Fecal Microbiota Transplantation: Potential Implications in Alcohol Use

by

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ABSTRACT

According to a recent survey, over half of adult Americans are current consumers of alcohol. Alcohol use is thought to alter the intestinal microbiota and increase the permeability of the intestinal epithelial barrier in a way that elicits a gut-mediated inflammatory response. This inflammatory response is associated with heightened levels of alcohol craving. Fecal microbiota transplantation (FMT) has been shown to successfully reestablish a balanced, healthy gut microbiota in human patients with gastrointestinal disorders. FMT may restore the disturbed microbiota in alcohol users, prevent gut-mediated inflammation, and ultimately reduce alcohol cravings. In the present study, adult, male, Sprague-Dawley rats were exposed to alcohol during multiple repetitions of a voluntary consumption, drinking-in-the-dark (DID) paradigm, shown to induce clinically relevant levels of alcohol consumption. Following DID, omeprazole was administered to increase bacterial survival in the stomach prior to FMT containing either donor fecal matter or their own. Subsequently, rats were given a two-bottle choice paradigm with water and 10% alcohol solution as a measure of alcohol craving. Due to the known relationship between anxiety and alcohol consumption as well as the known effects of anxiety on the gut microbiota, anxiety-like behavior was measured on an elevated plus-maze apparatus. A mixed model ANOVA was used to test the hypothesis that alcohol consumption levels in previously alcohol-exposed rats that received donor FMT would be significantly lower than in similar rats that received their own fecal matter. Findings revealed that there was a significant effect of day (1-14) and a significant effect of cohort (first and second) on consumption levels. However, there was no main effect of alcohol or FMT on two-bottle choice alcohol consumption, nor was there an interaction between FMT and alcohol. Additionally, analysis of time spent in the open arms of the elevated plus-maze failed to reveal a main effect of alcohol, FMT, or an
interaction on anxiety-like behavior. The results of the present study indicate a need for further research into the interactions between the gastrointestinal microbiota and alcohol consumption, with specific focus on underlying biological factors and future clinical applications. Future research along this line may yield a more thorough understanding of interactions between organ systems, the pathology of alcohol dependence, and effective treatments for alcohol dependence and other psychological disorders.

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Department of Psychology, 2018
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DEDICATION

In memory of my grandmother, Mildred Goad.
FMT: IMPLICATIONS IN ALCOHOL USE

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Chapter I: Purpose of Study

The present study investigated a potential treatment for alcohol cravings by intervening at the level of the gastrointestinal microbiota and tested the efficacy of fecal microbiota transplantation in mitigating alcohol cravings in a rodent model. According to a recent large-scale survey, over half of the adults in the United States are current alcohol users, having reported consumption in the past 30 days (Ahrensbrak, Bose, Hedden, Lipari, & Park-Lee, 2017). Alcohol use is linked to the development of alcohol cravings, which are thought to be a symptom of alcohol dependence. Recent surveys have shown that there are approximately 17 million incidences of reported alcohol dependence in the United States (Bose, Hedden, Lipari, & Park-Lee, 2016). Current pharmacological treatments for alcohol dependence are associated with low efficacy, high toxicity, and high rates of relapse (Johnson, 2008). Therefore, further research into alternative treatments for alcohol dependence is necessary.

Alcohol use is associated with alterations, or dysbiosis, in the gastrointestinal microbiota (Iyer & Vaishnava, 2016). The current study aimed to test a theoretical pathway, explained further in Chapter II, in which the gastrointestinal microbiota serves as a link in the causal chain between alcohol use and alcohol craving. To test this pathway, the microbiota of alcohol-exposed animals was restored through the use of fecal microbiota transplantation (FMT) and the subsequent effects on alcohol craving were observed. Confirmatory results of this study would have provided more evidence for the importance of the gut-brain axis in psychological disorders and broaden the understanding of the pathology of alcohol dependence. Though the results were contrary to the study hypotheses, they may inspire further research into the clinical uses of FMT for psychological disorders and alcohol dependence.
Chapter II: Overview of Previous Research

Alcohol in Psychology

Consumable alcohol, or ethanol, is a central nervous system depressant frequently used recreationally on a global level (Ahrnsbrak et al., 2017; Rehm et al., 2009). Alcohol can be administered in many ways, including intravenously, intraperitoneally, orally, and via vapor inhalation (Baraona et al., 2001; Correia et al., 2015). Most commonly, alcohol is diluted from its pure form and self-administered orally in the form of wine, beer, or liquor. While alcohol use is common in the human population, the dose, duration, and pattern of consumption are highly variable and approximately 17 million individuals meet the diagnostic criteria for alcohol dependence in the United States (Bose et al., 2016). For years, researchers have been working to fully understand the etiology of alcohol dependence as it is essential to determine what factors promote the development of alcohol dependence in alcohol users (Jacob et al., 2017).

One common theoretical model, the self-medication hypothesis, posits that the development of chronic alcohol use and alcohol dependence is due, in part, to the use of alcohol as a coping mechanism for psychological symptoms (Deykin, Levy, & Wells, 1987; Frone, 2016). Alcohol dependence is highly co-morbid with a variety of psychological disorders including anxiety and depression (Briere, Rohde, Seeley, Klein, & Lewinsohn, 2014; Ipser, Wilson, Akindipe, Sager, & Stein, 2015). The self-medication hypothesis suggests that the co-morbidity is due to the chronic use of alcohol to mitigate the effects of such disorders.

While the self-medication hypothesis is supported as a theoretical model, recent research is aimed toward understanding the direct biological pathways involved in alcohol use, chronic alcohol use, and the development of dependence. Such studies span a wide range of factors including neurotransmitter involvement, genetic predisposition, and more recently, the
involvement of the gut-brain axis (Hart & Kranzler, 2015; Hillmer, Mason, Fucito, O’Malley, & Cosgrove, 2015; Kashem et al., 2016; Leclercq et al., 2014a; Zhu et al., 2015).

**Overview of the Gut-Brain Axis**

Research suggests that the gastrointestinal system may play a role in the epidemiology of many psychological disorders through a bidirectional communication system between the gastrointestinal system and the brain (Mayer, 2011). This system, known as the gut-brain axis, is thought to allow gastrointestinal mechanisms to influence changes in the brain, as well as allow the brain to regulate gastrointestinal function (Carabotti, Scirocco, Maselli, & Severi, 2015).

Current understanding of the gut-brain axis began in the 1900s with the discovery of the enteric nervous system, a collection of over 100 million neurons located in the gut that are responsible for the neural regulation of the gastrointestinal system. While activity in the enteric nervous system is modulated by the central nervous system, it is able to operate independently if connections through the vagal nerve are severed. For this reason, it is sometimes called “the brain of the gut” or the “second brain” (Goyal & Hirano, 1996; Mayer, 2011). It is believed that the enteric nervous system, along with the central nervous system, autonomic nervous system, and hypothalamic pituitary adrenal axis make up the gut-brain axis (Carabotti et al., 2015).

Additional research on the gut-brain axis suggests that the microorganisms that populate the gastrointestinal tract, known as the gastrointestinal microbiota, play an important role in gut-brain communication (Cryan & O’Mahoney, 2011; Rhee, Pothoulakis, & Mayer, 2009; Turnbaugh et al., 2013). The gastrointestinal microbiota is thought to secrete various metabolites that signal the central nervous system, autonomic nervous system, and hypothalamic pituitary adrenal axis to produce modulatory effects (Mayer, Tillisch & Gupta, 2015). Evidence in this field suggests that the gastrointestinal or gut microbiota, through communication with the gut-
brain axis, is associated with psychological processes like stress, emotional behavior, and feeding; and may be involved in the pathology of psychological disorders like schizophrenia, autism spectrum disorder, depression, chronic pain, and anxiety (Berk et al., 2013; Evrensel & Ceylan, 2017; De Theije et al., 2014; Mayer et al., 2015). In regard to alcohol, a study by Leclercq and colleagues (2014a) found evidence that certain cytokines associated with the gastrointestinal microbiota, released in response to chronic alcohol consumption, lead to increased levels of alcohol craving.

**Overview of the Gastrointestinal Microbiota**

The mammalian microbiome consists of a community of microorganisms living in and on the human host. Even among healthy individuals, the microorganisms living in the microbiome vary widely (Human Microbiome Project Consortium, 2012). The largest subset of the human microbiome is the gastrointestinal microbiota, which houses a diverse population of bacteria, fungi, and viruses (Collins & Bersick, 2009; Salonen, deVos, & Palva, 2010).

Evidence from recent metagenomics sequencing studies suggests that, even though diverse, the gastrointestinal microbiota of mammals contain a common subset of microorganisms (Qin et al., 2010; Zhang et al., 2015). This common subset, known as the phylogenetic core, consists of microorganisms, like *Firmicutes* and *Bacteroidetes*, that are similar on the genus level in the majority of individuals (Zhang et al., 2015). The phylogenetic core is thought to be responsible for many of the essential functions that the gastrointestinal microbiota performs for its host organism (Brown, DeCoffe, Molcan, & Gibson, 2012; Tap et al., 2009).

Within the gastrointestinal tract, the healthy gastrointestinal microbiota is thought to be involved in many essential functions: extracting nutrients from food, synthesizing vitamins, preventing gastrointestinal infections, maintaining the intestinal barrier, and metabolizing drugs
(i.e., alcohol) (Collins & Bercik, 2009; Turnbaugh et al., 2013). At the organism level, the healthy gastrointestinal microbiota produces various metabolites that perform important functions for the host, such as aiding immune function, facilitating maturation and maintenance of microglia in the central nervous system, and preventing pre-term labor (Rooks & Garrett, 2016).

There are many factors implicated in determining the specific composition of the gastrointestinal microbiota within individual organisms such as; nurture in infancy, age, and dietary habits. In mammals, the original colonization of the gastrointestinal microbiota begins during birth when the organism comes in contact with external and maternal microbial populations (Brown et al., 2012). Additionally, a study by Mariat and colleagues (2009) revealed differences in the ratio of Firmicutes to Bacteroidetes in the human microbiota between infants, adults, and older adults, indicating that microbiota composition may shift with age. While comparing groups of children with distinct dietary habits, De Filippo and colleagues (2010) found that the composition of the gastrointestinal microbiota varied significantly between groups.

While slight fluctuations in the composition of the gastrointestinal microbiota are thought to be normal, more severe alterations are associated with an imbalance in phylogenetic core microorganisms (Chan, Estaki, & Gibson, 2013; Tap et al., 2009). In this unbalanced state, known as dysbiosis, the gastrointestinal microbiota may be unable to perform essential tasks for the host or may even produce harmful effects to the host (Hawrelak & Myers, 2004). Common causes of dysbiosis include the use of antibiotics, consumption of high-fat, high-sulfate, or high-protein diets, high levels of psychological stress or anxiety, and chronic alcohol consumption (Chan et al., 2013; Engen, Green, Voigt, Forsyth, & Keshavaszian, 2015; Hawrelak & Myers,
2004; Jernberg, Löfmark, Edlund, & Jansson, 2010; Wang, Zakhari, & Jung, 2010). Dysbiosis is not a term for a specific disease state, but is linked to the development of pathological conditions like irritable bowel syndrome, obesity, type 2 diabetes, impaired cognitive function, celiac disease, food allergies, cancer, and other chronic, degenerative, or psychological conditions (Bull & Plummer, 2014; Engen et al., 2015; Fröhlich et al., 2016; Hawrelak & Myers, 2004).

**Alcohol and the Gastrointestinal Microbiota**

One previously discussed function of the gastrointestinal microbiota is the maintenance of the intestinal epithelial barrier that functionally separates the intestinal microbiota from the internal human environment surrounding the gastrointestinal system (Iyer & Vaishnava, 2016; Turnbaugh et al., 2013). As mentioned previously, chronic alcohol use is linked to dysbiosis in the gastrointestinal microbiota. Alcohol-induced dysbiosis has been shown to cause a disruption of the tight junctions connecting the cells of the intestinal epithelial barrier, increasing its permeability (Engen et al., 2015; Wang et al., 2010). When the barrier is more permeable, microbes and metabolites can leave the intestinal tract and enter the bloodstream (Engen et al., 2015). Lipopolysaccharides, contained in the outer membrane of gram-negative bacteria, can also enter the bloodstream where it binds with Toll-like receptors (TLR-4 and TLR-2) to trigger a systemic immune response (Engen et al., 2015; Evrensel & Ceylan, 2017). The immune response results in the secretion of the pro-inflammatory cytokines interleukin-8, interleukin-1β, and interleukin-18 (Leclercq et al., 2014a).

According to Leclercq and colleagues (2014a), interleukin-8 and interleukin-1β, both cytokines secreted in response to the increased intestinal barrier permeability, are correlated with an increase in alcohol craving. In a later study, Leclercq and colleagues (2014b) reported that this increased permeability is also associated with increased anxiety and depression levels, two
psychological disorders that are often comorbid with alcohol use and dependence. Several recent studies have called for an investigation into re-establishing the biotic gastrointestinal microbiota as an intervention for the alcohol cravings associated with alcohol induced dysbiosis and subsequent inflammation (Engen et al., 2015; Everensel & Ceylan, 2017; Leclercq et al., 2014a).

**Re-establishing the Gastrointestinal Microbiota**

While the concept of manipulating the microbiota as a means for mitigating the effects of alcohol use is relatively new, there are currently several techniques for reestablishing or modifying the gastrointestinal microbiota being used to treat a range of disorders associated with dysbiosis, including prebiotics, probiotics, and fecal microbiota transplantation.

**Prebiotics.** Prebiotics are fermented non-living substances that serve as nutrients for specific bacterial populations and can produce helpful changes in the gastrointestinal microbiota. Prebiotics have not been tested in subjects with alcohol-induced dysbiosis and intestinal permeability, but there is research to suggest their efficacy in obesity- and diabetes-related gastrointestinal imbalances (Bull & Plummer, 2015; Engen et al., 2015). At this time, prebiotics have not been as well researched and are not thought to be as effective as the other reestablishment techniques.

**Probiotics.** Probiotics, such as *Lactobacillus* and *Bifidobacterium*, are live organisms that are administered to the host. Probiotics are thought to alter the contents of the native gastrointestinal microbiota, thus returning the colony to a more balanced state (Bull & Plummer, 2015). One specific, and relevant, mechanism through which probiotics ameliorate dysbiosis is by inhibiting apoptosis of intestinal barrier cells and creating proteins that strengthen the tight junctions (Abraham & Quigley, 2016).
In gastrointestinal disorders, the results of three recent meta-analyses reveal that while individual studies have produced mixed results, overall, probiotic treatment is beneficial in patients with irritable bowel syndrome, inflammatory bowel disorder, and *Clostridium difficile* infections (Bharat, Giri, Maharjan, Thapa, & Gang, 2017; Lau & Chamberlain, 2016; Mazurak, Broelz, Storr, & Enck, 2015). The primary challenge associated with the use of probiotics, though, is that each individual probiotic strain, or subset of strains, alters the microbiota differently. Therefore, efficacy must be tested with each strain in each potential disorder (Lyra et al., 2016).

In psychological disorders, while probiotics have not been as widely studied, initial investigations support their efficacy as a treatment for several disorders thought to be related to gastrointestinal dysbiosis. Ait-Belgnaoui and colleagues (2012) administered a probiotic strain to experimental mice as a treatment for stress-induced intestinal permeability. Following partial restraint stress, the treatment effectively reduced the hypothalamo-pituitary-adrenal stress response, as well as prevented intestinal permeability and subsequent neuro-inflammation. Additionally, one study found decreased levels of anxiety, depression, and stress levels in both rats and humans following a one-month course of probiotics (Messaoudi et al., 2011).

Currently, probiotic treatment is the primary technique that has been studied in the reversal of alcohol-related dysbiosis. Available research suggests that probiotics, particularly *Lactobacillus rhamnosus GG*, are effective in preventing dysbiosis of the gut microbiota and repairing intestinal permeability in alcohol-exposed or alcohol-dependent humans and rodents (Engen et al., 2015). While probiotics are generally considered effective for modifying the gastrointestinal microbiota, they do present a significant risk for sepsis, excessive immune
stimulation, and microbial resistance (Boyle, Robins-Browne, & Tang, 2006). Thus, other treatments, like fecal microbiota transplantation, are being investigated.

**Fecal Microbiota Transplantation.** Fecal microbiota transplantation (FMT) is a procedure in which fecal matter from a healthy donor’s gastrointestinal microbiota is administered to dysbiotic gastrointestinal microbiota, as a means for replenishing the diminished population of microorganisms (Borody, Brandt, & Paramsothy, 2014). FMT material can be administered to the recipient via fecal enema, colonoscopy, nasogastric gavage, duodenal tube, or oral administration (Borody & Khoruts, 2012; Youngster et al., 2014). FMT is unique from probiotics and prebiotics in its mechanism of action. Instead of supplementing or altering a dysbiotic microbiota with one particular living or fermented substance, FMT aims to provide a complete, healthy community of bacteria to replace or repair the existing dysbiotic community (Bull & Plummer, 2015).

While FMT has never been used to reverse alcohol-induced dysbiosis, it is currently used in both humans and animals as treatment for a variety of gastrointestinal disorders, including *Clostridium difficile* infection, inflammatory bowel disease, and irritable bowel syndrome with up to a 90% success rate (Brandt & Aroniadis, 2013; Bull & Plummer, 2015). Unlike probiotics and prebiotic treatments that require long-term use, evidence suggests that FMT is effective after a one-time treatment with relatively long-lasting results (Brandt & Aroniadas, 2013). In addition to gastrointestinal disorders, building evidence suggests that FMT may also be a viable treatment for some autoimmune disorders, metabolic disorders, neurodegenerative disorders, and psychological disorders (Borody et al., 2014).
Study Overview

The present study utilizes a between-subjects experimental design to reestablish the biotic gastrointestinal microbiota in alcohol drinking rats through the use of FMT to mitigate subsequent alcohol cravings. Alcohol use was established in an animal model through the use of a repeated drinking in the dark (DID) paradigm, modified from Rhodes and colleagues (2005). This particular paradigm was selected because it is a well-respected model, having been cited over 400 times, and is capable of producing significant levels of alcohol consumption. Additionally, the present study utilized FMT as a means of reestablishing the gastrointestinal microbiome due to its unique mechanism of action, the duration of results after one-time treatment, and its efficacy rates in previous studies. Subsequent alcohol cravings were operationalized as alcohol consumption levels in a two-bottle choice paradigm.

Due to the previously established relationship between alcohol use, dysbiosis in the gastrointestinal microbiota, and behavioral changes, the following primary hypotheses were formed within a 2 (FMT: self or donor) x 2 (Alcohol: exposed or not) x 2 (Cohort: first and second) x 14 (Days: Two-bottle choice) mixed model design:

\[ H1: \text{It was hypothesized that there would be a main effect of alcohol treatment on alcohol cravings. The rats in both the alcohol-exposed conditions would be familiar with alcohol consumption before the two-bottle choice measure of alcohol craving, and were therefore expected to behave differently toward alcohol than the non-alcohol exposed animals.} \]

\[ H2: \text{It was hypothesized that there would be a main effect of type of FMT treatment (healthy donor or own fecal matter) on alcohol cravings. For the animals that received their own fecal matter in a FMT, there were no expected changes. Additionally, for non-alcohol exposed animals that received a FMT from a healthy donor, it was expected that their gastrointestinal} \]

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microbiota would be relatively consistent with that of the donors and no subsequent changes were anticipated.

\textit{H3:} It was hypothesized that there would be no main effect of cohort (first or second) on alcohol cravings, as care was taken to ensure that handling, treatments, husbandry, and testing were kept consistent across both cohorts.

\textit{H4:} It was hypothesized that there would be an interaction between alcohol treatment and type of FMT treatment on alcohol cravings, such that previously alcohol-exposed rats that received a FMT from a healthy donor would exhibit lower levels of alcohol craving than previously alcohol-exposed rats that received a FMT containing their own fecal matter. It was expected that this FMT treatment containing healthy-donor fecal matter would reduce alcohol craving in previously alcohol-exposed rats to levels comparable to rats that were not previously exposed to alcohol, regardless of the type of FMT treatment.

Additionally, given the well-established relationship between alcohol consumption and anxiety, as well as the evidence suggesting that the gut-leakiness caused by alcohol consumption can lead to high levels of anxiety in addition to increased alcohol craving, the following secondary hypotheses were formed within a 2 (FMT: self or donor) x 2 (Alcohol: exposed or not) x 2 (Cohort: first and second) factorial design:

\textit{H5:} It was hypothesized that there would be a main effect of alcohol treatment on anxiety-like behavior.

\textit{H6:} It was hypothesized that there would be a main effect of type of FMT treatment (healthy donor or own fecal matter) on anxiety-like behavior.
FMT: IMPLICATIONS IN ALCOHOL USE

H7: It was hypothesized that there would be no main effect of cohort (first or second) on anxiety, as care was taken to ensure that handling, treatments, husbandry, and testing were kept consistent across both cohorts.

H8: It was hypothesized that there would be an interaction between alcohol treatment and type of FMT treatment on anxiety, such that previously alcohol-exposed rats that received a FMT from a healthy donor would exhibit lower levels of anxiety than previously alcohol-exposed rats that received a FMT containing their own fecal matter. It was expected that this FMT treatment containing healthy-donor fecal matter would reduce anxiety-like behavior in previously alcohol-exposed rats to levels comparable to rats that were not previously exposed to alcohol, regardless of the type of FMT treatment.
Chapter III: Methodology

Subjects

Fifty-four, post-natal day (PND) 200+, Sprague-Dawley rats were bred in house. All animals were housed individually in a humidity and temperature-controlled vivarium on a reversed 12-hour light/dark cycle, with lights turning on at 4 p.m. and turning off at 4 a.m. Animals were given ad libitum access to food and water throughout the study, except during the initial alcohol exposure period. Standard husbandry occurred weekly. Six unrelated animals were group housed in large plastic tubs, three per tub, and utilized as fecal donors. The donor animals were not subjected to any of the experimental treatments. The remaining 48 animals were semi-randomly assigned, to ensure genetic diversity, to one of four experimental conditions: alcohol-exposed rats that received a FMT from a healthy donor, alcohol-exposed rats that received a FMT containing their own fecal matter, rats that were not exposed to alcohol and received a FMT from a healthy donor, and rats that were not exposed to alcohol and received a FMT containing their own fecal matter.

Measures

Alcohol use. The alcohol treatment was a repeated 4-day drinking in the dark (DID) paradigm, modified from Rhodes, Best, Belknap, Finn, and Crabbe (2005). Three hours into the dark cycle, under red-light conditions, animals in the alcohol-exposure conditions had their water bottles replaced with a bottle of 20% ethanol solution. They then had access to the ethanol solution for 2 hours on the first, second, and third days. On the fourth day of the paradigm, animals had access to the solution for 4 hours beginning at the same time point. During this time frame, the animals in the no-alcohol conditions were given fresh water in place of the 20% ethanol solution. At the end of the 2 or 4-hour alcohol exposure time period, all bottles were
collected and animals were given back their original drinking water bottles. For 3 days following
the paradigm, animals did not receive any alcohol. This 4-day paradigm was repeated three
times, with a longer abstinence period following the second repetition (4 days on, 3 days off, 4
days on, 5 days off, 4 days on, 3 days off), totaling 20 days to ensure clinically relevant levels of
alcohol consumption and to establish an appropriate baseline for alcohol consumption in
individual alcohol-exposed animals. Total liquid consumption per animal was calculated every
day.

**Omeprazole.** During the abstinence period of the second DID repetition, all experimental
animals received a self-administered dose of approximately 50mg/kg of omeprazole suspended
in a 50% Ensure solution once a day for 3 days. This treatment, modified from Manichanh and
colleagues (2010), was used to reduce gastric acid secretions in the stomach and increase the
likelihood of survival of the bacteria in the fecal microbiota transplant.

**Fecal Microbiota Transplantation.** All animals, including donors, were moved into
sterilized containers approximately six hours prior to collection of fecal donations. Fecal matter
was collected directly from the containers and used fresh (Bakken et al., 2011). Animals in the
FMT from healthy donor conditions received a FMT containing donor fecal matter. Animals in
the FMT from self conditions received a FMT containing their own fecal matter. Each FMT stool
suspension was prepared by blending approximately 2g (within 0.05g) of assigned fecal matter
with 10 mL of non-preserved, 0.9% saline solution via vortex mixer until homogenous. Once
blended, the mixture was filtered through a gauze pad to remove larger particles. The stool
suspension was mixed thoroughly again immediately before each rat received 5ml of their
respective FMT stool suspension by way of oral gavage (Brandt & Aroniadis, 2013). During the
oral gavage procedure, the animals were lightly restrained around their shoulders with their head
immobilized. A metal cannula was inserted into the mouth, and guided through the esophagus to the stomach. Once the cannula was in place, the stool suspension dose was injected. Following administration, rats were monitored for medical complications such as respiratory distress, weight loss, swelling, bleeding, or visible discomfort.

**Alcohol craving.** Alcohol craving was measured, beginning 2 days after FMT administration, by providing the animals with a two-bottle choice paradigm. Animals in all experimental conditions were given *ad libitum* access to bottles containing water and a 10% alcohol solution for 14 days. Every 24 hours, the contents of each bottle was weighed to determine total liquid consumption. Higher levels of alcohol consumption represented higher levels of alcohol craving (*M* = .40, *SD* = .42).

**Anxiety.** Anxiety levels were measured using an elevated plus-maze apparatus, a standard measure of anxiety. The plus maze was constructed out of wood and painted white. It was elevated 62 cm above the floor and consisted of 4 arms, each measuring 60.5 cm long and 11.5 cm wide. A lip, approximately 2.4 cm high, surrounded each arm. The maze contained a center platform that measured approximately 12.5 x 12.5 cm. A wall enclosed the northwest and southeast arms. A video camera was mounted directly above the plus-maze to record the trial and convey the feed to behavioral tracking software. Each rat was marked on its back with a black dot using a non-toxic marker to provide visual contrast and facilitate tracking. Rats were then placed in the center of the maze facing the northwest arm and allowed to explore the maze for a 5-minute trial. Trials were run under red light conditions and precautions were taken to avoid white light exposure during transportation to the testing room, as rats are more active during their dark cycle. The percent time spent in the open and enclosed arms of the maze was recorded and
calculated via behavioral tracking software. Within the present study the subjects spent, on average, 27% of their time in the open arms ($SD = 14.07$).

**Procedure**

At the beginning of the experiment, rats were placed in appropriate housing for at least 14 days to allow habituation to a reversed 12-hour light/dark cycle. Experimental rats were housed in separate cages in order to facilitate individual alcohol consumption measurements and maintain the integrity of each rat’s intestinal microbiome. Throughout the study, all animals were monitored regularly for health status (i.e., signs of dehydration, increased porphyrin production, and weight loss). Efforts were made to maintain consistent microbial exposure for all subjects; these efforts included required use of dedicated gloves for each experimental group, reserved lab coats for animal caretakers, standardized cage cleaning procedures, and consistent bedding, water, and food for all subjects.

The alcohol treatment, a 4-day DID paradigm, began on day 15 of the study and was repeated until day 35. During the 3 days of abstinence following the first repetition, rats were handled and lightly restrained for 3 minutes each day to habituate them to the handling practices used during FMT administration. During the 3 days of abstinence following the third repetition, omeprazole was provided for self-administration once a day in 10ml of 50% Ensure solution.

FMT treatments were prepared and administered on day 37. Beginning on day 39 of the study, rats were given 24-hour access to water and a 10% alcohol solution for 14 days. On day 53, 2 days following the end of 24-hour alcohol access to allow all alcohol to clear their systems, rats were run on the elevated plus-maze apparatus to test for anxiety-like behavior. Due to researcher availability, rats were euthanized within 1 week of completing the elevated plus-maze. Euthanasia was carried out according to IACUC standards via sodium pentobarbital injection.
followed by transcardial perfusion. At this point, brain tissue was collected for further investigation. A timeline of procedures is provided in Figure 1.
Figure 1. This figure displays a timeline of experimental procedures for the present study.
Statistical Analysis Plan

The primary analysis was a $2 \times 2 \times 2 \times 14$ mixed model ANOVA to compare the levels of alcohol craving across the fourteen days of two-bottle choice among the alcohol treatment groups (alcohol-exposed or no alcohol exposure), FMT treatment conditions (healthy fecal matter or own fecal matter), and cohorts (first or second).

The secondary analysis was a $2 \times 2 \times 2$ factorial ANOVA to compare the levels of anxiety-like behavior among the alcohol treatment groups (alcohol-exposed or no alcohol exposure), FMT treatment conditions (healthy fecal matter or own fecal matter), and cohorts (first or second).
Chapter IV: Results

Description of Sample

Data was collected from 48 adult male Sprague-Dawley rats. During FMT administration, one subject from the no-alcohol exposure and healthy FMT condition sustained a fatal internal injury and was removed entirely from the data set prior to statistical analysis. The remaining 47 subjects ranged in age from PND 225 to 300 ($M = 271.64, SD = 21.02$) and ranged in weight from 630 to 1047 grams ($M = 853.45, SD = 93.40$) at the beginning of data collection.

Due to limitations on time and laboratory equipment, subjects were randomly assigned to one of two cohorts prior to the start of data collection. Data was collected from the two cohorts consecutively and semi-aggregated for analysis. Independent samples $t$-tests were run on age and weight, determining that there were no significant differences in mean beginning weights between the two cohorts, $t (45) = -1.05, p = .300, d = .31$. As expected, due to the passage of time between cohorts, the mean beginning ages were found to be statistically different with the average subject from the first cohort being 35.74 days younger, $t (45) = -11.26, p < .001, d = 3.36$. Since the average lifespan of Sprague-Dawley rats is approximately 3 years, with over PND 90 considered adulthood, and there are no major developmental changes that occur between PND 200 and 300, this difference in age is presumed to have little to no impact on the results of the present study (Sengupta, 2013).

Manipulation Check for Escalation of Alcohol Use during DID

Animals in the alcohol exposure conditions were subjected to three repetitions of a 4-day DID paradigm intended to escalate voluntary alcohol consumption over time. A $2 \times 3$ (Cohort: first or second) x (Repetition: first, second, or third) mixed model ANOVA revealed a significant main effect of repetition on alcohol consumption levels among the final day of each of the 3
repetitions, $F(2, 44) = 8.12, p = .001, \eta^2_p = .27$. Bonferroni adjusted post hoc analyses indicated a statistically significant increase in average alcohol consumption between the first and second repetitions from .312 g/kg to .403 g/kg ($SD_{First} = .30$, $SD_{Second} = .31; p = .044$), and a similar, though not statistically significant, increase between the second and third repetitions from .403 g/kg to .487 g/kg ($SD_{Second} = .31$, $SD_{Third} = .43; p = .245$). Overall, the repeated DID paradigm was successful in heightening alcohol consumption, as is evidenced by the significant increase in average alcohol consumption between the first and third repetitions of the paradigm from .312 g/kg to .487 g/kg ($SD_{First} = .30$, $SD_{Third} = .43; p = .005$).

Additionally, the mixed model ANOVA revealed a significant main effect of cohort, $F(1,22) = 4.67, p = .042, \eta^2_p = .18$. Though the pattern of alcohol consumption is similar between cohorts, subjects in the second cohort (C2) consumed significantly more alcohol than subjects in the first cohort (C1) on the final day of the first ($M_{C1} = .22$, $SD_{C1} = .19$, $M_{C2} = .40$, $SD_{C2} = .37$), second ($M_{C1} = .28$, $SD_{C1} = .27$, $M_{C2} = .53$, $SD_{C2} = .31$), and third ($M_{C1} = .30$, $SD_{C1} = .26$, $M_{C2} = .67$, $SD_{C2} = .49$), repetition of DID. There was no significant interaction between repetition and cohort on alcohol consumption levels. Visualization of the escalation in alcohol consumption levels of the first and second cohorts is provided in Figure 2.
Figure 2. This figure displays the mean alcohol consumption levels for subjects in the alcohol-exposure conditions on the final day of repetition 1, repetition 2, and repetition 3 of the drinking in the dark paradigm (DID). Error bars represent the standard error of the mean.
Analysis of Alcohol Craving Following Fecal Microbiota Transplantation

The primary analysis for the present study was a 2 x 2 x 2 x 14 mixed model ANOVA, used to compare the levels of alcohol craving during the 14 days of two-bottle choice voluntary consumption among the alcohol treatment groups (alcohol-exposed or no alcohol exposure), FMT treatment conditions (healthy fecal matter or own fecal matter), and cohorts (first and second). The initial results of this analysis suggest that there was a significant within-subjects main effect of day on alcohol craving levels \( F(13, 507) = 5.22, p < .001, \eta_p^2 = .12 \) and a significant between-subjects main effect of cohort on alcohol craving levels \( F(1,39) = 5.27, p = .027, \eta_p^2 = .12 \).

Subjects in the second cohort (C2) exhibited higher levels of alcohol craving than subjects in the first cohort (C1) overall \( (M_{C1} = .25, SD_{C1} = .27, M_{C2} = .54, SD_{C2} = .49) \) and on each of the 14 days of two-bottle choice. Further investigation into the main effect of day on alcohol craving, via post hoc analyses with a Bonferroni adjustment, revealed that the first day of two-bottle choice \( (M = .76, SD = .91) \) yielded significantly higher levels of alcohol craving than the 10th day \( (M = .31, SD = .41; p = .044) \), the 11th day \( (M = .294, SD = .41, p = .027) \), and the 13th day \( (M = .311, SD = .41, p = .046) \), though levels remained otherwise relatively stable throughout the consumption period (see Figure 3). There were no other significant main effects or interactions.

A supplemental analysis showed that among the animals in both alcohol exposure conditions, the amount of alcohol consumed on the final day of the alcohol treatment was strongly correlated with the average amount of alcohol consumed per day following FMT treatment, \( r = .66, p < .001 \). Among only the subjects in the alcohol exposure condition that received their own fecal matter, this relationship is even stronger, \( r = .81, p = .001 \). However, in
the alcohol exposure condition that received donor fecal matter, the correlation between these variables remains large but no longer reaches statistical significance, $r = .45, p = .142$. The varying strength of the relationship between previous levels of alcohol consumption and levels of alcohol craving post-FMT indicated a pattern that may correspond to and support the foundational assertion of this study: that FMT from a healthy donor may disrupt the pathway from prior alcohol use to future alcohol craving.
Figure 3. A. This figure displays the average alcohol craving levels for each condition in the first cohort across each of the 14 days of two-bottle choice measurement, with an inset displaying the average alcohol craving level for each group collapsed across all 14 days of two-bottle choice measurement. B. This figure displays the same information as described above, but for the second cohort. Error bars represent the standard error of the mean.
Analysis of Anxiety-like Behavior Following Fecal Microbiota Transplantation

A 2x2x2 factorial ANOVA was utilized to compare levels of anxiety-like behavior, as measured by percent time spent in open arms on the elevated plus-maze, among the alcohol treatment groups (alcohol-exposed or no alcohol exposure), FMT treatment conditions (healthy fecal matter or own fecal matter), and cohorts (first and second). This analysis revealed no significant main effect of alcohol treatment ($F(1, 38) = .05, p = .833$), FMT treatment ($F(1, 38) = .08, p = .777$), or cohort ($F(1, 38) = .05, p = .826$) on levels of anxiety-like behavior. Similarly, there were no significant interactions among the variables (see Figure 4).

A supplemental bivariate correlation was run to test the existence and strength of the pre-established relationship between levels of anxiety-like behavior and levels of alcohol craving, revealing no statistically significant relationship between these variables within the data ($r = .03, p = .823$).
Figure 4. A. This figure displays the average percent time in open arms, a measure of anxiety-like behavior, for each of the four conditions in the first cohort. B. This figure displays the same information as described above, but for the second cohort. Error bars represent the standard error of the mean.
Chapter V: Discussion

The present study aimed to establish fecal microbiota transplantation (FMT) as a viable means of attenuating alcohol cravings in a rodent model and to provide evidence for the role of the gut-brain axis in alcohol use. While the overall findings contravene the study hypotheses, they should not be interpreted without consideration for potential limitations and confounds within the study design. Likewise, the potential implications of gastrointestinal interventions for alcohol craving are so critical that, despite present results, future research should continue to pursue this line of inquiry.

Due to their prior voluntary exposure to alcohol during DID, animals in both of the alcohol-exposed conditions were expected to crave subsequent alcohol-containing fluids more than the non-alcohol exposed animals during the two bottle choice period. Further, it was expected that the type of FMT treatment (healthy donor or own fecal matter) given would have an effect on later levels of alcohol craving. Additionally, it was expected that FMT treatment containing healthy-donor fecal matter would reduce alcohol craving in previously alcohol-exposed rats to levels comparable to both groups of rats that were not previously exposed to alcohol. Contrary to these hypotheses, the results revealed no main effect of alcohol treatment, FMT treatment, nor an interaction between the two on subsequent alcohol craving levels. Without clarification via specific microbial analysis, these findings may suggest that the microbiome was not significantly altered over the course of the study or that changes in the microbiome did not produce the expected behavioral outcomes. Additionally, it is important to consider other factors that may account for these results.

FMT from healthy donors has been previously shown to effectively restore dysbiotic microbial populations in the gastrointestinal tract within 24-hours of administration (Borody et
al., 2014; Bull & Plummer, 2015). Since the exact composition of the healthy rat microbiota is unknown, as are any potential strain differences, there are currently several accepted and effective methods for donor selection in rats. Some studies utilize donors that are similar to the FMT recipients, while others use donor animals of different strains in an attempt to increase exogenous microbiota diversity (Manichanh et al., 2010). The donors used in the present study were randomly selected from the same 17 litters as the experimental animals to ensure that they, and their microbiota, were directly comparable to their FMT recipients and to those rodents receiving their own fecal matter during FMT.

Prior to administration of FMT in clinical settings, human donors are rigorously screened via self-report questionnaires, inspection of medical records, and testing of blood and fecal samples for a range of risk factors and communicable diseases (Bakken et al., 2011). The donors in the present study, as in other similar rodent studies, were not subjected to rigorous testing, but presumed healthy based on inspections of weight, appearance, and lack of observable symptoms conducted before the beginning of the study and prior to FMT sample collection (Manichanh et al., 2010). Since social housing is considered best practice for rodents, the donor animals were housed together in two large tubs. However, since rats are coprophagic, if one of the donor animals had an undetected, pre-existing condition or gastrointestinal imbalance, it could potentially have impacted half of the available donors.

Additionally, evidence of restored microbiota following FMT may not be apparent in the present study if the alcohol treatment used was insufficient to disrupt the gastrointestinal microbiota. DID is a well-accepted model of voluntary alcohol self-administration, capable of producing clinically relevant levels of alcohol consumption that are comparable to chronic alcohol consumption in humans. Though many previous studies have focused on the impact of
heavy intoxication on the microbiome, there is literature to suggest that chronic consumption of low doses of alcohol does sufficiently alter the gastrointestinal microbiota. One such study, conducted in humans, found that chronic consumption, daily ethanol doses of 0.31g/kg/day (the approximate equivalent of two glasses of wine) over a 20-day period, caused significant microbial changes (Queipo-Ortuño et al., 2012). In the present study, the average consumption levels among the alcohol-exposed subjects were escalated from 1.87 g/kg/day in the first round to 2.92 g/kg/day following the third repetition.

Other rodent models of alcohol consumption are capable of producing higher levels of intoxication (>10 g/kg/day) using breeding for genetic preference for alcohol drinking or methods of forced consumption, like gavage or vapor inhalation (McBride & Li, 1998). However, these methods were deemed inappropriate for use in the present study. Since much of the population of the gastrointestinal microbiota is thought to be established in infancy from maternal contact, the microbiota of animals born to mothers with a genetic preference for alcohol may not be adequately generalizable (Brown, DeCoffe, Molcan, & Gibson, 2012). Additionally, alcohol vapor chambers are capable of producing high levels of intoxication, but the alcohol is absorbed through mucus membranes in the lungs and airways. Since this route does not directly involve the gastrointestinal system like oral consumption, it is unlikely to produce the necessary dysbiosis (Gilpin, Richardson, Cole, & Koob, 2008). Since stress is known to have independent effects on the microbiota and gavage procedures are inherently stressful, oral gavage was intentionally used sparingly in the present study. Utilizing a method known to produce higher levels of intoxication may have eliminated any doubt that the alcohol treatment adequately disrupted the gastrointestinal microbiota. However, DID was chosen because it would introduce the fewest experimental confounds and produce steadily increasing consumption levels that are
easily comparable to chronic alcohol consumption in humans. The three repetitions of DID did significantly increase the amount of alcohol consumed, however, continuing to repeat the paradigm may have elicited even higher consumption levels over time (Thiele & Navarro, 2014).

Though the levels of alcohol craving did not differ among the four conditions, there was a day effect such that overall levels of alcohol craving during the first day of two-bottle choice were significantly higher than levels on several later days. This finding is consistent with the well-established tendency for rats to consume higher levels of alcohol after approximately a week of deprivation, known as the alcohol deprivation effect (Sinclair & Senter, 1968; Vengeliene, Bilbao, & Spanagel, 2014). In this case, the abstinence from alcohol during the 6 days after DID likely served as a deprivation period that encouraged heightened craving levels on the first day of the two-bottle choice measurement for the previously alcohol-exposed subjects. This effect does not, however, account for higher early levels of consumption among the subjects that were not previously exposed to alcohol. For these subjects, the levels may be attributable to the exploration of the novel substance.

Due to the requirements of this procedure and limitations of the research facility, all animals were housed in one room in wire-topped cages with shared sources of food, bedding, water, and animal care. This may not have been ideal, since studies in bacterial survival have shown that both Gram-positive and Gram-negative bacteria can survive on laboratory surfaces and cleaning equipment for up to 24 hours (Scott & Bloomfield, 1990). Care was taken to maintain sterility through use of dedicated gloves per group, use of reserved lab coats, thorough cleaning of equipment with an alcohol solution between subjects, sterilization of gavage needles, and rigorous researcher training. Despite these precautions, microbial mingling between groups may have occurred.
In addition to precautions taken to maintain sterility, care was taken to ensure that handling, treatment, husbandry, and testing of animals remained consistent across both cohorts. Therefore, no cohort effect was anticipated. However, the second cohort exhibited consistently higher levels of alcohol consumption than the first across all 14 days of two-bottle choice measurement as well as during the earlier repetitions of DID. Despite efforts to ensure consistency in methodology, there were small differences in the subjects and schedules of the two cohorts. These differences may account for part of the effect of cohort on alcohol craving. Since the subjects were all born around the same time, but were run in two consecutive cohorts, the subjects in the second cohort were older, by an average of 35.74 days compared to their counterparts in the first cohort. Previous literature suggests that there is a relationship between age and alcohol consumption levels, although the evidence is conflicting on whether consumption increases or decreases with age (Wallgren & Forsander, 1963; Walker, Roth & Ehlers, 2008). However, rats reach young adulthood at around PND 70 and are not considered geriatric until over PND 600. Thus, the subjects of the present study are all within the range of adulthood and the minor age variation is not thought to constitute a practical difference between cohorts (Sengupta, 2013).

Further, due to changes in researcher availability, two-bottle choice measurements were taken each day at 11 a.m. for the first cohort and at 4 p.m. for the second. This change was not expected to have an effect, since measurements were still being taken every 24-hours and the same measurement protocol was followed. At 11 a.m., the subjects in the first cohort were seven hours into their dark cycle. At 4 p.m., the subjects in the second cohort were just beginning their light cycle. According to an article by Walker et al. (2008), rodents that had access to alcohol only during their light cycle drank consistently higher levels than those that received alcohol
during their dark cycle. In the present study, rodents were given alcohol for 24-hour periods that contained both their light and dark cycle. However, having their attention drawn to the bottles, as they were removed and refilled, during their light cycle may have prompted higher levels of consumption in the second cohort. This timing difference may partially explain the cohort difference in alcohol craving measurements but does not account for similar differences in measurements of alcohol consumption during DID.

Another possible explanation for the cohort effect is that the first and second cohorts were housed in the same room for the duration of the experimental procedures beginning with the first cohort. Because rats are thought to experience empathy (Bartal, Decety, & Mason, 2011), witnessing the moderate distress caused during the oral gavage procedure may have caused additional stress to the subjects in the second cohort. Similarly, research in interspecies communication suggests that rats, like in the subjects in the first cohort, may be capable of relaying some degree of complex information to their conspecifics via ultrasonic vocalizations (Brudzynski, 2005). Based on these findings, the cohort differences in alcohol craving may be due, in part, to information being intentionally or unintentionally exchanged between the two cohorts during the time they spent housed in close proximity.

Despite the unexpected alcohol craving results, a supplemental assessment of anxiety was conducted, given it has been well established in previous literature that alcohol consumption is positively correlated with anxiety levels (Spanagel et al., 1995). Evidence also suggests that the gut-leakiness caused by alcohol consumption can lead to high levels of anxiety, in addition to alcohol craving (Leclercq, 2014b). This analysis was conducted in an attempt to tease apart the influences of alcohol and anxiety on one another in the present study. Unexpectedly, anxiety-like behavior and alcohol consumption were not found to be related in the present study and
results revealed no effect of original alcohol exposure or FMT treatment on levels of anxiety-like behavior.

The task used to measure anxiety-like behavior, the elevated plus-maze, utilized data from a 5-minute trial; percent time spent in the open arms of the maze was considered representative of non-anxious behavior. In previous studies, Sprague-Dawley control rats have spent approximately 20% of their time in the open arms, while rodents treated with diazepam, an anti-anxiety medication, spend between 40 and 50% of their time in the open areas of the arena (Braun, Skelton, Vorhees, & Williams, 2011; Mechan et al., 2002). In the present study, subjects in all conditions exhibited slightly lower levels of anxiety than previous controls, and higher levels than those previously treated with diazepam (approximately 30% time in open arms). These anxiety levels among all groups, when compared to previous literature, may suggest that some aspect of the experimental procedure had unexpected anxiolytic effects. According to a study by Padovan and Guimarães, prior restraint, experience with stressors, and alcohol withdrawal are all associated with alterations in elevated plus-maze results a significant time later (2000). Though no signs of alcohol withdrawal were observed during the present study, subjects in all conditions were lightly restrained and distressed during the administration of FMT via gavage, just over two weeks before being tested on the elevated plus-maze (Brown, Dinger, & Levine, 2000). Thus, the similarity in anxiety-like behavior levels across conditions may be attributable to prior experience with identical stressors.

Because the primary analyses of the present study were unable to provide evidence for the study hypotheses, a series of exploratory correlational analyses were run within the alcohol-exposed conditions in order to shed light on the relationships between the variables. These analyses indicated that while the relationship between alcohol consumption and alcohol craving
was very strong among subjects that received their own fecal matter during FMT, it was somewhat weaker and dropped below statistical significance among those that received a FMT from a healthy donor. This pattern is consistent with the proposed utility of healthy-donor FMT as a means of reducing alcohol cravings. Although not strong enough to cast doubt upon the findings of the primary analysis, this correlational discrepancy does offer hope that the proposed theoretical pathway may exist and be detectable in future studies. It also introduces the possibility that the FMT was initially restorative, but alcohol consumption during the two-bottle choice access reintroduced dysbiosis across all groups.

At several points throughout this study, fecal samples from all animals were collected and stored for future analysis. These samples were collected prior to experimentation, after the second repetition of DID, immediately prior to FMT treatment, and 24 hours after FMT administration. Future analysis of these samples may provide insight into how the gastrointestinal microbiota of the subjects changed over the course of the study and provide key information regarding whether the alcohol treatment sufficiently disrupted the microbiota, the donor animals were truly healthy, and the healthy-FMT treatment led to any behaviorally imperceptible improvement in the microbiota of recipients.

Additionally, at the time of sacrifice, brain tissue samples were collected from all subjects and may reveal more detail about the impact of alcohol consumption and FMT on rodent neurobiology. Future studies should endeavor to reinforce the relationship between gut leakiness and alcohol craving, establish and validate a standardized FMT procedure for rodents, expand the scope of inquiry to encompass other gastrointestinal-based interventions, expose the specific bacterial ecotypes impacted by alcohol consumption, and investigate the proposed pathway in humans or other mammalian species (Leclercq et al., 2014a).
Though understanding the underlying biology may reveal key information, the clinical value of this line of research lies primarily in the potential to alter cognitive and behavioral patterns in individuals who experience reduced quality of life due to alcohol cravings. The theoretical foundation that a healthy gastrointestinal microbiota serves an important role in alcohol consumption remains well-founded in previous psychological, microbiological, and gastroenterological literature. Though not independently groundbreaking, the present study provided exposure for this relatively novel line of research and may inspire future investigations, all with promising potential implications.
References


FMT: IMPLICATIONS IN ALCOHOL USE


