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Effects of Vancomycin on Persistent Pain-Stimulated and Pain-Depressed Behaviors in Female Fischer Rats With or Without Voluntary Access to Running Wheels

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Abstract: **The present experiments determined the effects of the narrow-spectrum antibiotic vancomycin on inflammatory pain-stimulated and pain-depressed behaviors in rats. Persistent inflammatory pain was modeled using dilute formalin (0.5%). Two weeks of oral vancomycin administered in drinking water attenuated Phase II formalin pain-stimulated behavior, and prevented formalin pain-depressed wheel running. Fecal microbiota transplantation produced a non-significant trend toward reversal of the vancomycin effect on pain-stimulated behavior. Vancomycin depleted *Firmicutes* and *Bacteroidetes* populations in the gut while having a partial sparing effect on *Lactobacillus* species and *Clostridiales*. The vancomycin treatment effect was associated with an altered profile in amino acid concentrations in the gut with increases in arginine, glycine, alanine, proline, valine, leucine, and decreases in tyrosine and methionine. These results indicate that vancomycin may have therapeutic effects against persistent inflammatory pain conditions that are distal to the gut.**

Perspective: ***The narrow-spectrum antibiotic vancomycin reduces pain-related behaviors in the formalin model of inflammatory pain. These data suggest that manipulation of the gut microbiome may be one method to attenuate inflammatory pain amplitude.***

Key words: *Pain-depressed behaviors, wheel running, rats, gut microbiome, antibiotics, amino acids.*

The gut microbiome is implicated in health and the pathogenesis of disease states, including neuroinflammation and intestinal inflammatory pain.[36,60] Modulation of the gut microbiome is possible via a wide variety of procedures that include antibiotic intervention and fecal microbiota transplantation (FMT). An example of the former strategy is administration of narrow- or broad-spectrum antibiotics with the goal of selective or global depletion of specific taxa, and the latter strategy of FMT from healthy donors, allows for replenishment of these bacteria in recipient hosts.[2,56,59,73] As an example of the former strategy,

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Gram-positive antibiotics that target cell wall synthesis selectively deplete *Firmicutes*, one of the prominent bacterial phyla found in the gut. Classically, this intervention produces dysbiosis, induces neuroinflammation, and increases symptoms of central or peripheral nervous system disease states.[25,47] However, the impact of such antibiotics is often severe across additional bacterial phyla, including Gram-negative *Bacteroidetes* and *Proteobacteria*, suggesting a complex ecological response.[19,61,69] Contrasting literature reports that Gram-positive antibiotics can reduce inflammation, attenuate pain-related behaviors, and reduce progression and/or severity of arthritis pain,[27,44,57,58] and more recent papers have begun to summarize potential mechanism, with pathways that include immune cell function/signaling and microbial metabolites.[8,24,51] Evidently, more work must be done to ascertain under what conditions antibiotics produce beneficial or detrimental effects, as well as the specific mechanisms that may be driving these divergent results.

Finally, although gut microbiome-pain interactions have been characterized in rodent models of pain-stimulated behavior, they have not yet been assessed in assays of pain-depressed behavior.[42,64] Advantages of including assays of pain-depressed responding in preclinical research are: (1) clinicians often quantify pain by the degree to which it depresses normally performed behaviors including exercise, (2) complementary use of both pain-depressed and pain-stimulated behaviors may provide more efficient bench-to-bedside translation, (3) occurrence of pain-depressed behaviors are often less subject to experimenter interpretation and (4) the neural pathways that underlie antinociception in pain-stimulated vs pain-depressed behaviors may be different. For example, reversal of pain-depressed behaviors may recruit overlapping but distinct pathways that include not only descending and ascending pain pathways,[3,4,49] but regions involved in organization and planning of movement including visual, motor, and association cortex, and medial reticular formation, mesencephalic locomotor region, and cerebellum.[29]

To address the potential link between the gut microbiome and distal site inflammatory pain, the present experiments sought to elucidate the effects of gut microbiome perturbation on persistent inflammatory pain-stimulated and pain-depressed behavior, in Fischer female rats. We profiled gut bacterial populations using 16S rRNA marker gene sequencing, which provides a DNA-based fingerprint of bacteria and their abundances. Following DNA sequencing, bacterial taxa were assigned by alignment to an extensive 16S rRNA gene database.[37] Liquid chromatography was used to quantify amino acids, which have previously been linked to gut dysbiosis,[45] and are elevated in humans following antibiotics treatment.[66] The narrow-spectrum antibiotic vancomycin was used as a tool to perturb the gut microbiome and deplete obligately anaerobic, fermentive bacteria in the *Firmicutes* and *Bacteroidetes* phyla, which typically dominate the gut microbiota. Formalin was used to model persistent inflammatory pain, and the acute biphasic pain-stimulated responses for licking behavior and posture were measured across 60 min. The formalin model of tissue injury and inflammation was chosen because it allows for assessment of two dichotomous conditions: nociception (interpreted to be manifested during Phase I) and inflammation (interpreted to be manifested during Phase II) in a single animal model.[10,26] Formalin was also assessed for its ability to depress voluntary wheel running for 3 consecutive days. This pain-depressed behavioral endpoint was chosen due to the ability to automate data collection and minimize experimenter bias, and the clinical relevance of exercise as a behavioral measure.[9,11,28,65,67] Exercised rats were compared to sedentary controls, and fecal amino acid concentrations were compared between vancomycin-treated rats and untreated controls. Finally, FMT was utilized with the goal of reversing any vancomycin-induced modulation to pain-related behaviors. Female rats were tested because (1) females, on average, have higher prevalence rates and are at increased risk for chronic pain in general,[12,17,21] and (2) historically, the majority of rodent work is biased toward male subjects.[46] Our working hypothesis was that formalin would produce pain-stimulated and pain-depressed behaviors, that pretreatment with vancomycin would modulate these pain-like behaviors as well as amino acid concentrations, and that FMT would reconstitute populations of bacteria back to baseline levels and/or block vancomycin-induced modulation to pain-related behaviors.

Methods

Subjects

Female Fischer rats (Charles River Labs 125−150 g at beginning of experiments) were utilized for all studies. Rats were individually housed, in separate Plexiglass chambers contained in an animal facility maintained on a 12 hour light / dark cycle in a temperature-controlled animal colony. Food and water were available ad libitum. Rats were assigned to either a sedentary or exercise condition following one week of habituation. Those in the exercise condition were moved to a separate wheel running room housing individual single activity wheel chambers (Lafayette Instruments, Lafayette, IN) for the duration of the experiment. Each rat was singly housed in a chamber and had 23 hour voluntary access to its running wheel, with one hour per day allocated for health checks, food/water replenishment, and cleaning bedding/equipment. All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals[41] and all methods and procedures were approved by the University of New England Institutional Animal Care and Use Committee. The health and safety of the rats were evaluated by research technicians and periodically monitored by a veterinarian.

Vancomycin Treatment

All sedentary and running wheel rats were randomly assigned to antibiotic or control conditions (n = 7 per

treatment). Rats in the antibiotic group received the narrow-spectrum, cell wall synthesis-inhibiting antibiotic vancomycin, which selectively targets Gram positive bacteria. Vancomycin was administered via their home cage water bottles at 500 mg/L in autoclaved water.[25,53,27] Controls were treated similarly but had access only to autoclaved normal drinking water without antibiotic, in their home cage water bottles. Vancomycin was delivered for 2 consecutive weeks + 4 days for post-injury recording for running wheel studies (Rakoff-Nahoum et al., 2004) and for 2 consecutive weeks for FMT studies. For running wheel rats, Days 8 to 14 of vancomycin was concurrent with the 7 day running wheel acquisition time period. Sedentary rats also had 2 consecutive weeks of vancomycin in drinking water but did not have access to running wheels. Both sedentary and running wheel rats were housed in identical cages the only difference being that running wheel rats had the external running wheel "door" open, and sedentary rats had the external running wheel "door" closed and locked.

Formalin Pain Model

The formalin pain model[26] was adopted in our study to evaluate pain-stimulated and pain-depressed behaviors. This model was selected because it allows for measurement of both an acute, nociceptive "early" phase (Phase I) and a tonic, inflammatory "late" phase (Phase II) pain-like behaviors in the same subject after a single noxious event. We defined Phase I as occurring 0 to 10 minutes post-injection, and Phase II as >20 minute post-injection.

Pain-Stimulated Behavior

The formalin test was utilized to quantify pain-stimulated behaviors in both sedentary and exercised rats. Rats were treated with vancomycin for fourteen days (Days 1−14), and formalin (0.5% in 50 μl) was injected subcutaneously (s.c.) into the dorsal surface of the right hind paw using a 50 μl micro syringe with a 30-gauge needle, on the morning of Day 15. Rats were then returned to Plexiglass observation chambers post injection, and behavioral observations commenced immediately. Total time (in seconds) spent licking the injured hind paw as well as pain scores were recorded for 60 minutes. Pain scores ranged from 0 to 3, where 0 = the injected paw is not favored; 1 = the injected paw has little or no weight placed upon it; 2 = the injected paw is elevated and not in contact with any surface; 3 = the injected paw is licked, bitten or shaken. Between-subjects design was utilized such that each rat received only 1 single injection at 1 single concentration into the hind paw.

Pain-Depressed Wheel Running

Rats were treated with vancomycin for eighteen days (Days 1−18), and formalin (0.5% in 50 μl) was injected s.c. as described above, on the morning of Day 15 (Fig 1B). Running wheel acquisition (7 consecutive days) overlapped with vancomycin Days 8 to 14, and vancomycin Days 15 to 18 overlapped with formalin injection (Day 15) followed by post-formalin days: PDs 1, 2, 3 (Days 16, 17, 18). Rats were removed from running wheel cages in the morning (during which time running behavior is negligible or non-existent in our lab) and taken to a behavioral analysis room, placed into Plexiglas chamber (dimensions), and allowed to habituate for 15 minute or until exploratory behavior stopped. Following habituation to chambers, the formalin test was performed as described above. Following formalin administration and 60 minute behavioral recording of pain-stimulated behaviors, rats were carefully removed from Plexiglass chambers and returned to the wheel running room and carefully placed back into their designated wheel running chambers. Finally, the greatest degree of pain-depressed wheel running in our laboratory is typically observed during the first 4 hours of dark cycle, and thus we report this time frame in results and figures below.[11,65] All sedentary and running wheel

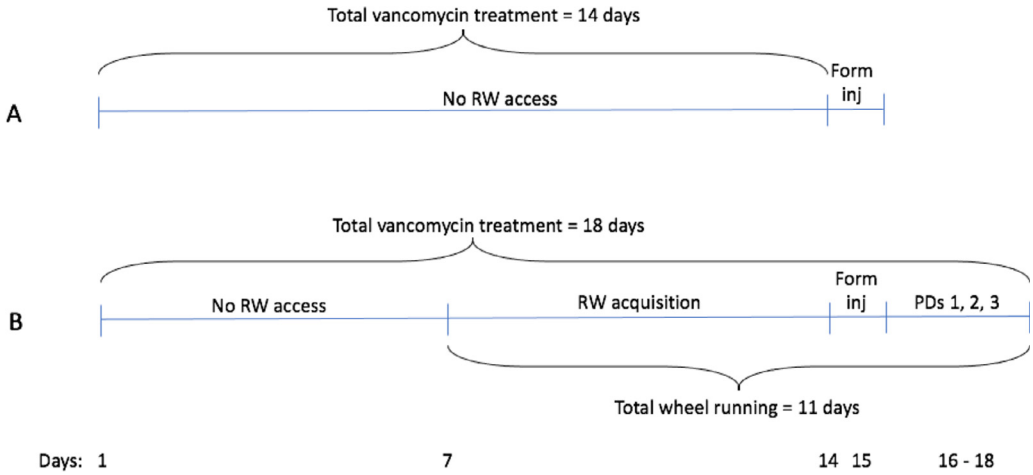

Figure 1. Experiment timeline for vancomycin experiments. Panel A shows the timeline for sedentary rats and Panel B shows the timeline for running wheel rats.

experiments were carried out with a total n = 7 to 8 per group, and random assignment for groups. As running rates vary widely, running wheel rats were matched according to distance traveled during acquisition phase. Thus, sedentary and exercised (running wheel) protocols were as follows (see Experiment Timeline in Fig 1, Panels A and B, respectively):

Sed: Days 1 to 14 on vancomycin (no access to running wheels) → Day 15 formalin injection (+formalin test)

RW: Days 1 to 18 on vancomycin (Days 8−14 = running wheel acquisition) → Day 15 formalin injection (+formalin test) → Days 16 to 18 post-formalin wheel running

Fecal Pellet Collection

Baseline fecal pellet collection occurred after habituation to the animal facility, but prior to the start of vancomycin administration, with a single pellet collected per subject. Pellet collection also occurred on vancomycin Day 14 and days 1, 3, 5, 7 of a 7-day oral gavage (FMT) regimen, as well as immediately before and after the formalin pain manipulation, and formalin PD1 and PD2. Once collected, pellets were immediately stored in a -80°C freezer[33,71] for 16 S ribosomal RNA sequencing.[33,63]

Fecal Microbiota Transplantation

Fecal material from healthy naÿve donor rats was collected, pooled and incorporated into a single sample following collection. Combined fecal pellets were suspended in sterile phosphate-buffered saline (PBS) at a concentration of 0.1 g/mL. Rats were administered either FMT homogenate or sterile saline (bodyweight) via oral gavage once per day for seven consecutive days. Groups were as follows: (a) Normal H2O (2 weeks) + vehicle (1 week) + formalin, (b) Vancomycin (2 weeks) + vehicle (1 week) + formalin, (c) Vancomycin (2 weeks) + FMT (1 week) + formalin (See Experiment Timeline in Fig 2).

Data Analysis for Behavioral Studies

The two dependent variables for the acute pain-stimulated behaviors in the formalin test were (1) time spent licking the injured hind paw and (2) overall pain score, and both measures were quantified for a 60 minutes duration in 5-minute blocks. The primary dependent variable for pain-depressed running wheel experiments was total distance traveled in the wheels (in meters) during the first 4 hours of dark cycle (7−11 pm), a time period when rats exhibit high rates of running behavior in our lab,[65] in a 24 hour voluntary access daily period. Statistical analysis was accomplished with one- or two-factor ANOVA as appropriate. Significant one-way or two-way ANOVAs were followed by Duncan post hoc test, and significance was set a priori at $P \leq .05$. Cohen's *d* and associated Confidence Intervals were calculated to determine the magnitude of effect and the precision of that *effect size* estimate.[40,74] This use of *p* values is standard and the subsequent effect size statistic allowed for high resolution characterization of the magnitude of effect and the precision of that estimate, in the samples of interest.

Determination of the Intestinal Microbiome

Rat fecal pellets were collected fresh for each group condition (n = 3−5), and frozen at -80°C until further processing. Total DNA was isolated using the Qiagen DNeasy PowerSoil kit (Germantown, Maryland) and quantified with the Quant-iT PicoGreen Assay (Turner BioSystems, Inc., Sunnyvale, CA). The V1-V2 region of the bacterial 16S rRNA gene was amplified in quadruplicate by polymerase chain reaction (PCR) using barcoded

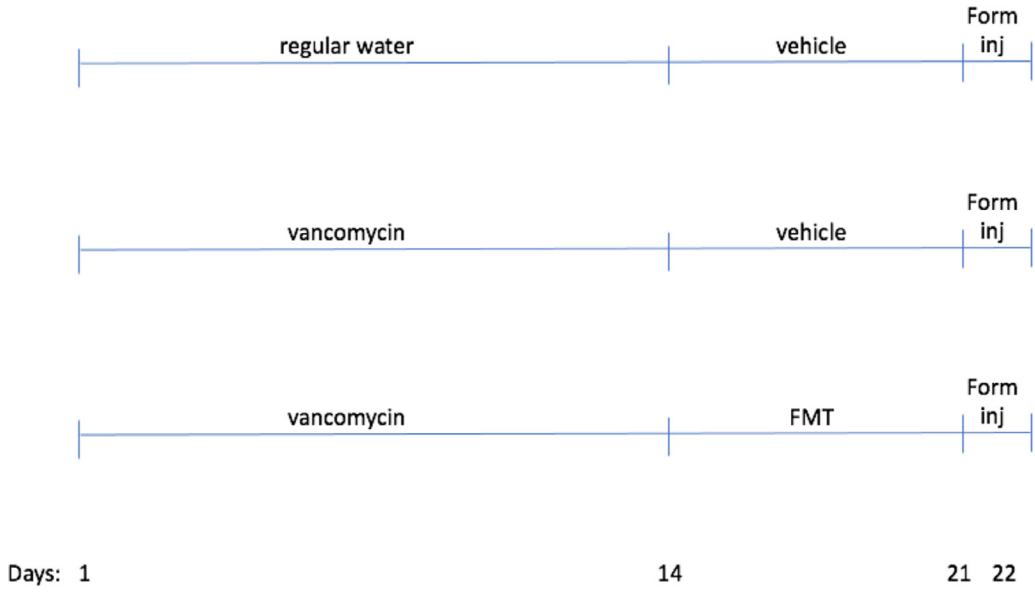

Figure 2. Experiment groups and timeline for FMT experiments.

primers reactions and Accumprime Taq (Invitrogen) reagents. Purified products were sequenced on the Illumina MiSeq (Illumina, Inc., San Diego, CA). Extraction blanks and DNA free water were subjected to the same amplification and purification procedure to allow for empirical assessment of environmental and reagent contamination. Positive controls, consisting of eight artificial 16S gene fragments synthesized in gene blocks and combined in known abundances, were also included. Sequence data were processed using QIIME2 (http://qiime2.org/). Read pairs were error corrected and joined using DADA2.[6] Taxonomic assignments were generated by comparison the Greengenes reference database.[37]

Liquid Chromatography

Amino acids (AAs) were quantified using a Waters Acquity uPLC System (Waters Corp., Milford, MA) with an AccQ-Tag Ultra C18 1.7 μm 2.1 $\times$ 100 mm column and a Photodiode Detector Array, according to previously published method.[45] Briefly, post-FMT fecal samples were homogenized in methanol (5 μL/mg stool) and centrifuged twice at 13,000 g for 5 minutes. AAs in the supernatant were derivatized using the Waters AccQ-Tag Ultra AA Derivatization Kit (Waters Corporation, Milford, MA) and analyzed using the UPLC AAA H-Class Application Kit (Waters Corporation, Milford MA) according to manufacturer's instructions. Quality control checks (blanks and standards) were run every eight samples. All chemicals and reagents used were mass spectrometry grade.

Intestinal Microbiome Data Analysis

Similarity between samples was assessed by weighted and unweighted UniFrac distance between sample.[34,35] Data files from QIIME were analyzed in the R environment for statistical computing, using the QIIMER library (http://cran.r-project.org/web/packages/qiimer). Global differences in bacterial community composition were visualized using Principal Coordinates Analysis. Community-level differences between sample groups were assessed using PERMANOVA, which tests for a difference in centroid position using a distance matrix between samples.[1] Differential abundance of specific taxa were assessed using nonparametric rank tests or general linear mixed effects models. The generalized linear models were selected when within vs between-subject effects were characterized.

Quantitative Detection of Key Taxa Following FMT

Frozen fecal pellets were cut in half with a sterile scalpel blade under sterile conditions. Total DNA was extracted using the GenElute Stool DNA Isolation Kit (Sigma-Aldrich, St. Louis, MO), following the manufacturer's protocol. DNA concentrations were determined using a Nanodrop One-C (Thermo-Fisher, Waltham, MA), and all sample concentrations were equalized at 5 ng/μL in nuclease-free water. Quantitative PCR (qPCR) was performed on a StepOne Plus RT-PCR system, using Power SYBR Green Master Mix (Applied Biosystems, Foster City, CA). All samples were run in triplicate using 10 ng of template. The optimized qPCR annealing temperatures for each primer pair were: 42.0°C (Universal 16S rRNA), 47.7°C (*Lactococcus* spp. 16S rRNA), 58.7°C (*Gammaproteobacteria* spp. 16S rRNA), 60.3°C (*Clostridiales* spp. 16S rRNA), 62.0°C (Fischer rat 18S rRNA), and 59.0°C (Fischer rat *cypA*). The qPCR cycling conditions were: stage 1, 95°C for 10 minutes; stage 2 (40 cycles), step 1, 95C for 30 seconds; step 2, optimized variable annealing temperatures; step 3, 74°C for 30 seconds; stage 3, 4°C infinite hold. The comparative C_T ($2^{-\Delta\Delta C(T)}$) method was used to calculate fold changes relative to experimental controls. Primer sequences are described in Table 1.

Results

Vancomycin Effects on Formalin Pain-Stimulated Behaviors

Fig 3 shows vancomycin (VANC) effects on Phase II (20–60 minute) formalin-induced pain scores for wheel runners (Panel A) and sedentary rats (Panel B). For runners (Panel A), One-Way ANOVA revealed that formalin alone produced a significant increase in pain scores relative to saline or VANC alone, and that VANC pretreatment (green bar) produced a significant decrease in formalin pain scores relative to formalin alone (blue bar) ($F(3, 23) = 13.15$; $P < .0001$). For sedentary rats (Panel B) One-Way ANOVA revealed that formalin produced a significant increase in pain scores relative to saline alone ($F(3, 23) = 3.94$; $P = .023$), and that there was no significant difference between vancomycin pretreatment (green bar) and formalin alone (blue bar). Phase I was unaltered by VANC (data not shown). Groups sizes as articulated in Methods, were n = 7 to 8.

Table 1. Primer Sequences used for qPCR

Target	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
Universal 16S	GTGYCAGCMGCCGCGGTAA	CCGYCAATTYMTTTRAGTTT
Lactococcus	TTCCCTTCGGGGACATGGATA	TGACGGGCGGTGTGTACAAG
Gamma-proteobacter	CGTGTTGTGAAATGTTGGGT	TGACGGGCGGTGTGTACAAG
Clostridium	ATTACCCTTAATCGGGGAAGC	TGACGGGCGGTGTGTACAAG
Fisher rat ribosomal 18S	ACGGACCAGAGCGAAAGCAT	TGTCAATCCTGTCCGTGTCC
Fisher rat cyclophilin-A	AGCATACAGGTCCTGGCATC	TTCACCTTCCCAAAGACCAC

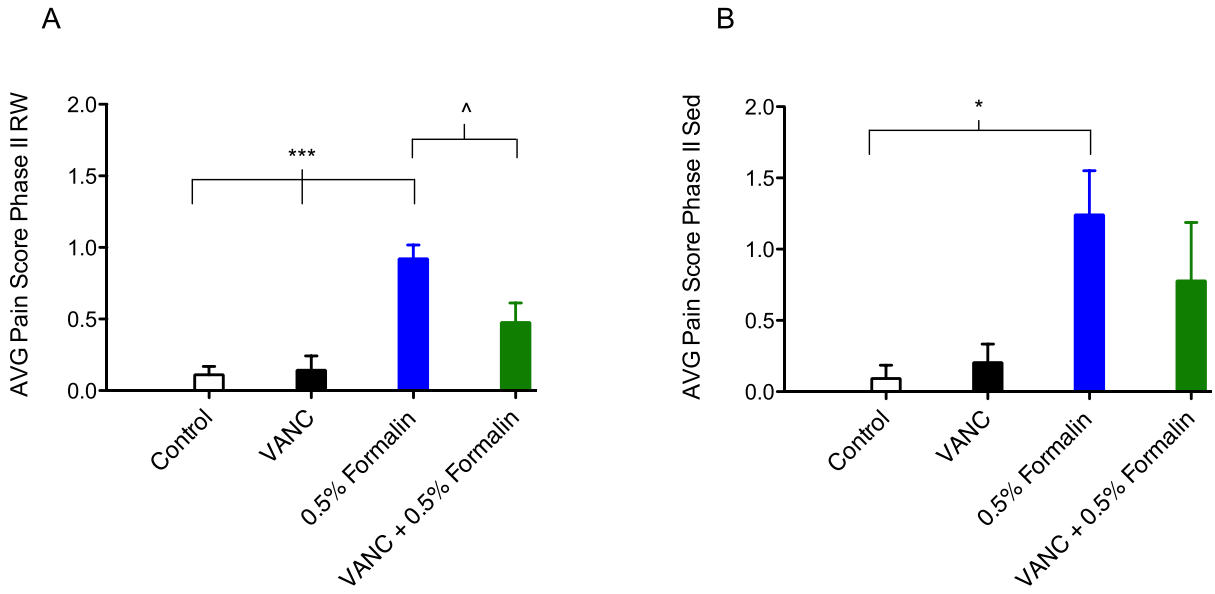

Figure 3. Average Phase II pain scores for saline controls, VANC, formalin alone, and VANC + formalin cohorts in the 0.5% formalin test for rats that had 24 hour voluntary access to running wheels (panel A) or were in sedentary condition (panel B). *, *** = significantly greater than saline controls ($P \leq .05, .001$). ^ = significantly decreased relative to formalin alone.

Vancomycin Effects on Formalin Pain-Depressed Wheel Running

Fig 4 shows the effects of vancomycin (VANC) pretreatment against 0.5% formalin pain-depressed wheel running. Panel A shows that the formalin decreased wheel running on PD2 and PD3 and 2-Way ANOVA revealed significant depression of running on PD3 (blue line), and that the 2-week course of vancomycin pretreatment prevented formalin pain-depressed wheel running on PD3 (green line) ($F(2, 48) = 21.61$, $P < .0001$). For the Panel A data, there was a significant main effect on time only, and no interaction occurred. Panel B shows effect sizes (Cohen's *d* statistic) for formalin vs saline (blue circles) and formalin + vancomycin (VANC) vs formalin (green triangles) at PD1, PD2, and PD3, for running wheel rats. Data indicate large effect size (defined as $d \geq 0.8$ and CI that do not overlap with zero) for formalin vs saline at PD3, and large effect size for

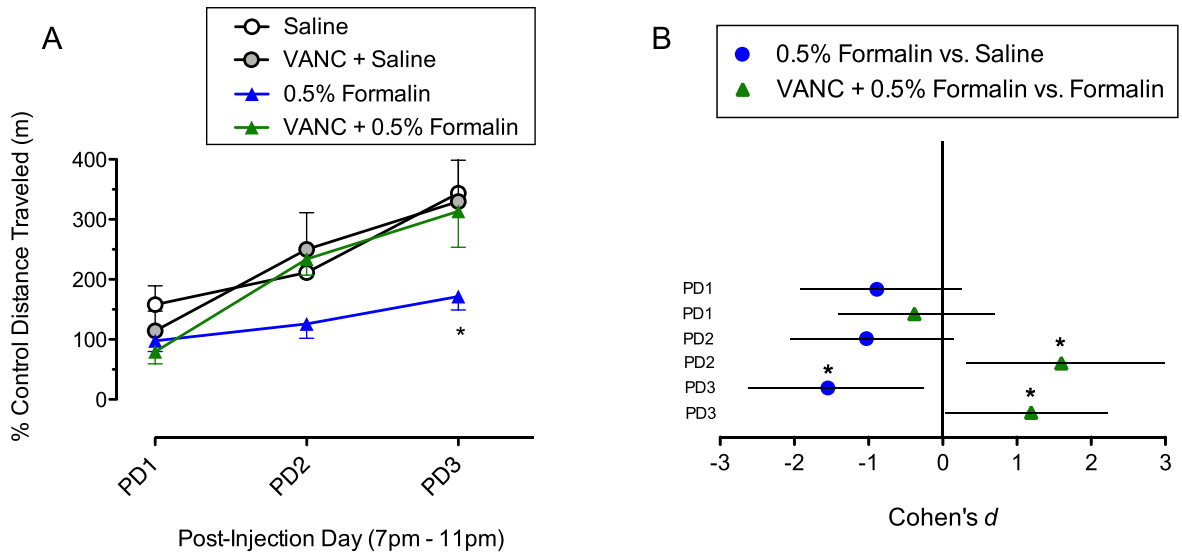

Figure 4. Effects of saline controls, VANC, formalin, and VANC + formalin on voluntary wheel running. Panel A ordinate: % control distance traveled in meters in running wheels. Panel A abscissa: Post-Days 1 thru 3. Panel A shows formalin produced significant depression of wheel running on PD3 and that vancomycin prevented this effect. * = significantly decreased relative to saline controls ($P < .05$). Panel B shows magnitude of effect (Cohen's *d* effect sizes and confidence intervals) for formalin vs saline (blue circles) and for VANC + formalin vs formalin (green diamonds). * = Effects sizes are large defined as ≥ 0.8 and confidence intervals do not overlap with zero (color version of figure is available online.).

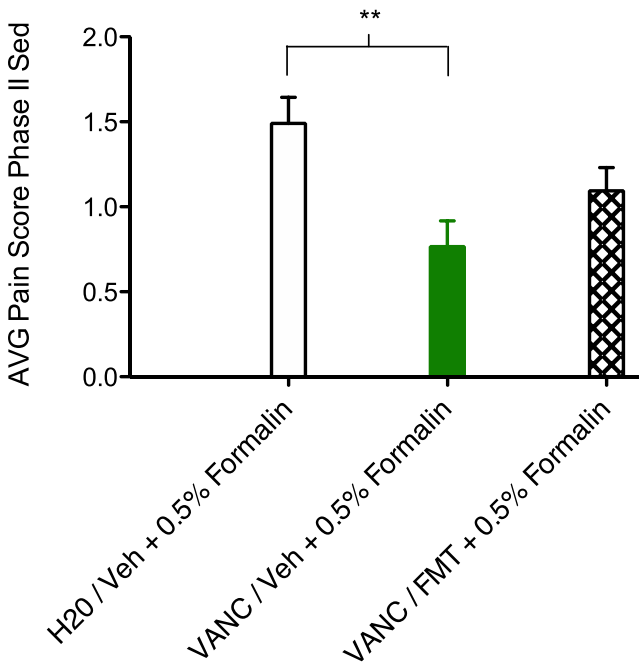

Figure 5. Average Phase II pain scores for formalin alone, VANC + formalin, and pretreatment effects of fecal microbiota transplantation (FMT) against formalin Phase II. VANC produced significant decreases in pain score relative to formalin group. FMT pretreatment did not significantly block the VANC effect, although there is a trend toward restoration to formalin baseline. ** = significantly decreased relative to formalin ($P < .01$).

VANC + formalin vs formalin at PD2 and PD3. Wheel running experiments were run with a total n = 8 for all groups.

FMT Effects on Formalin Pain-Stimulated Behaviors

Fig 5 shows the effects of FMT from non-formalin treated sedentary donor rats to recipient rats that received formalin on pain scores. The columns show, left-to-right: the effects of formalin alone (white bar); 2 weeks antibiotic + 1 week vehicle followed by formalin (green bar); 2 weeks antibiotic + 1 week FMT followed by formalin (checkered bar) during Phase II. One-Way ANOVA revealed that vancomycin produced a significant decrease in pain scores relative to formalin alone during Phase II (compare 2nd bar to 1st bar) ($F(2, 22) = 7.48$; $P = .004$). FMT did not significantly restore antibiotic-decreased pain scores (green bar), but FMT pain scores did trend upward. Experiments were run with total n = 7 or 8 for all groups.

Vancomycin Effects on Alpha Diversity and Amino Acids

Rat fecal microbiome profiles were generated by 16S marker gene sequencing (Fig 6A). The 2-week vancomycin (VANC) regimen depleted nearly all *Firmicutes* and *Bacteroidetes* in runner rats (runner VANC group, white squares) and sedentary rats (sedentary VANC group, white squares) relative to controls. Shannon diversity indices were drastically reduced ($P < .001$) in runner and sedentary groups with VANC alone or VANC + formalin (PD1 and PD2), relative to saline alone or formalin alone controls. There were no differences in Shannon index between sedentary and runner rats, although there is a trend toward greater reduction of abundance in sedentary rats (Fig 6B) (compare sedentary VANC to runner VANC; sedentary VANC + formalin to runner VANC + formalin). VANC administration was associated with significant increases in arginine (↑ 356%; $P = .0146$), glycine (↑ 293%; $P = .0023$), alanine (↑ 144%; $P = .004$), proline (↑ 574%; $P = .0001$), valine (↑ 198%; $P = .0001$) and leucine (↑ 137%; $P = .0005$), and significant decreases in tyrosine (↓ 52%; $P = .0003$) and methionine (↓ 53%; $P = .0043$), relative to pre-VANC baseline control conditions (Fig 6C). FMT fecal samples for amino acid Groups were n = 9 for both BL and post-vancomycin (post-VANC) conditions.

Abundance of Specific Taxa Across Study Days

Fig 7 shows the log abundance of taxa and study group for two distinct genera (*Lactobacillus, Klebsiella*) and one distinct order (*Clostridiales*) that are correlated with VANC and/or running effects on pain. Each panel characterizes how PD1 and PD2 differ ($P < .05$) from baseline (BL) in each group. Fig 7 thus shows in which taxa there were differences either between runners and sedentary rats and/or differences between BL vs formalin and VANC + formalin. Relative to other *Firmicutes* genus (see Heatmap, Fig 6A), VANC did not completely

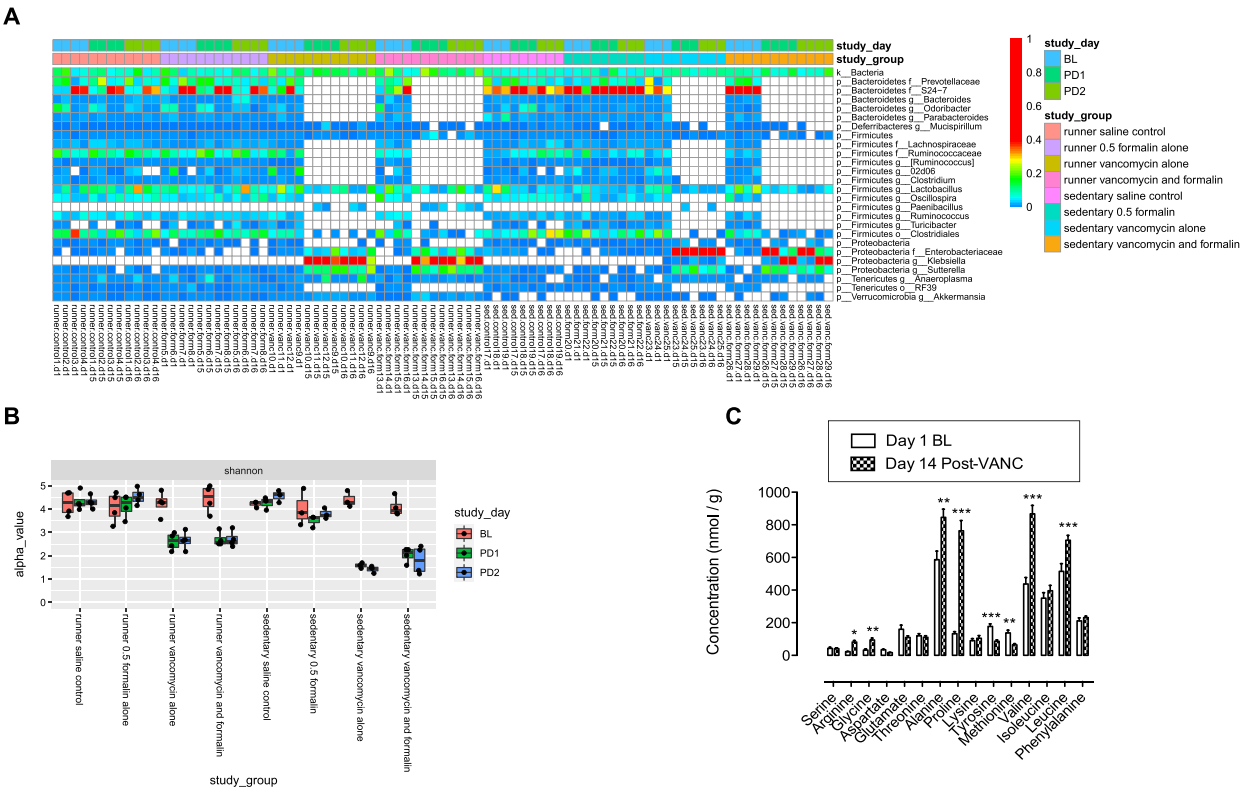

Figure 6. Panel A: 16S marker-gene sequencing Heatmap for Phyla across all groups. Top row shows study day. Each column represents one sample (rat) and each row represents one taxon (genus or best assignment). White boxes = absence of taxa in sample. Blue = low abundance; red = up to 40% abundance. Panel B: the Shannon Index showing even-ness of microbiome distribution. Study days are color coded: BL = red, PD1 = green, PD2 = blue. Formalin and VANC + formalin groups had less abundance relative to controls for both runners and sedentary rats. Panel C: Concentrations of AAs in nmol/gram at baseline (Day1) and following 2-week administration of VANC (Day14). *, **, *** = significantly decreased / increased relative to baseline (BL) controls ($P < .05, .01, .001$) (color version of figure is available online.).

deplete *F. Lactobacillus* for both sedentary compared to runners. VANC produced increases in the Gammaproteobacterial genus *Klebsiella* that varied as a function of addition of sedentary vs runners. Relative to other *Firmicutes* (see Heatmap, Fig 6A) VANC produced decreases in *Clostridiales* for both runners and sedentary groups. Fecal pellet samples were n = 3 to 4 / Group.

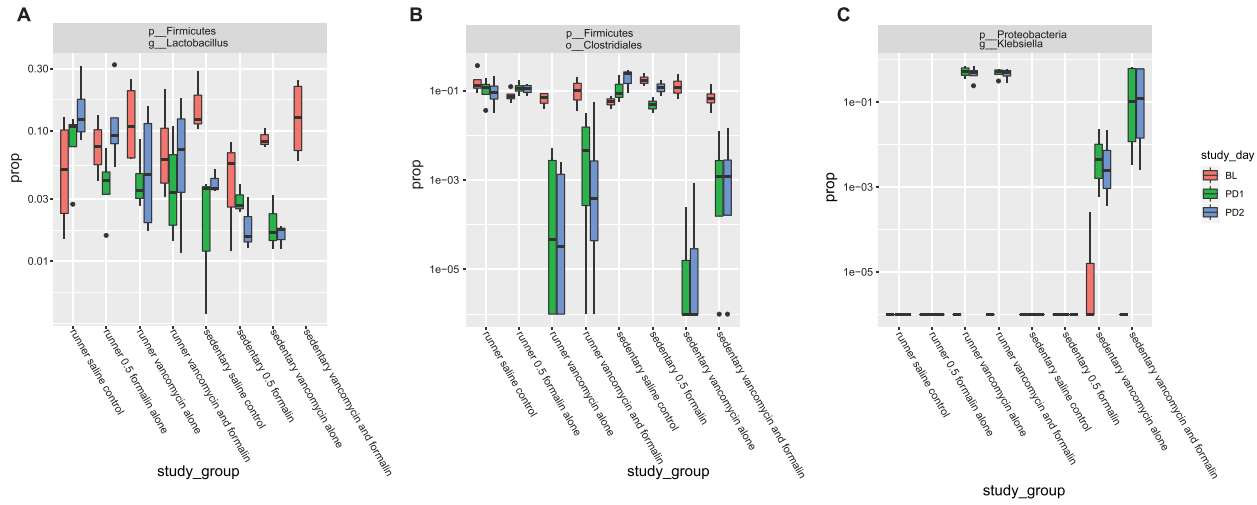

Figure 7. Log abundance of taxa and study group. Relative to other *Firmicutes* genus (see Heatmap, Fig 6A), VANC did not completely deplete *F. Lactobacillus* for both sedentary compared to runners. VANC produced increases in the Gammaproteobacterial genus *Klebsiella* that varied as a function of addition of sedentary vs runners. Relative to other *Firmicutes* (see Heatmap, Fig 6A) VANC produced decreases in *Clostridiales* for both runners and sedentary groups.

Beta Diversity Across Study Days

Fig 8 shows beta diversity within formalin alone groups across exercise or sedentary conditions, and across study day (BL, PD1, PD2). Differences in composition of the fecal microbiota are visualized with principal coordinates analysis of weighted (Panel A) and unweighted (Panel B) UniFrac distance. Unweighted UniFrac distance indicated different beta diversity when comparing runners to sedentary rats ($P \leq .002$), and differing beta diversity when comparing across study days, BL, PD1, and PD2 ($P \leq .005$). Sedentary condition was associated with a non-significant but decreased diversity of the gut microbial community (Panel C) and there were also no significant differences in diversity across baseline, post-day 1, and post-day 2 (Panel D) as assessed with the Shannon Index. Fecal pellet samples were n = 3 to 4/Group.

Fig 9 shows alpha and beta diversity within vancomycin (VANC) + formalin groups across exercise or sedentary condition, and across study day (BL, PD1, PD2). Differences in composition of the fecal microbiota are visualized with principal coordinates analysis of weighted (Panel A) and unweighted (Panel B) UniFrac distance. Both weighted and unweighted UniFrac distance indicated different beta diversity when comparing runners to sedentary rats ($P \leq .001$), and differing beta diversity when comparing across study days, BL, PD1, and PD2 ($P \leq .001$). Relative to baseline, there were significant decreases in diversity on post-day1 and post-day 2, (Panel C) as assessed with the Shannon

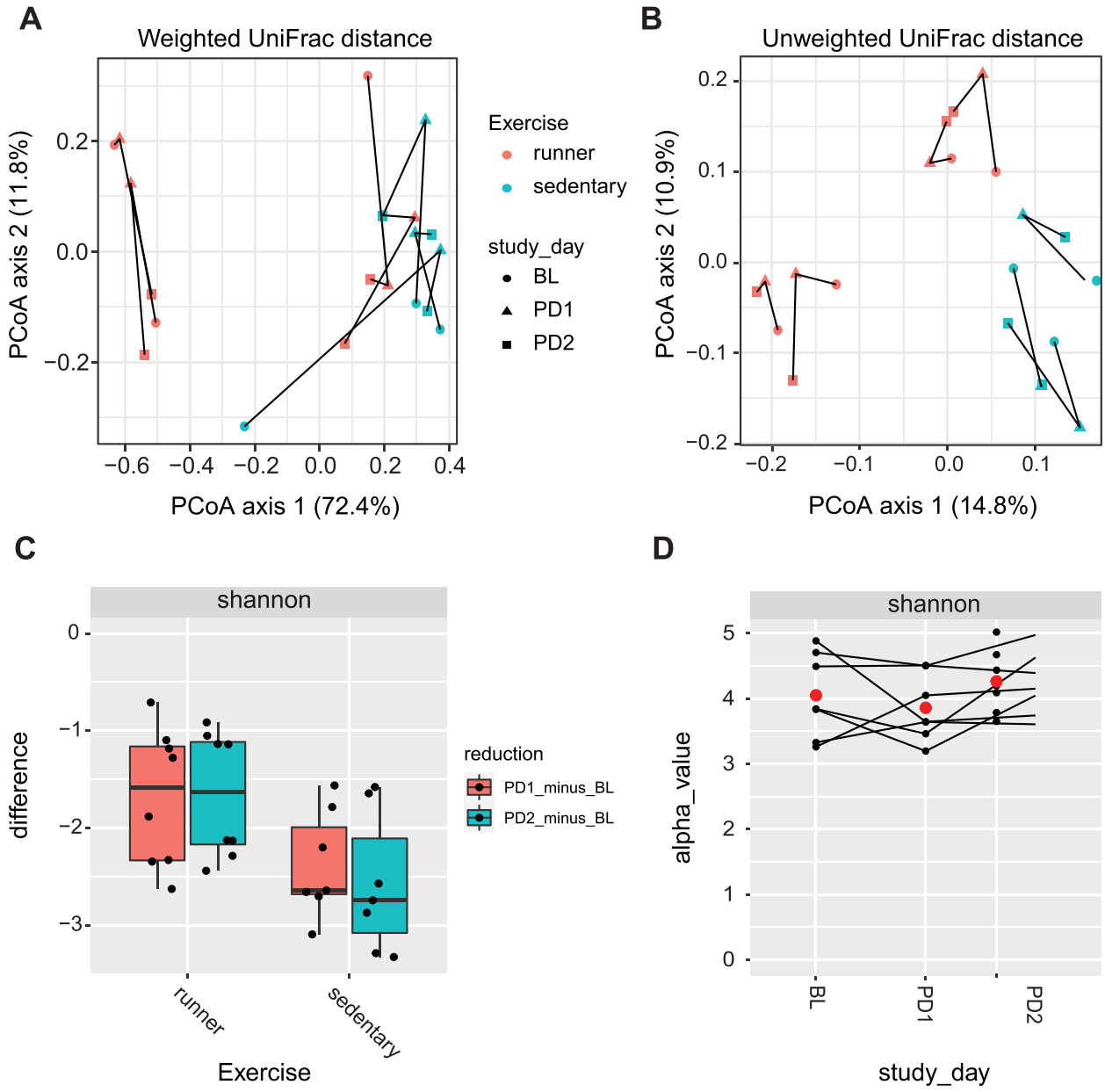

Figure 8. Beta diversity within formalin alone for both weighted (panel A) and unweighted (panel B) UniFrac distance. * = significant difference in distance between Phyla in sedentary vs running wheel rats ($P \leq .05$).

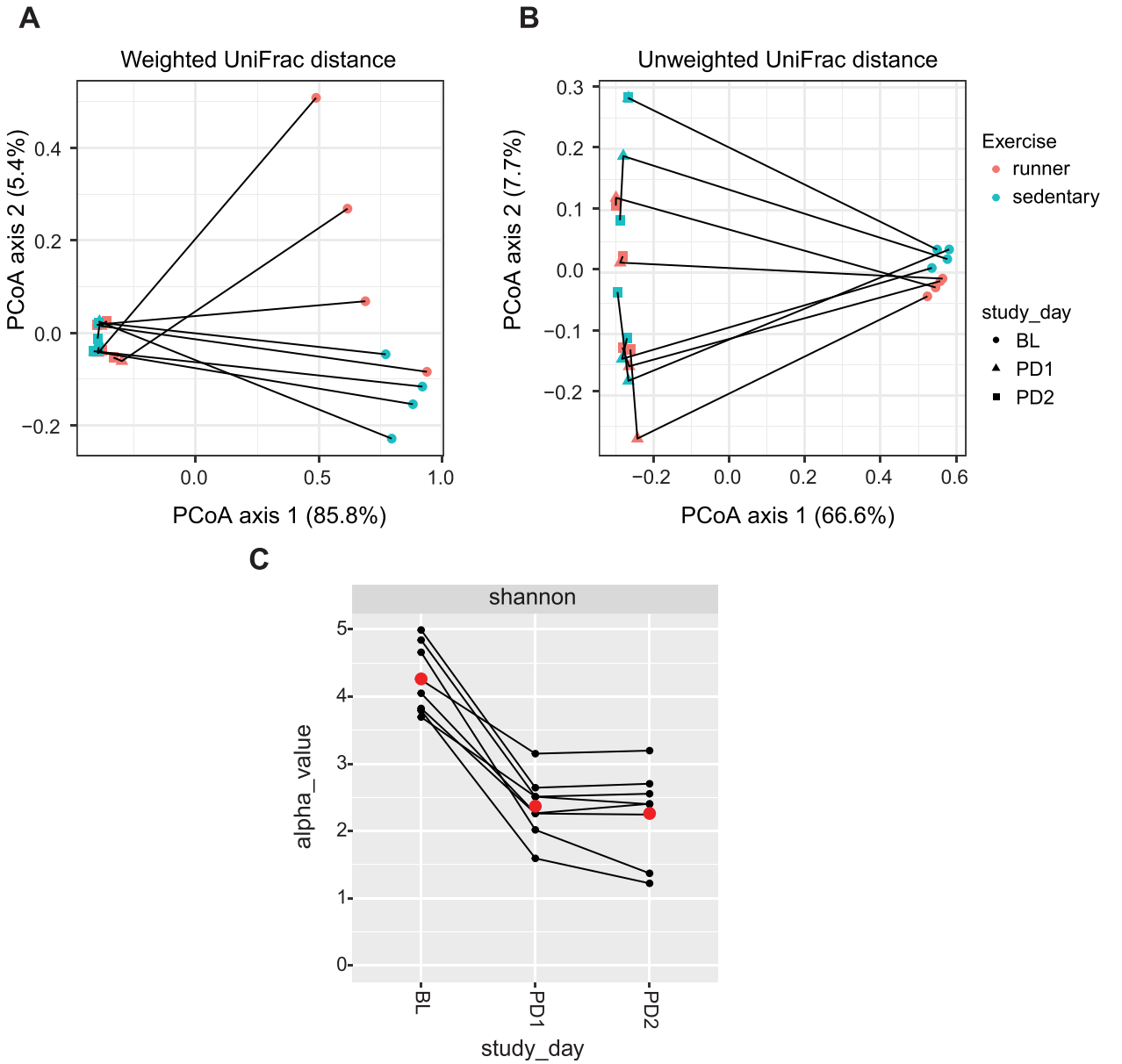

Figure 9. Beta diversity within vancomycin + formalin for both weighted (panel A) and unweighted (panel B) UniFrac distance. There are no differences in distance between Phyla in sedentary vs running wheel rats.

Index ($P \leq$ 2.377e-07; $P \leq$ 1.232e-07). Fecal pellet samples were n = 3 to 4/Group.

Detection of Key Taxa Post-FMT

The ability of FMT to reconstitute populations of *Lactobacillus, Clostridiales*, and *Gammaproteobacteria* to baseline levels (i.e., that of untreated control rats gavaged with PBS) was evaluated by qPCR. Consistent with microbiome sequencing, *Lactobacillus* species were significantly diminished by VANC treatment, and *Gammaproteobacteria* species significantly increased. Fecal pellets collected post-gavage, PD1, and PD2 indicated that FMT to VANC-treated rats restored populations of *Lactobacillus* and *Clostridiales* to baseline levels of the control animals (Fig. 10A, 10B). Similarly, populations of *Gammaproteobacteria* were diminished to baseline levels (Fig 10C). Rats administered PBS or a second dose of VANC rather than FMT remained dysbiotic relative to controls (Fig. 10A-10C). Fecal pellet samples were n = 3 to 4/Group.

Discussion

The present manuscript assessed the ability of the narrow-spectrum antibiotic vancomycin (VANC) to modulate formalin pain-stimulated and pain-depressed behaviors. The main findings were that pretreatment with VANC attenuated formalin Phase II pain-stimulated behaviors, and prevented formalin pain-depressed wheel running. Fecal microbiota transplantation from control donor rats to VANC-treated rats restored populations of *Lactobacillus, Clostridiales*, and *Gammaproteobacteria* back to baseline levels and produced a non-significant trend toward restoration of pain-stimulated

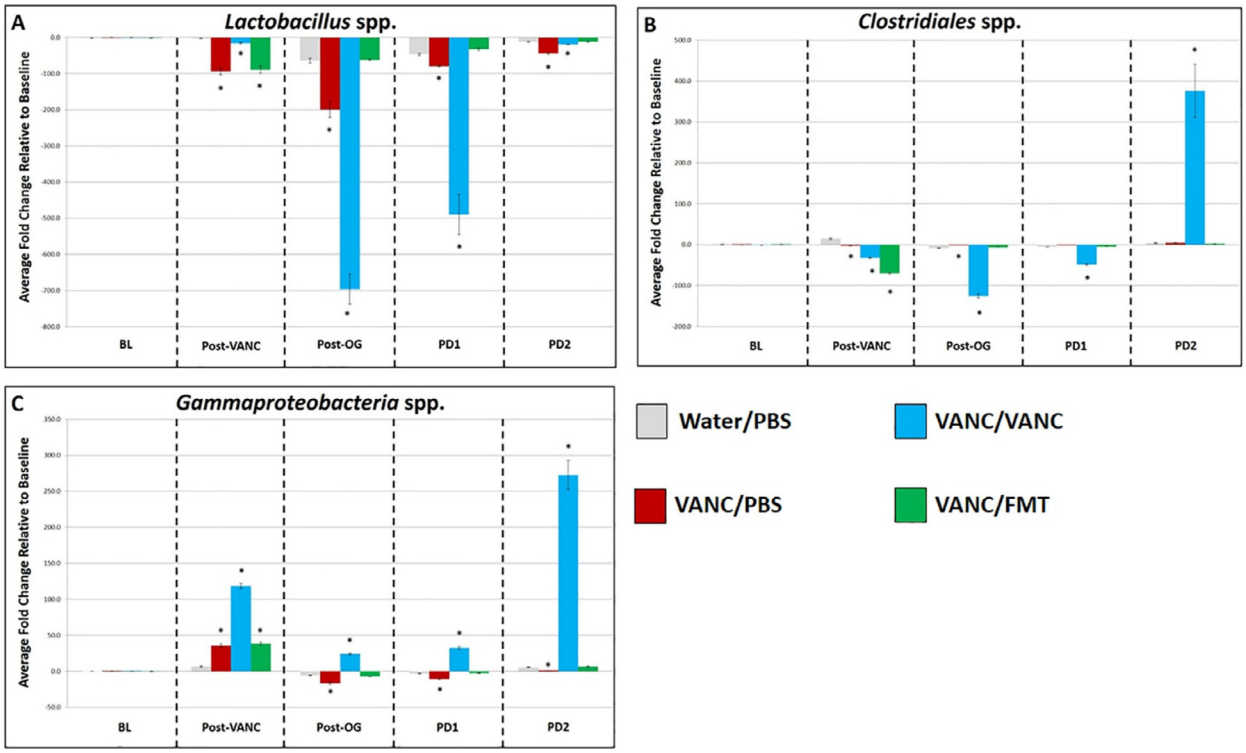

Figure 10. FMT to VANC-treated rats restored populations of *Lactobacillus* and *Clostridiales* to baseline levels of the control animals (panel A, panel B). Populations of *Gammaproteobacteria* were diminished to baseline levels (panel C).

formalin Phase II scores, indicating that the VANC effect may also include mechanisms distinct from alterations in the gut microbiota. An additional finding was that VANC produced distinct changes to a small set of AAs in the gut, consistent with previous reports of an anti-inflammation profile.

This is the first report to show antibiotic-induced modulation of pain-depressed behaviors in rodents. The antibiotic VANC was used as a selective tool to perturb the gut microbiome by depleting Gram positive bacteria, and the 2-week administration regimen prevented formalin pain-depressed wheel running on PD3. VANC also decreased Phase II pain-related scores (20–60 minutes). The utilization of a pain-depressed behavioral endpoint was useful in light of the relevance of these dependent measures as manifestations of overall function and the fact that veterinary and human clinical pain is quantified by the degree to which pain depresses normally adaptive functioning.[43] Additionally, wheel running has been used as both an independent variable to assess the impact of exercise duration on pain,[15,20,50,31,62] and also as a dependent variable to assess the impact of acute and chronic pain states on distance traveled.[9,11,20,28,39,65] The threshold concentration of 0.5% formalin was chosen for subsequent antibiotic – running wheel experiments in order to allow for detection of either antibiotic-increased or antibiotic-decreased effects on formalin pain-related behaviors, and to minimize "floor" effects. Gut microbial alpha and beta diversity were also quantified to determine the extent to which antibiotic administration changed the diversity and abundance of gut microbial taxa (see below).

The delivery of VANC depleted most of the *Firmicutes* and *Bacteroidetes* in the gut, as confirmed by 16S marker gene sequencing technology. This adversely impacted the Shannon Index, which is large if more taxa are present and have a more even abundance distribution. The robust depletion of *Firmicutes* and *Bacteroidetes* was associated with attenuated pain-stimulated behaviors and prevention of pain-depressed behavior. The seemingly therapeutic effects of VANC are consistent with recent reports on antibiotics reducing progression and/or severity of pain scores in rodent models of inflammatory and neuropathic pain.[27,44,58] A closer examination of taxa abundance for our PD1 and PD2 data relative to baseline, indicates that some taxa showed unique profiles across cohorts. For example, abundance of *Firmicutes* and *Bacteroidetes* is predominantly down, although the specific taxa *Lactobacillus* and *Clostridiales* were partially spared. In contrast, abundance of *Enterobacteriaceae*, and notably the genus *Klebsiella* was increased. There is a rich literature on the beneficial effects of Lactobacillus and the inflammation-reduction properties of Class IV and XIV *Clostridiales*. Thus, it is possible that the incomplete suppression of these taxa may in some way be linked to the therapeutic effects of VANC in our data. However, a more granular approach is necessary to ascertain the degree to which this may or may not be true, as the interactions among remaining taxa are unknown and little explored in the literature.

An emerging literature suggests that, under some conditions, different antibiotic classes decrease the amplitude, frequency and/or duration of pain-like behaviors.[5,44,58,70] The present study adds to the

literature that it is possible to attenuate pain behaviors with use of an antibiotic. The data reported here using a persistent inflammatory pain manipulation are consistent with the findings of Shen *et al*. showing VANC-induced reduction in pain-stimulated behaviors using a neuropathic chemical pain manipulation.[58] In that report, vincristine produced tactile sensitivity and VANC was found to block this pain-stimulated behavior. Their findings that FMT blocked the antibiotic effect on vincristine-related pain behaviors is consistent with the trend of the present FMT results reported below, although our FMT reversal on pain-related behavior was not significant.

The present experiments utilized both exercised rats and sedentary rats. It is reasonable to assume that exercise would produce distinct changes to the gut and/or behavior and potentially be a confounding variable. Although VANC produced no significant differences to gut microbes in exercised vs sedentary conditions, VANC produced decreases to Phase II scores only in runners but not sedentary controls, although there was a trend toward pain score attenuation in the latter group. Principle Coordinates Analysis was used to determine the differences in taxa abundance and diversity between runners and sedentary groups. Principle Coordinates Analysis showed that untreated rats have a significant difference in their Beta diversity depending on their conditioning (runners versus sedentary), but that this difference disappears for rats treated with VANC. It is also possible that longer duration wheel running protocols (e.g., 2 or 3 weeks acquisition; 7 or more days post-formalin running) would yield distinct changes to the gut microbiota relative to sedentary controls, and therefore yield more divergent pain-depressed outcomes.

Antibiotic-induced dysbiosis can be reversed by FMT.[2,56,59,73] In the present experiments, FMT was performed with the goal of reversing the VANC-induced modulation to formalin pain-related behaviors, regardless of the direction of antibiotic-induced change. Reconstitution of key taxa (*i.e*., the genus *Lactobacillus*, the order *Clostridiales*, and the phylum *Gammaproteobacteria*) following FMT was confirmed by qPCR. VANC attenuated Phase II pain-stimulated scores and there was a trend toward reversal by FMT. Consistent with the trend toward FMT reversal, the literature indicates that the antibiotic VANC is not absorbed systemically following oral administration.[13,14,16,22,72] Changes in intestinal AA concentrations can be evidence of one or more of the following: (1) membrane changes to bacteria, (2) membrane changes to host gut wall, or (3) changes to AA consumption or production commensurate with changes to bacterial populations in the gut. In the present set of experiments, we determined the degree to which an oral regimen of VANC modulated AA concentrations in the gut. Our findings indicated that of the 16 AAs quantified, VANC increased the concentrations of six AAs (arginine, glycine, alanine, proline, valine, and leucine), and decreased the concentrations of two AAs (tyrosine and methionine). Branched-chain AAs including leucine and valine have been reported to promote intestinal development, immune function, and improve overall gut health.[32,54,55] Similarly, arginine and glycine have been documented to improve the integrity of gut tight junctions, reduce inflammation and pro-inflammatory cytokines, and maintain mucosal barrier function in several rodent models of inflammatory bowel disease.[38] Mechanisms for these effects are still under exploration, but several reports show the byproducts of AA catabolism (most commonly short-chain fatty acids) can have anti-inflammatory effects.[52,76] Catabolism of arginine leads to an inhibition of proinflammatory cytokines IL-1 and TNF,[23,30] and production of butyrate, propionate, and acetate from alanine, proline, and valine catabolism have been shown to induce production of the anti-inflammatory cytokines TGF-β, IL-10, and IL-18.[48] Our findings of VANC-induced increases of these AAs against the backdrop of attenuated inflammatory pain severity are highly consistent with these reports, and point toward at least one physiological mechanism by which microbiome changes could impact inflammatory pain states. However, in order to determine whether the markedly changed AAs are truly "functional AAs" defined as AAs that are involved in improvements to host health,[75] will require additional and more granular study.

There are at least two limitations to this study. First, mechanical sensitivity was measured in running wheel rats at PD 1, 2, 3 using von Frey monofilaments (data not shown). Although there was no evidence of mechanical sensitivity at PDs 1-3, it is possible that the probing of the paw with this behavioral measure, at least in part, resulted in further suppression of wheel running across post-injection days. Second, although wheel running and treadmill exercise have been well utilized as independent and dependent variables in the pain field, in the context of this study, exercise via wheel running may be a confounding factor in terms of its ability to restore or protect against a pain-depressed behavioral phenotype and/or enhance overall fitness.[7,11,18,20,68,77]

This report provides evidence that the antibiotic VANC attenuated pain-stimulated and pain-depressed behaviors in a rodent model of persistent inflammatory pain, and that this reduction in pain amplitude was associated with a distinct profile of AA concentrations in the gut. Given that formalin phase II is interpreted as an inflammatory pain model, the present results may suggest that narrow-spectrum antibiotics have some utility in reducing pain amplitude in inflammatory pain conditions in humans. Future studies will explore the potential link between bacterial taxa abundance, AA concentrations, and innate immune cell function. Additional sources for future mechanistic study could include SCFA communication within the gut,

antibiotic-mediated changes to glial cell phenotype and resident immune cells as well as cytokine concentrations at multiple sites in the host.

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References

1. Anderson MJ: A new method for non-parametric multivariate analysis of variance. Austral Ecol 26:32-46, 2001

2. Bafeta A, Yavchitz A, Riveros C, Batista R, Ravaud P: Methods and reporting studies assessing fecal microbiota transplantation: a systematic review. Ann Intern Med 167:34-40, 2017

3. Basbaum AI, Fields HL: Endogenous pain control systems: brainstem spinal pathways and endorphin circuitry. Annu Rev Neurosci 7:309-338, 1984

4. Bee LA, Dickenson AH: Rostral ventromedial medulla control of spinal sensory processing in normal and pathophysiological states. Neurosci 147:786-793, 2007

5. Boyette-Davis J, Dougherty PM: Protection against oxaliplatin-induced mechanical hyperalgesia and intraepidermal nerve fiber loss by minocycline. Exp Neurol 229:353-357, 2011

6. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP: DADA2: high-resolution sample inference from Illumina amplicon data. Nat Methods 13:581-583, 2016

7. Chew C, Sengelaub DR: Neuroprotective effects of exercise on the morphology of somatic motoneurons following the death of neighboring motoneurons. Neurorehab Neural Re 33:656-667, 2019

8. Chriswell ME, Kuhn KA: Microbiota-mediated mucosal inflammation in arthritis. Best Pract Res Clin Rheumatol 33:1-12, 2019

9. Cobos EJ, Ghasemlou N, Araldi D, Segal D, Duong K, Woolf CJ: Inflammation-induced decrease in voluntary wheel running in mice: a non-reflexive test for evaluating inflammatory pain and analgesia. Pain 153:876-884, 2012

10. Coderre TJ, Abbott FV, Melzack R: Behavioral evidence in rats for a peptidergic-noradrenergic interaction in cutaneous sensory and vascular function. Neurosci Lett 15:113-118, 1984

11. Cormier J, Cone K, Lanpher J, Kinens A, Henderson T, Liaw L, Bilsky EJ, King T, Rosen CJ, Stevenson GW: Exercise reverses pain-related weight asymmetry and differentially modulates trabecular bone microarchitecture in a rat model of osteoarthritis. Life Sci 180:51-59, 2017. PMID: 28504116

12. Craft RM: Modulation of pain by estrogens. Pain 132: S3-S12, 2007

13. DeStefano IM, Wayne AS, Rozanski EA, Babyak JM: Parenterally administered vancomycin in 29 dogs and 7 cats (2003 −2017). J Vet Intern Med 33:200-207, 2019

14. Drennan PG, Begg EJ, Gardiner SJ, Kirkpatrick CMJ, Chambers ST: The dosing and monitoring of vancomycin: what is the best way forward? Int J Antimicrob Agents 401-407, 2019

15. Duman CH, Schlesinger L, Russell DS, Duman RS: Voluntary exercise produces antidepressant and anxiolytic behavioral effects in mice. Brain Res 1199:148-158, 2009

16. Elbarbry F: Vancomycin dosing and monitoring: critical evaluation of the current practice. Eur J Drug Metab Pharmacokinet 43:259-268, 2018

17. Fillingim RB, King CD, Ribeiro-Dasilva MC, Rahim-Williams B, Riley JL: Sex, gender, and pain: a review of recent clinical and experimental findings. J Pain 10:447-485, 2009

18. Fonseca H, Moreira-Goncalves D, Esteves JL, Viriato N, Vaz M, Mota MP, Duarte JA: Voluntary exercise has long-term in vivo protective effects on osteocyte viability and bone strength following ovariectomy. Calcif Tissue Int 88:443-454, 2011

19. Gao H, Shu Q, Chen J, Fan K, Xu P, Zhou Q, Li C, Zheng H: Antibiotic exposure has sex-dependent effects on the gut microbiota and metabolism of short-chain fatty acids and amino acids in mice. mSystems 4::e00048-19, 2019

20. Grace PM, Fabisiak TJ, Green-Fulgham SM, Anderson ND, Strand KA, Kwilasz AJ, Galer EL, Walker FR, Greenwood BN, Maier SF, Fleshner M, Watkins LR: Prior wheel running attenuates neuropathic pain. Pain 157:2012-2023, 2016

21. Greenspan JD, Craft RM, LeResche L, Arendt-Nielsen L, Berkley KJ, Fillingim RB, Gold MS, Holdcroft A, Lautenbacher S, Mayer EA, Mogil JS, Murphy AZ, Traub RJ: Studying sex and gender differences in pain and analgesia: a consensus report. Pain 132:S26-S45, 2007

22. Haak BW, Lankelma JM, Hugenholtz F, Belzer C, de Vos WM, Wiersinga WJ: Long-term impact of oral vancomycin, ciprofloxacin and metronidazole on the gut microbiota in healthy humans. J Antimicrob Chemother 74:782-786, 2019

23. Haskó G, Kuhel DG, Marton A, Nemeth ZH, Deitch EA, Szabó C: Spermine differentially regulates the production of interleukin-12 p40 and interleukin-10 and suppresses the release of the T helper 1 cytokine interferon-gamma. Shock Augusta Ga 14:144-149, 2000

24. Hernandez G, Mills TS, Rabe JL, Chavez JS, Kuldanek S, Kirkpatrick G, Noetzli L, Jubair WK, Zanche M, Myers JR, Stevens BM, Fleenor CJ, Adane B, Dinarello CA, Ashton J, Jordan CT, Di Paola J, Hagman JR, Holers VM, Kuhn KA, Peitras EM: Pro-inflammatory cytokine blockade attenuates myeloid expansion in a murine model of rheumatoid arthritis. Haematologica 105:585-597, 2020

25. Hoban AE, Moloney RD, Golubeva AV, McVey Neufeld KA, O'Sullivan O, Patterson E, Stanton C, dinan TG, Clarke G, Cryan JF: Behavioral and neurochemical consequences of chronic gut microbiota depletion during adulthood in the rat. Neurosci 339:463-477, 2016

26. Hunskaar S, Fasmer OB, Hole K: Formalin test in mice, a useful technique for evaluating mild analgesics. J Neurosci Methods 14:69-76, 1985

27. Jubair WK, Hendrickson JD, Severs EL, Schulz HM, Adhikari S, Ir D, Pagan JD, Anthony RM, Robertson CE, Frank DN, Banda NK, Kuhn KA: Modulation of inflammatory

arthritis in mice by gut microbiota through mucosal inflammation and autoantibody generation. Arthritis Rheum 70:1220-1233, 2018

28. Kandasamy R, Calsbeek JJ, Morgan MM: Home cage wheel running is an objective and clinically relevant method to assess inflammatory pain in male and female rats. J Neurosci Methods 263, 2016. 155-122

29. Kandel ER, Schwartz JH, Jessel TM, Siegelbaum SA, Hudspeth AJ: Principles of Neural Science, 5th edn. NY, McGraw Hill, 2013

30. Kibe R, Kurihara S, Sakai Y, Suzuki H, Ooga T, Sawaki E, Muramatsu K, Nakamura A, Yamashita A, Kitada Y, Kakeyama M, Benno Y, Matsumoto M: Upregulation of colonic luminal polyamines produced by intestinal microbiota delays senescence in mice. Sci Rep 4:45-48, 2014

31. Leung A, Gregory NS, Allen LA, Sluka KA: Regular physical activity prevents chronic pain by altering resident muscle macrophage phenotype and increasing interleukin-10 in mice. Pain 157:70-79, 2016

32. Liu H, Wang J, He T, Becker S, Zhang G, Li D, Ma X: Butyrate: a double-edged sword for health? Adv Nutr 9:21-29, 2018

33. Loftfield E, Vogtmann E, Sampson JN, Moore SC, Nelson H, Knight R, Chia N, Sinha R: Comparison of collection methods for fecal samples for discovery metabolomics in epidemiologic studies. Cancer Epidemiol Biomarkers Prev 25:1483-1490, 2016

34. Lozupone C, Knight R: UniFrac: a new phylogenetic method for comparing microbial communities. Appl Environ Microbiol 71:8228-8235, 2005

35. Lozupone CA, Hamady M, Kelley ST, Knight R: Quantitative and qualitative beta diversity measures lead to different insights into factors that structure microbial communities. Appl Environ Microbiol 73:1576-1585, 2007

36. Martin CR, Osadchiy V, Kalani A, Mayer EA: The brain-gut-microbiome axis. Cell Mol Gastroenterol Hepatol 6:133-148, 2018

37. McDonald D, Price MN, Goodrich J, Nawrocki EP, DeSantis TZ, Probst A, Andersen GL, Knight R, Hugenholtz P: An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. ISME J 6:610-618, 2012

38. Mcgaha TL, Huang L, Lemos H, Metz R, Mautino M, Prendergast GC, Mellor AL: Amino acid catabolism: a pivotal regulator of innate and adaptive immunity. Immunol Rev 249:135-157, 2012

39. Miller LL, Picker MJ, Schmidt KT, Dykstra LA: Effects of morphine on pain-elicited and pain-suppressed behavior in CB1 knockout and wildtype mice. Psychopharmacol 215:455-465, 2011

40. Nakagawa S, Cuthill IC: Effect size, confidence interval and statistical significance: a practical guide for biologists. Biol Rev 82:591-605, 2007

41. National Research Council: Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research. Washington, DC

42. Negus SS, Vanderah TW, Brandt MR, Bilsky EJ, Becerra L, Borsook D: Preclinical assessment of candidate analgesic drugs: recent advances and future challenges. J Pharmacol Exp Ther 319:507-514, 2006

43. Negus SS: Core outcome measures in preclinical assessment of candidate analgesics. Pharmacol Rev 71:225-266, 2019

44. Nieuwenhuis EES, Visser MR, Kavelaars A, Cobelens PM, Fleer A, Harmsen W, Verhoef J, Akkermans LMA, Heijnen CJ: Oral antibiotics as a novel therapy for arthritis. Arthritis Rheum 43:2583-2589, 2000

45. Ni J, Shen T-CD, Chen EZ, Bittinger K, Bailey A, Roggiani M, Sirota-Madi A, Friedman ES, Chau L, Lin A, Nissim I, Scott J, Lauder A, Hoffman C, Rivas G, Albenberg L, Baldassano RN, Braun J, Xavier RJ, Clish CB, Yudkoff M, Li H, Goulian M, Bushman FD, Lewis JD, Wu GD: A role for bacterial urease in gut dysbiosis and Crohn's disease. Sci Transl Med 9: eaah6888, 2017

46. NIH Policy on Sex as a Biological Variable. National Institutes of Health, Office of Research on Women's Health, searched on 9-27-20. Available at: https://orwh.od.nih.gov/sex-gender/nih-policy-sex-biological-variable. Accessed June 14, 2021.

47. O'Mahony SM, Felice VD, Nally K, Savignac HM, Claesson MJ, Scully P, Woznicki J, Hyland NP, Shanahan F, Quigley EM, Marchesi JR, O'Toole PW, Dinan TG, Cryan JF: Disturbance of the gut microbiota in early-life selectively affects visceral pain in adulthood without impacting cognitive or anxiety-related behaviors in male rats. Neurosci 277:885-901, 2014

48. Oliphant K, Allen-Vercoe E: Macronutrient metabolism by the human gut microbiome: major fermentation by-products and their impact on host health. Microbiome 7:1-15, 2019

49. Ossipov MH, Lai J, Malan Jr TP, Porreca F: Spinal and supraspinal mechanisms of neuropathic pain. Ann NY Acad Sci 909:12-24, 2000

50. Patten AR, Sickmann H, Hryciw BN, Kucharsky T, Parton R, Kernick A, Christie BR: Long-term exercise is needed to enhance synaptic plasticity in the hippocampus. Cold Spring Harbor Laboratory Press 20:642-647, 2013

51. Picchianti-Diamanti A, Rosado MM, D'Amelio R: Infectious agents and inflammation: the role of microbiota in autoimmune arthritis. Front Microbiol 8:1-9, 2018

52. Portune KJ, Beaumont M, Davila A-M, Tomé D, Blachier F, Sanz Y: Gut microbiota role in dietary protein metabolism and helath-related outcomes: the two sides of the coin. Trends Food Sci Technol 57:213-232, 2016

53. Reikvam DH, Erofeev A, Sandvik A, Grcic V, Jahnsen FL, Gaustad P, McCoy KD, Macpherson AJ, Meza-Zepeda LA, Johansen F-E: Depletion of murine intestinal microbiota: effects on gut mucosa and epithelial gene expression. PLoS ONE 6:e17996, 2011

54. Ren M, Zhang SH, Zeng XF, Liu H, Qiao SY: Branched-chain amino acids are beneficial to maintain growth performance and intestinal immune-related function in weaned piglets fed protein restricted diet. Asian Austral J Anim Sci 28:1742-1750, 2015

55. Ren M, Zhang S, Liu X, Li S, Mao X, Zeng X, Qiao S: Different lipopolysaccharide branched-chain amino acids modulate porcine intestinal endogenous β-defensin

expression through the Sirt1 /ERK / 90RSK pathway. J Agric Food Chem 64:337-3379, 2016

56. Sartor RB, Wu GD: Roles for intestinal bacteria, viruses, and fungi in pathogenesis of inflammatory bowel diseases and therapeutic approaches. Gastroenterol 152:327-339, 2017

57. Seifert HA, Benedek G, Nguyen H, Gerstner G, Zhang Y, Kent G, Vandenbark AA, Bernhagen J, Offner H: Antibiotics protect against EAE by increasing regulatory and anti-inflammatory cells. Metab Brain Dis 33:1599-1607, 2018

58. Shen S, Lim G, You Z, Ding W, Huang P, Ran C, Doheny J, Caravan P, Tate S, Hu K, Kim H, McCabe M, Huang B, Xie Z, Kwon D, Chen L, Mao J: Gut microbiota is critical for the induction of chemotherapy-induced pain. Nat Neurosci 20:1213-1216, 2017

59. Shen TC, Albenberg L, Bittinger K, Chehoud C, Chen YY, Judge CA, Chau L, Ni J, Sheng M, Lin A, Wilkins BJ, Buza EL, Lewis JD, Saikhin Y, Nissim I, Yudkoff M, Bushman FD, Wu GD: Engineering the gut microbiota to treat hyperammonemia. J Clin Invest 125:2841-2850, 2015

60. Sherwin E, Sandhu KV, Dinan TG, Cryan JF: May the force be with you: the light and dark sides of the microbiota-gut-brain axis in neuropsychiatry. CNS Drugs 30:1019-1041, 2016

61. Singh V, Yeoh BS, Abokor AA, Golonka RM, Tian Y, Patterson AD, Joe B, Heikenwalder M, Vijay-Kumar M: Vancomycin prevents fermentable fiber-induced liver cancer in mice with dysbiotic gut microbiota. Gut Microbes 11:1077-1091, 2020

62. Smith MA, Pitts EG: Wheel running decreases the positive reinforcing effects of heroin. Pharmacol Rep 64:960-964, 2012

63. Song SJ, Amir A, Metcalf JL, Amato KR, Xu ZZ, Humphrey G, Knight R: Preservation methods differ in fecal microbiome stability, affecting suitability for field studies. mSystems 1, 2016. e00021-16

64. Stevenson GW, Bilsky EJ, Negus SS: Targeting pain-suppressed behaviors in preclinical assays of pain and analgesia: effects of morphine on acetic acid-suppressed feeding in C57BL/6J mice. J Pain 6:408-416, 2006

65. Stevenson GW, Mercer M, Cormier J, Dunbar C, Benoit L, Adams C, Jezierski J, Luginbuhl A, Bilsky EJ: Monosodium iodoacetate-induced osteoarthritis produces pain-depressed wheel running in rats: implications for preclinical behavioral assessment of chronic pain. Pharmacol Biochem Behav 98:35-42, 2011

66. Tanes C, Bittinger K, Gao Y, Friedman ES, Nessel L, Paladhi UR, Chau L, Panfen E, Fischbach MA, Braun J, Xavier RJ, Clish CB, Li H, Bushman FD, Lewis JD, Wu GD: Role of dietary fiber in the recovery of the human gut microbiome and its metabolome. Cell Host Microb, 2021. S1931-3128 (20)30674-0

67. Tappe-Theodor A, King T, Morgan MM: Pros and cons of clinically relevant methods to assess pain in rodents. Neurosci Biobehav Rev 100:335-343, 2019

68. Thompson RS, Roller R, Greenwood BN, Fleshner M: Wheel running improves REM sleep and attenuates stress-induced flattening of diurnal rhythms in F344 rats. Stress 19:312-324, 2016

69. Uribe-Herranz M, Bittinger K, Rafail S, Guedan S, Pierini S, Tanes C, Ganetsky A, Morgan MA, Gill S, Tanyi JL, Bushman FD, June CH, Facciabene A: Gut microbiota modulates adoptive cell therapy via CD8α dendritic cells and IL-12. JCI Insight 3:e94952, 2018

70. Valdes C, Bustos G, Martinez JL, Laurido C: Antinociceptive antibiotic-loaded into solid lipid nanoparticles of prolonged release: measuring pharmacological efficiency and time span on chronic monoarthritis rats. PLoS ONE 13: e0187473, 2018

71. Vogtmann E, Chen J, Amir A, Shi J, Abnet CC, Nelson H, Knight R, Chia N, Sinha R: Comparison of collection methods for fecal samples in microbiome studies. Am J Epidemiol 185:115-123, 2017

72. Waineo MF, Kuhn TC, Brown DL: The pharmacokinetic/pharmacodynamic rationale for administering vancomycin via continuous infusion. J Clin Pharm Ther 40:259-265, 2015

73. Weingarden AR, Vaughn BP: Intestinal microbiota, fecal microbiota transplantation, and inflammatory bowel disease. Gut Microb 8:238-252, 2017

74. Wilson DB: Meta-analysis macros for SAS, SPSS, and Stata, and effect size calculator. 2004. Available at: http://mason.gmu.edu/~dwilsonb/ma.html. Accessed June 14, 2021.

75. Wu G: Functional amino acids in nutrition and health. Amino Acids 45:407-411, 2013

76. Yao CK, Muir JG, Gibson PR: Review article: insights into colonic protein fermentation, its modulation and potential health implications. Ailment Pharmacol Ther 43:181-196, 2016

77. Zidon TM, Park WM, Welly RJ, Woodford ML, Scroggins RJ, Britton SL, Koch LG, Booth FW, Padilla J, Kanaley JA, Vieira-Potter VJ: Voluntary wheel running improves adipose tissue immunometabolism in ovariectomized low-fit rats. Adipocyte 7:20-34, 2018