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SHORT COMMUNICATION



Common commercially available parasiticides do not cause fatal changes in the microbiome of the dung beetle *Onthophagus binodis*—a pilot study

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ABSTRACT

Dung beetles perform vital ecosystem functions, but their survival is threatened by parasiticide use in veterinary practices. Currently, it's unclear if parasiticides directly harm dung beetles or cause damage via secondary effects on crucial microbiome components. We investigated the impact of three commercial parasiticides (P1–levamisole and oxfendazole; P2–abamectin, levamisole and oxfendazole; P3–moxidectin) on *Onthophagus binodis* dung beetle gut and exoskeleton microbiomes. Dung beetles were subjected to parasiticides mixed with fresh dung for 14 days, and we used 16S rRNA genes to detect bacterial diversity changes. The tested parasiticides didn't alter dung beetle gut communities, but slightly affected the exoskeleton microbiota composition. No mortality difference was observed. *Onthophagus binodis* microbiomes are mainly diet-influenced, and their exoskeleton is more adaptable than their gut. Our study suggests that adjustable dung beetle microbiomes aren't susceptible to parasiticides due to their innate resilience.

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Introduction

Dung beetles in the genus *Onthophagus* (Coleoptera; Scarabaeidae) are known for their unique ecological roles and primarily feed on ruminant dung and restrict their ingestion to fine particles consisting of easily digestible, high-quality food such as bacteria and epithelial cells from the herbivore's gut (Holter and Scholtz 2007). The dung beetle microbiome is therefore likely strongly influenced by the gut microbiome of their host. Their gut microbiota also has an impact on survival and longevity (Suárez-Moo et al. 2020; Ebert et al. 2021). The transmission of specific bacteria from mother to offspring occurs through maternal secretions in the brood ball, known as the maternal gift (Estes et al. 2013; Shukla et al. 2016; Parker et al. 2020). The maternal gift significantly

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influences host development, with the experimental exchange of maternal gift between different species of dung beetles having adverse effects on growth, survival, and developmental time (Parker et al. 2020; Parker et al. 2021).

One of the primary threats to dung beetles is excessive use of veterinary products, such as parasiticides, which are excreted in mammalian dung (Beynon 2012; Sánchez-Bayo and Wyckhuys 2019). Studies have found that the use of these veterinary compounds on farms reduces dung beetle richness and diversity. Tunnelling dung beetles are most at risk of these compounds (Sands and Wall 2018). In *Euoniticellus intermedius*, decreased fecundity was observed after the use of ivermectin in cattle (Kadiri et al. 2017) and in *Scarabaeus cicatricosus* that same parasiticide caused decreased olfactory and locomotor capacity (Verdú et al. 2015). Longevity studies also suggest that the effect of parasiticides on dung beetles persists longer than the cattle treatment itself, increasing negative impacts (Pérez-Cogollo et al. 2017).

At present, the mode of impact of parasiticides on dung beetles' microbiome is unknown. Agrochemical exposure causes microbial shifts in other insects, and if that shift is in favour of detrimental bacteria, then this can result in host mortality (Voulgari-Kokota et al. 2020; Shi et al. 2021). In this study, we investigated the effect of three veterinary parasiticides used in cattle on the *Onthophagus binodis* microbiome. This species is a tunnelling dung beetle that buries dung into the soil and was introduced to New Zealand in 2013. To better understand the ecology of *O. binodis* microbiome, we also explored the effect of diet and compared their gut and exoskeleton microbial composition. Exoskeleton was included because of the dung beetle's lifestyle, which is more like that of intestinal parasites rather than other insects.

Materials and methods

Dung beetle rearing and experimental design

Onthophagus binodis beetles were obtained from a commercial rearing facility (Dung Beetle Innovations, New Zealand), where they were fed only cow dung from non-medicated stock from a farm local to the rearing facility in Whenuapai, Auckland (= Diet 1). Ten individuals were immediately stored at -20°C on arrival and represent our Diet 1 samples. The remainder were used in our experiment and were denoted as Diet 2, since they were fed dung originating from non-medicated stock from a farm near Christchurch (1130 km from the farm used as Diet 1). To test the effect of parasiticides on *O. binodis*' microbiome, three different commercially available parasiticide treatments (P1, P2 and P3) were mixed with cow dung (from the Diet 2 farm) at two different concentrations (Table S1). Specifically, treatment P1 was double combination Coopers® Scanda® Salinised (Wellington, New Zealand), P2 was triple combination Coopers® Alliance® (Wellington, New Zealand) and P3 was the single active ingredient compound Cydectin® S by Zoetis (Auckland, New Zealand). To achieve the treatment concentration, a known amount of stock parasiticide solution was thoroughly mixed into the 100 g of fresh cow's dung, absent of any veterinary medical products. Low concentration was determined based on manufacturer's recommended dose (P1–1 g levamisole and 0.57 g oxfendazole; P2–0.03 g abamectin, 1 g levamisole and 0.57 g oxfendazole; P3–5 g moxidectin) and the high dose was 10x the low dose. These concentrations were

selected based on previous reports, that 80% of oxfendazole gets excreted in the dung and is detrimental to dung dwellers in these concentrations (McKellar 1997).

Seven 20L plastic buckets were filled halfway with a coarse vermiculite and ten *O. binodis* beetles were introduced to each bucket. Each bucket represented a distinct treatment. The control treatment contained untreated dung, while the buckets of treatments were supplied with the respective mixture of dung and parasiticide. The experimental design is described in Figure S1. This was repeated every 4 days for 21 days during the experiment. After 21 days, dung beetles were collected and stored at -20°C , and adult dung beetle mortality was recorded for each bucket.

DNA extraction and sequencing

Five dung beetles per treatment were used in DNA extractions, each dung beetle was as an individual sample. Frozen dung beetles were thawed and dissected to separate their gut from the exoskeleton (comprising the washed body integument, legs, and head using sterile H_2O , minus mouthparts), with gut and exoskeleton representing our two analysed sample types. DNA was extracted from the beetles using MN Soil DNA manual extraction kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. Deionised water was used as a sample for negative DNA extraction control. To amplify bacterial 16S rRNA genes, PCR was performed using Bact_F515 (5'-GTGCCAGCMGCCGCGGTAA) and Bact_R806 (5'-GGACTACGVGGGTWTCTAAT) primers (Caporaso et al. 2011). Each 20 μL reaction contained 1 μg rabbit serum albumin, 10 μL 2 x KAPA Plant 3G Buffer (Kapa Biosystems, Wilmington, MA, USA), 0.2 μL KAPA Plant 3G enzyme, 500 nM of each target primer and 2 μL of the DNA extract as template or 2 μL of deionised water as negative control. The cycling conditions were 95°C for 3 min, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s; with a final elongation step of 72°C for 2 min. This 16S rRNA gene PCR product was used as template in a second PCR reaction to prepare samples for Illumina sequencing. These subsequent 25 μL reactions contained 2 μg rabbit serum albumin, 12.5 μL 2 x KAPA HiFi ReadyMix Buffer (Kapa Biosystems), 500 nM of Illumina-tagged sequencing adaptors, and 1 μL of the first PCR reaction as template. The cycling conditions were 95°C for 3 min then 5 cycles of 98°C for 15 s, 65°C for 15 s, and 72°C for 15 s; followed by 72°C for 1 min. Amplicons were treated with SPRIselect magnetic beads (Beckman Coulter, Brea, CA, USA) to remove primer dimers and high molecular weight DNA, according to the manufacturer's instructions. The amplicons were quantified using Qubit assay (ThermoFisher Scientific, Waltham, MA, USA) and pooled by equimolar amounts. The pooled library was sequenced in a 2×300 bp Illumina MiSeq run (Otago Genomics, University of Otago, NZ).

Bioinformatics and statistical analysis

Bioinformatic analysis of Illumina sequences followed a pipeline described by Dopheide et al. (2023). In short, demultiplexed sequences were merged, adaptors and primers were trimmed, sequences were quality filtered and denoised into amplicon sequence variants (ASVs), and ASVs were filtered for chimeras using VSEARCH (Rognes et al. 2016) and cutadapt (Martin 2011). The RDP naïve Bayesian classifier (Wang et al. 2007) was used

for taxonomic classification of 16S rRNA gene-based ASVs. We read the output tables (ASV, taxonomic) and the metadata into a phyloseq object (McMurdie and Holmes 2013). Contaminants from the negative controls were identified using the prevalence method with a threshold of 0.5 and removed using the decontam R package (Davis et al. 2018). We then transformed the data to relative abundance (McKnight et al. 2019) using the microbial R package (Guo and Gao 2021). This resulted in 76 dung beetle microbiome samples with a total of 1801 bacterial ASVs. Preliminary analysis investigated whether concentration of parasiticide influenced dung beetle microbiome. We used adonis PERMANOVA from the vegan R package (Oksanen et al. 2019) to analyse the effect of concentrations of each parasiticide on gut or exoskeleton microbiome. For further analyses, we pooled data from the two concentrations ('low' and 'high') to represent the three parasiticide treatments for the gut and exoskeleton samples.

To understand the effects of the three parasiticide treatments on the bacterial community composition on dung beetles' exoskeletons and guts, we visualised each of these treatments and the control group using PCoAs with Bray–Curtis dissimilarity matrices. We used adonis PERMANOVA from the vegan R package (Oksanen et al. 2019) to assess differences in community composition based on Bray–Curtis dissimilarity for each of the parasiticides compared to the control communities for gut and exoskeleton samples. As compositional differences can be driven by dispersion, we also used the betadisper function from the vegan R package (Oksanen et al. 2019) to calculate the distance to group centroids and ANOVA to test for differences in dispersion between the control and each of the parasiticide treated groups.

To understand the effect of diet on bacterial community composition, we considered samples from the Diet 2 control group and Diet 1. We visualised the community composition using PCoA with Bray–Curtis dissimilarity and adonis PERMANOVA to test for effects of diet, sampling location (gut and exoskeleton) and their interaction on community composition. We again used betadisper and ANOVA to calculate and test for differences in dispersion between communities in the Diet 2 no treatment and Diet 1 groups.

To assess which bacterial Classes may underly the changes in composition, we visualised the relative abundance of Classes across Diet 1, Diet 2, and Diet 2 + parasiticide treatments (P1, P2, P3) for the gut and exoskeleton groupings. For Classes that represented less than 5% of total relative abundance, we grouped these as 'low abundance'. Finally, to assess the relative abundance of putative parasiticide degraders across these groupings, we characterised the relative abundance of *Pseudomonas*, *Stenotrophomonas*, and *Burkholderia*. All analyses were performed in R v.3.6.3 (R Core Team 2020).

Results

Mortality of dung beetles was similar across all treatment groups (Table S2). Since there were no differences in community composition based on concentration within parasiticide treatment, except for P3 in the gut (Supplemental Table S3), the 'low' and 'high' concentration samples were pooled for each parasiticide treatment. When those pooled samples were compared to the control samples (Diet 2), all three parasiticides significantly affected the bacterial community composition on the dung beetles' exoskeletons (Figure 1A, Table S4, $p < 0.05$), but not their gut bacterial community compositions

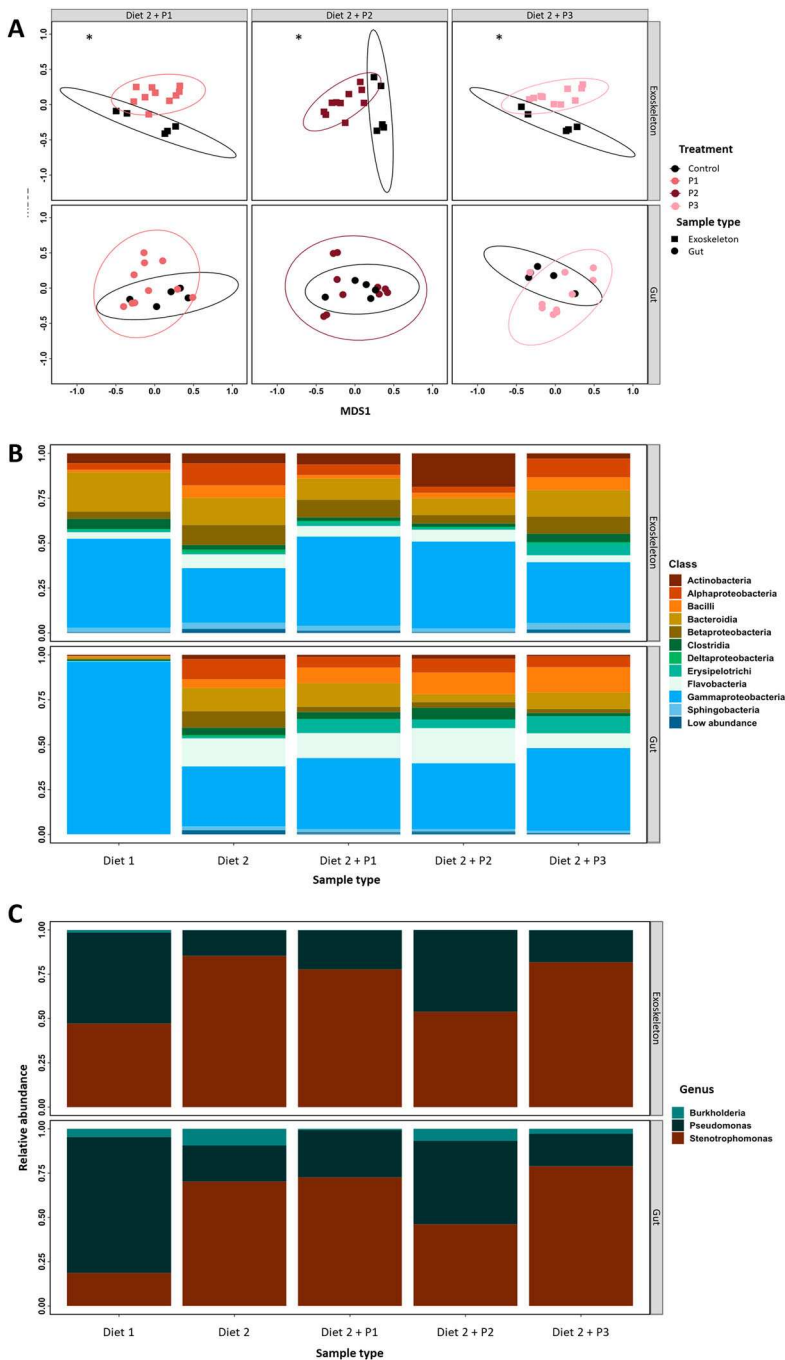


Figure 1. Effect of parasiticide on dung beetle's microbiome. **A**, Compositional comparison of bacterial taxa with a PCoA from the Diet 2 (black) and Diet 2 + parasiticide (red and pink colours) from gut (circle) and exoskeleton (square). The ellipses represent 95% confidence intervals. **B**, Relative abundance of bacterial taxa across the two diets and the different parasiticide treatments. **C**, Relative abundance of bacterial genera that include putative parasiticide degraders across the two diets and different parasiticide treatments.

(Figure 1A, Table S4, $p > 0.05$). These compositional changes in the exoskeleton bacterial communities were not due to differences in dispersion, as the dispersions of the parasiticide treated communities were not significantly different from that of the no-treatment communities (Table S5, $p > 0.05$). In Diet 2 treatments, bacterial classes *Erysipelotrichia* and *Gammaproteobacteria* increased on the exoskeleton, across all three parasiticides (Figure 1B). When comparing only Diet 1 and Diet 2 without parasiticides, the relative abundance of *Gammaproteobacteria* decreased drastically in the gut in Diet 2. *Alphaproteobacteria* and *Flavobacteria* increased in both gut and exoskeleton when moving from Diet 1 to Diet 2 (Figure 1B). These described changes observed in relative abundance were likely the driver of the significant shift in the bacterial community associated with the exoskeleton (Figure 1A).

Considering samples from Diet 1 and the control group of Diet 2, we found that diet is a key driver of the bacterial community composition (Figure 2) ($R^2 = .17$, $F = 2.99$, $df = 1$, $p = 0.003$), and that sample type (gut vs exoskeleton) was not a significant predictor of the bacterial composition ($R^2 = 0.07$, $F = 1.2$, $df = 1$, $p = 0.238$). Neither was their interaction ($R^2 = 0.06$, $F = 1.02$, $p = 0.404$) (Figure 2) or the dispersion of the communities in these two diet groups ($F = 0.03$, $df = 1$, $p = 0.86$).

We also identified differences in the relative abundances of three genera of putative parasiticide degraders—*Stenotrophomonas*, *Pseudomonas*, and *Burkholderia* (Ali et al. 2010; Wang et al. 2015; Xu et al. 2023). These three genera were present across the sample groups (Figure 1C). When focussing on only those genera, *Burkholderia* was the least abundant of the three, and it was more abundant in the gut than the exoskeleton. *Pseudomonas* was predominant in Diet 1 and *Stenotrophomonas* in Diet 2 (Figure 1C).

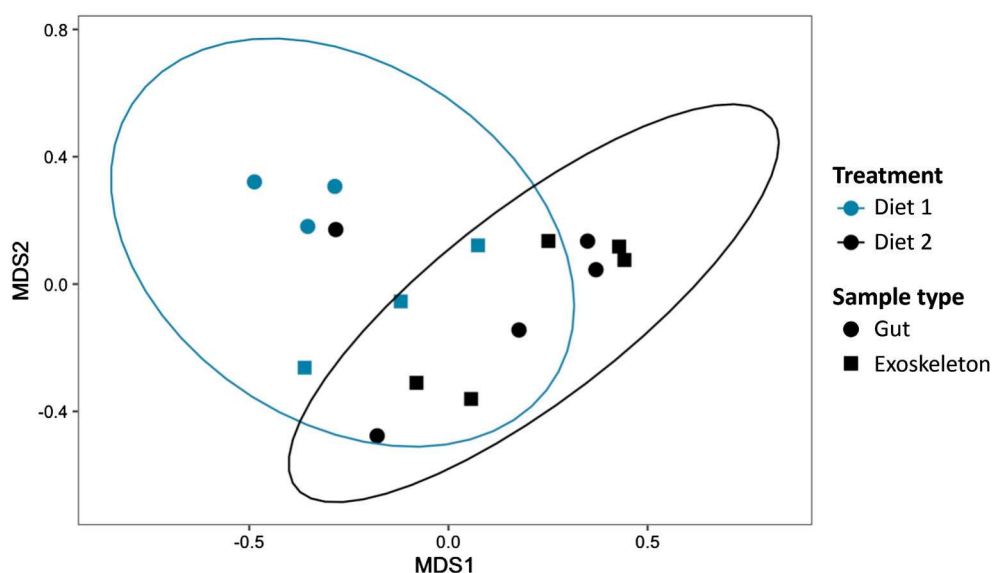


Figure 2. Compositional comparison of bacterial taxa with a PCoA of Diet 1 (blue) and Diet 2 (black) from the exoskeleton (square) and gut (circle). The ellipses represent 95% confidence intervals.

Discussion

Effect of parasiticide on the dung beetle's microbiome

Parasiticides have a devastating effect on dung beetle populations, especially those of our study organism *O. binodis* (Dadour et al. 2000), yet the mode of action is not understood. Currently there are known detrimental consequences to non-target organisms, however that risk is deemed acceptable due to the high commercial value of livestock production (Lumaret and Errouissi 2002). In a recent review Vokřál et al. (2023) highlighted that parasiticides circulate in the environment and in food chains and that the impact on non-target organisms requires more thorough investigation (Vokřál et al. 2023). In this pilot study we investigated whether the applied parasiticides cause a microbiome shift that contributes to heightened mortality. Our results did not support this hypothesis; while some change was detected in the exoskeletal microbiota when comparing a parasiticide-containing vs non-parasiticide diet, this did not cause heightened mortality in any of the parasiticide treatments. All three parasiticides significantly affected the bacterial community composition of the *O. binodis* exoskeleton but not their gut microbiota; however, the variation explained was relatively small (adonis R2 range: 8.7% to 14.3%). Interestingly, the abundance of *Erysipelotrichia* increased in both the gut and exoskeleton, but mostly in the gut, of those beetles exposed to parasiticide. In mice, levels of *Erysipelotrichia* have been demonstrated to increase in individuals treated with antibiotics (gentamicin and ceftriaxone) (Zhao et al. 2013). It is unclear whether this increase is opportunistic or protective and highlights an area of future research. In general, the most significant changes in community composition were on the exoskeleton rather than the gut. This is not surprising as this tissue is in contact with the changing environment i.e. the dung. Similarly, it was observed in human skin, when the environment changed from air to the ocean water, the skin microbiome changed significantly within 10 min of exposure (Nielsen and Jiang 2019).

It is possible that microbial parasiticide degraders are providing the beetles with tolerance to the toxins. To explore this, we searched for known abamectin and ivermectin degraders within dung beetle's gut and exoskeleton. Abamectin degrading bacteria *Stenotrophomonas maltophilia* and ivermectin degrading *Aeromonas taiwanensis* have been identified and isolated from soil (Wang et al. 2015; Wang et al. 2020). Ali et al. (2010) isolated abamectin degrading *Burkholderia cepacia*-like strain also from soil and we considered all three of these genera as candidate parasiticide degraders. We also included *Pseudomonas*, as it is well known for its bioremediation capabilities (Cycoń et al. 2009; Xu et al. 2023). We identified both *Pseudomonas* and *Stenotrophomonas* in the gut and exoskeleton of the dung beetles. We found no exact matches to the previously reported abamectin-degrading bacteria, and the potential for abamectin degradation within the dung beetle in our study remains unknown. It is also unknown whether the detected bacterial genera in our pilot study can degrade parasiticides; there is thus fertile ground for future studies to amend the gut of the dung beetle with known parasiticide degraders and to study the *in-situ* activity of the dung beetle gut microbiota. Alongside, it is important to also study the microorganisms that might already have a protective role, especially in the dung beetle's gut.

It is important to note that this pilot study was only performed for 3 weeks, while the normal *O. binodis* lifecycle lasts for 6–8 weeks. To fully understand the effects of

parasiticides on beetle fitness and mortality a longer, generational study with multiple replicates needs to be performed.

Other factors impacting the dung beetle microbiome

When we investigated the effect diet had on the *O. binoids* microbiome, diet was by far the most significant predictor of the microbial composition. We investigated this further to gain insight into their microbiome dynamics. In this study we had two sample types—dung beetle gut and dung beetle exoskeleton. Exoskeleton was included because of the dung beetle's lifestyle, which is more like that of intestinal parasites rather than other insects. They live in and eat the dung and their brood balls are comprised of dung. Therefore, their holobiont (dung beetle host plus all their associated microbes) is impacted by the microbiome of the mammals from which the dung originated. We expected to observe some differences in the microbiome between the exoskeleton and the gut, but also to see some overlap, since they are both exposed to the same medium throughout the life cycle.

Interestingly, the change in diet impacted on bacterial composition in both the gut and exoskeleton. In three weeks, the composition of the dung beetles' microbiota shifted significantly when their diet was changed from NZ North Island cattle dung to NZ South Island cattle dung. This indicates high plasticity within the dung beetle microbiome. Current literature indicates that insects with broad diets typically harbour more diverse gut communities, with hundreds of taxa (Yun et al. 2014; Gontang et al. 2017). Dung beetles appear to be an exception as they have a narrow diet (dung) but harbour a more diverse gut microbial community than other beetles (Kolasa et al. 2019). Our results were compatible with those of Parker et al. (2020) who found that the gut microbial communities of *Onthophagus taurus* shared some core elements between populations but were significantly influenced by their local environments and changes in diet.

In conclusion, dung beetles offer an interesting study system in that their own microbiome is impacted by the microbiome of another donor animal. *Onthophagus binodis* microbiome is mostly influenced by diet, and their exoskeleton is more plastic than that of the gut microbiome. To understand the complicated dynamics more fully at play, further research on how the mammalian diet (and therefore their gut microbiome) impacts the dung beetles is required. Such insights could conceivably then be transferred into other fields of research such as parasite infections and their survival within the host's gut.

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