

1 **HAM-ART: An optimised culture-free Hi-C metagenomics pipeline for tracking**
2 **antimicrobial resistance genes in complex microbial communities**

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21

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24

25 **Abstract**

26 Shotgun metagenomics is a powerful tool to identify antimicrobial resistance (AMR)
27 genes in microbiomes but has the limitation that extrachromosomal DNA, such as
28 plasmids, cannot be linked with the host bacterial chromosome. Here we present a
29 laboratory and bioinformatics pipeline HAM-ART (Hi-C Assisted Metagenomics for
30 Antimicrobial Resistance Tracking) optimised for the generation of metagenome-
31 assembled genomes including both chromosomal and extrachromosomal AMR
32 genes. We demonstrate the performance of the pipeline in a study comparing 100 pig
33 faecal microbiomes from low- and high-antimicrobial use pig farms (organic and
34 conventional farms). We found significant differences in the distribution of AMR
35 genes between low- and high-antimicrobial use farms including a plasmid-borne
36 lincosamide resistance gene exclusive to high-antimicrobial use farms in three
37 species of *Lactobacilli*.

38

39 **Author Summary**

40 Antimicrobial resistance (AMR) is one of the biggest global health threats humanity is
41 facing. Understanding the emergence and spread of AMR between different bacterial
42 species is crucial for the development of effective countermeasures. In this paper we
43 describe a user-friendly, affordable and comprehensive (laboratory and
44 bioinformatics) workflow that is able to identify, associate and track AMR genes in
45 bacteria. We demonstrate the efficiency and reliability of the method by comparing 50
46 faecal microbiomes from pig farms with high-antibiotic use (conventional farms), and
47 50 faecal microbiomes from pig farms with low-antibiotic use (organic farms). Our
48 method provides a novel approach to resistance gene tracking, that also leads to the
49 generation of high quality metagenomic assembled genomes that includes genes on

50 mobile genetic elements, such as plasmids, that would not otherwise be included in

51 these assembled genomes.

52

53 Introduction

54 The emergence of resistance to antimicrobials in bacteria can occur by spontaneous
55 mutation or by the acquisition of mobile genetic elements carrying antimicrobial
56 resistance (AMR) genes[1] (for example, plasmids *via* natural transformation or
57 conjugation, or bacteriophages *via* transduction[2]). Over the last decade,
58 metagenomic studies have revealed that bacterial communities comprising gut flora
59 or soil microbiota possess a diverse arsenal of AMR genes, termed the resistome[3],
60 some of which can be transferred between related or unrelated species. A limitation
61 of next-generation sequencing metagenomics is the identification of species
62 harbouring a particular AMR gene when that gene is present in extra-chromosomal
63 DNA. Alternative approaches based on traditional culture of bacteria have provided
64 direct experimental evidence of plasmid-mediated AMR gene transfer from enteric
65 pathogens to commensal *Escherichia coli* in rodents[4, 5], chickens[6] and
66 humans[7]. *Salmonella*-inflicted enteropathy has been shown to elicit parallel blooms
67 of the pathogen and of resident commensal *E. coli*. These blooms boosted horizontal
68 gene transfer (HGT) in general, and specifically, the transfer of a conjugative colicin-
69 plasmid p2 from an introduced *Salmonella enterica* serovar Typhimurium to
70 commensal *E. coli*[8]. It has been shown that the use of in-feed antimicrobials leads
71 to a bloom in AMR genes in the bacteriophage metagenome recovered from treated
72 pigs[9], although it is unclear what the sources or destinations of these genes are.
73 These observations suggest that HGT between pathogenic and commensal bacteria
74 is a common occurrence in humans and animals and is likely to contribute to the
75 persistence and spread of AMR. Moreover, many previous studies on the spread of
76 AMR from animal sources have focused on AMR of pathogens, with less emphasis

77 on genes within indigenous microbiota that may also pass to humans from animals
78 (and vice versa) but be difficult to culture.

79

80 To overcome the inability of next-generation metagenomic sequencing to identify
81 where extra-chromosomal genes of interest reside, a number of chromosome
82 conformation technologies (such as 3C, Hi-C), originally designed for the study of
83 three-dimensional genome structure in eukaryotes, have been used[10-12]. These
84 techniques exploit the ability to create artificial connections between strands of co-
85 localised DNA by cutting and re-ligating the strands. The techniques differ in their
86 manner of detection, and the scope of interactions they can probe. Marbouty *et al.*
87 describe the application of robust statistical methodology to 3C sequence data
88 (meta3C) derived from a river sediment microbiome[12]. Hi-C, a technical
89 improvement on the 3C method has been shown to successfully disambiguate
90 eukaryotes and prokaryotes[11], and to differentiate closely related *E. coli* strains
91 from microbiomes[10]. Both these techniques offer great potential to define the
92 dynamics of an introduced AMR gene (both chromosomal and extra-chromosomal),
93 in particular the nature and frequency of transfer events, including into microbiota
94 constituents that are not readily detectable by culture in the laboratory. We showcase
95 the performance of a novel laboratory and bioinformatic pipeline (HAM-ART),
96 optimised for tracking AMR genes, in a study comparing 100 faecal microbiomes
97 from UK conventional and organic pig farms.

98

99 **Results**

100

101 We developed a laboratory and bioinformatics pipeline (HAM-ART) that: (i)
102 assembles bacterial genomes with high reliability; (ii) associates mobile genetic
103 elements to the host genome; and (iii) annotates and associates AMR genes with
104 high specificity and sensitivity. As HAM-ART is built on traditional metagenomics
105 sequencing methodology, combined with Hi-C sequencing from the same bacterial
106 pellet, it could be applied to any complex microbial community. HAM-ART utilises a
107 widely used sequencing platform, Illumina paired-end sequencing, with standard
108 library sizes and affordable amounts of sequencing per sample. The bioinformatics
109 pipeline was designed to be user friendly, and in addition to generating a set of final
110 metagenomics assembled genomes (MAGs) it outputs results tables reporting
111 assembly quality, taxonomy and AMR gene association.

112

113 **Proof-of-concept study undertaken to validate HAM-ART**

114 The HAM-ART methodology was tested in a study comparing AMR in two groups of
115 farms; 5 organic (OG1-5) pig farms farming to organic certification standards with low
116 antibiotic use, and 5 conventional (CV1-5) pig farms with higher antibiotic use. Ten
117 faecal samples were taken from each farm for metagenomic analysis as described in
118 the methods section. The organic farms had lower population corrected use (PCU) of
119 antibiotics (average 3.0 mg/PCU, range 0-9.8 mg/PCU) over the year prior to
120 sampling compared to conventional farms (average 85.7 mg/PCU, range 3.9-170.1
121 mg/PCU). Similarly, the number of different classes of antibiotics used on each farm
122 ranged from 0-4 for organic farms and 4-9 for conventional farms. The results of
123 metagenomic analyses are described below.

124

125 **Generation of MAGs using the HAM-ART pipeline**

126 The pipeline scaffolded *de novo* assemblies using approximately 500k contigs from
127 each faecal sample, coupled with 0.2-3.4M informative binary connections from the
128 Hi-C pairs (a Hi-C connection is informative if it connects two different contigs as
129 opposed to a connection within the same contig). The initial products of HAM-ART
130 are the consensus clusters (CCs); a collection of contigs that are clustered together
131 during the network resolution step, solely based on Hi-C contacts, that approximate
132 to a genome of a constituent bacterial species. The total number of CCs for each
133 sample varied between 6k-54k, of these we focussed on CCs comprising >250kb
134 (representing about 1/10th of an average prokaryotic genome) for further pipeline
135 processing. After the splitting and extension of the large CCs (as described in the
136 methods section) the number of MAGs varied between 5 and 131 (mean: 62,
137 median: 60) per faecal sample.

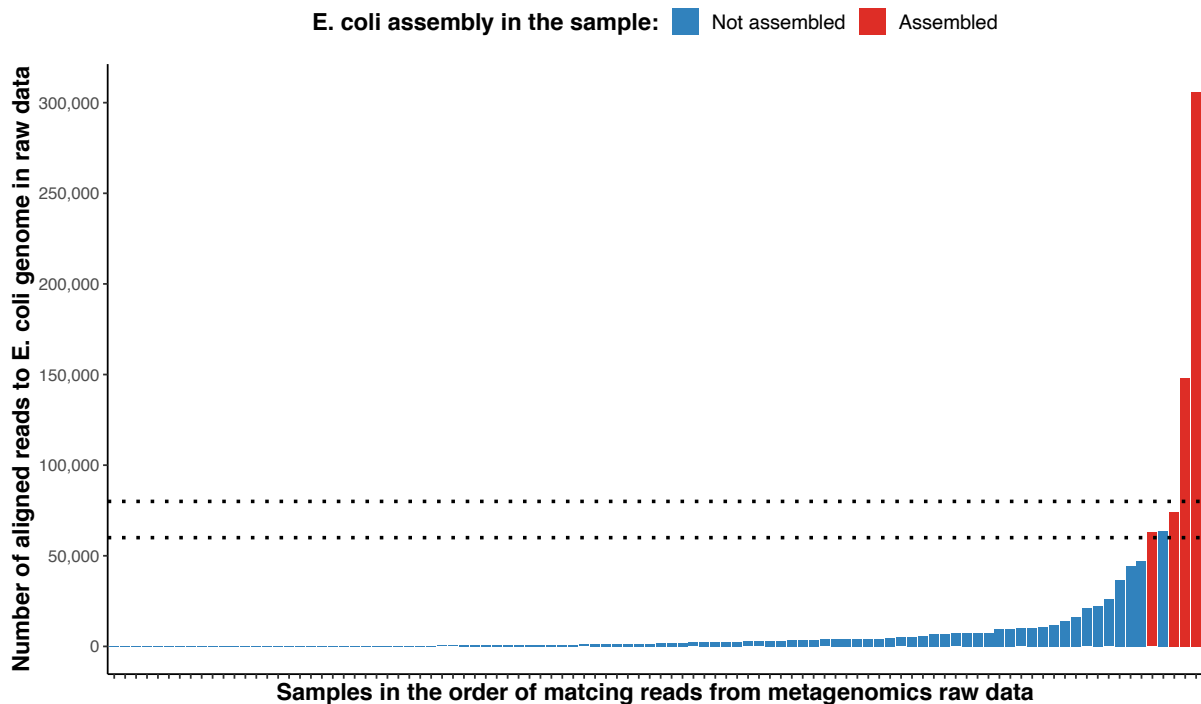
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139 A total of 6184 MAGs were identified from the 100 samples which were distributed
140 into 1555 clades based on pairwise genetic distance, indicating groups of MAGs
141 which were likely to represent the same species or genus. The number of members
142 for each clade varied between 1 and 79 (mean: 3.97, median: 2). All clades were
143 subjected to clade refinement that resulted in 553 clades with at least one MAG over
144 500kb in size. After the clade refinement we ended up with 6164 best quality MAGs.

145

146 **Validation of a Hi-C MAG with the matching genome generated from culture of** 147 **a single isolate**

148 We noted that *E. coli* were relatively rarely assembled in our samples (4% of
149 samples). One possible explanation was that *E. coli* were present, but in low
150 abundance. We investigated this by determining the number of reads in the shotgun
151 libraries from each sample that mapped to an *E. coli* reference genome (Figure 1).



152

153 **Figure 1. Presence of *E. coli* DNA in all samples.**

154 Metagenomic shotgun sequencing raw reads from each faecal sample were aligned
155 to a reference *E. coli* genome (*Escherichia coli* O157:H7, GCF_000008865.2) by
156 bowtie2 (—fast option) and the number of reads "aligned concordantly exactly 1 time"
157 were extracted from the output log file. Results were plotted in rank order by the
158 number of aligned reads. In samples plotted as red columns (n=4) *E. coli* MAGs were
159 successfully assembled using HAM-ART, while those plotted as blue columns were
160 not. Dotted horizontal lines represent the potential threshold range for successful
161 assembly of a MAG (60,000-80,000 reads, representing ~0.2% of the total number of
162 reads for this sample). Repeated analysis using different *E. coli* reference genomes

163 (including an *E. coli* cultured and sequenced from a farm included in this study) gave
164 similar results.

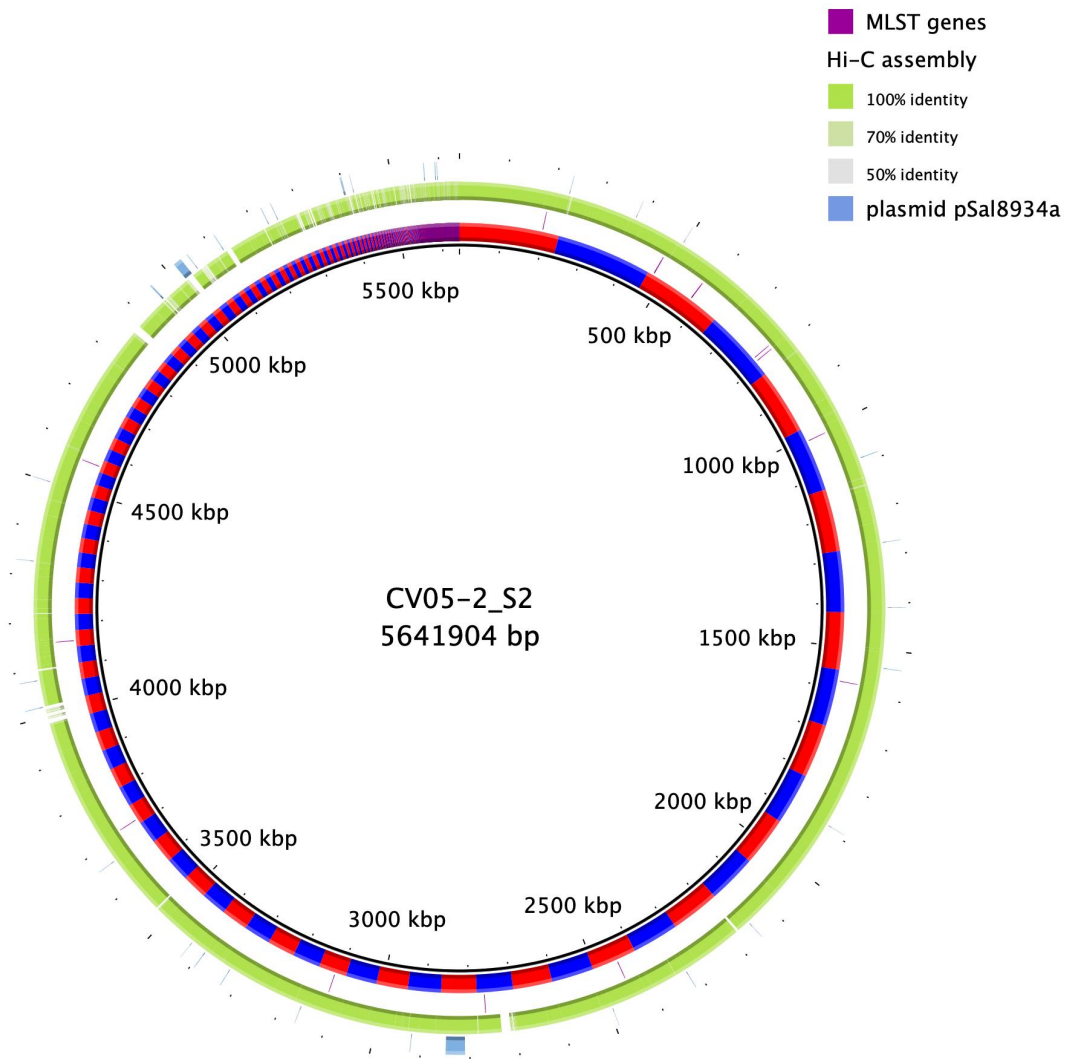
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166 This shows that although reads were present in the majority of shotgun libraries, it
167 was only when there were $\geq 50k$ *E. coli* reads that a MAG could be created. This
168 observation suggests that 60-80k reads (representing about 9-12 Mbp),
169 approximating to 2x coverage of an *E. coli* genome are required in order to generate
170 a MAG. In this study, which generated approximately 35M reads (5.25×10^9 bp) for
171 each sample, an individual species would need to represent $\sim 0.2\%$ of the total
172 bacterial community in order to generate a MAG.

173

174 We examined the quality of a single Hi-C MAG by performing conventional bacterial
175 culture and sequencing of an *E. coli* from the same faecal sample (CV5_05) that
176 generated the *E. coli* MAG. DNA was extracted and the genome obtained using DNA
177 sequencing and assembly (Illumina MiSeq and Spades). The MiSeq data yielded a
178 5.7 MBp assembly (CV05-2_S2) that was identified as ST20, and harboured 8 AMR
179 genes (*aadA1*, *aadA2*, *blaCFE-1*, *cmlA1*, *dfrA12*, *mdf(A)*, *sul3* and *tet(34)*). A BLAST
180 comparison of the MiSeq genome with the Hi-C MAG visualised using BRIG is shown
181 in Figure 2.

182



183

184 **Figure 2. BLAST comparison of an *E. coli* MAG with a corresponding *E. coli***
185 **assembly obtained using culture, followed by Illumina MiSeq sequencing and**
186 **assembly.**

187 The innermost ring shows the MiSeq assembly with contig boundaries indicated by
188 alternate red and blue colouring. The position of the MLST genes (both MLST
189 schemes 1 and 2) are indicated in the second ring. The matching Hi-C MAG's identity
190 levels are shown in the third ring. The presence of a possible plasmid is illustrated in

191 pale blue in the outermost ring. This ring contains the comparison results for a S.
192 Typhimurium plasmid pSal8934a (NCBI accession number JF274993). This plasmid
193 has 99.6% identity and a query coverage of 79% compared to one of the MiSeq
194 assembly contigs (and also to the matching MAG). This plasmid contains the *aadA1*,
195 *aadA2*, *cmlA1*, *dfrA12* and *sul3* AMR genes.

196

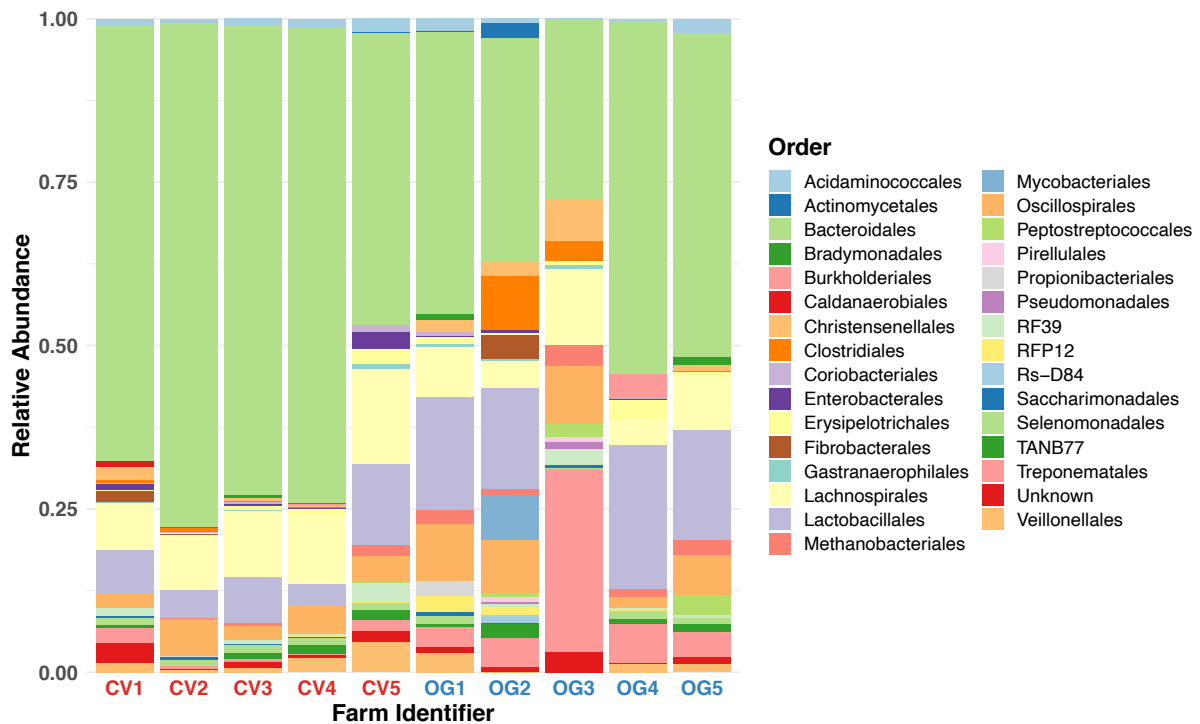
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198

199 **Taxa composition of the pig microbiomes from conventional and organic farms**

200 The distribution of taxa between CV and OG farms (Figure 3 and Supplementary
201 Figure S1) were broadly similar with the possible exception of OG3. On all farms the
202 diversity included common intestinal bacterial orders, dominated by *Bacteroidales*,
203 *Lachnospirales*, *Lactobacillales*, *Oscillospirales*, which is consistent with previous pig
204 faecal microbiome studies[9, 13-15]. The relative paucity of *Enterobacteriaceae* and
205 the presence of a substantial number of treponemes appear to be characteristic of
206 the pig faecal microbiome[16, 17].

207



208

209 **Figure 3. Order-level relative composition of the pig faecal microbiota in the**
210 **study farms.**

211 The average relative abundance of different orders identified by GTDB-tk in the final
212 assemblies were calculated from 10 samples in each of the 10 farms included in the
213 study. Plots of different taxonomic levels are shown in Supplementary Figure S1.

214

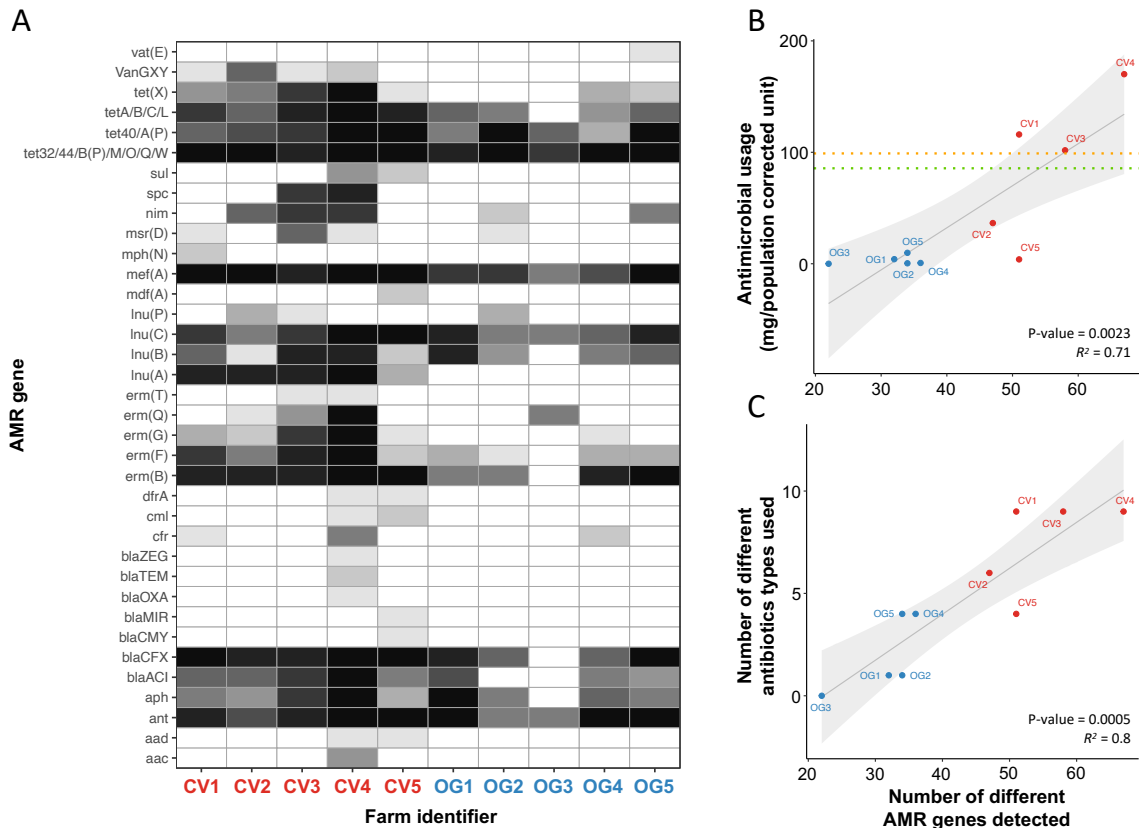
215

216 **AMR gene distribution in faecal samples from CV and OG farms**

217 We identified 66 different AMR genes (in 36 resistance gene groups, as described in
218 methods – analysis of assembly data) using the ResFinder AMR gene database
219 within our final 6164 MAGs (Supplementary Table S2). A comparison of the
220 distribution of AMR genes (Figure 4A) indicates that a greater diversity of AMR genes
221 were found in CV farms compared to OG farms.

222

223



224

225 **Figure 4. AMR gene distribution in faecal samples from CV and OG farms and**
 226 **the correlation with levels of antimicrobial used.**

227 Panel A: The heatmap shows the number of samples from which MAGs were
 228 generated containing different AMR genes, with the intensity of shading ranging from
 229 0/10 samples (white) to 10/10 samples (black). Conventional (CV1 to 5) and organic
 230 (OG1 to 5) are labelled using red and blue text respectively. Panel B: A scatter plot
 231 of the amounts of antimicrobial used (mg/population corrected unit (PCU)) in the year
 232 prior to sampling, against the number of different AMR genes detected for each farm.
 233 The orange line indicates the 2020 target set by the Responsible Use of Medicines in
 234 Agriculture Alliance for antibiotic use (99 mg/PCU), and the green line represents the
 235 average calculated from the 5 CV farms in our study (85.7 mg/PCU). Panel C: A
 236 scatter plot showing the number of different antimicrobial types used in the year prior

237 to sampling, against the number of different AMR genes found. Spearman correlation
238 coefficients and significance values were calculated by fitting a linear regression
239 model on the data points in R (line of best fit and 95% confidence intervals are
240 shaded in grey).

241

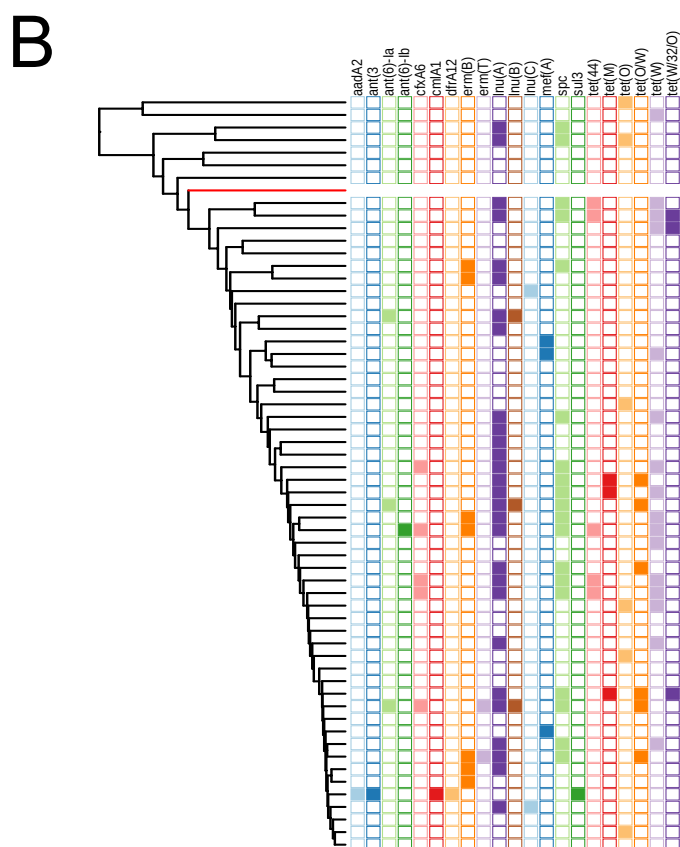
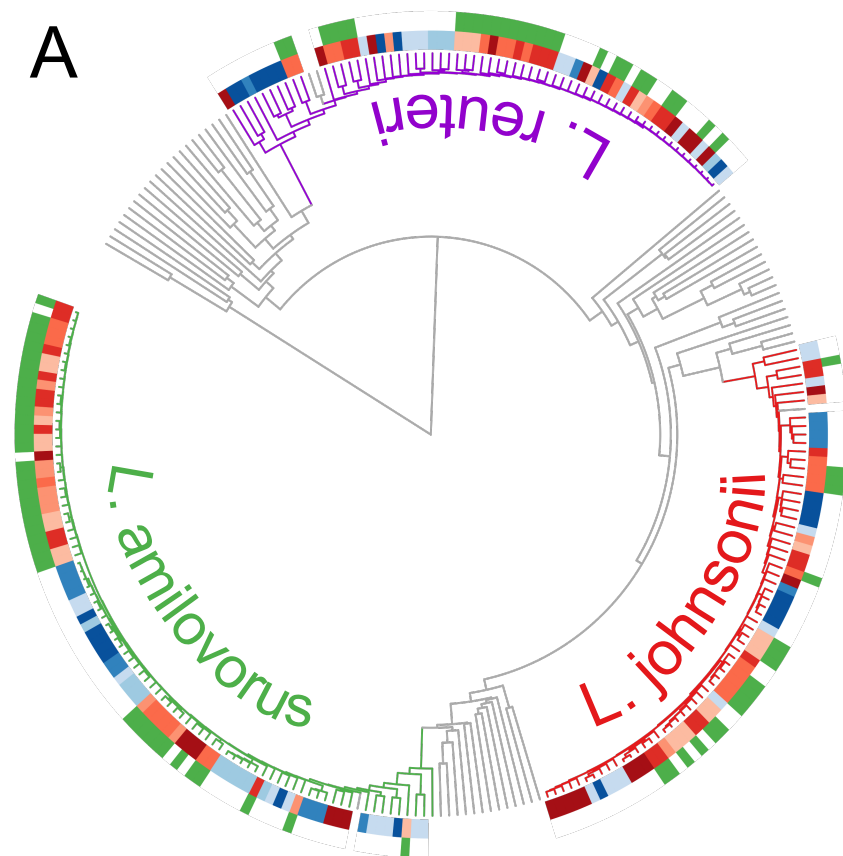
242 Genes that encode proteins potentially able to confer resistance to β -lactams and
243 chloramphenicol were present at greater numbers in samples from CV farms. A
244 number of genes were present solely in samples from CV farms (*aac*, *aad*, *blaCMY*,
245 *blaMIR*, *blaOXA*, *blaTEM*, *blaZEG*, *cml*, *dfrA*, *erm(T)*, *Inu(A)*, *mdf(A)*, *mph(N)*, *spc*,
246 *sul*, *van(GXY)*), whereas the gene *vat(E)* was found solely in one OG farm.

247 Comparing the number of different AMR genes found in the faecal microbiomes to
248 the antimicrobial use on each farm (using PCU, and the number of different
249 antimicrobials used), we observed statistically significant correlations (Figure 4
250 panels B and C). The correlation of AMR genes with PCU had an R squared value of
251 71% and P value of 0.0023; the correlation of AMR genes with the number of
252 different antimicrobials used had an R squared value of 80% and P value of 0.0005.

253

254 **Association of *Inu(A)* gene harbouring plasmid to *Lactobacilli* species**

255 An analysis of the distribution of resistance genes among their host MAGs revealed
256 that the lincosamide resistance gene, *Inu(A)*, was found in three clades
257 corresponding to *Lactobacillus amylovorus*, *Lactobacillus johnsonii*, and *Lactobacillus*
258 *reuteri*. All three clades were present in the majority of samples from both OG and
259 CV farms, however the *Inu(A)* gene was only present in CV farms (Figure 4 panel A
260 and Figure 5).



261

262

263

264 **Figure 5. Association of different AMR genes to *Lactobacilli* species.**

265 Panel A: A distance tree based on sequence comparisons of *L. amylovorus*, *L.*
266 *johnsonii* and *L. reuteri* assemblies found in farms (coloured branches) together with
267 genomes of the corresponding *Lactobacilli* species (grey branches inside clades) and
268 other known *Lactobacilli* species (grey branches outside the clades) from the NCBI
269 RefSeq collection (<https://www.ncbi.nlm.nih.gov/refseq/>). The blocks of colour
270 adjacent to the branch-tips indicate the farm type and number (light to dark red: CV1
271 to 5; light to dark blue: OG1 to 5). The outer circle shows the presence of the *Inu(A)*
272 gene within the MAG (green: present, none: absent). Panel B: A distance tree based
273 on sequence comparisons of all *L. reuteri* MAGs found in the farm samples showing
274 the presence/absence (filled/empty square) of all the AMR genes found in this clade
275 (black branches: MAGs from farm samples, red branch: *L. reuteri* reference genome
276 from NCBI RefSeq collection).

277

278

279 The *Inu(A)* gene was found in all of the CV farms and did not appear to be restricted
280 to a single lineage or species on individual farms. The number of *L. amylovorus*, *L.*
281 *johnsonii* and *L. reuteri* MAGs obtained from CV farms were 47, 36 and 42
282 respectively, and from OG farms were 36, 23, and 25. Examination of the contigs
283 which harboured the *Inu(A)* gene indicated that an identical 5.6 kb sequence was
284 present in 27/34 *Inu(A)* positive *L. amylovorus*, 17/28 *Inu(A)* positive *L. johnsonii* and
285 18/18 *Inu(A)* positive *L. reuteri* MAGs. The sequence was often present in a single
286 contig of approximately the same length but with different sequencing origins

287 suggesting that it was present as a plasmid. In the *Inu(A)* positive lactobacilli MAGs
288 that did not appear to contain the entire sequence, the majority (15/18) had a short
289 contig that was identical to part of the putative plasmid sequence. A BLAST search of
290 the NCBI database showed that this sequence had 99.8% identity with an 884bp
291 section of a plasmid from a *L. johnsonii* (CP021704) and had 83.2% identity with a
292 1399bp section of a plasmid from a *L. amylovorus* (CP002560).

293

294 **Discussion**

295 Conventional shotgun metagenomics sequencing can generate lists of AMR genes
296 and lists of species contained in a microbiome but is not capable of consistently
297 identifying which bacteria carry which plasmid. The application of Hi-C metagenomics
298 in this study demonstrates that this technique is able to place AMR genes carried on
299 plasmids with their host genomes. The HAM-ART pipeline was tested using a
300 challenging experimental design involving 100 faeces samples from 10 different
301 farms. The results from this study show that it is possible to obtain high resolution,
302 good quality results by performing relatively modest amounts of sequencing on
303 samples of varying quality. While there are other pipelines capable of analysing
304 combined chromosome-capture based assembly and AMR gene association[18-23],
305 HAM-ART is the first method that is designed to cope with large sample numbers,
306 using the most common Illumina based sequencing platform and delivering results
307 from affordable amounts of sequencing depth.

308

309 Unsurprisingly this study shows that farms with lower use of antimicrobials (typically
310 OG farms, who are members of an assurance scheme that strongly regulates the
311 amount of antimicrobials to which the animals are exposed) are associated with

312 smaller numbers and lower diversity of AMR genes, as has been shown in previous
313 studies[24, 25]. The statistically significant correlation between the amount of
314 antimicrobial used, and the number of different AMR genes detected for each farm
315 clearly demonstrates this relationship and supports this as a driver of AMR. The use
316 of Hi-C metagenomics allows a deeper investigation of the relationship between the
317 use of antimicrobials, AMR genes and the bacteria that harbour those genes.

318

319 Of note from this study, is the demonstration of a particular AMR gene, *Inu(A)* that
320 was only found in samples from CV farms. Within CV farms we found this gene to be
321 harboured in three different species of *Lactobacillus*. All three species of
322 *Lactobacillus* (*L. amylovorus*, *L. Johnsonii* and *L. reuteri*) were also found in OG
323 farms and the distance tree would suggest that similar levels of diversity are present
324 for each species, whether present on an OG or a CV farm. There is good evidence
325 that the *Inu(A)* gene is carried on the same plasmid for all three *Lactobacillus*
326 species, suggesting that any selection pressure selects for the mobile plasmid rather
327 than the host bacteria. The small number of farms, and potential confounders such
328 as geographical bias may have influenced the observed distribution and so this result
329 needs to be confirmed. Nonetheless, the detection of AMR genes, carried on a
330 plasmid, in multiple species without culture could only be performed using
331 chromosome conformation metagenomics techniques such as Hi-C.

332

333 A direct assessment of the quality of a Hi-C MAG was afforded by the parallel culture
334 and sequencing of an *E. coli* isolate from the same sample. The homologous Hi-C
335 MAG contained the same MLST and AMR genes as the assembly obtained from

336 conventional culture and sequencing, including AMR genes likely present on a
337 plasmid.

338
339 Taxonomic identification of shotgun metagenome assemblies is widely recognised as
340 problematic. We used GTDB-Tk[26], a method based on a significantly larger
341 genome set than previous algorithms (*e.g.* CheckM) but were still not able to resolve
342 the taxonomy of many large clades of interest beyond the class level. The
343 chromosome conformation methodology has the potential to generate better quality
344 MAGs by generating links between contigs to improve binning or scaffolding. Greater
345 use of Hi-C metagenomics will enable the production of better quality MAGs for rare
346 or difficult to culture bacteria. Use of the HAM-ART pipeline should also give a lower
347 likelihood of generating mixed or contaminated MAGs.

348
349 We performed further investigations to confirm that the use of pooled Hi-C libraries (2
350 per farm) did not lead to artefactual assembly of MAGs from all 5 of the shotgun
351 libraries that used the same pooled Hi-C library to identify connection pairs. The use
352 of pooled Hi-C libraries reduced costs considerably in terms of staff time and finance.
353 It is clear from an examination of the distribution of taxa among the samples that
354 there are numerous examples of clades/taxa which we only found in a single sample
355 from a set of 5 sharing the same Hi-C library. Out of our total of 6164 MAGs we
356 would have expected equal distribution between OG and CV farms but only 2176
357 came from OG farms and 3988 from CV farms. While this may have occurred due to
358 a lower species diversity present in the OG farms, it is likely to be a consequence of
359 the larger number of lower yielding Hi-C libraries generated from the OG farms.

360

361 The sensitivity threshold for the creation of a MAG from a species contained within a
362 microbiome using Hi-C metagenomic sequencing will be affected by three things.
363 Firstly, the size of the genome of the species of interest (which is likely to be a minor
364 effect); secondly, the amount of sequencing undertaken; and thirdly, the relative
365 abundance of the species of interest which is probably the most significant influence.
366 The relative abundance of a particular bacterial species may limit the power of the
367 technique when a species of interest may only be present in low numbers. It is likely
368 that there will be some species harbouring AMR genes of interest that are present
369 below a threshold of 1:500 (that we estimate as our theoretical threshold from the *E.*
370 *coli* content comparison). We used ARIBA to independently assemble AMR genes
371 from our short-read sequencing data and did not find any significant discrepancy
372 between the genes assembled with this method and those found in the MAGs.
373 Indicating that where a gene can be assembled, the Hi-C technique is able to place it
374 in a MAG.

375
376 In summary, we successfully established a laboratory and bioinformatics Hi-C
377 metagenomics pipeline HAM-ART and used it to address a research question using a
378 set of 100 separate samples. We optimised HAM-ART to deal with mixed MAGs, to
379 exploit reference MAGs from within the experimental data set, and to assign AMR
380 genes to the correct MAGs with maximum sensitivity and specificity. While the
381 pipeline focusses on AMR gene tracking for this study, it could be used on other
382 dedicated gene sets (for example a library of virulence-associated genes) to
383 associate these to the host genome. Moreover, it provides a cost-effective strategy to
384 assess the dynamics of AMR transfer longitudinally following treatment with specific
385 antibiotics or doses, and following experimental infection. We validated our assembly

386 quality and AMR gene associations by comparing a MAG to one obtained from a
387 cultured *E. coli* from the same faecal sample. We have also shown that the method is
388 robust and affordable when processing large number of samples and provide data
389 illustrating the operational characteristics of both the wet laboratory and bioinformatic
390 protocols involved.

391 **Methods**

392 **Study population, sampling and data collection.** Ethical approval for the sampling
393 and the collection of data was obtained (CR295; University of Cambridge,
394 Department of Veterinary Medicine). All of the pig farms sampled were located in
395 southern England and were selected arbitrarily from a list of volunteering farms. The
396 farm descriptors are shown in Supplementary Table S1. We sampled five CV pig
397 farms and five OG pig farms that were members of the Soil Association farm
398 assurance scheme (which stipulates strict controls on the use of antimicrobials). Ten
399 fresh faecal droppings per farm were collected from different groups of fattening pigs
400 aged between 4-20 weeks, transported on ice/cold packs and stored at -80°C within
401 6 h of collection. Information on the use of antimicrobials in the one-year period prior
402 to sampling was collected by questionnaire informed by the farm records. The annual
403 use of antimicrobials in mg/number of Population Correction Unit (PCU) was
404 calculated by dividing the total amount of each antibiotic used over the course of a
405 year by the total average liveweight of the animals on the farm taking into account
406 the numbers of pigs and their ages.

407
408 **Enrichment of the microbial fraction from pig faeces.** The microbial fraction from
409 a faecal sample was enriched using an adaptation of a previously described method
410 by Ikeda *et al.*[27]. Prior to the enrichment process, 0.5 g of faeces was re-
411 suspended in 9 ml of saline and homogenised for 2 min in a Stomacher 80 (Seward)
412 at high power. Debris was removed from the homogenised sample by centrifugation
413 at 500 g for 1 min. The supernatant was then transferred on top of 3.5 ml of sterile
414 80% (w/v) Histodenz (Sigma) and centrifuged in a Beckman ultracentrifuge using a
415 JLA 16.250 rotor at 10,000 g for 40 min at 4°C . After centrifugation, the layer on top

416 of the insoluble debris was recovered into a new 15 ml tube (Falcon) and centrifuged
417 at 500 g for 1 min to remove debris. The supernatant was moved to a new 15 ml tube
418 (Falcon) and centrifuged at 10,000 g for 20 min at 4°C. The bacterial pellet was
419 washed in 10 ml of TE buffer (Merck) and used for the generation of Hi-C libraries.

420

421 **Fixation of bacterial cells with formaldehyde.** The isolated bacterial fractions from
422 faeces (described in the previous section) were mixed with 2.5% (v/v) formaldehyde
423 (16% methanol-free formaldehyde, Sigma) and incubated at room temperature (RT)
424 for 30 min followed by 30 min at 4°C to facilitate cross-linking of DNA within each
425 bacterial cell. Formaldehyde was quenched with 0.25 M glycine (Merck) for 5 min at
426 RT followed by 15 min at 4°C. Fixed cells were collected by centrifugation (10 mins,
427 10000 rpm, 4°C) and stored at -80°C until further use. We pooled bacterial pellets of
428 five samples from the same farm to generate Hi-C libraries, thereby obtaining two Hi-
429 C libraries per farm.

430

431 **Generation of Hi-C libraries.** The method for the construction of bacterial Hi-C
432 libraries was adapted from Burton *et al.*[11]. Briefly, DNA from the fixed cells was
433 isolated by lysing bacterial pellets in lysozyme (Illumina) followed by mechanical
434 disruption using a Precellys Evolution bead beater (Bertin Technologies, France).
435 Isolated chromatin was split into four aliquots and digested for 3 h at 37°C using
436 *HpyCH4IV* restriction enzyme (New England Biolabs). Restriction fragment
437 overhangs were filled with biotinylated dCTP (Thermo Scientific) and Klenow (New
438 England Biolabs) as described by van Berkum *et al.*[28]. Biotin labelled digested
439 chromatin was diluted in 8 ml of ligation buffer (New England Biolabs, T4 ligase kit)
440 and proximity ligation was performed at 16°C for 4 h. De-cross-linking was performed

441 at 65°C overnight (o/n) with 250 µg/ml proteinase K (QIAGEN). DNA was recovered
442 upon precipitation with 50% (v/v) isopropanol (Fischer Scientific) in the presence of
443 5% (v/v) 3M sodium acetate (pH 5.2) (Merck) and then treated with RNase A
444 (QIAGEN). Finally, DNA from each sample was recovered in 50 µl TE buffer (Merck)
445 upon phenol-chloroform (Merck) extraction. For Hi-C libraries, biotin from the un-
446 ligated DNA ends was removed by T4 Polymerase (New England Biolabs). DNA was
447 purified using the Monarch PCR and DNA Clean-up Kit (New England Biolabs).

448
449 **Generation of Hi-C Illumina sequencing libraries.** Illumina sequencing libraries
450 were constructed from purified DNA obtained after Hi-C library preparations using
451 NEBNext Ultra II DNA library prep kit (New England Biolabs). Approximately, 100 ng
452 of DNA of Hi-C libraries was sheared to 400 bp using a Covaris M220 (duty cycle
453 20%, 200 cycles per burst, peak incident power 50W, treatment time 40 s; Covaris
454 Ltd., UK). Ends of the sheared fragments were repaired, adaptors ligated, and
455 samples were indexed as described in manufacturer's protocols. Before the indexing,
456 we performed semi-quantitative PCR to determine the optimal cycle range for
457 indexing.

458
459 **Metagenome sequencing.** Metagenomic DNA was isolated from 0.25 g of faeces
460 using Precellys Soil DNA kit (Bertin Technologies, France). Libraries for shotgun
461 metagenome Illumina sequencing were prepared using the NEBNext Ultra II DNA
462 library prep kit (New England Biolabs) upon shearing 250 ng of metagenomic DNA to
463 400 bp with Covaris M220 (duty cycle 20%, 200 cycles per burst, peak incident
464 power 50W, treatment time 50 s; Covaris Ltd., UK).

465

466 **Illumina sequencing of shotgun metagenomic and Hi-C libraries.** Following DNA
467 library preparation, the library size was determined with a Bioanalyzer 2100 (Agilent),
468 quantified using the Qubit dsDNA BR kit (Thermo Scientific), pooled appropriately,
469 and analysed with the NEBNext library quant kit (New England Biolabs). The pooled
470 library was subjected to 150 bp paired-end sequencing on the HiSeq 4000 platform
471 (Genomics core facility, Li Ka Shing Centre, University of Cambridge – as 4 shotgun
472 libraries per Illumina HiSeq lane, 1 Hi-C library per Illumina HiSeq lane).

473
474 **Bioinformatics pipeline – pre-processing and de-novo assembly.** Next
475 generation sequencing raw data files were pre-processed in different ways according
476 to the sequencing library they were derived from. Shotgun metagenomics
477 sequencing data passed through two filtering / quality control steps: (i) optical and
478 PCR duplication removal by using clumpify.sh script from the BBMap software
479 package (<https://sourceforge.net/projects/bbmap/>); (ii) removal of read pairs matching
480 with the host genome using Bowtie2[29] and the pig reference genome (Sscrofa11.1:
481 GCA_000003025.6). As we performed bacterial cell enrichment during the Hi-C
482 library preparation, we only filtered the raw reads for optical and PCR duplications by
483 using the above-mentioned method. Both raw datasets passed through a merging
484 step, where overlapping (at least 30 nucleotide) reads were merged to one single
485 read, using FLASH software[30]. After merging, metagenomic sequencing reads
486 were passed to the assembly step as paired-end (un-merged) or single-end (merged)
487 reads. All Hi-C sequencing reads were processed further by a Perl script that
488 detected the modified restriction site (in our case A|CGT is modified to ACGCGT)
489 and re-fragmented reads accordingly. This step ensured that hybrid DNA fragments
490 were not used in the assembly step. The pre-processed sequence reads from both

491 libraries were used in *de novo* metagenomic assembly to build up contigs from
492 overlapping reads by using metaSPAdes[31]. To avoid the introduction of any bias
493 towards known species, we did not use any reference sequence-based assembly
494 method.

495

496 **Bioinformatics pipeline – post-processing.** Re-fragmented and unmerged Hi-C
497 reads were realigned to the contigs from the assembly by Bowtie2[29] to extract the
498 binary contact information between DNA fragments. The complete list of binary
499 contacts was then transformed to a weighted list and fed into the Louvain algorithm
500 (<https://sourceforge.net/projects/louvain/>) for 100 iterations of network resolution.
501 Contigs that were clustered together in all 100 iterations were put in the same
502 consensus cluster (CC).

503

504 This network resolution method means that a contig can only be assigned to one
505 cluster which may have two unwanted consequences. Firstly, contigs from two or
506 more closely related species may be assigned to the same CC due to sequence
507 homology. The separation of mixed CCs is first addressed using a coverage
508 distribution-based separation algorithm for each CC which splits the CC if the
509 distribution of sequencing coverage was clearly multimodal. The second
510 consequence is that contigs that are shared may not be correctly assigned to all of
511 the CCs that should contain copies (*e.g.* a plasmid possessed by two or more
512 species as a result of HGT). An iterative CC extension step was built into the pipeline
513 at this point to extend clusters based on the Hi-C inter-contig contacts and cautiously
514 identify contigs that should be allocated to multiple CCs.

515

516 Final MAGs were annotated for AMR genes using BLAST[32] using the ResFinder
517 database[33] and taxonomically profiled by GTDB-Tk[26]. AMR genes were also
518 identified from the raw metagenomics sequence reads using ARIBA[34] and
519 compared to the MAG assembly AMR associations to identify the absence of any
520 AMR genes in the final MAGs.

521
522 A further clade refinement step in the pipeline exploits the availability of data from
523 multiple samples of the same type (e.g. the other faeces samples from the same
524 study).

525
526 **Bioinformatics pipeline – clade refinement.** This part of the pipeline undertakes a
527 new scaffolding iteration using reference genomes from the previous scaffolding
528 attempt. The main steps of this process were: (i) performing pairwise sequence
529 comparisons between all MAGs (from all samples) by using MASH[35, 36]; (ii) using
530 the UPGMA (unweighted pair group method with arithmetic mean) algorithm on
531 pairwise distance data to form clades of closely related MAGs (distance threshold in
532 UPGMA for clade definition: 0.12); (iii) select an exemplar MAG in the clade to use as
533 a within-clade reference sequence; (iv) use the exemplar reference sequence to
534 extract highly similar contigs (using BLAST[32]) from the original full contig collection
535 of the *de novo* assembly for each of the other samples; (v) use Hi-C contact data to
536 refine the collection of contigs extracted by reference search and exclude contigs
537 with no Hi-C contact to other contigs within the MAG; (vi) use Hi-C contacts to extend
538 MAGs with AMR gene containing contigs; (vii) perform a final extension on the MAGs
539 (with the same method as used in the post-processing). We found that the most
540 crucial step during the refinement was the selection of the clade exemplar in the

541 clade that potentially had the most complete genome with minimal contamination.
542 After several attempts of using physical parameters (e.g. using the largest, the
543 median size, the most unimodal coverage distribution) we found that mixed MAGs
544 (mixture of more than one closely related genomes) were also selected as exemplars
545 many times. Therefore, instead of using the above mentioned parameters alone or in
546 combination, we used the core single copy gene set of the GTDB-Tk[26] by running
547 the toolkit “identify” module and looking for: (i) the MAG with the highest number of
548 unique single copy genes (maximum completeness); and (ii) the MAG with the
549 highest unique single copy genes / multiple single copy genes ratio (minimum
550 contamination).

551
552 **Bioinformatics pipeline - analysis of MAG sequence data:** A set of custom scripts
553 were written to perform AMR gene searches, undertake taxonomic identification,
554 identify closely related reference genomes, and generate paired distance trees for
555 the clades. AMR gene searching was performed using a local installation of
556 BLAST[32] using the ResFinder database[33]. AMR genes were defined as being
557 present where >60% of the length of the target gene was present with an identity of
558 >80%. For AMR gene grouped analysis: (i) the aminoglycoside modifying enzymes
559 were grouped by the modifying group which was attached (aminoglycoside
560 nucleotidyl transferases were grouped together, as were phosphotransferases,
561 acetyltransferases and adenytransferases); (ii) due to increasing interest in the role
562 ESBL plays in disease, beta-lactamases were grouped by homology; (iii) gene
563 families which were represented by different alleles were considered one gene type;
564 (iv) the dihydrofolate reductase genes *dfrA12* and *dfrA14* are considered as *dfrA*; (v)
565 nitrofurantonin reducing genes were grouped together into the *nim* group; (vi)

566 sulfonamide resistance genes *sul1-sul3* were grouped as *sul*; (vii) tetracycline
567 resistance genes were grouped by function and sequence homology, with
568 homologous genes combined into groups; and (viii) vancomycin resistance clusters
569 *vanGXY* and *vanG2XY* were considered as one group. Taxonomic identification and
570 the search for closely related reference genomes was performed using GTDB-Tk[26].
571 Pairwise distances between clade member MAGs and other genomes were
572 determined using MASH[35, 36], and the distance tree generated by the UPGMA
573 algorithm. The Newick formatted tree files were annotated using iTOL[37]. Summary
574 text files were automatically created for all clade members with taxonomic
575 identifications and AMR gene associations. A summarised output with all MAGs and
576 AMR gene associations was generated together with a filtered version where
577 incomplete MAGs (filtered out by the default settings of GTDB-Tk[26]) were
578 excluded. For a detailed workflow of the bioinformatics pipeline see Supplementary
579 Figure S2.

580
581 **Bioinformatics pipeline - Quality control:** We created custom scripts to extract
582 quality information from almost every step during the pipeline: (i) ratio of duplicated
583 raw reads (detecting low concentration libraries); (ii) ratio of merged raw read pairs
584 (verifying library sizes); (iii) ratio of merged Hi-C reads without detectable ligation site
585 (pointing to problems with Hi-C library preparation); (iv) number of contigs in the *de*
586 *novo* assembly; (v) Hi-C reads alignment ratio; (vi) number and ratio of informative
587 Hi-C read pairs (a Hi-C read pair is informative if it connects two different contigs);
588 (vii) average modularity during the Louvain network resolution; (viii) single copy gene
589 ratios during clade refinement; (ix) final CheckM-like MAG parameters analysed by
590 GTDB-tk[26] (MAG size, contig number, N50, average coverage, GC-content,

591 taxonomy, completeness). We performed traditional metagenomics assembly on a
592 set (n=11) of randomly selected samples using the MetaWRAP pipeline (default
593 threshold setting, MetaSpades assembler) [38] and compared the result with the
594 HAM-ART output. While we generally got a higher number of final MAGs from the
595 HAM-ART pipeline (average number of final MAGs 29.5 vs 50.1), due to a few
596 potentially lower quality Hi-C libraries, we had higher variation among the HAM-ART
597 final sets (standard deviation of the mean 13.9 vs 41.2).

598

599 **Statistical analysis.** Simple linear regressions were performed using R (ggplot2 and
600 ggpmisc packages). Spearman's method was used to determine the P value and
601 correlation coefficient.

602

603 **Sequencing data.** Chromosome conformation capture and metagenome sequencing
604 data have been deposited in the European Nucleotide Archive
605 (<http://www.ebi.ac.uk/ena>) and are available *via* study accession number [To be
606 deposited before final submission].

607

608

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611

612 **Author contributions**

613 SPWdeV, AWT, MPS, JLNW, DJM, AJG, and MAH designed experiments.

614 LK, SG, IRLK, AJG, and MAH wrote the manuscript.

615 LK, SG, IRLK, SPWdeV, MB, OR, AWT, MPS, JLNW, DJM, AJG, and MAH edited
616 the manuscript.

617 SG, IRLK, XB, NH, EL, SPWdeV, HB, and JH-G performed the experimental work
618 and collected field data.

619 LK designed the HAM-ART pipeline; IRLK, MB, and MAH performed the
620 bioinformatic analyses.

621 LK, SG, IRLK, SPWdeV, OR, AJG, and MAH analysed results.

622

623 **Competing financial interest**

624 The authors declare no competing financial interests.

625

626

627 **Supplemental information**

628

629 **Table S1** | Characteristics of the farms used in the study. The conventional or high-
630 antimicrobial use farms are labelled CV_1 to 5 and the organic, or low antimicrobial
631 use farms are labelled OG_1 to 5.

632

633 **Table S2** | Complete list of MAGs and their AMR gene associations from 100 pig
634 faecal samples. Columns in the tab-separated table are: Farm and sample
635 identification (*e.g.* CV3_2 stands for sample 2 from conventional farm 3); Type of the
636 farm (organic / conventional); Clade identifier; Size of the assembly (in kilobases);
637 Number of contigs in the MAG; N50 of the MAG; Weighted mean coverage of the
638 contigs; GC content of the MAG; GTDB-tk taxonomy string; percentage of the
639 multiple sequence alignment (by GTDB-tk) spanned by the genome; The rest of the
640 columns indicate the presence (1) or absence (0) of the particular AMR gene within
641 the MAG.

642

643 **Figure S1** | Composition of the microbiota on the studied pig farms in domain,
644 phylum, class, order and family levels.

645

646 **Figure S2** | Detailed bioinformatics pipeline workflow separated to pre-assembly,
647 post-assembly and clade refinement. Text is coloured black for descriptions and
648 white for the used software / script background.

649

650

651

652

653 **References**

- 654 1. Stokes HW, Gillings MR. Gene flow, mobile genetic elements and the
655 recruitment of antibiotic resistance genes into Gram-negative pathogens. *FEMS*
656 *microbiology reviews*. 2011;35(5):790-819. Epub 2011/04/27. doi: 10.1111/j.1574-
657 6976.2011.00273.x. PubMed PMID: 21517914.
- 658 2. Baharoglu Z, Garriss G, Mazel D. Multiple Pathways of Genome Plasticity
659 Leading to Development of Antibiotic Resistance. *Antibiotics (Basel)*. 2013;2(2):288-
660 315. Epub 2013/01/01. doi: 10.3390/antibiotics2020288. PubMed PMID: 27029305;
661 PubMed Central PMCID: PMCPMC4790341.
- 662 3. Wright GD. The antibiotic resistome: the nexus of chemical and genetic
663 diversity. *Nature reviews Microbiology*. 2007;5(3):175-86. Epub 2007/02/06. doi:
664 10.1038/nrmicro1614. PubMed PMID: 17277795.
- 665 4. Faure S, Perrin-Guyomard A, Delmas JM, Chatre P, Laurentie M. Transfer of
666 plasmid-mediated CTX-M-9 from *Salmonella enterica* serotype Virchow to
667 *Enterobacteriaceae* in human flora-associated rats treated with cefixime. *Antimicrobial*
668 *agents and chemotherapy*. 2010;54(1):164-9. Epub 2009/11/11. doi:
669 10.1128/AAC.00310-09. PubMed PMID: 19901088; PubMed Central PMCID:
670 PMC2798505.
- 671 5. Guan J, Liu S, Lin Z, Li W, Liu X, Chen D. Severe sepsis facilitates intestinal
672 colonization by extended-spectrum-beta-lactamase-producing *Klebsiella pneumoniae*
673 and transfer of the SHV-18 resistance gene to *Escherichia coli* during antimicrobial
674 treatment. *Antimicrobial agents and chemotherapy*. 2014;58(2):1039-46. Epub
675 2013/11/28. doi: 10.1128/AAC.01632-13. PubMed PMID: 24277046; PubMed Central
676 PMCID: PMC3910833.

- 677 6. Dheilly A, Le Devendec L, Mourand G, Boudier A, Jouy E, Kempf I. Resistance
678 gene transfer during treatments for experimental avian colibacillosis. *Antimicrobial*
679 *agents and chemotherapy*. 2012;56(1):189-96. Epub 2011/10/12. doi:
680 10.1128/AAC.05617-11. PubMed PMID: 21986830; PubMed Central PMCID:
681 PMC3256041.
- 682 7. Doi Y, Adams-Haduch JM, Peleg AY, D'Agata EM. The role of horizontal gene
683 transfer in the dissemination of extended-spectrum beta-lactamase-producing
684 *Escherichia coli* and *Klebsiella pneumoniae* isolates in an endemic setting. *Diagnostic*
685 *microbiology and infectious disease*. 2012;74(1):34-8. Epub 2012/06/23. doi:
686 10.1016/j.diagmicrobio.2012.05.020. PubMed PMID: 22722012; PubMed Central
687 PMCID: PMC3427399.
- 688 8. Stecher B, Denzler R, Maier L, Bernet F, Sanders MJ, Pickard DJ, et al. Gut
689 inflammation can boost horizontal gene transfer between pathogenic and commensal
690 *Enterobacteriaceae*. *Proceedings of the National Academy of Sciences of the United*
691 *States of America*. 2012;109(4):1269-74. Epub 2012/01/11. doi:
692 10.1073/pnas.1113246109. PubMed PMID: 22232693; PubMed Central PMCID:
693 PMC3268327.
- 694 9. Allen HK, Looft T, Bayles DO, Humphrey S, Levine UY, Alt D, et al. Antibiotics
695 in feed induce prophages in swine fecal microbiomes. *mBio*. 2011;2(6). Epub
696 2011/12/01. doi: 10.1128/mBio.00260-11. PubMed PMID: 22128350; PubMed Central
697 PMCID: PMC3225969.
- 698 10. Beitel CW, Froenicke L, Lang JM, Korf IF, Michelmore RW, Eisen JA, et al.
699 Strain- and plasmid-level deconvolution of a synthetic metagenome by sequencing
700 proximity ligation products. *PeerJ*. 2014;2:e415. Epub 2014/06/12. doi:

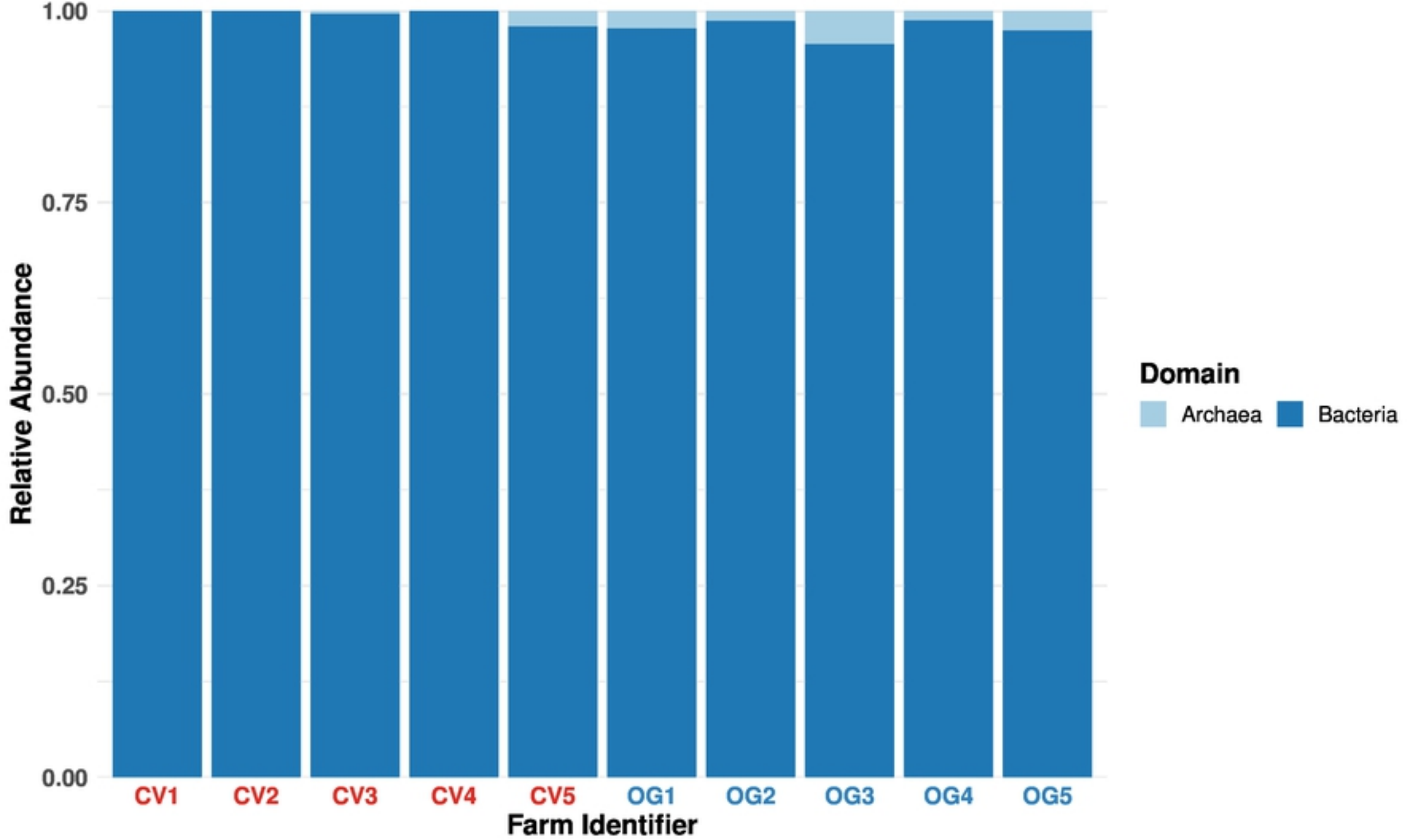
- 701 10.7717/peerj.415. PubMed PMID: 24918035; PubMed Central PMCID:
702 PMC4045339.
- 703 11. Burton JN, Liachko I, Dunham MJ, Shendure J. Species-level deconvolution of
704 metagenome assemblies with hi-C-based contact probability maps. *G3*.
705 2014;4(7):1339-46. Epub 2014/05/24. doi: 10.1534/g3.114.011825. PubMed PMID:
706 24855317.
- 707 12. Marbouty M, Cournac A, Flot JF, Marie-Nelly H, Mozziconacci J, Koszul R.
708 Metagenomic chromosome conformation capture (meta3C) unveils the diversity of
709 chromosome organization in microorganisms. *Elife*. 2014;3:e03318. Epub 2014/12/18.
710 doi: 10.7554/eLife.03318. PubMed PMID: 25517076; PubMed Central PMCID:
711 PMCPMC4381813.
- 712 13. Lamendella R, Domingo JW, Ghosh S, Martinson J, Oerther DB. Comparative
713 fecal metagenomics unveils unique functional capacity of the swine gut. *BMC*
714 *Microbiol*. 2011;11:103. Epub 2011/05/18. doi: 10.1186/1471-2180-11-103. PubMed
715 PMID: 21575148; PubMed Central PMCID: PMCPMC3123192.
- 716 14. Looft T, Johnson TA, Allen HK, Bayles DO, Alt DP, Stedtfeld RD, et al. In-feed
717 antibiotic effects on the swine intestinal microbiome. *Proceedings of the National*
718 *Academy of Sciences of the United States of America*. 2012;109(5):1691-6. Epub
719 2012/02/07. doi: 10.1073/pnas.1120238109. PubMed PMID: 22307632; PubMed
720 Central PMCID: PMCPMC3277147.
- 721 15. Xiao Y, Kong F, Xiang Y, Zhou W, Wang J, Yang H, et al. Comparative
722 biogeography of the gut microbiome between Jinhua and Landrace pigs. *Sci Rep*.
723 2018;8(1):5985. Epub 2018/04/15. doi: 10.1038/s41598-018-24289-z. PubMed PMID:
724 29654314; PubMed Central PMCID: PMCPMC5899086.

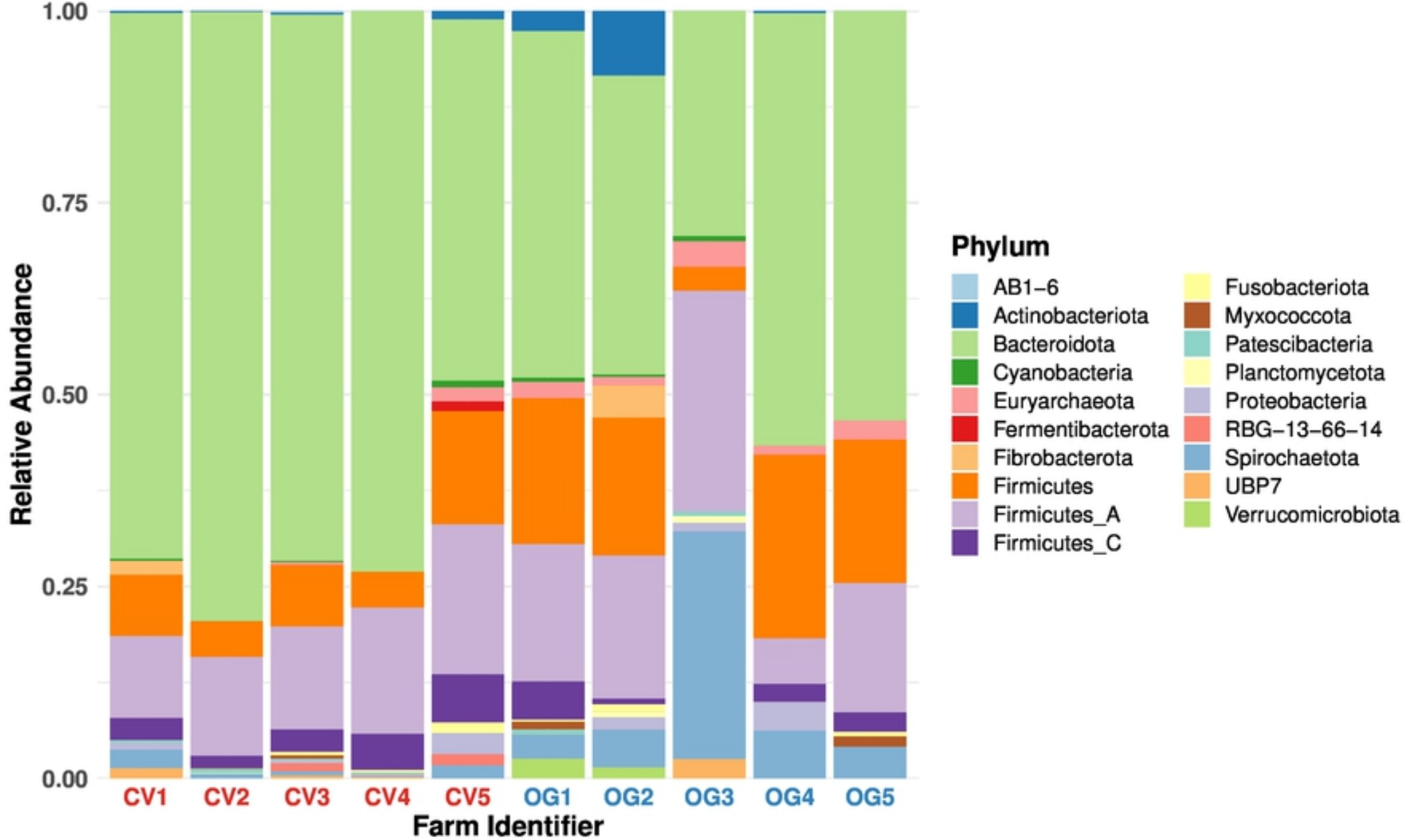
- 725 16. Gresse R, Chaucheyras Durand F, Duniere L, Blanquet-Diot S, Forano E.
726 Microbiota Composition and Functional Profiling Throughout the Gastrointestinal Tract
727 of Commercial Weaning Piglets. *Microorganisms*. 2019;7(9). Epub 2019/09/25. doi:
728 10.3390/microorganisms7090343. PubMed PMID: 31547478; PubMed Central
729 PMCID: PMCPMC6780805.
- 730 17. Le Sciellour M, Renaudeau D, Zemb O. Longitudinal Analysis of the Microbiota
731 Composition and Enterotypes of Pigs from Post-Weaning to Finishing.
732 *Microorganisms*. 2019;7(12). Epub 2019/12/05. doi:
733 10.3390/microorganisms7120622. PubMed PMID: 31795103.
- 734 18. Baudry L, Foutel-Rodier T, Thierry A, Koszul R, Marbouty M. MetaTOR: A
735 Computational Pipeline to Recover High-Quality Metagenomic Bins From Mammalian
736 Gut Proximity-Ligation (meta3C) Libraries. *Front Genet*. 2019;10:753. Epub
737 2019/09/05. doi: 10.3389/fgene.2019.00753. PubMed PMID: 31481973; PubMed
738 Central PMCID: PMCPMC6710406.
- 739 19. Bickhart DM, Watson M, Koren S, Panke-Buisse K, Cersosimo LM, Press MO,
740 et al. Assignment of virus and antimicrobial resistance genes to microbial hosts in a
741 complex microbial community by combined long-read assembly and proximity ligation.
742 *Genome Biol*. 2019;20(1):153. Epub 2019/08/04. doi: 10.1186/s13059-019-1760-x.
743 PubMed PMID: 31375138; PubMed Central PMCID: PMCPMC6676630.
- 744 20. Kent AG, Vill AC, Shi Q, Satlin MJ, Brito IL. Widespread transfer of mobile
745 antibiotic resistance genes within individual gut microbiomes revealed through
746 bacterial Hi-C. *Nat Commun*. 2020;11(1):4379. Epub 2020/09/03. doi:
747 10.1038/s41467-020-18164-7. PubMed PMID: 32873785; PubMed Central PMCID:
748 PMCPMC7463002.

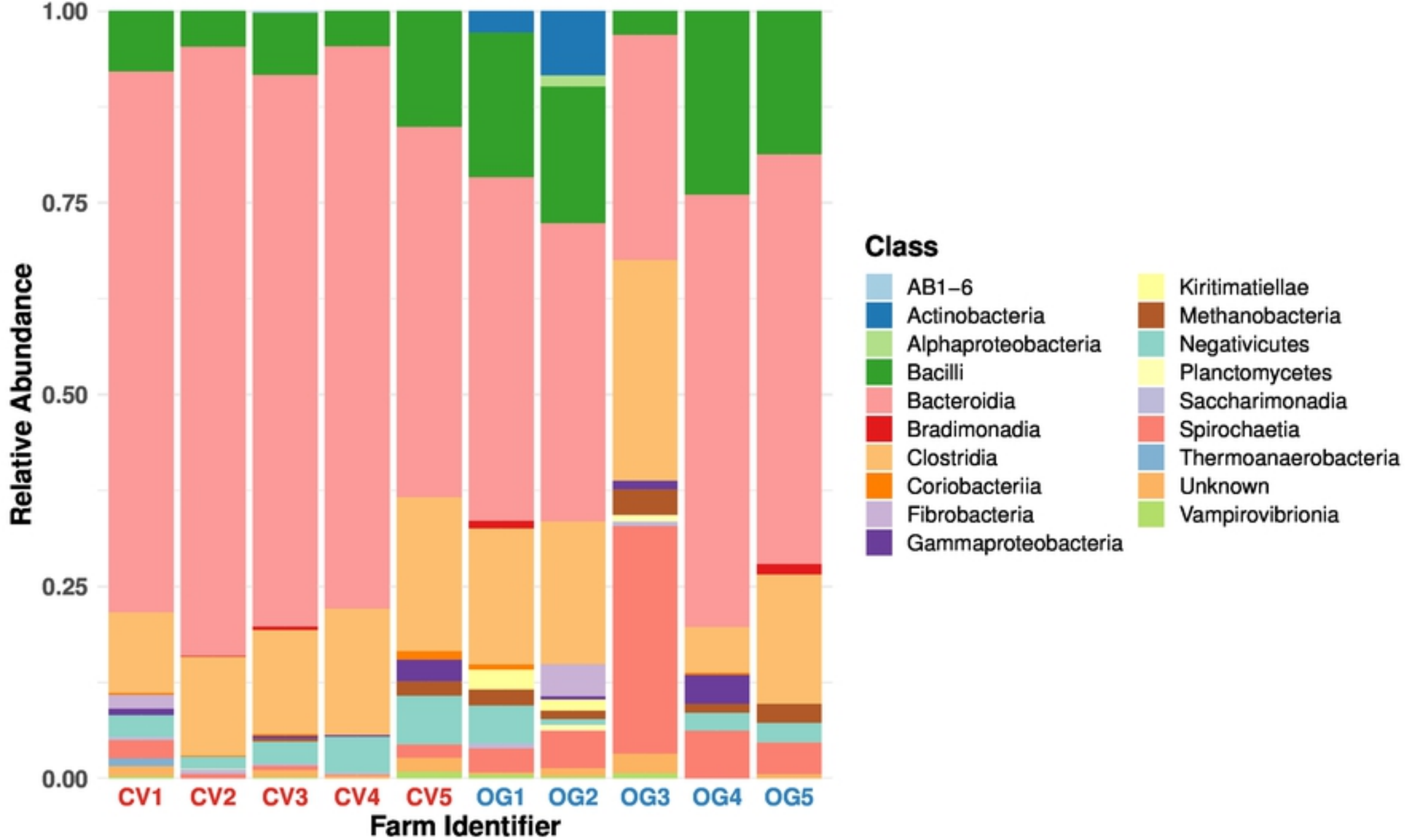
- 749 21. Yaffe E, Relman DA. Tracking microbial evolution in the human gut using Hi-C
750 reveals extensive horizontal gene transfer, persistence and adaptation. *Nat Microbiol.*
751 2020;5(2):343-53. Epub 2019/12/25. doi: 10.1038/s41564-019-0625-0. PubMed
752 PMID: 31873203; PubMed Central PMCID: PMC6992475.
- 753 22. Marbouty M, Baudry L, Cournac A, Koszul R. Scaffolding bacterial genomes
754 and probing host-virus interactions in gut microbiome by proximity ligation
755 (chromosome capture) assay. *Sci Adv.* 2017;3(2):e1602105. Epub 2017/02/25. doi:
756 10.1126/sciadv.1602105. PubMed PMID: 28232956; PubMed Central PMCID:
757 PMC65315449.
- 758 23. Stalder T, Press MO, Sullivan S, Liachko I, Top EM. Linking the resistome and
759 plasmidome to the microbiome. *ISME J.* 2019;13(10):2437-46. Epub 2019/05/31. doi:
760 10.1038/s41396-019-0446-4. PubMed PMID: 31147603; PubMed Central PMCID:
761 PMC6776055.
- 762 24. Cameron A, McAllister TA. Antimicrobial usage and resistance in beef
763 production. *J Anim Sci Biotechnol.* 2016;7:68. Epub 2016/12/22. doi: 10.1186/s40104-
764 016-0127-3. PubMed PMID: 27999667; PubMed Central PMCID: PMC5154118.
- 765 25. Schmidt VM, Pinchbeck G, McIntyre KM, Nuttall T, McEwan N, Dawson S, et
766 al. Routine antibiotic therapy in dogs increases the detection of antimicrobial-resistant
767 faecal *Escherichia coli*. *J Antimicrob Chemother.* 2018;73(12):3305-16. Epub
768 2018/09/15. doi: 10.1093/jac/dky352. PubMed PMID: 30215725.
- 769 26. Chaumeil PA, Mussig AJ, Hugenholtz P, Parks DH. GTDB-Tk: a toolkit to
770 classify genomes with the Genome Taxonomy Database. *Bioinformatics.* 2019. Epub
771 2019/11/16. doi: 10.1093/bioinformatics/btz848. PubMed PMID: 31730192.
- 772 27. Ikeda S, Kaneko T, Okubo T, Rallos LE, Eda S, Mitsui H, et al. Development of
773 a bacterial cell enrichment method and its application to the community analysis in

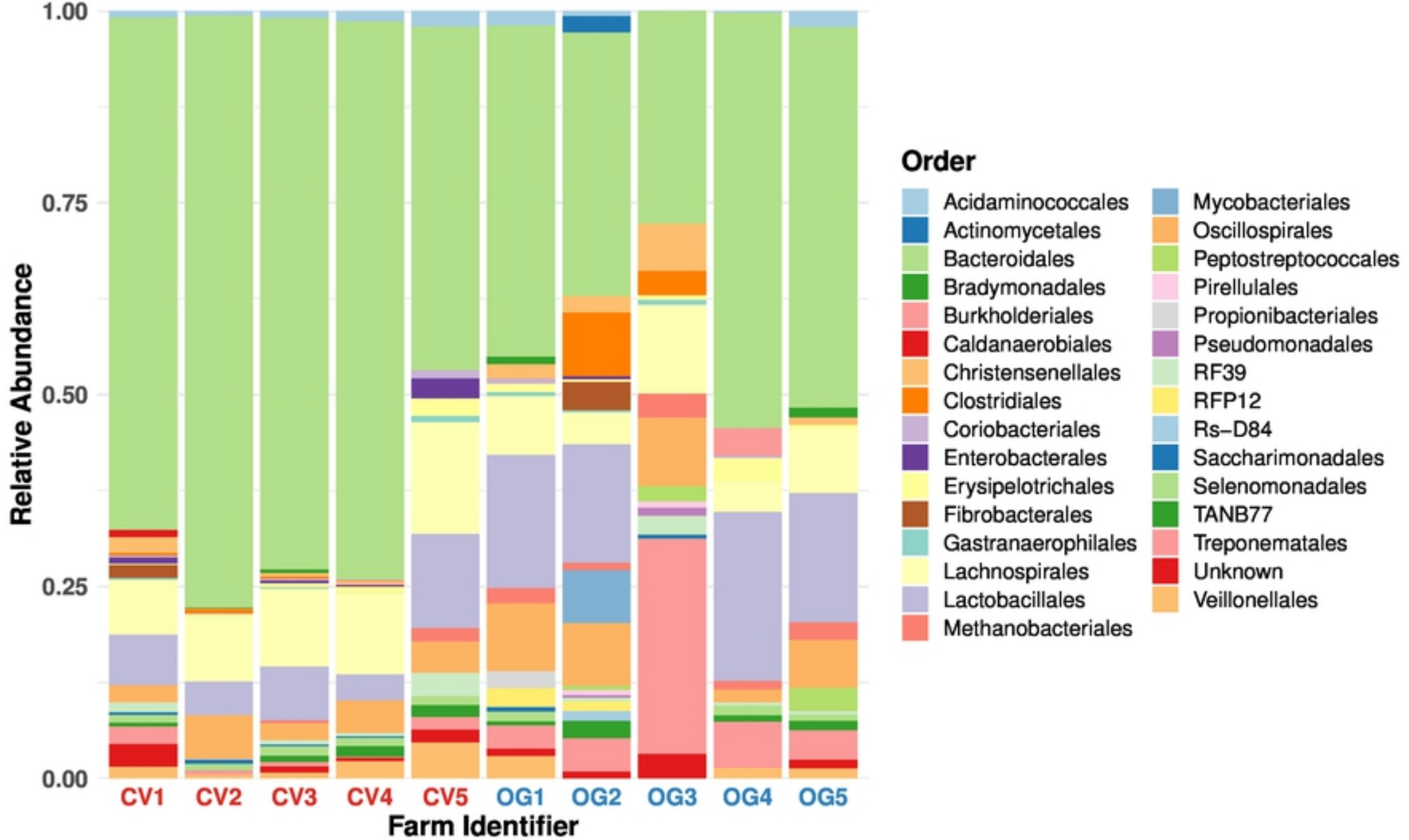
- 774 soybean stems. *Microb Ecol.* 2009;58(4):703-14. Epub 2009/08/08. doi:
775 10.1007/s00248-009-9566-0. PubMed PMID: 19662454.
- 776 28. van Berkum NL, Lieberman-Aiden E, Williams L, Imakaev M, Gnirke A, Mirny
777 LA, et al. Hi-C: a method to study the three-dimensional architecture of genomes.
778 *Journal of visualized experiments : JoVE.* 2010;(39). Epub 2010/05/13. doi:
779 10.3791/1869. PubMed PMID: 20461051; PubMed Central PMCID: PMC3149993.
- 780 29. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. *Nature*
781 *methods.* 2012;9(4):357-9. Epub 2012/03/06. doi: 10.1038/nmeth.1923. PubMed
782 PMID: 22388286; PubMed Central PMCID: PMC3322381.
- 783 30. Magoc T, Salzberg SL. FLASH: fast length adjustment of short reads to improve
784 genome assemblies. *Bioinformatics.* 2011;27(21):2957-63. Epub 2011/09/10. doi:
785 10.1093/bioinformatics/btr507. PubMed PMID: 21903629; PubMed Central PMCID:
786 PMCPMC3198573.
- 787 31. Nurk S, Meleshko D, Korobeynikov A, Pevzner PA. metaSPAdes: a new
788 versatile metagenomic assembler. *Genome Res.* 2017;27(5):824-34. Epub
789 2017/03/17. doi: 10.1101/gr.213959.116. PubMed PMID: 28298430; PubMed Central
790 PMCID: PMCPMC5411777.
- 791 32. Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, et al.
792 BLAST+: architecture and applications. *BMC bioinformatics.* 2009;10:421. Epub
793 2009/12/17. doi: 10.1186/1471-2105-10-421. PubMed PMID: 20003500; PubMed
794 Central PMCID: PMC2803857.
- 795 33. Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, et
796 al. Identification of acquired antimicrobial resistance genes. *J Antimicrob Chemother.*
797 2012;67(11):2640-4. Epub 2012/07/12. doi: 10.1093/jac/dks261. PubMed PMID:
798 22782487; PubMed Central PMCID: PMC3468078.

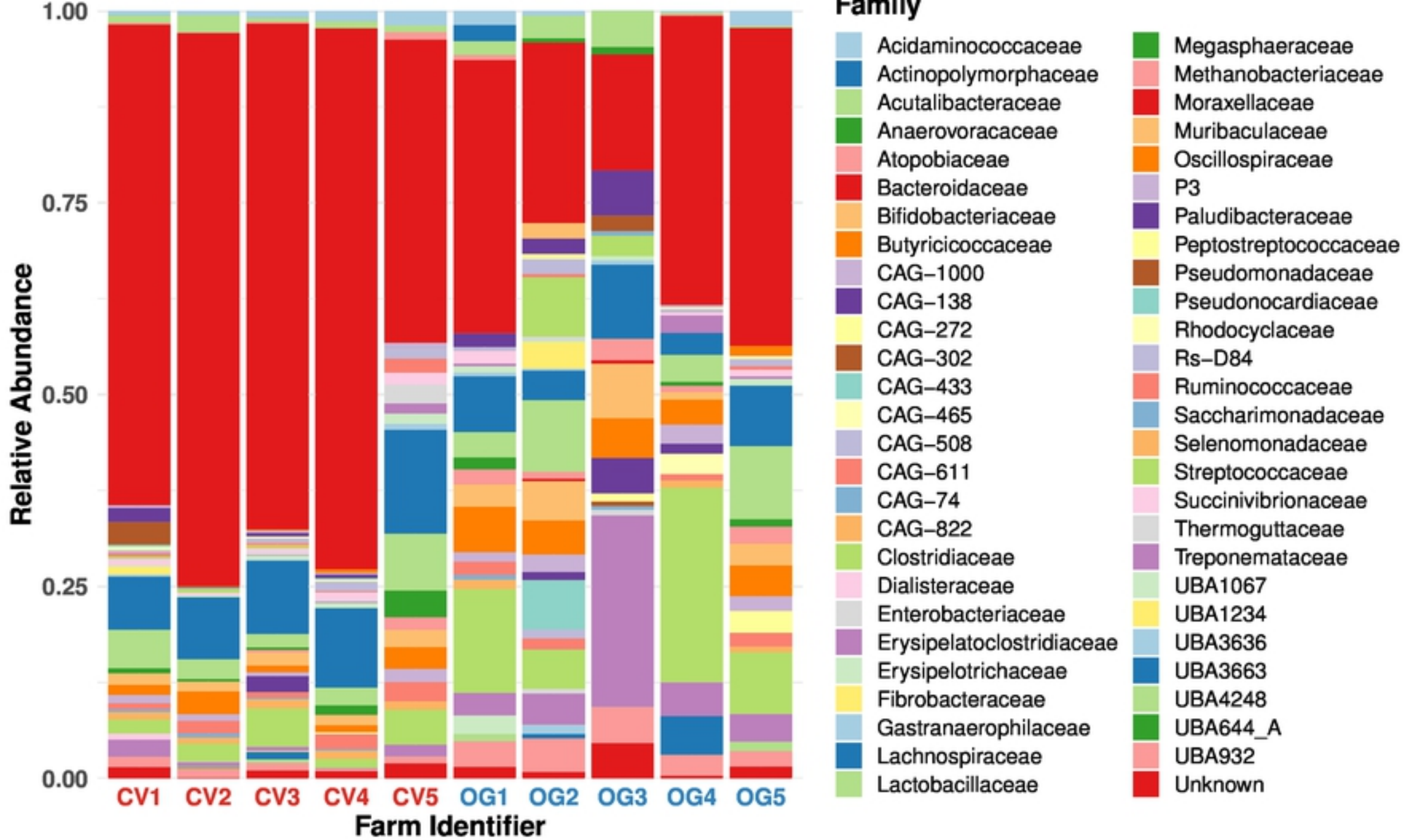
- 799 34. Hunt M, Mather AE, Sanchez-Buso L, Page AJ, Parkhill J, Keane JA, et al.
800 ARIBA: rapid antimicrobial resistance genotyping directly from sequencing reads.
801 Microb Genom. 2017;3(10):e000131. Epub 2017/11/28. doi: 10.1099/mgen.0.000131.
802 PubMed PMID: 29177089; PubMed Central PMCID: PMC65695208.
- 803 35. Ondov BD, Starrett GJ, Sappington A, Kostic A, Koren S, Buck CB, et al. Mash
804 Screen: high-throughput sequence containment estimation for genome discovery.
805 Genome Biol. 2019;20(1):232. Epub 2019/11/07. doi: 10.1186/s13059-019-1841-x.
806 PubMed PMID: 31690338; PubMed Central PMCID: PMC6833257.
- 807 36. Ondov BD, Treangen TJ, Melsted P, Mallonee AB, Bergman NH, Koren S, et
808 al. Mash: fast genome and metagenome distance estimation using MinHash. Genome
809 Biol. 2016;17(1):132. Epub 2016/06/22. doi: 10.1186/s13059-016-0997-x. PubMed
810 PMID: 27323842; PubMed Central PMCID: PMC4915045.
- 811 37. Letunic I, Bork P. Interactive Tree Of Life (iTOL) v4: recent updates and new
812 developments. Nucleic Acids Res. 2019;47(W1):W256-W9. Epub 2019/04/02. doi:
813 10.1093/nar/gkz239. PubMed PMID: 30931475; PubMed Central PMCID:
814 PMC6602468.
- 815 38. Uritskiy GV, DiRuggiero J, Taylor J. MetaWRAP-a flexible pipeline for genome-
816 resolved metagenomic data analysis. Microbiome. 2018;6(1):158. Epub 2018/09/17.
817 doi: 10.1186/s40168-018-0541-1. PubMed PMID: 30219103; PubMed Central PMCID:
818 PMC6138922.
- 819

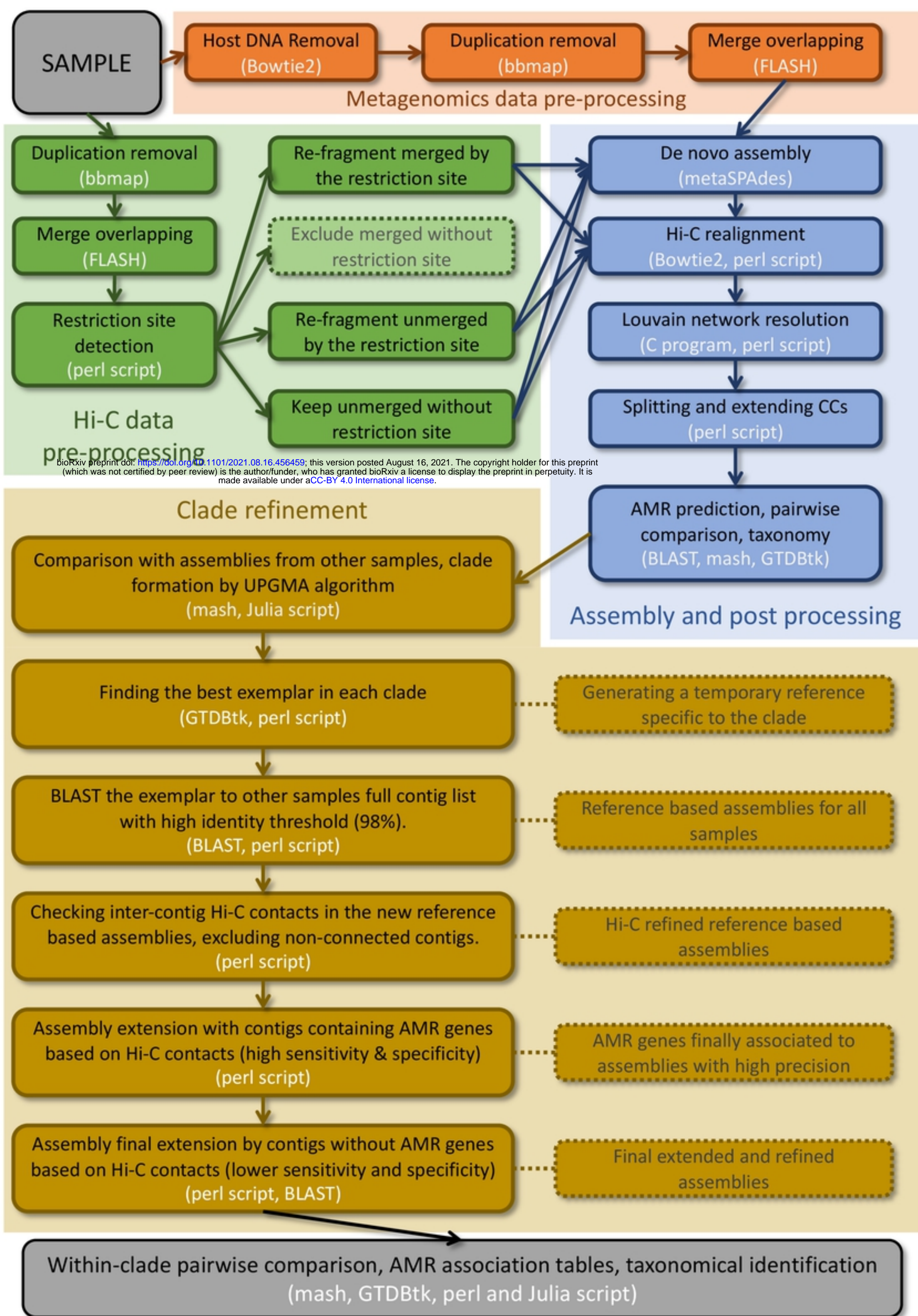












Supplementary figure 2

Supplementary Table S1. Characteristics of the farms used in the study. The conventional or high-antibiotic use farms are labelled CV_1 to 5 and the organic, or low antibiotic use farms are labelled OG_1 to 5

Farm ID	Location	Farm type	Total pigs	Date of sampling
CV_1	Suffolk	Finishing farm	1300	02/02/2017
CV_2	Norfolk	Finishing farm	1000	09/02/2017
CV_3	Norfolk	Finishing farm	1700	16/02/2017
CV_4	Suffolk	Finishing farm	1990	16/02/2017
CV_5	Suffolk	Farrow to finish	1000	17/03/2017
OG_1	Buckinghamshire	Farrow to finish	564	26/01/2017
OG_2	Sussex	Farrow to finish	808	21/02/2017
OG_3	Hampshire	Farrow to finish	604	21/02/2017
OG_4	Wiltshire	Farrow to finish	1266	27/02/2017
OG_5	Gloucestershire	Farrow to finish	700	27/02/2017

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