THE EFFECTS OF NICOTINE ON THE GUT MICROBIOME AND THE SERUM METABOLITE PROFILE OF MICE

by

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(Under the Direction of Kun Lu)

ABSTRACT

Nicotine exposure from tobacco smoke and electronic cigarettes has become a major public health problem. As both nicotine and gut microbiome are known to mediate metabolism in the body, it is of interest to know how nicotine exposure can affect the gut microbiome and the serum metabolite profile. Male and female C57BL/6 mice were exposed to 6 mg/L (low dose) and 60 mg/L (high dose) nicotine in drinking water for 13 weeks. High dose had a more different gut microbial profile and more changes to the serum metabolite profile than low dose. 16S rRNA sequencing found nicotine changed gut microbial community structure mainly at the family and genus level, with some gender differences. Gas-chromatography mass spectrometry serum profiling found gender-differences to changes in several metabolites. Our results are the first to exclusively look at the effects of nicotine on the mice gut microbiome and the serum metabolite profile.

INDEX WORDS: Nicotine, Environmental Exposure, Metabolomics, Gut Microbiome

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DEDICATION

I dedicate this thesis to all scientists and researchers.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Tobacco, Smoking, and Nicotine

Tobacco (genus: *Nicotiana*), having been cultivated as a cash crop in the Americas as early as 1615, is currently a 2.3 billion dollar/year industry in the United States with approximately 7% of the total world leaf tobacco originating from U.S. fields ([Anonymous] 2014a). Tobacco leaves are typically cured and then formed into cigarettes or cigars to be smoked, but can also be made into smokeless products such as chewing tobacco ([Anonymous] 2014a).

Smoking of tobacco is considered to be the "leading preventable cause of disease, disability, and death" in the United States, with 16 million Americans per year suffering from the effects of tobacco smoke and 1 in 5 of all yearly U.S. deaths being attributed to smoking (National Institute on Drug Abuse). According to the most recent published data, the estimated health care cost of smoking in the United States was found to be approximately $72.7 billion (Miller et al. 1998).

Clear epidemiological and experimental evidence suggests that tobacco smoke can cause lung cancer and stroke (Cornfield et al. 2009) (Hankey 1999) and can place smokers at risk for other disorders such as osteoporosis, blindness, and autoimmune diseases (U.S. Department of Health and Human Services)

A major reason for why smoking is so harmful is due to a component in tobacco known as nicotine (1-Methyl-2-(3-pyridyl)pyrrolidine), a colorless to pale-yellow oily chemical that is

known to make tobacco addictive (National Institute for Occupational Safety and Health (NIOSH) Education and Information Division) ([Anonymous] 2014b). The amount of nicotine within cigarettes and cigars can vary, with anywhere between 6 to 28 milligrams per cigarette and 6 to 335 mg per cigar (Henningfield et al. 1999) (Taghavi et al. 2012). Moreover, a recent study has shown that the nicotine yield of American cigarettes has been increasing in the period between 1998 and 2012 (Land et al. 2014), which increases the risk for nicotine dependence.

Electronic Cigarettes and Nicotine

Electronic Cigarettes (E-Cigs), a battery-operated product that vaporizes fluid containing nicotine and other chemicals, are a relatively new product that was introduced to the American market in 2007 (U.S. Department of Health & Human Services) (Orellana-Barrios et al. 2015). The average nicotine content of E-Cigs was previously found to be approximately 11 mg/ml, and the average exposure to nicotine from an E-Cig was found to be about 0.38 mg/kg bw/day (Hahn et al. 2014).

E-Cigs have become a major problem for regulators, especially due to the perception that E-Cigs are a healthier alternative to cigarettes and/or may even support smoking cessation (Oh and Kacker 2014). In fact, several high-profile studies have found that E-Cigs which deliver nicotine lowered the desire to smoke (Bullen et al. 2010) (Polosa et al. 2011), and one study found no adverse change in lung function as a result of exposure to E-Cigs (Flouris et al. 2013). However, the evidence of any human health effects and especially the beneficial effects of E-Cigs is inconclusive (Callahan-Lyon 2014). There is considerable variation in the components, design, and type of aerosol fluid amongst the various E-Cigs currently on market which could result in differences between E-Cigs in the amount of nicotine delivery; these variations present a serious consumer risk of exposure to high amounts of nicotine (Brown and Cheng 2014).

Currently, the United States Food and Drug Administration (FDA) has not approved E-Cigs for any kind of a therapeutic use (U.S. Department of Health & Human Services).

Acute Effects of Nicotine

Nicotine is known to harm human health. For example, eye or skin exposure to nicotine can lead to irritation and redness all the way up to inflammation. Inhalation and Ingestion of nicotine can initially lead to symptoms such as nausea and vomiting and progress to much more serious disorders such as abnormal heart rhythms and paralysis (National Institute for Occupational Safety and Health (NIOSH) Education and Information Division).

The toxic lethal dose of nicotine has been controversial, especially because there isn't any inhalation toxicity data available for nicotine (National Institute for Occupational Safety and Health Education and Information Division 2014). The Centers for Disease Control states that the fatal human dose for nicotine in humans is estimated to be between 50-60 mg, from which an IDLH (immediately dangerous to life or health) value of 5 mg/m^3 was calculated (National Institute for Occupational Safety and Health Education and Information Division 2014). However, an editorial challenged this lower dose and suggested that published cases of nicotine toxicity show the actual fatal toxic dose in humans to most likely be greater than 0.5 g (Mayer 2014).

Neural and Carcinogenic Effects of Nicotine

It is well known that chronic exposure to nicotine acts on nicotinic acetylcholine receptors in the brain, creating nicotine dependence which causes harmful psychological and physiological effects (Gurusamy and Natarajan 2013) (Buisson and Bertrand 2001). Specifically, a *C. elegans* model showed an upregulation of several genes related to the nicotinic acetylcholine receptors (Polli et al.) due to nicotine exposure, while physical neurological changes were

observed after chronic nicotine exposure in both rats and mice (Morud et al. 2015) (Renda and Nashmi 2014). Nicotine dependence has also been shown to be transferrable from nicotine-exposed mothers to their offspring, making nicotine teratogenic (Vaglenova et al. 2004) (Schneider et al. 2010).

Additionally, several studies have shown how nicotine and its metabolites can induce mutagenesis and promote cancer cell survival (Grando 2014). One study in specific showed how pancreatic ductal adenocarcinoma (PDAC) cells chronically exposed to nicotine triggered a nicotinic-acetylcholine receptor (nAChR)-driven autocrine catecholamine loop that stimulated proliferation of these cancerous cells (Al-Wadei et al. 2012) while another study in the rat brain had found that chronic exposure to nicotine negatively altered genes that had to do with cellular apoptosis and neuroprotection (Campain 2004). In a study using a xenograft model, nicotine was found to promote colon cancer tumorigenesis by activation of phosphorylated epidermal growth factor receptor and c-Src proto-oncogene (Ye et al. 2004).

Protective Effects of Nicotine Exposure

Though nicotine has a lot of negative effects, there are several important protective or beneficial effects of nicotine exposure that should be pointed out. For one, nicotine has been shown to reduce body weight by increasing the rate of metabolism and by reducing appetite (Yildiz 2004). Nicotine also has been tested as a treatment for stress and anxiety and has been discussed as a potential treatment for Parkinson's disease (Yildiz 2004) (Barreto et al. 2014), and a recent study described an increase in lifespan and decrease in olfactory and motor deficits in a Drosophila Parkinson's model (Chambers et al. 2013).

Several studies show that nicotine has a beneficial effect towards ulcerative colitis. In a DSS (dextran sodium sulfate) mouse model of ulcerative colitis, low doses of exposure to

nicotine showed anti-inflammatory effects as well as decreases to the severity of the colitis (AlSharari et al. 2013). Another study with an ulcerative colitis mouse model found that nicotine exposure suppressed colitis-associated tumorigenesis with observed decreases in the number and size of colonic tumors in nicotine-treated mice that had colitis-associated cancer compared to controls (Hayashi et al. 2014).

Nicotine Metabolism in the Body

Nicotine metabolism in humans has been exhaustively reviewed (Hukkanen et al. 2005) (Benowitz et al. 2009). The major site for nicotine metabolism is in the liver (Hukkanen et al. 2005). Most (around 70-80%) of absorbed nicotine is transformed in a two-step process to cotinine, while the remaining amount of absorbed nicotine is transformed into 5 other "primary" metabolites, including nicotine N'-oxide, whose metabolism was found to be mediated by intestinal bacteria (Hukkanen et al. 2005) (Beckett et al. 1970). Six metabolites that originated from cotinine were observed in urine, with 3′-Hydroxycotinine and its glucuronide conjugate being the major metabolites detected (Hukkanen et al. 2005)

Various studies have determined that CYP2A6, a cytochrome P450 enzyme, is the most important enzyme involved in the metabolism of nicotine in humans, with CYP2A5 being the analogue in mice (Ingelman-Sundberg 2004) (Siu and Tyndale 2007). CYP2A6 inactivates nicotine by turning it into cotinine; thus, genetic variations to CYP2A6 that may knock-out or hinder its function can influence the amount of nicotine in the body and thus there is significant inter-individual variability in nicotine toxicity (Mwenifumbo and Tyndale 2007; Nakajima 2007). In fact, treatments to induce the production of CYP2A6 have shown promise in detoxifying nicotine to treat the harmful metabolic effects that occur (Kim et al. 2014).

Nicotine and Metabolic Disease

Nicotine exposure can have both negative and positive metabolic effects, and it is well known that oxidative stress is a major characteristic of metabolic syndrome (Roberts and Sindhu 2009). A 2004 review detailed how nicotine could decrease the number of free-radical scavenging enzymes which in turn could promote the generation of harmful free-radicals that can cause oxidative stress (Yildiz 2004). In both humans and mice, nicotine can also mediate the activation of oxidases, enzymes that produce reactive oxygen species, to promote cellular and mitochondrial oxidative stress (Zanetti et al. 2014) (Cano-Domínguez et al. 2008).

Nicotine is known to induce weight loss, and the mechanism for this was found in a rat model to be through the inactivation of hypothalamic AMP-activated protein kinase (HAMPK), which is involved in maintaining energy balance and whose activation promotes feeding and suppresses energy expenditure in brown fat tissue (Martínez de Morentin et al. 2012). In an obese rat model, the same group confirmed that nicotine reduced weight but that it also improved obesity-linked metabolic disorders as shown in a decrease in fatty liver and improved serum lipid profile (Seoane-Collazo et al. 2014). Another study also found that nicotine enhanced insulin sensitivity (Xu et al. 2012). On the other hand, recent studies have shown how prenatal nicotine exposure (PNE) could promote metabolic disorders, with one study showing an increase in circulating and hepatic triglycerides due to PNE (Ma et al. 2014) and another study showing how PNE further exacerbated increases to serum glucose and blood lipids (trigylcerides and total cholesterol) in male mice that had been stressed and fed a high-fat diet (Xu et al. 2013). Though normal exposure to nicotine appears to create a protective effect, it has been suggested that PNE

alters metabolic programming to increase the susceptibility to metabolic disorders (Xu et al. 2013).

Nicotine-derived products may also have various metabolic effects. Though some studies have suggested that the formation of cotinine essentially detoxifies nicotine (Mwenifumbo and Tyndale 2007) , a higher plasma cotinine level itself has been associated with metabolic disorders such as insulin resistance and hyperinsulinemia, and high levels of "bad" cholesterol and triglycerides(Eliasson et al. 1996) (Kang and Song 2015). Another nicotine-derived product, Tobacco-specific nitrosamines (TSN) are carcinogenic metabolites which have been found to inhibit insulin signaling and promote inflammation in conjunction with ethanol exposure, induce cellular apoptosis, and reduce lung phospholipids that serve as a surfactant to make breathing easier (Veldhuizen and Possmayer 2004) (Vijayaraj et al. 2014) (Zabala et al. 2015) (Wei et al. 2015). Additionally, metabolites derived from TSN are also problematic, with some of them having been shown to have the ability to bind to macromolecules and promote cancer development (Hecht et al. 1994).

Nicotine Changes the Metabolite Profile

An early study using a rat model found that nicotine administration increased the concentration of cholesterol, phospholipids and triglycerides in the serum, creating a risk for the development of atherosclerosis (Latha et al. 1993). Interestingly, the authors of this study stated how such changes were similar to lipid profile changes seen after cigarette smoke exposure (Latha et al. 1993)

The development of metabolomics has spurred some studies which shed light on how nicotine can affect the metabolite profile and thus metabolism in the body. Metabolomics involves using technology such as nuclear magnetic resonance (NMR) or mass spectrometry

(LC-MS, GC-MS) to detect for metabolites in a biofluid or organic sample and to then develop a metabolite profile from this (Kang et al. 2015) (Bujak et al. 2014). Though serum/blood is commonly profiled, other biofluids or samples such as urine, saliva, and feces can also be profiled using metabolomics (Bujak et al. 2014). A metabolite profile can allow for researchers to see how toxicants or other kinds of environmental exposure may change the body, and in this function can serve as a useful biomarker for many kinds of disease states (Cox et al. 2014).

Some studies have used metabolomics profiling to find out how nicotine exposure can change the metabolite profile in the body in ways that can either promote or exacerbate metabolic disorders. The first was a study from a German human cohort where the intent was to look at metabolomic changes due to cigarette smoke but specifically where the effects of nicotine in cigarette smoke was analyzed (Wang-Sattler et al. 2008). A total of 198 metabolites were analyzed from the sera of 283 individuals who either smoked, used to smoke, or never smoked at all; several glycerophospholipids, which are nicotine-dependent metabolites, were changed in smokers compared to the other groups (Wang-Sattler et al. 2008). Though most of these glycerophospholipids were upregulated, three acyl-alkyl-phosphatidylcholines were downregulated, which the authors surmised occurred due to the downregulation of an enzyme known as alkylglycerone phosphate synthase (alkyl-DHAP) which was previously found to be downregulated in smokers (Wang-Sattler et al. 2008). Another study of the serum of a "smoker" mouse model not only detected an upregulation of nicotine metabolites such as pyrrolidine and 4-hydroxy-4-(3-pyridyl)-butanoic acid but also found similar upregulation of glycerophopholipid metabolites (Cruickshank-Quinn et al. 2014). The increase in these lipids could be a result of cellular membrane damage, which smoking has been found to do (Yildiz et al. 1998); nicotine itself, through lipid peroxidation, was found in another study to induce damage to red blood cell

membranes at lower concentrations while cotinine was found to cause dose-dependent increases in red-blood cell damage (Asgary et al. 2005).

Nicotine has also been found to affect the metabolite profile of the brain. An early study in a rat model employed NMR to find that acute nicotine exposure caused the decrease of several phospholipids such as glycerophosphocholine and phosphocholine (Pettegrew et al. 2001). In this case, a decrease could suggest breakdown of these substances to form choline and eventually acetylcholine, a neurotransmitter which is important to signaling in the brain and which has been found to be increased in the brain as a result of nicotine exposure (Armitage et al. 1969) (Rowell and Winkler 1984) (Pettegrew et al. 2001). To support this claim, other literature has suggested that during nicotine exposure, free choline required for acetylcholine formation is primarily recruited from membrane phospholipid metabolism (Lockman et al. 2006), meaning that nicotine exposure may cause a breakdown in brain cellular membrane phospholipids. Additionally, a recent NMR metabolomics analysis of a nicotine-exposed mouse model confirmed these previously researched increases to acetylcholine as well as metabolites suggesting brain cellular membrane degradation, and additionally found decreases to anti-oxidative metabolites and changes to energy and amino acid metabolites (Li et al. 2014).

Introduction to the Gut Microbiome

The gut microbiome is emerging as a "hidden metabolic organ" whose functions have an immense importance to the body (Khan et al. 2014). The human microbiome is composed of approximately 10-100 trillion microorganisms, primarily bacteria but which also include archaea, viruses, fungi, and protozoa (Ley et al. 2005) (Sommer and Backhed 2013). The development and refinement of culture-free genetic profiling techniques, specifically 16S rRNA sequencing, has spurred studies into the gut microbiome in the past 10 years (Robinson and Young 2010).

These techniques allowed for the determination that the two dominant bacterial phyla in the gut microbiome are the Bacteroidetes and the Firmicutes (Turnbaugh et al. 2006). An international sample of the gut microbial profile classified the gut microbiome as primarily comprising of three major enterotypes, named by the genus that dominates each enterotype: *Bacteroides*, *Prevotella*, and *Ruminococcus* (Arumugam et al. 2011).

The Gut Microbiome and Disease

The gut microbiome has approximately 150-fold more genetic capacity than the human genome, making it an attractive but understudied candidate as very important to the development of various diseases (Tilg and Kaser 2011). The gut microbiome in particular has been implicated in diseases such as obesity, diabetes, inflammatory bowel disease (IBD), and even autism.

Obesity has been associated with a decrease in the diversity of the gut microbiome (Turnbaugh et al. 2009), but also creates other important changes to the gut microbial profile. The cecal microbiota of obese mice contains more Firmicutes and fewer Bacteroidetes than non-obese controls (Ley et al. 2005) and similar results have been shown in humans suggesting that obesity has a microbial aspect to it (Ley et al. 2006). Other studies have furthered this claim, showing that the gut microbiome of both humans and rodents have an increased capacity for energy harvest which promotes obesity development (Turnbaugh et al. 2006) and that this kind of gut microbiome, when transplanted into germ-free mice, can promote energy harvest and obesogenic conditions within those mice (Turnbaugh et al. 2006).

With diabetes, studies have shown a decrease in the beneficial gut-microbe *Faecalibacterium prausnitzii* in the presence of type-2 diabetes in humans (Furet et al. 2010). The gut microbiome is also associated with the development of type-1 diabetes, where immune-comprised mice that were germ-free developed diabetes but specific-pathogen-free mice did not,

suggesting that the composition of the commensal gut microbiome has a role in influencing the development of diabetes (Tai et al. 2015). This further makes sense in light of a study that found transferring the microbiota from lean human donors to those with diabetes decreased insulin resistance in the recipient patients (Vrieze et al. 2012).

Irrritable bowel syndrome (IBD) is a condition in which there is chronic inflammation of the gastrointestinal tract which causes symptoms such as persistent diarrhea and abdominal pain, and can even put patients at risk for colon cancer (National Center for Chronic Disease Prevention and Health Promotion 2014). There are two kinds of diseases that IBD encompasses, one which is Crohn's disease (CD) and the other which is ulcerative colitis (UC); CD primarily affects the distal ileum and the colon while UC primarily affects the colon (Chandel et al. 2015). Animal models show some specific perturbations to the gut microbiome as a result of IBD, such as a decrease in gut microbial biodiversity and a specific decrease in *Bacteroides* in ulcerative colitis patients, and though several animal studies support a causal role of the microbiota in IBD, it has been hard to observe a clear relationship in humans (Nell et al. 2010) (Hansen and Sartor 2015) (Noor et al. 2010). Nevertheless, treatments such as probiotics are being examined as a way to modulate the gut microbiome to potentially cure or lessen the effects of IBD (De Greef et al. 2014).

Emerging evidence even suggests that the gut microbiome can modulate the brain, and several studies have suggested an association between autism and the gut microbiome (Louis 2012). Gut-microbiome metabolites such as proprionate were shown to elicit autistic-like symptoms in mice, while human studies reported differences in total short-chain fatty acids (metabolites formed by the gut microbiome) and some bacterial species between autistic and control patients and found that antibiotics and probiotics resulted in neuropsychological

improvements (Louis 2012). Additionally, compared to controls, elevated levels of p-cresol (another gut microbial metabolite) has been found in a sample of autistic children (Altieri et al. 2011).

The Gut Microbiome and Metabolism

With the gut microbiome playing a major role in disease, it only makes sense that the gut microbiome is also involved in metabolic processes within the body. The gut microbiome plays a huge role in the breakdown of plant polysaccharides such as starch, xylan, and psyllium hydrocolloid (Musso et al. 2011). The major products of plant polysaccharide metabolism are the short-chain fatty acids (SCFA), such as butyrate, proprionate, and acetate. SCFAs have several roles in the body: they can themselves be used in providing energy to the body, but they can also change the pH of the colon and affect gut microbial composition, modulate gut epithelial cell growth and differentiation, and can even have cancer-protective, anti-inflammatory effects as in the case with butyrate (Zimmerman et al. 2012) (Macfarlane and Macfarlane 2012; Musso et al. 2011). Generally, SCFAs are considered to serve a protective, beneficial effect to the body (Louis et al. 2014).

However, there are also gut-microbiome produced metabolites which can be harmful or are associated with harmful disorders. For example, the gut microbiome is also involved in metabolizing bile acids, which amongst other roles regulate lipid, glucose, and cholesterol homeostasis (Staels and Fonseca 2009), into secondary bile acids such as deoxycholic acid and lithocholic acid (Musso et al. 2011). Secondary bile acids (SBAs) have been implicated in carcinogenesis in several parts of the body (Louis et al. 2014) and have specifically been implicated in colon cancer, with secondary bile acids inducing DNA damage in colonic cells and causing cell membrane damage in the intestinal epithelium which activate repair mechanisms

which promote colorectal tumorigenesis (Ajouz et al. 2014). Additionally, the gut microbiome can metabolize some amino acids into harmful products. For example, lysine is metabolized by the gut microbiome into ammonia, and ammonia is known to be carcinogenic (Louis et al. 2014) (Dai et al. 2015). Various aromatic amino acids transformed by the gut microbiome into products which have associations with specific diseases. For example, tyrosine can be formed by the gut microbiome into 4-cresol, which has a known association with the development of autism (Clayton 2012), where gut-bacteria that can catabolize tryptophan to kynurenine via the kynurenine pathway were upregulated in patients with human immunodeficiency virus (HIV) (Vujkovic-Cvijin et al. 2013). Catabolism of tryptophan through the kynurenine pathway itself is associated with several health disorders such as malaria, Alzheimer's disease, and cancer (Chen and Guillemin 2009) and kynurenine itself is a metabolite known to be associated with depression (Oxenkrug 2010).

The gut microbiome can also regulate metabolism in the body by affecting the expression of genes. For example, the gut microbiome suppresses a gene known as *Fiaf* (fasting-induced adipocyte factor), which is translated into a protein inhibitor for lipoprotein lipase, an enzyme which increases triglyceride accumulation in fat cells and promotes fatty acid uptake by cells (Tilg and Kaser 2011) (Bäckhed et al. 2004). The gut microbiome can also indirectly regulate metabolism in the liver, with one study showing 112 differentially expressed genes related to the metabolism of xenobiotics in a comparison between germ-free and conventionally raised mice (Björkholm et al. 2009). Additionally, they found that germ-free mice challenged with a pentobarbital (an anesthetic agent) had a quicker recovery from anesthesia than conventional mice, which they attributed to efficient metabolism of the chemical in the germ-free mice and

which further lends support to the effect that the gut microbiome has in affecting metabolism in the body (Björkholm et al. 2009).

Effect of Xenobiotics on Gut Community Structure

Xenobiotics generally refer to chemicals that can be present in an organism, but which are not normally part of the organism and cannot be synthesized by the organism (Grace et al. 2008). Several xenobiotics , such as polychlorinated biphenyls, some pesticides, and some heavy metals are known to create changes to the gut microbiome by perturbing the gut community structure (Summers et al. 1993) (Choi et al. 2013) (Joly et al. 2013) (Lu et al. 2014a) (Liu et al. 2014). Xenobiotics perturbations create dysbiosis in the gastrointestinal tract, which can lead to internal dysfunction and promote the formation of the aforementioned diseases such as obesity and IBD (Mondot et al. 2013). In this section, we will briefly review a few effects that xenobiotics can have on perturbing the gut community structure.

Polychlorinated biphenyls (PCBs) are a synthetic chemical that have been used in various manufactured substances, such as protective coatings, adhesives, paint, and fluorescent lights, mostly because of the attractive physical properties of PCBs such as high boiling point, heat resistance, and low solubility in water (Coyne 2015). However, the toxicity of PCBs on both humans and animals started to become recognized in the 1970s and they have been considered persistent organic pollutants and efforts have been taken to limit the use of PCBs (Antonijevic´ et al. 2012) (Coyne 2015). Though there is evidence that show PCBs are involved in metabolic disorders in the same way as the gut microbiome is (Baker et al. 2015), currently there has been only one study on the effect of PCBs on the gut microbiome. In a mouse model, PCBs were shown to decrease gut bacterial abundance by 2.2% of baseline and specifically decreased levels of Proteobacteria (Choi et al. 2013). Interestingly, exercise by the mice was shown to attenuate

PCB-associated dysbiosis, and the authors suggested that exercise may have promoted the release of anti-microbial bile acids to the gastrointestinal tract which may have selectively inhibited growth of some bacterial species while promoting growth of others (Choi et al. 2013). However, the authors did not perform any further analysis in an attempt to understand what these gut bacterial changes signify.

Arsenic exposure is a major problem in the United States, with approximately 25 million people consuming water containing arsenic levels greater than the 10 μg/L guideline of the World Health Organization and U.S. Environmental Protection Agency (EPA) (Lu et al. 2014a). We have recently studied the effects of arsenic on the mouse gut microbiome community structure and found that arsenic exposure via drinking water significantly decreased several species within the Firmicutes phylum (Lu et al. 2014a). In an attempt to understand the significance of these changes, metabolic profiling was performed to connect changes to the gut microbiome to the metabolic effects these would have in the host organism. For example, fatty-acid carnitines, involved in fatty acid oxidation, were reduced in the urine of arsenic-treated mice, suggesting that an arsenic-altered GM could decrease energy metabolism by the host (Lu et al. 2014a). We also found the reduction of several glucuronide metabolites in the urine, which offers the possibility that gut-microbial perturbation could also negatively affect phase-II detoxification within the body (Lu et al. 2014a). Such changes to the metabolic profile could promote or exacerbate disease.

Effect of Gut Microbiome on Xenobiotics Biotransformation

The gut microbiome has been known to have the ability to biotransform xenobiotics since the 1970s (Soleim and Scheline 1972), and a detailed review of gut-bacterial xenobiotics biotransformation has been published (Sousa et al. 2008). This section will focus on two

examples where xenobiotics biotransformation by the gut microbiome can have a harmful effect on the body.

The metabolism of sorivudine is probably the most serious example of how the gut microbial biotransformation can be deadly to human health. Sorivudine, an antiviral drug which treats herpes zoster, is converted by the GM into (E)-5-(2-bromovinyl)uracil (BVU) (Nakayama et al. 1997). In 1993, there were 18 deaths in Japan of people who had been co-administered sorivudine with an anticancer drug 5-flurouracil (5-FU) (Li-Wan-Po 2013). Later studies in a rat model provided a possible mechanism for this occurrence: the gut-floral microbial metabolite BVU is reduced in the liver by an enzyme known as dihydropyrimidine dehydrogenase (DPD) (Okuda et al. 1998). DPD also mediates the hydrogenation of 5-FU into other metabolites. However, the rat model showed that reduced-BVU can inactivate DPD which promotes the buildup of 5-FU and leads to toxic conditions in the body (Okuda et al. 1998). Since people have been shown to have differential activities of DPD (Watabe et al. 2002), the co-administration of these drugs could cause significantly increased toxicity presents a great risk since some people may be more adversely affected by the drugs than others.

The biotransformation of arsenic by the gut microbiome has been studied in several labs, including our own. Generally, inorganic arsenic (iAsv) is detoxified in the body to dimethylarsinic acid (DMAV) (Conklin et al. 2006). However, the gut microbiome has been found to be involved in the biotransformation of inorganic arsenic to toxic metabolites such as monomethylarsonous acid (MMAIII) and a thiolated arsenical known as monomethylmonothioarsonic acid (MMMTAV), (Van de Wiele et al. 2010). Recently, our lab has expanded the understanding of gut-microbial mediated arsenic biotransformation by examining the effects of environmental and genetic-driven perturbations to the gut microbiome

on the biotransformation of arsenic. In the first study, mice were infected with *Helicobacter trogontum* and also administered arsenic in drinking water. Levels of DMAV decreased, while iAsV increased, suggesting that gut-microbial perturbation by environmental factors could inhibit the detoxification of arsenic (Lu et al. 2013). In another study, we found that arsenic-exposed, immunocompromised IL-10-/- mice [previously associated with gut microbial dysbiosis (Maharshak et al. 2013)] showed an increase in the ratio of MMAV/DMAV, which means a decreased biotransformation of arsenic to DMAV, which also has suggested that an abnormal genetic background is another factor that can contribute to an altered gut microbiome which can affect the biotransformation of arsenic (Lu et al. 2014b).

CHAPTER 2

INTRODUCTION TO PROJECT

So far, there have been no published studies that have clearly stated a link between nicotine exposure and its effects on the gut microbiome, though several studies showing harmful effects of nicotine on promoting harmful oral microbiota have been previously described (Huang et al. 2014; Li et al. 2014). Additionally, one study in a Swiss cohort found a link between smoking and the gut microbiome, with an increase in microbial diversity and specific increases to Firmicutes and Actinobacteria and decreases to Bacteroidetes and Proteobacteria in those who stopped smoking (Biedermann et al. 2013). However, since the study was not designed to provide a causative reason for these changes, it is unclear as to how these changes could make a difference in the host (Biedermann et al. 2013). With both nicotine and the gut microbiome being individually known to affect metabolic pathways in the body, and the well-known connections that several studies have shown between xenobiotics affecting the gut microbiome, it is of interest to know how nicotine may be affecting the gut microbiome, which may provide further insights into how nicotine changes to the gut microbiome could be affecting metabolism within the body. Thus, the first aim of our study is to use 16S rRNA sequencing in order to profile what changes can occur to the gut microbial community structure of a mouse model as a result of nicotine exposure.

Though previous studies have found that smokers have a distinct plasma metabolite profile compared to non-smokers (Hsu et al. 2013) and there has been a metabolomics analysis of the effects of nicotine exposure on the mice brain (Li et al. 2014), there has so far been no

studies exclusively analyzing the effects of nicotine on the serum metabolite profile in mice. The changes that we observe may also play a role in affecting metabolism within the mice. It would be interesting to see if there are any changes to any gut-microbial metabolites in the serum, which could signify a connection between an altered gut microbiome and changes to specific gut-bacterial related metabolites as seen in previous studies after exposure to other xenobiotics (Lu et al. 2014a).

Previous studies have shown that males and females have differences in reaction to either smoking or nicotine (Quick et al. 2014) (Wray et al. 2015) (Lenoir et al. 2015). As our study will use both male and female C57BL/6 mice, it will be interesting to see any gender differences in our results. Also, our mice will be orally administered either 60 mg/L (high dose) or 6 mg/L nicotine (low dose) in drinking water for 13 weeks. A drinking-water concentration of 60 mg/L was previously found to produce a steady-state nicotine plasma concentration of 34 ng/mL in mice, comparable to a steady-state plasma concentration of 40 ng/mL observed in chronic cigarette smokers (Rowell et al. 1983). Additionally, it has also been found that nicotine can be administered in the drinking water at concentrations below 100 mg/L with no decrease in fluid intake compared to non-nicotine exposed mice, signifying that the concentrations of nicotine we are using is appropriate for long-term nicotine exposure studies such as ours (Rowell et al. 1983). A 10 times dilution of our high dose will be used as a low dose to observe if there are any differences in effects between low and higher doses of nicotine in both the gut microbiome and serum metabolite profile.

With the current discussion amongst government regulators and the general public on the potential public-health risks to nicotine-based E-Cigs, as well as the risks of nicotine already

established from cigarette smoke, our study provides a timely analysis in further exploring the effects of nicotine on the body by using a relevant animal model.

CHAPTER 3

MATERIALS AND METHODS

Animal Husbandry and Experimental Design

5-7 week old C57BL/6 mice (Jackson Laboratory) were housed in the University of Georgia animal facility for a week before the start of experimentation. Mice were divided into 1 control group and 2 treatment groups (6 mg/L nicotine (hereafter referred to as "low dose") and 60 mg/L nicotine (hereafter referred to as "high dose")), 10 mice (5 male and 5 female) per group [Figure 3.1]. Mice were separated into cages based on both their treatment group and gender, with 5 mice per cage. Before experimentation, all mice were allowed to consume tap water *ad libitum*. Before and throughout the experimental period, mice were housed under environmental conditions of 22°C, 40–70% humidity, and a 12:12 hr light:dark cycle and were provided with standard pelleted rodent diet. The animal protocol was approved by the University of Georgia Institutional Animal Care and Use Committee.

Mice Monitoring, Feces Collection, and Serum Preparation

Experimental groups were allowed to consume nicotine-adulterated water (see Nicotine Administration) while control mice were allowed to consume unadulterated tap water *ad libitum*. Mice were observed for 13 weeks and body weight measurements were obtained at the start of the study (baseline) and at 13 weeks. Mice fecal pellets were collected for 16S rRNA analysis at 13 weeks, before mice necropsy, and stored under dry-ice before being transferred to a -80°C freezer until further analysis. After the study period, mice were euthanized with carbon dioxide and necropsied.

During necropsy, blood was collected into serum-separating tubes using cardiac puncture and was allowed to sit upright at room-temperature in serum-separating tubes for approximately 30 minutes in order to promote clotting. Blood was then centrifuged at 18,407 RCF for 10 minutes. Serum was extracted into a separate tube and was stored along with the fecal pellets into a -80°C freezer until further analysis. Other major organs and tissues were collected but are not included in this analysis.

Nicotine Administration

98% nicotine (Pfaltz & Bauer, Inc.) was diluted in tap water to either a low dose (6 mg/L) or high dose (60 mg/L) concentration and administered in the water bottles of the respective treatments who were allowed to consume it *ad libitum*. Drinking water with nicotine was made fresh every week.

16S rRNA Sequencing

DNA was isolated from fecal pellets collected at 13 weeks using a PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA) according to the manufacturer's instructions. The resultant DNA was quantified by Nanodrop and stored at –80°C until further analysis. A primary polymerase chain reaction (PCR) was performed on the DNA to amplify 16S genes, followed by normalization procedures. The resultant DNA was finally quantified by Qubit 2.0 Fluorometer using Qubit dsDNA HS Assay kit (Life Technologies, Grand Island, NY) according to manufacturer's instructions and pooled to be sequenced. Sequencing was performed on an Illumina MiSeq to generate pair-end 250 base reads. The resulted sequences were merged and analyzed to map the gut bacterial profiles.

Plots Depicting Bacterial Fold Changes

GraphPad Prism was used to produce plots depicting specific gut microbiota changes at the genus level based off the datasets obtained from 16S sequencing data.

Statistical Analysis of Mice Weight

Statistical analysis of mice weight was done separately for each gender. A repeated-measures two-way ANOVA was used to assess any statistically significant difference in mice weight at the end of the study (13 weeks) compared to the beginning of study (baseline). Bonferroni's multiple comparisons test was used to assess the effects of treatment on the weight of the mice both at baseline and at 13 weeks. GraphPad Prism was used for all statistical analysis.

Metabolomics analysis by Gas-Chromatography Mass Spectrometry (GC-MS)

15 µL of serum was added to 100 µL of methanol, vortexed, and placed at 4°C for 20 minutes. Subsequently, serum-methanol mixture was centrifuged at 11,752 RCF for 10 minutes. Supernatant was removed, placed into a 2 mL HPLC vial, and dried using a vacuum concentrator for 90 minutes until no liquid was present in the vial. After drying, metabolites were subsequently derivatized. Extracted metabolites were derivatized using N,O-Bis(trimethylsilyl)trifluroacetamide (Fluka Analytical, St. Louis, MO). The derivatized samples were analyzed using an Agilent Technologies 6890N Network GC System/ 5973 Mass-Selective Detector (Agilent Technologies, Santa Clara, CA). The temperature of the injector, ion source, and MS Quadrupole were set at 275°C, 230°C, and 150°C respectively. The mass spectrometer was operated in full scan mode from 50 to 600 m/z and the solvent delay was set at 7 minutes. The resultant data were processed by peak fitting and alignment. The data was processed in SIMCA 13.5.0 using the following parameters: par scaling and log-transformation for all data

features. Partial least squares-discriminant analysis (PLS-DA) plots were created following software instructions. Due to poor data quality, one replicate for female, low-dose nicotine was excluded from our metabolomics analysis.

Identification of Differentially Changed Serum Metabolites

To identify the metabolite represented by a particular feature, it was queried using NIST MS 2.0 database. Only identifications having a percent confidence above 50% were accepted. Since the database provided the silylated (i.e. derivatized) name for a particular metabolite, NIST Standard Reference Material and Data for Metabolites in Human Plasma (http://srm1950.nist.gov/srm_search.php?gc=on) and NIST Chemistry Webbook (http://webbook.nist.gov/chemistry/) were queried in order to find out what the actual identity of the metabolite was. Each identified metabolite was queried through the Human Metabolome Database (HMDB; http://www.hmdb.ca/) and their particular metabolic functions were noted. This information is listed in Appendix B.

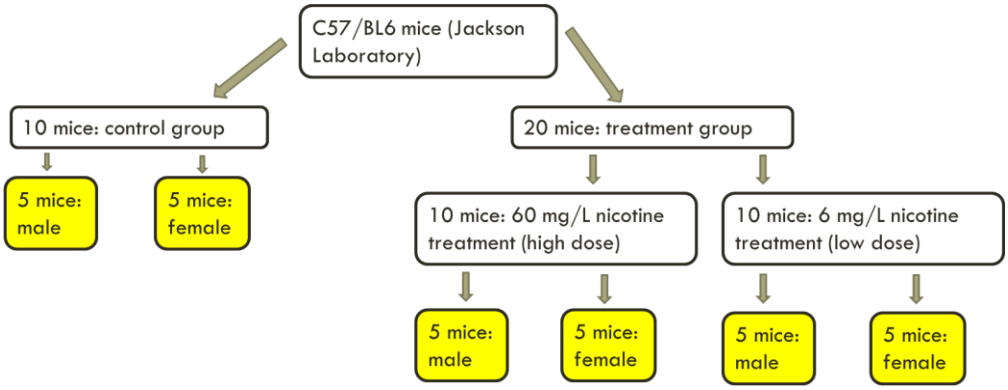

Figure 3.1: Mice Study Design. 10 mice (5 male and 5 female) were used as controls, while 10 mice each (5 males and 5 females) were used for high dose and low dose nicotine treatments. Mice were separated into cages based on both their gender and their treatment, so that each cage contained a total of 5 mice.

CHAPTER 4

RESULTS

High-dose treatment is associated with a lower weight in males

A repeated measures 2-way ANOVA of all treatments showed that timepoint had a significant effect on the weight of both male and female mice ($p<0.0001$), with a statistically significant weight increase in all mice from the beginning to the end of the study. At 13 weeks, the weight of both control and treatment female mice showed a statistically significant ($p<0.05$) increase compared to baseline, with control weight increasing by 4.214 g (95% CI: 3.179, 5.249), low dose by 3.402 g (95% CI: 2.367, 4.437), and high dose by 3.586 g (95% CI: 2.551, 4.621). In male mice, the weight of both control and treatment mice showed a statistically significant ($p<0.05$) increase compared to baseline, with control weight increasing by 7.508 g (95% CI: 5.068, 9.948), low dose by 5.930 g (95% CI: 3.490, 8.370), and high dose by 4.526 g (95% CI: 2.086, 6.966).

To see if treatment had an effect on the weight of male mice, Bonferroni's multiple comparisons test was performed. In both males and females, there was no statistically significant difference in weight between controls and treatment groups at baseline. However at the end of the study (13 weeks), high dose mice had a weight decrease of 3.904 g (95% CI: 0.6456, 7.162) compared to controls ($p<0.05$). There were no other statistically significant weight changes at 13 weeks.

High dose nicotine mice have a more different gut microbial profile from low dose and controls

A principal coordinates analysis (PCoA) plot of all samples do not show any difference in the gut-microbial community structure between control and treated mice [Figure 4.1]. However, gender-stratified PCoA plots show that in both genders, high-dose treatments are well separated from low-dose and control [Figure 4.2, Figure 4.3]. Both of these findings suggest that a high-dose of nicotine is associated with a more distinct gut microbial profile compared to low dose and control. Additionally, the male PCoA plot shows a greater separation between the control and low dose groups than observed in females [Figure 4.2 and Figure 4.3], suggesting a greater difference in the gut bacterial community profile between low dose and controls in males than in the females.

Gut microbiome community structure changes are mainly at the family and genus level

Both a phylum-level histogram and skiff-plot of all samples, as well as gender-stratified skiff plots at the phylum level, do not show any distinct patterns to changes to the gut microbial community structure [Figure 4.4, Figure 4.5, Figure 4.6], although there was a slight statistically significant increase in the TM7 phyla in high-dose male mice and a statistically significant decrease in the Tenericutes phyla in high-dose female mice compared to their respective gender controls [Appendix A]. Thus, further analyses into gut-microbiome community structure changes were not performed at the phylum level. Additionally, all further analyses at the family and genus level were performed using gender-stratified data.

As shown by the family-level skiff plot for females [Figure 4.7], the replicates within each treatment and control group clustered together, meaning that the gut microbial community structure between replicates in each treatment was similar. Both controls and high dose clustered

under the same branch, suggesting that the gut community profile between these two treatments had greater similarity to each other and were distinct from the gut community profile of low dose mice. Males do not exhibit a clear clustering pattern amongst replicates and a clear separation pattern between treatments at the family-level [Figure 4.8], suggesting that the effects of treatment cannot be detected in male mice at the family level.

Gender-stratified skiff plots at the genus level show treatment-associated differences in the gut microbial community structure. In males, the replicates of each treatment group clustered together, suggesting that the gut community profile between replicates in each treatment was similar [Figure 4.9]. Treatments clustered under the same branch distinct from that of controls, suggesting that nicotine altered the gut-microbial community structure for males at the genus-level in a similar manner [Figure 4.9]. As in males, female replicates also clustered together; however, controls and low-dose were clustered under the same branch separate from that of high-dose nicotine, suggesting that the gut community structure of high dose females was more different at the genus level compared to low dose and control females [Figure 4.10].

Changes to Peptostreptococcaceae and Erysipelotrichaceae at the family level

Appendix A shows all of the statistically significant changes to specific bacterial families in each treatment compared to control. There was a small, but statistically-significant increase in Peptostreptococcaceae in low dose female mice, with no detection of this bacterial family in either high dose females or any of the male treatment groups [Figure 4.7].

Interesting trends were observed with gender differences in the changes to the bacterial family Erysipelotrichaceae. In both female low-dose and high-dose mice, there is an approximately two-fold statistically significant decrease in the relative abundance of this family compared to controls, while the trend is the opposite in male high-dose mice, where there is an

approximately two-fold statistically significant increase compared to controls [Appendix A]. This suggests that high dose nicotine exposure affects the Erysipelotrichaceae family in a gender-dependent manner.

Specific bacterial changes at the genus level

16S rRNA analysis detected several statistically significant differences to several bacterial genera, with the greatest number of detected changes in female high-dose mice [Appendix A]. Fold-changes of these genera (compared to the respective gender control) have been presented for female high dose, male high dose, female low dose, and male low dose [Figure 4.11, Figure 4.12, Figure 4.13, and Figure 4.14, respectively].

In both female treatments as well as in low dose males, several genera in the family Lachnospiraceae were increased compared to controls of the respective gender. Female high-dose mice had smaller fold increases but to a more varied group of genera in the Lachnospiraceae family [Figure 4.11]., though there was approximately a 600-fold increase in the *Lachnobacterium* genus in low-dose males and an approximately 61,000-fold increase of the genus *Johnsonella* in low-dose females [Figure 4.14, Figure 4.13].

Several genera of Erysipelotrichaceae were detected to have been changed: there was a small fold-decrease in *Allobaculum* in female high-dose treatments while this same genus almost quadrupled in male high-dose mice [Figure 4.11, Figure 4.12]. Additionally, there was an approximately 2.5-fold increase in *Turcibacter* in male high-dose mice and approximately 2-fold decrease of *Turcibacter* in female low-dose mice [Figure 4.12, Figure 4.13]. The detected changes to genera in the Erysipelotrichaceae family reflect the trends seen at the family level.

Serum metabolite profile differences between treatments and control

PLS-DA plots comparing all treatments with control do not provide clear evidence of any difference in the serum metabolite profile between the treatments or between both treatments and control (data not shown). However, plots comparing each treatment individually with control showed that both treatments clustered and separated into their own groups [Figure 4.15]. Gender-stratified PLS-DA plot for females shows that all treatments separate and cluster into their groups [Figure 4.16], though there is no similar pattern in males [Figure 4.17].

Cloud plots depicting features that were changed compared to respective gender control (fold change>1.5, $p<0.05$) each have been provided for female high dose, male high dose, female low dose, and male low dose [Figure 4.18, Figure 4.19, Figure 4.20, and Figure 4.21 respectively]. Out of these changed features, a list of all metabolites that were positively identified, as well as their metabolic functions, have been listed in Appendix B. The cloud plots show that there were a greater number of changes to features with a fold upregulation/downregulation greater than 1.5 (compared to respective gender controls) in high-dose mice (188 features in female high dose mice and 169 features in male high dose) compared to the lower dose (103 features in male low dose and 62 features in female low dose), suggesting that high-dose nicotine created more change to the serum metabolite profile than low dose. Nicotine exposure in general led to metabolites being downregulated, though male low dose mice had a greater number of upregulated features than other treatment/gender groups and also had a greater number of identified upregulated metabolites compared to other treatment/gender groups [Figure 4.21, Appendix B].

Gender-differences in the changes to specific metabolites as well as changes to gut-microbiome related metabolites

There were some interesting patterns with changes to metabolites between genders and treatments [Figure 4.22]. For example, alanine was upregulated in high-dose females but downregulated in high-dose males. There were also gender differences in the upregulation of serine and adenosine: serine was upregulated in low-dose males but downregulated in all female treatments, while adenosine was downregulated in all male treatments but upregulated in female low dose nicotine treated mice [Figure 4.22]. Additionally, there was a downregulation of two gut-microbiome related metabolites, phenylalanine and tyrosine, in female high dose mice, the details of which will be expounded upon in the Discussion [Figure 4.22].

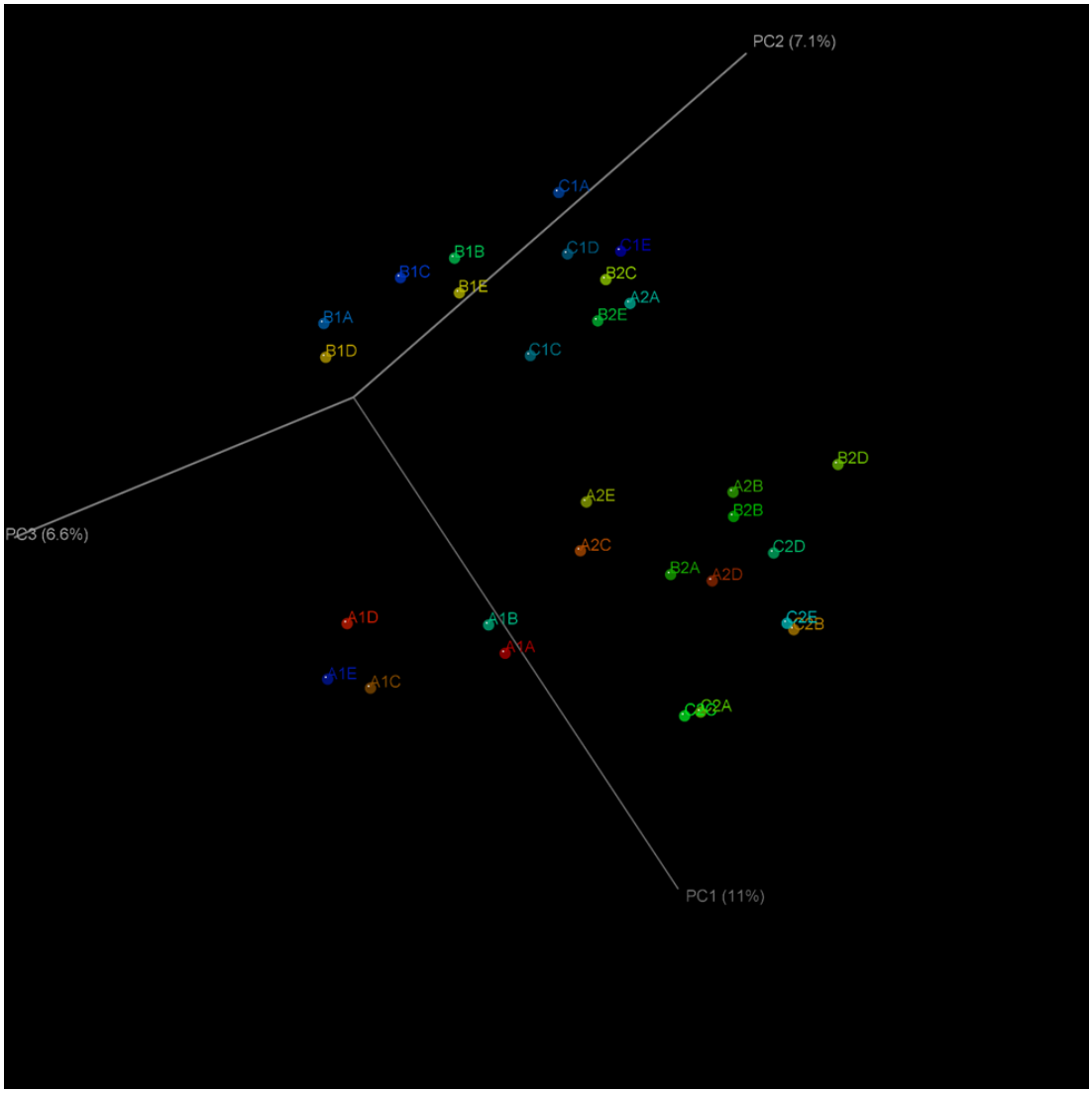

Figure 4.1: Principal Coordinates Analysis (PCoA) Unweighted Unifrac 3D Continuous Plot of Differences in Gut Microbial Community Structure in All Samples. This PCoA plot shows no clear trend to suggest that the gut-community structure of control mice is different from that of treatment mice. Each dot represents one replicate that is included in the analysis. The first letter for each replicate corresponds to the treatment group (A=control, B=low dose nicotine, C=high dose nicotine), the second number for each replicate corresponds to the gender (1= male, 2=female), and the third letter represents a particular unique replicate in a particular treatment and gender group.

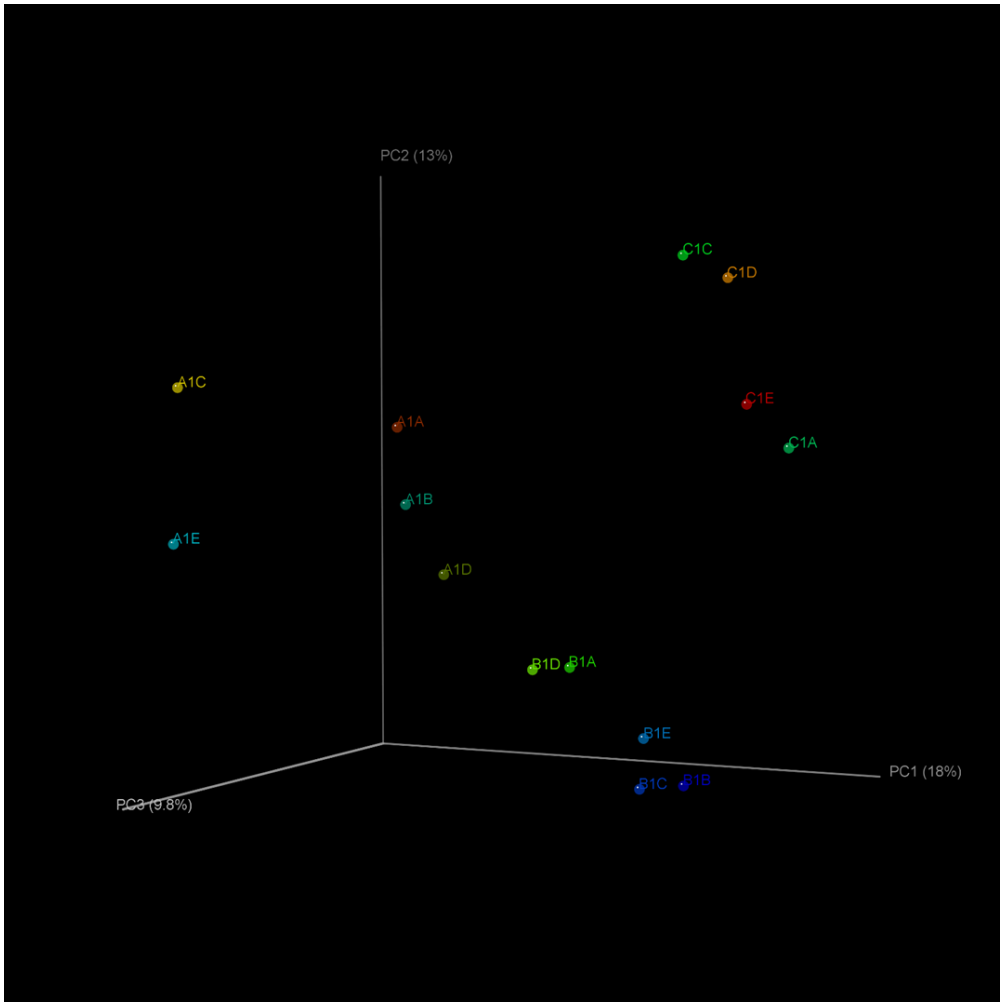

Figure 4.2: Principal Coordinates Analysis (PCoA) Unweighted Unifrac 3D Continuous Plot of Differences in Gut Microbial Community Structure for Male Mice Only. This PCoA plot shows that male mice exposed to high dose nicotine cluster together and are separated from other treatment groups; lower dose nicotine also has separation from controls. Each dot represents one replicate that is included in the analysis. The first letter for each replicate corresponds to the treatment group (A=control, B=low dose nicotine, C=high dose nicotine), the second number for each replicate corresponds to the gender (1= male, 2=female), and the third letter represents a particular unique replicate in a particular treatment and gender group.

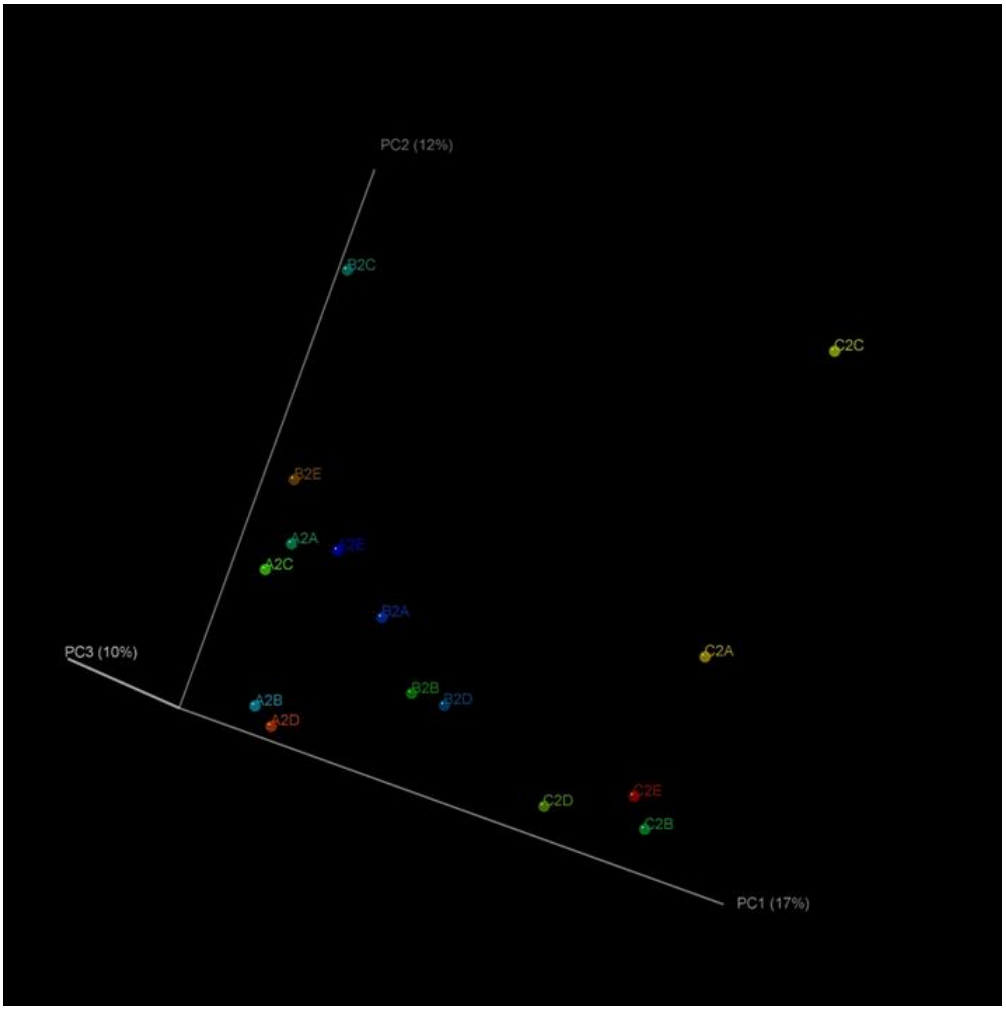

Figure 4.3: Principal Coordinates Analysis (PCoA) Unweighted Unifrac 3D Continuous Plot of Differences in Gut Microbial Community Structure for Female Mice Only. This PCoA plot shows that female mice exposed to high dose nicotine generally cluster together and are separated from other treatment groups. Each dot represents one replicate that is included in the analysis. The first letter for each replicate corresponds to the treatment group (A=control, B=low dose nicotine, C=high dose nicotine), the second number for each replicate corresponds to the gender (1= male, 2=female), and the third letter represents a particular unique replicate in a particular treatment and gender group.

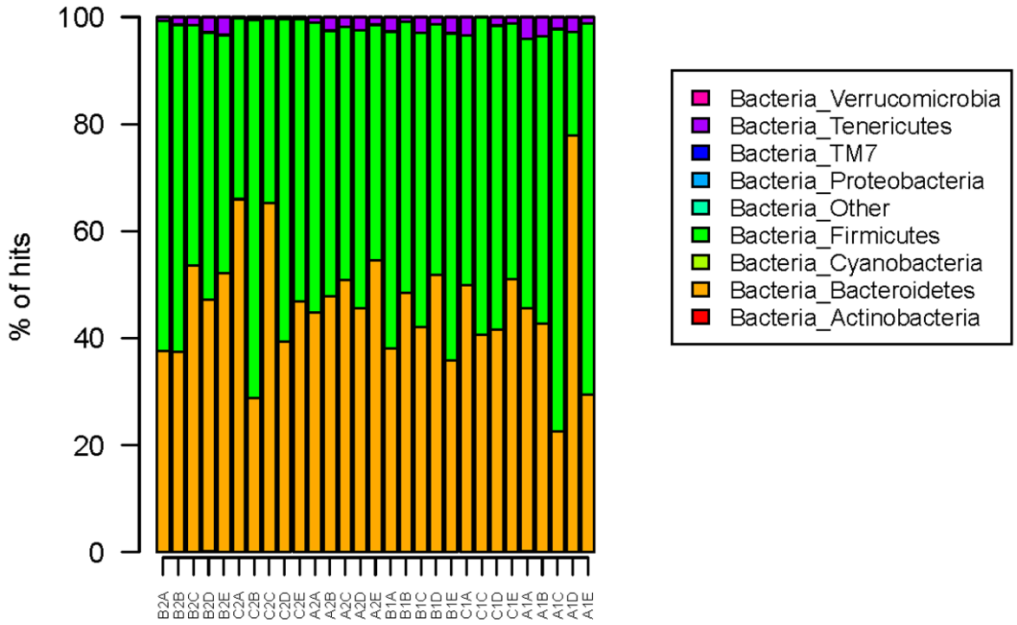

Figure 4.4: Phylum Level Histogram for All Samples. The first letter for each replicate corresponds to the treatment group (A=control, B=low dose nicotine, C=high dose nicotine), the second number for each replicate corresponds to the gender (1= male, 2=female), and the third letter represents a particular unique replicate in a particular treatment and gender group.

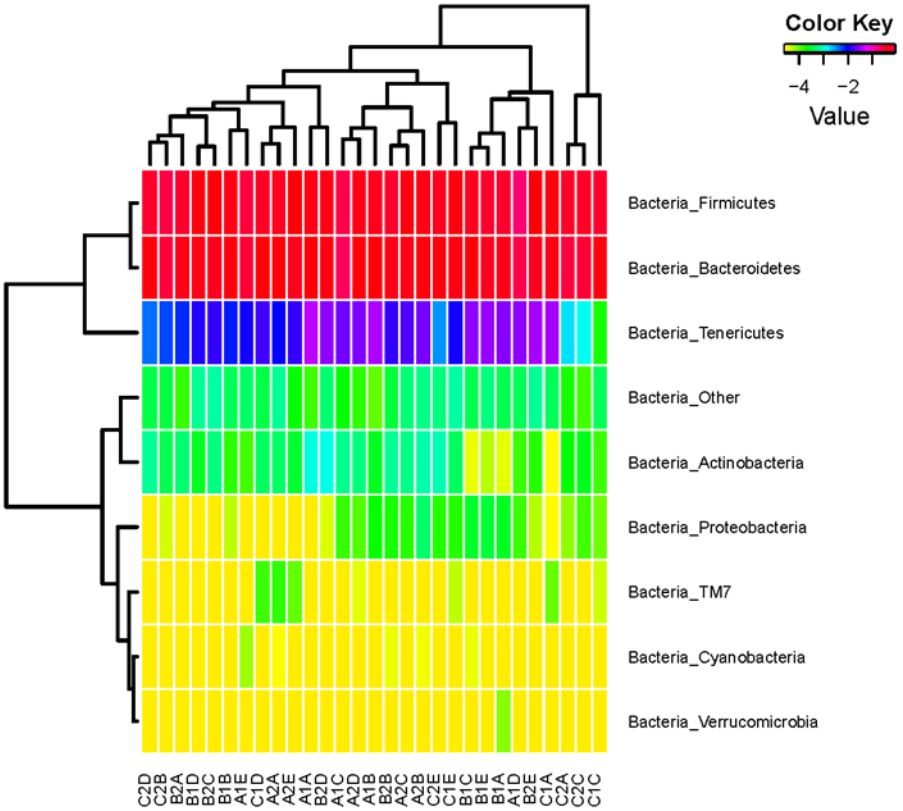

Figure 4.5: Phylum Level Log-Normalized Skiff Plot. The scale for the Color Key is as follows: -1~10%, -2~1%, -3~0.1%, and so forth. The first letter for each replicate corresponds to the treatment group (A=control, B=low dose nicotine, C=high dose nicotine), the second number for each replicate corresponds to the gender (1= male, 2=female), and the third letter represents a particular unique replicate in a particular treatment and gender group.

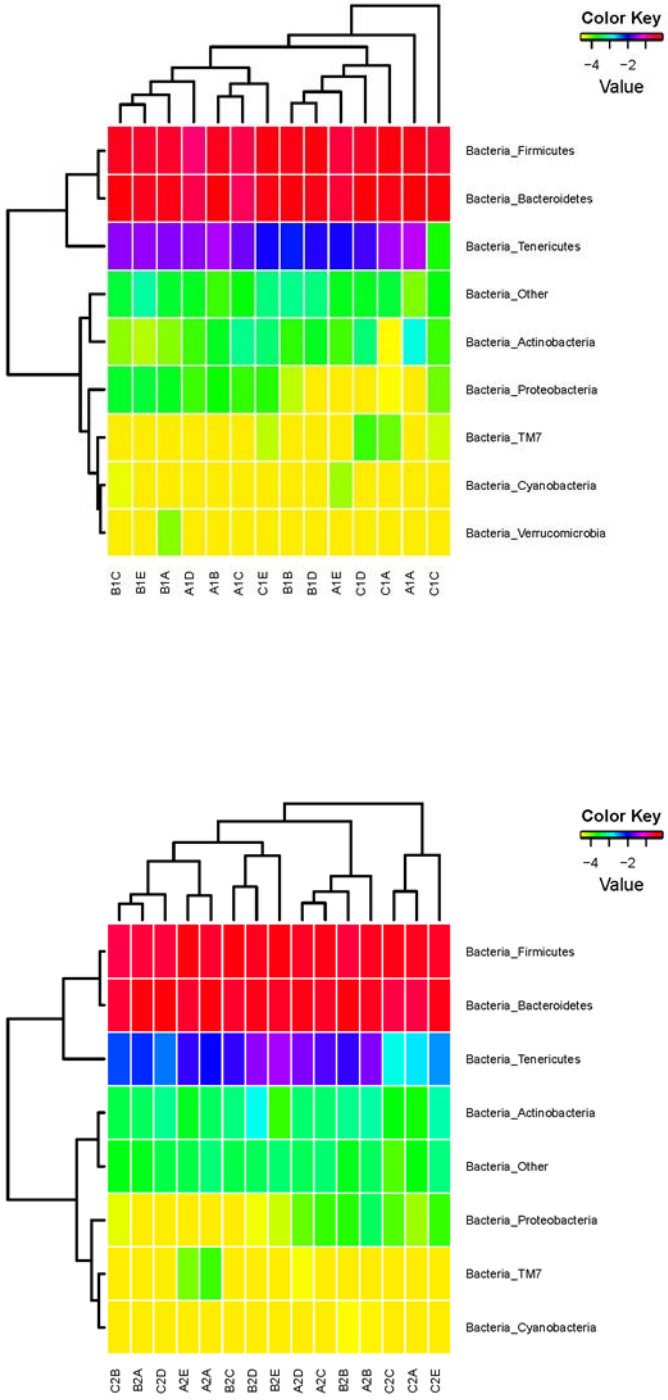

Figure 4.6: Gender-Stratified Log-Normalized Skiff Plots at the Phylum level. The scale for the Color Key is as follows: -1~10%, -2~1%, -3~0.1%, and so forth. The first letter for each replicate corresponds to the treatment group (A=control, B=low dose nicotine, C=high dose nicotine), the second number for each replicate corresponds to the gender (1= male, 2=female), and the third letter represents a particular unique replicate in a particular treatment and gender group.

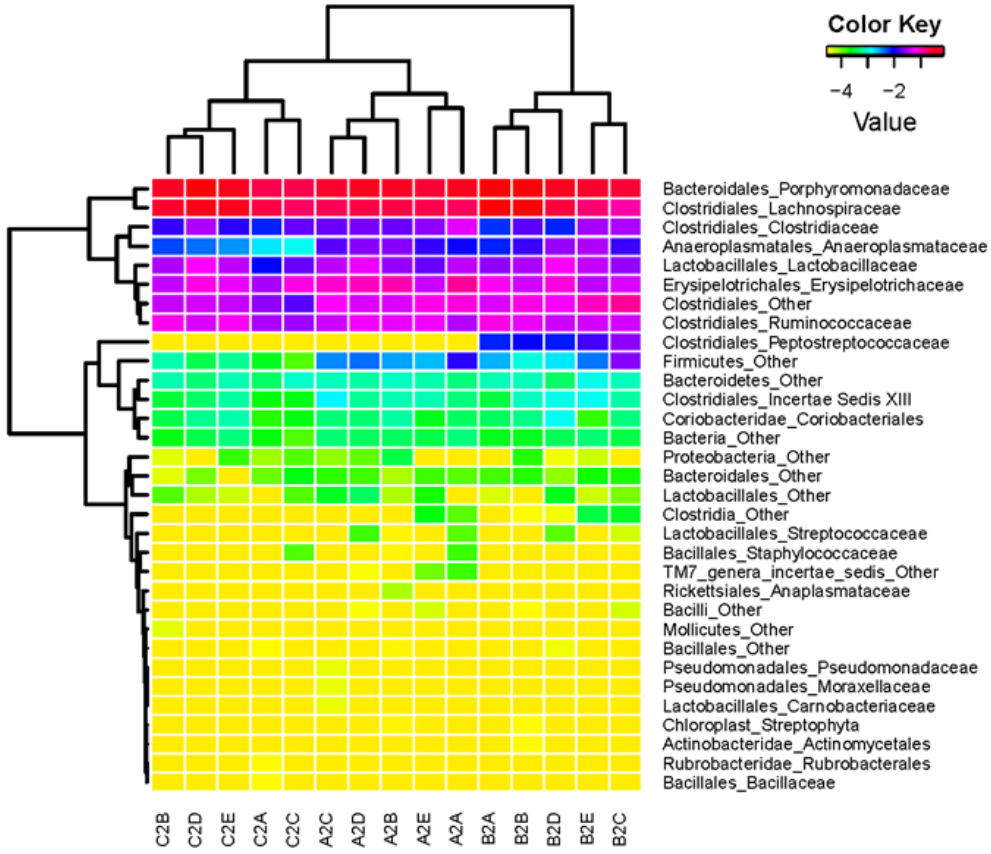

Figure 4.7: Family Level Log-Normalized Skiff Plot for Females. The scale for the Color Key is as follows: -1~10%, -2~1%, -3~0.1%, and so forth. The first letter for each replicate corresponds to the treatment group (A=control, B=low dose nicotine, C=high dose nicotine), the second number for each replicate corresponds to the gender (1= male, 2=female), and the third letter represents a particular unique replicate in a particular treatment and gender group.

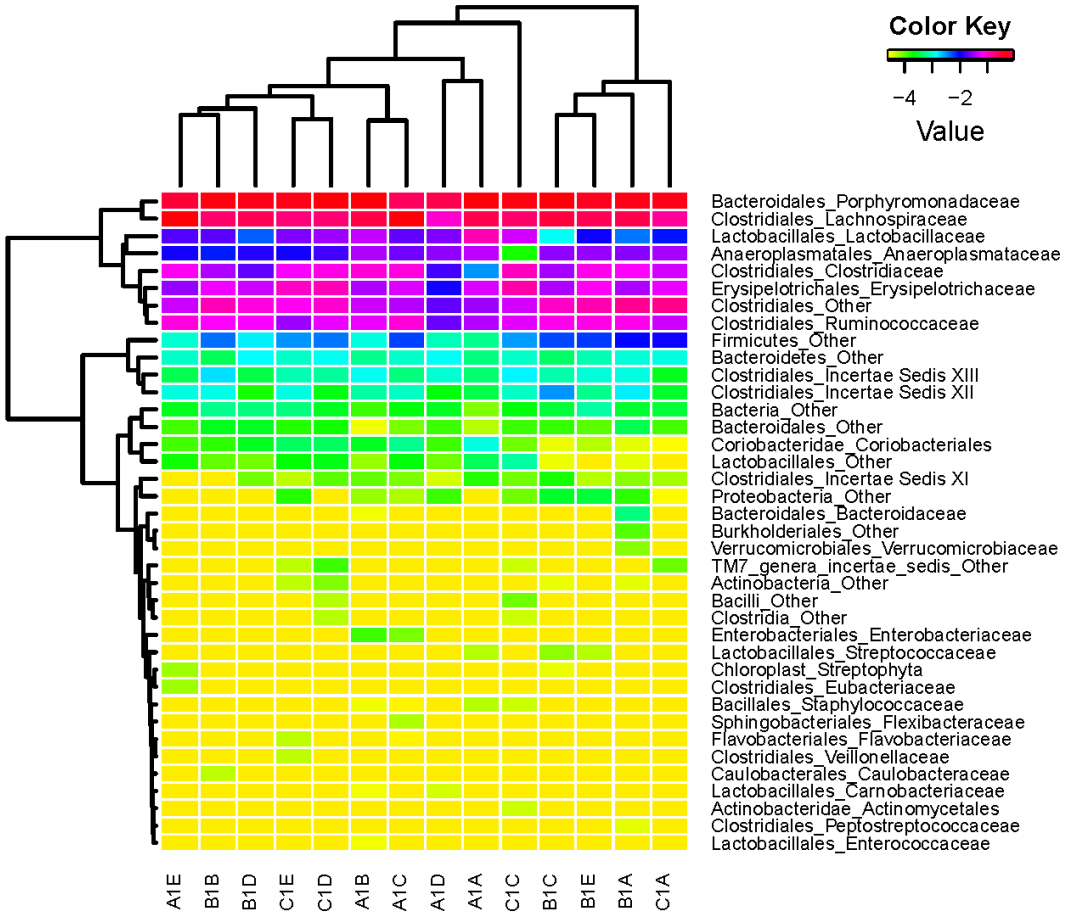

Figure 4.8: Family Level Log-Normalized Skiff Plot for Males. The scale for the Color Key is as follows: -1~10%, -2~1%, -3~0.1%, and so forth. The first letter for each replicate corresponds to the treatment group (A=control, B=low dose nicotine, C=high dose nicotine), the second number for each replicate corresponds to the gender (1= male, 2=female), and the third letter represents a particular unique replicate in a particular treatment and gender group.

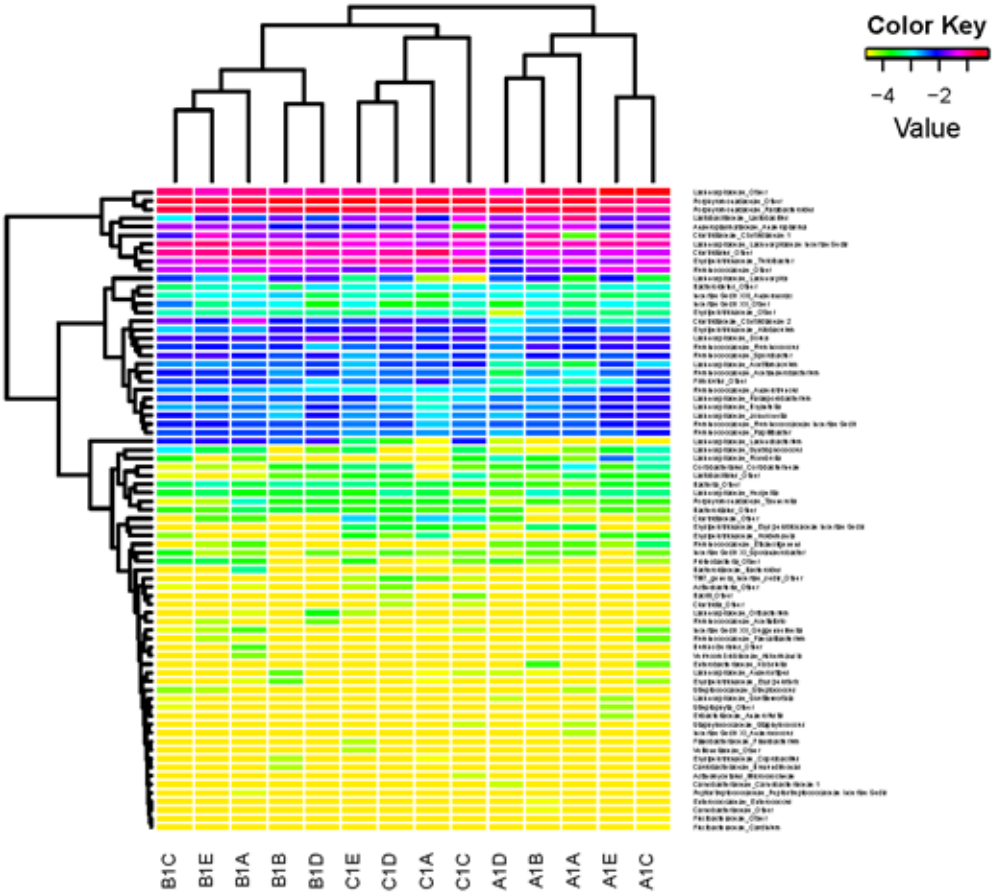

Figure 4.9: Genus Level Log-Normalized Skiff Plot for Males. The scale for the Color Key is as follows: -1~10%, -2~1%, -3~0.1%, and so forth. The first letter for each replicate corresponds to the treatment group (A=control, B=low dose nicotine, C=high dose nicotine), the second number for each replicate corresponds to the gender (1= male, 2=female), and the third letter represents a particular unique replicate in a particular treatment and gender group.

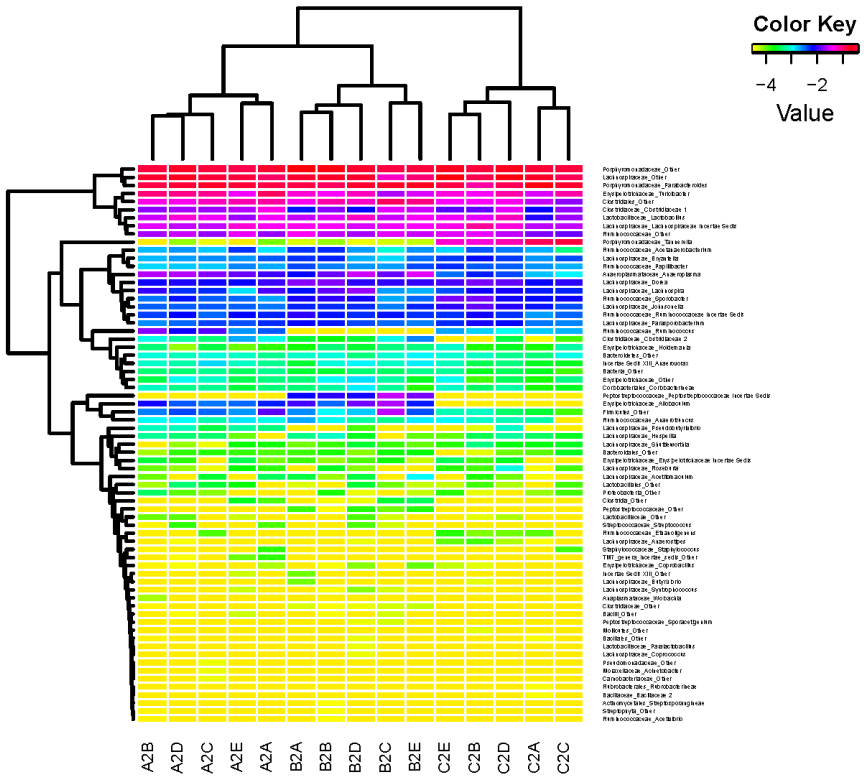

Figure 4.10: Genus Level Log-Normalized Skiff Plot for Females. The scale for the Color Key is as follows: -1~10%, -2~1%, -3~0.1%, and so forth. The first letter for each replicate corresponds to the treatment group (A=control, B=low dose nicotine, C=high dose nicotine), the second number for each replicate corresponds to the gender (1= male, 2=female), and the third letter represents a particular unique replicate in a particular treatment and gender group.

Code	Family (Genus)
B1	Lachnospiraceae (Anaerostipes)
B2	Lachnospiraceae (Bryantella)
B3	Lachnospiraceae (Johnsonella)
B4	Lachnospiraceae (Pseudobutyrivibrio)
B5	Lachnospiraceae (Shuttleworthia)
B6	Ruminococcaceae (Papillibacter)
B7	Ruminococcaceae (Ruminococcus)
B8	Ruminococcaceae (Sporobacter)
B9	Anaeroplasmataceae (Anaeroplasma)
B10	Clostridiaceae (Clostridiaceae 2)
B11	Clostridiales (Other)
B12	Erysipelotrichaceae (Allobaculum)
B13	Firmicutes (Other)
B14	Incertae Sedis XIII (Anaerovorax)

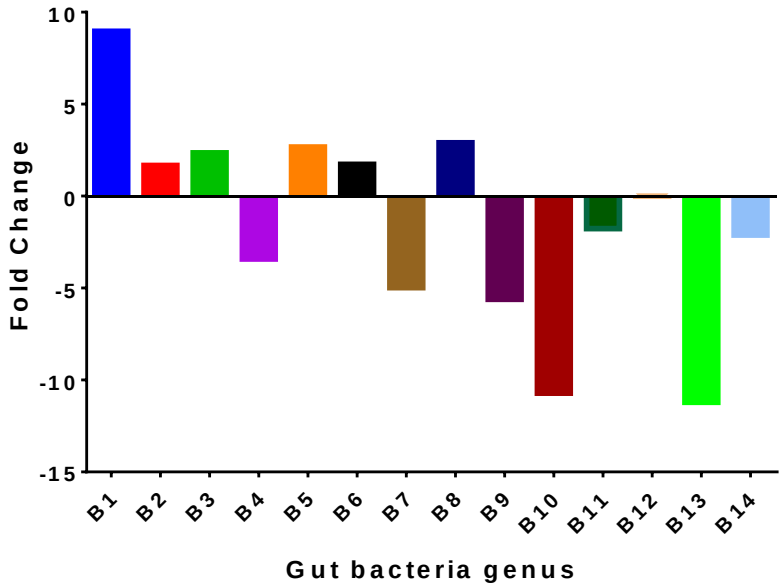

Figure 4.11: Fold Changes to Gut Bacteria in Female High Dose Mice Compared to Female Controls.

Code	Family (Genus)
B1	Ruminococcaceae (Ethanoligenens)
B2	Bacteroidales (Other)
B3	Clostridiaceae (Clostridiaceae 2)
B4	Clostridiaceae (Other)
B5	Erysipelotrichaceae (Allobaculum)
B6	Erysipelotrichaceae (Turicibacter)
B7	Porphyromonadaceae (Tannerella)
B8	TM7_genera_incertae_sedis (Other)

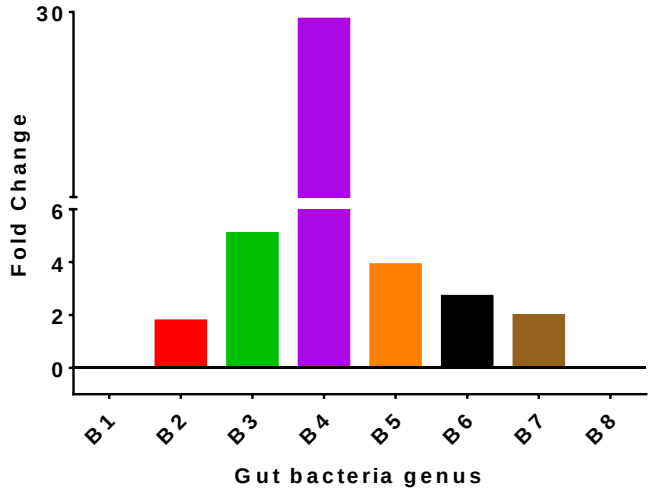

Figure 4.12: Fold Changes to Gut Bacteria in Male High Dose Mice Compared to Male Controls.

Code	Family (Genus)
B1	Lachnospiraceae (Johnsonella)
B2	Lachnospiraceae (Pseudobutyrivibrio)
B3	Peptostreptococcaceae (Other)
B4	Ruminococcaceae (Papillibacter)
B5	Ruminococcaceae (Ruminococcus)
B6	Clostridiaceae (Other)
B7	Erysipelotrichaceae (Turicibacter)

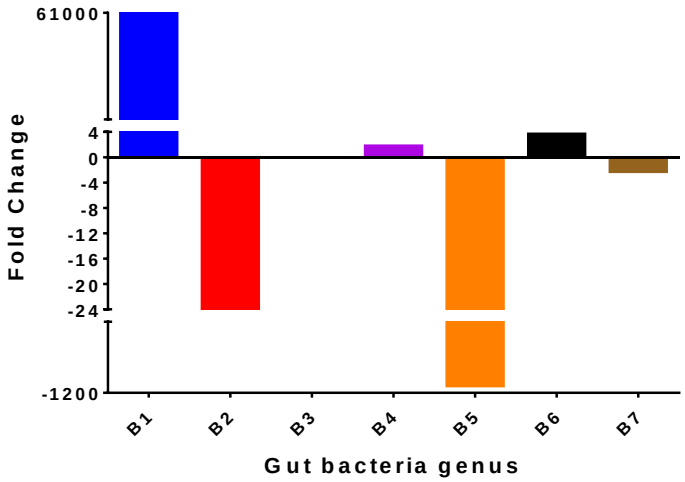

Figure 4.13: Fold Changes to Gut Bacteria in Female Low Dose Mice Compared to Female Controls.

Code	Family (Genus)
B1	Lachnospiraceae (Dorea)
B2	Lachnospiraceae (Lachnobacterium)
B3	Lactobacillales (Other)
B4	Bacteria (Other)
B5	Bacteroidales (Other)
B6	Firmicutes (Other)

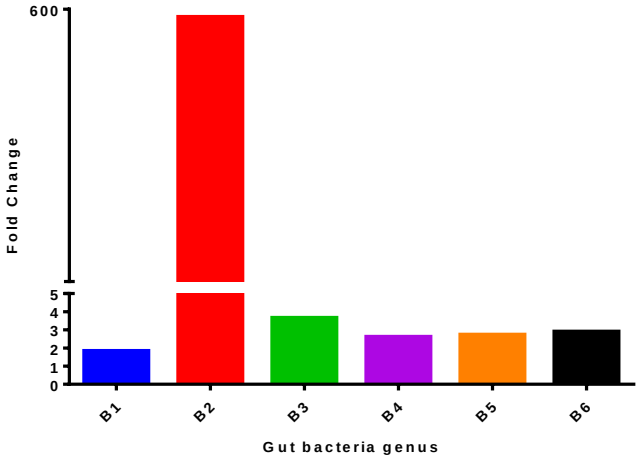

Figure 4.14: Fold Changes to Gut Bacteria in Male Low Dose Mice Compared to Male Controls.

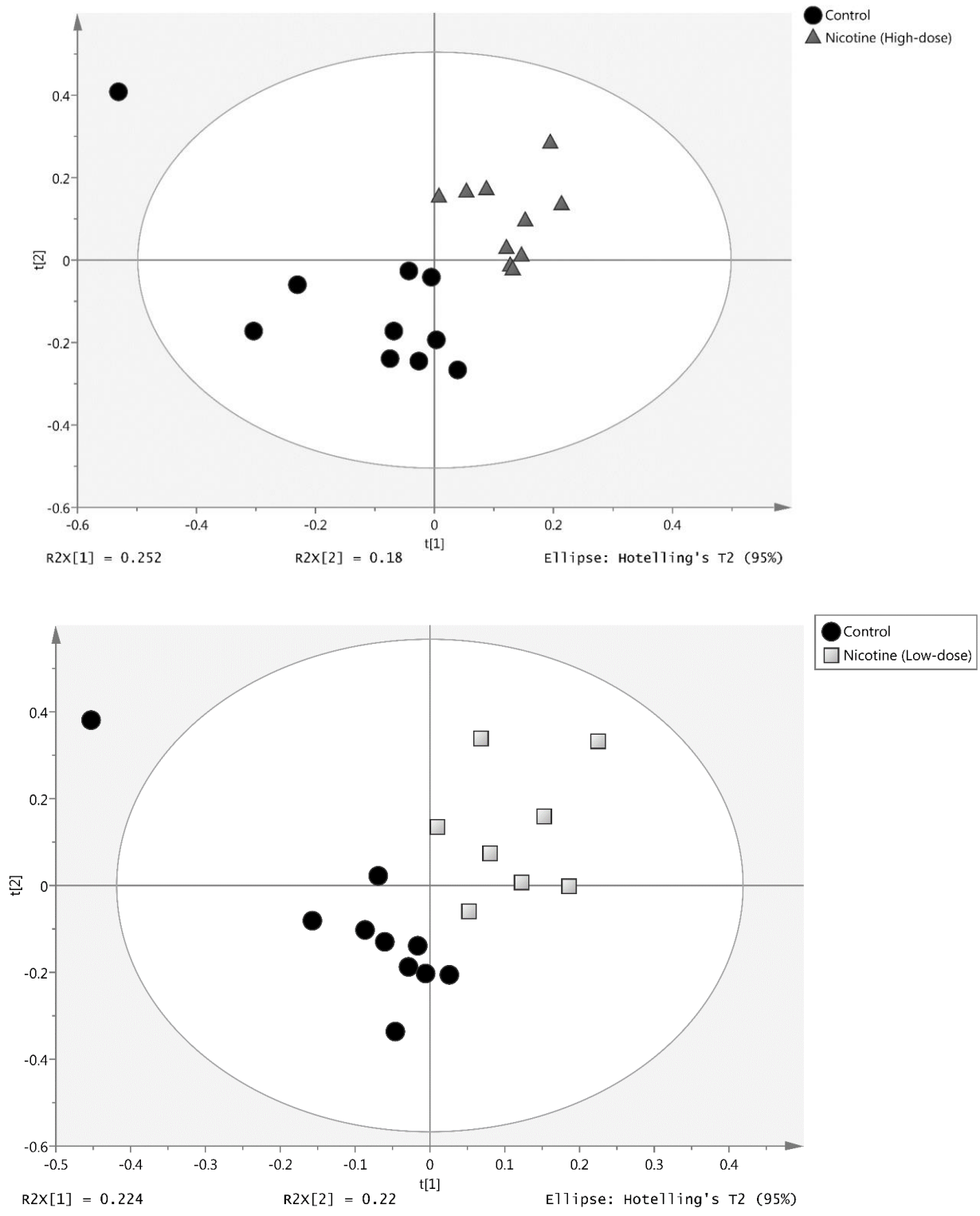

Figure 4.15: Individual PLS-DA Plots for Both Treatments. Top Plot: Control vs High Dose Score Plot (R2Y=0.996 Q2=0.763); Bottom Plot: Control vs Low Dose Score Plot (R2Y=0.773, Q2=0.237)

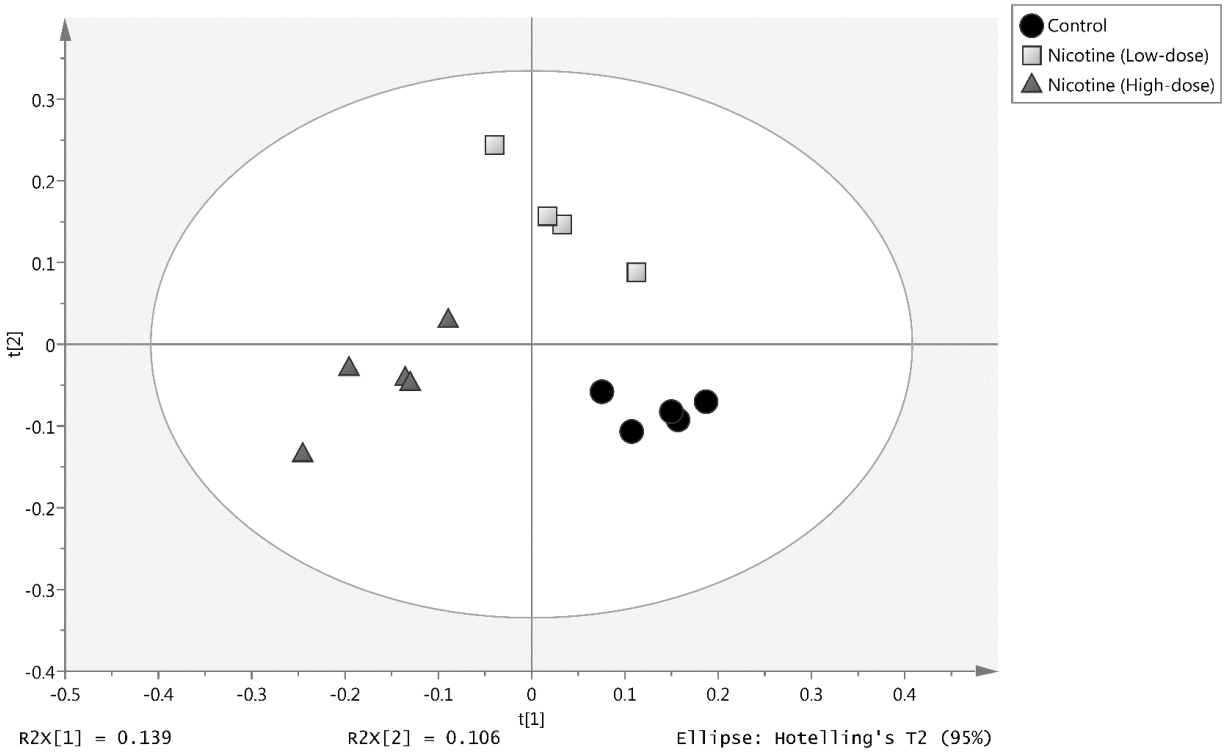

Figure 4.16: Serum Metabolomics PLS-DA Plot for Females (R2Y=0.852, Q2=0.226)

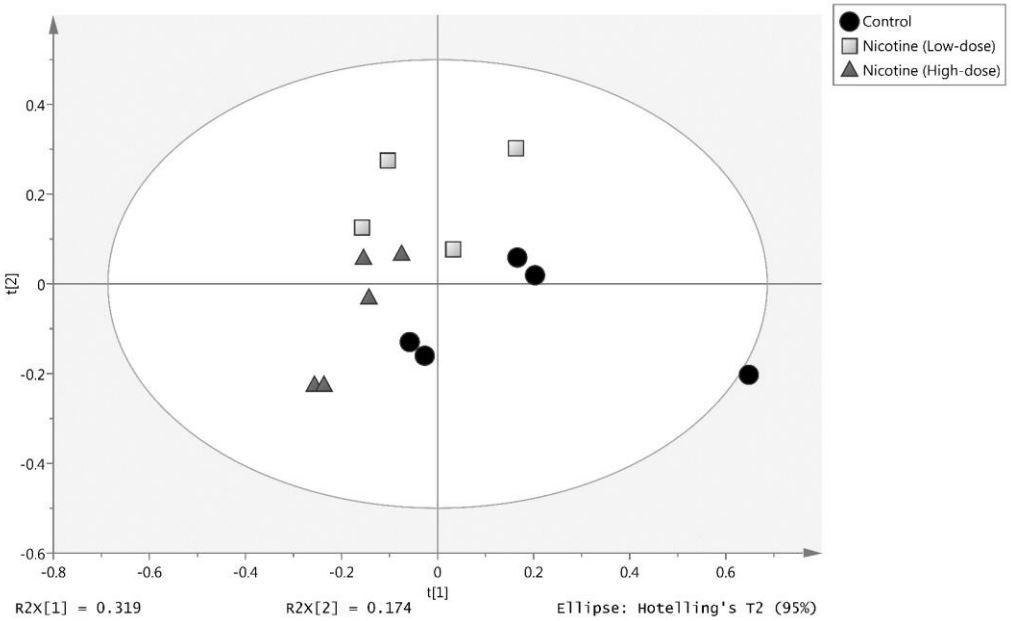

Figure 4.17: PLS-DA Plot for Males (R2Y=0.497, Q2= -0.0461)

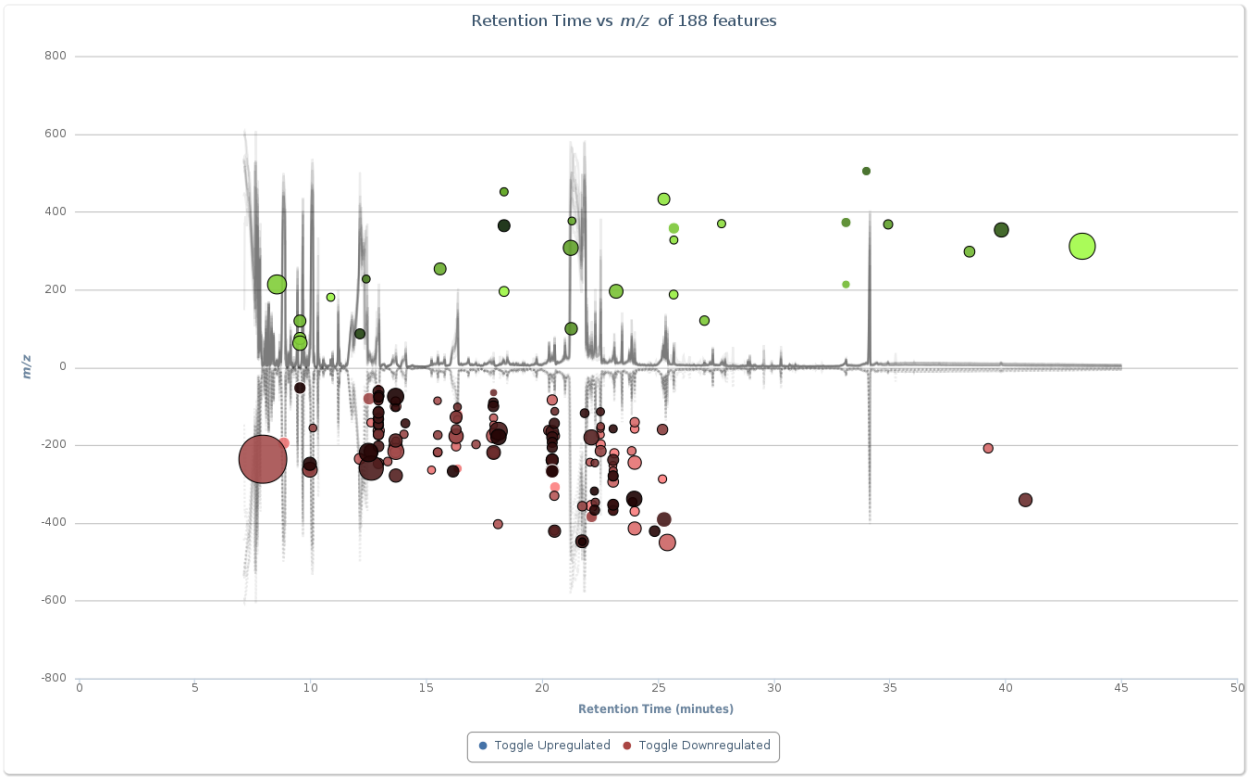

Figure 4.18: Cloud Plot of Changes to Features in Female High Dose Mice. All features have p<0.05; Fold Change > 1.5; GREEN=increased metabolites, RED=decreased metabolites; darker color=lower p-value; bigger circle=bigger log fold change of feature. In this plot, there are 158 downregulated features and 30 upregulated features.

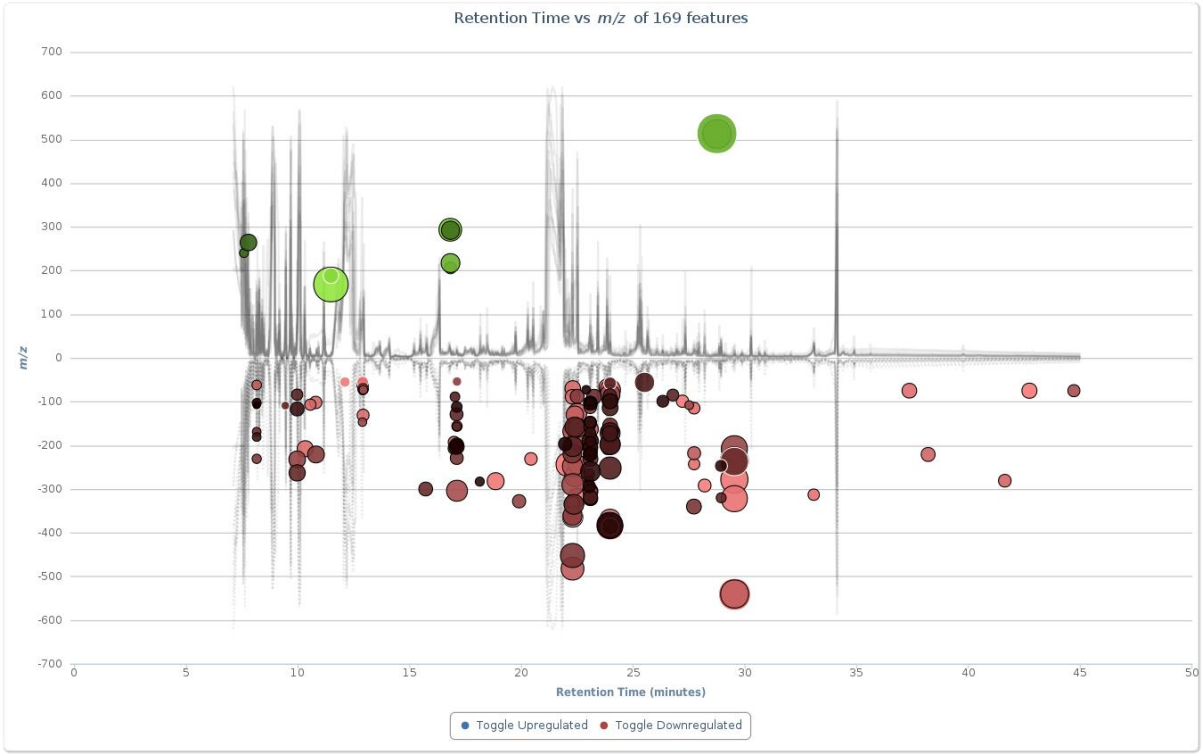

Figure 4.19: Cloud Plot of Changes to Features in Male High Dose Mice. All features have $p<0.05$; Fold Change > 1.5; GREEN=increased metabolites, RED=decreased metabolites; darker color=lower p-value; bigger circle=bigger log fold change of feature. In this plot, there are 10 upregulated features and 159 downregulated features

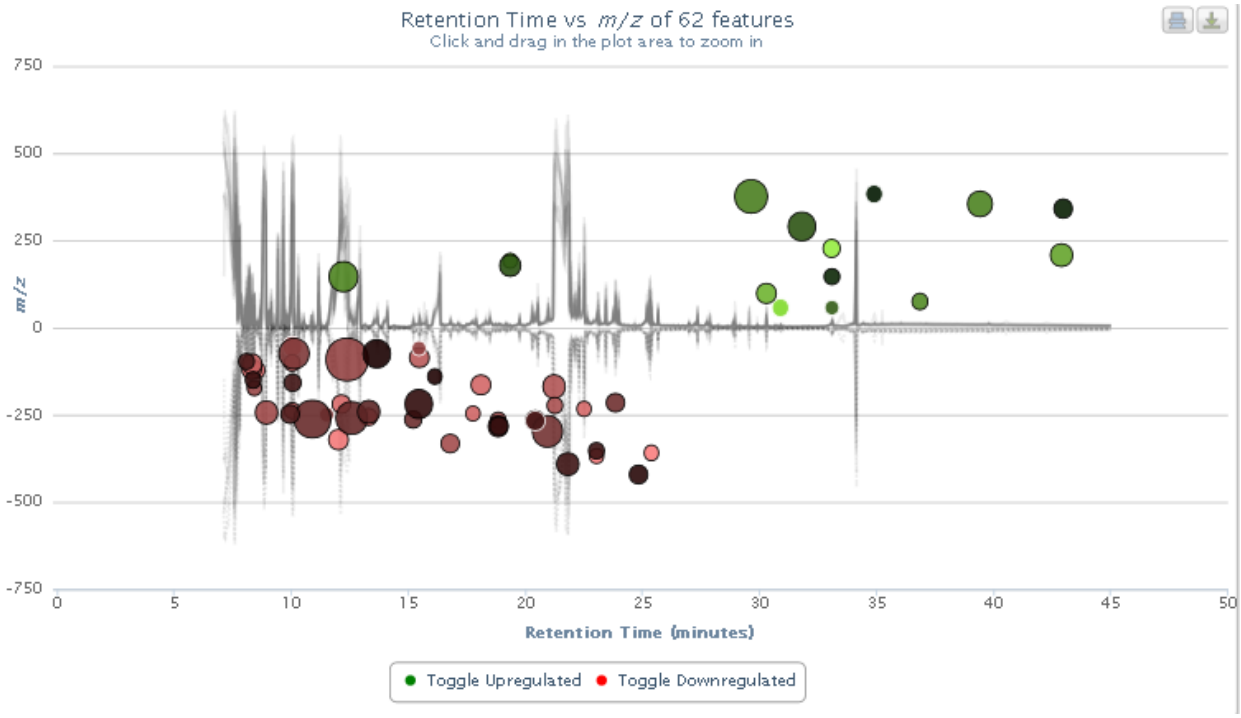

Figure 4.20: Cloud Plot of Changes to Features in Female Low Dose Mice. All features have $p<0.05$; Fold Change > 1.5; GREEN=increased metabolites, RED=decreased metabolites; darker color=lower p-value; bigger circle=bigger log fold change of feature. In this plot, there are 16 upregulated features and 46 downregulated features.

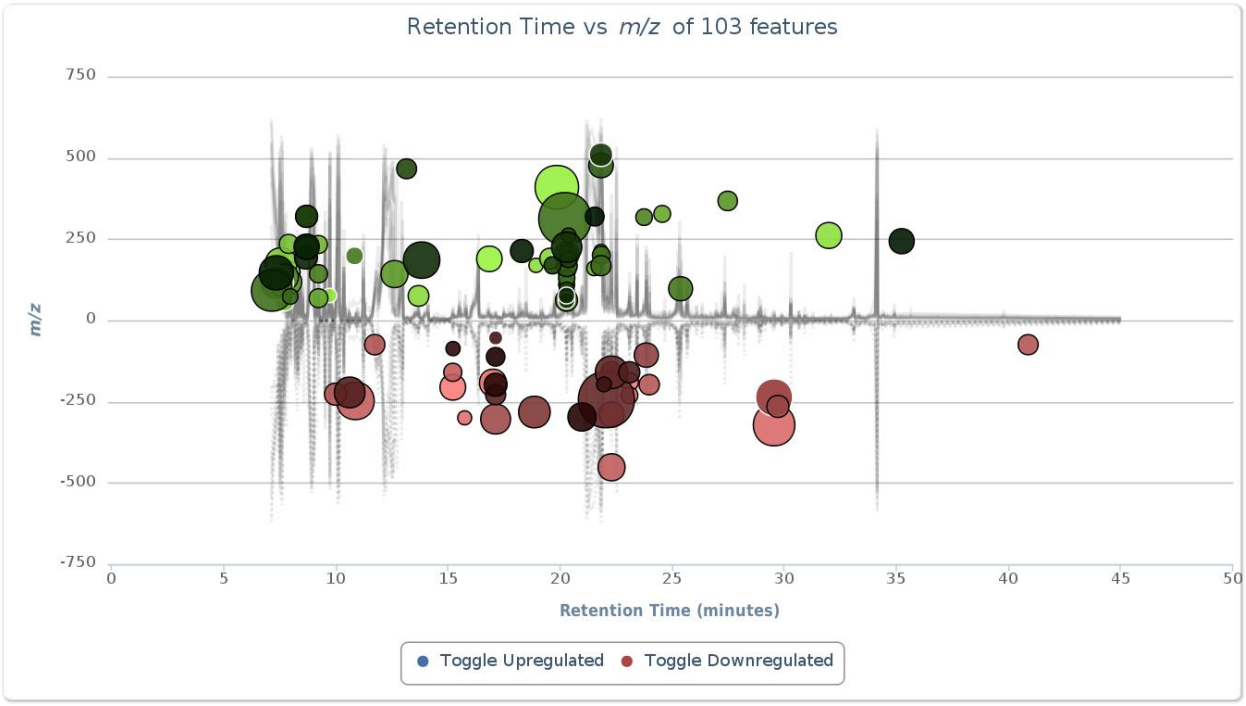

Figure 4.21: Cloud Plot of Changes to Features in Male Low Dose Mice. All features have $p<0.05$; Fold Change > 1.5; GREEN=increased metabolites, RED=decreased metabolites; darker color=lower p-value; bigger circle=bigger log fold change of feature. In this plot, there are 70 upregulated features and 33 downregulated features.

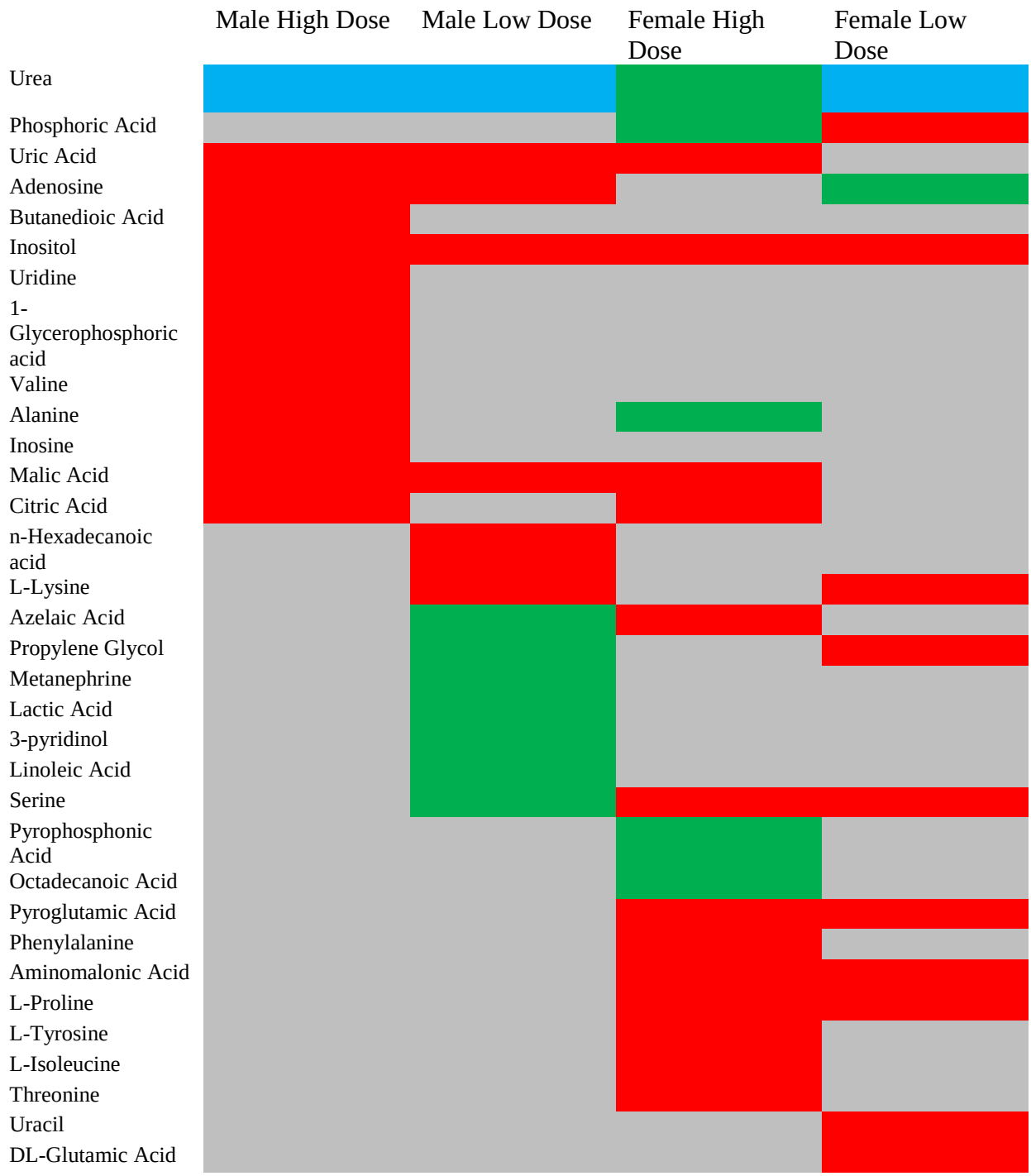

Figure 4.22: Gender Differences in Changes to Specific Metabolites. This chart shows the metabolites that were positively identified from the mice serum in each of the gender/treatment groups and how they compare between genders. Green=Upregulated compared to controls of the same gender; Red bars=Downregulated compared to controls of the same gender; Blue=Regulation Status not clear; Grey=Metabolites not identified in particular gender/treatment group.

CHAPTER 5

DISCUSSION

Mice body weight

In general, our data found that the body weight in all mice increased over the study period; however, this could be due to the mice growing up as opposed to the effects of treatment. Thus, statistical tests at both the beginning and end of the study were performed in order to ascertain the effects of nicotine treatment on body weight. Nicotine consumption has been previously ascribed in several studies to decreases in body weight in female rats and humans (Grunberg et al. 1986) (Cabanac and Frankham 2002). However, we only found a statistically significant decreased body weight of approximately 4 grams in high-dose male mice compared to male controls. Our results coincide with previous results that were found in a short-term nicotine exposure study using C57BL/6 male mice (Hur et al. 2010). However, another study using a larger sample of both female and male C57BL/6 mice that were provided 50 mg/L of nicotine in drinking water found no effects of the treatment on body weight (Abreu-Villaca et al. 2007). Since our sample size for each gender of mice was relatively small (n=5 for each), it is possible that our observed weight changes may be a statistical artifact rather than a change due to treatment.

High dose nicotine mice have a more different gut microbial profile from low dose and controls

High dose appeared to create a more dramatic change to the gut microbiome community structure in all the mice than low dose exposure to nicotine, as evidenced by clearer clustering

and separation from other doses in each of the gender-stratified PCoA plots [Figure 6.2 and Figure 6.3]. Though a lower dose may induce changes to the gut microbiome, especially since there was some separation of lower dose male mice from controls, this trend is not seen in a consistent manner over all genders. Since there have been no previous reports on the effects of nicotine on the gut microbiome, it is unclear what the observed changes signify.

Major changes to Peptostreptococcaceae, Erysipleotrichaceae, and Lachnospiraceae

It is interesting that members of the Peptostreptococcaceae family were increased in female low dose mice, as this species was associated with protective effects in previous studies. In one study, hydroxypropyl methylcellulose, a non-fermentable probiotic fiber previously found to have beneficial metabolic effects, was found to increase Peptostreptococcaceae almost 3.4-fold (Cox et al. 2013), while a study in female C57BL/6 mice that were treated with eugenol, a phytonutrient protective against infection, found to selectively increase the abundance of Peptostreptococcaceae (Wlodarska et al. 2015). Peptostreptococcaceae was also found to be increased in colitis-susceptible C57BL/6 mice that were treated with cellulose, a dietary fiber associated with protective effects against colitis (Nagy-Szakal et al. 2013). Interestingly, nicotine was also previously shown to have a protective effect against colitis (AlSharari et al. 2013). Possibly, nicotine and other similar substances could be acting through the gut microbiome in order to induce their protective effects, such as increasing families like Peptostreptococcaceae that are involved in offering protective effects to metabolism and the immune system in the body. Since previous studies have found that females tend to consume more nicotine as a percentage of fluid intake compared to males, probably due to nicotine being more rewarding to females due to higher psychostimulant sensitivity (Isiegas et al. 2009), it may be that the female mice consumed a greater amount of nicotine than males in order to induce the growth of

Peptostreptococcaceae observed at the end of the study. However, a high-dose concentration may have inhibited the growth of Peptostreptococcaceae in high-dose females, since nicotine was previously found to cause a dose-dependent inhibition of several bacterial organisms (Pavia et al. 2000).

Erysipelotrichaceae has a role in energy production and adiposity and higher numbers of this family has been previously associated with colorectal cancer, dyslipidemia, and body weight increase (Chen et al. 2012) (Martinez et al. 2013) (Choi et al. 2013). It was previously found to be dramatically decreased in male C57BL/6 exercised mice whose gut microbiota was perturbed by PCBs (Choi et al. 2013). However, high-dose males in our study showed an approximately two-fold increase in Erysipelotrichaceae compared to high-dose females, who had a two-fold decrease in this family.

However, it must be understood that Erysipelotrichaceae is a family with different genera, and previous studies have shown how some species within the genus *Incertae sedis* increased in the presence of high-fat diet (Turnbaugh et al. 2008) and how the genera *Turcibacter* is associated with ulcerative colitis and infection in pigs (Breton et al. 2013). However, species within the *Allobaculum* genus were found to be increased as a result of weight loss and its decrease was associated with a colitic-prone microbiome (Mir et al. 2013) (Cox et al. 2013). Our data found very a negligible decrease to *Allobaculum* in high-dose female mice and an approximately 2-fold decrease to *Turcibacter* in low-dose females. At the same time, there was a four-fold increase of *Allobaculum* and 2.5-fold increase of *Turcibacter* in male high-dose mice.

Possibly, there may have been different genera of Erysipelotrichaceae in both males and females, with females having a greater share of the “harmful” genera in Erysipelotrichaceae.

Since females tend to consume more nicotine than males, nicotine exposure may have provided a protective effect against "harmful" Erysipelotrichaceae genera that were unique to females, though bacterial inhibition by nicotine may have also played a role in the decrease observed in higher-dose mice. *Turcibacter*, which was decreased almost 2-fold in lower-dose females, was one of the detected Erysipelotrichaceae genera that may have decreased as a result of a protective effect from nicotine exposure. In males, nicotine exposure may have moderately provided some protective effects, as seen in the four-fold increase to *Allobaculum* in high-dose mice. However, as evidenced by the 2.5-fold increase in *Turcibacter* and 2-fold increase in Erysipelotrichaceae in high-dose males, males might not have consumed as much of the nicotine in order to get as strong of a inhibitory and protective effect as females, and/or may have had a different genera profile on which nicotine had a different effect.

At the genus level, there were few but very large fold-increases to Lachnospiraceae detected in low-dose mice and several smaller fold increases in high-dose females. Lachnospiraceae are known to be producers of butyrate, an anti-inflammatory short chain fatty acid implicated in several protective effects in the body (Meehan and Beiko 2014) and several of the genera detected in our samples, such as *Anaerostipes, Lachnobacterium*, *Bryantella, and Johnsonella*, are known to produce butyrate (Vos 2009). It is unclear why the lower-dose mice had huge fold-increases of Lachnospiraceae genera, but possibly there may have been inhibition of genera at the higher doses so that females had less dramatic increases to Lachnospiraceae and males had no detection.

Serum Metabolite Profile

In all samples, both low and high dose mice were shown to have a different serum metabolite profile compared to controls, though our PLS-DA analysis did not find that a higher

dose of nicotine induced a greater change to the serum metabolite profile than the lower dose in all samples. Possibly inherent differences of the mice serum metabolome between genders may have confounded our results within the PLS-DA plot and prevented us from seeing any changes between treatments, especially since cloud plots of feature changes with fold changes > 1.5 (compared to respective gender control) show that high dose was associated with changes to more features in both genders than low dose, suggesting that high dose has more of an impact on changing the serum metabolite profile compared to low dose. Additionally, we see in gender-stratified PLS-DA plots that females had a distinct metabolite profile for each treatment while males did not have any clear trend, possibly lending to the idea that gender difference is important to the effects of nicotine on the serum metabolite profile, which is supported by the gender-differences in changes to several metabolites in the profile as mentioned below. The potential reasons for the gender differences in our data await further studies.

Changes to features presented from the cloud plot show a general downregulation of features though male low dose mice had more upregulated features compared to other gender-treatment groups. However, since all of these features could not be identified, it is unclear if these changes confer a beneficial or harmful effect.

Gender Differences in Changes to Detected Serum Metabolites

There were gender differences in the changes between several detected metabolites, including two amino acids, alanine and serine, and adenosine, which is involved in purine metabolism.

Increased alanine has been associated with metabolic disorders such as increased body mass index, higher dietary cholesterol, an increased energy consumption (Holmes et al. 2008). Exercise, which is important to modulating metabolic disorders, was previously found to decrease plasma alanine in male C57BL/6 mice (Nobakht et al. 2015) (Monleon et al. 2014);

alanine is a gluconeogenic substrate which could signify modifications to glucose metabolism (Monleon et al. 2014). High dose males showed a decrease to alanine in our study, which may suggest that nicotine could be involved in metabolic processes that affect the presence of alanine and could potentially be protective in males. A downregulation of alanine in high dose males also makes sense in light of the decrease in male high dose body weight at the end of the study. However, female high dose shows an upregulation of alanine. Potentially, nicotine could be affecting metabolism in a sex-specific manner, especially since a previous study found gender-specific effects of smoking on serum metabolite profiles in humans (Xu et al. 2013). Currently, it is unclear what an upregulation of alanine in high-dose females may signify.

Previous studies have shown decreases in serine in both male and female obese mice (Won et al. 2013), which signifies that it could be involved in promoting metabolic disorders. However, serine was previously found to be upregulated in the sera of male current smokers (T Xu et al. 2013) so it is interesting to see this upregulated in male low-dose nicotine treatments. Possibly, nicotine could be modulating energy metabolism in males to offer a protective effect; however, since we did not see any statistically significant decrease in body weight in low dose males nor did we detect this metabolite in high dose males, this is only speculation. However, we found a downregulation of serine in both female treated mice. Similar to alanine, sex-specific differences of nicotine's effects on the metabolite profile might be the reason for our observations. Further mechanistic studies are needed to better understand gender-differences in serine as a result of nicotine exposure.

Adenosine has been linked to energy metabolism in cells (Porkka-Heiskanen and Kalinchuk 2011), has been known to mediate anti-inflammatory responses in the digestive tract, and to control colitis (Kurtz et al. 2014). A previous study found that cigarette smoke decreased

adenosine in male mice plasma (Cruickshank-Quinn et al. 2014). We also observed a decrease in adenosine in all male treatments, which brings up the question on whether or not nicotine within tobacco may be affecting the metabolism of adenosine. However, low-dose females show an upregulation of adenosine, which would suggest a protective effect. Possibly, nicotine is affecting the metabolite profile in different ways in different genders; the same ideas as mentioned above for alanine and serine may also apply for adenosine. Also, previous authors have discussed how the short half-life of adenosine in the body may explain contradictory observations (Cruickshank-Quinn et al. 2014). More studies specifically targeting adenosine metabolism in different genders in response to nicotine are needed to ascertain our thoughts.

Gut Microbiome and Serum Metabolites

In female high-dose mice, both phenylalanine and tyrosine were downregulated. Phenylalanine is known to be converted by the gut microbiome to 3-phenylpropionic acid and phenylacetic acid, while tyrosine can be converted to metabolites such as indolecarboxaldehyde and p-cresol, respectively, by the gut microbiome (Clayton 2012) (Gertsman et al. 2015). The downregulation of these metabolites could signify that high-dose exposure of nicotine in females may have stimulated gut-microbial metabolism, but since we were not able to detect any known downstream metabolites of these two amino acids, it is hard to say if a nicotine-altered gut microbiome affected the metabolism of these amino acids in order to decrease them in female high-dose treatments. However, this is a possibility.

It is well known that the composition of the gut microbiome affects the serum metabolite profile, as evidenced by differences in serum metabolites between germ-free and conventional mice (Wikoff et al. 2009). However, our study thus far has not been able to make clear connections between changes to the gut microbiome with the serum profile, mostly since several

metabolites were not able to be identified. Future studies will expand from just an analysis of the serum into profiling other metabolites, such as feces and liver, and may employ other analytical techniques, such as liquid-chromatography mass spectrometry, in order to better determine the metabolome that could be altered as a result of a nicotine-altered gut microbiome.

CHAPTER 6

CONCLUSION

High-dose was associated with a more distinct gut microbiome community structure as well as changes to more features in the serum metabolite profile, suggesting that nicotine exposure similar to that of smokers can change the gut-microbial community structure and the serum metabolite profile, though lower doses were also found to have a distinct gut microbiome profile in males and distinct serum metabolite profile in all samples, suggesting that even smaller doses of nicotine may induce changes to the gut microbiome and serum metabolite profile. The mechanistic basis for these changes await future studies. We also observed several differences to the gut microbiome community structure at the family and genus level, with gender-differences in changes to some bacterial families and genera. There were also several gender differences in the changes to specific metabolites in the serum, though not all metabolites could be identified and our study could not link metabolite changes in the serum to changes in the gut microbiome. However, our study provides the first look into the effects of nicotine on the gut microbiome and the serum metabolite profile, and could further the development of mechanistic hypotheses as well as provide specific biomarkers for the effects of nicotine on metabolism.

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APPENDIX A

STATISTICALLY SIGNIFICANT CHANGES TO THE GUT MICROBIOME

Notes:

Data was imported and has been presented directly in the format as was provided by the 16S sequencing pipeline. To interpret the data, use the following key:

```
[Bacteria Identifier] [mean(treatment)]     [variance(treatment)]
     [std.err(treatment)]  [mean(controls)]  [variance(controls)]
     [std.err(controls)]   [pvalue]   [qvalue]
```

- Mean= Average relative abundance of all replicates for a particular treatment or control
- Std. err = standard error of the relative abundance
- Variance= variance of the relative abundance
- p-value= the specific p-value of the difference between treatment and controls (α=0.05)

All statistically significant bacterial changes have been grouped below by gender, taxonomic classification level, and treatment type. Low dose= 6 mg/L nicotine, High dose= 60 mg/L nicotine.

Any classification of a bacterial phyla, family, or genus by as "Other" means that there is some ambiguity when the RDP classifier, a tool to classify 16S rRNA sequences, tries to classify a sequence at a desired level.

Males- Phylum Level

Low Dose Nicotine

```
Bacteria_Other   0.000506078242127414  3.22641669814822e-08
     8.03295300390612e-05  0.000196127036709133  6.7869624324812e-09
     3.68428077987582e-05  0.00428571428571429   0.0991408669608829
```

High Dose Nicotine

```
Bacteria_TM7     8.22099474946811e-05  1.76638317814345e-09
     2.10141807962115e-05  0     0     0     0.003
     0.0639756312363832
```

Females- Phylum Level

Low Dose Nicotine

none

High Dose Nicotine

Bacteria_Tenericutes 0.0196173786048648 0.000118632077753238
0.00487097685794621 0.00328773117346298 3.35398220903899e-06
0.000819021636959487 0.009 0.17514205433711

Males-Family Level

Low Dose

Lactobacillales_Other 5.44821961218534e-05 1.8784583390964e-09
1.93827672900254e-05 0.00019769704994918 1.77192842930807e-08
5.95303020201993e-05 0.0394285714285714 1

Bacteria_Other 0.000506078242127414 3.22641669814822e-08
8.03295300390612e-05 0.000196127036709133 6.7869624324812e-09
3.68428077987582e-05 0.00404761904761905 0.217048575742202

Bacteroidales_Other 0.000236885382108169 1.28266789637567e-08
5.06491440475685e-05 8.78908637982134e-05 1.95341276230973e-09
1.97656912973452e-05 0.0154761904761905 0.553261075421298

High Dose

Erysipelotrichales_Erysipelotrichaceae 0.0994137354264584
0.000990536994771284 0.0157363988476659 0.0361332019381365
0.000269161301629096 0.00733704711214391 0.00456521739130435
0.253729626353793

TM7_genera_incertae_sedis_Other 8.22099474946811e-05
1.76638317814345e-09 2.10141807962115e-05 0 0 0
0.00239130434782609 0.253729626353793

Females-Family Level

Low Dose

```
Clostridia_Other 0.000149776626023063  2.98942607900999e-08
      7.73230376926565e-05  7.5457476998454e-05   1.26815518117772e-08
      5.0361794669724e-05   0.417545454545454     1


Erysipelotrichales_Erysipelotrichaceae 0.0546018943364957
      0.000287304030155453  0.0075802906297246    0.0932461186724748
      0.00108673835321636   0.0147427158503199    0.0335     1


Lactobacillales_Lactobacillaceae 0.0377431558714485
      0.000177208683535462  0.00595329628920755   0.0355352045104163
      0.000138057717839903  0.00525466874008064   0.781818181818182
      1

Clostridiales_Peptostreptococcaceae    0.0134903952602267
      6.48862248795013e-05  0.00360239433931105   0     0     0
      0.00240909090909091   0.225237429187338



                                High Dose

Erysipelotrichales_Erysipelotrichaceae 0.0546018943364957
      0.000287304030155453  0.0075802906297246    0.0540823768387278
      0.000392261030000208  0.00885732499121724   0.904894736842105
      1


Anaeroplasmatales_Anaeroplasmataceae  0.0196173786048648
      0.000118632077753238  0.00487097685794621   0.00327893455136586
      3.29899350903814e-06  0.000812279940542439  0.007
      0.256892934908272



Clostridiales_Incertae Sedis XIII     0.000946699357539835
      1.52572299241501e-07  0.000174683885485468  0.000421716755042454
      4.28513924176169e-08  9.25757985843134e-05  0.0261052631578947
      0.479018404941741
```

Firmicutes_Other 0.00711543095969389 0.000112068559957022
0.00473431219834565 0.000467372203019862 7.60919535580889e-08
0.000123362841697238 0.185631578947368 1

Males-Genus Level

Low Dose

Lachnospiraceae_Dorea 0.0136950713347456 1.20519529517228e-05
0.0015525432652086 0.00761549924320866 2.18498499722284e-05
0.00209044731922277 0.0326041666666667 0.972289732946028

Lachnospiraceae_Lachnobacterium 0.00861779464101372
1.09366012147551e-05 0.00147895917555253 1.48160792789757e-05
4.41072727574139e-10 9.39225987262e-06 0.000583333333333333
0.0480049006078368

Lactobacillales_Other 5.44821961218534e-05 1.8784583390964e-09
1.93827672900254e-05 0.00019769704994918 1.77192842930807e-08
5.95303020201993e-05 0.0357291666666667 0.972289732946028

Bacteria_Other 0.000506078242127414 3.22641669814822e-08
8.03295300390612e-05 0.000196127036709133 6.7869624324812e-09
3.68428077987582e-05 0.00475 0.260598031871114

Bacteroidales_Other 0.000236885382108169 1.28266789637567e-08
5.06491440475685e-05 8.78908637982134e-05 1.95341276230973e-09
1.97656912973452e-05 0.0181875 0.748362111261456

Firmicutes_Other 0.00546967318507042 8.60635846787163e-06
0.00131197244390815 0.00190961155771361 5.02103593928543e-06
0.00100210138601695 0.0465625 0.972289732946028

High Dose

Ruminococcaceae_Ethanoligenens 0 0 0 0.000155302606032549
2.07288294524089e-08 6.43876221837846e-05 0.0363265306122449
0.897834641782359

Bacteroidales_Other 0.000152741863390901 8.34353836875249e-10
1.44425918456076e-05 8.78908637982134e-05 1.95341276230973e-09
1.97656912973452e-05 0.0254489795918367 0.718844172264912

```
Clostridiaceae_Clostridiaceae 2  0.00916067736440431
     2.442404579968O5e-05  0.00247103448982812   0.00181458302936682
     8.7397748784806e-07   0.000418085514661309  0.0153673469387755
     0.506419090083982


Clostridiaceae_Other  0.00113117910536299   4.70490713219109e-07
     0.000342961628035524  3.85759024091276e-05  3.50145144281068e-09
     2.64629984801824e-05  0.0112040816326531    0.44306626367507

Erysipelotrichaceae_Allobaculum  0.0128502500831121
     2.46170147375422e-05  0.00248077683083053   0.0033219394037327
     4.74378364527574e-06  0.00097404144113849   0.00681632653061225
     0.44306626367507

Erysipelotrichaceae_Turicibacter 0.0853205114908597
     0.000780408527119144  0.0139678964693967    0.0320022328643043
     0.000211236674954142  0.00649979499606168   0.00785714285714286
     0.44306626367507

Porphyromonadaceae_Tannerella    0.000210119713712675
     2.21226826799236e-09  2.35173779788073e-05  0.000108019775830635
     1.92717747005298e-09  1.96325111488723e-05  0.00910204081632653
     0.44306626367507

TM7_genera_incertae_sedis_Other  8.22099474946811e-05
     1.76638317814345e-09  2.10141807962115e-05  0    0    0
     0.00457142857142857   0.44306626367507
```

Females- Genus Level

Low Dose

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Lachnospiraceae_Johnsonella 0.00492557688702376   1.32918753065613e-06
     0.000515594323214701  0.00355368072050797   8.0833005381679e-08
     0.00012714794955616   0.03128    0.469733016683308

Lachnospiraceae_Pseudobutyrivibrio    2.45730433714216e-05
     3.01917230266882e-09  2.45730433714216e-05  0.00058804901965266
     3.14969404818574e-08  7.9368684607794e-05   0    0

Peptostreptococcaceae_Other 0.000120564930207885  2.89699837757587e-09
     2.40707223721095e-05  0    0    0    0.00078
     0.0468531653469284


Ruminococcaceae_Papillibacter    0.00328170011924911
     8.72995586646256e-07  0.00041785059211308   0.00202234876698326
```

2.81611224650229e-07 0.000237323081325955 0.02888
0.469733016683308

Ruminococcaceae_Ruminococcus 8.19101445714052e-06
3.35463589185425e-10 8.19101445714052e-06 0.00876949372556322
4.06894523735502e-05 0.00285269880546651 0.01482
0.356084056636656

Clostridiaceae_Other 4.56124055756584e-05 4.65023893964141e-11
3.0496684867839e-06 1.29935846739211e-05 3.1723121761255e-10
7.96531502970912e-06 0.00366 0.139958814433773

Erysipelotrichaceae_Turicibacter 0.0414717635643814
0.000257038873150052 0.00716992152188645 0.0874258705393499
0.00100651678874377 0.0141881414480105 0.01854
0.371221233133356

High Dose

Lachnospiraceae_Anaerostipes 6.82367432650858e-05
2.81240522970159e-09 2.37166828612333e-05 7.61208799573723e-06
2.89719418274234e-10 7.61208799573723e-06 0.0295
0.315086164472037

Lachnospiraceae_Bryantella 0.00446600723177212 1.87137867186906e-06
0.000611780789477581 0.00268561819825595 4.88687597801516e-07
0.000312630004254715 0.0219791666666667 0.315086164472037

Lachnospiraceae_Johnsonella 0.00834270997457932 1.22597535652538e-05
0.00156587059268982 0.00355368072050797 8.0833005381679e-08
0.00012714794955616 0.0102708333333333 0.189293890898133

Lachnospiraceae_Pseudobutyrivibrio 0.000171880544152656
1.15425503488595e-07 0.000151937818523628 0.00058804901965266
3.14969404818574e-08 7.9368684607794e-05 0.03175
0.315086164472037

Lachnospiraceae_Shuttleworthia 0.000270605439669845
1.54680832906385e-08 5.56202899860087e-05 0.000101457436505272
8.0061236179923e-09 4.00153061165157e-05 0.0269375
0.315086164472037

Ruminococcaceae_Papillibacter 0.00348592725100497
1.61652347353032e-06 0.00056859888735915 0.00202234876698326
2.81611224650229e-07 0.000237323081325955 0.0370625
0.328800544771673

Ruminococcaceae_Ruminococcus 0.00176187108951227
2.07878056026073e-07 0.000203900983826009 0.00876949372556322
4.06894523735502e-05 0.00285269880546651 0.0282708333333333
0.315086164472037

Ruminococcaceae_Sporobacter 0.0108272829781644 2.09328554017207e-05
0.00204611120918296 0.00373936396428086 2.05717727296642e-06
0.000641432346076563 0.00729166666666667 0.162608443803975

Anaeroplasmataceae_Anaeroplasma 0.00327893455136586
3.29899350903814e-06 0.000812279940542439 0.0184073263476775
4.18863629998082e-05 0.00289435184453474 0.000916666666666667
0.0591303432014453

Clostridiaceae_Clostridiaceae 2 9.82183645459707e-05
1.88021509729776e-08 6.13223466168372e-05 0.00105289618354541
4.13154537610648e-07 0.000287455922746653 0.0075625
0.162608443803975

Clostridiales_Other 0.0342855613497661 0.00012236965671993
0.00494711343552844 0.0606039763121214 0.000180564712164157
0.00600940449901914 0.00652083333333333 0.162608443803975

Erysipelotrichaceae_Allobaculum 0 0 0 0.00477615382029546
3.57202274732797e-06 0.000845224555645181 0.0005625
0.0591303432014453

Firmicutes_Other 0.000467372203019862 7.60919535580889e-08
0.000123362841697238 0.00524187336398777 1.91241116396282e-05
0.0019557152982798 0.0293333333333333 0.315086164472037

Incertae Sedis XIII_Anaerovorax 0.000421716755042454
4.28513924176169e-08 9.25757985843134e-05 0.000895575680262389
1.5895680485426e-07 0.000178301320720997 0.0382291666666667
0.328800544771673

APPENDIX B

LIST OF IDENTIFIED SERUM METABOLITES

Female High Dose

Downregulated

Name	Fold Change*	HMDB ID	Mass	Retention Time	Gut Microbiome Related	Pathways
L-Malic Acid	1.679	HMDB00156	134.0874	12.898, 12.905, 12.911		citric acid cycle; pyruvate metabolism
Serine	1.913	HMDB00187	105.0926	13.642, 13.648		amino acid metabolism
•Pyroglutamic Acid	1.842	HMDB00267	129.114	16.098,16.242, 16.248, 16.31		glutathione metabolism
•Phenylalanine	1.823	HMDB00159	165.1891	17.854, 17.860, 17.866	Y	amino acid metabolism
•Uric Acid	1.869	HMDB00289	168.1103	23.940, 23.947, 23.953, 23.959, 23.965		purine metabolism
•Citric Acid	1.737	HMDB00094	192.1235	20.491, 20.497, 20.516		citric acid cycle; energy metabolite
•Aminomalonic Acid	1.57	HMDB01147	119.0761	15.454		Protein synthesis, amino acid biosynthesis
•L-Proline	2.447	HMDB00162	115.1305	12.574, 12.58, 12.586		amino acid metabolism
•L-Tyrosine	1.925	HMDB00158	181.1885	22.091, 22.097	Y	amino acid metabolism

•L-Isoleucine	2.311	HMDB00172	131.1729	12.424, 12.461		amino acid metabolism
•Threonine	1.587	HMDB00167	119.1192	14.061, 14.067		amino acid metabolism
•Inositol	1.587			23.822		
•Azelaic Acid	1.696	HMDB00784	188.2209	20.247		

Upregulated

Name	HMDB ID	Fold Change*	Mass	Retention Time	Gut Microbiome Related	Pathways
Pyrophosphonic Acid	HMDB00250	1.59376	173.94	18.316		Oxidative phosphorylation
Octadecanoic Acid	HMDB00827	1.663	284.47	25.64, 25.646		Fatty acid biosynthesis
Alanine	HMDB00161	1.8961	89.09	9.412, 9.424		Amino acid metabolism
Urea	HMDB00294	1.705	60.05	12.111		Arginine and proline metabolism; urea cycle
Phosphoric Acid	HMDB02142	1.531	97.99	12.392		Oxidative phosphorylation

Male High Dose

Upregulated

Name	HMDB ID	Fold Change*	Mass	Retention Time	Gut Microbiome Related	Pathways

Urea	HMDB00294	2.099035	60.0553	11.461		Arginine and proline metabolism; Urea cycle

Downregulated

Name	HMDB ID	Fold Change*	Molecular Weight	Retention Time	Gut Microbiome Related	Pathways
Urea	HMDB00294	1.635455	60.0553	12.092		Arginine and proline metabolism; Urea cycle
Uric Acid	HMDB00289	2.28372	168.1103	23.941,23.947, 23.953, 23.959,		Purine metabolism
Adenosine	HMDB00050	3.317586	267.2413	29.514,29.515, 29.521, 29.527		Purine metabolism
Butanedioic Acid (Succinic Acid)	HMDB00254	1.621875	118.088	12.874, 12.892, 12.899, 12.905, 12.911, 12.917		Citric acid cycle, oxidative phosphorylation, tyrosine and phenylalanine metabolism
Inositol	HMDB00211	1.81261	180.1559	23.809, 23.815, 23.828		Galactose metabolism; inositol phosphate metabolism
Uridine	HMDB00296	1.753462	244.2014	27.727		Pyrimidine metabolism

1-Glycerophosphoric acid (Glycerol 3-phosphate)	HMDB00126	1.89629215	172.0737	19.735		Glycolysis
L-Valine	HMDB00883	1.718692	117.1463	9.98		Aminoacyl t-RNA biosynthesis; leucine and isoleucine degradation and biosynthesis
Alanine	HMDB00161	1.512268	89.0932	9.43		Amino acid metabolism
Inosine	HMDB00195	1.868108562	268.2261	28.914		Purine metabolism
Malic Acid	HMDB00156	1.93999209	134.0874	15.698		Citric Acid Cycle; Pyruvate Metabolism
Citric Acid	HMDB00094	1.83103664	192.1235	20.503		Citric Acid Cycle; Energy Metabolite

Female Low Dose

Downregulated

Name	HMDB ID	Fold Change*	Mass	Retention Time	Gut Microbiome Related	Pathways
Urea	HMDB00294	1.64963	60.0553	11.543, 12.005, 12.161		Urea Cycle; Arginine and Proline Metabolism

Phosphoric Acid	HMDB02142	3.25255638	98.00	12.367		Oxidative Phosphorylation
Serine	HMDB00187	2.031995196	105.0926	13.624, 13.648		Gycline, Serine, and Threonine Metabolism
Uracil		1.618090887	112.08676	13.286		
Inositol	HMDB00211	1.675342726	180.1559	23.384		Galactose and Inositol Metabolism
L-5-oxoproline (Pyroglutamic Acid)	HMDB00267	1.541653902		16.129		Glutathione Metabolism
L-Proline	HMDB00162	2.464959187		12.611		Amino Acid Metabolism
L-Lysine	HMDB00182	1.87497755	146.1876	21.916		Aminoacyl tRNA Biosynthesis; Biotin Metabolism
DL-Glutamic Acid		1.520687302	147.1293	17.741		
Aminomalonic Acid	HMDB01147	1.818869894	119.0761	15.442, 15.454		Protein synthesis; Amino Acid Biosynthesis
Propylene Glycol	HMDB00784	1.586154458	76.0944	8.024		Pyruvate Metabolism

Upregulated

Name	HMDB ID	Fold Change*	Mass	Retention Time	Gut Microbiome Related	Pathways
Urea	HMDB00294	2.299240724	60.0553	11.736		Urea Cycle; Arginine and Proline Metabolism

Adenosine	HMDB00050	2.508863837	267.2413	29.546		Purine Metabolism

Male Low Dose

Downregulated

Name	HMDB ID	Fold Change*	Mass	Retention Time	Gut Microbiome Related	Pathways
Adenosine	HMDB00050	3.168215	267.2413	29.515, 29.521		Purine Metabolism
Malic Acid	HMDB00156	1.553421096	134.0874	15.717		Citric Acid Cycle; Pyruvate Metabolism
n-Hexadecanoic acid	HMDB00220	1.964925401	256.4241	23.409		Fatty Acid Biosynthesis and Metabolism
L-Lysine	HMDB00182	1.591632559	146.1876	21.934		Aminoacyl tRNA Biosynthesis; Biotin Metabolism
Inositol	HMDB00211	2.153902565	180.1559	23.815		Galactose and Inositol Metabolism
Urea	HMDB00294	1.886849	60.0553	11.717		Urea Cycle; Arginine and Proline Metabolism
Uric Acid	HMDB00289	1.910491225	168.1103	23.947		Purine Metabolism

Upregulated

Name	HMDB ID	Fold Change*	Mass	Retention Time	Gut Microbiome Related	Pathways

Azelaic Acid	HMDB00784	1.734171054	188.2209	20.254, 20.260, 20.266, 20.291		
Propylene Glycol	HMDB01881	1.875827	76.0944	7.924, 7.937		Pyruvate Metabolism
Metanephrine	HMDB04063	3.208841525	197.231	7.118, 7.325, 7.381		Tyrosine Metabolism; Epinephrine Metabolite
Lactic Acid	HMDB00190	1.817014	90.0779	9.193		Gluconeogenesis; Pyruvate Metabolism
3-Pyridinol		1.915615999		8.662		
Linoleic Acid	HMDB00673	2.14738	280.4455	25.296		Linoleic Acid Metabolism
Serine	HMDB00187	1.928971819	105.0926	13.667		Glycine, Serine, and Threonine Metabolism
Urea	HMDB00294	2.365899402	60.0553	12.592		Urea Cycle; Arginine and Proline Metabolism

* These fold-changes represent an average fold change (compared to respective gender control) of all the positively identified features that were identified to represent the presence of a specific metabolite. They were not used in our analysis.