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

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Abstract

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

Author Summary

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

mobile genetic elements, such as plasmids, that would not otherwise be included in these assembled genomes.

Introduction

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

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

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

Results

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

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

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

Generation of MAGs using the HAM-ART pipeline

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

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

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

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

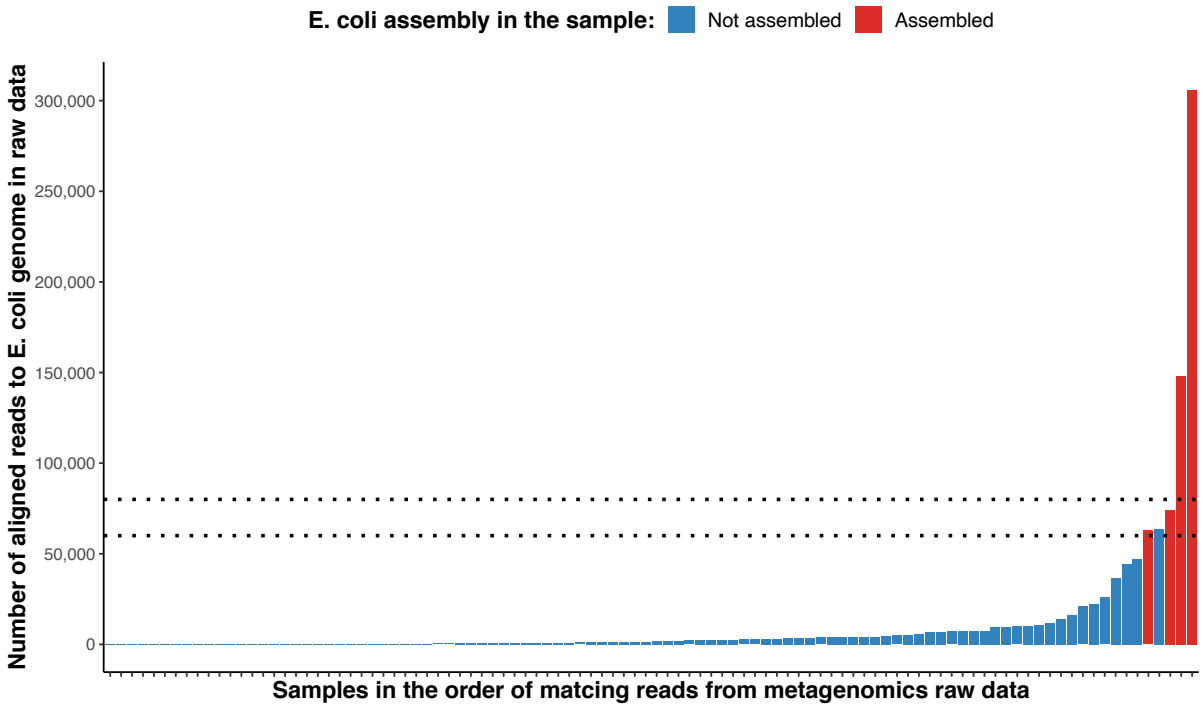


Figure 1. Presence of *E. coli* DNA in all samples.

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

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

This shows that although reads were present in the majority of shotgun libraries, it was only when there were ≥50k *E. coli* reads that a MAG could be created. This observation suggests that 60-80k reads (representing about 9-12 Mbp), approximating to 2x coverage of an *E. coli* genome are required in order to generate a MAG. In this study, which generated approximately 35M reads (5.25 x10^9 bp) for each sample, an individual species would need to represent ~0.2% of the total bacterial community in order to generate a MAG.

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

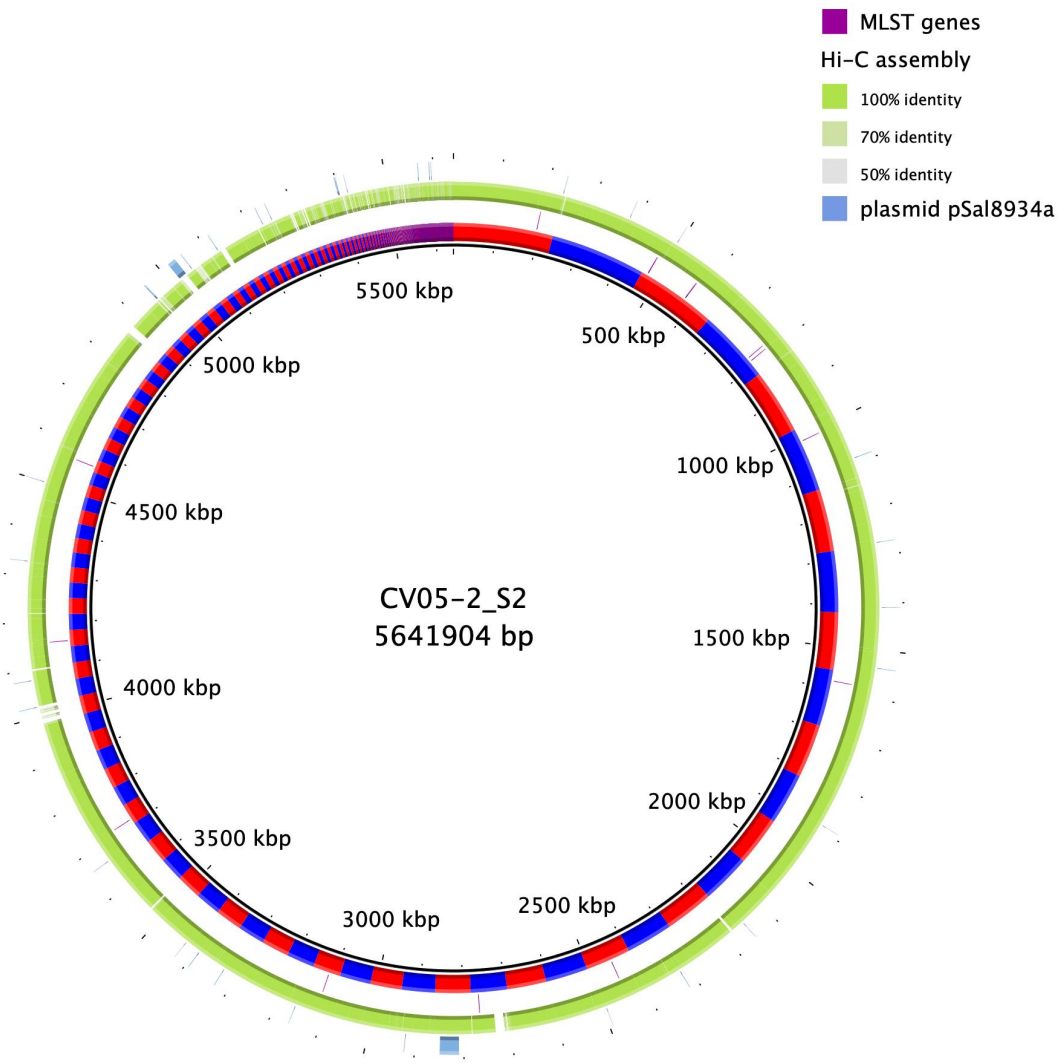


Figure 2. BLAST comparison of an *E. coli* MAG with a corresponding *E. coli* assembly obtained using culture, followed by Illumina MiSeq sequencing and assembly.

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

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

Taxa composition of the pig microbiomes from conventional and organic farms

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

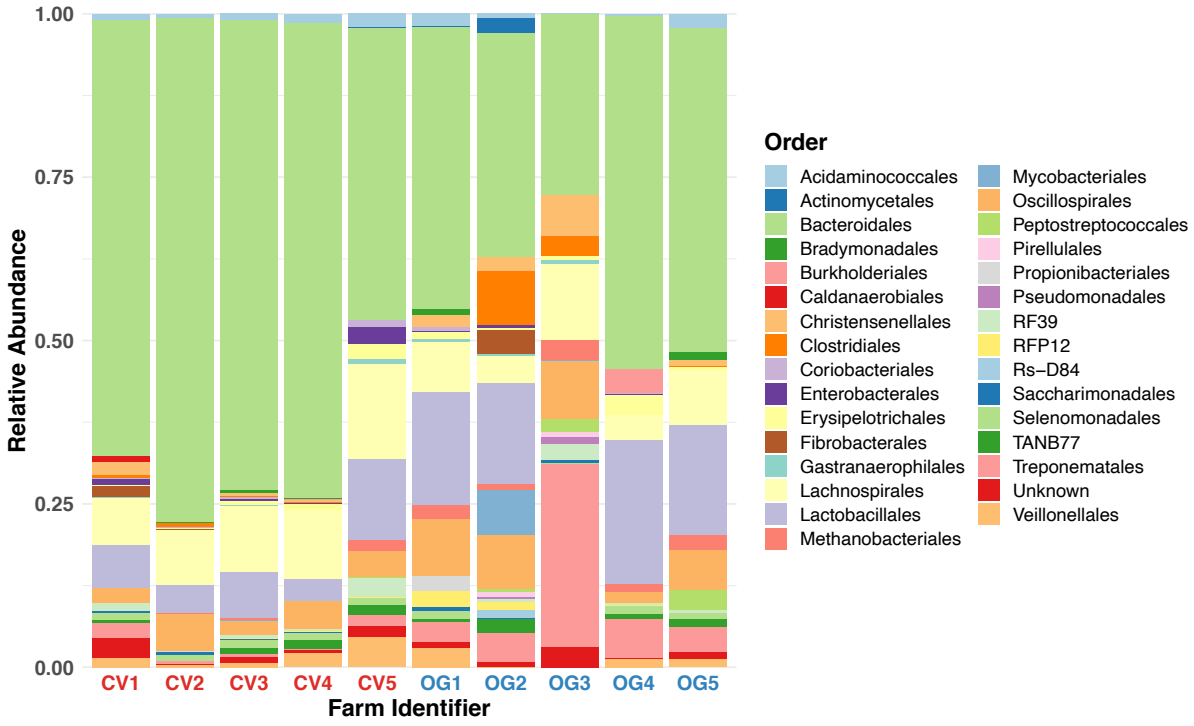


Figure 3. Order-level relative composition of the pig faecal microbiota in the study farms.

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

AMR gene distribution in faecal samples from CV and OG farms

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

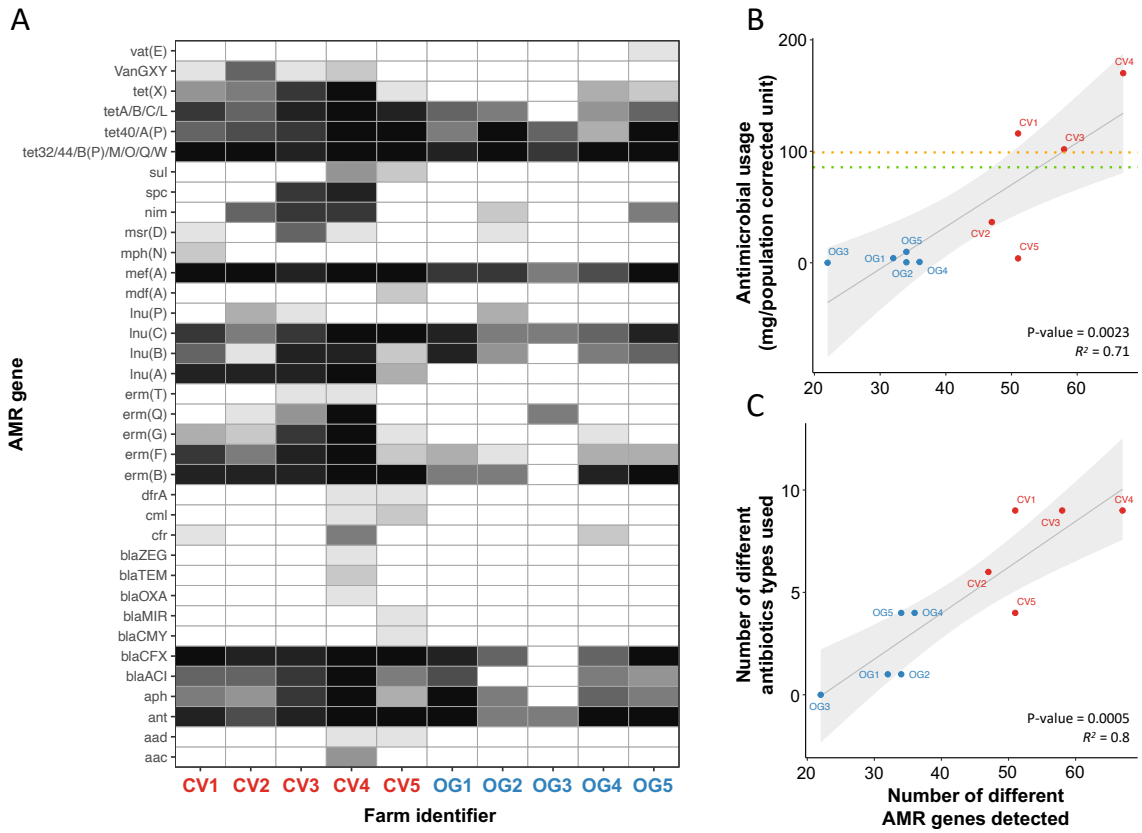


Figure 4. AMR gene distribution in faecal samples from CV and OG farms and the correlation with levels of antimicrobial used.

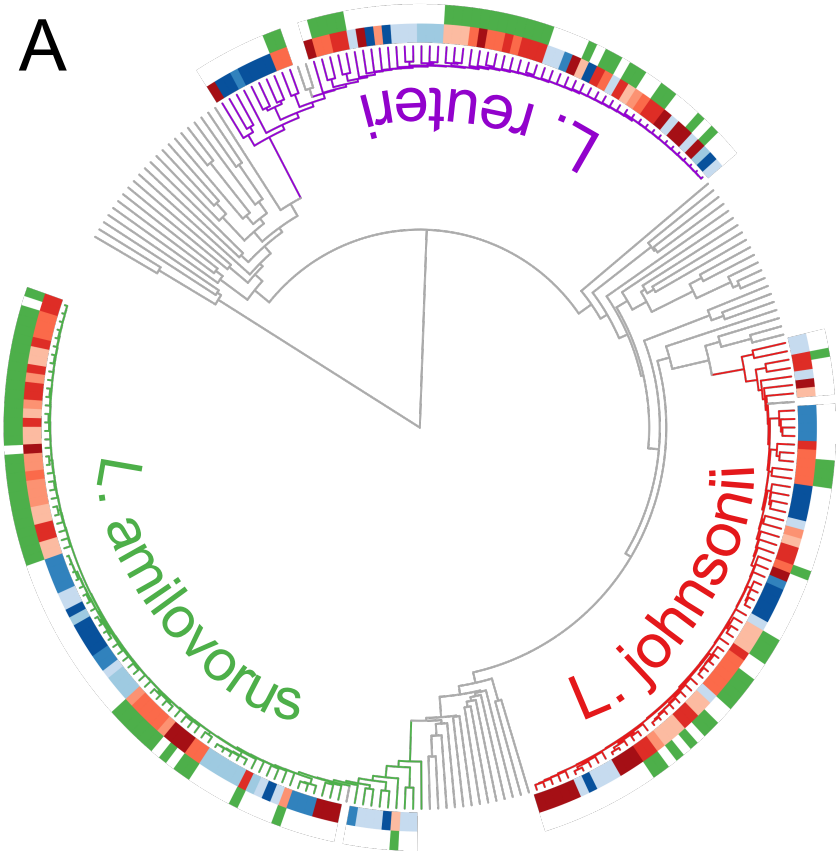
Panel A: The heatmap shows the number of samples from which MAGs were generated containing different AMR genes, with the intensity of shading ranging from 0/10 samples (white) to 10/10 samples (black). Conventional (CV1 to 5) and organic (OG1 to 5) are labelled using red and blue text respectively. Panel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

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

Genes that encode proteins potentially able to confer resistance to β-lactams and chloramphenicol were present at greater numbers in samples from CV farms. A number of genes were present solely in samples from CV farms (*aac*, *aad*, *blaCMY*, *blaMIR*, *blaOXA*, *blaTEM*, *bla*ZEG, *cml*, *dfrA*, *erm(T)*, *lnu(A)*, *mdf(A)*, *mph(N)*, *spc, sul*, *van(GXY)*), whereas the gene *vat(E)* was found solely in one OG farm. Comparing the number of different AMR genes found in the faecal microbiomes to the antimicrobial use on each farm (using PCU, and the number of different antimicrobials used), we observed statistically significant correlations (Figure 4 panels B and C). The correlation of AMR genes with PCU had an R squared value of 71% and P value of 0.0023; the correlation of AMR genes with the number of different antimicrobials used had an R squared value of 80% and P value of 0.0005.

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

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



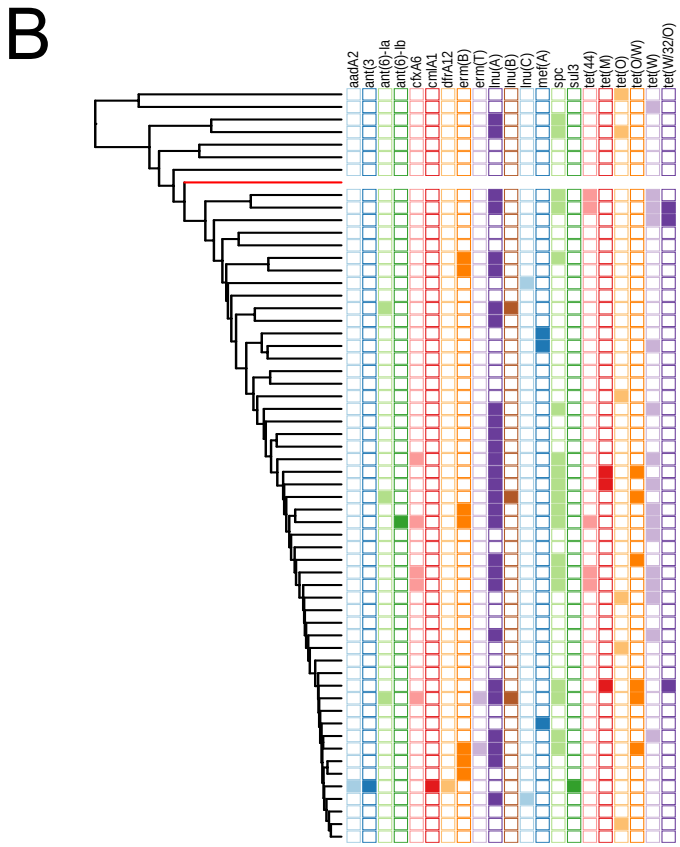


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Figure 5. Association of different AMR genes to *Lactobacilli* species.

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

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

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

Methods

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

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

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

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

Generation of Hi-C libraries. The method for the construction of bacterial Hi-C libraries was adapted from Burton *et al.*[11]. Briefly, DNA from the fixed cells was isolated by lysing bacterial pellets in lysozyme (Illumina) followed by mechanical disruption using a Precellys Evolution bead beater (Bertin Technologies, France). Isolated chromatin was split into four aliquots and digested for 3 h at 37°C using *HpyCH4*IV restriction enzyme (New England Biolabs). Restriction fragment overhangs were filled with biotinylated dCTP (Thermo Scientific) and Klenow (New England Biolabs) as described by van Berkum *et al.*[28]. Biotin labelled digested chromatin was diluted in 8 ml of ligation buffer (New England Biolabs, T4 ligase kit) 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

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

Bioinformatics pipeline – pre-processing and de-novo assembly. Next generation sequencing raw data files were pre-processed in different ways according to the sequencing library they were derived from. Shotgun metagenomics sequencing data passed through two filtering / quality control steps: (i) optical and PCR duplication removal by using clumpify.sh script from the BBMap software package (https://sourceforge.net/projects/bbmap/); (ii) removal of read pairs matching with the host genome using Bowtie2[29] and the pig reference genome (Sscrofa11.1: GCA_000003025.6). As we performed bacterial cell enrichment during the Hi-C library preparation, we only filtered the raw reads for optical and PCR duplications by using the above-mentioned method. Both raw datasets passed through a merging step, where overlapping (at least 30 nucleotide) reads were merged to one single read, using FLASH software[30]. After merging, metagenomic sequencing reads were passed to the assembly step as paired-end (un-merged) or single-end (merged) reads. All Hi-C sequencing reads were processed further by a Perl script that detected the modified restriction site (in our case A|CGT is modified to ACGCGT) and re-fragmented reads accordingly. This step ensured that hybrid DNA fragments 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

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

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

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

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

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

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

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

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

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Author contributions

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

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

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

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

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

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

Competing financial interest

The authors declare no competing financial interests.

Supplemental information

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

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

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

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

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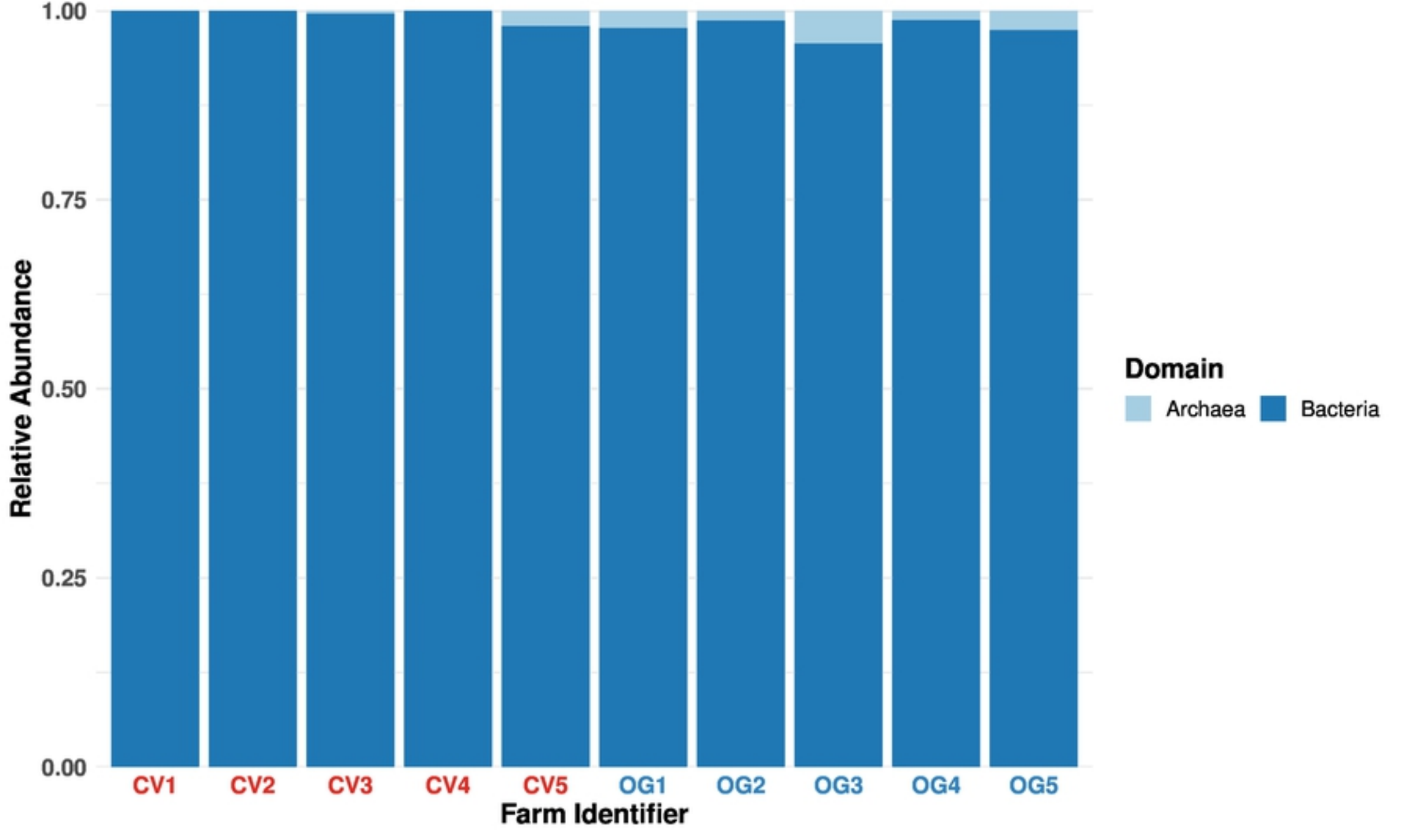
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OG4
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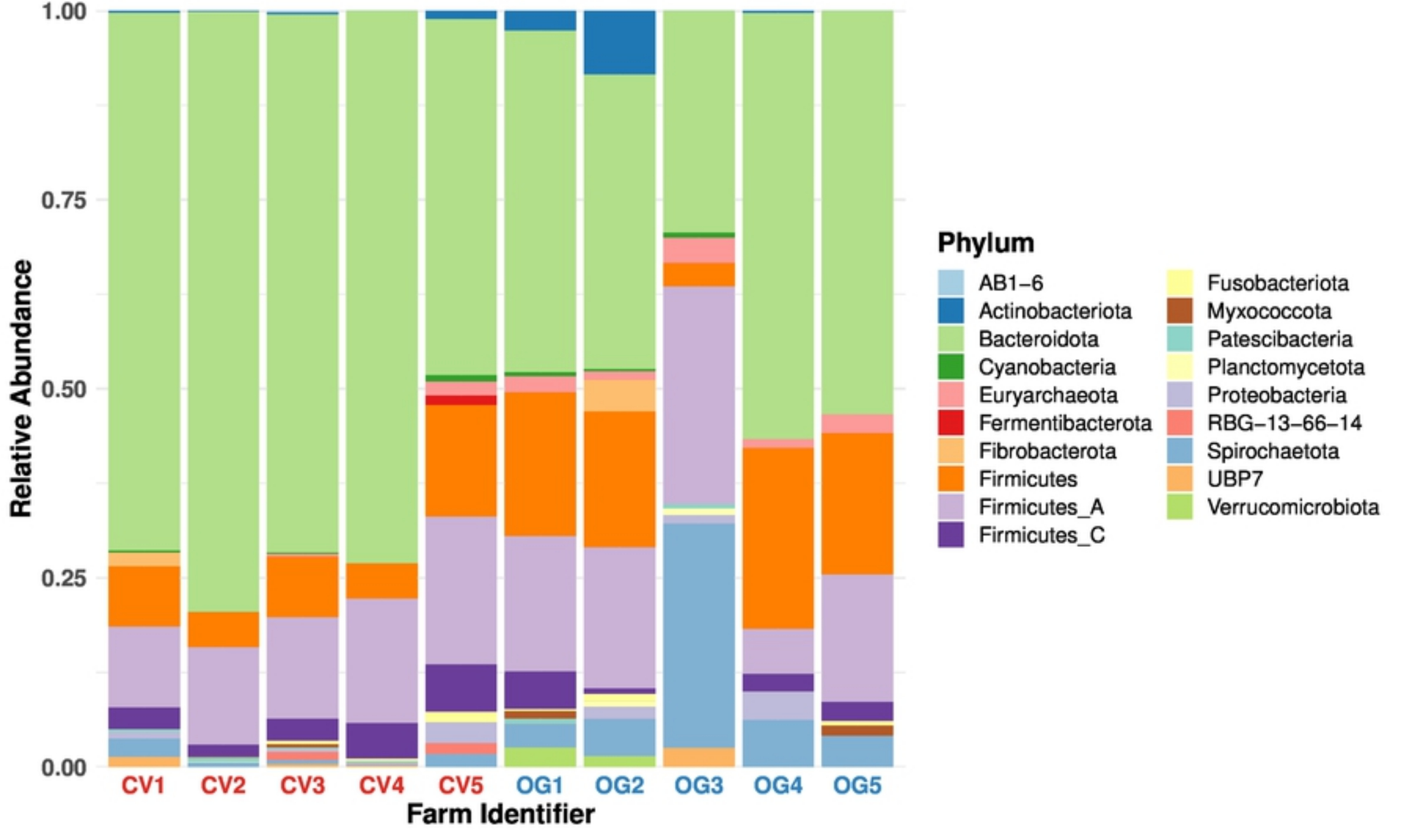
Bacteria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Fermentibacterota
Fibrobacterota
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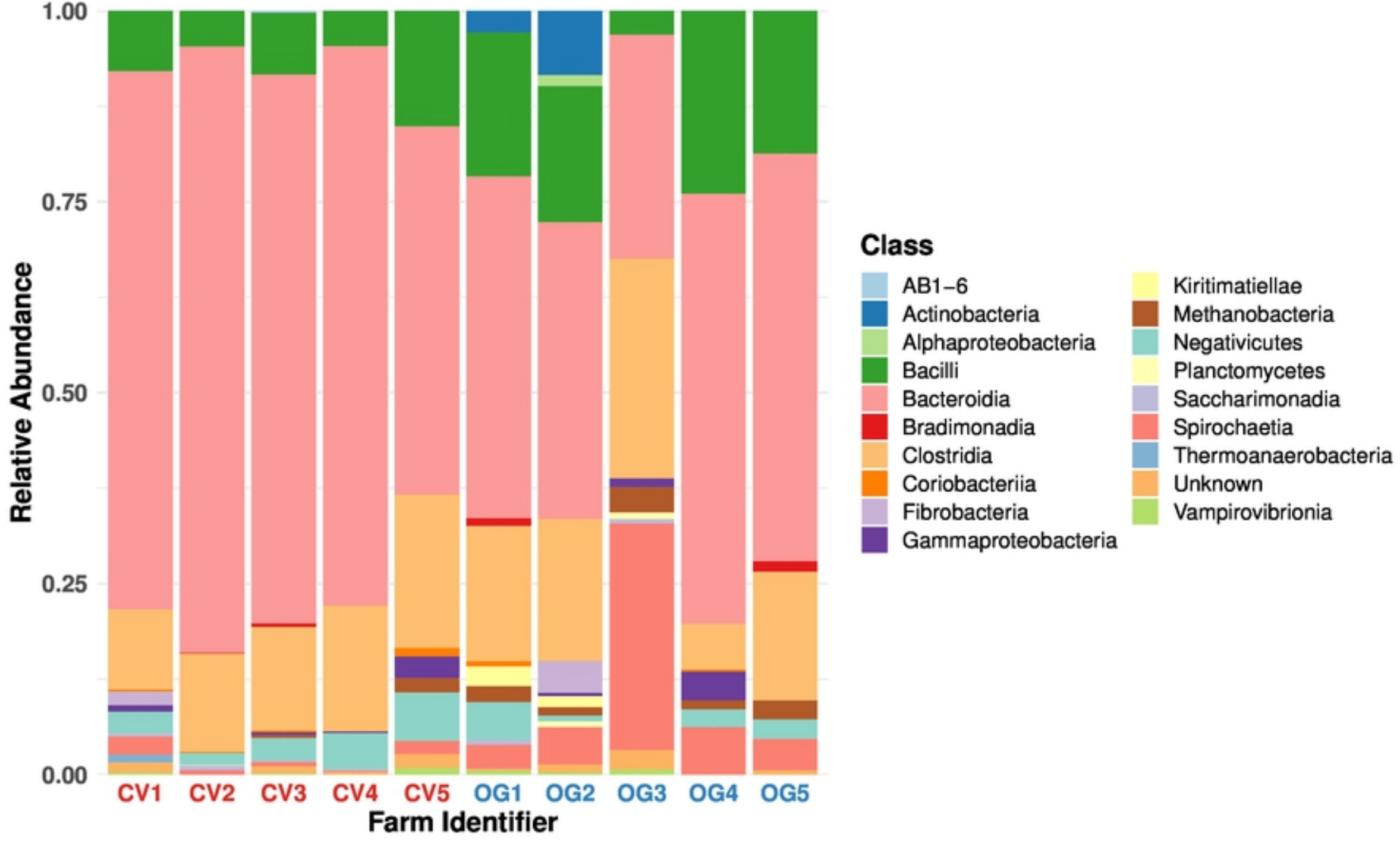
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Relative Abundance
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CV2
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CV4
CV5
OG1
OG2
OG3
OG4
OG5
Farm Identifier
Class
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Alphaproteobacteria
Bacilli
Bacteroidia
Bradimonadia
Clostridia
Coriobacteriia
Fibrobacteria
Gammaproteobacteria
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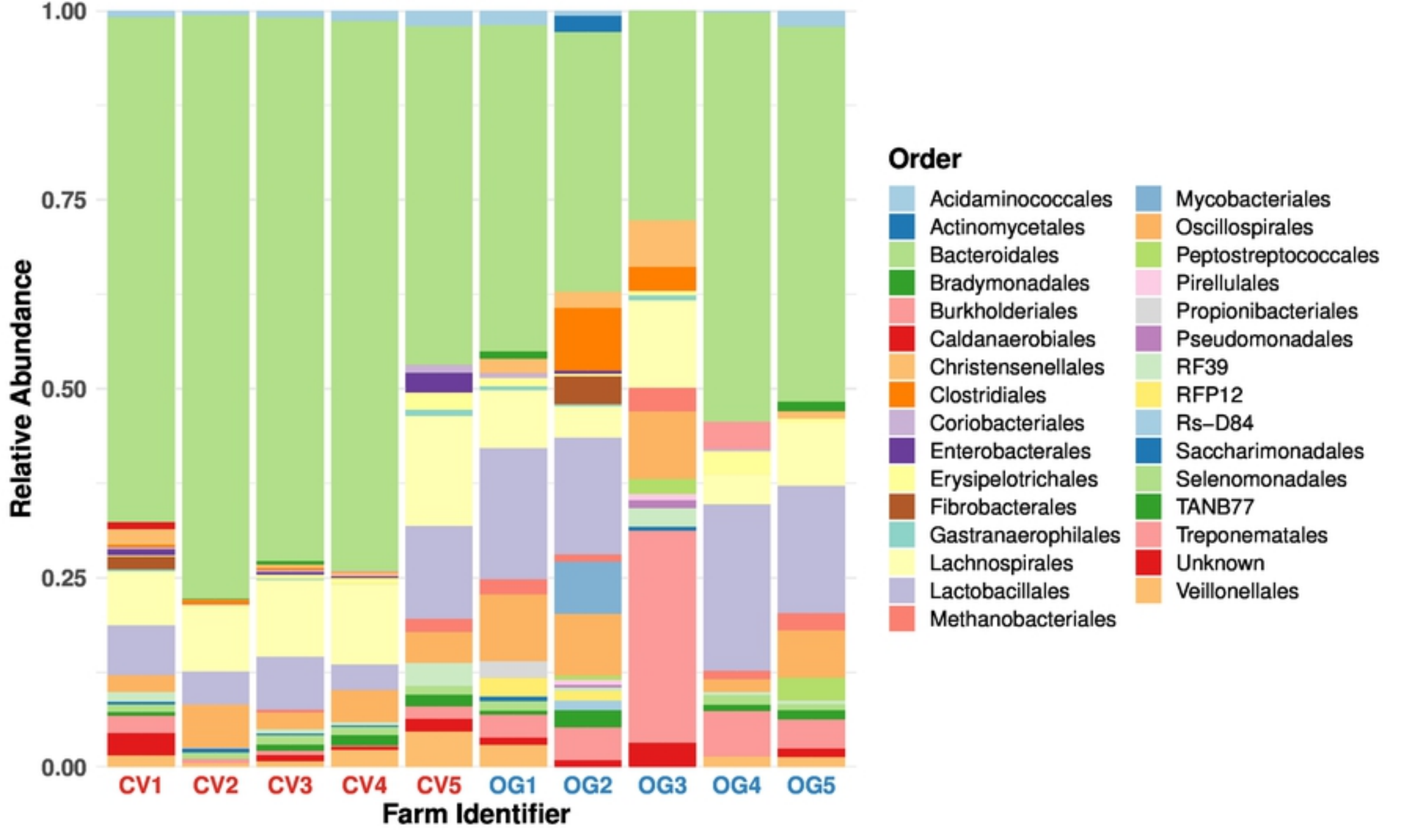
1.00
0.75
0.50
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Relative Abundance
CV1
CV2
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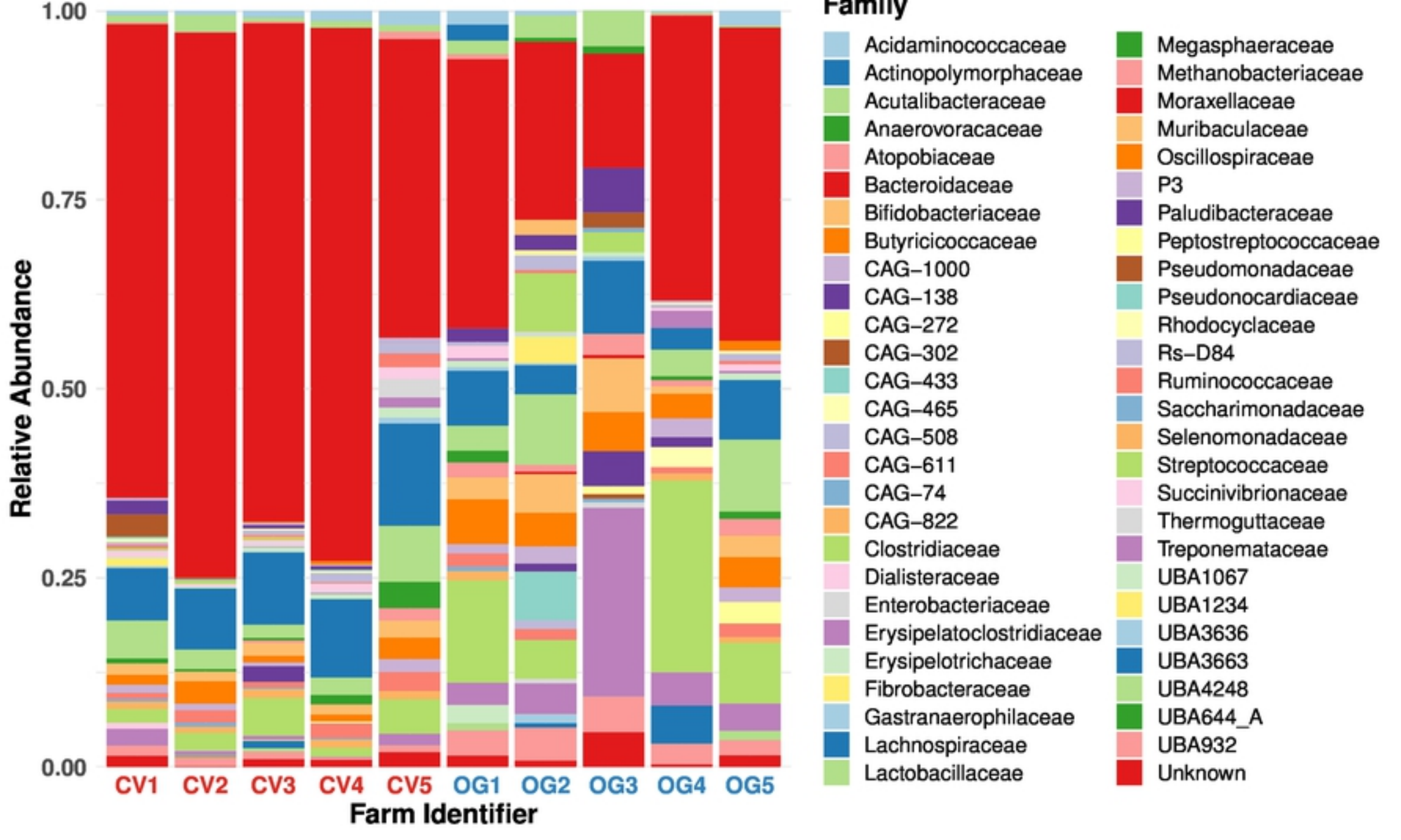
CV4
CV5
OG1
OG2
OG3
OG4
OG5
Farm Identifier
Order
Acidaminococcales
Actinomycetales
Bacteroidales
Bradymonadales
Burkholderiales
Caldanaerobiales
Christensenellales
Clostridiales
Coriobacteriales
Enterobacterales
Erysipelotrichales
Fibrobacterales
Gastranaerophilales
Lachnospirales
Lactobacillales
Methanobacteriales
Mycobacteriales
Oscillospirales
Peptostreptococcales
Pirellulales
Propionibacteriales
Pseudomonadales
RF39
RFP12
Rs−D84
Saccharimonadales
Selenomonadales
TANB77
Treponematales
Unknown
Veillonellales


1.00
0.75
0.50
0.25
0.00
Relative Abundance
CV1
CV2
CV3
CV4
CV5
OG1
OG2
OG3
OG4
OG5
Farm Identifier
Family
Acidaminococcaceae
Actinopolymorphaceae
Acutalibacteraceae
Anaerovoracaceae
Atopobiaceae
Bacteroidaceae
Bifidobacteriaceae
Butyricicoccaceae
CAG−1000
CAG−138
CAG−272
CAG−302
CAG−433
CAG−465
CAG−508
CAG−611
CAG−74
CAG−822
Clostridiaceae
Dialisteraceae
Enterobacteriaceae
Erysipelatoclostridiaceae
Erysipelotrichaceae
Fibrobacteraceae
Gastranaerophilaceae
Lachnospiraceae
Lactobacillaceae
Megasphaeraceae
Methanobacteriaceae
Moraxellaceae
Muribaculaceae
Oscillospiraceae
P3
Paludibacteraceae
Peptostreptococcaceae
Pseudomonadaceae
Pseudonocardiaceae
Rhodocyclaceae
Rs−D84
Ruminococcaceae
Saccharimonadaceae
Selenomonadaceae
Streptococcaceae
Succinivibrionaceae
Thermoguttaceae
Treponemataceae
UBA1067
UBA1234
UBA3636
UBA3663
UBA4248
UBA644_A
UBA932
Unknown

SAMPLE

Host DNA Removal (Bowtie2)

Duplication removal (bbmap)

Merge overlapping (FLASH)

Metagenomics data pre-processing

Duplication removal (bbmap)

Merge overlapping (FLASH)

Restriction site detection (perl script)

Re-fragment merged by the restriction site

Exclude merged without restriction site

Re-fragment unmerged by the restriction site

Keep unmerged without restriction site

Hi-C data pre-processing

De novo assembly (metaSPAdes)

Hi-C realignment (Bowtie2, perl script)

Louvain network resolution (C program, perl script)

Splitting and extending CCs (perl script)

AMR prediction, pairwise comparison, taxonomy (BLAST, mash, GTDBtk)

Assembly and post processing

Clade refinement

Comparison with assemblies from other samples, clade formation by UPGMA algorithm (mash, Julia script)

Finding the best exemplar in each clade (GTDBtk, perl script)

Generating a temporary reference specific to the clade

BLAST the exemplar to other samples full contig list with high identity threshold (98%). (BLAST, perl script)

Reference based assemblies for all samples

Checking inter-contig Hi-C contacts in the new reference based assemblies, excluding non-connected contigs. (perl script)

Hi-C refined reference based assemblies

Assembly extension with contigs containing AMR genes based on Hi-C contacts (high sensitivity & specificity) (perl script)

AMR genes finally associated to assemblies with high precision

Assembly final extension by contigs without AMR genes based on Hi-C contacts (lower sensitivity and specificity) (perl script, BLAST)

Final extended and refined assemblies

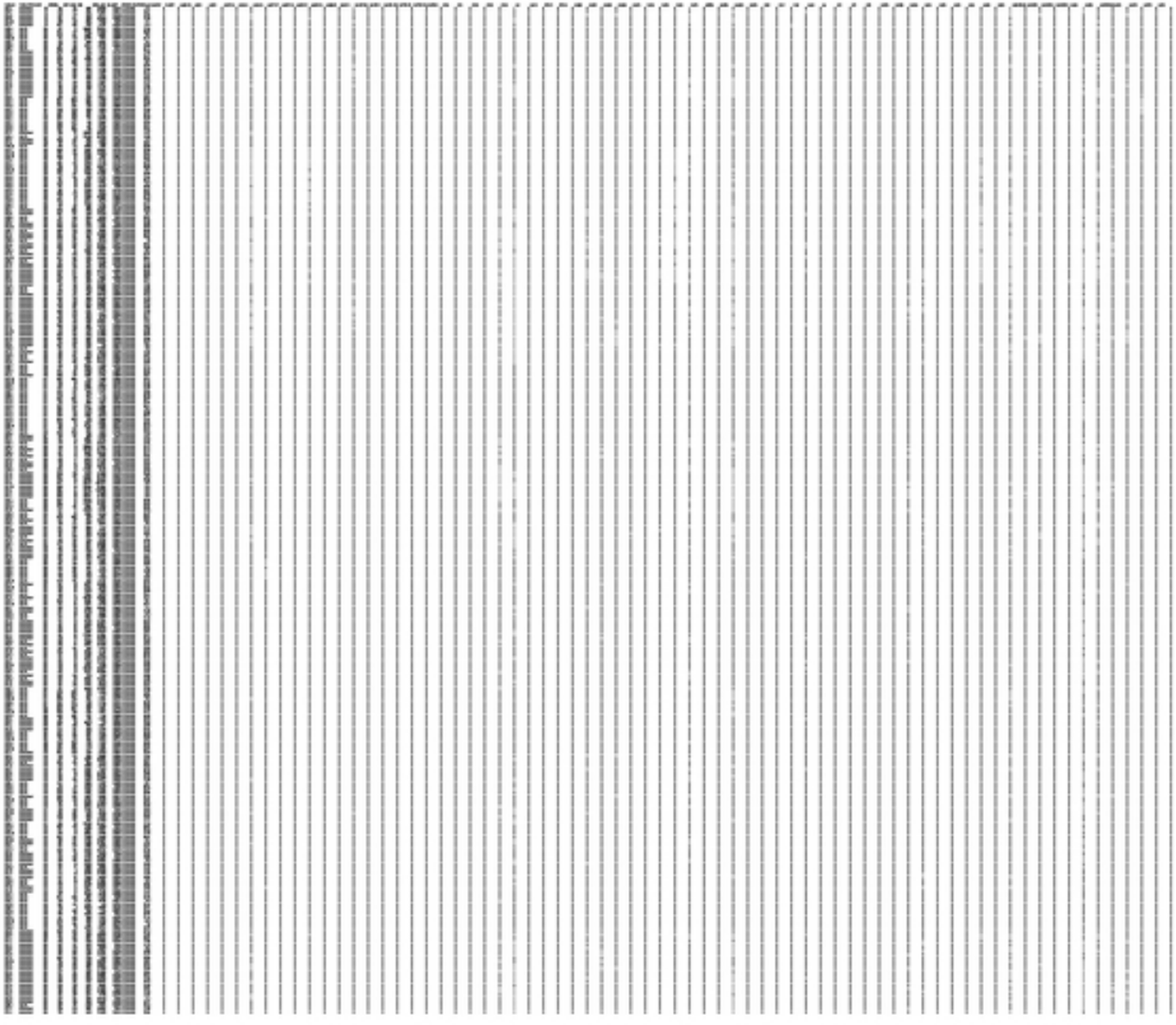
Within-clade pairwise comparison, AMR association tables, taxonomical identification (mash, GTDBtk, perl and Julia script)

Suplementary 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

Suplementary Table 1



Suplementary Table 2