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Abundance and diversity of the fecal resistome in slaughter pigs and broilers in nine European countries

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Abstract

Antimicrobial resistance (AMR) in bacteria and associated human morbidity and mortality is increasing. Use of antimicrobials in livestock selects for AMR that can subsequently be transferred to humans. This flow of AMR between reservoirs demands surveillance in livestock as well as in humans. As part of the EFFORT project (www.effort-against-amr.eu), we have quantified and characterized the acquired resistance gene pools (resistomes) of 181 pig and 178 poultry farms from nine European countries, generating more than 5,000 gigabases of DNA sequence, using shotgun metagenomics. We quantified acquired AMR using the ResFinder database and a database constructed for this study, consisting of AMR genes identified through screening environmental DNA. The pig and poultry resistomes were very different in abundance and composition. There was a significant country effect on the resistomes, more so in pigs than poultry. We found higher AMR loads in pigs, while poultry resistomes were more diverse. We detected several recently described, critical AMR genes, including *mcr-1* and *optrA*, the abundance of which differed both between host species and countries. We found that the total acquired AMR level, was associated with the overall country-specific antimicrobial usage in livestock and that countries with comparable usage patterns had similar resistomes. Novel, functionally-determined AMR genes were, however, not associated with total drug use.

Introduction

Antimicrobial resistance (AMR) is considered one of the largest threats to human health.[1] In addition to the use of antimicrobial agents for humans, livestock is considered an important source of AMR, potentially compromising human health.[2] Besides AMR in zoonotic pathogens, AMR in commensal bacteria is worrisome because of its ability to spread horizontally to pathogens.

Multiple studies have shown that use of antimicrobials in livestock will lead to increased occurrence of AMR and that reduction of usage will eventually lead to reduced resistance.[3–8] A number of national surveillance programs have been implemented to monitor the occurrence of AMR in different reservoirs and follow trends over time.[1,9–11] There are major differences in antimicrobial consumption patterns between different countries globally and also within Europe.[12] Major differences in the occurrence of AMR have also been observed among indicator organisms (e.g. *E. coli*) isolated from different European countries.[3,13] Current monitoring efforts are mainly based on culturing indicator bacteria followed by phenotypic AMR determination.[13,14] This procedure only targets a limited number of species present in the gut microbiota

31   and thus likely represents only a fraction of its resistome (the collective pool of AMR genes). Such

32   metagenomic approaches have been used in a number of recent studies and it has been shown that

33   metagenomic read mapping describes AMR abundance in bacterial communities more accurately than

34   commonly used technologies on selected indicator organisms.[15–17] A recent study focused on sampling a

35   diverse group of individual pigs from eleven farms in three countries showed that genetics, age, diet and

36   country all likely influence the pig microbiota, but little information is available for poultry.[16]

37   As part of the EU-funded EFFORT project (www.effort-against-amr.eu), we sampled over 9000 animals in

38   181 pig and 178 poultry herds in nine European countries, generating herd-level composite samples as

39   previously described.[17] This gives us a unique insight into the diversity and structure of the acquired pig and

40   broiler resistomes across Europe. We sampled animals as close as possible to slaughter to elucidate the

41   potential consumer exposure to AMR associated with meat production in Europe. Association between

42   AMR gene abundance and country level antimicrobial usage was analyzed. We hereby provide an overview

43   of AMR in the two most intensively raised European livestock species. To our knowledge, this study

44   represents the single largest metagenomic AMR monitoring effort of livestock: both in terms of countries

45   (9), herds included (359), individual animals sampled (over 9,000) and sequencing effort (5,000+ gigabases).

46

Methods

Farm selection and sampling

49   The sampling protocol for pig and broiler farms that has been agreed on by the EFFORT consortium is

50   described below. Selection of farms and sampling procedure followed these guidelines to the extent

51   possible, but some deviations from the protocol were occasionally necessary. A detailed description of the

52   sampling conducted in the individual countries is provided in supplementary material.

Selection of pig and poultry farms

54   In each participating country, 20 conventional integrated pig farrow-to-finisher non-mixed farms were

55   selected. The farms needed to have a minimum of 150 sows and 600 fatteners and employ batch

56   production to ensure that the majority of the animals of the sampled group originated from the same birth

57   cohort. All-in all-out production at compartment level was preferred and all fatteners sampled were

58   required to have been on the same site during their entire life. Selected farms should have no contact

59   through livestock trade, and have a random regional distribution.

60 In each country, 20 conventional broiler farms (no breeders) were selected. The farms should have all-in all-

61 out production, with a thinning procedure from day 30 onwards allowed. All selected farms should have no

62 intended slaughter age higher than 50 days, no slow growing breeds (intended growth rate less than 55

63 gram/day) and no stocking density lower than 10 birds/m². Only one flock/house per holding should be

64 sampled and the flock should be between 20,000 and 40,000 birds. If possible, selected farms should have

65 a random regional distribution.

Procedure for sampling

67 We sampled pig farms between May 2014 and December 2015, and tried to minimize seasonal influences.

68 The sampled fatteners should be as close to slaughter as possible (i.e. within the last week). A total of 25

69 fresh, still warm and undisturbed fecal droppings were sampled from pen floors (a minimum of 10 g of

70 feces per sample) randomly divided over all eligible compartments/stables of fatteners close to slaughter.

71 Broilers were sampled between May 2014 and June 2016, and we tried to minimize seasonal influences. On

72 each farm, 25 undisturbed, fresh main bowel droppings were collected from the floor of the house (a

73 minimum of 3 g feces per sample). The flocks should be sampled as close to slaughter as possible (last week

74 before the final depopulation).

75 All samples were collected aseptically in plastic containers and were stored at 4°C and transported to the

76 laboratory within 24 hours after sampling.

Pooling and handling of samples

78 Upon arrival in the laboratory, individual fecal samples were homogenized by stirring thoroughly with a

79 sterile tongue depressor/ spoon for a few minutes. From each pig sample, two 2 ml cryotubes were filled

80 and frozen immediately at -80°C (alternatively at -20°C for maximum 4 days, before transferring to -80°C).

81 For broiler samples, at least 0.5 g feces was added to two cryotubes. Sample pooling was either done

82 immediately or the frozen tubes were shipped to the Technical University of Denmark (DTU) on dry ice for

83 pooling. Individual samples from the same herd were defrosted and placed on ice briefly before weighing.

84 Following weighing, they were pooled with 0.5 g of feces from each sample and stirred for a few min with a

85 sterile device (e.g. disposable wooden tong depressor). All samples were only thawed once shortly before

86 DNA extraction.

87 After removing two mis-labeled samples, we ended up with composite fecal samples from 178 broiler

88 flocks and 181 pig herds.

Sampling to estimate the effect of random sampling

To study the potential effect of sampling randomness and the reproducibility of our sampling protocol, two of the pig herds were chosen for triplicate sampling. These two herds were sampled three times on the same day (25 samples x 3 sampling rounds), resulting in six pooled samples (2 herds x 3 sampling rounds), from which the within-farm variation was assessed. A table with all samples and their metadata is included as Supplementary Table 1.

DNA extraction and sequencing

From each of the pooled, herd-level fecal samples, DNA was extracted using a modified QIAamp Fast DNA stool mini kit protocol (Qiagen, cat. no. 51604), as previously described.[18] One major modification is the addition of a bead beating step in the beginning of DNA extraction. The protocol can be found at https://figshare.com/articles/SOP_-_DNA_Isolation_QIAamp_Fast_DNA_Stool_Modified/3475406. DNA-purification of all pooled samples was processed centrally at Technical University of Denmark (DTU), and the DNA was stored in duplicates at -20°C until further use.

DNA was shipped on dry ice for library preparation and sequencing at the Oklahoma Medical Research Foundation (OMRF). There, DNA from all samples was mechanically sheared to a targeted fragment size of 300bp using ultrasonication (Covaris E220evolution). For pooled pig samples, library preparation was performed with the NEXTflex PCR-free library preparation kit (Bioo Scientific). For poultry samples, due to a lower DNA availability, the minimal amplification-based KAPA Hyper kit (Kapa Biosystems) was used. For all samples, the Bioo NEXTflex-96 adapter set (Bioo Scientific) was used. In batches of roughly sixty samples, the libraries were multiplexed and sequenced on the HiSeq3000 platform (Illumina), using 2x150bp paired-end sequencing per flow cell. A total of 17 Belgian, Danish and Dutch pig fecal samples were sequenced on the HiSeq2500 platform (Illumina), using 2x100bp paired-end sequencing, before converting to HiSeq3000 for the remaining samples (See Supplementary Table 1).

Bioinformatics processing

The FASTQ files with sequencing data for each sample were analyzed following the principles from the previously described MGmapper tool.[15] The reads were first cleaned by cleaning out adaptors using BBduk (BBMap v39.92 - Bushnell B. -https://sourceforge.net/projects/bbmap/), and by removing reads that aligned to the internal sequencing control phi-X174 as determined by the BWA-MEM algorithm.[19] Trimmed read pairs were aligned using the BWA-MEM algorithm Prokaryotic RefSeq genomes from NCBI GenBank (downloaded on Nov. 18, 2016) with the "reference" or "representative" genome tags. The BWA-MEM algorithm (v0.7.15) was slightly modified such that the insert size was estimated for the entire sample unaffected by the number of CPU cores used. This estimate was then used for re-analyzing the cleaned

5

121   read pairs using BWA-MEM, ensuring that the thresholds for a read pair to map as a 'proper pair' were the

122   same for all read pairs, and avoiding bad insert size estimates when few read pairs aligned.

123   The read pairs were aligned to the bacterial NCBI genomes again, and to the AMR genes present in the

124   ResFinder database (accessed Nov. 17, 2016).[20] ResFinder is a manually curated database of horizontally

125   acquired AMR genes and thus does not include intrinsic AMR genes and mutated housekeeping genes

126   providing AMR by changing the drug target. Technical duplicate read pairs were then removed using

127   'MarkDuplicates' from the Picard command line tools (v2.8.3; http://broadinstitute.github.io/picard/).

128   For each ResFinder reference sequence, we counted the number of read pairs that properly aligned with at

129   least 50 bp aligning from both the forward and reverse reads. Each read pair matching a ResFinder

130   reference was assigned to the first highest-scoring reference, as done in MGmapper. The same was done

131   for NCBI microbial genomes in order to quantify the bacteriome and get a measure of the microbial

132   proportion within each sample. This total was used to normalize the ResFinder counts, by computing an

133   FPKM-value (fragments per kilobase reference per million bacterial fragments) for each ResFinder

134   reference sequence. The FPKM values were computed by dividing the mapping count on each reference

135   with its gene length and the total number of bacterial read pairs for the samples and multiplying by 10^9.[21]

136   Raw mapping count data and their associated FPKM values can be found in Supplementary Table 2 and 3.

137   Because ResFinder contains many representatives of certain gene families, a high degree of homology

138   exists, with long stretches of the references being identical. This causes unspecific mapping between high-

139   identity sequences. To eliminate this random noise, we chose to aggregate read counts and relative

140   abundances post-mapping at higher levels based on sequence identity. We clustered all the ResFinder

141   genes using CD-HIT-EST (v4.6.6) at a 90% identity level and otherwise default settings.[22] The resulting gene

142   clusters were manually inspected and named to reflect their contents while avoiding conflicts with other

143   clusters (Supplementary Table 4). Abundances were aggregated according to these clusters of high-identity

144   genes and the resistance-class-level as annotated in ResFinder. These two levels, "gene" and "class", were

145   used for all downstream analysis.

146   **Functional resistance database**

147   Previous studies have identified a wide array of novel AMR genes in various reservoirs using functional

148   metagenomics, referred to as functional AMR genes.[23–26] By cloning random DNA fragments from complex

149   microbiomes into an expression vector expressed in a host (typically *E. coli*) and selecting for growth in the

150   presence of certain antibiotics, they have been found to provide AMR to many antibiotics.[23–26] We

151   constructed a functional resistance database (FRD) from 3,416 AMR gene variants identified in four major

152   studies, using 23 different antimicrobials for selection.[23–26]

153   Briefly, in each of these studies, DNA was extracted from environmental and human fecal samples,

154   fragmented and cloned into a plasmid vector and screened for AMR functionality in *E. coli* cultured with

155   one of multiple antimicrobials. AMR-granting plasmid inserts were then sequenced and the responsible

156   open reading frame was identified. The protocol for the database construction can be found at

157   https://cge.cbs.dtu.dk/services/ResFinderFG/. Genes were quantified using MGmapper as was done for

158   ResFinder. Genes with more than 90% identity to ResFinder genes were removed post mapping to obtain

159   the new AMR genes without overlap with ResFinder. The resulting data was aggregated to 90% gene

160   clusters, using CD-HIT-EST, as was done for ResFinder.[22] The most frequent gene clusters remaining were

161   derived from genes selected using: trimethoprim, chloramphenicol, co-trimoxazole, cycloserine, amoxicillin,

162   gentamicin, penicillin and tetracycline.

Principal coordinate analysis and resistome clustering

164   For principal coordinate analysis (PCoA), the gene-cluster level FPKM matrix was Hellinger-transformed and

165   the Bray-Curtis (BC) dissimilarities between all samples were calculated using the R package *vegan*.[27] PCoA

166   was carried out for both pigs and poultry; combined and separately, using the *vegan* function 'betadisper'.

167   The same analysis was used to test whether host animal and country were significant predictors of within-

168   group beta diversity dispersion. The effects of country on sample dissimilarities was determined using

169   'permutational multivariate analysis of variance using distance matrices' ('adonis2' function in *vegan*

170   package), separately for pig and poultry.

Antimicrobial use in livestock

172   Data for national livestock antimicrobial usage (AMU) was obtained from the European Medicines Agency's

173   2014 European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) report and was stratified by

174   major drug family.[28] The mass of active compound sold for use in animals in 2014 was divided by the

175   Population Correction Unit (PCU) in 10^6 kg - approximating the biomass. The PCU is a unit that allows inter-

176   species integration by adjusting for import/export and differences in average weight between species when

177   they are most likely to receive antimicrobial treatment. The estimate was multiplied by 1000 to obtain drug

178   mg/kg PCU livestock. The country-specific veterinary drug use can be found in Supplementary Table 5.

Procrustes analyses

180   In order to test the association between country-specific AMU patterns and the resistomes, we performed

181   Procrustes analysis using the *vegan* R package as follows. A PCoA was generated from Euclidean distances

182   between the samples in the PCU-corrected AMU (Supplementary Table 5, Supplementary Figure 1). The

183   AMU PCoA was tested against the previously mentioned Hellinger-transformed, resistome BC dissimilarity

184   PCoA using the 'protest' function with the default 999 permutations, again separately for pigs and poultry.

185   The gene-cluster FPKM ResFinder matrix and the genus-level FPKM taxonomy matrix were Hellinger

186   transformed and BC dissimilarities were calculated. They were ordinated using non-metric

187   multidimensional scaling (NMDS) with the 'metaMDS' *vegan* function (999 permutations) for pig and

188   poultry samples separately. The symmetric Procrustes correlation coefficients between the bacteriome and

189   resistome ordinations, p-values and plots were obtained using the 'protest' and 'procrustes' functions in

190   *vegan*.[29]

Alpha diversity

192   For all samples, we computed the within-herd resistome diversity using Simpson diversity index (1-D),

193   Chao1 richness estimate and Pielou's evenness.[30] The raw read count matrix was rarified to 10,000 hits for

194   all samples for alpha diversity estimation, leading to the exclusion of 10 samples.

Visualization

196   Heatmaps were produced using the *pheatmap* R package. For heatmaps showing individual gene

197   abundances, the BC dissimilarities between samples were used. For all other dendrograms, the Pearson

198   product-moment correlation coefficients (PPMCC) were used. Complete-linkage clustering was used for all

199   dendrogram clustering. For sample similarities, BC dissimilarity was converted to a similarity percentage,

200   i.e., 100*(1- BC).

201   The circular BC sample dendrogram was exported in Newick format using the *ape* package and further

202   annotated with the Interactive Tree of Life tool.[31,32] Bar-, box- and scatter plots were produced using the

203   *ggplot2* R library.[33] The R library *RcolorBrewer* was used to generate the color palettes used. The library is

204   based on work by Cynthia A. Brewer (www.ColorBrewer.org).

Statistical analyses

206   All statistics was done in Microsoft R Open (MRO) 3.3.2, using the libraries and procedures detailed below.

207   Exact package versions can be found here: https://mran.revolutionanalytics.com/snapshot/2016-11-

208   01/bin/windows/contrib/3.3/. For statistical tests, only the first sampling from triple-sampled herds was

209   included (see Supplementary Table 1). Unless otherwise mentioned, all statistical analyses were performed

210   on pigs and poultry separately.

Effect of AMU on total AMR

For testing the effect of total AMU on total metagenomic AMR abundance (sum of all genes), we used the *lme4* 1.1-12 package to make linear mixed effects regression models with total livestock drug usage as the independent variable, total AMR abundance (FPKM) as the dependent variable and country as a mixed effect intercept, adjusting for the fact that AMR abundance observed in farms from the same country is correlated due to factors that are not included in this study.[34] The effect of AMU was modelled on a logarithmic scale, which resulted in lower Akaike's information criteria compared with modelling AMU on a linear scale. Country- and sample-level residuals were plotted and inspected for normality and homoscedasticity. Pig sample residuals and country residuals were normal and so were poultry country residuals. Poultry sample residuals had a longer right tail. Square-root transforming the poultry AMR data, gave more normal residuals and a similar conclusion ($p<0.05$). The effect and significance of drug usage was assessed using likelihood-ratio tests, comparing the random effect models with and without the AMU effect.

Differential abundance analysis

To identify differentially abundant AMR genes per country, we analyzed the read pair mapping count matrix using the *DESeq2* package as previously recommended for metagenomic read count data.[35,36] This was done on the raw read pair matrix, following recommendations that rarefying is not warranted in metagenomic studies.[36] The read-pair count matrices for pigs and poultry were analyzed separately. The number of mapped bacterial pairs was divided by the minimum number of mapped bacterial pairs and was used as the size factor. For each gene, we used a two-sided Wald test to determine whether the fold change between countries differed from 0 and extracted all the country-versus-country results. P-values were adjusted for the false discovery rate (FDR) using the Benjamini-Hochberg approach and we used a significance threshold of alpha: 0.05.[37]

Core resistome

The core resistomes determined here, were the set of AMR gene clusters with mapping read pairs in at least 95% of the samples. The core resistomes were determined separately for the pig and poultry reservoirs.

Results

In total, DNA from 365 pooled samples was extracted and shotgun sequenced, resulting in more than 36 billion sequences (18 billion paired-end (PE) reads), comprising more than 5 terabases of DNA. This yielded

242   an average of 50 million (SD: 18*10^6) PE reads per pooled sample. This was similar for pig and poultry

243   samples, though the former varied more than the latter.

244

Acquired resistome characterization

246   The total AMR gene level varied significantly across samples, both depending on host animal and country of

247   origin. In general, pigs had a higher AMR level than poultry (Figure 1a). The highest AMR levels were found

248   in Italian pigs from where the top four resistance-scoring samples originated, all in excess of 10,000 FPKM

249   AMR. At the lower end of the spectrum were Danish poultry samples that occupied the eleven samples

250   with least AMR, all below 500 FPKM.

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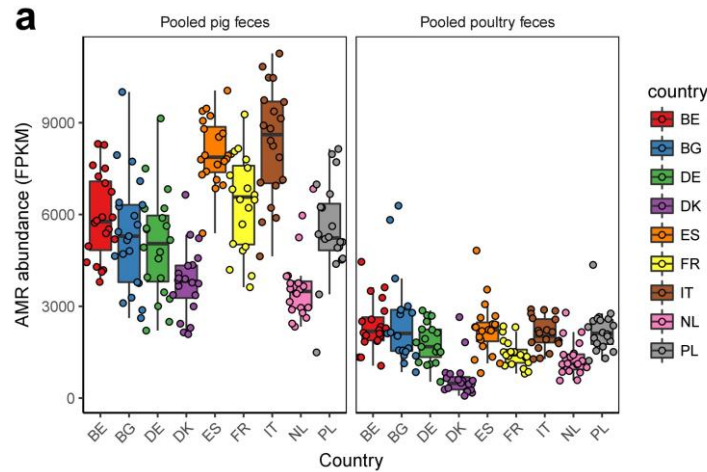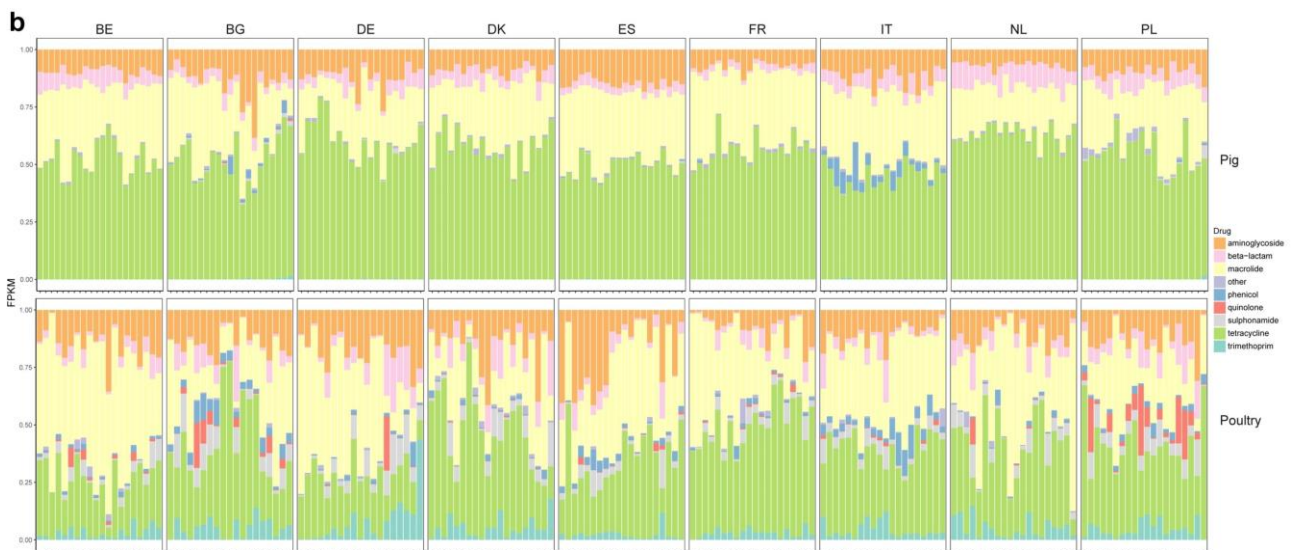
Figure 1. Overview of AMR abundance and composition. From the read mapping results to the ResFinder database, AMR abundance was calculated for each reference gene in each sample. (a) Boxplots showing the total AMR level per sample, stratified by host species and country. Each herd is also represented by a dot with sideways jitter to minimize overplotting. Horizontal box lines represent Q1, median and Q3. Whiskers denote range of points within Q1-1.5*IQR and Q3+1.5*IQR. (b) Stacked barchart of AMR abundance per type (colors) per sample (x-axis), proportional to total AMR within each sample.

We summed the relative abundance of AMR to the corresponding drug class level for each sample to look for major trends across species and countries (Figure 1b). When considering the proportion of the total resistome that each AMR type takes up, the pig samples were relatively homogenous: tetracycline AMR was by far the most common, followed by macrolide AMR. Beta-lactam and aminoglycoside AMR genes followed with other kinds of AMR being rare. Italian pigs had a notably larger proportion of phenicol AMR compared with other countries and it seemed to be consistent across all Italian farms. A subset of Bulgarian pig farms had a similar proportion of phenicol AMR.

11

266 Among the poultry farms, there was less consistency: both within and between countries, the relative

267 proportions of AMR per drug class varied more. Tetracycline, macrolide, beta-lactam and aminoglycoside

268 AMR made up the majority of AMR, as in pig samples, but the two latter classes had very minimal

269 contributions in a subset of herds. Sulfonamide and trimethoprim AMR were more abundant in poultry

270 samples compared with pig samples, across all countries. In many Polish poultry herds, quinolone AMR

271 made up a sizeable fraction of the combined resistome. This was also true for a few non-Polish herds,

272 notably in Bulgaria. For non-proportional graphical representations of the AMR load stratified by sample

273 and drug class, see the supplementary material for an unscaled, stacked bar chart (Supplementary Figure 2)

274 and a heatmap (Supplementary Figure 3). Class level AMR relative abundances can be found in

275 Supplementary Table 6.

276 To characterize the individual components of the resistome, we summed relative abundance to the gene-

277 cluster level as we had done at drug class level. We found evidence for 407 different gene clusters across all

278 pig and poultry samples (Supplementary Table 2).

279 We calculated the BC dissimilarities between all samples' gene-level resistomes and visualized it in a

280 dendrogram (Figure 2a). There was a perfect host separation, with all pig samples clustering separately

281 from all poultry, suggesting pig and poultry resistomes are very different. Within the pig cluster, the

282 country separation was more pronounced than for poultry. An exception was Danish poultry, where 18/20

283 samples clustered.

284 To assess the reproducibility of our protocol, from sampling through sequencing, we evaluated the

285 similarities between resistomes of two triple-sampled swine herds. Dutch triple-sampled herd pools had

286 the highest similarities of all samples, ranging from 93.6% - 93.7% BC similarity. The Belgian triple-sampled

287 herd pools had values ranging from 91.5% - 93.3% similarity. No replicated sample pool had higher

288 similarity to other herds than to its own replicates and the two sets of three samples can thus be seen

289 clustering as expected (Figure 2a). A sample similarity heatmap is found in Supplementary Figure 4.

290

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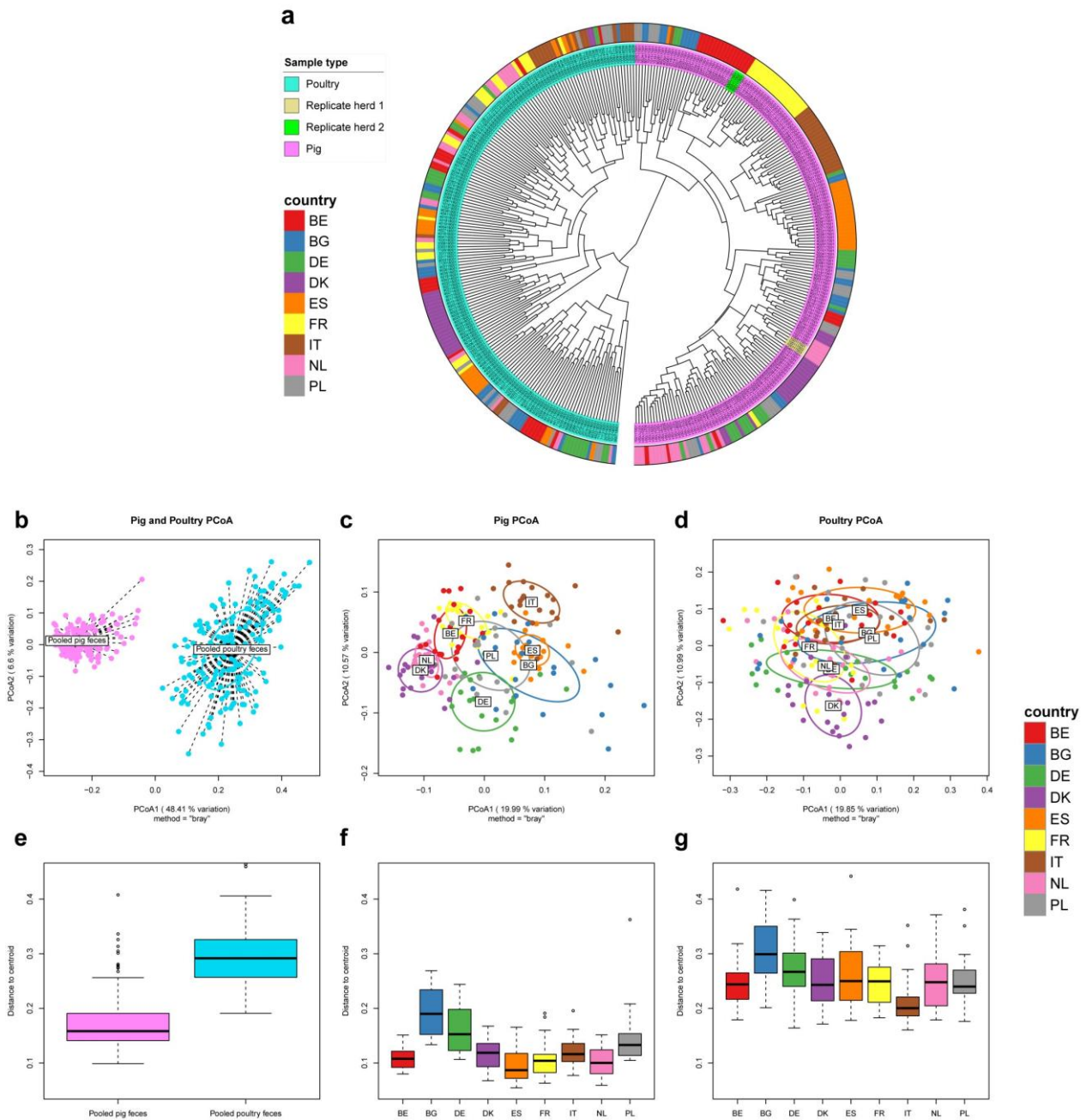
Figure 2. Resistome clustering is influenced by both host animal and country. (a) Dendrogram showing complete linkage clustering of BC dissimilarities between all pig and poultry resistomes. Triple-sampled pig herds are highlighted in separate colors. (b-d) PCoA plots for pig and poultry samples combined (b), pig samples (c) and poultry samples (d), respectively. Ellipses denote standard deviation for distance of each member to its group centroid (labeled). (e-g) Boxplots of distances for each group's samples to its centroid for pig and poultry samples combined (e), pig samples (f) and poultry samples (g), respectively. Horizontal box lines represent Q1, median and Q3. Whiskers denote range of points within Q1-1.5*IQR and Q3+1.5*IQR.

298

299   We ordinated the gene-level resistomes for all samples (Figure 2b) and pig and poultry samples separately

300   (Figure 2cd). As with hierarchical clustering, there was a clear separation of pig and poultry samples, along

301   the first principal coordinate, which explained 48% of the variation across all resistomes.

302   When analyzing the two reservoirs separately, we observed clustering according to country of origin in pigs

303   (Figure 2c), while clustering was more diffuse for poultry (Figure 2d). We tested for the country effect and

304   found it to be significant in both pigs (adonis2 p<0.001) and poultry (adonis2 p<0.001). In poultry however,

305   the country effect only explained roughly a quarter of the variation, while country explained roughly half of

306   the variation in pigs (data not shown). In the pig resistome ordination, the Danish and Dutch samples

307   clustered closely together. The same could be seen for French and Belgian resistomes and to a lesser

308   degree, Italian and Spanish samples. Bulgaria, Germany and Poland showed larger dispersions than the

309   other countries. Poultry samples had a higher dispersion than pig samples (Figure 2e, beta-dispersion

310   p<0.001). Beta-dispersion levels varied significantly between countries in both pigs (Figure 2f, beta-

311   dispersion p<0.001) and poultry (Figure 2g, beta-dispersion p<0.001).

312   We visualized the AMR gene abundances in a heatmap to look at the overall structure and composition of

313   the resistomes and the co-occurrence of AMR genes (Supplementary Figure 5). Some AMR genes were

314   more abundant in one species, while others, including *tet(W)* and *erm(B)* were ubiquitous in all samples, for

315   both species. Among the pig samples, the Italian samples stood out: several chloramphenicol AMR genes,

316   including *cat(pC194)*, *catP*, and *cat_2*, were much more abundant in Italy, compared to the other countries,

317   consistent with our inspection of AMR at class level (Figure 1). Several AMR genes known to be co-located

318   indeed co-occurred across samples. The genes in the vancomycin AMR *VanA* cassette were co-located in a

319   number of poultry samples. This was also true for the *VanB* cassette members, clustering together, but

320   separately from *VanA*, showing ability to distinguish variants of homologous genes. As earlier indicated, the

321   poultry samples showed less country-based clustering than pigs. An exception were the Danish poultry

322   samples. These had noticeably lower abundance of many AMR genes that were widespread in other

323   countries.

Core resistome

325   To determine whether specific genes were unique to each of the host animals, we examined the set of AMR

326   genes that was consistently observed within each animal species (evidence for it in 95% of samples). We

327   identified 33 core AMR genes in pigs and 49 core AMR genes in poultry, with 24 being shared between the

328   two hosts (Supplementary Figure 6). Hence, only nine AMR genes were pig-core genes without also being

329   poultry-core genes. These included the genes making up the Van-G vancomycin cassette, *tet(C)*, *bla*$_{ACI}$ and

330   *cfxA*. Twenty- five AMR genes were poultry-core genes without also being pig-core genes and include the

331   Enterobacteriaceae-associated *strAB*, *sul2*, *blaTEM* and *tet(A)* genes.

332

Differential abundance analysis

334   In order to test which specific genes differed in abundance between countries, we carried out a differential

335   abundance analysis for ResFinder gene read pair counts. Heavy overrepresentation of low unadjusted p-

336   values indicated a large effect of country in both in the pig and poultry datasets (Supplementary Figure 7).

337   Of special interest was the newly characterized *Enterococcus*-associated linezolid-resistance gene *optrA*,

338   that had a significantly higher abundance in Bulgarian poultry farms, compared with poultry farms in all

339   other countries (FDR < 0.05) (Figure 3b). A single Spanish farm did, however, have even higher *optrA*

340   abundance than any other farm. Among the pig herds, the *optrA* gene was more abundant in Bulgarian and

341   Italian herds than anywhere else (except for two farms in Spain) (FDR<0.05).

342   The newly identified colistin-resistance gene *mcr-1* was significantly more abundant in Bulgarian and Italian

343   poultry farms, compared with most other countries (FDR<0.05). France, Poland and Spain had intermediate

344   levels, while Denmark, the Netherlands and Germany had the lowest levels (Figure 3b).

345   As previously noted from visual inspection of heatmaps, multiple chloramphenicol AMR genes including

346   *cat(pC194)* were much more abundant in Italian pigs than other pigs. The ESBL *bla*$_{CTX-M}$ gene cluster 1 also

347   showed country dependency, being significantly more abundant in poultry samples from Spain, Poland,

348   Italy, France and Belgium than Germany (FDR<0.05). Differential abundance analysis results can be found in

349   Supplementary Table 7 and 8 for pig and poultry respectively.

15
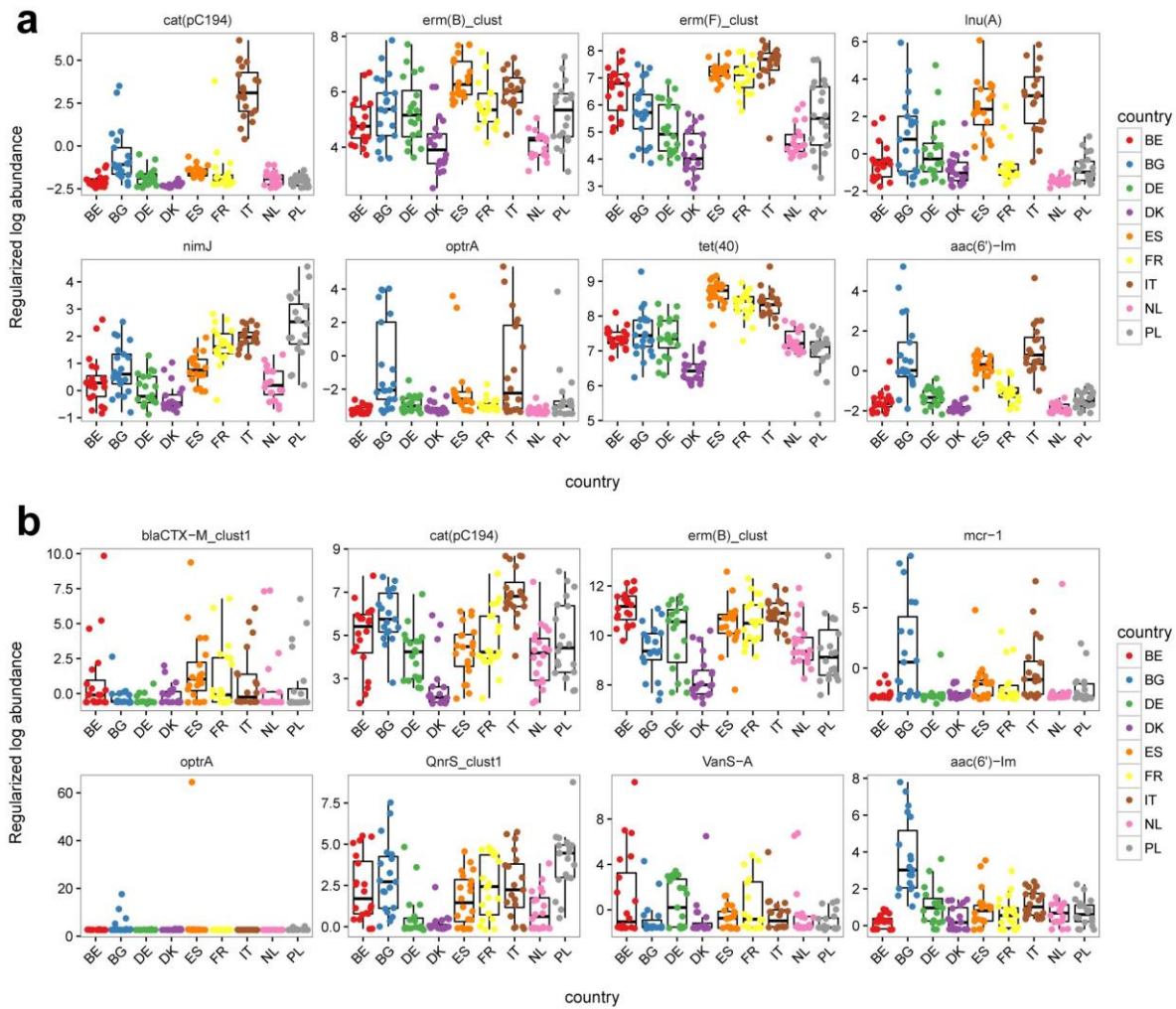
Figure 3. AMR genes differ in abundance between countries. A handpicked subset of genes that differed significantly in abundance between at least two countries' pig farms (a) or poultry farms (b). The regularized log abundance (rlog) is shown on the y-axis in boxplots and points. Points were sideways jittered to reduce overplotting. Horizontal box lines represent Q1, median and Q3. Whiskers denote range of points within Q1-1.5*IQR and Q3+1.5*IQR.
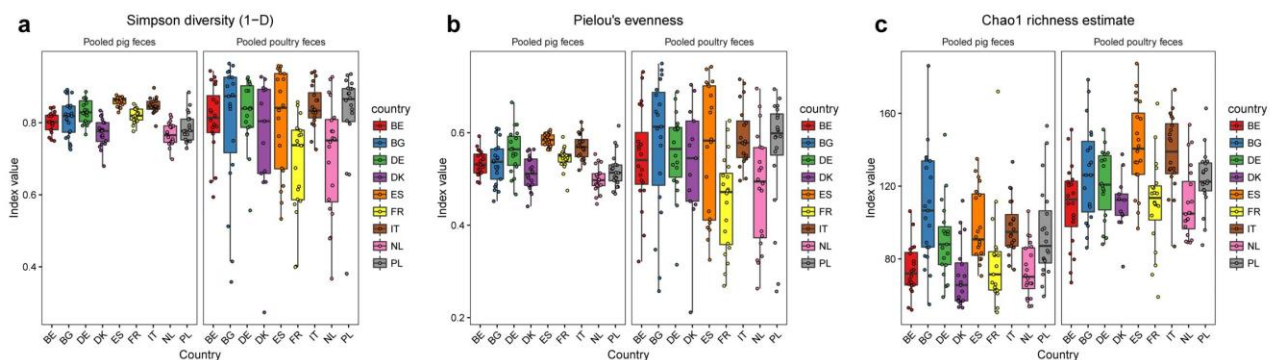
Alpha diversity and richness

We calculated several alpha diversity indexes for each farm resistome (Figure 4) The range of AMR diversity was generally much larger for poultry samples, having both lower and higher diversity than pig samples, which had a tighter spread of diversity. The poultry samples had a higher estimated richness than pigs (i.e. a higher number of unique AMR genes per sample). Alpha diversity indexes can be found in Supplementary Table 9.

Interestingly, countries with high AMR richness in pigs also tended to have high AMR richness in poultry. Spain had the highest median richness in both reservoirs, followed by Italy. Poland and Bulgaria together had the third and fourth highest AMR richness in pigs and poultry. Overall, the median estimated Chao1 richness per country correlated significantly between the reservoirs (Spearman's rho: 0.88, p < 0.01). This was neither true for evenness nor diversity (p>0.05). Rarefaction curves for pig and poultry resistomes can be found in Supplementary Figure 8.

Figure 4. Resistome alpha diversity and richness differs between animal host and countries. From the read count pair matrix, several indexes were calculated. (a) Simpson diversity index, (b) Pielou's evenness, and (c) the Chao1 richness estimate. Horizontal box lines represent Q1, median and Q3. Whiskers denote range of points within Q1-1.5*IQR and Q3+1.5*IQR.

Association between bacteriome and resistome

To test the degree to which bacterial genus composition of the microbiota dictates the resistomes, Procrustes analyses were performed. We compared ordinations of the pig microbiome with the pig resistome and the poultry microbiome with the poultry resistome (Figure 5). We found that for both pig

17

385 and poultry, the bacterial composition correlated significantly with the resistome (p<0.001). Samples with

386 similar taxonomic compositions thus tended to have similar resistome compositions.

387
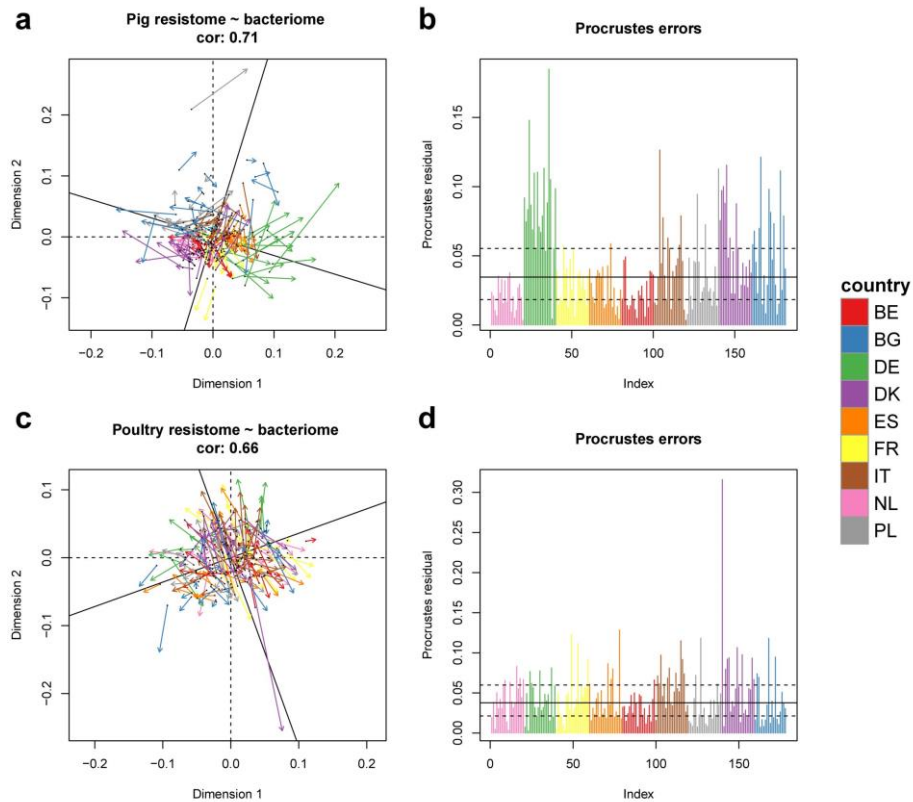
388

389 **Figure 5. Microbial composition dictates the resistome. Procrustes analysis plots for pig (a/b) and poultry (c/d). Procrustes**

390 **rotations of bacterial composition onto their corresponding resistome composition (a/c). Bar charts of Procrustes residuals**

391 **(b/d).**

392

393 The correspondence between the two datasets was slightly stronger in pigs (Procrustes symmetric

394 correlation: 0.71) than in poultry (Procrustes symmetric correlation: 0.66). Interestingly, in pig samples we

395 saw a country effect on the strength of association between the bacteriome and the resistome. In Dutch

396 and Belgian pig herds, ordinations based on bacterial genera and AMR genes gave similar results (Figure

397 5b). For samples from Bulgaria, Denmark, Italy and especially Germany however, the Procrustes residuals

398 were larger. This was less evident for poultry, though a single Danish poultry herd had a very unusual

399 resistome, considering its taxonomic composition (Figure 5d). Stress plots for NMDS can be found in

400 Supplementary Figure 9.

401

18

AMR and drug use association

We found that the total country-level veterinary AMU was positively associated with AMR in both pigs and poultry. The AMR abundance increased by 1736-3507 (95% CI, β=2621) FPKM in pigs when the AMU increased by 1 $\log_e$ unit (36.8% increase in AMU) (Figure 6a) and to a lesser degree in poultry, where the AMR abundance increase by 68 - 1330 FPKM (95% CI, β =700) when the AMU increase 1 $\log_e$ unit (Figure 6b). For pigs, the variance between farms within countries was 7 times larger than the variance between countries in general, whereas in poultry the variance was 4 times larger within-country than between countries.
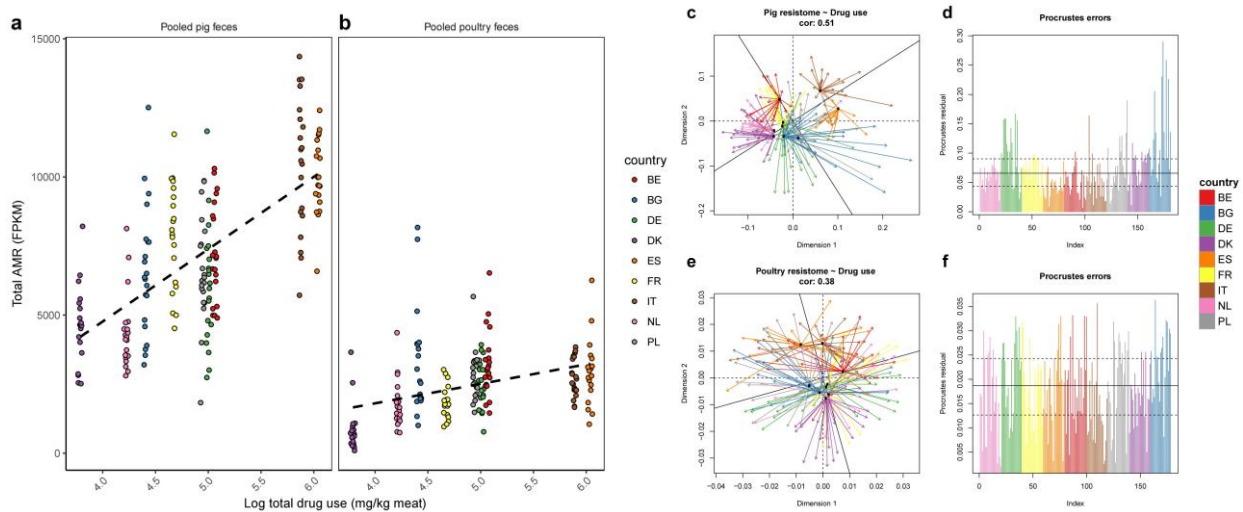
Figure 6. National veterinary AMU affects total metagenomic AMR. (a-b) Scatter plots of country AMU and pooled sample total AMR. A slight sideways jitter was added to the points to minimize overplotting. Trend lines are shown for (a) pigs and (b) poultry. (c-f) Procrustes errors from rotation of resistome principal coordinate analysis (PCoA) onto country AMU PCoA (c-d) and poultry (e-f). (c/e) Procrustes rotations with arrows starting in AMU and ending in corresponding AMR ordination. (d/f) Line plots showing length of each residual arrow in Procrustes rotation.

To test if the AMU pattern was associated with AMR gene profiles, we compared the AMR abundance matrix with AMU matrix, comprised of the 15 recorded AM classes (Supplementary Table 5). We found an association between the veterinary AMU pattern and the pig resistomes (Procrustes correlation: 0.51, p<0.001) (Figure 6cd). The poultry resistomes were also significantly associated with AMU, albeit with a lower correlation, likely due to their larger beta-diversity and lower degree of country clustering (Procrustes correlation: 0.38, p<0.001) (Figure 6ef).

19

Functional AMR genes

In addition to using ResFinder, we also ran most analyses with the FRD database, to elucidate whether the functionally determined AMR genes behave similarly to the acquired AMR genes in ResFinder. If FRD genes serve similar AMR functionality as the acquired ResFinder genes, we would expect similar results.

Using the FRD database, we found both similar and different patterns, compared with using ResFinder. There was still a perfect separation between pig and poultry samples, but the country separation in pigs was less distinct than when using ResFinder (Supplementary Figure 10). Though less variation could be explained by two axes, the PCoA plot of pig samples now clustered German and Spanish samples, with the remaining countries being more similar. The resistome richness showed similar patterns to ResFinder: Spanish, Italian, Polish and Bulgarian samples had a higher estimated richness in both pig and poultry, compared to the other countries. The Procrustes correlation between the resistome and drug usage was lower (0.40 for pig and 0.25 for poultry). This result was echoed by the lack of association between total AMR and total AMU, for both pig and poultry (p>0.05, Supplementary Figure 11).

Discussion

Using a metagenomic shotgun sequencing strategy, we were able to detect and quantify more than 400 AMR genes across 181 pig and 178 poultry herds in 9 European countries.

A recent study including Chinese, Danish and French pigs showed the Chinese pig resistomes clustered separately, while Danish and French overlapped.[16] Here we demonstrate that even among European countries, the livestock resistomes differ in a country-specific manner that might be explained by differential AMU so that countries with similarly high and diverse AMU (Spain, Italy) have similar resistomes, the same way as countries with similarly low AMU (Denmark, Netherlands) also have similar pig resistomes.

We found that the beta diversity dispersion seems to be country dependent, particularly in pigs, with Bulgarian, German and Polish pig herds having more dispersed AMR. While we cannot currently explain this, we consider possible causes as differences in trade and management, among others.

We found the recently discovered plasmid-borne colistin resistance gene *mcr-1* in a number of poultry herds, especially in Bulgaria, Spain and Italy. Spain and Italy had the highest reported veterinary colistin usage among the surveyed countries, whereas Bulgaria has a low reported usage, uncharacteristic for the

454 high *mcr-1* level found here.[13] This gene was recently discovered in China and identified throughout the

455 world and has been identified in pigs, poultry and human clinical infections alike.[38]

456 A newly characterized enterococcal linezolid-resistance gene, *optrA* was detected in a subset of pig

457 samples, with Bulgaria, Italy and Spain having the highest abundances. The *optrA* gene provides AMR to

458 both oxazolidinone and amphenicols, including the veterinary-used florfenicol.[13,39] The high abundance of

459 this gene in these countries can likely be explained by the fact that they have the highest veterinary

460 amphenicol usage (together with Croatia, which we did not sample) among the 26 countries surveyed by

461 ESVAC.[12] This explanation fits well with the fact that Bulgaria, Italy and Spain also had the highest

462 abundances of chloramphenicol AMR genes such as *cat(pC194)* in poultry.

463 Another AMR gene of special interest, the bla_{CTX-M}, was also observed in the poultry herds. The higher

464 abundance of bla_{CTX-M} cluster 1 in Spain, Italy, Poland and Belgium, could possibly be explained by co-

465 selection by fluoroquinolones, which is used more in Spain, Poland, Italy and Belgium than other sampled

466 countries. *qnr* and bla_{CTX-M} genes are frequently co-located on large ESBL plasmids. Veterinary

467 cephalosporin usage did not seem to explain the observed levels.

468 Poland and Spain use far more fluoroquinolones veterinarily than other countries included in this study. We

469 found that plasmid mediated quinolone AMR (*qnr*-genes) was frequently abundant in Polish, but not in

470 Spanish, poultry. In Bulgaria, quinolone AMR was also frequently observed, although their reported AMU

471 did not follow the same trend.

472 Interestingly, we observed that country-wise estimated AMR richness significantly correlated between pig

473 and poultry. Also, the countries with a high AMR richness were also the ones with higher AMR abundance

474 (Italy, Spain, Bulgaria and Poland). While having higher abundance of the ubiquitous core genes, these

475 countries had a higher number of unique genes in both pig and poultry production. The fact that countries'

476 AMR abundance and gene richness in pig and poultry tend to follow each other, could perhaps be explained

477 with policy: if a country has strict AMU regulations in one livestock species, chances are that similar

478 regulations are in place for other livestock species. Better host-separated, preferably herd-level, AMU data

479 is needed to further explore this.

480

481 It has previously been reported that soil community composition structures the soil resistome.[26] Using a

482 wide array of environmental matrices, including fecal and waste water samples, this has also been shown

483 for human habitats.[40] We found the same to be true for pig and poultry resistomes, and additionally, we

484 showed that the taxa-AMR association strength differs between countries. Horizontal gene transfer (HGT)

485 could explain this phenomenon, if a larger proportion of certain countries' resistome is mobile and AMR

486 genes are more frequently introduced and re-introduced to genera. On the other hand, vertical AMR

487 transmission can also play a role; if e.g. one country's livestock is more isolated from trade. This would

488 mean more livestock generations for the microbiome and resistome to diverge from those in other

489 countries.

490 The determination of a set of core AMR genes for pig and poultry likely represents an underestimate of the

491 true core resistomes. Requiring a certain percentage of samples to show evidence for a gene does not

492 account for differential sequencing depth. The core genes discovered here thus likely represent a

493 conservative subset of the true core resistomes.

494 In contrast to ResFinder, when using FRD we found no relationship between total drug use and total

495 functional AMR abundance. This suggests that while many genes can provide AMR when cloned into e.g. *E.*

496 *coli* in functional metagenomic assays, they might not provide AMR functionality in their natural hosts with

497 natural expression levels. If most of them did, we could expect to see AM-based selection and an

498 association to drug usage, like it is observed for the AMR genes in ResFinder - a database of genes known to

499 provide AMR to their natural hosts. This finding echoes previous sentiments that one should carefully

500 consider the risk to human health imposed by individual AMR genes.[41] Some FRD genes might represent

501 high risk, but we currently do not know what subset that is. Creating the FRD is a first step in trying to

502 catalog the many AMR genes found in functional metagenomic studies. Screening sequenced pathogenic

503 isolates and metagenomic assemblies for FRD genes, would be a good start for assessing their host range

504 and risk potential.

505 The AMU data used in this study is not optimal. There is variation in drug use within each country's farms

506 that we did not account for by using a national average and we are unable to accurately distinguish

507 between drugs used in different livestock species. Moreover the PCU denominator used by ESVAC may vary

508 greatly between countries with differences in livestock populations and imports/exports that contribute to

509 the biomass as calculated. Furthermore, the integrated herds enrolled in this study might represent only a

510 limited subset of the overall livestock production in some countries. However, even with our crude AMU

511 estimates, we found significant associations with total AMR abundance. The similar conclusion when

512 considering the specific drug usage profile of each country indicates that the resistome is responding to

513 AMU. The AMR-AMU association is well-documented for specific cultured indicator species and certain AM

514 drugs, but relatively unknown when considering the whole microbiota and resistome and the newer

515 approach of metagenomic shotgun sequencing.[3,8] We do not know why the pig samples had a large within-

516   country spread of total AMR, but perhaps the more heterogeneous production system and production

517   management is responsible.

518   DNA extractions from the pooled poultry samples resulted in relatively low DNA yields. The protocol used

519   was optimized for pig feces, human feces and sewage, but not poultry feces.[18] The lower yields

520   necessitated the use of a PCR-based library preparation kit, that can influence downstream analysis of

521   shotgun sequencing.[42]. While the large difference between pig and poultry resistomes in our study is likely

522   real, we caution the use of sensitive, quantitative analyses when comparing between samples prepared

523   using different library preparation kits. For this reason, we have mostly tested within each reservoir. For

524   future studies, one might consider using larger volumes of bird feces or otherwise optimize the protocol to

525   ensure PCR-free library preparation is possible for all samples.

526   The sensitivity of metagenomic approaches does not yet rival phenotypic alternatives such as selective

527   enrichment. We relied on read-mapping as opposed to a metagenomic assembly-based strategy for greater

528   sensitivity. Still, there are AMR genes in important pathogens that we know are likely present but are below

529   our detection limit. For example, we only found evidence for bla_{CTX-M} in three pig herds, whereas in

530   phenotypic studies, the prevalence is high even among farms with no cephalosporin usage.[43] Another

531   tradeoff with the use of reference-based read mapping is our inability to identify mutated housekeeping

532   genes granting AMR or distinguish between close homologs of the same AMR gene, e.g., the non-ESBL

533   bla_{TEM} genes from those encoding ESBL variants.

534   The primary concern with read-mapping techniques, the lack of genomic context, can be solved using

535   metagenomic assembly and binning approaches.[16,44,45] In this way, AMR alleles in full length, their genomic

536   context and their associated taxa have been identified in both pig, poultry and human fecal samples.[46] As

537   shown previously, the association between AMR and AMU is similar for metagenomics and traditional

538   phenotypic methods, but several aspects make metagenomics an intriguing monitoring tool.[17] The fact that

539   both types of analyses (quantitative, sensitive read mapping and qualitative, context-giving binning) use the

540   same raw data, makes metagenomics an attractive tool. In addition, the digital nature of shotgun data

541   would also allow future re-use and form the basis of an invaluable historical archive, potentially usable for

542   both AMR and pathogen tracking worldwide. While it is possible to identify thousands of AMR genes from

543   environmental samples using functional metagenomics, and then track them using shotgun sequencing,

544   their association to drug use and potential risk to human and animal health requires much further work to

545   inform effective drug policies.

546 We found that the metagenomic resistome varied significantly in livestock, with large differences between

547 the pig and poultry reservoir, but also within each livestock species, in a country-dependent manner.

548 Within each country, we found different levels of variation, with some countries having more homogenous

549 herds than others. Differences were seen both in total AMR abundance, but also abundances of AMR types

550 and specific genes, including clinically relevant AMR genes. Some of this variation, we attributed to

551 differential drug usage between the countries. We also identified the microbiome background as an

552 important factor in determining the resistome in livestock, but found the strength of the association was

553 country-dependent, at least in pigs. Interestingly, we found that AMR richness in one livestock species in a

554 country is linked to the abundance in another livestock species. Finally, we observed some indications that

555 newly described AMR genes from functionally metagenomic studies, might not provide AMR functionality

556 when expressed in their natural host, even though they have the potential at the right expression levels in

557 the right organism.

Data availability

559 The DNA sequences (reads) from the 363 metagenomic samples from the 359 herds are deposited in the

560 European Nucleotide Archive (ENA) under the project accession number PRJEB22062.

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Author contributions

FMA, DH, JAW, TH, DM and the EFFORT group designed the study. FMA and BEK detailed the sampling and sequencing. ASRD and the EFFORT group carried out sampling. BEK and SJP conducted the DNA purification and organized with PM the sequencing. RBH, OL and TNP created the read-mapping pipeline. ER created the functional resistome database. PM, OL, RECL, LC, LAMS, HS, AB and HV carried out the bioinformatics and statistical analysis. PM created the figures and drafted the manuscript. All authors helped review, edit and complete the manuscript.

Competing interests

The authors declare no competing financial interests.

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