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S-Equol: A Novel Therapeutic for HIV-1 Induced Gut Dysbiosis

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S-EQUOL: A NOVEL THERAPEUTIC FOR HIV-1 INDUCED GUT DYSBIOSIS

by

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DEDICATION

I dedicate this thesis to my parents, Prue and Ray. They have always had my back and if it wasn't for them, I would not have made it to this in my life. With their support, I was able to pursue my dreams to the fullest. I also dedicate this thesis to my wife, Lauren. Throughout the all-nighters, early mornings, and honestly some of the toughest of times, she was always there to motivate me and keep me steady. I thank you and hope to make you all proud with this work and with everything to come.

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ABSTRACT

HIV-1 infection affects approximately 38 million people around the world. The advent of cART has greatly improved the quality of life of infected individuals; however, roughly 50% of these individuals will still experience HIV-1 associated neurocognitive disorders (HAND). Additionally, the gut microbiome has been reported to be dysbiotic in HIV-1 infected individuals, regardless of adherence to cART. Current research has pointed to the gut-brain-microbiota axis as a potential target to treat both cognitive deficits and microbial changes. The present study investigated S-equol (SE) as a potential therapeutic for HAND by modulating the gut microbiome. The study included 21 HIV-1 Tg rats and 21 F344 control animals to test the effect 0.2mg SE has on cocaine-maintained responding on a PR schedule of reinforcement. Gut microbiome alterations between genotypes were found at the phylum and genus level, regardless of treatment group, and treatment had both main effects and interactions with genotype. Prevotella_UCG_001 was found to significantly covary with lever presses for drug, suggesting a possible effect on motivation for cocaine. Alloprevotella was found to significantly differentiate between genotype by treatment effects, indicating that SE may differently affect genotypes.

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CHAPTER 1

INTRODUCTION

Human immunodeficiency virus 1 (HIV-1) has infected an estimated 38 million people around the world as of 2019 (WHO, 2020). Combination antiretroviral therapy (cART) is the main treatment for HIV and works to control the replication of HIV, resulting in improved CD4 T cell counts and preventing transmission by those compliant with the cART regimen to others (WHO, 2020). The use of cART has also greatly decreased the prevalence of HIV-1 associated dementia (HAD), but HIV-1 associated neurocognitive disorders (HAND) continue to persist, affecting approximately 50% of HIV-1 seropositive individuals regardless of cART treatment (Heaton et al., 2011). Treatment is usually started as soon as a diagnosis is made but by then the infection has already established latent, viral reservoirs, that prevent full eradication of the virus by antiretrovirals, due to an inability of cART to reach these latent reservoirs. (Koay et al., 2018). The infection enters the body through the exchange of bodily fluids (blood, semen, and vaginal secretions) with an infected individual. Once inside the body, HIV-1 initially binds to CD4+ T cells via the C-C chemokine receptor type 5 (CCR5) co-receptor. The gastrointestinal (GI) tract contains the largest mucosal immune system, making it one of the initial sites of HIV-1 infection. The GI tract is also one of the most damaged by the initial infection, indicated by a greater reduction in CD4+ T cells than other tissues (Brenchley et al., 2004). After prolonged infection, the GI tract maintains the lowest level

of CD4+ T cells, which is accompanied by alterations to the composition of the gut microbiome (Mutlu et al., 2014; Ling et al., 2016; Tincati et al., 2016; Rocafort et al., 2019).

It is well understood that dysbiosis, or negative alterations, of the gut microbiome can cause cognitive deficits through interactions with the gut-brain-microbiota axis (Cryan et al., 2019). In HIV-1 seropositive individuals, dysbiosis of the gut microbiome is seen throughout infection (Ling et al., 2016), with alterations in the microbiota composition found less than 6 months post-infection (Rocafort et al., 2019). HIV-1 infection results in an overall reduction in microbiota diversity, with a significant decrease in Akkermansia Muciniphila and an increase in Prevotella (Ling et al., 2016). Reduced Akkermansia Muciniphila and increased Prevotella is an important observation for two reasons, first Akkermansia Muciniphila. It is a vital bacterium that is responsible for maintaining the integrity of the gut microbiome, and more specifically the epithelial barrier (Ouyang et al., 2020). Second, Prevotella is a gram-negative bacterium, meaning it possesses lipopolysaccharide (LPS) on the outer membrane, an endotoxin that increases immune activation and is commonly used to assess microbial translocation due to its ability to compromise the blood-brain barrier (BBB) (Mutlu et al., 2014; Ling et al., 2016; Wang, Y., et al., 2020). The combination of these two alterations allows HIV-1 to break down the beneficial bacteria in the gut microbiome, compromising the integrity of the epithelial barrier in the process and opening the pathway for harmful microbes from the gut to leak out. One of these harmful microbes is Prevotella, carrying LPS through the gut-brain-microbiota axis to the BBB where it simultaneously weakens the BBB as it passes through and binds to the surface of microglia (Wang, E. J., et al., 2003; Wang, H.

et al., 2008). Changes in biomarkers of gut epithelial barrier function or microbial translocation, such as circulating LPS, are correlated with immune dysfunction and found to strongly predict mortality in HIV-1 seropositive individuals (Hunt et al., 2014; Ouyang et al., 2020). Once LPS binds to the microglia, it puts them into an overactive state, damaging the cells over time and inadvertently causing the microglia to shed HIV-1 proteins from their latent reservoirs. Interestingly, elite controllers (people whose replication of HIV-1 is controlled without treatment) have microbiomes resembling uninfected individuals (Koay et al., 2018). Elite controllers are a unique example that illustrates how dysbiosis of the gut could be influencing the progression of HAND. Many different infections and diseases have been associated with dysbiosis, indicating either the disease has altered the microbiome directly or it is the body's response to compensate for the ongoing infection. There is a need to find a treatment that can simultaneously restore the gut microbiota and prevent over-activation of microglia, preventing shedding of the HIV-1 proteins into the brain.

The development of a treatment to prevent dysregulation of gut microbiota has led to an increase interest in phytoestrogen compounds as a potential therapeutic for HAND. Phytoestrogens refer to plant-derived compounds that mimic mammalian estrogen and act on estrogen receptors (ERs). The main groupings of phytoestrogens are polyphenols, flavonoids, and isoflavonoids. These can be further broken down, with the most studied being lignans, flavonols, and isoflavones (Patisaul & Jefferson, 2010). Phytoestrogen compounds can be found in most fruits and vegetables with the most abundant being in soybeans and other legumes (Kuhnle et al., 2009). Phytoestrogen compounds were originally investigated as a possible supplement for women who were postmenopausal

and had low estrogen levels (Casini et al., 2006; Basaria et al., 2009). Phytoestrogen treatment was found to improve self-reported quality of life and cognitive symptoms among postmenopausal women. These initial successful findings on phytoestrogen treatments has led to an increased interest in phytoestrogens, particularly if these improvements would translate to men as well. Improvements in both psychomotor speed and spatial memory were found in two separate studies looking at adult men (Thorp et al., 2009) and older, overweight adults (Anton et al., 2018) receiving isoflavone supplementation. Follow-up studies have found evidence for microglia inhibition as a mechanism by which phytoestrogens improve cognition and modulate brain activity (Baez-Jurado et al., 2019; Ariyani et al., 2019). To address the efficacy of phytoestrogens for treating symptoms of HAND, we investigated SE's therapeutic affects in HIV-1 Transgenic (Tg) rats.

HIV-1 Tg rats express 7 of the 9 genes associated with the HIV-1, providing a model for long-term HIV-1 viral protein exposure without active infection, resembling a state similar to HIV-1 positive individuals undergoing long-term cART therapy. Most if not all phytoestrogens require some metabolization by the gut for there to be any health benefit, and a metabolite that is of particular interest is SE. It is generally ingested as Daidzein or Genistein and is then broken down by gut bacteria where it eventually ends up as SE, which is one of the metabolites important for the health benefits associated with phytoestrogen consumption (Matthies et al., 2012). The term for someone who can metabolize daidzein into SE is equol producer and only encompasses ~40% of individuals who eat a primarily western diet with low soy (Igase et al., 2017). Due to the possibility of the HIV-1 Tg rats not having the bacteria required to metabolize specific

phytoestrogens, SE was selected as the treatment to bypass any confound that metabolization could cause. Additionally, the animals were ovariectomized to eliminate the potential confounding effects of endogenous estradiol. Estradiol is the natural estrogenic compound produced which can potentiate the reinforcing efficacy of cocaine (Hu et al., 2004), prevents HIV-1 induced neuronal damage (Bertrand et al., 2014, 2015; Heron et al., 2009), suppresses HIV-1 transcription (Cabrera-Munoz et al., 2012), and possibly affects the efficacy of SE treatment. The main goal was to investigate if SE protects or restores the microbiome from alterations associated with HIV-1. A secondary goal was to assess if these microbial changes in the gut microbiome were associated with changes in motivation. Additionally, intestinal tissue samples were taken at the end of the experiment, these were used to assess differences in microbial composition between genotypes. The current hypothesis was that SE will alter responding to cocaine on a PR schedule of reinforcement by protecting against HIV-1 associated alterations in microbial composition. Specifically, HIV-1 will be discriminated by an increase in the bacteria Prevotella and a decrease in the bacteria Akkermansia. SE treatment will restore these bacterial levels in HIV-1 Tg rats to control levels, and these alterations will be associated with behavioral changes in the rats.

CHAPTER 2

MATERIALS AND METHOD

2.1 ETHICS STATEMENT

Experiments were conducted in accordance with the recommendations listed in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The research protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of South Carolina (assurance number: A3049-01).

2.2 SUBJECTS

Animals (n=42; HIV-1 Tg=21, F344/N=21) were all ovariectomized (OVX) female rats purchased from Harlan Laboratories, Inc. (Indianapolis, IN). Ovariectomies were done at Harlan Laboratories before arrival at our lab. Animals were housed at targeted conditions of 21±2ºC, 50±10% relative humidity and a 12-hour light: dark cycle with lights on at 7:00am. Throughout the experiment animals had *ad libitum* access to a minimal phytoestrogen diet food (≤20ppm of phytoestrogen; Teklad 2020X Global Rodent Diet; Harlan Laboratories, Inc., IN, USA) and water, unless otherwise specified. Changing the rodent diet was done because standard rodent chow contains ~350ppm of soy and alfalfa (Harlan Laboratories, Inc., IN). Both soy and alfalfa contain phytoestrogens, soy specifically contains daidzein which can be converted by gut bacteria to SE, which is the drug intervention being used (Setchell & Cassidy, 1999; Setchell et al., 2005).

2.3 DATA COLLECTION

All microbiome data was collected as part of another study that sought to examine how SE impacts choice behavior in HIV-1 Tg animals when allowed to respond to either cocaine or sucrose. In brief, HIV-1 Tg and control F344/N animals were trained to lever press for sucrose and then cocaine while being treated with either SE or sucrose pellets. A mixed-design ANOVA was used to analyze the impact SE and genotype had on responding behaviors using both a fixed and progressive ratio schedule of reinforcement. At the end a choice behavior task was given to determine which reinforcer was preferred. Dendritic spine analysis was also conducted to determine differences in dendritic length and morphology between genotypes and treatment groups. Microbiome samples were collected at baseline and the end of the study.

2.4 DRUGS

Cocaine hydrochloride (Sigma-Aldrich Pharmaceuticals, St. Louis, MO) was weighed and dissolved in saline (0.9%). The solutions were made before the animals entered the operant chambers each day. Sucrose solutions were made fresh each testing day as well. SE (0.05mg) was purchased from Cayman Chemical (Ann Arboer, MI) and sucrose pellets (100mg) were purchased from Bio-Serv (Flemington, NJ). The SE pellets were added into 100mg sucrose pellets by Bio-Serv prior to sending them to the University of South Carolina, the combination of SE and the sucrose pellets were done to make sure both treatments were similar when administering SE or sucrose.

Additional drugs required for the use and maintenance of IV catheters included Heparin, purchased from APP Pharmaceuticals (Schamburg, IL), Gentamicin sulfate

from VEDCO (Saint Joseph, MO), butorphanol (Dolorex) from Merk Animal Health (Millsboro, DE), and Sevoflurane, USP from Baxter (Deerfield, IL).

2.5 TREATMENT

Animals were randomly assigned based on genotype to either SE (HIV-1-E = 11, F344/N-E=11) or sucrose (HIV-1-S=10, F344/N-S=10) treatment groups. Treatment groups received either 0.2mg SE (4 pellets) or 4 sucrose pellets per animal for 70 days. The dose of 0.2mg SE was used because it is consistent with what is used in human studies (~20mg for ~60kg human) and lower than the typical daily intake of phytoestrogens by elderly Japanese individuals (Akaza, 2012). Treatment started once daily for one week before the start of testing, and every day until catheterization. Following surgery, animals did not receive treatment for one week. Treatment resumed every other day after until the end of the 14-day cocaine self-administration progressive ratio task. Treatment did not occur during the cocaine dose-response or choice behavior tasks. Animals were pair-housed during the duration of the sucrose tasks but were placed in separate cages to ensure the consumption of the pellets. After catheterization took place, animals were single-housed and provided treatment in their home cage.

2.6 OPERANT CHAMBERS

Sound-attenuating enclosures housed operant chambers (ENV-008; Med Associated, St. Albans, VT) and were controlled by Med-PC computer software. Stainless steel was used for the front and back panels while the sides and top consisted of polycarbonate. The front stainless-steel panel contained a magazine that allowed a recessed 0.01cc dipper cup (ENV-202C) to deliver a solution through 5cm x 5cm opening following completion of a response requirement (ENV 202M-S). Two retractable active

metal levers (ENV-112BM) on either side of the receptacle were located 7.3cm above the metal grid floor. The cue light was a 28-V white light, 3cm in diameter and located above each active response lever but was never illuminated. Head entries into the magazine were detected using an infrared sensor (ENV 254-CB). There was another non-retractable lever located on the center of the back panel and a 28-V house light located above the lever. Responses on the center back lever were recorded but not reinforced. A syringe pump (PHM-100) was used to deliver intravenous cocaine infusions through a water-tight swivel (Instech 375/22ps 22GA; Instech Laboratories, Inc., Plymouth Meeting, PA), connected to the back mount of the animal using Tygon tubing (ID, 0.020 IN; OD, 0.060 IN) enclosed by a stainless-steel tether (Camcaths, Cambridgeshire, Great Britain). The infusion times of the pump were calculated by a Med-PC program according to the animal's bodyweight (weighed daily).

2.7 16S rRNA GENE SEQUENCING

A total of 81 fecal samples and 33 intestinal tissue samples were collected and sent to the Alkek Center for Metagenomics and Microbiome Research (CMMR) at Baylor College of Medicine in Houston, Texas for the microbiome analysis. Samples were collected in sterile tubes and stored at -80ºC until being shipped. For shipping, samples were placed in order and shipped on dry ice over-night with a sample manifest that included de-identified sample IDs and the tube positions. The analysis pipeline for the gene sequencing uses custom packages created by the CMMR to provide summary statistics, quality control measurements, multi-run reports and characterization of microbial communities across large numbers of samples. In brief, DNA was extracted from fecal samples using PowerMag Soil DNA Isolation Kit (MoBIO Laboratories, CA)

and intestinal tissue samples using Power Lyser Ultra Clean Tissue and Cell RNA kit (MoBio Laboratories, CA) both according to the manufacturer's protocol. 16S rRNA gene sequencing was done using the V4 primer region on the Illumina MiSeq program (Illumina, CA) to generate a baseline and the microbiome's response to SE. Gene sequences were clustered into operational taxonomic units (OTUs) based on the 16Sv4 region, and phylogenetic, alpha and beta-diversity changes were all reported.

2.8 COCAINE-MAINTAINED RESPONDING

Animals were first trained on an FR1 schedule of reinforcement (0.2mg/kg/inj) for 5 consecutive days, each session lasting 1 hour. Following completion of the response, the requirement resulted in a 20s time-out where the animals could not respond. In the next phase of the project, animals responded for IV cocaine on a PR schedule of reinforcement (0.75mg/kg/inj) for 14 consecutive days, each session lasting a maximum of 120 minutes. Completion of each ratio resulted in a 20s time-out.

2.9 DATA ANALYSIS

Data analysis was performed using SPSS (IBM Corporation, Armonk, NY) and Graphpad (Graphpad Software, Inc., La Jolla, CA). A 2x2 factorial design was used to analyze the bacterial changes of the microbiome with genotype (HIV-1 Tg vs. F344/N control) and treatment (SE vs. Sucrose) as between-subject factors. Diversity measures were calculated at baseline and after completion of treatment to determine pre- and post-changes from SE and if there was an interaction between genotype and treatment group. The alpha diversity analysis examined the bacterial richness and evenness within samples, which included OTUs (richness), Chao1 (estimator of diversity), and Shannon Diversity Index (richness and evenness). Beta diversity was assessed via weighted

UniFrac, which takes into account phylogenetic differences and taxonomic abundance, and unweighted UniFrac, which takes into account phylogenetic differences but not abundance. Next a principal coordinates analysis (PCoA) was used to summarize the microbiome compositional differences of each sample. A follow-up ANCOVA analysis was done to determine if the effect of SE on cocaine-maintained responses covaried with the microbiome composition. The ANCOVA analysis was done with day as the within-subjects factor, genotype and treatment as between-subject factors, and Prevotella_UCG_001, Alloprevotella, and Akkermansia as covariates. Lastly a discriminate function analysis was done to determine if the bacterial differences in Prevotella_UCG_001, Alloprevotella, and Akkermansia could be used to discriminate groups based on genotype and treatment received. Animals with potential patency issues (back mount leakage or inability to flush) were excluded from the analysis. Significant differences were set at $p \leq 0.05$.

CHAPTER 3

RESULTS

3.1 16S rRNA GENE SEQUECNING

The alpha diversity analysis suggested baseline differences between HIV-1 Tg and F344/N control rats ($p<0.051$) based on observed OTUs (Figure 3.1). PCoA was performed using the weighted UniFrac analysis of the beta diversity between genotypes and was found to be non-significant ($p\geq0.21$) at baseline (Figure 3.2). Beta diversity using the unweighted UniFrac analysis was found to be significant ($p<0.001$) at baseline, indicating that there is a phylogenetic difference but not a difference in abundance (Figure 3.3). Alpha diversity for the tissue samples was found to be nonsignificant ($p\geq0.37$) based on observed OTUs. The beta diversity of the tissue samples was also found to be non-significantly different between genotypes based on the weighted UniFrac ($p\geq0.28$) and unweighted UniFrac ($p\geq0.296$).

Alpha diversity measures for before and after treatment indicated no significant difference between HIV-1 Tg animals ($p\geq0.464$) or F344/N controls ($p\geq0.235$) based on observed OTUs (Figure 3.4). The results for the weighted UniFrac beta diversity measures were found to be significantly different for both the HIV-1 Tg animals ($p\leq0.007$) and the F344/N control animals ($p\leq0.047$), indicating a shift in phylogenetic makeup and taxonomic abundance in both genotypes after SE treatment (Figure 3.5). Similarly, when looking at the unweighted UniFrac beta diversity measures, both HIV-1

Tg animals ($p \leq 0.035$) and F344/N control animals ($p \leq 0.022$) had a shift in phylogenetic makeup after treatment.

3.2 MICROBIOME ALTERATIONS

The bacterial makeup of the gut microbiome was analyzed at the phylum and genus level. Baseline samples were found to be non-significant between genotypes at the phylum level. There were, however, baseline differences at the genus level between genotypes when looking at the top 20 abundant bacteria. Bacteroides, Alloprevotella, Streptococcus, Lachnoclostridium, and Tyzzerella all were found to be significantly elevated in HIV-1 Tg animals at baseline compared to F344/N control animals (Figure 3.6).

SE treatment had a main effect at the phylum level by significantly decreasing Bacteroidetes and increasing Tenericutes in both genotypes (Figure 3.7). Additionally, at the genus level SE treatment significantly lowered Alistipes in both genotypes. Within genotypes, F344/N control animals treated with SE experienced a significant increase in Streptococcus while HIV-1 Tg animals had a significant increase in Intestinbacter. HIV-1 Tg animals treated with SE also had significant decreases in Alloprevotella and Tyzzerella (Figure 3.8). Tissue samples were found to be non-significant the phylum level but at the genus level, Fusobacterium was found to be significantly higher in HIV-1 Tg animals compared to F344/N control animals (Figure 3.9).

3.3 BACTERIAL COVARIATES OF COCAINE USE IN S-EQUOL TREATED ANIMALS

Specific bacteria were chosen based upon the relevance they have to HIV-1 status. The ANCOVA analysis indicated that there were no within-subjects main effects of treatment, $F(13, 195)=1.372$, $p \geq 0.176$, genotype, $F(13, 195)=.106$, $p \geq 1.00$, or day,

$F(13, 195)=1.577$, $p\geq0.094$, at baseline samples. Prevotella_UCG_001, a subtype of Prevotella, was found to be a significant covariate of reinforcers per day, $F(13,169)=3.008$, $p\leq0.001$, after Greenhouse-Geisser corrections. However, Prevotella_UCG_001 was a significant covariate when comparing between-subjects factors, $F(1,15)=4.981$, $p\leq0.041$, indicating that Prevotella_UCG_001 covaried with genotype and treatment at baseline.

After treatment, bacterial samples were analyzed as well via an ANCOVA where a main effect of day was found to be significant, $F(13, 169)=4.120$, $p\leq0.001$. There were no main effects of genotype, $F(13, 169)=1.458$, $p\geq0.138$, or treatment, $F(13, 169)=1.583$, $p\geq0.094$. Within-subjects measures of Prevotella_UCG_001 was found to be significant, $F(13, 169)=1.979$, $p\leq0.025$. When looking at the between-subjects factors, there was a significant difference of intercept, $F(1,13)=4.783$, $p\leq0.048$, but no bacteria was found to significantly covary with the intercept.

Additionally, the change in bacteria abundance was used to see if the treatment by genotype effects on cocaine usage covaried with the difference in bacteria between the first and last sample collection. The analysis revealed no main effect for day but there was significance for an interaction of day by treatment by genotype, $F(13, 169)=3.426$, $p\leq0.001$. Prevotella_UCG_001 differences were also found to significant as a within-subjects covariate, $F(13,169)=4.316$, $p\leq0.001$. When looking at between-subjects, results were nearing significance for an interaction of treatment by genotype, $F(1, 13)=4.581$, $p\geq0.052$. Prevotella_UCG_001 differences however were found to significantly covary between-subjects, $F(1, 13)=5.568$, $p\leq0.035$, indicating that Prevotella_UCG_001 significantly varies across groups based on treatment and genotype.

3.4 BACTERIAL DISCRIMINANTS OF GROUP MEMBERSHIP

A discriminate function analysis was done to determine the best bacteria to differentiate between genotype x groups. A discriminant function analysis of the baseline bacteria revealed no discriminatory bacteria when looking at the sucrose treated groups, but when performing the discriminate function analysis on the SE treated groups, Alloprevotella was found to be significant, $F(1,10)=5.976$, $p\leq0.035$ (Figure 3.10), with 83.3% of original grouped cases classified. The significant discriminant function analysis indicates a significant difference in their abundance of Alloprevotella between SE groups at baseline.

When investigating the post-treatment samples, no bacteria were found to differentiate between sucrose-treated animals. There also wasn't a significant difference between SE treated animals, indicating a change in Alloprevotella towards similar abundances after treatment (Figure 3.11). To further investigate the change in Alloprevotella, a follow-up discriminant analysis was performed using the difference in pre- and post- bacterial compositions between genotype x treatment groups, which revealed Alloprevotella to be significantly different between the SE treated groups, $F(1,10)=5.301$, $p\leq0.044$ (Figure 3.12). The change in Alloprevotella was useful for classifying 83.3% of selected original grouped cases correctly between genotype x treatment groups.

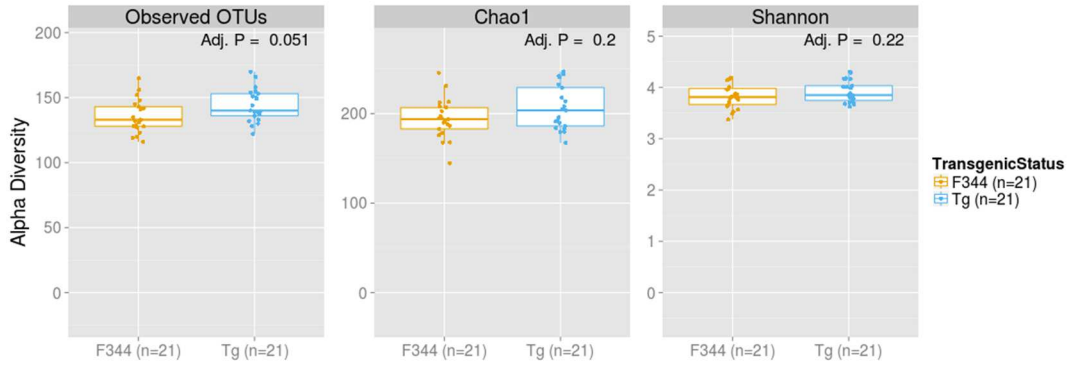

Figure 3.1 Baseline alpha diversity of gut microbiome between genotypes. Baseline differences in overall bacterial composition of the gut microbiome was measured via stool samples. Observed operational taxonomic units (OTUs) neared significance (p=0.051), indicating a difference in taxonomy between genotypes. Chao1 (p=0.2) and Shannon Index (p=0.22) estimates the overall diversity and richness, respectively. Both were found to be non-significant at baseline between genotypes.

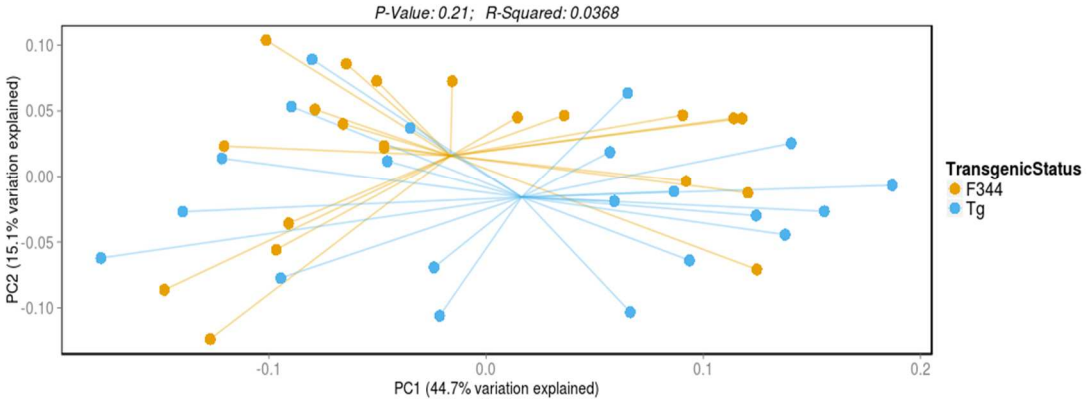

Figure 3.2 Baseline beta diversity of gut microbiome between genotypes. Beta diversity was represented via principle coordinates analysis using weighted unifrac measures of baseline bacterial composition. Weighted unifrac takes into account dissimilarities based on phylogenetic differences and taxonomic abundance, with no differences being found at baseline measures between genotypes (p=0.21).

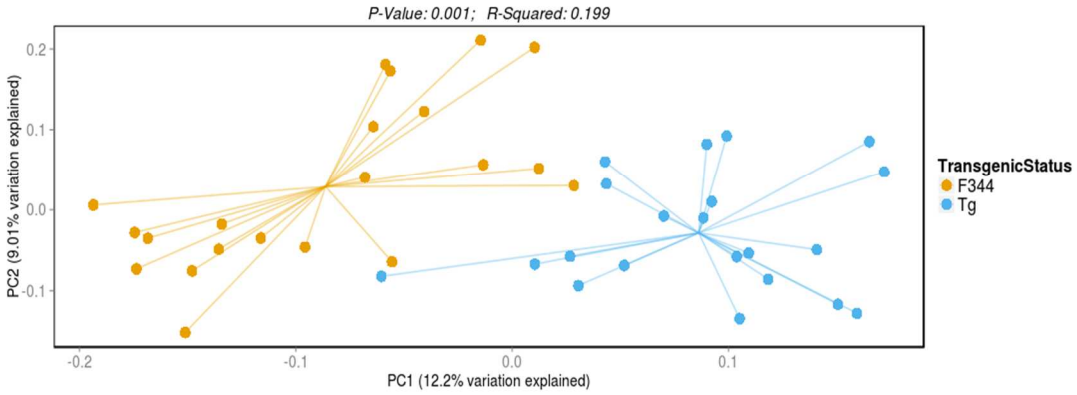

Figure 3.3 Unweighted unifrac baseline beta diversity of gut microbiome between genotypes. Beta diversity was represented via principle coordinates analysis using unweighted unifrac measures of baseline bacterial composition. Unweighted unifrac takes into account dissimilarities based on phylogenetic differences only, not abundance, and results indicate a significant dfferences being found at baseline measures between genotypes (p=0.001).

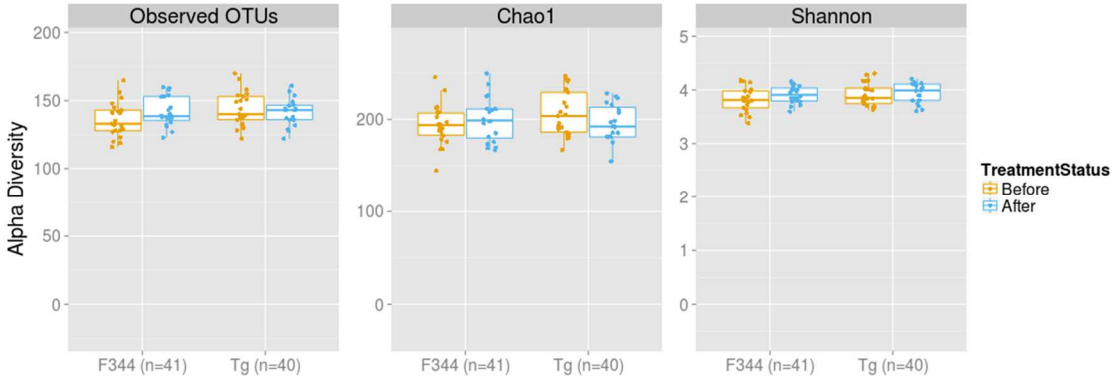

Figure 3.4 Alpha diversity differences of gut microbiome after treatment. Follow-up stool samples were analyzed to reveal treatment effects on the alpha diversity between genotypes. F344 animals' bacterial composition did not significantly differ after treatment, indicated by non-significant OTUs (p=0.235), Chao1 (p=0.404), and Shannon Index (p=0.481). Similarly, HIV-1 Tg animals did not alter in alpha diversity after treatment, OTUs (p=0.464), Chao1 (p=404), and Shannon Index (p=0.597).

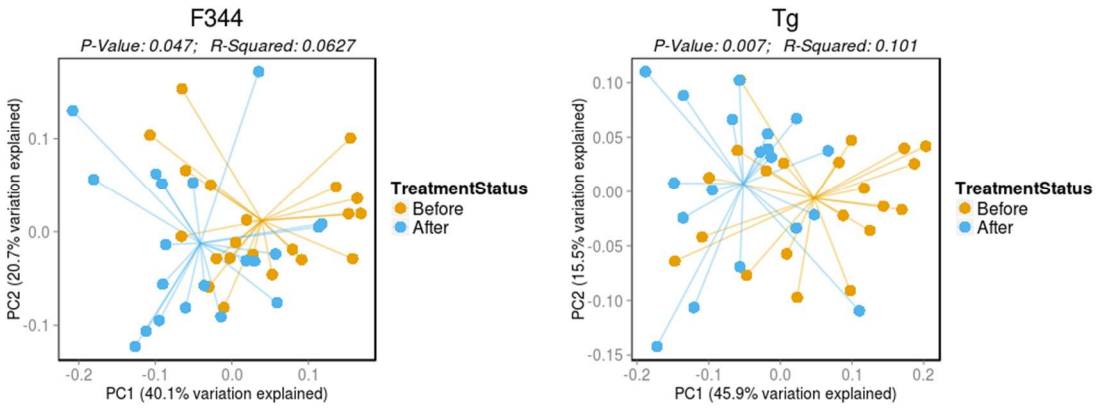

Figure 3.5 Weighted unifrac beta diversity of gut microbiome after treatment. Principle coordinates analysis of beta diversity change after treatment was done using weighted unifrac measures of bacterial composition. Results indicate significant alterations in both F344 (p=0.047) and HIV-1 Tg animals (p=0.007) after treatment, suggesting SE affects both genotypes' microbiome composition.

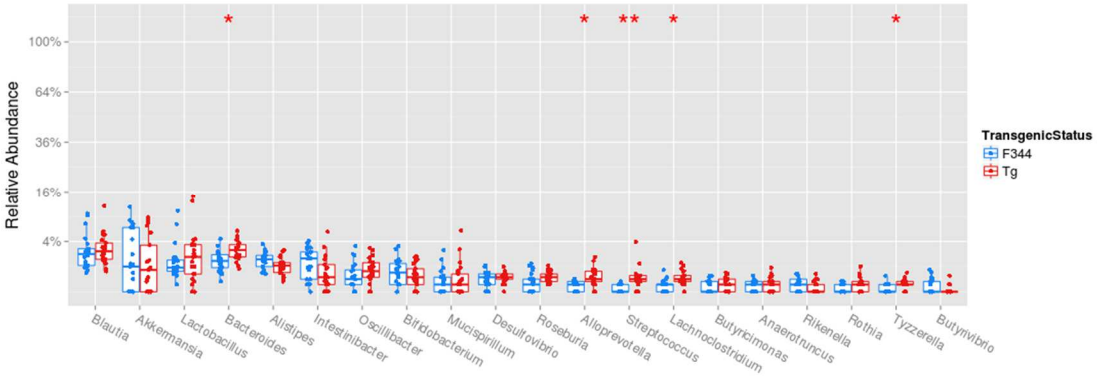

Figure 3.6 Baseline genus level taxonomic abundance in gut microbiome between genotypes. Genus level taxonomic abundance was summarized according to relative abundance of the top 20 genus from both genotypes. Significant baseline differences were found in Bacteroides, Alloprevotella, Streptococcus, Lachnoclostridium, and Tyzzerella with alpha at 0.05. Interestingly all bacteria were found to be higher within the HIV-1 Tg animals, indicating that these bacteria may be due to the HIV-1 transgene.

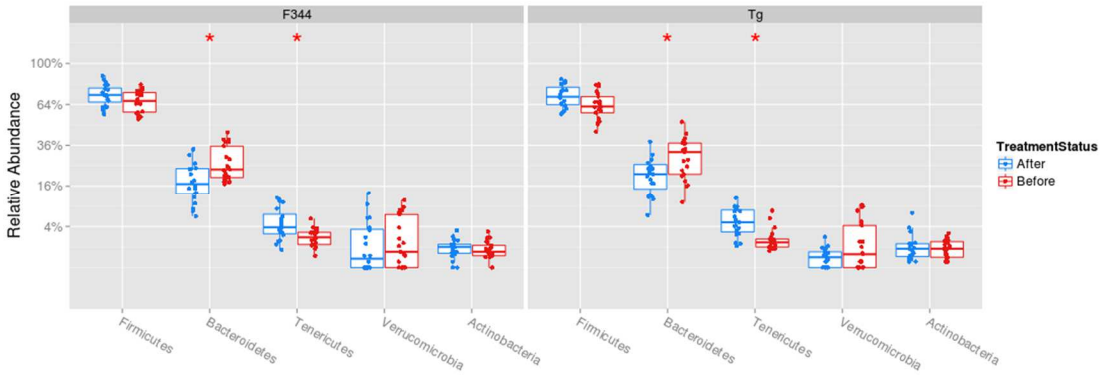

Figure 3.7 Phylum level taxonomic abundance summarized after treatment. Significant alterations were found in Bacteroidetes and Tenericutes with alpha at 0.05. The change in Bacteroidetes and Tenericutes was found in both genotypes, with Bacteroidetes being lower and Tenericutes being higher after treatment.

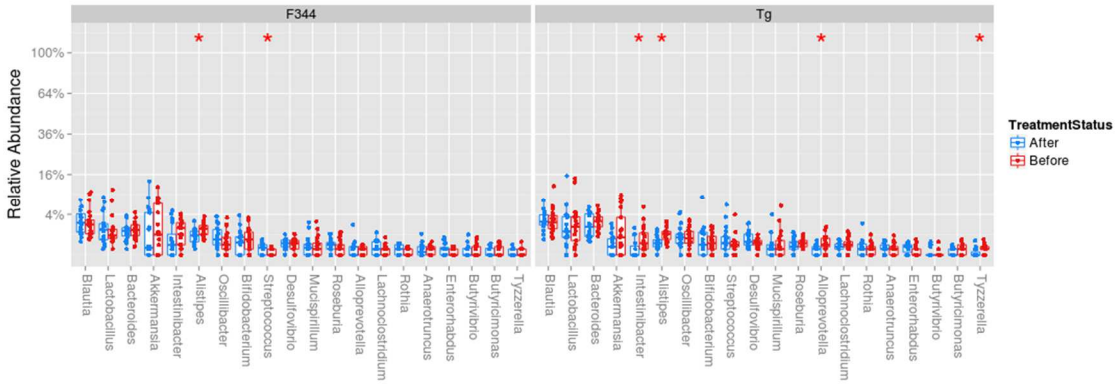

Figure 3.8 Genus level taxonomic abundance summarized after treatment. Significant alterations in Allistipes was found in both genotypes after treatment, both resulting in a reduction after treatment. Genotype x treatment differences were found with Streptococcus being increased in F344 animals while Intestinbacter, Alloprevotella, and Tyzzerella were reduced in the HIV-1 Tg animals, all at an alpha of 0.05.

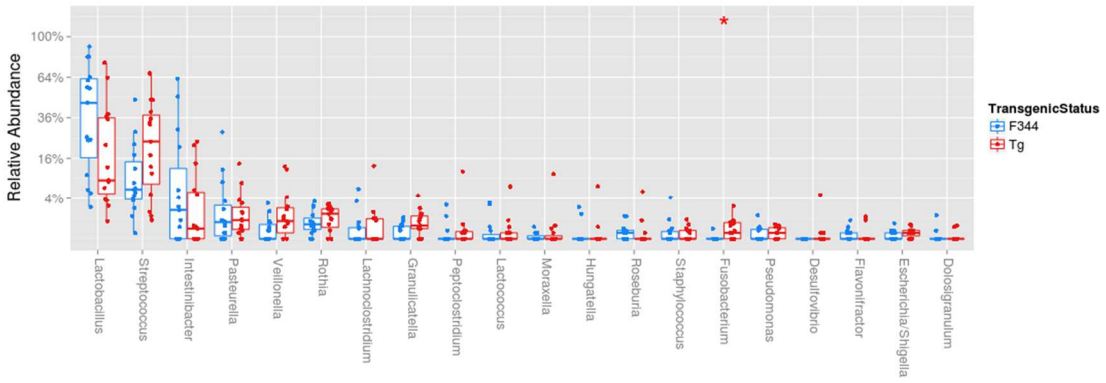

Figure 3.9 Genus level taxonomic abundance of tissue samples summarized. Significant differences were found in Fusobacterium between genotypes with HIV-1 Tg animals having increased abundance of Fusobacterium compared to the F344 animals at an alpha level of 0.05.

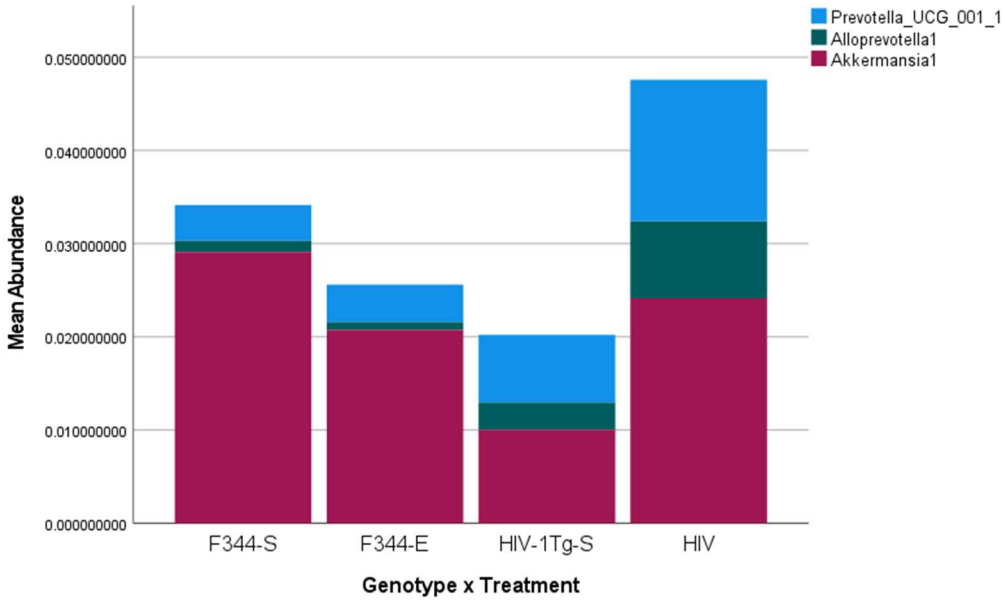

Figure 3.10 Baseline abundance differences in Prevotella, Alloprevotella and Akkermansia. Discriminant analysis was done to determine if HIV-1 relevant bacteria could be used to separate genotype x treatment groups. At baseline, Alloprevotella was found to be nearing significance between genotypes x treatment groups (p=0.035). Prevotella_UCG_001 and Akkermansia were not significant even though the graph suggests that Prevotella_UCG_001 could be significant between genotypes.

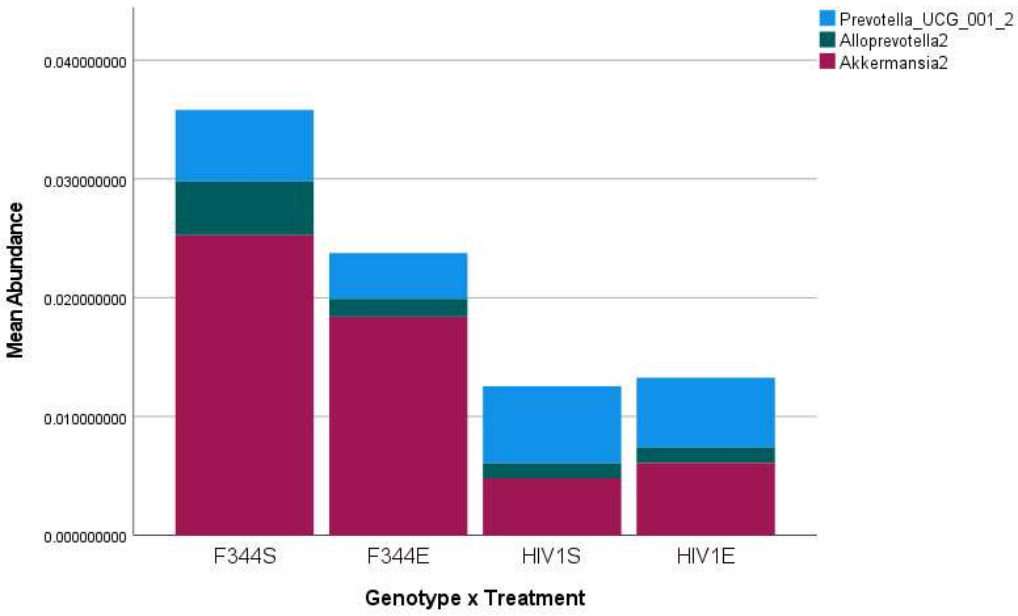

Figure 3.11 Abundance differences in Prevotella, Alloprevotella, and Akkermansia after treatment. Discriminant analysis was done to determine if HIV-1 relevant bacteria could be used to separate genotype x treatment groups at the end of the study. At the end point, no difference was observed between Prevotella_UCG_001, Alloprevotella, or Akkermansia. The graph suggests that Akkermansia lowered based on HIV-1 Tg status and SE did not alter this change.

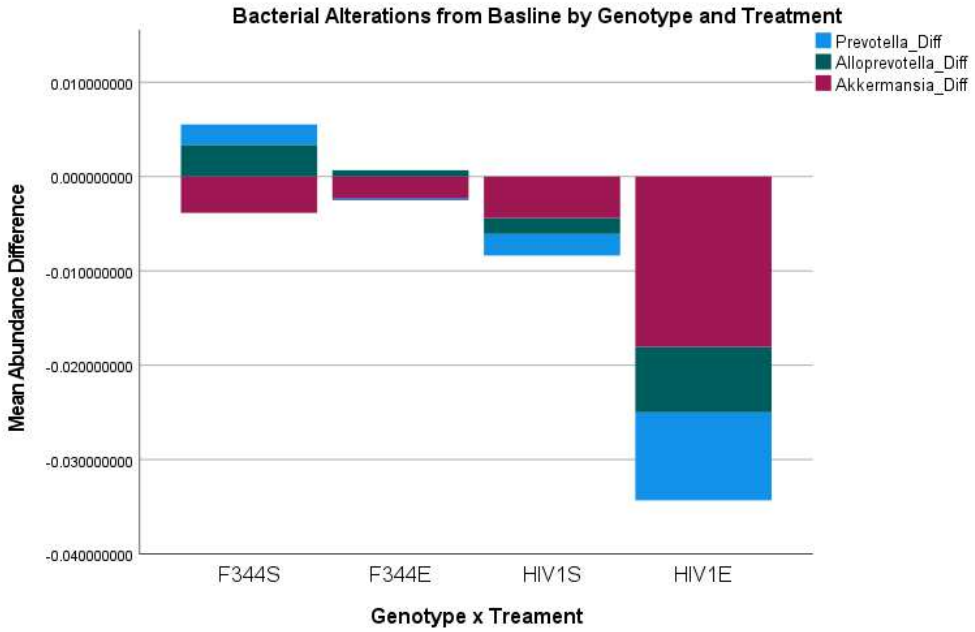

Figure 3.12 Change in abundance of Prevotella, Alloprevotella, and Akkermansia after treatment. Discriminant analysis was done to determine if pre- and post- alterations in HIV-1 relevant bacteria could be used to separate genotype x treatment groups at the end of the study. Alloprevotella was found to be significant indicating that it could be used to discriminate genotype x treatment effects, at an alpha of 0.05. The graph suggests that bacterial changes were mostly observed in HIV-1 Tg animals, with Akkermansia experiencing a large decrease in the SE treated HIV-1 Tg group, indicating a genotype x treatment effect of SE.

CHAPTER 4

DISCUSSION

The gut microbiome of HIV-1 seropositive individuals is characterized by an overall reduction in microbiota diversity, and at the genus level, a significant reduction in Akkermansia and an increase in Prevotella when compared to healthy individuals (Mutlu et al., 2014; Ling et al., 2016). In the present study, HIV-1 Tg rats were found to have a similar increase with a higher abundance of Prevotella_UCG_001 in the gut microbiome, but Akkermansia was not found to be significantly different even though there seems to be a reduction occurring based on figure 3.12. Previous studies have reported that even with cART, long-term HIV-1 infection leads to a significant loss of Akkermansia compared to healthy individuals (Brenchley et al, 2004, 2006; Mutlu et al., 2014). The finding of reduced Akkermansia in humans may indicate that given more time, HIV-1 Tg animals could eventually have a statistically significant reduction in Akkermansia. The trending reduction in Akkermansia ultimately is an important finding as it suggests that the HIV-1 Tg rat could be a useful model for studying a state of dysbiosis similar to that found in HIV-1 seropositive humans.

HIV-1 Tg rats exhibit neurocognitive deficits in prepulse inhibition, learning, sustained and selective attention and are associated with synaptodendritic alterations of medium spiny neurons (Roscoe, Mactutus, & Booze, 2014; McLaurin, Booze, & Mactutus, et al., 2016, 2017; McLaurin et al., 2020). The current study highlights that in

addition to these deficits, there are changes in the microbiome composition between HIV-1 Tg rats and F344 control animals that could be influencing these deficits through interactions in the gut-brain-microbiota axis. HIV-1 Tg rats were found to have elevated Prevotella levels, a bacteria that possess LPS on its outer membrane, an endotoxin that can damage the epithelial barrier and weaken the BBB (Mutlu et al., 2014; Wang, Y., et al., 2020). Increased LPS could be one of the main potential mechanisms that HIV-1 Tg-associated dysbiosis is worsening symptoms of HAND. LPS, once pass the BBB, can bind to the surface of microglia, increasing immune activation and putting the cell in an overactive state (Wang, E. J., et al., 2003; Wang, H., et al. 2008).

The current study investigated whether specific bacteria covaried with the behavior of lever pressing on a PR schedule of reinforcement that lasted 14 days. Prevotella_UCG_001, a subset of Prevotella, covaried with lever pressing for cocaine when taking into account genotype and treatment. The change in bacteria depended on the genotype, their treatment, and their cocaine intake, which suggests that there is an interaction between SE, cocaine, or both with the genotype due to the change not being consistent between groups. The study also sought to determine if any of the specific bacteria could be used to differentiate the effects of genotype and treatment on lever pressing. Interestingly, for the discriminant function analysis, a different bacteria differentiated between the groups, Alloprevotella, still another subset of Prevotella. The present finding indicated a genotype x treatment effect due to the groups treated with sucrose not being able to be differentiated between, but the SE treated groups were able to be. Differentiating SE treated groups by Alloprevotella abundance remained true for

only the baseline data, as SE appeared to return Alloprevotella in the HIV-1 Tg back to an abundance that was indistinguishable from the control animals.

Together these findings indicate that SE can modulate the gut microbiome composition, and these alterations have a direct link to the behavioral changes being observed in the animals. It also reiterates that phytoestrogens appear to interact differently with ill vs. healthy animals, meaning that the use of phytoestrogens for a cognitive or behavioral treatment may not benefit everyone the same (Neese et al., 2012; St. John et al., 2014). It seems that if individuals are already experiencing a deficit in cognition, then phytoestrogens may be restorative, but if it is a healthy individual then phytoestrogen treatment may impact them negatively. Potentially it could worsen cognition for healthy individuals, indicating a biphasic action of phytoestrogens that depends on the health status of the individual being treated.

Limitations to the present study are that sample collections were done only as pre and post samples, meaning that there is no way to separate the effects of SE vs. cocaine on the microbiome. Ideally a sample collection between the onset of SE and cocaine would allow understanding of how SE affected the microbiome's composition before the start of cocaine. Additionally, the antibiotic Gentamicin from APP Pharmaceuticals (Schamburg, IL) was given following the implantation of the catheter, causing another confound as antibiotics can quickly alter the microbiome's composition. Notably, all antibiotic treatment was administered to all rats, and there were no instances of antibiotic treatment (e.g., Cefazolin) for illness during the study.

Overall, SE appears to be a potential therapeutic for HIV-1 associated dysbiosis by modulating Prevotella_UCG_001 and Alloprevotella towards an abundance similar to

the control animals. Reducing Prevotella would lead to less surrounding LPS circulating in the gut and therefore lower the potential escape of LPS to the blood and the BBB. The bacterial alterations indicated an interaction between SE and the genotype, supporting the need for follow-up analysis on the efficacy of phytoestrogen use, specifically in diseased states. Further investigations also need to be done to discover the specific mechanism of action that SE uses to (1), alter the microbiome composition, and, (2), modulate neurocognition and behavior. It will also be important to address, (3), why an individual's state of health seems to modulate SE's efficacy in regard to microbiotic gut health and neurocognition.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

The current study investigated the effect SE has on both cocaine-maintained responding and gut microbiome composition in HIV-1 Tg rats and F344 control animals. The study found specific bacteria that covary along with lever pressing for cocaine and alterations strong enough to allow for accurate classification of group membership. Taken together, there seems to be an important interaction between the HIV-1 transgene and the gut microbiome, and it appears to be similar to what is seen in human individuals. The gut-brain-microbiota axis has been reported to influence many behavioral and cognitive functions, suggesting the importance of improving dysbiotic states that goes beyond possible local damage to the gut but to wide arching effects.

Phytoestrogen compounds such as SE have gained much interest in recent years for their efficacy in improving cognition and wellbeing in individuals suffering from an illness. In regard to HIV-1, SE and other phytoestrogens have the potential to migrate from the gut microbiome to the brain where they can bind to microglia via estrogen receptors and g-protein-coupled receptors, with the most common one being GPR30 (Bertrand et al., 2014; Ariyani et al., 2019). Suggestions have been made as to how phytoestrogens can improve cognitive function and most include something to deal with inflammation and/or microglia activation. Phytoestrogens bind to microglia to reduce

overactivation and inflammatory markers, but how the reduction occurs is still up for debate.

Future directions would include a follow-up cell culture study to investigate the effects of Prevotella bound LPS on microglial function. It would then subject the cells to SE treatment, allowing for an investigation into how SE can directly modulate microglial function and by what mechanisms is SE's effect occurring. Having a greater understanding of the mechanism may allow for more optimal treatment paradigms, either through altering concentrations or possibly allowing for better selection of phytoestrogen compounds that may have higher affinity for estrogen receptors. It would also provide a novel treatment for HIV-1 associated dysbiosis and symptoms of HAND, which as of now has no supported treatment and continues to affect approximately 50% of seropositive individuals, regardless of adherence to cART.

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