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Effect of Husbandry Practices on the Fecal Microbiota of C57BL/6J Breeding Colonies Housed in 2 Different Barrier Facilities in the Same Institution

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Evidence showing a relationship between the mouse gut microbiome and properties such as phenotype and reaction to therapeutic agents and other treatments has increased significantly over the past 20 to 30 y. Recent concerns regarding the reproducibility of animal experiments have underscored the importance of understanding this relationship and how differences in husbandry practices can affect the gut microbiome. The current study focuses on effects of different barrier practices in 2 barrier facilities at the same institution on the fecal microbiome of breeding C57Bl/6J mice. Ten female and 10 male C57Bl/6J mice were obtained in one shipment from Jackson Laboratories and were housed under different barrier conditions upon arrival. Fecal samples were collected on arrival and periodically thereafter and were sent to TransnetYX for microbiome analysis. Mice used for collection of feces were housed as breeding pairs, with a total of 5 breeding pairs per barrier. An additional fecal sample was collected from these mice at 8 wk after arrival. One F1 female and one F1 male from each breeding cage were housed as brother-sister breeding pairs and a fecal sample was collected from them at 8 wk of age. Brother-sister breeding colonies were continued through F3, with fecal samples for microbiome analysis were collected from each generation at 8 wk of age. Breeding colonies in the 2 barriers showed differences in relative abundance, α-diversity, and β-diversity. Our data indicate that differences in barrier husbandry practices, including the use of autoclaved cages, the degree of restricted access, feed treatment practices, and water provision practices, can affect fecal microbiome divergence in both the parental and filial generations of different breeding colonies. To our knowledge, this is the first study to examine the effect of barrier husbandry practices on the microbiome of breeding colonies through the F3 generation.

Abbreviations and Acronyms: FM, fecal microbiome; GM, gut microbiome; HB, high barrier; LB, low barrier; PCoA, principal coordinate analysis; OTU, observable taxonomic units; PERMANOVA, Permutational Analysis of Variance; SIMPER, Similarity Percentages; UBA, uncultivated bacteria and archaea

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Introduction

The community of microorganisms that live in the human gastrointestinal tract, including bacteria, archaea, and fungi, is known as the gut microbiome (GM), and affects many aspects of human health including immune, metabolic, and neurobehavioral traits.[5,16,30,31,35,37] As mice continue to be the most widely used model of most human disease processes and treatments, detailed knowledge about the mouse gut microbiota and the effect of different husbandry practices on the composition of the mouse GM is increasingly important. Environmental factors related to diet, drugs, and anthropometric measures are known to be key determinants of human GM.[13,27] The amount of research dedicated to studying the effect of husbandry practices on the mouse GM has increased significantly over the past 20 to 30 y, resulting in a continuously growing list of evidence supporting the influence of multiple factors in shaping the GM of mice used in research.[8,9,20,23,33]

In response to increasing attention to the validity of experiments that use animal models as predictors of the efficacy of human therapeutics, agencies like the National Institutes of Health have increased their efforts to improve the reproducibility of animal experiments.[6,11] With increasing evidence that the human and animal GM can have important effects on various phenotypes, including metabolism, and various human and animal health conditions,[14] we sought to evaluate both effects of different husbandry practices on the GM and the stability of the GM across generations. Therefore, we analyzed the fecal microbiome (FM) of generations F1, F2, and F3 as compared with that of the Parental generation (P generation) on arrival to a new facility and with brother-sister breeding colonies that were maintained in rooms with different husbandry practices but in the same institution.

Due to its noninvasive nature and the ability to collect repeated samples for longitudinal studies, the most commonly used method for evaluation of GM tests feces as proxies for the composition of the GM.[32] In this study, we compared the FM of mice from the same shipment immediately after receipt at our institution and again later 8 wk after being housed in

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2 separate barriers that were located in the same building but were maintained with different husbandry practices We also compared the FM of the F1, F2, and F3 generations to the FM of the parental generation (P) under the same husbandry practices as that of their parents. We hypothesized that the FM of mice from the same shipment would change after housing under different barrier conditions for a period of 8 wk. We further hypothesized that the FM of subsequent generations bred and maintained in the same room (that is, F1, F2, and F3) would remain similar to the FM of that of the parental generation if husbandry conditions were unchanged.

Materials and Methods

The mice used for this study were housed at the University of California Berkeley, an AAALAC-accredited facility. The study was performed in strict accordance with the National Research Council's *Guide for the Care and Use of Laboratory Animals* and the Public Health Service policy on the Humane Care and Use of Laboratory Animals. The protocol was approved by the University of California Berkeley IACUC.

A total of 20 C57BL/6J mice, 10 females and 10 males, were obtained from Jackson Laboratories (Sacramento, CA) and received at the institution at 3 wk of age. Mice were shipped in 4 shipping containers containing 5 mice each, 2 containing females and 2 containing males. Immediately upon arrival, one shipping container of 5 female mice and one shipping container of 5 male mice were moved into each barrier facility, resulting in 10 mice per barrier. Once inside the barrier, each mouse was ear-tagged for identification, and a fecal sample was collected directly from the anus and placed in a DNA Stabilizing buffer (TransnetYX, Cordova, TN). After fecal collection, mice were set into breeding pairs, resulting in 5 breeding pair cages in a low-barrier facility (LB) and 5 in a high-barrier facility (HB; Figure 1).

Husbandry. All mice in this study were housed on the same floor of the same facility, but in different rooms, and used the same cage processing center. All mice were observed daily and were maintained on a 14:10-h light cycle with lights on from 0600 to 2000, between 68 and 79 °F (20 to 26 °C) and relative humidity between 30% and 70%. Food and water were provided ad libitum. The differences between the 2 barrier rooms are listed in Table 1 below. All mice were housed in IVC caging (Lab Products [Lab Products, Seaford, DE] or Tecniplast [Tecniplast, Milan, Italy]).

The HB is a shared, restricted-access facility, whereas the LB is a shared facility that is often used by only one research group yet has more increased multidirectional movement between rooms in the barrier. The HB provides lab coats that are laundered by a dedicated laundry service (Aramark, Philadelphia, PA) and requires the use of disposable hair bonnets, face mask, and gloves for entry, whereas disposable gowns, bonnets, and gloves were required in the LB. Different disinfectant solutions were used in the 2 barrier rooms. The LB used Process NPD (Steris, Mentor, OH), a quaternary ammonium, for daily disinfection and cage change, whereas the HB used MB-10 (Quip Laboratories, Wilmington, DE), a chlorine dioxide. Both facilities followed the same cage changing practices, which included saturating gloved hands, cage changing supplies, and equipment in disinfectant during procedures.

7090 Teklad Sani-chips bedding (Envigo, Madison, WI) was used in both barriers. The main difference between the two was that the bedding was autoclaved for the HB but was not for the LB. Cages for both barriers were sanitized via automatic cage wash and filled with bedding in the same cage wash area using

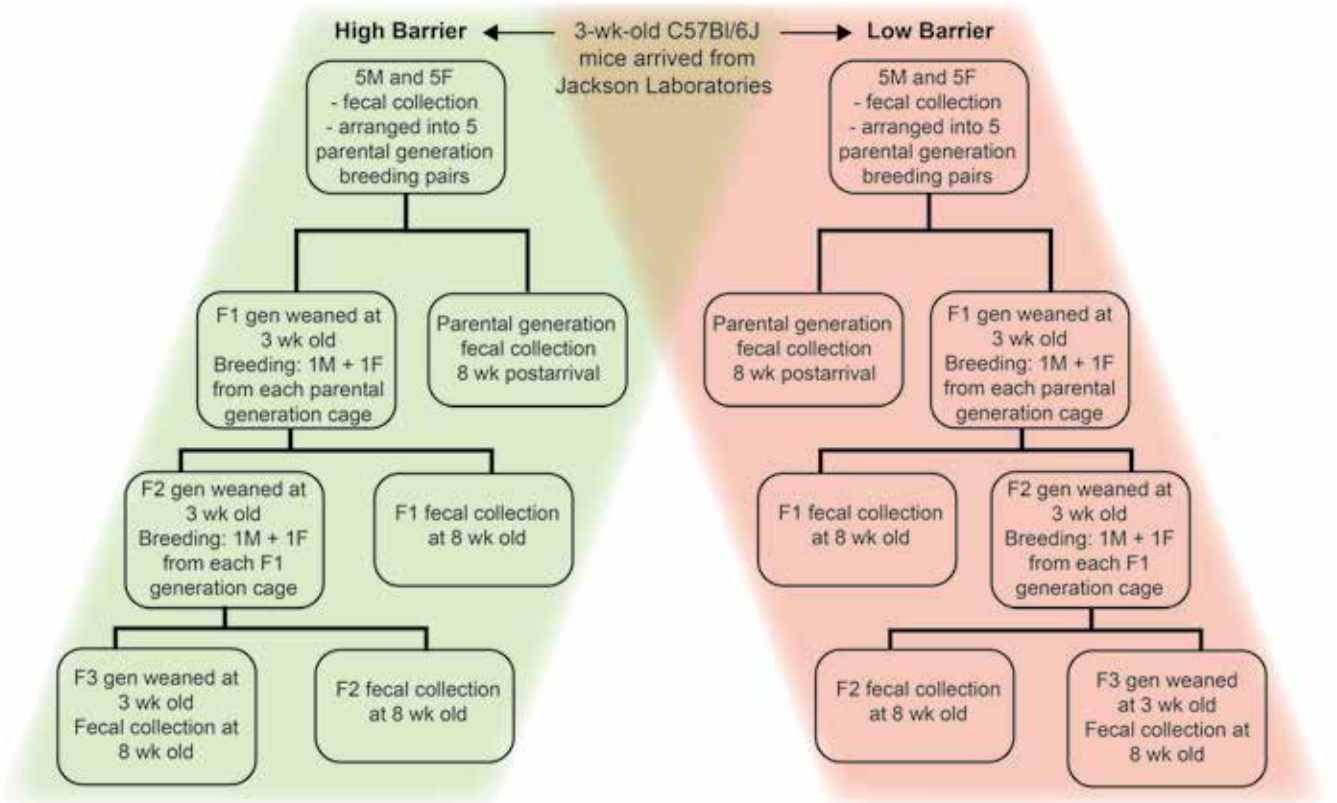

Figure 1. Flowchart of experimental setup. Upon arrival, shipping containers were moved into the HB represented by the green shaded area, and the LB represented by the red shaded area. Fecal collection and establishment of breeding pairs at weaning are represented by individual boxes.

Table 1. Key variables between both barrier facilities

Variable	Low Barrier	High Barrier
Caging	Tecniplast Blueline IVC	Lab Products IVC
Cage reprocessing	Automatic Cage Wash	Automatic Cage Wash + Autoclave
Water	Reusable water bottle with chlorinated water	Hydropacs chlorinated water
Diet	Irradiated	Irradiated Vacuum sealed
PPE	Disposable isolation gown or personal lab coat, hair bonnet, and gloves	Facility Provided Reusable Lab coat, Hair bonnet, gloves, and face mask
Disinfectant used within the facility	Process NPD (Steris)	MB-10 (Quip Laboratories)
Surveillance Exclusion List	MNV and *Helicobacter* permitted	MNV and *Helicobacter* excluded

the same supplies and equipment. However, bedding-filled cages were autoclaved before entering the HB facility but not before entering LB facility.

Mice in both barriers received hyperchlorinated (2 to 5 ppm) unfiltered municipal water. However, in the HB water was provided in sterile disposable Hydropacs (Avidity Science, Waterford, WI), whereas in the LB water was provided in reusable sanitized water bottles that were manually filled by the husbandry staff.

Mice in the LB were fed Lab Diet PicoLab@ Rodent Diet 20 5053 (LabDiet, St. Louis, MO) while mice in the HB were fed Lab Diet Pico-Vac@ Rodent Diet 20 (LabDiet, St. Louis, MO). The composition of these diets was identical, with the main difference being that the Lab Diet Pico-Vac@ Rodent Diet 20 was vacuum sealed into 5-lb units before irradiation. In the HB, a bag of feed is opened under a cage changing station immediately before use, and feed is dispensed directly from the sterile bag. In the LB, a larger bag of feed was emptied into a food-grade plastic container until empty, with open access to multiple users and not maintained sterile.

Sentinel mice that had been exposed to dirty bedding were tested in both rooms via serology on a semiannual basis for Group A rotavirus, Mouse hepatitis virus, Mouse parvovirus, Murine norovirus, Pneumonia virus of mice, and Theilovirus. Sentinel mice were tested annually via serology for Polyomavirus, Pneumonia virus of mice, Orthoreovirus, Murine Norovirus, Mouse T lymphotropic virus, Mouse parvovirus, Mouse cytomegalovirus, Mouse adenovirus 2, Mouse adenovirus 1, Minute virus of mice, Lymphocytic choriomeningitis virus, Group A rotavirus, Ectromelia, Mouse hepatitis virus, Theilovirus, *CAR Bacillus*, and *Mycoplasma pulmonis*. Mouse Norovirus and *Helicobacter* were both excluded from the HB but were permitted in the LB. Surveillance for both agents was conducted semiannually by PCR testing of sentinel mice exposed to dirty bedding.

Breeding. Brother-sister inbreeding was used for this study. The first breeding pairs were established on arrival. All litters from the original pairs were weaned at 21 d of age ± 2 d. At weaning, when sufficient male and female pups were available from a single litter, brother-sister breeding pairs were established. The same procedure was followed for F2 litters. F3 litters were also weaned at 21 d but breeding pairs were not established for this generation. For each filial generation, fecal samples for microbiome testing were collected at 8 wk of age ± 3 d (Figure 1).

Fecal sample collection and sequencing. In order to minimize circadian effects, fresh fecal samples were collected between 1200 and 1400. The second fecal collection from the founder pairs was performed at 8 wk after arrival (11 wk of age) which should allow sufficient time for the GM to stabilize.[21] The F1, F2, and F3 generations were tested as 8-wk of age because the GM is relatively stable at this age.[28,34]

We also collected fecal samples from established colonies (EC) in each barrier. We sampled 5 nonbreeding females and 5 nonbreeding males from 4 different cages in each barrier. All mice were between 8 and 10 wk of age, and all were of C57BL/6J background. These EC had been housed in the same room for at least 10 generations.

Two different methods were used to collect fecal samples. For the first method, mice were restrained by the scruff of the neck or base of the tail, and fecal pellets were collected directly from the anus. If needed, gentle pressure was applied to the base of the tail or abdomen. If fecal collection directly from the anus was unsuccessful, the mouse was placed in a clean cage lined with a clean disposable towel, and a sterile tooth pick was used to collect the sample as soon as defecation was observed. Samples were immediately placed in a barcoded DNA Stabilizer collection tube (TransnetYX, Cordova, TN).

Two fecal pellets were collected per mouse per time point. Care was taken to ensure that each fecal pellet was submerged in the Stool Nucleic Acid Preservative (Norogen, Thorold, ON, Canada). Each tube was closed securely and tapped firmly to ensure that samples were covered by the buffer solution. Samples were sent to TransnetYX (Cordova, TN) for DNA extraction, library preparation, and shallow shotgun whole-genome sequencing. Sequencing data were uploaded automatically onto the One Codex microbiome analysis platform (San Francisco, CA).

Data and statistical analysis. We used the One Codex microbiome analysis platform (San Francisco, CA) to organize and extract the fecal microbiome relative abundance data (a taxonomy list is provided as Supplemental Table S1). Shannon α-diversity data was extracted using the embedded Jupyter Notebooks option. Alpha and β diversity indices were used to assess differences between Time Points in each Barrier. Differences in β-diversity for all groups were tested by using one-way PERMANOVA of ranked Bray-Curtis (shared abundances of OTUs) similarity index using the open-access Past 4.10 software package (Hammer, Ø., Harper, D.A.T., Ryan, P.D. 2001.) Principal coordinate analysis (PCoA) was performed using the Past software package and the relative abundance data was square-root transformed. SIMPER test using Bray-Curtis measure was also performed using Past software package to compare overall average dissimilarity between time points in each barrier. Differences in Shannon α-diversity between time points within each barrier were assessed via the Kruskal–Wallis test (setting K = 1000) using Microsoft Office Excel (2016) with XL-Stat software (Addinsoft, Paris, France). A significance threshold of ≤ 0.01 was used for all comparisons.[18]

Results

Evaluation of the generation P fecal microbiome (FM) on arrival. The subjective evaluation of the fecal microbiome composition of each cohort at arrival demonstrated differences between mice assigned to the 2 housing locations, with *Alistipes* sp. UBA6068 and *Akkermansia muciniphila* having a greater relative abundance in samples collected from the mice in the HB group and *Porphyromonadaceae bacterium* UBA7213 and *Porphyromonadaceae bacterium* UBA7053 having the higher

relative abundance in samples from the LB mice (Figure 2A). Application of SIMPER test to the relative abundance data revealed an apparent difference between the 2 cohorts with an overall average dissimilarity of 23.8%, with *Porphyromonadaceae*, *unclassified Porphyromonadaceae*, *Alistipes* sp. UBA6068 and *Alistipes* having the highest percentage contribution to those differences (4.7%, 4.7%, 4.4%, and 4.4% respectively). One-way PERMANOVA test revealed no significant differences between the 2 cohorts with Bonferroni-corrected *P* value of 0.0748. PCoA analysis of the FM of all samples in the generation P on arrival showed a clear overlap between the 2 clusters representing each cohort with an Eigenvalue 1 of 0.29161 (46.8% of variance) and Eigenvalue 2 of 0.10571 (17% of variance) (Figure 2B).

Evaluation of the generation P fecal microbiome (FM) at 8 wk after arrival. Subjective evaluation of the FM relative abundance of generation P at 8 wk after arrival showed that both *Porphyromonadaceae bacterium* and *Alistipes* sp. remained the most abundant microbes in both cohorts. However, *Akkermansia muciniphila* and *Lachnospiraceae bacterium* A4 had a visibledecrease in abundance, with *Lachnospiraceae bacterium* A4 showing an abundance of less than 1% on its most abundant sample (Figure 3). We observed barrier-dependent differences, with *Bacteroidales bacterium* M7 having a visible increase in relative abundance among LB mice (mice 913, 915, and 920) and *Bacteroidales bacterium* M1 having a visible increase in relative abundance in 2 mice in the same barrier (mice 915 and 920). In mice 915 and 920, *Bacteroidales bacterium* M7 and *Bacteroidales bacterium* M1 were the most abundant members of their microbiota, accounting for 65.7% and 62.5% of total reads respectively. *Bacteroidales Bacterium* M7 was detected in 2 samples collected on arrival (relative abundance of 0.18% for mouse #903 in the HB cohort and 0.25% for mouse # 912 in the LB cohort. *Bacteroidales Bacterium* M1 was also detected on arrival in mouse #912, accounting for 0.02% of abundance. Both *Bacteroidales bacterium* M1 and *Bacteroidales bacterium* M7 are part of the FM of established mouse colonies in both barriers. SIMPER test was applied to the relative abundance data for this time point and revealed an overall average dissimilarity of 20.05%. A one-way PERMANOVA test revealed no significant differences between the two barriers with a Bonferroni-corrected *P* value of 0.1823.

Evaluation of the fecal microbiome of filial generations F1 through F3 at 8 wk old. Subjective evaluation of the FM relative abundance of the F1 generation at 8 wk of age showed a barrier-dependent pattern that was similar to that previously seen for generation P at 8 wk after arrival, with the HB cohort having their most abundant species being *Alistipes* sp. followed by *Porphyromonadaceae bacterium,* and samples in the LB cohort having *Bacteroidales bacterium* M7 and *Bacteroidales bacterium* M1 as their 2 most abundant species. Fecal samples collected from the F1 generation in the LB cohort had *Bacteroidales bacterium* M7 as the most abundant species in 60% of the samples. *Bacteroidales bacterium* M7 was also found in 2 samples from the HB cohort and was the most abundant operational taxonomic unit (OTU) in one of those (mouse 924) (Figure 3). The relative abundance in the F2 and F3 generations had the same pattern, with *Alistipes* sp. and *Porphyromonadaceae bacterium* continuing to be the most abundant OTUs in the HB cohort and *Bacteroidales bacterium* M7 and *Bacteroidales bacterium* M1 the most abundant OTUs in the LB cohort (Figure 3).

Evaluation of the fecal microbiome of established colonies (EC). Subjective evaluation of the FM of EC in each barrier facility showed differences between the 2 barriers,with *Porphyromonadaceae bacterium* and *Bacteroidales bacterium* M1 being visually dominant in samples collected from the mice in the HB group and *Lachnospiraceae bacterium*, *Porphyromonadaceae bacterium*, *bacterium J10*, and *Akkermancia muciniphila* all showing the higher relative abundance in the LB samples (Figure 4). Application of the SIMPER test to the relative abundance data revealed an apparent difference between the 2 cohorts, with an overall average dissimilarity of 22.3%, with the FCB Group, *Bacteroidia*, *Bacteroidales*, *Bacteroidetes Chloribi* group, and *Bacteroidetes,* which had the highest percentage contribution to those differences (3.3% each). A one-way PERMANOVA test revealed a significant difference between the 2 cohorts, with a Bonferroni-corrected *P* value of 0.0001. PCoA analysis of the FM of all samples in the HB and LB EC showed a clear overlap between the 2 clusters representing each cohort, with an eigenvalue 1 of 0.18227 (38.3% of variance) and eigenvalue 2 of 0.14127 (29.7% of variance). However, the convex hull region representing the LB samples covered a much wider area of the plot (Figure 4).

Alpha diversity of all time points. A Kruskal–Wallis test was used to determine the significance of changes in Shannon α-diversity index between each time point in each barrier. The Kruskal–Wallis test revealed no significant differences when comparing LB and HB breeding colony samples for all time points (*P* value > 0.05). However, pairwise comparisons of time points in the 2 barriers revealed a significant difference ($P = 0.001$) as compared with the LB P generation at 8 wk after arrival and the F3 from the same cohort. Although the Kruskal–Wallis test showed no significant differences overall, the box plot of α-diversity indices grouped by time point showed wider interquartile ranges and greater standard deviation values in the LB cohort for all time points except for P on arrival. This indicates a wider variety of α-diversity indices among members of the LB group when compared with the same time points in the HB cohort (Figure 5).

Beta diversity of all time points. A Bray-Curtis PCoA Plot of LB and HB cohorts comparing time points shows that clusters at each time point in the LB group start diverging from the baseline samples beginning in the P generation at 8 wk after arrival, with an eigenvalue 1 of 0.8325 (43.9% variance) and an eigenvalue 2 of 0.2785 (14.7% variance). In contrast, the clusters for each time point on the HB cohort maintain overlap through all time points, with an eigenvalue 1 of 0.3671 (33.9% variance) and an eigenvalue 2 of 0.1693 (15.6% variance) (Figure 6). The Bray-Curtis dissimilarity indices were used to compare samples within each cohort. Permutational Multivariate Analysis of Variance (PERMANOVA) test was applied to each time point and revealed significant differences between the generation P on arrival and the F1 and F3 generations in the HB cohort (Bonferroni-corrected *P* values of 0.023 and 0.037 respectively) (Table 2). PERMANOVA test in the LB cohort revealed significant differences between generation P on arrival and every other time point (Bonferroni-corrected *P* values < 0.05). A significant difference was also found between the generation P samples collected at 8 wk after arrival and those from the F3 generation. Significant comparisons (at $P < 0.05$) are shown in Table 3. When the HB and LB groups were compared using the same test, significant statistical differences were detected between the 2 barriers (Table 4).

The PCoA plot for the LB shows that the FM of the generation P at 8 wk after arrival has some similarity to the FM of an established colony in the same room. A similar pattern was observed when the FM of the F1, F2, and F3 generations were compared with the FM of the same established colony. In contrast, the PCoA plot of the FM data of our test breeding colony maintained in the HB showed no similarity to the FM of an established colony in the HB when all time points were compared.

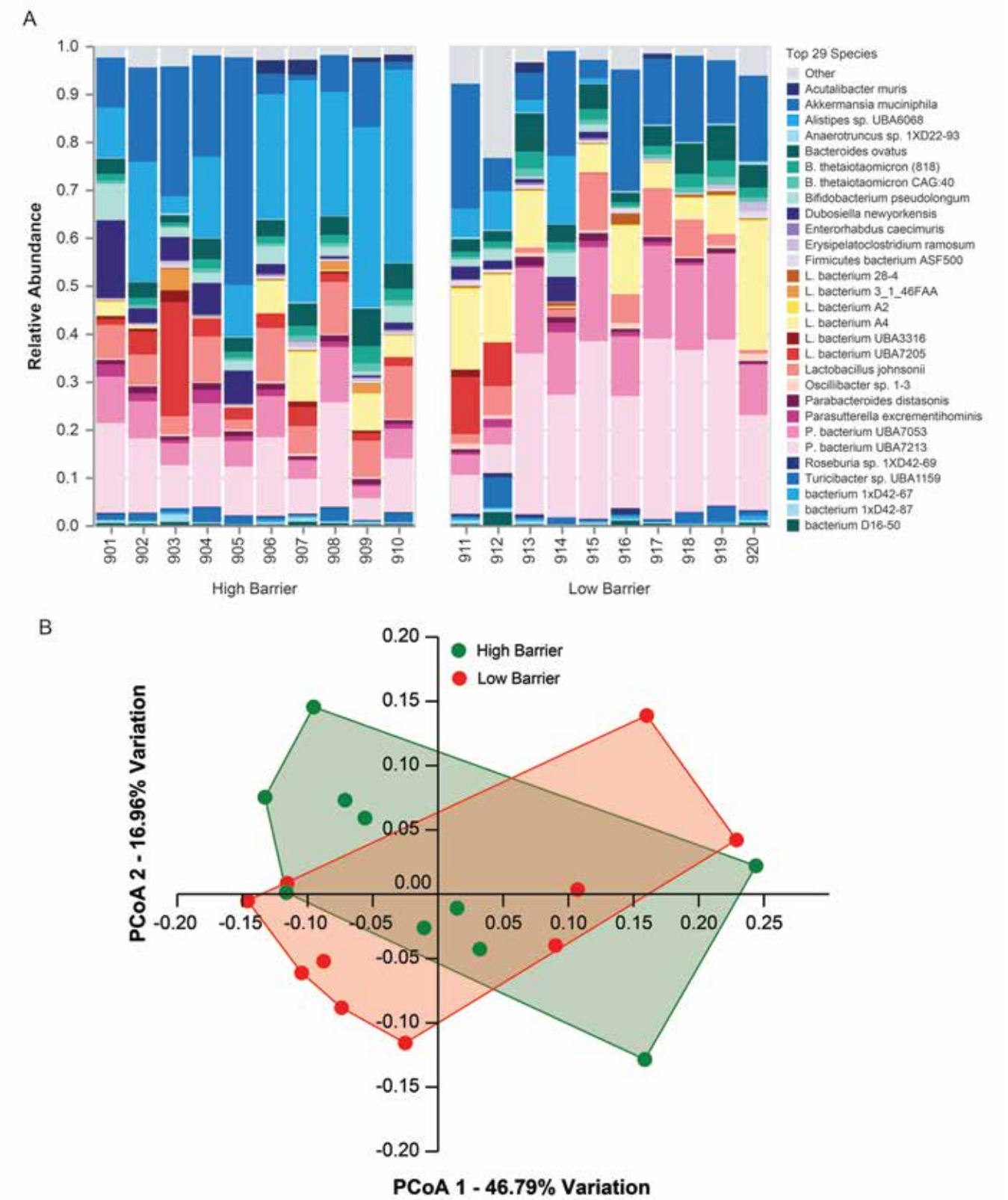

Figure 2. Gut microbiome composition upon arrival in 2 barrier facilities. A) Relative abundance plot generated using the OneCodex platform shows the top 29 species among all generation P samples on arrival comparing HB compared with LB cohorts. (B. bacterium = *Bacteroides bacterium*, L. bacterium = *Lachnospiraceae bacterium*, and P. bacterium = *Porphyromonadaceae bacterium*) B) Bray–Curtis PCoA Plot of Fecal Microbiome comparing generation P HB and LB on arrival. PCoA plots were generated using Bray-Curtis dissimilarity and a Transformation exponent of c = 2. Eigenvalue scaling was used to scale each axis using the square root of the eigenvalue. Convex hulls with filled regions connecting outermost points for each cohort are used to illustrate within compared with among variability (Past4.10.exe).

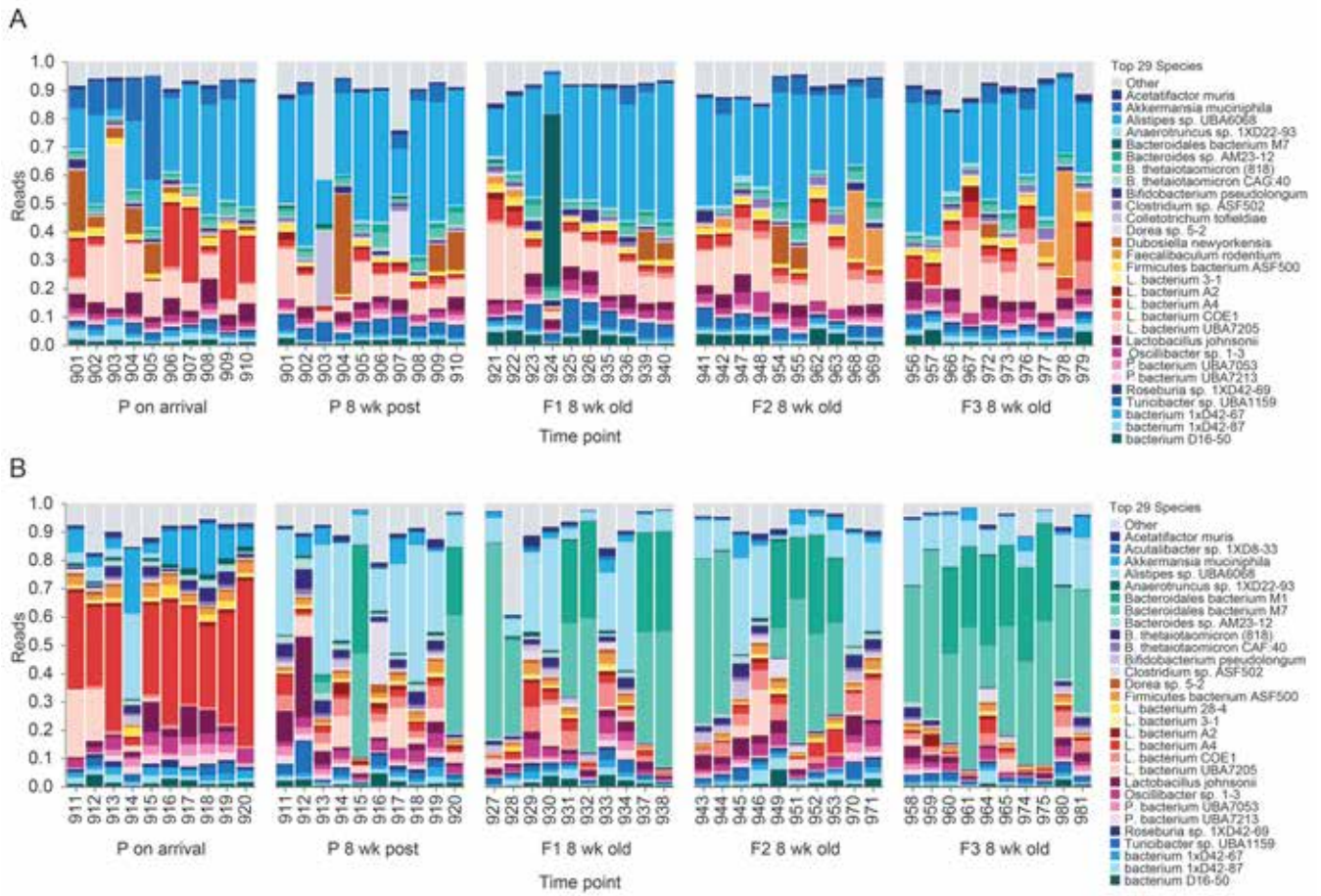

Figure 3. Relative abundance levels in 2 barrier facilities across generations. A) Relative abundance plot generated using the OneCodex platform shows the top 29 species among all HB samples comparing all time points in the cohort. (B. bacterium = *Bacteroides bacterium*, L. bacterium = *Lachnospiraceae bacterium*, and P. bacterium = *Porphyromonadaceae bacterium*) B) Relative abundance plot generated using the OneCodex platform shows the top 29 species among all LB samples comparing all time points in the cohort. (B. bacterium = *Bacteroides bacterium*, L. bacterium = *Lachnospiraceae bacterium*, and P. bacterium = *Porphyromonadaceae bacterium*).

Discussion

This study examined the FM of 2 groups of C57BL/6J mice from the same source that were maintained in 2 different rooms that were managed with different husbandry practices at the same institution. The cohorts were set up as breeding pairs to see if the differences between the 2 barriers had any impact on the divergence of the microbiome composition from the P generation on arrival or of their filial generations to F3. Our results showed statistically significant differences in the FM relative abundance, α-diversity, and β-diversity of 2 C57BL/6J breeding colonies maintained in 2 different rooms that had different husbandry practices.

Our use of shallow shotgun metagenomics sequencing allowed us to consistently identify organisms up to the species level; this is not always possible with alternative methodologies like 16s RNA sequencing. Our method captures the DNA sequences of all of the microbes in the sample including bacteria, fungi, DNA viruses, and other microbes. In comparison, 16S rRNA sequencing relies on sequencing only a single component of a single prokaryotic gene to determine microbial composition.[12,15] In contrast to Shallow shotgun metagenomics sequencing, the 16S rRNA sequencing method is highly dependent on the primer sequence used and is often limited to the analysis of bacteria at a family or genus level. Shallow shotgun metagenomic sequencing allowed us to obtain a highly specific and accurate FM profile.[22,25,38]

Although not statistically significant (Bonferroni corrected P values > 0.05) the 23.79% difference noted between the 2 cohorts on the SIMPER test upon arrival could be due to the fact that mice in both cohorts originated from 4 different rooms within the same barrier at the vendor facility. Several scientific publications have described similar vendor-dependent differences, as well as differences between mice in the same institution housed in different rooms.[7,21,26] These data highlight the importance of conducting microbiome analysis on mice on arrival or even before shipment whenever possible, especially for studies in which GM is an important variable (for example, gastrointestinal and metabolic studies, among others). Vendors commonly send mice from different rooms in the same barrier to accommodate client-specific requests, including but not limited to animal numbers, age, and gender. Retrospectively, we would ideally have obtained all P-generation mice from the same room. However, as shown in a previous study,[19] the microbiome should retain enough overlap to remain statistically similar.

Given that the differences in cohorts on arrival were not statistically different, we speculate that the patterns seen on PCoA analysis for each cohort (Figure 6), combined with the SIMPER test results comparing all time points from each barrier facility (Table 4), suggest a correlation between the housing facility and the speed with which divergence develops, wheras the speed of divergence does not depend on differences in the P generation on arrival. Ideally, we could have conducted randomization

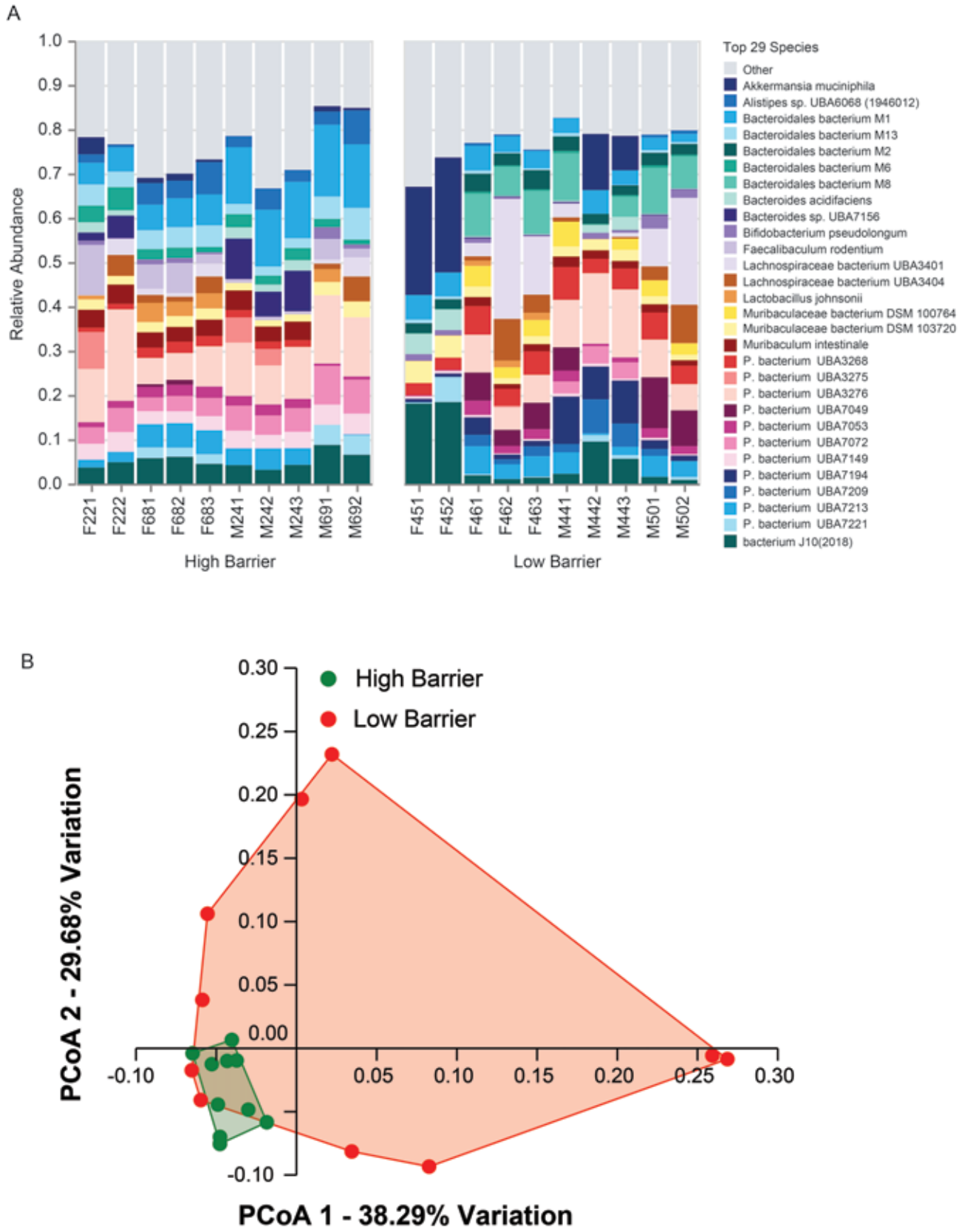

Figure 4. Gut microbiome composition of Established Colonies (EC) in the 2 barrier facilities. A) Relative abundance plot generated using the OneCodex platform shows the top 29 species among all EC samples as compared High- and Low-Barrier cohorts. (P. bacterium = *Porphyromonadaceae bacterium)*. B) Bray–Curtis PCoA Plot of Fecal Microbiome comparing EC with High Barrier and Low Barrier samples. PCoA plots were generated using Bray–Curtis dissimilarity and a Transformation exponent of c = 2. Convex hulls with filled regions connecting outermost points for each cohort are used to illustrate the comparison of 'within' and 'among' variability (Past4.10.exe).

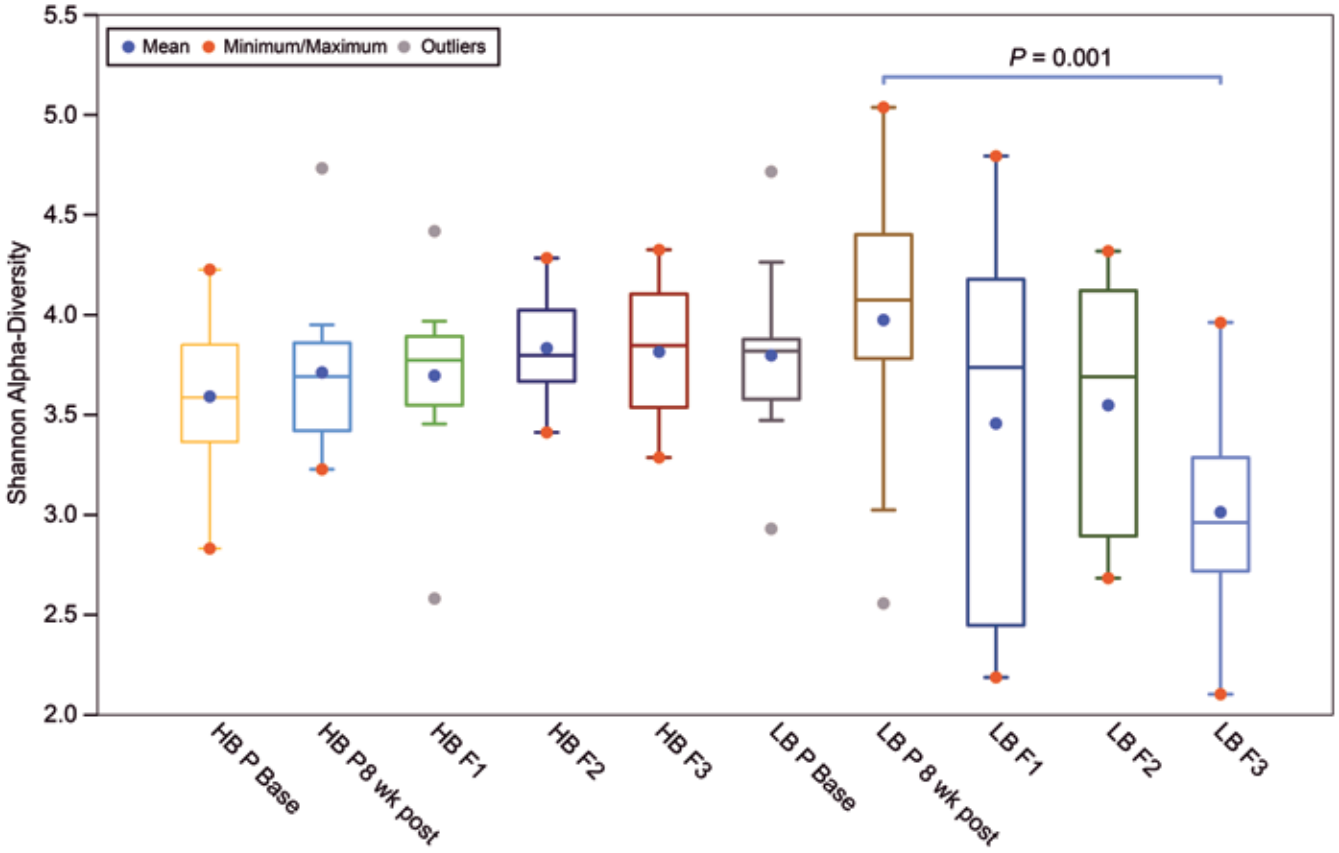

Figure 5. Shannon α-diversity box plot for all time points in both High Barrier (HB) and Low Barrier (LB) cohorts. Significant Bonferroni-corrected *P* value between LB at 8 wk after arrival and LB F3 shown with bracket.

of individual mice on arrival. However, because we wanted to limit mouse exposure to any external environments before introduction into each barrier, randomization on arrival was less practical due to our facility setup and the lack of a biosafety cabinet in our animal receiving area.

We observed a visible difference between housing areas in the divergence of FM from generation P on arrival as compared with F3. The subjective evaluation of all relative abundance plots for the LB showed a clear trend toward a divergence of the FM that began with generation P at 8 wk after arrival and continued through all subsequent generations. The most obvious increase occurred in the estimated relative abundance of organisms that were not previously abundant in arrival samples from generation P, specifically *Bacteroidales bacterium* M1 and *Bacteroidales bacterium* M7. In comparison, the generations kept in the HB had smaller changes in relative abundance from P on arrival to F3, and the divergence, although present, was less marked across time points and/or generations. Thus, all generations remained similar to each other.

Changes in FM of mice after arrival to a research institution have been previously described.[4] In one study, changes in the FM were detected as soon as 9 wk after arrival.[21] In that study, mice were housed in 2 separate conventional housing rooms in different buildings, with equivalent husbandry practices except for minor variations in ambient light levels and temperature and humidity. In contrast, our study revealed effects related to different husbandry practices between the 2 rooms located in the same building. Although the LB FM diverged rapidly, the HB FM maintained a closer resemblance to the FM of its P generation on arrival. These findings indicate that stricter husbandry practices can facilitate a higher level of control of the FM for the duration of studies in which generations are compared up to F3. Specifically, the processing of all materials that come in contact with the mice, including caging, water, bedding, and the rest of the microenvironment, appears to be critically important.

Both the LB and the HB in our study used irradiated diets. However, the 5-lb, vacuum-sealed packets of diet used in the HB ensured diet sterility for a longer period of time because the diet was not accessed by multiple people and was not subjected to the environmental air as compared with the larger communal bag kept in the LB. Furthermore, differences in the type of disinfectant used in research facilities can also affect the mouse FM.[29] In our case, the amount of direct contact of the mouse with disinfectant was minimal in light of husbandry practices in both rooms; mice were handled using disinfected thumb forceps or gloved disinfected hands typically every other week for less than 60 s at a time. Although the amount of mouse handling used in our study was minimal, resulting in reduced contact time for mice with disinfectant, the potential effects of using different disinfectants in the 2 barriers cannot be overlooked. These disinfectants may be more effective against some microbes tan others, which in theory could lead to a difference in the ability to maintain cage level barrier and allow some microbes to move among mice in the same colony.[3]

The PCoA plots for each cohort not only supported the findings observed in the relative abundance plots but also showed that the LB breeding colony seemed to acquire an FM that resembled the FM of the EC in the room much faster than did the HB breeding colony. When examining the PCoA plot for the LB, we saw a pattern of divergence of time points away from the

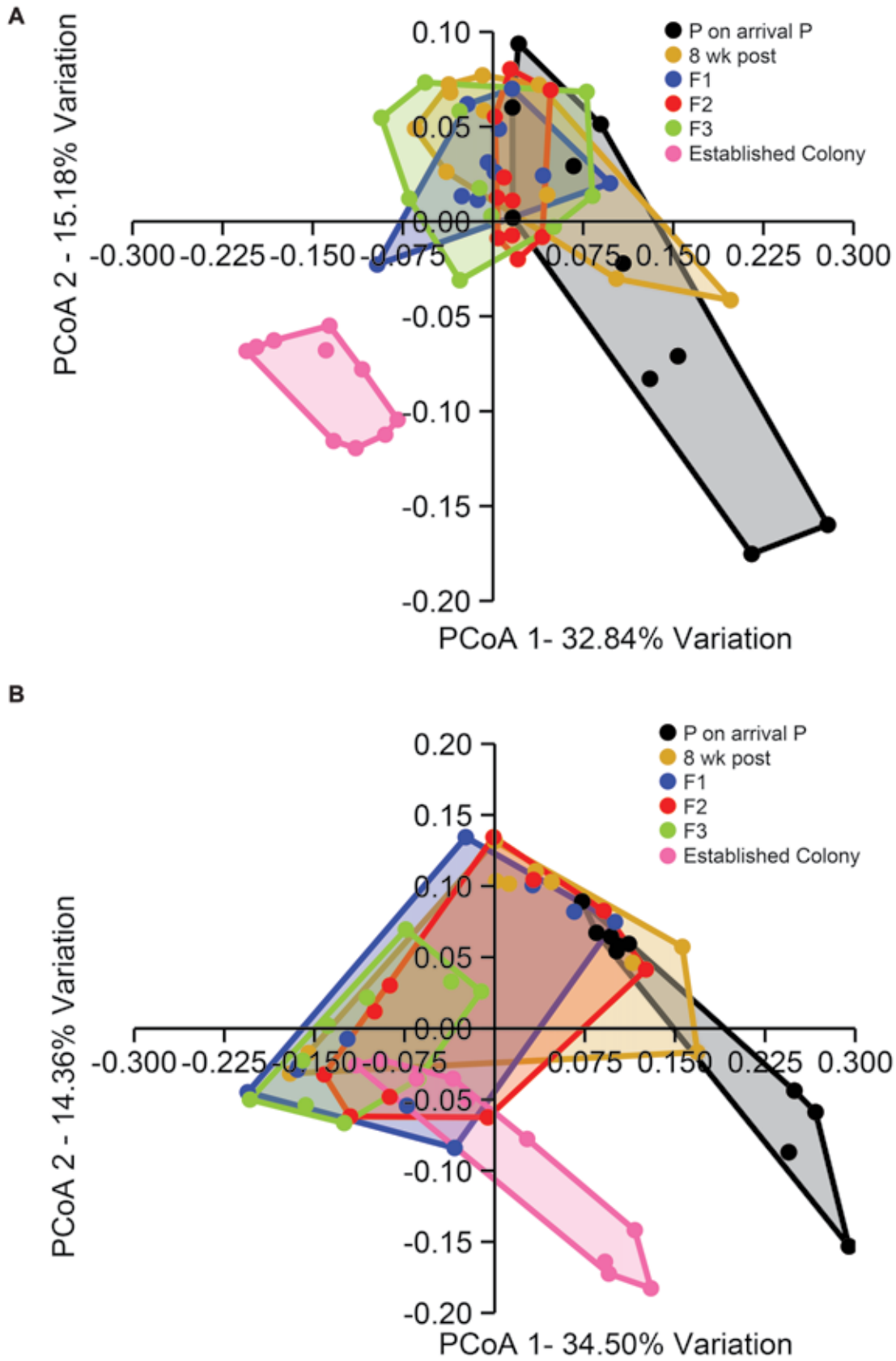

Figure 6. Beta diversity across generations. A) Bray–Curtis PCoA Plot comparing all time points in the High-Barrier cohort. B) Bray–Curtis PCoA Plot comparing all time points in the Low-Barrier cohort. PCoA plots were generated using Bray–Curtis dissimilarity and a Transformation exponent of c = 2. Eigenvalue scaling was used to scale each axis using the square root of the eigenvalue. Convex hulls with filled regions connecting outermost points for each time point are used to illustrate 'within- compared with 'among' variability (Past4.10.exe).

FM of P at arrival beginning as soon as P at 8 wk after arrival, with each subsequent time point showing a closer similarity to the EC. However, the HB clusters for each time point seemed to diverge less from each other as evidenced by the consistent overlap of clusters representing each time point. Although we saw a slight pattern of movement at each subsequent time point in the HB toward the cluster representing the EC samples, we never found overlap between the HB and EC clusters at any of the time points. These findings support a possible correlation between the husbandry practices in one room and the speed at which a new group of mice in the same room will assimilate the microbiota of its established colony.

The differences in husbandry practices also seem to affect both α-diversity and β-diversity, as evidenced by statistically significant differences in Bray-Curtis β-diversity observed on the PERMANOVA test and by the trends observed in the Shannon α-diversity box plots. Although the results from the Kruskal–Wallis test indicated that differences in Shannon α-diversity between the 2 barriers were not significantly different when compared at all time points, significant differences were detected between the LB P mice at 8 wk after arrival and F3. Wider interquartile ranges were seen among the LB box plots at all time points except for P on arrival, together with greater standard deviations among these groups, indicated a wider variety of α-diversity indices among members of the LB group as compared with the same time points in the HB cohort. Taken together, our findings suggest barrier-dependent difference in FM changes across time points, with stricter husbandry practices that include more restricted access, a wider exclusion list, and autoclaving of microenvironment components, including caging and bedding, seem to support conservation of similarity in the FM of breeding mice from 8 wk after arrival until the F3 generations.

A comparative study of the microbiome of common bedding materials before and after use on commercial dairy farms has been previously published.[24] We piloted the TransnetYX Microbiome assay to capture differences in the microbiota of microenvironmental samples, including bedding, diet, and water, in each barrier facility before their use to correlate with the observed changes in the respective mouse FM data. However, the TransnetYX Microbiome assay has not been validated for such samples and therefore the results we obtained are not included in this study. Nonetheless, we believe that using a validated shallow shotgun whole-genome sequencing assay to compare microenvironmental samples in each barrier could add value to our findings. Previous studies have demonstrated the presence of biologically active microbes in various types of unautoclaved rodent bedding by measuring coliform counts and lipopolysaccharide (LPS) levels respectively.[10,36] We would like to study the possibility that the process of autoclaving the bedding-filled cages used in the HB killed or inactivated most of the microbes present in the bedding used in this facility, therefore altering the number and type of live microbes to which mice in each barrier were exposed from the bedding. Furthermore, the use of disposable Hydropacs that are filled directly from the source via an automated system minimizes human interaction, reducing the human factor effect as compared with the process of refilling reusable water bottles and potentially leading to fewer chances of introducing unwanted microbes. We believe that in addition to the differences in diet packaging described above, differences in water delivery method and bedding treatment could also contribute to the effects we saw in the FM of mice in each cohort. The effect of water decontamination methods and bedding material on the mouse GM has been studied previously.[2,14]

Although we would ideally have data on established C57BL/6 colonies in our barrier for comparison, we do not have maintain inbred colonies in house. Limitations of time and funding prevented us from maintaining our breeding colonies for longer than F3. In addition, additional studies could examine the independent effects of diet, bedding, and water delivery methods and their relationship to effects on the FM, as well as potential effects of different cage changing techniques in each room.

An important consideration is that fecal metagenomics provides only a taxonomical profile of each sample at a given time. To obtain a more complete picture of the significance of the observed differences between the 2 barrier types, future

Table 2. High Barrier Pairwise One-Way PERMANOVA Bonferroni-corrected *P* values. *, $P < .05$.

	P Base	P Post	F1	F2	F3
P Base		0.198	0.023*	0.132	0.037*
P Post	0.198		1	1	1
F1	0.023*	1		1	1
F2	0.132	1	1		1
F3	0.037*	1	1	1	

Table 3. Low Barrier Pairwise One-Way PERMANOVA Bonferroni-corrected *P* values. *, $P < .05$.

	P Base	P Post	F1	F2	F3
P Base		0.016*	0.001*	0.002*	0.001*
P Post	0.016*		0.848	1	0.041*
F1	0.001*	0.848		1	1
F2	0.002*	1	1		0.639
F3	0.001*	0.041*	1	0.639	

Table 4. High Barrier and Low Barrier Pairwise One-Way PERMANOVA Bonferroni-corrected *P* values. *, $P < .05$.

	HB P Base	HB P Post	HB F1	HB F2	HBF3	LB P Base	LB P Post	LB F1	LB F2	LB F3
HB P Base		0.9675	0.1395	0.54	0.162	1	0.1845	0.009*	0.018*	0.0045*
HB P Post	0.9675		1	1	1	0.018*	1	0.0135*	0.054	0.0045*
HB F1	0.1395	1		1	1	0.0045*	1	0.369	0.4005	0.0045*
HB F2	0.54	1	1		1	0.0045*	1	0.018*	0.0675	0.0045*
HB F3	0.162	1	1	1		0.009*	1	0.2205	0.171	0.0045*
LB P Base	1	0.018*	0.0045*	0.0045*	0.009*		0.0495*	0.0045*	0.009*	0.0045*
LB P Post	0.1845	1	1	1	1	0.0495*		1	1	0.2025
LB F1	0.009*	0.0135*	0.369	0.018*	0.2205	0.0045*	1		1	1
LB F2	0.018*	0.054	0.4005	0.0675	0.171	0.009*	1	1		1
LB F3	0.0045*	0.0045*	0.0045*	0.0045*	0.0045*	0.0045*	0.2025	1	1	

studies should concentrate on conducting metatranscriptomics to obtain a functional profile, and metabolomics to complete the picture by determining which byproducts are being released.[1,17]

Finally, previous studies have suggested that cecal contents may be a better indicator of environmental influences on the GM, and the use of fecal samples may lead to "false negatives" when screening for effects on GM.[2,9] Therefore, comparing differences between the cecal and fecal microbiome with the same barrier-specific variables could provide a better understanding of dissimilarities between the information provided by the 2 different sampling methods.

Conclusion

This study compared the divergence of the FM of mice of the same genetic background when housed under 2 different barrier conditions, one with stricter husbandry practices than the other. Our findings strongly suggest that the differences in the husbandry practices between the 2 barriers had a direct effect on whether the FM of filial generations in a breeding colony remained relatively constant up to F3 as compared with each other and with the founder generation on arrival. In this case, stricter husbandry practices seemed to support a higher level of FM homogeneity among members of the same breeding colony across generations when compared with our LB practices.

Our findings also support previous reports of differences in the FM of mice from different rooms in the same institution.[7,21,26] However, our 2 cohorts were similar on arrival from the vendor, even though the vendor had housed the mice in different rooms in the same barrier.

Our findings also highlight the importance of understanding that differences in husbandry practices can directly affect the FM of breeding colonies. This possibility should be considered when obtaining unexpected results in attempts to replicate mouse experiments.

Supplementary Materials

Table S1. Taxonomy list. Taxonomy list including all OTUs detected in the samples included in this study.

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