UNIVERSITY OF CALIFORNIA,
IRVINE

Preterm birth conditions, including hypoxia and prenatal antibiotics, impact the gut bacteria and fungi in rats more extensively than voluntary exercise

THESIS

submitted in partial satisfaction of the requirements
for the degree of

MASTER OF SCIENCE

in Biomedical Engineering

by

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2019

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ACKNOWLEDGMENTS

I would like to express the deepest appreciation to my advisor, Dr. Katrine Whiteson, who has been so encouraging during my tenure as a student in her lab. Her wisdom has been integral to my pursuit of question driven science and her trust in my experience has allowed me to progress as a researcher.

I would like to thank my committee chair, Dr. Wendy Liu for permitting me to pursue the computational microbiome research I desired at the start of the program. I would also like to thank Dr. Michelle Digman for her instruction and encouragement throughout the duration of my tenure.

My colleagues in the Whiteson Lab, particularly, Stefan Riedel, Stephen Wandro, and Andrew Oliver helped me improve my computer literacy, programing capacity, and biostatistical skills. Also, Dr. Greg Adams, Paul Bodell and Pediatric Exercise Research Center for conducting the animal experiments and procuring the samples.

In addition, a thank you to Dr. David Underhill of Cedars-Sinai Medical Center, who introduced me to the fungal communities of the gut.

Finally, I want to thank my wonderful partner Mindy for her patience and support.

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ABSTRACT OF THE THESIS

Preterm birth conditions, including hypoxia and prenatal antibiotics, impact the gut bacteria and fungi in rats more extensively than voluntary exercise

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The animal gut hosts a complex community of microorganism, including bacteria, fungi and viruses, referred to as the microbiome. The composition of the microbiome is influenced by an ever-expanding number of factors including antibiotics, diet, sleep, exercise and birth route. Exercise has been shown to increase the diversity of gut communities in both animals and humans- a generalized metric of good gut health. It was hypothesized that with the introduction of exercise, the microbial community composition profile of rats previously exposed to hypoxia would shift in a healthy direction. The bacterial and fungal compositions were examined in groups of rats that were exposed to hypoxia before subgroups were given access to a running wheel for voluntary exercise. DNA was extracted from cecal contents for metagenomic shotgun sequencing. This experimental setup was also repeated in rats exposed to prenatal antibiotics in order to consider the large proportion of mothers who receive a series of antibiotics before delivery. Presented here is a multivariate analysis of the bacterial and fungal compositions in the gut of rats exposed to preterm birth conditions (hypoxia and/or maternal antibiotics) to evaluate if there is measurable dysbiosis and to what extent exercise can ameliorate it. There was

an observed expansion in microbial diversity in rats that voluntarily exercised, while inverse trends were observed when compounded with exposure to prenatal antibiotics. Additionally, there was a novel observation of an encompassing expansion of fungi in all rat pups that were exposed to prenatal antibiotics.

INTRODUCTION

Under-development of the lungs can be a consequence of premature birth, resulting in hypoxia. During early development hypoxia can stunt growth, decrease heart rate, and decrease immune cell function [Zasada et. al. 2018]. The long-term consequences of premature birth in adults is considered a clinical blind spot, as physicians generally do not consider it as the patient enters adulthood, but respiratory and cardiac complications have been reported along with an increased risk of infection [Bolton et. al. 2015]. Some of these consequences are better understood in more severe cases where asthma or bronchopulmonary dysplasia have developed [Bolton et. al. 2015]. The long-term consequences of preterm birth on the composition of the gut microbiome is not well understood, but the impact of the gut microbiome on the function of distant organs is increasingly in focus by the research community.

The gut microbiome is comprised of commensal bacteria, fungi and viruses that compete with one another for a niche in an incredibly dense ecosystem. Some microorganisms reside in the lumen while others are more associated with the mucosal lining of the gastrointestinal tract. The gut has a gradient of oxygen and nutrient availability between not only different regions of the colon, but also from the center of the lumen, where generally more anaerobic microbial species reside, to the mucosal lining, where generally more aerobic microbial species reside [Alenberg et. al. 2015]. It remains unclear if a hypoxic environment for the host shifts the composition of the gut to favor anerobic microorganisms.

Bacteria and their rival bacteriophages represent the vast majority of gut residents, while larger eukaryotes, like fungi, are many fold less abundant [Illiev et. al. 2012]. Microbes produce necessary metabolites for the host and together possess a quantity of genes that outnumber the

human genome 100 to 1 [Qin et. al. 2010]. These genes are responsible for producing metabolites for the host. The glut of microbiome research in the last decade has primarily focused on the composition of the microbiota, the metabolites they produce, and immunity, whether adaptive or innate. This research has led to some labelling the gut as the body's "second brain" and more generally as good indicator of overall health [Gershon 1995].

The composition of the gut microbiome is determined by two methods of next generation sequencing. The targeted amplicon sequencing method is well established in microbiome studies and extensive databases exist to which acquired data can be aligned. The16S rRNA gene is used for identifying bacteria, while either of the two Internal Transcribed Spacer (ITS) regions in eukaryotes are used to identify fungi. Amplicons offer very rich taxonomy data, but samples can only be compared relative to the amplicon. This means that 16S and ITS2 data cannot be compared relative to one another and estimating the number of bacteria and fungi present at a cellular level is not possible. Shotgun sequencing outputs many different genomic fragments and requires large genome databases to identify those sequences- the downside being that many phyla do not have sequenced genomes and that for microorganisms, like fungi, no meaningful database exists. On the other hand, shotgun sequencing does offer depth in the variety of information one can glean from the microbiome including metabolomic data and relative abundances microorganisms in the environment.

The unique composition of microorganisms in the gut possessed by each individual has been the focus for diagnostics linking certain species or taxonomic profiles to a particular clinical pathology. While there is very little consensus on the composition of a healthy microbiome, most studies aiming to link the microbiome to a disease state have instead chosen a case-control study design, defining the disease-specific dysbiosis of gut composition in comparison to non-disease

control groups. There is evidence that dysbiosis in the gut can affect the health and function of distant organs, notably the lungs [Ranucci et. al. 2017, Skalski et. al. 2018], the heart [Tang et. al. 2018], and the brain [Hsiao et. al. 2013]. The gut has been demonstrated to influence the function of these distant organs through commensally produced metabolites, as well as, circulating immune cells and inflammation [Hsiao et. al. 2013, Sampson et. al. 2016].

Therapies for dysbiosis in the gut are diverse, although limited within each approach. They include probiotic regimens that supplement desirable live bacteria, those that provide prebiotic dietary fibers that help support desirable bacteria, phage therapies that eliminate specific bacterial pathogens or fecal microbial transplants (FMT) that introduce a complete donor microbiome altogether. Although negative side effects have not been reported, there is debate over the effectiveness of probiotics and prebiotics. Phage therapies have been used successfully in isolated incidences to combat strains of bacteria resistant to antibiotics, but are still used as a last-ditch effort in clinic [Kortright et. al. 2019]. Fecal microbial transplants (FMT) have been very effective at alleviating the worst cases of Irritable Bowel Disease and *Clostridium difficile* infections, but have yet to provide a clear understanding of the underlying mechanisms. Conservatively, the field is still requires years of basic research before clinically actionable therapies or bioengineering of the gut becomes precision medicine.

Recent studies examining the gut microbiome have shown the bacterial composition to be altered in premature infants when compared to healthy counterparts [Chernikova et. al. 2018]. The specific effect hypoxia may have on the developing microbiome of the newborn is not well established. It is hypothesized that the intervention of exercise has the potential to normalize the underdevelopment of the lungs in premature infants [Martin et. al. 2018]. Similarly, it is thought that with the introduction of exercise, the microbial community composition and metabolic

profiles of the hypoxic infants may become more similar to full term infants, but studies have reported only modest compositional and immunological changes due to exercise [Chernikova et. al. 2018]. The impact of hypoxia on the gut microbiome remains poorly understood as the scope of mechanisms expands. This is complicated further by a very loose definition of what qualifies as a healthy microbiome.

Another important consideration when studying the newborn gut microbiome is that nearly a third of pregnant mothers are Group B *Streptococcus* positive and undergo at least one prophylactic antibiotics treatment before labor [Reed et. al. 2018]. The antibiotic treatments are to prevent passing the *Streptococcus* infection to the newborn. There is evidence that maternal antibiotics can alter the bacterial composition of offspring [Reed et. al. 2018, Zou et. al. 2018]. Acute and chronic use of wide spectrum antibiotics reduces the bacterial burden many fold and alters the composition of remaining gut flora, which in some cases, can exacerbate disease pathology [Reed et. al. 2018]. It is believed that antibiotics select for the expansion of some resistant bacteria and allows other microorganisms of the gut, such as fungi, to expand [Wheeler et. al. 2016]. It remains unknown what long-term impact prenatal antibiotics may have on the gut microbiome of offspring, but it warrants further investigation since mother-offspring microbial transmission is recognized, prenatal antibiotics are given so commonly, and acute gut dysbiosis from wide spectrum antibiotics is so well established.

This study attempts to determine the effect of early-life hypoxia on the bacterial and fungal compositions into adulthood and the extent to which exercise can normalize these alterations. Shortly after birth newborn rat pups were sorted into hypoxic or normal control environments for 10 days. Pups from both environments were then weaned into groups that either could or could not access a large running wheel for voluntary exercise. Therefore, each

experiment was comprised of four arms: a normal condition (normoxic) and hypoxic group that remained sedentary, referred to as NCSED and HXSED, respectively, in addition to, a normal condition and hypoxic group that could access a running wheel, referred to as NCRW and HXRW, respectively. The experiment was repeated, giving the pregnant dams daily antibiotics 3 days leading up to labor, but not within 48 hours of delivery. These experiments aimed to investigate whether there was a distinct change in the composition of microorganisms in the gut due to hypoxia during early development and if exercise could normalize any resulting dysbiosis. Cecal content DNA was extracted at the end of the 60 day experiments. The results of a multivariate analysis are presented from metagenomic shotgun sequencing.

Chapter 1: Methods

An outline of the animal groups and experimental timeline are provided (Fig. 1A, B), as well as, an outline describing the pipeline for analyzing the sequencing data (Fig. 2).

i. Animals

All experiments and sample procurements were performed by approved IACUC personnel on the protocols of Dr. Greg Adams and the Pediatric Exercise Research Center (PERC). Pregnant wild type Sprague-Dawley rats were purchased from Charles River (Wilmington, MA). Immediately post-partum, the litters were randomly cross fostered and sexed for female pups. Post-partum, litters were randomized into the hypoxia treatment group, culled to four female pups per litter, and housed with the dam, in standard cages placed in a Biospherix (Parish, NY) normobaric chamber. The small litter size was adopted to ensure adequate nutrition and minimize the maternal stress associated with full litter size in the hypoxic environment [Del et. al. 2009, Bruder et. al. 2008]. The litters randomized to the normal control (NC) groups were also culled to four pups and housed in standard cages in the same room.

ii. Hypoxia.

To induce hypoxia, a feedback controller senses O_2 levels in the chamber and feeds N_2 into the chamber to maintain the preset level of 12% or 10% O_2 [Radom-Aizik et. al. 2013]. PERC had modeled hypoxia at an FiO_2 of 12% and 10% that represents moderate hypoxic exposure,

equivalent to the ambient FiO_2 found at 4000 m and quite compatible with life. In humans, inhalation of 12.5% O_2 has been reported to decrease systemic PO_2 to below 50 Torr [Naeije R et. al. 1987]. Using the alveolar gas equation, $P_aO_2 = F_IO_2\ (P_B - P_{H_2O}) - \frac{P_aCO_2}{RQ}$, where P_aO_2 is the alveolar oxygen partial pressure, F_IO_2 is the fraction of inspired oxygen, P_B is the barometric pressure, P_{H2O} is the partial pressure of water, P_aCO_2 is the partial pressure of carbon dioxide, and RQ is the respiratory quotient, this exposure should produce approximately 80-85% oxygen saturation of hemoglobin in the rat. PERC and others have found that this level of hypoxia is tolerated by the young rats [Moromisato et. al. 1999, Del et. al. 2009]. The chamber used in this study allows exchange with the environment so that pressure remains unchanged. Humidity and CO_2 were monitored and regulated. After 10 days of hypoxia, the rats were removed from the hypoxia chamber. On day 22, all groups were weaned. At this time, the two groups, normoxic controls (NC) and Hypoxia (HX), were further subdivided into sedentary (SED) or exercise (RW) cohorts. The exercise cohorts with access to the running wheel are designated: NCRW and HXRW. The sedentary cohorts without access to the running wheel are designated as: NCSED and HXSED. Any hypoxia percent preceding NC labels simply designates which experiment those rats are controls for.

iii. Running wheel exercise.

After the removal from hypoxic or normoxic environments, pups were weaned and grouped into exercise (RW) or sedentary (SED) treatment groups. Rats placed in exercise groups, were provided a running wheel for 30 days within their cage. Wheels contained distance counters and the distances were recorded daily. PERC had initially determined that female rats voluntarily

exercised much more than male rats and therefore female rats were exclusively used for these experiments

iv. Antibiotics

For the final experiment, antibiotics were administered to (n= 11) dams as 100,000 units/kg/day of Penicillin G (Pen-G) injected subcutaneously for three consecutive days ending 48 hours before birth in order to mimic treatment for Group B *Streptococcus* during labor and delivery in humans [Reed et. al. 2018, Milliken et. al. 2019]. Antibiotic treatment ceased before labor to assure the dams would not be stressed before delivery. Pregnant dams that did not receive antibiotics (n= 3) were used as controls for mothers for this experiment, but their pups were not used for any component of the experiment. The dams that did not receive antibiotics are labeled No AB Dam.

v. Cecal content collection

Cecal contents are from the endpoint of the experiments, 60 days from birth. Rats were sacrificed, the cecum to anus of the GI track was removed, and given to the Whiteson Lab for storage at -80° C. Cecums were thawed on ice and ~70 mg of cecal contents were removed sterilely for DNA extraction.

vi. DNA extraction

DNA was isolated from fecal samples using the MoBio Laboratories' (Carlsbad, CA) PowerFecal DNA Isolation kit (catalogue # 12830-50). This protocol incorporates bead beating for rapid homogenization during the lysis and heating step. Upon centrifugation, a high-concentration salt solution is added to the supernatant containing the DNA. The DNA is bound to the silica membrane of the spin filter column in which the DNA is then washed and subsequently purified. The purified DNA is then eluted using a low-concentration salt solution.

vii. Library preparation, Next-Generation sequencing

Using the extracted DNA, sequencing libraries were prepared using Illumina's (San Diego, CA) Nextera Library Kit (catalogue # FC-131-1096) along with the protocol outlined in Baym et al. 2015. Tagmentation enzyme was used to attach unique barcodes to each sample. Barcodes were ordered from IDT. Amplification was carried out using the program on the thermocycler: 1) 72°C for 3 min 2) 95°C for 30 sec [3) 95°C for 10 sec 4) 55°C for 30 sec 5) 72°C for 30 sec] x 15 cycles 6) 72°C for 5min. Ampure XP magnetic beads (catalogue # A63880) from Beckman Coulter (Brea, CA) were used to remove any residual primers remaining free in the samples, per the manufacturer protocol. DNA concentrations were uniformly adjusted to 2 ng/ul after measuring DNA concentrations using Thermo-Fisher's (Waltham, MA) Quant-iT Pico Green dsDNA (catalogue # P11496) assay, per the manufacturer's protocol. Pooled PCR reactions were then analyzed by a bio-analyzer to confirm a band size of ~370 bp. Generated

libraries were loaded onto an Illumina Next-Seq at 1.8 picomolar concentration using Illumina's mid-output kit for 75 bp paired end sequencing.

viii. Sequencing Data

Fastq Sequencing data was analyzed using the High-Performance Computer (HPC) cluster provided by the University of California, Irvine. The initial Illumina Nextseq run for the 10% and 12% hypoxia experiments yielded 193 million reads with an average of 1.4 million reads per sample. The second Illumina Nextseq run with the prenatal antibiotic experiment yielded 166 million reads with an average of 2 million reads per sample. A combination of the tools Fastqc and Prinseq were used to filter or trim the raw sequence paired reads. The minimum length of reads was 70bp with a minimum quality mean Phred score of 30. A total of 16% of reads were lost.

ix. Microbial profiling

The metagenomic phylogenetic analysis tool, MetaPhlAn2, was used with the sequence alignment tool, Bowtie 2, to profile the composition of the microbial communities within each sample. MetaPhlAn2 uses clade-specific marker genes to unambiguously assign reads to microbial clades [Segata et. al. 2012]. The marker genes MetaPhlAn2 uses have been identified from 13,500 bacterial genomes, 3,500 viral genomes, and 110 eukaryotic genomes. It can provide accurate species-level resolution and relative abundance of each identified taxon.

x. Fungal profiling

For this project, a custom in-house fungal database was created using NCBI reference genomes of 14 commonly reported fungal species. The composition of those species for each sample could be determined using BLAST to allow alignment of the metadata. The following fungal species are contained in the database: *Alternaria alternata, Aspergillus fumigatus, Aspergillus niger, Candida albicans, Candida glabrata, Candida parapsilosis, Candida tropicalis, Cyberlindnera jadinii, Debaryomyces hansenii, Malassezia globose, Malassezia restricta, Malassezia sympodialis, Saccharomyces cerevisae, Saccharomyces pastorianus*. The GeneBank assembly accession numbers are listed in Table 1. All genomes were downloaded on 3/28/18.

xi. Microbial Diversity and Machine Learning

Alpha diversity represents the diversity within specific treatment groups. The Shannon diversity index was used to determine the alpha diversity. Beta diversity represents the diversity between different treatment groups. Principle Coordinate Analysis PCO plots were generated using the Bray-Curtis similarity matrix. Plots were generated using PRIMER-E as well as RStudio's vegan package [Dixon 2003]. PERMANOVAs were performed in PRIMER-E. The R^2 values reported here to explain the variance in PERMANOVAs were determined by dividing the estimated component of variation (ECV) of the factor by the total sum.

RandomForest is an ensemble machine learning algorithm that fits many decision trees on random subsamples of original data, and then aggregates the results of each decision tree to

improve the prediction accuracy [Breiman 2001]. The rfpermute package in RStudio was used to generate the variable of importance plots as well as the proximity plots.

Chapter 2: Results

Gut microbial diversity increased due to exercise, while hypoxia best explained the variation between groups of rats that were not exposed to prenatal antibiotics

A collaborative effort with PERC included the procurement of whole guts dissected from rat pups in a translational hypoxia and exercise study. Rat pups were born, then reared in a hypoxic chamber in a model designed to mimic hypoxia induced by pre-term birth. While previous studies had already demonstrated an increase in gut microbial diversity due to exercise in adult rodents [Monda et. al. 2017] and in people [Petersen et. al. 2017, Keohane et. al. 2019] we wanted to know if a hypoxic environment at birth alters the microbiome and to what extent can voluntary exercise normalize it.

Metagenomic shotgun sequencing was performed on the rat cecal contents from separate 10% O_2 (n=36) and 12% O_2 (n=36) experiments. Hypoxia exposure for the designated groups was for 10 days post-natally before weaning and ultimately separation at adulthood (30 days) into exercising (n=18) and sedentary groups (n=18) for each of the hypoxia studies. The reads were aligned against the MetaPhlan2 database and the microbial composition of each rat was determined at the phylum level (Fig. 3A). Similar to results from other studies [Albenburg et. al. 2014], Bacteriodetes and Firmicutes were the dominate phyla in the cecum, occupying ~75% of the bacterial composition in most subjects. No clear compositional differences were observed between exercise groups in either study (PERMANOVA 10% O_2, $r^2 = 0.028$, $p < 0.242$) (PERMANOVA 12% O_2, $R^2 = 0.023$, $p < 0.210$). Similarly, there were no clear compositional

differences between the rats exposed to 10% O_2 and 12% O_2 beyond varying non-significant average proportions of Bacteroidetes and Firmicutes (PERMANOVA 10% O_2 cohort vs. 12% O_2 cohort $r^2 = 0.027$, $p < 0.123$). There was no measurable cohort effect when comparing the control (NCSED) rats from the two experiments (PERMANOVA 10% O_2 NCSED vs. 12% O_2 NCSED $R^2 = - 0.099$, $p < 0.851$).

Across both cohorts and independent of the O_2 concentration (10% or 12%), the rats that exercised had a significantly higher alpha diversity by the Shannon Diversity Index (hypoxic $p < 0.0172$, normoxic $p < 0.002$) (Fig. 3B). No complete clustering resulted from the exercising hypoxic and normal condition groups based off the Bray-Curtis beta diversity matrix, obscuring the extent that exercising can normalize the microbiome of hypoxic rats (Fig. 3C) ($R^2= 0.074$, $p< 0.118$). To determine what may be driving any differences between groups, a supervised RandomForest analysis was performed. An unclassified *subdulogranulum* species was determined to be driving just over 11% of the difference between hypoxic and normal control groups when considering the combined results of both experiments (Fig. 3D). What is striking, is the growth difference of the rats at 10% and 12% oxygen environments compared to their normal condition counterparts, highlighting the profound physiological effect the hypoxia had on the rats regardless of exercise (Fig. 3 E, F).

Gut microbial diversity decreased due to exercise while hypoxia best explained the variation between groups of rats that were exposed to prenatal antibiotics

Since more than a third of pregnant mothers are given prenatal antibiotics [Reed et. al. 2018], we investigated the effect prenatal antibiotics may have on the microbiome under the

same hypoxia and exercise conditions as the previous experiments. Since no analysis could distinguish a difference between the 10% and 12% oxygen environments in the previous experiment, designated rat pups were only exposed to a 10% oxygen environment for the prenatal antibiotic, hypoxia, and exercise experiment (n = 36). Pregnant dams (n=11) were administered 100,000 units/k/day of Penicillin G subcutaneously for three consecutive days ending two days before giving birth.

Bacteroidetes and Firmicutes were again the dominant phyla when rat pups had been exposed to prenatal antibiotics (Fig. 4A). Interestingly, alpha diversity corresponding to the Shannon Diversity Index appeared to demonstrate an inverse trend when compared to the previous experiments with no prenatal antibiotics (Fig. 4B). Specifically, the sedentary mice in the normal environment had a non-significant trend of higher microbial diversity ($p<0.277$), but the sedentary hypoxia exposed rats did not. As in the previous experiments with no prenatal antibiotic exposure, no differences in beta diversity could be determined between exercising and sedentary groups exposed to hypoxia ($R^2 = 0.066$, $p <0.054$) (Fig. 4C). Random forest analysis determined *Lactobacillus reuteri* was driving just over 9% of the difference between the hypoxic and normal condition rat gut microbiomes independent of exercise. The proximity chart from random forest analysis was able to cluster the hypoxic and normal groups. This demonstrates there is a degree of predictive power when distinguishing between rats that have and have not been exposed to hypoxia when prenatal antibiotics are involved (Fig. 4D). Growth curves show a growth deficit in mice exposed to a 10% oxygen environment and prenatal antibiotics regardless of exercise (Fig. 4E).

Relative fungal abundance in the gut increased in adult rats exposed to prenatal antibiotics

Fungi and larger eukaryotes are increasingly scrutinized members of the microbiome, so the fungal composition of the rats was determined using the shotgun metadata from all experiments. There is currently no widely used reference database in host fungal research for shotgun metadata, therefore an in-house database including the genomes of the 14 most common commensal fungi species reported by groups studying the gut fungal community, with particular attention to those that have attempted to do so with shot gun sequencing [Nash AK et. al. 2018, Underhill et. al. 2017]. Previous studies have demonstrated that species of *Candida*, *Aspergillus*, and *Saccromyces* make up well over 50% of the total population of the fungal community of the mouse gut when the ITS1 or ITS2 amplicon is sequenced from stool [Illiev 2013, Wheeler 2016]. The dominance of a small number of widely reported species of fungi allowed for an approximation of differences in the community of rats exposed to hypoxia and exercise.

The fungal composition in the cohort of rat pups that were exposed to prenatal antibiotics before hypoxia and exercise treatments was vastly different from the gut fungal communities of rats that were not exposed to prenatal antibiotics using our in-house reference database (Fig. 5A). Not only did the prenatal antibiotic cohort have more fungal counts when compared to the cohorts that did not receive prenatal antibiotics ($p << 0.001$) (Fig. 5B), it was dominated by *Alternaria alternata* and *Aspergillus fumigatus*. PCA considering the variance between the three cohorts demonstrated that the cohort that received prenatal antibiotics was distinguishable in a clear cluster (Fig. 5C) compared to the cohorts that did not receive prenatal antibiotics (PERMANOVA of AB vs. NO AB $R^2 = 0.710$, $p < 0.001$). Nonetheless, no clusters could be discerned due to exercise (PERMANOVA exercise vs. sedentary $R^2 = -0.007$, $p < 0.502$) or

hypoxia (PERMANOVA R^2 = -0.014, $p < 0.906$) treatment regarding the cohorts considered together, or separately.

DISCUSSION

Although early-life exposure to hypoxia was able to alter the composition of microbes in the rat gut when compared to normal counterparts, the potential of exercise to normalize that alteration or expand diversity remains less clear. In the case where antibiotics were not administered to pregnant dams, exercise facilitated a more diverse microbiome in the pups, indicated by the increased alpha diversity in exercising rat pups. In the case where antibiotics were administered to the dams before labor, the alpha diversity appeared to increase in sedentary mice that were not exposed to hypoxia, although the trend was not significant. Prenatal antibiotics appear to be a substantial variable in reducing the variance of gut bacteria between the samples and groups.

Random forest analysis was also able to predict *Lactobacillus reuteri* as a species driving the difference in composition between normal and hypoxic rat pups with prenatal antibiotic use. *L. reuteri* is one of the few consistently protective gut microbes in the literature and has been incorporated into many probiotics [Hoang et. al. 2018, Forsberg et. al. 2014]. It is important to consider that the metagenomic analysis of the cecal samples is at the endpoint of experiments at day 60, highlighting the potential long-term impact exercise and hypoxia may have on the diversity of the bacterial community in the gut during development and into young adulthood. A longitudinal study would be able to determine if the impact of exercise persists in adulthood or if transmission is transient.

One exciting and novel finding reported here is the expansion of gut fungi in the cohort of rat pups exposed to prenatal antibiotics. Similarly, the dominance of *Alternaria alternata* and *Aspergillus fumigatus* in the mycobiome of rats in the prenatal antibiotics cohort is unique. A

recent report suggests *Candida albicans* and *Saccharomyces cerevisae* were still the dominant fungal species in the guts of infants in the neonatal intensive care unit using amplicon data [Heisel et. al. 2019]. The study did not focus on the use of prenatal antibiotics, but studies like this aim to illustrate how the newborn gut fungal composition is established. Infants have the potential to be exposed to antibiotics before, during, and after labor. The latter has been explored most extensively, but short-term studies do not offer much insight when it is understood wide spectrum antibiotics alter the microbiome extensively. Corroborating the prenatal antibiotic results in rodents with preterm human infants whose mothers had similar antibiotic treatments will be necessary to determine the translational significance of this study.

The fungal database used in this study to evaluate the metagenomic shotgun data was limited, but reasonable in order to estimate the relative abundances of fungi. Expanding this in-house database to include more genomes will make it a more useful in-house tool. Amplicon sequencing of procured stool samples over the course of the experiment is already in progress to validate taxonomic data reported from the metagenomic shotgun data. Similarly, a dedicated experiment to investigate the impact of prenatal antibiotics within the same cohort of control rats will be needed to confidently address any cohort differences between the experiments. A caveat of this study is that the antibiotic treatments were conducted in separate cohorts. Future experiments will help us investigate mother-offspring transmission of the microbial and fungal communities.

The impact commensal fungi in the gut have on health and disease states is much less understood than that of the bacterial community. New immunology research is emerging implicating gut commensal fungi like *Malassezia restricta* with Irritable Bowel Disease (IBD) [Limon et. al. 2019] and *Wallemia mellicola* with asthma [Wheeler et. al. 2016, Skalski et. al.

2018]. The immune system recognizes fungi via the innate immune receptor Dectin-1 [Iliev et. al. 2012] and depending on patient polymorphisms, can elicit a strong immune response in the colon and distant organs. Together these findings highlight the potential further exploring fungal dysbiosis in subjects that were exposed to prenatal antibiotics may have.

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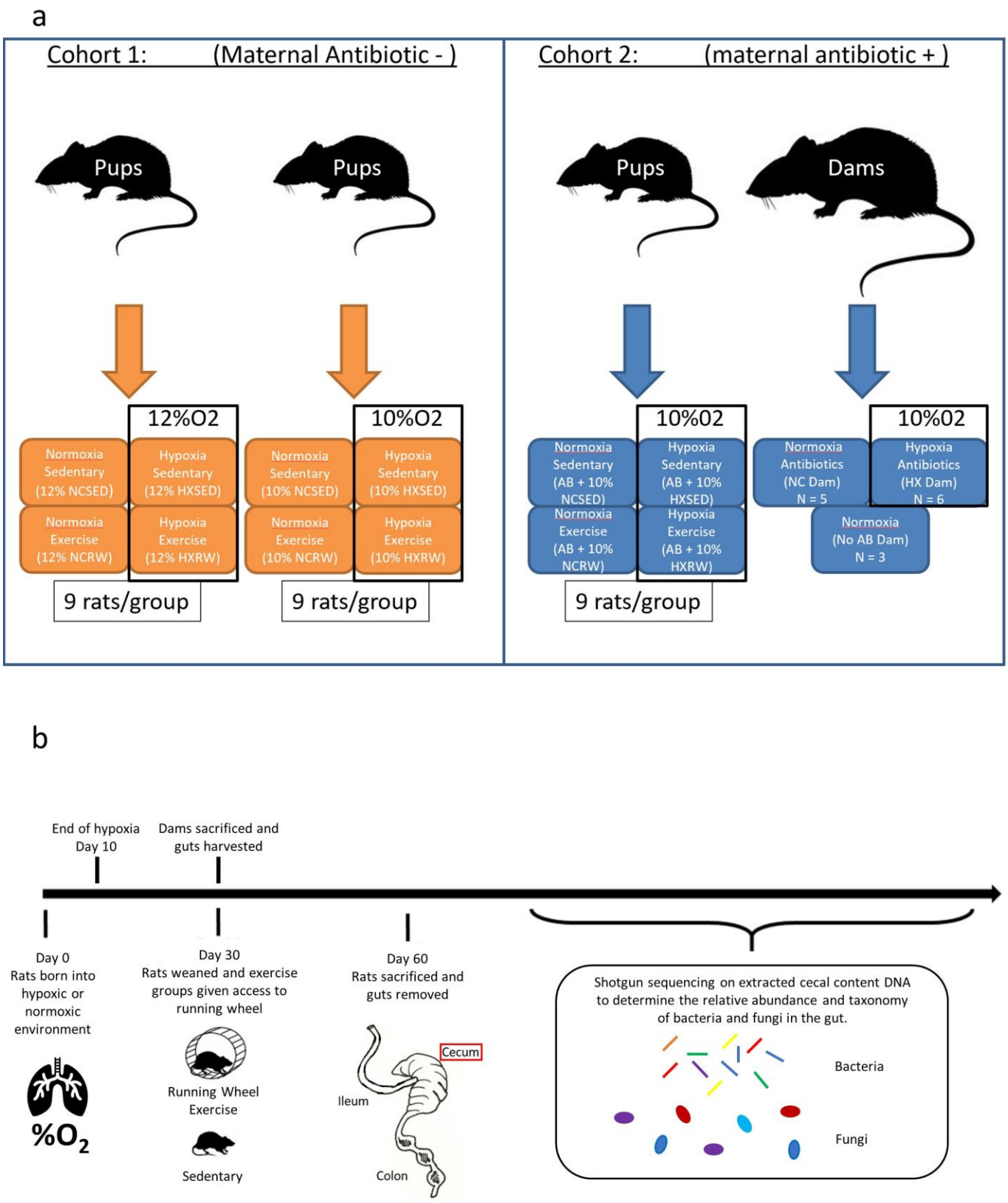

Figure 1: A. Experimental summary depicting rat subjects that were analyzed for this study. Rat pups were sorted into hypoxia and exercise groups for experiments without prenatal antibiotic use. For the experiment with antibiotics rat pups and dams were analyzed. B. A timeline of the 60 day hypoxia and exercise experiment.

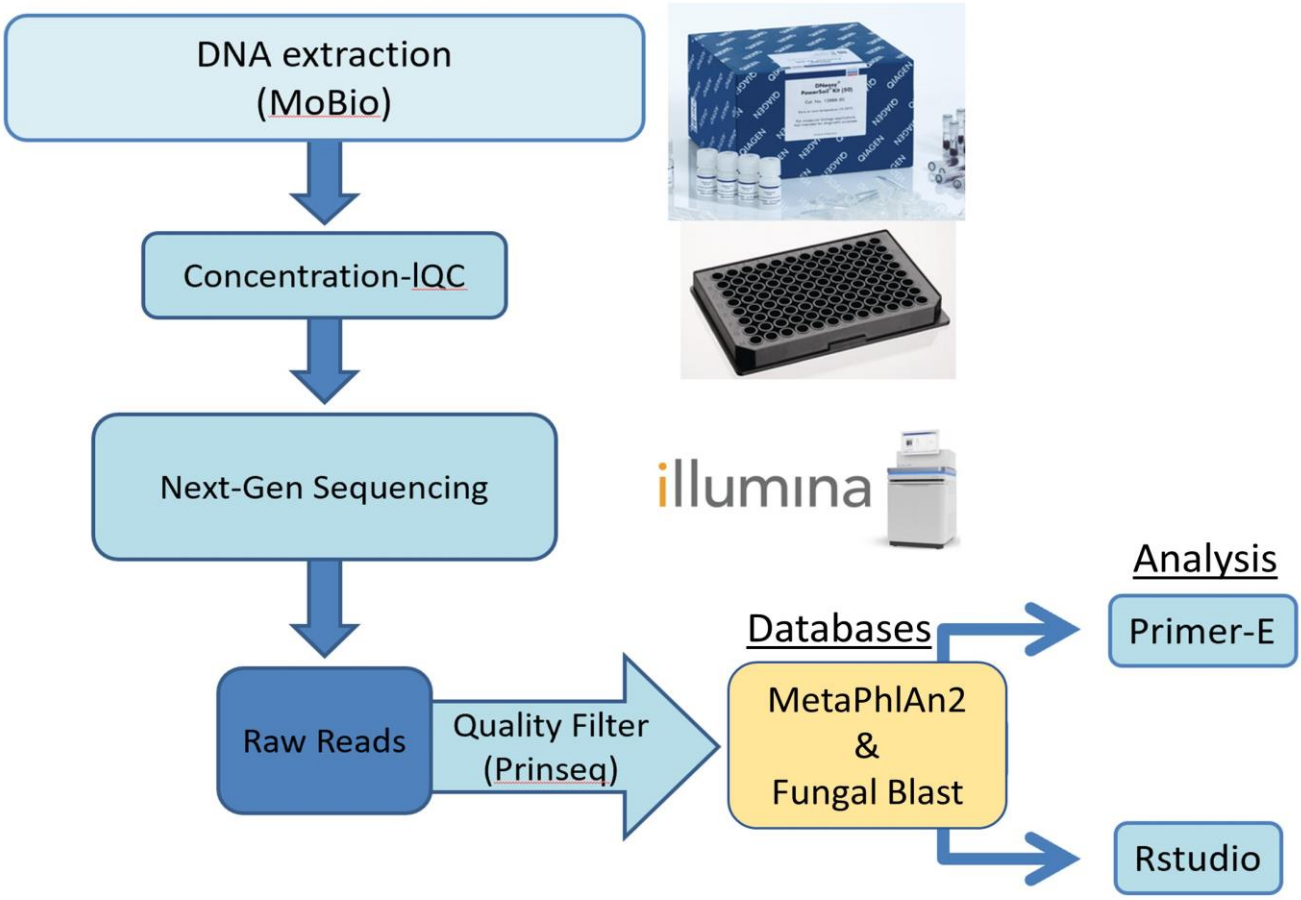

Figure 2: A flowchart of the workflow used to analyze the metagenomic shotgun data.

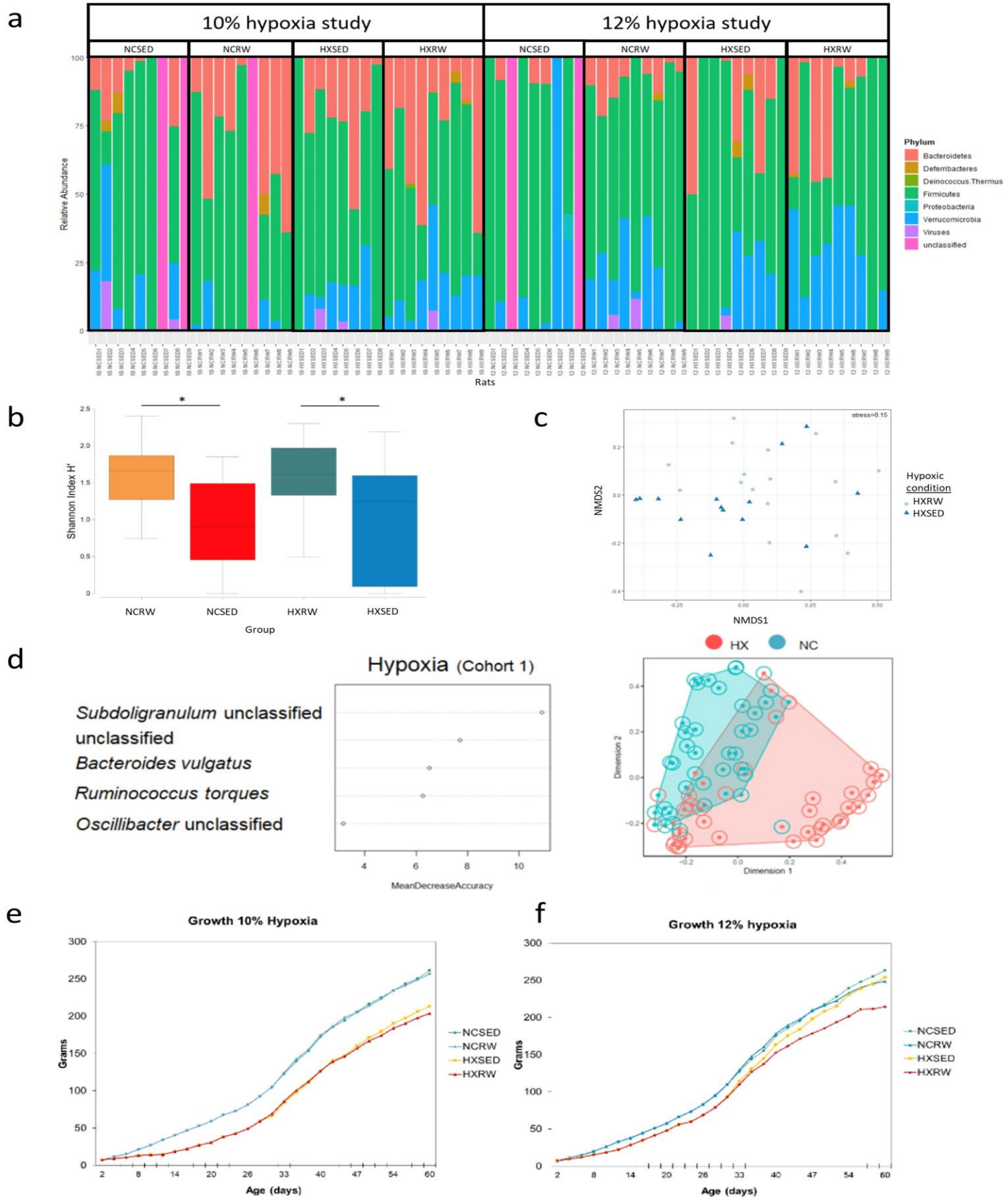

Figure 3: A. Bacterial composition of rat cecal contents raised in 10% (left) and 12% (right) hypoxia and their corresponding exercise groups B. Alpha diversity of the exercise groups from both hypoxic and normal conditions C. Beta diversity of exercising and sedentary hypoxic rats D. Random forest variable of importance and proximity plot hypoxic and normal rats from both studies E. Growth curve for the 10% hypoxia study F. Growth curve for the 12% hypoxia study.

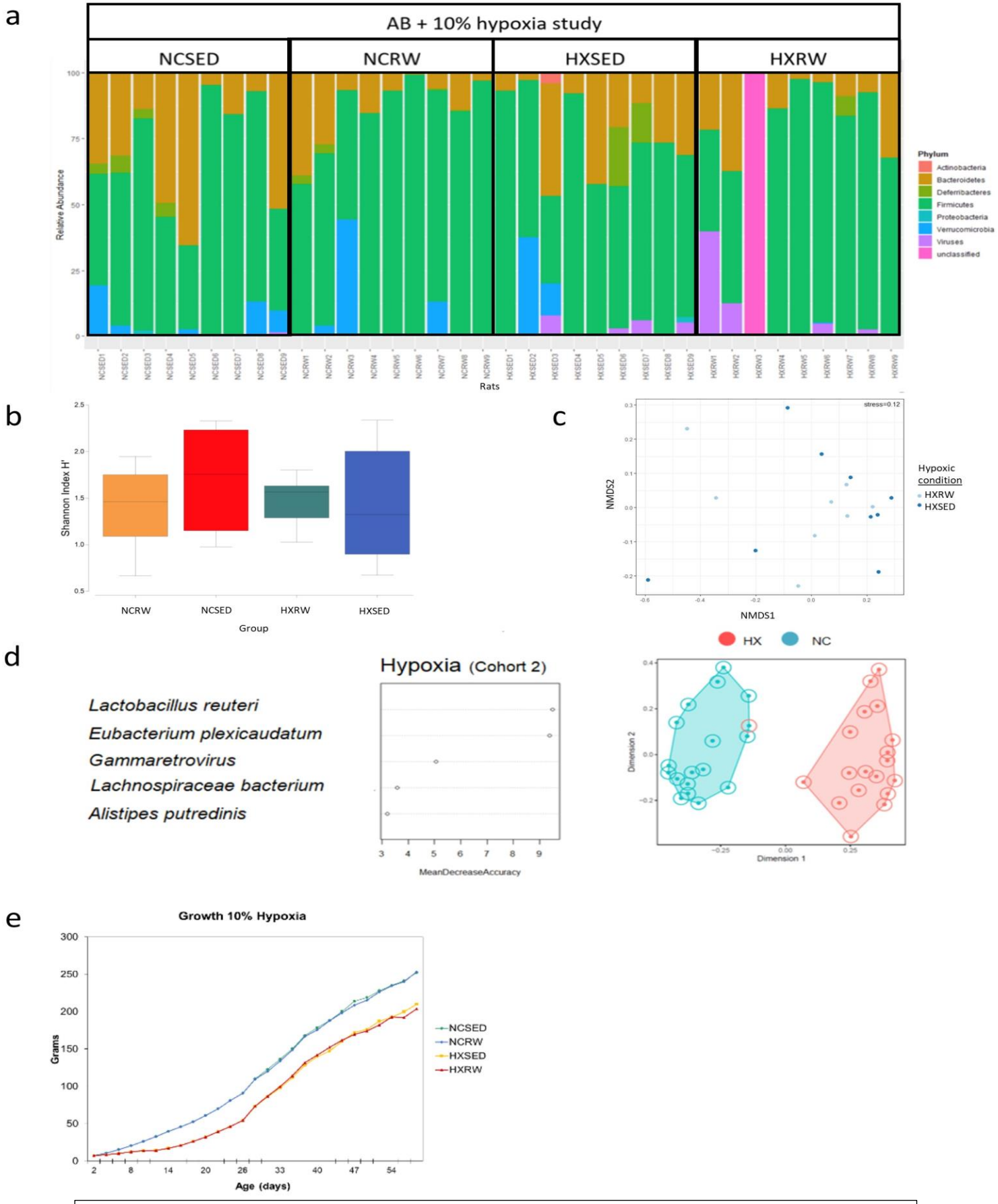

Fig. 4: A. Bacterial composition of the cecal contents of rat pups exposed to prenatal antibiotics B. Alpha diversity of exercise and hypoxia treatment groups C. Beta diversity among the sedentary and hypoxic rat pups D. Random forest variable of importance and proximity plots for the hypoxic and normoxic groups E. Growth curve

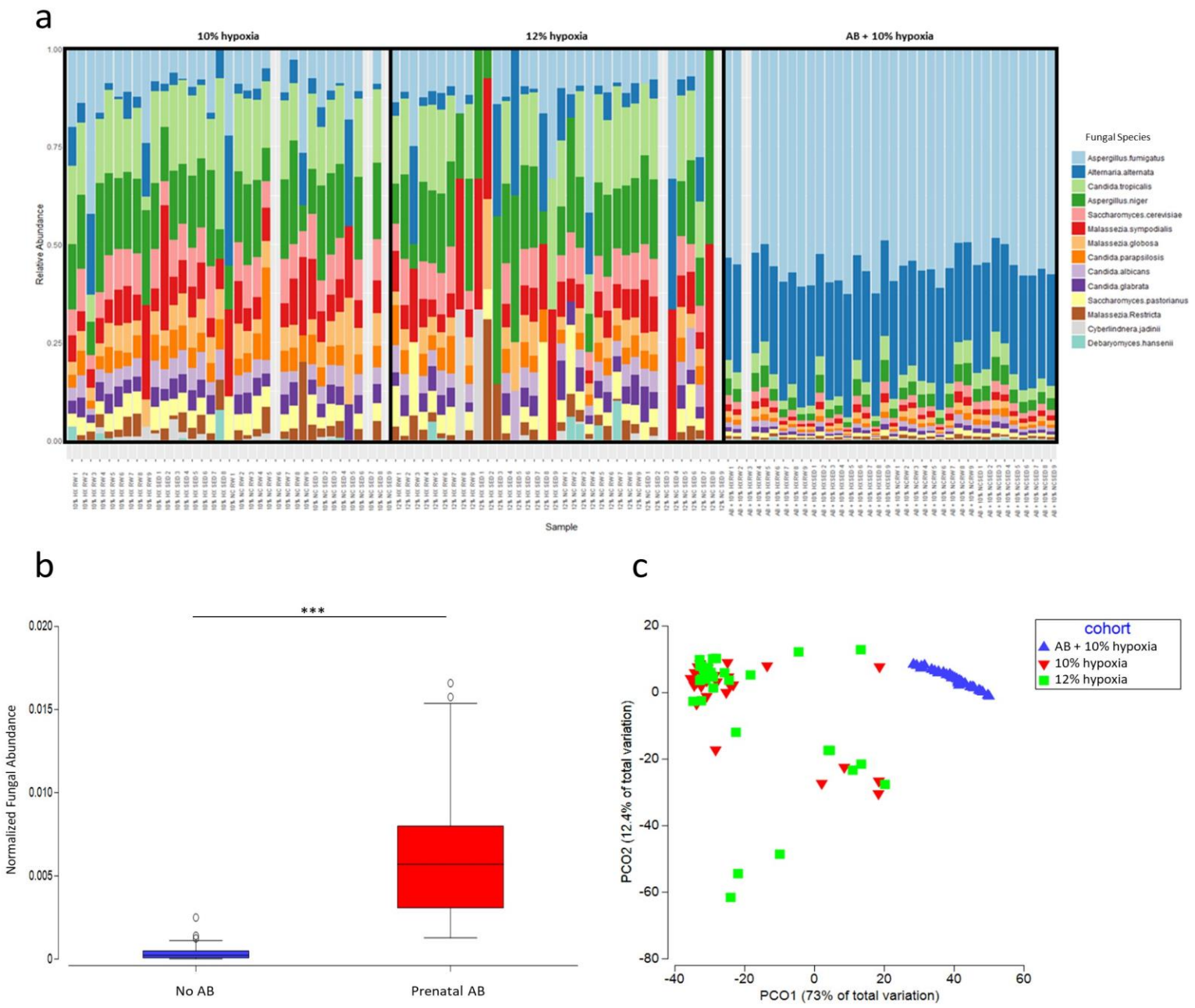

Figure 5: A. fungal composition standardized by total counts for the three experiments. Experimental cohort is labeled and boxed B. Average normalized fungal abundance of no AB and prenatal AB studies C. PCA of the three studies as wholes excluding treatment as a factor

Fungal Species	GeneBank assembly accession
Alternaria alternata	GCA_001642055.1
Aspergillus fumigatus	GCA_000002655.1
Aspergillus niger	GCA_000002855.2
Candida albicans	GCA_000182965.3
Candida glabrata	GCA_000182745.2
Candida parapsilosis	GCA_000182765.2
Candida tropicalis	GCA_000006335.3
Cyberlindnera jadinii	GCA_001661405.1
Debaryomyces hansenii	GCA_000006445.2
Malassezia globosa	GCA_000181695.1
Malassezia restricta	GCA_003290485.1
Malassezia sympodialis	GCA_000349305.2
Saccharomyces cerevisae	GCA_000146045.2
Saccharomyces pastorianus	GCA_000805465.1

Table 1: List of fungal species used in the in-house database and their accompanying GeneBank accession numbers.