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The Effect of Repeated Opioid Dosing on the Gut Microbiome

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The Effect of Repeated Opioid Dosing on the Gut Microbiome

A thesis submitted in partial fulfillment for the requirements for the degree of Master of Science in Physiology and Biophysics at Virginia Commonwealth University

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Abstract

Opioids are prescribed to millions of people each year, especially to help patients cope with chronic pain, something from which more than a fifth of U.S. adults suffer (CDC). Unfortunately, opioid use and abuse has become a national emergency as the number of opioid prescriptions, opioid misuse, and opioid-related drug overdoses and death have drastically increased in the last twenty years (HHS). Because of the emergent state surrounding opioid use and misuse, the effects of opioids on all aspects of the human body has been an increasingly large focus of research scientists. This study focuses on the possible effect of repeated opioid administration on the gut microbiome.

The human gut microbiome has also been a significant focus for researchers recently as more evidence is unveiling the effects of the gut microbiome on several organs of the body, especially the brain (Galland). It remains unclear the mechanism by which opioids affect humans beyond pain management, particularly cognitive function, mood and behavior. Given the similarities of side effects between gut microbial dysbiosis and chronic opioid use, including decreased gut motility, increased inflammation, altered cognitive function, and behavioral changes, it is possible that some of the effects of opioids on the brain's cognitive functions are mediated through microbial effectors due to alterations in gut microbial composition upon prolonged usage of opioids. Therefore, our lab took special interest in the possible link between opioids, the gut, and the brain.

It was our aim to provide evidence that repeated Buprenorphine, a partial μ-opioid receptor agonist, dosing alters gut microbial composition. By first isolating DNA from non-human primate fecal samples and analyzing DNA quality we were able to prepare DNA libraries

and perform DNA Illumina Next-Generation shotgun sequencing. At the phylum level we observed a fairly common trend in treated subjects of increased Firmicutes during dosing followed by a decrease toward baseline after one week post-dosing. We also observed increases and decreases in the sub-dominant phylum in treated subjects at two weeks of dosing followed by a respective decrease or increase toward baseline after one week post-dosing; however, these trends were less consistent. At the genus level we were unable to observe any trends as a result of opioid administration. Upon performing a non-parametric Wilcoxin Signed-Rank Test, it was determined that there was no significant differential abundance between time points in treated subjects. Quantitative PCR was also performed to validate our sequencing results, but considering the lack of trends we observed, it proved to be difficult to validate anything.

In the end, we were unable to provide significant evidence for our hypothesis. The gut microbiome varies so greatly among and within individuals that finding a significant and consistent alteration in bacterial abundance across all treated subjects proved nearly impossible. What we were able to take away from the study was the observation of some alteration in the microbiome which will need to be studied further by incorporating predicted experimental improvements gleaned from our pilot studies. Despite our results, it is still our hypothesis that opioids affect the microbiome and we encourage future researchers to use our findings as a guide for their experimental design.

Chapter 1 – Background

1.1 - Gut Physiology Overview

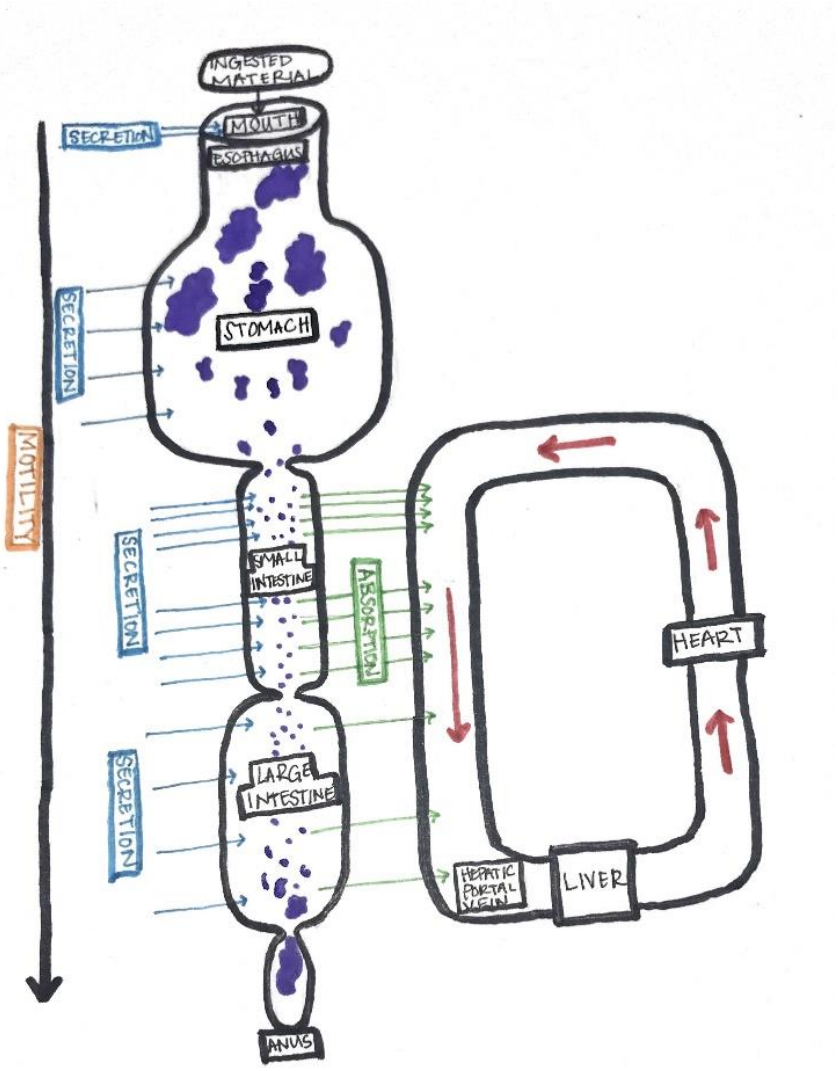


Figure 1. Schematic of gastrointestinal activity including motility, secretion, digestion, and absorption. Purple indicates ingested material.

The gastrointestinal tract encompasses a pathway consisting of multiple organs that allow for the digestion of ingested material. Beginning at the oral cavity where material is first ingested, mechanical digestion (chewing) occurs. This allows for the formation and passage of a bolus to the esophagus which utilizes peristalsis to move said bolus to the stomach. In the stomach, acids and other enzymes transform the bolus to chyme until it is

liquid enough to be passed to the small intestine. The small intestine is where most secretions are added and absorption occurs allowing for the removal of nutrients that can be supplied to the body. From the small intestine, the remainder enters the large intestine where some secretions are added such as water and mucus followed by absorption of vitamins, electrolytes, and water, resulting in the production of feces. Generally, absorbed materials flow through the hepatic portal system to enter the liver first so that nutrients can be removed and stored, and toxic materials can be filtered out. While this process is successful in a healthy gut, an "unhealthy gut" can have detours caused by gut permeability. Gut permeability is caused by a decrease in the protective bacterial barrier that generally exists along the gut walls. This permeability allows partially digested food, toxins, and microorganisms to escape to the bloodstream or impact the underlying gut tissue rather than follow the process detailed above.

The walls of the gastrointestinal tract are comprised of many different tissues. The epithelial cells lie on the mucosa, the innermost part of the gut wall, closest to the lumen. There are different types of muscle that make up the gut wall that assist in gut motility and digestion. In addition, the myenteric and submucosal plexuses make up the enteric nervous system, which is specific to the gut. The general makeup of the gut wall can be seen in Figure 2. However, each region of the gut has unique characteristics of the gut wall that enable it to function differently from its counterparts; therefore, Figure 2 does not accurately represent all regions of the gut. For instance, regions of the small intestine include glands that allow for secretions, Peyer's patches for protection from pathogens, and villi and crypts for absorption; while the stomach has an extra layer of muscle to assist in the breakup of chyme.

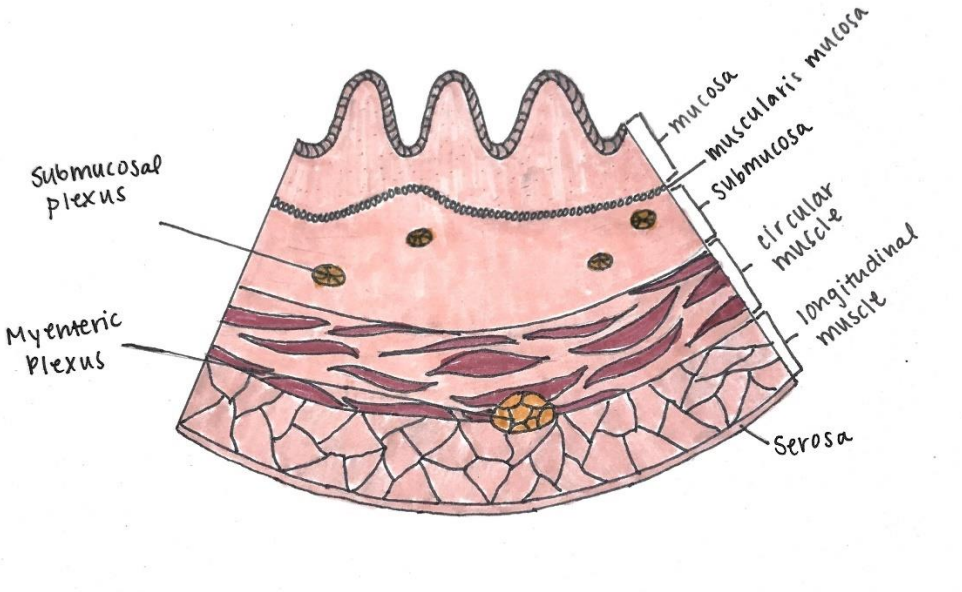


Figure 2. Layers of the gut wall

1.2 - The Gut Microbiome

The gut microbiome is comprised of the Bacteria, Archaea, and Eukarya that live in the gut (Thursby). The microbiome is formed at birth, as the baby travels through the vaginal canal or, in cases of caesarian section, is exposed to other humans and surrounding air. There are two phyla that typically dominate the human gut, Firmicutes and Bacteroidetes. Under Firmicutes falls *Clostridium, Enterococcus, Lactobacillus,* and *Ruminococcus* while *Bacteroides* and *Prevotella* fall under Bacteroidetes. After birth, Enterobacteriaceae, *Streptococci,* and *Staphylococci* dominate the microbiome and as time proceeds the three genera that dominate become *Bacteroides, Prevotella,* and *Ruminococcus* (Power).

1.2a – Diversity in the Microbiota

Proceeding from the stomach to the large intestine, the amount of diversity in the microbiota increases significantly. Based on metagenomic sequencing of fecal matter from 124 individuals, it was found that there are between 1,000-1,500 bacterial species present in the gut microbiome, 160 of such species belonging to each individual (Qin). Building on what was

previously stated, the most common genera of bacteria found in humans are *Bacteroides, Bifidobacterium, Eubacterium, Clostridium, Peptococcus, Peptostreptococcus,* and *Ruminococcus*. However, every person has a different variety of species related to these genera within them and generally have a unique combination of these species that dominate (Gaurner).

The different bacteria in the gut can be characterized by their function and the conditions in which they can function. Facultative anaerobes and aerobes are organisms that can function aerobically in the presence of oxygen or anaerobically in the absence of oxygen, but prefer the conditions indicated by their name. Obligate (strict) aerobes require oxygen to function while obligate (strict) anaerobes cannot survive in conditions in which oxygen is present. The colonic region of the gastrointestinal tract contains higher levels of obligate anaerobes and lower levels of facultative aerobes compared to other regions (Rastall). Of the dominant bacteria in the gut, *Bacteroides, Eubacterium, Bifidobacterium,* and *Peptostreptococcus* are all examples of strict anaerobes (Holzapfel). Among the subdominant genera are facultative anaerobes such as *Escherichia, Enterobacteria, Enterococcus, Klebsiella, Lactobacillus, Proteus*, and *Streptococcus* (Gaurner).

Pathogenic bacteria include those that have the ability to cause disease. Examples of pathogenic bacteria that can be found in the gut are those of the genus *Clostridia* as well as species such as *Eschericia coli*. More specifically, it is known that *Clostridium difficile* can cause inflammation of the colon and *E. coli* causes diarrhea. The gut microbiome is generally balanced so that pathogenic bacteria remain at low levels, however disruption of the balance can lead to both infection caused by exogenous pathogens and increased growth of endogenous pathogens which can lead to increased susceptibility to disease (Gorbach).

Probiotics are beneficial bacteria whose ingestion should enhance colonization of such bacteria resulting in strengthened defense against pathogens along the gastrointestinal tract. *Lactobacilli* are considered beneficial bacteria that are often used as probiotics to strengthen pathogen defense (Rastall). In addition, *Bacteroides fragilis* is another known probiotic (Galland).

Figure 3 depicts common bacteria found in the gut as either potentially pathogenic or beneficial (Kamada, Rastall, Rolhion).

Pathogenic	**Beneficial**
Clostridia (OAn*)	*Lactobacillus* (FAn)
Escherichia coli (FAn)	*Bifidobacterium* (OAn)
Citrobacter (FAn)	*Bacteroides* (OAn)
Salmonella (FAn)	Proteobacteria
Proteobacteria	Eubacterium (OAn)

Figure 3**.** Health negative (pathogenic) and health positive (beneficial) bacteria in the human gut. OAn indicates Obligate anaerobe, Fan indicates Facultative anaerobe. OAn* indicates a bacterial genus that sometimes acts as an obligate anaerobe but not always. Proteobacteria appears in both columns as genera from this phylum can be either potentially pathogenic or beneficial.

1.2b - Function of the Gut Flora

The gut flora has been linked to many regulatory processes in the body. Some important roles of the gut bacteria include that of metabolic processes, immune responses, cell development, hormone regulation, and mental health (Gaurner, Galland, Clarke). In addition, the gut flora are partially responsible for the degradation of certain food components and production of some vitamins and digestive or protective enzymes (Holzapfel). The gut microbiota also plays a role in the transformation of primary bile acids and thus may have involvement in lipid and glucose metabolism (Clarke). Largely, the gut flora can be described as a protective barrier between the gut lumen and the rest of the body. The intestinal epithelium is the path through which absorption of nutrients occurs, therefore the flora residing in the intestinal epithelium acts

as a barrier against uptake of pathogenic microorganisms, antigens and anything else that could be harmful to the body from the gut lumen (Holzapfel). Ideally, harmful substances would be successfully blocked from absorption by these intestinal bacteria and then degraded or excreted from the body.

1.2c - Microbial Dysbiosis

Microbial eubiosis refers to the state of the gut in which the bacterial environment is balanced. In this state, beneficial bacteria are able to compensate for harmful bacteria so that the gut remains healthy. More specifically, the dominating, beneficial bacteria generally belong to the phylum Firmicutes and Bacteroides, while the harmful bacteria, of phylum Proteobacteria or Enterobacteriaceae, is present in a much lower concentration. In microbial dysbiosis, the bacterial environment becomes unbalanced as the beneficial bacteria are overcome by the harmful bacteria (Iebba).

Microbiota can affect intestinal function in several ways including motility, digestion, permeability, and secretion. In addition, components of the microbiota have the ability to leave the gut and enter circulation which allows for dispersal to other organs of the body like the brain, liver, and pancreas affecting their function (Iebba). Therefore, microbial dysbiosis poses a potential threat to intestinal function as well as other organs of the body.

1.2d - The Gut Microbiome and the Brain

There are several ways in which the gut microbiome can affect the brain. First, there is a direct link between the gut bacteria, the enteric nervous system, and the brain via the vagus nerve. This connection is given the name the gut-brain axis and can be seen in Figure 4 (Galland, Clarke). Signaling molecules, secreted or controlled by gut microbiota, talk to vagal afferent nerves, both motor and sensory, which transmit signals to the brain. This innervation can affect satiety, nausea, visceral pain, sphincter operation, and peristalsis (Clarke).

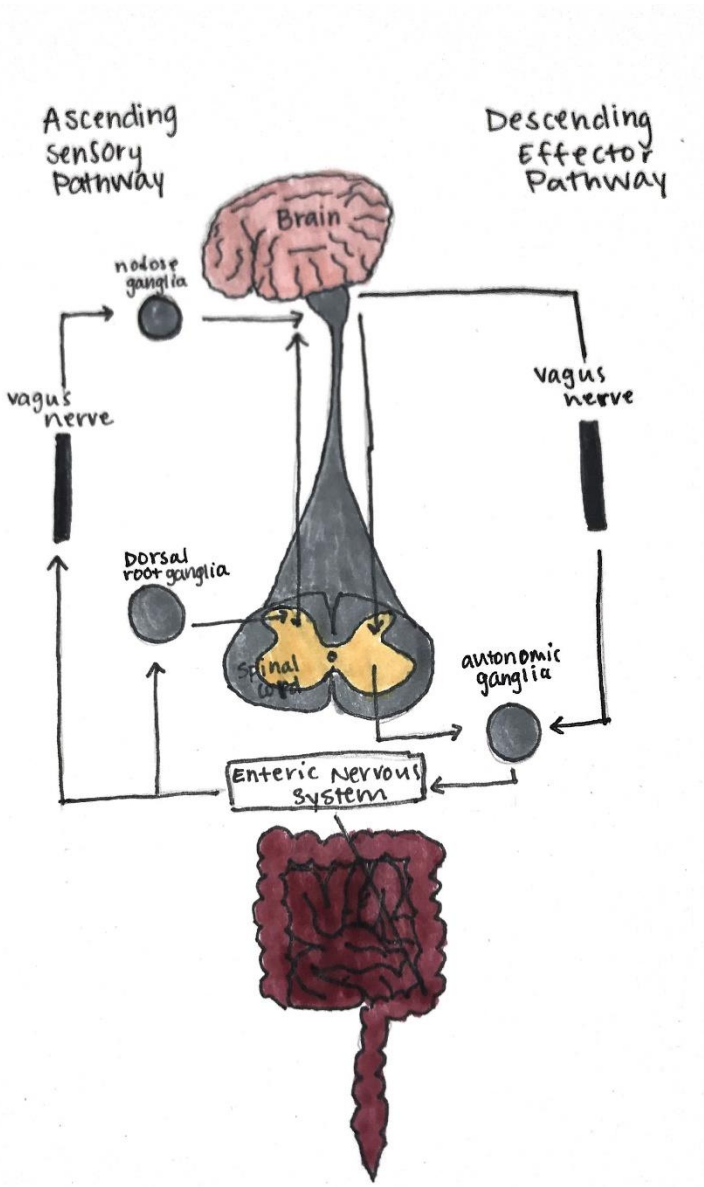


Figure 4. The relationship between the gut's enteric nervous system and the central nervous system

In addition, there is an indirect relationship between the gut and brain via the immune system. Gut bacteria can trigger stimulation of the innate immune system through gut permeability or dysbiosis which acts further to cause systemic or central nervous system inflammation (Galland). In a dysbiotic state, the gut wall's protective barrier becomes compromised so that harmful materials are able to pass through the wall and elicit an immune response.

In further detail, a component of the bacterial cell wall, lipopolysaccharides (LPS), induces synthesis of cytokines by the innate immune system. Therefore, increased bacteria leads to increased LPS which causes increased synthesis of pro-inflammatory cytokine, IL-6 and TNFα, anti-inflammatory cytokines, IL-10 and IL-1 receptor antagonist, as well as cortisol and epinephrine resulting in depressed mood, increased anxiety, impaired long-term memory, and interrupted sleep (Galland). In a study of chronic alcoholics, Leclercq et al. used Cr51-EDTA to determine intestinal permeability and split individuals into two groups based on high or low permeability. As stated previously, intestinal permeability is a factor that contributes to immune response and thus inflammatory response. Those with high permeability showed greater signs of depression, anxiety, and alcohol dependency than those with low permeability. In addition, those with high permeability had decreased colonization of *Bifidobacterium*, a known anti-inflammatory bacteria (Leclercq). This study provides evidence that bacterial dysbiosis can cause decreased protection of the gut wall leading to gut permeability and an immune response that leads to CNS inflammation and ultimately altered CNS function. The adaptive immune system also plays a role in that antigens may react with these bacteria to cause an immune response.

Lastly, these bacteria produce potentially neurotoxic metabolites like D-lactic acid, ammonia, and short-chain fatty acids. Again, increased permeability of the gut wall leads to increased production of these neurotoxic metabolites which have been linked to disorders such as Autism Spectrum Disorder (Galland).

Gut Microbial dysbiosis has been linked to several mental disorders. The aforementioned relationships provide a connection between the gut and the brain which allow the gut microbiome to impact memory, mood, and cognition (Galland). Wikoff et al. performed a study in germ-free mice to look at this gut-brain relationship. They found that in GF mice, synthesis of

most of the chemicals in the blood relies on the gut microbiome and these chemicals influence behavior and/or neuroendocrine response. Additionally, they determined that the microbiome affects brain development as they saw that in GF mice, developmental abnormalities were reversible by intestinal bacterial colonization only in early life (Wikoff).

The gut can also be viewed as an endocrine organ because of its ability to produce and control compounds that enter circulation which travel to and affect distant organs (Clarke). Neuroendocrine mechanisms affect the CNS in a variety of ways depending on the neurotransmitter or hormone that is produced by the gut. Large amounts of GABA (γ-amino butyrate), the most important inhibitory neurotransmitter in the brain, are produced by intestinal bacteria, like *Lactobacillus* and *Bifidobacterium* (Clarke, Galland). GABA regulates the hypothalamic-pituitary-adrenal (HPA) axis, which is activated in response to stress. The HPA system is set up so that stress induces the release of corticotrophin releasing hormone (CRH) from the hypothalamus which stimulates the synthesis and release of adrenocorticotropic hormone (ACTH) from the anterior pituitary which stimulates the synthesis and release of glucocorticoids from the adrenal cortex. The goal of HPA axis stimulation is production of glucocorticoids like cortisol which functions to regulate cardiovascular, metabolic and immunologic changes during stress events (Crowley). GABA inhibits CRH release meaning altered GABA release can alter glucocorticoid production during stress events (Barden). It is theorized that dysregulation in GABAergic transmission is linked to stress-related psychiatric disorders (Crowley). Evidence already exists that patients suffering from severe depression have HPA system alterations (Barden).

Escherichia are known to induce production of norepinephrine, serotonin and dopamine, examples of monoamine neurotransmitters. These neurotransmitters are involved in a variety of

processes including cognitive function, mood alteration, sympathetic nervous system action, regulation of movement, and more (Kema, Ressler, Tzchentke). *Streptococcus* and *Enterococcus* also produce serotonin and *Bacillus* produces norepinephrine and dopamine. *Lactobacillus* produces acetylcholine, the neurotransmitter used at the neuromuscular junction (Galland). Bacterial fermentation of carbohydrates and proteins in the gut lead to the production of short chain fatty acids (SCFAs), like butyrate or propionate. Many bacteria are known to produce SCFAs including *Bacteroides, Bifidobacterium, Propionibacterium, Eubacteria, Lactobacillus, Clostridium, Roseburia,* and *Prevotella*. SCFAs have several functions including modulation of enteroendocrine serotonin (5-HT) secretion and peptide YY (PYY) release and they affect several mechanisms in the body including epithelial cell transport, metabolism, growth and differentiation, hepatic control of lipids and carbohydrates and are a source of energy for muscles, kidneys, the heart and the brain (Clarke). Figure 5 summarizes some of the bacteria that produce or release important neurotransmitters.

Neurotransmitter	**Bacteria**
Serotonin	*Lactobacillus plantarum* *Streptococcus thermophilus* *Escherichia coli* *Klebsiella pneumoniae*
Dopamine	*Bacillus subtilis* *Escherichia coli*
Noradrenaline	*Bacillus subtilis* *Escherichia coli*
GABA	*Lactobacillus brevis* *Bifidobacterium*

Figure 5. Bacterial strains that produce common neurotransmitter

1.2e - Diet on the gut microbiome

Bacteroides dominant microbiomes are associated with high protein and animal fat diets, while *Prevotella* dominant microbiomes are associated with high carbohydrate diets.

Additionally, there is an increase in Firmicutes and decrease in Bacteroidetes correlated with the high-fat and high-sugar Western diet (Clarke).

Diet includes not only food consumption but also consumption of any foreign substance, including drugs. Just as probiotics are ingested to alter the gut microbiota to increase abundance of beneficial bacteria, other drug consumption, like that of antibiotics, anti-inflammatories, or analgesics may alter the microbiota either positively or negatively. It is expected that individuals following the same diet could maintain more similar gut microbial composition. However, age, gender, and other factors must also be considered.

1.3 - Opioids and Pain Management

According to the CDC's analysis of the 2016 National Health Interview Survey (NHIS) date, nearly 20% of U.S. adults, or 50 million people, suffer from chronic pain (CDC MMWR). Opioids are one of the most effective drugs for pain management, both acute and chronic (Rosenblum). However, it is estimated that 11.4 million people misused prescription opioids in 2017 and unfortunately, almost 80% of heroin users claim that they misused prescription opioids prior to using heroin (HHS Opioids). In 2017 the HHS declared a public health emergency as a result of the opioid epidemic and thus research on this topic is pertinent to understanding opioid effects on the human body as well as finding alternative pain treatment to reduce the opioid misuse/overdose statistics.

1.3a - Pharmacology of Opioids and Opioid Metabolism

It is important to note that opioids can be produced endogenously or taken in the form of a drug, the latter being the focus of this project. Opioid metabolism refers to the process by which the body breaks down the drug so that it can be eliminated from the body. Ingested opioid drugs must travel through the gastrointestinal tract before entering the liver's portal system. Once in

the liver, the first phase of metabolism occurs after which the opioids may enter systemic circulation to travel to target tissues (Smith).

The purpose of opioids is to inhibit pain sensation. To do this, opioids activate opioid receptors, which are among the g protein-coupled receptor family (GPCRs), on nerve cells, mostly found in the brain (NIH How opioid drugs activate receptors). While these receptors are largely found in the central nervous system, they are also found in the peripheral nervous system, as well as the gastrointestinal tract and the myenteric plexus of the enteric nervous system (Farzi). When opioids activate opioid receptors, like the μ-opioid receptor, they inhibit the signals produced by neurons as a result of painful stimuli from reaching the brain, in addition to increasing the release of dopamine, resulting in decreased pain and a euphoric state (ATrain Education).

There are three known opioid receptors, μ, δ, and κ which belong to the rhodopsin family of g protein-coupled receptors. They couple with G_i/G_o proteins which cause a cascade of intracellular events including inhibition of adenylyl cyclase activity, inhibition of voltage-gated Ca^{2+} channel opening and thus decreased neurotransmitter release from presynaptic terminals, stimulation of K^+ channels such as GIRKs causing hyperpolarization and inhibition of postsynaptic neurons, and activation of PKC and PLCβ. Opioids are most effective in reducing pain for acute nociception and tissue injury and can work supraspinally, at the level of the spine, and peripherally. Supraspinally, μ-opioid receptor agonists inhibit the release of GABA from periaqueductal gray (PAG) matter. At the level of the spine, opioids decrease the release of spinal neurons typically discharged as a result of pain stimuli to reduce sensitivity to somatic and visceral stimuli that evoke pain as well as reducing release of neurotransmitters. Peripherally,

opioid application produces local anesthetic-like action and reduces over-sensitization in regions suffering from inflammation (McGraw Hill).

1.3b - Buprenorphine

This study will focus on Buprenorphine, a partial μ-opioid receptor agonist, which has been compared to other commonly prescribed opioids such as morphine and methadone. In one study it was shown that buprenorphine lasts longer than morphine, requiring fewer doses for the same analgesic effects, and has similar side effects (Tigerstedt). In a different study, buprenorphine was compared to methadone and results showed similar efficacy in the treatment of opioid dependence for both drugs (Strain). Buprenorphine is generally prescribed for one or more of three reasons: opioid detoxification, opioid maintenance, and pain management. It is considered a semi-synthetic opioid derived from the alkaloid, thebaine, from the opium poppy, *Papaver somniferum*. As a partial μ-agonist, this drug has no effect after a certain dosage when receptor activation plateaus. It also has a high affinity for the μ-opioid receptor and dissociates slowly, allowing it to act for a longer period than other opioid drugs (Welsh).

1.3c - Common Side Effects of Opioids

Some of the most common side effects of opioid usage are constipation, nausea, vomiting, confusion, depression, and susceptibility to disease (CDC Opioid Overdose).' Opioids decrease intestinal motility by inhibiting myenteric activity. The decreased motility increases the likelihood of bacterial translocation as substances are forced to remain in certain regions of the GI tract longer than normal (Balzan). This promotes a link between opioids and susceptibility to disease. In addition, decreased intestinal motility encourages constipation because fecal matter sits in the large intestine longer, allowing more absorption to occur than usual, causing compressed and hardened feces. It is understood that a change in the composition of the gut microbiota is associated with chronic constipation. Generally, this would include a decrease in

obligate bacteria, *Lactobacillus*, *Bifiodbacterium,* and *Bacteroides* and an increase of pathogenic microorganisms like *Psuedomonas aeruginosa* and *Campylobacter jejuni* (Zhao).

The effects of opioids on mental health have been a more recent focus of research. In 2016, a long-term study determined that opioid use lasting greater than 30 days increases the risk of new-onset depression. The study was comprised of over 100,000 patients age 18-80 over a 12-year time frame. Each patient was a new opioid user and did not present with depression at the time of opioid treatment. Results concluded that between 9-12% of patients reported new-onset depression after opioid use. In addition, this percentage was increased in cohorts of patients using for greater than 30 days: 10.7% of patients using 1-30 days reported depression compared to 14.8% of patients using 31-90 days and 19% of patients using longer than 90 days (Scherrer). This study provides support for the link between opioids and mental health, furthering the appeal to study the mechanisms responsible for such results.

1.4 – The Link Between the Gut Microbiome and Opioid Use

There are limited studies relating the gut microbiome and opioid use, but there are a handful that provide evidence for a possible link between opioid use and the gut microbiome. In a study by Acharya et al. chronic opioid use was related to altered gut microbiota in patients with cirrhosis. This study was composed of two cohorts, one with 200 cirrhotic patients both with and without opioid use and the other with 72 cirrhotic chronic opioid users and 72 cirrhotic non-opioid-users. Using stool, they determined the composition of the microbiota of these patients and discovered that opioid users in cohort 2 had significant microbial dysbiosis, specifically a decrease in autochthonous taxa and Bacteroidaceae abundance. In cohort 1, they saw an increase in “all cause” readmission in cirrhotic opioid users, indicating a possible link to opioid use, microbial dysbiosis, and pathogen susceptibility. In a study by Banerjee et al. it was shown that

opioid-induced gut microbial disruption leads to gut barrier compromise and systemic inflammation in C57B16/j mice. Using bacterial 16srDNA sequencing they found that chronic morphine treatment significantly altered the gut microbial composition. They saw an increase in gram-positive Firmicutes phyla, specifically families *Enterococcaceae, Staphylococcacaea, Bacillaceae, Streptococcaceae,* and *Erysipelotrichaeceae,* and a decrease in phyla Bacteroidetes as a result of opioid dosing. Because of a lack of research on this topic, especially in animals closely related to humans, a pilot study is necessary for providing evidence for the link between the gut microbiome and opioid use. Researchers are limited in their ability to gather willing participants for such a study. Even if willing human participants are available, like in the Archarya study, the presence of other health conditions creates compounding factors that may influence results. Therefore, healthy animal models are most likely the most available and reliable participants of such a study.

Phylogenetically, humans, non-human primates, and rodents all stem from a common mammalian ancestor. Rodents, rather than non-human primates, are often chosen as the subjects in scientific studies that aim to provide insight into human microbiology, biochemistry, physiology, etc. However, because the gut microbiome varies so greatly, even among humans, to provide the most significant results, it was pertinent to choose subjects most closely related to humans, that being non-human primates i.e. rhesus monkeys. In 2007, the genome of the rhesus macaque monkey was discovered, and it was determined that rhesus monkeys and humans share about 93% of the same DNA (Gibbs). Chen et al. performed a study looking at the diversity in the rhesus macaque microbiota compared to that of humans and found that similarly to humans the microbiota is rich in Bacteroidetes, Firmicutes, and Proteobacteria, but phylotypes varied at different body sites. Importantly, the anal community, indicative of the gut microbiome had an

increased abundance of *Prevotella* which differs from humans whose gut microbiome is dominated by *Bacteroides*. Despite the differences, they concluded that rhesus monkeys are a good animal model for characterization of microbes. Yet, it is important to understand that despite the genomic and microbial similarity between humans and rhesus monkeys, it remains possible that the baseline gut microbial composition of these animals is different than what we expected to find given our knowledge of the human gut microbiome.

Chapter 2 - Hypothesis and Aims

2.1 - Hypothesis

We hypothesize that repeated opioid dosing alters the gut microbiome such that beneficial bacterial abundance decreases while potentially pathogenic bacterial abundance increases. We expect that this alteration influences other organs in the body, most importantly the brain, and plays a role in the mechanism by which opioids alter the psychological state of individuals.

2.2 - Aims

Aim 1. To characterize the baseline gut microbial composition of the subjects and determine the extent of variation in the baseline composition among the different subjects.

Aim 2. To determine if opioid dosing affects the gut microbiome using shotgun sequencing and qPCR methods. If there is a change, we aim to characterize said change in terms of beneficial/pathogenic bacterial abundance.

Sub-Aim 2-1. To compare the gut microbial composition before dosing to that at one week of dosing.

Sub-Aim 2-2. To compare the gut microbial composition before dosing to that at two weeks of dosing.

Aim 3. If there is a change in the gut microbiome from pre-dosing to during-dosing, we aim to determine if the microbiome returns to the baseline following the end of opioid dosing.

Chapter 3 – Materials and Methods

3.1 - Overview

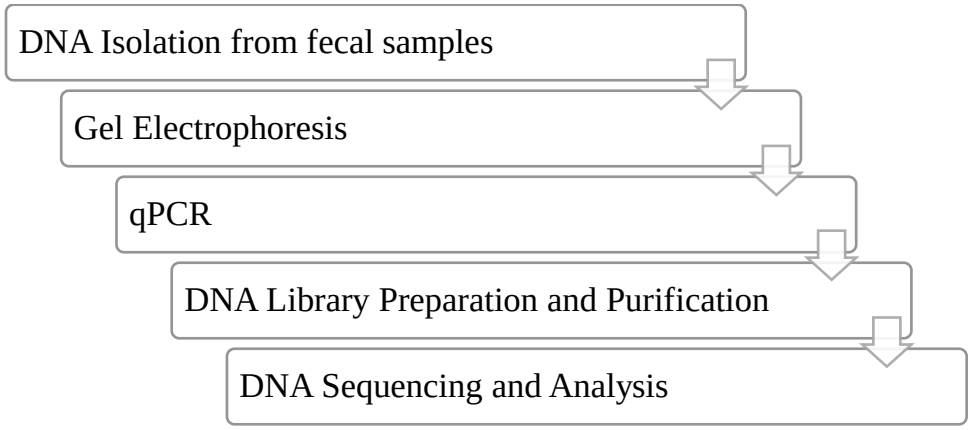


Figure 6. Materials and methods workflow

3.2 - Subjects

Nine rhesus monkeys were selected to participate as subjects in this study, four females and five males. The four females are subjects 1533, 1542, 1543, and 1544 and the five males are subjects 1496, 1497, 1500, 1501, and 1532. Of these nine, four were in the control group and five were in the Buprenorphine treatment group as shown in Figure 7.

Control Group	Buprenorphine Treatment Group
1496 (M)	1497 (M)
1501 (M)	1500 (M)
1533 (F)	1532 (M)
1542 (F)	1543 (F)
	1544 (F)

Figure 7. Control group subjects versus treatment group subjects. M represents males and F represents females.

3.3 - Drug of choice

Buprenorphine was chosen as the opioid used in this study. Buprenorphine, a partial agonist, has a lower efficacy as it is less effective at stimulating the μ-opioid receptor compared

to full agonist opioids used for pain management like Oxycodone or Morphine. The half-life of Buprenorphine is also longer than that of stronger analgesics. While Buprenorphine is a less intense opioid drug, it is still known to cause similar gastrointestinal problems as other opioids, such as inflammation, constipation, and nausea.

3.4 - Dosing and Sample Collection

Cohort 1 Subject	6-Aug	8-Oct	12-Oct	26-Oct	5-Nov	6-Nov **Start dosing**	12-Nov	13-Nov 1 week	18-Nov	19-Nov 2 weeks	24-Nov **Last Dose**	26-Nov	30-Nov	1-Dec 1 week post	2-Dec	7-Dec
1532	**X**				**X**			X		X		X		X		
1533*	**X**				**X**			X		X		X		X		
Cohort 2 Subject																
1542*	**X**				**X**			X		X		X	X			
1543	**X**				**X**			X		X		X		X		
1544	**X**				**X**			X		X		X		X		
Cohort 3 Subject									1 week			2 weeks	**Last Dose**			1 week post
1496*	**X**	**X**					**Start dosing**		X			X			X	X
1497		**X**	**X**						X			X			X	X
1500		**X**		**X**					X			X			X	X
1501*	**X**	**X**							X			X			X	X

Figure 8. Treatment and sample collection schedule. * indicates control subjects. X indicate sample collection.

Dosing occurred over a seventeen-day period and Buprenorphine/Saline was administered via intramuscular injections. Subjects 1532 and 1500 were administered 0.1mg/kg daily in divided doses while the remaining subjects were administered 0.03mg/kg daily in divided doses. Control subjects were administered saline in place of Buprenorphine. During dosing, lab assistants constantly checked for precipitate withdrawal.

Stool was collected from each subject at different time points before dosing, during dosing, and after dosing. Stool samples were stored in a -80° freezer and thawed when necessary for DNA isolation.

3.5 - DNA Isolation

Stool samples were thawed and a 200mg (+/- 50mg) portion was aliquoted into a clean microcentrifuge tube to be used for isolation as directed by the PureLink Microbiome DNA Purification Kit User Guide for Stool Samples (Invitrogen), the protocol chosen for DNA isolation. The concentration (ng/µL) of isolated DNA was found via the Nanodrop Spectrophotometer and the purity of the sample was observed using the "Purity A260/A280" ratio given by the machine, which quantifies the absorbance maxima of nucleic acids, at 260 nm and 280 nm. A A260/A280 ratio nearing 2.0 is the aim for such samples to indicate minimal impurities. The A260/A230 ratio is also used to assess solvent contamination in the DNA samples and this ratio should be greater than 1.8.

3.6 - Gel Electrophoresis

Following DNA isolation, gel electrophoresis was performed on the DNA samples to observe possible degradation. The apparatus was prepared with a .8% agarose gel made from TAE buffer, agarose, and red dye. Upon hardening of the gel, it was submerged in TAE buffer. 5µL of DNA sample was combined with 1µL loading dye and inserted into the gel wells and ran at 75V for 45 minutes.

3.7 - Quantitative PCR (qPCR)

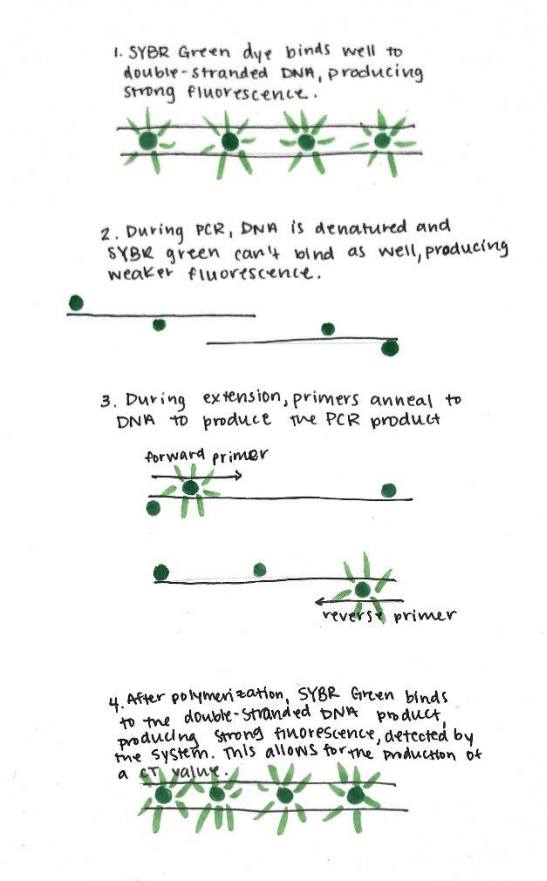


Figure 9. Depiction of SYBR Green based qPCR

For each 96-well qPCR plate that was run, each well was filled accordingly with 10µL SYBR Green Master Mix, 9µL nuclease free water, 0.1µL forward primer, 0.1µL reverse primer, and 1µL 10ng/µL diluted DNA sample. Plates were run in triplicate, meaning each DNA sample was added to three wells containing the same primer. Additionally, each primer was added to a control well in which no DNA sample was added so that primers could be assessed for contamination. Each qPCR plate was run on the QuantStudio Real-Time PCR machine which provided average threshold cycle values for each DNA sample with each primer which were used to quantify bacterial levels in the DNA samples. The thermocycler was set to forty cycles, therefore CT values only go as high as forty.

3.7a - qPCR Primers

We originally selected nine primers designed by other researchers. The two Domain Bacteria primers were used as universal primers to determine the abundance of bacteria as a whole in the DNA sample. The remainder of the primers were chosen because they are known as typically abundant bacteria in humans. *Clostridium* and Spirochaete were chosen because their abundance often increases in inflammatory states. After numerous attempts it was found that the *Bifidobacteria* primer was ineffective and due to extraneous circumstances, we were unable to replace the primer. Therefore, in the end we utilized eight primers to verify sequencing results provided by Hermann-Bank et al.

Target	**Primer sequences (5'→ 3')**		**Positions (bp)**
Spirochaetes	Forward	GTYTTAAAGCATGCAAGTC	294
	Reverse	TGCTGCCTCCCGTAGGAG	
Domain Bacteria A V2-V3	Forward	AGAGTTTGATCCTGGCTCAG	336
	Reverse	CTGCTGCCTYCCGTA	
Domain Bacteria B V4-V5	Forward	CAGCAGCCGCGGTAATAC	389
	Reverse	CCGTCAATTCCTTTGAGTTT	
Genus *Lactobacillus*	Forward	GCGGTGAAATTCCAAACG	216
	Reverse	GGGACCTTAACTGGTGAT	
Species *Clostridium perfingens*	Forward	TGAAAGATGGCATCATCATTCAAC	258
	Reverse	GGTACCGTCATTATCTTCCCCAAA	
Genus *Bacteroides*	Forward	AAGGTCCCCCACATTGG	300
	Reverse	GAGCCGCAAACTTTCACAA	
Species *Escherichia Coli*	Forward	GTTAATACCTTTGCTCATTGA	320
	Reverse	ACCAGGGTATCTAATCCTGTT	
Phylum Fusobacteria	Forward	GATCCAGCAATTCTGTGTGC	292
	Reverse	CGAATTTCACCTCTACACTTGT	

Figure 10. qPCR primers

3.8 DNA Library Preparation

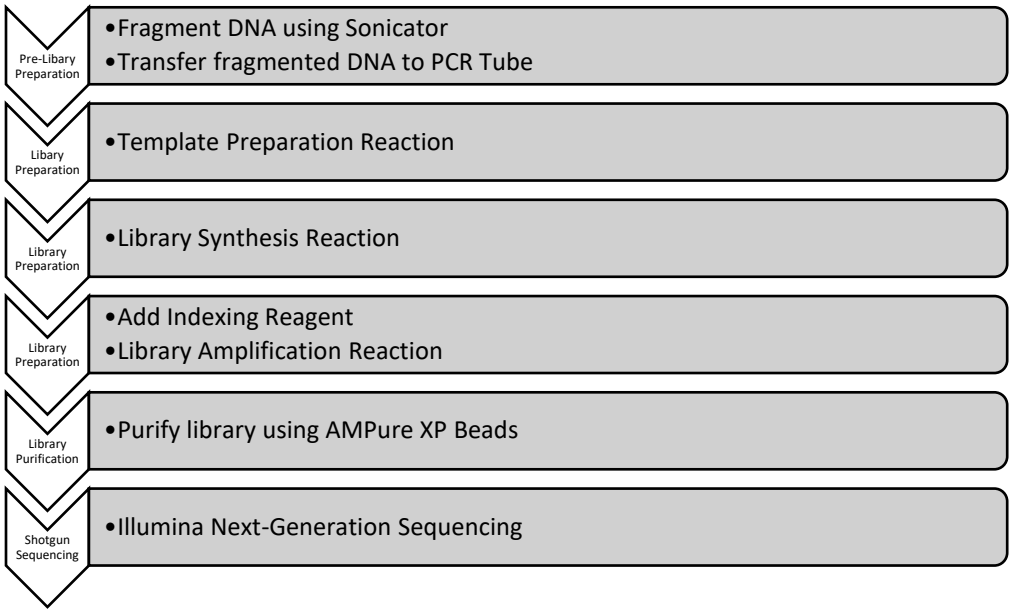


Figure 11. DNA library preparation, purification, and sequencing workflow

DNA libraries were created using the Takara Bio ThruPLEX DNA-seq Kit and User Manual. Samples were diluted so that 1 µg DNA went into 52.5 µL H_2O making each sample equal to 20 ng/µL. Following dilution, each sample was transferred into a covaris tube and then fragmented using the Covaris S2 Sonication machine. To do so, the sonication machine's tank was filled with dH_2O, degas was turned on, water was cooled to 4-6°C and each sample was fragmented for 50s. Subsequently, 1 µL fragmented DNA was mixed with 9 µL H_2O into PCR tubes rather than 10 µL fragmented DNA as suggested by the ThruPLEX DNA-seq Kit User Manual. This was done so that in the Library Amplification step, the amount of input DNA was 20 ng/µL, therefore the thermocycler was set for six PCR cycles. In addition, we used a dual index 96-well plate.

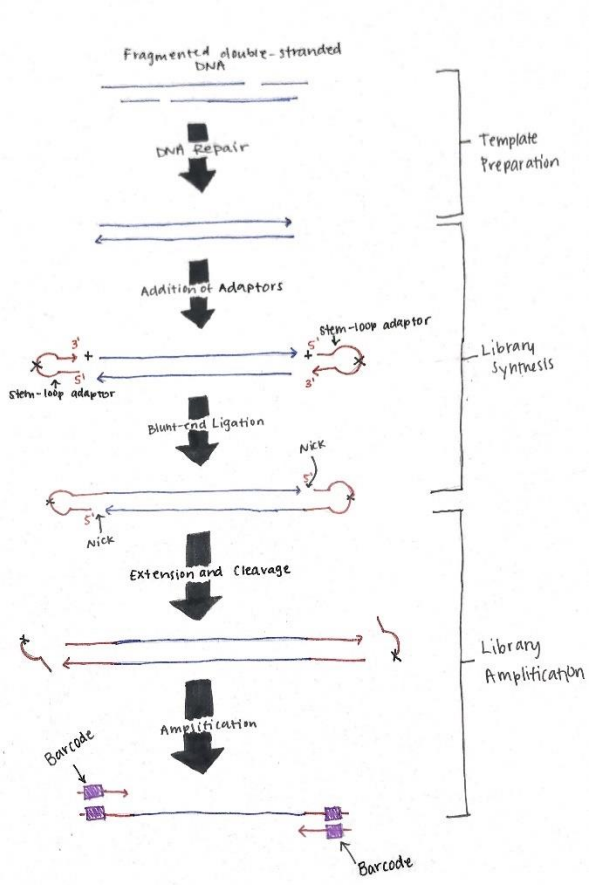


Figure 12. Overview of DNA library preparation with Takara Bio ThruPLEX DNA-seq kit

3.9 - DNA Library Purification

AMPure beads were used to purify DNA libraries. Beads were vortexed and incubated at room temperature for 10 minutes. Following incubation, 48 µL beads were mixed with 32 µL H_2O and this mixture was added to the 50 µL library and incubated for 10 minutes. Following incubation, the mixture was placed on the magnet stand for 5 minutes. The supernatant (130 µL) was saved and added to a clean microcentrifuge tube in which 15 µL beads was mixed in and incubated for 10 minutes. Following incubation, the mixture was placed on the magnet stand for 5 minutes. The supernatant was removed and the beads were given an 80% ethanol wash twice. Following the second wash, the ethanol was completely removed from the tube and the beads were left to dry for 15 minutes. The tubes were taken off the magnet stand and 30 µL of H_2O was

mixed with the beads by pipetting and the mixture was incubated at room temperature for 10 minutes. Following incubation, the mixture was placed on the magnet stand for 5 minutes. 25 µL of the supernatant was removed and added to clean microcentrifuge tubes and this was the purified DNA library.

3.10 - DNA Sequencing

Following DNA library preparation and purification, samples were sent for bioanalysis. Ideally, to succeed to the sequencing step, bioanalyzer results for each sample must show sharp peaks at 35 base pairs and 10380 base pairs and a curved peak at about 400-500 base pairs. Following bioanalysis, samples were re-purified to eliminate any unwanted adapter dimers and qPCR was performed on all samples. Illumina Next-Generation Sequencing was performed.

3.11 - Analysis

Microbiome Analysis

MetaPhlAn (metagenomic phylogenetic analysis) was performed from our sequencing results to profile the composition of the microbial community from each DNA library.

Statistical Analysis

A Wilcoxon signed-rank test was performed at the genus and species levels given the data provided by the MetaPhlAn output. The control group was compared to the treated group at different time points. A Wilcoxon test is used to determine if any given genus was differentially abundant between time points. In addition, a Kruskal-Wallis test was performed to determine significant alterations in the microbiome when comparing the control group and treated group prior to dosing and during dosing. This test revealed an LDA (linear discriminant analysis) score was provided for genera that were

significantly altered which describes ranked discriminative features consistent across the study.

Chapter 4 - Results

4.1 - DNA Isolation Nanodrop Results (Spectrophotometric Analysis)

4.1a - DNA concentration

DNA was isolated from fecal samples using the protocol mentioned in the materials and methods. Following isolation, DNA concentrations were determined using the Nanodrop machine and concentrations for each sample are listed in the charts below. Concentrations varied as some samples had concentrations <100 while others had concentrations >100 and a few had concentrations >50 ng/ul. All samples listed had reasonable concentrations for proceeding to the library preparation and sequencing step.

Subject	Collection Date	Concentration (ng/ µL)
1496	8/6/2018	159.5
	10/8/2018	75.4
	11/18/2018	126.5
	11/26/2018	115.2
	12/2/2018	84.1
	12/7/2018	87.3
1497	10/8/2018	126.2
	10/12/2018	103.4
	11/18/2018	110.5
	11/26/2018	63.7
	12/2/2018	88.3
	12/7/2018	88.6
1500	10/8/2018	124.6
	10/26/2018	75.1
	11/18/2018	141.5
	11/26/2018	115.7
	12/2/2018	87.7
	12/7/2018	34.8
1501	8/6/2018	145.6
	10/8/2018	46.4
	11/18/2018	84.1
	11/26/2018	57.6
	12/2/2018	107.6
	12/7/2018	108.4
1532	8/6/2018	105.9
	11/5/2018	47.1
	11/13/2018	127.3
	11/19/2018	97.5
	11/26/2018	64.1
	12/1/2018	51
1533	8/6/2018	121.5
	11/5/2018	107
	11/13/2018	134.9
	11/19/2018	126.7
	11/26/2018	114.2
	12/1/2018	48.4
1542	8/6/2018	99.3
	11/5/2018	53.7
	11/13/2018	127.8
	11/19/2018	89.4
	11/26/2018	60.6
	11/30/2018	73.1
1543	8/6/2018	90.6
	11/5/2018	78.6
	11/13/2018	103.5
	11/19/2018	69.3
	11/26/2018	91.2
	12/1/2018	91.9
1544	8/6/2018	117.3
	11/5/2018	70.2
	11/13/2018	83.6
	11/19/2018	100.5
	11/26/2018	56.3
	12/1/2018	68.1

Figure 13. DNA concentrations of isolated DNA from fecal samples

4.1b - Ratios (DNA Quality)

While DNA concentration was obtained following DNA isolation, so was the A260/A280 and A260/A230 ratios. These ratios are indicative of DNA sample quality

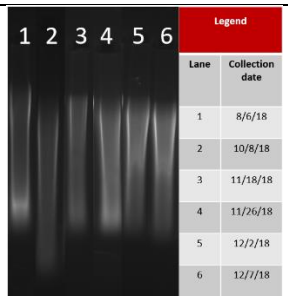
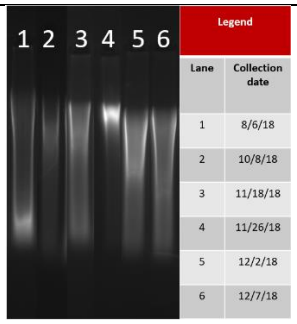
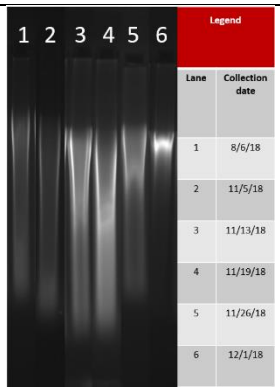
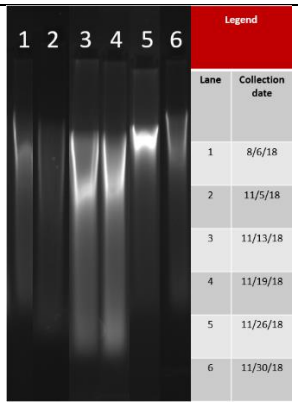
and contamination. It was imperative that the A260/A230 ratio fell within the 1.7 to 2.1 range otherwise the DNA needed to be isolated again. An A260/A230 ratio that did not fall in this range was indicative of DNA contamination, which did occur during some DNA isolations. From abnormal results of the A260/A230 ratios, it became apparent that the wash buffer from the DNA isolation kit was unusable and a new wash buffer needed to be implemented in order to maintain quality DNA. Following this wash buffer exchange, samples were isolated and ratios returned to normal. The poor quality DNA isolated using the subpar wash buffer was not recorded and do not appear in the figures below as this DNA was discarded.

Subject	Collection Date	A260/A280	A260/A230
1496	8/6/2018	1.79	1.91
	10/8/2018	1.81	1.96
	11/18/2018	1.84	1.83
	11/26/2018	1.85	2.01
	12/2/2018	1.85	1.8
	12/7/2018	1.83	1.74
1497	10/8/2018	1.83	1.91
	10/12/2018	1.84	1.84
	11/18/2018	1.86	1.83
	11/26/2018	1.82	1.78
	12/2/2018	1.86	1.8
	12/7/2018	1.83	1.86
1500	10/8/2018	1.85	1.88
	10/26/2018	1.84	1.79
	11/18/2018	1.85	1.87
	11/26/2018	1.83	1.86
	12/2/2018	1.81	1.82
	12/7/2018	1.85	1.55
1501	8/6/2018	1.79	2.03
	10/8/2018	1.83	1.68
	11/18/2018	1.86	1.79
	11/26/2018	1.82	1.91
	12/2/2018	1.84	1.8
	12/7/2018	1.84	1.94
1532	8/6/2018	1.78	1.91
	11/5/2018	1.76	1.62
	11/13/2018	1.81	1.88
	11/19/2018	1.81	1.76
	11/26/2018	1.79	1.87
	12/1/2018	1.84	1.63
1533	8/6/2018	1.8	1.78
	11/5/2018	1.83	2.11
	11/13/2018	1.85	2.03
	11/19/2018	1.84	1.91
	11/26/2018	1.85	1.87
	12/1/2018	1.87	1.74
1542	8/6/2018	1.77	1.66
	11/5/2018	1.79	1.78
	11/13/2018	1.84	1.98
	11/19/2018	1.85	1.97
	11/26/2018	1.83	2.01
	11/30/2018	1.93	1.93
1543	8/6/2018	1.78	1.82
	11/5/2018	1.82	2.05
	11/13/2018	1.87	2.09
	11/19/2018	1.84	1.84
	11/26/2018	1.83	1.65
	12/1/2018	1.83	1.98
1544	8/6/2018	1.78	2.02
	11/5/2018	1.82	2.12
	11/13/2018	1.85	2.13
	11/19/2018	1.83	1.95
	11/26/2018	1.85	2.02
	12/1/2018	1.83	2.08

Figure 14. Spectrophometric analysis results from isolated DNA from fecal samples

4.2 - Gel electrophoresis (DNA quality)

Following DNA isolation and Nanodrop analysis, DNA samples were run on an agarose gel to depict DNA quality. Stool samples typically have a less defined band on gels. We looked for a bright band located closer to the well as a marker for high quality samples. Most samples did maintain this result, however, a few samples resulted in bright bands toward the end of the gel. While this indicates more degraded DNA, it was still determined that these samples were of high enough quality to be used for sequencing. It should be noted that due to technological difficulties, we were unable to provide photographic evidence of some gels. Therefore, these gels were run again (lanes 1 and 2 for each subject) at a much later date, in order to provide photographic evidence for the thesis. It is possible that some DNA that appears more degraded than others had been stored in the freezer longer or thawed a greater number of times.

Sample	Gel	Sample	Gel
1496		1501	
1533		1542	

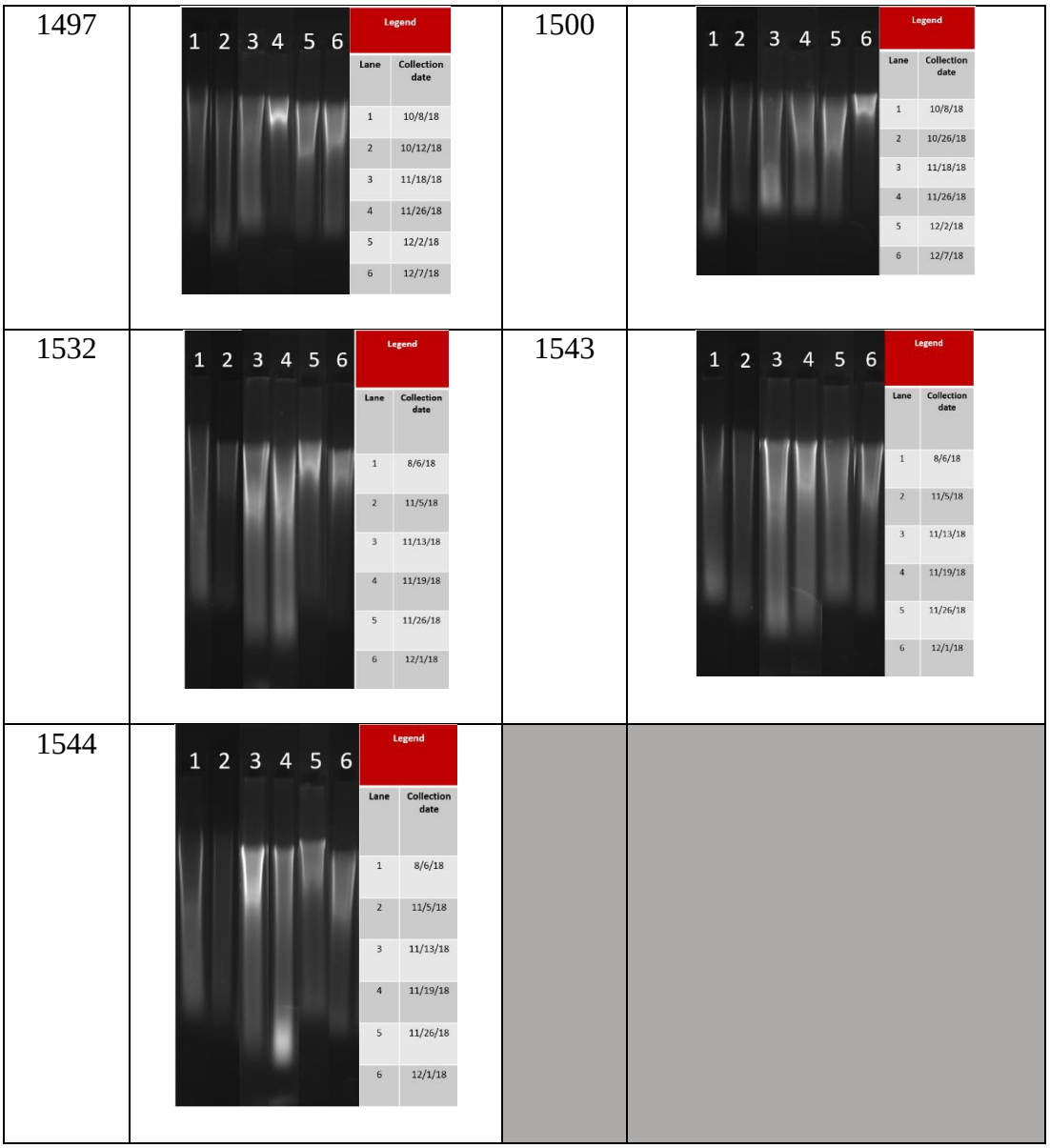


Figure 15. Gel electrophoresis of isolated DNA from fecal samples

4.3 - qPCR Analysis Using Primers for Gut Microbiota

Our first approach to examine changes in gut microbiota was via qPCR using bacterial primers that we predicted would be abundant or result in altered abundance upon opioid administration. It is important to first note that we performed qPCR using nine primers, but only included results from eight of the primers as the *Bifidobacteria* primer maintained low CT values (high abundance) in wells that were meant as a control i.e. wells that lacked any DNA sample. In

addition, the *Spirochaetes* primer was relatively unreliable as it resulted in low CT values in control wells on some plates.

CT values indicate abundance of whichever primer was used in the DNA sample. Values ≥ 40 are considered undetermined, meaning that the bacteria of the primer is essentially non-existent in the sample. Values between 30-40 indicate moderate abundance levels and values ≤29 indicate highly abundant bacteria, with decreasing values indicating increasing abundance.

CT values for both primers bacterial universal 1 and bacterial universal 2 remained in a range from 9-12 for all subjects at all dates, meaning there was consistently very abundant bacteria levels in the samples. Figure 16a depicts the CT values at chronological dates of each of the eight primers for each of the control samples. According to these results, there is much variability at the different dates, however, overall it can be see that pathogenic bacteria such as *Escherichia coli* and *Clostridium* remained close to undetermined CT values (depicted as nearing forty on the graphs), meaning these bacteria were not abundant in the subjects throughout the study. On the other hand, bacteria such as *Lactobacillus* and Spirochaetes maintained much lower CT values, in the range of 15-25, meaning these bacteria were much more abundant in the subjects throughout the study. *Bacteroides* maintained CT values in the range of 25-28, meaning it was abundant in the subjects as well. Lastly, Fusobacteria resulted in CT values ranging somewhere between the non-existent bacteria, *E. coli* and *Clostridium*, and the abundant bacteria, *Bacteroides*, meaning it was relatively, but not very, abundant in the subjects throughout the study.

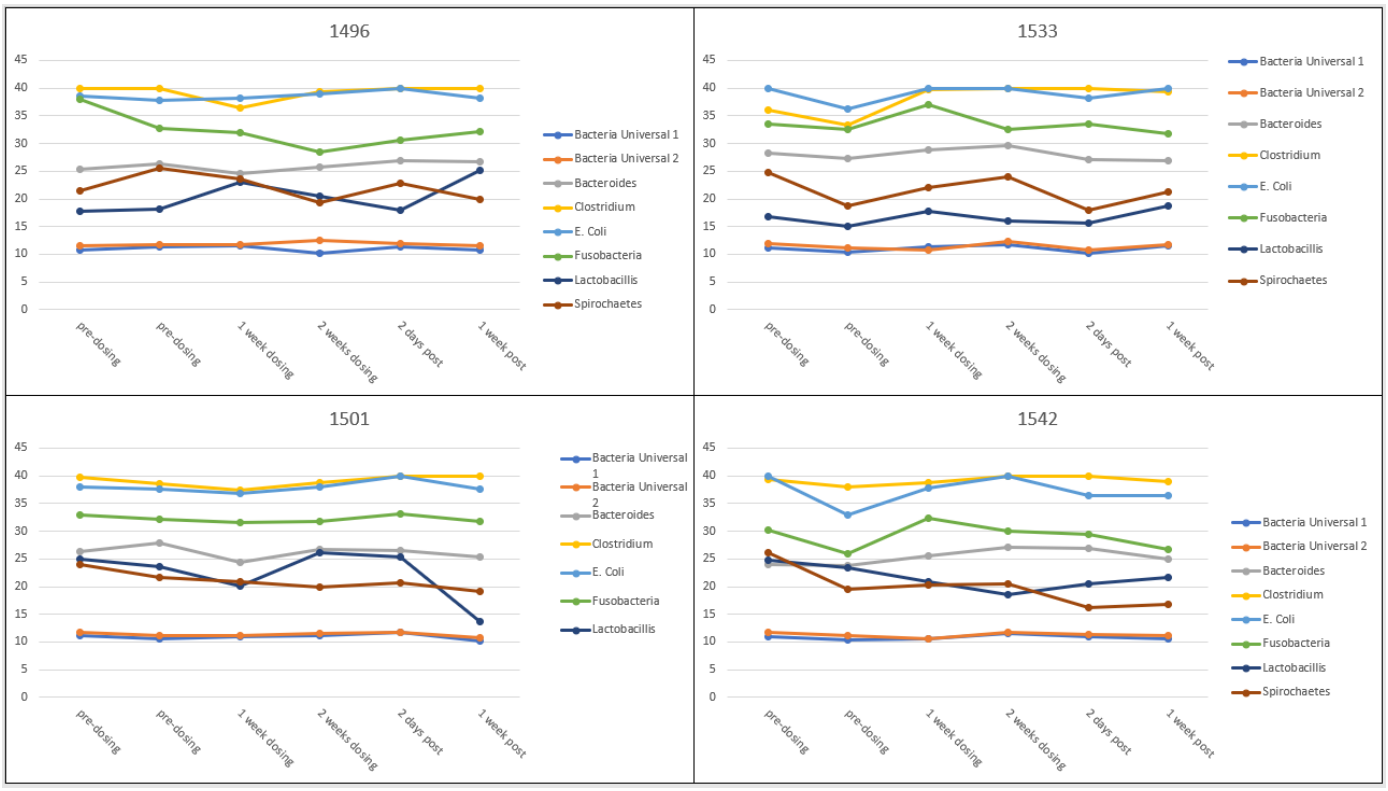


Figure 16a. qPCR results showing CT values for 8 primers of control subjects at each time point in the study. Y-axis values represent CT values.

Figure 16b depicts the CT values at each date for the treated group. Similar relative abundances are found in these subjects as found in the control subjects. However, it is important to look at the changes in the CT values for the bacterial primers during dosing compared to before dosing. For example, in subjects 1497 and 1532 there is an increase in the CT values for Fusobacteria during dosing and post-dosing, meaning there was a decrease in Fusobacteria abundance, possible as a result of the opioid dosing. However, in subjects 1500, 1543, and 1544 the same trend cannot be seen, in which Fusobacteria maintains fairly constant CT values, indicating that the change in abundance in subjects 1497 and 1532 may have been spontaneous and not as a result of opioid dosing. Subject 1532 has a unique profile in which the CT values for Fusobacteria, Spirochaetes, and *Bacteroides* increased at the second week of dosing, indicating a

decrease in their abundances. Unfortunately, it is difficult to observe similar changes in the other subjects.

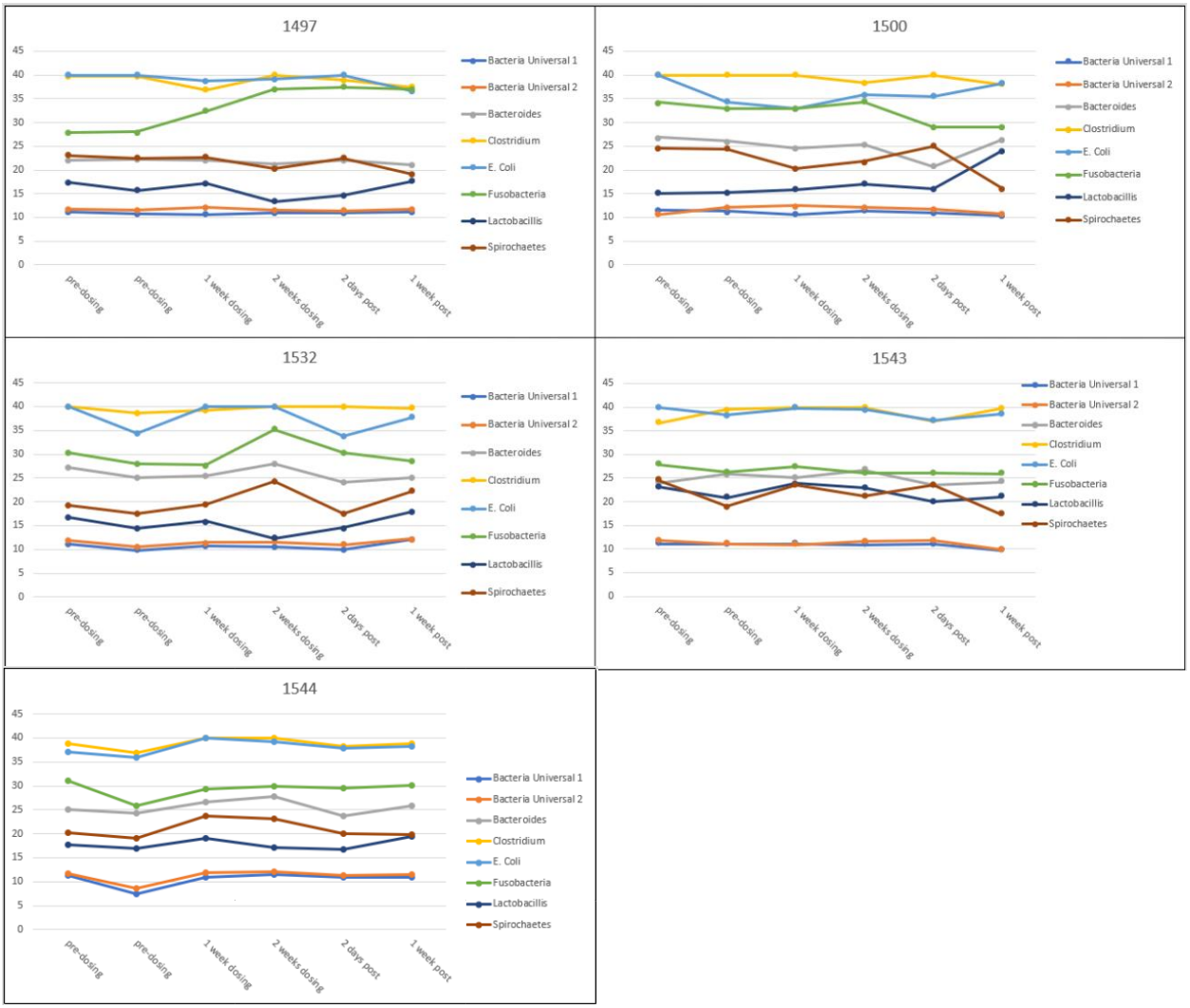


Figure 16b. qPCR results showing CT values from 8 primers for Buprenorphine-treated subjects at each time point in the study. Y-axis values represent CT values.

Figures 19 (a-i) illustrate a more in depth look at the change in CT values for a specific bacterial primer in each subject at the different time points. Error bars were added to each bar in the graph using the standard deviation of the results from the three wells used for each sample and primer.

Control subject 1496, shown in Figure 17a had variability especially in bacteria *Lactobacillus,* Fusobacteria, and Spirochaetes. However, *Bacteroides, Clostridium,* and *E. coli* remained mostly constant across all time points. Figure 17b illustrates the CT values for control subject 1501. This subject had less variability than subject 1496, but it was still apparent especially in *Lactobacillus*. However, the levels of the remaining primers were consistent with those found in subject 1496. CT values for control subject 1533 are shown in figure 17c. There is variability in the CT values in this subject as well, but the levels of each bacteria remain consistent with the previous control subjects. Spirochaetes resulted in greater variability, similar to the variability seen in subject 1496. These results could be inaccurate given the sometimes-dysfunctional primer. CT values for *Clostridium* also showed variation as the pre-dosing values are lower than the dosing and post-dosing values. However, the standard deviations for the pre-dosing results are large enough that one of the wells could have skewed the results because of contamination. Figure 17d depicts the CT values for control subject 1542. Like the other controls, the bacterial abundances varied at each date, but remained consistent with the overall abundance of each bacteria. Subject 1497, shown in Figure 17e was dosed with buprenorphine. The abundances remained constant according to the CT values from all the primers, excluding Fusobacteria for which the CT values increased during dosing from around 27 to 37. This means Fusobacteria likely became much less abundant after opioid dosing. Figure 17f depicts CT values for bacterial primers in Buprenorphine treated subject 1500. CT values for primers *Clostridium* and Fusobacteria remained constant from pre-dosing to two weeks dosing. For primers *Bacteroides,* Spirochaetes, and *E. coli* CT values decreased from pre-dosing to two weeks dosing while CT values for *Lactobacillus* increased. However, variability in the post-dosing CT values makes it difficult to assert whether or not these changes were a result of treatment. Aside from this, relative

abundances of each primer were similar according to CT values as compared to the other subjects. Subject 1532 shown in Figure 17g was also administered Buprenorphine. CT values were not conclusive of any change as a result of dosing because of variability in the pre-dosing results. Although, results for Fusobacteria showed a greatly increased CT value after two weeks of dosing, indicating decreased abundance in the subject. Ignoring variability in pre-dosing results, it does appear that *Lactobacillus* abundance increased during dosing and Spirochaetes abundance decreased during dosing, as indicated by their CT values. Figure 17h depicts the CT values for bacteria in subject 1543. Values remained fairly constant across all time periods, indicating no significant change in these bacteria's abundances as a result of dosing. However, there does appear to be a slight increase in CT values of *Bacteroides* at two weeks dosing, meaning a possible decrease in *Bacteroides* abundance. *Lactobacillus* CT vales also appear to vary during dosing; however, the first pre-dosing CT value is too close to those of during dosing to be able to make a valid inference about the effect of dosing on the abundance of *Lactobacillus.* Spirochaetes CT values vary greatly across the time points, again indicating a possible dysfunction in the Spirochaetes primer. Figure 17i depicts CT values for Buprenorphine treated subject 1544. CT values for Spirochaetes and *Bacteroides* increased during dosing as compared to pre-dosing or post-dosing meaning their abundances likely decreased during dosing. While there was some variation, CT values for *Clostridium, E. coli*, Fusobacteria, and *Lactobacillus* remained constant at all time points. Additionally, abundances as explained by the CT values were similar to those found in the other subjects.

It is difficult to definitively state whether the qPCR results match the sequencing results. There were some consistencies in that *Escherichia coli* and *Clostridium* proved to be nearly nonexistent in most subjects according to qPCR results and sequencing results. However,

phylum Fusobacteria and genus *Bacteroides* were at least moderately abundant in most subjects according to qPCR results but was at 0% relative abundance for most subjects according to sequencing results. *Lactobacillus* and Spirochaetes were abundant in most subjects according to qPCR results and their abundances varied depending on the collection date, but it is difficult to determine if this variation is consistent with the sequencing results' variation.

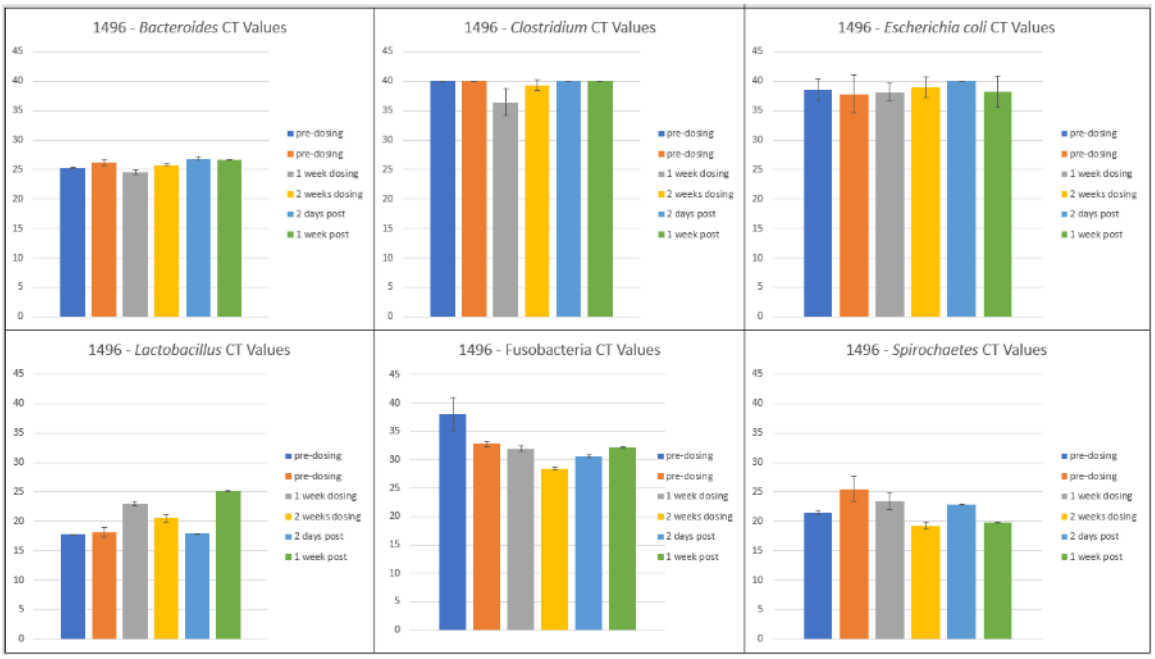


Figure 17a. CT values from 6 primers for control subject 1496 at each time point in the study

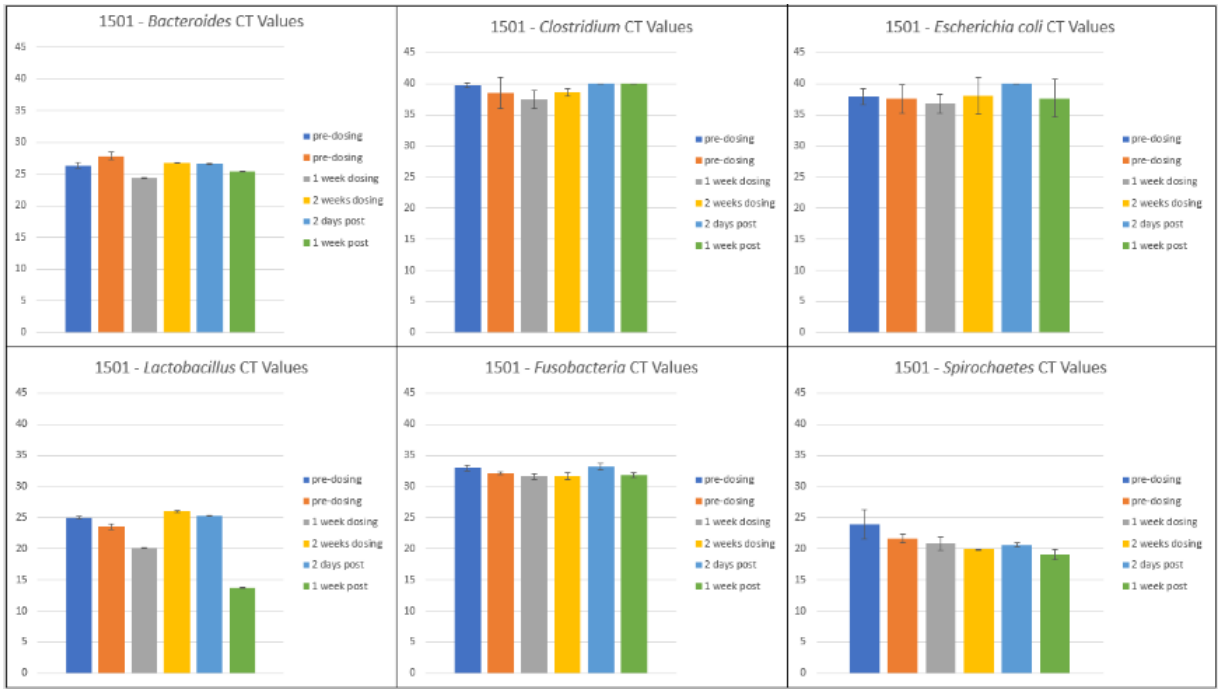


Figure 17b. CT Values from 6 primers for control subject 1501 at each time point in the study

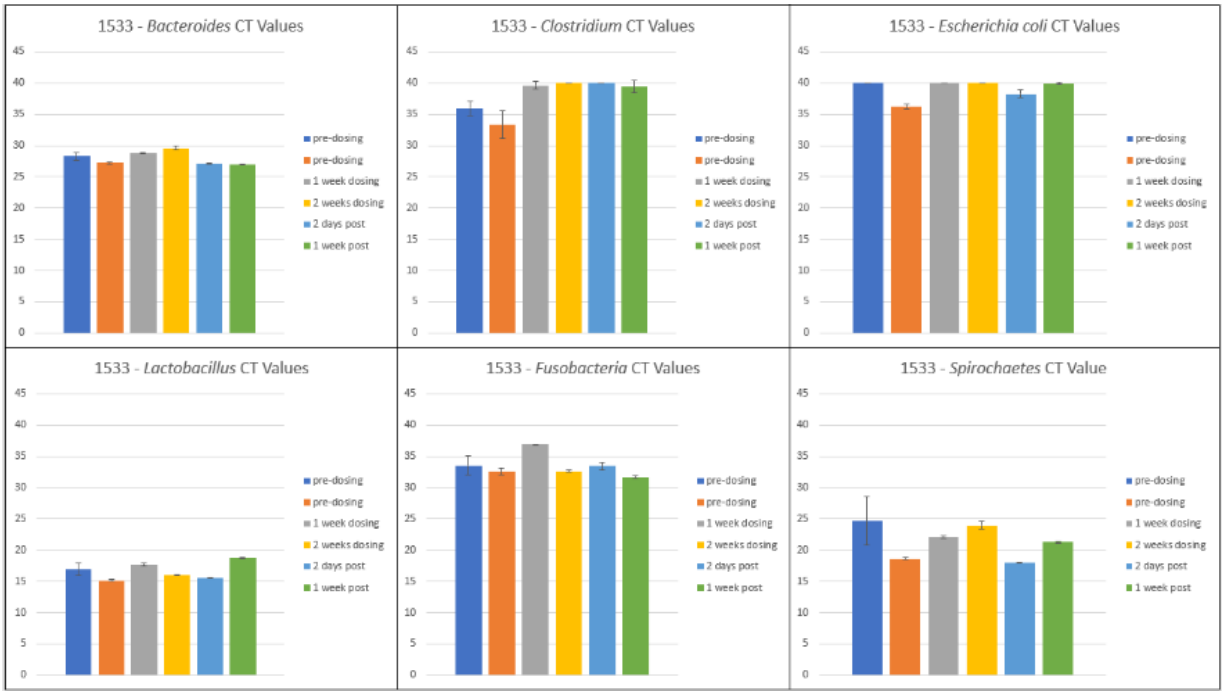


Figure 17c. CT Values from 6 primers for control subject 1533 at each time point in the study

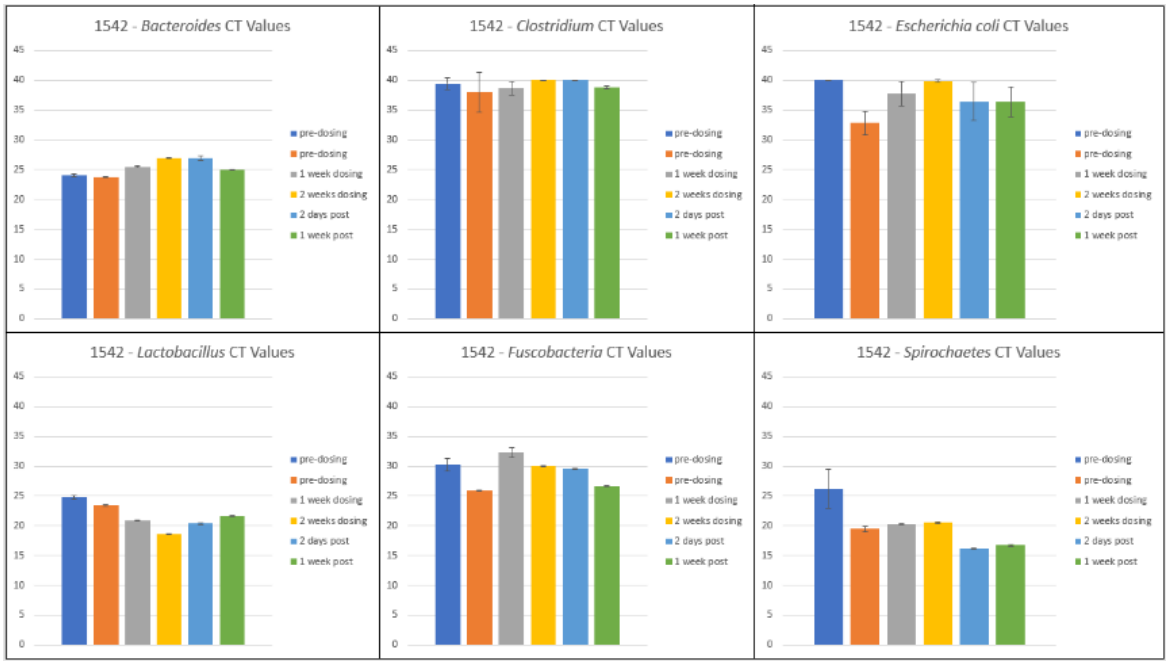


Figure 17d. CT values from 6 primers for control subject 1542 at each time point in the study

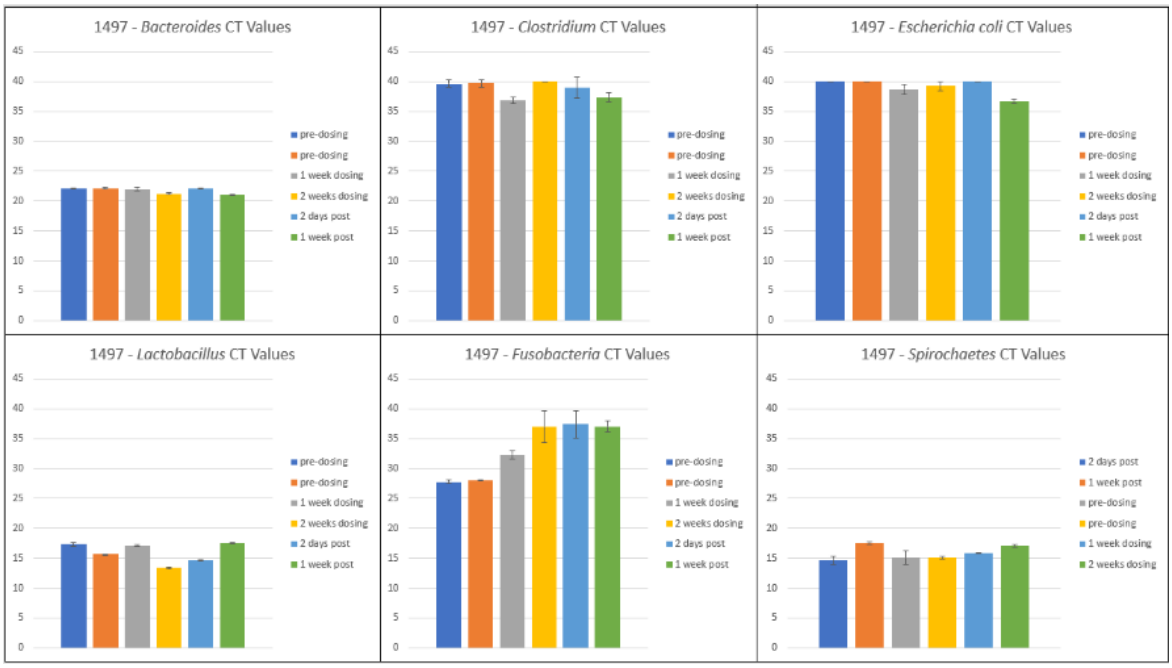


Figure 17e. CT values from 6 primers for Buprenorphine-treated subject 1497 at each time point in the study

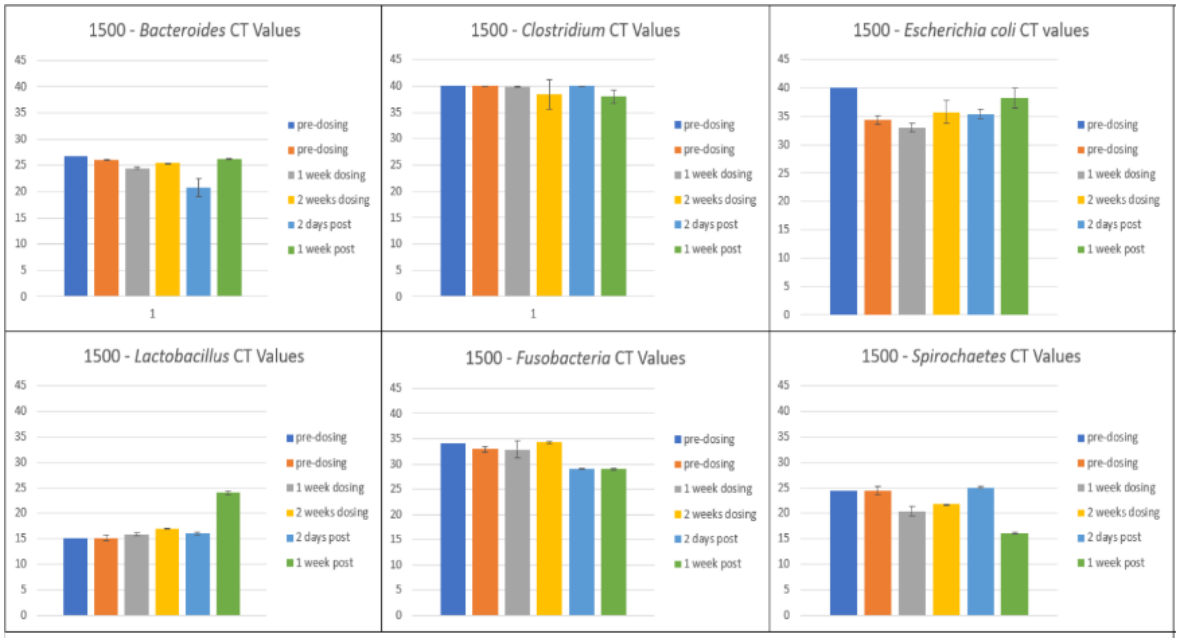


Figure 17f. CT values from 6 primers for Buprenorphine-treated subject 1500 at each time point in the study

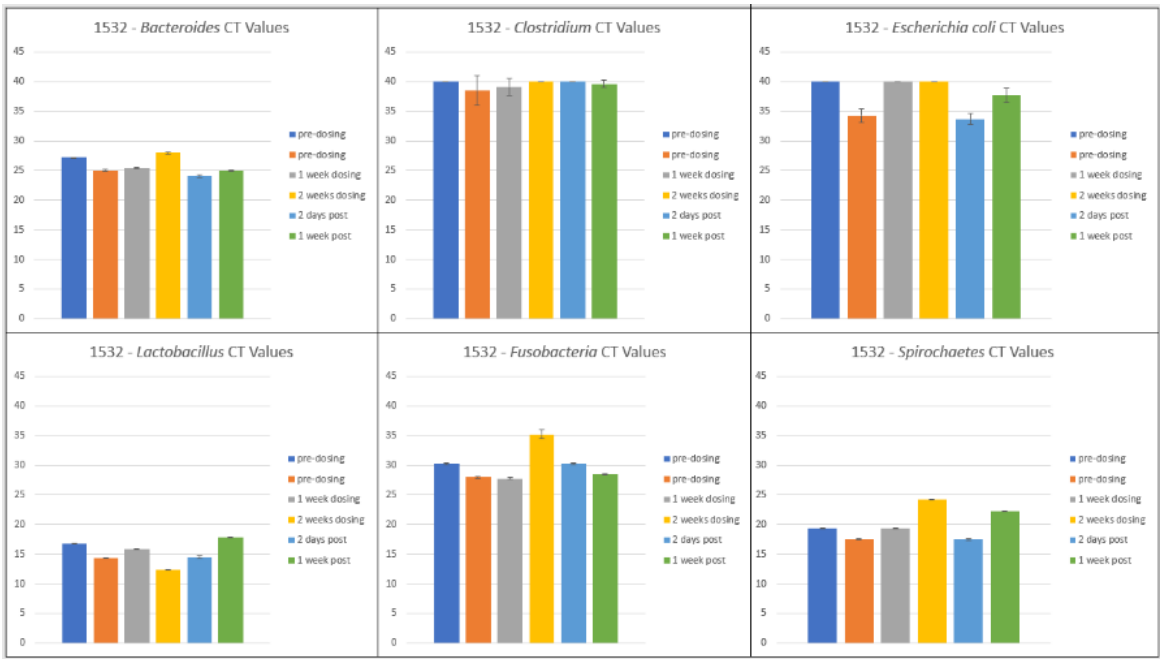


Figure 17g. CT values from 6 primers for Buprenorphine-treated subject 1532 at each time point in the study

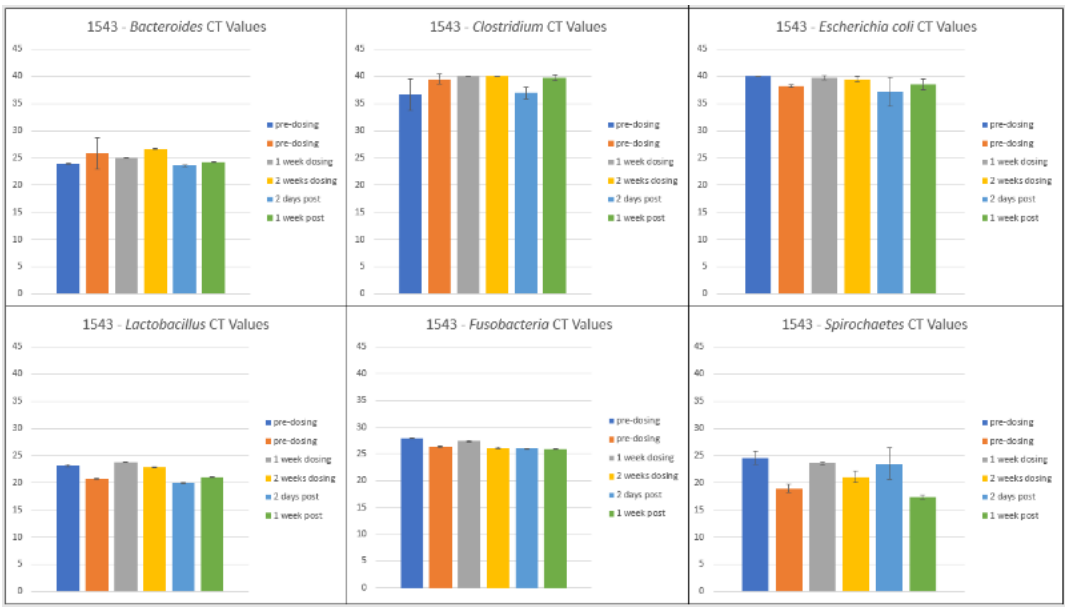


Figure 17h. CT values from 6 primers for Buprenorphine-treated subject 1543 at each time point in the study

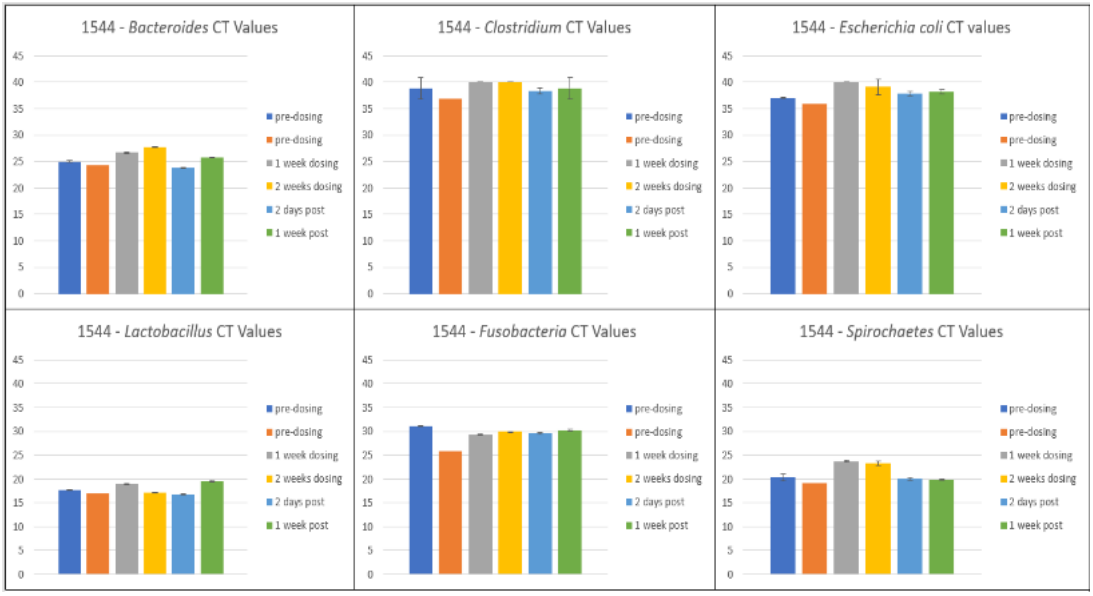


Figure 17i. CT values from 6 primers for Buprenorphine-treated subject 1544 at each time point in the study

4.4 - Library Preparation

4.4a - Nanodrop Results

DNA concentration

DNA concentrations following library preparation were significantly lower than that of the original isolated DNA samples. Most samples fell within a range of 3-12 ng/µL. Concentrations below 3 were not ideal, however, their bioanalyzer results offered the most insight into whether or not the sample could be used for sequencing. If the concentration was low but had normal bioanalyzer results, the sample was accepted for sequencing. There were a handful of samples with poor bioanalyzer results indicating low concentrations that were not accepted for sequencing and had to be prepped again. Following this, only one sample (1501 collection date 12/2) maintained the low concentration and poor bioanalyzer result, but was sent for sequencing regardless due to time constraints. Concentrations were found via the nanodrop machine, but Vladimir Lee, who performed the bioanalysis, also determined concentrations of the libraries as a verification.

Ratios

Ratios provided by the nanodrop machine were less accurate following library preparation because of the significantly lower concentrations of the samples. At low concentrations, the A260/A230 ratio can be much higher or lower than expected because the nanodrop machine cannot perform adequately when samples are at concentrations below 10ng/µL.

4.4b - Bioanalyzer results

The bioanalyzer determined whether the prepared DNA libraries were of good quality before continuing to the sequencing step. The bioanalyzer provides the average

size and concentration of DNA fragments. The sizes of our libraries were between 400-500 bp and the concentrations were between 1-15 nM, with higher concentrations being more desirable. Ultimately, the bioanalyzer produces a large linear range that indicates any impurities in the DNA purifications. The two sharp peaks are size markers, one low and one high, which are used to align the ladder. The high marker is also used to quantify the size of the middle, more broad peak, which represents the DNA fragments in the library. Any small peaks between the first sharp peak and the middle broad peak indicates unwanted adapter dimers which implied the need for further purification. Samples resulting in a lower middle peak generally corresponded to libraries of very low concentration and those libraries with nearly flat peaks were successfully reproduced to result in higher concentration and better sequencing results down the line. Our bioanalyzer results, shown in Figure 18, are all examples of how good results should appear.

Subject	Collection Dates					
1496	8/6/18	10/8/18	11/18/18	11/26/18	12/2/18	12/7/18
1497	10/8/18	10/12/18	11/18/18	11/26/18	12/2/18	12/7/18
1500	10/8/18	10/26/18	11/18/18	11/26/18	12/2/18	12/7/18
1501	8/6/18	10/8/18	11/18/18	11/26/18	12/2/18	12/7/18
1532	8/6/18	11/5/18	11/13/18	11/19/18	11/26/18	12/1/18
1533	8/6/18	11/5/18	11/13/18	11/19/18	11/26/18	12/1/18
1542	8/6/18	11/5/18	11/13/18	11/19/18	11/26/18	11/30/18
1543	8/6/18	11/5/18	11/13/18	11/19/18	11/26/18	12/1/18
1544	8/6/18	11/5/18	11/13/18	11/19/18	11/26/18	12/1/18

Figure 18. Bioanalyzer results for each DNA library from each sample at each collection date

4.5 - Sequencing Results

MetaPhlAn output provided relative abundances of bacteria from the level of kingdom down to the level of species. Because we performed shotgun sequencing rather than just 16s, the MetaPhlAn output showed bacterial abundance as well as relative abundances of other kingdoms such as Archaea, Eukarya, and Viruses. Bacteria were vastly more abundant than each of the other three kingdoms. Viruses and Eukarya were found only in one subject at one time point and although Archaea was found at varying levels in different subjects at different time points, it was still significantly less abundant than bacteria. Focusing on bacterial abundances, we were able to create figures based on phylum and genus level to depict alterations in the microbiota. For a more in-depth understanding of the abundances shown in each heat map, one can refer to supplemental figures 1, 2a, 2b, and 2c as they contain the numerical value for each of the relative abundances.

Figure 19 illustrates the relative abundances of the bacteria found in the stool sample DNA, which is representative of the gut microbiota at the phylum level, at the pre-dosing time point. Our sequencing results only include data for the pre-dosing time point from the date most closely associated with the beginning of the dosing period. In general, Firmicutes is the most dominant phylum, followed by subdominant phyla Euryarchaeota, Spirochaetes, Actinobacteria, Bacteroidetes, and Proteobacteria are the subdominant phylum. Each subject varies greatly with the relative abundances among the subdominant phyla. Subject 1496 is unique in that Euryarchaeota is not apparent. Subjects 1500, 1542, and 1543 are unique in that Spirochaetes is not apparent. Subject 1532 is unique in that Proteobacteria is not apparent.

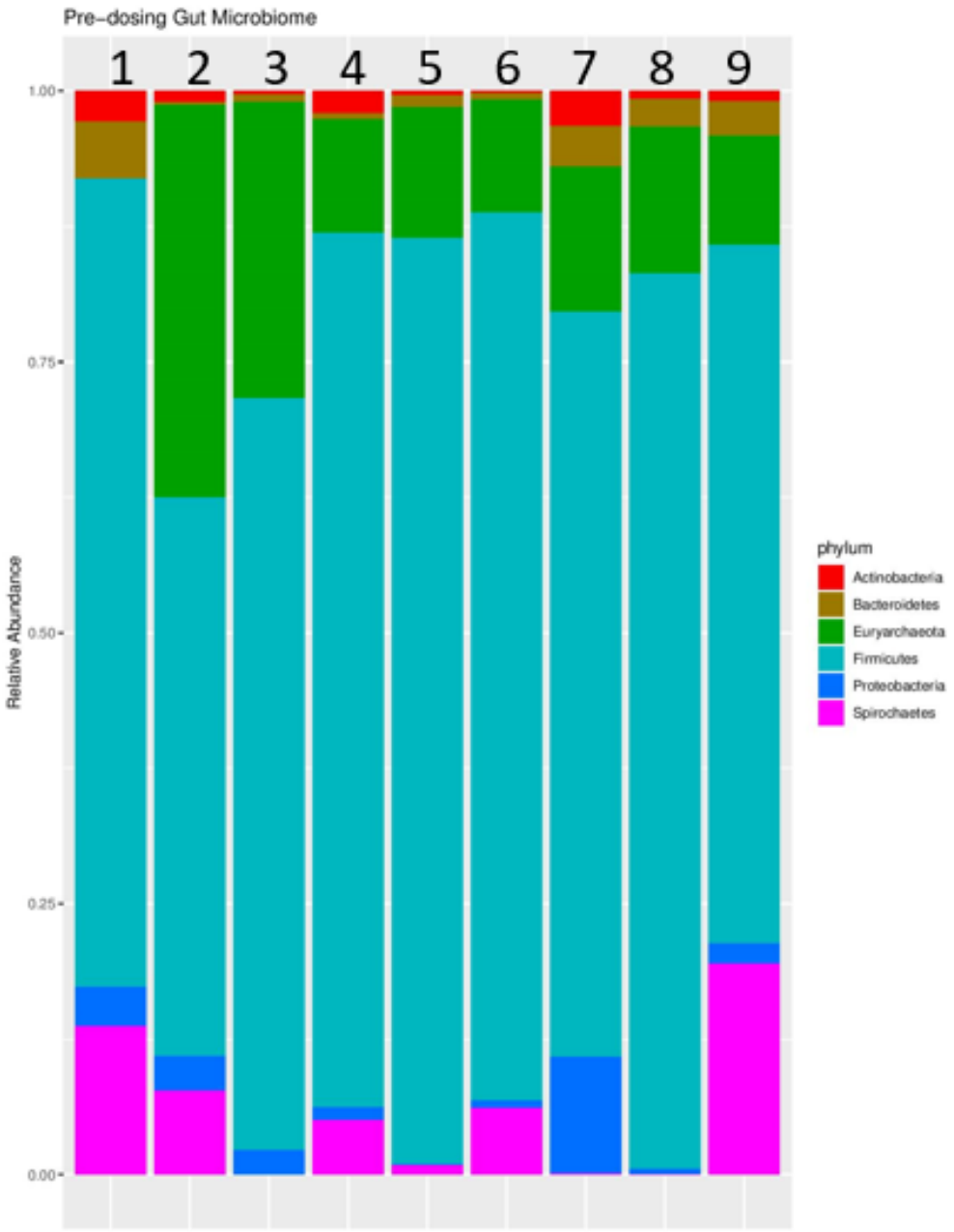


Figure 19. Phylum level gut microbiome composition in each subject pre-dosing. Each column indicates an individual sample in chronological order: 1496 (lane 1), 1497 (lane 2), 1500 (lane 3), 1501 (lane 4), 1532 (lane 5), 1533 (lane 6), 1542 (lane 7), 1543 (lane 8), 1544 (lane 9).

Figure 20 depicts the relative abundances of bacteria in the gut of all subjects during pre-dosing at the genus level. As shown, there is a lot of variation in the gut microbial composition across subjects. However, on average the most abundant bacteria is *Lactobacillus*, followed by *Eubacterium, Methanobrevibacter* (under the Kingdom Archaea), *Subdoligranulum,*

Phasolarctobacterium, Treponema, Ruminococcus, Methanosphaera (Kingdom Archaea), *Coprococcus*, and *Helicobacter*. Thirty-six other genus came back with some relative abundance (>3) and eleven genera had a relative abundance of zero across all subjects. In sample 1496, the most abundant bacteria include *Eubacteria* (20%) and *Coprococcus (16.6%)*, both falling under the phylum Firmicutes and the class Clostridia. *Treponema* was also 13% abundant and the remaining genus were less than 10% abundant. More specifically, *Subdolingranulum, Blautia, and Prevotella* ranged between 7.5 and 5% abundant, respectively. *Lactobacillus* and *Ruminococcus* were only about 4% abundant. In sample 1497, *Methanobrevibacter* and *Subdolingranulum* are almost equally abundant, at 20.3% and 20.1% abundance, respectively. *Methanosphaera* and *Lactobacillus* are also almost equally abundant at 16% and 15.7% abundance. *Phascolarctobacterium* was 12.7% abundant, *Treponema* was 7.8% abundant, and *Helicobacter* was 3.3% abundant. The remaining genus were <1% abundant. In subject 1500, *Lactobacillus* made up almost half of the genome at 44%. *Methanobrevibacter* was 14.6% abundant and *Subdoligranulum* was 11.8% abundant. *Phascolarctobacterium* was 8.4%, *methanosphaera was 5.8%, Helicobacter* was 5.2%, *Peptostreptococcaceae* was 3%, and *Blautia* was 1.4% abundant. The remaining genus were <1% abundant. In subject 1501, *Eubacterium* made up more than half of the genome at 56.3% abundance. *Streptococcus* was the next most abundant bacteria at only 8.3% and *Mathnaosphaera* fell close behind at 7.4%. *Treponema, Subdolingranulum, Methanobrevicater, Dorea, Blautia, Collinsella, Peptostreptococcae, Phascolarctobacterium, and Helicobacter* were between 1-5% abundant, listed in order of their relative abundances. All remaining genus were <1% abundant. In subject 1532, *Lactobacillus* is the most dominant genus at 36.8%, similar to subject 1500. Additionally, *Methanobrevibacter* is the next most abundant, like in subject 1500, at 12%. However, following these genera are

Phascolarctobacterium at 8.4%, *Eubacterium*at 7.3% and *Subdoligranulum* at 5.1%. *Faecalibacterium, Dorea, Coprococcus, Erysipelotrichaecea,* and *Catenibacterium* were all around 4% abundant. Following these were *Blautia, Clostridium, and Prevotella* at 2.8%, 2%, and 1.2%, respectively. The remaining genera were <1% abundant. Like subjects 1500 and 1532, the microbiome of subject 1533 is dominated by *Lactobacillus* at 39.5%. However, this subject is unique in that *Ruminococcus* is nearly as abundant at 31.2%. *Methanobrevibacter* falls behind these genera at 10.4% and *Treponema* was 6.2% abundant. *Subdoligranulum* and *Phascolarctobacterium* were about 3% abundant and the remaining genera were <1% abundant. Subject 1542 is unique in that it essentially does not have a dominant genus. *Lactobacillus* and *Helicobacter* are the most abundant at only 10.8% each. Nine other genera including *Eubacterium, Subdoligranulu, Faecalibacterium, Methanosphaera, Erysipelotrichaceae, Methanobrevibacter, Blautia, Ruminococcus,* and *Coprococcus* are between 5-9% abundant, listed in order of decreasing abundances, making up almost 70% of the microbiome. Subject 1543 is similar to 1542 in that the most abundant species are not relatively that abundant with *Phascolarctobacterium* at 21.4% and *Subdoligranulum* at 21.2%. *Methanobrevibacter* was 13.5%, *Ruminococcus* was 10.6%, *Eubacterium* was 7.3%, *Faecalibacterium* was 5.8%, and *Enterococcus* was 4.1% abundant. *Enterococcus, Coprococcus, Prevotella, Erysipelotrichaeceae* and *Lachnospiraceae* were all about 2% abundant. Subject 1544 similarly had lower abundance of dominant bacteria with *Treponema* at 19.5%. *Phascolarctobacterium, Eubacterium,* and *Methnaobrevibacter* were all about 10% abundant, followed by *Subdoligranulum, Enterococcus, Dorea,* and *Lactobacillus* ranging from 5-8% abundance. *Coprococcus, Prevotella,* and *Peptostreptococcaceae* were around 2-3% abundant. According to the relative abundances given

by MetaPhlAn, the variety in the pre-dosing microbiomes is apparent and makes analysis of microbiome alterations difficult as result of opioid dosing.

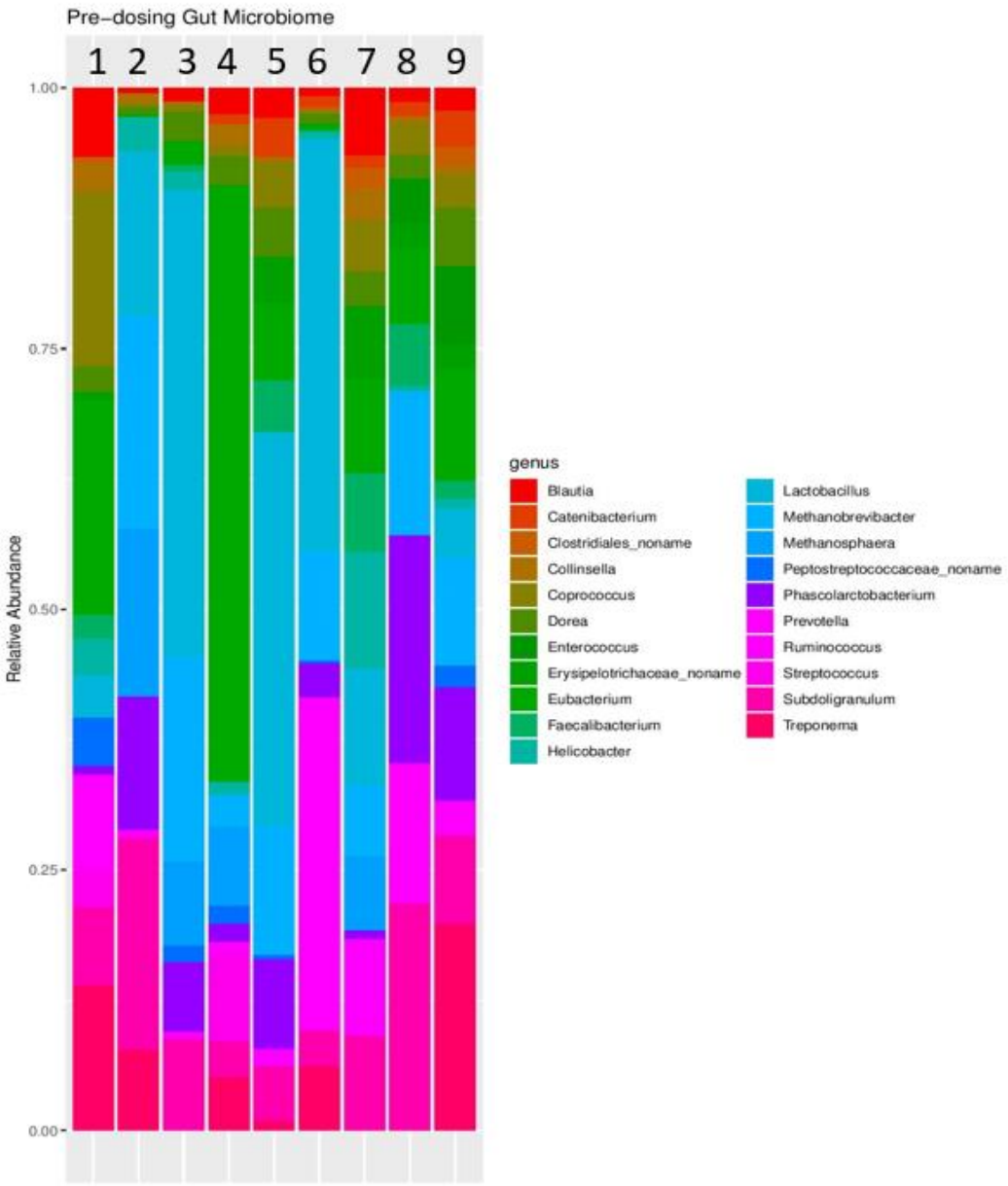


Figure 20. Genus level gut microbiome composition in each subject pre-dosing. Each column indicates an individual sample in chronological order: 1496 (lane 1), 1497 (lane 2), 1500 (lane 3), 1501 (lane 4), 1532 (lane 5), 1533 (lane 6), 1542 (lane 7), 1543 (lane 8), 1544 (lane 9).

In figure 21, the change in bacterial abundance at the genus level is characterized in each subject from pre-dosing to one week of dosing. Genera are clustered together using Euclidian

distances to indicate bacteria that acted similarly during the study. This figure indicates which genera increased, decreased, or remained the same after 1 week of dosing. Genera that remained at a relative abundance of zero were excluded from the figure.

Eubacterium decreased in abundance in control subjects 1496 and 1542, increased in abundance in control subject 1501, and remained constant in control subject 1533. Meanwhile its abundance remained constant in treated subjects 1497 and 1500, decreased in treated subjects 1532 and 1543, and increased in treated subject 1544. The remaining genera resulted in similar unpredictable variations in abundance at the two time points, regardless of the subject being in the control or treated group. We chose to focus more heavily on the pre-dosing to two-week dosing relative abundance results as this is more representative of prolonged opioid administration.

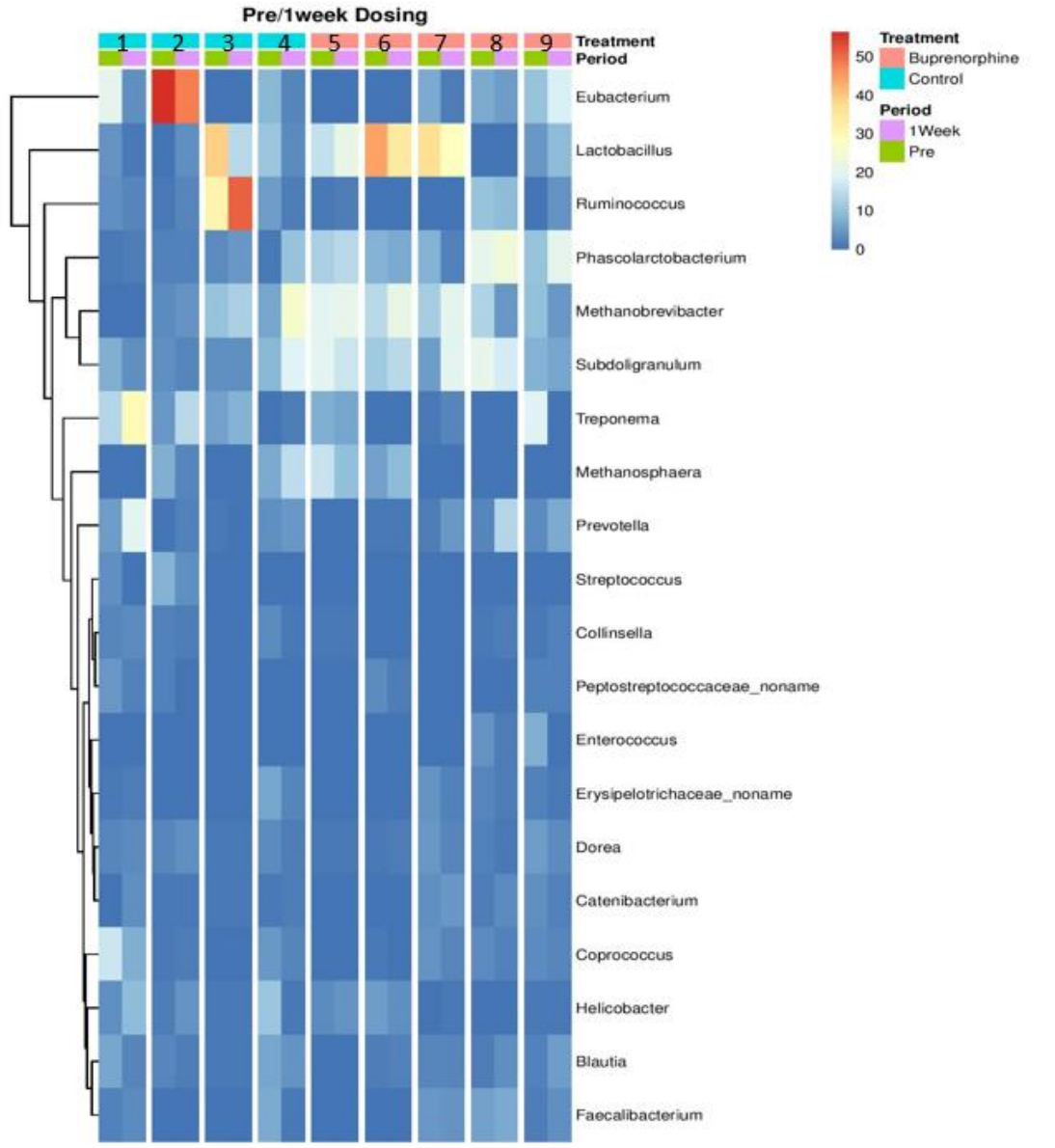


Figure 21. Genus level gut microbial composition pre-dosing and at one week of dosing for each subject. Control subjects are shown on the left: 1496 (lane 1), 1500 (lane 2), 1533 (lane 3), 1542 (lane 4) while Buprenorphine-treated subjects are shown on the right: 1497 (lane 5), 1501 (lane 6), 1532 (lane 7), 1543 (lane 8), 1544 (lane 9).

Figure 22 depicts the change in bacterial abundance at the genus level from pre-dosing to two weeks of dosing in each subject. Again, genera were clustered using Euclidian distances. Control groups are shown on the left of the figure while Buprenorphine treated groups are shown on the right.

In control subject 1496, the three most dominant genera before dosing were *Eubacterium* at 20.3%, *Coprococcus* at 16.6%, and *Treponema* at 13.8%. At the two week "dosing" time point, the three most dominant genera were *Treponema* at 21.7%, *Coprococcus* at 20.6%, and *Prevotella* at 12.3%. *Eubacterium* fell from 20.4% to 3.3% abundance and *Prevotella* increased from 5.3% to 12.3% abundance after two weeks of dosing. Additionally, there was almost a tenfold increase in genus *Desulfovibrio* from .028% to .297% and more than a seven fold change in the genus *Brachyspira* from .001% to .009%. While these are very low relative abundances compared to the other genera, the size of fold change is significant when addressing the variability in the microbiome, especially considering this being a control subject. In control subject 1501, the three most dominant genera before dosing were *Eubacterium* at 56.3%, *Streptococcus* at 8.3%, and *Methanosphaera* at 7.4%. At the two week "dosing" time point, the three most dominant genera were *Eubacterium* at 60.4%, *Treponema* at 8% and *Methanobrevibacter* at 7.1%. *Streptococcus* decreased from 8.3% to 0.08%, *Methanosphaera* decreased from 7.4% to 3.2%, *Treponema* increased from 5.1% to 8%, and *Methanobrevibacter* increased from 3% to 7.1% at the two week "dosing" time point. In control subject 1533, the dominant genera prior to dosing were *Lactobacillus* at 39.4%, *Ruminococcus* at 31.2%, and *Methanobrevibacter* at 10.4%. At the two week "dosing" time point, the dominant genera were *Lactobacillus* at 42.3%, *Ruminococcus* at 28.2%, and *Treponema* at 9.8%. *Methanobrevibacter* decreased from 10.4% to 8% and Treponema increased from 6.2% to 9.8%. This subject maintained reasonably consistent abundances at these two time periods. In control subject 1542, the dominant genera before dosing were *Lactobacillus* at 10.8%, *Helicobacter* at 10.8%, and *Eubacterium* at 8.7%. At the two week dosing mark, the dominant genera were *Methanobrevibacter* at 43.5%, *Subdoligranulum* at 17.2% and *Lactobacillus* at 9.2%.

Methanobrevibacter increased from 6.6% to 43.5%, *Subdoligranulum* increased from 8.7% to 17.2%, *Helicobacter* decreased from 10.8% to .12%, and *Eubacterium* decreased from 8.7% to .9%. This subject resulted in highly varied abundances despite it being a control subject. However, it is interesting to note that while other abundances varied, *Lactobacillus* remained fairly constant.

In Buprenorphine treated subject 1497, the three dominant genera before dosing were *Methanobrevibacter* at 20.3%, *Subdoligranulum* at 20.1%, and *Methanosphaera* at 16%. After two weeks of dosing, the three most dominant genera were *Lactobacillus* at 67.8%, *Methanobrevibacter* at 10.5%, and *Subdoligranulum* at 6.4%. *Lactobacillus* increased from 15.7% to 67.8%, a more than threefold increase, while *Methanosphaera* dropped from 16% to 4.2% after two weeks of dosing. *Treponema* also dropped from 7.8% to 2.4% and *Phascolarctobacterium* dropped from 12.7% to 5.3% after two weeks of dosing. Additionally, there was nearly a threefold increase in *Peptostreptococcaceae* from .015% to .056%. In Buprenorphine treated subject 1500, the three most dominant genera before dosing were *Lactobacillus* at 44%, *Methanobrevibacter* at 14.6%, and *Subdoligranulum* at 11.8%. After two weeks of dosing, the most dominant genera were *Lactobacillus* at 22.6%, *Methanobrevibacter* at 22.6%, and *Phascolarctobacterium* at 17.3%. *Subdoligranulum* decreased from 11.8% to 6.8% and *Phascolarctobacterium* increased from 8.4% to 17.3% after two weeks of dosing. The dominant genera in subject 1532 before dosing were *Lactobacillus* at 36.8%, *Methanobrevibacter* at 12.1%, and *Phascolarctobacterium* at 8.4%. After two weeks of dosing, the dominant genera were *Lactobacillus* at 91.6%, *Methanobrevibacter* at 1.8%, and *Catenibacterium* at 1%. *Phascolarctobacterium* decreased from 8.4% to .04% and although *Catenibacterium* became one of the three dominant genera after dosing, it actually decreased

from 3.6% to 1%. This subject resulted in a large increase in *Lactobacillus* while all other genera were depleted at the two-week dosing mark. In subject 1542, the dominant genera before dosing were *Phascolarctobacterium* at 21.4%, *Subdoligranulum* at 21.2%, and *Methanobreibacter* at 13.5%. After two weeks of dosing, the dominant genera were *Subdoligranulum* at 21.5%, *Phascolarctobacterium* at 16.8%, and *Prevotella* at 9.4%. *Methanobrevibacter* decreased from 13.5% to 6.1% and *Prevotella* increased from 2.6% to 9.4%. It should be noted that the results indicated 0 *Lactobacillus* before or after dosing. In subject 1544, the dominant genera before dosing were *Treponema* at 19.5%, *Phascolarctobacterium* at 10.7%, and *Eubacterium* at 10.6%. After two weeks of dosing, the dominant genera were *Lactobacillus* at 30.9%, *Eubacterium* at 13.8%, and *Phascolarctobacterium* at 13.2%. *Treponema* decreased from 19.5% to .36% and *Lactobacillus* increased from 4.7% to 30.9% after two weeks of dosing. Additionally, *Enterococcus* decreased from 7.4% to .05% and *Methanobrevibacter* decreased from 10.1% to 4.8%.

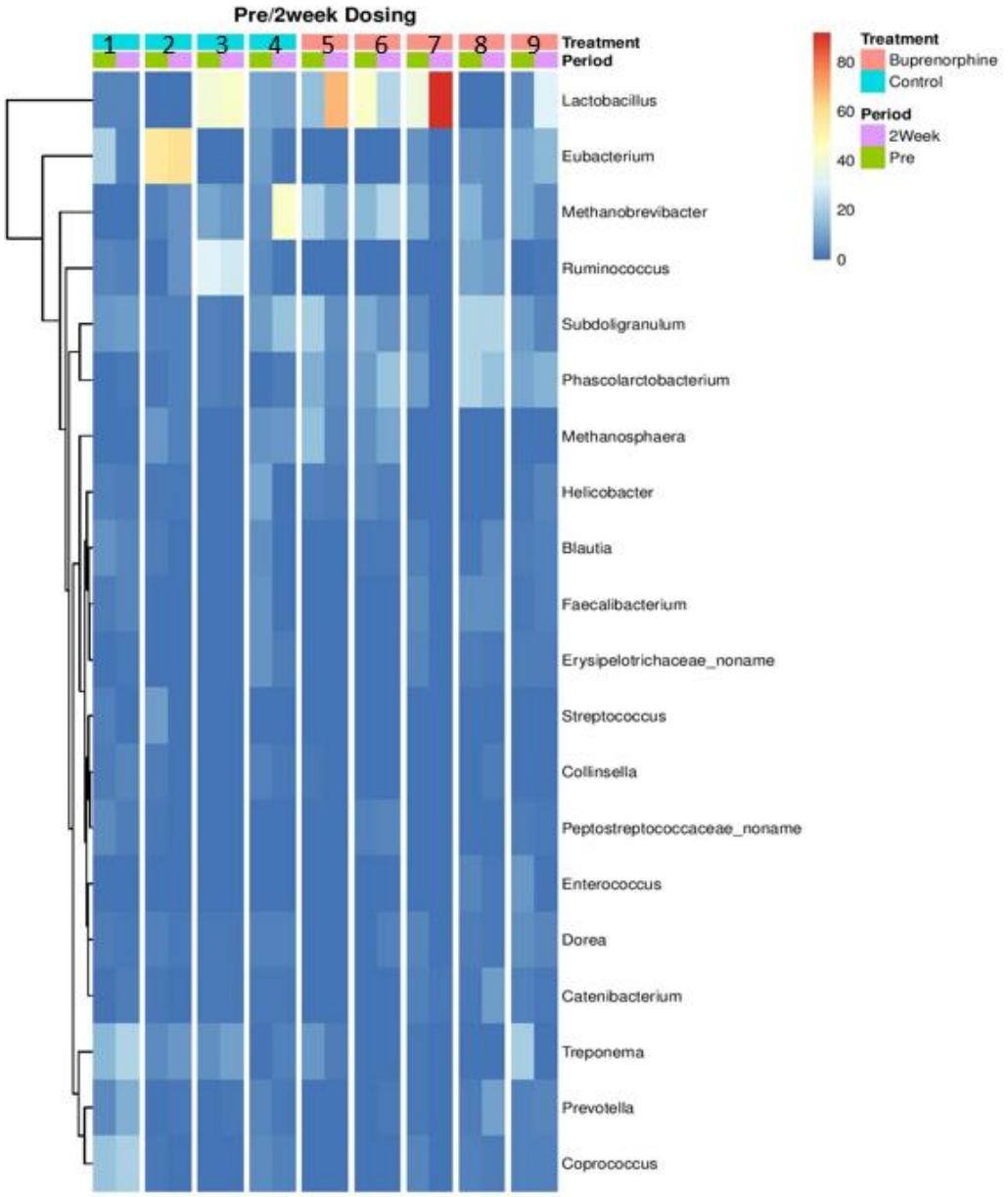


Figure 22. Genus level gut microbial composition pre-dosing and at two weeks of dosing for each subject. Control subjects are shown on the left: 1496 (lane 1), 1500 (lane 2), 1533 (lane 3), 1542 (lane 4) while Buprenorphine-treated subjects are shown on the right: 1497 (lane 5), 1501 (lane 6), 1532 (lane 7), 1543 (lane 8), 1544 (lane 9).

Figure 23 depicts the change in bacterial abundance at the genus level in each substance from pre-dosing to two weeks of dosing to one week post dosing.

In control subject 1496, at the pre-dosing mark the three most dominant genera were *Eubacterium, Coprococcus,* and *Treponema*. At the during dosing mark and the post dosing mark, the most dominant genera were *Treponema, Coprococcus,* and *Prevotella*. This data indicates some consistency in the mirobiome given the similarity in the microbiome at two of the three time points. In control subject 1501, the three most dominant genera at the pre-dosing mark were *Eubacterium, Streptococcus,* and *Methanosphaera*. At the two week dosing mark, the most dominant genera were *Eubacterium, Treponema,* and *Methanobrevibacter*. At the one week post dosing mark, the most dominant genera were *Lactobacillus, Methanobrevibacter,* and *Subdoligranulum.* There is some consistency from pre-dosing to during dosing, but there is great variation at the post-dosing mark. In control subject 1533, the dominant genera at the pre-dosing mark were *Lactobacillus, Ruminococcus,* and *Methanobrevibacter*. At the two week dosing mark the most dominant genera were *Lactobacillus, Ruminococcus,* and *Treponema*. At the post-dosing mark the most dominant genera were *Ruminococcus, Lactobacillus,* and *Treponema.* Subject 1533 shows the most consistency in the microbiome across subjects, implying that great variation does not always exist. However, while the dominant genera remained fairly consistent, the relative abundances of such genera did vary. While the relative abundances of *Methanobrevibacter* and *Treponema* remained constant over all the time points, *Lactobacillus* and *Ruminococcus* abundances varied. *Lactobacillus* decreased greatly after one week post dosing as compared to pre-dosing or during dosing time points. Additionally, *Ruminococcus*

relative abundance increased after one week post dosing as compared to pre-dosing or during dosing time points. In control subject 1542, the most dominant genera at the pre-dosing mark were *Lactobacillus, Helicobacter,* and *Eubacterium.* At the during dosing mark, the most dominant genera were *Methanobrevibacter, Subdoligranulum,* and *Lactobacillus.* At the post-dosing mark the most dominant genera were *Phascolarctobacterium, Subdoligranulum,* and *Prevotella.* Again, variation in the microbiome is shown in this sample as the dominant genera change at the different time points.

In Buprenorphine treated subject 1497, the dominant genera prior to dosing were *Methanobrevibacter, Subdoligranulum,* and *Methansosphaera*. After two weeks of dosing, the dominant genera were *Lactobacillus, Methanobrevibacter,* and *Subdoligranulum*. After one week post dosing, the dominant genera were *Methanobrevibacter, Subdoligranulum,* and *Phascolarctobacterium.* It is interesting to note that prior to dosing *Lactobacillus* was 15.7% abundant, at two weeks of dosing it was 67.8% abundant, and after one week post dosing it was 8.7% abundant. *Methanobrevibacter* was 20.3% abundant before dosing, reduced to 10.5% abundant after twoo weeks of dosing, and returned to 22.2% abundant one week after dosing. *Subdoligranulum* followed a similar pattern to the abundances of *Methanobrevibacter* at the different time points. Buprenorphine administration resulted in a marked decreased in Archaea, an increase in the bacteria *Lactobacillus* and a decrease in the bacter *Subdoligranulum*. In Bupreonorphine treated subject 1500, the dominant genera pre-dosing were *Lactobacillus,*

Methanobrevibacter, and *Subdoligranulum*. After two weeks of dosing, the dominant genera were *Lactobacillus,* although reduced by almost half its original relative abundance, *Methanobrevibacter,* and *Phascolarctobacter*. After one week post-dosing, the dominant genera were *Eubacteria*, *Treponema,* and *Methanobrevibacter*. *Lactobacillus* was reduced from nearly 44% abundance before dosing, to 23% during dosing, to only 2% at one week post dosing. *Eubacterium* remained nearly the same level of abundance pre-dosing and after two weeks of dosing at just .3% and .4%, but vastly increased after one week post dosing, making up over half of the genome at 56% relative abundance. In Buprenorphine treated subject 1532, the most dominant genera prior to dosing were *Lactobacillus, Methanobrevibacter,* and *Phascolarctobacterium.* After two weeks of dosing, the dominant genera were *Lactobacillus, Methanobrevibacter,* and *Catenibacterium.* After one week post dosing, the dominant genera were *Lactobacillus, Faecalibacterium,* and *Prevotella. Lactobacillus* increased from 37% abundance to nearly 92% abundance during dosing and after one week post dosing, it returned to about 36% relative abundance. The relative abundance of *Methanobrevibacter* decreased during dosing and returned to similar abundance after one week post dosing. Similarly, *Subdoligranulum* decreased during dosing and returend to similar relative abundance at one week post dosing. In Buprenorphine treated subject 1543, the dominant genera prior to dosing were *Phascolarctobacterium, Subdoligranulum,* and *Methanobrevibacter*. After two weeks of dosing, the dominant genear were *Subdoligranulum, Phascolarctobacterium,* and *Prevotella* and

after one week post dosing the dominant genera were *Subdoligranulum, Phascolarctobacterium,* and *Methanobrevibacter*. The relative abundance of *Methanobrevibacter* decreased during dosing and returned to original abundance after one week post dosing. *Subdoligranulum* actually remained at a fairly consistent relative abundance across all time points. *Phascolarctobacterium* had decreased relative abundance during dosing compared to pre-dosing, but maintained the lower abundance after one week post dosing. In Buprenorphine treated subject 1544, the pre-dosing dominant genera were *Treponema, Phascolarctobacterium,* and *Eubacterium.* After two weeks of dosing, the dominant genera were *Lactobacillus, Eubacterium,* and *Phascolarctobacterium* and after one week post dosing the dominant genera were *Phascolarctobacterium, Eubacterium,* and *Methanobrevibacter*. The relative abundance of *Treponema* greatly decreased during dosing from almost 20% to .4% and there was a slight increase after one week post dosing to become 6% abundant. *Lactobacillus* greatly increased from pre-dosing to during dosing starting at just 5% abundance to 31% abundance and returned to 8% abundance after one week post dosing. *Phascolarctobacterium* and *Eubacterium* gradually increased across the time points. The relative abundance of *Methanobrevibacter* decreased from pre-dosing to during dosing and returned to a relative abundance higher than the original after one week of dosing.

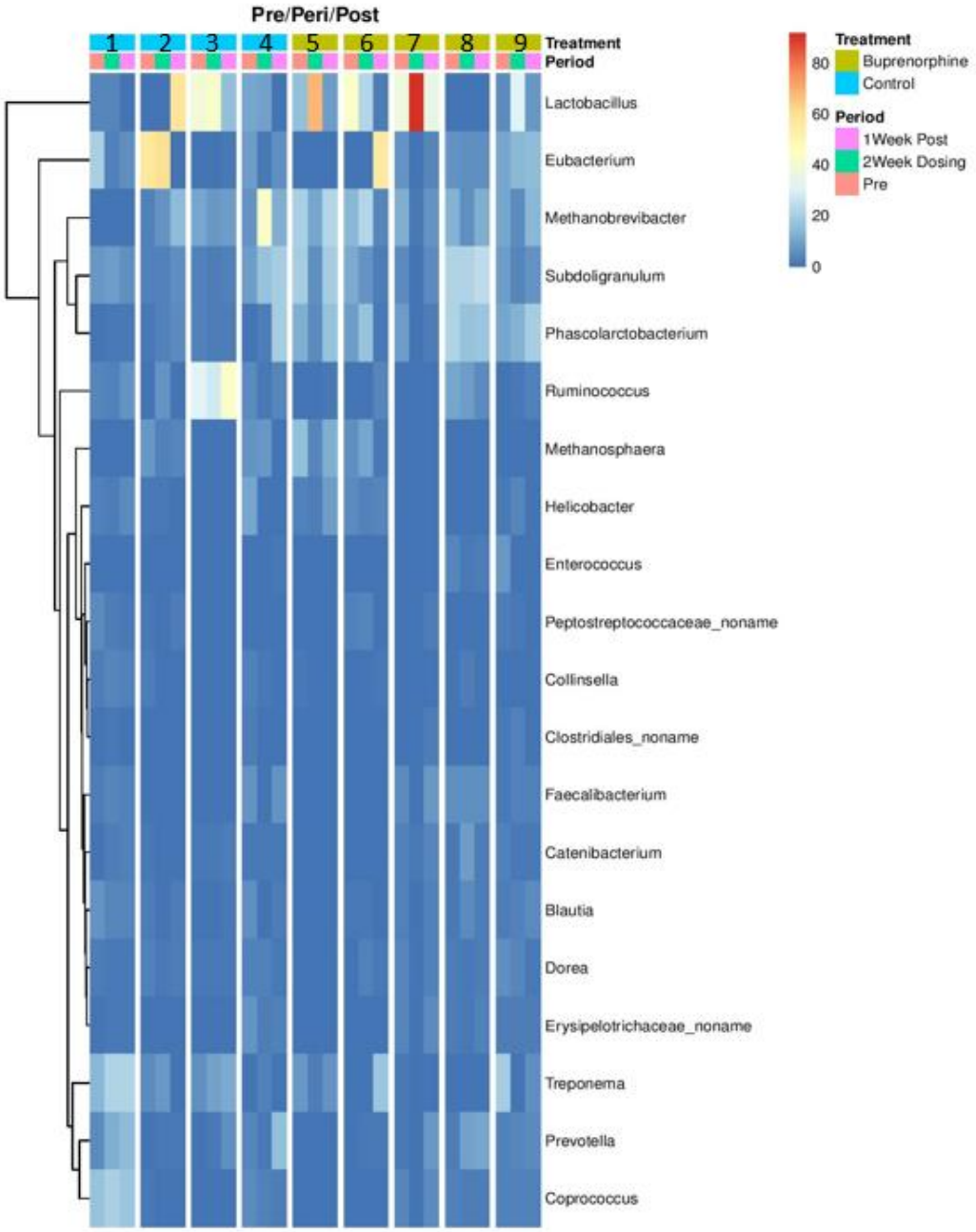


Figure 23. Genus level gut microbial composition at the pre-dosing, two-weeks of dosing, and one-week post dosing time points for each subject. Control subjects are shown on the left: 1496 (lane 1), 1500 (lane 2), 1533 (lane 3), 1542 (lane 4) while Buprenorphine-treated subjects are shown on the right: 1497 (lane 5), 1501 (lane 6), 1532 (lane 7), 1543 (lane 8), 1544 (lane 9).

Figure 24 depicts the change in bacterial abundance at the phylum level in each subject from pre-dosing to two weeks of dosing to one-week post dosing. As shown by the heat map, while there appear to be changes in the relative abundances in the control group subjects,

changes in the abundances are more apparent in the treatment group subjects, indicated by the more noticeable changes in color.

In control subject 1496, the three most dominant phylum were Firmicutes, Spirochaetes, and Bacteroidetes before dosing and at both the two-week dosing mark and one week post dosing mark. The relative abundances of each remained fairly consistent, especially between the two week dosing and one week post dosing marks. In control subject 1501, the two most dominant phylum were Firmicutes and Euryarchaeota at the pre dosing, two-week dosing, and one-week post dosing marks. The pre-dosing and two-week during dosing samples had extremely similar relative abundances of all phyla. The one-week post dosing sample showed some variation especially in the relative abundances of Euryarchaeota, Bacteroidetes, and Spirochaetes. In control subject 1533, the three most dominant genera were Firmicutes, Euryarchaeota, and Spirochaetes at the pre-dosing, two-week dosing, and one-week post dosing marks. However, while the pre-dosing sample followed this exact order of abundance, both the two-week dosing and one-week post dosing samples had slightly higher relative abundances of Spriochaetes compared to Euryarchaeota. Overall, the relative abundances of each phyla remained fairly similar at each time point, except Bacteroides which was slightly increased in the one-week post dosing sample. In control subject 1542, the dominant phyla were more varied than in the other control subjects. At the pre-dosing mark, the dominant phyla were Firmicutes, Euryarchaetoa, and Proteobacteria, listed in order of relative abundances. At the two-week dosing mark, the dominant genera were Euryarchaeota and Firmicutes. At the one-week post dosing mark, the dominant phyla were Firmicutes, Bacteroidetes, and Euryarchaeota. The relative abundances of Firmicutes and Euryarchaeota were most similar at the pre-dosing mark and one-week post dosing mark, but were altered in the two-week dosing sample. Overall, we

saw much more consistency in the control group subjects individually at each time point at the phylum level than at the genus level.

In Buprenorphine treated subject 1497, the dominant phyla were Firmicutes and Euryarchaeota at the pre-dosing, two-week dosing, and one-week post dosing time points. However, Firmicutes significantly increased during dosing from 52% to 81% and returned to 50% one week post dosing. Euryarchaetoa decreased during dosing from 36% to 15% and returned to 35% one week post dosing. Phylum Spirochaetes decreased during dosing from 8% to 2% and returned to 7% one week post dosing. In Buprenorphine treated subject 1500, the dominant phylum pre-dosing and at two weeks of dosing were Firmicutes, Euryarchaeota, and Proteobacteria. The relative abundance of Firmicutes decreased from 72% to 60%, that of Euryarchaetoa increased from 20% to 33%, and that of Proteobacteria remained fairly constant at 6% and 5%. At one-week post dosing, the dominant phyla were Firmicutes, Spirochaetes, and Euryarchaeota. Firmicutes returned to 70% relative abundance. Spirochaetes increased from almost non-existent levels to 17% abundance and Euryarchaeota decreased to 6% relative abundance. In Buprenorphine treated subject 1532, the dominant phylum was Firmicutes, making up between 83% to 98% of the genome at the different time points. Before dosing, the relative abundance of Firmicutes was 85% which increased to 98% at two weeks dosing and decreased to 83% after one week post dosing. The relative abundance of Euryarchaeota started at 12%, decreased to 2% during dosing and increased to 7% after one week post dosing. Bacteroidetes interestingly was nearly nonexistent before dosing and during dosing at just 1.2% and .4%, but its relative abundance increased to almost 8% after one week post dosing. In Buprenorphine treated subject 1543, the dominant phylum at all time periods was again Firmicutes and it remained at a relative abundance between 76% and 83% throughout.

Euryarchaeota was more abundant than Bacteroidetes prior to dosing, but became less abundant than Bacteroidetes at two weeks of dosing and after one week post dosing, Euryarchaeota returned to being more abundant. In Buprenorphine subject 1544, the dominant phylum was again Firmicutes. However, prior to dosing, the relative abundance was 64.5% and increased to 86% at two weeks of dosing and began to decrease to 74% at one-week post dosing. Spirochaetes was 19.5% relatively abundant prior to dosing, but was almost completely depleted at two weeks of dosing at just .4%. At one week post dosing its relative abundance began climbing back up to 6%.

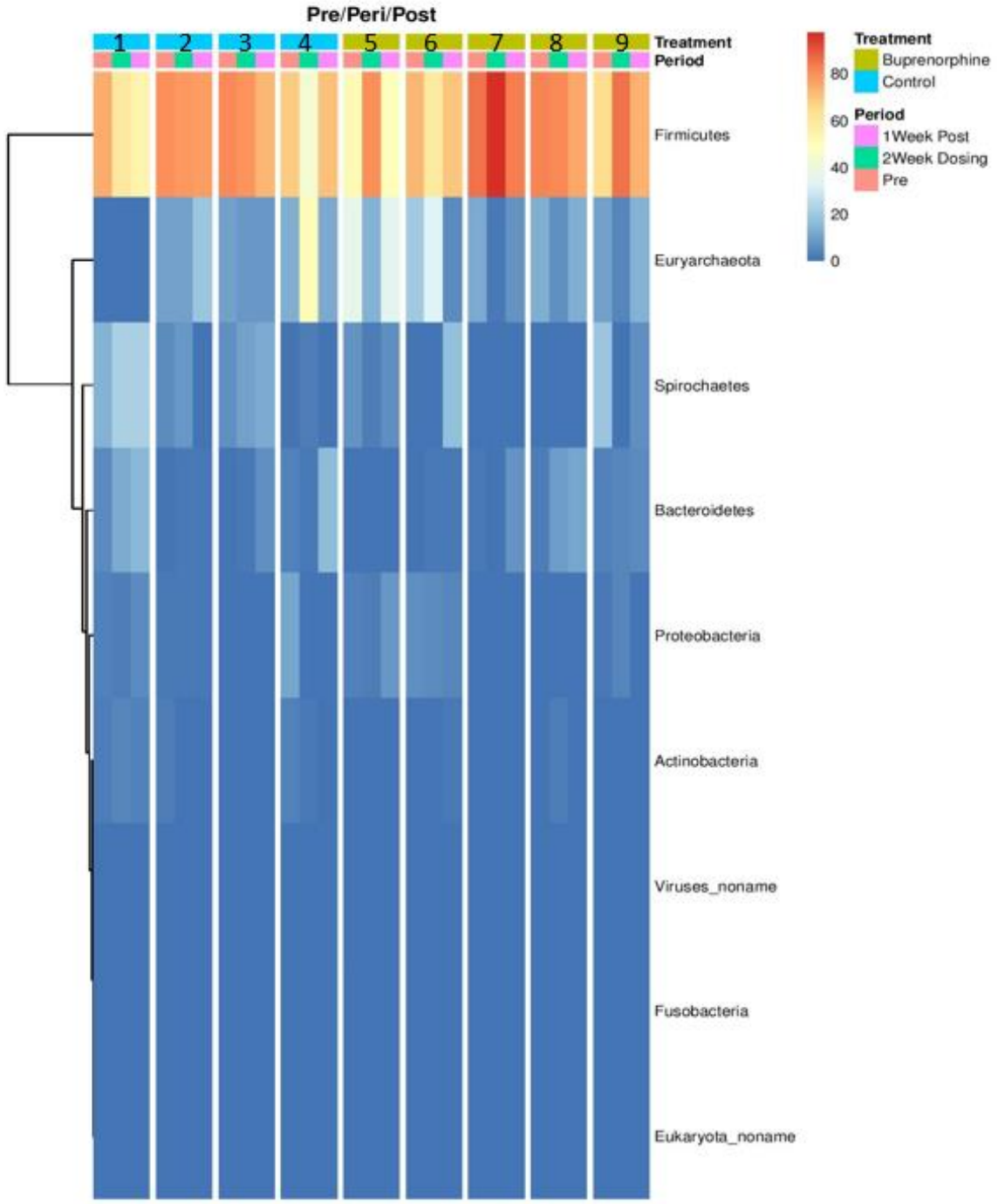


Figure 24. Phylum level gut microbial composition at the pre-dosing, two weeks of dosing, and one week post dosing time points for each subject. Control subjects are shown on the left: 1496 (lane 1), 1500 (lane 2), 1533 (lane 3), 1542 (lane 4) while Buprenorphine-treated subjects are shown on the right: 1497 (lane 5), 1501 (lane 6), 1532 (lane 7), 1543 (lane 8), 1544 (lane 9).

4.5a - Statistical Analysis

Statistical analysis was performed at both the genus level and species level in the control group versus the Buprenorphine treated group. We ran a non-parametric Wilcoxin

signed-rank test at both genus and species levels which provided p values to determine whether there was a significant differential abundance between time points, either pre-dosing to two weeks dosing or two weeks dosing to one week post-dosing, in treated subjects. According to the results, there was no significance alteration in the gut microbiome. While individually, subjects' microbiomes did obviously change, the Wilcoxin test suggested that there was no significant difference in abundance for all genus/species included in the study when comparing pre-dosing to two week dosing samples as well as two week dosing to one week post dosing. While there were p-values that did indicate significance for certain genera (a p-value <.05), these genera resulted in a zero for relative abundance at at least one time point, as shown in the third column of Figure 25. Unfortunately, the presence of a zero in the data for these genera causes the p-value results to be untrustworthy.

Pre vs 2 Week Dosing

Genus	pval	zeros
Methanobrevibacter	0.0715	non_zero
Methanosphaera	0.054474035	zero
Methanocaldococcaceae_unclassified	0.036888426	zero
Actinomyces	0.04771488	zero
Corynebacterium	0.036888426	zero
Rothia	0.036888426	zero
Propionibacterium	0.054474035	zero
Collinsella	0.0620	non_zero
Olsenella	0.057907265	zero
Bacteroides	0.036888426	zero
Parabacteroides	0.054474035	zero
Prevotella	0.0625	non_zero
Gemella	0.036888426	zero
Aerococcus	0.036888426	zero
Enterococcus	0.054474035	zero
Lactobacillus	0.0625	non_zero
Streptococcus	0.036888426	zero
Clostridium	0.036888426	zero
Clostridiales_Family_XIII_Incertae_Sedis_unclassified	0.04771488	zero
Clostridiales_noname	0.057907265	zero
Eubacterium	0.0625	non_zero
Anaerostipes	0.036888426	zero
Blautia	0.0625	non_zero
Butyrivibrio	0.04771488	zero
Coprococcus	0.0625	non_zero
Dorea	0.0625	non_zero
Lachnospiraceae_noname	0.0625	non_zero
Roseburia	0.0625	non_zero
Oscillibacter	0.0625	non_zero
Peptostreptococcaceae_noname	0.0625	non_zero
Faecalibacterium	0.0625	non_zero
Ruminococcus	0.0625	non_zero
Subdoligranulum	0.0625	non_zero
Catenibacterium	0.0625	non_zero
Erysipelotrichaceae_noname	0.0625	non_zero
Acidaminococcus	0.036888426	zero
Phascolarctobacterium	0.0625	non_zero
Megasphaera	0.04771488	zero
Mitsuokella	0.04771488	zero
Veillonella	0.057907265	zero
Fusobacterium	0.036888426	zero
Oligella	0.036888426	zero
Burkholderia	0.036888426	zero
Sutterellaceae_unclassified	0.036888426	zero
Neisseria	0.04771488	zero
Bilophila	0.04771488	zero
Desulfovibrio	0.04771488	zero
Helicobacter	0.0625	non_zero
Citrobacter	0.036888426	zero
Enterobacter	0.036888426	zero
Actinobacillus	0.04771488	zero
Acinetobacter	0.036888426	zero
Brachyspira	0.04771488	zero
Treponema	0.057907265	non_zero
Pyramidobacter	0.036888426	zero
Entamoeba	0.036888426	zero
Viruses_noname	0.036888426	zero

Figure 25. P values indicating significance or insignificance between the pre-dosing time point and the two-week dosing time point in treated subjects.

2week Dosing vs 1week Post

Genus	Pval	zeros
Methanobrevibacter	0.0625	non_zero
Methanosphaera	0.054474035	zero
Methanocaldococcaceae_unclassified	0.036888426	zero
Actinomyces	0.04771488	zero
Corynebacterium	0.036888426	zero
Rothia	0.036888426	zero
Propionibacterium	0.054474035	zero
Collinsella	0.0625	non_zero
Olsenella	0.057907265	zero
Bacteroides	0.036888426	zero
Parabacteroides	0.054474035	zero
Prevotella	0.0625	non_zero
Gemella	0.036888426	zero
Aerococcus	0.036888426	zero
Enterococcus	0.054474035	zero
Lactobacillus	0.0625	non_zero
Streptococcus	0.036888426	zero
Clostridium	0.036888426	zero
Clostridiales_Family_XIII_Incertae_Sedis_unclassified	0.04771488	zero
Clostridiales_noname	0.057907265	zero
Eubacterium	0.0625	non_zero
Anaerostipes	0.036888426	zero
Blautia	0.0625	non_zero
Butyrivibrio	0.04771488	zero
Coprococcus	0.0625	non_zero
Dorea	0.0625	non_zero
Lachnospiraceae_noname	0.0625	non_zero
Roseburia	0.0625	non_zero
Oscillibacter	0.0625	non_zero
Peptostreptococcaceae_noname	0.0625	non_zero
Faecalibacterium	0.0625	non_zero
Ruminococcus	0.0625	non_zero
Subdoligranulum	0.0625	non_zero
Catenibacterium	0.0625	non_zero
Erysipelotrichaceae_noname	0.0625	non_zero
Acidaminococcus	0.036888426	zero
Phascolarctobacterium	0.0625	non_zero
Megasphaera	0.04771488	zero
Mitsuokella	0.04771488	zero
Veillonella	0.057907265	zero
Fusobacterium	0.036888426	zero
Oligella	0.036888426	zero
Burkholderia	0.036888426	zero
Sutterellaceae_unclassified	0.036888426	zero
Neisseria	0.04771488	zero
Bilophila	0.04771488	zero
Desulfovibrio	0.04771488	zero
Helicobacter	0.0625	non_zero
Citrobacter	0.036888426	zero
Enterobacter	0.036888426	zero
Actinobacillus	0.04771488	zero
Acinetobacter	0.036888426	zero
Brachyspira	0.04771488	zero
Treponema	0.057907265	non_zero
Pyramidobacter	0.036888426	zero
Entamoeba	0.036888426	zero
Viruses_noname	0.036888426	zero

Figure 26. P values indicating significance or insignificance between the two-week dosing time point and the one-week post-dosing time point in treated subjects.

We also ran a Kruskal Wallis test at the genus level and the genera that resulted in significant LDA scores can be seen figure 27. The Kruskal Wallis test was set up using LefSe software with Saline vs. Treated as the class and time points as the subclass. The genera that are present on the graph are the only genera that resulted in differential abundance. The red bars in the graph indicate genera that are most closely associated with the saline group while the green bar indicates the genus that is most closely associated with the treatment group.

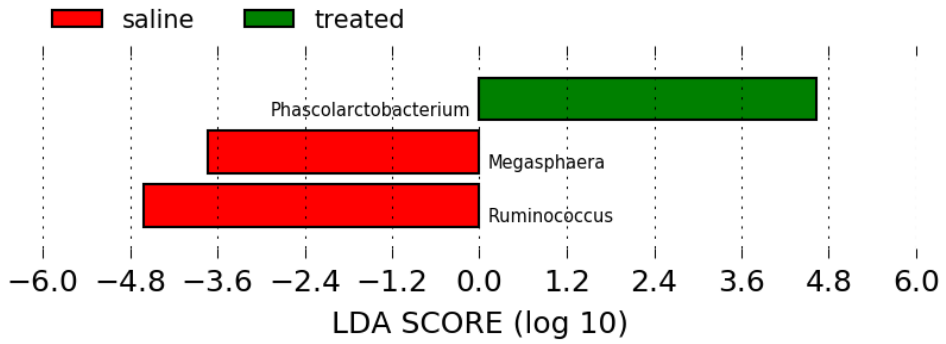


Figure 27. Kruskal Wallis test results for significant alterations in genera abundance in the gut microbiome in the control group versus the treatment group.

Some difficulty in running statistical analysis was a result of our smaller sample size. P values were calculated but many were unreliable given the amount of zeros for a specific genus across samples. While there are probability models that can be used to combat sparsity in data caused by zeros, we were unable to utilize such methods because of our small sample size.

Chapter 5 - Discussion

The purpose of our study was to provide evidence for the hypothesis that the gut microbiome alters in response to repeated opioid administration. Opioids cause a wide range of side effects including gut motility problems, inflammation, cognitive and behavioral changes, among others. In recent studies, the gut microbiome has been linked to a growing amount of physiological problems for humans, including many of the same side effects caused by opioids. Our study was comprised of nine animals, divided into a control group and treatment group. The treatment group was administered the partial agonist opioid drug, Buprenorphine, daily for a two week time span. Fecal samples were collected from each subject over the course of the study. DNA was isolated and analyzed for quality and DNA libraries were prepared and purified. Shotgun sequencing was performed to provide an in-depth look into the composition of the gut microbial community of each subject.

From this, we discovered that the gut microbiome is highly variable among individuals. This variation proves it difficult to identify trends in alterations in the microbiome among groups of individuals. The hope was that despite individual variation, each microbiome would maintain similar dominant genera, making it possible to identify alterations in dominant genera as a result of opioid administration. However, variation among individuals proved to be so vast that it became nearly impossible to find significant alterations in the microbiome as a result of opioid administration. While our subjects were fed the same diet and kept in the same environment, it's possible that variation was a result of gender, age, or other biological differences.

It was expected that Bacteroidetes, Firmicutes, and Proteobacteria would be the dominant bacterial phylum in the microbiome before dosing (Chen). As indicated by Figure 19, Firmicutes

is by far the most dominant phylum found in each subject. Bacteroidetes and Proteobacteria are apparent in each subject; however, they are much less abundant relative to Firmicutes. In seven of the nine subjects (1497, 1500, 1501, 1532, 1533, 1543, 1544), Euryarchaeota is significantly more abundant than Bacteroidetes or Proteobacteria, however, Euryarchaeota is a phylum falling under the kingdom Archaea rather than Bacteria.

We did not expect to find such high levels of Archaea in the fecal samples, as many papers discussing the gut microbiome focused heavily on bacterial species, with little or no mention of Archaea. However, it is known that Euryarchaeota are typically found in the gut. More specifically, *Methanobrevibacter* and *Methanosphaera* reside in the gut with the former usually being more abundant. Both of these Euryarchaeota are considered methanogens and there are two mechanisms by which methanogens might affect health. The first mechanism is their designation as a hydrogenotrophic group, meaning it plays a role in hydrogen transfer and thus a role in anaerobic fermentation of organic matter. This implies that methanogens encourage the growth of fermenting bacteria which can be either pathogenic or commensal bacteria. The second mechanism is their ability to transform heavy metals or metalloids into volatile methylated derivatives, which are toxic. It has been theorized that methanogens play a potential role in GI disorders but there have been conflicting results, leading to an inability to confirm this hypothesis (Horz). Based on this information, we would expect that methanogen abundance would increase as a result of repeated Buprenorphine administration because of the predicted increase in pathogenic bacteria as well as gut related issues, such as constipation and inflammation, as a result of opioid administration. However, in most, but not all, of the treated subjects, the relative abundance of *Methanobrevibacter* decreased after two weeks of dosing and in some of the control subjects, *Methanobrevibacter* relative abundance actually increased.

Therefore, there is no clear correlation between opioid administration and methanogen abundance.

Ignoring Euryarchaeota, there was only one subject, 1500, in which Firmicutes, Proteobacteria, and Bacteroidetes were the three most dominant phylum. Again, ignoring Euryarchaetoa, several subjects including, 1496, 1497, 1501, 1533, and 1544, had a genome comprised mostly of Firmicutes, unexpectedly followed by Spirochaetes rather than Proteobacteria or Bacteroidetes. Additionally, Actinobacteria was more abundant in several subjects than either one of or both Proteobacteria and Bacteroidetes.

It was expected that genera such as *Clostridium, Enterococcus, Lactobacillus* and *Ruminococcus* would be found in the microbiome under the phylum Firmicutes as well as *Bacteroides* and *Prevotella* under the phylum Bacteroidetes with *Bacteroides, Prevotella,* and *Ruminococcus* being the most abundant overall. In addition, *Prevotella* was expected to be the most dominant under the phylum Bacteroidetes (Power).

Sequencing results showed a wide variety of genera under Firmicutes depending on the subject. As expected, *Lactobacillus* was highly or moderately abundant in most subjects and *Ruminococcus* was at least somewhat abundant in all subjects and highly or moderately abundant in some. However, contrary to expectations, there were not genera that were equally highly abundant in all subjects. Additionally, *Clostridium* and *Enterococcus* were minimally abundant in some subjects, but nonexistent in most. *Eubacterium* was found in several subjects and was highly abundant relatively in some.

It was expected that *Prevotella* would be more abundant than *Bacteroides* because of the difference between the Rhesus monkey genome and human genome. *Prevotella* was the most

abundant genus under Bacteroidetes according to our results. However, the CT values from the *Bacteroides* primer ranged between 20-30 which did indicate at least moderate abundance within the subjects, yet the sequencing results did not result in any *Bacteroides* abundance.

It was expected that opioid administration would alter the microbiome to increase pathogenic bacteria such as Staphylococci, Clostridia (*Ruminococcus, Butyrovibrio, Fusobacterium, Eubacterium,* and *Peptostreptococcus*), and Veillonellae and decrease beneficial bacteria such as *Lactobacilli* and *Bifidobacteria*. Interestingly, *Bifidobacteria* was nonexistent in any subject at any time, going against previous understanding that such bacteria plays a very important role in the gut barrier (Rastall). This could be a result of the difference between the human microbiome and rhesus monkey microbiome. *Lactobacillus* abundance did change during dosing, but in many subjects its abundance actually increased. It is possible that this change in *Lactobacillus* could have an effect on GABA regulation, leading to changes in the brain affecting mood and behavior, despite its classification as a beneficial bacterium. Potentially pathogenic bacteria, especially under the class Clostridia were abundant in subjects. However, Staphylococci and Veillonellae were nonexistent even after dosing, another possible result of the difference between human and rhesus monkey microbiomes. It was determined that alterations in the microbiome were found to be insignificant based on a Wilcoxin signed-rank test.

Some possible limitations in the study leading to the insignificant results could be the DNA quality, sample size (both or either the number of subjects or the amount of sample collections), the use of a weaker analgesic as opposed to Morphine or the like, and the duration of the dosing period. DNA from fecal samples as illustrated by electrophoretic analysis appears more degraded than normal. While this is expected for fecal samples, it is possible that our DNA quality was lower than anticipated because DNA isolation and library preparation were not

performed immediately after the other, allowing time for DNA to further degrade before use. Additionally, it is possible that significant alterations in the gut microbiome occur only after an extended period of opioid administration, greater than two weeks. Furthermore, statistical analysis may have been more powerful had the sample size been larger.

The qPCR performed in this study was limited as far as validating sequencing results. First, it would have been beneficial to sequence the DNA samples prior to choosing qPCR primers so as to include more representative bacterial primers. However, this would not have been possible given the circumstances of the project including time restraints and resources. In addition, it was more efficient to choose primers that had already been designed and included in previous publications. Designing our own primers would have been difficult because of the amount of primers we wanted to use. Because we relied on pre-designed primers we could not be sure of the effectiveness prior to purchasing and buying said primers, leading to circumstances like that of the *Spirochaetes* primer in which results may be invalid and the *Bifidobacterium* primers whose results we could not include because of its ineffectiveness throughout the study.

Ultimately, the gut microbiome variation among and within individuals is so great that we could not successfully provide evidence for our hypothesis. In the future, it would be ideal to create an experimental design that studied gut microbiota alterations in parallel to mood/behavior alterations, inflammatory response, and any other host responses. Additionally, this would be more reliable given a longer dosing period, as depressed mood has been correlated to opioid administration following greater than thirty days, and a larger sample size. However, this experimental design is very difficult to execute given limitations in subject and resource availability, and the unknown possible variation among individuals.

Bibliography

1. Acharya C, Betrapally NS, Gillevet PM, et al. Chronic opioid use is associated with altered gut microbiota and predicts readmissions in patients with cirrhosis. *Aliment Pharmacol Ther*. 2017;45(2):319-331. doi:10.1111/apt.13858
2. Balzan S, de Almeida Quadros C, de Cleva R, Zilberstein B, Cecconello I. Bacterial translocation: Overview of mechanisms and clinical impact. *J Gastroenterol Hepatol*. 2007;22(4):464-471. doi:10.1111/j.1440-1746.2007.04933.x
3. Banerjee S, Sindberg G, Wang F, et al. Opioid-induced gut microbial disruption and bile dysregulation leads to gut barrier compromise and sustained systemic inflammation. *Mucosal Immunol*. 2016;9(6):1418-1428. doi:10.1038/mi.2016.9
4. Barden N. Implication of the hypothalamic-pituitary-adrenal axis in the physiopathology of depression. *J Psychiatry Neurosci*. 2004;29(3):185-193. http://www.ncbi.nlm.nih.gov/pubmed/15173895. Accessed April 1, 2019.
5. Bio USA T. *SMARTer® ThruPLEX® DNA-Seq Kit User Manual SMARTer ThruPLEX DNA-Seq Kit User Manual*. https://www.takarabio.com/assets/documents/User Manual/SMARTer ThruPLEX DNA-Seq Kit User Manual_080818.pdf. Accessed April 15, 2019.
6. Brooner RK, King VL, Kidorf M, Schmidt CW, Bigelow GE. Psychiatric and Substance Use Comorbidity Among Treatment-Seeking Opioid Abusers. *Arch Gen Psychiatry*. 1997;54(1):71. doi:10.1001/archpsyc.1997.01830130077015
7. Buprenorphine. DrugBank. https://www.drugbank.ca/drugs/DB00921. Accessed April 1, 2019.

8. Chen Z, Yeoh YK, Hui M, et al. Diversity of macaque microbiota compared to the human counterparts OPEN. *Nature*. 2018. doi:10.1038/s41598-018-33950-6

9. Clarke G, Stilling RM, Kennedy PJ, Stanton C, Cryan JF, Dinan TG. Minireview: Gut Microbiota: The Neglected Endocrine Organ. *Mol Endocrinol*. 2014;28(8):1221-1238. doi:10.1210/me.2014-1108

10. Coe MA, Lofwall MR, Walsh SL. Buprenorphine Pharmacology Review. *J Addict Med*. 2019;13(2):93-103. doi:10.1097/ADM.0000000000000457

11. Crowley SK, Girdler SS. Neurosteroid, GABAergic and Hypothalamic Pituitary Adrenal (HPA) Axis Regulation: What is the Current State of Knowledge in Humans? *Psychopharmacology (Berl)*. 2014;231(17):3619. doi:10.1007/S00213-014-3572-8

12. Farzi A, Halicka J, Mayerhofer R, Fröhlich EE, Tatzl E, Holzer P. Toll-like receptor 4 contributes to the inhibitory effect of morphine on colonic motility in vitro and in vivo. *Sci Rep*. 2015;5(1):9499. doi:10.1038/srep09499

13. Foster JA, McVey Neufeld K-A. Gut–brain axis: how the microbiome influences anxiety and depression. *Trends Neurosci*. 2013;36(5):305-312. doi:10.1016/j.tins.2013.01.005

14. Galland L. The Gut Microbiome and the Brain. *J Med Food*. 2014:12601-1272. doi:10.1089/jmf.2014.7000

15. Gibbs RA, Rogers J, Katze MG, et al. Evolutionary and Biomedical Insights from the Rhesus Macaque Genome. *Science (80-)*. 2007;316(5822):222-234. doi:10.1126/science.1139247

16. Gorbach SL. *Microbiology of the Gastrointestinal Tract*. University of Texas Medical Branch at Galveston; 1996. http://www.ncbi.nlm.nih.gov/pubmed/21413258. Accessed April 1, 2019.

17. Guarner F, Malagelada J-R. Gut flora in health and disease. *Lancet*. 2003;361(9356):512-519. doi:10.1016/S0140-6736(03)12489-0

18. Hermann-Bank ML, Skovgaard K, Stockmarr A, Larsen N, Mølbak L. *The Gut Microbiotassay: A High-Throughput QPCR Approach Combinable with next Generation Sequencing to Study Gut Microbial Diversity*.; 2013. http://www.arb-home.de/. Accessed April 1, 2019.

19. Holzapfel WH, Haberer P, Snel J, Schillinger U, Huis in't Veld JH. Overview of gut flora and probiotics. *Int J Food Microbiol*. 1998;41(2):85-101. doi:10.1016/S0168-1605(98)00044-0

20. Horz H-P, Conrads G. The Discussion Goes on: What Is the Role of *Euryarchaeota* in Humans? *Archaea*. 2010;2010:1-8. doi:10.1155/2010/967271

21. How opioid drugs activate receptors. National Institutes of Health. https://www.nih.gov/news-events/nih-research-matters/how-opioid-drugs-activate-receptors. Published June 5, 2018. Accessed April 1, 2019.

22. Iebba V, Totino V, Gagliardi A, et al. Eubiosis and dysbiosis: the two sides of the microbiota. *New Microbiol*. 2016;39(1):1-12. http://www.ncbi.nlm.nih.gov/pubmed/26922981. Accessed April 1, 2019.

23. Kamada N, Chen GY, Inohara N, Núñez G. Control of pathogens and pathobionts by the gut microbiota. *Nat Immunol*. 2013;14(7):685-690. doi:10.1038/ni.2608

24. Kema IP, de Vries EG, Muskiet FA. Clinical chemistry of serotonin and metabolites. *J Chromatogr B Biomed Sci Appl*. 2000;747(1-2):33-48. http://www.ncbi.nlm.nih.gov/pubmed/11103898. Accessed April 15, 2019.

25. Leclercq S, Matamoros S, Cani PD, et al. Intestinal permeability, gut-bacterial dysbiosis, and behavioral markers of alcohol-dependence severity. *Proc Natl Acad Sci*. 2014;111(42):E4485-E4493. doi:10.1073/pnas.1415174111

26. Machelska H, Mousa SA, Brack A, et al. *Opioid Control of Inflammatory Pain Regulated by Intercellular Adhesion Molecule-1*.; 2002. http://www.jneurosci.org/content/jneuro/22/13/5588.full.pdf. Accessed April 1, 2019.

27. Olivares M, Ben ıtez-P aez A, de Palma G, et al. Increased prevalence of pathogenic bacteria in the gut microbiota of infants at risk of developing celiac disease: The PROFICEL study. *Gut Microbes*. 2018;0. doi:10.1080/19490976.2018.1451276

28. Opioid Overdose. Centers for Disease Control and Prevention. https://www.cdc.gov/drugoverdose/opioids/prescribed.html. Published August 29, 2017. Accessed April 1, 2019.

29. Opioids, Analgesia, and Pain Management. In: Hilal-Dandan R, Brunton LL. eds.*Goodman and Gilman's Manual of Pharmacology and Therapeutics, 2e* New York, NY: McGraw-Hill; . http://accesspharmacy.mhmedical.com.proxy.library.vcu.edu/content.aspx?bookid=1810&sectionid=124491233. Accessed April 01, 2019.

30. Pathan H, Williams J. Basic opioid pharmacology: an update. *Br J Pain*. 2012;6(1):11-16. doi:10.1177/2049463712438493

31. Power SE, O'Toole PW, Stanton C, Ross RP, Fitzgerald GF. Intestinal microbiota, diet and health. *Br J Nutr*. 2014;111(03):387-402. doi:10.1017/S0007114513002560

32. Prevalence of Chronic Pain and High-Impact Chronic Pain Among Adults — United States, 2016. Morbidity and Mortality Weekly Report (MMWR). https://www.cdc.gov/mmwr/volumes/67/wr/mm6736a2.htm. Published September 13, 2018. Accessed April 1, 2019.

33. Public Affairs. "HHS.gov/Opioids: The Prescription Drug & Heroin Overdose Epidemic." *HHS.gov*, Https://Plus.google.com/+HHS, www.hhs.gov/opioids/.

34. Qin J, Li R, Raes J, et al. A human gut microbial gene catalogue established by metagenomic sequencing. *Nature*. 2010;464(7285):59-65. doi:10.1038/nature08821

35. Rastall RA. Bacteria in the Gut: Friends and Foes and How to Alter the Balance. *J Nutr*. 2004;134(8):2022S-2026S. doi:10.1093/jn/134.8.2022S

36. "Real-Time PCR/Quantitative PCR (QPCR) – An Introduction." *QPCR | ABM Inc.*, www.abmgood.com/marketing/knowledge_base/polymerase_chain_reaction_qpcr.php #sybr).

37. Ressler KJ, Nemeroff CB. Role of norepinephrine in the pathophysiology and treatment of mood disorders. *Biol Psychiatry*. 1999;46(9):1219-1233. http://www.ncbi.nlm.nih.gov/pubmed/10560027. Accessed April 15, 2019.

38. Rittner HL, Brack A, Machelska H, et al. *Opioid Peptide-Expressing Leukocytes Identification, Recruitment, and Simultaneously Increasing Inhibition of Inflammatory Pain*. Vol 95.; 2001. www.anesthesiology.org. Accessed April 1, 2019.

39. Rolhion N, Chassaing B. When pathogenic bacteria meet the intestinal microbiota. *Philos Trans R Soc Lond B Biol Sci*. 2016;371(1707). doi:10.1098/rstb.2015.0504

40. Rosenblum A, Marsch LA, Joseph H, Portenoy RK. Opioids and the Treatment of Chronic Pain: Controversies, Current Status, and Future Directions. *Exp Clin Psychopharmacol*. 2008:405-416. doi:10.1037/a0013628

41. Scherrer JF, Salas J, Copeland LA, et al. Prescription Opioid Duration, Dose, and Increased Risk of Depression in 3 Large Patient Populations. *Ann Fam Med*. 2016;14(1):54-62. doi:10.1370/afm.1885

42. Smith HS. Opioid Metabolism. *Mayo Clin Proc*. 2009;84(7):613-624. doi:10.4065/84.7.613

43. Stoeber M, Jullié D, Lobingier BT, et al. A Genetically Encoded Biosensor Reveals Location Bias of Opioid Drug Action. *Neuron*. 2018;98(5):963-976.e5. doi:10.1016/j.neuron.2018.04.021

44. Strain EC, Stitzer ML, Liebson IA, Bigelow GE. Comparison of buprenorphine and methadone in the treatment of opioid dependence. *Am J Psychiatry*. 1994;151(7):1025-1030. doi:10.1176/ajp.151.7.1025

45. Thursby E, Juge N. Introduction to the human gut microbiota. 2017. doi:10.1042/BCJ20160510

46. Tigerstedt I, Tammisto T. Double-blind, multiple-dose comparison of buprenorphine and morphine in postoperative pain. *Acta Anaesthesiol Scand*. 1980;24(6):462-468. http://www.ncbi.nlm.nih.gov/pubmed/7018155. Accessed April 1, 2019.

47. Turnbaugh PJ, Ridaura VK, Faith JJ, Rey FE, Knight R, Gordon JI. The Effect of Diet on the Human Gut Microbiome: A Metagenomic Analysis in Humanized Gnotobiotic Mice. *Sci Transl Med*. 2009;1(6):6ra14-6ra14. doi:10.1126/scitranslmed.3000322

48. Tzschentke TM. Pharmacology and behavioral pharmacology of the mesocortical dopamine system. *Prog Neurobiol*. 2001;63(3):241-320. http://www.ncbi.nlm.nih.gov/pubmed/11115727. Accessed April 15, 2019.

49. Valles-Colomer M, Falony G, Darzi Y, et al. The neuroactive potential of the human gut microbiota in quality of life and depression. *Nat Microbiol*. 2019;4(4):623-632. doi:10.1038/s41564-018-0337-x

50. Wang F, Roy S. Gut Homeostasis, Microbial Dysbiosis, and Opioids. *Toxicol Pathol*. 2017;45(1):150-156. doi:10.1177/0192623316679898

51. Welsh C, Valadez-Meltzer A. Buprenorphine: A (Relatively) New Treatment For Opioid Dependence. *Psychiatry (Edgmont)*. 2(12):29. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2994593/. Accessed April 1, 2019.

52. Wikoff WR, Anfora AT, Liu J, et al. Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. *Proc Natl Acad Sci*. 2009;106(10):3698-3703. doi:10.1073/pnas.0812874106

53. Zhao Y, Yu Y-B. Intestinal microbiota and chronic constipation. *Springerplus*. 2016;5(1). doi:10.1186/S40064-016-2821-1

54. 220_Opioid Epidemic: Module 04. ATrain Education. https://www.atrainceu.com/course-module/3644610-220_opioid-epidemic-module-04. Accessed April 1, 2019.

Appendix

	Firmicutes	Euryarchaeota	Spirochaetes	Proteobacteria	Actinobacteria	Bacteroidetes	Fusobacteria	Synergistetes	Eukaryota	Viruses_
1496 pre	74.57135	0	13.78755	3.58272	2.78711	5.27126	0	0	0	0
1496 1 wk dosing	38.16266	0	29.52673	9.3872	3.04818	19.87523	0	0	0	0
1496 2 wk dosing	59.57853	0	21.68886	2.15435	4.26458	12.31368	0	0	0	0
1496 2 days post	46.28882	0	34.24135	5.31021	3.19243	10.96719	0	0	0	0
1496 1 wk post	54.1699	0	21.91332	5.62442	3.20595	15.0864	0	0	0	0
1497 pre	67.48564	21.85133	7.18459	2.66205	0.62774	0.18865	0	0	0	0
1497 1 wk dosing	57.01044	31.28334	6.43999	4.07086	0.9201	0.27525	0	0	0	0
1497 2 wk dosing	80.72991	14.64148	2.36144	1.97497	0.17619	0.11601	0	0	0	0
1497 2 days post	74.94096	13.94803	7.43521	3.03052	0.48248	0.16281	0	0	0	0
1497 1 wk post	49.5089	34.50094	6.67662	8.52999	0.45721	0.32633	0	0	0	0
1500 pre	72.30366	20.41079	0.00771	6.16569	0.39181	0.72034	0	0	0	0
1500 1 wk dosing	63.22424	30.80377	0.00852	4.88111	0.25635	0.82601	0	0	0	0
1500 2 wk dosing	59.29088	32.88794	0	5.43123	0.89508	1.49488	0	0	0	0
1500 2 days post	63.8916	26.03789	0.00143	7.43759	0.92367	1.70782	0	0	0	0
1500 1 wk post	70.20105	5.74318	17.42389	4.1488	1.11416	1.36893	0	0	0	0
1501 pre	80.72365	10.4738	5.06122	1.20005	2.03546	0.50582	0	0	0	0
1501 1 wk dosing	70.54785	7.1755	14.55558	4.31508	1.65545	1.75054	0	0	0	0
1501 2 wk dosing	78.5849	10.32136	8.02215	1.13768	0.60964	1.32428	0	0	0	0
1501 2 days post	78.10198	2.77277	15.20629	1.38783	1.4824	1.04873	0	0	0	0
1501 1 wk post	77.84066	19.16797	0	1.31621	0.34978	1.32538	0	0	0	0
1532 pre	85.43492	12.06356	0.9256	0.09463	0.33099	1.1503	0	0	0	0
1532 1 wk dosing	71.10043	20.38211	2.55724	1.07499	0.29158	4.59365	0	0	0	0
1532 2 wk dosing	97.62603	1.79521	0.09367	0.02033	0.0736	0.39116	0	0	0	0
1532 2 days post	85.94126	9.31245	0.53717	0.32637	0.64942	3.23333	0	0	0	0
1532 1 wk post	83.26123	6.85268	0.73406	0.42713	0.95406	7.75193	0	0	0.01891	0
1533 pre	81.97856	10.40369	6.19463	0.69464	0.12001	0.60849	0	0	0	0
1533 1 wk dosing	77.16996	12.9586	8.30667	0.9544	0.1422	0.46817	0	0	0	0
1533 2 wk dosing	79.9828	8.10853	9.84633	0.78676	0.19284	1.08274	0	0	0	0
1533 1 wk post	82.42932	7.34905	8.58952	0.22156	0.24793	1.16262	0	0	0	0
1533 2 wk post	72.80473	8.67865	11.84835	0.12672	0.49386	6.0477	0	0	0	0
1542 pre	68.67822	13.39742	0.13136	10.80376	3.19314	3.74205	0.05406	0	0	0
1542 1 wk dosing	50.5287	40.97924	1.39723	1.01179	1.07126	5.01178	0	0	0	0
1542 2 wk dosing	43.21889	50.80545	2.87753	0.6601	1.24099	0.9876	0	0	0	0.2094
1542 2 days post	51.41388	45.15033	1.00609	0.29048	0.77003	1.3692	0	0	0	0
1542 1 wk post	70.71572	12.44264	0	0.3051	0.7086	15.82795	0	0	0	0
1543 pre	82.65318	13.49408	0.09203	0.47528	0.69666	2.58877	0	0	0	0
1543 1 wk dosing	79.33105	4.98811	0	0.48955	1.43237	13.72249	0	0.03643	0	0
1543 2 wk dosing	81.78543	6.06215	0	0.18121	2.45669	9.51452	0	0	0	0
1543 2 days post	64.56556	30.15412	0.27666	0.18526	0.94035	3.87804	0	0	0	0
1543 1 wk post	75.67617	12.76928	0	0.06345	0.63635	10.85475	0	0	0	0
1544 pre	64.47221	10.08301	19.50734	1.85995	0.89723	3.18026	0	0	0	0
1544 1 wk dosing	85.24226	4.56479	0.3732	1.21491	1.7995	6.80534	0	0	0	0
1544 2 wk dosing	85.74842	4.82189	0.36217	4.15865	0.89044	4.01844	0	0	0	0
1544 2 days post	78.73653	4.44503	0.70722	1.32365	0.98065	13.80692	0	0	0	0
1544 1 wk post	73.66483	14.32172	6.06116	0.78525	0.1921	4.97495	0	0	0	0

Supplemental Figure 1. Relative abundances of subjects at each time point (excluding the second pre-dosing day) at the phylum level.

	1496 pre	1496 1 wk dosing	1496 2 wk dosing	1496 1 wk post	1496 2 days post	1497 pre	1497 1 wk dosing	1497 2 wk dosing	1497 2 days post	1497 1 wk post	1500 pre	1500 1 wk dosing	1500 2 wk dosing	1500 2 days post	1500 1 wk post
Lactobacillus	3.96	0.57	4.21	0.34	7.84	15.72	21.65	67.81	52.56	8.74	43.97	34.27	22.56	29.36	2.07
Methanobrevibacter	0.00	0.00	0.00	0.00	0.00	20.25	21.16	10.46	10.87	22.16	14.58	21.52	22.55	21.72	4.24
Eubacterium	20.35	3.48	3.32	6.09	2.03	0.22	0.10	0.02	0.02	0.04	0.31	0.18	0.42	2.14	55.80
Subdoligranulum	7.44	3.63	8.30	6.26	6.44	20.09	16.60	6.37	12.88	19.71	11.79	14.45	6.85	10.97	2.48
Phascolarctobacterium	0.85	1.14	1.72	1.44	1.24	12.65	14.59	5.31	6.63	16.88	8.41	6.86	17.29	14.72	1.29
Treponema	13.79	29.46	21.68	21.89	34.22	7.76	6.44	2.36	7.44	6.67	0.00	0.00	0.00	0.00	17.42
Ruminococcus	3.83	2.47	3.57	6.66	1.80	0.57	1.65	0.39	1.44	1.48	0.25	0.31	0.75	1.55	4.06
Prevotella	5.27	19.82	12.28	15.07	10.93	0.28	0.28	0.12	0.16	0.33	0.72	0.83	1.49	1.22	1.37
Methanosphaera	0.00	0.00	0.00	0.00	0.00	15.97	10.13	4.18	3.08	12.32	5.83	9.28	10.34	4.32	1.50
Coprococcus	16.60	7.87	20.61	17.75	9.95	0.28	0.16	0.08	0.15	0.44	0.69	0.34	0.46	0.21	0.57
Helicobacter	3.51	9.15	1.86	5.06	4.40	3.25	4.07	1.97	3.03	8.53	5.22	3.54	2.94	4.72	4.13
Faecalibacterium	2.25	3.38	4.24	3.08	1.72	0.04	0.10	0.04	0.02	0.01	0.38	0.35	0.45	0.31	0.23
Blautia	6.49	2.34	3.95	4.07	3.35	0.48	0.32	0.20	0.24	0.36	1.38	2.10	1.81	1.22	0.84
Dorea	2.43	3.17	1.03	1.27	2.97	0.74	0.63	0.24	0.36	0.72	0.87	1.58	3.19	0.85	1.30
Catenibacterium	0.00	3.59	1.85	3.01	1.47	0.00	0.16	0.04	0.14	0.11	0.09	0.00	0.07	0.00	0.54
Erysipelotrichaceae	0.79	1.42	1.15	1.18	1.40	0.04	0.04	0.01	0.01	0.04	0.10	0.00	0.01	0.00	0.20
Collinsella	2.64	2.89	3.99	3.02	2.92	0.96	0.91	0.17	0.47	0.46	0.38	0.24	0.89	0.88	0.94
Peptostreptococcaceae	4.59	2.10	2.57	1.70	4.38	0.01	0.04	0.06	0.00	0.06	2.96	1.43	3.83	0.76	0.58
Clostridiales	0.62	1.56	1.41	0.27	0.65	0.00	0.09	0.00	0.00	0.00	0.03	0.00	0.08	0.06	0.09
Roseburia	0.65	0.88	0.89	0.57	0.58	0.28	0.42	0.09	0.23	0.56	1.00	1.30	1.43	1.60	0.09
Enterococcus	0.02	0.02	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Streptococcus	3.44	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bilophila	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.93	1.35	2.49	2.72	0.00
Megasphaera	0.14	0.25	0.53	0.24	0.17	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Mitsuokella	0.00	0.03	0.02	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Clostridium	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lachnospiraceae	0.10	0.18	0.15	0.12	0.16	0.29	0.39	0.04	0.20	0.30	0.06	0.03	0.05	0.05	0.01
Desulfovibrio	0.03	0.23	0.30	0.55	0.87	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02
Veillonella	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Parabacteroides	0.00	0.06	0.03	0.01	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Actinomyces	0.13	0.12	0.23	0.16	0.26	0.00	0.01	0.00	0.01	0.00	0.01	0.02	0.01	0.05	0.10
Oscillibacter	0.02	0.02	0.03	0.03	0.03	0.06	0.08	0.03	0.02	0.05	0.02	0.03	0.04	0.08	0.03
Acinetobacter	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Olsenella	0.02	0.04	0.04	0.03	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Sutterellaceae	0.00	0.01	0.00	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00
Bacteroides	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.48	0.00
Butyrivibrio	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.02	0.03	0.00	0.00	0.00	0.00	0.00	0.00
Acidaminococcus	0.02	0.06	0.04	0.03	0.09	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Clostridiales_Family_XIII_	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Actinobacillus	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Brachyspira	0.00	0.06	0.01	0.02	0.02	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.00	0.00	0.00
Viruses	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Burkholderia	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Propionibacterium	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02
Rothia	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Aerococcus	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03
Fusobacterium	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Corynebacterium	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04
Citrobacter	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Pyramidobacter	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Neisseria	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Enterobacter	0.00	0.00	0.00	0.01	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Entamoeba	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Methanocaldococcaceae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00
Anaerostipes	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00
Gemella	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Oligella	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Supplemental Figure 2a. Relative abundances for subjects 1496, 1497, and 1500 at the genus level.

	1501 pre	1501 1 wk dosing	1501 2 wk dosing	1501 2 days post	1501 1 wk post	1532 pre	1532 1 wk dosing	1532 2 wk dosing	1532 2 days post	1532 1 wk post	1533 pre	1533 1 wk dosing	1533 2 wk dosing	1533 2 days post	1533 1 wk post
Lactobacillus	0.00	3.40	0.00	0.00	58.21	36.84	28.09	91.63	47.63	35.54	39.41	14.40	42.33	26.98	16.10
Methanobrevibacter	3.04	4.45	7.13	2.47	14.75	12.06	20.38	1.80	9.31	6.85	10.40	12.96	8.10	7.35	8.68
Eubacterium	56.34	48.30	60.37	62.88	0.02	7.26	1.45	0.74	3.06	3.95	0.51	0.39	0.45	1.15	1.02
Subdoligranulum	3.41	2.50	3.17	1.48	5.70	5.10	20.13	0.91	5.78	5.40	3.39	3.63	2.60	2.16	3.29
Phascolarctobacterium	1.71	1.86	3.25	2.31	5.15	8.39	2.11	0.04	0.13	2.15	3.25	4.85	2.21	2.58	2.13
Treponema	5.06	14.56	8.02	15.21	0.00	0.93	2.56	0.09	0.54	0.72	6.19	8.31	9.85	8.59	11.85
Ruminococcus	0.64	2.43	6.93	9.10	0.39	0.52	0.35	0.04	2.36	0.23	31.24	50.86	28.15	44.77	43.60
Prevotella	0.48	1.75	1.31	1.05	1.33	1.15	4.59	0.39	3.19	7.64	0.61	0.47	1.08	1.16	6.05
Methanosphaera	7.43	2.72	3.20	0.30	4.42	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Coprococcus	1.00	1.47	0.54	0.42	0.64	4.37	2.52	0.56	3.95	4.61	0.29	0.27	0.44	0.34	0.19
Helicobacter	1.20	4.32	1.13	1.38	0.55	0.09	0.98	0.02	0.19	0.31	0.69	0.95	0.79	0.22	0.13
Faecalibacterium	0.09	0.18	0.27	0.08	0.34	4.81	4.34	0.45	7.63	7.95	0.12	0.25	0.23	0.31	0.32
Blautia	2.48	1.33	0.77	0.64	1.91	2.80	2.43	0.82	3.55	3.10	0.79	0.57	0.66	0.73	1.76
Dorea	2.77	3.71	1.53	0.63	2.11	4.58	2.36	0.57	1.43	3.52	1.01	0.69	1.06	0.88	0.94
Catenibacterium	0.97	1.02	0.82	0.26	0.10	3.65	4.70	0.99	4.74	4.48	1.00	0.87	1.18	1.37	2.23
Erysipelotrichaceae	0.12	0.13	0.06	0.00	0.00	4.35	1.96	0.82	3.00	4.86	0.12	0.05	0.11	0.15	0.11
Collinsella	1.98	1.52	0.53	1.36	0.35	0.32	0.25	0.06	0.59	0.87	0.12	0.13	0.18	0.23	0.49
Peptostreptococcaceae	1.75	0.47	0.64	0.19	2.46	0.37	0.32	0.02	0.71	1.84	0.29	0.03	0.06	0.14	0.00
Clostridiales	0.00	0.02	0.00	0.02	0.00	0.05	0.00	0.01	0.16	2.19	0.20	0.04	0.33	0.63	0.20
Roseburia	0.18	0.12	0.07	0.06	0.70	0.34	0.19	0.00	0.16	0.46	0.33	0.20	0.16	0.20	0.92
Enterococcus	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Streptococcus	8.28	3.56	0.08	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bilophila	0.00	0.00	0.00	0.00	0.76	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Megasphaera	0.94	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Mitsuokella	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Clostridium	0.00	0.00	0.00	0.00	0.00	1.96	0.00	0.00	1.45	2.74	0.00	0.00	0.00	0.00	0.00
Lachnospiraceae	0.00	0.01	0.01	0.00	0.03	0.00	0.00	0.02	0.02	0.07	0.00	0.00	0.00	0.01	0.00
Desulfovibrio	0.00	0.00	0.01	0.00	0.00	0.00	0.10	0.00	0.13	0.11	0.00	0.00	0.00	0.00	0.00
Veillonella	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.09	0.00	0.00	0.00	0.00	0.00
Parabacteroides	0.02	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.05	0.11	0.00	0.00	0.00	0.00	0.00
Actinomyces	0.05	0.11	0.06	0.09	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.00	0.00
Oscillibacter	0.03	0.03	0.06	0.03	0.00	0.05	0.13	0.00	0.09	0.08	0.02	0.02	0.02	0.03	0.00
Acinetobacter	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00
Olsenella	0.00	0.01	0.01	0.01	0.00	0.01	0.04	0.01	0.05	0.06	0.00	0.00	0.00	0.01	0.00
Sutterellaceae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00
Bacteroides	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Butyrivibrio	0.00	0.00	0.00	0.00	0.09	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00
Acidaminococcus	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.03	0.00	0.00	0.00	0.00	0.00
Clostridiales_Family_XIII_	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Actinobacillus	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Brachyspira	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00
Viruses	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Burkholderia	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Propionibacterium	0.00	0.01	0.01	0.01	0.00	0.00	0.00	0.00	0.01	0.03	0.00	0.00	0.00	0.00	0.00
Rothia	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Aerococcus	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.00
Fusobacterium	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Corynebacterium	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Citrobacter	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Pyramidobacter	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Neisseria	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Enterobacter	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Entamoeba	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00
Methanocaldococcaceae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Anaerostipes	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Gemella	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Oligella	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Supplemental Figure 2b. Relative abundances for subjects 1501, 1532, and 1533 at the genus level

	1542 pre	1542 1 wk dosing	1542 2 wk dosing	1542 2 days post	1542 1 wk post	1543 pre	1543 1 wk dosing	1543 2 wk dosing	1543 2 days post	1543 1 wk post	1544 pre	1544 1 wk dosing	1544 2 wk dosing	1544 2 days post	1544 1 wk post
Lactobacillus	10.81	3.04	9.17	12.73	0.00	0.00	0.00	0.00	1.27	0.00	4.66	9.12	30.85	31.68	8.21
Methanobrevibacter	6.59	25.89	43.47	41.15	12.44	13.49	4.99	6.06	30.15	12.77	10.08	4.56	4.82	4.45	14.32
Eubacterium	8.72	2.58	0.93	0.97	6.40	7.30	5.40	6.19	2.66	6.34	10.56	18.27	13.82	13.35	15.31
Subdoligranulum	8.68	19.11	17.20	21.61	19.29	21.24	17.68	21.53	28.37	24.28	8.29	6.46	3.71	3.73	6.95
Phascolarctobacterium	0.81	10.40	2.33	0.97	19.53	21.35	24.05	16.81	20.90	17.79	10.66	20.76	13.23	8.82	20.10
Treponema	0.10	1.37	2.88	1.01	0.00	0.09	0.00	0.00	0.28	0.00	19.51	0.37	0.36	0.71	6.06
Ruminococcus	5.19	1.37	1.15	0.31	4.06	10.59	9.17	8.55	3.28	4.88	0.31	4.21	1.45	0.82	3.00
Prevotella	3.74	4.98	0.99	1.37	15.59	2.56	13.53	9.35	3.22	10.62	2.99	6.77	4.00	13.78	4.70
Methanosphaera	6.81	15.09	7.34	4.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Coprococcus	4.66	2.53	3.28	4.46	2.10	2.95	1.88	1.86	0.87	1.94	3.11	2.33	3.17	1.69	3.65
Helicobacter	10.80	1.01	0.12	0.17	0.19	0.48	0.44	0.15	0.19	0.00	0.96	0.78	3.90	1.03	0.47
Faecalibacterium	7.26	0.89	0.24	0.51	7.15	5.83	6.86	6.15	1.30	6.27	1.68	3.41	3.60	5.52	3.61
Blautia	6.23	3.96	0.73	2.40	2.19	1.31	3.46	4.69	2.13	1.95	2.08	5.32	3.06	1.74	4.59
Dorea	3.21	1.21	3.24	0.80	1.49	2.22	0.91	1.38	0.57	2.04	5.54	2.88	4.13	4.22	1.70
Catenibacterium	1.10	1.38	1.40	0.28	1.70	1.31	3.08	8.54	0.29	2.10	3.41	1.81	1.21	1.26	1.33
Erysipelotrichaceae	6.65	2.30	2.11	5.50	2.86	2.31	1.48	1.49	2.11	3.64	2.25	0.73	1.90	1.71	2.51
Collinsella	2.92	1.01	1.20	0.69	0.69	0.66	1.41	2.40	0.84	0.64	0.90	1.76	0.88	0.95	0.18
Peptostreptococcaceae	0.00	0.11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.09	2.06	1.36	0.45	0.71
Clostridiales	1.98	0.35	0.74	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.71	6.07	3.25	2.57	0.26
Roseburia	0.74	0.06	0.24	0.47	0.58	0.11	0.99	0.52	0.09	0.44	0.44	1.29	0.42	0.66	1.02
Enterococcus	0.10	0.00	0.00	0.00	1.79	4.07	1.58	1.47	0.19	2.36	7.39	0.00	0.05	0.17	0.00
Streptococcus	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bilophila	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Megasphaera	1.41	0.96	0.39	0.00	0.53	1.38	0.21	0.00	0.00	0.00	0.00	0.29	0.29	0.00	0.00
Mitsuokella	0.14	0.00	0.00	0.00	1.01	0.66	1.85	2.22	0.01	1.65	0.00	0.00	0.00	0.12	0.00
Clostridium	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lachnospiraceae	0.11	0.13	0.00	0.29	0.00	0.00	0.33	0.04	0.36	0.00	0.00	0.07	0.02	0.12	0.70
Desulfovibrio	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.30	0.20	0.12	0.31
Veillonella	0.81	0.00	0.06	0.11	0.03	0.00	0.39	0.31	0.03	0.01	0.21	0.15	0.22	0.13	0.01
Parabacteroides	0.00	0.03	0.00	0.00	0.24	0.03	0.20	0.16	0.66	0.24	0.19	0.03	0.02	0.03	0.27
Actinomyces	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Oscillibacter	0.00	0.01	0.00	0.00	0.00	0.01	0.02	0.00	0.01	0.00	0.01	0.02	0.01	0.00	0.00
Acinetobacter	0.00	0.00	0.54	0.12	0.00	0.00	0.00	0.00	0.00	0.00	0.43	0.00	0.00	0.00	0.00
Olsenella	0.16	0.07	0.04	0.08	0.02	0.03	0.02	0.05	0.10	0.00	0.00	0.04	0.01	0.03	0.02
Sutterellaceae	0.00	0.00	0.00	0.00	0.11	0.00	0.05	0.00	0.00	0.06	0.31	0.13	0.00	0.00	0.00
Bacteroides	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Butyrivibrio	0.00	0.14	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Acidaminococcus	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Clostridiales_Family_XIII_	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.11	0.00	0.05	0.00	0.00	0.00	0.00
Actinobacillus	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.18	0.00
Brachyspira	0.03	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Viruses	0.00	0.00	0.21	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Burkholderia	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.16	0.00	0.00	0.00	0.00
Propionibacterium	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Rothia	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Aerococcus	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Fusobacterium	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Corynebacterium	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Citrobacter	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Pyramidobacter	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Neisseria	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Enterobacter	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Entamoeba	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Methanocaldococcaceae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Anaerostipes	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Gemella	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Oligella	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Supplemental Figure 2c. Relative abundances for subjects 1542, 1543, and 1544 at the genus level

Vita

Anne Rowe was born on April 12th, 1995 in Portsmouth, Virginia. She graduated from Tabb High School in 2013, after which she attended the University of Virginia, graduating with a Bachelor of Arts in Biology in 2017. Following her graduation from this program, Anne will attend the Virginia Commonwealth University School of Dentistry as part of the class of 2023.