Antimicrobial resistance in the farm environment: determinants of the resistome of animal feces and airborne dust

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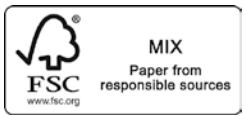

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Antimicrobial resistance in the farm environment: determinants of the resistome of animal feces and airborne dust

Antimicrobiële resistentie in de boerderijomgeving: determinanten van het resistoom van dierlijke mest en stof in de lucht

(met een samenvatting in het Nederlands)

Proefschrift

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General introduction

Antimicrobial resistance (AMR) is the phenomenon that infectious microorganisms acquire genetic information which results in a reduced response to treatments. In this thesis, AMR specifically refers to bacteria resistant to antibiotics. Antibiotics normally kill or inhibit the target bacteria (and many others as collateral damage of the therapy), and thereby stop or prevent infection. In general, soon after the introduction of a new antibiotic product on the market, resistance to this substance has been described [1]. The exact genetic factors and associated mechanisms leading to resistance differs between different types of antibiotics and bacteria, but in most cases, the information causing resistance is encoded on a small piece of DNA, acquired or intrinsically present, inside the bacterium; a resistance gene [2]. These genes are not new, for example genes similar to current vancomycin and beta-lactam resistance genes have been found in ancient reservoirs long before the introduction of antibiotics on the market took place [3, 4]. However, abundance and circulation has increased rapidly after the introduction of large-scale treatment with antibiotics and also then appeared in clinically relevant pathogens [5, 6]. The current challenge of antimicrobial resistance is thus an unwanted side-effect of an impressive development in our health care system: infections that once were a cause of high morbidity and mortality have become relatively easy to treat. However, exactly this use led to the problems of AMR we are facing now. Some of these 'easy to treat' infections have become not so easy to treat anymore [7, 8]. And since we do not have many treatment alternatives yet, controlling the development of AMR is a top priority.

The role of livestock farms

a. AMU and AMR in livestock

One of the main consumers of antimicrobials, apart from human healthcare, is livestock farming. Over the past 70 years, livestock farming has scaled up immensely in most places of the world. This upscaling was accompanied by a continuously increasing use of antimicrobials for treatment, but also for prevention of infections and/or growth promotion of animals. [1] Antimicrobial usage (AMU) and AMR in livestock, and their relation, has been a research topic since 1969, when the Swann report on the use of Antibiotics in Animal Husbandry and Veterinary Medicine was issued [9]. Since then, bust mostly since 2000, many initiatives were initiated to monitor AMU and AMR. Denmark was the first to have broad, systematic and continuous AMR surveillance and AMU monitoring programs in livestock in place [10, 11]. Other countries like France, Norway, Sweden, UK, the Netherlands and Canada followed with smaller or larger programs in AMR and AMU or both [12, 13]. Worldwide, for most countries, structural surveillance and monitoring (and reporting thereof) is in development, just starting, or non-existent [7, 14, 15] and often knowledge on AMR in the past can only be extracted out of (scarce) scientific papers [16, 17]. All these different sources do, however, show a roughly similar trend: a constantly increasing AMR prevalence in bacteria sampled from animals. In the Netherlands, where first recording started in 1998, AMR profiles of commensal *E. coli* isolated from animal feces to most types of antibiotics increased until 2009 [18]. In 2009, a nationwide plan was started with the aim to drastically lower AMU in the major livestock systems; pigs, broilers, dairy cattle and veal calves [19], and as a result since 2009 almost all types of resistance in *E. coli* started decreasing [18]. This type of aggregate or ecological

analyses, relating general AMU with a random selection of resistance in indicator organisms from the same region/country, has been undertaken at European level as well [20, 21]. The (data) situation per country differed, but positive associations were found between AMU and AMR, within one sector, for example between polymyxin use and resistance in *E. coli,* in both poultry and pig farming, and between the sectors, for example between total quinolone use in food producing animals and resistance in invasive *E. coli* in humans. The latest ESVAC report shows a decline of antimicrobial sales since 2014 in many European countries, while before sales generally increased [22].

Over the past 70 years, indeed AMU seems to be a primary driver in the emergence of AMR in livestock [1, 16]. However, the initial emergence of resistance is one aspect, but the subsequent impact of AMU on AMR depends most likely on more factors, like the prescribing practices and the involved bacteria. This was, for example, illustrated by a study on fluoroquinolone resistance in *E. coli*, in which resistance prevalence was influenced by a higher dose of enrofloxacin, oral versus parental administration, and lack of fitness of the *E. coli* strain [23]. Additionally, the impact of AMR depends heavily on the potential to spread and transmit among animals and between bacterial species [24, 25].

b. Relevance of farms for humans and the wider environment

AMR is a complex problem due to, among others, the many actors involved. Figure 1 shows one of the multiple attempts to visualize all the potential interactions and transmission routes of AMR from one human or animal or environmental reservoir to another.

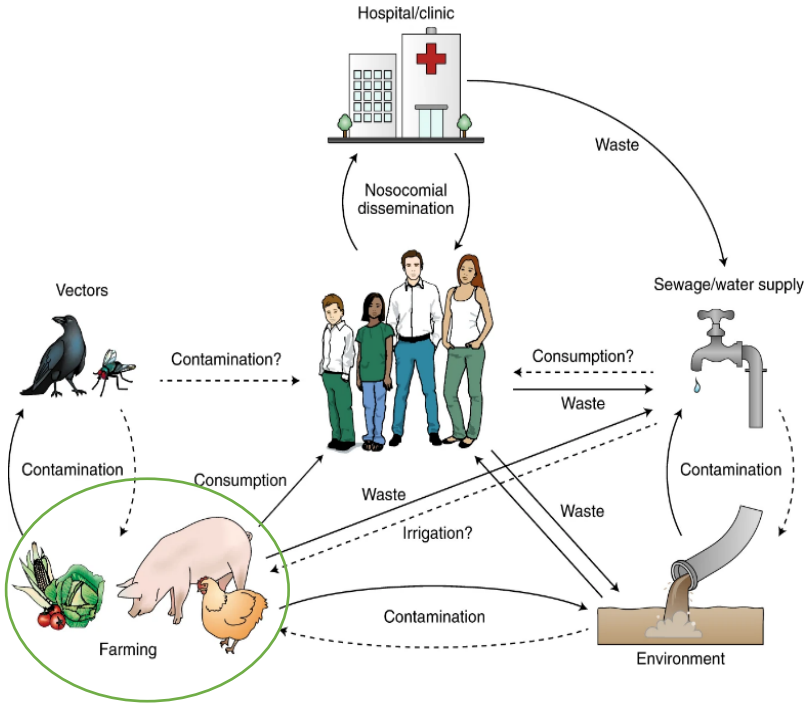

Figure 1 – Complex AMR interactions across one health sectors. Dashed lines indicate putative transmission paths. From Walsh et al. 2018 [26].

In the left bottom corner, 'farming' is visualized. Although resistant pathogens form a problem in veterinary medicine, (carriership of) resistant bacteria in animals seems, up to date, not often an immediate health threat to the livestock themselves [27-29] and resistance in livestock is mostly studied in relation to human health [30]. The ever increasing pressure, by AMU and ARGs, on the bacterial population in and around livestock farms is worrying for multiple reasons. Human pathogenic resistant bacteria stemming from farm animals can reach and harm the human population in a direct way via consumption of products of animal origin [31]. Transmission pathways can also be more indirect and include environmental compartments such as soil or water. Fecal waste from the animals can reach the outside environment with manure application on agricultural land [32], and with discharge of waste water[33] and runoff[34] fecal waste can reach surface or ground water. Lastly, vectors such as birds, rodents but also larger wild life, can play a role in connecting one reservoir to another [35, 36]. An important mechanism to simultaneously consider, is that resistance genes present in non-harmful bacteria can be transferred from one organism to another by horizontal gene transfer, of which the second (or third or, …., tenth) recipient might be pathogenic [25, 37]. This process can happen in any reservoir or between reservoirs. Not (explicitly) depicted in figure 1, however potentially relevant, is direct transmission via animal contact of farmers and their family members [38] and, next to the discharge (contamination) of resistant genes and bacteria in the environment, the discharge of products which put pressure on the bacterial composition favoring resistant over non-resistant strains from the farm, such as antibiotic residues, metals and biocides [39].

Lastly, not depicted and indeed also less studied, another environmental reservoir potentially involved in the dissemination of AMR in and from the farm is air. Air contains small dust particulates with bacterial material, so called bioaerosols [40, 41]. In the farm, animal fecal particles can become airborne and spread after transfer to outdoor air, through wind [42]. Some evidence already points to the relevance of aerial transmission of AMR related to livestock farms. Increased MRSA carriage of farmers as a result of aerial exposure has been postulated [43]. Additionally, viable resistant bacteria have been measured up to 150m from the farm [44]. The first attempts of metagenomic analysis of farm airborne dust reveals that dust consists of many different resistance genes making it a relevant reservoir [45].

All these human, animal and environmental dimensions of AMR and the interaction between, make AMR indeed a 'quintessential One Health issue', as Robinson and colleagues stated [46]. However, this is not only applicable for the total picture of AMR, specifically within farming systems this is also very accurate.

The next steps in livestock related AMR research

Although much knowledge on AMR is gained over the past decades, pressing relevant questions remain, partly due to several technical innovations and subsequent new opportunities. More refined knowledge on the extent, spread and determinants of AMR in and around the livestock farm is an area very relevant but still with many puzzling issues and

research opportunities. Some of these issues are addressed in this thesis and introduced hereafter.

a. The use of qPCR and metagenomics

Although the fraction of bacteria which can be cultured is estimated to be small (Amann et al 1995), AMR research has long been focused on specific (culturable) pathogens or indicator organisms, as other techniques were not available yet or affordable or not yet of interest [47]. However, in order to broaden the analysis of AMR beyond resistance in single species, the use of molecular techniques was introduced in the field in the eighties and took off in the nineties [47, 48]. (Quantitative) Polymerase Chain Reaction ((q)PCR) enables identification of specific antimicrobial resistance genes (ARGs). Due to constant development of this technique, it can now be used with almost any sample type and in both isolated bacteria or total bacterial communities. By relating the outcome to bacterial abundance (targeting 16S rRNA genes) in a sample a quantitative measure of the ARG relative to the bacterial concentration (relative abundance) is obtained. However, it still analyzes only a limited number of resistance genes (or other genes), that also need to be chosen (and known) beforehand. Metagenomic sequencing is overcoming those problems. With shotgun metagenomic analysis, all DNA extracted from a sample is sequenced and identified. Metagenomics thus bears the promise to detect everything, the full 'bacterial microbiome', without prior knowledge needed on what bacterium is in the sample. By 2010 the costs of metagenomics had dropped significantly so it became a research technique available for more and more researchers [49]. With the development of resistance gene sequence databases, such as ResFinder [50], it is possible to describe the 'resistome', the collection of all resistance genes, of the bacterial microbiome. The use of metagenomic analyses in samples from livestock sectors will definitely shed a whole new light on the extent of AMR in the farm [51], However, there are many challenges with this type of 'big data'. Challenges related to bioinformatics like sequencing depth and sensitivity, related to analysis, like normalization and suitable statistical methods, and related to interpretation of the results [52]. More experience should to be gained in this field to overcome these challenges, while uncovering the largely unexplored resistome of the farm.

b. Determinants for AMR at farm level

As stated above a positive relation between AMU and AMR has been shown on country or region level for certain bacterial species [16, 20, 53]. Studies to determine the relationship between AMU and AMR based on more refined epidemiological data, e.g. at farm level, are less common than one might think and again have mainly focused on one bacterium (commensal or pathogen) often in a single sector and country [54-61]. For example, fluoroquinolone use on broiler farms has been shown to be associated with increased prevalence of fluoroquinolone resistant *E. coli* and *Campylobacter* spp. [55] or higher AMU in general with higher MRSA prevalence on pig farms [57].

A study design at farm level, also allows for inclusion of other potential farm and animal related determinants for AMR abundance in the farm. Such a determinant is for example farm biosecurity. Farm biosecurity involves all measures possibly taken to reduce the entry (external biosecurity) and spread (internal biosecurity) of infectious agents [62, 63]. Many aspects of biosecurity, such as hygiene protocols, animal density or disease management, could, imaginably, also relate to (the spread of) AMR or interfere with the relation between AMU and AMR. Some earlier studies into AMR at pig and poultry farms have shown significant associations with biosecurity related factors such as hygiene locks or type of farm (open or closed) [55, 57-59] and resistant bacteria. Epidemiological studies based on farm (or flock) level data, and with sufficient sample size, have the potential to provide more detailed knowledge on the link between (current) antimicrobial usage and antimicrobial resistance in livestock production systems, and give opportunities to explore other determinants, modifying factors and confounders. However, the practical situation on the farm can differ a lot between and within livestock systems, which make these type of determinant analyses challenging as well.

c. The role of the farm environment

The role of the environment, in general, in AMR epidemiology has gained attention in the past 10-15 years [64, 65], but is still not as fully imbedded in the AMR field compared to the study of human and animal reservoirs [66]. As described the first paragraphs, farming has an important part in AMR epidemiology. To gain more insight in AMR dynamics on the farm and the potential exposure of animals, farmers, nearby residents and the natural environment to AMR through farming, we will need to assess the farm environmental reservoirs, on top of the more common animal fecal sampling. Next steps consist of describing the farm environmental resistome, quantifying resistance and determining factors that influence these concentrations and transmission, in order to ultimately quantify the attribution of farming systems as a whole to AMR abundance or human carriership [65, 67].

One of the focal points in this thesis is the farm environmental compartment air. Sampling bioaerosols is a challenging procedure. Typical complexities within bioaerosol sampling (techniques) are related to, capturing the relevant dust particle size, collecting a representative sample, because of limitations to sampling time, and achieving a sufficient sample size, limited by logistics and complex laboratory analyses, and last but not least, if one aims for culture dependent results, the challenge of keeping bacteria viable during sampling and storage [68]. There are therefore many ways of sampling air for microbiological measurements with which these complexities are addressed to a smaller or greater extent [69-71], and used in earlier studies [44, 72, 73]. However, none of the available sampling approaches has solved each of the aforementioned problems. Sample collection through active sampling, i.e. including pumping defined amounts of air through collection devices, is, time consuming and asking for a high level of technical expertise and thus expensive. Importantly, it is also the question if this type of detailed sampling is necessary to answer all questions regarding AMR transmission via air. Other less demanding methods of bioaerosol

sampling consist of passive dust collection methods e.g. through dust scrapings from window sills (which will result a mixture of old and fresh material) or through electrostatic dust collectors (which will result in mainly fresh airborne dust) [43, 74, 75]. The latter methods can more easily be implemented in large and/or multi-country studies and might give already sufficient information to gain relevant insights into AMR in the animal and farm environment and determinants thereof.

EFFORT

Until the start of the EU-FP7 EFFORT project, large studies on the resistome of animals let alone the resistome of their direct environment and link with AMU were missing and are to date still scarce. The European 'Ecology from Farm to Fork of microbial drug Resistance and Transmission' project (EFFORT) is a research program which started in 2013 as a collaboration between 10 European countries and included 20 partners. The overall objective was to study the (complex) epidemiology of AMR in the European food chain of the major livestock sectors. The use of metagenomics for resistome analysis in livestock on a large scale was unprecedented. Next to performing extensive fecal sampling, it was the aim to also study the animal environment, which led to a large-scale dust sampling campaign. Aside from the metagenomics part, across the project over 10.000 samples have been analyzed with qPCR. On the website (http://www.effort-against-amr.eu/) the collection of, thus far, published results from the EFFORT project can be found.

The general setup of the EFFORT project (and therewith for this thesis) was a cross-sectional design with sample collection in nine countries (Belgium, Bulgaria, Denmark, France, Germany, Italy, the Netherlands, Poland and Spain). In every country, 20 (conventional farrow to finish) pig and 20 (conventional broiler) poultry farms were included, by collecting 25 fresh fecal samples of the animals closest to slaughter age. Additionally, 3-4 freshly settled dust samples were collected from the same stables. During the farm visit information was gathered by conducting a questionnaire with the farmer. Collected data included AMU and biosecurity data and other farm and flock characteristics such as, age at sampling, type of feed and number of animals at the farm. In one country, the Netherlands, an additional stool sample of farmers from the same farms was collected.

Main objectives of this thesis

This thesis describes the investigation of the fecal and dust resistome and bacterial microbiome of pig and broiler farms in nine European countries with the following objectives:

- To describe the fecal resistome of pigs and poultry from nine European countries
- To determine the relationship between the fecal resistome and antimicrobial usage and biosecurity
- To describe the farm dust resistome and bacterial microbiome of pig and poultry farms, as part of the animal environment
- To determine the relationship between the farm dust and animal fecal resistome

- To determine/explore the relationship between the farm dust resistome or individual ARGs and several farm and animal related characteristics including antimicrobial usage and biosecurity
- To explore to which extent airborne ARGs reach the outdoor environment of the farm

Outline of the thesis

Part I - Poultry and pig fecal resistome and the role of antimicrobial usage

Chapter 2 describes the resistome, i.e. all known resistance genes, of one pooled fecal sample per farm of all 360 pig and poultry (broiler) farms and the relationship with some general determinants thereof such as country level AMU, bacterial microbiome and country of origin. A detailed analysis of the resistome abundance in poultry and its relationship with antimicrobial usage in the sampled flock and on the total farm is presented in chapter 3. The association with farm biosecurity is explored as well.

Part II - The poultry and pig aerial resistome in and around the farm

Chapter 4 describes the pig and poultry farm dust resistome and bacterial microbiome and its mutual relationship. For analysis of the farm dust resistome, one pooled sample per farm was studied with metagenomic analysis. The relation with the animal fecal resistome of the animals inside the same stable and with AMU was determined. Lastly the relation with the farmers resistome was explored. A detailed description of four of these resistance genes in the total resistome and a broad analysis of determinants for absolute and relative gene abundances is presented in chapter 5. For this larger association study, up to 3 samples per farm were included and resistance gene levels in dust were determined with qPCR.

Chapter 6 describes, not earlier published data of active air measurements to determine resistant gene levels upwind, inside the farm and up to 150m downwind of the farm, around a selection of EFFORT farms from 3 countries. The quantification of resistance gene levels in outdoor air around the farms was also done with qPCR.

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2

Abundance and diversity of the faecal 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. The 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 and in humans. We quantified and characterized the acquired resistance gene pools (resistomes) of 181 pig and 178 poultry farms from nine European countries, sequencing more than 5,000 Gb of DNA using shotgun metagenomics. We quantified acquired AMR using the ResFinder database and a second 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 in poultry. We found higher AMR loads in pigs, whereas 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 between 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. However, functionally determined AMR genes were 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 in 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 the use of antimicrobials in livestock will lead to an increased occurrence of AMR and that the reduction of usage will eventually lead to reduced resistance[3–8]. Several national surveillance programmes 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 (for example, *Escherichia coli*) isolated from different European countries[3,13]. Current monitoring efforts are mainly based on culturing indica- tor bacteria followed by phenotypic AMR determination[13,14]. This procedure only targets a limited number of species present in the gut microbiota and, therefore, probably represents only a fraction of its resistome (the collective pool of AMR genes). Metagenomic approaches have been used in several recent studies and have shown that metagenomic read mapping describes AMR abundance in bacterial communities more accurately than commonly used technologies on selected indicator organisms[15–17]. A recent study focused on sampling a diverse group of individual pigs from 11 farms in 3 countries and showed that genetics, age, diet and geography all probably influence the pig microbiota, but little information is available for the poultry microbiota[16].

As part of the European Union-funded EFFORT project (www. effort-against-amr.eu), we sampled >9,000 animals in 181 pig and 178 poultry herds in 9 European countries, generating herd-level composite samples as previously described[17]. Metagenomic sequenc- ing of these samples gives us a unique insight into the abundance, diversity and structure of the acquired pig and broiler resistomes in Europe. An association between AMR gene abundance and national veterinary antimicrobial usage (AMU) was also analysed. The results and raw data presented here can be used as a baseline for future metagenomic AMR monitoring. To our knowledge, this study repre- sents the single largest metagenomic AMR monitoring effort of livestock: both in terms of countries (9), herds included (359), individual animals sampled (>9,000) and sequencing effort (>5,000 Gb)[16].

Methods

Farm selection and sampling. The sampling protocol for pig and broiler farms that has been agreed on by the EFFORT consortium is described below. The selection of farms and thesampling procedure followed these guidelines to the extent possible, but some deviations from the protocol were occasionally necessary. The selection and sampling goals are described below, whereas a detailed description of the sampling conducted in the individual countries and exceptions is provided in the Supplementary Material.

Selection of pig and poultry farms. In each participating country, 20 conventional integrated pig farrow-to-finisher non-mixed farms were selected. The farms needed to have a minimum of 150 sows and 600 fatteners and use batch production to ensure that most of the animals of the sampled group originated from the same birth cohort. All-in all-out production at compartment level was preferred, and all fatteners sampled were required to have been on the same site during their entire life. Selected farms should have no contact through livestock trade and should have a random regional distribution.
In each country, 20 conventional broiler farms (no breeders) were selected. The farms had all-in all-out production, with a thinning procedure from day 30 onwards allowed. All selected farms should have no intended slaughter age higher than 50 days, no slow-growing breeds (intended growth rate of less than 55 g per day) and no stocking density lower than 10 birds per m^2. Only one flock per house per holding should be sampled and each flock should be between 20,000 and 40,000 birds. If possible, the selected farms should have a random regional distribution.

Procedure for sampling. We sampled pig farms between May 2014 and December 2015 and tried to minimize seasonal influences. The sampled fatteners were as close to slaughter as possible (that is, within the last week). A total of 25 fresh, still-warm and undisturbed faecal droppings were sampled from pen floors (a minimum of 10 g faeces per sample) randomly divided over all eligible compartments or stables of fatteners close to slaughter. Broilers were sampled between May 2014 and June 2016 and we tried to minimize seasonal influences. On each farm, 25 undisturbed, fresh main bowel droppings were collected from the floor of the house (a minimum of 3 g faeces per sample). The flocks were sampled as close to slaughter as possible (the last week before the final depopulation). All samples were collected aseptically in plastic containers and were stored at 4 °C and transported to the laboratory within 24 hours after sampling.

Pooling and handling of samples. Upon laboratory arrival, individual faecal samples were homogenized by stirring thoroughly with a sterile tongue depressor or spoon for a few minutes. From each pig sample, two 2-ml cryotubes were filled and frozen immediately at −80 °C (alternatively at − 20 °C for a maximum of 4 days, before transferring to − 80 °C). For broiler samples, two cryotubes were prepared with at least 0.5 g faeces each. Sample pooling was either done immediately or the frozen tubes were shipped to the Technical University of Denmark (DTU) on dry ice for pooling. Individual samples from the same herd were defrosted and placed on ice briefly before weighing. Following weighing, they were pooled with 0.5 g faeces from each sample and stirred for a few minutes with a sterile device (for example, a disposable wooden tongue depressor). All samples were only thawed once shortly before DNA extraction.
After the removal of two mislabelled samples, composite samples from a total of 178 broiler flocks and 181 pig herds remained.

Sampling to estimate the effect of random sampling. To study the potential effect of sampling randomness and the reproducibility of our sampling protocol, a Belgian and a Dutch pig herd were chosen for triplicate sampling. These two herds were sampled three times on the same day (25 samples × 3 sampling rounds), resulting in 6 pooled samples (2 herds × 3 sampling rounds), from which the within-farm resistome variation was assessed. A table with all the samples and the associated data is included as Supplementary Table 9.

DNA extraction and sequencing. From each of the pooled, herd-level faecal samples, DNA was extracted using a modified QIAamp Fast DNA Stool Mini Kit protocol (51604, Qiagen), as previously described[23]. One major modification is the addition of a bead-beating step at the beginning of DNA extraction. The protocol can be found at figshare.com/articles/SOP_-_DNA_Isolation_QIAamp_Fast_ DNA_Stool_Modified/3475406. DNA purification of all the pooled samples was processed centrally at the 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; Oklahoma City, OK, USA). At the OMRF, DNA from all samples was mechanically sheared using ultrasonication to a targeted fragment size of 300 bp (Covaris E220evolution). For pooled pig samples, library preparation was performed with the NEXTflex PCR-Free library preparation kit (Bioo Scientific). For poultry samples, owing 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 60 samples, the libraries were multiplexed and sequenced on the HiSeq3000 platform (Illumina), using 2 × 150-bp paired-end sequencing per flow cell. A total of 17 Belgian, Danish and Dutch pig faecal samples were sequenced on the HiSeq2500 platform (Illumina), using 2 × 100-bp paired-end sequencing before it became unavailable at the OMRF (see Supplementary Table 9). In total, DNA from 365 pooled samples was extracted and shotgun sequenced, resulting in >36 billion sequences (18 billion paired-end reads), comprising > 5,000 Gb of DNA. The sequencing yielded an average of 50 million (s.d.: 18×10^6) paired-end reads per pooled sample. This was similar for pig and poultry samples, although the sampling depth was more varied in pig samples.

Bioinformatics processing. The DNA sequences (FASTQ reads) from each sample were analysed following the principles from the previously described MGmapper tool[15]. To avoid PCR copies in the poultry data, identical read pairs were removed using 'MarkDuplicates' from the Picard software (v2.8.3; broadinstitute.github.io/picard). Adaptor sequences were removed using BBduk (BBMap software)[29].

Sequences from phiX174, which is an internal sequencing control, were removed using the BWA-MEM algorithm[30]. Trimmed read pairs were aligned using the BWA-MEM algorithm (Burrows-Wheeler aligner) to the prokaryotic RefSeq genomes from the NCBI GenBank with 'reference' and 'representative' tags (downloaded on 18 November 2016). The BWA-MEM algorithm (v0.7.15) normally estimates the insert size individually per computer CPU core. We used a per-sample estimate to increase the robustness of the estimated insert sizes and, therefore, the acceptable mapping distances for read pairs.

The read pairs were aligned to the prokaryotic genomes again and to the AMR genes present in the ResFinder database (accessed 17 November 2016) using the robust insert size estimates[31]. ResFinder is a manually curated database of acquired AMR genes and, therefore, does not include intrinsic AMR genes and mutated housekeeping genes.

Properly paired read pairs, with at least a 50-bp alignment in each read were accepted. ResFinder mapping counts were adjusted for differences in both gene lengths and bacterial sequence abundances by computing FPKM values for each ResFinder reference sequence[32]. Raw mapping count data and their associated FPKM values can be found in Supplementary Tables 2 and 10.

Genes with many alleles in ResFinder result in unspecific mapping and randomly assigned read pairs. To avoid sensitivity loss and wrong assignments, we kept ambiguous hits, but aggregated their abundances to higher levels, corresponding to 90% gene identity clusters. To determine these clusters, we used CD-HIT-EST (v4.6.6) at a 90% identity level and otherwise default settings[33].

The resulting gene clusters were manually inspected and named to reflect their gene members (Supplementary Table 11). In addition to this 'gene cluster' level, we summed the FPKMs to resistance phenotype levels, as annotated in the ResFinder database.

FRD. Previous studies have identified a wide array of AMR genes in various reservoirs using functional metagenomics, referred to as functional AMR genes[20,34–36]. By cloning random DNA fragments from complex microbiomes into an expression vector expressed in a host (typically *E. coli*) and selecting for growth in the presence of certain antibiotics, they have been found to provide AMR to many antibiotics[20,34–36]. We constructed a FRD from 3,416 AMR gene variants identified in four major studies using 23 different antimicrobials for selection[20,34–36].

Briefly, in each of these studies, DNA was extracted from environmental and human faecal samples, fragmented and cloned into a plasmid vector and screened for AMR functionality in *E. coli* cultured with one of several antimicrobials.

Cloned fragments in plasmids that were found to confer AMR were sequenced and the AMR genes were identified. The protocol for the database construction can be found at cge.cbs.dtu.dk/services/ResFinderFG. Genes were quantified using MGmapper, as was done for ResFinder. Genes with >90% identity to ResFinder genes were removed post-mapping to obtain the set of FRD genes that was absent from ResFinder. The reference gene abundances were summed to 90% gene clusters, using CD-HIT-EST, as was done for ResFinder[33]. The most frequent gene clusters remaining were derived from genes selected using trimethoprim, chloramphenicol, co-trimoxazole, cycloserine, amoxicillin, gentamicin, penicillin and tetracycline.

PCoA and resistome clustering. For PCoA, the gene cluster-level FPKM matrix was Hellinger transformed and the Bray–Curtis dissimilarities between all samples were calculated using the R package vegan[37]. PCoA was carried out or both pigs and poultry, combined and separately, using the vegan function 'betadisper'. The same analysis was used to test whether host animal and country were significant predictors of within-group dispersion. The effects of country on sample dissimilarities were determined using 'permutational multivariate analysis of variance using distance matrices' (the 'adonis2' function in the vegan package), separately for pig and poultry.

AMU in livestock. Data for the national livestock AMU were obtained from the ESVAC report and were stratified by major drug family[12]. The mass of active compound sold for use in

animals in 2014 was divided by the PCU in 10^6 kg, approximating the biomass. The PCU is a unit that allows inter-species integration by adjusting for import/export and differences in the average weight between species when they are most likely to receive antimicrobial treatment. The estimate was multiplied by 1,000 to obtain drug per mg per PCU livestock. The country-specific veterinary drug use can be found in Supplementary Table 6 and Supplementary Fig. 15.

In addition to the national veterinary AMU, we obtained data from collaborating researchers on the average treatment incidents in the sampled farms, stratified by antimicrobial class, country and livestock species. The treatment incidence was calculated as the antimicrobial dose per defined daily animal doses (DDDvet) per 1,000 animals at risk, adjusting for 200-day and 40-day production cycles for pigs and poultry, respectively[38]. These average AMU values for pigs (S. Sarrazin et al., manuscript in preparation) and poultry (P. Joosten et al., manuscript in preparation), stratified by drug group, are presented in Supplementary Tables 7 and 8, respectively. Data are visualized in Supplementary Figs. 16 and 17.

Procrustes analyses. To determine the effect of the underlying microbiota on the resistome, we used Procrustes analysis. The gene cluster FPKM ResFinder matrix and the genus-level FPKM taxonomy matrix were Hellinger transformed and Bray–Curtis dissimilarities were calculated. Each dissimilarity matrix was ordinated using PCoA. The symmetric Procrustes correlation coefficients between the bacteriome and the resistome ordinations, *P* values and plots were obtained using the 'protest' function in vegan[39]. To test the association between AMU patterns and the resistomes, we also used Procrustes analysis as follows. A PCoA was generated from Euclidean distances between the samples in the AMU data. The AMU PCoA configuration was tested against the AMR gene cluster PCoA configuration using the 'protest' function with the default 999 permutations. This was done separately for pig and poultry samples.

Alpha diversity. For all samples, we computed the within-herd resistome diversity using the Simpson's Index of Diversity (1-D), the Chao1 richness estimate and Pielou's evenness[40]. The gene cluster count matrix was rarified to 10,000 hits per sample for alpha diversity estimation, leading to the exclusion of samples with fewer hits to the AMR database.

Visualization. Heatmaps were produced using the pheatmap R package. For heatmaps showing individual-gene abundances, the Bray–Curtis dissimilarities between samples were used for hierarchical clustering. For all other dendrograms, the Pearson product-moment correlation coefficients were used. Complete-linkage clustering was used for all hierarchical sample clustering. For sample similarities, Bray–Curtis dissimilarity was converted to a similarity percentage, that is, 100 × (1 −Bray–Curtis).

The circular Bray–Curtis resistome dendrogram was constructed by exporting the dendrogram in Newick format using the ape package and further annotating it using the Interactive Tree of Life tool[41,42]. Bar plots, box plots and scatter plots were produced using the ggplot2 R

library[43]. The R library RcolorBrewer was used to generate the colour palettes used for the figures. This library is based on work by C. A. Brewer (www.ColorBrewer.org).

Statistical analyses. All statistics were done in Microsoft R Open 3.3.2, using the libraries and the procedures detailed below. The exact package versions can be found here: mran.revolutionanalytics.com/snapshot/2016-11-01/bin/windows/ contrib/3.3. For statistical tests, only samples from the first visit to the triple- sampled herds were included (see Supplementary Table 9), meaning n_{pig} = 181 and $n_{poultry}$ = 178. Unless otherwise mentioned, all statistical analyses were performed on pigs and poultry separately.

Association between AMU and AMR. To test the effect of the total AMU on the total metagenomic AMR abundance (the sum of all genes), we used the lme4 1.1- 12 package to make linear mixed-effects regression models, with the total livestock drug usage (the sum of the ESVAC PCUs) as the independent variable, the total AMR abundance (the sum of FPKM) as the dependent variable and country as a mixed-effect intercept[44]. The total AMU was log transformed, which resulted in lower Akaike's information criteria. Pig sample residuals and country residuals showed normality and so did the poultry country residuals. Poultry sample residuals had a longer right tail, but square-root transformation of the poultry AMR data gave more-normal residuals and a similar conclusion ($P < 0.05$). The effect and significance of drug usage were assessed using likelihood-ratio tests, ResFinder-treatment incidents and FRD-PCU tests were done in the same way as the ResFinder-PCU tests.

Differential abundance analysis. To identify AMR genes that differ in abundance between countries, we analysed the gene cluster count matrix using the DESeq2 package as previously recommended for metagenomic read count data[45,46]. This was done on the full count matrix, based on recommendations that rarefying is not warranted in metagenomic studies[46]. The read-pair count matrices for pigs and poultry were analysed 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 zero and extracted all the country- versus-country results. *P* values were adjusted for the FDR using the Benjamini–Hochberg approach and we used a significance threshold of alpha: 0.05 (ref. [47]).

Core resistome. To determine the set of AMR genes consistently found within each livestock species, we used a soft threshold. AMR gene clusters with mapping read pairs in at least 95% of samples from a livestock species were considered part of the core resistome.

Results

Acquired resistome characterization. The total AMR load varied significantly across samples, depending on both the host animal and the country of origin. In general, pigs had a higher AMR level than poultry (Fig. 1a). The highest AMR levels were found in Italian pigs, where the top four resistance-scoring samples originated, all in excess of 10,000 fragments per kilobase reference per million bacterial fragments (FPKM) AMR. At the lower end of the spectrum were Danish poultry samples that occupied the 11 samples with the least AMR, all below 500 FPKM.

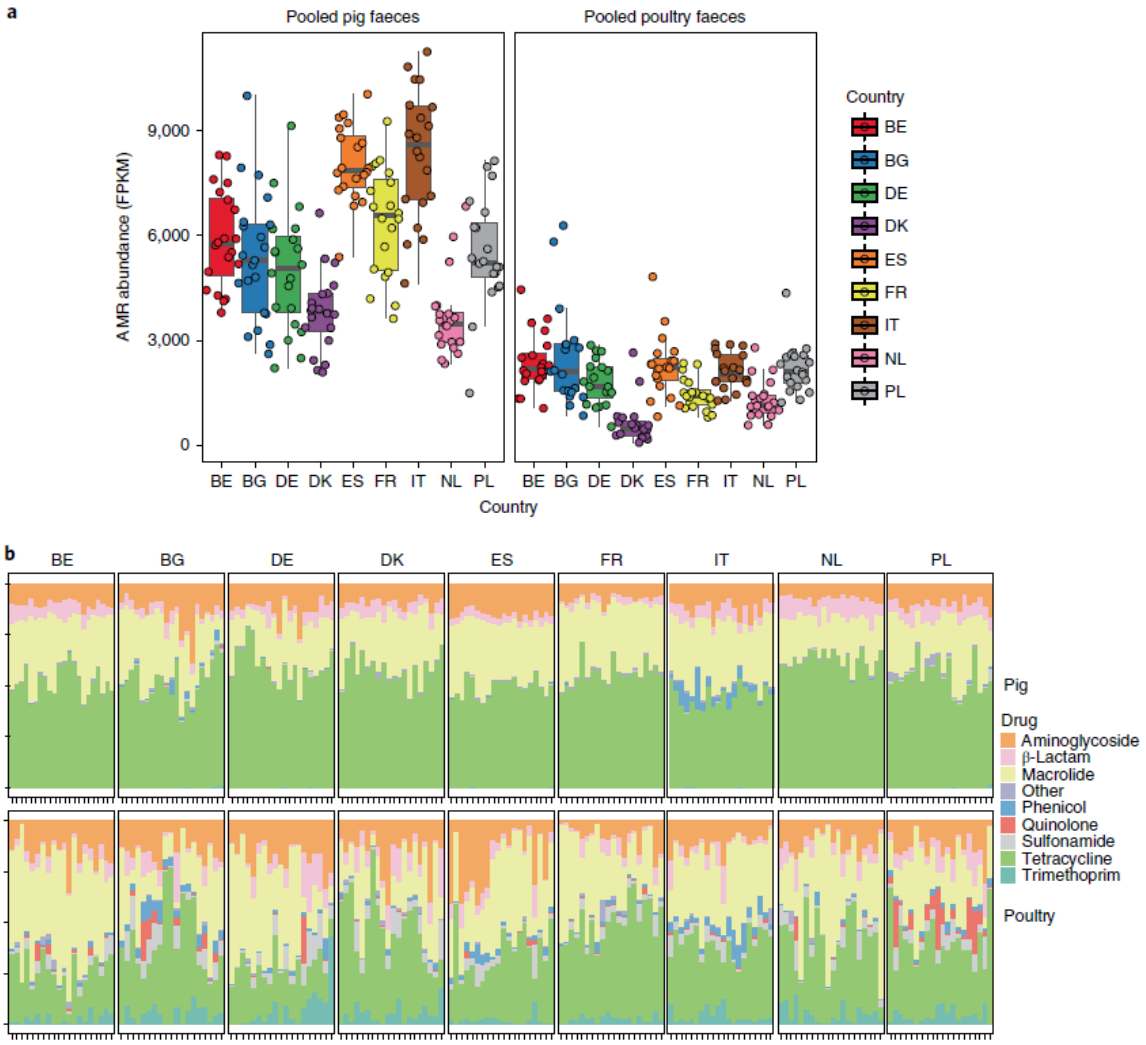

Fig. 1 | Overview of AMR abundance and composition. From read mapping to the ResFinder database, AMR abundance was calculated for each reference gene in each sample. **a**, Box plots showing the total AMR level per sample, stratified by host species and country. Each herd is also represented by a dot with sideways jitter to minimize overplotting. The horizontal box lines represent the first quartile, the median and the third quartile. Whiskers denote the range of points within the first quartile − 1.5× the interquartile range and the third quartile + 1.5× the interquartile range. *n* = 359 metagenomes from independent herds. Twenty metagenomes per livestock species per country were used, with the exception of Bulgarian pigs (21), Bulgarian poultry (19) and German poultry (19). **b**, Stacked bar chart of AMR abundance per type (colours) per sample (*x* axis), proportional to the total AMR within each sample. Note that the two-letter country code is used in all figures: BE, Belgium; BG, Bulgaria; DE, Germany; DK, Denmark; ES, Spain; FR, France; IT, Italy; NL, the Netherlands; PL, Poland.

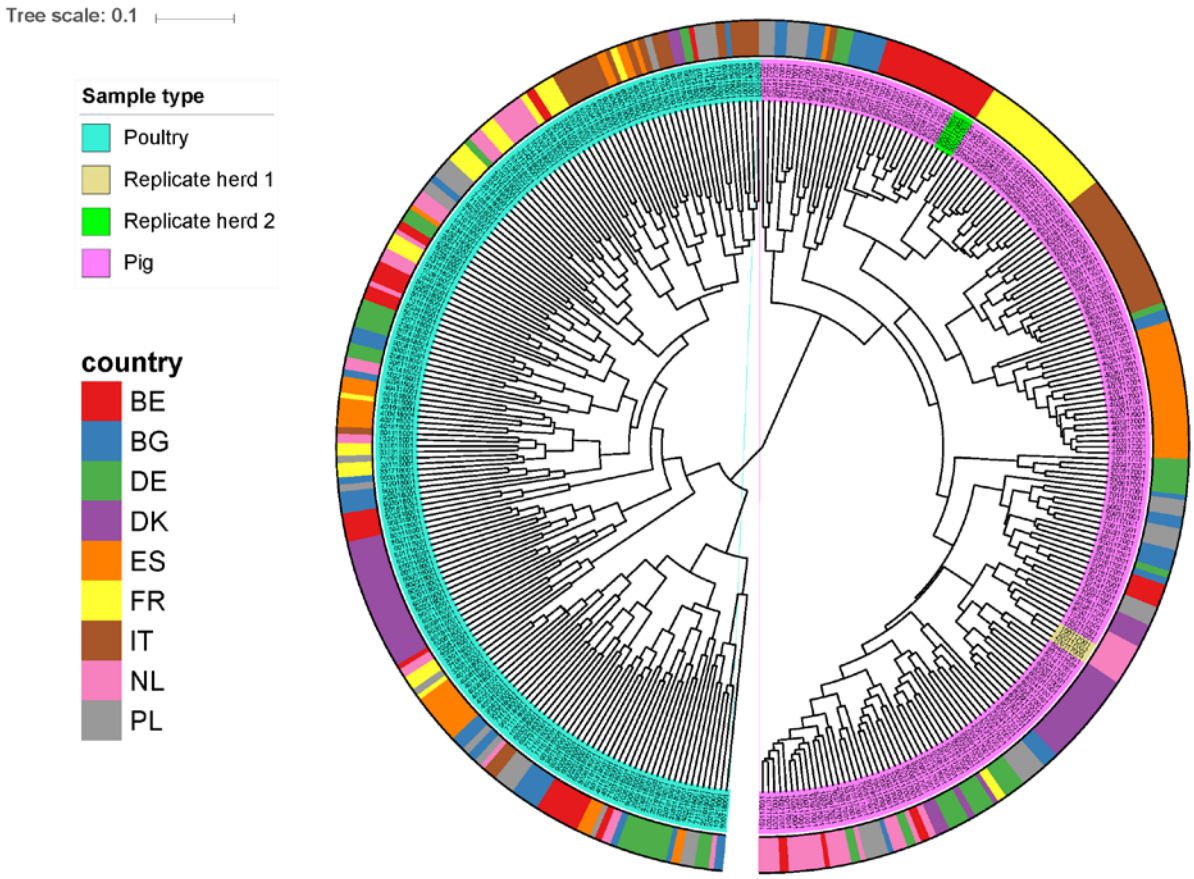

Fig. 2 | Resistome clustering is influenced by both host animal and country. A dendrogram showing the complete linkage clustering of Bray–Curtis dissimilarities between all pig and poultry resistomes. Triple-sampled pig herds are highlighted in separate colours. *n* = 363 metagenomes from 359 independent herds.

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

Among the poultry farms, there was less consistency. Both within and between countries, the relative proportions of AMR per drug were more varied. As in pig samples, tetracycline, macrolide, β-lactam and aminoglycoside AMR made up the majority, but the two latter classes had very minimal contributions in a subset of herds. Sulfonamide and trimethoprim AMR was more abundant in poultry samples than in pig samples across all

countries. In many Polish poultry herds, quinolone AMR made up a size- able fraction of the combined resistome. This was also true for a few non-Polish herds, notably in Bulgaria. For non-proportional graphical representations of the AMR load stratified by sample and drug class, see the Supplementary Material for an unscaled, stacked bar chart (Supplementary Fig. 1) and a heatmap (Supplementary Fig. 2). Class-level AMR relative abundances can be found in Supplementary Table 1.

To characterize the individual components of the resistome, we summed the relative abundance to the gene level, as was done for the phenotypic level. We found evidence for 407 different genes across all pig and poultry samples (Supplementary Table 2).

We calculated the dissimilarities between the gene-level resistomes of all samples and visualized it in a dendrogram (Fig. 2). There was a perfect host separation, with all pig samples clustering separately from all poultry, suggesting that pig and poultry resistomes are very distinct. In the pig cluster, the country separation was more pronounced than in the poultry cluster. An exception was Danish poultry, where 18 out of 20 farm resistomes clustered.

To assess the reproducibility of our protocol, from sampling through to sequencing, we evaluated the similarities between the resistomes of two triple-sampled swine herds. The Dutch triple- sampled herd had the highest similarities between composite samples, ranging from 93.6% to 93.7% Bray–Curtis similarity. The Belgian triple-sampled herd pools had values ranging from 91.5% to 93.3% similarity. No replicated sample pool had a higher similarity to other herds than to its own replicates, and the two sets of three samples can therefore be seen clustering separately, indicating reproducibility in both sampling and sequencing (Fig. 2). A farm resistome similarity heatmap is included in the Supplementary Material (Supplementary Fig. 3).

We ordinated the gene-level resistomes for all samples (Supplementary Fig. 4) and pig and poultry samples separately (Supplementary Fig. 5a,b). As with hierarchical clustering, there was a clear separation of pig and poultry samples, along the first principal coordinate, which explained 48% of the variation across all resistomes.

When analysing the two species separately, we observed clustering according to the country of origin in pigs (Supplementary Fig. 5a), whereas clustering was more diffuse for poultry (Supplementary Fig. 5b). We tested for the country effect and found it to be significant in both pigs (adonis2, $P < 0.001$) and poultry (adonis2, $P < 0.001$). However, in poultry, the country effect only explained 24% of the variation, whereas the country effect explained 41% of the variation in pigs. In the pig resistome ordination, the Danish and Dutch samples clustered closely together. The same could be seen for the French and Belgian resistomes and to a lesser degree, the Italian and Spanish samples. Bulgaria, Germany and Poland showed larger dispersions than the other countries. Beta-dispersion levels varied significantly between countries in both pigs (betadispersion $P < 0.001$; Supplementary Fig. 5c) and poultry (betadispersion $P < 0.001$; Supplementary Fig. 5d).

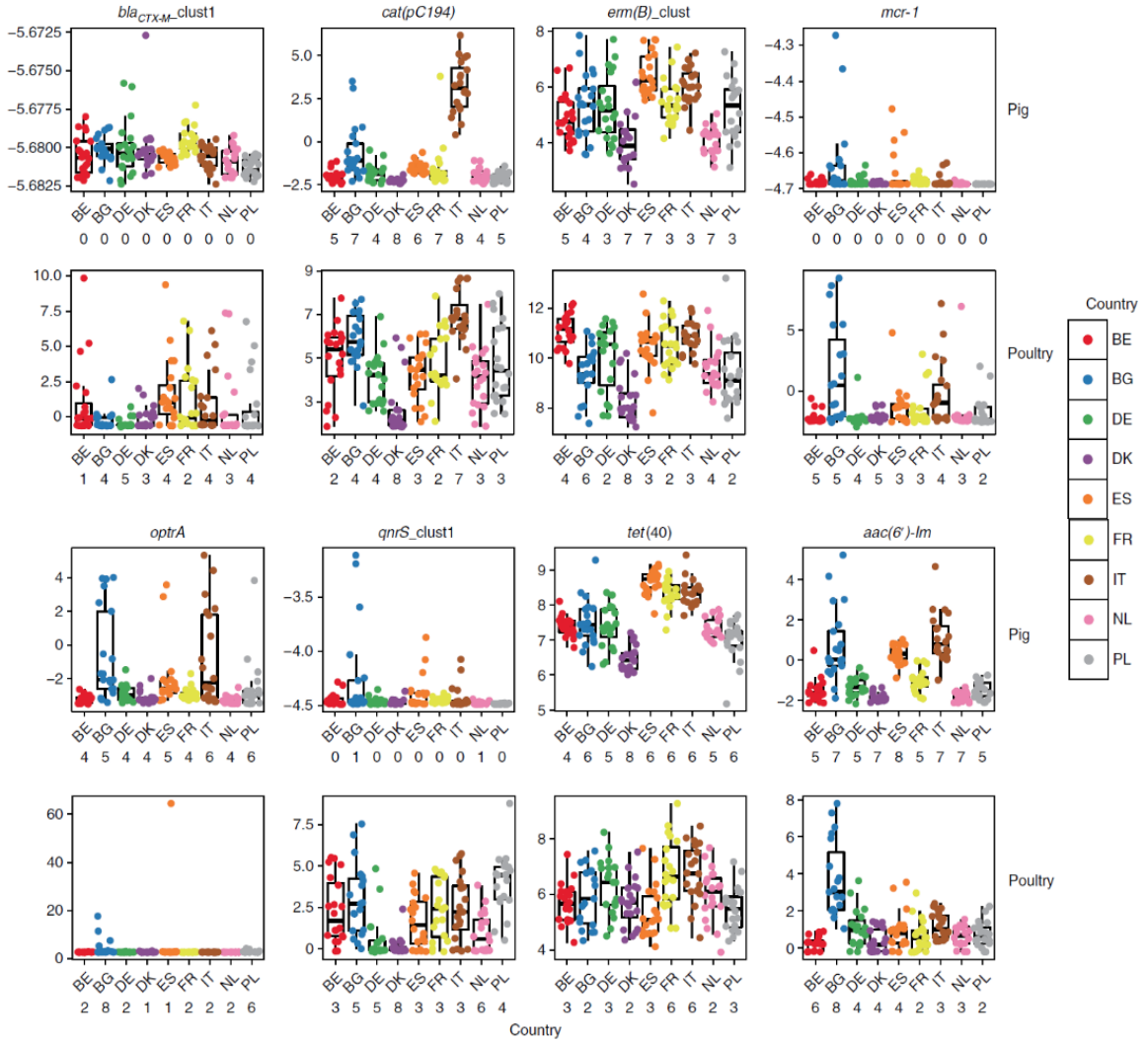

Fig. 3 | AMR genes differ in abundance between countries. A handpicked subset of genes that differed significantly in abundance between at least two countries in either pig or poultry farms is shown. The regularized log abundance (rlog) is shown on the *y* axis in box plots and points. Points were sideways jittered to reduce overplotting. The numbers along the *x* axis denote the number of countries with a significantly different mean abundance (DESeq2 Wald test, two-sided, α= 0.05). Testing was done for all ResFinder genes, and *P* values were adjusted for multiple testing (FDR) for all country comparisons. The horizontal box lines represent the first quartile, the median and the third quartile. Whiskers denote the range of points within the first quartile − 1.5× the interquartile and the third quartile + 1.5× the interquartile. *n* = 359 metagenomes from independent herds. Twenty metagenomes per livestock species per country were used, with the exception of Bulgarian pigs (21), Bulgarian poultry (19) and German poultry (19).

We visualized the AMR gene abundances in a heatmap to look at the overall structure and composition of the resistomes and the co-occurrence of AMR genes (Supplementary Fig. 6). Some AMR genes were more abundant in one species, whereas others, including *tet(W)* and *erm(B)*, were ubiquitous in all samples for both species. Among the pig samples, the Italian samples stood out: several chlor- amphenicol AMR genes, including *cat(pC194)*, *catP* and *cat_2*, were much more abundant in Italy than in the other countries, consistent with our inspection of AMR at the class level (Fig. 1). Several AMR genes known

to be co-located indeed co-occurred across samples. The genes in the vancomycin AMR *VanA* cassette were co-located in several poultry samples. This was also true for the *VanB* cassette members, clustering together but separately from *VanA*, showing an ability to distinguish variants of homologous genes. As indicated earlier, the poultry samples showed less country-based clustering than pigs. An exception was the Danish poultry samples; these had a noticeably lower abundance of many AMR genes that were wide-spread in other countries.

Core resistome. To determine whether specific genes were unique to each of the host animals, we examined the set of AMR genes that was consistently observed within each animal species (evidence for it in 95% of samples). We identified 33 core AMR genes in pigs and 49 core AMR genes in poultry, with 24 being shared between the two hosts (Supplementary Fig. 7). Hence, only nine AMR genes were pig-core genes without also being poultry- core genes. These included the genes making up the Van-G vancomycin cassette, *tet(C)*, *bla*$_{\text{ACI}}$ and *cfxA*. Twenty-five AMR genes were poultry-core genes without also being pig-core genes and include the Enterobacteriaceae-associated *strAB*, *sul2*, *blaTEM* and *tet(A)* genes.

Differential abundance analysis. To test which specific genes differed in abundance between countries, we carried out a differential abundance analysis for ResFinder gene cluster read counts. Heavy overrepresentation of low unadjusted *P* values indicated a large effect of country in both the pig and the poultry data sets (Supplementary Fig. 8). Of special interest was the newly characterized *Enterococcus*-associated linezolid-resistance gene *optrA*, which had a significantly higher abundance in Bulgarian poultry farms than in poultry farms in all other countries (false discovery rate (FDR) < 0.05) (Fig. 3). However, a single Spanish farm did have an even higher *optrA* abundance than any other farm. Among the pig herds, the *optrA* gene was more abundant in Bulgarian and Italian herds than anywhere else (except for two farms in Spain) (FDR < 0.05).

The newly identified colistin-resistance gene *mcr-1* was significantly more abundant in Bulgarian and Italian poultry farms than in most other countries (FDR < 0.05). France, Poland and Spain had intermediate levels, whereas Denmark, the Netherlands and Germany had the lowest levels (Fig. 3). The Bulgarian poultry farms enrolled in this study did not report any polymyxin usage, whereas Italian farmers reported the highest average treatment incidents.

As previously noted from visual inspection of heatmaps, multiple chloramphenicol AMR genes including *cat(pC194)* were much more abundant in Italian pigs than in other pigs. The extended- spectrum β-lactamase *bla*$_{\text{CTX-M}}$ gene cluster 1 also showed country dependency, being significantly more abundant in poultry samples from Spain, Poland, Italy, France and Belgium than in poultry samples from Germany (FDR < 0.05). Differential abundance analysis results can be found in Supplementary Tables 3 and 4 for pig and poultry, respectively.

Alpha diversity and richness. We calculated several alpha-diversity indexes for each farm resistome (Fig. 4 and Supplementary Table 5). The range of AMR diversity was generally much larger for poultry samples, having both lower and higher diversity, than for pig samples, which had a tighter spread of diversity. The poultry samples had a higher estimated number of different AMR genes (that is, a higher Chao1-estimated richness). Interestingly, countries with higher estimates of unique AMR genes in pigs also tended to have a high AMR richness in poul- try (Spearman's rho: 0.88, $P = 0.02$; Supplementary Fig. 9). Spain, Italy, Bulgaria and Poland had the highest estimated number of unique AMR genes in both pig and poultry. There was no such association for Pielou's evenness or Simpson diversity ($P > 0.05$). Rarefaction curves for pig and poultry resistomes can be found in Supplementary Fig. 10.

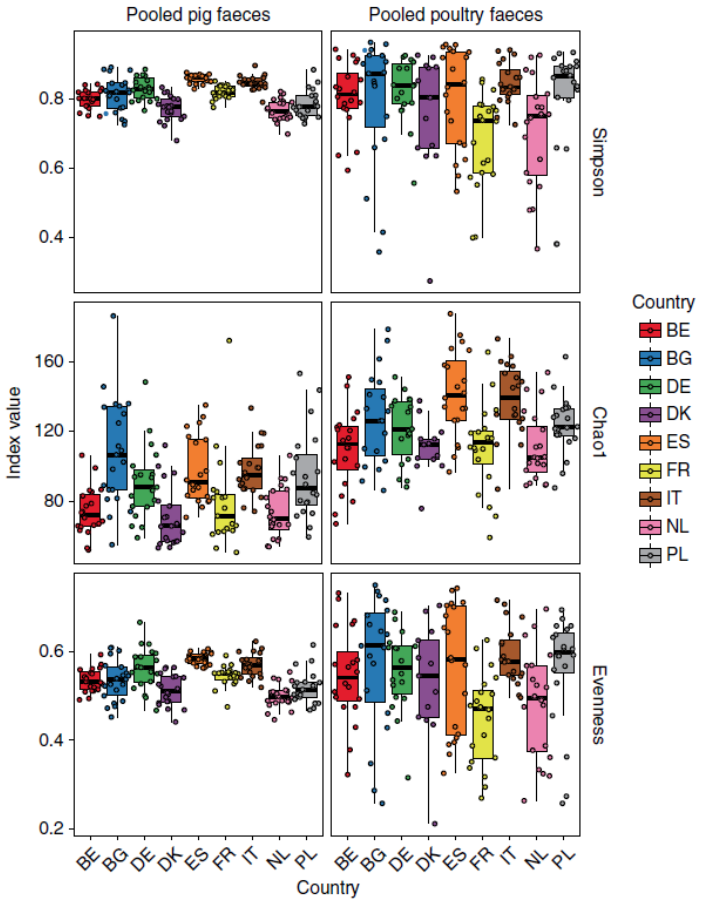

Fig. 4 | Resistome alpha diversity and richness differ between animal host and countries. From the read count pair matrix, several indexes were calculated: Simpson diversity index, Chao1-estimated richness and Pielou's evenness. The horizontal box lines represent the first quartile, the median and the third quartile. Whiskers denote the range of points within the first quartile − 1.5× the interquartile range and the third quartile + 1.5× the interquartile range. *n* = 359 metagenomes from independent herds. Twenty metagenomes per livestock species per country were used, with the exception of Bulgarian pigs (21), Bulgarian poultry (19) and German poultry (19).

Association between the bacteriome and the resistome. To test the degree to which the bacterial composition of the microbiota dictates the resistomes, Procrustes analyses were performed. We found that for both pig and poultry, the resistome correlated significantly with the bacterial composition (P = 0.001; Fig. 5). Thus, samples with similar taxonomic compositions tended to have similar resistome compositions. In addition, most of the between-country differences in resistomes seem to be explained by systematic between-country differences in bacteriomes.

The correlation between AMR and taxonomy was similar in pigs (correlation: 0.86) and poultry (correlation: 0.88). Interestingly, in the pig samples, we saw a country effect on the strength of association between the bacteriome and the resistome. In the Dutch and Spanish pig herds, ordinations based on bacterial genera and AMR genes gave similar results (Fig. 5b). In German farms, in particular, the resistome and bacteriome ordinations yielded more dissimilar results. This was less evident for poultry, although a single Danish poultry herd had a very unusual resistome, considering its taxonomic composition (Fig. 5d).

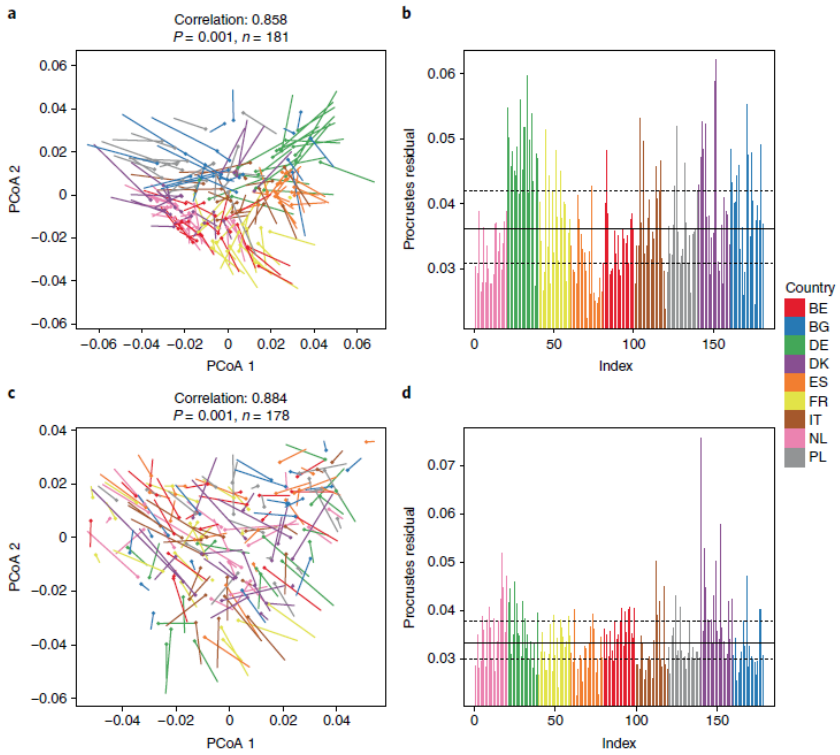

Fig 5 | Taxonomic variation explains resistome variation. Bacterial and AMR abundance profiles were correlated with each other using Procrustes analyses for pig (**a**) and poultry (**c**) herds, thus comparing the two multivariate data sets. The lines show the Procrustes residuals; the change in the ordination position when using the resistome (dotted ends) compared to the bacteriome (non-dotted ends) is displayed. The correlation coefficients and significance were derived using the protest function in vegan. The residual line plot for pig (**b**) and poultry (**d**) farms enables easier residual size comparison, showing the difference in the bacteriome–resistome association between farms. Horizontal lines denote the median (solid), 25% and 75%quantiles (dashed). n = 359

metagenomes from independent herds. Twenty metagenomes per livestock species per country were used, with the exception of Bulgarian pigs (21), Bulgarian poultry (19) and German poultry (19).

AMR and drug use association. We found that the total country- level veterinary AMU from the European Medicines Agency's European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) was positively associated with AMR in both pigs and poul- try. The AMR abundance increased by 1,736–3,507 FPKM (95% CI, β= 2,621) in pigs when the AMU increased by 1 $\log_e$ unit (a 36.8% increase in AMU) (Fig. 6a) and to a lesser degree in poultry, where the AMR abundance increased by 68–1,330 FPKM (95% CI, β= 700) when the AMU increased by 1 $\log_e$ unit (Fig. 6b). For pigs, the variance between farms within-country was seven-times larger than the variance between countries in general, whereas in poultry, the variance was four-times larger within-country than between countries. We repeated the regression with the treatment incidents data for the farms (Supplementary Fig. 11). Interestingly, these data were less associated with the AMR load than the national veterinary drug use data. The association remained for pigs, whereas poultry was not significant ($P > 0.05$). Bulgaria had low reported usage in both livestock species, whereas AMR was high. The Danish farms reported a higher average AMU in poultry than Bulgaria and the Netherlands, but the total AMR was far lower.

To test whether the AMU pattern across multiple antimicrobial classes was associated with AMR gene profiles, we compared the AMR gene cluster abundances for pig and poultry against both the ESVAC and the farm treatment incidents data (Supplementary Tables 6–8 and Supplementary Fig. 12). Using Procrustes analyses, all matrix–matrix correlations were significant (P = 0.001), although with low symmetric correlation coefficients (correlation: 0.34–0.45). As for the regression analysis, there was a better fit between pig AMR and the ESVAC data than between pig AMR and the farm treatment incidents data.

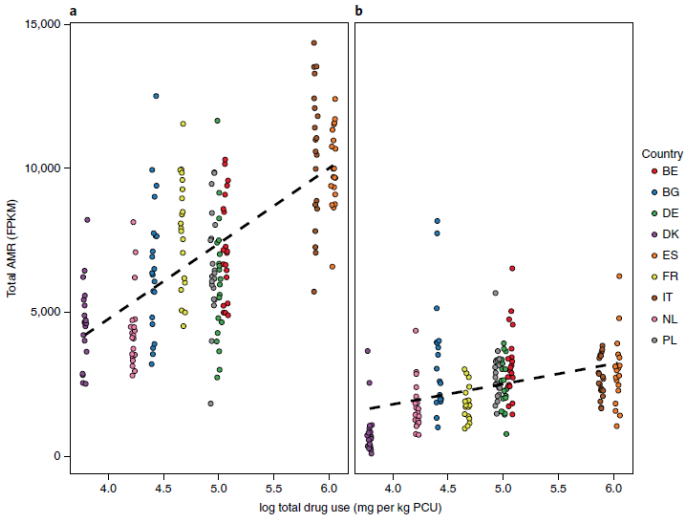

Fig. 6 | National veterinary AMu is associated with total metagenomic AMR. Scatter plots of the average total veterinary AMU (ESVAC) and the pooled sample total AMR. A slight sideways jitter was added to the points to minimize overplotting. **a**, The association between the average veterinary drug use and the total AMR load in pig

farms. *n* = 181 independent herds. **b**, The association between the average veterinary drug use and the total AMR load in poultry farms. *n* = 178 independent herds. See the main text for a description of the trend lines.

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

Using the FRD, we found both similar and different patterns than 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 Fig. 13). Although less variation could be explained by two axes, the principal coordinates analysis (PCoA) plot of pig samples now clustered German and Spanish samples, with the remaining countries being more similar. The resistome richness showed similar pat- terns to ResFinder: Spanish, Italian, Polish and Bulgarian samples had a higher estimated richness in both pig and poultry than 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 the total AMR and the total AMU, for both pig and poultry ($P > 0.05$; Supplementary Fig. 14).

Discussion

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

A recent study including Chinese, Danish and French pigs showed that the Chinese pig resistomes clustered separately, whereas the Danish and French pig resistomes 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 and Italy) have similar resistomes, the same way as countries with similarly low AMU (Denmark and the Netherlands) also have similar pig resistomes.

We found that within-country resistome dispersion is country dependent, particularly in pigs, with Bulgarian, German and Polish pig herds having more dispersed AMR. Although we cannot currently explain this, we consider the possible causes as differences in trade and management, among others.

We found the recently discovered plasmid-borne colistin resistance gene *mcr-1* in numerous 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, which is uncharacteristic for the high *mcr-1* level found here[13]. This gene was recently discovered in China and identified throughout the world and has been identified in pigs, poultry and human clinical infections alike[18].

A newly characterized enterococcal linezolid-resistance gene, *optrA*, was detected in a

subset of pig samples, with Bulgaria, Italy and Spain having the highest abundances. The *optrA* gene pro- vides AMR to both oxazolidinone and amphenicols, including the veterinary-used florfenicol[13,19]. The high abundance of this gene in these countries can probably be explained by the fact that they have the highest veterinary amphenicol usage among the nine countries investigated. This explanation fits well with the fact that Bulgaria, Italy and Spain also had the highest abundances of chloramphenicol AMR genes, such as *cat(pC194)*, in poultry.

Another AMR gene of special interest, the *bla*$_{\text{CTX-M}}$, was also observed in the poultry herds. The higher abundance of *bla*$_{\textit{CTX-M}}$ cluster 1 in Spain, Italy, Poland and Belgium could possibly be explained by co-selection by fluoroquinolones, which is used more in Spain, Poland, Italy and Belgium than in other sampled countries. *qnr* and *bla*$_{\text{CTX-M}}$ genes are frequently co-located on large extended- spectrum β-lactamase plasmids. Veterinary cephalosporin usage did not seem to explain the observed levels.

Poland and Spain use far more veterinary fluoroquinolones than other countries included in this study. We found that plasmid- mediated quinolone AMR (*qnr* genes) was frequently abundant in Polish, but not in Spanish, poultry. In Bulgaria, quinolone AMR was also frequently observed, although their reported AMU did not follow the same trend.

Interestingly, we observed that the number of unique AMR genes predicted (Chao1) significantly correlated between pig and poultry farms across countries. In addition, countries with a high estimated number of unique AMR genes also have a high AMR abundance (Italy, Spain, Bulgaria and Poland). The fact that countries' AMR abundance and the predicted number of unique AMR genes in pig and poultry tend to follow each other, could be explained with policy: if a country has strict AMU regulations in one livestock species, the chances are that similar regulations are in place for other livestock species. Indeed, the treatment incidents data showed that countries with higher AMU in pigs, had higher AMU in poultry. It might also be speculated that an ecological country effect plays a role; for example, the total country AMU might influence AMR abundance in all reservoirs. This, might explain why the ESVAC data correlate better with the observed AMR than the treatment incidents data. Better AMU data, at the herd and country level and over time, are needed to further explore the specific AMU–AMR associations. It has previously been reported that the composition of the bacterial community structures the resistome[20,21]. We found the same to be true for pig and poultry resistomes; in addition, we showed that the taxa–AMR association strength differs between countries. Horizontal gene transfer could explain this phenomenon, if a larger proportion of certain countries' resistome is mobile and AMR genes are more frequently introduced and re-introduced to genera. Conversely, vertical AMR transmission can also play a role, if, for example, one country's livestock is more isolated from trade. As we found that a large part of the observed resistome is dictated by the taxonomic composition, we expect much of the country resistome differences to be explained by systematic differences in feed and management.

In contrast to ResFinder, when using FRD, we found no relationship between the ESVAC total drug use and the total functional AMR abundance. This suggests that, although many genes can provide AMR when cloned into, for example, *E. coli*, in functional metagenomic assays, they might not provide AMR functionality in their natural hosts with natural expression levels. If most of them did, we could expect to see antimicrobial-based selection and an association to AMU, as it is observed for the AMR genes in ResFinder. This finding echoes previous sentiments that one should carefully consider the risk to human health imposed by functionally determined AMR genes[22]. Some FRD genes might represent high- risk AMR genes, but we currently do not know what subset that is. Creating the FRD is a first step in trying to catalogue the many AMR genes found in functional metagenomic studies. Screening sequenced pathogenic isolates and metagenomic assemblies for FRD genes would be a good start for assessing their host range and risk potential.

The AMU data used in this study are not optimal. There is variation in drug use within each country's farms that we did not account for by using the available country-wide averages per drug class. Moreover, the population correction unit (PCU) denominator used by the ESVAC may vary greatly between countries, and no independent validation of the data reported by the national competent authorities have been performed. Furthermore, the integrated herds enrolled in this study might represent only a limited subset of the overall livestock production in some countries. However, even with the crude ESVAC-based total veterinary AMU, we found significant associations with the total AMR abundance. The similar conclusion when considering the specific drug usage profile of each country indicates that the resistome is responding to AMU. The AMR–AMU association is well documented for specific cultured indicator species and certain antimicrobial drugs, but is relatively unknown when considering the whole microbiota and resistome and the newer approach of metagenomic shotgun sequencing[3,8]. We do not know why the pig samples had a larger within-country spread of total AMR, but perhaps the more heterogeneous production system and production management are responsible. Curiously, the treatment incidents data, which are specific to the sampled farms, was less associated with the resistomes and total AMR than the ESVAC data. Instead of reporting that AMU does not affect AMR after all, we think that it is worthwhile considering whether there are some AMU reporting biases between countries.

DNA extractions from the pooled poultry samples resulted in relatively low DNA yields. The protocol used was optimized for pig faeces, human faeces and sewage, but not for poultry faeces[23]. The lower yields necessitated the use of a PCR-based library preparation kit that can influence downstream analysis of shotgun sequencing[24]. Although the large difference between pig and poultry resistomes in our study is probably real, we caution the use of sensitive, quantitative analyses when comparing between samples prepared using different library preparation kits. For this reason, we have mostly tested within each reservoir.

The sensitivity of metagenomic approaches does not yet rival phenotypic alternatives such as selective enrichment. There are AMR genes in important pathogens that we know are probably present but are below our detection limit. For example, we only found evidence for *bla*$_{\text{CTX-M}}$ in three of the pig herds, whereas in phenotypic studies, the prevalence is high even among farms with no cephalosporin usage[25].

The primary concern with read-mapping techniques is the lack of genomic context, which can be solved using metagenomic assembly and binning approaches[16,26,27]. In this way, AMR alleles in full length, their genomic context and their associated taxa have been identified in both pig, poultry and human faecal samples[28]. As shown previously, the association between AMR and AMU is similar for metagenomics and traditional phenotypic methods, but several aspects make metagenomics an intriguing monitoring tool[17]. The fact that both types of analyses (quantitative, sensitive read mapping and qualitative, context-giving binning) use the same raw data makes metagenomics an attractive tool. In addition, the digital nature of sequence data would also allow future re-use and form the basis of an invaluable historical archive, potentially usable for both AMR and pathogen-tracking worldwide.

We found that the metagenomic resistome varied significantly between the pig and poultry reservoirs, but also within each species, in a country-dependent manner. Within each country, we found different levels of variation, with some countries having more homogenous herds than others. Differences were seen both in the total AMR abundance, but also the abundances of AMR types and specific genes, including clinically relevant AMR genes. Some of this variation we attributed to differential drug usage between the countries. We also identified the microbiome background as an important factor in determining the resistome in livestock, but found that the strength of the association was country dependent, at least in pigs. Interestingly, we found that the AMR richness in one livestock species in a country is linked to the abundance in another livestock species. Finally, we observed some indications that newly described AMR genes from functionally metagenomic studies might not provide AMR functionality when expressed in their natural host, even though they have the potential at the right expression levels in the right organism.

Data availability. Drug use data are attached as Supplementary Tables. The DNA sequences (reads) from the 363 metagenomic samples from the 359 herds are deposited in the European Nucleotide Archive under the project accession number PRJEB22062.

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Author contributions F.M.A., D.H., J.A.W., T.H., D.M. and the EFFORT group designed the study. F.M.A. and B.E.K. detailed the sampling and sequencing. A.S.R.D. and the EFFORT group carried out the sampling. B.E.K. and S.J.P. conducted the DNA purification and, with P.M., organized the sequencing. R.B.H., O.Lund. and T.N.P. created the read-mapping pipeline. E.R. created the FRD. P.M., O.Lukjacenko., R.E.C.L., L.V.G., L.A.M.S., H.S., A.B., A.D.G. and H.V. carried out the bioinformatics and statistical analysis. P.M. created the figures and drafted the manuscript. All authors helped review, edit and complete the manuscript.

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

Herd selection and sampling

Here we describe the selection of pig and broiler farms and corresponding sampling procedures, as they were performed. Comparisons to the original criteria described in the sampling protocol were made using data collected at the farms through a questionnaire. This overview is based on data from 181 broiler farms (one country sampled one extra farm) and 178 pig farms (one farm with unavailable metagenomic data and one farm with a mislabeled sample were not included in the present study).

Selection of pig farms

In each of the 9 participating countries, we planned to include 20 conventional integrated farrow-to-finisher, non-mixed farms. Seven farms (BG-3, DE-2, IT-2) had introduction of piglets, and therefore cannot be considered strictly integrated.

The farms needed to employ batch production, so that the majority of the animals of the sampled group originated from the same birth cohort. An all-in all-out production at compartment level was required, however not always possible to fulfill; only 159 and 141 farms had all-in all-out at the fattener's and nursery's compartments, respectively (Nursery: BE-18, DE-16, DK-12, ES-18, PL-15; Fattener: DE-16, DK-10, ES-11, FR-19, IT-12, NL-18, PL-15).

Occasionally, animals may have been raised on different sites, provided fatteners had been on the same site during their entire life. In 31 farms (DE-7; DK-1; ES-12; FR-2; IT-6; NL-3), production was split between different sites (most often pre-fattening period in one site and fattening period in another).

The farms needed to have at least 150 sows and 600 fatteners on average. Overall, the average number of sows per farm in all the farms visited was 496. All countries fulfilled this criteria, with a country-wise minimum average of 322 (FR) and maximum of 822 (ES). The total number of fatteners was unfortunately not investigated in the visited farms; however, the overall median number of fatteners set-up per year was 5,100**,** with a country-wise minimum median of 3,250 (BG) and maximum of 13,700 (IT).

The selected herds had no contact through trade, and each herd had only one owner. We planned to randomly select farms to participate in the study from a list of eligible farms, using regional stratification whenever possible. This protocol was not possible to fulfill in all cases. Below, we describe the selection procedure applied in each country.

BE- A random list of pig farms was obtained from the government. From this list, we contacted farmers for participation.
BG- The farms were selected from two geographic regions – Northern- and Southern-Bulgaria. We visited farms where the owners/managers agreed to participate.

DE- We contacted three veterinary practitioners and one slaughterhouse distributed over Germany to help us contact farmers. We contacted the suggested farmers and asked if they agreed to participate.

DK – We obtained a list of eligible farms fulfilling the selection criteria in terms of number of fatteners and sows from the national husbandry registration database. From this list, we randomly drawn farms using regional stratification (based on zip code). We contacted the randomly selected farms by phone, informed the farm managers about the project and invited them to participate. We kept a record of all reasons for declining.

ES- First, we contacted veterinary practitioners distributed over the country, who proposed eligible farms. Then we selected farms randomly from this list and coordinated the sampling with the veterinarians. The stratification was based on the answers of the practitioners and the number of farms available per area of work. Regional stratification was not possible because we did not obtain feedback from practitioners of all regions.
FR- Farms were all designated by veterinary practitioners we contacted. We managed to involve different production organizations located in the west part of France (conveniently located within maximum 2h30min to the laboratory, to respect requirements regarding samples' transportation conditions).
IT- The selection of farms was partially randomized. A regional stratification based on the production volumes (regions concentrating 90% of the Italian pig production) was first implemented. For the selected regions/areas, a list of farms with the desired characteristics was extrapolated from the National Animal Database. Local Veterinary Services and veterinary practitioners working in the area where these farms were located were contacted and informed about the project, and then appointed to contact farms with the required characteristics to obtain their availability. Farms giving their consent were sampled.
NL - We recruited farms in collaboration with 9 veterinary practices and 1 pig farming organization spread over the Netherlands. If the practice agreed to participate, they were asked to select 1 to 3 farms (depending on the size of the veterinary practice) that fulfilled the selection criteria. When a farmer showed interest, we contacted the farmer and explained the research, after which the farmer decided to participate or not. We kept a record with reasons for not participating.
PL- We contacted veterinary practitioners by phone from a list of specialists in pig diseases and asked them for participation in the project. To those who accepted the invitation and did work with pig farms, we asked for a list of farms complying with the selection criteria. The practitioners nominated the farm(s) of interest.

Sampling of pig farms

Among all countries, the sampled fattening animals had a mean age of 179 days, with a minimum of 95 and a maximum of 320 days.

To avoid seasonal influences we planned to divide sampling over the year, however, most farms were sampled in the fall (58) and summer (68) and least in winter (25) and spring (29). BG did not collect any samples in spring, and ES and NL did not sample in winter.
Per farm, we collected 25 fresh, still warm and undisturbed fecal droppings from the floor of separate pens (a minimum of 10g of feces/sample), divided over all present compartments/stables of fatteners close to slaughter. When there were less than 25 pens, we divided the 25 samples over all pens.

Selection of broiler farms

We planned to include 20 broiler farms per country. One country (BE) visited 21 farms, therefore samples were collected in a total of 181 farms.

The farms should have all-in all-out production, with a thinning procedure from day 30 onwards allowed. The duration of the production cycle on each farm was calculated using the age of the birds at the moment of sampling and the last slaughter date for the sampled flock. The overall estimated average cycle duration was 40 days. While we could not determine exactly the existence and duration of thinning, we estimated that only a maximum of 4 of the 181 farms had a cycle shorter than 30 days, which can be interpreted as a possible indication of a thinning procedure in the remaining 177 farms. This results are however highly uncertain and should be strictly used as a proxy.

The selected farms needed to be conventional, with no intended slaughter age higher than 50 days, no slow growing breeds (intended growth rate less than 55 gram/day), and no stocking density lower than 10 birds/m². All sampled farms were conventional. The average slaughter weight across all farms visited was 2,372 kg, with minimum 1,300 kg and maximum 3,700 kg. One country (FR) had a particularly low average weight at slaughter compared to the others (1,743 kg).The country-specific stocking density in birds/m² was calculated using the stocking density in kg/m² and the country's average weight at slaughter in kg. Among all countries, the stocking density varied from a minimum of 13.91 and a maximum of 17.10, with an average of 16.01 birds/m².
We included only one flock/house per holding, and the flock size criterion was minimum 20,000 and maximum 40,000 broilers/house. The average size of the flocks across the 181 farms was 28,044 birds at set-up. One country did not fulfill the criterion for maximum flock size (DK- 53,300), whereas most countries did not fulfill the criterion for minimum flock size (BE- 16,500; BG- 8,000; DE- 17,200; ES- 14,000; IT- 11,340; NL- 17,550; PL- 18,500).
We planned to randomly select farms to participate in the study, using regional stratification whenever possible. This protocol was not fulfilled in all cases. Below, we describe the selection procedure applied in each country:

BE- A random list of broiler farms was obtained from the government. From this list farmers were contacted for participation.

BG- The farms were selected from two geographic regions – Northern- and Southern-Bulgaria, covering 90% of the territory of the country. We included registered farms working for the two largest poultry producers in the country, and visited the ones where the owners/managers agreed to participate.

DE-We contacted two of the biggest integrations in Germany and tried to sample farms according to the poultry density of each country area. We contacted the farmers and sampled in the date that suited them best, while always trying to sample between 10 to 7 days before the date of slaughter.

DK - We obtained a list of eligible farms fulfilling the selection criteria in terms of flock size from the national husbandry registration database. From this list, we randomly drawn farms using regional stratification (based on zip code). We contacted the randomly selected farms by phone, informed the farm managers about the project and invited them to participate. We kept a record of all reasons for declining.
ES- First, we contacted veterinary practitioners distributed over the country, who proposed eligible farms. Then we selected farms randomly from this list and coordinated the sampling with the veterinarians. The stratification was based on the answers of the practitioners and the number of farms available per area of work. Regional stratification was not possible because we did not obtain feedback from practitioners of all regions.

FR- Half of the selected farms were contacted through veterinary practitioners, and the remaining half were farms participating in another study, which fulfilled the inclusion criteria and have accepted additional samples to be taken and questions to be completed..

IT- The selection of farms was partially randomized. A regional stratification based on the production volumes (regions concentrating 90% of the Italian pig production) was first implemented. For the selected regions/areas, a list of farms with the desired characteristics was extrapolated from the National Animal Database. Local Veterinary Services and veterinary practitioners working in the area where these farms were located were contacted and informed about the project, and then appointed to contact farms with the required characteristics to obtain their availability. Farms giving their consent were sampled.

NL-We recruited farms in collaboration with 7 veterinary practices spread over the Netherlands. If the practice agreed to participate, they were asked to select 1 to 6 farms (depending on the size of the veterinary practice) that fulfilled the selection criteria. When a farmer showed interest, we contacted the farmer and explained the research, after which the farmer decided to participate or not. We kept a record with reasons for not participating.

PL- We contacted veterinary practitioners by phone from a list of specialists in poultry diseases and asked them for participation in the project. To those who accepted the invitation and did work with broiler farms, we asked for a list of farms complying with the selection criteria. The practitioners nominated the farm(s) of interest.

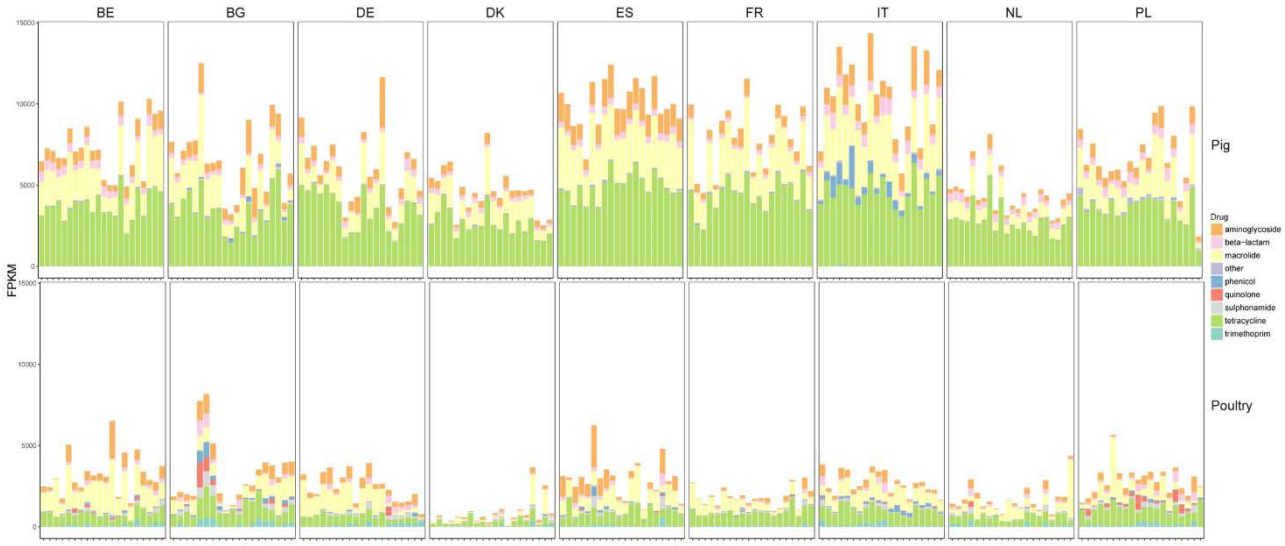
Sampling of broiler farms

To prevent seasonal influences, we tried to distribute sampling of farms over the year to the extent possible, however, most farms were sampled in fall (58) and least in winter (36). In summer and spring, 44 and 43 farms were sampled, respectively. Two countries (NL and ES) did not sample in winter.

The flocks should be sampled as close to slaughter as possible (last week before the final depopulation). Among the 181 farms, the average age at the sampling date was 33.8 days, with a minimum of 16 and a maximum of 54 days. Using the estimated duration of cycle, we estimated that in a total of 64 farms (BE- 3; BG- 15; DE- 3; DK- 13; ES- 10; FR- 3; IT- 8; NL- 2; PL- 7) sampling was performed more than one week (7 days) before final depopulation. These estimates should be only interpreted as a proxy, due to the uncertainty in the analysis.

In each farm, we collected 25 fresh undisturbed main bowel fecal droppings from the floor of the house (a minimum of 3g of feces/sample).

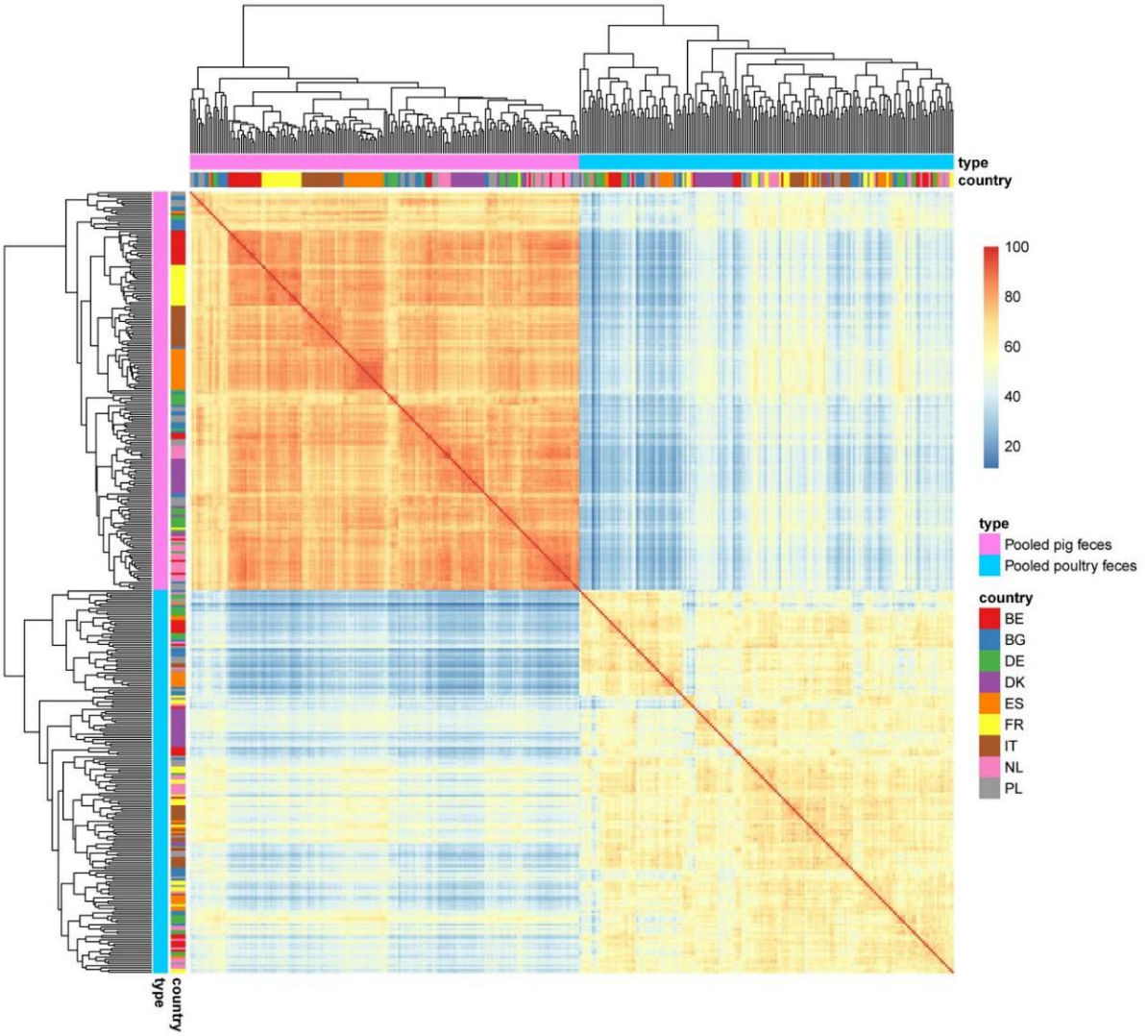
Supplemental figures



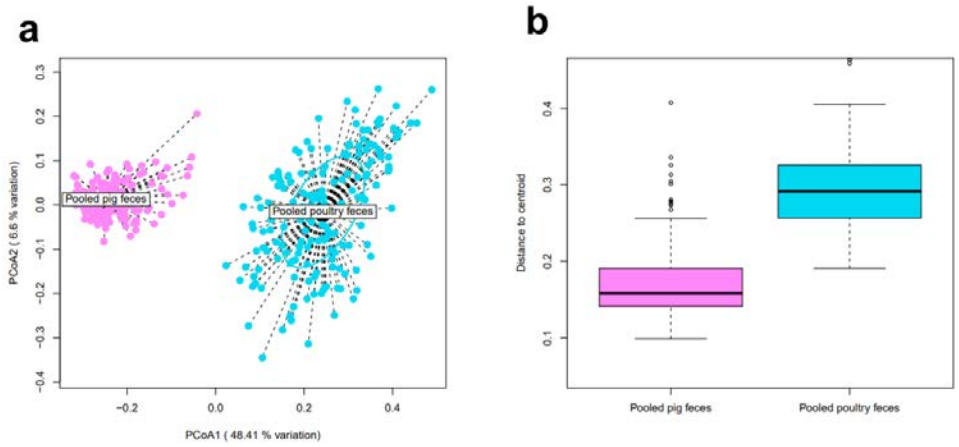
Supplementary Figure 1. Total AMR per sampled herd. Stacked bar chart of total relative AMR abundance in FPKM, separated by colour (AMR phenotype), sample (x-axis) and host species (top/bottom). This is an unscaled version of Figure 1b, where the height of each bar corresponds to the FPKM ResFinder AMR in a sample, rather than being proportional to within-sample AMR.

Please view Supplementary Figure 2 in the digital version of the paper.

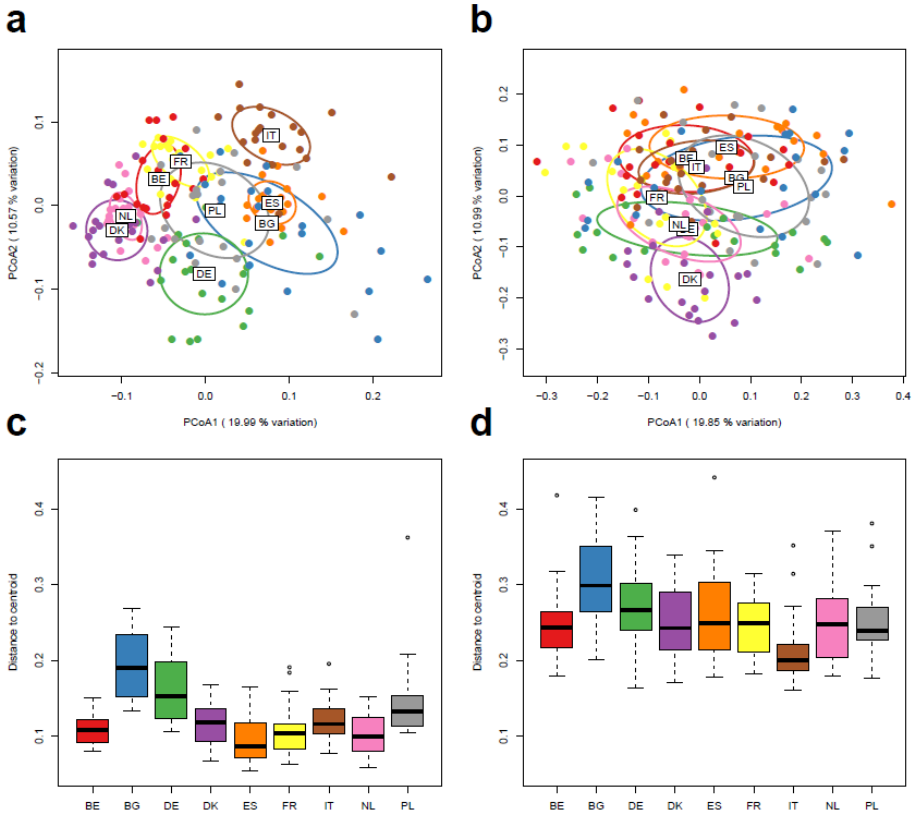
Supplementary Figure 2. AMR phenotype heatmap. Relative abundances (FPKM) of ResFinder reference genes were summed to their ResFinder-annotated AMR phenotypes and log-transformed. Both rows and columns were clustered according to Pearson correlation coefficients.



Supplementary Figure 3. Resistome sample similarity heatmap. Bray-Curtis dissimilarities were converted to similarities (%). Pearson correlation coefficients between samples were used for complete-linkage clustering of all samples. Sidebars colour-annotate the host animal and country of each sample.



Supplementary Figure 4. Pig and poultry farms have distinct resistomes. (a) PCoA plot of pig and poultry resistomes based on ResFinder gene clusters. n=359 metagenomes from independent herds. 20 metagenomes per livestock species per country was used, with the exception of BG pig (21), BG poultry (19) and DE poultry (19). (b) Distribution of sample-wise distances to their group (livestock species) centroid. npig=181, npoultry=178. Horizontal box lines represent Q1, median and Q3. Whiskers denote range of points within Q1-1.5*IQR.



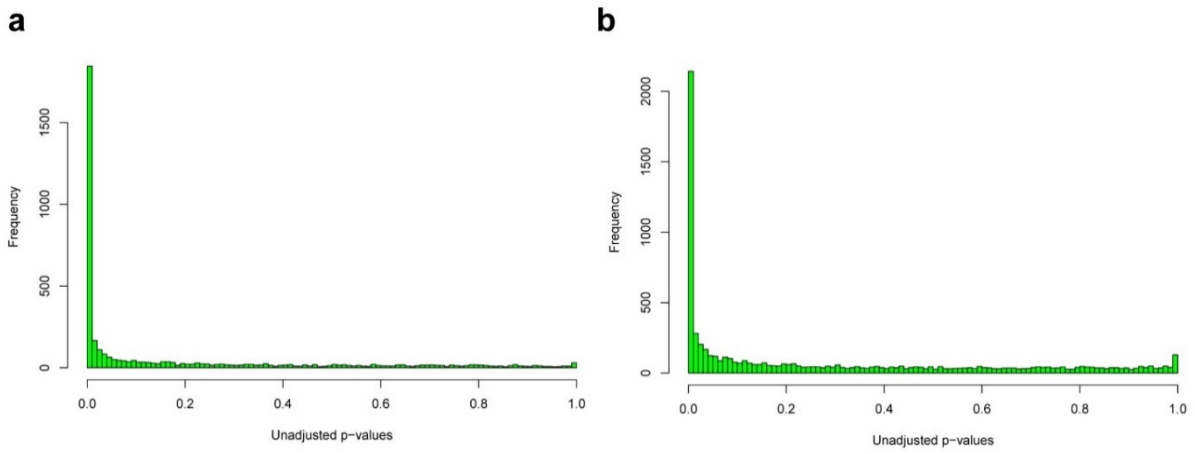
Supplementary Figure 5. Resistomes cluster according to country. (a-b) PCoA plots for pig (a) and poultry (b) resistomes. Ellipses denote standard deviation for distance of each resistome to its country centroid (labelled). (c-d) Boxplots of distances for each country's resistomes to their centroid for pig resistomes (c) and poultry resistomes (d). Horizontal boxplot lines represent Q1, median and Q3. Whiskers denote range of points within Q1-1.5*IQR and Q3+1.5*IQR. (b-e) n=359 metagenomes from independent herds. 20 metagenomes per livestock species per country was used, with the exception of BG pig (21), BG poultry (19) and DE poultry (19).

Please view Supplementary Figure 6 in the digital version of the paper.

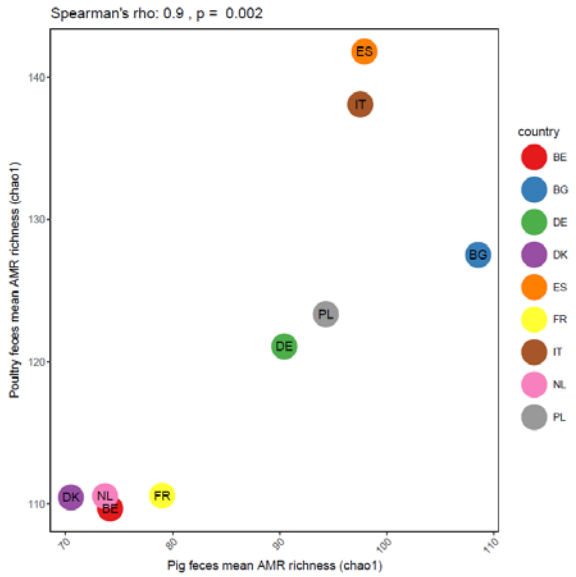
Supplementary Figure 6. AMR gene heatmap. Relative abundances (log FPKM) of most abundant AMR genes are shown with blue (low) to red (high). Gene dendrograms are based on Pearson correlation coefficients between gene abundances, while samples were clustered according to their Bray-Curtis dissimilarity indexes. The two coloured bars in the top indicate country and host species membership of each sample.

Please view Supplementary Figure 7 in digital version of the paper.

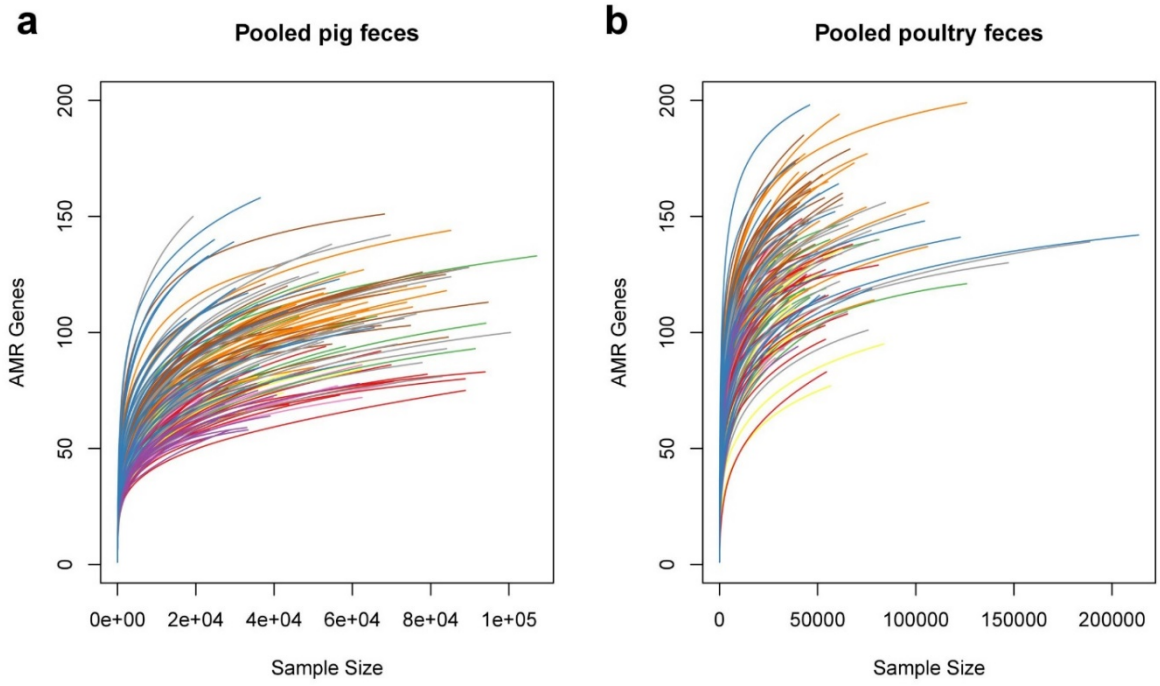
Supplementary Figure 7. AMR core gene heatmap. Heatmap of relative abundances (FPKM) for AMR genes detected in at least 95% of pig or poultry samples. Gene dendrograms are based on Pearson correlation coefficients between gene abundances, while samples were clustered according to their Bray-Curtis dissimilarity indexes. The two coloured bars in the top indicate country and host species membership of each sample. The two coloured bars on the left indicate whether a gene is core to pig and/or poultry.



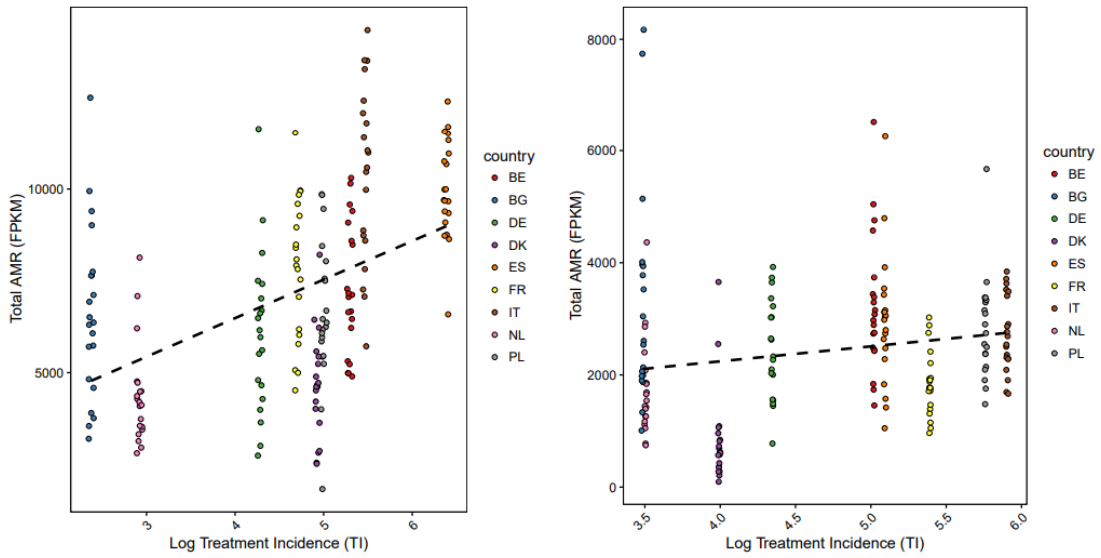
Supplementary Figure 8. Strong country effect on differential abundance of AMR genes. DESeq2 Wald tests were performed on the AMR mapping paired reads, testing for non-zero log2 fold change for each gene between countries. All country-versus-country unadjusted p-values were extracted and visualized for (a) pig (n=181 independent herd) and (b) poultry (n=178 independent herd) metagenomes. 20 metagenomes per livestock species per country was used, with the exception of BG pig (21), BG poultry (19) and DE poultry (19).



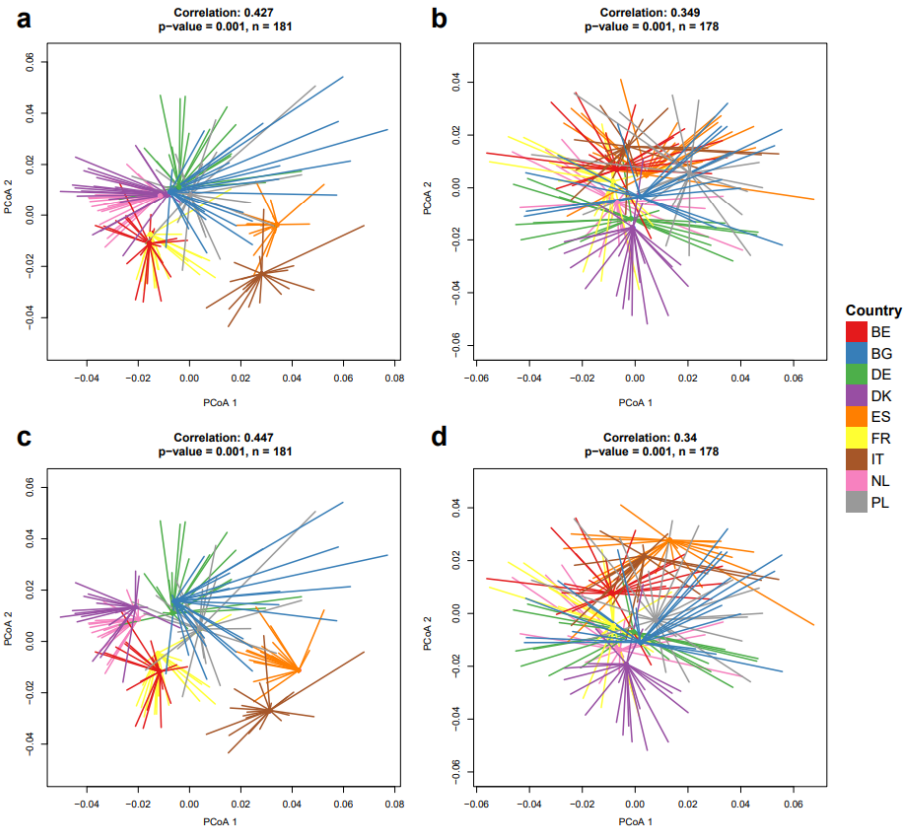
Supplementary Figure 9. Number of unique AMR genes in pig and poultry are associated. Country-wise average AMR Chao1 indexes are plotted and were used for correlation (Spearman rank, n=9).



Supplementary Figure 10. Resistome sampling effort. Rarefaction curves with each line representing a sample. The ResFinder count matrix was randomly subsampled without replacement (x-axis) to measure richness at each sampling level (y-axis). Shown for (a) pig and (b) poultry samples, with each line representing a sample.

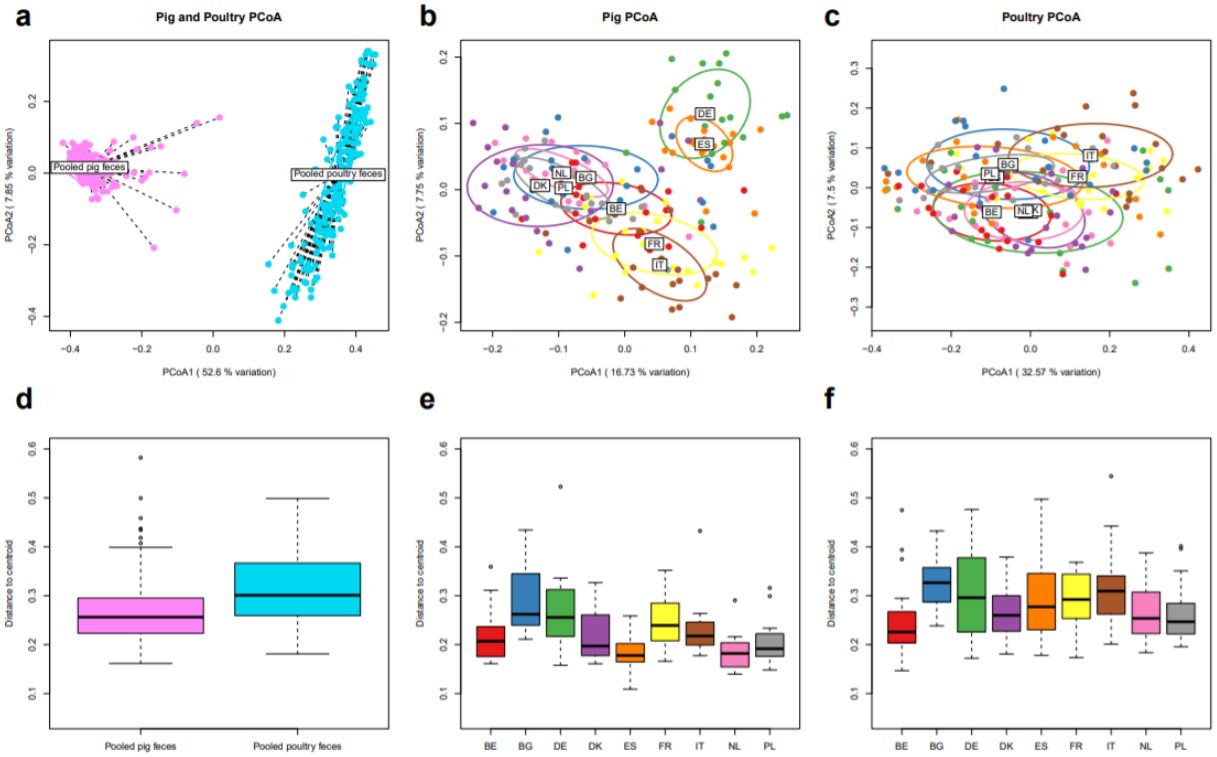


Supplementary Figure 11. Association between drug treatment incidence (TI) and total AMR in sampled farms. Scatter plots of average AM treatment incidence (TI-DDDvet) and pooled sample total AMR. A slight sideways jitter was added to the points to minimize overplotting. (a) Association between TI and total AMR load in pig farms was significant when analysed with a two-tailed, linear mixed effects regression model (p=0.03187, n=181 independent herds). (b) Association between TI and total AMR load in poultry farms was not significant (p=0.3718, n=178 independent herds). P-values for the two tests were not adjusted for multiple testing.

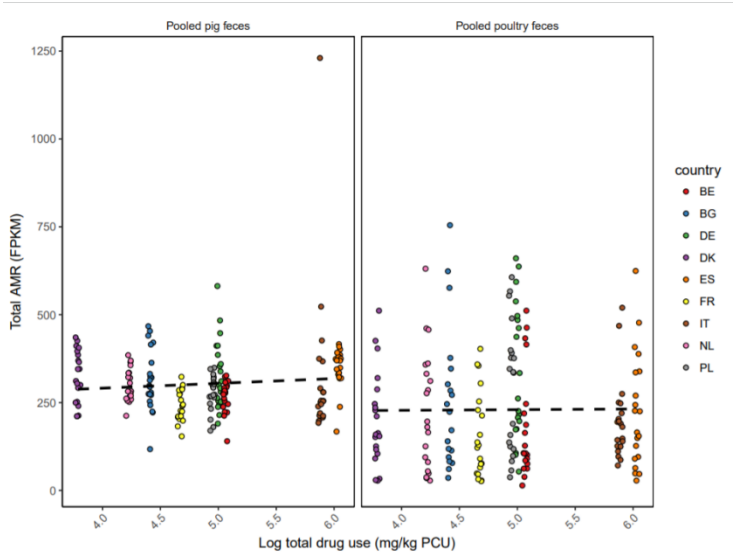


Supplementary Figure 12. Procrustes analyses comparing pig and poultry resistomes with country-specific drug usage. Procrustes superimposition plots showing the AMU configuration (dotted centres) rotated and

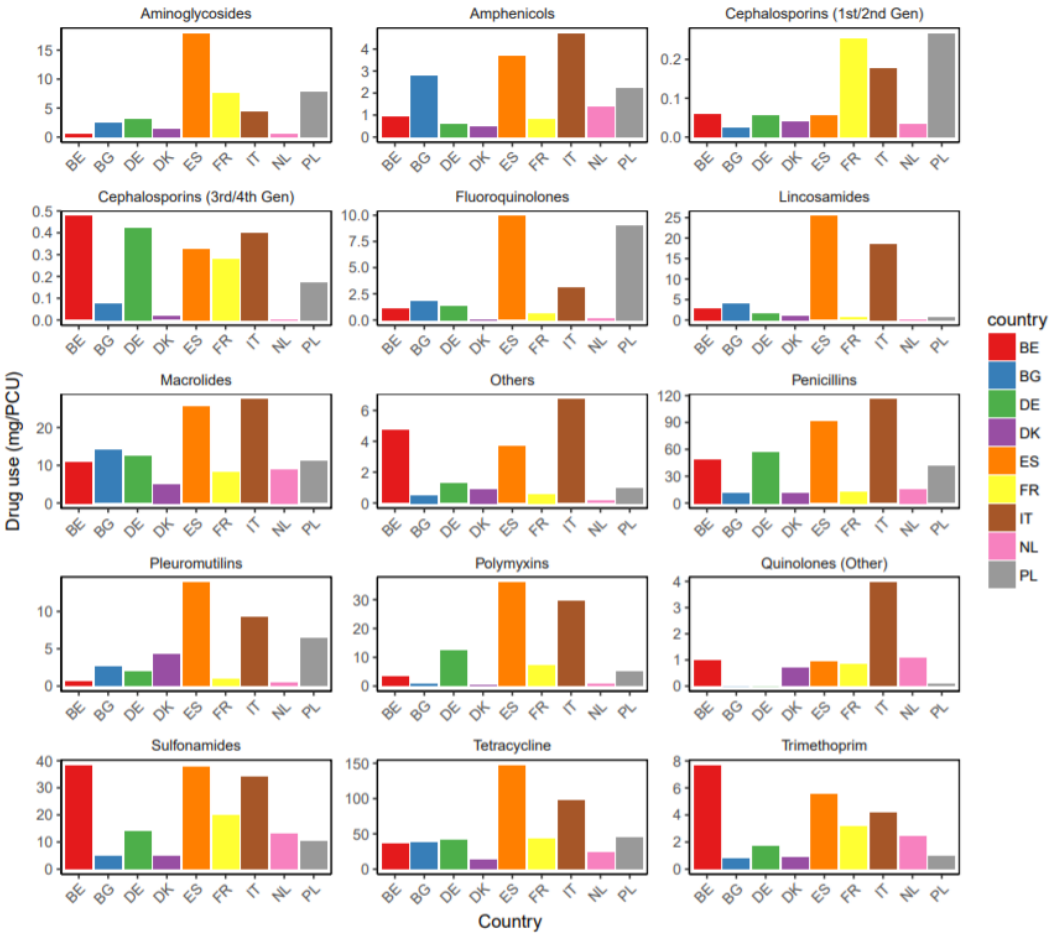
scaled to the resistome configuration. The vegan function 'protest' was used to derive the matrix-matrix correlations coefficients and p-values. (a) Association between pig sample resistomes (n=181 independent herds) and treatment incidence in sampled pig farms (n=9 national averages). (b) Association between poultry sample resistomes (n=178 independent herds) and treatment incidence in sampled poultry farms (n=9 national averages). (c) Association between pig sample resistomes (n=181 independent herds) and veterinary national drug usage (n=9 national averages, ESVAC). (d) Association between poultry sample resistomes (n=178 independent herds) and veterinary national drug usage (n=9 national averages, ESVAC).



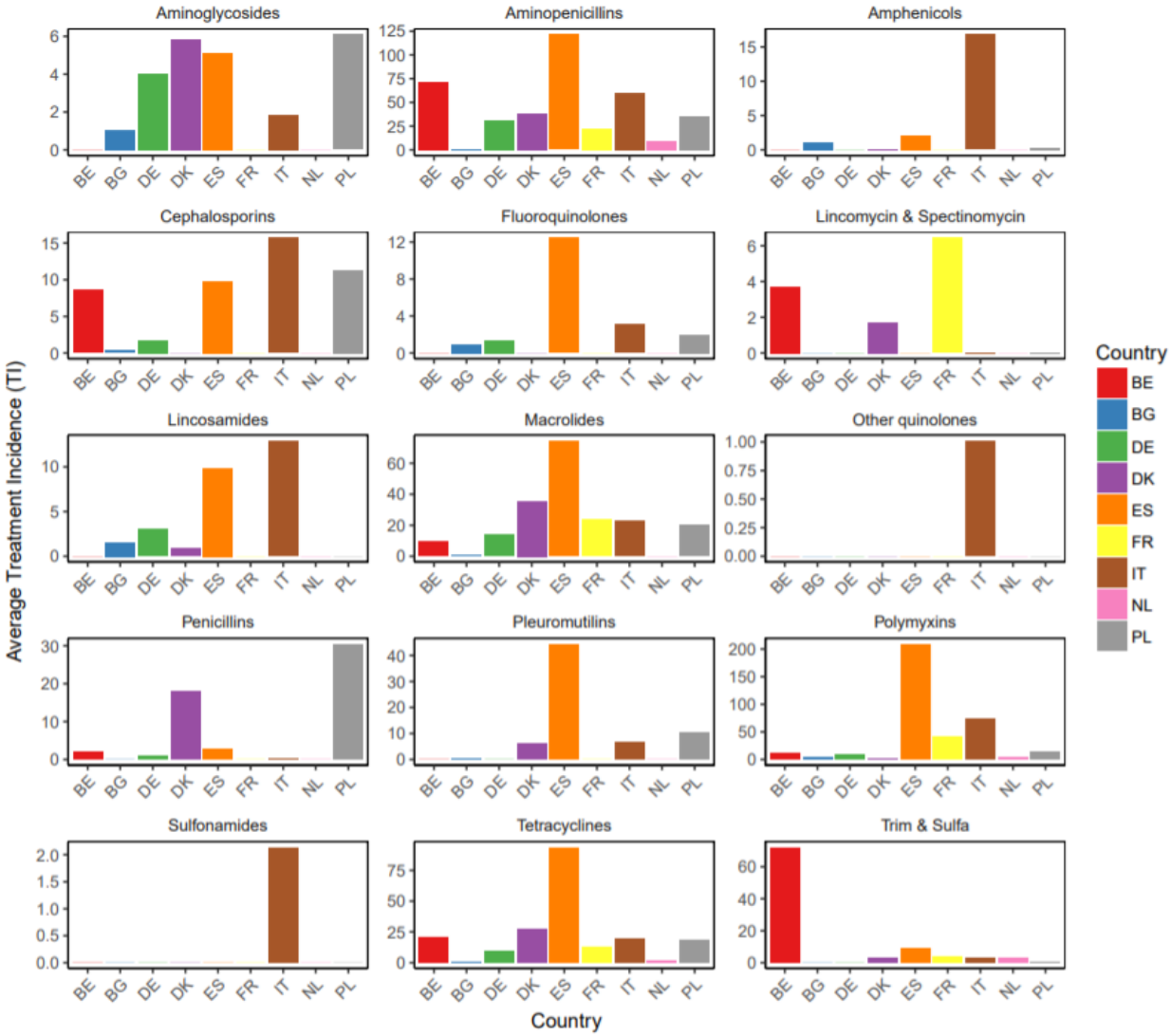
Supplementary Figure 13. Resistome clustering for the functional resistance database (FRD). The AMR genes present in FRD, but absent from ResFinder, were subjected to the same ordination analysis performed on the ResFinder genes. n=359 independent herds. 20 metagenomes per livestock species per country was used, with the exception of BG pig (21), BG poultry (19) and DE poultry (19). This figure is analogous to Supplementary Figure 7 and Figure 2, which should be referenced for details. Horizontal box lines represent Q1, median and Q3. Whiskers denote range of points within Q1-1.5*IQR and Q3+1.5*IQR.



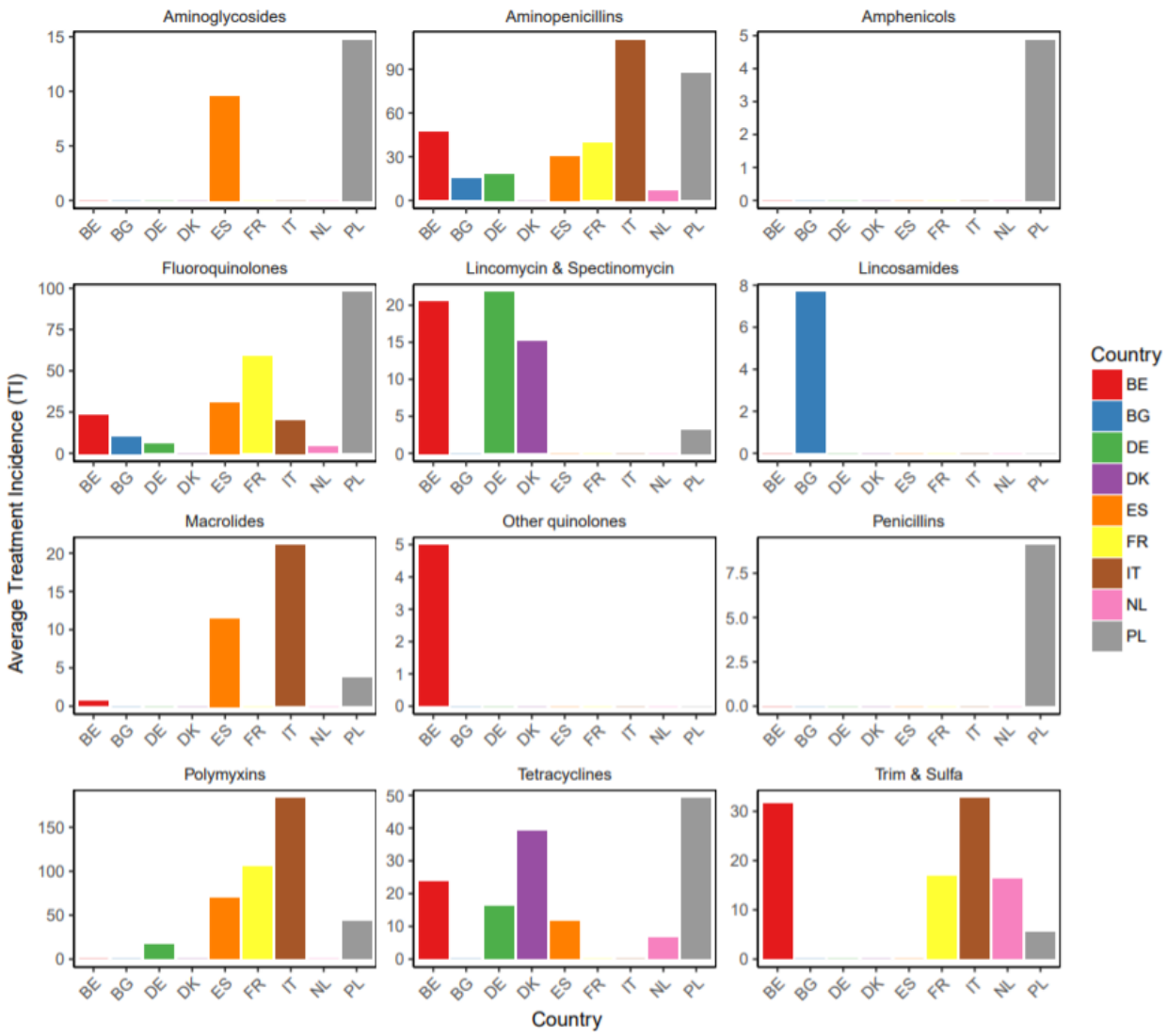
Supplementary Figure 14. National veterinary AMU is not associated with FRD AMR. Analogous to Figure 6 in main text (ResFinder). No association was found between the FRD genes' total abundance and ESVAC national veterinary AMU when analysed with a two-tailed, linear mixed effects regression model for pigs (p=0.3785, n=181 independent herds) or poultry (p=0.9392, n=178 independent herds).



Supplementary Figure 15. National veterinary drug use per country. Bar charts showing the antimicrobial usage (AMU) per country with each panel representing a separate drug class. 'Others' captures antimicrobials not included in the other categories, primarily colistin usage. Data is in mg/kg PCU from ESVAC and is not exclusive to use in pig and poultry.



Supplementary Figure 16. Average drug use in sampled pig herds. The average treatment incidence in the sampled herds (TI), stratified by drug class and country. The TI (TI-DDDvet) was calculated as the AM doses per Defined Daily Doses Animal (DDDvet) per 1000 animals at risk, adjusting for a 200 day production cycle.



Supplementary Figure 17. Average drug use in sampled poultry herds. The average treatment incidence in the sampled herds (TI), stratified by drug class and country. The TI (TI-DDDvet) was calculated as the AM doses per Defined Daily Doses Animal (DDDvet) per 1000 animals at risk, adjusting for a 40 day production cycle.

Due to the large size, part of the supplement is not printed. Please go to the online available Excel-file with supplementary tables 1-11

https://doi.org/10.1038/s41564-018-0192-9

3

Associations between antimicrobial use and the faecal resistome on broiler farms from nine European countries

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Abstract

Objectives To determine associations between farm and flock level antimicrobial usage (AMU), farm biosecurity status, and the abundance of faecal antimicrobial resistance genes (ARGs) on broiler farms.

Methods In the cross-sectional pan-European EFFORT study, conventional broiler farms were visited and feces, AMU and biosecurity records were collected. The resistomes of pooled faecal samples were determined by metagenomics analysis for 176 farms. A meta-analysis approach was used to relate total and class-specific ARGs (expressed as Fragments Per Kilobase reference per Million bacterial fragments, FPKM) to AMU (Treatment Incidence per Defined Daily Dose, TIDDDvet) per country and subsequently across all countries. In a similar way, the association between biosecurity status (Biocheck.UGent) and the resistome was explored.

Results Sixty-six (38%) flocks did not report group treatments but showed a similar resistome composition and roughly similar ARG levels as AM-treated flocks. Nevertheless, we found significant positive associations between beta-lactam, tetracycline, macrolide and lincosamide, trimethoprim and aminoglycoside antimicrobial flock treatments and ARG clusters conferring resistance to the same class. Similar associations were found with purchased products. In a gene level analysis for beta-lactams and macrolides, lincosamides and streptogramines (MLS), a significant positive association was found with the most abundant gene clusters *bla*$_{TEM}$ and *ermB*. Little evidence was found for associations with biosecurity.

Conclusions The faecal microbiome in European broilers contains a high diversity of ARGs, even in the absence of current antimicrobial selection pressure. Despite this, the relative abundance of genes and composition of the resistome is positively related to AMU in European broiler farms for several antimicrobial classes.

Introduction

Antimicrobial usage (AMU) is considered an important driver for the selection of antimicrobial resistance (AMR) in human, animal and environmental bacteria.[1] AMR in pathogenic bacteria hampers treatment and results in increased healthcare costs.[2] Next to human healthcare, one of the main users of antimicrobials is the intensive livestock industry. Resistance development in livestock is a great concern for the animal population and could be a source of bacteria transferring AMR to the human population.[3]

Broiler production is a major industry within livestock farming.[4] Broilers are produced in a highly-optimized way characterized by a pyramidal structure, consisting of a small number of pedigree and great-grandparent stock farms at the top of the pyramid and a large number of broiler farms at the bottom.[5] Broilers are raised for consumption within 6-7 weeks on average, which results in over 10 million tons of chicken meat produced in 2014 in the European Union.[6] In these conditions, antimicrobials are regularly administered to the whole flock to prevent or control infectious diseases.[7, 8]

AMU in broiler production is in Europe mostly reported on country level and based on national sales data monitoring systems. When national sales data are related to national AMR data, mostly based on MIC determinations for the bacterial indicator *E. coli*, positive associations have been observed for several antimicrobial classes.[1, 9, 10] Evidence beyond these 'ecological' associations is limited due to the absence of more detailed epidemiological data within countries and species at the farm level. Research at farm or even flock level enables analyzing such relations in the same epidemiological unit, and allows adjustment for potential confounding variables, which are generally not available for country-level analyses and might potentially lead to ecological fallacy.[11, 12] For broilers, only few association studies have been performed at farm level. These studies provide evidence for a positive association between flock or farm level AMU and AMR in specific commensal or pathogenic bacteria.[13, 14]

One possibly related (risk) factor or confounder that can be addressed with farm or flock level data is farm biosecurity. Farm biosecurity has been defined as the total of all measures taken to prevent both introduction and spread of infectious agents[15] and thus represents a collection of many potential factors that might influence introduction and further spread of AMR. [13, 14, 16]

In this study, metagenomic shotgun sequencing is applied for the analysis of the resistome, in DNA from the total community of faecal bacteria. Metagenomic sequencing enables a broad, culture independent and semi-quantitative reflection of resistance present in a broiler flock.[17] The aim of this study is to determine the relation between the broiler faecal resistome and farm and flock level usage of antimicrobials and farm biosecurity status in nine European countries.

Materials and methods

Study design

This cross-sectional study relates potential risk factors to the resistome of a pooled faecal sample from one flock of each broiler farm. In total 181 flocks from 181 farms from nine European countries (Belgium, Bulgaria, Denmark, France, Germany, Italy, the Netherlands, Poland and Spain) were sampled. In this paper, samples from 176 of the 181 farms were included. Five samples were excluded due to errors made during processing or incomplete data. All farms have been anonymized to ensure that results cannot be traced back to individual farms. Country was anonymized as this was required by the farming organization in one participating country.

Selection of farms and sampling

In the nine collaborating countries 20 non-mixed conventional broiler farms per country were visited between May 2014 and June 2016. Eligible farms needed to have, among other criteria, all-in all-out production (thinning from day 30 onwards allowed), no intended slaughter age higher than 50 days and no production of animals other than broilers. Further farm characteristics and (country specific) deviations from the selection protocol can be found in the supplement of Munk *et al*.[18] Per farm, 25 fresh faecal droppings from the floor from one flock (one batch) were collected, transported at 4˚C and stored at -80˚C within 24 hours. In this study a pooled sample of the 25 individual samples was used, resulting in one feacal pool per flock and per farm.

Laboratory analysis and bioinformatics analysis

After DNA extraction and metagenomic shotgun sequencing (Illumina HiSeq3000, 50 million paired-end reads per sample), the cleaned reads were mapped to the Resfinder antimicrobial resistance gene (ARG) database (accessed November 17th, 2016) of the Centre for Genomic Epidemiology.[19] The output was clustered at an ARG sequence identity level of 90%. The unit of outcome is a normalized read count FPKM (Fragments Per Kilobase reference per Million bacterial fragments). FPKM was calculated by dividing the mapped resistance fragments by the length of the respective resistance gene and the total number of bacterial fragments per sample and multiplying by 10^9. In the analyses the following outcomes per flock were used: 1) the sum of FPKM of all resistance gene clusters, 2) the sum of FPKM per antimicrobial class, and 3) the FPKM per 90% identity level gene cluster for two antimicrobial classes.

More details on the laboratory analysis and metagenomic shotgun sequencing can be found in the supplement and in Munk *et al*.[18]

Quantification of antimicrobial usage and farm biosecurity

Information on AMU, biosecurity status and several other characteristics of the farm and flock was collected through a questionnaire by interviewing the farmer on the day of the visit. The quantification of AMU is described in detail by Joosten *et al*.[7] Two measures of AMU have

been derived: 1) antimicrobials administered via group treatment to the flock from which the samples were taken (during its life span until sampling close to the age of slaughter) and 2) antimicrobials purchased for the whole farm (which may contain more flocks then the sampled flock) in the year before sampling. The treatment incidence (TI) of defined daily dosages (DDDvet) was calculated by dividing the amount of antimicrobials administered or purchased by the dose times days at risk and times kg animal. TIDDDvet can be read as the percentage of the life of a broiler for which it is treated. In the analyses the following explanatory variables were used: 1) the total sum of TIDDDvet per flock (group treatment data) or farm (purchase data), and 2) the sum per antimicrobial class.

The questionnaire also contained items relevant for the calculation of the biosecurity score with the Biocheck.Ugent method.[16] More details are provided in the supplement. The biosecurity score is expressed as a value between 0 (no biosecurity measures are in place) and 100 percent (all biosecurity measures are in place and used). In the analyses the external and internal biosecurity scores were tested as explanatory variables.

Data analysis

A country effect appears in both AMR and AMU data and has been described before.[7, 18] For example the country of origin of the samples is significantly associated with the resistome and explains roughly 25% of the variation observed. To address this effect, considering the total number of farms per country included in the analysis (18 or 20), we used country-specific models as input for a random-effects meta-analysis (R package Metafor, DerSimonian-Laird heterogeneity estimator).[20] Meta-analysis allows to obtain and visualize results in a transparent way.[21] Outcome and AMU-data were $\log_{10}$ transformed because of skewness (1 was added to keep zeros), and the outcome was standardized (mean 0, SD 1). Thus, associations were first calculated with linear regression per country and subsequently a meta- or overall association was calculated across countries. Concurrent usage and observations of the corresponding resistance did not occur at all farms and occasionally not in each country, resulting in specific meta-analyses with data from less than 9 countries. The analysis was performed stepwise: first the association between AMU and ARGs was calculated for each corresponding antimicrobial class (e.g. tetracycline resistance versus tetracycline use), followed by non-corresponding classes. Confounding by biosecurity status of the farm and sampling age of the broilers was tested. A sensitivity analysis was performed by calculating the association between corresponding ARGs and AMU as binary variable; 0 meaning no AMU reported on flock or farm level, 1 meaning (any) AMU reported on flock or farm level.

The same meta-analysis approach was used to test the association between ARGs and internal and external biosecurity status, with and without adjusting for AMU. For two antimicrobial groups that showed a robust association with corresponding ARGs, an additional analysis was performed to test which gene clusters drive the association with the respective antimicrobial class. Again, random-effects meta-analysis was used with individual gene clusters as outcome and corresponding AMU as explanatory variable.

Table 1 – General characteristics of the sampled farms and flocks by country and over all countries

Country	Number of farms included in the analyses	Average number of broilers present at the farm		Average number of rounds per year		Number of broilers set up in the sampled barn		Age broilers during sampling (days)		Average weight broilers during sampling (grams)		Average weight of broilers at slaughter (grams)	
		mean	median	mean	median	mean	median	mean	median	mean	median	mean	median
		(min - max)		(min - max)		(min - max)		(min - max)		(min - max)		(min - max)	
A	20	77322	80000	7	7	29952	28300	34	35	2042	2125	2529	2500
		(24530 - 180000)		(3 - 8)		(16500 - 46700)		(27 - 39)		(1300 - 2490)		(2385 - 2750)	
B	18	106059	76600	8	8	29827	32750	31	33	1861	1900	2320	2400
		(17200 - 240000)		(7.3 - 8.5)		(17200 - 41400)		(19 - 40)		(1350 - 2700)		(1550 - 2700)	
C	20	46255	35150	8	8	35035	34450	26	27	1361	1322	2236	2193
		(25000 - 144000)		(7 - 8.5)		(25000 - 53300)		(16 - 32)		(530 - 2000)		(2000 - 2800)	
D	20	96390	60000	5	5	23398	20950	42	43	2440	2450	2645	2600
		(24000 - 400000)		(4 - 5.5)		(11340 - 50000)		(34 - 51)		(1600 - 3650)		(2100 - 3700)	
E	20	54810	47500	6	6	30605	29580	31	29	1479	1214	1744	1625
		(21400 - 216000)		(3 - 9)		(21420 - 42886)		(21 - 48)		(575 - 3000)		(1300 - 2700)	
F	20	108258	110000	7	7	30849	31200	36	36	1939	1940	2375	2388
		(32000 - 200000)		(6 - 8.5)		(17550 - 49700)		(29 - 42)		(1500 - 2500)		(2065 - 2721)	
G	20	56135	41500	6	6	31473	33565	36	36	2009	2070	2528	2500
		(19000 - 150000)		(5 - 6)		(18500 - 41800)		(30 - 42)		(1300 - 2500)		(2050 - 2900)	
H	20	41680	30210	5	6	23219	22440	36	37	1907	1900	2693	2750
		(20000 - 114141)		(2 - 6.5)		(14000 - 33864)		(22 - 44)		(859 - 2500)		(1750 - 3000)	
I	18	54873	20000	6	6	16413	16725	31	29	1529	1645	2264	2200
		(8000 - 250000)		(4 - 7)		(8000 - 27000)		(19 - 54)		(730 - 2600)		(1950 - 2700)	
overall	176	71101	50000	6,35	6,15	27971	26500	33,8	34	1844	1850	2372	2450
		(8000 - 400000)		(2 - 9)		(8000 - 53300)		(19 - 54)		(575 - 3650)		(1300 - 3700)	

To control for multiple testing we applied a False Discovery Rate of 0.1, using the Benjamini Hochberg procedure[22]. This was done separately for the six analyses described above. All descriptive and statistical analyses were done in R (version 3.3.1).[23] An explanation of the interpretability of our results can be found in the supplement.

Results

Farms

The average flock size at setup over all nine countries was 27,971 one-day-old chicks (Table 1). On average, the smallest flocks were sampled in country I (16,413 one-day-old chicks) and the largest flocks in country C (35,035 one-day-old chicks). Age of sampled flocks was 34 days on average, with the youngest broilers sampled in country C (26 days) and the oldest in country D (42 days). The average weight at slaughter of the broilers from the sampled farms was 2,372 grams (range: 1,744 grams in country E and 2,693 grams in country H).

Associations between usage and resistance

Of the 176 analyzed flocks, 66 (38%) did not report any use of group treatments in the sampled flock up to the day of sampling. In total, 22 farms (13%) reported not to have purchased any antimicrobials in the year before sampling. Most of the non-users (47 of 66) were present in 3 countries (country C, F, I) However, these untreated flocks show similar diversity and only slightly reduced ARG clusters (overall mean of 1677 FPKM) as treated flocks (overall mean of 1880 FPKM) (Figure 1a and 1b).

Both possible confounders (farm biosecurity and sampling age of the broilers) were not significantly associated with the outcome and, when added to the models, estimates of the associations did not change more than 10%. Therefore, these variables were not included in the final and presented models. For discussion of our results we applied a FDR of 0.1, the FDR per comparison is given in all tables.

Although high levels of resistance were present in flocks or farms without antimicrobial use in the sampled rearing period, we did find associations between AMU and the corresponding ARGs (Table 2). Significant positive associations were found between flock group treatments and ARGs for MLS-antibiotics, tetracyclines, aminoglycosides, beta-lactams (Figure 2a) and trimethoprim and their respective resistance. MLS antibiotics used include macrolides and lincosamide treatments. The MLS resistance group includes macrolide, lincosamide and streptogramin gene clusters.

For AMU defined as products purchased by the whole farm, significant associations were found for total, MLS antibiotics (Figure 2b), tetracycline, amphenicol and trimethoprim products and respective resistance. The analysis between corresponding resistance and AMU as binary variable gave the same results as the analysis with AMU as continuous variable except for the association with total purchased products (Table S1).

To investigate co- or cross-resistance, associations between total ARGs or ARGs per antimicrobial class and total and non-corresponding usage or purchased products was tested. After controlling for the FDR, none of these associations remained significant (Table S2).

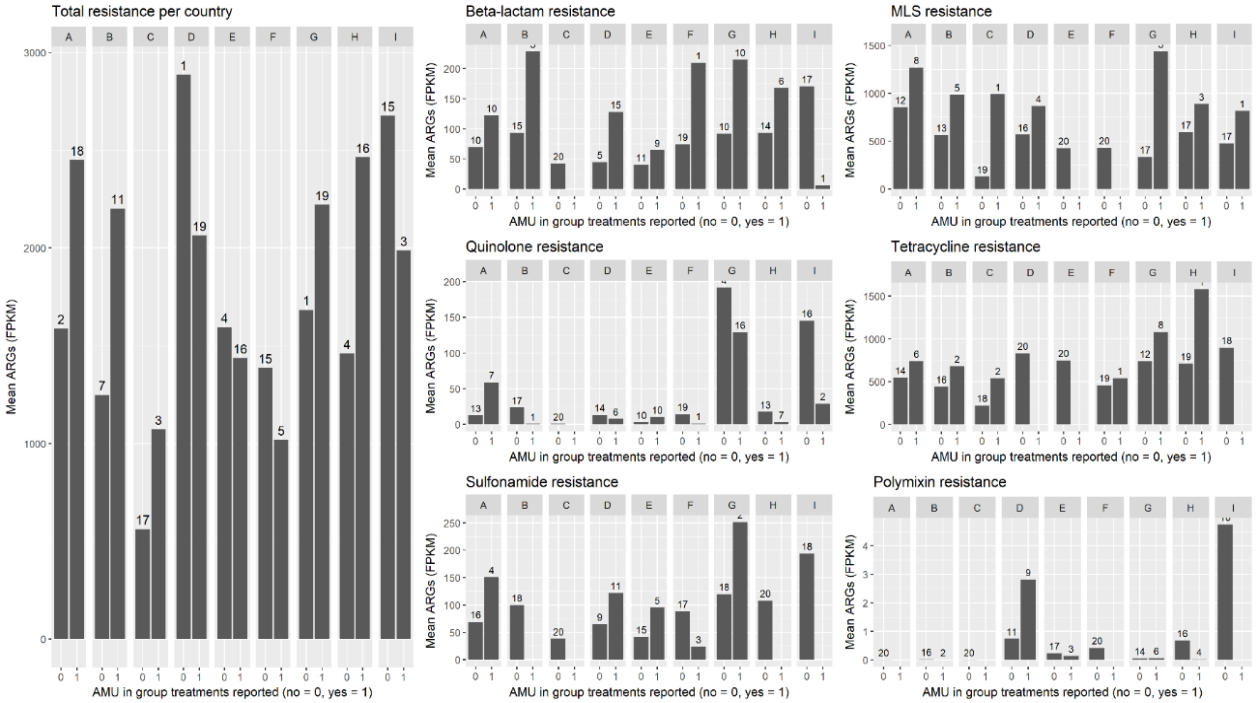

Figure 1a – Mean sum of antimicrobial resistance genes (ARGs) in Fragments Per Kilobase reference per Million bacterial fragments (FPKM) of farms that did or did not report antimicrobial use (AMU) in group treatments for the sampled flock, grouped by country. Left: total ARG's versus total AMU per flock with number of farms shown above the bars. Right: ARG's of several (handpicked) antimicrobial classes/groups versus corresponding AMU per flock with number of farms shown above the bars. MLS = macrolides, lincosamides and streptogramines.

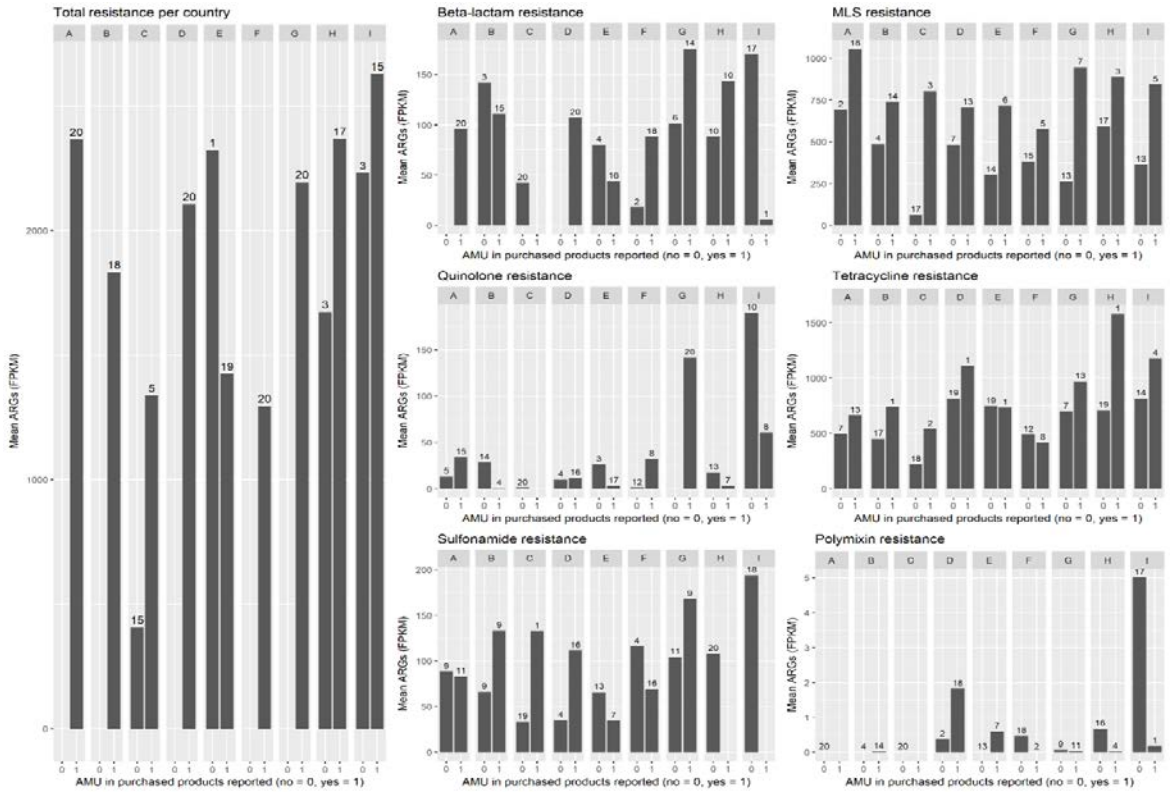

Figure 1b – Mean sum of antimicrobial resistance genes (ARGs) in Fragments Per Kilobase reference per Million bacterial fragments (FPKM) of farms that did or did not report antimicrobial use (AMU) in purchased products by the whole farm in the year before sampling, grouped by country. Left: total ARG's versus total AMU per farm with number of farms shown above the bars. Right: ARG's of several (handpicked) antimicrobial classes/groups versus corresponding AMU per farm with number of farms shown above the bars. MLS = macrolides, lincosamides and streptogramines.

The resistance reported per antimicrobial class is the sum of FPKM of many different resistance gene clusters. For most classes the contribution of single resistance gene clusters to the overall class-level ARG is highly skewed (with few genes largely determining the sum of ARG per class) (Table 3). A detailed analysis was performed of the association between beta-lactam group treatments and individual beta-lactam resistance gene clusters and between MLS purchased products and MLS resistance gene clusters (Tables S3 and S4). Within both these antimicrobial classes/groups we observed a significant positive association between the most abundant gene cluster (*bla*$_{TEM}$ and *ermB*). For beta-lactam group treatments the only other significant positive association is with the *bla*$_{ACT}$ cluster. For MLS purchased products we see also significant positive associations with several different *erm* gene clusters and *lsaA and mefB.*

Biosecurity

We found 2 statistically significant associations between ARGs (analyzed in total and per class) and internal or external biosecurity (Table S5). We observed a significant, positive association between oxazolidinone resistance and internal biosecurity (i.e. higher internal biosecurity is associated with more oxazolidinone resistance genes) and a significant, negative association

between tetracycline resistance and internal biosecurity. After adjustment for AMU, only the former association remained.

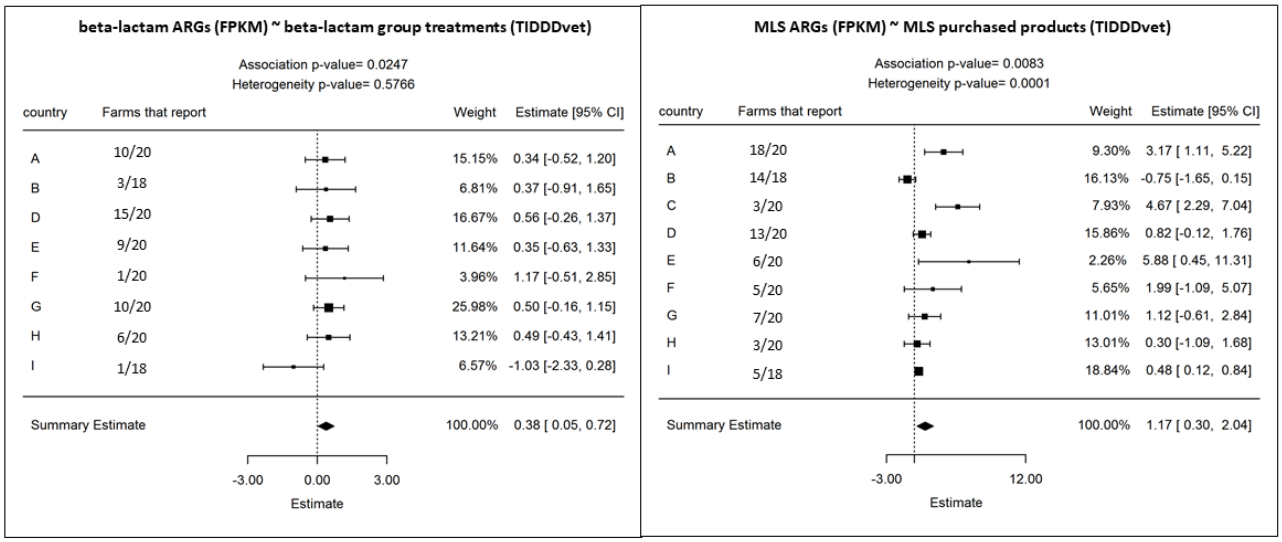

Figure 2 – Two example forest plots of the country specific associations and meta-analysis results. Left: beta-lactam antimicrobial resistance genes (ARGs) in Fragments Per Kilobase reference per Million bacterial fragments (FPKM) and beta-lactam group treatments as Treatment Incidence of Defined Daily Dosages (TIDDDvet). Right: Macrolides, lincosamides and streptogramines (MLS) ARGs (FPKM) and MLS purchased products (TIDDDvet). With number of farms that report AMU, the weight of the individual association in the summary estimate and the 95% confidence intervals (CI) per country. At the bottom the summary estimate with the confidence interval for the overall association.

Discussion

In this study we quantified resistance using the resistome of pooled faecal flock samples obtained by metagenomic analysis and related this to antimicrobial use data of the broiler flocks and farms from different countries. Our results confirm the hypothesis that higher antimicrobial exposure on flock or farm level is associated with more antimicrobial resistance.

Positive associations between AMU and ARGs

Our AMR and AMU data showed country specific differences[7, 18] and therefore random effects meta-analysis is used to test the relation between ARG and AMU. Almost all associations between ARGs and AMU of corresponding antimicrobial classes were positive. The abundance of genes encoding for tetracycline, MLS, trimethoprim and aminoglycoside resistance was significantly positively related to the corresponding flock treatments and corresponding products purchased by the farm. Our data thus showed that current use in a flock is associated with a higher abundance of resistance genes in the same flock, although antimicrobial products from these classes have been used in broilers for a long time now. An increase in

Table 2 – Results of meta-analysis between antimicrobial resistance genes (ARGs) in Fragments Per Kilobase reference per Million bacterial fragments (FPKM) and antimicrobial usage (AMU) as Treatment Incidence of Defined Daily Dosages (TIDDDvet) of corresponding antimicrobial classes/groups. Associations in bold have an False Discovery Rate <0.1. (group) = AMU in group treatments of the sampled flock, (purchased) = AMU in purchased products by the farm in the year before sampling

Class/group ARGs	Class/group AMU	Estimate	p-value	FDR	Confidence Interval	Country + number of farms with reported AMU
MLS (macrolide, lincosamide, streptrogramin)	**MLS (macrolide, lincosamide) (group)**	**1.18**	**<0.001**	**<0.001**	**[0.74-1.62]**	**A-8,B-5,C-1,D-4,G-3,H-3,I-1**
Tetracycline	**Tetracycline (group)**	**0.98**	**<0.001**	**<0.001**	**[0.57-1.39]**	**A-6,B-2,C-2,F-1,G-8,H-1**
Trimethoprim	**Trimethoprim&Sulfonamide (purchased)**	**1.19**	**0.004**	**0.026**	**[0.37-2.01]**	**A-11,B-9,C-1,D-16,E-7,F-16,G-9**
Tetracycline	**Tetracycline (purchased)**	**0.83**	**0.005**	**0.026**	**[0.25-1.41]**	**A-13,B-1,C-2,D-1,E-1,F-8,G-13,H-1,I-4**
Total	**Total (purchased)**	**0.58**	**0.008**	**0.028**	**[0.15-1.00]**	**A-20,B-18,C-5,D-20,E-19,F-20,G-20,H-17,I-15**
MLS (macrolide, lincosamide, streptrogramin)	**MLS (macrolide, lincosamide) (purchased)**	**1.17**	**0.008**	**0.028**	**[0.30-2.04]**	**A-18,B-14,C-3,D-13,E-6,F-5,G-7,H-3,I-5**
Amphenicol	**Amphenicol (purchased)**	**7.76**	**0.016**	**0.045**	**[1.47-14.04]**	**D-2,G-2,I-1**
Beta-lactam	**Beta-lactam (group)**	**0.38**	**0.025**	**0.058**	**[0.05-0.72]**	**A-10,B-3,D-15,E-9,F-1,G-10,H-6,I-1**
Trimethoprim	**Trimethoprim&Sulfonamide (group)**	**0.86**	**0.026**	**0.058**	**[0.10-1.61]**	**A-4,D-11,E-5,F-3,G-2**
Aminoglycoside	**Aminoglycoside (group)**	**1.23**	**0.031**	**0.063**	**[0.11-2.35]**	**G-2,H-7**
Aminoglycoside	**Aminoglycoside (purchased)**	**1.04**	**0.047**	**0.086**	**[0.01-2.07]**	**G-4,H-7**
Sulfonamide	Trimethoprim&Sulfonamide (purchased)	0.72	0.106	0.177	[-0.15-1.59]	A-11,B-9,C-1,D-16,E-7,F-16,G-9
Total	Total (group)	0.33	0.207	0.319	[-0.18-0.85]	A-18,B-11,C-3,D-19,E-16,F-5,G-19,H-16,I-3
Sulfonamide	Trimethoprim&Sulfonamide (group)	0.47	0.238	0.340	[-0.31-1.26]	A-4,D-11,E-5,F-3,G-2
Amphenicol	Amphenicol (group)	1.10	0.266	0.354	[-0.84-3.04]	G-1
Beta-lactam	Beta-lactam (purchased)	0.30	0.298	0.373	[-0.26-0.86]	A-20,B-15,D-20,E-16,F-18,G-14,H-10,I-1
Polymyxin	Polymyxin (purchased)	-0.07	0.668	0.786	[-0.38-0.24]	B-14,D-18,E-7,F-2,G-11,H-4,I-1
Quinolone	Quinolone (purchased)	0.20	0.738	0.820	[-0.96-1.36]	A-15,B-4,D-16,E-17,F-8,G-20,H-7,I-8
Quinolone	Quinolone (group)	-0.02	0.915	0.964	[-0.41-0.36]	A-7,B-1,D-6,E-10,F-1,G-16,H-7,I-2
Polymyxin	Polymyxin (group)	0.00	0.987	0.987	[-0.35-0.35]	B-2,D-9,E-3,G-6,H-4

several *tet*-genes and the use of chlortetracycline has also been shown by others.[24] In the frequent – and expected – case that not all classes of antibiotics are used in a specific flock in its lifespan, and when this occurs country wide, this country will not be included in the meta-analysis. The results per antibiotic class were therefore often based on less than nine countries. Beta-lactams, quinolones and polymyxins were the classes used most in this study.[7] For quinolones and polymyxins no significant relation was found with their corresponding ARGs. For polymyxins this is probably due to relatively low gene abundances in the samples (Figure 1). For quinolones usage is reported in almost all countries and an association with resistance has been described before (though not with metagenomic analysis).[13, 25] One likely reason for this lack of association is that quinolone resistance is partly due to point mutations that could not be detected sufficiently with the resistance gene database used here, and is difficult to detect in metagenomic studies altogether.[26] The association between beta-lactam resistance and use in the sampled flock was significant in our study. Within the class of beta-lactam resistance, *bla*$_{TEM}$ turns out as the gene cluster with the highest FPKM, in agreement with the significant positive association between *bla*$_{TEM}$ and beta-lactam use in the flock. The *bla*$_{TEM}$ cluster is large: it includes 223 TEM type beta-lactamases,[27] of which a large part have an ESBL-or inhibitor-resistant phenotype. Genes of special interest like bla$_{ctx-m}$ and bla$_{cmy}$ were observed in these flocks. Probably due to the fact that these genes were restricted to relatively rare species and that usage of the respective antibiotics was low, we did not find significant associations. Within the MLS gene cluster, there was a number of genes that were significantly positively associated with MLS-purchased products by the farm in the year before sampling, including not only the expected highly abundant gene clusters *ermB* and *ermC*, but also less prevalent genes. All in all we conclude that higher reported AMU is associated with higher relative gene abundance, while the resulting veterinary and public health implications are yet difficult to conclude upon.

Flock versus farm level AMU

Our results show a similar, but not identical, picture of associations between ARGs and usage on flock level (use in the sampled flock specifically) as for usage on farm level (purchased products over one year). Flock level data is considered to be superior to data on purchased products if associations between AMU and AMR are thought to occur by selection in the actual flock. The overall correlation between TIDDDvet of group treatments and purchased products over one year is moderate (0.54[7]) and data on these purchased products might resemble general and/or historic use by the farm and thereby give an additional perspective on the association between usage and resistance, which might also occur through recirculation of resistant bacteria within a farm from flock to flock. Moreover, the presence of residual amounts of antimicrobials might be sufficient to maintain the presence of resistant bacteria.

ARGs without current antimicrobial pressure

Overall, the observed positive associations between ARG and AMU were relatively weak, and the presence of many of the measured resistance genes seems not to be explained by current

use. This can also be concluded from the roughly similar abundance of resistance genes in the flocks and farms that do not report any AMU. Several reasons for resistance genes being present without current antimicrobial pressure have been suggested in literature. Roughly since the 1950's increasing amounts and types of antimicrobials have been used in the livestock industry exerting selective pressures on the development and spread of AMR.[28] Also, usage in other (higher) sections of the broiler pyramid, might influence AMR in lower sections through vertical transmission.[5, 29]

Once resistance genes are present at a farm, recirculation of resistance genes via the (farm) environment is possible.[30, 31] Furthermore, resistance gene carriage does not necessarily compromise microbial fitness which makes presence of resistance genes in the absence of AMU pressure more likely.[32, 33] Taken together, the drivers for resistance genes to be present in poultry samples are diverse, complicating quantifications of the associations between AMU and AMR. From these results, it can also be questioned to which extent resistance can be reduced only through reducing the use in specific flocks.

Biosecurity

External and internal biosecurity include all possible measures to minimize the introduction and spread of disease at the farm. Possibly, the introduction and spread of ARGs could also be influenced by these measures. No data exist on the association between internal and external biosecurity scores and AMR yet, but associations with a few individual measures, have been reported.[14, 34] Within the EFFORT study the same associations have been explored within European pig farming. This resulted in a positive association between internal biosecurity and macrolide gene clusters.[21] Our analysis, after adjusting for AMU, resulted in one association: a higher internal biosecurity was associated with a higher relative abundance of oxazolidinone ARGs. Oxazolidinone antibiotics are not used in broiler production though and we do not have an explanatory hypothesis for this specific association. Due to the limited degree of associations overall, we conclude that our data is not sufficient to support the hypothesis that introduction and spread of ARGs is influenced by biosecurity measures.

Co- or cross-resistance

The analysis of the relation between non-corresponding antimicrobial classes of resistance and use did not result in significant associations. The analysis is based on short metagenomics reads, which implies that the actual origin and genomic context is unknown, hindering focused searches for co- or cross-resistance within one species or genomic context. However, within the data generated in this study, the role of co- or cross-resistance is minor as compared to usage of the corresponding class.

Strengths and limitations of the study

With 176 broiler flocks included in these analyses this is, to our knowledge, the largest metagenomic cross-country study that has been performed in poultry which enabled us to look at the whole faecal resistome instead of specific resistances in specific bacteria. Despite

this large number of samples insufficient power might still be a reason for not detecting certain associations in our study.

Table 3 – Ten most abundant gene clusters per antimicrobial class/group (which gave an overall significant association with antimicrobial usage) and their contribution to the total sum of antimicrobial resistance genes in percentages. MLS = macrolides, lincosamides and streptogramines.

	Beta-lactam	**%**	**MLS**	**%**	**Aminoglycoside**	**%**	**Tetracycline**	**%**	**Amphenicol**	**%**	**Trimethoprim**	**%**
1	blaTEM	84,65	ermB	41,11	aadA cluster 1	23,06	tetW	57,35	cmx	29,90	dfrA1	47,50
2	blaCMY - blaBIL - blaLAT	2,43	lnuA	21,65	spc	12,73	tetA	8,67	catpC194	23,49	dfrD	15,34
3	blaSHV	1,70	lnuC	17,97	strB	10,5	tetL	6,71	cml	16,25	dfrK	7,90
4	blaOXA-61	1,67	ermC	3,80	aadE 1 KF864551	9,78	tetM	6,06	floR	12,92	dfrA12	7,14
5	blaACT-9	1,65	ermX	2,40	strA	9,27	tetQ	3,55	catA1	5,26	dfrA14	5,17
6	blaACT	1,43	vatE	1,86	aph3 III	8,15	tetZ	2,94	cat3	2,71	dfrG	4,35
7	cfxA	1,35	ermF	1,59	ant6 la	3,24	tetO	2,66	cat2	1,64	dfrA16	3,39
8	blaCTX-M cluster 1	1,26	lnuB	1,49	aac3 lva	3,08	tet4	1,92	cat	1,51	dfrA7 - dfrA17	3,25
9	cepA	0,59	ermG	0,95	aph4 la	2,98	tetB	1,75	fexA	1,17	dfrA15	2,96
10	mecA cluster 1	0,53	lnuF	0,91	aadE 1 KF421157	2,98	tet33	1,51	catpC221	1,06	dfrA5 - dfrA30	1,90

Although sampling was performed in nine countries, all information concerning AMU and biosecurity was collected in a harmonized way with the use of protocols and close collaboration between the researchers. Despite this, bias might be introduced by misclassification of biosecurity and underreporting of AMU. Bias might also be introduced by the DNA extraction procedure and the library preparation. It often favors certain bacteria, thereby biasing retrieved gene frequencies, however this bias should be consistent across all samples and countries. The current selection of farms in each country is based on preset inclusion criteria and in agreement with local farming organizations, and partially also based on convenience (e.g. distances to farms). As a result, the sample of farms in each country cannot be considered representative for the livestock sector in that country.

With respect to the methodology, another limitation of the focus on similarity of short reads to known resistance genes is that the function of the assumed resistance genes can only be assigned with a certain probability and it is unknown if their presence implies functional/expressed resistance. However, it has been shown that tetracycline resistance measurements in the same sample in cfu counting of aerobic bacteria and metagenomics do correlate significantly.[35] Another limitation is the fact that due to the although large but still limited sequencing depth relatively rare genes might be underrepresented in the results. Also, resistance genes from unculturable bacteria are probably underrepresented in the Resfinder database and therewith in our analysis.

Conclusions

This study applied metagenomics to establish associations between AMU and the resistome on European broiler farms. Clearly positive associations between corresponding antimicrobial usage and resistance genes were observed. Significant results were shown for both, flock-level and farm-level usage, highlighting that both actual and historic use can contribute to AMR presence. Our data did not support associations with ARGs and non-corresponding AMU or biosecurity status of the farm. We however show that the faecal microbiome harbours many resistance genes in the absence of current antimicrobial usage.

Data availability

The DNA sequences (reads) from 363 metagenomic samples from 359 herds are deposited in the European Nucleotide Archive under the project accession number PRJEB22062.

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Supplement of Associations between antimicrobial use and the faecal resistome on broiler farms from nine European countries

Laboratory analysis and bioinformatics

From each of the 25 individual samples equal amounts (0.5 gram) were collected to form a pooled sample. DNA was extracted from 0.2 gram of each faecal pool with the QIAamp Fast DNA stool mini kit, with a slightly modified protocol including an initial bead beating step, all at the same lab.[1] Samples were thawed only once before DNA extraction. Metagenomic deep sequencing was done at the Oklahoma Medical Research Foundation. The library preparation included a PCR amplification step (minimal amplification-based KAPA Hyper kit), because of low DNA concentrations (mean: 5.24 ng/ul, min: 1.24 ng/ul, max:28.6 ng/ul) in the poultry feces. DNA was sequenced on the Illumina HiSeq3000 platform, which resulted in 50 million paired-end reads per sample on average. The cleaned reads were mapped to the Resfinder database of AMR genes and to the NCBI RefSeq bacterial genome database, with at least 50 bp aligning from both forward and reverse reads and 80% similarity.

Biosecurity score

The Biocheck.Ugent consists of a risk-based weighted biosecurity scoring system and aims at objectively evaluating and comparing farms and regarding their biosecurity status. The total biosecurity is subdivided in 2 categories, external biosecurity and internal biosecurity with respectively 8 and 3 subcategories. These subcategories are composed of individual questions and each have a weight in the scoring which is based on the relative importance (risk-based). The external score accounts for 70% and the internal score for 30% of the total biosecurity score. [2] The website http://www.biocheck.ugent.be/ describes the current content of the scoring system in detail.

Interpretation of the results

The estimates of the associations shown in this paper are not intuitively interpretable for several reasons. The first is that we have standardized the outcome (mean is 0 and SD is 1) per country. This means that the estimate shows an increase or decrease in units of SD per model. Due to the fact that we report and conclude on the overall associations across countries and standardization is done per country, calculating the absolute increase or decrease is not possible. Another reason the results cannot be interpreted as an absolute association is the fact that the outcome (relative abundance of resistance genes in FPKM) is semi-quantitative. For these reasons we conclude on the direction (positive or negative) and significance of the association only.

Due to the large size, part of the supplement is not printed. Please go to the online available Excel-file with supplementary tables 1-5
https://doi.org/10.1093/jac/dkz235

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4

Farm dust resistomes and bacterial microbiomes in European poultry and pig farms

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Abstract

Background - Livestock farms are a reservoir of antimicrobial resistant bacteria from feces. Airborne dust-bound bacteria can spread across the barn and to the outdoor environment. Therefore, exposure to farm dust may be of concern for animals, farmers and neighboring residents. Although dust is a potential route of transmission, little is known about the resistome and bacterial microbiome of farm dust.

Objectives – We describe the resistome and bacterial microbiome of pig and poultry farm dust and their relation with animal feces resistomes and bacterial microbiomes, and on-farm antimicrobial usage (AMU). In addition, the relation between dust and farmers' stool resistomes was explored.

Methods - In the EFFORT-study, resistomes and bacterial microbiomes of indoor farm dust collected on Electrostatic Dust fall Collectors (EDCs), and animal feces of 35 conventional broiler and 44 farrow-to-finish pig farms from nine European countries were determined by shotgun metagenomic analysis. The analysis also included 79 stool samples from farmers working or living at 12 broiler and 19 pig farms and 46 human controls. Relative abundance of and variation in resistome and bacterial composition of farm dust was described and compared to animal feces and farmers' stool.

Results - The farm dust resistome contained a large variety of antimicrobial resistance genes (ARGs); more than the animal fecal resistome. For both poultry and pigs, composition of dust resistomes finds (partly) its origin in animal feces as dust resistomes correlated significantly with fecal resistomes. The dust bacterial microbiome also correlated significantly with the dust resistome composition. A positive association between AMU in animals on the farm and the total abundance of the dust resistome was found. Occupational exposure to pig farm dust or animal feces may contribute to farmers' resistomes, however no major shifts in farmers resistome towards feces or dust resistomes were found in this study.

Conclusion - Poultry and pig farm dust resistomes are rich and abundant and associated with the fecal resistome of the animals and the dust bacterial microbiome.

Introduction

Exposure to fecal antimicrobial resistance genes (ARGs) via dust is considered to be one of the routes of transmission of antimicrobial resistance from livestock to humans (McEachran, Blackwell et al. 2015, Li, Cao et al. 2018, Mbareche, Veillette et al. 2019). Intensive livestock farms are environments with a high load of bacteria combined with high selective antimicrobial pressure, a combination favoring the occurrence of resistant bacteria. Dust-bound resistant bacteria can become airborne and spread across the barn, and can be emitted via forced or natural ventilation to ambient air, exposing animals, farmers and neighboring residents and the surrounding environment (Casey, Kim et al. 2015, Woolhouse, Ward et al. 2015).

Dust sampling in air is time consuming, costly and often results in low total dust mass and DNA load. Nevertheless, some attempts to describe air resistomes are reported, often using different sampling techniques (Hu, Zhao et al. 2018, Li, Cao et al. 2018, Xie, Jin et al. 2019). In these, impact of geographical region, climate or air pollution on the aerial resistome have been shown. Also, the influence of livestock has been explored, and first reports show an elevated and distinct ARG abundance in farms compared to other places such as city residences or a waste-water-treatment plant (Yang, Zhou et al. 2018, Li, Liao et al. 2019).

In the farm, an important source of dust is animal feces (Cambra-López, Torres et al. 2011). Particles are continuously aerosolized, and this process is influenced by many factors like stable design, hygienic measures, ventilation, animal activity, type of feed and bedding material, and climate conditions (Basinas, Sigsgaard et al. 2013). Although the animal fecal resistome and bacterial microbiome have been described previously (Waite and Taylor 2014, Holman, Brunelle et al. 2017, Kers, Velkers et al. 2018, Munk, Knudsen et al. 2018), and some first attempts to describe the farm dust resistome and bacterial microbiome have thus been undertaken, studies including both dust and fecal samples and addressing their relation in the same environment across multiple farms are absent.

Farmers are exposed to the animal fecal and dust resistome on a daily basis either via hand-to-mouth contact resulting in ingestion or via inhalation. Correspondingly, studies have linked human carriage of specific resistant bacteria to aerial exposure (Bos, Verstappen et al. 2016, Dohmen, Schmitt et al. 2017). Exploration of the relation between resistomes of material (e.g. dust, animal feces) collected on farms and farmers' resistomes is, to the best of our knowledge, lacking. There have been some early studies on the bacterial microbiomes of the nasal or nasopharyngeal cavity of pig farmers and farm air. These are clustered, pointing to greater similarities between the compositions of the two as compared to other bacterial microbiomes such as dairy farmers or waste water treatment plant workers (Kraemer, Ramette et al. 2018, Mbareche, Veillette et al. 2019).

Antimicrobial resistance (AMR) is recognized as a problem which needs a One Health approach as the way to assess and tackle the problems that arise from the presence of antimicrobial resistant bacteria (Robinson, Bu et al. 2016). Within the European EFFORT

(Ecology from Farm to Fork Of microbial drug Resistance and Transmission) project, the animal resistome (Munk, Knudsen et al. 2018) and farmers' resistome and bacterial microbiome (Van Gompel, Luiken et al. 2020) have been described. The present study aims to address the resistome and bacterial microbiome of airborne dust, as determined by shotgun metagenomic sequencing. Specifically, we describe the dust resistome and bacterial microbiome, compare it with the poultry and pig fecal resistome and bacterial microbiome, and associate it with on-farm antimicrobial usage (AMU) in poultry and pig farms from nine European countries. In addition, the relation between the resistomes of dust, animal feces, and farmers' stool on Dutch farms is explored.

Materials and methods

Study design and farm population

In a cross-sectional study, conventional broiler farms and integrated farrow-to-finish pig farms were visited between 2014 and 2016 in nine European countries (Belgium, Bulgaria, Denmark, France, Germany, Italy, the Netherlands, Poland and Spain). In each country, three farms per animal species were sampled (animal feces and farm dust samples), except for one country that sampled two poultry farms and four pig farms instead (country 5). For an in-depth analysis on Dutch farms that included animal feces and farm dust samples, as well as human stool samples from the same farm, 12 poultry farms and 19 pig farms were sampled. This resulted in samples from 35 poultry farms and 44 pig farms for the present study. The current study represents a sub-selection of farms from the EFFORT study in which 20 farms were included per country. The selection of farms was described before (Munk, Knudsen et al. 2018). The most important inclusion criteria for all farms were: no other animals for production present at the farm, and all-in all-out production (for pigs at fattening compartment level and for poultry at stable level). All farms have been anonymized to ensure that results cannot be traced back to individual farms. Country was anonymized as this was required by the farming organization in one participating country, with one exception for country 1 = the Netherlands.

An overview of the number of included samples can be found in supplemental table 1.

Farm dust collection

During farm visits indoor farm dust was collected by use of Electrostatic Dust fall Collectors (EDCs) (Noss, Wouters et al. 2008) from compartments with broilers or fattening pigs close to slaughter age. The electrostatic cloths were sterilized and gamma irradiated (50 kGy) to remove possible bacterial contamination before the EDCs were assembled and packed in re-sealable bags. Per farm three EDCs were horizontally placed in the compartment at a height of about 150 cm above the ground, at a location were the air has already passed over the animals, distant from heating or cooling systems. For poultry the compartment consisted of one stable with animals close to slaughter age. For pigs all (with a maximum of four) compartments with pigs close to slaughter age were sampled. The farmer was asked to collect

and ship the EDCs after minimally 2 and maximally 7 days in the compartments, the latest before thinning or removing the animals for slaughter. The farmer packed the EDCs and sent them by regular mail to one central lab, from nine countries this took on average 11 days (10^{th}-90^{th} perc.: 2-25 days). One sample that consisted of DNA pooled from three separate DNA extracts extracted from the three samples taken at each farm was included in this study.

Blank samples were taken during the sampling period and consisted of unopened EDCs in a sealable bag which remained at randomly selected farms across three countries for the same time that EDCs were in the barn. The blanks were shipped together with the used EDCs and were processed in the same way as the other samples. In total six blanks were analyzed.

Animal feces collection

During the farm visits, 25 fecal samples were collected from animals in the same compartment(s) as the EDC's. Fresh fecal droppings were collected from the floor from one flock while walking through the whole stable (poultry) or from the floor of as many pens as possible in the fattening compartments or by catching feces while defecating (pigs), to ensure samples came from different animals and were roughly equally distributed over the compartment(s and pens). These samples were immediately cooled at 4°C and transported to the local lab where they were processed and frozen within 24 hours at -80°C (alternatively at -20°C for a maximum of 4 days, before transferring to −80°C). DNA extracted from one pool of the 25 samples was included in this study. From one Dutch pig farm there was no animal fecal sample available for analysis. More details on the feces sampling has been described before (Munk, Knudsen et al. 2018).

Farmers and control population and stool collection

Data collection among farmer and control populations are described in detail elsewhere (Van Gompel, Luiken et al. 2020). At all Dutch farms, farmers, partners, family members and workers (further addressed as 'farmers') were invited to participate in the study. A fresh stool sample of consenting farmers, was collected by self-sampling as close as possible to the collection by the researchers. All samples from adults (18 years and older) were included in the study. This resulted in 25 stool samples from 12 poultry farms and 54 stool samples from 19 pig farms. One stool sample from a poultry farm was removed for technical reasons. Stool samples were frozen at -20°C immediately after collection and transported to the lab on dry ice and were further processed following a single thaw cycle. As control, a total of 46 human stool samples were selected from the Dutch Lifelines Cohort Study (Stolk, Rosmalen et al. 2008). The most important inclusion criteria for control subjects were: 18 years and older, not living or working on a farm and no AMU or hospitalization in the 3 months prior to the sample collection. These samples were processed in the same way as the farmers' stool samples.

DNA extraction and library preparation of farm dust

All EDCs were processed centrally. After arrival at the lab the envelope was stored for maximally 6 days, subsequently opened in a flow cabinet and electrostatic cloths were

removed from the folder, folded and put into a small re-sealable bag with sterile tweezers and frozen at -80°C. Directly before DNA extraction, cloths were thawed, washed in sterile 0.05% Tween20 water (for better dust yield) and blended with a stomacher. Thereafter the remaining fluid was frozen in plastic tubes at -20°C, subsequently freeze dried for 3-4 days and the remaining material was stored again at -20°C. After thawing, the dust was weighed and 35 mg (+/- 1 mg) was collected for DNA extraction. From each dust sample, DNA was extracted using the Nucleospin 8 plant II kit (Machery-Nagel) using the standard protocol with an additional bead-beating step (30 sec at 5.5G with Fastprep-24). DNA of three EDC's of each farm were pooled for metagenomic analysis in an equi-volume manner and stored at -80°C until further processing.

Due to relatively low DNA yields (mean total dust DNA weight poultry = 11.7 ng and pig = 26 ng) amplification-free library preparation was not possible. Minimal (3) amplification cycles for library preparation (Kapa Hyper Prep Kit, Kapa Biosystems) were used according to manufacturer procedures. If the library yield was still insufficient for sequencing then a minimum number of cycles were added (up to max 10). The low levels of amplification are known to introduce minimal bias if any (https://sequencing.roche.com/en/products-solutions/by-category/library-preparation/dna-library-preparation/kapa-hyperprep.html).

Total number of bacterial hits of pig blank EDC samples unexposed to dust was 16 to 240 times lower than pig farm dust samples, for poultry this was 11 to 73 times lower for two blank samples. One poultry blank sample had a similar amount of total bacterial hits as the lower poultry farm dust samples (results not shown).

DNA extraction and library preparation of animal feces and farmers' and controls stool

DNA extraction and library preparation of animal fecal and human stool samples is described in short in the supplement.

Bioinformatics processing

Bioinformatic processing is described in short in the supplement. Resistome data was explored at two levels, clustered at a 90% identity level (named 'resistance gene' in this paper) and clustered per antimicrobial class (named 'AM class' in this paper) similar as for previous published work (Munk, Knudsen et al. 2018). Bacterial microbiome data was explored at bacterial class level (named 'bacterial class' in this paper).

Of these, four randomly selected poultry and pig dust samples were further explored to get more insight in the unclassified (i.e. nonbacterial) genes. The unclassified reads from the used pipeline were annotated by BLAST against the non-redundant nucleotide database at NCBI.

Collection of meta-data

Additional information on the farm was collected with the use of standardized field forms. Farm antimicrobial usage (AMU) data were collected through a questionnaire by interviewing the farmer on the day of the visit and/or through the veterinarian as described previously

(Joosten, Sarrazin et al. 2019, Sarrazin, Joosten et al. 2019). AMU was expressed as Treatment Incidence of Defined Daily Dosages (TI_{DDDvet}) of either group treatments of the sampled animals or purchased products by the whole farm in the year before sampling. Additional information about the farmers, e.g. age, job type and work hours on the farm, was collected through a personal questionnaire filled out by the participant her/himself.

Data analysis

The data analyses were performed in R (version 3.4.3) (R-Core-Team 2017). All analyses were done across all included countries, unless indicated otherwise. For all ARG-based analysis, Fragments Per Kilobase ARG-reference per Million bacterial fragments (FPKM) results were used and for bacterial class count-based analysis, genome-length-corrected-counts per million, which subsequently were divided by the sum of abundances for compositional analysis.

We performed our analyses in the following sequence. Firstly, resistome and bacterial microbiome composition of poultry and pig farm dust samples were described and compared with these of animal fecal samples. Secondly, associations between dust resistomes and AMU were explored. Thirdly, for the in-depth analysis in the Netherlands that included human samples from the same farms, the relation between the farm (dust and animal fecal) resistome and farmers' stool resistome was explored and compared with human controls.

Resistome and bacterial microbiome composition analysis

To visualize the (dis)similarities in sample resistome and bacterial microbiome compositions, Non-Metric Dimensional Scaling (NMDS) was performed. NMDS ordinations (in two dimensions) were calculated from a Bray-Curtis dissimilarity matrix after square root transformation and Double Wisconsin standardization (R vegan function metaMDS). For all NMDS analyses described in this paper, stress levels were below 0.2. To test the effect of determinants (such as animal species, country or sample type), Permutational Multivariate Analysis of Variance (PERMANOVA) including checks on homogeneity of dispersion was employed (R vegan functions adonis, betadisper).

Procrustes analyses were performed to determine symmetric rotation correlation between individual NMDS ordinations of resistome and bacterial microbiome compositions and/or different types of samples (e.g. animal feces vs farm dust) (R vegan functions procrustes and protest). In case of multiple farmer stool samples, farmers (which could technically be either the main farmer or a family member that works on the farm) with the greatest exposure, i.e. most working hours in the farm per week, were chosen for the analysis.

Visualization of the resistome and bacterial microbiome

Total relative abundance of the resistome was computed and visualized in boxplots. Relative abundances of resistance genes clustered per AM class as percentage of the total of resistance genes were computed and visualized per farm and per country with stacked bar

charts. The same was done for the abundance of bacteria. The abundance of ARGs in farm dust and animal feces was visualized with heatmaps with clustering of samples on the Bray-Curtis dissimilarity index. Alpha diversity (i.e. Richness and Evenness) was calculated for all samples after rarefaction and visualized in boxplots. Resistome data was rarefied by subsampling the data proportionally to the bacterial content per sample as follows: A rarefaction cut-off for the bacterial read counts was chosen such that at least 95% of the samples were preserved. Subsequently the relative subsampling rates between samples for bacterial counts were applied to each of the resistome per sample counts since the resistome per definition is measured as a fraction of the bacterial microbiome. Total presence, shared and unique ARGs between the different sample types were counted for the Dutch farms n = 11 for poultry or n = 18 for pigs, for which all sample types were available after rarefaction, and visualized binarily (i.e. based on absence/presence) in a Venn-diagram. For fair comparison with the control group we randomly selected a sub sample of all controls to match the number of farms included in the analysis (11 or 18).

Association with AMU

To explore the relationship between AMU and the dust resistome, linear regression was performed between total AMU and total AMR. AMR was expressed as the total of resistance genes in FPKM. AMU was expressed for broilers as total TI_{DDDvet} of group treatments or purchased products and for pigs as the total TI_{DDDvet} of group treatments or purchased products for either the group of (sampled) fatteners or for a standardized lifespan of 200 days. AMU data was log10(x+1) transformed and AMR data was log10 transformed before modeling and regression across all countries. The relation was explored with and without adjustment for the overall abundance of ARGs in animal feces.

Results

The composition of the farm dust resistome

This study included 79 farms with an average size of 77944 chickens (10-90th perc.: 28840-148400) or 5071 pigs (10-90th perc.: 1682-9339). Total AMR levels in dust of poultry and pig farms were similar and had means of 3,045 and 3,168 FPKM, respectively. This is in contrast to the total levels of ARGs in poultry and pig feces, with poultry feces having mostly lower values than farm dust and pig feces having mostly higher levels than farm dust (Figure 1).

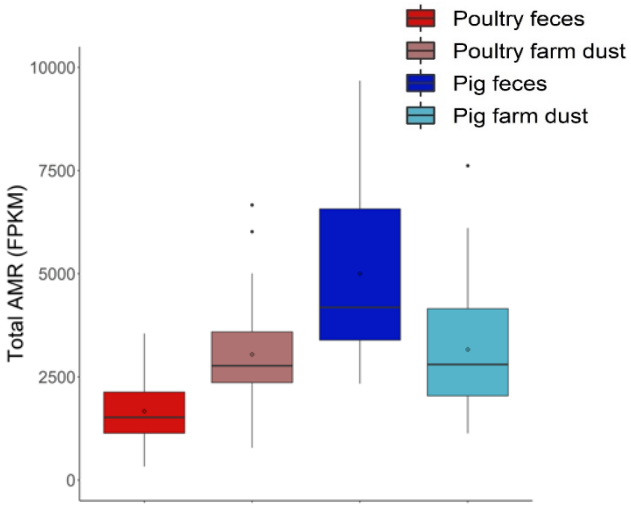

Figure 1 – Total AMR levels of farm dust and animal feces.
Boxplots for 35 poultry farms and 43 (feces) or 44 (dust) pig farms from nine countries. The horizontal line in the boxplots depicts the median, the empty circle the mean.

The resistome composition shows significantly distinct clusters of dust and feces for the two animal species (Figure 2). Pig and poultry dust resistomes both cluster closer to feces from their respective species. Pig and poultry dust bacterial microbiomes cluster less distinctly than dust resistomes (Figure 2), although for both dust resistomes and bacterial microbiomes the variance explained by species is 25% ($p < 0.05$, beta-dispersion $p > 0.05$).

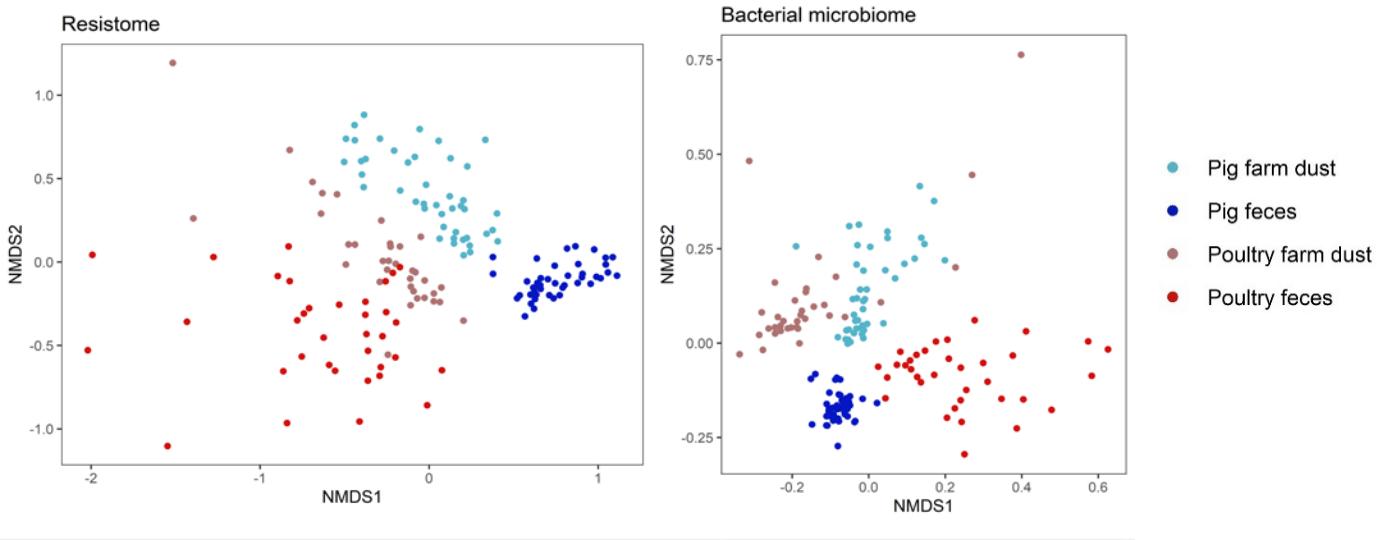

Figure 2 - Compositional differences of the resistomes and bacterial microbiomes of farm dust and animal feces.

NMDS plots of 35 poultry and 44 pig farms from nine countries. PERMANOVA results for comparison of dust and feces; Resistome: poultry, $R^2 = 0.19$, $p = <0.001$, beta dispersion $p = 0.012$. Pig, $R^2 = 0.44$, $p = <0.001$, beta-dispersion $p = <0.001$. Bacterial microbiome: poultry, $R^2 = 0.33$, $p = <0.001$, beta-dispersion $p = 0.50$. Pigs, $R^2 = 0.42$, $p = <0.001$, beta-dispersion $p = 0.05$. For 3 of 4 tests the assumption of homogeneity of variance was not met which may partly explain PERMANOVA results.

Poultry and pig farm dust resistomes showed many similarities at AM class level (Supplemental figure 1). Both were dominated by genes encoding for resistance to tetracyclines, aminoglycosides and macrolides, but a larger proportion of tetracycline resistance was present in pig farm dust. Beta-lactam resistance genes were relatively less abundant in farm dust compared to feces. Inspection of the heatmaps (Supplemental figure 2) showed that distinction between dust and feces was driven by genes from all classes with for example in poultry dust more *dfrD, tetK* and *str* genes in dust than in feces. In pig farm dust, many resistance genes are moderately abundant, while in pig feces fewer genes are highly abundant. This is confirmed by the Richness and Evenness calculations (Supplemental figure 3). The bacterial microbiome analysis also showed that the distribution of bacterial classes in poultry and pig dust is more similar than in pig and poultry feces (Supplemental figure 4). An increase in the proportion of Clostridia was seen in poultry dust compared to poultry feces, while Bacilli took up a large proportion in both sample types. For pigs, Bacteroidia had a much smaller and Bacilli and Betaproteobacteria a much larger contribution to the dust bacterial microbiome than to the feces bacterial microbiome. Pig feces samples

from all farms were less diverse in its bacterial composition, similar to its resistome composition.

For poultry farms no differences existed between country specific dust resistomes, for pig farm dust the differences were statistically significant but explained very little variation (R^2 = 0.067 p = 0.002 , beta-dispersion p=0.07). Therefore, subsequent analyses were performed across countries.

The effect of the fecal resistome, dust bacterial microbiome and AMU

The resistome composition of farm dust was significantly correlated with the resistome composition of animal feces across all countries and farms (correlation coefficient 0.49 (p < 0.001) for poultry and 0.65 (p < 0.001) for pigs) (Table 1A, figure 3 and supplemental figure 5). Dust bacterial microbiomes and resistomes were also significantly correlated in both poultry and pig farms, indicating that dust samples with a similar bacterial composition have a more similar resistome (correlation coefficient 0.65 (p < 0.001) for poultry and 0.50 (p = 0.001) for pigs) (Table 1B, figure 3 and supplemental figure 5). In contrast, dust bacterial microbiomes of pig farms were less strongly correlated with fecal bacterial microbiomes, in poultry farming there was no (significant) correlation.

Table 1 – Results of Procrustes correlation analysis

*Table displays the symmetric Procrustes correlation coefficient (cor), significance level (p-value), the number of countries and farms included in the analysis (n countries, n farms). Bold results have a p-value below 0.05. *correlations are plotted in figure 3. ^stress nearly zero, probably due to a too small sample size*

				Poultry			Pig		
		n countries		*cor.*	*p-value*	*n farms*	*cor*	*p-value*	*n farms*
A)	Animal feces - farm dust	*9*	*Resistome*	**0.49***	**<0.001**	35	**0.65***	**<0.001**	43
		9	*Bacterial microbiome*	0.14	0.76	35	**0.34**	**0.02**	43
B)	Bacterial microbiome dust - resistome dust	9		**0.65***	**<0.001**	35	**0.43***	**<0.001**	44
C)	Animal feces - farmer stool	*1*	*Resistome*	0.49	0.12	12	0.31	0.34	18
		1	*Bacterial microbiome*	0.41	0.28	12	0.21	0.71	18
	Farm dust - farmer stool	*1*	*Resistome*	0.03	0.98	12	0.39	0.11	19
		1	*Bacterial microbiome*	NMDS dust not possible^			0.25	0.54	19

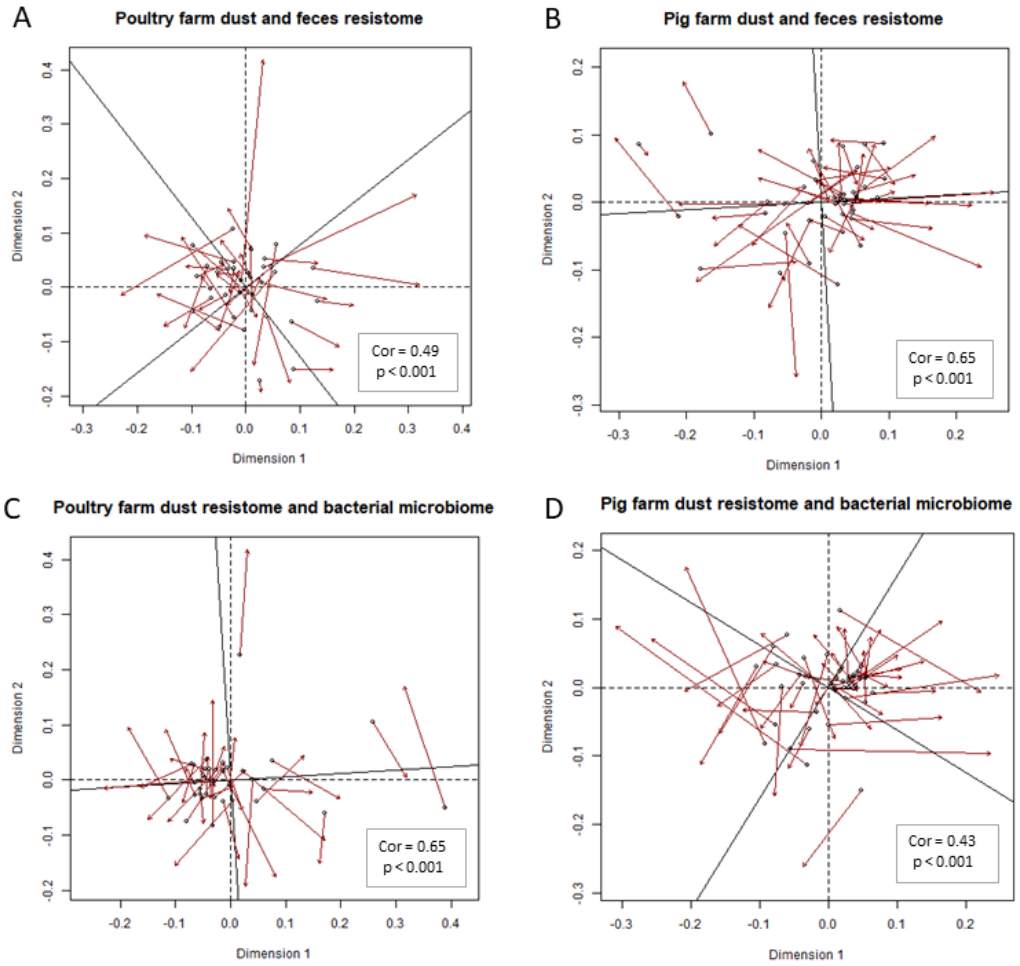

Figure 3 – Correlation between fecal and dust resistome and between dust bacterial microbiome and resistome.

Upper: Superimposition plots of Procrustes correlation of feces and farm dust resistomes of poultry (A) and pig (B) farms. Arrowheads point towards the dust ordination.

Lower: Superimposition plots of Procrustes correlation of dust bacterial microbiomes and resistomes of poultry (C) and pig (D) farms. Arrowheads point towards the resistome ordination.

Corresponding Procrustes error plots in supplemental figure 5. Corresponding coefficients in the boxed text and Table 1.

While only a part of the farms used antibiotics during the life span of the sampled animals, we found, for both poultry and pig farms, a significant positive association between AMU in the animals and AMR in dust for both poultry and pig farms, from the same stables/compartments (Figure 4). This association is likely greatly mediated through the association between AMU and AMR levels in feces. For both pigs and poultry the strength of the association of AMU and the dust resistome decreased while including resistome levels in feces, but the association was no longer significant (Supplemental table 2)."

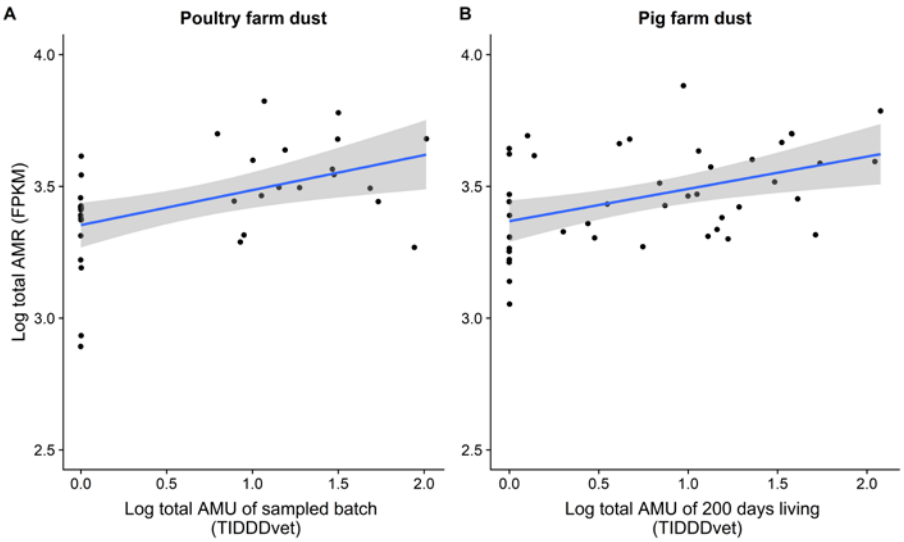

Figure 4 – Relation between total AMU and total AMR in farm dust.
A) Scatterplot for poultry farm dust. AMU = total group treatments of sampled chickens, coefficient = 0.13, p = 0.004 in non-adjusted model. B) Scatterplot for pig farm dust. AMU = total group treatments of pigs in standardized 200 days, coefficient = 0.12, p = 0.003 in non-adjusted model.

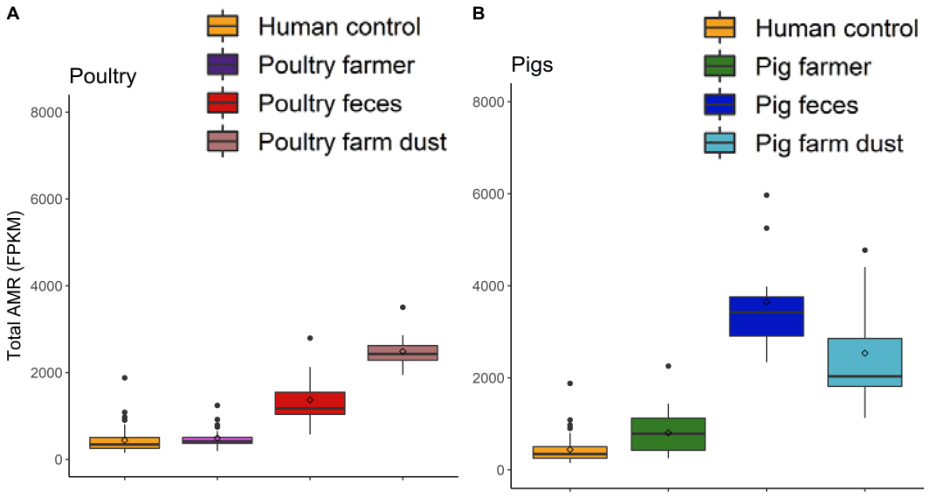

Figure 5 - Total AMR levels of farm dust, animals, farmers and controls.
Boxplots for 12 poultry farms (A) and 19 pig farms (B) from one country (the Netherlands). The horizontal line in the boxplots depicts the median, the empty circle the mean.

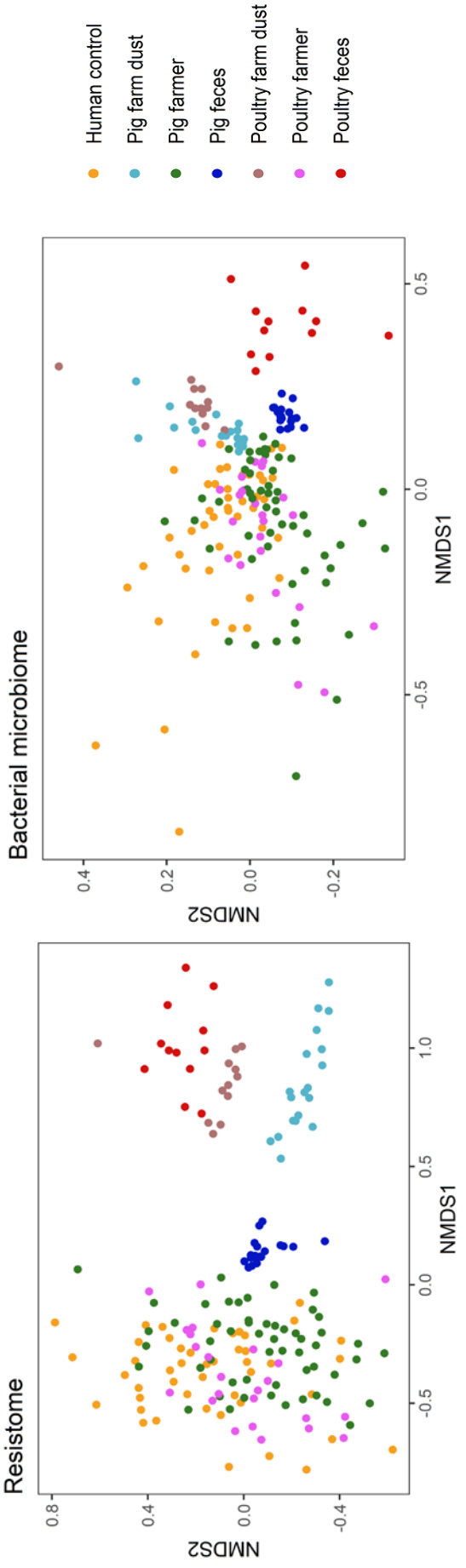

Figure 6 – Compositional differences of the resistomes and bacterial microbiomes of farm dust, animals, farmers and controls.
NMDS plots of all samples from the 12 Dutch poultry and 19 Dutch pig farms plus 46 human controls.

The relation between farm dust, animal feces and farmers

Human stool of either poultry and pig farmers or controls have less abundant resistomes compared to farm dust and animal feces (Figure 5). Clear clusters consisting of farm dust and animal feces per animal species and a clear human cluster which included all farmers and controls were observed using NMDS analysis (Figure 6).

The bacterial microbiome composition of farmers, human controls, animal feces and farm dust shows less distinct clustering, in particular poultry and pig farm dust (Figure 6). Bacterial microbiomes of the different human groups overlap even more than the resistomes and are close to or even overlap with dust clusters and the pig feces cluster. Differences in bacterial microbiome composition between human stool and poultry feces concern, among others, the proportion of Bacilli (large in poultry, small in farmers) and Bacteroidia (small in poultry, large in farmers) (Supplemental figure 4). The resistomes of farmers and controls consist of a much larger share of beta-lactam genes than the farm sources do (Supplemental figure 1) and are relatively less rich, as is pig feces compared to poultry feces and pig and poultry dust (Supplemental figure 3).

Correlation (Procrustes) analyses showed low to moderate correlations between farm (dust and animal feces) and farmers' stool resistome and bacterial microbiome compositions within each farm type; however they were not significant.

The majority of all resistance genes was found to be shared between animal, human and environmental samples, all from one country (Figure 7). For these analyses, one farmer was included per farm that had most working hours per week in the stables. Dust had the highest number of different resistance genes (i.e. highest richness) and the largest 'unique gene pool': of all dust resistance genes 20% (38/186, poultry) and 26% (49/186, pig) were not found in other sample types included in the study. These unique dust genes code for resistance to a variety of AM classes and have a moderate to low abundance. Examples consist of the *cfr* gene, coding for multi-resistance, and the *blaBRO* gene, coding for beta-lactam resistance, which were measured in dust but not in pig feces nor farmers' or controls' stool (supplemental table 3).

We conducted an exploratory analysis of unclassified reads to identify potential other sources than feces in a random subset of dust samples (data not shown). These unclassified reads were shown to be mainly linked to feed sources (e.g. wheat, barley, maize and grasses), hosts (poultry and pigs), other mammals (e.g. sheep and horses) and fungi.

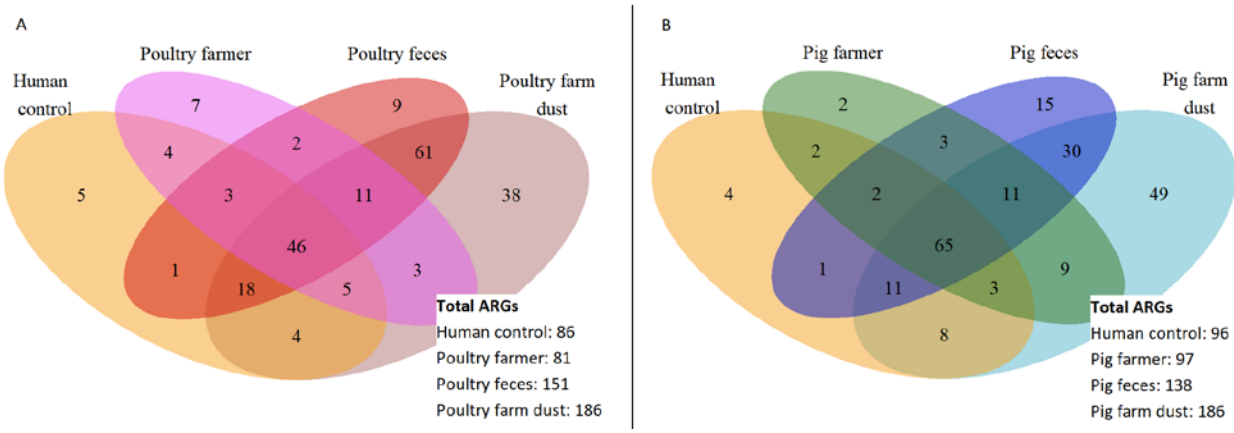

Figure 7 – Overlap in resistomes of the different farm sources and human controls.
A – Venn-diagram of 11 Dutch poultry farms, including 11 controls. B – Venn-diagram of 18 Dutch pig farms, including 18 controls. Supplemental table 3 lists the individual resistance genes per sample type per animal species.

Discussion

This study describes the abundance and diversity of 'the resistome' of farm dust in relation to that of animal and farmer feces from poultry (broilers) and pig farms (fatteners) from nine European countries. We discovered that resistome compositions are more similar between dust and feces samples from the same animal species, both on AM class level as on gene level. In addition, the composition of dust resistomes is correlated with underlying dust bacterial microbiomes, and farms with higher AMU have more abundant dust resistomes. Lastly, farm dust exposure may have an effect on the farmers' resistome, however this was not reflected in significant changes in the total resistome (nor bacterial microbiome) studied here.

Farm dust and its relation with feces

To the best of our knowledge, the farm air resistome has only been studied by Yang et al.(Yang, Zhou et al. 2018) and Li et al. (Li, Liao et al. 2019), showing that airborne dust in Chinese pig and chicken (laying hens) farms has a high diversity of ARGs compared to a waste water treatment plant, hospital or urban areas. In agreement with these findings, the dust resistome in this study was also found to have the largest richness of ARGs of all sample types (Supplemental figure 3). Both pig and poultry farm dust showed 186 different ARGs (after rarefaction), twice as many as farmers' stool and pig feces (results for one country). Part of the dust resistome probably originates from animal feces: 63% and 73% of dust-borne resistance genes are also detected in animal feces from their respective pig and poultry farms (Figure 7). Also, patterns of fecal and dust resistomes between farms are significantly correlated for both poultry and pig farms (Table 1). Correlation analysis does not inform on directionality of associations, however it is likely that the fecal resistome determines the dust resistome because aerosolization of dried feces results in airborne dust (Cambra-López,

Torres et al. 2011, Winkel 2016). In turn, dust exposure might also alter animal fecal resistomes.

On the other hand, the higher resistome richness and the large group of non-overlapping ARGs in dust suggest a substantial contribution of microbial sources other than animal feces. Animals and their non-fecal microbiota, such as bacteria stemming from skin, saliva, hairs and feathers, are potential sources, as has been hypothesized before (Vestergaard, Holst et al. 2018). For example, Strube et al. (Strube, Hansen et al. 2018) showed a large share of *Lactobacillus* and *Aerococcus* in the pig nose and on its skin, which could, after shedding and potential aerosolization, explain the increased share of Bacilli in pig farm dust. In addition, feed represents an important source of farm dust (Cambra-López, Torres et al. 2011, Winkel 2016) of which genetic signatures (e.g. barley, wheat, carp) have been found in dust samples in this study as well. Resistance genes might thus also stem from (traces of) feed-associated bacteria. Other sources might be (other) animals around the stable (e.g. sheep DNA has been identified) or soil. Soil microbiomes vary a lot between locations, it is therefore difficult to assign specific taxa to possible soil origin (Fierer 2017). All these sources potentially carry specific bacteria and probably ARGs and can explain the many other dust-specific ARGs and species we have found in our samples.

The abundant bacterial classes seen in our pig and poultry farm dust samples are consistent with previous studies on farm air, although with a different distribution (Vestergaard, Holst et al. 2018, Yang, Zhou et al. 2018, Mbareche, Veillette et al. 2019). The significant correlation for both animal species between the dust bacterial microbiome and resistome indicates that the composition of a dust bacterial microbiome mediates the composition of the resistome. The same has been shown in other environments such as pig and poultry feces, human stool and soils (Forsberg, Patel et al. 2014, Pehrsson, Tsukayama et al. 2016, Munk, Knudsen et al. 2018).

The role of antimicrobial usage for resistance in dust

Farms on which more antimicrobials are used in the sampled animals have a higher relative abundance of ARGs in indoor farm dust collected in the same compartments. This effect is likely to be largely mediated through AMR levels in feces of the animals. Indeed, positive associations between AMU and abundance of resistance genes in animal feces, determined with metagenomic analysis, has been shown before in a larger study including the same farms (Luiken, Van Gompel et al. 2019, Van Gompel, Luiken et al. 2019). Similar significant associations were found with AMU expressed as purchased products by the whole farm in the year before sampling. This AMU data might resemble more overall farm historic treatment patterns. The historic use of antimicrobials and the presence of residues can possibly affect the development and spread of ARGs and resistant bacteria not only within the treated animals themselves but also in the farm environment, as was also already suggested by others (Larsson, Andremont et al. 2018, Filippitzi, Devreese et al. 2019). Associations between ARGs in dust and historic use are not maintained after correction for fecal ARGs however. Thus, the

association between feces and dust might be so strong that it is difficult to conclude whether AMU has an effect on ARGs in dust additional to the effect of feces.

The relation between farm and farmers' resistomes

Pig farmers showed an increased resistome abundance compared to control subjects, this was not seen for broiler farmers. No significant correlations were found between farm (dust and animal feces) and farmers resistome or bacterial microbiome composition when analyzed within the pig and poultry domain. Van Gompel et al. (Van Gompel, Luiken et al. 2020) demonstrated resistome dissimilarities between pig and pork exposed workers (i.e. farmers and slaughterhouse workers), and broiler farmers and control subjects. Moreover, the number of on-farm working hours and living or working on a pig versus broiler farm was found to be positively associated with resistome abundance. Although our analysis of the resistome composition did not result in significant correlations, studies based on classical detection methods have indicated transmission of resistant bacteria from pigs to farmers via air/dust. (Bos, Verstappen et al. 2016, Dohmen, Schmitt et al. 2017) Thus, while farmers are exposed to farm dust and animal feces as shown in previous studies, possible effects of this exposure in terms of an overall change of the total resistome or bacterial microbiome composition within the studied populations could not be observed here. Both the small sample size and the complexity of this possible relation are possible reasons. Exposure to the farm air resistome goes beyond those who live and work on a farm, as it has been shown that the abundance of certain ARGs in air near homes is related to the number of farms in the vicinity (de Rooij, Hoek et al. 2019). There is however little evidence for airborne transmission to humans around farms, as only a small effect on MRSA (Methicillin resistant Staphylococcus aureus) carriage in the nose (Zomer, Wielders et al. 2017) and no increase in ESBL (Extended-Spectrum β-Lactamases) carriage in stool was found in residents in the proximity of animal farms (Wielders, van Hoek et al. 2016).

Study limitations

This study is unique in combining high quality data from three different reservoirs, two animal species and nine countries. The inevitable consequence consists of differences in sample processing and DNA extraction between reservoirs, and a relatively small sample size. Confirmation of the overlap and differences we observe between the different farm reservoirs is therefore needed. The bacterial hits seen in blanks can be related by several factors, we however find it most likely to be related to a small degree of cross-contamination during freeze drying, results of the samples were therefore not corrected. While human health hazards are expected to be predominantly determined by the presence of combinations of ARGs in specific pathogens (Bengtsson-Palme, Kristiansson et al. 2018), we investigated only ARG distributions. With the short read sequencing methods applied, it was not feasible to determine the bacterial context of ARGs nor their relation with mobile genetic elements which could facilitate their spread between species (von Wintersdorff, Penders et al. 2016). Arguably, the transmission of genes and bacteria between different hosts and the

environment is complex and therefore difficult to disentangle in a cross-sectional design. To better understand transmission of genes between hosts and environmental reservoirs, a longitudinal design with greater sample size is preferred, and/or other methods like Whole Genome Sequencing of bacterial isolates or long read sequencing can shed light on transmission and the role of mobile genetic elements for resistance gene mobility.

Conclusions

In conclusion, the results provide new insights in the resistome and bacterial microbiome of the farm environment characterized by a high antimicrobial selective pressure (Larsson, Andremont et al. 2018). The farm dust resistome from European poultry and pig farms is equally or more abundant and rich than the resistome of poultry and pig feces and farmers. The farm dust resistome is clearly, but not only, determined by the animal fecal resistome from the animals in the same stable and by the underlying farm dust bacterial microbiome. The higher the antimicrobial usage on the farm, the more abundant is the farm dust resistome. Farm dust exposure may have an effect on the farmers' resistome, however this was not reflected in significant changes in the total resistome (nor bacterial microbiome) studied here.

Data availability

Shot-gun metagenomic DNA sequence reads of the dust samples are deposited at National Center for Biotechnology Information (NCBI) under BioProject number: PRJNA623064. The reads of the animal fecal samples are deposited at the European Bioinformatics Institute Nucleotide Archive (ENA) under accession number: PRJEB22062.

The DNA sequence reads from the human samples are deposited in the European Nucleotide Archive (EGA) under project accession number: S00001003944. Access to the metadata from the control group ('Lifelines' cohort: e.g. age, gender, antimicrobial use, animal contact) was purchased from Lifelines for a period of 6 months (data access agreement OV19_0483) and can only be retrieved through www.lifelines.nl/researcher. This excludes the variable gender which can be retrieved through the EGA repository.

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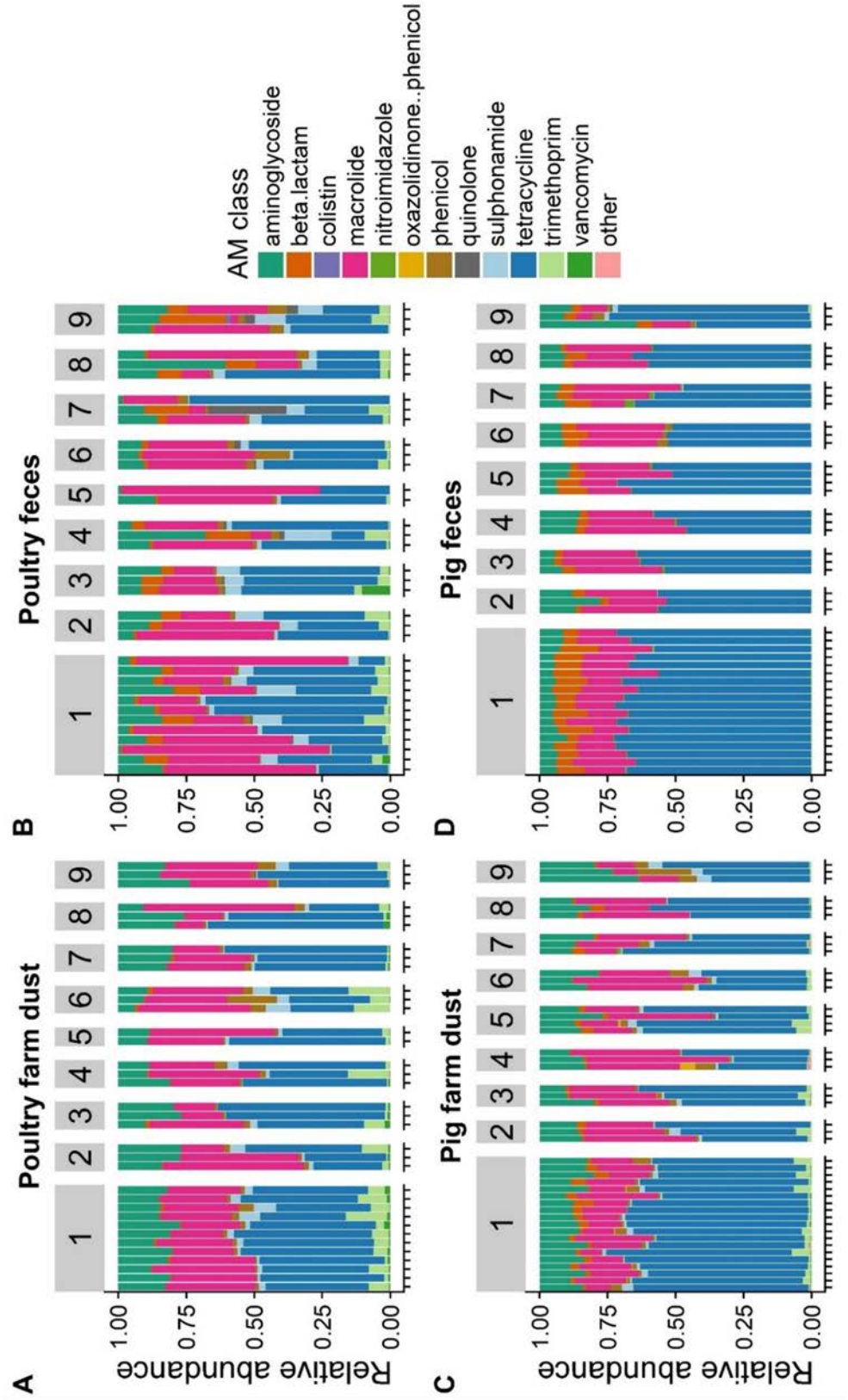
Supplement of Farm dust resistomes and bacterial microbiomes in European poultry and pig farms

Supplemental table 1 - Overview of number of samples included in the study

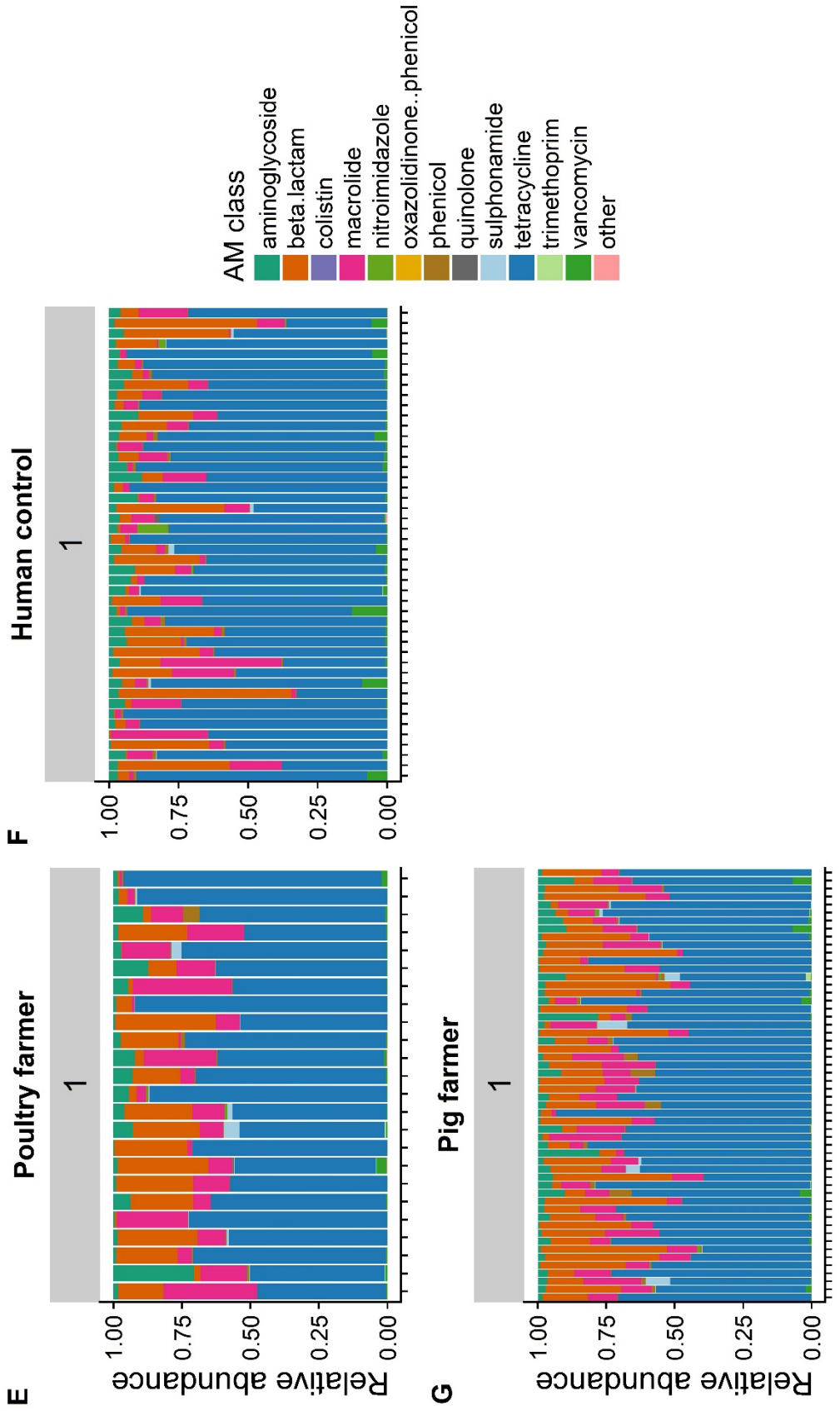
This table gives an overview of the number of samples included in the study described in the paper. Between brackets the number of farms the samples originate from. For one country, the Netherlands (NL), numbers are displayed in a separate row. The other eight countries are summed and in displayed in one row.

	Poultry			Pig					Total samples
Country	Farm dust samples (number of farms)	Animal feces samples (number of farms)	Farmers stool samples (number of farms)	Farm dust samples (number of farms)	Animal feces samples (number of farms)	Farmers stool samples (number of farms)	Control stool samples	Dust blanks	
NL	12 (12)	12 (12)	24 (12)	19 (19)	18 (19)	54 (19)	46	2	187
8 other countries	23 (23)	23 (23)		25 (25)	25 (25)			4	
Total samples (total farms)	35 (35)	35 (35)	24 (12)	44 (44)	43 (44)	54 (19)	46	6	287

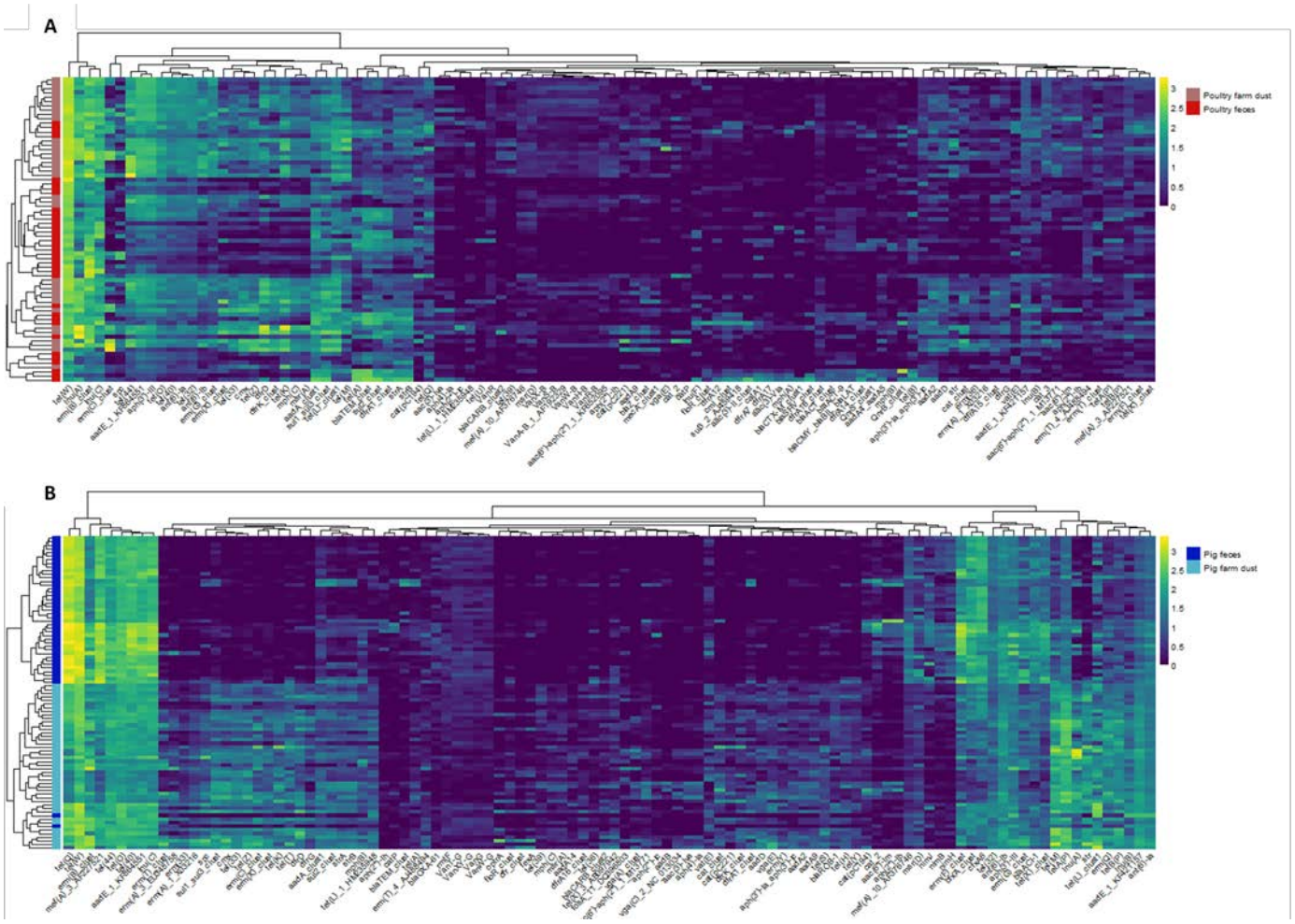


Supplemental figure 1 – Relative abundance of genes encoding for the top-12 most abundant antimicrobial classes in farm dust, animal feces and farmers' and control stool samples.

Barcharts of poultry farm dust (A) and feces (B) of 35 farms and pig farm dust (C) and feces (D) of 44 farms from nine countries (1-9). Results for poultry farmers (E), pig farmers (G) and human controls (F) from one country (country 1 = the Netherlands) (Van Gompel et al. 2020).

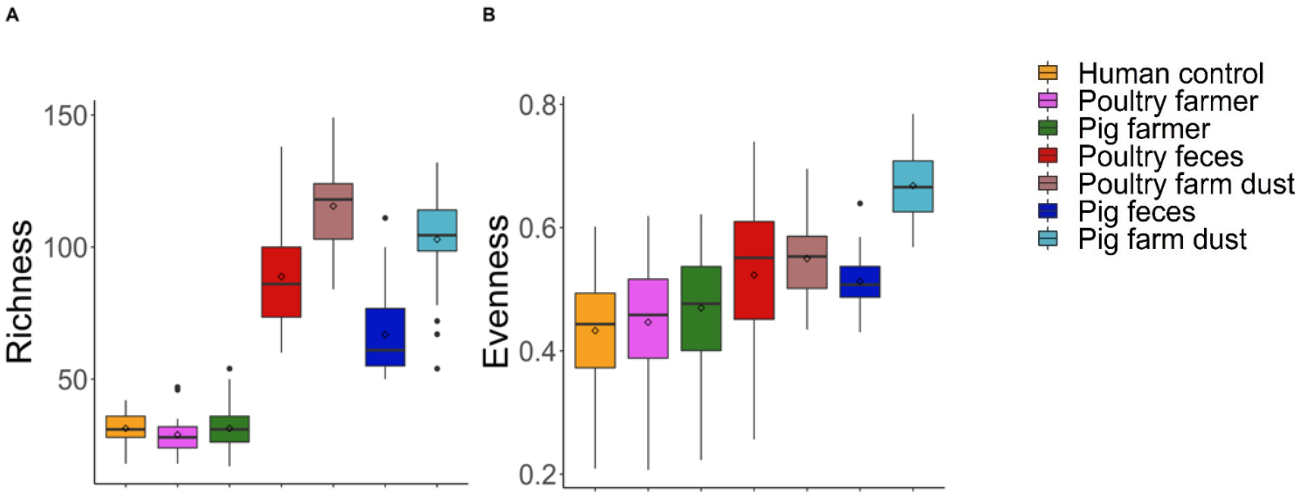

E
Poultry farmer
1
Relative abundance
1.00
0.75
0.50
0.25
0.00
F
Human control
1
1.00
0.75
0.50
0.25
0.00
G
Pig farmer
1
Relative abundance
1.00
0.75
0.50
0.25
0.00
AM class
aminoglycoside
beta.lactam
colistin
macrolide
nitroimidazole
oxazolidinone..phenicol
phenicol
quinolone
sulphonamide
tetracycline
trimethoprim
vancomycin
other

Supplemental figure 2 – Abundance of the ~100 most abundant AMR genes in farm dust and animal feces.
Heatmap for 35 poultry (A) and 43 pig (B) farms from nine countries. Abundance is shown on a log10 scale and samples (rows) are clustered based on their Bray-Curtis dissimilarity index.



Supplemental figure 3 – Alpha-diversity of the resistome of farm dust, animal feces and farmers and controls.

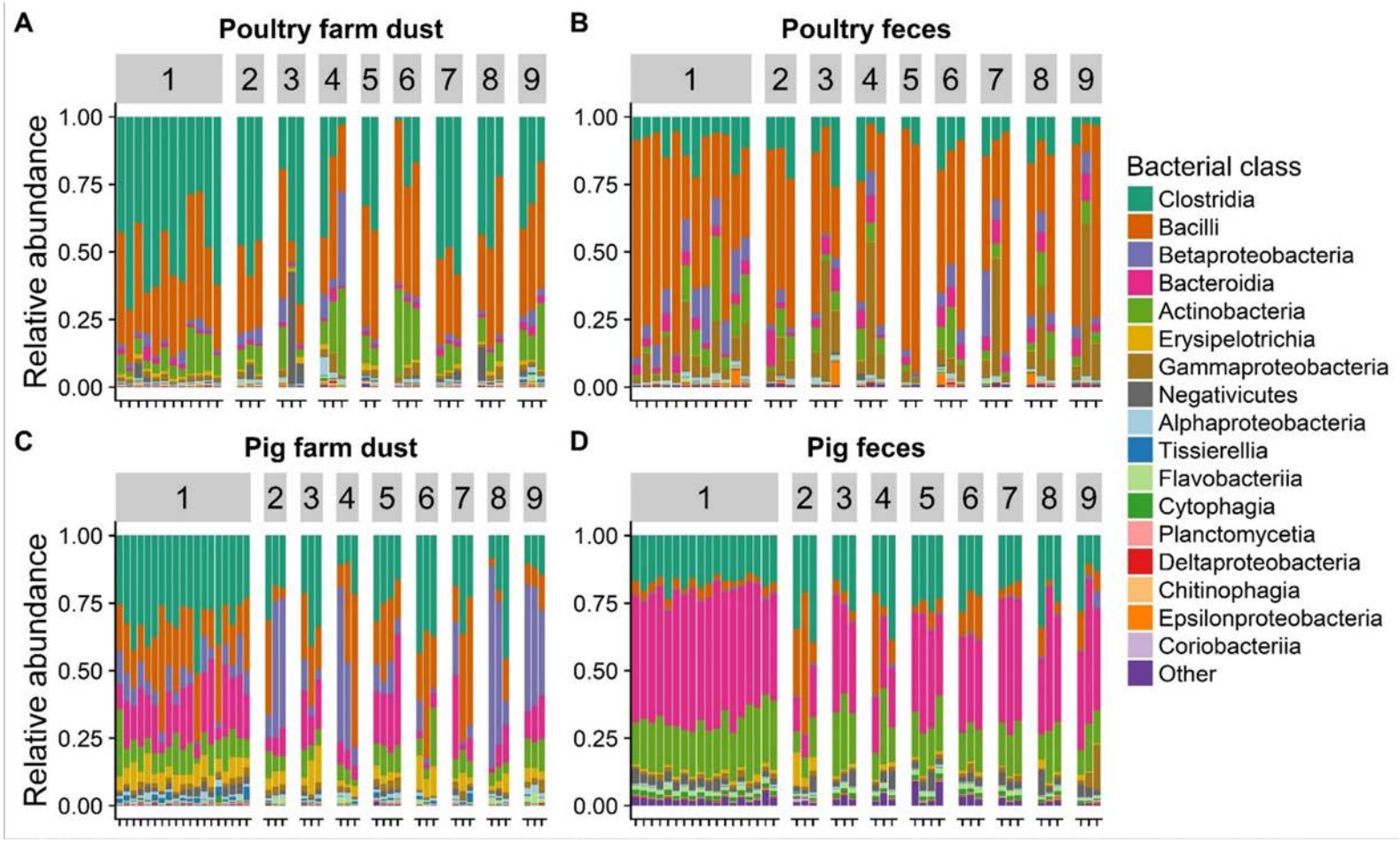
Boxplots showing Richness (A) and Evenness (B) of samples from 35 poultry and 43 (feces) or 44 (dust) pig farms from nine countries. Results for poultry farmers (n=24), pig farmers (n=54) and human controls (n=46) from one country (country 1 = the Netherlands).

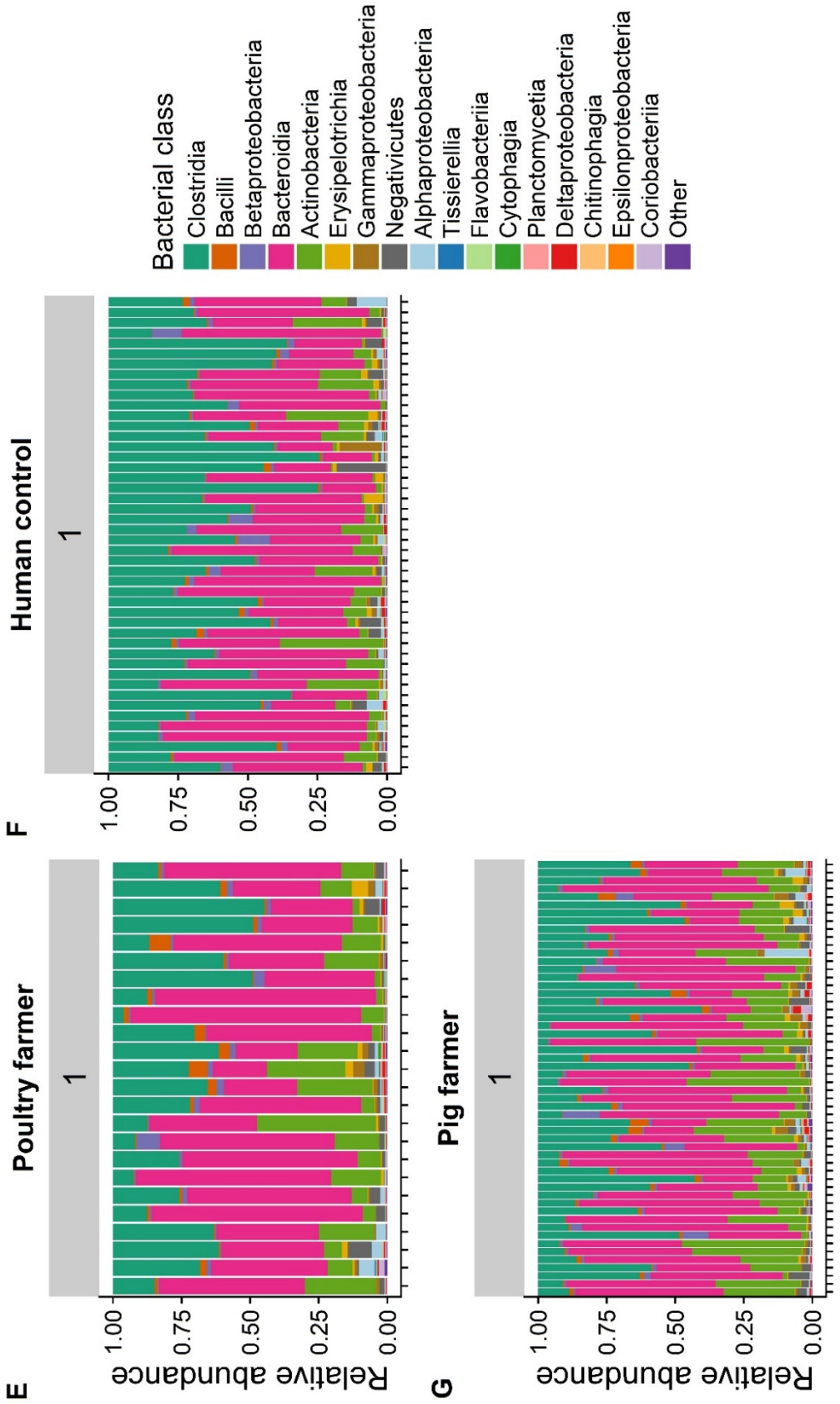


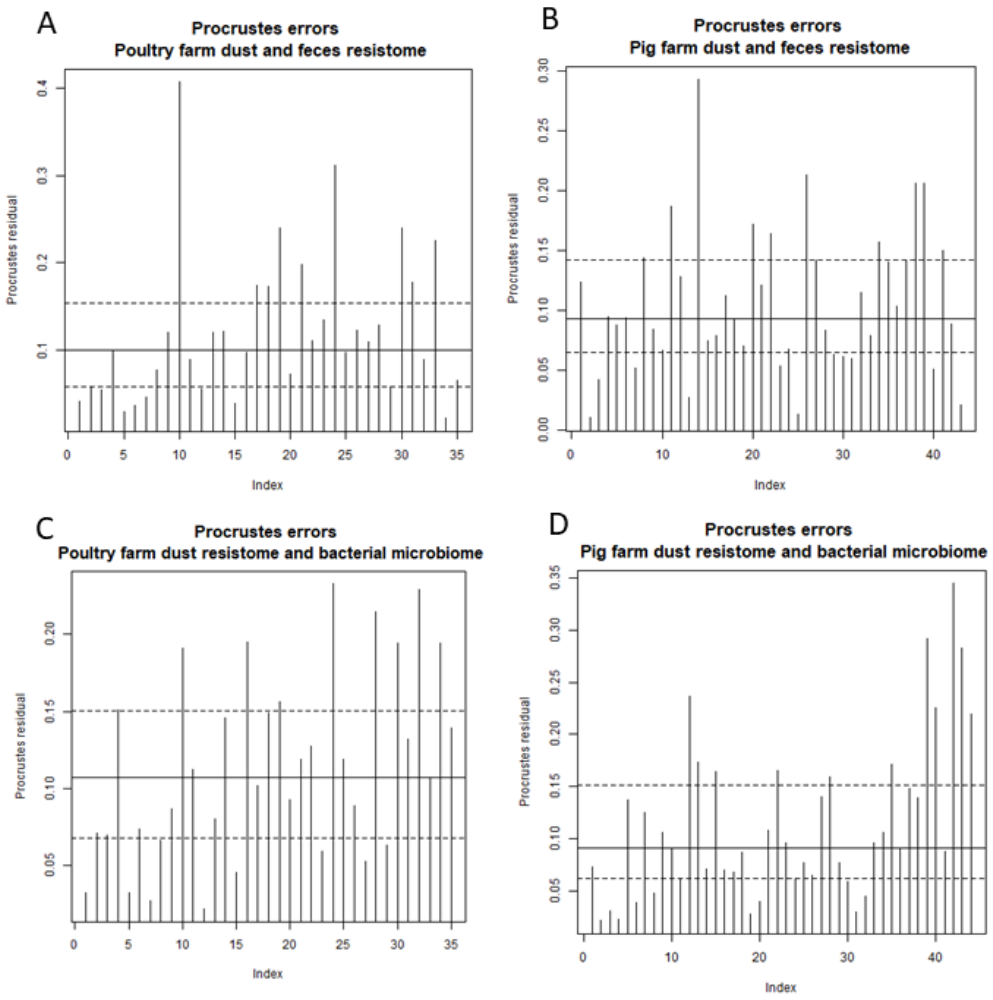
Supplemental figure 4 – Relative abundance of the top-17 most abundant bacterial classes in farm dust, animal feces and farmers' and control stool samples.

Barcharts of poultry farm dust (A) and feces (B) and pig farm dust (C) and feces (D), from nine countries (1-9).

Results for poultry farmers (E), pig farmers (G) and human controls (F) are from one country (country 1 = the Netherlands) (Van Gompel et al. 2020).




E
Poultry farmer
1
Relative abundance
1.00
0.75
0.50
0.25
0.00
F
Human control
1
G
Pig farmer
1
Bacterial class
Clostridia
Bacilli
Betaproteobacteria
Bacteroidia
Actinobacteria
Erysipelotrichia
Gammaproteobacteria
Negativicutes
Alphaproteobacteria
Tissierellia
Flavobacteriia
Cytophagia
Planctomycetia
Deltaproteobacteria
Chitinophagia
Epsilonproteobacteria
Coriobacteriia
Other



Supplemental figure 5 – Residual errors of superimposition plots of Procrustes correlation.
Residual errors of correlation of feces and farm dust resistomes of 35 poultry (A) and 43 pig (B) farms and of the correlation of bacterial microbiomes and resistomes of poultry (C) and pig (D) farm dust. Plots are corresponding to plots in figure 3 in the main text.

Supplemental table 2 – Linear regression results of AMU versus total AMR levels in farm dust.

*AMU is expressed a treatment of the sampled herd (group) and antibiotics purchased by the complete farm (purchased). Unadjusted and adjusted for total AMR levels in the feces of animals. Models with star * are plotted in figure 4.*

		Poultry				Pigs							
		Total AMU group		Total AMU purchased		Total AMU fatteners group		Total AMU fatteners purchased		Total AMU in 200 days group		Total AMU in 200 days purchased	
		Estimate	p-value	Estimate	p-value	Estimate	p-value	Estimate	p-value	Estimate	p-value	Estimate	p-value
Total AMR farm dust		**0.13***	**0.004**	**0.12**	**0.008**	**0.10**	**0.034**	**0.17**	**0.020**	**0.12***	**0.003**	**0.12**	**0.050**
Total AMR farm dust adjusted for total AMR animal feces		0.07	0.057	0.04	0.314	0.03	0.415	0.06	0.327	0.03	0.379	0.01	0.889

Supplemental table 3 *– Lists of resistance genes (90%ID cluster level) present in the different farm sources and human controls as depicted in the Venn-diagrams (figure 7, main text), all from one country plus the list of unique resistance genes present in farm dust from these poultry and pig farms.*

This table can be found in the separate Excel-file online.

Due to the large size, part of the supplement is not printed. Please go to the online available Excel-file with 'supplementary data 2'
https://doi.org/10.1016/j.envint.2020.105971

Supplemental methods

DNA extraction and library preparation of animal feces and farmers' and controls stool

DNA of the animal feces pools and human stool samples (controls and farmers) was extracted with a modified protocol of the QIAamp Fast DNA Stool Mini Kit (Qiagen) with an additional bead beating step (3X30 sec at 30Hz with TissuelyserII). For poultry feces samples, due to lower DNA yields, a PCR poor library preparation was necessary (Kapa Hyper Prep Kit, Kapa Biosystems). For pig feces samples, library preparation was done with NEXTflex PCR-Free library preparation (Bioo Scientific) and for farmer and control stool samples, the PCR poor NEBNext Ultra DNA library preparation kit (New England BioLabs inc.) was used. Minimal amplification cycles were used (3) unless the library was insufficient for sequencing then a minimum number of cycles were added (up to max 10). The low levels of amplification are known to introduce minimal bias if any (https://sequencing.roche.com/en/products-solutions/by-category/library-preparation/dna-library-preparation/kapa-hyperprep.html).
For further detail on DNA extraction and library preparation we refer to (Munk et al. 2018, Van Gompel et al. 2020)

Bioinformatics processing

Bioinformatics processing was similar to earlier published work using the same fixed versions of software and databases (Munk et al. 2018). In short, metagenomic sequencing of the farm dust, farmers stool and control samples was all done on the Illumina HiSeq4000 platform using 150 bp paired-end sequencing at a minimum of 40 million PE clusters (80 million reads) per sample (GenomeScan, Leiden, the Netherlands). For the animal feces samples this was done similarly but with the HiSeq3000 platform (Oklahoma Medical Research Foundation, Oklahoma City, OK, USA).

All DNA sequences were adapter-cleaned and classified. Briefly, reads were mapped using BWA against the NCBI microbial refseq genome and Resfinder database (Zankari et al. 2012). Fixed software and database versions were used (Resfinder database 17 November 2016 and NCBI RefSeq bacterial genome database 18 November 2016). Classified ARG reads were expressed as Fragments Per Kilobase ARG-reference per Million bacterial fragments (FPKM). FPKM is computed by dividing the mapped resistance fragments by the length of the

respective resistance gene and the total number of bacterial fragments per sample and multiplying by 10^9. For further detail on bioinformatics processing we refer to (Munk et al. 2018, Van Gompel et al. 2020).

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5

Determinants for antimicrobial resistance genes in farm dust on 333 poultry and pig farms in nine European countries

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Under review

Abstract

Livestock feces with antimicrobial resistant bacteria reaches the farm floor, manure pit, farm land and wider environment by run off and aerosolization. Little research has been done on the role of dust in the spread of antimicrobial resistance (AMR) in farms. Concentrations and potential determinants of antimicrobial resistance genes (ARGs) in farm dust are at present not known. Therefore in this study absolute ARG levels, representing the levels people and animals might be exposed to, and relative abundances of ARGs, representing the levels in the bacterial population, were quantified in airborne farm dust using qPCR. Four ARGs were determined in 947 freshly settled farm dust samples, captured with electrostatic dustfall collectors (EDCs), from 174 poultry (broiler) and 159 pig farms across nine European countries. By using linear mixed modeling, associations with fecal ARG levels, antimicrobial use (AMU) and farm and animal related parameters were determined. Results show similar relative abundances in farm dust as in feces and a significant positive association (ranging between 0.21-0.82) between the two reservoirs. AMU in pigs was positively associated with ARG abundances in dust from the same stable. Higher biosecurity standards were associated with lower relative ARG abundances in poultry and higher relative ARG abundances in pigs. Lower absolute ARG levels in dust were driven by, among others, summer season and certain bedding materials for poultry, and lower animal density and summer season for pigs. This study indicates different pathways that contribute to shaping the dust resistome in livestock farms, related to dust generation, or affecting the bacterial microbiome. Farm dust is a large reservoir of ARGs from which transmission to bacteria in other reservoirs can possibly occur. The identified determinants of ARG abundances in farm dust can guide future research and potentially farm management policy.

Introduction

Antimicrobial usage (AMU) in livestock farms is indicated for treatment of diseased animals but has clear effects on the development of antimicrobial resistant bacteria (ARB) [1]. The relationship between AMU and ARB, mainly in the gut of the animals, has been studied extensively [2, 3]. However, development and spread of ARB goes beyond the gut of the animals. Feces with ARB reaches the farm floor, manure pit, farm land and wider environment by run off and aerosolization [4-6]. Fresh animal feces and manure have been demonstrated to be major (microbiological) sources of farm dust [7, 8]. Viable (resistant) bacteria, bacterial DNA and antimicrobial resistance genes (ARGs) are transported as part of dust particles through the air[9-14]. Sequencing based studies have shown that ARGs are part of the airborne dust microbiome from different urban and agricultural environments [15-17]. Farm dust in particular has a relatively rich and abundant resistome [16, 18].

Most probably the role of farm dust in the epidemiology and ecology of antimicrobial resistance (AMR) is complex and multilevel. On one hand it can act as a potential transmission route of resistant bacteria within and between animals and humans [11, 19]. On the other hand it is a potential reservoir of ARGs which might be transferred to bacteria, including potentially pathogenic bacteria in other reservoirs, by e.g. horizontal gene transfer [5, 20, 21]. Work on the quantification of ARGs in poultry or pig farm dust is, to the best of our knowledge, scarce and either small scale or focused on single pathogens [17, 19] and literature on determinants is lacking. Studies on farm dust levels, regardless of AMR, point out the potentially high concentrations indoors, especially in poultry and pig farms [22], resulting in adverse respiratory health effects in farmers [22, 23] and thus potentially for animals. Demonstrated determinants of high dust concentrations in farms are, among others, a low ventilation rate in pig farms [24], age of the broilers [25], or presence of a slatted floor system in pig farms[26]. Previous research on fecal AMR in livestock mainly focused on the association with AMU [2, 3]. Yet some studies also identified farm or animal characteristics, or farm biosecurity measures (taken to reduce the entrance and spread of bacteria) to be related to increased fecal ARB levels, such as decreased farm hygiene [27-29], herd size [30], number of suppliers [30], or straw and flax as litter material [28, 31]. It is still unknown whether these parameters are also relevant determinants for AMR in airborne dust.

Our earlier study that used shotgun metagenomics sequencing to quantify ARGs in dust samples[18] gave many new insights in the farm dust resistome, but the sample size hampered a thorough quantitative analysis of risk factors for ARG abundance in dust. The current study describes the presence of four different ARGs in 947 freshly settled indoor poultry and pig farm dust samples from 333 farms in nine European countries. In this large-scale analysis, we used qPCR to quantify ARG levels. We present the distributions of absolute ARG levels in farm dust, representing the levels people and animals might be exposed to. In addition we present the relative abundance (normalized over 16S) of ARGs, representing the levels in the bacterial population. We assume that determinants may contribute differently to these two outcomes and therefore discuss results of associations with determinants for both endpoints. First, we

determined the relation with ARGs in what we expected to be the most important source, that is animal feces. Subsequently, associations with AMU and farm and animal related characteristics, including biosecurity levels of the farm, were analyzed.

Materials and methods

Study design

In this cross-sectional study, conventional broiler (names poultry throughout the text) and conventional farrow-to-finish pig farms were visited between 2014 and 2016 in nine European countries (Belgium, Bulgaria, Denmark, France, Germany, Italy, the Netherlands, Poland and Spain). In each country, 20 farms per animal species were visited and indoor farm dust, animal feces and meta-data were collected. The whole farm population and its selection criteria were described before [32]. Important inclusion criteria were regular/conventional production type, all-in all-out procedures on compartment level and no other farm animals kept at the farm for production goals. The study focused on animals closest to slaughter (broilers and fattening pigs) and is part of the EFFORT-project (http://www.effort-against-amr.eu/).

Farm dust collection

Dust sampling and lab processing has been described before [18]. Dust was sampled with the use of Electrostatic Dustfall Collectors (EDCs) [33]. These are sampling devices for 'passive' airborne dust sampling, consisting of plastic frame equipped with two (sterilized) electrostatic cloths. The cloths were gamma-radiated before use, to break down as much DNA as possible. Four EDCs were placed in and spatially spread over the poultry house or fattening pigs compartments. The EDCs were positioned horizontally at about 150 cm above ground level, distant from heating or cooling systems.

Blank samples were taken during the sampling period and consisted of unopened EDCs in a sealable bag, which remained at randomly selected farms across all countries for the whole time that EDCs were in the stable. In total 111 blanks were analyzed (56 from pig farms, 55 from poultry farms).

Farmers were asked to collect and ship the EDCs after two to seven days of placing in the compartments, at the latest just before any thinning or removing of animals for slaughter. The farmer folded the frame and packed the EDCs in sealable bags and an envelope and sent them by regular mail to a central lab (alternatively first to a local partner lab, and then to the central lab). The blanks were shipped together with the used EDCs and were processed in the same way and at the same time as the other samples.

Animal feces collection

During the farm visits, 25 fresh individual fecal samples were collected from animals in the same compartment(s) as the EDCs were placed in. Fresh droppings were collected, evenly divided over the compartment (often multiple compartments for pigs), with a sterile plastic spoon and cup. Feces was immediately stored at 4°C and transported to the local lab where

they were stirred, divided in smaller portions and frozen within 24 hours at -80°C (alternatively at -20°C for a maximum of 4 days, before transferring to −80°C) [32]. From the 25 individual samples, five poultry and seven pig fecal samples were randomly selected for further analysis using qPCR. The earlier metagenomic study, which was based on the same samples, used a pooled sample composed of all the 25 individual samples [18].

Meta-data collection

During the farm visit, information on farm and animal characteristics and antimicrobial usage (AMU) data was collected through a questionnaire filled out together with the farmer. Farm and animal characteristics included age of animals, animal density, type of ventilation systems, feed type and others. AMU data was collected per antimicrobial class and in total (sum of all classes). From these records Treatment Incidence for Defined Daily Dosages (TIdddvet) per 100 animals were calculated [34, 35]. Afterwards farm biosecurity scores were calculated with the Biocheck scoring system (www.biocheck.ugent.be). Results are expressed on a scale from 0 to 100, with 100 meaning that all possible biosecurity measures are present (100%). See also earlier works using these biosecurity scores [36, 37].

Lab processing and DNA extraction

After arrival at the central lab the EDCs were stored up to 6 days in the envelope used for transport, and subsequently opened in a flow cabinet. Electrostatic cloths were removed from the folder, transferred to a sealable bag and frozen at -80°C. Maximally three EDCs per farm were selected for further processing. EDCs showing traces of water damage or other signs of unintended contamination were excluded from further processing. In some cases farmers did not return EDCs or corresponding records were missing and therefore, in total, 947 samples from 333 farms were included in the analysis (500 samples from 174 poultry farms and 447 samples from 159 pig farms).

Directly before DNA extraction, EDC cloths were thawed, washed, and blended with the use of a stomacher. The resulting solution was freeze dried for 3-5 days. The resulting lyophilate was weighted to determine total amount of dust. The lyophilate was kept at -20°C until DNA was extracted using the Nucleospin 8 plant II kit (Machery-Nagel) following the standard protocol with an additional bead-beating step (30 sec at 5.5G with Fastprep-24). Extracted DNA was stored at -80°C until further processing.

Fecal samples were sent from local labs to the central lab on dry ice and processed as described earlier [32]. DNA was extracted using a modified protocol of the QIAamp Fast DNA Stool Mini Kit (Qiagen) with an additional bead beating step (3X30 sec at 30Hz with TissuelyserII) [38]. DNA was stored at -80°C until further processing.

qPCR protocol

qPCR was performed to quantify the abundance of the antimicrobial resistance genes *tetW*, *ermB*, *aph(3')-III* and *sul2*, coding for tetracycline, macrolide, aminoglycoside and sulfonamide

resistance, respectively, along with the bacterial *16S* rRNA gene. Targets were chosen based on expected abundance (i.e. quantifiable in the majority of samples) and variety in antimicrobial classes. qPCR was performed in two labs, namely for *16S*, *aph(3')-III* and *sul2* in Poland (National Veterinary Research Institute, PIWet, Puławy), and for *tetW* and *ermB* in The Netherlands (IRAS, Utrecht). qPCR was performed with a CFX384 Real-Time System (Bio-Rad, USA). Details on the qPCR-protocol, primers, quality control, calibration curves and LOD and LOQ can be found in the supplemental material and in earlier works [39, 40].

The initial results were expressed as gene copies per PCR reaction. These were recalculated into gene copies in dust per square meter surface per day, taking into account the amount of sample extracted, dilution factors, surface area of EDC cloths and number of days of exposure of the EDCs in a stable. For samples with PCR results below the limit of quantification (LOQ) the initial result was replaced by a value 2/3 of the lowest initial result, calculated per gene per animal species.

Data analysis

Selection of determinants

ARG levels in feces were analyzed as mean number of gene copies per gram of feces based on 5 (poultry) or 7 (pigs) individual samples per farm. Relevant AMU measures were selected. First, total and antimicrobial class specific group treatments given to the animals sampled in this study, second, total and specific purchased products for the whole farm in the year before sampling. Collected farm and animal related determinants were selected for data analysis based on literature and expert opinion. Out of a total of 105 (poultry) or 150 (pig) questions (including sub questions), roughly 20 individual questions were considered potentially relevant for dust exposure. Biosecurity information was summarized in scores (total, internal and external) and one internal biosecurity sub score ('cleaning and disinfection'). Potential determinants were included in further analysis when the missing value level was <10% and the determinant was present at, at least, 10% of the farms. AMU was tested as a dichotomous variable in case of scarce use of a specific group (use in less than 10% of the farms). This resulted in a collection of around 25 variables divided in several subcategories, as potential determinants or source for ARG levels in farm dust (see supplemental table for full list).

Statistical analysis

To obtain a comprehensive picture we analyzed ARG concentrations in farm dust in two ways; log10 of absolute level of gene copies per square meter surface per day in the stable and log10 of the relative abundance of genes, as retrieved from normalization by 16S rRNA. Additionally, total absolute levels of the *16S* rRNA gene were analyzed as general bacterial marker. The full selection of determinants was tested on both types of outcomes but described by subcategory. The subcategories are presented in the following order: 1) fecal ARG concentrations, 2) antimicrobial usage, 3) total dust weight and 4) all other determinants including biosecurity. Antimicrobial usage (TIdddvet) was log10(x+1) transformed. Feces ARG

concentrations were expressed on a log10 scale, as absolute level per gram feces and as relative abundance (normalized by 16S).

For the determinant analysis a linear mixed effect model was used to account for within farm and within country effects by including a nested random effect (R package *nlme [41]*). All model results are presented as regression coefficients with accompanying 95% confidence intervals. In the main text we choose to present only results from determinants presenting significant associations with at least two genes within one outcome. All results, including p-values, are included in the supplemental material.

All data handling and analyses were done in R software (version 4.0.2) [42]. All graphs were created with R package *ggplot2 [43]*.

Results

We analyzed 947 farm dust samples from 174 poultry and 159 pig farms from nine European countries. Table 1 gives an overview of some major characteristics of the farms and the group of animals in the poultry house or pigs compartments which were sampled.

Table 1. Three major characteristics of the included pig and poultry farms. All results are rounded to whole numbers.

	mean	median	10th – 90th percentile
Poultry farm size (total n broilers present)	70715	50000	23000 - 150000
Age of broilers at sampling (days)	34	35	26 - 42
Number of broilers present in sampled barn	25386	24558	14000 - 36801
Pig farm size (total n pigs present)	4571	3000	1350 - 9600
Age of fatteners at sampling (days)	180	173	135 -259
Number of fatteners present	562	300	100 - 1021

Antimicrobial resistance genes (ARGs) and the *16S* rRNA gene were quantifiable in almost all samples (figure 1). The percentage of samples below LOQ or LOD was below 0.01% for all genes except for *aph(3')-III* (<LOQ: 10%, <LOD: 3%) and *sul2* (<LOQ/LOD: 19%) in poultry dust. Field and procedural blanks contained only traces of the ARGs investigated. Field blanks had ARG abundances which were about 10000 (pigs) till 500 (poultry, probably more but limited by LOQ) times lower than actual samples (Supplemental figure 1) indicating that contamination of samples because of transport, field and laboratory procedures did not likely occur.

Absolute ARG abundances were higher in pig farm dust than in poultry farm dust, while relative levels of ARGs in poultry farm dust were slightly higher than in pig farm dust across genes (Figure 1). All ARG and *16S* gene levels were positively and significantly associated with each other, with a weaker association in pigs than in poultry (data not shown).

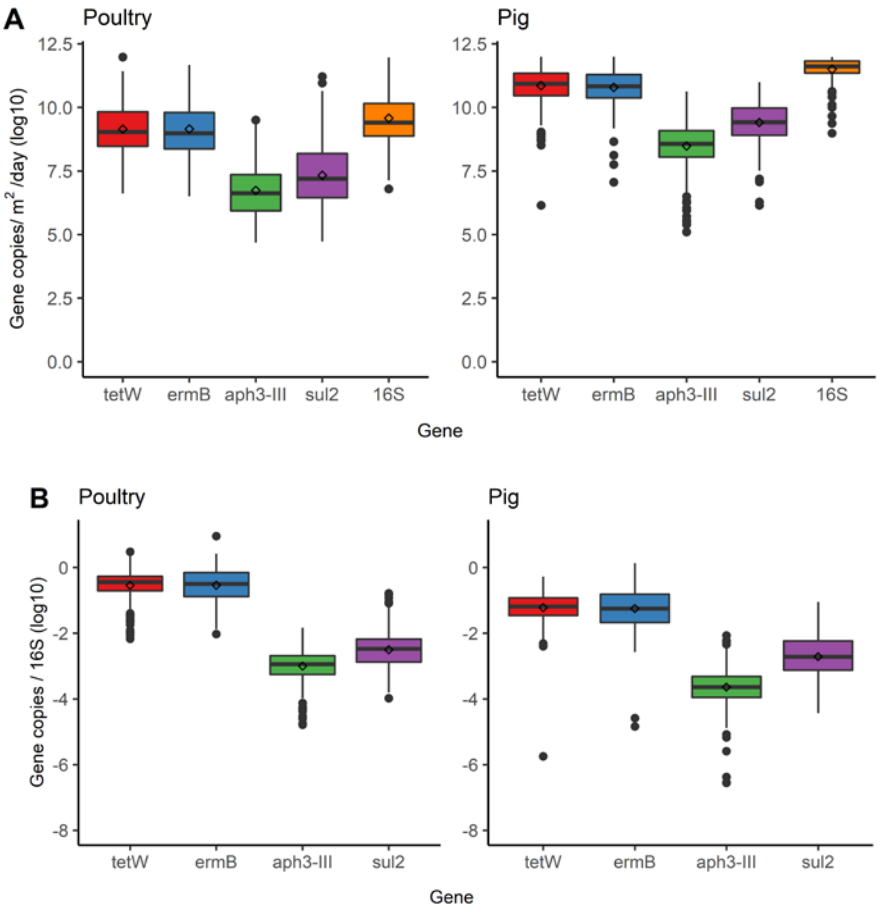

Figure 1. Farm dust ARG abundances in poultry and pig farms from nine countries. A) Absolute abundance per m^2 per day of four ARGs and 16S. b) Relative abundance (normalized over 16S) of four ARGs. The middle line in the (25-75 percentile) boxplot represents the median, the diamond the mean. Abundances of ARGs per country can be found in the Supplemental Figure 2.

Fecal ARG levels

Positive associations were found between ARG abundances of farm dust and animal feces. Generally, relative abundances were more often statistically significantly associated and 4 out of the 5 associations had a higher coefficient (Table 2). Similar results were observed when absolute and relative abundance models were compared while variables were standardized to an equal scale.

Table 2. Results of regression* between ARG abundances in dust and animal feces from the same compartment. Bold results have p < 0.05. *across participating countries analyzed using a mixed model nested by country and farm. Est = estimate, 95% CI = 95% Confidence Interval.

	Absolute abundance (log10 ARG copies/m²/day)					Relative abundance (log10 ARG copies normalized over 16S)			
	tetW Est (95% CI)	*ermB* Est (95% CI)	*aph(3')-III* Est (95% CI)	*sul2* Est (95% CI)	*16S* Est (95% CI)	*tetW* Est (95% CI)	*ermB* Est (95% CI)	*aph(3')-III* Est (95% CI)	*sul2* Est (95% CI)
Poultry									
	0.12 (-0.16 - 0.40)	**0.27 (0.04 - 0.50)**	0.18 (-0.09 - 0.44)	0.15 (-0.09 - 0.39)	0.02 (-0.40 - 0.44)	0.13 (-0.00 - 0.27)	**0.44 (0.34 - 0.54)**	0.03 (-0.09 - 0.15)	**0.22 (0.11 - 0.33)**
Pigs									
	0.30 (-0.16 - 0.75)	**0.67 (0.47 - 0.88)**	0.12 (-0.09 - 0.33)	**0.31 (0.11 - 0.51)**	0.11 (-0.20 - 0.42)	0.13 (-0.10 - 0.36)	**0.82 (0.68 - 0.96)**	**0.21 (0.06 - 0.35)**	**0.30 (0.15 - 0.44)**

Table 3. Results of regression* between absolute and relative ARG abundances in dust and total AMU administered as group treatment to the sampled animals in their life (poultry) or fattening phase (pigs). Bold results have p < 0.05. *analyzed across participating countries using a mixed model nested by country and farm. Est = estimate, 95% CI = 95% Confidence Interval. An overview of all model results, including other AMU measures, can be found in the supplemental table 3.

	Absolute abundance (log10 ARG copies/m2/day)					Relative abundance (log10 ARG copies normalized over 16S)			
Poultry	*tetW* Est (95% CI)	*ermB* Est (95% CI)	*aph(3')-III* Est (95% CI)	*sul2* Est (95% CI)	*16S* Est (95% CI)	*tetW* Est (95% CI)	*ermB* Est (95% CI)	*aph(3')-III* Est (95% CI)	*sul2* Est (95% CI)
TIdddvet total treatments in the sampled broilers	0.03 (-0.21 - 0.26)	0.04 (-0.20 - 0.28)	-0.07 (-0.32 - 0.19)	0.07 (-0.24 - 0.37)	-0.06 (-0.33 - 0.22)	0.09 (-0.01 - 0.18)	0.10 (-0.00 - 0.21)	-0.01 (-0.12 - 0.10)	0.13 (0.00 - 0.26)
Pigs									
TIdddvet total group treatments in sampled fatteners	**0.31 (0.10 - 0.52)**	**0.36 (0.15 - 0.57)**	**0.39 (0.15 - 0.62)**	**0.37 (0.16 - 0.59)**	0.09 (-0.08 - 0.25)	**0.23 (0.11 - 0.35)**	**0.29 (0.11 - 0.47)**	**0.32 (0.16 - 0.49)**	**0.30 (0.13 - 0.46)**

Antimicrobial usage

Absolute and relative abundance of ARGs in dust were significantly positively associated with AMU in pigs (Table 3). To test for a direct effect of AMU on dust ARG levels, thus next to mediation through feces, we adjusted the model for fecal ARG levels. The associations became slightly weaker, but a statistically significant effect remained for *tetW, aph(3')-III* and *sul2*. More frequent tetracycline treatments in fatteners was associated with higher absolute and relative *tetW* abundances in pig farm dust (Supplemental table 3). For poultry these associations were weaker and mainly seen for the relative abundance with p-values just above 0.05 (Table 3 and supplemental table 3).

Total dust levels

For both animal species the total amount of dust measured (per square meter per day) was significantly and positively related to absolute abundances of ARGs and 16S in dust, but not for relative levels (Table 4).

Table 4. Results of regression* between absolute and relative ARG abundances in dust and total dust levels. Bold results have p < 0.05. *analyzed across participating countries using a mixed model nested by country and farm. Est = estimate, 95% CI = 95% Confidence Interval.

	Absolute abundance (log10 ARG copies/m2/day)						**Relative abundance (log10 ARG copies normalized over 16S)**			
Poultry	*tetW* Est (95% CI)	*ermB* Est (95% CI)	*aph(3')-III* Est (95% CI)	*sul2* Est (95% CI)	*16S* Est (95% CI)		*tetW* Est (95% CI)	*ermB* Est (95% CI)	*aph(3')-III* Est (95% CI)	*sul2* Est (95% CI)
Total dust (gr/m2/day)	**0.161 (0.104 - 0.219)**	**0.168 (0.106 - 0.230)**	**0.164 (0.101 - 0.227)**	**0.184 (0.097 - 0.270)**	**0.197 (0.126 - 0.268)**		-0.026 (-0.054 - 0.001)	-0.022 (-0.049 - 0.004)	-0.009 (-0.038 - 0.021)	-0.019 (-0.065 - 0.027)
Pigs										
Total dust (gr/m2/day)	**0.022 (0.015 - 0.030)**	**0.024 (0.016 - 0.033)**	**0.022 (0.014 - 0.030)**	**0.021 (0.014 - 0.029)**	**0.024 (0.017 - 0.030)**		-0.001 (-0.007 - 0.004)	0.001 (-0.006 - 0.007)	-0.001 (-0.007 - 0.005)	-0.001 (-0.007 - 0.004)

Other determinants

a. Animal and farm related parameters

In pig farms, animal and farm related parameters (i.e. animal density, feed type and more farms in a 500m radius) were significantly related to absolute ARG abundances but not to relative abundances. For poultry this was observed as well (i.e. bedding and broilers present in the stable). Shredded straw as bedding material had an opposite effect direction for absolute and relative ARG abundance in poultry. For both pig and poultry, significantly lower absolute ARG levels were observed during the summer season (Table 5).

b. Biosecurity

Biosecurity scores (total, external and internal), and 'cleaning and disinfection' were predominantly related to relative ARG abundances in dust for both pig and poultry farms (Table 5). In poultry farms, higher levels of biosecurity measures were related to lower relative abundances, whereas biosecurity measures in pig farms were related to higher relative abundances. To test for an effect of biosecurity directly related to ARG dust levels, thus besides a pathway through feces, fecal ARG abundances were included in the models. This resulted in similar results. Absolute ARG abundances were not associated with the level of biosecurity measures taken at a farm. For pig farms a significant negative association was found between 16S i.e. total bacterial load of a dust sample and external biosecurity scores.

Table 5. Results of regression* between absolute and relative ARG abundances in dust and animal and farm related determinants, including biosecurity, for a) poultry and b) pigs. Bold results have $p < 0.05$ * analyzed across participating countries using a mixed model nested by country and farm. NB Only results with at least two genes significantly associated are shown. Est = estimate, 95% CI = 95% Confidence Interval. An overview of all model results can be found in the supplemental table 3

.

a) Poultry											
		Absolute abundance (log10 ARG copies/m2/day)						**Relative abundance (log10 ARG copies normalized over 16S)**			
Variable	Category	*tetW* Est (95% CI)	*ermB* Est (95% CI)	*aph(3')-III* Est (95% CI)	*sul2* Est (95% CI)	*16S* Est (95% CI)		*tetW* Est (95% CI)	*ermB* Est (95% CI)	*aph(3')-III* Est (95% CI)	*sul2* Est (95% CI)
Animal and farm related parameters											
Broilers present in the stable (per 10000)		**0.24 (0.08 - 0.39)**	**0.27 (0.11 - 0.43)**	**0.27 (0.10 - 0.44)**	**0.23 (0.03 - 0.43)**	**0.21 (0.03 - 0.39)**		0.03 (-0.03 - 0.09)	0.05 (-0.02 - 0.12)	0.08 (0.01 - 0.15)	0.03 (-0.06 - 0.12)
Bedding material (ref = sawdust)	other	-0.36 (-0.83 - 0.11)	-0.28 (-0.76 - 0.19)	-0.25 (-0.78 - 0.28)	-0.58 (-1.17 - 0.00)	-0.42 (-0.95 - 0.12)		0.03 (-0.16 - 0.21)	0.05 (-0.16 - 0.27)	0.03 (-0.18 - 0.25)	-0.25 (-0.52 - 0.01)
	shredded_straw	**-0.68 (-1.22 - -0.14)**	**-0.84 (-1.38 - -0.29)**	-0.51 (-1.11 - 0.09)	**-1.16 (-1.84 - -0.47)**	**-1.00 (-1.62 - -0.38)**		**0.27 (0.06 - 0.49)**	0.17 (-0.08 - 0.42)	**0.28 (0.03 - 0.52)**	-0.22 (-0.52 - 0.08)
Season (ref = winter)	autumn	0.20 (-0.15 - 0.54)	0.07 (-0.28 - 0.43)	0.13 (-0.25 - 0.51)	-0.09 (-0.55 - 0.36)	-0.04 (-0.45 - 0.37)		0.23 (0.09 - 0.37)	0.12 (-0.04 - 0.29)	0.12 (-0.04 - 0.28)	-0.12 (-0.32 - 0.09)
	spring	-0.26 (-0.66 - 0.14)	-0.33 (-0.74 - 0.08)	-0.38 (-0.82 - 0.06)	-0.49 (-1.01 - 0.03)	-0.49 (-0.96 - -0.02)		0.22 (0.06 - 0.38)	0.16 (-0.03 - 0.34)	0.09 (-0.10 - 0.27)	-0.01 (-0.24 - 0.23)
	summer	**-0.45 (-0.84 - -0.07)**	**-0.48 (-0.87 - -0.08)**	**-0.62 (-1.04 - -0.20)**	**-0.51 (-1.01 - -0.01)**	**-0.62 (-1.07 - -0.17)**		**0.18 (0.03 - 0.33)**	0.13 (-0.04 - 0.31)	-0.13 (-0.30 - 0.04)	0.07 (-0.15 - 0.29)
Length ventilation present (ref = no)	yes	0.15 (-0.16 - 0.46)	0.19 (-0.13 - 0.51)	0.17 (-0.17 - 0.51)	-0.02 (-0.42 - 0.38)	0.01 (-0.35 - 0.37)		**0.13 (0.01 - 0.25)**	**0.15 (0.01 - 0.28)**	0.13 (-0.01 - 0.27)	-0.03 (-0.20 - 0.14)

Biosecurity measures											
Biosecurity external (per 10pts)		0.013 (-0.160 - 0.187)	0.055 (-0.127 - 0.237)	0.060 (-0.125 - 0.245)	0.101 (-0.125 - 0.327)	0.125 (-0.073 - 0.324)		**-0.099 (-0.167 - - 0.030)**	-0.041 (-0.123 - 0.041)	-0.064 (-0.144 - 0.016)	-0.001 (-0.099 - 0.097)
Biosecurity internal (per 10pts)		0.009 (-0.103 - 0.121)	0.010 (-0.106 - 0.126)	0.051 (-0.070 - 0.171)	0.067 (-0.079 - 0.212)	0.084 (-0.045 - 0.213)		**-0.057 (-0.101 - - 0.013)**	**-0.051 (-0.0102 - - 0.000)**	**-0.052 (-0.102 - - 0.001)**	-0.010 (-0.072 - 0.053)
Biosecurity in total (per 10pts)		0.017 (-0.159 - 0.192)	0.047 (-0.136 - 0.231)	0.083 (-0.103 - 0.269)	0.124 (-0.105 - 0.353)	0.154 (-0.045 - 0.354)		**-0.113 (-0.182 - - 0.044)**	-0.069 (-0.150 - 0.013)	**-0.085 (-0.165 - - 0.005)**	-0.008 (-0.107 - 0.091)
Internal biosecurity sub score: cleaning and disinfection (per 10pts)		0.018 (-0.073 - 0.110)	0.014 (-0.082 - 0.110)	0.035 (-0.064 - 0.133)	0.058 (-0.061 - 0.177)	0.089 (-0.015 - 0.194)		**-0.051 (-0.087 - - 0.015)**	**-0.050 (-0.092 - - 0.008)**	**-0.064 (-0.105 - - 0.023)**	-0.016 (-0.068 - 0.035)

b) Pigs											
		Absolute abundance (log10 ARG copies/m2/day)						**Relative abundance (log10 ARG copies normalized over 16S)**			
Variable	Category	*tetW* Est (95% CI)	*ermB* Est (95% CI)	*aph(3')-III* Est (95% CI)	*sul2* Est (95% CI)	*16S* Est (95% CI)		*tetW* Est (95% CI)	*ermB* Est (95% CI)	*aph(3')-III* Est (95% CI)	*sul2* Est (95% CI)
Animal and farm related parameters											
Animal density (ref = high)	low	**-0.39 (-0.66 - - 0.11)**	**-0.03 (-0.32 - 0.26)**	-0.27 (-0.59 - 0.06)	-0.22 (-0.52 - 0.08)	-0.25 (-0.47 - -0.03)		-0.08 (-0.25 - 0.10)	0.25 (-0.01 - 0.50)	-0.09 (-0.32 - 0.15)	0.07 (-0.17 - 0.31)
Feed type (ref = dry)	wet-dry	**-0.52 (-0.91 - - 0.13)**	-0.36 (-0.76 - 0.05)	**-0.79 (-1.24 - - 0.34)**	-0.36 (-0.76 - 0.05)	**-0.52 (-0.82 - -0.22)**		0.06 (-0.18 - 0.31)	0.20 (-0.14 - 0.55)	-0.28 (-0.59 - 0.04)	0.27 (-0.05 - 0.59)
	wet	-0.10 (-0.34 - 0.13)	-0.09 (-0.33 - 0.15)	**-0.37 (-0.64 - - 0.10)**	-0.08 (-0.33 - 0.16)	-0.02 (-0.20 - 0.15)		-0.11 (-0.24 - 0.03)	-0.09 (-0.29 - 0.11)	-0.36 (-0.54 - -0.18)	-0.05 (-0.23 - 0.14)
Other farm in 500mbuffer around the farm (ref = no)	yes	**0.27 (0.07 - 0.48)**	0.18 (-0.03 - 0.39)	**0.30 (0.05 - 0.54)**	**0.24 (0.03 - 0.46)**	**0.25 (0.10 - 0.41)**		0.00 (-0.12 - 0.12)	-0.06 (-0.24 - 0.11)	0.07 (-0.10 - 0.23)	-0.03 (-0.19 - 0.14)

Season (ref = winter)	autumn	-0.03 (-0.34 - 0.27)	-0.22 (-0.54 - 0.10)	0.18 (-0.19 - 0.54)	0.07 (-0.26 - 0.39)	0.06 (-0.16 - 0.29)		-0.06 (-0.24 - 0.12)	-0.29 (-0.54 - -0.03)	0.11 (-0.12 - 0.35)	0.01 (-0.22 - 0.25)
	spring	0.06 (-0.28 - 0.39)	0.09 (-0.26 - 0.43)	0.06 (-0.34 - 0.45)	0.09 (-0.26 - 0.45)	0.11 (-0.13 - 0.36)		-0.03 (-0.23 - 0.17)	-0.04 (-0.32 - 0.24)	-0.08 (-0.34 - 0.18)	-0.03 (-0.29 - 0.23)
	summer	-0.31 (-0.62 - 0.00)	**-0.35 (-0.67 - -0.03)**	-0.36 (-0.73 - 0.00)	-0.07 (-0.40 - 0.26)	**-0.27 (-0.50 - -0.05)**		-0.00 (-0.18 - 0.18)	-0.12 (-0.38 - 0.14)	-0.13 (-0.37 - 0.11)	0.19 (-0.05 - 0.43)
Different ventilation system then all other ventilation system options (ref = no)	yes	0.27 (0.00 - 0.55)	-0.04 (-0.32 - 0.24)	0.28 (-0.04 - 0.59)	0.24 (-0.04 - 0.53)	0.07 (-0.14 - 0.28)		**0.17 (0.01 - 0.33)**	-0.12 (-0.36 - 0.11)	**0.25 (0.03 - 0.47)**	0.17 (-0.05 - 0.39)
Valve ventilation system (ref = no)	yes	-0.15 (-0.40 - 0.11)	0.03 (-0.24 - 0.29)	-0.15 (-0.45 - 0.15)	**-0.31 (-0.57 - -0.05)**	**-0.24 (-0.44 - -0.05)**		0.14 (-0.01 - 0.29)	**0.31 (0.09 - 0.52)**	0.13 (-0.08 - 0.33)	0.05 (-0.15 - 0.25)
Biosecurity measures											
Biosecurity external (per 10pts)		-0.090 (-0.187 - 0.008)	-0.018 (-0.117 - 0.081)	-0.102 (-0.209 - 0.005)	-0.073 (-0.176 - 0.030)	**-0.090 (-0.165 - -0.016)**		0.018 (-0.039 - 0.074)	**0.087 (0.005 - 0.169)**	0.007 (-0.071 - 0.084)	0.021 (-0.056 - 0.099)
Biosecurity internal (per 10pts)		0.011 (-0.071 - 0.094)	0.069 (-0.012 - 0.150)	-0.026 (-0.118 - 0.066)	0.057 (-0.028 - 0.143)	-0.039 (-0.101 - 0.023)		**0.054 (0.006 - 0.102)**	**0.111 (0.042 - 0.179)**	0.022 (-0.044 - 0.088)	**0.084 (0.018 - 0.149)**
Biosecurity in total (per 10pts)		-0.041 (-0.145 - 0.062)	0.046 (-0.058 - 0.149)	-0.075 (-0.188 - 0.038)	0.005 (-0.104 - 0.114)	**-0.083 (-0.160 - -0.005)**		0.053 (-0.008 - 0.113)	**0.138 (0.051 - 0.224)**	0.022 (-0.062 - 0.105)	0.079 (-0.004 - 0.162)
Internal biosecurity sub score: cleaning and disinfection (per 10pts)		0.012 (-0.039 - 0.064)	0.039 (-0.012 - 0.091)	-0.016 (-0.074 - 0.043)	0.021 (-0.032 - 0.075)	-0.024 (-0.063 - 0.015)		**0.037 (0.007 - 0.066)**	**0.060 (0.017 - 0.103)**	0.016 (-0.025 - 0.057)	0.037 (-0.003 - 0.077)

Discussion

This study determined the abundance of four different antimicrobial resistance genes in freshly settled farm dust in pig and poultry farms and quantified the relation with farm and animal related determinants across nine countries by including 333 European livestock farms. Two types of outcomes were assessed: the absolute and the relative number (normalized over 16S) of gene copies. Both parameters give complementary insights into the dynamics of dust in the epidemiology of AMR in the livestock farm.

Tetracycline (*tetW*) and macrolide (*ermB*) resistance genes were abundant in all samples both absolutely, and relatively to the total number of bacteria. The aminoglycoside (*aph(3')-III*) and sulfonamide (*sul2*) resistance genes were roughly 2-3 units lower on a log10 scale of absolute counts. ARGs in animal feces were positively related to ARGs in dust for most ARGs in both poultry and pig farms. Higher dust ARG abundance was observed in pig farms that reported higher AMU. Several farm and animal related determinants were significantly associated with lower absolute ARG levels such as, summer season, wet pig feed or shredded straw as poultry bedding material. This study is pointing towards different types of determinants and pathways able of shaping the dust resistome in livestock farms.

AMR studies involving airborne or settled dust are still relatively scarce but the available evidence indicates that ARGs are omnipresent in dust [10, 16, 44]. Having assessed ARGs in all dust samples in the current study is therefore not a surprise, however the relative levels of these genes are not very different from other (AMU exposed) samples such as animal feces or waste water from treatment plants [45]. This could be explained by the fact that animal feces is very likely the most important source of dust through aerosolization of fecal particles. Indeed, our data shows a positive relation between ARG levels in feces and dust, which was more pronounced for relative ARG abundances. However, although feces is an important source for farm dust, it is not the only organic/microbiological source [8]. Other sources, such as skin, mucus, feed, litter and outdoor air or soil are additional sources of bacterial DNA and potentially ARGs. The contribution of each source to the dust composition depends on animal species and the farming system [46, 47] and therefore, imaginably, differs per ARG. This might explain the different coefficients between feces and dust seen in this study per resistance gene.

Higher total AMU in fattening pigs from the fattening unit sampled for dust was positively associated with resistance gene abundances in dust. This was observed earlier for the summed abundance of all ARGs (the resistome) present in dust, determined with metagenomics, in a selection of the same pig and poultry farms [18]. A significant association was also observed between tetracycline usage and *tetW* dust levels, despite the smaller association between fecal and dust *tetW* levels. It is expected that at least a part of the relationship between AMU and dust is mediated by the effect of AMU on fecal resistance genes. After adjustment for ARG levels in feces, a significant positive effect remained for all genes except one: *ermB*. Fecal *ermB* concentrations had the largest association with dust

ermB concentrations. These results seem to point towards an independent effect of AMU on dust resistance genes in addition to the feces pathway. Another related issue here could be the excretion of antibiotic residues via feces or through the administration route, which is mainly by feed and water [34, 35], that may lead to local environmental selective effects [48, 49]. The association between corresponding usage and resistance genes for other classes than tetracycline (e.g. macrolide use and *ermB*) was hampered by limited AMU in fatteners or broilers in the sampled animals. Interestingly for poultry we found consistently lower coefficients (than for pigs) and borderline significant associations. This might partly be explained by a relatively small number of sampled poultry batches in which antimicrobials were used.

Differences between the two studied animal species, with known different farming systems, was observed often in this study. Biosecurity is the domain of all measures possibly taken to reduce the influx and spread of bacteria and other microorganisms on the farm [50] and thus possibly also affects the bacterial composition of the farm environment (i.e. dust). On poultry farms, higher internal biosecurity, including specifically a higher 'cleaning and disinfection score', and external biosecurity led to lower relative ARG abundances (mainly *tetW* and *ermB)* in dust. In pig farms, however, the opposite was observed. This opposite effect has been seen before for ARG in pig feces in the EFFORT project [37]. This underpins that interpreting biosecurity scores in relation to AMR, rather than to specific pathogens, is challenging, and the role of specific farm practices for AMR might deserve further research [51]. Some negative associations with fecal levels of certain bacteria and biosecurity measures have been already shown [29, 52], however, an effect of biosecurity on bacterial/ARG levels in airborne dust has not been observed earlier. None of the associations with biosecurity (except one) was statistically significant when ARGs were expressed as absolute levels (or 16S*)* in dust for both farm types. This suggest that biosecurity scores such as those used in this project are currently not capturing airborne dust forming processes. Of all other investigated determinants, associations differed between relative and absolute ARG levels, confirming that there are pathways increasing dust ARGs through processes influencing the total level of dust generation (absolute ARG levels), and processes affecting the bacterial microbiome (relative ARG levels). For example, we observed a reduction in absolute ARGs abundance in the summer season compared to winter for poultry and pig farms. These results are in accordance with studies reporting reduced ventilation in winter (due to cold weather) which in turn led to higher dust levels in the stables [22, 53]. Some associations found in this study have not been described before. For example, significant associations in poultry stables between bedding type and dust ARGs, however with an opposite direction of the relation for absolute and relative levels. Another interesting finding is that other livestock farms in a 500m buffer around pig farms resulted in higher absolute ARGs abundance in the dust.

This study was performed with data from nine different countries and identified determinants are thus important across countries. Nevertheless, ultimately the local AMR and AMU situation determines the relevance of animal and farm related drivers for AMR in the farm

environment. Therefore, results need to be confirmed with sufficiently powered studies in each country, or for example through intervention studies. Although this study involved a sufficiently high number of farms, it was complicated by between country differences, which required a tailor made analysis leading to some loss of power. Tested determinants in this study were not an ideal aggregation of variables potentially relevant for investigating dust formation, due to the fact that the broader project was not only set up for the objectives of the current study. For example, while effects of ventilation techniques and intensity are also expected to influence dust formation [22] and therefore absolute ARG levels, it was difficult to collect ventilation related determinants in a practical matter during field work, because it is time consuming, costly and expertise is needed. Across genes, no clear relations with absolute ARG levels were seen, possibly because the ventilation levels rather than the applied technique (tested here) determine aerosolization and dust formation. Since this study was set up as hypotheses generating research no multiple testing adjustment was applied.

The exact relevance of ARG transmission via farm dust is complex, still largely unknown but expected to play a role next to other transmission pathways [54, 55]. Livestock farms are an important reservoir of ARGs and ARBs and a source for environmental AMR [6]. Exposure to airborne AMR from farms will probably be more relevant for persons working in and around farms [11, 19] compared to the general population, which is, at least for specific resistant bacteria, dominated by human-human contact [56]. While the general population is probably considerably lower exposed to airborne dust from farms, exposure to resistance genes frequently occurring in animal feces is however possible in the vicinity of farms [9]. Transmission of ARGs and ARBs through air and dust between animals is most likely to occur as well [57]. With the methods used here we were able to not only detect, but also quantify ARG dust levels in farms. The use of EDCs enabled us to do large scale sampling with relatively little effort (compared to air sampling using pumps), while keeping relevance, as EDCs collect freshly, airborne, settled dust. We however do not know the ARG fraction that was part of viable bacteria nor do we know the magnitude of potential transmission to other bacteria. Determinants of ARGs in farm dust can guide future research and potentially farm management policy. Clearly any dust related intervention needs to be animal specific due to the different dynamics uncovered on the currently studied farms.

Conclusion

Antimicrobial resistance genes are widespread in European pig and poultry farm dust, and their relative abundances (relative to 16S) are similar to what has been found in animal feces. Higher animal fecal ARG abundance was predictive for higher ARG abundances in dust sampled in the same compartment. In pig farms we found an additional effect of antimicrobial usage in animals on dust ARG levels. Dust related determinants, such as summer season and wet-dry feed type, were related to lower absolute ARG levels. In conclusion, farm dust can be considered a large reservoir of ARGs from which transmission to bacteria in other reservoirs possibly can occur.

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Supplement of Determinants for antimicrobial resistance genes in farm dust on 333 poultry and pig farms in nine European countries

Further qPCR analysis details

References of followed qPCR protocols, primer and probe concentrations and annealing temperatures and time are shown in supplemental Table 1.

Supplemental Table 1 – qPCR protocol details

Gene	Primer reference	Primer concentration	Probe concentration	Annealing temperature / time
16S	Fierer et al.[1]	200nM	NA	60°C /45 sec
ermB	Koike et al.[2]	400nM	250nM	61°C/1 min
tetW	Walsh et al.[3]	600nM	200nM	59°C/45 sec
aph(3')-III	Woegerbauer et al.[4]	400nM	250nM	60°C/20 sec
sul2	Heuer et al.[5]	100nM	100nM	60°C/60 sec

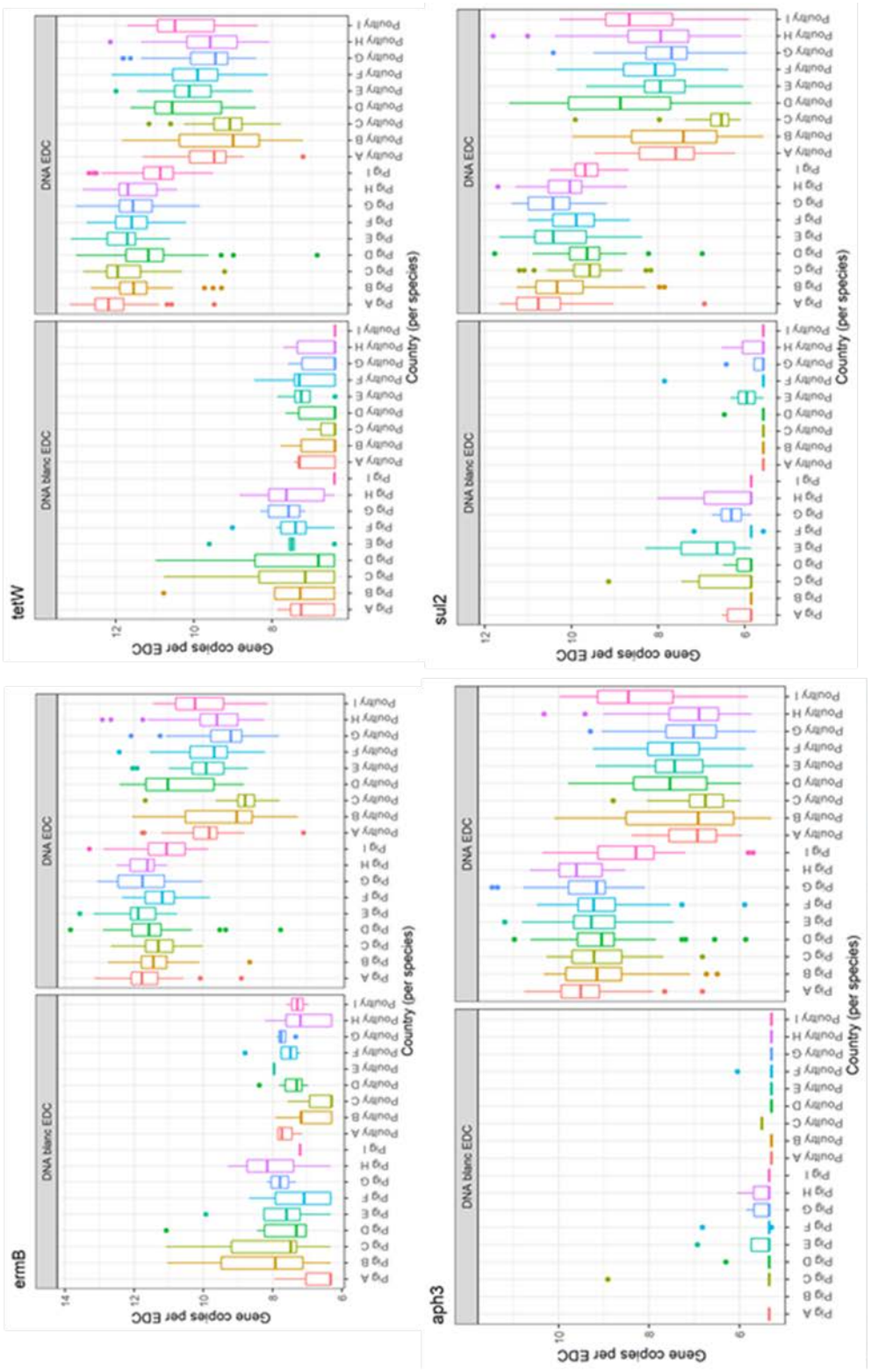
Standard curves were constructed by synthetic DNA on each PCR plate, 8 dilutions of the standard curve were run. TE-buffer was used as a negative control and mixtures of DNA from several fecal and dust samples were used as positive controls. To control PCR inhibition, DNA was diluted with TE-buffer in the following ratio: dust - 1:50, feces - 1:100. Additionally, a noncompetitive internal amplification control (IAC) was used to control the presence of qPCR inhibition for all genes except *16S*. IAC consisted of a gene that encodes for the blue fluorescence protein (bfp) and was added to the qPCR mastermix [6]. All samples were run in two technical duplicates.

Using a receiver operating characteristic (ROC) curve approach [7], the limit of detection (LOD), based on the number of false-positive results of the negative controls and on the number of false-negative results of the standard curve samples with low DNA concentration, was determined. Limit of quantification (LOQ) values were determined by defining a tolerable absolute deviation in log copies from true concentrations, and determining the highest Ct value below which absolute deviation was tolerable. The tolerable absolute variation was computed from the joint calibration curve constructed from all PCR plates and defined as 95% of all samples showing a log copy deviation from the true concentration of less than 1 log unit within a 2 Ct window. The resulting LOD and LOQ values per gene are shown in supplemental Table 2.

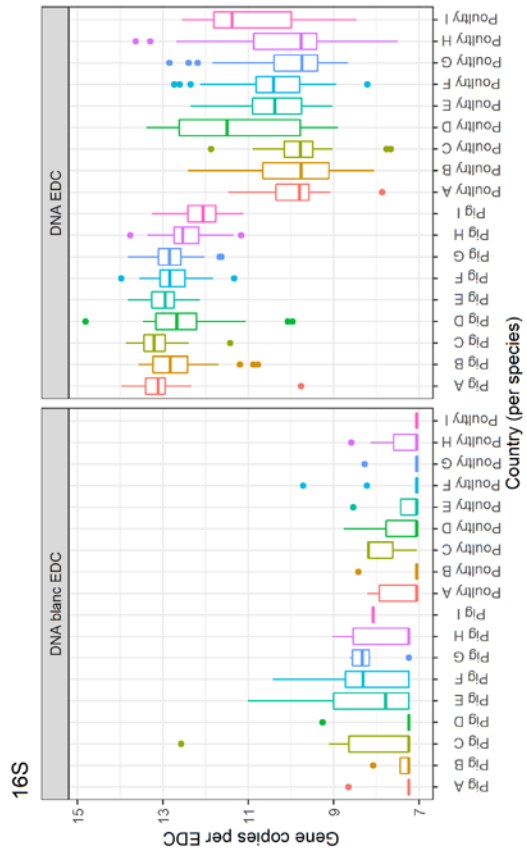
Supplemental Table 2 – LOD and LOQ expressed as initial result (= gene copies per PCR reaction on log10 scale) of the five PCR targets

Gene	**LOD**	**LOQ**
16S	3.11	1.98 / 3.11
ermB	1.52	2.02
tetW	0.64	2.16
aph(3')-III	0.22	0.52
sul2	0.91	0.92

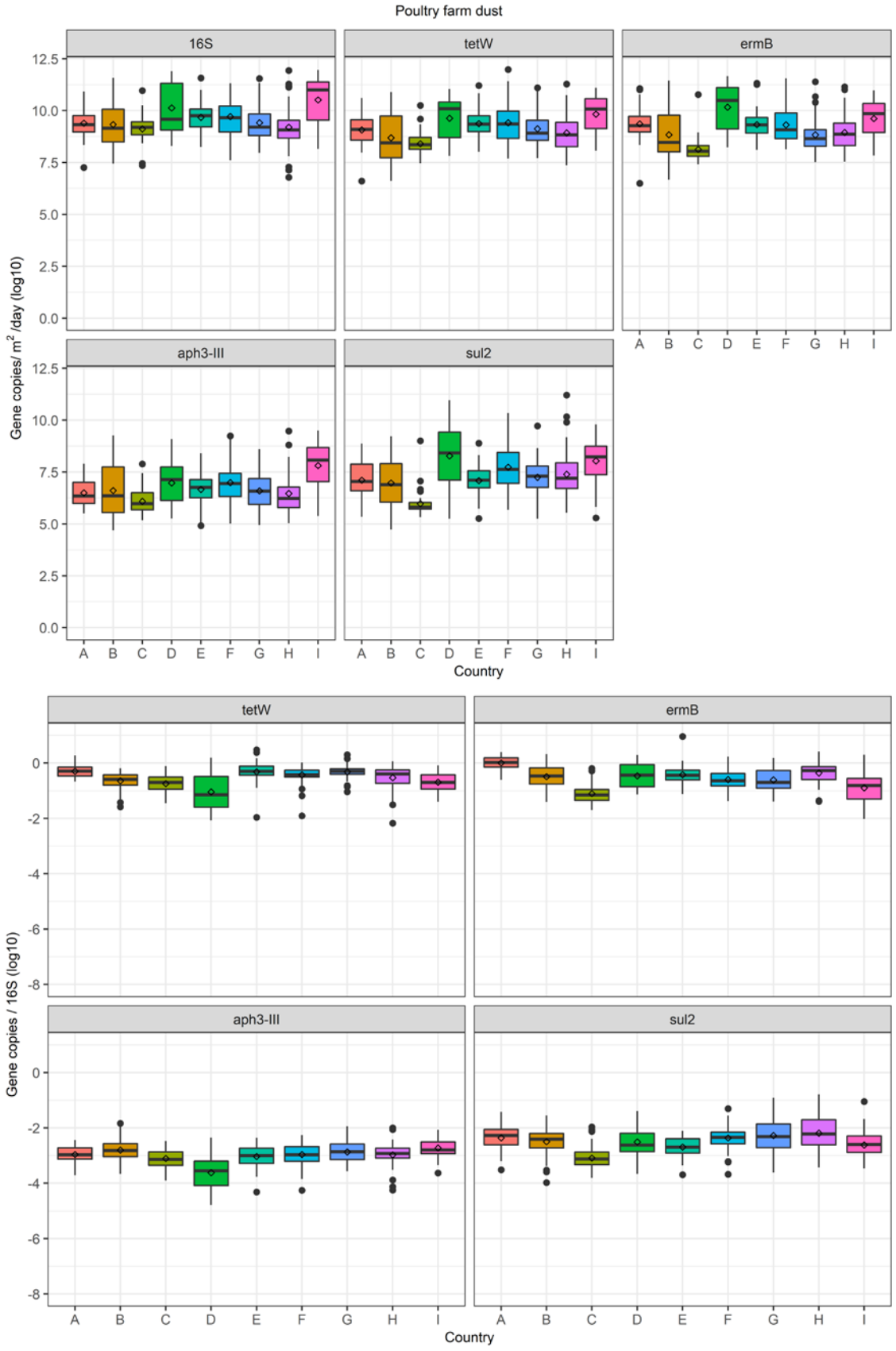
qPCR results were qualified as 'failed' when the technical duplicates deviated too much (acceptable deviations were derived for different Ct ranges, based on the distribution of deviations observed in all samples) or when the IAC had a Ct value greater than the mean+2 standard deviations of the IAC results of the calibration curves. In total 5% of samples failed in the *aph(3')-III* qPCR and 5% and 18% in the *sul2* qPCR for pig and poultry, respectively. For *16S, ermB* and *tetW* the percentage of samples failed was below 2%.

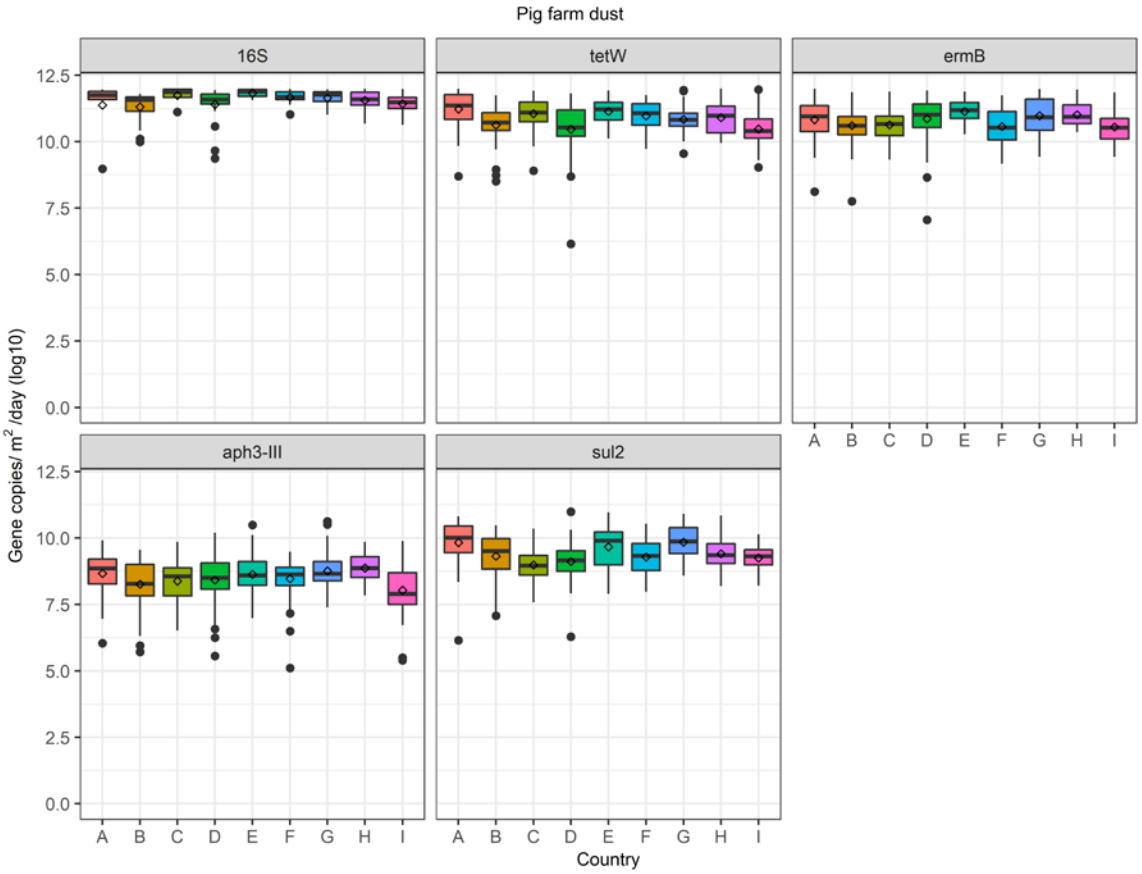


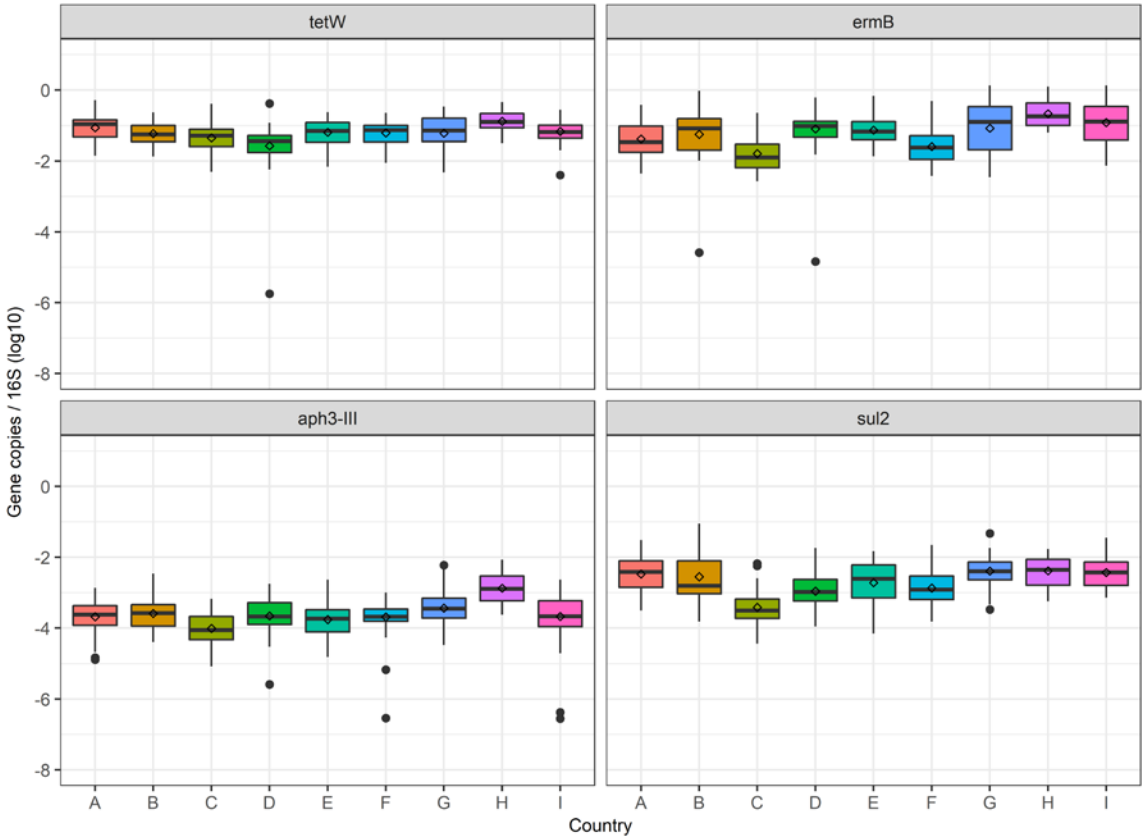
Supplemental Figure 1 – ARG abundance per EDC in field blanks ('DNA blanc EDC') compared to the samples ('DNA EDC') plotted per gene per country for poultry and pig farms. The middle line in the (25-75%) boxplot represents the median. All country names have been replaced by the letters A-I.


16S
DNA blanc EDC
DNA EDC
Gene copies per EDC
15
13
11
9
7
Country (per species)
Pig A
Pig B
Pig C
Pig D
Pig E
Pig F
Pig G
Pig H
Pig I
Poultry A
Poultry B
Poultry C
Poultry D
Poultry E
Poultry F
Poultry G
Poultry H
Poultry I

Supplemental Figure 2 – Absolute and relative abundances per antimicrobial resistant and 16S gene per country for poultry and pig farms. The middle line in the (25-75%) boxplot represents the median, the diamond the mean. All country names have been replaced by the letters A-I.




Pig farm dust
16S
tetW
ermB
aph3-III
sul2
Gene copies/ m² /day (log10)
12.5
10.0
7.5
5.0
2.5
0.0
A B C D E F G H I
Country


tetW
ermB
aph3-III
sul2
Gene copies / 16S (log10)
0
-2
-4
-6
-8
A B C D E F G H I
Country

Supplemental Table 3 - all regression model results* for absolute and relative ARG abundances for poultry and pig.

* analyzed across participating countries using a mixed model nested by country and farm.

Presented are the coefficient (=Est) and the p value.

Yellow marked variables are also presented in main text as indicated in the materials & methods (determinants presenting significant associations with at least two genes within one outcome).

Green marked cells are significant model results (p <0,05).

POULTRY

Variable	Category	Absolute abundance (log10 ARG copies/m2/day) tetW Est	pvalue	ermB Est	pvalue	aph(3')-III Est	pvalue	sul2 Est	pvalue	16S Est	pvalue
FECES											
fecal aph(3')-III abundance (absolute)						0,176319	0,1848				
fecal ermB abundance (absolute)				0,268659	0,0231						
fecal 16S abundance										0,0219	0,9177
fecal sul2 abundance (absolute)								0,147	0,2249		
fecal tetW abundance (absolute)		0,12377	0,3828								
AMU											
treatment of animals in the sampled compartment											
Tidddvet Total group treatments		0,02756	0,8176	0,039462	0,7494	-0,06746	0,603	0,06915	0,6553		
Tidddvet Tetracyclines group treatments		-0,17417	0,3456								
Tidddvet Macrolides group treatments binary				0,328117	0,223						
Tidddvet Aminoglycosides group treatments binary						0,270637	0,4353				
Tidddvet Sulfonamides group treatments binary								0,16964	0,5354		
products purchased by the whole farm in the year before sampling											
Tidddvet Total purchased products		-0,06067	0,5857	0,012443	0,9133	-0,03775	0,7558	0,13499	0,3488		
Tidddvet Tetracyclines purchased products		-0,12342	0,5196								
Tidddvet Macrolides purchased products				0,189114	0,1769						
Tidddvet Aminoglycosides purchased products						0,354078	0,4623				
Tidddvet Sulfonamides purchased products binary								0,00075	0,997		
Other DETMERMINANTS: farm and animal related											
Total amount of dust											
total dust levels (mg/m2/day)		0,00016	8E-08	0,000168	2E-07	0,000164	5E-07	0,00018	4E-05	0,0002	9E-08
size of the farm											
average animals present at farm		-1,7E-06	0,155	-1,4E-06	0,2313	-2,2E-06	0,0833	-2,6E-06	0,0943	-2E-06	0,148
size of the sampled compartment											
broilers present in the sampled stable		2,4E-05	0,0028	2,69E-05	0,0009	2,71E-05	0,0017	2,3E-05	0,0256	2E-05	0,0236
animal related											
age broilers at sampling		0,0187	0,1164	0,016055	0,1929	0,012416	0,3413	0,02336	0,132	0,0183	0,1908
weight broilers at sampling		5,7E-05	0,6963	8,96E-05	0,5509	-3,7E-05	0,8152	0,00012	0,5127	2E-05	0,8981
farm(management) related											
animal density ref = high	low	-0,29784	0,0593	-0,3536	0,0291	-0,2808	0,1049	-0,3008	0,1392	-0,306	0,095
bedding material	other	-0,35833	0,1341	-0,28325	0,2403	-0,24679	0,3576	-0,58187	0,0505	-0,418	0,1268
ref = saw dust	shredded_straw	-0,68162	0,0135	-0,83534	0,0031	-0,50718	0,0971	-1,15609	0,0011	-1,003	0,0017
fertilizing land around farm ref. =	yes	0,18773	0,2036	0,314897	0,036	0,305347	0,0585	0,04418	0,8157	0,2216	0,1959
manure storage at farm ref. = no	yes	0,15664	0,367	0,058757	0,7395	0,128138	0,5	0,14001	0,5258	0,1195	0,5529
other farms in 500m buffer ref. =	yes	0,06497	0,6449	0,106653	0,4579	0,109489	0,4786	-0,02545	0,8878	0,1019	0,5328
season											
season	autumm	0,1969	0,2652	0,073302	0,6855	0,133133	0,4919	-0,09343	0,686	-0,038	0,856
ref = winter	spring	-0,26141	0,1989	-0,33149	0,1128	-0,38271	0,0863	-0,49017	0,0662	-0,485	0,043
	summer	-0,45365	0,0203	-0,4758	0,0175	-0,61917	0,0041	-0,5116	0,0453	-0,622	0,0069
ventilation (yes present, ref. = no)											
cross ventilation present		-0,3629	0,0301	-0,31823	0,0634	-0,33815	0,065	-0,29472	0,1722	-0,308	0,1142
length ventilation present		0,15012	0,342	0,187162	0,2467	0,171066	0,3201	-0,01565	0,9385	0,0108	0,9533
natural ventilation present		-0,03851	0,8497	-0,09813	0,6355	-0,1232	0,5791	-0,05113	0,8432	-0,028	0,9067
roof ventilation present		-0,17107	0,2565	-0,20031	0,1928	-0,14888	0,3656	-0,23852	0,222	-0,076	0,6632
tunnel ventilation present		0,19166	0,3523	0,289952	0,1662	0,165129	0,4696	0,24352	0,3493	0,2714	0,2576
different ventilation present		0,12287	0,5677	0,07264	0,7405	-0,05942	0,7998	0,09405	0,7318	0,1185	0,6353
BIOSECURITY (summary measures plus 1 sub score)											
biosecurity external		0,00133	0,8795	0,005489	0,5518	0,006031	0,5204	0,01008	0,3793	0,0125	0,2146
biosecurity internal		0,00092	0,8719	0,001014	0,8633	0,00505	0,4106	0,00666	0,3672	0,0084	0,2024
biosecurity total		0,00166	0,8517	0,004741	0,6111	0,008323	0,3781	0,01241	0,2864	0,0154	0,1281
internal biosecurity sub score 'cleaning and desinfection'		0,00184	0,6929	0,001407	0,7719	0,00348	0,4858	0,0058	0,3368	0,0089	0,094

Variable (if different from column A)	Relative abundance (log10 ARG copies normalized over 16S) tetw Est	pvalue	ermb Est	pvalue	aph(3')-III Est	pvalue	sul2 Est	pvalue
fecal aph(3')-III abundance (relative)					0,02778	0,6513		
fecal ermB abundance (relative)			0,44313	1E-15				
fecal sul2 abundance (relative)							0,217302	0,000103505
fecal tetW abundance (relativ	0,134828	0,0547						
	0,086685	0,0682	0,1035	0,0583	-0,00986	0,8562	0,132677	0,045981946
	0,026686	0,7119						
			0,20955	0,0812				
					0,05002	0,7301		
							0,14092	0,212493905
	-0,04705	0,2811	0,0436	0,3859	-0,00424	0,9325	0,1179	0,055620708
	0,110208	0,1485						
			0,10222	0,1014				
					0,11718	0,3706		
							0,261646	0,057841772
	-2,6E-05	0,0626	-2,2E-05	0,0993	-8,6E-06	0,5743	-1,9E-05	0,40863579
	1,11E-07	0,8122	5,1E-07	0,3394	-5,2E-07	0,3331	-2,1E-07	0,758622068
	3,33E-06	0,2885	4,7E-06	0,1889	7,7E-06	0,0307	3,18E-06	0,474311809
	0,005277	0,2575	0,00322	0,5464	-0,00066	0,9007	0,008627	0,189320395
	6,98E-05	0,2131	0,00012	0,0696	-1,9E-05	0,7706	0,000119	0,133419369
	-0,01438	0,8154	-0,06561	0,3535	0,02215	0,7535	-0,04178	0,630882045
	0,028496	0,7599	0,05118	0,6395	0,03349	0,7591	-0,25275	0,059322718
	0,272888	0,012	0,17026	0,1799	0,27726	0,0279	-0,21855	0,153677847
	-0,04951	0,3992	0,03746	0,5783	-0,01155	0,8636	-0,20313	0,013458095
	0,027969	0,6816	-0,09907	0,2032	-0,00456	0,9533	0,022006	0,820214848
	-0,02208	0,6882	-0,00768	0,9032	-0,04394	0,4864	-0,08495	0,277920916
	0,227532	0,0015	0,12192	0,1425	0,12403	0,1285	-0,11719	0,257500596
	0,222649	0,0069	0,15586	0,1042	0,08729	0,3515	-0,00806	0,946104176
	0,180783	0,0182	0,13145	0,1407	-0,12896	0,1422	0,069907	0,530084216
	-0,06099	0,3517	-0,03699	0,6221	-0,01918	0,7982	0,000592	0,994909073
	0,129295	0,0341	0,14584	0,0374	0,12931	0,0639	-0,03008	0,728004959
	-0,00249	0,9748	-0,06993	0,4371	-0,14786	0,0983	-0,06369	0,564292944
	-0,10711	0,0709	-0,12166	0,074	-0,09806	0,1488	-0,15521	0,068735635
	-0,02898	0,7239	0,10598	0,2573	0,03002	0,7516	0,085991	0,456704028
	0,026603	0,751	-0,07472	0,4375	-0,16607	0,0815	-0,08388	0,47723486
	-0,00986	0,0053	-0,0041	0,3238	-0,0064	0,1172	-0,00011	0,981964016
	-0,00569	0,0109	-0,0051	0,0487	-0,00515	0,0451	-0,00095	0,763997108
	-0,0113	0,0014	-0,00689	0,0971	-0,00849	0,0384	-0,0008	0,873012124
	-0,00512	0,0056	-0,00497	0,0203	-0,00636	0,0026	-0,00164	0,529043597

PIG

Absolute abundance (log10 ARG copies/m2/day)

Variable	Category	tetW Est	pvalue	ermB Est	pvalue	aph(3')-III Est	pvalue	sul2 Est	pvalue	16S Est	pvalue
FECES											
fecal aph(3')-III abundance (absolute)						0,11877	0,2647				
fecal ermB abundance (absolute)				0,6723644	1,3E-09						
fecal 16S abundance										0,11244	0,4737
fecal sul2 abundance (absolute)								0,31059	0,00243		
fecal tetW abundance (absolute)		0,296748	0,20021								
AMU											
sampled fatteners treatment											
Tidddvet Total group treatments		0,310729	0,00449	0,3620025	0,0008	0,38561	0,0013	0,3713	0,00082	0,08658	0,3007
Tidddvet Aminoglycosides group treatments binary						-0,13301	0,6452				
Tidddvet Macrolides group treatments binary				0,2993064	0,43452						
Tidddvet Sulfonamides group treatments binary								0,12664	0,60943		
Tidddvet Tetracyclines group treatments		0,440103	0,00235								
purchased by the whole farm in the year before											
Tidddvet200 Total purchased products		0,037018	0,74213	-0,021386	0,85113	0,17941		0,14103	0,2216		
Tidddvet200 Tetracyclines purchased products		0,162226	0,24018								
Tidddvet200 Macrolides purchased products				0,4385707	0,03144						
Tidddvet200 Aminoglycosides purchased products						0,69215	0,1594				
Tidddvet200 Sulfonamides purchased products								5,94779	0,46069		
DETERMINANTS: animal and farm related parameters											
Total amount of dust											
mg_dust_m2_pday		2,22E-05	1,4E-08	2,415E-05	6,6E-08	2,2E-05	5E-07	2,1E-05	3E-08	2,4E-05	2E-11
size of the farm											
average animals present at farm		-1,2E-06	0,89595	9,982E-06	0,30508	-4,9E-06	0,655	-2,8E-05	0,01299	-6E-07	0,928
size of the sampled compartment											
number of fatteners		1,92E-05	0,68409	5,145E-05	0,28089	3,2E-05	0,5629	-6,5E-05	0,18055	-3E-06	0,9322
animal related											
age fatteners at sampling		-0,00067	0,70214	0,0004687	0,78773	0,00069	0,7083	0,0005	0,78665	0,00059	0,6789
farm (manegement) related											
all in out in fattening compartments ref. = no		0,003247	0,98151	0,0055481	0,9688	0,11422	0,4802	-0,03709	0,79636	-0,0486	0,6467
animal density	low	-0,38658	0,00626	-0,027955	0,84975	-0,26549	0,1096	-0,2207	0,1504	-0,2519	0,0242
ref = high											
feed type	wet-dry	-0,5207	0,00959	-0,355026	0,08399	-0,79205	0,0006	-0,3564	0,08255	-0,5187	0,0009
ref = dry	wet	-0,10418	0,37954	-0,087826	0,47131	-0,37094	0,0067	-0,08348	0,50003	-0,0246	0,7818
fertilizing land around farm ref. = no		0,059487	0,65328	0,0451694	0,73811	0,0247	0,8735	-0,09025	0,50874	-0,0372	0,7116
manure handeled at the farm ref. = no		-0,1798	0,13175	-0,206224	0,08832	-0,19272	0,1621	-0,13323	0,27707	-0,0743	0,4091
other farms in 500m buffer ref. = no		0,272069	0,01003	0,1801327	0,09789	0,29873	0,0169	0,24438	0,02415	0,25361	0,0014
season											
season	autumn	-0,03451	0,8249	-0,219944	0,17201	0,17524	0,3396	0,06589	0,69037	0,06487	0,5678
ref = winter	spring	0,056599	0,73883	0,0865921	0,61829	0,05663	0,7759	0,09437	0,59939	0,11451	0,3533
	summer	-0,30842	0,05094	-0,35355	0,03033	-0,3639	0,0508	-0,06988	0,67905	-0,2736	0,0176
ventilation (yes present, ref. = no)											
door ventilation present		0,126222	0,39755	0,1301288	0,39332	0,04257	0,8086	0,24564	0,10127	0,02562	0,8202
natural ventilation present		-0,27059	0,05774	-0,239597	0,12285	-0,10908	0,5113	0,02437	0,87674	-0,0675	0,5685
roof ventilation present		-0,04981	0,65632	-0,100629	0,37947	-0,10647	0,4215	-0,00754	0,94734	0,06181	0,4669
valve ventilation present		-0,1476	0,25505	0,0264304	0,84256	-0,15053	0,3252	-0,30886	0,01867	-0,2448	0,0123
different ventilation present		0,274945	0,0462	-0,044418	0,75425	0,27745	0,0837	0,24095	0,0964	0,06696	0,5331
way of air removing from sta air_removed2		0,144611	0,3233	0,1168442	0,441	-0,04219	0,8005	-0,0941	0,55975	-0,0624	0,6149
BIOSECURITY (summary measures plus 1 sub score)											
biosecurity external		-0,00896	0,07205	-0,001793	0,72146	-0,01023	0,0603	-0,00728	0,16319	-0,009	0,0177
biosecurity internal		0,001144	0,78338	0,006911	0,0946	-0,00259	0,5766	0,00574	0,18663	-0,0039	0,2142
biosecurity total		-0,00413	0,42978	0,0045862	0,38295	-0,00754	0,1893	0,00052	0,92491	-0,0083	0,0379
internal biosecurity sub score 'cleaning and desinfecti		0,001223	0,64029	0,0039169	0,13463	-0,00155	0,6037	0,00213	0,42893	-0,0024	0,2185

Relative abundance (log10 ARG copies normalized over 16S)

Variable (if different from column A)	tetw Est	pvalue	ermB Est	pvalue	aph(3')-III Est	pvalue	sul2 Est	pvalue
fecal aph(3')-III abundance (relative)					0,20581	0,0049		
fecal ermB abundance (relative)			0,81992503	1,4272E-21				
fecal sul2 abundance (relative)							0,29587745	9,0773E-05
fecal tetW abundance (rela	0,136011	0,2499						
	0,234106	0,0002	0,28643651	0,00194526	0,32205	0,0002	0,29819225	0,000421
					0,15963	0,4377		
			0,21669301	0,49054773				
							0,13188516	0,48011799
	0,277615	0,0009						
	-0,03328	0,6133	-0,1514118	0,11674499	0,08407	0,3503	0,0451565	0,61229713
	0,102191	0,2067						
			0,21416665	0,20328124				
					0,37796	0,2441		
							1,84583459	0,76167709
	-1,2E-06	0,6667	7,4954E-07	0,81629864	-6E-07	0,8292	-1,275E-06	0,63190283
	8,31E-07	0,879	1,3098E-05	0,09863861	-1E-06	0,8845	-1,683E-05	0,04529319
	2,79E-05	0,3123	5,3591E-05	0,17664446	3,6E-05	0,3291	-6,637E-05	0,06943764
	-0,001	0,3303	-0,0007823	0,63671181	-0,0009	0,5622	0,00032481	0,83052893
	0,058489	0,4665	-0,0113172	0,9224402	0,11675	0,2821	-0,0358992	0,73739295
	-0,07661	0,3808	0,24577336	0,05564352	-0,0864	0,4741	0,06883134	0,56699296
	0,062896	0,6125	0,20232219	0,25046008	-0,2763	0,0826	0,27067436	0,09277637
	-0,10669	0,1271	-0,0888106	0,38210487	-0,3571	0,0001	-0,0492121	0,59878587
	0,067611	0,3874	0,09746264	0,387726	0,05403	0,6114	-0,071745	0,49073047
	-0,09086	0,1946	-0,1258493	0,2117782	-0,0478	0,6121	-0,0768523	0,40956888
	0,000973	0,9875	-0,0637955	0,47634992	0,06879	0,4134	-0,0254498	0,7564528
	-0,05792	0,529	-0,2873253	0,02875772	0,11453	0,346	0,01415963	0,90653676
	-0,03107	0,7565	-0,0378514	0,79003852	-0,0786	0,5538	-0,0270984	0,83707581
	-0,00177	0,9847	-0,1222226	0,35515504	-0,1306	0,2897	0,19068471	0,12395222
	0,087544	0,317	0,11320019	0,36895159	-0,0203	0,8631	0,18579585	0,10163814
	-0,1374	0,1235	-0,2447119	0,07198448	-0,1256	0,3198	0,10113969	0,41277479
	-0,10695	0,1026	-0,1549017	0,10150655	-0,1339	0,1328	-0,0924642	0,28477182
	0,139536	0,0663	0,30615511	0,00481359	0,12653	0,2191	0,0494827	0,62017756
	0,168732	0,0402	-0,1248749	0,29976115	0,24624	0,0278	0,17044821	0,12518955
	0,096211	0,2903	0,19926724	0,15492421	-0,0423	0,7421	-0,077181	0,54353201
	0,001768	0,5391	0,00870251	0,03763065	0,00065	0,8686	0,00210888	0,59146395
	0,005397	0,0272	0,01107109	0,00168438	0,00221	0,5095	0,00836714	0,01257461
	0,00528	0,0862	0,01376228	0,00198202	0,00217	0,6077	0,00788557	0,0632844
	0,003661	0,0162	0,0060007	0,00623618	0,00161	0,4369	0,00370127	0,07114186

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6

Inhalable dust levels of *16S* rRNA and antimicrobial resistance genes *ermB, tetW, sul2, aph(3')-III* in air around European poultry and pig farms

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Unpublished results

Background and objective

Concerns exist on negative health impacts of livestock farm related exposure on occupationally exposed farmers, nearby residents and the environment[1]. One of the concerns is the transmission and dissemination of antimicrobial resistant bacteria and genes from animals or animal excreta to humans[2, 3]. In the current study the main interest was to look into the potential of aerial transmission of antimicrobial resistance genes outside livestock farms. Earlier work on pig and cattle farms has shown culturable levels of (antimicrobial resistant) bacteria downwind of farms[4-8]. Data on resistance genes in the air around farms is scarce[9]. This Dutch study determined AMR genes (*mecA*) in and outside a few pig and poultry farms to compare downwind and upwind concentrations. With a similar approach, the current study was set up.

The objective was to detect and quantify four antimicrobial resistance genes (*ermB, tetW, sul2, aph(3')-III*) and *16S rRNA* gene upwind, inside and downwind of pig and poultry farms. Samples were collected using active air sampling of all inhalable dust.

Materials and methods

Sampling design

From June 2015 until February 2017 a total of 20 measurements were performed on 12 farms. These 12 farms were a selection of eligible farms, from a collection of previously visited poultry (broiler) and pig (farrow- to-finish) farms enrolled for participation in the EFFORT project. Measurements were performed in the Netherlands, Germany and Spain.

The main inclusion criteria were: no buildings or other obstacles between the farm and potential downwind measurement positions, no livestock farms within 500 m upwind, no industrial activities with bio-aerosol generating activities at or near the farm (e.g. compositing, waste water treatment). Criteria for weather conditions on a sampling day were: no heavy rain, snow or storm. Wind speeds in a range between 2 – 4 m/sec (= 7.2 km/h – 14.4 km/h) and temperatures above 7°C and under 40°C.

A measurement lasted 5 hours and was done during day time. The equipment used was a Gilair5 pump set at 3,5L/min. The pump was connected to a GSP inhalable dust sampling-head containing a Teflon filter and placed at 1.5m height above the ground. In order to determine the right positioning of measurements around the farm, before sampling the wind direction was estimated with publicly available local weather forecasts. Air was sampled at 5 positions on and around the farm namely: 1 upwind (somewhere between 90 and 30 meter upwind of the farm), 1 in a stable (stable with eldest broilers or fattening pigs), 3 downwind of the farm at approximately 30m, 90m and 150m. During the measurement the actual wind direction and speed were continuously recorded with a small weather station at the farm. Afterwards the results of 4 poultry farms were excluded for current analyses because actual wind direction was more than 90 degrees (45 degrees both ways) different from the estimated wind direction and thus the measurement was not set up in line with the wind.

Lab processing and qPCR

After the measurement run, the GSP head including the filter was packed and stored at 4°C and transported to the lab within 12 hours. Filters were removed from the sampling head in the lab, stored in sterile petri dishes and frozen at -80°C. Filters were thawed and put into 15 mL tubes to which 5 mL extraction liquid (sterile water + 0.05% Tween20) was added. Tubes were placed on a roller for one hour and centrifuged for 15 minutes. Filters were removed and remaining fluid was frozen overnight at -20°C. Samples were freeze-dryed for 2-4 days, until all fluid was vaporized and thereafter frozen at -20°C. DNA was extracted with the Macherey Nagel Nucleospin 8 plant II kit (cat. No. MN 740669.5, Machery-Nagel, GmbH & Co. KG, Germany) according to the instruction of the manufacturer with an additional beat beating step.

qPCR was performed to quantify the abundance of the antimicrobial resistance genes *tetW*, *ermB*, *aph(3')-III* and *sul2*, coding for tetracycline, macrolide, aminoglycoside and sulfonamide resistance, respectively, along with *16S* rRNA gene. For a technical description of the qPCR analysis, including defining the limit of detection (LOD) and the limit of quantification (LOQ) and PCR quality control measures, refer to chapter 5 (Luiken et al. 2021, *submitted*).

Data analysis

The raw measurement results were expressed as gene copies per PCR reaction. These were subsequently recalculated into log10 gene copies per m^3 of air. The total number of measurements, total successful PCRs and total results above LOD were calculated and tabulated, per gene, per animal species. Abundances above LOD were visualized in a dot-line plot. The ARG abundances between LOD and LOQ were included in the graphs, separately marked as such. These results were included as quantitative results with the following justification. Other sample types (mainly animal feces) of the EFFORT project had much higher abundance of ARGs than air filters. Furthermore, differences of ARG concentrations between different sampling points are often larger than variation within other sample types, therefore, a higher variability in the obtained concentrations is more tolerable for air samples. We therefore chose to still calculate and present the results for air filters which often have abundances near the LOD. The results between LOD and LOQ potentially have larger uncertainty, but the measured value still gives an indication of the order of magnitude of the ARG abundance.

All data is presented across countries. Data handling was done in R software (version 3.6.3)[10]. Graphs were made with R package *ggplot2*[11]**.**

Results

The presence of 16S, *tetW*, *ermB*, *aph(3')-III* and *sul2* has been confirmed (results above LOD) outside both pig and poultry farms, up to 150m downwind, except for *aph(3')-III* in pigs, which was confirmed up to 90m downwind (Table 1).

The measured levels of *tetW* and *ermB* were higher than *aph(3')-III* and sul2 outside, as was also the case inside the farm (Figure 1). Most genes in both pig and poultry farms showed a concentration which was high inside the farm and decreased as the distance to the farm

increased in the downwind direction. Differences were visible between farms (Figure 1). A number of samples, mostly collected further away from the farm, resulted in PCR outcomes below our LOD (Table 1).

Table 1 – Overview of number of tested samples, number of successful qPCRs* and results above LOD of four ARGs and 16S, in total and per position around the poultry and pig farms. Numbers are presented per gene per animal species.

a) Poultry farms

GENE	TOTAL SAMPLES	SUCCESSFUL PCR	>LOD**	UPWIND	FARM	DOWNWIND 30M	DOWNWIND 90M	DOWNWIND 150M
16S	30	30	30	6/6/6	6/6/6	6/6/6	6/6/6	6/6/6
ERMB	30	29	23	4/6/6	6/6/6	4/6/6	5/5/6	4/6/6
TETW	30	29	22	3/5/6	6/6/6	5/6/6	5/6/6	3/6/6
SUL2	30	26	11	0/4/6	5/6/6	4/6/6	1/5/6	1/5/6
APH(3')-III	30	26	12	0/3/6	5/6/6	3/6/6	1/5/6	3/6/6

b) Pig farms

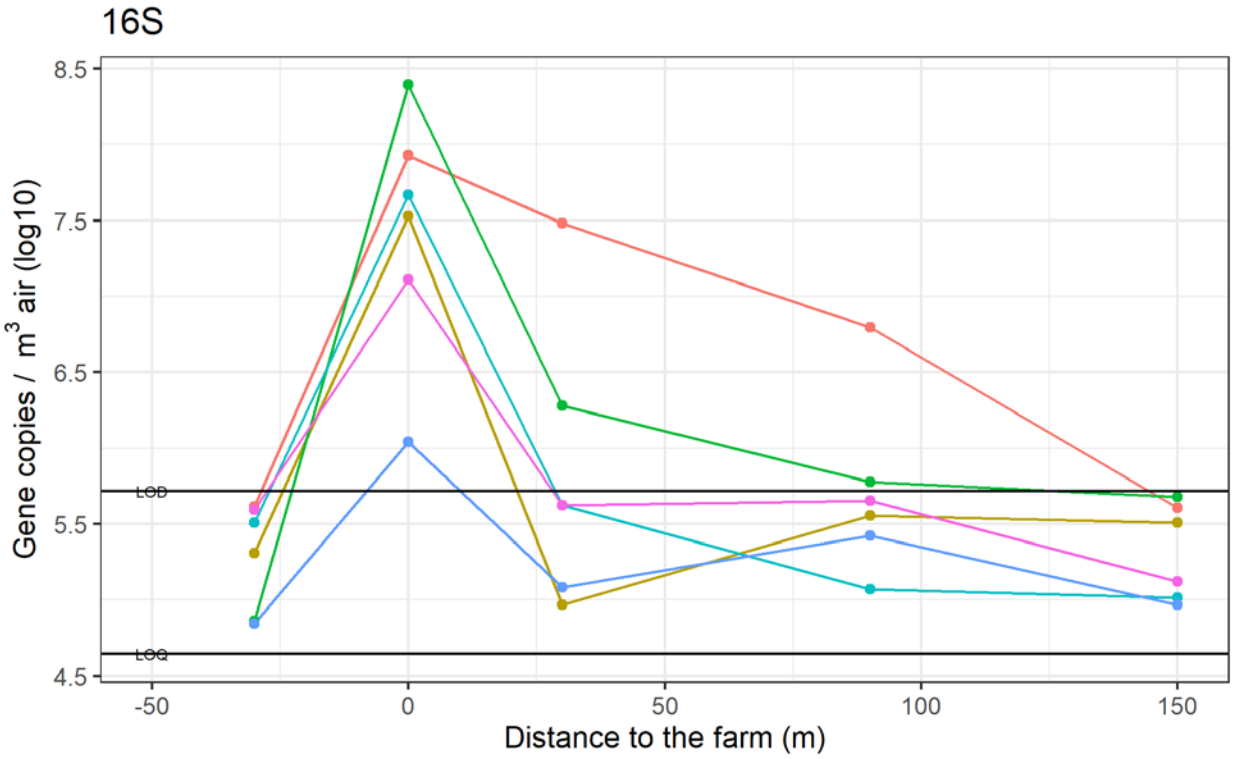
GENE	TOTAL SAMPLES	SUCCESSFUL PCR	>LOD**	UPWIND	FARM	DOWNWIND 30M	DOWNWIND 90M	DOWNWIND 150M
16S	48	48	48	10/10/10	9/9/9	10/10/10	10/10/10	9/9/9
ERMB	48	34	20	2/4/10	8/9/9	4/6/10	3/8/10	3/7/9
TETW	48	46	39	6/9/10	8/9/9	10/10/10	9/9/10	6/9/9
SUL2	48	34	20	2/7/10	8/8/9	4/6/10	3/6/10	3/7/9
APH(3')-III	48	42	13	0/9/10	8/9/9	4/9/10	1/7/10	0/8/9

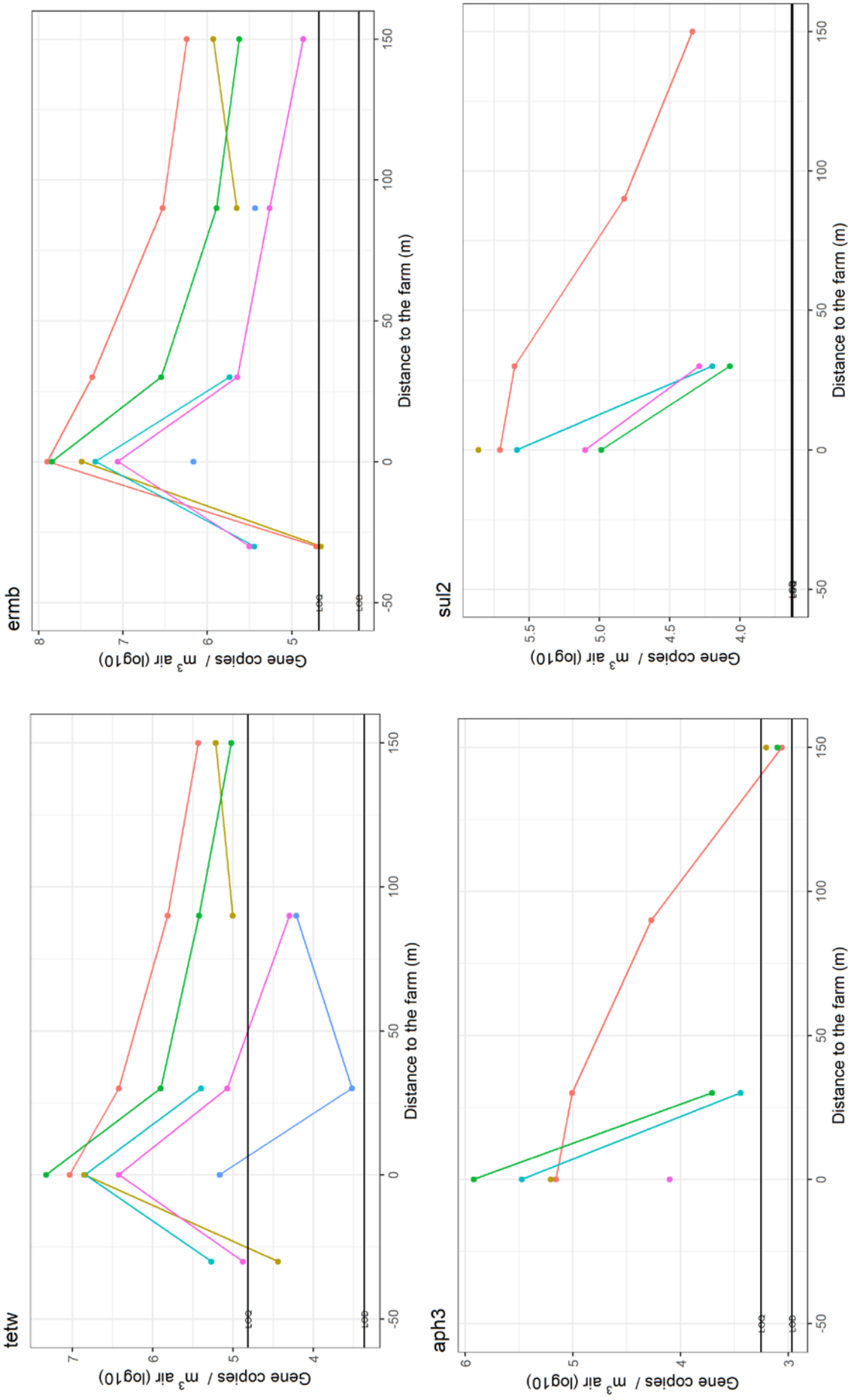
*a successful PCR is defined as a PCR result with acceptable deviations between technical duplicates and the ct value of the Internal Amplification control was within acceptable limits.

**for 16S results above LOQ are shown as, due to different ways of calculation, LOQ turned out lower then LOD.

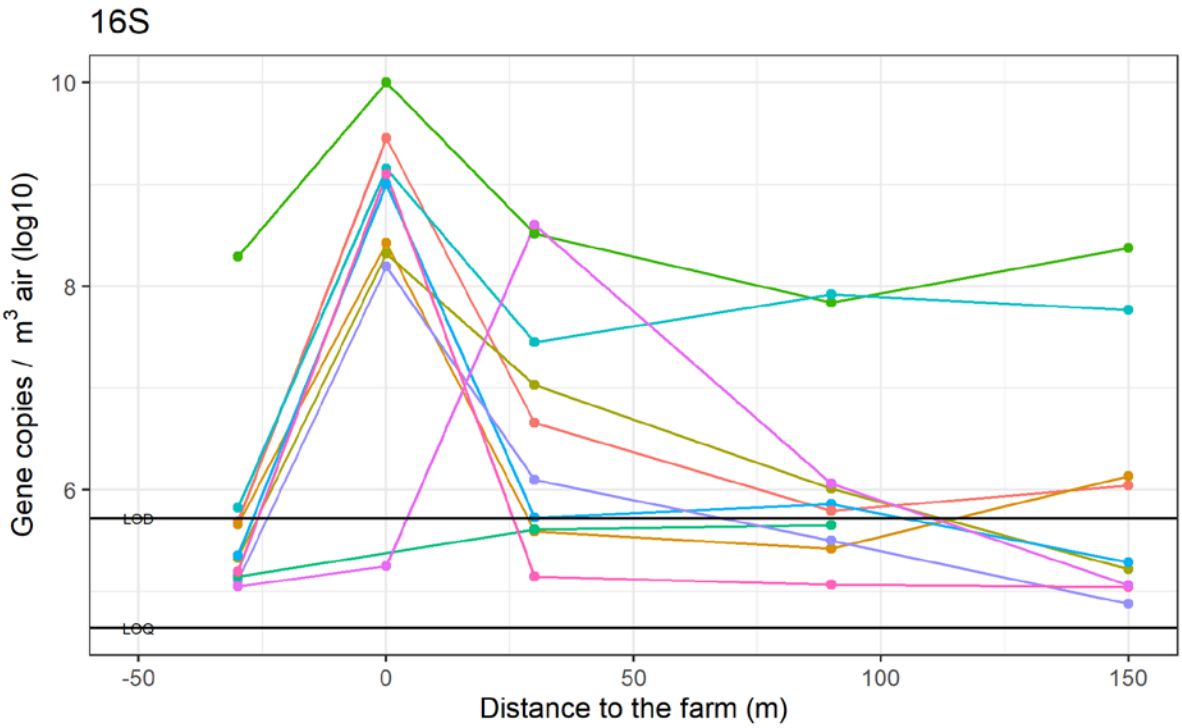
Figure 1 - Quantifiable amounts of 16S, *ermB, tetW, sul2 and aph(3')-III* upwind, inside and downwind of poultry and pig farms. NB the upwind measurement has been set in the graph at -30m but actually differed per measurement between -90m and -30m. Every colored line represents a measurement day at a farm. The upper horizontal line represents the LOQ, the lower the LOD (for *sul2* LOQ=LOD).

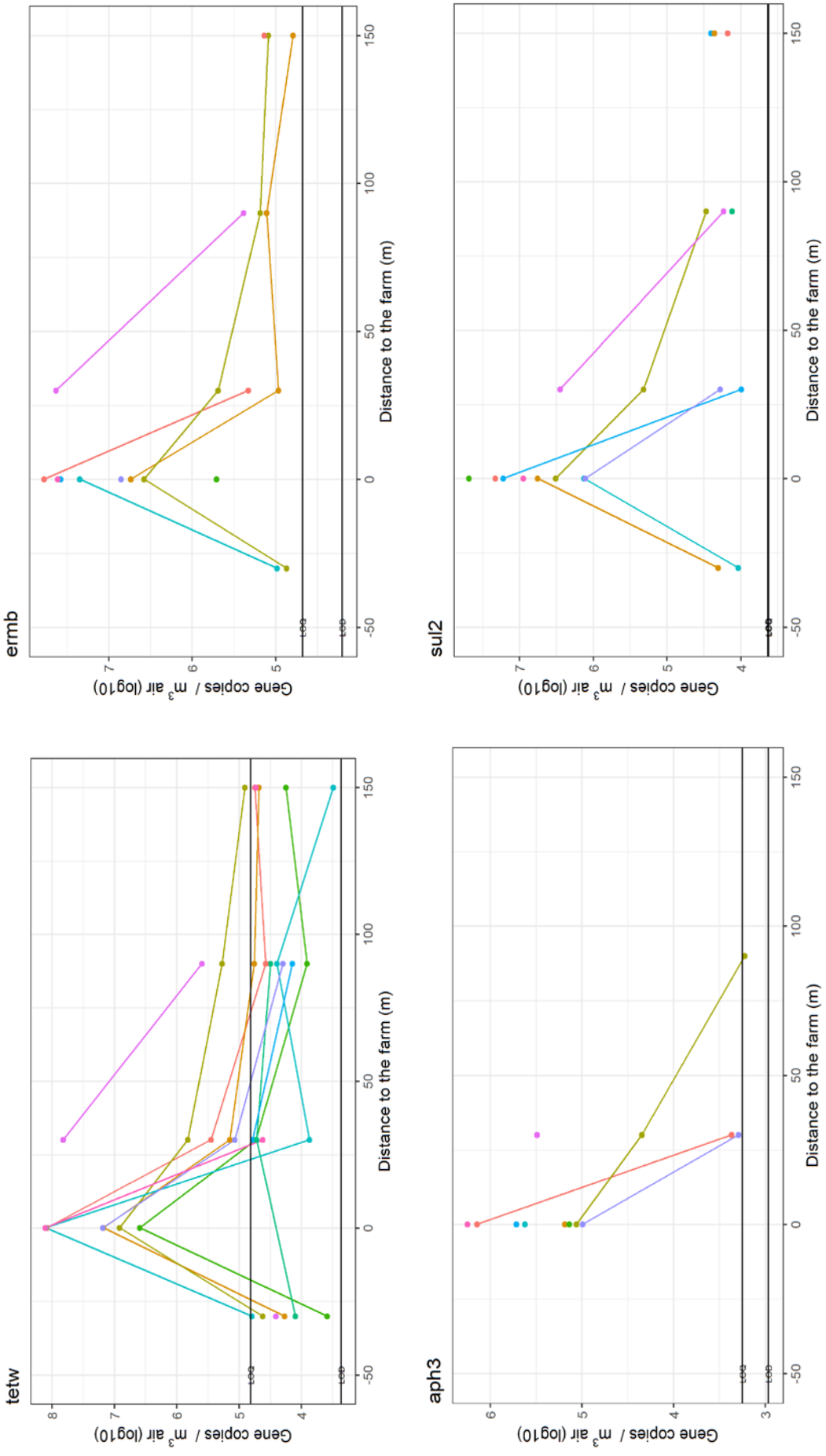
a) Poultry farms




ermb
sul2
tetw
aph3
Gene copies / m³ air (log10)
Distance to the farm (m)
-50
0
50
100
150
LOD
LOQ

b) Pig farms


16S
Gene copies / m3 air (log10)
10
8
6
LOD
LOQ
-50
0
50
100
150
Distance to the farm (m)


ermb
sul2
tetw
aph3
Gene copies / m³ air (log10)
Distance to the farm (m)
-50
0
50
100
150
LOQ
LOD

Discussion and conclusion

The data from active air measurements showed that detectable levels of 16S rRNA gene and resistance genes can be found upwind, inside and up to 150 downwind of pig and poultry farms. ARG levels inside the farm were a maximum of $10^8/m^3$ air for *ermB* and *tetW* and about 3 log units lower for *sul2* and *aph(3')-III*. Outside the farm, genes showed a similar gradient. For example, *ermB* genes at poultry stables decreased roughly 1.5 log units at the first downwind location compared to inside levels and roughly another 1.5 log units in the next 120m downwind. Downwind levels of *tetW* near pig stables returned to upwind levels at a distance of 90m for most farms. The decline as a function of downwind distance to a farm was similar to earlier results on antimicrobial resistant bacteria or gene air concentrations around livestock farms[4-9], however variation between farms was observed. The current study showed that also upwind concentrations were measurable and were similar to the downwind levels of the locations farthest away from the farm.

It can be concluded that measured patterns of antimicrobial resistance genes in and around the pig and poultry farm were as observed in the early studies based on shorter viable sampling.

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7

General discussion

This thesis provides new insights in antimicrobial resistance (AMR) occurrence and epidemiology in poultry and pig livestock farms in Europe, by the use of novel detection methods and a large multi-country study set up. The fecal bacterial microbiome and resistome of broilers and fattening pigs, analyzed with metagenomics, is described from different angles (Chapters 2 and 3). The fecal resistome inside these livestock farms consists of many different resistance genes covering a wide range of antimicrobial classes. This resistome is driven by, amongst others, farm selection per country and differences in antimicrobial usage at country and farm level. The direct environment of animals inside farms was studied by describing the bacterial microbiome and resistome of indoor airborne farm dust (Chapter 4). Dust showed even more richness in resistance genes than feces, which likely is explained by other microbiological sources for dust than fresh animal feces alone. Subsequently, Chapter 5, in which qPCR was used to overcome some of the difficulties related to analysis of metagenomic data, revealed that the abundance of antimicrobial resistance genes (ARGs) in farm dust is on one hand driven by dust generating processes and on the other hand by (antimicrobial) pressure on the microbiological community inside the farm. First steps have been taken to quantify the levels of antimicrobial resistance genes in air outside the farm, and first results are described in Chapter 6. Emitted resistance genes could be measured and quantified downwind of the farm, just near the stables and up to 150m distance.

AMR inside the farm

a. Description of the farm resistome

Shotgun metagenomics in combination with large reference databases of resistance genes added a whole new level to livestock related AMR research [1, 2], of which this research is an example. To date interpretation of this data remains challenging, not in the last place because of many involved environmental, animal and human reservoirs, from which genes can often be transferred from one to the other, in both directions [3, 4]. This web of reservoirs is, with current techniques and subsequently gained knowledge, showing to be even more complex than previously thought [5]. Farms appear to have a prominent role [3, 5, 6], however, the exact chain of exposure events from one point to another is still not known or complicated. Especially the role of the farm environment, in particular farm dust, is puzzling [7].

This thesis showed that resistomes of animal feces and farm dust from inside European poultry (broilers) and pig farms were rich (high number of different genes) and abundant (high sum of relative abundances), with dust being even richer than feces. It appeared that the type of livestock farm (poultry or pig) and reservoir (animal feces, farm dust, farmers stool) are important determinants of the specific characteristics of the resistome, such as, richness, evenness and most abundant genes. The resistomes of both feces and dust are dominated by genes encoding for resistance to tetracyclines, macrolides and aminoglycosides. A recent study on Spanish intensive livestock farms (compared to extensive farms) showed similar differences between reservoirs, with higher richness found again in farm environmental reservoirs. Additionally, the role of the ‘mobilome’ on the spread of resistance genes through the farm was emphasized [8]. We showed that farm dust and animal feces resistomes from

the same farms were correlated, which supports the idea of animal feces being an important source for AMR in farm dust. Given the abundance and diversity of AMR observed in this study, our results substantiate the view of the farm as a (potential) hotspot for AMR development and probable AMR dissemination beyond the farm environment.

b. The role of AMU

The positive relation between AMU and fecal AMR, which had previously been established in various ways [9-11], has been confirmed here while using metagenomics: both on a country scale (comparing country AMU data and farm resistome date) as on a farm scale (AMU and resistome data determined for the same unit). A relation with AMU was found for the total resistome (sum of relative abundances of all genes), for specific antimicrobial class gene clusters (e.g. beta-lactam group treatments with beta-lactam resistance genes) and even for specific ARGs. However, the relatively small differences between treated and non-treated poultry flocks, i.e. the almost similar fecal concentration of resistant genes in non-treated and treated poultry flocks was surprising. This may be a demonstration of the fact that in broilers, which have short production cycles, current usage of antimicrobials is not the most important driver for AMR. The evolution of resistance is an intricate process and thus will probably be its determinants; meaning there will not be one single determinant that drives the abundance and diversity of the resistome.

One other possible route in forming the resistome within the poultry (broiler) sector is transmission from higher up the production pyramid (parent and grandparent animals), downwards to broilers, which has been described for ESBL *E. coli* in the Dutch production pyramid [12]. Similar results have been shown for the close relatedness of quinolone and cephalosporin resistant strains from broilers and the grandparents and thus vertical transmission seems likely to occur [13-16]. Considering the larger number and high abundance of resistance genes in dust described in this thesis another route might be recirculation of ARGs in the farm environment. Earlier studies on broiler farms have shown ESBL producing strains persisting between rounds after cleaning and disinfection and a positive association between ESBL status of previous flocks with current ESBL status [12, 17, 18]. Both observations point to a role of the indoor farm environment in spread, transmission or recirculation or antimicrobial resistant bacteria (ARBs) or ARGs.

The existence of factors affecting the resistome next to current antibiotic use does not mean that aiming for reduced and prudent antimicrobial use is not important. The evidence for vertical transmission and farm recirculation points to a need for reduction and prudent use in all parts and steps of the production chain. Lastly, when investigating determinants for the diversity and abundance of the animal fecal resistome, one must take into consideration that most of these ARBs/ARGs emerged and spread over the course of years or even decades in these reservoirs [19] and that the decline due to (among other) reducing AMU will probably show its full effect only over the years to come. To be able to demonstrate this long term effect, longitudinal studies designs are required. Cross-sectional study designs, as were used in the EFFORT project, have limitations in this context. This idea is substantiated by insights we got from surveillance programs, such as trend analyses in the Netherlands, in which the time component is included, despite the fact that present surveillance systems involve limited

numbers of samples, lack contextual data for each sample and have a simplistic design [20, 21].

c. Determinants for AMR in dust

Reduced and prudent AMU will not only affect the animal gut microbiome but also the immediate animal environment, characterized by fecal contamination. For the first time, we intensively explored freshly settled airborne dust inside poultry and pig farms across Europe in relation to AMR. By using passive airborne dust collectors it was feasible to perform such a large-scale study on air in 360 farms from nine countries, but the data collection process is still much more time consuming than fecal sampling due to the manual assembly of the EDCs. We see farm dust, when collected with EDCs, as a representation of the air load with dust. What is found in dust is what we expect to find in the air of farms as well [22, 23]. Dust containing (viable) bacteria can transmit DNA from one reservoir to another (e.g. from broiler to broiler or from pig to farmer) [24]. Additionally, it could be studied as a (open) reservoir of genes and bacteria on its own [25, 26]. Feces from animals from the same compartments appeared to be an important source for resistance genes in the air, however the formation of the farm dust resistome seems to be affected by more sources and different processes. Firstly, by processes related to pressure on the bacterial microbiome such as AMU or biosecurity, and secondly, by processes related to microbiological dust generation such as dry versus wet feed or animal density. The latter group of determinants seems to be related to an increase of aerosolization of AMR also from other sources than feces, i.e. organic dust from feed, animal skin or feathers.

Davies and Wales [27] recently reviewed studies on the (probably interrelated) potential determinants for AMR on farms, with extra attention for the evidence of the role of farm biosecurity. Just a few studies have looked at other drivers than AMU of farm AMR. The few identified factors are mostly farm biosecurity (and often more specifically hygiene) related. This thesis also shows the importance of biosecurity, however, not focused primarily on the relation with animal fecal AMR levels, but instead focusing on associations with animal environmental (dust) AMR levels. The opposite effects of biosecurity indices found for pig and poultry farms emphasize that maybe not all measures taken at a farm have the effect hoped for, but more insights on relevant causal pathways is yet to be established.

AMR outside the farm

During animal raising and production in livestock farms, different forms of waste are formed. All these waste materials, fecal or contaminated with feces, contain microorganisms, which potentially carry resistance genes leave the farm in various ways [6, 28]. Indeed, in the chapters of this thesis on indoor farm dust it is assumed that genes or bacteria found inside the farm also reach the outdoor environment, which is relevant for the interpretation of findings. After being emitted from the farm (via air ducts) genes most likely disperse in ‘a plume’. The exact characteristics of the plume of ARGs transported through the air from livestock barns, similar to atmospheric dispersion of chemical pollutants, are affected by, among others, gene concentration in the exhaust air, meteorological parameters (wind speed, direction, precipitation) and terrain characteristics (roughness of the earth surface,

presence of wind obstructing objects) [29, 30]. The practical result of all factors combined; our quantification of this plume of resistance genes, showed a gradient of gene concentration downwind of the farms: high inside the stables and a decline downwind of the farm, reaching background levels mostly at 90m distance from the farm. These results confirm that farmed animals are a source for elevated aerial bacterial and ARG levels directly around the farm. What the exact contribution of farms is to the overall background bacterial and ARG levels in rural areas is unknown, but high density of farms is related to higher ARG abundance in air [31].

This type of data is difficult to obtain due to the weather-dependent and complicated (and thus costly and time-consuming) field work and levels of genes per sample are close to the detection limits because of the high dilution with increasing distance from the source. However, an environmental quantification study like this is an important step in further risk assessment attempts of AMR around farms, especially when combined with clinically relevant microorganisms [6]. As measurements of important and culturable bacteria can be even more difficult, measurement of genes is also a proxy which informs on the presence of potentially viable bacteria. Earlier work showed that ARGs/ARBs from pig and poultry farms indeed leads to exposure of farmers and increased carriage via air [32-34]. Studies on ESBL and MRSA carriage by nearby residents around farms showed, however, little to nearly no differences with the general population [35-38], although elevated aerial resistance gene levels near homes surrounded by a high density of livestock farms have been shown [31]. Although the role of aerial transmission of microbes can be (very) relevant for some bacterial diseases [30, 39-42], inclusion of bacterial exposure as stressor for human health in exposure studies is still scarce and deserves more attention [43].

Airborne ARGs, disseminated from farms, also contribute to the broader environmental resistome, by reaching soil [44] and, imaginably, surface water near the farm. To separately measure attribution of aerial dissemination of AMR to other environmental compartments is challenging. Studying the potential subsequent health risks or risks of increased carriage for humans and animals from exposure to AMR in the environment is also still complicated [6, 45]. In the meantime, while we work on risk assessment, it has been suggested that AMR in the environment should best be managed (i.e. mitigated) under the ‘precautionary principle’ [45].

AMR determination

Metagenomics has been shown, for some years now, to be very promising for descriptions of bacterial composition and patterns therein [46], as is also shown in this thesis. However as described several times, AMR epidemiology is complex and multi-factorial, which asks for well thought through studies of large populations and/or longitudinal designs [47]. Although the costs of metagenomics have dropped significantly, metagenomics is often still too expensive to fully answer the current (complex) research questions raised. Additionally, if finances would not be a problem, challenges remain such as bioinformatics being time consuming and statistical methods being not adequate enough for the required study designs [48]. An example of the latter is accounting for dependencies among the samples (e.g. in a longitudinal sampling scheme), while analyzing the full microbial composition.

This thesis also shows advantages of qPCR, a method which is still relevant. qPCR is affordable in large studies and can be used for actual quantification, opposed to the compositional nature of metagenomic data [49], and is therefore suited for association studies and exposure assessments. What also became clear is the high detection limit for metagenomics and the resulting difficulty to detect clinically relevant but relatively rare genes. For qPCR the detection limit also played a role, especially for environmental sampling. Developments on targeted sequencing, like ResCap, that can increase the sensitivity of the resistome by metagenomics by 200-fold, are promising [50]. Common to all molecular detection methods, the viability and thus infectious potential of (resistant) bacteria is unknown when merely DNA is sampled. Accessible (molecular) techniques to overcome this hurdle are not yet there. This is one of the strengths of culturing, especially for known (culturable) clinically relevant strains, despite the many disadvantages and logistical challenges of culturing in larger scale (epidemiological) studies. Subsequently, for example with the use of whole genome sequencing, knowledge of the exact strains can provide understanding of which clones circulate in certain reservoirs and, very relevant, if actual transmission took place between reservoirs. In short, studies are needed that combine techniques. Combining results of several methods and interpretation of the results by different experts will provide more functional meaning and health relevance [5, 51].

AMR, a regional and global issue

Our studies underpin another important issue within AMR research and (mitigation) policy. While the large number of countries involved in the EFFORT-project led to a diverse and unique dataset from which we could draw broad and relevant new insights, this work also showed that AMR occurrence and AMU had clear regional patterns. Although 20 farms per country are insufficient to representatively determine country status, country-wise clustering of resistomes was evident. Even within Europe clear differences existed in antibiotic subscribing patterns between the included countries [52, 53], and additional factors are expected to play a role, some not directly measured within EFFORT, though covered by the term ‘country differences’. Also factors which were measured and appear to affect development of the farm resistome like farm biosecurity or season, are with no doubt regionally different.

On the other hand, livestock farms and their potential as AMR hotspots are a global issue [54-56], and spread of resistant bacteria does not stop at country borders and difficult to treat infections can affect all humans [57]. The effect of AMR on animal health has had much less scientific attention compared to its potential effect on human health [58]. Since 2014 WHO has acknowledged antimicrobial resistance as a serious threat to human health and has called for global action to tackle the problem including a One Health approach, recognizing that human, animal and environmental health cannot be addressed individually [59].

Part of the necessary knowledge can be gained at any place in the world, by doing research to better understand fundamental and universal mechanisms involved in AMR, e.g. the relation between bacterial microbiomes, their resistomes and mobilomes. Also, there are clearly some drivers relevant across countries as was seen from this multi-country study with suitable design of the analysis which accounted for potential country effects. However, a local

situation will determine the total set of actual determinants for elevated AMR levels and also the magnitude of exposure to these levels (e.g. due to hygiene standards). The same treatment in a batch of animals may have a different effect on dissemination of and exposure to ARBs and ARGs in different regions. Therefore, the possibilities for action including (environmental) surveillance and mitigation are mostly determined by local circumstances and so should the research be to determine this. Some parts of the world, for instance countries in Southeast Asia and Africa, that are expected to suffer the most from AMR are however under-represented in scientific research [54, 60]. Applying the One Health approach on AMR research and other work is, luckily, becoming more and more the standard [61-65].

Epilogue

This thesis presents research on the epidemiology of AMR in pig and poultry farms from nine countries in Europe. By combining shotgun metagenomics and qPCR with antimicrobial usage data at farm and flock level, biosecurity scores and several other farm and animal characteristics, we were able to describe the animal fecal and airborne dust resistome and some of its determinants. The current research can hopefully spark new initiatives and render deeper understanding of the role of farm dust (and other environmental reservoirs) in the dynamics of AMR on the farm. At the same time, actions to reduce antimicrobial pressure on bacteria and slow down AMR transmission must keep going. The results in this thesis show that the environment of humans and animals, including dust and air, forms an important aspect in AMR (exposure) research, surveillance and mitigation.

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Introduction

Antibiotics are the most effective way of treating bacterial infections and their use has saved millions of lives in roughly the last 70 years. But when an infection is caused by antimicrobial-resistant (AMR) bacteria, treatment is (severely) hampered because standard antibiotics can no longer inhibit the infection. AMR is a global, significant, and growing health problem for humans and animals. Because humans and animals occupy an, often shared, environment where resistant bacteria can also persist, it is important to approach AMR as a "One-Health problem", emphasizing that the different domains are fundamentally linked and require interdisciplinary and integrated research and solutions.

The main driver for the emergence of AMR is high and incorrect use of antibiotics. In addition to use in human health care, many such pharmaceuticals are also used in veterinary health care, particularly in farm animals. This means that livestock can also be a reservoir of resistant bacteria and/or resistance genes, which are excreted with feces and then spread in and around the farms and enter the surrounding environment via water and soil. Under the right conditions, resistant bacteria can persist for a long time within but also beyond the intestine. When the bacteria themselves die, their genetic material (including resistance genes) can remain. Small particles of manure, including resistant bacteria, can also aerosolize and spread through air. Animals and humans, especially livestock farmers and family members and potentially also local residents, are exposed to these airborne particles by breathing.

For a long time, culture techniques were used in AMR research in which a sample was allowed to grow on a nutrient medium in order to detect specific bacterial species. But the fraction of (pathogenic or commensal) bacteria that can be cultured is small and it is now known that resistance genes can be found in all kinds of bacteria and are transferred between bacteria. In this thesis, two molecular techniques are used to detect antimicrobial resistance genes (ARGs) in order to overcome the shortcoming of these cultural assays. The first technique is shotgun metagenomic analysis, an advanced sequencing technique that has become affordable for broader application within research projects in the last decade. In metagenomics, all DNA from a sample is sequenced; the DNA code is determined, which in turn is compared to bacterial and ARG databases. This enables determination of the bacterial microbiome (collection of all known bacterial species) and resistome (collection of all known resistance genes) of a sample, without requiring prior knowledge on what species might be present in the sample. The second technique is qPCR, a much cheaper analysis technique, which can also be used on all DNA from a sample. With qPCR, one can detect and quantify preselected resistance targets, and due to the lower costs qPCR can be applied to large numbers of samples making it possible to do other types of epidemiological analyses.

This thesis describes the resistome and the bacterial microbiome of the feces of broilers and fattening pigs and of their direct environment, in the form of fresh airborne dust, in livestock

farms from nine European countries. In addition, the relationship of determinants, such as antibiotic use, animal and farm characteristics, and biosecurity (a composite index of factors that influence the introduction and transmission of disease on the livestock farm, such as extensive hygiene protocols or restriction of suppliers) with the composition and concentration of the resistome is examined.

Study design

The results described in this thesis are part of the EFFORT project, a cross-sectional study in which 360 pig and broiler farms were sampled in nine European countries (Belgium, Bulgaria, Denmark, France, Germany, Italy, the Netherlands, Poland and Spain). In each country, 20 conventional farrow-to-finish pig farms and 20 conventional broiler farms were visited, 25 fresh manure samples were collected and information on the biosecurity status of the farm and antibiotic use was gathered using of a questionnaire. In addition, 3-4 EDCs (Electrostatic Dust Collectors) were placed in the stables for several days to sample freshly settled dust. In one country (the Netherlands), fecal samples were also collected from the farmers and family members of a selection of the farms. In a selection of farms from three countries (Spain, Germany and the Netherlands) active air samples were collected with pumps in and around the stables. The presence of resistance genes was determined by metagenomic analysis (investigating all known resistance genes) and qPCR (focusing on 4 resistance genes - *tetW, ermB, sul2, aph(3`)-III* and 16S). The resulting data were analyzed with various univariate and multivariate techniques using R open software.

Main results

Chapter 2 describes that the resistome of fresh feces from broiler and fattening pig farms consists of 407 different ARGs in total and that concentrations vary between, circa, 500 to 10.000 FPKM. FPKM, is a measure of the frequency of resistance genes normalized by the amount bacterial genetic material in the sample. The resistance in pig samples was dominated by genes encoding for tetracycline resistance (around 50%). In broiler samples, genes encoding for tetracycline and macrolide resistance both had a share of around 30%. The distribution of resistance genes in pig samples was more consistent between farms and countries than was the case for broilers, which was more diverse between samples. Statistical analyses showed that the composition of the resistome were determined by the composition of the bacterial community, the animal species and antibiotic use at the country level. **Chapter 3** contains a detailed analysis of the relationship between antibiotic use at the level of single stables or the complete farm, and the amount of resistance genes in feces collected from broiler farms. Thirty-eight percent of the sampled flocks did not report any antibiotic use, but the resistome concentrations were still roughly comparable to those of the flocks that did report antibiotic use. Nevertheless, a positive association was demonstrated between, among others, usage of tetracyclines, beta-lactams and macrolides and lincosamides and the relative frequency of ARG clusters encoding for the corresponding resistance. A gene-level analysis showed that there was also a significant positive association

between antibiotic use and the most common ARGs per antimicrobial class. Only few associations were found between the resistome and the biosecurity status of the farm.

The immediate environment of the animals was studied in this thesis by the analysis of freshly settled dust from the air of the same stables as in which the feces was collected. The metagenomic analysis of dust is described in **Chapter 4**. The resistome of this dust is correlated with the resistome of the feces, which makes it plausible that manure is the major source for the dust, however dust also contains additional different resistance genes. The resistome composition of poultry and pig dust shows more similarities than the resistome of feces from the two species. The additional genes that were found in dust but not in feces may have originated from other microbiological sources such as feed or animals, as was shown in the small additional study (described in chapter 4) on the origin of the DNA found in the dust samples. Again, in the dust reservoir, the resistome was shaped by the underlying microbiome. No evidence of major resistome changes was found in livestock farmers as a result of work-related dust exposure in a smaller sub-study. By using qPCR on 947 dust samples in **Chapter 5**, a broad analysis of a variety of animal- and farm-specific determinants for a selection of four resistance genes in dust could be performed, focusing on resistance to tetracyclines (*tetW*), macrolides (*ermB*), aminoglycosides (*aph(3`)-III*) and sulfamides (*sul2*). Similar to what was observed for the resistome, the concentrations of the resistance genes in feces and dust showed a positive correlation. On the other hand, the absolute amount of resistance genes in dust was also shown to be determined by processes that affect the aerosolization of particles, such as animal density (pigs), bedding materials (poultry), and season (pigs and poultry). Last, they were determined by processes that exert pressure on the bacterial population such as antibiotic use (pigs) and the biosecurity status of the farm, in particular the hygiene status. The latter was positively associated with resistance genes on pig farms and negatively associated on poultry farms (ie improved hygiene does not always lead to reduced AMR), indicating that the relationship between hygiene and AMR is complex and needs more research. **Chapter 6** presents results of a study to determine the concentrations of resistance genes in the air surrounding livestock farms. Resistance genes, the same selection as in Chapter 5, were measured upwind, inside the farm and downwind and all show a similar picture: the concentration is low upwind, high inside the farm and shows a gradual decrease in the downwind direction. Between 90m and 150m downwind of the livestock farm, concentrations reach background (upwind) levels again.

Conclusions

Chapter 7, the general discussion, describes the complexity of the AMR problem with the many reservoirs and domains involved. For example, the gut microbiome of broilers and fattening pigs contains many different resistance genes even in the absence of current antibiotic use. In addition, the immediate environment of the animals, measured in the form of airborne dust, also contains even more different resistance genes. Metagenomics has proven useful to open a new world of uncultivable bacteria and the many resistance genes present in these livestock farm environments. However, these techniques also have

limitations, e.g. due to high costs, designing sufficient powered studies remains a challenge and detection of less prevalent but potentially very relevant genes is difficult. Therefore, studies that combine different methods are needed to answer the various remaining questions. One such question consists of the exact contribution of airborne or dust borne transmission to the broad AMR problem. However it has become clear that the direct environment of the animals can contain many resistance genes. Therefore, dust (or air) should not be forgotten when considering policy options to control AMR as it can act as reservoir from which transmission to humans and animals can occur. Finally and importantly, the risks associated with AMR, are location dependent due to the diversity in livestock farming, antibiotic use and exposure levels in Europe and beyond, and this should be reflected in research and policy.

Nederlandse samenvatting

Introductie

Antibiotica zijn de meest effectieve middelen om bacteriële infecties te bestrijden en het gebruik ervan heeft miljoenen levens gered in, grofweg, de laatste 70 jaar. Maar wanneer een infectie wordt veroorzaakt door antimicrobieel resistente (AMR) bacteriën is de behandeling (ernstig) bemoeilijkt, omdat standaard antibiotica de infectie niet meer kunnen remmen. AMR is een wereldwijd, belangrijk, en groeiend gezondheidsprobleem voor mensen en dieren. Omdat mensen en dieren zich in een, vaak gedeeld, milieu bevinden waar resistente bacteriën zich ook kunnen handhaven is het belangrijk om AMR te benaderen als een 'One-Health probleem', wat benadrukt dat de verschillende domeinen fundamenteel aan elkaar verbonden zijn en onderzoek en oplossingen op een interdisciplinaire en integrale manier moeten worden aangepakt.

De belangrijkste determinant voor het ontstaan van AMR is hoog en onjuist gebruik van antibiotica. Naast het gebruik in de humane gezondheidszorg, worden er ook veel van dit soort middelen gebruikt in de veterinaire gezondheidzorg, in het bijzonder bij landbouwhuisdieren. Dit betekent dat ook veehouderijen een reservoir kunnen vormen van resistente bacteriën en/of resistentiegenen. Deze worden namelijk uitgescheiden in de mest en vervolgens verspreid in en rond de bedrijven en ze kunnen via water en grond in het verdere milieu terecht komen. Als de omstandigheden goed zijn kunnen resistente bacteriën zich lang handhaven in de darm, maar ook daarbuiten, en wanneer de bacteriën zelf dood gaan kan het genetisch materiaal (inclusief resistentiegenen) aanwezig blijven. Kleine mestdeeltjes, inclusief resistente bacteriën, kunnen ook aerosoliseren en zich via de lucht verder verspreiden. Aan deze stofdeeltjes in de lucht zijn dieren en mensen, in het bijzonder de veehouders en familie en potentieel ook omwonenden, blootgesteld doordat ze deze lucht inademen.

Lang werd binnen onderzoek naar AMR gebruik gemaakt van kweekmethoden waarin men een monster bepaalde tijd op een voedingsbodem liet groeien om specifieke bacteriesoorten te detecteren. Maar de fractie van (ziekmakende of commensale) bacteriën die gekweekt kunnen worden is klein en inmiddels is bekend dat resistentiegenen zich in allerlei bacteriën kunnen bevinden en zelfs tussen bacteriën worden overgedragen. In dit proefschrift worden twee moleculaire technieken gebruikt voor het aantonen van antimicrobiële resistentiegenen (ARGs) in allerlei reservoirs. Ten eerste, *shotgun metagenoom analyse*, een geavanceerde sequencing techniek die sinds ongeveer 10 jaar betaalbaar is geworden voor een breder spectrum aan onderzoeksprojecten. Met *metagenomics* wordt al het DNA uit een monster gesequenced; de DNA code wordt bepaald, die weer wordt vergeleken met DNA gegevens in bacteriële en ARG databases. Dit maakt het mogelijk het bacteriële microbioom (verzameling van alle bekende bacteriesoorten) en resistoom (verzameling van alle bekende resistentiegenen) van een monster te bepalen, zonder van tevoren te moeten weten wat er mogelijk in het monster aanwezig is. Ten tweede, kwantitatieve PCR (qPCR), een veel

goedkopere analyse, die ook op al het DNA uit een monster gebruikt kan worden. Hiermee is het mogelijk om vooraf gekozen genetische resistentietargets aan te tonen alsmede ook te kwantificeren, waardoor het mogelijk is om andere type epidemiologische analyses te doen.

Dit proefschrift beschrijft het bacteriële microbioom en het resistoom van de mest van vleeskuikens en vleesvarkens en van hun directe omgeving, in de vorm van vers stof uit de lucht van veehouderijen uit negen Europese landen. Daarnaast wordt de relatie van determinanten, zoals antibioticumgebruik, dier- en bedrijfsgebonden karakteristieken en bioveiligheid (een samengestelde index van factoren die van invloed zijn op de insleep en transmissie van ziekte op het veehouderijbedrijf, zoals uitgebreide hygiëne protocollen of beperking van toeleveranciers) onderzocht met de samenstelling en concentratie van het resistoom.

Studieopzet

De resultaten beschreven in dit proefschrift zijn een onderdeel van het EFFORT-project, een cross-sectionele studie waarin 360 varken- en vleeskuikenbedrijven zijn bemonsterd in negen Europese landen (België, Bulgarije, Denemarken, Frankrijk, Duitsland, Italië, Nederland, Polen en Spanje). In ieder land werden 20 reguliere gesloten varkensbedrijven en 20 reguliere vleeskuikenbedrijven bezocht, waar telkens 25 verse mestmonsters werden verzameld plus een vragenlijst over o.a. de bioveiligheidsstatus van het bedrijf en antibioticumgebruik. Daarnaast werden 3-4 EDCs (Electrostatic Dust Collectors) enkele dagen neergelegd waarmee vers neergedaald stof uit de lucht bemonsterd kon worden. In één land (Nederland) zijn ook van een selectie van de bedrijven ontlastingsmonsters van de veehouders en familie verzameld. In drie landen (Spanje, Duitsland en Nederland) zijn op een selectie van de bedrijven actieve luchtmonsters (m.b.v. pompen) in en rondom de stallen genomen. De aanwezigheid van resistentie genen is bepaald met metagenome analyse (alle bekende resistentie genen) en qPCR gericht op *tetW, ermB, sul2, aph(3`)-III* en 16S. De resulterende data zijn geanalyseerd met diverse univariate en multivariate technieken met behulp van R open software.

Belangrijkste resultaten

In **hoofdstuk 2** staat beschreven dat het resistoom van verse mest van vleeskuiken- en vleesvarkenbedrijven uit in totaal 407 verschillende resistentiegenen bestaat met concentraties variërend van, circa, 500 tot 10.000 FPKM. FPKM is een maat om de hoeveelheid resistentiegenen uit te drukken ten opzichte van de totale hoeveelheid bacteriën in een monster. Het varkensresistoom werd gedomineerd door genen coderend voor tetracyclineresistentie (rond de 50%) en bij vleeskuikens hadden genen coderend voor tetracyclineresistentie en macrolideresistentie beiden een aandeel van rond de 30%. Het resistoom van varkens was meer overeenkomstig tussen bedrijven en landen dan het geval was bij vleeskuikens, dat meer divers was. Het is duidelijk geworden dat de compositie van het resistoom worden bepaald door het onderliggend microbioom, de diersoort en antibioticumgebruik op landniveau. **Hoofdstuk 3** beschrijft een gedetailleerde analyse van de

relatie tussen antibioticumgebruik op stal- en bedrijfsniveau en de hoeveelheid resistentiegenen in mest verzameld op de vleeskuikenbedrijven. Achtendertig procent van de bemonsterde koppels rapporteerde geen enkel antibioticumgebruik, terwijl de resistoomconcentraties grofweg vergelijkbaar waren met die van de koppels die wel antibioticumgebruik rapporteerden. Toch werd er een positieve associatie aangetoond tussen, onder meer, tetracyclines, beta-lactams, macroliden en lincosamidengebruik en ARG clusters die voor corresponderende resistentie coderen. Een analyse op gen-niveau liet zien dat er ook een significante positieve associatie bestaat tussen antibioticumgebruik en de meest voorkomende ARGs per antibioticaklasse. Er zijn maar weinig associaties gevonden tussen het resistoom en de bioveiligheidsstatus van het bedrijf.

De directe omgeving van de dieren is in dit proefschrift bestudeerd door de analyse van vers neergedaald stof uit de lucht in dezelfde stallen als waar de mest van afkomstig was. De metagenome analyse van stof wordt beschreven in **hoofdstuk 4**. Het resistoom van dit stof is gecorreleerd met het resistoom van de mest, wat aannemelijk maakt dat mest een belangrijke bron voor het stof is, maar stof bevat nog meer verschillende resistentiegenen. De compositie van het resistoom van stof uit kuiken- en varkensstallen heeft meer overeenkomsten dan dat van mest van de twee diersoorten. De additionele genen in stof zijn mogelijk afkomstig van andere microbiologische bronnen zoals voer of de dieren, zoals bleek in uit een kleine additionele studie (beschreven in hoofdstuk 4) naar de afkomst van het gevonden DNA in de stofmonsters. Ook in dit reservoir was het resistoom afhankelijk van het onderliggende microbioom. Er zijn, in een kleinere substudie, geen aanwijzingen voor grote resistoomveranderingen bij veehouders door werkgerelateerde blootstelling aan stof. Door gebruik te maken van de kwantitatieve determinatietechniek qPCR op bijna 947 stofmonsters in **hoofdstuk 5**, was er een brede analyse mogelijk van allerlei dier- en bedrijfsgebonden determinanten van een selectie van vier resistentie genen, die coderen voor resistentie tegen tetracyclines (*tetW*), macroliden (*ermB*), aminoglycosides (*aph(3`)-III*) en sulfamiden (*sul2*), in stof. De concentraties van de resistentiegenen in mest en stof hadden een positieve onderlinge relatie. Duidelijk werd dat de concentraties van resistentie genen in stof enerzijds bepaald worden door processen die invloed hebben op de aerosolisatie van deeltjes, zoals dierdichtheid (varkens), strooisel (kuikens) en seizoen (varkens en kuiken). Anderzijds werden ze bepaald door processen die druk uitvoeren op de bacteriële populatie zoals antibioticumgebruik (varkens) en de bioveiligheidsstatus van het bedrijf, in het bijzonder de hygiënestatus. De laatstgenoemde was positief geassocieerd met de concentratie van resistentiegenen op varkensbedrijven en negatief op vleeskuikenbedrijven, wat aangeeft dat het verband tussen verbeterde hygiëne en verminderd AMR complex is en meer onderzoek nodig heeft. **Hoofdstuk 6** bevat de opzet en resultaten van een studie om de concentraties van resistentiegenen in de lucht rondom de veehouderijen te bepalen. Resistentiegenen, dezelfde selectie als in hoofdstuk 5, zijn gemeten bovenwinds, in het bedrijf en benedenwinds en laten allemaal een vergelijkbaar beeld zien: de concentratie is laag voor het bedrijf, hoog in het bedrijf en laat een geleidelijke afname in de benedenwindse richting. Tussen de 90m

en 150m benedenwinds van de veehouderij bereiken de concentraties weer de achtergrond (bovenwindse) niveaus.

Tot slot

In **hoofdstuk 7**, de algemene discussie, wordt de duidelijke complexiteit van het probleem AMR benadrukt met de vele reservoirs en domeinen die betrokken zijn. Dit proefschrift beschrijft dat het darmmicrobioom van vleeskuikens en vleesvarkens veel verschillende resistentiegenen bevat, zelfs wanneer geen antibioticumgebruik wordt gerapporteerd. Daarnaast bevat de directe omgeving van de dieren, hier gemeten in de vorm van stof in de lucht, ook veel, zelfs meer, verschillende resistentiegenen. Metagenomics hebben hun nut bewezen en een nieuwe wereld geopend van niet kweekbare bacteriën en de vele resistentiegenen die aanwezig zijn in deze veehouderijomgevingen. De beperkingen van deze techniek zijn ook duidelijk geworden, bijvoorbeeld door de relatief hoge kosten is een juiste studiegrootte nog steeds een uitdaging en genen die weinig voorkomen maar mogelijk wel heel relevant zijn, zijn lastig te detecteren. Daarom zijn studies die methoden combineren nodig om de verschillende vragen die er nog liggen te beantwoorden. Verder onderzoek moet uitwijzen wat het precieze aandeel is van transmissie via de lucht of uit stof binnen de brede AMR problematiek, maar dat de directe omgeving van het dier ook vol resistentiegenen kan zitten is duidelijk geworden. Deze mag dus niet vergeten worden in het beleid voor bestrijding van AMR als reservoir waar via of vanuit transmissie naar mens en dier kan plaatsvinden. Een belangrijke kanttekening daarbij is dat de risico's verbonden aan AMR plaats afhankelijk zijn door de diversiteit in soorten dierhouderij, antibioticumgebruik en blootstellingsniveaus in Europa en daarbuiten en deze verschillen moeten gereflecteerd worden in het onderzoek en beleid.

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And the seasons, they go round and round, And the painted ponies go up and down.
We're captive on the carousel of time, We can't return, we can only look
Behind, from where we came - uit The Circle Game van Joni Mitchell

Ik ben eigenlijk niet zo goed in afsluiten. Ik hecht me aan plaatsen, mensen, dingen die me op een manier hebben geraakt. Maar de *carousel of time* gaat door. Er ontstaan constant nieuwe avonturen, waardoor loslaten toch grotendeels vanzelf gaat en veel ervaringen en verbindingen neem ik gewoon elke keer weer mee. *I look behind from where I came*, de tijd waarin dit proefschrift is ontstaan, een tijd met veel mooie kansen. Eén van die kansen is, hier en nu, hardop uitspreken dat ik dankbaar ben voor alle gebeurtenissen en alle mensen die mijn onderzoek en dit proefschrift mogelijk hebben gemaakt.

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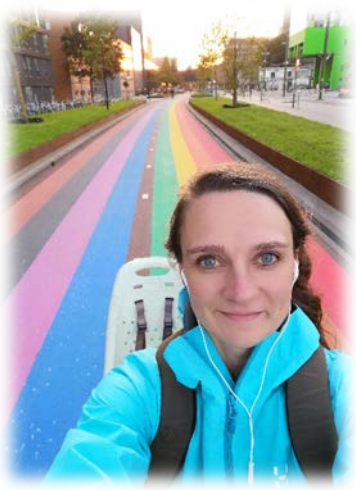
The painted ponies go up and down, op naar volgende avonturen!

Curriculum vitae



Roosmarijn Luiken was born in Roosendaal, the Netherlands, on July 4, 1986. In 2004 she graduated from secondary school with a VWO degree from Het Gertrudis college in Roosendaal. Later that year she continued her education at the Faculty of Veterinary Medicine at Utrecht University. Soon she discovered that becoming a veterinary practitioner was not the best choice for her and she chose to follow the path of Veterinary Public Health within the Farm Animal Health specialization, which consisted of more education and (research) internships in the One Health field. She developed herself outside of university by being actively involved in student organizations, including the presidency of multiple boards.
Roosmarijn graduated in January 2013, after which she worked in the university clinic to help with the implementation of antimicrobial reduction policies in livestock farming. In the beginning of 2014 she started her PhD research at the Institute for Risk Assessment Sciences (Utrecht University). The research was part of the large European EFFORT project on the epidemiology of antimicrobial resistance in the European food chain. Alongside her research she obtained her MSc degree in Epidemiology at Utrecht University. In 2018, Roosmarijn was interim program coordinator of the BMS master program One Health.

In 2021 she will defend her PhD thesis at Utrecht University. From the summer of 2020 onwards, she works as a postdoc at the group of Clinical Infectiology of the Utrecht Faculty of Veterinary Medicine, where she continues to do research on microbiomes and AMR. Roosmarijn lives in Utrecht with her husband and two daughters.

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