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Treating cattle with antibiotics affects greenhouse gas emissions, and microbiota in dung and dung beetles

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Antibiotics are routinely used to improve livestock health and growth. However, this practice may have unintended environmental impacts mediated by interactions among the wide range of micro- and macroorganisms found in agroecosystems. For example, antibiotics may alter microbial emissions of greenhouse gases by affecting livestock gut microbiota. Furthermore, antibiotics may affect the microbiota of non-target animals that rely on dung, such as dung beetles, and the ecosystem services they provide. To examine these interactions, we treated cattle with a commonly used broad-spectrum antibiotic and assessed downstream effects on microbiota in dung and dung beetles, greenhouse gas fluxes from dung, and beetle size, survival and reproduction. We found that antibiotic treatment restructured microbiota in dung beetles, which harboured a microbial community distinct from those in the dung they were consuming. The antibiotic effect on beetle microbiota was not associated with smaller size or lower numbers. Unexpectedly, antibiotic treatment raised methane fluxes from dung, possibly by altering the interactions between methanogenic archaea and bacteria in rumen and dung environments. Our findings that antibiotics restructure dung beetle microbiota and modify greenhouse gas emissions from dung indicate that antibiotic treatment may have unintended, cascading ecological effects that extend beyond the target animal.

1. Introduction

Antibiotics are extensively used in agriculture to promote growth and to treat or prevent livestock disease [1–7]; yet they may have major consequences for human and environmental health [4,8,9]. For example, the use of antibiotics in agriculture can favour the evolution of antibiotic resistance among pathogens and the spread of antibiotic resistance genes to surrounding environments (e.g. [10–12]). In addition, this practice can have other ecosystem-level ramifications that are likely to be important, but less appreciated. In particular, antibiotic treatment could affect two distinct, but potentially interacting ecological processes: the removal and recycling of livestock dung by decomposer organisms, and the release of greenhouse gases from dung.

Livestock dung provides a source of nutrients, organic matter and microbes to pasture ecosystems [13–15]. Dung can also act as a source of pathogens [16] and emit significant quantities of greenhouse gases, including methane [17,18]. However, these effects can be modified by the diverse communities that inhabit,

consume and/or interact with dung, of which dung beetles have been particularly well studied [19–23]. In general, dung beetles play a critical role in carbon and nitrogen cycling, and the maintenance of soil fertility in both natural and agricultural pasture ecosystems [24,25]. Veterinary pharmaceuticals can harm beetles and other downstream consumers of livestock dung, which may depress dung decomposition and reduce the diversity of dung-based communities. But while the effects of antiparasiticides have been relatively well studied in this context (e.g. [26–29]), the effects of antibiotics on dung beetles remain poorly known [30].

In contrast to antiparasiticides, which may directly act on the physiology of dung consumers (e.g. [31]), broad-spectrum antimicrobial compounds could have far-reaching, microbially mediated ecological effects. For example, as microbial symbionts (microbiota) are often critical to insect health and reproduction [32,33], antibiotics retained in dung could affect beetle performance by altering beetle microbiota. Furthermore, antibiotic-induced restructuring of livestock gut microbiota could change the nutritional, chemical and microbiological properties of dung, the diet of dung beetles.

Antibiotics could also directly or indirectly modulate greenhouse gas emissions from livestock dung. Antibiotics have been shown to alter the structure and activity of mammalian gut and faecal microbiota [34–36]. In livestock such as cattle, certain members of the gut microbiota are responsible for producing key greenhouse gases such as methane, nitrous oxide and carbon dioxide [37–39]. Despite interest in reducing emissions of these microbially mediated greenhouse gases, and the frequent use of antimicrobial compounds in agriculture, the relationship between the two remains unclear. Furthermore, the effects of antibiotics on greenhouse gas fluxes and on dung beetles could be linked. For example, dung beetles can reduce methane production from dung to an extent that could have impacts in some agricultural systems [40]. As methanogenesis is thought to be inhibited by oxygen entering the dung pat through beetle tunnels [20], it could be sensitive to the size and number of dung beetles. In turn, if beetle performance and fitness are influenced by antibiotic-sensitive microbiota, the effect of dung beetles on greenhouse gas emissions could depend upon the antibiotic treatment.

We hypothesized that antibiotics administered to cattle alter dung beetle microbiota and, as a consequence, depress beetle fitness. In addition, we hypothesized that antibiotics modulate gas emissions from dung and the extent to which beetles influence emissions. To experimentally test these hypotheses, we treated cattle with and without a broad-spectrum antibiotic, and assayed microbial communities in cow dung and in field-collected dung beetles (*Aphodius fossor* L.). We also tested the effect of antibiotics on beetle size and numbers, and on fluxes of multiple greenhouse gases (carbon dioxide, nitrous oxide and methane) from dung. Our findings demonstrate that the common practice of administering antibiotics to livestock [1–7] can have important, unintended impacts on dung biota and the biogeochemical processes they mediate in agroecosystems.

2. Material and methods

(a) Experimental set-up

Ten cows were randomly assigned to two treatments: five were given a standard 3-day course of tetracycline and five were left as controls. None of the cows had undergone antibiotic treatment within the previous six months, except for one cow in the antibiotic group, which had received a course of penicillin six weeks prior to the experiment (for the full history of each cow, see the electronic supplementary material, Methods). From each cow, we collected fresh dung on a single day, beginning 1 h after the last administration of antibiotics. That afternoon, we separated the dung from each cow into six 1 l pats and placed each pat in a mescosm (an open-bottom, mesh-covered plastic bucket) in the field. We also included four control mesocosms without dung to measure background fluxes of carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) from the soil. More detail on experimental design is given in the electronic supplementary material, Methods.

To examine the effects of antibiotics on dung beetle microbiota, on the performance of beetles and on beetle-mediated effects on gas fluxes from cow pats, we focused on the dung beetle *Aphodius fossor* (L.). *Aphodius fossor* is a regionally widespread and locally common species [41], and its ecology and interactions with dung have been extensively studied (e.g. [20,21,42]). The beetles were collected in the field in early June from different localities and stored at 4°C until they were added to dung pats.

Dung beetles were added to four of the six dung pats produced by each cow (randomly chosen). Gas measurements and dung samples for microbial analysis were taken from two intact pats with beetles and two without beetles. The two additional pats with beetles were used for more invasive sampling of beetles for microbial characterization and to measure beetle reproduction and development. Based on beetle densities recorded in the field and on a previous study [42], we added 12 beetles to each pat, maintaining a sex ratio of 1:1.

(b) Gas flux measurements

Gas fluxes of CO_2, N_2O and CH_4 were measured from dung mesocosms in the field at five time points over the course of the experiment (more information given in the electronic supplementary material). Net gas fluxes emitted from each dung pat were calculated as in Penttilä *et al.* [20].

(c) Dung and beetle sampling

All dung and beetle samples were preserved for subsequent characterization of microbiota using 95% ethanol, which is an effective storage medium for microbial community analysis [43]. On days 2, 11 and 23 of the experiment, dung samples of approx. 1 ml were taken and stored in ethanol (see the electronic supplementary material, Methods).

To test whether antibiotic treatment affected beetle microbiota, two parent beetles from each pat were preserved in ethanol on day 7 of the experiment. To measure antibiotic effects on beetle size, reproduction and survival, we sampled pats on day 43 (for half-grown larvae) and days 71 and 73 (for the next generation of adult beetles). Larvae recovered were weighed while fresh and the width of their head capsule was measured. Total offspring counts by the end of the experiment were used as an integrated measure of both adult reproduction and offspring survival.

(d) Molecular protocol and sequence data processing

To characterize the overall beetle-associated microbial community, whole adults were homogenized and DNA was extracted from approximately 100 mg of homogenate with the MoBio PowerSoil kit, following similar studies [43,44]. Approximately 100 mg subsamples of dung were used for DNA extraction with the same kit. Using barcoded primers, we PCR-amplified the V4 region of the bacterial and archaeal 16S rRNA gene, with the amplicons sequenced on an Illumina MiSeq platform
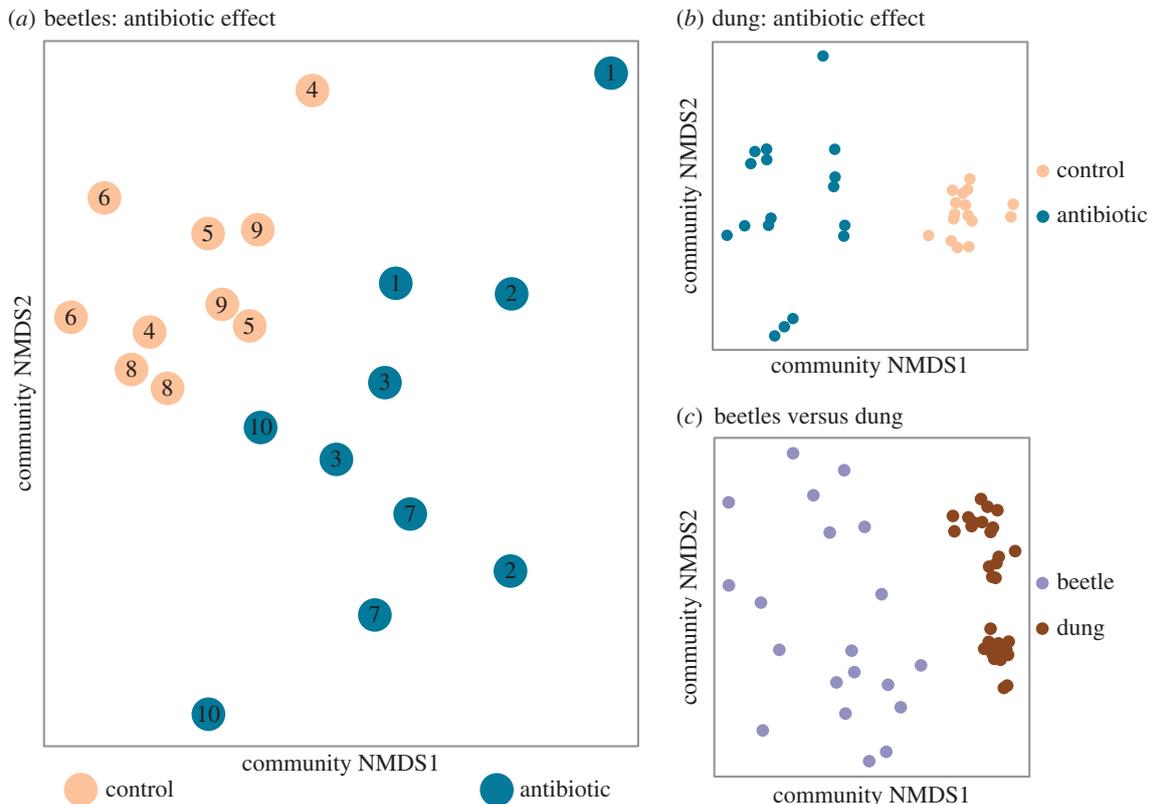
Figure 1. Microbial communities diverge upon antibiotic treatment and cluster according to sample habitat (beetle versus dung). Non-metric multidimensional scaling ordinations of microbiota in (*a*) parental dung beetles, where cow individual is indicated by numbers and treatment by colour, (*b*) dung samples only and (*c*) beetles versus dung. The ordinations visually represent Bray–Curtis dissimilarities among samples in two dimensions. (Online version in colour.)

as previously described [43,45]. Detail regarding sequence data processing and taxonomic identification is given in the electronic supplementary material, Methods.

(e) Statistical analysis

All statistical analyses were performed in R v. 3.2.2 [46], and plots were constructed with the ggplot2 package [47]. The vegan package [48] was used to analyse microbial community data. Following previous work (e.g. [43–45]), we used the Bray–Curtis dissimilarity metric to quantify variation in overall microbial community structure among samples. Non-metric multidimensional scaling plots were constructed to visualize Bray–Curtis dissimilarity in two dimensions. To avoid pseudo-replication of beetle data, dissimilarities (multivariate analyses) or performance variables (univariate analyses) were averaged between the beetles in each mesocosm; thus, multilevel statistical tests were conducted on the effects of antibiotic treatment and cow individual.

The effects of antibiotic treatment and cow individual on beetle and dung microbiota were tested using nested permutational multivariate ANOVA in the BiodiversityR package [49], with cow nested within treatment (999 permutations). Unless noted otherwise, below we report results from dung sampled on day 2 of the experiment, which we expected to most closely represent microbiota in the cow gut, but analyses of dung sampled on days 11 and 23 are shown in the electronic supplementary material. With each of the gases measured, we used ANOVA to assess effects of antibiotics and beetle presence on cumulative gas fluxes over the course of the experiment, as well as the interaction of the two factors. We analysed the proportion of methanogens in dung microbiota (all time points) with the same procedure, but with prior log transformation to meet the assumption of normality.

To assess antibiotic effects on the performance of larval offspring, we chose to focus on weight, which was highly

correlated with head size (Spearman's rank correlation, $\rho = 0.68$, $p = 0.005$). Weight can be considered as a proxy for performance as it is correlated with adult fecundity in both *Aphodius* [50] and other insects (e.g. [51,52]). A nested ANOVA (cow individual within antibiotic treatment) was used to test for effects on larval weight; normality of model residuals was confirmed visually. The effect of antibiotic treatment on total counts of beetle offspring recovered from each dung pat was analysed by fitting a generalized linear mixed model with cow individual as a random effect, Poisson-distributed errors and a log link function.

3. Results and discussion

(a) Microbial community structure in beetles

Dung beetles are extraordinarily diverse [53], and both scientifically [54] and ecologically important [25]; yet only one previous study has used DNA sequence-based methods to characterize their microbiota (*Onthophagus taurus* [55]). Despite feeding on a microbe-rich substrate, we found that *A. fossor* adults transform dung microbiota during digestion or host symbionts not present in their diet. Overall, beetles and dung had compositionally distinct microbiota ($F_{1,51} = 17.32$, $p = 0.001$; figures 1*c* and 2), regardless of the dung sampling time point (electronic supplementary material, figures S1*a*,*b*), and beetles contained lower microbial diversity than their diet ($F_{1,51} = 45.12$, $p < 0.0001$; electronic supplementary material, figure S2). While the dung communities were dominated by Bacteroidia, Clostridia and Spirochaetes, the dung beetle microbiota were dominated by Gammaproteobacteria and Bacilli. Less than 25% of the operational taxonomic units (OTUs) that were relatively abundant in beetle communities (overall proportion greater than or equal to 1%) were
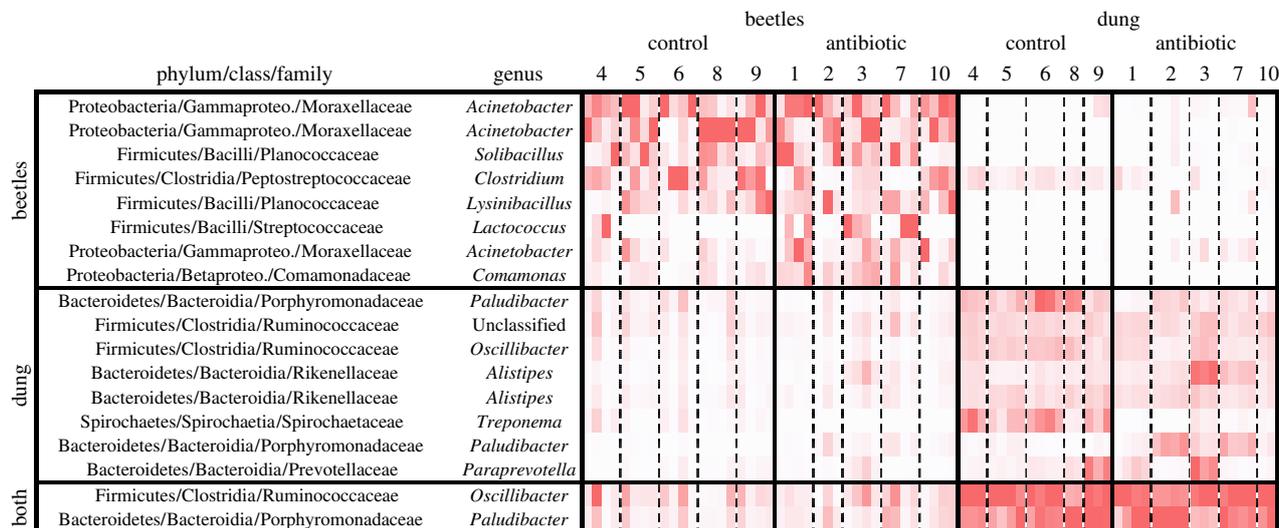
Figure 2. Variability in dominant microbial OTUs between individuals, sample habitats and treatments. The heatmap shows OTU relative abundance in each sample (columns) where darker shading indicates a higher proportion; the range of values is scaled separately for each sample. Numbers above columns denote cow individuals. For clarity, only the top 10 OTUs for each sample type are shown (two OTUs were in the top 10 of both beetles and dung). All OTUs shown are bacteria, as archaea were less abundant overall. (Online version in colour.)

also abundant in dung, implying that the dominant beetle microbes are either vertically transmitted or rare in dung. Indeed, the genus *Acinetobacter* was highly abundant in *A. fossor* (figure 2) and also found in *O. taurus* fed sterilized dung [55], suggesting that *Acinetobacter* could be vertically transmitted symbionts common among dung beetles.

(b) Effects of antibiotics on dung and dung beetle microbiota

As expected from previous work [34–36,56], we found a clear effect of antibiotic treatment on dung microbiota ($F_{1,23} = 4.84$, $p = 0.01$; figure 1*b*; electronic supplementary material, figure S2), and this effect persisted even 23 days after defecation and sample collection (electronic supplementary material, figure S1*c,d*). As in other animal taxa that maintain individual-specific microbiota (e.g. [56,57]), we also found that dung microbial communities clustered by cow individual ($F_{8,23} = 4.87$, $p = 0.001$; figure 2). Antibiotic treatment administered to cattle also affected dung beetle microbiota ($F_{1,10} = 2.39$, $p = 0.007$; figure 1*a*; electronic supplementary material, figure S2), and mirroring the patterns found for dung, the microbial communities in beetles also clustered by cow individual ($F_{8,10} = 1.38$, $p = 0.001$; figure 1*a*).

Antibiotics could alter beetle microbiota by multiple mechanisms. For example, antibiotic-induced changes in dung microbiota (figure 1*b*) could change the nutritional quality of the beetles' diet, or provide an altered inoculum to the beetle community. Alternatively, antibiotic residues present in dung and consumed by beetles may act on beetle gut microbiota. Concern has been raised over the persistence of antiparasiticides in the environment, where—as eukaryote-specific pharmaceuticals—they are directly toxic to animals [30,31,58]. Our data suggest that even antibacterials such as tetracycline may have similar far-reaching effects, by altering the microbiota of non-target animals.

(c) Beetle size and numbers

Despite clear antibiotic effects on beetle microbiota (figure 1*a*), and the often critical role of microbiota in insect biology [32,33],

antibiotics did not influence dung beetle size, reproduction or survival. Larval weight was unaffected by antibiotic treatment ($F_{1,13} = 0.018$, $p = 0.90$), and the total number of beetle offspring recovered from dung pats by the end of the experiment was similar between treatments (Poisson GLMM, $z = -0.35$, $p = 0.73$). An average of 4.2 ± 1.5 versus 3.0 ± 0.71 offspring were collected from dung pats of control and antibiotic treatments, respectively (mean $\pm$ s.e.m., $n = 10$ per treatment). As not all members of the beetle microbiota responded to antibiotics (figure 2), one possible explanation is that the antibiotic effect on overall microbiota was driven by commensal and not mutualistic species. For example, *Acinetobacter* abundance was not sensitive to antibiotic treatment ($F_{1,10} = 0.35$, $p = 0.57$; figure 2), and tetracycline resistance has been documented among *Acinetobacter* in other environments [59,60]. Alternatively, microbial symbionts may simply not be important to the nutrition or development of dung beetles [61]. However, given the wide variety of processes that can be influenced by microbiota (e.g. [62–64]), the antibiotic-induced microbial restructuring we observed could affect unmeasured aspects of dung beetle biology.

(d) Greenhouse gas emissions from dung

The presence of beetles decreased methane fluxes from dung ($F_{1,36} = 7.49$, $p < 0.01$; figure 3*a*), an effect reported previously and probably due to oxygenation of the pat caused by beetle tunnels [20,40]. By contrast, antibiotic treatment consistently increased methane emissions ($F_{1,36} = 22.21$, $p < 0.0001$; figure 3*a*), which are probably related to changes in dung microbiota (figure 1*b*). Contrary to our expectations, the two effects were unrelated (interaction $F_{1,36} = 0.004$, $p = 0.95$), suggesting that beetle tunnelling activity is not affected by antibiotic modification of dung (and its impact on the beetle microbiome).

Carbon dioxide emissions were similar between treatments ($F_{1,36} = 0.001$, $p = 0.98$), and there was no effect of beetle presence ($F_{1,36} = 0.02$, $p = 0.88$) nor an interaction between the two factors ($F_{1,36} = 0.22$, $p = 0.64$), suggesting that antibiotics do not affect overall microbial decomposition rates. This result indicates that the antibiotic effect on methane production is not simply due to an increase in overall microbial
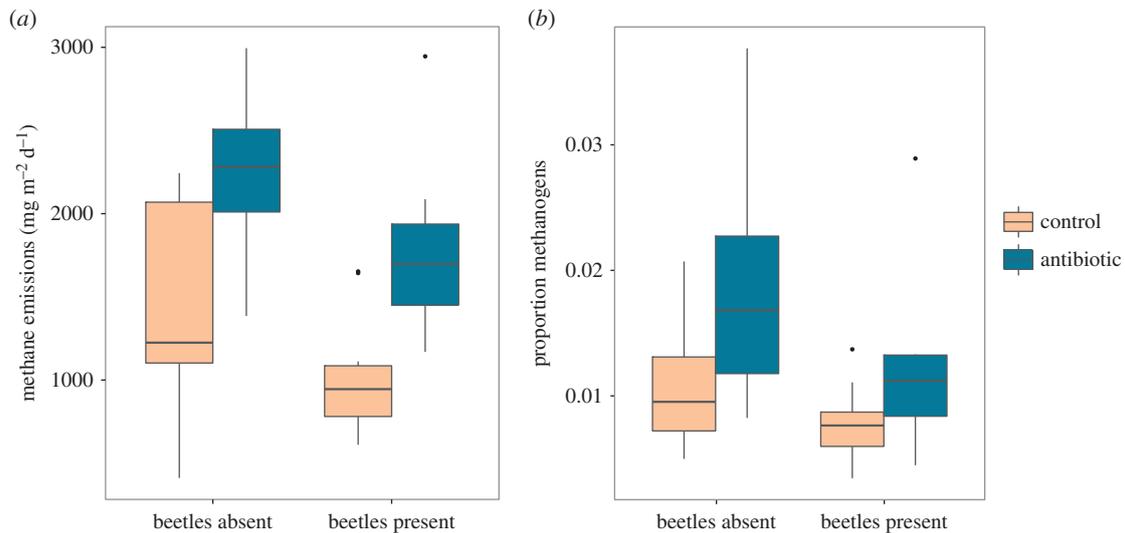
Figure 3. Dung methanogenesis is raised by antibiotic treatment and lowered by the presence of dung beetles, a pattern matched by methanogen abundance in dung microbiota. (*a*) Boxplot showing the factors affecting total methane emissions quantified from dung pats. (*b*) Boxplot showing the proportion of methanogenic archaea in DNA sequence libraries from the same dung samples. Bold horizontal lines indicate medians, box limits show first and third quartiles, whiskers extend to the most extreme values within $1.5 \times$ the inter-quartile range, and dots show outlying data points. (Online version in colour.)

activity. By contrast, fluxes of nitrous oxide, another potent greenhouse gas, were influenced by beetles in an antibiotic-dependent manner (electronic supplementary material, figure S3). Specifically, the presence of beetles raised nitrous oxide emissions ($F_{1,36} = 17.31$, $p < 0.001$), in agreement with a previous report [20] (but see [40]). In support of our original hypothesis, antibiotic treatment appeared to weaken this beetle-mediated increase (interaction $F_{1,36} = 5.85$, $p = 0.02$). There was no main effect of antibiotics alone ($F_{1,36} = 1.99$, $p = 0.17$), despite clear antibiotic-induced changes to dung microbiota (figure 1*b*). It remains to be determined whether antibiotics modulate the effect of beetle presence on nitrous oxide specifically through their impact on beetle microbiota (figure 1*a*).

(e) Implications and potential mechanism of methane effect

The large (1.8-fold) increase in methane emissions from the dung of cattle treated with antibiotics (figure 3*a*) has not been documented, despite the considerable literature on methane production from agricultural systems (e.g. [17,37–39,65,66]), and the long-standing and increasing administration of antibiotics to livestock. Previous studies have found either a short-lived decrease [65], or no effect following antibiotic treatment [66,67]; to the best of our knowledge, this is the first report of antibiotics increasing methane emissions. While dung emissions of methane are typically lower than those released from belching [68], they still comprise a substantial proportion of total agricultural methanogenesis in pasture systems [40]. Moreover, as the effects of antibiotics apparently derive from microbial interactions within the gut (explained below), they probably extend to gas emissions from enteric fermentation as well. Hence, we suggest that future research should be focused on antibiotic effects on methane emissions from belching.

The pattern of methane emissions (figure 3*a*) was qualitatively similar to that of the relative abundance of methanogens in the same dung pats (figure 3*b*). As with gas fluxes, antibiotics raised the proportional abundance of methanogens ($F_{1,35} = 8.72$, $p = 0.006$), and there was no interaction of

antibiotics with beetle presence ($F_{1,35} = 0.26$, $p = 0.62$). We suggest that these patterns may be explained by competitive dynamics among gut microbiota. In the gut of ruminants and other mammals, methanogenic archaea may compete with bacteria for hydrogen, which is often scarce [69,70]. Tetracycline and some other broad-spectrum antibiotics are generally less effective against archaea, including methanogens isolated from mammalian digestive tracts [71,72]. Therefore, we propose that by specifically suppressing bacteria in the gut and subsequently in dung, antibiotic treatment enables methanogens to outcompete bacteria for hydrogen, increasing their concomitant methane output.

4. Conclusion

The routine practice of administering antibiotics to livestock [1–7] can have unexpected consequences for dung biota and greenhouse gas emissions from agriculture. First, antibiotics altered the composition of microbial communities associated with dung beetles, an ecologically important group of insects in many environments. This finding highlights a unique feature of antibacterial pharmaceuticals: even if not directly toxic to non-target animals, they may have a range of unanticipated effects by altering the microbiota of both livestock and wildlife. Second, we provide the first demonstration that antibiotics can increase dung emissions of methane, a potent greenhouse gas. Our findings call for analyses at larger scales (e.g. [40]) that take other factors into account, such as the relative importance of dung versus belching in gas emissions, and the global extent and purpose of antibiotic use in livestock production. Improved monitoring and estimates of agricultural antibiotic use will be necessary to identify whether antibiotics may impact the overall contribution of livestock production to global warming. Finally, further research into this effect will require unravelling the ecological interactions between microbes in the gut of livestock and their susceptibilities to antibiotic disturbance.

6

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7

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