ORIGINAL ARTICLE

Colony but not social phenotype or status structures the gut bacteria of a eusocial mammal

Connor R. Fitzpatrick[1,2] · Ilapreet Toor[3] · Melissa M. Holmes[3,4,5]

Abstract

Group living occurs across the animal kingdom and can shape fundamental aspects of individual biology, including the microbes inhabiting the animal gut. The naked mole-rat, *Heterocephalus glaber*, exhibits extreme cooperative breeding (eusociality) and presents an ideal opportunity to study the effects of social structure on the mammalian gut microbiota. Within colonies reproduction is limited to few animals, while the remaining members are non-reproductive, form linear dominance hierarchies, and show evidence of task specialization with stable yet plastic behavioral phenotypes. Here, we sequenced the gut bacteria of naked mole-rats across 6 replicate captive colonies and longitudinally during a colony removal experiment. Colony had much larger effects than social phenotype or status on the diversity and composition of gut bacteria. Our longitudinal experiment revealed that over the course of 2 months, the gut bacteria of previously unfamiliar, newly paired animals did not become more similar. In comparison to mice housed in the same facility, the naked mole-rat gut microbiome exhibited substantial compositional differences including the near absence of Lactobacillaceae and the enrichment of Prevotellaceae. Our study provides new insight into the factors shaping gut bacterial communities in social animals and indicates a prominent role of social group membership but not social phenotype.

Significance statement

The social groups to which animals belong can be strong determinants of the composition of their associated gut microbial communities (i.e., gut microbiome). However, less is known about the link between social status within animal groups and the gut microbiome. We studied this relationship in the naked mole-rat, *Heterocephalus glaber*, which exhibits extreme cooperative breeding (eusociality). Animals within naked mole-rat colonies show clear social hierarchies and evidence of social phenotypes. Across replicate colonies, we found that colony but not social phenotype or status had large effects on the diversity and composition of gut bacteria. During a colony removal experiment, the gut bacteria of newly paired but previously unfamiliar animals did not become more similar over the course of 2 months. Our results show that when decoupled from differences in diet or exposure to microbes, within colony social roles and their accompanying unique physiology and social experience may have little effect on gut microbiota. In contrast, gut bacterial diversity and composition was strongly shaped by colony membership despite being exposed to identical environmental conditions.

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Introduction

Living in groups occurs frequently across the animal kingdom and can shape fundamental aspects of animal biology such as behavior, physiology, reproduction, and nutrition (Alexander 1974). This is, in part, because animal groups often exhibit social hierarchies, and an individual's position within a hierarchy is typically accompanied by unique behavior and social interactions and differential access to mates, food, and other resources. Emerging evidence suggests that

the social environment can also alter the microbes inhabiting the animal gut (Sherwin et al. 2019; Sarkar et al. 2020; Raulo et al. 2021). For example, numerous studies of various non-human primates have demonstrated that the composition of gut microbiota is more similar between individuals that belong to the same social group versus different social groups (Degnan et al. 2012; Tung et al. 2015; Moeller et al. 2016; Amato et al. 2017; Grieneisen et al. 2017; Perofsky et al. 2017; Wikberg et al. 2020). A similar pattern has been observed among groups of feral horses (Antwis et al. 2018; Stothart et al. 2021). These patterns appear to be most strongly driven by the degree of social contact among individuals and only weakly driven by genetic relatedness or shared diet, suggesting that social contact provides a route of transmission for host-associated microbiota across animal species (Sarkar et al. 2020).

However, our understanding of how the social phenotypes found within animal social groups impacts the diversity and composition of individual gut microbial communities is limited. Social insects including ants, termites, and honeybees can exhibit phenotype-specific differences in gut microbial community diversity and composition (Shimada et al. 2013; Tarpy et al. 2015; Inagaki and Matsuura 2016; Jones et al. 2018; Otani et al. 2019; Sinotte et al. 2020). In social insects, differences in gut microbial communities among social phenotypes, including reproductive and non-reproductive subcastes, tend to be strongly associated with differences in diet and environmental exposure (Hongoh et al. 2006; Mikaelyan et al. 2015; Jones et al. 2018). However, given the bidirectional relationship between the animal brain and gut microbiota (i.e., the microbiota-gut-brain axis), it is plausible that different social phenotypes harbor unique microbiota independent of differences in diet or exposure (Sherwin et al. 2019). For example, social stress can induce compositional changes in the gut bacteria of lab mice via elevated host immune responses (Bailey et al. 2011), and various social disorders in humans are associated with altered gut microbiota (Kelly et al. 2016), although in these cases differences in diet or exposure are likely still at play (Yap et al. 2021). Furthermore, social stress induced by isolation in voles is accompanied by altered neuronal activity, neurochemical expression, and gut microbiota (Donovan et al. 2020). Thus, differences in gut microbiota among social phenotypes may be driven by the behavioral and physiological differences among animals or by different diets or exposure to different microbial colonists.

The naked mole-rat, *Heterocephalus glaber*, exhibits extreme cooperative breeding (eusociality) and presents an ideal opportunity to study the effects of social structure on the mammalian gut microbiota (Jarvis 1981). This species is native to sub-Saharan East Africa where it lives in underground colonies averaging between 60 and 80 individuals that form linear dominance hierarchies with individual differences in social phenotype (Jarvis 1981). Reproduction is limited to a single breeding female and one to three reproductive males, who are typically socially dominant and remain in these roles for life (Jarvis et al. 1994). The subordinate members of the colony show evidence of task specialization with some individuals, often called workers, performing a number of duties including nest maintenance, litter care, and foraging (Jarvis 1981; Lacey and Sherman 1991). Other larger non-breeders, which have been called soldiers, can be highly aggressive to unfamiliar individuals and presumably participate in colony defense (Holmes and Goldman 2021). An additional phenotype, termed disperser, is characterized by larger animals with a higher fat content, increased tendency to exit colonies, and reduced aggression towards unfamiliar animals (O'Riain et al. 1996; Toor et al. 2020).

Substantial social contact across all members of the colony exists, yet individual differences in social decision-making, and thus social behavior, are a key component of social cohesion and colony success (Holmes and Goldman 2021). While there is some overlap in behavior among the non-reproductive phenotypes (Gilbert et al. 2020), task specialization appears stable in the short term (months) though task switching does occur (Mooney et al. 2015). Sex differences in morphology and behavior are nearly absent in non-breeding animals, yet different social phenotypes exhibit variation in neural anatomy and endocrine function that are shaped by individual social experiences rather than genetic differences (Holmes et al. 2007, 2009; Coen et al. 2021; Holmes and Goldman 2021). However, when non-reproductive animals are removed from the colony, they can rapidly transition from their previous non-breeding phenotype to a reproductively active animal. One of the most striking differences in neural anatomy among social phenotypes is the increased size of the paraventricular nucleus of the hypothalamus (PVN) found in the reproductive colony members (Holmes et al. 2007). The hypothalamus, in turn, is connected to the gut via the vagus nerve, which is one of the main mechanisms of communication between the brain and gut microbiota (Bravo et al. 2011). Consequently, physiological differences among social phenotypes, including such central components in the microbiota-gut-brain axis, could be linked with differences in gut microbiota.

Previous studies have characterized the fecal, cecal, and respiratory bacterial communities of either captive or wild naked mole-rats (Debebe et al. 2017; Cong et al. 2018), but to date, no study has examined whether different social phenotypes or colonies vary in gut microbiota. Unlike social insects where different social phenotypes are associated with large differences in diet and environmental exposure (Jones et al. 2018; Sinotte et al. 2020), members of different social phenotypes in naked mole-rat colonies have the same diet and are exposed to largely the same

physical environment. Moreover, while the nature of contact can vary between social phenotypes (e.g., genital nuzzling occurs only between reproductive members), social contact in general, including exposure to a common toilet chamber, is abundant among all members within a colony. Therefore, differences in gut microbiota among social phenotypes in naked mole-rat colonies would be the result of differences in intrinsic physiology and not diet, environmental exposure, or social contact.

In this study, we used 6 experimental colonies, to perform a cross-sectional experiment testing the effect of social phenotype, status, and colony on the gut bacterial communities of *H. glaber*. Next, we performed a longitudinal study where we tracked the gut bacteria of 10 newly formed pairs of animals originating from different colonies during their transition from non-reproductive to reproductive social phenotype. Finally, we investigated how the unique ecology of naked mole-rats shape their gut microbiota by performing a comparative analysis with the gut microbiota of mice, *Mus musculus*, housed in the same facility and exposed to similar environments. Mice present a useful comparison as they serve as a model system for understanding the interplay between gut microbiota and animal behavior (Buffington et al. 2016; Sgritta et al. 2019), and both mice and naked mole-rats belong to the order Rodentia. Furthermore, sample collection from the two host species under very similar environments affords a more direct comparison of their gut bacteria and eliminates the confounding factors of captivity status or facility effects often present in comparative gut microbial studies. Studying the gut microbiome of naked mole-rats across and within social groups, in addition to tracking gut microbial changes occurring as individual transition between reproductive phenotype, provides insight into how social roles and structure within a mammalian species can shape the gut microbiome.

Materials and methods

Experimental system

Cross-sectional study

Six captive colonies of naked mole-rats maintained in the University of Toronto Mississauga vivarium were used in this study (see Supplemental Methods for details). All colonies originated from the same founding population of wild animals and have been in captivity for the same length of time. Animals were fed hydrated sweet potato daily and wet Teklad Global 19% protein extruded rodent diet three times a week. Individuals were micro-chipped at a minimum of 6 months of age with a subcutaneous microchip (Avid, Cat. No. 2125, 12 mm) for identification purposes.

Social phenotype of individual animals was determined using a battery of tests including an out-pairing test to measure aggression (Toor et al. 2020), a disperser test to measure motivation to leave the colony (O'Riain et al. 1996; Toor et al. 2020), and a social dominance test (Toor et al. 2015). All testing took place between 12:00 P.M. and 5:00 P.M., and all behavioral scoring was performed by an experimenter blind to the familiarity status of stimulus animals as well as sex and phenotype, but not colony, of the experimental animals. See Supplemental Methods for full details. All fecal samples from the cross-sectional experiment were collected over the course of 1 month from October to November 2018.

To compare the gut microbiota between the two host species, mouse feces were also collected from the same animal facility. Ten mice (5 male and 5 female) from 4 different group housed cages were used to collect fecal samples. The mice were wildtype C57BL and were inbred due to in-house breeding. One cage was made up entirely of siblings, and the other 3 mixed housed with siblings and cousins. All mice were fed Teklad Global 19% protein extruded rodent diet and were 80 days old upon collection. Thus, with the exception of the sweet potato, the naked mole-rats and mice had identical diets.

Longitudinal study

We selected animals from experimental colonies based on the availability of opposite sexed individuals. Naked mole-rats were paired by choosing a male and a female of similar size and weight from two different experimental colonies and placing them in a small cage. Note that all animals selected for pairing exhibited no aggression and were non-reproductive, thus belonging to either the worker or disperser social phenotype. Baseline fecal samples were collected, and opposite-sex pairs were established in September 2018. Fecal samples were collected again at the 1-month and 2-month timepoints from the paired animals.

Sample harvest

To collect fecal samples, individuals were placed in an empty small cage. The hind legs of the individual were raised using the base of the tail, and its genitalia were lightly physically stimulated to help produce a bowel movement. Upon defecating, the fecal sample was collected from the clean caging, placed in microcentrifuge tubes, and immediately frozen in liquid nitrogen, and the naked mole-rat was returned to its regular caging. After collections were complete, the microcentrifuge tubes were removed from the liquid nitrogen and placed into a -80C freezer until processing.

DNA extraction and 16S amplification

Gut bacterial communities were characterized using amplification and partial sequencing of the 16S rRNA gene. DNA was extracted using the IBI stool DNA isolation kit (IBI Scientific, Dubuque, Iowa USA) using between 50 and 250 mg of fecal material for each sample. We amplified the V4 region of the 16S rRNA gene using a dual-index approach to barcode amplified DNA at the 3′ and 5′ ends (see Supplemental Methods for details). Pooled libraries were sequenced on an Illumina MiSeq using 2×250 bp paired-end reads. We recovered on average 38,415 (range: 16,478–75,382) high quality bacterial sequences from each individual. A rarefaction curve revealed that ASV richness plateaued well before the total number of reads in all samples (Fig. S1).

Bioinformatic analyses

We used the R package 'DADA2' to process our sequencing reads (Callahan et al. 2016), which infers amplicon sequence variants (ASV), and the R package 'phyloseq' to further process our samples (McMurdie and Holmes 2013) (see Supplemental Methods for full details). We constructed a phylogenetic tree of our ASVs with the phylogenetic placement method outlined in Janssen et al. (2018) and implemented using the SILVA 128 reference alignment in QIIME2 (Bolyen et al. 2019). We simplified our dataset to include only common ASVs for downstream analyses of community composition and differential abundance testing of bacterial taxa. We define common as occurring in at least 5 samples at an abundance of at least 25 sequences (Lundberg et al. 2012), which yielded 553 ASVs and accounted for 98% of the total number of sequences in the dataset. However, we also repeated analyses with a threshold of 2 samples at an abundance of at least 25 sequences and found near identical results. We performed a proportional abundance normalization (sequencing reads for an ASV in a given sample were divided by the total number of sequencing reads in that sample) on this common set of ASVs for our analysis of community composition (McMurdie and Holmes 2014). We also rarefied samples to the lowest read count in the dataset (16,478 reads) and found near identical results to the proportional abundance normalized analyses. We report results with the proportional abundance normalized dataset with the 5×25 threshold.

Statistical analyses

Diversity and composition

We used linear models (R function 'aov') to analyze the effects of social phenotype, colony, sex, rank, age, weight,

and technical factors on bacterial diversity in the cross-sectional study. In the longitudinal study, we used paired t-tests to analyze whether the diversity of individual animals or the difference in diversity between animals within a pair changed between sampling times. We calculated α-diversity as ASV richness (R), inverse Simpson's diversity ($D^{-1}: 1/\sum_{i=1}^{R} p_i^2$), and evenness ($D^{-1}/R$), where p is the proportional abundance of an ASV within a sample. Richness is strongly affected by sampling effort, whereas D^{-1} is less sensitive to rare taxa and indicates the number of species in a theoretical community with the observed level of diversity but with a uniform proportional abundance of each species. High values of evenness indicate communities that have uniform proportional abundance of each species, and low values indicate communities that have uneven proportional abundance of each species.

We used permutational multivariate ANOVA (R function 'adonis2' from the 'vegan' package v. 2.5–7 [Oksanen et al. 2020] marginal significance of terms computed) to analyze gut bacterial composition using the common ASV dataset described above. We used three different measures of community distance, weighted UniFrac, Bray–Curtis, and Jaccard's (note that ASV counts were converted to presence/absence before computing Jaccard's distance). These three measures of community distance are complementary. First, Jaccard's distance is calculated from presence/absence data and places equal weight among rare and abundant taxa that might differentiate two samples, whereas both weighted UniFrac and Bray–Curtis are abundance weighted and are affected less by rare taxa. Two samples that exhibit high dissimilarity with Jaccard's distance but not Bray–Curtis or weighted UniFrac are likely differentiated by low abundance taxa. Second, the weighted UniFrac distance is phylogenetically informed, whereas the Bray–Curtis distance is not. Two samples that exhibit high dissimilarity with Bray–Curtis distance but not weighted UniFrac are differentiated by closely related taxa. For visualization purposes, we performed principal coordinate analyses (PCoA) using weighted UniFrac distance matrices. Additionally, we tested whether compositional variance differed among colonies or social groups (function 'betadisper' from the 'vegan' package v. 2.5–7 [Oksanen et al. 2020]). We also used paired t-tests to ask whether the compositional difference between animals within a pair decreased between sampling times.

Differential abundance

We used the raw read counts of ASVs from the common ASV dataset, as well as the read counts of these ASVs aggregated at each higher bacterial taxonomic rank to analyze differential abundance of individual phyla, classes, orders, families, genera, and ASVs. We used three different methods to analyze differential abundance 'DESeq2' v. 1.20.0 (Love et al. 2014), 'ALDEx2' v. 1.26.0 (Fernandes et al. 2014),

and 'ANCOM-BC' v. 1.4.0 (Lin and Peddada 2020). Benchmarking studies demonstrate that all three methods exhibit acceptable false discovery rates (Lin and Peddada 2020; Nearing et al. 2022), and here we adopt the recently recommended practice of taking a consensus approach, whereby we require taxa to be called as significant by at least two of the three methods (Nearing et al. 2022). A full description of the parameters and models used across these methods can be found in the Supplemental Methods. All results from the differential abundance analysis are reported in Dataset S1.

See Supplemental Methods for a full description of statistical analyses.

Results

Colony not social phenotype or status shapes the naked mole-rat gut microbiome

Colonies significantly differed in the diversity (ANOVA: $F_{1,5} = 4.73$, $P < 0.001$; Table S2) and composition (PERMANOVA: weighted UniFrac, pseudo-$F_{1,5} = 6.13$, $R^2 = 0.25$, $P = 0.001$; Table S3) of gut bacteria (Fig. 1a, c, e). The average diversity among colonies differed by up to 50% (Fig. 1a), and compositional dissimilarity among colonies accounted for up to 37% of the total variation in community variation among individuals (Fig. 1c, e). The compositional variance explained by colony was 25% using the weighted UniFrac distance, 32% using the Bray–Curtis distance, and 37% using Jaccard's distance. Colonies also significantly varied in their beta-dispersion, meaning some colonies had greater compositional differences in gut bacteria among members than others, but this result depended on the distance measure used (ANOVA: weighted UniFrac distance $F_{1,5} = 2.76$, $P = 0.02$; Bray–Curtis distance $F_{1,5} = 1.91$, $P = 0.10$; Jaccard's distance $F_{1,5} = 6.31$, $P < 0.001$; Table S4). In accordance with the observed differences in composition, large numbers of bacterial taxa across all taxonomic ranks were differentially abundant across colonies (Fig. 3; $P_{adj} < 0.05$: 192/366 ASVs, 46/74 genera, 23/36 families, 16/24 orders, 12/20 classes, 9/14 phyla; Dataset S1).

Fig. 1 Colonies but not social phenotypes differ in gut bacterial diversity and composition. **A** and **B** Diversity and **C** and **D** composition of gut bacterial communities among every individual colored according to colonies and social phenotype. The ordinations in (**C** and **D**) depict the first two coordinate axes of a PCoA of weighted UniFrac distance among every individual sample in the cross-sectional study. Panels E and F depict the relative abundance of bacterial phyla and families within the Bacteroidetes and Firmicutes across colonies (**E**) and phenotypes (**F**). Colors refer to colony in panels **A**, **C**, and **E** and phenotype in panels **B**, **D**, and **F**
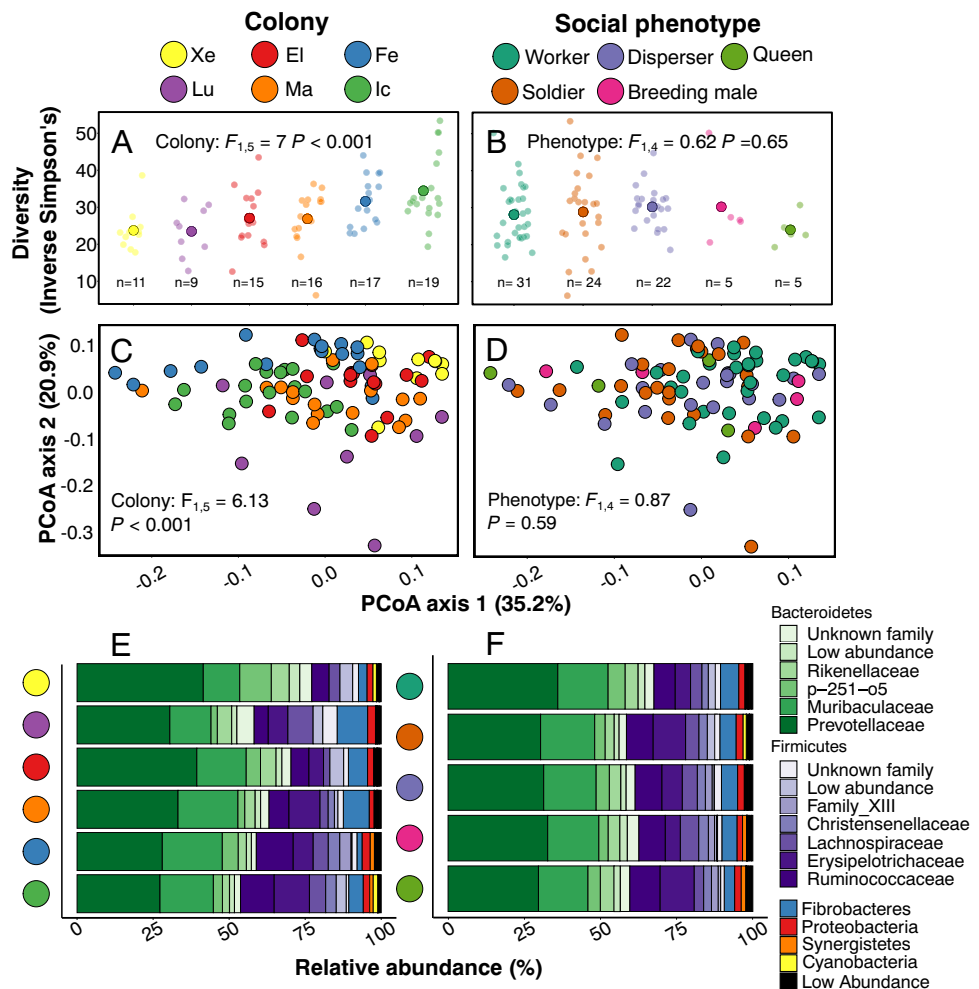
In contrast to colony, social phenotype had no effect on the diversity (ANOVA: $F_{1,4} = 0.62$, $P = 0.65$; Fig. 1b, Table S2) or the composition of naked mole-rat gut bacteria (PERMANOVA: weighted UniFrac, pseudo-$F_{1,4} = 0.87$, $R^2 = 0.03$, $P = 0.59$; Fig. 1d, f, Table S3). We also found no differences among social phenotypes in levels of beta-dispersion among members regardless of the distance measure used (ANOVA: weighted UniFrac distance $F_{1,4} = 1.59$, $P = 0.18$; Bray–Curtis distance $F_{1,4} = 2.01$, $P = 0.10$; Jaccard's distance $F_{1,4} = 0.36$, $P = 0.83$; Table S4). In addition to social phenotype, we also included a quantitative measure of social status, the linear rank of individual animals within each colony measured using pairwise dominance assays. However, rank had no effect on gut bacterial diversity (ANOVA: $F_{1,1} = 0.09$, $P = 0.76$; Table S2) or composition (PERMANOVA: weighted UniFrac, pseudo-$F_{1,1} = 0.76$, $R^2 = 0.01$, $P = 0.58$; Table S3). We also included animal age and weight as covariates in our analyses and found no effects of either on gut bacterial diversity or composition (Table S2, S3). Across all bacterial taxonomic ranks, we found only a single taxon, an ASV belonging to the Bacteroidales, which differed in abundance among phenotypes (Fig. 3; Dataset S1).

Isolated pairs do not exhibit increased microbiome resemblance over 2 months

Given the large effect of colony from the cross-sectional study, we predicted that over time the diversity and composition of gut bacteria would increase in similarity between individuals within isolated pairs due to transmission via social contact. Conversely, since we found no effect of social phenotype on gut bacteria, we predicted that the transition to reproductive status would not lead to convergent shifts in gut bacterial composition across pairs.

Diversity of separated males or females did not consistently change over the course of the pairing (paired t test: T1 vs. T2, $t = -1.27$, $P = 0.22$; T1 vs. T3, $t = -0.89$, $P = 0.39$; T2 vs. T3, $t = 0.74$, $P = 0.47$; Fig. 2a; Table S5). Similarly, we found that the difference in gut bacterial diversity between animals within a pair did not change over time (paired t test: T1 vs. T2, $t = -0.18$, $P = 0.57$; T1 vs. T3, $t = 1.37$, $P = 0.11$; T2 vs. T3, $t = -0.95$, $P = 0.81$; Fig. 2b; Table S6). Gut bacterial composition varied among pairs (Fig. 2c; PERMANOVA: Bray–Curtis, pseudo-$F_{1,9} = 1.48$, $R^2 = 0.21$, $P = 0.006$; Table S8) and sex (Fig. 2c; PERMANOVA: Jaccard's, pseudo-$F_{1,1} = 1.69$, $R^2 = 0.03$, $P = 0.03$; Table S8); however, these results depended on which measure of community distance

Fig. 2 The gut bacteria of newly paired individuals do not resemble one another over time. Gut bacterial diversity (**A**) of paired individuals and the difference in diversity between paired animals (**B**) over the course of isolation. Shapes in panel **A** refer to sex. (**C**) The ordination depicts the first two coordinate axes of a PCoA of weighted UniFrac distance among every individual sample in the longitudinal study. Colors refer to unique animals pairs, shape refers to timepoint, and outline refers to sex. The inset panel in **C** illustrates the within-pair compositional dissimilarity among pairs across the time course
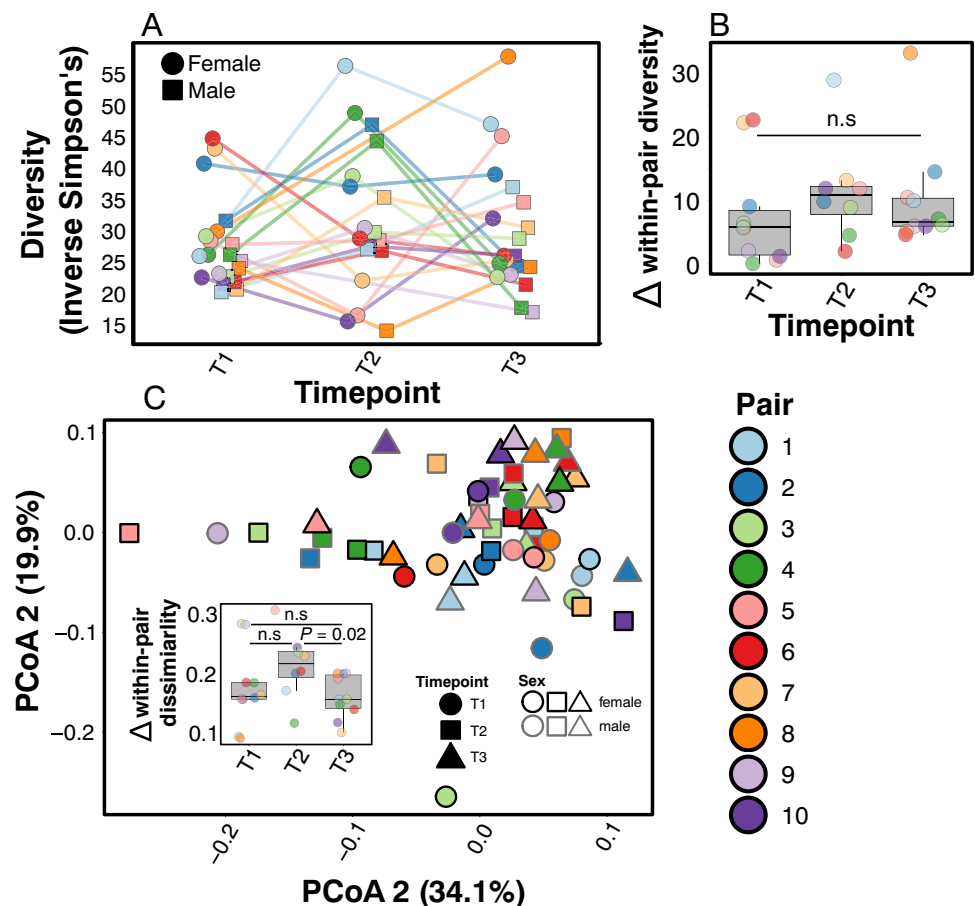
Fig. 3 Experimental factors affecting the abundance of the naked mole-rat core gut bacterial community. The phylum assignment of each ASV represented in the phylogenetic tree is shown in the first column of panel **A**. Columns 1–6 show the effects of different factors within the cross-sectional study on the abundance of individual ASVs (column numbers refer to the factors listed on the x-axis in panel **B**). Columns 7–9 show the effects of different factors within the longitudinal study. We used a consensus approach using three differential abundance analysis methods (see "Materials and methods"). Cells colored yellow, blue, or purple represent significant effects of experimental factors on taxon abundance found with one, two, or all three methods, respectively (false discovery rate adjusted P values < 0.05). We summarize the consensus distribution among all ASVs for each experimental factor in panel **B**. Full differential abundance results can be found in Supplemental Dataset S1
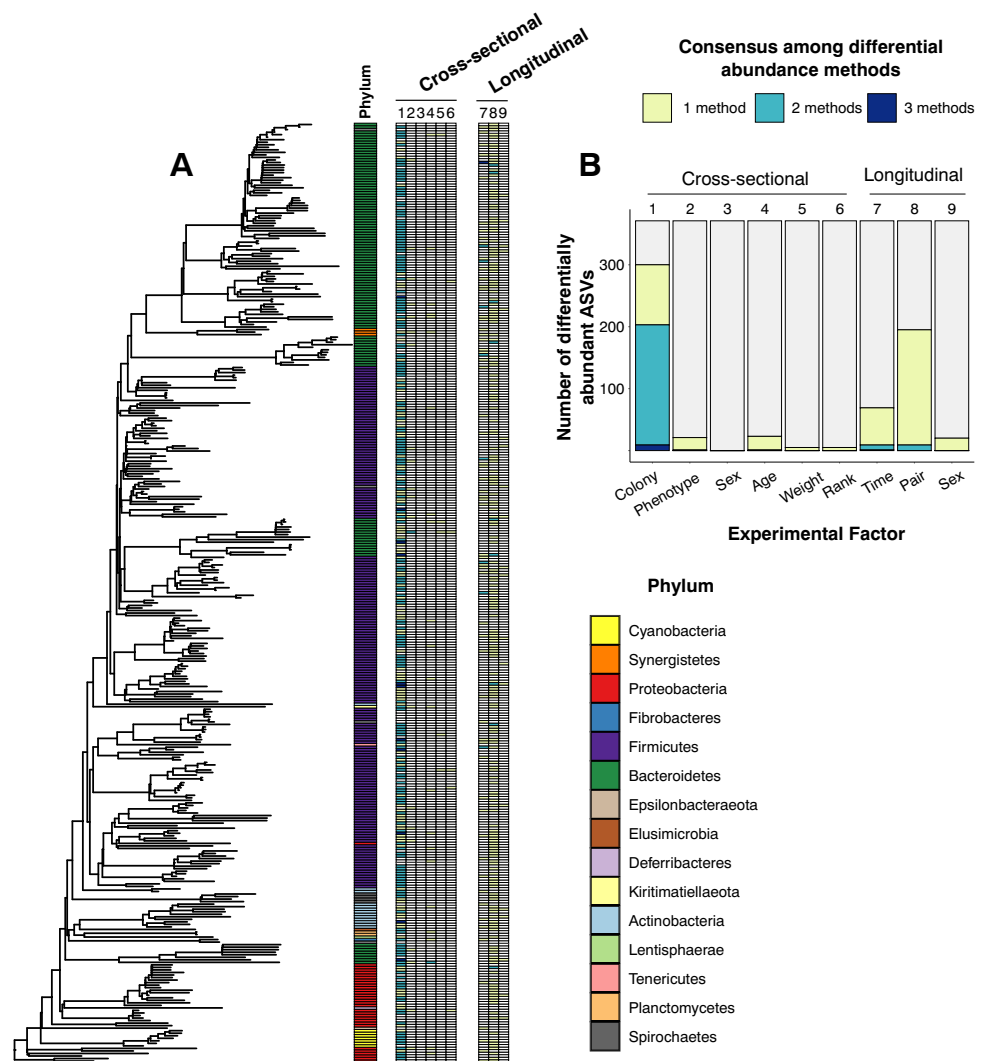

we analyzed. The effect of pair was not evident when using the weighted UniFrac distance, and the effect of sex was only evident when using Jaccard's distance. Neither the effect of pair nor sex changed over time (pair × time PERMANOVA: Bray–Curtis, pseudo-$F_{1,18} = 0.87$, $R^2 = 0.24$, $P = 0.92$; sex × time PERMANOVA: Bray–Curtis, pseudo-$F_{1,2} = 0.52$, $R^2 = 0.02$, $P = 0.99$; Table S8). All pairs exhibited a consistent shift in gut bacterial composition over time regardless of the distance measure used (Fig. 2c; PERMANOVA: Bray–Curtis, pseudo-$F_{1,2} = 2.79$, $R^2 = 0.08$, $P = 0.001$; Table S8), but animals within a pair did not become more compositionally similar to one another with the exception of timepoints 2 and 3 (paired t test using weighted UniFrac distance: T1 vs. T2, $t = -1.58$, $P = 0.16$; T1 vs. T3, $t = 0.64$, $P = 0.54$; T2 vs. T3, $t = 2.86$, $P = 0.02$; Fig. 3c inset; Table S7). Our differential abundance analysis revealed that 8 ASVs were differentially abundant over time, which included 4 ASVs from the Prevotellaceae (Fig. 4). Additionally, 7 ASVs were differentially abundant across pairs (Fig. 4; Dataset S1).

The naked mole-rat gut bacterial community differs markedly from mice

We compared the diversity and composition of gut bacteria of the naked mole-rat to that of mice housed in the same facility. Mice exhibited slightly elevated ASV diversity than naked mole-rats (ANOVA: $F_{1,1} = 12.56$, $P < 0.001$; Fig. 4a; Table S9); however, the bacterial communities of mice and naked mole-rats were compositionally distinct (PERMANOVA: weighted UniFrac, pseudo-$F_{1,1} = 81.86$, $R^2 = 0.42$, $P < 0.001$; Fig. 4b; Table S10). We found that high proportions of bacterial taxa across all taxonomic ranks exhibited differential abundance between the host species (Fig. 4c; Dataset S1), indicating that naked mole-rats and mice harbor fundamentally different gut microbiota from the level of phylum to ASV. At the phylum level, the naked mole-rat gut microbiome was characterized by an enrichment of Bacteroidetes and depletion of Firmicutes relative to mice (Fig. 4c, d; Dataset S1). At

Fig. 4 The **A** diversity and **B** composition of gut bacteria across host species and sex. The ordination in (**B**) depicts the first two coordinate axes of a PCoA of weighted UniFrac distance among every individual sample in the dataset. The compositional differences between the gut bacteria of *H. glaber* and *M. musculus* are largely driven by **C** shifts in the relative abundance of the two major bacterial phyla colonizing mammalian guts, Firmicutes and Bacteroidetes. **D** A number of abundant families in addition to Prevotellaceae and Lactobacillaceae exhibit differential abundance between *H. glaber* and *M. musculus* (note results from the DESeq2 analysis are shown but all three methods used to analyze differential abundance yield qualitatively identical results; Supplemental Dataset S1)
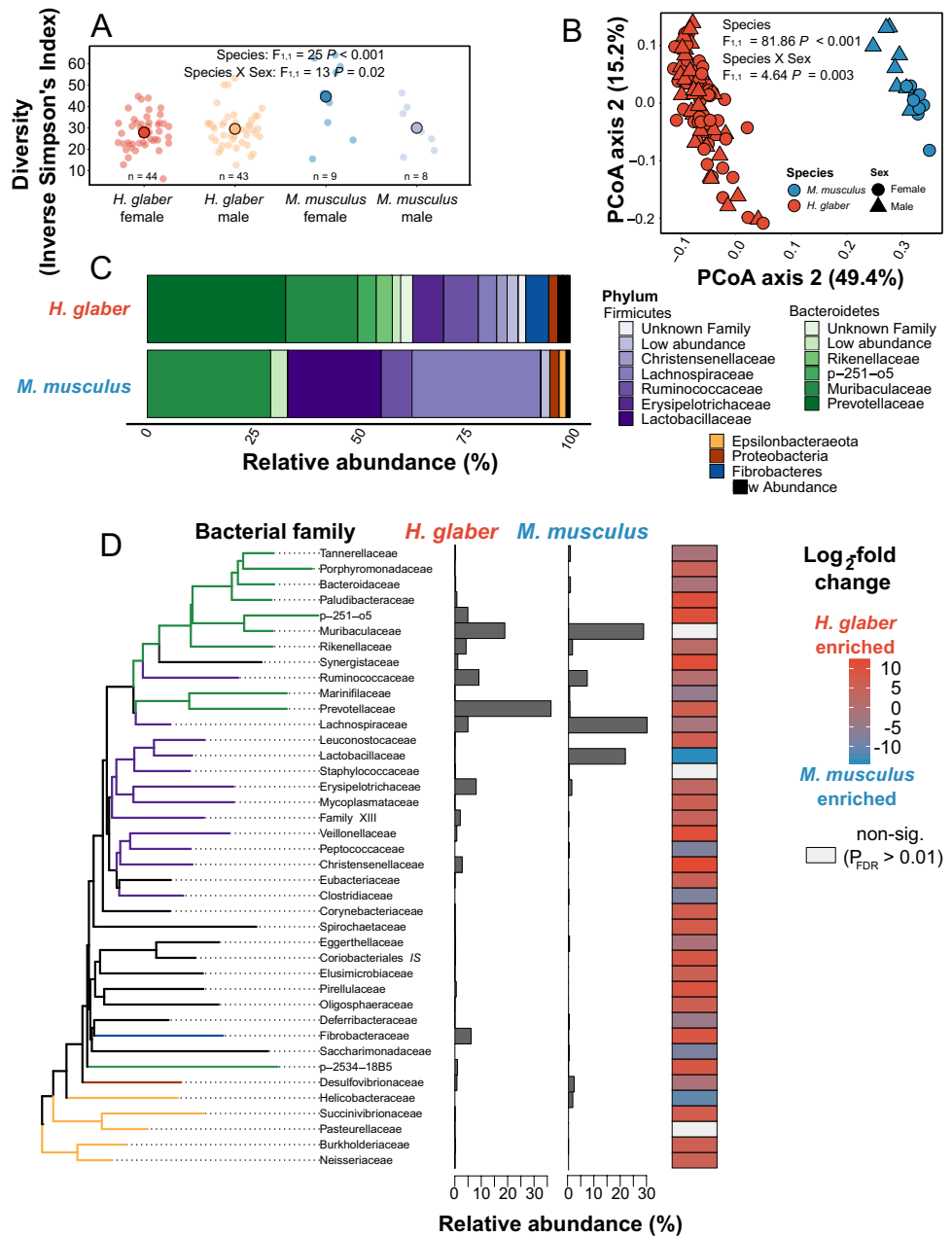
the family level, members of the Lactobacillaceae (Firmicutes) accounted for 25% of the mouse gut microbiome but were almost absent from naked mole-rats (Fig. 4d), while members of the Prevotellaceae (Bacteroidetes) and Erysipelotrichaceae (Firmicutes) made up over 40% of the naked mole-rat gut microbiome but were near absent from mice (Fig. 4d).

Interestingly, we also observed significant sex differences in both the diversity (ANOVA: species X sex, $F_{1,1} = 13.12$, $P < 0.001$) and composition (PERMANOVA: species × sex, weighted UniFrac, pseudo-$F_{1,1} = 4.64$, $R^2 = 0.02$, $P = 0.003$) of gut bacterial communities in mice but not naked mole-rats (Fig. 1a, b; Table S9, S10).

Discussion

In the present study, we performed a cross-sectional experiment to investigate the role of colony, social phenotype, and individual attributes on the diversity and composition of naked mole-rat gut bacteria. We found strong effects of colony but no detectable effects of social phenotype, rank, or other individual attributes. Next, we performed a longitudinal experiment, whereby we tracked the individual microbiota of pairs of newly isolated and unfamiliar animals. While composition changed over time, we found little evidence that pairs exhibited convergence in their gut bacteria over the course of 2 months. Lastly, we sought to

uncover the unique features of the naked mole-rat gut bacteria by comparing it to mice exposed to very similar environmental conditions. We found notable compositional differences and a lack of sex differences in gut bacterial diversity and composition in naked mole-rats versus mice.

Despite being housed in the same facility, under identical environmental conditions, and consuming a common diet, naked mole-rat colonies harbor distinct microbiota that vary in both diversity and composition. The compositional differences we observed across colonies were independent of the measure of community distance used though were largest with the measure which was neither informed by bacterial abundance nor phylogeny (Table S3). This indicates that while rare bacterial taxa from across the observed phylogenetic diversity contribute to differences between colonies, much of these differences are driven by abundant taxa from particular bacterial clades. Variation in gut microbiota between different colonies or social groups occurs across animal life from insects to primates (Antwis et al. 2018; Segers et al. 2019; Sarkar et al. 2020; Sinotte et al. 2020; Stothart et al. 2021). Animal groups in the wild can have access to different resources and may be exposed to different environments, both of which could contribute gut microbial variation. How such differences in gut bacteria arose in the captive naked mole-rat colonies is unclear but could simply be the result of stochastic changes in gut bacterial populations in founding colony members (i.e., community drift (Zhou 2017)). While stochastic in origin, these differences could then be amplified and propagated throughout the colony by various forms of social contact and coprophagy (Miller et al. 2018). Importantly, the colonies used in the current study all originate from the same collection of wild animals from a single locality and are the result of 3–5 generations of captive breeding (see Supplemental Material for greater detail). Due to the extreme cooperative breeding in naked mole-rats, it is also possible that genetic differentiation between colonies could be contributing to differences in gut bacteria; however, past studies have found little to no effect of genetic distance in mice on caecal content bacteria (Linnenbrink et al. 2013). Further study is required to determine the source of colony level differences in naked mole rat gut microbiota. Colony-specific differences in microbiota play a role in social recognition in honeybees by modulating cuticular hydrocarbon profiles (Vernier et al. 2020). Given the role of colony-specific odors and olfaction in social cohesion in naked mole-rats (O'Riain and Jarvis 1997; Toor et al. 2015), it is plausible that the gut microbiome may be contributing to such recognition via unknown chemical cues (Theis et al. 2012; Ezenwa and Williams 2014; Barker et al. 2021).

While naked mole-rat colonies differed in the diversity and composition of gut microbiota, we found limited effects of social phenotype or status. This contrasts with studies of social insects where subcastes can differ in gut bacterial composition. However, these differences are primarily driven by differences in diet and environmental exposure instead of intrinsic factors associated with different subcastes (Kapheim et al. 2015; Benjamino and Graf 2016; Sinotte et al. 2020). For example, in the highly structured Termitid termite colonies, gut bacteria can differ substantially between breeding and non-breeding subcastes, but this seems to driven by strict dietary differences and greatly reduced environmental exposure between the royal pair and the rest of the colony (Otani et al. 2019). In the case of honeybees, the largest difference is between queens and worker bees, which is thought to be driven by different modes of microbial transmission and diet (Kapheim et al. 2015; Jones et al. 2018). In primates, the largest differences in gut microbiota occur between members of different groups, while the effect of social contact within groups on gut microbial similarity is much more limited (Degnan et al. 2012; Tung et al. 2015; Moeller et al. 2016; Wikberg et al. 2020). By uncoupling social group and/or phenotype from large differences in diet, social contact, and environmental exposure, our study of replicated naked mole-rat colonies demonstrates that variation in intrinsic physiology associated with social phenotypes and status in a mammalian species has little effect on gut bacterial communities.

Collectively, our results from the longitudinal study demonstrate that the resultant social contact of pairing did not increase similarity in gut bacteria of animals within pairs and the compositional differences among pairs did not change over 2 months. These results are seemingly at odds with recent findings. For example, as primates immigrate into new social groups, the immigrant microbiome rapidly transitions to resembling that of the resident (Degnan et al. 2012; Grieneisen et al. 2017; Vangay et al. 2018; Goodfellow et al. 2019). Such changes can occur within 6 months of arrival and grow in magnitude with time since immigration (Grieneisen et al. 2017; Vangay et al. 2018). Furthermore, recently weaned rhesus monkeys converge in gut bacterial composition after 2 weeks of being introduced into social groups (Amaral et al. 2017). Finally, Donovan et al. (2020) found that prairie voles in complete isolation exhibited alterations to their gut bacteria in comparison to animals caged with an opposite-sex individual. However, there are important differences between our longitudinal experiment and those reported above. Here, animals did not immigrate into a large group, where potential for gut colonization by resident microbiota would be high and the new environments of the paired animals were nearly identical to those of the colonies from which they came. Furthermore, though our pairs were isolated from their respective natal colonies, they were not experiencing complete social isolation. However, we note that the pairing performed in our

experiment has been shown to elevate blood and fecal cortisol levels indicating that such pairing is stressful (Peragine et al. 2016; Edwards et al. 2020). Given that acute stress can alter gut microbiota, including *Prevotella* spp. (Maslanik et al. 2012), perhaps convergence between pairs may occur after the effects of acute stress diminish. This is supported by our observation that gut bacterial similarity increases between the middle and last timepoints (paired t test: T2 vs. T3, $t = 2.89$, $P = 0.02$; Fig. 2c inset; Table S7). Or perhaps as colonies grow with the birth of new pups, consistent colony-specific differences might emerge. Such a result could mean that similarity among colony members (e.g., Fig. 1c) is driven by pups born in the colony, while breeding animals remain distinct. However, we found no evidence that community dissimilarity between breeders was any greater than the community dissimilarity among non-breeding animals from our cross-sectional study (Table S11).

Naked mole-rats have a markedly different gut microbiome versus mice, driven by differential abundance of bacterial taxa across all taxonomic ranks. Additionally, we found differences between sexes in the diversity and composition of microbiota in mice but not naked mole-rats. In mice, sex differences in gut microbiota vary across strains and environmental conditions (Org et al. 2016). The lack of observable differences in gut microbiota between sex in naked mole-rats could be driven by the repression of sexual maturity in the bulk of our naked mole-rat sample population. However, when considering only breeding naked mole-rats, we were still unable to detect sex differences in diversity (ANOVA: $F_{1,1} = 0.19$, $P = 0.67$) or composition (PERMANOVA: pseudo-$F_{1,1} = 1.04$, $R^2 = 0.01$, $P = 0.35$). The observed differences between the gut microbiota of mice and naked mole-rats could be driven by any number of innate traits. Both mice and naked mole-rats engage in coprophagy and have abundant social contact among individuals. As with most comparative gut microbiome studies that investigate interspecific differences (Ley et al. 2008), we acknowledge that differences in diet between naked mole-rats and mice in our study could be contributing to the observed variation in gut microbiota. In our vivarium, the mice and naked mole-rat colonies were both fed standard mouse chow, but the naked mole-rats were also fed sweet potato. However, environmental conditions were otherwise very similar; consequently, our common environment design allows us to compare the gut bacteria of these two host species more directly than past studies that use wild animal or captive zoo collections (Debebe et al. 2017). Many of the taxa enriched in the gut microbiota of naked mole-rats (members of the Fibrobacteres, Spirochaetes, and Elusimicrobia; Dataset S1) are also prominent members of the gut bacteria found in higher termites (Mikaelyan et al. 2015), which feed on diverse lignocellulosic diets. Thus, it seems likely that some of the observed differences in the gut microbiota between mice and naked mole-rats are also driven by diet differences in captivity and/or naked mole-rat traits associated with adaptation to a diet high in cellulosic material in the wild (Lacey and Sherman 1991; Dyer 1998).

Perhaps the most striking difference between the gut bacteria of mice and naked mole-rats is the shift in dominance of the two major phyla in the mammalian gut microbiome, Firmicutes and Bacteroidetes. These differences at the phyla level were driven by members of the Lactobacillaceae (Firmicutes), which accounted for 25% of the mouse gut microbiome but were almost absent from naked mole-rats, and members of the Prevotellaceae (Bacteroidetes), which made up 30% of the naked mole-rat gut microbiome but were near absent from mice. Lactobacillaceae taxa are common gut microbiome members across animal species, and inoculation of particular strains can alter social behavior and ameliorate a wide range of social deficits (Vuong et al. 2017; Sherwin et al. 2019). For example, social deficits induced by maternal-derived gut microbial perturbations in mice were restored by inoculation with a single strain of *Lactobacillus reuteri* (Sgritta et al. 2019). The near absence of Lactobacillaceae and *Lactobacillus* from the gut microbiome of naked mole-rats is notable given the strong associations of this genus with host social behavior from fruit flies to humans. Given their prominence in the naked mole-rat gut microbiome and the fact that some members exhibit differential abundance across colonies and during our longitudinal study, we speculate that Prevotellaceae may harbor gut symbionts with close association to naked mole-rat gut and environmental attributes. Interestingly, taxa within Prevotellaceae were found to be among the most heritable members of the baboon gut microbiome (Grieneisen et al. 2021) and in human populations are rapidly lost in southeast Asian immigrants to the USA (Vangay et al. 2018). Additionally, the genus *Prevotella* exhibits shifts in abundance across human populations and is associated with a fiber-rich diet (Yatsunenko et al. 2012; O'Keefe et al. 2015). These results mirror our finding that Prevotellaceae are much more abundant in the host with the fiber-rich diet, naked mole-rats versus mice and exhibit variation across naked-mole rat colonies.

Our results are remarkably consistent with recent studies (Debebe et al. 2017; Cong et al. 2018), which characterized the gut bacteria of wild or other captive naked mole-rat individuals. In these studies, naked mole-rats exhibited enrichment of Bacteroidetes, particularly Prevotellaceae. Collectively, these findings indicate the existence of a distinctive bacterial community colonizing the guts of naked mole-rats independent of captivity status, environmental exposure, and diet. To fully understand how the ecology of naked mole-rats has shaped gut microbiota, future studies will need denser sampling of the mole rat-lineages within the infraorder Phiomorpha, including another eusocial mammal species, *Fukomys damarensis* (Holmes et al. 2009).

Conclusions

We used six replicate colonies in our cross-sectional study to demonstrate that colony (i.e., social group) plays a large role in structuring gut microbiota. Such colony-specific microbiome signatures could contribute to colony recognition via olfactory signals, as observed in other social animals. In contrast, individual rank and social roles within colonies (i.e., subgroups or phenotypes) had limited effects on gut microbiota. Therefore, our results indicate that beyond influencing diet or exposure to microbes, social roles and their accompanying unique physiology and social experience may have little effect on gut microbiota. Our inability to detect large changes in the gut microbiota of animals transitioning from non-breeding to breeding during our longitudinal study further supports this conclusion. Paired animals did not converge in gut microbial similarity over the course of 2 months, tempering the notion that social contact exclusively drives microbiome similarity between interacting individuals. The large compositional differences between the gut bacteria of naked mole-rats and mice likely reflect the peculiarities of naked mole-rat ecology, which includes a diet rich in lignocellulosic material. Given the role of members of *Lactobacillus* in the microbiota-gut-brain axis, their absence from the naked mole-rat gut microbiome is striking.

Declarations

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