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Assessing the efficacy of antibiotic treatment to produce earthworms with a suppressed microbiome.

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Abstract

Earthworms are an integral part of soil ecosystems, especially for their role in soil functions such as organic matter (OM) decomposition and nutrient cycling. Earthworms and microorganisms are interdependent, and a considerable portion of the contribution earthworms make to influencing OM fate is through interactions with microorganisms. However, the importance of the earthworm-associated microbiome is not fully understood, because it is difficult to separate the direct influence of the earthworms from the indirect influence of their microbiome. Here, we evaluated an antibiotic-based procedure to suppress the microbiome of individuals of ecologically-contrasting earthworm species (*Eisenia fetida*, *Lumbricus terrestris, Allolobophora chlorotica*) as the first step towards soil studies aimed at understanding the importance of the earthworm microbiome for host health and function. Individual earthworms were exposed to antibiotics: cycloheximide (150 μg ml^{-1}), ampicillin (100 μg ml^{-1}), ciprofloxacin (50 μg ml^{-1}), nalidixic acid (50 μg ml^{-1}), and gentamicin (50 μg ml^{-1}) either singly or in a cocktail via culture (96 h) in a semi-solid agar carrier. Compared to the non-antibiotic treated control, the cocktail (for all three species) and ciprofloxacin (for *E. fetida* and *A. chlorotica*) treatments significantly reduced ($P<0.05$) culturable microbial abundance on nutrient agar and potato dextrose agar. The microbial counts were reduced to below detection (<50 CFU $individual^{-1}$) for *E. fetida* and *A. chlorotica* receiving the cocktail. Illumina 16S rDNA amplicon sequence analysis of culturable *L. terrestris* -associated bacteria showed that antibiotic treatment influenced community composition revealing putative sensitive (*Comomonas*, *Kosakonia* and *Sphingobacterium)* and insensitive *(Aeromonas, Pseudochrobactrum)* taxa. Overall, we report a rapid, with minimal earthworm-handling, process of creating suppressed-microbiome *E. fetida*, *A. chlorotica* and *L.*

48 *terrestris* as a tool to be used in future ecological studies of earthworm microbial interactions
49 affecting host health and function.

50

51 **Keywords**: Suppressed-microbiome, axenic,16S rDNA, Earthworm-gut associated,
52 *Lumbricus terrestris*, culturable microbiome

53 **1. Introduction**

54 Earthworms are one of the most dominant soil invertebrates in terms of biomass [1,2] and are
55 frequently referred to as 'ecosystem engineers' due to their effects on soil structure and
56 nutrient availability [3]. Earthworms have been classified into three main ecological
57 categories (epigeic, endogeic and anecic groups) by Bouché (1977) [4] based on ecological
58 and morphological characteristics as well as their vertical distribution in the soil profile [4–6].
59 Epigeic species are surface dwelling, non-burrowing and consume decaying plant residues on
60 the soil surface. Anecic worms build permanent vertical burrows but feed on plant litters at
61 the surface or dragged into burrows to be pre-decomposed by microorganisms; endogeic
62 worms inhabit and feed in organo-mineral and deeper mineral horizons [2,4]. Recently,
63 Bottinelli et al. 2020 [6] applied a numerical approach to the classification of earthworms to
64 the ecological categories. This approach enabled a given species to be defined by three
65 dimensions of membership to the three main categories and allowed for species to belong to
66 supplemental intermediary categories (e.g., epi-anecic or epi-endo-anecic).

67 Earthworms are major players in determining soil organic matter (SOM) dynamics [7,8].
68 Earthworms not only stimulate organic matter (OM) decomposition, but they also promote
69 SOM stabilization within soil aggregates [9,10]. Decomposition is enhanced both by
70 increasing the access of microbial decomposers to OM substrates through mixing and
71 fragmentation of litter [9,11–14] and by stimulating the activity of the ingested soil-derived

72 earthworm gut microbiome, which accelerates the breakdown of earthworm-ingested OM
73 during gut passage. This latter is referred to as ‘the sleeping beauty paradox’ [3,15]. It
74 involves the production of intestinal C-rich mucus (‘the kiss’) by the earthworm (‘Prince
75 Charming’). This process awakens ingested dormant microflora (‘sleeping beauties’) and
76 thereby increases the decomposition of ingested organic matter because of a ‘priming’ effect
77 [15–18].

78 It has long been suggested that most earthworm species are not capable of secreting the full
79 set of enzymes that are required for the depolymerization of plant-derived polymers. Whilst
80 the possession of endogenous endocellulase genes by some earthworm species has been
81 reported [19], indicating the ability to digest cellulose, it is thought that even when
82 earthworms can produce endocellulase, their ability to digest and acquire nutrients from plant
83 litter lies fundamentally in their relationship with microorganisms [20]. This is because
84 efficient degradation of a complex polymer such as lignocellulose requires the synergistic
85 action of suites of enzymes, such as hemicellulase, endocellulase, lignin peroxidase and
86 exocellulase, that are primarily secreted by microorganisms [21]. The role of the
87 aforementioned ‘kiss’ may therefore be to stimulate microbial depolymerase production
88 during gut passage to aid acquisition of nutrients from ingested plant litter. However,
89 depolymerase activity in soil is a function of recently secreted enzymes, and those produced
90 in the past and stabilized through association with the soil matrix [22,23]. Therefore, it is not
91 clear if earthworms rely on the microbial production of enzymes during gut transit, or, if
92 already produced enzymes (before ingestion) are sufficient for complete depolymerisation. In
93 the latter case, earthworms would not depend on ingested microorganisms themselves, but
94 only on their pre-produced enzymes that were obtained through ingestion.

95 In addition to a role of an active, soil-derived, gut microbiome for host nutrition, it is possible
96 that the earthworm microbiome is also vital for other purposes. For example, many studies
97 have suggested that gut microbiomes of various hosts such as humans, *Drosophila*
98 *melanogaster* (fruit fly), *Riptortus pedetris* (bean bug) and termites, play essential roles in
99 different physiological processes. This includes immunity [24–27], reproduction [28], and
100 resistance to pesticide-induced stress [29]. The earthworm gut microbiome, and indeed the
101 microbiome associated with the other organs (such as skin and the nephridia), may confer
102 additional functions that extend beyond roles in digestion and provision of nutrients to the
103 host such as functions that affect host sexual maturity and reproduction [30,31].

104 Despite the uncertainties regarding the role of the earthworm microbiome in providing
105 nutritional and non-nutritional benefits to the host, comprehensive studies on this topic, and
106 on the role played by the earthworm host-microbiome interaction for ecosystem processes,
107 are lacking. These uncertainties are due to our inability to separate the contribution of the
108 microbiome to host processes. Whilst microorganisms associated with ingested soil and plant
109 material that are transient during gut passage might be removed via depuration of earthworm
110 individuals prior to experiments, distinct microbiome components known to be more tightly
111 host-associated (for example, with the intestinal wall; [5,32–34]) would not be removed in
112 this way. Therefore, we need a method to eliminate the non-transient microbiome to allow
113 the understanding of the contributions of the host, the microbiome (and host x microbiome
114 interactions) to functional effects.

115 Previous studies have attempted to produce suppressed-microbiome or ‘axenic’ (where
116 ‘axenic’ was used as the term to describe earthworm individuals that harbour no cultivable
117 microorganisms as detectable by the method employed) earthworm cultures through the
118 passage of individual animals via sterile solutions or suspensions containing antibiotics, both

119 single antibiotics and cocktail of antibiotics [35,36]. These studies used *Eisenia fetida* as the
120 'model' organism; presumably because it can easily be reared on a variety of organic
121 substrates [37] using standard protocols [38]. However, *E. fetida,* an epigeic species, is not a
122 typical soil dwelling earthworm species [38], preferring organic-rich habitats. Hence to
123 understand microbiome effects, there is a need to extend studies to other species of
124 earthworms occupying different niches within the soil.

125 In this present study, we developed and evaluated an antibiotic-based procedure for
126 producing suppressed-microbiome specimens of earthworms belonging to the epi- anecic (*L.*
127 *terrestris*) and epi-endo-anecic (*A. chlorotica*) groupings as well as *E. fetida* as a
128 comparatively well-studied comparison. The study, thus, provides a first step towards
129 understanding the importance of the earthworm microbiome for earthworm health and
130 ecological functional roles. We evaluated the effects of antifungal and anti-bacterial antibiotic
131 treatments (individually and in a cocktail) on culturable earthworm-associated colony
132 forming units (CFUs). To further understand how antibiotic exposure influenced the *L.*
133 *terrestris*-associated culturable bacterial diversity, we used 16S rDNA amplicon sequencing.
134 This provided insights into the taxa specific changes associated with specific treatment
135 knockdowns.

136 **2. Material and methods**

137 ***2.1 Earthworm collection and culture***

138 *E. fetida* and *L. terrestris* were purchased from Worms Direct (Essex, UK). *A. chlorotica*
139 specimens were collected from the University of Reading dairy farm at Shinfield (grid
140 reference 51.408580, -0.927353) by hand sorting for adult *A. chlorotica*, identified using the
141 guide of Sherlock [39]. Identified earthworms were washed with autoclaved de-ionised water
142 before transport back to the laboratory in a cool box. Each earthworm species was acclimated
143 to laboratory conditions in the dark at 20 ± 2 ^{o}C for two weeks [40,41] prior to the start of the

144 experiment in a culture made from Kettering loam and Irish moss peat (2:1 ratio) [42] and
145 the earthworms were fed Irish moss peat at approximately 1 g (dry matter) earthworm^{-1} week^{-1}
146 after one week of acclimation [41].

147

148 ***2.2 Antibiotic exposure***

149 The adult earthworm individuals selected for antibiotic exposure were responsive to stimuli
150 and visibly healthy. Selected individuals were of similar sizes and weights (within the same
151 species) to avoid the potential for size-specific and weight-specific effects. Following initial
152 depuration (48 h on moist filter paper in the dark), single earthworm specimens were
153 incubated in Duran bottles of either 250 ml (*E. fetida* and *A. chlorotica*) or 500 ml (*L.*
154 *terrestris*) in volume, containing either 50 ml (*E. fetida* and *A. chlorotica*) or 150 ml (*L.*
155 *terrestris*) of sterile 0.65 % (m/v) technical agar medium (Fisher Scientific, UK) made with
156 deionised water. The technical agar concentration used resulted in a medium that, as
157 determined in a preliminary experiment, was of a consistency that allowed the earthworms to
158 burrow within the agar. The agar volume ensured that there was an agar depth of at least 10
159 cm, as this was found to be a suitable depth, especially for the anecic earthworms, to form
160 vertical burrows[43]. The agar medium was supplemented with antibiotics (Sigma-Aldrich)
161 added individually or as a cocktail of the five antibiotics in the concentrations shown in Table
162 1. The concentration of each antibiotic in the cocktail was the same as the concentration used
163 when a single antibiotic was applied. Hence when combined this treatment provides both a
164 more complex and greater total antibiotic exposure treatment. The anti-bacterial antibiotics
165 belonged to the classes beta-lactam (ampicillin), (fluro)quinolone (ciprofloxacin, nalidixic
166 acid) and aminoglycoside (gentamicin) and were chosen considering: (i) their reported
167 bactericidal, as opposed to bacteriostatic, activity ([44]; to eliminate the possibility of the
168 resumption of bacterial growth following removal from antibiotic exposure): and, (ii) broad

169 spectrum of activity, targeting both Gram negative and Gram positive bacterial species
170 (ampicillin, ciprofloxacin, gentamicin; [45]; (iii) their usage in previous regimes for antibiotic
171 treatment of earthworms (Whiston and Seal [36], nalidixic acid, gentamicin). Cycloheximide
172 was chosen as the antifungal antibiotic as also based on Whiston and Seal [36].

173 Antibiotics that were not purchased as already-made solutions but in solid form were
174 dissolved in either 0.1 M hydrochloric acid (ciprofloxacin) or distilled water (nalidixic acid)
175 as required to make up stock solutions.
176 For each earthworm species, triplicate samples for each antibiotic treatment were incubated at
177 20 ± 2 °C in darkness for 96 hours following earthworm addition. Control samples with
178 technical agar but without antibiotics added were included (n = 3). The bottles were covered
179 with aluminium foil to prevent earthworm escape, with pin holes in the cover to ensure
180 aeration.
181

182 ***Table 1***

183

184 **2.3 Assessment of the abundance and diversity of earthworm-associated culturable**
185 **microorganisms**

186 ***2.3.1 Microbial abundance***

187 After 96-hours of antibiotic exposure, the earthworms were removed from the agar medium
188 with sterile tweezers. No earthworm mortality was recorded during the incubation period and
189 all earthworms had burrowed and were responsive to a physical stimulus. Following removal
190 from the antibiotic medium, earthworms were washed with autoclaved de-ionised water and
191 placed in 10 ml sterile centrifuge tubes. Earthworms were euthanised when placed in a 4°C
192 cold room for 1 hr and then crushed using sterile glass rods. One ml of autoclaved de-ionised
193 water was added to the tube, followed by vigorous shaking (250-rev min^{-1} for 2 min). The

194 resulting suspension was serially diluted in triplicate with autoclaved de-ionised water in a
195 ten-fold dilution series (10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8}). To determine the
196 number of colony-forming units (CFUs) of culturable earthworm-associated microorganisms,
197 20 µl (*E. fetida* and *A. chlorotica*) or 200 µl (*L. terrestris*) of the dilutions were plated on to
198 agar plates following the Miles and Misra method [46] or using the spread plate method,
199 respectively. The differences in the volume plated were due to the drop/spread plating
200 method adopted. The spread plate method was used for *L. terrestris* to facilitate the extraction
201 of DNA from colonies in subsequent analysis (section 2.3.2). Nutrient agar (NA), that
202 predominantly favours bacterial growth, and potato dextrose agar (PDA), normally used for
203 culturing fungi, were the agar media used. The agar plates were incubated in the dark at 26
204 °C under oxic conditions. The emerging colonies were observed after 24 hrs and then at two
205 weeks when the colonies were counted. The limit of detection of the plate count method was
206 determined using the volume plated and the dilution factor [47].

207

208 ***2.3.2 DNA Extraction, 16S-rDNA sequencing***

209 Out of the three earthworm species studied, *L. terrestris* (as the only species that had CFUs
210 above detection limits for all antibiotic treatments and both agars) was carried forward for
211 DNA-based analysis of associated microorganisms that were cultured on plates arising from
212 the dilution spread plate estimation of microbial abundance.

213 For each antibiotic treatment, earthworm individual and agar type, colonies growing across
214 all dilutions were harvested using a sterilised cell scraper. Harvested cells from each plate
215 were initially suspended in 1 ml sterile de-ionised water in a 2 ml centrifuge tube and then the
216 different dilutions of the same replicates were pooled and stored at -20 °C prior to DNA
217 extraction.

Total genomic DNA was extracted from the samples using DNeasy Ultraclean Microbial Kit (Qiagen) according to the manufacturer's protocol. The quality and concentration of the extracted DNA sample was measured using a NanoDrop spectrophotometer (ND-2000/2000c, NanoDrop Technologies).

A ~550 bp fragment of the V3-V4 hypervariable region of the bacterial 16S-rRNA gene was amplified by PCR with 5'-CCTACGGGAGGCAGCAG-3' as the forward primer and 5'-GGACTACHVGGGTWTCTAAT-3' as the reverse primer. Each reaction was done in a 50 µl reaction using four ng of genomic DNA. Each sample was dual index barcoded following Kozich et al.[48]. The amplification thermal cycling consisted of an initial denaturing step at 95 °C for 2 minutes, followed by 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 55 °C for 15 seconds and extension at 72 °C for 40 seconds, with a final extension step at 72 °C for 10 minutes. All PCR reactions were performed using Q5® High-Fidelity DNA Polymerase (New England BioLabs, USA). Quality and verification of fragment size was performed using gel electrophoresis. Samples were normalised using a SequalPrep Normalisation Plate Kit (Thermo Fisher Scientific, UK) and subsequently pooled. The pooled samples were subsequently run on a 1.2% agarose gel and a ~550 bp fragment was gel extracted using a QIAquick Gel Extraction Kit (Qiagen, the Netherlands). The gel extracted samples were quantified using a Qubit HS DNA Kit (Thermo Fisher Scientific, USA) and diluted to 7 pM using HT buffer. The final library was the run with 10% PhiX using a MiSeq Reagent Kit v3 (600 cycles) on a MiSeq (Illumina, USA). Nucleotide sequence data have been submitted to NCBI and are available under submission number SUB9306713 as part of bioproject ID PRJNA715719.

2.4 Statistical and bioinformatics analyses

The effect of antibiotic treatment on the number of CFUs for each earthworm species (*E. fetida, A. chlorotica,* and *L. terrestris*) was assessed using one-way analysis of variance

(ANOVA) followed by post hoc Tukey comparisons, where appropriate ($P<0.05$). Normality of residuals (Anderson-Darling test) and equal variance (Levine's test) assumptions were verified, and data was square root transformed where required. All analyses for the plate count data were performed with Minitab 19.1.1.

MiSeq reads were demultiplexed using BaseSpace (Illumina, USA). Amplicon sequence variant (ASV) tables were generated using the DADA2 pipeline [49]. Briefly, in this procedure, the forward and the reverse reads were inspected for quality. The Phred quality score of the reverse reads was below 30 from 200 bases onwards. This prevented sufficient merging of the forward and reversed reads, and hence only the forward reads were used in further analysis. The last ten bases of the forward reads were trimmed, and trimmed reads were subsequently filtered applying a maxN, maxEE and truncQ value of 0, 2 and 2, respectively. After sample inference, reads were subjected to chimera removal. Filtering of low-quality reads and removal of chimera led to removal of on average 18% of the forward reads per sample. Taxonomy was assigned using the Silva version 132 dataset [50].

ASVs assigned to eukaryotes, archaea, chloroplasts, and mitochondria or to an unknown phylum or kingdom were removed from the dataset.

All statistical analyses of ASVs data and data visualisations were performed in R v.3.6.3 [51]. The diversity analysis was carried out using the packages 'phyloseq' [52] and 'vegan' [53]. observed and Chao1 richness and phylogenetic diversity measures were used to estimate the alpha diversity. The normality of the dataset was checked using Shapiro-Wilk normality test and the significance of differences between alpha diversity and relative abundance of taxa was evaluated using analysis of variance (ANOVA). For the beta diversity, the principal coordinate analysis (PCoA) based on Jaccard distances using the binary data was used to visualise the similarity of individual replicates based on the presence and absence of ASVs.

The effect of antibiotic treatment on bacterial community patterns were further analysed by permutation analysis of variance (PERMANOVA) based on Jaccard distances with the Adonis function (999 permutations) of the 'vegan' package. The effect of antibiotic treatment on bacterial community patterns was also examined using ANOSIM. 'VennDiagram' was used to construct a logical visualisation of relationships between the bacterial genera present in the antibiotic treatments [54].

3. Results.

3.1 Effect of antibiotic treatment on earthworm-associated culturable microbial abundance.

The aim was to develop and evaluate an antibiotic-based procedure to eradicate earthworm-associated microorganisms and create suppressed-microbiome earthworms, as far as could be verified using culture-based methods. For the NA plates (Figure 1a, c, e), ANOVA revealed an overall significant effect of antibiotic treatments on the number of colonies forming for *E. fetida* ($P < 0.001$), *A. chlorotica* ($P < 0.001$) and *L. terrestris* ($P < 0.001$). The post hoc Tukey test showed that, when comparing the effect of the individual antibiotic treatments on earthworm-associated microbial abundance across all three earthworm species, cycloheximide and ampicillin had no significant effect on colony formation compared to the non-antibiotic-amended control. All other antibiotic treatments, however, did significantly reduce the microbial burden for at least one earthworm species. The cocktail treatment was the most effective with CFUs on NA reduced to below the limit of detection (<50 CFU/worm) for *E. fetida* and *A. chlorotica* and by more than 2 orders of magnitude for *L. terrestris.* Although the cocktail of antibiotics resulted in the lowest number CFUs, it did not result in a statistically significant different number of CFUs when compared to the

ciprofloxacin-only treatment in *E. fetida* and *A. chlorotica* (PDA) and the ciprofloxacin-, gentamicin- and nalidixic acid-only treatments for *L. terrestris*.

For the PDA plates (Figure 1 b, d, f), ANOVA indicated a significant effect of antibiotic treatment on the number of CFUs for *E. fetida* ($P < 0.001$), *A. chlorotica* ($P < 0.001$), and *L. terrestris* ($P < 0.011$). Post hoc Tukey test indicated that it was only the cocktail treatment that reduced CFUs compared to control consistently across species. CFU numbers for the cocktail were, however, not statistically different when compared to ciprofloxacin, gentamicin (all three species), and (for *E. fetida* and *L. terrestris*) nalidixic acid treatments.

Figure 1

3.2 Effect of antibiotic treatment on *L. terrestris*-associated culturable bacterial diversity

3.2.1 16S rDNA amplicon sequencing

Illumina 16S rDNA amplicon sequencing of DNA extracted from colony forming units harvested from NA and PDA dilution series plates from *L. terrestris* generated 1044826 high quality forward reads with an average of 24965 reads per sample. In total 524 ASVs were identified with an average of 31.5 ASVs per sample. Taxonomy was assigned using Silva database version 132 which resulted in the detected bacteria being classed into 10 phyla, 17 classes, 45 orders, 71 families and 143 genera.

3.2.2 Alpha diversity

The observed and estimated (Chao1) ASV richness between individual *L. terrestris* replicates had a large variation for control (e.g., for Chao1, the coefficient of variation was 82.31 % for

NA plates and 39.5% for PDA plates) and some antibiotic-amended treatments (Figure 2a-d). Against this variable background, one way ANOVA revealed that these alpha diversity measures were not significantly influenced by antibiotic treatment (P>0.05; Figure 2a-d). Similarly, there was no overall effect of antibiotic treatment on Faith's phylogenetic diversity (P>0.05; Figure 2e, f).

Figure 2

3.2.3 Beta diversity

PCoA based on Jaccard distances was used to visualise the similarity in the data from bacterial community composition for *L. terrestris* samples subjected to the different antibiotic treatments (Figure 3). For bacterial communities culturable on NA, the non-antibiotic-treated control samples overlapped with those in the ampicillin- and cycloheximide- treated samples. These clusters appeared distinct from other antibiotic treatment clusters (Figure 3; NA). The PERMANOVA analysis (P = 0.037; [Adonis]) and weakly, the ANOSIM analysis (P = 0.053) supported that the NA-culturable *L. terrestris* bacterial communities were significantly affected by the antibiotic treatments. The data from the PDA-cultured bacterial community (Figure 3: PDA), also showed that communities from control, ampicillin- and cycloheximide-treated *L. terrestris* clustered together and were separated from the clusters of bacterial communities from *L. terrestris* treated with nalidixic acid, ciprofloxacin, gentamicin, and cocktail. Both PERMANOVA ($P = 0.024$) and ANOSIM analysis ($P = 0.009$) revealed an overall significant difference between treatment groups. However, for both NA and PDA it is

notable that, with few exceptions (ampicillin and control for PDA), individual within-treatment replicates did not group closely together within the ordination space.

Figure 3

3.2.4 Cultivable shared and unique genera of *L. terrestris* individuals

Given the variability in both alpha and beta diversity at the individual *L. terrestris* level (Figure 2; Figure 3), Venn diagrams were used to visualise genera that were unique or common to more than one *L. terrestris* individual within the same treatment, with a focus on the non-antibiotic-treated control [to understand the initial variability in the culturable *L. terrestris* microbiome (Figure 4a, b)] and the cocktail-treated (Figure 4c, d) *L. terrestris* individuals [as the treatment that most significantly impacted culturable *L. terrestris*-associated bacterial abundance (Figure 1e, f)]. For nutrient agar, one genera (*Lelliottia*), was consistently detected across control *L. terrestris* individuals (Figure 4a). Whilst *Lelliottia* could still be detected in 2 out of 3 cocktail-treated individuals (Figure 4c), two other genera, *Aeromonas* and *Pseudochrobactrum*, were core in cocktail-treated individuals (Figure 4c). Whilst *Aeromonas* was present in the microbiome of two of the NA controls (Figure 4a) (and in all individuals for both control and cocktail treatments for PDA, Figure 4b, d), *Pseudochrobactrum* was not present in any other situation. In addition to *Aeromonas*, 7 other genera were core to control *L. terrestris* individuals on PDA (Figure 4b). Out of these, *Pseudomonas*, *Raoultella* and *Verminephrobacter* were still detected in two of the individuals treated with the antibiotic cocktail (Figure 4d). However, *Comomonas*, *Kosakonia*, *Pedobacter* and *Sphingobacterium* could not be detected in cocktail PDA plates (Figure 4d), and, except for *Pedobacter*, were similarly not present in the cocktail treatment for NA plates when they were detected in at least one NA control individual (Figure 4a, c).

Figure 4

4. Discussion

Earthworms are key soil organisms contributing to ecosystems processes and associated services [55]. It is recognised that earthworms harbour an abundant and diverse microbiome [56]. However, there are considerable uncertainties regarding the role of the earthworm microbiome in providing nutritional and non-nutritional benefits to the host and the consequences of the earthworm host-microbiome interaction for ecosystem processes such as OM decomposition. In this study we investigated the potential of antibiotics to create suppressed-microbiome earthworms for subsequent use in experiments aiming to improve our understanding of the role that the earthworm microbiome plays in host health and function. Previous studies have been carried out to produce 'axenic' *Eisenia fetida* [35,36]. However, the applicability of this method to species that can be considered more typical soil inhabitants was uncertain. Accordingly, here we extend the previous studies to consider species representing different earthworm ecotypes covering epi-anecic (*L. terrestris*) and epi-endo-anecic ('intermediate'; *A. chlorotica*) ecological groups (according to Bottinelli et al.'s [6] re-classification).

As well as examining the impact of the various antibiotic treatments on the earthworm-associated microbial abundance, we additionally report on the diversity (richness) and composition of the culturable microbiome of *L. terrestris* and its response to antibiotic treatment.

Overall, we have shown that it is possible to significantly reduce the abundance of earthworm-associated culturable microorganisms through the treatment of earthworm individuals with antibiotics or antibiotic cocktail. Our approach is suitable for use in *E. fetida*

386 and the soil dwelling species *L. terrestris* and *A. chlorotica*. However, the efficacy of
387 antibiotic treatment depended upon the antibiotic(s) used and the earthworm species.
388 Evaluation of the efficacy of antibiotic treatment also depended on the agar medium used for
389 microbial enumeration. In relation to the agar medium, we noted that colonies forming on
390 PDA, like those for NA, were small and smooth resembling bacterial growth. Although PDA
391 is associated with the cultivation of fungi (not bacteria), the composition of the medium
392 (potato extract, glucose) does not select against bacterial growth. It contains glucose as a
393 readily utilised C source. Given this colony appearance and also the observation that CFU
394 abundance on PDA was not affected by the antifungal cycloheximide treatment (Figure 1),
395 we assumed that colonies forming on PDA were bacterial.
396
397 Only the cocktail of five antibiotics (ampicillin, ciprofloxacin, cycloheximide, gentamicin
398 and nalidixic acid) resulted in culturable numbers significantly lower than the control for both
399 NA and PDA agar across all earthworm species (Figure 1), whilst ampicillin and
400 cycloheximide mostly showed no significant differences when compared to the control.
401 Resistance to ampicillin, a beta-lactam antibiotic, is known to be naturally prevalent among
402 soil bacteria [57,58], and cycloheximide, an antifungal, is expected not to be effective on
403 most bacteria [59].
404
405 It was possible, however, through the treatment of *E. fetida* (NA) and *A. chlorotica* with the
406 antibiotic cocktail to reduce the abundance of earthworm-associated microorganisms from ≥
407 10^5 CFU per earthworm individual to below the limit of detection (50 CFU/ earthworm in our
408 study). This agrees with previous studies [35,36] that have also applied antibiotics to create
409 earthworms (*E. fetida*) deemed 'axenic' with no associated microorganisms detectable by
410 culture.

411

412 Whilst the application of the antibiotic cocktail [and ciprofloxacin applied singly for *E. fetida*
413 (PDA)] reduced culturable microbial abundance to below detection in *E. fetida* and *A.*
414 *chlorotica*, microbial numbers were not reduced to below detection limits for *L. terrestris*,
415 although a significant >100-fold knockdown was recorded in this species for the cocktail. To
416 be exposed to antibiotics, through both dermal and gut contact, earthworm individuals needed
417 to burrow and ingest agar. Differences in burrowing behaviour between species may
418 influence the degree to which earthworms are exposed to antibiotics, and therefore the
419 effectiveness of the antibiotic treatment. Also, there may also be different exposure levels in
420 different bacterial populations. Bacteria in the gut are likely to receive a high dose, whereas
421 the nephridial symbionts may be more 'protected' against antibiotics due to their embedment
422 in an organ that may be more 'sealed' from antibiotics. *L. terrestris*'s natural behaviour is to
423 create permanent vertical burrows, travelling to the surface to feed on partially decomposed
424 plant litters and other organic matters [60,61]. Although we scaled up agar volumes to
425 accommodate the larger *L. terrestris* size and burrowing behaviour, it is possible that *L.*
426 *terrestris* individuals did not explore and ingest the antibiotic-containing agar to the same
427 extent, resulting in reduced exposure. In this case, increasing the concentration of antibiotics
428 in the agar or the time of exposure might have improved the effectiveness of the antibiotic
429 treatment. Alternatively, the *L. terrestris* microbiome may harbour a larger number of
430 culturable antibiotic-resistant microorganisms [62,63]. Earthworms are known to produce
431 their own antimicrobial agents [62] which might lead to earthworm species-specific selection
432 of antibiotic resistance traits within the microbiome.

433

434 Although based on methods of Hand and Hayes [35] and Whiston and Seal [36], our
435 approach differed from previously published work in terms of the spectrum of antibiotics

436 applied. Nalidixic acid, gentamicin, a penicillin (ampicillin) and cycloheximide [36] or
437 cycloheximide [35] were in common with the previous studies, but, additionally
438 ciprofloxacin (a fluoroquinolone) was included as an antimicrobial not tested previously. In
439 most cases ciprofloxacin, when applied alone, was just as effective in reducing culturable
440 numbers as the cocktail treatment. This effectiveness may be related to its particularly broad-
441 spectrum DNA gyrase inhibitory activity against both Gram-negative and Gram-positive
442 bacteria [64].
443
444 As well as differences in the choice of antibiotics used, our method also varied from
445 previously published work in terms of the methodology and duration of antibiotic exposure.
446 We used sterile semi-solid technical agar as the 'carrier' for antibiotic exposure. In contrast,
447 previous studies used aqueous solutions [35] or sterile suspensions of microcrystalline
448 cellulose [36]. Our exposure period (4 days) was comparable to that employed by Whiston
449 and Seal [36] (5 days), but shorter than the 35 days adopted by Hand and Hayes [35] and
450 consisted of a single exposure step as opposed to one [36] or several [35] transfers of
451 earthworm individuals between different antibiotic-containing media. Reducing the timescale
452 of exposure and the degree of earthworm handling reduces the risk of earthworm mortality.
453 In our trial, all earthworm specimens survived after the exposure to the antibiotic when using
454 response to touch stimuli as a superficial measurement of health condition. The lack of
455 mortality indicates low acute stress (but chronic impacts may have occurred undetected) and
456 provides viable earthworm individuals for use in future experiments.
457
458 For *L. terrestris*, 16S rDNA amplicon sequencing of the NA- and PDA- grown bacterial
459 communities was applied to characterise the richness and composition of the culturable
460 microbiome of *L. terrestris* and its response to antibiotic treatment. For reasons previously

461 discussed, PDA-grown colonies were assumed to be bacterial and were included in the 16S
462 rDNA-based sequencing effort. This enabled the characterisation of potentially different, agar
463 specific, microbiomes due to the selective nature of bacterial growth [65].
464
465 Whilst cognisant that the bacteria that can be cultured on laboratory media are only a very
466 small proportion of the total diversity and therefore that we have not captured what might be
467 a significant non-culturable fraction [66], we focussed on culturable microbiomes (i.e.,
468 amplicon sequencing from colony-extracted DNA). This was because we were concerned that
469 amplification of bacterial DNA directly extracted from earthworm tissues would not be able
470 to distinguish between DNA from living bacterial cells surviving the biocidal treatments and
471 those that had been recently killed [67]. Amplification of DNA from dead microorganisms
472 would undermine the identification of bacterial taxa that escaped the effect of the antibiotic
473 treatment. Since this culture-based approach will mean that the relative read abundance of a
474 given ASV in a sample will depend not only on the original cell abundance in the earthworm
475 sample but also confounded by the subsequent rate of multiplication on agar, the subsequent
476 analysis of diversity and taxonomic composition was based on presence/absence, not relative
477 abundance.
478
479 Comparison of estimated Chao1 ASV richness and observed richness suggested that the
480 sequencing depth covered the richness of ASVs present. However, there was substantial
481 within-treatment variation in ASV richness, including for the non-antibiotic-treated controls.
482 Due to the destructive nature of sampling, it was not possible to examine the impact of
483 antibiotic treatment on the microbiome for a given earthworm individual (i.e., before and
484 after treatment). That there was no significant effect of antibiotic treatment on either ASV
485 richness (observed and Chao1) or phylogenetic richness, even for antibiotic treatments that

significantly reduced the number of culturable bacteria (Cocktail (NA & PDA) and ciprofloxacin (NA); Figure 1), might be partly due to initial variability in bacterial richness between *L. terrestris* individuals (Figure 1) going into the incubation. This variability is in agreement with other reports of high variability in host -associated microbiomes [68–70]. When compared to other studies on earthworm-associated bacterial richness [69–71], our analysis revealed a low number of ASVs per worm (e.g., ~30 ASVs for the NA control). However, this is expected due to the focus on only those bacteria that formed colonies on the NA and PDA medium. In addition, the *L. terrestris* individuals in the current trial were depurated before the plating of earthworm samples. This means that the culturable microbiome in our study was likely not dominated by diverse transient microbes associated with the ingested loam: peat substrate but those more tightly-associated with the gut and other organs [70].

Whilst there was no significant impact on the richness of ASVs, PERMANOVA and ANOSIM analysis suggested an impact of antibiotic treatment on community composition. The PCoA (Figure 3) highlighted the variability between within-treatment replicates but suggested that the bacterial community compositions for the antibiotic treatments (cocktail, ciprofloxacin) that caused the most significant reduction in culturable abundance (Figure 1) were among the most dissimilar to the control.

Genera common to more than one *L. terrestris* individual within the same treatment were visualized by Venn diagrams (Figure 4) to identify core members of the culturable microbiome and those genera sensitive or tolerant to antibiotic treatment. The core bacterial diversity (phyla level) of the *L. terrestris* culturable microbiome composed of members of the *Proteobacteria* (*Aeromonas, Comomonas, Kosakonia, Lelliottia, Pseudomonas, Raoultella,*

511 *Verminephrobacter spp.*) and *Bacteroidetes* (*Pedobacter, Sphingobacterium spp.*). This
512 composition is in broad agreement with the earthworm-associated phyla described in other
513 earthworm microbiome studies [72–74]. In particular, members of the genus
514 *Verminephrobacter* are known symbionts found in Lumbricid earthworms and have a known
515 nephridial association [31,75,76]. *Aeromonas*, a genus consisting of facultative anaerobic
516 species, are a further taxa that are frequently earthworm- associated including with *L.*
517 *terrestris* [77,78].
518
519 Among taxa indicating potential resistance, the near ubiquitous detection of *Aeromonas* in the
520 culturable microbiome of both control and antibiotic cocktail treated individuals suggests that
521 representatives of this genus were resistant to antibiotic treatment. *Aeromonas* are considered
522 to be naturally resistant to β-lactam antibiotics, such as ampicillin [79,80] and resistance to
523 ciprofloxacin and nalidixic acid has also been reported for environmental strains, including
524 multi-antibiotic resistance [80]. In contrast, resistance of this genera to gentamicin appears to
525 be rare [80,81]. Further characterization of the antibiotic resistance profile of our *Aeromonas*
526 isolates would be required to discern if these strains were indeed gentamicin resistant as may
527 be suggested by their presence in the cocktail exposure or, alternatively, evaded exposure.
528 *Aeromonas hydrophila* has been isolated from the coelomic cavity of *L. terrestris* [82]. If
529 *Aeromonas* were in this compartment, their exposure may be more limited than for bacteria in
530 the gut. The organ-specific location of *Verminephrobacter* may similarly result in a lower
531 exposure for members of this genus.
532
533 In contrast to the apparent tolerance of *Aeromonas* species to the antibiotic exposure, bacteria
534 belonging to the genera *Comomonas*, *Kosakonia* and *Sphingobacterium* that were part of the
535 core in control *L. terrestris* were not detected in cocktail-treated individuals. This absence

suggests a possible antibiotic sensitivity of these strains. No antibiotic resistance genes have been annotated in environmental isolates of *Comamonas* [83] and we could not find reports of resistance traits in environmental *Kosakonia* and *Sphingobacterium* strains. The genus *Pseudochrobactrum*, however, was not detected in control individuals but was present in all cocktail-treated individuals (NA) suggesting that antibiotic treatment potentially promoted the growth of this putatively multi-antibiotic resistant group to densities above the limit of detection of the spread plate. We could not find any previous descriptions of the resistance of *Pseudochrobactrum* to the antibiotics used here. Further characterization is required to verify the antibiotic resistance profile of this group and to explore the earthworm as a bacterial environment conducive to acquisition of antibiotic resistance genes, particularly under the pressure of antibiotic selection [84].

4.1 Conclusion

We have shown that is it possible, across three ecologically-contrasting earthworm species (*E. fetida*, *L. terrestris, A. chlorotica*), to significantly reduce the abundance of earthworm-associated culturable microorganisms through a 96 h exposure of earthworm individuals to a cocktail of antibiotics containing cycloheximide (150 μg ml^{-1}), ampicillin (100 μg ml^{-1}), ciprofloxacin (50 μg ml^{-1}), nalidixic acid (50 μg ml^{-1}), and gentamicin (50 μg ml^{-1})) in a semi-solid agar carrier. Abundance was reduced to below detection limits (50 CFU $individual^{-1}$) in *E. fetida* and *A. chlorotica* and by >100-fold for *L. terrestris* with accompanying shifts in *L. terrestris* bacterial community composition. The culturable bacterial microbiome of control (non-exposed) and antibiotic cocktail-exposed *L. terrestris* individuals revealed between-individual variability in richness and diversity but also 'core' genera that were putatively sensitive (*Comomonas*, *Kosakonia* and *Sphingobacterium)* or resisted *(Aeromonas, Pseudochrobactrum)* antibiotic exposure. This characterization of the efficacy of antibiotic

561 treatment in suppressing the microbiome of *E. fetida*, *A. chlorotica* and *L. terrestris*
562 individuals provides the foundation for future experiments aimed at understanding the
563 importance of earthworm-associated microorganisms, be they transient gut inhabitants or
564 more permanently-associated, for host health and ecosystem functioning.
565
566 **5. References**

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Figures 1-4

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Table 1. Antibiotic types and concentrations used to amend agar media for the production of suppressed-microbiome earthworms

Antibiotic	**Antibiotic concentration (µg ml^{-1} agar)**
Cycloheximide	150[a]
Ampicillin	100
Ciprofloxacin	50
Nalidixic acid	50 [a]
Gentamicin	50 [a]

[a] Antibiotic concentration used in Whiston and Seal (1988)

Conflict of Interest

Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

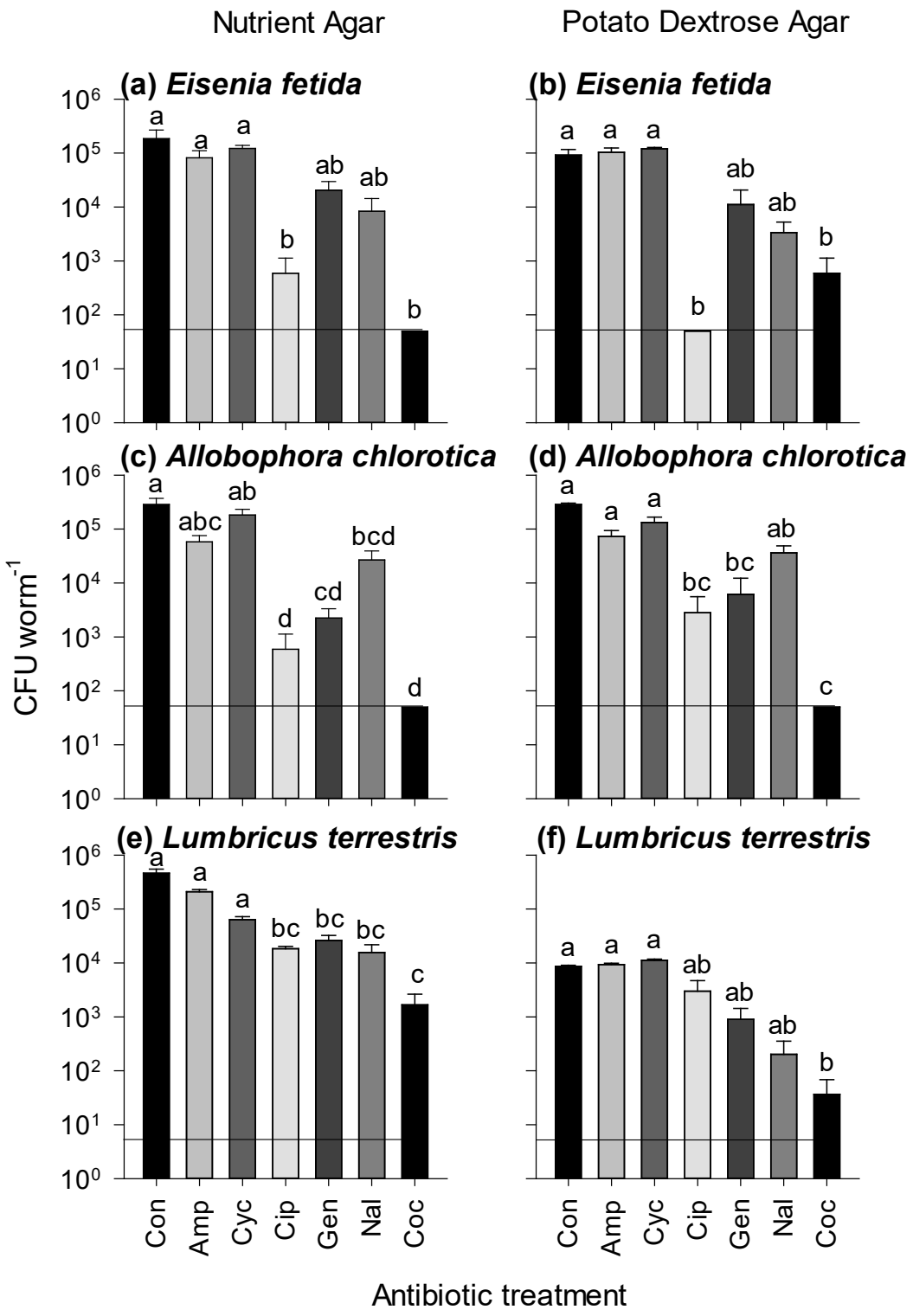


Figure 1. The effect of antibiotic treatment on culturable microbial abundance (Colony Forming Units; CFU) associated with: (a,b) *Eisenia fetida*; (c,d) *Allolobophora chlorotica* ; and, (e,f) *Lumbricus. terrestris* on nutrient agar and potato dextrose agar plates. Con = control; Amp = ampicillin; Cyc = cycloheximide; Cip = ciprofloxacin; Gen = gentamicin; Nal = nalidixic acid; Coc = Cocktail. Values are means ± SE (n = 3). Different letters indicate significant differences between antibiotic treatments applied to the same agar media and the same earthworm species (Tukey HSD test; $\alpha = 0.05$). The horizontal line represents the limit of detection for the method of 50 CFU worm^{-1} (*E. fetida* and *A. chlorotica*) or 5 CFU worm^{-1} (*L. terrestris*).

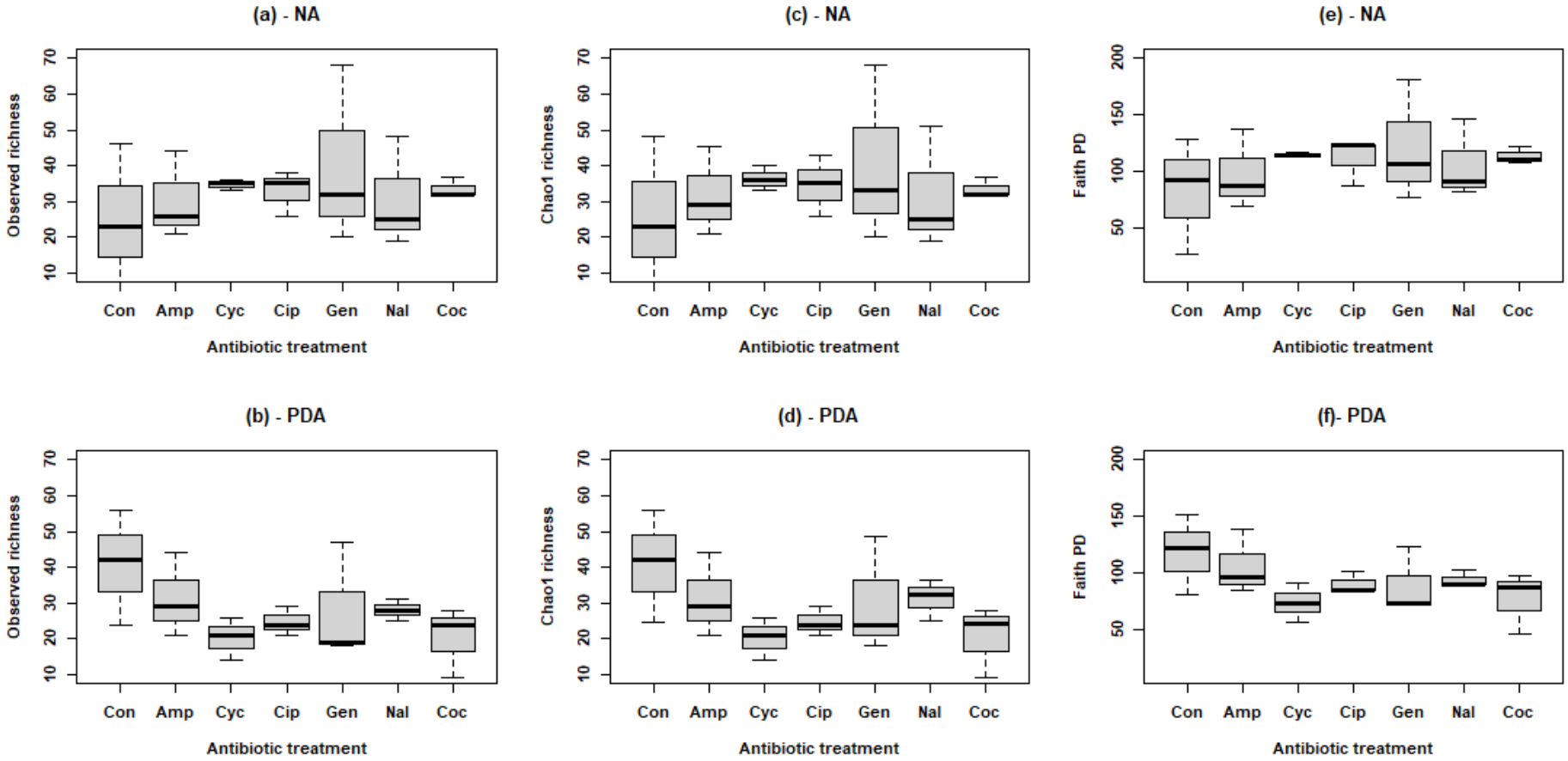
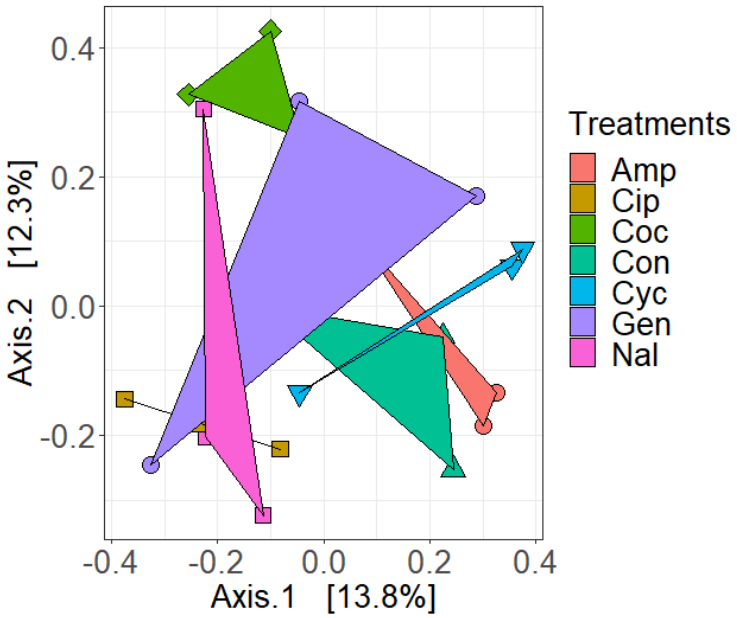


Figure 2. Box plots of Chao1 estimated (a, b) and observed (c, d) Amplicon Sequence Variant (ASV) richness and Faith's Phylogenetic Diversity (e, f) for *L. terrestris*-associated culturable bacterial communities for control and antibiotic-treated earthworm individuals (n=3) as cultured on nutrient agar (NA; a, c, e) and potato dextrose agar (PDA; b, d, f). Con = control; Amp = ampicillin; Cyc = cycloheximide; Cip = ciprofloxacin; Gen = gentamicin; Nal = nalidixic acid; Coc = Cocktail.

NA



PDA

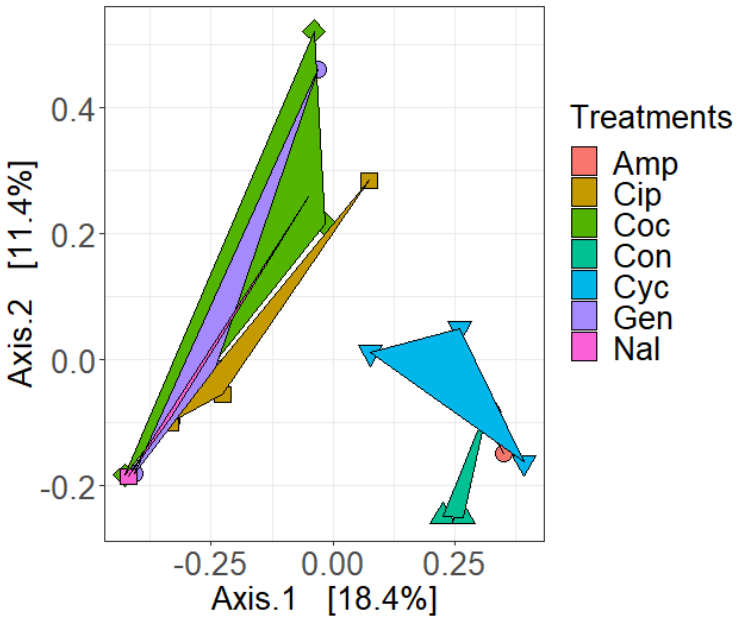


Figure 3. Principal coordinate analysis ordination based on Jaccard distances examining the similarity of composition of culturable bacterial communities for control and antibiotic-treated *L. terrestris* individuals (n=3) as determined by 16S rDNA amplicon sequencing of colonies cultured on NA and PDA. Con = control; Amp = ampicillin; Cyc = cycloheximide; Cip = ciprofloxacin; Gen = gentamicin; Nal = nalidixic acid; Coc = Cocktail.

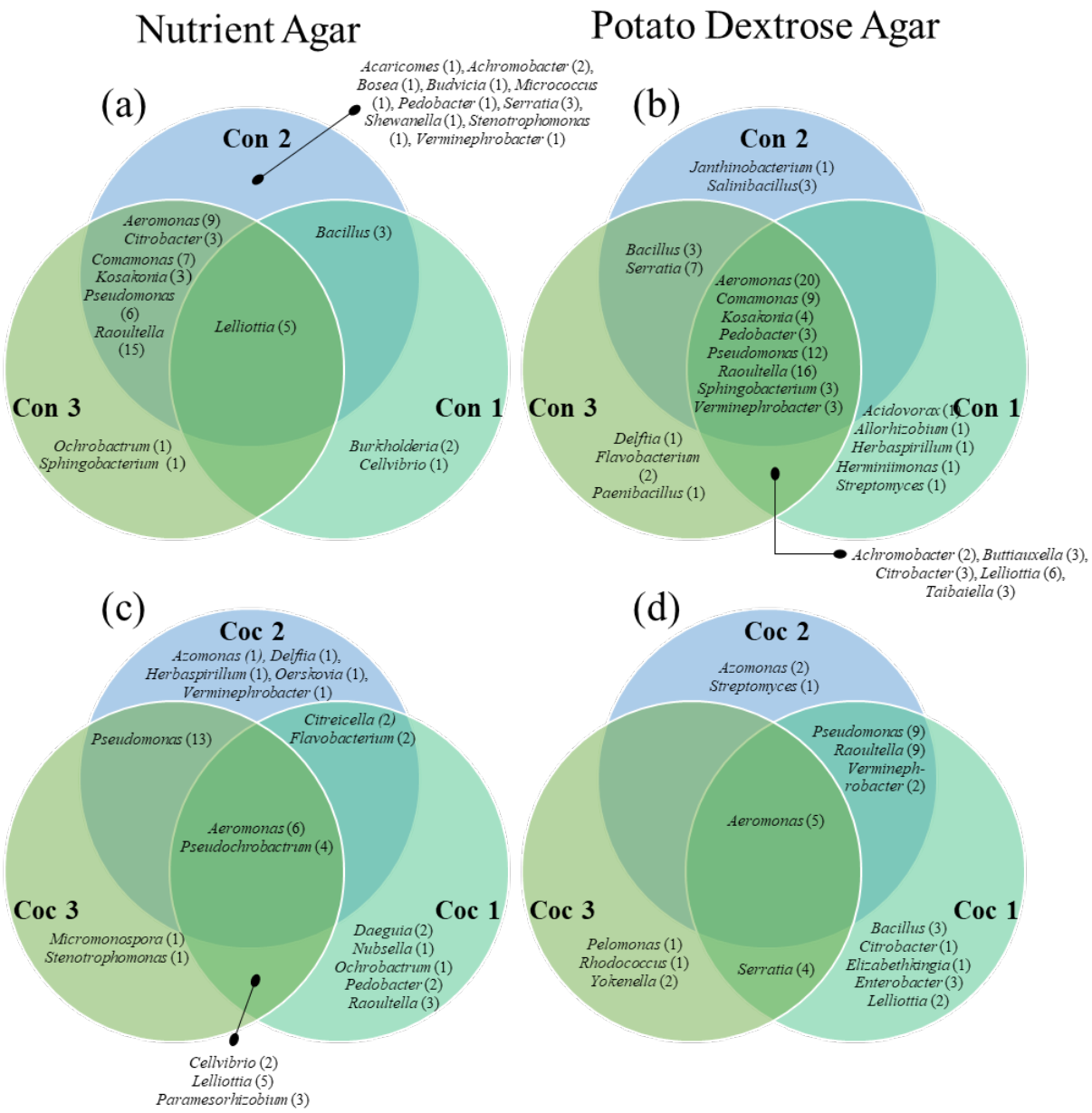


Figure 4. Venn diagram visualisation of genera that are unique or common to more than one *L. terrestris* replicate individual within the control (a, b; replicates Con 1, Con 2, Con 3) and cocktail (c, d; replicates Coc 1, Coc 2, Coc 3) treatments on nutrient agar (NA; a, c) and potato dextrose agar (PDA; b, d). The numbers in the brackets are the number of ASV representatives within each genera