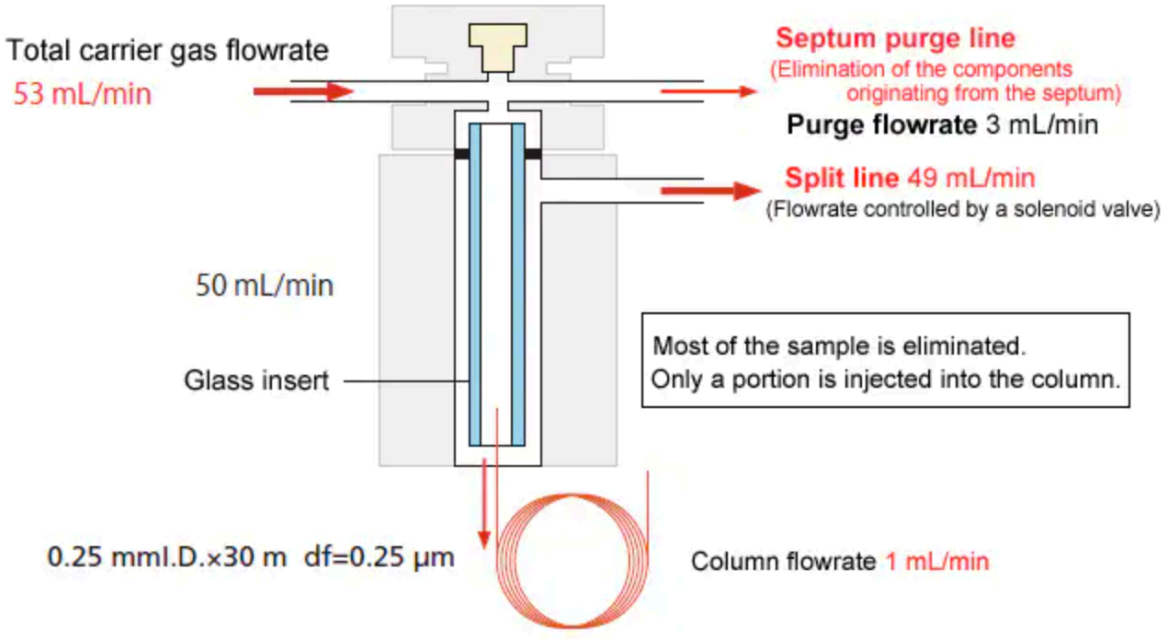

Figure 2-8:Schematic of gas flow when injector is set to split mode (SHIMADZU, n.d.)

The alternative splitless mode is engaged when the analyte concentration is low (trace samples). Retaining as much of the sample as possible is of the utmost importance. Within this mode the split ratio is still comprised of the two flow rates—column flow (1mL/min) and combined septum purge and split vent—but the split vent is completely shut off, forcing the vaporized sample on to the column (Figure 2-9).

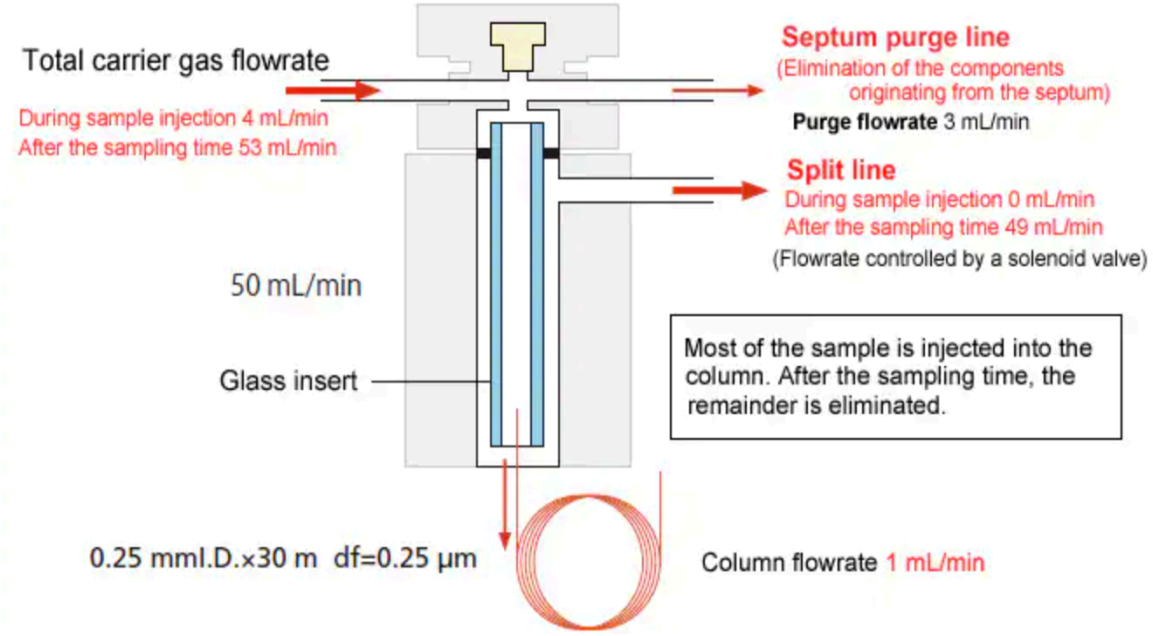

Figure 2-9:Schematic of gas flow when injector is set to splitless mode (SHIMADZU, n.d.)

Following the injection of the sample brings us back to the second component of the GC that will push the vaporized analyte down and through the column. It is referred to as the mobile phase in all chromatography techniques, but for GC it is known as the carrier gas. The type of carrier gas used has multiple factors to be considered: inertness, affinity to the column, and affinity to the analyte. Two gases are often employed for those reasons: Hydrogen and Helium. The diffusivity and viscosity properties of the gas chosen play a

major role in the GC's speed and efficiency, by creating better separations and linear velocities to carry the individual components through the stationary phase **(Heseltine et al., 2010)**. Although the diffusivity of helium and hydrogen are similar and the viscosity of hydrogen is half of helium, making hydrogen the most ideal carrier gas it has safety concerns of explosion **(Heseltine et al., 2010)**. Therefore, Helium has been the go-to carrier gas in all GC methods, and the gas utilized in this study.

Once the analyte has been injected through the injector port and vaporized by the carrier gas, the separation of the sample begins with the stationary phase–column. There are two types of columns (packed and capillary) that exist that impact the quality of the chromatogram observed at the end of analysis. Packed columns are stainless steel, or glass tubes filled with a diatomaceous earth surrounded by a solid phase that can be 2-4mm in diameter and 0.5-5m in length **(SHIMADZU, n.d.)**. Capillary columns can be represented in two forms: a porous solid referred to as Porous Layer Open Tubular Column (PLOT) and a liquid phase referred to as Wall Coated Open Tubular Column (WCOT) with increments of diameter from 0.1-0.53mm and lengths ranging from 5-100m **(SHIMADZU, n.d.)**. The capillary column is the most widely used in GC because of its efficiency and sensitivity dictated by the chemical composition of the stationary phase (polarity), column internal diameter (I.D.), film thickness, and column length. The commercially available stationary phases outside of the fused silica and their corresponding polarities are listed in table 2 **(SHIMADZU, n.d.)**. The remaining factors of a capillary column are interdependent of each other as the column I.D. impacts the film thickness and length. All together they contribute to how efficiently the GC column can separate the components of

the mixture, increase the resolution of the compound peaks, and maximize the ng amount of each analyte loaded.

Table 2: Stationary phases characteristics (SHIMADZU).

Stationary Phase	Polarity	Separation Characteristics	Application	Temperature Range (ºC)
Methyl Silicone	Non-polar	Boiling point	Petroleum, solvents, high boiling point compounds	-60 to 360
Phenylmethyl	Slightly/ moderately polar	Retention of aromatic compounds	Perfumes, environmental and aromatic compounds	-60 to 340
Cyanopropylphenol	Moderately/ Strongly polar	Oxygen-containing compounds, isomers, etc.	Agriculture chemicals, oxygen-containing compounds	-20 to 280
Trifluoropropyl	Moderately/ Strongly polar	Retention of halogens	Solvents, polar and halogen-containing compounds	-20 to 340
Polyethyleneglycol	Strongly polar	Retention of polar compounds	Perfumes, solvents, fatty acid methyl esters, and polar compounds	40 to 250

The recent technological advancements in stationary phase development have increased the thermal stability and improved inertness of various columns to cast a wider net of GC applications **(Jennings et al., 1997)**. The chemistry of developing the SolGel-WAX^{TM} column is a reaction of polycondensation on hydrolyzed monomers of a metal alkoxide (Figure 2-10) establishing the columns good retentive characteristics, inertness to the analyte being carried by the mobile phase, and stability at extreme temperatures **(SGE Analytical)**.

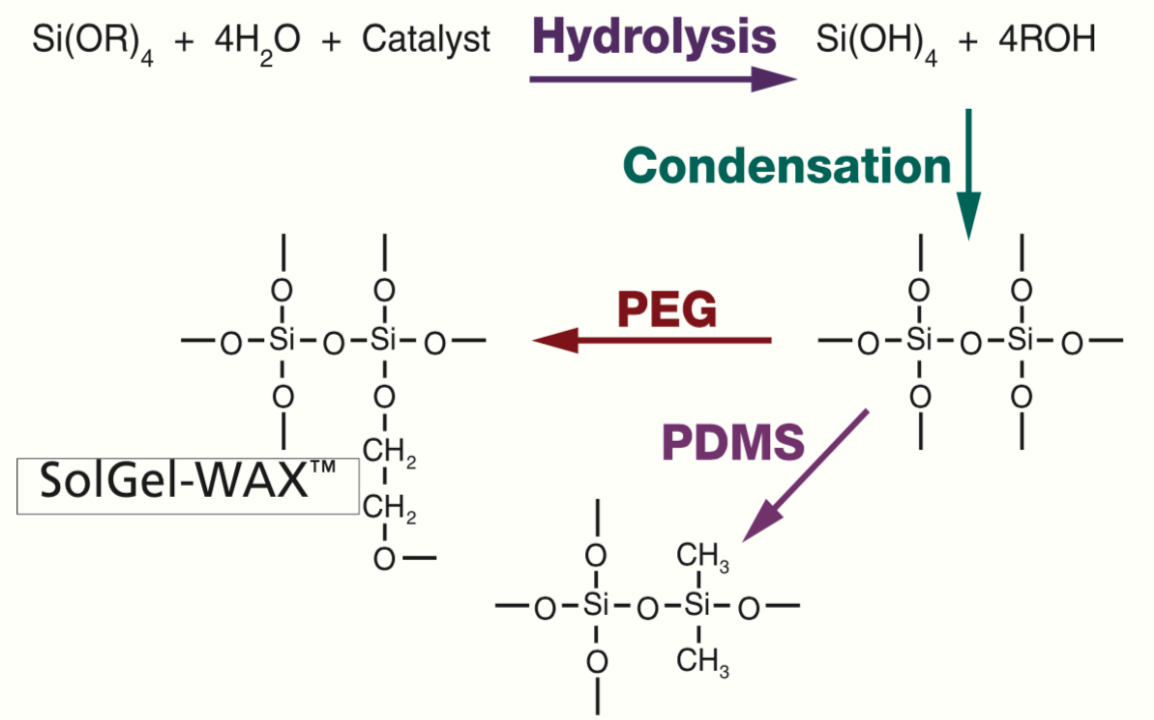

Figure 2-10:Chemistry for SolGel column development (SGE Analytical)

Once the analyte is vaporized onto the GC column, the mixture interacts and interchanges between the mobile and stationary phase to separate into its individual components. Dependent upon the affinity of the individual components to the stationary phase, components will release either quickly or slowly as they transfer to the detector **(Colon-crespo., 2017).** The detector detects, identifies, and translates the ionized fragments of compounds separated by their mass to charge ratios (m/z) into a chromatogram **(Urban 2016)**. The most common detector combined with the GC is the mass spectrometer because of its ability to simultaneously identify and quantify unknowns of a mixture **(Kabir et al.,)**.

The gas chromatography-mass spectrometry (GC-MS) combination can provide a dual set of information (retention time and mass spectra) for each individual compound

(Smith et al., 2013). Although the GC can be interchanged with many detectors, including but not limited to the following: flame ionization detector (FID), atomic emission detector (AED), electron-capture detector (ECD), ion mobility spectrometry (IMS), and time-of-flight mass spectrometry (TOFMS) **(Kabir et al., and Poole, 2015)**. Individual components separated via the GC are transferred through the transfer line through the ion source to be ionized by various techniques. More commonly within GC-MS ionization methods; electron and chemical are executed to ionize molecules in the gas-phase through high-energy electrons and reagent gas, respectively **(smith et al., 2013)**. After the ion source, fragmented ions are accelerated and streamlined to the mass analyzer according to m/z. Like ionization techniques, there are multiple mass analyzers that utilize different techniques such as magnetic fields (sector), kinetic energy (time-of-flight), radiofrequency with direct current generators (quadrupole), and oscillations (ion trap) **(smith et al., 2013)**. In figure 2-11, the two most common mass analyzers within GCMS, the quadrupole mass analyzer and quadrupole ion trap, arrangements are illustrated from ion source to ion detector.

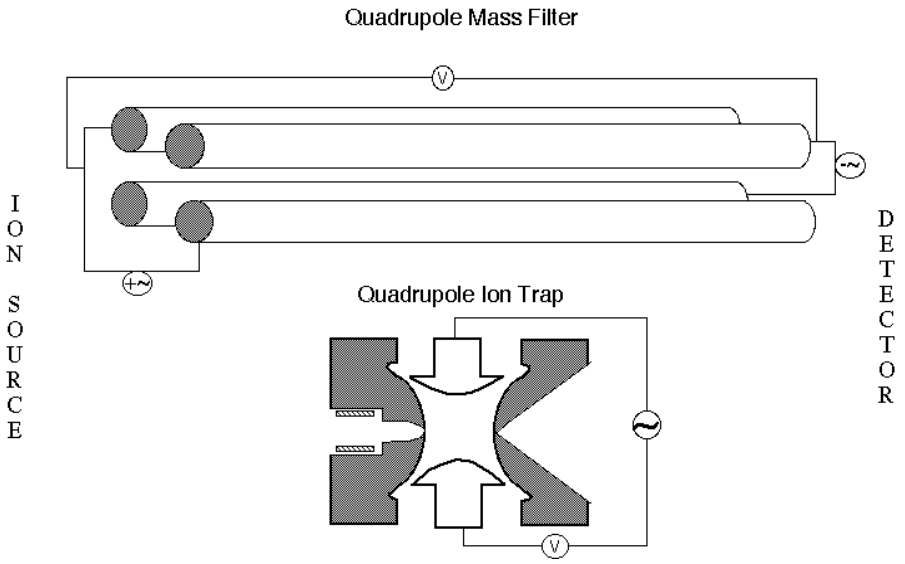

Figure 2-11:Schematic of Quadrupole and Ion Trap Mass Spectrometers (Gas Chromatography)

As a result of efficient design, compartmentalized nature, and the signal amplification ability of the electron multiplier, it makes the quadrupole mass spectrometer the most common ion detector used in GCMS instruments **(Smith et al., 2013)**. The overall schematic of the GCMS system from sample injection to chromatogram development is what makes the analytical technique ideal for this study (Figure 2-12).

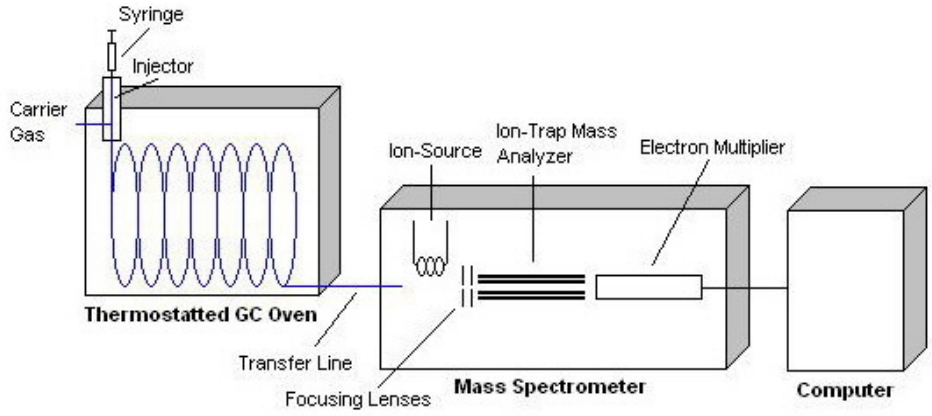

Figure 2-12:Full schematic diagram of a GC-MS instrument (Gas Chromatography)

2.7 Biological Analysis of Human Hand Odor

2.7.1 Volatilome

The "volatilome" is known as the overall collective of VOCs released by an organism. The human skin releases a variety of volatile metabolites with different biological origins that are subject to bacterial action **(Curran et al, 2010).** The exploration of the bacterial contribution to VOCs must be further investigated. Shelley et al. (1953) stated that human sweat has no odor until the skin microbiota begin to break down non-volatile compounds into volatile compounds **(Shelley et al, 1953)**. This has continued to be supported in recent research as freshly secreted sweat is sterile, but due to biotransformation by microorganisms (aerobic coryneforms, propionibacteria and Micrococcaceae), odoriferous VOCs are produced **(Lemfack et al., 2013)**. In the most basic unit of life, the cell, specific microorganisms such as bacteria are metabolizing specific metabolites to manifest odor. For example, common genus level microbiota and their species (spp) such as *Staphylococcus* spp, *Corynebacterium* spp, *Bacillus* spp, and others are responsible for transforming long-chain fatty acids into short and medium-chained products that can be further metabolized and volatilized. These volatiles are often associated with malodor. Skin microbiota, including fungi and viruses, occupy dynamic but unique niches within and on the human body **(Schommer and Gallo, 2013).** This 'uniqueness' can be exploited and applied as a novel approach to human scent and microbiome forensic studies. The knowledge gap of the presence or absence of certain organisms, and how they correspond to the production of odors for forensic identification of an individual, must be explored.

2.7.2 The Human Microbiome Project

Microbes are an invisible world of bacteria, viruses, and fungi that live within our water, soil, and air. In reference to the human body, the millions of microbes that live inside and outside are referred to as microorganisms. The human microbiome is described as an aggregation of microorganisms that form complex communities at various sites of the human body **(Shreiner et al. 2015)**. The National Institutes of Health (NIH) spearheaded the Human Microbiome Project (MHP) from 2008-2016 to understand the microbial genes and genomes of healthy individuals as related to human health and disease prevention **(Dekaboruah et al., 2020).** The MHP targeted the microbiome on five areas of the human body: Skin, oral cavity, gastrointestinal (gut), vaginal, and respiratory **(Peterson et al.,)**. It has been established that the bacterial genome contributes to the overall human genome by ten-fold, providing a high copy number genetic marker that can be targeted for forensic human identification investigations **(Woerner et al., 2019)**.

2.7.3 Human Skin

Complex microbial communities are exchanged to and from our surroundings with the use of our hands, as we open doors, shake hands, and hold objects. Considered the largest organ of the human body and the natural barrier that keeps all skeletal and muscular structures together, the skin provides primary protection against pathogens, radiation, and injury **(Yousef et al., 2021)**. In a review conducted by Dekaboruah et al., (2020) the human skin harbors four principle phyla: Actinobacteria, Firmicutes, Bacteroidetes and Proteobacteria **(Dekaboruah et al., 2020)**.

Table 3: Microorganisms that inhabit the human body and respective regions (Dekaboruah et al., 2020)

Region	Predominant organism
Scalp	*Staphylococci* (*S. captis*, *S. epidermidis* etc.), *Propionibacterium acne*, *P. granulosum, P. avedum, Malassezia* spp.
Toe interspace	*Staphylococcus epidermidis, S. haemolyticus, S. cohnii, S. hominis, S. warneri, Micrococcus* spp., *Malassezia* spp.
Perineum	*P. acne, P. granulosum, P. avedum, S. epidermidis, S. hominis, S. aureus, Coronybacterium minutissimum, C. xerosis, C. jeikeium, Malassezia* spp., *Strepyococci, E. coli*
Axillae	*P. acne, P. avidum, P. granulosum, S. aureus, S epidermidis, S. saprophyticus, C. xerosis, C. minutissimum*, Gram-negative rods (*E. coli, Klebsiella, Proteus, Enterobacter* spp, *Actinobacter* spp)
Sole of the foot	*S. epidermidis, S. hominis, S. haemolyticus, S. cohnii, S. warneri, Malassezia* spp, *Micrococcus* spp, aerobic coryneforms, Gram-negative organism
Forearm and leg	*Staphylococci* (*S. haemolyticus, S. epidermidis, S. aureus, S. hominis), coryneform* and *propionibacteria*
Hands	*S. aureus, S. epidermidis, S. hominis, C. xerosis, C. minutissimum,* yeast and other fungi (*Candida parapsilosis, Rhodotorula rubra*), Gram-negative *bacilli* (*Pseudomonas* spp, *Enterobacter* spp)
Outer ear	*S. auricularis, S. epidermidis, S. captis, S. aureus, S. caprae, Brevibacterium* spp, *Turicella otitidis, Alloiococcus otitis*

The human skin is comprised of three layers: epidermis, dermis, and hypodermis, in which large amounts of bacteria inhabit, which can be easily dislodge from the skin's surface and transferred onto objects via shed human epithelial cells **(Fierer et al., 2010).** Epithelial cells are constantly shed into the environment through the glands of outer (epidermis) layer of the skin via secretions, oil, and sweat (Figure 2-13) **(Syrotuck, 2000).** The chemical breakdown of body odor is heavily attributed to VOCs, but the influence on those compounds is correlated to the microorganisms that inhabit the skin (table 3) **(Ross et al., 2013 and Dekaboruah et al., 2020)**. The ease of sample collection and the integral role the microbiome plays in odor production makes the human skin a common site within microbiome investigations **(Castelino et al, 2017).**

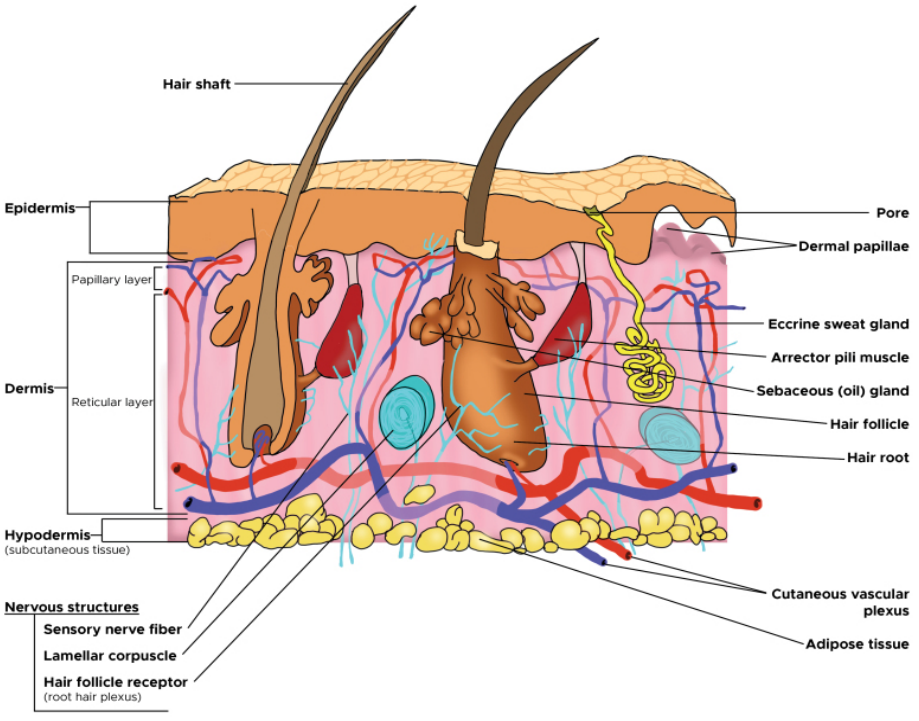

Figure 2-13:Cross-section diagram of the human skin contributed by Chelsea Rowe (Yousef et al.)

2.7.4 16S rRNA Gene

One of the primary focuses of microbiome investigations is the 16S rRNA gene located within the ribosome. Ribosomes are known primarily for their translation function by converting messenger RNA (mRNA) into amino acids. The prokaryotic ribosome is comprised of a Large Subunit (LSU), Small Subunit (SSU), and the space in between the two subunits' genes is called the Internal Transcribed Spacer (ITS). All three areas play a significant role in the function of the ribosome and are conserved across all three kingdoms. Additionally, the genes can be used as DNA markers. The LSU is the 50S subunit of the prokaryotic and archaea ribosome (60S in eukaryotes) which encodes ribosomal genes 5S and 23S (5S, 5.8S, and 25S/28S in eukaryotes) **(Lafontaine et al., 2001)**. These genes are responsible for peptidyl-transferase activity, which catalyzes the formation of peptide bonds (Figure 2-14) **(Lafontaine et al., 2001)**.

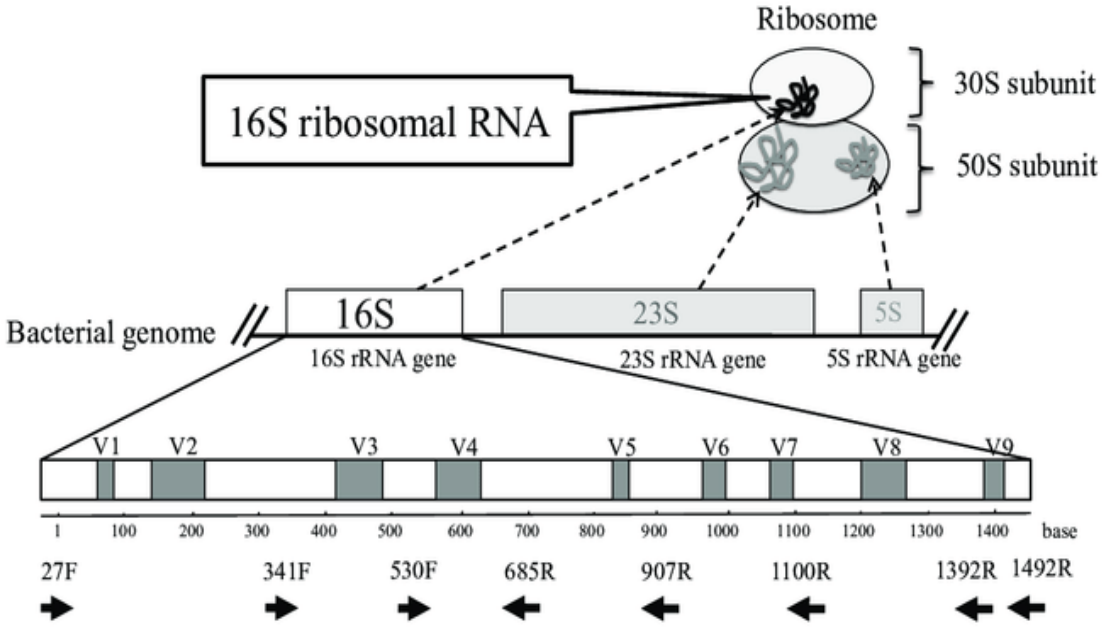

Figure 2-14: Ribosome complex of subunits and bacterial genome 16S rRNA gene and the nine hypervariable regions (V1-V9) denoted with grey boxes (Fukuda et. al 2016)

The SSU is the 30S subunit of the prokaryotic and archaea ribosome (40S in eukaryotes) which encodes the 16S rRNA gene (18S rRNA in eukaryotes) **(Lafontaine et al., 2001)**. Through translation of mRNA, the gene is responsible for converting genetic messages to functional cell components (Figure 14) **(Byrne et al, 2018)**. The 16S rRNA gene is specifically used for sequencing because it is ubiquitously present within all prokaryotes, easy to amplify through PCR because of its highly conserved primer sites, and the retrievability of phylogenetic information because of its short 1,500bp length (Figure 14). However, the 16S rRNA gene is also present within mitochondria and chloroplast of eukaryotes, and during sequencing techniques unspecified amplification can be avoided with proper selection of primers (Table 4).

Table 4: Primer sequences for 16S rRNA gene

Primer Name	**Target Region**	Primer Sequence (5'-3')	Source
27F	V1-V2	AGAGTTTGATCMTGGCTCAG	Suzuki et al., 1998
355R	V1-V2	GCTGCCTCCCGTAGGAGT	Suzuki et al., 1998
PCR1F	V3-V4	TCGTCGGCAGCGTCAGATGTGTATAAGAGAC AGCCTACGGGNGGCWGCAG	Illumina
PCR1R	V3-V4	GTCTCGTGGGCTCGGAGATGTGTATAAGAGA CAGGACTACHVGGGTATCTAATCC	Illumina
1507F	ITS	GTCGTAACAAGGTAGCCGTA	Ruegger et al., 2014
23SR	ITS	GCCAAGGCATCCACC	Ruegger et al., 2014

The majority of microbiome studies sequence the 16S rRNA gene because of its structural make up, which allows for the use of universal primers on the conserved regions, thereby commencing the research of phylogenetic relationships of bacterial taxa, and utilization for identification purposes **(Woese, 1987 and García-López et al., 2014).** The only downfall for 16S rRNA gene hypervariable regions is that different hypervariable regions sequence diversity at different levels, limiting the information that can be retrieved from one region **(Chakravorty et al., 2007)**. Often, multiple regions are chosen (i.e., V1-V2 or V3-V4) to obtain as much information as possible about the microorganisms that may exist in the community of an extracted sample. Many techniques such as capillary electrophoresis has made the collection of this information possible.

2.8 Analysis of Biological Signatures of Human Hand Odor

2.8.1 Length Heterogeneity-Polymerase Chain Reaction (LH-PCR)

The evolution of electrophoresis techniques began with Alex Jeffreys utilization of restriction enzymes to cut DNA at specific polymorphic sites to observe their fragmentation and his ability to observe that the combination of those cuts was unique to an individual **(Thompson et al.,2007)**. The basis of examining sequence and fragment length differences has been well established through capillary electrophoresis (CE). CE basic principles uses DNA's negative charge to migrate DNA from the anode through a capillary towards the cathode while the fluorescently tagged DNA fragments are excited via a laser and recognized by a detector.

From the discovery of Restriction Fragment Length Polymorphism and the advancements of modern technology, other methodologies evolved to observe the separation of DNA fragments. In a review by Jordan et al. (2020), the advantages and disadvantages of the past and present CE techniques and the basis of their differentiation was explained (Table 5) **(jordan et al., 2021)**. The principles of CE remain the same for all techniques, but the workflow and data generated vary by technique. Figure 2-15 illustrates the workflow of sanger sequencing and fragment analysis and their respective data analysis. Within this study LHPCR was designated as a quick and efficient screening of bacterial diversity and amplicons were further sequenced by Next Generation Sequencing (NGS) on the Illumina MiSeq® platform.

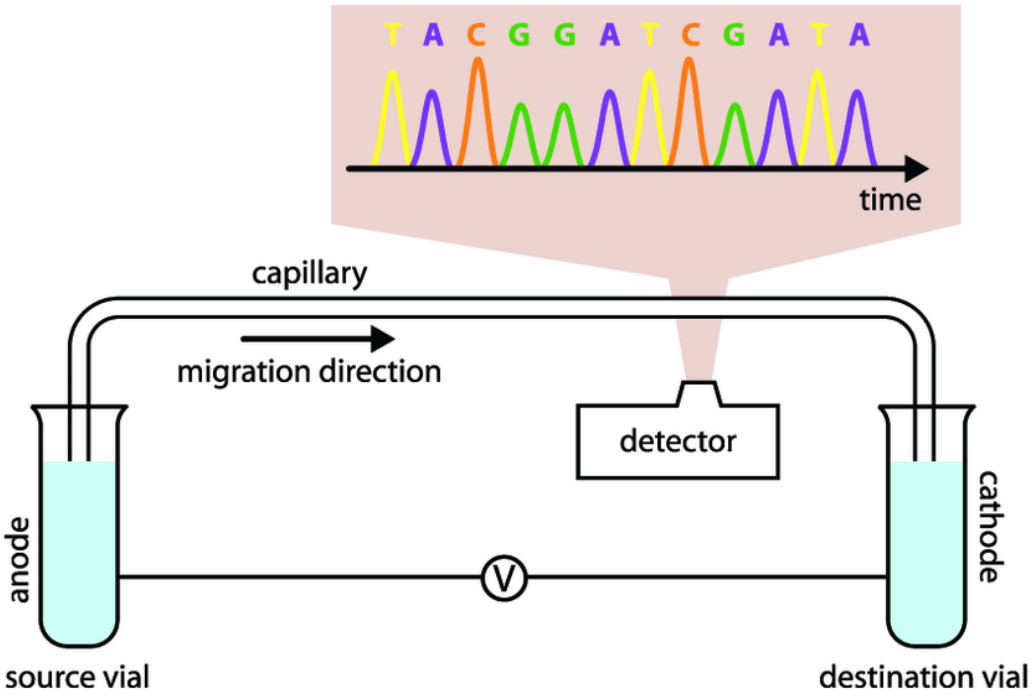

Figure 2-15:Capillary electrophoresis schematic of DNA migration vis Sanger sequencing (Gauthier, 2007)

Table 5:Advantages and disadvantages of capillary electrophoresis techniques (Jordan et al., 2021)

Analysis technique	Basis of differentiation	Advantages	Disadvantages
Restriction Fragment Length Polymorphism (RFLP)	Restriction site sequence and fragment length	– High power of discrimination – Reproducible – No prior sequence information required – Can differentiate between homozygotes and heterozygotes	– Time-consuming – Partial digests – Need at least 10–25 ng of DNA – Genetic mutations only identified at restriction cut sites – Not ideal for whole genome variation identification – Requires radioisotopes
Amplified Restriction Fragment Length Polymorphism (AFLP)	Restriction fragment length	– Reproducible – Amplify small amounts of DNA – Species ID not known – Detects dominant bi-allelic markers – No prior sequence information required – Uses capillary electrophoresis techniques	– Time-consuming – Partial digests – Multiple steps can lead to unreproducible results – Genetic mutations only identified at restriction cut sites – Not ideal for whole genome variation identification – Unable to differentiate between heterozygous and homozygous alleles
Terminal-Restriction Fragment Length Polymorphism (TRFLP)	Restriction fragment length	– Less complex results – Amplicon based PCR – Uses capillary electrophoresis techniques	– Partial digests – Genetic mutations only identified at restriction cut sites – Peaks can be representative of more than one species – Not ideal for whole genome variation identification
Length Heterogeneity-PCR(LH-PCR)	Gene fragment length	– Easy, fast results – Reproducible – Uses universal primers – Uses capillary electrophoresis techniques – Provides a quick screening or monitoring tool for community changes	– May underestimate community/mixture complexity – Lack of database hinders species identification – PCR bias can reduce detection of lower DNA template concentrations
Short Tandem Repeat (STR)	STR fragment length	– Fast – Highly reproducible – High level of discrimination, codominant alleles – Standardized across forensic laboratories – Uses low DNA amounts for amplification – Database of genetic profiles and allelic frequencies for statistical comparisons	– Mixture deconvolution not easy – PCR artifacts can complicate results – Challenges with highly degraded or low template DNA
Sanger Sequencing	Sequences every base	– Gold standard for sequence analyses – Uses capillary electrophoresis techniques	– Low throughput – Only 500–700 bases sequenced at a time – Cannot sequence mixtures without cloning
SNaPshot™	Single base changes	– Detects bi-allelic and multi-allelic SNP markers – Able to distinguish between heterozygotes and homozygotes – Human SNP database for statistical comparisons	– Time-consuming – Need to know SNP sequence in advance to design primers – Multiple markers required for high level of discrimination
Single-strand Conformational Polymorphism (SSCP)	2° structure of single-stranded DNA caused by base changes alters strand migration on CE	– Simple – High specificity – Screen potential variations	– Short fragments – Temperature-and mutation sensitive – Nucleotide change not identifiable – DNA strands can reanneal after denaturation affecting mobility during electrophoresis
Next-Generation Sequencing (NGS)	Massive parallel sequencing using various technologies	– High throughput – Deconvolve mixtures – Sequence entire genomes/metagenomes – Simultaneous detection of STR amplicon lengths and SNPs within the amplicon – Used for any DNA (human, non-human, viral, microbes)	– Massive data output that may be challenging to analyze – Analysis algorithms not standardized – Difficult with some technologies to analyze metagenomes to species level

2.8.2 Next Generation Sequencing (NexGen)

Early studies used amplification, sub-cloning, and Sanger sequencing of the highly conserved 16S rRNA gene, which can provide sequence information over the entire length of the 16S rRNA gene in a single reaction **(Cho et al., 2021)**. Although, these methods are still the most comprehensive of bacterial identification, they are expensive and time consuming. Next Generation Sequencing (NGS) is a process of developing a personalized pooled library, which allows for simultaneous sequencing of multiple samples and taxa and/or genes of choice all at the same time. The NGS library is prepared by fragmenting 16S rDNA amplicons and ligating specialized adapters to both fragmented ends utilizing the forward primer 27F (5'- AGAGTTTGATCMTGGCTCAG-3'), labeled with 6-FAM and reverse primer 355R (5'-CTGCTGCCTCCCGTAGGAGT-3'**) (Suzuki et al., 1998, Castillo et al., 2006, Wu et al., 2007, Doud et al., 2009, Doud et al., 2010)**. The library is loaded into the flow cell, allowing fragments to hybridize to the flow cell through bridge amplification. Each fragment attached to the flow cell is amplified to create what is known as clonal clusters. These clusters are sequenced with a multitude of reagents that include fluorescently labeled nucleotides. The flow cell is imaged and the emission from each cluster is recorded (Figure 2-16). The emission wavelength and intensity are used to identify the base and this process repeats until the specified read length is achieved. The results generated are translated into operational taxonomic units (OTUs) to analyze the gene sequence data **(He et al., 2015).** Microbial community identification may now be done at a higher throughput and at a cheaper cost thanks to NGS technology and bioinformatics. **(Castelino et al, 2017).**

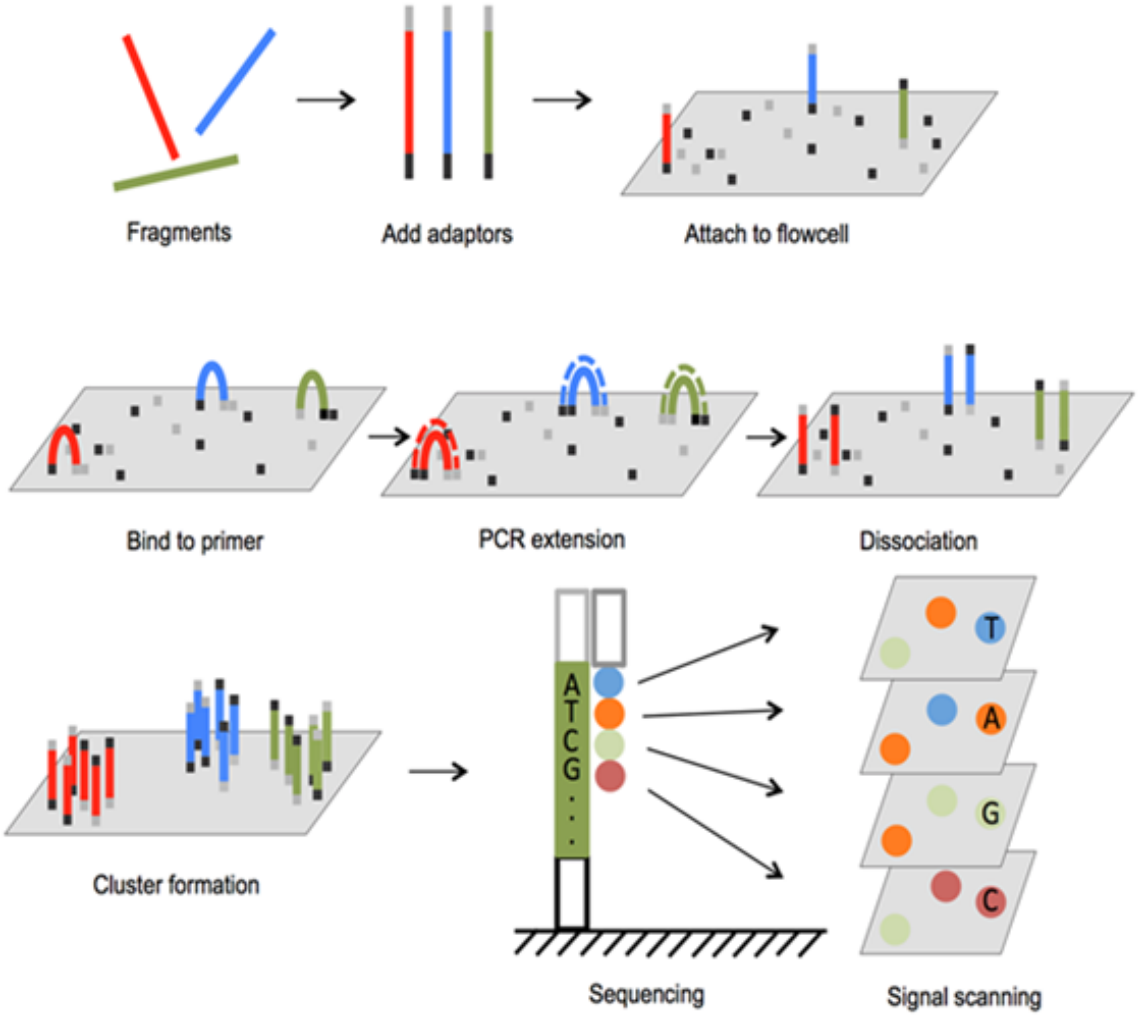

Figure 2-16:Workflow of Next Generation sequencing (Lu et al., 2016)

2.9 Statistical Analysis

Statistics is a quantitative science that deals with data gathering, analysis, presentation, and interpretation. Statistical analysis is the process of assessing data from a sample in order to evaluate a hypothesis. Within this study various open-format sources were utilized to examine the individual data sets of both the chemical and biological data. VOC peak areas were transformed and normalized in MetaboAnalyst software and statistically analyzed with supervised linear regression techniques: PLS-DA, OPLS-DA, and LDA. Fragment analysis relative abundance ratios were analyzed via Thermo Fisher Connect™ Microsatellite Analysis Software (MSA). Operational Taxonomic Units (OTUs) and Taxonomic information generated from NGS was processed via Mothur and statistically evaluated with MicrobiomeAnalyst and PRIMER v7 software examining alpha-diversity and beta-diversity metrics. In addition, statistical analyses similarity of percentages (SIMPER) and analysis of similarities (ANOSIM) was performed. Supervised machine learning technique Random Forest (RF) was also executed on OTU and taxonomic information.

3 RESEARCH OBJECTIVES

This dissertation aims to acquire information on the chemical and biological signatures of human odor profiles released from the palms of the hands of individuals from various genders and ethnicities. The evaluation of these profiles was meant to determine the applicability of human hand odor for forensic identification.

The objective of this research was to explore various bioinformatic modeling techniques that could indicate features of individual hand odor profiles that classify and discriminate for specific traits: gender and/or race/ethnicity. A thorough evaluation and development of analytical and biological methods was executed.

The tasks performed to satisfy the dissertation's goals are listed below.

a. Optimization of research methods, materials, and statistical analysis for the collection and analysis of volatile organic compounds released from the palms.
b. Multi-linear regression analysis with supervised techniques: PLS-DA, OPLS-DA, and LDA of HS-SPME-GCMS hand odor profiles for gender prediction
c. Optimization of research methods, materials, and statistical analysis for the collection and extraction of microbiota existing on the palms.
d. Screening and statistical analysis of community diversity with Length Heterogeneity-Polymerase Chain Reaction of 16S rDNA V1-V2 region bacterial microbiome profiles.
e. Next Generation Sequencing and statistical analysis of 16S rDNA V1-V2 region sequences for identification and classification of bacterial microbiome profiles.

4 MULTI-LINEAR REGRESSION MODELLING FOR GENDER PREDICTION USING VOLATILE ORGANIC COMPOUNDS FROM HAND ODOR

4.1 Introduction

Criminal activities involving robberies, assaults (sexual, simple, or aggravated), and rape are often executed with the use of the perpetrator's hands. As a result, hands are a focal point of investigations as trace amounts of evidence can be deposited on everyday objects through touch interactions. There is an exchange of biological and inorganic material between the perpetrator and the crime scene during these interactions. In accordance with *Locard's Exchange Principal,* the perpetrator will leave behind trace evidence in these moments **(Saferstein et al., 2011; Caraballo et al., 2016; Curran et al., 2007).** Fingerprints and DNA are biometrics commonly utilized to identify a suspect or victim of a crime. However, these forms of evidence can be degraded or found in quantities that are too small to be used. Even in these cases human scent evidence may be recovered and used as an individualizing feature in an investigation.

Well-trained canines (*canis familiaris*) operate as specialized sentient detectors able to distinguish and identify personal human odor and other chemicals of interest **(Filetti et al., 2019; Curran et al., 2010; Furton et al., 2001)**. Human odor is a complex of volatile organic compounds (VOCs) secreted from the body that are impacted by host genetics, environmental factors, and physiological secretions **(Prada et al., 2014)**. VOCs are organic compounds, often with high vapor pressures, that are emitted into the environment as gases. The persistence of an individual's odor in the environment is attributed to the constant shedding of the epidermis (outer layer) of the skin; this process leaves epithelial cells in the environment, along with sweat, oils, and other glandular secretions **(Baker, 2019;**

Syrotuck et al., 2000). Many compound classes are present in human emanations, including acids, alcohols, aldehydes, hydrocarbons, esters, and ketones **(Curran et al., 2007).**

An individual's odor is comprised of primary, secondary, and tertiary odors (**Curran et al., 2007; Syrotuck et al., 2000; Cuzuel et al., 2017).** Primary odor has been determined to be stable over time and distinct to an individual. One contributing factor to this distinctiveness is attributed to a polymorphic gene family known as the Major Histocompatibility Complex (MHC). The MHC contribution to human odor has been explained in three hypotheses. (1) The first hypothesis focuses on the presence of MHC-produced molecules found in sweat. (2) The second hypothesis states that MHC molecules may bind to specific peptides and present them to the surface of the cell/tissue and that these volatile metabolites may be the origin of skin odor VOCs. MHC molecules would therefore act as "odor carriers," with peptides functioning as precursors of VOCs (**Cuzuel et al., 2017).** (3) A final hypothesis suggests that MHC proteins/ peptides/metabolites may have a direct influence on the microbial flora. It is likely a combination of these hypotheses that truly explains how the MHC contributes to various roles in human odor production.

The genetic influence on microbial diversity leads to microbiota being another contributory factor of primary odor. As stated in a study by Shelley et. al, human perspiration has no odor until the microbiota in the skin begin to break down non-volatile chemicals into volatile molecules that are distinctive of human 'scent.' **(Shelley et al., 1953)**. Although genetics and microbial diversity aid in the stability of odor, there is a secondary odor composition that has been determined to be variable and endogenous via the skin's multi-layer composition. Physiological secretions produced from the dermis to

epidermis are excreted through three types of glands: eccrine, apocrine, and sebaceous. Eccrine glands are distributed all over the human body but more densely in the palms, forehead, and soles of the feet, which is the focus of this study **(Coutinho-Abreu et al., 2021)**. Tertiary odor has the highest variability due to exogenous compounds such as non-resident bacteria, cosmetic products, soaps, and perfumes.

Though canines have been proven to reliably identify persons based on their odor profile, laboratory based subject identification using analytical instruments has been difficult due to the lack of robust datasets and sufficiently developed analytical techniques. This work demonstrates the ability to predict donor gender based upon the VOCs present in a collected hand odor sample via Headspace-Solid Phase Microextraction-Gas Chromatography-Mass Spectrometry (HS-SPME-GC-MS). SPME is a solvent free method that integrates sampling, extraction, and concentration of analytes **(Gherghel et al., 2018)**. Existing human odor research has identified VOCs of interest that are characteristic of gender **(Zeng et al., 1996; Penn et al., 2006)**, age **(Mitro et al., 2012)**, and racial/ethnic groupings **(Bates et al., 2007; Prokop-Prigge et al., 2014; Colón-Crespo et al., 2017).** These works indicated a capacity for predictive classification of individuals using odor samples. The specific presence and abundance of VOCs in human hand odor creates a chemical profile that can characteristically be applied to classify individuals based on class characteristics, increasing the utility of human hand odor when other biological evidence is lacking. Our analyses utilized three linear regression modelling approaches for the classification of donor gender, creating a potential route to identifying donor characteristic based upon collected human odor samples.

4.2 Materials and Methods

4.2.1 Cohort

Sixty subjects of African American, Hispanic, and Caucasian race/ethnicity, between the ages of 18-46 years old volunteered to participate in this study (Table 6). Before participation in the study, all subjects signed a consent form and filled out a questionnaire providing information about race/ethnicity, country of origin, gender, age, diet restrictions, and current state of health at time of sampling. The sampling procedures and protocols involving human subjects were authorized by Florida International University's Institutional Review Board (IRB-19-0277) prior to commencement of the study. This study was conducted on a strict volunteer basis, therefore no participants received compensation.

Table 6: Demographics of participants in study

Race/ethnicity	Female	Male
African American	10	10
Hispanic	10	10
Caucasian	10	10

4.2.2 Chemical and Materials

Methanol (Fisher Scientific, Pittsburgh, PA) was used for the pretreatment of the collection material, 2 in. x 2 inch, 12ply, 100% cotton, sterile gauze pads (DUKAL Corporation, Syosset, NY). The glass vials for collection of hand scent samples were 10-mL, clear, screw top with PTFE/Silicone septa, respectively (SUPLECO, Bellefonte, PA). 10-mL vials were placed in Fisherbrand™ Isotemp™ Digital Dry Bath Incubators for HS-SPME equilibration and extraction. The Solid Phase Microextraction (SPME) fibers used were 50/30µm Divinylbenzene/Carboxen/Polydimethylsiloxane and 24ga needle size (50/30µm DVB/CAR/PDMS; SUPELCO, Bellefonte, PA).

4.2.3 Collection material pretreatment

Although human collection materials were biologically sterile, the absorbent material contained compounds such as Decanal and Nonanal, also found in human hand odor **(Curran et al., 2005)**. A pretreatment process of the storage vials and cotton gauze was used to remove any background interferents. The 10-mL vials and caps were cleaned with a mildly basic soap solution (Contrex AL®, Decon Labs, Inc.), rinsed with warm tap water, followed by deionized (DI) water, and a final acetone rinse, before baking in an oven at 105°C for 1 hour. The cotton gauze was pre-treated by laying the gauze flat on a sterilized watch glass, spiking the gauze pad with 1mL of methanol, placing the gauze in a cleaned 10-mL vial, and baking in an oven at 105°C for one hour **(Prada et al., 2011)**. The background levels of VOCs on the pretreated cotton gauze and vial were monitored by HS-SPME-GC-MS prior to use in sample collection.

4.2.4 Hand Odor Sample Collection and Analysis

The hand odor collection protocol was modified from the original protocol published by Curran et al. in efforts to resemble a more realistic collection of hand volatiles (**Curran et al., 2007).** Each subject was instructed to *not* wash their hands for a minimum of one hour prior to sampling. Hand odor was collected from each subject while sitting indoors. A pretreated 2 in. x 2 in., 12ply, 100% cotton DUKAL gauze was swiped on each palm of the hands and then squeezed between the palms for 10 min. Upon completion of the 10 min hold, the sampled gauze was placed back into its respective 10 mL vial and capped. The 10 mL vial containing sampled gauze was placed into a digital bath (set to 50°C) for 24 hours. After 24 hours, a clean 50/30µm DVB/CAR/PDMS SPME (Grey) fiber was placed into the sample vial and exposed for 15 hours. After exposure, the SPME fiber was subsequently desorbed into the inlet of an Agilent 6890 GC coupled with an Agilent 5973 MSD. Fibers were desorbed at 250°C for (5) minutes in the GC inlet. A SolGel-WAX™ 30M x 0.25mm ID x 0.25µm phase thickness (SGE Analytical Science) column was used with ultra-high purity helium (Airgas) as the carrier gas. The parameters displayed in table 7, were applied when analyzing each sample.

Table 7: HS-SPME-GC-MS analytical parameters for desorption, separation, and detection of VOCs

HS-SPME	
Fiber	Divinylbenzene/Carboxen/Polydimethylsiloxane (50/30µm)
Exposure time	15 hours
Exposure temperature	50°C
Gas Chromatography	
Column	SGE Analytical Science SOL-GEL-Wax™ (30m x 0.25mM, 0.25 µm)
Carrier gas	Helium (He)
Constant flow	1.0 mL/min
Inlet temperature	250°C
Ramp	40°C for 1.25 min 10°C/min until 135°C for 4 min 10°C/min until 185°C for 4 min 10°C/min until 205°C for 4 min 30°C/min until 280°C
Oven run-time	32 min
Mass detector	
Source temperature	230°C
Ionization mode	Electron Ionization
Scan range	40-400m/z

4.3 Data Analysis

4.3.1 Data Pre-Processing

The collected GC-MS data files for the 30 male and 30 female subjects were submitted to a proprietary software program currently under development by the authors for pre-processing. Data files were aligned by retention time and evaluated for reoccurring peaks present in the total ion chromatogram (TIC). All compounds were assigned numbers instead of names and corresponding peak areas were organized into tables for supervised dimensional reduction analysis.

4.3.2 Partial Least Squares- Discriminant Analysis (PLS-DA)

Partial Least Squares-Discriminant Analysis (PLS-DA) is a latent variable regression method based on covariance between the predictors and the response **(Thévenot et al., 2015).** PLS-DA represents a quantitative relationship between a matrix, X, usually comprising spectral or chromatographic data of a set of calibration samples, and another matrix, Y, containing quantitative values **(Trygg et al., 2006)**. This supervised method uses multivariate regression techniques to extract via a linear combination of the original variables and information that can predict class membership **(Mendel et al., 2021)**. The raw peak area data from the pre-processing was transformed with $\log_{10}$ transformation and auto-scaled (mean-centered and divided by the standard deviation of each variable) to be submitted to MetaboAnalyst software version 5.0 (https://www.metaboanalyst.ca) an analyzed with the PLS-DA algorithm (Appendix 2). The implementation of this method as described by Wehrens et al. produced a PLS-DA 2D and 3D model (Figure 4-1) **(Mevik et al., 2007)**.

4.3.3 Orthogonal-Projections Latent Structures Discriminant Analysis (OPLS-DA)

Orthogonal-Projections to Latent Structures Discriminant Analysis (OPLS-DA) is a supervised dimensional reduction tool with the predictive capacity of PLS but the combined orthogonal methodology is superior for predicting variables contributing to class separation **(Pinto et al., 2012)**. The main idea of OPLS is to separate the systematic variation in X into two parts, one that is linearly related to Y and one that is unrelated (orthogonal) to Y **(Trygg et al., 2006)**. The algorithm is modified to model separately, the variations of the predictors correlated and orthogonal to the response, minimizing a two or more predictive components analysis to a single predictive component **(Trygg et al., 2002)**. The $\log_{10}$ transformed data set was further analyzed in MetaboAnalyst software version 5.0 (https://www.metaboanalyst.ca) with the OPLS-DA algorithm as described by Thévenot et al. **(Thévenot et al., 2015).** Leave one out cross validation was ran concurrently with the OPLS-DA algorithm to ensure data was not overfitted within the model. The model produced a resulting graph that demonstrated a significant difference amongst the predictive component and its orthogonal response to separate male and female subjects (Figure 4-2).

4.3.4 Linear Discriminant Analysis (LDA)

Linear Discriminant Analysis (LDA) is a supervised learning technique that searches for those vectors in the underlying space that best discriminate among classes (rather than those that best describe the data) **(Martinez et al., 2001)**. In its application to the present dataset, LDA was used for dimensional reduction and supervised modelling of gender classification (Female or Male). Prior to the pre-processed data submission to JMP®, Version 16.1.0. SAS Institute Inc., Cary, NC, 1989–2021, the processed files were filtered

to contain all peaks present in more than 20% of submitted samples (12/60), as determined through retention time-based peak matching. The peak areas of the aligned samples were $\log_{10}$ transformed; the LDA model was validated using LOOCV (leave-one-out cross-validation). The resulting graph demonstrated separation of male and female subjects and the predictive ability of the model via cross-validation (Figure 4-3)**.**

4.4 Results and Discussion

4.4.1 Partial Least Squares- Discriminant Analysis (PLS-DA)

Various studies have examined and detected (in the headspace profiles) the presence of several compounds that have been articulated as human scent compounds **(Curran et al., 2005)**. The produced 2D PLS-DA (two component) and 3D PLS-DA (three component) score plots were created using log-transformed TIC peak areas. The 2D scores plot (Figure 4-1A) was comprised of the two principal components that illustrated clustering of the male and female subjects but no separation of the two classes. The green and red ellipses surrounding the male and female clusters represented 95% confidence region. The same principal was applied to the 3D scores plot, the incorporation of a third component revealed clustering of the male and female subjects with separation of the two classes (Figure 4-1B). While the PLS-DA model illustrates variations that may exist in the measurements for group prediction, these variations may be uncorrelated. Therefore, OPLS-DA is often implemented in order to disentangle group-predictive and group-unrelated variation in measured data **(Mevik et al., 2007)**.

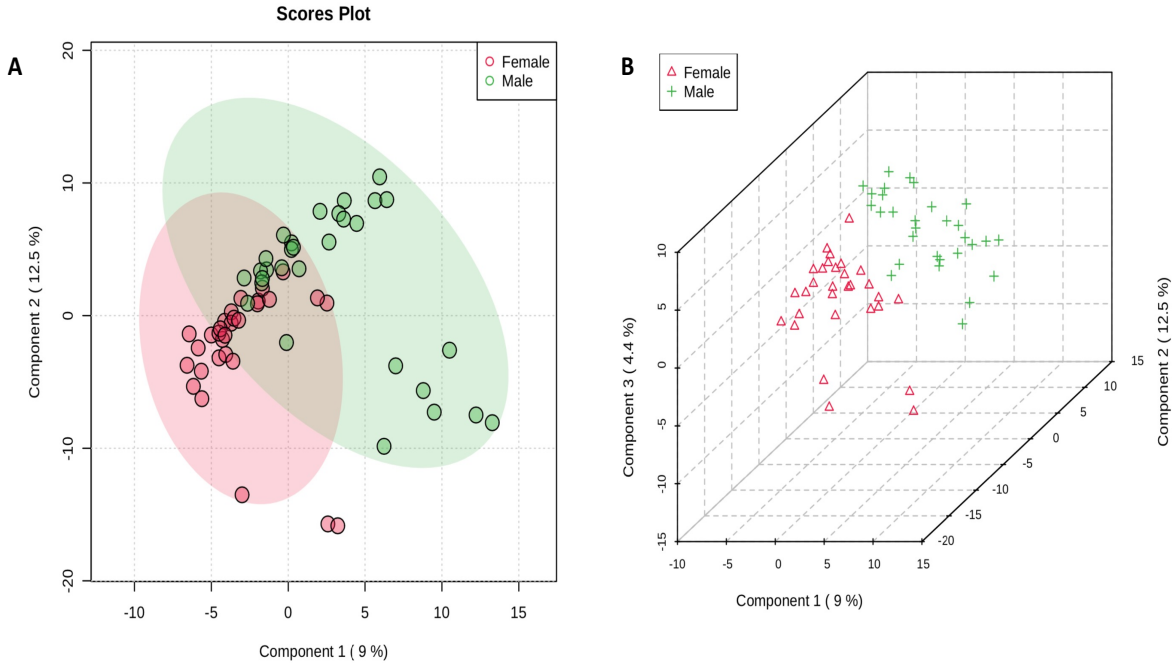

Figure 4-1:Gender(A) 2D PLS-DA plot (B) 3D PLS-DA plot

4.4.2 Orthogonal-Projections Latent Structures Discriminant Analysis (OPLS-DA)

The transformed peak data table was further analyzed in the researchers' attempt to determine whether HS-SPME-GCMS could reveal class separation of odor profiles into male and female clusters. Components are contributed by variable importance projections (VIPs), comprised of both the loading weights and the variability of the response explained **(Trygg et al., 2006)**. The T-score reflects the predictive component of the data set and orthogonal T-score represents the component unrelated to predictive component **(Thévenot et al., 2015).** The calculated covariance is explained within the respective parentheses (16.3% and 2.5%). The ellipses correspond to 95% of the multivariate normal distributions with the covariances for each class being shown. The supervised classification method OPLS-DA was employed utilizing all peak areas detected and the resulting graph

demonstrated sufficient variations in the chemometric data, such that, clustering and separation of female and male subject data was observed without the requirement of compound identification (Figure 4-2).

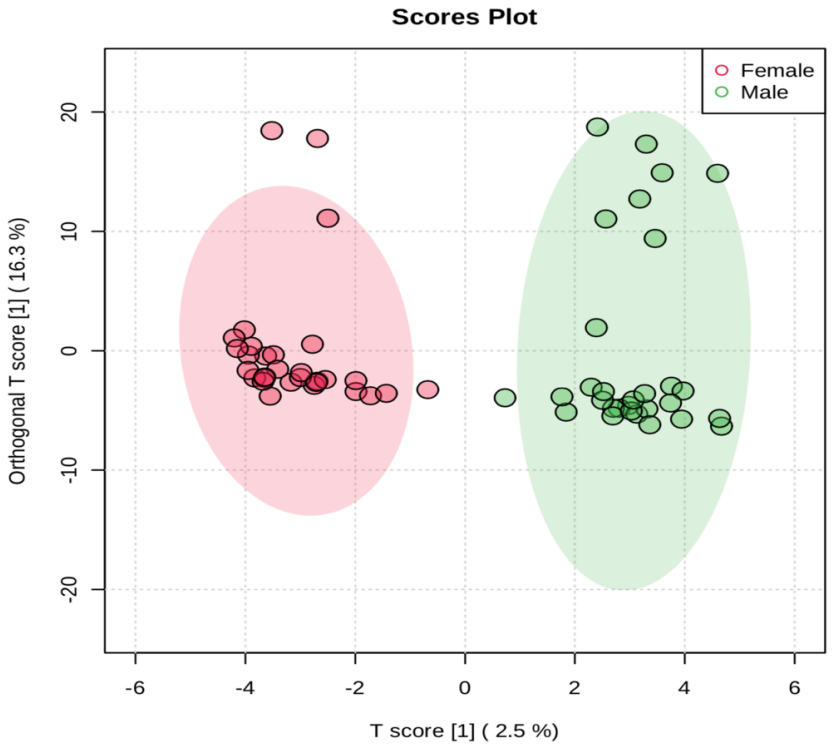

Figure 4-2:Gender OPLS-DA plot

4.4.3 Linear Discriminant Analysis (LDA)

Linear discriminant analysis was used as a dimensional reduction and modelling technique for the predictive classification of donor samples into "Male" or "Female" classes. Due to the composition of the DVB/Car/PDMS SPME fibers utilized and the makeup of the polar SolGel-WAX™ capillary column, highly abundant siloxane peaks were observed in the obtained chromatograms and spectra. Peaks appearing in less than 20% and more than 95% of samples were removed to minimize the effect of background interferents. This procedure was chosen to remove both consistently present background signals and sporadically present interferents. The filtered peak table was log-transformed and used to create an LDA model, the model was validated using LOOCV (leave-one-out cross-validation).

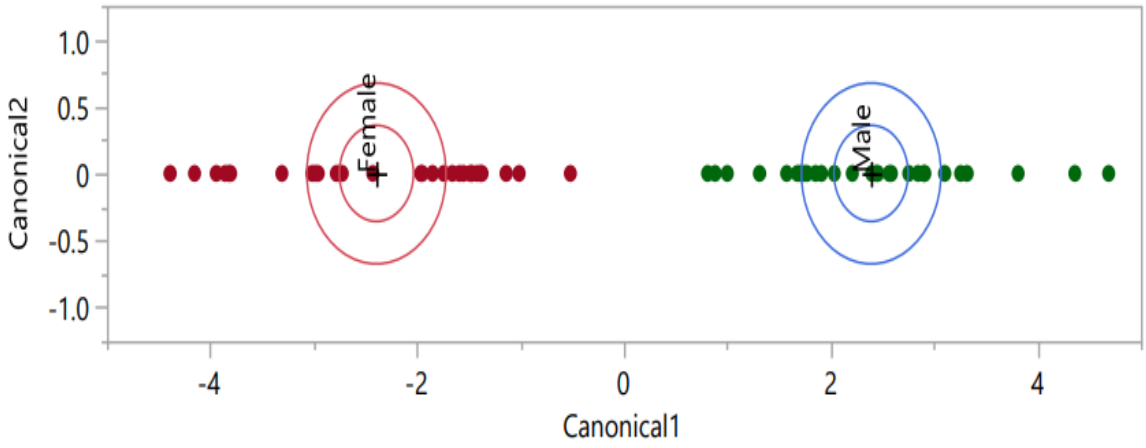

Figure 4-3:Gender LDA plot

The classification and separation of male and female subjects illustrated by the LDA model (Figure 4-3) was cross-validated using leave-one-out cross-validation. The LDA model's performance was determined to have a 29/30 (96.67%) rate for predicting samples from male donors as male. The LDA model also predicted the source of female donated samples as female at a 29/30 (96.67%) rate. One male sample and one female sourced sample were misclassified using the produced model.

4.5 Conclusion

The exploration of human odor to discriminate individuals through various class characteristics (gender, race/ethnicity, etc.) for forensic application has been conducted analytically via GC-MS. However, the previous analysis of hand odor profiles has been constricted to a visual (Appendix 1), unsupervised multivariate analysis (i.e., PCA), and a minute exploration of linear discriminant analysis. In this study, hand VOC odor profiles from 60 self-identifying participants were evaluated with supervised multivariate regression models for gender classification. Higher discrimination and classification of subject gender were observed with orthogonal projections latent structures- discriminant analysis (OPLS-DA) and linear discriminant analysis (LDA) as confidence level ellipses of both models were not seen to intercept. The cross validation of the LDA model demonstrated its efficacy with a 96.67% correct prediction of male and female subjects. The partial least squares-discriminant analysis (PLS-DA) 2D model displayed lesser discrimination of gender but exhibited the ability to cluster female and male subjects. With the addition of a third component greater classification and discrimination of gender could be observed in the 3D model.

This work displays a small component of statistical analysis necessary for human scent research to be applied for forensic identification. Further work is needed in the exploration of the feature selection provided by the VIP scores and loading plots of the PLS-DA and OPLS-DA model that are indicative of compounds that aid in discrimination and gender prediction of hand odor profiles. The overall statistical workflow could be applied to other identification factors such as ethnicity/race and age when other discriminatory evidence (e.g., DNA) may be lacking. Overall, the application of the discussed models throughout this paper can be applied to various forensic data sets regardless of the brand of instrumentation used for data collection and paves way for a tool that can perform standardized VOC comparisons.

5 A MOTHUR'S PERSPECTIVE ON 16S RDNA BACTERIAL MICROBIOME DIVERSITY ANALYSIS OF HANDS FOR FORENSIC IDENTIFICATION

5.1 Introduction

The various structures that make up the human hand—bones, phalanges, metacarpals, palmar fascia, and muscles—allow us to have stability and movement to feel, touch, and handle various objects within our everyday environments. These structures are all connected by the largest organ of the human body—skin. The skin provides a microenvironment for microorganisms to inhabit, the majority of which are bacteria. The influence of microbiota on the skin is contributed by a variety of factors: pH, temperature, moisture, and sebum content **(Grice et al., 2008).** The research of skin-associated microbiota through the Human Microbiome Project has been focused on understanding the microorganisms that contribute to a healthy individual's microbiome. The limitations on identification of the collective microbial community have been contingent upon the ability to culture organisms in the laboratory **(Cox et al., 2013)**. Evolving sequencing techniques have allowed for the identification of difficult and/or unculturable microbiota. Massively parallel sequencing (MPS) methods such as next generation sequencing (NGS) are the premier tools to use in microbial studies because they can simultaneously detect length variations and identify sequences **(Jordan et al., 2020).** However, the speed and inexpensive cost of amplification-based methods, such as length heterogeneity polymerase chain reaction (LHPCR), have become the standard of 16S rRNA gene microbiota studies **(Eisenhofer et al., 2019).**

These technological advancements have sparked interest in fields outside of health and disease research, underscoring the potential of examining the microbiome for future applications **(Knight et al., 2018; Budowle et al., 2019; Clarke et al., 2017; Carter et al., 2020).** Specifically in forensics, the microbiome has been evaluated as a form of trace evidence **(Knight et al., 2018).** Dependent upon skin location, bacterial communities can have a high degree of interindividual variability, specifically notating that only 13% of bacteria present on the palm surface is shared between two individuals **(Fierer et al., 2010; Fierer et al., 2008).** Thus, skin microbiota can be viewed as a form of evidence that can aid in forensic identification. Moreover, we examined the 16S rRNA gene V1-V2 hypervariable regions from skin microbiota of the palms surface. We used cotton swabs for collection of resident bacteria; then genomic DNA (gDNA) was extracted and amplified with 27F/355R 16S rRNA primers, followed by capillary electrophoresis with fragment analysis (LHPCR). Further analysis of the 16S rRNA V1-V2 amplicons was sequenced with next generation sequencing technologies and examined statistically via alpha-diversity (Shannon diversity), beta-diversity (Bray-Curtis), and Random Forest (RF) of the operational taxonomic units (OTUs) information processed in mothur **(Schloss et al., 2009; Kozich et al., 2013)**.

5.2 Materials and Methods

5.2.1 Sample preparation and collection

The skin of 60 healthy individuals (30 female and 30 male) was sampled by swabbing the palms surface for thirty (30) seconds with a HydraFlock 6" Sterile Standard Flock Swab (Puritan®, USA), which was dipped into an aliquot of 1X Phosphate buffer solution (PBS). The swab was rubbed back and forth in a crosswise, rotating manner in the defined area in the same fashion for each subject to maintain consistency. After 30 seconds, the head of each swab was placed into a sterile microcentrifuge tube and aseptically cut from the breakpoint of the handle before closing the tube lid. Additionally, a blank swab was dipped in PBS without collection of any epithelial cells from the skin was saved in a sterile microcentrifuge tube. Individuals were required to fill out a consent form and questionnaire following the Florida International University's Institutional Review Board (IRB-19-0277) approved protocol. Participants of the study self-identified their gender and ethnicity that was denoted with identifiers (F = Female, M = Male) and (AA = African American, C = Caucasian, H = Hispanic), respectively. Collected epithelial swabs from the palms surface were immediately frozen at -20°C until DNA extraction.

5.2.2 DNA isolation, quantification, and amplification

The genomic DNA (gDNA) was extracted with Maxwell® 16 Cell LEV DNA Purification Kit (Promega, Madison, WI, USA) as per manufacturer's instructions. The eluted DNA was quantified with the Qubit® 2.0 Fluorometer and Qubit® dsDNA HS Assay fluorometric quantitation kit (Invitrogen, Life Sciences, Carlsbad, CA). The 16S rRNA gene 1500bp contains nine hypervariable regions that are flanked by conserved primer sites that can be amplified with universal primers **(Chakravorty et al., 2007)**. PCR amplification was performed with the 27F (5′- AGAGTTTGATCMTGGCTCAG-3′), modified with 6-FAM, and 355R (5′-GCTGCCTCCCGTAGGAGT-3′) primer set which is known for its bacterial taxa identification targeting the 16S rRNA gene V1-V2 region **(Suzuki et al., 1998; Wu et al., 2007; Doud et al., 2009; Doud et al., 2010)**. For the distinction of the different 16S rDNA amplicon lengths, the 5′ end of 27F was labeled with 6-FAM™ (Integrated DNA Technologies, Coralville, IA, USA). **(Wu et al., 2008)**. A standard PCR protocol was conducted on a AppliedBiosystems ProFlex Thermocycler (Thermo Fisher Scientific, Waltham, MA, USA), in a 20µL volume consisting of Thermo Scientific™ Phire Hot Start II PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), 0.5µM of each forward and reverse primer, ~1ng of DNA, and water added to volume if necessary. The PCR conditions were carried out as follows: initial denaturation at 98°C for 5 min, 34 cycles of denaturation at 98°C for 5 secs, annealing at 55°C for 5secs, extension at 72°C for 5secs, and a final extension step at 72°C for 1min and 4°C for 1min. Confirmation of amplified PCR product was visualized on a 1% agarose gel prior to length heterogeneity (fragment analysis) with capillary electrophoresis (CE).

5.2.3 Fragment Analysis

Amplified V1-V2 region 16S rRNA bacterial DNA underwent fragment analysis according to protocol established by *Mills et al.* (2003) **(Mills et al., 2003)**. The labeled PCR products were loaded onto the AppliedBiosystems SeqStudio™ Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA) as a mixture of 1µL amplified PCR product with a 12µL master mix solution of 11.5µL Hi-Di™ Formamide and 0.5µL GeneScan™ 600 LIZ™ size standard (Thermo Fisher Scientific, Waltham, MA, USA). The SeqStudio™ Genetic Analyzer allows for simultaneous separation of four samples in 45 min. via capillary electrophoresis with universal polymer POP-1™ (Thermo Fisher Scientific, Waltham, MA, USA). Laser-induced fluorescence of amplicons was detected with module DS-33 (6-FAM™, VIC®, NED™, PET™) and G5 filter.

To screen diversity of fragment sizes of V1-V2 amplicons, a marker range from 300-500bp was curated to scan all the peaks present within the sample. Historically, the minimum threshold has been maintained at 50 RFUs **(Moreno et al., 2011)**. However, because of the increased sensitivity of the SeqStudio™ Genetic Analyzer, the minimum default threshold was elevated to 175 RFUs. Peaks below this threshold could be present within the electropherogram but would not be recognized as a true peak. The increased RFU threshold is important as abundance of certain fragment peaks could max out on the fluorescence scale causing pull-up of peaks that represent noise. The resulting electropherogram profiles were analyzed using Thermo Fisher Connect™ Microsatellite Analysis Software (MSA).

5.2.4 Bacterial 16S rRNA Gene Sequencing

Amplification, library preparation, and sequencing were conducted at the Forensic DNA core facility (Florida International University, Miami, FL, USA). The genomic DNA was amplified following the amplification protocol mentioned in section 2.2. Next Generation Sequencing (NGS) was performed on the Illumina MiSeq® instrument (Illumina, San Diego, CA, USA) with *Quick*-16S™ NGS library Prep Kit (Zymo Research Corp. Irvine, CA, USA) of amplicons. The size of amplicons of the 16S V1-V2 region with the inclusion of primers is ~350bp and the addition of barcoded primers the final amplicon size is ~486bp. Purification of prepared library was conducted with Select-a-Size MagBeads provided in library prep kit and loaded into the MiSeq® cartridge per manufacturer's instructions. According to the integrated index primer set, each paired-end sequence was assigned to its respective sample.

5.3 Statistical Analysis

Generated amplicon data and respective peak areas from electropherogram profiles analyzed with Thermo Fisher Connect™ Microsatellite Analysis Software (MSA) were exported to excel for calculation of relative (abundance) ratios. The ratio between the peak area at a specific amplicon length and the overall peak area of all DNA amplicon lengths in the sample was used to calculate the relative abundance of each DNA amplicon. Only the amplicons with a relative abundance larger than 0.01 were included in subsequent analyses to eliminate the mistake introduced by the collection and analysis tools. Peaks separated by 1 base pair (bp) or less were grouped together and used in all statistical analyses as one amplicon length. The square root transformation and graphical depiction

of relative abundance values for each length of DNA amplicon was followed as written by *Wu et al*. (2007) **(Wu et al., 2008)**.

The transformed relative ratios were analyzed with alpha and beta diversity metrics in PRIMER v7 (PRIMER-E Ltd, Lutton, Ivybridge, UK). Within PRIMER v7 software, all nonparametric multivariate studies, including the creation of cluster analysis, non-metric MDS, and ANOSIM on relative ratios of DNA amplicons from LHPCR was performed. Within cluster analysis, clusters are merged depending on their distance from one another, and different sorts of links are used to compute that distance. Ward, Average, Single, and Complete linkage are common distance measures that can be implemented to specify the distance between clusters based on various criteria. Ward examines cluster distances as a variance issue, disregarding distance metrics or association measures. Average linkage measures the distance between two clusters is the average of the distances between all the points in those clusters. Also known as the nearest neighbor technique, single linkage measures the smallest distance between two members of the two clusters. Complete linkage is the opposite of Single linkage by measuring the maximum distance between two members of the two clusters. Single linkage was chosen as the distance measure for cluster analysis within this study.

Non-metric MDS plots are developed from the cluster analysis Bray-Curtis dissimilarity matrix which is statistically evaluated with ANOSIM. Simultaneously, SIMPER (Similarity of Percentages) analysis was used to determine the differences (or distances) between ethnicity or gender factors and their contributions from each of the amplicon fragments. **(Clarke et al., 2015)**. Leading to indication of specific amplicon lengths that drive distances among samples that can later be identified.

All amplicon reads (FASTQ files) generated from NGS were processed in the platform Mothur **(Schloss et al., 2009)** according to the MiSeq standard operation procedures as described by Kozich et al., (2013) **(Kozich et al., 2013).** Graphical curation and statistical analyses (alpha and beta diversity) were performed in MicrobiomeAnalyst (https://www.microbiomeanalyst.ca/) **(Dhariwal et al., 2017; Chong et al., 2020)**.

5.3.1 Alpha-diversity

Microbiome studies frequently use alpha diversity evaluation to measure diversity within a sample or ecosystem. Analyzation of alpha-diversity is a normalized first approach to assessing the differences between sample groups when using amplicon sequencing data **(Willis, 2019)**. Through alpha diversity, the structure of an ecological community can be summarized in terms of its richness (number of taxonomic groupings), evenness (distribution of group abundances), or both **(Willis, 2019)**. Observed, ACE and Chao1, Shannon, Simpson, and Fisher are common metrics utilized to calculate diversity within the sample groups **(Fisher et al., 1943; Shannon, 1948; Simpson, 1949; Chao et. al., 2003)**. Observed diversity examines the richness of the sample's unique amount of OTUs. ACE and Chao1 also evaluate richness of the sample through a combined perspective of Observed OTUs and unobserved species correlated to low abundance OTUs. The Shannon, Simpson, and Fisher metrics take in consideration of both richness and evenness to evaluate the diversity within the sample **(Morris et al., 2014)**. Despite the close links between these diversity indicators, they are not interchangeable, and there has been much discussion over which is more appropriate in certain situations.

For this study Shannon diversity (H') was employed, which can be computed by (equation 3):

$$H' = -\Sigma P_i ln(P_i)$$

Equation 3: Shannon Diversity Index

Where "P_i" represents the proportion of individuals belonging to species "i" **(Shannon, 1948)**. An unknown individual in a highly diversified (and evenly distributed) system could belong to any species, resulting in a high level of uncertainty in predicting its identity. It is easier to anticipate the identification of unknown individuals in a less diversified system dominated by one or a few species, and there is less ambiguity in the system **(Shannon, 1948)**. Moreover, the evaluation of alpha-diversity alone does not paint the entire picture of microbial diversity and implores the necessity of additional analyses such as beta-diversity. Graphical descriptions of ethnic and gender-based groups were visualized with Primer v7.

5.3.2 Beta-diversity

To assess bacterial community structure between samples, beta-diversity indices such as Bray-Curtis, Jaccard index, and UniFrac distances can be employed. Within this study Bray-Curtis was chosen as the distance based method. Bray-Curtis works in conjunction with statistical methods such as Analysis of Similarities (ANOSIM) as a non-parametric test of significant differences between two or more groups **(Clarke et al., 1993)**. The dissimilarity or distance statistic (R) can be computed as (Equation 4):

$$R = 1 - (\frac{2w}{a+b})$$

Equation 4: Bray-Curtis Dissimilarity

Where "*w"* represents the sum of the lesser counts exclusively for species present within all communities, "*a"* is the sum of the counts for taxa present in one community, and *b* is the sum of the counts absent within the "*a"* community **(Bray et al., 1957).** The statistic compares the mean of ranked dissimilarities between groups with the mean of ranked dissimilarities within groups demonstrated with R values that can range from -1 to 1 **(Buttigieg et al., 2014).**

Random Forest, a machine learning technique for categorizing data and discovering predictive features (biomarkers), was used to investigate the prediction and classification of microbial communities after alpha and beta diversity evaluation of OTUs was completed. It works by training a large number of decision trees (or "forests") and predicting the class based on the individual trees' majority vote.

5.4 Results and Discussion

5.4.1 Fragment Analysis

Length heterogeneity fragment length plots (Appendices 3-8) were transformed into their relative ratios. Hierarchical clustering findings of various factors (gender and ethnicity) from transformed relative ratios are represented using dendrograms with single linkage distance measure (Figure 5-1). Non-metric MDS (NMDS) plots were visualized from calculated Bray-Curtis dissimilarity matrix established from single linkage cluster analysis. NMDS plots indicate that samples that are ordinated closer together are more likely to be similar than those that are ordinated further apart. The ellipses within each NMDS plot denoted 60% and 80% similarity amongst subjects. At minimum a 60% similarity was observed amongst each ethnic group regardless of gender with few outliers at an observed

stress of 0.01 or (Figure 5-2 B-D). The stress values of 0.1 or less are regarded fair, while values of 0.05 or less imply a good fit **(Buttigieg et al., 2014)**. However, within figure 5-2A, the samples clustered tightly together making interpretation of the NMDS difficult. This is in agreeance with the clusters of the dendrogram observed for all subjects being denoted with predominantly red lines. Red lines signify sub-structures that lack statistical support, which should avoid interpretation, whereas black lines denote divisions that have statistical support **(Clarke et al., 2015)**. The single linkage approach sometimes can present a disadvantage of causing early merging of groups with close pairs, even if those groups are otherwise highly dissimilar.

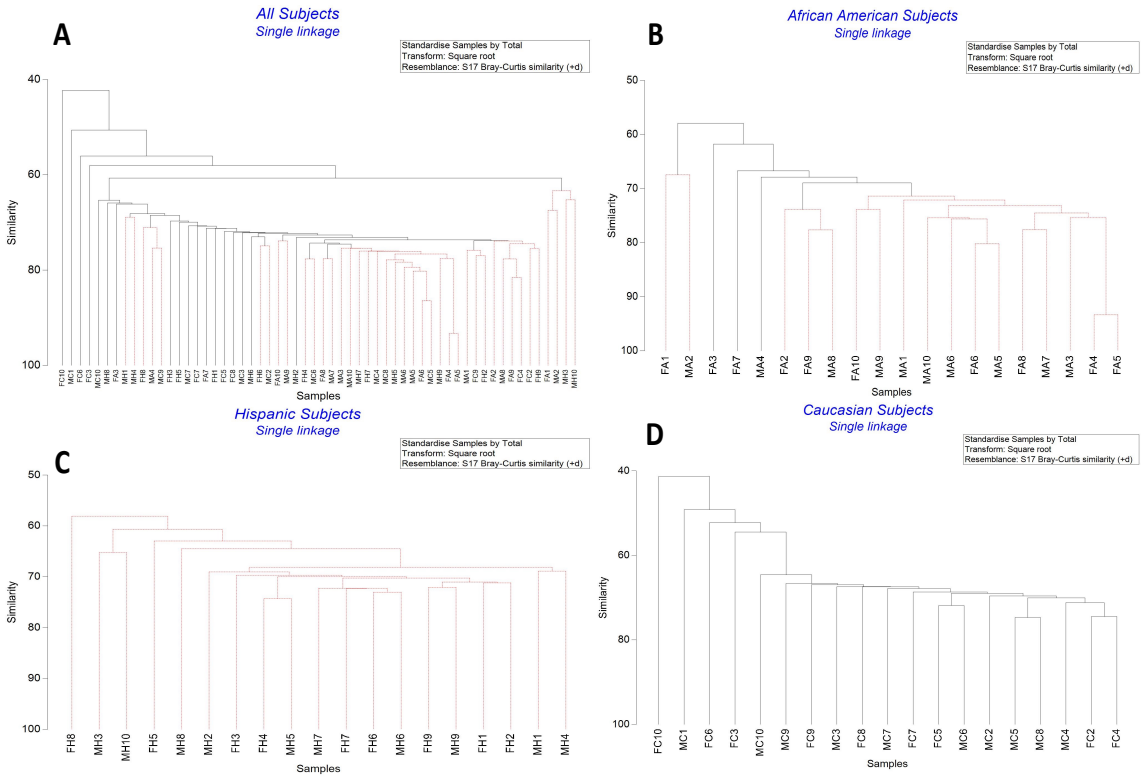

Figure 5-1:Dendrograms of single linkage hierarchical clustering for (A): All subjects (B) African American subjects (C) Hispanic subjects (D) Caucasian Subjects

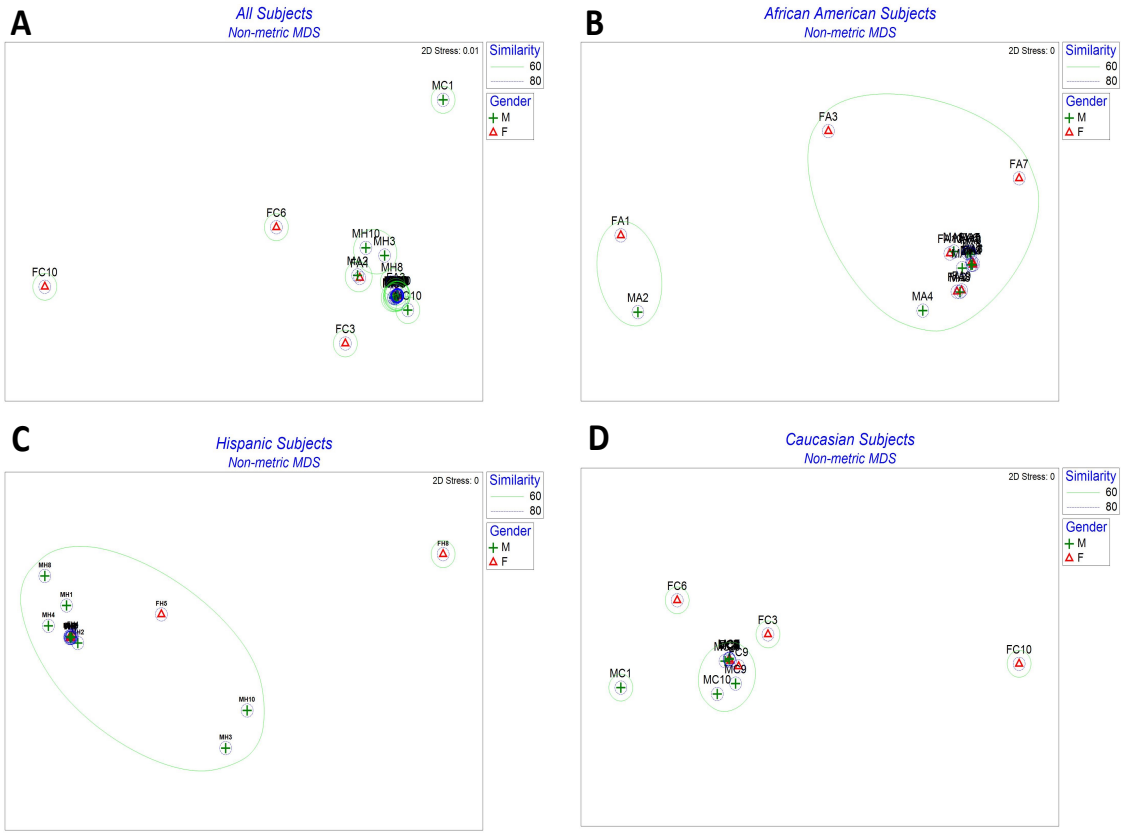

Figure 5-2:NMDS plots for (A): All subjects [stress: 0.01] (B) African American subjects [stress: 0] (C) Hispanic subjects [stress:0] (D) Caucasian Subjects [stress:0]

Therefore, further statistical analysis of Bray-Curtis dissimilarity matrices curated prior to hierarchical clustering underwent ANOSIM analysis to distinguish if cluster differences were distinct. The ANOSIM analysis of gender and ethnicity of subjects within their perspective groupings was conducted to see if any significant differences in mean dissimilarities could be observed across subjects described by the R statistic produced. A positive R value up to "1.0" indicates that groupings are distinct, whereas a R value near "0" indicates that high and low ranks are distributed evenly within and between groups. R values less than "0" indicate that differences within groups are bigger than differences

between groups **(Buttigieg et al., 2014)**. The significance of dissimilarities amongst groups is established with ANOSIM analysis. Each gender ANOSIM plot produced R values lesser then "0" indicating the genders differences within each ethnicity are greater in comparison to females and males within another ethnicity (Figure 5-3). Ethnicity ANOSIM average R statistic of male and female subjects was near 0, which demonstrates there is an even distribution of high and low dissimilarities of ethnicities within and between groups (Figure 5-4).

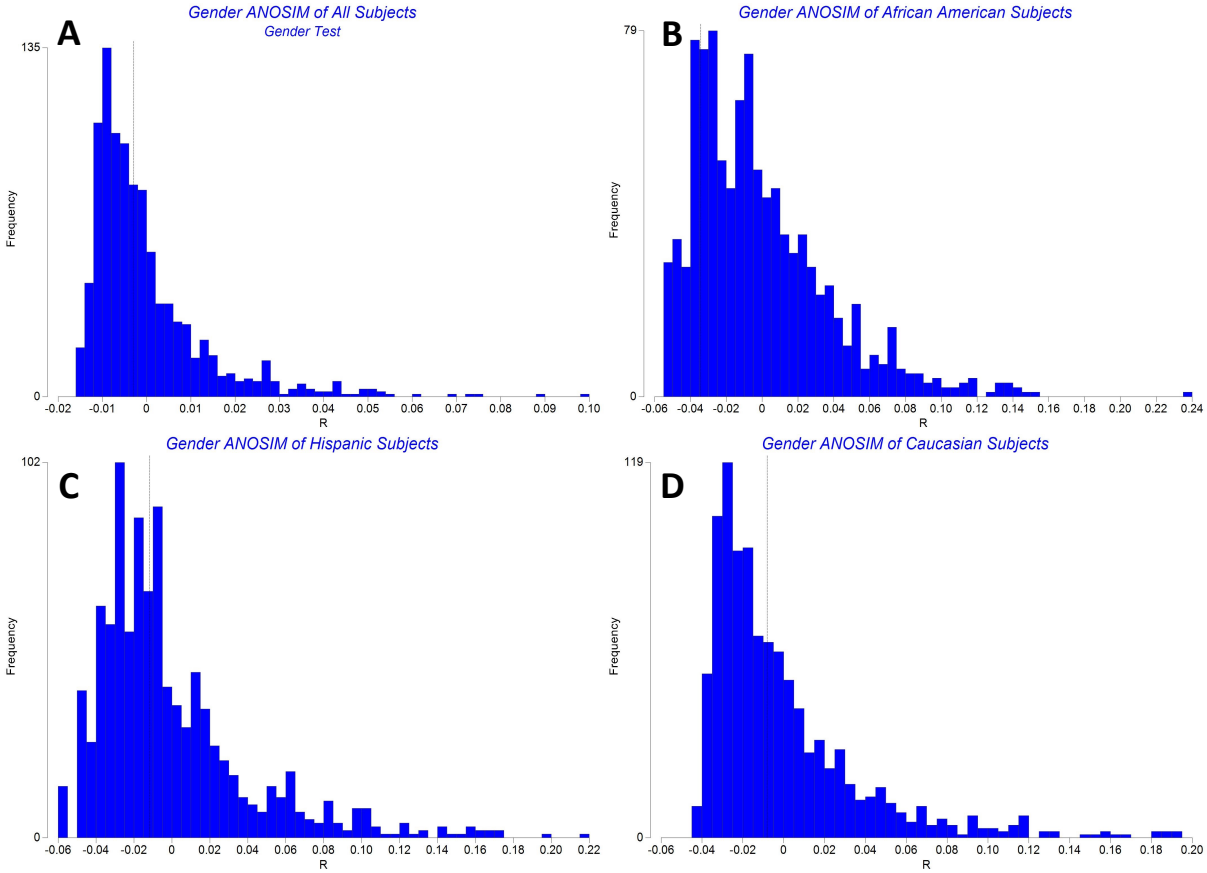

Figure 5-3: Gender ANOSIM of (A): All subjects [R:0.014] (B) African American subjects [R: -0.034] (C) Hispanic subjects [R: -0.012] (D) Caucasian Subjects [R: -0.008]

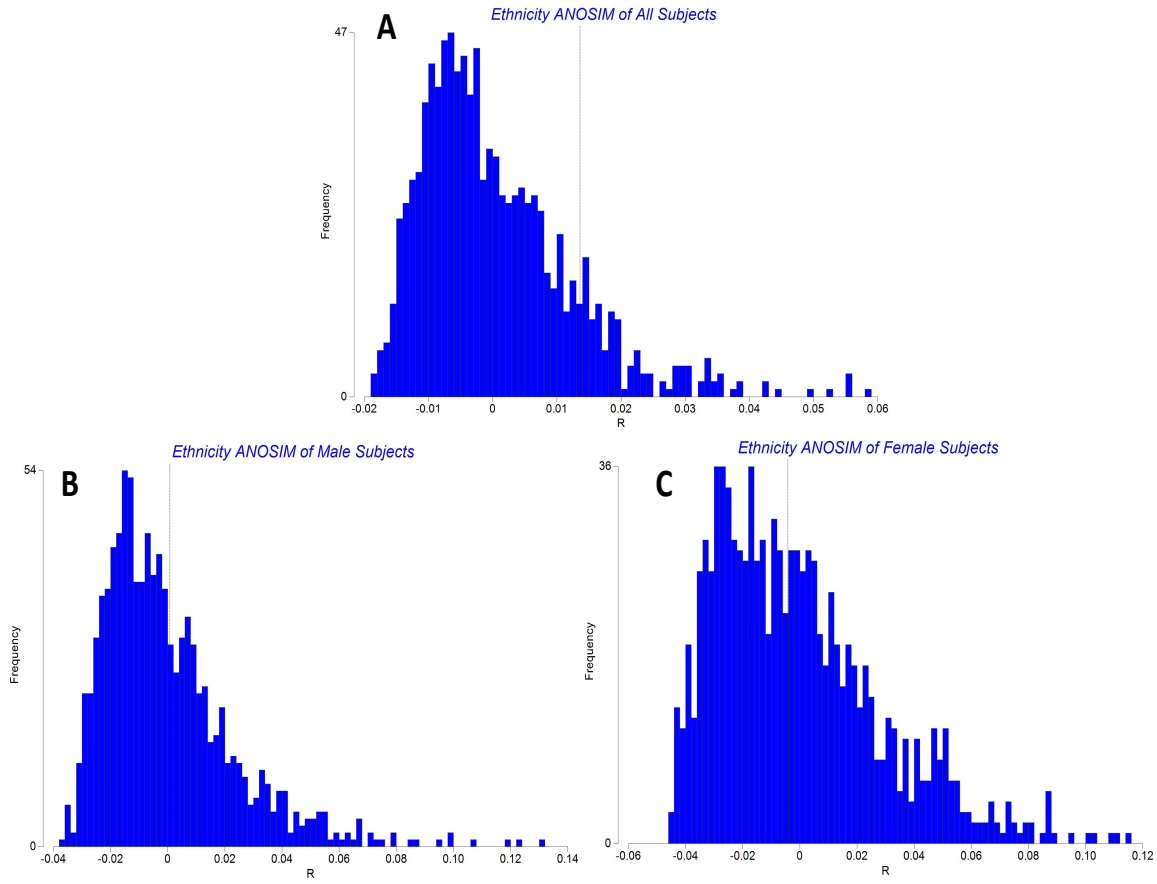

Figure 5-4: Ethnicity ANOSIM of (A) All subjects [R: -0.003] (B) Male subjects [R:0.001] (C) Female subjects [R: -0.004]

To understand the contributing species driving the distinction can be examined with SIMPER. According to SIMPER a core 24 amplicon lengths (species) were influential to the distinct differences amongst group differences as mentioned in the previous ANOSIM results (Table 8). In ethnic group comparison between AA and C subjects, species 358 held above a cumulative 60% contribution to between group differences and was not prevalent in other comparisons. This was also observed with species 365 and 374 in the AA and H subject comparison, species 339 in the H and C subject comparison, and species 367 in the F and M group comparison. The identity of these species could be discovered with the analysis of generated taxonomic data from sequencing of these amplicons with NGS.

Table 8:SIMPER analysis of cumulative contributions of most influential species in ethnicity and gender comparison

Species	Cumulative Contribution (%)			
	AA_C	AA_H	H_C	F_M
313	65.7	57.3	55.4	56.6
314	63.4	62.3	60.5	59.1
315	19.8	18.4	12.7	12.7
316	61.1	67.2	65.1	61.5
324	58.7		58.0	65.8
331		64.8	67.4	63.7
336		59.8	69.6	
339			62.8	
340	24.7	44.6	22.8	27.6
341	38.0	48.2	36.2	36.2
342	45.8	51.5	52.7	50.9
343	29.5	23.8	27.6	23.0
344	49.5	40.9	46.4	47.5
345	42.2	28.4	49.7	40.2
346	13.4	7.3	7.0	6.9
347	56.2	54.5	43.1	54.1
348	53.1	37.0	39.7	43.9
350	33.7	33.0	32.4	32.1
352	6.8	13.1	17.9	18.3
353	70.1		71.8	70.1
358	67.9			
365		69.5		
367				68.0
374		71.6		

5.4.2 16S rRNA Sequencing Abundance Profiling

With the palms surface being the source of microbial community collection for resemblance of forensic trace samples, the community abundance was expected to be low. Although, underclustering was observed in the quality control evaluation of NGS sequencing run, which often results in lesser data output; good data quality is often maintained because the camera can focus on each cluster. Operational Taxonomic Units (OTUs) and Taxonomic information generated from mothur was input into MicrobiomeAnalyst software to further summarize and compare OTUs. The OTU matrix data table (Appendix 9) was comprised of 56 samples in the columns and the corresponding 1263 OTUs in the rows. OTUs that contain zero counts within all samples or present a count in only one sample were automatically removed from further analysis in MicrobiomeAnalyst.

Prior to abundance profiling, the OTUs matrix underwent additional data filtering. This is because during the NGS process sequencing errors can occur, leading to OTUs with small counts **(Schloss et al., 2011)**. The MicrobiomeAnalyst software defaults to a minimum count of 4 OTUs; if a minimum 10% of the individual OTU does not contain the minimum 4 counts across all samples (prevalence) it was filtered out of the remainder of downstream analysis. Furthermore, a low variance filter was applied to OTUs that reflect minimal variation remaining constant throughout the matrix. After removal of 307 low abundant OTUs and 2 low variance OTUs, the remaining 9 OTUs was navigated to normalization. Although, normalization tries to address sample depth variability and data sparsity so that biologically meaningful comparisons can be made **(Weiss et al., 2017)**. No normalization was applied after filtering, due to the minute amount of OTUs remaining.

Visual exploration of the actual OTU abundance within each sample can be seen in figure 5-5. The abundance profiles showed that bacterium *Staphylococcus, Cutibacterium, Enhydrobacter, Streptococcus, Lawsonella, Fusobacterium, and Micrococcus* at the genus taxonomic level are represented, with the lowest total abundance at 3 counts for subject MC1 and the highest total abundance at 71 counts for subject MA6 and varying amounts in between for other subjects (Figure 5-5). Without statistical evaluation individuality amongst subjects can be observed.

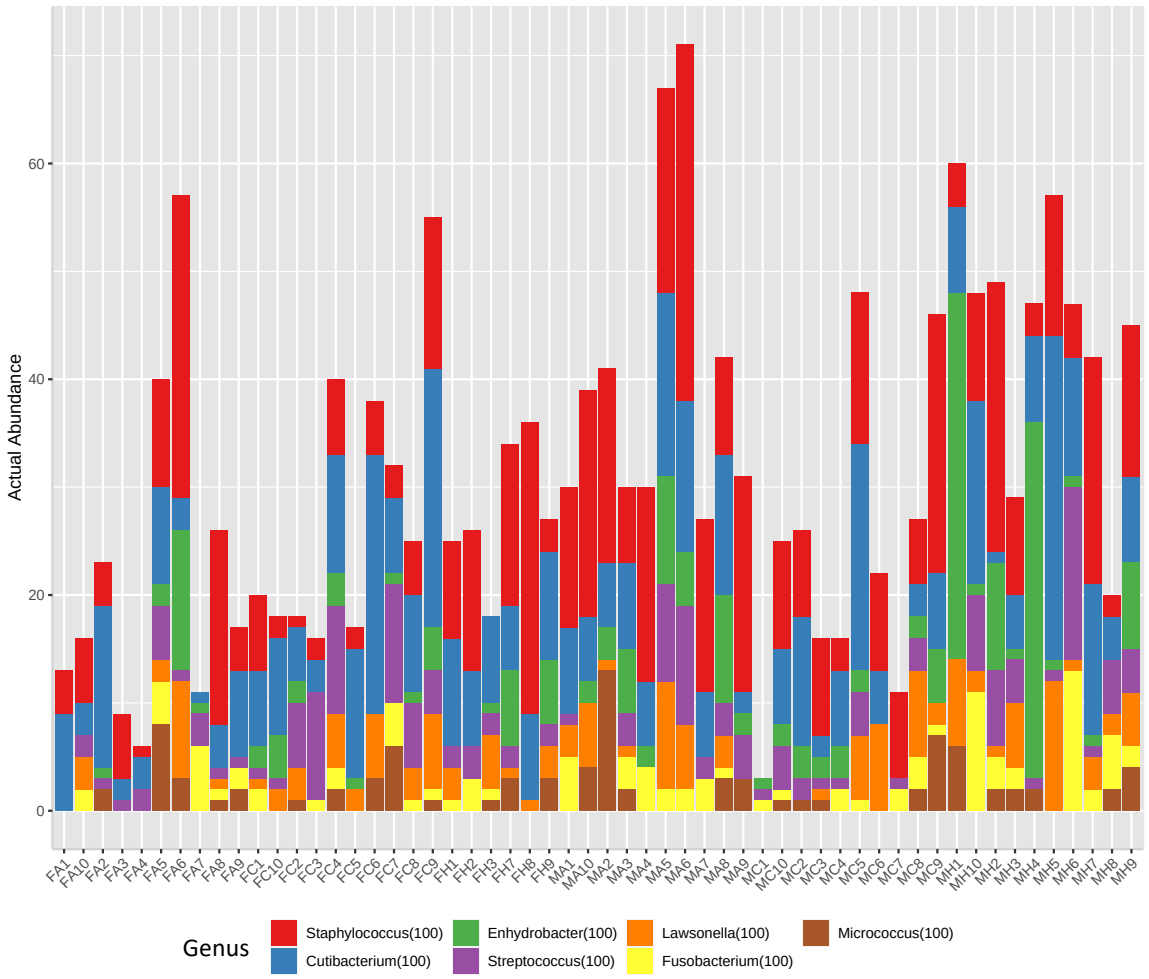

Figure 5-5:Actual abundance profiling of the genus taxonomic level of all samples

Additional observations of abundance profiles from an individual and group level with graphs oriented to compare factors gender to ethnicity (Figure 5-6) and ethnicity to gender (Figure 5-7). This revealed that male subjects of African American (AA) and Hispanic (H) ethnic groups were double that of their female counterparts (Figure 5-6B and Figure 5-7B). Whereas within Caucasian (male and female) subjects on the individual and group level their total abundances were almost even of each other.

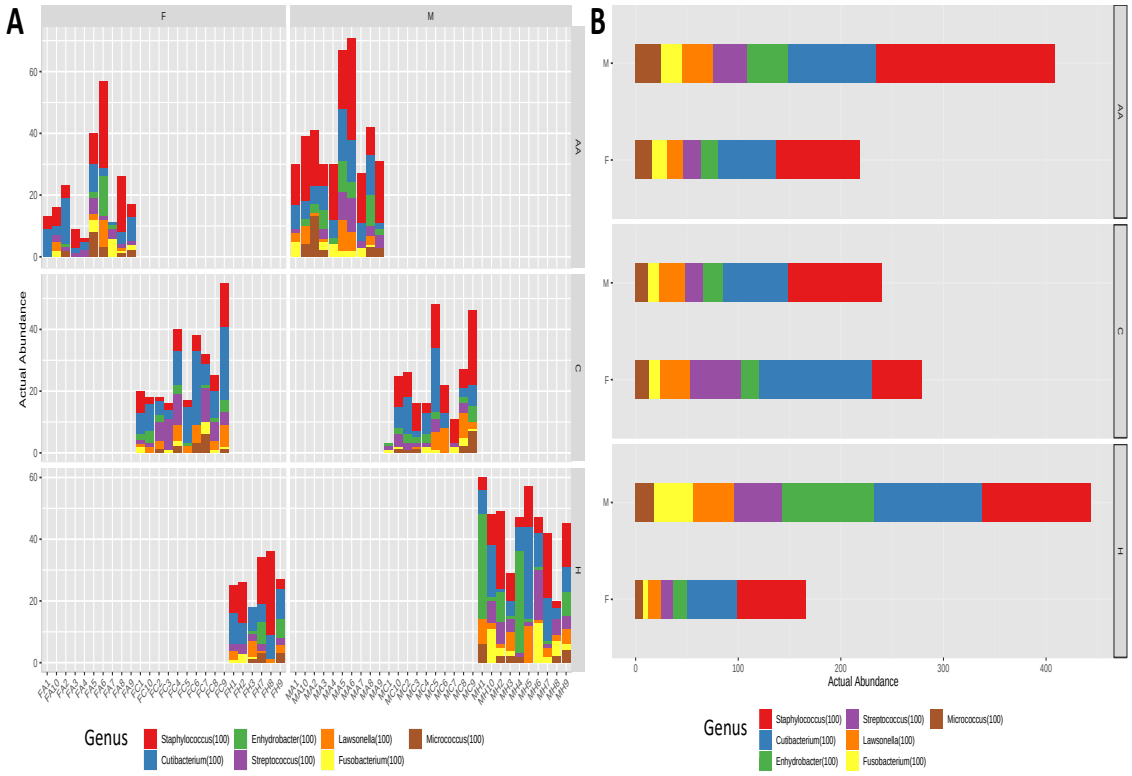

Figure 5-6: Abundance profiles of genus level comparison of gender to ethnicity of (A) Individual samples and (B) Grouped samples

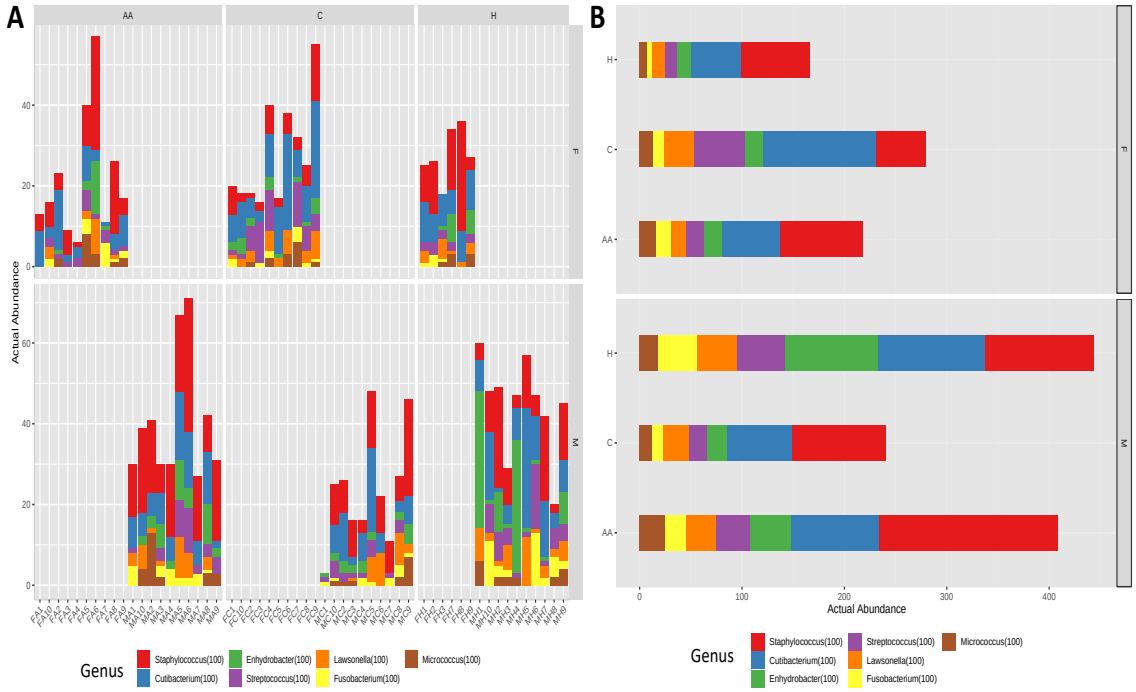

Figure 5-7: Abundance profiles of genus level comparison of ethnicity to gender of (A) Individual samples and (B) Grouped samples

The final visual exploration of abundance profiles was observed as percentages that represented each variation for grouping subjects. The bacterium *Staphylococcus* and *Cutibacterium* accounted for 59% of abundance in all subjects, with a remaining 41% being contributed by *Enhydrobacter, Streptococcus, Lawsonella, Fusobacterium, and Micrococcus* (Figure 5-8 A). A 63% abundance for Female subjects only (Figure 5-8 B) and 59% abundance for Male subjects only (Figure 5-8 C) was solely contributed by bacterium *Staphylococcus* and *Cutibacterium*. Observing abundance percentages with African American (Figure 5-8 D) and Hispanic subjects (Figure 5-8 E) *Staphylococcus* and *Cutibacterium* were still the leading contributors at 64% and 53%, respectively, with *Staphylococcus* having the largest proportion of the two. This was reversed within

Caucasian subjects as *Cutibacterium* contributed 34% of the combined 61% abundance (Figure 5-8 F). The heavy abundance of gram-positive *Staphylococcus* and *Cutibacterium* is expected because of their highly researched presence in the skin microbiome **(Barka et al., 2016; Reichmann et al., 2011).** Understanding most of the abundance being dictated by the two organisms begs for continued analysis of taxonomic information from a statistical perspective to determine if the pair or the lesser abundant bacterium discussed *Enhydrobacter, Streptococcus, Lawsonella, Fusobacterium, and Micrococcus* could have potential use as biomarkers for identification purposes.

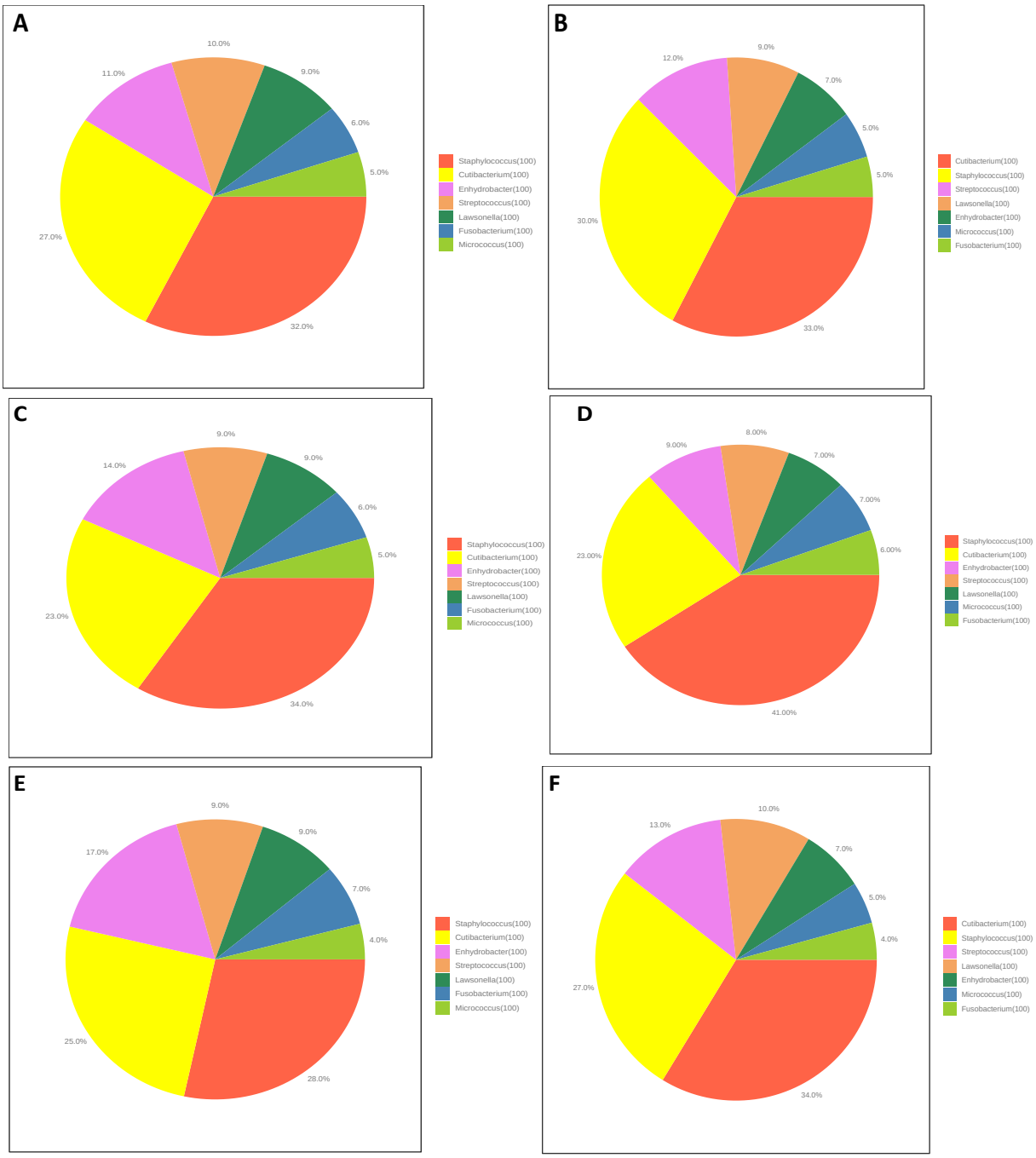

Figure 5-8: Abundance percentage profiles of (A) All subjects (B) Female subjects only (C) Male subjects (D) African American subjects (E) Hispanic subjects and (F) Caucasian subjects. Each color represents a bacterium at the genus taxonomic level.

5.4.3 Alpha diversity profiling and significance testing

Concluding abundance profiling, filtered OTUs were further evaluated with Shannon Index for community richness and diversity combined with statistical methods ANOVA for ethnicity and t-test for gender. For ANOVA the f-value statistic is inversely proportional to the p-value of the graph. Therefore, a high f-value correlates to a significant difference amongst the data set. In figures 5-9 and 5-10, samples are represented on the X-axis and their estimated diversity on the Y-axis. As well as each boxplot represents the diversity (high, median, and low) distribution of samples within the group.

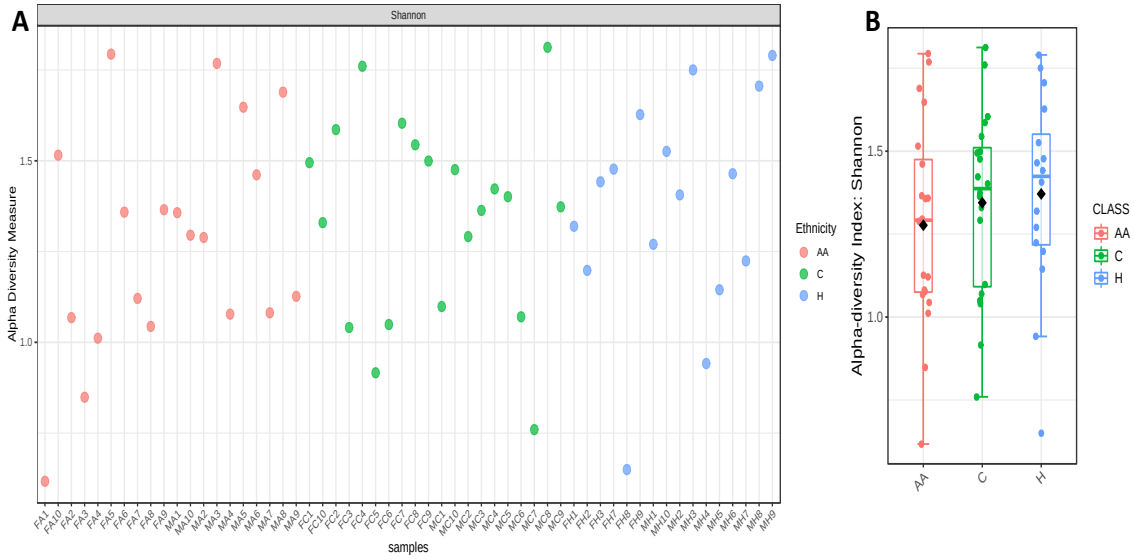

Figure 5-9: Alpha-diversity measure using Shannon (H') diversity index across (A) all subjects (B) Ethnicity groups. Each boxplot represents the diversity distribution of the group [Statistical significance: p-value: 0.61204; [ANOVA] f-value: 0.49554]

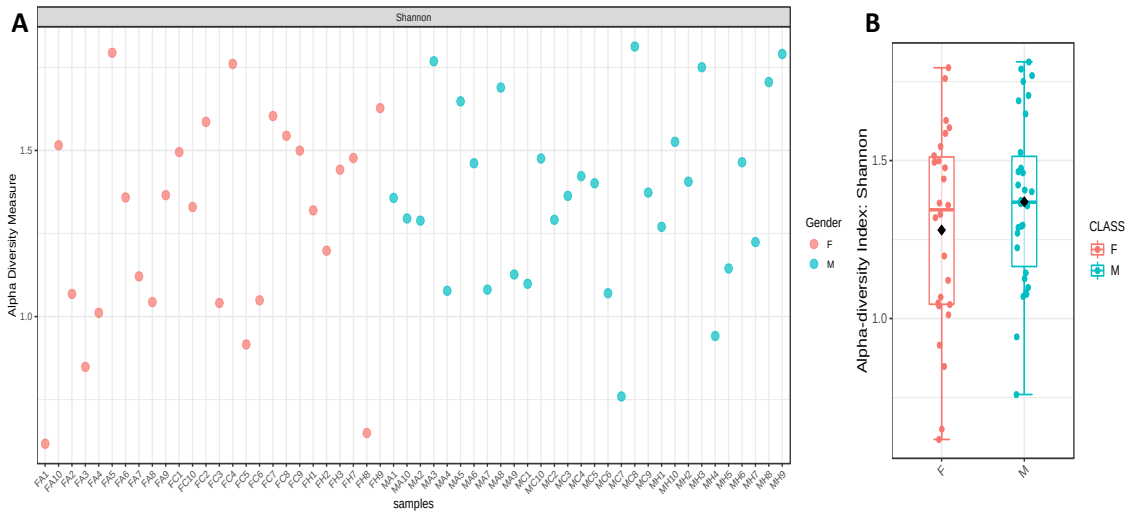

Figure 5-10: Alpha-diversity measure using Shannon (H') diversity index across (A) All subjects (B) Gender groups. Each boxplot represents the diversity distribution of the group [Statistical significance: p-value: 0.26761; [T-test] statistic: -1.1214]

The literature was examined to understand the core microbiome correlation to the production of odor (VOCs). The core microbiome—*Staphylococcus, Cutibacterium, Enhydrobacter, Streptococcus, Lawsonella, Fusobacterium, and Micrococcus* – was evaluated at the genus level with a minimum relative abundance threshold of 0.01 and prevalence of 20% (Figure 5-11). *Staphylococcus* was noted in the literature to contribute to the release of 37 VOCs comprised of aldehydes, alcohols, ketones, acids, esters, and hydrocarbons (Filipak et al., 2012). Moreover, *Cutibacterium* showed a consistent association with higher malodor and inter-bacterial competition with *Staphylococcus*, specifically during post-puberty (Lam et al., 2018). *Streptococcus* has been evaluated for its release of acetaldehyde, ethanol, acetone, and dimethyl sulfide (Allardyce et al., 2006). Furthermore, both *Enhydrobacter* and *Micrococcus* were examined for the transference of microorganisms from the skin to various textiles that produce malodor (Callewaert et al., 2014). *Lawsonella* has not been documented to be a precursor to odor (VOCs) but plays a

role in Atopic dermatitis (eczema) (Boxberger et al., 2021). Lastly, *Fusobacterium* and other bacteria release Propionic acid, valeric and isovaleric acid, butyric and isobutyric acid, and acetic acid (Kurita-Ochiai et al., 1995).

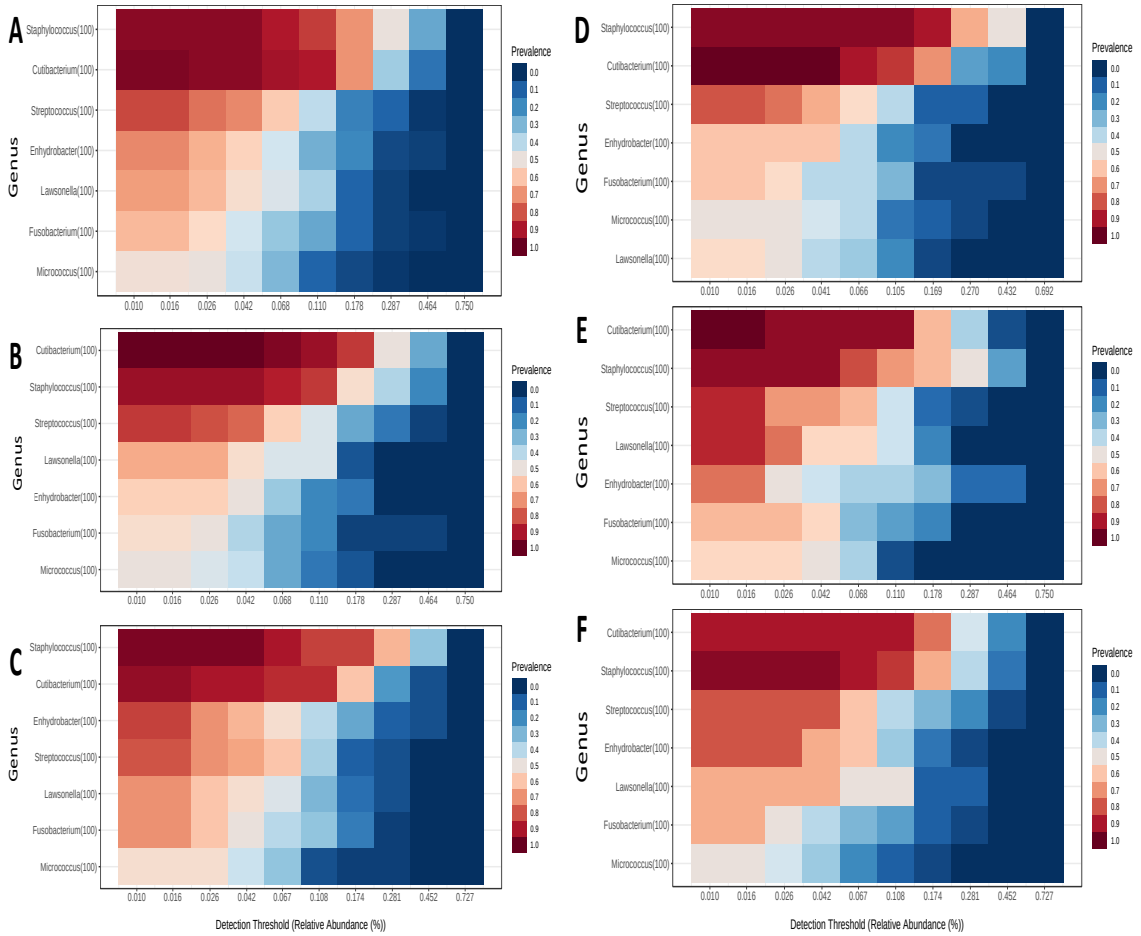

Figure 5-11: Core microbiome of (A) all samples (B) Female samples only (C) male samples (D) African American samples (E) Hispanic samples and (F) Caucasian samples on the genus taxonomic level

5.4.4 Beta diversity profiling and Random Forest

The difference in community makeup between samples is measured by beta diversity. The beta-diversity estimations obtained can be put into a distance matrix and utilized for pattern ordination. The ordination methods PCoA and NMDS calculated with Bray–Curtis dissimilarity index were used to visualize beta-diversity analysis, which was then assessed using ANOSIM. PCoA maximizes sample-to-sample linear correlation, whereas NMDS maximizes sample-to-sample rank-order correlation **(Ramette, 2007)**. The R and p-values of each plot suggests that there are no significant differences between sample diversity from each factor observed. In addition, the PCoA and NMDS ordination of the data was not clearly defined into two clusters or three clusters for gender and ethnicity analysis, respectively (Figure 5-12 B-C and Figure 5-13 B-C). Although, the gender PCoA and NMDS plots showcased a p-value < 0.041 and R-value 0.053207; along with a stress value greater then 0.2, determined the plots were poorly fit. Deeper analysis is required to understand where the true between sample differences may lie. Often when classification and identification of specific taxa may contribute to this understanding, machine learning techniques such as Random Forest are implemented.

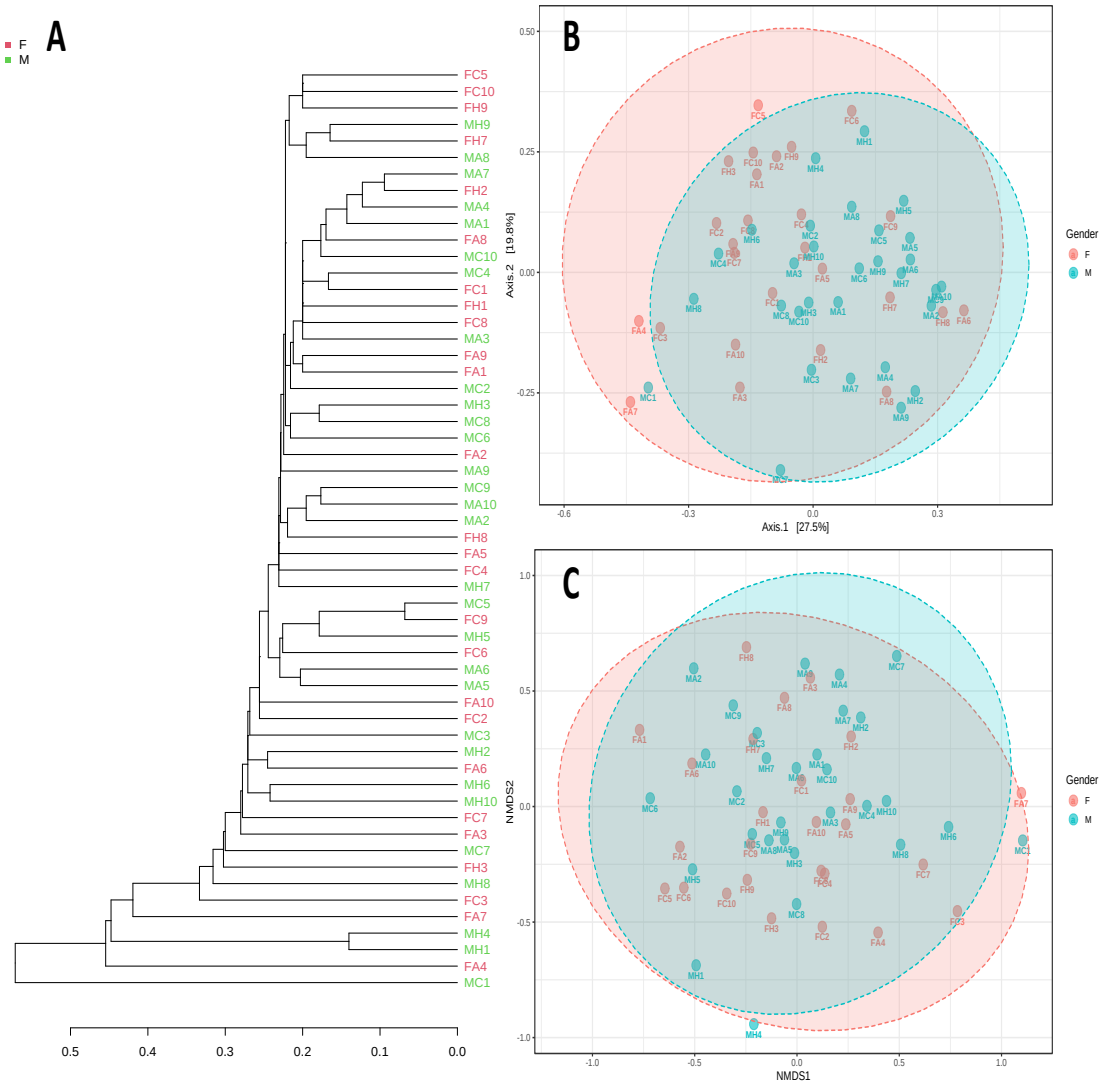

Figure 5-12: Beta diversity gender (A) Dendrogram (B) PCoA [R:0.053207, p-value<0.041] and (C) NMDS plot [R:0.010358, p-value<0.299, stress = 0.21358]

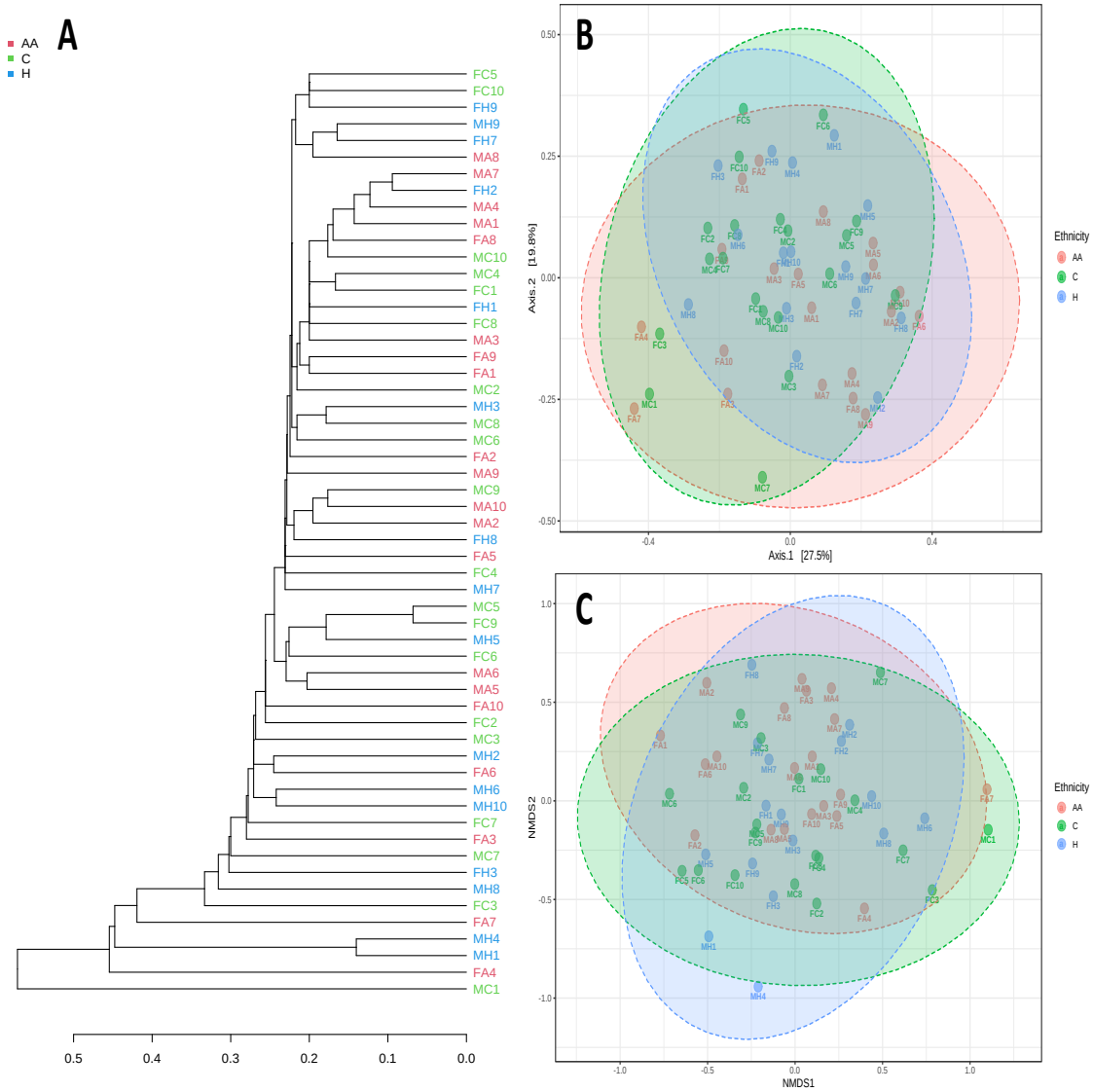

Figure 5-13: Beta diversity ethnicity (A) Dendrogram (B) PCoA [R:0.053207, p-value<0.042] and (C) NMDS plot [R:0.010358, p-value<0.304, stress = 0.21358]

Random Forest was executed to further assess if the microbial data could be observed for classification prediction and potential identification of contributing taxa. Because it can discover non-linear correlations, deal with unpredictable interactions, and can be resistant to overfitting, Random Forest is well suited for huge and noisy data like those from the microbiome **(Touw et al., 2013)**. To test the accuracy of classification, 1/3 of the samples are excluded during tree construction and then categorized using the models to calculate the out-of-bag (OOB) error rates **(Chong et al., 2020)**. When a variable is shuffled, the mean decrease in accuracy across all trees is used to determine its relevance. Out of the two random forest algorithms conducted, the gender factor analysis retained the lowest OOB error rate (42.9%) compared to the ethnicity random forest analysis OOB error rate (58.9%) (Figure 5-14 and Figure 5-15). However, the removal of outliers or increase to the amount of decision trees could potentially decrease this error and provide a better classification of individuals.

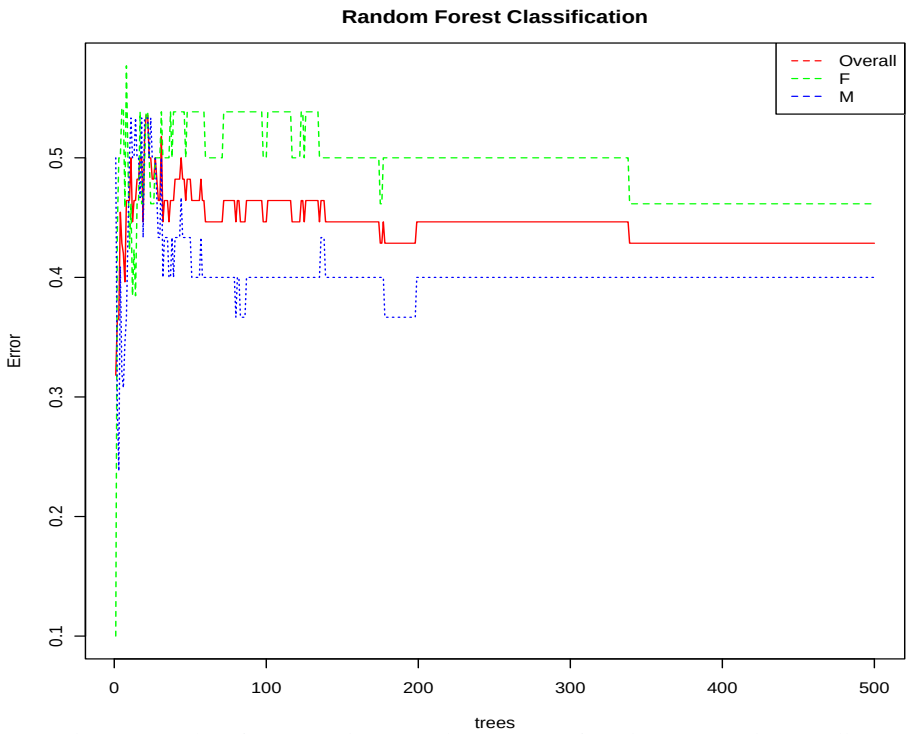

Figure 5-14: Random Forest classification with 500 prediction trees of gender groups. The overall error was 42.9%.

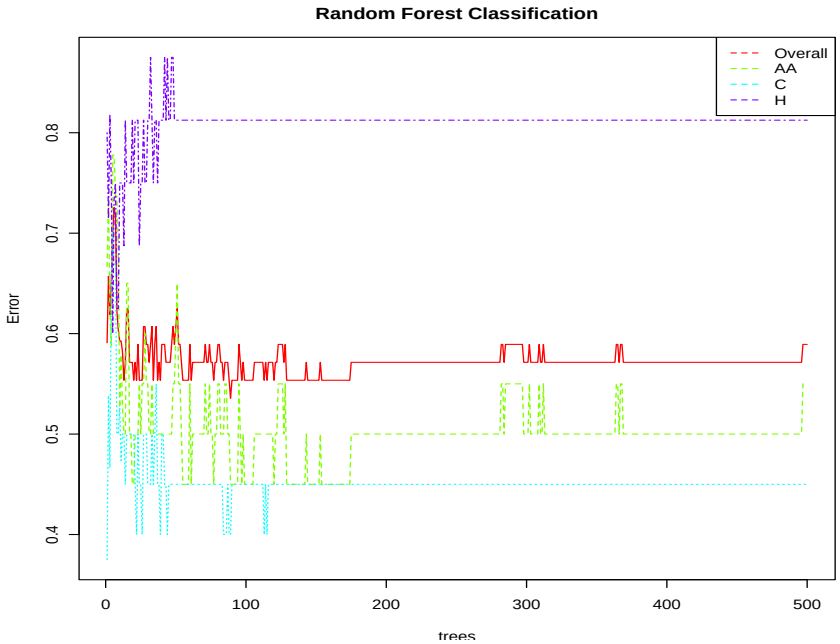

Figure 5-15: Random Forest classification with 500 prediction trees of ethnic groups. The overall error was 58.9%.

5.5 Conclusion

In conclusion, the evaluation of the palms surface microbiome for identification purposes was observed with capillary electrophoresis (LHPCR) technologies and massively parallel sequencing (NGS) technologies. The advantages and disadvantages of both LHPCR and NGS were showcased throughout this study. LHPCR while has the advantage of a quick efficient screening of community diversity, it does not have the resolution to provide the taxonomic information required for identifying the driving forces behind differences amongst subjects. Utilizing the DNA amplicon lengths to screen for diversity of the microbiome led to ANOSIM results that specified there was an even distribution of high and low dissimilarities within a group and between groups. Additional SIMPER analysis provided us with an idea of species that were influential in the deciphering of groups when compared against each other via ethnicity or gender. NGS on the other hand has the great advantage of providing resolution down to the species level, the redundancy of certain OTUs can hinder meaningful downstream analysis. The amplified DNA of the V1V2 16S rRNA was sequenced. A total of 5055 paired end sequences were processed via Mothur to generate specific OTUs and taxonomic information from the Kingdom to genus level. The initial visualization of abundance profiles for samples individually and grouped led to understanding the core microbiome present. The recognized core bacterium *Staphylococcus, Cutibacterium, Enhydrobacter, Streptococcus, Lawsonella, Fusobacterium, and Micrococcus* are known for their commensal and beneficial influence on the skin. Although alpha and beta-diversity could not implicate distinct difference among various analysis factors, the ANOSIM values continued the message provided during fragment analysis that there was an even

distribution of high and low dissimilarities within a group and between groups. The classification and identification of Taxa were explored with machine learning algorithm Random Forest, which indicated that it could correctly predict gender with a 42.9% error rate in comparison to ethnicity at 58.9%. The identification of LHPCR amplicon lengths specified in SIMPER and deeper evaluation of the core bacterium provide from NGS could lead to potential biomarkers in the use of forensic human identification.

6 OVERALL CONCLUSIONS

Our biological indicators, such as chromosomes (sex), DNA, and fingerprints, are all characteristic of an individual and distinctively differ from other individuals. These factors can be observed through various scientific methodologies. More importantly, the use and identification of current biometrics have laid the foundation upon which forensic human identification is built. Although these biometrics have become well-established and optimized for forensic analysis, their trace or degraded presence at a crime scene has become a bane of the forensic community.

Over the last 20 years, there has been an overwhelming accumulation of scientific literature on the application of human odor, both chemically and biologically. Within the health field, it has been evaluated as a biomarker for deciphering between a healthy individual's odor profile and the odor profile of an unhealthy (cancer, seizures, COVID-19, etc.) individual. In the field of pathology, research has been garnered to understand the various volatile organic compounds (VOCs) that contribute to the attraction or repelling of mosquitoes. Our mammalian counterparts, canines, are forensically trained on VOCs for the tracking/trailing of missing/fleeing individuals or the identification (alert) of explosives and drugs. Our lab has been a part of the forefront in establishing human odor as an additional biometric for forensic application in human identification. The works of Dr. Furton, Brown, Curran, Prada, Colón-Crespo, Caraballo and DeGreff have made this a reality by optimizing the protocols and methodologies that evaluate the stability, reproducibility, and individuality of human odor. However, these methods reveal the gap of developing practical models for input of an unknown sample to describe the potential class characteristics the odor profile may display. The capabilities to develop supervised

models as a tool for the characterization and discrimination of gender and ethnicity have been underrepresented through visual representation of VOCs with stacked bar graphs and unsupervised statistical applications such as principal component analysis (PCA).

This study approached the evaluation of hand odor profiles for forensic identification through a cross-disciplinary scope. The chemical and biological examination of hand odor VOCs has subsequently been analyzed individually with the understanding that microbiota is a precursor to odor production, but the compilation of the two has been limited. The VOCs collected and extracted from the palm's surface were detected via HS-SPME-GCMS. The chemometric data was processed with statistical software, MetaboAnalyst and JMP, to showcase a practical tool for discrimination and prediction of gender.

The collected and extracted bacterial DNA was processed through fragment analysis (LHPCR) and NGS. The microbiome data was evaluated with multiple open-format coding software. The fragment analysis of DNA amplicons' relative abundance ratios was visualized and analyzed in Thermo Fisher Connect™ Microsatellite Analysis and Primer-E v7. NGS sequencing reads were processed in Mothur to generate OTU matrix and Taxonomic information table, which was processed for alpha and beta diversity in MicrobiomeAnalyst. The fragment analysis data was interpreted with Bray-Curtis dissimilarity, providing nMDS plots that illustrated clustering amongst all ethnic groups regardless of gender with minor outliers. The ANOSIM statistics determined that differences among ethnicities were evenly distributed within and between groups. The SIMPER analysis indicated the amplicon lengths that drove the differences amongst these two factors.

The initial foundation of utilizing scientific methodologies to resolve civil and criminal disputes in forensics has exploded with advancing technologies being developed for the analysis of trace, biological, organic, and inorganic samples. The techniques, protocols, and instrumentation optimized for those purposes have begun to integrate other areas of science (microbiology, ecology, pathology, etc.). The discriminatory power of human odor and its efficacy for forensic identification purposes was explored throughout this study. From a bioinformatic evaluation of volatiles and microbiota, VOCs have the ability to not only cluster male and female subjects but discriminate from a trained algorithm. Microbiota community screening and alpha and beta-diversity evaluation of OTU taxonomic information revealed core bacterium among all individuals—*Staphylococcus, Cutibacterium, Enhydrobacter, Streptococcus, Lawsonella, Fusobacterium, and Micrococcus.* These bacterium are notated in literature to be precursors to the production of human odor recognized on the palms of the hand. The core microbiome community could have potential use in identification of race or ethnicity.

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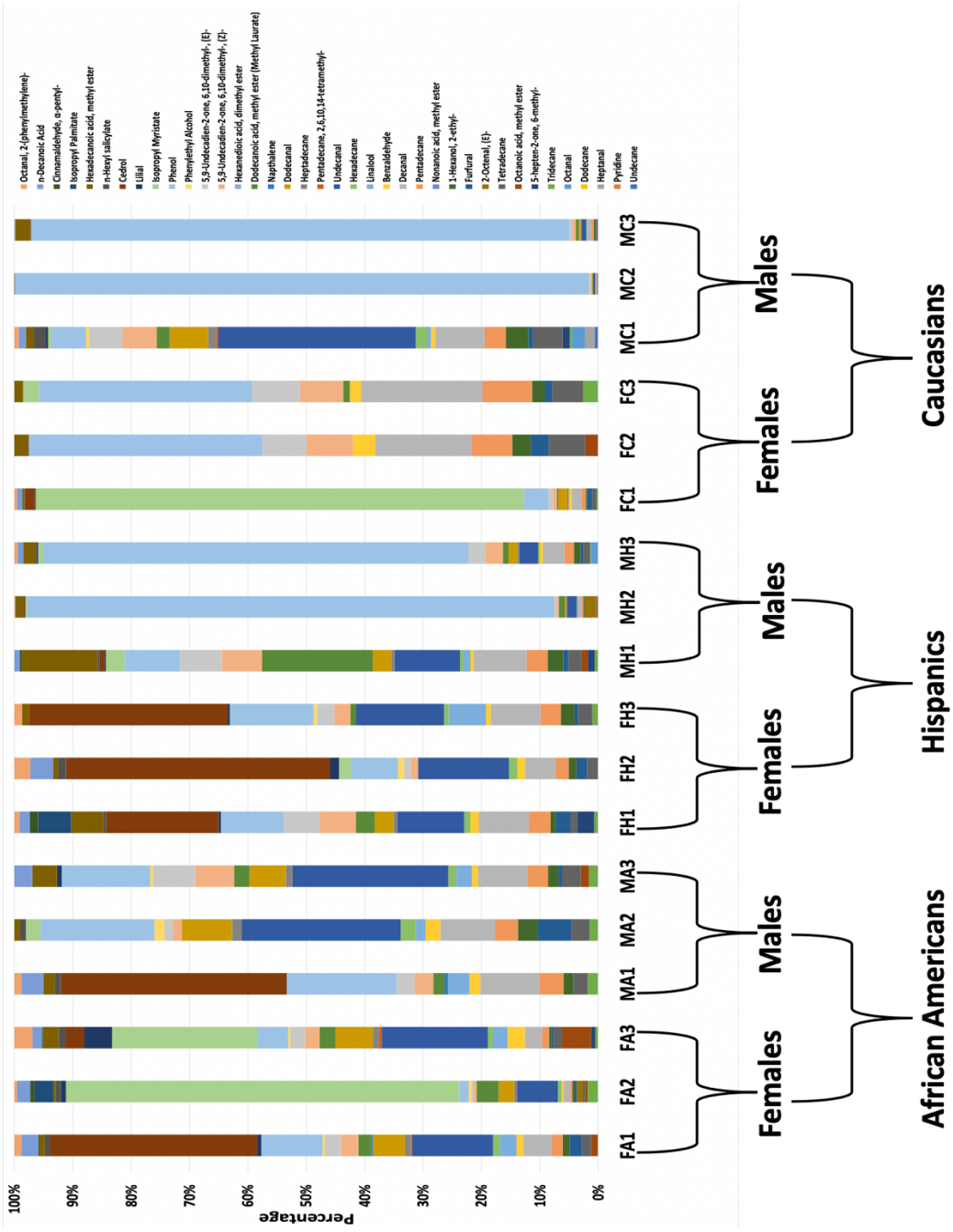
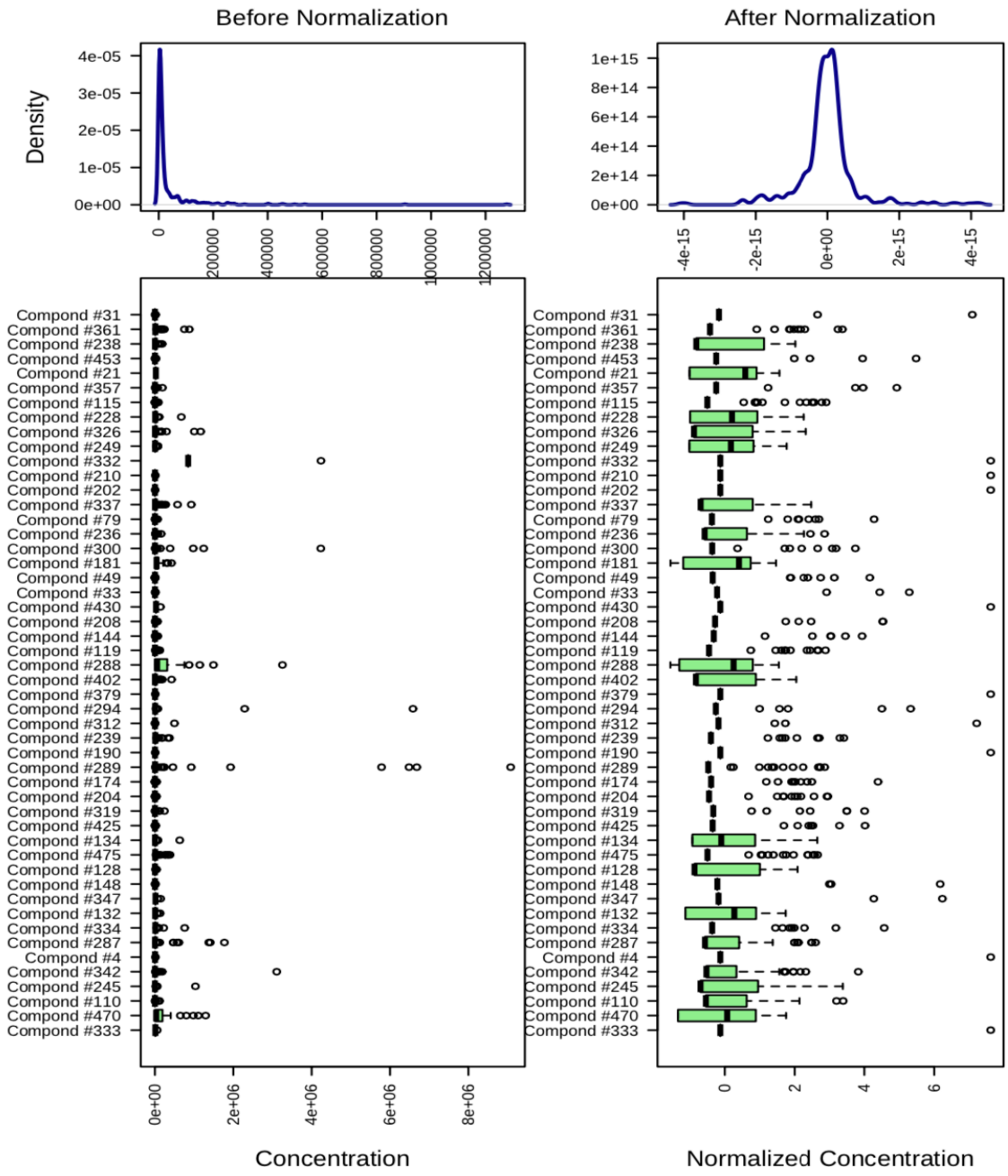
APPENDICES

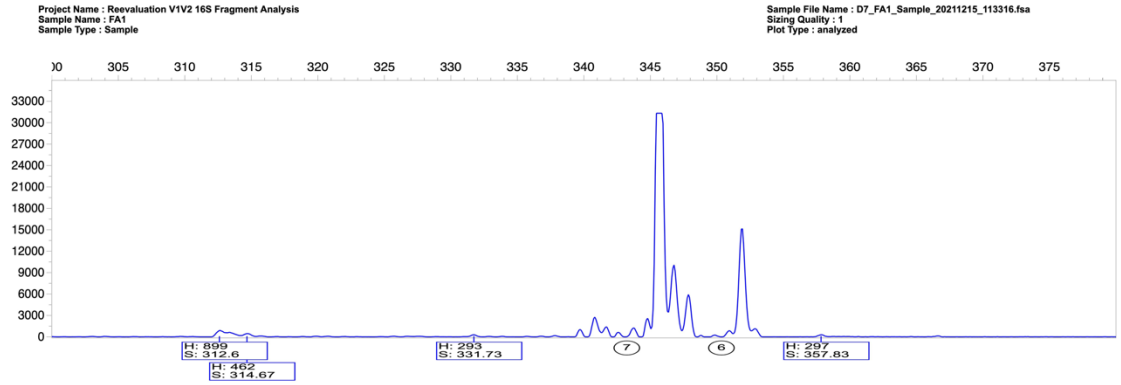
Appendix 1: A visual representation of quanti individual differences amongst a subset of sampled subjects (9 female and 9 male subjects from each ethnicity) when the abundance of compounds present in standard mixture are quantified.

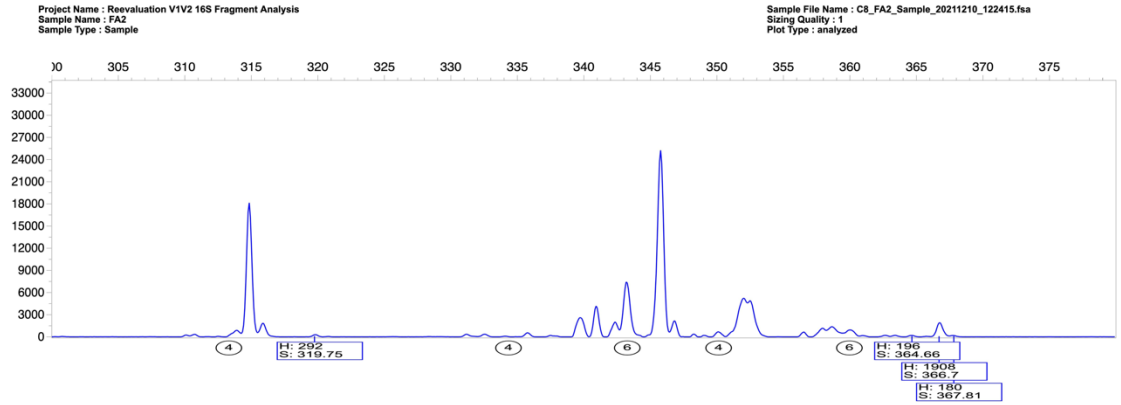


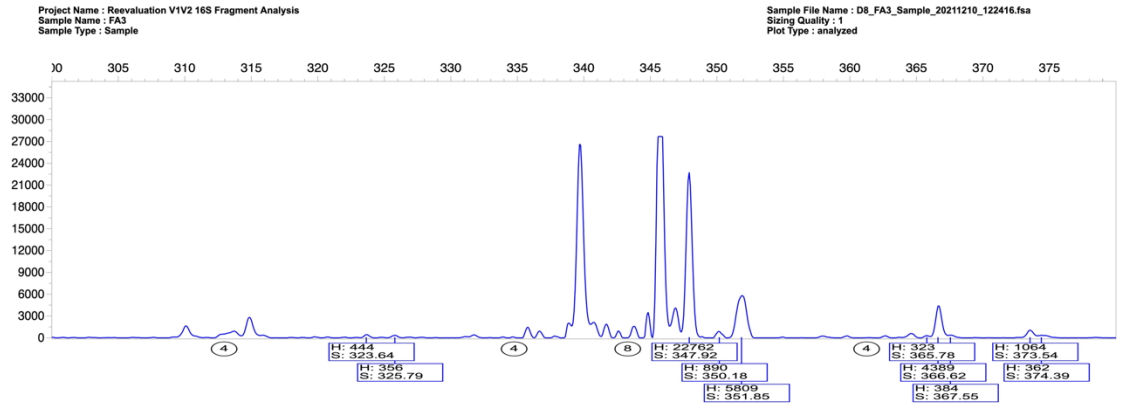
Appendix 2: Row-Wise Normalization and Log_{10} Transformation of Compound Peak Areas

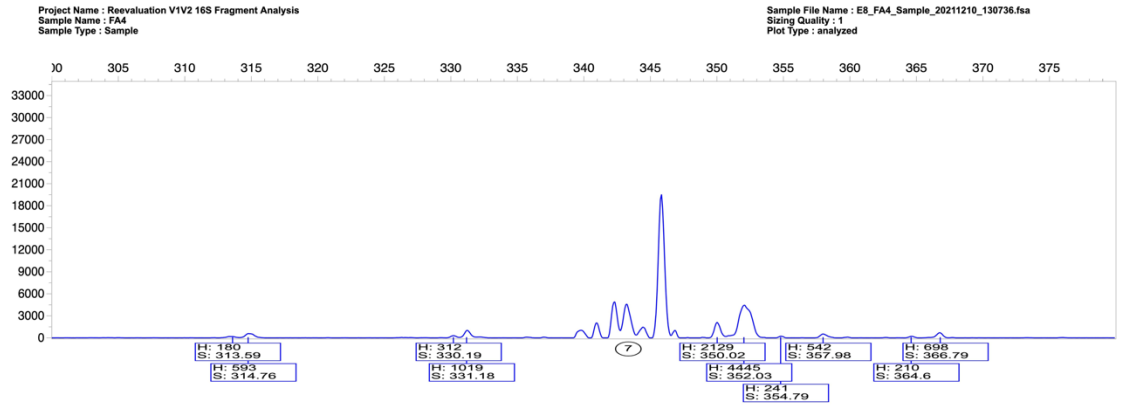


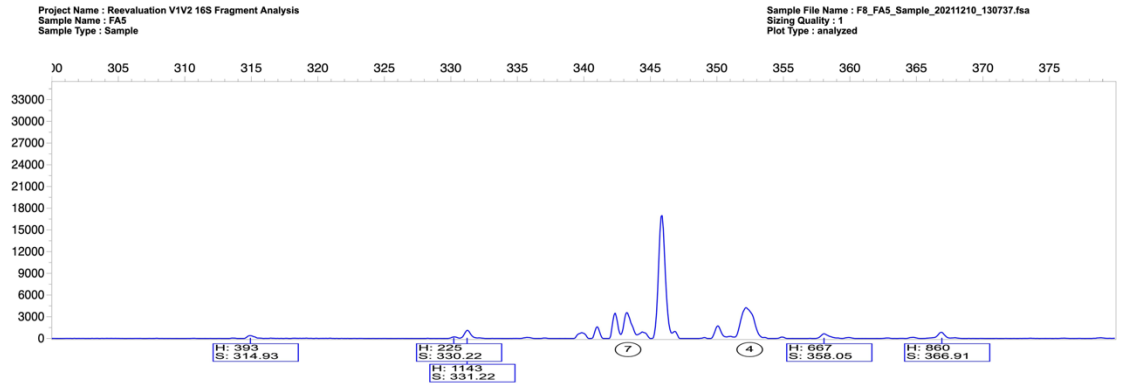
Appendix 3: Fragment Analysis (LHPCR) Plots of African American Female Subjects

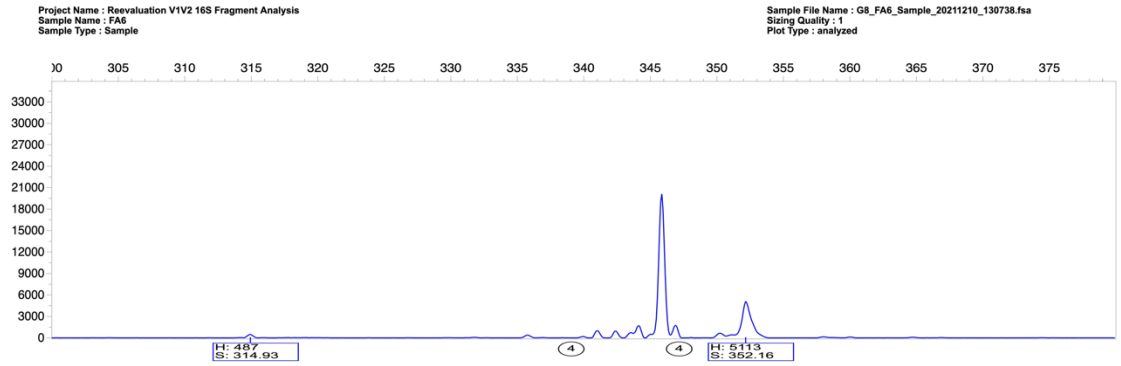


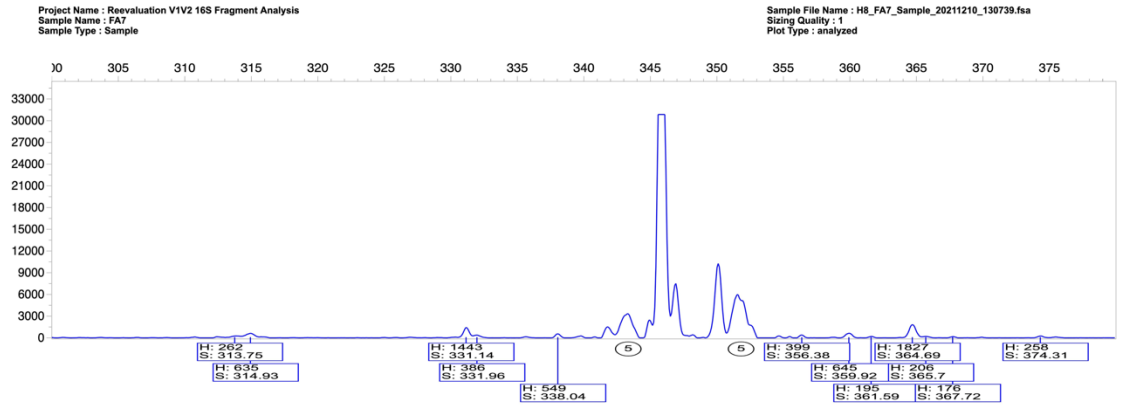


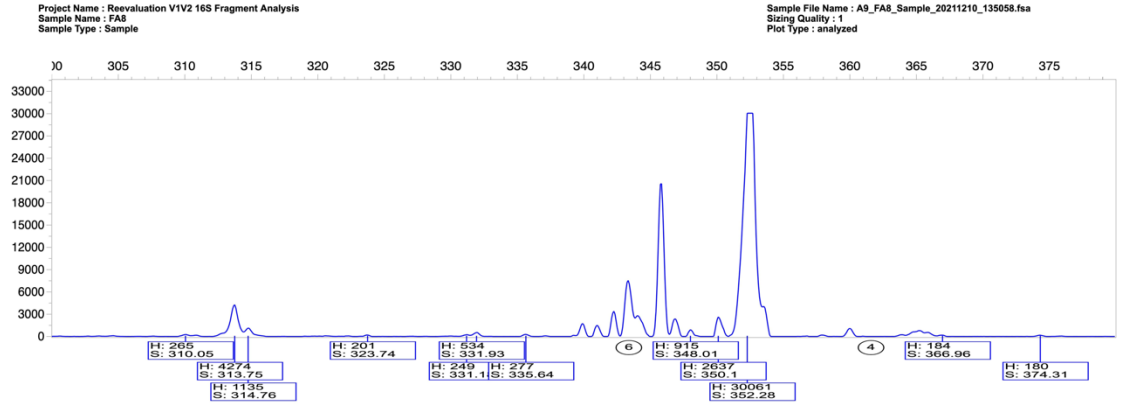


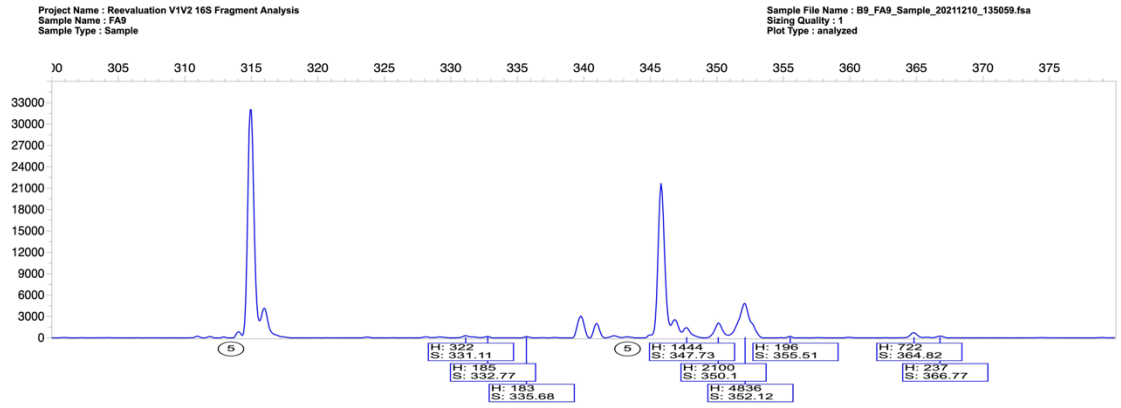

Project Name : Reevaluation V1V2 16S Fragment Analysis
Sample Name : FA4
Sample Type : Sample
Sample File Name : E8_FA4_Sample_20211210_130736.fsa
Sizing Quality : 1
Plot Type : analyzed
H: 180
S: 313.59
H: 503
S: 314.76
H: 312
S: 330.19
H: 1019
S: 331.18
H: 2129
S: 350.02
H: 4445
S: 352.03
H: 241
S: 354.79
H: 542
S: 357.98
H: 210
S: 364.6
H: 698
S: 366.79

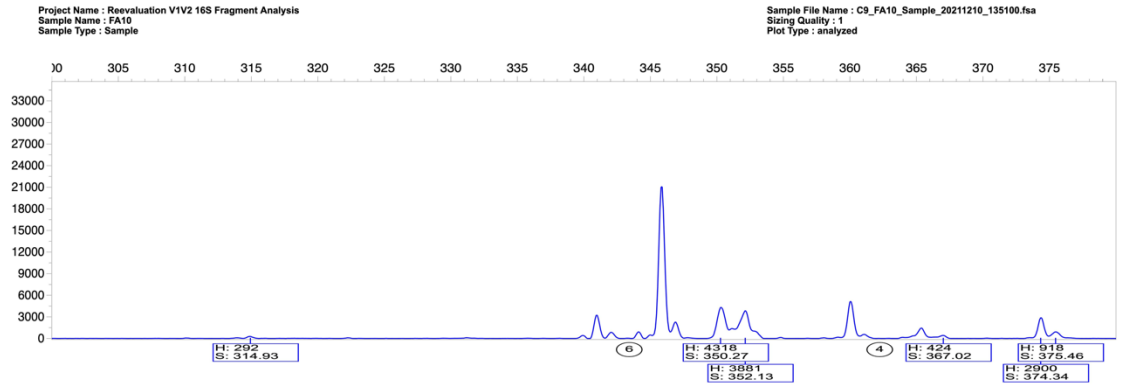

Project Name : Reevaluation V1V2 16S Fragment Analysis
Sample Name : FA5
Sample Type : Sample
Sample File Name : F8_FA5_Sample_20211210_130737.fsa
Sizing Quality : 1
Plot Type : analyzed
H: 393
S: 314.93
H: 225
S: 330.22
H: 1143
S: 331.22
H: 667
S: 358.05
H: 860
S: 366.91


Project Name : Reevaluation V1V2 16S Fragment Analysis
Sample Name : FA6
Sample Type : Sample
Sample File Name : G8_FA6_Sample_20211210_130738.fsa
Sizing Quality : 1
Plot Type : analyzed
H: 487
S: 314.93
H: 5113
S: 352.16

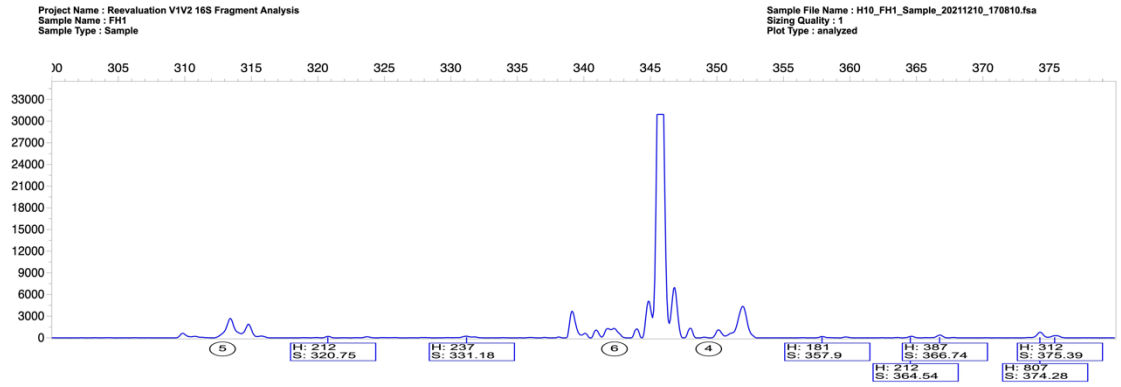

Project Name : Reevaluation V1V2 16S Fragment Analysis
Sample Name : FA7
Sample Type : Sample
Sample File Name : H8_FA7_Sample_20211210_130739.fsa
Sizing Quality : 1
Plot Type : analyzed
H: 262 S: 313.75
H: 635 S: 314.93
H: 1443 S: 331.14
H: 386 S: 331.96
H: 549 S: 338.04
H: 399 S: 356.38
H: 645 S: 359.92
H: 195 S: 361.59
H: 1827 S: 364.69
H: 206 S: 365.7
H: 176 S: 367.72
H: 258 S: 374.31

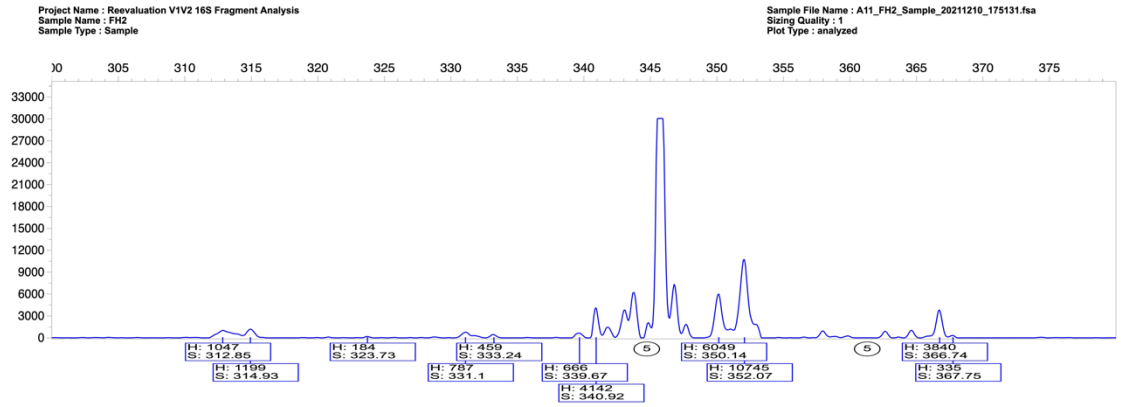

Project Name : Reevaluation V1V2 16S Fragment Analysis
Sample Name : FA8
Sample Type : Sample
Sample File Name : A9_FA8_Sample_20211210_135058.fsa
Sizing Quality : 1
Plot Type : analyzed
H: 265 S: 310.05
H: 4274 S: 313.75
H: 1135 S: 314.76
H: 201 S: 323.74
H: 534 S: 331.93
H: 249 S: 331.1
H: 277 S: 335.64
H: 915 S: 348.01
H: 2637 S: 350.1
H: 30061 S: 352.28
H: 184 S: 366.96
H: 180 S: 374.31

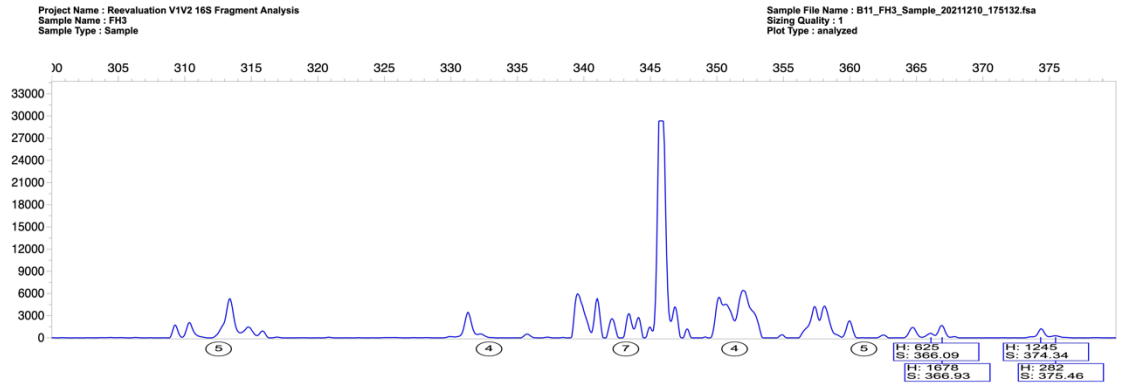

Project Name : Reevaluation V1V2 16S Fragment Analysis
Sample Name : FA9
Sample Type : Sample
Sample File Name : B9_FA9_Sample_20211210_135059.fsa
Sizing Quality : 1
Plot Type : analyzed
305 310 315 320 325 330 335 340 345 350 355 360 365 370 375
33000 30000 27000 24000 21000 18000 15000 12000 9000 6000 3000 0
5
H: 322
S: 331.11
H: 185
S: 332.77
H: 183
S: 335.68
5
H: 1444
S: 347.73
H: 196
S: 355.51
H: 2100
S: 350.1
H: 4836
S: 352.12
H: 722
S: 364.82
H: 237
S: 366.77

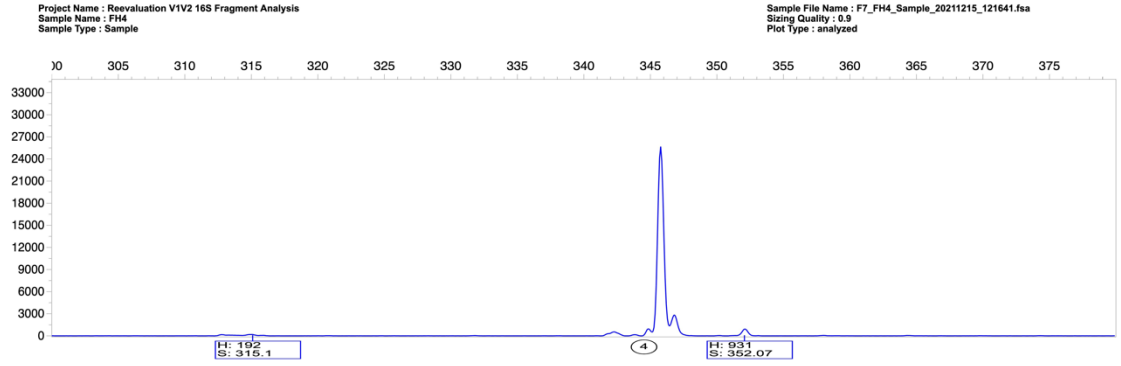

Project Name : Reevaluation V1V2 16S Fragment Analysis
Sample Name : FA10
Sample Type : Sample
Sample File Name : C9_FA10_Sample_20211210_135100.fsa
Sizing Quality : 1
Plot Type : analyzed
305 310 315 320 325 330 335 340 345 350 355 360 365 370 375
33000 30000 27000 24000 21000 18000 15000 12000 9000 6000 3000 0
H: 292
S: 314.93
6
H: 4318
S: 350.27
H: 3881
S: 352.13
4
H: 424
S: 367.02
H: 918
S: 375.46
H: 2900
S: 374.34

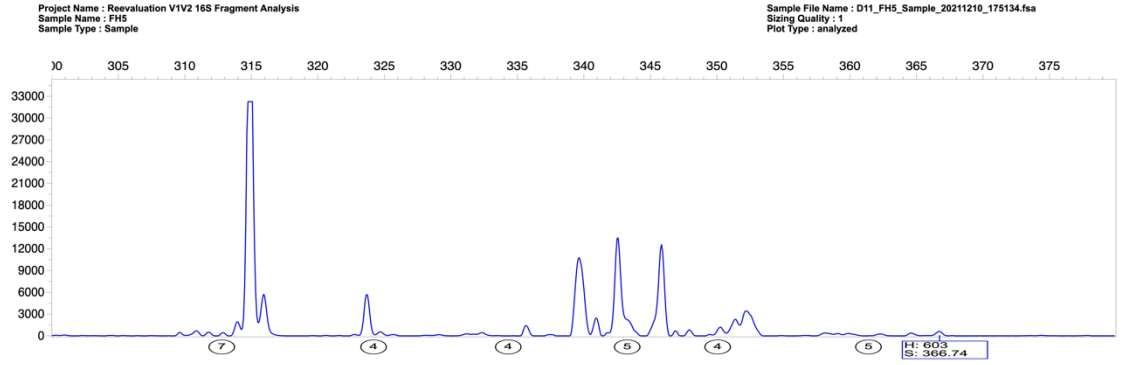
Appendix 4: Fragment Analysis (LHPCR) Plots of Hispanic Female Subjects

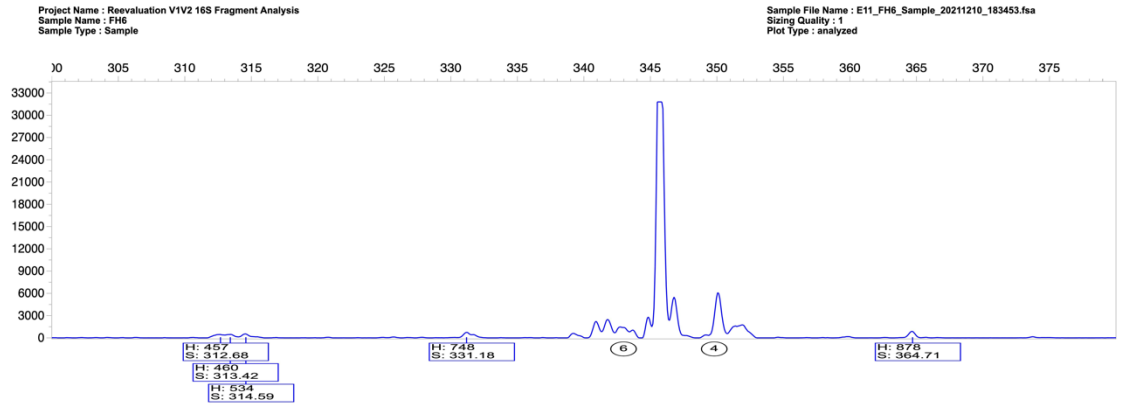


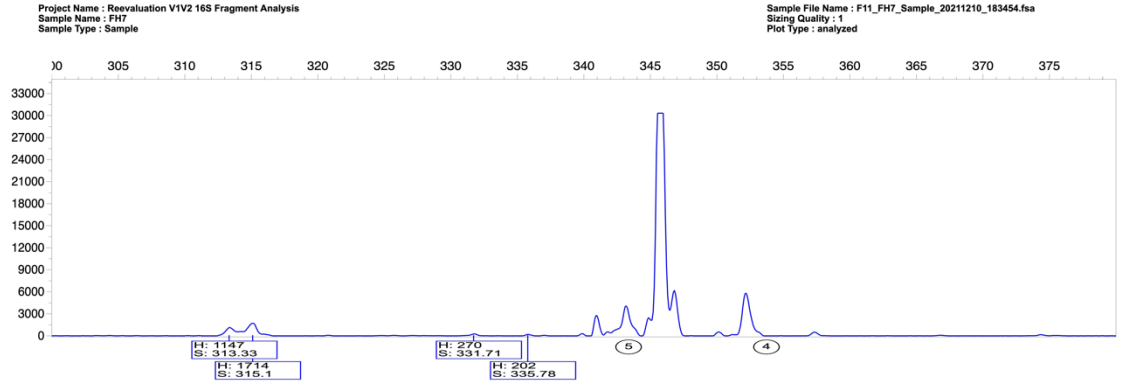


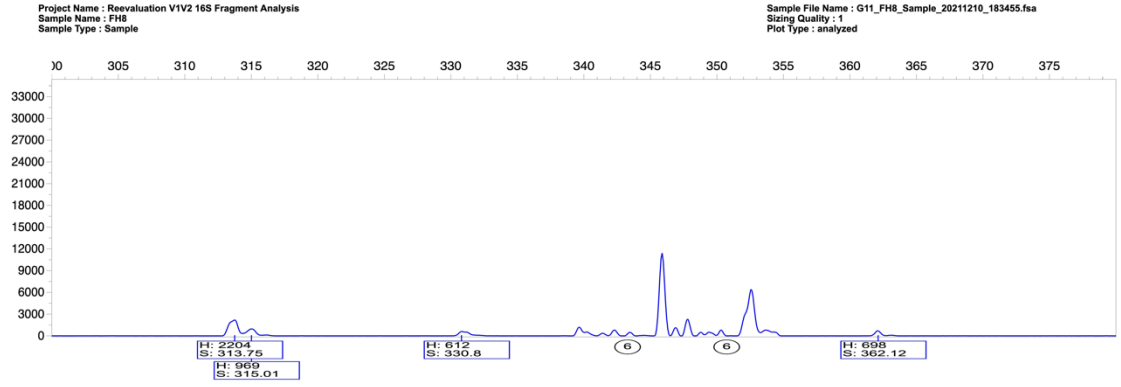

Project Name : Reevaluation V1V2 16S Fragment Analysis
Sample Name : FH3
Sample Type : Sample
Sample File Name : B11_FH3_Sample_20211210_175132.fsa
Sizing Quality : 1
Plot Type : analyzed
H: 625
S: 366.09
H: 1245
S: 374.34
H: 1678
S: 366.93
H: 282
S: 375.46

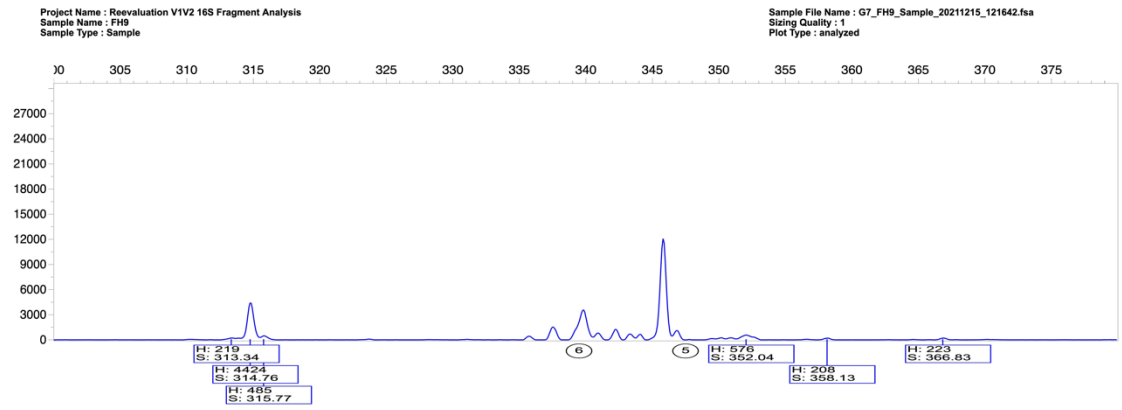

Project Name : Reevaluation V1V2 16S Fragment Analysis
Sample Name : FH4
Sample Type : Sample
Sample File Name : F7_FH4_Sample_20211215_121641.fsa
Sizing Quality : 0.9
Plot Type : analyzed
H: 192
S: 315.1
H: 931
S: 352.07


Project Name : Reevaluation V1V2 16S Fragment Analysis
Sample Name : FH5
Sample Type : Sample
Sample File Name : D11_FH5_Sample_20211210_175134.fsa
Sizing Quality : 1
Plot Type : analyzed
H: 603
S: 366.74

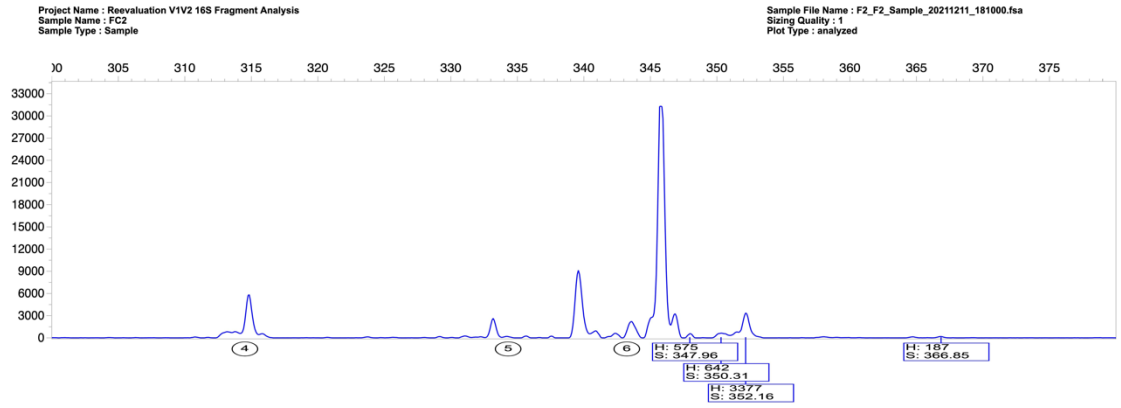

Project Name : Reevaluation V1V2 16S Fragment Analysis
Sample Name : FH6
Sample Type : Sample
Sample File Name : E11_FH6_Sample_20211210_183453.fsa
Sizing Quality : 1
Plot Type : analyzed
H: 457
S: 312.68
H: 460
S: 313.42
H: 534
S: 314.59
H: 748
S: 331.18
H: 878
S: 364.71

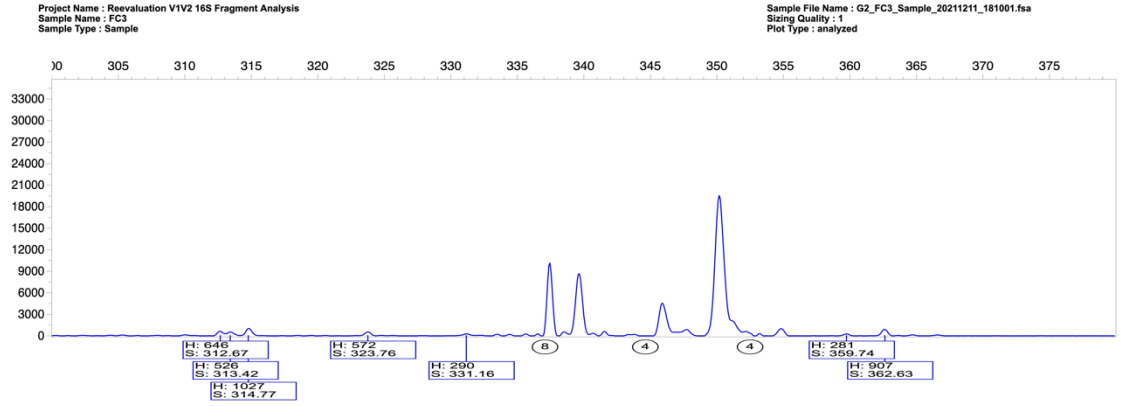

Project Name : Reevaluation V1V2 16S Fragment Analysis
Sample Name : FH7
Sample Type : Sample
Sample File Name : F11_FH7_Sample_20211210_183454.fsa
Sizing Quality : 1
Plot Type : analyzed
H: 1147
S: 313.33
H: 1714
S: 315.1
H: 270
S: 331.71
H: 202
S: 335.78

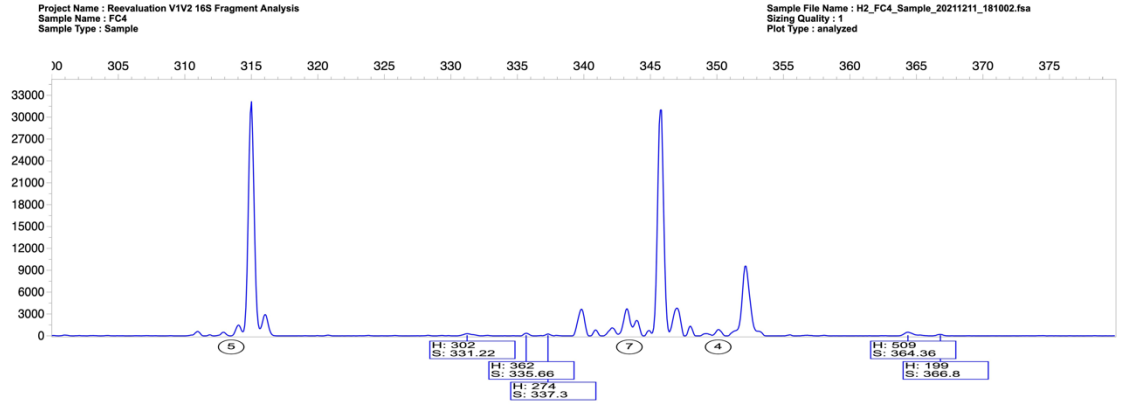

Project Name : Reevaluation V1V2 16S Fragment Analysis
Sample Name : FH8
Sample Type : Sample
Sample File Name : G11_FH8_Sample_20211210_183455.fsa
Sizing Quality : 1
Plot Type : analyzed
H: 2204
S: 313.75
H: 969
S: 315.01
H: 612
S: 330.8
H: 698
S: 362.12

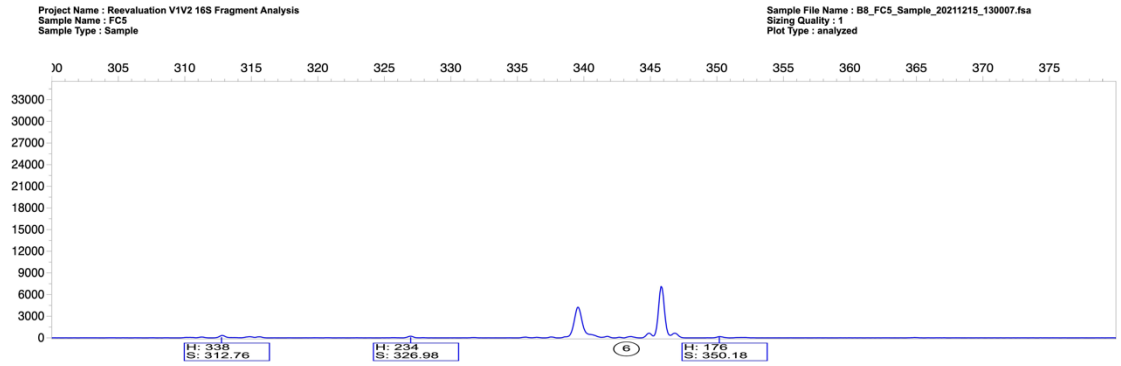


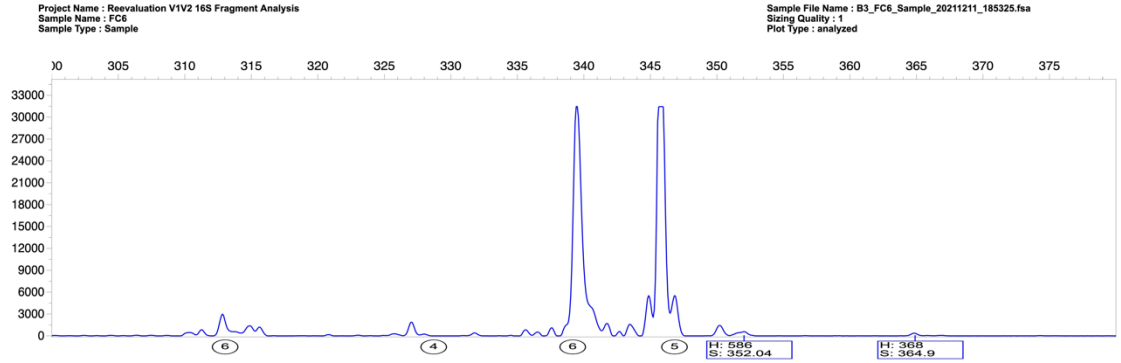
Appendix 5: Fragment Analysis (LHPCR) Plots of Caucasian Female Subjects

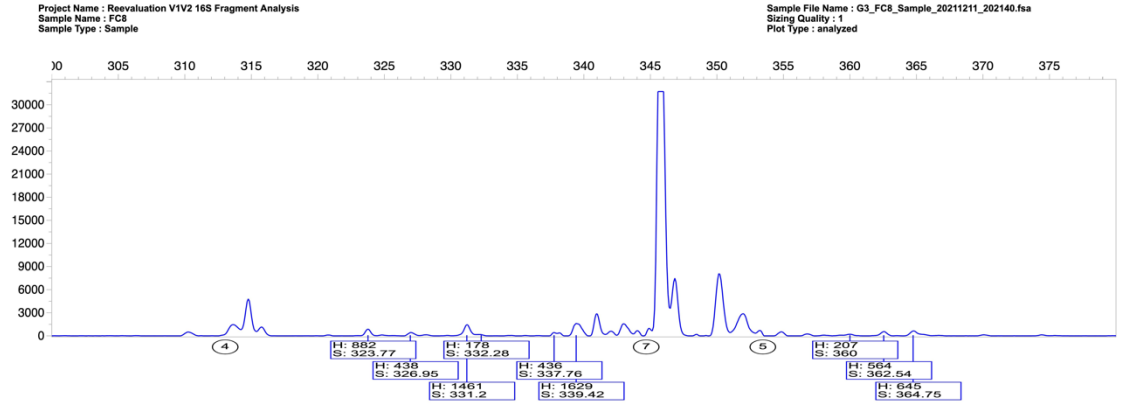

Project Name : Reevaluation V1V2 16S Fragment Analysis
Sample Name : FC2
Sample Type : Sample
Sample File Name : F2_F2_Sample_20211211_181000.fsa
Sizing Quality : 1
Plot Type : analyzed
H: 575
S: 347.96
H: 642
S: 350.31
H: 3377
S: 352.16
H: 187
S: 366.85

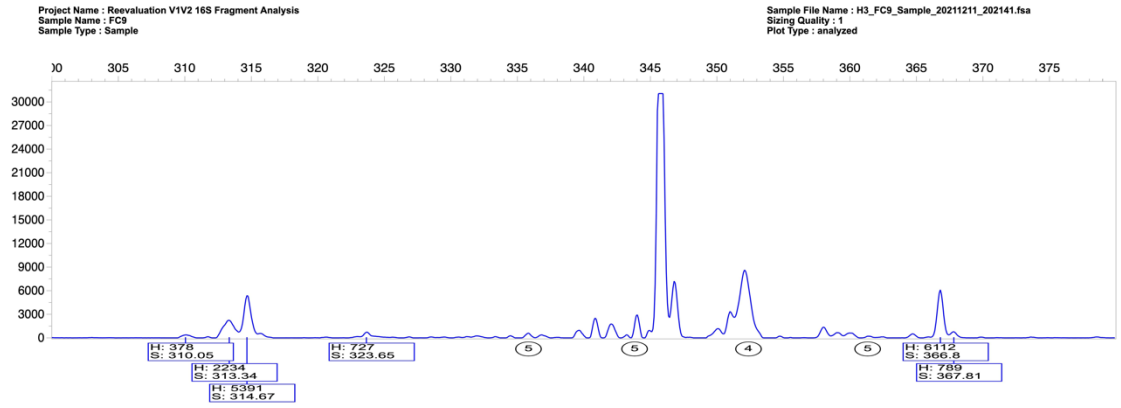

Project Name : Reevaluation V1V2 16S Fragment Analysis
Sample Name : FC3
Sample Type : Sample
Sample File Name : G2_FC3_Sample_20211211_181001.fsa
Sizing Quality : 1
Plot Type : analyzed
H: 646
S: 312.67
H: 526
S: 313.42
H: 1027
S: 314.77
H: 572
S: 323.76
H: 290
S: 331.16
H: 281
S: 359.74
H: 907
S: 362.63

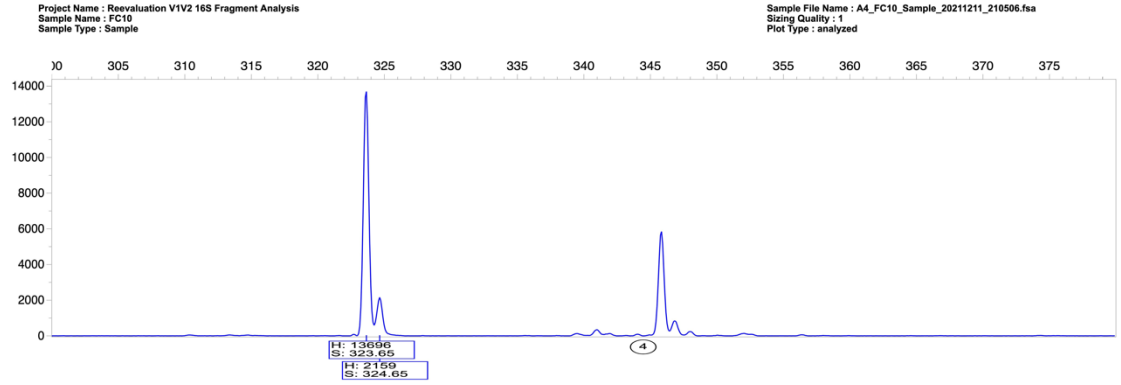

Project Name : Reevaluation V1V2 16S Fragment Analysis
Sample Name : FC4
Sample Type : Sample
Sample File Name : H2_FC4_Sample_20211211_181002.fsa
Sizing Quality : 1
Plot Type : analyzed
H: 302
S: 331.22
H: 362
S: 335.66
H: 274
S: 337.3
H: 509
S: 364.36
H: 199
S: 366.8


Project Name : Reevaluation V1V2 16S Fragment Analysis
Sample Name : FC5
Sample Type : Sample
Sample File Name : B8_FC5_Sample_20211215_130007.fsa
Sizing Quality : 1
Plot Type : analyzed
H: 338
S: 312.76
H: 234
S: 326.98
6
H: 176
S: 350.18

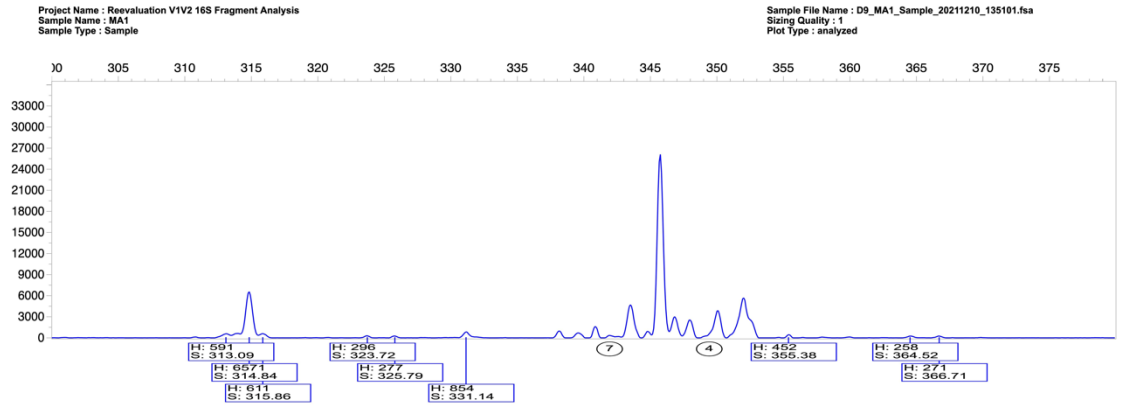

Project Name : Reevaluation V1V2 16S Fragment Analysis
Sample Name : FC6
Sample Type : Sample
Sample File Name : B3_FC6_Sample_20211211_185325.fsa
Sizing Quality : 1
Plot Type : analyzed
6
4
6
5
H: 586
S: 352.04
H: 368
S: 364.9

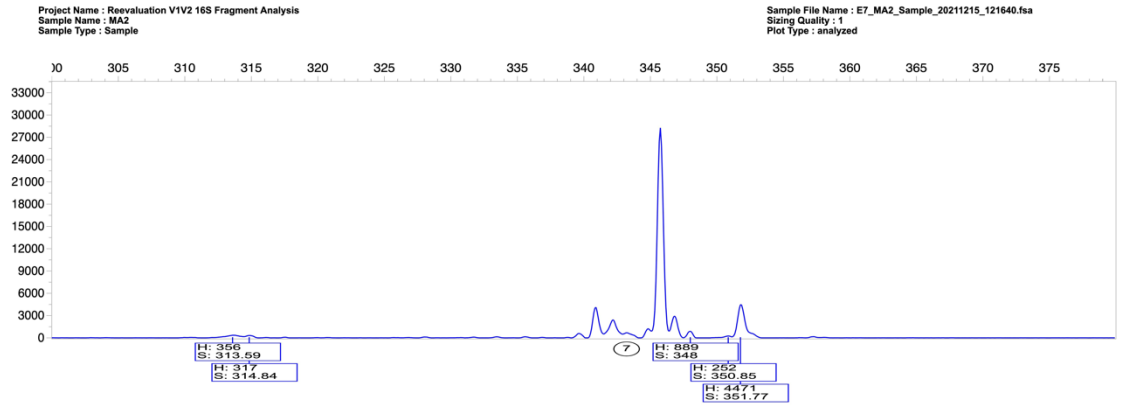

Project Name : Reevaluation V1V2 16S Fragment Analysis
Sample Name : FC8
Sample Type : Sample
Sample File Name : G3_FC8_Sample_20211211_202140.fsa
Sizing Quality : 1
Plot Type : analyzed
4
H: 882
S: 323.77
H: 178
S: 332.28
H: 438
S: 326.95
H: 436
S: 337.76
H: 1461
S: 331.2
H: 1629
S: 339.42
7
5
H: 207
S: 360
H: 564
S: 362.54
H: 645
S: 364.75

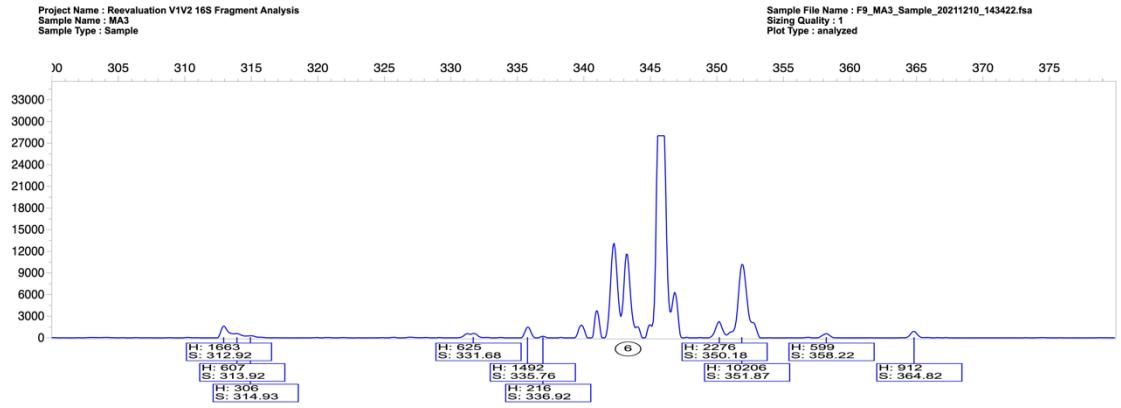

Project Name : Reevaluation V1V2 16S Fragment Analysis
Sample Name : FC9
Sample Type : Sample
Sample File Name : H3_FC9_Sample_20211211_202141.fsa
Sizing Quality : 1
Plot Type : analyzed
305 310 315 320 325 330 335 340 345 350 355 360 365 370 375
30000 27000 24000 21000 18000 15000 12000 9000 6000 3000 0
H: 378
S: 310.05
H: 2234
S: 313.34
H: 5391
S: 314.67
H: 727
S: 323.65
5
5
4
5
H: 6112
S: 366.8
H: 789
S: 367.81

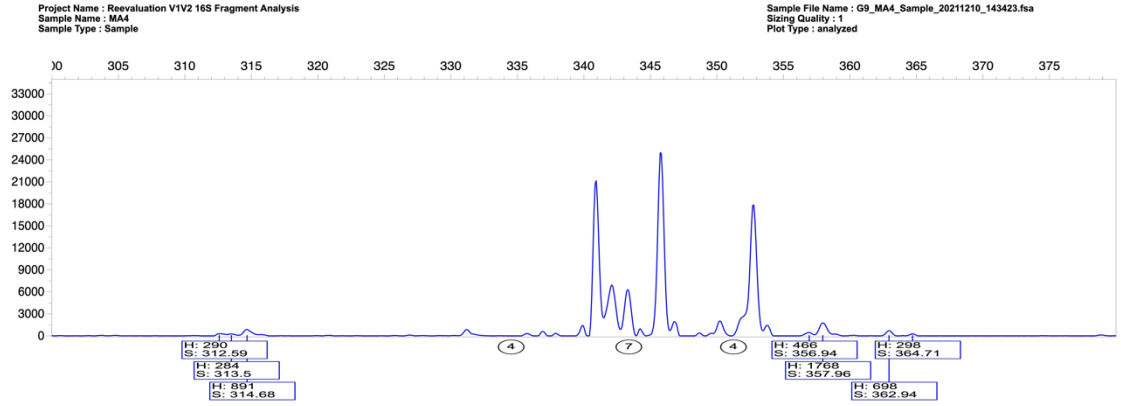

Project Name : Reevaluation V1V2 16S Fragment Analysis
Sample Name : FC10
Sample Type : Sample
Sample File Name : A4_FC10_Sample_20211211_210506.fsa
Sizing Quality : 1
Plot Type : analyzed
305 310 315 320 325 330 335 340 345 350 355 360 365 370 375
14000 12000 10000 8000 6000 4000 2000 0
H: 13696
S: 323.65
H: 2159
S: 324.65
4

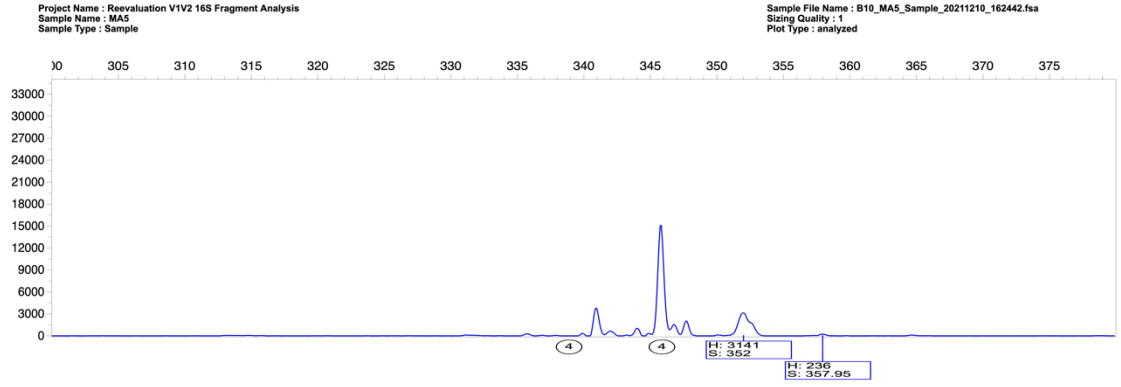
Appendix 6: Fragment Analysis (LHPCR) Plots of African American Male Subjects

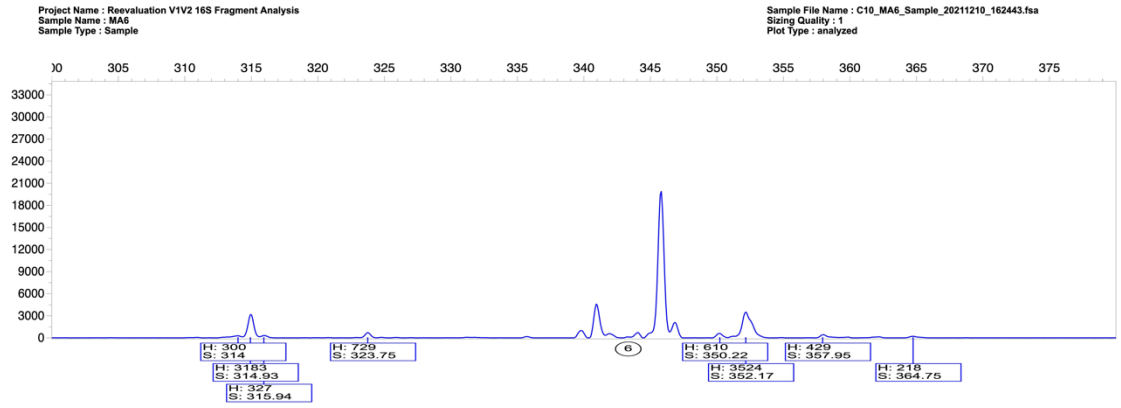


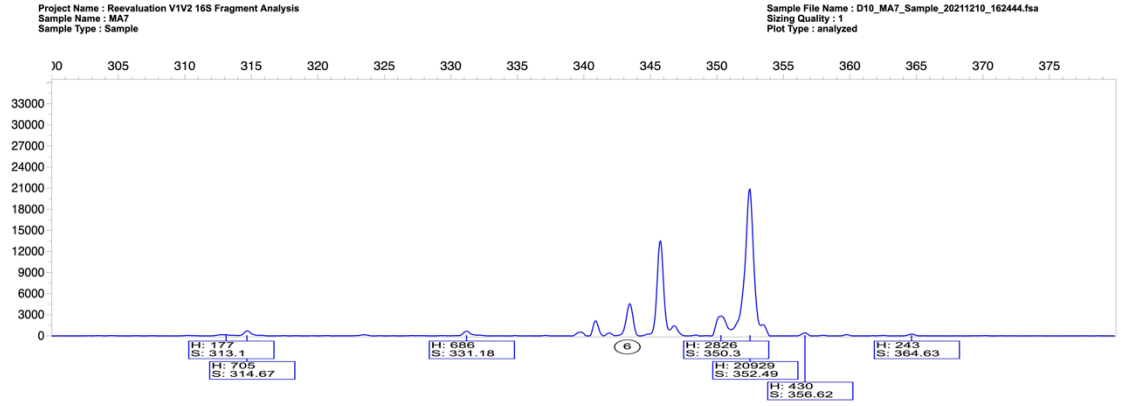


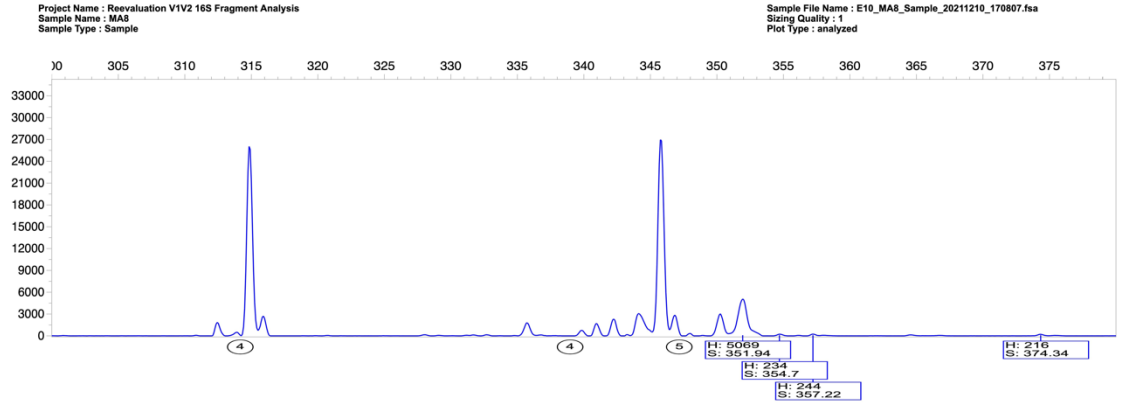

Project Name : Reevaluation V1V2 16S Fragment Analysis
Sample Name : MA3
Sample Type : Sample
Sample File Name : F9_MA3_Sample_20211210_143422.fsa
Sizing Quality : 1
Plot Type : analyzed
H: 1663
S: 312.92
H: 807
S: 313.92
H: 306
S: 314.93
H: 625
S: 331.68
H: 1492
S: 335.76
H: 216
S: 336.92
H: 2276
S: 350.18
H: 10206
S: 351.87
H: 599
S: 358.22
H: 912
S: 364.82

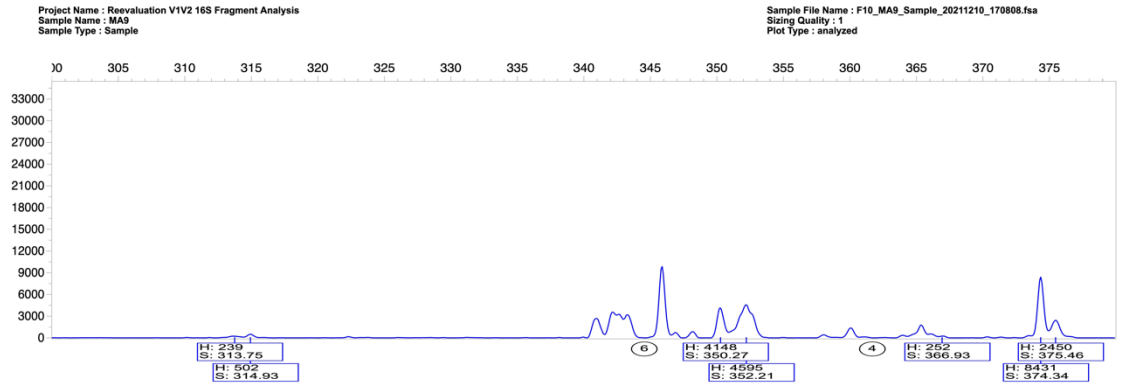

Project Name : Reevaluation V1V2 16S Fragment Analysis
Sample Name : MA4
Sample Type : Sample
Sample File Name : G9_MA4_Sample_20211210_143423.fsa
Sizing Quality : 1
Plot Type : analyzed
H: 290
S: 312.59
H: 284
S: 313.5
H: 891
S: 314.68
H: 466
S: 356.94
H: 1768
S: 357.96
H: 298
S: 364.71
H: 698
S: 362.94

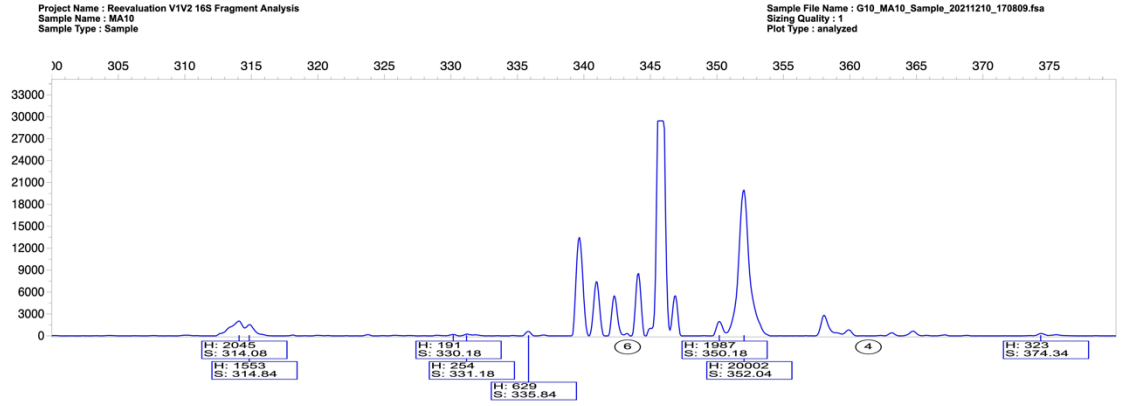

Project Name : Reevaluation V1V2 16S Fragment Analysis
Sample Name : MA5
Sample Type : Sample
Sample File Name : B10_MA5_Sample_20211210_162442.fsa
Sizing Quality : 1
Plot Type : analyzed
H: 3141
S: 352
H: 236
S: 357.95


Project Name : Reevaluation V1V2 16S Fragment Analysis
Sample Name : MA6
Sample Type : Sample
Sample File Name : C10_MA6_Sample_20211210_162443.fsa
Sizing Quality : 1
Plot Type : analyzed
H: 300
S: 314
H: 3183
S: 314.93
H: 327
S: 315.94
H: 729
S: 323.75
6
H: 610
S: 350.22
H: 3524
S: 352.17
H: 429
S: 357.95
H: 218
S: 364.75

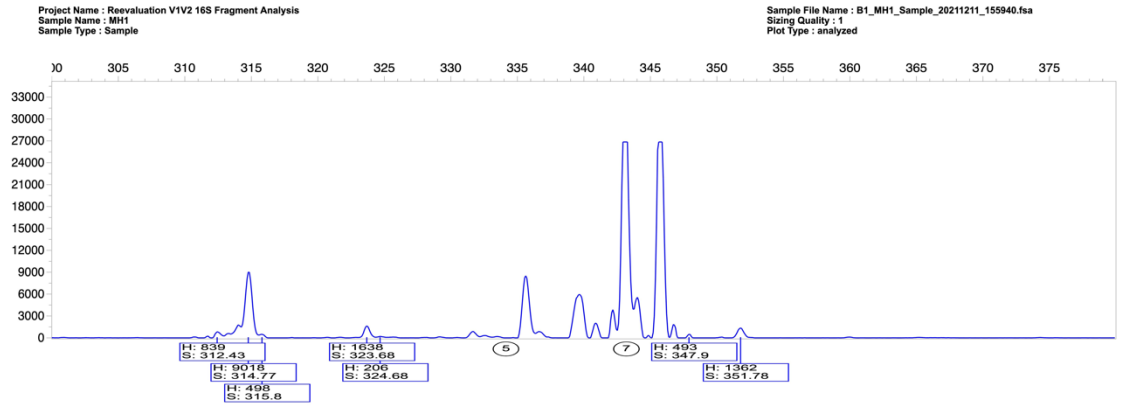

Project Name : Reevaluation V1V2 16S Fragment Analysis
Sample Name : MA7
Sample Type : Sample
Sample File Name : D10_MA7_Sample_20211210_162444.fsa
Sizing Quality : 1
Plot Type : analyzed
H: 177
S: 313.1
H: 705
S: 314.67
H: 686
S: 331.18
6
H: 2826
S: 350.3
H: 20929
S: 352.49
H: 430
S: 356.62
H: 243
S: 364.63

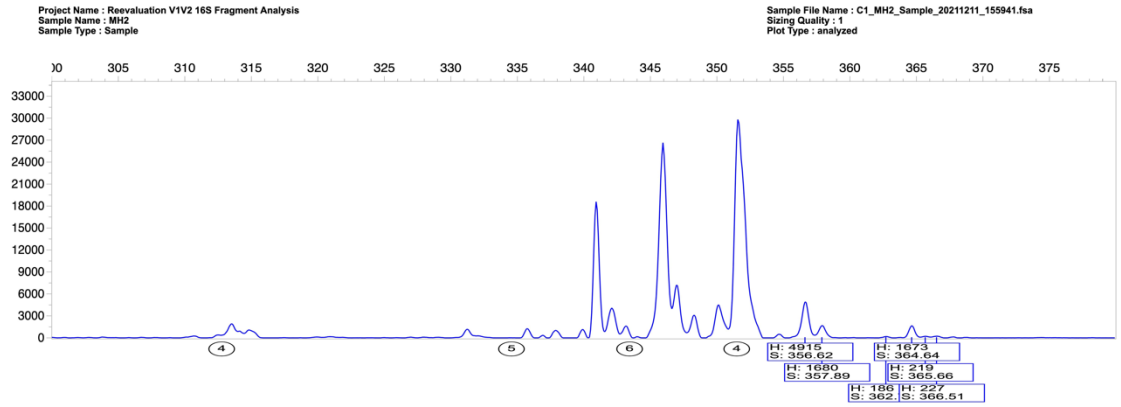

Project Name : Reevaluation V1V2 16S Fragment Analysis
Sample Name : MA8
Sample Type : Sample
Sample File Name : E10_MA8_Sample_20211210_170807.fsa
Sizing Quality : 1
Plot Type : analyzed
4
4
5
H: 5069
S: 351.94
H: 234
S: 354.7
H: 244
S: 357.22
H: 216
S: 374.34

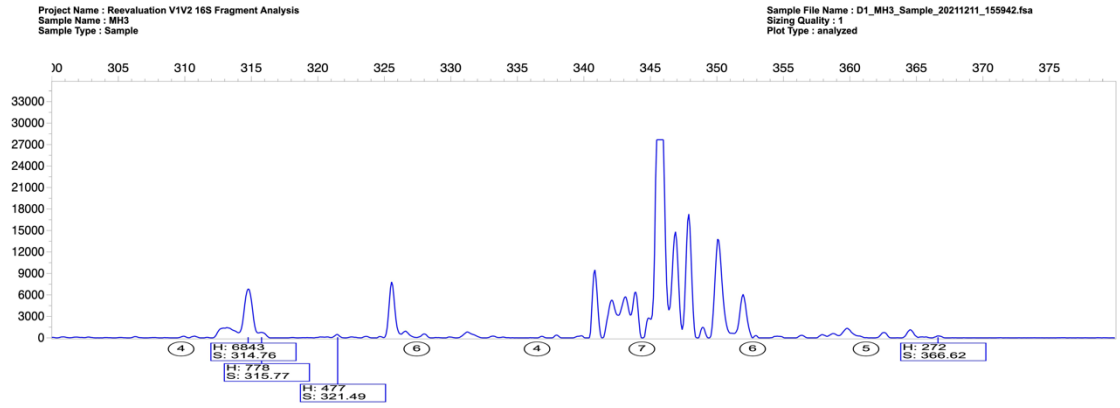

Project Name : Reevaluation V1V2 16S Fragment Analysis
Sample Name : MA9
Sample Type : Sample
Sample File Name : F10_MA9_Sample_20211210_170808.fsa
Sizing Quality : 1
Plot Type : analyzed
305 310 315 320 325 330 335 340 345 350 355 360 365 370 375
33000 30000 27000 24000 21000 18000 15000 12000 9000 6000 3000 0
H: 239
S: 313.75
H: 502
S: 314.93
6
H: 4148
S: 350.27
H: 4595
S: 352.21
4
H: 252
S: 366.93
H: 2450
S: 375.46
H: 8431
S: 374.34

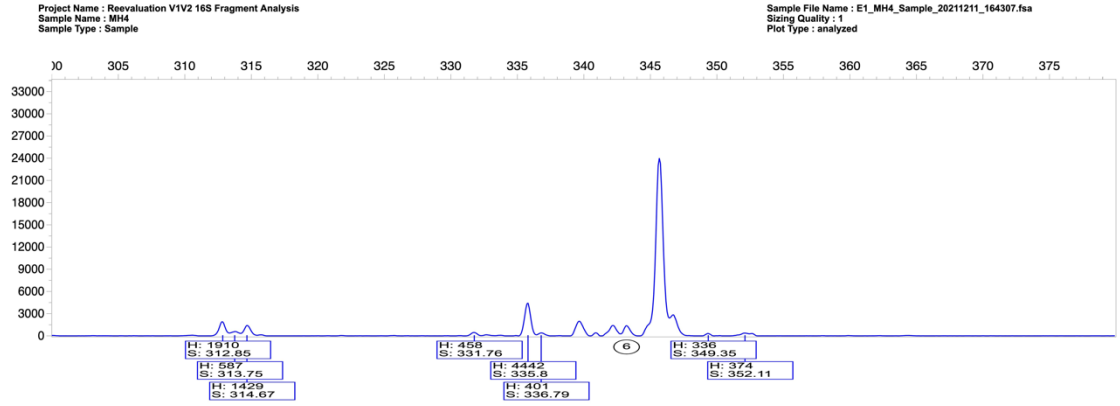

Project Name : Reevaluation V1V2 16S Fragment Analysis
Sample Name : MA10
Sample Type : Sample
Sample File Name : G10_MA10_Sample_20211210_170809.fsa
Sizing Quality : 1
Plot Type : analyzed
305 310 315 320 325 330 335 340 345 350 355 360 365 370 375
33000 30000 27000 24000 21000 18000 15000 12000 9000 6000 3000 0
H: 2045
S: 314.08
H: 1553
S: 314.84
H: 191
S: 330.18
H: 254
S: 331.18
H: 629
S: 335.84
6
H: 1987
S: 350.18
H: 20002
S: 352.04
4
H: 323
S: 374.34

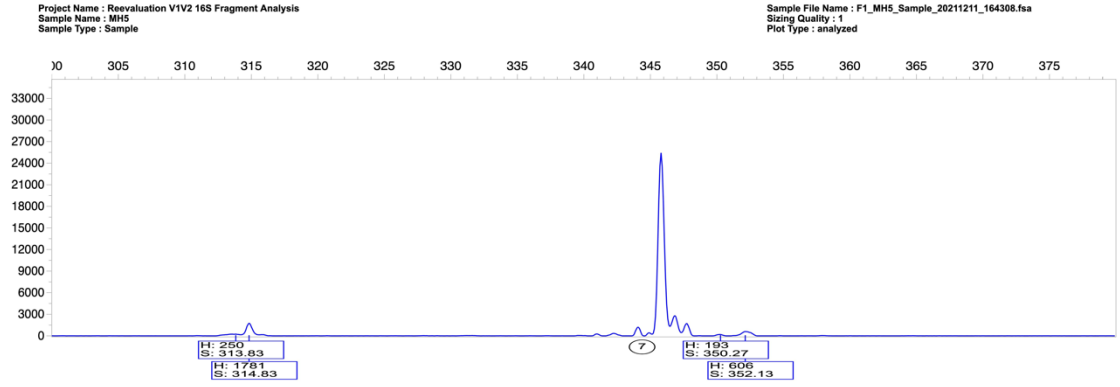
Appendix 7: Fragment Analysis (LHPCR) Plots of Hispanic Male Subjects

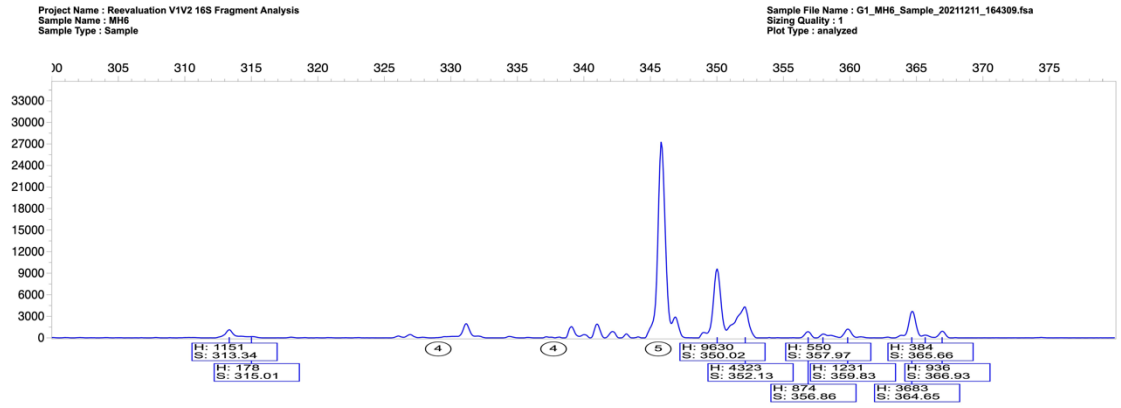


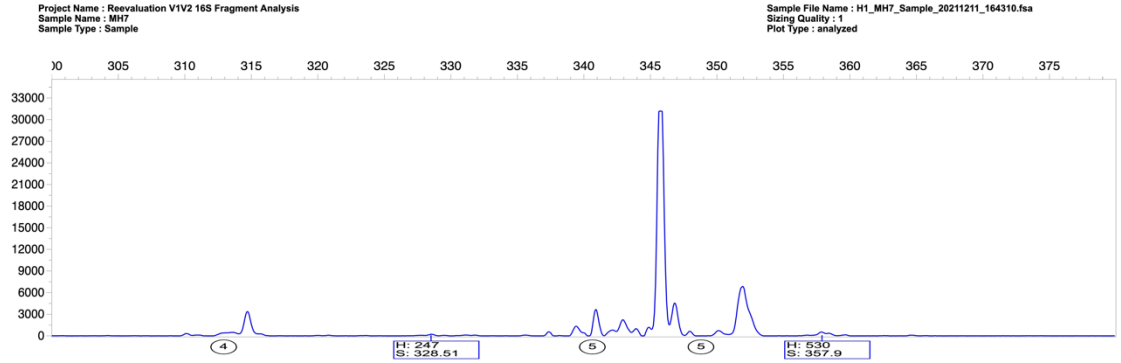


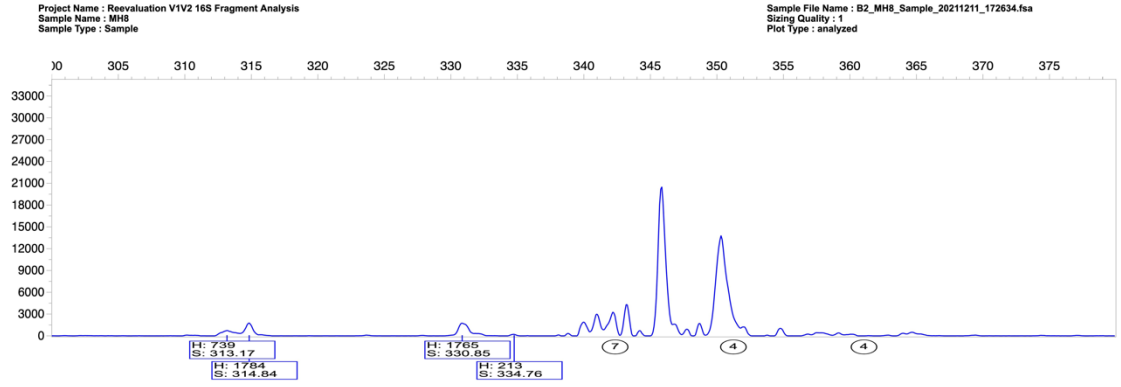

Project Name : Reevaluation V1V2 16S Fragment Analysis
Sample Name : MH3
Sample Type : Sample
Sample File Name : D1_MH3_Sample_20211211_155942.fsa
Sizing Quality : 1
Plot Type : analyzed
H: 6843
S: 314.76
H: 778
S: 315.77
H: 477
S: 321.49
H: 272
S: 366.62

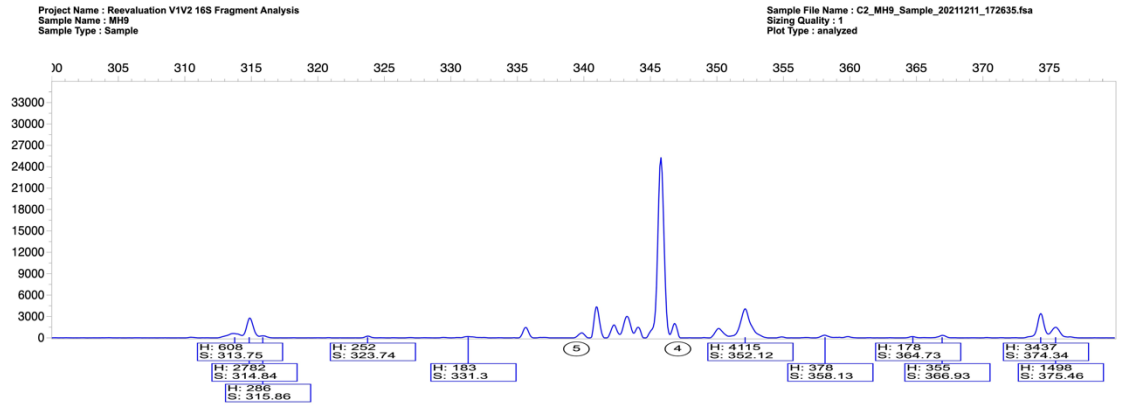

Project Name : Reevaluation V1V2 16S Fragment Analysis
Sample Name : MH4
Sample Type : Sample
Sample File Name : E1_MH4_Sample_20211211_164307.fsa
Sizing Quality : 1
Plot Type : analyzed
H: 1910
S: 312.85
H: 587
S: 313.75
H: 1429
S: 314.67
H: 458
S: 331.76
H: 4442
S: 335.8
H: 401
S: 336.79
H: 336
S: 349.35
H: 374
S: 352.11

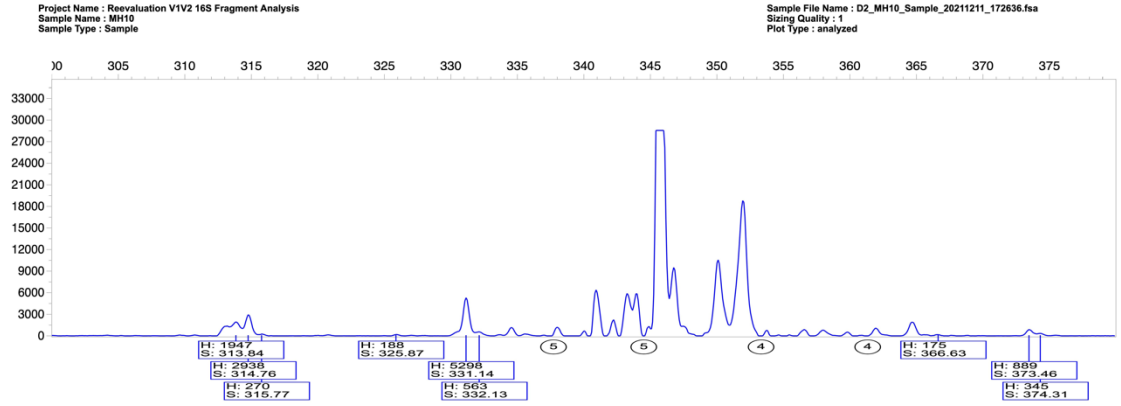

Project Name : Reevaluation V1V2 16S Fragment Analysis
Sample Name : MH5
Sample Type : Sample
Sample File Name : F1_MH5_Sample_20211211_164308.fsa
Sizing Quality : 1
Plot Type : analyzed
H: 250
S: 313.83
H: 1781
S: 314.83
H: 193
S: 350.27
H: 606
S: 352.13


Project Name : Reevaluation V1V2 16S Fragment Analysis
Sample Name : MH6
Sample Type : Sample
Sample File Name : G1_MH6_Sample_20211211_164309.fsa
Sizing Quality : 1
Plot Type : analyzed
H: 1151
S: 313.34
H: 178
S: 315.01
H: 9630
S: 350.02
H: 4323
S: 352.13
H: 550
S: 357.97
H: 1231
S: 359.83
H: 874
S: 356.86
H: 384
S: 365.66
H: 936
S: 366.93
H: 3683
S: 364.65


Project Name : Reevaluation V1V2 16S Fragment Analysis
Sample Name : MH7
Sample Type : Sample
Sample File Name : H1_MH7_Sample_20211211_164310.fsa
Sizing Quality : 1
Plot Type : analyzed
H: 247
S: 328.51
H: 530
S: 357.9


Project Name : Reevaluation V1V2 16S Fragment Analysis
Sample Name : MH8
Sample Type : Sample
Sample File Name : B2_MH8_Sample_20211211_172634.fsa
Sizing Quality : 1
Plot Type : analyzed
H: 739
S: 313.17
H: 1784
S: 314.84
H: 1765
S: 330.85
H: 213
S: 334.76


Project Name : Reevaluation V1V2 16S Fragment Analysis
Sample Name : MH9
Sample Type : Sample
Sample File Name : C2_MH9_Sample_20211211_172635.fsa
Sizing Quality : 1
Plot Type : analyzed
305 310 315 320 325 330 335 340 345 350 355 360 365 370 375
33000 30000 27000 24000 21000 18000 15000 12000 9000 6000 3000 0
H: 608 S: 313.75
H: 2782 S: 314.84
H: 286 S: 315.86
H: 252 S: 323.74
H: 183 S: 331.3
5
4
H: 4115 S: 352.12
H: 378 S: 358.13
H: 178 S: 364.73
H: 355 S: 366.93
H: 3437 S: 374.34
H: 1498 S: 375.46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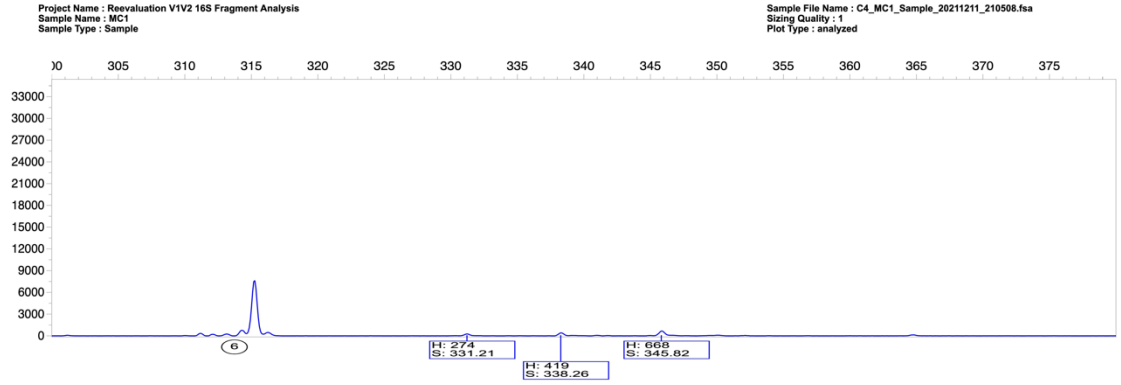
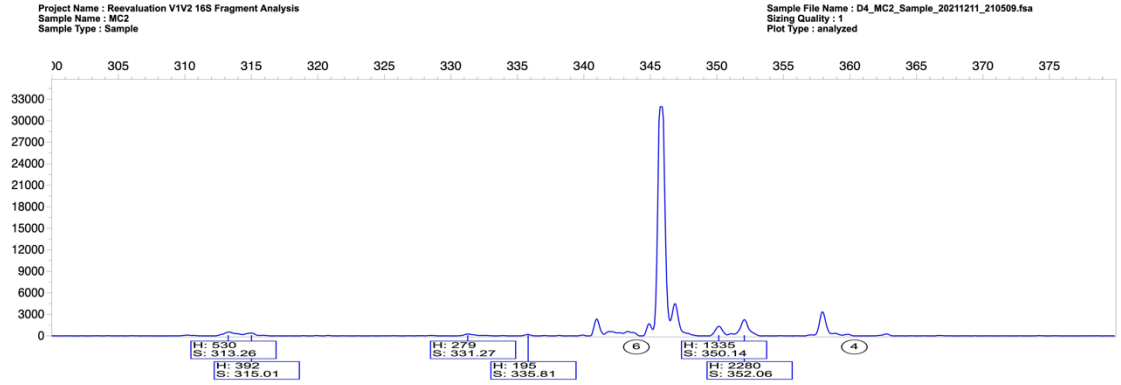
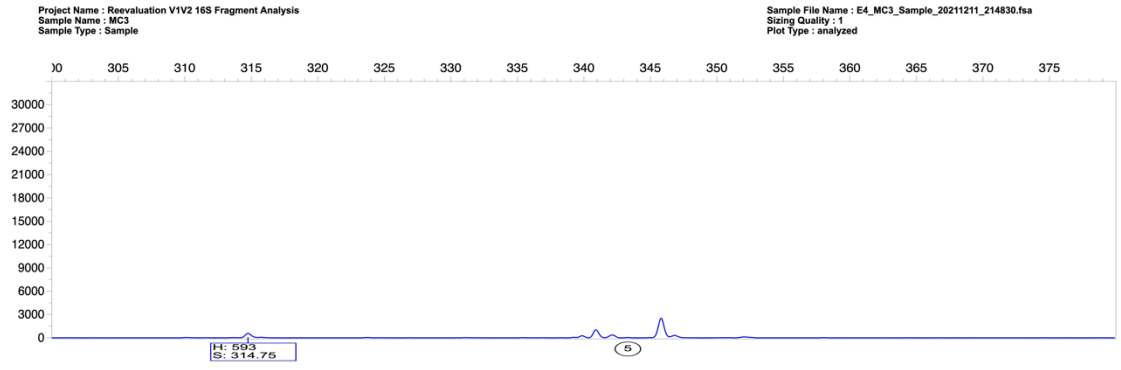
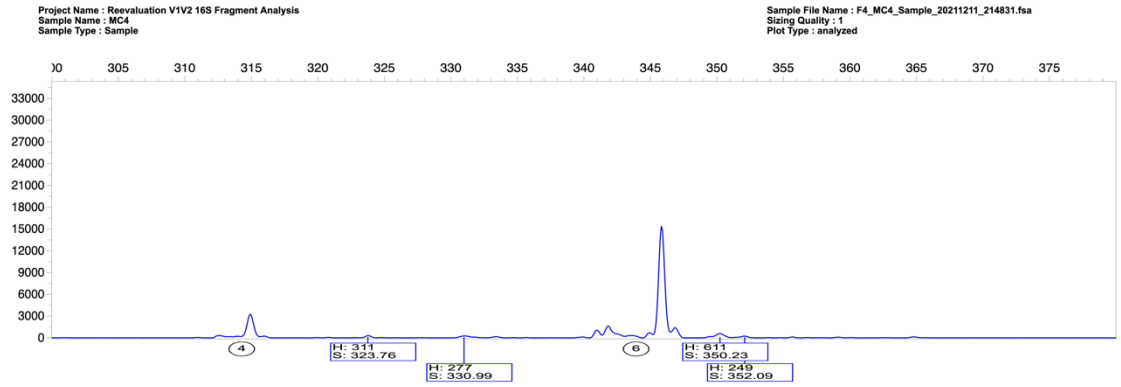
Sample Name : MH10
Sample Type : Sample
Sample File Name : D2_MH10_Sample_20211211_172636.fsa
Sizing Quality : 1
Plot Type : analyzed
305 310 315 320 325 330 335 340 345 350 355 360 365 370 375
33000 30000 27000 24000 21000 18000 15000 12000 9000 6000 3000 0
H: 1947 S: 313.84
H: 2938 S: 314.76
H: 270 S: 315.77
H: 188 S: 325.87
H: 5298 S: 331.14
H: 563 S: 332.13
5
5
4
4
H: 175 S: 366.63
H: 889 S: 373.46
H: 345 S: 374.31

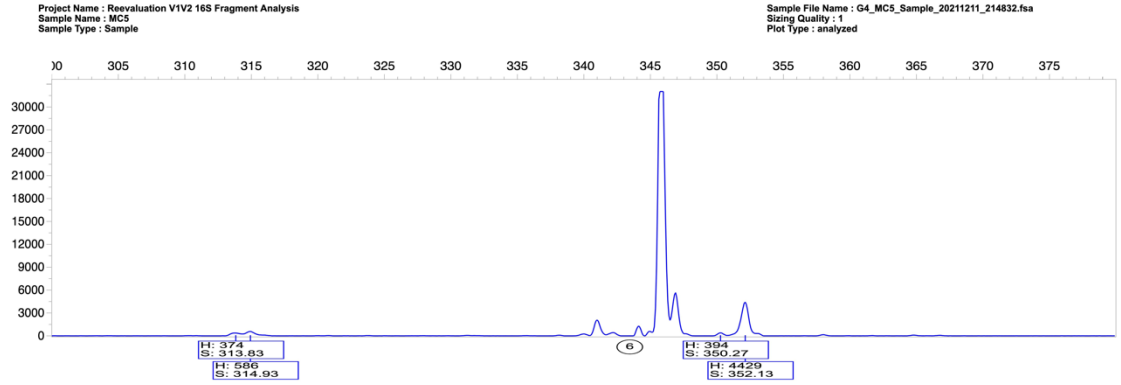
Appendix 8: Fragment Analysis (LHPCR) Plots of Caucasian Male Subjects

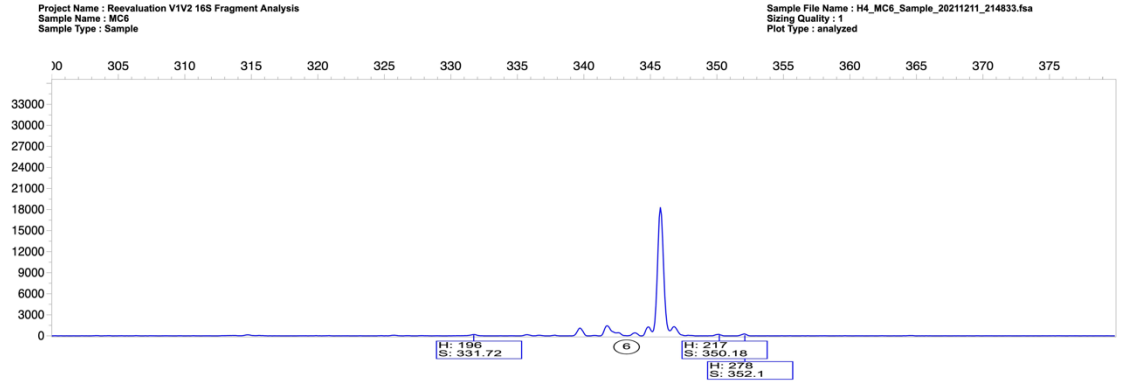


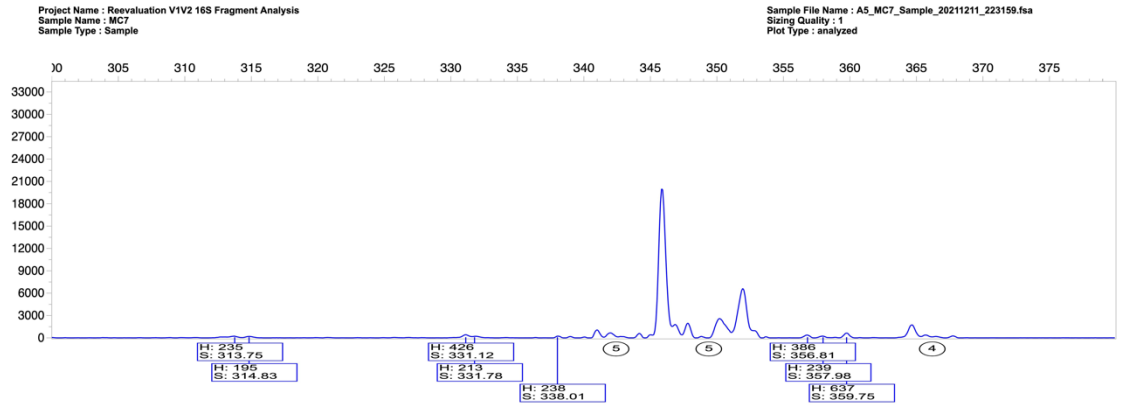


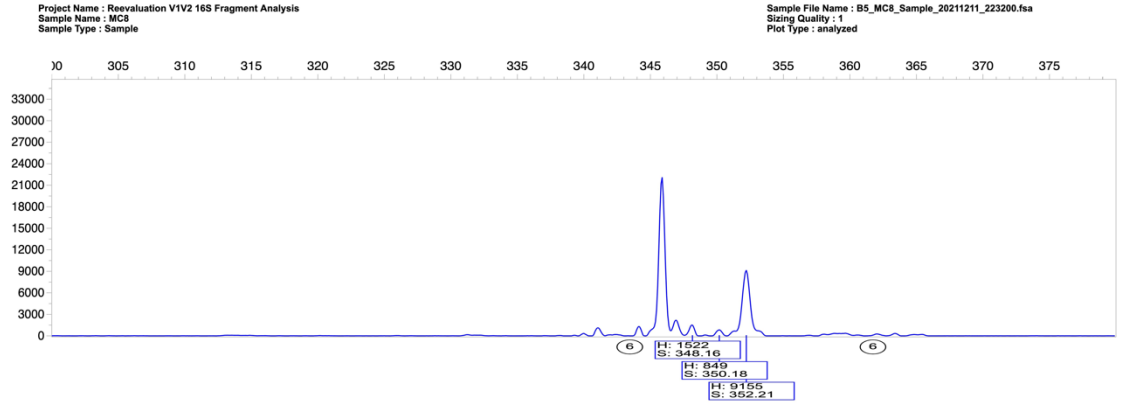


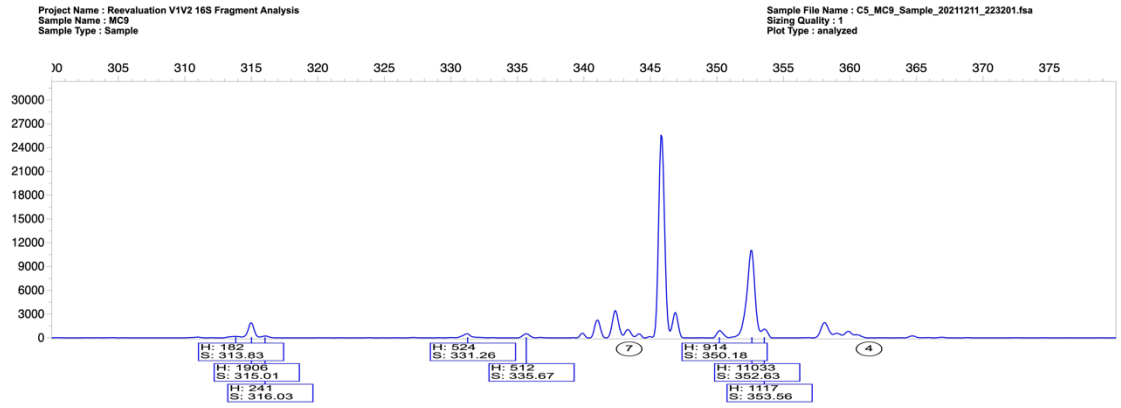

Project Name : Reevaluation V1V2 16S Fragment Analysis
Sample Name : MC4
Sample Type : Sample
Sample File Name : F4_MC4_Sample_20211211_214831.fsa
Sizing Quality : 1
Plot Type : analyzed
305 310 315 320 325 330 335 340 345 350 355 360 365 370 375
33000 30000 27000 24000 21000 18000 15000 12000 9000 6000 3000 0
4
H: 311
S: 323.76
H: 277
S: 330.99
6
H: 611
S: 350.23
H: 249
S: 352.09

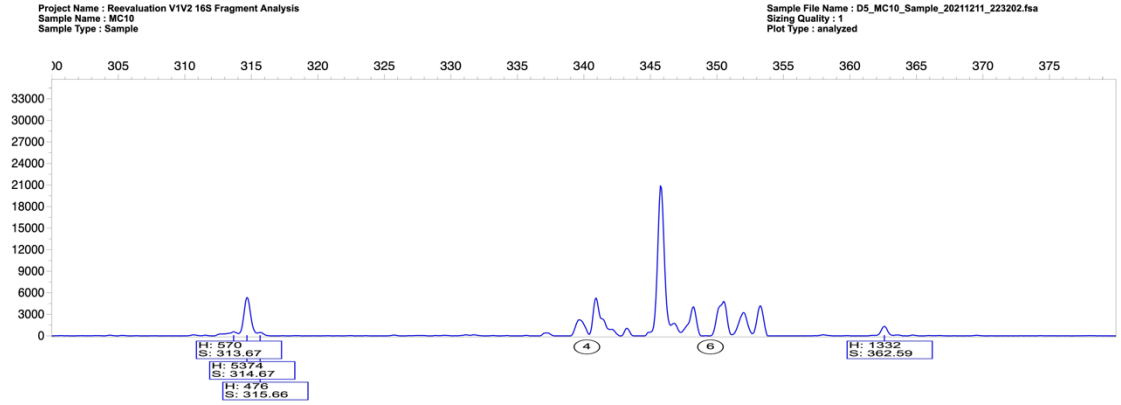

Project Name : Reevaluation V1V2 16S Fragment Analysis
Sample Name : MC5
Sample Type : Sample
Sample File Name : G4_MC5_Sample_20211211_214832.fsa
Sizing Quality : 1
Plot Type : analyzed
305 310 315 320 325 330 335 340 345 350 355 360 365 370 375
30000 27000 24000 21000 18000 15000 12000 9000 6000 3000 0
H: 374
S: 313.83
H: 586
S: 314.93
6
H: 394
S: 350.27
H: 429
S: 352.13


Project Name : Reevaluation V1V2 16S Fragment Analysis
Sample Name : MC6
Sample Type : Sample
Sample File Name : H4_MC6_Sample_20211211_214833.fsa
Sizing Quality : 1
Plot Type : analyzed
305 310 315 320 325 330 335 340 345 350 355 360 365 370 375
33000 30000 27000 24000 21000 18000 15000 12000 9000 6000 3000 0
H: 196
S: 331.72
6
H: 217
S: 350.18
H: 278
S: 352.1


Project Name : Reevaluation V1V2 16S Fragment Analysis
Sample Name : MC7
Sample Type : Sample
Sample File Name : A5_MC7_Sample_20211211_223159.fsa
Sizing Quality : 1
Plot Type : analyzed
H: 235
S: 313.75
H: 195
S: 314.83
H: 426
S: 331.12
H: 213
S: 331.78
H: 238
S: 338.01
H: 386
S: 356.81
H: 239
S: 357.98
H: 637
S: 359.75


Project Name : Reevaluation V1V2 16S Fragment Analysis
Sample Name : MC8
Sample Type : Sample
Sample File Name : B5_MC8_Sample_20211211_223200.fsa
Sizing Quality : 1
Plot Type : analyzed
H: 1522
S: 348.16
H: 849
S: 350.18
H: 9155
S: 352.21


Project Name : Reevaluation V1V2 16S Fragment Analysis
Sample Name : MC9
Sample Type : Sample
Sample File Name : C5_MC9_Sample_20211211_223201.fsa
Sizing Quality : 1
Plot Type : analyzed
305 310 315 320 325 330 335 340 345 350 355 360 365 370 375
30000 27000 24000 21000 18000 15000 12000 9000 6000 3000 0
H: 182 S: 313.83
H: 1906 S: 315.01
H: 241 S: 316.03
H: 524 S: 331.26
H: 512 S: 335.67
7
H: 914 S: 350.18
H: 11033 S: 352.63
H: 1117 S: 353.56
4


Project Name : Reevaluation V1V2 16S Fragment Analysis
Sample Name : MC10
Sample Type : Sample
Sample File Name : D5_MC10_Sample_20211211_223202.fsa
Sizing Quality : 1
Plot Type : analyzed
305 310 315 320 325 330 335 340 345 350 355 360 365 370 375
33000 30000 27000 24000 21000 18000 15000 12000 9000 6000 3000 0
H: 570 S: 313.67
H: 5374 S: 314.67
H: 476 S: 315.66
4
6
H: 1332 S: 362.59

Appendix 9: OTU Count Abundance Table of All Subjects

Sample	Cutibacterium(100)	Enhydrobacter(100)	Fusobacterium(100)	Lawsonella(100)	Micrococcus(100)	Staphylococcus(100)	Streptococcus(100)
FA1	9	0	0	0	0	4	0
FA10	3	0	2	3	0	6	2
FA2	15	1	0	0	2	4	1
FA3	2	0	0	0	0	6	1
FA4	3	0	0	0	0	1	2
FA5	9	2	4	2	8	10	5
FA6	3	13	0	9	3	28	1
FA7	1	1	6	0	0	0	3
FA8	4	0	1	1	1	18	1
FA9	8	0	2	0	2	4	1
FC1	7	2	2	1	0	7	1
FC10	9	4	0	2	0	2	1
FC2	5	2	0	3	1	1	6
FC3	3	0	1	0	0	2	10
FC4	11	3	2	5	2	7	10
FC5	12	1	0	2	0	2	0
FC6	24	0	0	6	3	5	0
FC7	7	1	4	0	6	3	11
FC8	9	1	1	3	0	5	6
FC9	24	4	1	7	1	14	4
FH1	10	0	1	3	0	9	2
FH2	7	0	3	0	0	13	3
FH3	8	1	1	5	1	0	2
FH7	6	7	0	1	3	15	2
FH8	8	0	0	1	0	27	0
FH9	10	6	0	3	3	3	2
MA1	8	0	5	3	0	13	1
MA10	6	2	0	6	4	21	0
MA2	6	3	0	1	13	18	0
MA3	8	6	3	1	2	7	3
MA4	6	2	4	0	0	18	0
MA5	17	10	2	10	0	19	9
MA6	14	5	2	6	0	33	11
MA7	6	0	3	0	0	16	2
MA8	13	10	1	3	3	9	3
MA9	2	2	0	0	3	20	4
MC1	0	1	1	0	0	0	1
MC10	7	2	1	0	1	10	4
MC2	12	3	0	0	1	8	2
MC3	2	2	0	1	1	9	1
MC4	7	3	2	0	0	3	1
MC5	21	2	1	6	0	14	4
MC6	5	0	0	8	0	9	0
MC7	0	0	2	0	0	8	1
MC8	3	2	3	8	2	6	3
MC9	7	5	1	2	7	24	0
MH1	8	34	0	8	6	4	0
MH10	17	1	11	2	0	10	7
MH2	1	10	3	1	2	25	7
MH3	5	1	2	6	2	9	4
MH4	8	33	0	0	2	3	1
MH5	30	1	0	12	0	13	1
MH6	11	1	13	1	0	5	16
MH7	14	1	2	3	0	21	1
MH8	4	0	5	2	2	2	5
MH9	8	8	2	5	4	14	4

VITA

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2012-2016	B.S., Forensic Science Savannah State University Savannah, Georgia
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PUBLICATIONS AND PRESENTATIONS

Frazier, C. (2021, November) "Human Odor to Forensic Identification" conducted at 3MT College of Arts and Sciences (CASE) competition, Miami, FL. *2nd Place for Presentation*

Frazier, C., Mills, D., Furton, K. (2021, June) "Statistical Analysis of Volatile Organic Compounds from Hand Odor" conducted at the Virtual 10th Annual Forensic Science Symposium, Miami, FL.

Frazier, C., Mills, D., Furton, K. (2020, February) "Forensic Identification: An Investigation to Corroborate Volatile and Biological Profiles for Subject Identification" conducted at the 2020 McKnight Mid-Year Research and Writing Conference, Tampa, FL.

Frazier, C., Mills, D., Furton, K. (2020, February) "Forensic Identification: An Investigation to Corroborate Volatile and Biological Profiles for Subject Identification" conducted at the American Academy of Forensic Science 72nd Annual Scientific Conference, Anaheim, CA.

Frazier, C., Mills, D., Furton, K. (2019, April) "Human Scent Forensics: A cross-disciplinary study using SPME-GC/MS VOC Analyses and Length Heterogeneity PCR" conducted at the Florida International University Graduate Student Appreciation Week, Miami, FL.

Frazier, C., Mills, D., Furton, K. (2019, March) "Human Scent Forensics: A cross-disciplinary study using SPME-GC/MS VOC Analyses and Length Heterogeneity PCR" conducted at University South Florida 17th Annual Castle Conference, Tampa, FL.

Frazier, C., Mills, D., Furton, K. (2019, March) "Human Scent Forensics: A cross-disciplinary study using SPME-GC/MS VOC Analyses and Length Heterogeneity PCR" NIJ Symposium conducted at Pittcon, Philadelphia, PA.

Frazier, C., Mills, D., Furton, K. (2019, January) "Human Scent Forensics: A cross-disciplinary study using SPME-GC/MS VOC Analyses and Length Heterogeneity PCR" conducted at the Florida International University Biosymposium, Miami, FL. *1st Place Poster Presentation Award*

Frazier, C., Mills, D., Furton, K. (2018, October) "Human Scent Forensics: A cross-disciplinary study using SPME-GC/MS VOC Analyses and Next Generation DNA Sequencing" conducted at the SciX Conference, Atlanta, GA. *2nd Place Poster Presentation Award*

Frazier, C. Nawaz, Z., El Hokayem, J. (2015, November) "E6-Associated Protein and its Transcriptional Co-activation of Estrogen Receptors in Neurons" conducted at the Annual Biomedical Research Conference for Minority Students conference, Seattle, WA.

Frazier, C. Nawaz, Z., El Hokayem, J. (2015, October) "E6-Associated Protein and its Transcriptional Co-activation of Estrogen Receptors in Neurons" conducted at the 10th Peach State Louis Stokes Alliance for Minority Participation (PLSAMP) conference, Athens, GA. *2nd Place for Poster Presentation in Molecular and Cellular Biology*

Frazier, C., Nawaz, Z., El Hokayem, J. (2015, July) "E6-Associated Protein and its Transcriptional Co-activation of Estrogen Receptors in Neurons" conducted at the University of Miami Summer Undergraduate Research Fellowship program, Miami, FL.

Frazier, C. Marriott, K., (2014, October) "Synthesis of Sigma-1 Receptor Ligands" conducted at the Peach State Louis Stokes Alliance for Minority Participation (PLSAMP) conference, Atlanta, GA.