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Probiotics Attenuate Cocaine-seeking Behavior in Adult but not Adolescent Male Rats

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PROBIOTICS ATTENUATE COCAINE-SEEKING BEHAVIOR IN ADULT BUT NOT ADOLESCENT MALE RATS

by

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ABSTRACT

Perturbations to the gut microbiome may boost the use of addictive substances such as cocaine. In animals, antibiotic-induced gut microbiome depletion heightens cocaine-seeking, although adolescent rats appear less sensitive than adults to these perturbations. The purpose of this study was to use adolescent vs. adult male Wistar rats to test two hypotheses: 1) probiotics will reduce cocaine-seeking that has been heightened by antibiotic treatment; and 2) probiotics administered throughout behavioral testing will reduce cocaine-taking and/or -seeking. Rats were catheterized and self-administered cocaine (0.36mg/kg i.v.), then tested for extinction and reinstatement of cocaine-seeking after abstinence. Some rats received antibiotics during self-administration followed by probiotics during abstinence, while others received probiotics throughout testing. Probiotics during abstinence attenuated antibiotic-heightened cocaine-seeking in adults but did not decrease adolescent responses. Prophylactic probiotics did not attenuate cocaine behaviors significantly. Probiotics may be useful in addiction treatment, but these results do not support their use in prevention.

INDEX WORDS: Cocaine, Gut-brain-axis, Adolescent, Reinstatement, Abstinence, Probiotics

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by

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DEDICATION

I dedicate this thesis to the special people in my life who have been greatly influential. First, my mother, Priscilla Ngwese, you have been with me every step of the way, and I cannot say thank you enough. To my grandmother, Pauline Mesape, and my recently deceased grandfather, Isaac Mesape, you both have been exemplary role models, who raised me to be the person I am today, taught me to be kind, compassionate, and driven. And my younger sister, Britney, your admiration pushes me every day to improve and better myself to earn the role model status you have bestowed upon me. All of you have encouraged me to strive to become a better scientist, and I look forward to sharing the fruits of the sacrifices you have made and continue to make for me.

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LIST OF ABBREVIATIONS

CNS	Central nervous system
GBA	Gut-brain axis
SUD	Substance use disorder
FR	Fixed ratio
i.v.	Intravenous
AAD	Antibiotic-associated diarrhea
CDAD	Clostridium difficile-associated diarrhea
IBS	Irritable bowel syndrome
SCFA	Short chain fatty acids
PND	Postnatal day
HPA axis	Hypothalamus-pituitary-adrenal axis

1 INTRODUCTION

Substance use disorder (SUD) is defined as a cluster of cognitive, behavioral, and physiological symptoms indicating that an individual continues using a pharmacological substance despite significant substance-related problems (DSM-5, 2013). SUD is the second leading cause of death and disability combined in the United States, with an 80 % increase in prevalence between 2009 and 2019 (IHME, 2020). A major issue in treating SUD is the vulnerability to relapse, which can persist even after long periods of abstinence (O'Brien, 2005). Whether heightened vulnerability to drug relapse is linked to earlier age of onset of drug intake, e.g., during adolescence, remains incompletely understood (Mooney-Leber & Gould, 2018). Recent findings reveal that the earlier teens start using substances, the greater the chances they will continue and classify themselves as addicted (NCBDDD, CDC, 2020), making research on both adolescent and adult drug use and abuse crucial to identifying effective treatments and solutions.

Adolescence is a developmental period characterized by significant brain maturation that may underlie vulnerability to stressors that can impact the brain (Breslau et al., 2017). With regard to drug reward and reinforcement, preclinical models of SUD have reported age differences in vulnerability to behavioral reinforcement by various drugs. For example, Shahbazi et al used an intravenous (i.v.) amphetamine self-administration model to show that adolescent rats acquired amphetamines faster than adults at low doses, and had a higher overall amphetamine intake than adults, regardless dose, on a fixed ratio (FR) schedule of reinforcement, over a 14-day period (Shahbazi et al., 2008). In addition, rats that acquired cocaine self-administration as adolescents reinstated their cocaine-seeking after abstinence at higher levels than adults, in a stress-induced reinstatement procedure (Wong and Martinelli, 2016). While

these studies suggest adolescent sensitivity to drugs of abuse, research from our own team suggests that some conditions are associated with the converse: adolescent resistance to some enduring effects of cocaine (Li & Frantz, 2009 & 2017) and heroin (Doherty & Frantz, 2012; Doherty et al., 2013), such as lower rates of cue-induced reinstatement after forced abstinence and lower levels of prefrontal cortical activation associated with re-exposure to drug.

Recent evidence suggests that the gut microbiome can exert great impact on behavior via the gut-brain axis (GBA) (Martin & Mayer, 2017) and a role of the gut microbiome in modulating drug reward and reinforcement is under investigation (Meckel & Kiraly, 2019). The GBA is a bidirectional communication system between the gut microbiota and the central nervous system (CNS) (Holzer & Farzi, 2014) and has been the subject of intense study in recent years. Perturbations of the gut microbiota are linked to many diseases including obesity (Abenavoli et al., 2019), irritable bowel syndrome (IBS) (Raskov et al., 2016), Alzheimer's disease (Angelucci et al., 2019; Pluta et al., 2020), Parkinson's disease (Sampson et al, 2016), autism spectrum disorder (Sgritta et al., 2019), and other neuropsychiatric conditions (Generoso et al., 2020). A high comorbidity between SUD and several neuropsychiatric diseases (Lee et al., 2011; Khokhar et al., 2018) suggests that the gut-brain axis might also be implicated in SUD. To that effect, the use of drugs such as alcohol, opioids and psychostimulants is associated with significant changes in the gut microbiota profile (Chivero et al., 2019; Meckel & Kiraly, 2019). For example, Mutlu and colleagues showed that chronic alcohol intake among adults is associated with dysbiosis in the colonic microbiome (Mutlu et al., 2019). Research with animal models shows that antibiotic-induced depletion of the gut microbiome results in an elevation of cocaine conditioned place preference (Kiraly et al., 2016). However, the conditioned place preference assay, a classical conditioning paradigm, does not provide the animal with control

over cocaine intake. Thus, the current study extends from existing research by using the intravenous (i.v.) drug self-administration, extinction, and reinstatement model, which is a more translatable model of the acute and enduring effects of cocaine use. Specifically, it tests the effects of antibiotic-induced gut bacterial depletion on cocaine-related behaviors by providing antibiotic treatment to male rats before and during i.v. cocaine self-administration, followed by a 30-day period of forced abstinence and culminating in a within-sessions extinction and cue-induced reinstatement test of cocaine-seeking. Testing both adolescent and adult rats may reveal age differences in antibiotic impact.

While gut microbiota perturbations have been shown to exacerbate behavioral deficits, treatment with probiotics can reduce these deficits (Sivamaruthi et al., 2020). Probiotics consists of live bacteria that confer beneficial health effects on the host when ingested (Butel, 2014). Probiotics are present in many dietary components and are known to attenuate the adverse effects of IBS (Raskov et al., 2016), autism spectrum disorder (Sgritta et al., 2019; Sivamaruthi et al., 2020), and cardiovascular conditions (Jin et al., 2019), to name a few instances. Yet, direct comparisons of probiotic-mediated rescue of antibiotic-induced depletion of gut microbiota have not been explored in the context of drug use or comparisons across adolescent and adult developmental stages. Thus, in the present study, we tested whether probiotic treatment during abstinence from cocaine could rescue normal cocaine-related behavior that had been disrupted by antibiotics in adolescent or adult male rats. Specifically, this study tests the hypothesis that a CereBiome probiotic preparation from Lallemand Health Solutions Inc. will reduce cocaine seeking that has been heightened by antibiotic treatment. Previous work by our lab has shown that antibiotic treatment increased cocaine seeking in adult but not adolescent rats (Suess et al., in preparation). Therefore, in the current study, we predict similar age differences.

Probiotics can confer natural health benefits in preventing disease onset and/or severity, not only as a treatment regimen but also in a prophylactic approach. Gao and colleagues showed that prophylactic probiotics reduced the incidence and symptom duration of antibiotic-associated diarrhea (AAD) and *Clostridium difficile*-associated diarrhea (CDAD) (Gao et al., 2010). Prophylactic probiotics have also been implicated in a reduction in incidences of food allergies by modulation of the gut microbiota (Aitoro et al., 2017). Probiotic pretreatment has also been shown to attenuate lipopolysaccharide-induced memory deficits in rats (Mohammadi et al., 2019). We are not aware of any other work exploring the prophylactic effects of probiotics on drug-related behaviors. Therefore, the present study also examines whether probiotics given before and during cocaine self-administration as well as during forced abstinence can reduce cocaine intake and/or subsequent extinction and reinstatement of cocaine-seeking in adults and adolescents. We hypothesize here that probiotics given throughout testing will reduce cocaine-taking and -seeking in male rats. As discussed above, adolescents have displayed resilience to cocaine seeking in the context of gut microbiome manipulations, so we predict that probiotics will reduce cocaine intake and cocaine seeking in adult but not adolescent rats.

2 MATERIALS AND METHODS

2.1 Experiment 1: Antibiotic-induced gut microbial depletion and probiotic rescue

2.1.1 Subjects

Adolescent (n = 25) and adult (n = 27) Wistar male rats (Charles River Laboratories, Inc, Raleigh, NC, USA) arrived at Georgia State University's animal housing facility at postnatal day (PND) 22 and 70-74, respectively. Animals acclimated to pair-housing in humidity and temperature-controlled ACS cages (Optirat Gen II by Animal Care Systems; Centennial, CO) on

a reverse light cycle (12 hr, lights on at 19:00 hr) for three days prior to catheter surgery. Animals were given *ad libitum* access to food in water in the home cage for the duration of the experiment, were assessed daily for general health, and weighed periodically. All procedures were conducted in adherence to the Principles of Laboratory Animal Care and the National Institute of Health Guide for the Care and Use of Laboratory Animals (8th edition, 2011) and approved by Georgia State University's Institutional Animal Care and Use Committee.

2.1.2 Surgery

Intravenous catheters were assembled as previously described (Roberts and Koob, 1982) with minor modifications including a shorter length of tubing inserted into the jugular vein for adolescents compared with adults (2 vs. 4 cm) (Shahbazi et al., 2008). Incisions were made at midscapular and ventral neck locations, the right jugular vein was isolated, and drug delivery tubing was inserted into an incision in the vein. A catheter portal and backplate were implanted subcutaneously at the midscapular location, about 5 cm from the base of the neck. Catheter patency was maintained by intravenous (i.v.) administration of Timentin (ticarillin disodium and clavulanate potassium; 100 mg/ml) and heparinized saline (100 USP units / 1ml) twice daily for three days post-operative, then daily for the rest of self-administration, using approximately 0.2 ml of each solution for adults and 0.1 ml for adolescents. A subset of randomly selected animals (n = 3 adolescents, n = 4 adults) underwent sham surgery, during which incisions were made then closed, but no catheter was implanted.

2.1.3 Administration of antibiotics and probiotics

Following 3 days of post-surgical recovery, the animals received a cocktail of antibiotics (abx) in the drinking water, including Bacitracin (0.5 mg/ml), Neomycin (2 mg/ml), and Ampicillin (1 mg/ml), as adapted from prior reports (Kanhere et al. 2018; Kiraly et al. 2016) and

based on preliminary experimentation in this laboratory. Animals received abx during the 4 days of lever-press training and throughout cocaine self-administration but were switched back to H_2O at the start of forced abstinence (see below). An experimental timeline is included in Figure 1.

Following cocaine self-administration, animals were assigned into experimental groups counterbalanced for body weight and cocaine intake: one group received a probiotic solution and the other received a placebo each day over the 30 days of forced abstinence. The probiotic formulation, CereBiome (Lallemand Health Solutions Inc., Montreal, QC, Canada; formerly known as Probio'Stick), consisted of *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175, along with malic acid and xylitol, mixed with phosphate buffered saline (PBS) to form a suspension that contained 10 $x10^9$ colony forming units (CFUs). Probiotic and placebo were prepared daily and administered to subjects at a volume of 0.5 mL at approximately 09:00 h, per previous reports (Tillmann et al., 2018) with some alterations. Briefly, subjects were lightly restrained with a cloth towel in red-light illumination. A syringe containing the preparation was presented at the animal's mouth with the tip just touching the animal. If the animal did not consume the solution after a few seconds, the tip was inserted into the animal's mouth behind the teeth. Contents were expelled over approximately 10 sec. As animals habituated over 3-10 days to the preparations and procedures, each of them transitioned to approaching the syringe tip placed at the lip of the home cages and consuming freely without being handled by an experimenter. An experimental timeline for probiotic intake is included in Figure 1.

2.1.4 Cocaine self-administration

Cocaine self-administration procedures were adapted from previous experiments (Suess et al., in revision). Animals acquired lever pressing behavior over four days via a negative

reinforcement procedure (removal of a white noise stimulus was contingent on lever-pressing) in daily 2-hr sessions in operant conditioning chambers (Med Associates, Inc., St. Albans, VT, USA), after which cocaine infusions (0.36 mg/kg/ infusion) were paired with white noise removal for two sessions, then lever-pressing resulted in cocaine infusions only for eight additional sessions conducted over ten days (with a two-day weekend recess). Only one lever was active, while presses on the inactive lever were recorded but had no scheduled consequence. Sessions were conducted under a fixed ratio (FR)-1 schedule of reinforcement with a timeout after each drug infusion (20 sec). Reinforced lever-presses also triggered a cue-light above the active lever to illuminate for 2 sec, and a house light to go off for 20 sec. Locomotor activity was assessed by crossings of photocell beams at the front and rear of the chambers. During cocaine sessions (but not the first four white noise training sessions), the catheter portal was attached to polyethylene tubing that led to a variable-speed syringe pump (Med Associates, Inc., St. Albans, VT, USA) which delivered drug solution via a stainless-steel swivel (Instech Laboratories, Inc., Plymouth Meeting, PA, USA). Sham animals were not attached to the drug delivery tubing and no cocaine was loaded in the pump.

2.1.5 Abstinence, extinction, and cue-induced reinstatement

At the conclusion of self-administration, animals entered a 30-day forced abstinence period during which they were maintained in their home cages and thus did not have access to cocaine or the operant conditioning chambers. Body weight and water intake data were collected periodically throughout abstinence.

Subsequently, animals underwent 5 consecutive 1-hr extinction in which they were placed back into the operant conditioning chambers where they had previously earned cocaine infusions, but no drug-paired cues nor drug infusions were presented. Five minutes later, animals

underwent a single 1-hr cue-induced reinstatement in which presses on the active lever yielded drug-paired cues including changes in the cue light, house light, and activation of the syringe pump, although no syringe was loaded, and cocaine was still not available. Active lever presses were recorded.

2.2 Experiment 2: Prophylactic probiotic

As above, adolescent (n = 29) and adult (n = 29) male rats arrived, acclimated to Georgia State University's animal housing facility and underwent catheterization surgery in preparation for lever-press training and i.v. cocaine self-administration. Following surgical recovery, the animals were randomly assigned to either a probiotic or placebo group and syringe-fed the corresponding formulation, as described above, throughout training, cocaine-self administration, and forced abstinence. A subset of randomly selected animals received sham surgeries (n = 3 adolescents; n = 2 adults) while others underwent no surgery at all (n = 2 per age group). All experimental procedures were conducted as described above. After reinstatement, animals were euthanized, and tissue samples (blood, brain, cecum and distal colon) were collected and frozen for future analyses not described herein.

2.3 Statistical analysis

Self-administration behavior during antibiotic treatment in Experiment 1 was analyzed via ANOVA, with drug (cocaine vs. sham) and age (adolescent vs. adult) as the independent variables and total number of infusions (or reinforced lever-presses) as the dependent measure. Independently, the effect of age on number of infusions per session was analyzed using a repeated measures ANOVA, with the sessions as the repeated measure. Similarly, results from Experiment 2 on prophylactic probiotic administration were analyzed using a univariate

ANOVA, with age and probiotic treatment as the independent variables, and number of infusions as the dependent variable. For both Experiments, total extinction responding summed over the 5 1-hr sessions and reinstatement responding during the single 1-hr session were analyzed using two-way ANOVAs (age x probiotic treatment group) on active lever presses. Targeted independent samples t-tests (two-tailed) with Bonferroni's corrections were also used to assess differences of a priori interest. Active lever presses during extinction sessions were also subjected to a repeated measures ANOVA separately within each age group to test the effects of probiotic treatment over sessions. A repeated measures ANOVA was also used to assess the effects cocaine self-administration probiotics on body mass, with days as the repeated measure. Alpha was set at 0.05 in all cases. Figures were generated using GraphPad Prism 9.1.0 (221).

3 RESULTS

3.1 Experiment 1: Antibiotic-induced gut microbial depletion and probiotic rescue

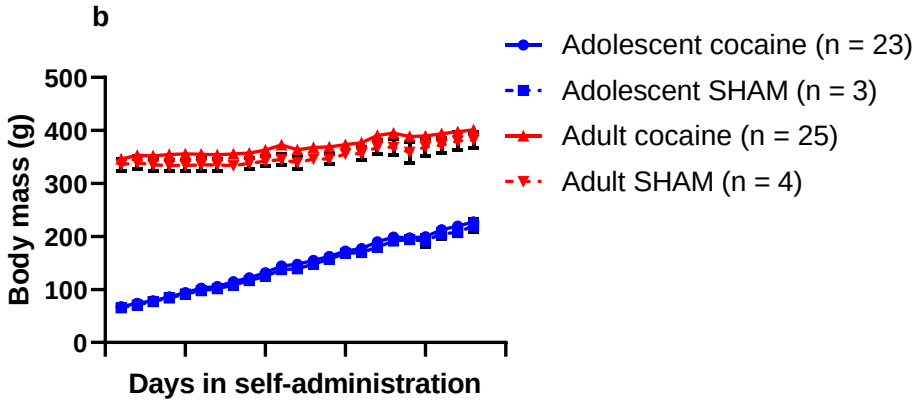
3.1.1 Body mass and fluid intake reflect normal growth and fluid consumption

Cocaine self-administration did not alter the growth rates of adolescents or adults. Specifically, two-way treatment (cocaine vs. sham) x session ANOVAs failed to reveal a main effect of treatment in adolescents ($F(3.142, 75.413) = 0.28$; $p = 0.849$) or adults ($F(4.073, 105.903) = 0.953$; $p = 0.438$) (Figure 1b). Similarly, probiotics given during forced abstinence did not alter growth in that phase of experimentation, with a two-way ANOVA failing to show a main effect of treatment in adolescents ($F(3.603, 86.472) = 0.681$; $p = 0.592$) or adults $F(3.642, 98.332) = 0.401$; $p = 0.79$) (Figure 1c). An independent samples t-test (two-tailed) showed no difference in water intake between rats that self-administered cocaine and their sham controls in both adolescents ($t(26) = 0.40$; $p = 0.692$) and adults ($t(28) = 0.146$; $p = 0.885$) (Figure 1d).

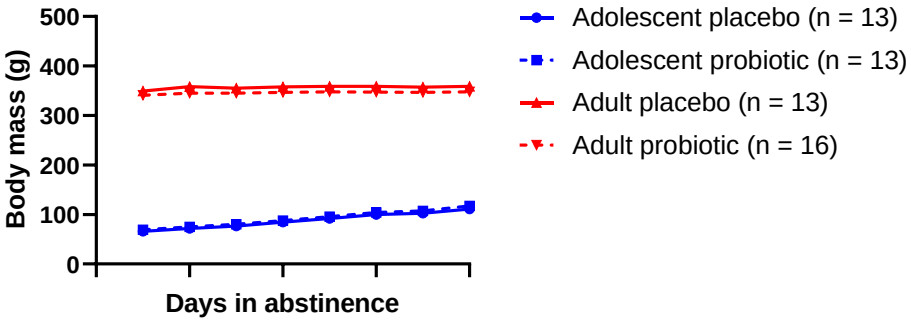
a


Cocaine self-administration
Procedure:
Animal arrival, catheterization, recovery
White noise training
Loud SA
Quiet SA
Forced abstinence
Extinction, reinstatement, & sacrifice
# of days:
7
4
2
8
30
Abx in drinking water
Probio'stick/ Placebo

b


Body mass (g)
500
400
300
200
100
0
Days in self-administration
Adolescent cocaine (n = 23)
Adolescent SHAM (n = 3)
Adult cocaine (n = 25)
Adult SHAM (n = 4)

c


Body mass (g)
500
400
300
200
100
0
Days in abstinence
Adolescent placebo (n = 13)
Adolescent probiotic (n = 13)
Adult placebo (n = 13)
Adult probiotic (n = 16)

d

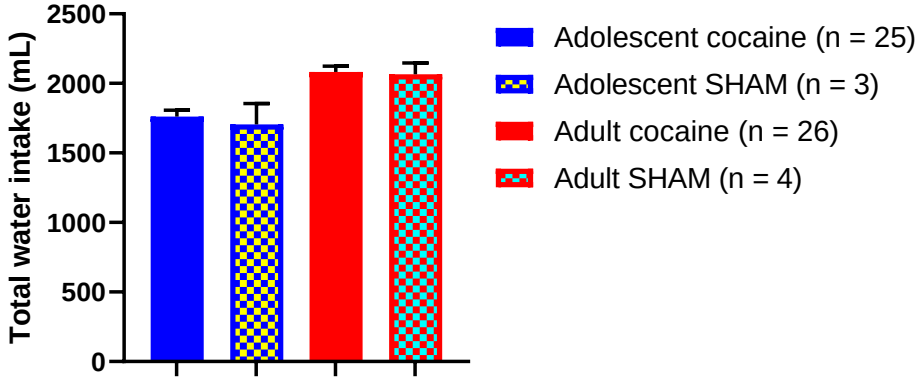

Total water intake (mL)
2500
2000
1500
1000
500
0
Adolescent cocaine (n = 25)
Adolescent SHAM (n = 3)
Adult cocaine (n = 26)
Adult SHAM (n = 4)

Figure 1: Body mass and fluid intake reflect normal growth and consumption
a. Representative experimental timeline showing the phases of experimentation and the duration of each (in days). **b**. Body mass during self-administration and **c.** forced abstinence, and **d**. water intake among rats that self-administered cocaine and age-matched controls. All error bars are ±S.E.M.

3.1.2 Adolescent and adult rats self-administer cocaine similarly

Confirming that cocaine reinforced lever pressing in the self-administration model, rats that self-administered cocaine had more lever presses than the sham rats that did not have access to cocaine ($F(4.625, 231.258) = 4.644$; $p = 0.001$). The sham rats were excluded from further data analysis.

In terms of age differences during the training and cocaine self-administration phases, repeated measures ANOVA revealed a significant effect of sessions ($F = 40.205$; $p < 0.001$), but not age ($F = 0.372$; $p = 0.545$) and no interaction between sessions and age of rats ($F = 1.212$; $p = 0.306$).

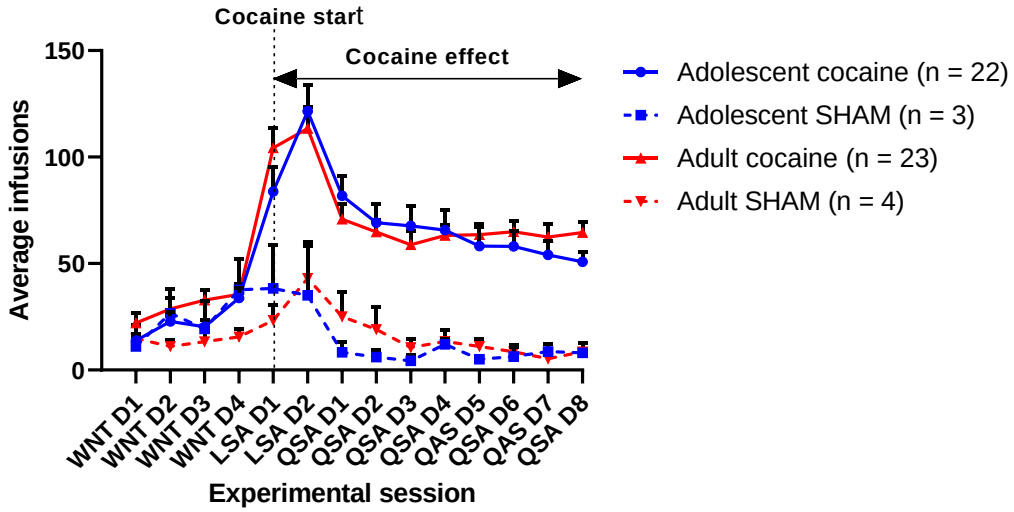


Figure 2: Cocaine influence and age difference on active lever pressing

Rats that self-administered cocaine had more active lever presses that resulted in cocaine infusions and/or environmental cue consequences than rats that did not receive cocaine. Adolescent and adult rats self-administered cocaine at similar rates. WNT D1 – D4 = white noise training days 1 to 4, LSA D1 & D2 = loud cocaine self-administration days 1 and 2, QSA D1 – D8 = quiet cocaine self-administration days 1 to 8. All error bars are ±S.E.M.

3.1.3 Probiotics attenuate cocaine-seeking behavior in adults but not adolescents

During the tests of extinction of cocaine-seeking behavior after 30 days of probiotic or placebo treatment, a two-way ANOVA (age x probiotic treatment) revealed a main effect of age on total extinction responding ($F(1,40) = 17.386$; $p = 0.000159$), but no effect of probiotic treatment ($F(1,40) = 0.98$; $p = 0.328$), and no interaction between age and probiotic treatment. Among adolescents, a repeated measures ANOVA revealed a significant effect of extinction sessions ($F(1.653, 31.41) = 20.427$; $p < 0.001$), but no interaction between extinction sessions and probiotic treatment ($F(1.653, 31.41) = 0.537$; $p = 0.557$). Similarly in adults, there was a significant effect of extinction sessions ($F(1.732, 36.366) = 60.986$; $p < 0.001$) but no interaction between extinction sessions and probiotic treatment ($F(1.732, 36.366) = 2.727$; $p = 0.086$). An independent samples t-test (two-tailed) revealed no difference in total extinction responses between the probiotic or placebo treated rats combined across age groups ($t(42) = 1.079$; $p = 0.29$) (figure 3a and 3b). However, probiotic-treated adults showed lower extinction responses during the first hour (ext 1) compared to their age-matched controls ($t(21) = 2.078$; $p = 0.050$) (figure 3a).

On reinstatement responding, a two-way ANOVA revealed a significant main effect of probiotics ($F(1,40) = 4.363$; $p = 0.043$) such that probiotic treatment was associated with lower rates of responding than placebo treatment, but no effect of age was observed ($F(1,40) = 3.104$; $p = 0.086$) and no interaction ($F(1,40) = 1.286$; $p = 0.264$). With focus on reinstatement among adults, the probiotic-treated rats showed significantly less reinstatement of cocaine-seeking than

their age-matched placebo-treated controls ($t(21) = 3.097$; $p = 0.006$) (figure 3c). With the adolescents, however, the probiotics failed to attenuate reinstatement responding ($t(19) = 0.57$; $p = 0.575$) (figure 3c). To account for the failure of some rats to extinguish their lever-pressing behavior fully over the 5 extinction sessions (which is commonly observed), we calculated a change score by subtracting the number of active lever presses in the last extinction session from those of the single reinstatement session. A two-way ANOVA revealed a significant main effect of probiotics ($F(1,40) = 11.251$; $p = 0.002$), but no effect of age ($F(1,40) = 1.048$; $p = 0.312$), and no interaction ($F(1,40) = 1.171$; $p = 0.286$). Targeted t-tests revealed that the probiotic-treated adults again showed a significantly lower change score ($t(21) = 3.644$; $p = 0.003$) than their age-matched counterparts, but no effect of probiotic treatment was observed among adolescents (figure 3d). Regarding age differences, placebo-treated adults showed significantly higher total extinction ($t(18) = -4.3$, $p < 0.001$) and reinstatement ($t(18) = -2.187$, $p = 0.042$) responses compared to their placebo-treated adolescent counterparts (figure 3a and 3b). Probiotics attenuated these differences such that the probiotic-treated adults did not differ from the probiotic-treated adolescents.

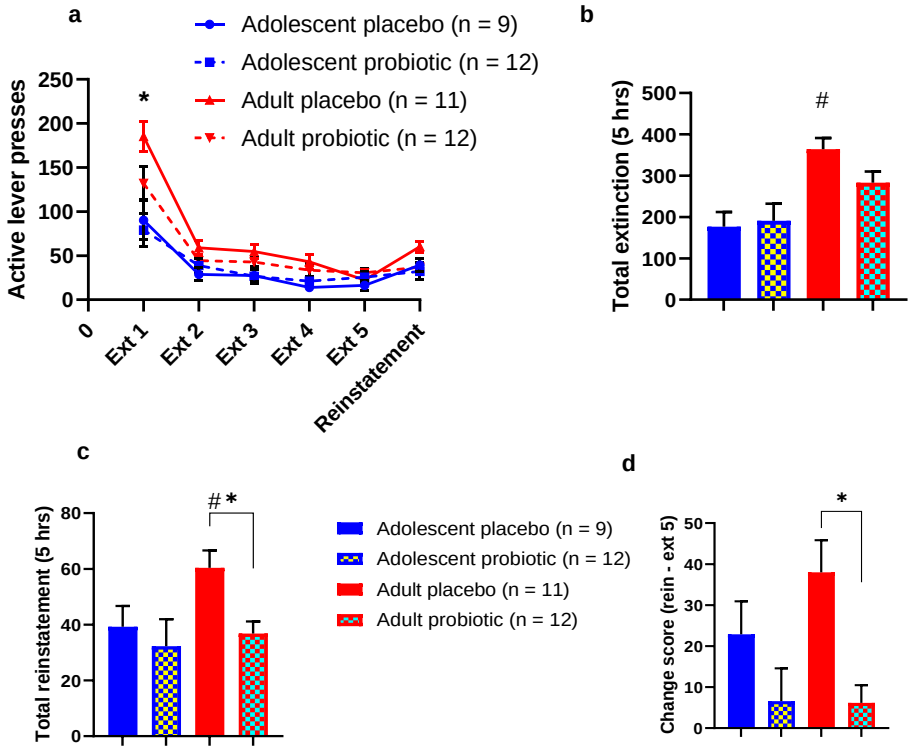


Figure 3: Probiotic effects on extinction and reinstatement of cocaine seeking
a. Probiotic-treated adults show lower extinction responding compared to their age-matched placebo controls in the first hour of extinction testing. **b.** Probiotic-treated adolescent and adult rats extinguish cocaine-seeking behavior similarly compared to their age-matched placebo controls, but placebo adults press more than placebo adolescents. **c.** Probiotics attenuate reinstatement in adult but not adolescent rats and placebo adults press more than placebo adolescents. **d.** Probiotic-treated adult rats showed a significantly reduced change score compared with their age-matched controls. * Denotes significant probiotic effects; # denotes significant age differences within placebo-treated groups (b and c) $p<0.05$ All error bars are ±S.E.M.

3.2 Experiment 2: Prophylactic probiotic

3.2.1 Daily probiotic administration did not alter normal weight gain

Probiotics and cocaine self-administration did not alter adolescent or adult body mass. A two-way repeated measures ANOVA failed to show a main effect of cocaine ($F(3.053, 76.321) = 0.247$; $p = 0.867$) or probiotic ($F(3.053, 76.321) = 1.449$; $p = 0.235$) on body mass in adolescents during self-administration (figure 4b). Probiotics during forced abstinence did not

further alter body mass (F (1.739, 46.951) = 0.141; p = 0.841) (figure 4c). Similarly, there was no effect of cocaine (F (1.407,32.359) = 0.349; p = 0.633) or probiotics (F (1.407, 32.359) = 0.34; p = 0.638) on body mass during self-administration, and probiotics during forced abstinence did not alter body mass (F (2.649, 66.236) = 1.238; p = 0.302) (figure 4c).

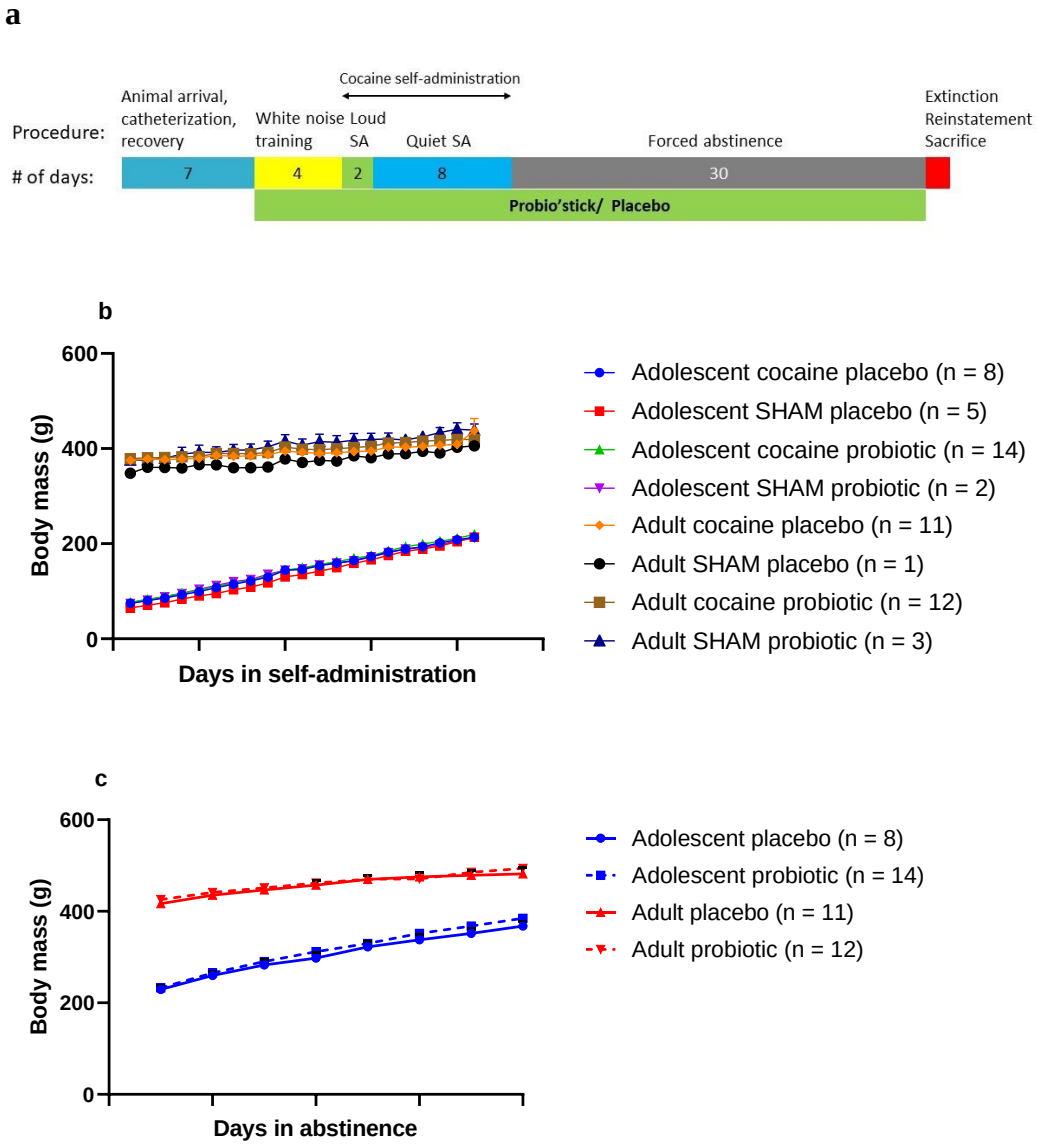


Figure 4:Effects of cocaine and probiotics on body mass

a. Diagram showing experimental timeline. **b.** Cocaine and probiotics did not alter body mass in both adolescents and adults during self-administration. **c.** Similarly, probiotics alone during abstinence from cocaine did not alter body mass among adolescent or adult rats. All error bars are ±S.E.M.

3.2.2 Adolescent and adult rats self-administer cocaine similarly, and probiotics did not alter pattern of drug intake

Rats that self-administered cocaine had more lever presses than those without access to cocaine regardless of probiotic (or placebo) treatment ($F(5.003, 270.166) = 8.024$; $p < 0.001$). The sham rats were excluded from further data analysis. Furthermore, a repeated measures ANOVA revealed that probiotics did not influence cocaine self-administration in adolescents ($F(13, 260) = 0.452$; $p = 0.948$) or adults ($F(13, 273) = 1.492$; $p = 0.12$). There was no effect of age on cocaine self-administration ($F(13, 702) = 0.891$; $p = 0.562$).

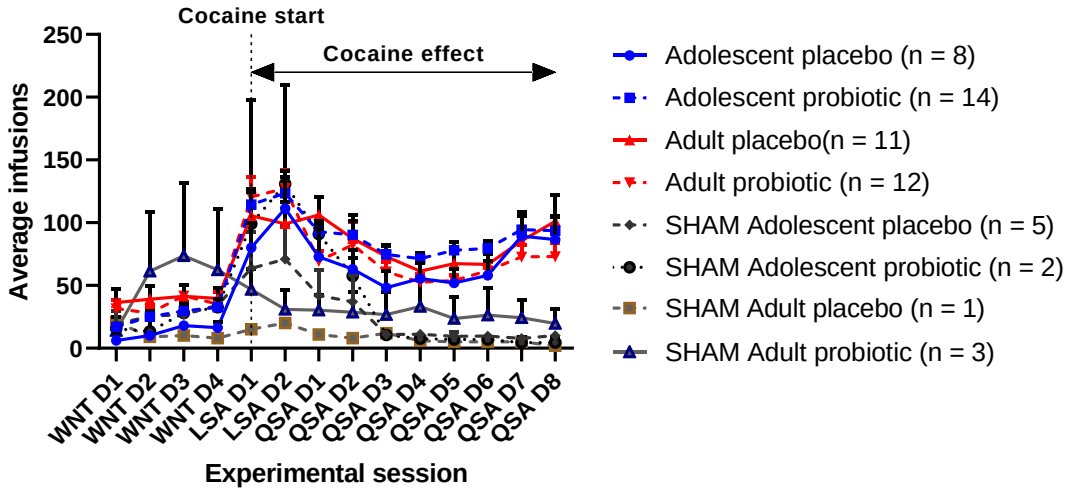


Figure 5: Effects of cocaine and probiotics on cocaine self-administration

Rats that self-administered cocaine showed increased reinforced lever-pressing compared with their age-matched sham controls. Adolescent and adult rats self-administered cocaine similarly. Probiotics did not significantly alter the number of infusions per session in either age group. WNT D1 – D4 = white noise training days 1 to 4, LSA D1 & D2 = loud cocaine self-administration days 1 and 2, QSA D1 – D8 = quiet cocaine self-administration days 1 to 8. All error bars are ±S.E.M.

3.2.3 Probiotics did not attenuate cocaine-seeking significantly in adolescent or adult rats

Following 30 days of forced abstinence from cocaine, a two-way ANOVA on total extinction responding revealed a significant main effect of age ($F(1,41) = 15.797$; $p < 0.001$) and an interaction between age and probiotic treatment ($F(1,41) = 4.943$; $p = 0.032$). An independent samples t-test revealed that probiotics had no effect on total extinction response for adolescents ($t(20) = -1.923$; $p = 0.069$) or adults ($t(14.945) = 1.343$; $p = 0.199$) (figure 6 a and b), although adult placebo-treated rats had higher extinction responding than their treatment-matched adolescents ($t(14.969) = -3.769$; $p = 0.002$; figure 6b). For adolescents, a repeated measures ANOVA revealed a significant effect of extinction sessions ($F(1.191, 23.827) = 12.828$; $p = 0.001$) but no interaction between extinction sessions and probiotic treatment ($F(1.191, 23.827) = 3.695$; 0.06). Similarly in adults, there was a significant effect of extinction sessions ($F(1.337, 28.078) = 48.114$; $p < 0.001$), but no interaction between age and extinction sessions ($F(1.337, 28.078) = 1.024$; $p = 0.344$). A two-way age x probiotic treatment ANOVA showed no effect of probiotics on reinstatement responding ($F(1,41) = 0.872$; $p = 0.356$), but a main effect of age ($F(1,41) = 4.986$; $p = 0.031$), and no interaction ($F(1,41) = 3.223$; $p = 0.08$). Also, probiotics did not influence cue-induced reinstatement of cocaine-seeking in adolescents ($t(20) = -0.902$; $p = 0.378$) or adults ($t(21) = 1.603$; $p = 0.124$) (figure 6c). A two-way ANOVA showed no effects of age or probiotics, and no interaction between age and probiotics. Targeted t-tests also failed to reveal an effect of probiotics on change score in adolescents ($t(20) = 0.199$; $p = 0.844$) or adults ($t(21) = -0.72$; $p = 0.943$) (figure 6d).

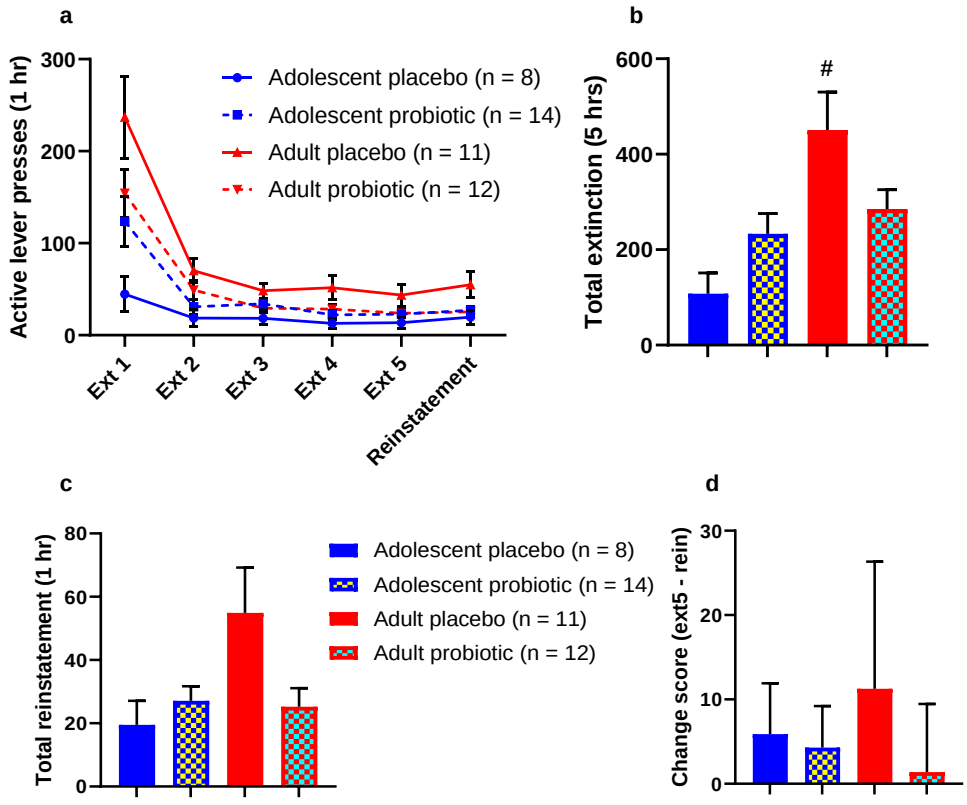


Figure 6: Probiotics did not attenuate cocaine-seeking behavior

a. and **b.** Probiotics did not affect extinction responding in adolescent or adult rats, although placebo-treated adults had higher extinction responding compared with placebo-treated adolescents. **c**. Probiotics did not attenuate reinforcement responding in neither adolescent nor adult rats. **d.** Probiotics did not affect change score in adolescent or adult rats. # denotes age difference between place-treated rats. All error bars are ±S.E.M.

4 DISCUSSION

This study tested two hypotheses. First, probiotics will reduce cocaine seeking that has been heightened by antibiotic treatment. This hypothesis was clearly supported, as probiotics given during forced abstinence reduced cocaine seeking in adult rats that had shown significantly elevated reinstatement levels when antibiotics were presented in the drinking water during cocaine self-administration. Reinstatement responding among adolescents had not been elevated by antibiotics, so the lack of probiotic impact on this lower-level responding remains consistent

with the hypothesis. Second, we tested whether probiotics given during the entirety of behavioral testing would reduce cocaine intake and/or seeking. This hypothesis was not clearly supported, as probiotics did not affect cocaine intake or cocaine seeking significantly in either adolescent-onset or adult age groups.

The present results are based on well-tolerated antibiotic and probiotic treatments, along with well-validated methodological approaches. No adverse effects from antibiotic or probiotic treatments were observed regarding body weight and fluid intake, suggesting normal growth and fluid consumption. With regard to validating the cocaine self-administration procedures, rats that self-administered cocaine had significantly higher active lever presses than their age-matched, sham-operated controls, which supports the conclusion that cocaine was the reinforcer of lever-pressing in the self-administration procedures. Moreover, presses on the inactive lever throughout behavior testing were lower than those on the active lever (data not shown), lending further support for the effectiveness of the behavioral model.

The present results are somewhat consistent with prior reports on the impact of gut microbial perturbations on cocaine-related behavior. Antibiotic-induced gut microbial depletion has been shown to increase sensitivities to the rewarding and sensitizing properties of cocaine in rodent models. Kiraly and colleagues demonstrated that antibiotic-induced gut bacterial depletion resulted in an enhanced sensitivity to cocaine reward, as seen in a conditioned place preference paradigm (Kiraly et al., 2016). Results from our experiments extend this outcome to the enduring effects of cocaine reinforcement in the i.v. self-administration model, such that antibiotics during self-administration decreased reinstatement responding after a 30-day abstinence period (Suess et al., in preparation). On the other hand, the level of cocaine intake during self-administration in the present experiment was similar to levels previously observed

among rats that did not have antibiotics in their drinking water throughout self-administration procedures. This observation may suggest that antibiotic-induced gut microbial depletion did not exert strong enough impact to alter the robust responding observed under these self-administration conditions, which are optimized for reliable acquisition and maintenance of drug intake, rather than revealing sensitivities to the acute reinforcing properties of the drug. A benefit of these self-administration parameters is assuring that most animals will acquire and proceed into extinction and reinstatement testing after abstinence, which is a critical element of the present behavioral testing, due to its high relevance to the human condition of SUD, in which vulnerability to relapse is a defining factor. The extinction responding and cue-induced reinstatement tests are the critical components of the present experiments, and they corroborate the association between gut microbial depletion and heightened sensitivity to cocaine-paired environmental and discrete sensory cues.

With regard to probiotic effects, the finding that probiotics rescued normal cocaine seeking behavior in adults after it had been elevated by antibiotics lends support to the conclusion that the antibiotic effect was related to disruptions in the gut microbiome. The data on adults are consistent with other studies and expand the beneficial impact of CereBiome which has been shown to be effective at alleviating major depressive disorder (Kazemi et al., 2019), stress-induced HPA axis dysfunction (Ait-Belgnaoui et al., 2018), gastrointestinal symptoms (Diop et al., 2008), and cardiovascular conditions (Jin et al., 2019). The mechanisms through which antibiotics and probiotics alter reward- and reinforcement-related behaviors, however, remains unclear. Exogenous short chain fatty acid treatment did restore normal cocaine CPP previously elevated by antibiotics in the drinking water (Kiraly et al., 2016) and decreased propionate was reported in fecal samples from rats that showed significant methamphetamine

conditioned place preference (Ning et al., 2017). These results suggest that bacterial production of SCFA might provide a signal out of the gut into the bloodstream to reach receptors in the brain or otherwise alter brain function, e.g., through alterations in gene expression. SCFA receptors include free fatty acid receptors FFAR2, FFAR3 and G-protein-coupled receptor GPR109a, and have been identified in the hippocampus and hypothalamus (Silva et al., 2020), as well as immune cells (He et al., 2020). Numerous other gut-brain axis signaling mechanisms may play additional or alternative roles, broadly including vagus nerve projections, modulation of neurotransmitter synthetic pathways, and indirect impacts through the immune system. Future experiments on the functional consequences of *Lactobacillus*- and *Bifidobacterium*-loading in the gut via the present CereBiome treatment is likely to narrow focus to some candidate mechanisms.

Regarding the relationship between age and drug use, age of onset of drug use may contribute to the risk of relapse, and adolescent onset of substance use appears to increase the likelihood of further drug use and addiction (NCBDDD, CDC, 2020). Adolescence is a developmental period characterized by active changes in the gut microbiome composition and function, which is more complex and less stable than the adult gut microbiome (Hollister et al., 2015), with adult gut environments marked by increased stability and evenness, and better adaptation to withstand insult from infection or antibiotics (Spor et al., 2011). Interestingly though, there was no effect of probiotics on extinction responding or reinstatement among adolescents. This is consistent with a prior experiment indicating that overall levels of gut bacteria had returned to normal levels 30 days since the last day of antibiotic intake, while bacterial levels remained lower among antibiotic-treated adults compared to their water-treated controls (Suess et al., in preparation). Overall, these results showing some adolescent resilience

to manipulations that significantly affect adult behavior and/or physiology contribute to a growing body of research showing that adolescents are not always more sensitive than adults, despite the higher levels of neural and thus behavioral plasticity they may show in certain domains (Doherty et al., 2009).

Results from the prophylaxis experiment revealed that probiotic prophylactic was not effective in reducing cocaine intake or seeking, as the probiotic-treated rats self-administered cocaine and reinstated cocaine-seeking at basically the same rate as their placebo-treated age-matched controls. Myriad potential explanations exist for this lack of robust impact of the prophylactic probiotic approach. First, higher variability in behavior was observed in the prophylaxis experiment 2 compared with experiment 1, which is not readily explained by observed factors in the laboratory but likely affected statistical outcomes. Second, cross-contamination between probiotic vs. placebo treatment groups could have occurred, bringing their behaviors closer to the mean across treatment groups. Yet careful experimental procedures were followed to avoid contamination, including different experimenters handling the probiotic formulation and rats vs. placebo formulation and rats each day of experimentation. Also, under these normal gut conditions at the start of experimentation, these bacteria perhaps did not grow to become a significant portion of the microbial profile in terms of abundance and function. Alternatively, these particular bacterial groups may not be the appropriate species to alter gut-brain signaling in this context. Finally, perhaps the simple fixed ratio 1 (FR1) schedule of reinforcement and the short access (2 hr) daily sessions provided inadequate levels of cocaine reward and reinforcement to elucidate the prophylactic effects of probiotics.

Future studies are certainly warranted to further investigate the potential benefits of probiotics to reduce cocaine-intake and -seeking. One approach is to use alternate probiotics.

Prior work in our lab has shown that the species *Akkermansia municiphila* is associated with resilience while the family *Ruminicoccaceae* is associated with vulnerability to cocaine intake in adult male rats (Suess et al., in preparation). Furthermore, different models of cocaine intake should be explored such as a daily long-access model, which simulates escalation of drug intake over time, or a progressive ratio schedule of reinforcement that measures motivation to take drugs, or a binge model which provides unlimited access to a drug over long periods.

An additional limitation of this study is that only male rats were tested. Sex differences in cocaine intake have been recognized for many years, e.g., Kawa & Robinson revealed that female Sprague Dawley rats showed increased motivation and cocaine consumption than males (Kawa & Robinson, 2019). Sex differences in the long-term impact of antibiotic exposure also are known, e.g., Zou and colleagues found that first-year antibiotics exposure was more associated with increased odds of lifetime-ever pneumonia, croup, wheeze, asthma, food allergies, allergic rhinitis and atopic dermatitis in girls aged 4 to 6 years old than boys (Zou et al., 2020). Just these two sample studies underscore the importance of testing females on all aspects of the present experiments.

In conclusion, this study shows that probiotics may be useful in addiction treatment, but these results do not support their use in prevention. With future research, targeted probiotic formulations could provide individualized gut therapy, thereby maximizing the impact of probiotic treatment for drug addiction or other disorders and diseases.

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