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1 **Identification of diverse antibiotic resistant bacteria in agricultural soil with $H_2^{18}O$ stable isotope**
2 **probing and metagenomics**
3
4 Marcela Hernández[a,b], Shamik Roy[b], C. William Keevil[a], Marc G. Dumont[a*]
5
6 [a] School of Biological Sciences, University of Southampton, Southampton SO17 1BJ, UK
7 [b] School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, UK
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10 *Running title: Multi-drug resistant bacteria in agricultural soil*
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17 *Corresponding author:
18 Marc G. Dumont, University of Southampton, Life Sciences Building 85, Highfield Campus,
19 Southampton SO17 1BJ, UK, m.g.dumont@soton.ac.uk
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27 **Abstract**

28 **Background:** In this study, we aimed to identify bacteria able to grow in the presence of several
29 antibiotics including the ultra-broad-spectrum antibiotic meropenem in a British agricultural soil, by
30 combining DNA stable isotope probing (SIP) with high throughput sequencing. Soil was incubated
31 with cefotaxime, meropenem, ciprofloxacin and trimethoprim in ^{18}O-water. Metagenomes and the
32 V4 region of the 16S rRNA gene from the labelled "heavy" and the unlabelled "light" SIP fractions
33 were sequenced.

34 **Results:** After incubations, an increase of the 16S rRNA copy numbers in the "heavy" fractions of the
35 treatments with ^{18}O-water compared with their controls was detected. The treatments resulted in
36 differences in the community composition of bacteria. Members of the phyla Acidobacteriota
37 (formally Acidobacteria) are highly abundant after two days of incubation with antibiotics. Several
38 Pseudomonadota (formally Proteobacteria) including *Stenotrophomonas* were prominent after four
39 days of incubation. Furthermore, a metagenome-assembled genome (MAG-1) from the genus
40 *Stenotrophomonas* (90.7% complete) was retrieved from the heavy fraction. Finally, 11 antimicrobial
41 resistance genes (ARGs) were identified in the unbinned-assembled heavy fractions, and 10 ARGs
42 were identified in MAG-1. On the other hand, only two ARGs from the unbinned-assembled light
43 fractions were identified.

44 **Conclusions:** The results indicate that both non-pathogenic soil- dwelling bacteria as well as
45 potential clinical pathogens are present in this agricultural soil, and several ARGs were identified
46 from the labelled communities, but it is still unclear if horizontal gene transfer between these groups
47 can occur.

48

49 **Keywords:** antimicrobial resistant bacteria, soil, antibiotics, pathogens, DNA stable isotope probing,
50 high-throughput sequencing, metagenomics.

51

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53 **Introduction**

54 Soil represents a natural reservoir of antimicrobial resistance genes (ARG) that has originated as a
55 defence mechanism for the microbes to deter antimicrobial products secreted by competing
56 microbes in the same niche. However, the abuse and/or misuse of antibiotics among humans to
57 treat diseases, and livestock production systems to increase yield has initiated the alteration of
58 natural antimicrobial resistance (AMR) and subsequent spread across all terrestrial ecosystems [1].
59 Indeed, no soil environment can be now considered pristine as the ARGs are present in garden soils
60 [2], agricultural soils [3], forests [4], grasslands [5], and even Antarctic soils [6]. So much so that soil
61 can harbour up to 32% of the overall ARG diversity [7]. In addition, a previous study has reported the
62 importance of low abundance antibiotic-resistant microbes in soil-plant systems for the spread of
63 AMR [8].

64 Transmission of AMR back to humans through soil-microbe-animal-plant nexus endangers
65 public health, since the spread of AMR could push us to the pre-antibiotic era. We now know what
66 can happen to AMR in soils due to rise in antibiotic diversity and abundance in the environment but
67 how AMR will spread in soil remains to be seen. In other words, the drivers, or mechanisms of the
68 inevitable spread of AMR in soils when challenged with antibiotics remains to be determined.
69 Deciphering this knowledge gap is crucial for us to develop strategies to alleviate the spread of AMR
70 in terrestrial ecosystems.

71 It has been hypothesised that the spread of AMR in soil is primarily driven by two non-
72 independent processes that can operate in tandem to alter the antibiotic resistome in soil [1,9]. One
73 process is horizontal gene transfer (HGT) of antimicrobial resistance genes (ARG) between microbial
74 community members. Secondly is the directional selection of antibiotic resistant microbes that can
75 grow in the presence of antibiotics. This could be either due to incorporation of microbiomes
76 derived from anthropogenic sources (e.g., organic fertiliser), or selection and proliferation of
77 naturally resistant microbiota. We are now beginning to understand how HGT can facilitate the
78 spread of AMR in pristine environments [6,10]. For instance, the $bla_{\text{NDM-1}}$ gene that confers

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79 resistance to carbapenem (last resort antibiotic) is now ubiquitous due to successive and distinct
80 HGT events [11,12]. On the other hand, there is limited knowledge about the community
81 composition of the microbiome that can resist antibiotic in soil. One of the main reasons could be
82 the large abundance of extracellular DNA (eDNA) in soil, which cannot distinguish active antibiotic
83 resistant microbes from dead/dormant antibiotic sensitive microbes [13,14]. This could be the
84 reason why studies have reported contradictory results of no change in microbiomes to complete
85 change upon antibiotic addition [5,15].

86 Agricultural ecosystems represent 38% of the Earth's ice-free terrestrial surface — the
87 largest use of land on the planet [16]. Sustainable agricultural practice envisions the widespread
88 adoption of organic fertilisers instead of chemical fertilisers as a source of nutrients to maintain or
89 increase crop yield [17]. This is essential to achieve climate-change goals and concomitantly meet
90 the dietary demands of 9 billion people by 2050. However, the build-up of antibiotic concentrations
91 and ARG abundance in environmentally sustainable organic fertilisers, such as livestock manure and
92 sewage sludge, permeates agricultural soils to spread AMR by altering the microbiome [1,9,13,18].
93 Since AMR microbes are one of the major determinants of AMR spread, it is therefore crucial for us
94 to identify the active fraction of the soil microbial community that can grow in the presence of
95 antibiotics.

96 Stable isotope probing (SIP) with [^{18}O]-water presents a unique approach to identify the
97 active AMR microbes [19,20]. SIP is a cultivation-independent approach that requires the addition of
98 stable-isotope-enriched substrates (e.g., ^{13}C-methane, ^{18}O-water) to environmental samples
99 followed by analyses of labelled DNA or RNA [20]. SIP techniques can target phylogenetically
100 constrained metabolic processes (e.g., ethane oxidation) where from a diverse pool of active
101 microbial community only those microbial guilds that can assimilate and subsequently incorporate
102 the labelled substrate into their biomolecules such as DNA and RNA are identified. In contrast, SIP-
103 $H_2^{18}O$ as a substrate can potentially label all metabolically active or growing microbes since water is
104 a prerequisite for growth and cellular maintenance [19,21]. Here, fast growing microbes are labelled

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105 first, but eventually all active microbes are expected to contain isotope-enriched DNA. Additionally,
106 ^{18}O has two more neutrons than naturally abundant ^{16}O, whereas ^{2}H, ^{13}C and ^{15}N has only one
107 additional neutron compared to their naturally abundant counterparts (^{1}H, ^{12}C and ^{14}N). This can
108 potentially increase the degree of physical separation of labelled ^{18}O-DNA from unlabelled DNA
109 during isopycnic centrifugation in SIP. As a result, the SIP-$H_2{}^{18}O$ has been used as a robust method to
110 identify the active microbes in a multitude of treatment set-ups such as nutrient addition to soil [22],
111 soil rewetting [21,23], and soil warming [24].
112 In this study we combine antibiotic selection and SIP-$H_2{}^{18}O$ to elucidate active and growing
113 microbial communities in an agricultural soil. Antibiotic selection or continuous presence of
114 antibiotics in the experiment will select only AMR microbes to grow, and simultaneously kill or
115 inhibit the growth of sensitive microbes. We also use agricultural soil with no history of antibiotic
116 addition either directly, or indirectly via organic fertilisers. This was done to reduce the bias in
117 identification that can be introduced from long-term exposure of microbes to anthropogenic derived
118 antibiotics as it may already have selected for a resistant microbial community with no difference
119 between the antibiotic challenged and unchallenged communities. Our objectives for this study
120 were to investigate whether microbes in agricultural soil with no-antibiotic history can grow if
121 challenged with antibiotic; secondly, if they are present to identify the microbes and the metabolic
122 machinery and/or AMR genes that confer resistance. We hypothesise that the addition of antibiotics
123 to agricultural soils in the presence of $H_2{}^{18}O$ will unravel the identity of antibiotic resistant microbes
124 and this will help to understand the drivers of AMR spread.
125
126 **Results**
127 To evaluate whether microbes in an agricultural soil with no-antibiotic history can grow if challenged
128 with antibiotic, agricultural soils were incubated with an antibiotic cocktail of meropenem (mem),
129 cefotaxime (ctx), ciprofloxacin (cip) and trimethoprim (tmp), along with $H_2{}^{18}O$ or natural isotope
130 abundance water (referred to as $H_2{}^{16}O$). Here we report the results after 4-days of incubation with

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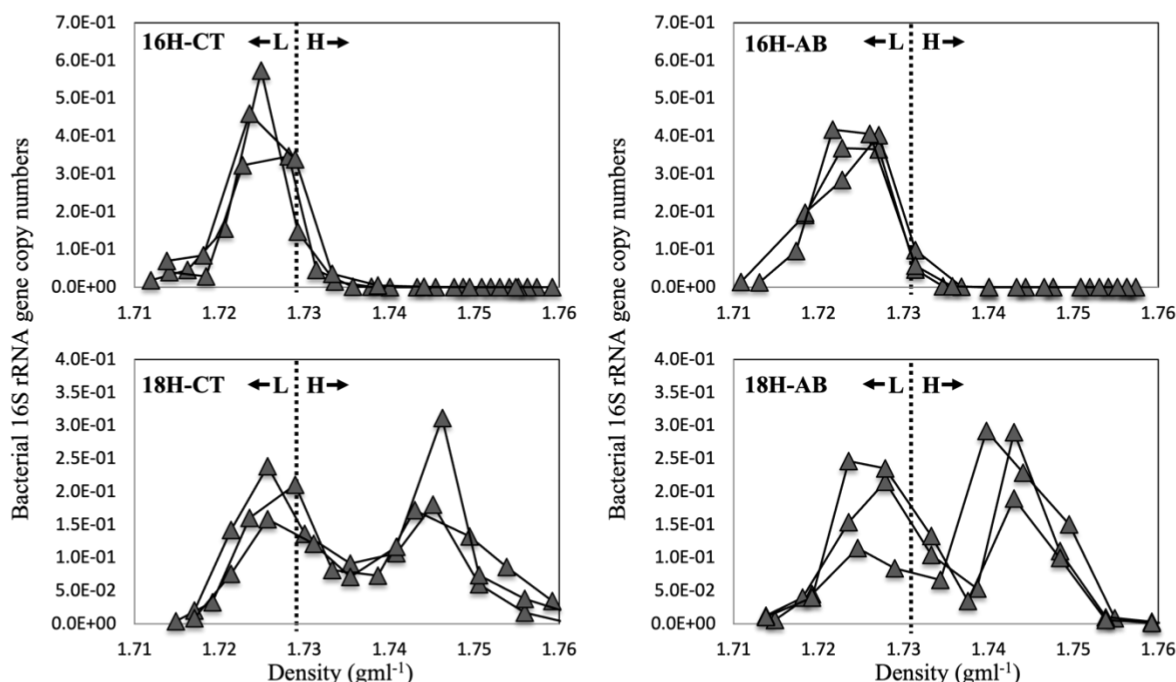
131 antibiotic addition at 0 and 48 hr time-points. A total of 18 CsCl gradient fractions were collected
132 following ultracentrifugation and the 16S rRNA gene copy numbers (bacterial abundance) were
133 analysed for each experimental setup.

134 Incubation with $H_2^{18}O$ increased the overall buoyant density of the extracted DNA as
135 compared to the $H_2^{16}O$ controls (Figure 1). ^{18}O-labelled DNA (heavy fractions) resided in fractions
136 with densities 1.73 g ml^{-1} and above, whereas unlabelled DNA (light fractions) resided in fractions
137 with densities 1.729 g ml^{-1} and lower (Figure 1). This indicates that bacteria were actively
138 incorporating ^{18}O into their DNA. Here, the heavy fraction indicates active or growing microbes,
139 whereas the light fraction indicates dormant or dead microbes.

140 After 4-days of incubation with $H_2^{18}O$ there was a large abundance of bacterial 16S rRNA
141 gene copies in the heavy fraction as compared to the heavy fraction of samples incubated with $H_2^{16}O$
142 (Figure 1). This was the case for both the antibiotic treatment and no-antibiotic controls suggesting
143 there was substantial bacterial growth in the presence of antibiotics.



144
145
146 **Figure 1.** Abundance of bacterial 16S rRNA genes in CsCl density gradients after ^{16}O-/^{18}O-H_2O
147 incubation. Vertical dotted lines demarcate the heavy (H) fractions from light (L) fractions. Each line
148 represents a sample. 16H-CT: soils incubated in the presence of natural isotope abundance water

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149 ($H_2{}^{16}O$) without antibiotics (control); 16H-AB: incubation in the presence of $H_2{}^{16}O$ and antibiotics; 18H-
150 CT: incubation in the presence of $H_2{}^{18}O$ without antibiotics (control); 18H-AB: incubation in the
151 presence of $H_2{}^{18}O$ and antibiotics.
152
153 Species richness was significantly lower in the heavy fractions (2278 ± 142, mean ± 95% confidence
154 interval) than light fractions (5025 ± 99) for antibiotics (AB) treated soil ($p<0.001$). Similarly for CT
155 treatment (i.e., control: soil without antibiotics), the species richness was significantly lower ($p=0.013$)
156 in the heavy fractions (2926 ± 314) than in the light fractions (3980 ± 370). Shannon diversity (*H*) was
157 lower ($p<0.001$) for heavy fractions (1.99 ± 0.36) than light fractions (6.50 ± 0.37) for AB treated soil.
158 However, for CT, Shannon diversity (*H*) did not differ ($p=0.157$) between heavy fractions (4.37 ± 0.12)
159 and light fractions (5.12 ± 0.68). Evenness (*J*) indexes were lower ($p<0.001$) in heavy fractions (0.42 ±
160 0.07) than in light fractions (0.98 ± 0.01) for AB treated soil. Contrarily, for control treatments (CT),
161 evenness (*J*) did not differ ($p=0.219$) between heavy fractions (0.93 ± 0.01) and light fractions (0.93 ±
162 0.05) (Figure 2).
163 When comparing the heavy fractions of AB and CT treatments, species richness was lower
164 ($p=0.039$) for heavy fractions for AB treatments (2278 ± 142) than CT treatments (2926 ± 314).
165 Similarly, Shannon diversity was lower ($p=0.003$) for AB treatments (1.99 ± 0.36) than CT treatments
166 (4.37 ± 0.12). Evenness was also lower ($p=0.005$) for AB treatments (0.42 ± 0.07) than CT treatments
167 (0.93 ± 0.01) (Figure 2). Finally, the coefficient of variation (CV) for all alpha diversity indices across
168 all the treatments ranged from 0.4% to 16.0% (Figure 2).
169

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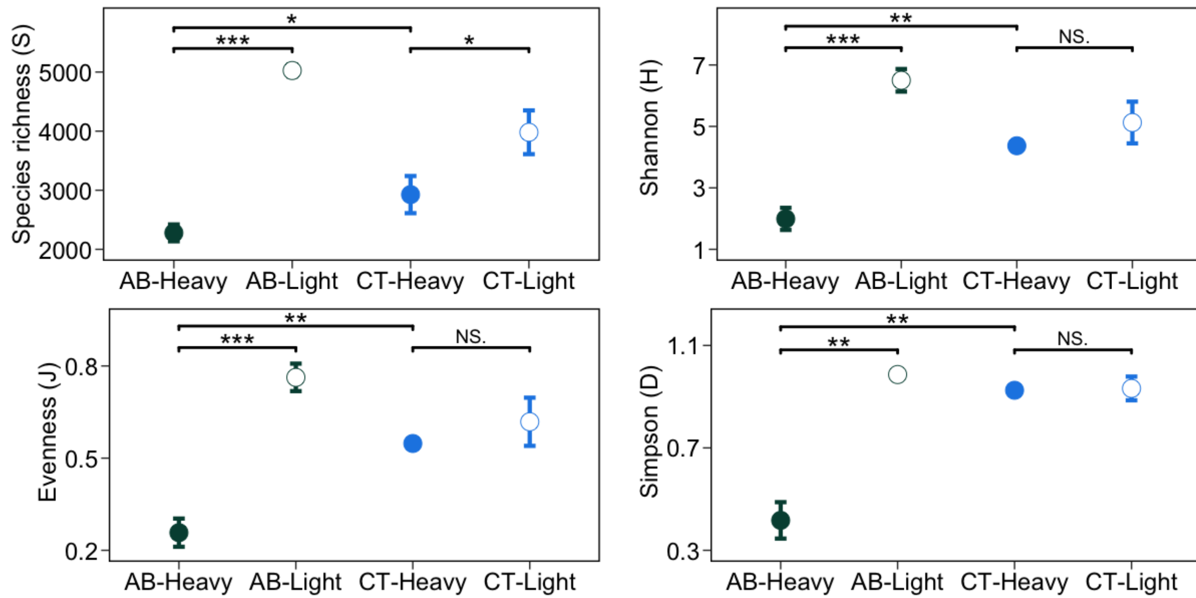

170

171 **Figure 2.** Alpha-diversity of 16S rRNA gene sequences from the “heavy” and “light” fractions of DNA

172 extracted from soils incubated with $H_2^{18}O$ in the presence (AB) or absence (CT) of antibiotics. Alpha-

173 diversity is summarised as species richness (S), Shannon diversity (H), evenness (J), and Simpson (D).

174 “*” corresponds to p-value <0.05 from pairwise t-test; “**” corresponds to p-value <0.01; “***”

175 corresponds to p-value <0.001; “NS.” corresponds to p-value >0.05

176

177 The microbial community composition was consistent for all replicates of both heavy and light

178 fractions across all the treatments. The community composition for heavy and light fractions of AB

179 and CT when incubated with $H_2^{18}O$ were different as they clustered separately (Figure 3).

180 Community composition of the light fraction in $H_2^{18}O$ incubated CT soil (18H-CT-Light) was similar to

181 both heavy (16H-CT-Heavy) and light (16H-CT-Light) fraction in $H_2^{16}O$ incubated CT soils as shown by

182 their proximity in the PCoA plot and the relative abundance profile (Figure 4, S3). Together, these

183 three fractions (18H-CT-Light, 16H-CT-Heavy, 16H-CT-Light) along with light fraction of $H_2^{16}O$

184 incubated AB soil (16H-AB-Heavy) were similar to the composition of the original soil.

185

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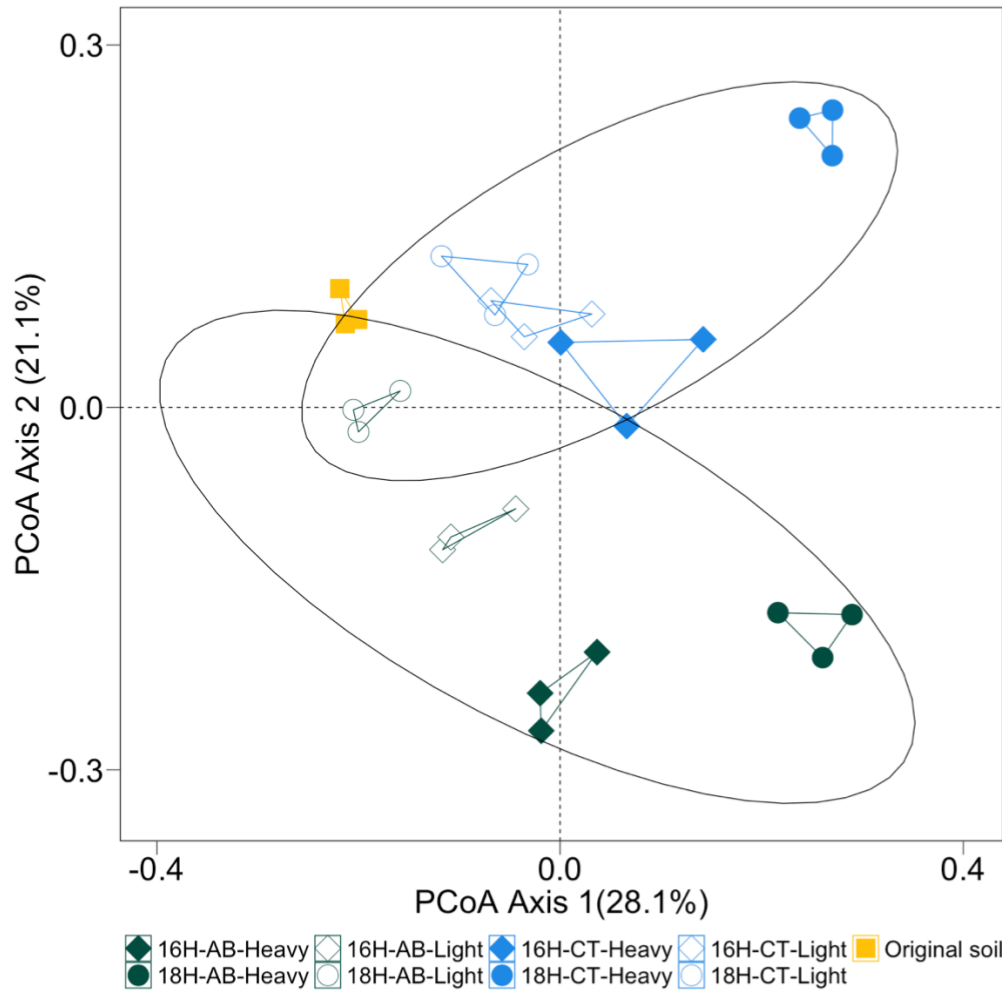

186

187 **Figure 3.** Principal coordinate analysis (PCoA) plots of bacterial OTUs (97% sequence similarity)
188 derived from 16S rDNA extracted from soil. The legend indicates the origin of the samples. 16H:
189 incubation with $H_2{}^{16}O$; 18H: incubation with $H_2{}^{18}O$; AB: incubation with antibiotics; CT: incubation
190 without antibiotics; Heavy: "heavy" fractions of the extracted soil DNA; Light: "light" fractions of the
191 extracted soil DNA.

192 The community in the heavy fractions of AB incubated with $H_2{}^{18}O$ were dominated by high
193 relative abundances of Pseudomonadota (84.9 ± 2.86%), Actinomycetota (formally Actinobacteria,
194 4.8 ± 1.42%), Acidobacteriota (4.7 ± 0.93%), Planctomycetota (formally Planctomycetes, 1.9 ±
195 0.42%), Verrucomicrobiota (formally Verrucomicrobia, 1.2%), and Gemmatimonadota (formally
196 Gemmatimonadetes, 1.1 ± 0.10%). *Stenotrophomonas* (Pseudomonadota, 76 ± 4.67%) was the most
197 abundant genus (Figure 4). In contrast, the heavy fractions for CT treatments were dominated
198 mainly by Pseudomonadota (72.1 ± 7.9%), Bacteroidota (formally Bacteroidetes, 16.1 ± 5.03%),

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199 Acidobacteriota (3.9 ± 0.83%), Saccharibacteria (2.3 ± 2.22 %), Actinomycetota (1.5 ± 0.38%),
200 Planctomycetota (1.3 ± 0.15%), Verrucomicrobiota (1.24%), and Gemmatimonadota (1.1%). Here,
201 *unclassified* (Pseudomonadota, 17.18%), *Sphingomonas* (Pseudomonadota, 11.9%), *Thermomonas*
202 (Pseudomonadota, 10.8%), *Arenimonas* (Pseudomonadota, 5.28%), *Novosphingobium*
203 (Pseudomonadota, 7.03%) were the abundant genera (Figure 4).

204 The relative abundance in the light fractions of AB were dominated by Pseudomonadota
205 (38.7 ± 5.38%), Acidobacteriota (17.2 ± 0.62%), Actinomycetota (12.3 ± 4.24%), Verrucomicrobiota
206 (10.9 ± 1.01%), Planctomycetota (6.2 ± 1.17%), Bacteroidota (3.7 ± 0.29%), Chloroflexota (formally
207 Chloroflexi, 3.6 ± 0.68%), and Gemmatimonadota (1.9 ± 0.19%). The most abundant genera were
208 *Stenotrophomonas* (Pseudomonadota, 8.9%), *Bradyrhizobium* (Pseudomonadota, 2.3%, only in one
209 replicate), and *Acidibacter* (Pseudomonadota, 2.1%) (Figure 4). In contrast, the light fractions of CT
210 were dominated by Bacteroidota (57.5 ± 8.54%), Pseudomonadota (14.7 ± 2.46%), Acidobacteriota
211 (9.9 ± 2.59%), Actinomycetota (4.7 ± 1.79%), Verrucomicrobiota (4.4 ± 1.29%), Planctomycetota (3.1
212 ± 0.62%), Chloroflexota (1.52 ± 0.40%), and Gemmatimonadota (1.1 ± 0.12%). The most abundant
213 genus in this treatment was *Flavobacterium* (Pseudomonadota, 38.91 ± 4.67%) (Figure 4).
214

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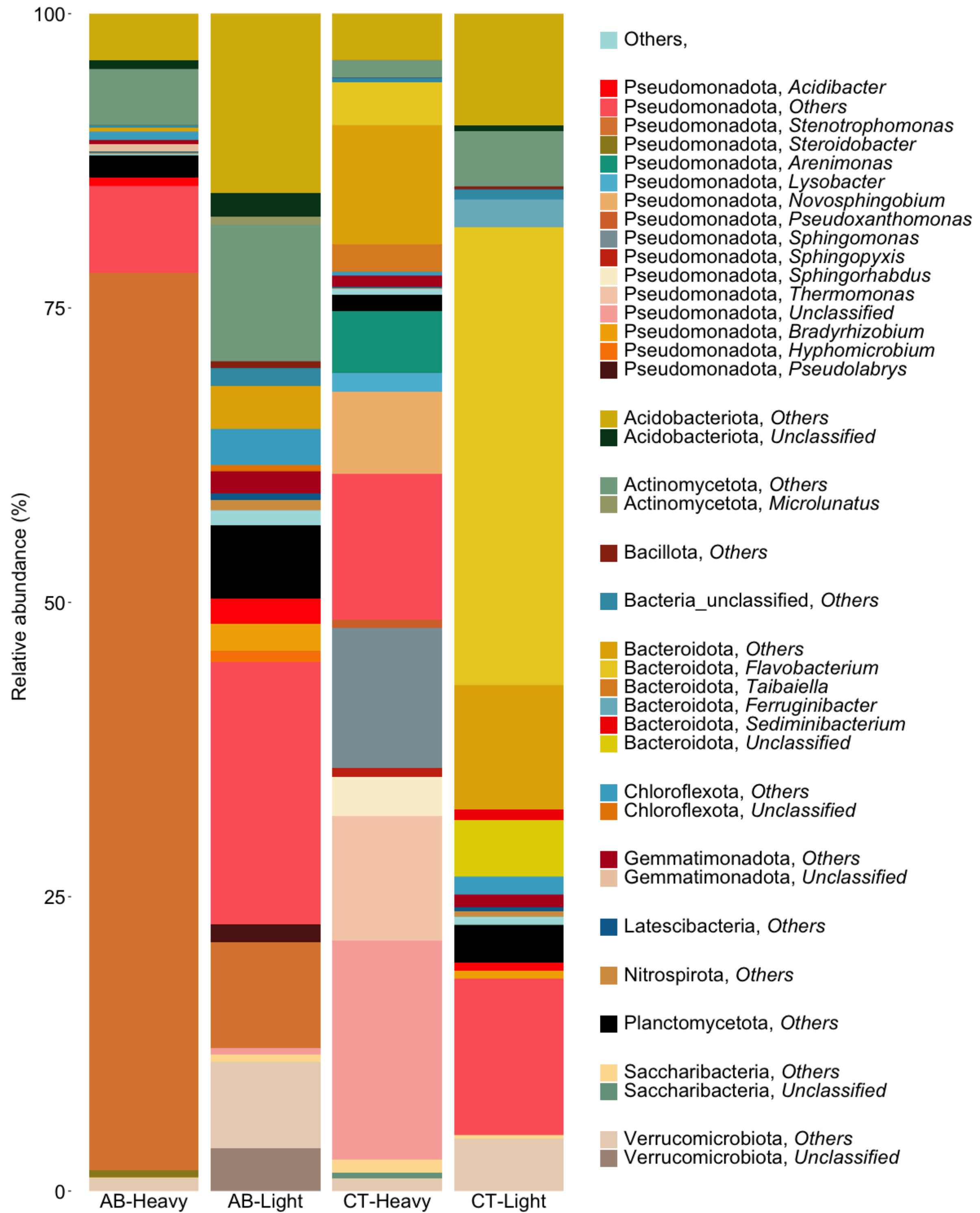

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216

217 **Figure 4.** Relative abundance of microbial communities at the phylum level identified in the “heavy”

218 and “light” fractions of DNA extracted from soils incubated with $H_2{}^{18}O$ in the presence (AB) or

219 absence (CT) of antibiotics.

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A heatmap was created to visualise and compare the abundance of the 20 OTUs that explains the most variation in the axis-1 and axis-2 of the PCA ordination. Out of a total of 28 OTUs selected, 19 OTUs belong to Pseudomonadota, followed by six OTUs of Bacteroidota, two OTUs of Verrucomicrobiota, and one OTU of Acidobacteriota. *Stenotrophomonas* (Pseudomonadota; OTU-7) was dominant in the heavy fraction of AB compared to heavy fraction of CT. On the other hand, *Sphingomonas* (Pseudomonadota; OTU-1065, OTU-1321, OTU-2509, OTU-488405, OTU-692415), *Lysobacter* (Pseudomonadota; OTU-12766), *Novosphingobium* (Pseudomonadota; OTU-14845), Xanthomonadaceae (Pseudomonadota; OTU-13089), *Arenimonas* (Pseudomonadota; OTU-1764) were dominant in heavy fraction of CT compared to AB. Additionally, *Pseudolabrys* (Pseudomonadota; OTU-1764), DA101 (Verrucomicrobiota; OTU-424), OPB35 (Verrucomicrobiota; OTU-8196) were dominant in light fractions of AB compared to heavy fractions of AB (Figure 5).

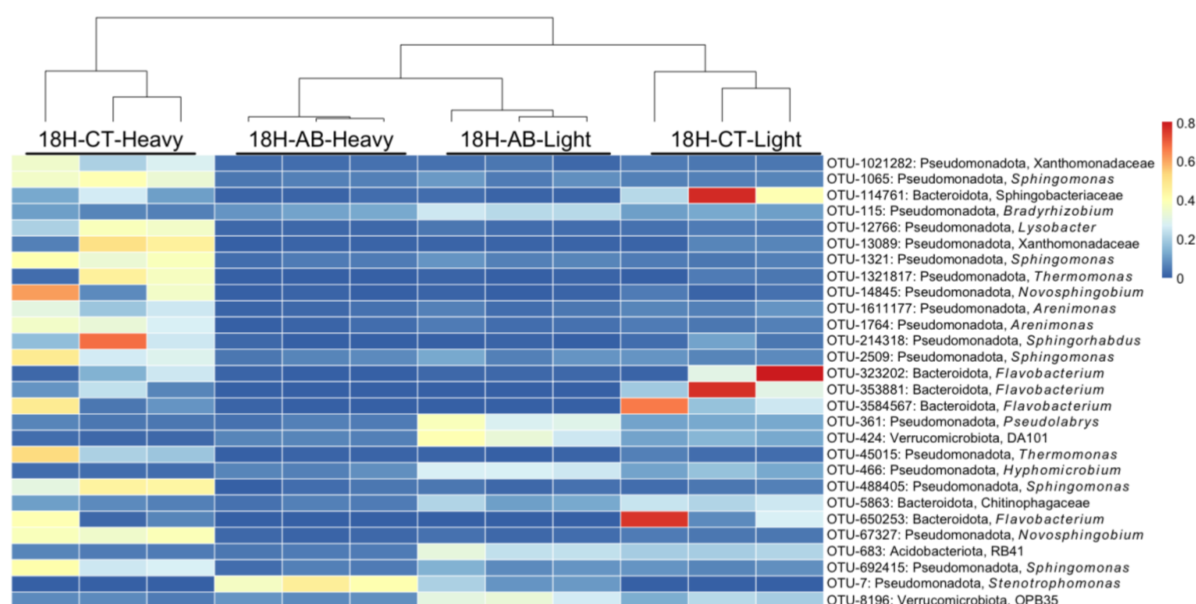

Figure 5. Heatmap of the most relevant bacterial OTUs identified in the "heavy" and "light" fractions of DNA extracted from soils incubated with $H_2^{18}O$ in the presence (AB) or absence (CT) of antibiotics. 18H-AB-heavy indicates "heavy" fractions treated with antibiotic;18H-AB-light indicates "light"

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238 fractions treated with antibiotic; 18H-CT-heavy indicates "heavy" fractions without antibiotic
239 treatment; 18H-CT-light indicates "light" fractions without antibiotic treatment. The coloured scale
240 represents the relative abundance of OTUs.
241
242 The DNA of both heavy and light fractions of soil when incubated with ^{18}O-labelled water in
243 the presence of antibiotics were sequenced individually using high-throughput sequencing. After
244 genome binning of both heavy and light fractions, one qualified metagenome-assembled genome
245 (MAG) was generated with 90.7% completeness and 0% contamination. The MAG was affiliated to
246 *Stenotrophomonas* (Pseudomonadota) (Figure 6). This is in-sync with the results of 16S rRNA gene
247 sequencing that also showed *Stenotrophomonas* (Pseudomonadota) as the dominant genus when
248 incubated with antibiotics (Figures 4, 5).

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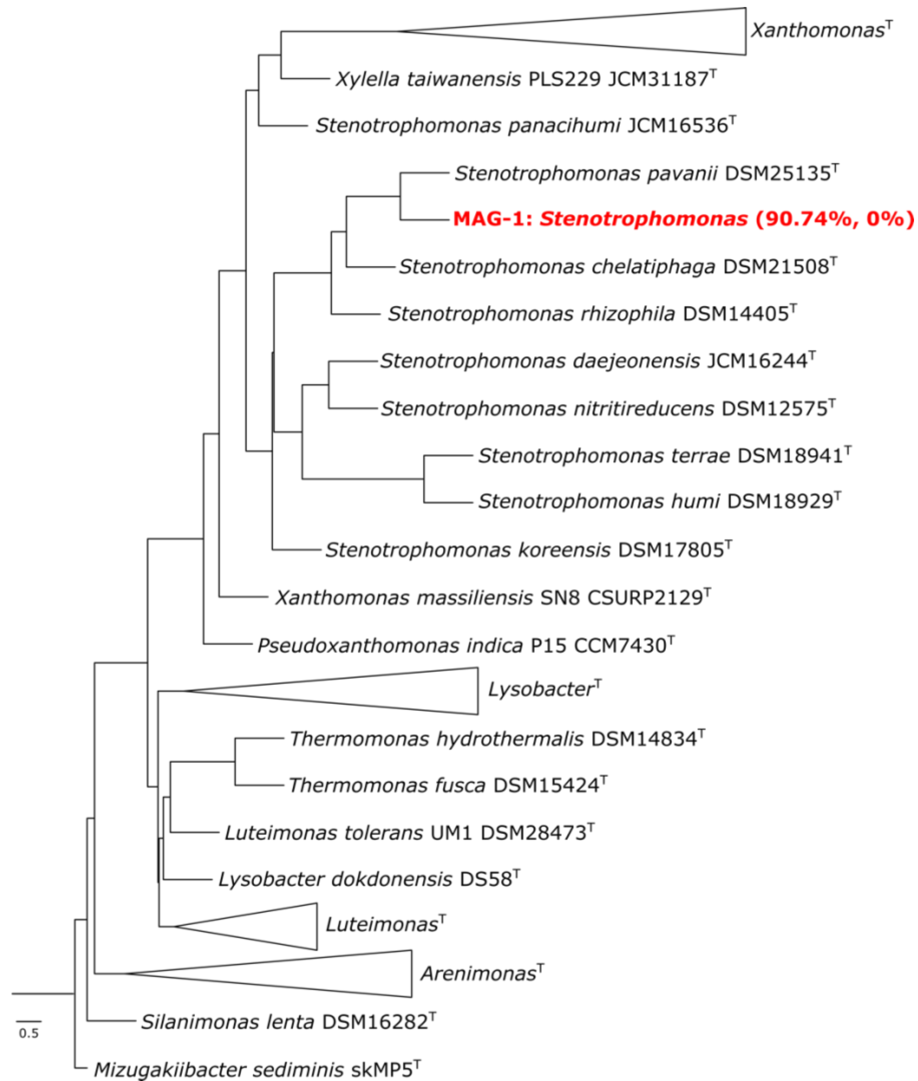

249

250 **Figure 6.** Multi-locus phylogenetic tree of the MAG-1 using autoMLST. 98 conserved housekeeping
251 genes were used for the analyses. MAG-1 is indicated in red together with their respective
252 completeness and contamination.

253

254 Analysis of the unbinned-assembled genomes of light and heavy fractions, along with the genome of
255 MAG-1 helped understand whether the microbial community exposed to antibiotic contained
256 antimicrobial resistance genes (ARGs) to survive the stress of antibiotics. The presence of *aph(3')-IIc*,
257 with 85.46% similarity to *Stenotrophomonas maltophilia* strain K279a was observed in the heavy
258 fraction and MAG-1, and no presence of this gene was observed in the light fraction. *aph(3')-IIc*
259 encodes for the aminoglycoside phosphotransferase enzyme that confers resistance to antibiotics in

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260 the aminoglycoside class (butirosin, paromycin, kanamycin, neomycin) among others. Similarly, the
261 presence of *oqxB*, with 76.28% similarity to *Escherichia coli* plasmid pOLA52 in heavy fraction and
262 MAG-1, which was also absent in the light fraction was also observed. The *oqxB* gene encodes for an
263 efflux pump that confers resistance to amphenicol class antibiotics (e.g., chloramphenicol),
264 disinfectants (e.g., benzalkonium chloride, cetylpyridinium chloride), quinolone class antibiotics (e.g.,
265 ciprofloxacin, nalidixic acid), trimethoprim, and others. However, *dfrB3*, which encodes for
266 dihydrofolate reductase that confers resistance to trimethoprim, was found with a 90.14% similarity
267 with the plasmid R751 in *Klebsiella aerogenes* only in the light fraction. The presence of ARGs that
268 confer resistance to beta-lactam were also found in both light and heavy fraction, but not in MAG-1.
269 For example, *blaTEM-181* in the light fraction was found with a 99.86% similarity with a vector pUC-
270 3GLA, and *blaL1* in heavy fraction with 85.84% of similarity with a beta-lactamase gene in
271 *Stenotrophomonas maltophilia* strain K1. No ARG conferring beta-lactam resistance was present in
272 MAG-1 (Table 1).
273
274 Table 1. Antimicrobial resistance genes found in the unbinned-assembled reads (from heavy and
275 light fractions) and MAG-1

Antimicrobial	Class	light	heavy	MAG-1	Gene (Similarity (%) / Reference*)		
					Light	Heavy	MAG-1
Butirosin	Aminoglycoside		X	X		aph(3')-IIc (85.46% / AM743169)	aph(3')-IIc (85.46% / AM743169)
Paromomycin			X	X			
Kanamycin			X	X			
Neomycin			X	X			
Chloramphenicol	Amphenicol		X	X		OqxB (76.28% / EU370913)	OqxB (76.28% / EU370913)
Benzylkonium chloride	Quaternary ammonium compound		X	X			
Cetylpyridinium chloride	Quaternary ammonium compound		X	X			

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Ciprofloxacin	Quinolone		X	X			
Nalidixic acid	Quinolone		X	X			
Trimethoprim	Folate pathway antagonist	X	X	X	dfrB3 (90.14% / X72585)	OqxB (76.28% / EU370913)	OqxB (76.28% / EU370913)
Unknown beta-lactam	Beta-lactam	X	X		blaTEM-181 (99.86% / KM977568)	blaL1 (85.84% / EF126059)	

* AM743169: *Stenotrophomonas maltophilia* strain K279a; EU370913: *Escherichia coli* plasmid pOLA52; X72585: *Klebsiella aerogenes* plasmid R751 genes from integron element; KM977568: Cloning and transformation vector pUC-3GLA; EF126059: *Stenotrophomonas maltophilia* strain K1 (beta-lactamase gene).

Discussion

In this study we used DNA-SIP with $H_2{}^{18}O$ to identify the antibiotic resistant microbes from the active pool of an agricultural soil microbiome that was not previously exposed to antibiotics. The results showed that microbes can grow in the presence of antibiotic even in the agricultural soil with no-antibiotic history (Figure 1). On the other hand, not all active microbes are antibiotic resistant, since community composition was different between antibiotic treated and untreated soil (Figures 2-5). The metagenomic analyses revealed the presence of ARGs in the active resistant microbial community (Table 1). Additionally, a MAG belonging to *Stenotrophomonas* was found in the heavy fraction after incubation with $H_2{}^{18}O$ and antibiotics. The study highlights the ability of DNA-SIP with $H_2{}^{18}O$ to identify active antibiotic resistant microbes.

The results showed that microbes in soils without prior exposure to antibiotic can harbour microbes that can become active and enriched in the presence of antibiotics during a short period of time, in this case after four days of incubation. This suggests that soils contain a seedbank of antibiotic resistance, and the microbiome can shift dramatically towards an enrichment of antibiotic resistant populations even after a short exposure to antibiotics. This also highlights the potential of soil to harbour native AMR bacteria, for these microbes to become dominant, and subsequently

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298 spread after exposure to antibiotics. The long-term consequence of shifts in community
299 composition, for example biogeochemical transformations, soil fertility, and disease risk is not clear
300 [25].

301 The AMR bacterial seedbank in soil could be a result of *in situ* selection as a consequence of
302 natural production of antimicrobials produced by the microbial community as microbes compete for
303 resources. Indeed, soils intrinsically harbour AMR bacteria and are a natural reservoir for ARGs
304 [7,12]. Alternatively, antibiotic resistant bacteria or ARGs could have been introduced to the soil
305 from external sources. This is common in soils exposed to livestock manure or sludge [9,18].
306 Moreover, the dispersal through unconventional sources such as birds can provide the initial seed
307 for the microbial communities to spread AMR. Birds have been shown to spread AMR through long-
308 distance and localised migration [26,27]. For example, Franklin's gulls (*Leucophaeus pipixcan*) in
309 Chile were found to have twice the prevalence of ESBL-producing *E. coli* compared to humans in the
310 same area along with high sequence similarity suggesting transmission. Interestingly, the gulls also
311 share sequences with drug-resistant human pathogens identified in clinical isolates from the central
312 Canadian region, which is a nesting place for these gulls [27]. However, more studies are needed to
313 decisively establish the roles of birds to encounter and acquire active antibiotic resistant microbes in
314 soils without prior exposure to antibiotic. Horizontal gene transfer (HGT) is another mechanism for
315 ARG accumulation within a community but there was no evidence for this in the present study.

316 The active pool of antibiotic resistant microbes was dominated by Pseudomonadota,
317 Actinomycetota, Acidobacteriota (Figures 4, 5). Pseudomonadota are known to be physiologically
318 and metabolically versatile with variable morphology that allows them to subsist in various
319 ecological niches [28–33]. This could be the reason why 72-84% of the OTUs labelled in the presence
320 of antibiotics were affiliated to Pseudomonadota (Figures 4, 5, S3, S4). Further, due to their
321 versatility, Pseudomonadota also contain the greatest number of bacterial pathogens to an extent
322 that this phylum has been proposed to be a potential diagnostic signature for disease risk [34,35].
323 Actinomycetota is another near ubiquitous phylum in soil that are known for their ability to

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324 synthesize diverse secondary metabolites and harbour different ARGs [33,36,37]. It is hypothesised
325 that in soils, ARGs of pathogenic Pseudomonadota originated from Actinomycetota through
326 horizontal gene transfer using conjugative plasmids [38–40]. These results reaffirm the role of
327 Actinomycetota in AMR spread and high abundance in AMR microbiomes. Similarly, Acidobacteriota
328 is also widespread in soil with phylogenetic depth and ecological importance comparable to
329 Pseudomonadota [33,41]. Acidobacteriota can harbour multiple integrative and conjugative
330 elements in their genome, a major determinant of horizontal gene transfer, that confers them a
331 major advantage to survive, resist, and persist in the presence of antibiotic [42,43].

332 In this study, *Stenotrophomonas* was found to be the dominant genus with a relative
333 abundance of 76% in the active resistant microbiome (Figures 4, 5, 6) and it possessed ARGs for
334 diverse antibiotics (see MAG-1 in Table 1). *Stenotrophomonas* is an antibiotic resistant opportunistic
335 pathogen that is commonly linked to respiratory infections in humans [44]. Possession of a wide
336 range of ARGs by *Stenotrophomonas* in an antibiotic unexposed soil is disturbing, but not unusual
337 and rare. For instance, on one hand, ARG in *Stenotrophomonas* strains has been reported from
338 deep-sea invertebrates [45]. On the other hand, multi-drug resistant *Stenotrophomonas* is a
339 common nosocomial and community-acquired infection [44].

340 The active pool of antibiotic resistant microbiota in this agricultural soil contains ARGs for a
341 wide variety of antibiotics (Table 1). Surprisingly, many of these antibiotics such as aminoglycosides,
342 chloramphenicol, were not even part of the experiment in this study. This highlights the potential
343 role of the seedbank of resistant bacteria in AMR spread. We hypothesize that these microbes are
344 present in soils at low abundance but with selection can become enriched increasing the probability
345 of causing disease outbreaks in livestock and human populations. Their enrichment may also spread
346 resistance within the microbial community through HGT.

347

348 **Conclusion**

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349 In this study, the active resistant soil microbiome from an agricultural field with no prior history of
350 antibiotic exposure using DNA-SIP with $H_2^{18}O$ was identified and differences in the composition of
351 active soil microbes and active antibiotic resistant soil microbes was observed. The metagenome
352 data shed light on the diversity of antibiotic resistant genes of the resistant microbiome. In this
353 study, we identified the prevalence of antibiotic resistant *Stenotrophomonas* in the soil, which might
354 be consequential for AMR spread and disease risk. Overall, this study makes a strong case for DNA-
355 SIP with $H_2^{18}O$ to identify the clinically important drug-resistant microbes in the environment.
356 Finally, this method can become gold standard to understand and identify the drivers of AMR spread
357 in any environment.
358
359 **Materials and methods**
360 **Soil sampling**
361 Agricultural soils from Chilworth Manor Experimental plots located in the Victorian Walled Gardens
362 (Southampton, U.K.) was sampled in October 2016. This soil does not have history of manure and no
363 antibiotic applications for over 20 years. Also, the last application of herbicide Round-up (Glyphosate
364 at a concentration of 360 g L^{-1} as active ingredient) was in 2007-2008 (M. Cotton, *pers. comm*.).
365 Samples were collected from 10-cm deep in a 10m triangular pattern as described previously
366 (Gomez- Alvarez et al., 2007). In total, three soil samples (not pooled) were transported to the
367 University of Southampton and storage at 4°C for further experiments. Physico-chemical parameters
368 were carried out at the Anglian soil analyses company (Lincolnshire, U.K.) and detailed in Table S1.
369 This soil is a sandy/loam soil with a pH of 6.17 (±0.006), organic matter of 7.73% (±1.43) and dry
370 matter of 85.99% (±3.58).
371
372 **Soil incubations**
373 Initial tests were performed to determine the concentration of antibiotic necessary to inhibit
374 bacterial growth in soil for up to 12 days. This is necessary due to potential attenuation of the

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375 antibiotic by soil (for methodology see Appendix S1). Since the attenuation of the antibiotic is as fast
376 as two days (Figure S1), a second preliminary experiment was carried out by incubating the soils with
377 several antibiotics to determine the suitable ones to be used for further labelling experiment.
378 Antibiotics were chosen because of their mechanism of action and described in Appendix S1. The
379 resistance genes for these antibiotics have been found in the genome of *Klebsiella pneumoniae* [46].
380 After performing the preliminary experiments, we decided to incubate the soils for up to 4 days due
381 to its fast decomposition (Figure S1) and four antibiotics were chosen for further incubations with
382 $H_2^{18}O$ (Table S2). One g of soil was incubated in 1.5 ml of with either label water ($H_2^{18}O$) or
383 unlabelled water (H_2O). Antibiotics [meropenem (mem), cefotaxime (ctx), ciprofloxacin (cip) and
384 trimethoprim (tmp)] at a concentration of 100 µg/ml each were added to the slurry at time 0 h and
385 48 h. Incubations were performed at 200 rpm, dark and room temperature. Sampling time for the
386 treatment was at day 2 and day 4. For controls, sampling was carried out after 4 days of incubation.
387 The experiments were performed in triplicate to provide statistically testable data (Figure S2).
388
389 **DNA extraction**
390 DNA was extracted from the soil at the end of the treatments by using the Power-Soil DNA isolation
391 kit (Mo Bio, UK) according to the manufacturer's recommendation. DNA purity and quantification
392 were determined using a NanoDrop® Spectrophotometer ND-1000 (Thermo Fisher Scientific, USA).
393 All DNA samples were stored at -80°C for further analysis.
394
395 **$H_2^{18}O$-SIP procedure**
396 A standard DNA-SIP protocol was used to resolve [^{18}O]-incorporation based on buoyant density [47].
397 1 µg of genomic DNA was loaded into 5.6-ml polyallomer quick-seal centrifuge tubes (Beckman
398 Coulter, USA) containing gradient buffer [20] and CsCl. The isopycnic centrifugation of DNA was
399 performed with an initial CsCl buoyant density of 1.725 g mL^{-1} subjected to centrifugation at 177,000

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400 × g for 36-40 h at 20 °C in an Optima XPN-80 ultracentrifuge (Beckman Coulter, USA). At the end of
401 the centrifugation, 18 fractions were separated from each gradient.
402

403 **Quantitative PCR**

404 The 16S rRNA gene was quantified in each of the fractions. All qPCR reactions were performed on a
405 StepOne Plus real-time PCR system (Applied Biosystems) and the data were processed using
406 StepOne software v2.3 (Applied Biosystems). For all assays, standards were prepared by PCR of
407 cloned genes. Standards were serially (10^1–10^7) diluted and used for the calibration curves in each
408 assay. Controls were run with water instead of template DNA. The assays were based on dual-
409 labelled probes using the primer–probe sets: BAC338F/BAC516P/BAC805R [48]. The probe was
410 synthesized with 6-Carboxyfluorescein (6-FAM) on their 5'end and Black Hole Quencher 1 (BHQ1) on
411 their 3'end. Each reaction was 20 mL in volume and contained the following mixture: 10 μL of
412 TaqMan fast advanced master mix (1X) (Applied Biosystems), 1.0 μL of of primer mix [18 μL BAC338F
413 (0.9 μM), 18 μL BAC805R (0.9 μM), 5 μL BAC516P (0.25 μM) and 59 μL of TE buffer], DNA template
414 (2.0 μL) and 7.0 μL of water. The program used was 95°C for 5 min, followed by 35 cycles of 95°C for
415 30 s and 62°C for 60 s for annealing, extension and signal acquisition respectively [49]. Efficiencies of
416 97 to 103% with R^2 values > 0.98 were obtained.
417

418 **High-throughput sequencing**

419 The 16S rRNA genes from SIP gradient fractions was amplified and sequenced by barcoded Illumina
420 sequencing. PCR primers 515FB (GTGYCAGCMGCCGCGGTAA) and 806RB
421 (GGACTACNVGGGTWTCTAAT) from the Earth Microbiome project
422 (http://press.igsb.anl.gov/earthmicrobiome/) targeting the V4 region of the 16S rRNA gene
423 (approximately 250 nucleotides) were used. Library preparation and sequencing was performed at
424 the National Oceanographic Centre (NOC) of the University of Southampton, UK, following

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425 methodologies described by [50]. Samples were pooled in an equimolar concentration and
426 sequenced on separate runs for MiSeq using a 2 bp × 300 bp paired end protocol.
427 The total metagenomic DNA of the heavy and light fractions from incubations with $H_2^{18}O$ (total of six
428 samples) were sequenced on an Illumina MiSeq at the University of Southampton (as described
429 above). The metagenome was analysed on a high-performance computing cluster supported by the
430 Research and Specialist Computing Support Service at the University of East Anglia (Norwich, UK).
431

432 **Bioinformatic Analyses**

433 For the 16S rRNA-sequencing, quality filtering of the sequences was carried out by using cutadapt
434 [51]. Forward and reverse reads were then merged by using the usearch fastq_mergepairs command
435 [52]. Downstream processing was performed by using UPARSE [52] and UCHIME pipelines [53].
436 Briefly, sequences shorter than 250 bp were discarded, singletons were retained, and operational
437 taxonomic units (OTUs) were defined at a sequence identity level of 97%.
438

439 For the DNA sequences, reads were checked using FastQC version 0.11.8 [54]. Low-quality reads
440 were discarded using BBDuk version 38.68 [55]. Afterwards, reads were merged into scaffolds using
441 de novo assembler metaSPAdes version 3.13.1 [56]. Binning of the assembled scaffolds from both
442 heavy and light fractions was carried out with the metaWRAP version 1.2.1 [57]. Completion and
443 contamination metrics of the extracted bins were estimated using CheckM [58]. The resulting bins
444 were collectively processed to produce consolidated metagenome-assembled genomes (MAGs)
445 using the bin_refinement module in wetaWRAP.
446

447 **Statistical analyses and OTU Classification**

448 Statistical analyses were performed using the 'vegan' package [59] in R software version 4.1.1. Tests
449 with $P<0.05$ were considered to be statistically significant. Shapiro-Wilk normality test was
450 performed for each analysis. ANOVA was performed when abundance data were normally

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451 distributed. A non-parametric Kruskal-Wallis one-way analysis of variance was performed when the
452 data were not normally distributed [60]. In parallel, to test the significance of the differences
453 between 2 samples (i.e., between heavy and light fractions), two-tailed independent t-tests were
454 done. For all OTU-based statistical analyses, the data set was normalized by a Hellinger
455 transformation [61] using the decostand function. For beta-diversity, principal coordinates analysis
456 (PCoA) ordination of Hellinger distances was carried out using the 'pcoa' function. Heatmaps were
457 constructed with 'pheatmap' package [62] for the OTUs explaining most of the differences between
458 samples. Principal component analysis (PCA) of the Hellinger transformed data was performed using
459 the prcomp function. The 20 OTUs explaining most of the differences between samples were
460 defined as the OTUs contributing the largest absolute loadings in the first and second dimensions of
461 the PCA [60], obtained from the rotation output file. Hierarchical clustering of the distance matrix
462 was carried out with the "ward.D2" method using 'hclust' function.
463
464 **Taxonomy Analysis**
465 A representative sequence of each OTU was aligned against the SILVA 16S rRNA gene database using
466 the naïve Bayesian classifier (bootstrap confidence threshold of 80%) by using the mothur software
467 platform [63].
468 The taxonomic classification of the MAG was performed as explained previously [64]. Briefly, DNA–
469 DNA hybridization (dDDH) was conducted using the Type Strain Genome Server (TYGS) [65]. Amino-
470 acid comparisons between the MAG retrieved in this study and their closest relative strains were
471 calculated based on reciprocal best hits (two-way amino acid identity AAI) using the enveomics
472 collection [66]. Finally, a phylogenomic tree was created using the automated multi-locus species
473 tree (autoM-LST) pipeline [67]. AutoMLST determines closely related genomes based on alignment
474 of >90 core genes, and the closest species were determined based on percent average nucleotide
475 identity (ANI).
476

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477 **Antimicrobial resistance genes**

478 Since only one MAG was recovered in this study, the unbinned-assembled reads (from heavy and
479 light fractions) were also analysed. Therefore, all (MAG-1, unbinned heavy fractions and unbinned
480 light fractions) reads were screened for antimicrobial resistance genes (ARGs) using the public
481 database Resfinder version 4.1 [68].

482

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488

489 **Authors' contributions**

490 MH, CWK and MGD planned the experiments. MH carried out experimental work. MH carried out
491 bioinformatic analysis. MH, SR and MGD analysed the data. MH and SR wrote the manuscript. All
492 authors read and approved the final manuscript.

493

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496

497 **Availability of data and materials**

498 Sequence data were deposited in the NCBI Sequence Read Archive (SRA) under accession number
499 PRJNA428598 for 16S rRNA gene sequences, PRJNA602606 for raw metagenome data, and
500 PRJNA778335 for MAG-1.

501

502 **Ethics approval and consent to participate**

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503 Not applicable.

504 **Consent for publication**

505 Not applicable.

506 **Competing interests**

507 The authors declare that they have no competing interests.

508

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