Fecal Concentrations of Short and Medium Chain Fatty Acids are Influenced by Postnatal Day, Antibiotic Exposure, and Cocaine Intake in Adolescent and Adult Male Rats.

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Fecal Concentrations of Short and Medium Chain Fatty Acids are Influenced by Postnatal Day, Antibiotic Exposure, and Cocaine Intake in Adolescent and Adult Male Rats.

by

Elizabeth Sambor

Under the Direction of Kyle J. Frantz, PhD

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science

in the College of Arts and Sciences

Georgia State University

2022
ABSTRACT

Cocaine addiction is a public health crisis without lasting treatments to prevent relapse, and the adolescent stage of development is associated with risky behavior, such as drug use. The gut-brain axis may be a new target for addiction treatments. Short and medium chain fatty acids (SMCFA) are produced by bacteria in the gut and communicate to the brain, thereby influencing drug reinforcement. This study hypothesized that SMCFA concentrations are reduced by antibiotic-induced gut bacterial depletion and altered by cocaine self-administration in adolescent and adult male Wistar rats. A new method of capillary electrophoresis coupled with indirect photometric detection (CE-IPD) was utilized to estimate SMCFA concentrations in fecal samples from ABX- and/or cocaine-experienced rats. Results suggest that some SMCFAs decline with bacterial depletion in adults, are influenced by cocaine in adolescents, and modestly increase over adolescence in treatment-naïve rats. SMCFA should be investigated as possible routes for gut-brain communication in addiction.

INDEX WORDS: Short chain fatty acid, Medium chain fatty acid, Antibiotic, Cocaine, Adolescent, Gut-brain axis
Fecal Concentrations of Short and Medium Chain Fatty Acids are Influenced by Postnatal Day, Antibiotic Exposure and Cocaine Intake in Adolescent and Adult Male Rats.

by

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August 2022
I dedicate this thesis to my husband, Adam Sambor and daughter, Charlie Sambor.

Adam, this would not have been possible without your support. You are my best friend, and I am so thankful for all the little things you do for me and Charlie so that I can work on this thesis. Without you, having a new baby and going to graduate school would not have been possible. From middle of the night feeds, doctors’ appointments, all the fun day care illnesses, you have been there all the way. You are my rock, and I am so thankful to have such an amazing partner.

Charlie, you were my inspiration to drive on and finish this. I thank you for being the light to guide me to be the best version of myself for you. I want to set an example that you can be proud of. It is important to me that you see your mother working toward a career that she loves by going to school, working hard, and succeeding.

Adam & Charlie, I love you both with all that I am.
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LIST OF ABBREVIATIONS

AA      Acetic acid
BA      Butyric acid
ABX     Antibiotics
CC      Calibration curve
CE      Capillary electrophoresis
FA      Fatty acid
GI      Gastrointestinal
HDAC    Histone deacetylase
HXA     Hexanoic acid
i.v.    intravenous
IBA     Isobutyric acid
IPD     Indirect photo detection
IVA     Isovaleric acid
PA      Propanoic acid
SCFA    short chain fatty acid
SMCFA   Short and medium chain fatty acid
SUD     Substance use disorder
TFA     Trifluoracetic acid
UV      Ultraviolet
VA      Valeric acid
1 INTRODUCTION

1.1 Substance Use Disorder

Substance use disorder (SUD) is the condition by which repeated use of an addictive substance causes physical and cognitive impairments that intervene with normal daily living (DSM-5 2013). According to the 2020 National Survey on Drug Use and Health, SUD affects over 40.3 million American citizens (NSDUH, et al. 2021). Among the most addictive substances, cocaine is a psychomotor stimulant that can lead to SUD, creating a public health crisis that affects over 5 million people in the United States (NSDUH, et al. 2021). Currently, there is no lasting treatment for cocaine addiction that effectively prevents relapse (Fischer et al., 2015). Novel approaches in investigating treatment options for those suffering from cocaine addiction are therefore necessary. Recent interest in the dramatic influence of the gut on the brain has suggested that the gut-brain axis may be a good target for new approaches to addiction treatment (Cryan et al., 2019; Meckel & Kiraly, 2019; Russell et al., 2021; Salavrakos et al., 2021; Simpson et al., 2021).

1.2 Gut-Brain Axis

The gut-brain axis is a bi-directional communication system between the gut and the brain. The gut is inhabited by trillions of bacteria and various other microbes such as fungi and viral particles (Cryan et al., 2019; Sender et al., 2016). Combined, these microbes make up the gut microbiome, which exerts considerable influence over many physiological processes within the host organism (Cryan et al., 2019). Disruptions to normal gut bacterial populations have been associated with numerous neurological disorders including (but not limited to) Alzheimer’s Disease, Parkinson’s Disease, anxiety, depression, autism spectrum disorder, attention deficit/hyperactivity disorder, and SUD (Fung, 2020; Gkougka et al., 2022; Perez-Pardo et al.,
Considering SUD, experience with cocaine abuse in particular is associated with altered gut bacterial populations in humans (Volpe et al. 2014). Research with rodent models also demonstrates that psychomotor stimulants alter the gut bacteria (Chivero et al., 2019; Ning et al., 2017; Scorza et al., 2019), and further demonstrates that manipulations to bacterial populations alter drug-associated behavior (Kiraly et al., 2016; Scorza et al., 2019; Suess, 2020). For example, Kiraly et al. demonstrated that antibiotic-induced gut bacterial depletion can lead to increased cocaine reward behavior (Kiraly et al., 2016). In our own lab, antibiotic-induced gut bacterial depletion did not affect intravenous (i.v.) cocaine self-administration, but adult male antibiotic-treated rats showed lower bacterial load in fecal samples and higher levels of cue-induced reinstatement of cocaine-associated lever-pressing after 30 days of forced abstinence, compared with water-treated controls (Suess, 2020). Combined, these studies implicate a connection between gut microbiota and drug-seeking behavior. However, there is limited research in this area and results vary, perhaps in part due to the diversity of experimental parameters such as which antibiotics are administered, the timing of antibiotic exposure (i.e., duration of exposure and whether antibiotics are given during or prior to drug exposure), method of drug delivery (i.e., intravenous, intraperitoneal, inhaled, self-administered via lever press, or passively exposed in conditioned place preference), animal model (rats or mice), etc. Therefore, the underlying mechanisms as to how the gut and brain interact with drug associated behavior has yet to be identified, but a mechanism of interest is short and medium chain fatty acids (SMCFA) synthesized by gut bacteria. This led our lab to investigate SMCFAs in fecal matter from subjects in our previous behavioral experiments.
1.3 SMCFAs Signal to the Brain

Short chain fatty acids (SCFAs) are the products of gut bacterial fermentation from dietary fibers and signal to the brain to carry out numerous biological roles (Dalile et al., 2019). For example, SCFAs influence gene transcription through the inhibition of histone deacetylases (HDACs) (Fattahi et al., 2020), and they alter immune responses by regulating immune cells such as neutrophils and macrophages (Corrêa-Oliveira et al., 2016). Locally in the gut, SCFAs influence gastrointestinal (GI) motility, maintain the GI barrier (Shaidullov et al., 2021), and exert anti-carcinogenic and anti-inflammatory properties (Carretta et al., 2021; Fattahi et al., 2020). Therefore, disruptions to SCFA concentrations will lead to disruptions in various physiological processes.

Three fatty acids are the most abundant as byproducts of bacterial fermentation in the mammalian gut: acetate, butyrate, and propionate (Macfarlane & Macfarlane, 2012). The genera of bacteria, *Bacteroides*, *Bifidobacteria*, and *Eubacteria* are among the main producers of acetate, while *Bacteroides* produce propionate, and *Eubacteria* produce butyrate (Macfarlane & Macfarlane, 2012). Additional SCFAs are formate and valerate, along with isomeric acids, known as branched chain fatty acids, that include methyl groups in their carbon chains (isobutyrate, isovalerate, and 2-methylbutanoate). These carboxylic acids vary in carbon chain length from 1-5. Medium chain fatty acids include caproate, caprylate, caprate, and laurate with chain lengths from 6-10. Less is known about medium chain fatty acids and their processes in the mammalian gut. The present study investigated caproate, also known as hexanoic acid.

With regard to psychostimulant-related behavior, Ning and colleagues, reported that methamphetamine alters gut bacterial composition, resulting in the observation that low levels of propionate are associated with methamphetamine conditioned place preference in rats (Ning et
Kiraly et al. demonstrated that exogenous administration of short chain fatty acids (acetate, butyrate, and propionate) reduces cocaine conditioned place preference, thereby reversing the effects caused by antibiotics mentioned above (Kiraly et al., 2016). The present study used fecal matter from male rats with antibiotic treatment and/or i.v. cocaine self-administration and cue-induced reinstatement experience from Suess et al. (2020) to explore gut levels of SMCFA concentrations in the context of antibiotic-induced depletion of gut bacteria.

1.4 Adolescents vs. Adults: Drug Abuse and Gut Microbiota

Adolescence is a developmental phase of increased risk-taking behavior, which can include illicit drug use, in part due to the relatively delayed maturation of the prefrontal cortex (Tervo-Clemmens et al., 2020). Early initiation of drug abuse can lead to dramatic long-term effects such as subsequent SUD along with other mental health comorbidities (Anthony & Petronis, 1995). Psychomotor stimulant abuse among adolescents, specifically cocaine, has been linked to disruptions to the normal development of the brain (Kuhn et al., 2013; Squeglia et al., 2009).

Thus, the present research also considers adolescent male rats and the potential impact of antibiotics and/or cocaine self-administration on SMCFA concentrations. In rats, cocaine intake during adolescence can lead to heightened levels of stress-induced reinstatement of drug-seeking behavior (Wong & Marinelli, 2016), but our own prior work shows numerous examples of resilience among adolescents compared with adults with regard to reinstatement of drug-seeking behavior (Doherty et al. 2013; 59 Doherty and Frantz 2013; Li and Frantz 2009; (Suess, 2020))

Furthermore, adolescent male Wistar rats demonstrate a gut resiliency to antibiotics, such that they recover faster from antibiotic-induced gut bacterial depletion and fail to show the heightened cue-induced reinstatement of cocaine-seeking after abstinence that was demonstrated by their adult counterparts (Suess, 2020). This dichotomy between adolescents vs. adults could be
due to the developing gut microbiota of adolescents, which is distinct from an adult’s (Agans et al., 2011). Research in adolescent gut development alongside the impact of cocaine is even more sparse and therefore a deeper examination of these components of the gut from a developmental viewpoint is warranted. Our lab sought to take preliminary steps better understand the disparity between adolescent and adult gut composition and the impact of antibiotic exposure and/or cocaine self-administration.

1.5 Gap in Current Knowledge: SMCFA Development, Antibiotics, and Cocaine

Adolescent development of the gut microbiota remains an overlooked area of scientific study. Even less explored is the development of SMCFA content in the gut. The current study hypothesized that there are developmental changes occurring in the gut during adolescence that can be observed through fecal SMCFA concentrations.

While our prior work shows that gut bacterial depletion with antibiotic administration is followed by a ‘rebound’ in bacterial load among adolescents but not adult male rats (Suess, 2020), we have yet to investigate the impact of antibiotics on SMCFAs in adolescent and adult rats. This led to the second of our present hypotheses; fecal concentrations of SMCFAs will mirror the bacterial abundance depletion induced by antibiotics in a prior experiment, such that the magnitude and/or duration of antibiotic-associated decline in SMCFAs will be less robust in adolescents than adults.

As summarized above, previous research has demonstrated that the drugs of abuse, such as cocaine and methamphetamine are associated with altered gut bacterial populations in both human participants and animal models. Additional studies show that cocaine has the ability to alter specific gut bacterial species that are known precursors to SMCFAs such as
Ruminococcaceae (Chivero et al., 2019; Scorza et al., 2019). This led us to our third hypothesis that SMCFAs will be altered with cocaine self-administration.

Lastly, we investigated the impact of both antibiotics and cocaine on fecal SMCFA concentrations with the fourth hypothesis that this double ‘insult’ will further alter SMCFA concentrations more than antibiotics or cocaine self-administration alone. Together these analyses will comprise a complete view of the impact of development, antibiotic intake, and cocaine self-administration on SMCFA concentrations in the gut.

2 MATERIALS AND METHODS

2.1 Subjects and Behavioral Experimentation

Fecal pellets from male Wistar rats were collected as a part of a prior behavioral experiment in our lab and stored at -80°C (Suess, 2020). Briefly, adolescent and adult rats (Charles River Laboratories, Inc, Raleigh, NC, USA) arrived at Georgia State University’s animal housing facility (postnatal day 22 for adolescents and 70-74 for adults). Animals habituated to vivarium conditions for at least 3 days prior to any procedures, were pair housed (2-3 per cage) in humidity and temperature-controlled cages (Optirat Gen II by Animal Care Systems; Centennial, CO) on a reverse light cycle (12:12 hr, lights off at 07:00 hr) and had ad libitum access to food and water. Diet consisted of Lab Diet 5001 (Standard Rodent Feed in a pellet form). All behavioral experimentation was conducted during the dark phase. All procedures were approved by the Georgia State University Institutional Animal Care and Use Committee.

Animals underwent catheterization surgery to the right jugular vein in order to receive cocaine infusions (Suess, 2020). After 3 days of surgical recovery, animals were placed in one of
eight experimental groups (Table 1), counterbalanced by body mass. Animals in antibiotic treatment conditions received an antibiotic ‘cocktail’ (ABX; Bacitracin 0.5 mg/ml, Neomycin 2mg/ml, and Ampicillin 1mg/ml) in their drinking water and simultaneously started their first day of white noise training (WNT). ABX was present in drinking water throughout cocaine self-administration but was removed at the start of a 30-day forced abstinence period. An experimental timeline is included in Figure 1. The protocol for antibiotic preparation and administration was adapted from prior reports (Kanhere et al., 2018; Kiraly et al., 2016). Estimated antibiotic intake was measured by the mass of the water reservoir every 2 days and then divided by the number of animals housed in the cage (2-3 per cage) (Figure 10).

Following the surgical recovery; animals were placed in operant conditioning chambers (Med Associates, Inc., St. Albans, VT, USA), and acquired lever pressing behavior via WNT for 4 days, such that reinforced lever-presses in daily two-hr sessions resulted in removal of a white noise stimulus on a fixed-ratio 1 schedule in a negative reinforcement procedure. After 4 days of WNT, two ‘loud’ cocaine-self administration sessions were conducted, in which lever-presses resulted in presentation of an i.v. cocaine infusion (0.36 mg/kg/infusion) coupled with removal of the white noise stimulus. Quiet administration (QA) conditions ensued for 10 sessions over 10 days with a drug paired cue (Figure 1 & 11). Patency was confirmed by way of i.v. infusion of 1% methohexital sodium and measured by complete loss of muscle tone within 5 s of administration. Catheter patency was tested prior to cocaine self-administration as well as 24 hr after the last self-administration session. Subjects that failed any patency test were eliminated from the study.

To control for the effects of ABX intake, control subjects had only H2O in their water reservoirs. To control for cocaine experience, some rats either underwent i.v. self-administration
procedures with saline instead of cocaine in the syringes, or they did not experience any chamber exposure at all.

After the self-administration phase, animals entered a 30-day forced abstinence period during which they remained in their home cages with neither ABX nor access to cocaine self-administration. Following the abstinence, animals underwent extinction testing during which the animals were placed back into the operant chambers for 5 consecutive 1-hr sessions with no drug cues or cocaine available. Immediately afterwards, animals underwent a single 1-hr reinstatement test, in which drug-paired cues were present, but no syringe was attached, and no cocaine was administered. Subjects were then sacrificed for organ harvesting for procedures outside the scope of this report.

<table>
<thead>
<tr>
<th>Peri-adolescent</th>
<th>Adult</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O/no cocaine</td>
<td>H₂O/no cocaine</td>
</tr>
<tr>
<td>ABX/no cocaine</td>
<td>ABX/no cocaine</td>
</tr>
<tr>
<td>H₂O/cocaine</td>
<td>H₂O/cocaine</td>
</tr>
<tr>
<td>ABX/cocaine</td>
<td>ABX/cocaine</td>
</tr>
</tbody>
</table>

*Table 1: Experimental Groups for Behavioral Testing*
2.2 Materials

Fecal samples were collected throughout the behavioral experimentation described above. Rats were held by the base of the tail and the back paws were gently lifted up and down while the front paws remained on a solid surface until a fresh fecal pellet was released. When animals were in operant chambers, fecal pellets were collected from the sterile tray under the animal. Samples were placed on ice in a 150 µL Eppendorf tube then moved to a -80 °C freezer for storage.

For the present analyses, fecal pellets were selected at 3 timepoints from each individual subject in each experimental condition. The number of samples per timepoint is listed in Table 2. To enable repeated measures analyses, each timepoint and experimental condition followed the same set of animals. For example, rat 977 was in the adult ABX/no cocaine treatment group and fecal samples from this specific animal were selected at each of the 3 timepoints listed.
Timepoint 1 was after arrival to university facilities, but prior to catheterization surgery, ABX, and/or cocaine exposure. Timepoint 2 was during cocaine (or saline) self-administration after 11-14 days of ABX treatment and/or 5-9 days of cocaine intake. Timepoint 3 was after the 30-day forced abstinence period on the day of extinction and reinstatement testing, which was immediately followed by sacrifice. See fecal collection timeline below (Figure 2).

Table 2: Number of Samples per Timeline and Postnatal (PND) Range

<table>
<thead>
<tr>
<th>Age group</th>
<th>Experimental group</th>
<th>Samples per timepoint</th>
<th>Timepoint 1 PND range</th>
<th>Timepoint 2 PND range</th>
<th>Timepoint 3 PND range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peri-adolescent</td>
<td>H₂O/no cocaine</td>
<td>7</td>
<td>24-26</td>
<td>41-51</td>
<td>68-76</td>
</tr>
<tr>
<td>Peri-adolescent</td>
<td>ABX/no cocaine</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peri-adolescent</td>
<td>H₂O/cocaine</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peri-adolescent</td>
<td>ABX/cocaine</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adult</td>
<td>H₂O/no cocaine</td>
<td>5</td>
<td>72-79</td>
<td>90-94</td>
<td>121-125</td>
</tr>
<tr>
<td>Adult</td>
<td>ABX/no cocaine</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adult</td>
<td>H₂O/cocaine</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adult</td>
<td>ABX/cocaine</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 2: Experimental Timeline for Fecal Analysis
2.3 Fecal Sample Desiccation Timeline Experiment

A preliminary study was conducted to determine the optimal duration of fecal pellet desiccation for SMCFA analysis. A single fecal sample from an adult H2O/no cocaine animal was divided into 6 subsamples of approximately 0.12 g each and placed in the desiccator. Every 2 hr for 12 hr, a subsample was taken out of the desiccator, re-weighed, and further prepared for SMCFA analysis (described below). This desiccation timeline revealed that a plateau of water loss occurs between 6 – 8 hr (Figure 3) and that the concentrations. The analysis also revealed that concentrations of propionate, butyrate, and acetate (in µmol/g fecal matter) showed significant changes between the 6 and 8 hr desiccation timepoints. No deterioration of sample was observed in this study. Thus, a desiccation of 8 hr was deemed optimal for analysis.

Table 3: Fecal Concentrations of SMCFA Observed in Desiccation Timeline Experiment

<table>
<thead>
<tr>
<th></th>
<th>HxA</th>
<th>VA</th>
<th>IVA</th>
<th>BA</th>
<th>IBA</th>
<th>PA</th>
<th>AA</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1 (2hr)</td>
<td>1.3±0.1</td>
<td>4.0±0.2</td>
<td>0.84±0.03</td>
<td>5.7±0.4</td>
<td>0.75±0.04</td>
<td>7.2±0.5</td>
<td>53±22</td>
</tr>
<tr>
<td>S2 (4hr)</td>
<td>1.48±0.03</td>
<td>6.2±0.2</td>
<td>1.10±0.09</td>
<td>7.3±0.1</td>
<td>1.0±0.03</td>
<td>9.8±0.1</td>
<td>74.7±0.1</td>
</tr>
<tr>
<td>S3 (6hr)</td>
<td>2.05±0.04</td>
<td>7.9±0.2</td>
<td>1.32±0.05</td>
<td>8.1±0.4</td>
<td>1.22±0.04</td>
<td>10.1±0.2</td>
<td>60.3±0.9</td>
</tr>
<tr>
<td>S4 (8hr)</td>
<td>3.9±0.3</td>
<td>14.0±0.8</td>
<td>2.4±0.2</td>
<td>16±0.4</td>
<td>2.2±0.2</td>
<td>19±0.1</td>
<td>130±25</td>
</tr>
<tr>
<td>S5 (10hr)</td>
<td>3.3±0.3</td>
<td>12.7±0.6</td>
<td>1.95±0.01</td>
<td>13.7±0.4</td>
<td>1.96±0.08</td>
<td>18.3±0.3</td>
<td>136±8</td>
</tr>
<tr>
<td>S6 (12hr)</td>
<td>3.1±0.2</td>
<td>12.1±0.2</td>
<td>2.4±0.2</td>
<td>14±0.2</td>
<td>2.4±0.2</td>
<td>18±0.1</td>
<td>138±10</td>
</tr>
</tbody>
</table>

2.4 Fecal Sample Preparation for SMCFA Analysis

After thawing, approximately 0.12 g of fecal sample was weighed, added to an Eppendorf tube, and placed in a desiccator with 2-5 mm blue silica gel beads for 8 hr. Fecal samples were then removed from the desiccator and weighed again to record final mass of analyzed sample.
Then 700 µL of deionized (DI) water was added to each sample tube, and vortexed for at least 3 min or until a homogenized solution was obtained. Each tube was then centrifuged at 4800 g (14.8x1000 rpm) at 4°C for 20 min. Next, 500 µL of the supernatant was transferred to a centrifuge filter tube and spun for 20 min at 4800 g (14.8x1000 rpm) at 4°C, with this step repeated to filter any solid fecal matter that might still be present in the collected supernatant. The filtered supernatant was then aliquoted into 3 separate Eppendorf tubes of approximately 150 µL each and immediately stored in -80°C freezer until further analysis.

2.5 Capillary Electrophoresis Coupled with Indirect Photometric Detection (CE-IPD)

As detailed in (Pham et al., 2021), fecal SMCFAs can be separated detected, and quantitated via Agilent 7100 Capillary Electrophoresis (CE) coupled with UV detection at 30kV using a UV-absorbing buffer (100 mM boric acid at pH 6.5), alongside a visualizing reagent (chromophore). The buffer and visualizing agent are needed because SMCFAs do not have any chromophore. Therefore, a UV-absorbing buffer [(7 mM adenosine monophosphate, (5’-AMP)] dissolved in 100 mM boric acid and 5 mM alpha cyclodextrin at pH 6.5 facilitates peak detection as negative peak of SMCFAs. However, the negative peak can be switched to positive peak by flipping the reference and detection wavelength on the software to allow accurate measurement of peak area. The capillary column used was bare fused silica with a capillary length of 64.5 cm, an average internal diameter of 75µm (356µm OD; Polymicro Technologies (Phoenix, AZ)). Each capillary column was rinsed with 1 M NaOH for 30 min, followed by triply deionized water for 15 min. Finally, the capillary was filled with 5-AMP buffer before injection and running each standard and sample. This is a newly developed and validated method of quantification that was used to determine the concentrations of several SMCFAs in rat fecal matter, including hexanoic acid (HXA), valeric acid (VA), isovaleric acid (IVA), butyric acid
(BA), isobutyric acid (IBA), propionic acid (PA), and acetic acid (AA). The quantification analysis for this study utilized the internal standard method with trifluoroacetic acid (TFA) as the internal standard to improve the precision of peak area. TFA was selected as the internal standard based on the elution time, which is after each of the target fatty acids (FA). To establish calibration curves, the following fatty acids were acquired: Acetic acid (AA, ACS reagent, ≥99.7%), propionic acid (PA, ACS reagent, ≥99.5%), butyric acid (BA, ≥99%), isobutyric acid (IBA), Valeric acid (VA, 99%), isovaleric acid (IVA, 98%) (purchased from AlfaAesar (Haverhill, MA)) and Hexanoic acid (HxA, 99%) (Acros Organics (Fair Lawn, NJ)).

During method development and sample testing, a calibration curve (CC) was used to estimate peak area and elution time for known quantities of each of the seven SMCFAs included in this analysis. The calibration curve contained five concentrations to create five peaks and was used to determine the concentration ratio of each FA, using standard mixtures (in order): deionized water (40 µL, 34 µL, 19.6 µL, 25 µL, and 9.4 µL accordingly), 2 mM of TFA for each point, AA (0.05, 1.70, 3.35, 5.00, and 6.65 mM), PA, BA, IBA, VA, IVA, and HXA (0.05, 0.20, 0.35, 5.00, and 0.65 mM). Each point also contained, 5 µL of fecal solution from an adolescent H₂O/no cocaine rat. The adolescent H₂O/no cocaine fecal sample was obtained from PND 25-27 (similar to timepoint 1) and was collected shortly after arrival to GSU and prior to any procedures and behavioral testing. This utilization an adolescent H₂O/no cocaine was determined during method development and was due to the low concentration levels of SMCFAs and therefore could be used for quantitation, allowing better matrix matching, and higher precision and accuracy of sample analysis. Each FA peak was then verified by an automated spiking method.
Once a CC curve was established, the previously prepared, aliquoted, and frozen fecal solutions were thawed and further prepped prior to loading. DI water, TFA, and fecal supernatant were added to an Eppendorf tube. The volume of DI water, TFA, and fecal supernatant was dependent on which treatment group: the mixture for adolescent and adult samples that were \( \text{H}_2\text{O/no cocaine} \) controls included 42 µL of DI water, 18 µL TFA, and 30 µL of fecal sample. For samples from ABX and/or cocaine self-administration groups, the mixture contained 30 µL DI water, 20 µL TFA, and 50 µL of fecal sample. Prior experimentation demonstrated that to optimize detection, ABX treated and/or cocaine self-administration fecal concentrations of TFA and fecal sample. To standardize calculations, these concentrations remained consistent for each timepoint.

2.6 Calculations and Statistical Analysis

SMCFA peak area ratios were calculated into \( \mu \text{mol/g} \) feces, as well as the limit of detection (LOD) and limit of quantification (LOQ) for each run were also calculated using Microsoft Excel version 2206.

To measure for the effect of time on each experimental group, SMCFA concentrations were analyzed using one-way repeated measures analyses of variance (ANOVAs) with the alpha cut-off set to 0.05 for significance. Significant one-way ANOVAs were followed up by three paired t-tests (T1 x T2, T1 xT3, T2 x T3). To analyze the different age groups for an extended view of the impact of time on fatty acid concentrations (i.e. peri-adolescent at T1 x adult at T3), unpaired T-tests were conducted. To determine the effects of treatment x time for the impact of antibiotics, impact of cocaine, and impact of antibiotics combined with cocaine, two-way mixed-measures (between-within) ANOVAs were utilized. If a two-way ANOVA resulted with a significant interaction of \( p <0.05 \), three unpaired t-tests were then preformed to identify at which
of the three time points was a main effect of treatment occurring (i.e. T1 H2O/no cocaine x T1 ABX/no cocaine, T2 H2O/no cocaine x T2 ABX/no cocaine, T3 H2O/no cocaine x T3 ABX/no cocaine). Moreover, the impact of time was pursued with follow-up one-way ANOVAs and specific t-tests as above. For the present analyses, Bonferroni’s Correction was not applied. All statistics on fecal concentrations, including arithmetic mean calculations, along with corresponding concentration graphs were generated using PRISM 7/GraphPad software (San Diego, California, USA) using the latest plugin application in Prism/GraphPad (v.9.3.1).

3 RESULTS

3.1 Limits of Detection and Limits of Quantification

Due to the number of samples that required analysis, this project was broken down into four runs of the CE-IPD machine on different dates. For each run, a limit of detection (LOD) and a limit of quantification (LOQ) was calculated. For all four of the runs, every fatty acid fell above the LOD and LOQ (Table 4).
<table>
<thead>
<tr>
<th>Run #</th>
<th>Limit of Detection</th>
<th>Limit of Quantification</th>
<th>SMCFA</th>
<th>LOD (mM) Results per Acid</th>
<th>LOQ (mM) Results per Acid</th>
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<td>1</td>
<td>0.019 - 0.026</td>
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<tr>
<td></td>
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<tr>
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<td></td>
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<td>IVA</td>
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<tr>
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<td></td>
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<tr>
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<tr>
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<td></td>
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<td>PA</td>
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3.2 Limited Change in SMCFA Concentrations Over Adolescent Development

Adolescent concentrations of propionic acid showed a significant difference over time, such that a one-way ANOVA was significant and follow-up t-tests showed a significant difference between timepoint 1 and timepoint 3 (t_{6}=2.771, p=0.0324; Figure 4.). No other acid showed any significant differences over time (Figure 4). Peri-adolescent concentrations at PND 68-76 at timepoint 3 were then compared to adult concentrations at PND 72-79 at timepoint 1 (Figure 5) using unpaired t-tests. These comparisons showed no significant difference for any of the 7 acids analyzed (Figure 5). Furthermore, SMCFA concentrations among peri-adolescents from PND 24-26 (timepoint 1) were compared to adults from PND 121-125 at timepoint 3 (Figure 6). This comparison showed significant differences between age groups for the medium chain fatty acid, hexanoic acid (t_{10}=4.841, p=0.0007; †††; Figure 6. a). There was also a significance found between age groups for short chain fatty acid, valerate (t_{10}=3.803, p=0.0035), as well as branched chain fatty acid, isobutyrate (t_{10}=3.027, p=0.0127; †; Figure 6. b & e).
Figure 4: SMCFA Concentrations of H₂O/No Cocaine  
Peri-adolescents (a.-g) Adults (h.-n)
Figure 5: Peri-adolescent H₂O/Control subjects at Timepoint 3 Compared to Adult H₂O/Control Subjects at Timepoint 1
Figure 6: Age Difference of Peri-adolescent H2O/Control Subjects at Timepoint 1 Compared to Adults H2O/Control Subjects at Timepoint 3
3.3 Antibiotic-induced Gut Bacterial Depletion is Associated with Lower SMCFA Concentrations in Adult Rats

To test the impact of ABX on SMCFA concentrations, two-way mixed measures ANOVAs were conducted, with treatment as the between-subjects variable (ABX vs. H2O) and time as the repeated measure. Among peri-adolescents, the two-way interaction was significant for 2 of the fatty acids: butyrate (F(2,16) = 7.903, p = 0.0041) and propionate (F(2,16) = 7.445, p = 0.0052; *; Figure 7. d & f). In preliminary one-way repeated measures ANOVAs on time, the adult antibiotic treated animals showed significant differences for all the acids except isovaleric: hexanoic acid (F(2,23) = 7.268, p = 0.0253), valeric acid (F(2,23) = 4.863, p = 0.0426), butyric acid (F(2,23) = 12.05, p = 0.0034), isobutyric acid (F(2,23) = 43.59, p < 0.0001), propionic acid (F(2,23) = 16.08, p = 0.001), and acetic acid (F(2,23) = 8.475, p = 0.0093). Follow-up t-tests indicated significant differences in timepoint 1 x timepoint 2, as well as timepoint 2 x timepoint 3, but not timepoint 1 x timepoint 3 for valeric acid, butyric acid, propionic acid, and acetic acid (# or ### for p < 0.001). In two-way mixed measures ANOVAs with treatment as the between-subjects variable (ABX vs. H2O) and time as the repeated measure, there was a significant interaction for valeric acid (F(2,22) = 3.453, p = 0.0496; *), isovaleric acid (F(2,22) = 4.18, p = 0.0289; *), isobutyric acid (F(2,22) = 15.63, p < 0.0001; ***), propionic acid (F(2,22) = 11.84, p = 0.0003; ***), and acetic acid (F(2,22) = 3.717, p = 0.0407; *).

Follow-up t-tests compared treatment groups at each timepoint to reveal significantly lower levels of SMCFA among ABX-treated adults compared to H2O controls for all the acids at timepoint 2. Other timepoints were not different, with one exception: an additional baseline difference was observed for isobutyric acid at timepoint 1, despite no intended differences in treatment protocols at that time.
With regard to the impact of ABX on gut bacterial populations, see supplemental figures (Figure 12) for decreases in gut bacterial abundance shown with qPCR for bacterial 16S rRNA. By the end of experimentation, decreased abundance of gut bacteria rebounded back to baseline for adolescents, but not adults. The HXA and IBA concentrations may reflect the bacterial changes with antibiotic treatment, given that they did not return to baseline either a shown in follow up t-test which revealed that timepoint 3 was significantly decreased compared to timepoint 1.
Figure 7: SMCFA Rat Fecal Concentrations of Antibiotic Treated Animals Compared to H2O Controls.
Peri-adolescents (a.-g.) Adults (h.-n.)
3.4 Cocaine Self-Administration Alters Few SMCFA Concentrations

Adolescents in the cocaine self-administration group showed a significant increase over time in valeric acid \( (F_{(2,14)}=4.907, p=0.0442) \), with timepoint 1 less than 3 and isobutyric acid \( (F_{(2,14)}=9.473, p=0.0316) \), with timepoint 2 less than 3 \( (#; \text{ Figure 8. b and e}) \). In light of results in Figure 6, which demonstrated an increase over time from timepoint 1 in adolescent controls lower than timepoint 3 in adult controls for both VA and IBA, the present elevations among cocaine-treated adolescents could reflect part of this developmental increase in acid concentrations. In two-way repeated measures ANOVAs with cocaine treatment as the between-subjects measure (cocaine vs. no cocaine controls) and time as the repeated measure, no significant interactions were observed for any fatty acids (Figure 8 a-g). Significant main effects of cocaine were recorded for hexanoic acid \( (F_{(1,10)}=108.1, p<0.0001) \) where cocaine was associated with higher levels than controls, as well as valeric acid \( (F_{(1,10)}=5.261, p=0.0447) \) and isobutyric acid \( (F_{(1,10)}=11.63, p=0.0067) \) where cocaine was associated with lower levels than controls \( \) (not marked). Adults in the cocaine self-administration group did not show any significant changes in SMCFA concentrations over time, according to one-way ANOVAs (Figure 8 h-n). In the two-way cocaine treatment x time ANOVAs, the interaction was significant for isobutryric acid \( (F_{(2,16)}=3.795, p=0.0448) \), with acid concentrations lower at each timepoint among cocaine-treated rats compared with controls \( (*; \text{ Figure 8 l}) \). Main effects of cocaine treatment were recorded for hexanoic acid \( (F_{(1,8)}=13.39, p=0.0064) \), isovaleric acid \( (F_{(1,8)}=8.814, p<0.0179) \), propionic acid \( (F_{(1,8)}=6.191, p=0.0376) \), and acetic acid \( (F_{(1,8)}=12.25, p=0.0081) \), where cocaine was associated with higher levels of hexanoic acid but lower levels of the other three acids.
Figure 8: SMCFA Concentrations of Rat Fecal Matter in Cocaine Self-administration Experimental Conditions Compared to H₂O Controls. Peri-adolescents (a.-g.) Adults (h.-n.)
3.5 Combined Insult of Antibiotic Treatment and Cocaine Self-administration

Adolescents that had both ABX exposure and cocaine self-administration experience showed a significant decrease over time for hexanoic acid, according to a t-test comparing the two available timepoints ($t_{(4)}=5.203$, $p=0.0065$, #). In two-way ANOVAs with treatment groups (ABX/cocaine, ABX/no cocaine, H2O/cocaine) x time (repeated measures), hexanoic acid again showed a significant interaction ($F_{(2,10)}=5.466$, $p=0.0249$), confirming a decrease over time in ABX/cocaine group but not others (Figure 9. a). Acetic acid also showed a significant interaction ($F_{(2,10)}=4.968$, $p=0.0318$), with significant differences between treatment groups at time 2 but not time 1. No other acid showed any significance over time, nor a significant interaction for adolescent animals (Figure 9). Adults that had both cocaine and ABX experience showed a significant decrease over time with isobutyric acid concentrations ($t_{(4)}=3.059$, $p=0.0377$; #; Figure 9. l). No other acid showed significant differences over time (Figure 9). A significant treatment x time interaction was identified in the level of propionic acid ($F_{(2,15)}=5.962$, $p=0.0124$), with unexpected treatment group differences at time 1 but not time 2 where significance was seen between ABX/cocaine x ABX/no cocaine (not shown). This is unexpected, as neither group has received any treatment at timepoint 1.
Figure 9 SMCFA Concentrations of Rat Fecal Matter in ABX/Cocaine Self-administration Experimental Conditions Compared to ABX/H₂O and H₂O/Cocaine Counterparts

Peri-adolescents (a.-g.) Adults (h.-n.)
4 DISCUSSION

4.1 Summary

This study shows effective use of a newly developed and validated CE-IPD method in analytical chemistry to consider the impact of adolescent development, antibiotic-induced gut bacterial depletion, and/or i.v. cocaine self-administration on concentrations of SMCFA in fecal matter from adolescent and adult male rats. In keeping with the new method validation (Pham et al. 2021), SMCFA concentrations were above the limits of detection and reliable across at least four analytical runs per fecal pellet. The behavioral assays of self-administration, extinction, and reinstatement reported previously are also validated, with the levels of lever-pressing and cocaine intake falling in expected ranges (Figure 12) (Suess 2020).

In terms of the present hypothesis-testing, the developmental analysis showed that only propionic acid significantly changed over three timepoints in adolescent development, providing partial support for the first hypothesis that developmental changes in bacterial populations will be reflected in changes in SMCFA concentrations in fecal samples. The antibiotic analysis demonstrated no significant decreases in any SMCFA concentrations over time for the peri-adolescent age group, but main effects of treatment (ABX vs. H₂O) on butyrate and propionate levels suggest impact of ABX to decrease some SMCFA when sample sizes are elevated and time is removed from the analysis. On the other hand, the adult age group showed significant decreases over time for 6 of the 7 fatty acids tested with one-way ANOVAs in this study and significant differences for 5 of the 7 acids when compared to adult water-controls. The adult ABX group also showed lasting effects for a few acids as well. These findings are consistent with the prior behavioral experiment performed in our lab, wherein adults showed longer gut bacterial deficits after ABX exposure than adolescents, and they provide partial support for the
second hypothesis that antibiotic-induced gut bacterial depletion is reflected in changes in SMCFA concentrations. The cocaine analysis showed significant differences over time in valeric acid and isobutyric acid for peri-adolescents but no significant changes in SMCFA concentrations among adults. Finally, the combined insult of both ABX and cocaine self-administration demonstrated some interesting findings where the effects of antibiotics may have been counteracted by cocaine experience in adult rats resulting in a less robust effect than associated with antibiotics alone.

4.2 Developmental Analysis

While propionic acid was the only fatty acid to show significant developmental differences in the present analysis of adolescents, valeric acid and isobutyric acid trended toward significance with p values of 0.052 and 0.0675, respectively. Both VA and IBA, as well as HXA, showed significantly higher levels in adults at PND 72-79 compared with adolescents at PND 24-26, suggesting a longer developmental trajectory of increasing some SMCFA. Propionic acid did not show a significant difference in this comparison of adults vs. adolescents, though, perhaps implying a possible peak that occurs around day 70 with a subsequent plateau. While not statistically significant, the individual means gradually rose for five of the seven fatty acids tested (HXA, VA, BA, IBA, PA) in adolescents, while IVA and AA rose from timepoint 1 to timepoint 2 but then fell from timepoint 2 to timepoint 3. This variation could imply that IVA and AA reach a developmental ‘peak’ earlier than the other acids. This developmental rise that plateaus around or by postnatal day 70 was also observed when comparing adolescents to adults at a similar PND, when the peri-adolescent age control group reached 68-76 PND/timepoint 3 and their adult counterparts were at 72-79 PND/timepoint 1. These comparisons showed no significant difference for any of the 7 SMCFA concentrations, perhaps indicating that the
adolescent rats had reached adult levels by the end of the present experiment. This also suggests that the experience in the behavioral experiment among adolescents did not affect SMCFA levels, given that the adults tested at approximately the same PND showed similar SMCFA concentrations before the start of their experimentation.

To further explore these developmental trends, future analysis would benefit from more experimental subjects (power of analysis) and more frequent sample collection (providing higher temporal resolution). Other considerations could include chamber exposure and amount of handling the animals were exposed to, i.e. whether the animal had a catheter and received saline, or underwent a sham surgery and had chamber exposure only, or did not have any chamber experience (housing controls). Another broad consideration is breeder handling and diet; the animals arrived at PND 22 (for adolescents) and PND 70-74 (for adults) and the developmental difference in breeder handling, GSU experimenter handling, and gut exposure to the breeder diet vs. GSU diet could affect age differences in SMCFA concentrations. Lastly, this analysis does not consider the possible differences in the development of females, and future studies would benefit from the incorporation of both genders.

For this developmental analysis and all other comparisons herein, it is critical to note that SCFAs are absorbed into the bloodstream via the hepatic portal vein for distribution and physiological impact (or excretion), as well as are found in the mucosa of the gut, whereas only a small portion of SMCFA molecules appear to be sequestered into fecal matter. Whether the concentrations in fecal matter are proportional to those distributed for physiological impact is not known. Sample concentrations in the blood from the portal vein as well as from the gut lining would provide another measure but is far more invasive. A future study may consider aligning fecal concentrations with gut and bloodstream concentrations. Moreover, any differences
observed in fecal concentrations of SMCFAs could be explained by lower rates of production (e.g. when SMCFA-producing bacterial populations decline) or by increased absorption of SCFAs into the bloodstream (via the hepatic portal). Further experimentation would also be required to differentiate these mechanisms. Finally, in terms of the new CE-IPD method, an extension of this study could seek to align the present results with a more traditional assays such as chemical separation by gas or liquid chromatography coupled with detection by mass spectrometry.

4.3 Impact of Antibiotics

Antibiotic treatment was associated with significant decreases in SMCFA concentrations that were more consistent and robust in adults, compared to adolescents. The adolescent ABX-treated rats showed no significant changes over the three timepoints, although butyric acid and propionic acid levels were lower among ABX-treated animals at timepoint 2 (during ABX-treatment) compared with water-treated controls at that timepoint. The lack of effect over time may be related to the initially low concentrations of SMCFAs in pre-adolescent rats upon arrival at PND 22. ABX-related declines in butyric and propionic acid likely reflect the impact of ABX to decrease bacterial abundance in the gut (Suess 2020), thereby eliminating bacterial producers of these acids, such as Bacteroides, the main producer of propionate, and/or Eubacteria, the main producer of butyrate (Macfarlane & Macfarlane, 2012). An extension of this study should consider analyzing the gut bacteria populations of these animals to see which bacteria were depleted by antibiotics and how this may align with the current findings.

The adult ABX-treated rats did show significant declines over time in concentrations of 6 of the 7 SMCFAs tested (all except IVA). While some acid concentrations ‘recovered’ to baseline levels by timepoint 3 (VA, BA, PA, AA), others remained lower (HXA and IBA). This
finding both complements and contradicts the prior experiment in this lab showing that bacterial abundance in these ABX adults did not recover to baseline by the time of extinction and reinstatement testing; HXA and IBA reflect this timeline, but the other five SMCFAs investigated here did return to baseline (Figure 12). It is possible that the fatty acid results provide clues about which bacterial populations recover to normal levels first, vs. those that remain compromised as bacterial flora recover from ABX-insult. One noteworthy limitation of this phase of the experiment was the potential for ABX intake to vary from subject to subject; the ABX solution intake was measured every other day, but for each cage of two-three rats. Future studies could consider food intake as well, examining for potential differences in overall physiology that contribute to results like the present outcomes. Our previous study did report daily body mass (Figure 16), which showed no significant differences across treatment groups, implying that overall energy balance was stable. However antibiotic and/or cocaine self-administration could potentially interrupt cycles of food consumption, etc. that exert secondary influence on production or utilization of SMCFA.

4.4 Impact of Cocaine Self-administration

Unlike the impact of ABX treatment alone, cocaine self-administration alone was associated with more robust effects in adolescents than adults. Cocaine experience was associated with increases in VA and IBA over time for the adolescent age group, perhaps implying that cocaine interrupts the normal developmental trajectory of fatty acid production and/or excretion. This could be related to the impact of cocaine on specific profiles of the bacterial populations in the gut, such that those producing VA and IBA are relatively more abundant during cocaine self-administration in rats of this age group. The adult cocaine-experienced group showed no significant changes in fatty acid concentrations over time, showing
that although cocaine shifts the overall gut bacterial profiles in adults (Figure 14), this drug does not interfere with the overall SMCFA production, at least not as seen in fecal concentrations.

### 4.5 Combined Insult of Antibiotic Treatment and Cocaine Self-administration

The combined treatments of ABX and cocaine self-administration resulted in some unexpected findings. First, given limitations in capacity to run samples for the CE-IPD analysis, we chose only two timepoints (1 and 2) for comparison to test the hypothesis that ABX and cocaine-combined will exert more robust effects on SMCFA concentrations. For both HXA and AA, significantly greater decreases were observed among ABX-cocaine-experienced treatment groups, when compared to ABX-alone or cocaine self-administration alone.

The adult group showed a significant decrease in IBA over time, and PA showed a significant interaction to both ABX alone or cocaine self-administration alone animals. Given that this analysis included only two time points, it was possible to observe the impact of cocaine and ABX compared to baseline, which was not as dramatic as antibiotics alone. This observation indicates the possibility that cocaine may have a stronger impact on the gut’s than antibiotics.

The fact that these results are less robust in adolescents than adults may contribute to our understanding of the mechanisms mediating the less robust effect of ABX-treatment on reinstatement of cocaine-seeking after abstinence, where we previously observed that adolescents did not show ABX-associated elevations in reinstatement responding, but adults did. In the absence of significant declines in most of these fatty acids, adolescent subjects may have maintained normal levels of cocaine-related reinforcement and enduring neurological changes that underlie reinstatement, whereas they were perhaps exacerbated by the loss of these fatty acids in adults. There is no information on adult recovery.
4.6 Conclusion: Interpretation of Findings

This study contributes important data on potential signaling mechanisms from the gut to the brain, with likely influence on drug reward and reinforcement. The fact that some developmental changes were observed further highlights the need for a better understanding of overall typical gut development. Adults show more robust effects than adolescents to antibiotic treatment, indicating that adolescents are more resistant to the effects of antibiotics. Adolescents therefore may have some form of gut protection and SMCFAs could play a role in this gut resiliency. The effects of cocaine may support this idea as well, in that adolescents show an increase in SMCFA concentrations with cocaine treatment. The observed increase in concentrations could indicate a protective effect, over-compensating from the cocaine insult and may reflect a potential mechanism for gut protection in adolescents. The combination of antibiotics and cocaine shows the powerful effects of cocaine on the gut outweighs the impact of antibiotics, at least as observed in fecal concentrations of SMCFAs. Put together, these findings suggest the possibility of clinical applications of SMCFAs as a potential target for treatment for drug addiction and relapse prevention.
5 SUPPLEMENTAL FIGURES

All supplemental figures (below) are adapted from Suess 2020. These figures were added with the purpose to provide a more in-depth understanding of the prior behavioral experiments performed to which the present study is an extension of.

**Figure 10: Fluid Intake**
Fluid intake was measured mass of water reservoir divided by number of animals per cage (2-3). This measurement provided an estimate of ABX intake for antibiotic treated animals.

**Figure 11: Cocaine Infusion Data**
Total cocaine infusions were measured by cocaine infusion per lever press.
Gut bacterial abundance was measured in fecal samples throughout experimentation and demonstrated depletion to bacterial abundance with antibiotic treatment that rebounded back to baseline for adolescents (blue), but not adults (red).

Figure 12: Extinction and Reinstatement Lever Press Data
Animals that self-administered cocaine showed higher levels of lever pressing during extinction and reinstatement compared to water/saline controls. Adults, but not adolescents, that received ABX and cocaine during self-administration showed higher numbers of lever pressing compared to no ABX groups.

Figure 13: Gut Bacterial Abundance
Gut bacterial abundance was measured in fecal samples throughout experimentation and demonstrated depletion to bacterial abundance with antibiotic treatment that rebounded back to baseline for adolescents (blue), but not adults (red).
Figure 14: Gut Bacterial Diversity in Adults
The gut bacterial profiles demonstrated an overall shift in diversity with cocaine self-administration (a.) and antibiotic intake (b.) compared to controls.

Figure 15: Gut Bacterial Diversity in Adolescents
Gut microbiota diversity shifts in cocaine self-administration adolescents compared to control counterparts. Antibiotic intake did not demonstrate a shift in bacterial profile.
Figure 16: Body Mass Data
Body mass was taken daily for the duration of experimentation.
REFERENCES


Suess, G. (2020). *COCAINE INTAKE AND THE GUT MICROBIOTA IN ADOLESCENT AND ADULT MALE RATS: A VICIOUS CYCLE?* (Publication Number 51) Georgia State University]. ScholarWorks@GeorgiaStateUniversity. https://scholarworks.gsu.edu/neurosci_diss/51


