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Original Research

Onthophagus taurus Increases Soil Microbes Associated with Nutrient Cycling in California Pastureland Soils[☆]

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ABSTRACT

When grazed intensively, grasslands can result in biodiversity loss and increased greenhouse gas emissions. However, when grasslands are maintained using agroecological practices, they can create diverse habitats and act as carbon sinks. Carbon sequestration in soil is mediated by the soil microbial community, which is largely affected by influxes of nutrients and the soil arthropod community. One such arthropod, the tunneling dung beetle *Onthophagus taurus* (Schreber 1759) incorporates animal dung into the soil, influencing both the soil microbial community and nutrient cycling. While dung beetles do affect the soil microbial community, there is a dearth of studies that examine the effect of tunneling dung beetles on soil microbial communities that may be associated with nutrient cycling. This study looks at the effect of the tunneling dung beetle *O. taurus* on the fungal, bacterial, and archaeal community of grassland soil in an in situ experiment in California's Central Coast region, using eDNA metabarcoding of the 16S locus for bacteria and archaea and the ITS1 locus for fungi. We find that *O. taurus* has a clear effect on the soil microbial community and its presence increases the abundance of soil microbes affiliated with degrading plant materials and carbohydrate metabolism.

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Introduction

Soil arthropods mediate the soil microbial community by influencing nutrient additions through their feeding and nesting activities (Bardgett and van der Putten 2014). In turn, changes in the soil microbiome can have a profound influence on nutrient cycling and carbon sources and sinks in soils (Nielsen et al. 2011; Schmidt et al. 2011). As such, arthropod-mediated microbial communities offer a means to regulate carbon and other nutrients and offer bio-control, which has economic importance for ranchers and climate mitigation efforts. An arthropod group with especially significant potential to influence these dynamics are the dung beetles.

Dung beetles are bioindicators of anthropogenic perturbations in different ecosystems (Barragán et al. 2011; Barragán et al. 2021; Noriega et al. 2021) and also offer a variety of ecosystem services via their nesting behaviors (Nichols et al. 2008). Dung beetles consume the liquid portion of dung and also brood their larvae

in dung (Nichols et al. 2008). There are three different functional types of dung beetles: dwellers, which live in the dung and deposit their eggs in the dung; tunnelers, which tunnel underneath dung piles, burying brood balls in the tunnels; and rollers, which roll dung balls away from dung piles, deposit their eggs in the dung, and bury them in the soil (Hanski and Cambefort 1991). The ecosystem services that dung beetles perform are particularly relevant in animal-grazed grasslands. Grazed grasslands, which occupy 26% of the world's terrestrial land (Carswell et al. 2019), can either be sources or sinks of greenhouse gases, depending on the emissions from standing manure (methane and nitrous oxide) and carbon storage in the soil (Hammer et al. 2016; Viglizzo et al. 2019; Arellano et al. 2023). Dung beetles affect greenhouse gas emissions in pasture systems by eating and burying dung in the soil (Slade et al. 2016a), and they also influence nitrogen, phosphorus, potassium, and carbon cycling in soils (Stanbrook et al. 2021). Through their activities, dung beetles alter the soil microbial community in grazed grasslands (Slade et al. 2016b), although the exact changes to the community and potential for improvements in global carbon and nutrient cycling and availability are unclear.

While many dung beetle studies characterize dung microbial communities or the microbiome of dung beetles themselves (Estes et al. 2013; Franzini et al. 2016; Parker et al. 2020), there is a

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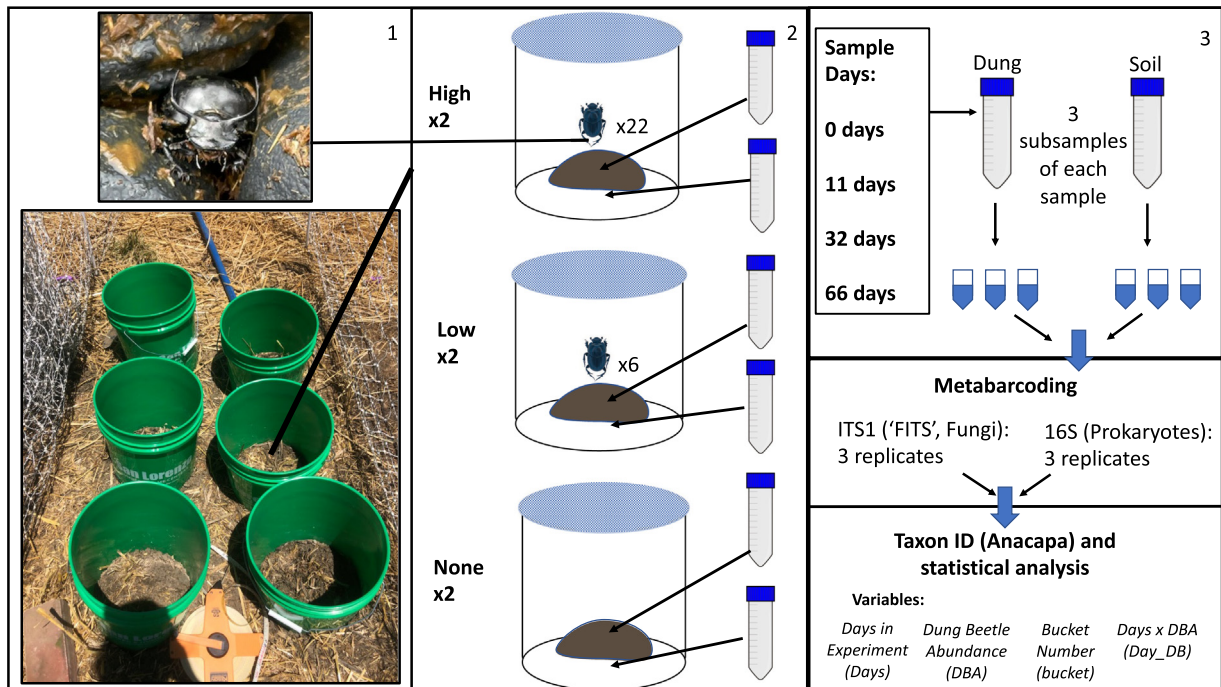


Figure 1. Experimental design and workflow. The first panel shows the dung beetle, *Onthophagus taurus*, and the setup of the enclosures. The second panel shows the inside of the enclosures, which were populated with 22, 6, or 0 (no) dung beetles, to which 400 g of dung was added, as well as where the dung and soil were sampled. Panel three shows sampling methodology. We collected the soil and dung on d 0, 11, 32, and 66 and extracted three subsamples from these enclosures. We assessed these samples for soil microbial composition using metabarcoding of the fungal ITS1 ("FITS") and 16S regions. Finally, we used Anacapa to identify taxa and did statistical analysis in R using the variables of Days in Experiment, Dung Beetle Abundance, Bucket Number, and Dung Beetle Abundance separated by Days in Experiment.

dearth of research on dung beetle influence on the soil microbiome using next-generation sequencing (NGS) metabarcoding. While not comprehensive, NGS metabarcoding allows for identification of alpha and beta diversity patterns, as well as identification of candidate microbes in nutrient cycling in natural systems, which can indicate soil processes in ecosystems (Fierer et al. 2013; Kroeger et al. 2018). The United States ceased importing dung beetles in the 1990s, but other countries have continued dung beetle inoculation programs, with great success for manure management (Noriega et al. 2020; Pokhrel et al. 2021). Microbiome NGS of soils can be a valuable tool for further evaluating dung beetle introduction programs and other management efforts to increase dung beetle populations on ranches in the United States and for helping ranchers understand the effects of dung beetles on soil health.

Here we present results from an in situ mesocosm experiment at a working cattle ranch in central California, the United States, coupled with metabarcoding to determine the influence of the exotic tunneling dung beetle *Onthophagus taurus* (Schreber 1759) on the soil bacterial, archaeal, and fungal community. This species is common in the United States because the US Department of Agriculture intentionally imported *O. taurus* in the 1970s in an effort to decrease fly populations, increase nutrient cycling, and aerate soils in pasture systems (Anderson and Loomis 1978). We asked if the presence of *O. taurus* influences the soil microbial community and, if so, if any specific bacterial, archaeal, or fungal families affected are associated with nutrient cycling in the soil, specifically carbon cycling.

Material and Methods

Field design

To determine how *O. taurus* abundance impacts soil microbial community composition, we established enclosures on recently grazed, irrigated pasture at Paicines Ranch in Paicines, California

(Fig. 1) from June–August 2020. This consisted of six total enclosures with two repetitions of three *O. taurus* abundance treatments: none, low (6 beetles), and high (22 beetles). Enclosures were constructed of 22.73 L plastic buckets with the bottoms removed, dug into the ground to a depth of 7 cm with minimal disturbance to the soil inside the enclosures. *Onthophagus taurus* beetles were collected from a nearby field using a small tin pan covered with mesh and cow dung, which prevents the beetles from flying away, and kept for 1–2 d in containers filled with dung and soil. Cow dung was collected fresh (within several hours of being dropped), partitioned into 400-g samples, and frozen for 1 wk prior at -20°C following the methods of Manning et al. (2016), to ensure that all soil invertebrates were dead while preserving the dung for *O. taurus* use. The pasture had been grazed 2 d before establishing the enclosures for 3 d with a mixed cow and sheep herd consisting of 96 animal units (one animal unit is equal to 1000 lb). Sixteen d before the enclosures were established, both the pasture and all the experimental enclosures were planted with a no-till drill with a cover crop mix of warm-season annual plants. This was not part of the study design, but rather a consequence of running this experiment on a working ranch. We established the enclosures on June 26, 2020.

We placed defrosted dung into the enclosures and added *O. taurus* to two low (6 beetles) and two high (22 beetles) treatment enclosures; two enclosures had no beetles. We chose the low and high treatments to mimic the range of abundance of beetles in dung in the study sites (0–22 beetles). The enclosures were covered with a fine window screen plastic mesh to prevent the beetles from flying away.

We took soil and dung samples four times from the center of each enclosure, at d 0, 11, 32, and 66. We collected approximately 20 mL from the first 0–1 cm of soil into 50 mL falcon tubes. We chose this shallow soil depth because changes in the soil microbial community due to environmental perturbations are most easily detectable at the surface (Barbour et al. 2022). All samples were stored in a -80°C freezer until DNA extraction.

Extraction, PCR, sequencing

DNA was extracted from soil and dung samples using the Qiagen DNeasy PowerSoil Kit (Qiagen, Germantown, MD) following the manufacturer's instructions. We included dung samples to ensure the microbial communities were consistent across treatments and not driving the differences we saw in the enclosures. A blank extraction was included with each batch of 8–24 samples. We amplified bacteria and archaea DNA communities with the 16S marker (515F and 806R; Caporaso et al. 2011) and amplified fungal DNA communities with the ITS1 marker (hereafter termed "FITS"; ITS5 and 5.8S; White et al. 1990; Epp et al. 2012). We constructed Nextera-indexed libraries following triplicate amplification methods described in Lin et al. (2021); more details are in Supplemental Methods 1 (available online at [10.1016/j.rama.2023.07.007]).

Bioinformatics and statistical analyses

We processed the Fastq files (sequence results, published in NCBI under project PRJNA993284) through the Anacapa Toolkit (Curd et al. 2019), then decontaminated results and filtered taxa with fewer than 10 reads (see Supplemental Methods 1 for a detailed description).

We plotted rarefaction curves for soil samples and dung samples and selected rarefaction levels for diversity analyses that were near the asymptote of the curves (Fig. S1, available online at 10.1016/j.rama.2023.07.007). Alpha and beta diversity analyses including community ordinations and permutational analysis of variance (PERMANOVA) were performed in R using *stats* (R Core Team 2013; v 4.0.4), *vegan* (Oksanen et al. 2022), *Adespatial* (Dray 2021), *Phyloseq*, and *ggplot2* (Wickham 2011). We measured differential abundance between experiments with the *DESeq2* package (Love et al. 2014) filtering samples to include taxa that have an abundance greater than three reads in at least 10% of samples. We made the *DESeq2* object using a Wald test, local fitType, poscounts sfType, and we set significance to be below an alpha of 0.01. Because we performed tests for each taxon, we adjusted all *P* values with Bonferroni correction before interpreting significance.

Results

Results produced a rich inventory of dung and soil microbiomes. We found 47 phyla and 417 families in 16S bacterial and archaeal taxonomic sequences, and we found 8 phyla and 159 families in FITS fungal sequences. Despite few biological replicates, samples from the same test groups were largely consistent in composition and dominant taxa (Figs. S2 and S3, and Table S2.1 and S2.2 available online at [10.1016/j.rama.2023.07.007]). Results showed that *O. taurus* drive differences in soil communities based on alpha diversity, beta diversity, and relative abundance of nutrient cycling candidate taxa.

Community alpha and beta diversity

Dung beetle abundance promoted *FITS* alpha diversity richness and evenness in soil, but not in dung (16S was not significant after Bonferroni correction). Increasing the days in experiment promoted 16S and *FITS* richness and evenness in dung but not soil (Table S2.3). We also found outliers based on local contribution to beta diversity in dung but not soil, demonstrating the soil compositional profiles were consistent within groups (Table S2.1 and S2.2).

We focused next on whether specific families were enriched in soil under different treatments over the course of the experiment, using *DESeq2*. *Onthophagus taurus* presence did change rela-

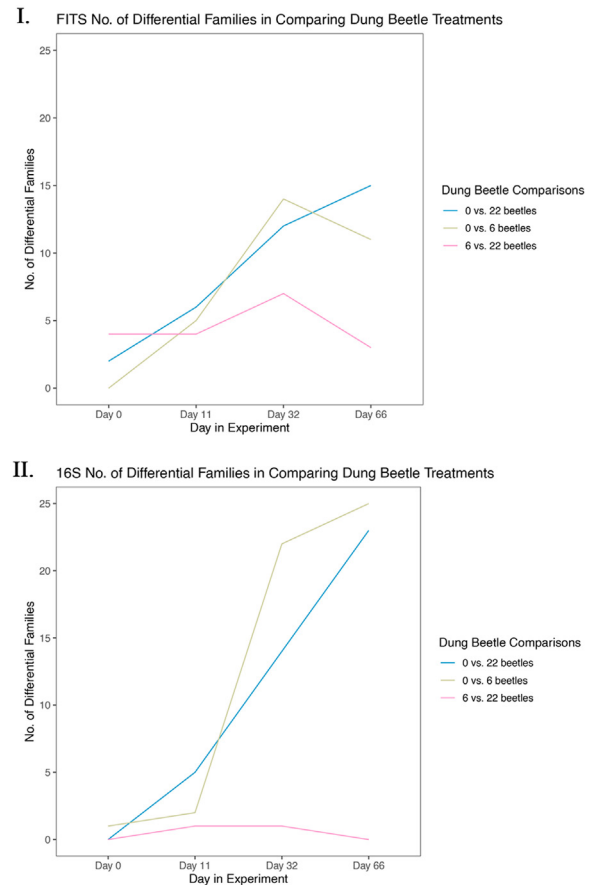


Figure 2. DESeq2 results. Number of families that are overrepresented between treatments (e.g., 0 vs. 6, 0 vs. 22, and 6 vs. 22 beetles) by days in experiment (0, 11, 32, 66) for i) *FITS* and ii) *16S*.

tive abundance of certain families, but density of 6 versus 22 beetles did not affect many bacterial families. Similarly for *FITS*, there were more differences in the presence of *O. taurus* rather than their abundance (Fig. 2, Figs. S4 and S5, Tables S2.4 and S2.5).

Community similarity analysis of beta diversity using Bray-Curtis ordination plots show that over the course of days in the experiment, the soil communities with *O. taurus* separate from the soil communities without *O. taurus* (Fig. 3). PERMANOVA test results show *O. taurus* abundance drives significant differences in both 16S and *FITS* microbial profiles (Table S2.6 and S2.7), as does days in experiment, and the interaction between days in experiment and beetle abundance. Explanatory power in these PERMANOVA results was consistently low ($R^2 < 0.2$).

Discussion

This study found changes in soil microbial abundance and composition that are sensitive to presence of the tunneling dung beetle, *O. taurus*. While this study was a pilot study, it is proof of concept that even with low replication, in one site, community changes are already significant on multiple levels. *Onthophagus taurus* presence affects soil fungal community richness and evenness, but not dung microbiomes. The presence of *Onthophagus taurus* drives changes in the composition of both bacterial/archaeal and fungal soil microbial communities in a similar magnitude to the effect of time (days in experiment). Another study that used DNA fingerprinting found that days in experiment outweighs the effect of dung beetles (Slade et al. 2016b), which is the case here

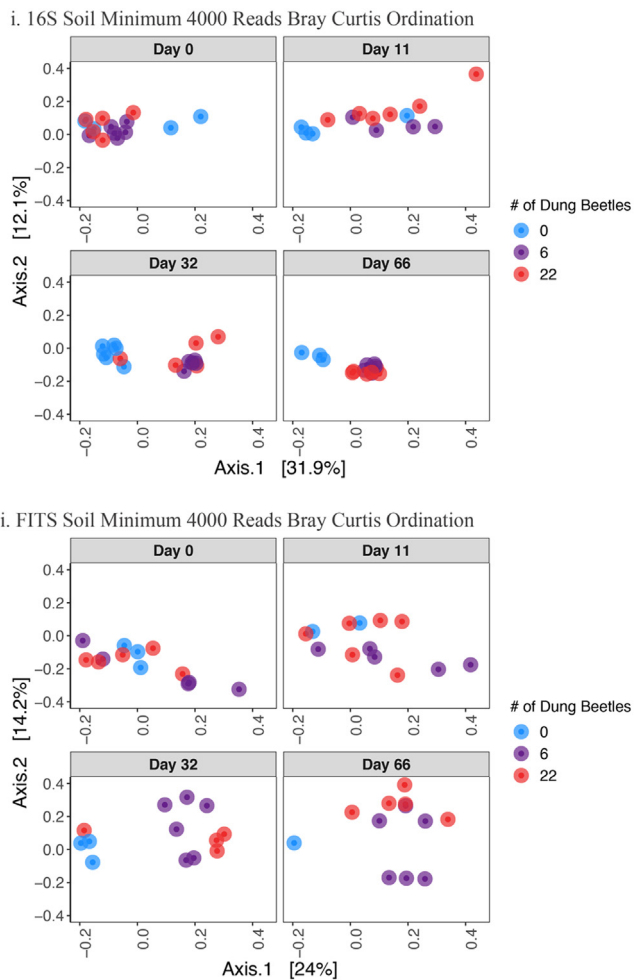


Figure 3. Bray Curtis ordination. i) is a Bray Curtis ordination plot of 16S soil samples with a minimum of 4000 reads, showing the dissimilarity between communities by sample. The ordination plot is split into the different sample times to show the clustering more clearly. Blue points are samples treated with no dung beetles, purple points are samples treated with 6 dung beetles, and red points are samples treated with 22 dung beetles.

for bacteria and archaeal communities, but not for fungal communities. This is likely because the turnover for bacteria and archaea is generally rapid: the same microbial community would rarely persist a month later (Hannula et al. 2019). Conversely, the fungal community is driven more by seasonal factors rather than time (Hannula et al. 2019). Our finding that both abundance and time effects are significant individually and have a significant interaction effect shows that *O. taurus* have an important impact on soil over time. While the explanatory power in the tests is low, the microbiome shifts explained by our PERMANOVA tests is consistent with other soil metabarcoding results (Lin et al. 2021). Also, we note that the *O. taurus* effect was seen even with the pre-experiment seeding and subsequent plant growth, even though plants have strong effects on the microbial community (Reese et al. 2018). Finally, while the sampling regime may have partially destroyed some of the soil material in each enclosure, we still see an effect based on *O. taurus* treatment.

Alpha diversity

Several soil bacterial families that increase in representation with *O. taurus* abundance by d 32 are predominantly related to

nutrient cycling or degrading plant materials. Although the abundance of none of these families surpassed 4.33% of total reads in any treatment (Table S2.10), several families experienced significant increases with *O. taurus*. Bacteria at d 32 included Bifidobacteriaceae, which was 17–29 times more common with *O. taurus*; Nannocystaceae, which was 26–55 times more common with *O. taurus*; Cyclobacteriaceae, which was 1.9–3.2 times more common with *O. taurus*; Polyangiaceae, which was 2.5–2.9 times more common with *O. taurus*; and Hyphomicrobiaceae, which was 3.3–3.6 times more common with *O. taurus*. These bacteria could be indicators of increased soil organic carbon content, given their role in plant degradation and carbohydrate metabolism, while many of the bacteria overrepresented in the no dung beetle treatment seem to be rhizobia or other denitrifying bacteria common to dry soils (Table S2.8). Cyclobacteriaceae contains species that degrade polysaccharides (Pinnaka and Tanuku 2014), Polyangiaceae is a soil microbe also found in decaying plant matter and may degrade cellulose (Garcia and Müller 2014), Hyphomicrobiaceae contains some nitrogen-fixing genera (Oren and Xu 2014), Bifidobacteriaceae breaks down complex carbohydrates (Eisenlord et al. 2013), and Nannocystaceae contains genera found in soils with decaying plant matter (Mohr et al. 2018). At d 66, samples from enclosures with 6 and 22 *O. taurus* both have a higher representation of Bifidobacteriaceae, which was not found in any samples without *O. taurus* and increased to 0.18% in low dung beetle treatments and 0.09% in high dung beetle treatments; Cytophagaceae, which was 1.6 to 2 times more common with *O. taurus*; as well as Cellulomonadaceae, which was 2.8–3.8 times more common with *O. taurus*; Isosphaeraceae, which was 32.1–53.6 times more common with *O. taurus*; and Verrucomicrobiaceae, which was 2.4–2.5 times more common with *O. taurus*. Cytophagaceae has been shown to increase with the addition of bioorganic fertilizer in apple orchards in China (Wang et al. 2016), Cellulomonadaceae decomposes plant derived macromolecules (Stackebrandt and Schumann 2014), Isosphaeraceae slowly decomposes plant matter (Ivanova et al. 2017), and Verrucomicrobiaceae shows a positive correlation with genes associated with carbohydrate metabolism in prairie soils of the Midwest (Fierer et al. 2013).

At the end of the experiment (d 66), the 6 and 22 *O. taurus* abundance enclosures have clear overrepresentation of the following fungi: Clavicipitaceae, which was 4.4–9 times more common with *O. taurus*; Lasiosphaeriaceae, which was 2.9–5.3 times more common with *O. taurus*; Bolbitiaceae, which was 248–710 times more common with *O. taurus*; and Pluteaceae, which increased from 0% with no dung beetles to 0.04% with low dung beetle treatment and 0.11% with high dung beetle treatment (Table S2.11). Clavicipitaceae is a soil saprophyte and potential insect pathogen (Torres and White 2009). Lasiosphaeriaceae is commonly found in herbivore dung (Melo et al. 2015). The largest genus in the family Bolbitiaceae, *Conocybe*, is commonly found in herbivore dung and grasslands (Amandeep et al. 2015; Joshi et al. 2021). Pluteaceae is common to wood and other decaying plant matter (Justo et al. 2011). Only one fungal family overrepresented in the no-*O. taurus* treatment was associated with dung: Sporormiaceae on d 32 (Phukhamsakda et al. 2016) (Table S2.9).

While many of the microbes that significantly differed between treatments have been implicated in roles that mitigate carbon and nitrogen cycling, we have not seen these mentioned in literature about soil carbon sequestration beyond Fierer et al. (2013), who discuss the importance of Verrucomicrobiaceae in carbon sequestration. Most members of the family Nannocystaceae are as of yet uncultivated, meaning that they can only be detected with metagenomics (Garcia and Müller 2014). The change in difference in FITS reads show that dung incorporated into the soil by *O. taurus* creates a noticeable impact on the soil fungal microbial community,

though the exact families do not seem to indicate a change in function.

Implications

Onthophagus taurus influence the soil microbial community. Some of these microbes contribute to nutrient cycling, specifically fluctuations in soil organic carbon and nitrogen content. We intended this experiment as a pilot experiment and as such it has low biological replicates. However, the clear compositional changes shaped by *O. taurus*, as well as the consistency of our results among samples from the same test groups, offer opportunities to discover the effects of dung beetles on the soil microbial community with larger studies in different kinds of environments. Although this is a pilot study with one species of dung beetle, grazed pastures cover 26% of the world's terrestrial land (Carswell et al. 2019) and there are 8000 species of dung beetles worldwide (Pokhrel et al. 2021), so noticing even small shifts with one species may be an indicator of the importance of this group of beetles on a global stage. Further, most conventional livestock systems use parasiticides, which can reduce or eliminate dung beetle populations (Li et al. 2023). Our research helps to validate that even a relatively low presence of dung beetles can influence the soil microbial community and that there may be a low population threshold required to activate these changes. Future in situ experiments will include assessments of soil organic carbon in addition to soil microbial composition. Results from this and subsequent experiments will inform management practices for ranchers who wish to improve nutrient cycling in their pastures.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:[10.1016/j.rama.2023.07.007](https://doi.org/10.1016/j.rama.2023.07.007).

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