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Effects of sample collection and storage methods on fecal bacterial diversity in California ground squirrels (*Otospermophilus beecheyi*)

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Measures of fecal bacterial abundance and taxonomic composition are commonly used as proxies for gut microbial diversity in studies of free-living mammals. Because methods of sample collection and storage may affect measures of bacterial diversity, we evaluated the effects of several procedures on fecal bacterial diversity in a free-living population of California ground squirrels (*Otospermophilus beecheyi*). Replicate fecal samples from 12 adult female squirrels were collected either from the soil beneath traps in which individuals had been captured or from tubs placed under squirrels during handling. Samples were then either frozen immediately in liquid nitrogen or stored on ice for several hours before being transferred to a −80°C freezer. Sequencing of the bacterial *16s RNA* gene revealed no differences among methods with regard to sequence read depth (number of sequences recovered per animal) or alpha (within-individual) diversity of bacterial taxa. While our collection-storage procedures had a significant effect on one of the metrics of beta (among-individual) bacterial diversity examined, this effect was small compared to that of individual identity. Date of sample collection affected alpha and beta diversity; samples collected only 1 week apart differed significantly in bacterial diversity. Overall, these findings suggest that the collection and storage methods employed yield generally comparable information and are capable of capturing potentially important patterns of fecal bacterial composition and diversity in free-living mammals.

Key words: fecal sampling, gut microbiome, noninvasive measures, *Otospermophilus beecheyi*, rodent

La abundancia de bacterias y composición taxonómica en muestras fecales son medidas comúnmente utilizadas para evaluar la diversidad de microbiomas intestinales en estudios de mamíferos de vida libre. Debido a que métodos de colección y almacenamiento de muestras pueden impactar las medidas de diversidad bacteriana, evaluamos los efectos de diferentes métodos en la diversidad bacteriana en muestras fecales en la población de vida libre de la ardilla de tierra de California (*Otospermophilus beecheyi*). Réplicas de muestras fecales de doce hembras adultas fueron colectadas tanto del suelo debajo de las trampas o de los contenedores de plástico donde se llevó a cabo el manejo de las ardillas. Las muestras fueron congeladas inmediatamente en nitrógeno líquido o almacenadas en hielo por varias horas, antes de transferirlas a un congelador a −80°C. La secuenciación del gen bacteriano *16s RNA*, no mostró diferencias en los métodos con respecto a la profundidad de secuenciación (número de secuencias recuperadas para cada animal) o la diversidad alfa de los taxa bacterianos (dentro de cada individuo). Sin embargo, nuestro protocolo de colecta-almacenamiento tuvo un efecto significativo en una de las métricas examinadas de diversidad bacteriana beta (entre individuos), siendo un efecto pequeño en comparación con la identidad individual. Asimismo, la fecha de la colección de la muestra afectó la diversidad alfa y beta; donde las muestras colectadas sólo con una semana de distancia muestran una significante diferencia en la diversidad bacteriana. En general, estos resultados sugieren que los métodos de colección y almacenamiento empleados producen información comparable y son capaces de potencialmente capturar importantes patrones de composición y diversidad bacteriana en muestras fecales en mamíferos de vida libre.

Palabas clave: muestra fecal, microbioma intestinal, medidas no-invasivas, *Otospermophilus beecheyi*, roedor

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Save Mount DiabloMills CollegeMills CollegeThe composition and diversity of the gut microbiome are associated with multiple adaptively important aspects of organismal function including digestive processes (Suzuki 2017), immune responses (Bäckhed et al. 2012), ontogenetic changes (Diaz Heijtz et al. 2011), and—in some species—social behavior (Ezenwa et al. 2012; Tung et al. 2015; Raulo et al. 2018). These relationships may be mediated by multiple intrinsic (e.g., glucocorticoid [GC] levels: Stothart et al. 2016) and extrinsic (e.g., diet: Suzuki 2017) factors. Greater gut microbial diversity is generally expected to be advantageous as low diversity can be linked to dysfunction or disease and more diverse communities are more resilient to perturbance (Bäckhed et al. 2012; Lu et al. 2014), though the relationship between microbiota and host is complex and can vary greatly depending on species and location of microbiota (Morgan et al. 2013; Suzuki 2017). Given the breadth and complexity of relationships between gut microbial diversity and other aspects of organismal biology, analyses of this diversity can generate important insights into the fitness consequences of variation in multiple phenotypic traits.

Measures of fecal microbiota provide an important proxy for gut microbial diversity in many studies of free-living animals (Reese and Dunn 2018). Although analyses of fecal samples do not capture information regarding the distinct microbial communities that occur in different portions of the digestive tract, these samples are relatively easy to obtain and are significantly less invasive than collecting portions of the gut; for these reasons, fecal samples are commonly used in field studies of gut microbial diversity (Hale et al. 2016). Methods used to obtain feces range from direct collection from known individuals (Hale et al. 2016; Turjeman et al. 2022) to opportunistic collection of material deposited in the environment (Guan et al. 2016; Table 1). Similarly, methods of sample storage vary, including differential use of storage buffers as well differences in time allowed between sample collection and freezing (Table 1). Because these sources of variation may affect subsequent estimates of fecal microbial composition and diversity (Choo et al. 2015; Blekhman et al. 2016; Hale et al. 2016), it is important to validate methods used to collect and to store samples intended for analysis, particularly for field-based studies that may be conducted under variable and unpredictable conditions.

California ground squirrels (*Otospermophilus beecheyi*) are abundant in many open habitats in central coastal California. These conspicuous, diurnal rodents are facultatively social, meaning that members of a local population display considerable variation in the nature and frequency of interactions with conspecifics (Smith et al. 2018). This variability has been linked to differences in foraging (Ortiz et al. 2019), which may influence acquisition of gut microbiota. At the same time, variable social relationships likely contribute to differences in physical contact among conspecifics (e.g., occurrence of burrow sharing; Smith et al. 2018), providing another avenue by which social behavior may shape gut microbial communities. These observations, combined with the relative ease of live-trapping and then collecting fecal samples from known individuals, make California ground squirrels an ideal system in which to explore behavioral and other correlates of fecal (and by extension gut) microbial diversity in natural populations of mammals.

Here, we examine measures of fecal bacterial diversity in samples obtained from members of a free-living population of California ground squirrels that is the subject of long-term studies of social behavior (Smith et al. 2018; Hammond et al. 2019; Ortiz et al. 2019; Ortiz-Jimenez et al. 2022). Specifically, we compare bacterial diversity and composition in replicate samples obtained from the same individuals but collected and stored following three different protocols. Our aim was to determine if collection protocol affects the measures obtained for the unique community of each individual. In addition to

Table 1.—Summary of microbiome collection and storage methods in studies of free-living animals.

Study	Taxon	Freezing	Storage buffer	Summary
Blekhman et al. (2016)	Rhesus macaques (*Macaca mulatta*)	Immediate or lyophilization	Ethanol or RNAlater	Signatures of individual identity in fecal microbiota were consistent across storage methods
Choo et al. (2015)	Human	4°C or ambient temperature	RNAlater, OMNIgene. GUT, Tris-ethylenediaminetetraacetic acid (EDTA)	Fecal microbiota varied with storage buffer, but refrigerated samples were similar to frozen samples
Guan et al. (2016)	Sable (*Martes zibellina*)	Immediately in LN2, stored at −80°C	Ethanol	Animals in captivity had different gut microbial composition than wild animals, perhaps due to diet
Hale et al. (2016)	Spider monkey, black-and-white snub-nosed monkey, gray snub-nosed monkey (*Ateles geoffroyi*, *Rhinopithecus bieti*, and *R. brelichi*)	Liquid nitrogen or Flinders Technology Associates (FTA) card	None	Time and method of preservation affected fecal microbiota across species
Kohl et al. (2015)	Desert woodrat (*Neotoma lepida*)	Immediately, stored at −80°C	None	Sherman trap-collected fecal samples were similar to samples collected from sterile surfaces
Turjeman et al. (2022)	Common crane (*Grus grus*)	Immediately at −20°C, transferred to −80°C	95% ethanol	Fecal samples collected noninvasively from soil differed from samples collected from captured animals, possibly due to handling stress

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validating the use of different collection and storage methods, our analyses generate preliminary insights into factors affecting the diversity of fecal bacteria in these animals. More generally, these analyses are intended to inform future studies that use fecal samples from free-living individuals as a proxy for gut microbial diversity in natural populations of mammals.

Materials and Methods

Study site and population.—We studied a free-living population of California ground squirrels located in Briones Regional Park, Contra Costa County, California. This population has been monitored annually since 2013, with data collection typically occurring from May to July, when the animals are most active above ground (Hammond et al. 2019). The habitat at the study site consists of open grassland containing scattered stands of oak and walnut trees, as well as riparian areas along seasonal creek beds (Ortiz et al. 2019). Ground squirrels occur primarily in open portions of the habitat, where members of the study population are routinely observed engaging in social interactions while above ground (Smith et al. 2018). Additionally, remote monitoring of burrow entrances suggests that individuals may share space while underground (Smith et al. 2018). Social network analyses indicate marked variation among individuals with regard to the number and strength of social connections (Smith et al. 2018), providing a critical foundation for exploring interactions between social behavior and other phenotypic traits, including fecal microbial diversity.

Animal capture and marking.—As part of long-term studies of behavior and demography, most members of the study population are captured several times per year using Tomahawk live traps baited with sunflower seeds and peanut butter (Smith et al. 2018). Once caught, individuals are transferred to a cone-shaped cloth handling bag for marking, weighing, and determination of reproductive status (Koprowski 2002). Upon first capture, each animal is permanently marked with a uniquely numbered Monel ear tag (National Band and Tag Co., Newport, Kentucky) and a passive integrated transponder tag (Biomark, Inc., Boise, Idaho). To allow visual identification of individuals, the fur of each animal is dyed black in a distinctive pattern (Nyanzol cattle dye; Greenville Colorants, Jersey City, New Jersey). Body mass is used to characterize animals as juveniles (weaned young of the year) or adults (at least 1-year-old; Hanson and Coss 1997). Because most individuals are first captured as juveniles, exact ages are known for many members of the study population. For animals of both sexes, visual inspection of the genitalia is used to determine reproductive status. Among females, palpation of the abdomen and inspection of the mammae are used to differentiate pregnant from lactating or nonreproductive animals.

Study subjects.—For this study, fecal pellets were collected from 12 squirrels for use in analyses of fecal bacterial composition and diversity. To reduce variation among the animals examined, all individuals sampled were adult females that were nonreproductive at the time of sample collection (mean ± *SD* age = 2.2 ± 1.1 years, range = 1–4 years, body mass = 620 ± 73 g; Supplementary Data SD1). Prior social network analysis indicates that all individuals are socially connected to the rest of the population to some degree, either directly or indirectly (Smith et al. 2018). Fecal samples from eight of these females were obtained from 3 to 5 June 2019 (Week 1); samples from the remaining four females were obtained on 17 and 18 June 2019 (Week 2). Samples were collected opportunistically; individuals included in this study were animals for which we were able to obtain fecal pellets using each of the collection and storage methods outlined below. To reduce temporal variation associated with repeated sampling of the same individuals, only adult females from whom all pellets were collected within a span of 3 days were analyzed.

Fecal sample collection.—California ground squirrels often defecate while in traps and during postcapture handling, providing a convenient means of collecting fecal samples that has been employed in multiple studies of natural populations of animals (Kohl et al. 2015; Murray et al. 2020). To evaluate the potential effects of different sources of fecal samples (i.e., direct vs. soil collection) on measures of bacterial composition and diversity, we used a repeated measures design in which fecal samples from each individual were collected using both of the following procedures:

(1) Direct collection.—Fecal samples were collected by placing a plastic tub under each ground squirrel such that fecal pellets produced during handling fell directly into the tub, preventing contact with soil or other surfaces; pellets were collected with gloved hands and stored as described below. Tubs were cleaned with 70% ethanol between individuals as part of our standard procedure for this long-term study (Smith et al. 2021). Spraying tubs with bleach was not possible for these samples due to use of ethanol to collect flea samples from the same animals; mixing of bleach and ethanol produces toxic chloroform. Direct collection of samples was completed the first time that an individual was captured during one of our June sampling periods.

(2) Soil collection.—Fecal samples were collected with gloved hands from the soil beneath the trap in which an individual was captured. Traps were checked approximately every 30 minutes, with captured animals typically producing multiple pellets during this interval. Traps containing squirrels were lifted and pellets beneath the trap were retrieved and stored as described below. Because pellets collected from soil on the first day that an animal was captured were reserved for endocrine analyses (see "Baseline GC concentrations" below), samples for microbiome studies were collected from beneath traps the second time that an individual was caught during a given June sampling period, typically 1–2 days after direct collection of fecal pellets.

Sample storage methods.—All pellets collected directly from an animal were placed in a cryotube containing 70% ethanol and frozen immediately in liquid nitrogen. Similarly, half of the pellets collected from the soil beneath traps were stored

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following this procedure; the remaining half of the pellets collected beneath traps were placed in a Ziploc plastic bag and then stored on wet ice packs in a cooler for 1–6 h until being transferred to a −80°C freezer at the end of the day. Because samples obtained directly from an animal rarely contained enough fecal material to be split between the two storage methods, we were unable to employ a fully balanced design that included all possible combinations of collection and storage methods; given this constraint, we emphasized rapid freezing of samples in liquid nitrogen because this storage method is thought to better preserve the microbial contents of samples (Fouhy et al. 2015). All samples placed in liquid nitrogen were transferred to a −80°C freezer at the end of the field season.

Ethics and permissions.—All procedures involving live animals were approved by the Animal Care and Use Committee at Mills College and were consistent with guidelines of the American Society of Mammologists for the use of wild mammals in research (Sikes et al. 2016). Permits to conduct this work were provided by the California Department of Fish and Wildlife and the East Bay Regional Parks District.

Baseline GC concentrations.—GC hormones are involved in regulation of multiple physiological processes associated with homeostasis and response to allostasis (Sapolsky et al. 2000). Because GC physiology can influence gut and fecal microbiota (Levin et al. 2016; Petrullo et al. 2022), we quantified baseline fecal GC metabolite (fGCm) concentrations using a fully validated corticosterone enzyme immunoassay (EIA) protocol developed for California ground squirrels (Hammond et al. 2019). As noted above, fecal samples for endocrine assays were collected from the soil beneath traps on the first day that animals were captured during this study; these samples were stored in a cooler until they could be transferred to a −80°C freezer later in the day on which they were collected. Corticosterone EIAs were conducted on the Mills College campus within approximately 6 months of sample collection.

Sequencing of microbial DNA.—Microbial DNA was isolated from 0.25 g of fecal material using the DNEasy PowerLyzer PowerSoil Kit for bead-based isolation (Qiagen LLC, Germantown, Maryland). To identify the bacterial taxa present in these samples and to quantify the abundances of the different taxa detected, the V4 region of the bacterial *16S rRNA* gene was PCR-amplified by Microbiome Insights (Vancouver, British Columbia, Canada) with dual-barcoded primers (515F 5′-GTGCCAGCMGCCGCGGTAA-3′, and 806R 5′-GGACTACHVGGGTWTCTAAT-3; Kozich et al. 2013). Amplicons were then sequenced on an Illumina MiSeq platform by Microbiome Insights.

Analyses of sequence data.—To assemble raw Illumina reads, remove low-quality reads, and remove nonbacterial and chimeric sequences (i.e., two or more reads that were incorrectly joined), we used the 'dada2' package (v1.16), as implemented in R (v4.1.0; Callahan et al. 2016). This package was also used to assemble an amplicon sequence variant (ASV) table (Callahan et al. 2016), which tallied the number of times that each distinct bacterial sequence occurred in our data set. We assigned ASVs to bacterial genera using the Silva reference database (v138.1), after which we used the R package “phangorn” (v2.10.0) to infer a maximum likelihood phylogeny for the bacterial taxa identified in fecal samples (Schliep 2011).

Read depth (the number of bacterial sequence reads per sample) was used to determine if different collection and storage methods affected the overall number of bacterial sequences recovered. To examine alpha and beta diversity, we rarefied our data to the minimum read depth obtained from our samples (8,536 reads). Following rarefaction, 62 (ca. 2% of) ASVs were lost. We used the 'decontam' package (v3.16; prevalence method, threshold: 0.05) in R to identify possible contaminant sequences based on nine negative controls (samples containing no fecal material) that were amplified and sequenced together with our target samples. Six ASVs were more abundant in the negative controls than in our samples; these ASVs were removed from the data set prior to further analysis (Davis et al. 2018). We also removed sequences ($n = 8$ ASVs) that could not be assigned to a phylum as well as sequences ($n = 6$ ASVs) that were classified as mitochondrial or chloroplast in origin or were not in kingdom Bacteria.

To determine if collection-storage method had an effect on read depth, we used the Friedman test for nonparametric repeated measures. To quantify within-individual (alpha) bacterial diversity in our study animals, we used Shannon’s diversity index, which accounts for both the taxonomic richness and evenness of a community (Hill 1973), after which we constructed a generalized linear mixed model (GLMM) using the “lme4” (v1.1-30) and “optimx” (v2022-4.30) packages in R (Nash and Varadhan 2011; Bates et al. 2015). The model included collection-storage treatment, body mass, fGCm concentrations at time of capture, and sampling week as fixed effects to explore potential effects of treatment as well as several key phenotypic traits. Corticosterone metabolite concentrations were natural log transformed and body mass was rescaled prior to use in the model to allow for comparison of effect sizes. We included individual ID as a random effect in the model to account for repeated measures from the same animals and to assess whether the measures of bacterial diversity employed were consistent within individuals across sampling and storage methods. Alpha diversity was not normally distributed and thus we used a Gamma distribution to construct the model. We performed an additional post hoc analysis (e.g., Mann–Whitney *U*-test) to examine the effects of predictor variables identified as significant by this model.

To examine potential among-individual (beta) differences in bacterial diversity, we conducted principal coordinate analyses using unweighted and weighted UniFrac distances, both of which provide a phylogenetically informed measure of the similarity among different bacterial communities. Unweighted UniFrac distances (i.e., community membership; Lozupone et al. 2007) consider the presence of bacterial taxa within a community but do not take into account the relative abundances of those taxa. In contrast, weighted UniFrac distances (i.e., community structure; Lozupone et al. 2007) consider both the presence and relative abundance of bacterial taxa. To compare measures of beta diversity among collection-storage treatments,

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individuals, and sampling periods, we employed permutational multivariate analysis of variance (PERMANOVA) tests as implemented in the "vegan" package in R (v2.6-4; Oskanen et al. 2022). To allow estimation of the effects of individual identity, two models were constructed for each type of UniFrac distance. The first model was estimated with individual as the only variable; the second model was estimated with treatment and sampling week as variables while controlling for individual identity to account for replication. We then used DESeq2 to identify taxa that differed in abundance among treatment groups (v1.32.0, Love et al. 2014). All figures were generated using the 'phyloseq' (v1.36.0), 'ggplot2' (v3.3.5), and 'cowplot' (v1.1.1) packages in R (McMurdie and Holmes 2013; Wickham 2016; Wilke 2020).

Results

A total of 2,685,793 reads were obtained for the 12 female ground squirrels sampled during this study. Following removal of low-quality and chimeric sequences as well as nonbacterial taxa and mitochondrial and chloroplast sequences, this number was reduced to 1,938,624 reads or 53,851 ± 34,035 (mean ± *SD*) sequences per sample. A total of 13 bacterial phyla and 159 bacterial genera were identified from these sequences. Overall, *Firmicutes* was the most abundant phylum, representing 61.7% of total reads, followed by *Bacteroidota* (20.5%) and *Actinobacteria* (6.7%;Supplementary Data SD2). Eight ASVs that together accounted for 0.011% of all high-quality sequences could not be assigned to a bacterial phylum and were treated as unidentified; these sequences were not included in downstream analyses of bacterial diversity.

Collection-storage treatment.—We found no effect of collection-storage treatment on read depth (number of sequences recovered per individual; Friedman test, $\chi^2 = 3.5$, $P = 0.174$; Fig. 1A). Similarly, we found no differences in alpha diversity among the three collection-storage treatments employed (GLMM, $z < 1.318$, $P > 0.1874$; Table 2, Fig. 1B), indicating that within-individual estimates of bacterial diversity were not affected by our sampling protocols. In contrast, we found a small but significant effect of collection-storage treatment on beta diversity (i.e., bacterial community composition) as measured by unweighted UniFrac distance (PERMANOVA, $R^2 = 0.061$, $P < 0.001$; Table 3, Fig. 2C). However, no effect of treatment on beta diversity was detected when weighted UniFrac distances (i.e., bacterial community structure) were examined (PERMANOVA, weighted UniFrac distance, $R^2 = 0.058$, $P = 0.088$; Table 3,; Fig. 2D), indicating that effects of collection-storage methods were not consistent across the estimates of among-individual diversity examined.

With regard to the specific bacterial taxa identified in our samples, three genera in family Oscillospiraceae (*Colidextribacter*, *Oscillibacter*, and *Oscillospira*) differed significantly in abundance between the two soil-collected treatments (frozen vs. stored on ice), as did four taxa that could only be identified to order (Table 4). All but the taxon in the order Clostridia UCG-014 were enriched in the treatment stored in liquid nitrogen. The same three genera and one additional genus (*Monoglobus*) differed significantly in abundance between the tub-collected treatment stored in liquid nitrogen and the soil-collected treatment stored on ice, as did three of the same taxa that differed in the previous comparison (Table 4). The genus *Monoglobus* and taxon in the order Clostridia UCG-014 were enriched in

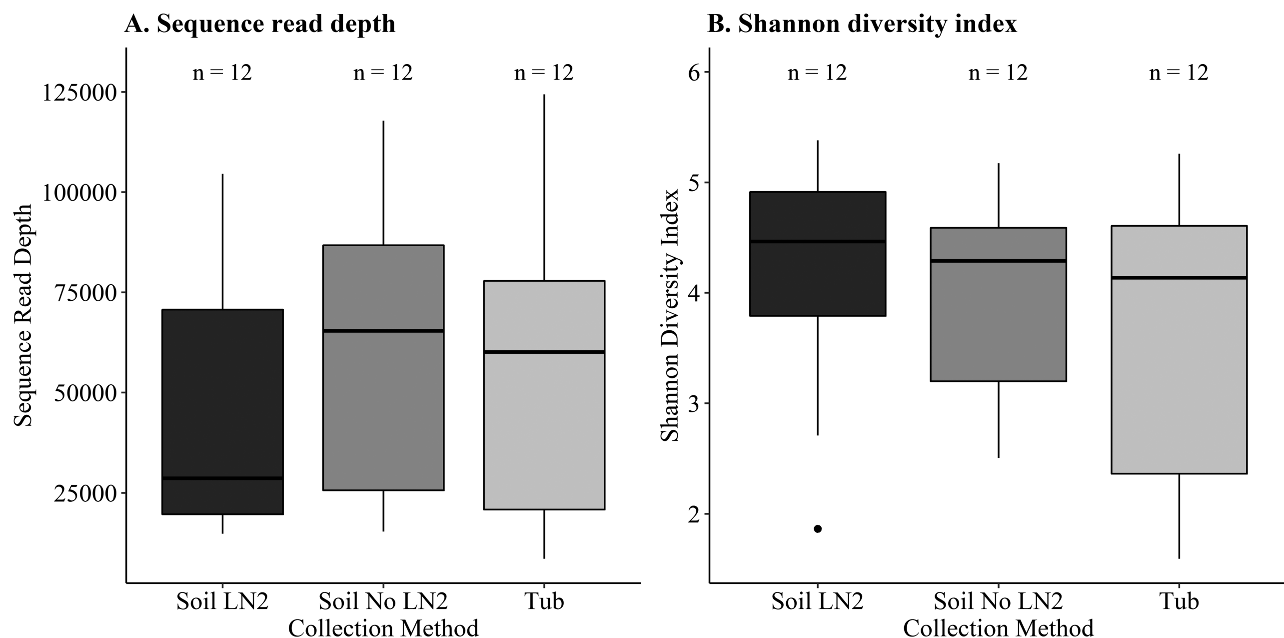

Fig. 1.—Box plots depicting the median and quartile of (A) read depth and (B) alpha diversity (Shannon index) of bacteria present in fecal samples from California ground squirrels as a function of sample collection-storage methods. No significant differences were found among treatments for either measure of bacterial diversity (read depth: Friedman test, $P = 0.174$; alpha diversity, generalized linear mixed model [GLMM], $P > 0.1874$).

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Table 2.—Results of generalized linear mixed model analyses of factors predicting alpha (within-individual) diversity in bacterial composition of fecal samples from California ground squirrels. Alpha diversity was quantified using Shannon's index. "Soil LN2" is the reference level for the treatment variable. Parameters identified as significant predictors of alpha diversity are shown in bold. *SD*: standard deviation; *SE*: standard error.

	Variance	*SD*	χ^2-value	Estimate	*SE*	*z*-value	*P*-value
Random effects							
Individual	0.000925	0.0304	1.98				0.1600
Residual	0.467146	0.2161					
Fixed effects							
(Intercept)				0.0354	0.6707	0.053	0.9579
Treatment (Soil No LN2)				0.0118	0.0206	0.571	0.5681
Treatment (Tub)				0.0282	0.0214	1.318	0.1874
Sampling week				**0.0859**	**0.0373**	**2.305**	**0.0212**
ln(Fecal Cort)				0.0143	0.0718	0.199	0.8422
Body mass				0.1201	0.2592	0.463	0.6432

Table 3.—Results of permutational multivariate analysis of variance (PERMANOVA) models of factors predicting beta (among-individual) diversity in bacterial composition of fecal samples collected from California ground squirrels. Beta diversity was quantified using UniFrac distances, with separate analyses run for unweighted and weighted distances. Models were run using 999 permutations. *R*-squared values denote the proportion of variation explained by each variable. To allow estimation of the effects of individual identity, two models were constructed for each type of UniFrac distance. The first model was run with individual as the only variable; the second model was run with treatment and sampling week as variables while controlling for individual identity to account for replication. *P*-values for significant predictors of beta diversity are shown in bold.

Predictor	R^2	*P*-value
Unweighted UniFrac		
Individual	**0.603**	**<0.001**
Treatment	**0.061**	**<0.001**
Sampling week	**0.072**	**<0.001**
Weighted UniFrac		
Individual	**0.620**	**<0.001**
Treatment	0.058	0.088
Sampling week	0.142	0.088

the treatment stored on ice, while the rest were enriched in the tub-collected samples stored in liquid nitrogen. No taxa differed in abundance between the two treatments that were stored in liquid nitrogen.

Phenotypic variation and other effects.—We found no effects of body mass or fGCm concentrations (Supplementary Data SD1) on estimates of alpha diversity (GLMM, $z < 0.199$, $P > 0.6232$; Table 2). In contrast, there was a significant effect of sampling week on alpha diversity (GLMM, $z = 2.305$, $P = 0.0212$; Table 2), suggesting possible temporal variation in individual-level bacterial diversity. Post hoc analysis indicated that alpha diversity was significantly lower during the second week of sampling (Mann–Whitney *U*-test, $W = 240$, $P = 0.00081$; Fig. 3).

Measures of beta diversity clustered significantly by individual ID for both unweighted and weighted UniFrac distances (PERMANOVAs, unweighted UniFrac, $R^2 = 0.602$, $P < 0.001$; Table 3; Fig. 2A; weighted UniFrac, $R^2 = 0.620$, $P < 0.001$; Table 3, Fig. 2B). Individual ID accounted for over 60% of variation in both measures of beta diversity, suggesting that individual hosts were an important source of variation in bacterial community composition. Beta diversity also clustered by sampling week for unweighted UniFrac distance (PERMANOVA, $R^2 = 0.072$, $P < 0.001$; Table 3, Fig. 2E), revealing possible temporal variation in bacterial diversity within our study population. In contrast, samples did not cluster by sampling week when weighted UniFrac distances were examined (PERMANOVA, $R^2 = 0.142$, $P = 0.088$; Table 3, Fig. 2F).

Discussion

We found no effects of sample collection or storage methods on most of the measures of fecal bacterial diversity and community composition considered in this study. The only significant effect of collection-storage procedure detected was for beta diversity, as measured using unweighted UniFrac distances. Treatment accounted for only 6.1% of among-sample variation in this metric compared to the 60–62% of among-sample variation explained by animal identity, suggesting that although collection-storage procedures had a statistically significant effect on beta diversity, other parameters were more important in explaining patterns of bacterial community composition in our study population. We found no relationship between read depth and treatment, confirming that there was no effect of collection-storage procedures on number of sequences recovered and thus no treatment-associated bias in our core data set that may have affected measures of diversity. With regard to alpha diversity, only sampling week emerged as a significant predictor; sampling week was also significantly associated with beta diversity as measured by unweighted UniFrac distances. Thus, in addition to validating the use of different sample collection and storage methods for analyses of fecal bacterial diversity in California ground squirrels, our analyses provide important preliminary information regarding factors affecting the diversity of fecal bacteria in free-living members of this species.

One potentially important caveat to our findings was the use of 70% ethanol to clean the tubs used to collect fecal samples from our study animals following our standard field protocol (Smith et al. 2021). While ethanol solutions are effective at

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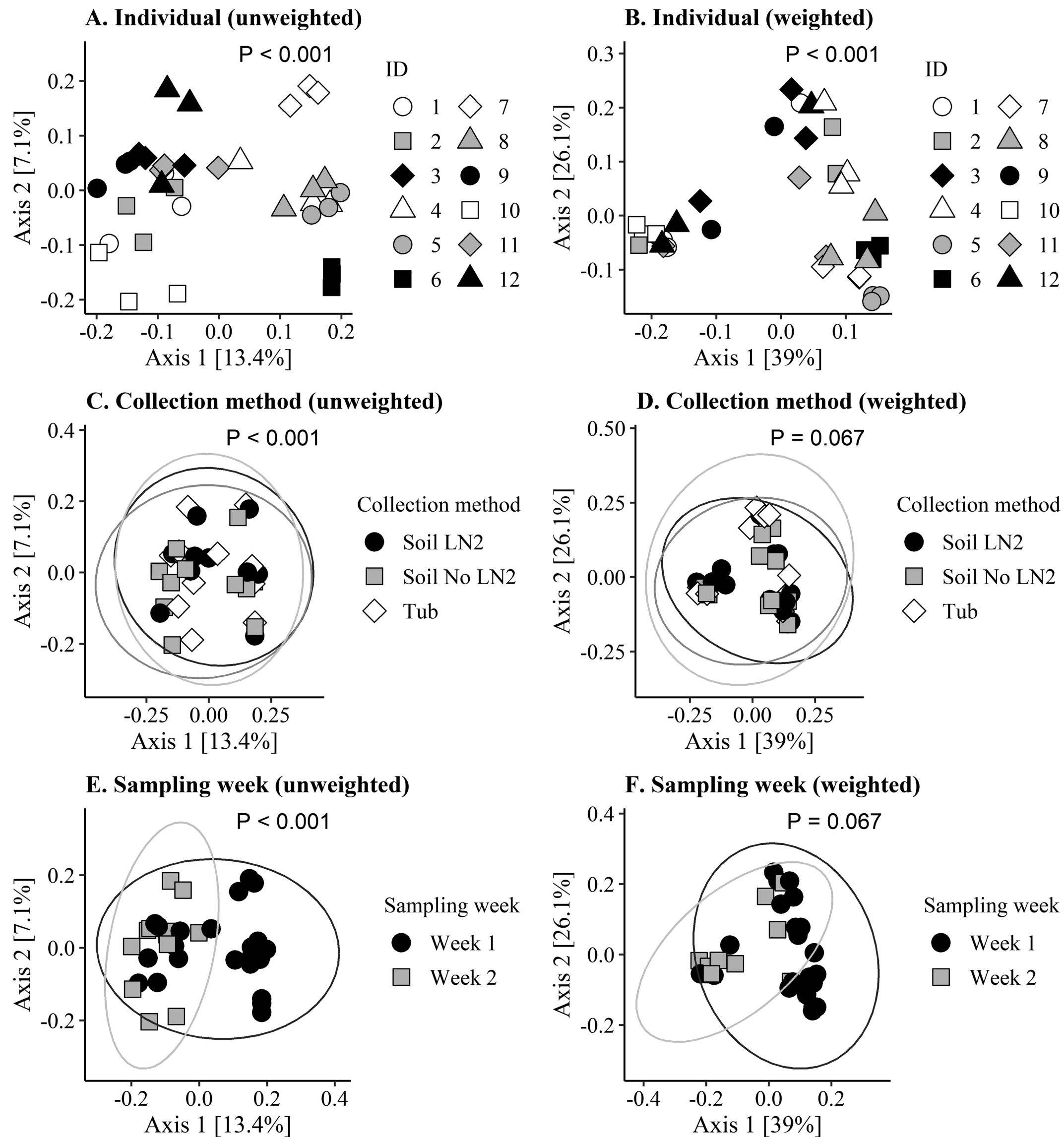

Fig. 2.—Results of principal coordinate analyses of beta (among-individual) diversity in bacteria present in fecal samples from California ground squirrels. In all panels, each symbol denotes estimated beta diversity for one individual. Diversity was estimated using either unweighted or weighted UniFrac distances. Color and symbol denote individuals in panels A (unweighted) and B (weighted), collection method in panels C (unweighted) and D (weighted), and sampling week in panels E (unweighted) and F (weighted). The percentage of total variation among samples explained by each axis is indicated. Ellipses denote 95% confidence intervals for samples characterized by the same predictor attribute (e.g., collection-storage treatment). *P*-values for each permutational multivariate analysis of variance (PERMANOVA) are shown in figure.

killing microbes, bleach solutions are more effective at denaturing DNA and preventing cross-contamination of microbial samples used for sequencing (Kemp and Smith 2005; Binetruy et al. 2019). To avoid toxicity, we were unable to use both solutions together. Accordingly, we cannot exclude the possibility that successive samples collected in tubs were contaminated with microbial DNA from samples collected previously. However, our finding that individual identity had a strong effect on microbial community regardless of collection method suggests that any contamination of samples resulting from our cleaning procedures did not lead to homogenization of the bacterial communities in our samples. Nevertheless, to reduce the potential for contamination, we suggest the use of dilute bleach solutions to clean surfaces or containers used during fecal sample collection.

Implications for storage and collection procedures.—The limited effects of our treatments on measures of microbial diversity suggest that fecal samples obtained from the soil beneath traps were not subject to significant environmental contamination. More specifically, bacterial taxa did not differ in abundance between treatments in which fecal samples stored in liquid nitrogen were collected from either the tub versus from the soil beneath traps. Collection of samples from beneath traps or from other portions of the environment is common in studies of wild mammals (Amato et al. 2017; Raulo et al. 2018) and our findings provide an important demonstration that materials

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Table 4.—Results of DESeq2 analysis for differentially abundant taxa. Taxa listed below differed significantly between the reference group ('Soil No LN2') and the comparison groups. No taxa differed significantly between the 'Soil LN2' treatment and the 'Tub' treatment. Negative log fold change values indicate taxa were decreased in the 'Soil No LN2' treatment. 'NA' indicates the taxon was not able to be classified to that level.

Soil No LN2 compared to Soil LN2				
Order	Family	Genus	Log fold change	*P*-value
Oscillospirales	Oscillospiraceae	*Colidextribacter*	−1.289	0.0002
Oscillospirales	Oscillospiraceae	*Oscillibacter*	−2.234	0.0002
Oscillospirales	Oscillospiraceae	*Oscillospira*	−2.340	<0.0001
Rhodospirillales	NA	NA	−3.041	0.0002
Gastranaerophilales	NA	NA	−3.149	<0.0001
Clostridia UCG-014	NA	NA	0.972	0.0010
Clostridia vadinBB60 group	NA	NA	−2.661	0.0002

Soil No LN2 compared to Tub				
Order	Family	Genus	Log fold change	*P*-value
Oscillospirales	Oscillospiraceae	*Colidextribacter*	−1.877	<0.0001
Monoglobales	Monoglobaceae	*Monoglobus*	1.430	0.0005
Oscillospirales	Oscillospiraceae	*Oscillibacter*	−2.789	<0.0001
Oscillospirales	Oscillospiraceae	*Oscillospira*	−2.643	<0.0001
Gastranaerophilales	NA	NA	−2.914	<0.0001
Clostridia UCG-014	NA	NA	0.948	0.0016
Clostridia vadinBB60 group	NA	NA	−2.133	0.0056

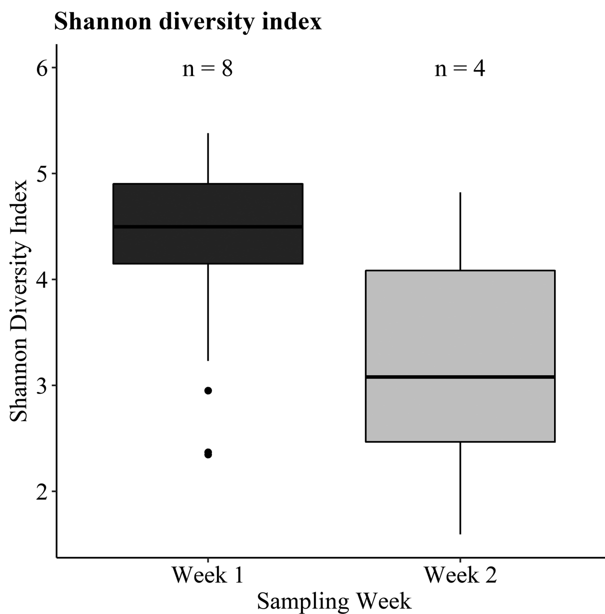

Fig. 3.—Box plot depicting the median and quartile of alpha diversity (Shannon index) of bacteria present in fecal samples from California ground squirrels as a function of sampling week. Alpha diversity decreased significantly from Week 1 to Week 2 (Mann–Whitney *U*-test, $P = 0.0008$).

obtained in this way can yield reliable estimates of bacterial abundance and diversity. However, because the potential for contamination likely varies as a function of the nature and duration of exposure to environmental conditions (Delsuc et al. 2014; Kohl et al. 2015; Blekhman et al. 2016; Hale et al. 2016; Turjeman et al. 2022), we encourage researchers to conduct similar comparisons that are specific to their study systems.

In comparison, although collecting samples directly from a sterile surface should reduce the probability of environmental contamination, direct collection of fecal material from wild mammals can be challenging. For example, not all members of our study population produced fecal pellets during handling, with the result that direct collection of samples was not possible for all animals. In other species, direct collection of fecal or other samples may not be feasible if the animals are difficult to capture or to handle. Thus, confirmation that samples collected from the environment can provide robust estimates of bacterial diversity may lead to larger sample sizes and more complete sampling of study populations than would be possible using only direct collection of fecal samples.

With regard to storage procedures, the limited effects of our treatments suggest that measures of bacterial diversity were not affected by a delay of several hours prior to freezing, again providing apparent validation of commonly used field procedures (Stothart et al. 2016; Grieneisen et al. 2017; Baniel et al. 2021). We did not evaluate all possible methods of sample collection and storage; the procedures examined reflect standard practices in studies of wild mammals (Table 1). In particular, immediate freezing of samples in liquid nitrogen may be challenging under field conditions, resulting in interim storage methods that vary with regard to use of preservative buffers, temperature, and duration. Of the bacterial taxa that differed in abundance between treatment groups (Table 4), the majority were enriched in treatments stored in liquid nitrogen compared to the treatment stored on ice. This suggests some taxa are more sensitive to temperature, which may be important for researchers intending to characterize fully the bacterial community composition of their study subjects. However, the taxa affected by storage procedure represent a relatively small proportion of all bacterial taxa identified and, again, differences associated with individual identity outweighed any effects due to storage treatment. We suggest that use of consistent storage methods is the most critical consideration for field studies, as this consistency will allow for comparisons among groups or individuals, even if not

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all bacterial taxa are preserved in full abundance. Confirmation that immediate freezing in liquid nitrogen is not required may increase the diversity of field situations in which collection of samples for analyses of fecal microbial diversity is possible.

Phenotypic correlates of fecal bacterial diversity.—We found no evidence that the phenotypic parameters examined affected alpha diversity of fecal bacteria in our study animals. This included consideration of fGCm concentrations, which are frequently used as a proxy for baseline measures of physiological stress (Hammond et al. 2019) and which have been shown to be correlated with key phenotypic differences (e.g., venom resistance; Holding et al. 2020) in this species as well as differences in gut microbial diversity and composition in other species (Levin et al. 2016; Stothart et al. 2016; Petrullo et al. 2022). The one phenotypic variable examined that was associated with significant differences in fecal bacterial composition (beta diversity) was animal identity. Because this parameter encompasses multiple aspects of phenotypic and genotypic variation, the specific variables contributing to this outcome remain unknown. Nevertheless, this finding suggests that differences among individuals contribute to fecal bacterial community composition in California ground squirrels and indicates that individual identity should be included in future analyses of bacterial diversity in these animals. Future studies of California ground squirrels will explore the effects of variation in other phenotypic traits (e.g., social network connections, reproductive status, familial relationships, shared burrow use) that have been shown to be correlated with microbial diversity in other mammalian species (Amato et al. 2017; Mallott et al. 2020).

Temporal variation in fecal bacterial diversity.—One of the few variables that was associated with differences in the abundance and diversity of fecal bacteria in our study animals was the timing of sample collection. Fecal samples were obtained from members of our study population during two distinct periods in June 2019. This difference in the timing of sample collection was a significant predictor of fecal bacterial diversity, with samples collected later in June characterized by decreased alpha diversity. Additionally, estimates of beta diversity (unweighted UniFrac distances) clustered significantly by sampling period, providing further evidence of a temporal effect of sample collection. This pattern was evident despite use of the same sample collection and storage methods throughout the study and despite a difference of no more than 2 weeks between sample collection periods. Possible explanations for this outcome include temporal changes in foods consumed by individuals (Orkin et al. 2018; Baniel et al. 2021), oscillations caused by circadian rhythms (Risely et al. 2021), or changes in social dynamics (Tung et al. 2015; Grieneisen et al. 2017; Raulo et al. 2018; Yarlagadda et al. 2021). Individual identity may also have played a role in this variation, given the strong effects of identity on beta diversity and given that different individuals were sampled during each time period. Finally, the small sample size for each temporal period (Week 1 $n = 8$; Week 2 $n = 4$) suggests that statistical artifacts, rather than biologically meaningful factors, may have contributed to the temporal difference in bacterial diversity reported here. While additional analyses involving larger sample sizes collected over a variety of time periods should clarify the effects of sampling period reported here, our finding that fecal bacterial diversity varied over even a relatively short interval suggests that future studies should consider both sampling date and temporally variable environmental parameters (e.g., diet) as part of analyses of the fecal microbiota of natural populations of mammals.

Recommendations for future studies.—Our comparisons of bacterial diversity in fecal samples collected and stored under different conditions have several implications for studies of free-living mammals. First, the lack of large treatment effects on read depth and both alpha and beta diversity suggests that these metrics are robust to the variation in field methods encompassed by our study. Although we did not explore the full range of sample collection and storage methods currently in use as part of field research, the methods employed here are fairly common in studies of free-living mammals and thus the lack of significant effects on measures of fecal bacterial diversity should enhance confidence in data obtained using these procedures. Second, our finding that fecal bacterial diversity varied over a relatively short temporal period (i.e., 2 weeks) suggests that date of collection should be incorporated into analyses of such diversity, particularly given the extended periods over which many field studies are conducted. Finally, although our experimental design included sampling and collection protocols that are common in field research, our results are limited to California ground squirrels and thus we cannot exclude the possibility that comparable variation in field methods would be associated with differences in fecal bacterial diversity in other species. Ultimately, the exact collection and storage methods employed will depend upon the specifics of the field site, study taxa, and research questions to be addressed. We suggest that within studies, consistency in the collection and storage methods employed is paramount. For situations in which variability in sample collection and storage methods cannot be avoided, taxon-specific validation of that variability, such as the analyses conducted here for California ground squirrels, is strongly recommended.

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Data Availability

All sequencing files have been archived with the National Center for Biotechnology Information Sequence Read Archive (NCBI SRA) under accession PRJNA865359. All code required to reproduce the analyses can be found at https://github.com/e-s-person/ground-squirrel-microbiome/blob/main/Person.et.al.analysis2022.R.

Supplementary Data

Supplementary data are available at *Journal of Mammalogy* online.

Supplementary Data SD1.—Table S1. Summary of attributes for the 12 California ground squirrels included in this study. The identity of each animal is given, as is the sampling period during which fecal pellets were collected. Individual values for body mass, fecal glucocorticoid metabolite (fGCm) concentration, and alpha diversity are shown, as is the total number of reads per animal that could be identified to bacterial phylum.

Supplementary Data SD2.—Table S2. Total abundance (number of sequences identified) for each bacterial phylum identified in fecal samples from 12 adult female California ground squirrels following removal of unidentified phyla, sequences of mitochondrial or chloroplast origin, and sequences not classified to kingdom Bacteria.

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