# The Wild Rat versus Outbred Laboratory Rat Microbiome

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***Abstract:***

Growing evidence suggests that the gut microbiota are important determinants of physiology, including immune function, and behavior. Previous studies have documented differences in the gut microbiota in model systems such as the laboratory mouse compared to their wild counterparts. However, no studies to date have examined the differences in the gut microbiomes of wild-caught rats and laboratory rats, including the presence of commonly-detected pathogens in wild-caught rats that are absent in laboratory rats. Here, we compared the microbiota of wild-caught rats in Boulder County, Colorado against outbred Sprague Dawley rats using 16S ribosomal RNA gene sequencing data from fecal samples collected over 4 weeks. PCR and serological analyses were also used to help profile influential pathogens found in the wild-caught rat cohort. We detected a wide range of pathobionts among the wild-caught rats, including keystone taxa such as *Helicobacter spp., Klebsiella pneumoniae,* and *Proteus mirabilis*. Firmicutes were more abundant in laboratory rats, while wild-caught rats had greater abundances of Bacteroidetes. These differences in microbial composition may be due to differences in diet and pathogen exposures in varying environments. These data underscore that compositional differences in the gut microbiome should be taken into account when considering the reproducibility, interpretation, and translatability of findings from studies of laboratory-reared rats.

## *1. Introduction:*

The microbiome-gut-brain axis plays an important role in determining risk of stress-related psychiatric disorders. Meanwhile, recent meta-analyses indicate that risk of developing affective disorders is greater for individuals living in urban, relative to rural environments (Peen et al. 2010), potentially due to selective vulnerability of the industrialized microbiota and dietary changes (Sonnenburg and Sonnenburg, 2019). Previous studies of the microbiome-gut-brain axis signaling in rodents have focused on relatively sterile, controlled and microbially deprived environments that are characteristic of laboratory animal facilities. Specific pathogen-free animals are known to have underdeveloped immune systems comparable to that of developing human neonates (Dobson et al., 2019). In the most extreme instances where model animal systems fail to accurately reflect clinical cases, such as in germ-free animals, the immune system is completely compromised and necessitates the use of sterilized equipment, animal housing, chow, water, and compartmentalized facilities, typically in isolators (Qv et al., 2020). In other studies, unplanned adventitial infection of laboratory rodents has caused problems for many research facilities which could affect the validity of the research and the health of the rodents (Ericsson et al., 2017). In addition to the health of the animals, certain zoonotic infections may also place the health of the animal handlers at risk (Roble et al., 2012). It is important to address the concerns and examine the differences in the gut microbiota between wild rats and laboratory rats to account for these problems.

To date, laboratory animals (mainly mice) have been the major study system for microbiota research. In some mouse-related studies, pet-store mice were shown to be exposed to or carry numerous pathogens that are absent in the laboratory rodents (Roble et al. 2012). While there have been several studies that have evaluated the infectious risks of wild and pet store mice; it is rare to find similar studies in rats despite rats having been historically more utilized in

neurological studies of behavior. However, it is becoming increasingly clear that clean, laboratory animals often poorly model aspects of the microbial biology of wild animals, and this concern extends to the study of gut microbiota. This study is designed to address that knowledge gap. Brown Norway rats (*Rattus norvegicus*) are distributed across the world and are frequently seen around Boulder County in various environments. In addition, many pathogens are commonly found in wild rats, which are often animal reservoirs for infectious and potentially zoonotic diseases and may pose a risk for introducing unwanted disease in animal research facilities. Common pathogens found in wild rats include keystone species such as *Helicobacter spp.* (*H. bilis*, *H. ganmani*, *H. hepaticus*, *H. mastomyrinus*, *H. rodentium*, *H. typhlonius*), *Klebsiella pneumoniae*, and *Proteus mirabilis* (Banerjee et al., 2018; Easterbrook et al., 2008).

We hypothesized the differences in laboratory and wild-caught rats' physiology may be due to differences in the composition and diversity of their gut microbiomes. To more fully understand the potential of commonly-detected pathogens' influence on the gut microbiomes of natural reservoir hosts, we designed a study to compare the microbiomes of wild-caught Brown Norway rats from Boulder County against outbred, specific pathogen-free Sprague-Dawley®□ rats from a common laboratory rodent vendor using 16S ribosomal RNA (16S rRNA) gene sequencing. Wild-caught rats after capture and laboratory rats after arrival from the animal vendor facility were tested for a broad-spectrum panel of pathogens in oral, fecal, and fur samples using both polymerase chain reaction (PCR) and serological (Opti-Spot®□) testing approaches. After characterization of all rats in the study for pathogen load, fecal samples were collected on a weekly basis starting on the day of capture or arrival (day 0) and continuing every 7 days (day 7, week 1; day 14, week 2; day 21, week 3; and day 28, week 4) to assess the potential impacts of acclimation to a novel controlled, disinfected/irradiated, and pathogen-free

research animal facility on the rat gut microbiome's composition. To do so, alpha- and beta-diversity analyses of 16S rRNA gene sequence data collected from fecal samples as described above were ran on cohort (wild-caught versus laboratory), sex (female versus male), time elapsed (0, 1, 2, 3, 4 weeks after arrival), and the two-way and three-way interactions between all three factors. Biplot analyses paired with analysis of composition of microbiomes (ANCOM-II) were performed to identify taxa that were differentially abundant between the wild-caught and laboratory rat microbiomes. Lastly, we used a supervised machine learning algorithm to predict cohorts (laboratory versus wild-caught rats) based on microbial features and identified specific pathobionts that may play an important role in the differential physiological and behavioral responses observed between wild-caught and laboratory rats (Taylor et al., 2019).

## *2. Materials and Methods*

### 2.1. Animals:

We used both naive, 6-8 week old Sprague-Dawley®□ rats (*n* = 5 males and *n* = 5 females, specific pathogen-free, Envigo Laboratories, Haslett, MI, USA) and wild-caught Brown Norway (BN) rats, described in more detail below, for these studies. Wild BN rats (*Rattus norvegicus*) were trapped using Sherman-style live traps (15.24 cm height x 15.24 cm width x 40.64 cm length) baited with a mixture of peanut butter (Skippy®□ peanut butter, Hormel Foods LLC), chocolate syrup (Hershey's, The Hershey Co.) and vanilla extract (Safeway Kitchens, Safeway™) placed in several locations throughout the city of Boulder, CO, USA, primarily next to burrows, water sources, and structures (i.e., wooded areas, hills, walls, and outbuildings). Traps were placed at dusk and evaluated at dawn only when ambient temperatures were above 4.44 °C to prevent hypothermia in trapped adult rats; all weanling and subadult rats were released. When handling trapped rats, personal protective equipment included the use of P100 respirators (Model No. 8233, P100 particulate respirator mask, 3M™), long sleeves, pants, nitrile

gloves, and leather gauntlets (Cat. No. CG-S, Humaniac™ Critter Gloves, Animal Care Equipment and Services LLC, Broomfield, CO, USA). Approval for trapping was provided by Integrated Pest Management Services at the University of Colorado Boulder (Boulder, CO, USA). The research described here was conducted in compliance with the *ARRIVE guidelines 2.0: Updated guidelines for reporting animal research* (Kilkenny et al., 2010; Percie du Sert et al., 2020), and all studies were consistent with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*, Eighth Edition (National Research Council, 2011). The Institutional Animal Care and Use Committee at the University of Colorado Boulder approved all procedures (IACUC, protocol no. 2469). All efforts were made to limit the number of animals used and their suffering.

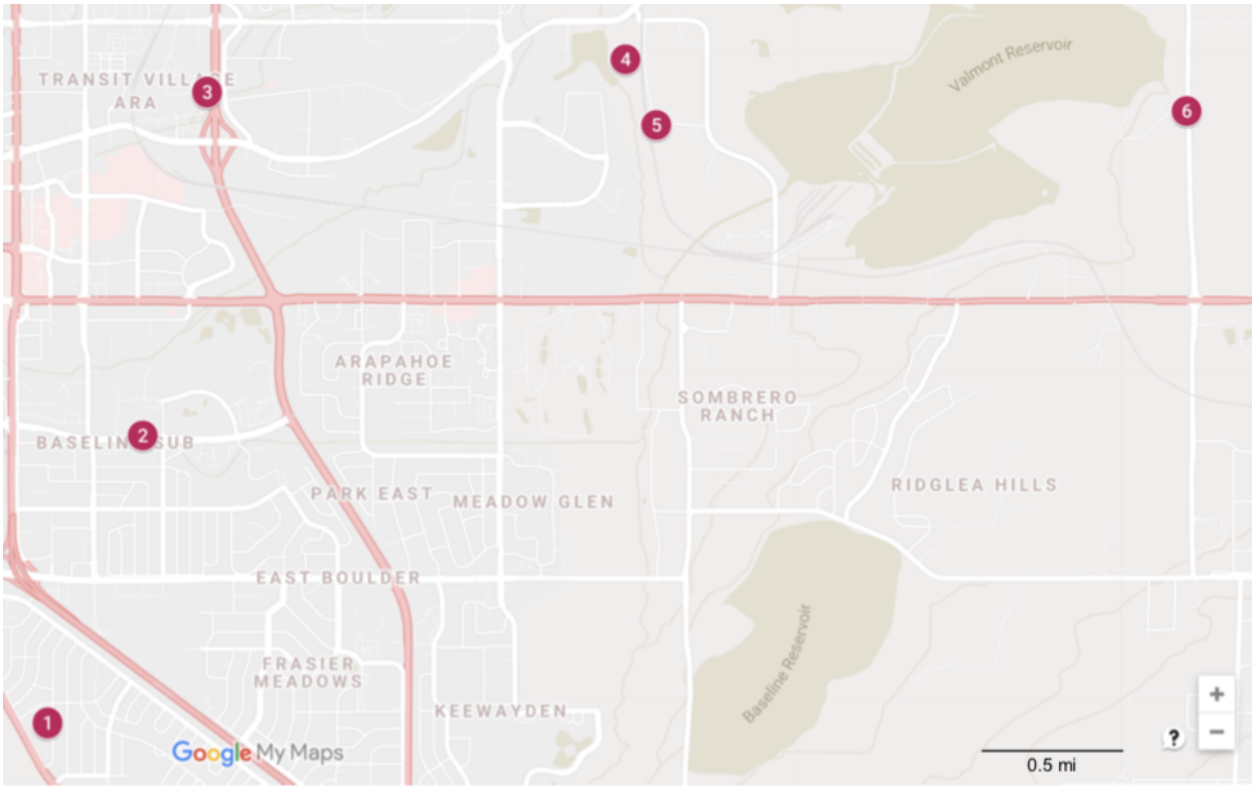


**Figure 1.** Location of adult wild Brown Norway rats caught in Boulder County using Sherman-style live traps for a total of n = 8 males and n = 7 females. Points marked by number in the map correspond to

catch locations as follows: (1) Halcyon, (2) BioFrontiers, (3) Goose Creek, (4) Western Disposal Facility, (5) Gerard Stazio Fields, and (6) Isenhart Farm.

**2.2. Husbandry**:

Wild adult rats caught at each site shown in Figure 1 ($n$ = 1 male, Halcyon; $n$ = 1 male and $n$ = 2 females, BioFrontiers; $n$ = 4 males and $n$ = 3 females, Goose Creek; $n$ = 1 male and $n$ = 1 female, Western Disposal Facility; $n$ = 1 female, Gerard Stazio Fields; $n$ = 1 male, Isenhart Farm) were secured in locked, ventilated and portable secondary containers and transported to the loading dock of the animal research facility, where the exterior was disinfected prior to housing the rats in biohazard containment (ABSL-2 level). Due to the aggressive nature of the wild adult rats, animals were lightly sedated with inhaled isoflurane (2–4%) before removal from the trap using a locking plastic tub as a secondary container. Rats were single-housed in static Allentown micro isolator filter-topped caging (25.9 cm wide × 47.6 cm long × 20.9 cm deep; Cage model No. PC10198HT, Allentown Plastics, Allentown, NJ, USA) containing a 1 cm-deep layer of bedding (approximately 300 g; Cat. No. 7090, Teklad® Sani-Chips, (Envigo, Huntingdon, United Kingdom). Microbarrier cage tops (Cat. No. MBT1019HT, Allentown Plastics) and stainless-steel wire cage lids (Cat. No. WBL1019RSMD, Allentown Plastics) were also used. Rats were given *ad libitum* access to food (Teklad 2918 irradiated rodent chow; Envigo) and sterile reverse osmosis water in bottles (Cat. No. PC16BHT, Allentown Plastics) with screw lids (Cat. No. SPL48RS, Allentown Plastics). Several forms of enrichment were provided to all experimental rats, including a combination of nyla-bones, red tunnels, wood blocks, sunflower seeds, and diamond twists.

Rats were evaluated for sex, weight, and physical condition before fecal and blood samples were collected for testing. Following acclimation, a subset of rats (with IDs 1, 2, and 11) were treated with topical selamectin on the nape of the neck (15 mg, MWI Animal Health,

Aurora, CO, USA), on day 0 and day 30; droncit (praziquantel, 5 mg/kg p.o., MWI Animal Health) on day 0; and daily metronidazole (10 mg/kg p.o., MWI Animal Health, Boise, ID, USA) from days 0-5 to minimize the risk of parasitic mites and helminths contaminating other animals in the vivaria. All other wild-caught rats were not treated for endo- or ecto-parasites in the manner described above to avoid iatrogenic disruption of the microbiome. Cages were changed weekly by the trained Office of Animal Resources (OAR) using inhaled isoflurane anesthesia (2–4%). Naïve, 6-8 week old female Sprague Dawley® rats (specific pathogen-free, Envigo Laboratories) were assigned as soiled-bedding sentinels in rooms where wild-caught rats were housed to assess shedding of pathogenic organisms. Fresh fecal pellets were collected from all rats at day zero (enrollment) and weekly at the time of cage change. Samples were stored in sterile Eppendorf tubes and immediately frozen at -70℃until analysis.

Naive, 6-8 week old Sprague Dawley® rats were pair-housed in the animal research facility under the same housing conditions (cage, bedding, cage top, food, water, temperature, and cage change cycles) as the wild-caught rats. All rats were maintained on a stable normal 12 h:12 h light:dark cycle (lights on at 0700 h, lights off at 1900 h), with temperature maintained at 22 ± 1 °C and relative humidity maintained between 30–50%.

**2.3. Serologic and PCR analysis:**

Evaluation of samples from wild-caught rats for serological reactivity against multiple pathogenic agents was performed by multiplex fluorescent immunoassay (MFI) by IDEXX BioAnalytics technicians (Opti-Spot®□ RADIL Advantage Global Profile, IDEXX BioAnalytics, Columbia, MO, USA).

Opti-Spot®□ Dried Blood Spot Global sampling: cilia-associated respiratory bacillus (CARB), *Clostridium piliforme, Encephalitozoon cuniculi,* Toolan's H-1 parvovirus (H-

1), Hantaan virus, infectious diarrhea of infant rats (IDIR), Kilham's rat virus (KRV), lymphocytic choriomeningitis virus (LCMV), mouse adenovirus strain 1 (MAV1) and 2 (MAV2), *Mycoplasma pulmonis,* generic parvovirus (NS1), *Pneumocystis carinii,* pneumonia virus of mice (PVM), rat coronavirus (RCV)/sialodacryoadenitis virus (SDAV), reovirus 3 (REO3), rat minute virus (RMV), rat parvovirus (RPV), rat polyomavirus 2 (RPyV2), rat theilovirus (RTV), Sendai virus (SV).

Additionally, oral swabs, fecal samples, and fur samples were analyzed by IDEXX BioAnalytics technicians using polymerase chain reaction (PCR) analysis and commercially available reagents (RADIL Advantage Global Profiles, IDEXX BioAnalytics).

Oral swabs: *Bordetella bronchiseptica*, *Bordetella hinzii, Corynebacterium kutscheri, Pasteurella multocida*, *Pasteurella pneumotropica* (biotypes Heyl and Jawetz), *Staphylococcus aureus*, *Streptobacillus moniliformis*, *Streptococcus pneumoniae*, *Streptococcus* sp. β-hemolytic Group A (BHGA), *Streptococcus* sp. β-hemolytic Group B (BHGB), *Streptococcus* sp. β-hemolytic Group C (BHGC), *Streptococcus* sp. β-hemolytic Group G (BHGG).

Fecal samples: Pinworms (*Aspiculuris tetraptera*, *Syphacia muris*, and *Syphacia obvelata*), Boone cardiovirus (BCV), *Campylobacter* spp. (*C. coli* and *C. jejuni*), *Cryptosporidium* spp., *Entamoeba muris*, *Giardia muris*, *Helicobacter spp.* (*H. bilis*, *H. ganmani*, *H. hepaticus*, *H. mastomyrinus*, *H. rodentium*, *H. typhlonius*), *Hymenolepis diminuta*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Rodentolepis nana*, *Salmonella* spp., *Spironucleus muris*, *Tritrichomonas muris.*

Fur samples: Fur mites (*Myocoptes*, *Myobia,* and *Radfordia* spp.).

In consultation with the Center for Disease Control (personal communication, Dr. Martin Schriefer, retired Chief of the Diagnostics Laboratory, Bacterial Diseases Branch, Division of Vector-Borne Diseases), any rat surviving past 3 days following quarantine was considered negative for *Yersinia pestis.*

**2.4. Bacterial DNA extraction and generation of 16S rRNA gene V4 amplicons:**

Bacterial genomic DNA extraction, hypervariable region 4 (V4) amplicon generation from the 16 small subunit ribosomal RNA (16S rRNA) gene, and amplicon preparation for sequences were performed according to protocols benchmarked for the Earth Microbiome Project (EMP) and can be found on the EMP website (http://www.earthmicrobiome.org/emp-standard-protocols/). Briefly, bacterial genomic DNA was extracted from samples using the PowerMag DNA isolation kit optimized for KingFisher Duo® (Cat. No. 27200-4, Mo Bio Laboratories, Carlsbad, CA, USA) according to manufacturer's instructions. Marker genes in isolated DNA were PCR-amplified in triplicate from each sample, targeting V4 of the 16S rRNA gene, modified with a unique 12-base pair (bp) sequence identifier for each sample and the Illumina adapter, as previously described by Caporaso et al. (2012).

The PCR mixtures contained 13 µl Mo Bio PCR water, 10 µl 5'-HotMasterMix, 0.5 µl each of the barcoded forward and reverse primers (515-bp forward: 5'-GTGCCAGCMGCCGCGGTAA-3'; 806-bp reverse: 5'-GGACTACHVGGGTWTCTAAT-3'; Caporaso et al., 2012; 10 µM final concentration, Integrated DNA Technologies, San Diego, CA, USA), and 1.0 µl genomic DNA. Reaction mixtures were held at 94 °C for 3 min, followed by 35 cycles of amplification (94 °C for 45 s, 50 °C for 1 min, and 72 °C for 1.5 min), followed by a final extension at 72 °C for 10 min. After amplification, the DNA concentration was quantified

using PicoGreen™ double-stranded DNA (dsDNA) reagent in 10 mM Tris buffer (pH 8.0) (Cat. No. P11496, Thermo Fisher Scientific). A composite sample for sequencing (16S rRNA gene library) was created by combining equimolar ratios of amplicons from the individual samples, followed by ethanol precipitation to remove any remaining contaminants and PCR artifacts.

**2.5. 16S rRNA gene sequence and data preparation:**

Pooled amplicons were sequenced at the Next Generation Sequencing Core Facility at the University of Colorado Boulder BioFrontiers Genomic Institute using the Illumina MiSeq® platform. The 16S rRNA gene library concentration was measured using the HiSens Qubit dsDNA HS assay kit (Cat. No. Q32854, ThermoFisher Scientific, Waltham, MA, USA). A total of 6 pM of the 16S rRNA gene library combined with 0.9 pM (15%) PhiX sequencing library control v3 (Cat. no. FC-110,3001, Illumina Inc., San Diego, CA, USA) was sequenced with 2 x 300-bp paired-end reads on an Illumina MiSeq® sequencing system using a MiSeq reagent kit V3 (300 cycles; Cat. no. MS-102-2002, Illumina Inc.). FASTQ files for reads 1 (forward), 2 (reverse), and the index (barcode) read were generated using the BCL-to-FASTQ file converter bcl2fastq (ver. 2.17.1.14, Illumina, Inc.).

Sequencing data were prepared and analyzed using the Quantitative Insights Into Microbial Ecology microbiome analysis pipeline (QIIME2 ver. 2020.2, http://qiime2.org; Bolyen et al., 2019). Mapping files and raw sequencing information are publicly available on the microbiome study management platform Qiita (http://qiita.ucsd.edu/study/description/15093; Gonzalez et al., 2018). Briefly, raw sequencing reads were quality-filtered and demultiplexed using the Divisive Amplicon Denoising Algorithm (DADA2) default parameters: expected error threshold of 2, trimming of 6 nucleotides from the start of the forward and reverse reads, truncating at the 243rd nucleotide at the end of the forward reads, and truncating at the 203rd

nucleotide at the end of the reverse reads. Filtered reads were then de-replicated and denoised using DADA2 default parameters to combine identical reads into unique amplicon sequence variants (ASVs) (Callahan et al., 2016) and construct consensus quality profiles for each combined lot of sequences; the consensus quality profiles then inform the de-noising algorithm, which infers error rates from samples and removes identified sequencing errors from the samples. Following processing of raw sequence reads through the DADA2 pipeline, the data were constructed into a feature table of 3,590 unique ASVs with an average read length of 240.93 $\pm$ 3.79 nucleotides in 121 samples submitted to the Next Generation Sequencing Core Facility.

After building the feature table and removing chimeras, phylogenetic trees were built using the SaTe-enabled phylogenetic placement (SEPP) fragment-insertion classifier implemented in QIIME2 using the q2-fragment-insertion plugin (Mirarab et al., 2012; Janssen et al., 2018), trained against the GreenGenes 13_8 99% ribosomal gene reference database built on the 16S rRNA gene V4 region using the same primers as above (Caporaso et al., 2012; McDonald et al., 2011; DeSantis et al., 2006). All samples were subsequently rarefied at 10,000 reads per sample, resulting in the removal of 3 samples (2 wild rat fecal and 1 laboratory rat fecal samples) prior to statistical analysis.

**2.6. Diversity and differential data analysis of 16S rRNA gene sequence data:**

Microbial community structure was characterized using measures of alpha-diversity (within-sample diversity) and beta-diversity (between-samples diversity). Metrics of alpha-diversity included number of distinct features and Pielou's J (Pielou, 1966) to represent species richness, Shannon's diversity index to quantify species abundance and evenness (Shannon et al., 1949), and Faith's phylogenetic diversity, which measures the total length of branches in a reference phylogenetic tree for all species in a given sample (Faith, 1992). Beta-diversity was

calculated using unweighted UniFrac distances (Lozupone and Knight, 2005; Lozupone et al., 2007; Lozupone et al., 2011) depicting community-wide differences in microbial composition amongst fecal samples from laboratory rats and wild-caught rats. Output distance matrices were ordinated using principal coordinate analyses (PCoA) and visualized using EMPeror (Vázquez-Baeza et al., 2013); vectors representing the magnitude and directionality of the top 5 bacterial feature loadings at the phylum level of taxonomic classification were added to each three-dimensional PCoA plot to represent their contributions to the clustering patterns observed.

Prior to statistical analysis of communities (ANCOM; Mandal et al., 2015) to identify taxa driving differences in microbial composition between rat cohorts over time, we pre-processed the ASV table to remove structural zeros from the data matrix and impute sampling zeros with a pseudo-count of 1 prior to analysis as described in the ANCOM-II methods (Kaul et al., 2017). No outlier zeros were identified in R as described in Kaul et al. (2017), so NA values were not substituted for any zero feature counts in the ASV table.

Additionally, we utilized a supervised machine-learning algorithm to predictively classify individual samples as belonging to laboratory or wild-caught rats based on microbial features. Using a nested cross-validated ($k$ = 5 folds) strategy, the receiver-operating characteristic (ROC) area-under-the-curve (AUC) values for the ASV-based models that were used to calculate feature importances were 1.00.

**2.7 Statistical approach:**

Statistical analysis of each alpha-diversity metric described above, using Kruskal-Wallis tests and post hoc pairwise Mann-Whitney *U* tests without adjustment for multiple tests, was conducted on all categorical variables in the metadata describing each sample submitted for 16S rRNA gene sequencing. These variables included rat sex, location caught (for a subset analysis

of wild-caught rats only), domestic versus wild rat status, interactions of status with time elapsed in animal housing, interactions of rat sex by status, and all of the pathogens screened for in the serologic and PCR analysis as described above (for a subset analysis of wild-caught rats at capture, or timepoint 0, only). Statistical significance of beta-diversity distances between groups was assessed using permutational analyses of variance (PERMANOVA) with 999 Monte Carlo permutations and post hoc pairwise permutational *t*-tests (Anderson, 2001). No continuous numerical variables were used in this analysis; subsequently, no Pearson's correlations of alpha-diversity metrics nor Mantel correlations of beta-diversity were performed.

To identify taxa driving differences in microbial composition between cohorts of rats, the pre-processed ASV table was then used in a linear mixed-effects model of rat cohort x elapsed time in weeks using the ANCOM-II implementation in R (source code: http://github.com/FrederickHuangLin/ANCOM). We chose a *W*-statistic cutoff of 0.8 where *p*-values obtained for each taxon was corrected for multiple testing against all other taxa using a Benjamini-Hochberg false discovery rate (FDR) adjustment with significant taxonomic abundance differences reported at an alpha-threshold of 0.05.

## *3. Results*

### 3.1. PCR and serological positivity profiles among wild-caught rats

Among wild-caught rats from Boulder County, PCR analysis of oral swabs taken at capture demonstrated that several rats were actively infected with the following pathogens (Table 1): *Corynebacterium kutscheri* (*n* = 3 positive, 20% prevalence), *Staphylococcus aureus* (*n* = 3 positive, 20% prevalence), and *Streptobacillus moniliformis* (*n* = 13 positive, 80% prevalence). PCR analysis of fur samples taken at capture demonstrated that no wild-caught rats were actively infected with fur mites (*Myocoptes*, *Myobia*, or *Radfordia spp.*; Table 2).

However, active gastrointestinal infection with nearly all of the pathogenic organisms screened by IDEXX Bioanalytics in fecal samples were detected by PCR (Table 3): *Campylobacter spp.* (*n* = 1 positive, 7% prevalence), *Klebsiella oxytoca* (*n* = 4 positive, 27% prevalence), *Klebsiella pneumoniae* (*n* = 4 positive, 27% prevalence), BCV (*n* = 9 positive, 60% prevalence), *Campylobacter coli* (*n* = 1 positive, 7% prevalence), *Campylobacter jejuni* (*n* = 10 positive, 67% prevalence), *Cryptosporidium spp.* (*n* = 7 positive, 47 % prevalence), *Helicobacter spp.* (*n* = 15 positive, 100% prevalence), *Helicobacter bilis* (*n* = 4 positive, 27% prevalence), *Helicobacter mastomyrinus* (*n* = 13 positive, 87% prevalence), *Helicobacter rodentium* (*n* = 15 positive, 100% prevalence), *Proteus mirabilis* (*n* = 3 positive, 20% prevalence), *Entamoeba muris* (*n* = 1 positive, 7% prevalence), *Giardia muris* (*n* = 6 positive, 40% prevalence), *Spironucleus muris* (*n* = 15 positive, 100% prevalence), *Tritrichomonas muris* (*n* = 4 positive, 27% prevalence), *Rodentolepis nana* (*n* = 7 positive, 47% prevalence), and *Syphacia muris* (*n* = 2 positive, 13% prevalence).

**Table 1.** PCR positivity occurrence rates of oral swab samples collected from wild-caught rats from Boulder County (*n* = 15).

| ID | 1 | 2 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sex | F | F | M | M | M | F | M | M | M | F | F | M | F | M | F |
| Catch site | BioFrontiers | BioFrontiers | Halcyon | BioFrontiers | Isenhart Farm | Goose Creek | Goose Creek | Goose Creek | Goose Creek | Goose Creek | Goose Creek | Goose Creek | George Stazio Fields | Western Disposal Facility | Western Disposal Facility |
| Pregnant? | NO | YES | NO | NO | NO | YES | NO | NO | NO | NO | NO | NO | YES | NO | NO |
| *Corynebacterium kutscheri* | NO | YES | NO | NO | NO | NO | NO | YES | NO | NO | NO | YES | NO | NO | NO |
| *Staphylococcus aureus* | YES | NO | NO | NO | NO | YES | NO | YES | NO | NO | NO | NO | NO | NO | NO |
| *Streptobacillus moniliformis* | YES | YES | YES | YES | YES | YES | YES | YES | NO | YES | YES | YES | YES | YES | NO |

**Table 2.** PCR positivity occurrence rates of fur samples collected from wild-caught rats from Boulder County (*n* = 15). All wild-caught rats were tested negative for fur mites.

| ID | 1 | 2 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sex | F | F | M | M | M | F | M | M | M | F | F | M | F | M | F |
| Catch site | BioFrontiers | BioFrontiers | Halcyon | BioFrontiers | Isenhart Farm | Goose Creek | Goose Creek | Goose Creek | Goose Creek | Goose Creek | Goose Creek | Goose Creek | George Stazio | Western Disposal | Western Disposal |

| | | | | | | | | | | | | | Fields | Facility | Facility |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Pregnant? | NO | YES | NO | NO | NO | YES | NO | NO | NO | NO | NO | NO | YES | NO | NO |
| *Myocoptes* | NO | NO | NO | NO | NO | NO | NO | NO | NO | NO | NO | NO | NO | NO | NO |
| *Myobia* | NO | NO | NO | NO | NO | NO | NO | NO | NO | NO | NO | NO | NO | NO | NO |
| *Radfordia spp.* | NO | NO | NO | NO | NO | NO | NO | NO | NO | NO | NO | NO | NO | NO | NO |

**Table 3.** PCR positivity occurrence rates of fecal samples collected from wild-caught rats from Boulder County (*n* = 15).

| ID | 1 | 2 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sex | F | F | M | M | M | F | M | M | M | F | F | M | F | M | F |
| Catch site | BioFrontiers | BioFrontiers | Halcyon | BioFrontiers | Isenhart Farm | Goose Creek | Goose Creek | Goose Creek | Goose Creek | Goose Creek | Goose Creek | Goose Creek | George Stazio Fields | Western Disposal Facility | Western Disposal Facility |
| Pregnant? | NO | YES | NO | NO | NO | YES | NO | NO | NO | NO | NO | NO | YES | NO | NO |
| *Campylobacter coli* | NO | NO | NO | NO | NO | NO | NO | YES | NO | NO | NO | NO | NO | NO | NO |
| *Campylobacter jejuni* | NO | YES | NO | NO | NO | YES | YES | YES | YES | YES | YES | YES | NO | YES | YES |
| *Cryptosporidium spp.* | YES | NO | NO | YES | NO | YES | YES | NO | YES | YES | NO | YES | NO | NO | NO |
| *Helicobacter spp.* | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES |
| *Helicobacter bilis* | YES | YES | NO | NO | NO | NO | NO | NO | NO | NO | NO | NO | YES | YES | NO |
| *Helicobacter mastomyrinus* | YES | YES | YES | YES | NO | YES | YES | YES | YES | YES | YES | YES | YES | NO | YES |
| *Helicobacter rodentium* | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES |
| *Proteus mirabilis* | NO | NO | NO | NO | NO | NO | NO | YES | NO | NO | NO | NO | YES | YES | NO |
| *Entamoeba muris* | NO | NO | NO | NO | YES | NO | NO | NO | NO | NO | NO | NO | NO | NO | NO |
| *Giardia muris* | NO | YES | YES | NO | NO | YES | YES | NO | NO | YES | YES | NO | NO | NO | NO |
| *Spironucleus muris* | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES |
| *Tritrichomonas muris* | NO | NO | YES | NO | YES | YES | NO | NO | NO | NO | YES | NO | NO | NO | NO |
| *Rodentolepis nana* | NO | YES | YES | YES | NO | YES | NO | NO | NO | NO | YES | NO | NO | YES | YES |
| *Syphacia muris* | NO | NO | YES | NO | NO | NO | NO | NO | NO | NO | NO | NO | NO | NO | YES |

Opti-Spot serological analysis detected antibodies produced against current or previous infections with the following pathogens (Table 4): CAR bacillus (*n* = 2 positive, 13% prevalence), *Mycoplasma pulmonis* (*n* = 1 positive, 7% prevalence), MAV2 (*n* = 2 positive, 13% prevalence), *Pneumocystis carinii* (*n* = 3 positive, 20% prevalence), RPV (*n* = 12 positive, 80% prevalence), RMV (*n* = 12 positive, 80% prevalence), KRV (*n* = 11 positive, 73% prevalence),

H-1 (*n* = 11 positive, 73% prevalence), RTV (*n* = 3 positive, 20% prevalence), *Clostridium piliforme* (*n* = 2 positive, 13% prevalence), and *Spironucleus muris* (*n* = 15 positive, 100% prevalence).

**Table 4.** Serological positivity occurrence rates among wild-caught rats from Boulder County (*n* = 15*)*.

| ID | 1 | 2 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sex | F | F | M | M | M | F | M | M | M | F | F | M | F | M | F |
| Catch site | BioFrontiers | BioFrontiers | Halcyon | BioFrontiers | Isenhart Farm | Goose Creek | Goose Creek | Goose Creek | Goose Creek | Goose Creek | Goose Creek | Goose Creek | George Stazio Fields | Western Disposal Facility | Western Disposal Facility |
| Pregnant? | NO | YES | NO | NO | NO | YES | NO | NO | NO | NO | NO | NO | YES | NO | NO |
| CARB | NO | NO | NO | NO | NO | NO | NO | NO | NO | NO | NO | YES | NO | NO | YES |
| *Mycoplasma pulmonis* | NO | NO | NO | NO | NO | NO | NO | NO | NO | NO | NO | NO | NO | NO | YES |
| MAV2* | NO | NO | NO | NO | NO | NO | NO | NO | YES | NO | YES | NO | NO | NO | NO |
| *Pneumocystis carinii* | NO | NO | NO | YES | NO | NO | YES | NO | NO | NO | NO | NO | NO | YES | NO |
| RPV | YES | YES | NO | YES | YES | YES | YES | NO | YES | NO | YES | YES | YES | YES | YES |
| RMV | YES | YES | NO | YES | YES | YES | YES | NO | YES | NO | YES | YES | YES | YES | YES |
| KRV | YES | YES | NO | YES | NO | YES | YES | NO | YES | NO | YES | YES | YES | YES | YES |
| H-1 | NO | YES | NO | YES | NO | YES | NO | YES | YES | YES | YES | YES | YES | YES | YES |
| RTV* | NO | YES | NO | NO | YES | NO | NO | NO | NO | NO | NO | NO | YES | NO | NO |
| *Clostridium piliforme* | NO | YES | NO | NO | NO | YES | NO | NO | NO | NO | NO | NO | NO | NO | NO |
| *Spironucleus muris* | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES |

*Soiled bedding was transferred from all wild-caught rats to sentinel animals as previously described in the Methods. Sentinel rats were tested once during the 4-week period in which this study took place. 1 sentinel rat tested positive for MAV2 and 2 sentinel rats tested positive for RTV during this study, demonstrating that these viral pathogens were shed in feces and could be transmitted to other animals in the facility.

### 3.2. Alpha-diversity differences in the gut microbiome are driven by cohort

In the alpha-diversity analysis of the number of distinct features, there was a notable difference in alpha-diversity between cohorts ($H = 27.1$, $p = 1.97E-07$; Figure 2A, 3A), where laboratory rats had greater alpha-diversity than wild-caught rats. We also observed a sex × cohort ($H = 31.1$, $p = 8.18E-07$; Figure 2A) interaction, in which both female and male laboratory rats were observed to have more distinct features than their wild-caught counterparts ($p = 5.81E-07$ in females, $p = 0.035$ in males; Figure 2A). There were no main effects of rat sex observed.

Alpha-diversity analysis of Faith's phylogenetic diversity reiterated that there was a difference among cohorts ($H = 4.88$, $p = 0.027$; Figure 2B, 3B), such that laboratory rats have higher alpha-diversity than wild-caught rats. We also found a sex × cohort interaction effect ($H = 11.48$, $p = 0.009$; Figure 2B), where female laboratory rats were observed to have higher Faith's phylogenetic diversity values than female wild-caught rats ($p = 6E-4$; Figure 2B) and male laboratory rats ($p = 0.012$; Figure 2B). No differences between male laboratory rats and male wild-caught rats was found. Additionally, no main effects of rat sex or interaction effects of cohort × time elapsed in the animal facility were found.

Shannon's diversity index analysis of cohort (H = 36.6, $p = 1.46E-09$; Figure 2C, 3C) also demonstrated higher alpha-diversity in laboratory rats than in wild-caught rats. In the analysis of sex × cohort (H = 43.0, $p = 2.44E-09$; Figure 2C), female laboratory rats had higher alpha-diversity than female wild-caught rats ($p = 8.29E-10$; Figure 2C) and male laboratory rats had higher alpha-diversity than male wild-caught rats ($p = 0.013$; Figure 2C). While we did observe that female laboratory rats had higher alpha-diversity than male laboratory rats ($p = 0.026$; Figure 2C), there were no main effects of rat sex in the non-parametric analysis shown here. In the analysis of Pielou's J, differences in alpha-diversity amongst cohorts (H = 27.2, $p = 1.86E-07$; Figure 2D) was reiterated, where laboratory rats had higher Pielou's J values against wild-caught rats. We found sex × cohort (H = 34.2, $p = 1.78E-07$; Figure 2D, 3D) interaction effects between female laboratory rats and female wild-caught rats, such that laboratory rats had higher alpha-diversity than wild-caught rats ($p = 4.02E-07$; Figure 2D) and male laboratory rats ($p = 0.048$). Here, we also showed that female wild-caught rats had lower alpha-diversity than male wild-caught rats ($p = 0.034$; Figure 2D). Likewise, there were no main effects of rat sex.

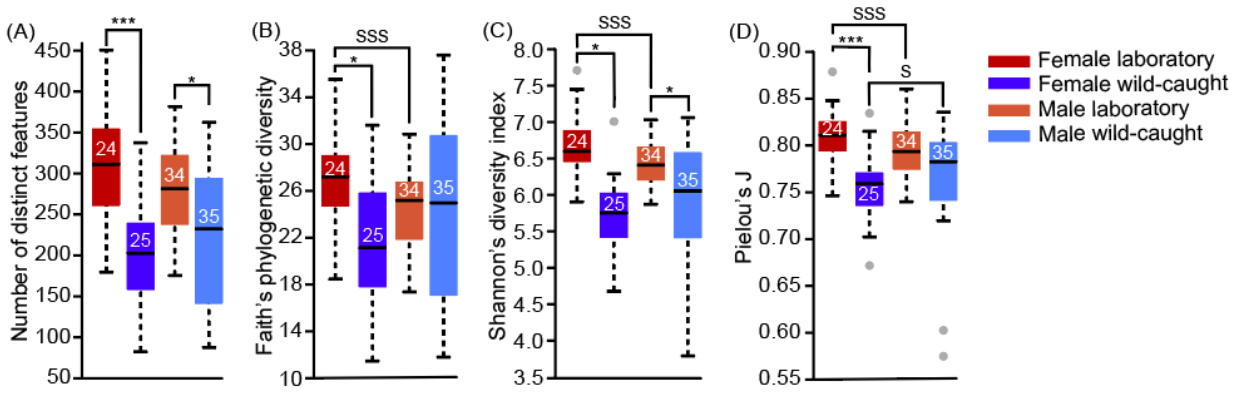


**Figure 2.** Alpha-diversity differences in the gut microbiome based on rat sex x cohort (female laboratory rats, dark red; female wild-caught rats, dark blue; male laboratory rats, orange; male wild-caught rats, light blue) are driven by cohort. (A) Number of distinct features shows that laboratory rats have higher alpha-diversity, regardless of sex, compared to wild-caught rats. (B) Faith's phylogenetic diversity shows higher alpha-diversity levels for female laboratory rats when compared to wild-caught females or male laboratory rats. (C) Shannon's diversity index, an alpha-diversity metric of both evenness and richness, shows the same relationship in alpha-diversity as panel A. Lastly, (D) Pielou's J shows that female laboratory rats have higher alpha-diversity than wild-caught females or laboratory males. Female wild-caught rats had lower alpha-diversity in a comparison against male wild rats. The bottoms and tops of boxes indicate the first and third quartiles, respectively; whiskers indicate the 1.5 interquartile range (IQR) beyond the upper and lower quartiles. Values outside the whiskers are indicated by grey circles; numbers in each box indicate sample sizes. Statistical significance in each categorical variable was evaluated using Kruskal-Wallis tests with post hoc pairwise Mann-Whitney U tests; $p < 0.05$ (*, S) and $p < 0.001$ (***, SSS). Abbreviations: IQR, interquartile range.

### 3.3. Differences in the gut microbial composition of wild-caught rats versus laboratory rats

Compositional biplot analysis of cohort differences in microbial features (Figure 3E) was conducted to examine the clustering of the dataset. We demonstrated that Firmicutes and Tenericutes are more prevalent in laboratory rats (Figure 3E), while Fusobacteria, Bacteroidetes, and Proteobacteria are more abundant in wild-caught rats (Figure 3E). These differences in the presence and relative abundances of specific phyla help explain the distinct clustering of wild-caught rat microbiomes (blue) against laboratory rat samples (red). Dimensionality reduction with PCoA emphasizes that wild-caught rats have greater variance among microbiomes than laboratory rats. Relative taxonomic abundance analysis was used to further illustrate the taxonomic composition and relative abundance differences between each cohort at the phylum level for each sample. From this representation of the data, we found that Firmicutes were the most dominant taxa (based on relative abundance) found in laboratory rats (Figure 3F). Other

dominant phyla in laboratory rat samples (>1% of community composition) included Bacteroidetes, Tenericutes, Actinobacteria, and Verrucomicrobia. In contrast, Bacteroidetes were the most dominant phyla in the microbiomes of wild-caught rats, followed by Firmicutes, Proteobacteria, Fusobacteria, and Spirochaetes (Figure 3F). In stark contrast to the wild-caught rats, there was a conspicuous lower relative abundance of Proteobacteria in fecal samples collected from laboratory rats.

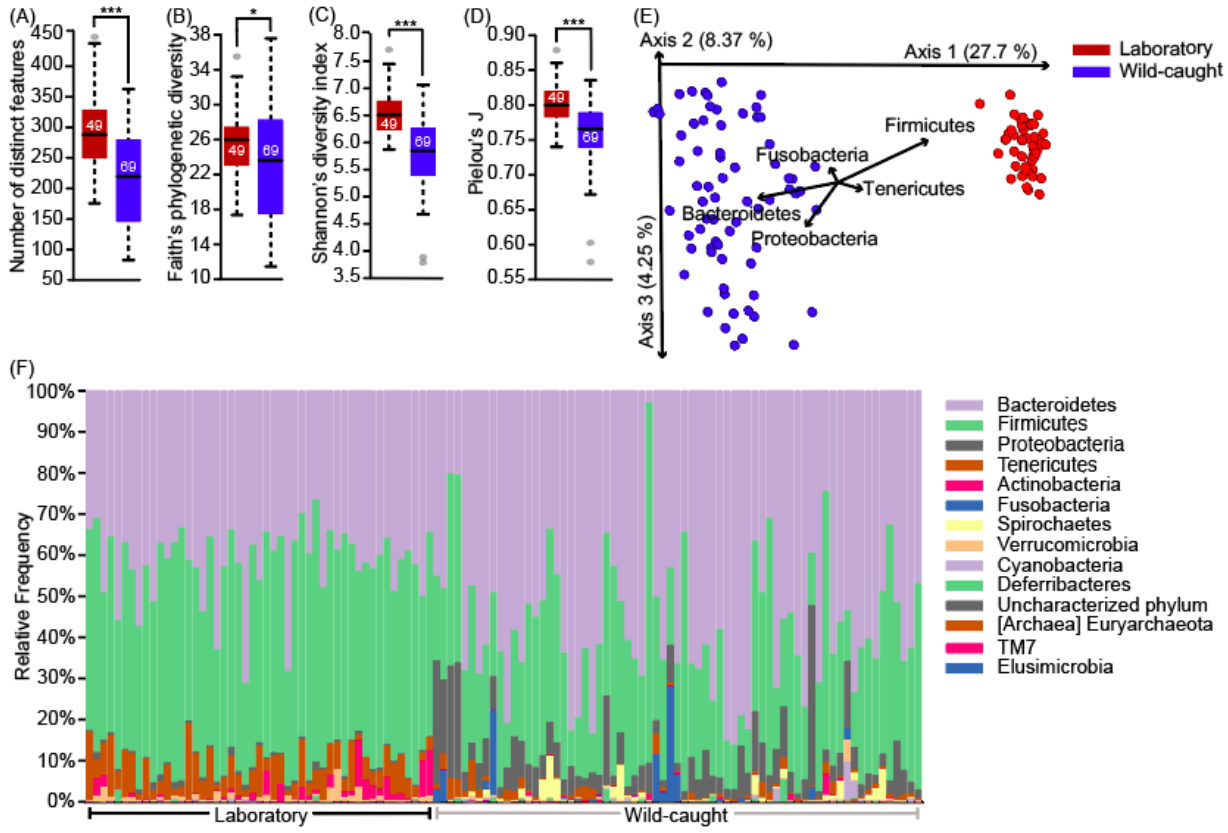


**Figure 3.** Microbiome analysis of the cohort effect on alpha-diversity demonstrates that laboratory rats (red bars) have higher alpha-diversity than wild-caught rats (blue bars). (A-D) Data represent all four alpha-diversity metrics most commonly reported in the literature collapsed by cohort: (A) Number of distinct features (B) Faith's phylogenetic diversity, (C) Shannon's diversity index, and (D) Pielou's J. The bottoms and tops of boxes indicate the first and third quartiles, respectively; whiskers indicate the 1.5 interquartile range (IQR) beyond the upper and lower quartiles. Values outside the whiskers are indicated by grey circles; numbers in each box indicate sample sizes. Statistical significance in each categorical variable was evaluated using pairwise Mann-Whitney U tests at $p < 0.05$. (E) Compositional biplot of the laboratory (red) versus wild-caught (blue) dataset using the unweighted UniFrac distance metric. Arrows indicate the amount and direction of variation of the ratio of each amplicon sequence variant (ASV) to all others in the dataset. The proportion of variation explained by components 1-3 in the principal coordinates analysis (PCoA) plot are also indicated. (F) Relative taxonomic abundance plot indicates that

the vast differences in laboratory (left) versus wild-caught rat (right) composition are driven by the phyla Firmicutes, Bacteroidetes, Proteobacteria, Tenericutes, and Fusobacteria. Laboratory rats show relatively higher relative abundances of Firmicutes, lower relative abundances of Bacteroidetes, and a lower relative abundance of Proteobacteria compared to samples obtained from wild-caught rats. Abbreviations: ASV, amplicon sequence variant; IQR, interquartile range; PCoA, principal coordinates analysis.

### 3.4. Differences in microbial composition driven by cohort × time elapsed in the animal facility

We noted a cohort × timepoint interaction effect in number of distinct features ($H = 27.8$, $p = 0.001$; Figure 4A), where laboratory rats had greater alpha-diversity than wild-caught rats on arrival (week 0; $p = 0.041$; Figure 4A), a difference that is sustained throughout the experiment from week 1 ($p = 0.0091$; Figure 4A), week 2 ($p = 0.035$; Figure 4A), week 3 ($p = 0.022$; Figure 4A), through week 4 ($p = 0.025$; Figure 4A). Similarly, we identified cohort × timepoint interactions in Shannon's diversity index calculations ($H = 39.0$, $p = 1.15\text{E-}5$; Figure 4B). Like in the number of distinct features analysis, we showed that laboratory rats on arrival also had higher Shannon's diversity index values against wild-caught rats on arrival (week 0; $p = 0.008$; Figure 4B), a difference that was sustained at week 1 ($p = 0.020$; Figure 4B), week 2 ($p = 0.006$; Figure 4B), week 3 ($p = 0.003$; Figure 4B), and week 4 ($p = 0.025$; Figure 4B). We also showed that laboratory rats had higher Shannon's diversity index values on arrival (week 0) compared to the end of the experiment (week 4; $p = 0.041$; Figure 4B), a within-cohort decrease in alpha-diversity which was supported by other evenness-based measures. Analysis of microbial evenness (Pielou's J) confirmed an interaction effect of cohort × timepoint ($H = 36.9$, $p = 2.8\text{E-}4$; Figure 4C). We showed that laboratory rats on arrival (week 0) had greater evenness than laboratory rats at subsequent timepoints, specifically at week 1 ($p = 0.027$; Figure 4C) and week 4 ($p = 0.006$; Figure 4C). Furthermore, laboratory rats on week 2 had higher Pielou's J values against laboratory rats on week 4 ($p = 0.019$; Figure 4C); likewise, laboratory rats on week 3 had higher Pielou's J values against laboratory rats on week 4 ($p = 0.049$; Figure 4C). We also showed that laboratory rats on arrival (week 0) had greater evenness compared against wild-

caught rats on arrival ($p$ = 0.002; Figure 4C). This cohort-based difference between laboratory and wild-caught rats was not apparent at week 1 but was apparent in weeks 2 ($p$ = 6.0E-4; Figure 4C) and 3 ($p$ = 0.004; Figure 4C).

Analysis of beta-diversity metrics of the gut microbiota using PERMANOVA demonstrated a cohort × timepoint interaction on gut microbial composition assessed using unweighted UniFac (Figure 4D), and weighted UniFrac (Figure 4E). An initial canvas of major contributors to clustering patterns observed in three-dimensional PCoA plots demonstrated that cohort (e.g., laboratory vs. wild-caught rats) had significant effects on global composition (unweighted UniFrac, *pseudo-F* = 42.4, $p$ = 0.001; Figure 4D). In alignment with the alpha-diversity analyses of cohort × timepoint interactions, we showed that the microbial composition of both laboratory rats and wild-caught rats significantly change over time while acclimating to the animal facility after arrival. For example, laboratory rats showed significant changes in beta-diversity by 1 week of acclimation (week 0 against week 1, $p$ = 0.048, $q$ = 0.062; Figure 4D), a change that persisted for the entire duration of the experiment (week 0 against week 2, $p$ = 0.004, $q$ = 0.038; week 0 against week 3, $p$ = 0.027, $q$ = 0.038; week 0 against week 4, $p$ = 0.007, $q$ = 0.011; Figure 4D). Similarly, wild-caught rats also experienced significant alterations to their gut microbiomes within 1 week of acclimation (week 0 against week 1, $p$ = 0.033, $q$ = 0.045; Figure 4D). This change for the wild-caught rats also persisted for the entirety of this experiment (week 0 against week 2: $p$ = 0.010, $q$ = 0.016; week 0 against week 3: $p$ = 0.014, $q$ = 0.021; week 0 against week 4: $p$ = 0.002, $q$ = 0.004; Figure 4D). Cohort differences in the unweighted UniFrac plot (Figure 4D) were evident at week 0, on arrival ($p$ = 0.001, $q$ = 0.002), and maintained through weeks 1 ($p$ = 0.001, $q$ = 0.002), 2 ($p$ = 0.001, $q$ = 0.002), 3 ($p$ = 0.001, $q$ = 0.002), and 4 ($p$ = 0.001, $q$ = 0.002).

In the weighted UniFrac PERMANOVA results, we demonstrated that laboratory rats on arrival (week 0) showed significant differences in microbial composition against laboratory rats at the final microbiota assessment timepoint, on week 4 ($p = 0.004$, $q = 0.007$; Figure 4E), but showed no differences against weeks 1-3. Wild-caught rats experienced changes in microbial composition on arrival in the animal facility compared to week 1 ($p = 0.034$, $q = 0.055$; Figure 4E), week 2 ($p = 0.045$, $q = 0.070$; Figure 4E), and week 4 ($p = 0.006$, $q = 0.010$; Figure 4E ). Cohort differences in the weighted UniFrac plot (Figure 4E) were maintained at week 0, on arrival ($p = 0.001$, $q = 0.002$), through week 1 ($p = 0.001$, $q = 0.002$), week 2 ($p = 0.001$, $q = 0.002$), week 3 ($p = 0.001$, $q = 0.002$), and week 4 ($p = 0.001$, $q = 0.002$). Despite the timepoint-dependent shifts in microbial communities, there were no points in which the gut microbiota composition of laboratory rats converged with the wild-caught rats.

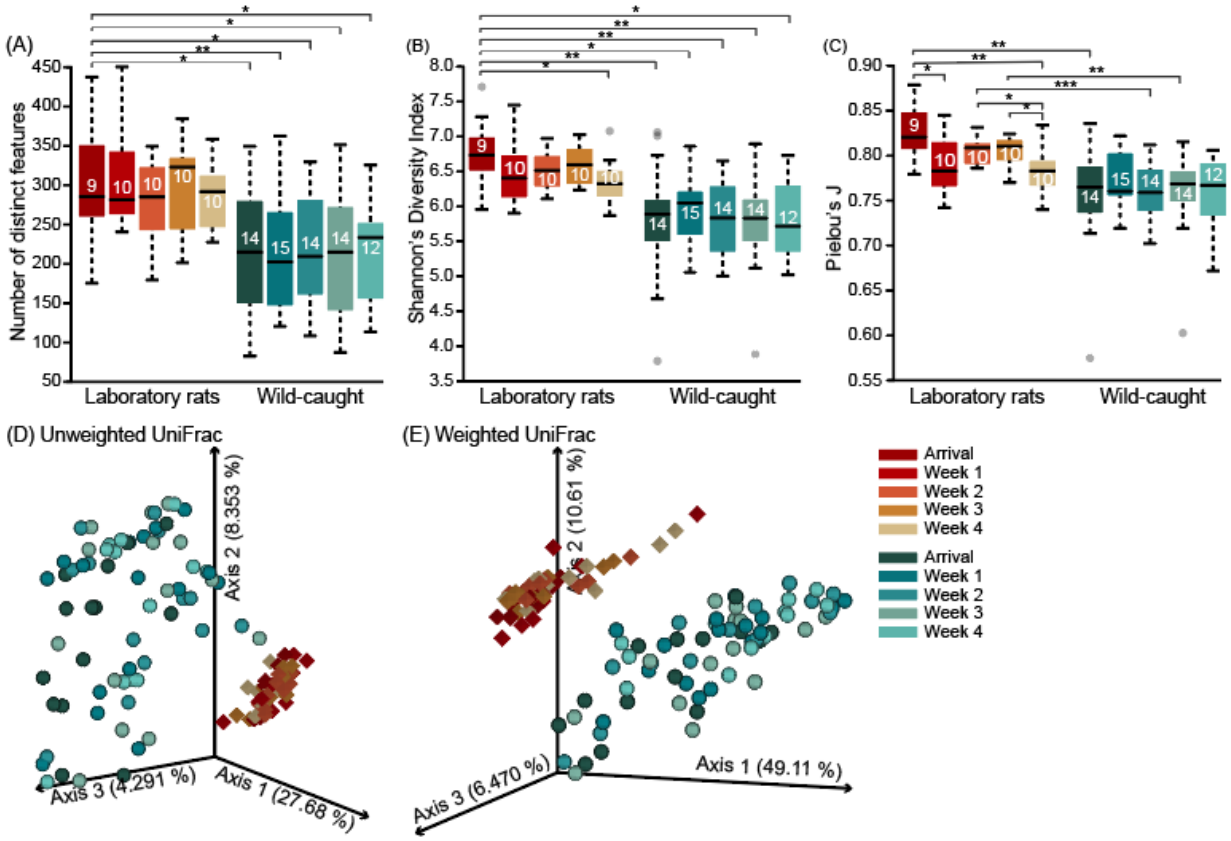


**Figure 4.** Alpha-diversity analysis of cohort (laboratory, shades of red; wild-caught, shades of blue) with time (bar colors lighten as time elapsed in the animal facility increased) where laboratory rats had greater

alpha-diversity than wild-caught rats on arrival. Alpha-diversity analysis is shown for (A) number of distinct features, (B) Shannon's diversity index, and (C) Pielou's J. The bottoms and tops of boxes indicate the first and third quartiles, respectively; whiskers indicate the 1.5 interquartile range (IQR) beyond the upper and lower quartiles. Values outside the whiskers are indicated by grey circles; numbers in each box indicate sample sizes. Statistical significance in each categorical variable was evaluated using Kruskal-Wallis ranks-sums tests with post hoc pairwise Mann-Whitney U tests at $p < 0.05$. Lastly, beta-diversity analysis of cohort x timepoint interactions reveals large-scale shifts in wild rat microbial composition on arrival (week 0) over time in the (D) unweighted UniFrac distance metric and (E) in the weighted UniFrac distance matrix. Bar colors lighten as time elapsed in the animal facility increased. Abbreviations: IQR, interquartile range.

### 3.5. ANCOM-II analysis of microbial community composition between laboratory and wild-caught rats

ANCOM-II identified multiple features above the coefficient of concordance threshold of 0.8 that were differentially abundant in the laboratory rat and wild-caught rat cohorts. To identify drivers of differentiation in microbial communities between the cohorts of rats, we collapsed the ASV table to the species level and re-ran ANCOM using the q2-ancom plugin in QIIME2. Using the ANCOM-II method, we found ten species to be differentially abundant in wild-caught and laboratory rats with a minimum *W*-statistic of 134. Of these species, *Adlercreutzia* sp. in the Actinobacteria phylum (*W*-statistic = 149), *Clostridium* sp. (*W*-statistic = 167), *Ruminococcus flavefaciens* (*W*-statistic = 171), and unknown member of the family Clostridiaceae (*W*-statistic = 169) and an unknown member of the Clostridiaceae sub-family 02d06 (*W*-statistic = 170) in the Firmicutes phylum were found to be more abundant in laboratory rats (Table 5). In contrast, species such as *Adlercreutzia* sp. were present in wild-caught rats but in very low abundance. Wild-caught rats were richly abundant in *Bacteroides plebeius* (*W*-statistic = 161), *Bacteroides eggerthii* (*W*-statistic = 167), *Bacteroides coprophilus* (*W*-statistic = 161), *Prevotella* sp. (*W*-statistic = 178), and *Prevotella copri* (*W*-statistic = 178) in the Bacteroidetes phylum (Table 5). Interestingly, *Prevotella* sp. and *Prevotella copri* were also present in laboratory rats, but in low counts. These findings supported the previous phylum-level biplot analysis shown in Figure 3E and 3F, where Bacteroidetes were demonstrated to be more prevalent in wild-caught rats and Firmicutes were more prevalent in laboratory rats.

**Table 5.** Differential abundance analysis of communities of microbiomes (ANCOM) based on rat cohort.[1]

| Taxonomic assignment | Laboratory rats | | | | | Wild-caught rats | | | | | *W*-statistic |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Number of reads in each quartile | 0 | 25 | 50 | 75 | 100 | 0 | 25 | 50 | 75 | 100 | |
| *Adlercreutzia* sp. (Actinobacteria) | 1 | 15.5 | 28 | 49.25 | 132 | 1 | 1 | 7 | 15 | 86 | 149 |
| *Clostridium* sp. (Firmicutes) | 1 | 21.75 | 54 | 136.5 | 750 | 1 | 1 | 1 | 1 | 160 | 167 |
| *Ruminococcus flavefaciens* (Firmicutes) | 10 | 83.25 | 183 | 341.75 | 719 | 1 | 1 | 1 | 1 | 566 | 171 |
| Unknown member of family Clostridiaceae (Firmicutes) | 1 | 14.5 | 147 | 731 | 1812 | 1 | 1 | 1 | 1 | 593 | 169 |
| Unknown member of family Clostridiaceae, sub-family 02d06 (Firmicutes) | 1 | 45.5 | 182 | 433.75 | 1144 | 1 | 1 | 1 | 1 | 720 | 170 |
| *Bacteroides plebeius* (Bacteroidetes) | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 51 | 289.5 | 6771 | 161 |
| *Bacteroides eggerthii* (Bacteroidetes) | 1 | 1 | 1 | 1 | 1 | 1 | 25 | 95 | 217 | 8104 | 167 |
| *Bacteroides coprophilus* (Bacteroidetes) | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 89 | 597.5 | 8885 | 161 |
| *Prevotella* sp. (Bacteroidetes) | 1 | 1 | 1 | 3 | 11 | 1 | 558 | 2028 | 4030 | 10646 | 178 |
| *Prevotella copri* (Bacteroidetes) | 1 | 1 | 1 | 1 | 12 | 1 | 436 | 1360 | 3747.5 | 11168 | 178 |

[1]ANCOM calculates the pairwise log ratios between all species and performs an ANOVA test to determine if there is a significant difference in the log ratios with respect to the cohort of interest (e.g. wild-caught versus laboratory rats). The *W*-statistic value represents the number of times the null hypothesis was rejected for a given species. Species rated above the coefficient of concordance threshold of 0.8 after a pseudocount of 1 was applied to the data shown here. Abbreviations: ANCOM, Analysis of composition of microbiomes; phylum is indicated in parentheses.

### 3.6. Supervised machine learning classification of samples based on microbial features:

Random forest is a supervised machine learning algorithm that was used to evaluate sample classification of laboratory and wild-caught rats based on microbial features in this analysis. With sufficient computational ability and a relatively small dataset of $n < 50$, a nested cross-validation approach ($k = 5$ folds) was employed to reduce bias and improve model performance (Raschka, 2018). Further unbiased classification of samples based on microbial composition allowed us to better understand how cohort differences affected the gut microbiomes of these rats beyond ANCOM analysis of taxa driving differences. Model

performance and prediction accuracy were based on receiver operating characteristic (ROC) scores that evaluated predictive true positive rates (TPR) against false positive rates (FPR).

Of all the factors previously identified that were associated with differences in alpha- or beta-diversity of wild-caught/ laboratory rats, model performance as shown by area-under-the-curve (AUC) macro- and micro-average values for rat cohort were greater than would have been predicted by chance alone (AUC = 1.00 and 1.00, respectively; Figure 5A). Per class ROC plots showed that the classification of samples to the rat cohort was 100% accurate (Figure 5A). This model shows that samples were always correctly predicted for wild-caught rats and for laboratory rats. The feature importance plot in Figure 5B shows that many bacteria drive the classification of cohort differences, including *Bilophila sp.*, *Bacteroides*, *Tenericutes* in the RF39 lineage, multiple genera in the S24-7 family, and an unknown member of the order Clostridales.

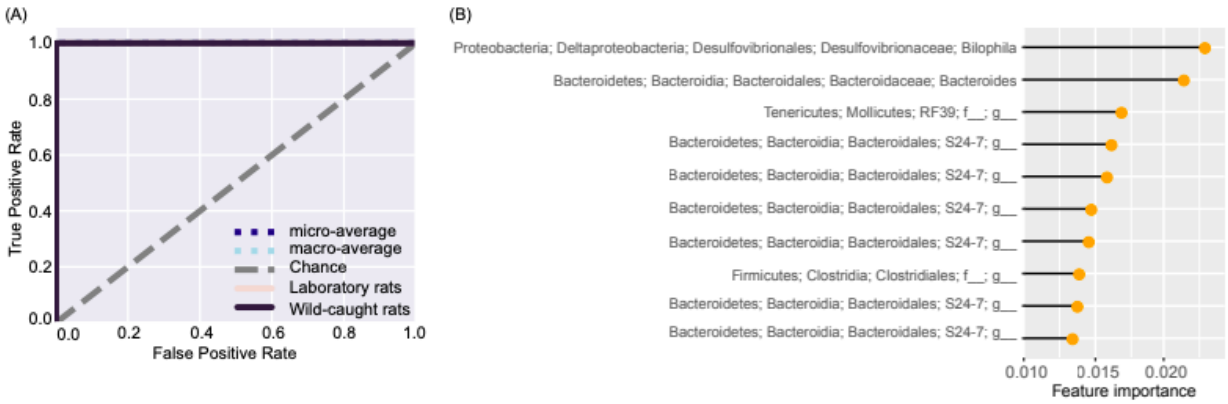


**Figure 5.** Machine learning classification accuracy in a nested cross-validation random forest model built to predict cohort (laboratory versus wild-caught rats) based on features from the gut microbiota dataset in this study. Feature inputs from the raw feature table included in this analysis consisted of all bacteria matched to the GreenGenes 13_8 ribosomal RNA reference database with 99% accuracy. (A) Per-class receiver operating characteristic (ROC) plot shows the macro-average precision (of each cohort equally-weighted, light blue dashed line, AUC = 1.00) and micro-average precision (averaged metrics across each sample, dark blue dashed-line, AUC = 1.00) at several true-positive rate (TPR) against false-positive (FPR) thresholds. Predictive accuracy results for laboratory (tan solid line) and wild-caught rats (black solid line) are also displayed against classification error rates achieved by random chance (grey dashed line). The overall accuracy in classifying cohort in rats was 100%, meaning that all samples were correctly identified. (B) Top 10 feature rankings in the random forest analysis. Abbreviations: AUC, area-under-the-curve; FPR, false-positive rate; ROC, receiver operating characteristic, TPR, true-positive rate.

***4. Discussion:***

Analysis of the gut microbiomes of laboratory rats and wild-caught rats revealed striking differences in microbial diversity, composition of microbial communities, and 'pathobiont load'. Richness metrics such as the number of distinct features demonstrated that laboratory rats have higher alpha-diversity, regardless of sex, compared to wild-caught rats. PCR and serological analyses identified the presence of pathogens in the gut microbiota of wild-caught rats that were completely absent from the laboratory rat population, which we refer to here as 'pathobionts'. In addition, we found that the community-wide compositional differences in gut microbiomes between laboratory rats and wild-caught rats were supported by differential abundance analyses such as ANCOM, with higher relative abundances of Firmicutes and Tenericutes characteristic of laboratory rats and higher relative abundances of Fusobacteria, Bacteroidetes, and Proteobacteria serving as a hallmark of wild-caught rat microbiomes. Finally, machine learning approaches identified specific taxa that allowed us to classify individual samples as belonging to either laboratory rats or wild-caught rats: *Bilophila* spp*., Bacteroides*, *Tenericutes* in the RF39 lineage, multiple genera in the S24-7 family, and an unknown member of the order Clostridiales.

Alpha-diversity analyses clearly demonstrated that laboratory rats have greater numbers of distinct features and Shannon's diversity (also known as Shannon's Entropy) compared to wild-caught rats. Previous examinations of wild and captive animal gut microbiomes used a similar approach to that shown here. In recent reports, inbreeding or captivity in many ungulate species (e.g., bison, musk deer, and rhinoceri) are not associated with any changes in microbial alpha-diversity (Prabhu et al., 2020; Li et al., 2017; Gibson et al., 2019), while other mammalian groups (e.g., primates and canids) experience sharp decreases in diversity due to captivity (McKenzie et al., 2017). Differences in microbial diversity observed between wild and captive populations can be attributed to domestication-associated factors such as artificial selection,

changes in immune status, dietary fiber availability, and stress (Prabhu et al., 2020; McKenzie et al., 2017). Few studies have examined the microbial diversity of laboratory and wild-caught rats despite their prevalence in preclinical research studies. The observed increase in alpha-diversity for laboratory rats may have been due to a lack of exposure to extreme environmental conditions that impact survivability, a complete lack of predation and exposure to pathogens, and *ad libitum* access to rodent chow throughout their lifespan (Reese and Dunn, 2018). Interestingly, pet store mice also possess a richer microbiota and have greater numbers of memory T cells due to more exposure of pathogens than their laboratory mice counterparts (Ericsson et al., 2017). Dietary diversity and gut morphology are important drivers of microbial diversity, and the presence of a simple gut (as opposed to a more complex system that is characteristic of a foregut ruminant or hindgut fermenter) has been shown to predict low alpha-diversity regardless of dietary classification (e.g., herbivore, omnivore, or carnivore) (Reese and Dunn, 2018). Low alpha-diversity in wild-caught rats may reflect the presence of pathobionts or keystone species that impact microbial community structure, described in more detail below.

PCR and serological analyses identified the presence of pathobionts in the gut microbiota of wild-caught rats that were completely absent from the laboratory rat population. 'Pathobionts' is a term that refers to host-associated organisms with the potential to or demonstrated evidence of causing reduced host health status (Bass et al., 2018). Genetic defects and environmental factors may predispose mammals to immune-mediated diseases that can be triggered by these potentially pathogenic members of the gut microflora (Chow et al., 2011). Under deleterious conditions, the presence of *Helicobacter pylori*, *Proteus mirabilis*, and *Klebsiella pneumoniae*, among other species widely found in the gut microbiome of wild rats, could promote the expression of virulence proteins, pathogenicity islands, and toxins that potentially cause disease in the host

(Chow et al., 2011; Polk and Peek, 2010). Some studies have suggested that *H. pylori* may have evolved to protect its host against inflammation. Infection with CagA-positive strains of *H. pylori* and subsequent inflammation-induced changes in the gastric mucosa have been shown to result in decreased acid output that, in turn, decreases the likelihood of reflux esophagitis (Straus et al., 2002). The presence of other pathobionts, such as *P. mirabilis* and *K. pneumoniae*, following spontaneous development of dysbiosis and colitis in TRUC mice, has been linked to increased risk of colorectal cancer (Chow et al., 2011; Garret et al., 2010). Here, the authors noted that natural infection of laboratory rats with pathobionts could confound carcinogenicity research; broadly speaking, heterogeneity in pathobiont presence/absence in population-wide studies of the gut microbiome impacts all research involving the gastrointestinal system (Baker, 1998). Given the prevalence of specific pathogen-free animal research, the selective screening of pathobionts out of wild-caught or captive populations of animal subjects has broadly impacted how we relate members of the gut microbiome to health status in the gastrointestinal system and beyond.

The community-level assemblages of the laboratory and wild-caught rat gut microbiota were remarkably different, with higher relative abundances of Firmicutes and Tenericutes in laboratory rats and higher relative abundances of Fusobacteria, Bacteroidetes, and Proteobacteria in wild-caught rats. Many studies show that diet, age, and other lifestyle factors play crucial roles in the development of a core gut microbiota, with the prevalence of specific phyla reflecting dietary status and availability. Specifically, a low-carbohydrate, fat-restricted diet is linked to the increase in relative abundance of Bacteroidetes and a concomitant decrease in the relative abundance of Firmicutes (Clarke et al., 2012; Turnbaugh et al., 2008). The prevalence of Bacteroidetes and large within-group beta diversity in wild-caught rats, in direct contrast with the

high abundances of Firmicutes and relatively homogeneous microbiota of laboratory rats, may have been due to the lack of dietary fat and carbohydrate availability in the wild. In addition, heterogeneous foraging sources employed by wild-caught rats in different habitats may also be a contributing factor. Certainly, when compared against the stable housing conditions and *ad libitum* access to a single source, nutritionally-defined rodent chow in the laboratory setting, it comes as no surprise that the wild-caught rat microbiome is profoundly different from the laboratory rat microbiome. Studies demonstrate that a low-fat, plant-based and polysaccharide-rich diet induces the proliferation of microbiota enriched for N-glycan degradation, sphingolipid metabolism and glycosaminoglycan degradation, all of which are metabolic pathways enriched in Bacteroidetes (Clarke et al., 2012; Sonnenberg and Sonnenberg, 2019; Turnbaugh et al., 2006). Interestingly, a high Firmicutes:Bacteroidetes ratio has been associated with obesity in adult Ukrainian populations (Koliada et al., 2017), monozygotic and dizygotic twins (Turnbaugh et al., 2008), and mice genetically predisposed to obesity as well as lean mice that receive fecal transplants from obese human donors (Turnbaugh et al., 2006). However, it is difficult to infer health status from a high Firmicutes:Bacteroidetes ratio alone.

Machine learning approaches identified specific taxa that were able to classify samples as belonging to either laboratory rats or wild-caught rats. Specifically, the presence of *Bilophila sp., Bacteroides*, *Tenericutes* in the RF39 lineage, multiple genera in the S24-7 family, and an unknown member of the order Clostridiales were ranked as important features in the classification of cohort. Using the ANCOM-II method, we determined that *Bacteroides plebeius*, *Bacteroides eggerthii*, *Bacteroides coprophilus*, *Prevotella* sp. and *Prevotella copri* in the Bacteroidetes phylum were richly abundant in wild-caught rats. We infer that the presence of these species in the genus *Bacteroides,* many of which play beneficial roles for the host

organism, are a direct reflection of differences in rearing conditions in the two cohorts of interest. For example, *B. plebeius* plays an important role in the production of carbohydrate-active enzymes (CAZymes), which obtain energy from dietary polysaccharides in humans that are otherwise nutritionally inaccessible and touches on the importance of symbiotic microbes in many digestive processes (Hehemann et al., 2012; Sonnenburg and Sonnenburg, 2019). The presence of *B. plebeius*, among other species in the genus *Bacteroides,* might suggest that there was a greater need for CAZymes in the wild-caught rat population due to the consumption of a diet rich in complex carbohydrates. However, some *Bacteroides* spp. can become opportunistic pathogens if they escape the gut (e.g., as a consequence of "leaky gut") and are known to cause sub-phrenic, hepatic, splenic and retroperitoneal abscesses (Wexler, 2007). It is also important to note that, aside from the gut microbiota, there are several immunological and behavioral differences between wild-caught and laboratory rats: laboratory rodents are bred to be relatively docile in comparison to their wild counterparts, and different stressors have been shown to affect each group's immune systems differently (Barth et al., 2019). Routine eradication of pathogens is also a normal process of modern laboratory practices, but the occurrence of pathogen-free rats in the wild are rare. However, the impact of the environmental factors should not be ignored in understanding the responses of animals used in the laboratory. One study suggests that differences in expression of complement regulators, adhesion proteins, and cellular signaling factors may be due to the environment-linked microbiome: wild-caught rats are known to have increased complement regulation and decreased sensitivity to complement, in comparison to laboratory rats (Trama et al., 2012).

We propose that targeted DNA extraction and sequencing methods to characterize and quantify non-bacterial members of the gut microbiome, such as protists, parasites, fungi, and

trans-kingdom interactions, will provide a more complete picture of the wild rat microbiome. Although PCR and serological analyses found that many different types of viruses and bacteria identified compositional differences in the gut microbiomes between laboratory and wild-caught rats, other undetected organisms might also contribute to these observed differences. The treatment of some wild-caught rats described in this study with anti-parasitic agents to reduce the risk of cross-facility mite and helminthic infection may have impacted the differences observed in wild-caught and laboratory rat microbiomes. Naturally occurring pathogens, environmental factors, and human activity at each catch site, which are known to impact other aspects of rat physiology, may also have had significant impacts on the microbiomes of wild-caught rats from Boulder County. To that end, expanding these techniques to other studies of wild-caught rats will certainly improve our understanding of natural rat physiology and behavioral phenomena, and how rat physiology and behavior have been impacted by domestication for use in laboratory settings.

***5. List of Abbreviations:***

BCV, Boone cardiovirus
BHGA, β-hemolytic Group A
BHGB, β-hemolytic Group B
BHGC, β-hemolytic Group C
BHGG, β-hemolytic Group G
BN, Brown Norway
CARB, cilia-associated respiratory bacillus
dsDNA, double-stranded DNA
EMP, Earth Microbiome Project
H-1, Toolan's H-1 parvovirus
IACUC, Institutional Animal Care and Use Committee
IDIR, infectious diarrhea of infant rats
KRV, Kilham's rat virus
LCMV, lymphocytic choriomeningitis virus
MAV1, mouse adenovirus strain 1
MAV2, mouse adenovirus strain 2
MFI, multiplex fluorescent immunoassay
NS1, generic parvovirus
OAR, Office of Animal Resources

PCoA, principal coordinates analysis
PCR, polymerase chain reaction
PERMANOVA, permutational analysis of variance
PVM, pneumonia virus of mice
RCV, rat coronavirus
REO3, reovirus 3
RMV, rat minute virus
RPV, rat parvovirus
RPyV2, rat polyomavirus 2
RTV, rat theilovirus
SDAV, sialodacryoadenitis virus
SV, Sendai virus/murine parainfluenza virus type 1
16S rRNA, 16 small subunit ribosomal RNA

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***7. Author contributions statement:***
CAL and JDR designed the research plan and experimental schema; AIE, JDH, TMM, and JDR conducted the experiments and acquired data for research analysis; CCL, CLF, CAL, and JDR analyzed and interpreted the data. All authors drafted, critically revised, and reviewed the manuscript for important intellectual content and agree to be held accountable to the accuracy and integrity of all work represented here.

***8. Conflict of interest statement:***
CAL serves on the Scientific Advisory Board of Immodulon Therapeutics, Ltd., is co-founder and Chief Scientific Officer of Mycobacteria Therapeutics Corp., and serves as an unpaid scientific consultant with Aurum Switzerland AG.

***9. Contribution to the field statement:***

Laboratory rats have become a commonly used model system for studies of physiology and behavior. Growing evidence suggests that the gut microbiome is an important determinant of physiology and behavior, yet no studies have investigated differences in the gut microbiomes of laboratory-reared rats and wild-caught rats. Here we assess differences in the diversity and community structure of the microbiomes of adult female and male laboratory-reared rats and wild-caught rats. Analysis revealed that adult female and male laboratory-reared rats have higher alpha-diversity and lower within-group dissimilarity of gut microbiomes. Furthermore, wild-caught rats have increased presence of pathobionts and keystone species, which may be important determinants of differences in microbiome compositions between laboratory-reared rats and wild-caught rats. Indeed, machine learning-based analyses were able to distinguish between laboratory-reared rats and wild-caught rats with one hundred percent accuracy. These data support the hypothesis that there are clear differences in the diversity and community composition of microbiomes of laboratory-reared rats and wild-caught rats, differences that should be taken into account when interpreting data from studies of laboratory-reared rats and translation to humans.

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